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Multiple analysis reprogrammable titration analyser for the kinetic characterization of nitrifying and autotrophic denitrifying biomass

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Abstract

The system Multiple Analysis Reprogrammable TItratioN Analyser (MARTINA) based on titrimetric techniques has been used to kinetically characterize different types of sludges: nitrifying, enriched ammonia oxidizing and autotrophic denitrifying biomass. The titration system employed, combines the addition of NaOH solution and H_2O_2 solution in the mixed liquor to keep the pre-established value of pH and the dissolved oxygen concentration, respectively. Results obtained from repeated experiments performed with nitrifying sludge from municipal and industrial origin present slight differences (coefficient of variation lower than 30%) indicating that the method is highly reproducible. Besides, the kinetic parameters of the enriched ammonia oxidizing sludge obtained using the MARTINA system are comparable to those obtained using the respirometry indicating the reliability of this methodology. Changes in the procedure may be easily implemented in order to estimate half saturation constants with high values. On the other hand, experiments in anoxic conditions applied to the estimation of the kinetic parameters of the autotrophic denitrifying biomass have been successfully performed, even if this process involves a reaction characterized by slight pH changes. The titration system MARTINA is a reproducible, reliable, versatile and precise alternative to the traditional respirometric and substrate monitoring tests for the characterization of kinetics for a wide range of sludges in aerobic or anoxic conditions.

Keywords: Autotrophic denitrification; Kinetic parameters; Nitrifying bacteria; Respirometry; Titrimetry

1. Introduction

In order to control and predict the quality of the effluent generated in wastewater treatment plants (WWTP), models describing the process and the estimation of kinetic parameters are useful. Different kinds of techniques can be used to determine these parameters (respirometry, calorimetry, ...). These techniques are also utilized to study the toxicity of some compounds which are possibly present in the effluents treated via biological processes. Besides, on-line measurements of the substrate consumption rate may be used to design a control strategy to optimize the performance of the WWTP.

The Monod equation has been found to be widely applicable in modelling the biodegradation rate of biological processes.

$$V = V_{\max}\left(\frac{S}{S + K_S}\right) \quad (1)$$

Thus, kinetics with reaction orders between 0 and 1 can be adequately described with a minimal number of parameters, maximum specific substrate consumption rate ($V_{\max}$) and Monod saturation constant for substrate (K_S). With regard to reactor design, $V_{\max}$ contains the necessary information about the limit of the maximum load, whereas K_S describes the achievable effluent quality for a specific substrate.

The estimation of these parameters using methodologies, such as the monitoring of concentrations of substrates in the liquid medium generally involves an analytical procedure, which requires time and is difficult to use on-line while

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Nomenclature

DO	dissolved oxygen concentration (mg O_2/l)
K_S	Monod saturation constant for substrate (mg substrate/l)
K_{SN}	Monod saturation constant for nitrate (mg NO_3^--N/l)
K_{SS}	Monod saturation constant for thiosulphate (mg $S_2O_3^{2-}$-S/l)
S	substrate concentration (mg substrate/l)
S_N	nitrate concentration (mg NO_3^--N/l)
S_S	thiosulphate concentration (mg $S_2O_3^{2-}$-S/l)
V	specific substrate consumption rate (mg substrate/(g VSS h))
V_{max}	maximum specific substrate consumption rate (mg substrate/(g VSS h))

other methods like calorimetry require expensive equipment. Respirometric methods are very simple and easy to use in order to determine kinetic parameters [1] and to control WWTP [2,3]. Nevertheless, these methods may be applied only to processes that involve the use of oxygen as the substrate.

As an alternative to these methods, the titration systems have been developed. Few experimental procedures related to these systems are available in literature. The measurement principle of these systems is based on the addition of small amounts of a titration solution to maintain the constant pH value at a fixed set point value during the reaction. This is the principle of the pH-stat (ANITA). The titrimetric systems were initially applied to the estimation of the kinetic parameters [4,5] and the evaluation of the inhibitory effects of different compounds on the activity of ammonia oxidizing bacteria [6,7]. Afterwards, this technique was further improved and applied to the on-line measurements of the nitrification rates from activated sludge [8,9]. The incorporation to this system of an additional dissolved oxygen probe, using an H_2O_2 solution as the source of oxygen is the base of the DO-stat (MARTINA). This new improved system made it feasible to evaluate the ammonia and nitrite oxidizing activities by two different and completely independent techniques [11] (MARTINA). Other variants of this system are proposed and systems based on the combination of titration and off-gas measurement techniques, to measure the changes in the pH value and the production rate of different compounds present in the gas phase [10] simultaneously are also used.

These titrimetric techniques have also been used with biological processes carried out under anoxic/anaerobic conditions, such as the anaerobic digestion [12,13], and as a special example to determine activities in Anammox processes [14].

In this study, the reproducibility of the titrimetric system MARTINA was tested and applied to the estimation of the nitrifying activities of sludge samples collected from industrial and urban wastewater treatment plants. A second objective was to study the sensitivity of the titrimetric method applied to enriched ammonia oxidizing and autotrophic denitrifying sludges in order to compare the values obtained for the kinetic parameters (V_{max} and K_S) of those obtained via the respirometric and substrate monitoring techniques.

2. Materials and methods

2.1. Sludge sources

2.1.1. Nitrifying sludge

Nitrifying sludge samples were collected from two Italian wastewater treatment plants: one located in San Giuliano (Milano), fed with industrial wastewater, and the other a municipal WWTP located in Peschiera del Garda (Verona).

Sludge enriched with ammonia oxidizing biomass was collected from a lab-scale single high ammonia removal over nitrite (SHARON) reactor of 5 l fed with a synthetic medium (1 g NH_4^+–N/l and 20 g NaCl/l). This reactor was operated for 30 days as a chemostate at a hydraulic retention time (HRT) of 1 day, 35 °C, pH 7.0–7.5 and oxygen concentration over 3 mg O_2/l.

2.1.2. Autotrophic denitrifying sludge

Autotrophic denitrifying biomass was pre-enriched by Kleerebezem and Méndez [15], from a granular anaerobic sludge collected from an UASB reactor treating fish canning wastewater. This biomass was placed in hermetically closed vials and periodically fed with a mixture of nitrate and thiosulphate to keep it active before use.

2.2. Titration system

Titrimetric assays were performed in the so-called Multiple Analysis Reprogrammable TItratioN Analyser (MARTINA) system, which is a combination of a pH-stat and a DO-stat titrator. This prototype was developed by the Politecnico di Milano in cooperation with SPES (Fabriano, AN, Italy) (Fig. 1). The MARTINA system consisted of an ANITA biosensor, described in detail by Rozzi et al. [16], provided with a titration unit to control the dissolved oxygen (DO) concentration to a constant fixed value. When the equipment is used to test anoxic or anaerobic activities, the oxygen probe may be replaced by an oxidation–reduction potential (ORP) probe, which is also connected to the MARTINA unit. The anoxic or anaerobic conditions are achieved by bubbling N_2 into the liquid media. The system is provided with a magnetic stirrer to homogenize the medium and temperature control device to keep then at the desired constant value.

2.2.1. Composition of the solutions

For the estimation of the biomass activities, two different titration solutions were used: an alkaline solution of sodium hydroxide (NaOH, 0.05N) to maintain the desired constant

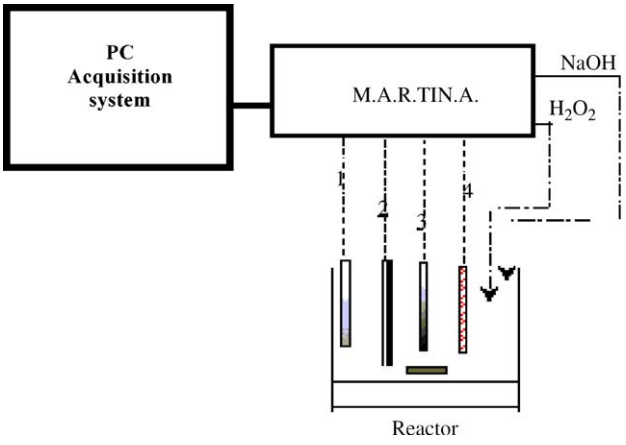

Fig. 1. Scheme of the titration unit: (1) ORP probe, (2) temperature probe, (3) pH meter and (4) oxygen probe.

pH (7.5 for the nitrifying sludge and 8.0 for autotrophic denitrifying sludge) and a solution of hydrogen peroxide (H_2O_2, 0.08N) to control the dissolved oxygen concentration around 8.0 mg O_2/l.

For the estimation of the ammonia and nitrite oxidizing activities, two solutions of ammonium chloride (2 g NH_4^+–N/l) and sodium nitrite (1 g NO_2^-–N/l) were used as the substrates, respectively. In the case of the determination autotrophic denitrification activities two solutions of sodium thiosulphate (1.4 g $S_2O_3^{2-}$–S/l) and sodium nitrate (0.6 g NO_3^-–N/l) were used as substrates.

2.2.2. Titration procedure

Fresh sludge samples were washed three times with a medium containing: 0.67 g/l of $NaHCO_3$ and 50 ml/l of the Winogradsky solution described by Ficara and Rozzi [7]. Biomass was re-suspended in this medium and a sample was transferred to the reaction vessel. The liquid media was gasified with air or nitrogen for aerobic and anoxic experiments, respectively. The stirrer and temperature control were connected.

The automatic titration system registers during the first 30 min of the experiment the endogenous respiration for the aerobic samples and the value of pH of equilibrium for both aerobic and anoxic samples. Then, the experiment starts by adding the substrate to each experiment at the corresponding concentration.

The titration process started and the NaOH solution was added to keep the pH value under the threshold value to neutralize the acidity produced by ammonia oxidation and CO_2 production by heterotrophic respiration or by autotrophic denitrification. The H_2O_2 solution was added when the dissolved oxygen concentration in the liquid medium decreased below the threshold value due to the ammonia and nitrite oxidation and heterotrophic respiration.

Five sets of experiments (A–E) were performed, the conditions of which are described in Table 1. The first set of experiments (A) was performed to estimate the ammonia and nitrite oxidizing activities of a nitrifying sludge collected from a WWTP. In this case, 2 l of nitrifying sludge were collected and concentrated to a final volume of 0.5 l, with a biomass concentration of about 2–3 g VSS/l, washed and re-suspended in a fresh washing medium. Temperature was controlled at 30 °C.

Once the endogenous respiration was obtained, 10 mg NO_2^-–N/l as substrate was added to the reaction vessel. Once nitrite was fully depleted, 3.5 mg NH_4^+–N/l was added, starting the estimation of the ammonia oxidation activity.

Table 1
Conditions of the experiments performed with the MARTINA, respirometric and monitoring substrate systems

Sludge sources	Repetitions	Activities	Sets	Substrate concentrations in the reaction vessel		g VSS/l	pH_{eq}	T (°C)
				mg NO_2^-–N/l	mg NH_4^+–N/l			
Titration system								
Nitrifying	4	NO	A	10	–	2–3	7.5	25
	5	AO	A	–	3.5	2–3	7.5	25
Ammonia oxidizing	2	AO	B	–	5–170[a]	∼2	7.5	35
				mg NO_3^-–N/l	mg $S_2O_3^{2-}$–S/l			
Autotrophic denitrifying	2	AD	C	3.2	12	0.5–0.8	8.0	30
	2		D	10	100	0.5–0.8	8.0	30
	2		E	100	10	0.5–0.8	8.0	30
				mg NO_2^-–N/l	mg NH_4^+–N/l			
Respirometric system								
Ammonia oxidizing	2	AO	F	–	5–100	∼1	7.5	35
				mg NO_3^-–N/l	mg $S_2O_3^{2-}$–S/l			
Monitoring substrate system								
Autotrophic denitrifying	2	AD	G	180	1200	0.5–0.6	8.0	30

AO, ammonia oxidizing activity; NO, nitrite oxidizing activity; AD, autotrophic denitrifying activity; pH_{eq}, pH of equilibrium.
[a] Successive additions in this range.

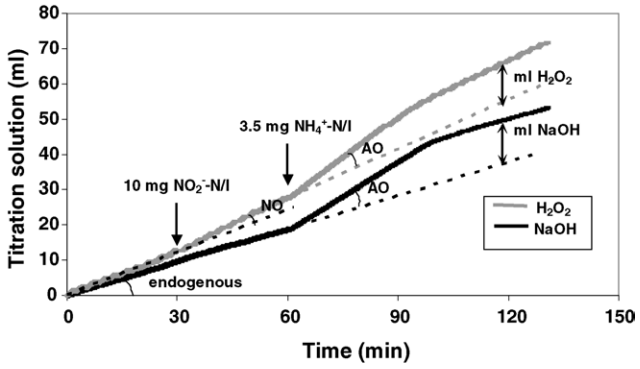

Fig. 2. Typical profile of the titration curves (NaOH and H_2O_2 solutions) to determine the ammonia oxidizing activity (AO) and nitrite oxidizing activity (NO).

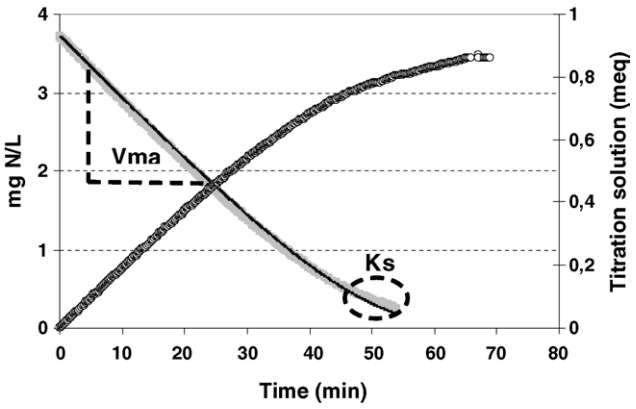

Fig. 3. Titration curve, (❍) milliequivalent of titration solution added and nitrogen profile fitted by a Monod model. (▬) Theoretical and (▭) experimental curves.

In the case of the experiments performed with enriched ammonia oxidizing sludge, set B, a sample of 0.25 l, containing 2 g VSS/l, of previously washed sludge in the washing medium, described later in Section 2.3, were added to the reaction vessel. The assays to determine the ammonia oxidizing activity were performed by spiking different and appropriate volumes of a solution of ammonium chloride (2 g NH_4^+–N/l) at intervals of 30 min in order to increase the concentration of ammonium chloride as substrate in the mixed liquor. Consecutively, the concentrations obtained in the mixed liquor were 5, 10, 30, 50, 80, 120 and 170 mg NH_4^+–N/l (Fig. 2). Temperature was fixed at 35 °C.

Sets C–E were performed with 0.25 l containing 1 g VSS/l of autotrophic denitrifying biomass washed and re-suspended with fresh washing solution up to a total volume of 0.25 l. N_2 gas was bubbled during 15 min to reach anoxic conditions. Temperature was controlled at 30 °C and the pH values of equilibrium were registered during the first 30 min of the experiment in endogenous conditions.

The maximum specific activity of the autotrophic denitrifying biomass was determined from the results obtained from the assays C, carried out in anoxic conditions using a substrate S/N ratio of 3.7, found experimentally by Oh et al. [17]. The assays from sets D and E were performed to determine the Monod saturation constant K_S of each substrate (K_{SN} and K_{SS} for nitrate and thiosulphate, respectively). The K_{SN} values were obtained from sets D, where concentrations of 10 mg NO_3^-–N/l and 100 mg $S_2O_3^{2-}$–S/l were tested. The K_{SS} values were obtained from sets E where concentrations of 100 mg NO_3^-–N/l and 10 mg $S_2O_3^{2-}$–S/l were used.

The titrimetric measurements in all cases ended after depletion of the limiting substrate and when the slope of the curves describing the concentrations of the titration solutions returned to the value initially obtained for the endogenous respiration.

2.2.3. Calculations

The ammonia oxidizing activity and Monod saturation constant were estimated from the volumes consumed of the NaOH and H_2O_2 titration solutions during the time of the assay. In this process, both parameters (pH and DO concentration) change during the reaction (equation (2)) (Fig. 3). When the kinetic parameters for the nitrite oxidizing biomass were estimated, only the volume consumed of the H_2O_2 titration solution was used to calculate V_{max} and K_S, because during this process no pH change took place (equation (3)). The maximum autotrophic denitrifying activity was determined using the NaOH titration solution and taking into account the stoichiometry of the reaction (equation (4)).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (2)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (3)$$

$$0.84S_2O_3^{2-} + NO_3^- + 0.35CO_2 + 0.09HCO_3^- + 0.09NH_4^+ + 0.43H_2O \rightarrow 1.7SO_4^{2-} + 0.50N_2 + 0.09C_5H_7O_2N + 0.069H^+ \quad (4)$$

During the MARTINA experiments, the volumes of each consumed titration solution were monitored chronologically. The slope corresponding to the endogenous respiration was subtracted from the total slope of the curve describing the amount of solution added after the substrate spiking. Using the stoichiometric equations corresponding to each process (equations (2)–(4)), the volumes can be expressed as mg substrate/l and this concentration graphically represented versus time.

In the case of the nitrifying activity measurements, these curves were fitted to a Monod type expression and the kinetic parameters, maximum biomass activity and Monod saturation constant, were estimated. The minimum error square sum (ESS) criteria were used as the fit criterion, indicating when the theoretical nitrogen profile closely reproduced the experimentally obtained values (Fig. 4). With this method,

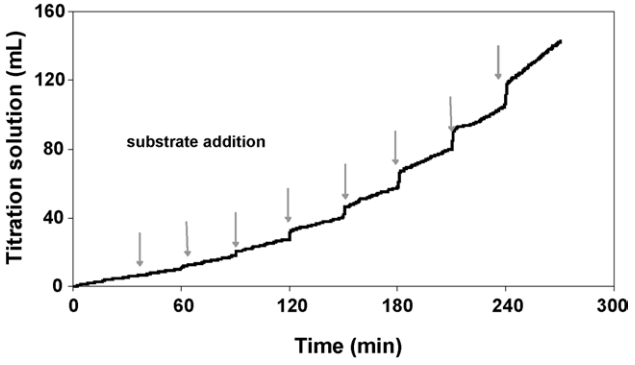

Fig. 4. Typical profile of titration curves to determine ammonia oxidizing activity (NaOH or H_2O_2 titration solutions). The arrow indicates the addition of a specific volume of ammonium.

the determination of the specific activities at different substrate concentrations was not necessary [18].

The kinetic parameters from the enriched ammonia oxidizing biomass collected from a SHARON reactor were determined, similar to the respirometric methodology that is described later, based on the estimation of the specific ammonia oxidizing activity of the biomass corresponding to different amounts of substrate.

The kinetic parameters for the autotrophic denitrifying biomass were estimated by deriving the experimental data corresponding to the curves of concentrations of S or N consumed versus time and fitting them to a Monod type equation for two substrates. The values of V_{max} and K_S for each substrate were obtained using the excess concentration method (equation (5)).

$$V = V_{max}\left(\frac{S_S}{S_S + K_{SS}}\right)\left(\frac{S_N}{S_N + K_{SN}}\right) \quad (5)$$

2.3. Respirometric system

Respirometric assays, set F, for the estimation of the kinetic parameters of the enriched ammonia oxidizing sludge were carried out using a biological oxygen monitor (BOM, Yellow Spring Instruments, Model 5300) with oxygen selective electrodes, equipped with a computer data acquisition system [19]. The BOM is a batch type of respirometer for oxygen uptake rate (OUR) measurements, with the possibility to inject the required substrate directly into the reaction chamber (10 ml).

Fresh biomass samples were washed four times with a medium containing: KH_2PO_4 (3.31 g/l), K_2HPO_4 (3.97 g/l), $MgSO_4{\cdot}7H_2O$ (1.84 g/l), $MgCl_2{\cdot}10H_2O$ (1.52 g/l), NaCl (0.80 g/l) and a nutrient solution (5 ml/l) prepared according to Strous et al. [20]. Biomass was re-suspended in this medium and $NaHCO_3$ (2.5 mM) was added.

A sample of 10 ml of the washed biomass suspended in the medium was transferred to the stirred BOM vessel resulting in a concentration about 1 g VSS/l and aerated for 15 min to obtain a dissolved oxygen concentration close to saturation. Temperature was controlled at 35 °C and the pH at 7.5. Finally, the oxygen probe was sealed in the BOM vessel in such a way that no air bubbles remained in the liquid. The decrease of dissolved oxygen concentration was monitored and recorded by a computer. After an initial period of 2 min, the endogenous respiration was obtained from the slope of the oxygen consumption curve. Then, different amounts of a concentrated ammonia solution were injected to obtain the desired concentration (5–100 mg NH_4^+–N/l). Each concentration was tested at least in duplicate. Biomass concentration in the vial was determined as volatile suspended solids (g VSS/l) and the specific activity was expressed as nitrogen consumption rate per biomass unit as mg NH_4^+–N/(g VSS h). The V_{max} was calculated from maximum slope of oxygen consumption during the assay. The nitrogen consumption rate was calculated from the amount of oxygen consumed according to the stoichiometric (equation (2)). The K_S values were obtained by fitting the experimental data of V_{max} to a linear expression of the Monod model (equation (1)).

2.4. Monitoring substrates system

The assays measuring the substrate concentrations in the liquid phase (set G) were performed in a completely stirred tank reactor with a useful volume of 3 l containing: $NaHCO_3$ (1.5 g/l), Na_2HPO_4 (1.5 g/l), KH_2PO_4 (0.3 g/l), $MgSO_4{\cdot}7H_2O$ (0.5 g/l), NH_4Cl (0.1 g/l), trace elements, $Na_2S_2O_3$ (2.96 g/l) and $NaNO_3$ (2.03 g/l). The initial S/N ratio in the liquid media was fixed at 3.70. The autotrophic denitrifying activity tests were carried out in similar conditions as the titrimetric assays, at 30 °C and the pH value was maintained at 8 through the addition of NaOH 1 M. Anoxic conditions required for the use of the nitrate as an acceptor of electrons were achieved through the continuous gasification with argon and the complete mixture was achieved by means of mechanical stirring at 150 rpm. The reactor was equipped with a water jacket connected to a thermostatic bath to keep the tests at a constant temperature in the system.

The maximum specific denitrifying activities were estimated from the maximum slope of the curve describing the concentration of nitrogen and sulphur compounds throughout time and related to the biomass concentration in the experiment.

2.5. Analytical methods

Ammonia concentration was measured by using the phenol–hypochlorite method [21]. Nitrite, nitrate and thiosulphate ions were determined by capillary electrophoresis using a Waters Quanta 4000 system with sodium sulphate as the electrolyte [22]. The suspended biomass concentrations were measured in terms of volatile suspended solid (VSS) according to the procedure described in the standard method 2540 E [23].

3. Results and discussion

3.1. MARTINA applied to the kinetic characterization of nitrifying biomass (set A): reproducibility and consistency

The kinetic parameters of the nitrifying sludges collected from an industrial and an urban wastewater treatment plants were determined using the NaOH (pH-stat) and H_2O_2 (DO-stat) titration solutions simultaneously.

Kinetic parameters for the nitrite oxidizing step were estimated from the DO-stat titration data due to the intrinsic characteristics of nitrite oxidation (equation (3)). The tests were performed in quadruplicate. The coefficients of variation were, in this case, lower than 22% (Table 2), which is also an acceptable value for the MARTINA methodology [11]. The values of K_S obtained in these assays are of 0.23 and 0.40 mg NO_2^--N/l for the industrial and municipal sludges, respectively. These values are similar to those of 0.35 mg NO_2^--N/l obtained by other authors [24,25]. The maximum nitrite oxidizing activities obtained were 2.8 mg NO_2^--N/(g VSS h) for industrial sludge and 1.0 mg NO_2^--N/(g VSS h) for municipal sludge.

The kinetic parameters for the ammonia oxidizing step were obtained from each titration solution and separately analysed (Table 3). Ficara and Rozzi [7] applying the titrimetric system ANITA to nitrifying sludge samples drawn from WWTPs obtained values of 0.11–0.57 mg NH_4^+–N/l, similar to those obtained in the present study and shown in Table 3. The maximum ammonium oxidizing activities obtained in these experiments were 2.4 mg NH_4^+-N/(gVSS h) for industrial sludge and 1.7 mg NH_4^+-N/(g VSS h) for municipal sludge.

Table 2
Kinetic parameters of nitrite oxidizing bacteria estimated from H_2O_2 consumption (set A)

	V_{max} (mg NO_2^--N/(g VSS h))	K_S (mg NO_2^--N/l)
Industrial sludge		
Mean	2.80 ± 0.60	0.40 ± 0.08
CV (%)	21.1	19.9
Municipal sludge		
Mean	1.00 ± 0.17	0.23 ± 0.02
CV (%)	17.3	6.71

Table 3
Comparison of kinetic parameters obtained for ammonia oxidizing biomass using the MARTINA system (set A)

	V_{max} (mg NH_4^+-N/(g VSS h))		K_S (mg NH_4^+-N/l)	
	NaOH	H_2O_2	NaOH	H_2O_2
Industrial sludge				
Mean	2.40 ± 0.11	2.50 ± 0.18	0.34 ± 0.04	0.36 ± 0.08
CV (%)	4.4	7.5	12.9	19.4
Municipal sludge				
Mean	1.70 ± 0.35	1.50 ± 0.30	0.68 ± 0.15	0.48 ± 0.14
CV (%)	20.5	26.6	22.2	29.7

The standard deviation values for the V_{max} and K_S obtained were relatively low with coefficients of variation comparable to those obtained by Rozzi et al. [11]. Only in the case of the K_S values corresponding to the municipal sludge, these values presented close to 30% of variation between the K_S value obtained from the addition of NaOH solution with respect to those obtained from of the addition H_2O_2 solution. This is a satisfactory result taking into account the well-known variability of the estimation of K_S values. Furthermore, the results obtained using both titration solutions were comparable indicating the consistency of the monitorization procedure of both solutions.

With the obtained maximum specific activities for both steps of the nitrifying reaction (equations (2) and (3)), it is possible to identify which one is the rate-limiting step of the reaction to determine the specific activity of the sludge. Using the industrial sludge, the ammonia oxidation to nitrate occurs without nitrite accumulation because the nitrite oxidizing activity is higher than the ammonia oxidizing one. In this case, the estimation of the ammonia oxidizing activity is enough to know the overall nitrifying activity of the sludge. In the case of the municipal sludge, the rate-limiting step is the nitrite oxidation, which occurs without pH change and it is not possible to be measured by means of the titrimetric ANITA system.

The ANITA pH-stat is limited to the estimation of biological activities where a pH change is involved. In the case of the nitrite oxidation activity, this system is not able to provide a measurement due to the non-pH change during the process. When the nitrifying sludge presents a limiting second step and nitrite is accumulated in the liquid media, it is important to determine this specific activity of the biomass. The MARTINA system allows the determination of the activity of the second step (equation (3)), which limits the overall reaction, by means of the DO-stat system.

3.2. Estimation of kinetics parameters of the ammonia oxidizing biomass (sets B and F): reliability and versatility

The kinetic parameters of the ammonia oxidizing biomass were estimated using two methods: MARTINA and respirometry (BOM), sets B and F, respectively. Due to the high K_S values of ammonia corresponding to the enriched ammonia oxidizing biomass, the titrimetric methodology was slightly modified and successive amounts of a solution of ammonia were added to the reaction vessel. With this procedure, the high ammonia nitrogen concentrations needed were reached. As a consequence, the duration of the experiment increased. Moreover, the fitting of the experimental data to a Monod expression was not used due to the high ESS values obtained. The K_S values obtained ranged from 15 to 18 mg NH_4^+-N/l, which are higher than those commonly obtained in the case of a nitrifying biomass [24]. These high values indicated the low

Table 4
Experimental kinetic parameters obtained from titration and respirometric methods (sets B and F)

Kinetic parameters	Titrimetric system		Respirometric system
	NaOH	H_2O_2	
V_{max} (mg NH_4^+–N/(g VSS h))	15.7	11.8	21.5
K_S (mg NH_4^+–N/l)	15.4	17.7	18.1

affinity of this biomass for the substrate, therefore, the effluent obtained from wastewater treatment systems that operate with this type of biomass always contain high ammonia concentrations. The effluent produced in the SHARON reactor where this biomass was collected from contained around 500 mg NH_4^+–N/l. The K_S values found in both methods are congruent with those obtained by Van Dongen et al. [26] of 26.2 mg NH_4^+–N/l in a reactor operated in similar conditions.

The kinetic parameters obtained using MARTINA were compared to those using the traditional respirometric system (Table 4). The largest difference corresponded to the maximum specific activities, 21.5 mg NH_4^+–N/(g VSS h) from the respirometry and 15.8 and 11.8 mg NH_4^+–N/(g VSS h) from the titrimetry. The averaged value of the obtained K_S values was 17.1 ± 1.5 mg NH_4^+–N/l using the pH-stat, DO-stat and respirometry. The low values of the variation coefficient (≈8%) indicate the high reliability of the method. Besides, the methodology of the titration system allows the determination of activity values at different substrate concentrations in a single experiment, which makes it versatile and simpler than the respirometric one.

3.3. Determination of kinetic parameters of the autotrophic denitrifying biomass (sets C–E and G): precision

Activity tests were carried out in similar conditions, using the titrimetric pH-stat and substrate monitoring methods in order to estimate the maximum specific activity for autotrophic denitrifying biomass. The specific nitrate activity values obtained from both methods, titrimetric and monitoring of the consumed substrate (sets C and G) were 51.04 and 38.75 mg NO_3^-–N/(g VSS h), respectively.

The K_S for nitrate and thiosulphate consumption were determined only using the titration method, working alternatively with one limiting substrate (nitrogen or sulphur) in sets D and E, respectively. The V_{max} and K_S relative to each substrate are shown in Table 5.

Table 5
Kinetic parameters obtained with the autotrophic denitrification biomass using the MARTINA system (sets C–E)

Assays	Kinetic parameters	
C	V_{max} (mg NO_3^-–N/(g VSS h))	51.04 ± 9.24
D	V_{max} (mg NO_3^-–N/(g VSS h))	25.5 ± 0.6
	K_S (mg NO_3^-–N/l)	1.30 ± 0.14
E	V_{max} (mg $S_2O_3^{2-}$–S/(g VSS h))	150.2 ± 32.6
	K_S (mg $S_2O_3^{2-}$–S/l)	32.4 ± 3.04

During the autotrophic denitrification, 0.069 moles of H^+ are produced per mole of nitrate nitrogen reduced (equation (4)). The MARTINA method appears to be a high precision method for the estimation of activities corresponding to processes with low pH changes. This methodology has already been successfully applied to Anammox biomass [14], which is also characterized by small pH changes.

4. Conclusions

- MARTINA is a suitable system to estimate the kinetic parameters of biological reactions, involving changes in alkalinity or oxygen consumption. This is the case in both steps of the nitrification process: ammonia oxidation occurs associated to changes in pH and dissolved oxygen concentration while the nitrite oxidation involves changes only in the dissolved oxygen concentration. For this second step, MARTINA is recommended.
- Results obtained from repeated experiments present slight differences (variation coefficients lower than 30%) indicating a good reproducibility of the method. Furthermore, the results obtained using both pH-stat and DO-stat in the experiments with the industrial and municipal WWTP were also similar indicating the consistency of the data.
- The kinetic parameters of the enriched ammonia oxidizing sludge obtained using the MARTINA system are comparable to those obtained by the respirometry indicating the reliability of this methodology. Besides, changes in the procedure are easily implemented in order to estimate high K_S values corresponding to the enriched ammonia oxidizing biomass. In one test, both values of V_{max} and K_S are estimated.
- The MARTINA system was successfully applied to the estimation of the kinetic parameters of the autotrophic denitrifying biomass. This process involves a reaction characterized by low pH changes, possible to measure using this system.
- The titration method is a reproducible, reliable, versatile and precise alternative to the traditional respirometric and substrate monitoring tests for the kinetic characterization of a wide range of sludges in aerobic or anoxic conditions.

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