# Determination of nitrification kinetics by the ANITA-DOstat biosensor

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**Abstract** The ANITA biosensor, which measures ammonium oxidation rate by alkaline titration at constant pH, has been recently coupled to open respirometry making it possible to evaluate $NH_4^+$ oxidation kinetics by two different and completely independent techniques. This paper describes the use of a new instrument, also derived from the titration biosensor ANITA and named ANITA-DOstat, which incorporates dissolved oxygen (DO) control by $H_2O_2$ addition. The peroxide is very rapidly decomposed to $H_2O_2$ and $O_2$ by bacterial catalase and supplies oxygen to the mixed liquor. The novelty lies not in the use of $H_2O_2$ addition as a source of oxygen for bacterial respiration, but in the measurement of kinetics by DOstat titrimetric respirometry which allows for evaluation of bacterial bio-oxidation parameters directly from the $H_2O_2$ flow rate at constant DO concentration. Preliminary results are presented on nitrification kinetics determination, for both ammonium oxidisers (AO) and nitrite oxidisers (NO), carried out by alkaline titration and by the new $H_2O_2$ DOstat titrimetric respirometry method.

**Keywords** Biosensors; nitrification; nitratation; nitritation; respirometry; titration; hydrogen peroxide

## Introduction

Due to the increasing concern over the issue of water resources protection, more rigorous legal restrictions on nutrients concentration in final effluents have been implemented in recent years, making instruments for nitrification control a fundamental tool for wastewater treatment plant (WWTP) operation. During the nineties, several techniques were developed to determine nitrification kinetics, e.g. Surmacz-Gorska *et al.* (1996), Massone *et al.* (1996). Among the instruments for process control, there are on the one hand classical probes for ammonia, nitrites and nitrates determination, and on the other biosensors which are able to directly assess the biological activity of nitrifying bacteria. Biosensors have the advantage of providing prompt information on nitrification capacity while in-reactor or effluent ammonia and nitrate concentrations are time delayed variables, indirectly correlated to biological activity.

The ANITA biosensor is basically a pHstat reactor which measures $NH_4^+$ oxidation rate, as well as $NH_4^+$ concentration, by determining the addition rate and total volume of an alkaline titrant required to neutralise the acidity produced by the metabolic activity of a nitrifying sludge. Taking account of nitrification reactions:

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + NO_2^- + H_2O \tag{1}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \tag{2}$$

it is possible to set up a procedure to completely characterise both ammonium oxidisers (AO) and nitrite oxidisers (NO) by coupling titration data by ANITA with open respirometric measurements, as shown by Devisscher (1997) and Ficara *et al.* (2000). However, although such tests are easy and readily performed, their results are affected by high variability due to the difficulty of obtaining accurate determinations of the oxygen mass transfer coefficient ($K_L a$), as described by Ficara *et al.* (2000). Moreover, the dynamics of the DO probe response to sharp DO variations make the interpretation of respirograms more complex.

In order to get over the drawbacks of open respirometry, a new instrument was designed. The novel idea was to work in pHstat *and* DOstat conditions by equipping the ANITA reactor with a DO control system, consisting of a titration unit fed on $H_2O_2$, and measure respiration rates through a new technique called DOstat titrimetric respirometry. Earlier investigations already indicated the feasibility of using $H_2O_2$ as an oxygen source in activated sludge systems (Cole *et al.*, 1974), in BOD tests (Chin and Hicks, 1970) and in tertiary nitrifying sand filters (Hunken *et al.*, 1973). In fact, catalase, the principal enzyme responsible for $H_2O_2$ decomposition to water and oxygen, is produced by almost all organisms to prevent the build-up of excessive levels of $H_2O_2$ which is detrimental to living cells. The rate of oxygen production from $H_2O_2$ may be related to the amount of biological solids present in activated sludge mixed liquor since it is proportional to the amount of catalase contained in the microorganisms (Cole *et al.*, 1974).

In this paper, the new titrimetric respirometry technique is described and its application to determine nitrification kinetics is presented. However, the DOstat titrimetric respirometry concept may be applied to determine the kinetics of any aerobic biological reaction, e.g. oxidation of organics by heterotrophic micro-organisms, as shown by preliminary-respiration tests on heterotrophic biomasses and rbCOD determinations.

## Materials and method

### Instrument description

The new instrument, called ANITA-DOstat, was obtained by equipping the ANITA biosensor (Austep, Milano, I and AKSiA Scientific, York, UK) with a DO concentration controller which consists of a $H_2O_2$ dosing system, structurally similar to the NaOH addition system (Massone *et al.*, 1998), capable of spiking impulsive dosages of 10–90 µl volume each, and a DO probe. Both the titrator and the DO probe are connected to a PC. A $H_2O_2$ solution (2 vol. $O_2$/vol.) was used, prepared by dilution from a commercial solution (10 vol. $O_2$/vol.). Stability of the diluted solution for short term periods (a few days) was verified. The controlled addition of the $H_2O_2$ solution, which is rapidly decomposed to $H_2O$ and $O_2$, supplies the oxygen consumed by microbial activity and allows for the maintenance of the DO constancy at a defined set point concentration. The latter is fixed at the value obtained when the endogenous respiration of the sludge (OURend) balances the oxygen supplied by a bubbling aeration system (air flow rate: 50–100 l/h). As described in detail by Vanrolleghem and Verstraete (1993), this equilibrium is:

$$OUR_{end} = K_L a * (DO_s - DO_e) \tag{3}$$

where $DO_s$ is the DO saturation value and $DO_e$ is the DO equilibrium value. Then, after the DO level is set equal to $DO_e$ by the operator on the PC control system, the substrate is added. The DO set point is kept constant by $H_2O_2$ addition and, as a consequence, the exogenous oxygen uptake rate (OUR$_{es}$) is balanced by $H_2O_2$ titration:

$$OUR_{es} = C_{H2O2} * Q_{H2O2} / V_s \tag{4}$$

where $C_{H2O2}$ is the concentration of the $H_2O_2$ solution (as g$O_2$/l), $Q_{H2O2}$ is the flow rate of titrated $H_2O_2$ and $V_s$ is the nitrifying sludge volume. Besides balancing endogenous respiration, the bubbling aeration makes possible $CO_2$ stripping, fixes the $CO_2/HCO_3^-$ ratio and avoids interference on the NaOH titration system. Thus, as shown by reaction (4), the biological reaction rate (in terms of oxygen demand) is directly measured by the mass flow rate of peroxide. In Figure 1, typical titration profiles obtained during a DOstat nitrification test are shown. The DO profile confirms that its level might be kept constant within ±0.25 mg/l.

**Nitrifying biomass**

Nitrifying sludge was sampled every month from the aeration basin of a nitrifying waste-water treatment plant in Northern Italy (Alto Seveso), fed on a mixture of industrial and municipal wastewater (70% and 30% as COD load respectively). The sludge was then kept in anoxic conditions at 4ºC. To restore nitrifiers activity, the required volume of sludge was brought back to room temperature and aerated, after addition of ammonium (10 mg N–$NH_4^+$/l), over a 16 hour period prior to use.

**Analytical methods**

Total and volatile solids, COD and pH analyses were performed according to official Italian methods (IRSA, 1994) which are similar to those described in *Standard Methods* (1995). Ammonium chloride and sodium nitrite solutions were prepared using pure grade chemicals.

**Experimental procedures and data elaboration**

Prior to getting a test underway, the nitrifying activated sludge was mixed, aerated and ther-mostated at constant temperature in the ANITA-DOstat 1 litre vessel until the stabilisation of temperature, pH and DO was reached. These parameters are considered as stable when their oscillation stays within 1/10 of the unit for temperature (ºC) and DO (mg/l) and within 1/100 of the unit for pH. The equilibrium values for pH and DO were fixed as set point val-ues to be maintained constant by NaOH and/or $H_2O_2$ titration respectively after substrate (ammonium and/or nitrite) addition. Tests were terminated when no further addition of either titrant was observed. Average experimental conditions are summarised in Table 1.

Four diverse types of experiments were performed:

*1. Nitritation tests.* These tests were carried out to compare nitritation ($NH_4^+ \rightarrow NO_2^-$) kinet-ics measured by classical alkaline titration and by titrimetric respirometry. A non-limiting concentration of $NO_2^-$ was added (25 mgN–$NO_2^-$/l) before the DO equilibrium was reached and fixed as the set-point value. In this case equation (3) becomes:

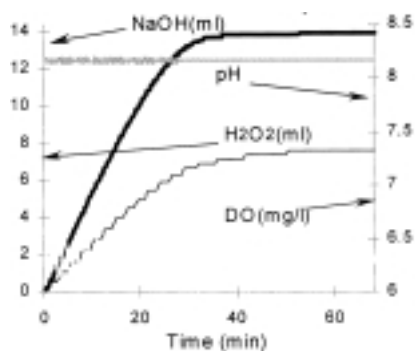$$OUR_{end} + OUR_{NO} = K_L a * (DO_s - DO_e) \tag{5}$$



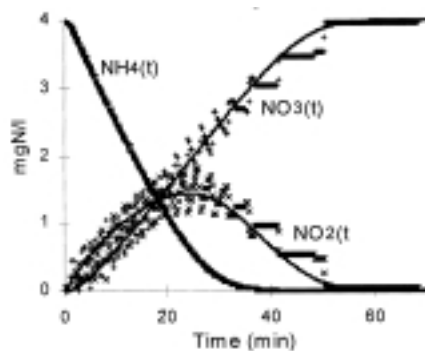**Figure 1** NaOH and $H_2O_2$ cumulated volumes, DO and pH versus time



**Figure 2** In-solution nitrogen forms profiles (experimental and interpolated data)

**Table 1** Range of experimental parameters

| DO set point (mg/l) | pH set point | TSS (g/l) | VSS/TSS (%) | Temperature (ºC) |
|---|---|---|---|---|
| 5–7 | 7.8–8.3 | 3–6 | 65–75 | 23–26 |

Subsequently NH₄Cl was added (4 mg N–NH$_4^+$/l) and H$_2$O$_2$ titration was actuated to balance the OUR of AO. Thus, in these tests both NaOH and H$_2$O$_2$ titrations were stoichiometrically related exclusively to the AO activity and were separately processed for the estimation of AO kinetic parameters. The following equations correlate NaOH and H$_2$O$_2$ titration data to ammonium oxidation:

$$(NH_4^+)_{t+1} = (NH_4^+)_t ( N_{NaOH} * [(V_{NaOH})_{t+1} - (V_{NaOH})_t] * 0.5 * 14 / V_s \qquad (6)$$

$$(NH_4^+)_{t+1} = (NH_4^+)_t ( CH_2O_2 * [(V_{H2O2})_{t+1} ( (V_{H2O2})_t] / (3.42 * V_s) \qquad (7)$$

where $N_{NaOH}$ and $V_{NaOH}$ are normality (meq/l) and volume respectively of the NaOH titrant solution, 14 (g/mol) is the nitrogen atomic weight, $V_s$ is the nitrifying sludge volume (l), $V_{H2O2}$ is the volume of H$_2$O$_2$ titrant solution and 3.42 is the stoichiometric conversion factor between nitrogen and oxygen for ammonium oxidation (see reaction (1)). $V_{max}$ and $K_s$ of the Michaelis-Menten model were estimated from either H$_2$O$_2$ or NaOH titration data (minimum square errors method).

*2. Nitratation tests.* The objective of these tests was to determine NO kinetics directly by the new titrimetric respirometry. After reaching pH and DO equilibrium in endogenous conditions, nitrite was added (4–6 mgN–NO$_2^-$ /l) as substrate and H$_2$O$_2$ titration data were used to calculate nitrite concentration versus time as follows:

$$(NO_2^-)_{t+1} = ( NO_2^-)_t ( C_{H2O2} * [(V_{H2O2})_{t+1} - (V_{H2O2})_t] / (1.14 * V_s) \qquad (8)$$

where 1.14 is the stoichiometric conversion factor between nitrogen and oxygen for nitrite oxidation (see reaction (2)). By fitting the H$_2$O$_2$ profile (minimum square errors method), $V_{max}$ and $K_s$ for NO were estimated.

*3. Nitrification tests.* These tests were performed to determine AO and NO bacteria kinetics at the same time. After reaching pH and DO equilibria in endogenous conditions, NH$_4$Cl (4 mg N–NH$_4^+$/l) was added. H$_2$O$_2$ and NaOH titration data (as shown in Figure 1) were used to estimate kinetic parameters of both AO and NO. In fact, NaOH data allow for the estimation of the ammonium profile (see equation (6)) while combining H$_2$O$_2$ and NaOH titration data, nitrate and nitrite profiles can be calculated according to the following equations:

$$(NO_3^-)_{t+1} = (NO_3^-)_t + [C_{H2O2} * ((V_{H2O2})_{t+1} ((V_{H2O2})_t)/V_s - 3.42 * ((NH_4^+)_t ( (NH_4^+)_{t+1}) / 1.14$$
$$(9)$$

$$(NO_2^-)_{t+1} = (NO_2^-)_t + [(NH_4^+)_t ( (NH_4^+)_{t+1}] ( [(NO_3^-)_{t+1} ( (NO_3^-)_t] \qquad (10)$$

Figure 2 shows the ammonium, nitrite and nitrate profiles as calculated from H$_2$O$_2$ and NaOH data plotted in Figure 1. By fitting (minimum square errors method) H$_2$O$_2$ and NaOH titration profiles with a Michaelis-Menten model, $V_{max}$ and $K_s$ for AO and NO were estimated.

*4. H$_2$O$_2$ potential inhibition evaluation.* AO and NO kinetics were determined after adding to the nitrifying sludge increasing volumes of H$_2$O$_2$ at different flow rates (0.24–2.86 ml/min) to check out the potential toxicity of H$_2$O$_2$ on both microbial populations.

*5. Estimation of initial ammonium and nitrite concentration.* Another application of this biosensor is the estimation of nitrogen oxygen demand (NOD). For nitrification tests, assuming that the oxidation reaction NH$_4^+$→NO$_3^-$ is completed, the final volumes of H$_2$O$_2$

$(V_{H2O2})_f$ and NaOH $(V_{NaOH})_f$ are related to the nitrogen oxygen demand according to equations (11) and (12).

$$NOD = (V_{NaOH})_f * N_{NaOH} * 0.5 * 14 * 4.57 / V_s \qquad (11)$$

$$NOD = C_{H2O2} * (V_{H2O2})_f / V_s \qquad (12)$$

For nitratation tests the coefficient 4.57 in equation (11) becomes 3.42 since nitratation is accounted for in endogenous respiration (see above).

## Results and discussion

### Nitritation experiments

NaOH and $H_2O_2$ titration data sets (14 tests) were processed to determine and compare AO kinetic parameters ($V_{max}$ and $K_s$) by the new DOstat titrimetric respirometry ($H_2O_2$ data) and by alkaline titration (NaOH data). The latter was used as a reference validation method for the former bacteria because it may be considered to be a well established and validated procedure (Massone *et al.*, 1996; Massone *et al.*, 1998). Figure 3 shows estimated AO kinetic parameters obtained from $H_2O_2$ data and from NaOH data, while in Table 2 (columns 1 to 4) related statistics are reported. It may be observed from both the figure and table that an excellent correlation was obtained. Moreover, repeatability of titrimetric respirometry was found to be very satisfactory, remarkable especially for $K_s$ values.

### Nitratation experiments

A series of 10 nitratation tests was performed with $NO_2^-$ as the only substrate (4 mg N-$NO_2^-$). In this case, NO kinetic parameters are directly derived from $H_2O_2$ data. The repeatability of the results (Figure 4 and Table 2, columns 5 and 6) was satisfactory, as confirmed by the low variation coefficients of all the estimates.

### Nitrification experiments

A series of 11 nitrification tests was performed with $NH_4Cl$ (4 mg N–$NH_4^+$/l) as the only substrate. Results, processed by the combined procedure (i.e. using Eqs. (6), (9) and (10)) are plotted in Figure 5, while statistics are reported in Table 2 (columns 7 to 10). The main advantage of this procedure is the feasibility of estimating AO and NO kinetic parameters in a single test. Once more, reproducibility of data was excellent. However, it should be mentioned that only 70% of the combined tests could be processed, while the remaining 30% were affected, after the complete oxidation of the substrate, by a residual base and/or $H_2O_2$
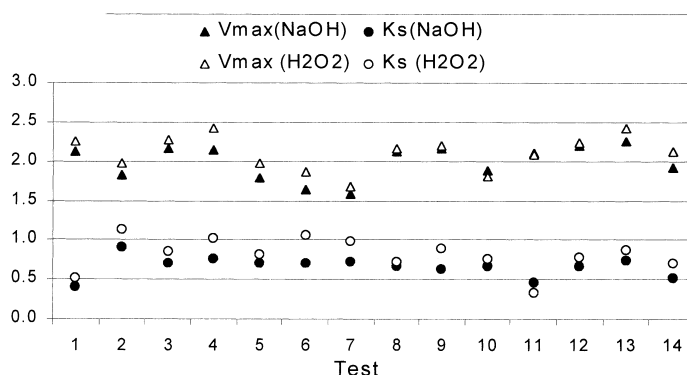


**Figure 3** Nitritation experiments: $V_{max}$ (mg N/(g TSS*h)) and $K_s$ (mg N/l) estimates for AO obtained from NaOH data and $H_2O_2$ data

**Table 2** Estimated values for $V_{max}$ and $K_S$ by nitritation, nitratation and nitrification tests. ($V_{max}$ and $K_S$ values are given as mg N/g TSS $*$ h and mg N/l respectively)

| | Nitritation tests | | | | Nitratation tests | | Nitrification tests | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Column | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| parameter | $V_{max}$ AO | $K_S$ AO | $V_{max}$ AO | $K_S$ AO | $V_{max}$ NO | $K_S$ NO | $V_{max}$ AO | $K_S$ AO | $V_{max}$ NO | $K_S$ NO |
| Titrant | $H_2O_2$ | $H_2O_2$ | NaOH | NaOH | $H_2O_2$ | $H_2O_2$ | NaOH | NaOH | $H_2O_2$ NaOH | $H_2O_2$ NaOH |
| mean | 2.1 | 0.80 | 2.0 | 0.67 | 2.1 | 1.2 | 2.3 | 0.50 | 2.2 | 1.08 |
| st.dev | 0.22 | 0.21 | 0.22 | 0.13 | 0.27 | 0.26 | 0.22 | 0.09 | 0.41 | 0.40 |
| No. tests | 14 | 14 | 14 | 14 | 10 | 10 | 11 | 11 | 11 | 11 |
| CV (%) | 10.6 | 26.0 | 11.0 | 19.0 | 12.6 | 21.2 | 9.5 | 17.0 | 19.2 | 36.7 |
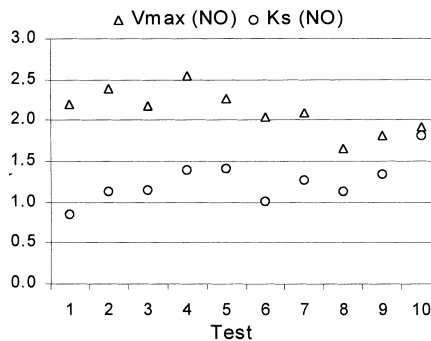


**Figure 4** Nitratation experiments: $V_{max}$ (mg N/(gTSS$*$h)) and $K_S$ (mg N/l) estimates for NO obtained from $H_2O_2$ data only
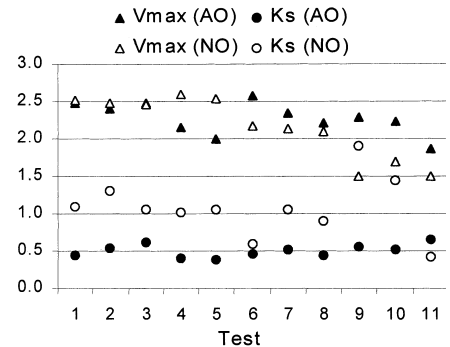


**Figure 5** Nitrification experiments: estimated values for $V_{max}$ (mg N/(gTSS$*$h)) and $K_S$ (mg N/l) for NO and AO (combined procedure)

dosage. The reason for this residual titration was not identified. It might well be due to a drift in DO and/or pH equilibrium value, following a change in temperature higher than 0.3–0.4°C during the test. Finally, it is worth noting that NO kinetic parameters obtained in nitrification experiments compare very well with those measured in nitratation tests (less than 5% and 11% difference for $V_{max}$ and $K_s$, respectively). However, NO kinetic parameters related to the former tests were characterised by a higher variability than those estimated from the latter. This is due to the fact that, in nitrification experiments, $V_{max}$ and $K_s$ estimates for NO bacteria are affected by measuring errors of both NaOH and $H_2O_2$ data, as already noted by Ficara *et al.* (2000) in ANITA-DO experiments.

**$H_2O_2$ potential inhibition evaluation**

It is well known that $H_2O_2$ inhibits microbial activity and it is reasonable to assume that its inhibitory effect depends on concentration factors. The rate of $H_2O_2$ decomposition depends on the concentration of catalase, which in turn is proportional to the activated sludge concentration. As the determination of $H_2O_2$ concentration is difficult and beyond the scope of this research, the $H_2O_2$ inhibitory effect was investigated as a function of $H_2O_2$ flow rate and time duration of the addition. The inhibitory effect of two different $H_2O_2$ addition rates (1 and 10 times the maximum rate used in the nitrification tests described above) was tested for both AO and NO and results are plotted in Figures 6 and 7. A remarkable inhibition was observed on AO at high addition rates (440 mgO$_2$/h), as shown in Figure 6. It is

**Table 3** Percentage of NOD estimated during the tests

| Titrant | Nitritation tests | | Nitrification tests | | Nitratation tests | Resuming results | |
|---|---|---|---|---|---|---|---|
| | NaOH | H$_2$O$_2$ | NaOH | H$_2$O$_2$ | H$_2$O$_2$ | NaOH | H$_2$O$_2$ |
| Mean | 95.6 | 101.3 | 97.0 | 112.1 | 94.8 | 96.2 | 102.8 |
| st.dev | 4.7 | 7.1 | 5.3 | 6.9 | 5.9 | 4.9 | 9.5 |
| No. tests | 14 | 14 | 11 | 11 | 10 | 25 | 35 |
| CV (%) | 4.9 | 7.0 | 5.4 | 6.2 | 6.2 | 5.1 | 9.3 |
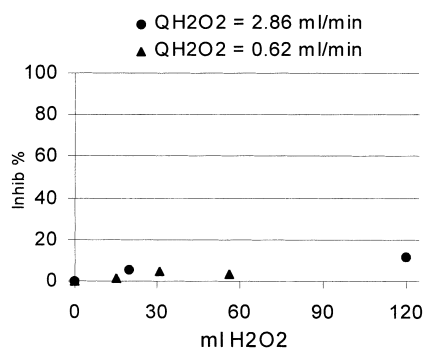


**Figure 6** Inhibition effect of H$_2$O$_2$ on AO at two H$_2$O$_2$ addition rates
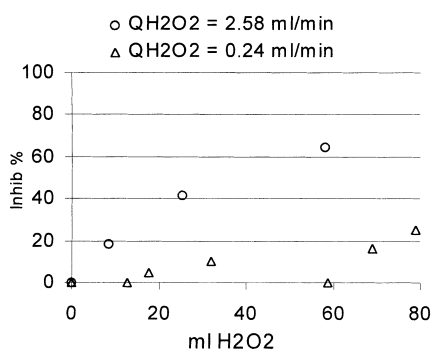


**Figure 7** Inhibition effect of H$_2$O$_2$ on NO at two H$_2$O$_2$ addition rates
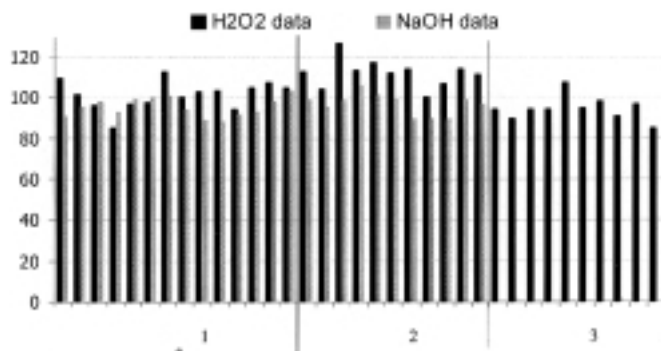


**Figure 8** Recovery of NOD estimated from H$_2$O$_2$ data and from NaOH data for nitritation tests (1), nitrification tests (2) and nitratation tests (3)

worth noting that the stock of nitrifying sludge used for that test had been stored in a refrigerator during a period three weeks longer than those used in the previous inhibition tests. A decrease in active biomass concentration and related catalase possibly induced an increase of the H$_2$O$_2$ concentration in the mixed liquor thus inhibiting the sludge activity. During the other test, carried out at the low peroxide flow rate addition, the fast bio-catalysed decomposition of H$_2$O$_2$ to H$_2$O and O$_2$ reduced its inhibitory effect below 25%. On the other hand, results plotted in Figure 7 indicate that H$_2$O$_2$ addition did not appreciably affect NO activity.

**Estimation of initial ammonium and nitrite concentration**

Figure 8 shows the percentage of NOD estimated for each of the tests belonging to the 3 series of experiments while Table 3 summarises these results. NOD recoveries are very accurate for both titrimetric methods. The overestimated values for the titrimetric respirometry technique were probably induced by the aeration system which stripped a small fraction of the oxygen produced by $H_2O_2$ conversion.

## Conclusions

A new biosensor, the ANITA-DOstat, was developed making use of titration respirometry by $H_2O_2$ addition combining a DOstat with a pHstat. The ANITA-DOstat biosensor was successfully applied to measure nitrification kinetics (for both AO and NO). Results indicated a very satisfactory reproducibility. Therefore, this biosensor appears to be a promising instrument to determine promptly, and with minimum analytical load (TSS or VSS determination only), kinetic parameters for nitrifying biomasses. Moreover, the same instrument can be used to substitute a classical respirometer for short term respiration tests. The following advantages may be claimed as compared to existing respirometric procedures:

- more accurate determinations are obtained, compared to open respirometers, because neither KLa determination nor DO data integration are required to evaluate the OUR;
- continuous OUR measurements may be recorded, a feature impossible in close respirometers;
- OUR can be measured at constant DO, which makes it possible to accurately determine the half saturation constant related to DO ($K_{DO}$);

Inhibition tests confirmed that $H_2O_2$ addition, at the flow rate required to supply the oxygen consumed by nitrifiers, did not appreciably affect their activity.

## Acknowledgements

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