Manometric monitoring of biological denitrification

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A simple, automated manometric method is here discussed and applied to perform batch experiments for the stoichiometric and kinetic characterization of biological denitrification. The main strength of the proposed methodology is that it requires simple instrumentation, which is usually available in wastewater treatment plant laboratories, being it used in BOD and in BMP tests. The experimental setup consists of a glass bottle, a mixing and termostated system and a manometric bottle-head which can measure and log the overpressure that is caused by denitrified N_2. At first, tests were conducted to determine the repeatability of the method; they were performed under low Food-to-Biomass ratio and with both endogenous and externally dosed carbon sources. Later, experimental procedures were performed to assess (1) the anoxic growth yield, (2) the endogenous anoxic decay rate; (3) the anoxic growth rate on acetate; (4) the fraction of anoxic active biomass in the sludge sample. Sludge samples for all these tests were taken from two WWTPs and 6 to 10 replicates were performed each time. Results indicated that the testing procedures is well repeatable and reliable and resulting estimates were within reported literature values.
Keywords denitrification, manometry, kinetic, stoichiometry.

INTRODUCTION

Design, monitoring and control of biological treatment are nowadays based on the knowledge of kinetics and stoichiometry of bioprocesses involved in the biological treatment. To this purpose, respirometric techniques have been developed to monitor and model aerobic processes, while much less efforts have been devoted to anoxic processes characterization. The NUR (Nitrate Uptake Rate) test is the most commonly applied one, however it requires manual sampling and determination of Nitrogen oxides (NO_x) concentration over time (Naidoo et al., 1998; Kujawa and Klapwijk, 1999). Automated methods include the on-line measurements of the off-gas (Larsen et al., 2000; Pratt et al., 2003); however, the required instrumentation and procedures are quite complex and more suitable for research purposes rather than for on-line process monitoring. As an alternative to the above mentioned methods, pH-stat titration can be applied to process monitoring since denitrification is a pH-affecting reaction (Sin and Vanrolleghem, 2004; Ficara and Canziani, 2007). The main drawback of these automated techniques is the need for sophisticated instrumentation that is rarely or never available on-site and require highly skilled operators.

This research aimed at developing a simple methodology for the characterization of the denitrification process that could be easily implemented at WWTPs laboratories and that would be helpful in process design, optimization or upgrading.

The applicability of manometry to assess denitrification rates was previously suggested in the literature, although limited experiences are reported (Sánchez et al., 2000). The basic idea is to monitor the denitrification process in batch experiments performed in closed bottles equipped with an automated manometric device that records the overpressure caused by the catabolic production of N_2 by denitrifiers and its released in the gas phase in the presence of an efficient CO_2 adsorbent.

MATERIALS METHODS

Principle of the method

In a closed bottle the denitrification process takes place and results in N_2 release that causes a pressure increase; the relationship between the overpressure generated, P(t), and the denitrification rate (r_d mgN L^{-1} h^{-1}) can be obtained by assuming that the N_2 transfer to the gas phase is not rate limiting (sludge mixing allows fast transfer of N_2 to the headspace of the bottle) and that N_2 does not remain in solution (being a poorly soluble gas). According to the gas law:

$$P(t) = n_{N_2}(t) \cdot \frac{R \cdot T}{V_{HS}} \qquad (1)$$

where V_{HS} (L) is the volume of the headspace in the bottle and n_{N2} (moles) is the number of moles of N_2 released into the headspace. The amount of the release of N_2 over time is thus the main output of the test from which relevant kinetic and stoichiometric information can be drawn.

Analytical methods

Sludge volatile suspended solids were determined according to standard methods (APHA AWWA WEF,1998). Nitrite and nitrate concentrations were determined according to the spectrophotometric method (APAT IRSA-CNR, 2003).

Testing procedure

Sludge samples were collected from two large WWTP plants, hereafter referred to as WW1 ($3.7 \cdot 10^5$ m^3 d^{-1}, mainly urban wastewater) and WW2 ($2.7 \cdot 10^5$ m^3 d^{-1}, about 80% of which from industrial origin). After collection, sludge samples were kept at 4°C for no more than 15 days before use.

Denitrification tests were performed with a widely used laboratory instrument (OxiTop Control® WTW), a manometric device consisting of a measuring head that records the overpressure values in the bottle. The measuring head is fixed on the top of a glass bottle which is provided with two lateral openings, sealed by rubber septa, that are used for substrate injections and for biogas discharge. A dedicated container allows NaOH pellets to be located in the headspace inside the bottle as a trap for the evolving CO_2, so that the measured overpressure is due to N_2 gas only. Efficacy of CO_2 adsorption was proven effective in preliminary tests (data not shown). Each bottle was placed in an incubator at 20°C and mixed by a magnetic stirrer. Up to six bottles were run in parallel.

Each bottle was prepared according to the following procedure. (1) A batch volume of sludge was taken from the fridge, aerated for 1-2 h, diluted to the desired concentration with a physiological washing solution (Winogradsky saline solution without micronutrients, Pochon and Tardieux, 1962) and allylthiourea (10 mgL^{-1}) was dosed to inhibit nitrification and prevent further nitrate production; (2) a known volume of sludge was poured into each test-bottle; N_2 was sparged into the headspace for 5 min before sealing the bottle and locating it inside the thermostatic chamber; (3) after 30-60 min, the pressure data-logger was started; (4) a known volume of a stock solution of nitrate and, if required, of the organic carbon source were added by injection through the rubber septum. At the end of the test, logged pressure values were processed to assess the mass of nitrogen released to the gas phase in the course of the experiment. At the beginning and at the end of each

test, pH in the sludge suspension was measured and it was found it always remained within a narrow and optimal range (from 8 to 8.5).

RESULTS

Tests at low F/M ratio

This first experimental campaign was performed to check the reliability and repeatability of the estimates of the specific denitrification rates as assessed from data of N_2 release. Each bottle was prepared according to the previously described procedure. Tests were conducted with sludge concentration of 2÷5 gVSS L^{-1}, sludge volume in the bottle of 1 L, initial electron donor concentration (either nitrous or nitric Nitrogen) of 15÷30 mgN·L^{-1}; the initial external carbon source was dosed at a COD/N mass ratio of 10÷15 (g·g^{-1}).

Figure 1a reports the output of a typical manometric test. Gaseous N_2 trend was used to back-calculate the expected in-solution NO_X concentration, as its initial value was known. As samples for nitrite and nitrate analyses were also taken during the test, both calculated and measured concentrations are compared in Figure 1b. A satisfactory agreement can be observed that supports the reliability of the method in the assessment of the denitrified nitrogen.
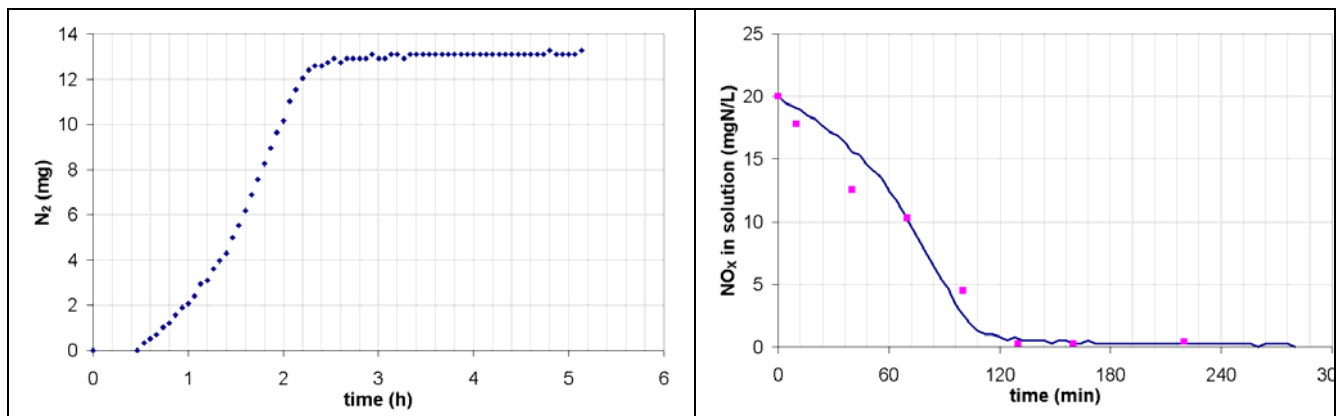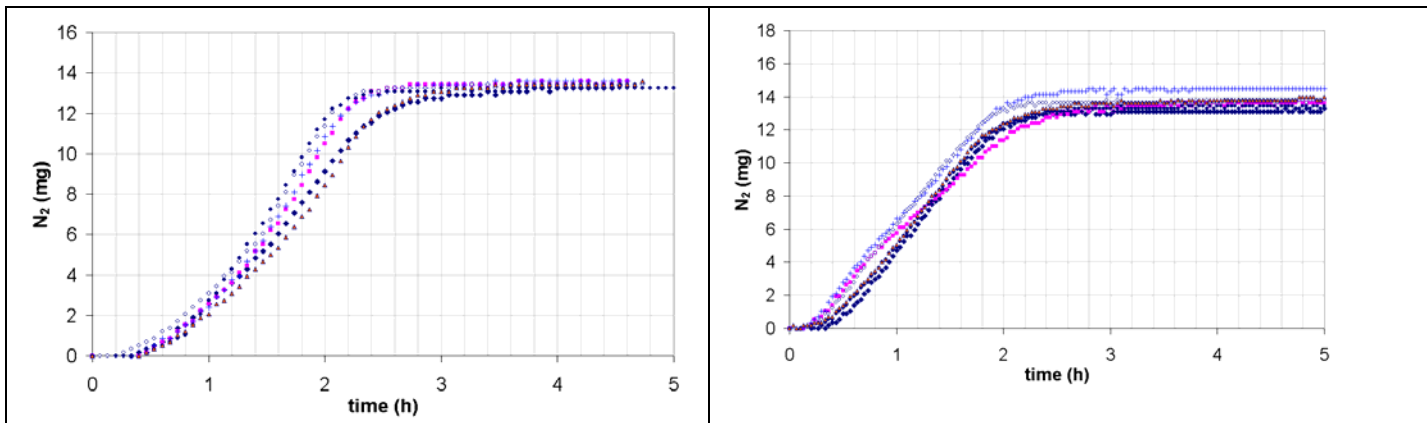

Figure 1 – Output of a denitrification test (a); comparison between in-solution NO_x concentration as measured by analytical methods (solid squares) and as back-calculated from N_2 evolution data (solid line) (b).

Typical replicates from tests conducted with various substrates are shown in Figure 2. Because of the low F/M ratio, the time required for completing the denitrification process was of the order of 2-4 h. An initial lag-phase was often observed before a constant gas production was evidenced. This lag phase can be attributed to:

- an imperfect headspace flushing and the presence of a residual oxygen in the gas phase; residual oxygen would be consumed during the lag-phase causing a decrease in the headspace pressure, corresponding to a negative N_2 consumption;
- the time biomass takes to switch from aerobic to anoxic metabolism after a long aeration phase (so called 'diauxic growth', Hamilton et al., 2005);
- the time N_2 produced in the liquid phase takes to be efficiently released to the gas phase.

This initial lag phase was not considered for denitrification rate calculations; to this purpose only the data referred to the linear N_2 production were fitted.

Figure 2. Typical output of low F/M denitrification tests on WW1 sludge samples; replicates of denitrification tests with acetate and nitrate (left) and nitrite (right) are shown.

The ultimate amount of N_2 evolved (i.e. the asymptotic value of each N_2 production curve) was compared to the amount of nitrate spiked in the bottle. Normally, cumulated N_2 was lower than expected from the amount of nitrate added and an average difference of 22% (mean value of about 160 repetitions) was observed. This is likely due to the residual presence of oxygen in the head space whose uptake for biomass respiration would result in a pressure decrease, corresponding to the previously discussed lag-phase. This pressure decrease would have masked the concomitant nitrogen production, eventually causing an underestimation of the total overpressure due to N_2 production. As a matter of fact, a small gaseous oxygen residue in the head space of the test bottle can cause a relevant error in the estimate. Let's consider a typical experimental conditions (headspace volume = 140 mL, sludge volume = 1 L; initial nitrate concentration = 20 mgN·L^{-1}; test temperature = 20°C): the observed average error of 22% could be caused by a residual O_2 partial pressure in the headspace of 2 to 3%, which is consistent with an imperfect headspace flushing or with a little oxygen entering the bottle after stopping nitrogen flushing. This suggests that much care has to be paid during this phase of the procedure.

According to the plots in Figure 2, all replicates led to very similar N_2 production curves, suggesting a satisfactory reproducibility of the methodology. In Table 1, specific denitrification rates (SDR) measured under various combinations of electron donor and acceptors are summarised. The reproducibility of SDR estimation is indeed satisfactory as demonstrated be the coefficient of variation that varied between 8 and 33 %.

Denitrification rates on acetate were found to be significantly higher in sludge samples taken from WW1 than those taken from WW2, as confirmed by a heteroschedastic t-test. Moreover, denitrification rates on acetate/nitrate were significantly lower than those with acetate/nitrite in both sludge samples, suggesting that nitrite build-up has not to be expected in the anoxic basins of both WWTPs.

Tests at high F/M ratio

This experimental set-up is similar to that used in typical respirometric tests for the assessment of the maximum growth rate (e.g. Kappeler J. and Gujer W., 1992). Each test is conducted by using a lower amount of sludge, a higher headspace volume and a higher initial nitrate concentration than in previously described tests. Moreover, the COD/N ratio is much lower so that the whole amount of soluble COD is used up in the course of the test and the endogenous denitrification rate can be assessed after acetate in fully depleted. In these tests, denitrifiers grow significantly and the N_2 production rate shows an exponential increase (Figure 3). With reference to the typical ASM1

notation (Henze et al., 1987), we can write the following equation for N_2 production rate (NPR):

$$NPR(mgN/h) = \frac{1 - Y_{HD}}{2.86 \cdot Y_{HD}} \cdot \mu_{HD} \cdot X_{BH,t=0} \cdot e^{(\mu_{HD} - b_{DH}) \cdot t} \qquad (2)$$

Table 1. Specific denitrification rates [mgN g^{-1}SSV h^{-1}], as measured in short term experiments.

Electron donor	Electron acceptor	WW1				WW2			
		mean	st. dev.	CV (%)	# of repetitions	mean	st. dev.	CV (%)	# of repetitions
Acetate	$N-NO_2^-$	5.00	0.46	9	6	2.18	0.72	33	29
	$N-NO_3^-$	2.61	0.77	29	9	1.76	0.48	28	47
Endogenous organic matter	$N-NO_2^-$	1.58	0.28	17	7	n.a.	n.a.	n.a.	5
	$N-NO_3^-$	1.70	0.13	8	9	0.66	0.21	31	6

n.a.: data not available

The shape of the NPR (Figure 3) is similar to that of the oxygen uptake rate (OUR) for the aerobic acetate degradation. An initial exponential growth phase is followed by a slow decrease down to endogenous levels, during which the internally stored organic substrate is used for further growth; this evidence is in agreement with the reported storage capacity of denitrifiers (Beun et al., 2000; Dionisi et al., 2001).
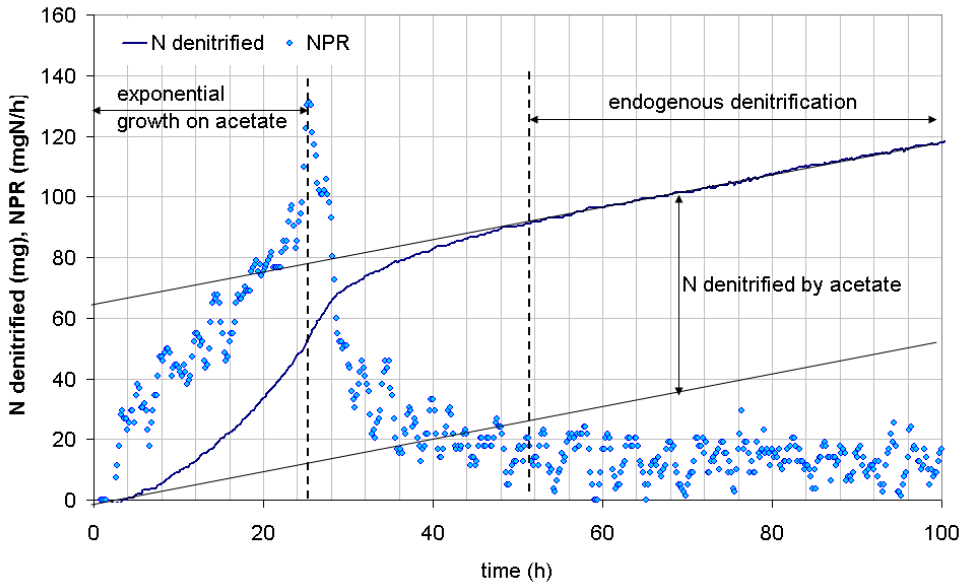
Figure 3. Typical output of a high F/M anoxic test with WW2 sludge.

From the output of these tests, the following information can be drawn:

- the **net growth rate** (μ_{HD} - b_{HD}): this is computed by plotting the N_2 production rate during the

exponential phase in a semi-log chart ($\ln(NPR/NPR_0)$ vs. time); the slope of the fitting line gives an estimate of the net growth rate;

- the **yield coefficient:** it is assessed by quantifying the amount of N_2 denitrified ($N_{denitrified}$) by the known amount of COD added as acetate (COD_0):

$$Y_{HD} = 1 - \frac{N_{denitrified} \cdot 2.86}{COD_0} \qquad (3)$$

- the amount of **active denitrifiers in the sludge**: this is computed by using eq. 2 and by considering the NPR measured at the beginning of the test (NPR_0):

$$X_{BH,t=0} = NPR_0 \cdot \frac{2.86 \cdot Y_{HD}}{\mu_{HD} \cdot (1 - Y_{HD})} \qquad (4)$$

Results of these estimations are summarized in Table 2.

Decay tests

In these tests, a more concentrated biomass is used, no external carbon source is dosed and pressure data acquisition is continued for 3 to 5 days. The slow decrease in the NPR is then fitted in a semi logarithmic plot to get the apparent decay rate constant (b'_{HD}). This value is then corrected for cryptic growth to get the net decay rate (b_{HD}) according to:

$$b_{HD} = \frac{b'_{HD}}{1 - Y_H(1 - f_p)} \qquad (5)$$

where f_p is the fraction of unbiodegradable biomass that is produced from biomass decay.

Results obtained from these tests are also summarized in Table 2.

Table 2. Estimated values for kinetic and stoichiometric parameters from high F/M tests.

Parameter	Sludge from WW1				Sludge from WW2			
	Mean	St. dev	CV(%)	# repetitions	Mean	St. dev	CV(%)	# repetitions
μ_{HD} (d^{-1})	2.26	0.03	2	8	2.74	0.21	8	8
Y_{HD} (gCOD g^{-1}COD)	0.56	0.03	6	4	0.66	0.02	3	4
X_{BH}/X_T (gCOD g^{-1}COD in VSS)	0.15	0.01	6	8	0.16	0.06	36	8
b_{HD} (d^{-1})	0.76	0.06	9	6	0.67	0.02	3	2

The values of the maximum growth rates appear in the range of values reported in the literature (e.g. 4 d^{-1} at 20°C, Mokhayeri et al., 2006) and measured Y_H values fall within the frequently reported interval of 0.5-0.7 gCODg^{-1}COD (Kujawa and Klapwijk, 1999; Muller et al., 2003; De Lucas et al., 2005). Calculated values of the decay rate are close to ASM1 default value of 0.62 d^{-1}.

Both WWTPs show similar values. Sludge samples from WW2 showed a slightly lower growth rate

and a slightly higher decay rate than WW1, while the average fraction of active denitrifiers was low in both cases; this evidence is well in agreement with their high sludge age (ranging from 29 and 44 d in WW1 and around 25 d in WW2).

Data reported in Table 2 can be also used to compute the specific denitrification rate (SDR):

$$SDR\left(\frac{mgN}{gVSS \cdot h}\right) = \frac{\mu_{HD} \cdot (1 - Y_{HD})}{2.86 \cdot Y_{HD}} \cdot \frac{1000}{24} \cdot \left(\frac{X_{BH}}{X_T} \cdot 1.42\right)$$ (6)

were a conventional COD content of 1.42 gCOD per gVSS was assumed.

The standard deviation reported in Table 2 for each parameter (maximum growth rate, growth yield and biomass active fraction) was propagated according to the theory of error propagation (Mood et al., 1974) under the assumption that all estimates were statistically independent. The following results were obtained: $SDR_{WW1} = 5.5 \pm 0.9$ mgN g^{-1}VSS h^{-1}; $SDR_{WW2} = 4.8 \pm 1.8$ mgN g^{-1}VSS h^{-1}. Both these values are much higher than those measured with acetate and nitrate in low F/M tests and already reported in Table 1. Apparently, if denitrifiers are previously kept under quiescent conditions (endogenous, low temperature storage) a lag-time is needed before their maximum growth rate can be fully expressed. It will matter for further research to elucidate which of these two values is more representative of actual SDRs under operating conditions in full scale plants.

CONCLUSIONS

The proposed batch bioassay for estimating stoichiometric coefficients and kinetics parameters of biological heterotrophic denitrification was proven to be simple, reliable and convenient, as it uses simple and widely employed manometric devices. Several replicates can be easily run in parallel, so that many replicates will allow to check the reliability of the results.

Denitrification tests performed under low F/M conditions, allowed to estimate the specific denitrification rate within few hours; a satisfactory reproducibility was obtained, with standard deviations from 8 to 33%. Better repeatability can be achieved by ensuring that no residual oxygen content is left in the head space.

High F/M, long-term tests were less affected by the initial lag-phase than low F/M tests. Fitting N_2 production rates allowed to estimate the maximum growth rate, biomass growth yield, and the fraction of active biomass under anoxic conditions. Results were usually within typical literature ranges. Long-term tests were also successfully performed to estimate the anoxic decay rate which was found to be similar to the typical ASM default value.

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