Microcalorimetric and manometric tests to assess anammox activity

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ABSTRACT

The present study compares two experimental methods to evaluate Anammox activity based on the assessment of (1) the N_2 production rate by a manometric device, as previously proposed, and (2) the heat production rate by a microcalorimeter. Two samples of Anammox suspended biomass were taken from a pilot-plant, and their specific Anammox activity measured by both techniques. Both methods were successfully applied. As for calorimetric tests, they were performed for the first time on Anammox enriched sludge samples. Comparisons between the specific Anammox activities estimated by manometry and calorimetry and between expected (from the reaction enthalpy) and measured heat productions were performed. Promising results were obtained.

Key words | anammox activity, manometry, microcalorimetry

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INTRODUCTION

Anaerobic ammonium oxidation is carried out by distinct bacteria, classified as Planctomycetes, whose metabolic reaction can be described by two proposed stoichiometric equation, the first one was proposed by Van de Graaf *et al.* (1996):

$$NH_4^+ + 1.31NO_2^- + 0.0425CO_2 \rightarrow 1.045N_2 + 0.22NO_3^- + 0.0425CH_2O + 1.87H_2O + 0.09OH^- \quad (1)$$

More recently, Strous *et al.* (1998), proposed this slightly different reaction stoichiometry:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (2)$$

In spite of the very recent microbiological identification and characterisation, the Anammox process has rapidly moved from lab-scale basic investigations to successful implementations in full-scale wastewater treatment systems, mainly for nitrogen removal from concentrated streams coming from anaerobic digesters. The most critical aspects in the process scaling-up are related, on the one hand, to the slow growth rate of Anammox bacteria, with doubling time as long as 14 days and, on the other hand, to their sensitivity to relatively moderate concentrations of their own substrate (nitrite, Strous *et al.* 1999); these aspects make the process potentially unstable and difficult to start-up. Simple monitoring techniques for the assessment of the specific Anammox activity are therefore needed to regulate the loading pattern thus avoiding potentially dangerous overloading conditions.

According to both stoichiometric reactions, the Anammox bacterial activity can be evaluated in batch tests by tracking:

- ammonium, nitrite and nitrate concentrations in time; this is a simple and conventional but time consuming method, requiring manual sampling and analysis;
- the acidity request to maintain the suspension pH at a constant level (pH-stat titration); this is a more

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automatised technique which however requires a controlling unit for the dosage of the acidic titrant (Remigi 2001); moreover, the stoichiometric relationship between the alkaline titration rate and the reaction rate is pH dependent (Ficara *et al.* 2002);

- the N_2 production rate;
- the heat power generation.

The last two options are here applied. It is worth noting that they do not need sampling nor dosing; therefore, external modifications of the reaction environment are minimized.

As for the first option, the N_2 production rate can be easily quantified by monitoring the pressure increase that occurs when the process takes place in closed bottles; this is feasible by using simple manometric devices. Manometric methods have a quite wide spectrum of potential applications since the majority of bioreactions that take place in the liquid phase implies the production/consumption of a poorly soluble gaseous species. Common applications include the heterotrophic aerobic degradation of organic matter (e.g. the assessment of the biological oxygen demand BOD) and anaerobic digestion (e.g. the assessment of biological methane potential, BMP). The applicability of this method to study the Anammox process was already proposed (Dapena-Mora *et al.* 2007) and its accuracy verified by comparing the N_2 gas production to the nitrogen consumption in the liquid phase (Bettazzi *et al.* submitted). A similar method was also applied to estimate the Anammox decaying constant (Scaglione *et al.* 2009).

As for calorimetric measurements, they are applicable to monitor all kinds of biological processes, since they measure the reaction entalphy (e.g. for nitrification is $359\,kJ/mol_{NH_4}$).

Very sophisticated instruments (named *micro*calorimeters) are required to achieve adequate sensitivities ($5 \div 10\,mW\,L^{-1}$) because biological reactions are characterized by much lower values and rates (Aulenta *et al.* 2002) compared to other chemical reactions (e.g. combustion).

When operating at constant temperature and pressure, the enthalpy variation is equal to the heat exchanged by the bioreaction, which is, in turns, mostly dependent on the catabolic activity, while the anabolic contribution is generally negligible (Aulenta *et al.* 2002). So far, biological microcalorimetry has been mostly applied to the studying of pure cultures, while very few studies are found in the literature concerning the use of microcalorimetry for biodegradation measurements in biological wastewater treatment processes (Buttiglieri *et al.* 2005; Bouju *et al.* 2008).

Microcalorimeters allow the heat power (Q_r, mW) generated by the bioreaction to be measured and recorded. This is related to the rate of substrate degradation by the following equation:

$$Q_r = Y_{q/s} \cdot \frac{dS}{dt} \cdot V_S \tag{3}$$

where:

S: is the substrate concentration ($mg\,L^{-1}$);
$Y_{q/s}$: is the energy generated for the unit of substrate degrade ($mJ\,mg^{-1}$),
V_S: is the volume of the reaction environment (L)

At constant temperature and pressure, the energy generated by the unit substrate degraded ($Y_{q/s}$) equals the specific reaction enthalpy (ΔH), therefore the latter can be calculated as follows:

$$\Delta H = \int_{t_0}^{t_f} Q_r \cdot dt \tag{4}$$

From Equations (3) and (4), it follows that:

$$\Delta H = \int_{t_0}^{t_f} Y_{q/s} \cdot \frac{dS}{dt} \cdot V_S \cdot dt = Y_{q/s} \cdot V_S \cdot \Delta S \tag{5}$$

When the metabolic Anammox reaction is considered, the overall expected enthalpy variation can be calculated according to the standard enthalpy of formation (Sawey *et al.* 2003), and it results $\Delta H_1 = -315.8\,J/mmol_{NH_4}$ by considering the stoichiometry proposed in Reaction (1). If the stoichiometry in Reaction (2) is considered, a ΔH_2 of $-321.4\,J/mmol_{NH_4}$ was estimated by assuming a standard enthalpy of formation of $-91\,kJ/mol$ for biomass, although this value refers to a slightly different biomass formulation ($CH_{1.8}O_{0.5}N_{0.2}$). Both values are quite similar to that of the nitrification reaction ($\Delta H = -359\,kJ/mol_{NH_4}$) for which microcalorimetry was already proven to be

applicable for activity measures (Daverio *et al.* 2003). Therefore, as soon as an Anammox culture is sufficiently enriched to achieve ammonium conversion rates (as $mgN\,L^{-1}h^{-1}$) of the same order of magnitudes of those that are typically recorded in nitrifying activated sludge samples, microcalorimetry is likely to be an applicable and effective monitoring method.

The present work aims to compare manometric and microcalorimetric techniques for Anammox activity measurements and to verify their applicability to monitor such slow-growing microorganisms.

MATERIALS AND METHODS

The anammox pilot plant reactor

Two Anammox-enriched suspended sludge samples (sample 1 and 2) were taken from a 40 liter-volume pilot-scale SBR (Sequencing Batch Reactor) operated according to a 24-hour cycle (Caffaz *et al.* 2008) The reactor was mixed and termostated at 35°C. It was fed with the effluent from a moving bed biofilm pilot-scale reactor operating the partial nitritation of the anaerobic supernatant coming from the Florence municipal waste water treatment plant (Italy). The average characteristics of the Anammox feed are summarized in Table 1. When sludge was sampled for activity measurements, the reactor loading rate was 0.044 kg N/kgSSV/d, its average hydraulic retention time was 8 d and the nitrogen removal efficiency was 99%. Sample 2 was taken 6 months after sample 1 (the reactor was running in stable operative conditions).

Manometry: apparatus and experimental procedure

Manometric determinations of the N_2 production rate were performed by means of the OxiTop® Control system (Figure 1). This is a manometric device consisting of a pressure transducer and data logger located inside a measuring head that is mounted on a glass bottle of 1,140 ml volume.

Table 1 | Characteristics of the Anammox bioreactor influent

NH_4^+-N [mg/l]	NO_2^--N [mg/l]	NO_2^--N/NH_4^+-N	COD [mg/l]
319 ± 51.4	311 ± 58.5	0.98 ± 0.12	444 ± 223

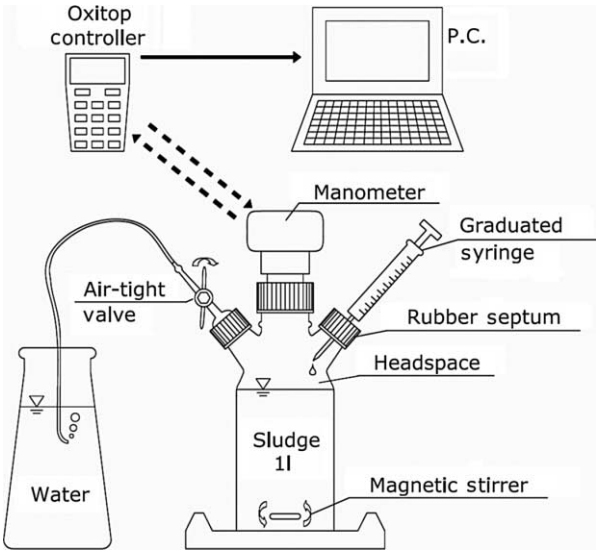

Figure 1 | Set-up of the manometric equipment for the assessment of the N_2 production rate.

The bottle has got two lateral holes; one is sealed by a rubber septum and is used for substrate injections, the second one is sealed by a teflon airtight valve and allows for biogas discharge. During tests, the overpressure (hPa) due to N_2 production was automatically registered by the measuring heads.

Manometric determinations of the Anammox activity were performed according to the following procedure. Each bottle was filled with 1,000 ml of sludge freshly taken from the Anammox pilot plant. The headspace was flushed with N_2 or helium gas to ensure anaerobic conditions. Bottles were located in a thermostated chamber at 35 ± 0.5°C and agitated by a magnetic mixer. After initial headspace pressure stabilisation, substrates were added by spike injections through the rubber septum. Then, the volume of N_2 cumulatively evolved (Nml) was calculated from over-pressure data by using the ideal gas law.

For each test, the maximum N_2 production rate ($mmolN_2/l/d$), the maximum specific Anammox activity, SAA_{max} ($mgN_2/gVSS/h$) and the nitrogen mass balance were verified.

Microcalorimetry: apparatus and experimental procedure

Microcalorimetric tests were performed by means of a 2 L Bio-RC1 microcalorimeter (Figure 2) developed by

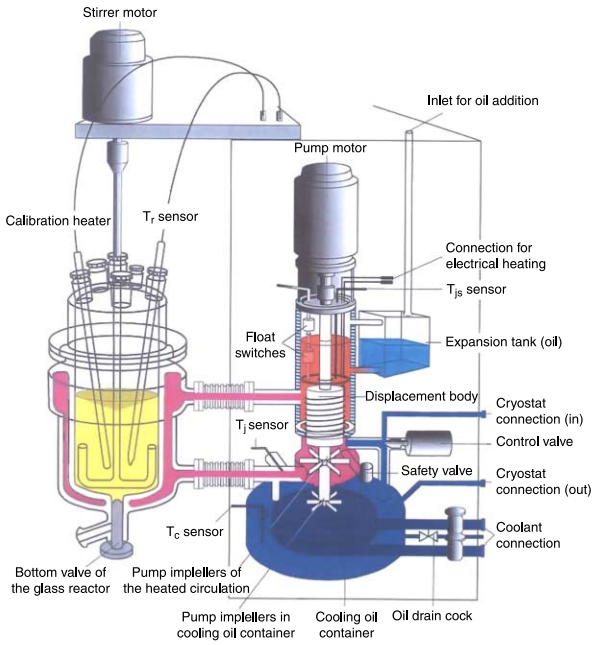

Figure 2 | Scheme of the microcalorimeter Bio-RC1 from Mettler-Toledo.

Mettler-Toledo (AG, Schwerzenbach, Switzerland). The reaction vessel is surrounded by a 2 cm thick Plexiglas box and featured by a thermostatic top-plate that plays a key role in limiting heat losses allowing the system to achieves a resolution of $5 \div 10\,\mathrm{mW\,L^{-1}}$.

In the isothermal mode, a low-viscosity silicone oil is pumped at a high rate ($2\,\mathrm{L\,s^{-1}}$) through the reactor jacket in order to maintain a constant temperature of the reaction medium (T_r) by adjusting the jacket temperature (T_j). The value of T_j is carefully controlled by blending oil from a hot and a cold oil circuit via an electronically controlled metering valve. When a process dissipates or takes up heat, T_j decreases or increases, respectively. Therefore, the temperature gradient through the side wall of the reaction vessel is proportional to the heat power (Q_r, mW) induced by the bioreaction that takes place inside, according to the following equation:

$$Q_r = U{\cdot}A{\cdot}(T_r - T_j) \tag{6}$$

where: U: is the overall heat transfer coefficient ($\mathrm{mW\,m^{-2}\,K^{-1}}$); A: is the heat transfer area, i.e. the surface of the reaction wall ($\mathrm{m^2}$); $(T_r - T_j)$: is the temperature gradient through the calorimeter side wall (K). The product $U{\cdot}A$ is assessed before each trial by means of a dedicated calibration test. Besides, pH and dissolved oxygen signals are also recorded with a high frequency ($3\,\mathrm{min^{-1}}$) during all experiments.

Tests for the assessment of the Anammox activity were performed according to the following procedure. A 2 L sludge sample was collected from the Anammox pilot-reactor and transferred to the lab and the test started after 2–3 days. When the sludge was poured into the reaction vessel, helium gas was flashed for approximately 30 min to strip the oxygen that had dissolved during sludge transfer. The test temperature was set at 35°C. Once the endogenous heat exchange stabilized at a baseline level, a known amount of nitrite was added to the sludge, while ammonium was kept at a non-limiting concentration. The heat power generation was then tracked until it went down to the previously measured baseline level.

Experimental conditions, sampling and analysis

For each sludge sample, manometric and calorimetric determinations of the Anammox activity were performed

Table 2 | Experimental conditions for manometric and calorimetric tests

Sludge sample		Manometric tests			Microcalometric tests		
Sampling time	SSV (g/L)	Test	Sludge volume (L)	NO_2 added (mgN/L)	Test	Sludge volume (L)	NO_2 added (mgN/L)
1	2	1	1	10	1a	2	10
					1b	2	5
2	1.7	2	1	10	2a	2	3.5
					2b	2	10
					2c	2	5
					2d	2	10

in parallel under similar substrates concentration. In Table 2, test conditions are summarized. For each sample, a single manometric test was performed while calorimetric tests were performed in 2 ÷ 4 replicates. Substrates were injected by using NH_4Cl, KNO_2 and KNO_3 (10 gN/l) concentrated solutions. NO_2/NH_4 ratio in the injection was equal to 1 in all the test, keeping ammonium in non-limiting conditions. At the beginning and at the end of each test the pH was measured (WTW probe) and was found to remain between 7.7 and 8.1. Concentrations of NH_4^+-N, NO_2^--N, NO_3^--N were measured by colorimetric methods in kit (Hach-Lange). Concentrations of TSS and VSS were measured according to Standard Methods (2005).

RESULTS AND DISCUSSION

As for manometric experiments, the typical trend of the cumulated gas production registered during a batch test is depicted in Figure 3. The feeding of 10 mg NH_4^+-N and 10 mg NO_2^--N was performed at the beginning of the test. An initial lag phase of approximately 20 min was observed in the N_2 production curve which was likely due to the initially limiting rate of N_2 transfer from the liquid to the gas phase. Then, a quite constant N_2 production was observed that continued for the following 2 h. A final N_2 production of 12.35 Nml was reached, which is very close to the stoichiometric value of 12.4 Nml. The maximum nitrogen production rate was estimated by linear regression on the gas production curve.

As for calorimetric tests, results from test 1 are reported in Figure 4. A clear heat response is evidenced, with limited

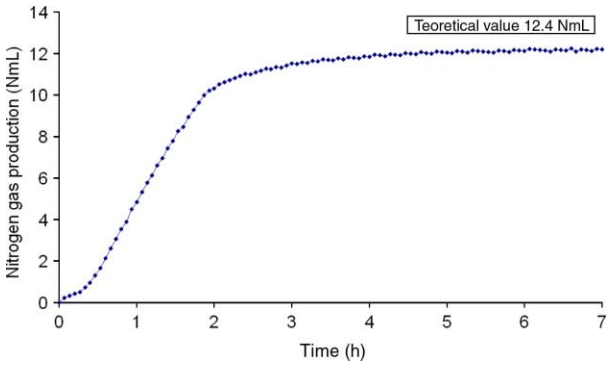

Figure 3 | Output of a manometric tests on Anammox biomass (test #1).

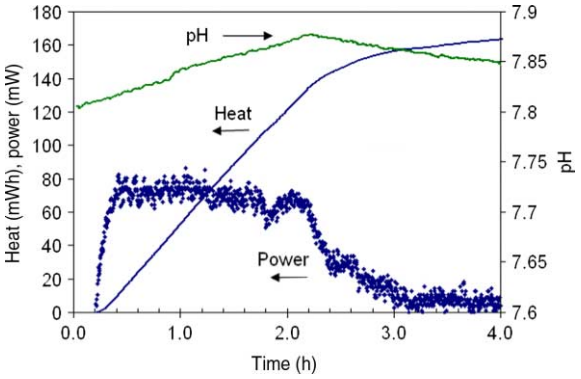

Figure 4 | Output of a microcalorimetric test on Anammox biomass (test #1a).

disturbance by signal noise. As a matter of fact, the minimum volumetric Anammox activity that would produce a distinct calorimetric response can be assessed by remembering the relationship expressed in Equation (3).

By assuming a $Y_{q/s} = \Delta H = 315.8\ \mathrm{J/mmol_{NH_4}} = 241\ \mathrm{J/mmol_{NO_2}}$, and a minimum resolution, $(Q_r/V_S)_{\min}$, of $10\ \mathrm{mW\,L^{-1}}$, then:

$$\left(\frac{dS}{dt}\right)_{\min} = \frac{1}{Y_{q/s}}\left(\frac{Q_r}{V_S}\right)_{\min} = \frac{1}{241}\times 10 \times \frac{3600}{1000}\times 14$$

$$= 2.2\,\frac{mgNO_2 - N}{L\cdot h}$$

This calculation evidences the importance of performing microcalorimetric tests with either sufficiently concentrated or sufficiently active sludge samples in order to work with a volumetric sludge activity higher than $(dS/dt)_{\min}$ and, thus, to get a clear heat production response. During this experimentation, sludge samples had a volumetric sludge activity higher than 4 mg NO_2^--N/L/h (calculated by the manometric test).

From Figure 4, it may be also noted that the first 20 min of measurement were disturbed by the slight cooling effect due to the injection of the concentrated nitrite solution. Later, the thermal power stabilized at its maximum (80 mW) along 0.5–3 h, depending on the amount of nitrite dosed; finally, the power decreased and reached its initial baseline value. It is also worth noting that as soon as heat production slowed down, the suspension pH had its maximum, well in accordance with the overall proton-request of the Anammox reaction (see Reaction 1).

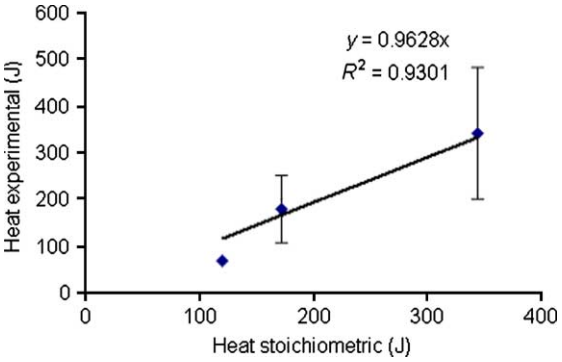

Figure 5 | Comparison between the heat exchanged and expected from the enthalpic variation.

It is now of interest to compare the cumulated heat production during the test, with the expected one, as calculated by multiplying the amount of nitrite added (being the test performed under nitrite limiting conditions) by the specific enthalpic variation (here assumed as the average value between ΔH_1 and ΔH_2, shown in Introduction). Note that the standard enthalpy variation is here considered and its correction with temperature according to the Kirchhoff law is neglected. In fact, no relevant variation in the heat capacity of reactants/products is expected from 25°C (standard temperature) to the operative temperature of 35°C. This comparison, presented in Figure 5, indicated a satisfactory agreement, since an average underestimation of the 4% between measured and expected value can be observed. However, further efforts should be devoted to limit the observed variability which is likely due to the complexity of the type of measure.

Finally, the SAA_{max} was calculated from the maximum heat production rate of the test by applying Equation (3). To allow the energy balance to be closed, the experimental enthalpic variation, calculated as the ratio between the experimental heat exchanged and the amount of substrate added, was used in this calculation. SAA_{max} estimates from calorimetric and manometric determination are presented in Table 3. Microcalorimetric results were found to be well reproducible.

Table 3 | Comparison between the maximum specific Anammox activity estimated by microcalorimetric and manometric tests

Sludge sample	From microcalorimetry SAA_{max} ($mgNO_2$-N/gSSV/h)	From manometry
1	3.39 ± 0.05	2.74
2	3.37 ± 0.54	2.58

Both methodologies gave very similar results for both sludge samples, however higher values (+23.7%) were obtained from microcalorimetric tests. This difference may be partially due to the low biomass activity which caused a difficult evaluation, in microcalorimetric test, of the endogenous heat exchange baseline level. However, more research is needed to fully clarify the origin of this difference, possibly testing biomasses with higher specific activity.

CONCLUSIONS

While manometry had already been proposed for the monitoring of the Anammox activity, this experimentation proved microcalorimetry to be an applicable alternative. Its resolution was sufficient to get a clear heat power response from the tested Anammox sludge samples, although they were drawn from a low loaded pilot plant.

The experimental heat exchanged was found to be averagely well comparable with what expected from the reaction enthalpy. Well reproducible estimates of the specific Anammox activity were obtained from microcalorimetric tests. However this activity was found to be slightly higher (+23.7% on average) that values obtained in manometric tests.

Although preliminary, these results are encouraging and further research will be devoted to elucidate differences on the observed SAA estimates.

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