Assessment of the endogenous respiration rate and the observed biomass yield for methanol-fed denitrifying bacteria under anoxic and aerobic conditions

Jamal Alikhani, Ahmed Al-Omari, Haydee De Clippeleir, Sudhir Murthy, Imre Takacs and Arash Massoudieh

ABSTRACT

In this study, the endogenous respiration rate and the observed biomass yield of denitrifying methylotrophic biomass were estimated through measuring changes in denitrification rates (DNR) as a result of maintaining the biomass under methanol deprived conditions. For this purpose, activated sludge biomass from a full-scale wastewater treatment plant was kept in 10-L batch reactors for 8 days under fully aerobic and anoxic conditions at 20 °C without methanol addition. To investigate temperature effects, another biomass sample was placed under starvation conditions over a period of 10 days under aerobic conditions at 25 °C. A series of secondary batch tests were conducted to measure DNR and observed biomass yields. The decline in DNR over the starvation period was used as a surrogate to biomass decay rate in order to infer the endogenous respiration rates of the methylotrophs. The regression analysis on the declining DNR data shows 95% confidence intervals of 0.130 ± 0.017 day^{-1} for endogenous respiration rate under aerobic conditions at 20 °C, 0.102 ± 0.013 day^{-1} under anoxic conditions at 20 °C, and 0.214 ± 0.044 day^{-1} under aerobic conditions at 25 °C. Results indicated that the endogenous respiration rate of methylotrophs is 20% slower under anoxic conditions than under aerobic conditions, and there is a significant temperature dependency, with an Arrhenius coefficient of 1.10. The observed biomass yield value showed an increasing trend from approximately 0.2 to 0.6 when the starvation time increased from 0 to 10 days.

Key words | anoxic/aerobic conditions, denitrification, denitrifying bacteria, endogenous respiration rate, methylotrophic biomass, observed biomass yield

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INTRODUCTION

Incorporating a biological nitrification/post-denitrification system into wastewater treatment plants (WWTPs) is one practical way to reduce the total nitrogen (TN) concentration in the effluent released into the surface water or the reclaimed water reuse network (Verstraete & Philips 1998; Khin & Annachhatre 2004; Lu *et al.* 2014). The post-denitrification process is carried out by heterotrophic bacteria that use nitrite/nitrate as electron acceptors under anoxic conditions, with an external carbon source – such as methanol, ethanol, or acetate – serving as an electron donor to compensate for the lack of organic carbon that has already been removed during the biochemical oxygen demand (BOD) removal stage. In the United States, methanol is widely used as an external carbon source, mainly due to its price and availability (Kristensen *et al.* 1992; Constantin & Fick 1997; Baytshtok *et al.* 2008; Mokhayeri *et al.* 2008; Lu *et al.* 2014; Rahman *et al.* 2016a).

When methanol is available as an electron donor over a long term, a new type of specialist heterotrophic bacteria is enriched in the activated sludge culture; these bacteria are collectively referred to as methylotrophs, and they only utilize methanol (Lu *et al.* 2014). Generalist heterotrophic bacteria, on the other hand, are capable of utilizing a broader spectrum of easily biodegradable substrates (Vasiliev & Vavilin 1991; Ginige *et al.* 2004; Baytshtok *et al.* 2008). From a modeling perspective, the stoichiometric and kinetic parameters of methylotrophs are different from those of the general heterotrophic cells considered in the

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widely used International Water Association (IWA) activated sludge models (ASMs), such as ASM1 and ASM3 (Henze *et al.* 1987, 2000). Although some studies have been performed to experimentally evaluate the specific growth rate and the yield coefficient of methylotrophs (Baytshtok *et al.* 2008; Dold *et al.* 2008; Mokhayeri *et al.* 2009), the endogenous respiration rate of methylotrophs under anoxic conditions has not been studied yet. Studies on the growth characteristics of methylotrophs have shown different bio-kinetic parameter values for methylotrophs compared to generalist heterotrophs. For example, the methylotrophs' yield on methanol is reported to be around 0.4 (mg chemical oxygen demand (COD) cell/mg COD methanol) (Baytshtok *et al.* 2008; Dold *et al.* 2008), whereas Henze *et al.* (2000) suggest a value of 0.54 for the growth of heterotrophic bacteria utilizing readily biodegradable COD under anoxic conditions. Mokhayeri *et al.* (2009) conducted a series of experimental batch tests and obtained a range of 0.8–1.3 day^{-1} for the maximum anoxic growth rate of methylotrophs. Sözen *et al.* (1998) measured a value of 1.8–3.4 day^{-1} for the anoxic growth of heterotrophs based on several wastewater samples.

Knowledge about the decay or endogenous respiration of methylotrophs is important for determining the minimum anoxic solids residence time (SRT) required to prevent methylotrophs' washout in low SRT systems, and more importantly for predicting the sludge production rate and the methanol consumption rate for biological methanol-feed nitrogen removal. A variety of microbiological mechanisms are involved in biomass decay pathways, including but not limited to maintenance, endogenous respiration, lysis, predation, and death (Van Loosdrecht & Henze 1999; Moussa *et al.* 2005; Manser *et al.* 2006). Considering each of these mechanisms separately in decay modeling is a complicated task, which results in over-parameterized models and possible non-identifiability of the parameters. For this reason, process modelers tend to use a simpler decay expression by lumping all the decay mechanisms together or by only considering those perceived to be the most important. For example, in the commonly used ASMs, ASM1 simply uses a single decay rate expression based on the lysis mechanism, while in ASM3 all the mechanisms in biomass loss are considered, but they are referred to as endogenous respiration (Henze *et al.* 2000).

The biomass yield coefficient indicates the amount of biomass growth relative to methanol consumption, and can be used to estimate the sludge production and methanol consumption rates. The total consumed methanol can be transferred to stored intracellular organic polymers, microbial growth, or energy production for microbial activity (Chen *et al.* 2008). Majone *et al.* (1998) performed storage response experiments in transient anoxic-aerobic denitrification rate (DNR) tests and measured the stored intercellular component – as well as nitrate, dissolved oxygen (DO), and acetate – and concluded that bacterial growth in transient conditions can start with the formation of intercellular polymers as stored compounds. After the available substrate runs out, the biomass begins using stored compounds to grow. In the storage and growth phases under anoxic conditions, denitrification provides the energy. The most common type of intercellular molecule is poly-hydroxy-alkanoates (PHAs) (Majone *et al.* 1998; Coats *et al.* 2011), which can be formed from different hydroxyl groups as well as methanol (Höfer *et al.* 2011). Methanol has been shown to be a proper substrate for the production of poly-hydroxy-butyrate in methylotrophic biomass (Yezza *et al.* 2006), which captures the interest of industrial bio-polymer production (Mokhtari-Hosseini *et al.* 2009; Khosravi-Darani *et al.* 2013).

The term 'observed biomass yield' refers to the total amount of consumed methanol regardless of whether it is stored or converted into biomass. In the denitrification process, it is straightforward to calculate the observed biomass yield, because the portion of the methanol that is utilized to generate energy can be inferred from the amount of nitrate consumed as electron acceptors. Chen *et al.* (2008) showed that both SRT and the endogenous respiration rate affect the observed biomass yield. They also showed that the inverse of the observed yield has a positive correlation with the system's SRT due to the physiological adaptation of bacteria to the SRT conditions. Friedrich *et al.* (2015) showed that physiological adaption of bacteria can significantly influence the growth and decay kinetics of the bacteria in general. Their investigation on the sludge taken from different plants with different SRTs (from 11 to 70 days) showed that the maximum growth and decay rates of sludge were higher in lower SRT systems. Physiological adaptation of methylotrophs to changes in SRT can occur at different electron acceptor conditions. Understanding this adaptation phenomenon can facilitate the identification of optimum SRTs and hydraulic retention times (HRTs) of nitrification-denitrification reactors, potentially resulting in lower methanol consumption.

One of the main cost factors in post-denitrification treatment systems is associated with the external carbon source; this can significantly contribute to the overall unit cost of the treatment process, and a small reduction in external carbon source loading can translate into significant cost savings.

More accurate quantitative understanding of the processes in the methanol-fed denitrification system can provide better ways to minimize the overall methanol loading rate while keeping the TN in the effluent below the regulatory required threshold. The goal of this study is to advance quantitative knowledge about the endogenous respiration rate and the observed biomass yield of methylotrophic bacteria by conducting laboratory experiments. Data analysis is performed based on the ASM3 model of the activated sludge process.

MATERIALS AND METHODS

Sludge preparation

Activated sludge samples were taken from nitrification-denitrification bioreactors at the Blue Plains Advanced WWTP in Washington, DC. At Blue Plains, the nitrification-denitrification phase with methanol addition as the external carbon source follows the COD removal stage, in which the SRT of the sludge in the system is on average 20 days, and the HRT of wastewater in the reactors is approximately 3.6 hours. A 50-liter sample of mixed liquor was taken from the nitrification stage, during which methanol was not present, and was allowed to settle. After the settling stage, the supernatant was extracted and the settled biomass was collected and divided into two 10-L reactors with an initial total suspended solids (TSS) and volatile suspended solids (VSS) of 6,300 mg/L and 5,150 mg/L, respectively, for the first run, and a TSS and VSS of 9,200 mg/L and 7,600 mg/L, respectively, for the second run. Each reactor was equipped with an impeller mixer, a digital pH meter, a thermometer, a DO meter, and an aquarium air-stone to diffuse air and nitrogen gas into the reactors. The reactors were placed in an incubator to maintain a temperature of 20 °C and 25 °C for the first and second runs, respectively. During the first run, one reactor was kept under aerobic conditions using the air-stone and pump, and the other one was maintained under anoxic conditions using nitrogen gas diffusion, both for a period of 8 days. For the second run, only one aerobic reactor was maintained at 25 °C for 10 days to evaluate the role of temperature in the endogenous respiration rate. As for the anoxic reactor, the nitrate concentration was manually maintained between 50–150 mg NO_3-N/L throughout the experiment period to prevent anaerobic conditions. To avoid pH reduction in the aerobic reactor due to nitrification, and pH increase in the anoxic reactor due to denitrification, an automatic pH controller was installed in each decay reactor to dose a solution of sodium bicarbonate ($NaHCO_3$) into the aerobic tank and diluted sulfuric acid (H_2SO_4) into the anoxic tank to maintain the pH in the range of 6.8–7.1. These two reactors are referred to as 'decay reactors' hereafter.

Batch experiment: DNR test

To perform the DNR test, a 500-ml mixed liquor sample was taken daily from each decay reactor, divided into two 250-ml portions, and placed into two (replicated) 2-L beakers, which served as batch test reactors. 750 ml of de-chlorinated tap water was added to each beaker to dilute the sludge. These reactors are referred to hereafter as 'batch DNR reactors'. Anoxic conditions were maintained in each DNR reactor by using nitrogen gas bubbling. A phosphate buffer ($H_2PO_4^- + HPO_4^{2-}$) was added to the DNR reactors to keep the pH in the range of 6.9–7.1 during the activity test. The methanol concentration was adjusted to approximately four to five times the nitrate concentrations to be stoichiometrically adequate for the denitrification reaction. Samples were collected from the DNR reactors during a 2-hour period at 30-minute intervals, and were immediately filtered using a 0.45 μm filter to separate the solids from the solution. Afterward, the nitrate, nitrite, ammonia, and soluble COD concentrations of the filtered sample were measured, the results of which are shown in Tables A1–A3 of the supplementary Appendix (available with the online version of this paper). For all the batch DNR tests (first and second runs), the COD concentration of each sample was measured by using Hach's COD digestion vials (Hach Co., Loveland, CO). These vials are commonly used in the WWTP's laboratories. However, to validate the COD values in the second run, in addition to the Hach's COD vials, methanol concentrations were directly measured by injecting samples into a gas-chromatography machine. A calibration line was already obtained by preparing standard samples in the range of 1 to 1,000 mg-COD/L. The results for the COD and methanol concentrations for the second run are shown in Table A3. It can be seen that the values obtained from the COD vials are slightly higher than those from the pure methanol concentrations due to the presence of other sources of COD in the samples, mainly from non-biodegradable organic matter. Nevertheless, the consumption rate of total COD and methanol is expected to be the same. Results obtained in the second run validate the results obtained by Hach's COD vials.

Henze *et al.* (2000) reported a value of 0.01 mg-N/L for the saturation coefficient for ammonia as a nutrient source for heterotrophic organisms. Alikhani *et al.* (2014) estimated

a value of 0.04 mg-N/L with a standard deviation of 0.012 mg-N/L for the ammonia saturation coefficient for methylotrophs based on data collected from the nitrification-denitrification system at Blue Plains WWTP (the same plant as used in this study). Based on this prior knowledge about the value of the ammonium saturation coefficient, before starting a batch DNR test, the ammonia concentrations were maintained above 0.1 mg-N/L to avoid ammonia limitation in the DNR tests. Overall, 32 DNR tests for the first run and 12 DNR tests for the second run were conducted.

Estimation of the endogenous respiration rate

The growth of methylotrophs is completely prevented under starvation conditions without methanol feeding into the decay reactors. Therefore, the only factor affecting the methylotrophs' biomass is loss due to decay. The endogenous respiration of methylotrophs in the decay reactors can be represented using a first order decay reaction:

$$\frac{dX_{B,M}(t)}{dt} = -\mathrm{b_M}.X_{B,M}(t) \tag{1}$$

where t is the starvation period time in the decay reactors, $X_{B,M}(t)$ denotes the methylotrophic biomass concentration as a function of decay time, and $\mathrm{b_M}$ is the endogenous respiration rate of methylotrophic biomass. Rearranging Equation (1) results in the following form of decay expression shown in Equation (2), allowing for estimation of the endogenous respiration rate as the slope of the natural logarithm of biomass with respect to time:

$$\frac{d\ln(X_{B,M}(t))}{dt} = -\mathrm{b_M} \tag{2}$$

which can be integrated to:

$$\ln\left(\frac{\mathrm{X_{B,M}}(t)}{\mathrm{X_{B,M}}(0)}\right) = -\mathrm{b_M}.\mathrm{t} \tag{3}$$

In practice, it is not feasible to directly measure the methylotrophic biomass concentrations of $\mathrm{X_{B,M}}(t)$ and $\mathrm{X_{B,M}}(0)$ in the decay reactors due to the presence of other types of biomass in the mixed liquor culture – such as autotrophs and heterotrophs – as well as the residue debris of dead biomass. This prevents the direct use of Equation (2) and/or Equation (3) to estimate $\mathrm{b_M}$. Nevertheless, it is possible to conceptualize that the loss of active methylotrophic biomass can decrease the DNR, which can be directly estimated by measuring the nitrate uptake rate (NUR). By applying the maximum growth of methylotrophs under fully anoxic conditions with high enough nitrate and methanol concentrations, the change in the concentration of nitrate over time can be defined as:

$$a\frac{dS_{NO}}{d\tau} = -\,\mu_{\mathrm{M}}.X_{B,M}(t) \tag{4}$$

where μ_{M} is the maximum growth rate coefficient of methylotrophs that can be assumed to stay constant during the DNR test, $a = (1 - Y_M)/2.86\,Y_M$ is the nitrate stoichiometry coefficient in the denitrification reaction, τ denotes the denitrification reaction time in the DNR test (which is different from the time scale in the decay reactor, shown as t), and $dS_{NO}/d\tau$ is NUR in the DNR test. If the change in methylotrophic biomass growth concentration ($\Delta\mathrm{COD_{XB,M}}$) relative to the change in methanol concentration ($\Delta\mathrm{COD_{methanol}}$) is assumed to be negligible, then DNR can be shown as a zero-order reaction:

$$DNR(t) = \frac{\mu_{\mathrm{M}}.X_{B,M}(t)}{a} \tag{5}$$

where $DNR(t)$ shows the DNR obtained from the DNR test reactor at each starvation time t, and $X_{B,M}(t)$ represents the active methylotrophic biomass concentration at the time of sampling from the decay reactors. Taking the derivative of both sides of Equation (5) after logarithmic transformation, while assuming a constant ratio of μ_M/a with respect to time t, yields:

$$\frac{d\ln(DNR(t))}{dt} = \frac{d\ln(X_{B,M}(t))}{dt} \tag{6}$$

where the right-hand side is equal to Equation (2), showing that the endogenous respiration rate ($\mathrm{b_M}$) can be obtained by a log-linear regression model on the obtained DNRs. The integrated form of DNR depletion rate can be represented as:

$$\ln\left(\frac{DNR(t)}{DNR(0)}\right) = -\mathrm{b_M}.\mathrm{t} \tag{7}$$

where the value of DNRs are measurable and can be used as a surrogate for biomass decay rate.

Observed biomass yield coefficient

The observed biomass yield coefficient of methylotrophs in each DNR test reactor can be obtained using Equation (8) (Dold *et al.* 2008; Rahman *et al.* 2016b):

$$Y_m^{obs}(t) = 1 - \frac{2.86}{dCOD/dN(t)} \quad (8)$$

where $Y_m^{obs}(t)$ is the observed biomass yield of methylotrophs as a function of decay time, and $dCOD/dN(t)$ represents the COD to nitrogen ratio, shown as scattered symbols in Figure 1. In Equation (8), the coefficient 2.86 is the electron equivalence of the NO_3^- to N_2 in the denitrification process step.

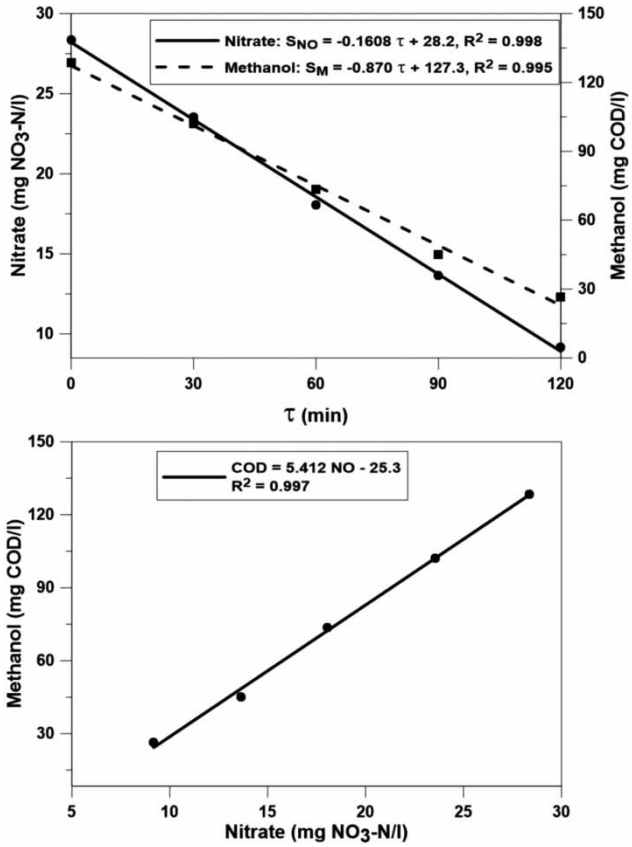

Figure 1 | Results of the 2-hour DNR test on day 2 of the first run for the sludge taken from the aerobic decay reactor with a VSS of around 1,300 mg/L. (Top) Nitrate and methanol uptake rates, where the time axis is the same as in Equation (4). The NUR in this particular DNR test is 0.1608 mg NO_3-N/L/min. (Bottom) The ratio of COD/N, which is 5.412 in this particular test, resulting in the observed biomass yield coefficient of 0.47 by applying Equation (8).

RESULTS AND DISCUSSION

Effect of starvation time on DNR

The results of the nitrate and methanol uptake rates, as well as the COD to nitrogen ratio (COD/N), on the second day of the first run for the sludge taken from aerobic conditions, are shown in Figure 1 as an example. For each DNR test, the DNR was obtained by performing a linear regression on the measured nitrate profile over time (Baytshtok *et al.* 2008; Dold *et al.* 2008). The DNR values rather than specific DNR were used to estimate the decay of methylotrophic biomass in order to avoid errors associated with the presence of other microorganisms, such as general heterotrophs, autotrophic nitrifiers, and dead biomass residue in the total VSS concentrations. The total VSS values of the DNR tests were around 1,300 mg/L and 1,900 mg/L for the first and second run, respectively. COD to nitrogen ratios (COD/N) were also estimated based on the slope of methanol (mg COD/L) versus nitrate (mg-N/L) concentrations, which can be further used to obtain the biomass yield coefficient for each test. As shown in the Appendix (supplementary data, available with the online version of this paper), the measured concentrations of nitrite for all the batch DNR tests were found to be less than 0.01 mg NO_2-N/L, and the measured concentrations of ammonia showed no statistically significant trend in any of the DNR tests.

The average values of DNR for the first and second runs are reported in the Appendix. A clear reducing trend of DNR with respect to starvation time is observed. Contrary to expectations, the DNR values show an increase after the first day (day one in comparison with day zero) in the first run. This did not happen in the second run. The first DNR point (day zero) shown in Table A1 is believed to be inaccurate and an artifact of either measurement or unconsidered temperature effects; therefore, this point was omitted from the regression analysis to avoid error. As shown in Table A1, for the sludge that was kept under aerobic conditions at 20 °C, DNR dropped from 246 to 113 mg NO_3-N L^{-1} d^{-1} over 7 days of starvation, showing roughly 54% reduction. For the sludge kept under anoxic conditions (Table A2) this reduction is from 258 to 144 mg NO_3-N L^{-1} d^{-1}, which constitute a 44% reduction during 7 days of starvation. The amount of DNR reduction for the 9 days of starvation for the sludge kept under aerobic conditions at 25 °C (Table A3) was significantly higher (85%): DNR dropped from 601 to 89 NO_3-N L^{-1} d^{-1}. DNR depletion is deemed to be related to the decay of methylotrophic biomass under starvation conditions. This decay can

be a result of maintenance, endogenous respiration, lysis, predation, death, or a combination of these.

Effect of aerobic-anoxic conditions and temperature on the endogenous respiration rate

The results of the DNR depletion profile under aerobic and anoxic conditions in the decay reactors and the two temperatures for the first and second run tests are shown in Figure 2, in which the y-axis shows the natural logarithm of DNR, and the x-axis shows the sludge decay time or the duration of starvation conditions in the decay reactors. Based on Equation (7), the methylotroph endogenous respiration rate (b_M) under aerobic and anoxic conditions is estimated from the slope of the linear regression between *ln*(DNR) and t. For the endogenous respiration rate coefficients of methylotrophic bacteria at 20 °C, the 95% confidence intervals of $0.130 \pm 0.017\ day^{-1}$ and $0.102 \pm 0.013\ day^{-1}$ are obtained under aerobic and anoxic conditions, respectively. As is seen from the results, the methylotrophic endogenous respiration rate under anoxic conditions is slower than under aerobic conditions. To test the statistical significance of the difference between the endogenous respiration rates under aerobic and anoxic conditions, a hypothesis test was performed by considering a null hypothesis of the slope (with negative value) of linear regression under anoxic conditions being smaller than or equal to the slope under aerobic conditions; as shown in Table A4 of the supplementary Appendix (available with the online version of this paper), ANOVA resulted in a *p*-value of 1.23×10^{-5}, which led to a rejection of the hypothesis, meaning that the difference is statistically significant. Under aerobic decay conditions at 25 °C, a 95% confidence interval of $0.214 \pm 0.044\ day^{-1}$ is obtained for the methylotrophic endogenous respiration rate, showing a significant increase as a result of the 5 °C increase in temperature. It was assumed that the temperature dependency of the methylotrophic endogenous respiration rate is independent of anoxic/aerobic conditions.

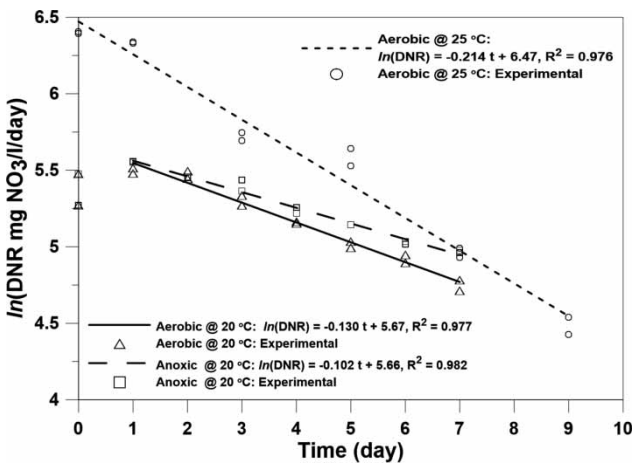

Figure 2 | DNR reduction profile under aerobic and anoxic starvation conditions in the decay reactors at 20 °C for the first run, and under aerobic starvation conditions at 25 °C for the second run. The initial biomass VSS was 1,300 mg/L and 1,900 mg/L in each DNR test for the first and second run, respectively. Scatter symbols show duplicate DNR results for each DNR test. The coefficient of *t* in the linear regression model shows the value of the endogenous respiration rate.

If the temperature dependence of b_M is defined based on the simplified version of the Arrhenius relationship: $b_M(T) = b_M(20)\theta^{(T-20)}$, then by comparing the endogenous respiration rates from the first and second run, a θ factor equal to 1.10 can be calculated for the b_M. However, it should be noted that obtaining a reliable estimate of the θ for the process requires further studies under a wider range of temperatures. Dold *et al.* (2008) also observed a strong temperature dependency for the maximum growth rate of methylotrophs with an Arrhenius coefficient of 1.13 when they conducted 22 DNR tests in the range of 10–25 °C. Henze *et al.* (2000) reported the maximum growth rate (μ_H) and endogenous respiration rate (b_H) of general heterotrophs values in ASM3 in the range of 10–20 °C, which denotes a temperature dependency of around 1.072. Koch *et al.* (2000) applied a value of 1.07 for both the maximum growth and decay rates of heterotrophs in the calibration of ASM3. The experimental findings in this study and in that of Dold *et al.* (2008) show higher temperature sensitivity of methylotrophs compared to general heterotrophs, implying that the temperature dependency coefficients must be applied with greater caution in the modeling and design of the denitrification system, with methanol as an external carbon source.

The value of the endogenous respiration rate for methylotrophs obtained in this study is not far from the decay rate coefficients of heterotrophic bacteria reported in other references. Based on an experimental study, McClintock *et al.* (1988) found decay rates of $0.111\ day^{-1}$ and $0.057\ day^{-1}$ for heterotrophs under aerobic and anoxic conditions, respectively. In ASM3, values of $0.2\ day^{-1}$ and $0.1\ day^{-1}$ (at 20 °C) have been suggested for the endogenous respiration rates of heterotrophs on oxygen and nitrate, respectively (Henze *et al.* 2000). Other studies have also experimentally shown that the anoxic endogenous respiration rate or decay rate for heterotrophic biomass in activated sludge is slower than that seen under aerobic conditions, mainly due to the fact that some of the decay mechanisms that can occur under aerobic conditions are

not effective under anoxic conditions (e.g., Siegrist *et al.* (1999) and McClintock *et al.* (1988)). For instance, the predation process by higher organisms in biomass decay is expected to be significantly reduced under anoxic conditions (Henze *et al.* 2000). Based on this understanding, Gernaey *et al.* (2004) suggest that controlling the excess aeration – besides reducing energy costs — can curb the decay of activated sludge and thus improve the nitrification-denitrification process.

An expression for endogenous respiration

In activated sludge modeling, ASM1 uses the death-regeneration approach, whereas ASM3 considers the endogenous respiration concept for biomass decay (Gernaey *et al.* 2004). However, the death-regeneration mechanism does not apply to methylotrophs because they do not regrow on multi-carbon decay products (Lu *et al.* 2014). Aerobic and anoxic conditions can significantly affect the decay process, particularly in the modeling of the nitrification-denitrification systems, in which the ratio of anoxic to aerobic zone is considerable (Henze *et al.* 2000). Following ASM3's endogenous respiration approach, a new expression for methylotrophic decay rate adjustable to both aerobic and anoxic conditions is proposed in Equation (9) (Alikhani *et al.* 2014):

$$\frac{dX_{B,M}}{dt} = -b_M\left(\frac{S_O}{K_{m,O}+S_O} + \eta_m \frac{K_{m,O}}{K_{m,O}+S_O}\frac{S_{NO}}{K_{m,NO}+S_{NO}}\right)X_{B,M} \quad (9)$$

where S_O and S_{NO} are DO and nitrate concentration, respectively, and $K_{m,O}$ and $K_{m,NO}$ are oxygen and nitrate half saturations, respectively. Combining both the aerobic and anoxic endogenous respiration in one single equation is based on the fact that in aerobic conditions, $S_O/(K_{m,O}+S_O)$ approaches 1 and the inhibitor ratio of $K_{m,O}/(K_{m,O}+S_O)$ approaches 0, and vice versa under anoxic conditions. The term $S_{NO}/(K_{m,NO}+S_{NO})$ represents the nitrate limitation effect. In Equation (9), the new parameter η_m (smaller than 1) is referred to as the anoxic to aerobic ratio of methylotroph endogenous respiration rate, for which a value of $0.10/0.13 \cong 0.77$ was obtained in this study based on the results of the first run. This indicates that endogenous respiration occurs at a slower rate under anoxic conditions than under aerobic conditions. It is worth noting that the value of η_m obtained here is close to the values suggested for the anoxic reduction factor η_h in ASM1, representing the ratio of growth rate of heterotrophic bacteria under anoxic conditions to the growth rate under aerobic conditions. The value of 0.8 is suggested for η_h by Henze *et al.* (1987), following the value obtained by Makinia & Wells (2000).

Effect of starvation time on the observed biomass yield coefficient

The observed biomass yield obtained through Equation (8) in each DNR test with respect to the starvation time is plotted in Figure 3. In all the cases of aerobic and anoxic decay tests at 20 °C as well as aerobic decay tests at 25 °C, a clearly increasing trend in the observed biomass yield coefficient with the starvation time can be observed (p-value = 0.0032). Yield increase as a result of the age of activated sludge was also reported by Liebeskind *et al.* (1996), who saw an increase from 0.59 to 0.68 for heterotrophic bacteria when the age of activated sludge was increased from 1.88 days to 24 days.

Based on the average flow, the methanol loading rate, and the concentration of ammonia and nitrate/nitrite in the influent and effluent of Blue Plain's nitrification-denitrification system, an average value of 0.39 is obtained for the observed yield of the plant over methanol. By comparing with Figure 3, it seems that Blue Plains' nitrification-denitrification system is not operating under starvation conditions.

In the methanol-feed denitrification system, methanol is used as a substrate for stored intercellular polymers, biomass production, and energy source. The energy production portion of methanol depletion can be directly linked to NUR. In Equation (8), the observed biomass yield is defined as the ratio of generated solid mass (either intercellular

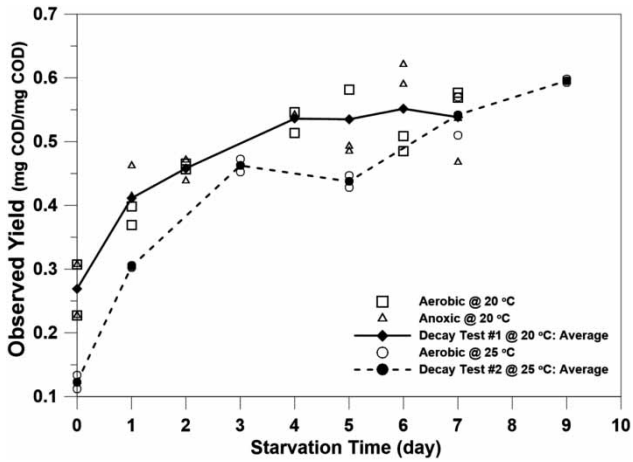

Figure 3 | The observed biomass yield coefficients during 2-hour DNR test with respect to starvation-induced time. The solid and dashed lines connect the average observed biomass yield for the first and second run, respectively.

polymers or biomass cells) relative to the consumed methanol. Specifically, the observed biomass yield encompasses two different factors including the stored yield (Y_{STO}) and growth yield (Y_m), that must be taken into consideration. As has been mentioned by other researchers, when heterotrophic cells are suddenly exposed to high substrate levels, two types of responses are perceivable: the cells can either instantaneously adapt to the new abundant food condition by increasing their growth rate (growth response), or they can initially store the available substrate (storage response) followed by delayed growth or a combination of both responses (Daigger & Grady 1982; Majone *et al.* 1998). It is speculated that the reason for the change in the observed biomass yield is that the longer methylotrophs are kept in the starvation conditions, the more methanol will be stored in their cells after being exposed to methanol. However, further studies are needed to rigorously evaluate this conclusion through directly measuring PHAs in the cells.

Under methanol-abundant conditions, the uptake rate may be faster than the consumption rate, resulting in an increase in storage, whereas during methanol-deprived conditions, storage decreases (Ni & Yu 2008; Kaelin *et al.* 2009). Majone *et al.* (1998) studied the behavior of generalist heterotrophs supplied with acetate as a carbon source in an alternate anoxic/aerobic conditions, and observed that intercellular PHAs can be consumed in the anoxic and aerobic conditions with almost the same rate of PHA consumption. Therefore, it can be concluded that in sequential nitrification-denitrification systems, if the stored PHAs are not completely consumed during the denitrification stage under anoxic conditions, they can be consumed in the nitrification stage under aerobic conditions after the sludge is returned, resulting in an ineffective utilization of methanol.

CONCLUSION

In this study, the denitrifying methylotrophic biomass from a full-scale WWTP was placed under conditions with no methanol addition under three different scenarios: (a) anoxic conditions with nitrate adjustment at 20 °C for 8 days, (b) aerobic conditions at 20 °C for 8 days, and (c) aerobic conditions at 25 °C for 10 days. A series of secondary batch tests over the period of operation of decay reactors were performed to measure DNR and observed biomass yields. The DNR reduction rate during the operation time of the decay reactors was then used to infer the endogenous respiration rates of the biomass in the decay reactors. The following results were obtained:

(1) A 95% confidence interval of $0.130 \pm 0.017\ day^{-1}$ and $0.102 \pm 0.013\ day^{-1}$ was estimated for the endogenous respiration rate coefficients of methylotrophic bacteria at 20 °C under aerobic and anoxic conditions, respectively. The anoxic endogenous respiration rate of methylotrophs was 20% lower than the aerobic endogenous respiration rate.
(2) A significant temperature dependency of the endogenous respiration rate of methylotrophs was observed. Specifically, a mean value of $0.214\ day^{-1}$ was obtained for the methylotrophic endogenous respiration rate under aerobic decay conditions at 25 °C compared to $0.130\ day^{-1}$ under 20 °C, indicating an Arrhenius coefficient of 1.1 based on the simplified version of the Arrhenius equation.
(3) The observed biomass yield coefficient of methylotrophs is increased as a result of a longer starvation time.

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