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Evaluation of Anoxic Heterotrophic Yield Using Multiple Calculation Methods

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ABSTRACT: The stoichiometric parameter namely "Yield Coefficient" associated with growth of certain organism can be determined by direct measurement and/or calculated indirectly. The focus of this paper was on the anoxic yield of heterotrophic organisms using ethanol as an external carbon source during the denitrification process. In the literature, it was observed that yield coefficients can vary for the same substrate, which can be referred to the relative acclimation to the substrate. The aim of this study was to evaluate the yields determined through various catabolic and anabolic estimations. This paper presents ten different yield coefficient calculation methods under anoxic conditions in a sequencing batch reactor using ethanol as an external substrate. The range of anoxic yield using different calculation methods was between 0.423±0.014 to 0.512±0.021 mgCOD/mgCOD at 20°C. It was concluded that there was no statistically significant difference between the yield values calculated from the different methods. Depending of what parameters can be measured correctly for a particular experiment or setup, a particular method can be selected using those parameters to calculate the yield.

Key words: Carbon to nitrate ratio, denitrification, nitrogen, specific denitrification rate, ANOVA

INTRODUCTION

The true yield coefficient (Y), associated with biomass synthesis, is an important stoichiometric parameter for calculating mass balances of biological reactions. For denitrifying heterotrophic microorganisms, the true anoxic yield (Y_{anox}) is important to determine mass of organic carbon required to remove nitrogen and the sludge production. More accurate estimation of the anoxic yield is beneficial to minimize the need for inflated safety factors resulting in smaller reactor volume.

The literature contains several studies using direct and indirect methods to measure Y_{anox} using different carbon sources. Copp and Dold (1998) designed an experimental method with two independent ways to calculate Y_{anox} (slope of particulate chemical oxygen demand, pCOD increase versus (vs). soluble chemical oxygen demand, sCOD reduction and slope of pCOD increase vs. nitrate reduction) for batch tests under anoxic conditions. However, the method has some assumptions that may impact the accuracy of determining the yield value. For example, the electron acceptor and electron donor must be sufficient for heterotrophic populations for small period of time interval. This will avoid endogenous decay and ensure maximum biomass growth rate. It also considered that consumption of soluble COD by microorganisms can only be utilized either to produce new cell or cell synthesis and thus assumed that there is no releases of soluble COD through metabolism of byproduct. In some studies Y_{anox} was calculated using only carbon to nitrate ratio (C/N) without considering nitrite production (Dold et al., 2008). Anoxic yield coefficient was also reported using the nitrate reduction, nitrite net reductions and consumption of sCOD considering a two-step denitrification process (Mokhayeri et al., 2010). McCarty et al. (1969) developed two stoichiometric equations (biomass production and methanol concentration requirement) on weight basis to measure biomass production and methanol

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requirement for denitrification. The ratio of the two equations gives yield value for methanol utilizing heterotrophs. According to Koike and Hattori (1975), a bath culture method was performed to measure *P. denitrificans* growth rate. Yield was determined by extinctions of regular interval at 660 nm (E_{660}) using a Spectronic 20 spectrophotometer (Bausch and Lomb). It was expressed in terms of E_{660} which represents dry weight/L, irrespective of substrates and culture conditions. Other studies reported yield coefficients that were estimated by direct laboratory measurements of volatile suspended solids produced per unit COD consumed (Hallin et al., 1996) and total suspended solids produced per unit COD consumed (Christensson et al. 1994). Muller at al. (2004) adopted a research approach to quantify the heterotrophic yield by equating aerobic and anoxic respirometries. The assumption was to consider the same soluble substrate concentrations utilized for aerobic and anoxic conditions. The readily biodegradable COD was eliminated as variable by equating those two equations to determine anoxic yield as a function of aerobic yield. In this case, aerobic yield has to be known for estimation of anoxic yield. Thermodynamic models (dissipation and energy approach) were also used to predict bacterial yields by developing stoichiometric reactions in the absence of empirical data. However, the high degree of variability in the behavior of different organisms growing on the same substrate and due to effect of environmental conditions on yield requires consideration for thermodynamic approaches (VanBriesen, 2002). Investigation of anoxic yield for different substrates (ethanol and acetate) was performed by Mokhayeri et al. (2008, 2010) and authors observed that the yield was variable (0.59 and 0.61 gCOD/gCOD, respectively). Earlier experiments were conducted by McCarty et al. (1969) at 20°C for ethanol, producing a yield of 0.513 gCOD/gCOD. These variations of yield coefficients were the motivation for this research to find an accurate yield value.

The objectives of this study were to: a) investigate the influence of acclimation of organisms from an activated sludge plant acclimated to wastewater, on yields for a single substrate (ethanol), b) evaluate the yields determined through various catabolic and anabolic estimations and develop appropriate balances. This paper investigated multiple calculation methods adopted from fundamental yield definition to compare those methods as best selective option for yield measurement.

MATERIALS & METHODS

Two 10 L sequencing batch reactors (SBRs) were operated in this study. The SBRs were seeded with mixed liquor suspended solids (MLSS) from a nutrient removing full-scale wastewater treatment plant (Piscataway Wastewater Treatment Plant, Accokeek, Maryland). The reactor was maintained at 20 °C temperature in an incubator and was operated for 24 h per cycle. The cycle included feeding 1 L of a mix of synthetic wastewater and ethanol, 23 h anoxic phase and 1 h reaeration phase. The purpose of using ethanol was that it considered as an interesting alternative carbon to the more commonly used methanol due its economy and process flexibility (Yong-zhen et al., 2007). The reactor was fed over a period of 1 h during anoxic cycle. The reactor was covered by a Styrofoam lid to reduce any oxygen intrusion. Influent feed composition (tap water, KNO_3, NH_4Cl, KH_2PO_4 and ethanol) was characterized to obtain 125 mgN/L nitrate, 15 mgN/L ammonia, 5 mgP/L ortho-phosphate and 500 mg/L sCOD in the SBR as initial concentration. Additional micronutrients were added to the feed solution. A mixture of mineral base stock solution was prepared for feed solution. The composition of mineral base consisted of 0.25 g $CoCl_2.6H_2O$, 4 g $FeCl_2.4H_2O$, 0.05 g $MnCl_2.4H_2O$, 0.025 g H_3BO_3, 0.025 g $ZnCl_2$, 0.005 g $NaMoO_4.2H_2O$, 0.025 g $NiCl_2.6H_2O$, 0.025 g Na_2SeO_4 and 0.007 g $CuCl_2.2H_2O$, respectively. A 2.8 gram of mineral base powder was added into the 1 L of de-ionized water to make concentrated nutrient stock solution. The formula used to make desired volume of mineral stock solution was: "add 2 mL mineral base stock solution to each liter diluted wastewater per 1000 mg/L COD (For example, for 1,000 mgCOD/L and 250 mL bottles, use 0.5 mL of each solution)". Therefore, 10 mL of mineral base stock solution was added in the 1 L synthetic feed solution. During anoxic cycle, nitrogen gas was used to strip out any dissolved oxygen (DO) from the reactor. The reactors were continuously mixed with a mechanical mixer. For reaeration, DO was maintained at 2.5±0.5 mg/L by a DO controller. The pH was maintained between 7.0 and 7.2 using acid and base solution. During the reaeration phase, 1 L of sample was wasted daily using a peristaltic pump. The wasting rate was adjusted daily to account for the solids lost in the effluent to maintain the operating solids retention time (SRT) of 10 days.

Samples were collected every 30 minutes for EtOH (ethanol), NH_3-N (ammonia nitrogen), NO_3-N (nitrate nitrogen), NO_2-N (nitrite nitrogen), OP (ortho-phosphate), sCOD and tCOD (total COD) measurement and analyzed using HACH test kits and HACH DR 2800 Spectrophotometer. Ethanol concentration was measured using a Gas Chromatography (GC) analyzer (GC – 2010 Plus, Shimadzu). Samples were filtered using a 0.45 μm syringe filter, except for tCOD. N_2O and NO gases were collected from head space of the reactor and analyzed using "Gas filter correlation N_2O analyzer

(Model - 320 E) and NO_x analyzer, (Model – T200M)". The total suspended solids (TSS) and volatile suspended solids (VSS) tests were analyzed as per Standard Method of 2540 D and 2540 E, respectively (APHA, 2012).

Specific denitrification rate (SDNR) was calculated from the slope of nitrate concentration over time during anoxic cycle (before endogenous period started) of SBR and divided by the reactor VSS concentration and reported with units of $mgNO_3$-N/gVSS as per equation:

$$SDNR\,(mgNO_3^- - N/gMLVSS/h) = \frac{Slope\ of\ NO_3^- - N\ during\ anoxic\ phase\ of\ ethanol\ consumption}{Reactor\ VSS\ concentration}$$

The carbon to nitrogen ratio (C/N) was calculated by plotting sCOD concentration versus nitrate concentration during the anoxic cycle of ethanol consumption in SBR and the slope is the ratio of carbon to nitrate nitrogen.

This study analyzed ten calculation methods to determine the heterotrophic yield coefficient and the concepts used for the methods are described below. All yield calculation methods for this paper are denoted from M-1 to M-10, where M-1 indicates Method 1 and so on.

The heterotrophic yield can be measured using particulate COD (pCOD) and soluble COD (sCOD) data (filtered with 0.45 μm syringe filter). The slope of the pCOD versus sCOD is an estimate of yield which is denoted in this paper as M-1 (Copp and Dold, 1998). The yield can also be calculated by dividing the net increase of pCOD by the net reduction of sCOD in a time interval for ethanol consumption and symbolized this method as M-2. Based on the same concept, the two methods mentioned above can be redeveloped by using ethanol data and can be presented as M-3 and M-4. The ethanol concentration needs to be multiplied by the stoichiometric COD to mass ratio (2.087) to calculate the COD unit value. Yield measurement using ethanol data gives more accurate value than using sCOD data. Ethanol detection by GC shows accurate carbon consumption for external substrate but sCOD measurement provides both carbon consumption plus decay and any COD release from by-product of metabolism which varies the yield. The equations for calculating yield coefficient using above principles are stating below:

$$M-1: Y = Slope\ of\ pCOD\ vs\ sCOD;$$

$$M-2: Y = \frac{Net\ increase\ of\ pCOD}{Net\ reduction\ of\ sCOD}$$

$$M-3: Y = Slope\ of\ pCOD\ vs\ (EtOH \times 2.08);$$

$$M-4: Y = \frac{Net\ increase\ of\ pCOD}{Net\ reduction\ of\ (EtOH \times 2.08)}$$

True growth yield (Y) and energy yield (1 – Y) can be expressed as follows:

$$Y = \frac{g\ biomass\ COD\ produced}{g\ substrate\ COD\ consumed\ or\ utilized}$$

$$1-Y = \frac{g\ COD\ oxidized}{g\ substrate\ COD\ consumed\ or\ utilized}$$

Total COD utilization is equal to COD consumption for growth plus oxidized COD to produce energy for cell growth. Dividing the first equation by the second equation gives the following equation:

$$\frac{g\ biomass\ COD\ produced}{g\ COD\ oxidized} = \frac{Y}{1-Y} \quad (1)$$

In this study, denitrification is considered as a 2-step process ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$) for yield measurement to simplify the calculation. For the first step, equation 1 can be represented by the following general equation for yield measurement:

$$\frac{g\ biomass\ COD\ produced}{g\ NO_2^- - N\ produced \times 1.14} = \frac{\frac{g\ biomass\ COD\ produced}{g\ substrate\ COD\ consumed}}{1-Y}$$

$$Or, \frac{[\Delta COD]_{NO_2^- \rightarrow NO_2^-}}{\Delta[NO_3^- \rightarrow NO_2^-]} = \frac{1.14}{1-Y} \quad (2)$$

Where, 1.14 = O_2 equivalent of $NO_2^- - N$ produced in g O_2 /g $NO_2^- - N$ (Metcalf and Eddy, 2003), $[\Delta COD]_{NO_3^- \rightarrow NO_2^-}$ = amount of substrate consumed to reduce nitrate to nitrite, mgCOD/L, $\Delta[NO_3^- \rightarrow NO_2^-]$ = amount of nitrate reduced to nitrite, mgN/L. This parameter can be calculated directly from experimental data.

For the second step, the yield coefficient can be represented as below:

$$\frac{\text{g substrate COD consumed}}{\text{g } NO_2^- - N \text{ removed}} = \frac{1.71}{1-Y} \quad (3)$$

$$\text{Or, } \frac{[\Delta COD]_{NO_2^- \rightarrow N_2}}{\Delta[NO_2^- \rightarrow N_2]} = \frac{1.71}{1-Y}$$

Where, 1.71 = O_2 equivalent of $NO_2^- - N$ removed, g O_2 /g $NO_2^- - N$ (Metcalf and Eddy, 2003), $[\Delta COD]_{NO_2^- \rightarrow N_2}$ = amount of substrate consumed to reduce nitrite to nitrogen gas, mgCOD/L, $\Delta[NO_2^- \rightarrow N_2]$ = amount of nitrite reduced to nitrogen gas, mgN/L. This parameter can be estimated using the following equation:

$$\Delta[NO_2^- \rightarrow N_2] = \Delta[NO_3^- \rightarrow NO_2^-] - \Delta NO_2^- \quad (4)$$

Where, ΔNO_2^- = change in nitrite concentration, mgN/L. Therefore total COD consumption for two-step process can be calculated as:

$$[\Delta COD]_{NO_3^- \rightarrow NO_2^- \rightarrow N_2} = [\Delta COD]_{NO_3^- \rightarrow NO_2^-} + [\Delta COD]_{NO_2^- \rightarrow N_2} \quad (5)$$

Simplifying the equations (2), (3), (4), (5) and hence,

$$\Delta COD_{NO_3^- \rightarrow NO_2^- \rightarrow N_2} = \left[\frac{1.14 \cdot \Delta[NO_3^- \rightarrow NO_2^-]}{1-Y}\right] + \left[\frac{1.71 \cdot \Delta[NO_2^- \rightarrow N_2]}{1-Y}\right]$$

$$\text{Or, } Y = 1 - \left[\frac{2.86\{\Delta[NO_3^- \rightarrow NO_2^-] - 0.6[\Delta NO_2^-]\}}{\Delta COD_{NO_3^- \rightarrow NO_2^- \rightarrow N_2}}\right] \quad (6)$$

Where, $\Delta COD_{NO_3^- \rightarrow NO_2^- \rightarrow N_2} = \Delta sCOD$. The experimental data of sCOD, $NO_3^- - N$ and $NO_2^- - N$ from anoxic test provides anoxic Y using above equation and is denoted as M-5. Y can also be measured using the ethanol concentration data. In that case, $\Delta COD_{NO_3^- \rightarrow NO_2^- \rightarrow N_2}$ in the yield equation can be replaced by ethanol (EtOH) to calculate yield coefficient (M-7). The mathematical term that is inside the parenthesis of yield equation can also be expressed as 2.86 times the inverse of the slope of sCOD versus nitrate (with nitrite correction) curve and this method is denoted as M-6. The same technique was also used to construct a method (M-8) using ethanol data. It is to be noted that the estimation of yield using M-5 to M-8 can give over-predictions due to possible production of unaccounted gaseous intermediates (NO and N_2O) (Mokhayeri et al., 2010). The equations for method M-5 to M-8 are presented here:

$$M-5: Y = 1 - \left[\frac{2.86[\Delta(NO_3 - 0.6NO_2)]}{\Delta sCOD}\right]$$

$$M-6: Y = 1 - \left[\frac{2.86}{\text{Slope of sCOD vs } (NO_3 - 0.6NO_2)}\right]$$

$$M-7: Y = 1 - \left[\frac{2.86[\Delta(NO_3 - 0.6NO_2)]}{\Delta EtOH \times 2.08}\right]$$

$$M-8: Y = 1 - \left[\frac{2.86}{\text{Slope of } (EtOH \times 2.08) \text{ vs } (NO_3 - 0.6NO_2)}\right]$$

A plot of pCOD versus NO_3-N utilization curve gives the slope which is equivalent to 2.86Y/ (1-Y) (Copp and Dold, 1998) and from this slope, the yield coefficient can be calculated (M-9). This coefficient can also be estimated by dividing the net increase of pCOD by net $NO_3^- - N$ reduction (between initial and final points) during ethanol consumption period in SBR which is also equal to the same parameter as indicated above and expressed as M-10. The following equations are used to calculate yield coefficient based on above principle:

$$M-9: \text{Slope of pCOD vs } NO_3 = \frac{2.86\,Y}{1-Y}$$

$$M-10: \frac{\text{Net increase of pCOD}}{\text{Net reduction of } NO_3} = \frac{2.86\,Y}{1-Y}$$

RESULTS & DISCUSSION

The typical COD, ethanol, nitrate, nitrite, ammonia and ortho-phosphorous concentrations during anoxic cycle of SBR test 2 on day seven is shown in Fig. 1. It explained that after feeding period, ethanol was consumed with a short period of time (due to acclimated biomass) and endogenous denitrification started once ethanol depleted completely. The nitrite concentration was gradually increased but started to go down while no external substrate available for microorganisms. Due to acclimation on ethanol, the utilization of ethanol was fast enough to produce nitrite with high concentrations. The SBR results (Fig. 2a) showed that specific denitrification rates (SDNR) gradually increased over a period of 8 days, suggesting a change in population and adaptation to ethanol. The initial and final SDNR was 2.22±0.17 and 46.6±4.47

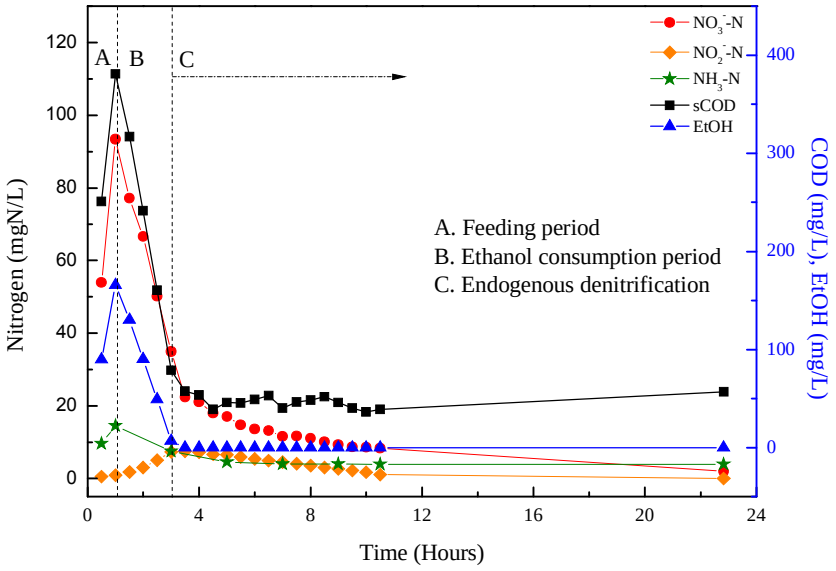

Fig. 1. Typical profile of COD, ethanol and nitrogen species observed on day 7 of sequencing batch reactor (SBR) test 2

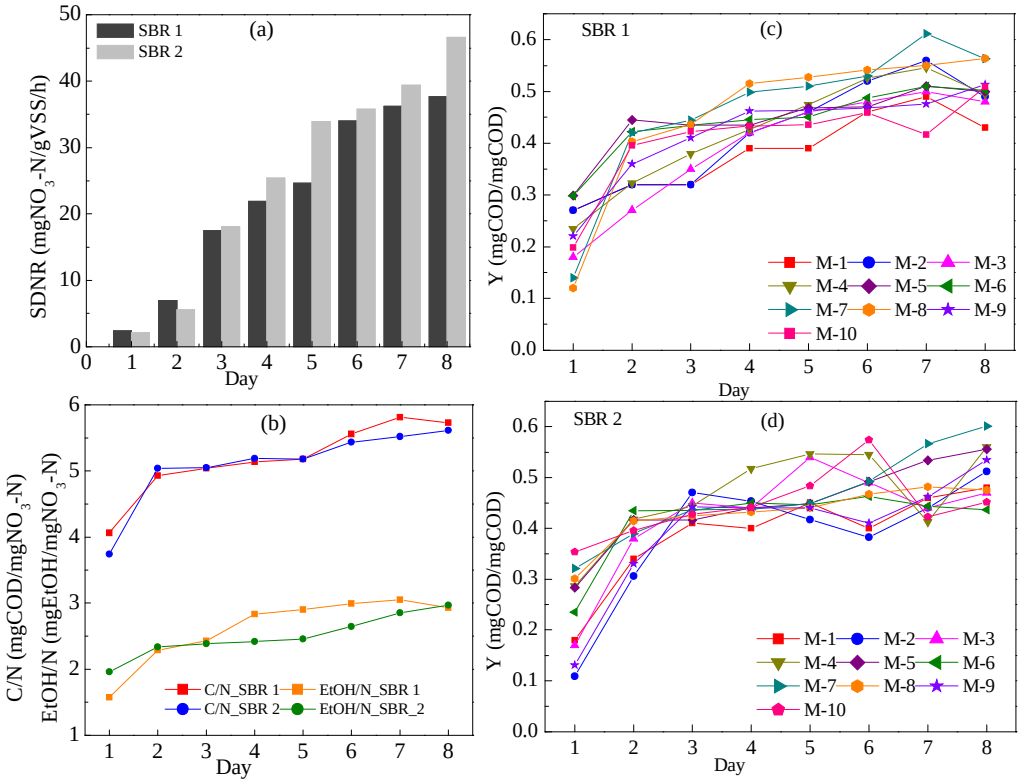

Fig. 2. (a) Specific denitrification rate (SDNR) profile (adaptation to acclimation period) for sequencing batch reactors (SBRs) from adaptation to a steady-state condition, (b) carbon to nitrate (C/N) ratio, ethanol to nitrate (EtOH/N) ratio, (c) and (d) anoxic yield profile using different calculation methods from adaptation to acclimation period for SBR 1 and 2, respectively

$mgNO_3^- - N$ /gVSS/hour, respectively. Fig. 2 illustrates the SDNR profile for two SBR system (SBR 1 and SBR 2).

The low initial SDNR value represents heterotrophs with poor ability to consume ethanol substrate. The reason for the low initial value is that mixed liquor was collected from a full scale WWTP where microorganisms were never exposed to any external substrate, and required time to adapt to a new environment. It is quite difficult to report a single SDNR value for acclimated biomass for SBR system.

Additionally, this SDNR represents only removal of nitrate rather than complete conversion to nitrogen gas. The results suggested that both SBR have similar denitrification rate characteristics. A wide range of values have been reported in literature for observed SDNR using ethanol as an external substrate. Christensson et al. (1994) conducted a pure culture batch study in 15 to 25°C temperature range and reported denitrification rate with the range of 46 to 139 mgN /g TSS/hour. Enrichment of *Hyphomicrobium* bacterium in that study on ethanol exhibited higher degree of exponential growth in the batch cultivations and showed higher denitrification rate.

Nyberg et al. (1996), Ramalingam et al. (2007) and Peng et al. (2007) estimated the SDNR for ethanol substrate to be 10, 5.6 and 9.6 $mgNO_3^- - N$ / gVSS/hour, respectively. Experimental results from this study showed similar SDNR values as Mokhayeri et al. (2010), who observed the SDNR to be 41.6 $mgNO_3^- - N$/g VSS/hour at 20°C. The variations in these rates are typically influenced by acclimated and non-acclimated sludge from full or laboratory scale systems, type of reactors, and environmental factors such as pH, temperature that affect biological processes.

The initial and final observed C/N ratio (Fig. 2b) was 3.9±0.16 and 5.71±0.10 mgCOD/mgNO_3-N, respectively. Results indicated that diverse group of heterotrophic microorganisms consume less ethanol to reduce nitrate during acclimation suggesting more catabolism compared to anabolism. In this case, the specific rate of nitrate reduction was also slower than for the adapted period. For SBR 1, the ratio was decreased from 5.81 to 5.73 mgCOD/mgNO_3-N on day 8 due to nitrite accumulation which could inhibit nitrate reduction. McCarty et al. (1969), and Mycielske et al. (1983) observed the C/N ratio for ethanol as 5.877 and 4.16 mgCOD/mgNO_3-N, respectively. Christensson et al. (1994) and Mokhayeri et al. (2010) reported the ratio as 3.85 (continuous experiment), 5.81 (pure culture batch study) and 6.9 mgCOD/mgNO_3-N at 25°C and 20°C, respectively. The values reported in this study agree with the literature reported values. The observed C/N depends on several factors. Majone et al. (1998) reported that microorganisms can produce internal polymers for faster adaptation to a new environment by storing the substrate which could change the C/N ratio. Naidoo et al. (2000) investigated that if activated sludge contains polyphosphorous-accumulating organisms (PAOs), then it could vary the C/N ratio due to PAOs possible activity. Oxygen intrusion is also an important factor which certainly differs the ratio but the concentration of DO in SBR system was zero for whole period of the anoxic conditions. The determination of the correct C/N ratio is crucial for the selection of alternative carbon sources, because it is an indicator of COD usage efficiency for denitrification. High operational costs and higher biomass production can be caused by C/N overestimation (Cherchi et al. 2009).

Ethanol to nitrate (EtOH/N) ratio was also determined to check the accuracy of sCOD measurement and vice-versa. The percentage of error can be calcualted by comparing measured COD to EtOH ratio with the ethanol stoichiometric COD to mass ratio (2.087). Based on the result of EtOH/N and C/N ratio (Fig. 2b), the range of percentage error was between 0.3 to 20%. This ratio determination ensured that both ethanol and sCOD concentration were measured in the experiment with good correlation.

The anoxic yield profile during the SBRs operation are shown in Fig. 2 c and 2d. For SBR test 1 (Fig. 2c), M-1 to M-7 showed a decrease in yield on final day of operation. This is possible due to nitrite accumulation (9.56 mgN/L) which could inhibit the growth of heterotrophic microorganisms. Ammonia was also accumulated during the test, but there was no relation found which could inhibit the biomass growth. It was also observed that the intermediate gases (N_2O and NO) had no effect on yield measurement as concentrations were negligible. The methods M-1 to M-4 exhibited similar trend of yield profile but the method M-1 had lower yield value (0.41±0.05 mgCOD/ mgCOD) than the other three methods. The method M-5 and 6 along with M-7 and 8 exhibited the same result. The methods M-5 to 8 are derived from basic stoichiometry, but they have two distinguishing parameters which are sCOD and EtOH.

For SBR test 2 (Fig. 2d), method M-1 and M-2 has similar outcomes for yield calculation. On day 6, both methods showed lower yield than previous day because of high nitrite concentration (3.2 mgN/L). The method M-3 and M-4 exhibited higher yield than method M-1 and M-2 due to dependency on two sensitive parameters, pCOD and EtOH. Thus any variability on those parameters changed the value significantly. The methods M-5 to 8 showed similar trend of yield whereas methods M-6 and 8 presented equal outcomes of yield coefficient. The methods M-9 and 10 depend on pCOD and nitrate data and exhibited identical results. The two SBR results were averaged and reported as a single anoxic yield value using all methods. The range of anoxic yield using ten different methods was between 0.423±0.014 to 0.512±0.021 mgCOD/mgCOD. The yield values with 95% confidence interval (CI), standard deviation (St. Dev),

source of error and associated artifacts of all catabolic and anabolic methods are explained in Table 1. Study results are in agreement with McCarty et al. (1969), Mokhayeri et al. (2010) and Peng et al. (2007) as researchers found the yield coefficient for ethanol as 0.513, 0.59 and 0.42 mgCOD/mgCOD, respectively. However, Hallin et al. (1996) reported a lowet yield value for ethanol which was 0.22 mgCOD/mgCOD.

Ten calculation methods were used in this study to estimate the yield coefficient with various parameter analyses. Method 1 to 4 depends on reliability of sCOD, tCOD and EtOH measurement. Based on laboratory performance, it is concluded that capturing the pCOD increasing trend is quite challenging. 'HACH COD high range' test kit was used for measuring the total COD. The kit needs 2 mL of unfiltered sample for digestion. This small volume of sample can alter the results as it can affect by some factors: collection method for sampling, mixing of sample and taking care of COD digestion procedure. For soluble COD measurement (filtered sample), same factors are applied. For COD sample analysis, there are key protocols that need to be maintained in the laboratory to obtain reliable values. It was vital to collect same amount of samples every time from reactor for analysis. It was required to keep same amount of unfiltered sample for filtration process to make sure consistent performance. Filtration process for sample analysis was done carefully to avoid high pressure on syringe filter for filtering the sample, otherwise high pressure could pass more particles (> 0.45 μm) through the filter. Hence, it is essential for yield experiment to have precise laboratory performance on COD measurement. Ethanol measurement can also depends on several factors: laboratory performance using GC analyzer, purity of ethanol solution, calibration of GC analyzer, standard ethanol solution and ethanol being used for reactor feeding, could vary the results. The first two methods (M-1 and M-2) assumed that decrease in soluble COD is converted to either biomass or is oxidized to CO_2 and water. That means there is no production of soluble COD as a by-product of metabolism (Copp and Dold, 1998). However this is not exactly true for biological denitrification. M-3 and 4 could give more correct result as ethanol measurement does not consider COD release from metabolism. Based on yield measurement, standard deviation is higher for ethanol than sCOD measurement.

It is not perfectly true to say that if standard deviation is large then the method is not best to select. Method 5 to 8 includes nitrate, nitrite, soluble COD and ethanol data which needs filtered sample and can give better result than other methods. M-6 and M-8 methods contain all sampling points for consumption of sCOD, nitrate reduction and nitrite concentration. M-5 and 7 only determines the yield based on initial and final point of anoxic experiment during ethanol consumption. Summary of the results clearly indicates that standard deviation for M-5 is smaller than other methods. This appear to be a more reliable method than other methods for estimation of biomass yield coefficient. Last two methods (M-9 and M-10) are depended on pCOD and nitrate concentration. Both methods only rely on measurement of tCOD and sCOD because nitrate measurement using HACH test kits are less sensitive based on laboratory performance than tCOD measurement.

For statistical analysis, one-way ANOVA (Analysis of Variance) model was used which provides a statistical test of whether or not the means of several methods are equal, and therefore generalizes t-test to more than two methods for statistical significance. For this study, one-way ANOVA was applied because of just one explanatory variable (yield) and the ANOVA was performed using MINITAB 17 Statistical Software. The independent variable (factor) is calculation method and the dependent variable is yield (response or level). The overall null hypothesis is that all of the population means are equal, without restricting what the common value is. The alternative hypothesis is that "the population means are not equal" and at least one mean is different. Equal variances were also assumed for the analysis and 5% significance level (α) was considered. After ANOVA analysis, the p-value for all yield methods is 0.098 (Table 2) which is greater than significance level of 5% indicating that calculation methods have no statistical significance for yield measurement.

When applying one way ANOVA, there are three key assumptions that requires to satisfy. So, it was necessary to check the assumptions which are: normality, constant variance and independence of errors (Pan, 2014). ANOVA also requires that observations should be randomly selected from the treatment population (yield) and assumptions can be checked with the residuals plot. The residual plots for yield using all methods are shown in Figure 3. The residual versus fits plot (Figure 3) showed a random pattern of residuals on both sides of zero satisfying the assumptions. However, the plot has more spread on the points of 0.49 fitted value with high error. Thus, it is difficult to reject the assumption of constant variance in the residuals. The residual versus order plot (Fig. 3) did not have any positive or negative correlation satisfying the assumptions of independence. The histogram appears to present a bell-shaped curve though two points in normal probability plot are outliers from the straight line. Since

Table 1. Anoxic yield (mgCOD/mgCOD) estimation by multiple calculation methods

Methods	Y_{anox} ±St. Dev (95 % CI)	Error source	Comments on method
M-1	0.423±0.014 (0.388, 0.458)	tCOD measurement technique: collection method for sampling, mixing of sample and taking care of COD digestion procedure	Difficult to get net increase of pCOD trend as tCOD is a sensitive parameter and its measurement depends on human skill performance
M-2	0.454±0.011 (0.419, 0.489)	tCOD measurement technique: same as above	Method is only two point depended which might increase the standard deviation and also involved with associated artifacts of tCOD measurement
M-3	0.468±0.028 (0.433, 0.503)	tCOD and EtOH measurement technique: performance of GC and purity of ethanol	Includes two sensitive parameter (tCOD and EtOH) but gives true measurement
M-4	0.490±0.212 (0.455, 0.525)	tCOD and EtOH measurement technique	Two points method increases the variability of the result
M-5	0.475±0.008 (0.439, 0.510)	sCOD measurement technique: filtration technique	Indirect method and only depends on sCOD measurement. Nitrate and nitrite are less sensitive than sCOD measurement
M-6	0.459±0.018 (0.424, 0.494)	sCOD measurement technique: filtration technique	Provides more accurate result due to considering full data points of anoxic test and simplicity of parameter measurement
M-7	0.512±0.021 (0.476, 0.547)	EtOH measurement technique	Depends only on Ethanol estimation and could vary the result
M-8	0.489±0.049 (0.453, 0.524)	EtOH measurement technique	Gives more accurate result than M-7
M-9	0.466±0.001 (0.431, 0.501)	tCOD measurement technique	Indirect method and solely depends on tCOD
M-10	0.456±0.013 (0.420, 0.491)	tCOD measurement technique	Depends only tCOD measurement and results vary due to two point calculation

Table 2. One-way analysis of variance table for anoxic yield calculation methods

Source	Degrees of freedom	Adjusted sum of squares	Adjusted mean squares	F-value	P-value
Method	9	0.010593	0.001177	2.37	0.098
Error	10	0.004971	0.000497		
Total	19	0.015565			

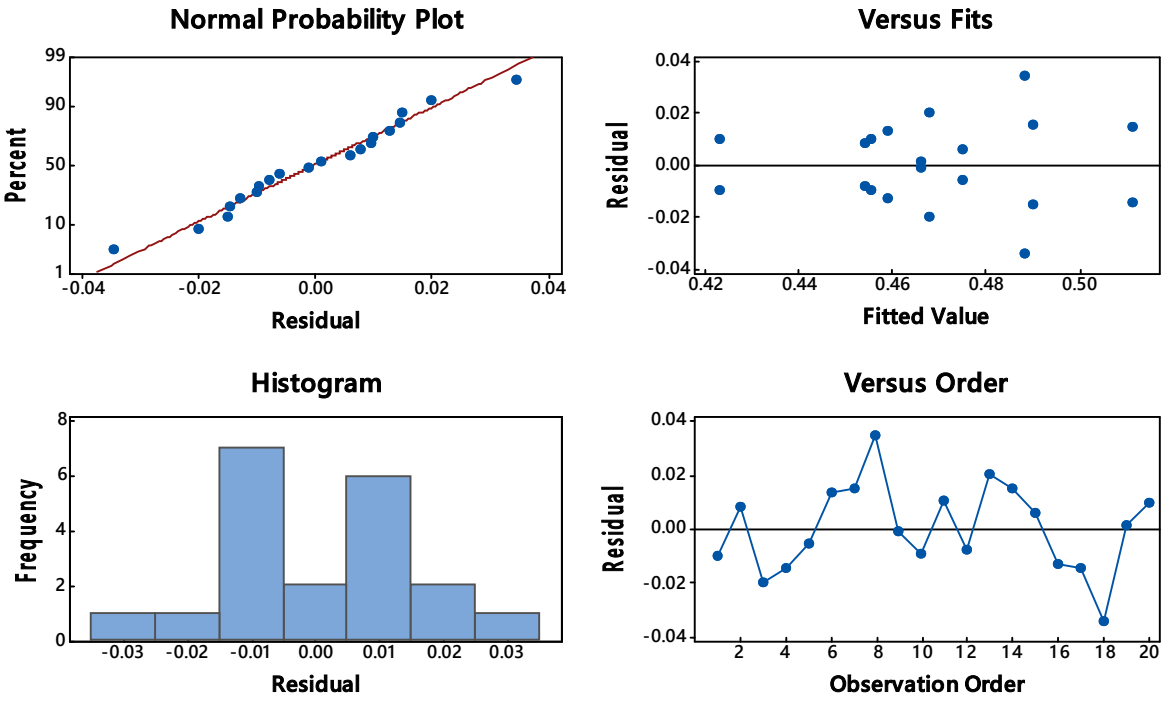

Fig. 3. Residual plot of yield coefficient: normal probability plot, residual versus fits plot to check constant variance, histogram plot to check normality and residual versus order (time order of data collection) plot to check independence

one-way ANOVA follows the linear model, there are two assumptions that need to be true to validate the model: normality and equality of variances. The distribution of the residuals or error terms can be checked by a residuals probability plot. In probability plot (not shown in the paper), larger p-value indicates larger support for the theoretical distributions, which is the normal distribution. In addition, the larger AD (Anderson-Darling)-value statistic indicates larger deviation from the fitted theoretical distribution. The probability plot illustrates that p-value is greater than 0.25 and AD statistic is 0.639. Based on analysis, it is concluded that the residuals are normally distributed. For equality of variances, Barlett's test can be used which is accurate for normal data only. The P-value for Barlett's test is 0.654 which is higher than α. Thus, the null hypothesis is failed to reject that the variances of the residuals of yield are constant across the treatments.

CONCLUSIONS

Experimental results confirmed that general heterotroph population was adapted with ethanol substrate by producing their special enzymes for ethanol. The final C/N ratio was obtained as 5.71±0.10 mgCOD/mgNO_3-N which is usually higher than methanol but lower than acetate as literature reported value. Yields can vary based on relative acclimation to a substrate and may explain the different anoxic yield observations in literature. A corollary to acclimation is that an organism or organisms that invest in resources to degrade many substrates (in wastewater) will have a lower yield than the same organism or specialized organisms that invests in fewer resources to degrade a

single substrate. The range of anoxic yield using all calculation methods was between 0.423±0.014 to 0.512±0.021 mgCOD/mgCOD at 20°C. However, the uncertainty in determination of the yield coefficient given that the standard deviation between the methods (highest to lowest) is around 23%. Estimation of anoxic yield using multiple calculation methods, after adding a specific substrate to a non-acclimated sludge and after steady state was achieved, provided a more reliable estimate than using a single method. It is also concluded that the different calculation methods has no statistically significant effect on yield determination suggesting that accurate measurements should provide no deviation from actual yield value and each of the methods provide a reasonable estimate of the yield. Depending of what parameters can be measured correctly for a particular experiment or setup, a particular method can be selected using those parameters to calculate the yield.

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