

# The effect of an initial anaerobic zone on the kinetics and stoichiometry of acetate removal during nutrient-limiting conditions

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## Abstract

I studied the stoichiometry and kinetics of acetate removal on anaerobic/aerobic (AnA) and completely aerobic (CA) sequencing batch reactors under nutrient-limiting conditions. CA acetate removal rates were 20 to 40% higher than AnA acetate removal rates when both nitrogen and phosphorus (P) were sufficient. When P was deficient, the acetate removal rate of both sludges was 0.8 mg acetic acid/g VSS/min. Anaerobic stoichiometry indicated that polyphosphate-accumulating organisms were present at a low-level, and that glycogen-accumulating organisms were dominant. I also found that the AnA sludge synthesised 2 to 5 times more polyhydroxybutyrate-C than the CA sludge.

**Keywords:** nutrient deficiency, anaerobic/aerobic, acetate removal, microbial storage products

## Nomenclature

AnA	- anaerobic/aerobic
CA	- completely aerobic
CH	- carbohydrate
COD	- chemical oxygen demand
GAO	- glycogen-accumulating organism
GLY	- glycogen
MCRT	- mean cell residence time
N	- nitrogen
P	- phosphorus
PAO	- polyphosphate-accumulating organism
PHA	- polyhydroxyalkanoate
PHB	- polyhydroxybutyrate
SBR	- sequencing batch reactor

## Introduction

It is common practice in biological treatment of nutrient-deficient wastewater to use completely aerobic (CA) activated sludge units. Recent laboratory-scale work has shown that the use of an anaerobic/aerobic (AnA) process may be a better alternative due to three key operating benefits. The first benefit is that the AnA process requires 20% less P to remove a given amount of influent chemical oxygen demand (COD) than a CA process because of the accumulation of glycogen (GLY) and polyhydroxyalkanoates (PHAs) (Harper and Jenkins, 2003). The second benefit is the suppression of viscous bulking, due to the accumulation of more intracellular carbohydrate (CH) (i.e. GLY) and less exocellular CH (Jobbagy et al., 2002). The third operating benefit is that the AnA process produces lower effluent P concentrations than the CA process, when both systems are subjected to variable influent organic loading (Harper and Jenkins, 2002).

These first two benefits are due mostly to glycogen-accumulating organisms (GAOs). These organisms synthesise and degrade storage products (PHB and GLY) as they are cycled between anaerobic and aerobic conditions. Under anaerobic conditions, GAOs remove soluble carbon and synthesise PHB, while degrading GLY. During the aerobic phase, they use a portion of the internal PHB pool to synthesise GLY, with the common result being net PHB and GLY accumulation in the biomass. GAOs are abundant in AnA systems operated at influent COD/P ratios > 60 (Liu et al. 1997, Schuler 1998).

The third benefit is due to another group of storage product-accumulating organisms, polyphosphate-accumulating organisms (PAOs). Like GAOs, PAOs also synthesise and degrade PHB and GLY, but unlike GAOs, PAOs rely on a third storage product, polyphosphate. As carbon is removed during the anaerobic phase, PAOs produce energy by hydrolysing polyphosphate, resulting in P release into the wastewater. Under the following aerobic phase, PAOs remove P to synthesise polyphosphate, resulting in net P removal from wastewater. Harper and Jenkins, 2002 exploited this characteristic P release and uptake profile to show the third operating benefit. When an AnA and CA SBR are both treating a P-limited wastewater with variable influent organic loading, the AnA SBR produces lower effluent P concentrations because of the P release and uptake characteristics of polyphosphate metabolism. During low influent COD loading periods, aerobic polyphosphate synthesis removes P to low levels, while under high influent COD loading periods, anaerobic P release provides P and prevents P deficiency. The key to this benefit is to constantly add enough P to stimulate the P release and uptake characteristics of polyphosphate metabolism.

Realising all three of these benefits means operating an AnA system in a way that sustains both PAOs and GAOs under nutrient-limiting conditions. Evaluating coexistence of PAOs and GAOs can be done by investigating the anaerobic stoichiometry, which depends on the relative abundance of PAOs and GAOs (Liu et al., 1997; Schuler 1998). The ratio of P released/acetate-C removed is the key measure of PAO activity, while the ratio of CH-C degraded/acetate-C removed is the key measure of GAO activity.

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The PHB-C synthesised/acetate-C removed ratio is also important because PHB plays a central role in the metabolisms of both the PAOs and GAOs. In this study these ratios are determined under nutrient-limiting conditions. Also, because it is current practice to use CA systems for nutrient-deficient wastewater treatment, I also investigated the acetate removal stoichiometry of a CA system to evaluate the effect of the initial anaerobic zone on stoichiometry.

Acetate can be removed very rapidly under aerobic or anoxic conditions, but likely not as quickly during an initial anaerobic phase. One possible reason is because of the aerobic function of the electron transport chain, which produces a great deal of energy that can be used to drive active transport of acetate and activation to acetyl-CoA. Thus, implementing an initial anaerobic zone may provide some benefits, but the potential disadvantages associated with acetate removal rates must be investigated. This need is included in the current work.

## Previous work

Most previous work on anaerobic acetate removal kinetics and stoichiometry has been conducted at the relatively low influent COD/N and COD/P ratios typical of municipal wastewater (e.g. influent COD/N  $\approx$ 10, COD/P  $\approx$ 20). Lui et al. 1997 and Schuler 1998 examined anaerobic acetate removal kinetics and stoichiometry at higher influent COD/P ratios by systematically adjusting the influent P content (from COD/P of approx. 10 to 130). As the influent COD/P ratio increased, they found the following trends:

- More GAOs and less PAOs were present, as determined by morphological comparisons.
- The acetate removal rates decreased linearly from approximately 3.5 to 1 mg acetate/g VSS/min, suggesting that PAOs remove acetate faster than GAOs.
- The P release/acetate-C removed ratio decreased from approximately 0.8 to 0.1 mol P/mol C, which shows decreasing PAO activity.
- The molar ratios of CH-C degraded per mole of acetate-C increased from approximately 0.3 to 0.8 mol C/mol C, because as GAOs became more dominant, anaerobic degradation of glycogen became more important.

Despite operating at influent COD/P ratios of up to 130, neither study reported P deficiency. This can likely be attributed to the operation of the GAOs, since the accumulation of internal CH reduces the amount of P needed for removal of carbon (Harper and Jenkins, 2003). There are currently no reports showing the effect of influent COD/N ratio on anaerobic acetate removal for AnA systems.

## Experimental methods

Laboratory-scale sequencing batch reactors (SBRs) with 1 l working volumes were used. One SBR was AnA; a second SBR was CA. The 6 h AnA SBR cycle was anaerobic (110 min), aerobic (180 min), settling (30 min), draw/fill and N<sub>2</sub>-stripping (40 min). The 6 h CA SBR was identical to the AnA SBR except that aerobic period was 290 min and there was no anaerobic period. The mean cell residence times (MCRTs) were 4 d to minimise nitrification, which could disrupt the operation of the GAOs and PAOs by creating an anoxic (and not anaerobic) zone. The hydraulic retention times were 12 h. pH was controlled between 7.0 and 7.3, and temperature was ambient (approx. 24°C). The feed included inorganic salts, acetate, casamino acids, and yeast extract (on a mg COD/l basis: acetate (200), casamino acids (20), yeast extract

(<1)). The inorganic salts content was (as mg/l total influent concentration): KCl (117), MgCl<sub>2</sub>-6H<sub>2</sub>O (219), MgSO<sub>4</sub>-7H<sub>2</sub>O (14.4), CaCl<sub>2</sub> (45.9), H<sub>3</sub>BO<sub>3</sub> (0.061), ZnSO<sub>4</sub>-7H<sub>2</sub>O (0.305), KI (0.015), CuSO<sub>4</sub>-5H<sub>2</sub>O (0.061), Co(NO<sub>3</sub>)<sub>2</sub>-6H<sub>2</sub>O (0.075), NaMoO<sub>4</sub>-2H<sub>2</sub>O (0.031), MnSO<sub>4</sub>-H<sub>2</sub>O (0.342), and FeSO<sub>4</sub>-7H<sub>2</sub>O (0.304). The influent P (supplied as NaH<sub>2</sub>PO<sub>4</sub>-2H<sub>2</sub>O) and N (supplied as NH<sub>4</sub>Cl) concentrations were intentionally varied.

Very few filaments were observed in the SBRs, and pin-point floc was a concern. Thus, a flocculation/settling aid was added daily to the SBRs to simulate the presence of filamentous organisms and help develop good settling floc. This was prepared by blending 20, 47 mm diameter Whatman glass-fibre filters (GFF) (Whatman International, Ltd., Maidstone, U.K.) in 1 l distilled water. Each day 19.5 mg GFF was added to both SBRs.

For both the AnA and CA SBRs, the influent COD/P or COD/N ratio was decreased until nutrient deficiency was observed. In practice, low effluent soluble P or N concentration (< 1.0 mg/l) is used as an indicator of activated sludge nutrient deficiency. For AnA activated sludge, this method is not feasible because the PAOs induced in AnA activated sludge produces low effluent soluble P levels when influent P is in excess. Therefore, the presence of effluent acetate was used as the criterion for both P and N deficiency. The details of effluent acetate measurements at various influent COD/P and COD/N ratios have been discussed previously (Harper and Jenkins 2003).

Influent P and N loadings were varied in separate experiments (one was a "low P loading" experiment and the other was a "low N loading" experiment). At each loading, the SBRs were operated for at least 3 MCRTs (12 d). Reactor TSS data were used to verify that steady state had been reached. Then measurements were made over a period of at least 2 MCRTs (8 d). Samples were taken for acetate, soluble orthophosphate, PHAs and CH during the first 110 min of the cycle, which was the length of the anaerobic phase for the AnA SBR.

During the low P loading experiments, the CA SBR influent COD/P was increased from 80 to 120 (effluent acetate was detected at an influent COD/P ratio of 110). The AnA SBR influent COD/P ratio was increased from 80 to 160 (effluent acetate was detected at an influent COD/P ratio of 140). For the CA SBR, P deficiency was observed at an influent COD/P of 110, while for the AnA SBR it was observed at 140. During the low N loading experiments, the CA and AnA SBR influent COD/N ratios were increased from 7 to 36 (effluent acetate detected at 30 for both). The influent COD/P ratio was constant at 90, which provided sufficient P. A high influent COD/P ratio was intentionally chosen for the low N loading experiments because a GAO-rich microbial community was intended for the AnA SBR.

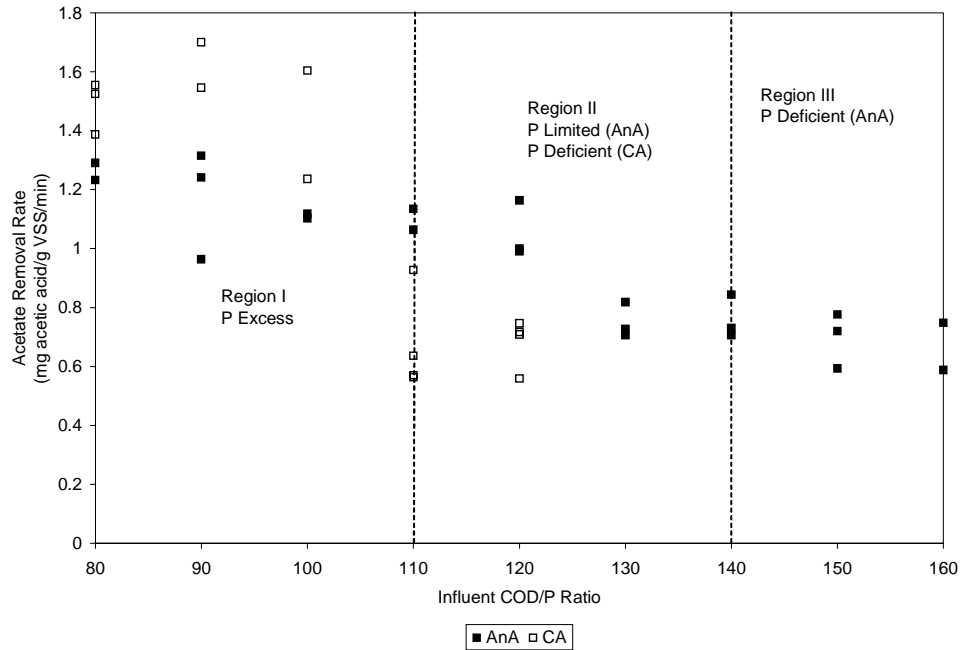
TSS, VSS, total and soluble orthophosphate were determined using *Standard Methods*, 1992. Acetate was measured on filtered, acidified samples by HPLC using an Aminex (No. 125-0140) organic acid analysis column. The solvent was 8.0 mM H<sub>2</sub>SO<sub>4</sub> at 0.9 ml/min. The array detector (Perkin Elmer LC 235) was operated at 205 nm. PHA was by a modified Riis and Mai GC method (Riis and Mai 1988; Schuler 1998). CH was by the anthrone method (ASM 1981). Non-storage product VSS was determined by subtracting the PHA and CH content from total VSS. Samples were taken in triplicate.

## Results

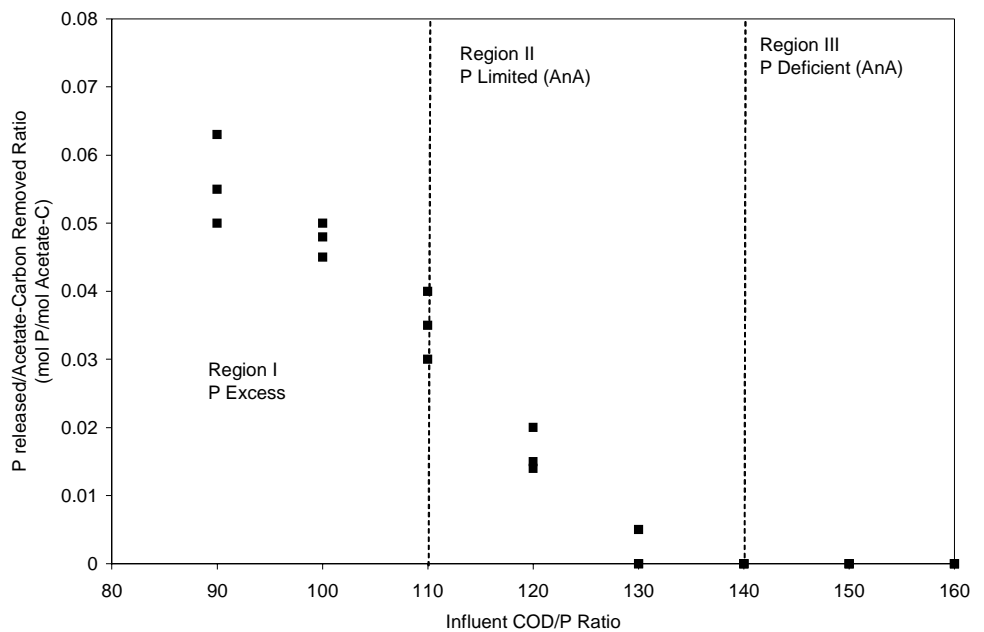
### Low P loading experiments

P limitation (< 1 mg P/l) was observed at an influent COD/P ratio of 110 for both the CA and AnA SBR, while P deficiency was

**Figure 1**  
Effect of influent COD/P ratio on acetate removal rate during the first 110 min of the cycle of an AnA SBR and a CA SBR



**Figure 2**  
The effect of influent COD/P ratio on P release/acetate-carbon removed ratio of AnA SBR



observed at an influent COD/P ratio of 110 for the CA SBR and 140 for the AnA SBR (Harper and Jenkins 2003). These points define the three operating regions of interest: (I) P excess, (II) P limiting, and (III) P deficient. In Region I, P was in excess for both SBRs (influent COD/P ratio < 110). In Region II, P was deficient for the CA SBR but not for the AnA SBR (influent COD/P ratio  $\geq 110$  and < 140). In Region III, both SBRs were P deficient (influent COD/P ratio  $\geq 140$ ). The following data are discussed with reference to these three regions.

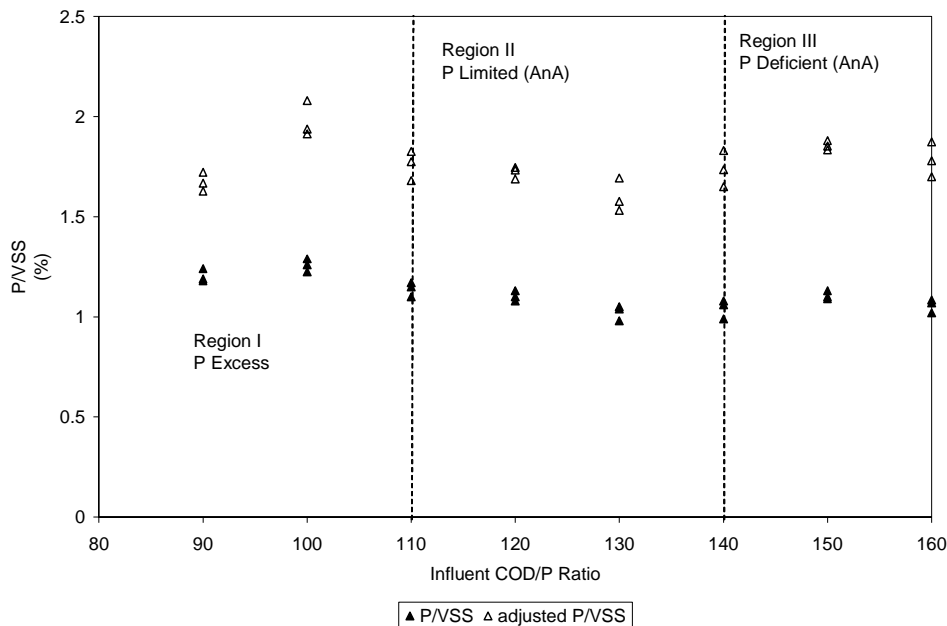
#### Acetate removal rates

Figure 1 shows that the AnA anaerobic acetate removal rate decreased as the influent COD/P ratio increased. In previous results, acetate removal rates linearly decreased as the influent COD/P ratio was increased from relatively low values (COD/P H=20) to an influent COD/P ratio of approximately 130 (Liu et al., 1997; Schuler 1998). The results presented in this work show that as the influent P loading approaches deficiency, the linearly de-

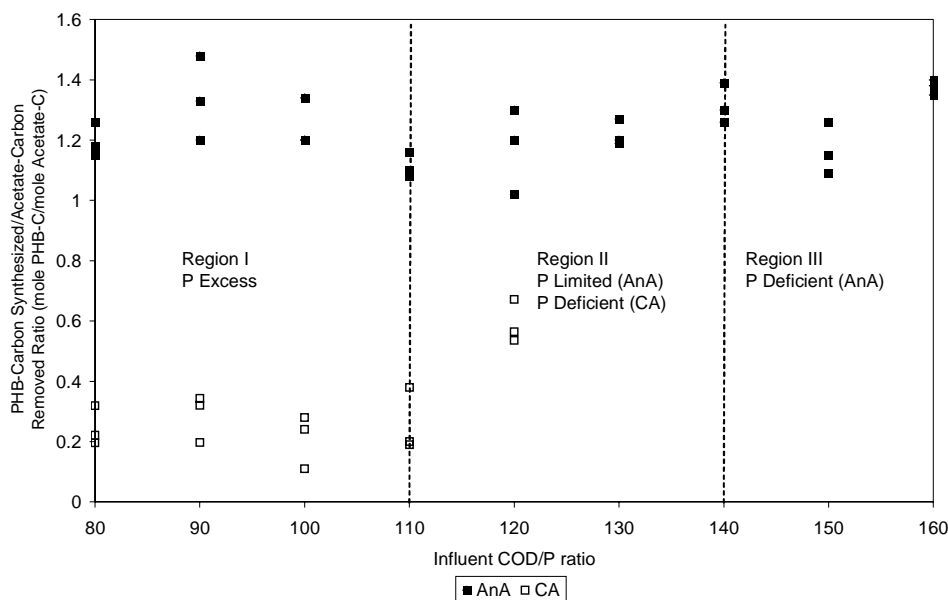
creasing trend found by Liu et al., 1997 and Schuler, 1998 continues until P deficiency is reached. The CA SBR acetate removal rates were approx. 20% higher than those of the AnA SBR when the influent COD/P ratio was  $\leq 100$ , but were lower than the AnA SBR acetate removal rates when influent COD/P ratio was  $\geq 110$ . The AnA acetate removal rate gradually decreased from approx. 1.3 mg acetic acid/g VSS/min at an influent COD/P ratio of 80 to 0.8 mg acetic acid/g VSS/min at an influent COD/P ratio of 140. The CA acetate removal rate was fairly constant when P was in excess (influent COD/P < 110), but decreased to approx. 0.8 mg acetic acid/g VSS/min when P was deficient.

#### Stoichiometry of P release and sludge P content

The P release/acetate-C removed ratio of the AnA SBR ranged from 0.06 to 0.01 mol P/mol acetate-C (Fig. 2). Because high PAO activity is associated with P release/acetate-C removed ratios of 0.3 to 0.8 mol P/mol acetate-C (Liu et al., 1997; Schuler 1998), the current data suggest that low-level PAO activity was present in the



**Figure 3**  
The effect of influent COD/  
P ratio on the P content of  
AnA SBR



**Figure 4**  
Effect of influent COD/P  
ratio on PHB-carbon  
synthesised/acetate-carbon  
removed ratio during the  
first 110 min of the cycle of  
an AnA SBR and a CA SBR

AnA SBR. The P release/acetate-C removed ratio decreased as the influent COD/P ratio increased, because at high influent COD/P ratios less P was available for polyphosphate synthesis. Figure 3 shows the sludge P content expressed as the fraction P/total end-of-aerobic VSS and as the fraction P/total end-of-aerobic non-storage product VSS. As a percentage of VSS, the sludge P content was between 1 and 1.3%, while the adjusted sludge P content ranged from 1.5% to 2.1%. Thus P release was observed even though the total P content of the active biomass was close to that of non-polyphosphate containing sludge. These results show that an AnA sludge being fed acetate can maintain anaerobic P release up to influent COD/P ratios of 120, even while the P content of the sludge is low.

#### **Stoichiometry of PHB formation and CH degradation**

During the AnA anaerobic phase, acetate-C taken into the cell is used to form PHAs. The reducing equivalents required for PHB formation are partially derived from intracellular CH degradation.

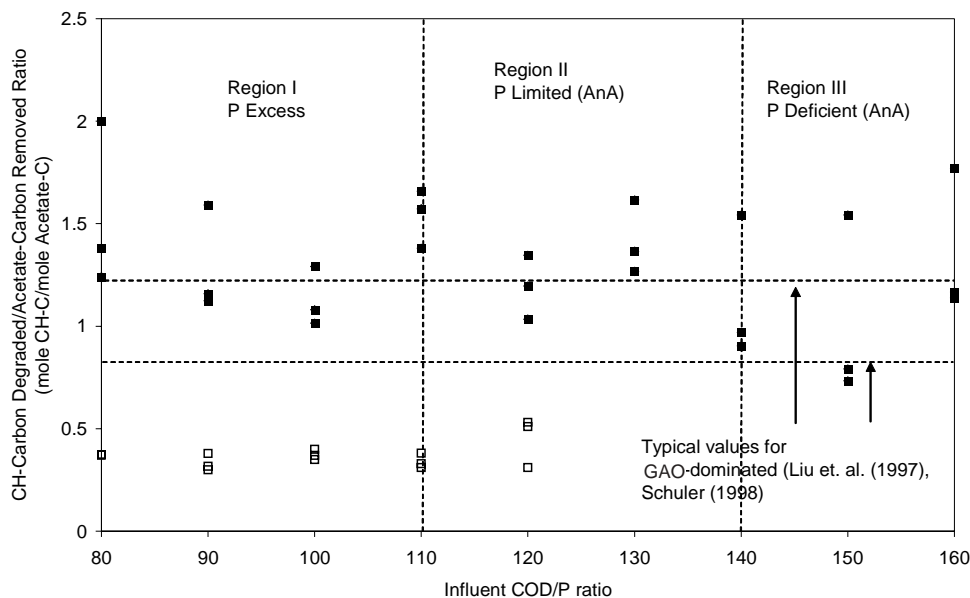
The PHB-C synthesised/acetate-C removed ratio for the AnA

SBR was 2 to 5 times higher than that of the CA SBR (Fig. 4). P deficiency had no effect on the PHB-C synthesised/acetate-C removed ratio for the AnA sludge, and the CA molar PHB-C synthesised/acetate-C removed ratio increased from approx. 0.2 to 0.6 mol PHB-C synthesised/mol acetate-C removed as the influent COD/P ratio increased from 80 to 120. Figure 5 shows that CH-C degradation in the AnA SBR was 2 to 5 times higher than that of the CA SBR. P deficiency had no effect on the CH-C degraded/acetate-C removed ratios. The AnA CH degradation stoichiometry was consistent with an AnA SBR dominated by GAOs.

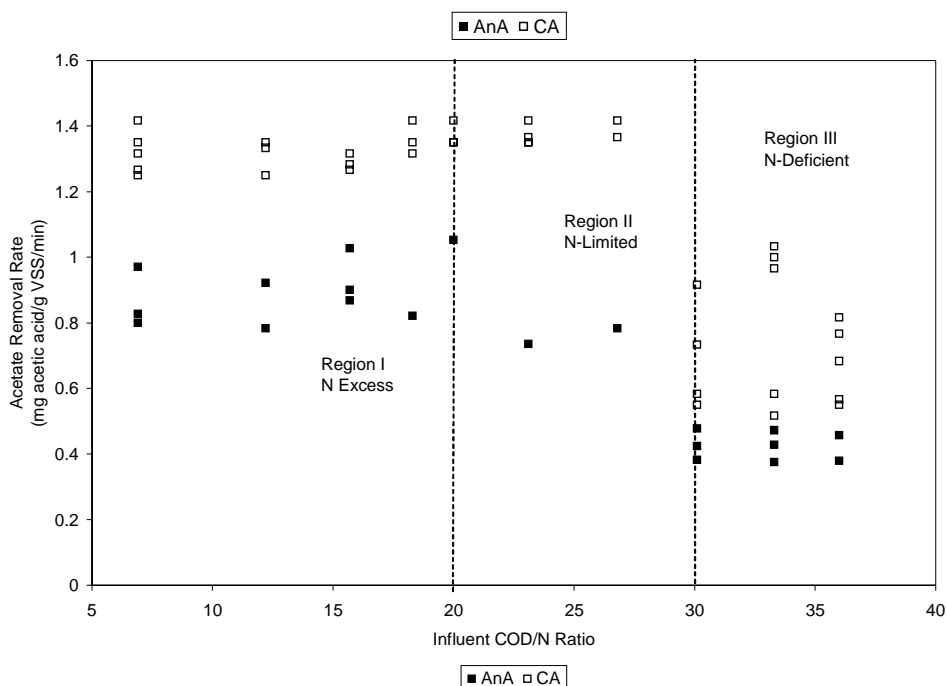
#### **Low N loading experiments**

During the low N loading experiments, effluent acetate and inorganic N concentrations were used to define three operating regions (Harper and Jenkins, 2003): Region I - N excess (effluent inorganic N > 1 mg/l; effluent acetate not detected), Region II - N-limited (effluent inorganic N < 1 mg/l; effluent acetate not detected), and Region III - N-deficient (effluent inorganic N < 1 mg/l; effluent

**Figure 5**  
Effect of influent COD/P ratio on CH-carbon degraded/acetate-carbon removed ratio during the first 110 min of the cycle of an AnA SBR and a CA SBR



**Figure 6**  
Effect of influent COD/N ratio on acetate removal rates during the first 110 min of the cycle of an AnA and a CA SBR



acetate detected). The following data are discussed with reference to these three regions.

#### Acetate removal rates

AnA and CA acetate removal rates were constant throughout Regions I and II (Fig. 6). The CA acetate removal rate was 30-40% higher than that of the AnA SBR for all COD/N ratios tested. Between influent COD/N ratios of 25 to 30 (onset of N deficiency), the acetate removal rate of both sludges decreased by approx. 50%. Thus, N deficiency, but not N limitation, greatly reduced the acetate removal rates of both sludges - a result previously found by others (Sawyer, 1941; Helmers et al., 1951; Jones, 1965). CA acetate removal rates were higher than those of the AnA SBR even beyond N deficiency.

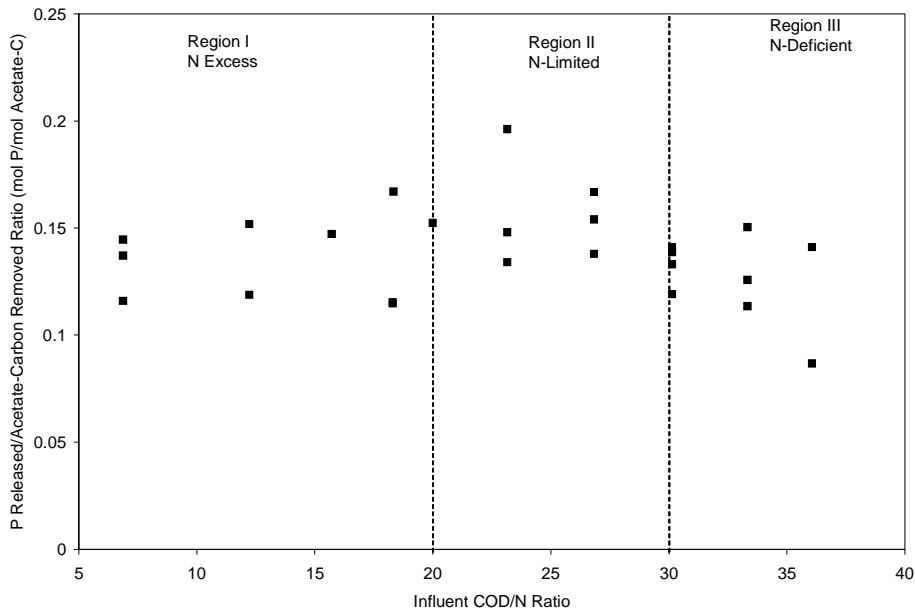
#### Stoichiometry of P release

Figure 7 shows that the anaerobic P release/acetate-C removed ratio was relatively constant (approx. 0.15 mol P/mol acetate-C

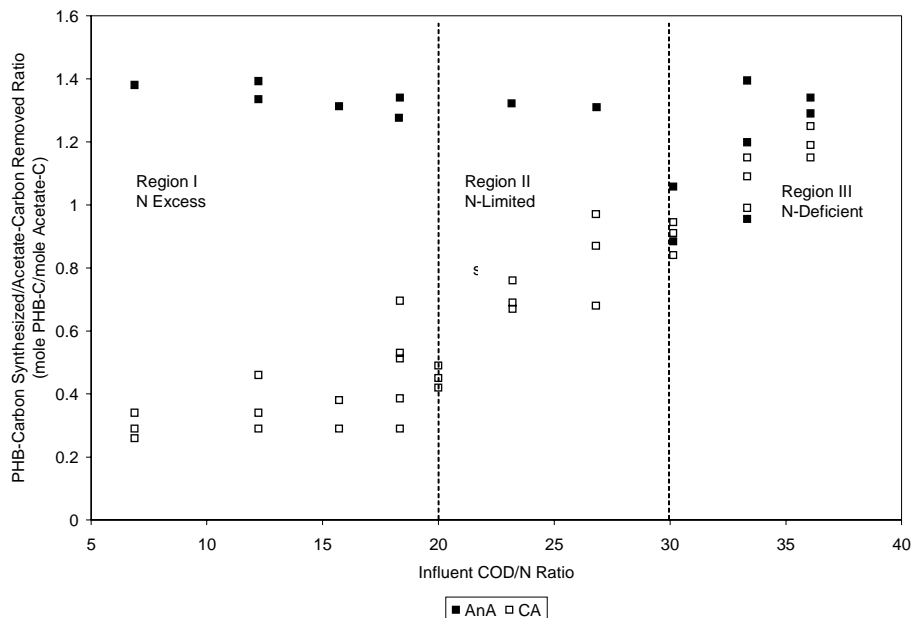
removed) across Regions I, II, and III. Neither N limitation nor deficiency appeared to affect P release stoichiometry. P release observed here is likely due to the action of polyphosphatases specific for short-chain polyphosphates. These enzymes are known to maintain relatively constant enzyme activity despite changes in specific growth rate (Lichko et al., 2002), and N is well known to cause a decrease in specific growth rates (Madigan et al., 1996).

#### Stoichiometry of PHB formation and CH degradation

Figure 8 shows the effect of influent COD/N ratio on the PHB-C synthesised/acetate-C removed ratio. The data shows that the fraction of influent acetate-C used to form PHB in the AnA was constant and close to previously reported values, but for the CA SBR the fraction of influent acetate-C used to form PHB depended on the operating region. In Region I, more PHB-C synthesis/acetate-C removed occurred in the AnA SBR, consistent with the results of the P requirement experiments. Under N-limiting conditions (Region II), the PHB-C synthesised/acetate-C removed



**Figure 7**  
The effect of influent COD/N ratio on P released/acetate-carbon removed ratio of an AnA SBR



**Figure 8**  
The effect of influent COD/N ratio on PHB-carbon synthesised/acetate-carbon removed ratio during the first 110 min of the cycle of an AnA and a CA SBR

of the CA sludge increased while that of the AnA sludge was relatively constant. In Region III, the PHB-C synthesised/acetate-C of the AnA and CA sludges were similar.

The influent COD/N ratio had no effect on the CH-C degraded/acetate-C stoichiometry for the AnA SBR, but the CA CH-C degraded/acetate-C removed ratio increased as the N loading was reduced (Fig. 9). This finding is consistent with the increased synthesis of PHB in the CA SBR because CH degradation provides reducing equivalents (i.e. NADH) required for PHA synthesis. The AnA CH degradation stoichiometry was consistent with that of a GAO-dominated system.

## Discussion

### Extending the study of anaerobic acetate removal

Liu et al., 1997 and Schuler 1998 investigated anaerobic stoichiometry up to an influent COD/P ratio of 130. The current work extended the study up to an influent COD/P ratio of 160 (with

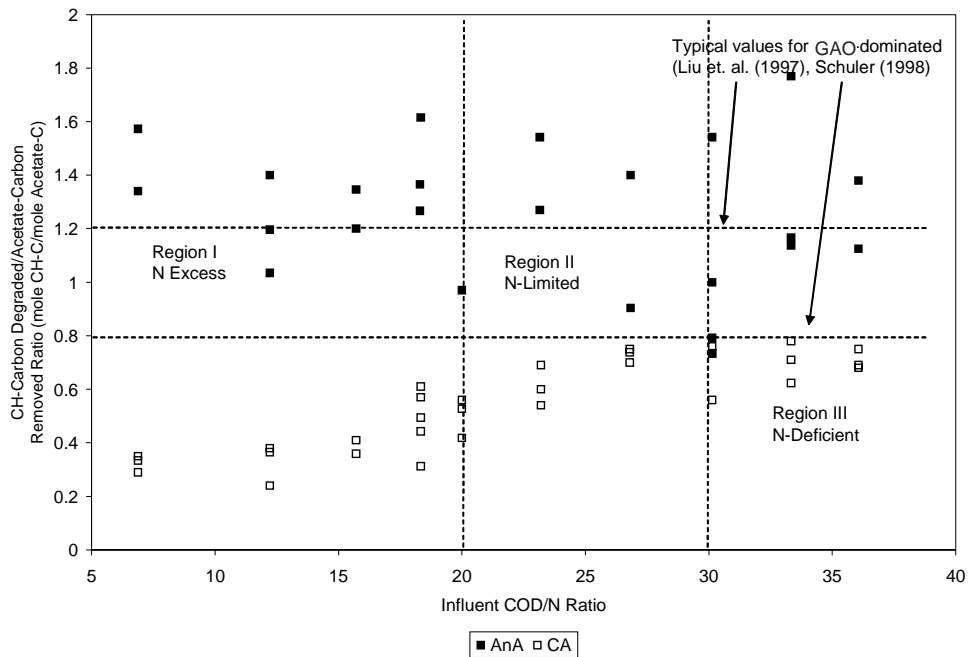
P deficiency occurring at 140). As the influent COD/P ratio is increased, anaerobic acetate removal rates linearly decrease to 0.8 mg acetic acid/g VSS/min – the value found when P deficiency is reached. Also, anaerobic stoichiometry showed that GAOs were dominant at high COD/P ratios, which is in good agreement with Liu et al., 1997 and Schuler 1998. Thus, this study helps form a more complete set of basic information about anaerobic acetate removal in AnA systems over a wider range of influent COD/P ratios.

### Anaerobic P release

The low P loading experiments showed that P release was maintained from influent COD/P ratios of 90 to 120, even though the PHA and CH stoichiometry showed that the GAOs were dominant in the AnA. This finding shows that P release characteristics can be maintained in a sludge that is P limited. In terms of using an AnA system for treating P deficient wastewater while meeting low effluent P limits, this finding means that treatment plants receiving



**Figure 9**  
Effect of influent COD/N ratio on CH-carbon degraded/acetate-carbon removed ratio during the first 110 min of the cycle of an AnA and a CA SBR



P deficient wastewater can observe PAO activity by carrying out P addition to adjust the influent COD/P ratio between 90 and 120. This influent COD/P range will allow AnA P release characteristics to be observed with a low biomass P content. In turn, this will allow low effluent total P levels. The low N loading experiments showed that P release can be maintained during N limitation, even while the GAOs were dominant. This finding shows that the advantages of operating an AnA system for P deficient wastewaters with effluent P limitations can be maintained if the wastewater is N limited.

### Storage product stoichiometry

Less PHB was synthesised per mole acetate-C removed in the CA SBR than in the AnA SBR under most of the influent COD/N and COD/P conditions tested. This may have occurred because a significant fraction of acetate taken up during the first 110 minutes in the CA SBR was used for cell growth; while the absence of oxygen (or nitrate) in the AnA SBR promoted more PHB synthesis. This result can be understood by considering that, during the anaerobic phase of the AnA SBR, PHA synthesis is used in place of respiration to reoxidise NAD(P)H (i.e. PHB synthesis replaces respiration as an electron sink during the anaerobic phase). The fact that less CH was degraded per mole acetate-C removed in CA SBR, which is consistent with less PHB being synthesised since CH degradation may be used as a source of reducing equivalents for PHA synthesis.

N limitation affected the amount of PHB synthesised per mole acetate-C removed for both sludges and the CH-C degraded per mole acetate-C removed ratios of the CA sludge. This finding shows that modeling of the AnA process must take N limitations into account when selecting stoichiometric values for PHB synthesis and CH degradation.

### Anaerobic removal rates

Aerobic acetate removal rates were 20 to 40% higher than anaerobic removal rates when N and P were in excess. This means that implementing an initial anaerobic zone will result in lower acetate removal rates. This may not affect the activated sludge basin volume required for substrate removal since the anaerobic stage is

followed by an aerobic stage, where acetate is removed more rapidly. Normally, AnA design criteria typically specify an anaerobic HRT in the range of 10 to 30% of the total aeration basin HRT (Water Environment Federation, 1998), and adherence to these criteria may prevent effluent acetate. However, there may also be cases where implementing an initial anaerobic zone will make it necessary to increase the aerobic residence time to avoid substrate breakthrough.

### Future research

This research used acetate as the primary carbon source. Carbohydrate should be used in future research, because it is an important carbon source in nutrient-deficient wastewaters from industries like pulp and paper or food and beverage. Higher temperatures, longer MCRTs, and different reactor configurations (i.e. completely-mixed continuous-flow system) should also be used in future efforts.

### Conclusions

The effect of an initial anaerobic zone on acetate removal kinetics and stoichiometry during nutrient-limiting conditions was investigated using an AnA and CA SBR. The conclusions are as follows:

- CA acetate removal rates were approximately 20 to 40% higher than AnA acetate removal rates when N and P were in excess. When P was deficient, the acetate removal rate was the same for both sludges (0.8 mg acetic acid/g VSS/min). N limitation had no effect on acetate removal rates for either system, but N deficiency decreased the acetate removal rates of both sludges by approximately 50%.
- Anaerobic P release and CH degradation stoichiometry showed that there was low-level PAO activity in an AnA sludge dominated by GAOs. The P release/acetate-C removed ratio decreased linearly as the P loading was decreased, but was unaffected by N limitation.
- The AnA SBR synthesised 2 to 5 times more PHB-C/acetate-C removed than the CA SBR. The CA PHB-C synthesised/acetate-C removed ratio was influenced by both P deficiency and N limitation.

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