Evaluating the Impact of Municipal Wastewater Strength on Granulation in Sequencing Batch Reactors

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Abstract

The impact of varying municipal wastewater strength on the formation and development of aerobic granular sludge (AGS) was investigated using three 5 L sequence batch reactors (SBRs). To maintain the same organic loading rate, cycle lengths and primary effluent (PE) volumes were adjusted. The average influent chemical oxygen demand (COD) concentration for Reactors 1, 2, and 3 was 140±36.0 mg/L, 344±89.2 mg/L, and 503±145 mg/L, respectively.

Reactor 1 was fed dilute PE and successfully developed dense, spherical granules with a De Brouckere mean diameter of at least 0.6 mm within three months of steady operation. Throughout the study, Reactor 2 (fed unaltered PE) and Reactor 3 (fed high-strength PE) both contained a higher presence of flocs rather than granules. Although Reactor 3 displayed the highest phosphorus removal efficiency amongst all three reactors, granules did not mature or stabilize.

At steady state, the average SVI_{30} for Reactor 1 was 85±8 mL/gVSS, whereas the average SVI_{30} for Reactors 2 and 3 exceeded 150 mL/gVSS for most of the study. The average soluble COD removal rates for Reactors 1, 2, and 3 were 83%, 89%, and 93%, respectively. The average NH_4-N removal for Reactors 2 and 3 was greater than 98% throughout the study. For Reactor 1, the removal rate declined from 96% to 65% after the aerobic period was shortened on day 159. For all reactors, the average PO_4-P removal was poor throughout the study. However, on day 126 onwards, Reactor 3's

orthophosphate removal was 99%. After extending Reactor 1's anaerobic period on day 159, the orthophosphate removal improved to 88%. From day 132 onward, Reactor 2 achieved an average PO_4-P removal of 70%.

Overall, this study demonstrated that shorter settling and decanting times (under 5 minutes), combined with an HRT of 6 hours, created a high selective pressure and led to the development of dense, round granules in Reactor 1. In contrast, extended settling, slow decanting, and longer HRTs favoured floc formation and granule destabilization. During early operation, aerobic granulation was achievable without the presence of phosphate-accumulating organisms, provided that strong feast–famine conditions were maintained. This study highlights that AGS can be developed and maintained in low-strength wastewater and its potential application in municipal wastewater treatment plants.

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List of Abbreviations

Activated sludge	AS
Aerobic granular sludge	AGS
Ammonia-oxidizing bacteria	AOB
Biological nutrient removal	BNR
Chemical oxygen demand	COD
Conventional activated sludge	CAS
Dissolved oxygen	DO
Extracellular polymeric substance	EPS
Flow injection analysis	FIA
Food-to-microorganism ratio	F/M
Glycogen-accumulating organism	GAO
Hydraulic retention time	HRT
Mixed liquor suspended solids	MLSS
Mixed liquor volatile suspended solids	MLVSS
Nitrite-oxidizing bacteria	NOB
Oxygen-reduction potential	ORP
Phosphate-accumulating organisms	PAO
Primary effluent	PE
Return activated sludge	RAS
Sequence batch reactor	SBR
Sludge retention time	SRT
Sludge volume index	SVI
Soluble chemical oxygen demand	sCOD
Total suspended solids	TSS
Volatile suspended solids	VSS
Volume exchange ratio	VER
Wastewater treatment plant	WWTP
West End Water Pollution Control Centre	WEWPCC

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Fly you fools!

Introduction

Background of the Study

Municipal wastewater effluent quality affects the environment, aquatic ecosystems, and Manitobans. Wastewater treatment plants adhere to regulations that outline discharge limitations for environmental compliance [1], [2]. Effluent that does not adhere to discharge limits can impact water bodies as excessive nutrient limitations of nitrogen and phosphorus may result in eutrophication [3]. As a result, nutrients enrich plants like algae to overtake the available oxygen for other aquatic species to survive. Algae bloom also affects the taste and odour of drinking water, directly affecting the health of users [4]. As Winnipeg's population and urban development are projected to continue growing, the issue of eutrophication is more prominent. Higher pressures are placed on existing wastewater collection systems and treatment plants to meet demand.

In Winnipeg, three wastewater treatment plants utilize the activated sludge (AS) process to remove contaminants and solids. This biological process is widely used in Canada, yet high operational costs are incurred during the aeration phase of treatment and a large footprint to operate is required [5]. The energy used in the aeration process is 40 – 60% of total energy demand at the treatment plant [5].

Aerobic granular sludge (AGS) is a type of microbial aggregate that has a compact structure with distinct layers of microbial communities [6]. Typically, the outer layer consists of aerobic bacteria, while deeper layers may contain anoxic or anaerobic bacteria

[7]. Compared to conventional activated sludge (CAS), nitrogen, carbon, and phosphorus removal can be achieved in the same biofilm [8]. Other advantages to an AGS process include higher biomass inventory, faster settling, and lower energy costs [9], [10]. A key advantage of reduced waste activated sludge generation is the reduction of solids fed to downstream digestion processes.

Many studies have shown the development of AGS in a sequence batch reactor (SBR) and factors that affect the growth and stability of granules [7]. The predominant factors include substrate source, organic loading rate (OLR), hydrodynamic shear force, settling time, feast and famine regime, hydraulic retention time (HRT), and volume exchange ratio (VER) [11].

Objectives of the Study

In this study, three distinct sequence batch reactors, Reactor 1 (R1), Reactor 2 (R2), and Reactor 3 (R3), were fed three varying strengths of municipal wastewater, primary effluent (PE), from Winnipeg's West End Water Pollution Control Center (WEWPPC). The objective was to determine the impact municipal wastewater strength has on developing and maintaining aerobic granular sludge. To evaluate the influence of wastewater characteristics on the formation of granules, the organic loading rate was kept constant among all reactors by adjusting the number of cycles and feed volume per day. This resulted in a different HRT per reactor.

R1 – fed diluted PE, 50% PE and 50% tap water.

R2 – fed unaltered PE, control reactor.

R3 – fed high-strength PE, dosed with sodium acetate trihydrate ($CH_3COONa \bullet 3H_2O$), ammonium chloride (NH_4Cl), and potassium dihydrogen phosphate (KH_2PO_4) to double the feed concentration of nutrients and food.

Research Questions or Hypotheses

Does municipal wastewater strength impact the formation and stability of aerobic granular sludge?

Hypothesis #1: The high-strength wastewater fed to Reactor 3 will result in granulation occurring first amongst the three reactors. As more nutrients are available for microbial growth, rapid formation of dense, round granules of at least 0.2 mm and $SVI_{30}/SVI_5 = 1$ is predicted.

Hypothesis #2: Reactor 3 is predicted to maintain robust granules when compared to R1 and R2, as higher nutrients are available in the feed. R1 is predicted to provide the least effective nutrient removal, as less food is available in the influent for biomass to achieve adequate phosphorus removal and denitrification.

Significance of the Study

The application of aerobic granular sludge in wastewater treatment plants could be significant for plants that have physical site constraints or fail to meet final effluent requirements. As municipal wastewater is considered low strength in terms of its organic concentration or chemical oxygen demand (COD), it would be interesting to see if further dilution or additional substrates impact the development or stability of granules. Although wastewater influent to WEWPCC is not collected via a combined water system, heavy rainfall or snowmelt affects the influent organic concentration. For the AGS process to be adopted by a treatment plant in Manitoba, the system must be resilient to diverse feed quality. Overall, retrofitting older treatment plants is more feasible with the AGS process as the footprint requirement is 40 – 50% less compared to the CAS process [12].

Scope and Limitations

The reaction phase consisted of two operating conditions: unaerated filling and aerated reaction. Dissolved oxygen was not controlled during the aerobic period, as the optimization of denitrification was not in the scope of the project. Similarly, pH and temperature were not controlled but occasionally monitored. Additionally, the effects of hydrodynamic shear force were not monitored during the study.

Literature Review

Aerobic Granular Sludge

Seed sludge

To initiate the experiment, inoculation with municipal activated sludge or mature granules from a prior study was considered. Many studies have used conventional activated sludge (CAS) as the inoculum [13], [14], [15], [9]. In many cases, filamentous bacteria are abundant in the activated sludge (AS), resulting in a high sludge volume index (SVI) over 150 mL/g [16]. Sometimes, AS is preconditioned with a specific substrate source, like phenol, to acclimate the sludge [15]. Other studies have utilized anaerobic granular sludge as a seeding source to cultivate aerobic granular sludge, resulting in an accelerated start-up period and stable treatment removal [17]. Another method used for inoculation is the mixture of floccular and crushed granules, which has also been found to speed up the granulation process [8]. The addition of mature granules provides an established microbial community which contributes to granular morphology and nutrient removal functions [8], [18].

In a case study at the Österröd wastewater treatment plant (WWTP) in Strömstad, Sweden, Reactor 1 was seeded with AS from a neighbouring WWTP. Reactor 2 was seeded with granules from an established AGS plant in the Netherlands. Throughout the study, Reactor 2 formed and maintained large, compact, and mostly spherical granules. Good settling was seen with SVI_{30} ranging between 50 – 80 mL/g and MLSS concentration increasing to 8 g/L. Contrarily, Reactor 1 formed slow, small, irregularly

shaped granules with finger-like outgrowths. Poor settling was noticed as seen in the measured SVI_{30}, ranging from 70 – 150 mL/g. Reactor 1 MLSS was maintained between 2 – 3 g/L throughout the study [2]. As a result, Reactor 1 was reseeded with granular sludge from Reactor 2. After reseeding, the abundance of polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) increased in Reactor 1, corresponding with improved SVI and the formation of large granules. Granules with irregular and filamentous outgrowths were common in both R1 and R2, possibly due to high concentrations of particulate organic matter, which can affect settling properties [19], [20]. As municipal wastewater was used to feed both reactors, the average COD fluctuated seasonally, with the highest concentration reported during July to September (312 ± 127 mg/L).

Selective pressure factors

Substrate source

Aerobic granular sludge can be cultivated using different substrate sources, such as acetate, glucose, phenol, synthetic, industrial, and municipal wastewater [21] [1] [15] [22] [23] [2]. Studies have investigated the impact different substrate sources have on the size, shape, and settling velocity of granules [24], [25], [26]. It is generally suggested that substrates not readily consumed during the anaerobic phase, like particulate COD in municipal wastewater, lead to flocculent growth in the aerobic phase, potentially hindering granulation structure [19], [20]. Further, the substrate source may have an impact on the microbial community, like the presence of PAOs [20]. The genus *Candidatus Accumulibacter* is a dominant PAO associated with the treatment of simple wastewater, whereas *Tetrasphaera* abundance is higher when treating complex monomeric wastewater [20].

For example, in a study utilizing synthetic wastewater feed and phenol as the sole carbon source, 80% by volume of granules were between 0.35 mm and 0.60 mm in diameter at steady state. There was no impact reported in terms of the granule's surface appearance, as granules were described as compact and smooth. From day 21 to the end of the study, the SVI was approximately 40 mL/g.

In another study, it was found that utilizing glucose as the sole carbon source led to the growth of filamentous bacteria as seen by the granule's fluffy surface [24]. Whereas the SBR using acetate as the substrate source developed a smooth surface with few

filaments and a very compact bacterial structure. The average size of glucose-fed and acetate-fed granules was 2.4 mm and 1.1 mm, respectively. The average SVI_{30} was similar between both reactors, ranging from 50 – 80 mL/g. Both reactors maintained an OLR of 6 kg/m³/d.

A separate study combined industrial and municipal wastewater as a substrate source. The composition of the industrial influent derived from the automobile, food production, and pharmaceutical sectors, which accounted for roughly 60% of total feed [27]. The average granule size was 1.2 mm, and the surface appearance was reported to be dense and spherical. Gradually, the granules changed from a tan to a dark brown colour. This colour change is attributed to pigment absorption. The peak SVI_{30} reported during the study was 56 mL/gTSS.

Organic loading rate

Higher organic loading rates (OLRs) have been found to encourage the formation and size of granules, shortening the start-up period [26]. As more food is available, accelerated growth is possible and can stimulate extracellular polymeric substances (EPS) [28]. This also indicates that AGS are resilient to a high OLR [29]. Generally, it is suggested that AGS can form within the OLR range of 2.5 – 15 kgCOD/m³/d [11]. However, high OLRs can lead to the disintegration of granules, affecting their dense structure to become fluffier and looser [26], [30], [21]. The long-term stability of granules is a limitation to the AGS process as excessive growth of filamentous populations contributes to granule disintegration and slow settling [7]. A recommended strategy is to

use a high OLR to quickly form granules, followed by a reduced OLR to ensure AGS stability [26].

A minimum influent COD concentration of 200 mg/L and an OLR of 0.6 kgCOD/m³/d has been recommended to maintain granules [31]. In one case study, the long-term stability of AGS was tested in an SBR with low-strength domestic wastewater (COD < 120 mg/L) from a WWTP in Florence, Italy. The selective pressure applied was metabolic, meaning the complete uptake of biodegradable COD (bCOD) was achieved during the anaerobic phase [31]. Using this method minimizes the formation of filamentous flocs and ensures that PAOs and GAOs do not compete with aerobic heterotrophic bacteria for food. The study was divided into two stages: Stage A and Stage B. To develop granules quickly, sodium acetate and phosphorus were added to municipal wastewater to obtain an influent COD of 290 ± 44 mg/L during Stage A. Once granules formed, additional chemical dosing to the influent stopped, and the SBR was fed low-strength wastewater of 115 ± 23 mg/L COD. During Stage B, granules remained stable and large, with an average granule diameter of 1.5 mm. The performance of the system was effective as phosphorus removal averaged 74 ± 24% and the SVI_{30} averaged 28 mL/g TSS during Stage B. This study challenged the conception that a minimum influent COD concentration was required. However, the start-up period or Stage A did not use low-strength municipal wastewater to determine if granules can form under nutrient-limited conditions.

Hydrodynamic shear force

Hydrodynamic shear force refers to the air velocity based on the cross-sectional area of the reactor during the aeration phase (superficial upflow air velocity). Generally, a higher

air flow rate results in a higher shear force, which is correlated to the formation of robust and denser granules [24]. During high turbulence, bacteria will release extracellular polymeric substances, which encourage microbial aggregation [3], [11]. Higher shear force may also limit the growth of faster-growing microorganisms, like ordinary heterotrophic organisms, from the granule's surface [22]. As a result, this promotes the roundness and smoothness of granules, preventing the formation of a filamentous surface layer [32]. However, excessively high aeration rates can lead to granule disintegration and the formation of filaments [27]. This indicates that there is an optimal range for the hydrodynamic shear force applied to the system. A minimum superficial upflow air velocity of 1.2 cm/s is reported for granule formation [26], [33]. Yet, the threshold is dependent on the OLR. One study found that high hydrodynamic shear force is not necessary for granulation to occur in low-strength synthetic wastewater. In the study, five SBRs were operated: three low-strength reactors, one medium-strength reactor, and one high-strength reactor. All reactors were operated with a constant low upflow superficial air velocity of 0.41 cm/s. The average COD of the low-strength, medium-strength, and high-strength wastewater was 340, 630, and 1300 mg/L, respectively. The reactors fed low-strength wastewater formed compact, smooth granules that achieved nitrogen and biological phosphorus removal. The medium-strength reactor developed granules with a filamentous surface layer. Although phosphorus removal improved during the study, nitrification remained poor. Evidently, the high-strength reactor did not develop granules, and nutrient removal performance was poor. This study showed that high hydrodynamic shear force is not a necessary selective pressure to develop AGS when feeding low-strength wastewater.

Settling time

A short settling time is believed to be a key strategy for forming quick settling granules [11]. During the decanting phase of an SBR process, lighter biomass is wasted from the reactor, and denser biomass remains [28]. The aggregation of heavier biomass leads to the formation of round, dense granules [34]. Short settling times can also promote the growth of EPS producing bacteria, aiding granule structure and stability [35]. The production of EPS is critical for increasing cell adhesion [35]. If the settling time is too long, fluffy flocs are not effectively wasted and can outcompete granules for nutrients, hindering the formation of AGS [11]. Conversely, if the settling time is too short, biomass washout of fast-settling sludge can occur [26]. The ideal reported settling time is 5 minutes [35], [10], [26].

However, one study found that once granules in a reactor have formed, a shorter settling time no longer acts as a strong selective pressure on the microbial community. During the first phase of the study, three reactors were operated under the same conditions (VER, OLR, reaction time) except for settling time. Reactor 1, 2, and 3 settling times were 10, 7, and 5 minutes, respectively. By the second week of phase 1, MLVSS increased, and granules with a diameter of 2.3 mm were present in Reactor 3. During the second phase, the settling time of all three reactors was 5 minutes. Consequently, the microbial community in Reactor 1 and 2 shifted, mirroring the same species found in Reactor 3, including EPS-producing bacteria like *Zoogloea resiniphila*, *Chryseobacterium gleum strain 2-1-9a*, and *Rhodocyclales bacterium TP411*. Additionally, it was reported that a shorter settling time encouraged the removal of non-flocculating species, like

Pseudomonas sp. HPC337 and *Flavobacterium sp. YL–10*. The washout of these non-flocculating species reduced the competition for strains contributing to granulation. These functional strains are known to produce EPS, which is critical to the formation of aerobic granules [35]. Yet in Reactor 3, the functional strains had already formed during phase 1, so minor changes in terms of the reactor's microbial community were noticed. As a result, it was observed that once granules are formed and the key microbial groups are present, the impact of quick settling as a selective pressure is negligible.

Feast and famine

The feast-famine regime involves a short feeding period, where readily biodegradable COD is quickly consumed by bacteria, and is followed by a longer famine phase, where food is depleted and bacteria rely on stored carbon reserves [7]. By extending the aerobic phase, bacteria become more hydrophobic and tend to aggregate more easily as the cell-to-cell affinity increases [24]. The continuous cycle of feast and famine periods, especially a long famine period after a short feast, promotes the production and consumption of EPS [7], [36]. During the famine phase, EPS serve as a carbon and energy source for endogenous respiration [37]. Also, the feast-famine regime controls the growth of fast-growing heterotrophic microorganisms that consume readily available carbon, which can lead to granule disintegration [26]. Ultimately, this microbial selection pressure influences granule density and compactness [11], [24].

One study used a 1:3 feast-famine ratio to promote the growth of slow-growing bacteria such as PAOs and to limit the growth of heterotrophic bacteria [7]. A 60-minute anaerobic feeding period duration was more effective compared to 47 minutes, as higher phosphate-

P removal was reported. In general, studies have recommended that the famine period be higher than 60% of the cycle time to control fast-growing heterotrophic bacteria and increase the production and consumption of EPS [36] [23].

Hydraulic Retention Time

Commonly, a shorter hydraulic retention time (HRT) or cycle time, between 6 to 13 hours, results in a higher selective pressure being applied on the granules [7], [38]. If the HRT is too long, then lighter flocs remain in the system instead of being washed out during the decanting phase. Conversely, a short HRT (2 – 6 hours) can lead to biomass washout, wasting slow-growing microorganisms, like PAOs [2]. It is suggested that HRT strongly influences cell hydrophobicity [39]. HRTs exceeding 12 hours can drastically decrease cell hydrophobicity as EPS is consumed during excessive starvation, impeding cell aggregation [39]. In terms of microbial community presence, shorter HRTs favour the growth of anammox bacteria as the volume of influent nitrogen and carbon is greater, yet lengthening HRT promotes the growth of ammonia-oxidizing bacteria (AOB) [40]. For long-term AGS stability, it is recommended to operate with a higher OLR until granulation is observed, and then lengthen the HRT to optimize the presence of EPS producers and AOBs [28], [41], [39].

The influence of HRT on AGS performance was investigated in an SBR treating rubber wastewater. At the beginning of the study, the HRT was 24 hours and over time, the HRT was reduced to 12 hours and finally to 6 hours. The physical characteristics and performance of AGS improved as HRT was lowered. A larger granule diameter was

attributed to the higher OLR and lower shear force during the aeration phase when HRT was 6 hours. In addition, sludge settleability improved at a lower HRT as granules were dense and compact in shape [3]. Overall, a higher hydraulic selective pressure was observed to enhance EPS production and form dense and stable granules.

Volume Exchange Ratio

The volume exchange ratio (VER) provides a hydraulic pressure on the system, influencing the formation of granules [42]. The selective pressure increases when the travelling distance to the decanting port is greater [43]. Dense granules will settle quickly, whereas light, fluffy flocs are wasted as they have a low settling velocity. The presence of spherical granules in a bioreactor increases with higher VERs [44] [45]. The diameter of granules tends to increase with the increasing minimum settling velocity, which is influenced by VER [43]. A decrease in VER may lead to poor settleability, resulting in a higher SVI [27]. In general, a VER between 50% and 75% is considered to promote sludge settleability and stability [27], [8], [26].

At higher VERs, a larger volume of substrate is introduced per cycle, increasing the OLR [27]. In one study, four reactors were operated at varying VERs: 20%, 40%, 60%, and 80%. It was reported that higher VERs accelerated the formation of granules. Specifically, granules formed within 2 days at an 80% VER, whereas formation took 20 days at a 20% VER. Overall, it was observed that a higher travel distance to the discharging port improves sludge settleability, produces larger, more spherical granules, and increases EPS production [43].

Food to Microorganism Ratio

Previous studies have proposed that a high F/M ratio promotes the rapid formation and growth of granules [28] [46]. Moreover, one investigation observed that the relationship between the F/M ratio applied and the average diameter of mature granules is almost linear [46]. Low F/M ratios are considered in the range of 0.2 – 0.4 gCOD/gVSS/d, whereas high F/M ratios have been reported upwards of 2.0 gCOD/gVSS/d [18], [47]. As the F/M ratio is directly related to OLR, a higher ratio indicates that more food is available, favouring fast-growing bacteria. However, high F/M ratios may lead to granular instability and disintegration [26]. The F/M ratio also has an impact on the abundance of bacteria found in SBRs. Denitrifiers like *Thauera* and *Dechloromonas*, and nitrifying bacteria such as *Nitrosomonas* are abundant in high F/M ratios [18]. Whereas the nitrifier *Nitrospira*, and EPS producers *Tahibacter*, *Ohtaekwangia*, and *Chryseobacterium* are more abundant in low F/M ranges [18]. It is suggested that as the F/M ratio increases, so does the quantity of EPS, ultimately encouraging the formation and structure of AGS [47].

Another investigation tested the long-term stability of AGS fed low-strength wastewater with a low COD/N ratio. By day 80, granules formed, and the peak size granule was reported at 471 µm on day 140. Between days 140 and 210, granules began to disintegrate, becoming smaller and fluffier in appearance. The study concluded that the low F/M ratio impacted the AGS stability, and a range between 0.3 to 1.0 gCOD/gSS/d is optimal for long-term stability [41].

Microbial Communities in AGS

As reported in other studies, a microbial community shift is noticed when seed sludge transitions to aerobic granular sludge [48], [2]. AGS is an aggregation of microorganisms creating a layered, spherical structure, which enables the granule to perform simultaneous carbon, nitrogen, and phosphorus removal in a single reactor [7], [9]. There are key microbial groups that contribute to the development and stability of AGS, including EPS producers, denitrifying and nitrifying bacteria, and PAOs. It has been reported that the microbial diversity of granules is related to the substrate source fed during development [49]. For example, the phylum *Proteobacteria* was dominant in granules developed from utilizing municipal wastewater [45].

Mature granules are embedded in a matrix of EPS, primarily composed of proteins and polysaccharides, which contribute to cell aggregation and structure [9]. Common EPS producers found in AGS include *Zoogloea*, *Thauera*, *Flavobacterium*, *Meganema*, and *Acinetobacter* [7], [50], [18], [9]. *Thauera* is typically found in municipal activated sludge, and is recognized as a heterotrophic denitrifier and EPS producer [7].

Ammonia-oxidizing bacteria (AOB) can reduce ammonia in influent wastewater to nitrite, and the growth of AOBs is suggested to improve AGS stability [7], [40]. AOBs are usually enriched in the outer granule layer where oxygen is available [17]. *Nitrosomonas* is a common AOB found in AGS reactors treating municipal wastewater [2], [49]. *Nitrosomonas* can treat wastewater of high ammonium concentrations [7].

Nitrite-oxidizing bacteria (NOB) can reduce nitrite to nitrate, and are often found in the aerobic outer layers of granules [41], [6]. Common NOBs found in AGS include *Candidatus Nitrotoga* and *Nitrospira* [2], [51].

Denitrifying bacteria can reduce nitrate and nitrite to nitrogen gas and are often found in the anoxic core of granules [34]. Many denitrifying bacteria are also EPS producers, contributing to the structural stability and aggregation of the granules [9]. Usual genera detected in AGS systems include *Thauera*, *Flavobacterium*, *Azoarcus*, *Paracoccus*, and *Pseudomonas* [7], [52], [53], [54].

PAOs are key bacteria for phosphorus removal and supporting granule density and stability, particularly under feast and famine conditions [55]. The PAO *Candidatus Accumulibacter* is suggested to contribute to granule formation for its role in transcribing genes related to EPS [14]. *Candidatus Accumulibacter* is known to utilize acetate as a substrate source [19]. *Tetrasphaera* is recognized as a fermenting PAO often found in municipal wastewater treatment plants [56], [57]. PAOs accumulate polyphosphate and have a higher density than GAOs, leading to better settling and higher biomass retention [25] [58]. *Dechloromonas* and *Gemmatimonas* are also noted for PAO activity [9], [59].

Identification of research gaps

Several studies have tested various selective pressures to determine the effectiveness in forming and maintaining AGS. This study will focus on short settling and decanting time and HRT as a selective pressure while maintaining a constant OLR. During the start-up phase, the settling time for all reactors will be set to 15 minutes and changed based on each reactor's performance during the study. Settling time will be lowered as MLVSS surpasses 3.5 g/L. Also, the target effluent withdrawal is under 5 minutes. A quicker decanting period is predicted to encourage the removal of light, fluffy biomass. During start-up, each reactor will be fed RAS from an existing WWTP, instead of using external matured granules for inoculum.

To consider the feasibility of utilizing AGS for biological nutrient removal in municipal WWTPs, it is important to test the formation and stability of granules when fed varying strengths of wastewater, especially when considering WWTPs that are impacted by a combined sewer system. Previous studies that report using low-strength real domestic wastewater as a substrate source contain a total COD of at least 200 mg/L [45] [41]. Whereas Reactor 1 will be diluted to closely resemble influent quality during wet weather flow, specifically periods of high snowmelt and rainfall. Although previous research has proven that granules can achieve long-term stability when feeding low-strength wastewater, during the start-up phase, the reactor was fed a higher COD concentration [8]. Only after granules had stabilized, reactors were fed low-strength wastewater. In this study, reactors will be fed varying-strength municipal wastewater throughout the whole operation. As a result, this study will address whether feeding diluted wastewater can

develop and sustain the growth of AGS. Moreover, the study will explore if PAOs can be sustained in nutrient-limited conditions.

Methodology

Reactor Design and Operation

The three SBRs were constructed from a 14.6 cm inner diameter acrylic rigid column. Each reactor had a working volume of 5 L. Reactors were inoculated with Winnipeg's West End Water Pollution Control Centre (WEWPCC) return activated sludge (RAS), with an approximate mixed liquor suspended solids (MLSS) of 9500 mg/L, to achieve a MLSS of 3000 mg/L. Primary effluent (PE) from WEWPCC was used for feeding each reactor. PE was collected in 25 L Jerry cans and stored for a maximum of 3 days at 4 °C. Wastewater was transferred to respective feeding containers daily, where it was maintained at ambient temperature 20 ±1 °C. The average quality of PE was 344 ± 89.2 mg/L COD, 230 ± 68.3 mg/L sCOD, 35.3 ± 7.04 mg/L NH_4-N, 3.72 ± 0.53 mg/L PO_4-P.

Reactor 1 was fed diluted PE, 50% tap water by feeding volume; Reactor 2 was fed unaltered PE; and Reactor 3 was fed high-strength PE, with the additional dosing of ammonium chloride, dihydrogen potassium phosphate, and sodium acetate trihydrate (99% purity and suppliers Sigma-Aldrich and Fisher Scientific). Chemical addition was used to double the incoming PE concentration of ammonium, orthophosphate, and sCOD. In other studies, chlorine has been shown to negatively impact biomass structure and nitrification EPS [60] [61]. Chlorine can inhibit nitrifiers, resulting in higher concentrations of ammonium and COD in the final effluent [60]. In this study, residual chlorine in Winnipeg's tap water was not measured. However, Winnipeg's water must contain at least 0.1 mg/L of free chlorine residual as per the City's Operating Licence [62].

Each reactor underwent the following phases: unaerated filling, aerobic reaction, settling, decanting, and idling, as depicted in Figure 1. All feeding and decanting pumps were peristaltic. During the aeration phase, dissolved oxygen was not controlled but maintained to be greater than 2.5 mg/L. Each reactor had two-cylinder air stones, air supplied by a 15 US-gallon air pump. 50% VER was achieved by placing tubing at the 2.5 L mark. Plug flow feeding or simultaneous feeding and effluent discharge was not implemented in the study. During the feeding phase, no aeration was introduced to the reactors. Nitrogen gas was not utilized to remove oxygen from PE, tap water, or any reactor. The feeding phase was un-aerated, and the presence of nitrite and nitrate was detected in influent PE. From day 0 to day 84, feeding was achieved via tubing secured on the top of each reactor, filling from top down with mixing. On day 85, tubing was secured under the decant level (roughly 2 L mark) to minimize splashing on the surface of the reactor and introduction of air. Reactors were placed on top of a benchmark magnetic stirrer. During the filling phase, mixing was achieved using a magnetic stirring bar. Mixing was set to the lowest setting possible to create a low-shear environment. Decanting pumps varied in speed. The highest decanting flow achieved (to remove supernatant) was 1250 mL per minute. The lowest decanting flow achieved was 250 mL per minute. Although fed varying strengths of PE, the goal of the project was to maintain the same OLR across all reactors. To achieve this, each reactor had varying phase durations as seen below in Table 1. Moreover, the number of cycles, feeding rates, and HRT varied across reactors, as seen in

Table 2. Cycles were controlled using 7-day timing outlet timers from McMaster Carr. Pump feeding lines were disinfected using a diluted Javex solution on a biweekly basis. The operational set-up is shown in Figure 2.

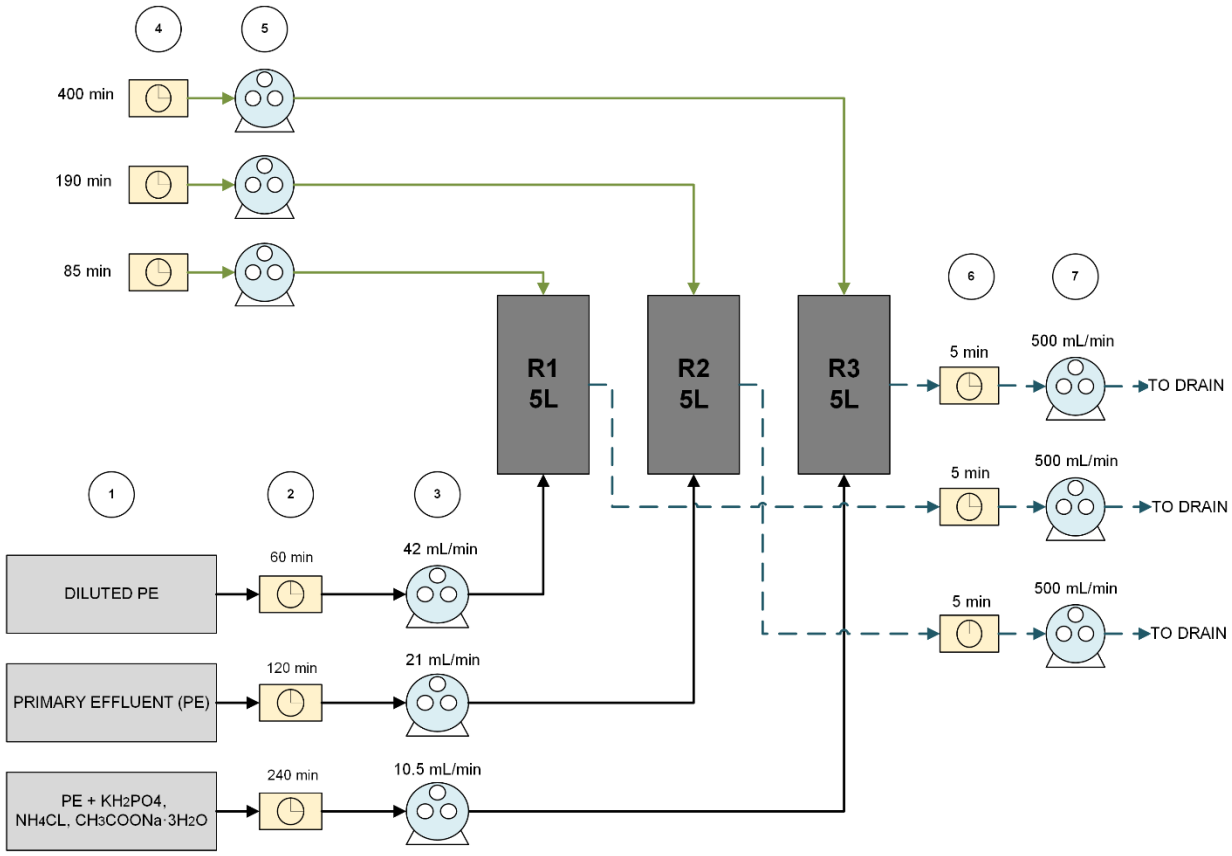


Figure 1: SBR set-up and major equipment. 1. PE feeding tanks; 2. feeding timers; 3. feeding pumps; 4. air timers; 5. air pumps; 6. decant timers; 7. decant pumps.



Figure 2: Lab space with operational set-up.

Table 1: Duration of each phase per reactor cycle. On day 159, the filling time was increased from 60 to 90 minutes, and the aerobic phase was decreased from 85 to 55 minutes for Reactor 1.

	Duration [min]		
Phase	**R1**	**R2**	**R3**
Filling	60*	120	240
Aerobic	85*	190	400
Settling	15	15	15
Decanting	5	5	5
Idle	15	30	60

Table 2: Reactor Feed Volume and HRT

	Filling Volume [L/d]	Cycles in 24 h	HRT [h]
R1	20	8	6
R2	10	4	12
R3	5	2	24

Data Collection and Analysis Techniques

Samples for MLSS, mixed liquor volatile suspended solids (MLVSS), and effluent total suspended solids (TSS) were analyzed according to Standard Methods [63]. Samples were quantified in duplicates. Sludge volume index (SVI) was calculated at both 5 and 30 minutes.

For nutrient analysis, all samples were filtered using 0.45µm paper before wet-chemical analysis. Flow injection analysis (FIA) was performed using the Lachat Instrument 19 QuikChem 8500. Nutrient results included ammonium-nitrogen, soluble orthophosphate, nitrite, and nitrate. FIA testing occurred once to three times a week to determine the nutrient concentrations of influent PE and final effluent per reactor. Based on FIA results for PE, the needed chemical addition was calculated, as seen in Equation 1, Equation 2, and Equation 3.

Dissolved oxygen (DO) and oxygen reduction potential (ORP) meters were used periodically, and pH was not consistently monitored. COD and sCOD were determined using Hach kits; sCOD samples were filtered (0.45µm) before analysis.

The SteREO Discovery (Zeiss, DE) microscope was used to take micrographs of samples periodically. Magnification varied based on the growth of biomass over time. Reactor samples were sent to the Richardson Centre for Food Technology and Research facility for particle size analysis using a Malvern Mastersize 3000 instrument. Samples were collected in centrifuge vials (50 mL) on three distinct days and analyzed that same day. Centrifuge vials were gently shaken before analysis to capture a representative particle size sample.

DNA analysis was not in the scope of this project until day 126 of the study. It would have been beneficial to collect samples from the start-up and monitor how the microbial community changed over time, especially the shift in Reactor 1 as granules formed.

Samples were taken on days 132, 156, 176, 190, and 196 of the study. Samples were taken to observe any changes in the microbial community after making process changes, such as changing the decant pumps in Reactors 2 and 3 and increasing the filling phase in Reactor 1. By day 132, granulation was observed in Reactor 1, but samples taken forward reflect changes made to optimize granulation. Samples were taken during the aerobic phase, when liquid and biomass were well mixed. Then, the sample was centrifuged, and only the biomass sludge was retained for analysis. From the settled biomass sample, 0.2 g was measured for DNA extraction. The analysis uses the Microbiome Helper pipeline based on QIIME2. DNA extraction was completed by utilizing the PureLink Microbiome DNA Purification kit and stored at –80 °C until all samples were collected. Samples were then shipped to the Integrated Microbiome Resource lab at Dalhousie University in Halifax, NB, Canada, for 16S rRNA sequencing analysis. Before shipping samples, NanoDrop and Qubit were used to confirm the quality and quantity of

the extracted DNA. Samples were analyzed using the Illumina NextSeq2000, which included 2x300 bp paired-end reads providing high taxonomic resolution for the V3-V4 regions of the 16S rRNA gene [64].

Equation 1: Ammonium chloride mass added to Reactor 3 feeding container.

$$Mass\ of\ NH_4Cl\ = \frac{[Target\ concentration\ -\ PE\ concentration] \cdot Feeding\ Volume}{\frac{Molar\ Mass\ NH_4}{Molar\ Mass\ NH_4Cl} \cdot 99\%\ Purity \cdot 1000\ \frac{mg}{g}}$$

Equation 2: Dihydrogen potassium phosphate mass added to Reactor 3 feeding container.

$$Mass\ of\ KH_2PO4\ = \frac{[Target\ concentration\ -\ PE\ concentration] \cdot Feeding\ Volume}{\frac{Molar\ Mass\ PO_4}{Molar\ Mass\ KH_2PO_4} \cdot 99\%\ Purity \cdot 1000\ \frac{mg}{g}}$$

Equation 3: Sodium acetate trihydrate mass added to Reactor 3 feeding container.

$$Mass\ of\ CH_3COONa \cdot 3H_2O = \frac{[Target\ concentration\ -\ PE\ concentration] \cdot Feeding\ Volume}{\frac{Molar\ Mass\ CH_3COO^-}{Molar\ Mass\ CH_3COONa \cdot 3H_2O} \cdot 99\%\ Purity \cdot 1000\ \frac{mg}{g}}$$

Results and Discussion

Timeline of Events

DAY	0	Inoculated reactors with WEWPCC RAS.
DAY	19	Lowered the settling time of all reactors from 15 to 10 minutes.
DAY	33	Changed Reactor 3's decant pump - slow flow rate.
DAY	44	Measured the sCOD uptake during the anaerobic phase of reactors. Negligible sCOD remaining in the aerobic phase.
DAY	51	Began adding sodium acetate trihydrate to Reactor 1 and 2. Additionally, increased Reactor 3 dosing by 13%.
DAY	54	Adjusted the cycle timings for Reactors 2 and 3, to ensure the decanting phase occurred while present.
DAY	62	Lowered the settling time of Reactor 1 to 8 minutes. Reactors 2 and 3 remained at 10 minutes.
DAY	65	Granules first noticed in Reactor 1.
DAY	70	Stopped adding sodium acetate trihydrate to Reactors 1 and 2. Returned Reactor 3 dosing to 2x PE.
DAY	72	Increased Reactors 1 and 3 VER from 50% to 60%.
DAY	79	Increased settling time of Reactor 2 to 14 minutes, high solids noted in effluent.
DAY	85	Extended feed tubing towards the bottom of all reactors. Increased settling time of Reactor 2 to 16 minutes.
DAY	93	Returned the VER to 50% for Reactors 1 and 2.
DAY	94	Decreased the settling time of Reactors 1 and 3 to 7 minutes.
DAY	120	Lowered the settling time of Reactor 1 to 4 minutes.
DAY	124	Changed Reactor 3's decant pump - slow flow rate.
DAY	156	Changed Reactor 3's decant pump, new pump decants 2.5 L in 4 minutes instead of 10 minutes (previously).
DAY	159	Increased the filling time of Reactor 1 to 90 minutes and decreased the aerobic phase to 55 minutes.
DAY	183	Changed Reactor 2's decant pump, new pump decants 2.5 L in 4 minutes. Lowered Reactor 2's settling time to 12 minutes.
DAY	196	End of study.

Figure 3: Timeline of key events during the duration of the study.

Biomass Characteristics

The SBRs were operated for 196 days. All three reactors were inoculated with WEWPCC RAS to target a mixed liquor suspended solids (MLSS) of 3000 mg/L. Throughout the study, MLSS did not increase beyond 3500 mg/L across all reactors. The average MLSS for Reactors 1, 2, and 3 was 2231 ± 322.6 mg/L, 2109 ± 682.4 mg/L, and 2423 ± 697.0 mg/L, respectively. The average mixed liquor volatile suspended solids (MLVSS) for Reactors 1, 2, and 3 was 1946 ± 255.2 mg/L, 1798 ± 547.1 mg/L, and 1965 ± 562.4 mg/L, respectively. From day 140 onward, MLVSS was estimated as the 550°C furnace was out of service. An average ratio of total suspended solids to volatile suspended solids (VSS/TSS) was used to estimate MLVSS.

Reactor 1 maintained a relatively stable MLSS concentration over the duration of the study compared to Reactors 2 and 3, which experienced higher fluctuations in MLSS. Although granulation was observed in Reactor 1 since day 65, the suspended solids concentration was never above 3500 mg/L, as seen in Figure 4. In contrast, typical MLSS concentrations for matured granules reported in other studies exceed 5 g/L [24], [34], [65].

Between days 51 and 70, sodium acetate trihydrate was added to increase COD concentrations by 10% in Reactors 1 and 2, and the sodium acetate trihydrate dose was increased by 12.5% in Reactor 3. The goal of adding more sodium acetate trihydrate was to observe if higher COD concentrations improved orthophosphorus removal and increased MLSS in each reactor.

The addition of sodium acetate trihydrate to Reactor 1's feed may have influenced a slight increase in MLSS and MLVSS on day 54, yet the impact of acetate appears minimal. Similarly, Reactor 1's final effluent total suspended solids (TSS) was relatively constant throughout the study, as seen in Figure 5. The average FE TSS during the study was 27 ± 14 mg/L. The highest FE TSS observed, 59 mg/L, occurred on day 68 during the addition of sodium acetate to Reactor 1's feed. After day 120, the FE TSS increased above the average because the settling time was shortened to 4 minutes.

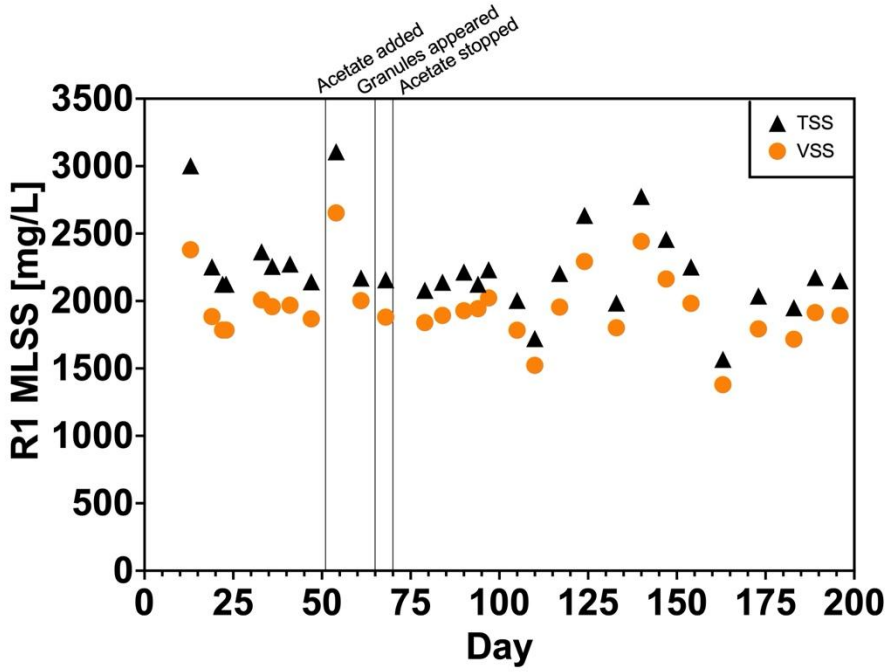


Figure 4: Reactor 1 MLSS (black triangle) and MLVSS (orange circle) throughout the study. Sodium acetate was added to the influent feed on day 51 and stopped on day 70. Granules were first observed on day 65.

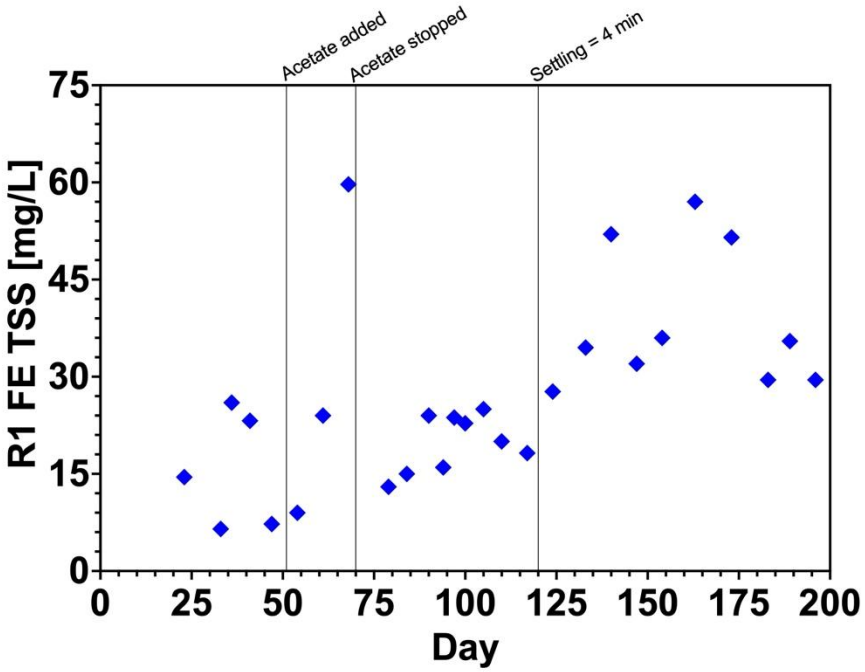


Figure 5: Reactor 1 FE TSS throughout the study. Sodium acetate was added to the influent feed on day 51 and stopped on day 70. The settling time was reduced to 4 minutes on day 120, resulting in a higher FE TSS to be reported for the remainder of the study.

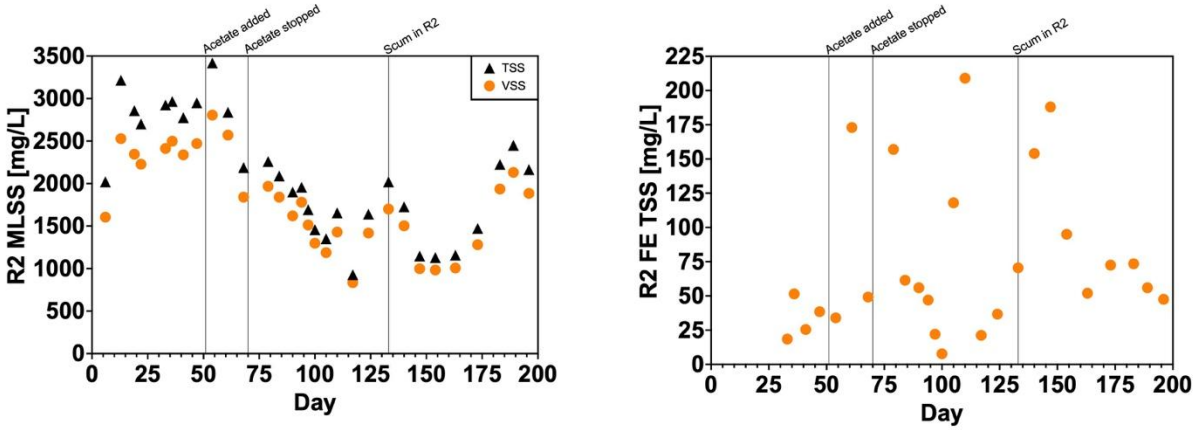


Figure 6: Reactor 2 MLSS and MLVSS, and FE TSS throughout the study. Sodium acetate was added to the influent feed on day 51 and stopped on day 70. Scum was observed on day 133 of the study.

Between days 68 and 173, low MLSS and MLVSS were reported in Reactor 2, as seen above in Figure 6. During this period, a high biomass washout was seen in the decanting

phase as settleability was low and SVI was high. The addition of sodium acetate appears to have influenced biomass washout, as the biomass remaining in the SBR was fluffy and light. The FE TSS averaged 75 ± 57 mg/L over the study. On days of poor settleability, Reactor 2's FE TSS exceeded 100 mg/L. On day 133, scum was observed in Reactor 2, further indicating poor sludge settleability. After day 173, MLSS increased, FE TSS decreased, and settling improved until the end of the study. Possibly, if the operation continued, Reactor 2 could have resulted in aerobic granule formation.

Once bulking and slow settling were noticed in Reactors 2 and 3, acetate dosing was terminated in Reactors 1 and 2 and reduced to its original concentration in Reactor 3. Ultimately, the addition of sodium acetate trihydrate contributed to biomass loss and low MLVSS concentrations. After day 140, Reactor 3 showed a continual decrease in biomass concentration until the end of the study, as seen below in Figure 7. Bulking was indicated by the high SVI values reported. After day 120, decanting was ineffective due to mechanical issues with the decant pump, requiring up to 10 minutes to remove 2.5 L of supernatant. As a result, the slow pumping rate lessened the selective pressure in Reactor 3. After the decant pump was swapped on days 124 and 156, higher FE TSS, upwards of 150 mg/L, was reported. The average FE TSS for Reactor 3 was 133 ± 164 mg/L during the study.

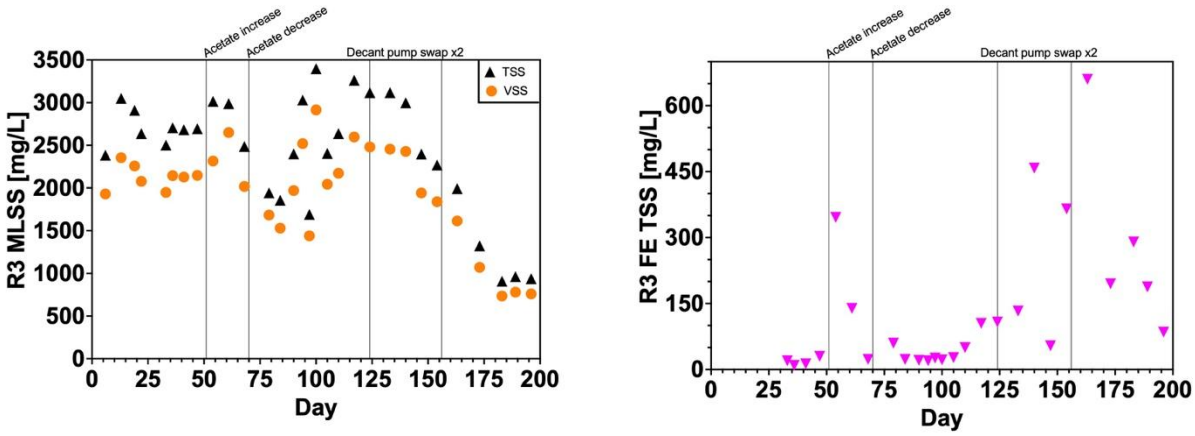


Figure 7: Reactor 3 MLSS and MLVSS, and FE TSS throughout the study. The sodium acetate dosage was increased in the influent feed on day 51 and returned to normal on day 70. Reactor 3's decant pump experienced mechanical issues after day 120, so the pump was swapped on days 124 and 156.

The sludge retention time (SRT) was not controlled during the duration of the study. Reactor wasting was solely via the decant sample each cycle. After the settling phase, the decant pump removed 2.5 L each cycle to achieve a volume exchange ratio (VER) of 50%. From days 72 to 93, the VER was increased in Reactors 1 and 3 from 50% to 60% to improve phosphorus removal performance. In one study, increasing the VER promoted the growth of polyphosphate-accumulating organisms (PAOs) [8]. By removing more effluent from reactors, a higher concentration of nitrates was wasted, allowing for PAOs to consume available COD, instead of denitrifying bacteria in the anaerobic phase. Yet, to maintain the same operational parameters as Reactor 2, the VER was reverted to 50%.

SRT was directly influenced by final effluent concentrations, so Figure 8 highlights the same events impacting FE TSS. For example, when a lower FE MLSS was measured for Reactor 3, an SRT exceeding 100 days was reported. Throughout the study, the average

SRT was 64 ± 72 days. After day 117, Reactor 3's SRT began to decrease. The average SRT between days 117 and 196 was 15 ± 14 days, and the lowest SRT reported during this period was 3 days. Before, the average SRT between days 36 and 110 was 103 ± 76 days. Clearly, SRT was heavily impacted by FE MLSS in the decant sample.

Reactors 1 and 2 had similar average SRT values, 28±22 days and 22±20 days, respectively. Scum was noticed in Reactor 2 on day 133, yet this was not wasted or removed from the system.

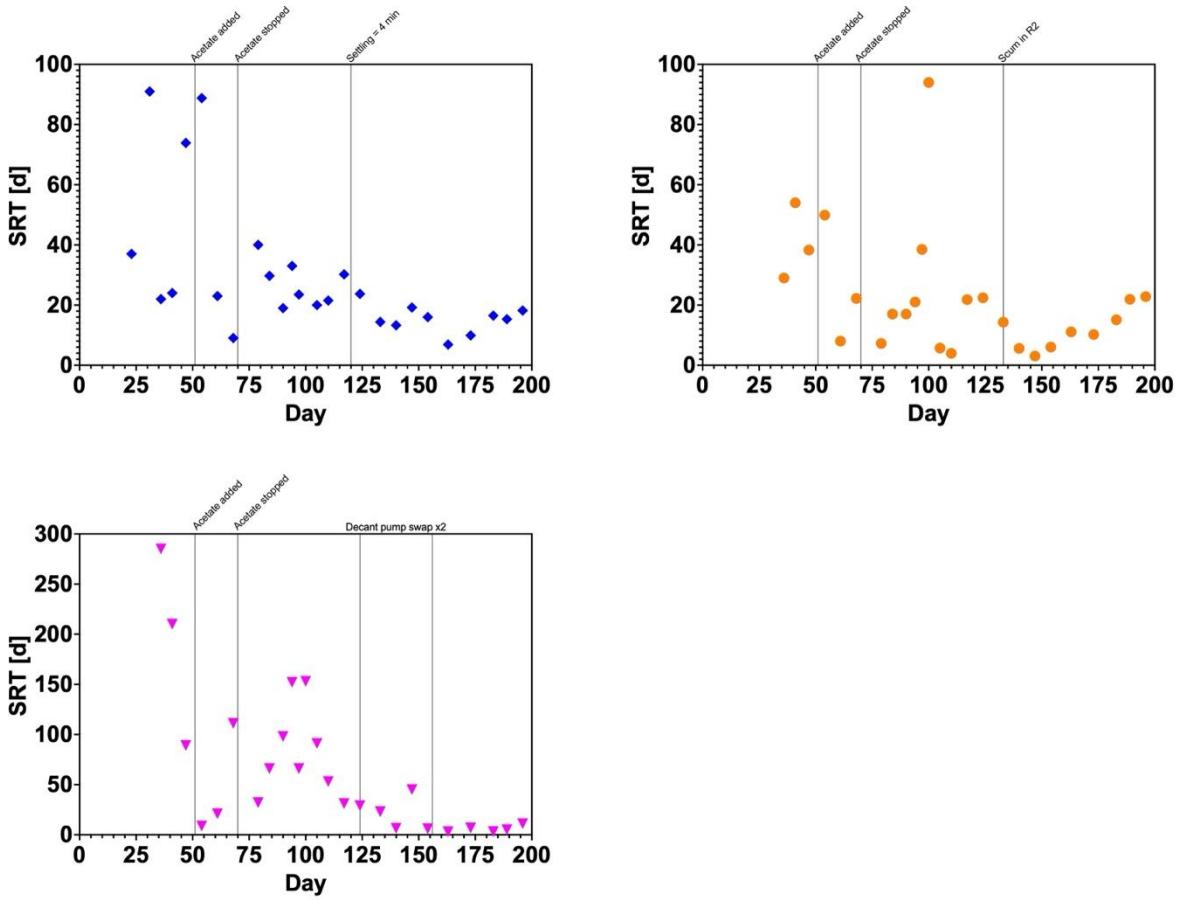


Figure 8: Reactors 1, 2, and 3 SRT throughout the study. Operational changes impacting the SRT of each reactor are shown.

The sludge volume index (SVI) was measured at 5 and 30 minutes for each reactor during the duration of the study. Generally, when MLVSS decreased, higher SVI_5 and SVI_{30} values were observed.

Granules were first observed in Reactor 1 on day 65. Once granules began to develop, the SVI_{30} decreased to under 100 mL/gVSS for the remainder of the study. As shown below in Figure 9, the SVI_{30} trend was relatively stable after the formation of granules. The SVI decreased as the settling time was shortened in Reactor 1. During days 65 to 196, the average SVI_{30} and SVI_5 were 85±8 mL/gVSS and 136±28 mL/gVSS, respectively. Additionally, MLVSS was consistent for most of the study, so a stable SVI value is expected. As mentioned previously, in fully matured AGS SBRs, biomass concentrations exceed 5 g/L [24], [34], [65]. However, the highest MLVSS reported was less than 2.7 g/L. Higher MLVSS may have resulted in lower SVI_{30} values. The lowest SVI_5/SVI_{30} ratio reported was 1.17 on day 183, yet the average ratio was 1.60 between days 65 and 196. Characteristically, the average SVI_5/SVI_{30} ratio for fully matured, dense granules is roughly 1 [8]. This metric signifies that Reactor 1 granules were not fully developed by the end of the study.

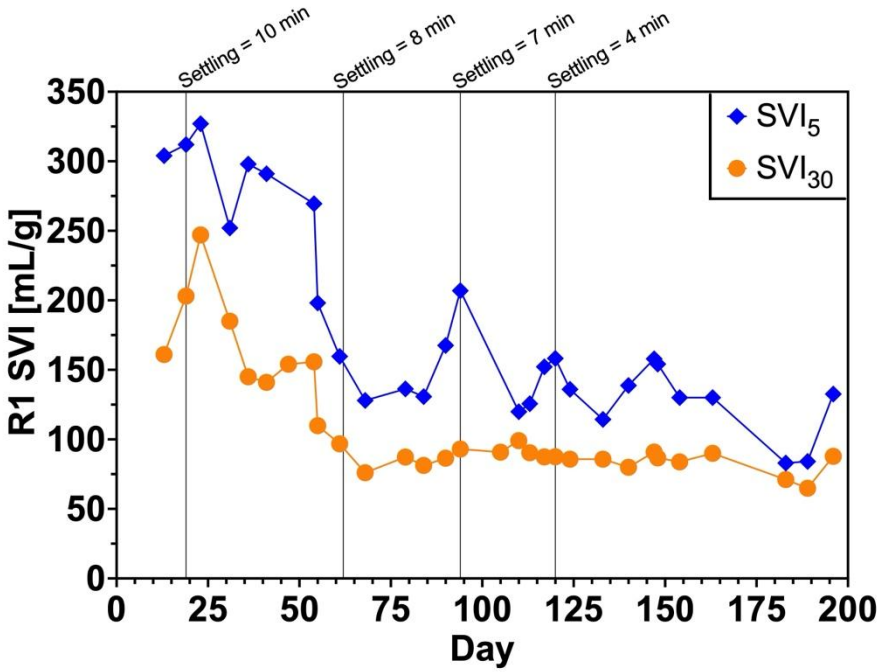


Figure 9: Reactor 1 SVI measured at 5 minutes (blue diamond) and 30 minutes (orange circle). The settling time was reduced on days 19, 62, 94, and 120.

It appears that Reactor 2 never formed stable or fully developed granules, only mixed liquor flocs, based on the high SVI_{30} and SVI_5 values seen in Figure 10. After the addition of sodium acetate to the influent feed, settleability in Reactor 2 reduced. To prevent high FE TSS, the settling time in Reactor 2 was progressively increased to 14 minutes on day 79, 16 minutes on day 85, and 18 minutes on day 91. By increasing settling time, the retention of light, fluffy biomass also increased, further worsening settleability. On days 94 and 120, settling time was decreased to 16 minutes and 12 minutes, respectively. During periods of low reported MLVSS, specifically under 1000 mg/L, bulking in Reactor 2 was evident as SVI_{30} was over 300 mL/gVSS. Generally, SVI_{30} values over 150 mL/gVSS are considered to indicate filamentous growth [66]. After day 183, the settleability improved as the average SVI_5 decreased from 733 mL/gVSS to 346

mL/gVSS, and SVI_{30} decreased from 330 mL/gVSS to 161 mL/gVSS. This observation coincides with increased MLVSS concentration in Reactor 2, from roughly 1200 mg/L to 1900 mg/L. Moreover, after day 183, the decanting phase period was shortened as a new pump provided faster removal of supernatant, removing a larger quantity of light, fluffy flocs.

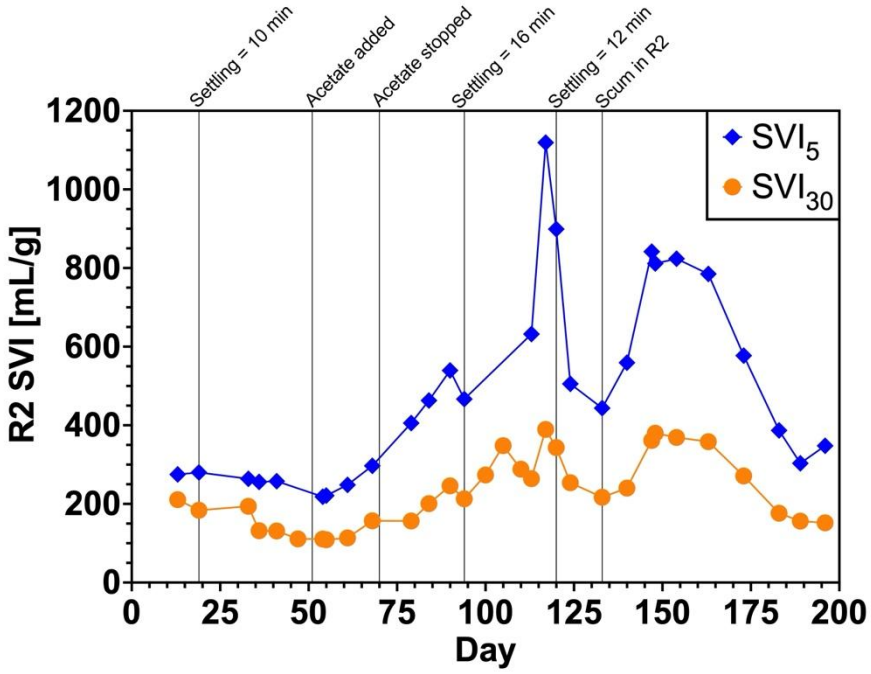


Figure 10: Reactor 2 SVI measured at 5 minutes (blue diamond) and 30 minutes (orange circle). The settling time was reduced on days 19 and 120 and increased on day 94. Other events affecting settleability include acetate addition to the influent feed and scum in Reactor 2.

On days 19, 70, and 94, the settling time of Reactor 3 was reduced as biomass settling improved. Between days 79 and 140, Reactor 3's average SVI_{30} was 107 ± 15.9 mL/gVSS. Yet, after day 140, SVI_{30} and SVI_5 worsened as seen in Figure 11. Between days 140 and 196, the reported SVI_{30} and SVI_5 were 247 ± 111 mL/gVSS and 570 ± 283 mL/gVSS, respectively. Between days 124 and 156, Reactor 3's decant pump operated

at a slower rate, removing 250 mL/min. The pump's low discharge rate extended the decanting period to 10 minutes and may have impacted both reactor performance and selective pressure as light, fluffy flocs remained in the system longer. Consequently, the main operational factors influencing settleability in Reactors 2 and 3 were decant pump speed and sodium acetate trihydrate addition.

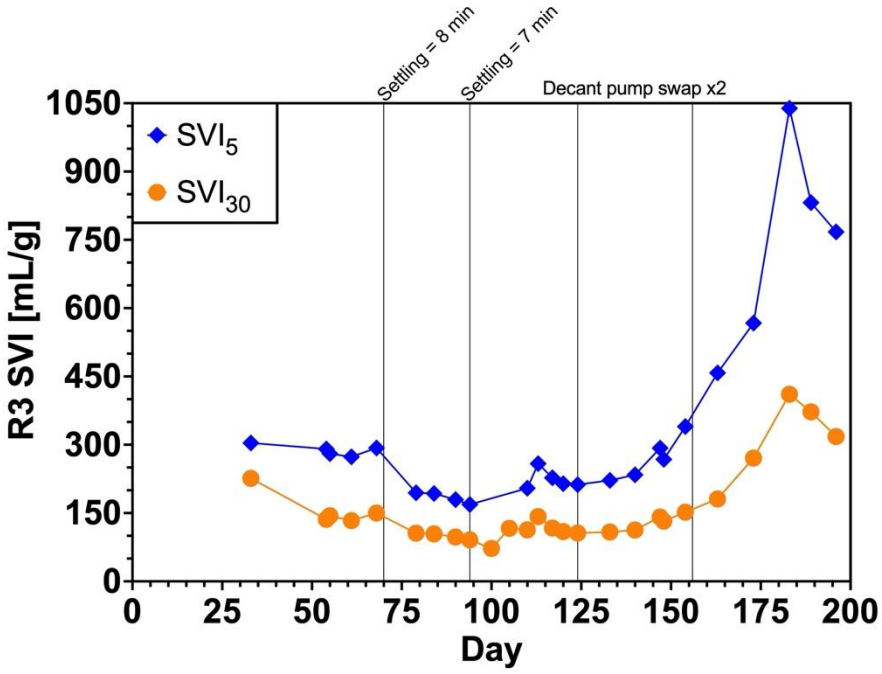


Figure 11: Reactor 3 SVI measured at 5 minutes (blue diamond) and 30 minutes (orange circle). The settling time was reduced on days 70 and 94. Other events affecting settleability include swapping the decant pump after mechanical issues.

Ammonium-Nitrogen Removal Performance

Seasonal fluctuations in ammonium concentrations were observed as the study began in early fall and ended in spring. Lower values of ammonium were reported after a heavy rainfall or snowmelt event. Higher ammonium concentrations were measured during days 75 to 120.

The average feed concentration of ammonium-nitrogen (NH_4-N) for Reactors 1, 2, and 3 was 17.8 ± 3.43 mg/L, 35.3 ± 7.04mg/L, and 51.6 ± 14.3 mg/L, respectively. Since the beginning of the study, all reactors demonstrated high removal of NH_4-N.

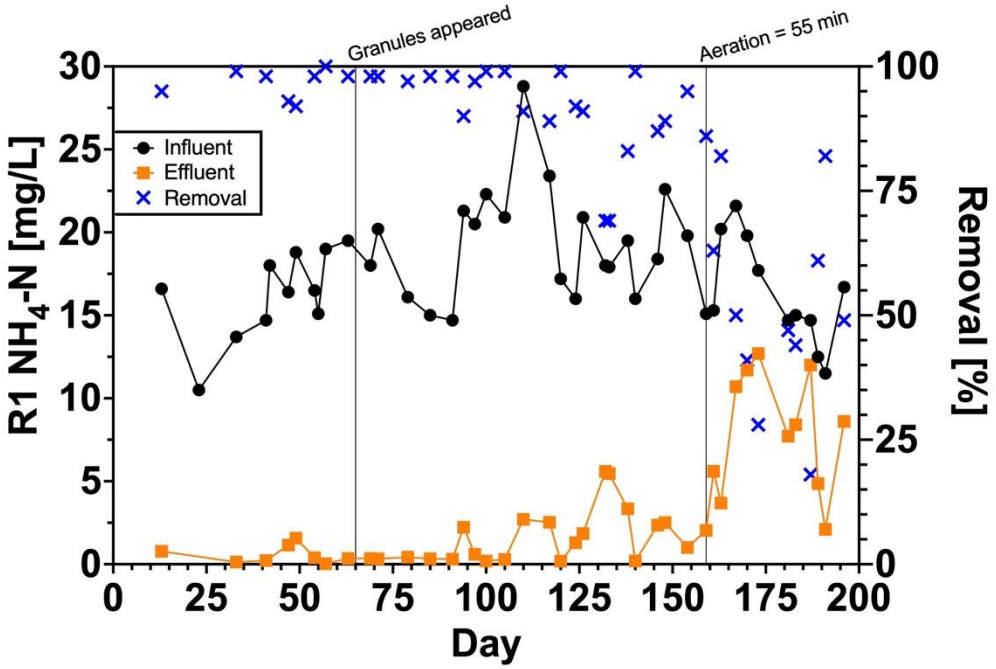


Figure 12: Reactor 1 influent NH_4-N (black circle), effluent (orange square), and removal (blue x) throughout the study. On day 159, the aeration phase was reduced from 85 to 55 minutes.

As seen in Figure 12, between days 13 to 126, Reactor 1 had an average removal rate of 96%. From day 127 to the end of the study, the average removal rate reduced to 65%, specifically decreasing after day 159. On day 159, the length of Reactor 1's aerobic phase

was reduced by 30 minutes to 55 minutes of reaction time, and the filling phase was increased by 30 minutes to extend the filling phase to 90 minutes. The aerobic phase was shortened to strengthen the reducing environment during filling. The oxygen reduction potential (ORP) was not measured until day 154, as the ORP probe had to be ordered and was delayed in delivery. The reported ORP during Reactor 1's filling phase was positive and ranged between 0 to 50 mV. Whereas Reactors 2 and 3 measured negative ORP readings below -100 mV. As nitrification performance was sufficient and stable in Reactor 1 for over 100 days, the optimization of phosphorus removal was prioritized, so the filling time was increased. As seen in other studies, DO concentrations decrease throughout a longer feeding period, creating a stronger anaerobic environment which improves the development of PAOs [7] [67] [26]. However, the reduction of the aeration phase hindered the nitrification performance of Reactor 1.

Reactors 2 and 3 maintained an average removal greater than 98% throughout their operation, as seen below in Figure 13.

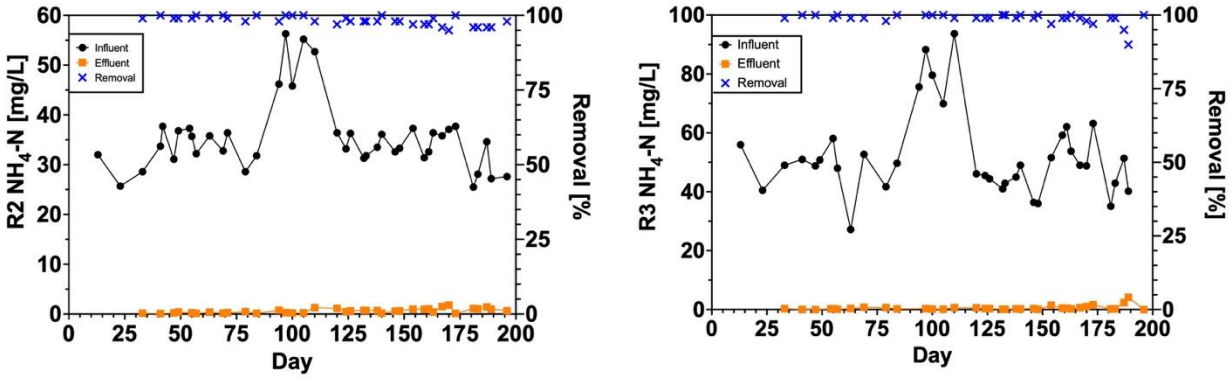


Figure 13: Reactors 2 and 3 influent NH_4-N (black circle), effluent (orange square), and removal (blue x) throughout the study.

Orthophosphate Removal Performance

The average feed concentration of orthophosphate (PO_4-P) for Reactors 1, 2, and 3 was 2.04 ± 0.39 mg/L, 3.72 ± 0.53mg/L, and 4.46 ± 1.31 mg/L, respectively. For most of the study, orthophosphate removal was negligible in Reactors 1 and 2. As mentioned previously, on day 159, Reactor 1's anaerobic period was increased by 30 minutes. This adjustment was made to ensure deep anaerobic conditions for PAOs to release phosphorus and store energy effectively. The oxygen-reduction potential (ORP) measured before and after extending the anaerobic phase time was 29 mV and –201 mV, respectively. Between days 173 and 196, the average PO_4-P removal was 88% in Reactor 1, as seen in Figure 14. Possibly, if the anaerobic phase period was longer since start-up, orthophosphate removal would have been higher earlier on.

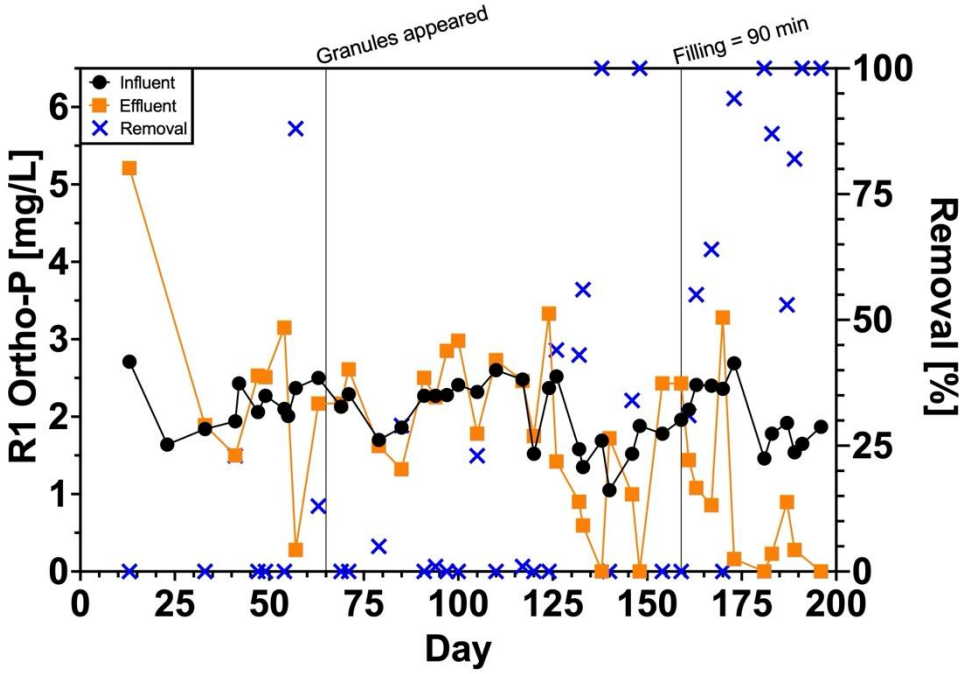


Figure 14: Reactor 1 influent PO_4-P (black circle), effluent (orange square), and removal (blue x) throughout the study. On day 159, the unaerated filling phase was increased from 60 to 90 minutes.

From day 132 onward, Reactor 2 achieved an average PO_4-P removal of 70%, as seen in Figure 15. This improved performance coincides with an increase in MLSS and MLVSS. Likely, biomass washout resulted in the loss of PAOs and reduced phosphorus removal effectiveness. Moreover, there were a few sampling days where the orthophosphate concentration in the effluent was higher than influent, indicating polyphosphate release but inefficient uptake.

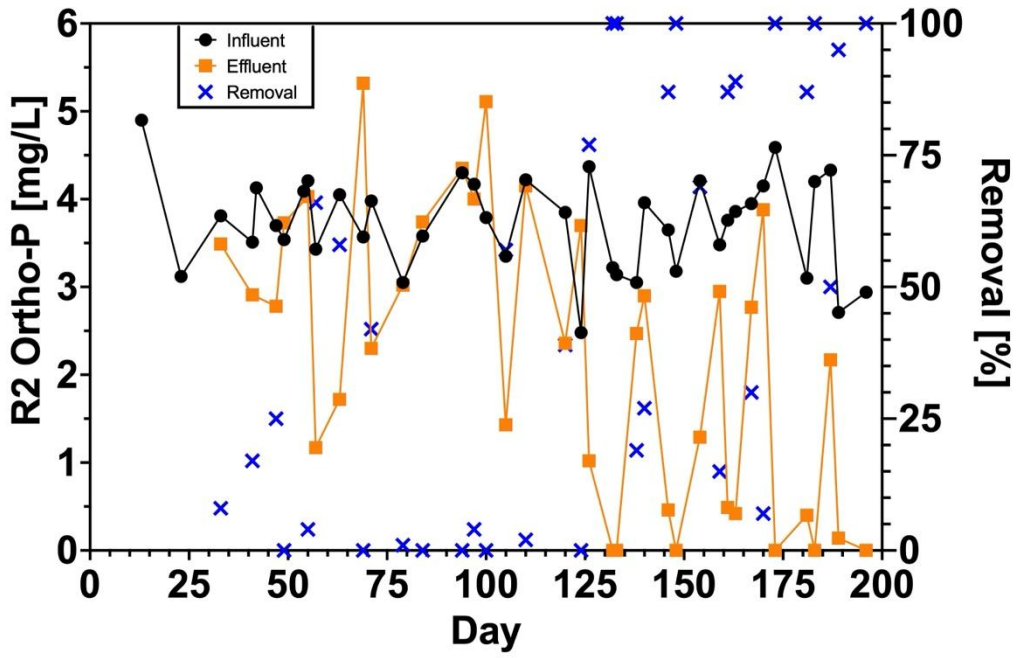


Figure 15: Reactor 2 influent PO_4-P (black circle), effluent (orange square), and removal (blue x) throughout the study.

From day 126 to the end of the study, Reactor 3 achieved an average removal rate of 99%, as seen in Figure 16. The higher influent COD concentration and longer anaerobic period may have favoured PAO activity in Reactor 3. Increasing the VER to 60% during days 72 and 93 possibly promoted the growth of PAOs, yet it is not certain. Although

calculated, Reactor 3's influent orthophosphate concentration was not double that of Reactor 2's.

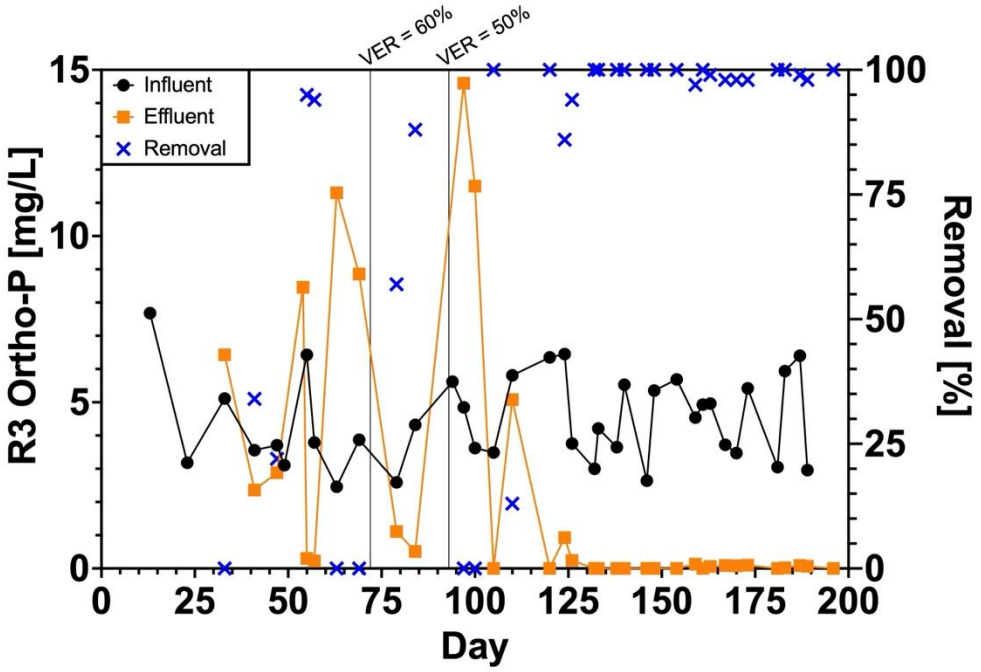


Figure 16: Reactor 3 influent PO_4-P (black circle), effluent (orange square), and removal (blue x) throughout the study. Between days 72 and 93, the VER was increased to 60%.

Denitrification Performance

In this study, the denitrification process was not optimized, so aeration was not controlled, and there was no dedicated anoxic phase. Throughout the operation of all reactors, final effluent contained negligible nitrite (NO_2^-) concentrations. In Reactor 1, the average nitrate removal was 46%. Upon increasing the timing of the filling phase of Reactor 1 on day 159, an increase in nitrate removal was observed, as seen in Figure 17. The average removal increased to 71%. This suggests that extending the unaerated filling phase promoted the activity of denitrifying bacteria, allowing for the complete reduction of nitrate to nitrogen gas.

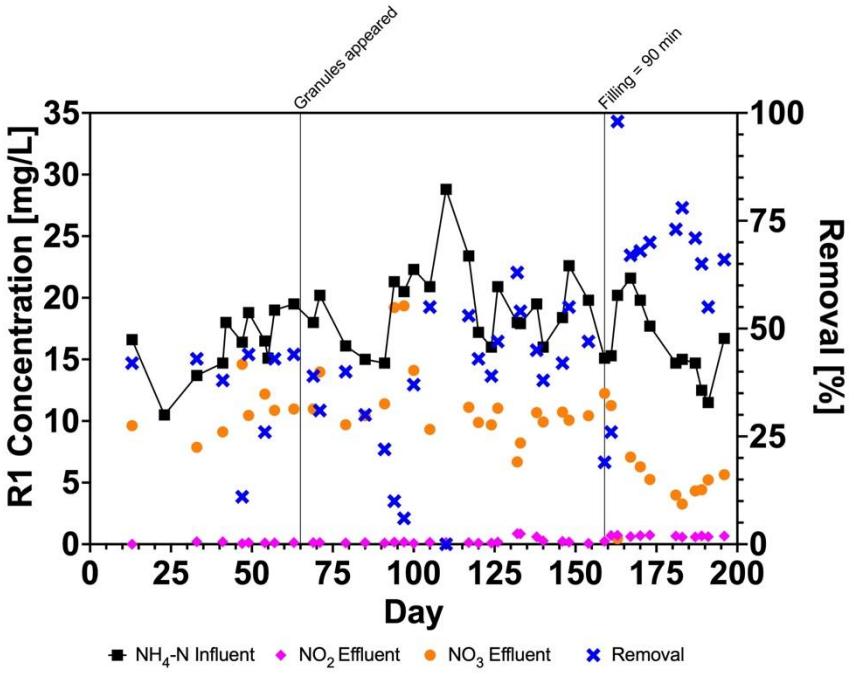


Figure 17: Reactor 1 influent NH_4-N (black square), NO_2 effluent (pink diamond), NO_3 effluent (orange circle), and removal (blue x) throughout the study. On day 159, the filling phase was increased to 90 minutes.

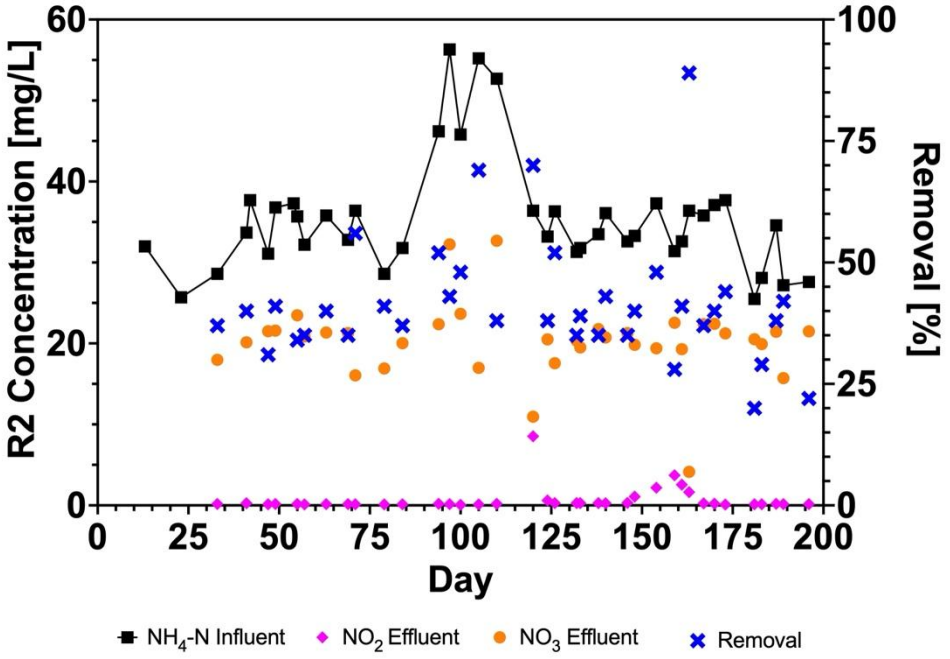


Figure 18: Reactor 2 influent NH_4-N (black square), NO_2 effluent (pink diamond), NO_3 effluent (orange circle), and removal (blue x) throughout the study.

The average removal rate in Reactor 2 was 42%, as depicted above in Figure 18. Lastly, during the study, the average removal rate in Reactor 3 was 54%. As shown in Figure 19, increasing the VER to 60% may have improved the denitrification in Reactor 3 after day 93. Yet, nitrate removal did not consistently exceed 75%.

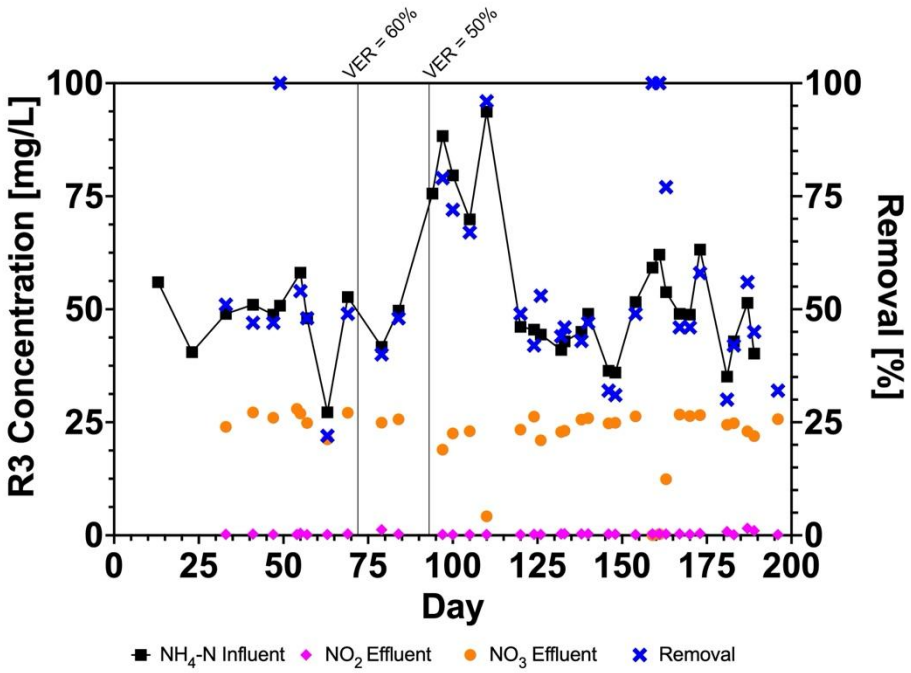


Figure 19: Reactor 3 influent NH_4-N (black square), NO_2 effluent (pink diamond), NO_3 effluent (orange circle), and removal (blue x) throughout the study. The VER was increased to 60% between days 72 and 93.

Organic Loading and Removal

During the study, Reactor 1 feed consisted of diluted PE, so approximately half the COD concentration of Reactor 2. Whereas, Reactor 3 feed was high-strength PE, dosed with sodium acetate trihydrate to double the COD concentration of Reactor 2. The average influent chemical oxygen demand (COD) concentration for Reactors 1, 2, and 3 was 140 ± 36.0 mg/L, 344 ± 89.2 mg/L, and 503 ± 145 mg/L, respectively. The average influent soluble COD (sCOD) concentration for Reactors 1, 2, and 3 was 86 ± 23 mg/L, 230 ± 68.3 mg/L, and 391 ± 129 mg/L, respectively. The COD and sCOD concentrations throughout the study can be seen below in Figure 20.

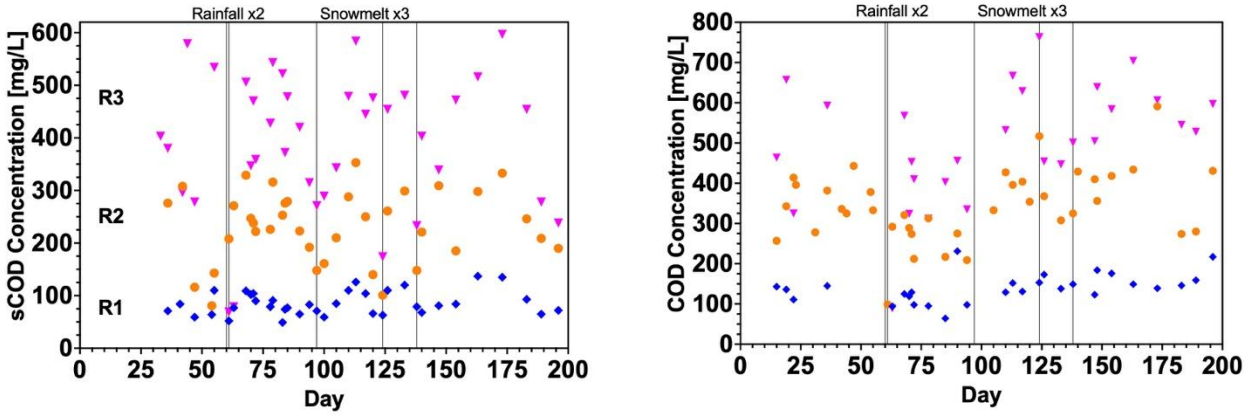


Figure 20: sCOD concentrations for Reactor 1 (blue diamond), Reactor 2 (orange circles), and Reactor 3 (magenta upside-down triangles). sCOD concentrations were impacted by seasonal fluctuations like rainfall on days 60 and 61 and snowmelt on days 97, 124, and 138.

Although Reactor 1 was intended to receive a feed diluted to half the concentration of the control reactor, the actual dilution was not exactly 50%. This discrepancy arose because the volume of water used for dilution was estimated manually, rather than precisely measured. As a result, slight variations in the added tap water volume led to fluctuations in the actual feed concentration.

Although Reactor 3 was intended to receive a feed with double the concentration of the control reactor, this target was not consistently achieved. This was mainly due to variability in how the control feed concentrations were determined. About 30% of the time, Reactor 2's feed was not directly measured. Instead, concentrations were estimated or based on previous data. These estimated values were then used to calculate the required mass of chemicals for Reactor 3, including ammonium chloride, potassium dihydrogen phosphate, and sodium acetate trihydrate. As a result, any inaccuracies in the control feed estimation propagated into Reactor 3's feed preparation. While Reactor 3 still received a higher loading than Reactor 2, the lack of precise, real-time measurements limited the ability to maintain a consistent double increase.

As per the study methods, the organic loading rate (OLR) was to be maintained equally across all reactors during the study. However, equal OLR was not observed, as seen in Figure 21 below. The average OLR for Reactors 1, 2, and 3 was 0.54 ± 0.16 kg/m^3/d, 0.68 ± 0.16 kg/m^3/d, and 0.49 ± 0.15 kg/m^3/d, respectively. On days 60 and 61, heavy rainfall was reported, and on days 97, 124, and 138, snowmelt was observed, causing PE to be diluted. This caused some fluctuations in primary effluent, affecting the OLR measured.

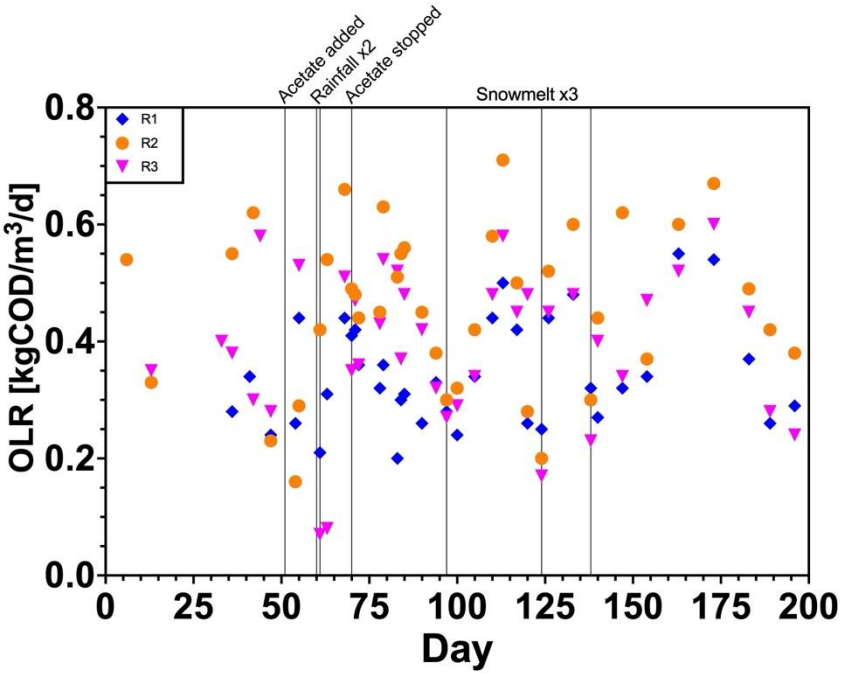


Figure 21: OLR throughout the study for Reactor 1 (blue diamond), Reactor 2 (orange circles), and Reactor 3 (magenta upside-down triangles). OLR was impacted by the addition of sodium acetate during days 51 and 70. Also, seasonal fluctuations like rainfall on days 60 and 61 and snowmelt on days 97, 124, and 138 affected the OLR.

This difference in food available per reactor is further noted in Figure 22, which highlights the food-to-microorganism ratio (F/M). The average F/M for Reactors 1, 2, and 3 was 0.18±0.08 gCOD/gVSS/d, 0.30±0.15 gCOD/gVSS/d, and 0.23±0.13 gCOD/gVSS/d, respectively. Besides the seasonal dilution discussed, periods of high biomass washout contribute to lower F/M ratios. So, the same operational events that impacted higher FE TSS are also highlighted in Figure 22. Utilizing volatile suspended solids (VSS) as a measure of microorganisms overestimates the active biomass in the reactor (dead, dormant, and EPS material are accounted for) and underestimates the organic load on biomass. Future work could include measuring the oxygen uptake rate to estimate the active granular biomass.

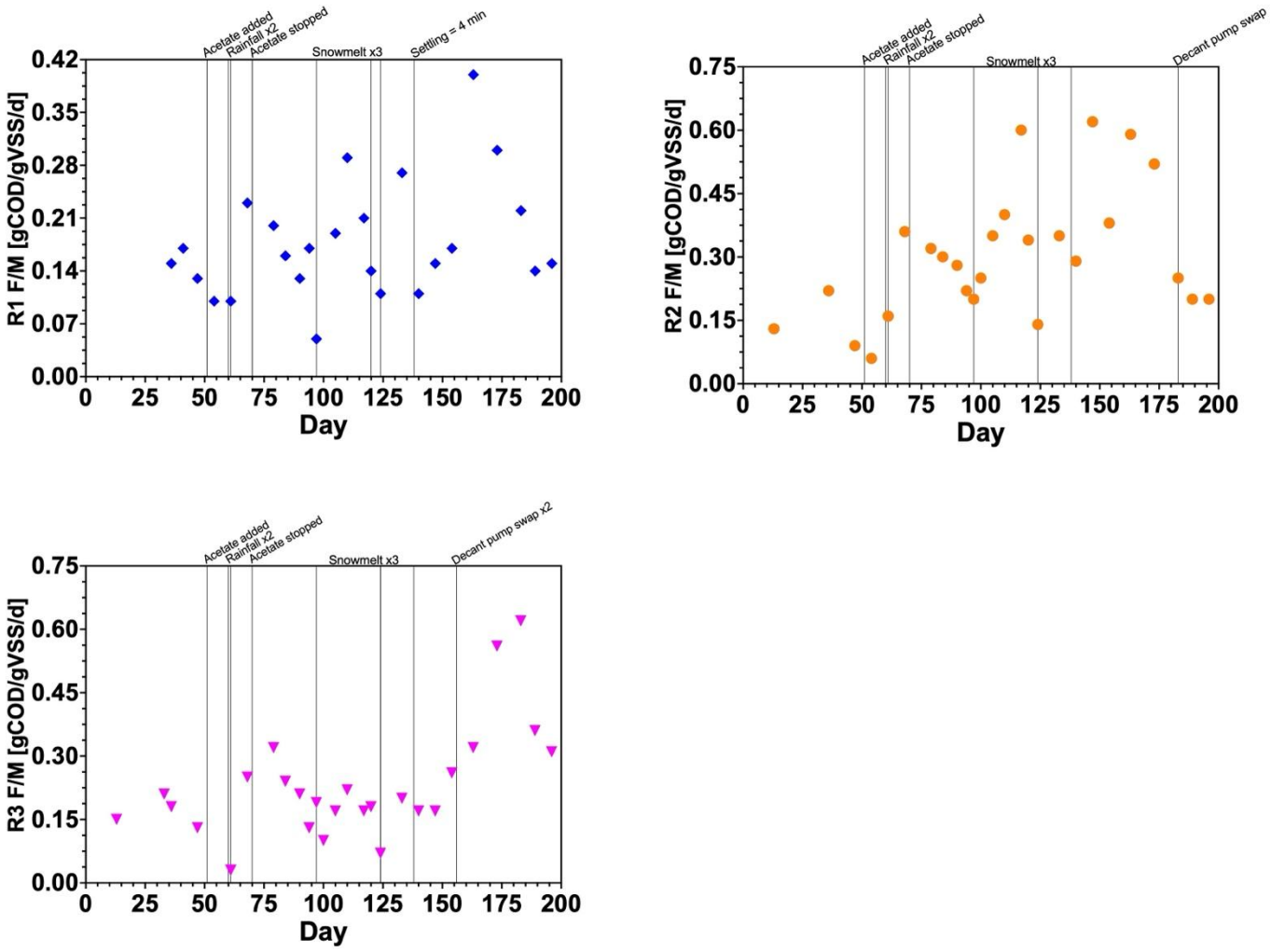


Figure 22: Food-to-microorganism ratio of each reactor throughout the study. Seasonal fluctuations, such as rainfall and snowmelt, impacted F/M. Moreover, periods of higher FE TSS increased the F/M.

The disparity between OLR and F/M is likely due to inaccurate dilution and chemical dosing, and degradation of ammonium chloride, potassium dihydrogen phosphate, and sodium acetate trihydrate at room temperature over a few days. Before adding the chemicals, they were dissolved in Reactor 3's primary effluent feed. The potential consumption or transformation of chemicals is estimated to be between 5 – 10% per day. As mentioned previously, between days 51 and 70, sodium acetate trihydrate was added to Reactors 1 and 2 feed to increase the supply of readily available food. The goal was to

increase the concentration of sCOD during the filling phase to promote the growth of slow-growing bacteria and increase MLSS. For Reactor 3, the sodium acetate trihydrate dosage was increased by 12.5% to target a concentration 2.25 times that of Reactor 2's feed. Throughout the study, pH was not controlled but occasionally monitored. The pH recorded for all reactors ranged between 7.2 to 7.7, so the addition of sodium acetate trihydrate did not appear to influence the pH of any reactor, especially Reactor 3.

The average effluent concentration of soluble chemical oxygen demand (sCOD) for Reactors 1, 2, and 3 was 14 ± 9.8 mg/L, 21 ± 12 mg/L, and 23 ± 12 mg/L, respectively. As seen in Figure 23, the average sCOD removal rate for Reactors 1, 2, and 3 was 83%, 89%, and 93%, respectively. After decreasing the aeration phase of Reactor 1, the sCOD removal decreased, lowering to 58% on day 196. It is possible that 55 minutes was insufficient time for aerobic carbon degradation or that there was an insufficient DO concentration during the aerobic phase.

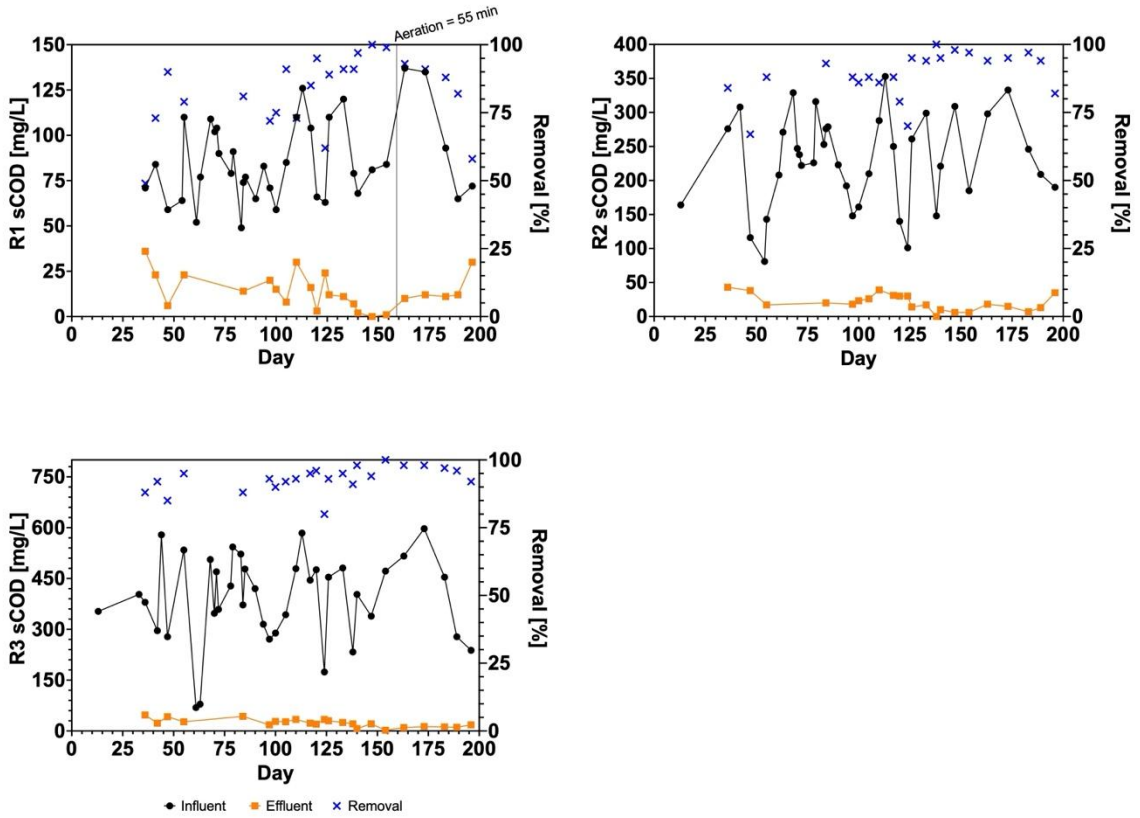


Figure 23: sCOD influent (black circle), effluent (orange square), and removal (blue x) per reactor throughout the study. On day 159, Reactor 1's aeration phase was reduced to 55 minutes.

Microbial Community

DNA was extracted from Reactor 1, 2, and 3 on days 132, 156, 176, 190, and 196 (Reactor 3 was not sampled on day 196). Taxonomic bar plots were generated to display the genus level. In terms of taxonomic resolution, the genus level provides more insight into microbial communities and their potential roles. Whereas the family level is more generalized, so large microbial shifts are detected, but specific microbial functions are less clear [68].

In Reactor 1, the relative abundance of the genus *Flavobacterium* and *Tahibacter* was high, over 5% on all sample days, as seen in Figure 24, Figure 25, Figure 26, Figure 27, and Figure 28. *Flavobacterium* is recognized as a heterotrophic nitrifier, and its functions include denitrification and floc formation [59], [52]. The removal of nitrate was not significant during this sampling period, as the average was roughly 45%. The presence of *Tahibacter* has been reported to emerge as granules mature, and the genus also plays a role in the denitrification process [18], [69], [70].

There was a consistent relative abundance, greater than 8% of Comamonadaceae (f) throughout all of Reactor 1's samples. Their role in nutrient removal is vital as *Comamonadaceae* contributes to phosphorus removal and denitrification when acetate is supplied as a carbon source [71], [17]. The development of *Comamonadaceae* has been shown to develop under shorter sludge retention times [57]. In Reactor 2, *Comamonadaceae* was present on all days, except 156. *Comamonadaceae* was also found in Reactor 3, yet the relative abundance of *Comamonadaceae* was lowest on day 156 compared to the other sample days, where frequency was greater than 10%. The reduced presence of *Comamonadaceae* in both reactors likely coincides with biomass

washout seen during days 120 to 175, as shown by the sudden decrease in SRT in Figure 8.

The presence of *Nitrospira* was detected on day 156 at 4.9% and became more abundant by day 190, at 14.5%. The genus was also detected in Reactor 2 on day 196. *Nitrospira* is a common nitrite-oxidizing bacterium found in AGS, and can reduce nitrite to nitrate by utilizing nitrite as an energy source [51]. Its abundance is suggested to be higher in low ammonium concentration environments [40]. As Reactor 1's influent feed was diluted, the detection of *Nitrospira* is expected to be higher than in Reactors 2 and 3.

On days 190 and 196, the genus *Dechloromonas* was found in Reactor 1, with a relative abundance of > 2%. *Dechloromonas* is recognized for its ability to reduce nitrate to nitrite [59]. Typically, the abundance of *Dechloromonas* is found in the inner anoxic core of granules [34]. As seen in Figure 17, the nitrate removal improved in Reactor 1 after day 159.

In Reactor 1, the genus *Terrimonas* was detected on day 156 at a high relative frequency of over 20%. Its presence lessened over time to 2.8% by day 196. The genus is known to tolerate low COD environments and can degrade organic matter [18]. Again, it appears Reactor 1's dilute feed encouraged the growth of *Terrimonas*.

The genus *Thiothrix* was detected in Reactor 1 on days 132, 156, and 176, with abundance decreasing until it was no longer present with a relative abundance of at least 1.5% on days 190 and 196. A similar trend was seen in Reactor 2, with the relative abundance of *Thiothrix* diminishing after day 156. The genus is recognized as a putative PAO, since they are suspected of performing phosphorus removal [8]. The decreased

abundance of *Thiothrix* could be explained by the increased presence of PAO *Candidatus Accumulibacter* on days 190 and 196.

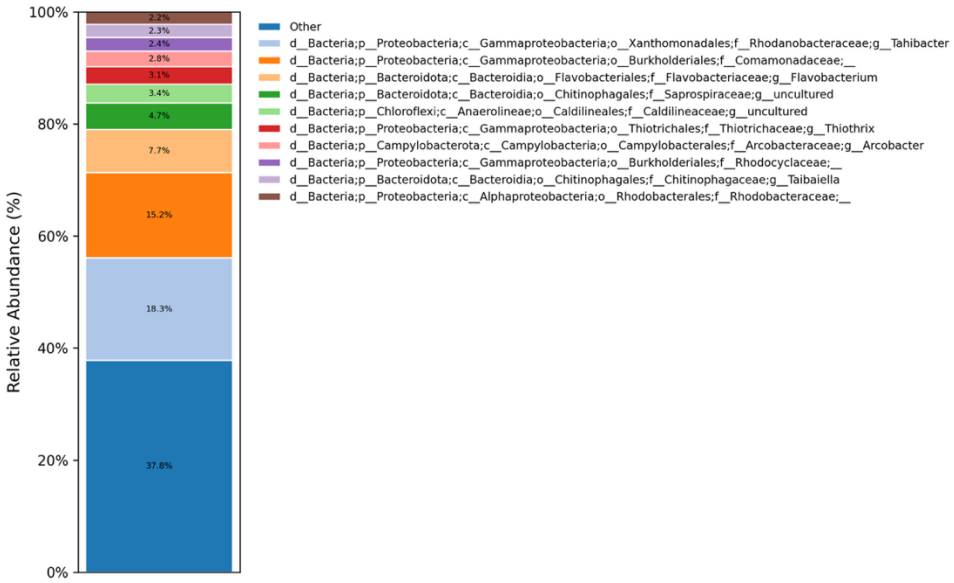


Figure 24: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 1 on day 132. Relative abundance below 1.5% is grouped as Other.

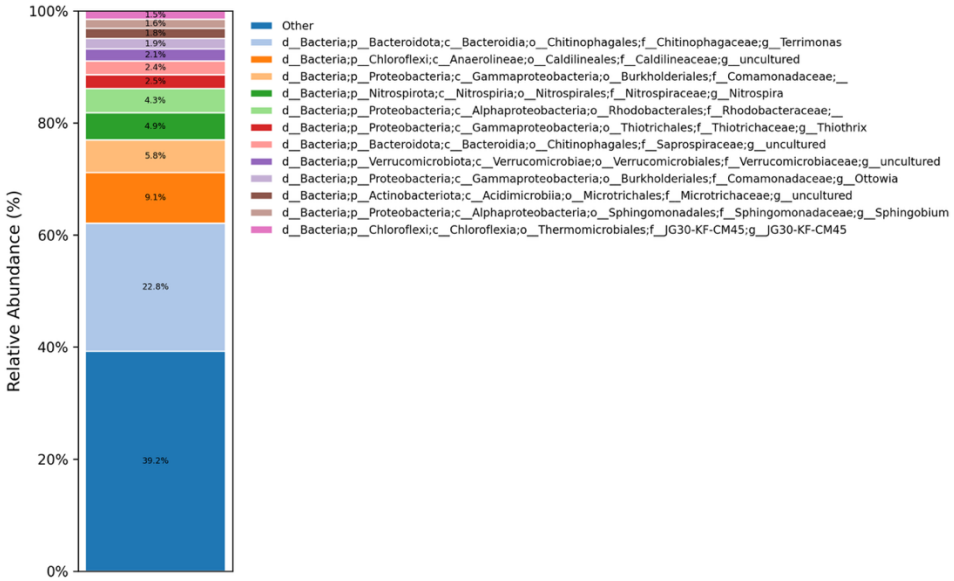


Figure 25: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 1 on day 156. Relative abundance below 1.5% is grouped as Other.



Figure 26: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 1 on day 176. Relative abundance below 1.5% is grouped as Other.

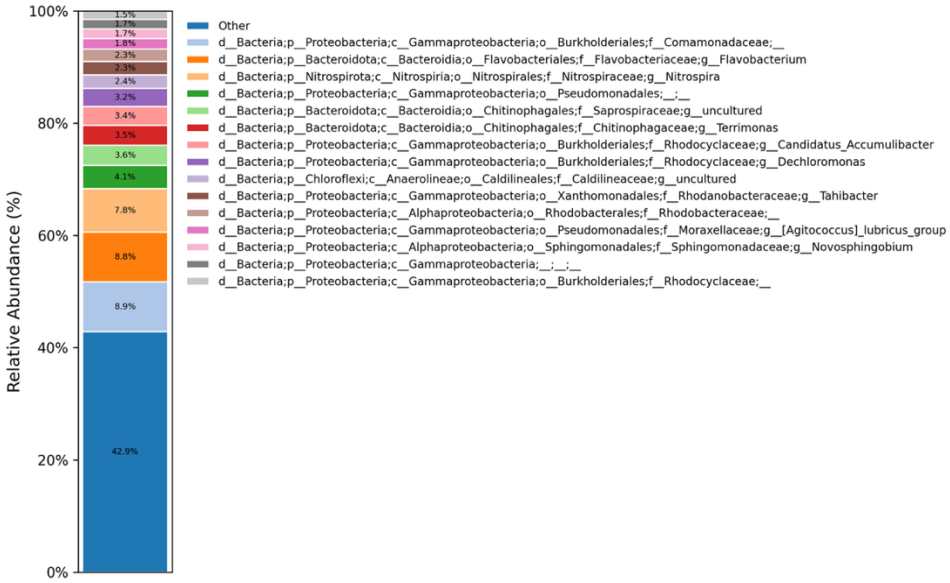


Figure 27: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 1 on day 190. Relative abundance below 1.5% is grouped as Other.



Figure 28: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 1 on day 196. Relative abundance below 1.5% is grouped as Other.

Reactor 2's microbial taxa for days 132, 156, 176, 190, and 196 are shown below in Figure 29, Figure 30, Figure 31, Figure 32, and Figure 33, respectively.

In Reactor 2, the genus *Thiothrix* was present in Reactor 2 on days 132 and 156, with the relative abundance increasing to over 9%. Also, a relative abundance of < 2% of *Candidatus Microthrix* was found on days 176, 190, and 196. *Candidatus Microthrix* was also detected at < 2% in Reactor 3 on day 156. The presence of *Thiothrix* and *Candidatus Microthrix*, both filamentous microorganisms found in wastewater systems, signifies sludge bulking and foaming [72]. As mentioned, scum was present in Reactor 2 on day 133, so the detection of filamentous bacteria is expected. So, the poor settling shown in Figure 10 and Figure 11 for Reactors 2 and 3, respectively, further highlights the impact of *Thiothrix* and *Candidatus Microthrix*.

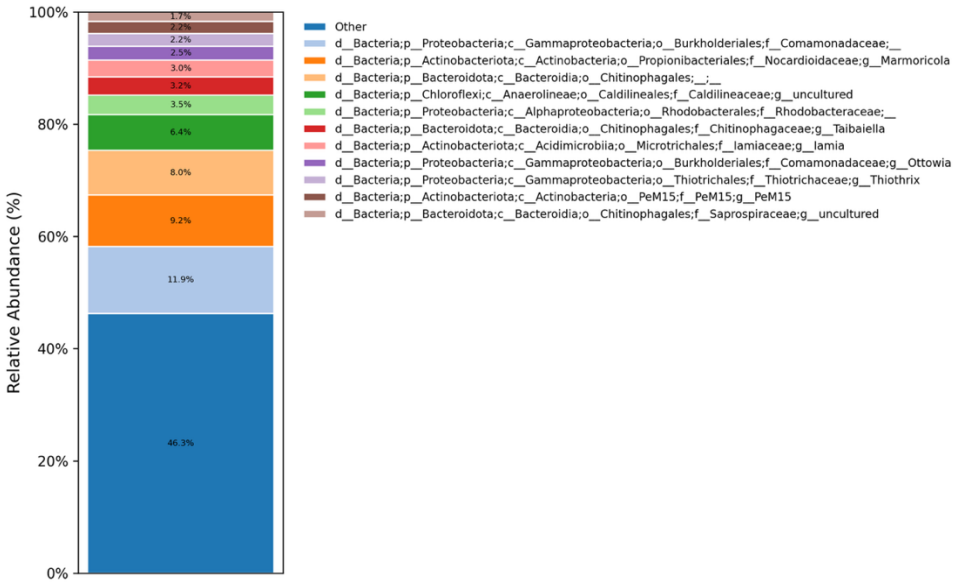


Figure 29: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 2 on day 132. Relative abundance below 1.5% is grouped as Other.



Figure 30: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 2 on day 156. Relative abundance below 1.5% is grouped as Other.

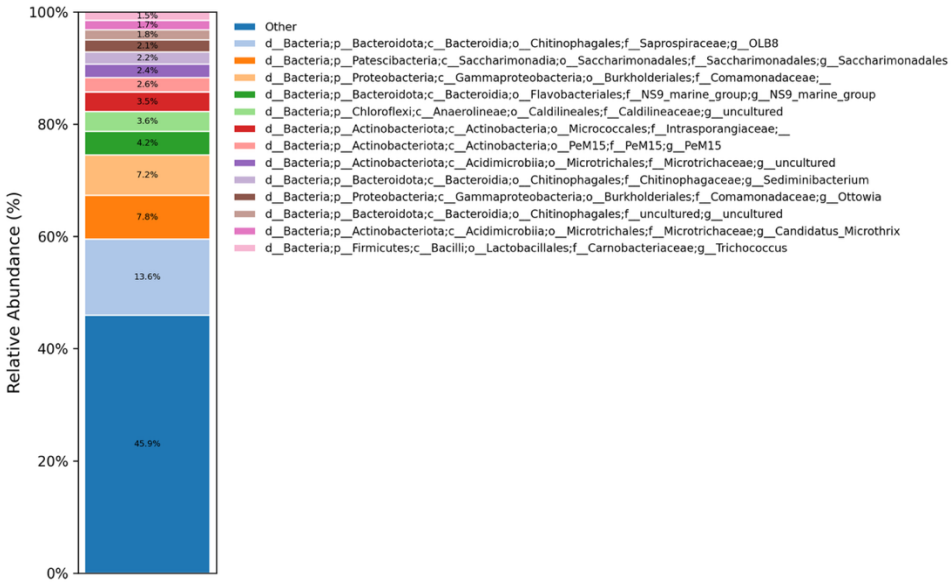


Figure 31: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 2 on day 176. Relative abundance below 1.5% is grouped as Other.



Figure 32: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 2 on day 190. Relative abundance below 1.5% is grouped as Other.

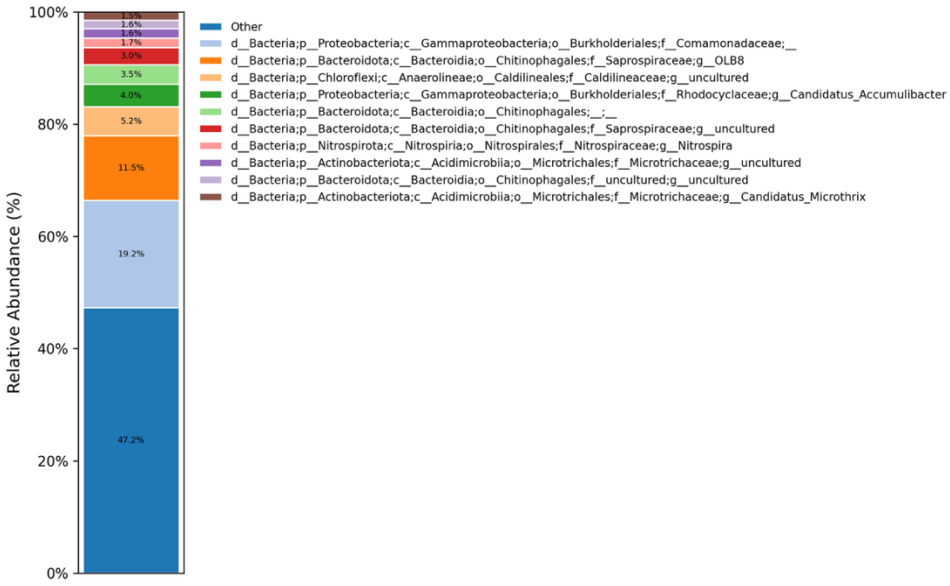


Figure 33: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 2 on day 196. Relative abundance below 1.5% is grouped as Other.

Reactor 3's microbial taxa for days 132, 156, 176, and 190 are shown below in Figure 34, Figure 35, Figure 36, and Figure 37, respectively. A microbial community shift was observed in Reactor 3 after day 156, potentially because of the high biomass washout seen and high FE MLSS measured, as depicted in Figure 7.

Bacteria of the order *Chitinophagales* were detected in Reactors 2 and 3. In Reactor 2, the relative abundance of *Chitinophagales* was 8% on day 132, but for subsequent days, the abundance was below 3.5%. Whereas the relative abundance in Reactor 3 was greater than 5% on all days, except day 190. It has been reported that members of *Chitinophagales* contribute to the denitrification process [73]. As noted in Figure 19, Reactor 3's nitrate removal on days 159 and 161 was 100%. The higher abundance of *Chitinophagales* on day 156 may be due to Reactor 3's shorter SRT, as members of *Chitinophagales* can hydrolyze proteins and utilize available substrates quickly [62].

The genus OLB12 was identified in Reactor 3 on days 132, 156, and 176. Its abundance lessened over time, from over 5% to under 2%. This genus is less studied but associated with anaerobic ammonia oxidation [59] [74].

Candidatus Accumulibacter was detected in all of Reactor 3's samples, except on day 176. In Reactors 1 and 2, the presence of *Candidatus Accumulibacter* was observed on days 190 and 196. *Candidatus Accumulibacter* is recognized as a PAO contributing to the removal of phosphorus in wastewater, preferring VFAs such as acetate as a carbon source [59], [75], [14]. As Reactor 3's feed was composed of sodium acetate, the higher abundance of *Candidatus Accumulibacter* is expected compared to Reactors 1 and 2. Generally, the presence of *Candidatus Accumulibacter* is seen in long-term operations [14]. Also, *Rhodocyclaceae* (f) was present on all days in Reactor 3. The genus *Candidatus Accumulibacter* is a member of this family, so this may indicate the presence of other PAOs.



Figure 34: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 3 on day 132. Relative abundance below 1.5% is grouped as Other.

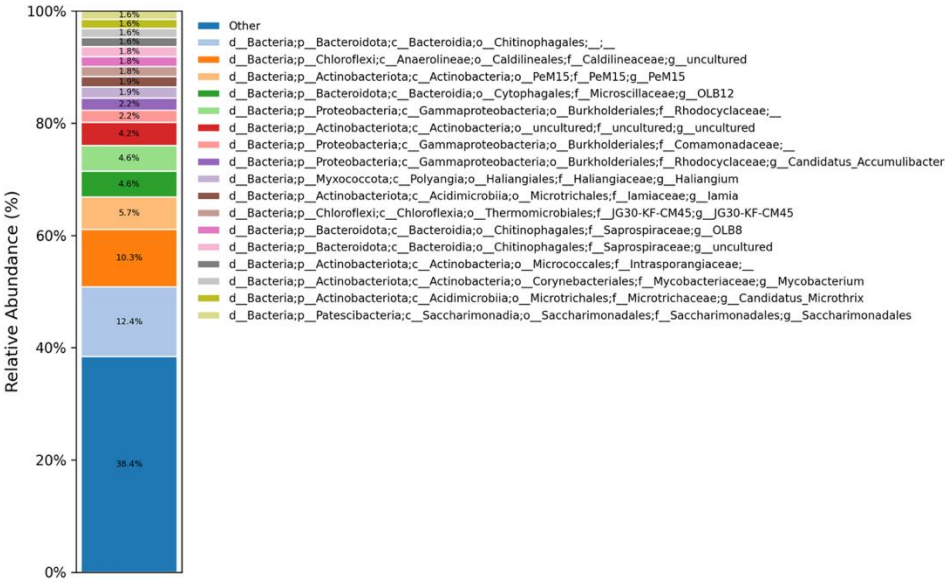


Figure 35: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 3 on day 156. Relative abundance below 1.5% is grouped as Other.

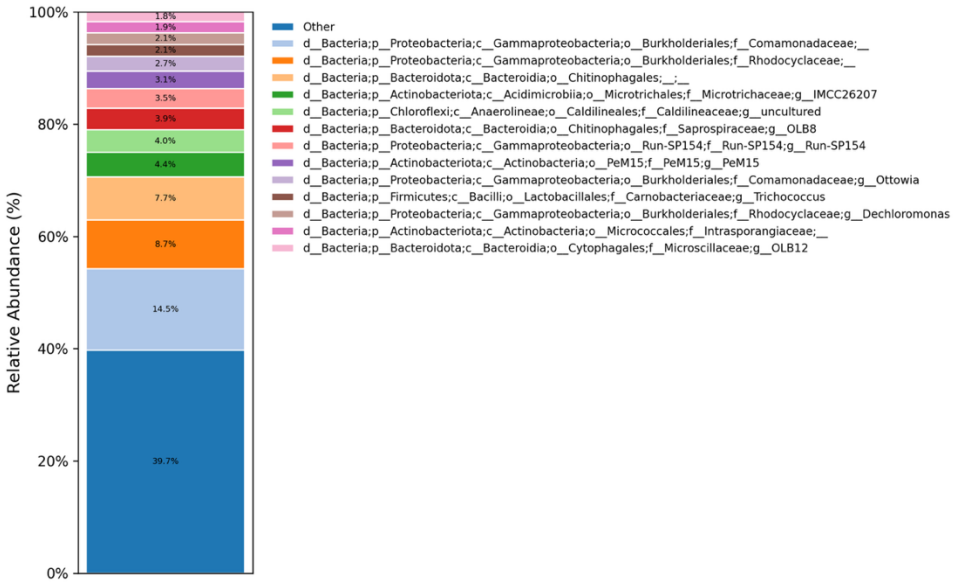


Figure 36: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 3 on day 176. Relative abundance below 1.5% is grouped as Other.

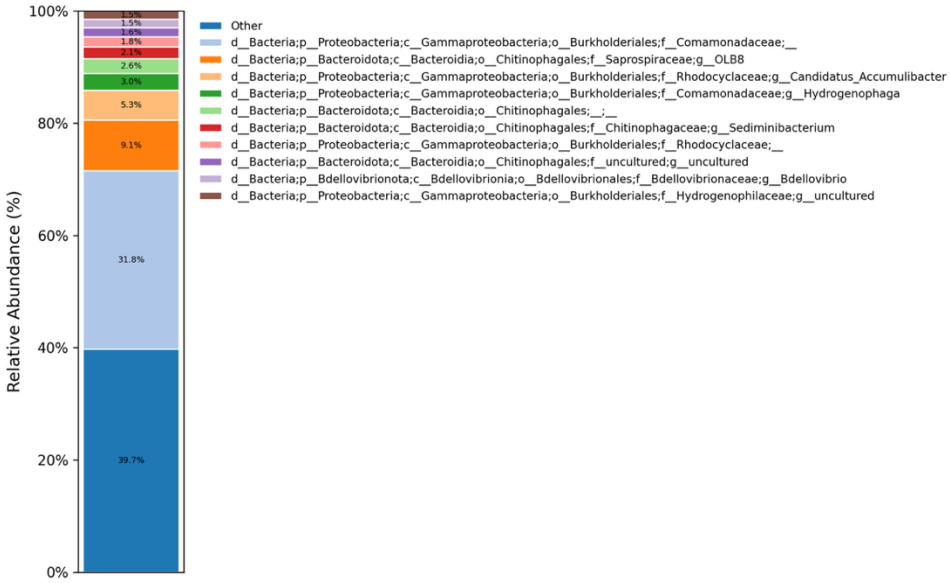


Figure 37: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in Reactor 3 on day 190. Relative abundance below 1.5% is grouped as Other.

As previously discussed, RAS from the WEPWCC was used to inoculate each reactor. Common bacteria found in higher abundance in both RAS and SBRs include *Candidatus Microthrix* and *Comamonadaceae* (f). A key distinction found in the RAS includes the

presence of *Tetrasphaera*. The genus is widely recognized as a PAO, capable of phosphorus uptake and release from wastewater and denitrification [56], [76]. At the WEWPCC, the BNR process utilizes fermentate as a carbon source. *Tetrasphaera* is primarily characterized as a fermenting PAO, so its presence in RAS is expected [20].

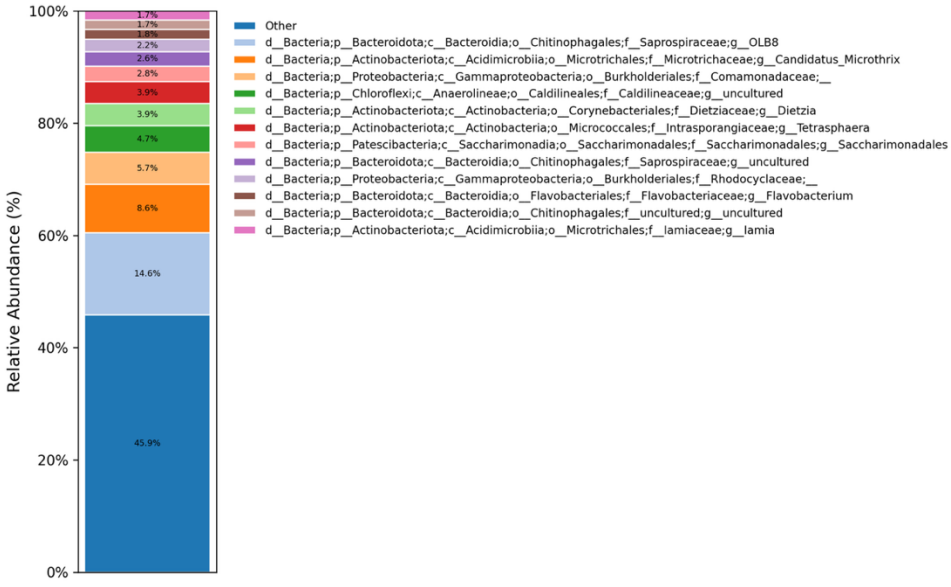


Figure 38: Stacked bar plot showing the relative abundance of the microbial taxa at the genus level (level 6) in RAS from the WEWPCC on day 167. Relative abundance below 1.5% is grouped as Other.

Particle Size Analysis

A particle size analysis was completed using a Malvern Mastersize 3000 instrument. As seen in Table 3, Reactor 1 maintained the largest granule diameter across all three sampling days. The Sauter diameter reports the mean diameter of a particle based on the total volume to surface area ratio, whereas the De Brouckere mean diameter is a volume-weighted mean diameter, so larger particles influence the reported mean. In both Reactor 1 and 3, the Sauter and De Brouckere mean granule diameter was largest on day 167

versus days 184 and 194. Whereas Reactor 2's Sauter diameter was reported to be the largest on day 194. Generally, the diameter of a granule must be at least 0.2 mm, else considered a floc [2], [6]. Accordingly, Reactors 2 and 3 contained more flocs than granules, whereas Reactor 1 developed and maintained smaller-sized granules under 1 mm.

Table 3: Particle size analysis obtained via Mastersizer 3000, where D[3,2] represents the Sauter mean; D[4,3] represents the De Brouckere mean; Dv(10), Dv(50), and Dv(90) represent the 10^{th}, 50^{th}, and 90^{th} percentiles of the cumulative volume distribution.

Particle Size [um]	Day 167			Day 184			Day 194		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
D[3,2]	290	79.9	84.5	75.5	40.2	29.2	183	97.7	75.4
D[4,3]	961	268	304	287	125	151	628	242	240
Dv(10)	143	53.8	47.8	36.4	20.6	17.1	92.4	55.9	45.4
Dv(50)	845	152	140	154	72.1	68.1	430	158	123
Dv(90)	2020	555	835	730	257	415	1440	516	567

Bright-field micrographs were taken over the course of the study and are presented in Figure 39 below. On day 70, loose aggregates were present in Reactor 1. By day 90, compact granules were noticed. From day 127 onward, although the physical appearance of the granules became more round, filamentous microorganisms were present on the surface of the granules. It is evident that there were more flocs than granules present in both Reactors 2 and 3. Yet, in Reactor 3, larger, irregularly shaped aggregates are noted, particularly between days 90 and 127. Previous studies have suggested that larger granules could destabilize as the structure is unstable [26]. Moreover, irregular and loose aggregates typically settle more slowly and can be the result of filamentous bacteria [11], [24].

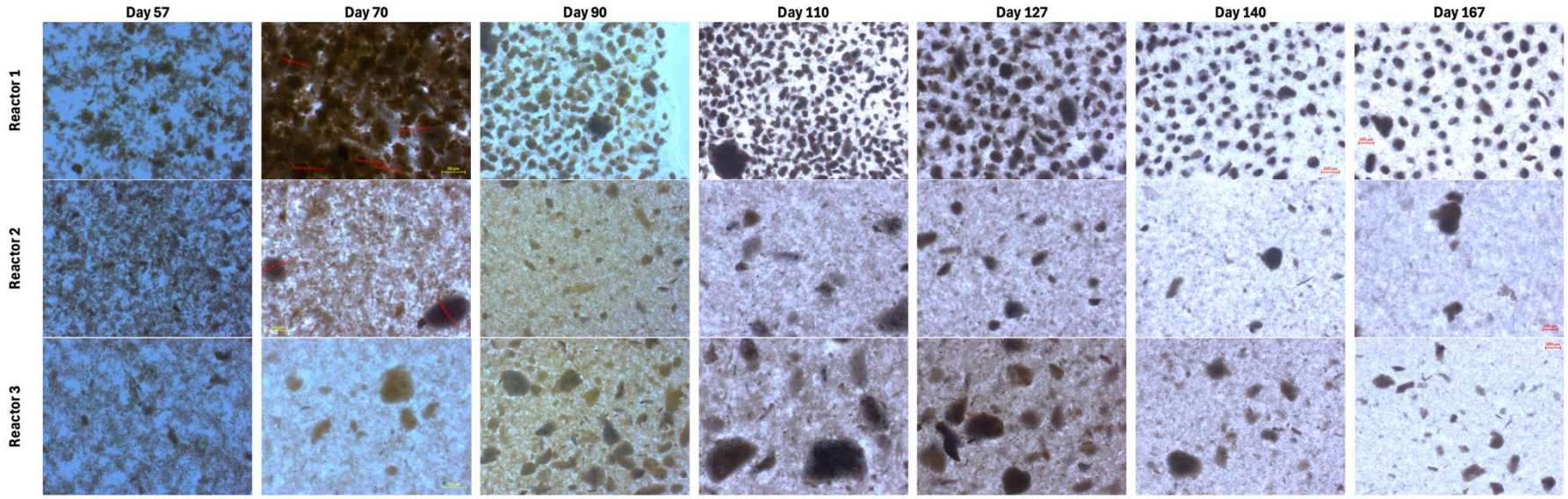


Figure 39: Representative brightfield images of biomass samples collected on days 51, 70, 90, 110, 127, 140, and 167 from bioreactors R1, R2, and R3.

Limitations of the study

Primary effluent (PE) was typically collected from WEWPCC three times per week. Flow injection analysis was not performed each time fresh PE was collected, so the concentration of nutrients was occasionally unknown, about 30% of the time. This uncertainty impacted the chemical addition of ammonium chloride, potassium dihydrogen phosphate, and sodium acetate trihydrate to Reactor 3's feed. To double the concentration of nutrients and sCOD, the most recent PE quality data was used to estimate chemical dosages. Lastly, PE quality fluctuated based on rainfall or snow melt events, as seen in Figure 20, making it challenging to maintain a consistent organic loading rate with daily changes in PE quality.

Feeding tanks were not covered or kept in the refrigerator, given space constraints and the large feeding volume required for Reactor 1 (20 L per day). At room temperature, PE can degrade as organic matter is broken down, leading to a decrease in COD and VFA concentrations. The estimated degradation of PE and the chemicals, ammonium chloride, potassium dihydrogen phosphate, and sodium acetate trihydrate, is 5 – 10% per day. Ultimately, the degradation of chemicals did affect the OLR of each reactor.

From day 140 to 196, mixed liquor volatile suspended solids (MLVSS) were not measured accurately as the muffle furnace was out of service. To maintain data collection, the MLVSS was estimated from historical VSS/TSS ratio data.

During the initial reactor setup, feed tubing was positioned at the top edge of each reactor. This placement was chosen to facilitate tube cleaning and adjusting the feeding pump speed, which was controlled using a dial. Yet, this configuration may have introduced air during the filling phase. So, on day 85, the feed tubing was extended and repositioned towards the bottom of the reactor, which promoted an up-flow feed. In other studies, bottom feeding is a more favourable configuration for the long-term stability of AGS [77]. No major improvements were noticed in terms of nutrient removal, specifically phosphorus removal, so it is unclear if the implementation of an up-flow feed system had a benefit on the SBRs.

Throughout the study, the decant pumps operated at varying speeds due to mechanical issues. The highest decanting flow rate achieved was 1250 mL/min, while the lowest was 250 mL/min. This speed variation significantly impacted the settling process, particularly in Reactors 2 and 3. The decant pump for Reactor 1 was the most effective, which promoted quicker settling and created a greater selective pressure on the system. In contrast, the decant pumps for Reactors 2 and 3 removed supernatants at a slower rate, resulting in the retention of light, fluffy flocs and reducing the selective pressure. Due to extended delivery times, the replacement of the decant pumps was delayed. Once the pumps were identified as a source of concern, the study was already underway and could not be paused to accommodate the swap.

Conclusions and Recommendations

Summary of Key Findings

Selective Pressure

A shorter settling time, ideally less than 5 minutes, strongly promotes the formation of aerobic granular sludge (AGS). Once Reactor 1 presented rapid settling, the settling time was gradually lowered to 4 minutes, as presented in Figure 9. As a result, the selective pressure was increased and led to the formation of round, compact granules. In contrast, when bulking was observed in Reactor 2, the settling time was extended, as seen in Figure 10. Consequently, the selective pressure on the system decreased, encouraging the overgrowth of filamentous bacteria. Although shorter settling times may lead to a high biomass washout, the removal of flocs and retention of dense granules is critical for the development of AGS.

Moreover, a decanting period of less than 5 minutes also increased selective pressure. As discussed, Reactor 1's decant pump was most efficient as the removal of supernatant occurred under 5 minutes. A quicker removal of supernatant removes a greater volume of lighter flocs, leading to a higher retention of denser biomass. Once the decanting pump was exchanged in Reactor 2 and achieved removal under 5 minutes, settling performance began to improve, as shown in Figure 10. Reactor 3's decanting pump required multiple replacements due to mechanical issues. The slowest decanting pump speed reported was 10 minutes, which aligned with slow settleability, as depicted in Figure 11.

An HRT of 6 hours was optimal (compared to 12 and 24 hours) for granule formation and long-term stability. The shorter the HRT, the higher the selective pressure is on the system. Reactor 1 experienced twice the selective pressure of Reactor 2, and four times that of Reactor 3. So, a shorter HRT promoted a greater washout of light, fluffy flocs, while retaining dense granules in Reactor 1. Additionally, a shorter HRT is linked to higher cell hydrophobicity, favouring cell aggregation and structure [24], [26]. At longer HRTs, poor settling flocs remain in the SBR longer and can lead to excessive starvation during the famine period. This may lead to the degradation of proteins, negatively impacting EPS production.

Across all reactors, the quick uptake of sCOD was observed during the anaerobic phase. This rapid consumption of food indicated a strong feast-famine regime, essential for EPS production and consumption [7]. A longer starvation period favours the development of slow-growing bacteria like phosphate-accumulating organisms (PAOs) and prevents competition with aerobic heterotrophic bacteria [41].

Development and Stability of AGS

Contrary to many studies, granules developed in Reactor 1 without the presence of PAOs until day 190, as seen in Figure 27 and Figure 28 [8], [14], [7]. Before day 159, Reactor 1's anaerobic zone was weak, as ORP was higher than -50 mV. Consequently, the unfavourable reduction environment limited PAO growth and reproduction, hindering phosphorus removal in Reactor 1.

Reactor 2 only developed mixed liquor flocs as shown by its surface appearance, high SVI_{30}, and small particle size. Factors that contributed to granule destabilization included a high F/M ratio, slow decanting rate, and a high settling time.

Although Reactor 3 developed a strong presence of PAOs, had a longer famine period, and received a higher concentration of nutrients and food, the granules destabilized over time. Perhaps, if operated with a shorter HRT, Reactor 3 would have maintained stability long-term. However, under the actual operating conditions, high-strength influent was not ideal for long-term operation, especially with short settling as the sole selective pressure applied throughout the study.

This study hypothesized that Reactor 3 would result in granulation first amongst the three reactors. Yet, as seen in Figure 11 and Figure 39, the settleability of biomass in Reactor 3 worsened, especially after day 140, and the physical appearance of biomass became more irregular and loose throughout the study. The second hypothesis predicted that Reactor 1 would provide the least effective nutrient removal. If the filling time on day 159 was not extended, phosphorus removal and denitrification performance would likely not have improved towards the end of the study. Since this type of operational change was not part of the Methods scope, the hypothesis was correct. Compared to Reactors 2 and 3, Reactor 1’s nutrient removal was less effective.

Contributions to the Field

Using low-strength wastewater as a substrate source did not impact the formation or stability of granules. This finding suggests that aerobic granular sludge (AGS) may be implemented in municipal WWTPs impacted by combined sewer systems or seasonal flow fluctuations such as heavy rainfall or snow melt. AGS technology offers superior settleability, biomass retention, simultaneous nutrient removal, and lower energy and footprint costs over the conventional activated sludge process [7], [8], [9], [10]. In summary, AGS should be considered as a feasible option for biological nutrient removal at municipal WWTPs, as diluted wastewater has no impact on AGS development when selective pressure is enforced and maintained.

Recommendations for Future Research

1. When bulking or foaming is noticed, the best practice is to waste biomass and continue to enforce the selective pressure. In Reactor 2, bulking was observed, and the settling time was lengthened. However, the settling time should have been shortened to wash out filamentous bacteria. As a result, the reduced selective pressure hindered the formation of dense granules.
2. Collect samples for 16S rRNA analysis from the start-up of the study to determine the microbial community shift between activated sludge to AGS. Moreover, the analysis would confirm if operational changes had an impact on the growth or disappearance of certain bacterial groups.
3. Maintaining the OLR across all reactors was challenging because of the PE quality fluctuations and inaccuracies in feed preparation for Reactors 1 and 3. So, future

studies should measure influent concentrations daily to ensure the precise addition of chemicals to the high-strength feed and the volume of tap water added to the low-strength feed.

4. Place the feed tubing towards the bottom of the reactor to limit the introduction of air during the filling phase.
5. Optimize the feast-famine lengths and hydraulic retention time to further prove which phase and cycle length promotes the growth of rapid settling, compact, and dense granules.
6. Addition of fermentate from the WEWPCC to all reactors to determine if additional volatile fatty acids enhance the development of AGS.
7. Implement nitrogen sparging in all reactors to provide consistent mixing and oxygen reduction during the anaerobic phase. Moreover, constantly measuring the ORP during the anaerobic phase would be beneficial.

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Appendix

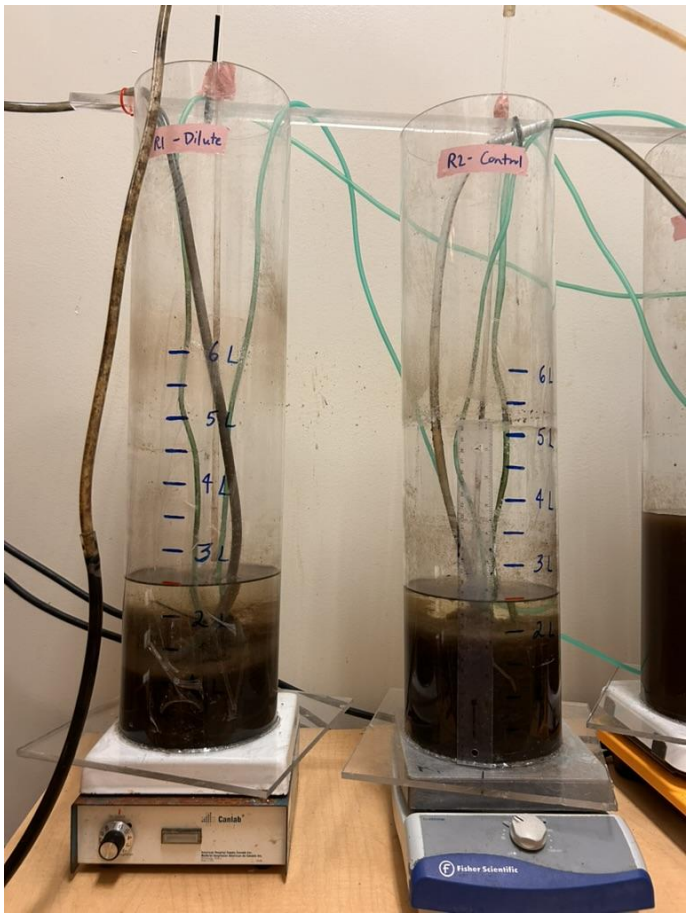


Figure 40: On day 124, during the idle phase of Reactors 1 and 2. Rapid settling was observed in Reactor 1 compared to Reactor 2.

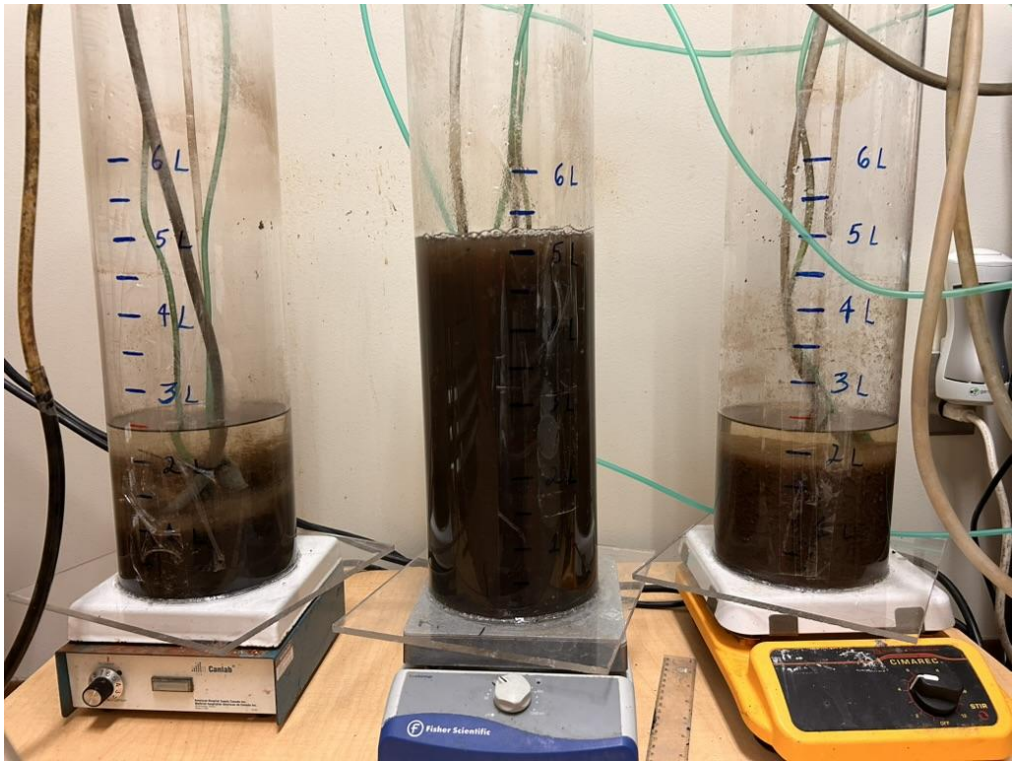


Figure 41: On day 124, during the idle phase of Reactors 1 and 3. Rapid settling was observed in Reactor 1 compared to Reactor 3.

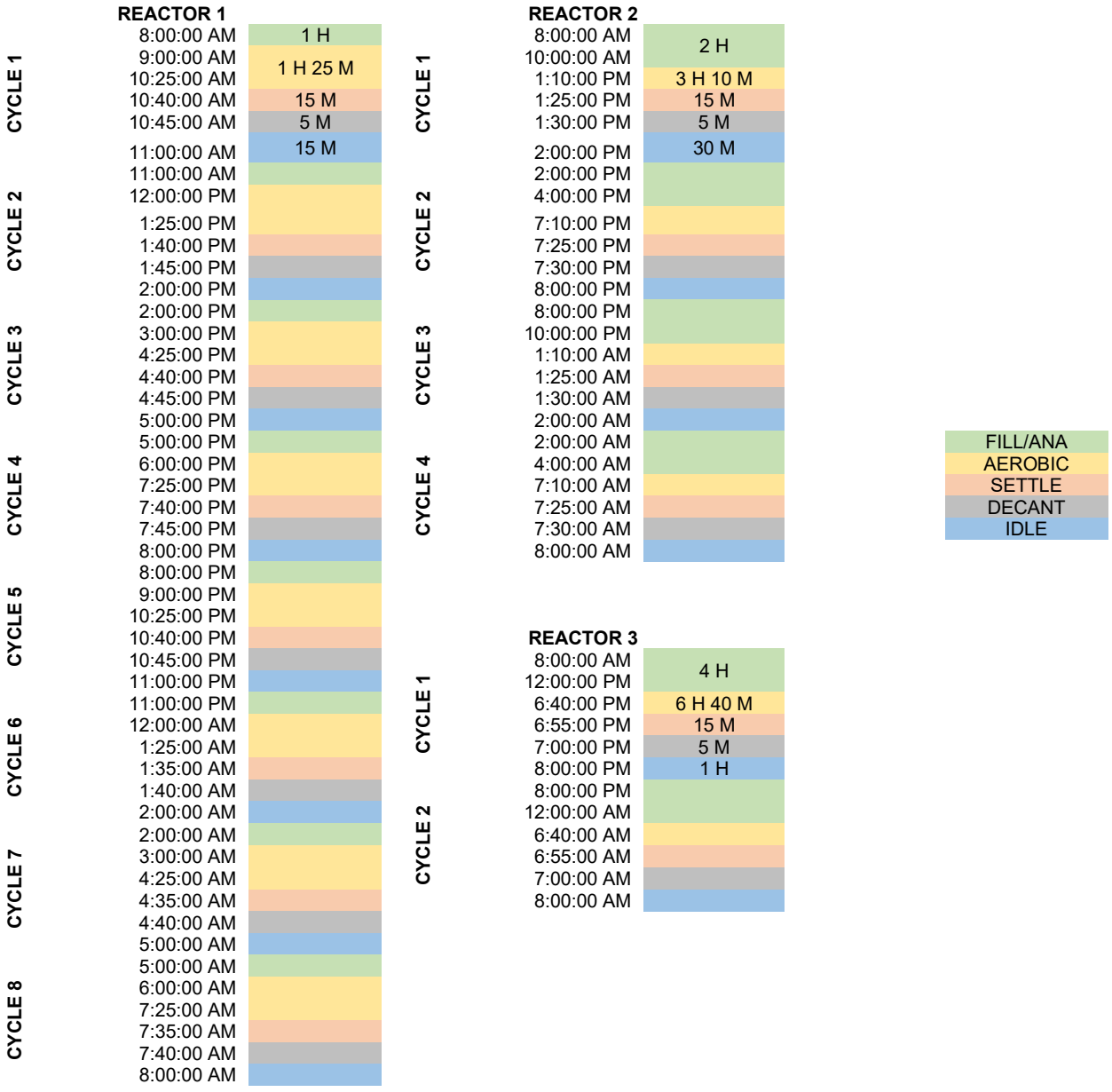


Figure 42: Daily cycle timing per reactor from day 0 to 54.