THE INTEGRATION OF RESOURCE RECOVERY STRATEGIES IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL

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— PhD Thesis —

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CERTIFIQUEM:

Que l'enginyera Congcong Zhang ha realitzat sota la nostra direcció, el treball que amb títol "THE INTEGRATION OF RESOURCE RECOVERY STRATEGIES IN ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL" es presenta en aquesta memòria, i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I per a què se'n prengui coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat.

Bellatera, 20 de juliol de 2022

Dr. Albert Guisasola i Canudas　　　　Dr. Juan Antonio Baeza Labat

A single spark can start a prairie fire *Mao Zedong*

星星之火，可以燎原 *毛泽东*

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Summary

As the global demand for water grows, the amount of wastewater produced and its overall pollution load are continuously increasing worldwide. Therefore, wastewater treatment is becoming a critical point in water management in view of its potential threat to public health as well as environmental problems. In this sense, wastewater treatment plants (WWTPs) are being transformed into water resource recovery facilities (WRRFs) with the aim to achieve a good effluent quality, as well as to recover resources (such as carbon (C), nitrogen (N) and phosphorus (P)), water and energy in a sustainable way.

Biological processes are considered as the most economical and sustainable processes for removing P and N to prevent eutrophication. Enhanced biological phosphorus removal (EBPR) is the most efficient way for P removal. The new paradigm in WWTP evolving to WRRF attempts to change P removal to P recovery from wastewater due to its significance on food production and the limited deposits on our planet. In EBPR-based WWTPs, the most reported location to precipitate/recover P is the digestate from the sludge treatment process. However, it can result in an undesired precipitation in reactors, tubes as well as the instrumentation. As an alternative, anaerobic reactor seems to provide an ideal scenario with high concentration of P: mainstream P-recovery. Due to the potential of high ammonium concentrations, struvite may seem a good precipitating agent. Apart from that, the integration of EBPR and mainstream P-recovery as struvite can also reduce N load of the mainstream. This thesis provides a critical evaluation of the existing mainstream P-recovery technologies reported under different configurations and thoroughly analyses the most critical parameters affecting the performance of mainstream P recovery. Then, this thesis studies, for the first time, an experimental evaluation of integration of mainstream P recovery to the recently designed process for novel WWTP, known as the two-stage A/B process. The two-stage A/B configuration is proposed with the aim of maximizing COD capture for biogas production and energy recovery by operating a high-rate active sludge system in A-stage, and nitrogen is removed in B-stage. The integration of A-stage and EBPR (A-stage-EBPR) is promising for simultaneous P and COD removal as well as the energy recovery.

The nature of the carbon source in the influent wastewater plays an important role in the EBPR process. Acetate and propionate are the most popular substrates for efficient lab-scale EBPR. Therefore, most of the reported experimental work has promoted the proliferation of *Accumulibacter* (which are, nowadays, the most common Polyphosphate Accumulating Organisms-PAO). However, recent microbiological advances in full-scale WWTPs have shown a high diversity of putative PAO other than *Accumulibacter* that are selected due the presence of other substances in the real wastewater (e.g. carbohydrates, proteins, alcohols…), which can’t be directly degraded by *Accumulibacter*. A fermentation process of these complex substances to VFA would be necessary for its

utilization by *Accumulibacter*. More perspectives on the different carbon source utilization need to be improved. Therefore, this thesis reviews the utilization of different carbon sources strategies with particular emphasis on the fermentation products from biosolids as additional carbon source. Secondly, a low concentration of COD is one of the main causes for EBPR failure in full-scale WWTP. The side-stream sludge fermenter (SSSF) is proposed to solve this problem by introducing part of the waste sludge for fermentation to provide additional COD for the mainstream process, which not only saves the cost but also decreases the carbon footprint dispensed with the external carbon addition.

Chapter 4 evaluates different P recovery strategies, especially the start of the art of the novel mainstream P-recovery. In addition, the effect of different carbon source on EBPR performance was comprehensively assessed to go beyond the knowledge of the common carbon sources (acetate and propionate) and specifically to the feasibility of solid waste as carbon source. A continuous pilot-scale A/O system (42 L) was operated in a low SRT and DO for P removal with the minimum of carbon mineralization for energy recovery (Chapter 5). Lastly, the performance of the incorporation of a SSSF into an A_2O configuration (S2EBPR) under a low COD influent condition was investigated and the microbial communities were evaluated (Chapter 6).

The most accepted strategy for P recovery is crystallization, and the preferred precipitation product is struvite (Chapter 4). When mainstream P-recovery is involved to EBPR, the amount of anaerobic supernatant extracted is the most important parameter to recover P for its successful implementation. According to the current investigations, 60% of influent P could be the maximum value for mainstream P-recovery in a long-term operation. A higher value may pose a potentially deleterious effect on PAO activity due to the decrease of the internal poly-P loads. Our primarily experimental results about the possibility of mainstream P- recovery on A_2O showed successful P, COD and ammonium removal regardless of aerobic or anaerobic purge. However, anaerobic purge improved P release by 27%. Further, 26% of input P could be recovered from the anaerobic supernatant under the SRT around 16 days with only 4.6 % of influent flow redirection.

The review on the potential EBPR carbon sources shows that the application of different carbon utilization strategies leads to more versatile metabolic ways of diverse putative PAOs (e.g. *Tetrasphaera*). *Tetrasphaera* show the ability to assimilate glucose, amino acids and waste sludge, which opens the door of more utilization possibility of diverse carbon source. Applying waste fermentation as additional carbon source shows successful system performance. It has been an attractive solution for solving the problem of insufficient COD of raw wastewater and the environmental pressure for the future WWTP.

In Chapter 5, the successful system performance of A-stage-EBPR was obtained with the SRT of 6, 5 and 4 days treating real wastewater. The best steady removal of P and COD (both around 95%) was achieved with the minimum SRT = 4d, and about 64% of input carbon could be diverted as biomass for energy recovery, about 69% of the influent N could be left for B-stage. The optimal biochemical methane potential was obtained was around 300 mL CH_4/gVSS, and the predominant position of *Accumulibacter* over *Competibacter* and *Defluviicoccus* was observed. Then the stability of this system was evaluated under different operational conditions with successful COD and P removal obtained in the range 0.5-1 mgDO/L without nitrification, but 0.2 mgDO/L led to system deterioration. 22% of influent P could be recovered by anaerobic purge for the mainstream P-recovery. With glutamate as sole carbon source, the system could maintain successful EBPR activity and COD removal for two months, and biomass loss happened with the sludge bulking problem afterwards. Microbial community analysis showed that *Propionivibrio* was the most favoured with glutamate, and *Propionivibrio*, *Thiothrix* and *Lewinella* were the most abundant species with propionic acid as sole carbon source.

In Chapter 6, the S2EBPR (with SSSF receiving 6% of the recycled activated sludge connected to the anaerobic reactor and HRT = 2 d) improved about 27% of P removal compared with A_2O under the same limited of influent COD (350 mg/L) condition. Full ammonium and COD removal was obtained but the fermentation effluent increased the P load to the plant and resulted in a higher P concentration in the effluent. The potential of energy production should be compromised, and both methane and energy recovery indexes were around 45% lower than those of A_2O. Sequencing analysis revealed a high abundance of PAO in S2EBPR, and an inferior GAO abundance compared with PAO. For further exploration of this novel configuration, the different combination possibilities were explored, and the combination of SSSF to anaerobic reactor showed superior EBPR performance than to anoxic and aerobic reactors.

Resumen

A medida que crece la demanda mundial de agua, la cantidad de aguas residuales producidas y su carga contaminante global aumentan continuamente en todo el mundo. Por lo tanto, el tratamiento de las aguas residuales se está convirtiendo en un punto crítico en la gestión del agua en vista de su potencial amenaza para la salud pública, así como los problemas ambientales. En este sentido, las plantas de tratamiento de aguas residuales (EDAR) se están transformando en instalaciones de recuperación de recursos hídricos (WRRF) con el objetivo de conseguir una buena calidad de los efluentes, así como de recuperar recursos (como el carbono (C), el nitrógeno (N) y el fósforo (P)), agua y energía de forma sostenible.

Los procesos biológicos se consideran los más económicos y sostenibles para eliminar el P y el N y evitar la eutrofización. La eliminación biológica mejorada de fósforo (EBPR) es la forma más eficiente de eliminar el P. El nuevo paradigma de las EDAR que evolucionan hacia los WRRF intenta cambiar la eliminación de P por la recuperación de P de las aguas residuales debido a su importancia en la producción de alimentos y a los limitados depósitos de nuestro planeta. En las EDAR basadas en la EBPR, la ubicación más conocida para precipitar/recuperar el P es el digestato del proceso de tratamiento de lodos. Sin embargo, puede dar lugar a una precipitación no deseada en los reactores, los tubos y la instrumentación. Como alternativa, el reactor anaeróbico parece proporcionar un escenario ideal con una alta concentración de P: la recuperación de P en la corriente principal. Debido al potencial de altas concentraciones de amonio, la estruvita puede parecer un buen agente precipitante. Además, la integración de la EBPR y la recuperación de P de la corriente principal, ya que la estruvita también puede reducir la carga de N de la corriente principal. Esta tesis proporciona una evaluación crítica de las tecnologías existentes de recuperación de P en la corriente principal bajo diferentes configuraciones y analiza a fondo los parámetros más críticos que afectan al rendimiento de la recuperación de P en la corriente principal. A continuación, esta tesis estudia, por primera vez, una evaluación experimental de la integración de la recuperación de P de la corriente principal en el proceso recientemente diseñado para una nueva EDAR, conocido como proceso A/B de dos etapas. La configuración A/B de dos etapas se propone con el objetivo de maximizar la captura de la DQO para la producción de biogás y la recuperación de energía operando un sistema de fangos activos de alta tasa en la etapa A, y el nitrógeno se elimina en la etapa B. La integración de la etapa A y la EBPR (A-stage-EBPR) es prometedora para la eliminación simultánea de P y DQO, así como para la recuperación de energía.

La naturaleza de la fuente de carbono en el agua residual afluente juega un papel importante en el proceso EBPR. El acetato y el propionato son los sustratos más populares para una EBPR eficiente a escala de laboratorio. Por lo tanto, la mayoría de los trabajos

experimentales reportados han promovido la proliferación de *Accumulibacter* (que son, hoy en día, los Organismos Acumuladores de Polifosfato-PAO más comunes). Sin embargo, los recientes avances microbiológicos en las EDAR a escala real han mostrado una gran diversidad de PAOs putativos distintos de *Accumulibacter* que son seleccionados debido a la presencia de otras sustancias en el agua residual real (por ejemplo, carbohidratos, proteínas, alcoholes...), que no pueden ser degradados directamente por *Accumulibacter*. Sería necesario un proceso de fermentación de estas sustancias complejas a AGV para su utilización por *Accumulibacter*. Es necesario mejorar las perspectivas de utilización de las diferentes fuentes de carbono. Por lo tanto, esta tesis revisa las estrategias de utilización de diferentes fuentes de carbono con especial énfasis en los productos de fermentación de los biosólidos como fuente de carbono adicional. En segundo lugar, una baja concentración de DQO es una de las principales causas del fracaso de la EBPR en las EDAR a gran escala. Se propone el fermentador de lodos lateral (SSSF) para resolver este problema introduciendo parte de los lodos residuales para su fermentación con el fin de proporcionar DQO adicional para el proceso principal, lo que no sólo ahorra el coste sino que también disminuye la huella de carbono prescindiendo de la adición de carbono externo.

En el capítulo 4 se evalúan diferentes estrategias de recuperación de P, especialmente el inicio de la novedosa recuperación de P en la corriente principal. Además, se evaluó exhaustivamente el efecto de diferentes fuentes de carbono en el rendimiento de la EBPR para ir más allá del conocimiento de las fuentes de carbono comunes (acetato y propionato) y, específicamente, la viabilidad de los residuos sólidos como fuente de carbono. Se operó un sistema continuo de A/O a escala piloto (42 L) en un SRT y DO bajos para la eliminación de P con el mínimo de mineralización de carbono para la recuperación de energía (Capítulo 5). Por último, se investigó el rendimiento de la incorporación de un SSSF en una configuración de A_2O (S2EBPR) bajo una condición de influente de baja DQO y se evaluaron las comunidades microbianas (Capítulo 6).

La estrategia más aceptada para la recuperación de P es la cristalización, y el producto de precipitación preferido es la estruvita (Capítulo 4). Cuando la recuperación de P en la corriente principal está involucrada en la EBPR, la cantidad de sobrenadante anaeróbico extraído es el parámetro más importante para recuperar P para su implementación exitosa. Según las investigaciones actuales, el 60% del P afluente podría ser el valor máximo para la recuperación de P en la corriente principal en una operación a largo plazo. Un valor más alto puede suponer un efecto potencialmente perjudicial para la actividad de la PAO debido a la disminución de las cargas internas de poli-P. Nuestros principales resultados experimentales sobre la posibilidad de recuperación de P en la corriente principal de la A_2O mostraron una buena eliminación de P, DQO y amonio, independientemente de la purga aeróbica o anaeróbica. Sin embargo, la purga anaeróbica mejoró la liberación de P en un 27%. Además, el 26% del P de entrada pudo recuperarse del sobrenadante anaeróbico bajo el SRT de unos 16 días con sólo un 4,6% de redirección del flujo afluente.

La revisión de las potenciales fuentes de carbono de la EBPR muestra que la aplicación de diferentes estrategias de utilización del carbono conduce a formas metabólicas más versátiles de diversas PAOs putativas (por ejemplo, *Tetrasphaera*). *Tetrasphaera* muestra la capacidad de asimilar glucosa, aminoácidos y lodos residuales, lo que abre la puerta a más posibilidades de utilización de diversas fuentes de carbono. La aplicación de la fermentación de residuos como fuente de carbono adicional muestra un rendimiento exitoso del sistema. Ha sido una solución atractiva para resolver el problema de la insuficiente DQO de las aguas residuales crudas y la presión ambiental para la futura EDAR.

En el capítulo 5, se obtuvo el rendimiento exitoso del sistema de la etapa A-EBPR con el SRT de 6, 5 y 4 días tratando aguas residuales reales. La mejor eliminación constante de P y DQO (ambas en torno al 95%) se consiguió con el SRT mínimo = 4d, y alrededor del 64% del carbono de entrada pudo desviarse como biomasa para la recuperación de energía, alrededor del 69% del N afluente pudo dejarse para la etapa B. El potencial bioquímico óptimo de metano obtenido fue de unos 300 mL CH_4/gVSS, y se observó la posición predominante de *Accumulibacter* sobre *Competibacter* y *Defluviicoccus*. A continuación, se evaluó la estabilidad de este sistema bajo diferentes condiciones operativas, obteniéndose un éxito en la eliminación de DQO y P en el rango de 0,5-1 mgDO/L sin nitrificación, pero 0,2 mgDO/L condujo al deterioro del sistema. El 22% del P afluente pudo recuperarse mediante una purga anaeróbica para la recuperación de P en la corriente principal. Con el glutamato como única fuente de carbono, el sistema pudo mantener una actividad exitosa de EBPR y la eliminación de DQO durante dos meses, y la pérdida de biomasa se produjo con el problema de abultamiento de los lodos después. El análisis de la comunidad microbiana mostró que *Propionivibrio* era el más favorecido con el glutamato, y *Propionivibrio*, *Thiothrix* y *Lewinella* eran las especies más abundantes con el ácido propiónico como única fuente de carbono.

En el capítulo 6, el S2EBPR (con SSSF recibiendo el 6% de los lodos activados reciclados conectados al reactor anaeróbico y HRT = 2 d) mejoró alrededor del 27% de la eliminación de P en comparación con A_2O bajo la misma condición limitada de DQO influente (350 mg/L). Se obtuvo una eliminación completa de amonio y DQO, pero el efluente de la fermentación aumentó la carga de P en la planta y dio lugar a una mayor concentración de P en el efluente. El potencial de producción de energía debía comprometerse, y tanto los índices de recuperación de metano como de energía eran alrededor de un 45% inferiores a los de la A_2O. El análisis de secuenciación reveló una alta abundancia de PAO en S2EBPR, y una abundancia inferior de GAO en comparación con PAO. Para profundizar en esta novedosa configuración, se exploraron las diferentes posibilidades de combinación, y la combinación de SSSF con el reactor anaeróbico mostró un rendimiento de EBPR superior al de los reactores anóxicos y aeróbicos.

Resum

A mesura que creix la demanda mundial d'aigua, la quantitat d'aigües residuals produïdes i la càrrega contaminant global augmenten contínuament a tot el món. Per tant, el tractament de les aigües residuals s'està convertint en un punt crític en la gestió de l'aigua en vista de la potencial amenaça per a la salut pública, així com els problemes ambientals. En aquest sentit, les plantes de tractament d'aigües residuals (EDAR) s'estan transformant en instal·lacions de recuperació de recursos hídrics (WRRF) amb l'objectiu d'aconseguir una bona qualitat dels efluents, així com de recuperar recursos (com el carboni (C)), el nitrogen (N) i el fòsfor (P)), aigua i energia de forma sostenible.

Els processos biològics es consideren els més econòmics i sostenibles per eliminar el P i el N i evitar l'eutrofització. L'eliminació biològica millorada de fòsfor (EBPR) és la forma més eficient d'eliminar el P. El nou paradigma de les EDAR que evolucionen cap als WRRF intenta canviar l'eliminació de P per la recuperació de P de les aigües residuals a causa de la seva importància a la producció d'aliments i als dipòsits limitats del nostre planeta. A les EDAR basades en l'EBPR, la ubicació més coneguda per precipitar/recuperar el P és el digestat del procés de tractament de llots. Això no obstant, pot donar lloc a una precipitació no desitjada en els reactors, els tubs i la instrumentació. Com a alternativa, el reactor anaeròbic sembla proporcionar un escenari ideal amb una alta concentració de P: la recuperació de P al corrent principal. A causa del potencial d'altes concentracions d'amoni, l'estruvita pot semblar un bon agent precipitant. A més, la integració de l'EBPR i la recuperació de P del corrent principal, ja que l'estruvita també pot reduir la càrrega de N del corrent principal. Aquesta tesi proporciona una avaluació crítica de les tecnologies existents de recuperació de P al corrent principal sota diferents configuracions i analitza a fons els paràmetres més crítics que afecten el rendiment de la recuperació de P al corrent principal. Tot seguit, aquesta tesi estudia, per primera vegada, una avaluació experimental de la integració de la recuperació de P del corrent principal en el procés recentment dissenyat per a una nova EDAR, conegut com a procés A/B de dues etapes. La configuració A/B de dues etapes es proposa amb l'objectiu de maximitzar la captura de la DQO per a la producció de biogàs i la recuperació d'energia operant un sistema de fangs actius d'alta taxa a l'etapa A, i el nitrogen s'elimina a l'etapa B. La integració de l'etapa A i l'EBPR (A-stage-EBPR) és prometedora per a l'eliminació simultània de P i DQO, així com per a la recuperació d'energia.

La naturalesa de la font de carboni a l'aigua residual afluent té un paper important en el procés EBPR. L'acetat i el propionat són els substrats més populars per a una EBPR eficient a escala de laboratori. Per tant, la majoria dels treballs experimentals reportats han promogut la proliferació d'*Accumulibacter* (que són, avui dia, els Organismes Acumuladors de Polifosfat-PAO més comuns). No obstant això, els recents avenços microbiològics a les EDAR a escala real han mostrat una gran diversitat de PAO putatius

diferents d'*Accumulibacter* que són seleccionats a causa de la presència d'altres substàncies a l'aigua residual real (per exemple, carbohidrats, proteïnes, alcohols). .), que no poden ser degradats directament per *Accumulibacter*. Caldria un procés de fermentació d'aquestes substàncies complexes a AGV per utilitzar-lo per *Accumulibacter*. Cal millorar les perspectives d'utilització de les diferents fonts de carboni. Per tant, aquesta tesi revisa les estratègies d'utilització de diferents fonts de carboni amb un èmfasi especial en els productes de fermentació dels biosòlids com a font de carboni addicional. En segon lloc, una baixa concentració de DQO és una de les principals causes del fracàs de l'EBPR a les EDAR a gran escala. Es proposa el fermentador de llots lateral (SSSF) per resoldre aquest problema introduint part dels llots residuals per a la seva fermentació per tal de proporcionar DQO addicional per al procés principal, cosa que no només estalvia el cost sinó que també disminueix l'empremta de carboni prescindint de l'addició de carboni extern.

Al capítol 4 s'avaluen diferents estratègies de recuperació de P, especialment l'inici de la nova recuperació de P al corrent principal. A més, es va avaluar exhaustivament l'efecte de diferents fonts de carboni en el rendiment de l'EBPR per anar més enllà del coneixement de les fonts de carboni comunes (acetat i propionat) i, específicament, la viabilitat dels residus sòlids com a font de carboni. Es va operar un sistema continu d'A/O a escala pilot (42 L) en un SRT i DO baixos per a l'eliminació de P amb el mínim de mineralització de carboni per a la recuperació d'energia (Capítol 5). Per acabar, es va investigar el rendiment de la incorporació d'un SSSF en una configuració d'A_2O (S2EBPR) sota una condició d'influent de baixa DQO i es van avaluar les comunitats microbianes (Capítol 6).

L'estratègia més acceptada per a la recuperació de P és la cristal·lització i el producte de precipitació preferit és l'estruvita (Capítol 4). Quan la recuperació de P al corrent principal està involucrada a l'EBPR, la quantitat de sobrenedant anaeròbic extret és el paràmetre més important per recuperar P per a la seva implementació amb èxit. Segons les investigacions actuals, el 60% del P afluent podria ser el valor màxim per a la recuperació de P al corrent principal en una operació a llarg termini. Un valor més alt pot suposar un efecte potencialment perjudicial per a l'activitat de la PAO a causa de la disminució de les càrregues internes de poli-P. Els nostres principals resultats experimentals sobre la possibilitat de recuperació de P al corrent principal de l'A_2O van mostrar una bona eliminació de P, DQO i amoni, independentment de la purga aeròbica o anaeròbica. Tot i això, la purga anaeròbica va millorar l'alliberament de P en un 27%. A més, el 26% del P d'entrada es va poder recuperar del sobrenedant anaeròbic sota l'SRT d'uns 16 dies amb només un 4,6% de redirecció del flux afluent.

La revisió de les fonts de carboni potencials de l'EBPR mostra que l'aplicació de diferents estratègies d'utilització del carboni condueix a formes metabòliques més versàtils de diverses PAO putatives (per exemple, *Tetrasphaera*). *Tetrasphaera* mostra la capacitat d'assimilar glucosa, aminoàcids i llots residuals, cosa que obre la porta a més possibilitats d'utilització de diverses fonts de carboni. L'aplicació de la fermentació de residus com a font de carboni addicional mostra un rendiment exitós del sistema. Ha estat una solució atractiva per resoldre el problema de la insuficient DQO de les aigües residuals crues i la pressió ambiental per a la futura EDAR.

Al capítol 5, es va obtenir el rendiment exitós del sistema de l'etapa A-EBPR amb l'SRT de 6, 5 i 4 dies tractant aigües residuals reals. La millor eliminació constant de P i DQO (totes dues al voltant del 95%) es va aconseguir amb el SRT mínim = 4d, i al voltant del 64% del carboni d'entrada es va poder desviar com a biomassa per a la recuperació d'energia, al voltant del 69% del N es va poder deixar afluent per a l'etapa B. El potencial bioquímic òptim de metà obtingut va ser d'uns 300 mL CH_4/gVSS, i es va observar la posició predominant d'*Accumulibacter* sobre *Competibacter* i *Defluviicoccus*. A continuació, es va avaluar l'estabilitat d'aquest sistema sota diferents condicions operatives, obtenint un èxit en l'eliminació de DQO i P en el rang de 0,5-1 mgDO/L sense nitrificació, però 0,2 mgDO/L va conduir al deteriorament del sistema. El 22% del P afluent es va poder recuperar mitjançant una purga anaeròbica per a la recuperació de P al corrent principal. Amb el glutamat com a única font de carboni, el sistema va poder mantenir una activitat d'èxit d'EBPR i l'eliminació de DQO durant dos mesos, i la pèrdua de biomassa es va produir amb el problema d'engreixament dels llots després. L'anàlisi de la comunitat microbiana va mostrar que *Propionivibrio* era el més afavorit amb el glutamat, i *Propionivibrio*, *Thiothrix* i *Lewinella* eren les espècies més abundants amb l'àcid propiònic com a única font de carboni.

Al capítol 6, el S2EBPR (amb SSSF rebent el 6% dels llots activats reciclats connectats al reactor anaeròbic i HRT = 2 d) va millorar al voltant del 27% de l'eliminació de P en comparació amb A_2O sota la mateixa condició limitada de DQO influent (350 mg/L). Es va obtenir una eliminació completa d'amoni i DQO, però l'efluent de la fermentació va augmentar la càrrega de P a la planta i va donar lloc a una concentració més gran de P a l'efluent. El potencial de producció d'energia s'havia de comprometre i tant els índexs de recuperació de metà com d'energia eren al voltant d'un 45% inferiors als de l'A_2O. L'anàlisi de seqüenciació va revelar una alta abundància de PAO a S2EBPR, i una abundància inferior de GAO en comparació amb PAO. Per aprofundir en aquesta nova configuració, es van explorar les diferents possibilitats de combinació i la combinació de SSSF amb el reactor anaeròbic va mostrar un rendiment d'EBPR superior al dels reactors anòxics i aeròbics.

摘要

随着全球对水需求的增长，全世界产生的废水量及其总体污染负荷不断增加。因此，考虑到废水对公众健康和环境问题的潜在威胁，废水处理正成为水管理的一个关键点。在这个意义上，废水处理厂（WWTPs）正在转变为水资源回收设施（WRRFs），目的是实现良好的出水质量，并以可持续的方式回收资源（如碳（C），氮（N）和磷（P）），水和能源。

生物工艺被认为是去除 P 和 N 以防止富营养化的最经济和可持续发展的工艺。强化生物除磷（EBPR）是最有效的除磷方式。污水处理厂发展 WRRF 的新模式，是试图将废水中的 P 去除改为 P 回收，因为 P 不仅对粮食生产有重要意义，而且在地球上的储量有限。在基于 EBPR 的污水处理厂中，被常见的回收 P 的位置是污泥处理过程中的消化液。然而，这个过程可能会导致反应器、管道和仪器中出现不希望有的沉淀。厌氧反应器作为一种选择，似乎提供了一种理想的高浓度 P 的情况：主流 P 回收。由于潜在的高铵根浓度，为鸟粪石（磷酸铵镁）成为沉淀剂提供很大的可能性。除此之外，EBPR 和主流 P 回收的整合也可以减少主流的 N 负荷。本论文对不同配置下的现有主流 P 回收技术进行了严格评估，并深入分析了影响主流 P 回收性能的最关键参数。然后，本论文首次研究了主流 P 回收与最近设计的新型污水处理厂工艺（即两级 A/B 工艺）整合的实验评估。提出两阶段 A/B 配置的目的是通过在 A 阶段运行高速活性污泥系统，最大限度地捕获 COD 以生产沼气和回收能源，并在 B 阶段去除氮。A 阶段和 EBPR 的整合对于同时去除 P 和 COD 以及能源回收是有希望的。

进水废水中碳源的性质在 EBPR 过程中起着重要作用。乙酸盐和丙酸盐是实现高效的实验室规模 EBPR 的最受欢迎的底物。因此，大多数报道的实验工作都促进了 *Accumulibacter*（最常见的 PAO-*Accumulibacter*）的繁殖。然而，最近在大规模污水处理厂的微生物学进展显示，除 *Accumulibacter* 外，还有很多假定的 PAO，它们是由于实际废水中存在其他物质（如碳水化合物、蛋白质、酒精......）而被富集的，而这些物质不能被 *Accumulibacter* 直接降解。这些复杂物质的发酵过程对于 *Accumulibacter* 的利用是必要的。关于不同碳源利用的更多观点需要被改进。因此，本论文回顾了不同碳源的利用策略，特别强调了生物固体的发酵产物作为额外的碳源。其次，低浓度的 COD 是导致大规模污水处理厂 EBPR 失败的主要原因之一。侧流污泥发酵池（SSSF）被提出来解决这个问题，通过引入部分剩余污泥进行发酵，为主流工艺提供额外的 COD，这不仅节省了成本，也减少了外部碳添加所带来的碳足迹。

第 4 章评估了不同的 P 回收策略，特别是新型主流 P 回收技术的发展。此外，全面评估了不同碳源对 EBPR 性能的影响，以超越对常见碳源（乙酸盐和丙酸盐）

的认识，特别是对固体废物作为碳源的可行性。一个中试规模的 A/O 连续系统（42L）在低 SRT 和 DO 下运行来去除 P，并将碳的矿化程度降到最低以便进行能源回收（第 5 章）。最后，研究了在低 COD 进水条件下，将 SSSF 纳入 A_2O 配置（S2EBPR）的性能，并对微生物群落进行了评估（第 6 章）。

最被接受的 P 回收策略是结晶，而首选的沉淀产品是鸟粪石（第 4 章）。当主流的 P 回收涉及到 EBPR 时，厌氧上清液的提取量是成功实施 P 回收的最重要参数。根据目前的研究，在长期运行中，60%的进水 P 可能是主流 P 回收的最高值。更高的值可能会对 PAO 的活性造成潜在的有害影响由于其内部多聚物存储的减少。我们关于在 A_2O 主流 P 回收的可能性的实验结果表明，不管是好氧还是厌氧排泥，都能成功去除 P、COD 和铵。然而，厌氧排泥提高了 27%的 P 释放。此外，在 16 天左右的 SRT 条件下，通过提取 4.6％进水量的上清液，就可以从厌氧上清液中回收 26％的进水 P。

对潜在的 EBPR 碳源的研究表明，不同的碳源利用策略的应用导致了不同的假定 PAO（如 *Tetrasphaera*）的更多的代谢方式。*Tetrasphaera* 显示出同化葡萄糖、氨基酸和固体有机物的能力，这为更多地利用不同碳源的可能性打开了大门。应用废物发酵作为额外的碳源表现出成功的系统性能，这对于解决原污水中 COD 不足的问题和未来污水处理厂的环境压力来说，是一个有吸引力的解决方案。

在第 5 章中，A 阶段 EBPR 的成功处理实际废水的性能可以在 SRT 为 6 天、5 天和 4 天的情况下得到。在最短的 SRT 为 4 天时，实现了对 P 和 COD 的最佳稳定去除（都在 95%左右），大约 64%的输入碳可以作为生物质转用于能源回收，大约 69%的进水 N 可以留给 B 阶段处理。获得的最佳生化甲烷潜力约为 300 mL CH4/gVSS，并观察到 *Accumulibacter* 相较于 *Competibacter* 和 *Defluviicoccus* 占主导地位。然后，在不同的操作条件下对该系统的稳定性进行了评估，在 0.5-1 mgDO/L 的范围内成功去除 COD 和 P，但 0.2 mgDO/L 会导致系统的恶化。22%的进水 P 可以通过厌氧排泥进行主流 P 回收。以谷氨酸为唯一的碳源时，该系统可以保持两个月的成功的 EBPR 活性和 COD 去除，之后会发生生物量损失与污泥膨胀的问题。微生物群落分析显示，*Propionivibrio* 是最喜欢用谷氨酸为碳源的物种，而丙酸为唯一碳源时，*Propionivibrio*、*Thiothrix* 和 *Lewinella* 是最丰富物种。

在第 6 章中，与 A_2O 相比，在相同的进水 COD（350mg/L）条件下，S2EBPR（SSSF 接受 6%的循环活性污泥并且连接到厌氧反应器中，HRT=2d）提高了约 27%的 P 去除率。铵根和 COD 得到了完全的去除，但发酵出水增加了系统的 P 负荷，导致出水的 P 浓度更高。能源生产的潜力也会受到影响，甲烷和能源回收指数都比 A_2O 低 45%左右。测序分析显示，S2EBPR 中 PAO 的丰度较高，而 GAO

的丰度则远低于 PAO。为了进一步探索这种新的配置，我们研究了不同组合的可能性，SSSF 与厌氧反应器的组合显示出比缺氧和好氧反应器更好的 EBPR 性能。

Contents

Chapter 1:

General introduction

1. General introduction

1.1. The importance of wastewater treatment

The issue of environmental pollution is of great significance for our planet. The problem of water pollution and the shortage of available drinking water poses a great challenge for the survival of human beings. As a result, the organic substances, nutrients, coagulants, metal or other contaminants in wastewater have to be removed to meet the discharge limitations in view of reducing the current pressure of water bodies. Table 1.1 shows the main compositions of raw municipal wastewater.

Table 1.1 The main compositions of raw municipal wastewater in terms of organic compounds, nutrient elements and solids (Tchobanoglous et al., 2013).

Compositions (mg/L)	High strength	Medium strength	Low strength
Total COD	1200	750	500
Soluble COD	480	300	200
VFA (mg-acetate/L)	80	30	10
Total nitrogen	100	60	30
NH_4^+-N	75	45	20
Total phosphate	25	15	6
Total suspended solids	600	400	250
Volatile suspended solids	480	320	200

Eutrophication is a globally widespread pollution phenomenon, which emerged from the dramatic development and expansion of the industries in the last century. The population expansion and food demand induce the overutilization of fertilization on agriculture, which result in the excess concentration of P and N discharging to the water bodies, and further the overgrowth of aquatic plant and the accompanying phenomenon of marine 'dead zone' (Ashley et al., 2011). Much stricter discharge rules limit the discharge of the P and N into the water. Table 1.2 and 1.3 show the discharge requirements of the pollutants in European and China. Nutrients should be removed by physical, chemical or biological methods before the discharging to lakes, rivers and oceans for a sustainable development (Perera et al., 2019; Rout et al., 2021).

1.2. Traditional biological nutrient removal based on activated sludge process

Numerous technologies have been proposed to track the problem of overload concentration of P and N in the wastewater. The dosage of chemicals has been proved to be efficient to remove the nutrients in the water. However, its collateral detrimental effects cannot be ignored (e.g. the high cost of the chemicals and the associated danger to its handling, the pollution to the environment and the increased operation and maintenance fees due to the higher sludge generation) (Moelants et al., 2011). Likewise, it is unavoidable that the expensive cost of membrane applications (e.g. forward osmosis) and the inefficient cleaning and maintenance of the membrane fouling (Mulkerrins et al., 2004; Shahid et al., 2020).

Table 1.2 Requirements for discharges for urban WWTPs of the Directive in European Union. The values for concentration or for the percentage of reduction shall apply (Council Directive, 1992).

Indicator (mg/L)	Concentration (mg/L)	Population equivalent	Minimum percentage of reduction[a]
COD	125	-	75
BOD_5	25	-	70-90 (or 40)[b]
TSS	35	more than 10000	90
	60	2000-10000	70
TN	15	10,000-100,000	70-80
	10	more than 100,000	70-80
TP	2	10,000-100,000	80
	1	more than 100,000	80

a Reduction in relation to the load of the influent
b Depend on different articles

Table 1.3. Discharge standard of pollutants for municipal WWTPs in China (GB 18918–2002) (Zhang et al., 2016).

Indicator	Grade I-A[c]	Grade I-B[c]	Grade I-C[c]
COD	50	60	100
BOD_5	10	20	30
TSS	10	20	30
TN	15	20	-
TP	0.5	1	3

c With the unit of mg/L

As a result, biological nutrient removal processes have been developed and evolved due to the disadvantages of the chemical and physical methods described above. Biological nutrient removal is based on activated sludge systems enriched with microorganisms under different conditions (anaerobic/anoxic and aerobic). The typical activated sludge systems could remove COD by forming new biomass or by oxidising it CO_2. The incorporation of activated sludge systems and biological nutrient removal allows the simultaneous biological P, N and COD removal (Henze et al., 2008; Metcalf andEddy et al., 2014). Biological nutrient removal based activated sludge process has been considered as the most sustainable and economical solution to solve the eutrophication, which is widely applied in real WWTPs.

1.2.1. *Nitrification/denitrification process*

Nitrification is the process of ammonium oxidation to nitrate by two steps: the first step is nitritation, where ammonium is oxidized to nitrite by ammonia oxidizing bacteria (AOB); the second step is the nitratation process, where nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). Denitrification requires the presence of an electron donor under anoxic conditions (generally a carbon source), and nitrite/nitrate is reduced to gaseous nitrogen (N_2), by ordinary heterotrophic organisms (OHO) (Henze M., Gujer W., Mino T., Matsuo T., Wentzel M. C., 1999). The conventional biological nitrogen removal is based on the above nitrification and denitrification process (Figure 1.1).

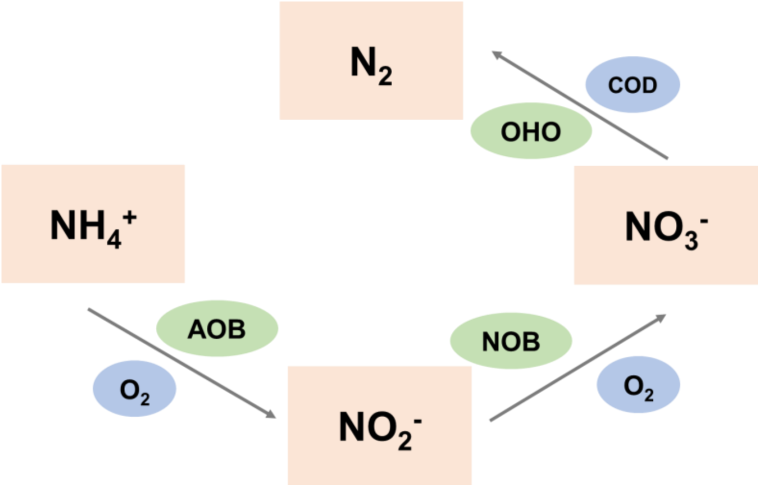

Figure 1.1. Conventional nitrification and denitrification process for biological nitrogen removal.

1.2.2. *Enhanced biological phosphorus removal process*

Enhanced Biological Phosphorus Removal (EBPR) has been proposed and widely applied for P and organic matter removal for several decades and, thus, it is regarded as one of the most sustainable and efficient technologies in WWTPs. During the EBPR process, polyphosphate accumulating organisms (PAOs) are considered as the functional bacteria for enabling P and COD removal.

Normally PAOs can uptake organic matter (mainly volatile fatty acids, VFA) and store them as polyhydroxyalkanoate (PHA) into the cell under anaerobic conditions. The energy required is supported by the hydrolysis of glycogen and intracellular polyphosphate (poly-P). Under anoxic/aerobic conditions, PHA is utilized for the replenishment of glycogen and the accumulation of phosphate into the cell to remove P, as well as the biomass growth, and P is removed from the liquid by stored in cell (Figure 1.2). Most of *Candidatus* Accumulibacter-related PAOs (generally referred as

Accumulibacter) have shown this traditional metabolic pathway. *Accumulibacter* are promoted with VFA (e.g. acetate and propionate) as electron donor and, generally, in lab-scale systems (Nielsen et al., 2019; Singleton et al., 2022). Some other PAO-relative bacteria were reported to show more versatile metabolic ways under some specific carbon sources. For instance, Nguyen et al., (2011) observed that most *Tetrasphaera* could take up acetate, casamino acid, glutamic acid and glucose as carbon sources anaerobically but are lack of the ability to form PHA. Likewise, *Thiothrix* was found to store poly-P for P removal with glutamate as carbon source but without PHA synthesis (Rey-Martínez et al., 2019).

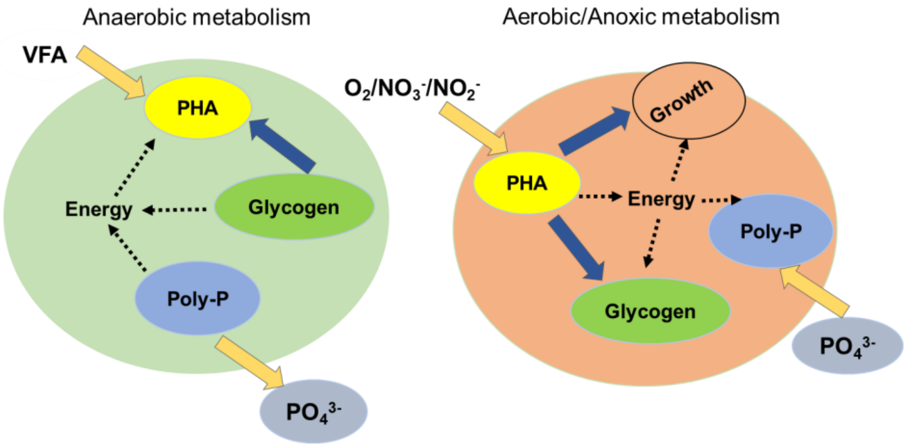

Figure 1.2. Conventional metabolic pathway for PAO (DPAO).

Denitrifying PAOs (DPAOs) were regarded as heterotrophic denitrifying phosphorus accumulating organisms, which is attributed to the removal of nitrogen and P simultaneously by the usage of nitrate or nitrite as electron acceptors for P uptake instead of oxygen (Comeau et al., 1987; Guisasola et al., 2009; Kerrn-Jespersen and Henze, 1993; Kuba et al., 1996) (Figure 1.2). Under aerobic condition, DPAOs were reported to conduct P uptake as PAOs due to the possession of enzymes for aerobic metabolism (Zeng et al., 2003a). For example, *Dechloromonas*-related PAOs have been shown to use nitrate and nitrite as electron acceptors (McIlroy et al., 2016). P removal by DPAOs shows advantages: i) the utilization of nitrate or nitrite instead of oxygen saves the cost for aeration. ii) less COD could be required for DPAOs compared with separate removal of P and N by PAOs. iii) 20%-30% lower cell yield could result in less sludge production (Oehmen et al., 2007; T. Kuba, A. Wachtmeister, M. C. M. van Loosdrecht, 1994).

Among the different reported failures of the EBPR process, the presence of glycogen accumulating organisms (GAOs) has been long studied. GAOs would utilize carbon sources to store as intracellular PHA anaerobically, and the glycogen hydrolysis provides the sole source for obtaining energy (Figure 1.3). For the aerobic condition, the PHA is consumed for the growth. The main difference is that the whole anaerobic and aerobic process they don’t exhibit P release and uptake (M.Sudiana et al., 1999; Oehmen et al., 2005a). Therefore, GAOs (e.g. *Candidatus* Competibacter, *Propionivibrio*, *Defluviicoccu*) could be the major competitor of PAOs for utilizing VFA and the dominant presence of GAOs could outcompete PAOs and further lead EBPR failure (Nittami et al., 2009; Oehmen et al., 2007; Roy et al., 2021; Wong et al., 2004). The successful EBPR performance is determined by the dominant PAOs over GAOs. Different operation conditions can affect the competition of PAOs and GAOs and adjust the relative abundances: the influent ratio of C/P, the type of carbon usage, aeration, SRT, temperature and so on (Nielsen et al., 2019; Oehmen et al., 2007; Roy et al., 2021; Shen and Zhou, 2016; Valverde-Pérez et al., 2016), which could be explained hereinafter.

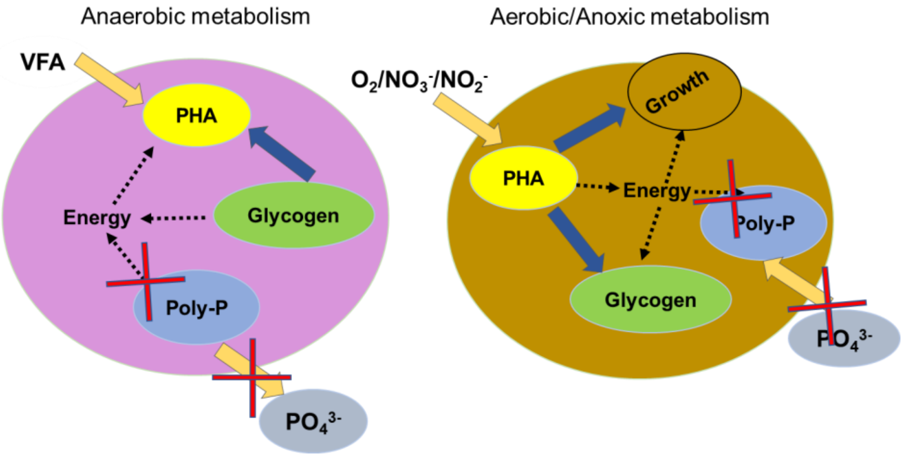

Figure 1.3. Metabolic pathway for GAO.

1.3. The configurations in wastewater treatment plant for EBPR

As mentioned above, EBPR process is involved with the recirculation of enriched PAO biomass by alternating anaerobic and aerobic state. In the application cases, biological N removal is normally incorporated by an anoxic reactor for simultaneous removal of P and N. Different configurations have been developed to realize that.

1.3.1. *Phoredox system (A/O)*

The Phoredox system is the most common system for biological P removal. It is a continuous process which is consisted of a separated anaerobic, aerobic reactor (A/O) and a sedimentation tank. Biological P removal comes from the P release in the anaerobic reactor and the P uptake in the following aerobic reactor. Phosphorus is removed by being stored as poly-P in PAOs, and carbon is removed under both anaerobic and aerobic conditions. The returned activated sludge (RAS) from the sedimentation tank is recirculated to the anaerobic reactor directly to maintain the biomass concentration levels (Grady Jr et al., 2011) (Figure 1.4).

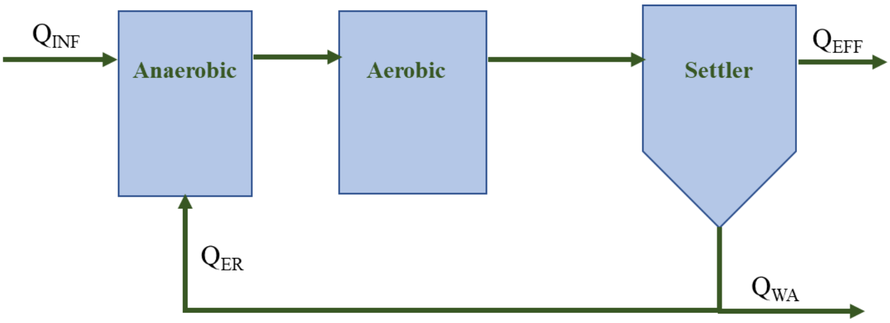

Figure 1.4. Process diagram for A/O system.

1.3.2. *Anaerobic/Anoxic/Aerobic system (A_2O)*

A_2O consists of an anaerobic, anoxic and aerobic reactor, which is the most conventional configuration for simultaneous P, COD and N removal (Figure 1.5). P is removed in the anoxic reactor (under the presence of nitrate or nitrite) and in the aerobic reactor (with the presence of oxygen), and N is removed by nitrification (aerobic reactor) and denitrification (anoxic reactor) with the help of the internal recycle of the mixed liquor from the aerobic reactor to the anoxic reactor.

The major drawbacks are the potential unstable performance due to: i) a low influent C/N or C/P ratio; ii) nitrate entering through the external recycle and iii) undesired struvite precipitation during anaerobic digestion of EBPR sludge (Guerrero et al., 2011; Oehmen et al., 2007; Yuan and Oleszkiewicz, 2010).

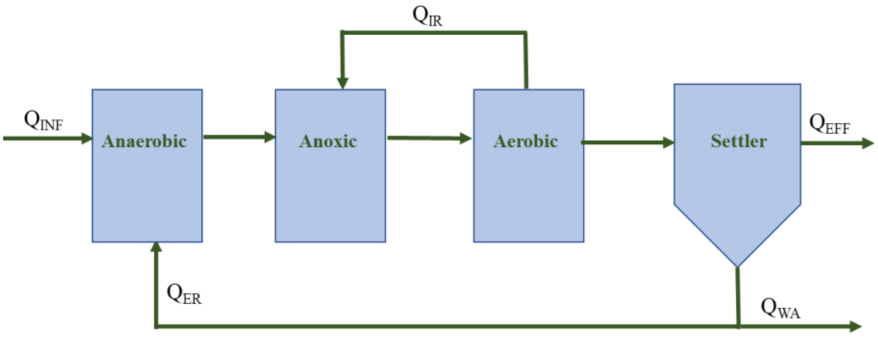

Figure 1.5. Process diagram for A_2O system.

1.3.3. *University of Cape Town (UCT) process*

Different from A_2O, the RAS from UCT returns to anoxic reactor, rather than anaerobic reactor, which receives the nitrate and nitrite from aerobic reactor and the denitrification happens quickly (Figure 1.6). The design of recirculation flow from anoxic reactor to anaerobic reactor was aimed to provide optimum conditions for the fermentation to form VFA, which could increase the carbon utilization and biomass recycling.

The disadvantage of UCT system is that the requirement of additional internal recycling and large fractions of anaerobic reactor volume increase the cost of pumping and maintenance (Tchobanoglous et al., 2013).

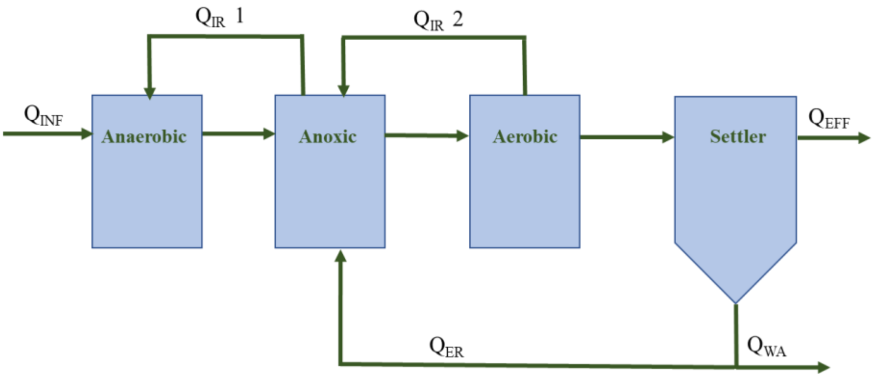

Figure 1.6. Process diagram for University of Cape Town (UCT) process.

1.3.4. *The five-stage Bardenpho process*

The five-stage Bardenpho process provides additional anoxic and aerobic reactors based on A_2O configuration (Figure 1.7). The incorporation of the second anoxic reactor allows denitrifying the residual NOx, which limits the NOx load in the stream of external recycle and reduces the undesirable presence of NOx in the anaerobic zone (Tchobanoglous et al., 2013). The second aerobic reactor promotes the P uptake.

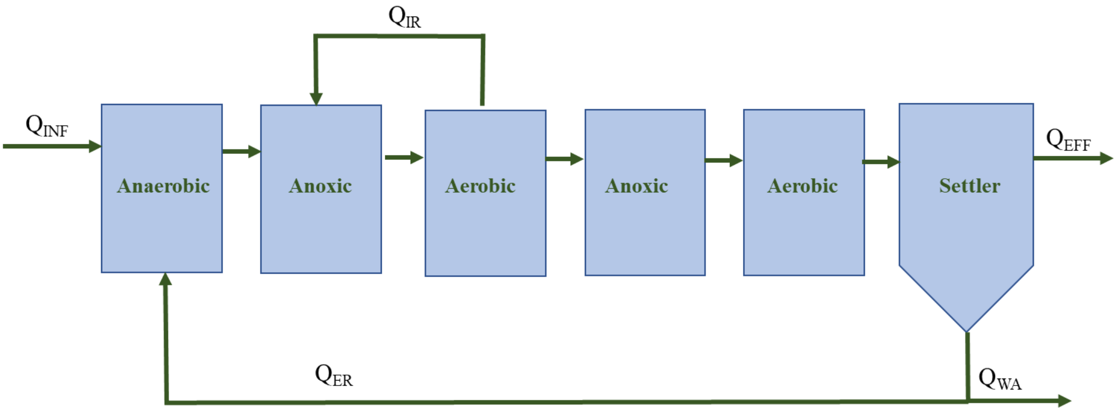

Figure 1.7. Process diagram for the five-stage Bardenpho process.

1.3.5. *Johannesburg process*

As an alternative of A_2O, Johannesburg was proposed by Osborn and Nicholls (1978) with an extra anoxic reactor connected to the stream of external recycle (Figure 1.8). The inclusion of the anoxic reactor is to pre-denitrify the NOx and reduce the NOx flowing to the anaerobic reactor, thus, minimize the detrimental effect on EBPR.

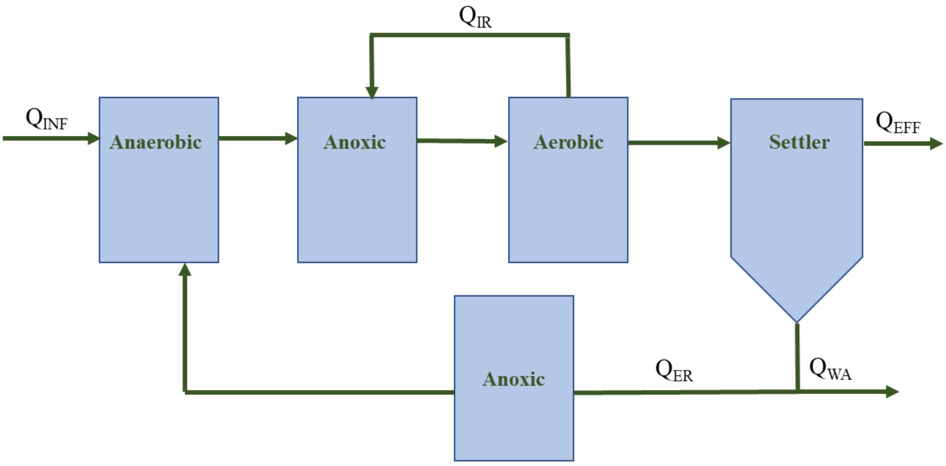

Figure 1.8. Process diagram for Johannesburg process.

1.4. The impact parameters on EBPR process

As is stated above, biological P removal is based on the sufficient uptake of VFA in the anaerobic phase for energy storage and P uptake in the aerobic or anoxic phase by PAOs. P removal is achieved by discharging the waste sludge with high poly-P content. A stable and reliable EBPR in WWTPs requires properly operational parameters to ensure successful performance. For example, the environmental parameters (e.g. pH, temperature), the wastewater compositions (VFA and other fermentable carbon sources) and the design parameters (e.g. SRT, HRT) based on different configurations applied.

1.4.1. *COD/P*

The influent COD/P ratio plays an important role in EBPR performance and the relative abundances of the microbial communities (Gu et al., 2008; Oehmen et al., 2007). However, the total amount of C is as important as its biodegradability. PAOs need VFA-like organic matter for their anaerobic metabolism. The reported observed stoichiometric COD requirement for per unit P removal was about 10-20 mg rbCOD/ mg P (Barnard et al., 2005; Tchobanoglous et al., 2013), and textbook knowledge recommends that the minimum ratio of rbCOD/P to maintain successful P removal in WWTPs is about 15:1 to 25:1 (Gu et al., 2008; Tetreault et al., 1986).

For example, a high ratio of COD/P input may favour the proliferation of GAOs and deteriorate EBPR systems (Shen and Zhou, 2016). Majed and Gu, (2020) showed that the relative abundance of GAOs increased by 40% with the increasing ratio rbCOD/P (from 20 to 50) but the percentage of total PAOs decreased.

1.4.2. *The utilization of substrates*

The nature of the carbon source is not only relevant for promoting EBPR or for selecting a certain type of PAOs but also for being a key agent in the competition between PAOs and glycogen-accumulating organisms (GAOs) (e.g. *Candidatus* Competibacter, *Propionivibrio, Defluviicoccus*). A successful EBPR performance is characterised by a PAO-enriched sludge with efficient carbon usage. GAOs outcompeting PAOs would lead EBPR failure and that depends on the operational conditions and on the quantity/biodegradabilty of the carbon source(Nittami et al., 2009; Oehmen et al., 2007; Roy et al., 2021; Wong et al., 2004). GAOs proliferation was observed with overload of VFA (Shen and Zhou, 2016) and some certain carbon sources (e.g. glucose, starch and methanol) (Randall et al., 1997; Rollemberg et al., 2019; Tayà et al., 2013b; Wei et al., 2014; Yazıcı and Kılıç, 2016). On the contrary, PAO-enrichment has been promoted by propionate (Oehmen et al., 2005; S. Wang et al., 2020), butyrate (Begum and Batista, 2014; Cai et al., 2019), glucose (Nguyen et al., 2011), amino acids (Nguyen et al., 2015; Qiu et al., 2020) or mixture carbon sources (Wang et al., 2021; Xie et al., 2017; Yang et al., 2018).

The carbon source can also affect the proliferation of denitrifying PAOs (DPAOs), which are able to simultaneously remove nitrogen (N) and P by using nitrate/nitrite as electron acceptors for PHA oxidation (Guisasola et al., 2009; Kerrn-Jespersen and Henze, 1993; Kuba et al., 1996). DPAOs are reported to be favoured by fermentation products of solid wastes (Ji and Chen, 2010; R. Xu et al., 2016; Yuan et al., 2016).

1.4.3. *Solids retention time*

Solids retention time (SRT) is of essential importance for the design and operation of EBPR process since SRT is related to the growth of the microorganism (Smolders et al., 1995). Normally around 10 days SRT of EBPR in full-scale WWTPs is adequate to promote PAO growth. A high SRT could result the decrease of biomass yield and the production of wasted sludge, which indicates a lower P removal efficiency by discharging wasted sludge (Li et al., 2016). A full-scale study of Onnis - Hayden et al.,(2019) showed that better and more consistent P removal was achieved at SRT below 10 days, with the optimum at SRT = 7d.

Some studies have focused on integrating in short-SRT in EBPR systems for removing carbon and P in view of energy efficiency and sustainability. Chan et al. (2017) operated sequencing batch reactor (SBR) with EBPR for treating synthetic wastewater with successful results with a SRT down to 3.6 d at 25ºC. Valverde-Pérez et al., (2016) showed P could be effectively removed with mitigated nitrification at a SRT of 3 days in a high-rate EBPR-SBR system.

However, too much lower SRT (lower than 3 days) may induce the proliferation of filamentous bacteria and the biomass washout (Chan et al., 2017; Valverde-Pérez et al., 2016; Zhang et al., 2021). Thus, a compromise scenario that a SRT is high enough for the

PAO growth and low enough for less carbon oxidization and for the washout of nitrifiers is of great importance.

1.4.4. *Oxygen*

The nature of PAOs indicates the importance of dissolved oxygen (DO) in the wastewater treatment process. The devoid of oxygen is necessary for anaerobic phase due to the interaction effect with the presence of oxidising substances such as oxygen and NOx. The aerobic condition with proper DO concentration allows efficient luxury P uptake and a DO between 3-4 mg/L in aerobic phase was recommended by Shehab et al., (1996). The upsets of EBPR performance could be led by overload DO. The reason could be due to that excessive DO could deplete the intercellular poly-hydroxybutyrate (PHB) and negatively affect the uptake of P, the formation of glycogen and the growth of PAOs (Brdjanvic et al., 1998).

Considering the economic cost and energy consumption due to the aeration, more and more investigations focus on the low DO for maintaining EBPR. Lower DO was reported to promote the selection of PAOs than GAOs (Carvalheira et al., 2014b; Chiu et al., 2007; Izadi et al., 2021a). Izadi et al., (2021) indicated that operating DO at 0.8 mg/L could remove 90% of P in an A/O SBR system with a high enrichment of PAOs.

In view of the simultaneous P and N removal, a sufficient DO should be considered due to the consumption of both P uptake and nitrification. Nuno R. Louzeiroa et al., (2002) indicated that more than 2 mg/L of DO was required for nitrification and carbon oxidation with activated sludge systems. A recent study of Keene et al., (2017) showed that a stepwise decreasing aeration to 0.33 mg/L of DO could lead to 90% of P and 70% of N removal in a pilot-scale system in a long operational period, and the estimated energy use reduction could be 25%.

1.4.5. *Temperature*

Temperature exerts a great influence on biological processes (e.g. fermentation, nitrification, growth and metabolism) (Henze et al., 2008). EBPR can be observed under a wide range of temperature (5-37 °C). Low temperature may challenge the P removal efficiency (Mulkerrins et al., 2004), and EBPR disturbances were reported in winter of the northern hemisphere (Helmer and Kunst, 1998). However, successful EBPR performance is still broadly reported under low temperatures (around 10 °C).

Previous investigations have shown that the fluctuation or even deterioration of EBPR happened under high temperatures (Lopez-Vazquez et al., 2009; Oehmen et al., 2007; Whang and Park, 2006), and it was considered that *Competibacter* showed advantage to outcompete PAO with the temperature above 30 °C (Lopez-Vazquez et al., 2009). However, some recent reports showed successful EBPR performance in full-scale WWTP of Malaysia and Singapore under high temperatures (28-32°C) and in lab-scale systems with enriched biomass from tropical EBPR plants (Nielsen et al., 2019; Ong et al., 2014; Qiu et al., 2019; Shen et al., 2017).

1.4.6. *pH*

Apart from the above operational parameters, pH can affect the competition of PAOs and GAOs and thus EBPR performance (Lopez-Vazquez et al., 2009). Normally a wide range of pH is proper for EBPR (6.5-8.0) and a relatively higher pH was reported to favour more PAOs than GAOs (Filipe et al., 2001). In view of simultaneous P and N removal in A_2O system, an optimum pH should be implemented for both PAOs, nitrifiers and denitrifiers since a relatively higher pH could be necessary for the optimum performance of nitrifiers.

1.5. The importance and potential to recover P in wastewater management

1.5.1. *The importance to recover P in wastewater management*

The new paradigm in wastewater treatment proclaims that WWTPs should evolve to water resource recovery facilities (WRRFs) where material and energetic resources should be recovered from wastewater. As a result, P arises as a perfect potential candidate since:

i) P has a vital role in life activities (i.e. growth and energy supply of human processes), particularly for the formation of DNA and RNA. P is also involved in photosynthesis.

ii) P demand is also growing since it is essential for the production of fertilizers (Chowdhury et al., 2017; Cieślik and Konieczka, 2017; Roy, 2017) and there is a current context of population and food demand growth. In addition, an increase on the demand of P due to new electric car battery developments as the lithium iron phosphate battery (LiFePO4 battery or LFP battery, lithium ferrophosphate) is currently a clear trend in this field.

iii) P is a limited and non-renewable resource and mainly is obtained from mined P rocks in some specific regions (about 75% of the P reserved in the world is controlled in Morocco) (Jasinski, 2006; Rosmarin, 2004). Table 1.4 shows global phosphorus production and consumption in different regions around the world. The main source of P is envisaged to be depleted in the next 50-100 years (Cordell et al., 2009; Desmidt et al., 2015; Rittmann et al., 2011). Approximately 20 million tons of phosphorus rocks are mined each year with rising price (Cordell et al., 2011). As easy-mining P rocks are gradually depleted, high-impurity and hard-mining rock would be employed, escalating production costs, and it is estimated that only 20% of the P can be extracted under techno-economically feasible conditions (Cieślik and Konieczka, 2017; Cordell et al., 2011).

iv) P discharge limits are expected to become more stringent. Some EU countries (e.g. UK and Germany) have set lower P limits for WWTP effluents (0.15-0.3 mg/L) and may decrease even further, while USEPA has established a recommended limit around 0.1 mg/L for water bodies sensitive to eutrophication.

Therefore, recovering P is extremely necessary, and extensive proposals have been suggested in view of slowing the pace of its depletion (Cardoso et al., 2019; Egle et al., 2015; Law and Pagilla, 2018; Yang et al., 2017; Zhang et al., 2022)

Table 1.4. Phosphorus production and consumption in different regions around the world (Data from U.S. Geological Survey, 2012; IFA, 2012) (Kuck, 2012).

Regions	Production (%) (2010)	Consumption (%) (2008)
Asia	38	42
Africa-Middle East	34	18
North America	15	20
Eastern Europe	7	7
Latin America	4	6
Western Europe	0	5
Oceania	2	2

1.5.2. *The potential to recover P in wastewater management*

As described above, P recovery is of great importance and urgently needed. The recent paradigm shift in the field of EBPR to remove P attracts the environmental engineering to the integration of more sustainable and novel P recovery strategies. It was estimated that 15-20% of the global P demand could be satisfied if most of the P contained in WWTPs could be recovered (Ruo hong Li and Li, 2017; Wu et al., 2019; Yuan et al., 2012). Egle et al., (2016) indicated that the recovered P in WWTPs could ensure 40-50% of annual application of mineral P fertilizer in agriculture.

1.6. The development trend and potential to recover organic matter in wastewater management

The issue of resource restrictions and the climate change accompanied with the increasing growth of the population drives us to shift the conventional configurations in WWTPs to a new paradigm of WRRFs. Enormous efforts have been made by scientists and water industries to achieve this ambition from concept to standard practice (Pikaar et al., 2020). Among all, the recovery of organic matter is a hot trend that has attracted extensive attentions by investigators.

The organic matter in municipal wastewater is consisted of VFA, proteins, lipids and carbohydrates, which are normally removed by EBPR and denitrification process. High amount of COD in municipal wastewater (500-1200 mg/L COD) (Table 1.1) and high quantity of annual production (about 312 million megaliters) show great potential for energy recovery (Pikaar et al., 2022) (Table 1.5). The theoretical energy is regarded as chemical energy (assumed to be transformed to methane through anaerobic digestion) and thermal energy (assumed to be transformed to electrical or thermal energy via cogeneration), and the energy recovered is normally reused to satisfy the energy requirements of the in-situ WWTPs. It is reported that the recovered energy could cover about half of the total energy demands (Capodaglio and Olsson, 2019).

Table 1.5. Overview of the potential recoverable resources in typical municipal wastewater (Modified from (Liu et al., 2019))

Compositions	Typical Concentration (mg/L)	Potential recoverable energy （kJ/m^3）
Total COD	500	6955
Biodegradable COD	320	4451
Soluble COD	145	2017
Suspended COD	175	2434

1.7. Actions moving forward wastewater treatment for recovering energy and P in current WWTPs

1.7.1. *Recovering energy from organic matter*

During the biological treatment process, part of the unrecoverable potential energy of organic substances in municipal wastewater is lost in different forms. For example, the mineralization of COD to CO_2, the conversion of COD to methane and the requirement for microbial assimilation (which accounts for about 15% of the potential energy in municipal wastewater) (McCarty et al., 2011a; Verstraete and Vlaeminck, 2011). In view of the loss of the energy, the paradigm shift proposes maximizing the energy recovery with advantages of reducing the aeration for less energy consumption and minimizing the sludge production (Wan et al., 2016). Anaerobic digestion (AD) process is adopted as a solution to minimize the energy loss, and it was reported that, with good practices, 81% of the biodegradable COD could be transformed to methane by minimizing the sludge production (Appels et al., 2008). Further, after the efficient conversion of the methane, about 32% of the potential energy could be harvested as electricity (Liu et al., 2019).

The high-rate activated sludge (HRAS) process is a novel technology for recovering as much energy as possible from the organic substances (including particulate, colloidal and soluble COD) in municipal wastewater based on the philosophy of biosorption and bioaccumulation. HRAS is a capture of organic matter process which indicates the potential of COD recovery prior to biological oxidation. To achieve this, a relatively low HRT and SRT under a limited oxygen conditions is required to obtain the bio-sludge called adsorptive or young-age sludge. This sludge is more digestible than the normal wasted sludge from the traditional activated sludge process. As a result, more influent

COD is captured in the biomass efficiently, rather than mineralization or hydrolyzation (Modin et al., 2016; Rahman et al., 2014). Further, this sludge could be subjected to AD for the subsequent biogas generation. It has been reported that about 60% of the input total COD could be captured by HRAS process, and the efficiency could be improved to even 81% under optimal conditions (Sancho et al., 2019; Wett et al., 2007). The efforts devoted to exploring the optimal conditions to obtain more efficient COD capture are concentrated on HRT, SRT and DO (Carrera et al., 2022; Sancho et al., 2019), and also it is necessary to tradeoff the harvested sludge for AD to produce more energy and the sludge returned back for long-term system stability.

1.7.2. *Recovering phosphorus in current WWTPs*

Recovering P from wastewater has been developed for the last two decades, and it could be recovered in various forms by different strategies. Different technologies have been proposed for P-recovery from flows containing phosphate in WWTPs (i.e. effluent, sewage sludge, sludge ash, dewatering liquor and digester supernatant) according to the operability, the costs and the potential uses of the recovered material (Cieślik and Konieczka, 2017; Desmidt et al., 2015; Egle et al., 2016; Law and Pagilla, 2018).

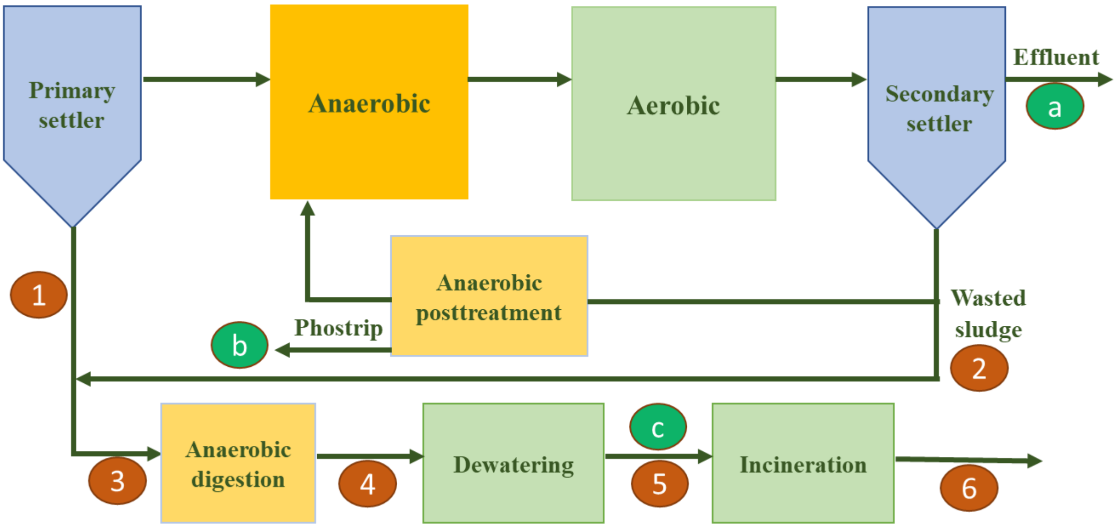

Figure 1.9. Potential positions for P recovery (Number: sludge line (1. primary sludge; 2. wasted sludge; 3. raw sludge; 4. sludge from digester before dewatering; 5. dewatered sludge; 6. sludge ash) Letter: Water line (a. effluent; b. side-stream supernatant; c. dewatering unit after anaerobic digestion) (modified from Desmidt et al., 2015).

Figure 1.9 shows the potential positions for P recovery from sludge line and water line. The most common location for P recovery in an EBPR-based WWTP is the supernatant of the AD. The substrate for AD is the purged biomass, i.e. biomass from the aerobic phase, which contains the maximum P content of all the EBPR cycle (Garcia-Belinchón et al., 2013; Geerts et al., 2015) and a high ammonium concentration. Both are released in the anaerobic digester and the digestate becomes a favourable option for struvite precipitation (Yuan et al., 2012; Zhang et al., 2022).

Besides the digestate, the anaerobic reactor is the spot of the plant with higher P concentration and, thus, an alternative location to apply P-recovery strategies (i.e. mainstream P-recovery). During the anaerobic phase, PAOs have taken up most of the readily biodegradable COD and most of the P has been released into the bulk liquid, reaching levels from 30 to 70 mg P/L (Acevedo et al., 2015; Izadi et al., 2021b, 2020; Zhang et al., 2021; Zou and Wang, 2016). Considering the major issue of undesired precipitation in the anaerobic digester or in the downstream tubing/instrumentation (Lizarralde et al., 2019; Parsons and Doyle, 2002), the anaerobic reactor appears as a good point to implement mainstream P-recovery. Figure 1.10 shows the general scheme for mainstream P-recovery in Phoredox process, whilst the digestate seems to be the ideal point for side-stream P-recovery.

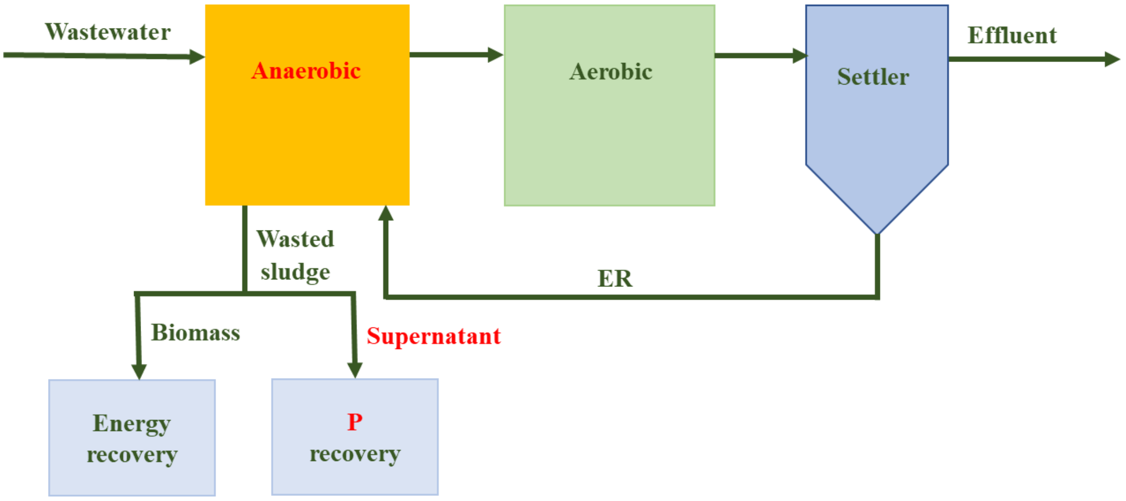

Figure 1.10. General scheme for mainstream P-recovery in Phoredox process

Apart from the above-mentioned locations and relative approaches, microalgae cultivation has been proposed as a promising technology for P-recovery from secondary effluents due to its low cost, eco-friendliness and low technology dependence (Yang et al., 2017; Bodin et al., 2006). P is taken up biologically by algal growth by assimilation, accompanied by oxygen generation that supports aerobic bacteria growth. Algae can also be used either as a feedstock for biofuel production or directly as a biofertilizer (Chisti, 2013, 2008). Xu et al (2016, 2015) also showed that algae could remove about 60% of influent phosphorus for secondary wastewater effluent. However, the application of algae for P-recovery is still limited in large-scale wastewater treatment (Gebremariam et al., 2011a).

1.7.3. *Recovering energy and phosphorus simultaneously with the integration of high-rate system into EBPR process*

A recent WWTP configuration to recover resources (i.e. nutrients and energy) from industrial/urban wastewater is the A/B strategy (Boehnke and Diering, 1997; Wan et al., 2016; Wang et al., 2009) (Figure 1.11). The first A-stage is employed for the maximum

COD capture by HRAS to redirect and concentrate carbon, rather than mineralization. 50-80% of influent COD can be recovered and redirected to AD as methane for energy recovery (Sancho et al., 2019). Subsequently, nitrogen removal is handled by B-stage that includes partial nitrification combined with anammox or shortcut nitrification-denitrification processes (Jenni et al., 2014; Rossle and Pretorius, 2001; G. Xu et al., 2015). This novel process makes the conventional WWTPs take benefits of the "waste" and aims to achieve the goal to recover water, resources and energy (Figure 1.12).

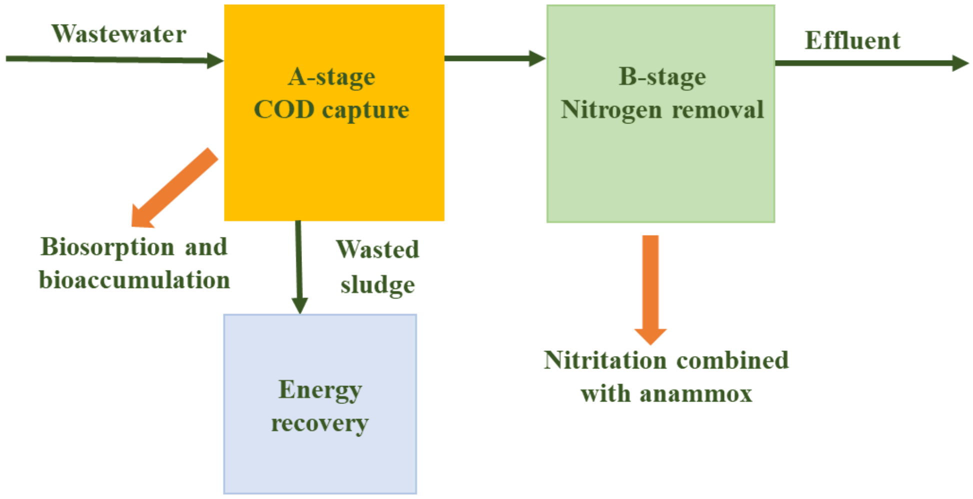

Figure 1.11. General scheme of two-stage A/B system (modified from Wan et al., 2016).

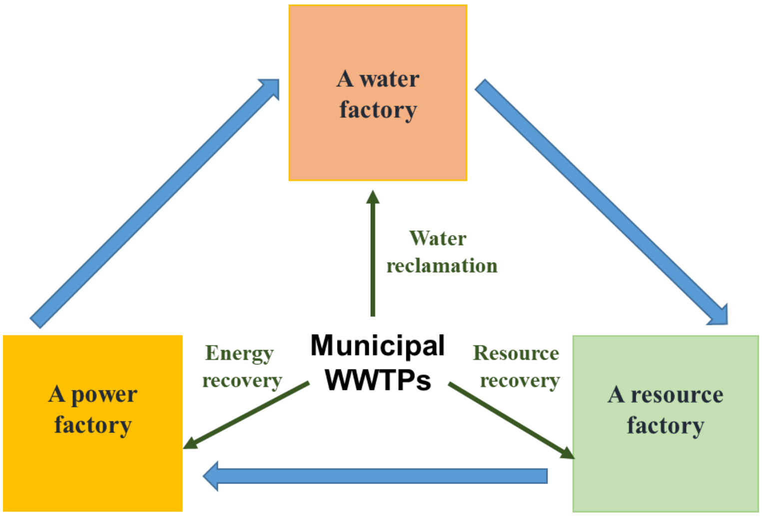

Figure 1.12. The philosophy of the removal of "waste" from conventional WWTPs to a recovery of water, resources and energy (modified from Liu et al., 2019).

The proposal of combining A/B strategy to EBPR aims at the removal of P and COD simultaneously and the maximized capture of carbon to biomass in the A-stage process, and the removal of N is realized by the subsequent B-stage (Figure 1.13).

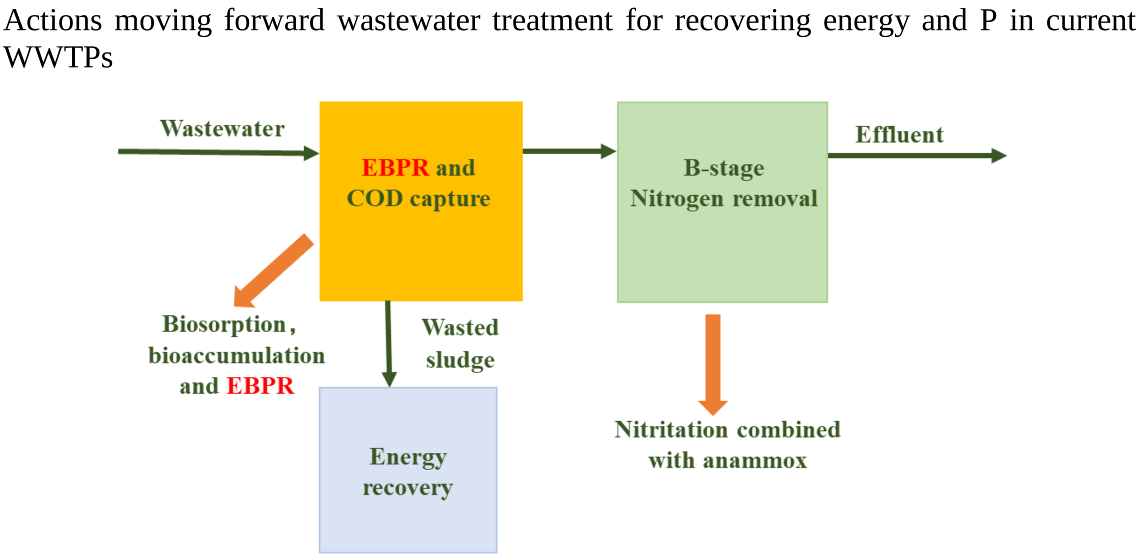

Figure 1.13. General scheme of EBPR +A/B process.

The integration of A-stage to EBPR process has been investigated broadly (Chan et al., 2017; Ge et al., 2013; Zhang et al., 2021). The key for A-stage EBPR is to find the proper conditions for removing P with the minimum of carbon mineralization. Exploring the minimum SRT, HRT and DO without compromising P removal is the point of this investigation. The minimum SRT has been studied by a lot of researchers. Successfully EBPR performance on SBR have been shown in less than 4 days of SRT (Chan et al., 2017; Ge et al., 2013; Valverde-Pérez et al., 2016). For the limitation of DO, 1 to 0.5 mg/L of DO has been exhibited to sustain successful P removal without nitrification in the A-stage-EBPR system (Zhang et al., 2021).

Mainstream P recovery could be also realized by the EBPR+A-stage process by anaerobic purging. Then, P and carbon can be recovered from the anaerobic supernatant with enriched P and anaerobic sludge with high concentration of PHA for biochemical methane production as energy, respectively.

1.7.4. *Recovering COD and phosphorus simultaneously with the integration of side-stream sludge fermenter into EBPR process*

As stated above, recovering the organic matter by AD in the form of energy has been well-developed, but the hydrolysis process as the rate-limited step has been a bottleneck due to a series of complex biological processes of AD (i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis) (Henze et al., 2008). Pretreatment the biosolids is normally employed to improve the hydrolysis to enhance energy recovery, e.g. thermal, mechanical or chemical pretreatment (Figure 1.14). However, additional energy and costs are necessary (Liu et al., 2019).

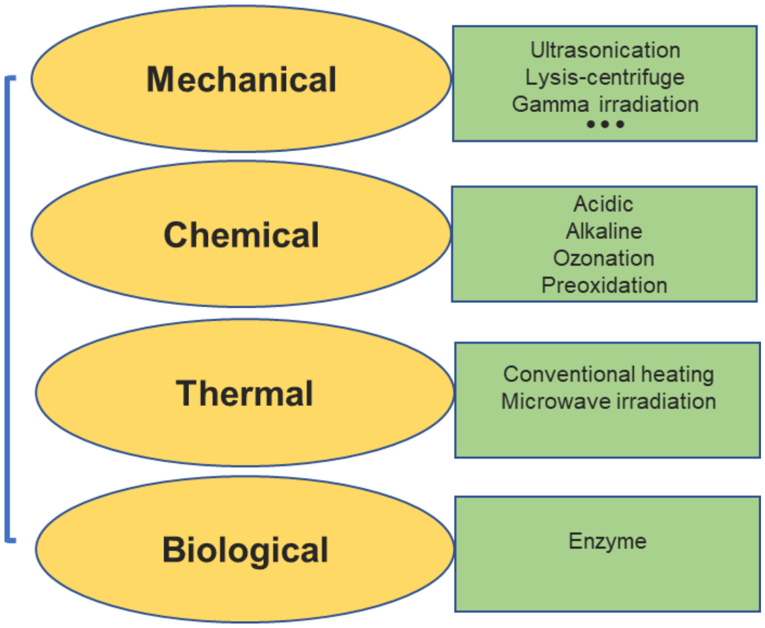

Figure 1.14. Pretreatment methods of the biosolids to improve the hydrolysis to enhance energy recovery.

The integration of a side-stream sludge fermenter (SSSF) could be another potential way for recovering organic matter from the system to produce VFA for the mainstream (Arabi and Lynne, 2019; Ferrentino et al., 2016; Goel and Noguera, 2006; Wang et al., 2019). The influent of the SSSF is a fraction of the anaerobic mixed liquor or of the RAS (Barnard et al., 2017; G. Li et al., 2020; Onnis-Hayden et al., 2020; Wang et al., 2019). Different from AD, SSSF has a moderate temperature and a relatively lower SRT, which allows the happen of hydrolysis and acidogenesis of bio-sludge to soluble organic molecules and then the release of VFA, rather than inducing the subsequent acetogenesis and methanogenesis (Figure 1.15). The enriched VFA in the supernatant of SSSF could be utilized as a promising carbon source for the mainstream nutrients removal. As a result, embracing SSSF to EBPR (S2EBPR) has been investigated to improve EBPR performance (Barnard et al., 2017; Coats et al., 2018). Figure 1.16 shows the general scheme of S2EBPR based on conventional A_2O system. The application of SSSF can avoid the addition of commercial carbon sources, reduce the cost and the footprint of carbon source, which was also reported to demonstrate more advantages to improve EBPR and stability compared to traditional EBPR configurations (Onnis-Hayden et al., 2020; Wang et al., 2019). There are more than 80 full-scale applications of S2EBPR facilities worldwide (Copp et al., 2012; Tooker et al., 2017; Vale et al., 2008; Vollertsen et al., 2006).

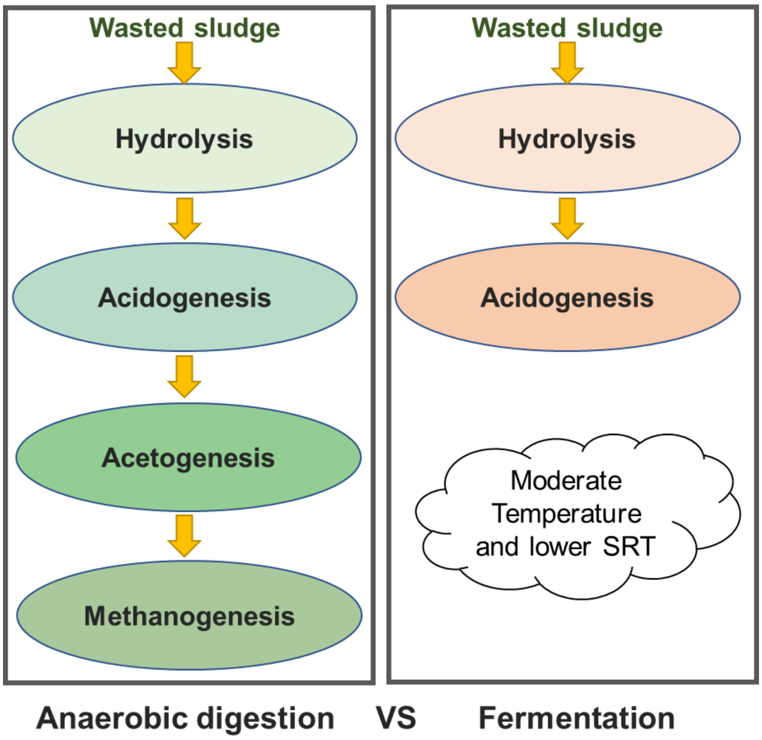

Figure 1.15. The comparison of anaerobic digestion process and the fermentation of SSSF with wasted sludge.

Mainstream P-recovery in S2EBPR system can be realized by extracting part of the anaerobic supernatant. As a result, the S2EBPR configuration with mainstream P recovery process not only takes more advantage of the internal carbon source by SSSF, but also shows great potential to remove and recover phosphorus.

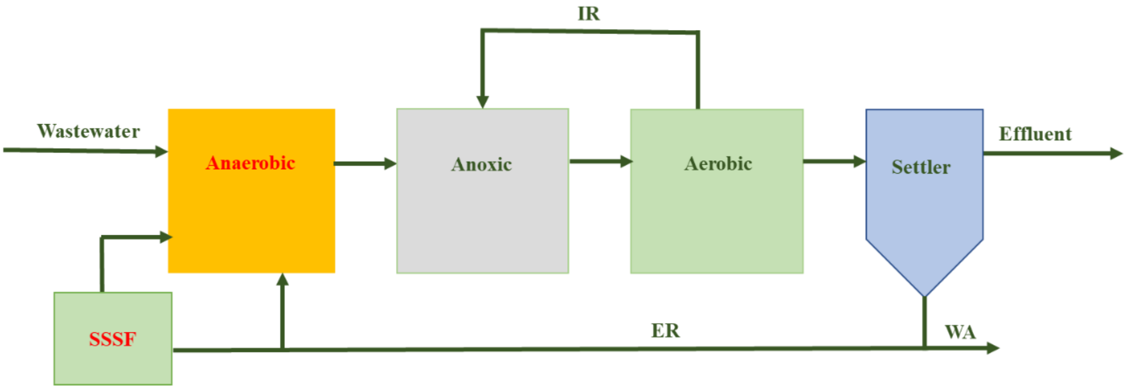

Figure 1.16. General scheme of S2EBPR based on conventional A_2O system.

1.8. Research motivations and thesis overview

1.8.1. *Research motivations*

Significant endeavors have been dedicated to the removal of organic matter as well as nutrients (phosphorus and nitrogen) in the last few decades. Faced with the challenge of resource shortage and environmental pollution, the new trends for EBPR aim at integrating the recovery of energy and P in the same wastewater treatment process. Secondly, carbon source plays significant role on EBPR performance, and improper utilization or insufficient COD in the raw wastewater may lead to the system failure. As a result, more and more efforts have been attempted to obtain suitable carbon sources from sustainable substrates such as the waste sludge and applied it to the low loaded COD wastewaters.

Thus, the first research motivation of this thesis has been devoted to investigating the integration of EBPR in HRAS, with the objective of treating real wastewater for simultaneous and efficient organic matter and P removal and energy recovery. The minimum SRT and DO for a long-term successful EBPR performance has to be explored to minimize the carbonization and aeration. The relative microbial communities promoted in this optimal scenario should be analyzed as well.

The thesis also aims at investigating the possibility of purging from the anaerobic reactor rather than from the aerobic reactor in view of harvesting biomass with high PHA content to maximize biogas production for energy recovery. Apart from that, anaerobic supernatant provides more advantages for P recovery. As a result, the related investigations could lead to the integration of mainstream P-recovery strategies.

The second research motivation of this work is understanding the effect of using different carbon sources EBPR system. The effect of diverse carbon source utilization strategies (sole, multiple or complex carbon sources) on the EBPR performance, microbial communities as well as the metabolic way of PAOs and GAOs need to be evaluated in view of finding the suitable carbon source for limited carbon influents. More importantly, the fermentation products from solid waste arise as a novel potential environmental-friendly carbon source.

Moving forward, the side-stream sludge fermenter (SSSF) has been employed to be expected as a VFA production site for providing carbon source for the mainstream EBPR process. The performance of this novel configuration with the integration of SSSF into A_2O (S2EBPR) under low COD influent needs to be comprehensively evaluated, and the developed corresponding microbial communities need to be analyzed to gain knowledge of the succession of communities under different operation conditions.

1.8.2. *Thesis overview*

This document is divided into eight Chapters. Chapter 1 comprises an overall introduction to the topic of EBPR and the state of the art of the current development in the field of the recovery of energy, P and COD. Chapter 2 presents the main objectives of the thesis. Chapter 3 describes the related configurations applied in the thesis and the chemical and microbial analysis methods. Chapter 4 reviews the advances on P-recovery and type of carbon source in EBPR systems. Firstly, it focuses on P recovery strategies, especially on the state of the art of the integration of mainstream P-recovery strategies with EBPR. Then, it evaluates the effect of different carbon sources on EBPR performance according to the investigations in recent years. Chapter 5 exhibits the A-stage-EBPR system to achieve simultaneous biological COD and P removal in a continuous anaerobic/aerobic system and the limits of operating parameters for obtaining a successful performance. Chapter 6 is focused on the integration of a SSSF on A_2O- EBPR performance with a low influent COD condition. Finally, Chapter 7 presents main conclusions extracted from the thesis and Chapter 8 lists all the references cited in this document.

Chapter 2:

Objectives

2. Objectives

The main objective of this thesis is to go beyond current knowledge on EBPR and advance in the sustainability of this P removal process. The focus is on three specific questions: make the process more energetically sustainable, improving its efficiency when treating wastewater with low organic matter concentration and integrating P-recovery strategies.

Guiding by these general objectives, the specific aims are shown below:

i) to review the possibilities of P recovery and specifically focus the state of the art on the integration of mainstream P-recovery strategies on EBPR.

ii) to gain insight into the possibility of purging from the anaerobic reactor of a high-rate-EBPR system and an A_2O configuration to implement mainstream P recovery strategies.

iii) to gather all the current information available on carbon sources that have been assessed for EBPR.

iv) to investigate the required SRT and DO to maintain successful COD and P removal in a continuous A-stage-EBPR system in the long term.

v) to explore under a low COD feed condition, the effects of integrating a side-stream sludge fermenter in an A_2O configuration (i.e. a side-stream EBPR configuration, S2EBPR) on the nutrient removal performance and microbial community.

Chapter 3:

Materials and methods

3. Materials and methods

3.1. A/O reactor description

The pilot plant used consisted of two continuous stirred tank reactors (CSTR) (one anaerobic V=19L and one aerobic V=23L) and a settler (25L) (Figure 3.1). The feed was connected to the anaerobic reactor. The returned activated sludge from the settler is recirculated to the anaerobic reactor by external recycle. The DO and pH probes were connected to a multimeter controller (HACH CRI44). DO was initially controlled with an on/off controller. Due to the significant DO fluctuations during the operation process, the uneven distribution of DO in the system could have led to a simultaneous nitrification/denitrification scenario. Hence, a proportional-integral algorithm manipulating the aeration flow rate with a mass flow controller (MFC F-201CV, Bronkhorst) was implemented in the aerobic reactor subsequently to maintain a controlled continuous aeration with a more stable DO value. The system was operated in a lab with roughly controlled room temperature (21±2ºC) through air conditioning. The whole system was controlled using an industrial PC (Advantech PPC-3190) with a data acquisition card (Advantech PCI1711) running our ADDcontrol software developed in LabWindows CVI (National Instruments).

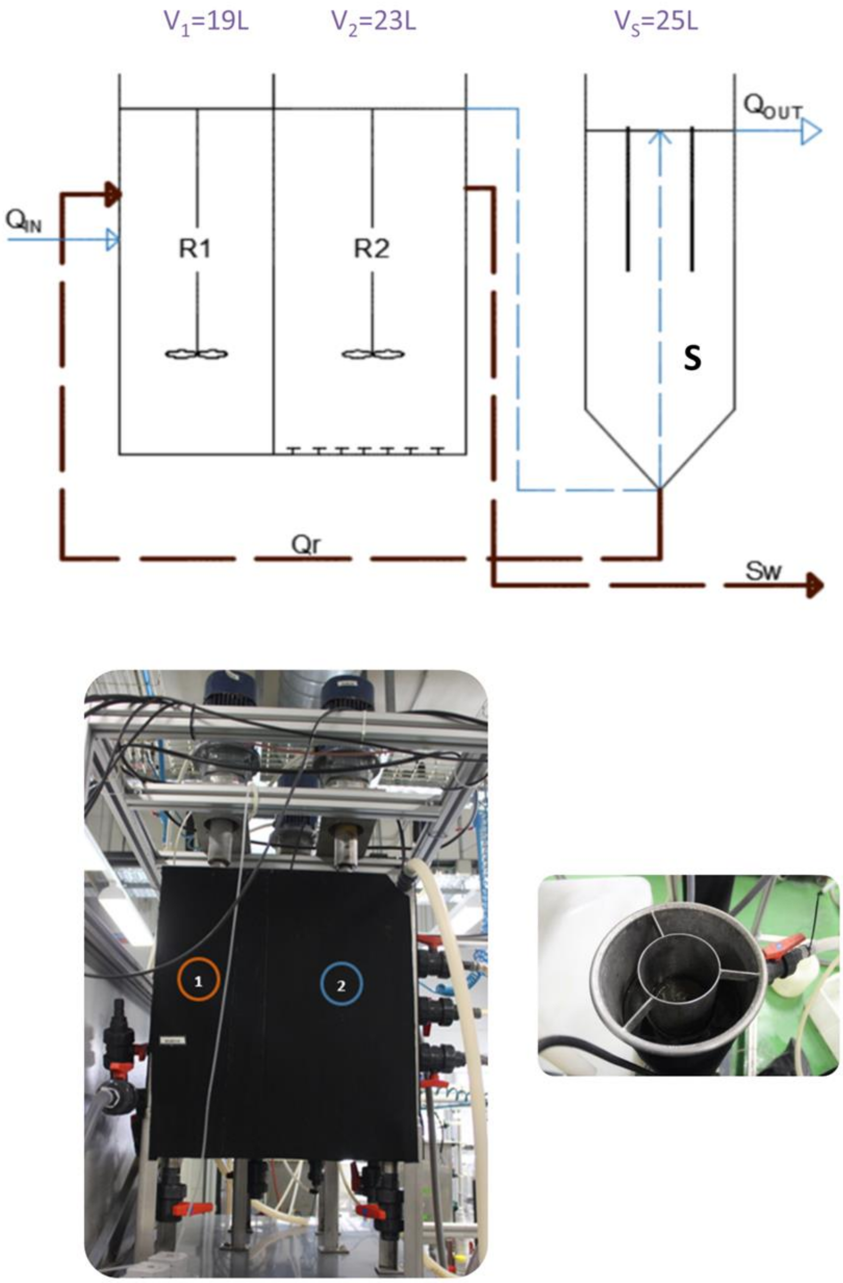

Figure 3.1. Schematics and pictures of the pilot plant used in this study. R1: anaerobic reactor R2: aerobic reactor, S: settler, Q_{IN}: inflow, Qr: recycle from the settler to R1, Sw: purge, Q_{OUT}: effluent.

3.2. A_2O and S2EBPR system description

The initial A_2O configuration consists of three continuous stirred tank reactors for simultaneous C/N/P removal, with an anaerobic reactor (R1, 28 L), anoxic reactor (R2, 28 L), aerobic reactor (R3, 90L) and settler (50 L) (Figure 3.2). The feeding solution introduced to the anaerobic reactor consisted of tap water (144 L/d) and a concentrated solution (7 L/d). The internal recycle (IR) from R3 to R2 (450 L/d) was used to keep the anoxic condition in R2. Waste sludge was discharged from R3 automatically with a flowrate selected to maintain the desired sludge retention time (SRT). The settler produced the effluent stream and an enriched biomass stream which was recycled to R1 with a flow rate of external recycle (ER) about 140 L/d.

The three reactors were monitored on-line with DO (HACH CRI6050), pH (HACH CRI5335) and temperature probes (Axiomatic Pt1000) connected to multimeters (HACH CRI-MM44). On-line data was acquired with a data acquisition card (Advantech PCI-1711), which was connected to a PC with the AddControl software (LabWindows CVI, National Instruments) developed in the research group for process monitoring and control. DO in R2 was controlled with a proportional-integral algorithm manipulating the aeration flow rate by a mass flow controller (MFC F-201CV, Bronkhorst) with the setpoint in A_2O of 2 mg/L and in S2EBPR of 2 or 3 mg/L. pH in R3 was controlled with an on-off controller dosing a sodium carbonate solution to adjust the pH about 7.5. The system was operated at room temperature (22±2 °C).

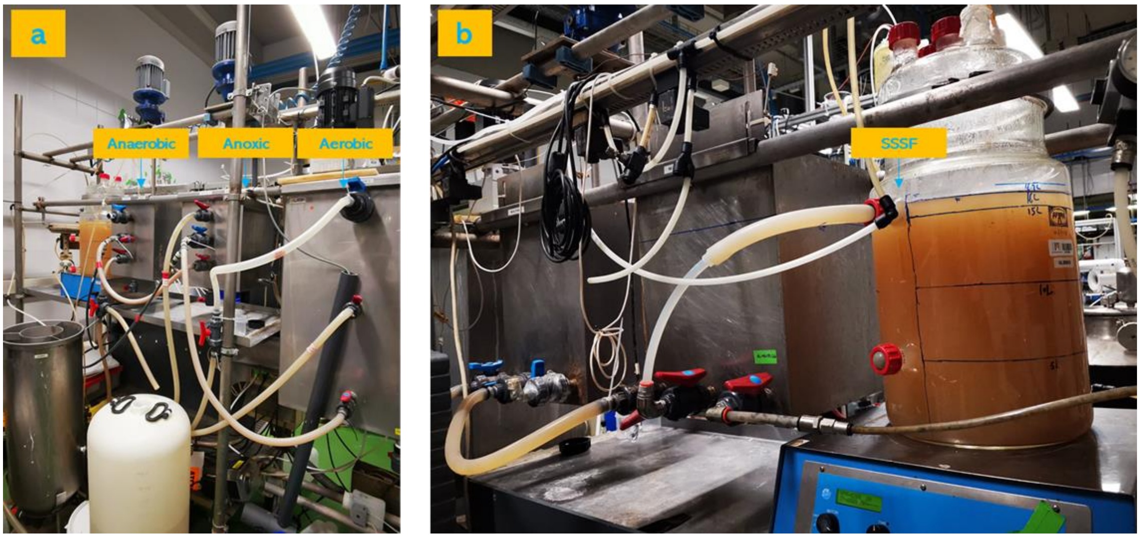

Figure 3.2. The configuration of the integration of A_2O (a) and SSSF (b) used in this study.

For the S2EBPR system, once the A_2O operation has been studied, an additional side-stream sludge fermentation reactor (SSSF) was installed to ferment part of the external recycle sludge which was introduced in anaerobic, anoxic or aerobic reactors depending on the investigation objectives (Figure 3.3). The SSSF worked with a working volume about 20 L with the glass cap tightened to provide anaerobic condition and a black plastic bag covered to avoid the growth of green alga. A magnetic rotor was equipped to provide a mixed fermentation condition.

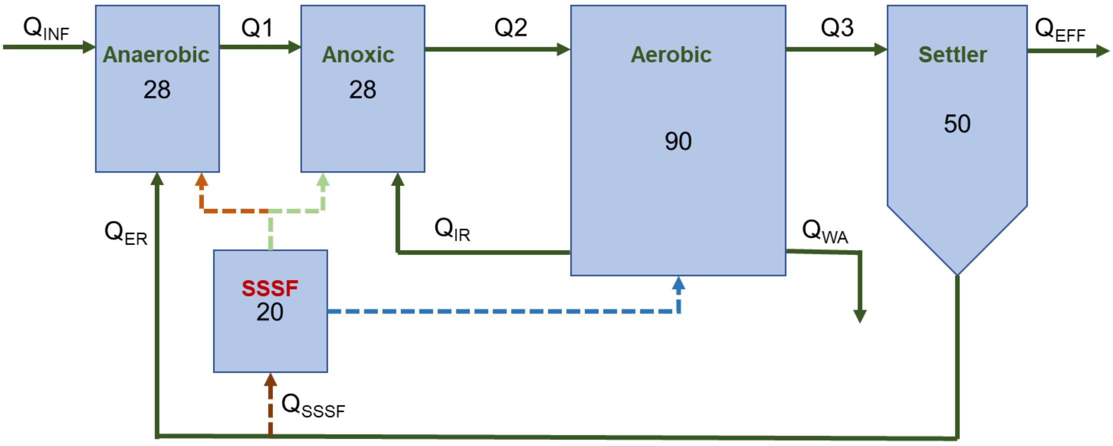

Figure 3.3. The diagrams of S2EBPR with different combination possibilities.

3.3. Chemical analysis

3.3.1. *Phosphorus*

Samples for phosphate were obtained from the reactors or the containers of batch tests. After the filtration with 0.22 mm filters (Millipore), the liquid samples were analysed by a phosphate analyser (115 VAC PHOSPHAX sc, Hach-Lange) based on the Vanadomolybdate yellow method, where the phosphate specific yellow was measured by a two-beam photometer with LEDs. The range of the PO_4^{3-}-P concentration was 0.05-15 mg/L.

3.3.2. *Nitrogen (ammonium, nitrate and nitrite)*

The samples of nitrogen species obtained from the reactors or the containers of batch tests were filtrated with 0.22 mm filters (Millipore) for further measurement. Ammonium concentration was measured with an ammonium analyser (AMTAXsc, Hach Lange), based on the potentiometric determination of ammonia.

The concentrations of nitrite and nitrate were detected by Ionic Chromatography (DIONEXICS-2000). Apart from that, strips of nitrate (A029835 MACHEREY-NAGEL), nitrite (A029985 MACHEREY-NAGEL), kits of nitrate (LCK 339 HACH LANGE GMBH) and nitrite (LCK 342 HACH LANGE GMBH) were employed to determine the corresponding concentration as well.

3.3.3. *COD*

COD was extracted and filtered with the same method as phosphorus and then determined by kits (HACH LCK 314 and LCK 714) and a spectrophotomer (DR2800 Hach Lange).

3.3.4. *Solids*

Sludge samples were withdrawn from the reactors or the containers of batch tests, and analysed for mixed liquor volatile suspended solids (VSS) and total suspended solids (TSS) according to Standard Methods (APHA, 1995). The sludge volume index (SVI) was calculated as the observed volume (mL) of sludge from the aerobic reactor after settling for 30 min divided by the TSS (g/L) measured on the same day.

3.3.5. *Biochemical methane potential (BMP) test*

BMP experiments were conducted to analyse the methane produced by anaerobic digestion of sludge (Angelidaki et al., 2009). Sludge from the anaerobic digester of an urban WWTP (Manresa, Barcelona) was used as inoculum, and degassed for at least 3 days at 37 ºC before use. The anaerobic, aerobic or SSSF sludge as substrates were taken from the anaerobic, aerobic reactors and SSSF when the system was at steady state. Control samples (i.e. without substrate) were used to obtain the net BMP. Both inoculum and substrate were kept at 2 gVSS/L of sludge. The anaerobic digestion tests were conducted in 160 mL serum bottles with 125 mL of effective volume and 35 mL headspace for biogas production. The pH of each bottle was initially set at 7.0, and N_2 was sparged into all these bottles for 2 min to obtain anaerobic conditions. Each sludge and control samples were tested in triplicate and kept in an oven at a constant temperature of 37ºC throughout the process. The content of each bottle was mixed every day. In each BMP test, the increase of pressure in the headspace volume of each bottle was measured by a gas pressure meter to obtain the gas production, and the volume of accumulated gas production was calculated expressed under standard conditions (25 ºC, 1 atm). Further details are provided by Chan et al. (2020). The biogas compositions (i.e. CH_4, CO_2 and H_2) were periodically measured by a Perkin Elmer gas chromatography equipped with a thermal conductivity detector (GC-TCD).

3.4. Microbiological analysis

3.4.1. *Fluorescence in situ hybridisation*

Fluorescence in situ hybridization (FISH) analyses coupled with confocal laser scanning microscopy (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) were performed as detailed in (Amann et al., 1995) to evaluate the biomass community. The distribution of *Accumulibacter* (PAO) and their competitors *Competibacter* (GAO), *Defluviicoccus* Cluster I (DFI) and *Defluviicoccus* Cluster II (DFII) were labelled (PAOMIX, GAOMIX, DFIMIX, DFIIMIX and EUBMIX probes) and quantified by using confocal microscopy and image analysis as described in Jubany et al. (2009). Each sample was observed by applying around 40 randomly chosen CLSM fields with 600 magnification of different x, y and z coordinates. All the probes (detailed in Table 3.1) were used with a formamide concentration of 35%.

Table 3.1. FISH probes used to quantify the amount of PAO and GAO

Probe	**Target**	**Reference**
Cy5-labelled EUBMIX	Most bacteria	(Daims et al., 1999)
Cy3-labelled PAOMIX, comprising PAO462, PAO651 and PAO846	*Candidatus* Accumulibacter phosphatis,	(Crocetti et al., 2000)
Cy3-labelled GAOMIX, comprising GAOQ431 and GAOQ989 probes	*Candidatus* Competibacter phosphatis	(Crocetti et al., 2002)
Cy3-labelled DFIMIX, comprising TFO_DF218 and TFO_DF618 probes	Cluster I of *Defluviicoccus vanus* GAO	(Wong et al., 2004)
Cy3-labelled DFIIMIX, comprising DF988 and DF1020 probes plus helper probes H966 and H1038	Cluster II of *Defluviicoccus vanus* GAO	(Nguyen et al., 2011)

3.4.2. *Illumina Sequencing*

The biomass community was evaluated by the Illumina amplicon sequencing of the 16S rRNA gene. The detailed process was as follows: the samples from the system were washed by PBS for three times, centrifuged and stored at − 20 ◦C for further DNA extractions. Soil DNA isolation plus kits (Norgen Biotek CORP, Ontario., Canada) were used for the genomic DNA extraction process. The extracted DNA was quantified by a DNA NanoDrop 1000 Spectrophotometer (Waltham, MA, USA), and analysed by the "Genomic and Bioinformatics service center" at Autonomous university of Barcelona (Barcelona, Spain). The universal primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify and sequence the V4 region of the 16S rRNA to ensure high sequence coverage of bacteria and archaea and to produce an appropriately sized amplicon for Illumina sequencing (Wu et al., 2015). The Greengenes database was used to classify the organisms and the sequence reads were analyzed through Usearch software.

Chapter 4:

The integration of mainstream P-recovery strategies with EBPR and the application of different carbon sources on EBPR

4. The integration of mainstream P-recovery strategies with EBPR and the application of different carbon sources on EBPR

4.1. The integration of mainstream P-recovery strategies with EBPR

4.1.1. *Abstract*

Phosphorus (P), an essential nutrient for all organisms, urgently needs to be recovered due to the increasing demand and scarcity of this natural resource. Recovering P from wastewater is a feasible and promising way widely studied nowadays due to the need to remove P in wastewater treatment plants (WWTPs). When enhanced biological P removal (EBPR) is implemented, an innovative option is to recover P from the supernatant streams obtained in the mainstream water line, and then combine it with liquor-crystallisation recovery processes, being the final recovered product struvite, vivianite or hydroxyapatite. The basic idea of these mainstream P-recovery strategies is to take advantage of the ability of polyphosphate accumulating organisms (PAO) to increase P concentration under anaerobic conditions when some carbon source is available. This work shows the mainstream P-recovery technologies reported so far, both in continuous and sequenced batch reactors (SBR) based configurations. The amount of extraction, as a key parameter to balance the recovery efficiency and the maintenance of the EBPR of the system, should be the first design criterion. The maximum value of P-recovery efficiency for long-term operation with an adequate extraction ratio would be around 60%. Other relevant factors (e.g. COD/P ratio of the influent, need for an additional carbon source) and operational parameters (e.g. aeration, SRT, HRT) are also reported and discussed.

4.1.2. *Introduction*

The new paradigm in wastewater treatment proclaims that wastewater treatment plants (WWTPs) should evolve to water resource recovery facilities (WRRFs), where resources and energy are recovered from water. Among all the potential resources to be recovered, P arises as a perfect candidate since: i) in the current context of population and food demand growth, P is essential for fertilizers production (Chowdhury et al., 2017; Cieślik and Konieczka, 2017; Roy, 2017), ii) P is currently a pollutant of our wastewater which may promote eutrophication in water bodies if it is not removed and iii) P is mainly obtained from non-renewable sources such as phosphate rocks (Van Vuuren et al., 2010), which are expected to be depleted in only 50-100 years (Cordell et al., 2009; Desmidt et al., 2015; Rittmann et al., 2011). For the full-scale application of P-recovery processes, one of the main current limitations is that the market price (Table 4.1) of rock phosphate is lower than recovered products such as struvite. However, if the additional benefits of P-recovery are accounted for, significant incentives to support it emerge: for example, the need to treat the impurities in phosphate rock (e.g. cadmium or uranium), which can be highly toxic (Cornel and Schaum, 2009), or to avoid the need for external dependence on a strategic resource such as phosphate rock. In addition, P in urban wastewaters has to be removed anyway to avoid eutrophication processes, making P-recovery a win-win scenario.

Table 4.1. The market values of P rock and P-recovery products.

P related species	Market price	Reference
P rock	~120 $/ton	(Mew, 2016)
Struvite	~500 €/ton	(Wu et al., 2019)
Vivianite	~10000 €/ton	(Wu et al., 2019)
Hydroxyapatite	~10000 $/ton	(Annisa et al., 2021)

WWTPs seem to be an adequate place to recover P, since 1.3 Mt of P are removed annually worldwide through wastewater treatment. Some estimates indicate that global demand for P could be covered by 15-20% with this recovery (Ruo-hong hong Li and Li, 2017; Wu et al., 2019; Yuan et al., 2012). Other studies indicate that in Central Europe, P-recovery from municipal wastewater would satisfy 40-50% of the mineral P fertilizer applied annually in agriculture (Egle et al., 2016).

Furthermore, from a life cycle assessment (LCA) point of view, it has been concluded that P-recovery from municipal wastewater sludge, liquor or ash of sludge mono-incineration would provide environmental benefits (Remy and Jossa, 2015). Although differences appear in the amount of P recovered, requirements of energy, chemicals and fuels, or in side effects in the process, P-recovery processes can reduce environmental impacts, mainly on global warming potential, freshwater eutrophication of and fossil energy demand (Remy and Jossa, 2015). P-recovery as struvite seems to be especially attractive for urban areas, to satisfy the growing trend of local crop production linked to the large volumes of wastewater treated in centralized WWTPs (Rufí-Salís et al., 2020). In this recent LCA study, three recovery technologies were analyzed for large WWTPs, showing that were able to recover 5-30 times the amount of P required to fertilize the agricultural area of the studied region annually (36.5 t). Although there was an increase of chemicals and energy requirements per m^3 of wastewater due to struvite recovery, it resulted in lower eutrophication potential.

4.1.2.1. Fate of P in WWTPs

The fate of the influent P in a WWTP depends on its configuration. Part of the influent P is removed with the primary sludge (around 11%) according to Cornel and Schaum (2009). If the plant does not implement enhanced biological phosphorus removal (EBPR) in the secondary step, approximately 15-30% of the P in the influent becomes part of the biomass (a conventional WWTP sludge contains around 1-2% of P according to mass basis) and removed with the purged sludge (Henze et al., 2008; Parsons and Smith, 2008).

Then, if complete P-removal is mandatory, the remaining P should be chemically removed with addition of Fe(III) and Al(III) salts in different dosing points such as the primary sedimentation, before and/or following biological treatment or in a tertiary treatment (Tchobanoglous et al., 2014). However, the chemical sludge produced is not recommended as fertiliser since these salts could reduce the mobility of P in soils where this chemical sludge is applied (Desmidt et al., 2015; Sartorius et al., 2012). The sludge generated is often accumulated in landfills or burned in incinerators, and may end up contaminating water bodies (such as aquifers, rivers or sea) (Mainstone et al., 2000). Alternatively, the use of ashes from sewage sludge incinerators is proposed for P-recovery (Ma and Rosen, 2021) and to produce fertilizers via a chemical extraction (Fang et al., 2021; J. Li et al., 2018).

The fate of P in WWTPs that include EBPR is very different. EBPR is based on the proliferation of polyphosphate accumulating organisms (PAO), which can accumulate phosphate internally as polyphosphate (poly-P) under aerobic (or anoxic) conditions after being exposed to anaerobic conditions with available carbon source. Then, P is removed as poly-P inside cell biomass by waste sludge (Tchobanoglous et al., 2014). PAO can incorporate up to 0.38 mg P/mg VSS compared to the 0.02 mg P/mg VSS incorporated by the ordinary heterotrophic organisms (OHO) (Gebremariam et al., 2011b; Henze et al., 2008; Yuan et al., 2012). EBPR-based WWTPs can typically remove more than 85% of the P from the influent that is stored within the biomass (Bunce et al., 2018; Gebremariam et al., 2011b). The reported range of the P content in EBPR-based sludge is around 0.06-0.15 mg P/mg VSS (Henze et al., 2008). However, the use of this sewage sludge in agriculture is nowadays limited in many areas because of the ecological and health threats involved. For example, it is estimated that only about 20% of the sewage sludge meets the EU regulations for fertilizers in terms of concentration of heavy metals or radioactive elements (e.g. radon and uranium) (European Commission, 2019; Pettersson et al., 2008; Weigand et al., 2013).

There is another reason to implement P-recovery strategies. In EBPR-based WWTPs, the clogging of tubes and pumps due to undesired struvite precipitation is observed when the bio-P sludge undergoes anaerobic digestion and P-recovery is not implemented (D.Wild et al., 1996; Parsons and Doyle, 2002). The repair of this clogging is costly due to the need to shut down and clean the clogged equipment. In a modelling study, Lizarralde et al. (2019) constructed a plant-wide model to understand the effect of several operational parameters on the uncontrolled struvite precipitation when EBPR was implemented. They concluded that struvite recovery has many benefits for the plant not only for its commercial value, but also for the reduction of sludge production, ferric chloride dosage and the prevention of uncontrolled struvite precipitation.

4.1.2.2. Most studied technologies for P-recovery in WWTPs

Different technologies have been proposed for P-recovery from flows containing phosphorus in WWTP (i.e. effluent, sewage sludge, sludge ash, dewatering liquor and digester supernatant), providing different operability, costs and potential uses of the recovered material (Cieślik and Konieczka, 2017; Desmidt et al., 2015; Egle et al., 2016; Law and Pagilla, 2018). Egle et al. (2016) indicated that an ideal technology should provide good P-removal from wastewater, maximize P-recovery rate, destroy hazardous substances and produce a material that could be applied with low environmental risks, good fertilizing effects and high economic efficiency. They also studied all these characteristics from 19 different P-recovery technologies and concluded that the choice of a specific recovery technology is a compromise among these factors. Furthermore, the LCA analysis by Amann et al. (2018) showed wide range of changes in gaseous emissions, energy demand and P-recovery potential of different technologies based on liquid phase, sewage sludge and ash, and concluded that LCA should be combined with additional environmental criteria to put the performance of the technologies into perspective.

Considering the wide range of existing P-recovery processes, this section presents the most studied ones classified in two groups, depending on whether they can be obtained from primary/secondary effluents or from the sludge line, and includes another subsection showing the crystallisation processes that could be applied to P-enriched streams to make P-recovery effective. Section 2 then presents the P-recovery processes related to mainstream strategies that are the focus of this review.

4.1.2.2.1. P-recovery from primary or secondary effluents

P-recovery from primary or secondary effluents using chemical precipitation does not seem to be a cost-effective alternative because it would require a large amount of chemicals (Cornel and Schaum, 2009; Desmidt et al., 2015; Egle et al., 2016). Several alternative methodologies have been described previously (Egle et al., 2015; Kabdaşlı and Tünay, 2018). For instance, Liberti et al. (1979) reported a methodology based on the combination of ion exchange (IX) for phosphate selective exchange and P-recovery from the concentrated stream by struvite precipitation, which was further evaluated in subsequent works (Liberti et al., 2010; Petruzzelli et al., 2003). Williams et al. (2015) studied P and N removal with media based on Fe, Cu, and Al and then precipitation columns with clinoptilolite IX. They achieved almost 84% of P-recovery and a regeneration eluate with concentration up to 560 mg $P \cdot L^{-1}$. Beaudry and Sengupta (2021) used a novel pyridine-based polymeric ion exchanger and three different fixed-bed columns, producing an effluent concentration with $P < 6$ μg/L and recovering about 90% of the phosphorus.

The hybrid anion exchanger (HAIX), which consists of a hybrid polymeric base dispersed with hydrated ferric oxide (HFO) nanoparticles, has also been reported for P removal (Martin et al., 2018, 2009; Sendrowski and Boyer, 2013). Phosphate uptake is based on the selective sorption sites with HFO nanoparticles, which form sphere complexes with Fe(III) and phosphate (BLANEY et al., 2007). After long-term sorption, phosphate breakthrough occurs, but the exhausted HAIX can be reused with a regeneration phase with NaOH/NaCl solution and, finally, a rinse with CO_2 sparged filtered water (BLANEY et al., 2007). A recent work by Guida et al. (2021) evaluated a large (10 m^3/d) HAIX system for 2.5 years using treated municipal wastewater, showing high P removal (95%, i.e. from 6 to <0.3 mg PO_4^{3-}-P/L) and recovery efficiency (95% of phosphate from the wastewater recovered as hydroxyapatite). In subsequent work, the regeneration of the media and the modelling of this system were also studied (Pinelli et al., 2022). However, the feasibility of this methodology at full-scale has not been proved yet (Egle et al., 2015; Rufí-Salís et al., 2020; Siciliano et al., 2020).

Apart from the above-mentioned approach, microalgae cultivation has been proposed as a promising technology for P-recovery from secondary effluents due to its low cost, eco-friendliness and low technology dependence (Yang et al., 2017; Bodin et al., 2006). P is taken up biologically by algal growth by assimilation, accompanied by oxygen generation that supports aerobic bacteria growth. Algae can also be used either as a feedstock for biofuel production or directly as a biofertilizer (Chisti, 2013, 2008). El Hamouri (2009) showed that 63% of influent P could be removed and incorporated into algae as a tertiary treatment using a high-rate algal pond. Xu et al (2016, 2015) also showed that algae could remove about 60% of influent phosphorus for secondary wastewater effluent. However, the application of algae for P-recovery is still limited in large-scale wastewater treatment (Gebremariam et al., 2011b).

4.1.2.2.2. P-recovery from the sludge line

As mentioned above, the maximum P-recovery potential is from EBPR-based sludge. However, this option is not straightforward. For instance, the agricultural application of stabilized sludge with high P content (Yuan et al., 2010) has to consider the strict national health and safety restrictions on nutrients and heavy metals. This hinders the agricultural application of sludge and, thus, this pathway of P-recovery (Law and Pagilla, 2018). Another option is the incinerated sludge ashes, which may contain 4 to 11% of P (Biswas et al., 2009; Donatello et al., 2010; Franz, 2008; Ottosen et al., 2013). However, as a result of the organic matter oxidation, the heavy metal content in these ashes may be even higher than the sludge itself. Therefore, a chemical (Franz, 2008; Ottosen et al., 2013) or thermochemical (Adam et al., 2007) process is needed to separate the P from the heavy metals of the ashes. Wet chemical treatment by acid, alkaline or both have been proposed for the dissolution of P (Law and Pagilla, 2018); acid leaching (sulphuric or nitric acid) is proposed for Fe-rich ashes (Ottosen et al., 2013), while the combination of both acid and alkaline treatment is proposed for Al-rich ashes (Petzet et al., 2012).

The most reported location to recover P is a liquid stream from the sludge line, either from the sludge treatment or the anaerobic digestion (Egle et al., 2015). The treatment of the purged secondary sludge releases soluble phosphate and ammonium, resulting in a supernatant with increased concentrations of these compounds. These streams can contain up to 30% of the influent P loads. Levlin and Hultman (2003) reviewed several side-stream based P-recovery strategies and concluded that P-recovery efficiency about 60-65% is achieved from these streams.

4.1.2.2.3. Crystallization processes for P-recovery

Crystallization is nowadays the most accepted strategy for recovering P from these enriched liquors, being struvite the preferred precipitation product (Le Corre et al., 2009; B. Li et al., 2019b; Rittmann et al., 2011). Struvite ($MgNH_4PO_4 \cdot 6H_2O$) can be applied directly to the field as a slow release fertiliser, also providing nitrogen (N) and magnesium (Mg) to the soil, and has been reported to be a good option for agricultural uses (Hu et al., 2016). Sometimes, ammonium in struvite can be replaced by potassium and then the precipitate is known as K-struvite (Ronteltap et al., 2007). The reactions to precipitate struvite and K-struvite and their corresponding solubility are presented in Table 4.2.

Table 4.2. Stoichiometry and solubility for the most usual P-recovery chemical compounds.

Compound	Stoichiometry	pKsp	Reference
struvite	$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6\ H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O$	13.26	(Ohlinger et al., 1998)
K-struvite	$Mg^{2+} + K^+ + PO_4^{3-} + 6\ H_2O \rightarrow MgKPO_4 \cdot 6H_2O$	11.7	(Luff and Reed, 1980)
vivianite	$3\ Fe^{2+} + 2\ PO_4^{3-} + 8\ H_2O \rightarrow Fe_3(PO_4)_2 \cdot 8H_2O$	29.03	(Chen and Faust, 1974)
hydroxyapatite	$5\ Ca^{2+} + 3\ PO_4^{3-} + OH^- \rightarrow Ca_5(PO_4)_3OH$	58.5	(Mcdowell et al., 1977)

Figure 4.1 presents a graphical representation of the minimum magnesium (Mg^{2+}) concentration needed to reach the struvite solubility product at different pH as a function of phosphate and ammonium concentration. As observed, significant concentrations of P, Mg^{2+}, NH_4^+ and high pH (around 9-10) are needed so that struvite crystallisation becomes thermodynamically favourable. Struvite precipitation in streams with low concentration of phosphate or ammonium or low pH (as the influent or effluent of WWTPs) becomes an unaffordable option because of the requirement of adding high amount of magnesium (e.g. see Figure 4.1 at the condition of pH = 7).

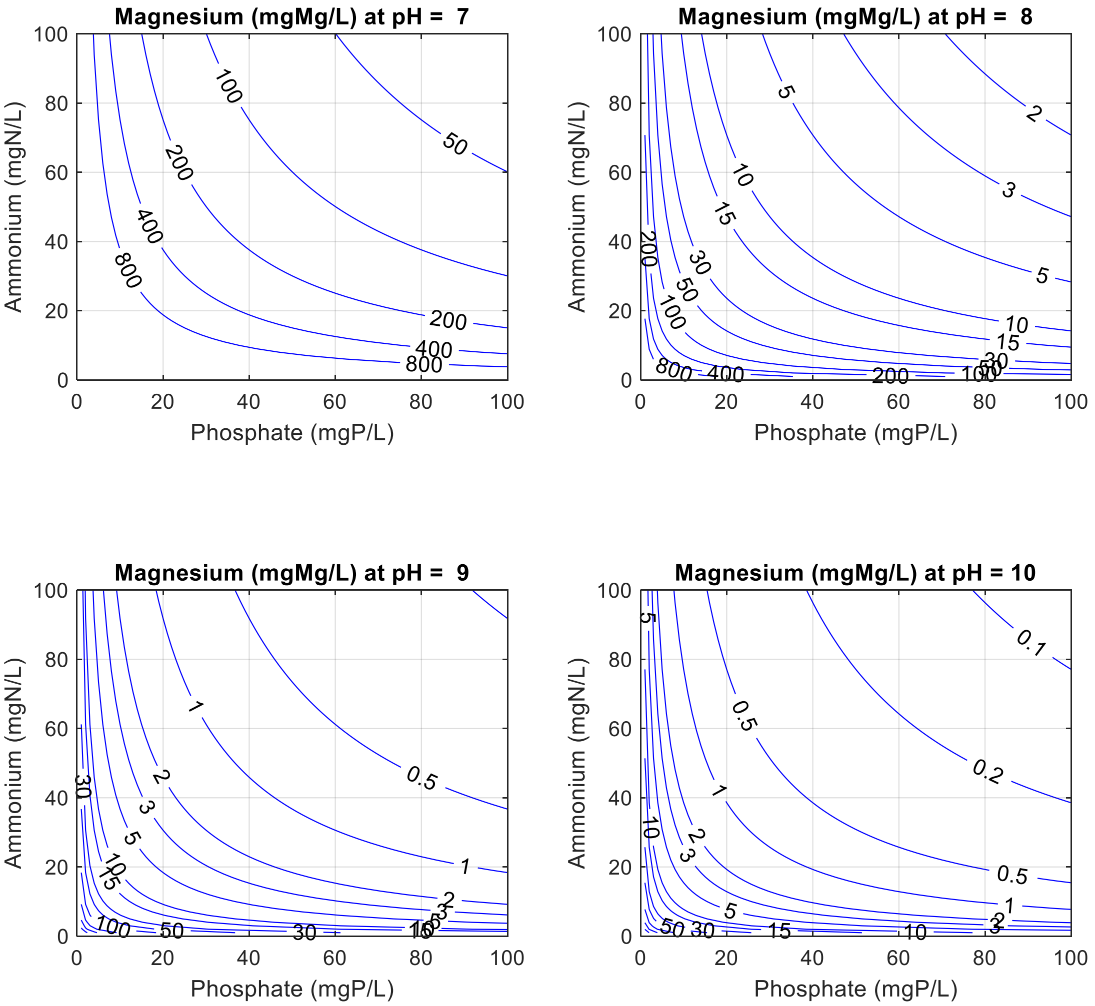

Figure 4.1. Minimum magnesium (Mg^{2+}) concentration to reach the K_{sp} value for struvite at different pH and as function of phosphate and ammonium concentration. Addition of magnesium beyond this value will drive struvite precipitation.

In any case, there are successful reports of P-recovery as struvite under full-scale conditions using side-streams obtained from the sludge line (Desmidt et al., 2015; Jabr et al., 2019; Law and Pagilla, 2018; Le Corre et al., 2009; Mayer et al., 2016) and successfully marketed technologies for the recovery of struvite include (among others) Ostara®, Airprex™, Multiform™, DHV Crystalactor® and NuReSys™ (Law and Pagilla, 2018). However, struvite crystallization also has some drawbacks in its practical application, for example, an unsatisfactory recovery efficiency related to the total P load in the raw sludge, in the range 45-80% in some full-scale projects for P-recovery from digested sludge or liquor, as reported by Remy and Jossa (2015).

Vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) is an alternative valuable product to struvite for P-recovery. It has a high market value (Table 4.1) as it is a relatively slow-release fertilizer widely found in soils, lake sediments, hydrothermal deposits or bogs (Wu et al., 2019). Moreover, the precipitating agent, iron, is a common dissolved mineral in natural water and Fe(III) salts are commonly used for improving sludge settleability (Li et al., 2014) and odour control (Zhang et al., 2009). The reaction to precipitate vivianite and its solubility is presented in Table 4.2.

The theoretical molar ratio of Fe:P to form vivianite is 1.5:1, but the optimal stoichiometric ratio range applied is 1.5-2 considering some of Fe(II) could be transformed to $Fe(OH)_2$ or oxidized to Fe(III) (Priambodo et al., 2017). The formation condition of vivianite is less dependent on pH (i.e. adequate range is 6-9) compared to struvite (8-9.5). Vivianite can be precipitated from the digested sewage sludge (Prot et al., 2020; Wilfert et al., 2018), wastewater (Wu et al., 2019) and aqueous solutions (J. Liu et al., 2018). Some researchers have shown that about 62% of P could be precipitated to vivianite from an anaerobic digestate with a pH around 8 (Li et al., 2018; Priambodo et al., 2017). However, the P-recovery process as vivianite also has disadvantages: i) the formation of vivianite in the sludge produces small crystals or aggregates (10-150 μm) resulting in a complex separation from sludge and ii) vivianite usually contains calcium or magnesium impurities (Robles et al., 2020). Impure vivianite is less stable, since it can be oxidized within 48 h, in contrast to the several weeks of stability reported for pure vivianite (Wilfert et al., 2018). In addition, separation and purification methods for vivianite still need further development (Robles et al., 2020).

Another alternative to struvite is hydroxyapatite (HAP, $Ca_5(PO_4)_3OH$). HAP can be directly used as a fertilizer in the field of agriculture (Johansson et al., 2017; Mayer et al., 2016) and as an adsorbent for removing dyes, emerging pollutants and heavy metals (Maity et al., 2018; Z. Zhang et al., 2018). It is of great potential for P-recovery since calcium phosphate has a large market (Driver et al., 1999) and, thus, it is expected to be of essential importance for the closure of P cycle (Leinweber et al., 2018). The reaction to precipitate HAP and its solubility is presented in Table 4.2.

HAP is thermodynamically the most stable phase for orthophosphate and calcium in solution within a broad range of pH of 4-12 (Cichy et al., 2019; Oubagha et al., 2017). When dealing with the common problem of low P concentration in municipal wastewater, HAP is a more attractive option than struvite since struvite demands higher enriched P. For example, although this is highly specific depending on the pH and species concentration, for a typical wastewater composition (8 mgPO_4^{3-}-P/L and 40 mgNH_4^+-N/L), and based on the HAP and struvite solubility, the addition of 10 mgCa^{2+}/L and 10 mgMg^{2+}/L at pH = 8 or higher leads to almost complete precipitation of Ca to produce HAP, whereas struvite is not formed even at a higher pH. Thus, some works have exploited this facility of HAP to precipitate and evaluated P-recovery strategies in lab-scale systems (Berg et al., 2005; Zou and Wang, 2016). However, HAP could lose advantage over struvite precipitation when aiming at a simultaneous recovery of N and P in real industrial wastewater treatment (Cichy et al., 2019). In addition, HAP has lower potential for fertilizer use (Robles et al., 2020).

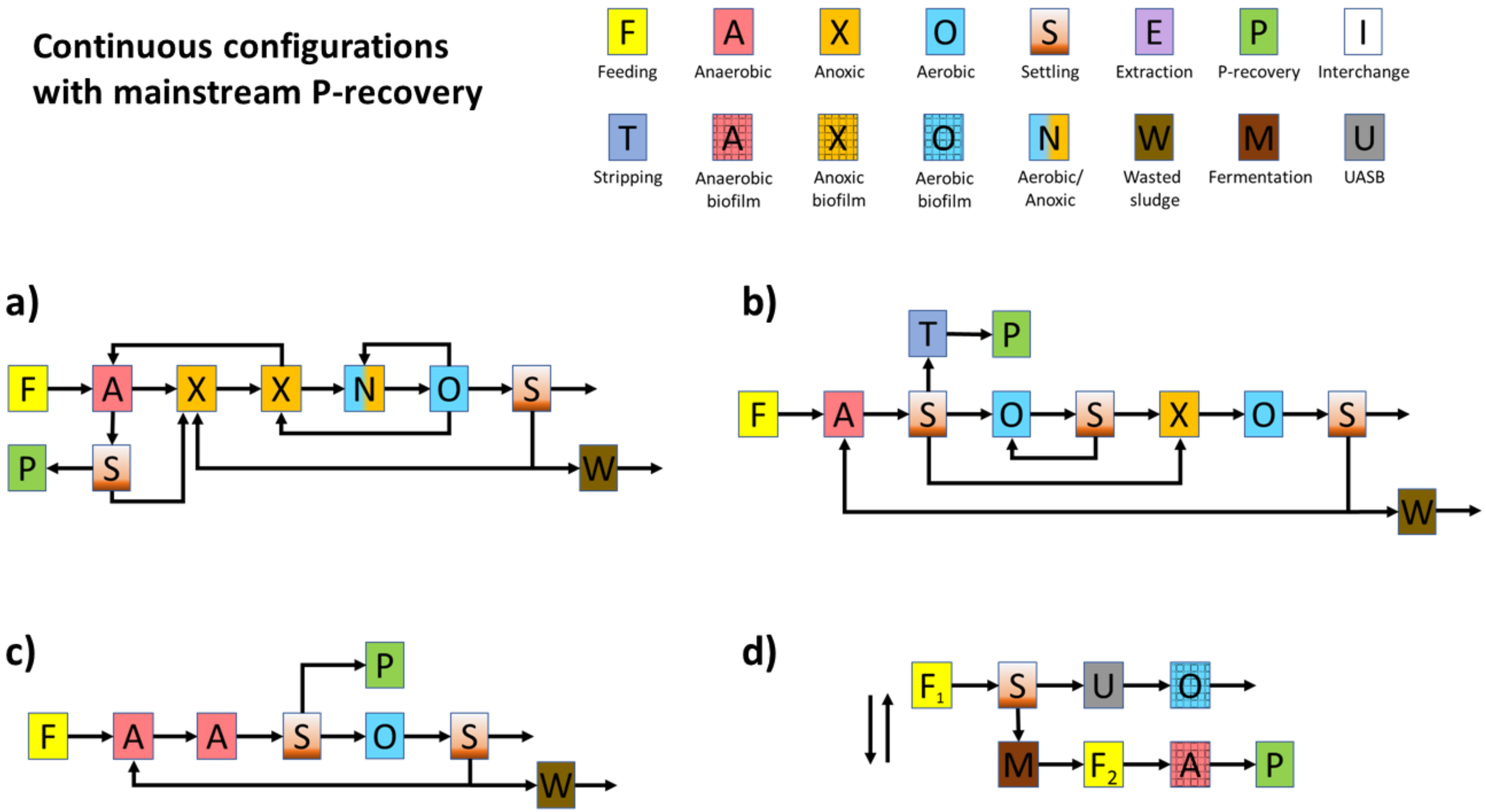

Figure 4.2. Schematic representations of the main continuous configurations with mainstream P-recovery found in the literature. a) BCFS process implemented in Hardenberg (Hao and van Loosdrecht, 2006), b) A2N-IC process (Shi et al., 2012), c) EBP2R system (Valverde-Pérez et al., 2015), d) trickling filter with alternative anaerobic and aerobic phases and sludge fermentation (Kodera et al., 2013). Please note that the symbols in the legend include those used in Figures 2 and 3.

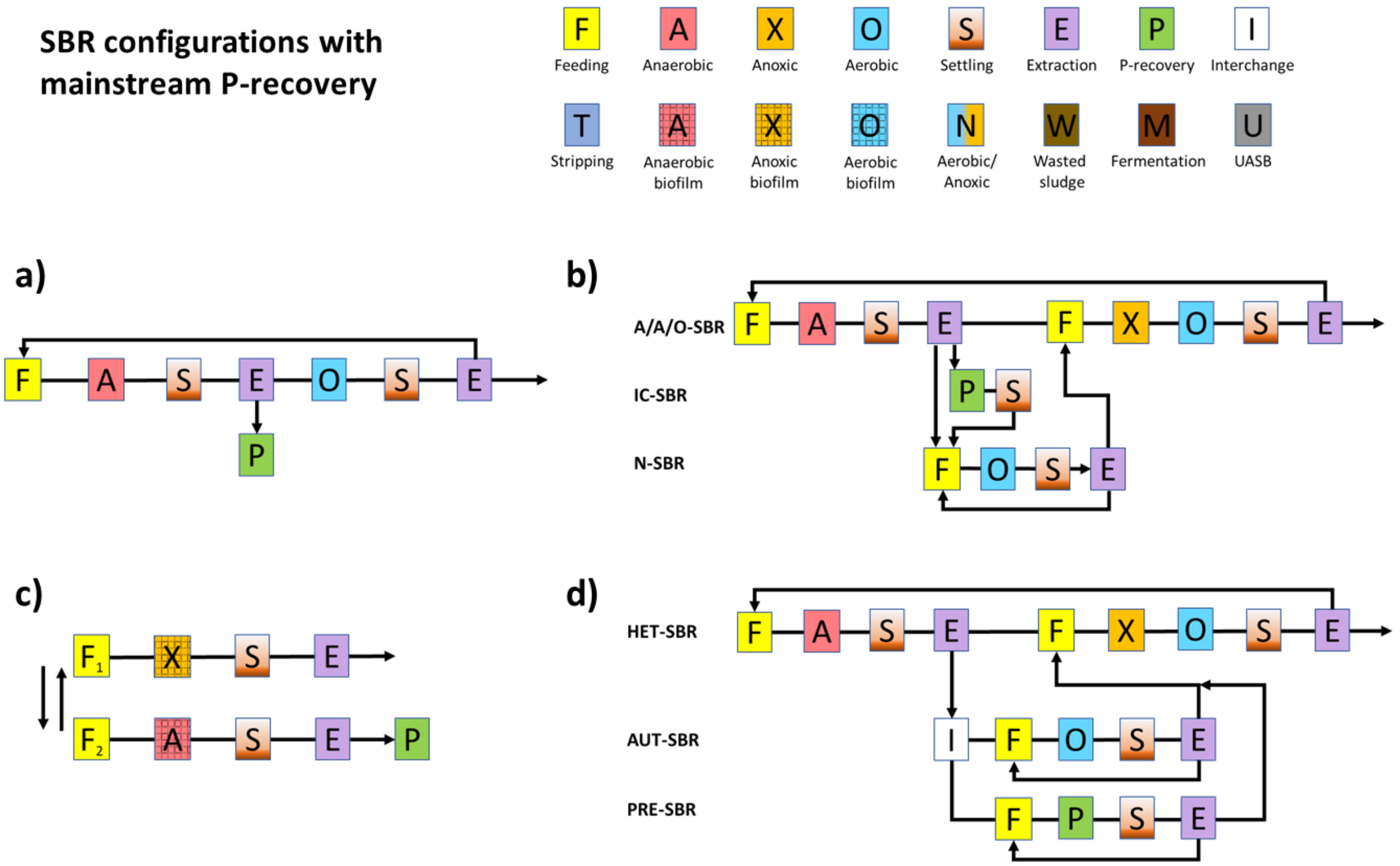

Figure 4.3. Schematic representations of the phases used in SBR configurations with mainstream P-recovery found in the literature. a) anaerobic/aerobic SBR EBPR^2 process (Baeza et al., 2017; Guisasola et al., 2019), b) anaerobic/anoxic/nitrifying-induced crystallization SBR process (Shi et al., 2016), c) two-step biofilm system alternatively exposed to a dilute wastewater stream to facilitate P uptake (under anoxic condition) and to a recovery stream to facilitate P release (under anaerobic condition with external carbon source) (Wong et al., 2013), d) mainstream shortcut enhanced phosphorus and PHA recovery (mainstream SCEPPHAR) configuration (Larriba et al., 2020). Please note that the symbols in the legend include those used in Figures 2 and 3.

4.1.3. *Potential P-recovery from mainstream*

P-recovery via precipitation (e.g. struvite, K-struvite, HAP, vivianite) requires a high concentration of P for displacing the solubility equilibrium to the precipitation side (Figure 4.1). As described before, in EBPR-based WWTPs, digestate is a common option to precipitate/recover P since the sludge entering anaerobic digestion contains a high amount of internally stored P as poly-P that can be easily released inside the digester and results in a high concentration of P into the digestate. The digestate also contains high ammonia concentration and, thus, seems to be a favourable option for struvite precipitation. However, these systems also bring disadvantages such as undesired precipitation in the anaerobic digester or in the downstream tubing and instrumentation (Parsons and Doyle, 2002). On the other hand, the high concentration of some ions in the anaerobic digestion sludge (e.g. calcium) could hinder the precipitation of P as struvite, since when P is released it can precipitate as other compounds such as HAP, brushite and other calcium phosphates. In any case, to determine the actual precipitation competition in a given anaerobic digestion scenario, not only solubility has to be taken into account, but also kinetics (D.Wild et al., 1996).

Apart from the digestate, the other location with the highest P concentration in an EBPR plant is the anaerobic reactor and, thus, it can be an alternative for P-recovery. During the anaerobic phase, PAO have taken up most of the readily biodegradable COD and most of the P has been released into the bulk liquid, reaching levels from 30 to 70 mg P/L (Acevedo et al., 2015; Izadi et al., 2021b, 2020; Zhang et al., 2021; Zou and Wang, 2016). Therefore, the anaerobic reactor appears as a good point to implement mainstream P-recovery whilst the digestate seems to be the ideal point for side-stream P-recovery. The integration of EBPR and mainstream P-recovery as struvite would be beneficial to prevent undesired precipitation throughout the plant and to reduce N load when treating wastewater with high ammonium concentration (1 g of P removed as struvite yields 7.92 g of struvite containing 0.45 g of N). Thus, it shows a good potential for improving system performance, and for this reason it has recently received the attention of researchers (e.g., Acevedo et al., 2015; Larriba et al., 2020; Shi et al., 2016; Zou and Lu, 2016).

The first proposal on the potential to obtain a P-rich liquor from the mainstream anaerobic reactor was reported by Van Loosdrecht et al. (1998): the Biological-chemical P and N removal (BCFS®) process (Figure 4.2a). BCFS was a modification of the UCT design where a stream from the anaerobic reactor (for instance, an average flow of 10% of the influent flow) was sent to a sludge thickener and a phosphate stripper where $FeCl_3$ was dosed for P precipitation. Thus, a continuous EBPR operation and a P chemical precipitation system were combined. Modelling was used to better understand this process and to assess its performance under different scenarios (Barat and van Loosdrecht, 2006; Hao and van Loosdrecht, 2006). Hao and van Loosdrecht, (2006) simulated the effect of anaerobic stripping on the P-removal and recovery performance within the BCFS® frame and reported an optimal stripping flow rate ratio (flow rate of supernatant /flow rate of influent) of 0.2 to favour P-recovery. Higher values resulted in decreased PAO activity and lower values in low P-recovery (see section 3.2 for the discussion of this effect). Moreover, they showed that the system could operate with a lower influent COD/P ratio without hindering PAO activity, the ratio could be decreased from 20 to 10 g COD/g P and the system could still meet the discharge requirements of P (1 mg P/L) and maintain 36% of P-recovery.

This first experimental attempt of mainstream P-recovery already showed that there is a maximum threshold value for the amount of P extracted. Barat and van Loosdrecht, (2006) implemented the model of the Hardenberg WWTP and simulated different control strategies to explore the P-recovery potential of the BCFS® configuration. They stated that the maximum potential P-recovery without affecting PAO activity was 60% of the influent P. Following this first report, several other works have presented configurations taking advantage of the PAO capability to increase P concentration under anaerobic conditions (Baeza et al., 2017; Shi et al., 2016; Wong et al., 2013).

The P-recovery efficiencies of the evaluated works were calculated according to equations 1-3:

$$P_{OUT} = P_{EFF} + P_{BIO} \quad (1)$$

$$P_{AS} = P_{INF} - P_{OUT} \quad (2)$$

$$PR = \frac{P_{AS}}{P_{INF}} * 100\% \quad (3)$$

where P_{OUT} represents the P output per day in the effluent (P_{EFF}) plus in the purged biomass (P_{BIO}), P_{AS} the P in the anaerobic supernatant (thus available for P-recovery), P_{INF} the total input of P in the influent to the secondary treatment per day and PR the P-recovery efficiency. P_{INF} is highly influenced by the configuration of the primary treatment. Typical P removal values around 11% have been reported for conventional primary treatments (Cornel and Schaum, 2009), but these values can be increased considerably to values of 40-99% if chemical dosing is used in the primary settler, as in the chemically enhanced primary treatment (CEPT) processes (Shewa and Dagnew, 2020).

The rest of the manuscript is organized as follows. The studies related to mainstream P-recovery currently available in the literature are briefly described in the following two subsections, classified by their operation mode: section 2.1 for sequencing batch reactors (SBRs) and section 2.2 for continuous systems. This classification separates SBR configurations, which offer greater configurability than continuous systems, although they are typically used for lab-scale evaluation of different operating strategies, from continuous systems, which offer more stable operation and are more suitable for full-scale implementation, although the achievable P concentration is typically lower. After this schematic description, section 3 critically discusses the most important factors affecting P-recovery: SBR vs. continuous configurations, extraction ratio, influent COD/P ratio, concentration of P in the recovered stream, carbon source needs and future outlook.

4.1.3.1. SBR configurations

Most of the configurations for mainstream P-recovery studied in previous works have been based on SBR operation (Table 4.3, Figure 4.3). The supernatant after the anaerobic phase is used for P-recovery since it has the highest P concentration in all the cycle. SBRs provide greater flexibility than continuous systems for research studies, and the fact that particular actions can be taken in a single cycle (such as extra COD dosing or supernatant extraction) facilitates the understanding of P-recovery performance under different operation modes. For example, some authors have evaluated the effects of sporadic extractions of anaerobic supernatant and others have tested the automated extraction every single cycle. Long-term operation is in general required to demonstrate the ability of these two different approaches to maintain stable PAO activity despite the extractions, as a decrease in P availability may hinder PAO metabolism.

Wong et al. (2013) reported a novel two-step biofilm (Figure 4.3c) process to facilitate anoxic P-recovery. In the first step, the PAO-enriched biofilm was immersed into a diluted wastewater stream (F_1) so that the biomass could take up P and nitrate to remove P and N of the wastewater. In a second step, the biofilm was subjected to a carbon-enriched stream (F_2) under anaerobic conditions, where acetate was taken up and P was released. Then, the biofilm acted under aerobic condition storing P from wastewater and under anaerobic condition releasing P and hence allowing the generation of an enriched P-recovery stream. The anaerobic supernatant volume (1.8L) was four times lower than the initial volume of wastewater (7.2L) and achieved a P concentration almost 4 times higher (28 vs 7 mg P/L). An additional experiment was conducted to improve the system efficiency. They applied a highly enriched carbon stream after 10 cycles of normal operation and they obtained an anaerobic supernatant (around 100 mg P/L) which is theoretically high enough for an efficient P-recovery by struvite (Rittmann et al., 2011). Nevertheless, they did not test the effect of extracting this P on PAO survival neither in a short-term nor a long-term basis.

Xia et al. (2014) studied a configuration where a fraction of the anaerobic mixed liquor from a parent EBPR-SBR was treated in a separate batch reactor, and intermittent external carbon source was dosed to stimulate P-release in view of P-recovery. The sludge from this batch reactor was separated with a non-woven cloth and recycled to the parent SBR, whereas the supernatant was treated with $MgCl_2 \cdot 6H_2O$ to recover P as $Mg_3(PO_4)_2$. Thus, dosing 10% of additional carbon source in the batch reactor resulted in the recovery of 79% of the P in the influent in single cycle. The authors applied this new P-recovery

Table 4.3. Summary of P-recovery from mainstream and the related operation parameters based on sequencing batch configurations

Reference	Configuration	Volume	Nitrification	SRT	HRT	Temperature	DO	pH	Recovery species	Wastewater	Influent COD/P	Influent P[a]	[P] in the recovery stream	Extraction percentage[b]	P-recovery efficiency[c]	P-removal efficiency[d]	Sporadic external carbon addition
		(L)		(d)	(h)	(℃)	(mg/L)				(gCOD/gP)	(mg/L)	(mg/L)	(%)	(%)	(%)	
(Xia et al., 2014)	anaerobic/aero-bic	8	no (ATU[e])	60	24	18-22	1.5-2.5	7.0 - 8.0	$Mg_3(PO_4)_2$	synthetic	22	9	240[f]	12.5	79	100	acetate 640 mg COD/L
(Acevedo et al., 2015)	anaerobic/aero-bic	7	no (ATU)	10	12	20	1.5-2.5	7.0 - 8.5	struvite	synthetic	13	7.5	60-72 110-200	85[g] 85[g]	59[h] 81[h]	100 100	acetate 100 mg COD/L acetate 350 mg COD/L
(Wong et al., 2013)	anaerobic/ano-xic + biofilm	10	no (ATU)	n.d.	n.d.	22 ± 2	5-8	6.8 - 7.2	n.d.	synthetic	22	8	>90	8	80	79 ± 6	acetate 370 mg COD/L
(Shi et al., 2016)	A_2O+induced crystallization	-	yes	15	n.d.	20	n.d.	8.2 - 8.6	HAP	synthetic /raw	41	6.1	20	20	33	100	no
(Lv and Yuan, 2015)	SBR+EBPR	10	n.d.	11	16	20 ± 1	> 2	7.1 - 8.3	vivianite	synthetic	40	10	39	50	50	95- 97	no
(Zou and Wang, 2016)	Anaerobic /anoxic	6	yes	20	n.d.	16-21	n.d.	>8.5	HAP	synthetic	40	5	15.8	20	59.3	91.6	no
(Baeza et al., 2017)	Anaerobic /aerobic	10	yes	15	n.d.	n.d.	3	n.d.	struvite	urban (model)	33	9	57	5	63	100	no
(Guisasola et al., 2019)	Anaerobic /aerobic	10	no (ATU)	10	12	25	2.5-3.5	7.50 ± 0.05	struvite	synthetic	15 10 10 8	20 30 30 40	40-60 71-123 52-95 59-78	10	54[c] 66[c] 69[c] 33[c]	98 ± 1 67± 10 82 ± 14 77± 6	no
(Larriba et al., 2020)	two sludge system	7800	yes	15	24-36	20 ± 6	3	n.d.	struvite	raw	63	3.8	35	8.5	63	93±9	no
(Yu et al., 2021)	anaerobic/oxic	5.4	yes	10	24	16 ± 0.5	1 0.6 0.5	n.d.	struvite	synthetic	400/8	8	26-19 25-16 21-6.5	8, 11, 17	10, 24, 50 4, 18,40 0, 0	>90 >90 60	no

a Influent entering the secondary treatment

b Calculated as the fraction of the volume of the anaerobic supernatant extracted with respect to the volume of the reactor

c Calculated as the percentage of P in the anaerobic supernatant extracted with respect to the total input of P (per day)

d Calculated as the fraction of P in the influent that is removed

e ATU: Allylthiourea

f P concentration of a parent batch reactor with extra dose of acetate. The anaerobic P concentration is around 80 mg/L

g Average

h Weekly average

Table 4.4. Summary of P-recovery from mainstream and the related operation parameters based on continuous configurations

Reference	Configuration	Volume	Nitrificat-ion	SRT	HRT	Temper-ature	DO	Recovery species	Wastewater	Influent COD/P	Influent P[a]	[P] in the recovery stream	Extraction percentage[b]	P-recovery efficiency[c]	P-removal efficiency[d]	Sporadic external carbon addition
		(L)		(d)	(h)	(℃)	(mg/L)			(gCOD/g P)	(mg/L)	(mg/L)	(%)	(%)	(%)	
(Barat and van Loosdrecht, 2006)	BCFS-TUD[e] model calibrated for Hardenberg WWTP	$1.215\cdot10^{7}$	yes	n.d.	3.8	10–20	21	P Stripper (stripping)	model	54	14.2	60 - 70 kg P/d	45	~60	> 90	no
(Shi et al., 2012)	BNR-IC[f] (A_2O-crystallization)	31	yes	15	n.d.	n.d.	2-3.8	HAP	synthetic	19.9 ± 1.5 31.0 ± 4.2 42.4 ± 4.4 67.4 ± 10.2	12.7 ± 0.7 7.82 ± 0.68 5.73 ± 0.51 3.75± 0.51	n.d.	20	8 9.1 10.8 12.6	> 99	no
(Kodera et al., 2013)	PAOs-enriched biofilm within a modified trickling filter	2.4	n.d.	n.d.	0.5	20	0.9 L/h air[g]	struvite	synthetic	200/5	5	125	10	~60	50-60	no
(Valverde-Pérez et al., 2015)	EBP2R green microalgae cultivation	$5.1\cdot10^{6}$	yes	5	4.25	n.d.	0-3	green microalgae	model	79	9	n.d.	30	70	n.d.	no
(Zou and Lu, 2016)	BNR-IC	12.4	yes	n.d.	n.d.	n.d.	>2	HAP	synthetic real domestic	25 20-30	10 7.08-7.69	28 12	35 35	70 74	95.2 ± 1.0 90.7 ± 1.2	no
(Dai et al., 2017)	BNR-IC	316	yes	12-16	n.d.	n.d.	n.d.	HAP	synthetic	50	5	24	30	83	95	no

a Concentration of P for the EBPR process
b Calculated as the the ratio of the flow rate of anaerobic supernatant diverted to the P-recovery unit and flow rate of the influent
c Calculated as the percentage of P in the anaerobic supernatant extracted for P-recovery per day in respect of the total input of P in the influent per day
d Calculated as the removal fraction of phosphate in the effluent with respect to the phosphate in the influent
e BCFS-TUD: Biological-chemical phosphorus and nitrogen removal system-Model system of Delft University Technology.
f BNR-IC: An anaerobic-anoxic/nitrifying (A2N) continuous configuration for biological nutrients removal (BNR) combined with an induced P-crystallisation (IC) column.
g Aeration flow rate

sequence for nearly one month in only one out of four cycles although recommended to evaluate this approach in a longer period.

Acevedo et al. (2015) investigated a comparable EBPR-SBR configuration but the extra carbon was dosed *in situ* in the anaerobic phase. The frequency of these extra carbon addition and, subsequent anaerobic extraction for potential P-recovery were varied in different sets of experiments: two times per week with low P extraction or once per week but with high P extraction. Their results were promising since they obtained 59% recovery of the influent P when extracting P two times per week and 81% when they extracted once per week. In the latter case, they used an extra VFA addition of 9% of the usual load. No P-removal deterioration was observed in the cycle after both extractions, but the long-term impact of a P-recovery strategy with continuous extraction of P was not investigated.

Lv et al. (2014) studied a 10 L EBPR-SBR system to investigate the effect of the extraction of 5 L of supernatant enriched in P for subsequent chemical precipitation, supernatant that was obtained after an additional anaerobic settling period. The supernatant was treated with $FeCl_3$ for P-precipitation and the treated supernatant was returned to the aerobic stage. This extraction was applied to the SBR once every 3 cycles for a long term and observed the decrease of P-release, P-uptake and the concentration of intracellular poly-P. EBPR activity was deteriorated, and P-removal efficiency decreased to about 75%. They disabled the P-recovery strategy and tried to recover the EBPR activity by operating under conventional anaerobic/aerobic conditions, but the system did not recover. After that work, Lv and Yuan (2015) demonstrated that the same system could have a successful short-term performance (40 d) when 50% of anaerobic supernatant (with a concentration of 39.4 mg P/L) was extracted. The recovery process was operated in another separated reaction-sedimentation basin with $FeCl_3$ as a precipitating agent, and half of the anaerobic supernatant was removed for P-recovery. EBPR activity was strongly limited because of the shortage of poly-P as a continuous stripping of 40 d, and it could not be recovered even though the stripping was stopped for 60 d.

In the same direction, Guisasola et al. (2019) proposed the operation with an automated extraction of anaerobic supernatant every cycle to understand the P-recovery capacity in a long-term basis but without any external dose of additional organic carbon (Figure 4.3a). Thus, the anaerobic P concentration was lower than in the previous reports since it was the routine anaerobic P concentration of the system. They demonstrated that a successful P-recovery strategy could be maintained with 1L (10% of reactor volume, 20% of the influent) of automated anaerobic extraction per cycle during more than 120 days without any deleterious effect on EBPR activity. They tried to push the system into the limits and operated with other COD/P ratios and different extraction conditions in order to investigate two different aspects: the minimum initial COD/P ratio and the maximum amount of P extraction that the system could tolerate without the loss of the PAO activity. They demonstrated that up to 60% of the influent P could be recovered (when extracting 10 % of the anaerobic volume per cycle) without any harmful effect on PAO activity and that the system could be operated under low COD/P ratios (i.e. 7.5 gCOD/gP).

Zu et al. (2020) also used a lab-scale SBR to study the effect of the volume of anaerobic extraction on the biological C and P-removal performance. Different extraction ratio of anaerobic supernatant (30%, 60% and 90%) were tested for P-recovery as vivianite. Extraction was carried out every fourth cycle. P-removal efficiency was improved when increasing from 30 to 60% of the extraction volume. However, the phosphorus balance and the PAO population severely deceased under the extraction ratio of 90%. The harm was irreversible even after a long-term normal operation without stripping process.

In addition to P-removal and P-recovery, some researchers have studied the simultaneous P and N recovery from mainstream in a batch system configuration. Shi et al. (2016) proposed an anaerobic/anoxic/nitrifying-induced crystallization (IC) SBR configuration (Figure 4.3b, named as A_2N-IC-SBR) for N, P-removal and P-recovery which was composed of three different SBRs (A/A/O-SBR, N-SBR, and IC-SBR, with 10, 10 and 1.3 L). Nitrifiers oxidised ammonium to nitrate, which was denitrified by denitrifying PAO (DPAO) linked to P uptake. During this process, 1.3 L of anaerobic supernatant extraction was sent to the IC column with calcite seeds for P-recovery. The percentage of chemical P-removal with respect to the total P-removal was 14.9-17.1%.

Similarly, Zou and Wang (2016) implemented a lab-scale EBPR-P-recovery process aiming at removing COD, nutrients and recovering P, which consisted of a DPAO-enriched SBR operated with anaerobic-aerobic/anoxic mode and an IC P-recovery column. The IC column received part of the supernatant obtained after a post-anaerobic settling phase and the IC supernatant was returned to the anoxic phase with external nitrate solution dosage. The system was operated with either synthetic (around 250 mg COD /L) or real domestic wastewater (COD concentration between 153-223 mg COD/ L). They achieved successful N and P-removal and P-recovery efficiency via HAP precipitation: the average removal efficiencies were 82.6%, 87.5% and 91.6% for COD, P and N, respectively. The flow ratio of anaerobic supernatant to influent (from 0 to 0.5) was varied and the maximum P-recovery as HAP (up to 59%) was achieved at a ratio of 0.3.

Salehi et al. (2019) explored the possibility of a simultaneous nitrification, denitrification and P-removal (SNDPR) process for nutrient removal and P-recovery. A lab-scale anaerobic/aerobic SBR was employed with synthetic wastewater feeding. They achieved anaerobic liquor with high P concentration (i.e. around 100 mg/L) in granular SNDPR process with an influent P of 10 mg/L and a low COD = 200 mg/L.

Yu et al. (2021) used an anaerobic-aerobic lab-scale SBR (5.4L) to promote biological C, N and P-removal. Biological nitrogen removal (BNR) was achieved with low DO during the aerobic phase and thus simultaneous nitrification and denitrification was promoted. Successful biological nutrient removal was maintained stable for a long period (310 d) under different anaerobic supernatant extraction ratios (0, 1/12, 1/9, 1/6) and low DO conditions (0.6 and 1 mg/L). More than 40% of P could be recovered as struvite due to these extractions. The reactor began to deteriorate and the efficiency of P-removal decreased to 60% when operating the system with the lowest DO of 0.2 mg/L and a relatively high extraction ratio (1/9), and no P-recovery was found afterwards. They proposed a DO of 0.6 mg/L and an extraction ratio of 1/6 in view of saving energy and an optimal condition of DO of 1 mg/L and extraction ratio of 1/9 to maximise the process performance.

Some modelling studies have been reported about mainstream P-recovery with SBR systems. Baeza et al. (2017) simulated an EBPR-based SBR configuration for P-recovery named $EBPR^2$. A typical EBPR anaerobic/aerobic cycle configuration was modified including, after the anaerobic phase, a settling and decanting phase where the P-enriched stream was extracted for P-recovery (Figure 4.3a). They simulated different scenarios and showed that it was possible to obtain a stable operation with a high amount of P recovered (up to 63%) when the supernatant extraction in each cycle was 5% of the reactor volume. Moreover, total P-removal was achieved regardless of COD fractionation in the wastewater. In addition, they proposed the inclusion of anaerobic sludge purging, which could be a good option in view of a higher energy recovery (i.e. increasing biochemical methane potential per gram of volatile suspended solid). Sludge obtained from the anaerobic phase contains a higher concentration of poly-hydroxyalkanoate (PHA), which would boost the production of methane during anaerobic digestion (Chan et al., 2020a). The highest PHA production was obtained by extracting 4.3% of the reactor volume per cycle.

Taking into account the previous simulations and the pilot plant experiments of a two-sludge configuration with EBPR and via-nitrite N-removal (Marcelino et al., 2011), a new two-sludge process including P-recovery was designed (Larriba et al., 2020). This novel configuration (Figure 4.3d) was named shortcut enhanced P and PHA recovery (mainstream SCEPPHAR) and was tested in a large demo pilot-scale (total volume of 7.8 m^3) fed with real primary influent (Larriba et al., 2020). It consisted of a two-sludge SBR system: HET-SBR was operated as a conventional Anaerobic/anoxic/aerobic (A_2O) EBPR-

SBR under alternating anaerobic/anoxic/aerobic conditions and AUT-SBR was aerobic and aimed at a complete or partial nitrification. A third crystallization reactor (PRE-SBR) was designed so that the supernatant extracted after the anaerobic phase of HET-SBR could be used for P-recovery. An additional interchange vessel was required to interchange supernatants among these reactors, in order to avoid idle phases and to ensure that all three reactors could operate simultaneously rather than sequentially. This configuration was operated successfully for a long term for C, N and P-removal (86 ± 12%, 93 ± 9% and 79 ± 6%) with either full nitrification or nitrite shortcut. P-recovery as struvite was carried out in this third reactor by Mg^{2+} dosing at a pH of 8.5, allowing the recovery of an average of 45% of the influent P as struvite precipitate (with a maximum peak of 63%).

4.1.3.2. Continuous configurations

This section details the reported alternatives for P-recovery from mainstream with continuous configurations, in addition to the seminal BCFS process discussed above (Van Loosdrecht et al., 1998). Continuous systems are often used to test the P-recovery strategies previously developed in SBR mode. Under these conditions, part of the anaerobic supernatant is diverted to a P-recovery equipment and, in some cases, the flow is returned to the system after P is recovered.

For instance, an anaerobic-anoxic/nitrifying (A_2N) continuous configuration combined with an induced P-crystallisation (IC) column was proposed by Shi et al. (2012) for mainstream P-recovery in addition to removal of COD, N and P (Figure 4.2b). Part of the P enriched anaerobic supernatant was extracted to precipitate calcium phosphate crystals in the IC column. The conventional A_2N system performance was compared to that of A_2N-IC. A_2N-IC showed a more stable performance than A_2N. The authors used a wide range of COD/P ratios (from 17.7 to 67.4 gCOD/gP) and found that the P-removal efficiency was consistently maintained at 99.2% for A_2N-IC, regardless of the influent COD/P ratio tested. However, the A_2N process reduced P-removal efficiency from 93.0% to 65.7% when the influent COD/P ratio was decreased from 67.4 to 17.7 gCOD/gP. P-recovery in the A_2N-IC process ranged from 8.9 to 12.9% when the influent COD/P ratio was reduced from 67.4 to 19.9 gCOD/gP. Later, Zou and Wang (2017) used the same configuration and studied the effect of various operational conditions on the IC performance and optimized the crystal dose acting as a seed, pH, temperature, DO, molar Ca/P ratio and reaction time in view of boosting P-recovery. They showed that on the optimal conditions (80 min, pH of 8.5, molar Ca/P ratio of 2.0, 60 g/L seed crystal loads and 4.0 L/min of aeration flow) the system reached an excellent P-recovery of 92.3%. In addition, they found that the temperature had a slight effect on induced HAP crystallization, thus benefiting the integration of EBPR with induced HAP crystallization.

In a similar configuration, Dai et al. (2017) evaluated the influence of the flow of the anaerobic supernatant to IC in a long run (over 180 d) and found that the optimal ratio of anaerobic supernatant extracted to influent was 0.3. Under these conditions, P-recovery efficiency reached 72.4% at the optimal HRT = 0.5 h in IC reactor. Higher extraction ratios could disrupt system stability without enhancing P-recovery and could induce a change of the activated sludge characteristics since it favoured the percentage of extracellular polymeric substances of the sludge. The same authors reported a modelling study about the continuous A2N-IC aiming at finding the optimal conditions to maximize energy savings and minimize emission reduction (Dai et al. 2019). Their results indicated that P-recovery decreased with the reduction of the extraction ratio and the IC volume.

Kodera et al. (2013) studied a configuration based on a PAO-enriched biotrickling filter, exposed alternatively to anaerobic and aerobic conditions (Figure 4.2d). P-uptake by PAO was promoted under aerobic conditions, when the filter treated by recirculation a synthetic stream emulating an urban wastewater, which contained a low concentration of P (5 mg P/L). For the anaerobic phase, the system required a stream with VFA to enhance P-release, with up to 2000 mg COD/L which could be generated by primary sludge fermentation, although in this work a synthetic stream was used. The effluent from the anaerobic phase had a high concentration of P (125 mg P/L) that was adequate for P-recovery. The system operated for a long period of time (250 d) with this configuration, obtaining a P-recovery of almost 60% of the influent P.

In the frame of the cultivation of microalgae for P-recovery, Valverde-Pérez et al. (2015) proposed a novel model-based design of P removal and recovery system consisting of a conventional continuous A_2O operation where nitrification was suppressed at low DO (Figure 4.2c). The objective was to produce a nutrient medium for microalgae cultivation to produce biofuel or a natural fertiliser for direct use in agriculture. The P recovered for algae cultivation was obtained from the anaerobic effluent as a stream with high P concentration. They investigated the potential P-recovery under different scenarios of DO and SRT and obtained a maximum of 70% P-recovery of influent P, when 30% of the influent flow was diverted as a P-stream and the system was operated at SRT = 5 d. At DO = 1.5 mg/L, nitrification was still being inhibited due to this low DO and P uptake was favoured, increasing P-recovery up to 75%.

4.1.4. *Discussion*

Among the different locations for P-recovery in EBPR-based WWTPs, the supernatant of the anaerobic reactor has recently gained attention since mainstream P-recovery would i) mitigate undesired precipitation of P in the sludge line, ii) reduce the COD requirements for P-removal, iii) enable the treatment of wastewater with lower COD/P ratio and iv) depending on the recovery agent, decrease the N load to the plant and, thus, its operational costs. As detailed above, mainstream P-recovery can be achieved with different configurations and, thus, several operation parameters are essential for an efficient P-recovery performance. Tables 4.3 and 4.4 summarize the different reported proposals for the implementation of P-recovery from mainstream and lists, for each case, the applied equipment and recovery methods, basic operation parameters (SRT, HRT, operation temperature, aeration, pH, extraction volume and frequency) and influent characteristics.

4.1.4.1. SBR vs continuous configurations for mainstream P-recovery

Mainstream P-recovery under SBR conditions is based on extracting part of the supernatant once the anaerobic phase is finished, whereas it consists of diverting part of the anaerobic liquid to a P-recovery unit under continuous conditions. Most reports on mainstream P-recovery employed lab-scale SBRs (Table 4.3), as SBRs have several advantages compared to continuous systems. On the one hand. it is easier to obtain a biomass-free anaerobic supernatant as a settling phase can be easily included after the anaerobic phase whereas in the case of continuous system a membrane or a combined settling reactor+biomass recycle would be needed (Sekine et al., 2018). Moreover, SBRs show a lower space requirement and lower configuration and installation costs, as well as , and the flexibility to face operational or influent variations (Jiang et al., 2016; Tomei et al., 2016). However, it can also lead to higher operational costs and capital expenditures due to its discontinuous operation, as reported in (Larriba et al., 2020) when comparing the mainstream SCEPPHAR configuration to a conventional continuous A_2O configuration.

4.1.4.2. Extraction ratio

As mentioned before, the key operational parameter for efficient P-recovery is the extraction ratio, i.e. the percentage of anaerobic volume extracted for an SBR or the flow diverted from the anaerobic reactor to the P-recovery unit for a continuous system. High anaerobic extraction volumes should be chosen to maximise P-recovery, but too high values may hinder P-storage activity of PAO. The rationale is that the effect of internal poly-P limitations is not observed with single periodic extraction or in the short-term, but after several cycles, high P extraction values would deplete the internal poly-P reserves and PAO would lose the competitive advantage versus other microorganisms such as glycogen accumulating organisms (GAO), which are able to uptake VFA as PHA without requiring poly-P reserves. For this reason, many authors have opted for sporadic extraction of anaerobic liquid. Thus, the reported P-recovery efficiencies for single/short-term experiments can be very high, particularly if an extra dose of carbon is added under anaerobic conditions to boost P-release. In this sense, as observed in Table 4.3, the P-recovery efficiency ranges from 30 to 90% as a function of the SBR operational conditions. For example, Acevedo et al. (2015) reported P-recovery efficiencies around 80% in a single cycle with extraction values of nearly 75% and an external carbon dosage of 350 mg COD/L as acetate. However, it must be carefully considered that even if 100% P-recovery from one out of four cycles was achieved, the average extraction per treated cycle in the plant would only be about 25% of the P input. Moreover, one should realise that these high P extractions are sporadic and could not be sustained in a routine basis due to loss of PAO activity. For example, Lv et al., (2014) operated an EBPR-SBR with an extraction ratio of 0.5 once every 3 cycles. After 83 days of P stripping, P-removal performance decreased, observing a metabolic shift from PAO to GAO and PAO declined to less than 1% of the population. In a subsequent study, Lv and Yuan (2015) extracted half of the anaerobic supernatant every cycle and reached a P-recovery efficiency of 50% in 40 days of operation without additional VFA dosage in the anaerobic stage, but again the internal poly-P levels decreased and the system failed in the next 20 days

On the other hand, if the extraction ratio is properly selected to avoid over-extraction, PAO activity can be maintained for a longer period of time. In this sense, a maximum automated cycle extraction ratio of 10% was suggested to ensure the stability of PAO activity and thus the feasibility of a long-term P-recovery process (Guisasola et al., 2019), which allowed the recovery of 60% of influent P without any harmful effect on PAO.

In a modelling study, Baeza et al., (2017) evaluated the impact of the anaerobic supernatant extraction ratio on the performance of an EBPR-SBR P-recovery system. If the objective was achieving the highest concentration of P in the supernatant to facilitate precipitation, an extraction ratio lower than 5% of the reactor volume (10% of the influent) was recommended, for which a concentration of 61 mg P/L was obtained. Lower ratios did not increase P concentration and resulted in a lower amount of P redirected to the P-recovery system, due to the lower volume. On the other hand, higher ratios led to a decrease of PAO activity and hence lower P concentration, hindering P-precipitation.

Regarding the experimental studies, the range of extraction ratios that led to a successful long-term P-recovery was 8-15% of reactor volume in batch mode and 20 to 35% of flowrate under continuous mode. Moreover, lower extraction ratios lead to higher anaerobic P concentrations, which from a practical point of view is very interesting since precipitation becomes more feasible (i.e. with less chemical dosage requirements). On the other side, higher extraction ratios enable a higher amount of P-recovery but at expenses of higher costs since the anaerobic P concentration is lower. In summary, choosing the optimal extraction ratio requires a balance between a) optimal PAO activity, high anaerobic

P concentration and a low amount of P recovered vs. b) hindering PAO activity, low anaerobic P concentration and a high amount of P recovered. Thus, it is difficult to suggest a general optimal value but, based on the results reported in the literature, we propose 10% as a first initial guess for the extraction ratio when designing batch systems and 25% when designing continuous systems.

4.1.4.3. Influent COD/P ratio and N-removal

Another significant parameter is the influent COD/P ratio. The theoretical studies on mainstream P-recovery indicate that wastewater with a low COD/P ratio could be successfully treated in systems where mainstream P-recovery is implemented due to a much better utilisation of the influent C. The range of influent COD/P ratios in Tables 4.3 and 4.4 is very wide (from 7.5 to 80 gCOD/gP), since few reports aimed at finding the minimum COD requirements for a successful mainstream P-removal/recovery. In general, continuous systems are reported with a higher COD/P ratio. In addition, some of the reports include simultaneous N and P-removal and, hence, a higher amount of COD is needed. Other reports somehow prevent nitrification from occurring either with a low SRT, with allylthiourea (ATU) addition, low DO setpoint or with a very low influent nitrogen load. For example, Valverde-Pérez et al. (2015) fixed a DO setpoint of 1.5 mg/L and P-recovery efficiency improved up to 75% of the influent P because the activity of nitrifiers was being limited at low oxygen concentrations and P uptake was favoured. In any case, the choice of the DO setpoint is not straightforward since too low DO values may also hinder PAO activity.

Guisasola et al. (2019) prevented nitrifying activity using ATU, tested different influent COD/P ratio and showed that the minimum initial COD/P ratio (7.5 gCOD/gP) allowed 60% of the P-recovery without any harmful effect on PAO activity, which is the value that agrees with the first experiments conducted with the BCFS® methodology (Barat and van Loosdrecht, 2006). This value also agrees with the modelling work of Baeza et al. (2017), where a supernatant extraction of 5% of the reactor volume in each SBR cycle led to a stable operation with up to 63% of P recovered. The rest of P removed was incorporated to the purged biomass, where additional P-recovery strategies reported for the sludge line (i.e. as sewage, sewage ash or digestate) could also be applied in view of boosting the global P-recovery efficiency.

The interaction between mainstream P-recovery and nitrogen removal deserves more attention. If P is recovered as struvite, part of the influent ammonium (1:1 molar P:N ratio) would be directly recovered without nitrification/denitrification needed. This would decrease the N load to the plant and, thus, the operation costs (e.g. those related to aeration or biomass handling). To the best of our knowledge, an experimental study that analyses these interactions has not been reported yet. When aiming at simultaneous N and P-removal, a higher COD/P ratio is needed in order to divert some electron donor to BNR. BNR results in a certain entrance of nitrate to the anaerobic reactor and, if COD limitations exist, that would hinder the amount of P to be released and, thus, the amount of P to be recovered. Guerrero et al. (2011) already demonstrated that PAO can outcompete denitrifiers under certain operational conditions. Salehi et al. (2019) showed that P-recovery could be achieved together with BNR with synthetic wastewater of only 200 mg COD/L and a COD/P ratio of 20 gCOD/gP.

4.1.4.4. Concentration of P in the recovered stream

A high P concentration is critical to guarantee a high P-recovery performance: the Phostrip and BCFS® processes report a minimum phosphate concentration in the stripper stream of 25 mg P/L for an efficient P-recovery during the crystallization process (Barat and van Loosdrecht, 2006; Hao and van Loosdrecht, 2006). Other works report a higher minimum P concentration (more than 50 mg/L) for the struvite precipitation (Cordell et al., 2009; Kodera et al., 2013). Most of the mainstream P-recovery processes listed in Tables 4.3 and 4.4report P concentrations higher than 50 mg/L in the recovery stream. Even so, some works report more than 100 mg/L of P in the enriched stream by repeated exposure of the biofilm to external COD source (acetate or propionate). The typical reported anaerobic P concentrations in EBPR systems are in the range of 30 to 70 mg P/L, so they are in the bottom limit and this is why some of these reports use an external carbon dosage under anaerobic conditions to increase anaerobic P-release. In a real environment, external COD dosage under anaerobic conditions boosts P-release at expenses of higher operating cost. Moreover, this addition should be sporadic or, otherwise, the internal poly-P reserves will be depleted and, thus, this operation would not be sustained in a long-term basis.

4.1.4.5. Carbon source needs

Most of the works in Table 4.3 and 4.4 were conducted at lab-scale and used synthetic wastewater to mimic urban wastewater. However, some of them prepared a highly loaded wastewater with an extra C inlet, normally acetic acid or propionic acid (Acevedo et al., 2015; Guisasola et al., 2019; Wong et al., 2018) and there was no need for a sporadic carbon dosage. In the case of the reports with real wastewater, the additional carbon source was only supplemented when the influent COD was relatively low (Zou and Lu, 2016). The only work with external COD amendment and a real environment is presented by Larriba et al. (2020). They treated the effluent from the primary settler of a full sale WWTP by the mainstream SCEPPHAR configuration aiming at P-recovery in a demo scale and studied different strategies (such as nitrite pathway) to improve the efficiency of C usage in view of decreasing the carbon footprint and the operational costs.

This external carbon can also be obtained in a more sustainable way from the own internal plant resources though the fermentation of waste sludge, which is an environmental way to obtain the VFA required to boost anaerobic P-release. In the work of Kodera et al. (2013), an additional fermentation tank was conceptually proposed to obtain enriched VFA by primary sludge fermentation, which was supposed to provide enough COD for anaerobic P-release and subsequent P-recovery. Nowadays, the use of side-stream sludge fermenters to provide COD to fulfil the carbon needs for biological nutrient removal is becoming a trend (Ali et al., 2021; R. Li et al., 2018; Wang et al., 2019). Thus, it could also be used as a source for extra carbon dosage in view of P-recovery.

4.1.4.6. Effect of other operational parameters

Regarding the effect of other operational parameters on the observed performance, the main general recommendation would be to select them to ensure good PAO activity. The same conditions that would maintain PAO activity in a conventional SBR or A_2O continuous system would be recommended in a mainstream P-recovery configuration. For example, (Baeza et al., 2017) reported the effect of SRT in an SBR configuration with P-recovery and found a minimum of SRT = 4 d was required to achieve complete P-removal, but to achieve maximum PHA production, SRT = 10 d was recommended. Therefore, for the specific wastewater composition used and the other particular experimental conditions (e.g. T, DO, pH, volume exchange ratio, operation objective) these results were obtained, but they are case-specific, and hence, for each scenario, they should be determined experimentally or using a proper modelling approach. In fact, mainstream P-recovery is adding an additional limitation to the typical needs that PAO microorganisms also require, such as a minimal T-dependent SRT (Brdjanovic et al., 1998; Chan et al., 2020b) , enough DO and aerobic phase length and typical aerobic and anaerobic HRT (Tchobanoglous et al., 2014).

4.1.4.7. Future outlook

P-recovery from the anaerobic supernatant of the mainstream EBPR process is a novel and promising process, as in addition to the advantages of other P-recovery configurations such as alleviating the problem of undesired P precipitation and recovering a valuable resource, it has additional advantages that help to reduce the COD requirements for P-removal, allowing the treatment of wastewaters with lower COD/P ratio, and maintaining high P-removal activity. However, most of the investigations concentrated on lab-scale SBR and almost all recovery processes were based on P crystallization.

In order to optimize these systems, the extraction ratio is the most basic and important operating parameter to achieve high P-recovery without impairing the long-term stability of the system. Anyway, proper simulation studies should be performed, as the operating parameters are case-specific and highly dependent on the operating objective.

To demonstrate that this is a reliable technology for P-recovery, future works should focus on continuous configurations, which seem more suitable for full-scale application, and using real municipal wastewater. The objective is challenging, as it is necessary to obtain an enriched stream with high P concentration to facilitate P-recovery, but without requiring the addition of an external carbon source that would compromise the economics of the process. Moreover, LCA and cost analysis should be used to demonstrate the overall positive effect of P-recovery systems in WRRFs. In any case, policy decisions will be of paramount importance for the implementation of P-recovery, as it has been shown that the costs are affordable, but like many environmental technologies, it is not cost-effective unless it is driven by policy (Nättorp et al., 2017).

4.1.5. *Conclusions*

This study critically reviews for the first time the performance of the current reported strategies for mainstream P-recovery in EBPR WWTPs and provides the main points to be addressed for its successful implementation. Furthermore, it shows that full-scale mainstream P-recovery is a medium-term possibility.

- It is based on the PAO capability of producing a P-enriched supernatant that enables P-recovery, and SBR and continuous configurations at different scales have been reported.

- It can improve P-removal performance and enable a successful treatment of influents with a relatively low COD/P ratio.

- Configurations combining EBPR with mainstream P-recovery are still at an early stage and their successful full-scale application has not yet been reported.

- The most important parameter to describe these systems is the extraction ratio: the amount of anaerobic supernatant to be extracted or derived to recover P. The choice of this value is a compromise between having high PAO activity and anaerobic P concentration (i.e. easing precipitation) but low amount of P recovered and a potential deleterious effect to PAO activity, low anaerobic P concentration but a high amount of P recovered.

- A maximum value for the P-recovery efficiency of 60% of influent P seems the most sensitive goal for a long-term operation of these systems. However, higher values can be found for sporadic or single extractions.

- Regarding the preferred agent for P-recovery, most of the works opted for struvite, while vivianite or HAP are less common at this point.

4.2. The mainstream P-recovery strategy on A_2O

4.2.1. *Abstract*

This chapter shows the opportunities for mainstream P-recovery in a pilot-scale continuous A_2O system for both anaerobic and aerobic purging. The system exhibited successful P, COD and ammonium removal under both aerobic and anaerobic purge. Anaerobic purge improved P release by 27% but N removal efficiency decreased from 87% to 72%. Decreasing SRT from 20.5±1.1 d (purge of 5L/d) to 16.3±0.6 d (purge of 7L/d) allowed 26% of input P recovered from the anaerobic supernatant with only 4.6 % of influent flow redirection. However, the denitrified N decreased to 27% with 12.4 mg/L of NO_3^--N detected in the effluent. COD mass balance showed that the COD contained in the biomass increased by 6%, which could be beneficial for energy recovery by biomethane production.

4.2.2. *Introduction*

As seen in Chapter 4.1, recovering P from the supernatant of the anaerobic reactor has attracted the attention of researchers due to its high P concentration. Mainstream P recovery reduces a potential of undesired precipitation and decreases the P and N load to the system, thus, the COD requirement and the operational costs. Anaerobic sludge has also a higher PHA concentration than that of aerobic reactor, which will lead to more biomethane production when submitted under anaerobic digestion condition (Chan et al., 2020; Larriba et al., 2020).

The amount of P extraction is the most important parameters for obtaining efficient P recovery, and overload of P extraction may hinder the amount of stored P in the cell and further, lead the system failure. Therefore, evaluating the volume of anaerobic purge is of great importance for high efficiency of P and energy recovery. Apart from that, the implementation of mainstream P-recovery in EBPR has been mostly reported in lab-scale SBR due to the less requirement for configuration and space and the flexibility of operation (Chapter 4.1). This chapter proposes a continuous operation. To our best knowledge, the continuous investigations reported were operated in an A_2O mode with the integration of induced crystallization (Dai et al., 2017; Shi et al., 2012). Moreover, there is a need to study the stability of the A_2O system about the effect of extracting a certain the volume of anaerobic purge continuously on a long-term basis. Thus, this work faces the integration of mainstream P-recovery in a continuous A_2O system. The two main objectives of this research are: 1) the effect of the different purge position and volume on the EBPR performance; 2) the potential of the anaerobic purge for the mainstream P-recovery.

4.2.3. *Materials and Methods*

4.2.3.1. Equipment and operation parameters

The A_2O configuration and the relative operation mode and parameters are explained in Chapter 3.2 (Figure 3.2). The compositions of the concentrated solution are shown in Table 4.5. The micronutrients composition was adapted from Smolders et al. (1994). The biomass for inoculation was obtained from the municipal WWTP of Baix Llobregat (Barcelona, Spain). Waste sludge was discharged from the corresponding reactor automatically with a flow rate selected to maintain the desired SRT.

The SRT was calculated with equation (1):

$$SRT = \frac{V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER}}{Q_{PUR} \cdot X_{AER} + Q_{EFF} \cdot X_{EFF}} \qquad (1)$$

The HRT in the A_2O plant was about 23 h considering only the reactors and 31 h taken into account the settler.

Table 4.5. COD feed compositions and the corresponding percentages

Compositions	Concentrations (g/L)
sodium propionate[a1]	2.8
sucrose[a2]	2.9
acetic acid[a3]	3.1
sodium glutamate[a4]	1.1
dipotassium phosphate [b1]	0.74
potassium phosphate [b2]	0.29
ammonium chloride [c1]	3.06

a1, a2, a3 and a4 provided with 30%, 30%, 30% and 10% of the COD_{inf}, respectively, and the relative concentrations expressed the COD_{inf} with 500 mg/L (76.1 gCOD/d)
b1 and b2 composed the influent concentration of PO_4^{3-}-P about 9 mg/L (1.4 gP/d)
c1 provided the influent concentration of NH_4^+-N about 37 mg/L (5.6 gN/d).

4.2.3.2. Chemical analysis

The liquid samples for the concentration analysis of phosphate, COD, ammonium, nitrate and nitrite were withdrawn from the three reactors almost daily. Sludge samples were withdrawn from the reactors and effluent for the analysis of VSS, TSS and SVI. The detailed process for the liquid and solid analysis was explained in Chapter 3.3.

4.2.3.3. Performance indicators

The load of P, N and COD to the system was 1.4 gP/d, 5.6 gN/d and 76.1 gCOD/d, respectively. In terms of the removal performance), $P_{REMOVAL\_ABSOLUTE}$, $N_{REMOVAL\_ABSOLUTE}$ and $COD_{REMOVAL\_ABSOLUTE}$ were calculated as equations 2, 3 and 4.

$$P_{REMOVAL\_ABSOLUTE} = P_{LOAD} - P_{DIS\_EFF} = P_{LOAD} - P_{EFF} * Q_{EFF} \tag{2}$$

$$N_{REMOVAL\_ABSOLUTE} = N_{LOAD} - N_{DIS\_EFF} = N_{LOAD} - N_{EFF} * Q_{EFF} \tag{3}$$

$$COD_{REMOVAL\_ABSOLUTE} = COD_{LOAD} - COD_{DIS\_EFF} = COD_{LOAD} - COD_{EFF} * Q_{EFF} \tag{4}$$

where P_{DIS_EFF}, N_{DIS_EFF}, COD_{DIS_EFF} are the P, N and COD discharge at the effluent (g/d), and P_{EFF}, N_{EFF}, COD_{EFF} are the concentration of P, total N (sum of NH_4^+-N, NO_3^--N and NO_2^--N) and COD in the effluent.

The fate of N_{LOAD} could be divided into: i) the N in the biomass exiting the system, the N in the liquid and the denitrified N, equations 5-7

$$N_{BIOMASS}\ (\%) = (N_{EFFB} + N_{PURB})\ / N_{LOAD} * 100 \tag{5}$$

$$N_{LIQUID}\ (\%) = (N_{EFF} \cdot Q_{EFF} + \cdot\, N_{PUR} \cdot Q_{PUR}) / N_{LOAD} * 100 \tag{6}$$

$$N_{DENITRIFIED}\ (\%) = \left(100 - N_{BIOMASS}\ (\%) - N_{LIQUID}\ (\%)\right) \tag{7}$$

Where N_{EFFB} (g/d) is the amount of nitrogen in the effluent biomass, and N_{PURB} (g/d) the amount of nitrogen in the purged biomass. The amount of nitrogen in the biomass was estimated using the factor 0.124 gN/gVSS obtained from the general formula of bacteria $C_5H_7NO_2$. N_{PUR} is the concentration of total N (sum of NH_4^+-N, NO_3^--N and NO_2^--N) in the aerobic or anaerobic reactor depending on the purge position.

Similarly, the fate of COD_{LOAD} was COD in the biomass, the COD in the liquid and the mineralized COD, equations 8 to 10:

$$COD_{BIOMASS}\ (\%) = (COD_{EFFB} + COD_{PURB})\ / COD_{LOAD} * 100 \tag{8}$$

$$COD_{LIQUID}\ (\%) = (COD_{EFF} \cdot Q_{EFF} + \cdot\, COD_{PUR} \cdot Q_{PUR}) / N_{LOAD} * 100 \tag{9}$$

$$COD_{MINERALIZED}\ (\%) = \left(100 - COD_{BIOMASS}\ (\%)\right) \tag{10}$$

Where COD_{EFFB} (g/d) is the amount of COD in the effluent biomass, and COD_{PURB} (g/d) is the amount of COD in the purge. The amount of COD in the biomass was calculated from the VSS and the general formula for bacteria: C5H7NO2 (1.416 gCOD/gVSS). COD_{PURB} is the concentration of COD in the aerobic or anaerobic reactor depending on the purge position.

4.2.4. *Results and discussion*

4.2.4.1. A$_2$O system performance

The A$_2$O plant was initially operated with the aerobic purge (Table 4.6). Figures 4.4 and 4.5 show the performance of P, N and COD and the solids evolution in the system under the different operation stages. Tables 4.7 to 4.9 present the performances and mass balance of P, N and COD and the evolution of solids during all the operational periods.

The system achieved a good P removal performance (period I) after 4 days of aerobic purge from 0 to 5 L/d (Figure 4.4a and Table 4.7). The P removal absolute reached to 1.38±0.01 g/d and the concentration of P in the effluent was about 0.1±0.1 mg/L. Full nitrification and almost full COD removal were obtained (Figure 4.4b, 4.4c, Table 4.8 and 4.9). The VSS in the reactor was stable at 2.37±0.10 g/L and SVI was 332±94 mL/g showing good settleability (Figure 4.5a, c and Table 4.10) with the average SRT of 20.6±1.0 days.

Table 4.6. The purge position and purge flow for each operational period.

Period	Day operation	Purge position	Purge flow （L/d）
Start-up	1-4	Aerobic	0 to 5
I	5-20	Aerobic	5
II	21-41	Anaerobic	5
III	42-54	Anaerobic	7

The purge position was moved from the aerobic to the anaerobic reactor on day 21 (period II), and the system was maintained at an SRT of 20.5±1.1 days. The P concentration in the effluent increased to 1.7 mg/L on the following day. However, full P removal was obtained on day 23 (0.1 mg P/L in the effluent), and the P in the anaerobic reactor increased from about 44 mg/L to almost 56 mg/L (27% increase), indicating that PAO activity was enhanced. Full ammonium and COD removal were observed. The average concentration of NO_3^--N in the effluent increased from around 4.8 to 10.4 mg/L with the relative N in the liquid increasing from 0.72±0.19 to 1.55±0.32 g/d, thus, the N removal efficiency decreased from 87% to 72%. No nitrite was detected during the whole operational process. The concentrations of VSS in the reactor and in the effluent were about 2.1 and 0.03 g/L

(Table 4.10 and Figure 4.5b, and SVI was 221±12 mL/g (Figure 4.5d). The successful nutrient and COD removal performance implied that changing the purge from aerobic to anaerobic phase improved PAO activity without affecting the system stability.

Further, the purge volume was increased to 7L/d on day 42 (period III) for obtaining more potential mainstream P-recovery. The SRT decreased from 20.5 to 16.3 d. Successful P removal was maintained with only 0.2 mg/L P in the effluent and P absolute kept similar (1.37 g/d) as those in period I and II (1.38 and 1.36 g/d). Again, full nitrification and COD removal efficiency were observed. The N removal efficiency decreased further to 67% and the concentration of NO_3^--N in the effluent reached to 12.4±2.4 mg/L. However, the sludge in the system kept a similar state as in period II, and SVI was 209±25 mL/g. The ratio of VSS/TSS was about 0.79 (Figure 4.5c), which indicated a good sludge state for PAO.

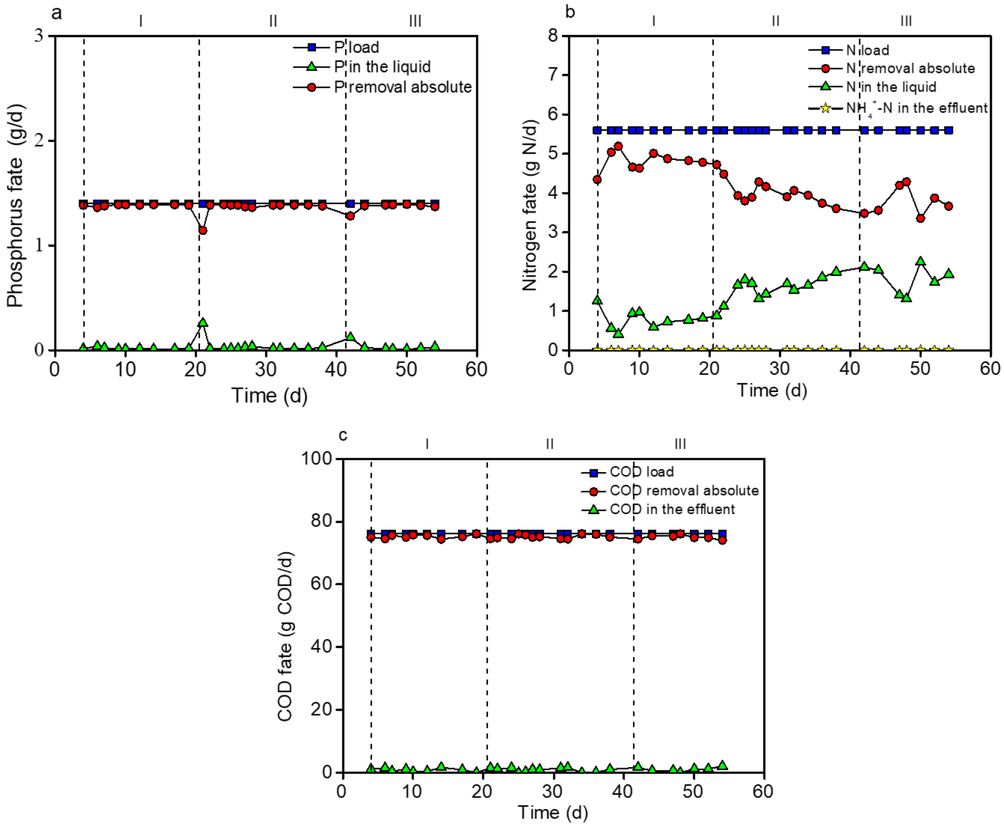

Figure 4.4. The system performance of phosphorus (a), nitrogen (b) and COD (c) in different operation stages.

Table 4.7. EBPR performance for each experimental period.

Period	P_{ana} (mgP/L)	P_{anox} (mgP/L)	P_{aer} (mgP/L)	P in anaerobic reactor for P recovery (g/d)	P removal absolute (g/d)	P in the liquid (g/d)
I	43.9±10.5	14.0±9.9	0.1±0.1	0.22±0.05	1.38±0.01	0.02±0.01
II	55.9±14.5	11.8±4.4	0.3±0.2	0.28±0.07	1.36±0.04	0.04±0.03
III	51.4±4.8	9.9±1.5	0.2±0.3	0.36±0.03	1.37±0.04	0.03±0.04

Table 4.8. N removal performance and mass balance of each experimental period.

Period	NH_4^+-N_{ana} (mg/L)	NO_3^--N_{eff} (mg/L)	NH_4^+-N removal efficiency (%)	N removal efficiency (%)	N removal absolute (g/d)	N in the liquid (g/d)	N in biomass (%)	N in liquid (%)	Denitrified N (%)
I	17.0±2.0	4.8±1.3	100±0	87±3	4.88±0.19	0.72±0.19	37±1	13±3	50±4
II	17.3±2.4	10.4±2.2	100±0	72±6	4.05±0.32	1.55±0.32	33±1	28±6	40±5
III	18.0±2.5	12.4±2.4	100±0	67±6	3.78±0.36	1.82±0.36	41±1	33±6	27±7

Table 4.9. COD removal performance and mass balance for each experimental period.

Period	COD load (g/d)	COD removal absolute (g/d)	COD in the liquid (g/d)	COD in the biomass (%)	COD in the liquid (%)	Mineralized COD (%)
I	76.1±0	75.3±0.6	0.8±0.6	31±1	1±1	68±2
II	76.1±0	75.2±0.7	0.9±0.6	28±1	1±1	71±2
III	76.1±0	75.0±0.7	1.1±0.7	34±1	1±1	64±1

Table 4.10. Average solids concentrations, VSS/TSS and settleability in the system for each experimental period.

Period	SRT (d)	VSS_{purge} (g/L)	$VSS_{effluent}$ (g/L)	TSS_{purge} (g/L)	$TSS_{effluent}$ (g/L)	VSS/TSS	SVI (mL/g VSS)
I	20.6±1.0	2.37±0.10	0.034±0.005	3.02±0.13	0.044±0.005	0.792±0.016	332±94
II	20.5±1.1	2.07±0.06	0.031±0.004	2.64±0.12	0.039±0.006	0.783±0.013	221±12
III	16.3±0.6	2.04±0.05	0.029±0.004	2.57±0.06	0.035±0.006	0.793±0.008	209±25

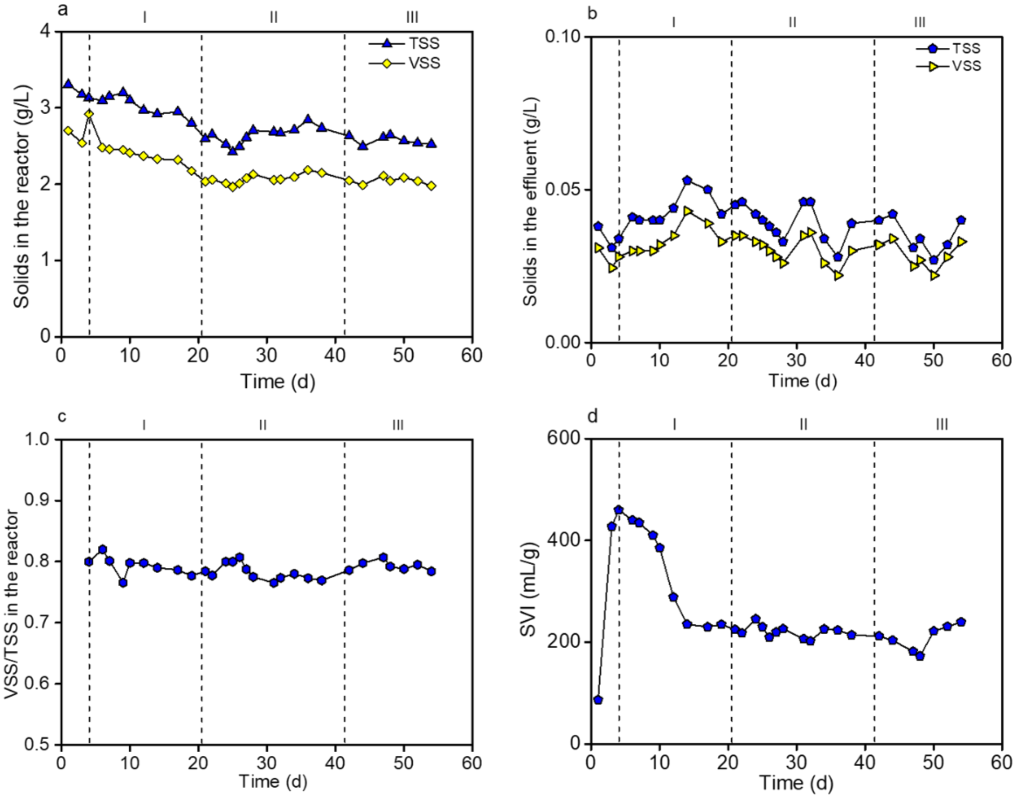

Figure 4.5. Solids concentration in the reactor (a), effluent (b), the ratio of VSS/TSS in the reactor (c) and SVI (d) of the solids in different operation stages.

4.2.4.2. The potential for P recovery for anaerobic purge condition

The potential for P recovery under anaerobic purge condition in this work can be calculated based on P_{ANA} during period II (55.9±14.5 mgP/L) with the purge flowrate (5 L/d) and period III (51.4±4.8 mgP/L) with the purge flowrate (7 L/d). Then, about 0.28 and 0.36 g/d of the P can be recovered from the anaerobic supernatant by mainstream P-recovery, respectively (Table 4.7). Considering a constant 1.4 g/d of P load to the system, about 20 % and 26% of the input P could be recovered with the 5 L/d and 7L/d of anaerobic purge, respectively. The increase of the anaerobic purge from 5 to 7 L/d improved 6% of P recovery without any detrimental side-effect to the plant operation. The anaerobic P concentration (i.e. P concentration in the recovery liquid) was almost 6 times compared to that in the input (9 mg/L), which can trigger off the possibility of P-recovery as struvite or vivianite can be favoured after a separation step for the biomass. The concentration of NH_4^+-N in the anaerobic supernatant was about 18 mg/L (Table 4.8), which is not high enough for struvite formation ($MgNH_4PO_4·6H_2O$) considering for the theoretical stoichiometric ratio of P and N with 1:1. In any case, the concentration of the other counterions has to be considered for a comprehensive evaluation of this P-recovery step: Mg and ammonium in the case of struvite and ferrous iron in the case of vivianite.

A model-based A_2O work was proposed by Valverde-Pérez et al., (2015) for P removal and recovery in the frame of the microalgae cultivation and nitrification was inhibited at low DO. P was recovered from the anaerobic stream for algae cultivation. The potential P-recovery was obtained with the maximum of 70% of influent P under the condition that 30% of the influent flow was diverted as a P-stream with SRT = 5 d.

Our previous work from Larriba et al., (2020) reported that an average of 45% the influent P can be recovered from anaerobic supernatant (redirecting about 9 % of the influent flow) in the form of struvite with a demo-scale pilot plant operated in two-sludge SBR mode in the long-term operation, and the SRT was 10-15 d. However, in this work 26% of P could be recovered by redirecting 4.6 % of the influent flow with 7L/d of purge (SRT about 16 d). The lower extraction volume could explain the lower P recovery efficiency. A higher anaerobic purge volume is necessary to attempt for obtaining a high efficiency of P recovery in this system. In Chapter 4.1, we suggested the possible maximum value for mainstream-P recovery could be 60% of the influent P in the long run, and a higher value may lead to the deterioration of the system.

*4.2.4.3. M*ass balances of nitrogen and carbon

Table 4.8 shows the N mass balances from period I to III. The input N fate could be: 1) effluent, as dissolved N or biomass, 2) purge, as dissolved N or biomass and 3) N mineralization. The percentage of N in the biomass showed no big difference when changing the purge from aerobic reactor (37±1%) to anaerobic reactor (33±1%). However, the percentage in the liquid increased from 13±3% to 28±6%. The N in the liquid comprises the amount of N in the effluent and in the purge. For the fraction of the purge, the output N was mostly the amount of ammonium (17.3 mgN/L) in the anaerobic purge whereas nitrite (4.8mgN/L) in the aerobic purge (Table 4.8). However, it only contributed to an insignificant percentage with less than 0.1% increase due to only 5L/d of purge volume. As a result, the main reason for the increase of N in the liquid was due to the increase of N (nitrate) in the effluent from 4.8 to 10.4 mg/L, which led to the percentage of N in the liquid increasing by 14.6% considering the effluent flow rate of 146 L/d, and further reason for this high residual of nitrate needs to be explored. Increasing the anaerobic purge from 5 to 7 L/d resulted in a much higher fraction of N in the liquid (33±6%). The N contained in the biomass increased to 41±1% due to the growth and it was reasonable since more purge was discharged. As a result, only 27±7% of input N was denitrified**.**

The COD fate is shown in Table 4.9 and the potential COD outlets are: 1) effluent, as dissolved COD or biomass, 2) purge, as dissolved COD or biomass and 3) COD mineralization. The COD in the liquid was kept the same among the three periods (around 1%). Mineralized COD increased from 68±2% to 71±2% with purge position changing from aerobic reactor to anaerobic reactor. However, it decreased to 64±2% with the anaerobic purge increasing to 7L/d. Decreasing the SRT results in less COD mineralized. The corresponding COD stored in biomass increased from 28±1% to 34±1% due to the increase of purge (i.e. a lower SRT and higher biomass growth), which can increase the potential biomethane production. Our A-stage-EBPR work showed that decreasing SRT from 6 to 4 d leading to the increase of COD stored in biomass from 36% to 58% (Chapter 5.1), resulting in 28% enhancement of biomethane production from the anaerobic sludge (Zhang et al., 2021).

More work is required for designing an efficient mainstream P recovery from the continuous configurations, which means maximizing the anaerobic purge to obtain a higher fraction of input P from the supernatant without compromising the system performance in the long term. In addition, the effect of different purge strategies on the relative microbial communities is needed for a more comprehensive understanding on this novel strategy.

4.2.5. *Conclusions*

This work explored the effect of different purging strategies in a continuous A_2O system on EBPR performance. The integration of mainstream P-recovery was investigated with the aim of P recovery due to the high P concentration in the anaerobic reactor. The main conclusions are:

Successful P and COD removal can be obtained independently of the aerobic or anaerobic purge under the SRT around 21 days. Full COD and ammonium removal could also be reached under these conditions.

Purging from the anaerobic reactor improved PAO activity: anaerobic P concentration increased by almost 27%. However, the total N removal efficiency decreased by 15% due to the increase of nitrate in the effluent.

26% of the input P can be extracted within the anaerobic supernatant with an anaerobic purge and SRT of 20.5±1.1 d compared with 20% under the SRT of 16.3±0.6 d.

N and COD balance showed that N contained in the biomass increased from 33±1% to 41±1% with the anaerobic purge increasing from 5 to 7 L/d, and COD stored in biomass increased by 6% which could favour biomethane production for energy recovery.

4.3. The effect of different carbon sources on EBPR performance

4.3.1. *Abstract*

Enhanced biological phosphorus removal (EBPR), as one of the most sustainable and economical technology for efficient P removal from wastewater, is widely applied in full-scale wastewater treatment plants (WWTPs). The types of carbon sources exert the key effects on the performance of EBPR, which leads to the diverse dominant microbial communities in the process. Polyphosphate accumulating organisms (PAO) contribute to the P removal. However, the dominance of glycogen accumulating organisms (GAO) under some carbon source conditions may outcompete PAO, which will deteriorate the stability of EBPR and even lead to system failure. One key aspect in the choice of the carbon source is the potential to alter the ratio of the groups of PAO/GAO, thus the EBPR performance. The advanced investigations show more versatile metabolic ways of diverse putative PAOs (e.g. *Accumulibacter*, *Tetrasphaera*, *Dechloromonas*, *Thiothrix*) with various carbon source strategies, which could benefit for the full-scale WWTPs to increase the resistance to unstable environment. This review carefully reevaluates the application of different carbon sources (sole, multiple or complex carbon sources) in the field of EBPR in recent years, and especially emphasizes on the fermentation productions from wastewater and waste solids as additional carbon source by different strategies. The applications of waste fermentation as additional carbon source not only show successful system performance, but also avoid the need of commercial carbon source input and relieve the waste disposition issue, which could be a promising development trend faced with the insufficient COD of raw wastewater as well as the environmental pressure problem.

4.3.2. *Introduction*

The overload of phosphorus (P) contained in the wastewater could lead to eutrophication, which is harmful to the aquatic environment (Hanhoun et al., 2011). Enhanced biological phosphorus removal (EBPR) has been proposed as one of the most efficient and sustainable process for treating wastewater containing P in full-scale wastewater treatment plants (WWTPs). During the EBPR process, polyphosphate accumulating organisms (PAO) are considered as the functional bacteria for P and COD removal. The most reported lineages of PAOs in full-scale EBPR systems include the β-*proteobacterial Candidatus* Accumulibacter (generally referred as *Accumulibacter*), *Tetrasphaera*, *Dechloromonas*, *Thiothrix* and *Comamonadaceae* (Ge et al., 2015; Kong et al., 2005a; Rey-Martínez et al., 2019; Rubio-Rincón et al., 2017; Seviour et al., 2003).

PAO can uptake organic matter (mainly volatile fatty acids, VFA) and store them as polyhydroxyalkanoate (PHA) under anaerobic conditions. The energy required is supported by the hydrolysis of glycogen and intracellular polyphosphate (poly-P). Under anoxic/aerobic conditions, PHA is oxidized and energy is obtained for the replenishment of glycogen and polyphosphate as well as for biomass growth. Then, provided P uptake is higher than P release, net P accumulation is observed and P is removed from the WWTP and most of it leaves the plant as part of the waste sludge.

Anaerobic substrate uptake is an energy-intensive process and not all organic compounds are suitable to be degraded under these conditions. Short organic compounds often require less energy to be transported through the membrane. Thus, organic substrates play an essential role on EBPR performance during PAO metabolism (Shen and Zhou, 2016). Most of the lab-scale experiments reported were conducted with VFA (e.g. acetic and propionic acids) and this has led to *Accumulibacter*-enriched sludge (Nielsen et al., 2019; Singleton et al., 2022). However, the recent microbiological advances on EBPR have identified some other PAO-relative bacteria at full-scale plants that show more versatile metabolic ways under a wider range of potential carbon sources (Close et al., 2021; Fan et al., 2022; F. A. Herbst et al., 2019; Petriglieri et al., 2021; Tian et al., 2022). For example, *Thiothrix* or *Tetrasphaera* showed the classical P-release/P-uptake phenotype of PAO with carbon sources other than VFA and without PHA synthesis (Close et al., 2021; Nguyen et al., 2011; Rey-Martínez et al., 2019; Tian et al., 2022).

The nature of the carbon source is not only relevant for promoting EBPR or for selecting a certain type of PAO but it is also a key agent in the competition between PAO and their competitors: glycogen-accumulating organisms (GAO) (e.g. *Candidatus* Competibacter, *Propionivibrio*, *Defluviicoccus*). A successful EBPR performance is characterised by a PAO-enriched sludge with an efficient carbon usage since GAO outcompeting PAO would lead EBPR failure. This competition is heavily dependent on the operational conditions and on the quantity/biodegradabilty of the carbon source (Nittami et al., 2009; Oehmen et al., 2007; Roy et al., 2021; Wong et al., 2004). GAO proliferation was observed with overload of VFA (Shen and Zhou, 2016) and some certain carbon sources (e.g. glucose, starch and methanol) (Randall et al., 1997; Rollemberg et al., 2019; Tayà et al., 2013b; Wei et al., 2014; Yazıcı and Kılıç, 2016). On the contrary, PAO-enrichment has been promoted by propionate (Oehmen et al., 2005; S. Wang et al., 2020), butyrate (Begum and Batista, 2014; Cai et al., 2019), glucose (Nguyen et al., 2011), amino acids (Nguyen et al., 2015; Qiu et al., 2020) or mixture carbon sources (Wang et al., 2021; Xie et al., 2017; Yang et al., 2018).

The carbon source can also affect the proliferation of denitrifying PAOs (DPAO), which are able to simultaneously remove nitrogen (N) and P by using nitrate/nitrite as electron acceptors for PHA oxidation (Guisasola et al., 2009; Kerrn-Jespersen and Henze, 1993; Kuba et al., 1996). DPAO are reported to be favoured by fermentation products of solid wastes (Ji and Chen, 2010; R. Xu et al., 2016; Yuan et al., 2016).

Experimental reports on the utilization of different carbon sources for EPBR have to be taken with care for two reasons. On the one hand, EPBR-based experiments are never conducted with pure cultures and, hence, when using complex sources other than VFA, the possibility of flaking species fermenting the complex substrates into VFA and PAO living off these fermentation products has to be considered. Predicting the type of fermentative bacteria and the fermentation products is not a straightforward issue and, it is difficult to estimate EBPR performance under different complex substrates. This issue is particularly significant when dealing with low COD/N/P wastewaters. A simple and sustainable solution to overcome potential COD limitations is an external addition of an organic waste to provide the required electron donors (note that adding a commercial organic compound is also possible but less efficient from an economic and sustainable point of view). In this context, the usage of fermented products (with abundant COD) of waste sludge as carbon source is a sustainable solution for EBPR and N removal. The solid waste is normally pretreated under chemical (alkaline, acid), thermal conditions for fermentation (Liu et al., 2020; Luo et al., 2019; L. Zhang et al., 2018), and it can be fermented by novel configurations implemented (i.e. side-stream sludge fermenter) or *Tetrasphaera* without pretreatment (Arabi and Lynne, 2019; Fan et al., 2021; Onnis-Hayden et al., 2020).

On the other hand, some of the reports on the utilization of carbon sources are based on batch tests with bio-P biomass fed for a long time with common substrates. An efficient/unsuccessful utilization of a certain substrate using batch or first-time experiments may result in the opposite results as the case of a step-wise replacement of the primary carbon source for the targeted substrate. The history of the biomass is very relevant when analyzing these experiments when the carbon source is used for the first time.

This work reviews the opportunities of different carbon source (sole, multiple or the fermentation products from wastewater and waste solids) on EBPR performance (Figure 4.6), the dominant microbial communities and the metabolic pathways of PAO and GAO. With the most studied VFA, i.e. acetic and propionic acid, more comprehensive and advanced information and perspectives are analysed under different conditions, and it also provides analytical orientation for others carbon sources. The application of others carbon sources are systematically evaluated, and specifically the solid waste as carbon source.

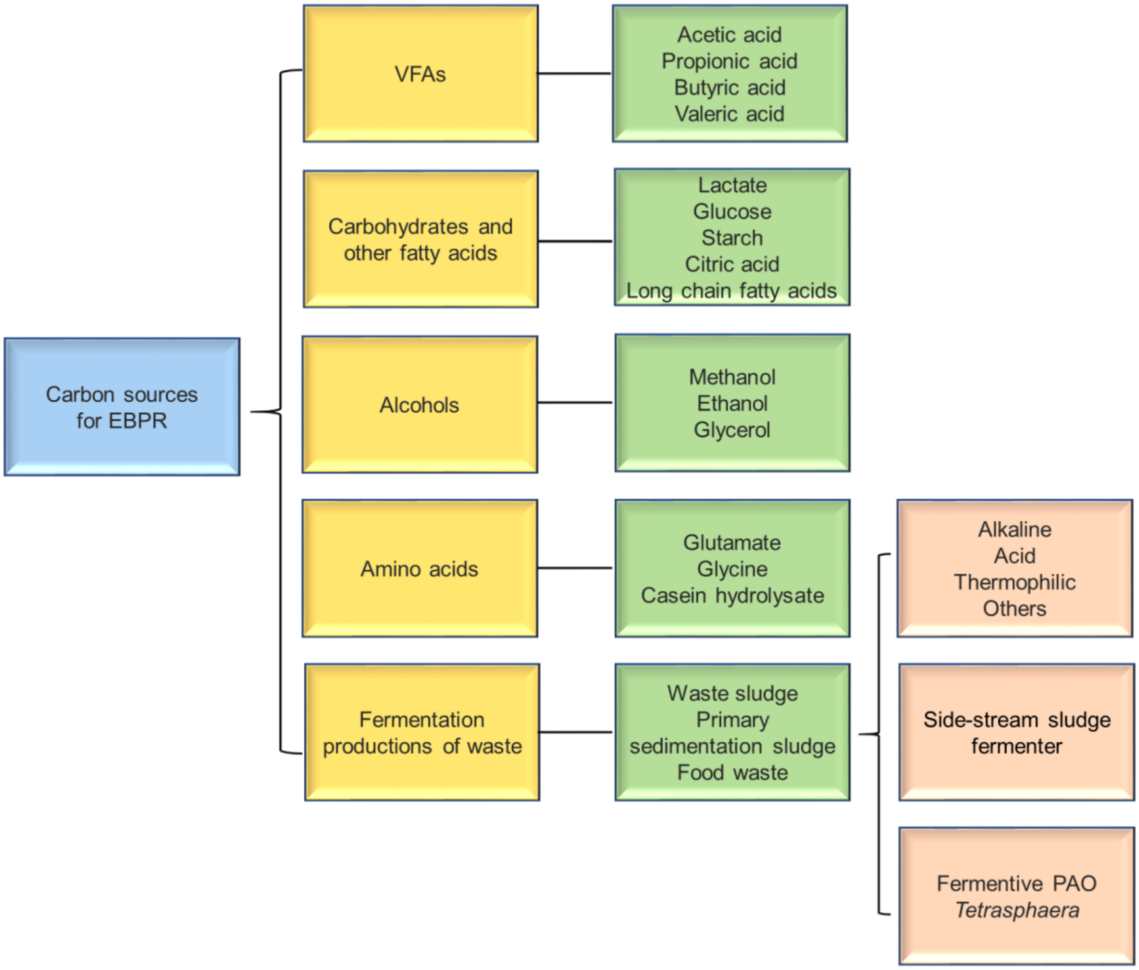

Figure 4.6. Carbon sources utilized by EBPR.

4.3.3. *VFA as carbon source for EBPR*

4.3.3.1. Acetate and propionate

Acetate and propionate are the two most common substrates present in real domestic wastewater and the most prevailing carbon sources in EBPR process, and they usually account to 60-80% of the total VFA (S. Wang et al., 2020). Lots of research has been conducted since the end of the 20th century to understand the EBPR performance of acetate- and propionate-fed systems and its effect on the metabolic pathways of PAO and GAO and their relative abundance, which are shown in Table 4.11 and 4.12. Generally, both of them can induce stable EBPR performance either by individual or mixed application (Oehmen et al., 2004; M. Pijuan et al., 2004; S. Wang et al., 2020; Zhang et al., 2020), or alternation of acetate and propionate strategies (Lu et al., 2006; Wang et al., 2010).

The different metabolic pathways of PAO and GAO under the two different carbon sources have been described by metabolic models and validated with experimental results (Acevedo et al., 2012; Oehmen et al., 2005b; M. Pijuan et al., 2004; Qiu et al., 2022; Smolders et al., 1994; Zeng et al., 2003) (Table 4.11). The P/C ratio (i.e. the molar ratio of P release to C-mol of VFA uptake) is an indicator of the PAO activity. Under acetate as carbon source, the theoretical P/C ratio was determined as 0.5 (Smolders et al., 1994). As seen in Table 4.11, there is a huge variety of reported ranges for PAO-enriched cultures using acetate as sole carbon source: 0.08-0.8 mmolP/ mmolC. Lower values than the theoretical one can be explained by the presence of other anaerobic carbon scavengers as for example GAO (Acevedo et al., 2012) whereas values higher than 0.5 are more difficult to understand from a theoretical point of view. There are also other ratios that can be used to investigate the enrichment of PAO/GAO in EBPR sludge such as the PHA/VFA ratio (PHA formation to VFA uptake), the Gly/PHA ratio (glycogen formation to PHA degradation) or the Gly/PHA ratio (glycogen formation to PHA degradation) as shown in Table 4.11. An increasing usage of glycogen can be a good indicator of predominance of the GAO metabolism (Acevedo et al., 2015, 2012; Zhou et al., 2008).

The PHA quantity and distribution depend on the carbon source used. Poly- β-hydroxybutyrate (PHB) is the dominant PHA proportion (60%-100%) in acetate-fed systems whereas poly- β-hydroxyvalerate (PHV) (35-85%) and poly- β–hydroxy-2-methylvalerate (PH2MV) (40%-60%) and are the most promoted when propionate is used (Nittami et al., 2017; Oehmen et al., 2005a; Pijuan et al., 2009; Shen and Zhou, 2016; Smolders et al., 1994).

Propionate is suggested to be a more preferable carbon source than acetate in view of PAO enrichment over GAO (Oehmen et al., 2005a, 2005b; Carvalheira et al., 2014b; Maite Pijuan et al., 2004): the P/C is lower than that of acetate (experimental and theoretical ratios) (Table 4.11) and, thus, less energy is required by PAO to uptake propionate (Pijuan et al., 2004; Seviour and Nielsen, 2010). It is worth to mention that some studies have stated that acetate showed better EBPR performance, specifically for the granule-sludge EBPR systems. Cai et al., (2016) reported larger and more stable granules and more bioavailable P content in acetate-fed granules rather than those with propionate. S. Wang et al., (2020) found that successful P and COD removal efficiency could be maintained in granular SBR with both VFA but PAO were favoured against GAO with acetate, whereas a mixed PAO/GAO culture was found when using propionate.

The role of the carbon sources when selecting PAO/GAO can be affected by temperature. At high temperatures (＞25 °C), GAO can be promoted independently of the carbon source, which poses a great threaten to EBPR (Lopez-Vazquez et al., 2009; López-Vázquez et al., 2008; Panswad et al., 2003; Whang and Park, 2006). However, recent studies unveiled that successful lab/full-scale EBPR performance can be maintained with the enrichment of PAO independently of the VFA used under temperatures higher than 25ºC (Law et al., 2016; Nielsen et al., 2019; Qiu et al., 2022, 2019), and some studies even mention that acetate could be a preferable option than propionate for PAO enrichment (Shen et al., 2017; Wang et al., 2020). Another key aspect in the PAO/GAO competition based on acetate and propionate as carbon sources is the concentration. Lower VFA concentrations may favour PAO over GAO and, thus, working in continuous stirred tank reactors could be a strategy to select PAO and/or to recover PAO activity (Ong et al., 2013; Tu and Schuler, 2013). Carvalheira et al., (2014c) hypothesised on PAO having a lower decay rate than GAO to describe this phenomenon since PAO preferably use PHA rather than glycogen for maintenance. A moderate VFA load assures the coexistence of PAO and GAO and thus, a successful EBPR performance. In fact, the presence of GAO has been appointed as an indicator of the excess of COD load for EBPR (Nielsen et al., 2019; Qiu et al., 2022). Finally, an excessive COD load, that is, a potential COD transfer to the aerobic phase has been reported to enhance the proliferation of filamentous bacteria which may hinder the sludge settleability, thus, EBPR performance (Haaksman et al., 2020; Li et al., 2016; Yu et al., 2014). Li et al., (2016) found that P removal efficiency decreased from 97% to 50% when doubling the COD (propionate) load due to settling issues (49% of *Thiothrix* detected and *Rhodocyclaeae* decreasing from 38% to less than 3%). EBPR performance, *Accumulibacter* abundance (＞30%) and decay of *Thiothrix* were observed when the normal load was restored. Similarly, Haaksman et al., (2020) reported that over dosage of acetate led to the loss of P removal and deterioration of sludge settleability in EBPR-granules system, and a relative lower dosage of acetate ratio (about 4mg COD/gVSS/h) was recommend by them for full P removal and good sludge shape. Qiu et al., (2022) pointed out that PAO could outcompete GAO under high temperatures (30 and 35 ℃) with a low COD load of a mixture of acetate and propionate.

Table 4.11. Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with VFA as carbon source

References	Carbon source (with the ratio based on COD quantity)	Anaerobic				Aerobic		
		VFA uptake (mmol C/g VSS/h)	P/C (mol/mol)	PHA/C (mol C/mol C)	Gly/VFA (mol C/mol C)	P uptake rate (mmol/g VSS h)	P/PHA (mol/mol C)	Gly/PHA (mol C/mol C)
Shen and Zhou, (2016)	Acetate	-	0.45-0.73	0.62-1.48	0.08–0.50	0.23–0.48	-	-
Smolders et al., (1994)	Acetate model	7.5	0.50	1.33	0.5	-	-	-
Acevedo et al., (2012)	Acetate [a]	-	0.08-0.8	0.8-2.0	0.3-1.1	-	0.4-0.7	0.4-0.6
(Acevedo et al., 2012)	Acetate [b]	-	0-0.02	1.5-2.0	1.0-1.3	-	-	0.65
Shen and Zhou, (2016)	Propionate	-	0.23-0.44	0.52-1.39	0.08-0.50	0.41-0.72	-	-
Oehmen et al., (2005b)	Propionate model	-	0.42	1.22	0.33	-	-	-
S. Wang et al., (2020) [T1]	Acetate	-	0.60	PHA/C 0.41 PHB/C 0.35 PHV/C 0.06	0.41	-	-	-
Shen et al., (2017) [T2]	Acetate	4.96	0.82	PHA/C 1.15 PHB/C 0.96 PHV/C 0.19	0.41	0.71	0.98	0.28
	Propionate	4.74	0.65	PHA/C 1.19 PHB/C 0.05 PHV/C 0.71 PH2MV/C 0.43	0.30	0.51	0.84	0.26
Qiu et al., (2019) [T2]	Acetate [c]	-	0.35-0.66	0.63-0.78	0.58-0.64	-	0.57-1.21	0.17-0.26
	Propionate [c]	-	0.38-0.60	0.56-0.61	0.48-0.55	-	0.31-1.06	0.35-0.52

Nittami et al., (2017)	Acetate	-	-	PHB (88-94%) [e] PHV (10-11%)	-	-	-	-
	Propionate	-	-	PH2MV (30-58%) [e] PHV (36-63%)	-	-	-	-
L. Wang et al., (2020) [T2]	Acetate [a]	4.92	0.51-0.71	PHA/C 1.54 PHB/C 1.33 PHV/C 0.21	-	0.67-0.89	0.76-0.90	0.22-0.37
	Propionate [a]	4.50	0.45-0.65	PHA/C 1.34 PHV/C 0.46 PH2MV/C 0.88	-	0.41-0.60	0.63-0.80	0.30-0.52
	Acetate or propionate [d]	6.13-9.17	0.47-0.57	PHA/C 1.21-1.57 PHB/C 0-1.21 PHV/C 0.21-0.54 PH2MV/C 0-0.88	0.26-0.42	0.36-0.65	-	0.27-0.98
	butyrate (or ISO butyrate) [d]	0.5-0.85	0.33-0.44	PHA/C 0.74-0.88 PHB/C 0.57-0.76 PHV/C 0.06-0.2 PH2MV/C 0.04-0.06	0.23-0.40	0.3-0.58	-	0-0.53
	Valerate [d]	0.33-0.36	0.39-0.42	PHA/C 1.35-1.47 PHB/C 0.04-0.11 PHV/C 1.08 PH2MV/C 0.16-0.34	0.33-0.74	0.31-0.36	-	0.26-0.44
	Mixture of four VFA [d]	5.84-9.62	0.37-0.63	PHA/C 0.88-1.42 PHB/C 0.34-0.67 PHV/C 0.35-0.76 PH2MV/C 0.05-0.15	0.16-0.36	0.37-0.47	-	0.18-0.29
Pijuan et al., (2009)	Butyrate [d]	-	0.17-0.22	0.46-0.92 [f]	0.43-0.49	-	-	-
Begum and (Batista, 2014)	Butyrate	-	0.20-0.80	-	-	-	-	-
Wang et al., (2021) [T2]	Acetate [a]	-	0.58	PHA/C 1.05 PHB/C 0.83 PHV/C 0.22	0.28	2.58	-	0.33

	Acetate: butyrate 1:1[a]	-	0.74	PHA/C 1.04 PHB/C 0.73 PHV/C 0.13 PH2MV/C 0.08 PHH/C 0.12[f]	0.13	1.12	-	0.17
	Butyrate[a]	-	0.59	PHA/C 0.69 PHB/C 0.52 PHH/C 0.17[f]	0.15	0.80	0.76	0.24
	Acetate[b]	-	1.55	PHAC 2.45 PHB/C 1.55 PHV/C 0.90	1.23	-	-	0.55
	Acetate: butyrate 1:1[b]	-	0.81	PHA/C 1.98 PHB/C 0.81 PHV/C 0.58 PHH/C 0.59[f]	0.72	-	-	0.34
	Butyrate[b]	-	0.24	PHA/C 0.99 PHB/C 0.24 PHV/C 0.40 PHH/C 0.35	0.48	-	-	0.54

a. with enriched-PAO
b. with enriched-GAO
c. diverse PAO in full scale
d. sporadic dosage to enriched-PAO
e. the relative terms are expressed with percentage
f. when butyrate was used as substrate, a novel PHA monomer was synthesised, and (Pijuan et al., 2009) indicated it was an unknown composition with quantified PHB+PHV=95%.
T1 low to 10 ℃
T2 high to 30 or 35 ℃

Table 4.12. Summary of microbial communities under different carbon sources.

Type of carbon source	References	Carbon source	Microbial communities
VFA	S. Wang et al., (2020)	Acetate	*Rhodocyclaceae* 32%, genus *Dechloromonas* 1%
		Propionate	R*hodocyclaceae* 72%, genus *Dechloromonas* 61%
	Shen et al., (2017); Wang et al., (2020)	Acetate [a]	Favours *Accumulibacter* IIC, *Accumulibacter* clade IIF
	L. Wang et al., (2020)	Acetate [a]	*Accumulibacter* (64%), *Defluviicoccus* (6%), *Competibacter* (1%)
		Propionate [a]	*Accumulibacter* (52%), *Defluviicoccus* (8%), no *Competibacter*
	Li et al., (2016)	Acetate [b]	*Thiothrix* (49%), *Rhodocyclaeae* (3%)
		Acetate [c]	*Accumulibacter* (> 30%), *Thiothrix* (17%)
	Zhang et al., (2020)	Acetate and propionate[d]	Favours *Accumulibacter, Acinetobacter, Dechloromonas* and *Pseudomonas*, less of *Competibacter* and *Defluviicoccus*
	Begum and Batista, (2014)	Butyrate	*Accumulibacter* (50%), *Defluviicoccus* (16%), *Competibacter* (2%)
	Wang et al., (2021)	Acetate change to butyrate [e]	*Accumulibacter* decreased from 37% to 14%, and *Rhodocyclaceae* increased from 2% to 15%
		Acetate change to butyrate [f]	*Competibacter* reduced from 27% to 6%, *Zoogloea* increased from 0.2% to 38%
	Cai et al., (2019)	Butyrate: glucose 1:1	*Rhodocyclus*- related bacteria (17.5%), *Actinobacteria* (1.4%)
		Valerate: glucose 1:1	*Rhodocyclus*- related bacteria (12.6%), *Actinobacteria* (0.9%)
Fermentable carbon sources	Rubio-Rincón et al., (2019)	Lactate	*Accumulibacter* and *Tetrasphaera*
	Jeon and PARK, (2000)	Glucose	Favours lactic acid producing organism and PAO
	Oehmen et al., (2005a); Wang et al., (2010); Zengin et al., (2010)	Glucose	Favours *Competibacter*
	Dockx et al., (2021)	Glucose	Favours GAO-*Saccharimonadaceae* and other GAOs, but no *Competibacter*
	He et al., (2018)	Glucose	Decreased PAO, favours *Nitrospira*

	Xie et al., (2017)	Glucose: acetate 1:1	Favours *Tetrasphaera* and *Microlunatus phosphovorus*
	Nguyen et al., (2011)	Glucose	Favours *Tetrasphaera*
	Li et al., (2019)	Starch	Favours filamentous bacteria *Thiothrix* (4%)
	Luo et al., (2018)	Starch	Favours lactic acid producing organism and PAO
	Li et al., (2019); S. Li et al., (2020)	Long chain fatty acids	Favour filamentous bacteria *Microthrix parvicella*
Alcohols	Tayà et al., (2013)	Methanol [g]	PAO MIX (11%), GAO MIX (5%), DFI and II (7%)
	Iannacone et al., (2021)	Acetate	PAO clades: *Thauera* (14%), *Hyphomicrobium* (10%), *Pseudomonas* (9%) and *Hydrogenophaga* (4%)
		Ethanol	PAO clades: *Acidovorax* (14%) and *Thaurea* (7%)
	Yang et al., (2018)	Acetate	PAOs 35%, GAOs 13%
		Acetate: glycerol 1:1	PAOs 40%, GAOs 10%
		Glycerol	PAOs 27%, GAOs 16%
	Zhao et al., (2016)	Acetate	PAOs 32%, GAOs 14%
		Acetate: glycerol 1:1	PAOs 40%, GAOs 10%
		Glycerol	PAOs 28%, GAOs 25%
Amino acids	Rey-Martínez et al., (2019)	Glutamate	Favours Family *Comamonadaceae* (16%), *Accumulibacter* (8%), genus *Thiothrix* (37%)
	Chua et al., (2006); Kristiansen et al., (2013); Zengin et al., (2011)	Glutamate	Favours *Actinobacterial* PAOs
	Nguyen et al., (2011)	Glutamate	Favours *Tetrasphaera*
	Marques et al., (2017)	Casein hydrolysate	*Accumulibacter* (22%) and *Tetrasphaera* (70%)

a with temperature around 30 ℃
b 200 mg COD/L
c 400 mg COD/L
d denitrifying P removal
e with enriched-PAO
f with enriched-GAO
g with the environment of coexistence of PAO and methanol-degraders

Table 4.12 shows the microbial communities under different carbon sources. S. Wang et al., (2020) showed different microbial communities as a function of the carbon source fed: PAO (*Rhodocyclaceae*) was around 32% and 72% in acetate- and propionate-fed systems respectively in family level. In addition, *Dechloromonas* (owning the ability to use nitrate as electron acceptors for P uptake) was detected to be much more enriched in propionate-fed (61%) than in acetate-fed (1%) systems in genus level. Likewise, Zhang et al., (2020) showed that changing the carbon source from acetate to the coexistence acetate and propionate (with a ratio of 1:1) allowed optimal P and N removals (91% and 85% respectively), and increased the percentages of the main responsible denitrifying P removal bacteria *Dechloromonas* from 1.5% to 4.8%. The responsible bacterial community for DPR process were *Accumulibacter*, *Acinetobacter*, *Dechloromonas* and *Pseudomonas* with a percentage of 14%-29%, which led to the disadvantage of *Competibacter* and *Defluviicoccus*.

4.3.3.2. Butyrate and valerate

In addition to acetic and propionic acids, butyric and valeric acids are major VFA species present in wastewater: they can account for 20-40% of the total VFA in the anaerobic fermentation liquor of waste sludge (Cai et al., 2019; Yuan et al., 2009). Butyrate can be

used as sole carbon source to drive EBPR but long term P removal cannot be supported (Begum and Batista, 2014; Machado, 2004) (Figure 4.7). Butyrate has shown lower P activity when compared to acetate and propionate in a *Accumulibacter*-enriched sludge (Begum and Batista, 2014; Oehmen et al., 2004; Pijuan et al., 2004) due to a slow butyrate uptake rate. A wide range of P/C ratio was reported (0.2-0.8) by Begum and Batista, (2014) during the first experiments but, in the long-term, P removal deteriorated after 6 weeks even though *Accumulibacter* (50%) and *Defluviicoccus* (16%) were favoured versus *Competibacter* (2%) (Table 4.12). The failure was observed in the second phase of aerobic P uptake, probably due to a decrease on the internal PHA levels. However, (Wang et al., 2021) showed successful and stable EBPR with butyrate as sole carbon source for more than 2 months and at a temperature around 30 °C despite they also observed that the total amount of PHA decreased when changing the carbon source from acetate to butyrate. The same replacement of the carbon source was conducted in a GAO-enriched system and it was observed that butyrate could be more detrimental to GAO metabolism and, in turn, favour PAO. Finally, the relative abundance of PAO species in the PAO-enriched SBR system experienced substantial changes (Table 4.12): *Accumulibacter* decreasing from 37 % to 14% and *Rhodocyclaceae* increasing from 2% to 15%. A reduction of microbial diversity and on the GAO percentage (from 27% to 6%) was observed in the GAO-enriched system and *Zoogloea* was favoured (from 0.2% to 38%). Butyrate was also tested as additional carbon source (to VFA or glucose) to boost EBPR performance with successful results (Cai et al., 2019; L. Wang et al., 2020), and *Rhodocyclus*-related bacteria and *Actinobacteria* as putative PAOs were favoured (Table 4.2). For the compositions of PHA (Table 4.12), Pijuan et al., (2009) showed 47% of PHB, 49% of PHV and 4% of PH2MV and a non-identified monomer. However, the novel PHA fraction poly- β-hydroxyhexanoate (PHH) was reported by Wang et al., (2021), with the percentage of 28% and 35% in PAO-enriched and GAO-enriched systems with butyrate as sole carbon source.

To the best of authors' knowledge, there is no research about valerate as sole carbon source for EBPR. The application of valerate as additional carbon source to glucose showed successful P removal and an enrichment of PAO (13% of *Rhodocyclus*-related bacteria and 12% of *Actinobacteria*) (Table 4.12). However, butyrate was more preferred to valerate and led to a much higher P removal ability and P content in the sludge (Cai et al., 2019). The ratio of P/C showed a relatively lower value compared with other three VFA (Table 4.11) (L. Wang et al., 2020), and the relative PHA compositions were mainly PHV and PH2MV.

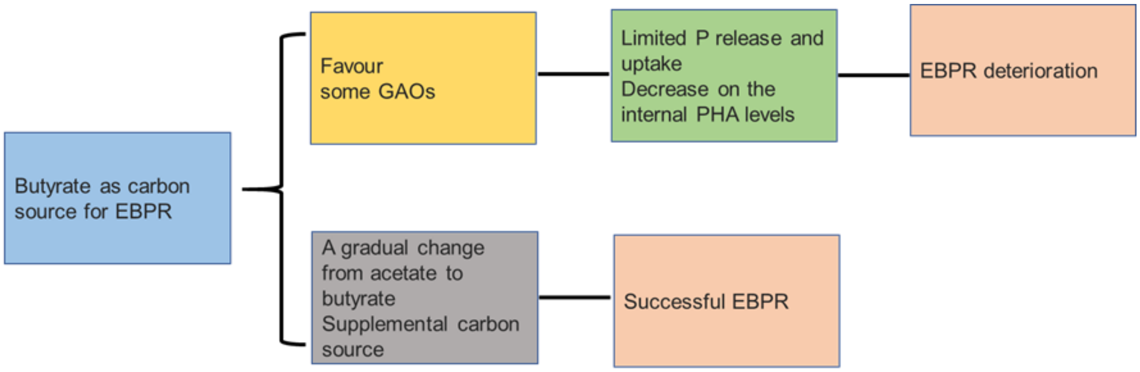

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Figure 4.7. The overall perspective of the feasibility of butyrate as carbon source for EBPR.

4.3.4. *The application of fermentable carbon sources*

VFAs are the most efficient and common carbon sources to drive and maintain the stability of the EBPR process (Oehmen et al., 2007). However, the commercial dosage of VFA not only increases the consumption of carbon source and carbon footprint of the plant, but also the operational costs. Thus, other fermentable carbon sources such as glucose, starch, lactate, ethanol, amino acids (e.g. glutamate, casein hydrolysate, casamino acid) as a source of VFA have been studied as potential carbon source for the EBPR process.

4.3.4.1. Lactate, glucose and starch

Lactic acid is the hydrolysed substance of glucose, and it is unproved to lead to successful EBPR (Figure 4.8). Rubio-Rincón et al., (2019) showed an EBPR failure when the feed was switched from a mixture of acetate/propionate/lactate to lactate as sole carbon source in a lab-scale EBPR-SBR system. The dominant metabolism experienced a shift from a PAO to GAO metabolism. Glycogen consumption and the percentage of PHV formation increased almost double (Table 4.13). The storage of PHA from lactate by *Accumulibacter* and *Tetrasphaera* did not seem to require poly-P hydrolysis and, thus, the PAO phenotype was lost. The EBPR activity could only be triggered by the fermentation of lactate to VFA (acetic acid or propionic acid). Zengin et al., (2010) and Baetens et al., (2002) also stated that lactate could not sustain EBPR because of sludge bulking problems (proliferation of filamentous bacteria) when switching from acetate to lactate/acetate.

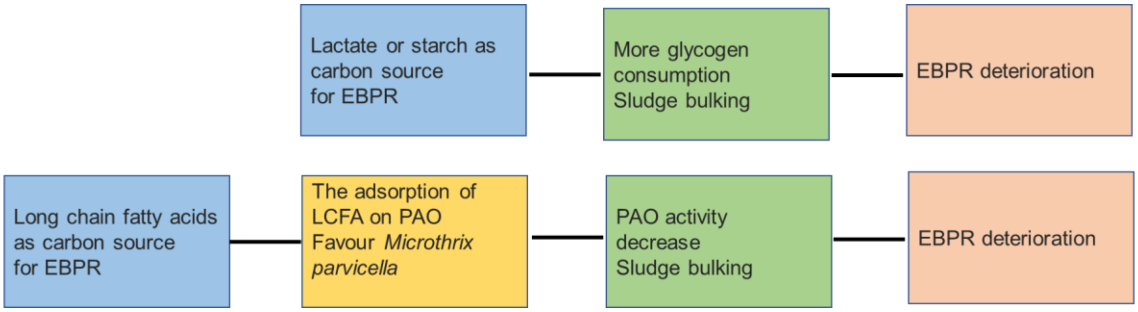


Figure 4.8. The overall perspective of the feasibility of lactate, starch or long chain fatty acids as carbon source for EBPR.

Glucose is a common substance in domestic wastewater that has also been reported to lead the deterioration of EBPR (Izadi et al., 2021; Wang et al., 2002; Zengin et al., 2010) due to the proliferation of GAO (Oehmen et al., 2005a; Wang et al., 2010; Zengin et al., 2010) (Figure 4.9). The common knowledge is that glucose cannot be directly used by PAO and that flanking species (e.g. lactic acid bacteria) should transform glucose to produce pyruvate through glycolysis and, afterwards, the lactic acid can be used under anaerobic conditions for glycogen storage. Zengin et al., (2010) reported initial P removal in glucose-fed SBR system (around 30 day) since the carbon source was lactic acid produced by lactic acid bacteria. The system failure happened on day 29 due to the significant increase of glycogen consumption and thus, GAO proliferation. Similarly, Yazıcı and Kılıç (2016) reported a 5 times higher glycogen with glucose than with acetate. Glucose favouring GAO in EBPR systems was confirmed by the presence of *Competibacter* was in most of the cases (Table 4.12). However, Dockx et al., (2021) showed that the enrichment of GAO-*Saccharimonadaceae* and other GAOs proliferated with glucose over *Competibacter*.

Table 4.13. Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with diverse carbon sources except for VFA.

References	Carbon source	Anaerobic				Aerobic		
		Carbon uptake (mmol C/g VSS/h)	P/C (mol/mol)	PHA/C (mol C/mol C)	Gly/C (mol C/mol C)	P uptake rate (mmol/g VSS h)	P/PHA (mol/mol C)	Gly/PHA (mol C/mol C)
Rubio-Rincón et al., (2019)	Mixture of acetate, propionate and lactate	3.06	0.70	PHA/C 0.9 PHB/C 0.5 PHV/C 0.4	0.18	1.06	-	-
	Lactate	2.71	0.11	PHA/C 0.76 PHB/C 0.1 PHV/C 0.66	0.47	0.21		
Pijuan et al., (2009)	Glucose [a]	1.32-4.68	0.05-0.12	PHA/C 0.36-0.44 PHB 30-44% [f] PHV 56-70%	0.12-0.28 [b]	-	-	-
Yazıcı and Kılıç, (2016)	Acetate	-	0.17-0.31		19 [c]	-	-	25 [c]
	Glucose	-	0.06-0.21		19 [c]	-	-	45 [c]
Wang et al., (2010)	Glucose	-	0.0059		0.128	0.19	-	-
Luo et al., (2018)	Starch	-	-0.08 [d]	0.03	2.64 [b]	-	41	68 (1.36/0.02)
Puig et al., (2008) Tayà et al., (2013)	Methanol	-	0.38-0.54 [e]	PHB 8%[f] PHV 92%	-	-	0.2	0
	Ethanol	-	0.2-0.4	PHB 18% [f]	0.8	0.05-0.22	0.2	-

				PHV 82%				
Guerrero et al., (2012)	Glycerol	-	0.22	PHA/C 0.31 PHB 26% [f] PHV 45% PH2MV 29%	0.25	-	-	-
Yang et al., (2018)	Acetate	-	0.38	PHA/C 1.21 PHB 95% [f] PHV 3% PH2MV 2%	0.42	-	-	-
	Acetate：glycerol 1:1	-	0.64	PHA/C 1.35 PHB 60% [f] PHV 30% PH2MV 10%	0.34	-	-	-
	Glycerol	-	0.24	PHA/C 1.03 PHB 30% [f] PHV 55% PH2MV 15%	0.49	-	-	-
Tayà et al., (2015)	Crude glycerol	-	0.3	PHA/C 0.43 PHB 40% [f] PHV 60%	-	-	-	-
	Long chain fatty acids	-	0-0.4	-	-	-	-	-
Zengin et al., (2011)	Glutamate	-	0.2-0.7	PHA/C 0-0.6 PHB 6% [f] PHV 47% PH2MV 35% PH2MB 12%	0.5-1.2	-	-	-
Rey-Martínez et al., (2019)	Glutamate	-	0.21	PHA/C 0.07 PHB 71% [f] PHV 29%	0.01 [b]	-	-	-
Marques et al., (2017)	Glycine [h]	-	0	PHA 0.12 PHB 17% [f] PHV 75%	0.56	-	-	-

				PH2MV 8%				
	Casein hydrolysate	-	0.35	PHA/C 0.15 PHB 20% [f] PHV 60% PH2MV 20%	0.38	1.76 [g]	2.23	1.84

a. sporadic dosage to enriched-PAO in VFA-fed system
b. glycogen was synthesised not degraded
c. the percentage of glycogen content in biomass at the end of anaerobic phase, and the same was in acetate-fed system
d. indicate P uptake, rather than common P release
e. with the environment of coexistence of PAO and methanol-degraders
f. the relative percentages under successful EBPR performance
g. P uptake with the unit of mmolP/L
h. sporadic dosage to enriched-PAO and *Tetrasphaera* in casein hydrolysate-fed system

Glucose extra addition to an EBPR system also decreased its microbial diversity (He et al., 2018). Rollemberg et al., (2019) also indicated that a glucose-amended feed induced the lowest microbial diversity compared with acetate and ethanol, as well as the worst P and N removal and granular formation (＜1mm). Yazıcı and Kılıç, (2016) found that changing carbon source from acetate to glucose had no significant effect on the settleability of biomass in SBR system but decreased P release and uptake ratios when compared to acetate-fed systems. A decrease trend of P/C ratio was observed from 0.21 to 0.06 (Table 4.13). However, some recent research showed that glucose could effectively be used as carbon source for EBPR under some certain conditions. For instance, Li et al., (2019) reported that glucose could act as proper carbon source for successful simultaneously P and nitrogen removal and good sludge settleability in anoxic/oxic SBR system, even though not as well as those of acetate as carbon source. Similarly, the investigation of Son et al., (2020) showed that improvement of simultaneous P and nitrogen removal with high organic loading (53-88 of the influent C/P ratio) in the device operated in anoxic (anaerobic) /oxic mode with glucose and other carbon sources.

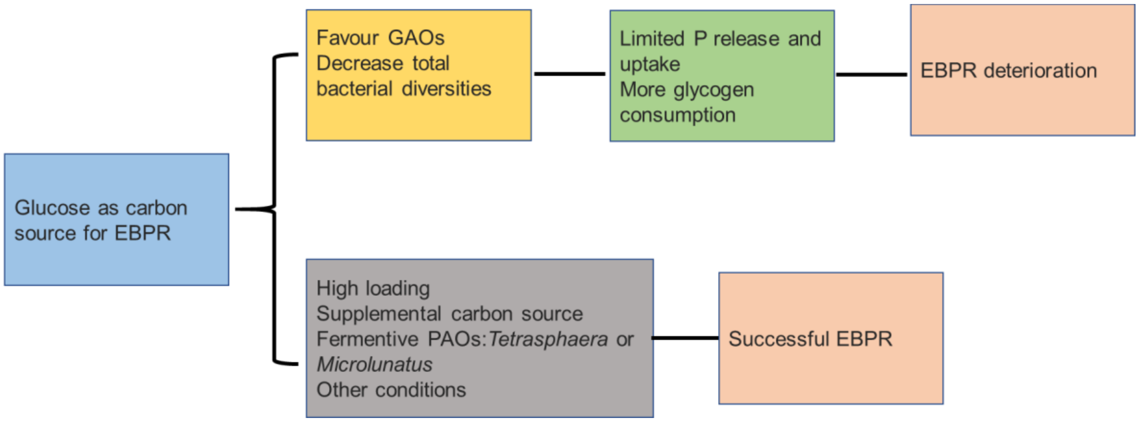


Figure 4.9. The overall perspective of the feasibility of glucose as carbon source for EBPR.

Glucose as supplemental carbon source was reported to result in successful P removal (Chuang et al., 2011). Xie et al., (2017) also showed the mixture of glucose and acetate as carbon sources (with a ratio of 1:1) showed the highest P removal (96.3%) in EBPR-SBR systems compared other different molar percentage of acetate and glucose. *Tetrasphaera*-related PAOs, *Microlunatus phosphovorus* and another isolated PAO candidate were detected as main functional P removal bacteria in these experiments. *Microlunatus* has been considered as fermentative PAO (Kawakoshi et al., 2012; Nielsen et al., 2019).

The unfavorable effect of glucose as sole carbon source to *Accumulibacter* may limit its application in some cases. However, *Tetrasphaera*, another putative PAO favoured by glucose (Nguyen et al., 2011), could provide a possibility for its broad application due to the high percentage detected in many WWTPs, which could be discussed afterwards.

Starch, as a polymer of glucose, is a common compound of wastewater that showed detrimental effect to EBPR as carbon source (Randall et al., 1997; Tam et al., 1992) and favoured the sludge bulking (S. Li et al., 2019) (Figure 4.8). Wei et al., (2014) showed lower EBPR efficiency (77%) with the mixture of starch and acetate (with a ratio of 1:1) compared with that of acetate as sole carbon source (94%) in anaerobic/aerobic SBR since the internal amount of PHA decreased and limited anaerobic P uptake. Luo et al., (2018) obtained 80% of P removal in an anoxic-aerobic SBR system with starch as sole carbon source. They reported a novel P removal process since no P release was observed and the sludge had more glycogen accumulation but less PHA. They proposed that starch was fermented to lactic acid and the lactic acid utilisation was responsible for the majority of P removal in anoxic phase.

4.3.4.2. Citric acid

Citric acid is an essential intermediate substance for the TCA cycle. It leads to the increase of ATP in the cells under aerobic conditions and enhances poly-P accumulation (Smolders et al., 1995). Mielcarek et al., (2015) showed the feasibility of citric acid as carbon source to perform EBPR with biofilm SBR operated in anaerobic/aerobic mode, and longer aerobic phase improved P removal.

4.3.4.3. Long chain fatty acids

Long chain fatty acid (LCFA) as carbon source to trigger EBPR is possible since it has been showed that they do not exert inhibition/toxicity on PAO (Figure 4.8). However, long term LCFA-fed is not feasible due to sludge bulking. Tayà et al., (2015) reported that a mixture of VFA (acetic acid and propionic acid) and LCFA (half of myristic and half of palmitic acid) as carbon source with a ratio of 2:3 showed successful P release and uptake performance with a ratio of P/C between 0.1 to 0.4. However, failure of P removal and a decrease of PAO activity were observed by LCFA as sole carbon source. They hypothesized that the reason was the adsorption of LCFA on the surface of PAO which increased the hydrophobicity of the biomass and substrate and caused sludge bulking. The EBPR recovered again when transformed LCFA to VFA with the ratio of P/C increasing from 0 to 0.3. S. Li et al., (2020) studied the combination of oleic acid (the most prevalent LCFA in the composition of wastewater) and acetic acid under different ratios. The best TN and TP performances were obtained with the ratio of acetate to oleic acid of 4:6. The increasing percentage of oleic acid led to sludge bulking problems due to the proliferation of the typical filamentous bacteria *Microthrix parvicella* (Dunkel et al., 2016; Fan et al., 2017). Similarly, Li et al., (2019) also showed that Tween 80 (a water-soluble emulgator that contains oleic acid) could be used as a carbon source with about 50% of P removal. Tween 80 favoured the production of extracellular polymeric substances (EPS) and also the proliferation of *Microthrix parvicella*. In a word, LCFA could be as a supplementary carbon source for EBPR but with a low ratio since it can lead to sludge bulking problem because of the proliferation of filamentous bacteria such as *Microthrix parvicella*.

4.3.4.4. Alcohols

Alcohols are normally considered as more economical and sustainable carbon sources as supplementary carbon source compared with VFA (Wang et al., 2013). Methanol and ethanol are used as supplements for COD-limited real wastewaters in view of enhancing denitrification. Thus, if added to the anaerobic phase, they could also enhance EBPR.

4.3.4.4.1. Methanol and ethanol

A long-term successful application of methanol as sole carbon source hasn't been reported yet, but the improvement of P removal and N removal efficiency with methanol as a supplemental carbon source was shown by (R. Xu et al., 2016). However, in most cases, methanol as sole carbon source has been reported to be unsuitable for EBPR (Shen and Zhou, 2016; Wang et al., 2013). The addition of methanol to EBPR system has been proven to be detrimental to the stability of the system since the methanol couldn't be directly degraded by PAO (Nuno R. Louzeiroa et al., 2002; Puig et al., 2008; Tayà et al., 2013b) (Figure 4.10). However, Tayà et al., (2013) managed to obtain methanol-based EBPR by with an sludge that contained PAO and methanol-fermenters. A mid-term EBPR performance (around 35 days) was sustained with the ratio of P/C about 0.38-0.54. Methanol was fermented to acetic acid and PAO lived off this VFA. PAO MIX (11%) showed a relative high percentage compared with GAO MIX (5%), DFI and II faded to a negligible amount regardless of the high initial percentage (7%) in the PAO-enriched inoculum (Table 4.12).

Ethanol has been reported as efficient additional carbon source for EBPR in the long run (Puig et al., 2008; Wang et al., 2013). When compared with VFA, ethanol enhanced the stability of granules than acetate as sole carbon source in a EBPR-SBR (Rollemberg et al., 2019), but acetate acted as preferable carbon source than ethanol in terms of N and P removal. Similar and successful P and N removal efficiency (more than 80%) was shown by Iannacone et al., (2021) with acetate and ethanol as carbon source individually in a moving bed biofilm reactor.

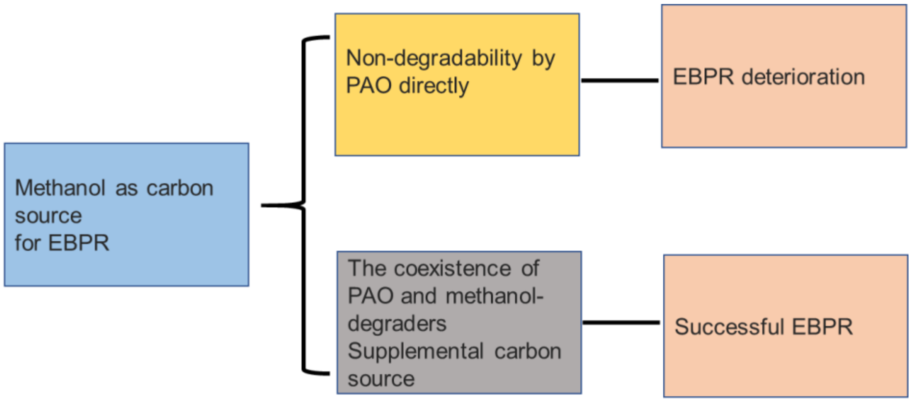


Figure 4.10. The overall perspective of the feasibility of methanol as carbon source for EBPR.

Iannacone et al., (2021) reported that adding ethanol reinforced the functional PAO: *Thauera* (14%), *Hyphomicrobium* (10%), *Pseudomonas* (9%) and *Hydrogenophaga* (4%), whereas acetate as sole carbon source selected PAO clades such as *Acidovorax* (14%) and *Thaurea* (7%) (Table 4.12**).** Skennerton et al., (2015) inferred that two *Accumulibacter* taxa from clade IIF may transform ethanol to acetate to boost EBPR since this clade contained acetaldehyde dehydrogenase, which may generate acetate as a final step in ethanol degradation. As a result, the fermentation of both alcohols led to acetic acid production. Puig et al., (2008) reported a high percentage of PHB (80-90%) in PHA with methanol or ethanol as carbon source (Table 4.13), which is similar to that of acetate.

4.3.4.4.2. Glycerol

Glycerol fermentation to VFA and its posterior biological utilisation is promising way to convert glycerol in a resource rather than a waste or a by-product of biodiesel fuel production (Johnson and Taconi, 2007; Akunna et al., 1993; Bodík et al., 2009; Grabińska-ńoniewska et al., 1985; Torà et al., 2011). Yang et al., (2018) showed that applying glycerol as additional carbon source to acetate (1:1 ratio) improved EBPR (PRE about 96%) in a lab-scale A/O SBR compared with pure acetate (PRE about 90%) and pure glycerol (PRE about 31%). The combination of acetate and glycerol also favoured the percentage of PAO, decreased the percentage of GAO and increased the amount of PHA synthesis (Table 4.12 and 4.13). Zhao et al., (2016) showed that EBPR performance decreased from 97% with mixture of acetate and glycerol as carbon source to 58% with glycerol as sole carbon source, and the corresponding percentage of PAO decreased from 40% to 28% and GAO inversely increased to 10% to 25%. As a result, it was economically and technically feasible to apply glycerol as auxiliary carbon source for nutrients removal.

However, the utilisation of pure glycerol as sole carbon source has led to EBPR failure. Pure glycerol led to less PHA synthesis and then less energy was available for a posterior

P uptake (Guerrero et al., 2012a; Yang et al., 2018; Zhao et al., 2016) (Figure 4.11). The most common explanation was insufficient anaerobic fermentation time. Note that two sequential processes are needed under anaerobic conditions: fermentation of glycerol to VFA (mainly propionic acid and acetic acid) and VFA utilisation by PAO (Barbirato et al., 1997; Salamah and Randall, 2019; Yuan et al., 2010). Therefore, allowing a higher anaerobic HRT or adding a side-stream reactor to ferment glycerol to VFA could be a solution to avoid the EBPR deterioration when pure glycerol was used as carbon source. Guerrero et al., (2012) showed promising EBPR performance with glycerol as sole carbon source under enough anaerobic HRT (4h) in a single-sludge anaerobic/aerobic SBR, and a high ratio of P/C (0.22) was obtained. Salamah and Randall, (2019) proposed two five-stage Bardenpho TM BNR pilot-scale systems for treating raw wastewater (anaerobic, anoxic I, aerobic I, anoxic II, and aerobic II) coupled with side-stream fermenter, which was connected to anoxic II. A substantial concentration of VFA was obtained by the co-fermentation of glycerol and primary solids (around 2500 mg COD/L). Similar PRE was obtained using two different configurations (direct glycerol addition to anoxic II and glycerol addition to a side stream fermenter connected to anoxic II, 82% and 89%, respectively). In terms of N and COD, both systems could reach complete denitrification and more than 90% of COD removal.

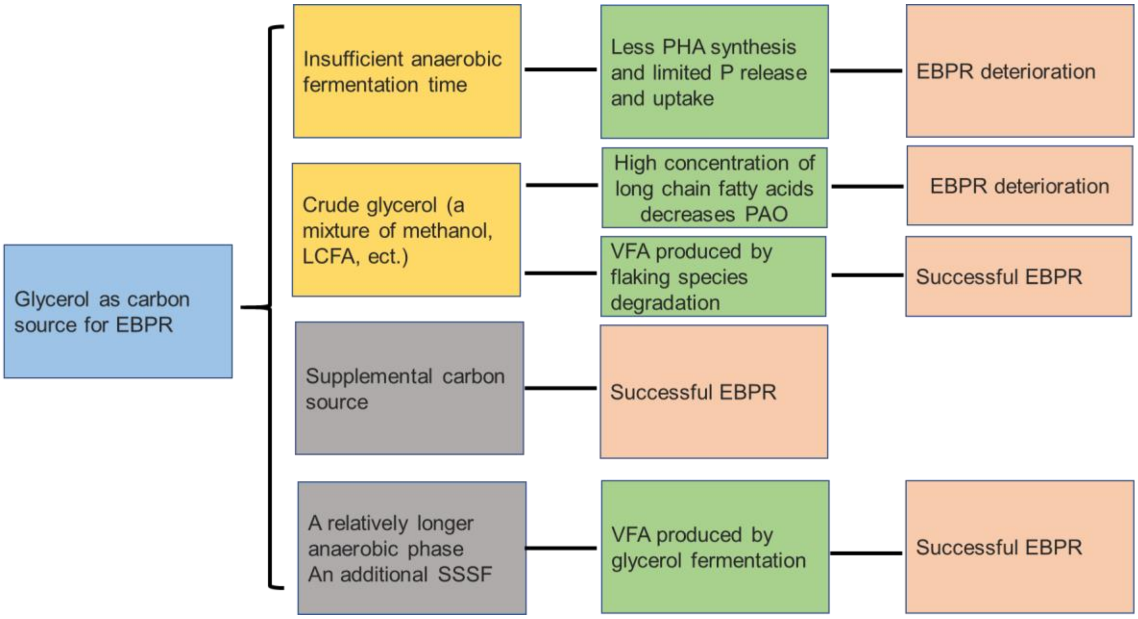


Figure 4.11. The overall perspective of the feasibility of glycerol as carbon source for EBPR.

Crude glycerol (a mixture of methanol, LCFA and salts) was also tested as sole carbon source and it sustained mid-term successful EBPR but not in a long-term (Tayà et al., 2015). They argued that the complex carbon compounds can be degraded to VFA by flanking species, but crude glycerol with high content of LCFA deteriorated PAO activity and led to the collapse of EBPR. Guerrero et al., (2015) also showed that crude glycerol can be as carbon source to improve P and N removal in A_2O system for alleviating the detrimental effect of the presence of anaerobic nitrate and nitrite.

Glycerol as carbon source could favour PAO over GAO. Specifically, the dominant GAO *Defluvicoccus*, and *Competibacter* was rarely detected since propionic acid is reported as the most significant fermentation production of glycerol, and *Competibacter* can only assimilate acetic acid. For the PHA production, PHV appeared to be the main specie (45%-60%) (Guerrero et al., 2012a; Tayà et al., 2015; Yang et al., 2018) (Table 4.12).

4.3.4.5. Amino acids

The proteins may account for more than 50% of all the organic substances in real wastewater (Shon et al., 2007). The hydrolytes of protein, amino acids, have been reported as promising carbon sources to induce EBPR (Fukushima et al., 2007; Marques et al., 2017; Qiu et al., 2020), and amino acid favoured the proliferation of *Accumulibacter*, *Thiothrix* and *Tetrasphaera* (Singleton et al., 2022; Zhang and Kinyua, 2020; Dionisi et al., 2004; Kong et al., 2005; Rey-Martínez et al., 2019, 2021b).

4.3.4.5.1. Glutamate

Glutamate as a common amino acid has been proved to support EBPR as carbon source and to favour a variety of PAO clades (Table 4.12) (Rey-Martínez et al., 2021b, 2019; Zengin et al., 2011). Zengin et al., (2011) showed that glutamate could boost EBPR with the range of P/C ratio of 0.2-0.7, and that *Actinobacterial* PAO were favored in the glutamate-fed system. However, the levels of internal PHA were decreasing in time, and EPBR activity was lost in the long-term. Glutamate contains a high fraction of nitrogen that, when glutamate is fermented, is released to the medium. Thus, Rey-Martínez et al., (2019) showed successful of P and N removal with glutamate as the sole carbon and nitrogen source in an anaerobic/anoxic/oxic continuous pilot system. Low PHA storage was also observed (with the ratio of PHA/C about 0.07) and anaerobic glycogen storage rather than consumption (Table 4.13). They proposed the possibility of other carbon storage routes different from the involvement of PHA and glycogen. The sludge was initially enriched in *Accumulibacter* and glutamate addition promoted the growth of *Thiothrix* and family *Comamonadaceae*. They indicated that *Thiothrix* showed ability to store poly-P with glutamate involvement but without PHA synthesis, *Comamonadaceae* was confirmed to degrade glutamate and denitrification, and the mechanisms for P uptake were unclear. In the study of Chua et al., (2006) and Zengin et al., (2011), *Actinobacterial*

PAOs were favoured in glutamate system, and *Actinobacterial* PAOs were shown to assimilate glutamate and poly-P simultaneously but without the ability to store PHA (Kristiansen et al., 2013). Qiu et al., (2020) also proved that the acetate-fed sludge enriched *Accumulibacte* of acetate-fed sludge can metabolize glutamate without the formation of PHA.

For the metabolic substances composition with glutamate as carbon source, Rey-Martínez et al., (2019) reported PHB (70%) and PHV (20%) while Zengin et al., (2011) indicated it was mostly composed with PHV (47%), PH2MV (35%) and PH2MB (12%).

4.3.4.5.2. Glycine

The results reported on glycine as carbon source for EBPR are also inconclusive (Figure 4.12). On the one hand, glycine was shown to induce the highest P release in batch tests with 11 different amino acids (Nguyen et al., 2015) wih the sludge from full-scale WWTPs. Qiu et al., (2020) and Tian et al., (2022) found that glycine induced P release without efficient uptake by biomass during anaerobic phase. Thus, PAO could release P under the presence of glycine (with a high P/C ratio of 0.87-5.20) but could not uptake P during aerobic phase. Thus, glycine cannot be considered as an effective carbon source for EBPR systems. However, the unique characteristic of glycine to induce P release without cellular uptake could provide possibility to recover P from P-enriched waste sludge.

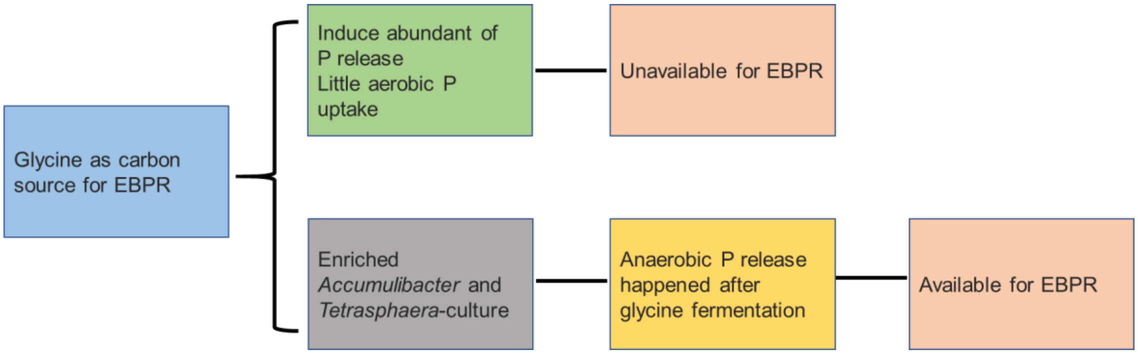


Figure 4.12. The overall perspective of the feasibility of glycine as carbon source for EBPR.

On the other hand, Marques et al., (2017) indicated that glycine might be an effective carbon source for the specific genus of *Tetrasphaera*, but not for *Accumulibacter*. They observed no P release with glycine as carbon source in an enriched *Accumulibacter* and *Tetrasphaera*-culture. P release only happened after the full consumption of glycine, which suggested the P release was not positively relative to glycine uptake. They further showed the energy for *Tetrasphaera* to take up P anaerobically is from glycine fermentation (other carbon source as well, e.g. glucose, glutamate and aspartate).

4.3.4.5.3. *Mixtures of amino acids*

The mixture of amino acids and other carbon sources may pose different effects on the performance of the system and on the microbial communities. Casein hydrolysate, as a mixture of amino acids and peptides, was used as sole carbon source with an enriched culture of *Tetrasphaera* and *Accumulibacter* in Marques et al., (2017), and more than 99% of P removal was observed. More than 90% of *Tetrasphaera* cells was verified to be responsible for the consumption of amino acid and participated in about 80% of P removal. However, *Accumulibacter* was likely to only survive on the fermentation productions such as acetate and propionate. The energy for anaerobic P release was provided by the fermentation of casein hydrolysate to VFA. Amino acid, sugars and some small amines were stored as intracellular substances to provide energy for aerobic metabolites. The PHA compositions were PHB (20%), PHV (60%) and PH2MV (20%) (Table 4.13), which is more similar to a propionate-fed system with the PHV as the most dominant PHA.

Adler and Holliger, (2020) showed that replacing the carbon source from VFA to a mixture of amino acids, VFA and glucose had little impact on the P and N removal and the settleability of the sludge, but affected the microbial communities and *Actinobacteria* became dominant. Qiu et al., (2020) proposed a mixture of acetate and amino acids as carbon source could save more than 17% of energy compared with that of sole individual carbon source due to the flexibility of metabolic way of *Accumulibacter* under different carbon sources. Close et al., (2021) showed that a highly enriched *Tetrasphaera* (95%) showed lower P removal (72%) with amino acids as sole carbon source compared with a sludge contained *Tetrasphaera* and *Accumulibacter* ($>$ 99%).

4.3.5. *The application of wastes to carbon sources*

The dosage of an external carbon source is often not feasible from an economic and environmental point of view and that limits its full-scale application. Therefore, environmental-friendly and economical carbon sources have drawn the attention of research. Carbon sources derived from waste materials, such as organic waste or waste sludge from side-stream, mainstream, primary settler are now studied. The overall perspective of the fermentation productions from wastes as carbon source for EBPR is shown in Figure 4.13.

4.3.5.1. *Pre-treated sludge and food waste*

Sludge disposal is of great concern for many wastewater treatment facilities. Waste sludge digestion or fermentation not only relieves this issue but also can be an efficient way of producing VFA. Soluble products from waste fermentation are mainly short-chain fatty acids with two to five carbon atoms, which can be directly used as carbon source for many bioprocesses (Moser-Engeler et al., 1998; Vázquez-Fernández et al., 2022). Pretreatment methods are usually applied to increase the VFA yield such as alkaline treatment (Gao et al., 2011; Li et al., 2011; Tong and Chen, 2009; Ye et al., 2020), acid treatment (Gao et al., 2011; Liu et al., 2020), thermophilic operation (Tang et al., 2019b), microwave-H_2O_2 (R. Xu et al., 2016) and mechanical disintegration (Kampas et al., 2009).

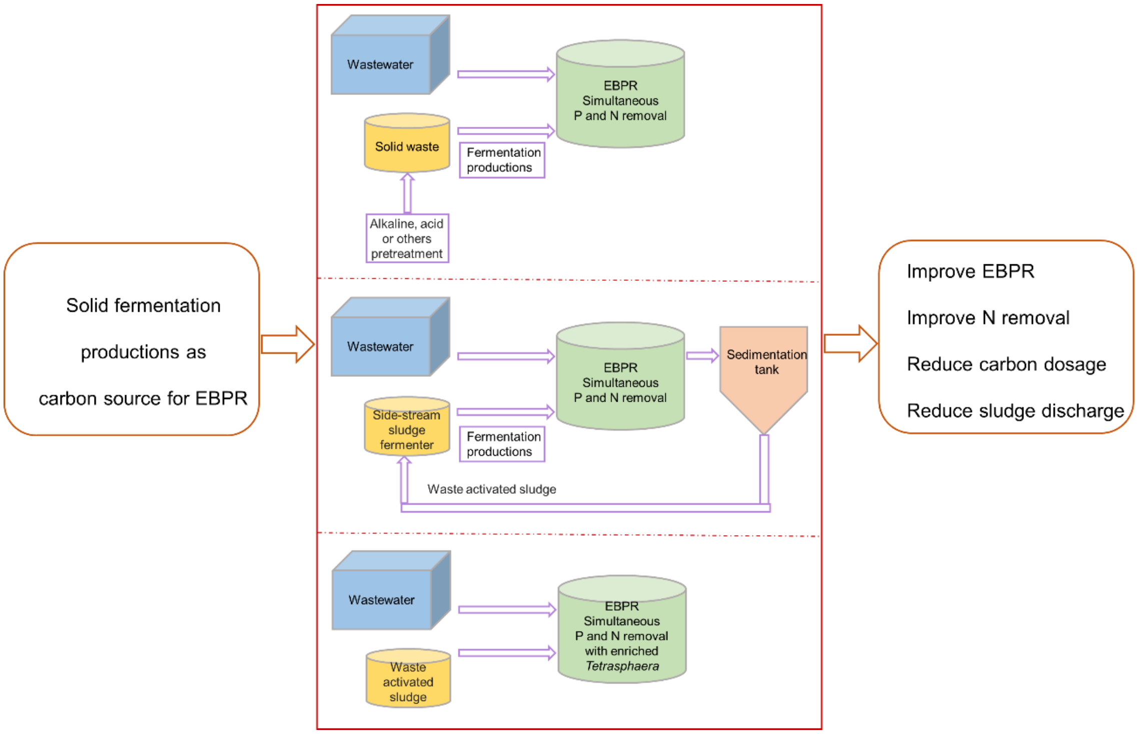


Figure 4.13. The overall perspective of the fermentation productions from wastes as carbon source for EBPR.

Liu et al., (2017) showed the fermentation products of the waste sludge could be used as carbon source to improve N and P removal in municipal wastewater with low C/N ratio, and the reduced sludge discharge was 44-52%. Ji and Chen,. (2010) demonstrated that the products of an alkaline fermentation of waste sludge as sole carbon source showed higher nutrient removal efficiency than that of acetic acid with the same amount of COD. The reason for the improvement of nutrients removal could be probably the dominant percentage of DPAO (61% of total PAO), much higher than acetic acid-fed system (7%) (Table 4.14). Zaman et al., (2019) also obtained more than 90% of P removal and about 70% of N removal with the effluent of the alkaline fermentation of solids from primary settling tank as carbon source. However, synthetic VFA showed advantage on nutrients removal and P release and uptake rate compared with the fermentation liquid due to the other carbon sources except for VFA contained in fermentation liquid.

Anaerobic alkaline fermentation liquid used as carbon source for improving BNR and sludge disposal process has already been applied in pilot-scale and full-scale processes (Gao et al., 2011; Liu et al., 2018). Gao et al., (2011) applied a pilot-scale alkaline continuous fermentation process which provided additional VFA to the A_2O system for treating domestic wastewater. They showed that TP and TN removal were about 90% and 80% respectively, and 42% of waste sludge was reduced simultaneously. Liu et al., (2018) operated a full-scale WWTP as an A_2O process and the waste sludge from the WWTP was fermented as VFA as additional COD. TP and TN removal were about 90% and 73% respectively, and 54% of sludge could be reduced.

Fe-enhanced primary sedimentation sludge is an efficient way to convert solids into VFA by the acidogenic sludge fermentation (Liu et al., 2020). It was applied by Li et al., (2021) in SBR system to provide COD for the system. The removal efficiencies of TP and TN reached to 89% and 83% treating the raw wastewater without any additional COD dosage, which showed extensive improvement (with P and N removal efficiency increased by 65% and 50%) compared with conventional SBR (Table 4.14).

Similar as the case of waste sludge, the effluents from the fermentation of food waste are enriched with VFA, alcohols and lactic acid and, therefore, they can be used as carbon source to enhance nutrient removal (Feng et al., 2021; Strazzera et al., 2018). Tang et al., (2019b) showed that anaerobic thermophilic food waste fermentation led to P and N removal about 98% and 90% respectively in a lab-scale SBR system. The EBPR performance increased due to the enrichment of *Rhodocyclacea* (7%) (Table 4.14). Tang et al., (2019a) also showed successful P (90%) and N (more than 80%) removal when feeding the system with the mesophilic fermentation of food waste (Table 4.14). Zheng et al., (2018) showed that the improvement of biological nutrient performance of alkaline fermentation products was caused by fermentation product containing a ratio of acetic acid to propionic acid about 1:1 under the optimal condition.

Table 4.14. The system performance and relative microbial communities of solids as carbon source with different pretreatment.

Biosolids type	References	Pretreatment	Carbon source and wastewater	Configuration and scale	P removal efficiency (%)	N removal efficiency (%)	Microbial community
Primary sedimentation sludge	Li et al., (2021)	Fe-based chemically enhanced pretreatment	Fermentation liquid + municipal wastewater	SBR 24L	89	83	-
Waste sludge	Liu et al., (2017)	Alkaline fermentation (pH=10)	Fermentation liquid + municipal wastewater	AOA-SBR 11.5 L	99	89	*Accumulibacter* 4%
	Ji and Chen,. (2010)	Alkaline fermentation (pH=10)	Fermentation liquid + synthetic wastewater	SBR 4L	98	99	PAO 59% DPAO 61% GAO 3%
			Acetic acid + synthetic wastewater	SBR 4L	73	79	PAO 37% DPAO 7% GAO 11%
	Gao et al., (2011)	Alkaline fermentation (pH=10)	Fermentation liquid + municipal wastewater	Pilot-scale A_2O 55L	90	80	-
	Liu et al., (2018)	Thermal-alkaline fermentation (pH 10-11)	Fermentation liquid + municipal wastewater	Full-scale A_2O with 40,000 m^3/d wastewater handling capacity	90	73	-
Kitchen wastewater	Zheng et al., (2018)	-	Acetic acid+ domestic wastewater	Full scale A_2O with 25,000 m^3/d wastewater handling capacity	88	70	-
		Alkaline fermentation (pH=8)	Fermentation liquid + municipal wastewater	Full scale A_2O with 25,000 m^3/d wastewater handling capacity	95	78	-
Food waste	Tang et al., (2019b)	Thermophilic fermentation (55 ℃)	Fermentation liquid+ domestic wastewater	5L SBR	98	90	*Rhodocyclaceae* 7%
	Tang et al., (2019a)	Mesophilic acidogenic fermentation (pH=4)	Fermentation liquid+ domestic wastewater	5L SBR	90	> 80	*Accumulibacter* 0.6% *Rhodocyclaceae* 5.6%

4.3.5.2. Waste sludge from a side-stream sludge fermenter

A recent proposal to reuse the surplus carbon generated in the plant is the integration of a side-stream sludge fermenter (SSSF) where part of the return activated sludge (4-30%) is hydrolysed and fermented to provide VFA for the system without any pretreatment (Barnard et al., 2017; G. Li et al., 2020; Onnis-Hayden et al., 2020; Wang et al., 2019). The integration of SSSF and conventional EBPR process (S2EBPR) has been broadly investigated and about 80 full-scale facilities worldwide have been proven successful (Coats et al., 2018; Copp et al., 2012; Tooker et al., 2017; Vale et al., 2008; Vollertsen et al., 2006). Compared with traditional EBPR configurations, the S2EBPR configuration was shown to enhance EBRP and the stability of system (Lanham et al., 2013; Onnis-Hayden et al., 2020).

Wang et al., (2019) showed that SSSF could provide a substantial VFA load and both P removal and denitrification performance were enhanced. The reported P release in SSSF increased 24.5 % in S2EBPR compared with A_2O (132 kg P/d vs 106 kg P/d), and P removal efficiency was also improved from 80% with A_2O to 94% with S2EBPR. The P/C ratio in S2EBPR (0.45) was even two times as that of A_2O (0.22), as well as the P uptake rate (Table 4.15). Onnis-Hayden et al., (2020) also reported higher P removal performance with S2EBPR vs A_2O (90% vs 82%). For the microbial communities, the relative abundances of *Accumulibacter* and *Tetrasphaera* showed no significant difference compared with those of traditional A_2O system. However, PAO exhibited superior EBPR activity and less decay in the SSSF, which probably enabled GAO outcompetition, and further improved the EBPR performance and stability. Other studies also showed that PAO could be promoted independently of the nature of the carbon source of the WWTP influent. For example, Vollertsen et al., (2006) showed P and considerable COD concentrations were detected in the SSSF of two WWTPs with HRT about 30 to 35 h, which made S2EBPR less dependent on the input wastewater quality compared with traditional EBPR. The reasons for the promotion of PAO activity in the S2EBPR could be due to: i) the biomass fermentation products are mostly VFA, which are the preferred electron donor for PAO (G. Li et al., 2020; Onnis-Hayden et al., 2020); ii) the SSSF contains extra readily biodegradable COD except for VFA, which could be further fermented to VFA under anaerobic condition by PAO fermenters such as *Tetrasphaera* (Barnard et al., 2017; Fernando et al., 2019; Nielsen et al., 2019); iii) the extended anaerobic phase of SSSF provides a competitive advantage for PAO with respect to GAO and other heterotrophic organisms (Barnard et al., 2017; Barnard and Abraham, 2006; Wang et al., 2019).

Table 4.15. Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with waste sludge as carbon source by SSSF or by *Tetrasphaera*-enriched culture (modified from (Wang et al., 2019)).

References	Configuration	Carbon source and wastewater	P/C (mol/ mol)	PHA/C (molC/ molC)	Gly/C (molC/ molC)	P uptake rate (mmol/ g VSS h)	P/PHA (molP/molC)	Gly/PHA (molC/molC)
Wang et al., (2019) [a]	Full-scale A_2O	Municipal wastewater	0.22	0.64	0.16	0.07	0.32	0.55
	Full-scale S2EBPR	Fermentation liquid + municipal wastewater	0.45	0.50	0.22	0.14	0.97	0.61
Fan et al., (2022) [a]	Continuous anaerobic/aerobic/anoxic system (10L) with enriched-*Tetrasphaera*	Fermentation liquid + municipal wastewater	0.26	0.36	0.34	-	0.99	-

a. acetate sporadic dosage to the sludge from the system

4.3.5.3. *Waste sludge by the fermentation of Tetrasphaera*

Tetrashpaera are reported to have the ability to ferment complex organics such as amino acids and glucose (Singleton et al., 2022; Zhang and Kinyua, 2020; Dionisi et al., 2004; Kong et al., 2005; Marques et al., 2017). The high amount of proteins and carbohydrate (30-40% of total COD) contained in the waste (Gao et al., 2011; Liu et al., 2017) allow *Tetrashpaera* to ferment them and to generate an effluent suitable for its application into EBPR (Fan et al., 2022, 2021; F. A. Herbst et al., 2019; Nguyen et al., 2015). Fan et al., (2021) investigated one lab-scale SBR- *Tetrashpaera* operated in anaerobic-aerobic mode with the only carbon source provided from the waste sludge of the other parent SBR system. Enriched *Tetrashpaera* (91.9%) was observed in SBR- *Tetrashpaera* system even though there was no *Tetrashpaera* detected from the parent system. Successful P removal (no P detected in the effluent) and sludge reduction (44%) were obtained simultaneously, and it also should be noted that there was no pretreatment with the waste sludge. Compared with traditional sludge fermentation, the slowly biodegradable organics (e.g. amino acids and soluble microbial by-product) from waste sludge in the *Tetrasphaera*-dominated reactor experienced better hydrolyzation and acidification and further to VFA, and final VFA concentration was about 5.46 times as that of traditional sludge. In terms of the metabolism of *Tetrashpaera*, they proposed that SBR-*Tetrasphaera* rely on amino acids as energy source for anaerobic storage and aerobic consumption in EBPR process, rather than glycogen and PHA, and glutamate was the most crucial intracellular substance for metabolites of *Tetrashpaera*.

The operation of this system under continuous anaerobic/aerobic/anoxic conditions was investigated in their posterior work (Fan et al., 2022). The only carbon source was provided by in-situ fermentation of waste sludge by *Tetrasphaera* through a prolonged anaerobic phase (increasing HRT from 2 h to 15 h) to treat low ratio of COD /N real domestic wastewater for EBPR and partial nitrification. P removal could be maintained to 100% and the sludge discharged reduction was reduced by 61.9% due to sludge fermentation. The abundance of *Tetrasphaera* accounted for 31.2% and 72.8% at genus level and transcriptional level, respectively. The prolonged anaerobic HRT for sludge fermentation favoured more *Tetrasphaera* to outcompete *Accumulibacter* and further improve the fermentation for VFA production, and it also benefited more stable partial nitrification.

4.3.6. *Discussions and remarks*

4.3.6.1. *The EBPR performance under a sole carbon source*

The general view of the availability of diverse carbon source for an efficient EBPR performance is shown in Figure 4.14. Acetate and propionate are still the most common carbon sources for EBPR, particularly at lab scale, and a moderate concentration of VFA ensures a successful system performance (Nielsen et al., 2019; Qiu et al., 2022). Other VFA (butyric and valeric acids) seem to be more suitable as additional carbon source to obtain stable EBPR (Begum and Batista, 2014; Cai et al., 2019; Machado, 2004). Lactate, glucose and starch can support EBPR its use in a long-term basis is controversial (Luo et al., 2018; Son et al., 2020; Yazıcı and Kılıç, 2016; Zengin et al., 2010). Glucose allows successful EBPR in enriched-*Tetrasphaera* culture (Nguyen et al., 2011). LCFA as sole carbon source could lead to a decrease of PAO activity and the proliferation of filamentous bacteria, thus, the failure of P removal (Dunkel et al., 2016; Tayà et al., 2015).

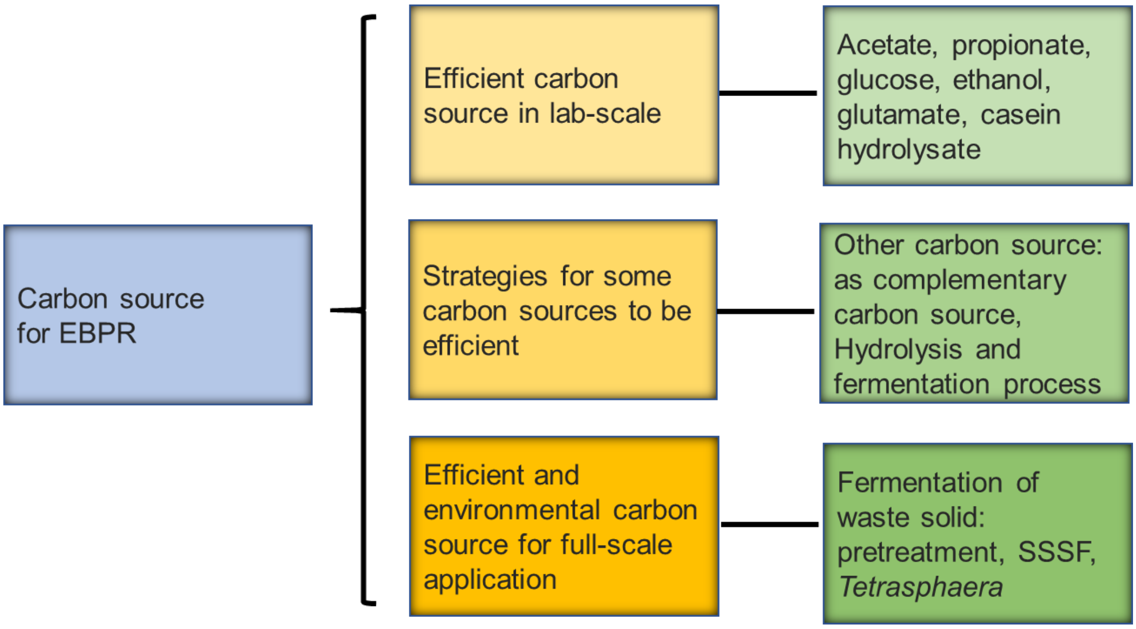


Figure 4.14. The general view of the availability of diverse carbon source for efficient EBPR performance.

Methanol as sole carbon source may lead successful P removal by promoting fermentative bacteria but ethanol are reported to be assimilated directly and allow successful EBPR (Shen and Zhou, 2016; Wang et al., 2013). Pure glycerol led to less PHA synthesis and thus to the EBPR failure(Guerrero et al., 2012a; Yang et al., 2018; Zhao et al., 2016). Glutamate have been proved to support EBPR. Glycine is probably not suitable for EBPR.

Other carbon sources (carbohydrates (e.g. glucose, starch), LCFAs, methanol, glycerol, protein (amino acids)) could act as complementary carbon source for EBPR. In addition, hydrolysis and fermentation process of complex carbon sources to VFA is suggested to take full advantage of these substances in real wastewater. For example, the fermentation of glucose by *Tetrasphaera*, the fermentation of starch to lactic acid, the acidification of methanol to acetic acid, and the longer anaerobic fermentation time for glycerol, for some amino acids or LCFAs to VFA.

4.3.6.2. *The EBPR performance under mixed substrate strategies*

EBPR performance is greatly influenced by the different feeding strategies of carbon substrates. A mixture of carbon sources can improve the EBPR performance with respect to single carbon source feedings to some extent even though the prevalent position of VFA. Other carbon sources (e.g. carbohydrate as glucose or starch, methanol or LCFA) that often lead to unstable EBPR performance as individual carbon source are shown to be good candidates as complementary carbon source to improve EBPR and N removal (Randall et al., 1997; Rollemberg et al., 2019; Tayà et al., 2013b; Wei et al., 2014; Yazıcı and Kılıç, 2016). These carbon compounds can be used as carbon sources if properly fermented to VFA as explained above but the main issue is that they promote the growth of flanking filamentous species that result in bulking issues and, thus, the decrease of the system performance. However, when combined to other VFA, the growth of undesired microorganisms can be mitigated and thus, these compounds can effectively be used as electron donors. For instance mixture of acetate and glycerol (Yang et al., 2018) or the mixture of acetate and glucose (Xie et al., 2017) even showed higher EBPR performance than pure acetate. A mixture of amino acids and acetate can save more than 17% of energy compared with that of sole individual carbon source due to the more flexible metabolic way of *Accumulibacter* with different carbon sources (Qiu et al., 2020). In the case of casein hydrolysate, the successful EBPR performance is dependent on the contribution of enriched-*Tetrasphaera* cells.

4.3.6.3. *Novel carbon sources coming from fermentation of waste solids*

The fermentation products of waste and food sludge have provided very good experimental results to enhance biological P and N removal and overcome potential C limitations in full-scale systems (Ji and Chen, 2010; Kampas et al., 2009; Zheng et al., 2018) (Figure 4.13 and 4.14).

1. The soluble COD concentration from the fermentation of waste sludge is reported with a range of 1000-6000 mg/L depending on the operation condition (Ji and Chen, 2010; Liu et al., 2017; Yuan et al., 2016), and the VFA concentration may account for half of the soluble COD. Liu et al., (2018) indicated around 30000 mg/L COD of the waste sludge fermentation liquid, and the obtained VFA was about 5000 mg/L (Table 4.16). In the case of food waste, much higher soluble COD was detected (40000-50000 mg/L) as well as a high concentration of VFA, about 8600 and 30000 mg/L from the investigations of Tang et al., (2019a) and Zheng et al.,(2018).

2. The other advantage for the application of waste sludge fermentation could be the reduction of sludge discharge, which could reduce the sludge disposal cost. The reported percentage for sludge discharge reduction is in the range of 40-55% in lab or full-scale configurations (Liu et al., 2017; Gao et al., 2011; Liu et al., 2018).

3. Potential economic savings can be obtained from the production of COD via sludge or waste fermentation. Li et al., (2021) reported that the total annual cost saved could be 1270,350 USD per year considering the cost reduction from decreasing acetate dosage and electricity for aeration (for the treatment capacity of 100,000 m^3/d), and H. Liu et al., (2018) indicated the net profit for VFA production reached to 9.12 USD/m^3due to the unnecessary addition of commercial carbon source.

Table 4.16. Summary of the COD concentrations from the fermentation liquid.

Biosolids type	References	SCOD (mg/L)	VFA (mg/L)
Waste sludge	Yuan et al., (2016)	~3400	~1500[a]
	Liu et al., (2017)	~3100	~1600[a]
	Ji and Chen,. (2010)	~5700	~1700
	Gao et al., (2011)	~1300 [b]	~330 [b]
	Liu et al., (2018)	~30000	~5000
Kitchen wastewater	Zheng et al., (2018)	~44000	~30000 [c]
Food waste	Tang et al., (2019a)	~48200	~8600

a. short-chain fatty acids
b. under the pH=10
c. 67% of VFA obtained under the optimal condition (pH=8, HRT=6d)

The utilisation of waste sludge reduces the sludge discharge and leads to significant economic savings and to a lower carbon footprint of the plant (Fan et al., 2022; Kampas et al., 2009; Li et al., 2021; Zaman et al., 2019). However, the potential disadvantage that can’t be ignored is that the physical or chemical pretreatment process for obtaining more suitable COD compositions for promoting EBPR will lead to additional cost on electrical energy input and a potential environmental threat due to the reagents addition. Kampas et al., (2009) reported that the COD compositions of SCOD and VFA are dependent on the disintegration time, which means the additional energy input for the sludge disintegration should be considered, and the additional cost could be due to the high temperature, pressure and pH (Neumann et al., 2016; Zaman et al., 2019). Too high pH would also result in high pH in the effluent, thus, the poor quality of effluent (Ahn and Speece, 2006).

The above disadvantages may constrain its techo-economic feasibility. As a supplementary solution, applying biological fermentation to the waste sludge provide another possibility for the application of full-scale WWTPs by implementing waste sludge to a SSSF. In addition, the fermentation ability of *Tetrasphaera* on waste sludge was reported to exert more efficient VFA production than traditional sludge. It also showed successful P and N removal for treating real wastewater without the additional carbon source compared with *Accumulibacter* dominated system which may demand additional dosage of external carbon source (Fan et al., 2022, 2021).

However, the significant disadvantage for the integration of SSSF is that the effluent of SSSF not only contains VFA, but also a significant amount of P due to less biomass purge and thus more P residual in the system. As a result, the extra P load may pose threat to the plant performance since not all the entering P could be always removed with continuous input of high concentration of P from the SSSF effluent to the system. Vale et al. (2008) reported 2.8 mg/L of P in the effluent in a full-scale S2EBPR with 6% of RAS to SSSF.

Secondly, the additional COD production is at expense of producing less purge for digestion. Thereby, the potential biogas production is decreased and the great biochemical methane potential by anaerobic digestion and its ability to recover the chemical energy is partly lost (Appels et al., 2008; Chan et al., 2020a; Zhang et al., 2021, 2011).

4.3.6.4. The microbial communities and metabolic way under different substrates

It is clear that the carbon source promotes certain PAO among all the putative PAO detected. Most of lab-scale studies have been conducted with acetate or propionate-based influents and that has led to the proliferation of *Accumulibacter*-the most common PAO of full-scale WWTPs. Apart from that, *Accumulibacter*-enriched sludge has also been reported to proliferate under the butyrate, lactate, glutamate and casein hydrolysate as individual or supplemental carbon source, as well as the fermentation liquid (with pretreatment or system or the integration of SSSF) as carbon source (Liu et al., 2017; Onnis-Hayden et al., 2020; Wang et al., 2019). The recent microbiological advances in full-scale systems fed with real wastewater have shown many other different microbial consortia able to conduct biological P removal, for example, *Tetrasphaera*. *Tetrasphaera*-related bacteria were reported to assimilate glucose, amino acids (e.g. glutamate, glycine, aspartate, casein hydrolysate), and to ferment waste sludge into short-chain fatty acids. They were even speculated to perform a more important role than *Accumulibacter*-PAO due to the high abundance and diversity in full-scale EBPR plants and the devise metabolic way (Fukushima et al., 2007; Qiu et al., 2020, 2019; Zhang and Kinyua, 2020). In addition, some genus *Dechloromonas*, as DPAO, were shown to appear in high proportions in some successful EBPR systems with VFA, or the mixture of amino acids and VFA as carbon sources and store them as PHA (McIlroy et al., 2016; S. Wang et al., 2020; Zhang et al., 2020). *Dechloromonas* are also shown to be enriched in the fermentation of waste sludge system for removing P and N, which could decrease the demand of aeration for aerobic P uptake (Yuan et al., 2016). In fact, the proliferation of DPAO by the fermentation productions of the sludge are reported to improve the removal of P and N, which may show great potential in full-scale WWTPs. Organisms in the *Rhodocyclaceae* are known to involve in P removal in full-scale WWTPs (He and McMahon, 2011; Wang et al., 2019), and it was reported to hold an abundant percentage in lab-scale VFA-fed system (even to 72%), as well as the food waste fermentation liquid-fed system (7%).

Also, the different carbon source uses determine the predominant microbial community when it comes to the competition between PAO and GAO for the substrate (He and McMahon, 2011; Oehmen et al., 2007). GAO could degrade glycogen and store VFA as PHA under anaerobic conditions without involving the poly-P economy. The glycogen consumption and regeneration are highly related to the activity of GAO (Zeng et al., 2002; Zheng et al., 2011). Lower VFA concentrations may favour PAO over GAO, but overload dosage of VFA may favour the proliferation of filamentous bacteria (Haaksman et al., 2020; Li et al., 2016; Ong et al., 2013; Yu et al., 2014). Temperature is also a sensitive parameter that can affect this competition. VFA could enhance the proliferation of *Accumulibacter* under high temperature conditions, and specifically, *Dechloromonas* could be much favoured by propionate (Ong et al., 2014; Qiu et al., 2022; S. Wang et al., 2020). Butyrate also favours more PAO than GAO in warm climates (Wang et al., 2021). In fact, butyrate was also shown to select more *Accumulibacter* than *Defluviicoccus* or *Competibacter* (Begum and Batista, 2014). With glycerol as carbon source, more percentage of *Defluvicoccus* was selected (Guerrero 2012), but Yang et al., (2018) showed glycerol favoured more PAO than GAO. Zhang et al., (2020) claimed that the coexistence of acetate and propionate (with a ratio of 1:1) favoured denitrifying P removal owing to the transformation of PHB and PHV. *Accumulibacter*, *Acinetobacter*, *Dechloromonas* and *Pseudomonas* were the most enriched clades and outcompeted *Competibacter* and *Defluviicoccus*.

Another important aspect is the change in the microbial distribution of the community when the carbon source is replaced. Changing acetate to butyrate gradually induced the increase of *Rhodocyclaceae* and the decrease of *Accumulibacter* and GAO, and a floc-forming specie *Zoogloea* led the dominant position (Wang et al., 2021). Yang et al., (2018) showed the ratio of Gly/VFA (and, thus, GAO) increased when the mixture of acetate and glycerol as carbon source with the ratio less than 1:1. Glycerol as sole carbon source led to the highest GAO abundance. Similarly, Yazıcı and Kılıç, (2016) found that the consumption of glycogen was 5 times higher after the changing of carbon source from acetate to glucose, which could be an indicator for the transformation of a PAO-enriched sludge to GAO.

Even though GAO was regarded as competitors of PAO evidently in EBPR, the coexistence was not proved to be threatened the system performance (Nielsen et al., 2019), and variable metabolic ways of different biomass under diverse carbon sources could allow them to sustain the complex environments and facilitate more robust EBPR.

4.3.7. *Conclusions*

This chapter systematically reviews the effects of carbon source on EBPR systems, especially the carbon utilization strategies and current developing trend due to the deficient COD in the influent. According to the above involved investigations, acetate and propionate are still as the most crucial and efficient substrates to promote the *Accumulibacter*-enriched sludge and assure successful EBPR, and a moderate load of VFA is necessary for favouring PAO. Other substances (e.g. methanol, glycerol, lactate, starch, LCFA…) that are not degraded by PAO directly as sole carbon source may lead the unstable performance and even the system failure. The longer fermentation time for the complex carbon sources to VFA or the mixture of these carbon sources with VFA can support successful lab-scale EBPR performance. However, the recent detection of other PAO-clades opens the door to more diverse carbon utilization. The fermentive PAO-*Tetrasphaera* have the ability to ferment glucose, some amino acids and waste sludge, and the VFA from the fermentation productions can be assimilated by *Accumulibacter*. Applying the fermentation productions from the waste as carbon source has been a popular and environmental solution for the EBPR process, which can not only lead to lower carbon footprint but also reduce large amount of sludge discharge. The full-scale application has shown the efficient P and N removal performance with the utilization of the fermentation products (with abundant VFA) from waste sludge or food waste by some pretreatment (mostly with alkaline pretreatment) strategies and the waste sludge by SSSF. The SSSF can also be a device for fermenting some complex carbon sources from the wastewater to VFA faced with the VFA deficiency problem in real wastewater. Apart from that, taking advantage of the fermentation ability of *Tetrasphaera* to ferment waste sludge as carbon source could be a promising way in the future for the full-scale WWTPs.

Chapter 5:

Overall evaluation of a high-rate EBPR system

5. Overall evaluation of a high-rate EBPR system

5.1. Achieving simultaneous biological COD and phosphorus removal in a continuous anaerobic/aerobic A-stage system

5.1.1. *Abstract*

Recovering energy from wastewater in addition to its treatment is a hot trend in the new concept of water resource recovery facility (WRRF). High-rate systems operating at low solid retention time (SRT) have been proposed to meet this challenge. In this work, the integration of Enhanced Biological Phosphorus Removal (EBPR) in an anaerobic/aerobic continuous high-rate system (A-stage EBPR) was evaluated. Successful P and COD removal were obtained operating at SRT 6, 5 and 4 days treating real wastewater, while a further decrease to 3 days led to biomass washout. The best steady state operational conditions were obtained at SRT = 4d, with high removal percentage of P (94.5%) and COD (96.3%), and without detecting nitrification. COD mineralization could be reduced to 30%, while 64 % of the entering carbon could be diverted as biomass to energy recovery. Regarding nitrogen, about 69±1% of the influent N was left as ammonium in the effluent, with 30% used for biomass growth. The aerobic reactor could be operated at low dissolved oxygen (DO) (0.5 mg/L), which is beneficial to decrease energy requirements. Biochemical methane potential (BMP) tests showed better productivity for the anaerobic sludge than the aerobic sludge, with an optimal BMP of 296±2 mL CH_4/gVSS. FISH analysis at SRT = 4d revealed a high abundance of *Accumulibacter* (33±13%) and lower proportion of GAO: *Competibacter* (3.0±0.3%), *Defluviicoccus* I (0.6±0.1%) and *Defluviicoccus* II (4.3±1.1%).

5.1.2. *Introduction*

Wastewater treatment using conventional activated sludge systems has been beneficial for the whole society and environment for over 100 years. This technology is robust and provides good effluent quality at the expense of high operating costs (due to aeration and sludge management), high organic matter losses (due to mineralization) and large areas for settlers to retain flocculent solids (McCarty et al., 2011b; van Loosdrecht and Brdjanovic, 2014). Moreover, these conventional systems may not recover the potential energy present in the organic compounds of wastewater (Akanyeti et al., 2010). The influent organic matter contains a significant amount of chemical energy stored in the chemical bonds: around 1.9 kWh/m^3 (Bowen et al., 2014; Heidrich et al., 2011; McCarty et al., 2011b), and the amount of energy required for aeration in municipal wastewater treatment plants (WWTPs) is usually in the range 0.3-0.7 kWh/m^3 of wastewater (Jimenez et al., 2015; Shizas and Bagley, 2004). Therefore, there is a great potential for improving energy recovery in classical wastewater treatment systems.

Current research focuses on proposing new WWTP configurations to recover resources (i.e. nutrients and energy) from industrial/urban wastewater, which represents a step forward from conventional treatment. The idea is to transform WWTPs into Water Resource Recovery Facilities (WRRFs). The integration of the bio-refinery concept could be beneficial to solve the current issues of energy-inefficient wastewater treatment processes and resource shortage under a circular economy scenario (Sheik et al., 2014).

One of the golden rules of this innovative concept is to promote the diversion of organic matter to anaerobic digestion to produce biogas. The most common way is to send the primary and secondary sludge from WRRFs to the anaerobic digester to produce methane (5-7 kWh/m^3). Some researchers have suggested that WRRFs could be transformed into self-sustained energy systems by maximizing the organic matter fed to the anaerobic digester (Kartal et al., 2010; Verstraete and Vlaeminck, 2011). In this sense, the most recent configurations have revisited an old concept: the two-stage A/B configuration (Böhnke, 1977; Böhnke et al., 1998). The A-stage is in charge of organic matter removal, while the B-stage faces autotrophic nitrogen removal, since the wastewater entering to the B-stage should not contain organic matter. The A-stage employs a high-rate activated sludge (HRAS) aiming at removing particulate and soluble organics through a combination of adsorption and degradation. Thus, carbon is redirected and concentrated, rather than mineralised, and the solids produced are diverted to energy recovery through anaerobic digestion. HRAS systems allow to recover an important fraction of the chemical oxygen demand (COD) in the influent, typically in the range 50-80% (Sancho et al., 2019), although new processes as the alternating activated adsorption are being developed to increase its performance (Wett et al., 2020). Hence, maximization of energy recovery could be obtained by applying HRAS systems with a relative low SRT (between 0.5 and 4 days) (Jimenez et al., 2015). Moreover, this stage acts as a shock absorber of unexpected toxic loads that could be very detrimental to the B-stage (Smitshuijzen et al., 2016).

Besides organic matter and nitrogen, the discharge of the phosphorus (P) contained in wastewater into the aquatic environment can lead to eutrophication, which is detrimental to both aquatic life and water supply for domestic and industrial fields (Hanhoun et al., 2011). However, the fate of phosphorus in the A/B configuration has not yet been fully studied and chemical precipitation in the tertiary step is currently the most typical option.

Enhanced Biological Phosphorus Removal (EBPR) has been widely used to remove P and organic matter from wastewater for some decades. In EBPR systems, polyphosphate accumulating organisms (PAO) have abilities to store polyhydroxyalkanoate (PHA) anaerobically from organic matter (mainly volatile fatty acids, VFA) and to accumulate P as polyphosphate under anoxic or aerobic conditions.

If EBPR needs to be integrated into an A-stage system, an additional anaerobic reactor has to be included. Building an integrated A-stage EBPR system is not only interesting for achieving simultaneous biological C and P removal (and thus, no further chemical precipitation of P would be required) but also enables the application of other novel advanced configurations. On the one hand, an A-stage EBPR system would be suitable for anaerobic wastage, as biomass purged from the anaerobic reactor would have a high PHA content and therefore a high potential for biogas production and energy recovery (Chan et al., 2020a; Guisasola et al., 2019; Huda et al., 2013). On the other hand, A-stage EBPR systems would be suitable for the implementation of mainstream P-recovery strategies, for instance by precipitating P from a stream from the anaerobic reactor (Acevedo et al., 2015; Guisasola et al., 2019; Larriba et al., 2020; Law and Pagilla, 2018). Then, PAO are excellent candidates for decreasing the degree of organic matter mineralization, for maximizing the amount of biogas produced during anaerobic digestion and for improving plant economics.

Hence, it would be extremely interesting to design and to operate EBPR systems (i.e. anaerobic-aerobic, A/O systems) at very low SRT. However, an A-stage EBPR system requires the inclusion of an anaerobic phase in the A-stage and operating at SRTs higher than those without EBPR. A recent full-scale study at different SRTs have shown a clear correlation between the EBPR performance and the SRT, achieving better and more consistent P removal at SRT below 10 days, with the optimum at SRT = 7d (Onnis-Hayden et al., 2019). Some studies have focused on integrating EBPR in short-SRT systems for removing carbon and P in view of energy efficiency and sustainability of the treatment process. Ge et al., (2013) found more than 80% reduction in COD and P with a high-rate Sequencing Batch Reactors (SBR) process (SRT = 2-3 d). Chan et al. (2017) operated SBRs with EBPR for treating synthetic wastewater with successful results with a SRT down to 3.6 d at 25ºC. Valverde-Pérez et al., (2016) investigated the high rate EBPR system with a SBR, and found that phosphate could be effectively removed with mitigated nitrification at the lowest SRT = 3 d.

On the other hand, one of the most common causes of EBPR failure is the presence of nitrate under anaerobic conditions due to the occurrence of nitrification in the aerobic reactor. Recycling of nitrate to the anaerobic reactor can result in denitrifiers using part of the carbon source and thus outcompeting PAO. Nitrification could be suppressed at low SRT and low DO, however, these two strategies can also affect PAO growth and, thus, a compromise scenario must be found: choosing a SRT high enough for the PAO growth and low enough for more efficient carbon removal and for the washout of nitrifiers. Moreover, the fraction of VFA in the influent wastewater is also a key factor since a low VFA content would require a high anaerobic residence time to convert the organic matter into VFA.

Considering this background, this work faces the integration of EBPR in a continuous A-stage system treating real wastewater. The main objectives of this work are i) to explore the feasibility of the continuous A-stage EBPR system, ii) to investigate the minimum SRT to maintain successful organic matter and P removal in the long-term, iii) to prevent nitrification under low SRT conditions without the addition of any nitrification inhibitor and iv) to gain insight into the possibility of purging from the anaerobic reactor of this novel configuration in view of harvesting biomass with high PHA content to maximize biogas production.

5.1.3. *Materials and methods*

5.1.3.1. Equipment

The pilot plant consisted of two continuous stirred tank reactors (CSTR) (one anaerobic V=19L and one aerobic V=23L) and a settler (25L) as explained in Chapter 3.1 (Figure 3.1). The wastewater used was the primary settler effluent of the WWTP of Manresa (Barcelona, Spain). The average characteristics of the raw wastewater are shown in Table 5.1. Because of the low COD concentration, additional propionic acid was added from a concentrated solution (2300 mgCOD/L) to increase the COD concentration of the influent up to 260±70 mg/L. Thus, the influent consisted of raw wastewater (102 L/d) and propionic acid solution (8.8 L/d). The sludge recycle flow from the settler to the anaerobic reactor was set at 1/3 times of the influent (37 L/d). The hydraulic retention time (HRT) was 9 h considering only the reactors and 14.5 h considering also the settler. The SRT was controlled by manipulating the purge flow rate from the aerobic reactor, although a period with anaerobic purge was also tested. Purging from a reactor instead of the recycle from the settler allows better control of SRT, since the biomass concentration in a reactor is more stable. The reported SRT values (d) (Table 5.2) were calculated with equation (1):

$$SRT = \frac{V_{ana} \cdot X_{ana} + V_{aer} \cdot X_{aer}}{Q_{pur} \cdot X_{aer} + Q_{eff} \cdot X_{eff}} \qquad (1)$$

where V_{ana} and a V_{aer} (L) are the volume of the anaerobic and aerobic reactors, X_{ana} and X_{aer} (g/L) the biomass concentration in these reactors, Q_{pur} and Q_{eff} the purge and effluent flow rate (L/d) and X_{eff} the biomass concentration in the effluent.

Table 5.1. Average composition of the real wastewater used.

Components	Concentration (mg/L)
PO_4^{3-}-P	5.4 ± 1.3
NH_4^+-N	51 ± 8
NO_3^--N	0.03 ± 0.07
NO_2^--N	0
COD_S	130 ± 30

The pH of the aerobic reactor was monitored (HACH CRI5335) but not controlled, being in the range 7.1-7.5. DO was controlled with an on/off controller for the first 40 days. Due to the significant DO fluctuations during the operation process, the uneven distribution of DO in the system could have led to a simultaneous nitrification and denitrification scenario (Chiu et al., 2007). Hence, a proportional-integral algorithm manipulating the aeration flow rate with a mass flow controller (MFC F-201CV, Bronkhorst) was implemented in the aerobic reactor on day 40 to maintain a controlled continuous aeration with a more stable DO value. The system operated at room temperature (21±2ºC) through air conditioning. The whole system was controlled using an industrial PC (Advantech PPC-3190) with a data acquisition card (Advantech PCI1711) running our ADDcontrol software developed in LabWindows CVI (National Instruments).

Table 5.2. SRT, DO and purge flow for the different operational periods.

Period	SRT (d)	DO	Duration (d)	Purge (L/d)
I	Start up	—	0-60	—
II	6	1	61-80	5
III	6	0.5	81-117	5
IV	5	0.5	118-135	7
V	4	0.5	136-148	9
VI	3	0.5	149-158	13
VII	4	0.5	159-166	9

5.1.3.2. Chemical and biochemical analyses

The liquid samples for the concentration analysis of phosphate, COD, ammonium, nitrate and nitrite were withdrawn from the anaerobic reactor, aerobic reactor and effluent almost daily. Sludge samples were withdrawn from both reactors and effluent for the analysis of VSS, TSS and SVI. The detailed process for the liquid and solid analysis was explained in Chapter 3.3.

5.1.3.3. Performance indicators

The P removal efficiency (PRE) was calculated as equation (2):

$$PRE(\%) = (P_{INF} - P_{AER})/P_{INF} \times 100 \qquad (2)$$

where P_{INF} and P_{AER} are the concentrations of P in the influent and the aerobic reactor.

COD removal after the anaerobic reactor (CRE_{ANA}) and total COD removal (CRE) were defined as equation (3) and (4):

$$CRE_{ANA}(\%) = (COD_{FEED} - COD_{ANA})/COD_{FEED} \times 100 \qquad (3)$$

$$CRE(\%) = (COD_{INF} - COD_{AER})/COD_{INF} \times 100 \qquad (4)$$

where COD_{FEED} is the COD concentration entering the anaerobic reactor, which also considers the external recycle contribution, COD_{ANA} is the COD concentration in the anaerobic reactor, COD_{INF} is the influent COD concentration (combination of the raw wastewater and the extra propionic acid dosage) and COD_{AER} is the COD in the aerobic reactor.

5.1.3.4. Fate of COD and nitrogen

The fate of inlet COD and N was calculated by considering the outlet COD versus inlet COD (%) and outlet N versus inlet N (%):

$$COD_{OUT} = COD_{EFF} + COD_{PUR} + COD_{EFFB} + COD_{PURB} \tag{5}$$

Where COD_{EFF} and COD_{PUR} are the filtered COD in the effluent and the purge, and COD_{EFFB} and COD_{PURB} are the COD due to biomass in the effluent and the purge. These values were calculated assuming that the biomass corresponds to the measured VSS and considering the general formula of bacteria $C_5H_7NO_2$, which leads to 1.416 gCOD/gVSS.

Similarly, the fate of the inlet N can be calculated as equation 6:

$$N_{OUT} = \left(NH_4{}^{+}\text{-}N + NO_3{}^{-}\text{-}N + NO_2{}^{-}\text{-}N\right)_{EFF} + \left(NH_4{}^{+}\text{-}N + NO_3{}^{-}\text{-}N + NO_2{}^{-}\text{-}N\right)_{PUR} + N_{EFFB} + N_{PURB} \tag{6}$$

Where $(NH_4^+\text{-}N+NO_3^-\text{-}N+NO_2^-\text{-}N)_{EFF}$ and $(NH_4^+\text{-}N+NO_3^-\text{-}N+NO_2^-\text{-}N)_{PUR}$ are the filtered concentration of ammonium, nitrate and nitrite in the effluent and the purge, and N_{EFFB} and N_{PURB} are the amount of nitrogen in the biomass (calculated from the VSS and the general formula for bacteria: $C_5H_7NO_2$).

5.1.3.5. Batch tests

Four batch activity tests were carried out to investigate the EBPR process activity at different SRTs. The sludge was withdrawn from the aerobic reactor after reaching steady state conditions at each SRT: 6 d (day 96), 5d (day 118), 4d (day 147), and 3d (day 158). The tests were performed in a magnetically stirred vessel (2 L) monitored with pH (Sentix 81, WTW) and DO (Cellox 325, WTW) probes. Anaerobic condition was maintained for the first 3 hours by supplying nitrogen gas, and aerobic condition was achieved with a constant air flow with a mass flow meter (MFC F-201CV, Bronkhorst) for the next 3 hours. The temperature was maintained constant (25°C) by means of a water bath. The pH was not controlled but was maintained at 7.6±0.3 throughout the process. Samples for phosphate, ammonium, nitrate, nitrite and COD were taken every 30 min and immediately filtered with 0.22μm Millipore filters.

5.1.3.6. *Biochemical methane potential tests*

Six sets of anaerobic digestion experiments were studied to investigate the BMP of sludge samples from the anaerobic and aerobic reactor at SRT = 6, 4 and 3 d, and the specific operation for analysis as explained in Chapter 3.3.5.

5.1.3.7. *Microbiological analyses*

FISH analyses coupled with confocal laser scanning microscopy (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) were performed to evaluate the biomass community in the A/O system under different SRT conditions as explained in 3.4.1.

5.1.4. *Results and discussion*

5.1.4.1. *System performance under different SRT*

The A-stage EBPR system was operated under five different SRT conditions by controlling the purge flow rate (Table 5.2). Figure 5.1 compares the aimed SRT to the measured SRT. When the system reached stable and successful simultaneous P and COD removal in Period II (Table 5.2), the SRT was gradually decreased in view of determining the minimum SRT possible with EBPR activity. The lower was the SRT, the shorter to reach stable conditions. The entire operation lasted for 166 days.

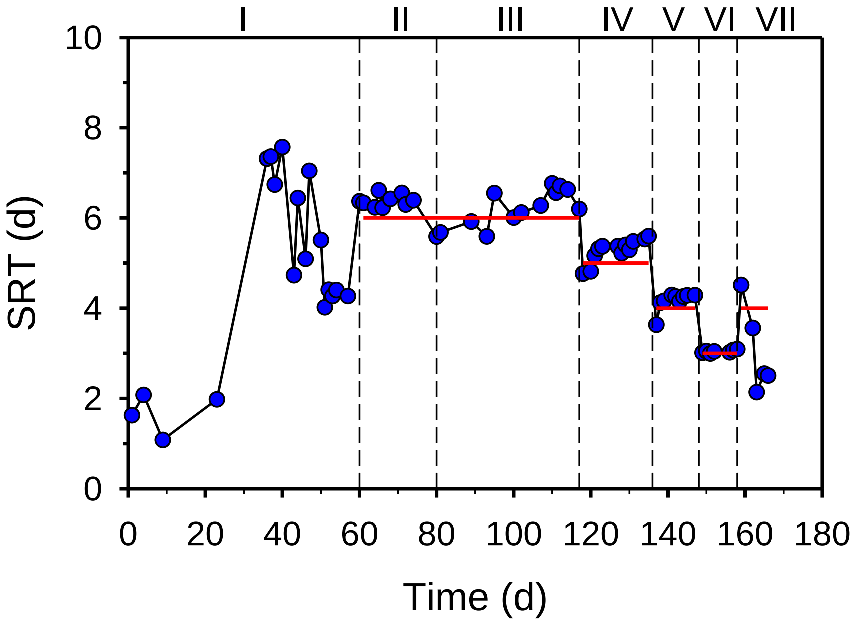


Figure 5.1. Target SRT (solid line) vs actual SRT (circles) throughout the operational period (separated by dashed lines).

Figure 5.2 shows the experimental C, N and P profiles and Table 5.3 the average performance during the whole experimental period. Figure 5.2a shows the experimental

P_{INF}, P_{ANA}, P_{AER} and, P removal efficiency (PRE). PRE increased to almost 60% during the first 20 days indicating the development of the PAO community, while C removal efficiency (CRE) (Figure 5.2b) was stable reaching values above 80%. Ammonia (Figure 5.2c) scarcely decreased in both reactors, nitrite was always below the detection limit and there was no nitrate presence until the day 30 (Figure 5.2d) when nitrification built up. Nitrate is detrimental for the EBPR process due to the competition between PAO and denitrifiers for the carbon source (Guerrero et al., 2012b) and, thus, nitrification should be avoided. Moreover, in the A/B concept, nitrogen is supposed to be removed in the B-stage with lower oxygen and COD requirements. Figure 5.3 shows the evolution of solids in the reactor during the experimental period. It shows that nitrate build-up and a non-adequate DO control led to a decrease in solids concentration. Hence, the DO control system was upgraded (see details in 5.1.3.1) on day 40 to maintain a more stable DO and the purge was stopped for some days to recover the biomass concentration. The reactor recovered and reached a steady state where nitrification was suppressed and PRE and CRE rose above 97% and 90% respectively. The purge was increased to meet the desired SRT = 6 d and the solids in the system began to decrease concomitantly.

From day 61 to 80 (Period II), the system was operated at a SRT = 6 d by maintaining the flow rate of purge at 5 L/d. P_{ANA} was most of the time above 25 mg PO_4^{3-}-P/L (the highest value of P_{ANA} was 34.7 mg/L), while P_{AER} was below 0.6mg/L, and PRE was 94±6% (Table 5.3). In terms of COD, most of the COD was consumed under anaerobic conditions (i.e. COD_{ANA} 38±18 mg/L was much lower than COD_{INF} 268±27 mg/L) and it was stored by PAO, achieving a high CRE (94±5%). Although very satisfactory PRE and CRE were obtained, nitrification appeared again and nitrate concentrations up to 5 mg/L NO_3^-- N were observed.

The DO setpoint was decreased from 1 to 0.5mg/L in view of suppressing nitrification at period III (day 81). Under certain SRT and T conditions, the DO setpoint should be low enough to hinder nitrification but not to prevent PAO growth. Moreover, textbook knowledge states that besides hampering nitrification, low DO favours PAO versus glycogen accumulating organisms (GAO) (Carvalheira et al., 2014a; Chiu et al., 2007).

Table 5.3. P and COD removal performance for each experimental period.

Period	SRT (d)	P_{INF} (mgP/L)	P_{ANA} (mgP/L)	P_{AER} (mgP/L)	PRE (%)	COD_{INF} (mg/L)	COD_{ANA} (mg/L)	COD_{EFF} (mg/L)	CRE_{ANA}(%)	CRE (%)
I	Start up	4.8±0.8	—	—	—	261±49	—	—	—	—
II, III	6	4.3±0.7	28.2±2.6	0.27±0.23	94±6	268±27	38±18	15±14	76±16	94±5
IV	5	4.9±0.4	25.4±3.5	0.15±0.09	97±2	292±10	42±13	11±10	81±6	97±3
V	4	6.7±0.2	32.5±2.6	0.40±0.26	95±3	275±27	46±28	14±14	83±7	96±4
VI	3	6.7±1.3	31.1±2.8	0.73±0.62	85±14	303±41	76±46	32±25	74±16	92±6
VII	4	6.9±0.1	24.8±1.5	0.52±0.67	93±9	326±22	99±10	33±10	58±3	93±9

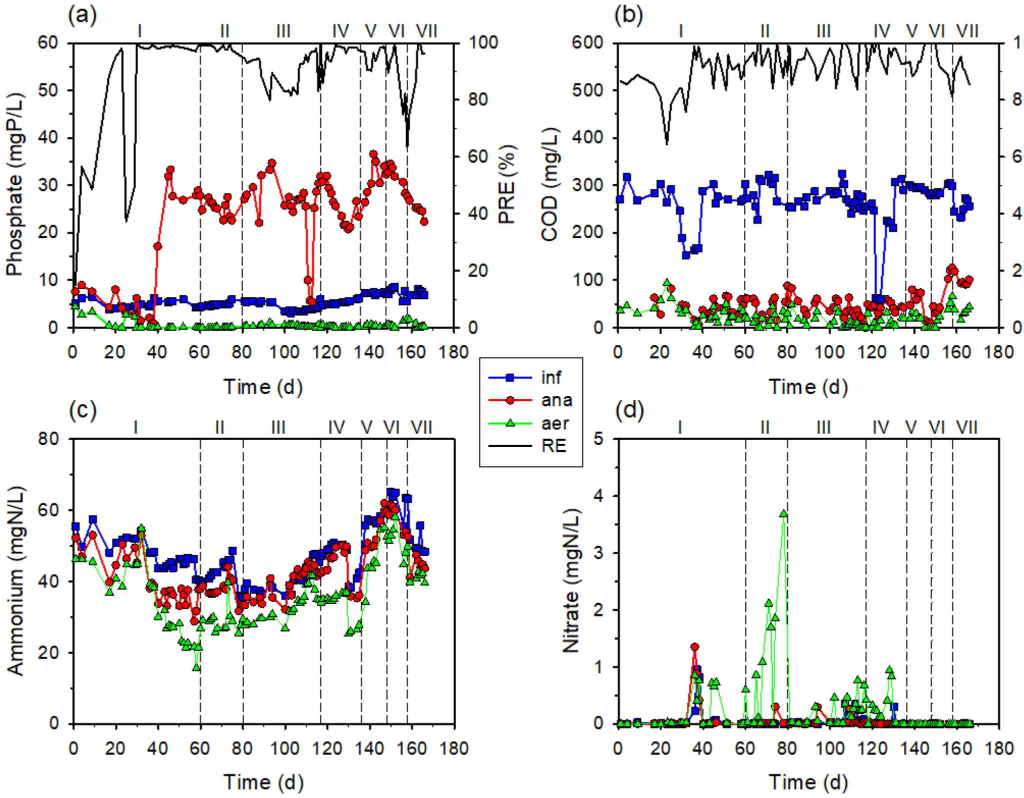


Figure 5.2. Evolution of the main operational parameters in the influent, anaerobic and aerobic reactors and removal efficiency. (a) Phosphate, (b) COD, (c) ammonium and (d) nitrate.

Further optimization of DO setpoint to find the proper operational range may be possible, although the difficulties to maintain a stable DO in full-scale WWTPs under dynamic conditions should also be considered. In our work, P_{ANA} in period III was maintained at 26.0±0.2 mg/L, and PRE was about 94%. Thus, decreasing DO from 1 to 0.5mg/L was not detrimental for PAO activity. The CRE decreased from 98% to 85% in the initial 2 days, but it recovered to 94% after the following 3 days. In conclusion, the absence of nitrate in the effluent indicated that a lower DO was beneficial for avoiding nitrification and a DO setpoint of 0.5 mg/L was adequate. Moreover, lower DO means less aeration and higher energy savings. For instance, Valverde-Pérez et al, (2016) could employ a high rate EBPR system but with a higher DO in the range 2 - 3 mg/L.

The extra addition of propionic acid was removed during days 110 to 113 to study the system performance at low COD_{INF} and without extra VFA. As shown in Figure 5.2a, P_{ANA} decreased dramatically, from 25.6 to 5.4 mg/L. However, due to the internal polymer reserves, it still showed good PRE (about 96%) during these 3 days. CRE_{ANA} showed a similar trend and it decreased sharply from 85% to 39%, but the total CRE was less affected, decreasing from 95% to 84%. Therefore, EBPR activity was still obtained with only raw wastewater but with much lower activity. Nitrate in the effluent showed a little increase during these 3 days. Given the poor P release activity, the external propionic acid dosage was restarted and the system recovered immediately. The concentration of TSS in the reactor was in a range of 1.5 g/L to 2 g/L, and SVI was about 181 mL/g.

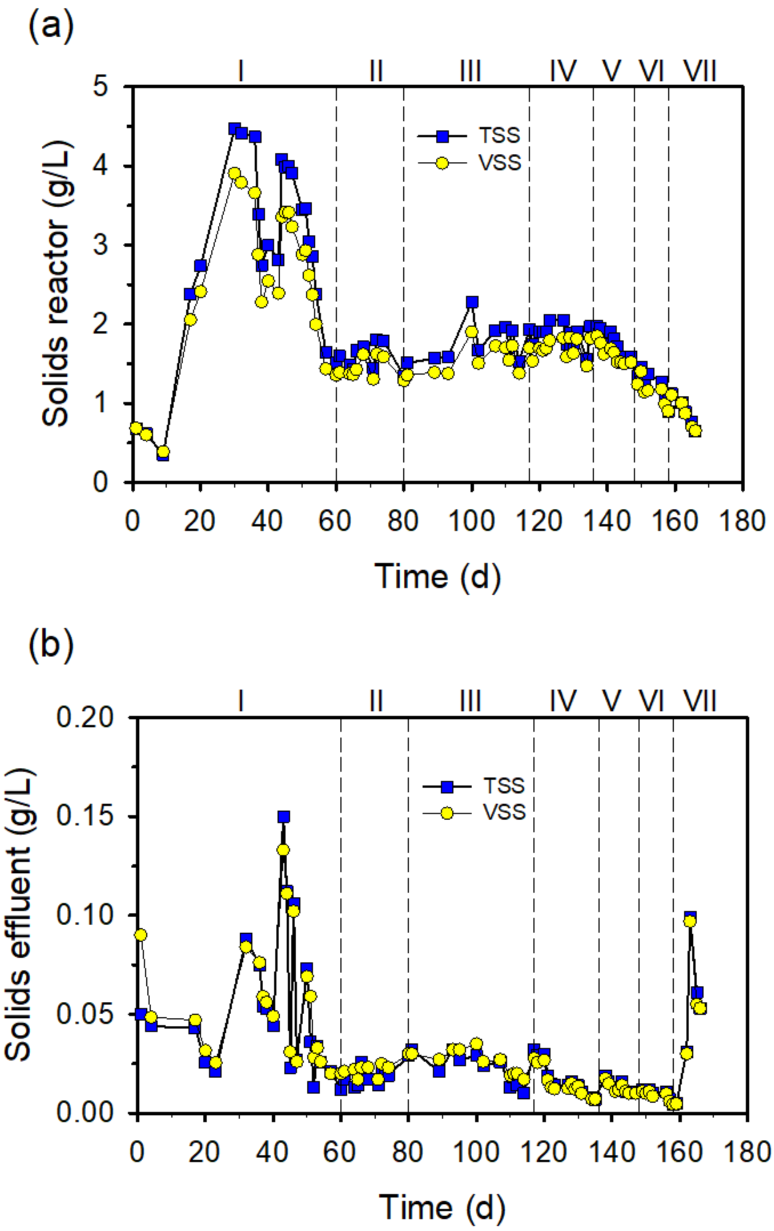


Figure 5.3. Concentration of solids in the reactor (a) and in the effluent (b) during the operational period.

External VFA addition is not feasible in a full-scale scenario but low-loaded wastewaters, such as in this case, are a drawback for implementing biological P removal since it requires organic matter as an electron donor. Then, other actions for *in-situ* VFA production could be applied such as fermentation of primary sludge or increasing the anaerobic reactor residence time for promoting fermentation of complex carbon sources (Guerrero et al., 2015, 2012a; Longo et al., 2015; Luo et al., 2019).

Subsequently, SRT was reduced to 5 days during the next 18 days (Period IV). P_{ANA} decreased from 32.0 to 20.8 mg/L during the first 10 days, and then increased gradually to 26.6 mg/L, but the PRE remained stable between 94% and 99%. Similarly, CRE kept stable with 97±3%. The concentration of TSS in the reactor stabilized at 1.9±0.1 g/L. Hence, decreasing SRT from 6 to 5 days had no significant effect on TSS. Neither nitrate nor nitrite build-up was observed from this point onwards. Therefore, SRT = 5 d was a strict frontier value for the growth of nitrifiers in our system. Valverde-Pérez et al. (2016) also pointed out that operating at SRT = 3.5 d their SBR system with EBPR also prevented nitrification.

In view of the good response of the system, SRT was further reduced to 4 days for the next 13 days (Period V). Successful PRE and CRE were achieved, with 95±3% and 96±4%, respectively. The SVI experienced an increase from 181 to 284 mL/g; however, the system was able to maintain a stable state. It is worth noticing that the objective of the A-stage EBPR is to remove as much P as possible but, considering that there is a subsequent biological B-stage, a minimum P concentration should be guaranteed for the growth of the microorganisms in the B-stage. The effluent P was kept low down to 0.3±0.2 mg/L, which is theoretically high enough to support the growth of the bacteria involved (AOB and anammox) when removing a concentration around 50 mg $N\text{-}NH_4^+$/L.

Finally, the SRT was decreased to 3 days in view of pushing the system to its limits (Period VI). P_{ANA} decreased from 34.5 to 27.6 mg/L, and P_{EFF} kept increasing from 0.4 to 2 mg/L, accompanied with a sharp CRE decay. A high decrease in TSS concentration down to only 0.9 g/L was observed during the first 10 days of Period VI, resulting in a decrease of PRE and CRE to 85.0±14.0% and 92±6% respectively. Meanwhile, SVI increased considerably to 932 mL/g showing very poor settleability, which indicated the excessive growth of filamentous bacteria leading to filamentous bulking. Other works studying EBPR have already reported filamentous bulking sludge (Vaiopoulou et al., 2007; Valverde-Pérez et al., 2016; Yang et al., 2013), which can lead to a poor system performance, inducing the loss of biomass and the failure of system if the clarifier is not over-dimensioned. The factors for filamentous bacteria proliferation could be variable, such as DO, F/M and SRT (Martins et al., 2004; Valverde-Pérez et al., 2016; Yang et al., 2013). Liao et al., (2006) indicated that filamentous bacteria proliferation could be facilitated by low SRT due to relatively unstable microbial communities. The increased SVI of our work was in agreement with the work of Valverde-Pérez et al. (2016), who reported an extreme increase of SVI to 1100 mL/g due to filamentous bulking at SRT = 3.5 d in an SBR.

Given the fragile condition observed at SRT = 3 d, the purge flow rate was decreased and SRT = 4 d was restored (Period VII). However, the system performance could not be recovered successfully because of the heavy loss of biomass. According to our previous experience, when the VSS in the effluent exceeds 0.06 g/L and SVI is higher than 1000 mL/g, the proliferation of filamentous bacteria leads to biomass washout and system failure. As the consequence of system failure, TSS in the effluent increased from 0.031 to 0.061 mg/L and the TSS in the reactor decreased to the minimum of 0.65 mg/L. On the other hand, the ratio of VSS/TSS was also affected by SRT. This ratio kept increasing gradually with the reduction of SRT (Table 5.4). Chan et al. (2017) also observed that low SRTs resulted in higher VSS/TSS in the reactor. In our case, operating at SRT = 3 d led to the failure of system with the maximum VSS/TSS of 0.97±0.03. The value of VSS/TSS tending to 1 was a red flag of the system that indicated that PAO were being washed out. In conclusion of the whole operational period, our compromise scenario of SRT = 4 d was the minimum SRT for PAO growth, low enough for the washout of nitrifiers and without high filamentous bulking under steady state. However, a warning should be given about the increased risk to observe bulking if a too low SRT is used in this type of A-stage EBPR system.

Table 5.4. Average VSS/TSS ratio and settleability at different SRT

Period	SRT (d)	VSS/TSS	SVI
II, III	6	0.89±0.03	135±25
IV	5	0.91±0.03	345±124
V	4	0.93±0.04	505±248
VI	3	0.94±0.02	1040±216
VII	4	0.97±0.03	—

5.1.4.2. *Batch tests under different SRT*

Figure 5.4 shows four batch tests with sludge obtained at SRT = 6, 5, 4 and 3 d. Similar trend lines were observed in the four experiments. Most of the COD was consumed after 180 min under anaerobic conditions, and the remaining COD was consumed after 60 min of aerobic phase. At the end of the aerobic phase, P was depleted. In terms of nitrogen, very slight ammonia consumption was observed in both the anaerobic and aerobic phases, but there were no significant changes in nitrite and nitrate concentration. Therefore, no nitrification was observed during the batch tests, which was in agreement with the performance of the A/O system.

Table 5.5 shows the PAO activity indices from the four batch tests under different SRT. P-release rate increased from 0.08 to 0.27 mgP/gVSS·min with the reduction of SRT from 6 d to 3 d. However, P-uptake rate showed a different behaviour. It increased from 0.12 to 0.26 mgP/gVSS min with the reduction of SRT from 6 to 4 d, and decreased dramatically to the lower value of 0.09 mgP/gVSSmin with SRT = 3 d. These results are consistent with the higher PRE and CRE values obtained under SRT = 4 d when compared to 3 d. Furthermore, the ability of PAO to release P under short SRT seems to be more robust than the P-uptake ability.

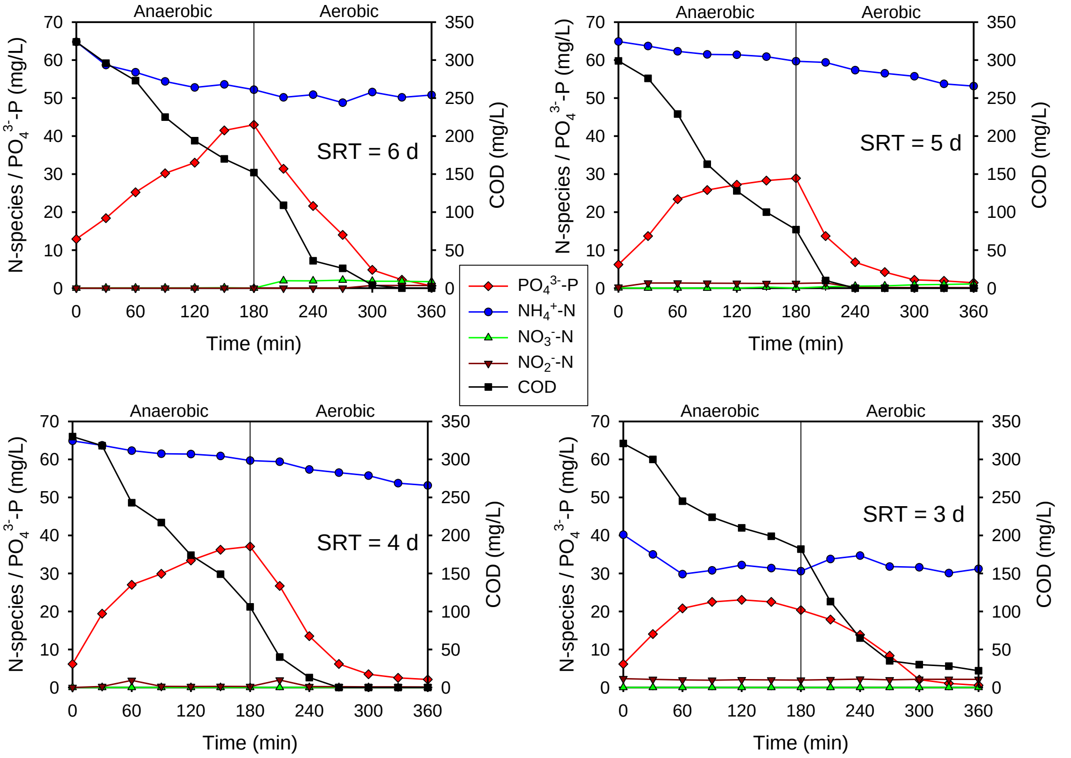


Figure 5.4. Profiles of P, N, and COD in batch tests with sludge withdrawn from the aerobic reactor at different SRT.

Table 5.5. PAO activity in batch tests with sludge withdrawn from the aerobic reactor at different SRT.

Period	Operation day	SRT (d)	PO$_4^{3-}$-P (mgP/L)	P release rate (mgP/gVSS min)	P uptake rate (mgP/gVSS min)
III	96	6	43	0.08	0.12
IV	118	5	29	0.19	0.24
V	147	4	37	0.23	0.26
VI	158	3	23	0.27	0.09

5.1.4.3. *Effect on BMP of SRT and aerobic or anaerobic biomass*

The A-stage EBPR integration enables the implementation of novel strategies in view of process optimisation. For instance, biomass could be purged from the anaerobic phase and, since it has a higher PHA content, it could be used to increase energy recovery as biogas rather than being oxidised in the aerobic phase. However, the stability of the A-stage EBPR system with this anaerobic purge should be demonstrated. Thus, anaerobic purging was implemented for 15 days to prove its feasibility (Figure 5.5). The system was operated with aerobic purging during the first 50 days with successful PRE (97-100%)

and CRE (around 98%) (Figure 5.5 a and b). The operation was stable with VSS = 2.8±0.1 g/L and SVI = 98±7 mL/g (Figure 5.5c). On day 50, the purge was switched to the anaerobic reactor and the system was operated at SRT = 6 d by maintaining the purge flow rate at 5 L/d. PRE remained stable at 97±2%. CRE was about 96% during the first days, then experienced fluctuations from day 57, which could be due to the increase of COD concentration of raw wastewater. However, CRE was always above 90% and COD_{EFF} was less than 45 mg/L. The concentration of VSS in the reactor showed a little decrease during the first days but it kept stable around 2.5 g/L. The SVI experienced an increase to 260 mL/g, but EBPR performance was not affected. Hence, steady biological P and COD removal without nitrification could be maintained despite the anaerobic purging. The COD mass balance (Table 5.6) showed that COD mineralization with anaerobic purging was 42±2%, which is in the same order than the 46±12% obtained with aerobic purge at the same SRT = 6 d.

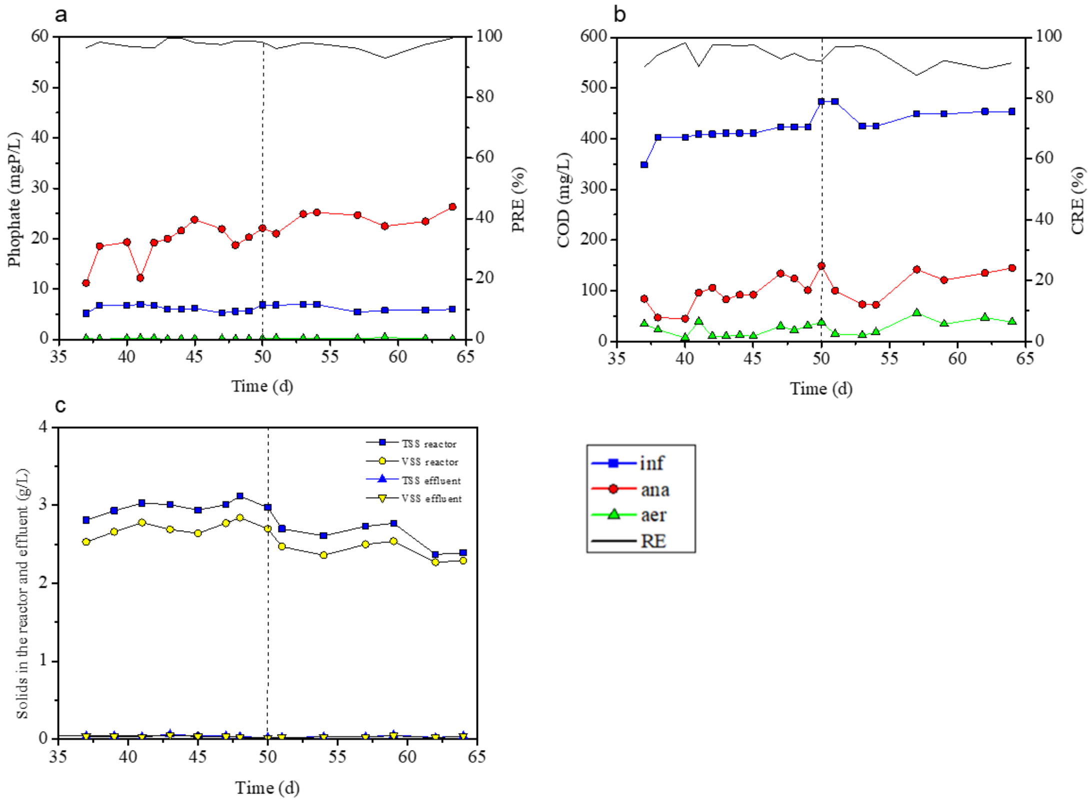


Figure 5.5. Evolution of the main operational parameters in the influent, anaerobic and aerobic reactors and removal efficiency during a restart operating period with anaerobic purge from day 50. (a) Phosphate, (b) COD, (c) VSS and TSS in the reactor and effluent.

Table 5.6. COD mass balance during a start-up operating period with anaerobic purge. All COD terms are expressed as a percentage with respect to the influent COD. EFF and PUR are the filtered COD in the effluent of the plant and in the purge stream; EFFB and PURB are the COD contained in the biomass of these streams; OUT is the total COD in these streams, calculated as the sum of the four previous terms; COD_{MINER} is the percentage of COD mineralized to CO_2.

Period	EFF (%)	PUR (%)	EFFB (%)	PURB (%)	OUT (%)	MINER(%)
COD	7±4	0.4±0.2	8±3	43±2	58±5	42±2

Once the stability of the system with anaerobic purge was demonstrated, the possible BMP enhancement that could be achieved with the different sludge available in the A-stage EBPR was evaluated. Figure 5.6 shows the BMP tests obtained with sludge purged from the anaerobic and aerobic reactors at different SRT conditions, while Table 5.7 details a summary of the average results obtained. The anaerobic sludge had the highest BMP (296±2 mL CH_4/gVSS) at SRT = 4 d, followed by SRT = 3 d (286±7 mL CH_4/gVSS) and 6 d (231±7 mL CH_4/gVSS) (Table 5.7). The same trend was obtained with aerobic sludge at each SRT. Theoretically, the lower the SRT, the more carbon can be stored as PHA instead of being mineralized in high rate systems (Bolzonella et al., 2005) and therefore more carbon is diverted to anaerobic digestion. Chan et al. (2020) observed large BMP variations under different SRT conditions, from 401 to 306 mL CH4/gVSS (at SRT = 5 to 10 d). Bolzonella et al. (2005) found that a reduction of SRT from 20 to 10 days resulted in an increase of biogas production of about 25%. In our work, the highest BMP was obtained at SRT = 4 d, since the amount of PAO in sludge at SRT = 3 d was not enough to uptake the initial organic matter, and hence, the PHA content was probably lower. This observation is consistent with the better system performance and higher PAO activity observed in the batch tests at SRT = 4 d.

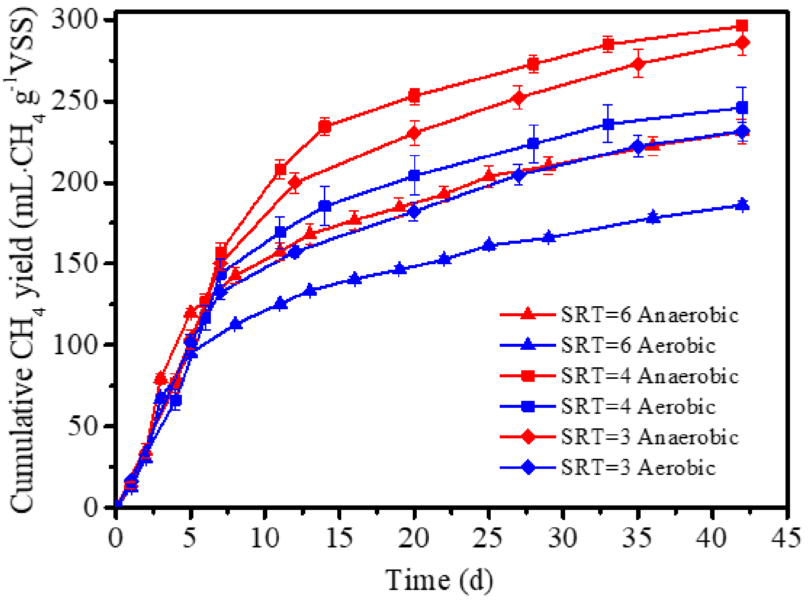


Figure 5.6. Biochemical methane potential obtained with sludge withdrawn from the anaerobic and aerobic reactors under different SRT conditions.

Our anaerobic sludge had 20.4% more BMP than aerobic sludge at different SRTs (Table 5.7) because of the higher PHA content. Wang et al. (2015) also found that higher PHA content led to higher BMP: their sludge with 14.3% of PHA had 150% more BMP than that with a 2.1% PHA content. Chan et al. (2020) also demonstrated with an EBPR-SBR system that BMP could be enhanced by 21.8% by using anaerobic sludge instead of aerobic sludge at SRT = 5 d.

Table 5.7 Biochemical methane potential tests using sludge from the anaerobic and aerobic reactors at different SRT.

Period	Day	SRT (d)	BMP (mL CH_4 / gVSS)		Mean Enhancement percentage[a] (%)	Initial methane production rate[b] (mL CH_4·/ gVSS·d)	
			Anaerobic sludge	Aerobic sludge		Anaerobic sludge	Aerobic sludge
III	102	6	231±7	186±2	22.6	19.0±0.6	15.0±0.1
V	147	4	296±2	246±13	20.4	22.0±0.8	20.0±1.3
VI	158	3	286±7	231±6	23.5	21.0±0.8	19.0±0.6

[a] Anaerobic sludge compared with aerobic sludge at the same SRT condition
[b] The first 10 days of anaerobic digestion

The initial methane production rate (IMPR) varied significantly with different SRT. When SRT was 6 d, the IMPRs were the lowest, 19.0±0.6 and 15.0±0.1 mL CH_4/gVSS·d with anaerobic and aerobic sludge. The highest values (i.e. 22.0±0.8 and 20.0±1.3 mL CH_4/gVSS·d) were obtained at SRT = 4 d. Chan et al. (2020) observed that the IMPR was relative to the PHA content because PHA could be more easily degraded than other compounds during anaerobic digestion, and proposed a positive correlation between BMP and PHA, irrespective of SRT and anaerobic or aerobic biomass. Wang et al. (2016) also pointed out higher PHA content had superior degradability in the initial 8 days. Hence, it is explainable in our work that applying SRT= 4 d the observed IMPR was higher.

5.1.4.4. The fate of COD, N and P in the system

The inlet COD had three different fates: i) as dissolved COD in the plant effluent and purge, ii) the formation of biomass exiting by either the effluent or the purge and iii) the carbon mineralization in the system. Table 5.8 summarises all these fractions for the different periods. The total fraction of (i) and (ii) was expressed as total outlet COD (COD_{OUT}) following the methodology detailed in section 2.4. COD_{OUT} increased with the decrease of SRT except for Period VII due to its unstable operation. When operating the reactor at SRT = 6 d, 54±12% of the inlet COD was quantified in the outlet and 46±12 was mineralized. However, when working at SRT = 4 d (Period V) only 30±5% was mineralised, while 58±5% could be recovered as biomass in the purge and an additional 6±1% as biomass in the effluent. Hence, if a better biomass separation system could be implemented in the effluent, a maximum of 64±6% of the COD in the influent could be recovered as biomass that could be redirected to biogas production. These results fit well in the frame of developing innovative technologies for WRRFs aiming at energy autarky and resource recovery, as the system was able to biologically remove organic matter and phosphorus and redirecting 64±6% of the COD to energy recovery, rather than being oxidized to CO_2.

Table 5.8. COD mass balance during the whole operating period. All COD terms are expressed as a percentage with respect to the influent COD. COD_{EFF} and COD_{PUR} are the filtered COD in the effluent of the plant and in the purge stream; COD_{EFFB} and COD_{PURB} are the COD contained in the biomass of these streams; COD_{OUT} is the total COD in these streams, calculated as the sum of the four previous terms; and COD_{MINER} is the percentage of COD mineralized to CO_2.

Period	SRT (d)	COD_{EFF} (%)	COD_{PUR} (%)	COD_{EFFB} (%)	COD_{PURB} (%)	COD_{OUT} (%)	COD_{MINER} (%)
II, III	6	5±4	0.2±0.2	13±3	36±9	54±12	46±12
IV	5	4±3	0.3±0.3	6±3	55±5	66±6	34±6
V	4	6±4	0.4±0.3	6±1	58±5	70±5	30±5
VI	3	8±7	1.0±0.9	4±1	68±16	80±10	20±10
VII	4	9±3	0.9±0.3	20±14	32±5	61±13	39±13

This carbon redirection improvement was in the range of previous works in A-stage system without EBPR implementation (Akanyeti et al., 2010; Jimenez et al., 2015), but in our case the system was also able to remove biologically P. When the SRT was decreased to 3 d, the COD_{MINER} decreased to 20±10% and COD_{OUT} increased to 80±10%; however, these results were obtained when the plant was operating with bulking and unstable performance with lower EBPR activity.

Regarding the implementation of P-recovery strategies in this configuration, the utilization of the anaerobic mixed liquor with a high concentration of P is the best alternative. In the case of Period V, if assumed an average P concentration in the anaerobic reactor of 32 mgP/L and a purge flow of 9 L/d, around 39% of the P in the influent would be contained in the purge stream, This P could be recovered, for example, via precipitation, as struvite (Jabr et al., 2019; B. Li et al., 2019a; Rittmann et al., 2011) or calcium phosphate (Law and Pagilla, 2018). Recovering P from the anaerobic supernatant, would also lead to a reduction of the P content in the side-stream from anaerobic digestion, which usually represent a significant fraction of the P treated in the secondary treatment (Kassouf et al., 2020). The long-term operation of SBRs with high EBPR activity and simultaneous P extraction from the anaerobic supernatant has been demonstrated in previous works, reaching values up to 60% of recovery (Guisasola et al., 2019; Larriba et al., 2020).

The fate of influent N is either nitrification or biomass assimilation. As shown in Table 5.9, the total outlet N (N_{OUT}) could account for all of the influent N at each SRT operation conditions when the system was at steady state. N_{OUT} was slightly higher than 100% in all the periods, probably because some organic nitrogen was not considered in the calculation of the N load in the influent. Importantly, no significant nitrification was observed in the system. Looking into detail to the results of Period V, 27±1% of the nitrogen was used for biomass growth, and 75±1% remained in the effluent, mostly as ammonium (69±1%). Therefore, a desired relative high concentration of ammonia in the A-stage effluent was left for the following B stage, which could be more energy-saving compared with conventional nitrification/denitrification processes. These values improved the results reported by Valverde-Pérez et al., (2016b), where about 40% of the ammonia was detected in the effluent when their EBPR system was operated at SRT = 3 d, while the rest of N removed was attributed to assimilation.

Overall, the results presented in this section demonstrate the feasibility of integrating EBPR into a continuous anaerobic/aerobic A-stage system, obtaining excellent COD and P removal with high redirection of COD to biomass (64±6%), phosphorus to a concentrated stream of phosphate (39%) and ammonium to the subsequent B-stage (69±1%).

Table 5.9. N mass balance during the whole operating period. All N terms are expressed as a percentage with respect to the influent N. N_{EFF} and N_{PUR} are the soluble N in the effluent of the plant and in the purge stream; N_{EFFB} and N_{PURB} are the N contained in the biomass of these streams; NH_4^+-N_{EFF} is ammonium nitrogen in the effluent; and N_{OUT} is the total N contained in the previous streams.

Period	SRT (d)	N_{EFF} (%)	N_{PUR} (%)	N_{EFFB} (%)	N_{PURB} (%)	NH_4^+-N_{EFF} (%)	N_{OUT} (%)
II, III	6	67±12	4±1	10±5	25±11	62±13	106±3
IV	5	66±4	5±0	3±1	29±4	62±4	103±5
V	4	75±1	6±0	3±0	24±1	69±1	106±1
VI	3	72±3	10±0	1±1	25±4	66±3	108±7
VII	4	77±4	7±0	8±5	17±4	69±3	108±6

5.1.4.5. Evolution of PAO and GAO presence in the microbial community

The biomass distribution of our system was evaluated by FISH labelling and quantification (Table 5.10). Four groups of probes were used with sludge withdrawn from the aerobic reactor at days 147 (SRT = 4 d, Period V) and 157 (SRT = 3 d, Period VI). *Accumulibacter* PAO (targeted by PAOMIX) led the predominant position (33±13%) at SRT = 4d. Regarding the PAO competitors, *Competibacter* (labelled by GAOMIX) accounted for 3.0±0.3%, and *Defluviicoccus* (targeted by probes DFIMIX and DFIIMIX) were 0.6±0.1% (Cluster I) and 4.3±1.1% (Cluster II). The presence of *Defluviicoccus* in our system was favoured by the use of propionic acid as additional carbon source, which is reported to be a proper substrate for these microorganisms (Oehmen et al., 2010; Tayà et al., 2013a). These results show that a certain amount of *Competibacter* and *Defluviicoccus* could coexist with *Accumulibacter* at SRT = 4 d without affecting the system performance. A similar microbial community was reported by Ong et al. (2014), who reported good EBPR performance under the coexistence of *Accumulibacter* and *Competibacter* at different temperatures from 24 to 32ºC. These coexistence has also been reported at full-scale EBPR WWTPs with stable performance (Lanham et al., 2013; Nielsen et al., 2019; Saunders et al., 2003; Stokholm-Bjerregaard et al., 2017) and at three full-scale tropical WWTPs (Qiu et al., 2019).

Table 5.10. FISH quantification in sludge samples withdrawn from the aerobic reactor at days 147 and 157. Four specific groups of probes were used: PAOMIX, GAOMIX, DFIMIX and DFIIMIX.

Period	SRT (d)	Operation day	PAOMIX (%)	GAOMIX (%)	DFIMIX (%)	DFIIMIX (%)
V	4	147	33±13 (n=70)[a]	3.0±0.3 (n=48)	0.6±0.1 (n=35)	4.3±1.1 (n=38)
VI	3	157	22±2 (n=58)	1.6±0.3 (n=36)	4.2±0.5 (n=53)	7.9±1.6 (n=27)

[a] The number in brackets indicates the number of images used for the quantification.

The microbial community experienced a remarkable shift when decreasing SRT from 4 to 3 days. A decrease of *Accumulibacter* and *Competibacter* was detected, with the percentages about 22±2% and 1.6±0.3% respectively at SRT = 3 d, which corresponded with the decrease in EBPR performance. Even faced with the washout of biomass at SRT = 3 d, *Accumulibacter* still showed the highest abundance. Interestingly, both *Defluviicoccus* Cluster I and *Defluviicoccus* Cluster II showed a great increase, with the proportions of 4.2±0.5 and 7.9±1.6% respectively, which could be correlated to the sludge bulking. Nittami et al. (2009) pointed out that some *Defluviicoccus* species are filamentous bacteria which are responsible for bulking problems in full-scale EBPR plants treating domestic wastewater.

An example of FISH images of the four microbial groups mentioned above are shown in Figure 5.7 and 5.8. The morphologies of the biomass at SRT = 3 d (a2 to d2) revealed more filamentous bacteria compared to the biomass at SRT = 4 d (a1 to d1), which was in accordance with the system performance and more sludge bulking at SRT = 3 d. Li and Stenstrom, (2018) pointed out the biomass population succession or the production of extracellular polymers substances could be induced by changing SRT, and further affected solid settleability. Moreover, *Defluviicoccus* Cluster II (d1 and d2) exhibited a filamentous overgrowth and owned more abundance compared with *Competibacter* and *Defluviicoccus* Cluster I. Burow et al., (2007) found that *Defluviicoccus* Cluster I and Cluster II could use propionic as substrate and *Defluviicoccus* Cluster II showed relatively higher than *Competibacter* and *Defluviicoccus* Cluster I in full-scale EBPR systems, which was consistent with our results. Though there was a limitation at SRT = 3 d in our case, the above results could provide some useful information to researchers who are keen on energy efficient, resource recovery and sludge bulking control of wastewater treatment. Specifically, the findings could establish some fundamental references for the application of EBPR in high-rate A-stage systems treating real wastewater.

5.1.4.6. Limitations and practical implications of the A-stage-EBPR

The A-stage of A-B configurations aims to divert the maximum amount of COD to anaerobic digestion to increase energy recovery. Nitrogen is autotrophically removed in the B-stage, but phosphorus is usually removed by chemical precipitation. This study proposes the use of a modified A-stage including an anaerobic reactor to implement EBPR (A-stage EBPR). The feasibility to operate with successful COD and biological P-removal is demonstrated under pilot plant operation with optimal operation at SRT = 4 d and DO setpoint of 0.5 mg/L. This stable operation was demonstrated at room temperature (21±2ºC) and accurate DO control.

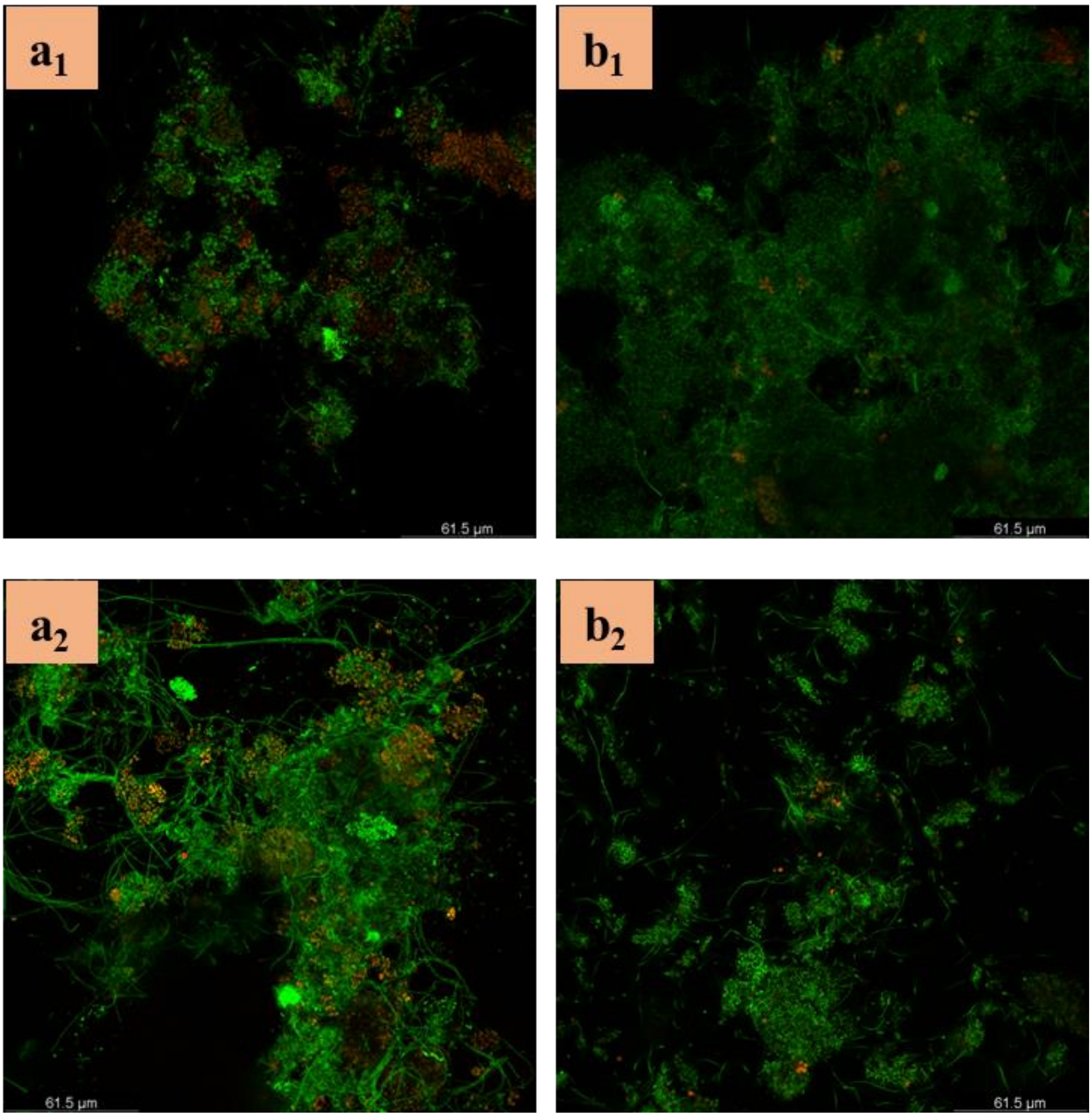


Figure 5.7. Example of FISH CLSM micrographs for the biomass of the continuous anaerobic/aerobic A-stage system after 147 days (SRT=4d, a1 and b1) and 157 days (SRT=3d, a2 and b2) of operation. Green: all bacteria; red: *Accumulibacter* (a1, a2) and *Competibacter* (b1, b2).

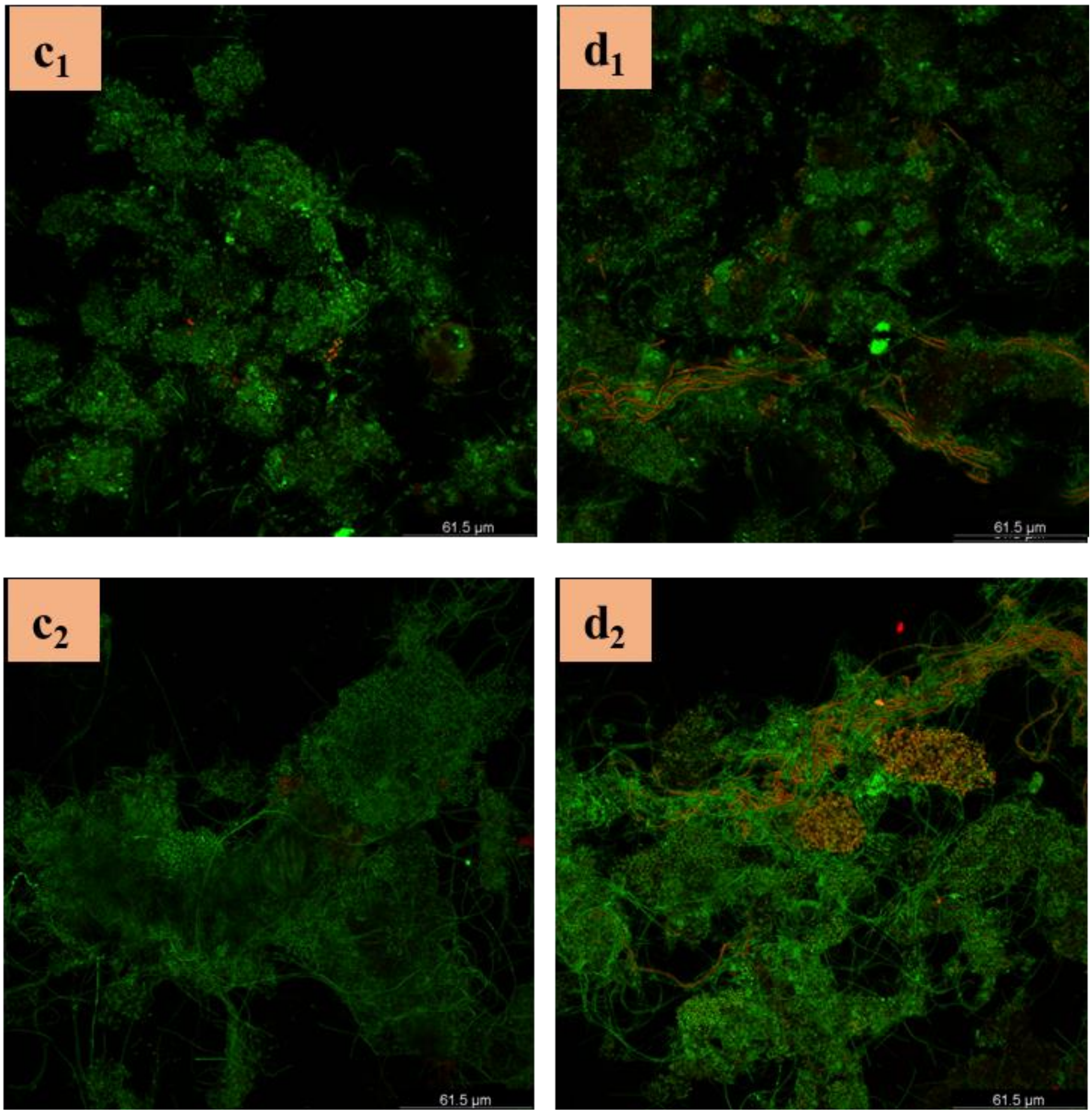


Figure 5.8. Example of FISH CLSM micrographs for the biomass of the continuous anaerobic/aerobic A-stage system after 147 days (SRT=4d, c1 and d1) and 157 days (SRT=3d, c2 and d2) of operation. Green: all bacteria; red: *Defluviicoccus* cluster I (c1, c2) and *Defluviicoccus* cluster II (d1, d2).

If applied in a full-scale environment, SRT should be seasonally adapted to the specific temperature, as PAO require a higher minimum SRT at lower temperature to avoid washout (Chan et al., 2020b). This selected SRT should be able to maintain PAO activity while preventing nitrifiers growth. Achieving these conditions at lower temperature does not seem difficult, since nitrifiers have a high degree of temperature dependency (Henze et al., 2000) but PAO temperature dependency has been reported to be medium (Henze et al., 2000) or low (Chan et al., 2020b). Operating at low DO of 0.5 mg/L also gives additional savings due to the lower aeration required, but a good DO control system should be implemented to avoid oxygen limitations in the aerobic reactor.

Settling problems are usually found in A-stage systems operated at low SRT and iron salts addition is a typical solution proposed for these systems. However, the proposed A-stage EBPR allows obtaining a good settleability, except in the case of working at very low SRT. SRT lower than 4 d should be avoided as it can lead to PAO washout by: inducing the proliferation of filamentous bacteria, reducing biomass settleability and unintentionally decreasing VSS and SRT.

When wastage from the anaerobic reactor is implemented, an additional settler would be needed to treat this low flowrate stream. The effluents of this settler would be a concentrated biomass stream to be diverted to the anaerobic digester and a P-concentrated stream to implement a P recovery strategies as for example using struvite precipitation (Larriba et al., 2020).

Implementing A-stage EBPR requires wastewater with enough organic matter to drive EBPR. Metcalf and Eddy (Tchobanoglous et al., 2013) recommend some threshold ratios for the design of WWTP configurations with COD, N and P removal: readily biodegradable COD (rbCOD) content of 6.6 g rbCOD/gNO3-N and 10 g rbCOD/g P. Since N removal is not required in the A-stage, COD requirements decrease. However, if these requirements are not satisfied, external carbon source addition or fermentation of primary sludge to produce extra VFA would be required.

A-stage EBPR is an evolution of the A-B systems that are currently under research. Lower energy recovery would be obtained *a priori* due to the higher SRT (4d) needed when compared to the conventional reported A-stage systems (0.1-2d) (Jimenez et al., 2015). However, mineralization for a conventional A-stage is reported to be in a wide range depending on SRT: 14% (0.1d), 37% (0.5d) and 67% (2d) (Jimenez et al., 2015), while a range from 30±5% (4d) to 46±12% (6d) was reported in this work for the A-stage EBPR. Hence, this theoretical decrease in energy recovery can be compensated by the higher PHA content in the sludge, with the additional advantages in the A-stage EBPR of higher biomass stability, avoidance of tertiary chemical P removal and the possibility to implement P-recovery strategies using the anaerobic purge stream.

Another possible implementation would be retrofitting an overloaded Ludzack-Ettigner configuration for COD/N removal into an A-stage system for COD and P removal that should be complemented with an additional B-stage for autotrophic N removal. This would lead to a configuration with lower COD requirements for nutrient removal and higher energy recovery.

5.1.5. *Conclusions*

This work shows the successful implementation of an innovative concept that integrates EBPR in a continuous anaerobic/aerobic A-stage system at low SRT conditions (A-stage EBPR).

- The best performance was obtained operating the system at SRT = 4 d and the aerobic reactor at a DO setpoint of 0.5 mg/L, achieving P and COD removal efficiencies of 94.5% and 96.3% and avoiding nitrification.
- Bulking problems can appear at low SRT. Biomass washout was observed when decreasing the SRT to 3 d due to the poor settleability of solids.
- Higher biogas production is obtained using anaerobic sludge, 20%-24% higher BMP than aerobic sludge under the same conditions. The highest BMP was 296±2 mL CH_4/gVSS using anaerobic sludge at SRT = 4 d.
- A high fraction of COD in the influent can be diverted to biomass for energy recovery (64±6% at SRT = 4 d).
- About 30% of inlet N is consumed for biomass growth, leaving a significant fraction 69±1% as ammonium for the subsequent B-stage.
- The FISH analysis at SRT = 4 d showed *Accumulibacter* predominance (33±13%), much higher than its competitors *Competibacter* (3.0±0.3%), *Defluviicoccus* Cluster I (0.6±0.1%) and *Defluviicoccus* Cluster II (4.3±1.1%). Both *Defluviicoccus* Clusters significantly increased when SRT was decreased to 3 d (4.2±0.5% for Cluster I and 7.9±1.6% for Cluster II).

5.2. Exploring the stability of an A-stage-EBPR system for simultaneous biological removal of organic matter and phosphorus

5.2.1. *Abstract*

This work evaluates the performance and stability of a continuous anaerobic/aerobic A-stage system with integrated enhanced biological phosphorus removal (A-stage-EBPR) under different operational conditions. Dissolved oxygen (DO) in the aerobic reactor was tested in the 0.2-2 mgDO/L range using real wastewater amended with propionic acid, obtaining almost full simultaneous COD and P removal without nitrification in the range 0.5-1 mgDO/L, but failing at 0.2 mgDO/L. Anaerobic purge was tested to evaluate a possible mainstream P-recovery strategy, generating a P-enriched stream containing 22% of influent P. COD and N mass balances indicated that about 43% of the influent COD could be redirected to the anaerobic digestion for methane production and 66% of influent NH_4^+-N was discharged in the effluent for the following N-removal B-stage. Finally, when the system was switched to glutamate as sole carbon source, successful EBPR activity and COD removal were maintained for two months, but after this period settleability problems appeared with biomass loss. Microbial community analysis indicated that *Propionivibrio*, *Thiothrix* and *Lewinella* were the most abundant species when propionic acid was the carbon source and *Propionivibrio* was the most favoured with glutamate. *Thiothrix, Hydrogenophaga, Dechloromonas* and *Desulfobacter* appeared as the dominant polyphosphate-accumulating organisms (PAOs) during the whole operation.

5.2.2. *Introduction*

Biological nutrient removal (BNR) processes are widely investigated and applied in wastewater treatment plants (WWTPs) for removing phosphorus (P), nitrogen (N) and COD from wastewater so that eutrophication caused by overloaded nutrient discharging to the water bodies is prevented (Welles et al., 2015; Yuan et al., 2012). Enhanced biological phosphorus removal (EBPR), based on the proliferation of polyphosphate-accumulating organisms (PAO), is an environmental-friendly and cost-effective technology for P removal in WWTPs (Oehmen et al., 2007; Tchobanoglous et al., 2014) PAO are promoted by alternating anaerobic and aerobic/anoxic conditions (Comeau et al., 1987). Under anaerobic conditions, PAO take up carbon sources (normally volatile fatty acids, VFA) and store them as polyhydroxyalkanoates (PHA). The required reductive power and energy for this process come from the degradation of glycogen and the hydrolysis of polyphosphate (poly-P) to phosphate, which is released into the mixed liquor. In the subsequent aerobic/anoxic condition, PAO oxidize their internal PHA reserves and obtain the required energy to grow, to regenerate their glycogen pools and to uptake phosphate as poly-P (Satoh et al., 1998; Smolders et al., 1994). EBPR outcompetes chemical P removal in terms of sustainability and costs at the expense of an increased degree of complexity of plant operation (Tchobanoglous et al., 2014).

The recent paradigm shift in the field of environmental engineering paves the way for the integration of novel P recovery strategies that are even more sustainable than EBPR, since P is a non-renewable resource that is expected to cause limitations in the next century (Cordell et al., 2009; Desmidt et al., 2015; Rittmann et al., 2011). Mainstream P-recovery from the anaerobic supernatant of EBPR systems is a promising methodology when compared to P-recovery from other side streams (e.g. effluent from anaerobic sludge digestion) because, theoretically, a higher percentage of P can be recovered (Zhang et al., 2022). Moreover, mainstream P-recovery can be linked to anaerobic biomass purging which would be beneficial in view of carbon recovery processes: i) anaerobic sludge shows higher biochemical methane potential when compared to aerobic sludge and ii) anaerobic sludge contains a higher percentage of PHA and, thus, could also be a precursor of bioplastics after an extraction process (Chan et al., 2020a; Larriba et al., 2020; Wang et al., 2016).

On the other hand, one of the latest configurations for energy recovery from wastewater is the two-stage A/B process (Boehnke and Diering, 1997; Wan et al., 2016; Wang et al., 2009). In a few words, the first A-stage is designed to capture as much COD as possible using a high-rate activated sludge (HRAS) system to redirect and concentrate carbon, rather than mineralization. 50-80% of COD can be recovered from the influent (Sancho et al., 2019) and can be redirected to anaerobic digestion for energy recovery as methane. Subsequently, autotrophic nitrogen removal is handled by a B-stage that includes, for example, partial nitritation (Isanta et al., 2015) combined with anammox (Jenni et al., 2014; Reino et al., 2018; G. Xu et al., 2015).

In our previous study (Zhang et al., 2021), the focus was on demonstrating the feasibility of the A-stage-EBPR concept, which integrates the traditional anaerobic/aerobic (A/O) EBPR configuration into an A-stage system to maximize the redirection of COD to anaerobic digestion, as well as to remove the P biologically. However, it was noted in that work that the A-stage-EBPR needed to be tested under a wider range of operating conditions to demonstrate its stability and performance, i.e. whether organic matter removal and PAO activity could be maintained and both the growth of nitrifying organisms and sedimentation problems (e.g. filamentous bulking) could be avoided.

On the one hand, the DO setpoint in the aerobic phase is essential. Lower DO can reduce the energy consumption of aeration at expenses of a decrease in PAO kinetics. Moreover, Izadi et al. (2021a) also reported that PAO could outcompete glycogen accumulating organisms (GAO) under lower DO concentrations. It is therefore relevant to explore the effect of the DO setpoint on this novel A-stage-EBPR system.

On the other hand, the nature of the influent carbon source is also essential when determining the minimum retention time needed under anaerobic conditions. Different carbon sources exert great influence during this process for the growth and metabolism of PAO and GAO (Nittami et al., 2017; Shen and Zhou, 2016). The application of VFA (e.g. acetate and propionate) is usual to promote PAO growth, obtaining microbial communities highly enriched in *Candidatus* Accumulibacter phosphatis (hereafter "*Accumulibacter*"). Then, a fermentation step may be needed for complex carbon sources (thus, enough anaerobic retention time) so that *Accumulibacter* PAO can live off the fermentation products. However, the different conditions and organic matter compounds in real wastewater may lead to the proliferation of fermentative types of PAO (e.g. *Tetrasphaera* or *Microlunatus*), GAO (e.g. *Micropruina*) or other facultative anaerobic bacteria which could produce substrate for PAO and GAO (Nielsen et al., 2019; Singleton et al., 2022). Proteins are also a kind of significant carbon source, accounting for example 25-35% of COD in real wastewater entering Danish EBPR plants (Nielsen et al., 2010). Hydrolysates of protein- amino acids have been applied in lab-scale EBPR studies (Marques et al., 2017; Shon et al., 2007; Zengin et al., 2011), where *Accumulibacter*, *Tetrasphaera*-related PAOs and *Thiothrix* were favoured. Glutamate, as a potential carbon source for EBPR has been specifically investigated by some researchers (Dionisi et al., 2004; Kong et al., 2005b; Marques et al., 2017; Rey-Martínez et al., 2021b, 2019). However, the feasibility of using glutamate as carbon source in an A-stage-EBPR system and its effect on the microbial community have not been studied.

Therefore, this work aims to evaluate the performance of a continuous A-stage-EBPR system under different operational conditions. The main objectives of this work are: i) to study the effect of different DO setpoint in the aerobic reactor to maintain long-term successful organic matter and P removal without nitrification, ii) to investigate the possibility to recover P by purging from the anaerobic reactor, iii) to gain insight of the performance of the system with different carbon sources (propionic acid and glutamate), and iv) to evaluate the changes in the microbial community under the different operational conditions.

5.2.3. Materials and methods

5.2.3.1. Equipment

The A/O configuration in explained in Chapter 3.1 (Figure 3.1). The system was inoculated with sludge collected from a municipal WWTP (Manresa, Spain) and the raw wastewater used was from the primary settler effluent of the same plant. The average characteristics of the raw wastewater are shown in Table 5.11 (period I and II). Because of the low concentration of COD in this wastewater, additional propionic acid was added from a concentrated solution (46000 mg/L COD) to increase it up to around 410 to 430 mgCOD/L. Thus, the influent was composed of raw wastewater (90 L/d) with propionic acid solution (0.45 L/d). Synthetic wastewater was applied in period III using glutamate sodium as sole carbon and nitrogen source, with a theorical concentration of 41 mgN/L. The external recycle flowrate (45 L/d) was set at 0.5 times the influent. Hydraulic retention time was 11.2 h considering only the reactors and 17.9 h also considering the settler. The sludge retention time (SRT) was controlled in different periods based on equation (1), considering the solids lost in the effluent and selecting the proper flow rate of wasted sludge.

$$SRT = \frac{V_{ana} \cdot X_{ana} + V_{aer} \cdot X_{aer}}{Q_{pur} \cdot X_{aer} + Q_{eff} \cdot X_{eff}} \qquad (1)$$

where V_{ana} and V_{aer} (L) represent the volume of the anaerobic and aerobic reactors, X_{ana}, X_{aer} and X_{eff} (g/L) mean concentration of the biomass in both reactors and the effluent, Q_{pur} and Q_{eff} (L/d) are the flow rate of purge and effluent.

The pH during the reported period was in the range 6.2-7.8. The system was operated at room temperature (21±2ºC).

Table 5.11. Average compositions of the real wastewater amended with propionic acid used in period I and II (0-56d) and synthetic wastewater applied in Period III (57-142d).

Components	**Units**	**Period I**	**Period II**	**Period III**
PO_4^{3-}	mgP/L	6.0 ± 1.0	6.2 ± 0.6	6.4±0.6
NH_4^+	mgN/L	58 ± 9	50 ± 9	41
$COD_S{}^a$ (raw wastewater)	mgCOD/L	186±68	197±30	_
$COD_S{}^a$ (external carbon source)	mgCOD/L	230 (propionic acid)	230 (propionic acid)	380±30 (glutamate)
COD_S (total)	mgCOD/L	414 ± 67	425 ± 29	380±30

a Soluble COD concentrations

5.2.3.2. Chemical and biochemical analyses

The liquid samples for the concentration analysis of phosphate, COD, ammonium, nitrate and nitrite were withdrawn from both reactors and effluent almost daily. Sludge samples were withdrawn from both reactors and effluent for the analysis of VSS, TSS and SVI. The detailed process for the liquid and solid analysis was explained in Chapter 3.3.

5.2.3.3. Performance indicators and fate of COD and nitrogen

The calculation of the P removal efficiency (PRE), the total COD removal efficiency (CRE), the fate of COD and N are as shown in Chapter 5.1.3.4.

5.2.3.4. *Batch tests*

Two batch tests (as explained in 5.1.3.5) were carried out to investigate the EBPR activity at different stages: i) day 52 in period IIb, with DO = 1 mg/L, raw wastewater amended with propionic acid and anaerobic purge; and ii) day 88 in period IIIa, with DO = 1 mg/L, glutamate as sole carbon source and anaerobic purge.

5.2.3.5. *Microbiological analyses*

Sludge samples were collected in the aerobic reactor during different stable operation periods ((a) propionic acid as carbon source under aerobic purge (period IIa) and (b) anaerobic purge (period IIb), and (c) with glutamate as carbon source under anaerobic purge (period IIIa)) to identify the bacterial population by Illumina amplicon sequencing of the 16S rRNA gene (as explained in 3.4.2).

5.2.4. *Results and discussion*

The experimental work conducted can be divided into three different periods (Table 5.12). Period I (Section 3.1) aimed at finding the minimum DO setpoint to run the A-stage-EBPR system. Period II (Section 3.2) was set to explore the A-stage-EBPR performance when operated with anaerobic purge instead of the conventional aerobic purge. Finally, glutamate was used in period III (Section 3.3) to study the effect of this carbon source on the A-stage-EBPR performance and on the microbial community. The profiles of C, N and P during the whole experimental period are shown in Figure 5.9 and the average values for each period are reported in Table 5.13.

Table 5.12. DO, purge position and carbon source used for the different operational periods.

Period	Duration (d)	DO (mg/L)	Purge position	Carbon source
Ia	0-9	1.0	Aerobic	Raw wastewater + propionic acid
Ib	10-18	0.5		
Ic	19-21	0.2		
Id	22-26	0.5		
IIa	27-39	1.0		
IIb	40-56		Anaerobic	
IIIa	57-88			Synthetic wastewater (glutamate)
IIIb	89-122			
IIIc	123-142			

Table 5.13. P and COD concentrations and removal performance obtained during different periods.

Period	Duration (d)	P_{INF}[a] (mgP/L)	P_{ANA}[b] (mgP/L)	P_{AER}[c] (mgP/L)	PRE[d] (%)	COD_{INF}[a] (mgCOD/L)	COD_{ANA}[b] (mgCOD/L)	COD_{AER}[c] (mgCOD/L)	CRE[e] (%)
Ia	0-9	7.2±0.9	26±2	0.2±0.1	98±1	331±91	70±80	15±19	95±7
Ib	10-18	5.4±1.2	26±2	0.3±0.1	94±3	445±14	119±47	23±10	95±2
Ic	19-21	5.7±0.7	19±7	3.0±1.9	50±29	434±0	149±91	14±11	97±3
Id	22-26	5.5±0.4	9±2	2.0±1.0	64±15	452±12	177±18	37±10	94±4
IIa	27-39	6.1±0.6	19±4	0.1±0.1	98±1	407±21	91±27	21±11	95±3
IIb	40-56	6.2±0.6	24±2	0.2±0.1	97±2	450±19	117±32	36±20	92±4
IIIa	57-88	6.8±0.8	26±4	0.1±0.1	98±1	360±76	69±74	8±15	98±4
IIIb	89-122	6.3±0.3	29±8	0.1±0.0	98±1	358±32	76±79	22±29	94±8
IIIc	123-142	6.2±0.6	12±4	2.4±3.9	65±41	396±21	210±53	60±47	85±12

a INF: concentration in the influent
b ANA: concentration in the anaerobic reactor
c AER: concentration in the aerobic reactor
d PRE: P removal efficiency
e CRE: COD removal efficiency

Table 5.14. Evolution of SRT, solids concentration, VSS/TSS ratio and settleability in the A-stage-EBPR system for different periods.

Period	Duration (d)	SRT (d)	VSS_{AER} (g/L)	VSS_{EFF} (g/L)	VSS/TSS_{ANA}	VSS/TSS_{AER}	SVI (mL/g)
Ia	0-9	6.3±0.1	2.63±0.35	0.03±0.02	0.89±0.06	0.85±0.05	77±20
Ib	10-18	5.4±0.6	2.29±0.28	0.05±0.03	0.94±0.04	0.91±0.01	134±29
Ic	19-21	2.9±0.9	1.42±0.35	0.14±0.04	0.96±0.03	0.93±0.00	617±169
Id	22-26	0.7±0.4	0.84±0.01	0.60±0.31	1.00±0.01	1.00±0.01	1143±24
IIa	27-39	6.9±0.2	2.70±0.10	0.03±0.01	0.93±0.02	0.91±0.01	91±12
IIb	40-56	6.4±0.3	2.45±0.15	0.03±0.01	0.94±0.02	0.93±0.02	178±64
IIIa	57-88	6.7±1.8	2.34±0.27	0.07±0.03	0.95±0.03	0.92±0.03	232±62
IIIb	89-122	1.9±1.0	1.72±0.26	0.42±0.20	0.97±0.01	0.93±0.05	518±115
IIIc	123-142	0.5±0.1	0.79±0.22	0.71±0.03	1.00±0.00	0.99±0.01	1139±352

5.2.4.1. *Performance under different DO setpoints*

The A-stage-EBPR system was operated under different DO setpoints in period I (Table 5.12) with raw wastewater amended with propionic acid. The starting value was a DO setpoint of 1 mg/L and it was gradually decreased (1-0.5-0.2 mg/L) when successful simultaneous P and COD removal were reached. Figure 5.9a shows the experimental P concentration in the influent, anaerobic reactor and aerobic reactor and its removal efficiency PRE, while Figure 5.9b shows COD concentration at the same sampling points. During period Ia (DO=1 mg/L, purge of 6 L/d and SRT =6 d), excellent P and COD removal efficiency were obtained (PRE = 98±1 % and CRE = 95±7 %, Table 5.13). Ammonia (Figure 5.9c), nitrate and nitrite (Figure 5.9d) profiles showed that after 6 days of operation nitrifying activity was negligible. The A-stage-EBPR system aims at suppressing nitrification since nitrogen is supposed to be removed by the subsequent B-stage and, besides that, nitrate entering the anaerobic reactor through the external recycle could be deleterious for PAO activity. VSS was about 2.63 ± 0.35 g/L (Table 5.14 and Figure 5.10 a) in the reactor and 0.03 ± 0.02 g/L (Table 5.14 and Figure 10 b) in the effluent. This low concentration of biomass in the effluent, in addition to a low SVI of 77 mL/g (Figure 5.10c and Table 5.14) was an indication of the good settleability of the sludge. The ratio of VSS/TSS in the aerobic reactor was 0.91 at the end of this period (Figure 5.10d), lower than the anaerobic ratio of 0.96. Thus, there was a significant change in poly-P concentration, a clear indication of good P-release and P-uptake activity. As a conclusion of period Ia, the system showed a stable performance with the selected operational conditions and DO = 1mg/L.

The DO setpoint was moved from 1 to 0.5 mg/L (Period Ib) to assess whether the same performance could be obtained with lower aeration requirements. Izadi et al. (2021) indicated that operating DO at 0.8 mg/L could remove 90% of P in an A/O SBR system with a high enrichment in PAO. Other studies also showed that lower DO values could promote the selection of PAO over GAO (Carvalheira et al., 2014b; Chiu et al., 2007). In our work, PRE and CRE were maintained at 94±3 % and 95±2 %, while nitrification activity remained suppressed as expected. The solids showed a little decrease (around 2.3 gVSS/L in the reactor) and SVI slightly increased to 134 ± 29 mL/g. Then, the system was able to maintain a good performance under the DO = 0.5mg/L condition.

The DO setpoint in the aerobic reactor was further decreased to 0.2 mg/L (period Ic) and, subsequently, PAO activity was severely damaged. P concentration in the anaerobic reactor (P_{ANA}) decreased from 26.4 to 12.9 mg/L in the first 2 days, and the corresponding P concentration in the aerobic reactor (P_{AER}) increased from 0.4 to 4.6 mg/L. Conversely, COD removal performance was not affected (CRE about 97%). Anaerobic carbon concentration shortly increased in this period, indicating a slight decrease of anaerobic

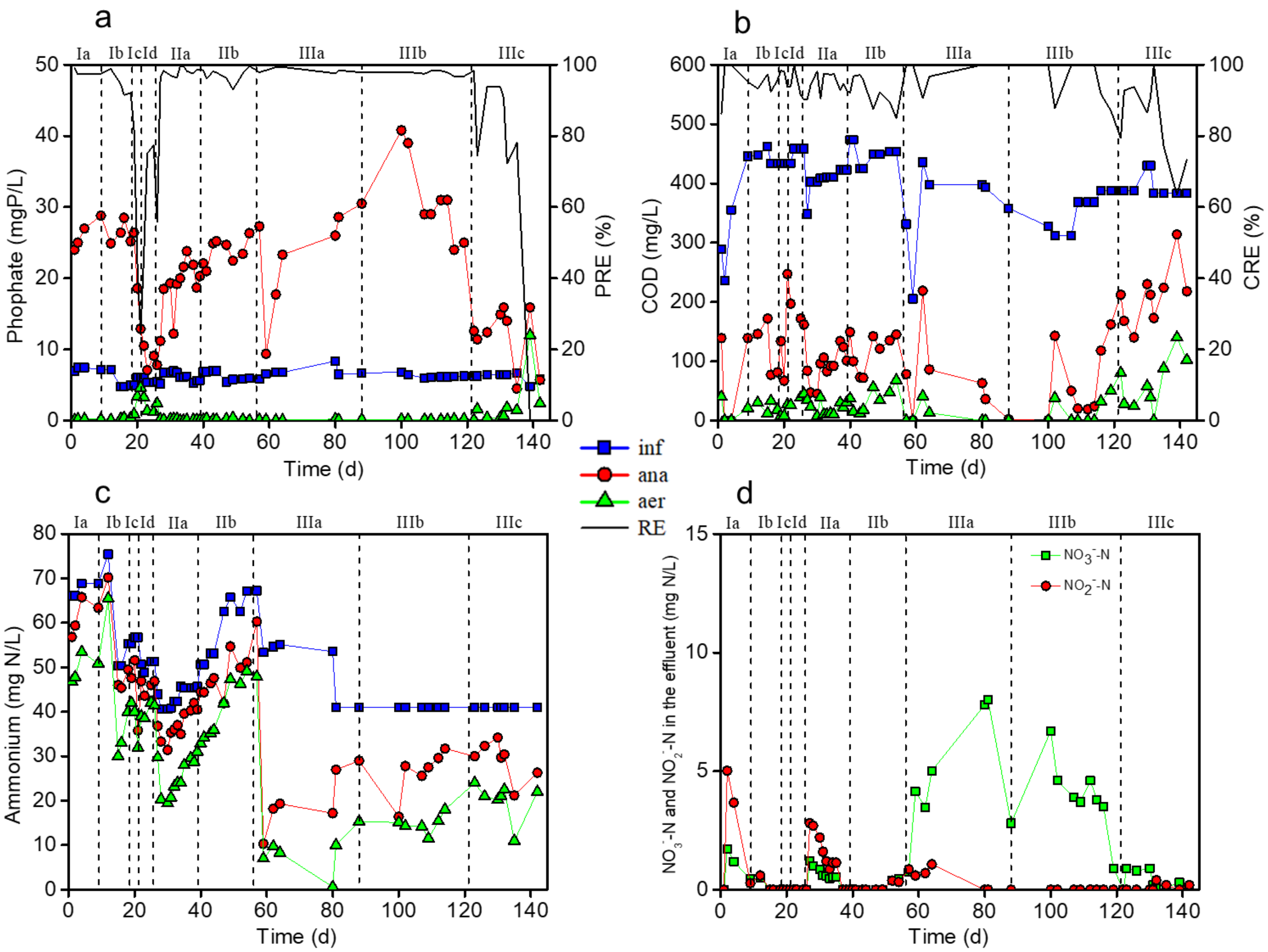


Figure 5.9. Evolution for different operational periods of the removal efficiencies and concentrations in the influent, the anaerobic reactor and the aerobic reactor. a) Phosphorus, b) COD, c) ammonium and d) nitrate and nitrite

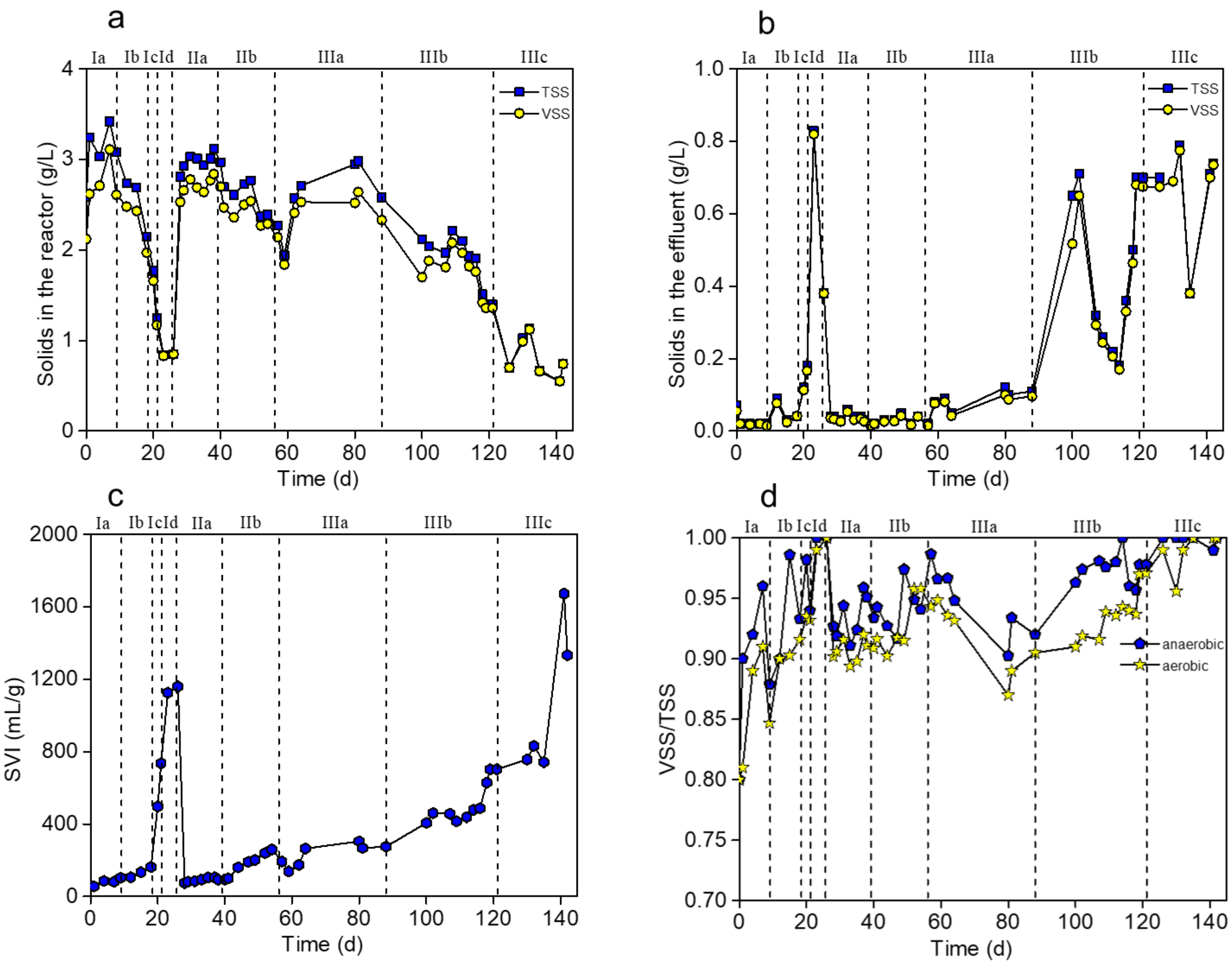


Figure 5.10. Solids related evolution for different periods. Solids concentration in (a) the reactor, (b) effluent, (c) SVI and (d) VSS/TSS ratio.

COD consumption. The reasons are either PAO reaching their maximum capacity for COD storage as PHA or its inability to restore the poly-P reserves under aerobic conditions due to oxygen limitation. Then, the excess of COD from the anaerobic phase was oxidized under limited aerobic conditions, which led to a severe decrease of the settleability (SVI increased to 617±169 mL/g), probably due to the promotion of filamentous bacteria, which is expected under these operational conditions (Jenkins et al., 2003). The high effluent VSS (around 0.14 gVSS/L) led to a decrease of the SRT to 3 days. Our previous studies showed that 4 days SRT was the minimum threshold of this A-stage-EBPR system (Zhang et al., 2021). Then, the performance was severely affected in period Ic (DO=0.2 mg/L).

After this unsuccessful operational period, efforts were made to recover lost activity by increasing the DO to 0.5 mg/L and drastically reducing the purge to increase SRT (period Id). Despite these changes, P removal performance showed no improvement, the biomass in the reactor decreased (0.84±0.01 gVSS/L) and SVI increased to 1143 mL/g, indicating a high proliferation of filamentous bacteria. Moreover, an increasingly high ratio of VSS/TSS about 1 was observed (Table 5.14 and Figure 5.10d), which showed the red flag of PAO washout (Chan et al., 2017; Oehmen et al., 2006; Zhang et al., 2021). As a summary of Period I, a setpoint of 0.2 mg/L DO was not able to support a stable performance of the A-stage-EBPR system, but operation at DO in a range 0.5-1 mg/L was feasible. Operating at a too low DO can lead to problems of poor settleability and loss of PAO activity.

5.2.4.2. System performance under different purge positions

The sludge from the anaerobic reactor could be an opportunity to increase energy recovery because of its higher PHA content and therefore higher potential for biomethane production (Chan et al., 2020a). In addition to that, the anaerobic liquor contains a high P concentration which could be adequate for P recovery by precipitation. However, the stability of the A-stage-EBPR operation under these operational conditions should be demonstrated.

To study this operation, the plant was reinoculated on day 27 and operated with a DO setpoint of 1 mg/L and a reduced purge of 1 L/d from the aerobic reactor to achieve a good P removal performance (period IIa). The concentration of P in the effluent was about 0.1±0.1 mg/L (PRE = 98±1% and CRE = 95±3 %). Some nitrification appeared at the start of period IIa due to the biomass reinoculation and a long SRT. The purge flowrate was gradually increased to decrease the SRT, operating at 6 days from day 36. Then, PRE remained stable between 97% and 100% and similarly, CRE was around 98% and nitrification activity ceased. The VSS in the reactor was stable at 2.7±0.1 g/L (Figure 5.10a) and SVI was 91±12 mL/g showing good settleability.

The purge position was moved from the aerobic to the anaerobic reactor on day 40 (period IIb), and the system was maintained at an SRT of 6 days. The performance of the plant was maintained using the anaerobic purge (Figure 5.9): PRE remained stable with 97±2% and CRE was around 92±4% even though there were fluctuations due to the changeable influent COD of the real wastewater. However, the effluent COD was always less than 40 mg/L. Neither nitrate nor nitrite was detected in the effluent, which implied that the nitrification was avoided. VSS in the reactor experienced a little decrease and with a concentration of 2.5±0.2 gVSS/L and SVI slightly increased from 161 to 260 mL/g.

5.2.4.2.1. *The potential for P recovery for anaerobic purge condition*

Anaerobic purging is not only beneficial for carbon recovery due to the higher biomethane production potential of the anaerobic sludge, but also provides an opportunity for mainstream P-recovery due to the enriched P concentration in the anaerobic supernatant (Acevedo et al., 2015; Guisasola et al., 2019; Zhang et al., 2022). The potential for P recovery under anaerobic purge condition in this work can be calculated based on P_{ANA} during period IIb (24±2 mgP/L) and the purge flowrate (5 L/d). Considering the influent flowrate of 90 L/d and an average P concentration of 6.2 mg/L, about 22 % of P in the influent was contained in the anaerobic supernatant. P concentration in this mixed liquid was increased by a factor of four compared to the input, which should favour its precipitation and recovery as struvite or vivianite after a separation step for the biomass. In a previous work, Larriba et al., (2020) obtained an average recovery of 45% of the influent P by struvite precipitation from the anaerobic supernatant in a demo-scale pilot plant for a long period operation (with higher SRT about 10-15 d). However, this higher percentage was obtained by redirecting 8.6 % of the influent flow to the P-recovery stream, whereas in the present work only 5.6% was proposed for redirection. A higher P-recovery percentage would be possible, but at the expense of adding a biomass separation and recycling stage able to separate the P-recovery stream from the anaerobic purge extraction. Otherwise, the SRT would be too low.

5.2.4.2.2. *Mass balances of carbon and nitrogen*

Table 5.15 shows the results of the COD mass balance in period I and II. The potential COD outlets are: 1) effluent, as dissolved COD or biomass, 2) purge, as dissolved COD or biomass and 3) COD mineralization. Mineralized COD decreased from 42±12% to 32±5% with the DO decrease from 1 mg/L (period Ia) to 0.5 mg/L (period Ib). These values are comparable to the mineralization observed in a non-EBPR A-stage reported by Jimenez et al. (2015) for SRT = 0.5 d (37%) and lower to that observed at SRT = 2 d (67%). They are close to the range of a continuous A-stage (41-58%) and an A-stage SBR (20-48%) reported in a previous work (Rey-Martínez et al., 2021a) and slightly higher than those reported for SRT = 1.0 d (22%) and SRT = 2.1 d (27%) in a pilot-scale continuous HRAS system (Carrera et al., 2022).

Table 5.15. COD mass balance during periods I and II. All COD items are represented as a percentage of the influent COD.

Period	COD_{EFF}[a] (%)	COD_{PUR}[b] (%)	COD_{EFFB}[c] (%)	COD_{PURB}[d] (%)	COD_{OUT}[e] (%)	COD_{MINER}[f] (%)
Ia	4±6	0.2±0.2	21±12	32±17	58±12	42±12
Ib	5±2	0.3±0.3	10±8	53±2	68±5	32±5
IIa	6±6	0.3±0	10±2	54±2	70±4	30±4
IIb	7±4	0.4±0.2	8±3	43±2	58±5	42±2

a COD in the effluent after filtration
b COD in the purge stream after filtration
c COD contained in the biomass of the effluent
d COD contained in the biomass of the purge stream
e Total COD output obtained as the sum of the previous four items: e=a+b+c+d
f Percentage of input COD mineralized to CO_2: f=100-e %

Regarding the COD content in the biomass of the purge, a much higher fraction of the input COD could be redirected to the anaerobic digestion under DO = 0.5 mg/L setpoint (53±2%) compared to that of DO = 1 mg/L (32±17%). When the purge was moved to the anaerobic reactor with DO = 1 mg/L (period IIb), the input COD stored in the biomass was increased up to 43±2%, showing the positive effect of the anaerobic purge. In the work of (Jimenez et al., 2015), the COD redirection in their A-stage system increased from 23 to 48 % when decreasing the SRT from 2 to 0.3 d, while Rey-Martínez et al., (2021a) reported values of 30 and 34% for the continuous A-stage at SRT = 1 and 2 d and up to 62% for the A-stage SBR at SRT = 1d. Carrera et al. (2022) in their HRAS system showed about 24% of COD stored in the biomass at SRT =2.1 d and increasing to 29% at SRT = 1.0 d with an additional percentage of COD adsorption in the range 25-30%.

Comparing the results of our work with previous results obtained in non-EBPR A-stage systems, it can be concluded that with the A-stage-EBPR system operating at SRT = 6 d and low oxygen concentration in the range 0.5-1 mg/L, it is possible to obtain COD redirection results to purged biomass and COD mineralization percentages that are comparable to non-EBPR A-stage systems operating at much lower SRTs even below 2d.

Table 5.16 shows the results of the N mass balance. Assuming no denitrification, the fate of the inlet N that is not in the effluent can be either biomass assimilation or nitrification (i.e. nitrite/nitrate). As can be observed, the total outlet N (N_{OUT}) covered all the input N in period I and II when the system was under stable operation, which means no significant nitrification occurred, and it was in accordance with the result of the system performance. All the N_{OUT} values higher than 100% are probably due to the hydrolysis of some organic

N in the feed that was not considered in the influent. The percentage of N_{PURB} was 15±12% under the DO of 1 mg/L in period Ia, and it increased to 36±5% under the DO of 0.5 mg/L which could be associated with the slight decrease of solids in the system under the DO of 0.5 mg/L.

Table 5.16. N mass balance during periods I and II. All N items are represented as a percentage of the influent N.

Period	N_{EFF}[a] (%)	N_{PUR}[b] (%)	N_{EFFB}[c] (%)	N_{PURB}[d] (%)	N_{OUT}[e] (%)	NH_4^+-N_{EFF}[f] (%)
Ia	76±7	3±2	8±4	15±12	101±8	71±3
Ib	67±12	5±1	8±7	36±5	115±4	66±11
IIa	62±2	4±0	7±1	42±1	115±2	62±2
IIb	66±3	4±1	7±3	26±5	102±9	66±2

a Total N in the effluent after filtration
b Total N in the purge stream after filtration
c N contained in the biomass of the effluent
d N contained in the biomass of the purge stream
e Total N output obtained as the sum of the previous four items: e=a+b+c+d
f Ammonium nitrogen in the effluent after filtration

N mass balances reveal that an average value of 66% of the influent nitrogen was present as ammonium in the effluent, showing a relatively high fraction of ammonium left for the following B-stage. The rest of N (34%) was mostly contained in the biomass due to growth and the rest was soluble ammonium in the purge stream. The 34% was higher than the 7-21 % range obtained in the A-stage SBR reported by Rey-Martínez et al. (2021a), probably due to the higher SRT in the A-stage-EBPR that could lead to higher biomass growth instead of other adsorption processes that can occur at lower SRT.

5.2.4.3. System performance with glutamate as carbon source

The anaerobic retention time is key when operating an A-stage-EBPR system. This value should be as low as possible to operate the system under low SRT conditions but high enough to maintain EBPR activity. Since PAO mainly use short-chain fatty acids, different processes coexist under anaerobic conditions: hydrolysis and fermentation of complex organic substrates to simple organic compounds and the posterior anaerobic uptake of these simple compounds by PAO. The rate of the limiting step will determine the minimum anaerobic residence time needed and, therefore, the nature of the carbon source (i.e. its biodegradability) is very important. Period III was operated with glutamate to better understand the link between the fractionation of the influent organic matter and the operation of A-stage-EBPR systems.

On day 57, the carbon source was switched from propionic acid to sodium glutamate under a DO of 1 mg/L and anaerobic purge (period IIIa). Glutamate contains nitrogen that is released as ammonium when hydrolysed, thus it acted both as carbon and nitrogen source. Unexpectedly, the use of glutamate led to nitrification and hence the appearance of nitrate and nitrite in the aerobic reactor (Figure 5.9d). However, both PRE and CRE were very high, around 98% (Table 5.13). VSS was around 2.34±0.27 g/L and, despite SVI was around 232±62 mL/g, the effluent VSS concentration was low. The SRT was 6.7±1.8 days and the A-stage-EBPR system showed successful P and COD removal performance in spite of the effluent nitrate (5.0 mg/L) and nitrite (1.1 mg/L). In fact, this period showed the higher P-release and uptake rates than that with propionic acid as carbon source (Table 5.17, see below). Glutamate as the sole carbon source seemed to be responsible for the overgrowth of filamentous bacteria. Similar problems of settleability were observed in a previous work, indicating that filamentous bacteria could be clearly favoured with a high content of glutamate in the feed (Rey-Martínez et al., 2019). This period showed successful removal of P and COD despite detecting nitrate and nitrite, indicating that PAO, filamentous bacteria and nitrifiers could coexist for more than 30 days in this A-stage-EBPR system.

Severe bulking issues happened in period IIIb, leading to low SRT (2 d) and a high SVI 518±115 mL/g. The purge was reduced from 7 to 3 L/d and bleach was stepwise dosed as recommended (Jenkins et al., 2003) to decrease sludge bulking, but this problem persisted. In any case, PRE and CRE could be maintained about 98% and 94% even under the bad settleability condition. From that moment onwards, the bulking problem increased (with SVI = 1139 mL/g) and caused a high concentration of biomass in the effluent (0.71 g VSS/L) and a big loss of biomass in the reactor (with 0.79 g VSS/L left) in period IIIc. The ratio of VSS/TSS increased up to 1 and the SRT decreased to 0.5 d which led to the system failure.

As a summary of period III, the use of glutamate as the only carbon source allowed to maintain successful EBPR activity and COD removal for 2 months (periods IIIa and IIIb), but with a progressive loss of biomass settleability, causing a decrease in biomass concentration in the reactor. EBPR was lost when the VSS concentration decreased below 1 g/L. In addition, the undesired occurrence of some nitrifying activity could not be avoided. Undesirable SVI increase due to poor settleability has already been reported in previous EBPR works at low SRT (Valverde-Pérez et al., 2016; Zhang et al., 2021). The change of the PAO microbial community due to the change of carbon source to glutamate, linked to the operation at low SRT may be the reason for this poor sedimentation. Nevertheless, the use of glutamate should not be problematic provided that it is the only carbon source, or if a significant glutamate concentration is maintained for long periods. In any case, a pilot-scale study using the real influent would be desirable before the implementation of this type of system in a full-scale plant.

Two batch tests were performed to study the PAO activity with different carbon sources at the end of period IIb on day 52 (a) and period IIIa on day 88 (b) with the sludge from the aerobic reactor (Table 5.17 and Figure 5.11a under propionic acid and 5.11b under glutamate as carbon source). P concentration in these tests reached 20 and 24 mg/L at the end of anaerobic phase and less than 1 mg/L at the end of aerobic phase, which indicates robust P removal activity. P-release and uptake rate showed higher values under the glutamate as carbon source than propionic acid, with P-release and uptake rate 0.19 vs 0.11 and 0.16 vs 0.12 mgP/gVSS·min, respectively. The P/C ratio didn’t exhibit major differences, with a value around 0.12. This value is much lower than those reported by Shen and Zhou (2016) (0.23–0.44) and than the theoretical value for propionic acid reported by Oehmen et al. (2005) of 0.42. The low values may indicate that our system had a fraction of GAO (which agrees with the results presented in the next section). There was some nitrite at the start of batch b, accompanied with denitrification during the anaerobic phase and some nitrification during the aerobic phase. However, the activity of PAO seemed to be unaffected, which is consistent with the high PRE and CRE values obtained in the plant with glutamate as carbon source and indicates the coexistence of PAO, nitrifiers and denitrifies in our system. The profiles of ammonium and COD showed similar trends.

Table 5.17. PAO activity and relative stoichiometric ratio in the anaerobic/aerobic batch tests carried out with the sludge from the aerobic reactor in two different periods.

Period	Carbon source	PO_4^{3-}-P_{max} (mgP/L)	PO_4^{3-}-P_{min} (mgP/L)	P release rate (mgP/gVSS min)	P uptake rate (mgP/gVSS min)	P/C (mol P/mol C)
IIb	Propionic acid	20	0.8	0.11	0.12	0.119
IIIa	Glutamate	24	0.4	0.19	0.16	0.121

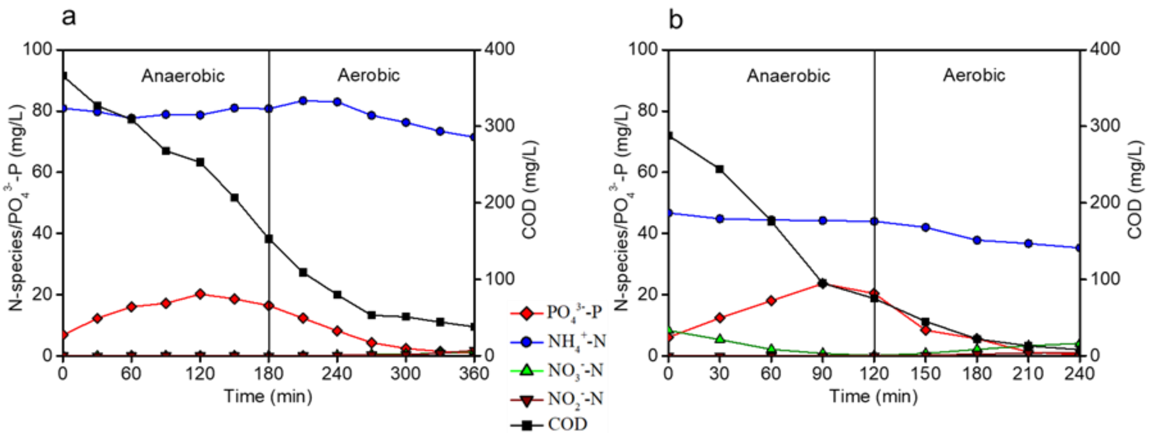
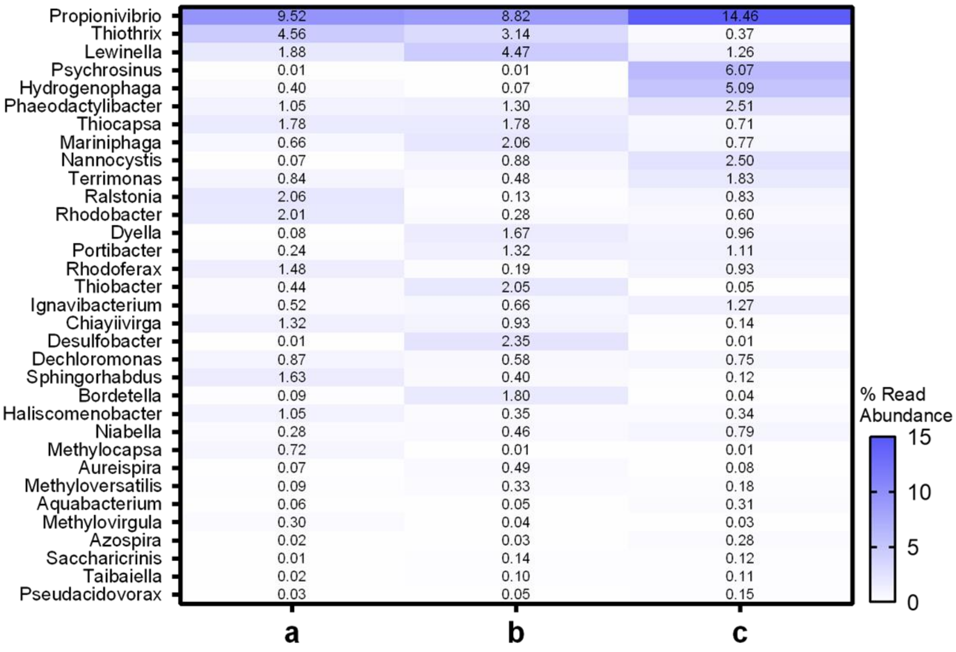


Figure. 5.11. Anaerobic/aerobic batch tests in terms of P, N, and COD with sludge withdrawn from the aerobic reactor on (a) day 52 (period IIb) and (b) day 88 (period IIIa).

5.2.4.4. Evolution of the microbial community

The variations and relative abundances of the functional bacteria were analysed by 16S rRNA gene sequencing at genus levels (Figure 5.12). *Propionivibrio* exhibited the higher percentage in all the conditions, increasing from 8.8 and 9.5 % with propionic acid to 14.5% when using glutamate. *Propionivibrio*, which has been referred as a GAO (Albertsen et al., 2016; Roy et al., 2021), has been reported to utilize acetate and propionate (Thrash et al., 2010) and can also ferment organic substrates to acetate and propionate (Albertsen et al., 2016). Some investigations considered certain strains of *Propionivibrio* as putative PAO (Coats et al., 2017). Li et al. (2019) proposed that *Propionivibrio* may harbour new strains belonging to PAOs due to the dominant position (48.9%) in a successful system for simultaneous N and P removal with propionate as carbon source. In our system, the high percentages of *Propionivibrio* seemed to be related to system performance, as high P removal efficiencies were observed under all the operating conditions. The high EBPR activity in period IIIa can be related to the ability of *Propionivibrio* to ferment glutamate to VFA that can be used by themselves or other PAO. *Propionivibrio* was also reported to be the most abundant species (11.6%) in the work of Rey-Martínez et al. (2019) in an A/O SBR system with glutamate and aspartate as carbon source.



	a	b	c
Propionivibrio	9.52	8.82	14.46
Thiothrix	4.56	3.14	0.37
Lewinella	1.88	4.47	1.26
Psychrosinus	0.01	0.01	6.07
Hydrogenophaga	0.40	0.07	5.09
Phaeodactylibacter	1.05	1.30	2.51
Thiocapsa	1.78	1.78	0.71
Mariniphaga	0.66	2.06	0.77
Nannocystis	0.07	0.88	2.50
Terrimonas	0.84	0.48	1.83
Ralstonia	2.06	0.13	0.83
Rhodobacter	2.01	0.28	0.60
Dyella	0.08	1.67	0.96
Portibacter	0.24	1.32	1.11
Rhodoferax	1.48	0.19	0.93
Thiobacter	0.44	2.05	0.05
Ignavibacterium	0.52	0.66	1.27
Chiayiivirga	1.32	0.93	0.14
Desulfobacter	0.01	2.35	0.01
Dechloromonas	0.87	0.58	0.75
Sphingorhabdus	1.63	0.40	0.12
Bordetella	0.09	1.80	0.04
Haliscomenobacter	1.05	0.35	0.34
Niabella	0.28	0.46	0.79
Methylocapsa	0.72	0.01	0.01
Aureispira	0.07	0.49	0.08
Methyloversatilis	0.09	0.33	0.18
Aquabacterium	0.06	0.05	0.31
Methylovirgula	0.30	0.04	0.03
Azospira	0.02	0.03	0.28
Saccharicrinis	0.01	0.14	0.12
Taibaiella	0.02	0.10	0.11
Pseudacidovorax	0.03	0.05	0.15

Figure 5.12. Microbial communities in the level of genus in the A-stage-EBPR system during stable operation: with (a) propionic acid as carbon source under aerobic purge on day 38 (period IIa) and (b) anaerobic purge on day 56 (period IIb), and (c) with glutamate as carbon source under anaerobic purge on day 81 (period IIIa). The microorganisms are ranked according to the sum of their abundance during the three periods.

Thiothrix has also been recognized as a putative PAO in some reports (Meng et al., 2020; Rey-Martínez et al., 2019; Rubio-Rincón et al., 2017) and it grows at low COD concentration as well as in a sulphur-reducing environment (Rubio-Rincón et al., 2017). It was observed in the A-stage-EBPR process in high proportions (about 4.6%, 3.1% and 0.37%). However, (Rey-Martínez et al., 2019) showed that *Thiothrix* ranked the most abundant position (37%) in a glutamate-fed anaerobic/anoxic/aerobic EBPR system, but in that case the system was operated at a higher SRT (10-15d), which could be the reason for the difference in abundance. *Lewinella* was also detected to have high proportions in all conditions, with 1.9%, 4.5% and 1.3%. *Lewinella* was shown to hold a 9.8% percentage in the glutamate-fed A_2O system by Rey-Martínez et al. (2021b), though no investigation demonstrated *Lewinella* to possess PAO metabolism.

Rhodobacter (HIRAIS et al., 1991) has been reported as an important bacteria in conventional EBPR systems and percentages about 2.0%, 0.3% and 0.6% were observed. *Hydrogenophaga* is assumed to be a putative PAO by some investigations, and is presented in systems using acetic, ethanol or real wastewater as carbon source (Ge et al., 2015; Iannacone et al., 2021, 2020). The highest abundance (5.1%) was observed in the glutamate system, being less than 0.5% with propionic acid. Ge et al. (2015) showed that *Hydrogenophaga* was promoted when treating a protein-rich wastewater system, and the work of Rey-Martínez et al. (2019) also detected its presence with glutamate and aspartate as carbon source. Thus, it is not surprising that *Hydrogenophaga* could be favoured in the glutamate-fed A-stage-EBPR system. *Dechloromonas*, which can use oxygen or NO_X as electron acceptors, has been reported as a functional PAO and appears extensively in full-scale WWTP (Petriglieri et al., 2021). The relative percentage of *Dechloromonas* in the propionic-fed periods was about 0.9% and 0.6% and about 0.8 % for the glutamate-fed period. *Desulfobacter* is closely related to organisms implicated in the sulfur-EBPR studies (Zhang, 2017), and appeared at a high percentage of 2.4 % in period IIb with anaerobic purge for treating the real wastewater. Finally, and in contrast to other investigations, *Tetrasphaera*-related organisms were only detected in very low concentration (not shown) throughout the operation. All in all, it can be observed that the appearance of different putative PAO assured the efficient utilization of the carbon sources for a successful operation of the A-stage-EBPR system.

5.2.5. *Conclusions*

This work explored the performance of an A-stage-EBPR system under different operational conditions, showing situations where the system operates stable but also some cases where stability problems can appear. High P and COD removal was reached (94-98% and 95%) under DO setpoints of 0.5 and 1 mg/L when treating raw wastewater amended with propionic acid. However, decreasing the DO setpoint to 0.2 mg/L led to the deterioration of the system.

Changing the purge position from the aerobic to the anaerobic reactor maintained good COD and P removal performance and stable settleability without nitrification. About 22% of the influent P could be recovered using the anaerobic purge and about 43% of the influent COD could be captured and recovered (rather than mineralized) according to the mass balances. Nitrogen mass balance showed that 66% of the input N was in the effluent as ammonium for further treatment in the B-stage.

Employing glutamate as sole carbon and nitrogen source allowed simultaneous COD and P removal but with a slight nitrification build-up. After two months of operation with glutamate, biomass settleability was progressively lost and EBPR activity disappeared, indicating that it may be only a suitable carbon source for short periods. The microbial community analysis showed that *Propionivibrio, Thiothrix* and *Lewinella* exhibited the highest abundances. *Propionivibrio* percentage seemed to be correlated to high P-removal. *Thiothrix, Hydrogenophaga, Dechloromonas* and *Desulfobacter* were detected as the dominant PAO during the whole operation.

Chapter 6:

Benefits and drawbacks of integrating a side-stream sludge fermenter into an EBPR system

6. Benefits and drawbacks of integrating a side-stream sludge fermenter into an EBPR system

6.1. Abstract

The implementation of a side-stream sludge fermenter (SSSF) has been identified as a possible solution to improve the performance of enhanced biological phosphorus removal (EBPR) when treating low COD wastewater.

This study systematically evaluated the effects of incorporating a SSSF into an anaerobic/anoxic/aerobic (A_2O) configuration (side-stream EBPR, S2EBPR) for P/N/COD removal under a limited influent COD (COD_{INF}) condition. The performance of the S2EBPR (with SSSF receiving 6% of the recycled activated sludge connected to anaerobic reactor and HRT = 2 d) and A_2O were compared under the same limited COD_{INF} (350 mg/L) condition. S2EBPR improved P removal (26.6%) and denitrification (11%) without compromising full ammonium and COD removal. However, it increased P_{LOAD} to the plant due to the P-release in SSSF, resulting in higher effluent P concentration. The methane and energy recovery indexes were around 45% lower than those of A_2O. Sequencing analysis revealed a high abundance of PAO, and a lower GAO/PAO ratio in the S2EBPR, in accordance to its higher P removal. The S2EBPR performance was also evaluated when the SSSF reactor outlet was connected to the anaerobic, anoxic or aerobic reactor, with the best results obtained for the first case. This study represents a comprehensive evaluation of the S2EBPR configuration and provides further enriched information to assess its suitability.

6.2. Introduction

The increasingly serious eutrophication problem led by over-discharging of phosphorus (P) and nitrogen (N) drives the research for an efficient biological nitrogen and phosphorus removal from wastewaters. Among the different configurations of wastewater treatment plants (WWTPs), the anaerobic/anoxic/aerobic (A_2O) configuration is the most common when aiming at removing nutrients in addition to organic matter (Tchobanoglous et al., 2013). P is removed by promoting the proliferation of polyphosphate-accumulating organisms (PAO) with alternative anaerobic and aerobic/anoxic conditions in the so-called enhanced biological phosphorus removal (EBPR) process (Comeau et al., 1987). PAO uptake organic matter, preferably volatile fatty acids (VFA) under anaerobic conditions and store it as polyhydroxyalkanoates (PHAs). The energy required for this process is mostly obtained from ATP hydrolysis and, thus, P is released into the liquid. Under the subsequent aerobic/anoxic phase, this PHA is used as energy and carbon source for, among other processes, P uptake and growth (He and McMahon, 2011; Satoh et al., 1998; Yuan et al., 2012).

Failures in the A_2O performance have been reported under undesired situations, for example a low COD/P ratio or an overload of nitrate to the anaerobic reactor via the external recycle (Barnard and Abraham, 2006; Gu et al., 2008; Neethling, 2015). Additional COD dosage from commercial organic chemicals can improve EBPR performance in both cases at expense of higher cost and higher carbon footprint (Shen and Zhou, 2016).

The integration of a side-stream sludge fermenter (SSSF) has been suggested as a potential strategy to overcome these issues, since the VFA production from sludge fermentation could cover the extra COD requirements. The integration of a SSSF into a conventional EBPR process (also known as S2EBPR) has been recently investigated (Coats et al., 2018). The influent of the SSSF can be a fraction of the anaerobic mixed liquor or the return activated sludge (RAS) (4%-30%) (Barnard et al., 2017; G. Li et al., 2020; Onnis-Hayden et al., 2020; Wang et al., 2019). There are more than 80 full-scale applications of S2EBPR facilities worldwide (Copp et al., 2012; Tooker et al., 2017; Vale et al., 2008; Vollertsen et al., 2006), and most of them are implemented in Europe (60) and United States (12) (Onnis-Hayden et al., 2020). The S2EBPR configuration has improved the P removal performance and stability compared to traditional EBPR configurations (Lanham et al., 2013; Onnis-Hayden et al., 2020; Wang et al., 2019). Moreover, PAO could be promoted independently of the nature of the carbon source of the WWTP influent. For example, Vollertsen et al., (2006) showed P concentrations about 40 mg/L and considerable COD concentrations in the SSSF of two WWTPs with HRT about 30 to 35 h, which made S2EBPR less dependent on the input wastewater quality compared with traditional EBPR. The reasons for the promotion of PAO activity with the integration of a SSSF could be ascribed to: i) the biomass fermentation products are mostly VFA, a preferred electron donor for PAO (G. Li et al., 2020; Onnis-Hayden et al., 2020); ii) the extended anaerobic phase gives a competitive advantage for PAO with respect to GAO and other heterotrophic organisms (Barnard et al., 2017; Barnard and Abraham, 2006; Wang et al., 2019) and iii) besides VFA, the SSSF can contain extra readily biodegradable chemical oxygen demand (rbCOD), which could be further fermented to VFA in the anaerobic reactor by PAO fermenters such as *Tetrasphaera* (Barnard et al., 2017; Fernando et al., 2019; Nielsen et al., 2019).

The comparison of microbial populations by Wang et al. (2019) showed that S2EBPR exerted a relatively higher PAO activity, higher total PAO abundance and lower GAO abundance. Nevertheless, there were no high differences in the abundance of putative PAOs, such as Ca. *Accumulibacter* and *Tetrasphaera*. To our knowledge, there is no previous report on the microbial evolution from a conventional A_2O to a S2EBPR configuration and on the major microbial communities present in a long-term operated SSSF reactor.

In this work, it was comprehensively evaluated the EBPR performance when a SSSF was integrated in an A_2O system under a low influent COD (COD_{INF}) scenario. The main objectives were: (1) to explore the COD_{INF} limits for the A_2O configuration, (2) to explore the S2EBPR system to achieve good performance with low COD wastewater, (3) to study the impact on the plant performance of integrating a SSSF reactor in the A_2O plant, (4) to investigate the difference of microbial communities in the S2EBPR under different operation conditions.

6.3. Materials and methods

6.3.1. *Equipment and operation parameters*

The initial pilot-scale A_2O configuration and the relative operation mode and parameters are explained in Chapter 3.2 (Figure 3.2). The compositions of the concentrated solution are shown in Table 6.1. The initial COD concentration was designed about 500 mg/L after the start-up state, and the COD concentration was decreased by 10% for each operation period when the system reached a steady state. The micronutrients composition was adapted from Smolders et al. (1994). The biomass for inoculation was obtained from the municipal WWTP of Baix Llobregat (Barcelona, Spain).

Once the limitation of COD in A_2O system was studied, the S2EBPR configuration was implemented by installing a SSSF (20 L) reactor treating an enriched biomass stream from the settler. The SSSF reactor was mixed with a magnetic stirrer at 200 rpm and was kept under anaerobic conditions to favour fermentation processes. The influent flowrate from the settler to the SSSF was set at 8.4 L/d, i.e. 6% of the influent flow rate, and HRT of 2 d. The effluent of the SSSF was fed to R1 most of the time, although it was connected to R2 or R3 for some shorter periods to investigate its effect. The relative configurations and diagrams are shown in Figure 3.2 and 3.3. HRT in the A_2O was about 23 h considering only the reactors and 31 h taken into account the settler. For S2EBPR, HRT was about 26 h considering only the reactors and 34 h with settler.

Table 6.1. Concentrated feed composition for COD_{inf} = 500 mg/L.

Composition	Concentration (g/L)
sodium propionate^{a1}	2.8
sucrose^{a2}	2.9
acetic acid^{a3}	3.1
sodium glutamate^{a4}	1.1
dipotassium phosphate ^{b1}	0.74
potassium phosphate ^{b2}	0.29
ammonium chloride ^{c1}	3.06

a1, a2, a3 and a4 provided 30%, 30%, 30% and 10% of the CODinf
b1 and b2 give a plant influent concentration of 9 mgP/L (1.4 gP/d)
c1 results in an influent concentration of 37 mgN/L (5.6 g N/d).

SRT was calculated with equation (1) for the A_2O plant and equation (2) for the S2EBPR:

$$SRT = \frac{V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER}}{Q_{PUR} \cdot X_{AER} + Q_{EFF} \cdot X_{EFF}} \tag{1}$$

$$SRT = \frac{V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER}}{Q_{PUR} \cdot X_{AER} + Q_{EFF} \cdot X_{EFF} + Q_{SSSF} \cdot \Delta X_{SSSF}} \tag{2}$$

where V_{ANA}, V_{ANOX} and V_{AER} (L) are the volume of the anaerobic, anoxic and aerobic reactors, X_{ANA}, X_{ANOX} and X_{AER} (g/L) the biomass concentration in these reactors, Q_{PUR}, Q_{EFF} and Q_{SSSF} mean the flow rate (L/d) of purge, effluent and SSSF. X_{EFF} is the biomass concentration in the effluent. ΔX_{SSSF} was calculated by the input biomass concentration minus the biomass concentration of SSSF, where the input biomass concentration is the theoretical biomass concentration from the external recycle ($X_{SETTLER}$) determined with equation (3):

$$X_{SETTLER} = \frac{(Q_{EFF} + Q_{ER} + Q_{SSSF}) \cdot X_{AER} - Q_{EFF} \cdot X_{EFF}}{Q_{SSSF} + Q_{ER}} \tag{3}$$

where Q_{ER} means the flow rate of external recycle.

SRT of the A_2O and S2EBPR was controlled around 13±3 days by manipulating the purge flowrate from the aerobic reactor. The actual SRT for each period is shown in Table 6.6.

6.3.2. *Chemical and biochemical analyses*

The liquid samples for the concentration analysis of phosphate, COD, ammonium, nitrate and nitrite were withdrawn from R1, R2, R3 and SSSF almost daily. Sludge samples were withdrawn from R1, R2, R3, SSSF and effluent for the analysis of VSS, TSS and SVI. The detailed process for the liquid and solid analysis was explained in Chapter 3.3.

6.3.3. *Performance indicators*

P_{LOAD} (g/d) comprised the P input of the concentrated solution and the amount of P from the SSSF returned to the system, equation (4):

$$P_{LOAD} = P_{CON} * Q_{CON} + P_{SSSF} * Q_{SSSF} \tag{4}$$

Where P_{CON} and P_{SSSF} are the P concentration of concentrated solution and SSSF, and Q_{CON} and Q_{SSSF} are the flow rate of concentrated solution and SSSF

Similarly, N_{LOAD} (g/d) and COD_{LOAD} (g/d) were calculated by equations (5) and (6):

$$N_{LOAD} = N_{CON} * Q_{CON} + N_{SSSF} * Q_{SSSF} \tag{5}$$

$$COD_{LOAD} = COD_{CON} * Q_{CON} + COD_{SSSF} * Q_{SSSF} \tag{6}$$

where N_{CON} *and* N_{SSSF} are the total N concentration of concentrated solution and SSSF (thereinto N_{SSSF} equals the concentration of NH_4^+-N since neither NO_3^--N no NO_2^--N were detected), and COD_{CON} and COD_{SSSF} are the soluble COD concentration of concentrated solution and SSSF.

In terms of nutrient removal (g/d), $P_{REMOVAL\_ABSOLUTE}$, $N_{REMOVAL\_ABSOLUTE}$ and $COD_{REMOVAL\_ABSOLUTE}$ were calculated as equations 7, 8 and 9.

$$P_{REMOVAL\_ABSOLUTE} = P_{LOAD} - P_{DIS\_EFF} = P_{LOAD} - P_{EFF} * Q_{EFF} \tag{7}$$

$$N_{REMOVAL\_ABSOLUTE} = N_{LOAD} - N_{DIS\_EFF} = N_{LOAD} - N_{EFF} * Q_{EFF} \tag{8}$$

$$COD_{REMOVAL\_ABSOLUTE} = COD_{LOAD} - COD_{DIS\_EFF} = COD_{LOAD} - COD_{EFF} * Q_{EFF} \tag{9}$$

where P_{DIS_EFF}, N_{DIS_EFF}, COD_{DIS_EFF} are the P, N and COD discharge at the effluent (g/d), and P_{EFF}, N_{EFF}, COD_{EFF} are the concentration of P, total N (sum of NH_4^+-N, NO_3^--N and NO_2^--N) and COD in the effluent.

The fate of N_{LOAD} could be divided into: i) the N in the biomass exiting the system, the N in the liquid and the denitrified N, equations 10-12:

$$N_{BIOMASS}\,(\%) = (N_{EFFB} + N_{PURB})\,/N_{LOAD} * 100 \tag{10}$$

$$N_{LIQUID}\,(\%) = N_{EFF} \cdot (Q_{EFF} + Q_{PUR})/N_{LOAD} * 100 \tag{11}$$

$$N_{DENITRIFIED}\,(\%) = \left(100 - N_{BIOMASS}\,(\%) - N_{LIQUID}\,(\%)\right) \tag{12}$$

Where N_{EFFB} (g/d) is the amount of nitrogen in the effluent biomass, and N_{PURB} (g/d) the amount of nitrogen in the purged biomass. The amount of nitrogen in the biomass was estimated by considering VSS concentration multiplied by the flowrate and by the factor 0.124 gN/gVSS obtained from the general formula of bacteria $C_5H_7NO_2$.

Similarly**,** the fate of COD_{LOAD} was COD in the biomass and the mineralized COD, since there was almost no COD detected in the effluent, equations 13-14:

$$COD_{BIOMASS}\,(\%) = (COD_{EFFB} + COD_{PURB})\,/COD_{LOAD} * 100 \tag{13}$$

$$COD_{MINERALIZED}\,(\%) = (100 - COD_{BIOMASS}\,(\%)) \tag{14}$$

Where COD_{EFFB} (g/d) is the amount of COD in the effluent biomass, and COD_{PURB} **(**g/d) is the amount of COD in the purge. The amount of COD in the biomass was calculated from the VSS and the general formula for bacteria: $C_5H_7NO_2$ (1.416 gCOD/gVSS).

Regarding the observed yield (Y_{OBS}) (gCODx/gCODs), the calculation of output of the biomass was obtained by formular (15):

$$P_X = V_{ANA} \cdot X_{ANA} + V_{ANOX} \cdot X_{ANOX} + V_{AER} \cdot X_{AER} \tag{15}$$

and the corresponding Y_{obs} in A_2O and S2EBPR systems was calculated by formular (16) and (17), respectively:

$$Y_{OBS} = \frac{P_X \cdot 1.416}{Q_{INF} \cdot COD_{INF}} \tag{16}$$

$$Y_{OBS} = \frac{P_X \cdot 1.416}{Q_{INF} \cdot COD_{INF} + Q_{SSSF} \cdot COD_{SSSF}} \tag{17}$$

Regarding energy recovery indexes: *methane recovery index* (*MRI*), an indicator of the mineralization and digestibility degree of the solids in the system, and *energy recovery index* (*ERI*), an indicator for the energy recovered by methane from the COD_{INF}, were calculated by equations (18) and (19) (Rey-Martínez et al., 2021a).

$$MRI = \frac{gCOD_{CH4}}{gCOD_{REM}} = Y_{obs}[\frac{gVSS}{gCOD_{REM}}] \cdot BMP[\frac{NLCH4}{gVSS}] \cdot ConvFact[\frac{gCOD_{CH4}}{NLCH4}] \tag{18}$$

$$ERI = \frac{kJ_{CH4}}{kJ_{INF}} = \frac{CH4\ prod\ [g\ CH4] \cdot Energy\ CH4\ [\frac{kJ}{g\ CH4}]\ CHP_{Efficciency}}{Influent\ load\ [gCOD_{INF}] \cdot Energy\ of\ Influent\ [\frac{kJ}{g\ COD_{INF}}]} \tag{19}$$

Where *ConvFact* in equation (18) was obtained as function of the T and P as 2.86 g COD_{CH4}/ NL CH_4. The theoretical value of the *Energy* CH_4 in equation (19) is 13.9 kJ/g CH_4, the value for $CHP_{Efficiency}$ was assumed as 35%, and the *Energy of the influent* was calculated as 15 kJ/gCOD (Heidrich et al., 2011; Korth et al., 2017; McCarty et al., 2011b).

6.3.4. *Batch tests*

6.3.4.1. PAO batch activity tests

Four A_2O batch tests were conducted to study the EBPR activities under different COD_{INF} conditions in A_2O system in a separate vessel. The biomass was obtained from the aerobic reactor when the system was at stable operation in period I c: (day 36), I e (day 57), I f (day 70), and I g (day 78). All of them were carried out in a system equipped with a magnetically stirred vessel of 2 L, DO probe (Cellox 325, WTW) and pH (Sentix 81, WTW) probe. The anaerobic, anoxic conditions were maintained 2 hours by nitrogen gas sparging, followed by 2 hours of aerobic condition with a mass flowmeter (MFC F-201CV, Bronkhorst). The carbon source for the anaerobic phase was the same composition as the feed for the A_2O pilot plant to reach a concentration about 200 mg/L and NO_3^--N was dosed at the end of anaerobic phase to reach the concentration about 10 mg N/L. The temperature and pH were controlled about 25°C and 7.5±0.3 throughout the process. Samples for phosphate, nitrogen species and COD were taken every 30 min and filtered with 0.22 µm filters (Millipore).

6.3.4.2. *Biochemical methane potential (BMP) batch tests in S2EBPR system*

Three sets of BMP experiments as in (Angelidaki et al., 2009) were conducted to investigate the methane produced from different sludge samples: from anaerobic, aerobic reactors and SSSF when the S2EBPR system had a stable performance. The inoculum sludge was from the anaerobic digester of an urban WWTP (Manresa, Barcelona) and degassed at 37 ºC for at least 3 days before use. The anaerobic digestion tests were conducted in 160 mL serum bottles with 125 mL of effective volume and 35 mL headspace for biogas production. All the tests lasted for 42 days. Further details are provided by Zhang et al. (2021).

6.3.5. *Microbiological analyses*

Sludge samples were collected from the aerobic reactor on day 36 (Period I c), 56 (I e), 69 (I f), 77 (I g), 105 (I j), and biomass from the aerobic reactor and SSSF on day175 (II c), 245 (period IIIc), 266 (period IIIe) and 283 (period IIIf) when the system reached to a stable operation. The bacterial population was identified by the Illumina amplicon sequencing of the 16S rRNA gene. The detailed process was as follows: the sample*s from* the system were washed by PBS for three times and centrifugated for further DNA extractions. Soil DNA isolation plus kit (Norgen Biotek CORP, Ontario., Canada) were used for Genomic DNA extraction process. Further, the obtained extracted DNA was detected and quantified by DNA NanoDrop 1000 Spectrophotometer (Waltham, MA, USA), and the purified DNA was performed in an Illumina MiSeq platform service center in Autonomous University of Barcelona (Barcelona, Spain). Universal primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were applied to amplify the V6–8 regions of the small subunit (SSU) rRNA prokaryote gene (16S) (Rey-Martínez et al., 2021b). The database used for the classification of organisms was based on the Greengenes database. The sequence reads were processed through Usearch software. Operational taxonomic units (OTUs) were generated with the open reference methodology.

6.4. Results

6.4.1. *Exploring the effect of influent COD limitation on the A_2O performance*

The first operational period I aimed at assessing the lower limit of the COD_{INF} under an A_2O configuration (Table 6.2). Period II investigated the performance of the S2EBPR configuration. Finally, period III dealt with the different potential locations for the SSSF effluent entering to the S2EBPR system. The input of P and N provided was 1.4 g/d and 5.6 g/d in the whole process (Table 6.1) whereas the COD input depended on the different operational periods (Table 6.2). Table 6.3 to Table 6.7 show the performance of the system regarding the different species (P, N, COD, solids and the solids relevant indicators) during the whole operation process. Accordingly, Figure 6.1 to 6.4 exhibit the removal performance of these parameters during the whole operation process.

Table 6.2. Operational parameters for each period: COD feed concentration, SSSF connection, purge flow position, and DO setpoint.

Period	Day operation	COD_{INF} (mg/L)	SSSF connection	Purge flow (L/d)	DO (mg/L)
I a	1	—	—	0 to10	2
I b	18	585	—	10	2
I c	32	500	—	10	2
I d	42	450	—	10	2
I e	50	400	—	10	2
I f	57	350	—	10	2
I g	70	300	—	10	2
I h	78	500	—	10	2
I i	84	400	—	10	2
I j	92	450	—	10	2
II a	107	450	anaerobic reactor	10	2
II b	119	350	anaerobic reactor	1	2
II c	133-175	350	anaerobic reactor	5	2
III a	206	350	anaerobic reactor	7	2
III b	217	350	disconnect SSSF	7	2
III c	232	350	anoxic reactor	7	2
III d	246	350	aerobic reactor	7	2
III e	255	350	aerobic reactor	7	3
III f	268-283	350	anaerobic reactor	7	3

After the start-up, it took 18 days for the reactor to reach a pseudo steady state under A_2O conditions (Period I a) with full P, ammonium and COD removal. Period I b had the highest influent COD concentration (585 mg/L) and it was successfully operated for two weeks. P_{ANA} and P_{AER} reached to 28.7 and 0.6 mg/L respectively with a $P_{REMOVAL\_ABSOLUTE}$ about 1.33 g/d (Table 6.3). In terms of N, there was neither ammonium, nor nitrite detected in the effluent. NO_3^--N was about 7.0 mg/L, indicating that all influent ammonium was oxidized into nitrate in aerobic reactor. Total $N_{REMOVAL\_ABSOLUTE}$ was about 4.79 g/d (Figure 6.1b and Table 6.4). Regarding organic carbon, total $COD_{REMOVAL\_ABSOLUTE}$ (76.1 g/d COD) was reached (Figure 6.1c, Table 6.5). VSS concentrations in the reactor and in the effluent were around 1.40 and 0.034 g/L (Figure 6.1d, Table 6.6), which indicated a period of stability in terms of biomass concentration. The A_2O plant with an excess of COD_{INF} could operate successfully.

COD_{INF} was decreased down to 500 mg/L in Period Ic for 10 days. Anaerobic P release was about 35.5 mg/L, and P in the anoxic and aerobic reactors was 11.6 and 0.3 mg/L, respectively. Again, all the performance indices were positive and COD_{INF} was successively decreased to 350 mg/L from Periods Id to If and, in all the cases, the high removal percentages of P, N and COD were maintained. The VSS concentration in the system showed an expected decrease trend with the decrease of COD_{INF} (Figure 6.2) (except for the Ib to Ic when undesired tap water fluctuations occurred).

The COD_{INF} was further decreased to 300 mg/L (Period I g) and P_{ANA} exhibited a decrease from 26.1 to 17.6 mg/L due to the COD limitations. An increased concentration of P_{AER} from 0.6 to 2.2 mg/L was observed in the last two days of this period. It was stated that the limit COD_{INF} to maintain successful EBPR activity in the A_2O system was 350 mg/L. Then, COD_{INF} was increased to 500 mg/L to temporarily recover the system and to avoid

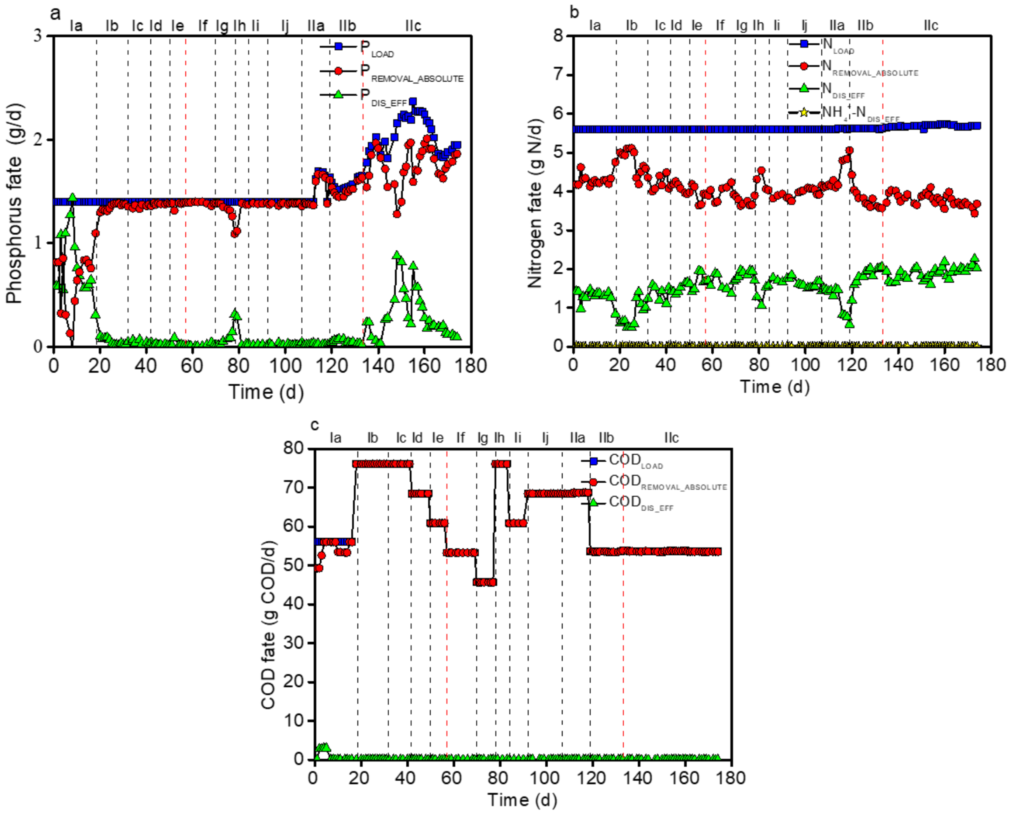


Figure 6.1. The fate of P (a), N (b) and COD (c) and the removal performance of A_2O (Period I) and S2EBPR (Period II) configurations.

Table 6.3. EBPR performance for each experimental period.

Period	$\mathrm{COD_{INF}}$ (mg/L)	$\mathrm{P_{ANA}}$ (mgP/L)	$\mathrm{P_{ANOX}}$ (mgP/L)	$\mathrm{P_{AER}}$ (mgP/L)	$\mathrm{P_{SSSF}}$ (mgP/L)	$\mathrm{P_{LOAD}}$ (g/d)	$\mathrm{P_{REMOVAL\_ABSOLUTE}}$ (g/d)	$\mathrm{P_{DIS\_EFF}}$ (g/d)
I a	—	—	—	—	—	—	—	—
I b	585	28.7±6.2	12.9±3.7	0.6±0.7	—	1.40±0	1.33±0.08	0.07±0.08
I c	500	35.5±7.5	11.6±2.3	0.3±0.1	—	1.40±0	1.36±0.02	0.04±0.02
I d	450	41.0±1.9	11.4±0.9	0.2±0.1	—	1.40±0	1.38±0.01	0.02±0.01
I e	400	32.9±3.8	10.7±1.3	0.2±0.2	—	1.40±0	1.37±0.03	0.03±0.03
I f	350	26.1±5.1	6.9±1.1	0.1±0.1	—	1.40±0	1.39±0.01	0.01±0.01
I g	300	17.6±6.0	4.1±1.4	0.6±0.7	—	1.40±0	1.34±0.05	0.06±0.05
I h	500	29.2±10.7	10.1±3.3	0.6±0.9	—	1.40±0	1.24±0.16	0.16±0.16
I i	400	32.7±4.7	9.6±3.4	0.1±0.0	—	1.40±0	1.38±0.01	0.02±0.01
I j	450	38.6±4.2	10.7±1.8	0.1±0.1	—	1.40±0	1.38±0.01	0.02±0.01
II a	450	34.7±5.2	11.8±1.7	0.2±0.1	—	1.50±0.13	1.47±0.14	0.02±0.01
II b	350	30.3±6.5	9.8±1.4	0.3±0.1	—	1.57±0.05	1.52±0.06	0.05±0.02
II c	350	37.5±6.5	14.4±2.5	2.0±1.6	88.7±15.9	2.07±0.15	1.76±0.14	0.29±0.23
III a	350	27.5±4.3	14.8±2.3	7.8±1.3	87.6±7.0	2.14±0.06	1.01±0.18	1.13±0.18
III b	350	20.5±5.0	11.8±1.8	6.8±1.0	89.3	1.40±0	0.42±0.14	0.98±0.14
III c	350	16.9±1.8	12.7±1.6	8.8±0.7	49.2±10.0	1.81±0.08	0.54±0.13	1.27±0.11
III d	350	21.7±2.6	12.8±1.2	5.1±1.3	44.6±4.4	1.77±0.04	1.04±0.22	0.73±0.18
III e	350	25.9±2.6	12.2±2.0	5.2±1.3	22.6±16.4	1.59±0.14	0.84±0.19	0.75±0.19
III f	350	24.3±4.1	10.7±1.8	2.3±0.6	16.2±5.0	1.54±0.04	1.20±0.11	0.34±0.09

Table 6.4. N removal performance and mass balance for each experimental period.

Period	COD_{INF} (mg/L)	NH_4^+-N removal efficiency (%)	N removal efficiency (%)	NH_4^+-N_{SSSF} (mg/L)	N_{LOAD} (g/d)	$N_{REMOVAL\_ABSOLUTE}$ (g/d)	N_{DIS_EFF} (g/d)	$N_{BIOMASS}$ (%)	N_{LIQUID} (%)	$N_{DENITRIFIED}$ (%)
I a	—	—	—	—	—	—	—	—	—	—
I b	585	100±0	84±6	—	5.60±0	4.79±0.31	0.81±0.31	44±2	16±6	40±7
I c	500	100±0	73±4	—	5.60±0	4.22±0.19	1.38±0.19	50±2	27±4	24±3
I d	450	100±0	71±2	—	5.60±0	4.11±0.12	1.49±0.12	45±3	29±2	27±3
I e	400	100±0	68±4	—	5.60±0	3.92±0.23	1.68±0.23	41±2	32±4	26±5
I f	350	100±0	69±3	—	5.60±0	3.96±0.17	1.64±0.17	33±4	31±3	35±5
I g	300	100±0	64±2	—	5.60±0	3.72±0.09	1.88±0.09	27±3	36±2	37±3
I h	500	100±0	73±5	—	5.60±0	4.20±0.28	1.40±0.28	28±3	27±5	45±5
I i	400	100±0	68±2	—	5.60±0	3.92±0.09	1.68±0.09	30±1	32±2	37±3
I j	450	100±0	69±2	—	5.60±0	3.96±0.11	1.64±0.11	36±4	31±2	33±4
II a	450	100±0	74±6	—	5.61±0.01	4.34±0.32	1.27±0.32	35±9	24±6	41±4
II b	350	100±0	68±8	—	5.63±0.00	3.93±0.43	1.70±0.43	15±7	31±8	54±4
II c	350	100±0	63±3	11.0±3.2	5.69±0.03	3.79±0.16	1.91±0.16	19±3	35±3	46±4
III a	350	100±0	62±2	21.4±1.4	5.78±0.01	3.81±0.11	1.97±0.11	23±1	37±2	40±2
III b	350	100±0	65±3	41.0	5.60±0.00	3.71±0.17	1.89±0.17	22±3	35±3	43±4
III c	350	100±0	63±3	25.3±6.7	5.81±0.06	3.81±0.12	2.00±0.12	26±3	37±2	36±5
III d	350	59±9	50±8	29.4±5.6	5.85±0.05	3.20±0.40	2.65±0.40	21±2	50±8	30±9
III e	350	92±11	69±7	12.1±7.9	5.70±0.07	4.04±0.36	1.66±0.36	17±1	31±7	52±8
III f	350	87±12	64±9	11.8±5.9	5.70±0.05	3.84±0.36	1.86±0.36	20±1	35±9	45±10

Table 6.5. COD removal performance and mass balance for each experimental period.

Period	COD_{INF} (mg/L)	COD/P (mg/mg)	COD_{SSSF} (mg/L)	COD_{LOAD} (g/d)	$COD_{REMOVAL\_ABSOLUTE}$ (g/d)	$COD_{BIOMASS}$ (%)	$COD_{MINERALIZED}$ (%)
I a	—	—	—	—	—	—	—
I b	585	54.4±0	—	76.1±0	76.1±0	37±1	63±1
I c	500	54.4±0	—	76.1±0	76.1±0	42±2	58±2
I d	450	48.9±0	—	68.5±0	68.5±0	42±3	58±3
I e	400	43.5±0	—	60.9±0	60.9±0	44±3	56±3
I f	350	38.0±0	—	53.3±0	53.3±0	40±4	60±4
I g	300	32.6±0	—	45.6±0	45.6±0	38±4	62±4
I h	500	54.4±0	—	76.1±0	76.1±0	24±2	76±2
I i	400	43.5±0	—	60.9±0	60.9±0	32±1	68±1
I j	450	48.9±0	—	68.5±0	68.5±0	34±4	66±4
II a	450	45.9±3.8	—	68.6±0.1	68.6±0.1	32±8	68±8
II b	350	34.1±1.1	—	53.5±0.1	53.5±0.1	19±8	81±8
II c	350	26.3±2.2	40±10	53.6±0.1	53.6±0.1	23±3	77±3
III a	350	25.2±0.7	69±11	53.8±0.1	53.8±0.1	27±1	73±1
III b	350	38.0±0	112	53.3±0	53.3±0	27±3	73±3
III c	350	38.0±0	62±15	53.8±0.1	53.8±0.1	31±2	69±2
III d	350	38.0±0	54±6	53.7±0.1	53.7±0.1	25±2	75±2
III e	350	38.0±0	28±7	53.5±0.1	53.5±0.1	21±1	79±1
III f	350	34.8±0.9	23±4	53.5±0	53.5±0	24±2	76±2

Table 6.6. Average solids concentration, SRT and output sludge production in the system for each experimental period.

Period	COD$_{INF}$ (mg/L)	SRT (d)	VSS$_{purge}$ (g/L)	VSS$_{effluent}$ (g/L)	VSS$_{SSSF}$ (g/L)	TSS$_{purge}$ (g/L)	TSS$_{effluent}$ (g/L)	TSS$_{SSSF}$ (g/L)	Output sludge production (g/d)
I a	—		—	—	—	—	—	—	—
I b	585	10.2±0.5	1.40±0.05	0.034±0.009	—	1.61±0.07	0.038±0.012	—	20.0±0.8
I c	500	10.8±0.7	1.65±0.10	0.030±0.006	—	1.87±0.11	0.034±0.007	—	22.5±1.0
I d	450	11.7±0.2	1.62±0.13	0.028±0.003	—	1.91±0.17	0.033±0.003	—	20.2±1.5
I e	400	11.3±0.6	1.45±0.06	0.027±0.009	—	1.67±0.07	0.032±0.010	—	18.8±1.1
I f	350	11.7±0.1	1.13±0.13	0.027±0.006	—	1.31±0.16	0.031±0.007	—	15.1±1.6
I g	300	11.8±0.8	0.99±0.07	0.017±0.006	—	1.14±0.07	0.018±0.006	—	12.3±1.4
I h	500	12.2±0.3	1.06±0.08	0.015±0.003	—	1.26±0.09	0.019±0.003	—	12.7±1.3
I i	400	12.3±0.6	1.16±0.04	0.016±0.005	—	1.40±0.05	0.019±0.006	—	13.8±0.6
I j	450	11.4±0.8	1.26±0.07	0.026±0.009	—	1.49±0.09	0.030±0.009	—	16.3±1.7
II a	450	9.0±1.9	0.98±0.11	0.041±0.034	1.78±0.07	1.16±0.13	0.049±0.038	2.07±0.14	15.7±3.9
II b	350	16.5±6.9	0.75±0.10	0.041±0.022	1.39±0.20	0.88±0.14	0.048±0.025	1.64±0.26	6.9±3.2
II c	350	12.4±0.6	0.95±0.12	0.025±0.009	1.73±0.21	1.15±0.16	0.031±0.010	2.02±0.30	8.5±1.2
III a	350	11.6±0.4	1.07±0.05	0.020±0.003	1.83±0.05	1.26±0.06	0.024±0.004	1.98±0.08	10.3±0.5
III b	350	10.3±1.6	0.96±0.04	0.023±0.009	1.89-1.13	1.12±0.06	0.026±0.009	1.98-1.13	10.0±1.2
III c	350	9.2±0.5	0.82±0.07	0.042±0.004	1.42±0.09	0.88±0.09	0.045±0.005	1.46±0.11	11.8±0.9
III d	350	10.2±0.3	0.77±0.02	0.028±0.005	1.28±0.04	0.87±0.03	0.031±0.004	1.31±0.05	9.3±0.7
III e	350	7.8±1.5	0.66±0.06	0.023±0.004	0.73±0.26	0.82±0.05	0.030±0.005	0.80±0.24	8.0±0.6
III f	350	7.8±0.9	0.76±0.04	0.026±0.004	0.84±0.15	0.96±0.06	0.033±0.005	1.05±0.22	9.0±0.6

the loss of EBPR activity (Period I h). In spite of the fast EBPR recovery, the system showed bulking issues. Dispersed growth was observed, and it was likely due to a sudden and large increase of COD_{INF}. Floc-forming species may grow in a non-settleable form when exposed to a sudden high organic loading. The best response to this problem is a reduction ratio in the F/M of the system (Eckenfelder and Musterman, 1998; Richard, 2003). Thus, the COD_{INF} was decreased to 400 mg/L (Period I i) to alleviate the disperse growth, and Period I j (450 mg/L) had the same objective. The corresponding P_{ANA} and P_{AER} recovered to the previous condition and the bulking problem improved slightly. The amount of $P_{REMOVAL\_ABSOLUTE}$ and P in the effluent were similar to those of Period I d with 1.38 g/d and 0.02 g/d respectively.

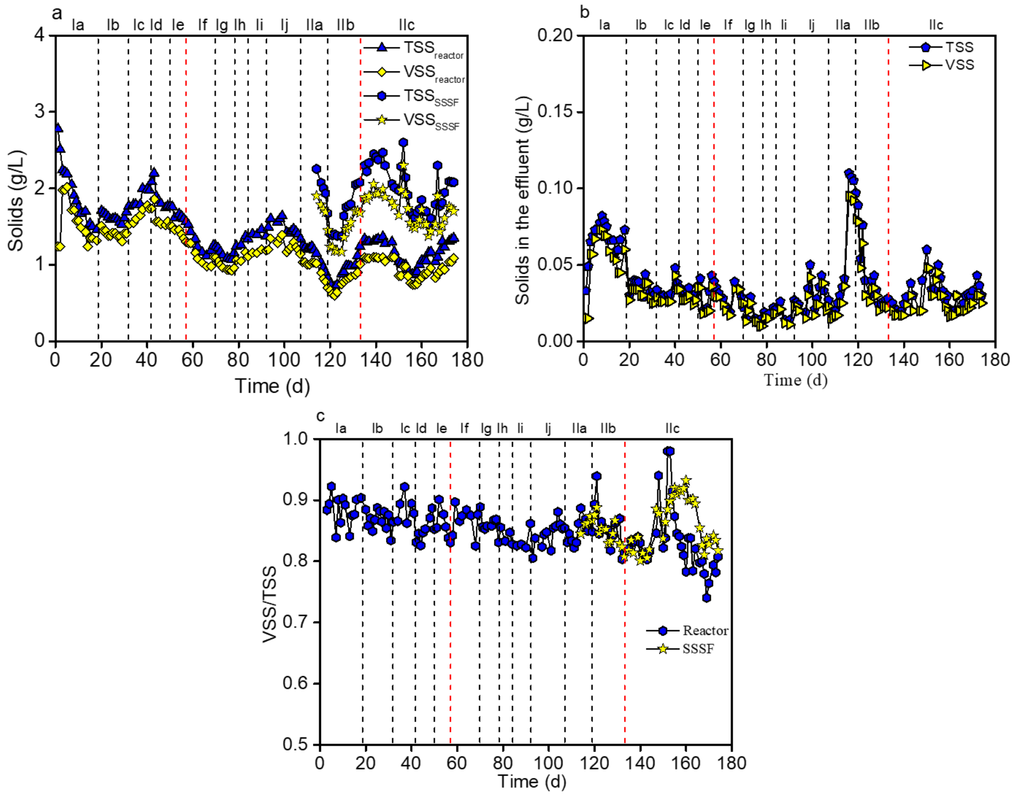


Figure 6.2. Solids concentration in the reactor (a), in the effluent (b), the ratio of VSS and TSS (c) of the A_2O and S2EBPR systems in Periods I and II.

Four batch tests were performed to evaluate PAO activity (Table 6.7). High P anaerobic release and aerobic uptake rates were obtained with the system operation of 500 mg/L COD_{INF} (0.4 and 0.2 mgP/gVSS min respectively). The lowest P uptake rate in the anoxic and aerobic phases was observed (0.022 and 0.024 mgP/gVSS min respectively) for the biomass withdrawn under the lowest COD_{INF} condition (300 mg/L). That led to incomplete P uptake detected at the end of aerobic condition. Hence, PAO were active during all the A_2O operations except for the period with limitations under COD_{INF} of 300 mg/L.

Table 6.7. PAO activity for A_2O respirometry tests with sludge from the aerobic reactor under different COD_{inf} conditions

Period	Operation day	COD_{inf} (mg/L)	PO_4^{3-}-P_{max} (mgP/L)	P_{ana} release rate (mgP/gVSS min)	P_{anox} uptake rate (mgP/gVSS min)	P_{aer} uptake rate (mgP/gVSS min)
I c	36	500	34.5	0.372	0.049	0.200
I e	57	400	28.4	0.176	0.055	0.069
I f	70	350	32.4	0.349	0.051	0.074
I g	78	300	21.6	0.133	0.022	0.024

6.4.2. *Performance of the S2EBPR configuration*

On day 107, the SSSF was installed and fed with a biomass enriched stream from the settler (8.4 L/d, i.e. 6% of the influent flowrate) which, in turn, was connected to the anaerobic reactor (Period II a). The initial integration of the SSSF with a high daily purge volume (10 L) and 450 mg/L of COD_{INF} exhibited bulking issues (VSS in the reactor decreasing from 0.98 to 0.75 g/L and in the effluent increasing from 0.023 to 0.095 g/L). Period II b was designed as a recovery period with lower COD_{INF} and lower purge flow to avoid biomass washing out and the solids concentration in the system recovered subsequently without affecting the P performance.

Then, the purge was increased again in Period II c to reach the targeted SRT (11.7±0.1 d) and the same COD_{INF} of 350 mg/L as Period I f to allow for a thorough comparison of S2EBPR vs A_2O. In fact, the comparison of S2EBPR vs A_2O in the Results sections 3.4 and 3.5 and the Discussion section is based on the system operation in Period I f and Period II c operating with the same COD_{INF}. The performance of the SSSF for the different periods is shown in Table 6.3 to 6.6 (the concentrations of P, N, COD and solids were indicated as P_{SSSF}, NH_4^+-N_{SSSF}, COD_{SSSF}, VSS_{SSSF} and TSS_{SSSF}) and Figure 6.3a. Despite the original objective of the SSSF was to promote VFA production, it has been reported that P release and carbon uptake can occur simultaneously (Onnis-Hayden et al., 2020; Vale et al., 2008; Vollertsen et al., 2006). Therefore, a high P release was observed in the SSSF reactor from the VFA produced from the sludge fermentation, which was directly stored as PHA by PAO. On average, the SSSF showed an absolute P release about 88.7 mg/L and a concentration of COD detection around 40 mg/L. Thus, the SSSF did not provide the expected extra organic matter to the anaerobic reactor, but the system began to receive a higher P_{LOAD} (Figure 6.1a and Table 6.3). As a result, the S2EBPR configuration showed higher P_{ANA} (37.5 vs 26.1 mg/L) compared with A_2O in period I f, P_{LOAD} (2.07 vs 1.40 g/d), and the effluent P was still very low, so a higher $P_{REMOVAL\_ABSOLUTE}$ was obtained (1.76 vs 1.39 g/d). The EBPR activity with the SSSF integration was enhanced by around 27%. However, it can’t be denied that the high increase of P_{LOAD} led to undesired and unstable effluent quality, with P_{AER} about 2.0±1.6 mg/L.

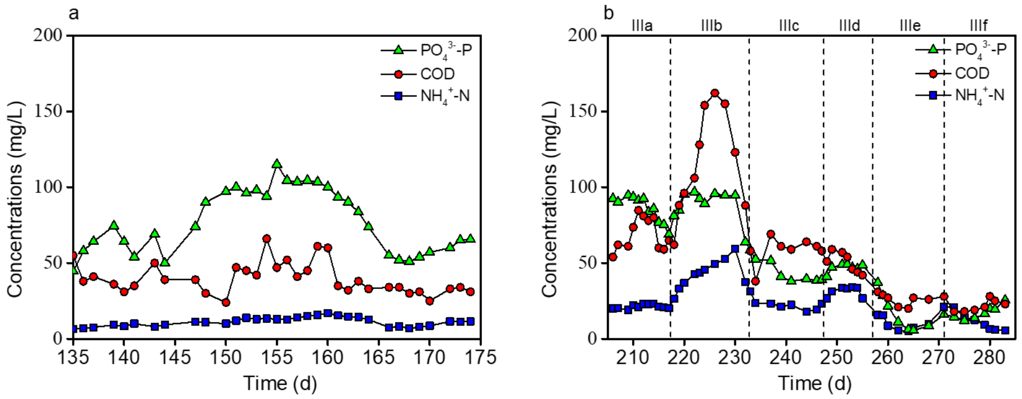


Figure 6.3. Profiles of PO_4^{3-}-P, COD and NH_4^+-N in SSSF during period IIc (a) and III (b).

In terms of N, the SSSF effluent provided around 11.0 mg NH_4^+-N /L (without nitrate and nitrite detection) (Table 6.4 and Figure 6.3a) due to the fermentation/hydrolysis of the biomass and cell lysis or decay in SSSF. Thus, the S2EBPR showed a higher N_{LOAD} than the A_2O system (5.69 vs 5.60 g/d) and, thus, a higher N in the effluent (1.91 vs 1.64 g/d) without any observed influence on nitrification. Mass balance showed that the percentage of influent N incorporated into the $N_{BIOMASS}$ decreased from 33% with A_2O to 19% with S2EBPR, and it was reasonable since less purge was discharged. Accordingly, the percentage of $N_{DENITRIFIED}$ improved from 35% (A_2O) to 46% (S2EBPR), which showed that the integration of SSSF improved the denitrification degree.

Regarding the COD, the SSSF effluent contained about 40 mg/L COD (Table 6.5 and Figure 6.3a), which only represented a very low increase of the COD_{LOAD} of S2EBPR from 53.3 to 53.6 g/d (0.5% improvement). Full COD removal efficiency indicated that the SSSF integration did not affect the COD removal since most of the COD produced was used *in situ* in the SSSF. The COD mass balance showed a higher percentage of $COD_{MINERALIZED}$ (77%) in S2EBPR compared to the A_2O (60%), which agreed with part of the theoretical purge being degraded in the SSSF. The solids in the reactors in both scenarios (S2EBPR and A_2O) were around 1g/L. However, the solids in the SSSF were always higher than that in the reactors (around 2g/L) due to the use of the concentrated biomass stream from the settler. The ratio VSS/TSS in SSSF tended to be higher than that of A_2O system, which indicated that the biomass in SSSF was releasing P and the internal polyphosphate levels were much lower. In addition, the sludge production decreased from 15.1 to 8.5 g/d because the purge flow of S2EBPR was half compared with A_2O to maintain a similar SRT (see below in the discussion section).

6.4.3. *The possibilities of different combination of SSSF in the S2EBPR*

Period III aimed at comparing the different combination locations for the effluent of the SSSF (Table 6.2 and Figure 6.4 and 6.5). Period III a had the SSSF connected to the anaerobic reactor and a purge flow of 7L/d from day 206 to maintain the targeted SRT. There was a high P release in the system with 27.5 mgP/L in the anaerobic reactor (Table 6.3). However, a much high quantity of P_{LOAD} and P in the effluent was detected respectively, 2.14 and 1.13 g/d. Ammonium and COD removal (Table 6.4 and 6.5) were not affected, and solids were maintained at 1.07 g VSS/L in the reactor with only 0.020 g VSS/L in the effluent (Table 6.6 and Figure 6.5).

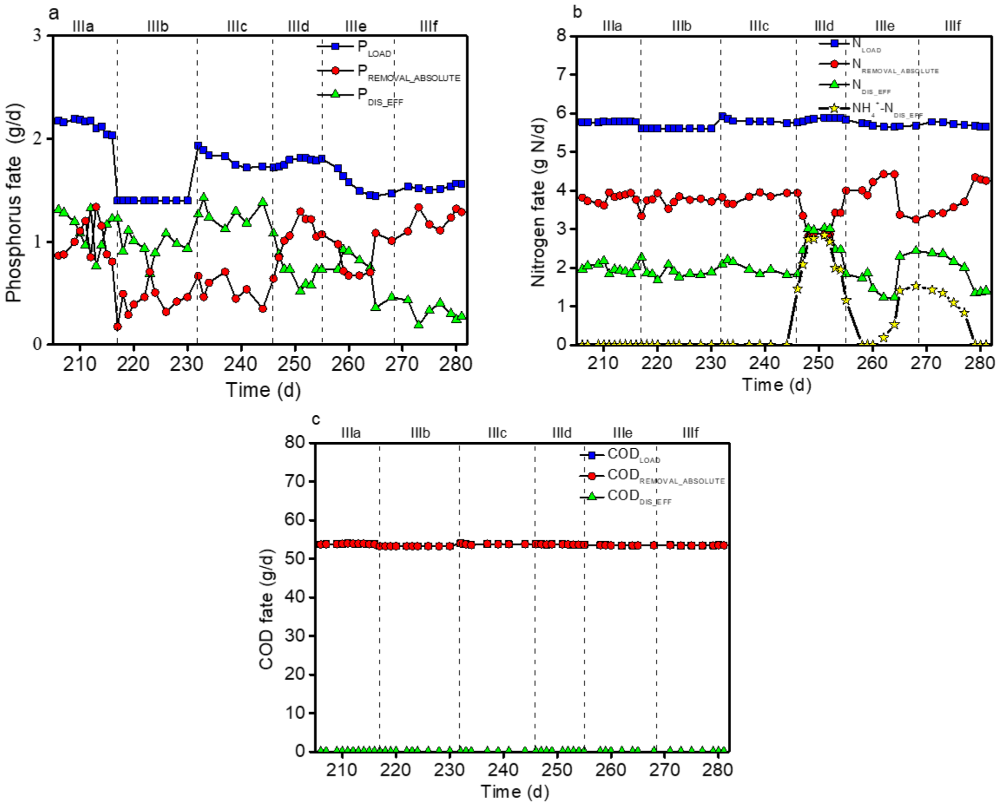


Figure 6.4. Fate of P (a), N (b) and COD (c) and the removal performances of the S2EBPR system in Period III.

SSSF was disconnected for a short period III b to investigate the performance without SSSF as in a conventional A_2O configuration. Even though the P_{LOAD} was reduced to 1.4 g/d, no improvement of $P_{REMOVAL\_ABSOLUTE}$ (with only 0.42 g/d) was detected. P_{ANA} was reduced from 27.5 to only 20.5 mg/L though a much higher influent COD to P ratio (38.0 vs 25.2) compared with that in period III a. A poorer EBPR performance was observed but N and COD removal performance showed no big difference between these two periods. It can be inferred that the transformation of S2EBPR to traditional EBPR process made no improvement to the system performance, even with a relatively lower P_{LOAD} and higher COD/P ratio.

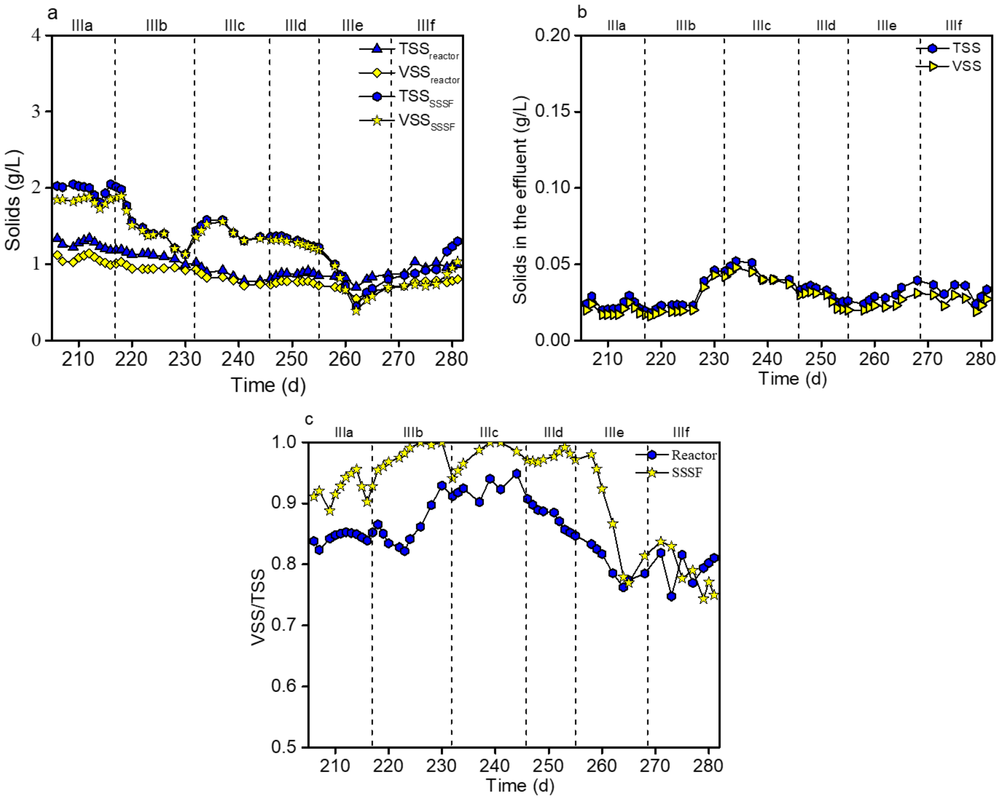


Figure 6.5. Solids concentration in the reactor (a), in the effluent (b), the ratio of VSS and TSS (c) of the S2EBPR in Period III.

The possibility of connecting the SSSF to the anoxic reactor was explored in period III c. That would be a preferred option if the influent COD has enough VFA fraction to remove P but there are some COD limitations in the anoxic reactor for nitrate removal. However, this combination led to the worst results in terms of P release with only 16.9 mg/L in the anaerobic reactor. $P_{REMOVAL\_ABSOLUTE}$ and P in the effluent were 0.54 and 1.27 g/d, respectively, and the P release in the SSSF also decreased to 49.2 mg/L (Table 6.3 and Figure 6.3b). Despite the decay of PAO performance, ammonium and COD were completely removed. Period I f proved that complete P removal could be achieved with a COD_{INF} of 350 mg/L. However, P_{LOAD} to the plant increased with the integration of a SSSF in period III c (1.81 vs 1.40 g/d) and that increase was too high to maintain complete P removal. Biological N removal was maintained since the SSSF effluent flowing to the anoxic reactor favored denitrification.

Finally, the SSSF effluent was connected to the aerobic reactor (period III d). The average indicators (P, N, COD and solids) of SSSF didn't show obvious change. However, for the whole system, EBPR performance improved as it showed high P release in the anaerobic reactor and P uptake in the aerobic reactor (with P_{ANA} about 21.7 mg/L and P_{AER} about 5.1 mg/L). $P_{REMOVAL\_ABSOLUTE}$ increased to 1.04 g/d, which was almost double of the last period with the SSSF effluent connected to the anoxic reactor. However, full nitrification was lost with only 59% of ammonium removal efficiency. This decrease was attributed to a decrease of oxygen availability for nitrifiers in the aerobic reactor. With this configuration, organic products generated in the SSSF were redirected to the aerobic reactor, increasing its organic load. Although the DO control was able to increase the aeration flow rate to maintain the desired DO setpoint, this increase of organic load probably led to a deficit of oxygen in the inner layers of the floccules, resulting in an overall lower nitrifying activity. Nevertheless, full COD removal was still maintained, but with higher aeration requirements. Then, the organic matter generation in the SSFF was detrimental since it was only oxidized under aerobic conditions (resource-consuming process) rather than being diverted to be used to enhance nutrient removal (i.e. P release or denitrification). The reason for the improvement of P removal could be the decrease of nitrate entering the anaerobic reactor.

In order to gain insight on the underlying causes of the system failure, DO was increased to 3.0 mg/L to obtain full nitrification in the subsequent period III e. Biological N removal was recovered (nitrification from 59% to 92% and the denitrification from 30% to 52%), but the surplus oxygen flowing to the SSSF showed a detrimental effect and P concentration in SSSF decreased to half (from 44.6 to 22.6 mg/L). The solids in the SSSF decreased from 1.28 g/L to 0.73 g/L (Table 6.6), which led to almost the same level of that in the aerobic reactor (0.66 g/L). Hence, nitrification was improved due to the high DO in the aerobic reactor at expense of a bad SSSF performance.

Finally, the combination of SSSF to the anaerobic reactor with higher DO condition were tested (period III f). The SSSF performance didn’t show improvement except for a slight increase of VSS concentration from 0.73 to 0.84 g/L. However, EBPR improved extensively with only 0.34 gP/d detected in the effluent without significant change of N and COD performance.

According to the above results, the optimum integration position of SSSF to A_2O for EBPR performance under a low COD_{INF} condition is to the anaerobic reactor. The worst EBPR performance was obtained by the combination of SSSF to anoxic reactor, and the reason needs to be further explored. The integration of SSSF to aerobic reactor could enhance the P activity at expense of a detrimental effect on nitrification. Finally, aerobic DO seemed to show a significant effect on the nitrification and the individual SSSF performance, which could be essential for the EBPR performance with the integration of SSSF.

6.4.4. *Energy recovery based on BMP in S2EBPR*

Recovering part of the chemical energy contained in the wastewater is a hot trend in current water resource recovery facilities. Considering that the SSSF integration decreased the amount of solids purged, the likelihood of biogas production should be compromised. The sludge coming from an EBPR system may have different methane production depending on the location of the purge because of the different PHA content (Chan et al., 2020a; Huda et al., 2016). Figure 6.6 and Table 6.8 compare the BMP of different sludge samples from the S2EBPR system: sludge from anaerobic, aerobic reactors and SSSF in Period II c. The highest BMP was obtained from the anaerobic sludge with about 250 mL CH_4 /gVSS, followed by aerobic and SSSF, with 221 and 215 mL CH_4 /gVSS, respectively. Anaerobic biomass showed a 16% higher BMP than aerobic sludge. Unexpectedly, the BMP of SSSF was closer to that in the aerobic reactor rather than being similar to the anaerobic BMP. It can be speculated that the internal levels of PHA in the biomass from the SSSF were lower to those from the anaerobic reactor. The initial methane production rates varied from different sludge samples, the anaerobic sludge exerted the highest value with 27.3 mL CH_4/(gVSS·d), followed by 24.7 and 22.3 mL CH_4/(gVSS·d) for aerobic and SSSF sludge, respectively.

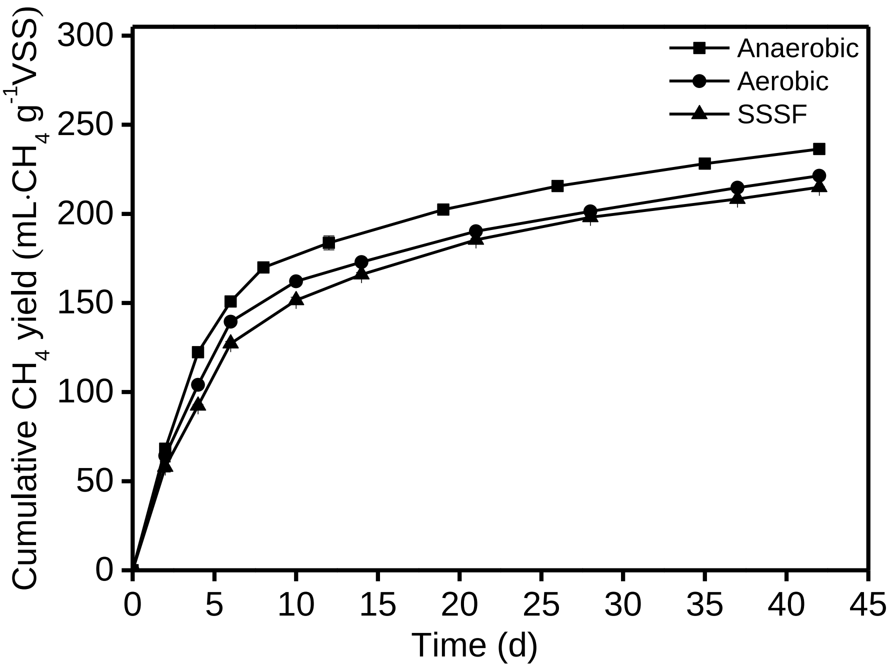


Figure 6.6. Biomass samples obtained from anaerobic, aerobic and SSSF reactors for anaerobic biochemical methane potential tests under the operation of S2EBPR.

Table 6.8. Biochemical methane potential (BMP) tests with biomass from anaerobic, aerobic reactor and SSSF

Sludge sample [a]	BMP [b] (mL CH_4 / gVSS)	Initial methane production rate [c] (mL CH_4·/ gVSS·d^{-1})
Anaerobic reactor	250±10	27.3±0.8
Aerobic reactor	221±3	24.7±0.6
SSSF	215±11	22.3±1.7

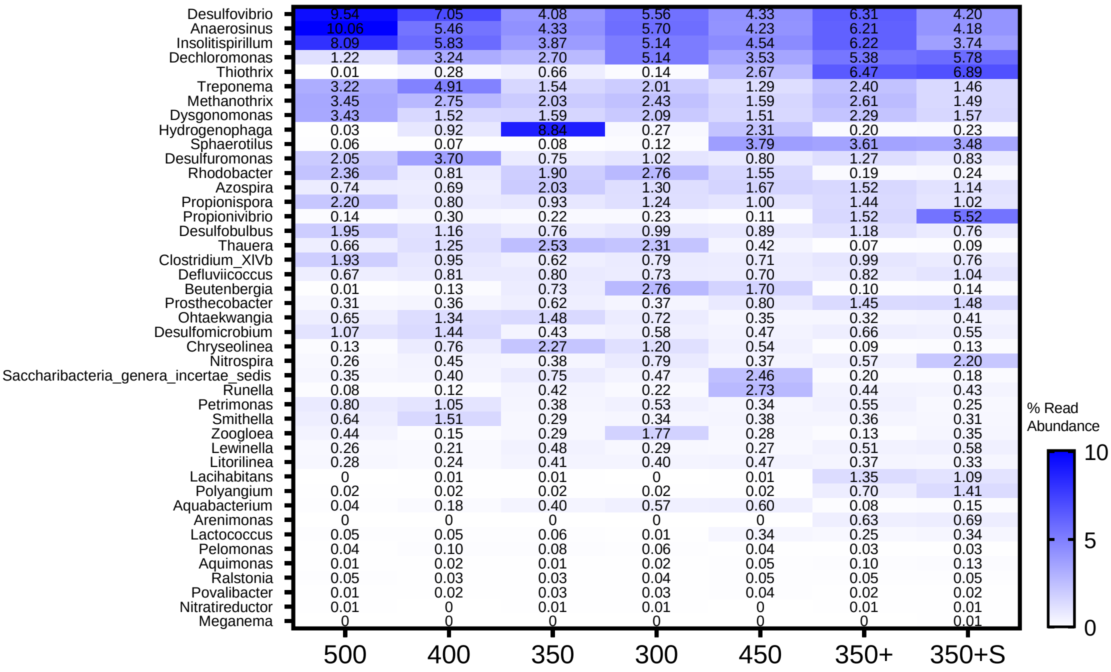
[a] The sludge samples were taken from anaerobic, aerobic reactor and SSSF in Period IIc during stable state

[b] The final cumulative CH_4 yield obtained from different sludge samples in 42 days

[c] The rates for the first 10 days of anaerobic digestion

6.4.5. *The microbiological community in A_2O and S2EBPR*

The variations and relative abundances of the bacteria selected in A_2O (period I) and S2EBPR (period II and III) were analysed by 16S rRNA gene sequencing, where Figure 6.7 compares the microbiological community observed at the genus level in the operation of periods I and II (identity derived at an OTU threshold of 96.5% similarity). The OTU was about 1414 in A_2O system in period I (Table 6.9), which suggested that the diversity of the microorganisms was enriched with the stable and successful EBPR performance. The subsequent S2EBPR process in period II and III had the same level of OTUs in the reactor (1430) and in the SSSF (1480) as A_2O.



	500	400	350	300	450	350+	350+S
Desulfovibrio	9.54	7.05	4.08	5.56	4.33	6.31	4.20
Anaerosinus	10.06	5.46	4.33	5.70	4.23	6.21	4.18
Insolitispirillum	8.09	5.83	3.87	5.14	4.54	6.22	3.74
Dechloromonas	1.22	3.24	2.70	5.14	3.53	5.38	5.78
Thiothrix	0.01	0.28	0.66	0.14	2.67	6.47	6.89
Treponema	3.22	4.91	1.54	2.01	1.29	2.40	1.46
Methanothrix	3.45	2.75	2.03	2.43	1.59	2.61	1.49
Dysgonomonas	3.43	1.52	1.59	2.09	1.51	2.29	1.57
Hydrogenophaga	0.03	0.92	8.84	0.27	2.31	0.20	0.23
Sphaerotilus	0.06	0.07	0.08	0.12	3.79	3.61	3.48
Desulfuromonas	2.05	3.70	0.75	1.02	0.80	1.27	0.83
Rhodobacter	2.36	0.81	1.90	2.76	1.55	0.19	0.24
Azospira	0.74	0.69	2.03	1.30	1.67	1.52	1.14
Propionispora	2.20	0.80	0.93	1.24	1.00	1.44	1.02
Propionivibrio	0.14	0.30	0.22	0.23	0.11	1.52	5.52
Desulfobulbus	1.95	1.16	0.76	0.99	0.89	1.18	0.76
Thauera	0.66	1.25	2.53	2.31	0.42	0.07	0.09
Clostridium_XIVb	1.93	0.95	0.62	0.79	0.71	0.99	0.76
Defluviicoccus	0.67	0.81	0.80	0.73	0.70	0.82	1.04
Beutenbergia	0.01	0.13	0.73	2.76	1.70	0.10	0.14
Prosthecobacter	0.31	0.36	0.62	0.37	0.80	1.45	1.48
Ohtaekwangia	0.65	1.34	1.48	0.72	0.35	0.32	0.41
Desulfomicrobium	1.07	1.44	0.43	0.58	0.47	0.66	0.55
Chryseolinea	0.13	0.76	2.27	1.20	0.54	0.09	0.13
Nitrospira	0.26	0.45	0.38	0.79	0.37	0.57	2.20
Saccharibacteria_genera_incertae_sedis	0.35	0.40	0.75	0.47	2.46	0.20	0.18
Runella	0.08	0.12	0.42	0.22	2.73	0.44	0.43
Petrimonas	0.80	1.05	0.38	0.53	0.34	0.55	0.25
Smithella	0.64	1.51	0.29	0.34	0.38	0.36	0.31
Zoogloea	0.44	0.15	0.29	1.77	0.28	0.13	0.35
Lewinella	0.26	0.21	0.48	0.29	0.27	0.51	0.58
Litorilinea	0.28	0.24	0.41	0.40	0.47	0.37	0.33
Lacihabitans	0	0.01	0.01	0	0.01	1.35	1.09
Polyangium	0.02	0.02	0.02	0.02	0.02	0.70	1.41
Aquabacterium	0.04	0.18	0.40	0.57	0.60	0.08	0.15
Arenimonas	0	0	0	0	0	0.63	0.69
Lactococcus	0.05	0.05	0.06	0.01	0.34	0.25	0.34
Pelomonas	0.04	0.10	0.08	0.06	0.04	0.03	0.03
Aquimonas	0.01	0.02	0.01	0.02	0.05	0.10	0.13
Ralstonia	0.05	0.03	0.03	0.04	0.05	0.05	0.05
Povalibacter	0.01	0.02	0.03	0.03	0.04	0.02	0.02
Nitratireductor	0.01	0	0.01	0.01	0	0.01	0.01
Meganema	0	0	0	0	0	0	0.01

Figure 6.7. Microbial communities at the genus level observed during the different operational periods: 500, 400, 350, 300, 450 mg/L/ of COD_{inf} in the A_2O system (period I) ; 350+ and 350+S are samples from the aerobic reactor and SSSF of the S2EBPR system (period IIc) obtained operating with COD_{inf} of 350 mg/L.

Among the observed OTUs, the clusters *Desulfovibrio*, *Anaerosinus*, *Insolitispirillum* and *Dechloromonas* were detected to be dominant along the whole operation process. Thereinto, *Desulfovibrio* were considered as heterotrophic denitrifying bacteria (Chu and Wang, 2013), and it has been observed in a system with the simultaneous removal of N and P (Sun et al., 2021). *Desulfovibrio* was generally the most abundant organism during the whole study, no matter if the system was A_2O (4%-10%) under the condition of COD_{INF} as 500, 400, 350, 300 and 450 mg/L respectively), or S2EBPR (about 6.3% and 4.2% under the condition of COD_{INF} as 350 mg/L in the aerobic reactor and SSSF respectively). *Dechloromonas*, which can use oxygen or NO_X^--N as electron acceptors, have been reported to exist extensively in full-scale WWTP and have been considered as functional PAO (Petriglieri et al., 2021). The relative abundance in the A_2O system was in the range of 1.22-5.15%, and in the S2EBPR system was found to be 5.38% in the aerobic reactor and 5.78% in the SSSF.

Table 6.9. OTU from Sequencing results at the level of family, genus, and species.

Configuration	A_2O^a	S2EBPR[b]	S2EBPR[c]
Family	289±49	276±19	279±13
Genus	1056±283	1043±120	1086±118
Species	1414±494	1430±260	1480±239
N[d]	10	8	8

a biomass from aerobic reactor in period I
b biomass from aerobic reactor of S2EBPR in period II and III
c biomass from SSSF of S2EBPR in period II and III
d the number of the samples

Rhodobacter (HIRAIS et al., 1991) and *Thauera* (Zhang et al., 2019) have been implicated as potential PAO in conventional EBPR systems. Interestingly, these organisms were found to be of higher abundance during the A_2O operation rather than S2EBPR. However, *Thiothrix* was much higher in abundance in the S2EBPR process (more than 6.5%) as compared to A_2O (from 0.01% to 2.7%). *Thiothrix* has been recognized as candidate PAO in a broad range of reports (Meng et al., 2020; Rey-Martínez et al., 2019; Rubio-Rincón et al., 2017) and grew in a low COD condition (Rubio-Rincón et al., 2017). This correlated well with the results of this study, since the ratio of C/P reached the lowest level under the operation of the S2EBPR system. *Thiothrix* was also considered as a typical filamentous bacteria which could lead to settling problems of activated sludge (Vaiopoulou et al., 2007). Notably, the percentage of *Thiothrix* began to increase in the A_2O process when the COD_{INF} was 450 mg/L and sludge bulking was observed (SVI around 571 mL/g). However, In S2EEBPR there was no sludge bulking with the SVI about 200 mL/g. In addition, fermenters *Dysgonomonas* and *Propionispora* were reported to ferment organics to VFA (Zhao et al., 2021), which accounted for significant abundances with 1.5%-3.4% and 0.8%-2.2%, respectively, during the whole operation. In terms of *Propionivibrio* and *Defluviicoccus*, known as GAO (Albertsen et al., 2016; Roy et al., 2021) they were less abundant in the A_2O system, less than 0.3% and 0.8% in the whole COD decreasing process, respectively, which corresponded with successful EBPR performance. However, the presence of GAO is not a necessary indicator of the deterioration of EBPR if PAO are favoured kinetically (G. Li et al., 2020; Nielsen et al., 2019). It is interesting that in the S2EBPR system, both of these organisms showed higher proportions. Especially, *Propionivibrio* accounted for 1.5% in the aerobic reactor and 5.5% in the SSSF. These results were contrary to that of (Wang et al., 2019), since they found *Propionivibrio* showed an inferior population in conventional EBPR than S2EBPR. Nevertheless, in our S2EBPR system, PAO still held a competitive advantage over GAO, both in terms of total population abundance (Table 6.10) and in terms of P removal performance.

6.5. Discussion

6.5.1. *The real effect of SSSF on S2EBPR*

The main objective of integrating a SSSF into an A_2O system is to increase the stability of biological P removal under potential detrimental situations (i.e. low influent rbCOD or excessive nitrate entering the anaerobic reactor). The SSSF should provide an extra source of VFA to the system. This VFA should allegedly come from the degradation of part of the biomass of the purge at expense of producing less purge for digestion. When the SSSF is applied in a non-EBPR system, the SSSF effluent contains a high amount of VFA that could be used, for example, to enhance denitrification. In this work, the first significant finding is that the effluent of the SSSF was not VFA-rich but it contained a significant amount of P. PAO had consumed the VFA produced *in situ* in the SSSF and have stored it as PHA linked to P release, with an average of P concentration about 88.7 mg/L in SSSF. This phenomenon has been already reported in other SSSFs (Vollertsen et al., 2006; Wang et al., 2019). Thus, in our case, the SSSF integration increased the average P_{LOAD} (47.9%) and concentration of P_{ANA} (43.7%) in the S2EBPR system (2.07 g/d and 37.5 mgP/L) when compared to A_2O (1.40 g/d and 26.1 mgP/L) under the same COD_{INF} conditions. The increase of P_{LOAD} led to 26.6% increase of the total P removal capacity in S2EBPR (1.76 vs 1.39 g P/d). Similarly, Wang et al., (2019) reported P release in SSSF increased 24.5 % in S2EBPR compared with A_2O (132 kg P/d vs 106 kg P/d), and P removal efficiency was also improved from 80% with A_2O to 94% with S2EBPR. Onnis-Hayden et al., (2020) also reported higher P removal performance with S2EBPR vs A_2O (90% vs 82%).

Regardless of the advantage, it is reasonable this extra P_{LOAD} can challenge the plant performance since an increase of effluent P could be observed if the ratio of RAS diverted to the SSSF was too high. P_{AER} was about 2.0±1.6 mgP/L in our system with the current operation situation since not all the entering P could be always removed due to the continuously abundant input of P from SSSF effluent. Similar phenomenon was observed in other full-scale S2EBPR systems. Vale et al. (2008) showed 2.8 mg/L of P in the effluent in a full-scale S2EBPR with 6% of RAS to SSSF (without reporting the specific extra P_{LOAD}), and they indicated that SSSF is not always a panacea for all EBPR cases due to the complex interactions (e.g. the decay of biomass leads to P and ammonia recycled to the mainstream, an overall increased SRT with less purge in S2EBPR poses more challenge to remove P and N...).

The COD in the SSSF effluent was low to around 40 mg/L and this observation is also observed in previous reports: the hydrolyzed VFA was up-taken by PAO and some other unknown bacteria (Jönsson and Jansen, 2006). Vollertsen et al., (2006) estimated that about half of the rbCOD was stored by PAO in the SSSF with HRT about 30 to 35 h, which left half of the COD in the effluent of SSSF (50-90 mg rbCOD/L). Andreasen et al., (1997) and Wang et al., (2019) reported that the full-scale S2EBPR could receive the increase of the input COD_{LOAD} about 14% and 16% from the effluent SSSF. The extent of the COD_{LOAD} increase depends on both the HRT of SSSF and the proportion of PAO in the sludge. Low HRTs (less than 1 day) and high amount of PAO lead to a low COD increase since all the potential COD released would be directly used in the SSSF.

In addition, the biomass fermentation also led to ammonium release and about 11 mgN/L was detected in the SSSF effluent, which resulted the N_{LOAD} of 5.69 g N/d. This resulted in a scarce increase of the total N_{LOAD} to the system of 1.6%. The VFA released by biomass fermentation was not only used by PAO, but also acted as an electron donor for denitrification since the nitrate recycled to the SSSF by external recycle was denitrified and there was no nitrate detected in the SSSF effluent. The concept is similar to that of the so-called Johannesburg WWTP configuration that aims at avoiding nitrate entering to the anaerobic reactor by providing an external carbon source as electron donor. Thus, the percentage of $N_{DENITRIFIED}$ increased from 35% to 46%. The work of Wang et al., (2019) also showed that S2EBPR could be beneficial for denitrifiers as well as for PAO. However, the $N_{REMOVAL\_ABSOLUTE}$ decreased from 3.96 to 3.79 g/d with the N removal efficiency decreased from 69% to 63%, which is due to the less purge of S2EBPR (5L/d) compared with A_2O (10L/d) despite of the same SRT (see equations 1 and 2). Equation 1 shows the normal SRT calculation but equation 2 includes the amount of biomass degraded in the SSSF. This biomass is theoretically extracted from the system (i.e. it should be included in the SRT calculation) but, at the same time, it is degraded inside the system. Hence, we need much less purge in the S2EBPR rather than in the A_2O system to have the same SRT. The SRT concept is only related to the fate of the solids: part of the COD as biomass was degraded in the SSSF, however, P and N were not degraded but released to the medium as a result of the biomass fermentation. As above mentioned, the SSSF integration decreased the amount of solids purged and increased the nutrient load to the plant, and these factors poses complex interactions to the system and may compromise the effluent quality.

6.5.2. *Influent C/P ratio and the COD requirement*

In our case, considering the increase of P_{LOAD} and the low increase of COD, the influent COD/P (g/g) was only 26.3 in the S2EBPR being 31% lower than that of A_2O system under the same of COD_{INF} (COD/P ratio about 38). It was even lower than that of A_2O system under the COD_{INF} = 300 mg/L (period I g; COD/P ratio about 32.6), which resulted in EBPR deterioration. However, the S2EBPR system could maintain EBPR activity at a much lower COD/P ratio (26.3), which indicated the promising advantage of S2EBPR compared with A_2O under low COD/P ratio scenario. Vale et al., (2008) indicated the unsatisfying effluent P concentration (2.8 mg/L) in S2EBPR should be ascribed to the low ratio of influent COD/P as well as COD/N (COD:TP=43 and COD: TN=8). The influent ratio of C/P plays an important role in EBPR performance (Gu et al., 2008). However, the amount of C is as important as its biodegradability/fractionation. PAO need VFA-like organic matter for their anaerobic metabolism. Calculating the minimum COD required for a successful N and P removal is not a straightforward issue and depends not only on the N and P loads but also on the COD biodegradability. Metcalf and Eddy (Tchobanoglous et al., 2013) suggest for simultaneous C, P and N removal in an A_2O configuration the following values of readily biodegradable COD (rbCOD): 10 g rbCOD/g P and 6.6 g rbCOD/gNO_3^--N. Then, to remove the influent concentrations of 9 mgP/L and 37 mgN/L in this work, 334 mg/L rbCOD would be required. This theoretical value agrees with the required COD_{INF} experimentally observed during the A_2O evaluation (350 mg/L). In the case of the S2EBPR in this study, P_{LOAD} and N_{LOAD} increased to 2.07 gP/d and 5.69 gN/d with the input from the SSSF, which would correspond to an influent concentration of 13.3 mgP/L and 37.6 mgN/L. Considering the P concentration in the effluent (2.0 mgP/L) during period IIc, 11.3 mgP/L were removed in the S2EBPR system with the same COD_{INF} = 350 mg/L instead of only 9 mgP/L. This would imply a decrease in the COD needs down to 9 g rbCOD/g P, 10% lower than for the A_2O configuration. Then, considering that the generated organic matter is mostly used by PAO in the anaerobic reactor and not for denitrification, it is reasonable to assume organic matter requirements of 9 g rbCOD/g P and 6.6 g rbCOD/g NO_3^--N for simultaneous C, P and N removal in a S2EBPR system.

6.5.3. *Energy recovery indices*

Table 6.11 shows the energy recovery indices of A_2O and S2EBPR at the same COD_{INF} conditions. The integration of the SSSF should decrease the potential energy recovery of the plant since not all the purged biomass is derived to energy recovery (i.e. biogas production) but part of it is degraded again in the SSSF. The purge in the S2EBPR was lower than in the A_2O for the same targeted SRT. We can assume similar BMP values from these two systems since the BMP value is mostly dependent on the internal PHA content and, thus, on the SRT (Chan et al., 2020a; Zhang et al., 2021). We adopted 221 mL CH_4/gVSS from aerobic sludge under the condition of S2EBPR for the calculation process (see section 3.4). Thus, both energy recovery indices for the S2EBPR system were around 45 % lower than those from the A_2O for the period with the same SRT since half volume of wasted sludge was discharged in S2EBPR with similar amount of *COD_{LOAD}*. MRI and ERI are about 0.253 $gCOD_{CH4}/gCOD_{REM}$ and 0.166 kJ_{CH4}/kJ_{INF} for the A_2O case and 0.139 $gCOD_{CH4}/gCOD_{REM}$ and 0.093 kJ_{CH4}/kJ_{INF} for the S2EBPR. MRI is also an indicator for mineralization degree, which was in accordance with the COD mineralization degree in these two systems (60% in A_2O and 77% in S2EBPR), and higher mineralization results in higher cost in S2EBPR. Literature reports show that the conversion efficiency from influent COD to methane is in the range of 15–35% and this energy could be recovered as a form of electricity by combined heat and power technologies. Assuming 35% of the transformation efficiency from methane to electricity, only 5% (S2EBPR) or 12% (A_2O) of the COD_{INF} could be recovered in the form of electricity (McCarty et al., 2011a).

6.5.4. *Key functionally populations- relevant PAO and GAO*

The S2EBPR showed more total known candidate PAO percentages than the A_2O system (Table 6.10), which was in accordance with the higher P removal capability of the S2EBPR. Specifically, under the same COD_{INF} condition of 350 mg/L, more PAO clades (e.g. *Thiothrix* and *Dechloromonas*) could be enriched in S2EBPR. GAO seemed to hold higher percentage in S2EBPR (2.3% in the aerobic reactor and 6.55% in the SSSF) than in A_2O (1.02%), in contrast to previous findings that S2EBPR showed lower GAO abundance than conventional EBPR (Onnis-Hayden et al., 2020; Wang et al., 2019). Different from other investigations (Nguyen et al., 2011; Onnis-Hayden et al., 2020; Wang et al., 2019), *Tetrasphaera* was not observed in this study, despite of its reported low decay rate and fermenting ability. Dold and Conidi (2019) also pointed out some potential conflicts for *Tetrasphaera*, such as the conflict in its abundance due to different quantification procedures, the implied importance in RAS fermentation processes and its importance in its ability to ferment in EBPR systems. Apart from that, it should be noted that in S2EBPR the microbial communities showed visible changes with a relatively lower percentage of PAO and higher percentage of GAO in SSSF compared with the aerobic reactor. SSSF was speculated to induce more decay of GAO and other OHOs due to the extended anaerobic condition, thus giving PAO a competitive advantage (Dold and Conidi, 2019; Herbst et al., 2019; Wang et al., 2019). In any case, the S2EBPR sustained the coexistence of PAO, GAO and other microbial bacteria.

Table 6.10. The read abundance of relative PAO and GAO at the genus level observed during different operational periods: 500, 400, 350, 300, 450 mg/L of CODinf in the A_2O system (period I); 350+ and 350+S are samples from the aerobic reactor and SSSF of the S2EBPR system (period IIc) obtained operating with CODinf of 350 mg/L.

COD_{inf}(mg/L)	500 (%)	400 (%)	350 (%)	300 (%)	450 (%)	350+ (%)	350+S (%)
Thiothrix	0.01±0	0.28±0.31	0.66±0.04	0.14±0.02	2.67±1.31	6.47±1.40	6.89±0.39
Dechloromonas	1.22±1.20	3.24±3.18	2.70±0.01	5.14±1.54	3.53±0.70	5.38±1.35	5.78±1.47
Desulfuromonas	2.04±0.57	3.70±3.72	0.75±0.02	1.02±0.06	0.80±0.17	1.27±0.31	0.83±0
Desulfovibrio	9.54±2.80	7.05±2.53	4.08±0.36	5.56±0.22	4.33±1.50	6.31±1.23	4.20±0.64
Rhodobacter	2.36±1.67	0.81±0.9	1.90±0.08	2.76±0.34	1.55±0.56	0.19±0.06	0.24±0.03
Desulfobulbus	1.95±0.82	1.16±0.29	0.76±0.09	0.99±0.07	0.89±0.33	1.18±0.26	0.76±0.13
Thauera	0.66±0.43	1.25±1.11	2.53±0.10	2.31±0.87	0.42±0.41	0.07±0.01	0.09±0.02
Desulfomicrobium	1.07±0.25	1.44±1.16	0.43±0.04	0.58±0.13	0.47±0.15	0.66±0.09	0.55±0
Total PAO	18.84±7.84	18.91±13.2	13.81±0.76	18.50±3.25	14.66±5.13	21.54±4.72	19.35±2.67
Propionivibrio	0.14±0.08	0.30±0.06	0.22±0.01	0.23±0.20	0.11±0.04	1.52±0.38	5.52±0.67
Defluviicoccus	0.67±0.60	0.81±0.92	0.80±0.15	0.73±0.28	0.70±0.31	0.82±0.14	1.03±0.01
Total GAO	0.81±0.69	1.11±0.98	1.02±0.16	0.96±0.48	0.81±0.35	2.34±0.52	6.55±0.69

Table 6.11. Comparison of energy recovery indices of A_2O and S2EBPR at the same COD_{inf} conditions.

Systems	Period	VSS (g/L)	Y_{OBS} (gCODx/gCODs)	Influent load (gCOD/d)	CH_4 production (gCOD/d)	MRI ($gCOD_{CH4}$)/($gCOD_{REM}$)	ERI (kJ_{CH4})/(kJ_{INF})
A_2O	If	1.13±0.13	0.40±0.04	53.3	9.544	0.253	0.166
S2EBPR	IIc	0.95±0.12	0.22±0.03	53.6	5.373	0.139	0.093

6.5.5. *Practical implications*

This work systematically evaluated the effect on the system performance of introducing a SSSF in an A_2O plant, showing the integration of SSSF is not always presenting net benefits to solve the EBPR problems. Implementing a SSSF into a conventional A_2O system would have the following implications:

1) The connection of a SSSF to the anaerobic reactor can allow higher SRT for fermentation processes and thus let to a lower COD/P requirements for EBPR, but at the expense of a lower purge. Thus, if an energy recovery process is implemented (i.e. anaerobic digestion), the energy recovery indices would decrease. The integration of a SSSF to the anaerobic reactor of A_2O showed the optimum EBPR performance compared with other conditions (to anoxic or aerobic reactor).

2) Combining the S2EBPR with P recovery provides a novel opportunity for P recovery by chemical precipitation due to the higher P concentrations attained. Moreover, P-recovery would be a perfect complement to S2EBPR, since it would allow removing part of the P accumulated in the system due to the lower purge required to maintain biomass concentration in this configuration and would decrease the potential of additional P load from SSSF to threaten the quality of mainstream effluent.

3) In this work, we have not considered another potential P input to the plant: the reject wastewater or the effluent from the waste sludge treatment. We only considered the P-load increase due to part of the RAS being diverted to the SSSF. In real plants, the whole reject water may be recycled to the plant. This effluent would increase the P-load to the plant without providing any extra COD and, thus, would even make more evident the need of novel configurations such as the SSSF. In fact, the A_2O configuration evaluated in this work has some advantage for the comparison made, as it is not receiving the significant load of P that would be recirculated in a full-scale WWTP.

Further investigations relative to the SSSF operation are necessary for a more comprehensive understanding of the limits for the integration of SSSF to the traditional EBPR system and the optimum operational conditions, in order to derive full benefits of this configuration, such as the reactor volume and the percentage of RAS redirected to this reactor.

6.6. Conclusions

The S2EBPR configuration with the connexion of the SSSF to the anaerobic reactor was studied by introducing 6% of the RAS with HRT = 2 d and COD_{INF} of 350 mg/L (minimum value for successful P/N removal in an A_2O configuration). The main benefits and drawbacks of S2EBPR compared to A_2O were:

Benefits:

Conclusions

1. Higher P and N removal capacity (26.6% and 11%) without compromising full COD and ammonium oxidation.

2. A promising superiority to maintain EBPR with the influent COD/P of only 26.3 compared with EBPR deterioration in A_2O with the ratio about 32.6. Organic matter needs decreased from 10 g rbCOD/gP to 9 g rbCOD/gP.

3. A higher abundance of functional PAO and a relatively lower GAO percentage were observed in S2EBPR, which gave PAO the advantage for EBPR activity.

Drawbacks:

1. Lower effluent quality (with 2.0±1.6 mgP/L) was observed due to the additional P_{LOAD}, accompanied with a relatively insignificant increase of residual COD (0.5%) and N (1.6%).

2. The energy recovery indices were around 45% lower than those of A_2O **(**with the same SRT), and less of the input COD could be recovered as electricity in S2EBPR (5%) compared with A_2O (12%).

Chapter 7:

General conclusions

7. General conclusions

The overall results obtained in this thesis have contributed to a deeper understanding of the EBPR process by i) studying the most important factors affecting the process performance of a continuous A-stage-EBPR system treating real wastewater and ii) investigating the effect of the integration of a side-stream sludge fermenter to an A_2O system under a low COD_{inf} scenario. In addition, this thesis has reviewed the potential of the mainstream P-recovery strategies on EBPR and gathered the most recent works on the effect of different carbon sources on EBPR performance. The most important conclusions of each specific Chapter are detailed below.

Chapter 4.1 critically reviews for the first time the performance of the current reported strategies for mainstream P-recovery in EBPR WWTPs and provides the main points to be addressed for its successful implementation. Furthermore, it shows that full-scale mainstream P-recovery is a medium-term possibility. It is based on the PAO capability of producing a P-enriched supernatant that enables P-recovery, and SBR and continuous configurations at different scales have been reported. It can improve P-removal performance and enable a successful treatment of influents with a relatively low COD/P ratio. However, configurations combining EBPR with mainstream P-recovery are still at an early stage and their successful full-scale application has not yet been reported. The main conclusions were:

- The most important parameter to describe these systems is the extraction ratio: the amount of anaerobic supernatant to be extracted or derived to recover P. The choice of this value is a compromise between having high PAO activity and anaerobic P concentration (i.e. easing precipitation) but low amount of P recovered and a potential deleterious effect to PAO activity, low anaerobic P concentration but a high amount of P recovered.
- A maximum value for the P-recovery efficiency of 60% of influent P seems the most sensitive goal for a long-term operation of these systems. However, higher values can be found for sporadic or single extractions.
- Regarding the preferred agent for P-recovery, most of the works opted for struvite, while vivianite or HAP are less common at this point.

Chapter 4.2 explored the effect of different purging strategies in a continuous A_2O system on EBPR performance. The integration of mainstream P-recovery was investigated with the aim of P recovery due to the high P concentration in the anaerobic reactor and the key findings were:

- Successful P and COD removal can be obtained independently of the aerobic or anaerobic purge under the SRT around 21 days. Full COD and ammonium removal could also be reached under these conditions.
- Purging from the anaerobic reactor improved PAO activity: anaerobic P concentration increased by almost 27%. However, the total N removal efficiency decreased by 15% due to the increase of nitrate in the effluent.
- 26% of the input P can be extracted within the anaerobic supernatant with an anaerobic purge and SRT of 20.5±1.1 d compared with 20% under the SRT of 16.3±0.6 d.
- N and COD balance showed that N contained in the biomass increased from 33±1% to 41±1% with the anaerobic purge increasing from 5 to 7 L/d, and COD stored in biomass increased by 6% which could favour biomethane production for energy recovery.

Chapter 4.3 systematically evaluates the effects of carbon source on EBPR systems, especially the carbon utilization strategies and current developing trend due to the deficient COD in the influent. The main conclusions were:

- According to the above involved investigations, acetate and propionate are still as the most crucial and efficient substrates to promote the *Accumulibacter*-enriched sludge and assure successful EBPR, and a moderate load of VFA is necessary for favouring PAO.
- Other substances (e.g. methanol, glycerol, lactate, starch, LCFA…) that are not degraded by PAO directly as sole carbon source may lead the unstable performance and even the system failure. The longer fermentation time for the complex carbon sources to VFA or the mixture of these carbon sources with VFA can support successful lab-scale EBPR performance.
- However, the recent detection of other PAO-clades opens the door to more diverse carbon utilization. The fermentive PAO- *Tetrasphaera* have the ability to ferment glucose, some amino acids and waste sludge, and the VFA from the fermentation productions can be assimilated by *Accumulibacter*.
- Applying the fermentation productions from the waste as carbon source has been a popular and environmental solution for the EBPR process, which can not only lead to lower carbon footprint but also reduce large amount of sludge discharge. The full-scale application have shown the efficient P and N removal performance with the utilization of the fermentation products from waste sludge or food waste by some pretreatment (mostly with alkaline pretreatment) strategies and the waste sludge by SSSF.
- The SSSF can also be a device for fermenting some complex carbon sources from the wastewater to VFA faced with the VFA deficiency problem in real

wastewater. Apart from that, taking advantage of the fermentation ability of *Tetrasphaera* to ferment waste sludge as carbon source could be a promising way in the future for the full-scale WWTPs.

High-rate system operating at low SRT has been proposed to meet the challenge for recovering energy from wastewater in addition to its treatment in the new concept of WRRF. Chapter 5.1 evaluated the integration of EBPR in an anaerobic/aerobic continuous high-rate system (A-stage-EBPR), and the main conclusions were:

- The best performance was obtained operating the system at SRT = 4 d and the aerobic reactor at a DO setpoint of 0.5 mg/L, achieving P and COD removal efficiencies of 94.5% and 96.3% and avoiding nitrification.
- Bulking problems can appear at low SRT. Biomass washout was observed when decreasing the SRT to 3 d due to the poor settleability of solids.
- Higher biogas production is obtained using anaerobic sludge, 20%-24% higher BMP than aerobic sludge under the same conditions. The highest BMP was 296±2 mL CH_4/gVSS using anaerobic sludge at SRT = 4 d.
- A high fraction of COD in the influent can be diverted to biomass for energy recovery (64±6% at SRT = 4 d).
- About 30% of inlet N is consumed for biomass growth, leaving a significant fraction 69±1% as ammonium for the subsequent B-stage.
- The FISH analysis at SRT = 4 d showed *Accumulibacter* predominance (33±13%), much higher than its competitors *Competibacter* (3.0±0.3%), *Defluviicoccus* Cluster I (0.6±0.1%) and *Defluviicoccus* Cluster II (4.3±1.1%). Both *Defluviicoccus* Clusters significantly increased when SRT was decreased to 3 d (4.2±0.5% for Cluster I and 7.9±1.6% for Cluster II).

The performance of the A-stage-EBPR system was evaluated in Chapter 5.2 under different operational conditions, showing situations where the system operates stable but also some cases where stability problems can appear, and the key findings were:

- High P and COD removal was reached (94-98% and 95%) under DO setpoints of 0.5 and 1 mg/L when treating raw wastewater amended with propionic acid. However, decreasing the DO setpoint to 0.2 mg/L led to the deterioration of the system.
- Changing the purge position from the aerobic to the anaerobic reactor maintained good COD and P removal performance and stable settleability without nitrification. About 22% of the influent P could be recovered using the anaerobic purge and about 43% of the influent COD could be captured and recovered (rather

than mineralized) according to the mass balances. Nitrogen mass balance showed that 66% of the input N was in the effluent as ammonium for further treatment in the B-stage.

- Employing glutamate as sole carbon and nitrogen source allowed simultaneous COD and P removal but with a slight nitrification build-up. After two months of operation with glutamate, biomass settleability was progressively lost and EBPR activity disappeared, indicating that it may be only a suitable carbon source for short periods.
- The microbial community analysis showed that *Propionivibrio*, *Thiothrix* and *Lewinella* exhibited the highest abundances. *Propionivibrio* percentage seemed to be correlated to high P-removal. *Thiothrix, Hydrogenophaga, Dechloromonas* and *Desulfobacter* were detected as the dominant PAO during the whole operation.

The implementation of a side-stream sludge fermenter (SSSF) has been identified as a possible solution to improve the performance of EBPR when treating low COD wastewater. The incorporating a SSSF into an A_2O configuration (side-stream EBPR, S2EBPR) with the connexion of the SSSF to the anaerobic reactor was studied in Chapter 6 by introducing 6% of the RAS with HRT = 2 d and COD_{INF} of 350 mg/L (minimum value for successful P/N removal in an A_2O configuration). The main benefits and drawbacks of S2EBPR compared to A_2O were:

Benefits:

- Higher P and N removal capacity (26.6% and 11%) without compromising full COD and ammonium oxidation.
- A promising superiority to maintain EBPR with the influent COD/P of only 26.3 compared with EBPR deterioration in A_2O with the ratio about 32.6. Organic matter needs decreased from 10 g rbCOD/gP to 9 g rbCOD/gP.
- A higher abundance of functional PAO and a relatively lower GAO percentage were observed in S2EBPR, which gave PAO the advantage for EBPR activity.

Drawbacks:

- Lower effluent quality (with 2.0±1.6 mgP/L) was observed due to the additional P_{LOAD}, accompanied with a relatively insignificant increase of residual COD (0.5%) and N (1.6%).
- The energy recovery indices were around 45% lower than those of A_2O (with the same SRT), and less of the input COD could be recovered as electricity in S2EBPR (5%) compared with A_2O (12%).

Sequencing analysis revealed a high abundance of PAO, and a lower GAO/PAO ratio in the S2EBPR, in accordance to its higher P removal. The S2EBPR performance was also evaluated when the SSSF reactor outlet was connected to the anaerobic, anoxic or aerobic reactor, with the best results obtained for the first case. This study represents a comprehensive evaluation of the S2EBPR configuration and provides further enriched information to assess its suitability.

Chapter 8:

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8. References

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List of Abbreviations

A/O	Anaerobic/oxic
A_2O	Anaerobic/anoxic/aerobic
AD	Anaerobic digestion
AER	Aerobic reactor
ANA	Anaerobic reactor
ANOX	Anoxic reactor
AOB	Ammonium Oxidizing Bacteria
AS	Activated Sludge
ATU	Allylthiourea
BMP	Biochemical Methane Potential
BNR	Biological Nitrogen Removal
AS	Activated sludge
BNR	Biological Nitrogen Removal
BOD	Biological Oxygen Demand
C	Carbon
COD	Chemical Oxygen Demand
CODs	Soluble COD after filtration
DF I	*Defluviicoccus* vanus cluster I
DNA	Deoxyribonucleic Acid
DO	Dissolved oxygen
DPAO	Denitrifying Poly-phosphate Accumulating Organisms
EBPR	Enhanced Biological Phosphorus Removal
FISH	Flurescence in situ Hybridization
GAO	Glycogen Accumulating Organism
GC	Gas Chromatography
Gly	Glycogen
HPLC	High-Performance Liquid Chromatography
HRAS	High-Rate Activated Sludge
HRT	Hydraulic Retention Time
IMBR	Initial methane production rate
N	Nitrogen
NOB	Nitrite Oxidizing Bacteria
OHO	Ordinary Heterotrophic Organisms
P	Phosphorus
PAO	Polyphosphate Accumulating Organisms
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoates
Poly-P	Poly-Phosphate
rRNA	Ribosomal Ribonucleic Acid

S2EBPR	Side-stream Enhanced Biological Phosphorus Removal
SBR	Sequencing Batch Reactor
SRT	Sludge Retention Time
SSSF	Side-stream Sludge Fermenter
SVI	Sludge Volume Index
TCA	Tricarboxylic Acid Cycle
TOC	Total Organic Carbon
TSS	Total Suspended Solids
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids
WRRF	Water Resource Recovery Facility
WWTP	Wastewater Treatment Plant
Y_{OBS}	Observed Biomass Growth Yield

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List of publications and author’s contributions

I. Zhang C., Guisasola, A., Baeza, J. A., Achieving simultaneous biological COD and phosphorus removal in a continuous anaerobic/aerobic A-stage system, Water Research, 2021, 190: 116703.

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II.	Zhang C., Guisasola, A., Baeza, J. A., A review on the integration of mainstream P-recovery strategies with enhanced biological phosphorus removal, Water Research, 2022, 212: 118102.

Author’s contribution: Article design, writing, discussion and editing. Research supervision, article design, writing, discussion and editing contribution from Guisasola, A., and Baeza, J.A.

III.	Zhang C., Guisasola, A., Baeza, J. A., Exploring the stability of an A-stage-EBPR system for simultaneous biological removal of organic matter and phosphorus. Chemosphere, Submitted.

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IV.	Zhang C., Guisasola, A., Adrian O., Baeza, J. A., Benefits and drawbackes of integrating a side-stream sludge fermenter into an enhanced biological phosphorus removal system. Water Research, Submitted.

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V.	Zhang C., Guisasola, A., Baeza, J. A., Re-evaluating the effect of different carbon sources on EBPR performance: a critical review. In preparation.

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Participation in Research Projects

Project title	**HIPATIA. Towards the implementation of the biorefinery concept and energy self-sustainability in an urban wastewater treatment plant.**
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