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ADVANCED REACTOR TECHNOLOGY FOR WASTEWATER TREATMENT

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ABSTRACT

Elevated stringency regarding discharges and an aging asset base represent challenges to modern wastewater treatment. This requires upgrade of existing wastewater assets for low energy nutrient removal for minimal cost. Rotating biofilm reactors can be used as a pre-treatment, high organic loading rate (OLR), low hydraulic residence time (HRT) treatment facilitating upgrade of existing wastewater treatment plant (WWTP). The threshold for stable nitrification in rotating biological contactors (RBCs) was assumed to be 15 $g.BOD_5.m^{-2}d^{-1}$ treating municipal wastewaters, however media modifications have shown that this value can be elevated to ~35 $g.sCOD.m^{-2}d^{-1}$ (73.5 $g.BOD_5.m^{-2}d^{-1}$) in rotating biofilm reactors (RBR). Mesh media was compared to two different reticulated foam media, the mesh media had similar porosities but elevated performance compared to the foam media. Elevated OLR resulted in lower bacterial viability suggesting inhibition at >100 $g.sCOD.m^{-2}d^{-1}$. Comparison of four different mesh media suggested that high porosity mesh media is best for performance and to prevent pore clogging. Bacterial activity increased with OLR, but performance at very high OLR decreased. Biofilm reactors can be operated in a 'hybrid' configuration which is where settled bacterial solids can be recycled into the biofilm reactor to improve performance by reducing the effective biofilm OLR. Studies at full scale revealed that extracellular enzyme activity was higher in biofilms compared to suspended growth bacteria. Hybrid upgrade of existing wastewater treatment works resulted in 52 and 40% increase in removal rate of COD and NH_4-N respectively. This allowed the reactors to operate at a higher OLR and provide better effluent quality. Comparing different solids type for hybrid reactors utilising activated sludge flocs had the greatest performance benefit compared to Humus Solids (HS) and final effluent (FE) respectively for sCOD and NH_4-N removal. Incorporating a solids feed in hybrid reactors improved nitrification and organics removal at lower loading. However the solids in the recycle feed reduced denitrification at very high OLR suggesting flocs inhibit denitrification. Hybrid RBRs have 4.8 fold increase in protein extracellular

enzyme activity (EEA) compared to single pass reactors under similar conditions. Recycling bacterial solids reduces the effective OLR on the biofilm and confers significant performance benefits. Upfront RBRs provide suitable upgrade for existing WWTP.

Keywords:

Biofilm, extracellular enzyme activity, HYBACS, HYFILT, hybrid activated sludge, hybrid filtration, microbial activity, organic loading rate, rotating biological contactor, rotating biofilm reactor, solids recycle, SMART unit, viability.

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ABBREVIATIONS

AFM	Atomic force microscopy
AHTN	6-acetyl-1,1,2,4,4,7-hexamethylteraline
ANOVA	Analysis of Variance
AOB	Ammonia oxidising bacteria
APHA	American public health association
ASM	Activated sludge model
ASP	Activated sludge plant
BNR	Biological nutrient removal
BOD_5	Biochemical oxygen demand (5 days)
CAS	Conventional activated sludge
CAPEX	Capital expenditure
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
DI	Deionised water
DO	Dissolved oxygen
EBPR	Biological phosphorus removal
EEA	Extracellular enzyme activity
EPS	Extracellular polymeric substance
EPSRC	Engineering and Physical Sciences Research Council
FE	Final effluent
F:M	Food to microorganism ratio
FOV	Field of view
HE	Hydrolytic enzyme
HRT	Hydraulic retention time
HS	Humus solids

HYBACS	Hybrid activated sludge
HYFILT	Hybrid filtration
IFAS	Integrated fixed film activated sludge
MC	Medium composition
MCRT	Mean cell residence time (see sludge retention time)
MLSS	Mixed liquor suspended solids
MRA	Multiple regression analysis
MT	Mass transfer
M_w	Molecular weight
NAPL	Non-aqueous phase liquids
NLR	Nitrogen loading rate
NOB	Nitrite oxidising bacteria
OD	Oxidation ditch
OLAND	Oxygen limited autotrophic nitrification and denitrification
OPEX	Operational expenditure
OTR	Oxygen transfer rate
PAO	Polyphosphate accumulating organisms
PE	Population equivalent
PP	Polypropylene
PPCP	Pharmaceuticals and personal care products
PVC	Polyvinylchloride-like material
RAS	Return activated sludge
RBC	Rotating biological contactor
rpm	Revolutions per minute
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SD	Standard deviation (relative)
SEM	Standard error of mean (SD/√n)

SMART	Shaft mounted advanced reactor technology (generally similar to RBR unless stated)
sp	Species
SRT	Sludge retention time (similar to MCRT)
STOWA	Stichting Toegepast Onderziek Waterbeheer
SUR	Substrate utilisation rate
SVI	Sludge volume index
tCOD	Total chemical oxygen demand
TF	Trickling filter
TLA	Three letter acronym
TN	Total nitrogen
TP	Total phosphorus
TS	Total solids (biofilm)
TSS	Total suspended solids
VBNC	Viable but non-culturable
vOLR	Volumetric organic loading rate
VRR	Volumetric removal rate
VS	Volatile solids (biofilm)
VSS	Volatile suspended solids
WAS	Waste activated sludge
WPCC	Water pollution control centre
WRF	White-rot funghi
WST-8	Water soluble tetrazolium salt #8
WWT	Wastewater treatment
WWTP	Wastewater treatment plant
4-FCA	4-fluorocinnamic acid

Note: to maintain integrity and flow of thesis and save ink, three letter acronym (TLA) abbreviations will be defined on first use only.

NOTATIONS

A_d	total disc surface area (L^2)
A_{exp}	area of exposed disc (L^2)
A_{sub}	area of submerged disc (L^2)
A_t	cross sectional area of tank (L^2)
C_{Bf}	compound concentration at the biofilm surface (ML^{-3})
C_{Lf}	compound concentration at liquid film surface (ML^{-3})
C_i	influent compound concentration (ML^{-3})
C_e	effluent compound concentration (ML^{-3})
C_T	compound concentration in the tank (ML^{-3})
C*	equilibrium compound concentration at a given temperature (ML^{-3})
D_L	diffusion coefficient of oxygen in water (L^2T^{-1})
e	distance from disc edge to the basin (L)
F:M	food to microorganism ratio
g	acceleration due to gravity (LT^{-2})
H	distance between the disc centre to the liquid free surface (L)
K_C	half saturation constant for compound (ML^{-3})
$K_L{}^{air}$	oxygen mass transfer coefficient film
K_L	overall oxygen mass transfer coefficient (LT^{-1})
$K_L a_t$,	volumetric oxygen mass transfer coefficient total (T^{-1})
K_m	Michaelis-Menten half saturation coefficient (ML^{-3})
N	number of discs
N_v	volume renewal number (T^{-1})
Q	reactor flow rate (L^3T^{-1})
R	radius of disc (L)
r_a	substrate removal rate ($ML^{-2}T^{-1}$)
s	half space between discs (L)
t_R	contact time per rotation (T)
V	wet volume of reactor (L^3)
V_{max}	Michaelis-Menten maximum enzymatic rate at saturation point ($ML^{-3}T^{-1}$)

V_{Lf}	volume of liquid film (L^3)
U_{max}	maximum substrate removal rate ($ML^{-2}T^{-1}$)
X_a	concentration of attached biomass (ML^{-3})
Y_a	yield coefficient for attached biomass
δ	liquid film thickness (L)
δ_{bf}	biofilm thickness (L)
μ	Absolute viscosity of a liquid (MLT^{-1})
μ_{max}	maximum specific growth rate (T^{-1})
ρ	fluid density (ML^{-3})
ω	rotational speed (RPM)
ω'	$\omega/60$
ϕ	disc diameter (L)
ϕ_0	wetted disc diameter (L)

Notations will only be used if words are not appropriate.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 BACKGROUND

The rotating biofilm reactor (RBR) is a fixed film wastewater treatment reactor with a plastic disc or mesh media where bacteria grow by utilising substrates from the wastewater and is similar in design to a rotating biological contactor (RBC). Gaseous exchange occurs as the media is rotated through the wastewater (Patwardhan 2003). The bacteria grow in a concentrated biofilm which is principally why the RBR can deliver low reactor volumes, high organic loading rates (OLR) (Najafpour et al. 2006; Cortez et al. 2008). The RBR can be integrated with a solids recycle which is referred to as a 'hybrid' reactor setup as the recycle contains active solids from an additional secondary treatment process (Hassard et al. 2015). The biggest challenge to the application of RBR-like technology is the mechanical engineering, for example shaft, bearing and media failures have been reported frequently (Mba et al. 1999). A commercial example of a RBR is called a shaft mounted advanced reactor technology (SMART) unit (Hoyland et al. 2010). The SMART unit utilises non-woven mesh media which has high porosity and an air-scour for biofilm control facilitating use under very high organic loadings (Hassard et al. 2014). The air scour in a SMART unit is similar to Biological Aerated Filters which utilise backwashing to remove excess bacterial growth to optimise performance, drawing analogies to mixed liquor wastage in activated sludge (Mendoza-Espinosa and Stephenson 1999) and could be utilised to manipulate microbial growth rates in the future (Shackle et al. 2000). The SMART unit can be integrated in a hybrid setup as an upfront 'roughing' reactor with conventional secondary treatment processes such as activated sludge (HYBACS) and trickling filters (HYFILT). The advantages of roughing filters is they facilitate upgrade of existing wastewater treatment works by providing a low footprint, retrofit option without significant additional operational or capital expenditure (CAPEX). Other technologies exist for upgrading existing wastewater treatment works e.g., membrane bio-reactors (Judd 2010), integrated fixed film activated sludge (IFAS) and hybrid moving bed biofilm reactors (Mannina and Viviani 2009). However membrane bioreactors

have high power consumption and IFAS-style systems have limited ability to control biofilm growth.

The mesh media used in the SMART is novel, therefore there is a requirement to benchmark the mesh media against available alternatives. Both media surface composition and architecture are important for biofilm formation and performance (Khan et al. 2013). Surface composition can be optimised to select for the adhesion, survival and activity of bacterial groups which provide performance benefit such as nitrifiers for better ammonia removal (Stephenson et al. 2013; Hassard et al 2014). Different media and operating conditions such as; rotational speed, organic loading, nitrogen loading, recirculation rate and submergence can be optimised to provide the physicochemical conditions which select for different bacterial groups (Cortez et al. 2008; Hassard et al. 2015).

Media properties can influence the adsorption and adhesion rates. Furthermore, electrostatic interactions can modify the microbial viability and hence activity of the population (van der Mei et al. 2008; Lackner et al. 2009). In addition operating conditions could modify the viability of bacterial populations (Okabe et al. 1996). Substrate loading rate influences the structure and function of wastewater biofilms (Wijeyekoon et al. 2004). However conventional design criterion utilising Monod kinetics are not appropriate in fixed biofilm reactors (Dutta et al. 2008). Canon et al. (1991) plotted dimensionless substrate removal rate against minimum effluent quality and suggested that biofilm modelling can be applied with similar confidence to suspended growth systems for predicting effluent quality. However fundamental parameters such as microbial growth rate and sludge retention time (SRT) remain undefined in most biofilm systems (Bryers 2000) and are confounded by the hybrid component of RBR, there is requirement to test hybrid RBRs under different operating conditions.

Biofilm systems degrade readily biodegradable substrates effectively. However slowly biodegradable soluble, colloidal and particulate substrates often limit removal rates that can be applied. This is because diffusion resistance prevents mass transfer (MT) into the biofilm for biocenosis and short HRTs inhibit prolonged extracellular hydrolysis to shorter more degradable substrates.

However around 50% of municipal wastewaters constitute polymers >1kDa in size which prevents transfer into bacterial cells (Burgess and Pletschke 2008). Enhanced extracellular enzyme activity has previously been noted in biofilms, (Jones and Lock 1989) due to natural gradients in substrates and electron acceptors conferring an advantage towards species with greater enzymatic activity (Allison 2005). Wastewater biofilms have been shown to regulate their enzymatic machinery according to process and physiochemical conditions (Shackle et al. 2000; Teuber and Brodisch 1977). It is important to understand how bacteria respond to recycling from a low to high substrate environments on enzyme activity and production of storage compounds (van Loosdrecht et al. 1997). There is a requirement to understand what impact process decisions make on performance and also the microbial community as a whole (Curtis et al. 2003). This Thesis aims to do so using RBRs as a novel experimental system utilising real wastewater where possible.

1.2 AIMS AND OBJECTIVES

This research aims to understand the performance of different mesh media for biofilm development, and removal of a diverse range of macropollutants from wastewater. In addition to understand the impact of operating conditions on biofilm reactors in a ‘hybrid’ configuration. Findings from bench scale studies will be tested at pilot scale and analysis of data from full scale works will provide additional information regarding hybrid reactors. Consequently, the following objectives were identified:

1. Review history, process engineering, microbiology, biological nutrient removal, organic pollution removal and modelling for rotating biological contactor technology, with focus on WWT.
2. Assess the impact of different available media for performance and biofilm viability.
3. Determine the impact of media type, OLR on microbial viability, activity and performance.
4. Assess the performance and microbial extracellular enzyme activity of hybrid biofilm reactors at a full scale hybrid WWTP.

5. Determine the impact of solids loading, OLR and solids type on performance and microbial extracellular enzyme activity in hybrid biofilm reactors.

1.3 THESIS PLAN

This thesis is presented in the style of thesis by publication. All papers were written by first author Francis Hassard and edited by Prof. Tom Stephenson with editorial support from Dr Jeremy Biddle (Bluewater Bio UK) and Prof. Elise Cartmell. The exception being chapter 2 where editorial support was made by Prof Bruce Jefferson and Dr Sean Tyrrel and chapter 5 where fieldwork contributions were made by Richard Harnett (Bluewater Bio UK) and Dr Garry Hoyland (Bluewater Bio UK). All laboratory work was undertaken by Francis Hassard with the exception of chapter 5, where extracellular enzyme activity validation experiments were undertaken through a Masters' thesis Fanny Hilaire (*Ecole Nationale Supérieure de Chimie de Rennes)*, all results featured in the publication of the study site and at bench scale were performed solely by the lead author.

The linkage between the chapters is represented in Figure 1. The thesis begins with a review of the state of the art of rotating biological contactors (on which the technology featured in this thesis is based) (*Chapter 2, published in Process Safety and Environmental Protection,* (2015) *94:* 285-306 – *Francis Hassard, Jeremy Biddle, Elise Cartmell, Bruce Jefferson, Sean Tyrrel, Tom Stephenson. Rotating biological contactors for wastewater treatment – A review.*

Chapter 3 compares three different media for biofilm development, performance and bacterial viability (*Chapter 3, published in Water Science and Technology (2014) 69: 1926-1931 – Francis Hassard, Elise Cartmell, Jeremy Biddle, Tom Stephenson. Performance of permeable media rotating reactors used for pretreatment of wastewaters*).

Chapter 4 investigates the impact of mesh media type and porosity on performance microbial viability and activity. (*Chapter 4, submitted to Bioresource Technology - Francis Hassard, Elise Cartmell, Jeremy Biddle, Tom Stephenson.*

Mesh rotating reactors for biofilm pre-treatment of wastewaters – influence of media type on microbial activity, viability and performance).

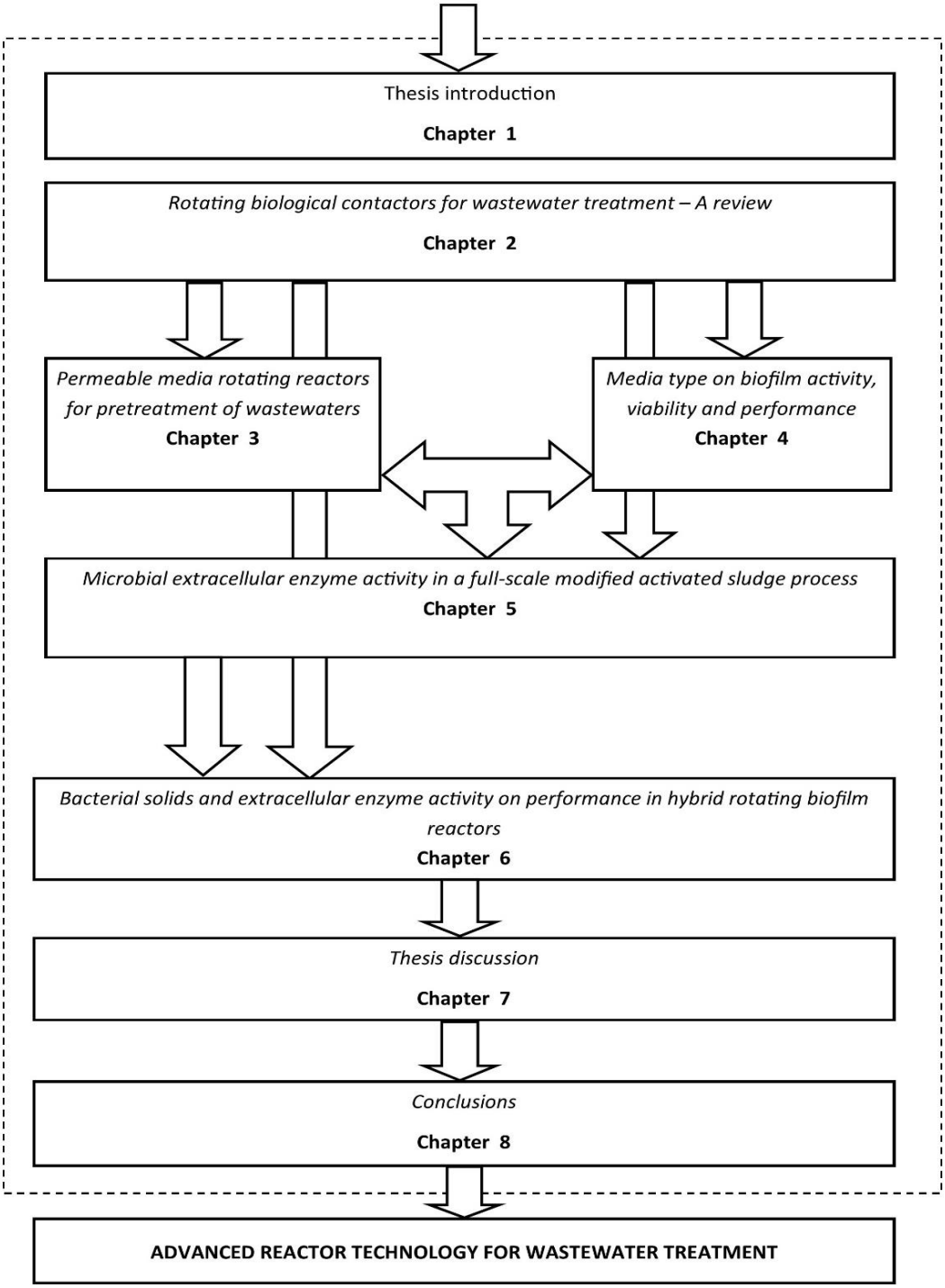
Chapter 5 investigates the microbial extracellular enzyme activity at a full scale modified 'hybrid' activated sludge process. (*Chapter 5, to be submitted to Water Research - Francis Hassard, Jeremy Biddle, Garry Hoyland Richard Harnett, Fanny Hilaire, Tom Stephenson. Microbial extracellular enzyme activity in a full-scale modified activated sludge process*).

Chapter 6 determines the impact of solids type, solids loading and organic loading on the performance of rotating biofilm reactors for wastewater treatment. (*Chapter 6, to be submitted to Water Research - Francis Hassard, Jeremy Biddle, Elise Cartmell and Tom Stephenson 6. Impact of bacterial solids on extracellular enzyme activity and performance in hybrid rotating biofilm reactors*).

Chapter 7 is the overall discussion of the research.

Chapter 8 is the conclusions of the thesis.

Figure 1 – Thesis structure as a flow chart



CHAPTER 2

LITERATURE REVIEW – ROTATING BIOLOGICAL CONTACTORS FOR WASTWATER TREATMENT – A REVIEW

PUBLISHED: *Process Safety and Environmental Protection, 94* (2015), 285-306.

2 ROTATING BIOLOGICAL CONTACTORS FOR WASTEWATER TREATMENT – A REVIEW.

2.1 ABSTRACT

The use of RBCs for WWT began in the 1970s. Removal of organic matter has been targeted within OLRs of up to 120 g.BOD.$m^{-2}d^{-1}$ with an optimum at around 15 g.$m^{-2}d^{-1}$ for combined BOD_5 and ammonia removal. Full nitrification is achievable under appropriate process conditions with oxidation rates of up to 6 g$m^{-2}d^{-1}$ reported for municipal wastewater. The RBC process has been adapted for denitrification with reported removal rates of up to 14 g.NH_4-N.$m^{-2}d^{-1}$ with nitrogen rich wastewaters. Different media types can be used to improve organic/nitrogen loading rates through selecting for different bacterial groups. The RBC has been applied with only limited success for enhanced biological phosphorus removal and attained up to 70% total phosphorus removal. Compared to other biofilm processes, RBCs had 35% lower energy costs than trickling filters but higher demand than wetland systems. However, the land footprint for the same treatment is lower than these alternatives. The RBC process has been used for removal of priority pollutants such as pharmaceuticals and personal care products. The RBC system has a 2.2±1.7 removal (log CFU/100 ml) of total coliforms and the majority of other wastewater pathogens. Novel RBC reactors include systems for energy generation such as algae, methane production and microbial fuel cells for direct current generation. Issues such as scale up remain challenging for the future application of RBC technology and topics such as phosphorus removal and denitrification still require further research. High volumetric removal rate, solids retention, low footprint, HRTs are characteristics of RBCs. The RBC is therefore an ideal candidate for hybrid processes for upgrading works maximising efficiency of existing infrastructure and minimising energy consumption for nutrient removal. This review will provide a link between disciplines and discuss recent developments in RBC research and comparison of recent process designs are provided. The microbial features of the RBC biofilm are highlighted and topics such as biological nitrogen removal and

priority pollutant remediation are discussed. Developments in kinetics and modelling are highlighted and future research themes are mentioned.

2.2 INTRODUCTION

Wastewater treatment processes should comply with standards that ensure environmental protection, whilst be efficient to minimise socio-economic burden (Ainger et al. 2009). The main priorities WWT are effluent quality, cost, energy efficiency and nutrient removal/recovery (STOWA, 2010). Regulatory agencies aim to improve local environmental health using advanced forms of WWT such as biological nutrient removal (BNR). To achieve tighter effluent standards, traditional biological treatment is largely reliant on increasing energy input through extended reactor aeration or retention time. Already, ~55% of the energy budget for sewage treatment is used in aeration (Ainger et al. 2009). The development of WWT technology is critical to improve the long term sustainability of necessary treatment capacity (Hoyland et al. 2010; STOWA, 2010).

Rotating biological contactors are called disc, surface, media and biofilm reactors and provide an alternative to the activated sludge process. The RBC has a solid media that encourages microbial growth in a static biofilm (Singh and Mittal 2012). The RBC media is arranged in a series of plates or discs which are rotated on a shaft through a biozone trough by motor or air drive (Patwardhan 2003). The rotation leads to bulk fluid mixing, convection through media/biofilm pores, compound diffusion to the film and subsequent product exchange with the reactor and surroundings (Rittmann and McCarty 2001). Biological processes occur inside a fixed microbial biofilm, which contains components of active/non-active biomass, biofilm extracellular matrix and debris (Arvin and Harremoës 1990). The RBC combines bacterial growth and substrate utilisation with a natural biomass separation system; however effluent quality and process stability is contingent on a distal sedimentation zone. The principal advantage of biofilm processes, such as RBCs, is that the mean cell residence time (MCRT) is uncoupled from HRT without the requirement of a clarification step. This could allow higher OLRs and resistance to toxic shocks than suspended culture systems (Najafpour et al. 2006; Cortez et al. 2008). Fixed RBC biofilms offer higher substrate affinity, resistance

to traumatic events and exhibit quicker recovery from starvation than suspended counterparts (Batchelor et al. 1997; Bollmann et al. 2005). This could be due to differential gene expression, physical or chemical isolation and the presence of stronger diffusion gradients (Cohen 2001). The RBC is especially useful for the degradation of refractory agents due to high bacterial density and compound immobilisation within the biofilm (Singh et al. 2006). The presence of gradients can promote aerobic, anaerobic and anoxic conditions within a single amalgamated system, which promotes different removal regimes (Dutta et al. 2007).

The RBC biofilm can undertake biochemical oxygen demand (BOD_5) removal and BNR for domestic and high strength sewage (Hiras et al. 2004; Vlaeminck et al. 2009) and limited enhanced phosphorus recovery (Yun et al. 2004). Mounting evidence suggests that the RBC consortia can offer specific contaminant remediation for certain aromatics molecules including hydrocarbons, heavy metals, xenobiotics and pharmaceuticals/personal care products (PPCP) under appropriate process conditions (Novotný et al. 2012, Jeswani and Mukherji 2012, Orandi et al. 2012 and Simonich et al. 2002). Rotating biological contactors are used for WWT requiring low land area, maintenance, energy or start-up costs and can facilitate a more decentralised water treatment network (Hiras et al. 2004; Dutta et al. 2007). Traditional RBC design, maintenance and operation relied on process theory; however the biochemistry, biofilm modelling and microbial ecology have received increased attention recently (Wuertz et al. 2004). Patwardhan (2003), reviewed the process design aspects of RBCs and Cortez et al. (2008) highlighted some performance related process parameters. However despite investment and research in areas such as enhanced biological phosphorus removal, denitrification, cost and scale-up, the RBC is yet to achieve full potential as fundamental changes are required to the design or flow-sheet are required and other approaches such as granular sludge have appeared more flexible.

2.2.1 Process development history

The RBC concept originated in Germany in 1920s where it was described as a ‘rotating aerobic mass’ fixed to a media support (Chan and Stenstrom 1981), although the first plant was registered in the United States and was named the ‘Contact Filter’ or ‘Biologic Wheel’ consisting of partially submerged rotating plates (Doman 1929). This device served as an alternative to the trickling filter with 1/10th the land area, and lower power cost than activated sludge (Allen 1929). Commercial interest in RBCs was minimal, until the modern emergence of the so called ‘rotating drip body immersion systems’ (Hartman 1960). The design was patented (Hartmann 1961) and the first recorded experimental pilot RBC was undertaken to test performance (Popel 1964). This landmark study informed future RBC design which progressed in the 1960s. For example the surface BOD_5 loading from this study of ~3 $gm^{-2}d^{-1}$ is similar to modern overall surface OLRs of 3–15 $g.BOD_5.m^{-2}.d^{-1}$ that have been applied recently (Rittmann and McCarty 2001). The availability of stronger, lighter and affordable materials such as plastics increased the stability of media and increased the surface area available for microbiological growth, which improved treatment capacity. This allowed a plethora of capital ventures in the 1960 and 1970s. The RBC was applied for biological treatment under a variety of influent types, organic and hydraulic regimes (Rittmann and McCarty 2001 and Cortez et al. 2008). A Japanese company known as Kubota submitted a patent application for an RBC capable of simultaneous nitrification and denitrification, using variable submergence to facilitate multiple nutrient removal regimes (Sim 1988). A series of process failures have been noted for RBCs, many were due to inappropriate mechanical design which did not account for biomass growth, often leading to shaft, bearing and media malfunctions (Mba et al. 1999). A report suggested that equipment warranty should protect the owner from failure (Weston 1985), however often liability contracts rarely exceeded 3 years which provided little stimulus to fix inherent mechanical issues (Griffin and Findlay 2000). Another challenge was supplier competition led to an exaggeration of possible removal rates (Rittmann and McCarty 2001); allowable loadings varied by a factor of 7 between suppliers (Ross et al. 1994). Unlike other major biological processes,

designers were initially reliant on proprietors design criterion for process control (Ross et al. 1994). Hydraulic loading was previously applied as a design parameter, but was usually inappropriate by not considering organic strength; biodegradability, toxicity and temperature which impact microbial process performance (Steiner 1997). Design criteria should be used that incorporate fundamental parameters including microbial growth rates, OLR and substrate utilisation rate.

2.3 PROCESS ENGINEERING OF RBCS

2.3.1 Types

There are two main types of RBC; integral and modular. Integral systems consist of a single unit combining primary settlement, RBC biozone and either a contained or separate final clarifier (Figure 2 a). Integral units are usually contained within a package plant and have a treatment capacity of ≤250 population equivalents (PE) (Findlay 1993). Conversely, modular systems have separate operations for primary, secondary, and solids treatment respectively and usually treat PE >1000 (Griffin and Findlay 2000), which allows more flexible process configurations (Figure 2 b and c). However size and weight constraints generally limit RBCs to a size of <3.5 m disc diameter. Modular RBCs can be operated using parallel flow separation between units allowing operation within acceptable loading limits (Figure 2 b). In contrast, if effluent quality is of principal concern, RBCs are often operated in series, with an n^{th} RBC operating distal in the flow sheet (Figure 2 c). Typically a submergence of 40% (wet disc level), is used (Cortez et al. 2008). By increasing the submergence (Figure 2 d), the conditions in the reactor become increasingly anaerobic which could favour processes that require reduced oxygen levels such as denitrification (Teixeira and Oliveira 2001). Hybrid systems operate a RBC combined with another unit

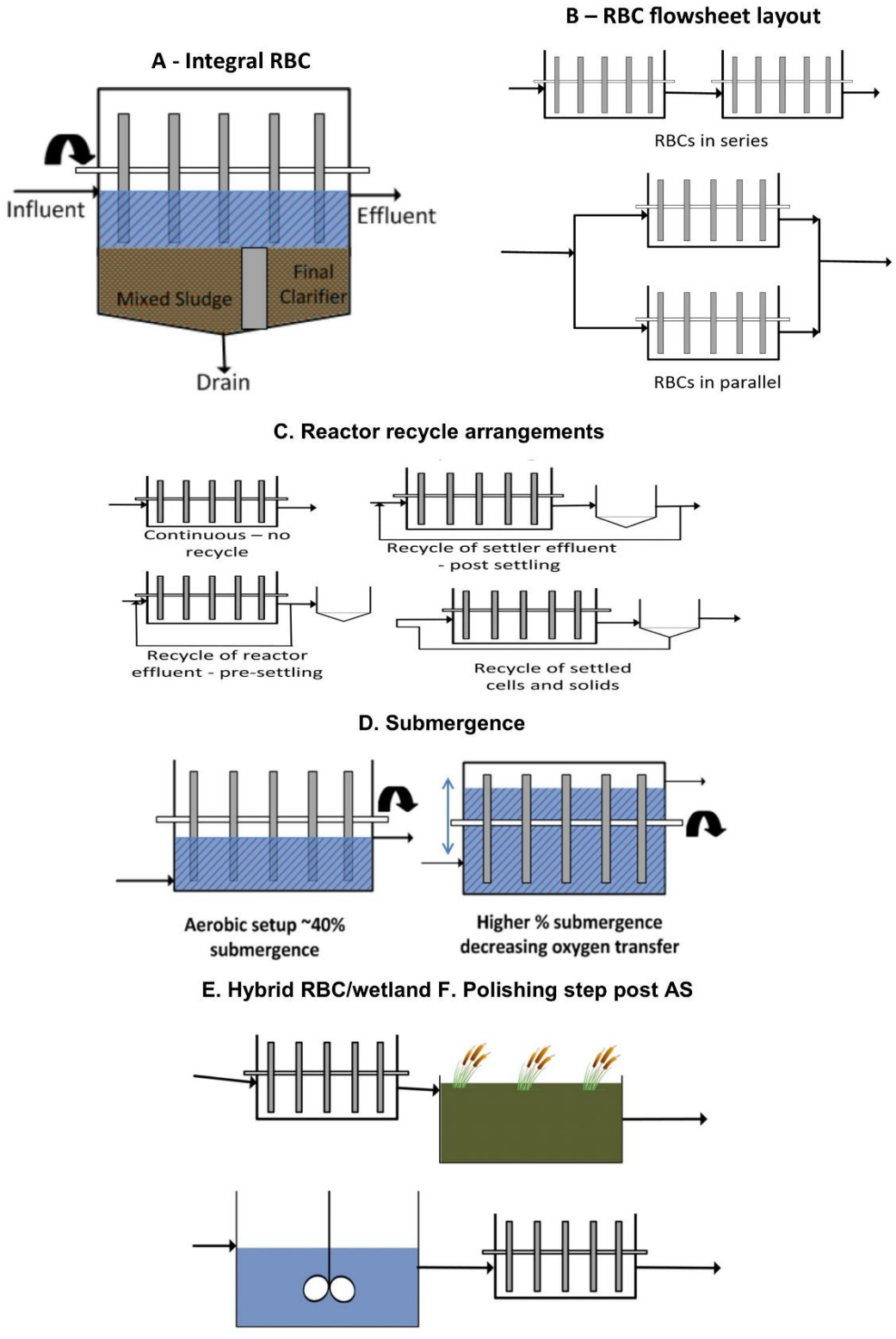


Figure 2 – Process configurations of RBC technology

operation to improve the stability of a process that has strong or variable loading, increase load capacity or improve the achievable effluent standard (Vesilind 2003; Hoyland et al. 2010). Common configurations include a RBC/wetland (Figure 2 e) or RBC/suspended growth combination which can be used for the upgrade of capacity (roughing) or provide tertiary treatment (Figure 2 f) (Vesilind 2003, Upton et al. 1995). The RBC/wetland combination has been applied to improve discharge consents for small works and provide a storm flow buffer (Griffin and Findlay 2000) (Figure 2 e). For longevity, the RBC is protected using ultra violet light resistant media (e.g. plastic with carbon black) or by covering the RBC within protective casing which can also reduce heat loss and flies/odour.

2.3.2 Cost

The CAPEX and operational expenditure (OPEX) of RBCs is low with reduced commissioning, monitoring and maintenance costs compared to activated sludge processes. In the UK, half the CAPEX for RBCs is related to mechanical and electrical components. The CAPEX cost per head in RBCs is inversely proportional to the PE for treatment. At PE >1000 the CAPEX cost decreased by up to 50% (Upton et al. 1995). For example Labella et al. (1972) compared the cost of an activated sludge plant and RBC system treating winery waste with a flow of 1.8×10^3 m^3d^{-1}. They noted that while CAPEX were similar, estimated power consumption was less than half that of a concrete tank aerated activated sludge plant (ASP). An RBC was found to be on average 35% cheaper per PE per year compared to trickling filters due to lower land area and running costs (Upton et al. 1995). However other authors have suggested that the OPEX of an RBC are similar to suspended growth systems and savings are only apparent with CAPEX (Ware et al. 1990). Fountoulakis et al. (2009) identified that RBCs had 29% lower and 44% higher CAPEX than packed bed filters and horizontal surface flow wetlands respectively. In addition RBCs were shown to have five times the power consumption than packed bed filters when operated within the OLR range of 0.53–2.01 kg COD $m^{-3}d^{-1}$. The power efficiency of a RBC operated a 7.5 horse power motor ranged from 72% to 88% at 25–100% load capacity respectively (Brenner and Opaken 1984). However RBCs are appropriate for

decentralised water treatment systems which generally have lower OPEX costs compared to a centralised approach which may require specialist labour and process control (Fountoulakis et al. 2009).

2.3.3 Substrate

Substrate dependent parameters in RBCs are staging (series or parallel), OLR, recycle ratio or rate and flowsheet position. The hydraulic considerations include HRT, tip speed, media specific surface area, substrate transfer rate and submergence. However there is considerable overlap between these parameters, for example the inverse relationship between HRT and OLR (Patwardhan 2003). Another example is the association between rotational speed, oxygen transfer rate (OTR) and biofilm thickness. A key criterion for RBC reactors is OLR which is defined as substrate (kg COD or N or pollutant) applied per square metre (specific or nominal) of media per day. In RBCs, the removal rate increases in proportion with OLR until another parameter limits reaction rates (Figure 3). For example Hiras et al. (2004) operated a two stage pre-denitrification and aerobic RBC for the treatment of settled municipal sewage. A decrease in the percentage removal of COD with increasing OLR was observed from 50% to 35% at OLR of 90 and 360 g.TOC.$m^{-2}d^{-1}$ respectively. Di Palma and Verdone 2009 showed that OTR limited the performance. Raising the rpm increased the total organic carbon (TOC) removal rate from 45 to 125 $gm^{-2}.d^{-1}$ suggesting that there was more capacity for bulk organics removal in the system. Therefore the highest substrate removal rate is achieved at the maximum loading before the transfer of rate limiting compound is exceeded (Figure 3). In RBC biofilms MT restrictions usually mask biological reaction kinetic limitations. As both substrates diffuse from the bulk fluid in the same direction and one or both will become limiting at a certain depth in the biofilm. In RBC biofilms there is an equilibrium between the rate of substrate consumption and diffusional transfer which influences the penetration depth (Stewart and Franklin 2008). Under constant loading the microbial community will attain steady state based on available substrates and competition for electron acceptors and space. In the biofilm there

is a layering of bacteria based on prevailing conditions with the lowest redox conditions proximal to the media (Okabe et al. 1999).

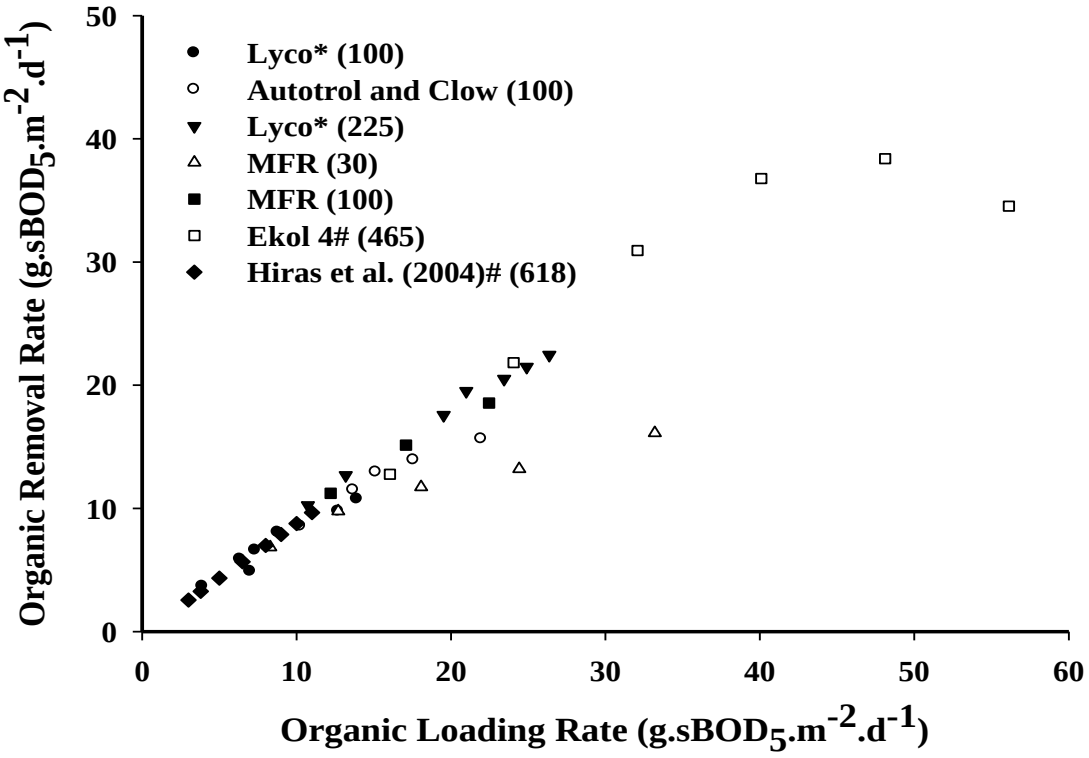


Figure 3 - Organic removal rate with loading rate of RBCs from different manufacturers for soluble BOD_5, *total BOD_5., #total COD, numbers in brackets indicate influent concentration mgL^{-1} (Brenner and Opaken 1984), Ekol 4 data adapted from Fountoulakis et al. (2009), data from Hiras et al. (2004) is an unspecified media/manufacturer.

Staging is a physical barrier employed to separate the wastewater chemistry within or between reactors (Figure 2 b), which leads to a stepwise reduction in the bio-available substrate to the point where the reactor approaches plug-flow (Ayoub and Saikaly 2004). This localisation selects for microbial populations adapted to the physiochemical conditions within each stage, which could be confirmed a metagenomic study to correlate species abundance to a parameter of interest such as substrate concentration. Staging improves removal rate, process stability and permits autotrophic nitrification at higher organic loads than normally possible (Tawfik et al. 2002, Kulikowska et al. 2010 and Najafpour et al. 2006). Staging can also permit enhanced ability to manage shock loads providing the biomass has sufficient substrate. The positive impact of staging on RBC performance was found to be negligible after four stages (Andreadakis 1987), although this is dependent on wastewater load and composition. Step feeding can be used to reduce the initial effective substrate concentration. Ayoub and Saikaly (2004) showed that step feeding had minimal impact on removal of RBC bulk COD removal rate, however NH_4-N removal increased by 18%, by staggering the organic load which reduced the likelihood of oxygen limitation (Rittmann et al. 1983). Recycling effluent permits greater portions of the biofilm to nitrify by diluting the influent organic concentration (Ayoub and Saikaly 2004). The recycle can be either pre, post or from the clarifier depending on treatment aim (Figure 2 c). Recycling settled solids helps aid bacterial retention as sloughed biomass is returned to the reactor. Other biomass associated products like extracellular enzymes may be recycled which could aid the breakdown of complex polymers, which constitute roughly half of domestic wastewater (Confer and Logan 1998).

2.3.4 Hydrodynamics

Understanding the hydrodynamics of RBCs is important to maintain appropriate biomass thickness, encourage compound MT and prevent unequal biomass distribution (Di Palma et al. 2003; Griffin and Findlay 2000). Rotation of media

creates a head difference leading to convective air/water exchange. Increasing tip speed increases the total OTR in a pseudo-linear fashion (Rittmann et al. 1983). However the energy usage for motor drive increases exponentially with increasing rotational speed. For minimal OPEX the lowest rpm should be selected and rotor speeds of 0.7–2.0 rpm are common (Mba et al. 1999). However, some high rate systems are known to exceed this speed, (Hoyland et al. 2010). Microscale biofilm structure can influence compound MT into the RBC biofilm. For example high biofilm roughness influenced the RBC biofilm boundary layer thickness by changing hydraulics and flow velocity perpendicular to the biofilm. This increased the rate of diffusion through the boundary layer and DO concentration in the biofilm (De la Rosa and Yu 2005).

2.3.5 Media composition

The RBC media can be present as discs, mesh plates, saddles or rings in a packed bed reactor, which resembles a partially submerged, rotating, moving bed biofilm reactor (Ware et al. 1990; Sirianuntapiboon and Chumlaong, 2013). The RBC media commonly has a specific surface area of 150–250 m^2 m^{-3} for biofilm growth which supports high removal rates at low HRTs. Lower surface area media is normally applied at the front-end of the works which typically has high organic loads (Cortez et al. 2008). Support media should be insoluble, have high mechanical and biological stability, and be cost effective (Leenen et al. 1996). The media physicochemical composition and architecture both impact on the microbial biofilm and the removal rate of substrates (Tawfik and Klapwijk 2010; Stephenson et al. 2013). A comparison between the oxygen transfer efficiency in RBCs was between 2–5 and 1–2 $kg.O_2.kWh^{-1}$ for comparable packed bed and disc RBCs respectively (Mathure and Patwardhan 2005). However previously it was noted that any performance gains from packed bed RBCs are usually offset by higher CAPEX costs and reliability issues (Ware et al. 1990). Polyurethane foam has been utilised to increase surface area for biofilm growth, reported specific surface areas range from 600 to 1000 m^2m^{-3} can provide greater solids retention, however careful management of both biofilm thickness and pore clogging is required (Windey et al. 2005; Tawfik and Klapwijk 2010). Chen et al.

(2006) used a ‘net-like’ media which increased the surface area of flat discs to facilitate a nitrification rate of 0.6 $g.N.m^{-2}.d^{-1}$ (Table 1). Liu et al. (2008), utilised a pyridinium type polymer sprayed to a non-woven carrier. They demonstrated the feasibility of autotrophic anaerobic denitrification. It was suggested that surface properties of the pyridinium facilitated attachment of nitrifiers permitting a nitrification rate >26 $kg.NH_4\text{-}N.m^{-2}d^{-1}$. Whereas Hassard et al. (2014) studied the impact of OLR on removal rates of biofilm cultivated on a polyvinylchloride-like (PVC) mesh, polyester and polyurethane foam in RBC-like reactors operated concurrently. They identified that under high loading conditions macroscale media pore size was the most significant parameter governing performance. As pore clogging leads to biomass inactivation and a decrease in effective surface area due to mass MT restrictions.

2.3.6 Scale up considerations

Appropriate scale up of RBCs is critical to validate whether performance will be comparable from bench/pilot to full scale (Arvin and Harremoës 1990). For RBCs, scale up should incorporate parameters of hydrodynamics, media active surface area, flow, OLR, OTR, bacterial growth rate, biofilm accumulation and detachment. However most models only accommodate one of these variables. For example Wilson et al. (1980) developed ‘generalised design loadings’ based on 12 months data at different scales. However, resulting models are not transferable to different operating/environmental conditions (Harremoës and Gönenc 1983). The use of tip speed is rarely a suitable parameter – as it increases (along with shear forces and mixer power) to the square of the diameter. To simulate full scale, bench scale reactors were previously operated at higher rotational speeds (to keep constant tip speed) which decreased the contact time per rotation (Spengel and Dzombak 1992). This also resulted in different shear distributions influencing erosion and sloughing processes in the biofilm, greater mixing and improved substrate removal. The empirical approach to scale-up involves constructing reactors of different sizes and is popular but is generally expensive. After sufficient development a mechanistic model can be developed, reducing the need for extensive testing. However these models are

usually appropriate for use with identical operating and wastewater conditions. Dutta et al. (2007) constructed three different sized RBCs to characterise the oxygen transfer coefficient at different scales. The model was based around existing ones: the Activated Sludge Model (ASM) No. 3 for biochemical reactions, a multiculture biofilm model and an RBC model. However the main limitation for this approach is that oxygen transfer should be suitably characterised on scale up, which can rarely be applied. An alternative approach is to design full scale reactors to have identical chemical, dynamic, geometric and kinematic to bench scale trials (Spengel and Dzombak 1992). The appreciation of scale up in RBCs is far from complete however models based on fundamental laws such as diffusion and mass transfer are less sensitive to scale up than empirical rule of thumb design parameters (Heath et al. 1990).

2.4 MICROBIOLOGY OF RBCS

The microbiology of RBC systems is governed by influent substrate conditions, seed population and hydrodynamic conditions. The biofilm which grows on RBC media is reliant on initial adhesion and the formation of glycoconjugate extracellular polymeric substance matrix for stability (Möhle and Langemann 2007). The most influential variable to the microbiology of RBCs is compound MT, which is dependent on operational parameters, biofilm structure and attachment/detachment mechanisms, and boundary layer thickness which have profound impact on the chemistry and microbial community structure, function and activity (Wuertz et al. 2004).

2.4.1 Structure

The growth rate and yield govern the spatial location of groups within multispecies RBC biofilms (Wuertz et al. 2004). Organisms with the highest maximum specific growth rate will be located towards the outside of the biofilm whereas slower growing organisms will be located towards the inside (Okabe et al. 1996). Ouyang (1980) reported an RBC biofilm with 74% VS, 95% water content and a chemical composition of $C_{4.2}H_8N_{0.6}O_2$. However RBC biofilm communities also exhibit distinct three dimensional organisation, for example Zahid and Ganczarczyk

(1994) found that early RBC biofilms are characterised by numerous fine pores, whereas mature biofilms have few large pores. This could reflect biofilm community regulation by quorum sensing, which is the extracellular communication by signal molecules between bacteria (Strous et al. 1999). Pores influence the convective flow and diffusive MT within the biofilm itself. De la Rosa and Yu (2005) found that a mature RBC biofilm had highly heterogeneous surface DO concentration from 3.8 to 0 $mg.L^{-1}$ which suggested that the biofilm oxygen consumption exceeded the rate of MT through the boundary layer. However, they identified pockets of high DO (>1 $mg.L^{-1}$) at depths of 760 µm, which is attributed to convective water flow through pores within the biofilm (Zahid and Ganczarczyk 1994) this suggests the microbial community is regulating itself analogous to multicellular organisms. The surface microbiota will be exposed to shear forces and the biofilm as an entity is subject to erosion. It is important to minimise mass sloughing events which negatively impact biofilm MCRT and process performance can ultimately suffer. Biofilm density is important to reduce sloughing frequency. Cell density increased from 3.3×10^9 to 3.9×10^{10} cells cm^3 with depth from 0 to 350 µm towards media surface (Okabe et al. 1996). The inner layers are protected from erosion and contain groups with a higher cell density (Arvin and Harremoës 1990). The rate of diffusion decreases with depth into the biofilm due to density, mineral formation and reduced mass driving force (Okabe et al. 1996 and Stewart 2003). Okabe et al. (1996) discovered that increasing the C:N ratio from 0 to 1.5 in an RBC biofilm created a distinct stratification in biofilm functional groups, where heterotrophs outcompeted nitrifiers for oxygen and space in the outer layers resulting in different niches. Further increases in the carbon ratio decreased nitrification rate and enhanced the biofilm functional stratification. The biofilm thickness also influences the performance of RBC reactors by providing a barrier to MT. Möhle and Langemann (2007) showed that RBC biofilm thickness increases with substrate concentration and decreases with surface shear forces. The cohesive strength of biofilms on RBC media was identified to be 6.1 and 7.7 Nm^{-2} at a biofilm thickness of 412 and 151 µm respectively, suggesting that biofilm stability is linked to thickness and density. Under high load and or low shear environments

filamentous groups proliferate at the surface RBC biofilm boundary. Alleman et al. (1982) showed that a distinct redox layering exists where the *Desulfovibrio* sp. reduce of sulphate to sulphide in the anaerobic sublayer and the *Beggiatoa* sp. dominate the outer aerobic layer where they oxidise hydrogen sulphide. This was confirmed by Kinner et al. (1985) identified bacteria containing poly-β-hydroxybutyrate and elemental sulphur inclusions. This situation develops under high OLR and low oxygen conditions in the biofilm which can result in reductions in RBC performance. Decreased OLR subsequently reduced the dominance of these organisms (Kinner et al. 1985).

2.4.2 Function and activity

Bacteria persistence within a biofilm does not necessarily indicate activity. Satoh et al. (2003) studied the influence of bioaugmentation and biostimulation on the efficacy of nitrification by RBC biofilms. Addition of nitrifying bacteria into the RBC resulted in elevated bacteria cell numbers at the surface of the biofilm. This resulted in higher NH_4-N/NO_2-N removal rates and 0.33 and 3 times lower start-up required for Ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) respectively compared to a control. Kindaichi et al. (2004) showed that a carbon limited RBC biofilm was comprised of 50% nitrifying bacteria composed of AOBs and NOBs consuming the influent ammonia and nitrite products respectively. However the remaining 50% were heterotrophic bacteria consuming soluble microbial products for nourishment from biofilm endogenous decay. A diverse heterotrophic community was present but sometimes inactive, however the majority of the carbohydrate and protein utilisation was by bacteria undertaking endogenous decay. Okabe et al. (2005) demonstrated that under substrate limitation the Chloroflexi group utilised 14C labelled products derived from RBC biofilm endogenous decay. In contrast the Cytophaga-Flavobacterium group accumulated 14C labelled reaction products from nitrifying growth, which suggested that each group specialised in utilising products from different biofilm growth phases. Heterotrophic turnover of utilisation and biomass decay products formed an equal contribution to the cell number and a greater contribution to the total diversity within a nitrifying RBC biofilm suggesting a role in community

regulation. Fountoulakis et al. (2009) demonstrated that an integral RBCs can remove up 2.2±1.7 log reduction in total coliforms from the influent. Tawfik et al. (2004) suggested that adsorption to the RBC biofilm could be a major mechanism for the removal of *Escherichia coli* although grazing by higher organisms or sedimentation could also contribute to pathogen removal in RBCs. Further research is warranted on the mechanisms of initial adhesion and bacterial incorporation in RBCs.

2.5 BIOLOGICAL NUTRIENT REMOVAL IN RBCS

2.5.1 Nitrification

Rotating biological contactors are used for nitrification and denitrification of a range of influent conditions (Cortez et al. 2008; De Clippeleir et al. 2011). Stringent rules govern nitrogen discharge and the energy cost/greenhouse gas emissions are a growing concern (Ainger et al. 2009). The RBC has potential benefits by reducing tank volume, HRT and aeration demand coupled with nitrogen removal at greater loadings compared to traditional treatments. Furthermore RBCs have been applied for refractory or contaminated wastes. For example Kulikowska et al. (2010) achieved a maximum nitrification rate of 4.8 g.NH_4-N.m^2.d^{-1} at a loading of 6.6 g.NH_4-N.m^2.d^{-1} (Table 1). Sequence analysis revealed microbial diversity decreased with time, suggesting a climax community was attained. Diversity indices were resistant to shock loading of >70% of normal flow and fluctuating performance, suggesting more sensitive measures of community change are required.

Table 1 – Impact of N loading rate on NH_4-N removal, data represent maximum removals achieved in each study.

Wastewater	Influent N concentration	N loading rate (g.N $m^{-2}d^{-1}$)	N reaction rate (g.N $m^{-2}d^{-1}$)	HRT (day)	Process	Reference
Synthetic high nitrogen	450	5.7	4.8	1.70	Anammox	Wyffels et al. (2003)
Synthetic sewage like nitrogen	280	5.4	3.5	1.00	Anammox	Lv et al. (2011)
Saline high NH_4-N	770	12.9	11.9	0.77	OLAND	Windey et al. (2005)
Synthetic high nitrogen	750	9.6	6.4	0.38	OLAND	Pynaert et al. (2004)
	1300	16.7	14.4	0.38		
Synthetic high nitrogen	1150	11.5	10.3	0.70	OLAND	Pynaert et al. (2003)
Synthetic high nitrogen	400	1.7	1.6	1.70	OLAND	Pynaert et al. (2002)
Digested black water	537	2.2	2.2	0.66	OLAND	De Clippeleir et al. (2011)
Synthetic high nitrogen	278	2.2	2.0	0.34		
	146	2.2	1.6	0.18		
	66	2.1	1.9	0.08		
Digested black water	1023	0.9	0.71	1.14	OLAND	Vlaeminck et al. (2009)
Landfill leachate	209	0.4	0.67	0.55	OLAND	Hippen et al. (1997)
Synthetic high nitrogen	60	0.5	0.5	0.20	Nitrification	Jang et al. (2005)

Digested real sewage	43	1.9 3.8 7.6	1.6 2.9 1.5	10.00 5.00 2.50	Nitrification	Tawfik et al. (2002)
Landfill leachate	130 244 332 451	1.9 3.6 4.8 6.6	1.9 3.6 3.6 4.8	6.6	Nitrification	Kulikowska et al. (2010)
Real settled sewage	24 36	3.5 10.3	0.2 6.3	0.16 0.08	Nitrification	Hassard et al. (2014)
Synthetic high nitrogen	110	1.1	1.1	0.63	SND	Gupta and Gupta (2001)
Synthetic sewage	30	0.7	0.6	0.33	SND	Chen et al. (2006)
Real settled sewage	42	0.06	0.1	0.25	SND	Hiras et al. (2004)

2.5.2 Denitrification

Denitrification is the dissimilarly reduction of nitrate to nitrite to dinitrogen gas under anoxic conditions (Paredes et al. 2007). Conventional heterotrophic denitrification is possible in wastewaters with a C/N ratio >2.5, without additional carbon sources (Hippen et al. 2001). As DO is consumed within a biofilm the community becomes oxygen limited. Thereby facilitating microenvironments where each consortia can develop. Helmer and Kunst (1998), found that under low DO conditions RBCs can remove up to 90% of the nitrogen load from landfill leachate. Odegaard and Rusten (1980) found that the NO_x-N recycle ratio in RBCs improved denitrification rate. Batch testing revealed that nitrogen removal was carbon limited, suggesting autotrophic degradation satisfied the nitrogen deficit. Cortez et al. (2011 a) achieved almost complete nitrogen removal from landfill leachate using conventional denitrification in an anoxic RBC, they identified that preozonation was required to remove refractory carbon compounds. Gupta and Gupta (2001) augmented a mixotroph known as *Paracoccus denitrificans* to undertake simultaneous aerobic carbon oxidation, nitrification and denitrification. *P. denitrificans* removed a maximum of 26 and 1.9 $gm^{-2}d^{-1}$ of COD and nitrogen respectively in an RBC. However, the aerobic denitrification rate was slower than conventional denitrification. At high nitrate concentrations (>500 mgL^{-1}) inorganic phosphorus can limit denitrification. Cortez et al. (2011 b) suggested that phosphorus addition improves overall biofilm denitrifying activity and nitrogen removal by promoting bacterial growth. Teixeira and Oliveira (2000) improved denitrification by 30% upon the addition of phosphorus. Hanhan et al. (2005) compared the nitrogen removal rates in full scale pre-denitrifying RBCs. The highest reported removal was ~2 $g.N.m^{-2}.d^{-1}$ with a HRT of 0.2 d (Table 1). The nitrogen removal rate decreased with increasing rotational speed, suggesting oxygen inhibition led to suppression of the denitrification pathway. Teixeira and Oliveira (2001) demonstrated that increased disc submergence from 64.5% to 100% improved the TN removal by 63% but had delayed start-up.

Anammox is an anaerobic pathway for denitrification involving bacteria which convert nitrite and ammonium into dinitrogen gas. The RBC is suitable for autotrophic denitrification as the anammox bacteria have low growth rates and therefore require reactors with a high MCRT (Siegrist et al. 1998). Initially the thin RBC biofilm is conductive for AOBs to proliferate and provide the colonisation matrix for slow growing anammox bacteria; providing the biofilm is oxygen limited or NOBs are supressed (Pynaert et al. 2004). De Clippeleir et al. (2011) showed that decreasing HRT from 0.66 to 0.18 d stimulated a decrease in removal rate from 2.2 to 1.6 $g.N.m^{-2}.d^{-1}$ (Table 1). This was attributed to increased nitratation by *Nitrospira* sp. which proliferated at DO concentrations of >1.2 mgL^{-1}. Stepwise loading increases allowed removal rates in excess of 1.8 $g.N.m^{-2}.d^{-1}$ (Pynaert et al. 2004). Vlaeminck et al. (2009) tested the feasibility of an oxygen limited autotrophic nitrification and denitrification (OLAND) process to treat digestate from source separated black water and achieved a removal rate of 0.71 $g.N.m^{-2}.d^{-1}$. The nitrite oxidising bacteria were supressed at free ammonia levels >3 mgL^{-1}, however, DO levels <0.3 mgL^{-1} are required for process stability. The effluent from this reactor had a N/P ratio of 1 suggesting struvite production and therefore nutrient recovery is possible. However facilitating struvite accumulating organisms in RBC biofilms has not received any research attention. Windey et al. (2005) showed that anammox bacteria could adapt to high salinity conditions of up to 30 gL^{-1}, providing the RBC biofilm acclimation was gradual. The removal rate of nitrogen decreased from 11.9 gL^{-1} using non-saline wastewater to 11.5, 9.6 and 9.6 at 5, 10 and 30 gL^{-1} of salt respectively. A similar study by Kartal et al. (2006), identified that 45 gL^{-1} of salt completely inhibited anammox bacteria. Liu et al. (2008) suggested that the anammox consortium on RBCs were relatively resistant to DO shocks. They found that a *Nitrosomonas* eutropha-like species protected the Planctomycetes by sequestrating potentially inhibiting DO levels.

2.6 BIOLOGICAL PHOSPHORUS REMOVAL

Attaining enhanced biological phosphorus removal (EBPR) is challenging in RBC systems, as it is difficult to control the sequential oxic and anaerobic conditions for growth of phosphorus accumulating organisms (PAO). Kenneth (1994) grew

PAOs in a modified RBC setup with an anaerobic clarifier and carbon addition for PAO growth, with subsequent sludge recycle to the RBC. This solids recycle allowed oxygen conditions for EBPR and increased the liquid phase MLSS improving organic removal rates. Sim (1988) varied the submergence in a RBC operated as a sequencing batch contactor. Initially full submergence and acetate addition created anaerobic conditions necessary for phosphorus release and fatty acid storage. Next half of the fluid was stored in a holding tank, the remaining liquid in the RBC was subjected to oxic conditions allowing enhanced phosphorus uptake. Yun et al. (2004) used a sequencing batch reactor (SBR) approach to undertake EBPR without an additional carbon source. The authors demonstrated that the maximum biofilm phosphorus uptake was at a C:P range of 13–18 where P ranged from 3% to 8% of biofilm VS. The biofilm thickness appeared to determine the TP removal with a maximum removal efficiency of total phosphorus of 70% was attained at a biofilm thickness <1.8 mm. This limitation is not apparent in suspended growth SBR. This could be a MT restriction preventing exchange of available phosphorus and organic substrates restricting TP uptake rate which is not present in suspended growth setups. Understanding mechanisms which govern EBPR in RBC biofilms warrants further attention.

2.7 PRIORITY POLLUTANT REMEDIATION IN RBCS

Priority pollutant remediation can require the bioaugmentation or retention of specialised strains. Bioaugmentation in RBC systems is usually achieved through addition of either suspended or freeze dried artificial cultures or freeze dried biomass to the RBC (Stephenson and Stephenson 1992). Alternatively cultures of microbes can be grown in a side stream reactor prior to addition. The natural solids retention of the RBC biofilm permits microbe retention without additional separation or recirculation. Many systems require acclimatisation periods and are sensitive to shock/variable loadings or intermittent feeding of the pollutant which is of import for the removal of priority substances from wastewaters (Stephenson and Stephenson 1992, Duque et al. 2011 and Amorim et al. 2013).

2.8 Organic pollutants

Dye wastewater is a challenging form of organic pollutant as the dyes or breakdown products can be toxic or mutagenic (Malachova et al. 2013). The RBC is ideal for dye treatment due to high biomass retention, low startup costs, and appropriate technology level for developing countries (Robinson et al. 2001). Wastewater dyes are initially absorbed to the biofilm but a continually exposed biomass will eventually saturate. Most dyes do not penetrate bacteria as they have a high molecular weight and contain hydrophobic groups, which are a barrier to biocenocis (Pearce et al. 2003). The bioaugmentation of white rot funghi (WRF), e.g. *Phanerochaete* sp. has been undertaken in RBC systems as they excrete non-specific extracellular hydrolytic enzymes with dye decolouring capacity (Pakshirajan and Kheria 2012). Novotný et al. (2012) found a surface decolourisation rate of 0.63, 0.19 and 0.01 $mgm^{-2}h^{-1}$ for Remazol Brilliant Blue R, Methylene Blue and Azure B respectively by the augmented fungus *Dichomitus squalens* (Table 2). Dye degradation is often undertaken as a secondary metabolism so allochthonous carbon sources are required to maintain activity. Novotný et al. (2012) identified that *D. squalens* has a minimum glucose concentration of 0.018 gL^{-1} for effective dye decolourisation. Pakshirajan and Kheria (2012) showed that the decolourisation rate of *P. chrysosporium* is proportional to glucose concentrations to a limit of 10 gL^{-1}. The use of molasses dosing decreased the decolourisation rate of *P. chrysosporium* by 20% compared to glucose control (Pakshirajan and Kheria 2012). Dye removal has been correlated with activity of manganese dependent peroxidase and lignin peroxidases. For full dye remediation from wastewater the dye should be decolourised and detoxified. Malachova et al. (2013) utilised an RBC bioaugmented with *Irpex lacteus 931*, and achieved a batch methyl blue decolourisation rate of 9.4 $mgm^{-2}d^{-1}$. Decolourisation resulted in reduced toxicity level of the wastewater. However the WRF are susceptible to bacterial stress which usually prevents application under real wastewater conditions. Nilsson et al. (2006) used an RBC augmented with *Trametes versicolor* to treat real textile wastewater and achieved 60–70% decolourisation efficiency. Research should identify if WRF can be utilised in RBCs with appropriate scale up.

Duque et al. (2011) inoculated a strain capable of degrading 2-fluorophenol and demonstrated increased removal efficiency under constant pollutant loading. Under variable loading the pollutant removal decreased even though the community remained in the biofilm. Amorim et al. (2013) studied the impact of shock loadings of 4-fluorocinnamic acid (4-FCA) on an augmented RBC. The removal efficiency was increased from 8% to 46% at surface loadings of 73–168 $gm^{-2}d^{-1}$ respectively (Table 3). Isolation of biofilm strains and batch testing revealed that two strains completely mineralised 4-FCA. Sequence analysis revealed a 97% similarity to the original augmented *Rhodococcus* strain, suggesting horizontal gene transfer or genetic drift had occurred (Singh et al., 2006). The RBC reactor has also been applied for removal of non-aqueous phase liquids (NAPL) (Mukherji and Chavan 2012). Chavan and Mukherji (2008 a) found that a mixed freshwater phototrophic community augmented with *Burkholderia cepacia* had a removal rate of >26 $gm^{-2}d^{-1}$ for removal of diesel NAPL. The NAPL component of the wastewater was likely sorbed onto the biofilm for subsequent biodegradation of the aliphatic fraction (Mukherji and Chavan 2012). Operation with the co-contaminant phenol slightly reduced the removal efficacy of NAPL but resulted in complete phenol removal (Chavan and Mukherji, 2010). Under constant pollutant loading in RBCs it is therefore important to promote proliferation of the augmented community at functional levels

In WWTPs micropollutants are usually eliminated through biotic degradation or abiotic sorption. Simonich et al. (2002) compared removal of fragrances in different WWTPs. Fragrances appeared to be removed typically in the biodegradable fraction of the wastewater. However sorptive non-biodegradable fragrance material removal is linked to solids disposal (Simonich et al. 2002). In contrast micropollutants which are non-sorptive and non-readily biodegradable are of greatest concern. In this study the RBC achieved 99% removal efficiency of methyl dihydrojasmonate compared to 98%, 93%, 82% for an ASP, trickling filter and carousel setup respectively. The removal of 6-acetyl-1,1,2,4,4,7-hexamethylteraline (AHTN) in the RBC was inferior compared to other secondary treatments which could be due to poor removal of particulate matter and therefore AHTN. Batt et al. (2007) compared four treatment works with similar influent

concentrations of Ciprofloxacin, Sulfamethoxazole, Tetracycline and Trimethoprim and found that the RBC had comparable removal of antibiotics of between 52–95% removal of Ciprofloxacin, Tetracycline and Trimethoprim to an extended aeration ASP but with lower HRT and presumably treatment cost. In contrast the RBC demonstrated 43% lower Sulfamethoxazole removal compared to the ASP. The degradation behaviour of this antibiotic could be due to physical differences between bacteria in biofilms and suspended growth.

Table 2 – Dye concentrations and decolourisation rates of dyes in bioaugmented RBCs.

Compound	Dye concentration mgL^{-1}	Growth Medium composition	Organism	Removal (%)	Dye surface loading $mgm^{-2}h^{-1}$	Surface Decolourisation rate $mg.m^{-2}.h^{-1}$	Reference
Remazol Brilliant Blue R	50	Mineral medium 10 gL^{-1} glucose	*Dichomitus squalens*	95	0.66	0.63	Novotný et al. 2011
Reactive Blue 4	200	Citric buffer 21.4 gL^{-1} glucose	*Trametes versicolor*	70	0.26	0.18	Nilsson et al. 2006
Reactive Blue 4	100	10 gL^{-1} glucose	*Bjerkandera* sp.	99	0.07	0.06	Axelsson et al. 2006
Methylene Blue	50	Mineral medium 10 gL^{-1} glucose	*Dichomitus squalens*	85	0.22	0.19	Novotný et al. 2011
	150	Malt extract glucose 10 gL^{-1} + 2% agar	*Irpex lacteus 931*	55	N/A	0.39	Malachova et al. 2013
Azure B	50	Mineral medium	*Dichomitus squalens*	42	0.03	0.01	Novotný et al. 2011

		10 gL^{-1} glucose					
Basic Blue 22	200	8 gL^{-1} glucose	*Phanerochaete sordida*	0.80	0.89	0.71	Ge et al. (2004)
Direct Red 80	200	13.46 gL^{-1} glucose	*Phanerochaete chrysosporium*	0.80	0.55	0.44	Pakshirajan and Singh 2010
Mordant Blue 9	200	13.46 gL^{-1} glucose	*Phanerochaete chrysosporium*	0.62	0.55	0.34	Pakshirajan and Singh 2010
Reactive Red 2	100	10 gL^{-1} glucose	*Bjerkandera* sp.	0.99	0.07	0.06	Axelsson et al. 2006
Acid Red 27	62.4	Kirk's medium 10.1 gL^{-1}	*Trametes versicolor*	0.58	1.64	0.96	Ramsay et al. 2006

Table 3 – Priority pollutant removal by RBC reactor communities

Organic pollutant	Initial pollutant concentration mgL^{-1}	Degrading species / consortia	Removal efficiency %	Pollutant surface loading rate $mg.pollutant.m^{-2}d^{-1}$	Maximum pollutant surface removal rate $mg.pollutant.m^{-2}d^{-1}$	HRT (d)	References
Benzene	1193	*Pseudomonas* sp., *Bacillus, Enterococcus* sp.	97.7	4.0	3.9	1.23	Sarayu and Sandhya 2012
Xylene	1226	*Pseudomonas* sp., *Bacillus, Enterococcus* sp.	98.5	4.1	4.1	1.23	Sarayu and Sandhya 2012
Phenol*#	250	*Exiguobacterium aurantiacum*	48.4	154.4	74.7	1.00	Jeswani and Mukherji, 2012
Pyridine*	280	*E. aurantiacum*	34.2	169.5	58.0	0.50	Jeswani and Mukherji, 2012
Quinoline*	280	*E. aurantiacum*	48.9	345.3	168.9	0.50	Jeswani and Mukherji, 2012
Benzene*	200	*E. aurantiacum*	35.0	246.7	86.3	0.50	Jeswani and Mukherji, 2012

Napthalene*	60	*E. aurantiacum*	59.8	36.3	21.7	0.50	Jeswani and Mukherji, 2012
Phenanthrene*	0.5	*E. aurantiacum*	53.2	0.3	0.2	0.50	Jeswani and Mukherji, 2012
Phenanthrene	1.73	*Phanerochaete chrysosporium*	41.0	2.5	1.0	12.16	Zheng and Obbard, (2002)
Fluoranthene*	0.2	*E. aurantiacum*	46.0	0.1	0.1	0.50	Jeswani and Mukherji, 2012
Pyrene*	0.12	*E. aurantiacum*	80.0	0.1	0.1	0.50	Jeswani and Mukherji, 2012
Pyrene	1.23	*P. chrysosporium*	65.9	1.8	1.2	12.07	Zheng and Obbard, (2002)
Benzol(α)pyrene	0.21	*P. chrysosporium*	96.9	0.3	0.3	11.72	Zheng and Obbard, (2002)
Trichloroethylene	30	Mixed culture (MC) augmented with *Thiosphaera pantotropha*	98.7	202.8	200.1	2.00	Brar and Gupta (2000)

2-fluorophenol	100	MC augmented with 2-fluorophenol degrader (FP1)	82.0	4.8	3.9	0.78	Duque et al. (2011)
1,5-pentanedial (Glutaldehyde)	180	MC from RBC treating glutaldehyde and RAS[#]	71.4	31468	22455	0.03	Laopaiboon et al. (2007)
4-chlorophenol	826	MC from settled sewage	51.3	37545	18300	0.35	Sahinkaya and Dilek,(2006)
2,4-dichlorophenol	424	MC from settled sewage	50.7	19272	9500	0.35	Sahinkaya and Dilek,(2006)
4-fluorocinnamic acid	80	*Rhodococcus* sp. S2	45.7	4660	2129	0.77	Amorim et al. 2013
4-fluorocinnamic acid	35	*Rhodococcus* sp. S2	7.9	2038	110	0.77	Amorim et al. 2013

*Mixed synthetic wastewater stream containing multiple organic pollutants, #removal from first stage only

#Recycle activated sludge (RAS)

2.8.1 Inorganic pollutants

Biological heavy metal removal relies on both the sorption of the metal species to biomass and the bioaccumulation by metabolic processes (Costley and Wallis 2001). The RBC microbial biofilm is suitable for biosorption as there is a high contact area for sorption and a long MCRT. However the metal removal rate will decrease with time, as the attraction sites become saturated (Matheikal et al. 1991). For example Sirianuntapiboon and Chumlaong (2013) found that an RBC had a decreased removal efficiency of 64–45% and 80–85% with increased loading which corresponded to a removal rate of between 255–400 and 255–480 $mgm^{-2}d^{-1}$ for Ni and Pb respectively (Table 4). This is similar to removal rates reported for Cu of ~450 $mgm^{-2}d^{-1}$ using activated sludge consortia (Costley and Wallis 2001) (Table 4). To prevent saturation it is necessary to remove the metal loaded biomass by suitable treatment. However this is costly and produces secondary waste issues (Costley and Wallis 2001). Costley and Wallis (2001) showed that multiple cycles of sorption/desorption, using a dilute (<0.5 M) acid did not impact the adsorption efficiency of a mixed culture RBC biofilm, suggesting reuse was possible. The removal rates demonstrated by Costley and Wallis (2001) of ~640, 450 and 320 $mgm^{-2}d^{-1}$ for Zn, Cu and Cd appeared dependent on loading and the availability of free sorption sites. Regression analysis reveals that the loading rate predicts removal rate between loads of 0.003–762.8 $mg.metal.m^{-2}\ d^{-1}$ ($R^2 = 0.9$, $P < 0.001$) (Based on data from Table 4).

Table 4 – Heavy metals and pollutant sequestration by RBC community

Trace pollutant	Initial metal concentration mgL^{-1}	Biosorbent species	Removal efficiency %	Metal loading rate $mg.metal.m^{-2}d^{-1}$	Metal removal rate $mg.metal.m^{-2}d^{-1}$	Adsorption capacity mg.metal. $biofilm.g^{-1}$	HRT d	References
Mn	45	*Ulothrix* sp.	36.7	18.243	6.695	-	1	Orandi et al. 2012
Co	0.5	*Ulothrix* sp.	5.7	0.203	0.012	-	1	Orandi et al. 2012
Cu	100	*Ulothrix* sp.	38	40.541	15.405	-	1	Orandi et al. 2012
Cu	100	Activated sludge consortium enriched by metal spiking	59	762.829	450.069	4484	1	Costley and Wallis 2001
Pb	30	Sedimentation tank biomass	80	600	480	-	4	Sirianuntapiboon and Chumlaong (2013)
			83	400	332		6	
			85	300	255		8	
Zn	20	*Ulothrix* sp.	29	8.108	2.351	-	1	Orandi et al. 2012
Zn	100	Activated sludge consortium enriched by metal spiking	84	762.829	640.777	3454.1	1	Costley and Wallis 2001
Se	0.04	*Ulothrix* sp.	35.2	0.016	0.006	-	1	Orandi et al. 2012
Sb	0.007	*Ulothrix* sp.	35.6	0.003	0.001	-	1	Orandi et al. 2012
Ni	3	*Ulothrix* sp.	35.7	1.216	0.434	-	1	Orandi et al. 2012
Ni	30	Sedimentation tank biomass	67	600	400	-	4	Sirianuntapiboon and
			71	400	284		6	

			74	300	222		8	Chumlaong (2013)
Cd	100	Activated sludge consortium enriched by metal spiking	42	762.829	320.388	1914.4	1	Costley and Wallis 2001
Cyanide	40	Sedimentation tank consortium	90	0.408	0.367	-	0.33	Sirianuntapiboon and Chuamkaew, (2007)

2.9 MODELLING OF RBC REACTORS

Process optimisation and scale-up are challenges for the efficient use of RBCs (Spengel and Dzombak 1992; Dutta et al. 2010). In contrast to most suspended growth processes, MT can often mask the impact of biokinetics on the performance of RBCs (Famularo et al. 1978). This is because thick biofilms and unidirectional transfer limit the rate of compound exchange. Previously, the derivations of MT were described within the context of penetration and surface renewal theory (Patwardhan 2003). Then focus was placed on the relationship between OTR and substrate utilisation biokinetics (Chavan and Mukherji 2008 b). However usage of empirical approaches are limited to wastewater and operational conditions similar to the derivative source of the models (Di Palma et al. 2003). Models can also be based on reaction order, substrate diffusion, microbial growth biokinetics and the identification of different oxygen and nutrient conditions (Clark et al. 1978; Patwardhan 2003). Finally, multiple substrate and species models have been applied to RBCs using biofilm models based on description of transformation and transport processes (Gujer and Boller 1990; Dutta et al. 2007). Historically RBC modelling has received significant research attention; however the inherent complexities of system hydrodynamics prevent application to other biological treatment processes.

2.9.1 Substrate utilisation in RBCs

The substrate utilisation in RBCs is separated into substrates and electron acceptors, model assumptions and output. Roberts (1973) developed a model incorporating substrate MT limitation to/from the biofilm and the kinetic considerations governing biodegradable substrate utilisation. Alternatively the removal of soluble substances is determined by the boundary layer diffusion resistance, into the biofilm prior to microbial degradation within the interior (Arvin and Harremoës 1990). An empirical relationship to predict effluent BOD_5 was determined by the US Environmental Protection Agency (Brenner and Opaken 1984) in which:

Equation 1 – Removal rate expressed as ratio of effluent to influent compound concentration.

$$\frac{C_e}{C_i} = e^{K(0.000125\,V/Q)^{0.5}}$$

In which:

C_i = compound concentration in the influent, C_e = compound concentration in the effluent, *K*= reaction rate constant (0.3) at 13°C. , V= media volume , Q = reactor flow rate

This model does not include parameters on microbial kinetics, substrate limitation or changes to influent/temperature. Clark et al. (1978) developed an RBC model where removal rate can be determined from influent/effluent conditions and microbial growth rate in which:

Equation 2 – Removal rate based on specific growth rate and yield of heterotrophic bacteria.

$$r_a = \left(\frac{\mu_{max}}{X_a}\right)/Y_a$$

In which: μ_{max} = specific growth rate, X_a = concentration of biomass, Y_a = yield coefficient of biomass

A modified version of the Kincannon and Stover (1982) model for RBC systems of removal rate integrated over disc area in which:

Equation 3 – Removal rates based on nominal surface area and growth kinetics.

$$r_a = \left(\frac{K_C}{U_{max}}\right).\left(\frac{A_d}{QC_i}\right) + \left(\frac{1}{\mu_{max}}\right)$$

In which: K_c = half saturation constant for compound, U_{max} = maximum substrate removal rate

The equations mentioned above are empirical or analytical in origin which predict removal rate per area as a function of a chosen suite of dependent variables. The removal rate constants and model coefficients are obtained by regression analysis with experimental data (Hansford et al. 1978). However providing the

system has been adequately described more complex models allow application to different treatment scenarios (Wanner et al. 2006). An RBC model was one of the first to describe simultaneous BOD_5 removal and nitrification. It was suggested that heterotrophic activity is the dominant process at earlier stages in RBC treatment and nitrification occurs once the BOD_5 concentration is below the threshold selecting against autotrophic nitrification (Mueller et al. 1978). Wanner and Gujer (1986) demonstrated that competition for space and electron acceptors between heterotrophs and autotrophs occurs in biofilms. Biofilm modelling was previously based on Fick's Law of diffusion, however, Wanner and Gujer (1986) also accounted for biofilm behavior and internal microbial distribution in a dynamic model. This facilitated the application of a modified version of ASM 1 to permit true dynamic modelling of RBCs for aerobic and anoxic degradation of organic constituents (Gujer and Boller 1990). The model revealed that the distal compartment of the RBC was substrate limited for nitrification, in which decay and inactivation outweighed growth (Dutta et al. 2007). This identified a potential risk to effluent quality under shock load scenarios. Model simulations demonstrated that periodic flow reversal restored the activity to the distal compartment by countering nitrifyer starvation. Dutta et al. (2007) developed a model incorporating the multi-species biofilm model after Gujer and Boller (1990) and the kinetics from the ASM 3 (Gujer et al. 1999).

Equation 4- Removal rate taking into account transfer from air to liquid film and subsequently the biofilm. Compounds with no gasesous component take into account only liquid phase.

$$\frac{dC^{Lf}}{dt} = K_L^{air}\frac{A_{exp}}{V_{Lf}}(C^* - C^{Lf}) + K_L\frac{A_{sub}}{V_{Lf}}(C^T - C^{Lf}) - K_L\frac{A}{V_{Lf}}\left(C^{Lf} - C^{Bf}\Big|_{x=\delta_{Bf}}\right)$$

Table 5 – Expressions for oxygen transfer in RBCs

Application / Derivation	Expression	Assumptions	Reference
Liquid film (LF) thickness	$\delta = \phi^{0.5}\omega^{1.5}s^{1}$		(Zhevalkink et al., 1978)
Overall oxygen transfer (OT) considering film theory	$K_L = -2(\frac{D_L}{\pi t_R})^{0.5}((1-4.21)\frac{\delta}{D_L T_R})^{0.5}$		Zeevalkink et al. (1979)
Overall OT to bulk	$lnK_L = 1.31\ln\omega + 14.78$	OT governed by disc rotation alone	Friedman et al. (1979)
	$K_L = 2(\frac{D_L}{\pi t_R})^{0.5}$	Where $\delta/D_L t_R \geq 1.7$	
Overall OT considering film theory	$K_L = 2\left(\frac{2\alpha}{\pi^{0.5}}\right).\frac{\delta}{t_R} \sim \frac{\delta}{t_R}$	Where $\delta/D_L t_R < 0.8$	Bintanja et al. (1975)
Overall OT	$K_L\frac{\emptyset}{D_L} = K\left(\frac{\omega'\emptyset^2\rho}{\mu}\right)^{l}\left(\frac{\omega'^2\emptyset}{g}\right)^{m}\left(\frac{\emptyset-\emptyset_0}{\emptyset}\right)^{n}$	K = 1.7, l = 0.8, m = 0.13, n =0.74	Sant' Anna (1980)
Volume renewal number	$K_L a = 0.0011\ (\phi^{0.5}\omega^{1.5}s^{-1})^{0.732}$	Sterile disks, *e/r* = 0.042 and H/t_R = 0.15	Kim and Molof (1982)

The OT dependence on volume renewal number	$K_L a = 0.0003\left(\frac{NA_d\delta\omega}{V}\right) + 0.0119$	Where N_v : <800	Kubsad et al. (2004)
	$K_L a = 0.0001\left(\frac{NA_d\delta\omega}{V}\right) + 0.1157$	Where Nv >800	
Non-dimensional model of $K_L\alpha$	$\frac{(K_L a\rho A_d)}{\mu} = \left(\frac{\emptyset}{{A_d}^{0.5}}\right)^{\psi}\left(\frac{\rho A_d\omega}{\mu}\right)^{\varepsilon}\left(\frac{A_d}{A_t}\right)^{\theta}\left(\frac{\delta}{V^{0.33}}\right)^{\lambda}$	Ψ=0.327, ε=1.018, θ=0.743, λ= 0.624	Chavan and Mukherji (2008 b)
Model of Oxygen transfer	$K_L a = \alpha.\omega^{1.5}.\phi^{0.5}.(\beta/\omega + \gamma)$	where α, β, γ are constants that need defining	Di Palma et al. (2003)
Experimentally verified model from above	$K_L a = 134.07.\omega^{1.5}.\phi^{0.5}.(2.15/\omega) + 0.006)$	Model only valid providing enhancement factor is described.	Di Palma et al. (2009)

2.9.2 Oxygen transfer in RBCs

The OTR determines the biofilm oxygen concentration and hence the selected removal regime in RBCs. Initially, oxygen must diffuse from the bulk water/gas phase across the boundary layer, into the film layer and eventually into the biofilm itself. The rate of diffusion is dependent on the diffusion coefficient of oxygen and the distance according to Fick's Law (Stewart 2003). Originally it was thought that the majority of transfer occurs with biofilm contact to the air phase and therefore bulk fluid concentration was less important (Hartman 1960). Other models were developed with the assumption that substrate alone rather than oxygen is limiting in RBCs: these are now deemed unsuitable (Clark et al. 1978; Spengel and Dzombak 1992). The OTR is related to the difference between the liquid phase and equilibrium concentration, in the liquid film and RBC biofilm (Chavan and Mukherji 2008 b). Hansford et al. (1978), presented one of the first attempts to include mass transport resistance to OTR. Initial models of OTR assumed that turbulence, wave generation and immersion dominate (Patwardhan 2003). An alternative method is that oxygenation occurs during film breakup and renewal. This is caused by the air/water cycling involving the interaction with rotational derived forces, which overcome film layer surface tension. The rate of renewal is dependent on the rotational speed, disc diameter, position and half spacing (Table 5) (Chavan and Mukherji 2008 b). A study suggested that the relationship between liquid film renewal and the OTR was linear under sterile conditions (Kim and Molof 1982). Attached biofilm increases the OTR, by enhancing concentration gradients due to consumption in the film and adsorption to the biofilm (Kim and Molof 1982; Zeevalkink et al. 1979). However biofilm growth can reduce OTR by clogging pores which reduces MT, Friedman et al. (1979) related OTR to rpm alone. Rittmann et al. (1983), identified the importance of adsorption for OTR at high rotational speed (>25) whereas diffusive film transport dominated during operation at normal rotor speed. Kubsad et al. (2004), compared two forms of the Kim and Molof (1982) model to alternatives and found appropriate predictive fit providing the volume renewal number can be estimated effectively

(Table 5). Di Palma and Verdone (2009) calibrated a previously defined model and found that the kLa_t increased in a linear fashion between the speeds of 3 and 10 rpm at bench scale. The majority of film renewal is thought to occur when the surface tension resistance is broken under the effect of gravity after the so called 'falling film' theory (Zhang et al. 2009) which can be experimentally determined through high resolution photography to measure rate of droplet formation with time.

2.10 NOVEL APPLICATIONS OF RBCS

The relatively simple engineering of RBC type systems promises to provide a platform for new energy generating processes that treat wastewater. There are a variety of RBC systems that have been applied for direct electricity generation or energy production through biogas and algae (Sayess et al. 2013, Cheng et al. 2011 and Paule et al. 2011). Sayess et al. (2013) coupled an RBC with a microbial fuel cell configuration which allowed for contaminant removal and electricity production. This RBC achieved between 6.9% and 20.9% higher denitrification rates compared to a control RBC setup where electron generation by anodic oxidation was used by denitrifiers for nitrate reduction at the cathode. In a similar system it was shown that the optimum current for nitrogen removal is 0.2 A^{-2} (Rodziewicz et al. 2011). Cheng et al. (2011), developed a bioelectrochemical RBC-type system for indirect energy generation. Each disc was split with regular 180° rotations which led to inversion of the anode and cathode allowing concurrent spatial acetate oxidation and methanogenesis respectively. Methane generation appeared proportional to electrical input with ~80% energy recovery. Christenson and Sims (2012) developed a method for indirect energy generation and removal of nitrogen and phosphorus utilising an algal RBC-type reactor. The reactor design consisted of a RBC drum with ropes and scraper blades which collected the algae. The maximum harvested biomass produced was ~30 $g.TS.m^{-2}.d^{-1}$. The algal RBC reactor achieved removal rates of ~14 and 2 $gm^{-2}d^{-1}$ of soluble nitrogen and phosphorus respectively. Paule et al. (2011) designed a vertical RBC with an intrinsic light source with removable polyethylene

plates produced 0.007 $gm^{-2}d^{-1}$ of volatile solids (VS) which could be used for energy generation.

2.11 CONCLUSIONS

The use of RBCs for conventional biological WWT to remove BOD_5 and ammonia has been well established for the last three decades (Mueller et al. 1978). Application has largely been at the lower end of the WWT scale, usually for up to 2000 P.E. (Griffin and Findlay 2000). The limits of organic carbon renewal have been thoroughly investigated, with maximum OLRs of up to 120 $g.sCOD.m^{-2}d^{-1}$ through using improved media optimised disc immersion and adjusted rotational speeds (Teixeira and Oliveira 2001, Hanhan et al. 2005, Chen et al. 2006 and Hassard et al. 2014). However, novel configurations of media – such as mesh types (Chen et al. 2006, Liu et al. 2008 and Hassard et al. 2014) – and careful selection of media to enhance growth of certain bacterial populations could increase applied OLRs and nitrogen loading rates (NLRs) incrementally (Khan et al. 2013; Stephenson et al. 2013).

Recent research has demonstrated that the process can be adapted to remove nutrients, both nitrogen and phosphorus, as with other biological processes (Yun et al. 2004 and Hanhan et al. 2005). Novel RBC type processes, such as Hybrid Activated Sludge (HYBACS), has shown that new combinations of suspended growth and fixed film on rotating media can provide higher organic removal rates and efficient denitrification (Hoyland et al. 2010). Solid and liquid phase bacterial interactions have been mentioned previously (Wanner et al. 1990; Kenneth 1994), a better understanding of these mechanisms merit further investigation in applying hybrid RBCs for energy efficient nutrient removal. Biofilm systems are suited to providing a range of redox environments, from wholly aerobic through anoxic to anaerobic conditions (Wuertz et al. 2004). Exploitation of this phenomenon in RBCs is in its infancy at full-scale: for example, anammox (Strous et al. 1999) has been demonstrated in RBCs (Siegrist et al. 1998, Vlaeminck et al. 2009 and De Clippeleir et al. 2011). Control of disc immersion can be used to stimulate denitrification (Courtens et al. 2014). Enhanced biological phosphorus removal requires alternating anaerobic and aerobic conditions (Yun et al. 2004);

however enforcing SBR type approaches in RBCs at full scale is challenging. Therefore manipulation of the gaseous headspace, submergence, rotational speed or recycle in RBCs could be explored to stimulate the EBPR process. Fully submerged processes such as Biological Aerated Filters use backwashing to remove excess bacterial growth to optimise performance, drawing analogies to mixed liquor wastage in activated sludge (Mendoza-Espinosa and Stephenson 1999). Deliberate removal of RBC biofilm, either by mechanical means or air scouring, to control the biomass growth rate, and therefore performance, has not been directly employed. A full scale exception is the air scour used to remove biofilm in RBRs, however, this is usually applied to prevent media clogging (Hoyland et al. 2010). Yun et al. (2004) suggested biofilm thickness should be controlled every 15 days to enable EBPR in a SBR type RBC, although this would be dependent on biofilm accumulation rate. Christenson and Sims (2012) used scraper blades to remove algal biofilm for harvesting providing new surfaces for biomass growth. Manipulating microbial growth rate to determine performance could allow greater process control of RBCs. The mechanical engineering of RBCs has proven to be the most problematic issue when applied at full scale, specifically shaft material selection, media robustness and construction and design and maintenance of bearings (Mba et al. 1999). 'Lightweighting' of these components through use of new materials, e.g. composites, provide opportunities for re-engineering and allowing further scale-up. Application of low resistance bearings, e.g. air or 'non-stick' bearings, may allow for lower energy, higher rotational speeds that could enhance treatment. The removal of dyes and other recalcitrant organic pollutants in RBCs appears linked to bioaugmentation and propagation of allochthonous microbial populations with pollutant degrading capacity (Novotný et al. 2012). The sensitivities and expense of these communities remains an issue for application under real scenarios with representative wastewater. Future research should focus on approaches suitable for scale-up or methods for upgrade or existing works which struggle to deal with organic pollutants containing wastewater. Costley and Wallis (2001) highlighted the potential of RBC biofilms for resource recovery, with the increasing price of metals and nutrient fertiliser new opportunities could be created for cost positive

WWT (STOWA 2010). The simplicity, adaptability, low land use and maintenance and high volumetric activity of the RBC suggest that it will continue to help meet our WWT requirements for years to come.

CHAPTER 3

PERFORMANCE OF ROTATING BIOFILM REACTORS USED FOR PRETREATMENT OF WASTEWATERS. PUBLISHED: *Water Science and Technology, 69* (2014) *1926-1931*

3 PERFORMANCE OF ROTATING BIOFILM REACTORS USED FOR PRETREATMENT OF WASTEWATERS.

3.1 ABSTRACT

The impact of OLR on carbonaceous materials and ammonia removal was assessed in bench scale RBR treating real wastewater. Media composition influences biofilm structure and therefore performance. Here, plastic mesh, reticulated coarse foam and fine foam media were operated concurrently at OLRs of 15, 35 and 60 g.sCOD.$m^{-2}d^{-1}$ in three bench RBR. The sCOD removal rate increased with loading from 6 to 25 g.sCOD.$m^{-2}d^{-1}$ ($p<0.001$). At 35 g.BOD_5.$m^{-2}d^{-1}$, more than double the arbitrary OLR limit of normal nitrifying conditions (15 g.BOD_5.$m^{-2}d^{-1}$); the removal efficiency of NH_4-N was 82±5, 27±19 and 39±8% for the mesh, coarse foam and fine foam media, respectively. Increasing the OLR to 35 gm decreased NH_4-N removal efficiency to 38±6, 21±4 and 21±6%, respectively. The mesh media achieved the highest stable NH_4-N removal rate of 6.5±1.6 g.$m^{-2}d^{-1}$at an OLR of 35 g.sCOD.$m^{-2}d^{-1}$. Viable bacterial numbers decreased with increasing OLR from 2×10^{10}–4×10^{9} cells per ml of biofilm from the low to high loading, suggesting an accumulation of inert non-viable biomass with higher OLR. Increasing the OLR in permeable media is of practical benefit for high rate carbonaceous materials and ammonia removal in the pretreatment of wastewater.

3.2 INTRODUCTION

The main priorities for WWT are effluent quality, cost, energy efficiency and nutrient removal/recovery (STOWA 2010). In traditional biological treatment, the achievable effluent standard is largely dependent on the energy applied through aeration and extended HRT. This increases the cost to the operator/consumer and the environmental impact of treatment (Vanrolleghem et al. 1996). These challenges are important when commissioning or upgrading WWTPs. To achieve discharge limits with financial constraints it is imperative to optimize process operation. An RBR technology known as SMART has shown promise in

addressing these challenges (Hoyland et al. 2010). These units are similar to RBCs but the media composition is permeable with heterogeneous architecture and a high porosity, which is thought to overcome the limitations of RBC-like reactors (Chen et al. 2006). The RBR units usually operate in conjunction with an activated sludge process collectively known as hybrid activated sludge. The RBR unit operate as a roughing bulk carbon oxidation step with nutrient removal followed by clarification in the activated sludge stage (Hoyland et al. 2010). In this study, the impact of novel media as a roughing stage was compared for the first time against other high specific surface area, high porosity, commercially available alternatives. The OLR is the principal parameter when deploying a RBR (Cortez et al. 2008) and impacts on biofilm treatment performance (Ayoub and Saikaly 2004). A traditional RBR process should have an overall maximum surface loading of 6 $g.BOD_5.m^{-2}d^{-1}$ to achieve simultaneous biochemical oxygen demand (BOD_5) removal and nitrification (Rittmann and McCarty 2001). However, RBR units are designed as a 'roughing step' to rapidly remove BOD_5, to reduce load for the secondary treatment processes and improve the nitrogen removal capacity of the treatment process as a whole. In RBRs the MCRT is independent of HRT, which allows greater flow rates, OLRs and process stability than is possible in suspended culture systems (Cortez et al. 2008). The addition of a roughing step to a WWTP can improve the stability of a process that has strong or variable loading, increase capacity or improve the achievable effluent standard (Hoyland et al. 2010).

Common to most biofilm processes is an inert solid support medium on which the microbial community grows (Stephenson et al. 2013). The physical composition and architecture of the medium has an impact on the biofilm and the removal rate of both BOD_5 and ammonia (Tawfik and Klapwijk 2010). Biofilm processes have unique features that affect their biofilm structure, microbial composition and therefore substrate utilization (Wuertz et al. 2004). The biofilm biomass, usually expressed through the concentration of VS does not measure biological information about the health, viability or activity of the biofilm (Ziglio et al. 2002). The use of viability as an indicator is useful for determining the impact of operating conditions on biofilm bacterial performance. The objectives were to

establish the impact of OLR on the substrate removal rate and to assess how the media type affects this dependency. To elucidate this relationship, the viability of the microbial population was used to evaluate the bacterial viability within the biofilm at different surface OLRs.

3.3 MATERIALS AND METHODS

3.3.1 Pilot studies at varying OLRs

Three bench scale RBR units were situated at Cranfield University WWTP; each consisted of a plastic vessel and a single rotating shaft with permeable plastic frames for housing the media. The media consisted of circular plates of a PVC-derived mesh, polyester reticulated foam and polyurethane reticulated foam with specific surface area of ±450,±800 and ±1,000 m^2m^{-3} — henceforth called mesh, coarse foam and fine foam, respectively to measure the influence of media selection on performance. The total media volume per reactor was 0.003 m^3 (d = 0.2 m, surface area = 0.19 m^2, disk n=2, wetted volume =3 L, submergence =40%). The RBR units were operated at a constant tip speed of 0.08 m/s (8 rpm); at this speed, the rpm itself is unlikely to limit the substrate removal rate (Di Palma and Verdone 2009). The RBR units were fed with real settled sewage and were operated for a minimum of 3 weeks prior to monitoring to ensure pseudo-steady state conditions. Different OLRs were applied to each reactor: 15, 35 and 60 $g.sCOD.m^{-2}d^{-1}$, which corresponded to 1.1, 2.2 and 4.4 L of settled sewage per hour (Table 6). To achieve stable nitrification, a surface loading of <15 $g.BOD_5.m^{-2}d^{-1}$ is recommended for biofilm processes (Rittmann and McCarty 2001). The equivalent BOD_5 loading rates were ~35, 81 and 140 $g.BOD_5.m^{-2}d^{-1}$ based on an average $sCOD:BOD_5$ ratio of 1:2.3 from influent wastewater from the study period (n=78) for low, medium and high OLRs, respectively. The impact of OLR on trial media for RBR units was assessed at~2X (low), 5X (medium) and 10X (high) this value, as these units have high voidage and are used in a roughing configuration (Table 6). The removal rates of sCOD and NH_4-N are compared to other process characteristics. The total operating time for the study was 9 months with,

approximately 3 months at each loading. The RBR reactors were temperature controlled to 15ºC using a 50 W thermostatic aquarium heater (Superfish, UK).

Table 6 - Operating conditions of the RBR units

Load	HRT (d)	Flow rate (Ld^{-1})	sCOD loading rate ($g.m^{-2}d^{-1}$)	NH_4-N loading rate ($g.m^{-2}d^{-1}$)	COD/N ($g.COD.g^{-1}N$)
Low	0.115	26	15±0.37	4±0.14	3.25±0.32
Med.	0.057	53	35±2.51	10±1.19	3.34±0.17
High	0.028	106	60±3.30	14±0.27	4.35±0.09

3.3.2 Wastewater analysis and calculations

Influent samples were collected at 09:00, with effluent samples collected at 1, 2 and 4 h post-influent sampling depending on the OLR studied. Wastewater was analysed using proprietary cell test kits (VWR, UK) for total chemical oxygen demand (tCOD) and TN. The wastewater was filtered through a 1.2µm glassfibre filter (Whatman, UK). The sCOD ammonia-nitrogen (NH_4-N), nitrite-nitrogen (NO_3-N) and nitrate-nitrogen (NO_3-N) were measured using a NOVA60 photometer (VWR, UK). Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to Standard Methods (APHA 2012). The DO of the effluent was measured using an HQ30d DO probe (Hach, Germany) and the pH of the influent and effluent was measured using a Jenway 320 pH meter (Bibby, UK). The COD was used to assess the OLR applied as it is the more fundamental parameter compared to the BOD_5 test (Roeleveld and van Loosdrecht 2002). The media nominal surface area ($A_{nominal}$) and OLR were calculated according to Equations (5) and (6). Nominal rates were selected, as specific surface area is less important under high biofilm growth conditions such as the OLRs studied. The removal efficiency and substrate removal rate were calculated normally.

Equation 5 – Nominal surface area of disc

$$A_{nominal} = 2\pi.r^2 + 2\pi.r.h$$

Equation 6 – Nominal OLR

$OLR = S_i \times Q_i / A_{nominal}$

In which: r = radius of the plate, h = plate thickness, Si =influent substrate concentration (sCOD or NH_4-N) and Q_i = influent flowrate.

Equation 7 – Volumetric OLR including mesh/disc volume only.

$vOLR = S_i \times Q_i / V_{nominal}$

3.3.3 Microbial viability

Wastewater biofilm was sampled from the media surface using a 15 mL sterile plastic bottle at the same time as effluent samples. A dilute, dispersed cellular fraction was obtained according to Ziglio et al. (2002). A 5 mL sub-sample was mechanically disaggregated using a homogeniser (Powergen 125, Fisherbrand, UK) for 10 min at speed setting 2 (12,250 1/min). Samples were diluted using 0.22µm filter sterilised NaCl solution (0.085%) (Boulos et al. 1999). The samples were then disaggregated for a further 5 min to obtain the maximum number of viable bacteria (Ziglio et al. 2002 and Appendix 8D.1). Differential centrifugation 180×g (r_{max}=3.5, microcentaur, MSEUK,UK) was used to separate the bacteria (in the supernatant) from the solids and extracellular debris (pellet) (Lunau et al. 2005). The bacteria were diluted to give approximate bacterial numbers per field of view (FOV). Bacterial samples were stained with LIVE/DEAD®BacLight™ test (Invitrogen, Glasgow, UK) according to the manufacturer's guidelines with modifications according to Boulos et al. (1999). The bacterial sample was then vacuum filtered onto a black polycarbonate membrane filter (0.22µm pore size, 25mm diameter; Nucleopore, Whatman, UK). The filter was washed with sterilised NaCl solution (0.085%) and mounted on a microscope slide with a drop of LIVE/DEAD® BacLight™ mounting oil, and a coverslip was placed over the filter and fixed with clear nail polish (Rimmel London, UK). Cells were viewed under oil immersion on an LSM 510 META confocal laser scanning microscope (CLSM) (Carl Zeiss, Inc., Germany) with Axiovision software. Images for cell counts were acquired using a Zeiss LSM camera. Image processing with the imageJ program (Abramoff et al. 2004) was used to prepare images for counting. The number of

viable (green) and dead (red) bacteria was then calculated, taking dilutions into account. Bacteria stained orange were considered to have intermediate dye penetration and were considered dead due to non-intact membrane.

3.3.4 Experimental design

The performance aspect of this study was based on a pilot (n=10); the averages and standard deviation (SD) were used to calculate the sample size using G*power3. There were 12 and 11 samples per group for sCOD and NH_4-N (95% confidence interval, 50% power), respectively. A balanced statistical design was used and the assumptions of parametric statistics were met. The grouped data of loading and media data were normally distributed (Kolmogorov Smirnov test sCOD $p>0.01$; NH_4-N $p>0.01$). To test whether there was a difference in the removal rate of sCOD/NH_4-N with both OLR and media type, separate one way analysis of variance (ANOVA) was undertaken (SPSS, IBM, USA). The viability test was performed on the last 3 days of performance data. Each sample was analysed in triplicate and incorporated recommendations by Lisle and Hamilton (2004) to achieve representative counts.

3.4 RESULTS AND DISCUSSION

3.5 Effect of OLR on sCOD removal

The removal rate of sCOD was ranked from greatest to least mesh>coarse foam>fine foam for all studied loadings except for the medium OLR, where the fine foam had a higher removal rate of 8 $g.m^{-2}d^{-1}$ compared to 6.5 $gm^{-2}d^{-1}$ for the coarse foam (Figure 4 a). The mesh and the coarse foam media had significantly higher removal rates at this loading ($p<0.05$). The mesh media achieved superior sCOD removal of 6, 14 and 26 $gm^{-2}d^{-1}$ at low, medium and high loads, respectively (Figure 4 a). The foam media sCOD removal rate did not increase in proportion to OLR, unlike the mesh media. Biofilm bridging probably reduced the active biofilm surface area and reduced the transfer of oxygen and substrates (Gujer and Boller 1990). At a higher OLR, the removal rate of the permeable media increased but the removal efficiency decreased, which has been noted

previously (Hiras et al. 2004). Sayess et al. (2013) achieved consistently high COD removal efficiency of >86% at loadings of 5 or 10 $gm^{-2}d^{-1}$ in RBC systems. Chen et al. (2006) obtained an increase in removal rate from 30 to 38 $g.tCOD.m^{-2}d^{-1}$ with increasing OLR. Di Palma and Verdone (2009) found that 23 $g.tCOD.m^{-2}d^{-1}$ was the OLR threshold, after which the removal rate of organic carbon decreased due to reduced oxygen transfer. However, increasing rotational speed reduced this effect. Most of these studies utilised synthetic sewage, which is more readily biodegradable than real sewage, so results should be compared with caution. At a volumetric organic loading rate (vOLR) of 4.0 $kg.sCOD.m^3d^{-1}$ the mesh media achieved a reactor volumetric removal rate (VRR) of 1.4 $kg.sCOD.m^3d^{-1}$, which is similar to the lower limit for high-rate suspended growth systems that have vOLR from 1.5–3 $kg.BOD_5.m^3d^{-1}$ (WEF 1998). Roughing trickling filters are operated with a vOLR of >1.5 $kg.m^{-3}d^{-1}$ with a reported removal rate of<1 $kgm^{-3}d^{-1}$ (WEF 2000). The RBR unit achieves a higher removal rate with a lower footprint, without excessive odour or flies – typical attributes for enclosed biofilm reactors.

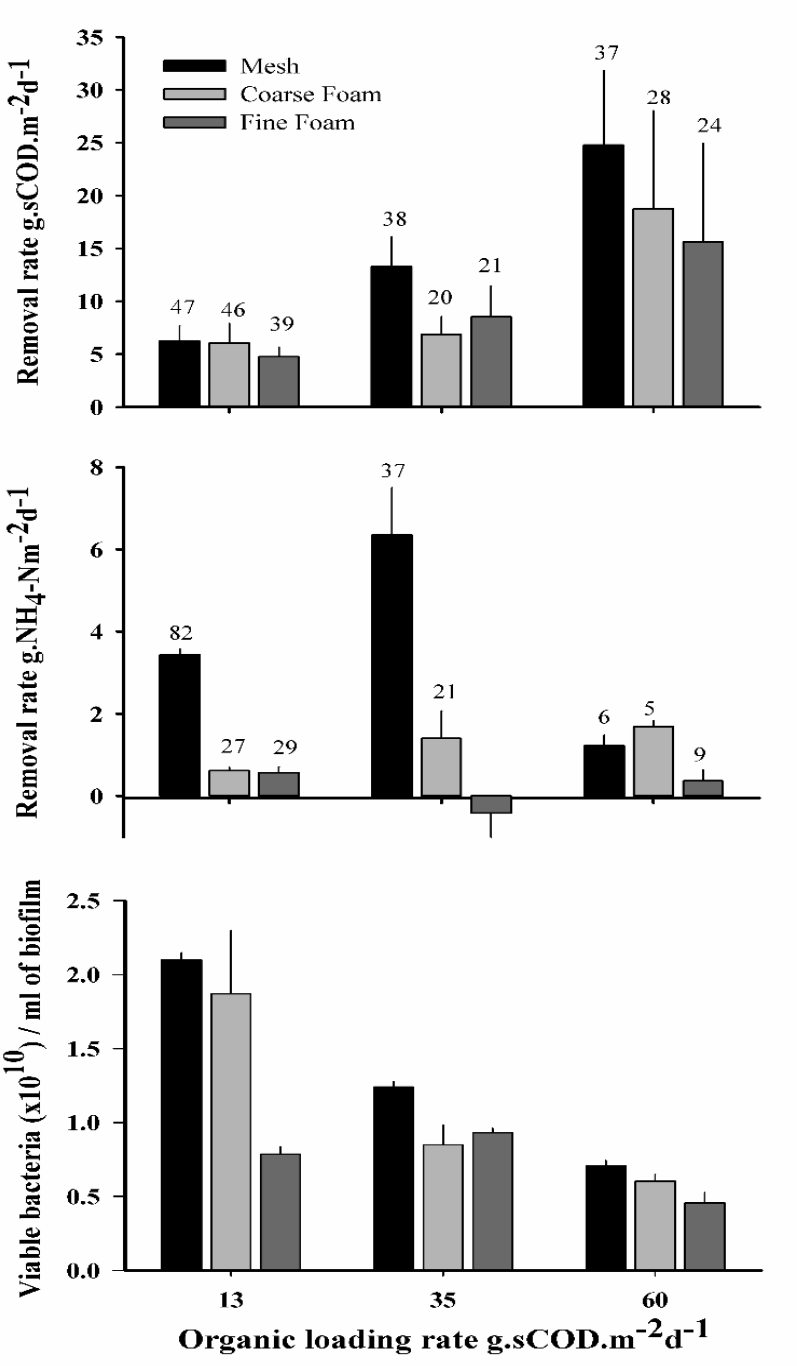


Figure 4 - Impact of OLR on performance: a. Surface removal rate of sCOD ($g.sCOD.m^{-2}d^{-1}$) b. Removal rate of NH_4-N ($g.NH_4\text{-}N.m^{-2}d^{-1}$) related to OLR. Error bars indicate ±95% confidence intervals (based on standard error mean (SEM) and n=11). (c) Viable 'membrane intact' bacterial counts per ml of biofilm with OLR. Error bars indicate ±1 SD from the mean.

Table 7 - Wastewater characteristics of influent feed and effluent of RBR units and performance running settled sewage at different OLR ± 95% confidence interval.

OLR	Low			Medium			High		
Parameter (mgL^{-1})	Mesh	Coarse Foam	Fine Foam	Mesh	Coarse Foam	Fine Foam	Mesh	Coarse Foam	Fine Foam
$tCOD_i$	450±82	440±60	459±67	297±33	268±39	300±41	345±43	347±53	385±60
$tCOD_e$	126±27	98±60	134±36	135±24	163±26	189±32	221±36	183±60	250±48
$sCOD_i$	85±13	80±10	85±9	117±15	109±35	129±21	106±15	99±17	93±13
$sCOD_e$	44±8	40±6	53.4±11	73±12	86±13	101±17	63±8	67±11	67±12
TN_i	45±4	44±4	42±4	52±9	50±8	53±8	42±3	42±2	42±2
NH_4-N_i	28±4	26±6	26±4	36±10	35±11	29±11	24±3	24±2	23±2
NH_4-N_e	5±1	22±7	23±7	14±6	30±9	39±12	23±3	23±2	21±2
NO_3-N_e	18.5±2	1.5±0.3	2.0±0.3	7±1.3	1.0±0.25	1.5±0.5	1±0.25	1±0.25	1±0.5
DO_e	8.4±1.3	8.6±0.4	7.0±1.0	8.3±0.3	7.4±0.2	6.4±0.6	4.5±0.3	4.9±0.5	3.4±0.4
TSS_i	213±56	222±38	154±23	150±33	138±23	124±23	177±29	161±21	187±35
TSS_e	71±13	56±16	77±44	25±15	34±11	53±15	93±17	82±15	103±23

3.6 Effect of OLR on nitrogen oxidation

The mesh media achieved the highest ammonia removal rate of 3.8 and 6.5 $g.m^{-2}d^{-1}$ at the low and medium OLRs, which was greater than the foam media ($p<0.05$). The mesh media achieved greater sCOD and NH_4-N removal and stable nitrification at OLR of up to 35 $g.sCOD.m^{-2}d^{-1}$ (Figure 5 a). Chen et al. (2006) achieved a first stage removal of 1.9 $g.NH_4\text{-}N.m^{-2}d^{-1}$ removal, at a COD loading of 51 $g.tCOD.m^{-2}d^{-1}$ at 2 hours HRT. There was little or no TN removal as no solids separation was incorporated in the RBR units. The mesh media exhibited three times the NH_4-N removal at double the sCOD concentration than previously reported for traditional solid disc RBCs fed with real municipal wastewater. An incremental increase in OLR reduced the NH_4-N removal rate by a factor of 4.3; however, an increase in COD/N ratio could have exacerbated this (Table 7). At the high OLR, nitrifiers incur greater mass transport resistance and physical competition from heterotrophs (Okabe et al. 1996); these performance data for novel media suggest that a washout, selection or inhibition mechanism occurred (Gujer and Boller 1990). Ayoub and Saikaly (2004) showed that the negative effects of OLR on NH_4-N removal efficiency can be reduced using step feeding and an internal recycle. To provide nitrification, a trickling filter OLR should be <0.5 $kg.BOD_5.m^{-3}d^{-1}$ (WEF 2000). The maximum NH_4-N reactor agreed VRR of the mesh media was 0.4 $kg.sCOD\ m^{-3}d^{-1}$at a vOLR of 2.1 $kg.sCOD.m^{-3}d^{-1}$.

3.7 CONCLUSIONS

- Increasing the OLR in all the permeable media resulted in greater removal rates, but lower percentage removal efficiency.
- The mesh media exhibited the highest (6 $g.NH_4\text{-}N.m^{-2}d^{-1}$) ammonium removal rates, even at sCOD loads of 35 $gm^{-2}d^{-1}$ and low HRT.
- Media porosity was similar for the permeable media studied; the architecture and sizes of the apertures appeared to impact the maximum removal rate (8Appendix C).

- The viability of the bacteria in the biofilm had a significant negative correlation with sCOD loading for the mesh and coarse foam media.
- Understanding the impact of process condition on bacterial viability could improve biofilm performance during WWT.

CHAPTER 4

MESH MEDIA ROTATING BIOFILM REACTORS FOR PRETREATMENT OF WASTEWATERS – INFLUENCE OF MEDIA TYPE ON MICROBIAL ACTIVITY, VIABILITY AND PERFORMANCE.

SUBMITTED: *Bioresource Technology.*

4 MESH MEDIA ROTATING BIOFILM REACTORS FOR PRETREATMENT OF WASTEWATERS – INFLUENCE OF MEDIA TYPE ON MICROBIAL ACTIVITY, VIABILITY AND PERFORMANCE.

4.1 ABSTRACT

Hybrid biofilm reactors are suitable for the upgrade of existing WWTPs as a small footprint, pre-treatment stage. The impact of OLR on carbonaceous oxidation and nitrogen transformation was assessed for low and high surface area PVC and polypropylene (PP) plastic mesh media (PVC-L, PVC-H, PP-L and PP-H). Mesh media was operated simultaneously under incrementally increasing OLR to test performance of candidate mesh media in RBRs treating real wastewater. The maximum VRR of 2.4 kg.sCOD.m^3d^{-1} occurred at the high OLR, but reduced under very high OLR: this effect was more pronounced for the lower porosity media due to pore clogging. The media surface OLR threshold for stable nitrification was 35 g.sCOD.$m^{-2}d^{-1}$ (~80 g.BOD_5.$m^{-2}d^{-1}$) where a VRR of 0.3 to 0.4 and 0.2 to 0.3 kg.NH_4-N.$m^{-3}d^{-1}$ was obtained for PVC and PP respectively. There a reduction in biofilm present under medium compared to low OLR, resulting in higher effective organic loading. This coincided with a ~ 2 fold decrease in the microbial viability from low to medium OLR on average. The high sCOD removal rates attained by the PVC-L and PP-L media at very high OLR (160 g.sCOD.m^{-2}d-1 or ~320 g.BOD_5.$m^{-2}d^{-1}$) suggested that porosity was a key parameter to maintain high biofilm performance under elevated OLR wastewater pre-treatments. In contrast operating within established OLR thresholds is more important for NH_4-N removal.

4.2 INTRODUCTION

Achieving more stringent effluent standards in conventional biological treatment is usually contingent on factors such as extended reactor retention times and aeration rates, both of which increase the cost of treatment (Ainger et al. 2009). To achieve discharge limits within financial constraints with low energy usage, it

is imperative to optimize process operation and develop new technology (STOWA, 2010). A RBR known as a SMART unit has shown promise for high OLR treatment (Hoyland et al. 2010). These units are similar to RBCs but with an open architecture media comprised of fibres arranged in a high porosity mesh, which overcomes the limitations of RBC-like reactors under high load conditions (Chen et al. 2006; Hassard et al. 2014). In order to minimise the cost of treatment and maximise value of existing assets, the RBR unit operates as a roughing biofilm for oxidation of bulk organics prior to existing secondary process such as a hybrid modification of activated sludge (e.g. HYBACS) or trickling filters (e.g. HYFILT) (Hassard et al. 2014; Hoyland et al. 2010).

The OLR is important when deploying a biofilm reactor as this impacts on biofilm treatment performance and effluent quality (Wijeyekoon et al. 2004; Chen et al. 2006). Previous research suggested that rotating biofilm processes achieved simultaneous BOD_5 removal and nitrification at an arbitrary surface loading of 15 $g.BOD_5.m^2d^{-1}$ although some high rate systems have exceeded this limit (WEF 2000; Hassard et al. 2014). Above 15 $g.BOD_5.m^{-2}d^{-1}$ biofilm/solids accumulation occurs, preventing nitrification. Increasingly, biofilm accumulation is seen as an advantage by facilitating a greater range of treatment regimens in a simultaneous volume (Lackner et al. 2014) as biofilm reactors have an undefined MCRT and growth kinetics and distinct gradients in substrate and electron acceptors (Bryers 2000). High biomass concentrations in biofilm systems constitute an opportunity to construct simple, cheap and compact reactors (Mendoza-Espinosa and Stephenson 1999; Elenter et al. 2007). In biofilm reactors the microbial population is attached on a solid media, which allows greater flow rates, OLR and process stability than is possible in most suspended culture systems (Stephenson et al. 2013).

Careful media selection has been suggested as a method to control biofilm thickness (Morgenroth and Wilderer 2000; Elenter et al. 2007), select for and against different strains (Khan et al. 2013; Stephenson et al. 2013) ensure biofilm stability, microbial viability or activity of microbial populations (van der Mei et al. 2008, Lackner et al. 2009, Jurecska et al. 2013 and Hassard et al. 2014). The

aim of these studies were to improve performance through harnessing different media/bacterial interactions. Research has suggested that the media physical composition and architecture affects the reactor microbial activity and the removal rate of BOD_5 and ammonia (Tawfik and Klapwijk 2010). Traditional RBC media are ineffective under high OLR as biofilms excessively grow on the media which can inhibit MT to the biofilm (Kim et al. 2010), leading to inactivation of some consortia (Matsumoto et al. 2012) or mechanical failure of media, shaft or bearings (Mba et al. 1999). Flexible fibres with a heterogeneous architecture propagate differential shear and boundary layer conditions and could maintain appropriate thickness to prevent clogging, whilst tortuosity provides heterogeneous microniches in hydrodynamics, substrate and electron acceptor conditions. This could result in different microbial communities and removal regimes in biofilms compared to suspended growth alternatives (Singer et al. 2010). In addition, media should have chemical stability in wastewater, be resistant to microbial degradation, extremes of temperature, pH, salinity and ultra-violet radiation whilst having appropriate tensile strength, reliability and life cycle costs (Jurecska et al. 2013). Hassard et al. (2014) tested a novel PVC mesh media in isolation against high porosity reticulated foam. This mesh media offered elevated removal of substrates and microbial resistance to high OLR treatments, despite similar porosities, which demonstrated the role of media architecture to performance. Jechalke et al. (2010) found that coconut fibres were more effective than polypropylene mesh for removal of a variety of micropollutants suggesting that material selection is important. Jurecska et al. (2013) compared PP to polyester fibres and showed PP had better biofilm attachment, performance and microbial activity despite similar macroscale media architecture.

This current research examined the effect of macroscale properties on the performance of mesh media under high OLR and representative operating conditions for the RBR process. To test this, the impact of OLR on substrate removal (sCOD, NH_4-N) was measured on different mesh media under incrementally increasing OLR. To understand the impact of micro/nanoscale differences in media, microbial activity and viability of the microbial population, was determined.

4.3 MATERIALS AND METHODS

4.3.1 Mesh media characterisation

Four different mesh media (Bluewater Bio, UK) were used in the RBR. Each mesh media was analysed for surface physical properties by atomic force microscopy (AFM). Surface roughness values were obtained after method Stephenson et al. (2013).

4.3.2 Pilot studies at varying OLR

Four bench scale RBRs were operated at Cranfield University WWTP and each consisted of a plastic vessel, single rotating shaft and permeable plastic frames for housing the media. The media consisted of two circular mesh plates in each reactor comprised of polyvinyl chloride-like and polypropylene each with low and high surface area (PVC –L, PVC –H, PP –L and PP -H) respectively (Table 8). The total media volume per reactor, surface area, number of disks, wetted volume, submergence and tip speed was set after 3.3.1. The RBRs were operated with real settled wastewater for a minimum of 3 weeks prior to monitoring for biofilm development and pseudo-steady state conditions. The RBR were temperature adjusted to 15 ºC using a 50W thermostatic aquarium micro heater (Superfish, UK). Different OLR runs were applied to each reactor, nominally 16, 35, 60 and 160 g.sCOD.$m^{-2}d^{-1}$ which corresponded to 1.1, 2.2 and 4.4, 8.8 L of settled sewage per hour (Table 8). To achieve stable nitrification, a surface loading of <15 g.BOD_5.$m^{-2}d^{-1}$ is recommended for rotating biofilm processes (Rittmann and McCarty 2001). The equivalent BOD_5 loading rates were ~32, 81, 138 and 368 g.BOD_5.$m^{-2}d^{-1}$ based on an average sCOD:BOD_5 ratios from influent wastewater from the study period (n=25) for low, medium, high and very high OLRs respectively (2.1±0.4). The impact of OLR on trial media for RBRs was assessed at ~1x (low), 2x (medium), 4x (high) and 10x (very high) this value (Table 8). The removal rate of sCOD and NH_4-N are compared along with the production of oxidised nitrogen. The total operating time for the study was 8 months with approximately 2 months at each loading.

Table 8 – Operating conditions of the RBRs.

Load	HRT (min)	sCOD loading rate ($gm^{-2}d^{-1}$)	NH_4-N loading rate ($gm^{-2}d^{-1}$)	Temp. °C
Low	195.8	16±3.2	5.7±0.9	14.7
Medium	100	35±3.7	6.7±1.1	13.6
High	50.4	66±6	20±0.5	15.6
Very High	24.5	160±14.1	30±4.3	17.8

4.3.3 Wastewater analysis and calculations

Influent samples were collected 09:00±1h, with effluent samples collected at 30 mins,1, 2 and 4 h post influent sampling depending on the OLR studied. Wastewater was analysed after 3.3.2. The media nominal surface area ($A_{nominal}$) and surface and volumetric OLR were calculated according to Equations (Equation 5), (Equation 6) and (Equation 7) respectively. The removal efficiency and substrate removal rate were calculated normally. The biomass NH_4-N removal rate was based on a mass balance of dissolved nitrogen species. The removal of TN and TP were not considered here as the RBRs in this configuration were used as a pretreatment.

4.3.4 Biofilm concentration measurements

Biofilm measures were undertaken at the end of each sampling run by sacrificial sampling. The biofilm was removed from the media to ensure complete biofilm removal (Regmi et al. 2011). Three fibres per sample were checked with SYTO-9 (Invitrogen, Glasgow, UK) staining with CLSM to ensure >95% biofilm removal. The removed biofilm was measured for total solids (TS) and VS by standard methods (APHA 2012). For simplicity a homogenous biofilm distribution on the mesh was assumed and the microbial activity/ viability analysis was undertaken by destructively sampling the biofilm from the mesh media.

4.3.5 Microbial viability of biofilm

Biofilm was harvested as above and diluted using BOD_5 water (1.25 x 10^{-4} mg l^{-1} ferric chloride; 0.028 mg l^{-1} calcium chloride; 0.025 mg l^{-1} magnesium sulphate in a buffered aqueous solution), handled by pipetting (Finntip™ Wide Orifice Pipette Tips, Thermofisher, UK) and mechanically disaggregated (Hassard et al. 2014). Microbial viability was measured by staining biofilm samples with LIVE/DEAD® BacLight™ test (Invitrogen, Glasgow, UK). The fluorochromes SYTO 9 and propidium iodide were added with biomass to a 96 well black flat bottom microtitre well (Porvair sciences, UK) and incubated in the dark for 15 minutes with intermittent orbital shaking according to manufacturer's instructions for a plate reader (Infinite M200, Tecan, UK).

4.3.6 Microbial activity of biofilm

Microbial activity as a proxy for bacterial activity was measured to ascertain the biofilm was affected by OLR and media type, after modified method Nocker et al. (2011). The biofilm was harvested, handled, diluted and disaggregated as above. The microbial activity was measured using a water soluble tetrazolium salt (WST-8) (2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H/tetrazolium monosodium) (Genscript, US) and electron mediator menadione (2-methyl-1,4-naphthoquinone) (Acros Organics, Fisher Scientific, UK). Diluted biofilm was added to a reaction mix of 2x Tryptic Soy Broth and WST-8 detection reagent in a ratio of 4:5:1. The change in absorbance was measured after an incubation time and the biofilm was removed by centrifugation at 10,000x g for 1 minute to minimise solids interference. The microbial activity was measured against appropriate controls at an absorbance of 460 nm in a 96 well clear flat bottom plate using a plate reader. The microbial activity (mol dye reduced per time) was calculated based on the molar absorption coefficient of WST-8 ($3.4x10^4$ $M^{-1}cm^{-1}$) taking into account dilutions. Standard curves could not be used in this instance, as a 'reduced' standard form of WST-8 is currently unavailable. The specific microbial activity was measured based reduction of WST-8 dye per gram of biomass (Appendix 8E.2).

4.3.7 Experimental design

A balanced parametric statistical approach was taken where appropriate. The grouped data of loading and media data were normally distributed Kolmogorov-Smirnov (sCOD $p > 0.01$; NH_4-N $p > 0.01$). To test if there was a difference in removal rate of sCOD/ NH_4-N with both OLR and media type separate one-way ANOVA were undertaken. To compare the non-parametric data (microbial activity, membrane integrity and biofilm concentration) separate Mann-Whitney U tests were performed (SPSS, IBM, USA). The activity and membrane integrity testing was undertaken on the same day as performance data was taken.

4.4 RESULTS AND DISCUSSION

4.4.1 Performance of mesh media for sCOD removal

The pH was on average 7.7±0.08, and the BOD:sCOD ratio was 2.1±0.4 suggesting wastewater conditions were similar during low, medium, high and very high OLR experimental runs. The VRR increased from 0.5-2.4 $kgm^{-3}d^{-1}$ from low through to high OLR, before decreasing to 0.9-2 $kgm^{-3}d^{-1}$ at very high OLR (Table 9). The removal efficiency decreased from low to very high OLR (Table 9). This has been reported previously and can be attributed to MT, kinetics and hydrolytic restrictions resulting in competition for resources or space (Hiras et al. 2004, Chen et al. 2006 and Hassard et al. 2014). Di Palma and Verdone et al. (2009) showed that loss in treatment efficacy can be offset by raising rotational speed, which increases oxygen transfer. The maximum removal rate of sCOD ($g.sCOD.m^{-2}d^{-1}$) ranked from greatest to least in the order: PVC-L > PP-H > PP-L > PVC-H (Figure 5 a). The removal rate of sCOD at the low and medium loadings were similar between media. The removal rate decreased by 15, 48 and 58% for PVC-L, PVC-H and PP-H respectively as OLR increased from high to very high. In contrast the PP-L media organic removal rate increased by 31%, although the consistency of this removal decreased (Figure 5 a). At the very high OLR, the higher porosity mesh exhibited double the removal rate of 31 $g.sCOD.m^{-2}d^{-1}$ compared to 16 $g.sCOD.m^{-2}d^{-1}$ for the lower porosity mesh ($p < 0.05$) (Students' t test on pooled removals) as media clogging reduced effective

media/biofilm contact for removal. Deliberate air scouring could allow incrementally higher OLRs, analogous to backwashing in submerged growth biofilm processes (Mendoza Espinosa and Stephenson 1999). Wijeyekoon et al. (2004) found that a wastewater biofilm had greater density and was increasingly resistant to shear under high OLR conditions resulting in clogging and reduced performance (Kim et al. 2010). Porous media clogging occurs under higher biofilm OLR and lower pore flow rate resulting in media bridging (Kim et al. 2010 and Gamri et al. 2014). The low surface area mesh media PVC-L, PP-L (specific surface area of ~150 m^2m^{-3}) were most appropriate for roughing application at the high and very high OLR which is experienced in full scale RBRs (Table 9 and Figure 5 a).

The biofilm mass increased with OLR independent of media type except at the highest OLR (Table 9). The proportion of VS ranged from 80.6 to 82% at the low OLR, which increased to >90% for higher OLRs. The PVC-L had significantly greater biofilm growth compared to other media tested ($p < 0.05$). The biofilm amount depends on media surface properties, packing density, shear conditions, attachment/detachment rates and grazing (Morgenroth and Wilderer 2000). The biofilm did not accumulate/grow in proportion to OLR as expected (Table 9). From low to medium OLR the biofilm mass increased by 78, 22, 39% on average, for PVC-L, PVC-H and PP-H respectively, whilst the PP-L biofilm mass decreased by 60%. Grazing pressure could have reduced biomass concentrations at this OLR (Bryers 2000). Visible aggregations of nematodes at this OLR suggest grazing by higher organisms contributed to the decreased biofilm VS. Low biofilm thickness and waste sludge production along with increased effective biofilm OLR are likely under this scenario (Salvadó et al. 2004; Chen et al. 2006). The impact of higher organisms reduced due to higher biofilm growth rates, and lower oxygen concentrations in the biofilm.

4.4.2 Performance of mesh media for NH_4-N removal

The mesh media had a maximum VRR at between 0.2-0.3 $kg.NH_4\text{-}N.m^{-3}.d^{-1}$ as OLR increased from low to medium OLR for most media tested (Table 9). However the PVC-H media volumetric removal increased from 0.2 to 0.4 $kgm^{-3}d^{-}$

[1] from low to medium OLR respectively (Table 9), suggesting this media was under-loaded for nitrifying biofilm growth. Incremental increases in OLR decreased NH_4-N removal significantly ($p<0.05$) for all media, suggesting nitrification was inhibited, which was confirmed by lack of NO_2-N and NO_3-N in the effluent (Table 9). The media removal efficiency decreased by 76.2, 59.1, 2.4 and 3.6% for low through to very high OLRs respectively (Figure 5 b): this has been observed previously in suspended growth and biofilm reactor systems (Hiras et al. 2004). The suppression of nitrifying bacterial growth by competition from heterotrophs and inhibition through inefficient oxygen transfer is widely accepted as the dominant mechanism in biofilm reactors (Wijeyekoon et al. 2004; Di Palma and Verdone et al. 2009).

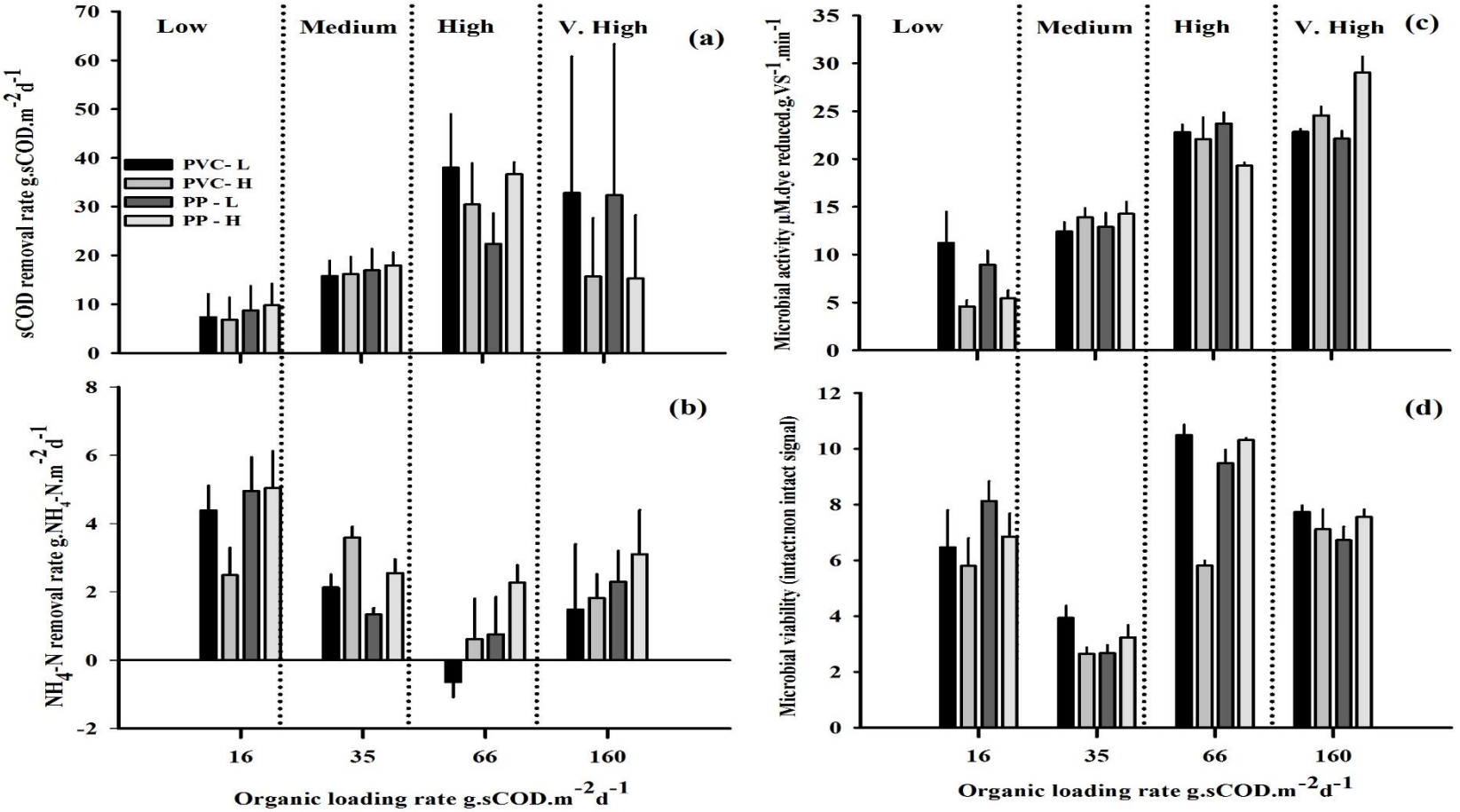


Figure 5 - Performance of mesh media for bulk organics and nitrification and biomass viability and activity measures (a) sCOD removal rate (b) NH_4-N removal rate (c) Microbial activity (moles dye reduced per minute) (d) Microbial viability (ratio intact:non intact signal) of biofilm from each media at different OLR. Error bars indicate ±1 SD from mean.

Table 9 - Wastewater characteristics, removal rates and biofilm development of the RBR media under incrementally increasing OLR. Values represent ±1 SD from mean.

OLR→	Low				Medium				High				Very High			
Parameter (mgL^{-1})↓	**PVC-L**	**PVC-H**	**PP-L**	**PP-H**	**PVC-L**	**PVC-H**	**PP-L**	**PP-H**	**PVC-L**	**PVC-H**	**PP-L**	**PP-H**	**PVC-L**	**PVC-H**	**PP-L**	**PP-H**
$tCOD_i$	564±134				403±70				394±42				485±104			
$tCOD_e$	306±53	297±76	378±122	381±193	263±39	306±47	300±20	285±38	180±26	173±33	258±44	155±70	335±55	359±68	279±46	325±50
$sCOD_i$	135±26.5				144.7±15.4				139±12.6				166±14.7			
$sCOD_e$	63.4±8	67.5±9	51.9±11	46.1±11	84.5±15	82.2±17	79.8±13	76.8±12	54.3±10	76.3±4	94.3±23	68.3±16	106.5±10	135.7±9	109.5±9	135.7±13
	53%	50%	58%	66%	42%	43%	45%	47%	61%	45%	32%	51%	36%	18%	34%	18%
TN_i	47.4±7				49.7±7				53.3±3				44.6±5			
TN_e	47.4±4	45.7±9	46.5±5	47±5	46.7±7	40.0±1	47±8	47±16	52.3±1	50±2	51±17	47±9	43.3±4	42±4	43±4	40±3
$NH_4\text{-}N_i$	43.1±7.1				37.7±4.8				41.8±1.0				31.3±4.5			
$NH_4\text{-}N_e$	4.7±3	26.5±8	6.0±3	5.2±2	15.4±2	4.2±3	23.3±4	13.0±4	43.6±1	41.7±3	40.4±3	37.5±2	29.8±6	31.6±5	31.6±5	32.5±5
	89%	39%	86%	87%	59%	88%	38%	685%	-4%	0.2%	3.3%	10%	4%	-0.9%	-1%	-4%
$NO_2\text{-}N_e$	3.4±0.2	2.8±1.4	2.6±0.8	3.9±1.9	2.9±1	2.9±0.8	2±1.2	3.0±0.4	1±0.2	1±0.3	1.2±0.2	1.6±0.5	1.3±0.2	1.8±0.2	1.9±0.3	1.5±0.2
$NO_3\text{-}N_e$	24.4±4	10.3±3	30.4±5	27.7±4	18.5±2	30.9±9	11.8±4	23.4±2	2.2±1	2.4±0.5	2.8±1	5±0.5	1.8±0.2	1.3±0.2	1.4±0.2	1.9±0.2

sCOD rem. $kg.m^{-3}.d^{-1}$	0.5 ±0. 2	0.5±0.2	0.6±0.2	0.6±0. 2	0.9±0 .3	0.9±0. 3	0.9±0. 3	1.0±0. 3	2.4±0. 5	1.8±0. 4	1.3±0. 7	2±0.3	2±0.6	0.9±0.3	1.9±0.7	0.9±0.3
NH4-N rem. $kg.m^{-3}.d^{-1}$	0.3±.0 6	0.2±.05	0.3±.06	0.3±.0 7	0.3±. 04	0.4±.0 4	0.2±.0 2	0.3±.0 5	- .05±.0 3	0±.06	.04±.0 6	0.1±.0 3	0.1±.09	0.02±.0 9	0.05±0.1 2	0.05±0. 12
Biofilm VS (gm^{-2})	36.9	30	65.8	40	157	38.4	26.6	65.8	569.7	290.8	74.7	308.4	1499.2	501.2	325.9	629.6
Biofilm TS (gm^{-2})	44.9	36.9	80.9	49.6	168.4	48.5	28.4	71.2	573.8	299.9	75.8	262.1	1514.8	528.4	351.9	661.9

The NH_4-N removal rate decreased from 4.3, 4.9, 5.0 $gm^{-2}d^{-1}$ by ~50% for PVC-H, PP-L and PP-H from low to medium OLRs respectively (Figure 5 b). Further inhibition of NH_4-N removal resulted minimal (<2 $g.NH_4\text{-}N.m^{-2}d^{-1}$) for PVC-H, PP-L and PP-H respectively at high OLRs (Figure 5 b). Media architecture could result in different OTR and MCRT and therefore different nitrification rates (Bryers 2000 and Singer et al. 2010). Slight increase in NH_4-N removal rate was found at very high OLRs possibly through heterotrophic nitrogen demand. The COD/NH_4-N ratios used this study were 2.8, 5.2, 3.3 and 5.3 for low, medium, high and very high OLR respectively due to natural variability in settled wastewater. Okabe et al. (1996) found that an increased C:N ratio from 0 to 1.5 led to stratification between functional groups in an RBC biofilm, whereby heterotrophs outcompeted the nitrifiers in the outer layers due to elevated growth rates. High NH_4-N concentrations allow high nitrifying growth and removal rates (Gujer and Boller 1990). Therefore nitrifiers within the biofilm in this study could be substrate starved at the lower OLR particularly for PVC-H (Table 9), where decay processes will dominate over growth (Gujer et al. 2010). Wijeyekoon et al. (2004) demonstrated that biofilms grown under high OLR conditions produced more

Table 10 – Properties of media used in RBR.

Mesh media name	PVC-L	PVC-H	PP-L	PP-H
Base material	PVC	PVC	PP	PP
Filament diameter[a] (µm)	544±84	300±46	578±36	394±22
Filament linear mass density[b] ($g.10^4m^{-1}$)	4400	2222	1100	504
Porosity[c] (%)	96	93	96	95
Surface area per unit weight[b] (m^2g^{-1})	0.26	0.41	0.84	0.98
Specific surface area of mesh[a] (m^2m^{-3})	~150	~360	~160	~277
Average roughness (R_a surface)[a] (nm)	8.72±4.2	11.75±3.08	26.75±6.8	23.2±4.2

a. Experimental results, b. Manufacturer data, c. Derived results

compact consortia with reduced microbial activity. Nogueira et al. (2002) demonstrated that a nitrifying reactor with a long HRT was more affected by shock COD loads than a similar reactor with a shorter HRT but comparable OLRs. This could be as organics are rapidly utilised by existing heterotrophs with greater microbial activity 'shielding' the nitrifiers. In contrast to the long HRT reactor where heterotrophic bacteria coated existing nitrifying biofilm reducing oxygen availability. This could explain why short HRT, mixed communities tend to be more resilient to process upsets.

4.4.3 Mesh media characterisation and biofilm growth

Biofilm reactor performance is thought to benefit from a more predictable biofilm growth (Oliveira et al. 2003). Media properties such as surface energy, charge density and wettability can influence biofilm adhesion and performance (Lackner et al. 2009; Khan et al. 2013). The media tested in this study varied in nanoscale topology and microscale physical structure including linear mass density, porosity and surface area (Stephenson et al. 2013). The media filament linear mass density was 4400, 2222, 1100, 504 g x 10^4 m^{-1} for PVC-L, PVC-H, PP-L and PP-H respectively (Table 10). The porosities were equivalent between PVC-L and PP-L and PVC-H and PVC-H respectively. The media dry weight is important when considering scale-up and the energetic costs of rotation (Mba et al. 1999). Microscale roughness and porosity can be important protecting biofilm from the effects of shear (Oliveira et al. 2003). The average roughness of the media was 8.7, 11.7, 23.2 and 26.7 nm for PVC-L, PVC-H, PP-L and PP-H respectively (Table 10) indicating that PP had the rougher surface.

The biofilm accumulation ranked from greatest to least in the order: PVC-L>PVC-H and PP-H >PP-L (Table 9). This could be due to higher surface energy values of ~45 compared to ~35 $mN.m^{-1}$ for PVC and PP respectively (DataPhysics Instruments, 2007 http://www.surface-tension.de/solid-surface-energy.htm). Gottenbos et al. (2001) found that bacteria adhered more rapidly to positively charged surfaces but strong electrostatic interaction impeded bacterial growth. Furthermore this interaction decreased the bacterial adenosine triphosphate content and proton motive force upon adhesion (Hong et al. 2009) justifying the

decreased cell viability identified by van der Mei et al. (2008). Conversely, negatively charged media could promulgate the opposite favouring proliferation of both Gram negative and positive strains. Hadjiev et al. (2007) found biofilm attachment is greatest at the maximum surface energy difference between biofilm and media. Biomass attachment, nitrification and estrogenic hormone removal rates were correlated with increased surface energy and more hydrophilic surfaces on polymer sheets immersed in a WWTP (Khan et al. 2013). The importance of the initial conditioning film on subsequent biofilm development was not clear. Busscher et al. (1995) suggested that biofilm failure is linked to the strength of the media/conditioning film, which if weak could result in sloughing, loss of treatment and poor effluent. Recent evidence suggested increased media roughness improved biofilm accumulation (Stephenson et al. 2013). In contrast Flint et al. (2000) suggested that this was contingent on the pore size being greater than the size of an average bacterial cell. Roughness could determine the properties of the initial conditioning film, in turn potentially impacting on adhering microbes. Singh et al. (2011) identified a threshold of ~20 nm where superior protein adsorption substantially decreased attachment rates and biofilm formation by clogging nanoscale pores on the material surface. Jucker and Clark (1994) suggested that decreasing zeta potential and increasing hydrophilicity could reduce adsorption. The PVC mesh media tested here had greater biofilm accumulation compared to PP. The biofilm mass was lower on the rougher materials (Table 9**;** Table 10), in synthesis rougher media could be utilised in reactors whereby thinner biofilms are preferred such as combined nitrification processes.

4.4.4 Effect of OLR on microbial viability and activity of biofilms

Research on microbial viability and activity could help in the understanding of the effects of operating conditions on performance (Oliveira et al. 2003). The OLR and media type can influence viability of bacteria, hence biofilm growth and decay processes (Okabe et al. 1996; van der Mei et al. 2008). In this study the microbial viability ratio ranged from 2.6 to 10.5 at medium and high OLR respectively. Interestingly the highest removal rates of bulk organics coincided with the

greatest microbial viability under high OLR. This confirmed the importance of viable microbial abundance for high performance in biofilm reactors. van der Mei et al. (2008) identified that very positively charged media decreased the viability of pure culture bacteria upon initial adhesion, whilst Lackner et al. (2009) showed that biofilm formation of a nitrifying community did not lead to a net reduction in performance and therefore viability. However surface properties may not modify the microbial viability post initial adhesion (Busscher et al. 1995). The presence of a conditioning film that is the primary layer that adheres, could mask the impact of surface properties by acting as a barrier to chemical and spatial heterogeneity. Alternatively the film could provide a link between the media surface and bacteria (Singh et al. 2011). In this study the microbial viability decreased from low to medium OLR by 39, 54, 67 and 52% for PVC-H, PVC-L, PP-H, PP-L respectively (Figure 5 d) which could be due to higher organism grazing resulting in cellular fragmentation. A shift from a nitrifying, carbon limited biofilm to a heterotrophic oxygen limited biofilm one could result in temporary suppressed viability as conditions became less conducive for the previous community (Figure 5 b, d). Further study could elucidate whether microscale deficiency in substrate or electron acceptor could account for greater microbial decay in wastewater biofilms (Okabe et al. 1996), which can precedes loss of treatment under very high OLRs.

The microbial activity of the biofilm increased with OLR. At low OLR the low surface area media (PVC-L and PP-L) had 2.4x and 1.9x more microbial activity than PVC-H and PP-H respectively; less surface area for initial colonisation probably resulting in locally denser communities (Jurecska et al. 2013). However as OLR increased from low to medium, this effect was muted as the microbial activity increased by 41% on average between media, although the increase was greatest for high surface area media. This suggested that the microbial activity in this study was dependent on OLR, not media properties. As OLR increased from medium to high OLR, the microbial activity increased from a minimum of 12.4 to a maximum of 23.7 μM.dye reduced.g.$VS^{-1}min^{-1}$ for PVC-H and PP-H respectively. However further OLR increases did not result in greater microbial activity for most media. In contrast the microbial activity of the PP-H media biofilm

increased by 34% (Figure 5 c); however, this was not mirrored by better performance (Figure 5 a, b; Table 9). Okabe et al. (1996) showed that after long term operation 30% of the cells in the biofilm become physiologically inert, probably due to inactivation, decay or competition effects. In contrast our study showed that more active microbial communities were selected for at higher OLRs, which rapidly utilise available soluble substrates. However, this maximum activity remained unrealised due to MT limitations, which has been documented in other biofilm reactors, previously (Truu et al. 2009). The key observation is that under high OLR differences between media were masked. This could be confirmed through community analyses to confirm similar populations were present. Differences in community could still yield differing responses to process upsets such as shock loadings due to lower community resilience (Harris et al. 2012). This study demonstrated high volumetric performance of mesh media under representative conditions of a WWTP. It would be interesting to study the impact of a solids recycle on the effective OLR, and how this influences the microbial viability and activity of biofilms for WWT.

4.5 CONCLUSION

This study tested the difference between PVC and PP mesh media and looked at the impact of physical architecture for their ability to remove soluble macropollutants (COD and NH_4-N) and accumulate biofilm under incrementally increasing OLR under representative conditions for RBRs.

- Biofilm accumulation increased with OLR for all media studied. The PVC-L media demonstrated enhanced ability to support biofilm compared to other media studied. Particularly low biofilm accumulation was found for PP-L.
- The reactor performance for sCOD removal was not significantly different between media under most OLRs.
- Under very high OLR conditions low mesh media specific surface area is required to maintain performance.
- The microbial viability was similar between media suggesting the long term effect of media properties is limited under operation with real wastewater.

- The microbial activity was similar between media, however the biofilm became more active with OLR suggesting selection for faster growing microbial community.
- Mesh media with a specific surface area of ~150 m^2m^{-3} performed the best for roughing application at high and very high OLR for RBRs.

CHAPTER 5

MICROBIAL EXTRACELLULAR ENZYME ACTIVITY IN A FULL-SCALE MODIFIED ACTIVATED SLUDGE PROCESS.

TO BE SUBMITTED: *Water Research.*

5 MICROBIAL EXTRACELLULAR ENZYME ACTIVITY IN A FULL SCALE MODIFIED ACTIVATED SLUDGE PROCESS.

5.1 ABSTRACT

Hybrid biofilm reactors are suitable to upgrade existing sewage treatment works as a small footprint, pre-treatment stage. A modified activated sludge process known as Hybrid Activated Sludge (HYBACS) was used to upgrade an overloaded WWTP. Full scale RBRs were added upstream of a suspended growth aeration stage and settled sludge was recycled to the head of the process. The treatment capacity of two aeration lanes was upgraded from 40,000 m^3 to 100,000 m^3 (140,000 PE to 500,000 PE). The modified activated sludge had higher volumetric removal of 2.27±0.52 $kg.COD.m^{-3}d^{-1}$ and 0.096±0.02 $kg.NH_4\text{-}N.m^{-3}d^{-1}$ compared to 1.08±0.27 $kg.COD.m^{-3}d^{-1}$ and 0.057±0.015 $kg.NH_4\text{-}N.m^{-3}d^{-1}$ in the conventional activated sludge (CAS) ($p<0.05$). Maximum microbial extracellular enzyme activity (V_{max}) was similar between sites, but modified activated sludge had greater rate of extracellular enzyme activity at low substrate concentrations ($p<0.05$). The RBRs had ~3, 10 and 15 x the V_{max} of carbohydrate, phosphate and amino-acid cleavage respectively compared to the most active suspended growth biomass studied. Pre-hydrolysis by RBR could facilitate greater removal rates in the activated sludge reactor. Controlled experimentation with bench-scale RBR revealed extracellular enzyme activity (EEA) increased with OLR to a maximum of 100 $g.sCOD.m^{-2}d^{-1}$ (227 $g.BOD_5.m^{-2}d^{-1}$). Further increases in OLR significantly reduced the specific EEA suggesting wastewater biofilms regulated EEA under different operating/physico-chemical conditions. Anaerobic conditions developed with a redox potential of -245 to -335 mV at OLR of 100-400 $g.sCOD.m^{-2}d^{-1}$ respectively suggesting that electron acceptor conditions limit EEA at high OLR. The modified activated sludge aeration lane had better affinities and EEA at lower substrate concentrations reducing the rate limiting step of WWT. Elevated EEA could contribute to better performance in RBR modified activated sludge compared to CAS.

5.2 INTRODUCTION

An aging asset base, more stringent discharge consents and a shift to whole life costs are challenges for the water industry (Ainger et al. 2009). To achieve effluent standards within financial constraints with low energy usage, it is imperative to innovate through new technology and optimize process operation (STOWA 2010). Numerous technologies exist for upgrading existing WWTP e.g., membrane bio-reactors (Judd, 2010) which treat high organic loads to appropriate effluent standards but have much increased power consumption (Fenu et al. 2010). Integrated fixed film activated sludge and 'hybrid' moving bed biofilm reactors have been successfully utilised to upgrade existing works (Mannina and Viviani 2009); however, they have limited ability to control biofilm growth and have high CAPEX costs. A modified activated sludge process known as HYBACS uses an upstream RBR for high OLR WWT (Hassard et al. 2014). These have a rotating semi-submerged open architecture media comprised of a high porosity mesh, combined with a daily air scour for biofilm control, which enables operation at high OLR (Chen et al. 2006; Hassard et al. 2015). This minimises the cost of treatment through reduced aeration requirements and maximises existing asset value (Hoyland et al. 2010). High OLR conditions in the RBRs present challenges for diffusion and biodegradation of high M_w compounds which limits the rate of WWT.

Extracellular enzymatic hydrolysis is required as a first step in the treatment of organic wastewater polymers (Cadoret et al. 2002). Domestic wastewater is a complex matrix of carbohydrates, proteins and lipids, ~50% of which has a molecular weight >1kDa, limiting MT in bacterial cells (Burgess and Pletschke 2008). The majority of EEA is associated with bacterial cell walls, floc, granule or biofilm. High enzyme stability has been noted previously, however maintaining EEA in the biofilm is ultimately reliant on continued enzyme synthesis as enzyme half-life is shorter than SRT (Confer and Logan 1998, Goel et al. 1999 and Morgenroth et al 2002). Polymers are utilised by bacteria through: first, transport/adsorption, second, stepwise depolymerisation and third, assimilation/storage; together this limits achievable removal rate in WWT and therefore OLRs that can be applied (Orhon and Çokgör, 1997, Martins et al. 2003 and de Kreuk et al. 2010). Conventional activated sludge bacteria regulate enzymatic activity and affinity based on available substrates (Li and Chróst 2006), electron acceptor conditions (Hauduc et al. 2013) and microbial growth rate (Shackle et al.

2000). Teuber and Brodisch (1977) identified a response time of <2 h for activated sludge bacteria to a source of polymeric substrate, suggesting the adaptive response is faster than changes to microbial population (Wingender and Jaeger 2002). This suggests that manipulation of bacterial growth and extracellular enzyme activities can be used to maximise the efficacy of WWT (Shackle et al. 2000). However understanding the impact of operating conditions on functional EEAs requires further attention in engineered biological systems (Curtis et al. 2003; Truu et al. 2009). In modified activated sludge, the return activated sludge (RAS) enters the head of the process, which is thought to improve treatment efficacy through enhanced bacterial solids contact and EEAs (Daigger and Boltz 2011). It is hypothesised that RBR and modified activated sludge bacteria have elevated EEA which could contribute to better performance observed previously (Hassard et al. 2014, Biddle et al. 2014 and Hoyland et al. 2010). The current study assessed the role of microbial EEA in an activated sludge process and the role of an RBR.

5.3 MATERIALS AND METHODS

5.3.1 Full scale study site

Tubli Wastewater Pollution Control Centre (WPCC) serves the city of Manama, Bahrain with a design capacity of 200,000 m^3d^{-1} and 41,000 $kg.BOD_5.d^{-1}$, representing ~700,000 PE (Table 11). The process comprised preliminary treatment (screening and grit removal), 10 aeration lanes and 12 clarifiers. Each aeration lane and clarifier had a design capacity of 20,000 m^3d^{-1} and 17,000 m^3d^{-1} respectively. The population of Bahrain has grown by 29.7% from 2005 to 2010, the average wastewater flow received was 303,000 m^3 and the BOD_5 load recorded was 53,200 kgd^{-1}, representing ~890,000 PE (Table 11). Some of the secondary effluent is ozonated and filtered for use in irrigation; the remainder is discharged into Tubli Bay. The effluent standard for discharge to sea is currently 15 mgL^{-1} BOD_5, 20 mgL^{-1} suspended solids and 3 mgL^{-1} NH_4-N, though new limits of 10 mgL^{-1} TN and 1 mgL^{-1} NH_4-N are due to be applied in the future

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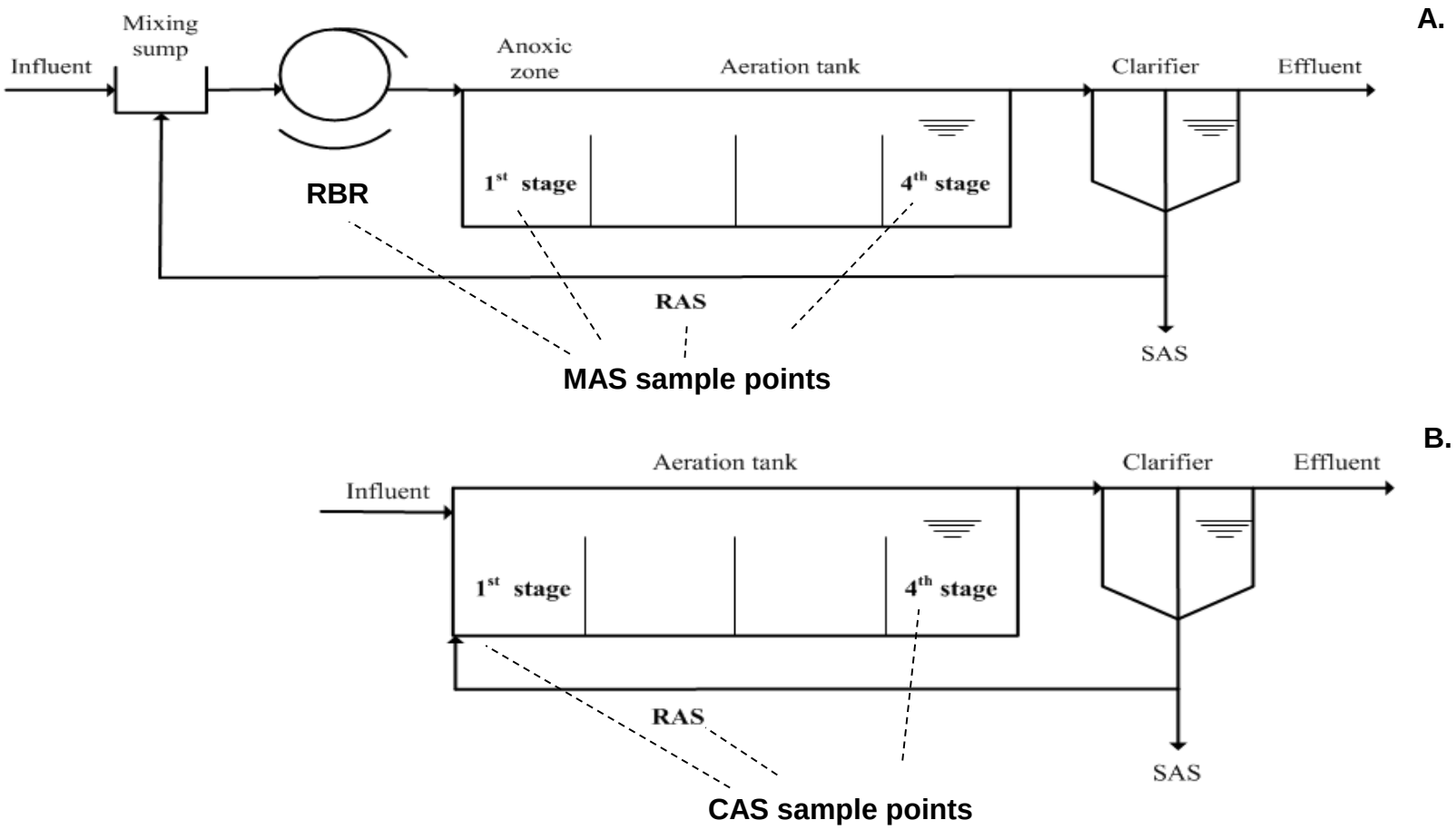


Figure 6 - Sampling points for enzyme study a. modified activated sludge (MAS) b. CAS at full scale plants.

Table 11 - Design flows and loadings for CAS and modified activated sludge full-scale plants after upgrade.

Parameter	CAS site (lanes 1-8)	Modified activated sludge (lanes 9-10)*
PE before upgrade	~890,000	-
PE after upgrade	~700,000	~540,000
Flow ($m^{-3}d^{-1}$)	250,928	100,505
COD load (kg d^{-1})	123,580	51,492
SS load (kg d^{-1})	76,882	32,034
NH_4-N load (kg d^{-1})	6038	2516
TP load (kg d^{-1})	528	220

* Modified activated sludge lanes have 20% greater volume per lane than CAS site.

The modified activated sludge upgrade involved installation of 42 RBRs upstream of two existing aeration lanes, conversion of the first aeration zones to a pre-anoxic zone and upgrade of surface aerators to a fine bubble diffused air system. A new clarifier distribution chamber and RAS pumping station separated the four modified activated sludge clarifiers from the eight conventional clarifiers. This study comprised the first 12 months of operation from commissioning of the first and second modified activated sludge upgraded lanes (10 and 9) which started in June and October 2013 respectively.

The RBRs were located downstream of the mixing point of wastewater with RAS, increasing bacterial/wastewater contact. Each RBR incorporated motor driven (2.2 kW, Sumitomo Buddybox, Japan) porous PVC-like mesh plates (Bluewater Bio, UK) for biofilm growth (n = 30, d = 2 m, thickness = 0.05 m, pitch spacing = 0.095 m, porosity = 95%, submergence = 40%, wetted reactor volume = 9 m^3). The RBR rotational speed was controlled at 2.0-5.8 rpm based on DO concentration and adjusted to a set point by a proportional integral derivative controller. The DO was measured by a DO probe (Hach, LDO model 1, Germany). The RBRs were operated in banks of 14 reactors operated with three reactors in series (3 x 14 =42). These RBRs had a disc radius 10x larger than bench RBRs but similar media composition. The surface area of a full scale RBR was 732 x larger than at bench scale.

The wastewater flowed from the RBRs to the two activated sludge lanes, each separated by baffles into four zones, the first of which was anoxic (Figure 6). The existing aeration lanes were 112.6 m x 22.5 m x 4.5 m (length x width x depth). The volume of the activated sludge lanes was ~25,000 m^3 for the modified activated sludge plant, of which 5,000 m^3 was anoxic. The original surface aerators (10 x 80 kW rated power) were replaced by a fine bubble diffused aeration system (EDI, Flexair 9" membrane diffusers, USA) and three turbo blowers operating as duty/ assist/ standby (471 kW) (Siemens, KA22SV, Denmark). The DO concentration was independently controlled in each zone to a setpoint of 1.5 mgL^{-1} in the first aerated zone, and 2 mgL^{-1} in subsequent zones. An internal recycle pump in the final zone of each aeration tank returned approximately 50% of the incoming wastewater flow to the anoxic zone for denitrification.

The wastewater was passed to the final clarifiers and the sludge was either recycled or wasted (Figure 6). For the period of study the RAS recycle ratio was 0.75. The target operating MLSS was 3,600 mgL^{-1} resulting in a clarifiers solids loading rate (SLR) of 8.1 $kgm^{-2}h^{-1}$ at the peak flow of 150,000 m^3 and a clarifier upflow velocity of 1.38 mh^{-1} (excluding RAS flow).

5.3.2 Wastewater analysis

Influent samples were collected at 10:00±1h. Wastewater was analysed using proprietary cell test kits (Hach-Lange, Germany) including nitrite nitrogen (NO_2-N) and (NO_3-N) using a Hach DR 2800 spectrophotometer (Hach-Lange, Germany). Other standard wastewater constituents such as COD and NH_4-N were measured after 3.3.2. Biochemical oxygen demand, mixed liquor suspended solids (MLSS), VSS, TS were measured according to standard methods (APHA 2012). Dissolved oxygen concentration was measured by DO probe (LDO model 1, Hach-Lange, Germany). The redox potential was measured by redox probe (HI-98201, Hanna Instruments, US). The removal efficiency, substrate removal rate, substrate utilisation rates (SUR), food to microorganism ratio (F:M) were calculated based on standards methods for suspended growth and biofilm reactors (APHA 2012).

The SRT which is similar to MCRT mentioned previously, sludge volume index (SVI), hindered settling velocity and upflow velocity were calculated normally (Qasim 1999).

The biofilm TS concentration was measured after the method in Regmi et al. (2011). The clarifier SLR was calculated after (Equation 8)

Equation 8 – Solids loading rate

$$SLR = (Q \times X)/ SA$$

Where Q = wastewater flow, X = total suspended solids concentration, SA = surface area

The volumetric power consumption of modified and conventional activated sludge was calculated after (Equation 9)

Equation 9 – Volumetric power consumption

$$V_{We} = W_e / Q$$

Where W_e = total daily power consumption (including RBR and aeration costs)

The removal rate per power consumption (R_p) was calculated after (Equation 10):

Equation 10 – Performance and power consumption

$$R_p = R/W_e$$

Where R = daily COD removal rate

Flocs were characterised under light microscopy (100X magnification) and subjectively quantified compared to a reference filamentous scale after Madoni et al. (2000).

5.3.3 Sample recovery and pre-treatment

The biofilm was harvested from mesh media (Hassard et al. 2014) and MLSS from each sampling point for modified activated sludge and CAS respectively (Figure 6 and Appendix 8B.3.1). At Bahrain WPCC the modified activated sludge EEA was measured in the influent, biofilm, biofilm reactor effluent, MLSS, RAS and plant effluent. This was compared to the influent, MLSS and RAS from the CAS site operated concurrently (Figure 6). All bench scale assays were undertaken using RBR reactors (identical in design to Chapters 3 and 4) operated with settled sewage from

Cranfield University WWTP (full experimental description in Chapter 3.3.1) at incrementally increasing OLRs from 12 g.sCOD.$m^{-2}d^{-1}$ to 400 g.sCOD.$m^{-2}d^{-1}$ (~27 g.BOD_5.$m^{-2}d^{-1}$ to 910 g.BOD_5.$m^{-2}d^{-1}$) with three separate experimental repeats all performed in triplicate. Samples were subjected to identical pre-treatment. To batch samples buffer (Li and Chróst, 2006) and methanol was added (10% v:v) (Lunau et al. 2005). The biomass was diluted and disrupted and handled by pipetting after method in 4.3.5.

5.3.4 Extracellular enzyme activity assays

Pre-treated biomass was incubated at ~25°C in the dark and mixed at 200 rpm (Minishaker, VWR, UK) with appropriate buffer (Fisher Scientific, UK) (for details of buffers see Li and Chróst, 2006) for each synthetic substrate (Sigma Aldrich, UK) to obtain the chromophore product of the extracellular enzyme reactions. Three EEA assays were for amino-peptidases, glucosidases and phosphatases henceforth identified as proxies protein hydrolysis, carbohydrate degradation and organic phosphate hydrolysis. This demonstrates potential for EEA not EEA per se as were assays were undertaken on artificial substrates only. Previous work by Logan et al. (1998) measured the EEA before and after filtration through a 0.22 µm filter, which enables distinction from membrane bound to 'extracellular' EEA. Minimal EEA has been noted in filtrate fraction in wastewater bacteria and therefore, we omitted this distinction. In this study the EEA was measured after catalysis of synthetic substrates p-nitrophenyl-α-d-glucopyranoside (Sigma N1377), l-leucine-p-nitroanilide (Sigma L9125) and p-nitrophenyl-phosphate (Sigma 104-0). These artificial substrates represent a substitute for actually measuring processes and indicates potential. Incubation times were optimised for each enzyme and location. The absorbance of p-nitroaniline (λ_{max}= 380 nm) and p-nitrophenol (λ_{max}= 348 nm) was measured at six substrate concentrations (ranging from 10-250 µM) using a spectrophotometer (Hach-Lange, DR 2800) and a microplate reader (M2000 infinite pro, Tecan, Austria) at full and bench scale respectively. Appropriate controls were: biomass with substrate, no substrate control, no biomass control and deionised water (DI) blanks. Standard curves were used to calculate the concentration of substrate liberated per time. First, the Michaelis-Menten equation (Equation 17) was solved using a non-linear least squares method for kinetic parameter estimation (V_{max}, K_m) after Kemmer and Keller

(2010); then the standard error of mean and significance of model fit were calculated using a Hessian matrix and t-test respectively (R statistical package) (Venables et al. 2011). The specific enzymatic activity was quoted as the maximum rate per gram of bacterial solids calculated after (Equation 11)

Equation 11 – The EEA per unit weight of bacterial solids

Maximum specific EEA = V_{max}/ (VSS or VS)

5.4 RESULTS AND DISCUSSION

5.5 Wastewater characteristics

During this study the modified activated sludge full-scale plant was operated within intended design capacity (Table 11). The influent wastewater had an average tCOD of 482 mgL^{-1}, sCOD of 175 mgL^{-1}, NH_4-N of 24 mgL^{-1} and TSS of 287 mgL^{-1}. Influent conditions were similar during the study period, except December 2013-February 2014 where the tCOD was significantly higher at 639±55 mgL^{-1} compared to normal operation of 396±48 mgL^{-1} ($p<0.05$), attributable primarily to a 40% increase in influent suspended solids concentration (Table 12). The wastewater pH, sCOD and NH_4-N did not differ significantly. The wastewater entering the modified activated sludge and CAS plants were from the same source and therefore the same in composition. The bench scale study was undertaken with settled municipal wastewater from Cranfield University WWTP. The influent wastewater characteristics had an average tCOD of 498 mgL^{-1}, a BOD_5 of 259 mgL^{-1} a NH_4-N of 29.7 mgL^{-1} and a TSS of 245 mgL^{-1} which was statistically similar from that characterised previously (Table 7; Hassard et al. 2014).

5.6 Performance of modified activated sludge compared to CAS

The first modified activated sludge lane was commissioned on 5th June 2013, with a flow of 30,000 m^3d^{-1}; this was increased gradually to ~60,000 m^3d^{-1} and the second lane was commissioned on 25th October 2013, after which the flow rate increased to 98,866 m^3d^{-1} on average (Figure 7 a). The remaining eight aeration lanes at Tubli WPCC continued to operate as a CAS plant, treating approximately 200,000 m^3d^{-1} of wastewater, operating at a F:M ratio of ~0.2 kg.COD.kg.$MLSS^{-1}d^{-1}$. The modified activated sludge had elevated removal rate of bulk organics of 2.27±0.52 kg.tCOD.$m^{-3}d^{-1}$ for modified activated sludge compared to 1.08±0.27 kg.tCOD.$m^{-3}d^{-1}$ for CAS ($p < 0.001$) (Figure 7 b). The SUR for tCOD was 0.35±0.19 compared to 0.57±0.07 kg.COD.kg.MLSS.d^{-1} for CAS and modified activated sludge respectively which resulted in better tCOD effluent quality of 27.9±12.5 mgL^{-1} for modified activated sludge compared to 65.3±48.7 mgL^{-1} for CAS despite 40% greater OLR and 31% lower HRT.

Table 12 - Wastewater characteristics for CAS and modified activated sludge full-scale plants during study period.

Parameter mgL^{-1}	Influent	CAS effluent	Modified activated sludge effluent	Difference significant at 95%
tCOD	482±171	65.3±48.4	27.9±12.5	Yes -
sCOD	175.3±72.7	-	-	-
TN	-	-	4.2±3.3	-
NH_4-N	24±5.4	13.1±2.8	0.06±1.2	Yes
NO_3-N		-	5.5±1.3	-
TP	4.1±1.3	-	2.1±0.6	-
TSS	288±139	-	15.2±10	-

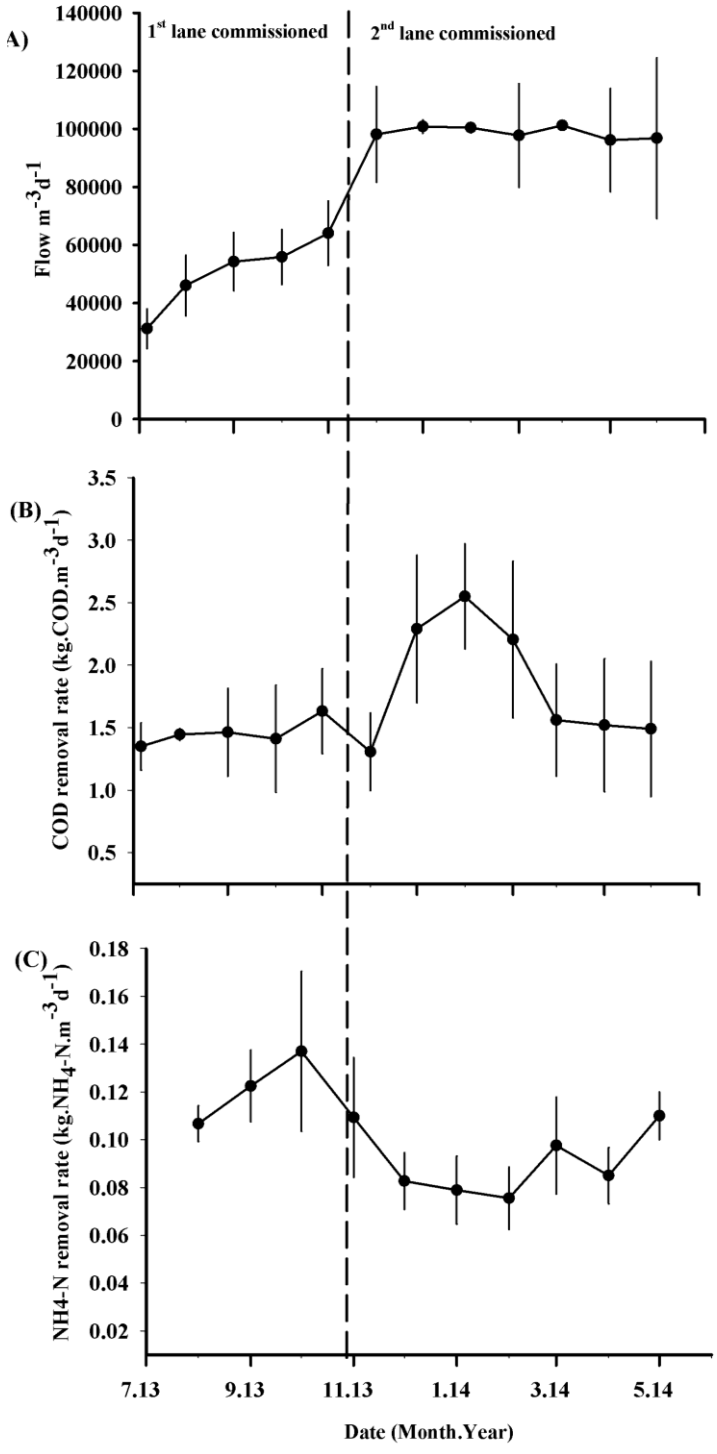


Figure 7 a. - Average monthly wastewater flow to modified activated sludge during commissioning, b. Average monthly COD volumetric removal rate. c. - Average monthly NH_4-N volumetric removal rate, averages comprised of daily wastewater data, error bars ± 1 SD from mean.

Table 13 - Aeration tank characteristics for CAS and modified activated sludge full-scale plants during study period.

Parameter	CAS	Modified activated sludge	Performance significant at 95%
MLSS (mgL^{-1})	3059±535	3254±767	No
SVI (mLg^{-1})	96.9±1.7	43.9±5.5	Yes
F:M ($kg.COD.kg.MLSS^{-1}$)	0.5±0.2	0.8±0.3	No
tCOD removal rate $kg.tCOD.m^{-3}d^{-1}$	1.08±0.2	2.27±0.52	Yes
NH_4-N removal rate ($kg.NH_4\text{-}Nm^{-3}d^{-1}$)	0.057±0.015	0.096±0.02	Yes
COD SUR (kg.COD.kg.MLSS)	0.35±0.19	0.57±0.07	Yes
NH_4-N SUR ($kg.NH_4\text{-}N.kg.MLSS$)	0.022±0.01	0.031±0.009	Yes

The modified activated sludge plant had 42% better NH_4-N VRR of 0.096±0.02 $kg.NH_4\text{-}N.m^{-3}d^{-1}$ compared to 0.057±0.015 $kg.NH_4\text{-}N.m^{-3}d^{-1}$for the CAS site ($p < 0.001$) (Figure 7 c). The CAS had an average nitrification efficiency of 54.7±10.5%, suggesting incomplete nitrification whilst, the modified activated sludge achieved an average of 97.5±4.2% (Table 12) efficiency despite significantly higher F:M (Table 13). The SUR of NH_4-N was 0.031 $kg.NH_4\text{-}N.kg.MLSS$, in the modified activated sludge compared to 0.022 $kg.NH_4\text{-}N.kg.MLSS$ for the CAS suggesting greater nitrification rates (Table 13). Addition of pre-denitrification anoxic zones in the modified activated sludge upgrade reduced the effective volume for nitrification, however improved performance could be attributed to alkalinity addition by denitrification, improved nitrifier abundance or activity (You et al. 2003) or improved MT in the aeration tanks. The effluent NH_4-N was on average 0.6 mgL^{-1} (Table 12) for the duration of the study, irrespective of F:M (Figure 8) suggesting spare nitrification capacity in the modified activated sludge plant. You et al. (2003) found that hybrid processes allow treatment at greater OLRs, nitrification at lower SRT and increased resilience to disruption of

nitrification performance compared to CAS. It was suggested that greater nitrifier abundance could play a determinant role governing performance in hybrid systems (You et al. 2003). Future studies could utilise genetic methods to determine whether nitrifier abundance increases upon upgrade to modified activated sludge targeting *Nitrospira spp*. and AOB- β proteobacteria specific sequences through qPCR. The nitrification activity could be assessed through controlled nitrification activity measures by measuring NO_x production with time.

The modified activated sludge had a better sludge SVI of 43±5.5 mLg^{-1} compared to 96.9±1.7 mLg^{-1} for CAS ($p < 0.05$) (Table 13). The hindered settling velocity of modified activated sludge at the full scale plant was between 6-8 mh^{-1}, which allowed higher clarifier SLR compared to the CAS reactor. This high SLR could select for larger, denser flocs, which could contribute to the formation and stability of modified activated sludge compared to CAS flocs (Lin et al. 2010). Sludge samples from 4th aerated zone

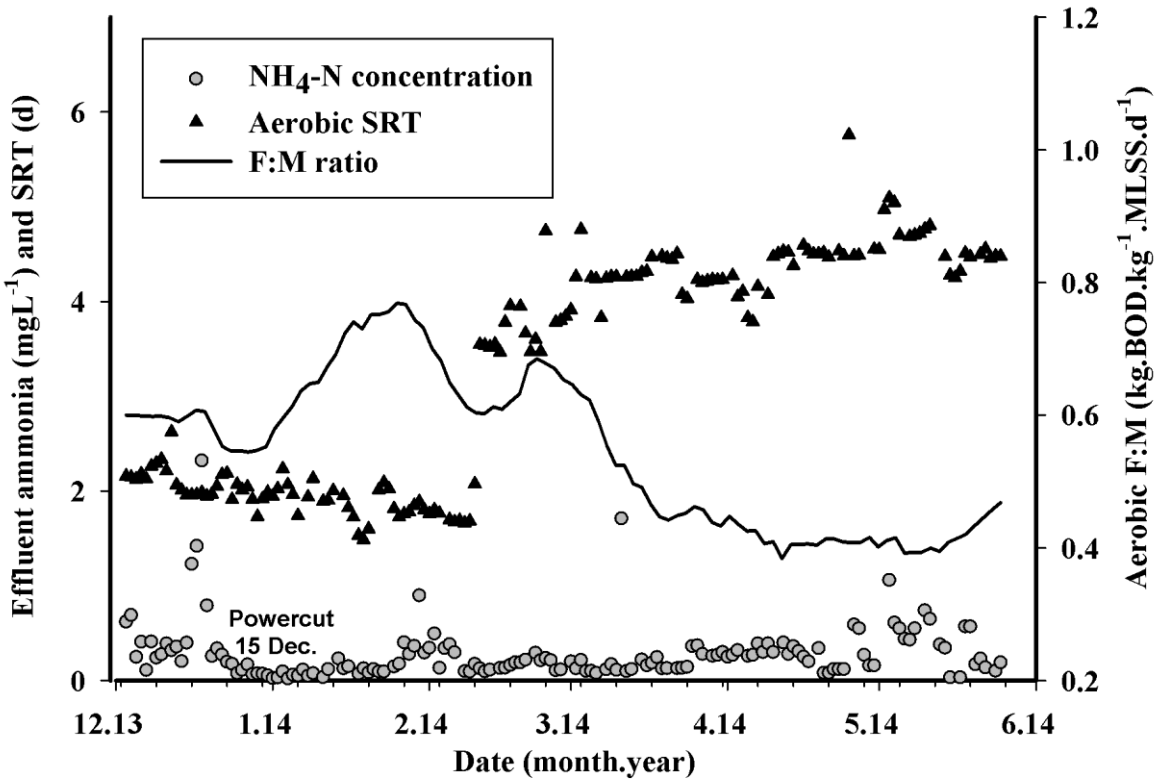


Figure 8 - Modified activated sludge effluent ammonia concentration, SRT and aerobic F:M ratio during study period.

had a filament index of between 0-1 revealed minimal filamentous bacterial groups in the modified activated sludge compared to 3-4 for a well maintained CAS (Jenkins et al. 2003). The EEA pretreatment by RBR could select against filamentous groups by facilitating greater substrate penetration depth and three dimensional floc growth (Liao et al. 2004; de Kreuk et al. 2010). Incorporation of dispersed solids from the biofilm reactor could contribute to floc density, elevated EEA and therefore performance (Costerton et al. 1995). The average total power consumption of the modified activated sludge plant (total plant including blowers and RBR) during the performance test was 0.23 $kWh.m^{-3}$ treated effluent compared to the CAS of 0.38 $kWh.m^{-3}$ treated effluent. The RBR acted as a pretreatment prior to the aeration lanes through biofilm growth and enhanced solids contact compared to the CAS plant (Daigger and Boltz 2011). Increased EEA in the RBR could aid degradation of the polymeric fraction wastewater, improving the degradability and reactions rates downstream.

5.7 Extracellular enzyme activity

Extracellular enzyme activity as measured by V_{max} for all three classes of substrate – proteins, carbohydrates and phosphatases was between 4 and 10 times higher in RBRs than the next greatest value for suspended growth (Table 14). However, the V_{max} was similar between suspended growth sections of the modified activated sludge and comparative sections of the CAS. The K_m was significantly higher for CAS ($p<0.05$), suggesting that modified activated sludge has a greater substrate affinity resulting in elevated EEA at lower substrate concentration. The RBRs had a K_m ranging from between 517-634, 375-243 and 531-553 µM for protein, carbohydrate and phosphate which was less than suspended growth for protein and carbohydrate but higher for phosphate (Table 14). Greater protein demand and EEA liberation has been noted for biofilms previously (Jones and Lock 1989), and could be due to higher cell densities, extra growth requirements or diffusion limitation (Allison and Vitousek 2005; Burns et al. 2013). The EEA in a WWTP may increase because; first, suitable substrates do not repress enzyme systems; second, EEA liberates more low M_w substrate which becomes available; third, microbial population growth and therefore enzyme quantity or activity increases; fourth, the enzymes are shed into the wastewater biofilm/floc matrix and remain active (Shackle et al. 2000). In the wastewater/activated sludge matrix it is likely that the EEA is balanced by natural

selection pressures and providing influent/operating conditions remain near constant, the EEA will also remain roughly stable. The EEA could however increase if the biodegradability of the influent changes or a principal design parameter such as SRT is altered. The modified activated sludge microbiota had increased carbohydrate EEA from 0.7, 4.1 and 4.5 $\mu M.min^{-1}$ for stage 1, stage 4 and RAS respectively suggesting greater requirement for sources of readily biodegradable carbon as treatment progressed. Heterotrophic scavenging in modified activated sludge could contribute to elevated depolymerisation and removal of long chain carbonaceous compounds compared to CAS (Table 14). During nutrient limitation many catabolic enzyme operons are expressed, although EEA is suppressed until suitable organic inducers are present (Konopka et al. 2000), therefore high EEA in RBRs could provide a mechanism for high substrate removal rates in modified activated sludge aeration lanes. These results suggest that enzyme expression in wastewater treatment systems is not uniform. This could be verified by measuring the ratio of abundance and expression of genes linked to EEA through metagenomics and metatranscriptomic analysis of DNA and copy DNA extracted from wastewater treatment systems with particular bias towards genes encoding the enzymes and secretion systems. San Pedro et al. (1994) suggested that the starch hydrolysis rate was independent of biomass concentration and that amylases were in excess in CAS. In this study using a different enzyme target we demonstrated higher carbohydrate EEA between RBR biofilm and modified activated sludge/CAS. High EEA with high K_m was found at numerous sample locations for both modified activated sludge and CAS. This is attributed either to low affinity for the artificial substrate and/or to concomitant high concentrations of natural substrates which competitively interfered with formation of artificial substrate/enzyme complex (Li and Chróst 2006). To elucidate the impact of OLR on EEA, controlled experiments were undertaken using bench scale RBRs. The V_{max} of protein increased from 124 $\mu M \cdot min^{-1}$ to 402 $\mu M.min^{-1}$ in a linear fashion as average OLR increased from 12.5 $g.sCOD.m^{-2}d^{-1}$ to 100 $g.sCOD.m^{-2}d^{-1}$. However at OLRs >100 $g.sCOD.m^{-2}d^{-1}$ the EEA decreased significantly ($p< 0.05$) (Figure 9 a). The phosphate EEA initially increased from 52 $\mu Mmin^{-1}$ to 98 $\mu Mmin^{-1}$ from 12 $g.sCOD.m^{-2}d^{-1}$ to 50 $g.sCOD.m^{-2}d^{-1}$ before decreasing at OLRs >100 $g.sCOD.m^{-2}d^{-1}$. The trend was similar for carbohydrates but with ~15 and 3 x lower EEA than protein and phosphate respectively. The specific EEA also yielded an identical trend, suggesting increased net activity not simply a gross increase microbiota or enzyme abundance

(Figure 9 c). The bench scale RBR data showed that the EEA were the same order of magnitude as the full scale data although the OLRs experienced at full scale were significantly greater. The experimental data did not differ significantly from the Michaelis-Menten model for all V_{max} and K_m treatments.

Table 14 – Extracellular enzyme kinetic characterisation for CAS and modified activated sludge full-scale plants, sampled at different sites in the treatment flow sheet.

		CAS		Modified activated sludge	
Site	**Extracellular Enzyme**	**Vmax (µmol.L^{-1}.min^{-1})**	**Km (µmol.L^{-1})**	**Vmax (µmol.L^{-1}min^{-1})**	**Km (µmol.L^{-1})**
RBR start of train	Protein	-	-	83.6±10.2^{b}	517±278^{d}
	Carbohydrate	-	-	38.2±1.2^{a}	375±48.1^{b}
	Phosphate	-	-	55.5±3.4^{a}	531±103^{b}
RBR end of train	Protein	-	-	84.6±5.5^{a}	634±151^{c}
	Carbohydrate	-	-	47.6±3.6^{a}	243±88.8^{c}
	Phosphate	-	-	54.7±4.8^{a}	553±156^{c}
1st Stage	Protein	12.3±1.5^{b}	1212±435^{c}	13.8±0.6^{a}	744±121^{b}
	Carbohydrate	1±0.05^{a}	990±155^{b}	0.7±0.05^{a}	354±104^{c}
	Phosphate	7.3±0.08^{a}	58.9±5.1^{a}	5.4±0.2^{a}	77±17.6^{c}
4th Stage	Protein	11.9±1.2^{a}	755±271^{c}	15.8±1.1^{a}	1013±231^{c}
	Carbohydrate	0.6±0.1^{b}	331±235^{c}	4.2±0.2^{a}	410±70.9^{b}
	Phosphate	7.6±0.5^{a}	297±68.4^{c}	9.2±0.2^{a}	45.8±6.7^{c}
RAS	Protein	18. 0±1.4^{a}	995±249^{c}	17.6±1.7^{a}	803±267^{c}
	Carbohydrate	2.8±0.1^{a}	263±60.3^{c}	4.3±0.2^{a}	206±56.6^{c}
	Phosphate	8.8±0.05^{a}	22.4±2.12^{a}	8.5±0.05^{a}	22.4±2.12^{a}

Significance of data fit to Michaelis-Menten model a= <0.001, b= <0.01, c= <0.05 d>0.05

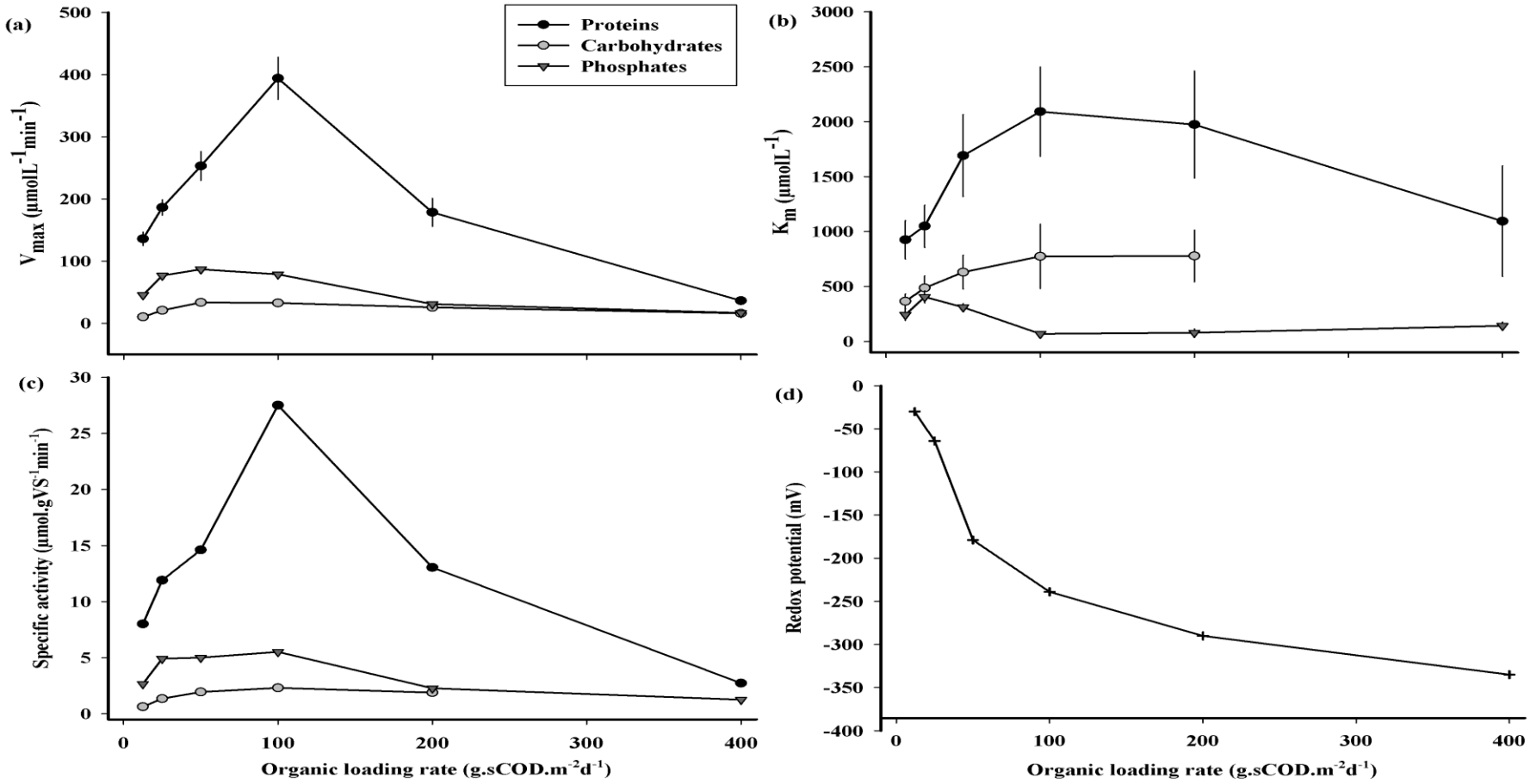


Figure 9 - Extracellular enzyme kinetic characterisation of bench RBR operated at incrementally increasing OLR a. V_{max}, b. K_m, c. specific EEA for protein, carbohydrate and phosphate enzymes respectively, d. redox potential (mV) of biofilm.

Table 15 – pH, DO, Redox potential of CAS and modified activated sludge (MAS)* processes measured during enzyme testing.

Parameter Sample site	Reactor type	pH	DO (mgL^{-1})	Redox potential (mV)
Rotating biofilm reactor	MAS only	7.2	-	-381
1st stage	CAS	7.2	0.46	-46
	MAS	7.2	0.2	-34
4th stage	CAS	6.6	3.2	62
	MAS	6	2.2	96
RAS	CAS	5	0.1	-22
	MAS	6.8	0.2	-12
Final effluent	CAS	7.35	-	7
	MAS	6.8	-	136

*The TLA for MAS is non-standard so is not used in the text.

The biofilm redox potential was -31 mV at 12 $g.sCOD.m^{-2}d^{-1}$ before decreasing to -245 mV at 100 $g.sCOD.m^{-2}d^{-1}$; further increases in OLR decreased redox potential further (Figure 9 d). Heterogeneity within the biofilm could contribute to fine scale variability in redox potential, electron acceptors, substrates and therefore potentially EEA. The impact of fine scale variability could be measured in future using microelectrode probes (De la Rosa and Yu 2005 and Matsumoto et al. 2010). The elevated protein EEA in the RBR suggests the biofilm has a greater intrinsic demand for amino acids compared to phosphate or sugars (Jones and Lock 1989) and that this demand is strongly influenced by OLR and/or prevailing electron acceptor conditions in the biofilm (Figure 9 d) (Hauduc et al. 2013). Goel et al. (1999) suggested that redox environment does not influence the activity, only expression/synthesis of extracellular enzymes. The redox/OLR linked EEA response of the RBR biofilm suggested a role for higher organisms, such as protozoa and metazoa, which decay under extended periods of anaerobiosis, but have a large impact on the EEA of the system (Morgenroth et al. 2002; Hauduc et al. 2013).

The K_m ranked in order protein> carbohydrate> phosphate, therefore the RBR biofilm had greater affinity (lower K_m) for phosphate despite significantly higher V_{max} (Figure 9 d). This trend was most striking between 100 g.sCOD.$m^{-2}d^{-1}$ and 200 g.sCOD.$m^{-2}d^{-1}$, possibly due to demand for phosphate storage under anaerobic conditions (Figure 9 d) (Hauduc et al. 2013). A lower particulate fraction at the full scale plant (due to high temperature and longer sewer HRT) compared to Cranfield WWTP (temperate conditions, very short sewer HRT) could explain the higher bench RBR EEA (Table 14; Figure 9 a) (de Kreuk et al. 2010; Tas et al. 2009). The air scour employed on full scale RBRs could prevent slow growing strains (Allison and Vitousek 2005) or significant higher organism growth (de Kreuk et al. 2010), although the air scour facility provides an opportunity to modify the biofilm growth rate and EEA in order to maximise the efficacy of WWT (Shackle et al. 2000). Further work could elucidate the impact of an air scour on biofilm systems and functional EEA.

The modified activated sludge produced dense activated sludge flocs with few filaments and low SVI of 43 compared to 96 mLg^{-1} for CAS, enabling high SLR to be applied to the clarification stage, facilitating a smaller clarifier area. High clarifier SLR, selects for larger, denser flocs with a SVI approaching that of granular systems (Table 13). The RBRs facilitate a greater EEA compared to the aeration tanks. This could facilitate greater substrate penetration depth, reducing outgrowth rate of filamentous groups (Martins et al. 2003). These features could contribute to better effluent quality compared to CAS systems (Table 12) by accelerating extracellular hydrolysis and solids settlement rate, two factors known to limit biological WWT

This study demonstrated the impact of a modified activated sludge upgrade on performance and microbial EEA of soluble substrates. Enzyme testing shed light on the regulatory effect microorganisms have on EEA in response to operating/physic-chemical conditions which is important for other biological processes and remains poorly characterised in WWT models.

5.8 CONCLUSIONS

- The VRR was 52% and 40% higher for tCOD and NH_4-N respectively for modified activated sludge compared to CAS ($p < 0.001$).
- The RBRs had between 4 and 10x the EEA (measured by V_{max}) compared to the highest suspended growth biomass studied.

- The modified activated sludge microbial enzymes displayed greater substrate affinity compared to a CAS for most sites and enzymes.
- Bench studies revealed distinct regulation of EEA with OLR which could be linked to prevailing redox conditions in the biofilm.

CHAPTER 6

IMPACT OF BACTERIAL SOLIDS ON EXTRACELLULAR ENZYME ACTIVITY AND PERFORMANCE IN HYBRID ROTATING BIOFILM REACTORS.

TO BE SUBMITTED: *Water Research.*

6 IMPACT OF BACTERIAL SOLIDS ON EXTRACELLULAR ENZYME ACTIVITY AND PERFORMANCE IN HYBRID ROTATING BIOFILM REACTORS.

6.1 ABSTRACT

Performance of final effluent, humus solids and RAS as hybrid component (bacterial solids flow) in RBRs was studied under incrementally increasing OLR and SLR. The performance for bulk organics removal, nitrification and denitrification was assessed by hierarchical multiple linear regression analysis for removal performance and microbial extracellular enzyme activity. The organic loading rate and solids type had the greatest positive impact and solids loading rate had a negative impact on sCOD removal rate model (R^2 = 56%). The solids type and recycle redox potential positively correlated and SLR negatively correlated with the NH_4-N removal rate model (R^2 = 36%). The nitrate loading rate and solids type positively correlated and the SLR and reactor DO concentration negatively correlated with the NO_x-N removal rate model (R^2 = 54%). The RAS reactor had the maximum removal rates of 231, and 31.1 $gm^{-2}d^{-1}$ for sCOD and NH_4-N respectively at OLRs of ~660 and ~200 $g.sCOD.m^{-2}d^{-1}$. The final effluent achieved the maximum NO_x-N removal rate of 71.3 $g.m^{-2}d^{-1}$ at an OLR of ~720 $g.sCOD.m^{-2}d^{-1}$ ($p<0.05$). Maximum protein EEA of ~120 $\mu mol.g.VS^{-1}.min^{-1}$ was attained at OLRs of ~ 424 $g.sCOD.m^{-2}d^{-1}$ for the final effluent and RAS reactors. The RAS reactor had higher phosphate EEA of 55.1 $\mu mol.g.VS^{-1}.min^{-1}$ compared to final effluent or humus solids ($p<0.05$) at OLR of ~400 $g.sCOD.m^{-2}d^{-1}$. The phosphate EEA determined 12% of the bulk organics removal rate. The EEA did not impact nitrification. The K_m of protein and phosphate EEA positively and negatively impacted denitrification respectively. Augmentation of humus solids and RAS enhanced nitrification at medium loadings and decreased denitrification at high loadings. Returning active nitrifying flocs facilitates nitrification but inhibits denitrification, although this was dependent on OLR. Operation of RBRs with a return solids allows bulk organics

removal and high nitrification rates at elevated OLRs than normally possible. High biofilm denitrification rates at HRTs of ~5 mins demonstrate pre-denitrification capacity of hybrid RBRs as suitable reactors for upgrade of existing secondary treatment assets.

6.2 INTRODUCTION

Incorporating a solids feed of active biological solids analogous to the roughing trickling filter (TF)/ASP (Daigger and Boltz 2011) or roughing TF/TF (Daigger et al. 1993) allows for pretreatment of wastewater prior to existing secondary treatment process at higher OLRs than normally permissible. The return of settled sludge to the head of the process is thought to improve treatment efficacy through enhanced bacterial contact, elevated suspended solids concentration and EEA (Daigger and Boltz, 2011). However this is balanced through reduction in HRT, which increases with higher OLR and SLR reducing time for biological degradation to occur. This could result in washout or inactivation of microbial community and extracellular enzymes. The OLR is usually the dominant factor controlling biofilm structure and function (Wijeykoon et al. 2004) although microbial growth rates and oxygen transfer limit removal rates and effluent quality.

Extracellular enzyme activity is required prior to treatment of wastewater polymers as the majority are too large to enter bacterial cells (Burgess and Pletschke 2008). This prevents degradation and therefore limits the removal rate of biological WWT (Orhon and Çokgör, 1997, Martins et al. 2003 and de Kreuk et al. 2010) and OLRs that can be applied whilst maintaining environmental quality standards (Hassard et al. 2014). The majority of the EEA is associated directly with bacterial cell walls or localised within floc or biofilm (Confer and Logan, 1998; Morgenroth et al, 2002) and wastewater bacteria regulate EEA based on available substrates, electron acceptor conditions and microbial growth rate (Li and Chróst 2006, Hauduc et al. 2013 and Shackle et al. 2000). Alterations in the enzyme and/or substrate concentration and bacterial metabolic state impact the kinetics of EEAs, the potential for extracellular degradation and process performance (Goel et al. 1997; Wingender and Jaeger 2002). Despite the relative

importance of EEA for biological processes and modelling purposes, understanding process or biochemical factors that affect expression, regulation and activity of EEAs in wastewater treatment remains unclear (Goel et al. 1997; Truu et al. 2009). Feast-famine conditions influences the uptake and utilisation of storage products. Recycling bacteria from end of a works (famine) to the front (feast) probably removes easily degradable sCOD through adsorption, EEA and subsequent storage and utilisation (van Loosdrecht et al. 1997).

In roughing hybrid biofilm systems, such as TF/ASP or TF/TF, nitrification principally occurs downstream of the biofilm reactor (Datta et al. 2011), aided in part by sloughed bacterial solids and reduced OLR on the secondary treatment process (Daigger et al. 1993; Hassard et al. 2015). At suitable OLRs, oxygen transfer restriction facilitates multiple removal regimes in a single volume, such as simultaneous nitrification or denitrification, or conventional pre-denitrification through heterotrophic nitrate utilisation in solids feed. Manser et al. (2003) demonstrated that floc size governs the substrate kinetics of nitrifying populations, which suggested that floc type influences prevailing conditionals and treatment capacity. You et al. (2003) found that hybrid processes allow treatment at greater OLRs, nitrification at lower SRT and increased resilience to nitrification performance upsets. Recycling solids (bacteria or enzymes) which improve the performance of hybrid biofilm systems would be of functional benefit (Truu et al. 2009). Solids could allow greater volumetric removal than single pass systems without significant extra aeration costs (Hassard et al. 2015). This study aims to elucidate the impact of different candidate solids types; final effluent (FE), humus solids (HS) and RAS on performance and microbial extracellular enzyme activity in hybrid RBR.

6.3 MATERIALS AND METHODS

6.3.1 Laboratory scale studies at varying OLR and SLR

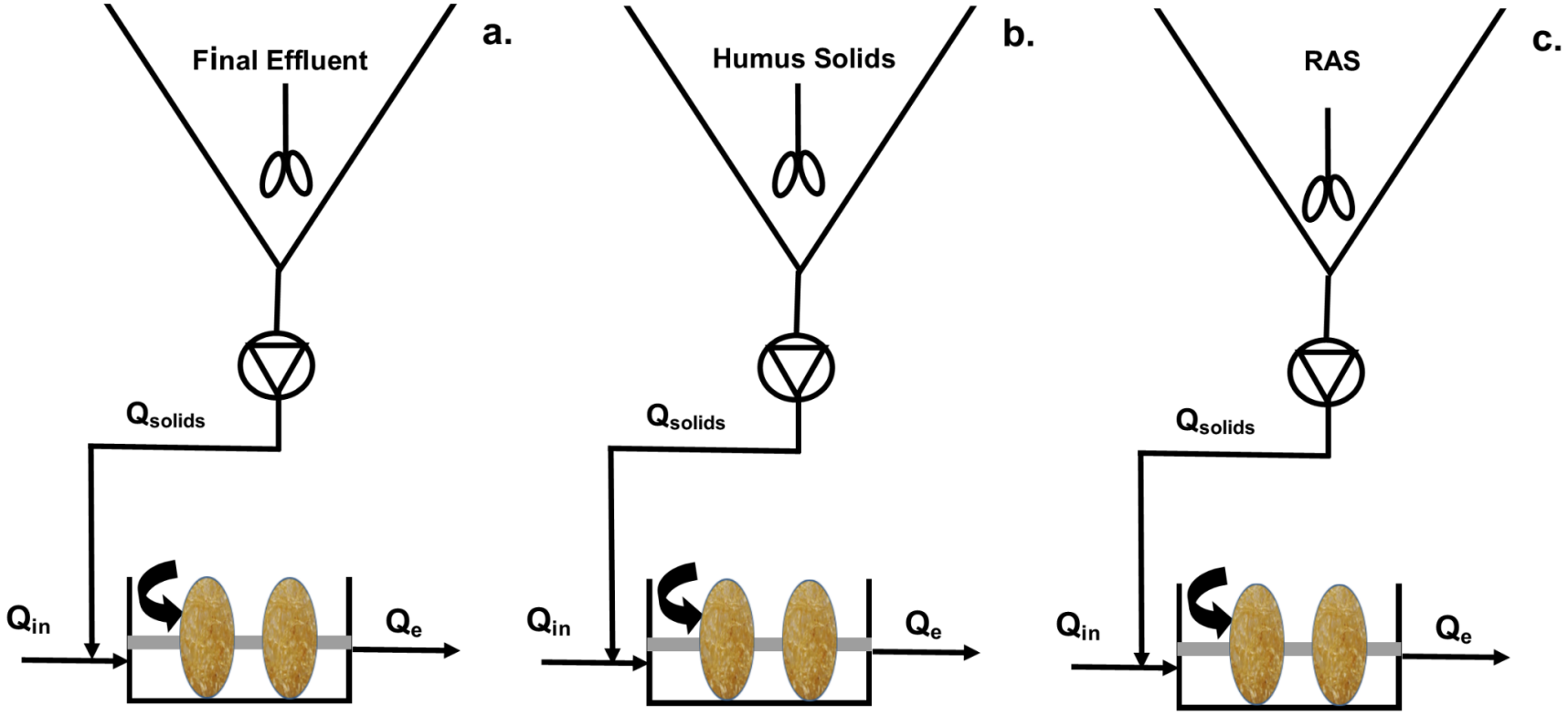
Six bench scale RBRs were situated at Cranfield University WWTP as characterised previously. The RBRs were operated at a constant tip speed (0.08 ms^{-1}) and fed with real settled sewage (representing influent feed) and operated

for a period of 5 months. Trickling filter HS were obtained from a clarifier (post-secondary treatment) of ~3000 PE situated at Cranfield University WWTP treating municipal wastewaters. The FE was obtained from the environmental discharge point. The HS and FE were fed into the RBRs by peristaltic pumps from 400L holding tanks (T425NA12GH, Tanks-Direct, UK) which were refreshed daily; the solids were stirred to prevent settlement (Figure 10 a, b). The RAS was provided by a pilot scale activated sludge plant characterised by Petrie et al. (2014) operated at a SRT of 20 day and a hydraulic retention time of 16 hours. The RAS was pumped from the final settlement tank of the pilot scale activated sludge plant as the same rate as FE and HS (Figure 10 c). The FE, HS and RAS represents the 'solids feed' component of hybrid RBRs. Different total OLRs and SLRs were applied to each FE, HS and RAS reactor to understand the impact and interaction of OLR, SLR and solids type on performance and microbial EEA of the biofilm reactor. Different nominal OLRs were applied to each RBRs at ~72, 152, 351, 546 g.sCOD.$m^{-2}d^{-1}$ corresponding to 50, 100, 200, 400 Ld^{-1} of settled wastewater per day. This was equivalent to a BOD_5 loading rates of 151.2, 262.5, 739.2, 1146.2 g.BOD_5.$m^{-2}d^{-1}$ (based on average BOD_5:sCOD ratio of 2.1 which did not change significantly during study). Two different solids recycle rates (50% and 100% of influent flow) were applied to each solids type reactor at each OLR treatment (2 x 3 x 4 = 24 treatments) resulting in low and high SLRs. The HRT therefore decreased incrementally from 57.6 mins at low OLR, low SLR to a minimum of 5.4 mins at very high OLR, high SLR (Table 16). Temperatures were recorded in the bulk fluid of RBR reactors and the aeration tank of the pilot scale activated sludge reactor (EL-WiFi-TP^{+}, Corintech, UK).

Table 16 - Study conditions of hybrid RBRs

Organic Loading	Recycle flow as ratio of influent	Influent flow rate (Ld^{-1})	Solids flow rate (Ld^{-1})	Total flow to RBR (Ld^{-1})	RBR HRT (mins)
low	0.5	50	25	75	57.6
	1	50	50	100	43.2
medium	0.5	100	50	150	28.8
	1	100	100	200	21.6
high	0.5	200	100	300	14.4
	1	200	200	400	10.8
very high	0.5	400	200	600	7.2
	1	400	400	800	5.4

Figure 10 - Experimental setup for lab scale hybrid rotating biofilm reactors. With influent (Q_{in}), effluent (Q_{eff}) and solids flows (Q_{solids}).



6.3.2 Wastewater analysis

Influent samples were collected at 9:00±1h daily. Wastewater was analysed for standard wastewater constituents. Dissolved oxygen concentration was measured by in reactor DO probe (LDO model 1, Hach-Lange, Germany). The redox potential of the influent and effluent was measured by redox probe (HI-98201, Hanna Instruments, US).

The removal efficiency (bulk organics, NH_4-N) was calculated taking into account influent flow, solids flow and respective concentrations:

Equation 12 – removal efficiency for hybrid RBRs

$$\% = (S_i + (R*S_{solids})-S_e)/ (S_i + (R*S_{solids}))*100$$

Where S_i = the influent substrate concentration, R = proportion of flow is solids, S_{solids} = the substrate concentration in the solids flow, S_e = effluent substrate concentration.

In Equation 12 the solids flow is similar and represents a return flow or recycle in conventional secondary treatment processes, such as activated sludge.

The substrate loading rate (X [either organics, NH_4-N, NO_x-N]-LR) was calculated taking into account influent and solids flows:

Equation 13 – Substrate loading rate for hybrid RBRs

$$X\text{-}LR_{nominal} = (S_i *Q_i) +(S_{solids}*Q_r)/SA_{nominal}$$

Where Q_i = influent flow rate, Q_r = solids flow rate, $SA_{nominal}$ = the nominal mesh surface area

The removal rate for sCOD and NH_4-N was calculated based Equation 12 and Equation 13.

The solids loading rate (SLR) was calculated taking into account solids flow after:

Equation 14 – Solids loading rate in hybrid RBRs

$SLR_{nominal} = (TSS_{ret} * Q_r)/SA_{nominal}$

Where TSS_{ret} = total suspended of solids flow.

The nitrogen removal efficiency (TN) was calculated taking into account influent and recycle NO_x concentrations (NO_2-N, NO_3-N) and accounting for internal nitrification after the simplified Equation 15

Equation 15 – TN removal rate for hybrid RBRs

$\mu TN = ((NO_x\text{-}N_i + NO_x\text{-}N_s + NO_x\text{-}N_n) - (NO_x\text{-}N)_e / (NO_x\text{-}N_i + NO_x\text{-}N_s + NO_x\text{-}N_n) * 100$

Where: NO_x-N_i = Influent NO_x concentration, NO_x-N_s = Solids NO_x-N concentration, NO_x-N_n = internal NO_x-N generation by nitrification.

6.3.3 Extracellular enzyme activity assays

The biofilm was harvested from mesh media and EEA assays were undertaken as described previously. Standard curves were used to calculate the concentration of substrate liberated per time and the Michaelis-Menten equation was solved using a non-linear least squares method for kinetic parameter estimation (V_{max}, K_m), then the standard error of mean and significance of model fit were calculated using a Hessian matrix and t-test respectively (R statistical package) (Venables et al. 2011).

The specific enzymatic activity was expressed as the maximum rate per gram of bacterial solids after equation 11.

6.3.4 Statistical analysis

Separate hierarchical multiple linear regression analysis (MRA) was undertaken to understand the impact of independent variables (Solids type, OLR, NH_4-N-LR, NO_3-N-LR, oxygen concentration, redox potential, pH, biofilm specific EEA and K_m on the dependent variables (sCOD removal rate, NH_4-N removal rate, NO_3-N removal rate). Standardised regression coefficients (β coefficient) allows

comparison of the impact of each independent variable on the same scale (Germain et al. 2004). Prior to MRA an independence of observation test (Durbin-Watson) was calculated, scores ~2 suggested minimal correlation between residuals. Correlation analysis between each independent variable showed that OLR, NH_4-N-LR, NO_3-N-LR were highly correlated with each other (R^2 >0.75) therefore only each variable was used represent 'loading' in each substrate specific MRA model. Tolerance collinearity statistics for each remaining independent variable were >0.1 suggesting collinearity did not impact eh model. Casewise diagnostics were used to detect outliers (studentised deleted residual ±3) three data points were excluded from the NO_3-N data. Leverage values and influence points (Cook's distance test) were calculated and any influential points were investigated, and no additional transformations were required. A frequency histogram of regression standardised residuals was plotted and the mean and standard deviation were ~0 and ~1 respectively for each MRA model suggesting normal distributions. Due to high correlations between OLR, NH_4-N-LR and NO_3-N-LR individual impact on performance of RBRs could not be characterised. In addition to MRA, separate one-way ANOVA was used to differentiate significant performance differences between solids type under each OLR and SLR for the performance data and enzyme kinetics with Posthoc Bonferroni corrected values test to attribute differences.

6.4 RESULTS

6.4.1 Performance

The influent wastewater was settled sewage with 630.1±505.5, 121.9±22.8, 31.6±6.2 and 331±203.6 mgL^{-1} tCOD, sCOD, NH_4-N and TSS respectively, identical in composition for each reactor and did not differ between OLRs treatments ($p<0.05$). The ambient air in the test facility was heated to 17.5±2.1, 18.8±1.8, 17.2±0.9 and 16.8±0.8 ºC for low, medium, high and very high OLRs at time of sampling. The reactor temperature in the RBRs and activated sludge pilot did not change by more than 12% through day/night cycle. The total OLRs increased as expected based on influent flow rates and were not significantly different between solids type reactors (Table 16; Table 17). The influent sCOD

and NH_4-N concentration did not exceed ± 2 standard deviation between different reactors and treatments. The NO_3-N concentration in the influent remained low (<2.5 mgL^{-1}) throughout the study whilst the recycle NO_3-N was on average 28.9±6.6, 29.3±5.8 and 24.9±5.2 for FE, HS and RAS respectively i.e. no statistical difference. The NO_3-N-LR increased as expected based on recycle flows and was similar for each solids type (Table 17) and the low SLR had half the NO_3-N-LR of the high SLR (Table 17) for all treatments as expected. The very high OLR treatment was the exception as the NO_3-N concentration in the RAS recycle feed decreased by 67% to 8.2±4.3 mgL^{-1}, due to denitrification in the final clarifier of the ASP. The reactor DO decreased from between 4 and 5 mgL^{-1} and between 1.4-1.9 mgL^{-1} at low and very high OLR respectively, for both FE and HS reactors. The RAS reactor DO was between 1 and 1.8 $mgL^{-1}$and low OLR and decreased to <0.7 mgL^{-1} at very high OLR (Table 17). In general the DO decreased with higher SLR (Table 16; Table 17) presumably due to less time for oxygen transfer. The β coefficients ranked in order of importance in predicting sCOD performance (greatest to least OLR>Solids type>phosphate EEA (sCOD model, R^2 = 52% and Table 18) with OLR predicting 82% of the sCOD removal rate. At low OLR, the RAS reactor had a removal rate of ~50 $g.sCOD.m^{-2}d^{-1}$: twice that of FE or HS reactors (Figure 11 a). The sCOD removal rate increased in a pseudo-linear fashion from low to very high OLR to a maximum of 231 $g.sCOD.m^{-2}d^{-1}$ for the RAS reactor attained at the low SLR treatment. This represents a 5.7 fold improvement on identical RBRs operated without a solids feed (data presented in Hassard et al. 2014). This suggests a solids feed improved bulk organics removal despite reductions in HRT (Table 16). In this study very high OLR/high SLR treatment reduced the sCOD removal rate decreased by 16.5, 8.5 and 25.6 % for FE, HS and RAS respectively (Figure 11 a).

Table 17 - Operating conditions in hybrid RBRs operated with different SLR set at 50% ¦ and 100% of influent flows.

Organic loading→	Low			Medium			High			Very High		
Return type →	**Final effluent**	**Humus solids**	**RAS**	**Final effluent**	**Humus solids**	**RAS**	**Final effluent**	**Humus solids**	**RAS**	**Final effluent**	**Humus solids**	**RAS**
Total OLR ($gm^{-2}d^{-1}$)	78 ¦ 88	78 ¦ 87	82 ¦ 95	180 ¦ 209	185 ¦ 218	181 ¦ 211	422 ¦ 442	396 ¦ 439	399 ¦ 446	670 ¦ 776	648 ¦ 731	683 ¦ 801
%OLR from solids	13 ¦ 32	11 ¦ 19	17 ¦ 28	16 ¦ 27	18 ¦ 30	16 ¦ 28	14 ¦ 24	12 ¦ 21	12 ¦ 22	16 ¦ 27	13 ¦ 22	18 ¦ 29
SLR ($kg.TSS.m^{-2}d^{-1}$)	0.05 ¦ 0.09	0.13 ¦ 0.27	2.88 ¦ 5.76	0.05 ¦ 0.11	1.68 ¦ 3.37	3.75 ¦ 7.49	.08 ¦ .16	5.1¦ 10.2	10 ¦ 21	0.25¦0.5	6.51¦13.02	14.59¦29.18
NH_4-N-LR ($gm^{-2}d^{-1}$)	17 ¦ 17	16 ¦ 16	17 ¦ 19	41 ¦ 41	41 ¦ 41	41 ¦ 41	84 ¦ 85	83 ¦ 84	84 ¦ 87	165 ¦ 166	164 ¦ 164	191 ¦ 219
% NH_4-N-LR from solids	1.0 ¦ 2.1	0.2 ¦ 0.4	0.6 ¦ 1.1	0.9 ¦ 0.8	0.3 ¦ 0.5	0.2 ¦ 0.3	2.3 ¦ 4.4	1.3 ¦ 2.5	2.7 ¦ 4.9	0.9 ¦ 1.8	0.2 ¦ 0.4	12.3 ¦ 21.1
NO_3-N-LR ($gm^{-2}d^{-1}$)	7 ¦ 14*	9 ¦ 17*	6 ¦ 12*	19 ¦ 37*	21 ¦ 41*	46 ¦ 64*	46 ¦ 89*	42 ¦ 81*	33 ¦ 64*	78 ¦ 153*	75 ¦ 148*	27 ¦ 50*

Reactor oxygen concentration (mgL^{-1})	5.2 ¦ 4.0	5.6 ¦ 4.6	1.8 ¦ 1*	3.6 ¦ 2.1	3.7 ¦ 3.1	3.1 ¦ 2.4	1.3 ¦ 1.2	2.6 ¦ 2.1	1.9 ¦ 1.9	1.5 ¦ 1.7	1.4 ¦ 1.9	0.8 ¦ 0.6
Reactor redox (mV)	72 ¦ 66	70 ¦ 63	68 ¦ 92	56 ¦ 39*	43 ¦ 40	43 ¦ 42	42 ¦ 23	29 ¦ 22	14 ¦ 12	25 ¦ 17	20 ¦ 16	14 ¦ 6*
Reactor pH ($-\log_{10}[H^+]$)	7.6 ¦ 7.3	7.6 ¦ 7.7	7.5 ¦ 7.5	7.5 ¦ 7.7	7.4 ¦ 7.4	7.4 ¦ 7.5	7.6 ¦ 7.7	7.7 ¦ 7.7	7.6 ¦ 7.6	7.7 ¦ 7.7	7.7 ¦ 7.4	7.7 ¦ 7.8

(*) = difference between solids recycle ratio of 0.5 & 1 significant

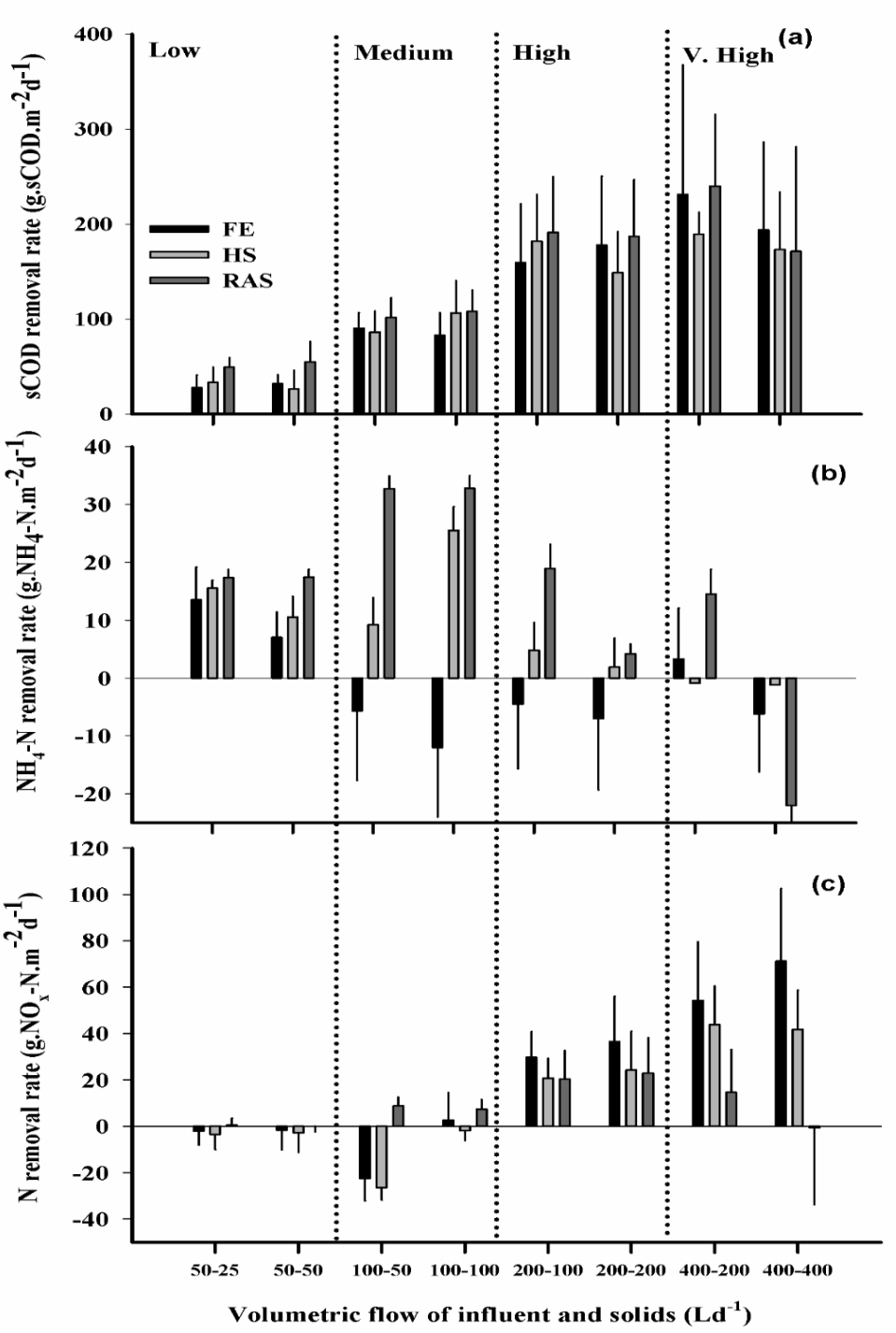


Figure 11 - Performance of hybrid rotating biofilm reactors for (a) soluble organics removal rate (sCOD) (b) nitrification (NH_4-N) (c) denitrification (NO_3-N) rates operated at 50, 100, 200,400 Ld^{-1} influent flow rate. Solids recycles [FE, HS, RAS] at 50% and 100% of influent flow rates were applied. Data represent 6 independent reactor experiments with averages ± SD of 8 replicates

The FE reactor only nitrified at low OLR/ low SLR whilst at the high SLR the removal decreased by 48% ($p<0.05$) (Figure 11 b). The HS reactor performed in a similar fashion for the low OLR, attaining 90.7 and 35.2% NH_4-N removal efficiency at the low and high SLR respectively (Figure 11 b). In contrast the RAS reactor had 94 and 78% NH_4-N removal rate at SLR of low and high respectively suggesting that HRT had less of an impact on nitrification when RAS was utilised as solids feed. At medium OLR the RAS reactor outperformed both the FE and HS reactors ($p<0.05$) with a maximum NH_4-N removal rate of ~31 $g.NH_4\text{-}N.m^{-2}d^{-1}$ at both the low and high SLR. This represents a 5.6 fold improvement compared to identical RBRs without solids feed (Hassard et al. 2014). In this study at high OLR nitrification rate reduced by 47.8 and 92.1% (low and high SLR) for HS and more gradually at 40.9 and 89.3% (low and high SLR) in the RAS (Figure 11 b). This suggests that augmentation of active bacterial solids (RAS or HS) is critical to maintain high NH_4-N removal rates at OLR >80.$g.sCOD.m^{-2}d^{-1}$ compared to the FE reactor and systems with no solids feed (Hassard et al. 2014). The NH_4-N β coefficients ranked in order from greatest to least solids type>recycle redox potential> reactor pH> reactor redox potential (NH_4-N model, R^2 = 36%, Table 18). The solids type and recycle redox potential impacted 44 and 31% of the nitrification performance. Both augmentation of active nitrifying solids and aerobic conditions are required for elevated nitrification (Figure 11 b; Table 18).

The hybrid RBRs did not remove NO_x-N at low OLR, and the FE and HS reactors had high effluent NO_x-N indicative of nitrifying conditions, which ranged between 19.2 and 26.2 mgL^{-1}. In contrast, the RAS reactor had low effluent NO_x-N (8.8 and 7.4 $g.m^{-2}d^{-1}$ for low and high SLR respectively) at medium OLR despite low effluent ammonia. This is indicative of the onset of simultaneous nitrification/denitrification at lower OLR (Figure 11 b, c) confirmed by high NH_4-N removal, and absence of NO_x-N in the effluent. At very high OLR the NO_x-N removal rate for FE and HS increased to a maximum of 41.8 and 71.3 $g.NO_x\text{-}N.m^{-2}d^{-1}$ respectively with FE outperforming HS and RAS ($p<0.05$) (Figure 11 c). The NO_3-N removal rate model had an R^2 of 0.54 therefore 54%, the β coefficients

ranked in order from greatest to least NO_3-N-LR > Solids type > protein EEA > Reactor O_2> SLR. Elevated denitrification in the FE reactor suggested that the type of solid changes the removal regime of the biofilm in hybrid systems by reducing effective OLR to the biofilm. The maximum NO_3-N removal rate of the RAS reactor was 22.9 $gm^{-2}d^{-1}$ at the high OLR and high SLR treatment, similar to the HS removal despite 21.1% lower NO_3-N-LR supplied to the RAS reactor (Figure 11 c; Table 17). The denitrification performance of the RAS reactor was restricted by experimental limitations notably NO_3-N-LR. Variables not deemed significant to hybrid biofilm reactors were summarised in Table 19.

Table 18 - Multiple linear regression (MLR) output β coefficients.

	β coefficient		
Model (dependent variable →) independent variables↓,	**sCOD[c] R^2 = 0.56**	**NH_4[b] R^2 = 0.33**	**NO_3[b] R^2= 0.54**
Solids type	0.197[a]	0.441[a]	0.136[c]
OLR	0.823[a]	-	-
SLR	-0.210[a]	-0.183[d]	-0.235[a]
NH_4-N-LR	-	-0.076[d]	-
NO_3-N-LR	-	-	0.66[a]
Reactor pH	-	-0.174[a]	-
Recycle pH	-	-0.039[d]	-
Reactor O2	-	-	-0.148[b]
Recycle O2	-	-	0.057[d]
Reactor redox potential	-	-0.133[c]	-
Recycle redox potential	-	0.310[a]	-
Biofilm protein specific EEA	0.026[d]	0.017[d]	-
Biofilm phosphate specific EEA	0.155[c]	-0.045[d]	-
Biofilm protein K_m	-	-	0.121[c]
Biofilm phosphate K_m	-	-	-0.011[d]

Significance of MLR model a= <0.001, b= <0.01, c= <0.05 d>0.05; (-) = variable excluded from regression model, as not correlated with performance.

Table 19 - oxygen concentration, redox and pH of influent and recycle flows in RBRs with different SLR at 50% and 100% of influent flows.

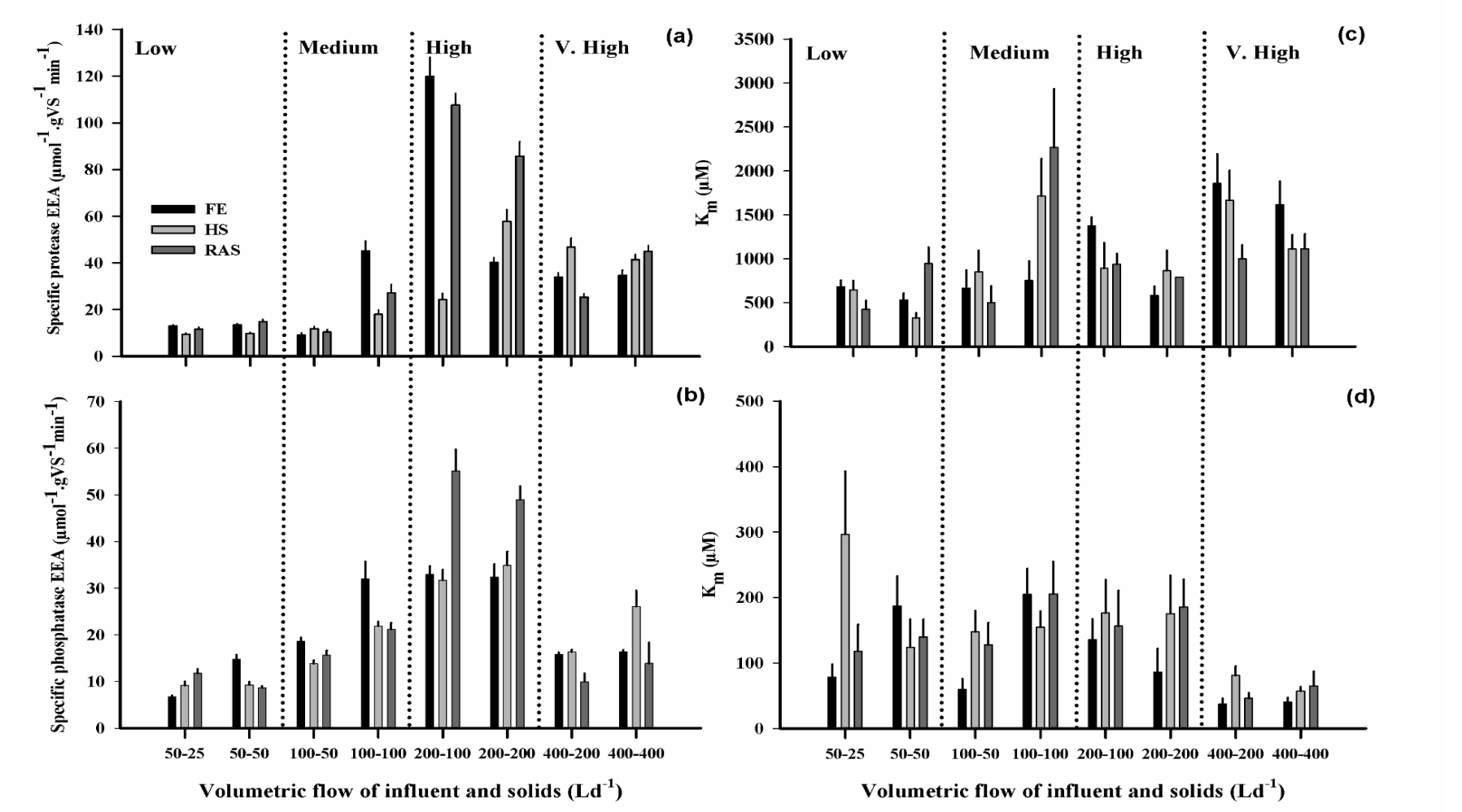
OLR →	**Low**			**Medium**			**High**			**Very High**		
Parameter↓ – solids type →	**FE**	**HS**	**RAS**	**FE**	**HS**	**RAS**	**FE**	**HS**	**RAS**	**FE**	**HS**	**RAS**
Influent oxygen concentration (mgL^{-1})	0.80			0.37			0.98			1.60		
Influent redox (mV)	-184			-258			-194			-270		
Influent pH ($-\log_{10}$ [H+])	7.65			7.76			7.60			7.74		
Recycle oxygen concentration (mgL^{-1})	5.6	4.3	3.0	3.0	4.5	5.0	6.1	6.1	3.7	4.6	6.3	4.5
Recycle redox (mV)	94	100	100	46	584	45	35	40	38	19	20	-13
Recycle pH ($-\log_{10}$ [H+])	7.5	7.4	7.5	7.1	7.1	7.1	6.8	6.9	7.2	7.6	6.4	7.5

6.4.2 Microbial extracellular enzyme activity

The ability of the biofilm to degrade bulk long chain polymers is limited by EEA (Burgess and Pletschke 2008). It is hypothesised that elevated EEA could correlate with better performance in hybrid biofilm reactors (Goel et al. 1997). Mechanisms governing EEA require further attention in most biological process. Direct hydrolysis of slowly biodegradable substrates, elevated utilisation of storage compounds in the bacteria and removal of particulates in the biofilm itself have been suggested to improve performance in biological processes (van Loosdrecht et al. 1997, Goel et al. 1997, Goel et al. 1998 and Goel et al. 1999). In this study, the data fitted the Michaelis-Menten model for each enzyme (V_{max} and K_m) at each treatment (t-test, $p<0.05$, data not shown), which allowed use of this model. The protein EEA was minimal and ranged from 9.4 to 14.7 $\mu mol.g.VS^{-1}.min^{-1}$ between low OLR (low and high SLR) and medium OLR/low SLR for all reactors suggesting low protein acquisition. Protein EEA increased by 76.2, 35.1, 62.9% for FE, HS and RAS respectively with high SLR (Figure 12 a, $p<0.05$), possibly due to enhanced competition for substrate under elevated SLR (Table 17) and elevated microbial growth rates. Alternatively recycling refractory polymers could induce of EEA particularly under nutrient limited conditions (low OLR) where there is lower overall readily biodegradable organics to facilitate growth and enzyme production (Molina-Muñoz et al. 2010). At high OLR/low SLR the protein EEA increased by ~3 and 4.5 x for both FE and RAS reactors ($p>0.05$). The EEA reached a maximum of 122 and 115 $\mu mol.g.VS^{-1}.min^{-1}$ at high OLR/low SLR for FE and RAS reactors respectively. This was not found in the HS reactor as protein EEA increased by <30%. The FE reactor protein EEA decreased to ~40 $\mu mol.g.VS^{-1}min^{-1}$ for at OLRs >400 $g.sCODm^{-2}d^{-1}$ and high SLR (Figure 11 a). The EEA in HS and RAS reactors declined rapidly to minimal at HRTs <14 mins (Figure 12 a, b; Table 16). The trend was similar for phosphate EEA with an increase with OLR to a maximum of 55.1 $\mu mol.g.VS^{-1}.min^{-1}$ at high OLR/low SLR treatment for the RAS reactor, however the EEA was ~50% of protein and activity in FE and HS reactors (Figure 12 b).

The protein K_m increased with OLR suggesting the biofilm reaches maximum rate and saturation more slowly, there was no difference with SLR. The RAS reactor had similar K_m of 789 and 937 µM despite EEA of 85.9 and 115.7 $\mu mol.g.VS^{-1}.min^{-1}$ at the high OLR for low and high SLR respectively. At the very high OLR the FE reactor had a K_m of 46.1 and 31.7% greater than the RAS reactor at low and high SLR (Figure 12 c). The phosphate K_m was similar between reactors under most conditions studied although a marked decline in K_m of 84.6 and 48.5% between high and very high OLRs for the RAS reactor at low and high SLR respectively, this trend was reflected in all reactors (Figure 12 d). The SLR negatively correlated to sCOD removal rate β coefficient = -0.210. Variables excluded in hierarchical regressions (-) and included non-significant variables are summarised in Table 18.

Figure 12 - Microbial EEA of hybrid RBRs (a) Specific protein EEA (b) Specific phosphate EEA (c) Protein K_m (d) Phosphate K_m. Solids recycles [black: FE, light grey: HS, dark grey: RAS] at 50% and 100% of influent flow were applied. Bars represent, averages ±1SD.



6.5 Discussion

The sCOD removal rate was found to increase with OLR and the RBRs exhibited 5.7x greater sCOD removal rates compared to identical RBRs without solids feed (Hassard et al. 2014), suggesting the solids recycle enables operation at greater OLRs. Addition of a recycle of reactor effluent or bacterial solids (FE, HS and RAS used in this study) has been shown to dilute the influent feed resulting in lower substrate concentrations, higher reactor DO concentration and reduction in filamentous microbiota (Ayoub and Saikaly 2004; De Kreuk et al. 2010) facilitating higher removal rates of bulk organics in identical reactor volumes. De Kreuk et al. (2010) showed that lower substrate gradients from water/granule interface reduced the competitive advantage of filamentous groups typically found in high OLR biofilm or granular systems (Ayoub and Saikaly 2004; Hassard et al. 2014). In this study the sCOD removal rate correlated 15.5% with phosphate EEA ($p<0.05$) suggesting a slight but significant impact on bulk organics removal. The presence of phosphate groups can prevent substrate from penetrating bacterial membranes. Ammerman and Azam (1985) suggested that phosphate EEA is required for dephosphorylation prior to substrates metabolism and elevated growth rates. Higher bulk organics removal identified in the RAS reactor, ~3000 PEcompared to FE and HS could be due to augmentation of active floc bound heterotrophs for substrate metabolism and EEA, resulting in an increase in the readily bioavailable fraction for the reactor community as a whole (Figure 12 a, b). Elevated surface area for wastewater/bacterial contact could further increase the EEA and removal rates achieved (Confer and Logan 1998). Decreased sCOD removals at the very high OLR/high SLR treatment suggested growth, kinetic or DO limitation (Nogueira et al. 2002). The highest OLR coincided with the lowest reactor DO (Ayoub and Saikaly 2004) and a reduction in the EEA at very high OLR compared to high OLR for all reactors studied. Decrease in sCOD removal rates at very high OLR and high SLR resulted in a HRT of 5.4 mins, below the established doubling time of most heterotrophic bacteria. Bacteria have been shown to enter a viable but non-culturable state (VBNC) under conditions of low

electron acceptor conditions, which are characterised by low growth rates, performance and EEA (Pinto et al. 2013). Inhibition of protozoa under anaerobic conditions has been suggested to contribute to low EEA (Hauduc et al. 2013). At high OLRs the viability of bacteria can be reduced, therefore resulting in decreased EEA (Hassard et al. 2014). More anaerobic conditions are also associated with low EEA (Goel et al. 1998).

High nitrification rates (>78%) were observed in the RAS solids RBRs at higher OLRs than previously established thresholds of 15 $g.BOD_5.m^{-2}d^{-1}$ for rotating biological contactor reactors (Rittmann and McCarty 2001), and 35 $g.sCOD.m^{-2}d^{-1}$ for mesh media reactors without solids feed (Hassard et al. 2014). Addition of a recycle has previously been observed to result in greater nitrification efficacy in rotating biological contactor (RBCs) reactors (Ayoub and Saikaly 2004). In this study OLRs >50 $g.sCOD.m^{-2}d^{-1}$ addition of FE (minimal solids) was not sufficient to maintain nitrification removal efficiencies. In contrast HS and RAS reactors achieve removal rates of >10 and>30 $g.NH_4\text{-}N.m^{-2}d^{-1}$ at sCOD loadings rates of ~180 $g^{-2}d^{-1}$ (BOD_5 loading rate of ~260 $g.m^{-2}d^{-1}$). The greater maximum NH_4-N removal rate in the RAS reactor could be attributed to the flocs or SLR, as other operating conditions in the FE and HS reactors were similar in other regards. The solids type and recycle redox potential most impacted the NH_4-N removal rate model in a positive and negative manner respectively. You et al. (2003) found that hybrid process allow treatment at greater OLRs, nitrification at lower SRT and increased resilience to nitrification performance upsets compared to conventional suspended growth systems. They suggested that nitrifier abundance or activity governs performance in hybrid systems. The origin of the solids could influence nitrifier abundance or activity, as the RAS comes from an ASP in which, the growth rate is controlled through wasting. Dead or slow growing bacteria therefore are outcompeted. In contrast the HS were from a trickling filter in which growth rate was not controlled and SRT is undefined (Bryers, 2000). In addition, the bacteria in the HS are sloughed or eroded bacteria which were likely inactive or decaying fraction and have a smaller impact to nitrification rates (Daigger and Boltz, 2011). Satoh et al. (2003) demonstrated that augmentation of nitrifiers into a RBC biofilm resulted in quicker start-up and elevated removal

rates through greater nitrifier density. The HS and RAS reactor in this study would have active nitrifiers bound within flocs, this could explain the higher nitrification rates identified, further study could elucidate whether accumulation/incorporation occurs. Goel et al. (1998) identified that extracellular enzyme synthesis was impacted by anaerobic conditions, but the activity remains roughly stable. In contrast regulation of EEA shown in our different system suggests that prolonged anaerobic conditions likely at very high OLR; could reduce the efficacy of EEA in biofilms. The greater NO_x removal rate identified in the FE reactor could be due to thicker biofilm present in the RBR (visual observation), elevated SLR negatively correlated with (Table 18), suggesting that the greater solids loadings present in HS and RAS reactors negatively impacted denitrification possibly by reducing biofilm thickness by substrate utilisation in the suspended phase. Active heterotrophs in the flocs could compete with the biofilm for substrate, resulting in a slower biofilm growth rate and therefore thickness. In turn, this resulted in greater oxygen penetration depth and inhibition of denitrification in HS and RAS respectively (Hanhan et al. 2005). This difference is most striking between FE and HS as the HS had 41% lower NO_3-N removal rate at very high OLR – high SLR treatment (Figure 12 c.) despite identical OLRs/NLRs. The reactor oxygen concentration negatively correlated with (β coefficient = -0.14) with NO_x-N rate (Table 17). If NO_3-N-LR is excluded from the MRA, relatively low total β coefficient <50% for other variables suggested that another parameter controls denitrification rate in RBRs such as biofilm DO concentration, which resulted in inhibition of denitrification and lower density of denitrifers in biofilm systems (Gómez et al. 2002). Wijeykoon et al. (2004) showed that competition for space limited stable nitrification under high OLR. Nogueira et al. (2002) showed that shorter HRT mixed community nitrifying biofilms were more resilient to high OLR conditions than longer HRT biofilms. Competition between floc and biofilm bound bacteria for resources has a determinate role governing the function of hybrid RBR reactors. Performance data showed that nitrification was stimulated but denitrification is inhibited. Operation of RBRs with a solids feed allowed bulk organics removal and high nitrification rates at elevated OLRs than normally possible. The protein K_m accounted for ~12% of the variation in denitrification

performance. The FE reactor had significantly higher protein K_m ($p<0.05$) despite similar EEA (Figure 12 a, b) suggesting a slowdown in protein turnover are requirements at elevated OLR. In contrast the phosphate K_m negatively correlated with denitrification performance, suggesting that the affinity for phosphate increased at higher OLRs as V_{max} was approached more quickly (Lehninger et al. 2005). Hanhan et al. (2005) found a denitrification rate of 2.06 $g.N.m^{-2}d^{-1}$ in an RBC system with a HRT if ~30 mins. In this study denitrification rates >60 $g.NO_x.m^{-2}d^{-1}$ was found in hybrid RBRs. High biofilm denitrification rates at HRTs of ~5 mins demonstrate pre-denitrification capacity of hybrid RBRs and therefore the potential for upgrade of existing WWTP for BNR. In light of these results utilising RAS as solids feed is preferred for upgrade of existing works by propagating better carbonaceous removal and nitrification rates the RBR unit.

6.6 Conclusions

1. Addition of a solids feed increased maximum sCOD and NH_4-N removal rates by 5.7 and 5.6 x respectively.
2. The RAS solids performance best for sCOD and NH_4-N removal at low OLR.
3. Maximum sCOD removal rate was 231 $g.sCOD.m^{-2}d^{-1}$ for RAS reactor. sCOD removal rate correlated with phosphate EEA.
4. Maximum NH_4-N removal rate was 30 $g.NH_4\text{-}N.m^{-2}d^{-1}$ for the RAS reactor. The NH_4-N removal rate did not correlate with EEA.
5. Maximum denitrification rate was 71 $g.sCOD.m^{-2}d^{-1}$ for the FE reactor at very high OLR. The denitrification rate correlated with increased phosphate affinity and decreased protein affinity.
6. OLR, Solids type and NO_x-N loading rate most important factors governing sCOD, NH_4-N and NO_3-N removal rate respectively.
7. The RAS solids biofilm had the greatest phosphate EEA and the most consistent elevated protein EEA which suggests a role of EEA and performance.

7 DISCUSSION

The use of RBCs for conventional biological WWT to remove BOD_5 and ammonia has been well established (Mueller et al. 1978). Application has largely been at the lower end of the WWT scale, usually for up to 2000 P.E. (Griffin and Findlay 2000). To upgrade existing wastewater treatment increasing effluent standards and reducing aeration costs are paramount (STOWA 2010; Ainger 2009), an approach is to utilise high OLR roughing reactors in a hybrid configuration. In this thesis the limits of removal were thoroughly investigated to a maximum of 40 and 5.5 $g.m^{-2}d^{-1}$ for sCOD and NH_4-N in single pass systems (Teixeira and Oliveira 2001, Hanhan et al. 2005, Chen et al. 2006, Hassard et al. 2014 and Chapter 4). Media choice did not enhance growth of certain bacterial populations at the organic loadings studied although high porosity mesh was better at very high OLR. However different solids type and loadings combinations facilitated nitrification and denitrification in an amalgamated reaction volume (Stephenson et al. 2013; Hassard et al. 2015). The high OLR conditions experienced by biofilm communities could decrease the viability or enzyme activities as electron acceptors usually limit growth (Okabe et al. 1996). To achieve high process efficiency under representative conditions experienced by upfront RBR reactors the maintenance of biofilm viability and enzyme activity is essential for biocenosis of wastewaters (Truu et al. 2009, Hassard et al. 2014 and Hassard et al. 2015). Polymeric fractions present a unique challenge, particularly for low HRT reactors (de Kreuk et al. 2010). Biofilm reactors have a poor track record at particulate remediation, presumably due to adsorption and diffusion resistance in the biofilm (Gujer and Boller 1990; Dutta et al. 2005). However roughly half of wastewater requires extracellular hydrolysis prior to treatment (Burgess and Pletschke 2008) which can limit achievable removal rates in wastewater treatment (Goel et al. 1997). In this thesis the specific EEA increased with organic loading. A negative association was noted with the biofilm redox potential suggesting more aerobic conditions favour extracellular hydrolysis. Additional of a solids recycle appeared to reduce the effective biofilm OLR delaying the onset of anaerobic conditions within the biofilm.

Operating suspended growth reactors such as activated sludge or membrane bioreactors at very high OLR usually results in washout or membrane clogging respectively. In biofilm systems such as RBRs washout is rarely an issue. However biofilm inactivation either through filamentous outgrowth (Kinner et al. 1985, Hassard et al. 2014, de Kreuk et al. 2010 and Appendix C) of nuisance species which can result in catastrophic mechanical failure (Mba et al. 1999) or poor effluent standards have been extensively reported (Cortez et al. 2008; Hassard et al. 2015). However the advantages of high OLR wastewater treatment are many such as; reduced power costs per unit of wastewater flow, reduced reactor volume; small unit operation footprint and process flexibility (Chapter 2; Hassard et al. 2015). Mechanical issues aside, inhibition of the biofilm community has been noted, usually due to localised deficiency of a nutrient which limits growth. Okabe et al. (1996) suggested that electron acceptor could result in reduced cell viability and performance respectively. To understand the impact of OLR on performance different media were operated at between 2 and 10 x the normal OLR threshold for combined organics removal and nitrification. Despite relatively high nitrification rates reported 6 $g.NH_4\text{-}N.m^{-2}d^{-1}$ and organics removal 25 $g.sCOD.m^{-2}d^{-1}$ the cell viability declined from a maximum of 2×10^{10}–4×10^{9} viable bacteria/ml biofilm. Although the mesh media had consistently higher removals compared to foam media the removal efficiency did decrease in agreement with previous works (Hiras et al. 2004, Chen et al. 2006). The build-up of inert or particulate matter (Orhon et al. 1997), reductions in convective flow through pore clogging (Kim et al. 2010), MT through the biofilm (Hassard et al. 2015) and decrease in bacterial metabolism (Pinto et al. 2013) have been suggested previously. This work highlighted that despite similar porosities between the foam and mesh media, architecture appeared critical to reduce pore clogging to maintain performance at high OLR (Chapter 3; Appendix C).

From Chapter 3 it was clear that mesh media offered performance and biofilm advantages over reticulated foam media. However due to architectural differences in the media, characterising differences in biofilm and or specific surface area was challenging. Therefore to investigate the impact of specific surface area and media type PVC and PP mesh media were operated

concurrently at low, medium, high and very high OLR. Two different specific surface area options were trialled for each material (PVC and PP) simultaneously corresponding to low and high specific surface area respectively (PVC-L, PCV-H, PP-L and PP-H). Biofilm was harvested and utilised for a series batch end point activity measures and viability tests. To augment performance and biofilm data surface properties of each media surface such as roughness was characterised for each media, alongside manufacturer data for filament linear density and porosity. Differences in removal rates were generally small between media at low, medium and high OLR. Inherent variability present in real wastewaters further exacerbated this effect. At very high OLR the high porosity mesh media (PVC-L and PP-L) significantly outperformed lower porosity alternatives of the same media type, by 51 and 54% for PCV and PP respectively. Porous media clogging has been attributed to cause this effect previously (Kim et al. 2010; Gamri et al. 2014). Broadly similar microbial activity and viability was to be expected as the media were operated concurrently with identical process conditions. Physical heterogeneity caused by media tortuosity and different flow patterns has been shown to increase fine scale microbial activity (Singer et al. 2010; Harris et al. 2012). Research largely on pure cultures has shown that surface interaction can strongly influence both the initial biofilm microbial population and viability (van der Mei et al. 2008). Gottenbos et al. (2001) found that bacteria adhered more rapidly to positively charged surfaces but strong electrostatic interaction impeded bacterial growth. Furthermore this interaction decreased the bacterial adenosine triphosphate content and proton motive force upon adhesion suggesting reduced microbial activity (Hong et al. 2009). Under real wastewater with media that had similar physiochemical properties (Section 4.4.3) it is likely that the presence of a conditioning film of proteins and colloidal matter masked differences in biofilm adhesions and viability (Busscher et al. 1995) and significant difficulty removing biofilm from mesh could have contributed to this. Batch studies using flat media using real wastewaters after Khan et al. (2013) and Stephenson et al. (2013) could shed light on this effect further. Singh et al. (2011) identified a threshold of ~20 nm where superior protein adsorption substantially decreased attachment rates and biofilm formation. This could

explain lower biofilm amounts present on the PP mesh media as it had an roughness ~25 nm (Table 10) by clogging nanoscale pores on the material surface and providing a barrier to adsorption; although further experiments would be required to elucidate this effect (Section 4.4.3).

Despite the clear links between microbial activity, viability and performance demonstrated in Chapter 3 and 4 the removal efficiency and therefore removal rates in single pass RBRs declined at OLRs >160 $g.m^{-2}d^{-1}$. It has been well documented previously that biofilm reactors are poor at remediation of polymeric and particulate fractions of the wastewater. Extracellular hydrolysis is required prior to treatment of >50% of wastewater components (Confer and Logan 1998, Cadoret et al. 2002 and Hassard et al. 2015). It was hypothesised that declining EEA could contribute to reduction in performance seen in single pass RBR systems at very high OLR (Hassard et al. 2014). In addition we further suspect that high EEA could contribute to better performance of wastewater treatment works upgraded with RBR. A full scale conventional wastewater treatment works was upgraded using hybrid RBRs. The modified activated sludge had 52 and 40% greater removal rates of COD and NH_4-N respectively compared to CAS. Greater tCOD removal is expected as the OLR increased by 40%, however elevated nitrification performance is not expected at this OLR as the HRT also decreased by 31%. Improved oxygenation of the aeration tanks could contribute to this effect. The biofilm RBR reactor had 3, 10 and 15 fold higher carbohydrate, phosphate and protein EEA compared to equivalent suspended growth stages in the modified activated sludge. Biofilm systems had elevated protein EEA in riverbed systems compared to suspended growth counterparts (Jones and Lock 1989). Higher cell densities, extra growth requirements or diffusion limitation has been suggested to account for this difference (Allison and Vitousek 2005). Biofilms by their nature propagate sharp gradients in substrates, electron acceptors and reaction products (Burns et al. 2013) with undefined SRT (Bryers 2000) which prevents the prevalence of ‘cheaters’ which utilise the short chain reaction products of bacteria which produce energetically costly extracellular enzymes (Allison 2005). This prevents outgrowth by one or a few dominant species which could occur in a mixed culture reactor where homogenous

substrate concentrations occur (de Kreuk et al. 2010). The maximum EEA was similar between modified activated sludge and CAS. This is unsurprising as the works were treating identical wastewaters. During nutrient limitation many catabolic enzyme operons are expressed, although EEA is suppressed until suitable organic are present (Konopka et al. 2000). Feast-famine conditions such as experienced in the modified activated sludge reactor could influence the bacterial community and activity but also the function of the system. Recycling bacteria from end of a works (famine) to the front (feast) likely removes easily degradable sCOD through adsorption, EEA and subsequent storage and utilisation (van Loosdrecht et al. 1997). Although it was not possible to elucidate the TN removal rate at full scale it is likely that recycling a solids stream high in oxidised nitrogen species could facilitate denitrification in the RBRs. At laboratory scale there was a stark increase in specific EEA with OLR suggesting that EEA was linked to growth of the viable biofilm community (Figure 5) although methods such as flow cytometry could improve quantification of 'specific' bacterial numbers (Boulos et al. 1999) as VS does not accurately reflect bacterial numbers. In spite of methodological limitations, the volumetric increase in EEA with OLR and decrease due to onset of anaerobic conditions has not been demonstrated previously in mixed culture biofilms to our knowledge. These data yield interesting insight into functional change in the biofilm due to changing process and physiochemical conditions. The return of settled solids to the head of the process is thought to improve treatment efficacy through enhanced bacterial contact, elevated suspended solids concentration and EEA (Daigger and Boltz 2011). However this is balanced through reduction in HRT, which increases with higher OLR and SLR reducing time for biological degradation to occur (Hassard et al. 2014, Hassard et al. 2015 and Chapter 5). This could result in washout or inactivation of microbial community and extracellular enzymes in conventional systems (Rittman and McCarty 2001). Immobilisation of microbial community within a biofilm usually prevents this fate in RBRs (Stephenson et al. 2013). Chapter 5 revealed the impact of OLR on EEA, however understanding the impact of 'hybrid' recycle component at full scale was compounded by lack of experimental controls. A paucity of information exists on the impact of recycling

large amounts of solids or effluent containing a myriad of bacteria, enzymes, refractory polymers, utilisation associated products and substrates such as NO_x-N on the function of microbial biofilms (Curtis et al. 2003). The laboratory study from Chapter 5 revealed that wastewater biofilms strongly regulate their EEA based on OLR and prevalent redox conditions. We hypothesised that incorporating a solids feed can prolong the period of elevated EEA in wastewater biofilms by reducing the effective OLR experienced by the biofilm which also delays the onset of anaerobic conditions. The maximum removal rates of 231 $g.sCOD.m^{-2}d^{-1}$ (~0.6 $kg.BOD_5 .m^{-2}d^{-1}$), 31 $g.NH_4\text{-}N.m^{-2}d^{-1}$ and 71.5 $g.NO_3\text{-}N.m^{-2}d^{-1}$ through use and a hybrid solids feed for permitting multiple removal regimes at high OLRs. The performance aspects of this study have been demonstrated previously in single pass RBRs (Hanhan et al. 2005; Chen et al. 2006) and similar conclusions were reached by Dutta et al. (2005) through works of modelling hybrid RBRs. The novelty of this work is that additional of a solids recycle appeared to reduce the effective biofilm OLR. As a consequence the maximum protein EEA was 4.4 fold higher in hybrid compared to single pass RBRs. The removal of sCOD and NH_4-N also increased 5.7 and 5.6 fold respectively and denitrification was achieved with addition of a solids feed. The major variables influencing removal rates of key wastewater constituents was loading (Table 18). However significant positive correlations for phosphate EEA with sCOD removal and protein K_m with nitrogen removal were found. This suggests that performance in RBRs is dependent in part on sustained EEA. Overall, it has been demonstrated that enhanced constituent removal can be achieved beyond normal thresholds with the addition of a solids recycle. The biofilm extracellular activity can be manipulated through effective process control. The RBR can be effectively utilised to modify existing secondary treatment assets for low energy nutrient removal.

8 CONCLUSIONS AND FUTURE WORK

- Rotating mesh biofilm reactors showed high COD and ammonia removal rate, low HRT treatment is permissible above the established OLR thresholds for RBCs.
- At very high OLR of >160 g.sCOD.$m^{-2}d^{-1}$, high porosity mesh media with a specific surface area ~ 150 m^2m^{-3} is required to maintain high removal rates to prevent biofilm inactivation. Biofilm activities and viability increased with OLR to a maximum of 30 µM.dye reduced.gVS^{-1}.min^{-1} and a intact:non-intact ratio of 10 respectively.
- Existing secondary treatment assets can be upgraded using hybrid RBRs. Improved removal rates of 52 and 40% for COD and NH_4-N respectively was achieved. Higher extracellular enzyme activity in the biofilm fraction of hybrid reactors could contribute to elevated performance. High substrate affinity was found in works which have RBRs as a pretreatment.
- The extracellular enzyme activity was found to increase with OLR to a maximum of 100 g.sCOD.$m^{-2}d^{-1}$ in single pass systems. The observed significant decreases in extracellular enzyme activity at higher OLRs could contribute to the declining performance for removal of bulk organics. A strong negative relationship between redox potential and extracellular enzyme activity suggests a role for higher organisms such as protozoa for hydrolysis of polymers in wastewater treatment.
- Incorporating a solids recycle into a hybrid reactors enables elevated sCOD and NH_4-N removal at low OLR and increases the threshold for which nitrification is achievable. Recycle activated sludge provided better performance than HS and FE for sCOD and NH_4-N. Active solids (RAS and HS) inhibits denitrification performance compared to FE under conditions studied.
- Incorporation of a recycle resulted in ~5 fold greater removal rate of both sCOD and NH_4-N compared to single pass systems.

- Provision of a recycle to an RBR enhanced extracellular enzyme activity at OLRs >400 g.sCOD.$m^{-2}d^{-1}$ and facilitated 4.4 fold greater protein extracellular enzyme activity compared to single pass systems.
- Investigating the impact of air scour on removal performance and biofilm viability, EEA and community structure would have benefit and allow comparison between bench and full scale RBRs. This harsh daily selection pressure would favour faster growing strains and reduce diversity of bacteria and higher organisms possibly impacting viability and EEA.
- Investigating the effect of submergence in RBRs could facilitate enhanced biological nutrient removal and the high organic loading treatment to enhance effluent quality and reduce energetic cost of wastewater treatment.
- Further fundamental research into EEA in biological wastewater treatment systems particularly but not limited to competition between biofilm and suspended growth bacteria, influence of species composition and impact of other process parameters on EEA.
- Research into how system architecture could result in fine scale heterogeneity in flow regime which directly influences mass transfer to the biofilm and activity of the populations within. The impact of process optimisation and resilience on mixed culture unit operations requires further research. The effect of the full scale ‘air scour’ requires research attention as this creates a harsh selection pressure against slow growing strains in favour of faster growing heterotrophs.
- For applying hybrid RBRs the final recommendation would be OLRs no greater than 180 g.sCOD.$m^{-2}d^{-1}$ for combined organics removal and nitrification. A recycle rate of 0.5 is preferred for nitrification and bulk organics removal as any benefit by recycling solids is offset by reduced HRT. In contrast elevated OLR and SLR is preferred for denitrification as NO_x availability principally limits achievable removal rates.

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APPENDICES

Appendix A Author addresses

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.

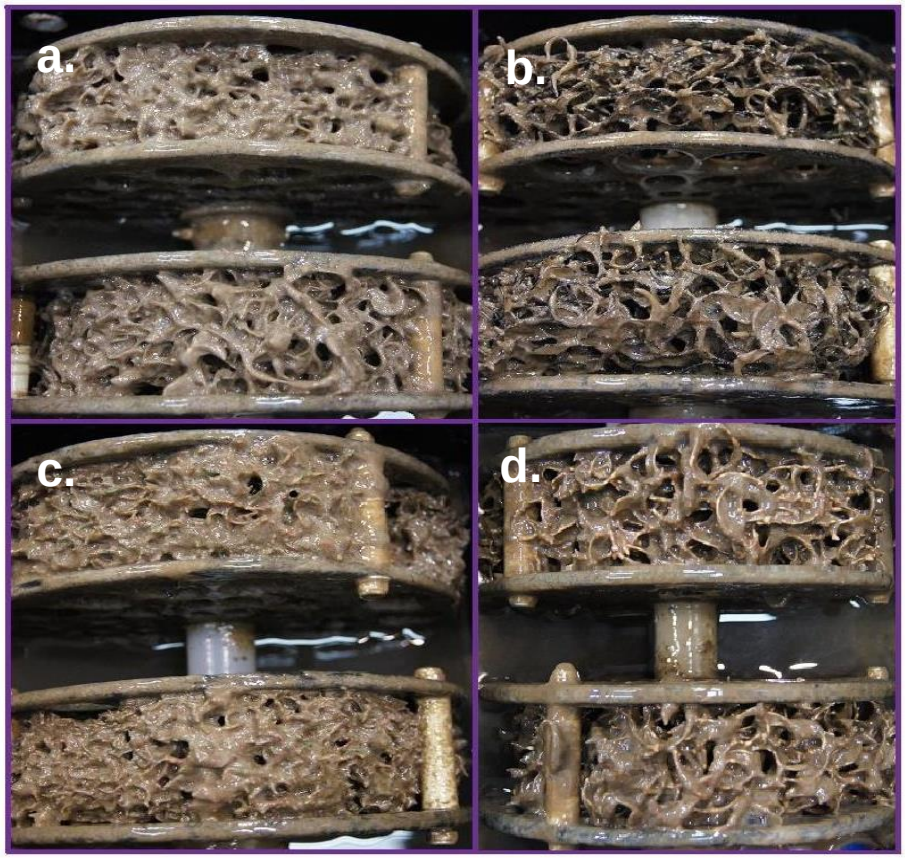
Appendix B Description of experimental setup

B.1 Chapter 3

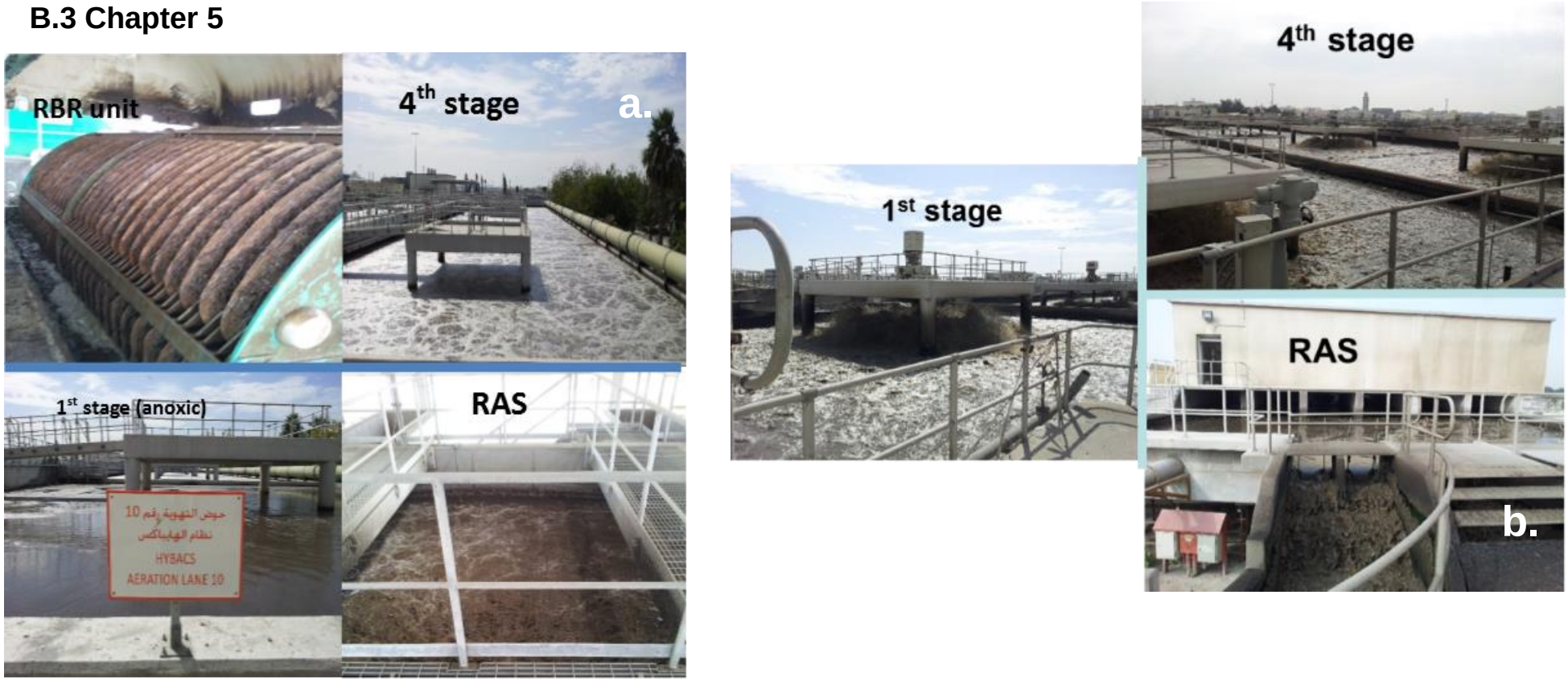
B.1.1 Mesh, coarse foam and fine foam media in single pass RBR setup

B.2 Chapter 4

B.2.1 Mesh media after biofilm development a. PVC-L, b. PP-L, c. PVC-H, d. PP-H single pass RBR reactors.



B.3 Chapter 5



B.3.1 Sample sites from Figure 6 a. Modified activated sludge with RBR upgrade b. CAS plant.

B.4 The impact of SLR, OLR and solids type on performance and EEA in hybrid biofilm reactors.

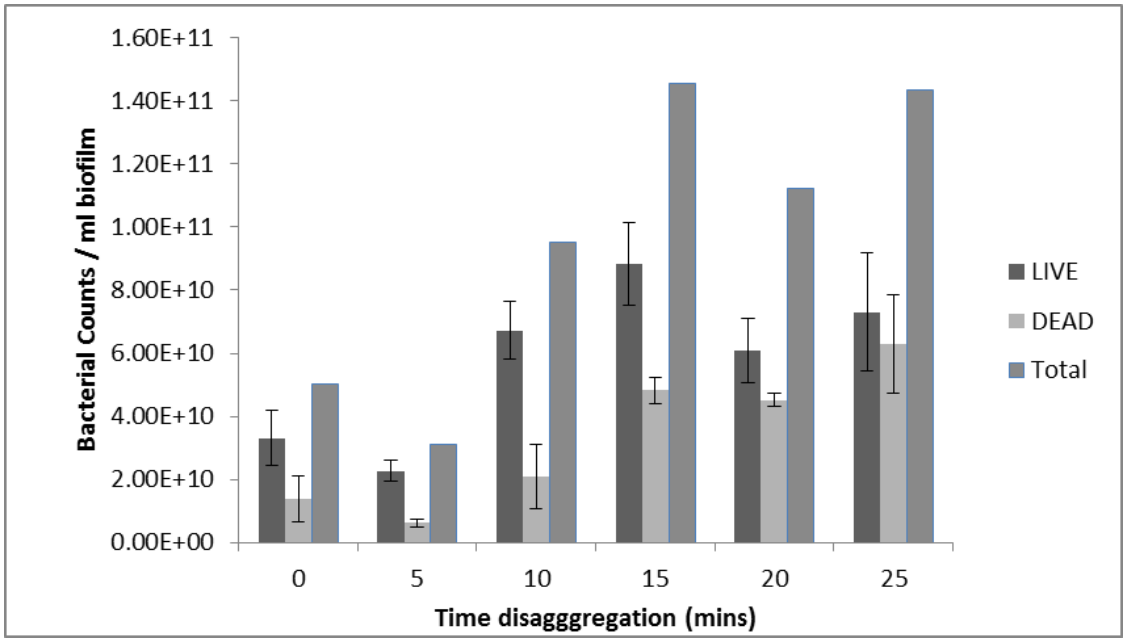
B.4.1 Experimental setup for Chapter 6 a. calibrated flow splitter for influent feed, b. Recycle pumps top of image and hybrid RBRs bottom, c. Feed tanks for HS and FE, d. FE sample point, e. HS sample point from bottom of clarifier, f. pilot scale ASP reactor.

Appendix C Biofilm development on mesh media during chapter 3

A. Mesh media, B. Coarse foam, C. Fine foam. Biofilm development and solids accumulation occurred with time. Pore clogging was noticed in coarse and fine foam prior to mesh media.

Appendix D Method development and validation for viability testing from chapter 3.

D.1 Determination of disaggregation rate for enumeration from complex samples



Ziglio et al. (2002) found that 10 minutes was optimum for full disaggregation of activated sludge flocs. Due to the delicate nature of the biofilm we hypothesised that the time required would be lower. However around 15 mins provided the maximum yield of live cells prior to increased abundance of dead cells found at 25 minutes (D.1).

D.2 Bacterial enumeration by CLSM

Optical density was used as a proxy to estimate bacterial numbers and thus, numbers of bacteria that would be visible under the microscope. The total numbers of bacteria should be ~60 cells per focal view. This was calculated after Equation 16.

Equation 16 – Calculation of cells per ml based on CLSM microscopy images

$$N = [(A_f/A_g)*x]/(V_s*d)$$

N= Average number of cells per grid

A_f = Area of filter covered by sample

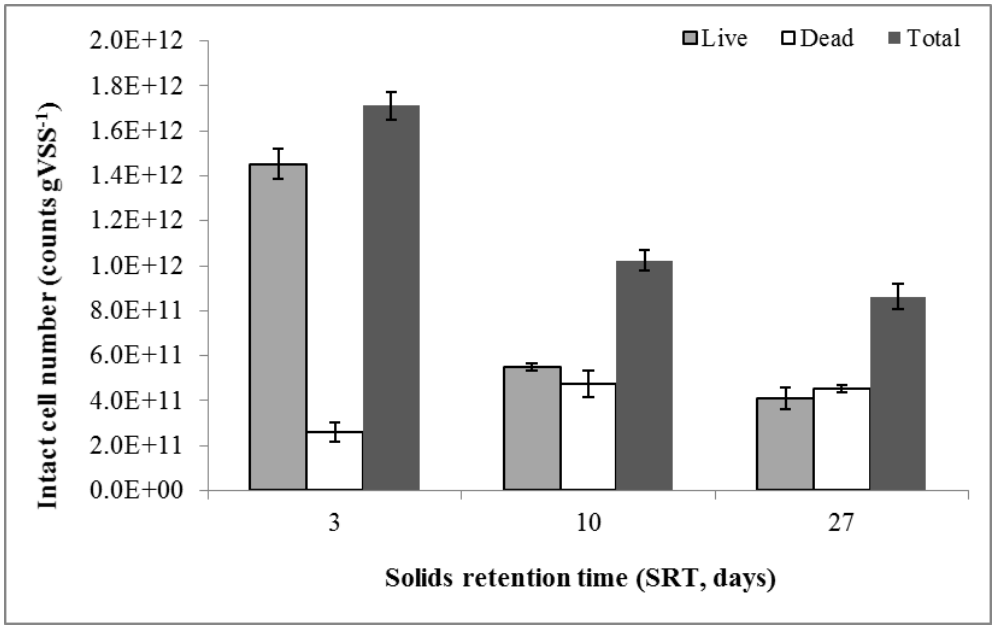
A_g = Area of grid

V_s = Volume sample

d = dilution factor (V_{final} / $V_{intitial}$).

D.3 Determination of viability methodology on activated sludge flocs – impact of SRT on viability.

To appreciate whether we have developed a sound methodology for testing the viability of wastewater bacteria a short study was commissioned. The study was to look at the impact of SRT on wastewater bacterial floc viability. The hypothesis was that elevated SRT could reduce the bacterial viability through endogenous decay and that being within a reactor systems for longer time could increase the number of dead cells within the floc. The highest bacterial viability (1.43 x 10^{12}.cells counts.$gVSS^{-1}$ was a 3 day SRT which decreased significantly at 10 and 27 day SRT).

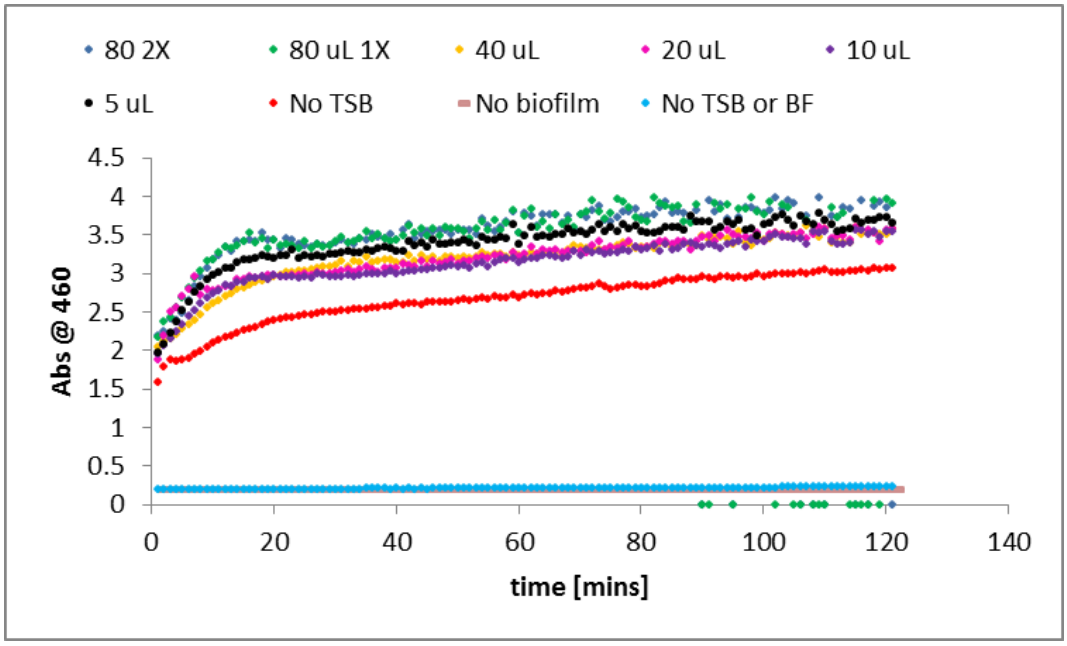


D.3.1 Impact of SRT on live, dead and total cells in a pilot scale activated sludge reactor (Chapter 6 and Petrie et al. 2014).

These data were presented in modified form in Petrie et al. (2014).

Appendix E Method development and validation for bacterial activity and rapid microbial viability measurement.

E.1 Rate based activity assay



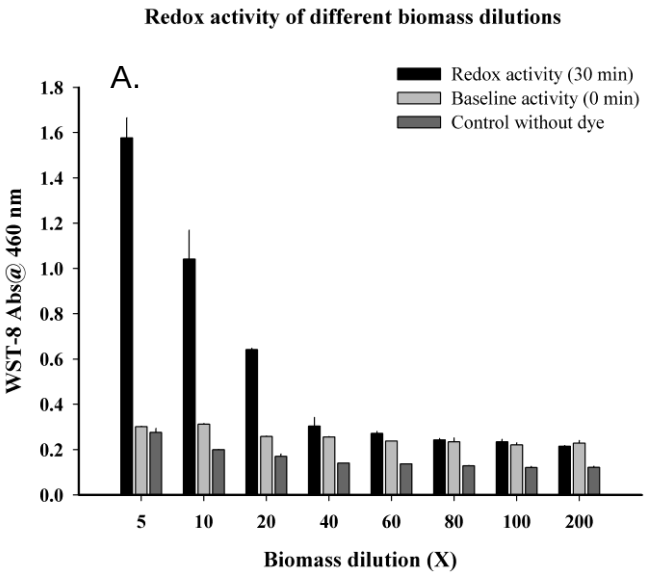
Legend represents volume of tryptone soy broth (TSB) utilised in each reaction well. Volume was made up with DI. The prefix 2X and 1X represent the relative strength of the TSB based on standard microbiological recipes. Unless stated the relative strength is 1X. The No TSB and No biofilm represent the negative controls. The NO TSB or BF represent a double negative control.

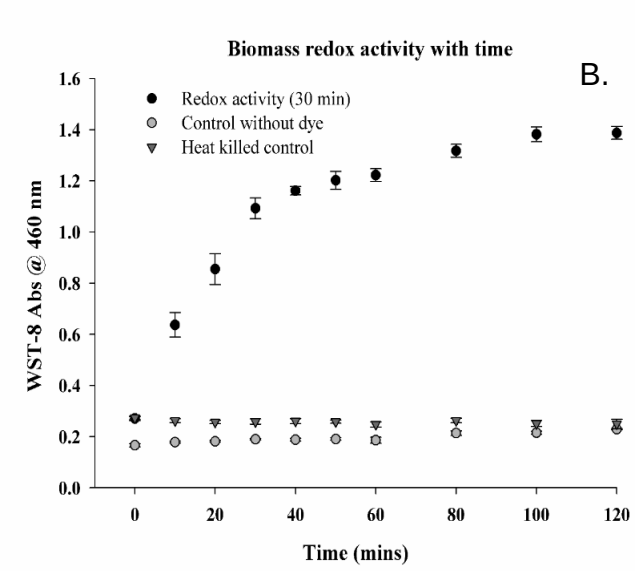
The aim of this work was to provide a rapid sensor for bacterial activity. The overarching null hypothesis being that performance is not dependent on specific bacterial activity. However measuring bacterial activity *in situ* is difficult in biofilm reactors and traditional biomass utilisation rate assays are rarely applied to solids state fixed media reactors such as RBCs and RBRs. The rate based assay which utilised the tetrazolium dye presented in Chapter 4 provided information on the

instantaneous biofilm activity in near real time. However inaccuracies caused by solids interference block the incident light on the spectrophotometer limited applicability beyond pure culture systems. E.1 shows that the highest volume of tryptone soy broth (uL) did not necessarily correspond to the greatest WST-8 linked increase in absorbance. Dilution of biomass resulted in activities being below detection limit of microplate reader.

E.2 End point activity assay method development

E.2.1 Microbial activity assay A. Redox activity of different biofilm dilutions, B. Biofilm redox activity with time.

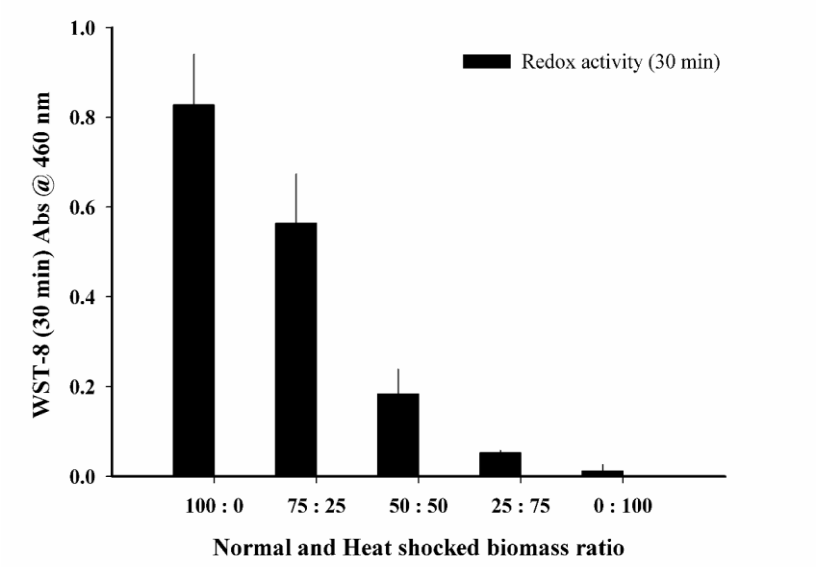




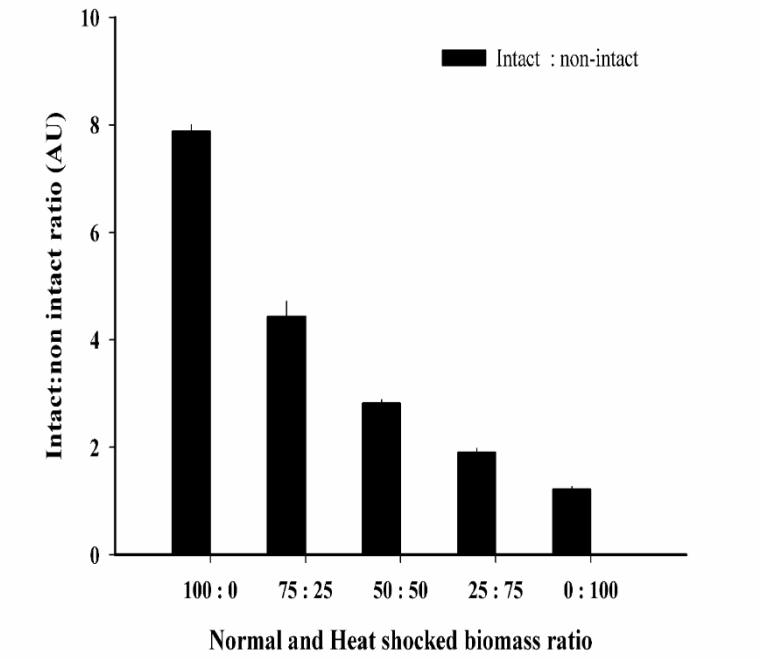
This section provides evidence for development of method used in section 4.3.6. Biofilm was harvested from mesh media after 3.3.3, 0 and 4.3.6. The samples were then diluted and centrifuged to remove the biofilm from solution. This subsequently reduced the solids interference and allowed accurate quantification of the microbiological redox activity signal. As expected diluting the biofilm 2 fold resulted in a 2 fold reduction in the net microbiological activity (8E.2.1 A) with 40x to 60x dilution being the lowest biofilm concentration that can be detected by the plate reader. Measuring the biofilm redox activity at different time points showed the state of the reaction. As the WST-8 detection dye and electron carrier were in excess it is likely that either substrate (TSB) or terminal electron acceptor was limiting. The end point was chosen at 20 minutes

as the reaction is still progressing at maximum velocity and as such provides a reference (8E.2.1 B).

E.2.2 Redox activity of different ratios of control and heat shocked biomass



To further validate the experimental method biofilm was subjected to 15 minutes at 75ºC in a heatblock. This inactivates the biomass and providing activity is linked to active microbial metabolism, the activity. Different ratios of normal and heat shocked biofilm were analysed for bacterial activity using the 20 minute end point assay. Stepwise reductions in microbial activity 8E.2.2) and viability (8E.2.3) were found.



E.2.3 Membrane integrity of different ratios of control and heat shocked biomass

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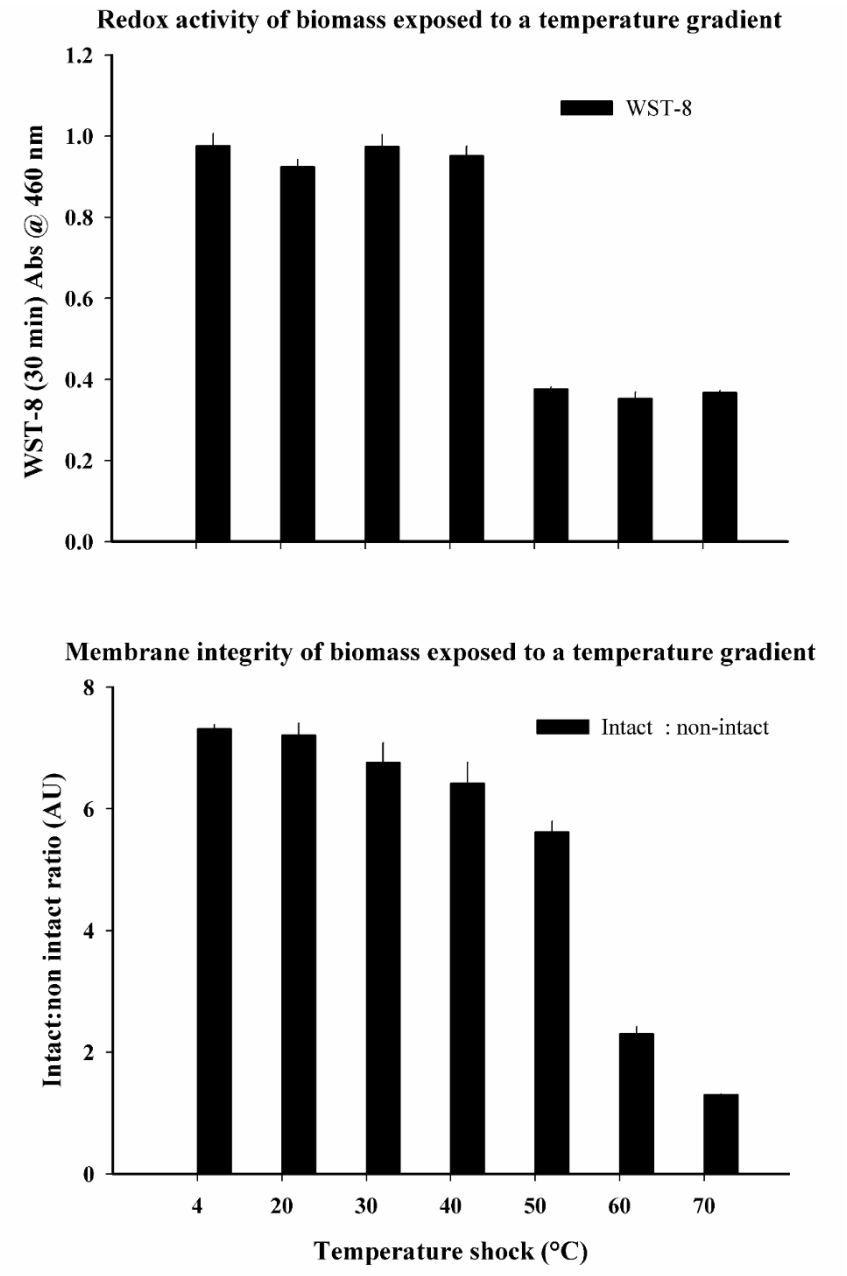
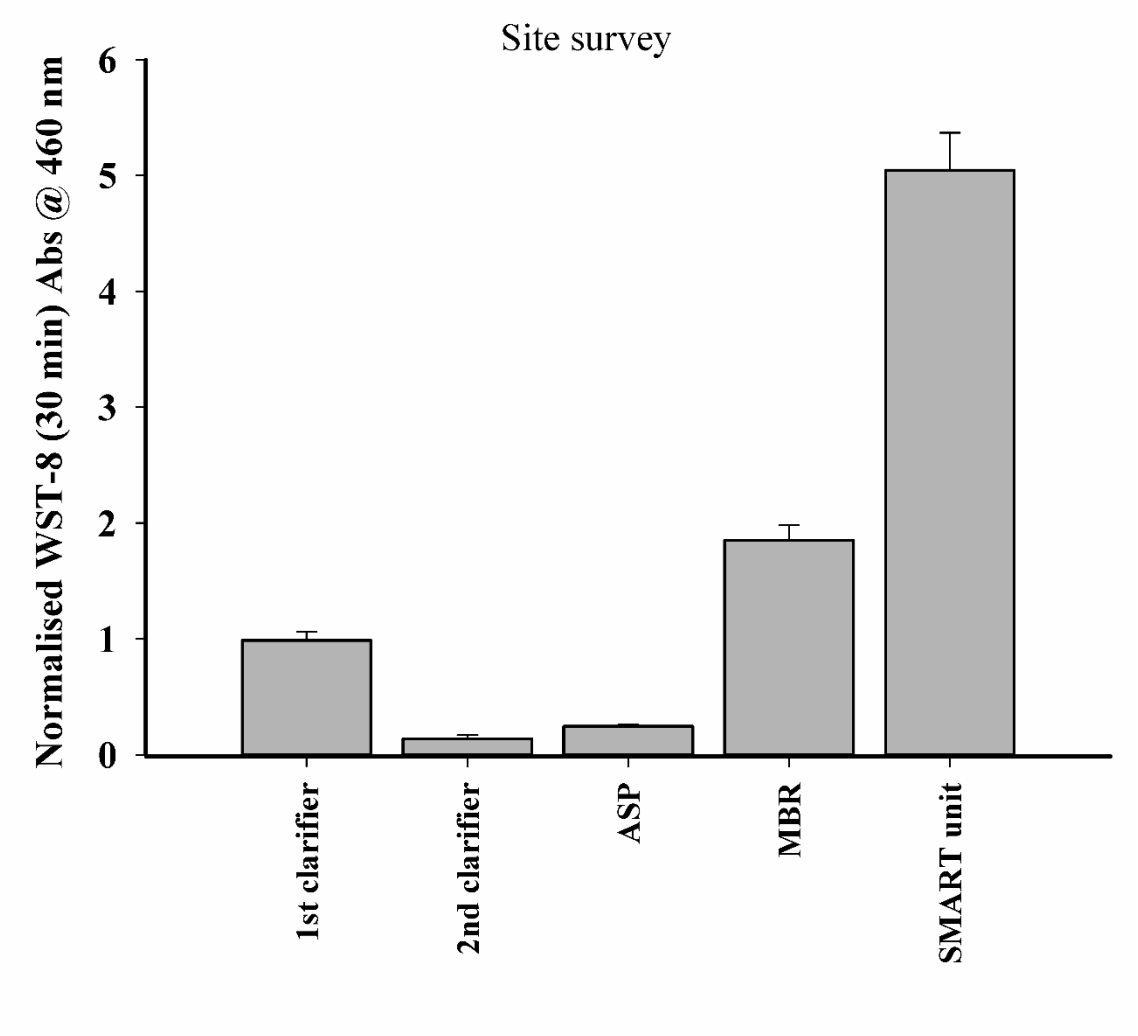
E.2.4 Effect of temperature on microbial activity and viability.



Biofilm was placed in a buffered solutions (1/4 strength ringers) the tubes were sealed and the outside sterilised with 70% ethanol. The tubes were placed in PCR gradient program ranging from 4-70 ºC. At >40ºC the RBR biofilm was inactivated presumably due to enzyme denaturing, however the viability assay did not demonstrate such decrease in intact:non intact cell ratio. This suggests that the WST-8 assay was more sensitive than viability for detecting a stress in mixed culture biofilms. Further highlighting that cells can be ‘viable’ but not ‘active’.

E.2.5 Site survey utilising WST-8 redox activity.

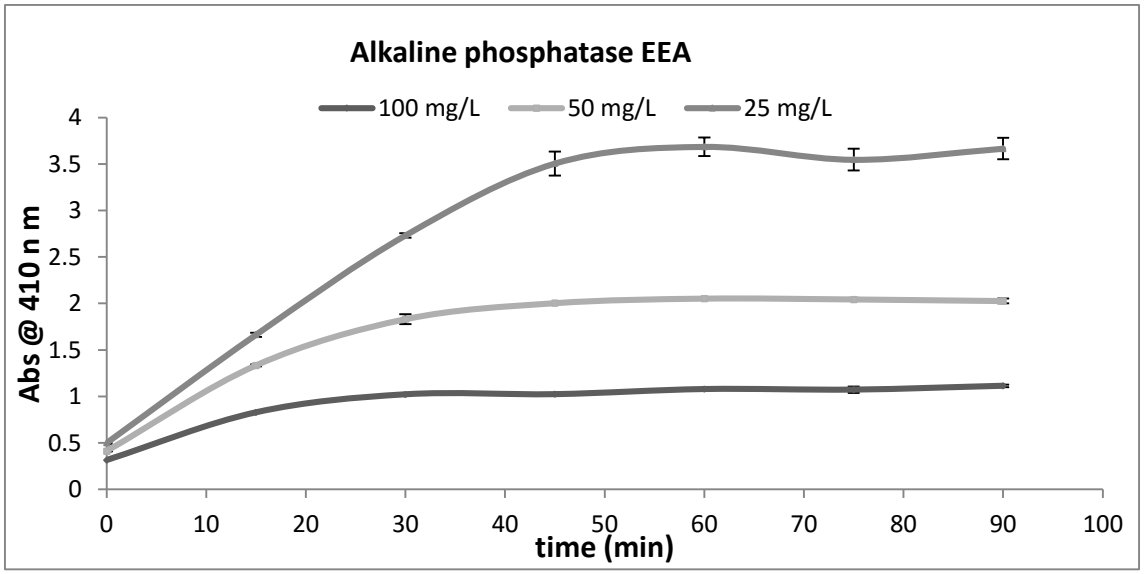


A site survey using the newly developed WST-8 end point assay was undertaken. As future works impinged on recycling 'solids' from sources (Chapter 6) there was benefit in understanding the relative microbiological activities of different systems. The SMART unit (RBR) biofilm was the most active which was 2.5x more active that MBR biomass (E.2.5). Relatively low activity in the pilot scale ASP could be due to dilute nature of the medium. Relatively low activity in the secondary clarifier could be due to lack of heterotrophs in this solids fraction or inactivation or decay towards the end of the Cranfield university wastewater treatment works.

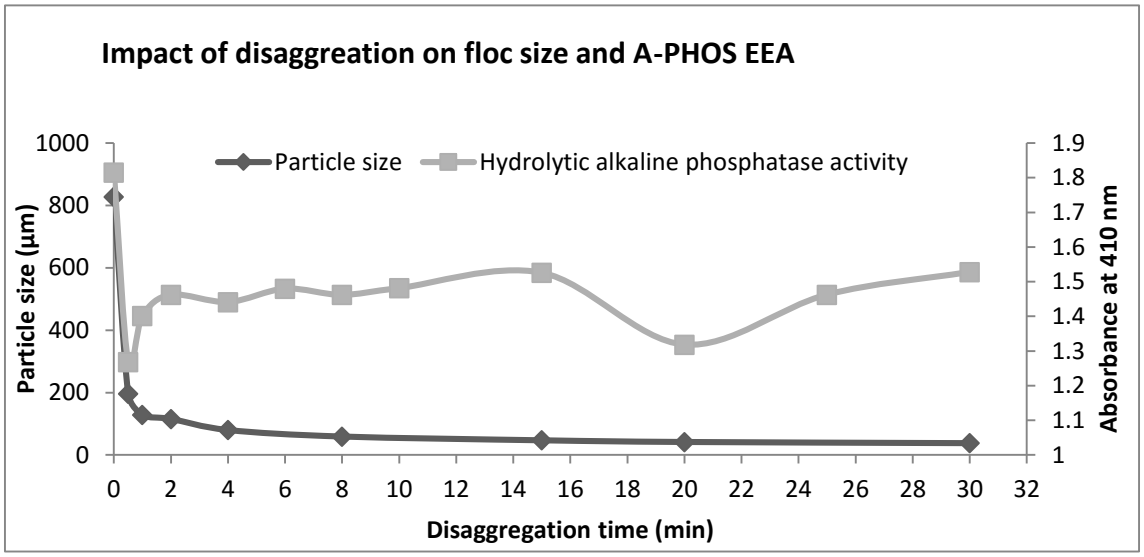
Appendix F Method development for extracellular enzyme activity measurements

The works undertaken in E.2 suggested that the activity of microbiological mixed culture systems can vary dramatically despite similar wastewaters being treated. However this approach suffers from similar limitations to biomass utilization rate assays in that laboratory medium i.e. TSB rarely contain the substrate heterogeneity experienced when utilising real wastewater. Furthermore it is generally accepted that the principal barrier to high rate wastewater treatment is degradation of polymeric or particulate components. As adsorption and subsequent MT represents a significant barrier prior to the relatively slow extracellular enzymatic hydrolysis that is required. The first step was to select and examine different polymeric extracellular artificial substrates and look at degradation rates.

F.1.1 Impact of substrate concentration on EEA



F.1.2 Effect of disaggregation rate on particle size and extracellular enzyme activity (EEA).



The EEA increased with time and roughly obeyed the Michaelis-Menten model for enzyme kinetics (F.1.1). To understand whether diffusion resistance impacts extracellular enzyme activity the biofilm was disaggregated for different amounts of time and compared to a control which was mixed but not dispersed by pipetting. Disaggregation was performed using method previously characterised in Hassard et al. (2014). Elevated alkaline phosphatase activity was found after disaggregation and the greatest different was between 0 and 2 minutes disaggregation time. This could be by liberating bound enzymes, removing the diffusion barrier to artificial substrates or increasing the effective surface area for EEA (F.1.2). Unlike the end point activity assay it was thought that whilst the maximum rate of hydrolysis could be similar the kinetics would provide more useful information to understand the different in EEA between different treatments or reactor types. The next step involved characterisation of the kinetics of the EEA of biofilms. This represented a significant technical challenge and was beyond the scope of the student project which began the work.

Appendix G Script for determining Michaelis-Menten kinetics of different substrates.

Equation 17 - The Michaelis-Menten equation

$V_o = (V_{max}[S])/ K_m + [S]$

Where V_0 is the initial velocity of a known substrate concentration, V_{max} represents the maximum EEA, [S] is the artificial substrate concentration, K_m is the Michaelis-Menten coefficient. Measuring the 'EEA' as determined by V_{max} and K_m required more than 'by eye' estimation. Routine statistical packages proved wholly inadequate for either estimating these parameters or statistically analysing any difference or deviations from the models. Venables et al. (2011) provide examples of how to analyse these data using 'R' statistical package. An example script which was modified to suit data presented in Chapters 5 and 6. The y data and axis represent the V_0 measured for each substrate concentration presented on the x axis.

Coding:

```
y <- c(21.97, 33.52, 63.28, 81.97, 94.65, 20.00, 41.93, 44.84, 80.25, 106.64)

x <- c(164.23, 328.63, 656.86, 1313.10, 2627.4, 164.23, 328.63, 656.86, 1313.10, 2627.4)

df <- data.frame(x=x, y=y)

fit <- nls(y ~ SSmicmen(x, Vm, K), df)

fit
summary(fit)
```

Example results:

```
Nonlinear regression model
  model: y ~ SSmicmen(x, Vm, K)
   data: df
  Vm     K
```

136.3 925.3
residual sum-of-squares: 307.5

Number of iterations to convergence: 0
Achieved convergence tolerance: 1.044e-06
> summary(fit)

The relative iteration convergence tolerance of <1e-4 was selected for all Michaelis-menten curve estimation. Convergence tolerance refers to the second order moment representation of the error associated with each iteration. Larger values may produce inaccurate results since convergence tolerance applies to each step, and error may propagate and accumulate over a series of iterations.

Formula: y ~ SSmicmen(x, Vm, K)

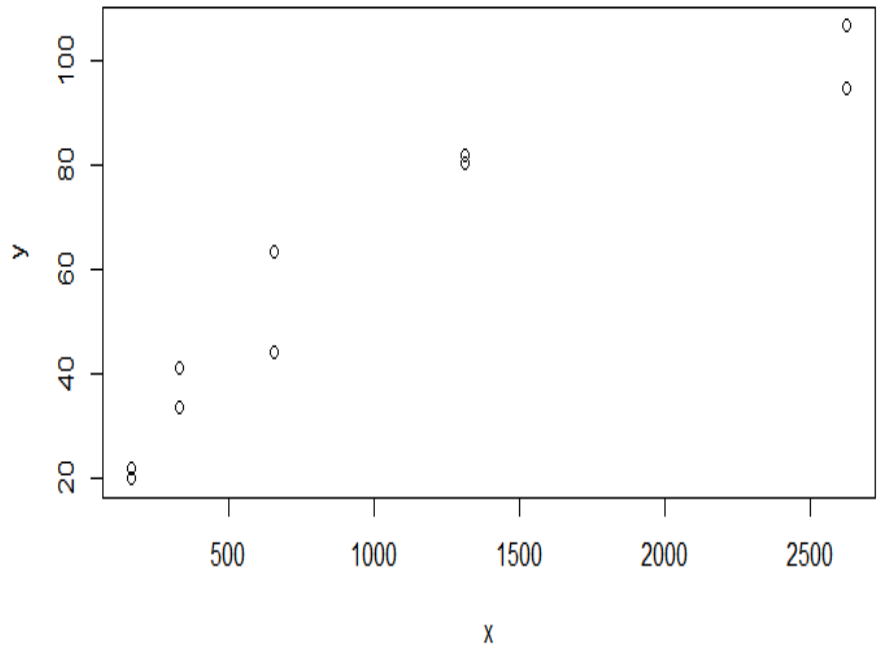
Parameters:
  Estimate Std. Error t value Pr(>|t|)
Vm   136.31   10.94 12.455 1.61e-06 ***
K   925.30   174.78   5.294 0.000734 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 6.199 on 8 degrees of freedom

Number of iterations to convergence: 0

Achieved convergence tolerance: 1.044e-06



In this example rate data (~200 values for each substrate concentration) are calculated based on standard curves for each substrate. Triplicate values from each concentration are plotted. The kinetic parameters are measured by a least squares method whereby the permutation with the lowest sums squares has the highest probability of being correct. A hessian matrix and t-test is used to calculate whether the data plotted deviated from the Michaelis-Menten ‘ideal’ model. Degrees of significance are highlighted in red and results are highlighted in yellow.