Science of the Total Environment 625 (2018) 1527–1534

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microbial extracellular enzyme activity affects performance in a full-scale modified activated sludge process

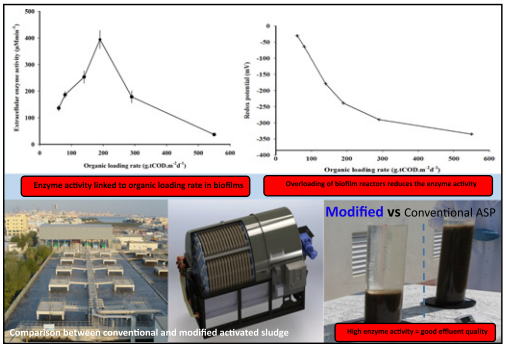
Francis Hassard [a,*], Jeremy Biddle [b], Richard Harnett [b], Tom Stephenson [a]

[a] Cranfield University, Cranfield MK43 0AL, UK
[b] Bluewater Bio, Winchester House, Old Marylebone Rd, Marylebone, London NW1 5JB, UK

HIGHLIGHTS

- Activated sludge (AS) process was modified using a rotating biofilm reactor (RBR).
- Extracellular enzyme activity (EEA) in the biofilm was 4.6–13.5 × greater compared to AS biomass.
- RBR EEA increased with increasing organic loading rate (OLR) to a maximum at 190 g·tCOD·m^{-2}d^{-1}.
- Modified activated sludge had 2 and 1.5 × the volumetric removal rate of COD and NH_4-N respectively.
- Elevated EEA correlated with tCOD removal performance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 September 2017
Received in revised form 8 January 2018
Accepted 8 January 2018
Available online xxxx

Editor: Simon Pollard

Keywords:
Biofilm
Microbial extracellular enzyme activity
Hybrid activated sludge
Hydrolytic activity
Performance

ABSTRACT

The rate-limiting step of wastewater treatment is the breakdown of polymers by extracellular enzyme activity (EEA). The efficacy of EEA on biomass from full scale conventional activated sludge (AS) and modified AS with bench scale and full scale rotating biofilm reactors (RBR) was compared. The maximum amino-peptidase EEA was 394 ± 34 μmolL^{-1} min^{-1} for the bench RBR which was 11.7 and 4.5 times greater than maximum α-glucosidase and phosphatase EEA in these reactors. At full scale the RBR gave ~4.6, 13.5 and 6.3 times the EEA for amino-peptidase, α-glucosidase and phosphatase (based on enzyme V_{max}) compared to the highest EEA in conventional AS biomass. Controlled overloading of the bench RBRs revealed that EEA increased with OLR up to 190 g tCOD m^{-2}d^{-1} and further increases in OLR reduced the EEA. Pretreatment of wastewater by EEA in the RBR was linked to better performance of the modified activated sludge process. Maintaining high EEA of biofilms is critical for the design of high OLR wastewater treatment systems.

1. Introduction

Extracellular enzymatic hydrolysis is required as a first step in the breakdown, utilisation and therefore treatment of organic wastewater polymers (Cadoret et al., 2002). Municipal wastewater is a complex matrix of carbohydrates, proteins and lipids, ~50% of which has a molecular weight > 1 kDa, limiting mass transfer into bacterial cells (Burgess and Pletschke, 2008). The majority of extracellular enzyme activity (EEA) is associated with bacterial cell walls, floc, granule and biofilm, depending on the biological process in operation (Wingender and Jaeger, 2002). High enzyme stability in the environment has been noted previously; however, the EEA in activated sludge systems is ultimately reliant on continued enzyme

* Corresponding author.
E-mail address: francis.hassard@cranfield.ac.uk (F. Hassard).

https://doi.org/10.1016/j.scitotenv.2018.01.073
0048-9697/

synthesis, due to wasting of bacteria and enzymes from biological reactors (Confer and Logan, 1998; Goel et al., 1999; Morgenroth et al., 2002). Polymers are utilised by bacteria through: (1), transport/adsorption, (2), stepwise depolymerisation and (3), assimilation/storage. Together, these factors limits achievable removal rates in wastewater treatment (WWT) and therefore organic loading rates (OLRs) that can be applied (Orhon and Çokgör, 1997; Martins et al., 2003; De Kreuk et al., 2010).

Conventional AS 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) showed that activated sludge bacteria increased their EEA to a source of polymeric substrate in <2 h, suggesting the adaptive response is faster than, but linked to, population level changes (Wingender and Jaeger, 2002). Therefore, manipulation of bacterial growth rates and EEAs, could be used to maximise the efficacy of wastewater treatment by controlling biomass/biofilm sludge age (Shackle et al., 2000). This is pertinent, considering the environment of increasingly stringent wastewater discharge consents, within a regulatory framework of whole life costs for commissioning and operation of wastewater treatment assets (Ainger et al., 2009). Therefore, to meet these challenges, improvements to the efficiency and operation of existing biological wastewater processes are required (Truu et al., 2009) which could be attained through greater understanding of EEA (Curtis et al., 2003).

To achieve effluent standards within financial constraints with low energy usage, it is imperative to innovate through new technology and optimise process operation (STOWA, 2010). Numerous technologies exist for upgrading existing wastewater treatment works (WWTW) e.g., membrane bio-reactors (MBRs) (Judd, 2010; Judd, 2017) which treat high organic loads to appropriate effluent standards but can have much increased power consumption per m^3 treated compared to conventional activated sludge systems (Fenu et al., 2010). Integrated fixed film activated sludge (IFAS) and 'hybrid' moving bed biofilm reactors (MBBRs) have been successfully utilised to upgrade existing works (Mannina and Viviani, 2009); however, they have limited ability to control biofilm growth and can have high capital expenditure (CAPEX) costs principally due to media purchase. A modified activated sludge (AS) process known as Hybrid Activated Sludge (HYBACS) uses an upstream rotating biofilm reactor (RBR) for high organic load treatment (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 organic loading rates (OLR) typically upstream of other secondary treatment reactors (Chen et al., 2006; Hassard et al., 2015; Hassard et al., 2016). The impact of overloading on RBR performance and the impact of these physicochemical conditions (e.g. anaerobic biomass, low oxygen concentration) on the biofilm EEA has yet to be demonstrated at full scale.

High OLR conditions in the RBRs present challenges for diffusion of high molecular weight compounds and electron acceptors which could limit expression or activity of the extracellular enzymes and therefore the rate of treatment which can be achieved. In modified AS, 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; Daigger et al., 1993). It is hypothesised that RBR and modified AS bacteria have elevated EEA which could contribute to better performance compared to conventional biological processes earlier observed (Biddle et al., 2014; Hassard et al., 2014). To test this, the microbial EEA and performance in a conventional AS and a modified AS system with an RBR were compared at a full scale WWTW. Controlled organic overloading was also performed with bench scale RBRs to assess the impact of high OLRs on EEA of amino-peptidase, α-glucosidase and phosphatase under controlled conditions.

2. Materials and methods

2.1. Full scale study site

Tubli WWTW serves the city of Manama, Bahrain with a design capacity of 200,000 m^3d^{-1} and 41,000 $kg \cdot BOD_5 \cdot d^{-1}$, representing ~700,000 PE (Table S1). The effluent standard was 15 mgL^{-1} BOD_5, 20 mgL^{-1} suspended solids and 3 mgL^{-1} NH_4-N at the time of the study. The WWTW had 8 lanes of conventional AS and 2 lanes of modified AS. The modified AS consisted of 42 RBRs upstream of 2 aeration lanes, with a single pre-anoxic zone and 3 aerated zones (EDI, Flexair 9″ membrane diffusers, USA) of total volume ~25,000 m^3. The RBRs were located downstream of the mixing point of wastewater with RAS. Each RBR incorporated motor driven (2.2 kW, Sumitomo Buddybox, Japan) porous polyvinyl chloride (PVC) 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 set to 5.8 rpm. The wastewater flowed from the RBRs to the anoxic stage of the 2 activated sludge lanes (Fig. 1). 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 (Fig. 1). For the period of study, the RAS recycle ratio was maintained at 0.75 as a proportion of influent flow rate.

The conventional AS consisted of eight lanes of aerated (40 × 80 kW surface aerators) operated with identical wastewater influent but without a pre-anoxic stage for biological nitrogen removal. The RAS recycle rate in the conventional AS was 0.75–0.9 as a proportion of influent flow rate. The full-scale study was commissioned between July 2013 and June 2014. Wastewater analysis was undertaken daily from the sampling points outlined in Fig. 1. The EEA study was undertaken at Tubli WTW in February 2014 for a period of 1 month.

2.2. Bench scale study

Four bench scale RBRs, each being a polycarbonate vessel with plastic frames for housing the media (Hassard et al., 2014), treating settled wastewater from Cranfield University wastewater treatment works (WWTW). The media consisted of 2 circular mesh plates (Bluewater Bio, UK) in each reactor contained PVC material with a specific surface area of 150 m^2 m^{-3} which was similar to that used in the full-scale study. The RBR operation is outlined in Hassard et al. (2016), with different surface organic loading rates (OLRs) selected based on soluble COD and ammonia (NH_4-N). The reactors were commissioned at a low flowrate of 1.1 Lh^{-1} and increased over time to 2.2, 4.4, 8.8, 17.2 and 34.4 Lh^{-1}. These flowrates corresponded to nominal hydraulic retention times of 200, 100, 50, 25, 12 and 6 min respectively. The bench scale study was conducted between March 2014 and June 2014, with the RBR operated for approximately 1 month at each nominal hydraulic retention time.

2.3. Sampling and pretreatment of biofilm and biomass

Mesh media biofilm was harvested from the three RBRs at the Tubli WWTW for analysis of EEA after the method outlined in Hassard et al. (2014, 2016) and mixed liquor suspended solids (MLSS) was taken from the sampling points outlined in Fig. 1. At Tubli WWTW, the EEA was measured in the modified AS in the biofilm, the MLSS, the RAS and in the plant effluent. This was compared to the influent, the MLSS, the RAS and the plant effluent from the conventional AS operated concurrently (Fig. 1). Biofilm from bench scale RBRs was harvested as above. Duplicate analysis of the EEA assays and biofilm solids were undertaken on three samples for each sampling event which occurred at each sample point. The biofilm total solids concentration was measured after the method of Regmi et al. (2011).

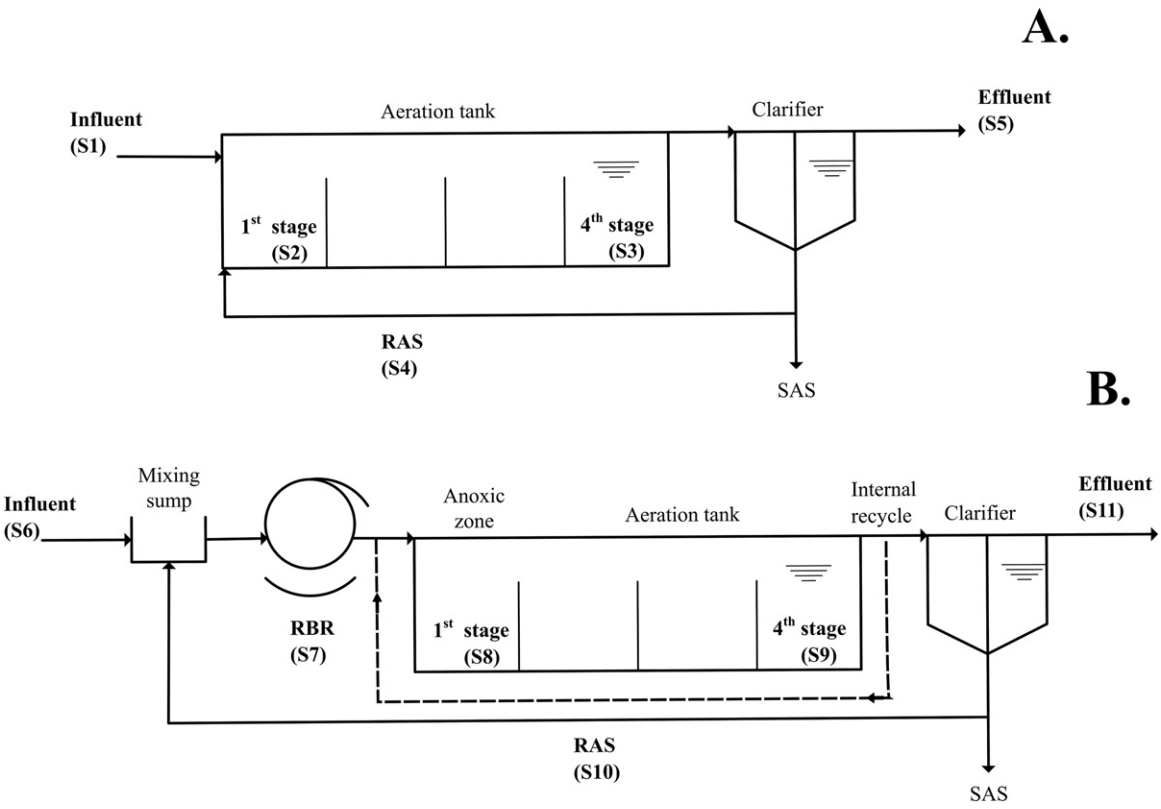


Fig. 1. Sampling points for enzyme study presented in brackets. A. conventional activated sludge at full scale plants B. modified activated sludge. Dashed line represents the an internal recycle of wastewater.

For EEA assays all batch samples were subjected to identical pretreatment: (1) to batch samples (100 mL for MLSS/RAS and 50 mL for biofilm) buffer (50% v:v) was added, (2) to this buffer/biomass mixture, methanol was added (10% v:v) (Lunau et al., 2005), (3) 100 mL of this biomass was disrupted mechanically using a homogeniser (T50 Ultra-turrax, IKA, Germany) for 1 min at 6000 rpm, to reduce mass transfer limitation (Hassard et al., 2014), (4) the disrupted biomass was handled by pipetting (Finntip™ Wide Orifice Pipette Tips, Thermofisher, UK) and 0.5 mL of this disrupted biomass was transferred to 48 well microtiter plates prior to the EEA assays.

2.4. Extracellular enzyme activity assays

Biomass samples (from the above pretreatment) were incubated in the dark at ~25 °C and mixed for 10 s at 200 rpm (Minishaker, VWR, UK) with appropriate synthetic substrate which was dissolved in the same buffer as the diluted biomass (50% biomass: substrate v:v) (Sigma Aldrich, UK). This resulted in a final volume of 1 mL for each biomass and substrate assay per microtiter well. The EEA was measured by analysing the production of the chromophore product of the extracellular enzyme reactions. Details of buffers are given in Li and Chróst (2006). Three different EEA assays tests were performed for each biomass sample, which together represent the majority polymeric degradation required for the heterotrophic removal of bulk organics. This is because the organic component of municipal wastewater is principally comprised of carbohydrates and proteins (Molina-Muñoz et al., 2010). The phosphatase enzyme group was selected for study due to the importance of phosphate accumulation for biological phosphorus removal and as an important step within lipid degradation. The three EEAs were performed for protein hydrolysis, carbohydrate degradation and organic phosphate hydrolysis i.e. the generic enzyme types amino-peptidases, α-glucosidases and phosphatases respectively. This was measured after catalysis of synthetic substrates: l-leucine-p-nitroanilide (Sigma L9125), p-nitrophenyl-α-d-glucopyranoside (Sigma N1377), p-nitrophenyl-phosphate (Sigma 104-0) respectively. The EEA assay run times was set to 2 h, which provided sufficient time for the absorbance to plateau, despite the dynamic variability of the enzyme activity between the different enzymes and sample points. The absorbance of p-nitroaniline ($\lambda_{max} = 380$ nm) and p-nitrophenol ($\lambda_{max} = 348$ nm) was measured at six substrate concentrations (ranging from 10 to 250 μM) using a spectrophotometer (Hach-Lange, DR 2800) and a microplate reader (M2000 infinite pro, Tecan, Austria) for samples taken at full and bench scale respectively. The experimental setup included of biomass with substrate and controls no substrate control, no biomass control and deionised water (DI) blanks to ensure there was no intrinsic change in absorbance or contamination of substrate stocks. Calibration curves were created using analytical grade 4-Nitrophenol (4-NP) 10 mM solution (N7660, Sigma-Aldrich, UK) and 4-Nitroaniline powder (4-NA, 31569, Sigma-Aldrich, UK). A stock solution of 4-NA was created by dissolving the power in 100% ethanol (1.38 mg/mL) which was stored at −20 °C. A dilution series was created by diluting the stock solutions with appropriate buffers to a final concentration of 10–100 μM. These calibration curves were used to calculate the concentration of substrate liberated per time. The initial rate of the enzyme substrate reaction was used to calculate the EEA. The V_0 is defined as the initial velocity of the enzyme/substrate reaction. The V_{max} is defined as the maximum enzyme velocity which can be achieved in a dynamic system, where substrate (S) itself does not limit the reaction rate which can be achieved. The K_m is the Michaelis–Menten constant and is defined as the substrate concentration at half the V_{max} (Chen et al., 2010). (1) the Michaelis–Menten equation (Eq. (1)) was solved using a non-linear least squares method for kinetic parameter estimation (V_{max}, K_m) after Kemmer and Keller (2010); (2) the standard error of mean and significance of model fit were calculated using a Hessian matrix and *t*-test respectively (Venables and Smith, 2011). The specific enzymatic activity was quoted as the maximum rate per gram of volatile

suspended or immobilised solids calculated after Eqs. (2) and (3) respectively:

$$V_0 = (V_{max} \times S)/(K_m + S) \quad (1)$$

$$\text{Maximum specific EEA suspended biomass} = V_{max}/VSS \quad (2)$$

$$\text{Maximum specific EEA biofilm} = V_{max}/TS \quad (3)$$

2.5. Wastewater analysis

A composite influent sample was analysed from two discrete grab samples which were collected at 10:00 ± 1 h within a two-hour period. A composite effluent sample was analysed from two discrete grab samples over a two-hour period after a single hydraulic residence time (HRT) from the point when the influent samples were taken. Wastewater was analysed using proprietary cell test kits (Hach-Lange, Germany) for total chemical oxygen demand (tCOD), total nitrogen (TN), ammonia-nitrogen (NH_4-N), nitrite nitrogen (NO_2-N) and nitrate-nitrogen (NO_3-N) using a Hach DR 2800 spectrophotometer (Hach-Lange, Germany). Soluble chemical oxygen demand (sCOD) was assessed during the intensive enzyme study (February 2014) only. 20 mL of wastewater was filtered using cellulose nitrate filters (0.45 μm, Millapore, UK) and the filtrate was analysed for COD as above. Biochemical oxygen demand (BOD), mixed liquor suspended solids (MLSS), volatile suspended solids (VSS) were measured according to standard methods (APHA-AWWA-WEF, 2012). The pH of the influent and effluent was measured using a Jenway 320 pH meter (Bibby, UK). 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 standard methods for suspended growth and biofilm reactors (APHA-AWWA-WEF, 2012). For the suspended growth reactors, the sludge retention time (SRT), sludge volume index (SVI) were calculated (APHA-AWWA-WEF, 2012). Flocs were characterised under light microscopy (100× magnification) and subjectively quantified compared to a reference filamentous scale after Madoni et al. (2000).

2.6. Statistical analysis

Data were analysed using SPSS v22 (IBM, USA) for calculation of analysis of variance and *t*-tests between sites/locations. To compare biological variables (e.g. Michaelis–Menten parameters) with operating variables (Biofilm TS, VS, OLRs, % removal, effluent quality) a non-parametric Spearman Rank Correlation Coefficient was used, as the data was not normally distributed after transformation was applied. Of these variables, the biofilm VS was removed due to strong auto-correlation with biofilm TS. Influent concentrations were not included due to strong auto-correlation with OLRs.

3. Results and discussion

3.1. Wastewater characteristics

The influent wastewater at Tubli WWTW had an average tCOD of 483 mgL^{-1}, sCOD of 175 mgL^{-1}, NH_4-N of 24 mgL^{-1} and TSS of 287 mgL^{-1} (Table 1). Influent conditions were similar during the study period, except December 2013–February 2014 when the tCOD was significantly higher at 639 ± 55 mgL^{-1} compared to normal operation of 396 ± 48 mgL^{-1} ($p = .03$), attributable primarily to a 40% increase in influent suspended solids concentration. The wastewater pH, sCOD and NH_4-N did not differ significantly. The wastewater entering the conventional AS and the modified AS plants were from the same source and therefore the same in composition. The volumetric loading rate was 1.3 times less in the conventional AS compared to the modified AS (Table S1). The performance of the conventional and modified AS prior to EEA study is described in the supplementary material. The bench scale study influent wastewater 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 to that characterised previously (Hassard et al., 2016). The effluent quality in terms of sCOD and NH_4-N of bench scale RBRs worsen with increased OLR. In contrast the tCOD effluent quality improved at OLRs >190 $g \cdot tCOD \cdot m^{-2} \cdot d^{-1}$ (Fig. S3).

Table 1
Wastewater characteristics for conventional AS and modified AS full-scale plants during study period.

Parameter (mgL^{-1})	Influent	Conventional activated sludge	Modified activated sludge
tCOD	483 ± 172	65 ± 48	27.9 ± 13
sCOD	175 ± 73	–	–
TN	–	–	4.2 ± 3.3
NH_4-N	24 ± 5.4	13 ± 2.8	0 .06 ± 1.2
NO_3-N	–	–	5.5 ± 1.3
TP	4.1 ± 1.3	–	2.1 ± 0.6
TSS	288 ± 139	–	15.2 ± 10

(–) = not measured.

The modified AS plant had a 42% better NH_4-N volumetric removal rate of 0.096 ± 0.02 $kg \cdot NH_4\text{-}N \cdot m^{-3} d^{-1}$ compared to 0.057 ± 0.015 $kg \cdot NH_4\text{-}N \cdot m^{-3} d^{-1}$ for the conventional AS site ($p < .001$) (Fig. S1C) although this could be due to incomplete nitrification in the conventional AS site. The tCOD OLR was equivalent between the RBR at bench and the modified AS (Table 2). The conventional AS had a nitrification efficiency of 54.7 ± 10.5% on average, suggesting incomplete nitrification whilst, the modified AS achieved an average of 97.5 ± 4.2% (Table 1) efficiency despite significantly higher F:M (Table 3). The SUR of NH_4-N was 0.031 $kg \cdot NH_4\text{-}N \cdot kg \cdot MLSS$, in the modified AS compared to 0.022 $kg \cdot NH_4\text{-}N \cdot kg \cdot MLSS$ for the conventional AS suggesting greater nitrification rates (Table 3). The predenitrification anoxic zones which are present in the modified AS but absent in the conventional AS, reduced the effective volume for nitrification in the modified AS. However, improved nitrification performance could be attributed to: alkalinity addition by denitrification, improved nitrifier abundance or activity (Hassard et al., 2015; You et al., 2003) or improved mass transfer in the aeration tanks due to difference in the method of aeration.

The effluent NH_4-N was on average 0.6 mgL^{-1} (Table 1) for the duration of the study, irrespective of F: M applied (Fig. S2). 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 conventional AS. Nitrifier abundance could play a determinant role governing performance in hybrid systems (Hassard et al., 2015).

The modified AS had a better sludge volume index (SVI) of 43 ± 5.5 mLg^{-1} compared to 96.9 ± 1.7 mLg^{-1} for conventional AS ($p < .05$) (Table 3). The modified AS had for larger, denser flocs, which could contribute to the formation and stability of modified AS compared to conventional AS flocs (Lin et al., 2010). A filament index of between 0 and 1 revealed minimal filamentous bacterial groups in the modified AS compared to 3–4 for a well maintained conventional AS (Jenkins et al., 2003). The enzymatic pretreatment by RBR EEA could select against filamentous groups (Liao et al., 2004) by facilitating greater substrate penetration depth and three-dimensional floc growth as described previously (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 RBR acted as a pretreatment prior to the aeration lanes through biofilm growth and enhanced solids contact compared to the conventional AS plant (Daigger and Boltz, 2011). Increased EEA in the RBR could aid degradation of the polymeric fraction of the wastewater, improving the degradability and reactions rates downstream.

Table 2
tCOD organic loading rate, organic removal rate and % removal data of conventional activated sludge, modified activated sludge and bench scale rotating biofilm reactors. Data presented as average and range.

Reactor	Organic loading rate ($kg \cdot tCOD \cdot m^{-3} \cdot d^{-1}$)	Effluent tCOD removal %	Organic removal rate ($kg \cdot tCOD \cdot m^{-3} \cdot d^{-1}$)
Conventional AS	1.1 (0.7–1.6)	81.8 (48.4–95)	0.87 (0.4–1.3)
Modified AS	1.9 (0.6–4.1)	91.9 (74.4–97.1)	1.8 (0.6–3.97)
Bench scale RBR	10.2 (2.2–33.4)	43.6 (19–62)	4.3 (0.6–14.2)

3.2. Extracellular enzyme activity

Extracellular enzyme activity as measured by V_{max} for all three types of enzyme – amino-peptidases, glucosidases and phosphatases was between 4.6 and 13.5 times higher in RBRs than the next greatest value for suspended growth at full scale (Table 4). The maximum amino-peptidase EEA of 394 ± 34 $\mu molL^{-1}\ min^{-1}$ was for the bench RBR which was 11.7 and 4.5 times greater than maximum α-glucosidase and phosphatase EEA at the same sample point (Fig. 2A). At equivalent very high organic loading rates (OLR) of ~10 $kg \cdot tCOD \cdot m^{-3}d^{-1}$ (Table 2) the full scale RBR had 2.1 times greater EEA than the bench scale RBR for amino-peptidases and α-glucosidases and 3.2 times greater EEA for phosphatases (Table 4). However, the V_{max} was similar between the suspended growth section of the modified AS and equivalent sections of the conventional AS. The modified AS has a greater affinity for amino-peptidases, glucosidases and phosphatases resulting in elevated EEA at lower substrate concentration. The RBRs had a K_m which averaged 518, 375 and 531 μM for amino-peptidases, α-glucosidase and phosphatase respectively which was less than suspended growth for amino-peptidases and α-glucosidase but higher for phosphatase (Table 4). Greater protein demand or diffusion limitation has been noted for biofilms previously which could be attributed to observed elevated amino-peptidase enzyme quantity and EEA per cell in biofilms compared to planktonic bacteria (Jones and Lock, 1989); however, this also could be linked to higher cell densities and the intrinsic extra growth requirements of biofilms (Allison and Vitousek, 2005). The EEA in a WWTW may increase because; (1) suitable substrates do not repress enzyme systems; (2) EEA liberate more low M_w substrate which becomes bioavailable; (3) microbial population growth and therefore enzyme quantity or activity increases; (4) the enzymes are shed into the wastewater biofilm/floc matrix and remain active (Shackle et al., 2000). The modified AS microbiota had increased α-glucosidase EEA of 0.7 and 4.5 $\mu M \cdot min^{-1}$ for the aeration tank and RAS respectively suggesting greater requirement for sources of readily biodegradable carbon, due to feast/famine conditions between the AS and RAS in the modified AS (Bengtsson et al., 2008). Heterotrophic scavenging in modified AS could contribute to elevated depolymerisation and removal of long chain carbonaceous compounds compared to conventional AS. During nutrient limitation many catabolic enzyme operons are expressed, although EEA is suppressed until suitable organic inducers are present (Konopka, 2000), therefore high EEA in RBRs could provide a mechanism for elevated substrate removal rates in modified AS aeration lanes. San Pedro et al. (1994) suggested that the starch hydrolysis rate was independent of biomass concentration and that glucosidases were in excess in conventional AS. In this study using a different enzyme target we demonstrated that the α-glucosidase EEA was greater in RBR biofilm compared to the AS biomass, although catabolite repression was identified at the substrate concentrations >125 μM. High EEA with high K_m was found at numerous sample locations for both modified AS and conventional AS. This is attributed either to low affinity of the wastewater bacteria enzymes for the 4-NA and 4-NP labelled substrates and/or to concomitant high concentrations of natural substrates which could have competitively interfered with formation of the substrate/enzyme complex in the batch tests (Li and Chróst, 2006).

The experimental data did not differ significantly from the Michaelis–Menten model for all V_{max} and K_m treatments (*t*-test between observed and expected, $p < .05$) which suggested this model was suitable. The maximum number of interations to convergence for the EEA models was <2 in all cases. The achieved convergence tolerance was $<5 \times 10^{-6}$, which is below the accepted upper limit of 1×10^{-4}, suggesting low error accumulation and therefore model accuracy to achieve convergence (Sacchi Landriani et al., 1983). To elucidate the impact of organic overloading on EEA, controlled overloading experiments were undertaken using bench scale RBRs. The V_{max} of amino-peptidases increased from 124 $\mu M\ min^{-1}$ to 394 $\mu M \cdot min^{-1}$ in a linear fashion as average OLR increased from 60 $g \cdot tCOD \cdot m^{-2}d^{-1}$ to 190 $g \cdot tCOD \cdot m^{-2}d^{-1}$ (Table 4, Fig. 2a). However, at OLRs >190 $g \cdot tCOD \cdot m^{-2}d^{-1}$ the EEA decreased significantly ($p < .05$) (Fig. 2 a). The phosphatase EEA initially increased from 52 $\mu Mmin^{-1}$ to 98 $\mu Mmin^{-1}$ from 60 $g \cdot tCOD \cdot m^{-2}d^{-1}$ to 140 $g \cdot tCOD \cdot m^{-2}d^{-1}$ before decreasing at OLRs >190 $g \cdot tCOD \cdot m^{-2}d^{-1}$. The trend was similar for α-glucosidase but with EEAs ~15 and 3 x than amino-peptidases and phosphatase respectively. The maximum specific EEA also yielded an identical trend, suggesting increased net activity and not simply an increase in microbial solids (Fig. 2 c). Significant correlations were observed between influent tCOD ($R_s = 0.52$, $p = .007$), sCOD ($R_s = 0.56$, $p = .003$) and NH_4-N ($R_s = 0.57$, $p = .004$) loading rates and phosphatase EEA (Table 4). The best tCOD effluent quality occurred when amino-peptidase ($R_s = -0.62$, $p = .002$) and phosphatase ($R_s = -0.66$, $p = .001$) extracellular enzymes were most active (Table 5). In addition there was a positive correlation between tCOD effluent quality and phosphatase K_m ($R_s = 0.63$, $p = .001$). This suggests removal performance for bulk organics and total solids is linked to the EEA in wastewater biofilms in this system.

Table 3
Aeration tank characteristics for conventional activated sludge and modified activated sludge full-scale plants during study period. Conventional AS and modified AS represent the average EEA from two sample points each.

Parameter	Conventional AS (S2 + S3)	Modified AS (S8 + S9)	Difference in performance significant at 95% ($p < .05$)
MLSS (mgL^{-1})	3059 ± 536	3254 ± 768	No
SVI (mLg^{-1})	96.9 ± 1.7	43.9 ± 5.5	Yes
SRT (d)			
F:M ($kg \cdot COD \cdot kg \cdot MLSS^{-1}$)	0.5 ± 0.26	0.8 ± 0.31	No
tCOD removal rate $kg \cdot tCOD \cdot m^{-3}d^{-1}$	1.08 ± 0.21	2.27 ± 0.52	Yes
NH4-N removal rate ($kg \cdot NH_4\text{-}Nm^{-3}d^{-1}$)	0.06 ± 0.02	0.09 ± 0.02	Yes
COD SUR ($kg \cdot COD \cdot kg \cdot MLSS$)	0.35 ± 0.19	0.57 ± 0.07	Yes
NH_4-N SUR ($kg \cdot NH4\text{-}N \cdot kg \cdot MLSS$)	0.022 ± 0.01	0.03 ± 0.01	Yes

Table 4
Extracellular enzyme activity (V_{max}) and substrate K_m for amino-peptidases, α-glucosidase and phosphatase enzymes. Biomass samples were taken from biofilm on RBR at bench and full scale and mixed liquor from conventional activated sludge and modified activated sludge full-scale plants. The RBR bench EEA data are from equivalent tCOD organic loading rates between bench RBR and modified AS. N.D = no data available due to poor model fit. Sample points from Fig. 1 are presented in brackets. Conventional AS and modified AS represent the average EEA from two samples points each.

Biomass	Amino-peptidase	α-Glucosidase	Phosphatase
Average EEA V_{max} (μM min^{-1})			
Conventional AS (S2 + S3)	12.3 ± 1.5^b	1 ± 0.05^a	7.3 ± 0.08^a
Conventional RAS (S4)	18.0 ± 1.4^a	2.82 ± 0.1^a	8.8 ± 0.05^a
Modified AS (S8 + S9)	13.8 ± 0.6^a	0.7 ± 0.05^a	5.4 ± 0.2^a
Modified RAS (S10)	17.6 ± 1.7^a	4.3 ± 0.2^a	8.5 ± 0.05^a
RBR modified AS full scale (S2)	83.6 ± 10.2^b	38.2 ± 1.2^a	55.5 ± 3.4^a
RBR bench	36.6 ± 6.1^b	16.2 ± 5.2^c	16.9 ± 1.2^b
Average EEA K_m (μM)			
Conventional AS (S2 + S3)	1212 ± 435^c	990 ± 155^b	58.9 ± 5.2^a
Conventional RAS (S4)	995 ± 249^c	263 ± 60^c	22.4 ± 2.1^a
Modified AS (S8 + S9)	744 ± 121^b	355 ± 104^c	77 ± 17^c
Modified RAS (S10)	803 ± 267^c	207 ± 56^c	22.4 ± 2.1^a
RBR modified AS full scale (S2)	518 ± 278^d	375 ± 48^b	531 ± 104^b
RBR bench	1094 ± 504^c	N.D	142.4 ± 36.7

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

The biofilm redox potential was −31 mV at 60 g·tCOD·$m^{-2}d^{-1}$ before decreasing to −245 mV at 190 g·tCOD·$m^{-2}d^{-1}$; further increases in OLR decreased redox potential further (Fig. 2 d). The elevated amino-peptidase EEA in the RBR suggests that the biofilm has a greater intrinsic demand for protein compared to phosphate or carbohydrate (Jones and Lock, 1989) and that this demand is strongly influenced by OLR and/or prevailing electron acceptor conditions in the biofilm (Fig. 2 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 decrease in EEA which occurred in the RBR biofilm at high OLR 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 significant correlation between α-glucosidase K_m and phosphatase V_{max} suggest that biofilm solids could be used to infer EEA in other biological systems. In terms of performance the amino-peptidase V_{max} appears a suitable parameter to help predict effluent quality. Performance in aerobic biological treatment processes is directly related to biological growth rates therefore the strong positive correlation between key effluent quality criterion and amino-peptidase V_{max} suggest this could be a global indicator of the health of biological treatment processes (Loukidou and Zouboulis, 2001).

The K_m ranked in order amino-peptidases > α-glucosidase > phosphatase, therefore the RBR biofilm had greater affinity (lower K_m) for phosphatase despite significantly higher V_{max} (Fig. 2 b). This trend was most striking between 190 g·tCOD·$m^{-2}d^{-1}$ and 290 g·tCOD·$m^{-2}d^{-1}$, possibly due to demand for phosphate storage under anaerobic conditions (Fig. 2 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 WWTW (temperate conditions, very short sewer HRT) could explain the higher bench RBR EEA (Table 5, Fig. 2 a) (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). It should be noted that the air scour facility provides an opportunity to modify the biofilm growth rate and EEA in order to maximise the efficacy of WWT through elevated EEA (Shackle et al., 2000).

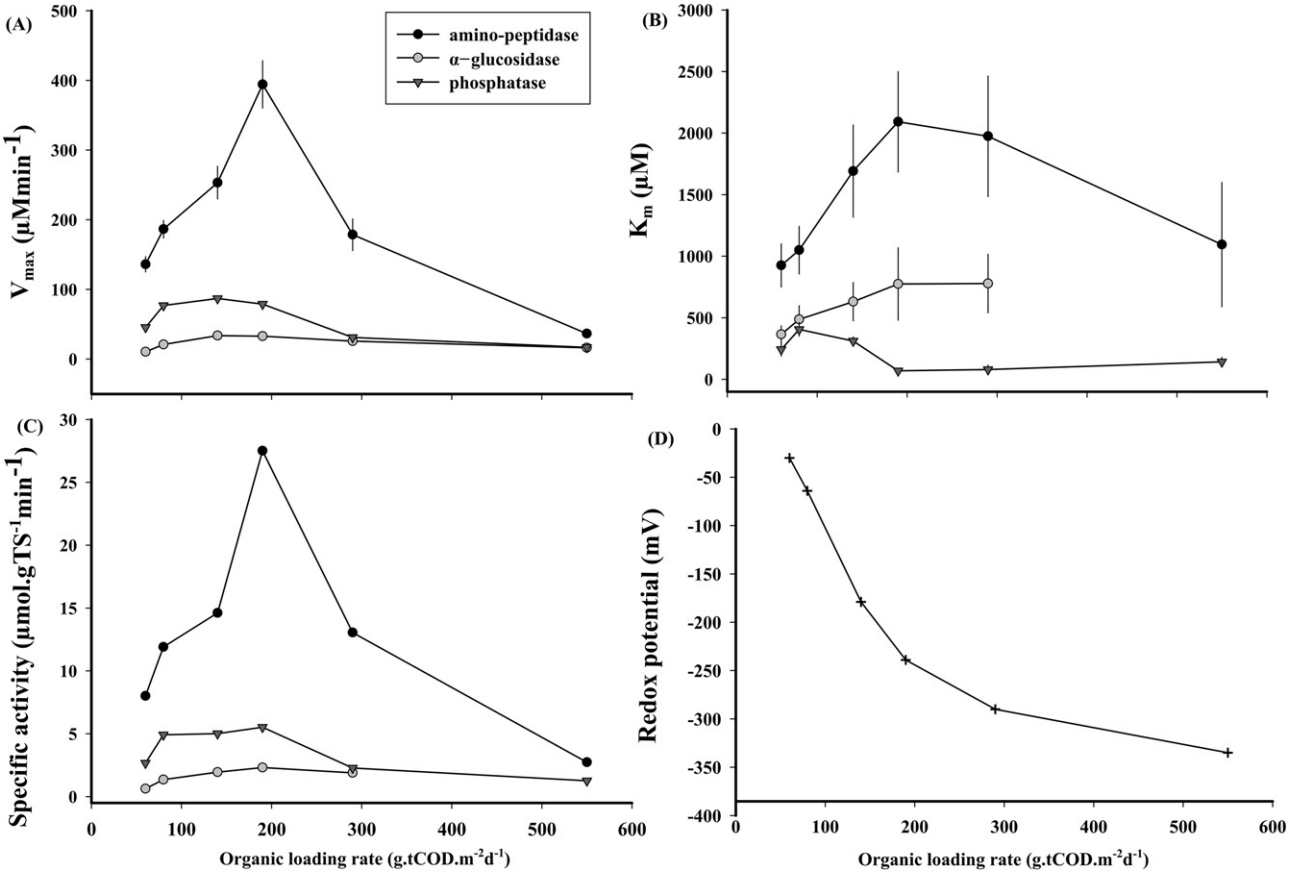


Fig. 2. Extracellular enzyme kinetic characterisation of bench rotating biofilm reactors operated at incrementally increasing OLR (based on average tCOD OLR for each experimental period). A. V_{max}, B. K_m C. specific enzyme activity (SEA) for amino-peptidases, α-glucosidase and phosphatase respectively, D. redox potential (mV) of biofilm. α-glucosidase EEA assay data could not be determined at 550 g·tCOD·$m^{-2}d^{-1}$.

Table 5
Spearman's correlation coefficients of extracellular enzyme activity with effluent quality and performance parameters.

Extracellular enzyme Michaelis–Menten parameter	Loading rates			Effluent quality			% removal	
	tCOD	sCOD	NH_4-N	tCOD	sCOD	NH_4-N	NH_4-N	Biofilm TS
Amino-peptidase V_{max}	0.1	0.16	0.11	−0.62**	−0.1	0.4*	−0.27	0.13
Amino-peptidase K_m	0.27	0.29	0.25	−0.29	0.25	0.21	−0.3	0.28
α-Glucosidase V_{max}	−0.28	−0.27	−0.33	−0.28	−0.26	−0.14	0.19	−0.29
α-Glucosidase K_m	0.66**	0.7**	0.67**	−0.64**	0.41*	0.78**	−0.76**	0.73**
Phosphatase V_{max}	0.52**	0.56**	0.57**	−0.66**	0.38	0.59**	−0.61**	0.61**
Phophatase K_m	−0.62**	−0.6**	−0.63**	0.63**	−0.32	−0.78**	0.7**	−0.68**

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level.
N = 23.
% removal of sCOD and tCOD did not correlate to Extracellular enzyme Michaelis–Menten parameters.

This study demonstrates the impact of a modified AS 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/physico-chemical conditions which is important for other biological processes and remains poorly characterised in wastewater treatment models.

4. Conclusions

- The RBRs had between 4.6 and 13.5 times the EEA (measured by V_{max}) compared to the highest suspended growth biomass studied.
- The modified AS microbial enzymes displayed greater substrate affinity compared to a conventional AS 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.
- The volumetric removal rate was 52% and 40% higher for tCOD and NH_4-N respectively for modified AS compared to conventional AS ($p < .001$).

Conflict of interest statement

The authors have no competing interests to declare.

Acknowledgements

This study was part funded by the UK Engineering and Physical Sciences Research Council (EPSRC, grant number: EP/J500203/1), through a Doctoral Training Award to Francis Hassard and part funded by Bluewater Bio Ltd. The raw data presented is subject to a client confidentiality agreement. The authors would like to thank Fanny Hilaire for her assistance in this project.

Role of funding source

The sponsors did not influence the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.073.

References

Ainger, C., Butler, D., Caffor, I., Crawford-Brown, D., Helm, D., Stephenson, T., 2009. A Low Carbon Water Industry in 2050 — Report: SC070010/R3. Environment Agency, Bristol, UK.
Allison, S.D., Vitousek, P.M., 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biol. Biochem. 37, 937–944.
APHA-AWWA-WEF, 2012. Standard Methods for the Examination of Water and Wastewater. 22nd edition. (Washington DC, USA).
Bengtsson, S., Werker, A., Christensson, M., Welander, T., 2008. Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresour. Technol. 99, 509–516.
Biddle, J.R., Hoyland, G., Harnett, R.J., Dvořáková, M., Huo, C.X., Hassard, F., Stephenson, T., 2014. HYBACS: future-proofing an aging generation of activated sludge assets. One Hundred Years of Activated Sludge Proceedings, Aqua Enviro Technology Transfer, Wakefield, UK, pp. 179–192.
Burgess, J., Pletschke, B., 2008. Hydrolytic enzymes in sewage sludge treatment: a mini-review. Water SA 34, 343–350.
Cadoret, A., Conrad, A., Block, J., 2002. Availability of low and high molecular weight substrates to extracellular enzymes in whole and dispersed activated sludges. Enzym. Microb. Technol. 31, 179–186.
Chen, Z., Wen, Q., Wang, J., Li, F., 2006. Simultaneous removal of carbon and nitrogen from municipal-type synthetic wastewater using net-like rotating biological contactor (NRBC). Proc. Biochem. 41, 2468–2472.
Chen, W., Niepel, M., Sorger, P., 2010. Classic and contemporary approaches to modeling biochemical reactions. Genes Dev. 1861–1875.
Confer, D.R., Logan, B.E., 1998. Location of protein and polysaccharide hydrolytic activity in suspended and biofilm wastewater cultures. Water Res. 32, 31–38.
Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. Annu. Rev. Microbiol. 49, 711–745.
Curtis, T.P., Head, I.M., Graham, D.W., 2003. Theoretical ecology for engineering biology. Environ. Sci. Technol. 37, 64–70.
Daigger, G.T., Boltz, J.P., 2011. Trickling filter and trickling filter-suspended growth process design and operation: a state-of-the-art review. Water Environ. Res. 83, 388–404.
Daigger, G.T., Norton, L.E., Watson, R.S., Crawford, D., Sieger, R., 1993. Process and kinetic analysis of nitrification in coupled trickling filter/activated sludge processes. Water Environ. Res. 65, 750–758.
De Kreuk, M.K., Kishida, N., Tsuneda, S., van Loosdrecht, M.C.M., 2010. Behaviour of polymeric substrates in an aerobic granular sludge system. Water Res. 44, 5929–5938.
Fenu, A., Roels, J., Wambecq, T., De Gussem, K., Thoeye, C., De Gueldre, G., Van De Steene, B., 2010. Energy audit of a full scale MBR system. Desalination 262, 121–128.
Goel, R., Mino, T., Satoh, H., Matsuo, T., 1999. Modelling hydrolysis processes considering intracellular storage. Water Sci. Technol. 39 (1), 97–105.
Hassard, F., Cartmell, E., Biddle, J., Stephenson, T., 2014. Performance of permeable media rotating reactors used for pretreatment of wastewaters. Water Sci. Technol. 69 (9), 1926–1931.
Hassard, F., Biddle, J., Cartmell, E., Jefferson, B., Tyrrel, S., Stephenson, T., 2015. Rotating biological contactors for wastewater treatment — a review. Process. Saf. Environ. Prot. 94 (C), 285–306.
Hassard, F., Biddle, J., Cartmell, E., Stephenson, T., 2016. Mesh rotating reactors for biofilm pre-treatment of wastewaters — influence of media type on microbial activity, viability and performance. Process. Saf. Environ. Prot. 103, 69–75.
Hauduc, H., Rieger, L., Oehmen, A., van Loosdrecht, M.C.M., Comeau, Y., Héduit, A., Gillot, S., 2013. Critical review of activated sludge modeling: state of process knowledge, modeling concepts, and limitations. Biotechnol. Bioeng. 110, 24–46.
Jenkins, D., Richard, M.G., Daigger, G.T., 2003. Manual on the Causes and Control of Activated Sludge Bulking, Foaming and Other Solids Separation Problems. IWA & CRC Press, Boca Raton, USA.
Jones, S.E., Lock, M.A., 1989. Hydrolytic extracellular enzyme activity in heterotrophic biofilms from two contrasting streams. Freshw. Biol. 22, 289–296.
Judd, S., 2010. The MBR Book: Principles and Applications of Membrane Bioreactors in Water and Wastewater Treatment. Second edition. Elsevier, Oxford, UK.
Judd, S.J., 2017. Membrane technology costs and me. Water Res. 122 (Supplement C), 1–9.
Kemmer, G., Keller, S., 2010. Nonlinear least-squares data fitting in excel spreadsheets. Nat. Protoc. 5, 267–281.
Konopka, A., 2000. Microbial physiological state at low growth rate in natural and engineered ecosystems. Curr. Opin. Microbiol. 3, 244–247.
Li, Y., Chróst, R.J., 2006. Microbial enzymatic activities in aerobic activated sludge model reactors. Enzym. Microb. Technol. 39, 568–572.
Liao, J.Y., Lou, I.C., de los Reyes, F.L., 2004. Relationship of species-specific filament levels to filamentous bulking in activated sludge. Appl. Environ. Microbiol. 70, 2420–2428.
Lin, Y., de Kreuk, M., van Loosdrecht, M.C.M., Adin, A., 2010. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. Water Res. 44, 3355–3364.

Loukidou, M.X., Zouboulis, A.I., 2001. Comparison of two biological treatment processes using attached-growth biomass for sanitary landfill leachate treatment. Environ. Pollut. 111 (2), 273–281.
Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., Simon, M., 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. Environ. Microbiol. 7 (7), 961–968.
Madoni, P., Davoli, D., Gibin, G., 2000. Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. Water Res. 34, 1767–1772.
Mannina, G., Viviani, G., 2009. Hybrid moving bed biofilm reactors: an effective solution for upgrading a large wastewater treatment plant. Water Sci. Technol. 60 (5), 1103–1116.
Martins, A.M.P., Heijnen, J.J., van Loosdrecht, M.C.M., 2003. Effect of feeding pattern and storage on the sludge settleability under aerobic conditions. Water Res. 37, 2555–2570.
Molina-Muñoz, M., Poyatos, J.M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., et al., 2010. Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions. Bioresour. Technol. 101, 696–704.
Morgenroth, E., Kommedal, R., Harems, P., 2002. Processes and modeling of hydrolysis of particulate organic matter in aerobic wastewater treatment — a review. Water. Sci. Technol. 45 (6), 25–40.
Orhon, D., Çokgör, E.U., 1997. COD fractionation in wastewater characterization — the state of the art. J. Chem. Technol. Biotechnol. 68, 283–293.
Regmi, P., Thomas, W., Schafran, G., Bott, C., Rutherford, B., Waltrip, D., 2011. Nitrogen removal assessment through nitrification rates and media biofilm accumulation in an IFAS process demonstration study. Water Res. 45, 6699–6708.
Sacchi Landriani, G., Guardabasso, V., Rocchetti, M., 1983. NL-FIT: a microcomputer program for non-linear fitting. Comput. Programs Biomed. 16, 35–42.
San Pedro, D.C., Mino, T., Matsuo, T., 1994. Evaluation of the rate hydrolysis of slowly biodegradable COD (SBCOD) using starch as substrate under anaerobic, anoxic and aerobic conditions. Water Sci. Technol. 30 (11), 191–199.
Shackle, V.J., Freeman, C., Reynolds, B., 2000. Carbon supply and the regulation of enzyme activity in constructed wetlands. Soil Biol. Biochem. 32, 1935–1940.
STOWA, 2010. STOWA NEWs: The Dutch Roadmap for the WWTP of 2030, STOWA Report 2010–24, Amersfoort, Netherlands.
Tas, D.O., Karahan, O., Insel, G., Orhon, D., 2009. The biodegradability and denitrification potential of settleable chemical oxygen demand in domestic sewage. Water Environ. Res. 81, 715–727.
Teuber, M., Brodisch, K.E.U., 1977. Enzymatic activities of activated sludge. Eur. J. Appl. Microbiol. Biotechnol. 4, 185–194.
Truu, M., Juhanson, J., Truu, J., 2009. Microbial biomass, activity and community composition in constructed wetlands. Sci. Total Environ. 407, 3958–3971.
Venables, W.N., Smith, D.M., 2011. An introduction to R: notes on R: a programming environment for data analysis and graphics. Version 2.14.1. R-Development Core Team. R Foundation for Statistical Computing, Vienna Available at. www.math.vu.nl/sto/onderwijs/statlearn/R-Binder.pdf (Accessed 2014).
Wingender, J., Jaeger, K.-E., 2002. Extracellular enzymes in biofilms. In: Bitton, G. (Ed.), Encyclopaedia of Environmental Microbiology. 3, pp. 1207–1222.
You, S.J., Hsu, C.L., Chuang, S.H., Ouyang, C.F., 2003. Nitrification efficiency and nitrifying bacteria abundance in combined AS-RBC and A2O systems. Water Res. 37, 2281–2290.

Microbial extracellular enzyme activity affects performance in a full-scale modified activated sludge process

Hassard, Francis

2018-01-12

Hassard F, Biddle J, Harnett R, Stephenson T. (2018) Microbial extracellular enzyme activity affects performance in a full-scale modified activated sludge process. Science of the Total Environment, Volume 625, June 2018, pp. 1527-1534

http://doi.org/10.1016/j.scitotenv.2018.01.073