

**Starch degradation and intermediate dynamics in flocculated and dispersed
microcosms**

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Submitted to Water Science and Technology on 15.03.17, revised version submitted on
01.08.17

Abstract

A large fraction of the organic substrate in municipal wastewater is particulate. Prior to uptake, particles have to be degraded through potentially a range of intermediates. However, research on intermediate dynamics during particle hydrolysis is limited. In this paper batch experiments on flocculated and dispersed biomass microcosms using starch as particulate substrate are reported. Overall hydrolysis rate was not significantly different between the two systems. Particle colonization, increased particle porosity in combination with particle breakup led to increased substrate availability over time. Particle breakup was more important for flocculated biomass, while increased particle porosity and particle colonization played a larger role for dispersed biomass. During particle degradation intermediates were formed, however, all intermediate polymer sizes were not formed to the same extent. This can be explained by non-random enzymatic degradation, where some products are preferred over others. Intermediates dynamics also depend on the biomass structure, and in a floc based system, diffusion limitations allow glucose to accumulate in the system.

30 [Keywords](#)

31 Particle colonization

32 Particle breakup

33 Intermediate dynamics

34 Hydrolysis

35 Starch

36 [Abbreviations](#)

37 DRI: differential refractive index detector

38 F/M-ratio: Food to mass ratio

39 HMW: High molecular weight

40 LMW: Low molecular weight

41 MALS: Multi angle light scattering detector

42 MMW: Medium molecular weight

43 OUR: Oxygen utilization rate

44 PBM: Particle break-up model

45 PSD: Particle size distribution

46 SEC: Size exclusion chromatography

47 SPM: shrinking particle model

48 ThOD: Theoretical oxygen demand

49 Introduction

50 Municipal wastewater consist of a large fraction of particulate organic matter (41-73 %;
51 Levine et al. 1991), hence, particle degradation dynamics is important for process
52 analysis in wastewater treatment. In general, particles cannot be directly taken up by
53 bacterial cells, but has to undergo extracellular depolymerisation until molecules small
54 enough for transport across the bacterial cell membrane are available. Size limit for
55 cellular uptake is generally assumed to be 0.6-1 kDa (White et al. 2012). Hydrolytic and
56 lytic depolymerisation are the dominant mechanisms of depolymerisation, and
57 theoretically allow for any sub-polymeric intermediate to be formed. Most work on
58 depolymerisation dynamics focus on easily biodegradable substrate formation kinetics,
59 while research on intermediate dynamics during particle hydrolysis is limited.

60 Starch is a common model substrate for slowly biodegradable substrate in wastewater
61 (Karahan et al. 2006). Being a natural constituent in municipal wastewater, starch-
62 hydrolysing organisms are abundant in activated sludge (Xia et al. 2008). Starch is also
63 a common biodegradable particle in industrial wastewater from the textile industry
64 (Feitkenhauer & Meyer 2002), in addition to food processing industrial wastewaters.
65 Native starch from various plant sources are composed of the α 1,4-linked glucosidic
66 polymers amylose and amylopectin (Ball et al. 1996; Dona et al. 2010; Oates 1997).
67 Amylopectin is the major component of starch, a highly α 1,6-branched water-soluble
68 polymer with a molecular weight of 10^4 - 10^6 kDa (Ball et al. 1996; Dona et al. 2010).
69 Amylose is a smaller linear insoluble polymer of molecular weight range 100-1000 kDa
70 (Ball et al. 1996).

Different mechanisms and modelling approaches have been proposed for particle hydrolysis (Morgenroth et al. 2002; Vavilin et al. 2008). In a model for anaerobic digestion proposed by Vavilin et al. (1996) solid waste particles are assumed to be colonized by hydrolytic bacteria, who subsequently produce hydrolytic enzymes. Colonization (biofilm growth covering the particle surface) was in a previous study observed by microscopy and proposed as the main mechanism for particle degradation in a biologically activated membrane bioreactor (Ravndal et al. 2015). In activated sludge processes, bacteria grow in flocs and the initial step of starch degradation has been proposed as adsorption of starch to the flocs (Ciggin et al. 2013; Karahan et al. 2006; Martins et al. 2011).

Regardless of whether degradation of particulates work through colonialization or flocs adsorption, degradation will depend on available particle surface area. In addition to the particle – biomass contact perspective, particle degradation also depends on the particle morphology. Two different models proposed are the shrinking particle model (SPM; Sanders et al. 2000) and the particle breakup model (PBM; Dimock & Morgenroth 2006). The SPM assumes particles to shrink gradually as they are degraded, hence available surface area decrease. In the PBM, particles break up as they are degraded leading to an increase in available surface area. Hence, in the PBM, surface area to volume ratio are included as a state variable in the model. An open question of the PBM is whether the kinetics observed also could be caused by increased particle porosity leading to increased surface area to volume ratio or increased particle colonization (Dimock & Morgenroth 2006).

The hydrolysis process is an enzymatic degradation process as discussed in both the activated sludge models (Henze et al. 2000) and anaerobic digestion model (Batstone et

al. 2002). Hydrolytic enzyme kinetics are independent of electron acceptor conditions (Goel et al. 1998), hence hydrolysis studied under aerobic conditions is also relevant for anaerobic conditions and vice versa. The concentration of hydrolytic enzymes, however, may depend on electron acceptor conditions (lower concentrations under anaerobic conditions) probably due to correlation to cellular yield (Kommedal 2003). For starch degradation, a range of extracellular enzymes are active (Robyt 2009), and a substantial collection of published articles on characteristics of starch degrading enzymes is available (Sun et al. 2010). However, less attention has been granted the combined substrate and biomass effect, and their influence on the substrate degradation dynamics. In this study we address the need for more detailed knowledge of particulate organic matter degradation by (i) investigating starch particle degradation and intermediate dynamics including the entire size range from micrometer scale particles, polymers, oligomers and monomers (substrate size intermediate dynamics), and (ii) evaluate whether there is a difference in particle and intermediate dynamics for biomass aggregates or dispersed biomass (the substrate - biomass size effects). Our hypothesis is that 1) Starch is degraded via potentially all intermediate size ranges and that the kinetics is size dependant; and 2) Intermediate dynamics depends on the biomass structure, especially during the particulate substrate phase.

Materials and Methods

Batch experiments with starch as sole substrate, inoculated with activated sludge and dispersed activated sludge were analysed over a period of 117 days. Oxygen utilisation rate (OUR) was monitored, and sampling was performed regularly for particle size distribution (PSD) and polymeric, oligomeric and monomeric intermediates formation.

Particle morphology and particle – biomass interaction were observed by light microscopy. Polymeric intermediate dynamics was followed using SEC in combination with multi angle light scattering detector (MALS) and differential refractive index detector (DRI), a technique with absolute determination of molar mass and mass concentration, allowing for molecular mass determination without using molecular standards (Cheong et al. 2015; Wyatt 1993).

Experimental setup

Batch tests were prepared at an initial volume of 500 mL and concentration of 2.00 g L⁻¹ of potato starch (Fluka Analytical 03967) in tap water. An initial high food to mass ratio (F/M-ratio) was chosen in order to emphasize the substrate size effects (comparably low initial biomass concentration). Inorganic nutrients were added (at concentrations 32.4 mg L⁻¹ K₂HPO₄, 1.6 mg L⁻¹ KH₂PO₄, 50 mg L⁻¹ NaNO₃, 1.2 mg L⁻¹ NH₄Cl, 0.1 mg L⁻¹ FeCl₃, 5 mg L⁻¹ CaCl₂ and 3 mg L⁻¹ MgSO₄) in addition to trace elements according to Balch et al. (1979). Amino acids (10 µL, R 7131 RPMI-1640 [50X], Sigma Aldrich) and vitamins (10 µL, B6891 BME [100X], Sigma Aldrich) were also added to each test bottle. Test bottles (five replicates) were inoculated with 500 µL activated sludge or 500 µL dispersed activated sludge (four replicates). Activated sludge was collected at Vik wastewater treatment plant (Rogaland, Norway), from the aerated bioreactor containing approximately 4 g L⁻¹ mixed liquor suspended solids, giving an estimated initial suspended total solids concentration in test bottles of 2.5 mg TSS L⁻¹ (F/M-ratio > 1000). Dispersed activated sludge was prepared by sonication (Branson 2510 Sonicator, 100W) of a sub-volume of collected sludge for a total of 20 min in 5 min intervals to minimise temperature increase. Sonication efficiency was confirmed by microscopy, and positive controls inoculated with glucose was used to validate viability of sonicated

cells. Sampling for particle and intermediate analysis was performed by drawing 5 mL samples from the batch tests throughout the experiment every 3-7 days until day 62, every 12-14 day until day 88 and a last sampling at day 117.

Oxygen utilization rate

OUR was monitored on-line by a Micro-oxymax dynamic respirometer (Columbus Instruments, Ohio, USA) measuring oxygen concentration in the gas phase of each test bottle (653 mL) 21.6 times per day. The respirometer was equipped with a paramagnetic oxygen sensor (Paramax-101, Columbus Instruments, Ohio, USA). To ensure aerobic conditions, the respirometer refreshed the gas phase when the O₂ concentration fell below 18.9 mole %.

Particle size measurements

PSDs were analysed by a Multisizer 4 coulter counter (Beckman Coulter) using 0.9 M% NaCl as electrolyte. Samples were vortexed before diluting 1-2.5 mL of the sample to 200 mL with 0.9 M% NaCl. Analysis was performed with a 200 µm aperture tube (measurement range 4-120 µm) in volumetric mode analysing 2 mL of the diluted sample. Electrolyte blanks were analysed for subtraction of background noise.

Light microscopy

A qualitative observation of particles and biomass in the batch tests was performed using light microscopy (Olympus BX61 microscope, 100x oil Plan Fluorite objective with iris) equipped with a CCD camera (Olympus DP72). Image acquisition and analysis was performed using cellSens Dimension 1.3 software (Olympus).

Polymer analysis

SEC in connection with MALS and DRI detectors was used to separate and analyse intermediates in the polymeric range of 1-10⁶ kDa. An Agilent 1260 Infinity HPLC system with a quaternary pump was equipped with a PL-aquagel-OH50 (Agilent) and a PL-aquagel-OH30 (Agilent) column in series. Sodium nitrate (50 mM) was filtered with 0.1 µm cellulose nitrate membrane filters (Whatman) and used as mobile phase. Two detectors were connected to the system in series, a MALS detector (Dawn 8⁺, Wyatt Technology) and a DRI detector (Optilab T-rEX, Wyatt Technology). Flowrate during analysis was 0.75 mL min⁻¹ and the column was kept at 30 °C by a column oven (Agilent 1260 column compartment). All samples were filtered through 0.45 µm Marcherey-Nagel Nanocolor 50 chromafil GF/PET membrane filters prewashed with deionized water. 100 µL sample was injected. Two parallel samples were withdrawn from all bottles at each sampling time, one sample was filtered, while the other was filtered and heated to 85 °C for 5 min to denature extracellular enzymes. Resulting mass and molar mass of the two samples were compared, and found to be comparable between samples. The universal refractive index increment (dn/dc) value of 0.15 for polysaccharides in water with low salt concentration was used (Cheong et al. 2015). Based on the chromatograms polymers were separated in three size fractions, low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW). Molecular weight of HMW fraction was measured by the MALS detector. LMW and MMW fractions had a low light scattering signal, and molecular weight was estimated based on dextran calibration standards. LMW polymers were in the size range from 1-12 kDa, and MMW polymers in the range from 12-350 kDa.

Monomer and oligomer analysis

Concentrations of glucose, maltose, isomaltotriose, maltotetraose and maltopentaose in the bulk liquid was measured using an Agilent 1100 series HPLC system with a quaternary pump connected to an ELSD-detector (3300 ELSD, Alltech). The system was equipped with a Shodex combined SEC and ion-exchange column (Sugar KS-802, Showa Denko Europe GmbH). Mobile phase was Milli-Q quality water at a flow rate of 0.75 mL min⁻¹. Column temperature was kept at 80 °C using a column oven (Eppendorf CH-30). The ELSD detector had a N₂-gas flow of 1.3 L min⁻¹ and held a temperature of 35 °C, gain was set at 16. Calibration standards used were D(+)-glucose (Merck), D-(+)-maltose monohydrate from potato (Sigma Aldrich M5885), isomaltotriose (Sigma M8378), maltotetraose (Supelco 47877), and maltopentaose (Supelco 47876).

Results

Oxygen utilization rate

OUR (figure 1) was monitored in five parallel batch tests inoculated with flocculated biomass, and four parallel batch tests inoculated with dispersed biomass. OUR trends were similar for flocculated and dispersed biomass tests. An initial fast increase in rate was observed between day 2 and 4. Between day 4 and 36 OUR was stable at 2.0 ± 0.4 mg L⁻¹ h⁻¹ and 1.8 ± 0.4 mg L⁻¹ h⁻¹ for flocculated and dispersed biomass batch tests, respectively. A steady decrease in OUR was observed after 36 days, before the rate stabilized at a low level after 87 and 78 days for respectively flocculated and dispersed biomass tests. After 97 days, accumulated oxygen consumption was 2978 ± 116 mg L⁻¹ for flocculated biomass and 2451 ± 102 mg L⁻¹ for dispersed biomass. Based on initial starch concentration, theoretical oxygen demand (ThOD) was 2380 mg L⁻¹, within the

range for the dispersed biomass, but lower than measured accumulated oxygen consumption for the flocculated biomass. The overestimation was due to large batch test variability and single batch instrumental errors during the experiment.

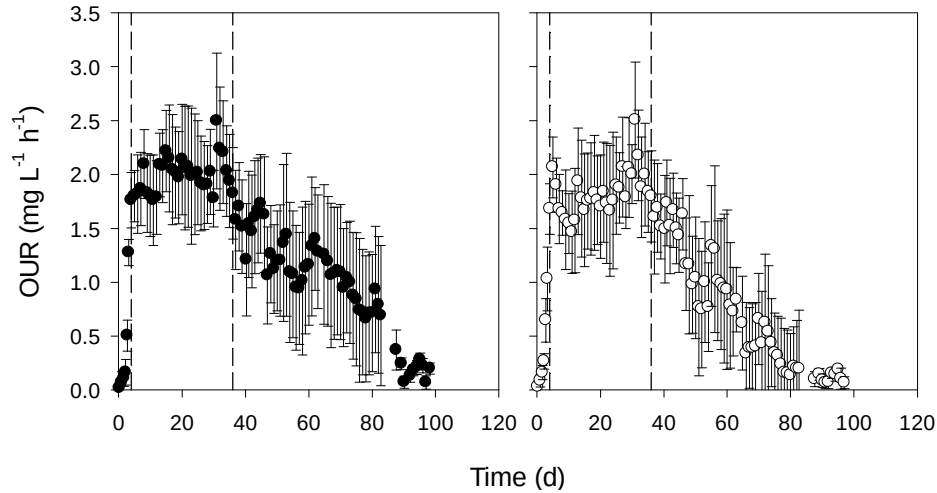


Figure 1: OUR in ● activated sludge and ○ dispersed activated sludge batch tests. Dashed lines indicate a shift in OUR trend at 4 and 36 days. Error bars indicate standard error.

Particle dynamics

Total particle number, volume and surface area were measured in all batch tests, and surface area to volume ratio was calculated (figure 2). Surface area to volume ratio increased before stabilizing after 32 days at $0.58 \pm 0.06 \mu\text{m}^{-1}$ and $0.56 \pm 0.06 \mu\text{m}^{-1}$ for flocculated and dispersed biomass tests, respectively. This corresponds to a mean spherical particle diameter of 10 μm . Total particle number, volume and surface area all had an early stage increase before a maximum was reached after 20 d, 6 d and 20 d, respectively, for both flocculated and dispersed biomass batch tests. After the early increase, all three variables decreased and reached a stable level after 44 days. Particle

number became constant at a higher level than the initial value, while particle volume and surface area arrived at lower than initial values. Change in PSD was monitored as a function of time (figure 3). Initially most of the particle volume detected was distributed between 10 μm and 70 μm . with a peak at 35 μm . Distribution shifted towards smaller particle sizes over time, and after 20 days peak maxima was at a particle diameter of 12 μm and 18 μm for flocculated and dispersed biomass tests, respectively. Flocculated biomass batch tests had an overall higher particle volume than dispersed biomass tests.

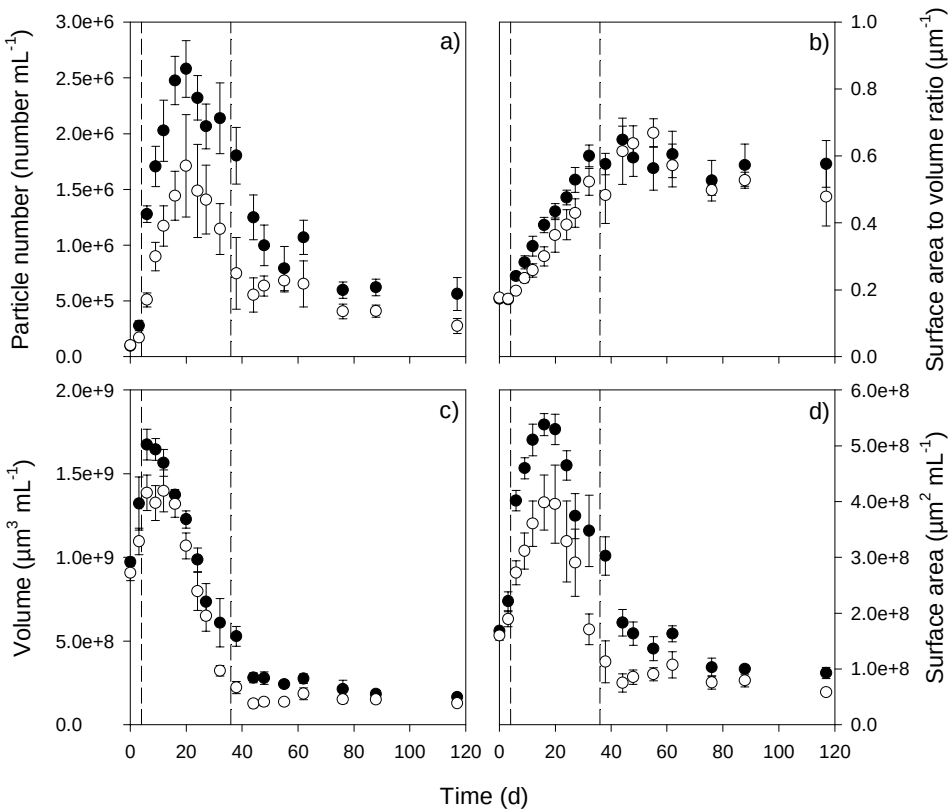


Figure 2: a) particle number (number mL⁻¹), b) surface area to volume ratio (μm⁻¹), c) particle volume (μm³ mL⁻¹), d) particle surface area (μm² mL⁻¹) measured over 117 days in ● activated sludge and ○ dispersed activated sludge batch tests. Dashed lines indicate a phase shift at day 4 and day 36. Error bars indicate standard error.

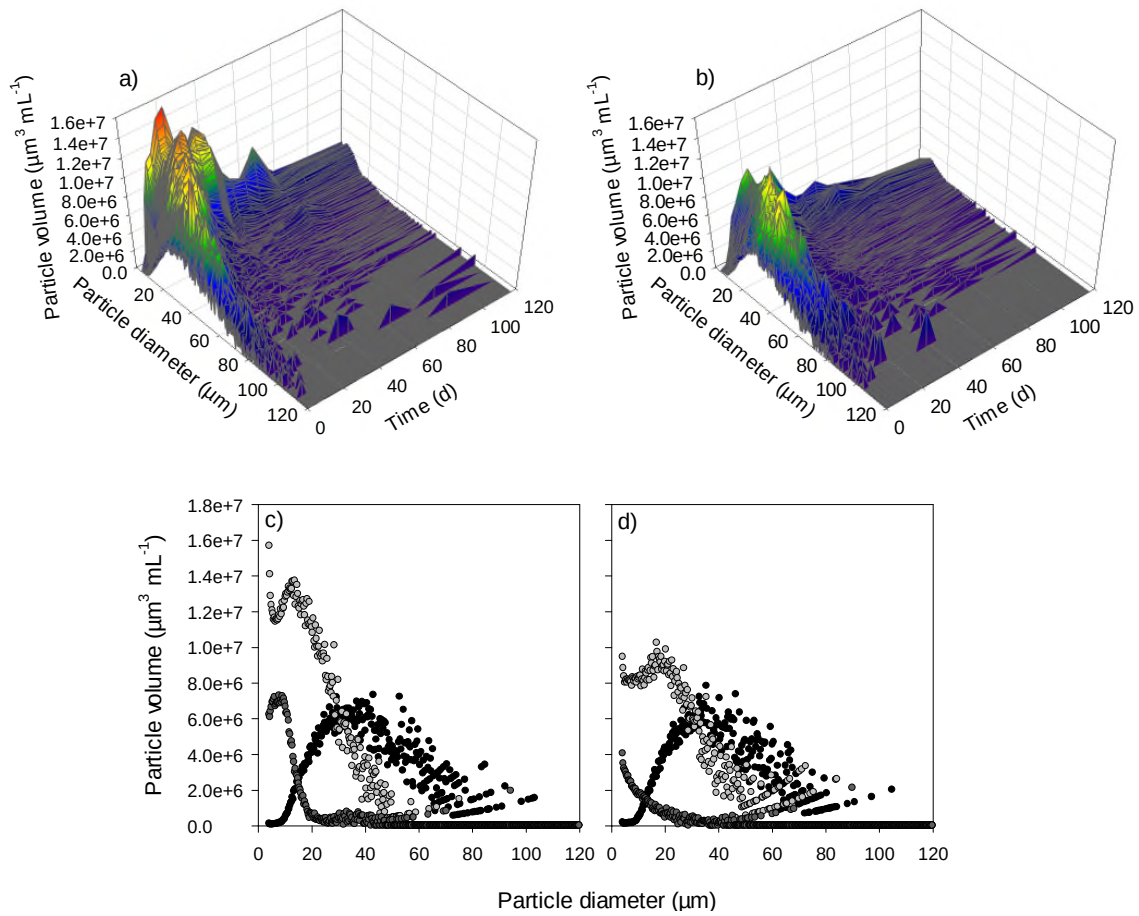
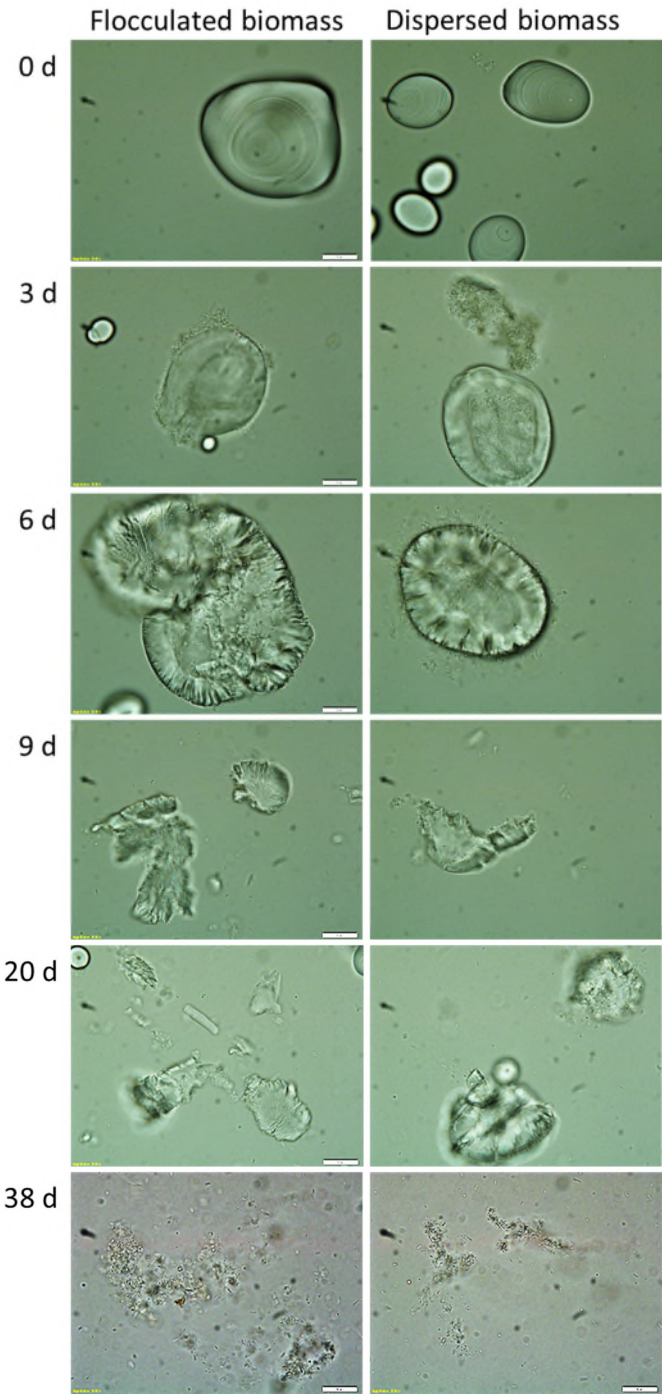


Figure 3: Change in PSD over time in a) activated sludge batch tests and b) dispersed activated sludge batch tests, and PSD at ● day 0, ● day 20, ● day 44 in c) activated sludge batch tests and d) dispersed activated sludge batch tests.

Brightfield microscopy images were collected for flocculated and dispersed biomass batch tests (figure 4). Due to the high F/M-ratio chosen, at day 0 mainly starch particles were observed. Starch particles colonized by microbial biomass was observed at day 3, and over time the particle surface cracked and particles broke up. After 38 days only microbial biomass was observed. Images from flocculated and dispersed biomass tests were similar, particle colonization, particle cracking and particle breakup was observed in both types of tests.



250

251 **Figure 4:** Brightfield microscopy images at 0, 3, 6, 9, 20 and 38 days for flocculated and dispersed
252 biomass batch tests. Bar length is 20 μm . Picture at day 0 show smooth starch particles, day 3 show
253 colonized starch particles (microbial biomass covering the surface of the starch particle), day 6 cracked

and colonized starch particles, day 9 and 20 show starch particles that have been broken up, and day 38 show microbial biomass.

Intermediate dynamics

Monomer, oligomer and polymer intermediates were measured in the bulk liquid of batch tests inoculated with flocculated and dispersed biomass (figure 5). When flocculated biomass was used as inoculum, glucose was detected in the bulk liquid the first two weeks of the experiment with a maximum measured concentration of 54 ± 21 mg L⁻¹. Glucose was not detected above 2 mg L⁻¹ in dispersed biomass tests. Maltose was measured between day 3 and 20 at a maximum concentration of 28 ± 14 mg L⁻¹ and 36 ± 31 mg L⁻¹, respectively, in flocculated and dispersed biomass tests. Isomaltose, maltotetraose and maltopentaose were detected at low levels in both flocculated and dispersed biomass tests between day 3 and 20. LMW polymers were present at a background level of about 20 mg L⁻¹ at the start of the experiment. Concentration of LMW polymers increased after day 3, reached a maximum of 142 ± 23 mg L⁻¹ after 12 days for flocculated biomass and 125-130 mg L⁻¹ between day 9 and 20 for dispersed biomass. After peak concentrations, a gradual decrease back to the background level after 60 days was observed. Concentration of MMW polymers in the bulk liquid increased between day 32 and 76, with maximum peak concentration of less than 20 mg L⁻¹ at day 48 for flocculated biomass and day 55 for dispersed biomass batch systems. HMW polymers were detected in the bulk liquid from day 9 to 88 with a maximum concentration of 70 ± 13 mg L⁻¹ after 48 days for flocculated and 48 ± 8 mg L⁻¹ at 55 days for dispersed biomass. In the period between day 6 to 88, average molecular weight of the HMW polymer fraction was 8222 ± 1210 kDa and 9496 ± 1408 kDa for flocculated and dispersed biomass tests, respectively (figure 6). Molecular weight in

278 flocculated biomass batch tests had an early increase with a peak at 12 days, before
279 steadily decreasing until the end of the experiment. Dispersed biomass tests had the
280 same early increase, but did not show the same decrease towards the end of the
281 experiment.

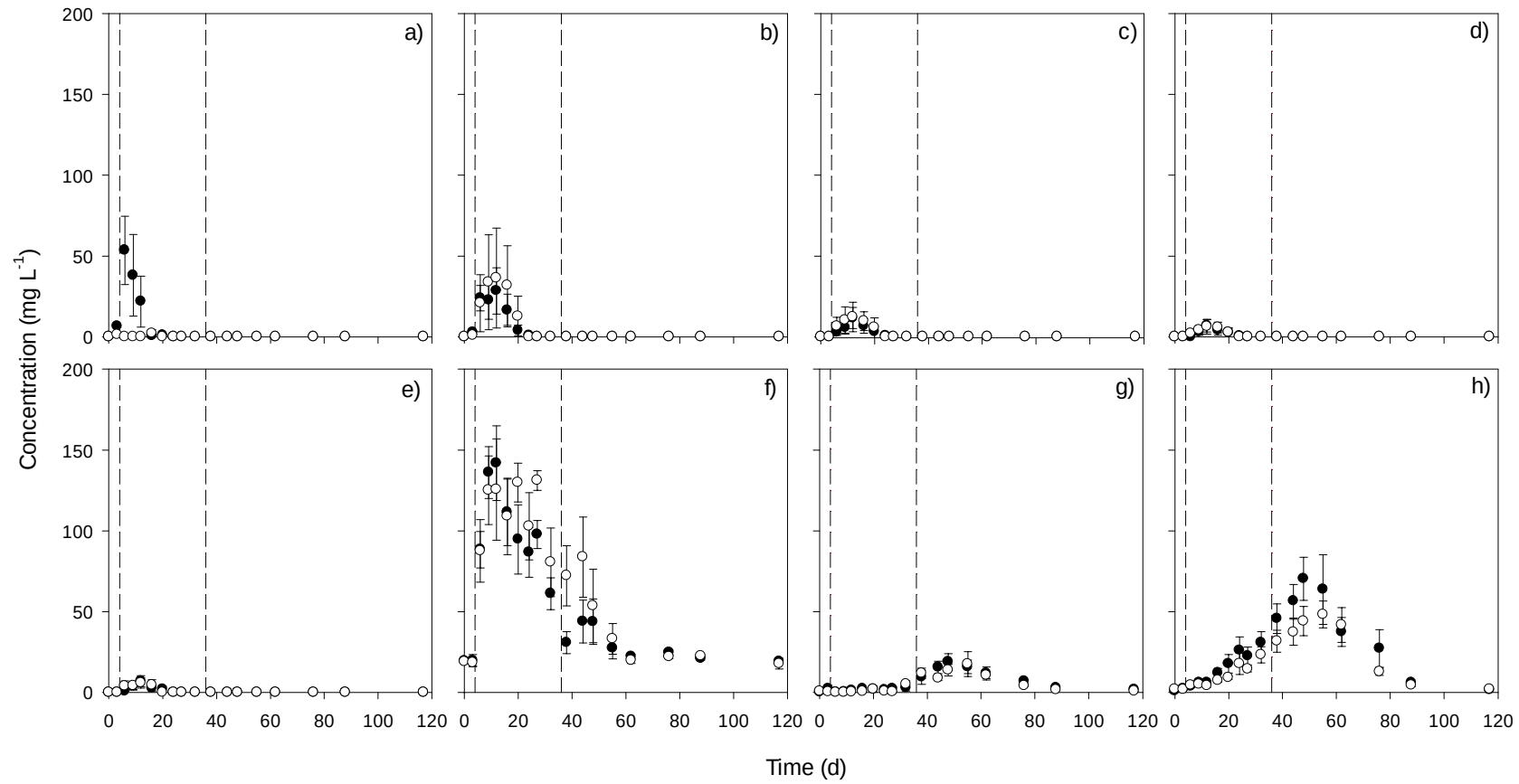


Figure 5: Concentration (mg L⁻¹) of a) glucose, b) maltose, c) isomaltotriose, d) maltotetraose, e) maltopentaose, f) LMW polymers, g) MMW polymers and h) HMW polymers in ● activated sludge and ○ dispersed activated sludge batch tests. Dashed lines indicate a phase shift at day 4 and day 36. Error bars indicate standard error.

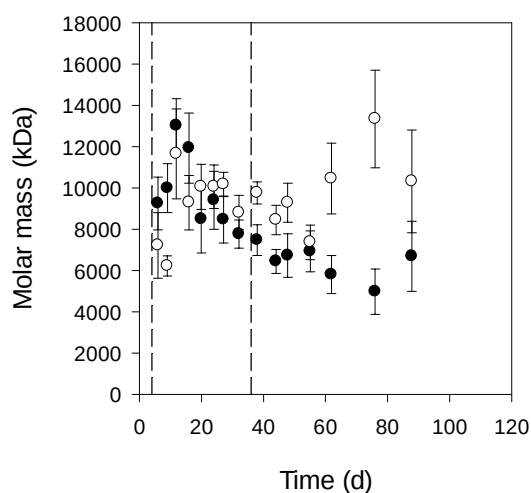


Figure 6: Molar mass (kDa) of polymeric fraction in ● activated sludge and ○ dispersed activated sludge batch tests. Dashed lines indicate a phase shift at day 4 and day 36. Error bars indicate standard error.

Discussion

Starch degradation in batch experiments

Starch particles and microbial biomass are both particulate and will not be distinguished by coulter counter analysis. In the first days, total particle volume increased (figure 2), likely due to microbial biomass growth and starch granule swelling. Swelling was also observed by microscopy showing larger and more heterogeneous starch particles after 3-6 days. Swelling of starch granules are normally studied during gelatinisation of starch occurring when starch is heated in the excess of water (Hoover 2001; Jenkins & Donald 1998; Singh & Kaur 2004). However, when starch granules were added to water low rate swelling is expected due to water binding even at lower temperatures. The early volume increase coincided with an increase in OUR indicating significant microbial growth (figure 1). This first microbial colonization and growth phase, is indicated by a dashed line at 4 days in figure 1. Earlier research has shown an initial fast adsorption of starch to activated sludge flocs at low F/M-

ratios (Ciggin et al. 2013; Karahan et al. 2006; Martins et al. 2011). However, microscopy (figure 4) did not show any flocculation of starch particles to activated sludge flocs in the early phases of this experiment with a high F/M-ratio. Hence, our results indicate a combined starch granule swelling and biomass growth effect on observed size distribution, and not a flocculation effect. Due to the low initial biomass content, absolute increase in biomass over the first days will be small even at maximum growth rate. After initial volume increase, particle number continued to increase until day 20 (figure 2), this number increase was likely the combined effect of biomass growth and particle breakup.

After approximately 40 days, particle distribution shifted away from initial starch granule distribution to smaller particle sizes (figure 3), surface area to volume ratio was constant (figure 2) and only flocculated biomass was observed (figure 4). This coincided with a shift in OUR from a stable high OUR to a linearly decreasing OUR over time indicated by dashed lines (figure 1). HMW polymeric substrate was measured in the bulk liquid at high levels at the time of this shift in OUR (figure 5). The system had at this point shifted from a starch particle, to a biomass particle dominated system, and the substrate shifted from microscale particle to dissolved polymers with high molar mass (figure 6). Hence, the stable OUR phase (between day 4 and 36) of the experiment was a period dominated by particle degradation, while the steadily decreasing OUR phase (after day 36) was a phase dominated by HMW polymeric intermediate degradation and biomass decay (figure 2).

An OUR peak of about $2 \text{ mg L}^{-1} \text{ h}^{-1}$ was measured in this experiment during the particle degradation phase (figure 1). This is 25-100 times lower than literature data of starch degradation in sequencing batch reactors (Ciggin et al. 2013; Karahan et al. 2006), and 3-4 times lower than OUR rates measured on egg white particles in batch reactors (Dimock & Morgenroth 2006) and settleable wastewater fractions (Ginestet et al. 2002). This difference

can be explained by an initially higher F/M-ratio used in this experiment compared to the other studies.

What is the mechanism and dynamics of starch particle degradation?

Starch particles were colonized by microbial biomass (figure 4), supporting colonization of particles as mechanism for particle degradation (Ravndal et al. 2015). All starch particles were not immediately colonized, or biomass intermittently detached as starch particles free of biomass were observed also at later stages in the experiment. In addition to particle colonization, particle cracking was observed by microscopy (figure 4), and starch granules became more heterogeneous over time. Cracking of particles could be a combined effect of free extracellular enzymatic activity, hydration and physical-chemical fragmentation.

Extracellular enzymes are able to attack brittle zones of the starch granules and lead to pit and pore formation on the particle surface (Gallant et al. 1992; Robyt 2009; Tang et al. 2006).

This results in an increased surface area. Finally, particle breakup leading to formation of smaller and more heterogeneous particles was microscopically observed (figure 4). This was supported by total particle volume and surface area measurements (figure 2), and by changes in PSD (figure 3). After the early volume increase, surface area continued to increase while volume started to decrease. This lead to an increasing surface area to volume ratio over time.

If degradation followed the SPM, surface area should gradually decrease throughout the particle degradation phase. On the other hand, in the PBM particle breakup lead to an early increase in surface area when the rate of particle breakup is larger than removal rate of particulate substrate due to further biodegradation (Dimock & Morgenroth 2006). Our results show an increase in surface area simultaneous as the overall volume of particles decrease (figure 2), hence, degradation follow the PBM and not the SPM. Dimock and Morgenroth (2006) proposed particle breakup as the main mechanism, but also hypothesized that increased particle porosity and increase in particle colonization could explain the kinetics of

the PBM model. Based on our results, all three factors seem to contribute to particle degradation.

Glucose, maltose, larger oligomers and polymers were detected in the bulk liquid, and it is hypothesised that these are intermediates formed during particle hydrolysis (figure 5). Maltose has earlier been detected as primary end-product for hydrolysis of starch by activated sludge (Karahan et al. 2006; Ubukata 1999), while we detected both glucose and maltose when batch tests was inoculated with activated sludge. Release of intermediates to the bulk liquid in this experiment confirms earlier studies showing release of dissolved organic carbon to the bulk liquid during activated sludge starch degradation (Karahan et al. 2006; Ubukata 1999). Contrary to our results, Martins et al. (2011) did not observe bulk phase intermediates during starch degradation in activated sludge sequencing batch reactors. If intermediates are not detected, they can be assumed to be consumed close to their production site (Martins et al. 2011). In systems with a low F/M-ratio, such as the study by Martins et al. (2011) it is also possible that diffusion into the bulk liquid is limited due to particulate substrate being fully surrounded by biomass. However, our results at an initially high F/M-ratio and several other studies with a low F/M-ratio (Confer & Logan 1997; Karahan et al. 2006; Ubukata 1999) report intermediate release to the bulk liquid during starch degradation. Thus, it is important to consider also degradation mechanism and dynamics of polymeric intermediates when modelling degradation of starch, and potentially any substrate particles. Most existing models, however, do not consider a soluble polymeric intermediate fraction (Morgenroth et al. 2002).

Another explanation of polymers being detected in the bulk liquid during the experiment is release of soluble microbial products (SMP). SMPs are defined as DOM released to the bulk liquid due to substrate metabolism/biomass growth and biomass decay (Barker & Stuckey 1999). Size distribution of SMP identified in earlier research and summarised in Barker and Stuckey (1999) show that SMP have a wide, but lower molecular weight distribution than

376 reported here. The analysis method used for molecular weight measurements were not
377 specific for starch intermediates, hence SMP were included in the total polymer data.
378 However, due to the large size of HMW polymers, we conclude that the majority of polymers
379 measured in the bulk liquid were in fact intermediates produced outside the bacterial cell due
380 to starch degradation.

381 Based on observed intermediate and particle dynamics, we propose a conceptual model
382 including intermediate dynamics for the extracellular enzymatic degradation of starch (figure
383 7a). Upon microbial colonization of starch particles, hydrolytic extracellular enzymes are
384 released in the contact zone between bacteria and starch particles. Polymeric, oligomeric and
385 monomeric intermediates formed during particle degradation may diffuse into the bulk liquid.
386 Polymeric and oligomeric intermediates are subsequently depolymerised into easily
387 biodegradable oligomers and monomers that are readily taken up by growing microbial cells.
388 All size intermediates may be expected, however based on our results and for
389 conceptualization, polymeric intermediates are grouped into HMW, MMW and LMW
390 fractions.

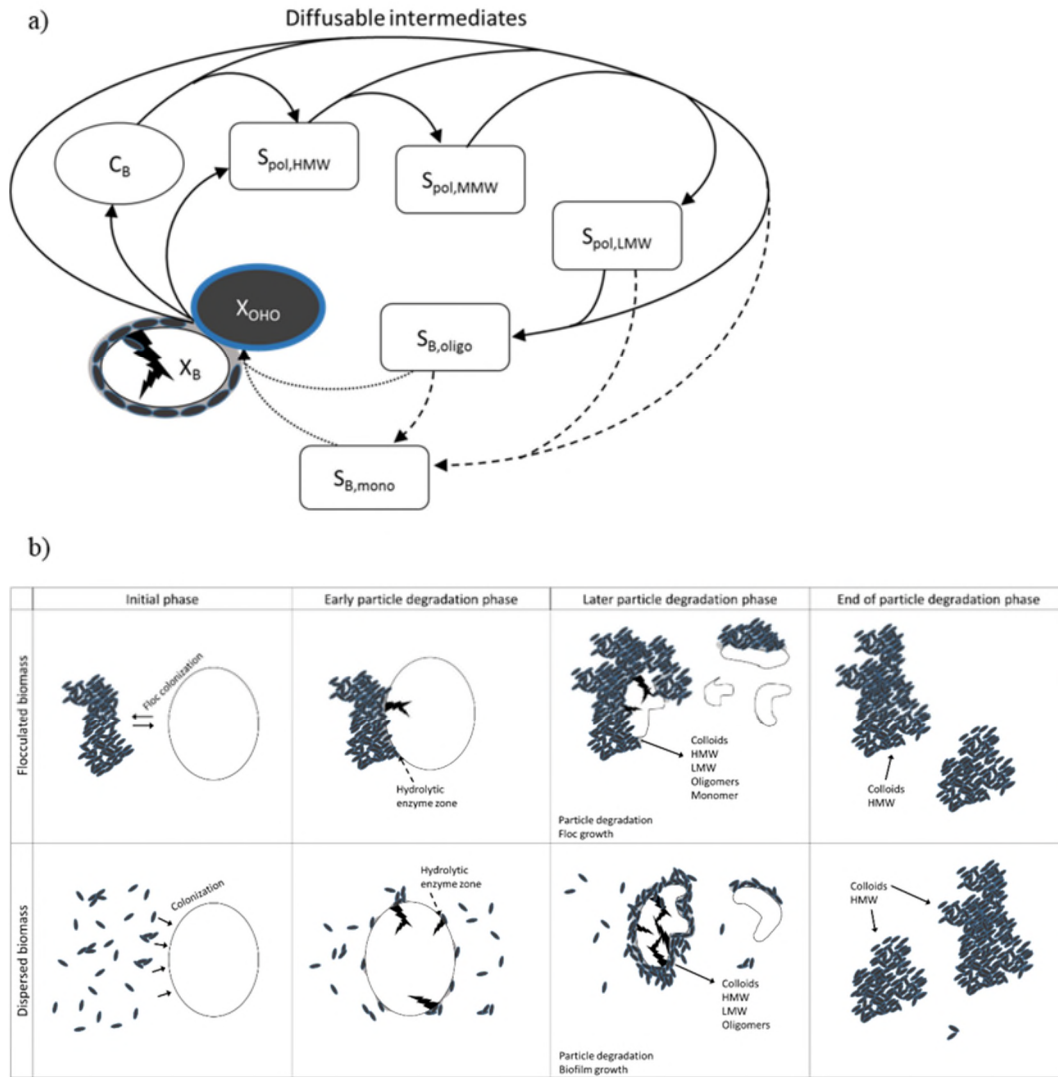


Figure 7: a) Conceptual COD flow model of starch (X_B) depolymerisation. The model assumes a colonized starch surface to be the hotspot of extracellular hydrolytic activity, whereby exo- (dashed lines) and endoenzymatic (solid lines) degradation of particulate (oval boxes) and dissolved polymers (Square boxes) leads to diffusible intermediates that undergo further depolymerisation to oligo ($S_{B,oligo}$) and monomeric ($S_{B,mono}$) easily biodegradable substrates that are readily taken up by growing cells (X_{OHO}) (dotted lines). The largest degradation product of starch are colloids (C_B). Polymeric intermediates are separated in HMW ($S_{pol,HMW}$), MMW ($S_{pol,MMW}$) and LMW ($S_{pol,LMW}$). New and existing model variables are implemented with standardised notation proposed by Corominas et al. (2010). b) Conceptual model of biomass substrate interactions during particle degradation with flocculated and dispersed biomass. Four different phases of degradation are differentiated.

Monomers and oligomers are expected to be released during enzymatic degradation of starch (Robyt 2009). In our study, monomers and oligomers formed during depolymerisation were detected in the bulk liquid only in the beginning of the experiment (figure 5), indicating monomer and oligomer formation and diffusion into the bulk liquid to be higher than uptake rate at that stage. Later, these were no longer measured in the bulk liquid, indicating limited diffusion into the bulk liquid due to starch particles being fully colonized by bacteria. Alternatively, this can also be explained by uptake of released easily biodegradable substrate in the bulk liquid under high suspended biomass concentrations following suspended growth or detachment of biomass from the particles.

By qualitative comparison to our data, intermediate polymers in the form of amylopectin, amylose and polymeric degradation products of the two were released to the bulk liquid (figure 5 and 6). LMW polymers were released at high concentrations early in the experiment, suggesting these to be formed directly from particle degradation. The LMW polymeric fraction include several of the known products of enzymatic degradation of starch (Robyt 2009). When starch particles no longer were detected in the bulk liquid, HMW polymer concentration was still increasing, indicating the presence of a colloidal fraction in between measured HMW polymer and particle fraction.

Low concentrations of MMW polymers were detected in the bulk liquid (figure 5), in addition the measured molar mass of HMW polymers were very high (figure 6). This was either due to difference in hydrolysis rate between different fractions, or it means that not all intermediate polymer sizes were formed. Earlier research has shown that hydrolysis rate increase as molecular weight decreases (Kommedal et al. 2006), potentially leading to faster removal than production of smaller polymer sizes. However, this can also be explained by a non random degradation pattern of starch and larger HMW polymers by extracellular enzymes. Others have shown that the enzymes degrading starch do not have a random degradation

pattern, but enzymes from different organisms will produce different products in variable amounts (Robyt 2009). Most enzymes will produce oligomers as end-products, while larger polymers would be a minor degradation product. This can be illustrated by the action of bacterial β -amylases, which act towards amylopectin and form about 50 % maltose and 50 % HMW polymers (Robyt 2009). β -amylases cannot pass α -1,6-branching points, hence HMW polymers are formed when the enzyme reaches a branching point. Another starch acting enzyme, α -amylases, normally lead to production of oligomers (Robyt 2009), and larger polymers would be minor degradation product formed when the overall polymer size are reduced. MMW polymers were only detected in the bulk liquid after HMW polymer concentration increased. This indicates that MMW polymers were a degradation product from HMW polymer hydrolysis, and not from starch hydrolysis. This support the hypothesis that this is a minor degradation product formed as overall polymer size decreases, and not a major product of enzymatic degradation of starch. Hence, even though hydrolysis rate increase with decreasing polymer size, size distribution of polymeric intermediates, and timing of the different size classes indicates that all potential intermediate sizes were not formed to the same amount.

Protozoa have been shown to be able to directly feed on starch (de Kreuk et al. 2010). In this experiment protozoa was seen by microscopy, but mainly late in the experiment (after 30 days). They therefore did not contribute to significant starch degradation, but probably affected biomass decay rates.

Can initial biomass composition have an effect on mechanism and observed dynamics of particle degradation?

Overall degradation was the same with little difference in accumulated oxygen consumption over 97 days for flocculated and dispersed biomass. The most distinctive difference observed

was detection of glucose in the bulk liquid only in batch tests fed with flocculated biomass (figure 5). Maltose was detected in the bulk liquid of both systems(figure 5). This could be due to a higher exo-enzymatic activity in flocculated biomass tests leading to a higher formation of glucose, or a difference in transport of glucose between flocculated and dispersed biomass tests. Sonication was performed on a sub-volume of collected activated sludge, hence the same microbes should be present in both tests and there should not be a genotypic exo-enzymatic difference. Therefore, a more likely explanation is that transport of glucose was different in dispersed and flocculated activated sludge batch test.

The differences in transport regimes and particle biomass interactions between the two systems are presented in a proposed conceptual model (figure 7b). In flocculated biomass tests, colonization can be assumed to be floc-based, and the initial high F/M-ratio means that the substrate was partially colonized. On the other hand, the dispersed biomass allow for the entire substrate particles to adsorb bacteria, and form an initial thin biofilm covering the entire surface of the substrate. After initial colonization, the colonized surface would be e a hot-spot for extracellular enzyme activity. However, truly extracellular enzymes would also be free to diffuse to uncovered areas of the particle surface. Similarly, glucose produced on the starch particle surface in the early particle degradation phase (figure 7b), would diffuse into the bulk liquid for the partially covered substrate in both systems. However, the diffusion distance for glucose back into the flocculated biomass is longer compared to the short diffusion distance required by homogenously distributed single cells (Stewart 2003). Hence, glucose accumulate in the bulk liquid due to diffusion limitations for the flocculated system. On the other hand, for the dispersed system, glucose are consumed fast by free cells and do not accumulate. In the later stages, a thin biofilm can fully cover the entire starch particle surface in the dispersed system and glucose produced on the surface would be directly consumed, and not diffuse into the bulk liquid. Hence, for the dispersed system the combination of biofilm formation and

non-limited transport explained why glucose did not accumulate. For larger intermediates, there is no difference between the two systems, an aspect explained by diffusion coefficients decreasing with increasing molecular weight leading to accumulation in both systems.

PSD shifted towards smaller diameters at a slower rate in the dispersed biomass tests, compared to the flocculated biomass system (figure 3 and figure 2b). This can be explained by either flocculation of the dispersed biomass and a difference in particle break-up between the two systems. Due to a very low F/M-ratio, the effect of biomass flocculation would be minimal. Hence, the difference in PSD, indicate that for flocculated biomass, particles were breaking up into smaller particles at a faster rate than for dispersed biomass tests. Hence, increased porosity and colonization played a larger role for dispersed biomass, while particle breakup was more important with flocculated biomass. This can be explained by the proposed substrate and biomass interaction model (figure 7b). Formation of a colonization biofilm over a larger surface area by dispersed biomass, lead to extracellular enzymes attacking a larger area of the particle. Enzyme attack lead to pit and pore formation on the particle surface (Gallant et al. 1992; Robyt 2009; Tang et al. 2006), and could explain particle cracking seen by microscopy. Pit and pore formation on the surface of the particles would again lead to increased particle porosity.

Towards the end of the particle degradation phase, the biomass will converge in the two systems as illustrated in the conceptual biomass model (figure 7b). This is supported by a comparable surface area to volume ratio (figure 2), by PSDs (figure 3a and 3b) and by microscopy pictures (figure 4). Biomass particles measured in the system after the particle degradation phase has a mean spherical particle diameter of 10 μm . This show that particulate substrate lead to floc-formation of the biomass due to colonization. Hence, during the degradation phase dominated by HMW polymers as substrate, the difference of a flocculated and dispersed biomass system cannot be evaluated.

The results and conclusions gained in this work have implications for the way we understand particle degradation in bioprocesses. For the general case, intermediates form during particle and polymer degradation, and the biomass transport regime could allow for considerable intermediate accumulation in the bulk phase. When adequate, models used for particulate/polymeric slow biodegradable analysis should reflect this aspect of the system, as indicated by the conceptual models proposed herein. For systems with short hydraulic retention times, like biofilm and granulated biomass processes, significant fractions of COD would be lost to effluents reducing treatment performance and bioproduct yields. This is relevant for water and wastewater treatment systems, as well as bioenergy and biofuels processes based on particulate substrates.

Conclusions

- All intermediate polymer sizes are not formed to the same extent during starch particle degradation indicating non-random enzymatic degradation, either low or ultra high molecular weight polymers are preferred.
- During starch particle degradation, intermediate dynamics depend on the biomass structure. In a floc-based system, diffusion limitations allow glucose to accumulate in the system. This is a generic effect of bioaggregates.
- The combination of particle colonization, increased particle porosity and particle breakup led to increased substrate availability during particle degradation. Particle breakup was more important for flocculated biomass, while particle colonization and increased particle porosity was more important for dispersed biomass.

523 Acknowledgments

524 The authors want to thank Leif Ydstebø and Lena Pedersen at IVAR for providing activated
525 sludge. Internal funding at University of Stavanger, Institute for Mathematics and Natural
526 Sciences, financed this work.

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2017-08-16

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<http://dspace.lib.cranfield.ac.uk/handle/1826/12908>

Kristin T. Ravndal and Roald Kommedal. Starch degradation and intermediate dynamics in flocculated and dispersed microcosms. Water Science and Technology, December 2017, Vol. 76(11), pp2928-2940