DELFT UNIVERSITY OF TECHNOLOGY

Modelling the influence of extracellular polymeric substances in aerobic granule morphology

by

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Abstract

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In this thesis the influence of physical properties of structural EPS on granule morphology is examined. Two types of structural EPS are found to be important. These types of structural EPS are: surface bound EPS and hydrogel forming EPS matrix. VirtualLeaf is used to model granules which contain only binding surface bound EPS. The TST model is used to find the influence of both structural EPS components. In VirtualLeaf compact granules are formed with slight bulging features, similar to granules found in literature. The TST shows that binding surface binding EPS increase the compactness of granules. Furthermore it is shown that porosity of the EPS matrix also determines compactness and maximum size of granules. The strength and measure of the EPS matrix is shown to influence granule stability.

Acknowledgements

To try something different than experimentation in the lab, I wanted to learn about modelling. I did not have extensive prior knowledge in this discipline and thanks to the enthusiasm of both Roeland and Yuemei for the project I have learned a great deal about both granular sludge and modelling. I would like to express my gratitude towards my supervisor Yuemei Lin for the guidance, enthousiasm and also the enjoyable conversations. Furthermore, I would like to thank Roeland Merks for his help in setting up the models, for guidance in using the models and altering the models.I have enjoyed coming to the CWI for discussions. I would especially like to thank Claudiu Antonivici for his help with setting up VirtualLeaf, which proved to be a challenging task. Moreover I would like to thank the EBT group for the help in gaining insights about the project. Lastly I would like to thank my parents and girlfriend for their support and love during the course of my thesis.

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Abbreviations

AGS	**A**erobic **G**ranular **S**ludge
ALE	**A**lginate **L**ike **E**xopolysaccharide
CPM	**C**ellular **P**otts **M**ethod
ECM	**E**xtra **C**ellular **M**atrix
EPS	**E**xtracellular **P**olymeric **S**ubstance
GAG	**G**lycos **A**mino **G**lycans
PDE	**P**artial **D**ifferential **E**quation
SRT	**S**olid **R**etention **T**ime
TST	**T**issue **S**imulation **T**oolkit
VFA	**V**olatile **F**atty **A**cids

Chapter 1

Introduction

In this thesis the use of cell-based modelling in order to simulate granule morphology is discussed. VirtualLeaf, a plant-cell based model was used for simulation of granular sludge with cells that are anchored to the granule. Furthermore the Tissue Simulation Toolkit was used for simulating cell growth, EPS production and adhesion between cells and extracellular matrix. Both models are based on the Cellular Potts Method a method originally used for cell sorting and diffusion-adhesion reactions.[1]Firstly the granules will be discussed, secondly the structural components in granular sludge, thirdly the models used and finally the hypothesis and research questions.

1.1 AGS for more compact wastewater treatment

Wastewater treatment plants use microorganisms to remove organic and inorganic components from wastewater. These organic components can be for instance VFA's, sugars and biomass. Nitrate and phosphate are examples of inorganic components. The drive for wastewater treatment is to recycle as much resources as possible for the lowest cost. For this end, wastewater plants are shifting to the use of granular sludge, instead of activated sludge. In granular sludge, organisms immobilize themselves by the secretion of polymers that form a rigid gel and anchors them to the gel. This causes interesting changes in the properties of the biological aggregations. By forming rigid granules, more cells can be compactly packed in a granule, increasing the settling capacity. This decreases the size needed for settler tanks and operating costs will therefore also decrease. Due to concentration gradients in the granules, different organisms can live in symbiosis in one granule. This makes it possible to have multiple reactions in a single tank. With these advantages tank size decreases dramatically and therefore the cost of treatment decreases. The granules are also more resilient to damaging influences, like toxins, that can be found in wastewater, due to absorption by the EPS matrix or decreased diffusion

inside the matrix. Although methods for granulation of activated sludge exist, it is not yet completely clear how it happens. The EPS content in floccular sludge is lower than in granular sludge, making the influence of EPS obvious. However, wastewater is not a homogeneous mixture and components can vary over time and place. Due to this complexity multiple product pathways are used to create various EPS.

1.2 Characterization of EPS

The most important function of granular EPS is the formation of a rigid gel that immobilizes the inhabitants. For this structural function certain polymers have a bigger impact than others. For this reason, latest studies have concentrated their efforts on characterizing the components of structural EPS.

1.2.1 Gelation in structural EPS

Exopolysaccharides are the major factor in forming the rigid gels that make the granular sludge. Strong gels are able to trap water inside, are able to resist compression and stretching. Gels can form when crosslinks between polymer chains are formed and when negatively charged molecules are attracted. In general, β-1,4 linkages and β-1,3 linkages make strong rigid gels, whereas α-1,2 and α-1,6 linkages make more flexible structures.[2][3]

Most exopolysaccharides are hetero-polysaccharides formed by neutral and charged monomers. These neutral monomers are mostly hexoses, like glucose or fructose, and the charged monomers are mostly uronic acids. The uronic acids are also commonly replaced with acetate ester, inorganic phosphate or sulphate. [4]

In 2010, Lin et al showed that granular EPS contains ALE, a polysaccharide resembling alginate in its gelling capacity.[3] Alginate and ALE are not fixed molecules, but are a family of polymers with differing properties. Subsequent research with ALE showed that the differences in composition of uronic acid blocks determine the strength of the gel. ALE containing more guluronic acids molecules was able to make more cross-links between chains, producing a stronger gel.[5]

Seviour found that extracted exopolysaccharide behaved more like chitosan than alginate. Chitosan is a linear polysaccharide that is randomly linked by glucosamine molecules. This shows that the EPS hydrogel can be a combination between linear polysaccharides with cross-linking glucosamines.[6] In 2010 Seviour et al. elucidated the chemical structure of a new structural polysaccharide, granulan. The structure was a complex heteropolysaccharide chain, with repeating hexoses and glucosamines, with

branching disaccharides containing uronic acids.[7]In 2017 a capsular EPS was found composed of hetero-polysaccharides when using an acid extraction. [8]

These studies show that different polymers can provide the gelling properties of granular EPS. Furthermore they show that not a single extraction method can be used for the extraction of structural EPS. It is therefore that new protocols and extraction methods have been found in the past 2 years. With these studies new types of structural EPS were found.

1.2.2 Similarities between ECM and EPS

For a long time bacterial EPS and animal ECM have been seen as different substances, yet in the recent years the likeness between them has increased as our understanding of EPS grows. The presence of GAG's was detected in structural EPS by using a GAG kit normally used for medical purposes. GAG's have a big influence on the gelation. This is because GAG's are able to span large volumes and trap lots of water molecules.(BSc Thesis: Stef Broenink, 2017)[9]In a MSc thesis performed by Swetha Umesh, the presence of sialic acid was detected in EPS granules grown under salinic conditions. Sialic acids are often found in glycoproteins and these function in the ECM as either opposing compression or stretching.(MSc Thesis: Swetha Umesh, 2017) The suggested glycoproteins were found in 2018, where Lin et al. proved the existence of glycoproteins in structural EPS.[10] For a more complete explanation of the animal tissue ECM see Appendix A1.

Following this analogy, it should be possible to find in granular EPS the functional groups found in ECM. Polyanionic linear polysaccharides linked together for the formation of hydrogels. Structural components as elastin-and collagen-like would contribute to the ability of the granules to withstand pressure and stretching forces. It is thus important to look at polymers that are similar to the polymer chains found in animal ECM or look at what polymers are found in different types of granular sludge.[9][11][12]

1.3 Modelling granular growth

The cells are seen as individual agents that consume substrate and grow, or have no access to substrate and die. The interactions between the cells is based on pushing back of other cells as they grow and the competition for substrate.Models used to model biofilms have not yet been found that model specifically for the different structural EPS. In some cases EPS is seen as a passive particle that can be secreted by the cell.[13][14][15] In 2014 the cells were examined as a mass spring system to investigate the effect of cell properties biological aggregate formation. [16] Most of these models are made for

modelling biofilms and study different properties, but do not study the effect of EPS on granule formation.

In recent years more information regarding EPS was found and multiple different structural EPS compositions were found. In light of the current studies showing resemblances between the ECM and EPS in granular sludge, the use of tissue models should proof to be an interesting step. In these model not only the competition for substrate is included, but also interactions between cells and the EPS in which they reside can be simulated This would provide a perspective on how EPS affects granule morphology. In this thesis two situations are examined. The granules that form compact granule with surface bound EPS, and other granules where a EPS matrix hydrogel is formed. [8][17][6][7][10] The granule found with the capsular EPS or surface bound EPS is an extreme example found, and is explained by only producing surface bound EPS. This looks similar as to how plant cells are anchored together by their cell wall. The bacterial EPS resembles the plant cell walls and therefore the VirtualLeaf can be used. VirtualLeaf is a vertex-based model, that simulates cells as a collection of nodes linked together by viscoelastic walls. The cells are anchored as neighbouring cells share cell walls. A similar method as in the CPM is used to have nodes be moved randomly and the stretching and compressing of the walls are included in the Hamiltonian energy function.[18] Biochemical rules can be included into the model to include substrate limited growth. Substrate is modelled to come in from the cell perimeter and diffuse into the cell. As this is an aerobic granule, both oxygen and substrate are used in the substrate limited growth and first substrate is fed and stored. After a certain period substrate feeding is stopped and aeration begins. This should simulate the feast-famine periods in the reactor and show the influence of this feeding regime. By changing parameters for growth rate, substrate concentration, rigidity, and perimeter EPS concentration influence of growth and EPS can be simulated with the VirtualLeaf model.

For granules producing both surface bound EPS, and hydrogel EPS a different model was used. Due to the similarities of EPS and ECM it was chosen to use the animal TST. In this model cells are seen as patches in a lattice site and with a Monte Carlo algorithm each patch is randomly moved. The form and linkage with other cells is controlled by an Hamiltonian function which is minimized using metropolitan dynamics. A random patch site is chosen and moved in a random direction a check is made to see if the energy function $dH < 0$. If so the move is accepted, if not it is rejected. To be able to stay out of local minima, moves with $dH \geq 0$ are accepted with a Boltzmann weighed probability.

The toolkit comes with possibilities to include cell growth, cell death, chemotaxis and adherence to other cells. An application for this method and toolkit was the simulation of vasculogenesis. [19][20]

Yet also other mechanisms can be tested by adding different biochemical rules. In the model used in this thesis, the discreticized field is used to have substrate enter from the sides and diffuse through the lattice site. When it reaches the cells it is taken up, triggering a substrate dependent growth reaction as well as a production of polymers in a second layer of the discreticized field. A penalty function was added when cells are in concentrations of EPS that exceed a certain value. Furthermore by changing the energy of binding of intercellular lattice sites or binding with the medium, the influence of these binding can examined.

After adjusting the models, a parameter search can be performed to see what kind of influence the parameters have on the granular morphology. This can be used to predict what kind of forms coincide with the parameters used. If these parameters and morphologies can be linked, to experimental data it can be possible to predict what kind of shape a granule should have when EPS composition is known. By doing a large scale parameter search all the possible morphologies can be found for this system, yet such a task does have some computational limitations. Finding one-dimensional correlations can be done, but finding multidimensional correlations is computationally heavy and can only be done to a certain extent. [21][22]

1.3.1 Hypothesis

In different kinds of granules different structural EPS can be found. The composition of the structural EPS varies, and so its chemical and physical properties will be different. This will have an influence on the form of the granules. VirtualLeaf can be used to simulate granule morphology of closely binding surface bound EPS. The TST can be used to model the more basic granules by a mix of surface bound EPS and hydrogel matrix formation. By using emergent models it will be possible to simulate the granule growth and morphological changes over time. The parameters in the model can be used to simulate the physical changes in structural EPS.

1.3.2 Research questions

The aforementioned hypothesis has led to the formulation of the main research question:

- How do physical properties of structural EPS influence granule morphology in granular sludge?

In order to answer this research question the following sub-questions have to be answered:

- Why do organisms produce EPS?
- What kind of physical properties could be important for granule morphology?
- Can we use existing models like VirtualLeaf and the tissue simulation toolkit?
- How can we link physical properties to model parameters?

1.3.3 Approach

The Tissue Simulation Toolkit and the VirtualLeaf model are used for the simulation of granular sludge. The experiment is performed on single granules that change in size over time by the addition of substrate from the outside. The models were modified to grow with substrate limited growth and in the case of the Tissue Simulation Toolkit also produced EPS. The connections between the cells are simulated by either shared cell walls, in VirtualLeaf, or adhesion energy changes, in the Tissue Toolkit. A parameter search is performed in combination with morphometrics to see the effect of parameter changes and to quantify the output.

Chapter 2

Materials & Methods

In this thesis VirtualLeaf and the TST are used to simulate granular morphology. In the first section the installation of both models is described briefly. In the following section the VirtualLeaf model is described. This is followed by the TST model description.

2.1 Model Installation

Both TST and VirtualLeaf are open-source projects meaning that the source code is available and alterable by anyone online. To download VirtualLeaf go to:

https://code.google.com/archive/p/virtualleaf/downloads.

To download the TST go to:

https://sourceforge.net/projects/tst/.

Tutorials for installation are included in the downloaded files. Yet a few important notes will be discussed. For the compilation of both models, qt 4.8.5 or higher is needed. In Linux this can easily be downloaded from the synaptic package manager, by searching for the package qt4-dev. The C++ compiler needed can also be found by looking for the g++ package. The models can be compiled from the src folder by typing >qmake -projectname.pro and then typing > make clean; make. The programs can be run by typing >./'executivefile' in either in the bin folder, or the src folder, respectively.

2.2 VirtualLeaf model description

VirtualLeaf is built with the idea of being a platform on which experimental biologists can test new hypothesis without having to build a whole new model. The basis of the model represents the cells as a collection of nodes and viscoelastic cell walls connecting

these nodes. The cells move using a Monte-Carlo energy minimization approach. In this approach a random node is chosen and moved in a random direction. If the energy of the system, displayed in the Hamiltonian, is lowered then the move is accepted, else it is accepted with a certain Boltzmann-weighed probability. The Hamiltonian shown in equation 1 is the basis of the model used. For further information see Merks et al. (2011)[18]

$$H = \lambda_A \sum_i (a(i) - A_T(i))^2 \tag{1}$$

For this model certain values were tested that can easily be added into the extended Hamiltonian. (Equation 2.)

$$H' = H + \lambda_B \tag{2}$$

In the model, plug-ins can be built that describe biochemical processes in the cell. For the double substrate limited substrate growth a few things were altered from the Tutorial0 template. The number of chemicals is set to 2, because two substrates will be used. In other tutorial codes, the second chemical is used to simulate the transporter protein PIN, and every cell will have a standard set. For us this is set to zero, as second chemical represents oxygen. The first cell rule that was added was the inclusion of division. When the cells reach an area that is bigger than rel-div-threshold*base area, they will divide. It is set that the chemical of the cell decreases to zero to mimic the energy requirements of growth and division. The cells grow according to a double substrate limited growth rate:

$$\mu = \mu_{max} * \frac{Cs}{(Ks + Cs)} * \frac{Co}{(Ko + Co)} \tag{3}$$

The area of the cell is increased with an amount equal to the growth rate, because the model tries to have the cells at the target area, the cells will grow. The influx is set to be according to equation (4)

$$Influx = (par_{concentration} - Cs) * par_{leaftipsource} * Length \tag{4}$$

Making the influx dependent on the difference in concentration, a parameter that can be changed for changes in inflow and the wall length. Inside the cells, only passive diffusion occurs dependent on the diffusion coefficient and the difference in concentration. Lastly part of the substrate that is taken up by the cells is destroyed to mimic maintenance. This is governed by parameters k1 and k2 for oxygen and substrate, respectively. The complete code for the model plugin can be found in Appendix B1.

2.2.1 Parameter explanation

For experimentation the parameters are already set for the right conditions yet a few need to be adapted. The diffusion coefficient is not set for oxygen so this is set at 1e-6 cm^2/s, the same as for substrate. The inflow concentration or par_concentration was set to be 0.0008 g/L and 0.001 g/L, respectively for the concentrations of oxygen and substrate. Oxygen was set slightly lower because it has a maximum solubility of 8 mg/L and substrate can reach higher concentrations. These values are switched on and off repeatedly.

The decay parameters of substrates due to maintenance was set to be 0.1% of substrate uptake. This was set so low because cells already lose substrate when dividing and will else lose to much of the stored substrate and not grow. The cells that can take up and store the nutrients are investigated. It was however not set at zero to see a concentration gradient. The leaftip source parameter was set to be 0.01, but this parameter in this case is another control parameter, and other values could be used. The full parameter settings can be found in Appendix B1 in Figure B.1

2.2.2 Double substrate limited growth

The experiment is started with a leaffile that can be set for the specific model plugin. The model plugin can be chosen, and from there the parameters can be changed. For the experiment the leaf file from auxin-dependent growth was used, as this contained all the chemicals. The granules start with 4 cells.

When the simulation is started, every two hours of simulation time the parameters of oxygen and substrate concentration inflow were changed. This was done to simulate the feast and famine cycles performed. From the settings, movie frame collection can be set.

2.3 TST model description

The Tissue Simulation Toolkit is a model that uses the Cellular Potts Method, introduced by Glazier & Graner.[1] The basics of this method, is that the system randomly moves parts of cells and checks if this lowers the Hamiltonian by a Monte Carlo step. The Hamiltonian in our model is based on the adhesion between cells and medium and the target area of the cells. When a move is made into an area with medium, it will assign this move with the energy correlating with the medium, with other cells the cell energy is assigned. The target area constraint keeps the cells at their target size, but

can also be used to make the cells grow.

$$H = \sum_{x^{\rightarrow},x'^{\rightarrow}} J_T(\sigma^{\rightarrow}),(\sigma^{\rightarrow\prime})(1-\delta_{\sigma,\sigma'}) + \lambda_A \sum_i (a(i) - A_T(i))^2 (5)$$

The TST has been fully described in Merks et al.(2005). [19] For the experimentation on granules, certain mechanisms were added to the model. First was the addition of substrate from the outside. At the system perimeter an inflow equal to the secretion rate parameter was set. It was assumed that no convective flow is present in the system, so transport of substrate only occurs due to diffusion. The diffusion was calculated by finite-elements. When the substrate reaches the cells, it gets taken up with a rate estimated with the Monod equation. (Equation 6)

$$qs = qs_{max} * \frac{Cs}{(Ks + Cs)} \tag{6}$$

With qs_{max} the second secretion rate parameter, Cs the concentration of substrate and Ks 0.1. This uptaken substrate would either be converted into EPS or into growth. The rate at which this happened depended on the fraction going to the mechanisms and the substrate uptake rate. Making it linearly dependent on the substrate uptake rate. This linear dependency was chosen as it is the easiest.

$$qs = a * Y_{xs} * qs + b * Y_{EPS} * qs \tag{7}$$

With a = fraction and b = $\frac{1}{a}$, the yield for both EPS and growth was set at 0.2 $gBiomass/gSubstrate$. Both were the same yield, because EPS and biomass have a very similar composition and therefore the same theoretical yield applies. From there the growth rate was determined and growth was approximated with an increase in target area. The target area was however an int and therefore to get the required growth rate the chance that the cell would grow 1 area is set to be proportional to the growth rate. EPS is secreted by cells but only at the cell border when they border the medium.

EPS was modeled by either the binding between cells due to the adhesion, or by the EPS that was excreted in the EPS production reaction. To simulate the rigid hydrogels that form, movements into EPS rich area got a penalty to the dH, making it less probable to happen. This was performed with an if statement checking of the moved lattice is in a field with concentration above 1e-5 g/L. If so then dH would be increased by 5000. This makes it highly unlikely for cells to move into EPS rich environments. The 1e-5 concentration can be changed based on the gelling concentration of EPS. For now it was set at 1e-5 g/L, but can be higher, around 1e-2 g/L, as was shown that gelling occurs at $\frac{w}{w}\%$ 0.1. [6]

Diffusion inside area with high EPS was set to be slower. When the concentration of

EPS was 1e-4 g/L or bigger, the diffusion rate would be the parameter diffusion rate given. It was expected that when the dense hydrogels are made, the diffusion is hindered and therefore the diffusion coefficient is lower. For the whole code see Appendix B2

2.3.1 Parameter explanation

For the experiments certain parameters are important. The adhesion to cells and medium was made by making a .dat file with the number of cell types, adhesion of medium to itself and adhesion of cell to other cells and medium. This is shown in Table 2.1. Table 2.2 and Table 2.3 show the most important parameters used for experimentation. For the full parameter file see Appendix B2.

TABLE 2.1: $J_{adhesion}$ table

	J_{cell}	J_{medium}
No. of cells	2	
Medium	0	
Cell	5	15

TABLE 2.2: Cellular Potts parameters

	Cellular Potts parameters
T	50
Target area	100
Lambda	200
J_{table}	'name'.dat
Connection dissipation	0
Border energy	100

TABLE 2.3: PDE parameters

	PDE parameters		
No. of chemicals	3		
Diff. coeff.	7.32e-9	1e-19	0
Substrate diff. coeff. in EPS	7.32e-9		
Secretion rate	1e-1	1e-1	
Fraction	1		

2.3.2 Parameter search

The parameter runs were performed by calling the model via a python script that creates a parameter file and Jtable.dat file and then runs the model using these files. Via for loops the experiments can be repeated for different values or for repeating experiments. In the python script a text file is written containing the morphometric values from

this simulation. Copying the executive folder makes it possible to run multiple similar experiments in parallel. Whereas nested for loops could test for multivariable changes although this is not recommended as multiple for loops are not efficient.

2.4 Morphometrics

The resulting granule morphology was quantified by looking at compactness, circumference and number of cells. these measurements were performed during the simulation and the output of this data was written to a .txt file. The resulting text files were concatenated and the resulting measurements were plotted using Matlab. The values between the data points were approximated by interpolation and non-linear regression.

2.4.1 Fitting of results

The experiments were repeated multiple times, varying from 4 to 10 times. This was done in order to get more realiable results. The model uses random steps and in general tries to move every patch a random distance. These random steps can have large effects if set in a certain direction, therefore completing a certain number of repeats is essential to get significant results. For the fitting of data gathered from the effect of different adhesion energies, it was found that by repeating ten times mean squared error of less than 5% could be reported.

To show how the parameter changes affected the morphometric variables, the data points were plotted using MATLAB. The amount of points measured was not extensive so for a better visualization of the effect the points were interpolated using MATLAB's function interp1. This interpolation function can either find linear relations between the data points using the 'linear' mode or can find cubic relations using 'cubic' or 'pchip'. Interpolation can be useful in showing the trend of the data, but does not give a dependency of data. Therefore a regression analysis was performed. Using non-linear functions the data was approximated, the best-fit criteria optimization was performed using MATLAB function lsqcurvefit. For an example of the matlab file and explanation see Appendix B3.

Chapter 3

Results

3.1 VirtualLeaf

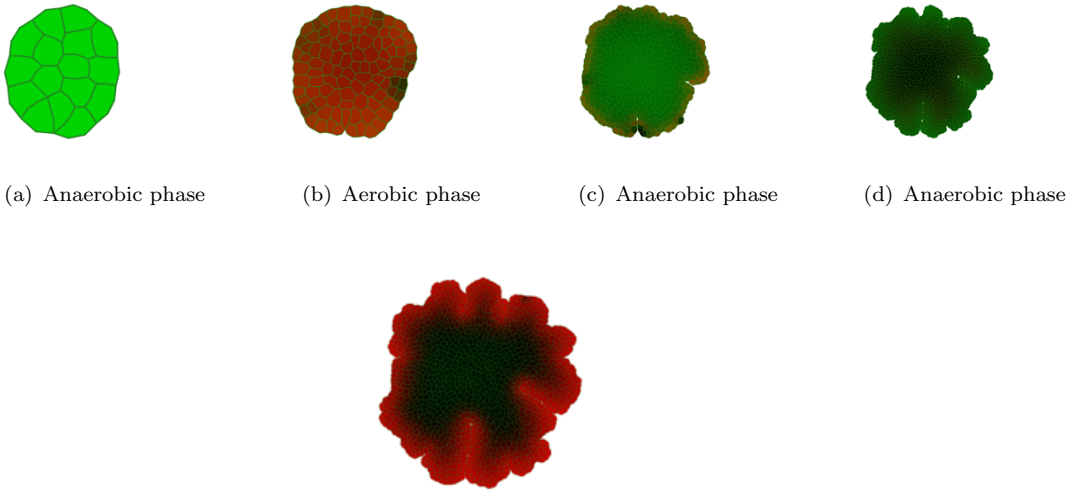
VirtualLeaf was used to simulate the granules obtained by Pronk et al. (2017)[8] The granules were found to be encapsulated with EPS and closely packed to each other. It is assumed that these granules contain bigger amounts of surface bound EPS and that EPS matrix can be neglected. To simulate granule growth with interconnected cells the VirtualLeaf model was used. In this model, cells are connected by cell walls, which simulates the surface bound EPS.

Merks et al. noted that because when a cell in the center of the granule wants to move, it has to move additional cell walls. Therefore it is more inhibited than a cell on the outer perimeter of the granule, making the cells bulge out more easily. In order to make smooth forms, this was counteracted by Merks et al. (2011) by having the outer perimeter walls have a stiffness that is twice the internal wall stiffness. [18] This could be explained by the fact that plant cells on the outer side of the plant normally produces more cellulose, making them stiffer. In granules it is not known of cells on the perimeter make relatively more EPS, therefore the relative perimeter is lowered. However when granules are sufficiently large a concentration gradient can be found in the granules. This means that cells on the outer perimeter of the granule will have relatively more substrate than cells in the center of the granule. Therefore it is assumed that cells on the perimeter produce relatively more EPS than cells in the middle. This creates a stronger connection between cells in the perimeter. In order to simulate this the outside perimeter wall stiffness was 1.5 times higher than other walls.

To recreate the conditions of AGS the feed was switched from anaerobic substrate feed, to aerobic without substrate feed. Substrate is taken up by the cell and stored inside. The anaerobic and aerobic phase was switched every 2 hours of simulation time to simulate the cycles. When the feed is switched to the aerobic phase, cells that have

stored substrate and receive oxygen will grow with a growth rate that is modelled to be a double substrate limited growth rate.

Substrate diffuse from the outside of the granule into the granule. This depends on the concentration of substrate in the bulk liquid and the area in contact with the bulk liquid. This inflow of substrate was approximated by having the outer cell walls be the sources of substrate. The influx from the walls is dependent on the concentration of substrate, a parameter to control the inflow and the length of the cell wall. This makes a known flux enter the granules from the outer cell wall, which can be used to calculate the diffusion inside the rest of the granule. Using these assumptions and conditions the model was run which resulted in Figure 3.1.



(a) Anaerobic phase (b) Aerobic phase (c) Anaerobic phase (d) Anaerobic phase

(e) Aerobic phase

FIGURE 3.1: Growth of aerobic granular sludge with anaerobic and aerobic phases. a) After anearobic phase cells are filled with substrate. (green) b) After aerobic phase, start of the anaerobic phase. Cells have used all the substrate and have oxygen concentration. c) After the anaerobic feed phase, start of aerobic phase. d) Start of the anaerobic substrate uptake phase, diffusion gradient seen. e)Start of aerobic phase, oxygen diffusion gradient.

The simulation results shown in Figure 3.1 shows that as time progresses, the bulging occurs and granule size increases. When the granule is big enough concentration gradients can be found. Making the assumption of higher EPS formation on the outside reasonable. Although it is hard to compare a 3-D granule with a 2-D simulation some analogies can be found. In Figure 3.2 a tubular structure is seen in the granule and, when zoomed in, closely packed cells can be seen in the granule. These features are very similar to the simulation found in VirtualLeaf.

In the figure an imperfection can be found in the model in respect to substrate uptake. This can be seen by the substrate inflow inside the granule. An assumption was made

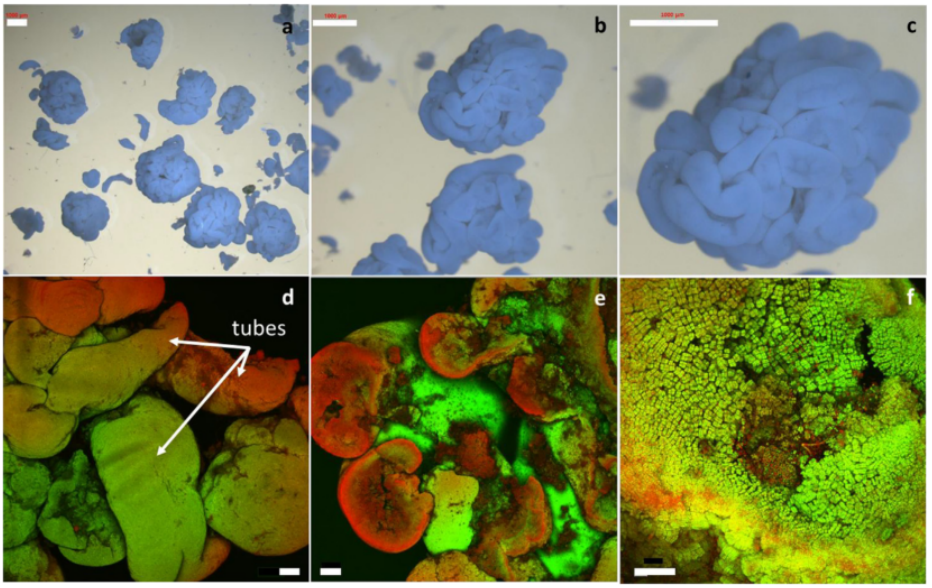


FIGURE 3.2: Adapted from Pronk et al.(2017)

that the influx in the perimeter walls is equal everywhere. In reality the influx is dependent on the concentration of substrate and cells compete for substrate. Cells that are closer to higher concentrations will have higher influxes of substrate and therefore take away resources for surrounding cells.

3.2 Tissue Simulation Toolkit

In most cases of granular sludge the amount of surface bound EPS is not higher than the amount of EPS matrix. This means that the effect of EPS matrix cannot be neglected. Therefore a different model is used to describe granules with surface bound EPS and EPS matrix. The TST model can simulate the surface bound EPS and can be adapted to include EPS matrix production.

With the adaptations the TST was used to test the influence of physical properties of structural EPS. This structural EPS can then be subdivided into the surface bound EPS and the EPS matrix. The surface bound EPS could be tested by varying two parameters. These were the adhesion between cells and the adhesion of cell to the medium. The adhesion between cells is in this model the same surface bound EPS as before it connects the cells anchoring them to each other. The adhesion to medium is a measure of how much cells want to be dissociated from other cells. This can be seen as surface bound EPS that does not bind other cells, or inhibits binding of cells.

With this basics the effect of changes in the surface bound EPS were investigated by changing the adhesion to cells and adhesion to medium parameter. Furthermore the effect of changes in the EPS matrix were examined in terms of production and decay, movement, and porosity of the matrix.

Lastly as granules can vary as substrate uptake vary, different experiments were performed on the effect of substrate availability in the granule. Here the substrate inflow concentration, maximum uptake rate and fraction of substrate going to growth and EPS are examined.

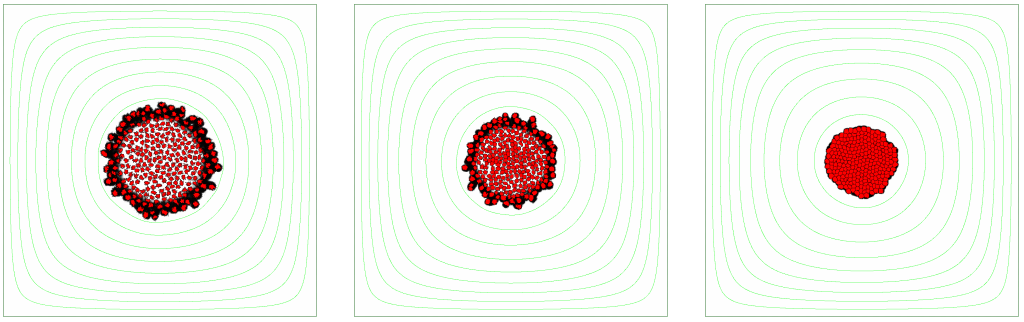
The morphology of the granules is examined by comparing granules from different parameter values, and by a morphometric analysis. In this analysis for three variables a value can be given to provide a quantitative measure for morphology. The three variables are, as mentioned before, the compactness, circumference and the number of cells.

3.2.1 Influence of surface bound EPS on granule morphology

As mentioned earlier, the surface bound EPS is simulated with the parameters adhesion to cell and adhesion to medium. It is assumed that the adhesion to cells is a measure for the relative amount of surface bound EPS that bind cell together. Furthermore the adhesion to medium is then assumed to be a measure for the relative amount of surface bound EPS that is non-binding or even inhibiting inter cellular binding. So when the adhesion to cell parameter is higher than the adhesion to medium, relatively more surface bound EPS are available for linking cells. Merks et al. (2005) advises that these parameters can be changed from 1 to 50, thus this was the range of experimentation.

[19]It was further assumed the cells start as a similar granule, regardless from the surface bound EPS to be experimented. For the morphometric analysis the data is fitted with a non-linear equation to be able to approximate unmeasured data points.

Firstly, only the effect of adhesion to cell was examined. The adhesion to cell was kept constant at 25 whilst the adhesion to cell was changed from 1 to 51. The granules formed with the different adhesion to cell values are shown in Figure 3.3. The morphometric values obtained from this examination are shown in Figure 3.4



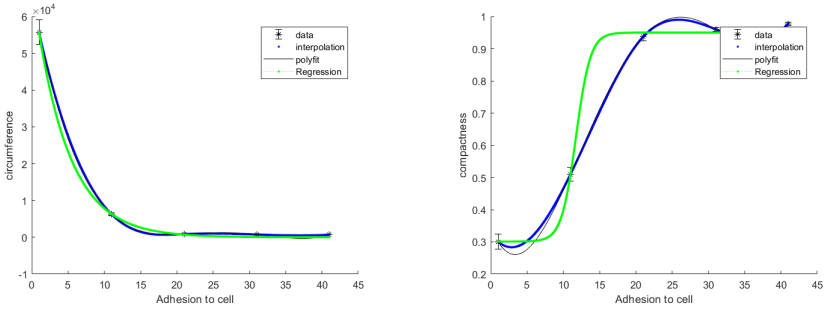
(a) Adhesion to cell 5 (b) Adhesion to cell 10 (c) Adhesion to cell 15

FIGURE 3.3: Effect of different adhesion energy between cells for a fixed medium adhesion energy of 25

From the results shown in Figure 3.3 can be seen that when the adhesion between cells increases the granules become more densely packed. This shows that when the number of binding surface bound EPS is relatively higher the granule will become more compact. With a lower adhesion to cell, more cells are able to move freely until the EPS matrix immobilizes the cells. This results in larger granules where slow diffusion can cause the granule cores to starve and die.

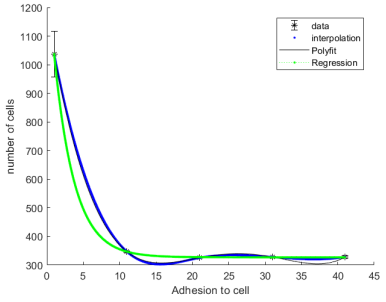
The results of the morphometric analysis shown in Figure 3.4 concur with the granules shown in Figure 3.3. The measured circumference seems to drop when the adhesion to cell is increased until it reaches a plateau value. This also happens with the number of cells, which drops to the starting number of cells as the adhesion to cell is increased. The compactness of the granule increases from approximately 0.3 to 1 when adhesion to cell rises. This shows that when the cell binding surface bound EPS is increased cells will form a more compact smooth granule, whereas when it is lower a bigger more lumped granule is produced.

The influence of the cell-binding surface-bound EPS was investigated in the previous paragraph for a fixed inhibitory surface bound EPS. For this experiment the assumption was made that the cell binding inhibitory EPS can vary in the granule. To test the influence of the inhibitory EPS together with the cell binding EPS, the same test was performed for varying adhesion to medium values. The data was interpolated to generate a smoother picture using the two-dimensional interpolation function of MATLAB



(a) Circumference

(b) Compactness

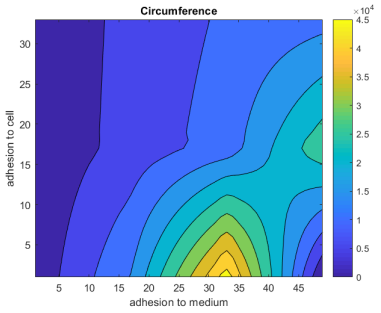


(c) No. cells

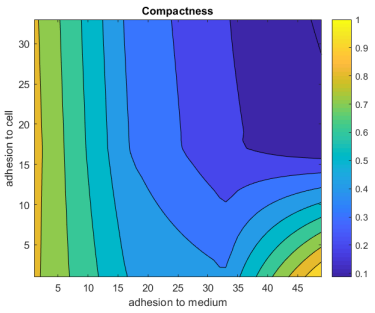
FIGURE 3.4: Effect of different adhesion energy between cells for a fixed medium adhesion energy of 25

interp2. This creates a matrix of values that is shown using a contour plot, which can be seen in Figure 3.5.

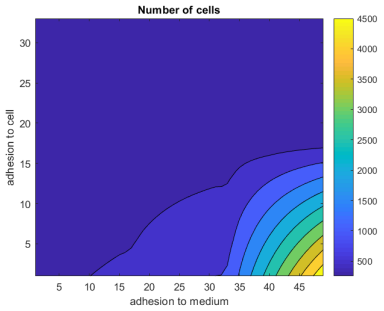
Two peaks are clearly visible in Figure 3.5a for the circumference value. These peaks are found when the adhesion to medium is relatively higher than the adhesion to cell. The biggest peak is seen when adhesion to cell is 1 and adhesion to medium is 33. The compactness is shown in Figure 3.5b and shows that compactness peaks at either low adhesion to medium or at maximum adhesion to medium. The number of cells are shown in Figure 3.5c and this shows that at adhesion to medium 50, the number of cells are the highest. This offers insight in the peaks of compactness at 50. The cells in the granules are not bonded and can therefore move more freely in substrate rich areas. This increases the number of cells so much that the system is overgrown. The same applies to the circumference, at adhesion to medium 50, the circumference drops due to complete overfill of the system. This shows that setting the adhesion to cell and adhesion to medium has to be done carefully as the cellular growth can overgrow the system. When comparing Figure 3.4 and Figure 3.5 the values do not align. This is because a different version of the model was used for the multivariable analysis. This analysis did not contain the EPS matrix production, but included a cellular EPS excretion with a negative chemotaxis constraint that made the cells move away from each other. Although the figures cannot be compared, Figure 3.5 shows how



(a) Circumference 2-dimensional field



(b) Compactness 2-dimensional field



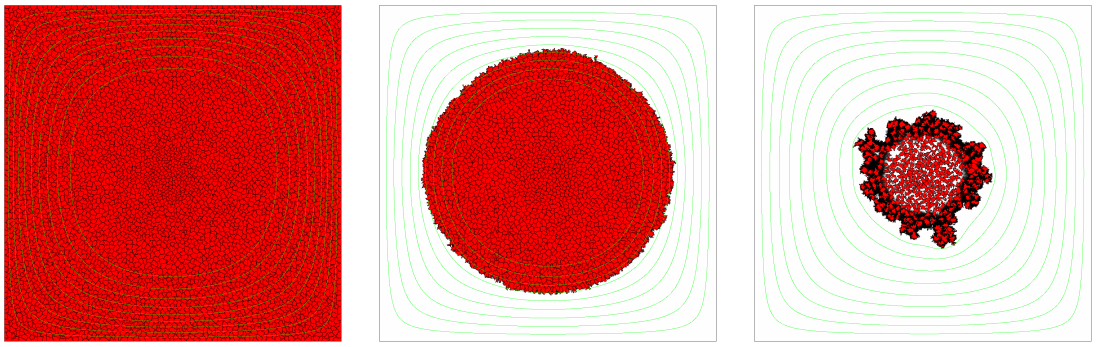
(c) No. cells 2-dimensional field

FIGURE 3.5: The different 2-dimensional analysis for varying J_{cells} and J_{medium}.

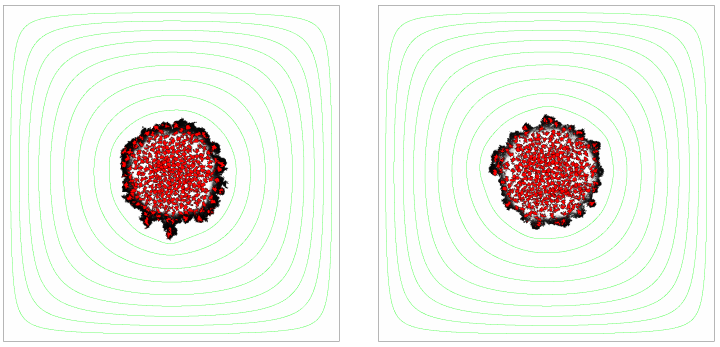
important setting the right adhesion values and how rapidly cells can overgrow at border values.

3.2.2 Influence of the porosity of the EPS matrix on granule morphology

The study performed by Xavier showed the concept of EPS production as a competitive advantage. Cells producing the EPS matrix push their progeny outwards into substrate rich environments. These cells can then take up the available substrate which suffocates their neighbours.[23] Building on this idea, it is proposed that the lower substrate diffusion coefficient of the dense EPS matrix is another evolutionary advantage for cells that produce EPS. An assumption is made that the substrate diffusion coefficient is mainly dependent on the EPS matrix. It is assumed that when an EPS matrix is made, the substrate diffusion coefficient is lowered inside that matrix. When the substrate diffusion coefficient is lower, cells have more time to uptake or hydrolyse the substrate diffusing into the granule. This experiment was tested for several different substrate diffusion coefficients. The beginning diffusion coefficient tested was in the same order as substrate diffusion coefficient in the medium. With every next diffusion coefficient becoming 10 times smaller. The results of this experiment are shown in Figure 3.6.



(a) Diffusion coefficient 1.32E-7 (b) Diffusion coefficient 1.32E-8 (c) Diffusion coefficient 1.32E-9



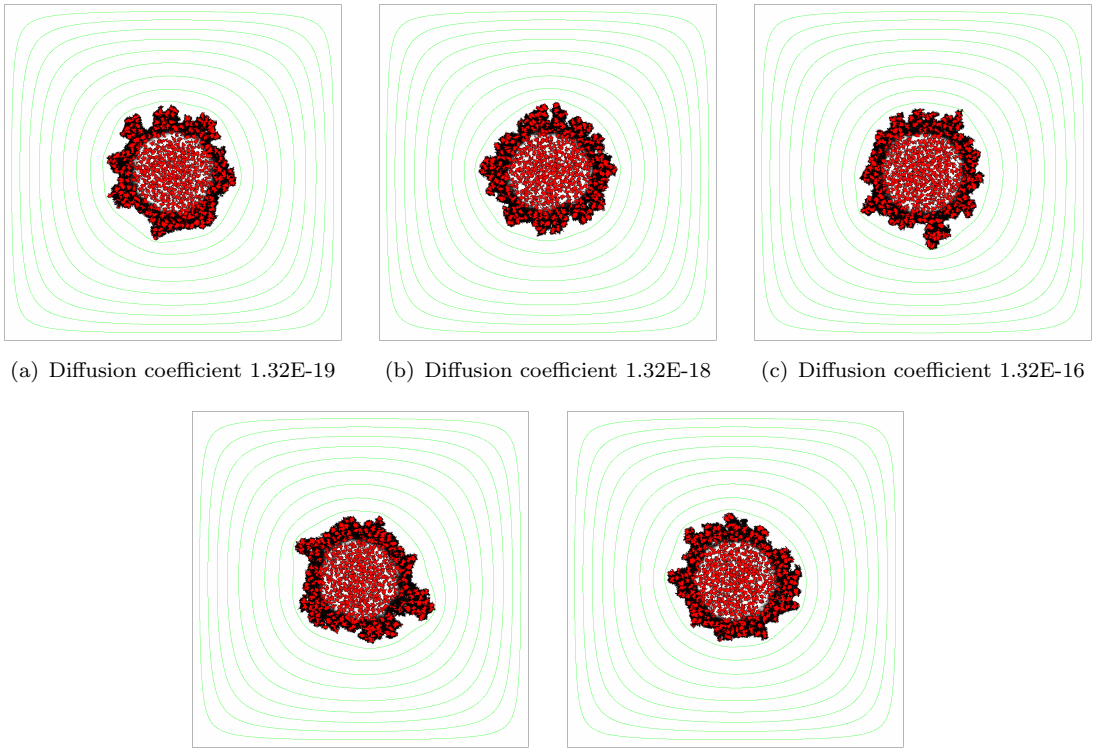
(d) Diffusion coefficient 1.32E-10 (e) Diffusion coefficient 1.32E-11

FIGURE 3.6: The influence of the diffusion coefficient of substrate in structural EPS

When the substrate diffusion coefficient in EPS is in the same order as the diffusion in medium, cells will grow out of bounds as can be seen in Figure 3.6a. When the diffusion coefficient is lowered by 10, the granule is smaller yet will grow out of bounds if run for a longer period of time. For the subsequent substrate diffusion rates seen in Figures 3.6c-e the size of the granule becomes smaller and the active area of the granule also decreases. In Figure 3.6a-b all or almost all the cells are receiving substrate and thus growing. In the other figures only the outside perimeter has access to substrate. This is also dependant on the substrate concentration, but this will be explained later on. Stable granules seem to have formed at diffusion coefficients of 1.32e-9 cm^2/s and lower. It is however apparent that, the inactive area in the center of the granule becomes bigger as the diffusion coefficient of the EPS becomes smaller. There is less growth and less EPS because only the outer cells have access to resources. At a certain point when this inactive area becomes too big, the granules can disintegrate, returning to smaller granules or flocs.[24]Therefore when a dense polymer matrix is produced, granule size is limited. Cells in granules are equipped with an arsenal of enzymes that can digest the matrix and will do so when experiencing stress or when they lyse.[2][25]This interaction can be an important factor in granule morphology. The effect of EPS secretion and digestion on granule morphology could be an interesting study in combination with the porosity of EPS.

3.2.3 Influence of diffusion of EPS matrix on granule morphology

The EPS hydrogels are considered as a sturdy network of polymer chains and therefore it is assumed that the movement speed of EPS is close to 0 (1e-19 cm^2/s).[6][7] In this experiment the assumption is made that EPS movement can be simulated by diffusion of EPS. It is assumed that EPS matrix can move, albeit slowly by means of diffusion. To see what kind of effect movement of EPS has on the granule morphology the diffusion coefficient of EPS is varied to see the effect of flow of EPS in the granule morphology. The EPS diffusion coefficient was varied from 1e-19 cm^2/s to 1e-13 cm^2/s and results are shown in Figure 3.7



(a) Diffusion coefficient 1.32E-19 (b) Diffusion coefficient 1.32E-18 (c) Diffusion coefficient 1.32E-16

(d) Diffusion coefficient 1.32E-15 (e) Diffusion coefficient 1.32E-13

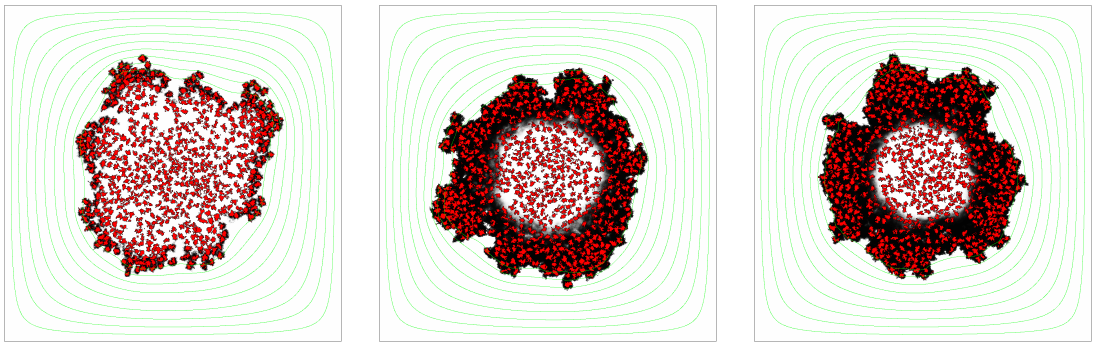
FIGURE 3.7: The influence of the diffusion coefficient of EPS

The influence of diffusion of EPS was not noticeable. The location of EPS is still dictated by the location of production and not by movement of EPS. The EPS diffusion coefficient can be increased further to see what kind of effect this has on the granule, but this was thought to be inappropriate. Furthermore modelling the movement of EPS by only using diffusion is using some big assumptions. Normally the flow of highly viscous fluids is a product of bulk flow.This can be explained by the production of EPS at the cell membrane that pushes away other EPS. For this it is assumed that the fluid is incompressible. It is assumed in the model that the EPS is compressible because the concentration of the EPS increases when EPS moves in a location where EPS is already

present. Therefore it is not possible to simulate advection or bulk flow of EPS. However before the gelling concentration is reached the strong and rigid gel is not formed, so for low concentrations of soluble EPS this movement of EPS can be used.

3.2.4 Influence of EPS matrix decay rate on granule morphology

Rigid gels will less likely lose EPS by shear stress or degradation because of the strong gel. It is assumed that the strength of the EPS matrix correlates with the resistance to degradation and loss of EPS. Furthermore it is assumed that this strength can vary because of differences in EPS compositions. To test the influence of this EPS matrix strength, the general decay rate of EPS was varied. The effect of changes in the decay rate can be seen in Figure 3.7.



(a) Decay rate 1E-3 (b) Decay rate 1E-5 (c) Decay rate 1E-6

FIGURE 3.8: Influence of decay rates on granular sludge

Figure 3.8a shows the highest decay rate and shows EPS only at the edges of the granule, in substrate rich environments. When the decay rate is lowered as shown in Figure 3.8b, larger EPS matrix areas can be seen. Only at the center of the granule no EPS matrix is present. When the EPS matrix decay rate is lowered further as shown in Figure 3.8c even more EPS can be seen. The core without EPS matrix is smaller than in the other figures shown.

The EPS matrix concentration is balanced between a production rate and a degradation or decay rate. When the decay rate is only slightly lower than the maximum production rate, the only place where EPS will accumulate is at the places with the highest production rates. This can be seen clearly in Figure 3.8a. When the decay rate is significantly lower the decay rate shall not affect the EPS at locations where EPS is produced. However when a concentration gradient is present in granules cells in the center have less substrate available and can therefore produce less EPS. As is clearly seen in Figures 3.8b-c. Granular sludge contains more EPS than flocular sludge, therefore either the degradation of EPS is low in granular sludge, or the production is high. [5] This would

mean that either a strong EPS matrix is formed in granules, or cells invest more energy in EPS production.

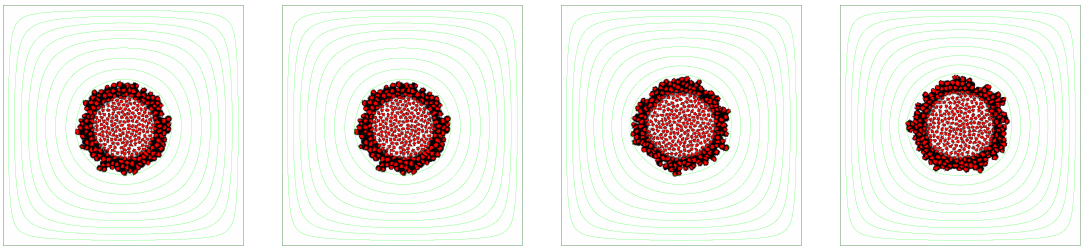
3.2.5 Effect of substrate availability in granular sludge

High cell growth rate produce unstable granules, thus slow growers must be selected when making granules. [26][27] To see the effects of growth rate, the maximum substrate uptake rate, substrate concentration in the bulk and fraction of substrate going to growth were varied. The growth rate and EPS production rate are assumed to be linearly dependent on the substrate uptake rate. The substrate uptake rate is assumed to be non-linearly dependent on the concentration of substrate available. This dependency is modelled to follow the classic Monod equation for substrate limited substrate uptake rate.

Granules are formed by setting selective pressure for slow growing bacteria.[27][26] This slow growth can be achieved in multiple ways. In this model it is assumed that slow growing bacteria either have a low substrate uptake rate, low substrate conversion rate, or have a significant portion of their energy diverted to other biomass like EPS. This is tested with the model in the form of fraction of energy going to the EPS production and part of the substrate going to the growth rate. When a bigger portion is going into the growth of EPS, it is expected that the granules stay compact and stable. This can be because less energy is put into growth.[5]

The substrate uptake rate was varied as well to see what kind of impact this has. With a higher substrate-uptake rate more EPS will be produced but also more cells. The expectation is that cells with a higher growth rate will grow out of bounds, whereas granules with a slower growth will grow to form better granules. In reactors,selection pressure is set for bacteria that have a high substrate uptake rate, but a low growth rate. This is because the cells that can uptake the substrate in the anaerobic condition and store it for later use in the aerobic phase will make the granules. This forms stable granules, capable of handling high loading rates.It has been seen that granulation also occurs in only aerobic conditions, but these granules are less stable and granulation depends on the substrate used.[26] In Figure 3.9 the effect of changing the maximal substrate uptake rate is shown.

In Figure 3.9e the morphometric variables are shown for the different rates. It can be seen that the maximal substrate uptake rate mostly influences the number of cells and the circumference and that no significant effect can be seen in the compactness variable. It seems that by increasing the substrate uptake rate linearly the number of cells and circumference also increases linearly. Yet qualitatively the morphology is not impacted by the increase. This can be explained by the small change imposed in the model. As

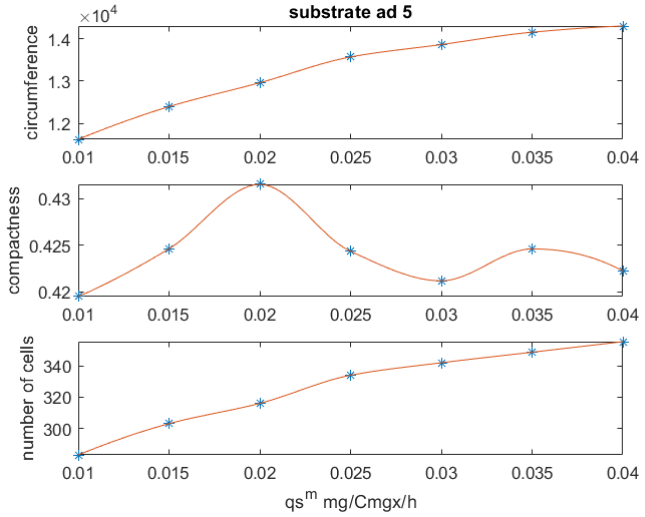


(a) Max substrate uptake rate 0.01

(b) Max substrate uptake rate 0.015

(c) Max substrate uptake rate 0.025

(d) Max substrate uptake rate 0.04



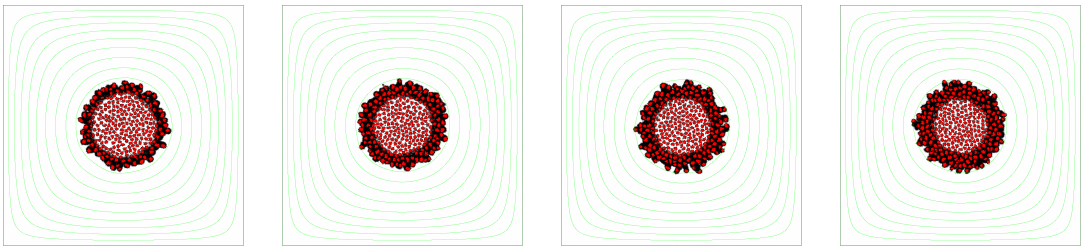
(e) Max substrate uptake rate 0.04

FIGURE 3.9: Influence of substrate uptake on the granule morphology

could be seen in the other experiments, changes in the model happen when there are big differences between the parameters. Lastly increasing the substrate uptake rate further in this simulation will not result in slow growth as the growth rate is linearly dependent on the substrate uptake rate. Still slow growers can be simulated by assuming a substrate inhibited growth rate. This can be found in bacteria that grow on acetate.[28]

The effect of substrate concentration on granular sludge is shown in Figure 3.10. Figure 3.9 shows the effect of substrate concentration on the granule morphology. The most important change in the granules is that the granular active zone is bigger when the substrate concentration increases. This can be seen by looking at the white inactive core. This is explained by deeper penetration of substrate in the granule. The maximum substrate uptake rate is set at a certain value and therefore when it is lower than the amount of substrate, the substrate will diffuse through the layers reaching deeper into the granules.

As mentioned earlier, granular sludge contains more EPS than floccular sludge. Therefore the effect of the fraction of substrate going to EPS production is varied. It was

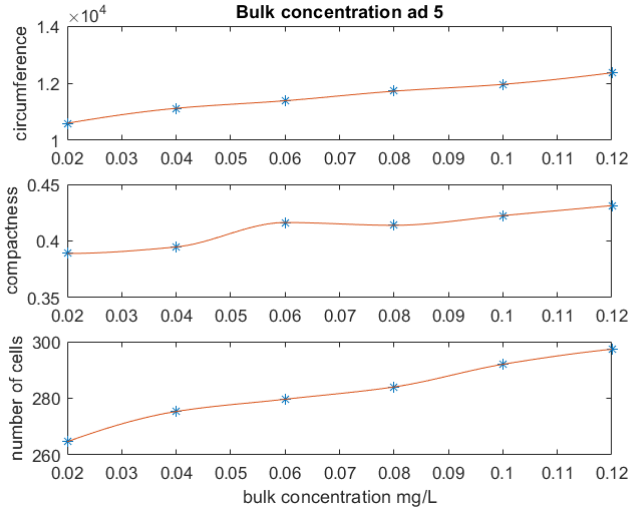


(a) Substrate concentration 0.02

(b) Substrate concentration 0.06

(c) Substrate concentration 0.08

(d) Substrate concentration 0.12

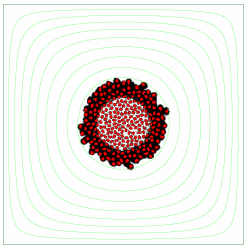


(e) Substrate concentration 0.12

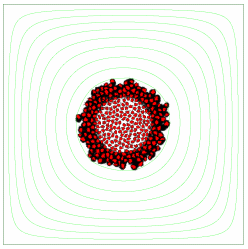
FIGURE 3.10: Influence of substrate concentration on granule morphology

assumed that the EPS production and growth rate are linearly dependent on the substrate uptake rate. The EPS production rate was varied by having a smaller or bigger fraction of substrate being available to the EPS production. The maintenance was assumed to be insignificant and no balances of substrate were assumed to be needed. For this assumption to be valid the EPS production rate and the growth rate should not exceed the substrate uptake rate. The results of this simulation can be seen in Figure 3.11.

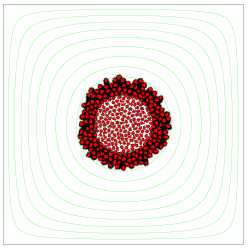
As is to be expected, when the fraction of substrate going towards growth is increased, the number of cells increase with it. This difference can clearly be seen between 3.10a and 3.10d. The form of the granules does not however, seem too differ. This is also reflected in the compactness measurement shown in Figure 3.11e, which does not change significantly.



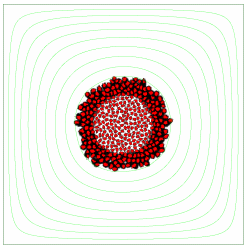
(a) Fraction of substrate (EPS:Growth) 4:1



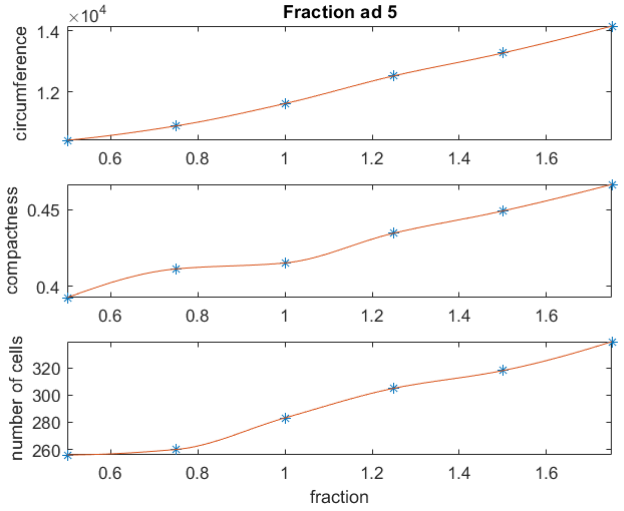
(b) Fraction of substrate (EPS:Growth) 1:1



(c) Fraction of substrate (EPS:Growth) 1:2.25



(d) Fraction of substrate (EPS:Growth) 1:3



(e) Morpometric variables for fraction analysis

FIGURE 3.11: Influence of concentration inflow on the granule morphology

Chapter 4

Discussion

4.1 Influence of physical properties of structural EPS on granule morphology

Based on model simulations the following physical properties of structural EPS influence the granule morphology: The physical properties of structural EPS are governed by the chemical composition of EPS. In latest studies of EPS characterization, the presence of proteoglycans and glycoproteins was found.[10] In comparison these polymers are the key components in animal tissue. Differences in the various animal tissues are mainly caused by variances in polymer composition. The functions of proteoglycans and glycoproteins in animal tissue has been highly investigated.[12][11] Due to similarities between EPS and the animal ECM. These functions were partly investigated in the experiments concerning physical properties of structural EPS.

When the cellular adhesion is increased, the granules become more condensed. Cellular adhesion increases as more binding surface bound EPS can be found in the model. By increasing non-binding surface bound EPS, granules become less compact and unstable. This is shown by the overgrowing of the system and can especially be seen when binding EPS is low and inhibiting EPS is high. With these measurements it is shown that the amount of surface bound EPS, simulated as varying binding energies between cells and the medium can show changes in the granule compactness. In animal tissue the most important connective polymers are found in the group of glycoproteins. These polymers have a relative high protein:carbohydrate ratio. Compact granules with high protein contents could therefore have a big chance of containing glycoproteins.[10]

Porosity of the EPS matrix is another important parameter for granular sludge. Although EPS production seems like an altruistic feat, EPS production is actually a competitive exploit. [23]Cells that produce EPS push their progeny into substrate-rich areas.

This ensures rapid growth of their progeny and suffocation of surrounding cells. When the porosity of structural EPS is lowered, cells on the granule perimeter will have more substrate at their disposal. Granules with increased substrate diffusion inside EPS, thus increased porosity, grew out of system bounds. Whereas when substrate diffusion in EPS was lowered, the granule was smaller and had a bigger inactive center.

In granules cells can counteract this kind of substrate-snatching by producing EPS-degrading enzymes.[2] These enzymes digest the EPS and thereby produce resources. Furthermore when the EPS matrix is digested the porosity increases. In animal tissue, these interactions with the ECM are sollicited by other cells and signalling chemicals. In bacteria, signalling molecules are found to be important for the formation and maintenance of granules. [29]Signalling factors can induce the production of EPS, whereas other signalling could induce for the degradation of enzymes. When cells lyse, all the enzymes are released into the granule and this can destabilize the granule.[2] In a study on long time stability of granular sludge it was seen that older granules with substrate limitations can get inactive cores which lowers the structural strength.[24] Therefore a matrix in which substrate diffusion is hindered will result in smaller granules or granules that break down in time. When substrate diffusion in the EPS matrix is completely unhindered, the granule can grow out of bounds unless lower substrate concentrations are used.

No effect was found by changing movement of EPS matrix. This was done by changing the diffusion coefficient of EPS. It was assumed that because a gel was formed EPS does not flow and thus diffusion of EPS was set to be near zero. To test if this assumption had any effect, the diffusion of EPS was increased. Only when the diffusion rate would go to orders of magnitude around the diffusion of substrate would there be a visible effect.The premise of this simulation was that diffusion could be used for EPS movement. This can however only be used for soluble materials and not particles.

The strength of the EPS matrix did influence the morphology of the granules. When the decay rate was high, the granule would only accumulate EPS at places with maximum EPS matrix production. When the EPS decay rate is low, accumulation occurs throughout the granule. It can be stated that if a shear force would be applied the granules would break into smaller pieces. This is similar to the results found in the porosity experiment.

Strength of the EPS matrix formed therefore has multiple effects. It can effect the porosity of the granule which decreases the possible size of stable granules. Substrate limitation can lead to the degradation of the granules by digestion enzymes making the granules break apart. When the EPS matrix is not easily digested, granular structure is more stable.

It was shown in the section above that the biggest influence on granule morphology were the amount of binding surface bound EPS, and EPS matrix porosity. Both of these properties changed the compactness and size of the granules.

4.2 Influence of substrate availability

What has been seen in literature is that when cells grow faster they will grow into activated, floccular or filamentous sludge. This is mostly performed by fast heterotrophic growers. When organisms in a system are fed with easily bio-degradable substrate, selection is in favor of those organisms that can take up and convert the most substrate to growth. Therefore bacteria that take up high amounts of substrate but have slow growing rate must be selected. The selection for slow growth is done by having a high SRT or growing at low concentrations.[24][26]

The effects of substrate bulk concentration, maximal substrate uptake rate and fraction of substrate going to EPS production was examined. It was shown that the substrate bulk concentration was important for the penetration of substrate in the granule. This means that when the substrate concentration is higher, a bigger portion of the granules received substrate. This was seen by the smaller inactive core. From the model it was seen that the fraction of substrate did not affect the form of the granule, for these conditions. What could be seen however was a decrease in cell counts when the fraction of substrate going to EPS production was higher. This confirms that when more substrate goes in the production of EPS, cell growth slows down.[5] In this section was seen that substrate concentration affects the penetration of substrate in the granules. Furthermore it was stated that when a higher fraction of substrate goes to EPS production, cells grow slower and that will cause stable granules to form.

4.3 Relevance of VirtualLeaf and Tissue Simulation Toolkit

The premise of this thesis was to investigate the influence of structural EPS on granule morphology. For this the use of two models was employed. The plant cell tissue model: VirtualLeaf and the TST. VirtualLeaf does not have EPS objects and thus the cell walls surrounding and connecting cells were used as a substitute. To simulate surface bound EPS. With this substitute not all the wide ranges of EPS could be examined, but only one type of granule found by Pronk et al.(2017)[8] Here the EPS formed capsular EPS making cells closely connected like in plant cells. Therefore when this model is used it should be able to copy the shape that the granules form. Which it did to a certain extent.

It was found that the substrate influx simulated in this model is not an accurate representation, as substrate influx varies on the concentration of the substrate.[14][30][31][24]

The TST model was useful as it is possible to imitate cellular interactions with the hydrogel. Either the cells would get a penalty for being in the hydrogel at certain EPS concentrations or got rewarded for connecting with other cells. Yet the penalty for moving into EPS rich areas was an approximation, as it is still possible for the cells to move into the rich area. This penalty was meant to simulate the sturdy EPS produced when the hydrogels are formed. In this case it shows the embedding of the cells as cells will move less when in the matrix.For these simulations a big penalty is chosen, and so this would coincide with a very rigid gel.

An improvement of this modelling approach could be found in the combination of both models. In the hybrid version the substrate field of the TST would be used, whereas the cells forms are projected unto it.

Chapter 5

Conclusions

5.1 Conclusions

In this thesis the influence of physical properties in structural EPS on granule morphology was tested. From literature it was shown that many different types of granule morphologies can be found and that these all contain different structural EPS. These different structural EPS, are created by varying compositions of key components. In the last year resemblance between animal ECM and EPS was found. This means that granular sludge could be represented by using the tissue models: VirtualLeaf and the Tissue Simulation Toolkit. VirtualLeaf was used to simulate the tightly bound connections found when cells would only produce binding surface bound EPS. And the TST was used to find the effect of binding surface bound EPS and hydrogel matrix on granular morphology. Here parameters that represented these changes in composition were tested.

From these observations it could be concluded that an increase of binding surface bound EPS in the structural EPS increases the compactness of the cells. The porosity of the EPS matrix was found to be important in determining compactness, and showed that a lower porosity ultimately lowers the possible granule size. The movement of EPS seemed to have no visible impact for the parameters tested. Lastly when the EPS matrix is stronger bigger groups of cells where shown to form the granule, whereas when it is weaker only small flocs of EPS and cells were seen.

When increasing the substrate available in the system, it was seen that the substrate penetrated deeper into the granules. Furthermore it was shown that the fraction of substrate going to EPS affected the number of cells inside the granule. This increase in number was also shown when the substrate uptake rate was increased.

VirtualLeaf and the TST could be used for this experimentation, for simulating certain aspects of the EPS. Yet it could be adapted for a better representation of the granule.

The links between model parameters and physical properties were made between EPS composition and model parameters. When more of a certain polymer were to be found, a change in the parameter should be implemented.

5.2 Outlook

In the future more experimentation in multiple directions can be made. For better representation of granular sludge VirtualLeaf can be modified to include a substrate field outside of the granules. This would be a sort of hybrid TST/VirtualLeaf.

The Tissue Simulation Toolkit can be further modified to include cell death, degradation of EPS and excretion of digestive enzymes. With this granulation and granule morphology can be examined as well as the cellular reactions inside the tissue when signalling factors accumulate signalling EPS production or signalling EPS degradation. Furthermore different morphometric values could be used to better represent the granule morphology, like granular compactness including EPS and concentration of EPS. Lastly continuation of the experiments on granule composition are very important. Finding more links between animal ECM and bacterial EPS could make more knowledge available from the medical field. Therefore it could be interesting to find signalling factors that trigger EPS formation or other cellular responses.

Appendix A

Appendix

A.1 Animal extracellular matrix

Most cells in multicellular animal tissue are surrounded by a network of polymers that form the ECM. The most important polymers in the ECM are proteoglycans and glycoproteins. The proteoglycans contain a core protein as a backbone with one or multiple long chains of polysaccharides called glycosaminoglycans covalently bonded. Glycosaminoglycans are long unbranched polymers composed of hexosamines and uronic acids. These can be substituted by sulfate groups at different positions.
The main groups of GAG's chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin, heparan sulfate and hyaluronan. Hyaluronan is not bound to the protein and can move freely through the ECM. The multiple charged groups in the polymer makes them polyionic and able to attract positively charged ions. This makes highly hydrophilic regions that can span a large volume and attract water.

The proteoglycans can interact with many different proteins that are used for cell signalling. This gives two main functions for the proteoglycans, they are either structural for the creation of hydrogels and trapping water, or they can be involved in cell signalling pathways. [11][9] Glycoproteins are structurally similar to proteoglycans, but contain less carbohydrates. The carbohydrates present are branched oligosaccharides instead of long linear polysaccharides and often contain sialic acid ends.[12]

Glycoproteins can be divided into two functional groups. The first one are the structural glycoproteins and comprises of collagen and elastin groups. Collagen oppose pressure on the structure whereas elastin is the opposite. Elastin groups will oppose stretching and will restore the structure to it original shape.
The second functional group are the adhesive glycoproteins, like fibronectin and laminin.

Fibronectin can bind with collagen and heparin, as well as with the cells surface, anchoring them to the network. Laminins bind to specific collagen types and some GAGs giving them a similar function as fibronectin. In the end due to highly variable proteoglycans and glycoproteins animal cell tissue can achieve distinct tissues ranging from tendons, muscles or even bone.

A.2 Theoretical Background

The various components of EPS will be discussed in detail in this chapter. In addition the morphological modelling will be discussed. The connection between the ECM and EPS will be made by showing that the same components are found in both. One of the most important components in ECM in animal tissue, GAGs, have also been found in EPS. This proves at the least that EPS and ECM have the same properties and that the cells inside the granules have a sort of cooperation. Or that cells in multicellular organisms also compete. This is very interesting stuff. To Do: Tell the structure of EPS. (That means subdivision into multiple large groups, saccharides, proteins DNA, lipids and humic substances. Explain the ECM matrix in multicellular organisms Make the link between EPS and ECM. Explain the morphological models Sum the physical properties of EPS. Different types of morpological models.

A.3 EPS at the basis of biological aggregates

EPS is important to determine the shape of biological aggregation. It was found that in floccular sludge the EPS content is lower than in aerobic granules. According to Flemming & Wingender, the EPS content in a biofilm can vary between 50 and 90%. Yet different yields are found in different studies, depending on the extraction methods. [25][4][32] The most important part of the aggregation is water, which can amount to 97% of the granule. This water is either inside the cells, or trapped by the EPS in the aggregation.[2] EPS is mostly a complex mixture of multiple organic and inorganic compounds. These can be subdivided into the following subsections.

A.3.1 Exopolysaccharides

Exopolysaccharides are the major factor in forming the rigid gels that make the granular sludge. Gels can form when crosslinks between polymer chains are formed. In general, β-1,4 linkages and β-1,3 linkages make strong rigid gels, whereas α-1,2 and α-1,6 linkages make more flexible structures. [2][3] Exopolysaccharides can either be homo-polysaccharides or hetero-polysaccharides. Homo-polysaccharides are made from

a single repeating monosaccharide, generally composed of glucose or fructose. As More et al. states, homo-polysaccharides can be subdivided into three main groups. The first group are α-D-glucans. These contain mostly α-1,6 glycosidic bonds, with mostly α-1,3 bonds as branching connections. The lactic acid bacteria of the species Leuconostoc and Streptococcus are known for producing dextran. This is a series of repeating glucose molecules bonded with α-1,6 glycosidic bonds with branching molecules starting from α-1,3.The second group are β-D-glucan. These contain β-1,2 bonds and β-1,3 linkages. The third group are fructans, which have mostly β-2,6 fructosyl links. Dextran and cellulose are examples of homo-polysaccharides that are commonly found. [4] Most exopolysaccharides however, are hetero-polysaccharides formed by neutral and charged monomers. These neutral monomers are mostly hexoses, like glucose or fructose, and the charged monomers are mostly uronic acids. The uronic acids are also commonly replaced with acetate ester, inorganic phosphate or sulphate. [4] In 2010, Lin et al showed that granular EPS contains ALE, a polysaccharide resembling alginate in its gelling capacity. ALE consists of varying uronic acids blocks. Alginate and ALE are not fixed molecules, but are a family of polymers with differing properties. Subsequent research with ALE showed that the differences in composition of uronic acid blocks determine the strength of the gel. ALE containing more guluronic acids molecules was able to make more cross-links between chains, producing a stronger gel. Seviour et al. found that the extracted exopolysaccharide behaved more alike chitosan than alginate. Chitosan is a linear polysaccharide that is randomly linked by glycosylated aminoacid molecules. This shows that the EPS hydrogel can be a combination between linear polysaccharides with cross-linking glycosylated proteins. From this information, it is apparent that exopolysaccharide chains that contain crosslinking polymers can produce strong rigid gels. A relatively higher amount of possible crosslinks makes a stronger gel. In ALE this is accomplished by having a higher amount of guluronic acid blocks. Evidence has been provided that the gels can be formed by linear polysaccharide chains, with proteins interlinking the polymer chains. The ability to make gels has the following functions for the granules: adhesion to surfaces, aggregation of bacterial cells, retention of water, adsorption of different compounds, binding of different enzymes, nutrient source and protective barrier.[25]

A.3.2 Proteins

The content of proteins in the EPS can vary widely, from 1 to 60% . They are present in the form of structural polymers or enzymes. Enzymes detected in biofilms are mainly involved in the degradation of EPS. These enzymes are retained in the matrix, in close proximity to the cells, due to their interactions with the polysaccharides. Flemming and Wingender state that during starvation cells excrete more EPS degrading enzymes.

These enzymes will either degrade own EPS or EPS from other species. Structural proteins in the form of glycoproteins are well known in eukaryotic organisms, but as stated earlier are also found in prokaryotic organisms. Examples of these structural proteins are the cell surface-associated and extracellular carbohydrate-binding proteins (known as lectins). They act as link between EPS and the bacterial surface. [4]

A.3.3 Extracellular DNA

Extracellular DNA is major component in the EPS matrix of some bacteria. However, it varies even between related species (1-10%). This DNA is responsible for horizontal gene exchange across species and within species. This diversifies the members of the colonies creating a more robust microcolony. It has also been seen that the presence of DNA is necessary for the formation of biofilms in some species. [25][4]

A.3.4 lipids

The EPS matrix also contains lipids and lipids derivate compounds. In some bacteria, lipopolysaccharides participate in the process of adhesion. There are also surface active EPS (biosurfactants) that are capable of dispersing hydrophobic substances in the medium. They have also antibacterial and antifungal properties. [25]

A.3.5 humic substances

Another group of important compounds in EPS is humic substances. They are not secreted by microorganisms. These substances get adsorbed by the matrix from the environment. Its presence in EPS matrix is important because it can influence some properties of the EPS as biodegradability and adsorption ability.[4][33][34][35][23]

A.4 Animal extracellular matrix

A.4.1 Overview of the extracellular matrix

Most cells in multicellular animal tissue are surrounded by a network of polymers that form the ECM. The most important polymers in the ECM are proteoglycans and glycoproteins. The proteoglycans contain a core protein as a backbone with one or multiple long chains of polysaccharides called glycosaminoglycans covalently bonded. Glycosaminoglycans are long unbranched polymers composed of hexosamines and uronic acids. These can be substituted by sulfate groups at different positions.

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The second functional group are the adhesive glycoproteins, like fibronectin and laminin. Fibronectin can bind with collagen and heparin, as well as with the cells surface, anchoring them to the network. Laminins bind to specific collagen types and some GAGs giving them a similar function as fibronectin. In the end due to highly variable proteoglycans and glycoproteins animal cell tissue can achieve distinct tissues ranging from tendons, muscles or even bone.

A.4.2 Similarities between ECM and EPS

For a long time it was thought that it was not possible for prokaryotic organisms to produce glycoproteins. Yet as stated before polysaccharides linked with protein polymers have been found in granular sludge EPS. Recent work (unpublished) found that also sialic acid are present in granular sludge. Furthermore sulphurylated proteins were also present. This provides evidence that granular sludge contains glycoproteins. This could also explain the gelling capacities of EPS. EPS extracted from granules has shown behaviour that coincide with GAG. This shows that bacterial EPS and animal ECM are not very different and possible coinciding molecules and functions can be found between them. Following this analogy, it should be possible to find in granular EPS the functional groups found in ECM. Polyanionic linear polysaccharides linked together for the formation of hydrogels. Structural components as elastin-and collagen-like would

contribute to the ability of the granules to withstand pressure and stretching forces. It is thus important to look at polymers that are similar to the polymer chains found in animal ECM or look at what polymers are found in different types of granular sludge.

A.5 Physical properties in EPS

The influence of physical properties of structural EPS on granule morphology will be investigated. Therefore it is imperative that the physical properties can be measured in a way, to get more data for better models or to check existing models. What becomes clear from literature is that the gelling capacity of EPS is an important characteristic for EPS. This kind of measurement was either performed as a qualitative test to see if a gel forms at all, or as quantitative test to see the amount of gelation. [17][6] By looking at the gelation capacity at different pH, statements can be made about the putative composition of the polymers. This would give a start in the chemical composition, which is also, to a certain extent, measurable property of EPS. The amount of crosslinks between polymer chains can be dependent on the amount of branching linkages that can be made. Therefore if EPS contains glycoproteins, or other crosslinking polymers. In order to make better models, the diffusion coefficient in EPS could be defined Absorption of certain chemicals could also give hints in what polymers are abundant in the EPS, especially relative to other types of EPS.

Appendix B

Appendix

B.1 Model plugin Virtualeaf

External attachment, sent in online version.

B.2 TST model

External attachments, sent online. The vessel.cpp is the main file, in which secretion and substrate uptake calculations are also performed. Diffusion is described in the pde.cpp

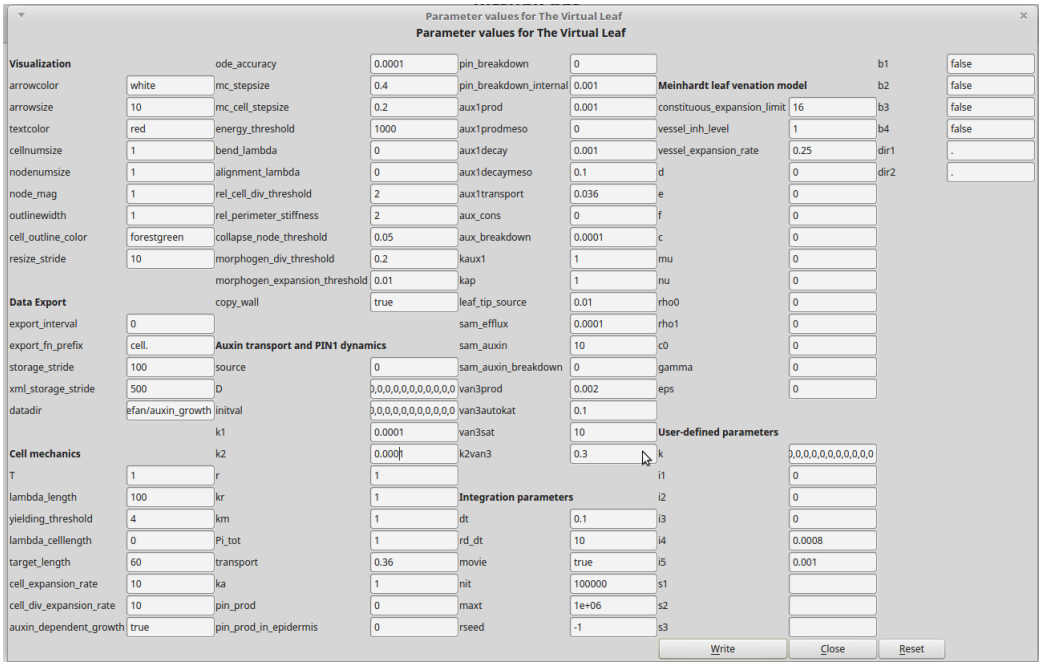

FIGURE B.1: The parameter settings for the double substrate limited growth, take in mind that s4 and s5 are interchanged every 2 hours of simulation time.

file and the cellular potts code can be found in ca.cpp. The fraction.par file shows one of the parameter files that was used for the fraction analysis as an example.

B.3 Matlab

Included in the online version is a matlab example file. Here a morphometric analysis is performed, including the morphometric regression. First data is collected and the different morphometric data points are plotted. Then the data is interpolated with interp1 or interp2. Finally, a non-linear regression is made.

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