The Physical Mechanisms of Microbiological Cellular Transport, Storage, and Metabolism of Aromatic Hydrocarbon-Degrading Soil Microbiota

by

Ioanna Isobel Malton

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Earth Science

Waterloo, Ontario, Canada, 2021

Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Ioanna I. Malton was the sole author for Chapters 1 and 3, which were written under the supervision of Dr. Andre J. Unger and were not written for publication.

This thesis consists in part of two manuscripts written for publication. Exceptions of sole authorship of material are as follows:

Model Description presented in Chapter 2 & Model Simulations presented in Chapter 4:

Dr. Robert W. Enouy and Dr. Andre J. Unger were the primary co-investigators on National Science and Engineering Research Council (NSERC) of Canada and Chevron Energy Technology grants which supported this work. Dr. Kenneth M. Walton, Dr. Mario A. Ioannidis, Dr. Kanwartej S. Sra, Dr. Natasha N. Sihota, and Dr. Eric J. Daniels were co-investigators on the grant and are co-authors on any publications relating to this work.

This research was conducted at the University of Waterloo by Ioanna I. Malton under the supervision of Dr. Andre J. Unger. Dr Robert W. Enouy, Dr. Andre J. Unger, and Dr. Kenneth M. Walton were primary coders of the model. Dr. Mario A. Ioannidis, Dr. Kanwartej S. Sra, Dr. Natasha N. Sihota, and Dr. Eric J. Daniels contributed to model research and development and intellectual input. Dr. Robert W. Enouy and Dr. Andre J. Unger wrote draft manuscripts, with input from all the above-mentioned co-authors.

Citations:

Chapter 2:

Enouy, R. W., Malton, I. I., Walton, K. M., Ioannidis, M. A., & Unger, A. J. A. (2021). *A Numerical Model for Simulating Cell Growth and Biodegradation Rates of Microbes Utilizing Dissolved Hydrocarbons*. [Unpublished manuscript].

Enouy, R. W., Walton, K. M., Malton, I. I., Kanwartej, S. S., Sihota, N. N., Daniels, E. J., & Unger, A. J. A. (2021). *A Mechanistic Derivation of the Monod Bioreaction Equation for a Limiting Substrate*. [Manuscript submitted for publication]. Earth and Environmental Science, University of Waterloo.

Chapter 4:

Enouy, R. W., Walton, K. M., Malton, I. I., Kanwartej, S. S., Sihota, N. N., Daniels, E. J., & Unger, A. J. A. (2021). *A Mechanistic Derivation of the Monod Bioreaction Equation For a Limiting Substrate*. [Manuscript submitted for publication]. Earth and Environmental Science, University of Waterloo.

Abstract

The Monod bioreaction equation, though justified from extensive practical use, has been found to have limited mechanistic basis (Enouy, Walton, et al., 2021). To attempt to gain a biological mechanistic representation of the equation, Enouy, Walton, et al. (2021) created a three-stage serial process by deriving Monod as a special case of aquifer phase diffusive transport rate of a substrate onto the surface of microbe, uptake rate across the microbial membrane, and the subsequent interior biodegradation rate. The derivation was parameterised with single-substrate depletion of benzene, phenol, and toluene, and biomass (*Pseudomonas putida* F1) growth data from Reardon et al. (2000). Though this derivation of Monod can fit the data adequately, it is simplistic, and sometimes deviates from the data. It is proposed that a detailed understanding of the biological processes involved in the biodegradation of aromatic hydrocarbons is necessary to begin to provide a meaningful mechanistic basis to the Monod coefficients, K_s and μ_{max}, and to support and embellish this derivation for the purpose of improving the fit of the data, and of future experimental data sets. To this purpose, a literature review of the four major steps in the biological degradation of aromatic hydrocarbons was undertaken. The processes studied are as follows: a) cellular transport; b) energy storage; c) metabolism of aromatic hydrocarbons (both aerobically and anaerobically); and d) cellular growth. The contributions of these processes are discussed alongside the fits of the Reardon et al. (2000) data to propose embellishments to the Monod derivation for improving the data fit, and future biological investigations for improving mechanistic understanding and contributing to biological systematic modelling.

Acknowledgements

I would like to first thank my supervisor, Dr. Andre Unger, for allowing me to have this excellent learning experience, and for providing constant guidance, mentorship, and support. I'm very grateful for the opportunity to collaborate on this project that involved multiple areas of study. I would also like to thank Dr. Robert Enouy for his many contributions to the project, and his helpful advice.

Thank you to my committee members, Dr. Barbara Butler and Dr. Yuri Leonenko, for their insights and support.

Thank you to the team at Chevron, especially Dr. Natasha Sihota, Dr. Eric Daniels, and Dr. Kammy Sra, for their knowledge, advice, and suggestions that helped move the project along.

Finally, thanks to my family, for their immensely patient love, especially my father who helped me by constantly discussing my work and encouraging me.

Dedication

I dedicate this thesis to my father and mother for their constant loving support and careful patient advice.

Table of Contents

List of Figures

List of Abbreviations

ADP: adenosine diphosphate

ATP: adenosine triphosphate

ATPase: ATP synthase

BTEX: benzene, toluene, ethylbenzene, xylene

CCCP: carbonyl cyanide 3-chlorophenylhydrazone

CMC: critical micellar concentration

CoA: coenzyme A

COG: cluster of orthologues group

CTV: constant transport velocity

DO: dissolved oxygen

ETC: electron transport chain

FAD/$FADH_2$: flavin adenine dinucleotide (oxidized/reduced forms)

FM: fluorescence microscopy

HOC: hydrophobic organic chemicals

LCFA: long chain fatty acid(s)

LPS: lipopolysaccharide(s)

mRNA: messenger ribonucleic acid

NAD/NADH: nicotinamide adenine dinucleotide (oxidized/reduced forms)

NADP/NADPH: nicotinamide adenine dinucleotide phosphate (oxidized/reduced forms)

OM: outer membrane

OMLCFA: outer membrane long chain fatty acid (transporter)

orf: open reading frame

PAH: polyaromatic hydrocarbon(s)

PDC: pyruvate dehydrogenase complex

PHB: poly-ß-hydroxybutyrate

PI: propidium iodide

PMF: proton motive force

RTM: reactive transport model

SMFM: single-molecule fluorescence microscopy

SPL: squamosa-promoter binding protein-like (gene family)

TCA: tricarboxylic acid cycle

Chapter 1 – Introduction

Environmental contamination is a growing concern in this age of industrial human activity. Soil, sediment and groundwater are frequently contaminated by various environmental contaminants, xenobiotics, and other pollutants, from lakes, improper disposal, and spills (Edwards et al., 1992). Xenobiotics are anthropogenic compounds which can include adhesives, dyes, flame retardants, lubricants, oil, petroleum products, organic solvents, pesticides, and gasoline and oil combustion products (Parker et al., 2018). Petroleum hydrocarbons, in particular, are an ever-growing source of environmental contamination, particularly of aquifers, during the human activity of recovery, refining, storage, use, and disposal of oil and gasoline (Schirmer et al., 1999). Though they occur naturally, anthropogenic activity greatly increases their environmental concentrations, making them grave pollutants. The aromatic compounds of such hydrocarbons are the second most widely distributed organic compounds in nature (Carmona et al., 2009). Nonoxygenated, homocyclic aromatic compounds, including benzene, toluene, ethylbenzene, xylene (BTEX) compounds, are of particular concern since they are suspected or confirmed carcinogens, even at low concentrations (Schirmer et al., 1999; Edwards et al., 1992). They are all hazardous and have a great environmental impact due to various water solubilities and mobilities allowing them to migrate through groundwater, which makes clean up difficult as they travel from the original contamination site (Schirmer et al., 1999; Edwards et al., 1992). Benzene is the main aromatic compound of concern, commonly found in crude oil, petroleum products, and oil pollutants; it is mutagenic and carcinogenic, and is classified as a class A human carcinogen under the United States Environmental Protection Agency (Aburto & Ball, 2009; Ladino-Orjuela et al., 2016). Its six-carbon ring is the defining feature in all aromatic compounds (Carmona et al., 2009). It is thermodynamically stable due to its resonance structure, and has widespread production and industrial usage, though it is the most toxic and most soluble of hydrocarbon pollutants (Carmona et al., 2009; Aburto & Ball, 2009). Due to such molecular stability, there is a great persistence of these aromatic ring-containing compounds in the environment (Carmona et al., 2009). They resist breakdown, and as such can quickly accumulate in the food chain, which can include eventual indirect consumption by humans (Parker et al., 2018). Since they are not readily degraded and persist for a long time in the environment, this allows them to be indicators of the effectiveness of clean-up procedures,

such as government agencies' regulation standards (Aburto & Ball, 2009; Ladino-Orjuela et al., 2016).

Despite there being various methods of clean-up, the one that shows the most promise for being cost-effective, environmentally friendly, efficient, and permanent, is biodegradation (Adams et al., 2015). That is, the use of microbial metabolic processes to destroy or immobilise waste material: a detoxification strategy that targets such harmful chemicals via mineralization, alteration, and transformation (Parker et al., 2018). Many microorganisms, particularly those ubiquitous to soils and groundwater systems, can utilise the aromatic hydrocarbons as their carbon and energy source, by degrading the ring structure and converting the products to compounds that can then directly enter their central metabolic pathways (Moat et al., 2002). Microorganisms can recycle carbon, maintaining the health of the biosphere, and there are many bacteria, archaea, and fungi that degrade most naturally occurring organic compounds, including these persistent aromatics (Carmona et al., 2009). The microbes produce their energy, via redox reactions, for internal respiration, cell maintenance, and reproduction (Parker et al., 2018). Individual microorganisms can only metabolise limited ranges of substrates, and have a preference for substrates that are easier to degrade over those that are more difficult; these differences between species lie in their particular enzymatic genes (mostly chromosomal genes, though sometimes found on plasmids), coding for the enzymes necessary for each reaction (Adams et al., 2015; Edwards et al., 1992; Parker et al., 2018). Since each individual species can only degrade a small range, often multi-species communities of microbes are needed to tackle pollutants which are often mixtures of many and varied substrates. Such communities adapt to the constant presence of hydrocarbons, and can show selective enrichment and genetic changes which allows them to respond in the presence of their preferred substrate within hours (Adams et al., 2015).

With all these species and substrates, there are two main methods of degradation: aerobic and anaerobic. Aerobic degradation utilises oxygen as the electron acceptor, and aromatics, such as benzene, are readily degraded aerobically and as such these processes have been extensively studied (Burland & Edwards, 1999; Edwards et al., 1992). Anaerobic processes,

by comparison, utilise various non-oxygen electron acceptors, such as nitrate, iron, or sulphate, however these processes are much slower and are less understood (Edwards et al., 1992). Though the aerobic processes look more promising, since they are faster, it is crucial not to overestimate them, for in many habitats where aromatic pollutants are found, oxygen is often rapidly depleted, leaving an anoxic environment (such as aquifers, aquatic sediments, submerged soils, sludge digesters, intestinal contents, or aerobic sites with high carbon concentration) (Burland & Edwards, 1999; Carmona et al., 2009). With all these various factors and parameters at play, *in situ* biodegradation and remediation quickly becomes a very complex issue. Each habitat or environment where pollutants are found may vary so widely that any intrinsic bioremediation must be demonstrated for each contaminated site individually (Schirmer et al., 1999). Therefore, the development and verification of a numerical model is necessary to predict and determine whether natural contaminant depletion will be sufficient under specific site conditions, to meet remedial goals (Schirmer et al., 1999). A model is a tool which allows one to determine the timeline and spread of an environmental pollutant, such as a spill, which is useful for legal and political legislation purposes. With any numerical model, the formulation and assumptions must be able to be explained by the actual mechanisms, so that the concepts of the model fit the real world.

The most common numerical formulation for microbial population growth is that of Monod (1949), however despite many attempts to interpret its kinetics within a microbial mechanistic framework, it has generally been accepted to have a limited mechanistic basis, justified only by its extensive use in practice (Enouy, Walton, et al., 2021). To attempt to show on what its coefficients are dependent, Enouy, Walton, et al. (2021) presents a special case of Monod described in Chapter 2, with a parameterisation of the model using biodegradation data of benzene, toluene, and phenol, with respective biomass growth data from Reardon et al. (2000), the simulations of which are presented in Chapter 4. This is an algebraic derivation of the Monod bioreaction equation using a simplified three-step serial process involving: aqueous phase diffusion transport of the substrate towards a spherical microbe; active transport across the membrane; and a zero-order reaction within the microbe (Enouy, Walton, et al., 2021). With the aim of recognising of what dependency the Monod coefficients have on the limitations

of the microbial processes involved, this study focuses on four key steps within the microbe, that are involved in the biodegradation process. They are 1) transmembrane transport, 2) interior energy storage, 3) energy production/metabolism, and 4) subsequent growth, survival mode, or death. Firstly, there is transport of the substrate across the cell membrane. The microbe needs to recognise whether there is substrate available in the near vicinity, and then needs to transport the substrate across the cellular membrane, either through passive, passive facilitated, or active transport (Hua & Wang, 2014). There is also sometimes internal storage of metabolites, especially when the microbe is in variable environments, where energy sources may be limited (Hua & Wang, 2014). Next, there is the metabolic process, either aerobic or anaerobic, in which the substrate is degraded for energy and/or for growth constituents, like carbon. This energy generation involves requiring the appropriate electron acceptor to be present in adequate quantity in the environment. Finally, the microbe may use such energy for cellular processes of maintenance, and cell growth, or if there is not enough energy supply in the environment, the cell may remain in a quiescent state, or may die off (Parker et al., 2018). These processes are all identified in microbiological literature, though there are various knowledge gaps which limit our current scope of understanding. The motivation of exploring these processes is to observe if fitting of these processes into the Monod derivation can improve the fit of experimental data sets. To develop and validate a model, with its coefficients and parameters, it is necessary to provide support from the literature, that explains the mathematical assumptions made. The objective of this thesis is to present the current state of scientific understanding of the bacterial degradation of aromatic hydrocarbons pertaining to the mechanisms of transport, storage, respiration, and replication, to provide support for the model concepts to better fit experimental data.

To reach this objective, an in-depth literature review was conducted. This involved searching for papers pertaining to the biodegradation of aromatic hydrocarbons by various bacteria, centering on benzene degradation in particular, and then organising them according to the portion of the process (identified above) that they were observing. As the model was being developed, new subtopics became necessary to be searched for, especially as more detail was considered for inclusion in the derivation. A description of the Monod derivation is presented in

Chapter 2, the literature review is presented in Chapter 3, and Chapter 4 discusses how the literature supports the model, as well as exploring possible ways to improve the model by including more detailed understandings of the processes involved. The discussion will also include a simulation of the Monod derivation using single-substrate depletion and biomass growth data from Reardon et al. (2000), a calibration effort of the model, to assist in parameterising the derivation and interpreting Monod kinetics. Several knowledge gaps in the research are identified, specifically regarding the quantifiable measures in biological experiments, such that there is a gap between the current understanding of microbiological degradation of hydrocarbons, and the ability to apply techniques of numerical modelling to model microbial behaviour. Though information on both subjects is widespread and detailed, there is limited connection between the two. The assumptions made in numerical modelling need to be supported and validated by microbiological research, however there is a lack of such research with a modelling mindset. As such, though there is much research about the microbial metabolic mechanisms at play, certain quantifiable components are not always measured to the fullest extent possible or desired. For example, experiments observing the prolonged degradation of a hydrocarbon slurry tend to limit the measuring of biomass growth (such as only before and after the experiment), however a numerical modelling mindset would require more frequent biomass measurements to observe the growth curve, especially where the exponential growth occurred, versus the points of plateauing, or of dying off (Schirmer et al., 1999; Parker et al., 2018; Reardon et al., 2000).

Chapter 2 – Background

2.1 Monod Kinetics

The Monod bioreaction equation is the mathematical representation of growth rate first introduced by Monod (1949). It is now commonly written as $\mu = \mu_{max} S/K_s + S$, where μ is the specific growth rate of the bacterium, μ_{max} is its maximum specific growth rate, S is the substrate concentration, and K_s is the Monod coefficient: a constant representing the substrate concentration at which growth rate is half the maximum rate (Alexander, 1999). K_s represents the affinity of the organism for the growth-supporting organic nutrient (i.e., a lower value would indicate a greater affinity of the bacterium for that molecule) (Alexander, 1999).

2.2 Reardon et al. (2000) Biodegradation Experiment

Reardon et al. (2000) conducted an experiment biodegradation of benzene, toluene, and phenol, by the microorganism *Pseudomonas putida* F1, to report the kinetics of these reactions in single and mixed batch reactors. They chose those three monoaromatic compounds because they are produced in huge amounts, are used in fuels, as solvents, and as starting materials in producing plastics, synthetic fibers, and pesticides, and are prevalent environmental contaminants due to spills and tank leaks (Reardon et al., 2000). *P. putida* F1 is one of the best characterised aromatic hydrocarbon-degrading bacteria, and this particular strain can use toluene, benzene, ethylbenzene, phenol, and other aromatics as its sole carbon and energy sources (Reardon et al., 2000). Multiple batch reactors were set up, to obtain the kinetics of each substrate's degradation singly, as well as all the combinations: benzene + toluene, benzene + phenol, toluene + phenol, and benzene + toluene + phenol (Reardon et al., 2000).

The cellular concentration of *P. putida* F1 was measured throughout the experiment using optical density at 600 nm, which corresponded to biomass concentration and therefore could be used to observe the population's growth over time (Reardon et al., 2000). The substrate aqueous and gaseous phase concentrations were measured using gas chromatography, and any aqueous intermediates that formed were detected by high-pressure liquid chromatography (Reardon et al., 2000). The biodegradation rate data was obtained from batch

cultivations in stirred-tank bioreactors, with controlled temperature (of 30 ° C) and regular agitation (pH was not controlled for but remained in the 6.7-6.9 range) (Reardon et al., 2000). There was no air sparging since two of the substrates were highly volatile, so the bioreactor was run as a closed system after inoculation, except when sampling (Reardon et al., 2000). Since the tanks were 3-L tanks, and only 1.6 L of liquid volume was used, that left an adequate headspace for sufficient oxygen for cellular growth (Reardon et al., 2000). The DO levels were monitored and never fell below 5 mg/L, and Reardon et al. (2000) cite several sources that state that aerobic bacterial growth is generally independent of oxygen at concentrations above 0.4 mg/L, so it is assumed that oxygen was not a growth-rate-limiting substrate in these experiments (Reardon et al., 2000). Each experiment was performed in duplicate, with repeated experiments run at separate times, for an accurate variability assessment (Reardon et al., 2000).

This experiment was considered ideal for model parameterization testing because it was a controlled, closed system, and offered multiple variations of substrate depletions (single, double, triple), as well as measuring the biomass concentration multiple times throughout the course of the experiment (Reardon et al., 2000). This last point is crucial since most other experiments only measure biomass concentration initially and at the end of the experiment, or not at all.

2.3 Model Background

The Monod bioreaction equation is widely used to describe microbial growth and substrate depletion with nutrient limitation, however its relation to the corresponding microbial mechanisms remains ambiguous (Enouy, Walton, et al., 2021). It is generally accepted to have limited mechanistic basis but is rather justified by extensive practice (Enouy, Walton, et al., 2021). There have been several attempts to derive an interpretation of Monod that is more representative of the biomass mechanisms involved (Liu, 2006; Liu, 2007). One attempt takes into account mass transfer considerations to provide an approximate prediction of the Monod coefficients K_s and μ_{max}, assuming that the microbe is the site of a zero-sink condition (Merchuk & Asenjo, 1995). This implies that the concentration of the substrate on the surface of the microbe is negligible compared to its bulk aqueous phase concentration (Merchuk & Asenjo, 1995). Other attempts suggest that K_s is governed by substrate-species pairs (Westerhoff et al., 1982; Tan et al., 1994), that it is concentration dependent (Alexander, 1999), or that it is the

direct result of thermodynamics and microbial metabolic processes (Heijnen & Romein, 1995; Jin & Bethke, 2003).

Enouy, Walton, et al. (2021) presents a conceptual mass transport and reaction model demonstration that the Monod equation can be derived as a special case of the following processes in series: aqueous phase diffusive transport of a substrate towards a spherical microbe; transport across the microbial membrane; and the depletion reaction within the microbe. The microbe is the initiator of transport and thereby depletes the substrate from the aqueous phase within its near vicinity, so the transport into the microbe causes a cascade of electron donor concentration gradient moving toward the bulk aqueous phase (the electron acceptor is considered to be in excess relative to the donor) (Enouy, Walton, et al., 2021). A couple assumptions govern this conception: 1) there exists flux continuity across the surface of the microbe, which means that the formulation allows for a continuous transition between limiting processes, for an ultimate control of the spatiotemporal evolution of the bioreaction process; and 2) the bulk concentration remains fixed at an infinite distance from the centre of the microbe [Figure 1] (Enouy, Malton, et al., 2021; Enouy, Walton, et al., 2021). The transport initiated by the microbe could be diffusive, or facilitated diffusive, or active transport (Hua & Wang, 2014). Since there is this possibility of passive or active transport, which could be replicated by a diffusive or an advective transport rate respectively, it is quantified by a defined macroscopic influence coefficient, $k_L a [s^{-1}]$, representing the upscale of transport onto the surface of a microscale microbe (Enouy, Malton, et al., 2021).

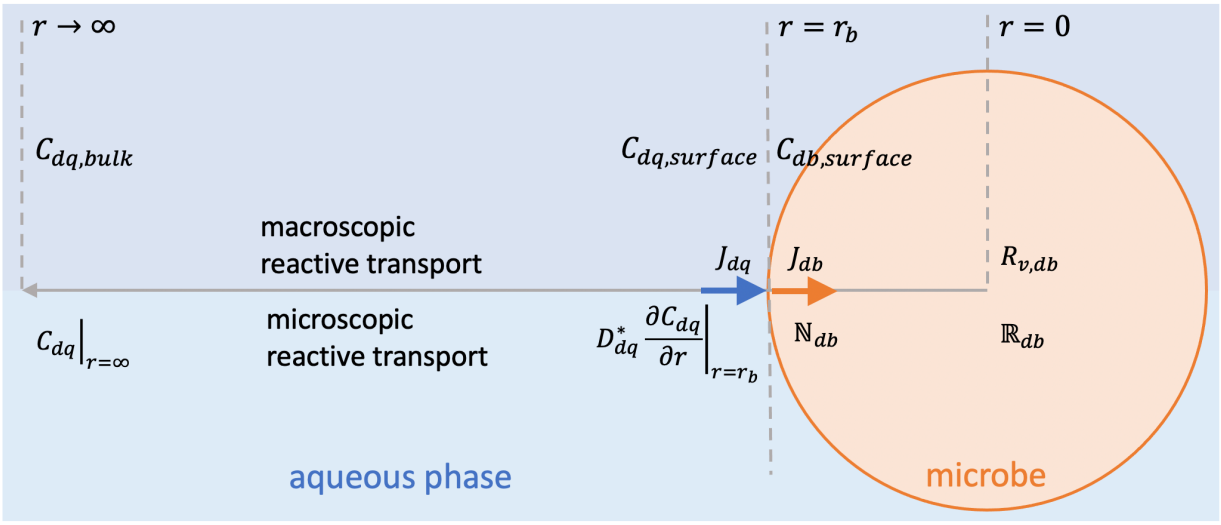

Figure 1: Conceptive Reactive Transport Model – The concentration of electron donor in the aqueous phase must transport from the bulk aqueous phase to the surface of the microbe, and then be taken up by the microbe so to be degraded internally.

In Enouy, Walton, et al. (2021), there are two substrate transport scenarios investigated to describe the flux of a substrate across the surface of a single microbe, which are generic representations for the flux: either constant and hence independent, or linearly dependent upon the substrate concentration on the surface of a microbe. The derivation serves the purpose of ascertaining what the Monod coefficients represent among the biological process involved (Enouy, Walton, et al., 2021). They show that K_s [kg/m^3] and μ_{max}, [m^3$_q$/kg$_d$/s], which are the Monod coefficient and the maximal microbial reproduction rate, respectively, are dependent on some limitation within the three above-mentioned processes, depending on the scenario (Enouy, Walton, et al., 2021). The derivation was parameterised with data of benzene, toluene, and phenol depletion, and biomass growth from Reardon et al. (2000), solving for the empirical values of substrate flux across the microbial membrane, and the transport velocity at which the substrate is taken up into the microbial cytoplasm (Enouy, Walton, et al., 2021).

The first scenario is the constant reaction model, where there is a function of a constant flux across the surface of a microbe, which is assumed to sustain the specific reaction process within the microbe and assumes both the flux and the interior reaction stay constant (Enouy, Walton, et al., 2021). It was suspected that either the reaction rate within the microbe, or the

transmembrane transport may be limiting, such that a constant flux of substrate across the surface of the microbe is necessary to sustain interior processes (Enouy, Walton, et al., 2021). Thus, the diffusion of the substrate is not considered to be limiting, and the Monod coefficients K_s and μ_{max}, solve to about constant and are a function of the physiochemical and mass transfer properties of the system (Enouy, Walton, et al., 2021). The key assumption is that the utilization of the substrate is independent of the microbe's surrounding environment (Enouy, Walton, et al., 2021). For some scenarios, this condition can predominate until the diffusive transport becomes the limiting step of biodegradation (Enouy, Walton, et al., 2021).

The second scenario is the linear reaction model, where substrate flux is assumed to be linearly dependent on the product of a constant transport velocity (CTV) and the concentration of the substrate on the surface of the microbe (Enouy, Walton, et al., 2021). CTV is the velocity at which the substrate moves across the microbial membrane (Enouy, Walton, et al., 2021). It was suspected that the substrate flux across the microbial membrane was the limiting process (Enouy, Walton, et al., 2021). As such, K_s and μ_{max} solve to be a function of the physiochemical and mass transfer properties of the system, but also to be linearly dependent on the substrate's bulk concentration (in the aqueous phase) (Enouy, Walton, et al., 2021). They cannot be constant for this substrate depletion scenario, since they are dependent upon the substrate concentration (Enouy, Walton, et al., 2021). The key assumption is that the substrate utilisation is proportional to the substrate concentration on the surface of the microbe, which means that the microbial consumption of the substrate is dependent upon the surrounding environmental conditions (Enouy, Walton, et al., 2021).

A key path to understanding Monod kinetics and in the development of groundwater biodegradation models is to observe the microbe's action throughout the degradation process. In such observations, it can generally be determined that the microbe utilises active transport pathways to move larger molecules (such as the aromatic hydrocarbons) across the microbial membrane. The rate of active transport should be dictated by the rate of internal biochemical processes able to decompose hydrocarbons to extract energy. There is also evidence of storage mechanisms, which would indicate that the microbe could be something of a delayed zero sink,

however these have yet to be considered in the model (Hua & Wang, 2014). The active transport pathway matches with the assumptions of Schäfer et al. (1998) such that the electron donors are distributed between aqueous and biomass phases solely based on the chemical potential gradient.

Chapter 3 – The Four Pillars of Microbiological Growth

In modelling development, there must be a mechanistic understanding as well as a suitable explanation for all parameters and processes inherent in the conceptual model including any numerical assumptions within the algorithm used to implement it. Observations of substrate depletion and biomass growth curves representing various substrate-species pairs from both laboratory experiments and field sites are essential both for determining how these processes occur, and moreover, how to represent these processes for modelling purposes. There are many aromatic hydrocarbons industrially utilized, and subsequently introduced into the environment as pollutants, the most chemically stable being benzene: a ring of six alternative double-bonded carbons (Ladino-Orjuela et al., 2016). The electrons are delocalized making this compound immensely stable, thus slow and difficult to degrade (Ladino-Orjuela et al., 2016). Since all aromatic compounds include this structure as a part of their whole molecule, they all encompass the most persistent environmental pollutants. Though they can be degraded both aerobically and anaerobically, the aerobic pathway has proved more efficient, though the anaerobic pathway is more commonly utilized since biodegradation tends to occur in more anaerobic environments (Chakraborty & Coates, 2004; Carmona et al., 2009).

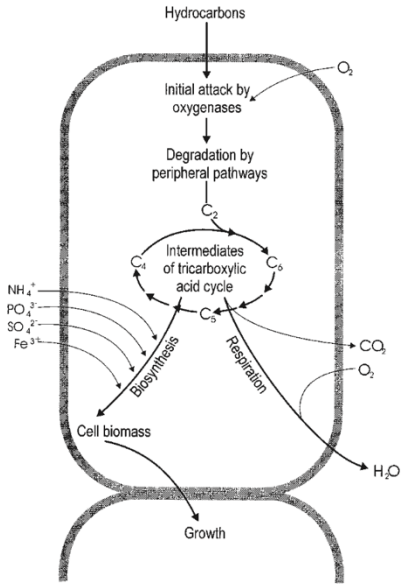

Figure 2: The growth-associated processes that make up the main principles of aerobic degradation of hydrocarbons, from Fritsche and Hofrichter, (2000).

Following below are insights into the four main steps in microbiological biodegradation of aromatic hydrocarbons and subsequent microbial growth. The steps involved are: cellular transport involving the uptake of substrates for metabolism; storage of energy in the form of lipid inclusions; metabolic degradation and energy production from the substrate (either aerobically or anaerobically); and finally, cellular growth [Figure 2].

3.1 Cellular Transport

The first major step involved in biodegradation is the sensing of hydrocarbons in the environment, and subsequent uptake of these substrates. For environmental remediation, the hydrocarbon substrate must pass the cell membrane, however the outer membrane (OM) of Gram-negative bacteria provides a barrier against just hydrophobic molecules (Hua & Wang, 2014). Gram-negative outer membranes contain lipopolysaccharides on their surface, requiring uptake channels, from proteins embedded in the OM, for hydrophobic molecules (Hua & Wang, 2014; van den Berg et al., 2015). Transmembrane transport is a sort of bottleneck, such that the uptake of hydrocarbons determines the efficiency of bioaccumulation and biodegradation (Jin et al., 2017). Membrane transport mechanisms must be able to maintain internal conditions within a prescribed range, despite external environmental changes (Parker et al., 2018).

The theories behind the various methods of uptake are unclear and controversial mainly due to difficulties in the current methods of investigation (Jin et al., 2017). However, optimising and enhancing these methods promotes degradation efficiency, so is essential in natural bioremediation (Jin et al., 2017). Jin et al., (2017) discusses these challenges, stating that they are poorly studied because there is no sensitive detection method to analyse transmembrane process *in vivo*. Though the usual detection and analysation methods of chromatography and mass spectrometry can analyse hydrocarbons in microorganisms, these tools require large sample sizes and complicated pretreatment procedures (extraction and concentration) which are error-prone and result in destroying the biological samples. As such, it is difficult to determine microscopic distribution and dynamic changes of hydrocarbon transport and accumulation *in situ*, not to mention that these methods only measure ensemble behaviour in microbial populations, ignoring cell-to-cell heterogeneity (Jin et al., 2017). One must always keep in mind that there is phenotypic heterogeneity in a population of cells with identical genome and that it can be quite broad due to epigenetic modifications, stochastic gene expression, variable mRNA stabilities and protein activities (Jin et al., 2017).

Nonetheless, there are three main hypotheses of uptake of a substrate. They are i) passive diffusion; ii) passive facilitated diffusion; and iii) energy-dependent or active uptake (Jin

et al., 2017; Hua & Wang, 2014). Passive diffusion involves uptake of molecules without the use of ATP. It is the simple diffusion of molecules moving from high to low concentrations (Parker et al., 2018). An ATP inhibitor in solution would have no effect on this passive process. However, this process always requires there to be a certain higher concentration of the substrate in the near vicinity of the microbe, otherwise active transport would be required (Hua & Wang, 2014). Charged molecules, or larger molecules, would need the assistance of carriers or channels via so-called facilitated diffusion (Parker et al., 2018). The outer membrane of Gram-negative bacteria will permit the diffusion of small hydrocarbons, however the corresponding role of Gram-positive bacterial membrane has not been reported (Hua & Wang, 2014). Gram-negative bacterial outer membranes contain many channels for mediating the uptake of nutrients and other small molecules necessary for respiration and growth (van den Berg et al., 2015). For example, Gram-negative *Escherichia coli*, has a lateral diffusion mechanism for the uptake of hydrophobic substrates, called the OMLCFA (outer-membrane long-chain-fatty-acid) transporter FadL (Hearn et al., 2009). This transporter is also present in other bacterial species, and its crystal structure has been visualised in *Pseudomonas aeruginosa* as a ß-barrel which creates a long hydrophobic tunnel for mediating the substrate from the extracellular environment through the polar lipopolysaccharide layer via a lateral opening in the barrel wall, into the lipid bilayer (Hearn et al., 2009). From there, the substrate diffuses into the periplasm where endoenzymes can begin the degradation process (Hearn et al., 2009). Organic compounds, such as PAHs, with low water solubility and high octanol-water partition coefficients tend to use this method, by partitioning into the cell wall structures, and travelling down the concentration gradient (Hua & Wang, 2014). There is also group translocation in which as the molecule moves into the cell, against the concentration gradient, it is chemically modified so it does not require transport against the unfavourable concentration gradient (Parker et al., 2018). Energy-dependent, or active transport involves hydrocarbons travelling across the bacterial membrane via the use of ATP "pumps" (Parker et al., 2018). This is especially necessary for moving molecules "uphill" against their concentration gradient (Parker et al., 2018). Some hydrocarbons are degraded before uptake, via exoenzymes, but the majority will travel across the bacterial membrane to reach the cytoplasm where endoenzymes will degrade them internally (Hua &

Wang, 2014). The outer membrane proteins' involvement in these energy-dependent transport mechanisms is poorly studied (Hua & Wang, 2014).

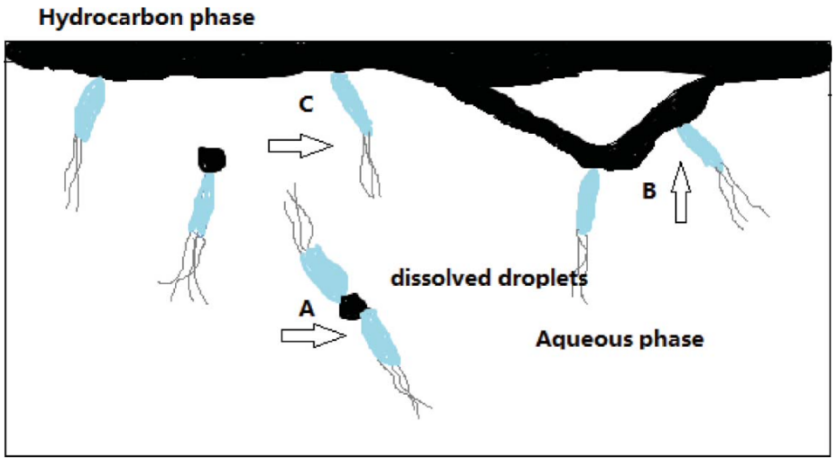

Figure 3: The three modes of uptake of a petroleum pollutant in aqueous phase, by microorganisms, from Hua & Wang (2014).

A hydrocarbon spilled in the environment could be dissolved in the aqueous phase or present as hydrophobic droplets on the surface of groundwater (Hua & Wang, 2014). There are three modes of uptake when a hydrocarbon pollutant is in the environment: a) uptake of the hydrocarbon dissolved in aqueous phase; b) contact of a microbial cell with a submicron oil droplet; or c) direct contact with a large oil droplet [Figure 3] (Hua & Wang, 2014). The uptake of hydrocarbons dissolved in aqueous phase allows for water-soluble aromatics and short-chain hydrocarbons to be utilised (Hua & Wang, 2014). If the dissolution rate of the hydrocarbon is less than the growth rate of the microorganism, than the microorganism will grow well in solution, however if the rates are reversed, then the biodegradation potential by the microbe is limited (Hua & Wang, 2014). For example, about half of bacterial strains found in petroleum-contaminated soils showed exclusive direct interfacial uptake, whereas the other half produced biosurfactants, indicating two main mechanisms for taking up alkanes (Hua & Wang, 2014).

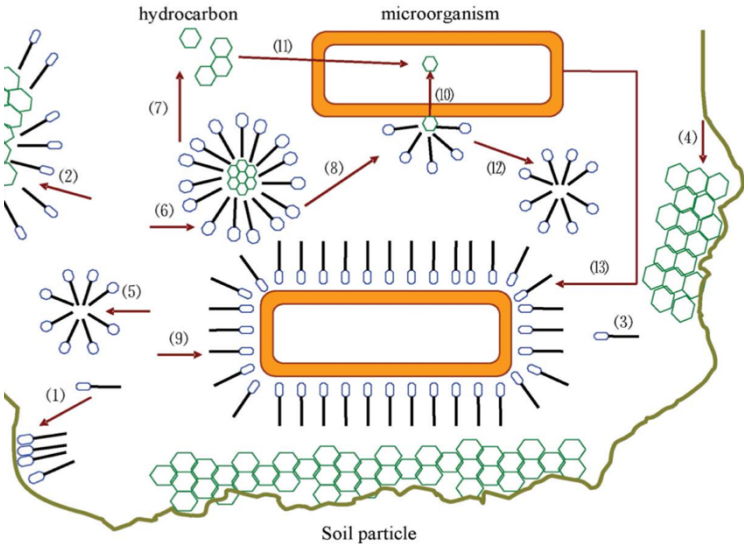

Figure 4: The uptake of hydrophobic hydrocarbons (green hexagons) by microorganisms (orange rectangles), with some utilisation of biosurfactants (blue hexagons with black tails), from Hua & Wang (2014).

Surfactants are emulsifying agents that will break down the large substrate droplets into microdroplets. Natural surfactants, usually produced by various microorganisms, are called biosurfactants, and there are many various classes, such as glycolipids, lipopeptides, fatty acid, and polymeric biosurfactants (Kaczorek et al., 2018). Their production has been well studied though the uptake mechanism by the microbe is still unknown (Hua & Wang, 2014). The uptake of pseudo-solubilised hydrocarbons involves the production and excretion of biosurfactants by the microbe, and this is a very common form of uptake [Figure 4] (Hua & Wang, 2014). The use of biosurfactants allows for an increase in dispersion of pollutants in the water phase, and a desorption of contaminants from the soil matrix, ultimately increasing bioavailability of hydrocarbon pollutants to microbial degraders (Kaczorek et al., 2018). The biosurfactant is made of one or more lipophilic and hydrophilic moieties. The lipophilic moieties include proteins, peptides with high proportion of hydrophobic side chains of usually about 10-18 carbons. The hydrophilic moieties include ester, hydroxyl, phosphate or carboxylate groups or carbohydrates. This makes the biosurfactant amphiphilic so that it can emulsify the hydrocarbon, enhance water solubility, decrease surface tension, increase displacement of oily substrates from soil particles, assist transport and translocation of insoluble substrates across

cell membranes, and help detach bacteria from oil particles after the utilisable hydrocarbon has been depleted (Hua & Wang, 2014).

There have been numerous studies on the effectiveness of the addition of synthetic surfactants to solutions where microbial uptake of emulsified agents is required, in the hopes of improving removal and cleanup efficiency of pollutant spills (Kaczorek et al., 2018). Although there is some promise that these synthetic surfactants can greatly increase bioavailability of pollutants, it has been observed that synthetic surfactants can have too much of a toxic effect on the microbial membrane fluidity which undesirably causes some cellular growth inhibition and might cause cell rupture and death, as well as often becoming another environmental pollutant itself (Kaczorek et al., 2018). Therefore, the natural surfactants produced by biodegrading microorganisms seem to be more effective. These natural surfactants are advantageous due to their biodegradability, high environmental compatibility, strong surface activity, and lower toxicity (Kaczorek et al., 2018). The use of biosurfactants has enhanced the rate of biodegradation of hydrocarbons, such as the observation by Bezza & Chirwa (2017) in which a lipopeptidal biosurfactant produced by *Bacillus cereus* SPL-4 enhanced the bioremediation of aged PAH-contaminated soils by a microbial consortium by 86.5% degradation efficiency (compared to 57% efficiency without biosurfactants and nutrient amendments) (Kaczorek et al., 2018). These biosurfactants could even be used to assist in non-biodegrading clean up techniques, though industrial production and implementation has yet to be very successful (Kaczorek et al., 2018).

Different microorganisms have different affinities to hydrocarbons. Some microbes grow in lipid-like environments rather than aqueous environments; the microbial cell directly attaches to liquid hydrocarbon drops that are much bigger than the cells, so that the microorganism forms an agglomeration with the hydrocarbon to bring the cell in close contact (Hua & Wang, 2014). For water-insoluble substrates, such as n-alkanes and PAHs, the hydrophobic nature of the bacterial cell surface plays a key role in uptake: the cells reach the oil-water interface by a thermal diffusion process, and then release surface-active compounds that result in formation of an interfacial visoelastic film which exerts control over all cell behaviour (Hua & Wang, 2014).

The cell's hydrophobicity increases in the presence of biosurfactants and if the substrate is slightly soluble, this can promote more attachment of the cell to the oil droplets, enhancing alkane degradation (Hua & Wang, 2014). The biosurfactant plays a key role in this process: it increases the chance of direct contact between a bacterial cell and an oil droplet, and affects the bioavailability and biodegradation kinetics of hydrophobic pollutants by stimulatory or inhibitory effects dependent on the chemical characteristics of the surfactant, the pollutant, and the physiology of the microorganism (Hua & Wang, 2014). Surfactants must be at critical micellar concentration (CMC; the concentration at which micelles form): if too low, then no enhancement or inhibition of biodegradation is observed, and if too high, than biodegradation is inhibited (the substrate would remain in micellular form and not be bioavailable) (Hua & Wang, 2014). Some microorganisms (in particular, strains of *Pseudomonas*) can form an agglomeration with the hydrocarbon to bring the cell in close contact (Hua & Wang, 2014). The cell simply adheres to the surface of the oil and directly transports the substrate into cells without the use of extracellular surfactant- or emulsifier-excretion (Hua & Wang, 2014).

There are some proteins and channels that have been identified for their involvement in the uptake of hydrophobic molecules, despite limited research into the precise uptake mechanism. The COG4313 proteins form the family of OM channel proteins involved in the uptake of hydrophobic molecules, such as hydrocarbons (van den Berg et al., 2015). For Gram-negative bacteria, proteins allow transport of molecules too large or too scarce to accumulate, by use of the TonB-dependent OM active transport system which is a ligand-specific active transport system that moves molecules against the concentration gradient (Hua & Wang, 2014). The crystal structure of such an active transport OM receptor has two distinct functional domains: 1) an even numbered 8-24-stranded ß-barrel spanning the OM, forming pore-like structures called porins, and containing large extracellular loops for ligand binding and 2) a globular N-terminal domain that folds into the barrel pore, inhibiting access to periplasm and contributing two additional loops for potential ligand binding (Hua & Wang, 2014). The presence of these loops could also indicate that there is also a signalling pathway between processes of ligand recognition and the TonB-mediated transport, whereas the blockage of the pore containing this channel suggests that the N-terminal domain of the protein must go

through a conformational change to allow ligand transport into the periplasm (Hua & Wang, 2014). Porins allow for the passive diffusion of molecules smaller than 600 Da on a downhill gradient through the barrel into the periplasm, which is key for nutrient uptake (Hua & Wang, 2014). In the Gram-negative biodegrader *Pseudomonas putida* F1 (PpF1), there is a channel coded by orf Pput2725 (van den Berg et al., 2015). Though its purpose is not yet established, the neighbouring gene Pput2724 and further genes nearby (Pput2727-2729) code for enzymes involved in monoaromatic hydrocarbon (MAH) degradation, which may suggest that Pput2725 codes for the channel used to uptake such substances (van den Berg et al., 2015). The only OM protein family with an established role in hydrophobic substrate uptake is the FadL family, which can laterally diffuse long-chain fatty acids (LCFA) from the barrel lumen of a channel through the OM, bypassing the polar layer of the LPS which otherwise prevents diffusion of hydrophobic molecules (van den Berg et al., 2015). They can cross through via diffusion or in an active manner (Hua & Wang, 2014). For example, in *Escherichia coli*, FadL mediates passive diffusion of hydrophobic molecules across the OM and conformational changes to the N-terminus opens up the channel for such substrate diffusion (Hua & Wang, 2014).

Each microorganism uses different methods to uptake necessary substrates. Yeast cells can utilise both direct and biosurfactant-mediated methods (Hua & Wang, 2014). Different microorganisms often work together in tandem through different strategies to access hydrocarbons (Hua & Wang, 2014). Edwards and Grbić-Galić (1992) observed that in the degradation of benzene, by a consortium of microorganisms from a contaminated soil site, that the cells appeared in close association with solid particles and with each other, allowing for a complex community structure able to live off electron donors and acceptors in a system that offers little energy.

To test whether the transport of a particular substrate is induced by active transport or not, Hua & Wang (2014) suggests the use of ^{14}C-labelled hydrocarbon and non-labelled hydrocarbon in solution with microbial degraders, and sodium azide (an oxidative phosphorylation uncoupler, which would reduce the uptake if active transport is occurring). If hydrocarbon uptake is reduced, then that would indicate that the uptake is dependent on proton

motive force (Hua & Wang, 2014). Other studies have suggested that substrate transport is related to the environmental concentration of the substrate, and as such, since energy-dependent mechanisms mediate the process of transport into the bacterial cells, then blocking these mechanisms would cause biosurfactant production to cease as well (Hua & Wang, 2014). Nonetheless, this all needs better and more research (Hua & Wang, 2014).

3.2 Storage

Bacteria accumulate many compounds for storage as a strategy to survive in variable environments, and these are called intracellular inclusions (Hua & Wang, 2014; Parker et al., 2018; Wilkinson, 1963). Though ATP can usually reach high concentrations within cells (in the millimolar range), with both a high rate of ATP-dependent process, and ATP's low stability in water, this concentration could be quickly depleted if not immediately replenished by glycolysis and oxidative phosphorylation (Bonora et al., 2012). Hence, the storage of carbon sources for ATP production is a necessity (Bonora et al., 2012). These intracellular inclusions can store excess nutrients, within cytoplasmic structures, in a polymerised form to reduce buildup of osmotic pressure which usually occurs during the accumulation of solutes (Parker et al., 2018). The most common such compounds are intracellular polysaccharides such as glycogen and starch, and lipids such as poly-ß-hydroxybutyrate (PHB) and triglycerides (Wilkinson, 1963; Bonora et al., 2012). Normally, lipids and polyglucoses act as alternative reserves, depending on the bacterial species, and the nature of the carbon and energy sources in the environment (Wilkinson, 1963). They uptake their electron-donor substrates when present in their near environment and oxidise them internally into fatty acids to store for when energy sources are low (Hua & Wang, 2014; Parker et al., 2018). There are several kinds of inclusions. Volutin (or metachromatic) granules store polymerised inorganic phosphate to be used in metabolism or in the formation of biofilms (Parker et al., 2018). Some are not used for nutrient storage, but rather for other mechanistic purposes: gas vacuoles for altering buoyancy, and magnetosomes filled with magnetic iron oxide or iron sulphide to align with the magnetic field to aid movement (Parker et al., 2018).

Hydrophobic organic chemicals (HOC), which include the hydrocarbons of environmental concern, can be incorporated and bioconcentrated into living tissue (Jin et al., 2017). Microorganisms significantly affect mobility and bioavailability of HOC in the environment via bioaccumulation and biotransformation (Jin et al., 2017). Oil production and processing generate significant portions of oily sludge, containing waste oil (40-60%), wastewater (30-90%) and mineral particles (5-40%) (Hoseinabadi et al., 2015). Accumulation of PHB under aerobic conditions occurs when a carbon source is in excess (e.g. provided from oil sludge), but when one or several other nutrients are limited (Hoseinabadi et al., 2015). The convection and active processes in the cellular cytoplasm allows for accumulation of HOC from water, when present in high concentrations (Jin et al., 2017). This does present a ecotoxicological concern since it can be biomagnified through the food chain via contaminated foodstuffs, but it also presents questions in regards to the processes involved in biodegradation (Jin et al., 2017). How do cells store extra energy and when? Is there a mechanism for ceasing uptake or redirecting to resume uptake? And what is the mechanism and pathway for directing the lipids to energy production?

To demonstrate storage function for a compound, one must show that the products of the breakdown are used for some advantageous purpose by the cell in its struggle for survival over other organisms (Wilkinson, 1963). For example, Wilkinson (1963) reports that glycogen and PHB (a polysaccharide and a lipid both able to act as alternative energy reserves) provide intermediates for the synthesis of proteins, allowing for an increment of growth and division when carbon and energy sources are sporadic. Such storage mechanisms may also allow rapid adaptation to different environments by production of appropriate enzymes (Wilkinson, 1963). Again, Wilkinson (1963) gives the example that the breakdown of PHB provides energy and intermediates for sporulation. Such breakdowns of reserves helps preserves the cell's viability by providing energy for maintenance (Wilkinson, 1963). A cell will tend to store any polymers that can be accumulated without decreasing its growth rate (Wilkinson, 1963). The nature of such polymers is dependent on the rate-limiting growth step, so upon the near environment, and the level of nutrients (Wilkinson, 1963). Wilkinson (1963) hypothesises that probably the rate-limiting factor will either be the synthesis of proteins and nucleic acids when sources of carbon,

hydrogen, and oxygen reserves accumulate, or the primary degradative pathway of carbon and energy when no such specialised reserves accumulate.

Jin et al. (2017) monitored real-time bioaccumulation and efflux of HOC in microorganisms at single-cell level using single-molecule fluorescence microscopy (SMFM) with a microfluidic device and using temperature control. They used the Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* since these are two species with distinct membrane structures, with the hypothesis that it will lead to different features of bioaccumulation. They used perylene (a five-ring PAH, $C_{20}H_{12}$) as the HOC since it has a high quantum yield (0.94) and an excitation spectrum in the visible-light region, making it ideal for FM (Jin et al., 2017). Incubation of the bacterial strains in perylene was followed by observation of the cellular perylene concentration (Jin et al., 2017). SMFM allows for observation of cell-to-cell heterogeneity, which was most notable in *E. coli*, as well as for differentiating between living and dead cells via a fluorescent dye, PI (cells with a higher than threshold fluorescence were considered dead) (Jin et al., 2017). However, not all cells with high perylene accumulation were dead, which indicates heterogeneity within live cells and homogeneity within dead ones (Jin et al., 2017). There was a high level of heterogeneity between *E. coli* cells, which was attributed to phenotypic differences within the isogenic population and their biological processes (Jin et al., 2017). Neither *E. coli* nor *S. aureus* have perylene degradation genes, so biodegradation was not a possible reason for cell-to-cell heterogeneity nor for perylene removal from the incubation solution (Jin et al., 2017). Jin et al., (2017) supposed the heterogeneity within *E. coli* cells to be due to epigenetic modifications, stochastic gene expression, variable mRNA stabilities, and protein activities. They propose *E. coli* to use its membrane efflux system to avoid accumulation too much perylene, whereas *S. aureus* did not have bioaccumulation correlated to survival state (Jin et al., 2017). In *S. aureus*, it was an entirely energy-independent passive process, with both living and dead cells having comparable perylene accumulation (Jin et al., 2017). *E. coli* cells were able to achieve steady state after about 10 minutes of incubation whereas *S. aureus* cells never reached steady state, indicating a passive trans-membrane diffusion in *S. aureus* cells, versus the membrane-efflux system active in live *E. coli* cells which effectively resists perylene accumulation (Jin et al., 2017).

Gram-negative bacteria have a thick polar lipopolysaccharide (LPS) layer in their OM which provides an effective barrier against hydrophobic molecules, but this is not the only aspect at play here; *E. coli* would then reach steady state much more slowly so there must be an additional mechanism: perhaps a multidrug efflux pump, known to exist in *E. coli* and other bacteria, which may play a role in the intrinsic resistance to perylene (Jin et al., 2017). Such transporters have multiple specificities for many relative lipophilic planar molecules (Jin et al., 2017). To demonstrate the role of this pump, Jin et al. (2017) used the Tol C efflux pump knockout strain of *E. coli* ($\Delta tolC$) and a proton motive force (PMF) inhibitor, CCCP. Tol C is a common channel protein of both major and minor efflux systems, enabling interactions with translocase systems (Jin et al., 2017). Deletion of the *tolC* gene largely abolishes efflux activity (Jin et al., 2017). PMF is the driving force of multidrug efflux pumps like Tol C, and CCCP dissipates PMF thus blocking the efflux system (Jin et al., 2017). When using the knockout strain and CCCP, the rate of perylene accumulation was constant during incubation and was much lower in dead *E. coli* cells, indicating that there are different membrane structures in live and dead cells, such as that the cytoplasmic membranes of dead bacteria are partly damaged, and that the live cells have integrated membranes which slow accumulation of perylene molecules (Jin et al., 2017). However, there was still a high heterogeneity which can be attributed to combined phenotypic differences in cellular sensitivity to the CCCP treatment, individual capability of recovering from CCCP, and individual membrane efflux activity (Jin et al., 2017).

Though only reporting the visualisation of *E. coli* and *S. aureus*, Jin et al. (2017) also visualised perylene accumulation by other Gram-negative (*Vibrio alginolyticus, Proteus vulgaris*) and Gram-positive (*Bacillus subtilis*) bacteria. *B. subtilis* had a similar result to *S. aureus*, both Gram-positive bacteria having high levels of perylene accumulation without reaching steady state during incubation (Jin et al., 2017). *V. alginolyticus* and *P. vulgaris* both excluded perylene completely (Jin et al., 2017). Another study observed nine different bacterial strains with the accumulation of phenanthrene (Stringfellow & Alvarez-Cohen, 1999). All Gram-positive bacteria, except *Micrococcus luteus*, had higher or better accumulation than Gram-negative

bacteria, which is consistent with (Jin et al., 2017; Stringfellow & Alvarez-Cohen, 1999). The observed effect is not just due to the difference in membrane structures, but more specifically due to the presence of the multidrug efflux systems associated with *Tol C* protein, driven by PMF (Jin et al., 2017). However, Gram-positive bacteria also have numerous membrane transporters for efflux of various drugs, so their inability to expel perylene is unclear and warrants further investigation (Jin et al., 2017).

Energy reserves can be determined via a process called microbial calorimetric analysis which involves measuring the heat coming from carbon sources inside bacteria (Boe & Lovrien, 1990). A rather underused tool, microbial calorimetry measures bacterial energy stores without breaking the cells open, and is a rapid and easy method though precision and accuracy may be unclear (Boe & Lovrien, 1990). Not all energy storage polymers are PHB, which would skew the calculations of dry weight of energy storage in the cells, since they would be of different molecular weights (Boe & Lovrien, 1990). However, it overall gives an easily obtained estimate of "heat content" in bacterial energy reserves, easily drawn on by adding oxygen (Boe & Lovrien, 1990).

If an organism is grown under nitrogen limitation, oxygen limitation, and in excess carbon, many accumulate storage polymers such as PHB, which can account for sometimes as much as 60-80% of their dry weight (Boe & Lovrien, 1990). Aerobic metabolism of glucose, by many bacteria, generates -305 ± 20 Kcal/mol for conversion of acetate and three CO_2 molecules (Boe & Lovrien, 1990). 45% of glucose's carbon forms CO_2, about 16% forms acetate or ethanol, and the remainder remains in the cell as biomass or other intermediates (Boe & Lovrien, 1990). Oxygen triggers the metabolism of stored carbon, and sustains it, though different species carry out metabolic consumption at different rates (Boe & Lovrien, 1990). For example, *Pseudomonas putida* can proceed for up to two hours with about twenty remixing cycles, at 25 °C, whereas *Alcaligenes eutrophus* lasts about forty minutes with forty remixing cycles at 25 °C, or more if remixing is more spread out (such as five-minute intervals) (Boe & Lovrien, 1990). The estimated average heat for metabolising a PHB molecule is 200 Kcal/mol so the total heat measured is divided by this value, to give an estimate of how much dry weight of the cell may

be PHB (Boe & Lovrien, 1990). Boe & Lovrien (1990) found that about 5-50% of the dry weight of these cells may be PHB, depending on species, growth conditions, and carbon source, but calorimetric estimates need to be compared to conventional extractive-chemical degradative methods.

There are still questions to be answered regarding energy storage and its relationship to metabolism. Boe & Lovrien (1990) observed that oxygen is the "trigger" but it is really just the ultimate electron acceptor, and part of the fuel. There could be other potential triggers or signals, such as uncoupling agents (i.e. dinitrophenol), that can call on glycolysis and make it accelerate, or other signals such as glutathione deficiency which demands restoration of ATP and NADPH (Boe & Lovrien, 1990). Such agents mixed with cells can amplify the effects, forcing cells to use extra oxygen and endogenous carbon reserves to produce large metabolic heats (Boe & Lovrien, 1990). Wältermann & Steinbüchel (2005) presented different kinds of lipids stored in prokaryotes, comparing their formations to similar structures in eukaryotes, however they noted many open questions which had not yet been fully answered, including: 1) how their synthesis and mobilization is regulated; 2) which enzymes catalyze intracellular mobilization and how they get access; 3) what regulates size and shape of these lipid inclusions.

3.3 Aerobic Cellular Respiration

Cellular respiration can fall into two main modes: in the presence or absence of oxygen. Both are important in the bioremediation of aromatic hydrocarbons since there are both aerobic and anaerobic bacteria and archaea with abilities involved in the processes of degradation. Bacterial catabolism of aromatics involves many peripheral pathways that transform the compounds into a limited number of common intermediates which are further processed into the central metabolism of the cell (Carmona et al., 2009). In aerobic respiration, oxygen is the electron acceptor and co-substrate for hydroxylation and oxygenolytic ring cleavage of aromatic hydrocarbons (catalysed by oxygenases) (Carmona et al., 2009).

Aliphatic hydrocarbons are oxidised readily by a variety of bacteria and fungi (Gaudy, Jr. & Gaudy, 1980). The major aliphatic mode of attack is: alkane → alcohol → aldehyde →

fatty acid, which then undergoes ß-oxidation processes that are the same as for fatty acids derived from lipids (Gaudy, Jr. & Gaudy, 1980). For aromatic hydrocarbons, there are two main steps. An upper pathway involving enzymatic ring activation, preparing the hydrocarbon to enter some central intermediate pathways, followed by a lower pathway, where dearomatisation and ring cleavage occurs, breaking the aromatic ring of the molecule [Figure 5] (Jiménez et al., 2004; Gaudy, Jr. & Gaudy, 1980). The conventional route involves activation of the aromatic ring, leading to the formation of a dihydric phenol which involves the addition of two hydroxyl groups (Jiménez et al., 2004). The final compounds funnel into the central metabolic systems, usually beginning with ß-oxidation, then entering the Krebs or TCA cycle (Jiménez et al., 2004). For example, benzene degradation begins with oxidation of the benzene ring by either a benzene dioxygenase, or a benzene monooxygenase and a phenol hydroxylase, which ultimately forms catechol, and then *ortho* or *meta* fission (*ortho* shown below; *meta* forms 2-hydroxymuconic semialdehyde) (Gaudy, Jr. & Gaudy, 1980; Rochman et al., 2017; Harayama et al., 2004). Most microbial benzene degraders have genes encoding for both monooxygenases and dioxygenases (Rochman et al., 2017). Dioxygenases are more efficient than monooxygenases, since they catalyze both steps of converting benzene to catechol, whereas monooxygenases must be coupled with a phenol hydroxylase in what is more clearly a two-step process (Rochman et al., 2017). This is the benzene degradation pathway with two-step ring activation and *ortho* fission ring cleavage: $benzene \rightarrow cis-1,2-dihydroxy-1,2-dihydrobenzene \rightarrow catechol \rightarrow cis,cis-Muconic\ acid \rightarrow ß-ketoadipic\ acid \rightarrow acetyl-CoA + succinic\ acid$, which enters the TCA cycle (Gaudy, Jr. & Gaudy, 1980; Fritsche & Hofrichter, 2000; Ladino-Orjuela et al., 2016) . However, benzene also has many satellite pathways which lead to the production of benzoate or benzyl-CoA, before ring reduction to enter the TCA cycle (Chakraborty & Coates, 2004). Its main pathway is the catechol branch of the ß-ketoadipate pathway (the *ortho*-cleavage pathway) coded by *cat* genes (*pcaD, pcaIJ, pcaF, catRBCA*), and catalysed by 1,2-dioxygenase (CatA coded by *catA* gene), often in two sets, such as catBC (coded by *catBC* gene), two subsequent enzymes that make lactone transform into succinyl- and acetyl-CoA (Jiménez et al., 2004; Moat et al., 2002). Common benzene degrading genera include *Acinetobacter, Pseudomonas,* and *Burkholderia* (Jiménez et al., 2004). Phenol follows a similar pathway to benzene: $phenol \rightarrow catechol \rightarrow cis,cis-muconic\ acid \rightarrow ß-ketoadipic\ acid \rightarrow acetyl-CoA + succinic\ acid$,

since phenol already has one hydroxyl group, so is one step closer to catechol (Neilson, 1994; Reardon et al., 2000). Many other aromatics degrade to benzene-like compounds which then enter pathways similar to benzene degradation, but benzene does not have extra substituent branches so its degradation process is somewhat simpler than more complex compounds (Jiménez et al., 2004). Toluene can undergo multiple biotransformations, but Reardon et al. (2000) suggests $toluene \rightarrow 3-methyl\ catechol \rightarrow 2-hydroxy-6-oxo-2,4-heptadienoate$ before entering the TCA cycle as the process undergone by *Pseudomonas putida* F1. Some of the degradation processes occur very slowly, and sometimes only part of the process can occur (Gaudy, Jr. & Gaudy, 1980). Often, one species can only do one part of the full degradation process, so a varied community is required for full degradation (Gaudy, Jr. & Gaudy, 1980).

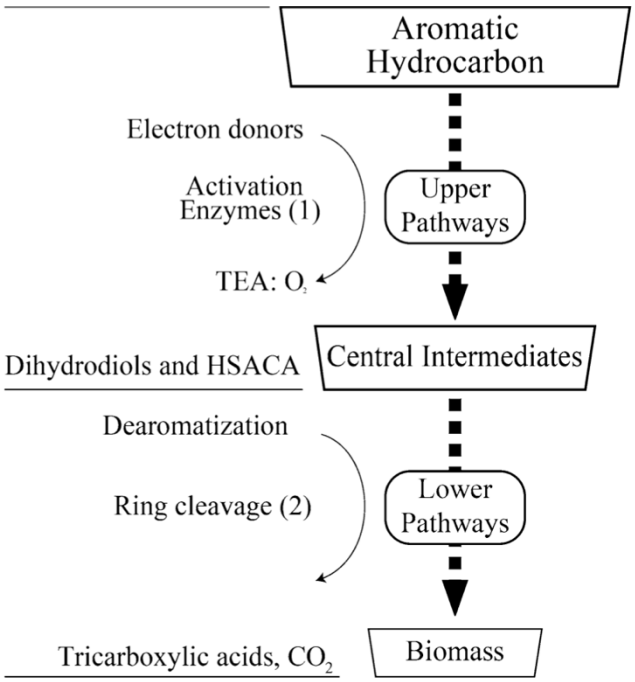

Figure 5: A schematic representation of the metabolic pathways of aromatic hydrocarbons by bacteria in aerobic conditions from Ladina-Orjuela et al. (2016).

Once the substrate has been transformed into a common intermediate, it can enter the further down the normal aerobic cellular respiration process, which normally begins with glycolysis and oxidative decarboxylation, then the TCA cycle, and finally, oxidative

phosphorylation and the electron transport chain (Parker et al., 2018). Aerobic cellular respiration normally begins with glycolysis, transforming a glucose molecule (the fundamental source of energy for most cells) into pyruvate ($glucose + 2\,NAD + + 2\,Pi + 2\,ADP \rightarrow 2\,pyruvate + 2\,NADH + 2\,ATP + 2\,H + + 2\,H_2O + heat$) (Parker et al., 2018). Pyruvate undergoes oxidative decarboxylation which oxidises the molecule into acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (PDC) (Parker et al., 2018). Acetyl-CoA, in the presence of oxygen, enters the TCA citric acid cycle (also known as the citric acid cycle or Krebs cycle) where it undergoes a series of transformations to produce energy in the form of ATP (the overall equation is: $acetyl - CoA + NAD^+ \rightarrow 6\,NADH + CO_2 + H_2O + 2\,FADH_2 + 2\,ATP$) (Parker et al., 2018). The NADH produced in the Krebs cycle is oxidised via oxidative phosphorylation and enters the electron transport chain travelling down a proton gradient to produce more ATP (via ATP synthase) and water (Parker et al., 2018).

There are many species that have been identified as aromatic hydrocarbon degraders. Many belonging to the *Pseudomonas* genus, especially *Pseudomonas putida,* contain many dioxygenases for aerobic degradation (Parker et al., 2018). *Pseudomonas* sp. are the best understood and very commonly studied as a quintessential γ-proteobacteria since it is a metabolically versatile genus, very adaptable both physiologically and genetically, and found in a vast array of environments such as soil, freshwater, and marine ecosystems, as well as being associated with some plants and animals (Jiménez et al., 2004). Other species include members of the *Rhodococcus* and *Acinetobacter* sp. (Parker et al., 2018).

There have been many developments in efficiency due to the desire to increase energy production by microorganisms for the purposes of industrial biotechnology (Zhou et al., 2009). The field of metabolic engineering has developed improvements in the microbial processes to gain increased product concentration, yield, and productivity of chemicals produced by such processes (Zhou et al., 2009). Though there has been great success, some tactics such as overexpression, deletion, or introduction of heterologous genes in specific metabolic pathways do not always achieve these targets (Zhou et al., 2009). For example, the glycolytic pathway, present in both eukaryotic and prokaryotic microorganisms, cannot increase its flux by

increasing genetic overexpression since the demand and supply of ATP play key roles in glycolysis as well as in the various cofactors (i.e. ATP/ADP, NADH/NAD, acetyl-CoA/CoA) (Zhou et al., 2009). ATP, in particular, is a nucleotide which can be a substrate, a product, an activator, or an inhibitor throughout the metabolic networks, and it is its supply and demand that affects active transportation, peptide folding, subunit assembly, protein relocation and phosphorylation, cell morphology, signal transduction, and the stress response (Zhou et al., 2009). Since it is involved in so many pathways and in the production of almost all metabolites, the manipulation of the ATP supply and demand is necessary to achieve higher product concentration, higher productivity, higher substrate yield, expanse of substrate spectra, and an enhancement of resistance to harsh environmental conditions (Zhou et al., 2009). Many of these would be important in improving the microbial efficiency of hydrocarbon biodegradation.

ATP supply in microorganisms begins in substrate-level phosphorylation and oxidative phosphorylation (Zhou et al., 2009). The latter's manipulation is more efficient to regulate intracellular ATP concentration under aerobic conditions, since most ATP production comes from the oxidative phosphorylation pathway (Zhou et al., 2009). Thus for regulating ATP availability, one would need to manipulate NADH availability, the electron transfer chain, the proton gradient, F_0F_1-ATPase, and oxygen supply (Zhou et al., 2009). Intracellular NADH converts to NAD via the ETC, with oxygen as the final electron acceptor, producing lots of ATP (Zhou et al., 2009). Or it can be directly oxidised into water and NAD through NADH oxidase (Zhou et al., 2009). So manipulation of these processes can be used to manipulate intracellular ATP (Zhou et al., 2009).

3.4 Anaerobic Cellular Respiration

Some bacteria and archaea lack the ability to respire aerobically, and as such, utilise other inorganic electron acceptors that are not oxygen (Parker et al., 2018). These microbes may not be able to use aerobic respiration for various reasons such as: 1) lacking cytochrome oxidase genes, which transfer electrons to oxygen at the end of the ETC; 2) lacking genes for enzymes which protect against dangerous oxygen radicals (i.e. hydrogen peroxide, superoxide); 3) lacking sufficient oxygen to carry out aerobic respiration (Parker et al., 2018). There are many options

for inorganic molecules other than oxygen, such as nitrate, sulphate, iron (III), carbon dioxide, manganese (II), selenate, and others (Parker et al., 2018; Carmona et al., 2009). For example, denitrifing microbes use nitrate and nitrite as their final electron acceptors, and produce nitrogen gas (Parker et al., 2018). Some common anaerobic biodegraders of benzene include *Thauera aromatica* (utilises iron as electron acceptor, can tolerate other aromatics but prefers benzene), members of the *Peptococcaceae* family (utilise iron, dominate in sulphate-reducing conditions), and the *Desulfobacteraceae* family (dominate in denitrifying, sulphate-reducing, and methanogenic conditions; can syntrophically interact with *Peptococcaceae* when iron(III) is the electron acceptor) (van der Zaan et al., 2012; Rochman et al., 2017). Another example is the facultative anaerobe *Thauera phenylacetica,* which can utilise oxygen or nitrate (Rochman et al., 2017). Such anaerobic respirers commonly have an intact Krebs cycle, so they can still access energy from NADH and $FADH_2$, but they use an altered ETC including distinct complexes for electron transfer to their final electron acceptors (Parker et al., 2018). Intracellular NADH converts to NAD via fermentative pathways (i.e. aldehyde dehydrogenase or lactate dehydrogenase), so mainly through substrate-level phosphorylation (Zhou et al., 2009). In practice, there are smaller electrochemical gradients therefore less ATP produced during anaerobic respiration (Parker et al., 2018).

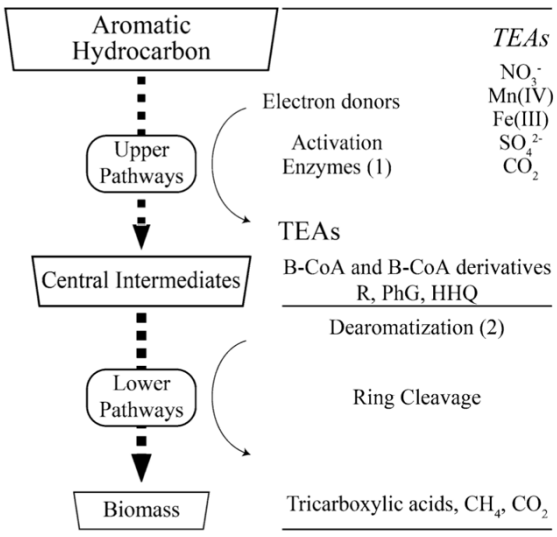

Figure 6: A schematic representation of the metabolic pathways of aromatic hydrocarbons by bacteria in anaerobic conditions from Ladina-Orjuela et al. (2016).

During anaerobic degradation of aromatic hydrocarbons, a major strategy is the reductive reactions attacking the aromatic ring [Figure 6] (Carmona et al., 2009). This strategy is more recently discovered despite the anaerobic microbial metabolism being the most ancient of life processes; the genetic and biochemical bases of anaerobic degradation are not well established due to difficulties in routinely growing and genetically manipulating aromatic-degrading anaerobic microbes (Carmona et al., 2009). Anaerobic pathways are far less understood than aerobic since the former are not as efficient, and soil allows for oxygen diffusion so aerobic pathways tend to be primarily used (van der Zaan et al., 2012). However, anoxic conditions *do* dominate many natural habitats and contaminated sites such that anaerobic biodegradation is the dominant process of natural biodegradation, despite being the less efficient process (Carmona et al., 2009). There is also the coexistence of multiple species to keep in mind; often mixtures of anaerobic and aerobic microbes, especially in water source zones where water circulates causing anoxic and oxic zones to mix, along with the ubiquitous microbial species (van der Zaan et al., 2012; Rochman et al., 2017). Local microbial communities use locally available electron donors (carbon sources) and acceptors to perform biodegradation rates according to particular redox parameters (Carmona et al., 2009). The anaerobic pathways that have best been observed and demonstrated are nitrate reducing, iron (III) reducing, sulphate reducing, and methanogenic (Chakraborty & Coates, 2004).

Benzene biodegradation, in particular, has been linked with multiple inorganic electron acceptors: sulfate reduction, iron reduction, methanogenesis, and nitrate reduction (Burland & Edwards, 1999). Benzene-degrading species work in syntrophic interactions since benzene is degraded by a collection of various electron acceptor pathways (van der Zaan et al., 2012). Edwards et al. (1992) confirmed the link between BTEX biodegradation (specifically xylene and toluene) and sulphate reduction, and Burland & Edwards (1999) confirmed the link between benzene biodegradation and nitrate reduction. Edwards et al. (1992) observed in sediment microcosms collected from the Amoco Cadiz oil spill site that toluene and m-xylene were degraded more rapidly than p-xylene and o-xylene; toluene always being the preferred substrate, however that in the presence of more easily degradable substrates such as lactate, glucose, or yeast extract, hydrocarbon degradation ceased entirely until the preferred substrates were

consumed. Degradation would diminish when there was excessive substrate concentrations (>30 mg/L or 300 µM), and was inhibited in the presence of free sulphide (via Na_2S), but would increase again if the cultures were flushed with N_2-CO_2 and replenished with fresh medium (Edwards et al., 1992). Edwards et al. (1992) utilised ^{14}C-labelled substrates to determine degradation rates and observed that 95-100% of the hydrocarbon was recovered as $^{14}CO_2$, indicating that only a very small portion was assimilated into cells. They deduced that there were probably not optimal conditions in the lab, suggested by extremely long doubling times, and low cell yields, plus variable degradation rates (experiment yield was half of the theoretical yield), but most especially, because there was an obvious order of preference in substrate degradation (toluene over xylenes, lactate/glucose/yeast extract over toluene), which suggests that degradation of BTEX and similar contaminants at contaminated sites may be prevented not due to lack of appropriate organisms or enzymes, but due to presence of more readily degraded substrates (Edwards et al., 1992).

Burland & Edwards (1999) confirmed the link between benzene biodegradation and nitrate reduction using batch microcosms of soil and groundwater from two different sites and evaluating a variety of electron acceptors to confirm nitrate as sole electron acceptor in this case. One site was a decontaminated retail gasoline station in Toronto, Ontario, and the other was an uncontaminated fresh water swamp near Perth, Ontario (Burland & Edwards, 1999). Nitrate reduction is the process of converting nitrate to nitrite and then finally to ammonia or nitrogen gas (Burland & Edwards, 1999). Burland & Edwards (1999) observed 92-95% of initial benzene (^{14}C-labelled) was recovered as CO_2 and 5-8% occurred in the non-volatile fraction which was presumed to be biomass. Denitrification to nitrogen gas was observed through gas bubble formation in the solution, and was measured and determined to be stoichiometrically equal to nitrate consumption (Burland & Edwards, 1999). Including other electron acceptors to determine whether they were being used or whether nitrate was the dominant electron acceptor, they observed no methane production (CO_2 is not utilised) (Burland & Edwards, 1999). FeS was present in the medium was rapidly oxidised to stoichiometric amounts of ferric iron and small amounts of sulphate when nitrate was added to the inoculated cultures (sulphate was not initially present in the culture medium), and this occurred regardless of whether benzene was

present or not, nor was iron and sulphate produced here re-reduced (Burland & Edwards, 1999). Of the various electron acceptors tested (CO_2, sulphate, iron, nitrate), all were measured initially and after 3.38 µmol of benzene was degraded: only nitrate was significantly consumed (˜3.6 µmol/vial) (Burland & Edwards, 1999). Nitrite accumulated in benzene-degrading cultures, and if nitrate reduction was slow, so was benzene degradation, and as nitrate reduced to nitrite, benzene degradation began to cease, indicating that nitrate is a better electron acceptor than nitrite, or that nitrite may be a benzene degradation inhibitor (Burland & Edwards, 1999).

3.5 Cellular Growth

The bacterial cellular growth cycle is well established and understood. Most bacteria replicate through the process of binary fission, where each cell duplicates its DNA and then cleaves in two, forming two identical cells (Parker et al., 2018). When there are the proper conditions, such as excessive energy supply or the most preferrable environmental conditions (pH, oxygen levels, temperature, nutrients, et cetera), then bacteria can grow comfortably, which can be observed with batch culture conditions in a "closed" system of a laboratory (Parker et al., 2018). Outside of their optimal preferred environment, they may still grow but not as efficiently. When a bacterial population begins to grow in a new environment, its adjustment begins with a period of no growth called the lag phase (Parker et al., 2018). As the cells adjust, they begin to grow exponentially, dividing by binary fission, in the period known as the log phase (Parker et al., 2018). The shortest possible generation time, and specific growth conditions are all genetically determined, though the actual generation time will be affected by the environmental conditions (Parker et al., 2018). After some time, the number of cells increases such that various factors in the environment require the growth rate to slow. These may include accumulation of waste products, depletion of nutrients or oxygen, adjustment of pH or temperature due to waste products or other factors (Parker et al., 2018). (In an "open" system, the population may act differently with the introduction of factors such as a renewal of resources, or the removal of waste products.) Then a population will enter a stationary phase, where the growth curve plateaus, and the rate of new cellular growth is equal to the rate of cellular death (Parker et al., 2018). Cells can persist in this phase for months to years. Bacteria begin a survival mode of

metabolism, where the synthesis of necessary proteins and nucleic acids are slowed or stalled, and they are not actively growing (Parker et al., 2018). If the environment becomes too toxic for the bacterial community, such as if the waste products are overwhelming, or the nutrients are exhausted, then the cells may begin to die off in exponential fashion, aptly named the death phase (Parker et al., 2018).

Burland & Edwards (1999) determined cell yield by measuring cellular protein before and after degradation of a known amount of benzene. Assuming 50% of the dry cell weight is protein, they calculated 8.8 g of cells generated per mole of benzene degraded (Burland & Edwards, 1999). They estimated the fraction of electrons from benzene that would be used for cell synthesis (f_s) by converting the measured yield (g/mol) to units of electron equivalents, based on the McCarthy prediction method, which is based on a correlation between free energy released during oxidation of a substrate, and experimental cell yield (Burland & Edwards, 1999). They calculated $f_s = 0.05 = 5\%$ of electrons in benzene were recovered as biomass which correlated with the 5-8% isotopic carbon recovered as the non-volatile fraction (Burland & Edwards, 1999). The assumed typical efficiency of electron transfer was 60% and the theoretical values of benzene oxidation were 0.45 when coupled with nitrate reduction to nitrogen, and 0.35 when coupled with nitrate reduction to nitrite (Burland & Edwards, 1999). However, the theoretical values were greater than the experimental value of 0.05 which indicates that the actual efficiency was far lower (Burland & Edwards, 1999). They hypothesised that this was due to suboptimal growth conditions, or the presence of inhibiting substances, or an inefficient pathway for benzene (Burland & Edwards, 1999).

As for the stoichiometry, theoretically 6 moles of nitrate should be needed to oxidise 1 mole of benzene (if nitrate is reduced to nitrogen), or 15 moles of nitrate if reduced to nitrite (Burland & Edwards, 1999). This stoichiometry does not incorporate new cell synthesis, though, which would require multiplying the coefficients for nitrate in the energy equations by a fraction of the benzene used for energy production ($f_e = 1 - f_s$), to determine the ratio of the amount of nitrate consumed to the amount of benzene degraded in individual culture vials at various enrichment stages (Burland & Edwards, 1999). For example, in the original soil culture,

the nitrate:benzene ratio was initially greater than the theoretical value presumably due to nitrate from any unknown electron donors in the soil sample (Burland & Edwards, 1999). But as carbon sources were depleted, the ratio stabilized to 10 moles of nitrate per mole of benzene (Burland & Edwards, 1999). The predicted nitrate:benzene ratio (for nitrate completely reduced to nitrogen gas) was 5.7 mol/mol, with the experimentally measured biomass yield of 8.8 g/mol (corresponding to an f_e value of 0.95), and 3.3 mol/mol (for $f_e = 0.55$) if the yield is predicted using the McCarthy method (Burland & Edwards, 1999). So, the observed ratio of 10 mol/mol is closer to the predicted ratio of benzene oxidation coupled to partial reduction of nitrate to nitrite (ratio should be about 14 mol/mol if $f_e = 0.65$ and was experimentally measured to be 9.8 mol/mol) which indicates that most of the cells' energy is derived during the first stage of nitrate reduction, and only a smaller amount is derived in subsequent stages (Burland & Edwards, 1999).

Chapter 4 – Discussion

Enouy, Walton, et al. (2021) showed that the Monod bioreaction equation can be represented as a three-stage serial process involving aqueous phase diffusive transport towards a spherical microbe, transmembrane transport, and reaction within the microbe. The transmembrane transport process can be further subdivided into two different scenarios: a constant rate model and a linear rate model. However, the three-stage process is rather simplistic. Connecting more considerations of mass transport, storage, and metabolic systems to the physical processes in the Monod model could improve its ability to fit data sets. The idea here is to expand the view of the Monod equation to include more processes than just internal microbial reaction. Though the data were able to fit fairly well within the two different scenarios, there is some deviation between the model and the data, as will be mentioned below, which implies that there could be other scenarios that better fit the data, especially as different substrate-species pairs would result in different degradation and growth curves. At this point, all the discussion of deviation between model and data from Reardon et al. (2000) is simply a visual exercise. The previous chapter explored detailed contributions of research to the understanding of the biological processes involved in biodegradation, some of which are involved in the representation of Monod presented by Enouy, Walton, et al. (2021), and some of which were not considered, such as any sort of storage capacities. This chapter will present a fit of single-substrate-depletion and biomass growth data from Reardon et al. (2000) using the Monod representation from Enouy, Walton, et al. (2021), and will discuss how the contributions explored in chapter 3 could embellish the Monod equation as represented by the three-stage process, to improve its fit of experimental data.

Enouy, Walton, et al. (2021) demonstrates that the Monod bioreaction equation can be derived as a special case of aqueous phase diffusive transport towards the surface of the microbe, and subsequent transport across its membrane in two scenarios: a constant rate model, and a linear rate model. The models were parameterised with benzene, toluene, and phenol depletion and biomass growth data from Reardon et al. (2000). The constant reaction rate model contains a constant transport flux characterised by constant Monod coefficients K_s and μ_{max}, which is consistent with the original interpretation of the Monod model Enouy, Walton, et al. (2021).

The linear reaction rate model, with its constant transport velocity, shows that K_s and μ_{max} could be dependent on bulk concentration (Enouy, Walton, et al., 2021). In both scenarios, K_s is dependent on 1) the physiochemical relationship of the diffusion coefficient for the substrate in the aqueous phase; 2) the biophysiochemical characteristic of the microbial radius; and 3) the microbe-specific biophysiochemical transport process (either flux or velocity) of the substrate (Enouy, Walton, et al., 2021). These attributes of the chemical species and of the microbial population together define K_s, giving it an inherent substrate-species dependency (Enouy, Walton, et al., 2021). In this simulation, the experimental conditions must be specified, which involved making adjustments to the numerical code to reflect biological mechanistic understandings mentioned above (Enouy, Walton, et al., 2021). The empirical values of the flux of the substrate across the surface of the microbe, and of the transfer velocity at which the substrate is taken up internally, and calibration of the model indicated a normalised surface-to-bulk-concentration ratio of approximate unity in all simulations (Enouy, Walton, et al., 2021). This implies that the process of biodegradation occurring in the experiments of Reardon et al. (2000) is not diffusion limited (Enouy, Walton, et al., 2021). The model shows that the surface concentration is nearly equal to bulk concentration when *P. putida* F1 is paired with benzene, toluene, and phenol as the limiting substrate (Enouy, Walton, et al., 2021). This indicates that the zero-sink surface condition, which is presumed by other models such as by Merchuk & Asenjo (1995), is not a valid assumption for the biodegradation processes, and it is expected that other biodegradation processes would exhibit similar kinetics (Enouy, Walton, et al., 2021). The verification exercise, using the data of Reardon et al. (2000), concludes that both benzene and toluene depletion, with their biomass growth curves, best fit with a constant reaction rate model, but phenol fits best with a linear reaction rate model (Enouy, Walton, et al., 2021). Each substrate-species pair requires unique metabolic processes given the difference in physical structures of substrates and in maximal yield coefficients (Enouy, Walton, et al., 2021).

Each substrate depletion curve and biomass growth curve give certain conclusions about the model's representation, and how best the numerical formulation fits. The benzene depletion and biomass growth curves [Figure 7] conclude that the constant reaction model is a better representation of substrate flux at the microbial surface, which then sustains the internal

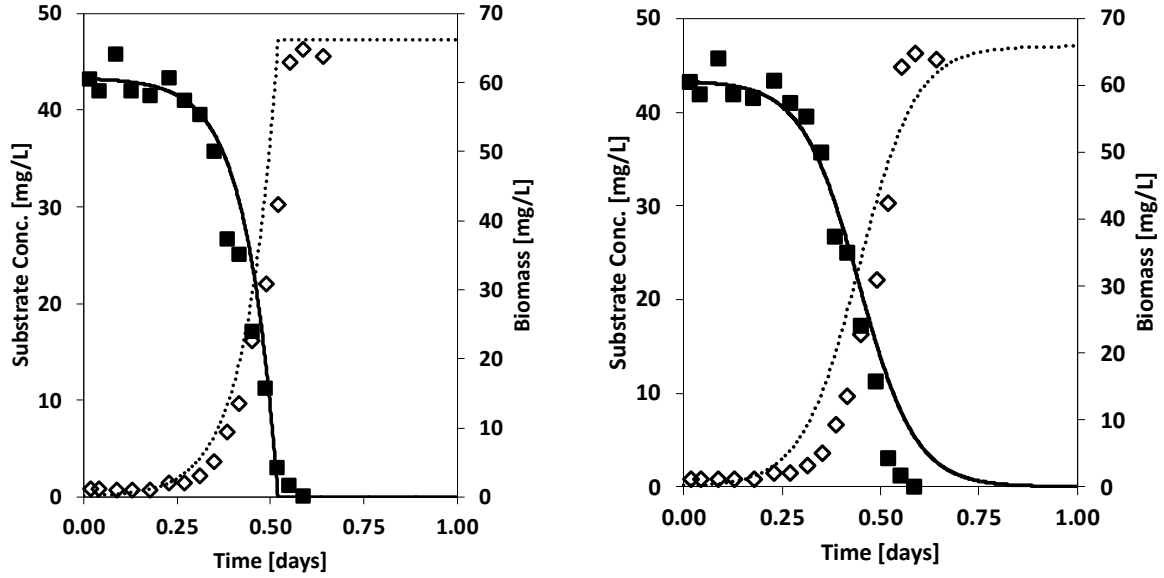

Figure 7: Benzene depletion and Biomass growth Simulation - (a) Benzene depletion curve (solid line) for the constant reaction rate model, with corresponding biomass growth curve (dotted line); (b) Benzene depletion curve (solid line) for the linear reaction rate model, with corresponding biomass growth curve (dotted line). Squares and diamonds represent Reardon et al. (2000) experimental substrate depletion and biomass growth respectively.

bioreaction process (Enouy, Walton, et al., 2021). Since the normalised surface concentration can be approximate unity throughout the depletion curve, K_s and μ_{max} can be considered constants (following the conventional Monod formulation), which implies transport processes are more than the reaction rate throughout the biodegradation process (Enouy, Walton, et al., 2021). Once the substrate is nearly depleted, the macroscopic transport rate becomes the limiting process, restricting the reaction rate to match the diffusive transport rate in the aqueous phase (Enouy, Walton, et al., 2021). For the toluene depletion [Figure 8], the constant reaction model is also a better representation of substrate flux. Normalised surface concentration is approximately constant and unity throughout the biodegradation process, keeping both K_s and μ_{max} as constants, again in keeping with conventional Monod formulation (Enouy, Walton, et al., 2021). Of the phenol depletion and biomass growth curves [Figure 9], simulations were conducted with constraint on growth coefficients to be consistent with the other curves. However, the linear reaction model appears to be the better representation of substrate flux at the microbial surface, therefore K_s and μ_{max} are dependent on bulk substrate concentration (Enouy, Walton, et al., 2021). Phenol and *P. putida* F1 as a pair deviate from the conventional

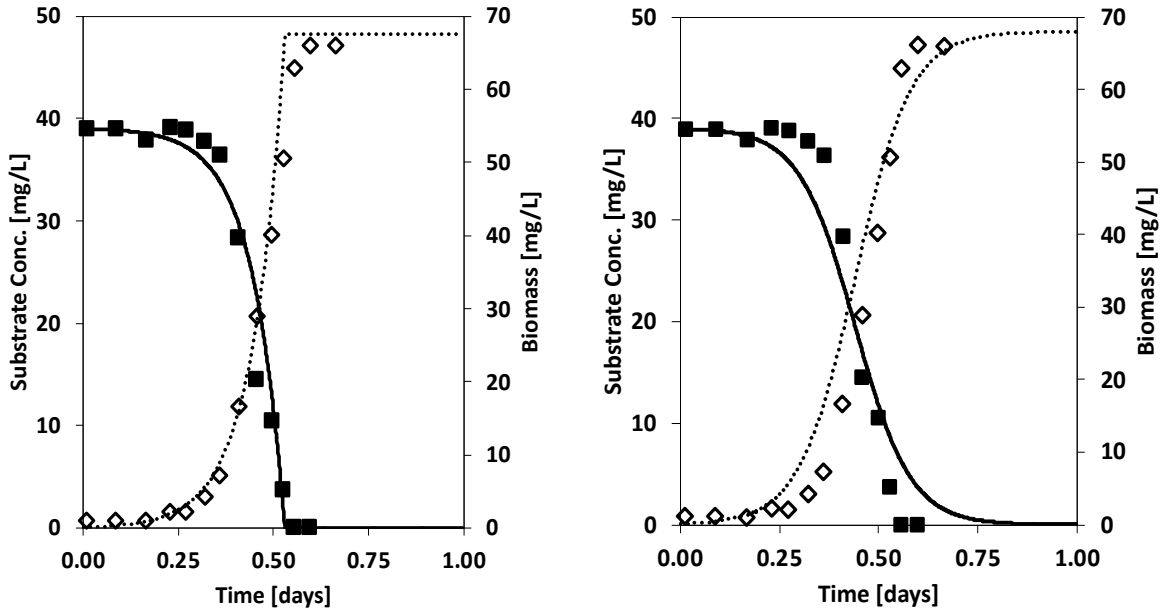

Figure 8: Toluene depletion and Biomass growth Simulation - (a) Toluene depletion curve (solid line) for the constant reaction rate model, with corresponding biomass growth curve (dotted line); (b) Toluene depletion curve (solid line) for the linear reaction rate model, with corresponding biomass growth curve (dotted line). Squares and diamonds represent Reardon et al. (2000) experimental substrate depletion and biomass growth respectively.

Monod formulation (which assumes K_s and μ_{max} are constant). Reardon et al. (2000) suggests the presence of an intermediary daughter product as the reason for model deviation from experimental results (at about t+2 d) and inferred that the degradation of the daughter product may compete with phenol, reducing biomass yield. Enouy, Walton, et al. (2021) hypothesize that the hydroxyl group on the phenol aromatic ring may inhibit or limit transmembrane transport, causing a low efficiency biodegradation due to the orientation of the molecule being crucial for contact with the transport site for uptake, since uptake transport plays a key role in the overall biodegradation rate. However, toluene also has a side group, though it is a methyl group (-CH_3), so perhaps the presence of a hydroxyl group (-OH) makes a difference compared to a methyl group. The linear reaction rate model is superior in capturing this phenomenon (Enouy, Walton, et al., 2021).

The costs of biodegradation and synthesis are determined by Button (1998) to be directly reflected in the transport and metabolic process kinetics. These combine to characterise cell interactions with the environment (Enouy, Walton, et al., 2021). Therefore, testing the key

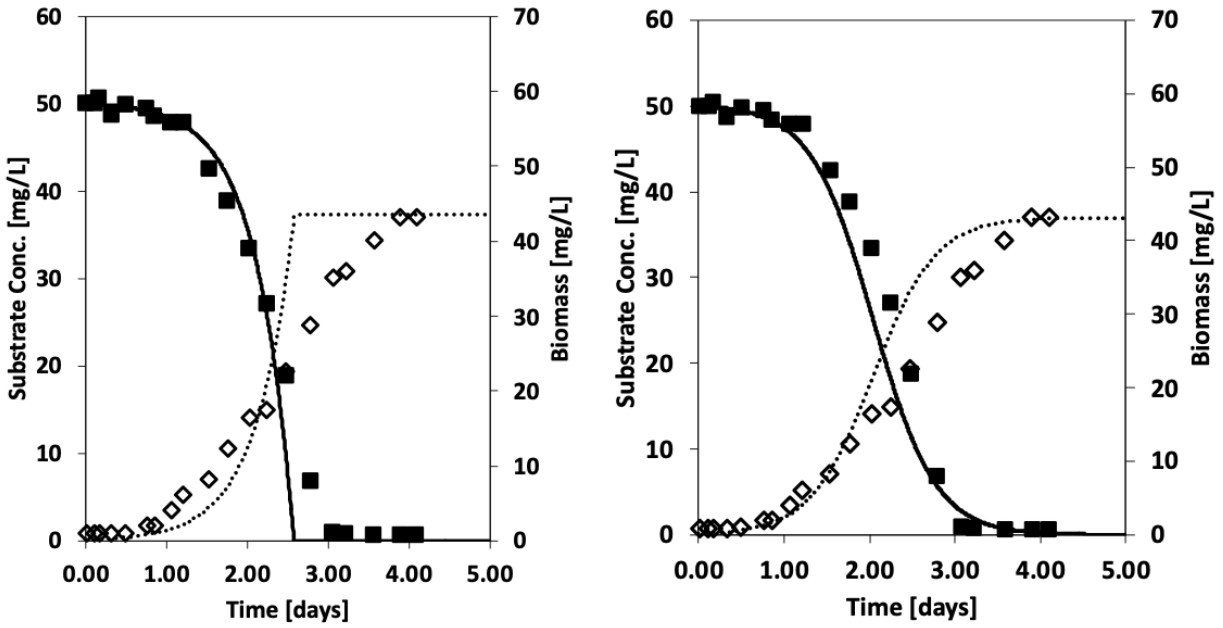

Figure 9: Phenol depletion and Biomass growth Simulation - (a) Phenol depletion curve (solid line) for the constant reaction rate model, with corresponding biomass growth curve (dotted line); (b) Phenol depletion curve (solid line) for the linear reaction rate model, with corresponding biomass growth curve (dotted line). Squares and diamonds represent Reardon et al. (2000) experimental substrate depletion and biomass growth respectively.

characteristics of known hydrocarbon-degrader species is essential for developing a model to include multiple biomass phases. However, often experiments use a mixture of different microorganisms without including observations about the various contributions of each species (Burland & Edwards, 1999; Edwards & Grbić-Galić, 1994; Schirmer et al., 1999; Rochman et al., 2017). Soil microbial communities often work in tandem, supporting each other, and creating symbiotic relationships, and there are often multiple substrates present in environmental spills, such that there is a preferential order of treatment by the microbial consortium, or that different members of the consortium maybe active under different circumstances (e.g. substrate concentrations, electron acceptor availability and concentrations, temperature, pH) (Reardon et al., 2000).

Regarding cellular transport and uptake, though there are various theories about the mechanisms involved (passive versus active transport, involvement of biosurfactants and exoenzymes), there is not a lot of clarity and precision, especially due to lack of efficient and careful measurement tools (Hua & Wang, 2014; Jin et al., 2017). Since uptake is the first

bottleneck in the biodegradation process within the microbe itself, it is essential to understand the various mechanisms, and how they may differ in varying environmental conditions. Hua & Wang (2014) state that the basic features of biosurfactants are well reported, as well as the uptake of dissolved droplets or uptake through direct contact to big oil droplets. They note that cell hydrophobicity is an important factor in influencing the physical contact between microorganisms and hydrocarbons, so increasing hydrophobicity could increase direct contact (Hua & Wang, 2014). However, further study is necessary especially in regards to how multiple uptake methods can occur at different times, when the microorganism is utilising specific hydrocarbons (Hua & Wang, 2014). And, though biosurfactants are necessary for uptake, since they make some hydrocarbons more bioavailable for the microorganism, there is also evidence of biosurfactants inhibiting biodegradation, so their overall effect requires more study (Hua & Wang, 2014; Kaczorek et al., 2018). Hua & Wang (2014) also state that there is limited research on the other key steps in how a hydrophobic hydrocarbon crosses the cell membrane, such that there are very few reports about the uptake of larger alkanes (C_{20}-C_{30}), and that active transport proteins are very poorly studied. Though there are some studies of OM proteins involved in transmembrane transport, this has mostly been focused on Gram-negative bacteria, leaving plenty of well-known hydrocarbons transported and biodegraded by Gram-positive microorganisms unstudied (Hua & Wang, 2014). Overall, further research is needed for a more detailed understanding of the molecular processes involved in the transmembrane transport of hydrocarbons, for it is a key step in the remediation of petroleum pollutions (Hua & Wang, 2014).

Enouy, Walton, et al. (2021) used the two scenarios of constant and linear rate models for describing the transmembrane transport. They appear very similar to the logarithmic and logistic kinetics curves described by Alexander (1985), who compares those two kinetics considerations with Monod, placing the Monod kinetics somewhat in the middle of these "extremes" [Figure 10]. So, the derivation of Monod by Enouy, Walton, et al. (2021) shows that Monod kinetics can be used to mimic logarithmic or logistic kinetics. This implies that other

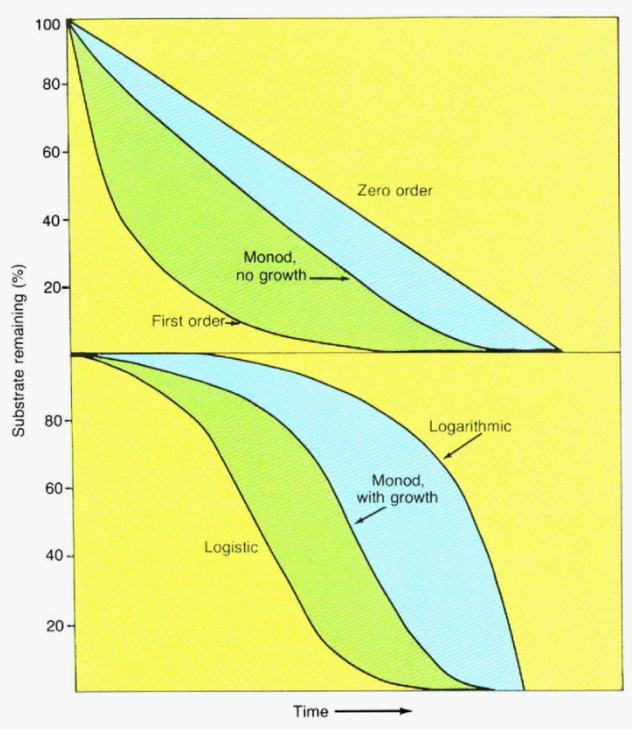

Figure 10: Disappearance curves for chemicals that are mineralised as related to individual kinetic models from Alexander (1985).

substrate-species may be modelled with curves that deviate from Alexander (1985), and that transport considerations are essential to such models of microbial biodegradation.

Regarding storage capacity, though there has been knowledge of a microbial ability to store energy in the form of lipid inclusions for decades, there are still details unknown. Current lab techniques make it difficult to visualise the formation of intracellular lipid inclusions (Jin et al., 2017). Measuring the metabolic capacity of lipid inclusions can either be invasive and destructive to cells, or else somewhat inaccurate based on the presumed lipid utilised as storage molecules in the method of calorimetry (Boe & Lovrien, 1990). Until better methods are found, or current methods are improved upon, it remains difficult to answer questions regarding the synthesis and regulation of lipid inclusions, the role of enzymes in their synthesis and usage, and other regulatory mechanistic details such as size and shape (Wältermann & Steinbüchel, 2005). Since there is also evidence of non-hydrocarbon-degrading microorganisms accumulating hydrocarbons, there needs to be greater understanding of the differences between general accumulation and its purpose, versus accumulation as an energy store (Jin et al., 2017). Especially since microorganisms are a fundamental component of the biosphere, and microbial

carriers can enhance the transfer of HOCs by two orders of magnitude resulting in bioaccumulation and biotransformation which affects HOCs' mobility and bioavailability (Jin et al., 2017). Jin et al. (2017) presented the first study of a single-cell visualisation of HOC accumulation. Further investigations of these mechanisms are required to build upon the current understanding of lipid inclusions as energy storage.

In Figures 7 and 8, the biomass growth curve overshoots in the constant rate model, and for the linear rate model, begins too quickly and then slows down too much (Enouy, Walton, et al., 2021). The benzene degradation curve is slower in the linear rate model, and the toluene degradation curve begins too slowly in both the constant and linear rate models, and then speeds up too much in the linear rate model (Enouy, Walton, et al., 2021). This could be due to limitations in transport processes either not yet identified or not incorporated into the numerical model, or due to storage capabilities of *P. putida* F1 that would slow down metabolic processes and subsequent growth. In Figure 9, the phenol degradation curves undershoot in both rate models, and the biomass growth curves deviate greatly from the actual growth. This could be due to transport difficulties, as Enouy, Walton, et al. (2021) hypothesizes, or again storage capabilities, toxicity, or slower metabolic reaction rates on the part of *P. putida* F1. Future investigations into the processes of this species, and general microbial biodegradation processes are suggested to further advance the support of models like this.

There is a very comprehensive understanding of the metabolic processes involved in both aerobic and anaerobic biodegradation of hydrocarbons, including the main metabolic pathways, how certain groups of hydrocarbons funnel into these pathways, and all the major steps of energy production (Parker et al., 2018; Carmona et al., 2009; Gaudy, Jr. & Gaudy, 1980). However, for the purposes of verifying a numerical model that involves these processes, it is necessary for laboratory experiments to have a modular mentality in terms of what is measured before, during, and after the experiment. Many publications presenting a biodegradation experiment often lack important data points for the purpose of verifying a hydrocarbon-degrading numerical model. For the conceptualisations to gain meaningful support for the Monod coefficients, it is necessary to have a modular approach. Several types of data need to be

measured: electron donor and acceptor depletion over time, biomass change over time, and preferably a measure of the production of outputs or waste products. Many papers measure the depletion of the electron donor, as it is degraded over the course of an experiment (Dou et al., 2010; van der Waals et al., 2018), but fewer include measures of biomass growth, or any sort of output or waste production (some include biomass as one measure at the beginning and one at the end, such as Schirmer et al. (1999); some included regular measure throughout the course of the experiment, such as Reardon et al. (2000)). The ideal for determining a maximal energy production and degradation capacity is to include as many of these measurements as possible, allowing for a calculation of the amount of moles consumed to yield a determinate amount of energy. These measurements are necessary to parameterise the model, for it to be able to represent specific species' physical growth characteristics, in order to then be capable of representing the physical processes especially for future developments, such as incorporating the interactions between multiple ubiquitous soil biomasses, or a storage component, particularly since it is also observed that microbes have a clear preference for the easiest-to-degrade substrate, and are often found in symbiotic multi-species consortia. Further investigations of these interactions are necessary to understand how bacteria and archaea are involved in multi-step degradation processes, and how to encourage a microbe to utilise a particular substrate *in situ*.

In the Monod derivation from Enouy, Walton, et al. (2021), the interior bioreaction stage was considered to be a zeroth-order reaction. Consequently, the reaction process remains constant until one of aqueous phase diffusive transport or transmembrane transport processes becomes limiting (such as when oxygen is depleted). This simple mathematical concept allowed for the reproduction of Monod to fit the Reardon et al. (2000) data sets, especially if regarding the mass transport across the microbial membrane to be the limiting process: if there is no substrate, there can be no reaction. However, this assumption may not be correct, or may not always be the case. The transport rate may be very fast, but the interior metabolism may be slower making it the controller of the overall reaction rate. Other substrate-species pairs may be better modelled with first-order reaction kinetics within the volume of a microbe, where the reaction rate is a function of some limiting nutrient, or non-constant zero-order kinetics, where

the reaction rate is dependent not on a reactant concentration, but on some other step in the entire process, such as the transport rate, or the exterior environmental conditions. A single microbe has many particular conditions and nutrient requirements for optimal growth, such as temperature, pH, water activity, oxygen presence and concentration, and sources of energy (Parker et al., 2018). And a soil microbiota usually contains multiple species, living symbiotically, and each separate species may be necessary for completing a different portion of biodegradation process. So, though a zero-order reaction could represent a single substrate-species degradation curve, there is much more complexity in upscaling to a multi-substrate multi-species model that would require dividing the entire process into more stages.

Chapter 5 – Conclusion

For the purpose of gaining insight into its mechanistic basis, a derivation of the Monod bioreaction equation was presented by Enouy, Walton, et al. (2021) as a special case of aqueous phase diffusive transport with series of three stages: transport rate of a substrate onto the surface of a microbe, uptake rate by the microbe across the microbial membrane, and a rate of interior degradation and energy production by the microbe, with zeroth-order kinetics. Two scenarios were included: a constant rate model, with constant transport flux, independent of bulk substrate concentration; and a linear rate model, with constant transport velocity, dependent on bulk substrate concentration. To parameterise the derivation, the model was used to fit single-substrate depletion of benzene, toluene, and phenol, and biomass growth curve from Reardon et al. (2000). This simplistic serial derivation of Monod fit the Reardon et al. (2000) data quite well for the benzene and toluene data with the constant rate model, with some derivation of the depletion curve from the data in the linear rate model, and more derivation in both biomass growth curves in both models. The phenol data was less successfully fit in both models, but the linear rate model was the more successful. The biomass growth curve however was very different from the data in both models.

A literature review of the biological processes involved in the biodegradation of aromatic hydrocarbons was presented, including the following steps: cellular transport and uptake, energy storage, aerobic and anaerobic biodegradation and metabolism, and cellular growth. The aim in exploring these processes was to support the Monod derivation, as well as suggest ways of improving the fit of experimental data by including other processes as part of the serial stages assumed in the model. The model only included three stages, which involved cellular transport and interior metabolism. However, to improve the fit of experimental data, suggestions include cellular transport limitations regarding passive versus active transport, the inclusion of a storage component, breaking up the interior reaction rate to reflect the complexity of the metabolic system, and including variations in cellular growth speeds as the environmental conditions may fluctuate. Further investigations with a modelling mindset into the understanding of these biological processes is suggested to improve the capabilities of modelling biodegradation systems.

Bibliography

Aburto, A., & Ball, A. S. (2009). Bacterial Population Dynamics and Separation of Active Degraders by Stable Isotope Probing During Benzene Degradation in a BTEX-Impacted Aquifer. *Revista Internacional de Contaminación Ambiental*, *25*(3), 147–156.

Adams, G. O., Fufeyin, P. T., Okoro, S. E., & Ehinomen, I. (2015). Bioremediation, Biostimulation and Bioaugmention: A Review. *International Journal of Environmental Bioremediation & Biodegradation*, *3*(1), 28–39. https://doi.org/10.12691/ijebb-3-1-5

Alexander, M. (1985). Biodegradation of Organic Chemicals. *Environmental Science & Technology*, *19*(2), 106–111. https://doi.org/10.1021/es00132a602

Alexander, M. (1999). *Biodegradation and Bioremediation* (Second Edition). Academic Press.

Bezza, F. A., & Chirwa, E. M. N. (2017). The Role of Lipopeptide Biosurfactant on Microbial Remediation of Aged Polycyclic Aromatic Hydrocarbons (PAHs)-Contaminated Soil. *Chemical Engineering Journal*, *309*, 563–576. https://doi.org/10.1016/j.cej.2016.10.055

Boe, I. N., & Lovrien, R. E. (1990). Energy Reserves and Storage Polymers in Intact Bacteria Analyzed by Metabolic Calorimetry. *Thermochimica Acta*, *172*, 115–122. https://doi.org/10.1016/0040-6031(90)80565-G

Bonora, M., Patergnani, S., Rimessi, A., De Marchi, E., Suski, J. M., Bononi, A., Giorgi, C., Marchi, S., Missiroli, S., Poletti, F., Wieckowski, M. R., & Pinton, P. (2012). ATP Synthesis and Storage. *Purinergic Signalling*, *8*(3), 343–357. https://doi.org/10.1007/s11302-012-9305-8

Burland, S. M., & Edwards, E. A. (1999). Anaerobic Benzene Biodegradation Linked to Nitrate Reduction. *Applied and Environmental Microbiology*, *65*(2), 529–533. https://doi.org/10.1128/AEM.65.2.529-533.1999

Button, D. K. (1998). Nutrient Uptake by Microorganisms According to Kinetic Parameters from Theory as Related to Cytoarchitecture. *Microbiology and Molecular Biology Reviews*, *62*(3), 636–645. https://doi.org/10.1128/MMBR.62.3.636-645.1998

Carmona, M., Zamarro, M. T., Blázquez, B., Durante-Rodríguez, G., Juárez, J. F., Valderrama, J. A., Barragán, M. J. L., García, J. L., & Díaz, E. (2009). Anaerobic Catabolism of Aromatic Compounds: A Genetic and Genomic View. *Microbiology and Molecular Biology Reviews*, *73*(1), 71–133. https://doi.org/10.1128/MMBR.00021-08

Chakraborty, R., & Coates, J. D. (2004). Anaerobic Degradation of Monoaromatic Hydrocarbons. *Applied Microbiology and Biotechnology*, *64*(4), 437–446. https://doi.org/10.1007/s00253-003-1526-x

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., & Wang, J. (2010). Anaerobic Benzene Biodegradation by a Pure Bacterial Culture of *Bacillus cereus* under Nitrate Reducing Conditions. *Journal of Environmental Sciences*, *22*(5), 709–715. https://doi.org/10.1016/S1001-0742(09)60167-4

Edwards, E. A., & Grbić-Galić, D. (1992). Complete Mineralization of Benzene by Aquifer Microorganisms under Strictly Anaerobic Conditions. *Applied and Environmental Microbiology*, *58*(8), 2663–2666. https://doi.org/10.1128/AEM.58.8.2663-2666.1992

Edwards, E. A., & Grbić-Galić, D. (1994). Anaerobic Degradation of Toluene and o-Xylene by a Methanogenic Consortium. *Applied and Environmental Microbiology*, *60*(1), 10.

Edwards, E. A., Wills, L. E., Reinhard, M., & Grbić-Galić, D. (1992). Anaerobic Degradation of Toluene and Xylene by Aquifer Microorganisms under Sulfate-Reducing Conditions. *Applied and Environmental Microbiology*, *58*(3), 794–800. https://doi.org/10.1128/AEM.58.3.794-800.1992

Enouy, R. W., Malton, I. I., Walton, K. M., Ioannidis, M. A., & Unger, A. J. A. (2021). *A Numerical Model for Simulating Cell Growth and Biodegradation Rates of Microbes Utilizing Dissolved Hydrocarbons*. [Unpublished manuscript].

Enouy, R. W., Walton, K. M., Malton, I. I., Kanwartej, S. S., Sihota, N. N., Daniels, E. J., & Unger, A. J. A. (2021). *A Mechanistic Derivation of the Monod Bioreaction Equation For a Limiting Substrate*. [Manuscript submitted for publication]. Earth and Environmental Science, University of Waterloo.

Fritsche, W., & Hofrichter, M. (2000). Aerobic Degradation by Microorganisms. In J. Klein (Ed.), *Biotechnology: Environmental Processes II Soil Decontamination* (2nd ed., Vol. 11b, pp. 145–155). Wiley-VCH.

Gaudy, Jr., A. F., & Gaudy, E. T. (1980). *Microbiology for Environmental Scientists and Engineers*. McGraw-Hill, Inc.

Harayama, S., Kasai, Y., & Hara, A. (2004). Microbial Communities in Oil-Contaminated Seawater. *Current Opinion in Biotechnology*, *15*(3), 205–214. https://doi.org/10.1016/j.copbio.2004.04.002

Hearn, E. M., Patel, D. R., Lepore, B. W., Indic, M., & van den Berg, B. (2009). Transmembrane Passage of Hydrophobic Compounds through a Protein Channel Wall. *Nature*, *458*(7236), 367–370. https://doi.org/10.1038/nature07678

Hearn, E. M., Patel, D. R., & van den Berg, B. (2008). Outer-Membrane Transport of Aromatic Hydrocarbons as a First Step in Biodegradation. *Proceedings of the National Academy of Sciences*, *105*(25), 8601–8606. https://doi.org/10.1073/pnas.0801264105

Heijnen, J. J., & Romein, B. (1995). Derivation of Kinetic Equations for Growth on Single Substrates Based on General Properties of a Simple Metabolic Network. *Biotechnology Progress*, *11*(6), 712–716. https://doi.org/10.1021/bp00036a018

Hoseinabadi, A., Rasooli, I., & Taran, M. (2015). Isolation and Identification of Poly β-Hydroxybutyrate Over-Producing Bacteria and Optimization of Production Medium. *Jundishapur Journal of Microbiology*, *8*(7). https://doi.org/10.5812/jjm.16965v2

Hua, F., & Wang, H. Q. (2014). Uptake and Trans-Membrane Transport of Petroleum Hydrocarbons by Microorganisms. *Biotechnology & Biotechnological Equipment*, *28*(2), 165–175. https://doi.org/10.1080/13102818.2014.906136

Jiménez, J. I., Miñambres, B., García, J. L., & Diaz, E. (2004). Genomic Insights in the Metabolism of Aromatic Compounds in *Pseudomonas*. In J. L. Ramos (Ed.), *Pseudomonas – Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism* (pp. 425–462). Kluwer Academic / Plenum Publisher.

Jin, Q., & Bethke, C. M. (2003). A New Rate Law Describing Microbial Respiration. *Applied and Environmental Microbiology*, *69*(4), 2340–2348. https://doi.org/10.1128/AEM.69.4.2340-2348.2003

Jin, X., Guo, X., Xu, D., Zhao, Y., Xia, X., & Bai, F. (2017). Single-Cell Real-Time Visualization and Quantification of Perylene Bioaccumulation in Microorganisms. *Environmental Science & Technology*, *51*(11), 6211–6219. https://doi.org/10.1021/acs.est.7b02070

Kaczorek, E., Pacholak, A., Zdarta, A., & Smułek, W. (2018). The Impact of Biosurfactants on Microbial Cell Properties Leading to Hydrocarbon Bioavailability Increase. *Colloids and Interfaces*, *2*(3), 35. https://doi.org/10.3390/colloids2030035

Ladino-Orjuela, G., Gomes, E., da Silva, R., Salt, C., & Parsons, J. R. (2016). Metabolic Pathways for Degradation of Aromatic Hydrocarbons by Bacteria. In W. P. de Voogt (Ed.), *Reviews of Environmental Contamination and Toxicology Volume 237* (Vol. 237, pp. 105–121). Springer International Publishing. https://doi.org/10.1007/978-3-319-23573-8_5

Liu, Y. (2006). A Simple Thermodynamic Approach for Derivation of a General Monod Equation for Microbial growth. *Biochemical Engineering Journal*, *31*(1), 102–105. https://doi.org/10.1016/j.bej.2006.05.022

Liu, Y. (2007). Overview of Some Theoretical Approaches for Derivation of the Monod Equation. *Applied Microbiology and Biotechnology*, *73*(6), 1241–1250. https://doi.org/10.1007/s00253-006-0717-7

Merchuk, J. C., & Asenjo, J. A. (1995). The Monod Equation and Mass Transfer. *Biotechnology and Bioengineering*, *45*(1), 91–94. https://doi.org/10.1002/bit.260450113

Moat, A. G., Foster, J. W., & Spector, M. P. (2002). *Microbial Physiology* (4th Ed.). Wiley-Liss, Inc.

Monod, J. (1949). The Growth of Bacterial Cultures. *Annual Review of Microbiology*, *3*, 371–394. https://doi.org/10.1146/annurev.mi.03.100149.002103

Neilson, A. H. (1994). *Organic Chemicals: An Environmental Perspective*. Lewis Publishers.

Parker, N., Schneegurt, M., Tu, A.-H. T., Forster, B. M., & Lister, P. (2018). *Microbiology*. Rice University.

Reardon, K. F., Mosteller, D. C., & Rogers, J. D. B. (2000). Biodegradation Kinetics of Benzene, Toluene, and Phenol as Single and Mixed Substrates for *Pseudomonas putida* F. *Biotechnology and Bioengineering*, *69*(4), 16.

Rochman, F. F., Sheremet, A., Tamas, I., Saidi-Mehrabad, A., Kim, J.-J., Dong, X., Sensen, C. W., Gieg, L. M., & Dunfield, P. F. (2017). Benzene and Naphthalene Degrading Bacterial Communities in an Oil Sands Tailings Pond. *Frontiers in Microbiology*, *8*, 1845. https://doi.org/10.3389/fmicb.2017.01845

Schäfer, D., Schäfer, W., & Kinzelbach, W. (1998). Simulation of Reactive Processes Related to Biodegradation in Aquifers 1. Structure of the Three-Dimensional Reactive Transport Model. *Journal of Contaminant Hydrology, 31*(1998), 167-186.

Schirmer, M., Butler, B. J., Roy, J. W., Frind, E. O., & Barker, J. F. (1999). A Relative-Least-Squares Technique to Determine Unique Monod Kinetic Parameters of BTEX Compounds using Batch Experiments. *Journal of Contaminant Hydrology*, *37*(1–2), 69–86. https://doi.org/10.1016/S0169-7722(98)00159-4

Stringfellow, W.T., Alvarez-Cohen, L. (1999). Evaluating the Relationship Between the Sorption of PAHs to Bacterial Biomass and Biodegradation. *Water Research, 33*(11), 2535-2544.

Tan, Y., Wang, Z.-X., Schneider, R. P., & Marshall, K. C. (1994). Modelling Microbial Growth: A Statistical Thermodynamic Approach. *Journal of Biotechnology*, *32*(2), 97–106. https://doi.org/10.1016/0168-1656(94)90173-2

van den Berg, B., Bhamidimarri, S. P., & Winterhalter, M. (2015). Crystal Structure of a COG4313 Outer Membrane Channel. *Scientific Reports*, *5*(1), 11927. https://doi.org/10.1038/srep11927

van der Waals, M. J., Pijls, C., Sinke, A. J. C., Langenhoff, A. A. M., Smidt, H., & Gerritse, J. (2018). Anaerobic Degradation of a Mixture of MtBE, EtBE, TBA, and Benzene under Different Redox Conditions. *Applied Microbiology and Biotechnology*, *102*(7), 3387–3397. https://doi.org/10.1007/s00253-018-8853-4

van der Zaan, B. M., Saia, F. T., Stams, A. J. M., Plugge, C. M., de Vos, W. M., Smidt, H., Langenhoff, A. A. M., & Gerritse, J. (2012). Anaerobic Benzene Degradation under Denitrifying Conditions: *Peptococcaceae* as Dominant Benzene Degraders and Evidence for a Syntrophic Process: Anaerobic Benzene Degradation. *Environmental Microbiology*, *14*(5), 1171–1181. https://doi.org/10.1111/j.1462-2920.2012.02697.x

Wältermann, M., & Steinbüchel, A. (2005). Neutral Lipid Bodies in Prokaryotes: Recent Insights into Structure, Formation, and Relationship to Eukaryotic Lipid Depots. *Journal of Bacteriology*, *187*(11), 3607–3619. https://doi.org/10.1128/JB.187.11.3607-3619.2005

Westerhoff, H. V., Lolkema, J. S., Otto, R., & Hellingwerf, K. J. (1982). Thermodynamics of Growth Non-Equilibrium Thermodynamics of Bacterial Growth the Phenomenological

and the Mosaic Approach. *Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics*, *683*(3–4), 181–220. https://doi.org/10.1016/0304-4173(82)90001-5

Wilkinson, J. F. (1963). Carbon and Energy Storage in Bacteria. *Journal of General Microbiology*, *32*(2), 171–176. https://doi.org/10.1099/00221287-32-2-171

Zhou, J., Liu, L., Shi, Z., Du, G., & Chen, J. (2009). ATP in Current Biotechnology: Regulation, Applications and Perspectives. *Biotechnology Advances*, *27*(1), 94–101. https://doi.org/10.1016/j.biotechadv.2008.10.005