Microbial Ecology of Methanogenic Sludge Granulation and its Application in the Reduction of Selenate and Selenite

A thesis submitted in partial fulfilment of the requirements for

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by

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Publications

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Publications in Review & Preparation

Methanogenic granule growth and development is a continual process characterized by distinct morphological features - Submitted to *The Journal of Environmental Management*

A Distinct Microbial Community is Involved in the Formation of Methanogenic Granules - Submitted to *Water Research*

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1st International Congress on Metals in Anaerobic Biotechnologies (2017). *Selenium-reducing organisms in anaerobic granular sludge.*

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Poster Presentations

International Society for Microbial Ecology (2018). *Selenium respiration in anaerobic sludge granules reveals different taxa reduce selenate and selenite*

Anaerobic Digestion 16 (2019) *Size-driven life-cycle of formation and maintenance of methanogenic granules*

Abstract

Up-flow anaerobic bioreactors are widely applied for high-rate treatment of industrial wastewaters and rely on formation and retention, of dense well settling biomass in the form of methanogenic granules (approx. 0.5-4.0 mm in diameter). Mechanisms of granule formation (granulation) have been proposed previously, but an ecological understanding of granulation is lacking. Additionally, much of the current granulation research examines the start-up phase of bioreactor operation only, rather than monitoring on-going granulation, over time. However, this thesis used laboratory-scale bioreactors inoculated with size-constrained granules (four bioreactors inoculated with small granules, four with large granules, and four with a full complement of naturally-size-distributed granules) to follow new granule formation, maturation, disintegration and re-formation. Constrained granule size profiles shifted toward the natural distribution, which was associated with maximal bioreactor performance. Temporal size profiles, volatile solids content, settling velocity, and ultrastructure of granules were determined. Distinct morphological features characterized different granule sizes and biofilm development stages, including 'young', 'juvenile', 'mature' and 'old' were proposed. 16S rRNA gene sequencing was used to identify a distinct "pre-granular" microbial community, with a high proportion of acidogenic organisms such as the *Streptococcaceae*, which was determined to be important for reactor operation and granule formation. This flocculent, acidogenic community may constitute primary consumers during community succession before making way for syntrophic and methanogenic organisms in newly formed granular biomass. Environmental pressure increased with granule size indicating deterministic community assembly. Cycles of granule growth and breakage may have led to divergent community evolution within individual granules and overall diversification in the meta-community of the bioreactors. The findings offer opportunities toward optimizing management

of high-rate, anaerobic digesters and provide a new ecological perspective on methanogenic sludge granulation.

The sheer metabolic diversity, which is inherent in granular sludge means that it is not only used for removal of organic compounds. Anaerobic ammonia oxidation (ANAMMOX) granules have also been applied for nitrogen removal and methanogenic granules have been applied to treat wastewaters laden with toxic selenium oxyanions. Selenium (Se) exists in a range of different oxidation states (+6, +4, 0, -2), with varying chemical properties. Selenium oxyanions – selenate and selenite – are bioavailable and toxic at higher concentrations and emission of selenium containing wastewaters from industries such as agriculture and mining has led to serious pollution incidents. Microorganisms capable of reducing soluble Se oxyanions are thought to be phylogenetically diverse and a deeper, and fuller, understanding of this phylogeny is required to exploit Se microbiology and develop new environmental biotechnologies. It is unclear whether microbial communities exposed to selenate or selenite might differ taxonomically. Enrichment cultures of anaerobic, granular sludge were set up with lactate and acetate as electron donors, and either selenate or selenite as electron acceptors. Microscopic observations revealed a diverse array of cell morphologies associated with selenium naonparticles. Microbial community dynamics were observed at each sub-culture using 16S rRNA gene amplicon sequencing and revealed distinct taxa associated with selenate and selenite reduction.

CHAPTER 1

Introduction and Scope of Thesis

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Introduction to Thesis

Growing populations and increasing levels of industrialization have put increasing pressure on wastewater treatment systems. Therefore, research into efficient wastewater treatment and resource recovery technologies is paramount. Wastewater treatment is primarily concerned with the removal of nutrients such as carbon, nitrogen and phosphorous, which can lead to eutrophication if they are discharged to surface waters. Traditional wastewater treatment techniques can be organized into three broad categories; physical, chemical or biological, all of which are typically applied to remove contaminants from wastewater prior to discharge. Physical treatment methods such as screening or sedimentation are usually applied first, with the goal of removing particulate matter. Biological wastewater treatment is usually applied as a secondary treatment and relies on complex microbial communities to remove organic contaminants from wastewaters by converting them into gaseous forms or additional microbial biomass (Grady et al., 2011). Tertiary treatments are often physical or chemical and include processes such as: filtration, reverse osmosis and ultraviolet-light oxidation (Plakas et al., 2015).

The development and application of renewable energy technology is also essential to facilitate sustainable economic growth. Since 2005 the EU has reduced its demand for fossil fuels by 12% and its greenhouse gas emissions by 10% (Capizzi et al., 2019). However, it is increasingly likely that Europe's climate goals for 2030, in relation to renewable energy and industrial emissions will not be achieved (European Environment Agency, 2019). Anaerobic Digestion (AD), has been established as a successful method of biological wastewater treatment and a renewable energy technology which is often applied to treat industrial wastewaters, laden with organic contaminants, in upflow anaerobic bioreactors (van Lier et al., 2015). Such bioreactors are underpinned by complex microbial communities which

sequentially breakdown organic molecules into simpler monomers and eventually biogas, consisting mainly of CH_4 and CO_2. Bioreactor performance is often dependent on the immobilization of the microbial communities inside, into spherical aggregates known as granules. Granules are dense and settleable, which allow high influent rates, without washout of essential microbial biomass. Granule formation (granulation) is also thought to facilitate syntrophic interactions between microbes and provide physiochemical gradients (e.g. oxygen) resulting in niche development, meaning that each granule contains all the necessary organisms for the complete mineralization of organic matter. Therefore, ecological interactions of microorganisms in actively forming and growing granules should be studied and included in any granulation mechanism.

Traditionally, microbiologists have studied microorganisms using culture-dependent techniques. However, it has been suggested that only 0.1-1% of all microorganisms can actually be cultured in laboratory conditions (Staley and Konopka, 1985). The development of culture independent techniques such as high throughput amplicon sequencing, targeting the 16S rRNA gene of bacteria and archaea was revolutionary for the field of microbiology and has allowed microbial ecology to be studied in situ, in a range of environments (Thompson et al., 2017). Vast amounts of amplicon sequencing data has now been generated on microbial communities in anaerobic digestion (McIlroy et al., 2017) but this has not provided concrete answers on ecological aspects of granulation. For example, the role of *Methanosaeta* species in initiating methanogenic granulation is well established (Wiegant et al., 1988), however, due to the complexity and syntrophic nature of methanogenic communities it is unlikely that one species is solely responsible for granulation. For example, the role of other trophic groups suges as fermenters or acidogens in granulation is unknown but could be essential in providing substrates for Methanogenic species such as *Methanosaeta.*

While granular microbial communities are most widely applied for methanogenic conversion of organic compounds into biogas, several other successful applications have been developed. Nitrogen removal with anammox granules or heterotrophic removal of organics coupled to phosphorous and nitrogen removal in aerobic granules are becoming more common (Milferstedt et al., 2017). The vast metabolic flexibility of the microbial communities in granular biomass means that it is not only applied for carbon, phosphorous and nitrogen removal, but it can also be used for other applications, including the removal of toxic compounds or for the recovery of valuable resources. Selenium oxyanions - selenate and selenite – can be present at toxic concentrations in wastewaters originating from several sources such as mining, coal fired power plants and agriculture. Several studies have assessed the effectiveness of methanogenic granular sludge for selenium removal. However, the microbial ecology of selenium reducing communities is not well studied, in particular how selenate and selenite reducing communities might differ from one another is unknown.

Scope of Thesis

This thesis attempts to address some of the knowledge gaps in the literature around methanogenic granulation. Much existing research focuses on granulation of flocculent biomass up to the point at which satisfactory granular biomass is obtained. However, granulation is a dynamic process, where continual granule formation, disintegration and reformation occurs throughout the period of bioreactor operation. In addition, much of the well cited literature on methanogenic granulation was published in the 1980s. While these articles provided a solid foundation for the area of granulation research much of the work was carried out prior to the advent of many culture independent techniques for microbial community analysis. Therefore, specifically designed bioreactor trials are required to capture data on actively, forming, growing and disintegrating granules, at different stages of development and microbial community analysis could provide significant advancements in the field of granulation research. This is precisely what this thesis attempts to achieve, through a novel lab-scale bioreactor trial outlined in Chapters 3 and 4. In addition, this thesis attempted to increase our understanding of selenate and selenite reducing communities in methanogenic granular sludge and obtain selenate and selenite reducing isolates from methanogenic granular sludge. Specifically, it was investigated whether the selenite reducing community constituted a sub-population of the selenate reducing community, since selenate reduction is generally believed to be a two-step mechanism where selenate is first reduced to selenite by anaerobic respiration and selenite is then reduced to elemental selenium.

Thesis Outline

Chapter 2

A comprehensive review of the literature relating to granulation of aerobic, anammox and methanogenic granular sludge granulation was carried out. This review had the specific goal of identifying unifying concepts in granulation theories across all sludge types and identifying areas which require further research in order to develop a comprehensive universal granulation mechanism.

Chapter 3

In Chapter 3 a unique bioreactor trial was specifically designed to study on-going granulation, in a stable system, rather than granulation from flocculent biomass during bioreactor start-up only. Size-constrained seed biomass, representing different stages of granule growth was used to inoculate 12 lab-scale bioreactors. Whole bioreactors were sacrificed sequentially to assess how granule size distributions evolved, relative to their size-constrained starting points. The impact of changing size distributions on performance was assessed and an in-depth physical characterisation of granules was carried out. A granule classification system, based on granule morphology was also proposed. It was hypothesized that granules of different sizes, representing stages of granule development, would be associated with defining physical characteristics and that granule size distributions in size-constrained bioreactors, would revert to the natural distribution, corresponding with maximal bioreactor performance.

Chapter 4

The size-separated granular biomass obtained in chapter three, which represented incremental stages of granule development, was analysed using 16S rRNA gene sequencing in order to understand microbial community dynamics in actively forming, growing and disintegrating granules. The “active” community was also assessed by sequencing complimentary DNA,

which was reverse transcribed from rRNA gene transcripts. It was hypothesized that distinct microbial communities would characterize developmental stages of granules and patterns of community succession would be apparent in growing granules.

Chapter 5

In Chapter 5 separate enrichments of methanogenic granular sludge, for selenate and selenite reducing microorganisms were carried out and changes in microbial community composition were monitored by 16S rRNA gene sequencing. Conversion of selenium oxyanions to elemental selenium nano-particles was confirmed by Transmission Electron Microscopy (TEM) and selenium content was monitored using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Isolation of selenium reducing organisms was attempted and a single isolate was obtained for further study. It was hypothesised that separate selenate and selenite reducing communities would form in each enrichment but that the selenite reducing community may constitute a sub-population of the selenate reducing community.

Chapter 6

Chapter 6 contains general findings, conclusions and future recommendations from the thesis

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CHAPTER 2

Unifying Concepts in Methanogenic, Aerobic and Anammox Sludge Granulation

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1 Introduction

A growing human population, and increasing industrialisation, along with the need for environmental sustainability, has led to a demand for efficient wastewater treatment systems. Biological treatment of wastewaters is widely applied and is generally considered to be "greener", with lower operational costs than physical/chemical treatment methods (Machineni, 2020). The removal of macronutrients such as carbon, nitrogen and phosphorous is of particular importance, in order to prevent eutrophication of surface waters and is generally carried out by microbial biomass in biological wastewater treatment systems. Thus the cultivation and retention of dense, well-functioning microbial biomass is essential. The most widely applied and successful biological treatment method is the activated sludge process which relies on the breakdown of organic contaminants by heterotrophic microorganisms (Sheik et al., 2014). Activated sludge is flocculent in nature and its cultivation requires aeration, this leads to some drawbacks, namely, large initial capital costs, energy requirements and land area requirements (van Loosdrecht and Brdjanovic, 2014). Granular bioreactors provide an alternative to the activated sludge process. In granular bioreactors, microbial biomass exists in the form of granules, small, self-immobilized, spherical, microbial aggregates, 0.5-4mm in size (Trego et al., 2020b). Granules are denser and more compact than activated sludge flocs and therefore granular bioreactors have a lower land area requirement and lower up-front capital costs. Settling velocities for granules range between 25m/h and 160m/h (Trego et al., 2020b) making them more settleable than activated sludge flocs (Liu et al., 2009), which typically have settling velocities of less than 2m/h (Dominiak et al., 2011; Feng et al., 2009; Schuler and Jang, 2007).This permits for hydraulic retention time to be uncoupled from biomass retention, in granular systems,allowing for high rate treatment of wastewaters.

Granules were first applied for anaerobic digestion (AD) in novel upflow anaerobic bioreactors such as the Upflow Anaerobic Sludge Blanket (UASB) (Lettinga et al., 1980). These reactors

utilize the naturally occurring, microbially-mediated AD pathway (McHugh et al., 2003) to convert organic contaminants in wastewaters to valuable biogas. Granular anaerobic bioreactors are now widely applied for the treatment of a variety of industrial wastewaters (van Lier et al., 2015) including brewery wastewater (Díaz et al., 2006a), dairy wastewater (Paulo et al., 2020) and sewage (Faria et al., 2019a) .

Aerobic granules are a more recent application of granular biomass (Mishima and Nakamura, 1991) and are similar to methanogenic granules. Aerobic granules are often used for heterotrophic removal of carbon from wastewater streams, coupled to nitrogen and phosphorous removal. Aerobic granules are typically applied in Sequencing Batch Reactors (SBRs), rather than the continuous systems applied for AD. However continuous operation of aerobic granular systems is an area of ongoing research (Li et al., 2019; Zou et al., 2018)

Anammox granular sludge is typically applied for ammonia removal from wastewaters. The anammox process involves the autotrophic conversion of ammonia to dinitrogen gas with nitrite as a terminal electron acceptor (Kartal et al., 2011) and is carried out by several genera of anammox bacteria (Manonmani and Joseph, 2018). The process is more energy efficient than traditional denitrification methods as less aeration is required and there is no requirement for the addition of external carbon sources (Kartal et al., 2010). However, anammox bacteria are very slow growing, with doubling times reported of up to two weeks (Kuenen, 2008), meaning that good biomass retention essential for good bioreactor performance, which can be achieved through the development of dense, stable anammox granules (Gonzalez-Gil et al., 2015). In contrast maximum specific growth rate for heterotrophs in activated sludge has been estimated as 6 day^{-1}.

The mechanisms by which different granule types form (granulation) have been extensively reviewed (Hulshoff Pol et al., 2004; Liu et al., 2003a; Manonmani and Joseph, 2018; Show et

al., 2020; Wilén et al., 2018). However, the various granule types are often considered in isolation and therefore, there is no universally accepted mechanism of granulation. The purpose of this chapter is to review the literature around granulation mechanisms of methanogenic, aerobic and anammox granules and identify commonalities, which may contribute to a unified granulation hypothesis. Other granule types such as photogranules (Abouhend et al., 2018) and hydrogenic granules (Milferstedt et al., 2017) have not been considered in this review as the volume of literature specific to granulation in these granule types is relatively small in comparison to methanogenic, aerobic and anammox granules.

2 Unifying Concepts in Granulation

2.1 Selection Pressure

The concept of selection pressure with regards to granulation refers to the continual selection of dense well settling sludge and washout of lighter sludge particles (Hulshoff Pol et al., 1983; Liu et al., 2005). In continuous, methanogenic bioreactors, selection pressure is mainly a function of liquid upflow velocity (V_{up}). In the UASB configuration V_{up} is entirely determined by influent rate or HRT, whereas V_{up} in EGSBs is a function of HRT and the recirculation rate. UASB reactors generally have V_{up} below 4m h^{-1} and are often operated below 1 m h^{-1} (Latif et al., 2011). Whereas EGSB reactors are typically operated at V_{up} of greater than 4m h^{-1} (Reino and Carrera, 2017). High V_{up} causes dispersed, light sludge flocs and particles to be washed out and heavier sludge to be retained, which then grows to form a well settling sludge bed (Hulshoff Pol et al., 1988). Increases of V_{up} and decreases of HRT (i.e. increasing selection pressure) has led to improved granulation on a number of occasions (Arcand et al., 1994; Noyola and Moreno, 1994; Wang et al., 2018; Xu et al., 2018). The anammox process is also generally conducted in continuous systems meaning that selection pressure is determined by upflow velocity. Increasing V_{up} and therefore selection pressure has been shown to correlate with increased granule size and stability in anammox granules (Ma et al., 2013; Reino and Carrera, 2017b; Shi et al., 2017) and lead to selective washout of smaller, less settleable biomass (Jin et al., 2012). However, much higher upflow velocities (>10 m h^{-1}) have been detrimental to reactor performance due to granule disintegration (Dries et al., 1998). However, due to the low growth rate of anammox biomass these reactors are generally operated under conditions of lower selection pressure, especially during start-up to avoid excessive washout (Manonmani and Joseph, 2018).

Since most aerobic granular systems are operated as SBR's, settling time and aeration rate rather than upflow velocity determine selection pressure. Settling time has been shown to be a

key factor in achieving fast aerobic granulation (Liu and Tay, 2015). Liu and Tay proposed a granulation mechanism whereby poorly settling flocs are washed out of reactors quickly due to high initial selection pressure. The remaining well settling sludge is then less concentrated and became overstressed by relatively high OLR biomass ratio. This stress, caused increased EPS production and changes in cell surface hydrophobicity, promoting granulation. Settling time has also been determined to be the main selection pressure in aerobic bubble column reactors (Liu et al., 2016) The causes (settling time or upflow velocity) and extent of selection pressure may vary with reactor and biomass type but the underlying principles remain the same; higher selection pressure, generally leads to better settling biomass and improved granulation.

2.2 Shear Force

Like selection pressure, shear force is essentially a function of reactor hydrodynamics, including liquid and gas upflow velocity. Strong shear forces have been shown to improve granulation in methanogenic bioreactors by stimulating the production of EPS rich in proteins (Wu et al., 2009). However, in the same study very strong or "violent" shear forces (12.42 s^{-1}) were shown to have a negative influence on granulation. Therefore, appropriate shear forces may vary, depending on the reactor conditions. Increased shear force has also been shown to cause an increase in the protein fraction of EPS in anammox granules and promote granulation (Tang et al., 2011). Numerous studies have also reported the rate of EPS production is dependent on shear force in aerobic granules (He et al., 2017; Tay and Liu, 2001; Zhu et al., 2012). Therefore, the influence of shear force on EPS production appears to be common among all granular sludge types. It may be the case that in response to the stress of increasing shear force, microorganisms respond by producing EPS in order to aid aggregation and remain in the reactor.

Liu and Tay (2002) emphasized the importance of hydrodynamic shear force in shaping granular sludge (aerobic and methanogenic) and provided a comprehensive 4 step mechanism

for granulation. It was proposed that the final step in the granulation process involved steady growth, where hydrodynamic shear forces in the reactor shape the three dimensional structure of the aggregate. A number of other studies have reported the role of shear force in shaping newly formed granules into dense spherical aggregates by preventing attachment of loose filamentous organisms on the granule surface (Chen et al., 2007; Kent et al., 2018; Zhang et al., 2016). Therefore, shear force may have an important role in the initial stages of granule formation (stimulation of EPS production) but also in the shaping of formed granules into dense settleable entities by removing filamentous growth at the granule surface.

2.3 Importance of EPS

Several studies state the importance of EPS for the formation of a matrix which facilitates granulation and provides structural stability in methanogenic (Wang et al., 2018), aerobic (Chen et al., 2019b) and anammox (Ni et al., 2015) granules.

Cui et al., (2014) proposed an aerobic granulation mechanism where heterotrophs grew quickly and established an EPS matrix as the basis for granule formation. Further aggregation of nitrifying organisms into the EPS matrix was then promoted by hydrophobic interactions between their cell surfaces and EPS (Cui et al., 2014; Kim et al., 2002). Increased cell-cell interaction and presence of an EPS support matrix along with external physical pressures (shear forces, etc.) then allowed formation of stable granules.

A comprehensive three-step theory on anammox granulation was proposed by Lin and Wang (2017) using a combination of microscopy techniques (SEM, TEM, CLSM) selective enzymatic hydrolysis and rheometry. The first step involves initial cell to cell contact of anammox bacteria followed by aggregation in a thin EPS matrix. Next, separate cell clusters aggregate together, along with other heterotrophic bacteria which consume the EPS secreted by anammox bacteria.

High protein content in EPS can increase cell surface hydrophobicity and facilitate better aggregation (Ding et al., 2015; Sheng et al., 2010). Xu et al (2018) proposed a granulation mechanism for methanogenic granules, which relied on a continually decreasing OLR and increasing V_{up} to stress the microbial community and stimulate EPS production, particularly proteins. The increased protein content of EPS resulted in greater cell surface hydrophobicity and therefore aggregation of cells into granules. Similar observations regarding increased protein content of EPS and granulation were seen in an Internal Circulation (IC) reactor treating leachate from a municipal waste incineration plant (Wang et al., 2018) and chitosan-supplemented reactors treating organic solvent containing wastewaters (Torres et al., 2018).

EPS protein content is also important for aerobic granule formation (Gao et al., 2011; Timothy R. Kent et al., 2018). Increases in the protein-polysaccharide ratio were shown to correlate with larger granular sludge and improved performance (Chen et al., 2019) was thought to play an important structural role (Lin and Wang, 2017).

A number of points are common among all EPS-based granulation theories; EPS production forms an initial matrix for the attachment of other cells and higher protein content is beneficial for granulation. Therefore, methods of stimulating EPS with a high protein content could be beneficial for granulation. As mentioned previously increasing shear forces has been shown to stimulate EPS production. Other environmental stresses can also have a similar effect such as starvation or feast-famine feeding (Corsino et al., 2017; Timothy R. Kent et al., 2018; Liu et al., 2005) and influent characteristics (Kodera et al., 2017; Lu et al., 2018). However, overproduction of EPS in response to stresses can also be detrimental to reactor performance by reducing granule settling velocity (Zhang et al., 2016), therefore additional research is required on the nature and extent of stressors required to adequately stimulate EPS production.

2.4 Microbial-Based Mechanisms

The idea that microorganisms form a matrix in which other cells can embed is common to all granule types. This may be via filamentous growth or production of an EPS matrix but there often appears to be one or several organisms present which are influential in initiating granulation. One widely accepted aspect of methanogenic granulation is the importance of filamentous *Methanosaeta* sp which are thought to provide a branched growth network within which, other cells can embed (Wiegant et al., 1988). Subsequent growth of the aggregate occurs via cellular multiplication and shear forces, caused by liquid and gas upflow, begin to shape the aggregate into a dense sphere (Hulshoff Pol et al., 2004; Liu et al., 2003b).

In contrast to methanogenic granules, no single organism has been universally found to dominate aerobic granules or suggested to be essentials for aerobic granulation (Trego et al., 2020b). This may be because they are often operated for multiple processes such as nitrogen, phosphorous and carbon removal, leading to diverse microbiomes which vary between reactors. Several EPS-producing bacteria have been suggested as important for aerobic granulation including; *Arcobacter, Aeromonas* and *Flavobacterium* (Fan et al., 2018); *Rhodanobacter* (Aqeel et al., 2016); *Thauera* and *Zoogloea* (Zou et al., 2019) and *Agrobacterium* (Gómez-Basurto et al., 2019). *Acintobacter sp* have also been suggested in multiple studies to facilitate initial aggregation of flocs into aerobic granules by acting as a "bridging" organism, through EPS production (Adav et al., 2008; Liébana et al., 2019; Malik et al., 2003)

Weissbrodt et al (2012) identified *Zoogloea sp* as being important for the formation of dense, well settling aerobic granules, whereas *Burkholderiales* were more predominant in "fluffy" slow settling granules in the same reactor. The same authors also identified *Candidatus Accumulibacter* and *Competibacter sp* as being important for aerobic granulation and proposed different mechanisms depending on the organism involved (Weissbrodt et al., 2013).

Candidatus Accumulibacter were also suggested to play a role in aerobic granulation by Barr et al (2010), who proposed an in depth granulation mechanism where white, dense, spherical granules were formed by flocs dominated by *Candidatus Accumulibacter*. Concurrently, yellow granules were also formed from smaller, more diverse micro-colonies resulting in larger more irregular granules. Fungi have even been suggested to play a role in aerobic granulation (Beun et al., 1999).

Temporal studies of anammox granular communities during the initial stages of granulation are lacking. Many studies have identified anammox bacteria, the Planctomycetes as the dominant organisms in anammox granules, with relative abundances as high as 80% (Cho et al., 2010). The Planctomycetes generally form an internal core suggesting that they may be involved in initiating granulation. However, Luo et al (2017) examined the microbial communities in size-resolved anammox granules and found that smaller granules (<1mm) were dominated by Ammonia Oxidising Bacteria (AOB), suggesting that they are important in the early stages of granule formation but become less relatively abundant as granules grow. Anammox bacteria, AOB and Nitrite Oxidising Bacteria (NOB), are typically the dominant groups in anammox granular sludge, however, *Chloroflexi* are also often found in relatively high abundance (Chen et al., 2019; Luo et al., 2017) and were proposed to have a role in maintaining granule structural integrity (Yang et al., 2018). More research is required, particularly on actively growing anammox granules in order to make stronger conclusions about which organisms initiate granulation.

Key to all of these granulation mechanisms is the presence of a key organism(s) at the outset of granule formation, often involved in production of a matrix of EPS or filamentous growth. While *Methanosaeta sp* are widely accepted to initiate methanogenic granulation, additional study is required to identify similar organisms in aerobic and anammox granules. However, it may be the case that no single organism is universally responsible for granulation in aerobic

systems due to the variety of operation strategies. It is also apparent EPS, rather than filamentous growth is more important for initiating granulation in aerobic and anammox granules than in methanogenic granules.

2.5 Layered Structure

Concentric layers are a common theme in many in-depth studies of granulation. A layered structure for methanogenic granules was first suggested by MacLeod et al (1990) and later by (Ahn, 2000) where aggregations of *Methanosaeta* sp formed a central core. Other functional groups such as fermentative bacteria, acidogens and acetogens are thought to form concentric layers around the archaeal core (Guiot et al., 1992b; Hulshoff Pol et al., 2004; McHugh et al., 2003). This theory was later corroborated in a number of studies using 16S rRNA-targeted Fluorescence In Situ Hybridization (FISH) (Collins et al., 2005; Sekiguchi et al., 1999; Tagawa et al., 2000).

Layers have also been observed in aerobic granules and it is generally accepted that aerobic granules contain aerobic outer layers, surrounding an anoxic middle layer with an anaerobic or inert core (Franca et al., 2018; Sarma et al., 2017; Tay et al., 2002). Lv et al (2014) carried out DGGE and high throughput amplicon sequencing on thin sections of aerobic granules and whole granules. The results revealed a core dominated by anaerobic organisms such as the *Rhodocyclaceae* family and an outer layer consisting of aerobic and anaerobic organisms such as *Microbacteriaceae*, *Sphingobacteriaceae*, and *Moraxellaceae* The spatial distribution of aerobic granules can be more complex than methanogenic granules as aerobic granular bioreactors are often operated for nitrogen or phosphorous removal along with Chemical Oxygen Demand (COD) removal. This leads to several functional groups of microorganisms carrying out separate processes in each granule. NOB and AOB are often located in the outer layer of aerobic granules, where oxygen is more readily available. Whereas denitrifying

bacteria and phosphate accumulating organisms are located in the anoxic layers (Nancharaiah and Kiran Kumar Reddy, 2018; Trego et al., 2020b; Winkler et al., 2015)

Vlaeminck et al. (2010) formed a comprehensive anammox granulation model based on the spatial organization of key anammox groups in three single stage OLAND reactors. In short, it was hypothesized that aggregation of single cells leads to the formation of flocks, which grow and develop an outer rim of aerobic ammonium oxidisers leading to an anoxic core of anammox bacteria (Vlaeminck et al., 2010). While there does often appear to be a layered structure in anammox granules, the anammox bacteria at the centre of the granule tend to be organised in "cell-cluster" type internal architecture which appears to be as a result of the aggregation of several smaller anammox granules into a larger unit, followed by subsequent concentric growth (Chen et al., 2019; Wang et al., 2020a). This is discussed in more detail in Section 2.8

The observation of similar concentric layer structures across all granule types implies that a similar formation mechanism may be occurring. Concentric growth suggests the formation of an initial nucleus is followed by either outward growth from the centre, or attachment of cells at the granule surface. Either way, the increasing granule size, leads to the development of gradients in physiochemical properties from the granule core to the surface for example, gradients in oxygen concentration. These gradients create different environmental niches allowing various organisms to proliferate at different granule depths.

2.6 Formation around a Nucleus

Inert particulate matter present in influent wastewater has been proposed to act as a nucleus for the initiation of methanogenic sludge granulation by providing a substratum for initial microbial attachment, followed by concentric growth and granule maturation (Hulshoff Pol et al., 1988; Pereboom, 1994). The availability of nuclei in the feedstock has even been suggested to influence the size distribution of granules (Pereboom, 1994).

Inorganic calcium and phosphate precipitates were observed at the core of mature aerobic granules, surrounded by microbial cells attached via an EPS matrix (Wan et al., 2015). These precipitates were proposed to act as nuclei for the initiation of granulation under alkaline conditions followed by cell multiplication and concentric growth leading to mature granules. A calcium carbonate core was also observed by Ren et al (2008) who suggested that this core provided increased rigidity and structural stability but lower activity levels.

Fernández et al (2008) investigated the effectiveness of inorganic salt precipitation on biomass retention in an anammox SBR and found that increasing NaCl concentrations in the influent led to precipitation of inorganic salts and improved biomass retention. Retained biomass had a higher inorganic content and increased density indicating that salt precipitates were acting as precursors to granule formation (Fernández et al., 2008).

The addition of various carrier materials to bioreactors has also been used to promote biomass growth. For example, zeolite addition has been shown to be effective in improving granulation and biomass retention in methanogenic bioreactors (Montalvo et al., 2014). Granular activated carbon (GAC) has been successfully used as a nucleating agent which accelerates the formation of aerobic granules (Zhou et al., 2015). Amendment of GAC as a support material for anammox granulation has also been proven to be very successful, achieving granulation in just 38 days (Zhang et al., 2015). The addition of carrier materials alters the initial steps of granule formation in that it circumvents the need for initial cell to cell contact and initial granule formation may proceed more similarly to that of traditional biofilm growth on surfaces. Addition of electrically conductive materials such as GAC may also promote Direct Interspecies Electron Transport in methanogenic granules as discussed in Section 3.2.

Several studies have highlighted the importance of inorganic nuclei in initiating the formation of granules and addition of support material has been successfully applied to improve biomass

retention. However, addition of growth nuclei does not appear to be a prerequisite for granulation.

2.7 Role of Cations

The presence of divalent cations such as Fe^{2+} Ca^{2+} and Mg^{2+} is generally thought to have a positive effect on granulation by counteracting the negative surface charges of microbial cells or negatively charged components of EPS (Liu et al., 2003a, 2002; Long et al., 2014). Their addition has often been shown to improve methanogenic granulation and overall reactor operation (Mahoney et al., 1987; Pevere et al., 2007; Schmidt and Ahring, 1993; Vlyssides et al., 2009; Yu et al., 2001). A range of other cationic polymers have also been shown to improve granulation in methanogenic systems (Show et al., 2004; Torres et al., 2018)

Several studies have also shown the effectiveness of divalent cations for improving anammox granulation. The addition of Fe^{2+} and Fe^{3+} to stirred bioreactors seeded with non-granular anammox sludge was shown to promote granulation and enhance anammox activity (Gao et al., 2014) and divalent cationic bridging has been observed to be an important aspect of EPS bridging in anammox granular sludge, leading to dense, stable granules (Lin and Wang, 2017)

Multiple studies have also implicated the importance of cations such as Ca^{2+} or Mg^{2+} in aerobic granulation due to their interaction with EPS (Caudan et al., 2014; Kończak et al., 2014). Addition of Ca^{2+} or Mg^{2+} has even been shown to enhance granulation in halophilic sludge, treating saline wastewater, which is a notoriously difficult type of system to achieve good granulation (Cui et al., 2021).

The role of divalent cations in linking negative charges on bacterial surfaces or in EPS appears to be common among all sludge types and addition of exogenous cations has proven to be an effective strategy for improving granulation. Therefore, any unified granulation theory must include the roles of cations in the early stages of granulation.

2.8 Granule Size and Granule Growth

Granule size is a frequently measured characteristic in granular sludge and is often used to assess granulation progress. Granules typically range between 0.5 and 2mm but can be as large as 5mm (Trego et al., 2020b). Granule growth is often associated with improved performance in aerobic (Liu et al., 2015), methanogenic (Faria et al., 2019a) and anammox granules (Vlaeminck et al., 2010). However, there does appear to be a limit at which increasing granule size does become detrimental and can lead to dead zones (Doloman et al., 2017a) breakage (Verawaty et al., 2013) and flotation (Lu et al., 2012; Song et al., 2017)

In general, there appears to be two types of granulation mechanism which have been formulated based on granule size: 1) micro-colony aggregation and 2) micro-colony growth. Micro-colony aggregation involves initial formation of small cell clusters often called flocs, micro-colonies or micro-granules etc. which aggregate and form a larger granule which subsequently grows via further cell attachment or cellular multiplication. This results in internal cell clusters within larger granules which were originally separate micro-colonies. Micro-colony growth, once again involves the initial formation of small cell clusters or micro-colonies but is followed by continual growth of each individual unit, without further aggregation and generally leads to the concentrically organised microbial communities discussed previously (Section 2.5).

Micro-colony aggregation appears to be more common in anammox granules and leads to mature granules with internal sub-units (Chen et al., 2019; Kang et al., 2019; Wang et al., 2020b, 2020a). For example, Lu et al (2012) proposed a granulation mechanism for anammox granules which involves aggregation of single cells into cell clusters, followed by further aggregation of clusters to form small granules with internal sub-units, which then grow into successively larger granules. These large granules then break and provide material for the formation of new granules in a life-cycle type pattern. A similar mechanism was also proposed by Wang et al (2020a). Some evidence does also point towards micro colony growth type

mechanisms and concentric architecture in anammox granules (Vlaeminck et al., 2010) as described in Section 2.5. Granule size is thought to play an important role in determining the level of anammox activity, with larger granules being more active and containing a higher relative abundance of anammox bacteria (Nielsen et al., 2005; Vlaeminck et al., 2009). It has been proposed, that as granules grow, more strict anaerobic conditions develop at the core of the granule, leading to proliferation of anammox bacteria (Winkler et al., 2011). Metatranscriptomic evidence has also supported this hypothesis as the expression of key anammox metabolic genes and production of transport proteins increased with granule size (Bagchi et al., 2016).

Granulation mechanisms proposed for methanogenic granules tend to be based more on micro-colony growth rather than aggregation of separate micro-colonies, resulting in the layered morphology described in Section 2.5. Granule size distributions in methanogenic reactors have been studied extensively and led to several "life-cycle" type granulation theories in which small granules are considered young and large granules are older (Díaz et al., 2006a; Trego et al., 2020a). Zheng et al (2006) proposed a granule life-cycle, where growth was initiated by clusters of *Methanosaeta concilli.* This was followed by a "coat" of syntrophic bacteria and subsequent concentric growth, resulting in mature granules. Substrate limitation at the centre of large granules then leads to decay and breakage and the resultant pieces provide anchors for new growth (Zheng et al., 2006). Granule size appears to have a large impact on microbial community composition in methanogenic granules indicating that as granules grow their microbial community shifts accordingly. Indeed Trego et al (2020c) concluded that granule size had a greater impact on the active microbial community of methanogenic granules than the availability of specific methanogenic substrates.

Zhou et al (2014) proposed a micro-colony aggregation type granulation mechanism for aerobic granules, whereby small "bioflocs" aggregated to form a small granule which then

grew successively larger. However micro-colony growth mechanisms resulting in concentric architecture have been proposed more frequently for aerobic granulation (Lv et al., 2014a; Sarma et al., 2017) Granulation via both micro-colony aggregation and micro-colony growth have been observed to occur side by side in an aerobic granular system operated for biological phosphorous removal (Barr et al., 2010), indicating that bot may contribute to granule formation. Cyclical "life-cycle" type granulation mechanisms have also been proposed for aerobic granules (Verawaty et al., 2013).

It is likely that a combination of many different granulation processes such as micro-colony aggregation, micro-colony growth and granule life-cycles occur simultaneously in bioreactors and to suggest that one process dominates may be over simplistic. There is a general consensus however that small granules are considered "young" and as they increase in size, breakage occurs for a number of reasons e.g. shear force, internally trapped gas, abrasion. It is then possible that granule pieces or single cells released from granules could then go on to form the basis for new granules. This cycle of growth and breakage along with the formation of new flocs from individual cells and subsequent micro-colony growth or aggregation could loosely explain the process of granulation across all three sludge types.

3 Unique Observations

Although all three sludge types share multiple characteristics and similar granulation mechanisms have been proposed, there are some differences which should be considered, such as: anammox granule morphology, The role of Direct Interspecies Electron Transfer (DIET) in methanogenic granules and different quorum sensing systems.

3.1 Anammox Granule Morphology

As mentioned previously, in contrast to aerobic and methanogenic granules anammox granules often have an internal structure which consists of separate cell clusters (Wang et al., 2020a). The outer surface of anammox granules also often appears to be rougher than other granule types with bumps and convex protrusions which have been quantified by Kang et al., (2019). A budding mechanism which may account for this structural difference has been proposed by Vlaeminck et al (2010), whereby growth of anaerobic ammonium oxidisers at the core of the granule cause protrusions at the granules outer surface, which are broken off by shear forces and collisions and form the basis for new granules. However, it appears more likely that this type of morphology is a result of a micro-colony aggregation type granulation mechanism such as those discussed in Section 2.8. These morphological differences in anammox granules may indicate that the mechanism of formation is slightly different than aerobic or anammox granules. Nonetheless, several aspects of granulation are common among all three granule types such as the importance of selection pressure, shear force and EPS.

3.2 DIET in Methanogenic Granules

The breakdown of organic compounds in anaerobic digestion often involves the syntrophic transfer of electrons between organisms via reduced intermediate molecules such as hydrogen or formate (Stams et al., 2006). For example, syntrophic propionate oxidising organisms often oxidise propionate, resulting in the production of molecular hydrogen which is in turn utilised by hydrogenotrophic methanogens (Dong and Stams, 1995; Li et al., 2012; McInerney et al.,

2009). In these partnerships the role of the methanogen is essential for maintaining low H_2 partial pressures, allowing VFA oxidation to be thermodynamically feasible (Stams and Plugge, 2009). These syntrophic interactions are also thought to be facilitated by aggregation of microorganisms (Ishii et al., 2005; Stams and Plugge, 2009) and are common in methanogenic granules (de Bok et al., 2004). It is also becoming increasingly apparent that electrons can be transferred directly from one organism to another, without the need for exogenous electron carriers, via Direct Interspecies Electron Transport (DIET) (Rotaru et al., 2014). Initial evidence for this process was observed in co-cultures of *Geobacter sp* and *Methanosaeta sp* (Summers et al., 2010). Close physical associations between DIET partners are required to facilitate electron transfer through membrane bound cytochromes or conductive pili (Baek et al., 2018) and organisms carrying out DIET have been shown to favour aggregated growth (Summers et al., 2010). DIET is now believed to be common in anaerobic granules (Morita et al., 2011; A.E. Rotaru et al., 2014b). Therefore, aggregation of organisms to facilitate DIET may actively play a role in enhancing granulation. A range of conductive additives, which microorganisms can attach to and exchange electrons through have been investigated for promoting DIET in bioreactors (Gahlot et al., 2020). These additives have been successful in terms of enhancing performance (Dang et al., 2016; Zhao et al., 2015) and undoubtedly have an effect on the granulation mechanism . The role of DIET in granulation of methanogenic communities must be taken into account in any granulation theory both in the presence and absence of conductive additives.

3.3 Unique Quorum Sensing Systems

Quorum sensing is an important aspect of granular biofilm formation (Huang et al., 2019) and is reasonably well studied in aerobic systems(Huang et al., 2016; Lin et al., 2020), where increases in AHL signal molecules have been shown to positively correlate with increased EPS production and improved granulation (Tan et al., 2014). Regulation of QS in aerobic systems

has been attempted through addition of exogenous AHL signal molecules (Tan et al., 2014), bioaugmentation with AHL producing strains (Gao et al., 2019) and addition AHL-containing supernatant from mixed cultures (Zhang et al., 2020). However some knowledge gaps still exist, such as the role of quorum quenching in granule disintegration as highlighted by Sarma et al. (2017).

QS systems are also active in anammox granules (Zhao et al., 2020) and levels of AHLs in reactors have been shown to positively correlate with improved anammox activity (Tang et al., 2015) and improved granule settleability (Zhang et al., 2019). Inactivation of AHLs and interference with AHL receptors in anammox granular sludge reduced anammox activity and influenced protein to polysaccharide ratios in EPS, resulting in granule disintegration (Zhao et al., 2016). This indicates that QS is essential for maintenance of stable anammox granules.

Bacterial AHL-based quorum sensing has also been studied in methanogenic systems (Lv et al., 2018; Ma et al., 2018) but to a lesser extent than aerobic or anammox granules. In addition, the role of archaea in quorum sensing is still largely unknown (Charlesworth et al., 2017). A methanogen specific quorum sensing system, with specific, modified AHLs has been identified in *Methanosaeta harundinacea* 6Ac (Zhang et al., 2012). Expression of these AHLs causes a shift from short cell type growth to filamentous growth (Zhang et al., 2012). Bioaugmentation of this strain along with its specific AHLs has been shown to improve granulation and reduce sludge washout (Li et al., 2015). As mentioned earlier, filamentous *Methanosaeta* sp are thought to be essential for methanogenic granulation by providing a filamentous matrix for aggregation of other organisms. Therefore, QS in methanogens may be essential for inducing this filamentous morphology and initiating methanogenic granulation. This mechanism is different from QS in aerobic or anammox granule which, as mentioned earlier promotes protein-rich EPS production, rather than a change in cell morphology. Therefore, methanogenic QS may provide a cellular matrix for granule formation whereas bacterial QS may provide and

EPS matrix rich in proteins. It is likely that both bacterial and archaeal QS occur in methanogenic granule formation and further research is required to elucidate the exact role of both in order to successfully apply approaches such as bioaugmentation and exogenous AHL addition.

3.4 SBRs to continuous systems

Another point of difference which needs to be considered is that aerobic granules have largely been studied in SBRs, whereas methanogenic and anammox granules have more often been applied in continuous reactors such as UASBs and EGSBs. In recent years there has been a movement to continuous systems aerobic granular systems (Kent et al., 2018) but it should be noted that much of the observations on aerobic granulation to date have been made in SBRs. The batch mode of operation in SBRs may lead to less prolonged exposure to shear forces, when compared to a continuous system resulting in less compact granules as filamentous outgrowth may be more likely to establish.

4 A Unified Granulation Theory

Several unifying concepts have been identified which are common across the three sludge types surveyed. It is clear that selection pressure, shear forces, EPS (particularly protein content), divalent cations and inorganic nuclei may play a similar role in all sludge types (Fig. 1). However, the presence of a concentrically layered structure may not be universal and a cell cluster like structure in anammox granules could indicate a possible different mechanism of formation. The importance of the filamentous growth of *Methanosaeta* sp for the initiation of methanogenic granule formation is already established. However, anammox and aerobic granules appear to be more reliant on establishment of an EPS matrix, rather than growth of filamentous organisms for initiating granulation. This difference could be induced by different QS systems with bacterial quorum sensing promoting EPS production and methanogenic QS promoting filamentous growth of *Methanosaeta* sp. In addition, the diversity of applications

for aerobic granular sludge results in diverse microbiomes, making it unlikely to find a single organism such as *Methanosaeta* which is universally important for granulation. Therefore, rather than searching for a *Methanosaeta*-like equivalents in aerobic or anammox granules, patterns of diversity or in ecological phenomena such as community assembly and community succession could be studied which may be common to all three sludge types.

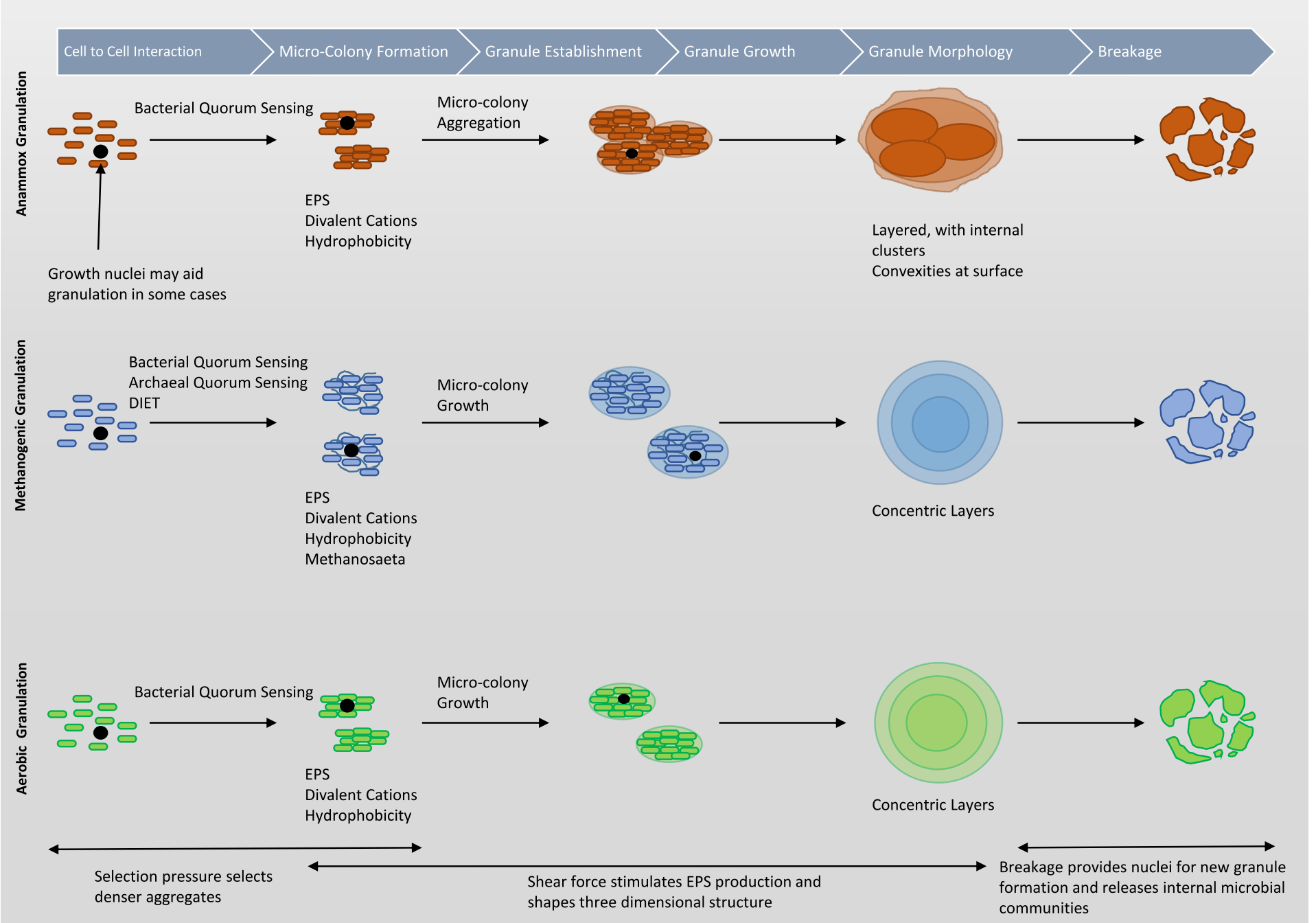

Figure 1. Graphical representation of similarities and differences between granulation mechanisms of the reviewed granule types

4.1 Implications for this Thesis and a Microbial Based Granulation Model

From examining the similarities and differences between granule types a number of questions arise, which may help in formulating a universal granulation theory. Most of these relate to microbial community dynamics:

1. Is a distinct community associated with the initial formation of granules?
2. What is the fate of broken granule pieces?
3. How does the release of internal microbial communities impact total reactor diversity?
4. How do microbial communities evolve as granules grow?
5. Is there a clear pattern of community succession in growing granules?

A granulation mechanism based on microbial ecology could answer some of these questions. Research in this area is lacking and few studies exist where the sole purpose is to examine microbial ecology during granulation, meaning that most observations on granulation are often secondary to other objectives. In order to really understand how microbial communities, assemble and influence granulation and bioreactor performance more data on actively forming, growing and disintegrating granules is required. It is generally accepted that once granules reach a certain size, they break apart (Trego et al., 2020a) due to factors such as substrate limitation or increased abrasion. Therefore, in depth microbiological analysis of granules at different growth stages is required to address the research questions outlined above. The experimental designs outlined in chapters 3 and 4 of this thesis were designed in order to answer the above experimental questions, with the ultimate goal of developing an ecological granulation model for methanogenic granular sludge

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CHAPTER 3

Methanogenic Granule Growth and Development is a Continual Process Characterized by Distinct Morphological Features

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Abstract

High-rate anaerobic digestion of industrial wastewaters is reliant on the formation of anaerobic granular sludge inside up-flow anaerobic bioreactors. Granular sludge consists of dense, highly settable microbial aggregates (approx. 0.5-4.0 mm in diameter) known as granules. Granules found in anaerobic digesters are essential to their successful operation as their unique properties allow high influent rates to be applied. The mechanisms of granule formation (granulation), and persistence, in anaerobic digesters, require further investigation. Previous studies have focused on granulation during start-up of bioreactors rather than monitoring how granules persist, and new granules emerge, over time. Granulation is a dynamic and continual process, and methanogenic granules likely disintegrate and, along with planktonic cells, provide founder material for new biofilms. Granular sludge was sourced from a full-scale anaerobic bioreactor treating dairy-processing wastewater. Size-separated granules were used to inoculate 12 laboratory-scale bioreactors in three sets of four replicates, as follows: four bioreactors (S1-S4) inoculated only with small (S) granules; four bioreactors (L1-L4) inoculated only with large (L) granules; and four bioreactors (ND1-ND4) inoculated with a full complement of naturally-distributed (ND) granules. One bioreactor from each of the three sets was sampled (sacrificed) after 30, 60, 90 and 150 days of operation. Temporal changes in granule size distribution, volatile solids content (VS), settling velocity, and biofilm ultrastructure (scanning electron microscopy (SEM)) were monitored. The experiment provided unique opportunities to study new granule formation, maturation, disintegration and reformation. Cyclical patterns of biofilm development were observed and significant shifts in granule size distributions were associated with improved bioreactor performance. SEM revealed distinct morphological characteristics associated with differently sized granules and different stages of biofilm development.

1 Introduction

High-rate, up-flow anaerobic bioreactors, such as the Expanded Granular Sludge Bed (EGSB), are widely used to treat industrial effluents (Kamali et al., 2016; Kong et al., 2019; Paulo et al., 2020; van Lier et al., 2015), allowing removal of organic contaminants and recovery of biogas energy (Appels et al., 2011), thus contributing to resource recovery and development of the circular bio-economy. Such reactor configurations are underpinned by aggregation of planktonic cells, flocculent biomass and suspended particles to form, and maintain, dense, well-settling, granular sludge comprised of small (0.2-4 mm diameter), spherical, extracellular polymeric substance (EPS)-embedded, microbial aggregates (Trego et al., 2020b). Each granule is thought to contain all of the microorganisms necessary for complete breakdown of organic contaminants in wastewater to biogas (McHugh et al., 2003). Granule size affects various granule properties, such as porosity (Wu et al., 2016), mass transfer (Afridi et al., 2017) and settling velocity (Bhunia and Ghangrekar, 2007), all of which are important for substrate conversion and bioreactor performance.

Sustained granulation is considered important for bioreactor performance (Hulshoff Pol et al., 2004; McHugh et al., 2003) and understood to be controlled by several factors, some of which can be directly controlled. For example, hydrodynamic shear force; organic loading rate (OLR); temperature and pH, whereas others can only be controlled indirectly such as, EPS production; substrate composition; availability of inorganic nuclei; and the microbial ecology of the starting biomass (Ding et al., 2015; Tiwari et al., 2006; Wu et al., 2009). Indeed, many studies report granule disintegration (degranulation) leading to biomass loss and even reactor failure (e.g., Ahn et al., 2001; Borzacconi et al., 2008; Macarie et al., 2018). Prolonged sludge loss and low microbial growth rates at full scale often necessitate re-seeding or ‘topping up’ of the sludge volume with externally sourced granular sludge, increasing operational costs. That degranulation and sludge loss can occur with such critical consequences indicates the need for

further study of granulation mechanisms. A seminal review on granulation mechanisms was published by Hulshoff Pol *et al.* (2004) but the field is still an active area of ongoing research (e.g., Xu *et al.*, 2018; Gagliano *et al.*, 2020). Many of the currently proposed granulation mechanisms are generally focused on the start-up phase of bioreactor operation when steadily improving performances align with increasing granule size and stability (Doloman et al., 2017; Faria et al., 2019; Sudmalis et al., 2018; Wang et al., 2018). However, granulation is likely a continual, dynamic process occurring over the entire operation of a bioreactor (Trego et al., 2020a), rather than only during start-up. Previous studies (Trego et al., 2020a; Zheng et al., 2006) proposed 'life-cycle' trajectories in which granules grow from small to large aggregates before eventually disintegrating and providing founder material for new granules. Insights to continual biofilm development could allow for smarter, precision management of such systems and prevent future bioreactor failure.

A newly-conceived, uniquely-designed, laboratory-scale, bioreactor trial was used to study growth, disintegration and re-formation of methanogenic granules. The granulation trajectory, and potential for wastewater treatment, of distinct, size-constrained fractions and a full complement of granule sizes was compared. It was hypothesized that granules sampled at different stages of growth would have defining physical characteristics, and that a full complement of granule sizes would be required for maximal bioreactor performance

2 Materials & Methods

2.1 Source of Biomass

Granular sludge was sourced from a mesophilic (37°C), anaerobic, 'internal circulation' (IC) bioreactor (650m^3) from a dairy-processing and ethanol-production plant in Ballineen, Co. Cork, Ireland. Sludge was separated into five distinct size bins based on granule diameter – 'extra-small' (XS) (<0.4 mm), 'small' (S) (0.4-1.18 mm), 'medium' (M) (1.18-1.8 mm), 'large' (L) (1.8-2.24 mm) and 'extra-large' (XL) (>2.24 mm) – by passing through stainless steel grading sieves. Initial size distributions and VS concentrations are provided (Table. 1) Bins were then further sieved into 14 highly resolved size fractions (A-N), as shown in Table 1. Non-sieved granules are hereafter referred to as Natural Distribution (ND) granules. Samples of each size fraction (A-N) from the seed sludge were physically characterized with respect to volatile solids (VS), settling velocity, and morphology using scanning electron microscopy (SEM).

Table 1. Size ranges of each of the five size bins (XS – XL) and their contribution to the total volume as well as the VS concentration. Size ranges for constituent fractions (A – N) are also displayed.

Bins	Fraction	Size (mm)	Bin Volumetric Contribution (%)	VS Concentration (%)
XS	A	< 0.2	12	37
	B	0.2 - 0.4		
S	C	0.4 - 0.6	21	42
	D	0.6 - 0.8		
	E	0.8 – 1		
	F	1 - 1.2		
M	G	1.2- 1.4	27	65
	H	1.4 - 1.6		
	I	1.6 - 1.8		
L	J	1.8 – 2	23	70
	K	2 - 2.24		
XL	L	2.24 - 3.15	17	69
	M	3.15 – 4		
	N	> 4		

2.2 Bioreactor Configuration and Sampling

Twelve, laboratory-scale (2 L), expanded granular sludge bed (EGSB) bioreactors were seeded with either S, L or ND granular sludge to a final concentration of 15gVSS/L (Fig. 1). Four bioreactors (RS1-RS4) were seeded with S granules, four bioreactors (RL1-RL4) were seeded with L granules and four bioreactors (RND1-RND4) were seeded with non-sieved ND granules. Bioreactors were operated identically (Table 2) for the duration of the trial (140 d) and supplied with a simple, entirely soluble, synthetic wastewater supplemented with macronutrients and trace metals (Shelton and Tiedje, 1984) and buffered with 5 g/L sodium bicarbonate

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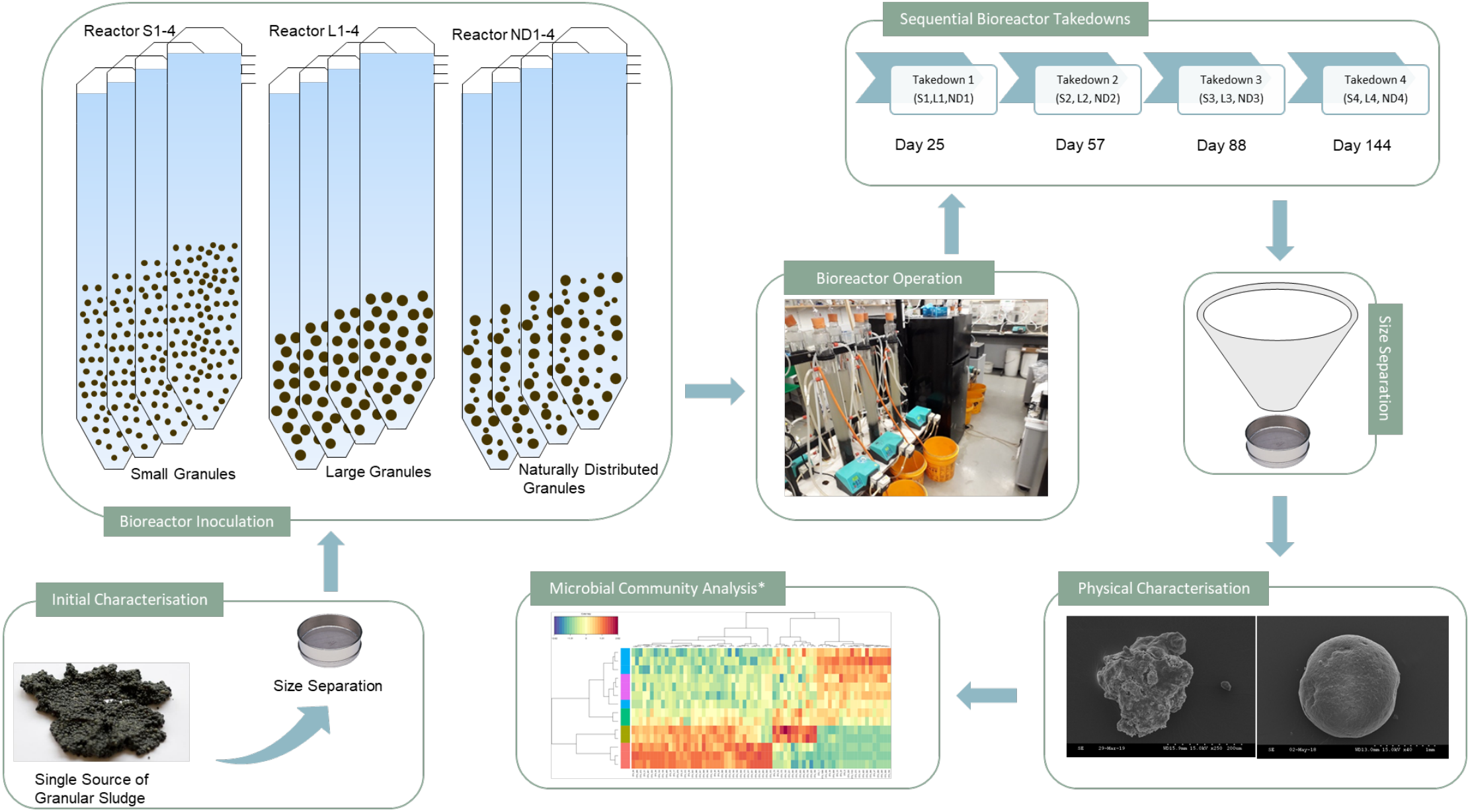

Figure 1. Experimental design of bioreactor trial.

*Results of microbial community analysis are presented in Chapter 4

Table 2. Bioreactor operating parameters, and synthetic feed composition

Operational Parameters		**Feed Component**	**Concentration (g/l)**
Hydraulic Retention Time (HRT)	21 h	Lactose Monohydrate	3.3
Organic Loading Rate (OLR)	9.5kgCOD/l/d	Ethanol	2.36
Upflow Velocity (V_{up})	5.5 m/h	Acetic Acid	0.47
Influent COD	10 g/L	Butyric Acid	0.28
pH	7-7.5	Propionic Acid	0.33
		Yeast Extract	0.1

One bioreactor from each of the three sets (RS1-4, RL1-4 & RND1-4) was decommissioned at approximately 1-2 month intervals. The first bioreactors taken down (takedown 1) were RS1, RL1 and RND1, followed by RS2, RL2, and RND2 (takedown 2), and so on. Upon takedown of each sacrificial bioreactor, all sludge was collected and bioreactor liquor was separated by decanting for initial sieving. A small sample (approx. 50-100ml) of sludge was sieved through 4 stainless steel sieves using only reactor liquor, in order to quickly obtain size separated granular sludge for microbial community analysis. All samples were snap frozen with liquid nitrogen in RNALater and stored at -80˚C. After snap freezing the remainder of the sludge was sieved, using tap water to determine the size distribution. After the new size distributions were obtained, all sludge was further sieved into 14 further size fractions (A-N) (Table. 1) for comprehensive physical characterization including TS/VS content, settling velocity and morphology.

2.3 Physical Characterization

2.3.1 Volatile Solids & Settling Velocity

The standard loss-on-ignition technique (APHA, 1998) was used to determine the concentration of total solids (TS) and volatile solids (VS) of sludge samples. Granule settling velocity, was measured by dropping individual granules vertically through a 1-m-tall, clear, acrylic tube, filled with de-ionised water. The time taken for granules to travel from the 0.6 m

mark to the 0.3 m mark was recorded and settling velocity was calculated as the distance (0.3 m) divided by settling time.

2.3.2 Scanning Electron Microscopy (SEM)

Approximately 1 g of sludge from each size fraction from each bioreactor at take-down was sampled and transferred to respective 1.5-ml microcentrifuge tubes. Granules were fixed overnight, at 4°C in 2.5% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer. The following day, the glutaraldehyde supernatant was removed using a pipette and granules were immersed in a graded ethanol series of 50, 70, 70 and 90% for ten min each. Four granules of each recovered size fraction from each bioreactor (where sufficient sample was available) were then mounted on aluminum stubs using carbon adhesive tabs. Finally, mounted granules were covered with 2 µl hexamethyldisilazane (HMDS) and left to dry overnight in a fume hood. Fixed samples were sputter coated with gold and visualised on a scanning electron microscope (Hitachi S-2600, Mountain View, CA, USA).

All granules were initially imaged at x40 magnification to compare granule structure at the same scale. Any physical features of note, such as cracks, breaks, flakes and bumps were then observed at higher magnification and noted. Commonly occurring features were recorded and assigned descriptors (Appendix 1) based on simple visual inspection of SEM micrographs, therefore all data is purely observational and should be taken with care. Once each feature was defined (Appendix 1), all images (Approx. 500) were then manually screened for presence/absence of the features, to construct a binary matrix which was later used for statistical analysis (Section 2.5.1). The occurrence of features was then associated with differently sized granules. Inevitably, some features are likely to have been caused by the fixation process, but several correlated strongly with size (Table 1) and, therefore, are likely influenced by underlying granule characteristics.

2.4 Analytical Methods

Biogas methane content; biogas volume; and effluent COD (total (tCOD) and soluble (sCOD)), metabolite (lactose, ethanol and volatile fatty acids (VFAs)) concentrations, and pH, were each monitored 2-3 times weekly during the trial. Biogas volume was measured using water displacement. Biogas methane concentrations were determined using a gas chromatograph (GC; Varian) equipped with a flame ionisation detector with nitrogen as a carrier gas, at a flow rate of 25 ml/min and a lower limit of detection of 30%. A benchtop probe (Hanna Instruments, Woonsocket, RI) was used to measure pH. COD concentrations were determined using a commercial COD kit (Reagacon, Shannon, Ireland) according to the manufacturer's instructions. To analyse total COD, bioreactor effluent samples were diluted to within an approximate range (<1500 mg/L) and an homogenous sample was added directly to the test vial provided by the manufacturer. In the case of soluble COD, samples were centrifuged at 8,000 rcf for 10 min and 2 ml supernatant were then added to the test vial. COD vials were incubated at 150˚C for 2 h and the COD concentration was measured on a Hach Dr/4000 Spectrophotometer at 435 nm. Lactose, ethanol, acetic acid, propionic acid and butyric acid were measured using a 1260 Infinity II liquid chromatograph (Agilent, Germany) equipped with a Hi Plex H 7.7 x 300 mm and 8 µm (p/n PL1170-6830) column (Agilent, UK) maintained at 60°C, and an RI detector at 55°C. The mobile phase was 0.005 M sulfuric acid with a flow rate of 0.7 mL/min. The lower limit of detection for all compounds was 0.02g/l

2.5 Statistical analyses

2.5.1 Granule feature analyses

In order to identify relationships between the granule features described in Appendix 1 and granule size, generalized estimation equation (GEE) models were used (Liang and Zeger, 1986). First, a binary matrix was constructed based on presence absence of each of the 13 identified features, which are listed in Appendix 1. Each feature (binary: Yes vs. no) was used

as response variable in the model, while the size of the granule was the predictor of interest. The distribution family was chosen to be binomial and the link function logistic. To check for potential nonlinear associations, a quadratic size term was included into the regression equation as well. The granule size was introduced as center of the size bins (e.g. 0.3mm for the bin 0.2-0.4mm) with assigning 0.1mm to granules smaller than 0.2mm and 4mm to granules larger than 4mm. Estimates at the lower and upper bound of granule sizes should therefore be treated with care. Statistical dependence of a feature on granule size was then determined by a Wald test, testing the linear and quadratic term of the regression simultaneously on zero. Additionally, we tested on departure from linearity by testing the quadratic term on zero. As we tested 13 different features, we corrected for multiple testing by Bonferoni correction setting the significance threshold to 0.05/13. A significant result means that the feature is dependent in its occurrence on the size of the granule. For visualization, the prediction scores of the GEE models were retrieved for each of the features and plotted against the size of the granules. This results in the predicted share of granules showing a specific feature in dependence on the granule size. All statistical analyses were performed with STATA 16/MP.

3 Results

3.1 Size Distribution

The XS bin was visually different from other bins in that it comprised flocculent biomass and particulates, rather than granules. The S, M and L granules were grey/black and spherical, with a hard external layer. XL granules had more cracks in the outer layer and were generally more fragile based on visual inspection. XS biomass recovered from the bioreactors after decommissioning was similar in appearance (Fig. 2) to the XS biomass of the seed, and appeared to originate from the surface of the sludge bed (Fig. 2). S, M, L and XL granules were darker in appearance but otherwise generally similar to the seed. The seed sludge (ND0) comprised approximately 12% XS, 21% S, 27% M, 22% L and 17% XL sludge, as proportions of the total sludge volume (Fig. 3).

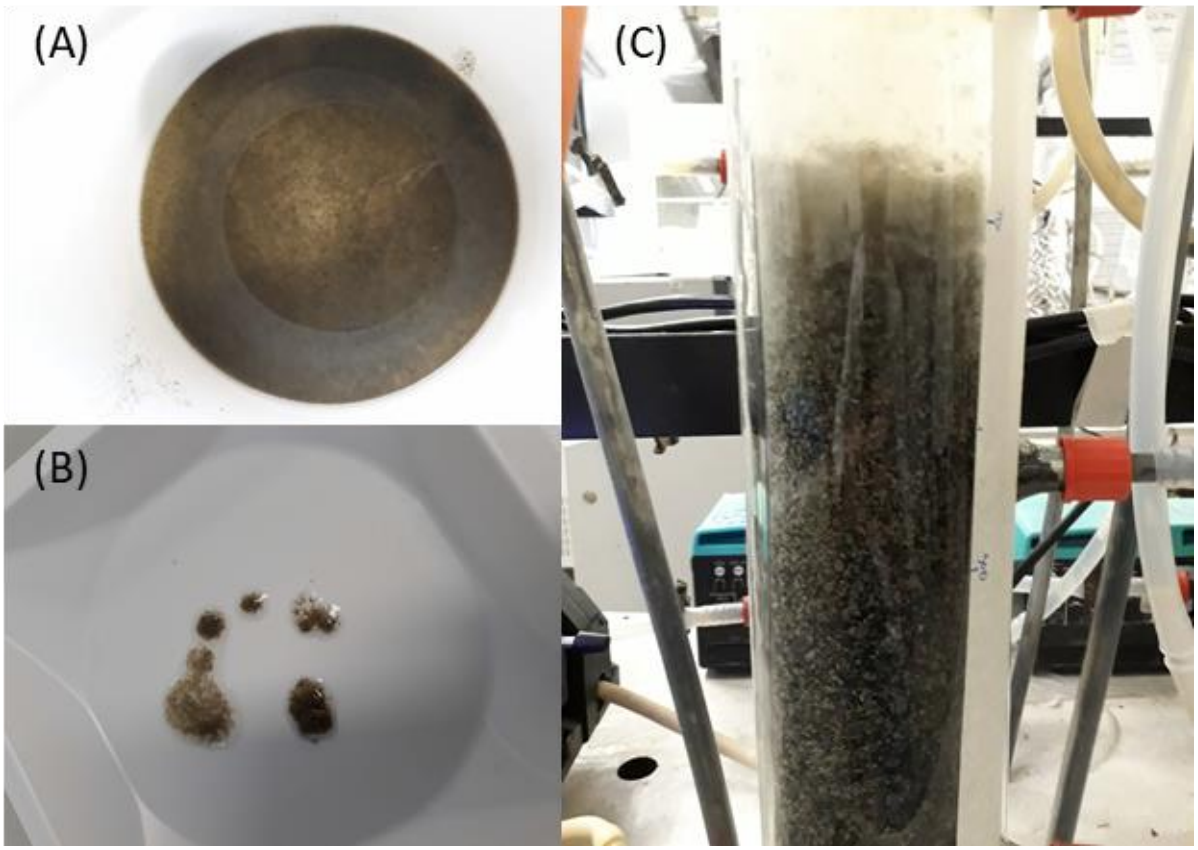

Figure 2. (A-B) Images depicting flocculent nature of XS biomass recovered from lab-scale reactors. **(C)** Apparent location of flocculent biomass on top of sludge bed

Nearly 13% of the total sludge volume in RS1, at the time of takedown (i.e. Day 25), consisted of XS biomass. Granule growth also occurred after just one 25 days of operation, as M, L and XL granules were recovered from RS1 as well (Fig. 3). More growth was observed in RS2 (57 Days) and RS3 (88 Days). By the takedown of RS4 (144 Days), approximately 32% of the sludge consisted of L and XL granules, and 41% comprised of M granules (Fig. 3). Fast, initial growth was also apparent in RL1, in which nearly 50% of the sludge volume comprised of XL granules, although that proportion was unexpectedly lower (about 25%) in RL2, RL3 and RL4. Simultaneously, a temporal increase in the proportion of XS granules was observed in each of those bioreactors.

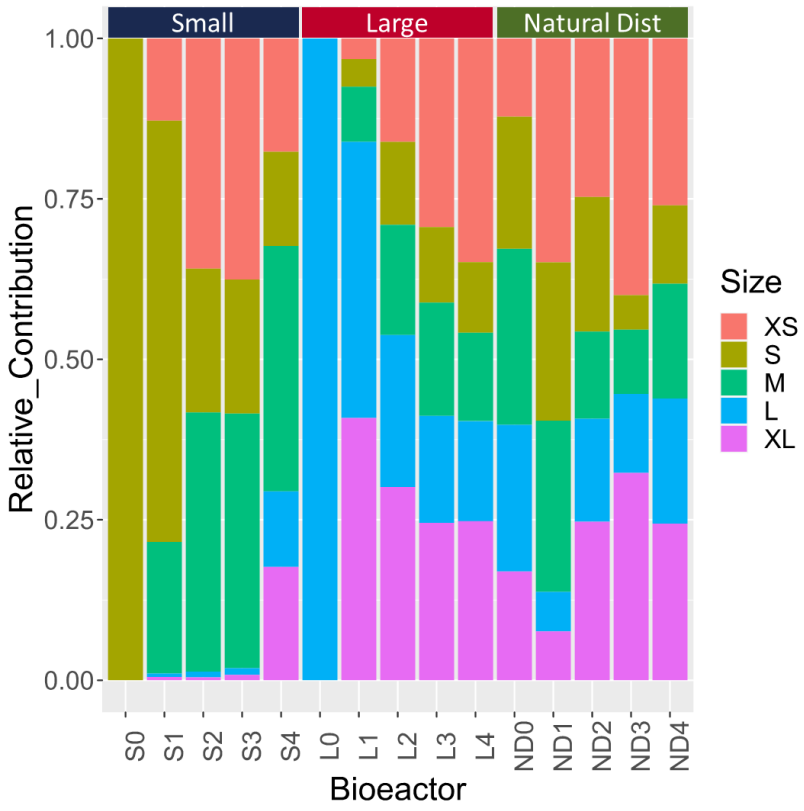

Figure 3. Stacked bar chart illustrating relative distribution of granule sizes in each reactor at takedown (S1-S4, L1-L4, ND1-ND4), and in the respective seed sludge (S0, L0, ND0).

3.2 Physical Characterisation

3.2.1 Scanning Electron Microscopy

Three mutually exclusive granule characteristics were identified, each relating to surface morphology of granules: 'open', 'partial skin' and 'skin' (Appendix 1). The occurrence of the surface morphology characteristics was significantly dependent on granule size (Table 3). An estimation of the predicted share of granules which would display a given characteristic was made by plotting the prediction scores of the GEE against the size of the granules (Figure 4). The open and partial skin features occurred with a much higher frequency in granules <1 mm (Fig. 4). The skin feature occurred with very high frequency once granule size reached 1.5 mm (Fig. 4).

Table 3. Summary statistics for dependence of features on granule size

Feature	**Occurrence (95%-CI)**	**p-value global for dependence on granule size**	**p-value for quadratic granule size term**	**Shape of association with granule size**
Open	0.06(0.04;0.08)	1.97e-04	0.64	↓
Partial skin	0.14(0.11;0.18)	5.22e-10	0.25	↓
Skin	0.80(0.76;0.84)	1.08e-16	0.86	↑
Irregular	0.19(0.16;0.23)	1.63e-18	0.75	↓
Chunk/flake	0.14(0.11;0.18)	1.01e-12	0.91	↓
Some rounding	0.18(0.15;0.22)	1.90e-06	0.10	↓
Rounded	0.55(0.51;0.60)	2.97e-29	7.85e-07	∩
Peeling	0.19(0.16;0.23)	6.25e-09	0.001	∩
Cracking Skin	0.32(0.28;0.37)	1.19e-10	0.04	↑
Wrinkled	0.32(0.27;0.36)	1.93e-15	2.23e-07	∩
Fissures	0.08(0.05;0.10)	1.19e-05	0.27	↑
Concave	0.09(0.06;0.12)	8.05e-04	0.003	∩
Hollow	0.02(0.01;0.04)	0.27	0.32	n.s.

P-values from general equation estimation models using binomial distribution as family and the logistic function as link. Threshold for significance was set to 0.05/13=0.0038 correcting for multiple testing *via* Bonferoni correction. Shape of association was determined by graphical inspection of prediction scores. CI=confidence interval, n.s.=not significant.

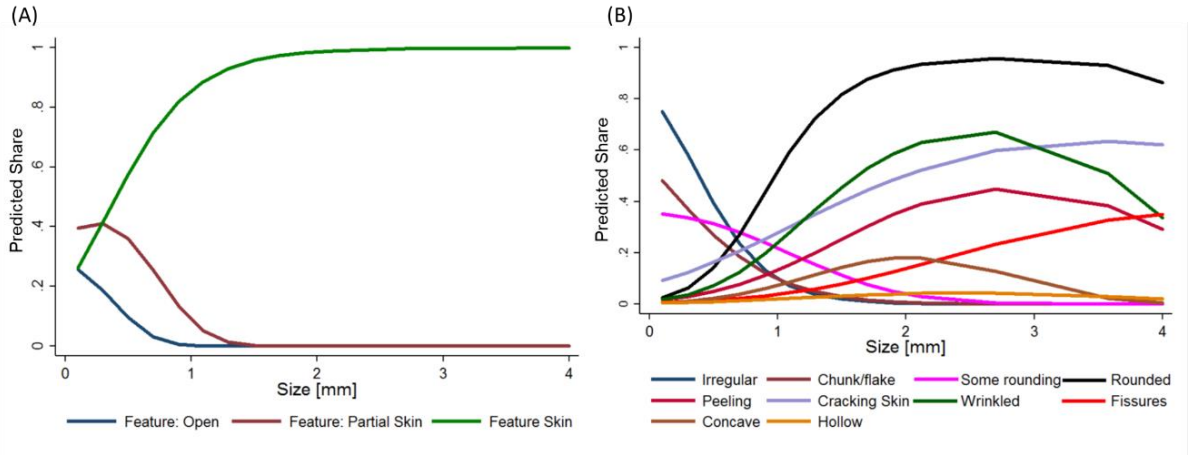

Figure 4. Predicted shares of particular granule features, identified by SEM, occurring in granules across all sizes tested (Fractions A-N). **(A)** Predicted share of mutually exclusive granule surface features; **(B)** predicted share of other features. Predicted shares were derived from a general estimation equation (Section 2.5.1) using the linear and quadratic granule size as predictors.

Aside from surface morphology, ten other features were identified (Appendix 1). Four of those features relate to overall granule shape: 'irregular', 'chunk/flake', 'some rounding' and 'rounding'. The remaining features related to granule damage: 'peeling', 'cracking skin', 'wrinkled', 'fissures', 'concave' and 'hollow' (Appendix 1). The occurrence of all features, except 'hollow', was significantly dependent on granule size (Table 3). Since the 'hollow' feature was rare, the statistical power to detect a dependency was also rather low and a higher sample size may be needed. Three features related to granule shape had a higher probability of occurring in granules <1 mm: 'irregular', 'chunk/flake' and 'some rounding'. Therefore, those features, along with the 'open' and 'partial skin' surface features were indicative of "young" flocs or granule chunks broken off from larger granules (Table 4). Granules of 1-2 mm tended to be 'rounded', and were likely to display the 'skin' surface morphology and had some features related to granule damage, such as wrinkling and cracking. Those granules were considered to be "juvenile" (Table 4). Granules of 2-3 mm almost exclusively had a 'skin' and were 'rounded' in shape. The 'wrinkled' and 'peeling' features also tended to be most prevalent in those granules (but were less common in granules >3 mm). The 2-3 mm granules were

considered “mature” (Table 4). Granules >3 mm were mostly ‘rounded’ and had a ‘skin’. The ‘cracking skin’ and ‘fissures’ features were most prevalent in granules >3 mm, indicating that larger, “old” granules are more fragile and prone to breakage (Table 4).

Table 4. Summary of size-associated granule features and proposed age classification

Size	Typical Features	Proposed Age	Implication
<1mm	‘Open’ or ‘partial skin’ surface morphology. ‘Irregular’, ‘chunk/flake’ or ‘some rounding’ in shape. Little damage.	Young	Prone to Washout
1-2mm	‘Skin’ or ‘partial skin’ surface morphology. Most samples ‘rounded’ in shape. Minimal ‘cracking skin’. Some ‘wrinkled’.	Juvenile	Optimal performance
2-3mm	‘Skin’ surface morphology. ‘Rounded’ in shape. More granule damage including ‘peeling’, ‘wrinkled’, more severe ‘cracking skin’	Mature	Optimal Performance
3-4mm	Large cracks in outer layer and deep ‘fissures’ into the center of the granule	Old	Prone to disintegration

3.2.2 Volatile Solids and Settling Velocity

Volatile solids content and settling velocity were determined for granule fractions A-N (Fig. 5). Volatile solids content of the seed sludge was much lower in smaller granules. This may be an anomaly of the sieving process; whereby small inorganic particles were included as part of the smaller fractioned biomass.

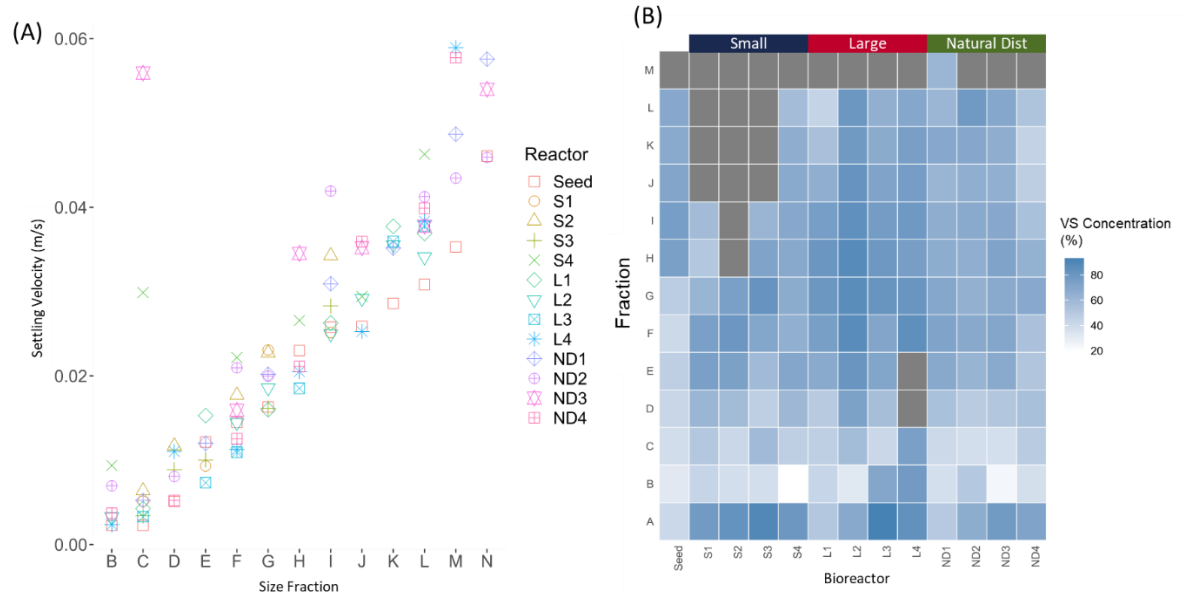

Figure 5. (A) Scatter plot with of granule settling velocity vs. size **(B)** heatmap showing the concentration of volatile solids, relative to total solids, for fractions A-N for all bioreactors.

The VS content of fraction A in newly emerged biomass in the S, L and ND bioreactors was much higher than in the seed sludge, indicating a higher ratio of organics to inorganics. The VS content was then lower fractions B and C, in all bioreactors, before increasing again in mid-size granules and decreasing in XL granules. Settling velocity increased in a roughly linear fashion with granule size (Fig.5).

3.3 Reactor Performance and Biomass Dynamics

Summary statistics for reactor performance were expressed as means and standard deviations (Table 5). After a short start-up, the efficiency of each of the bioreactors for removal of soluble COD remained generally high (95%-99%) throughout the trial (Fig. 6). sCOD removal by RS1-4 and RL1-4 was, however, generally more unstable (Fig. 6), with more drops in sCOD removal and larger standard deviations (Table. 5) throughout the trial. The performance of RS4 in particular, declined slightly towards the end of the trial (Fig.6) but sCOD was still relatively high (96.25% +/- 1.85%). On the other hand, the performance of RND1-4 was stable throughout.

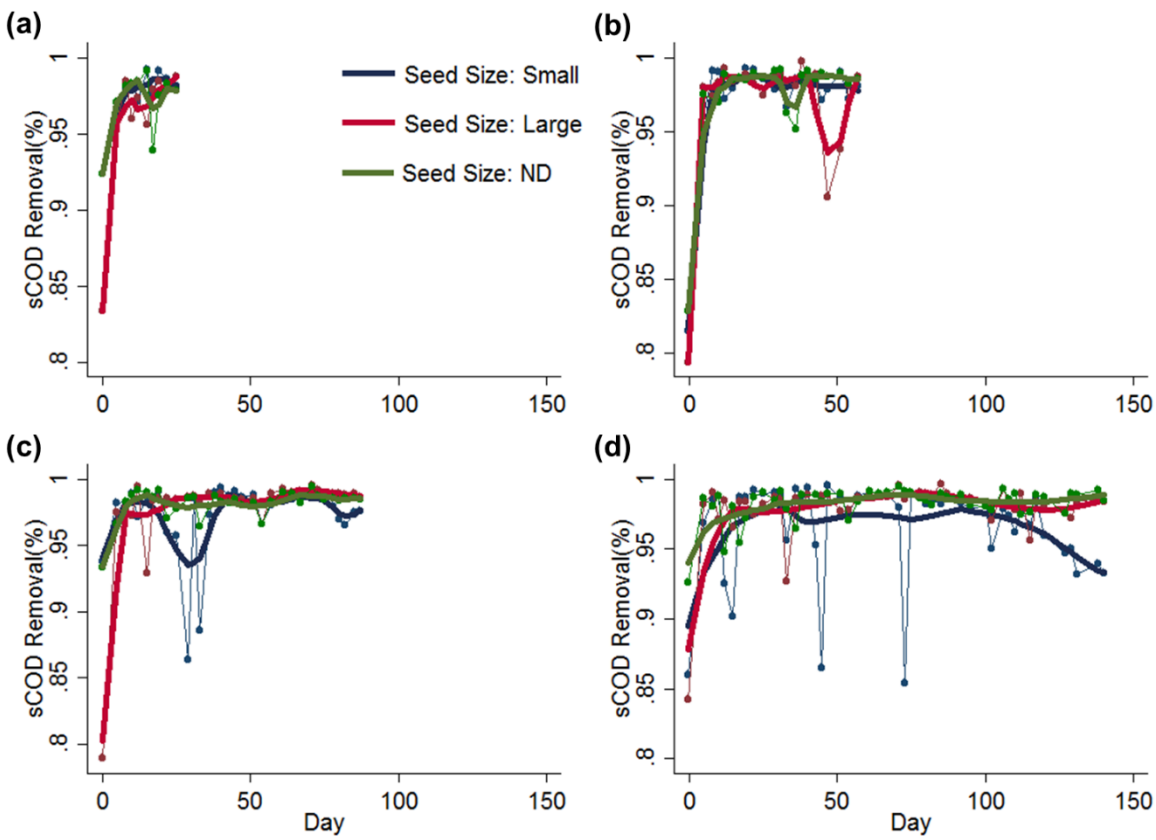

Figure 6. sCOD removal (%) in each bioreactor **(a)** S1, L1 & ND1. **(b)** S2, L2 & ND2. **(c)** S3, L3 & ND3. **(d)** S4, L4 & ND4. Figures show corresponding locally weighted scatterplot smoothing lines.

Table 5. Summary statistics regarding reactor performance

Day	S1	S2	S3	S4	L1	L2	L3	L4	ND1	ND2	ND3	ND4
sCOD Removal (%)												
0-25	97.19 (2.84)	96.65 (5.14)	97.24 (1.74)	95.74 (4.44)	95.88 (4.34)	96.53 (5.73)	95.62 (5.90)	96.32 (4.30)	96.80 (2.30)	95.40 (6.20)	97.15 (2.71)	96.78 (2.33)
26-57		98.09 (0.79)	96.60 (4.38)	97.28 (3.68)		97.70 (2.74)	98.48 (0.62)	98.04 (1.75)		98.30 (1.26)	98.04 (0.74)	98.31 (0.87)
58-88			98.09 (0.86)	97.43 (4.01)			99.02 (0.23)	99.00 (0.32)			98.56 (0.50)	98.88 (0.37)
89-140				96.25 (1.85)				98.09 (0.89)				98.47 (0.61)
Effluent pCOD (mg/l)												
0-25	1990 (1420)	1624 (964)	2454 (2703)	2419 (1667)	1112 (525)	1731 (1154)	1565 (915)	1841 (1650)	1294 (1144)	1486 (1473)	1311 (677)	943 (729)
26-57		1917 (1392)	1947 (742)	1848 (752)		1520 (716)	1749 (1448)	1679 (802)		1495 (438)	1768 (1695)	1010 (340)
58-88			1542 (551)	1956 (953)			1889 (587)	1523 (780)			1371 (730)	1213 (287)
89-140				1443 (420)				1403 (867)				950 (382)
Methane Production (ml/h)												
0-25	300 (90)	317 (132)	317 (134)	328 (98)	311 (168)	295 (83)	300 (75)	348 (135)	331 (133)	326 (110)	329 (108)	323 (81)
26-57		323 (79)	361 (79)	379 (43)		271 (128)	329 (42)	387 (34)		381 (56)	369 (55)	374 (99)
58-88			309 (63)	303 (41)			300 (54)	307 (51)			356 (50)	338 (44)
89-140				327 (56)				343 (59)				321 (74)

Mean values during period of bioreactor operation indicated in column 1. Standard deviations are presented in parenthesis beneath each value.

The pH was high (pH 8-9) in all bioreactors for the first 6 days of the but soon dropped, and remained consistently between 7 and 8 (Fig. 7). Concentrations of lactose, ethanol and VFAs were generally very low in the effluent but some VFA accumulation was observed in RS1-4 and RL1-4 (Table 6).

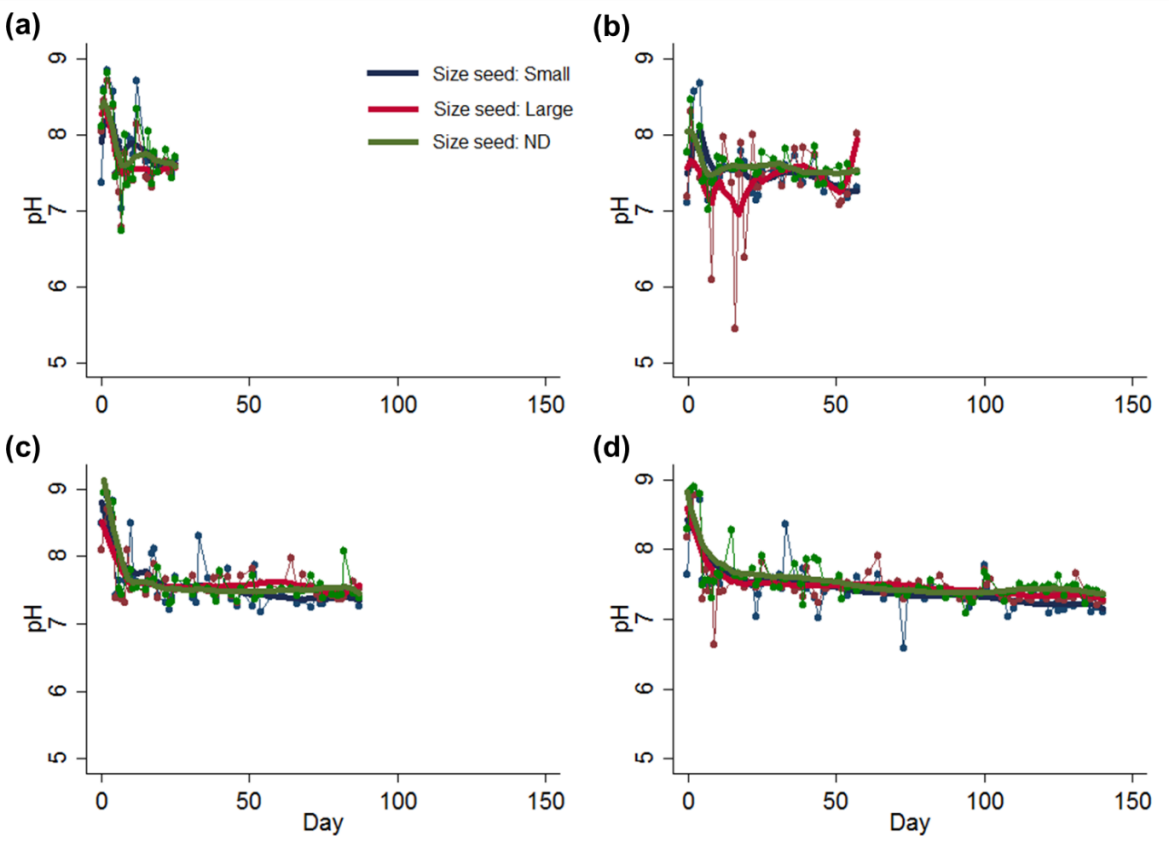

Figure 7. Effluent pH in each bioreactor **(a)** S1, L1 & ND1. **(b)** S2, L2 & ND2. **(c)** S3, L3 & ND3. **(d)** S4, L4 & ND4. Figures show corresponding locally weighted scatterplot smoothing lines.

Table 6. Effluent metabolite concentrations

Day	S1	S2	S3	S4	L1	L2	L3	L4	ND1	ND2	ND3	ND4
Lactose (g/l)												
8	*	*	*	*	*	*	*	*	*		*	
33		*	*	*		*	*	*		*	*	*
67			*	*			*	*			*	*
73			*	*			*	*			*	*
102				*				*				*
124				*				*				*
Ethanol (g/l)												
8	*	*	*	*	*	0.048	*	*	*		*	
33		*	0.086	0.048		*	*	*		*	*	*
67			*	*			*	*			*	0.069
73			*	0.134			*	*			*	*
102				0.091				*				*
124				0.066				*				*
Acetic Acid (g/l)												
8	*	0.050	*	*	*	0.073	0.130	*	0.026		*	
33		0.020	0.238	0.058		*	0.027	0.075		*	0.034	*
67			*	*			*	0.026			*	0.462
73			*	1.505			*	0.024			0.043	*
102				0.112				0.039				*
124				0.113				*				0.063
Propionic Acid (g/l)												
8	*	*	*	*	*	0.058	0.024	*	*		*	
33		*	0.351	0.155		*	*	0.087		0.034	*	*
67			*	0.038			*	*			*	*
73			*	0.739			*	0.020			*	*
102				0.040				*				*
124				0.038				*				*
Butyric Acid (g/l)												
8	*	*	*	*	*	*	*	*	*		*	
33		*	0.033	*		*	*	*		*	*	*
67			*	*			*	*			*	*
73			*	0.130			*	*			*	*
102				*				*				*
124				*				*				*

* represents concentrations below the limit of detection. L=Large; S=Small; ND=Natural Distribution.

The concentration of methane in the biogas remained at approximately 70% throughout the trial in all bioreactors (Fig. 8). The absolute volume of methane also remained stable (Fig. 9).

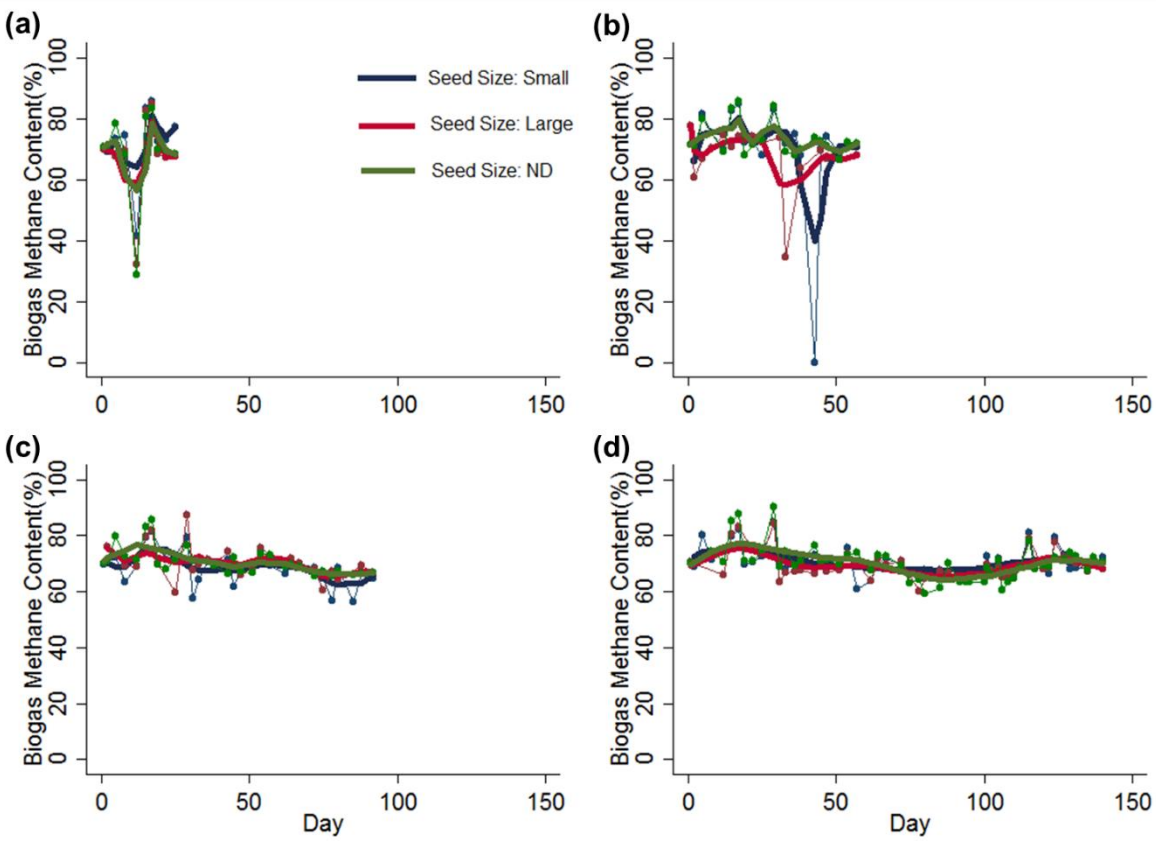

Figure 8. Biogas methane content in each bioreactor **(a)** S1, L1 & ND1. **(b)** S2, L2 & ND2. **(c)** S3, L3 & ND3. **(d)** S4, L4 & ND4. Figures show corresponding locally weighted scatterplot smoothing lines.

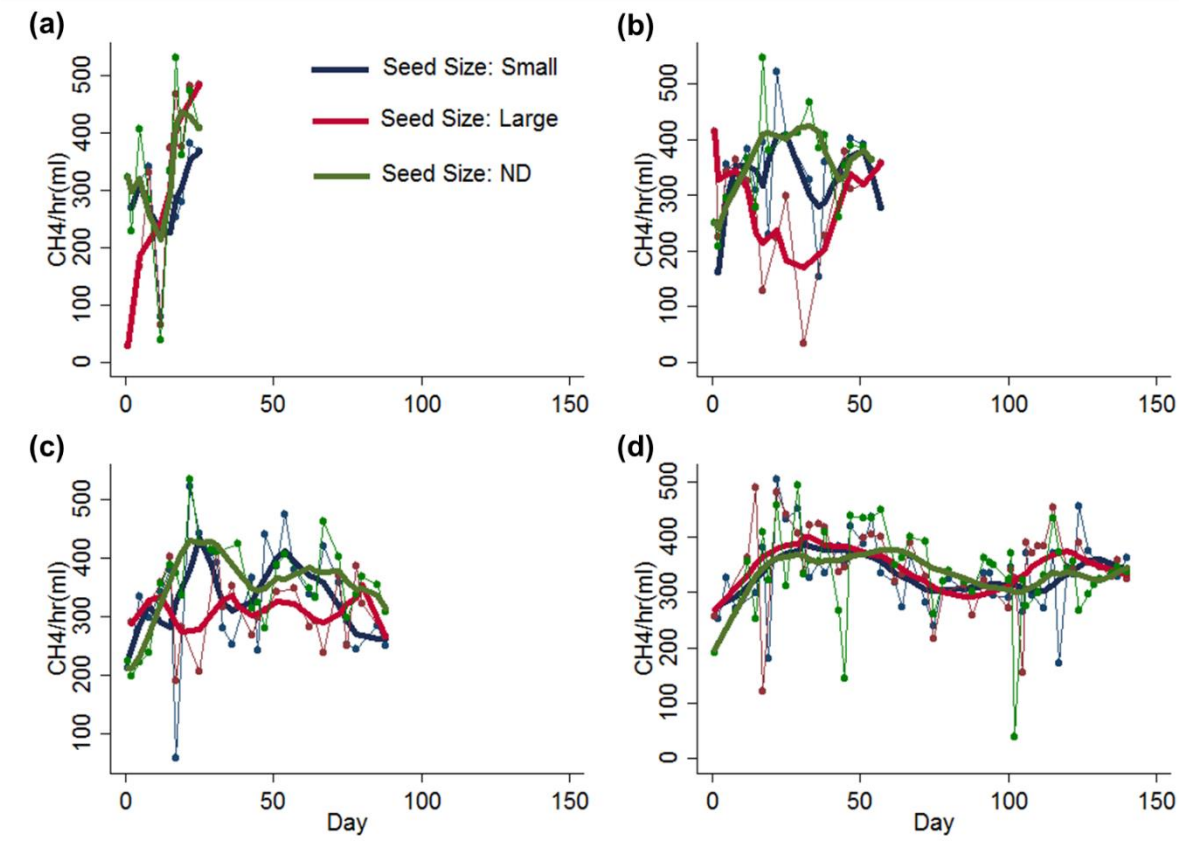

Figure 9. Absolute methane volume in each bioreactor **(a)** S1, L1 & ND1. **(b)** S2, L2 & ND2. **(c)** S3, L3 & ND3. **(d)** S4, L4 & ND4. Figures show corresponding locally weighted scatterplot smoothing lines.

Although the influent was almost entirely soluble, biomass washout, in the form of effluent particulate COD remained reasonably high (1000-2000 mg/L) across all bioreactors (Fig. 10). More variability in biomass washout was apparent in RS1-4 and RL1-4, which generally had higher standard deviations for mean pCOD values during each stage of the trial (Table. 5) but this improved over the course of the trial. Although the extent of biomass washout was substantial, a net increase in the sludge volume of RL1-4 and RND1-4 and a net loss of biomass in RS1-4 was observed (Fig. 10).

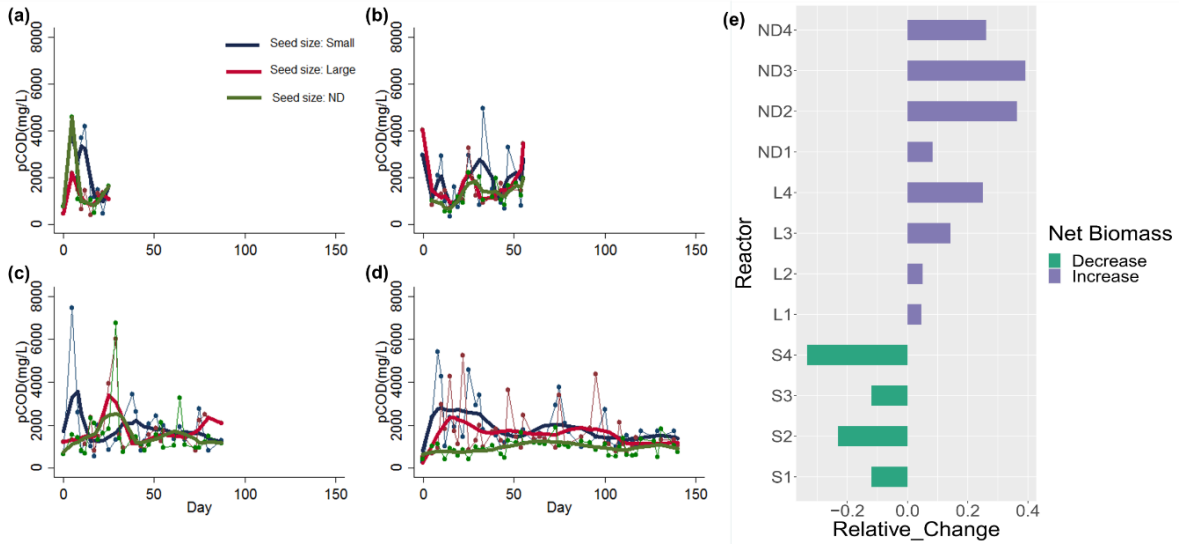

Figure 10. pCOD washout for all bioreactors **(a)** S1, L1 & ND1. **(b)** S2, L2 & ND2. **(c)** S3, L3 & ND3. **(d)** S4, L4 & ND4. Figures show corresponding locally weighted scatterplot smoothing lines. **(e)** Bar chart showing relative increase (or decrease) in biomass volume in each bioreactor.

4 Discussion

4.1 Size Distribution

The minimum size for microbial aggregates to be considered granules was previously suggested as 0.34 mm (Bhunia and Ghangrekar, 2007). Since the XS bin in the current study consisted of all biomass < 0.4 mm, this bin was considered as flocculent or "pre-granular" rather than granular. The XS sludge in RS1 after just 25 days of operation could have originated from granule breakage or from growth of planktonic organisms and indicates that granule growth is not strictly cyclical as it is unlikely that this fraction arose entirely from the break-up of XL granules, since there was not sufficient time for enough XL granules to develop (Fig. 3). It may be the case that at least some of the new XS biomass emerged from planktonic cells whilst others emerged following the break-up of larger granules (of any size). Nonetheless, some XL granules were recovered from RS1 (Fig. 3), which demonstrated rapid granule growth (from a maximum granule diameter of 1.2 mm at seeding to at least 3.15 mm in one month).

In addition to the formation of XS biomass from planktonic cells, the concurrent decrease of XL granules and increase of XS biomass in RL2-4 (Fig. 3) does indicate that the break-up of XL granules also contributed to the larger proportion of XS biomass. Breakage is likely caused by several factors, such as substrate limitation at the granule core (Doloman et al., 2017; Guiot et al., 1992b) or the increased collision force of larger individual granules. An increase in the proportion of XS and XL sludge was observed in RND1-4 (Fig. 3), which could indicate that these sizes are linked and the proportion of XS sludge is dependent on the volume of XL sludge available to provide initial nuclei after shearing.

Different granule sizes have been suggested to have separate roles in bioreactors – particularly smaller granules and planktonic biomass, which have been proposed to

specialize in acidogenesis (Guiot et al., 1992a; Zhu et al., 2017). Therefore, as granules grow, their function within the granule meta-community of the bioreactor may change, meaning that a full complement of granule sizes may be required for optimal performance. Granule growth has previously been hypothesized to be the result of an equilibrium between surface biomass sloughing caused by abrasion or shear forces, and internal microbial growth (Arcand et al., 1994). Therefore, overall granule growth would result in an increase in the abundance of microorganisms at the core of the granule, and lower relative abundance of organisms at the surface. Communities at the core of the granule are generally believed to be acetoclastic and methanogenic (Díaz et al., 2006a; Guiot et al., 1992b), whereas the outer layer of anaerobic granules is generally considered to comprise of acidogens (Batstone et al., 2004; McAteer et al., 2020; McHugh et al., 2003). Therefore, a granular system, started up with a size-constrained inoculum, such as in the current study, may be initially lacking in certain activities or skewed toward others. This potential dysbiosis caused by artificially altering the granule size distribution may have impacted performance.

4.2 Physical Characterisation

4.2.1 Scanning Electron Microscopy

Several characteristics were identified which were strongly associated with different granule sizes (Table 3) and a granule age classification system was proposed (Table 4). Young granules (<1mm) were often irregular in shape and had an open surface with visible individual cells and may be considdered to be more similar to flocs than traditional granules. Young granules also often resembled chunks of older granules indicating that they had arisen friom granule disintegration. The ‘partial skin’ or ‘skin’ features were a strong indicator for the formation of juvenile granules (1-2mm). It is unclear from initial

observation what the outer skin comprised. However a smooth, outer layer/skin has been previously reported in anaerobic granules, and was generally thought to consist of a layer of more densely packed cells and EPS (Baloch et al., 2008; Fang et al., 1995a; Fang et al., 1995b; Subramanyam and Mishra, 2013). Mature granules of 2-3mm were almost all rounded in shape and had begun to developm signs of granule damage such as cracking, peeling and wrinkling (Fig. 4). Old granules (3-4mm) were often significantly damaged with cracks and large fissures but with less occurences of the wrinkling or peeling features (Fig. 4). It is likely that the 'wrinkled' or 'peeling' features were not absent but were simply masked by significant breaking/cracking in the largest granules. It is also likely that some of these features were functions of the granule fixation process. However, their correlation with size is strong (Table 3) and therefore it is likely that underlying characteristics of granules, which have previously been proposed, such as a hollow or dead core (Doloman et al., 2017), led to the formation of the features. These data are observational and can be considered subjective. However, the development of such a classification system, perhaps using computational image analysis, could be extremely useful in determining the average "age" of granular sludge and providing information on the general health of the sludge bed in anaerobic bioreactors. For example, differently sized granules could have implications for reactor performance (Table 4). Such a methodology might provide an early warning system for the disintegration of granular sludge which is often an issue in full-scale applications (Macarie et al., 2018).

4.2.2 Volatile Solids and Settling Velocity

VS content was highest in Fraction A (<.2mm) which were also the most likely to indicate the 'irregular' and 'open' characteristics in SEM analysis (Fig. 5) . It may be the case that this fraction is mostly comprised of flocculent biomass from growth of planktonic, thus

cells accounting for its high VS content. This fraction also had the lowest settling velocity and, therefore, was likely to be located at the top of the sludge bed. The lower VS content of fractions B and C indicates those granules contained more inorganic material, however the proportion of organic to inorganic material then increased in fractions D-M. The higher VS content of fraction A may indicate that it is mostly comprised of flocculent growth. Whereas fractions B and C maycave been composed of the inorganic fractions of broken, larger granules which were heavy and were retained in the reactor.

4.3 Bioreactor Performance

All bioreactors performed well in terms of sCOD removal (>95%) throughout the trial (Fig. 6). However, some differences were observed which could be attributed to seed size, namely, RND1-4 performing in a more stable manner overall (Fig. 6) (Table 5). Some VFA accumulation also occurred in the size-constrained reactor sets (Table 6) , indicating that a full comliment of granule sizes is required for optimal performance. The performance of RS4 did decline towards the end of the trial (Fig. 6), with some VFA accumulation and decreased sCOD removal which was attributed to sustained biomass loss throughout the course of the trial. The high pH values observed during the first six days of the trial were attributed to naturally occuring buffering compounds in the seed sludge (caused by calcification in the full scale reactor) which were eventually washed out, resulting in the pH stabalising at 7-8. Metabolite concentrations in the effluent were often below the limit of detection (Table 6), indicating that most of the lactose added, and intermediary organic acids produced, were consumed. However, some VFA accumulation was observed, particularly in RS1-4 and RL1-4, underscoring their instability relative to RND1-4. RS4 especially indicated VFA accumulation toward the

end of the trial when sCOD removal was also lower, which may be have been due to the loss of less settleable biomass and resultant process instability.

Although the bioreactors were performing well, with efficient sCOD conversion to CH_4, biomass was persistently washed out over the course of the trial, particularly in RS1-4 and RL1-4 (Fig. 10). Based on the well-established theory of selection pressure (Hulshoff Pol et al., 1988), lighter, less settleable sludge is continually washed out of a bioreactor selecting for denser, compact aggregates in the sludge bed. The higher level of biomass loss in RS1-4 is therefore unsurprising as smaller granules were less settleable and, thus, prone to washout. On the other hand, however, it would have been expected to see less washout in RL1-4 than RND1-4, at least initially. The high levels of washout in RL1-4 could be due to the immediate (i.e. by Day 25) break-up of large granules and formation of smaller, less settleable sludge. This may also have provided smaller particles to act as nuclei for new granule formation.

The initial instability observed in RS1-4 and RL1-4 may have been due to microbial community dysbiosis associated with the constrained size distribution after sieving. The higher levels of washout in these reactor sets potentially indicates that fewer particulate granule pieces were retained and assimilated into the process of continual biofilm development.

4.4 Biomass Dynamics and the Granule Life-cycle

4.4.1 Biomass Dynamics and Influence of Seed Size

Over the course of the trial the size distribution of L4 had evolved to become more similar to that of ND4 (Fig. 11). Nonetheless, RS4 contained a much smaller proportion of XS granules, likely due to biomass washout. The poorer performance of RS4 toward the conclusion of the trial could indicate that XS biomass was important for bioreactor performance, and that washout resulted in less efficient sCOD removal and accumulation of some VFAs. The planktonic community of UASB bioreactors has been investigated previously and was suggested to have the primary functions of hydrolysis and acidogenesis (Zhu et al., 2017). This may also be the case for the XS biomass in this study and may explain the poorer performance of RS4, and the initially poorer performance of the S and L bioreactor sets since the constrained size profile of the seed sludge in those bioreactors excluded XS biomass.

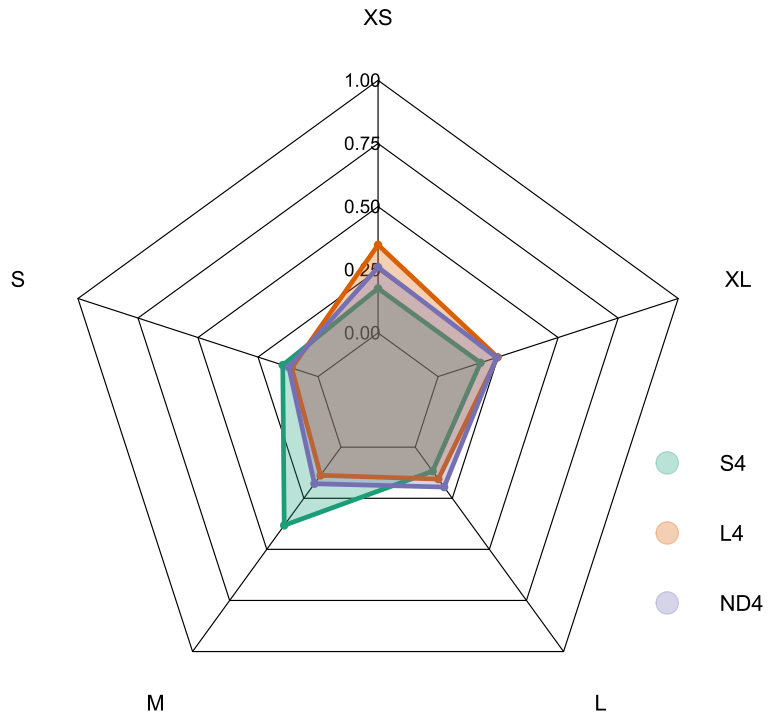

Figure 11. Radar chart illustrating granule size distributions of RS4, RL4 and RND4, where proportions of each sludge size are indicated on the XS axis.

A ‘critical size’ hypothesis for aerobic granules was proposed by Verawaty et al. (2013) in sequencing batch reactors (SBRs), which stated that granules in a given system will equilibrate to an optimal size distribution and this will be maintained by cycles of granule formation and breakage. Therefore, there may be an innate size distribution associated with distinct reactor conditions. The size distribution in RL4 and RND4 may be close to this innate equilibrium (Fig. 11). If this is the case, RS4 appeared to not reach such an equilibrium, possibly due to biomass washout or the short nature of the trial. However, it may also be the case that the artificial nature of the initial seeding, influenced the prevailing conditions in RS1-4, resulting in a different innate size distribution at equilibrium. Thus, although the size-constrained nature of the respective seed sludge’s in RS1-4 and RL1-4 was experimentally appropriate to test our hypothesis, it nonetheless likely set up differing prevailing conditions in the bioreactors. Specifically, for example, washout and net sludge loss in RS1-4 altered the sludge loading rate (SLR). Granule size was previously shown to correlate negatively with increasing SLR (Arcand et al., 1994), and it is therefore likely that changing SLRs impacted on size distributions. All bioreactors were seeded with 15gVS/L, providing a sludge loading rate of 0.76 gCOD g^{-1}VS d^{-1}. However, RS1-4 lost on average 20% biomass (Fig. 10), thus increasing the SLR. Assuming the same VS/TS concentration as at the point of seeding, this would have provided an SLR of 0.95 gCOD g^{-1}VS d^{-1}. The net biomass washout observed in RS1-4 and net biomass accumulation in RL1-4 and RND1-4, further compounded the differences in conditions between bioreactor sets.

Upflow velocity, hydrodynamics and sludge bed fluidization also play important roles in determining granule size (Arcand et al., 1994; Wang et al., 2016). In the present study, the upflow velocity was identical in all bioreactors, but differences in the seed size profiles provided for different fluidization patterns between the bioreactor sets. The RS1-4 sludge beds were more expanded, with more granule movement, influencing the extent and nature of

particle-particle contact (Fig. 12). This, coupled with changing SLRs, may have further shaped the innate size distribution in RS1-4. The results are similar to those of Verawaty *et al*. (2013) who found that bioreactors seeded only with small granules performed worse, and the size distribution did not converge with bioreactors seeded with large granules or a mixture of sizes. However, it may also be the case that a longer trial would allow for the size distribution of RS4 to equilibrate.

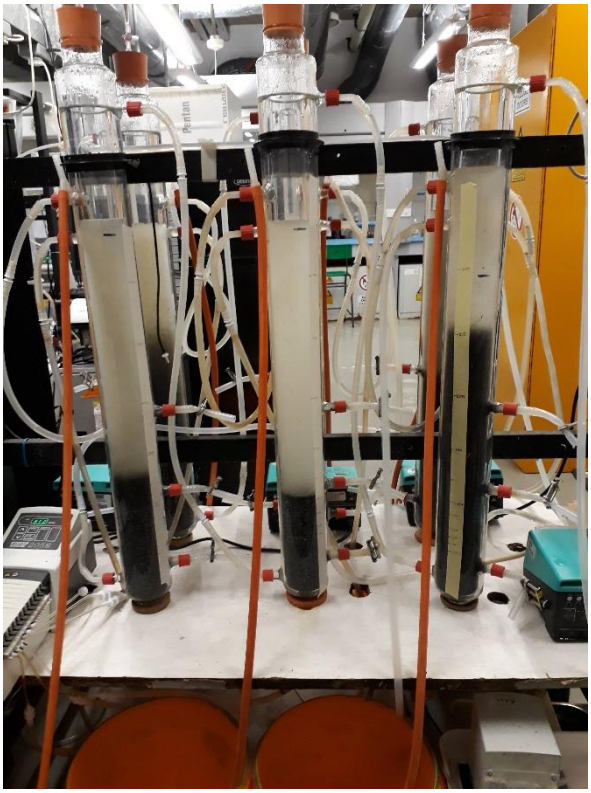

Figure 12. Differences in fluidization patterns of small (S) (right), large (L) (middle) and natural distribution (ND) (left) seed sludge.

4.4.2 A granule life-cycle?

Granule growth, and diversification of size-constrained sludge, was previously observed under stressed conditions (Trego et al., 2020a). In the present study, the development of the size profile in RL4 toward a distribution similar to RND4 indicates that the continual process of biofilm development results in a range of granule sizes at an equilibrium determined by reactor

conditions. Furthermore, the relatively better performance of the RND bioreactors suggests a full complement of granule sizes at equilibrium is required for optimal performance.

The concept of a strict granule life-cycle, where only XL granules break to form XS particles may be too simplistic to usefully describe the apparently continual process of granulation. It is likely that granulation occurs in several ways. For example, a granule of any given size may break to provide founder material for new granules, or new granules may originate from planktonic cells. In this study it appears that the XS bin comprised of planktonic and flocculent biomass, and small granule pieces. This biomass likely played a role in the formation of new granules perhaps by entangling broken granule pieces and initiating granule formation at the surface of the sludge bed. Indeed, the role of flocculent biomass in such bioreactors is often overlooked and more research is required on its role in granule formation.

Zheng et al. (2006) suggested a life-cycle for anaerobic granulation whereby a granule nucleus was initiated by *Methanosaeta sp* and subsequently colonized by syntrophic propionate oxidizers, and finally by an outer layer of fermentative bacteria. The authors proposed that once granules reached a certain size they decay and break, providing more *Methanosaeta*-based nuclei. This was based on observations of free-floating biomass, which contained filamentous, fermentative bacteria and small aggregates. It was also suggested that this fermentative community had a negative impact on granulation by reducing the settleability of granules, leading to washout. A similar mechanism was proposed by Trego et al. (2020a). We propose a similar mechanism with the exception that the role of the planktonic and flocculent organisms (XS) is of more importance than usually suggested. The XS biomass may have a role in entangling fragments of established granules and acting as primary consumers, providing substrates to methanogens. However, in-depth microbiological analysis will be required to assess this.

5 Conclusions

Granule growth in size-constrained bioreactors proceeded rapidly. RL1-4 achieved a size distribution similar to the naturally-distributed profile, but the profile of RS1-4 granules was constrained or delayed. Granule size strongly associated with morphological characteristics, and a potential classification system was proposed toward assessing the 'health' of granular sludge. Continual granule growth, disintegration and re-formation involving each of the sizes (or life-stage proxies), suggested a more complex process than a simple life-cycle revolving around breakage of XL granules into XS founding material. An innate size distribution is associated with bioreactor performance, which appeared to rely on a full complement of granule sizes.

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CHAPTER 4

A Distinct Microbial Community is Involved in the Formation of Methanogenic Granules

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1 Introduction

Chapter three identified several morphological characteristics strongly associated with granule size and confirmed that a full complement of granule sizes is required for optimal bioreactor performance. In addition, the XS community was hypothesized to be important for granulation and may be distinct from the granular community. However, a number of outstanding research questions still remain, specifically related to ecological aspects of granulation including knowledge gaps which were identified after an extensive literature review in chapter 2 (Chapter 2 Section 4.1). In depth ecological analysis via 16S rRNA gene and gene transcript sequencing may provide answers and aid in the formulation of an ecological granulation theory.

Methanogenic communities underpin efficient waste to energy conversion in upflow anaerobic bioreactors and are often present in the form of anaerobic sludge granules. Granules are spherical microbial aggregates approximately 0.5 - 4mm in size, each containing all of the necessary organisms for the complete conversion of organic contaminants in to methane (Trego et al., 2020b). Retention of well-functioning microbial biomass is essential for such bioreactors and the immobilization of microbial communities into dense, settlable granules allows high upflow velocities to be applied without biomass washout (van Lier et al., 2015). Difficulties in achieving granulation or even granule disintegration and washout can occur, with detrimental effects on bioreactor performance (Macarie et al., 2018). Therefore, the process of granule formation (granulation), granule disintegration and re-granulation requires further investigation. A comprehensive outline of early granulation mechanisms was published by Hulshoff Pol et al. (2004) and research on granulation is still underway (Faria et al., 2019a; Sudmalis et al., 2018; Wang et al., 2018). However, these mechanisms often lack an ecological understanding and also rarely take into account the fate of established granules. It is unlikely that a particular granule, once formed, remains intact in a bioreactor for the entirety of its operation and probably breaks apart at some point (Trego et al., 2020a). The fate of the resultant

granule particles and release of internal granule communities, which were previously isolated from other granule communities, could be important for granulation and a driver of microbial diversity in reactors.

The formation of traditional, surface attached biofilms is well defined and widely accepted (Flemming et al., 2016). However, even though granules are considered biofilms (Davey and O'toole, 2000; Flemming et al., 2016; Leventhal et al., 2018), the five main steps of traditional biofilm formation (initial attachment of cells to surfaces; irreversible attachment through EPS production; early formation of biofilm architecture; biofilm architecture maturation and; dispersal of single cells (Stoodley et al., 2002)) cannot be applied directly. The importance of EPS production has been established (Ding et al., 2015; Sheng et al., 2010), and the concentric layers (Sekiguchi et al., 1999) and channels (Wu et al., 2016) in methanogenic granules could be considered analogous to architectural features of traditional biofilm matrices. However, initial formation/attachment and dispersal steps clearly occur by different mechanisms in granules, due to their suspended nature. Therefore, it seems logical that to address these knowledge gaps it is essential to study growing granules of different sizes i.e. small and large. Granules of different sizes are rarely sampled and therefore it is unknown whether microbial communities of granules change during different stages of growth or how cycles of granule growth and breakage contribute to the ecology of the whole reactor. Studying granules undergoing active growth and disintegration would address this knowledge gap and also allow patterns in community succession to be identified, facilitating the formation of an ecological granulation model.

Patterns of community succession have previously been observed in complex microbial communities (Datta et al., 2016; J. Zhou et al., 2014). Datta et al (2016) identified stages of community succession on organic marine particles, whereby primary consumers initiated colonization and facilitated the succession of secondary consumers which utilized metabolic

products of primary consumers. Similar complementary metabolisms occur widely in anaerobic digestion (Zelezniak et al., 2015) and cooperation and mutualism have are considered drivers of diversity in methanogenic communities (Embree et al., 2015). It is possible that a sequence of community succession could facilitate granule formation, where planktonic organisms along with particulate matter such as pieces of old, disintegrated granules aggregate to initiate the development of small granules, which then grow through stages of community succession.

In the present study, differently sized granules were sampled from 12 laboratory scale (2L) bioreactors to assess community dynamics during the growth, maturation and break up of granules. The aims were to 1) determine if a distinct microbial community is associated with granule formation; 2) determine the fate of disintegrated granules 3) determine the impact of granule disintegration on total bioreactor diversity; 4) characterize the microbial communities associated with granules in different stages of development and observe patterns of community succession. It was hypothesized that separate microbial communities would be associated with granules at different stages of development, and different taxa would have distinct roles in community succession. Additionally, a distinct pre-granular community would be associated with granule formation.

2 Materials & Methods

2.1 Reactor Trial

Details relating to bioreactor configuration, set-up of the bioreactor trial and bioreactor performance were reported previously (Chapter 3). Temporal changes in granule size distribution, volatile solids content (VS), settling velocity, and biofilm ultrastructure (scanning electron microscopy (SEM)) were monitored and were also reported in detail previously (Chapter 3). Size distributions in each of the size-restricted bioreactor sets diversified significantly beyond their original range (Chapter 3, Fig. 3) and tended to revert back to a distribution similar to that of the natural distribution (Chapter 3, Fig. 10). XS biomass was generally flocculent and upon examination, contained chunks and pieces of larger granules indicating that this fraction was made up of both flocculent growth, and pieces of broken granules. For the purposes of this study the XS fraction is therefore considered pre-granular rather than granular.

2.2 DNA Extraction, cDNA Synthesis, PCR, Sequencing

Methodology for sampling of granular sludge from bioreactors was outlined previously (Chapter 3, Section 2.2). Genomic DNA and RNA were co-extracted according to the protocol described by Griffiths *et al* (2000). Samples previously snap frozen in liquid nitrogen (Chapter 3, Section 2.2) were crushed using a glass rod in a micro-centrifuge tube to obtain a homogeneous mixture. Cells were lysed by beat beating in a 1% cetyltrimethylammonium bromide (CTAB) lysis buffer followed by phenol-chloroform co-extraction. DNA and RNA concentrations were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

Samples from the final takedown (RS4, RL4 and RND4) were used for analysis of the "active community" by sequencing cDNA which was reverse-transcribed from 16S rRNA gene transcripts. Prior to cDNA synthesis contaminating DNA was removed from RNA samples

using the TurboDNase kit (AMBION – Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Complete removal of DNA was confirmed by PCR using the primer pair 515F 806R (Caporaso et al., 2011). cDNA synthesis was carried out with the Superscript IV (SSIV) Reverse Transcriptase Kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions.

PCR amplification of DNA and cDNA was performed using the 515F 806R primer pair (Caporaso et al., 2011). PCR cycles were as follows; Initial denaturation was performed for 3 minutes at 95˚C. Followed by 25 cycles of; denaturation at 95˚C for 30s; annealing at 55 ˚C for 30s; and extension at 72 ˚C for 30s. Library preparation and sequencing was carried out by The Foundation for the Promotion of Health and Biomedical Research of Valencia Region, (FISABIO) (Valencia, Spain). Sequencing was done on the Illumina MiSeq platform.

2.3 Bioinformatic Analysis

Amplicon Sequencing Variants (ASVs) were constructed using Qiime2 workflow. In the final analysis, 1 954 726 clean ASVs were extracted for n=240 samples on which different multivariate statistical analyses (Section 2.4) were performed using R software. The details of the bioinformatics and sequence processing steps are available at https://github.com/umerijaz/tutorials/blob/master/qiime2_tutorial.md.

2.4 Statistical Analysis

The vegan package (Oksanen et al., 2015) was used to assess alpha and beta diversity. Alpha diversity was assessed using the following indices: (i) rarefied richness – an estimation of the total number of ASVs in a rarefied sample (to minimum library size); (ii) Shannon entropy – a commonly used diversity index; (iii) Pilou eveness – a comparison of the actual diversity values to the highest possible diversity value, constrained from 0 to 1.0, whereby lower values indicate more variation in abundance between different ASVs in each group. Beta diversity was

assessed with Principal Coordinate Analysis (PCoA). ASVs were plotted with (i) the Bray-Curtis distance metric which considers only ASV abundance counts. Multivariate homogeneity of sample variability was assessed using Vegan's betadisper() function, this handles distances between objects and group centroids by reducing the original distances to principal coordinates and reporting significances based on Analysis of variance (ANOVA). ANOVA was carried out with Vegan's Adonis() function on distance matrices. This function, referred to as PERMANOVA, fits linear models to distance matrices contingent upon the explanatory variables (meta data) for a given study to assess how much of the variability in microbial community structure they can explain. Local Contribution to Beta Diversity (LCBD) analysis (Legendre and De Cáceres, 2013) was performed with the LCBD.comp() function from the adespatial package (Dray et al., 2016) with the Bray-Curtis distance metric. LCBD assesses how far the microbial community structure of one sample is from the average of all the samples, in this case in terms of ASV abundances.

Phylogenetic distances within each sample were assessed by calculating the nearest taxa index (NTI) and net relatedness index (NRI). This analysis determined if the community structure was stochastic (i.e. driven by competition among taxa) or deterministic (i.e. driven by strong environmental pressure). NTI was calculated with mntd() and ses.mntd(), and the mean phylogenetic diversity (MPD) and NRI were calculated using mpd() and ses.mpd() functions from the picante package (Kembel et al., 2010). NTI and NRI represent the negatives of the output from ses.mntd() and ses.mpd(), respectively. Additionally, they quantify the number of standard deviations that separate the observed values from the mean of the null distribution (999 randomisation using null.model-'richness' in the ses.mntd() and ses.mpd() functions and only considering taxa as either present or absent regardless of their relative abundance). A positive NTI value indicates that species co-occur with more closely related species than would be expected by chance, whereas negative values suggesting otherwise. NTI measures tip-level

divergences (putting more emphasis on terminal clades and is akin to "local" clustering) in phylogeny while NRI measures deeper divergences (akin to "global" clustering or "clumpedness"). For both NTI and NRI, values > +2 indicate strong environmental pressure, and values < -2 indicate strong competition among species as the driver of community structure

Sparse Projection to Latent Structure – Discriminant Analysis (sPLS-DA) was carried out using the MixOmics package (Rohart et al., 2017). Here, artificial latent components were constructed for predicted variables (ASVs) and response variables (sample groups) by factoring these matrices into scores and loading vectors in a new space to achieve a maximum covariance between the scores of these two matrices. Loading vectors (with piece-wise coefficient for each ASV) were constructed so that the coefficients indicate the importance of each variable to define the component. Non-zero coefficients for the loading vectors indicate ASVs which are significantly different between the categories and are deemed discriminants. The initial ASV table was prefiltered by removing 1% of ASVs with low counts according to the author's recommendations given at http://mixomics.org/mixmc/pre-processing/. Following this, the ASV table was normalised using Total Sum Scaling (TSS) on the ASVs and then Centered Log Ratio (CLR) (in conjunction these are referred to as TSS+CLR normalization) before applying the splsda() function. The perf.plsda() and tune.splsda() functions were initially used to predict the number of latent components (associated loading vectors) and the number of discriminants by initializing the perf.plsda() procedure with the total number components to be the number of groups used in the study. Then, the first two components were retained as the classification error rates were minimum for these using the centroid distance matrix in the procedure. The tune.splsda() function was then initialized with two components and using leave-one-out cross-validation.

Additionally, a study-wise comparison of reactors (RS1-4, RL1-4, and RND1-4) was done to find discriminating taxa between recovered granule sizes (XS, S, M, L, and XL). This was done

using the Multivariate Integration (MINT) algorithm (Rohart et al., 2017). The algorithm is an extension of the multi-group Projection to Latent Structure (mgPLS), similar to sPLS-DA and it attempts to find a common projection space across all studies (reactors as above), defined on a small subset of discriminative variables that consistently discriminate the outcome classes (granule sizes). In MINT, for every reactor set we have combined four datasets where all these datasets share the taxa whilst the number of samples differ. Similar to sPLS-DA, we have performed prefilteration of 1% of the lowest abundant taxa followed by TSS+CLR normalization. Differential taxa, identified by MINT analysis were visualized using differential heat trees (Foster et al., 2017) which compared the relative abundance of these taxa (using Wilcoxin p-value test) between different sample groups. The authors have applied these methods before with further details given in (Trego et al., 2020a).

Subset regression was performed against different microbiome metrics (Table 1) by testing all possible combination of the meta data in this study, and then selecting the best model according to some statistical criteria, with recommendations given in Kassambara (2018) with code available at http://www.sthda.com/english/articles/37-model-selection-essentials-in-r/155-best-subsets-regression-essentials-in-r/. Bioreactor performance data used in subset regression analysis is summarised as means and standard deviations from the final 21 days of operation of each bioreactor. This allowed bioreactor performance in the 21 days prior to bioreactor takedown and subsequent size distribution analysis to be analysed in the context of various microbiome metrics.

The R function regubsets() from the leaps package (Lumley and Miller, 2009) was used to identify different best models of different sizes, by specifying the option nvmax, set to the maximum number of predictors to incorporate the model. Having obtained the best possible subsets, the k-fold cross-validation consisting of first dividing the data into k subsets. Each subset (10%) served successively as test data set and the remaining subset (90%) as training

data. The average cross-validation error is then computed as the model prediction error. This was all done using a custom function utilising R's train() function from the caret package (Kuhn, 2005). Finally R's tab_model() function from sjPlot package (Lüdecke, 2019) was used to obtain the statistics for each model.

Table 1 Bioreactor performance data used in Subset Regression Analysis

Bioreactor Performance Metrics												
Day	**S1**	**S2**	**S3**	**S4**	**L1**	**L2**	**L3**	**L4**	**ND1**	**ND2**	**ND3**	**ND4**
sCOD Removal (%)	98 (0.93)	98 (0.72)	98 (0.95)	94 (1.07)	97 (1.25)	97 (3.14)	99 (0.25)	98 (0.55)	98 (1.51)	98 (1.22)	97 (0.35)	99 (0.58)
Effluent pCOD (mgCOD/l)	2178 (1369)	1673 (972)	1512 (670)	1551 (229)	1202 (530)	1685 (807)	1889 (643)	1141 (134)	1454 (1212)	1455 (439)	1145 (207)	1118 (468)
Methane Volume ($mlCH_4/h$)	294 (96)	323 (85)	277 (75)	361 (41)	343 (153)	319 (58)	318 (68)	348 (19)	354 (149)	364 (49)	344 (56)	324 (28)
Reported values averaged over final 20/21 days of reactor operation with standard deviations in parentheses.												

3 Results

3.1 Seed Sludge and Effect of Size-Separation

Bioreactor performance and granule characterisation was reported in detail previously (Chapter 3). The microbial communities of the starting (T0) size bins (XS, S, M, L & XL) were analysed to determine the potential impact of size separation on the evolution of subsequent microbial communities in each reactor. L seed sludge (T0 L DNA) was significantly lower in evenness and diversity than S seed sludge (T0 S DNA) but there was no significant difference in Richness (Fig. 1). L and S seed sludge also clustered separately based on principle component analysis using the Bray-Curtis distance metric (Fig. 1).

Discriminant taxa, which can be thought of as a subset of the community which explain most of the variation between samples, were identified by sPLS-DA (Fig. 2). Several of these were more abundant in the large granules than small granules including many methanogenic and syntrophic organisms (Fig. 2). The XS size fraction clustered separately from all other sizes in SPLS-DA and had higher abundances of genera within the *Firmicutes* and *Bacteroidetes* (Fig. 2).

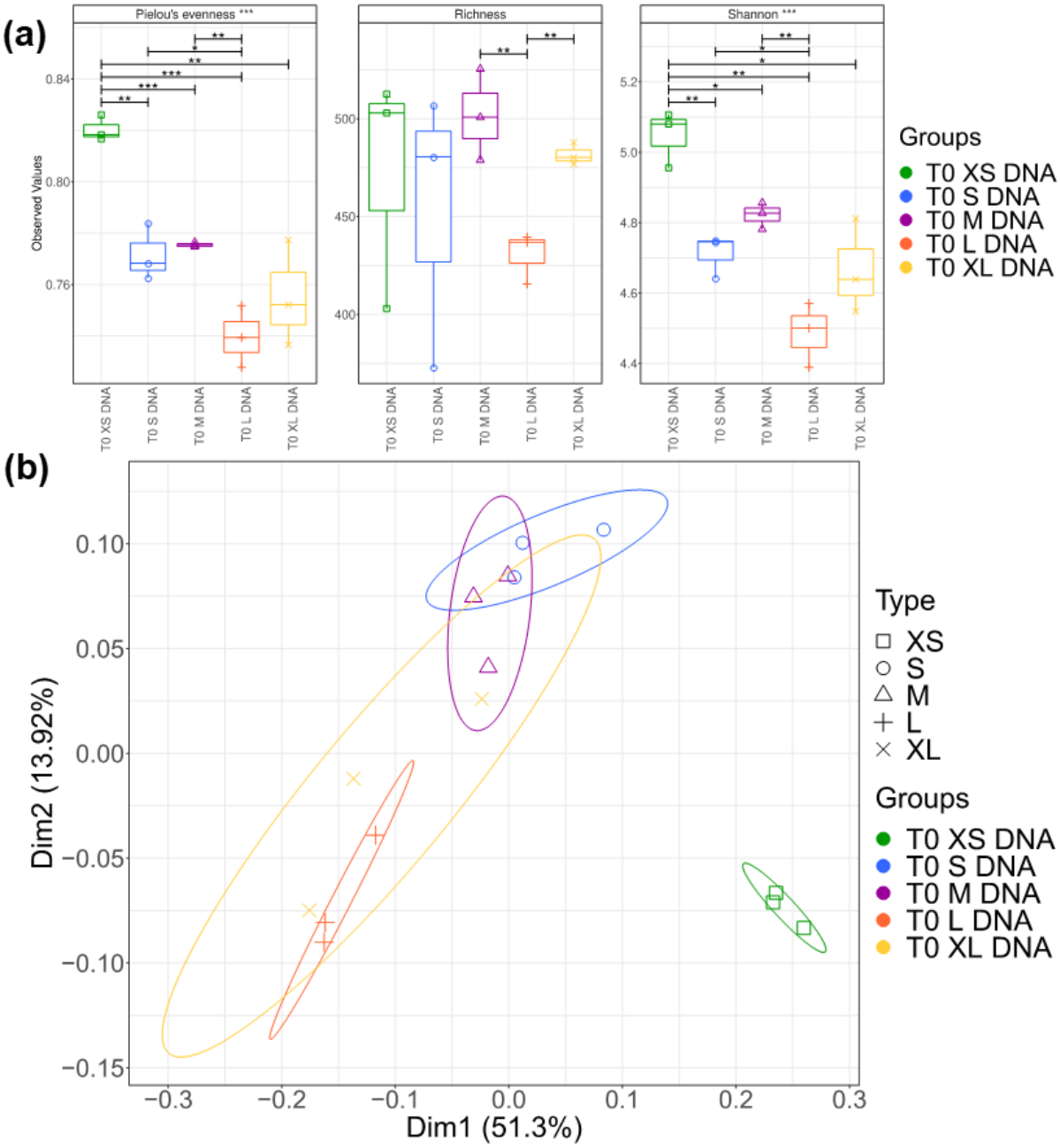


Figure. 1 (a) Alpha Diversity Indices; Pielou's Evenness, Rarefied Richness and Shannon Entropy (Lines of significance depict significant differences as follows: * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) based on ANOVA. **(b)** Principal Component Analysis (Bray-Curtis) of biomass of seed samples grouped by size, where ellipses were drawn using 95% confidence intervals based on standard deviation.

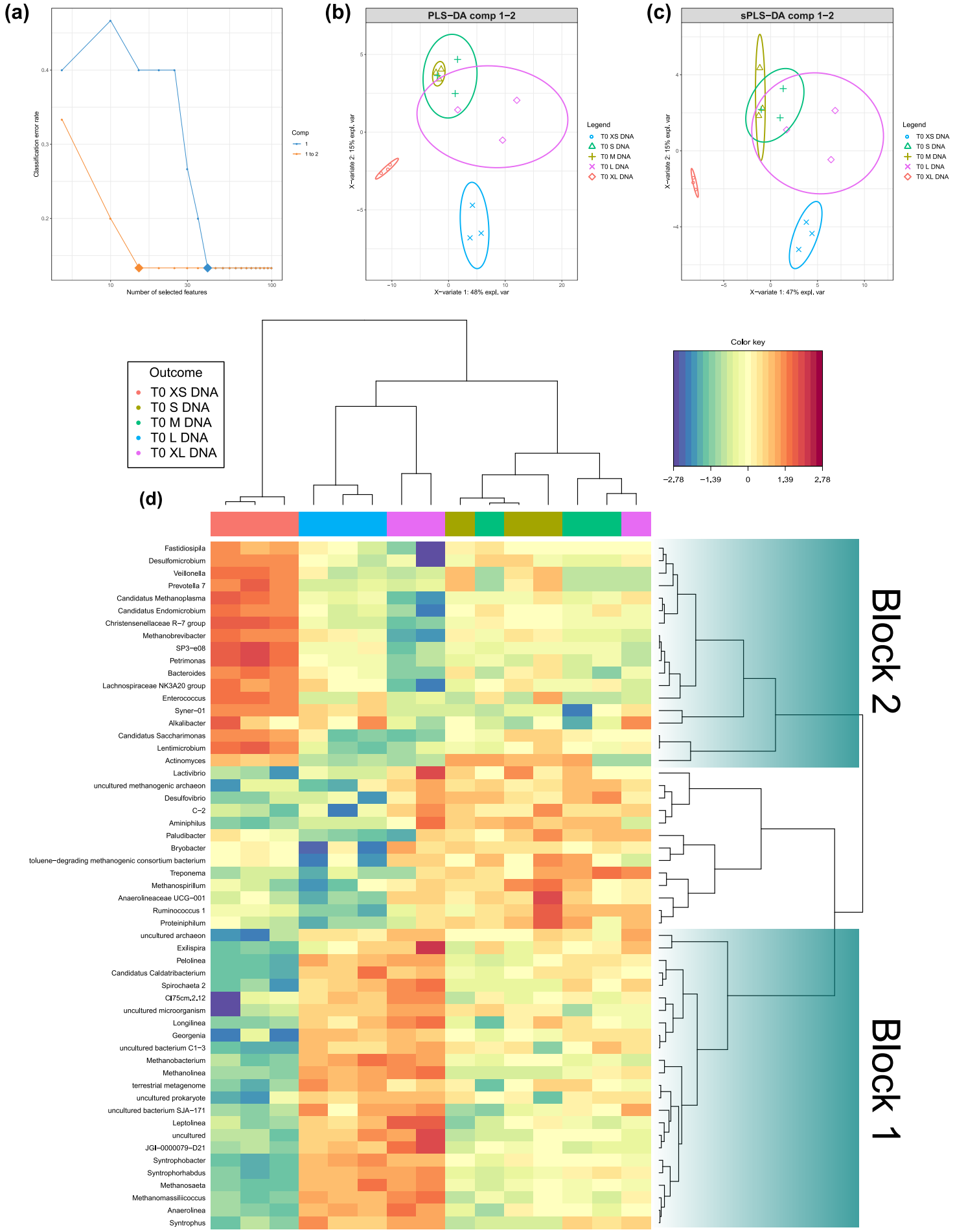


Figure. 2 (a) Classification error rates over the components and the numbers of optimal features (genera) in each component, included in the model, chosen by the lowest error rates which are denoted by diamonds. **(b)** Ordination of all genera **(c)** Ordination of discriminant genera only **(d)** Heatmap based on sPLS-DA of amplicon sequencing data depicting discriminant genera. Rows and columns are order according to hierarchical (average linkage) clustering to identify clusters of genera among groups. Block 1 contains discriminant taxa more abundant in L and XL granuls and Block 1 contains taxa more abundant in XS biomass

Once seeded, beta diversity in all reactors evolved in a similar fashion from takedown 1 to takedown 4, regardless of the seed source (Fig 3). Seed size had the least significant effect on the final microbial communities in comparison to other explanatory variables (reactor, granule size and takedown) according to PERMANOVA, indicating that effect of initial size separation was minor in comparison to other factors (Fig. 3).

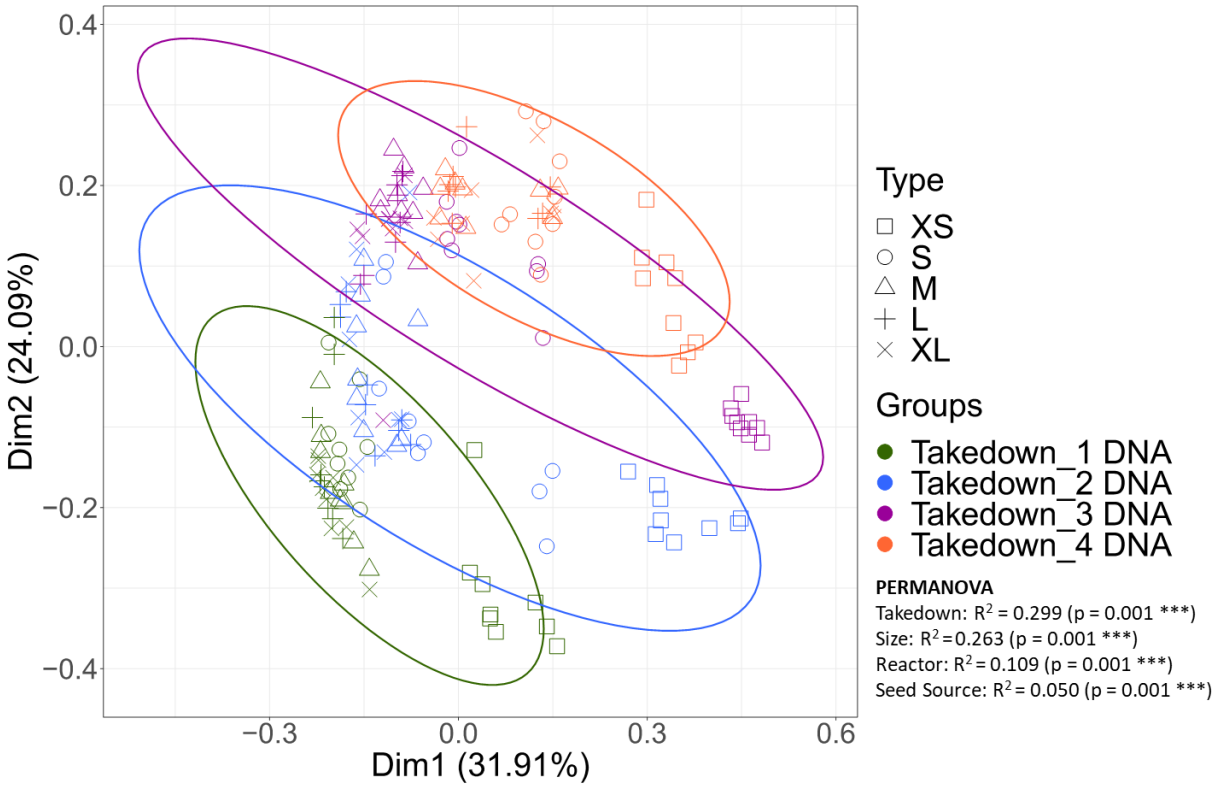


Figure. 3 Principle Component Analysis of Total Microbial Community. Reactors S1, L1 and ND1 are included in Takedown 1. Reactors S2, L2 and ND2 are included in Takedown 2 and so on.

3.2 General Trends in Diversity

Clear trends in alpha diversity were seen with each bioreactor takedown across all bioreactor sets (Fig. 4). Generally, species richness and Shannon entropy increased in granules of all sizes from Takedown 1 to Takedown 4, regardless of seed size (Fig. 4). However, diversity and richness was lower in S, M, L and XL granules from RS4 than the equivalent sizes in RS3. Beta diversity analysis showed community succession over time and clustering with respect to reactor and granule size (Fig. 4). High levels of environmental pressure (>2 NTI) were observed in all samples, and a general trend of increasing environmental pressure with granule size was apparent. (Fig. 4). Environmental pressure also decreased with each takedown, as alpha diversity increased.

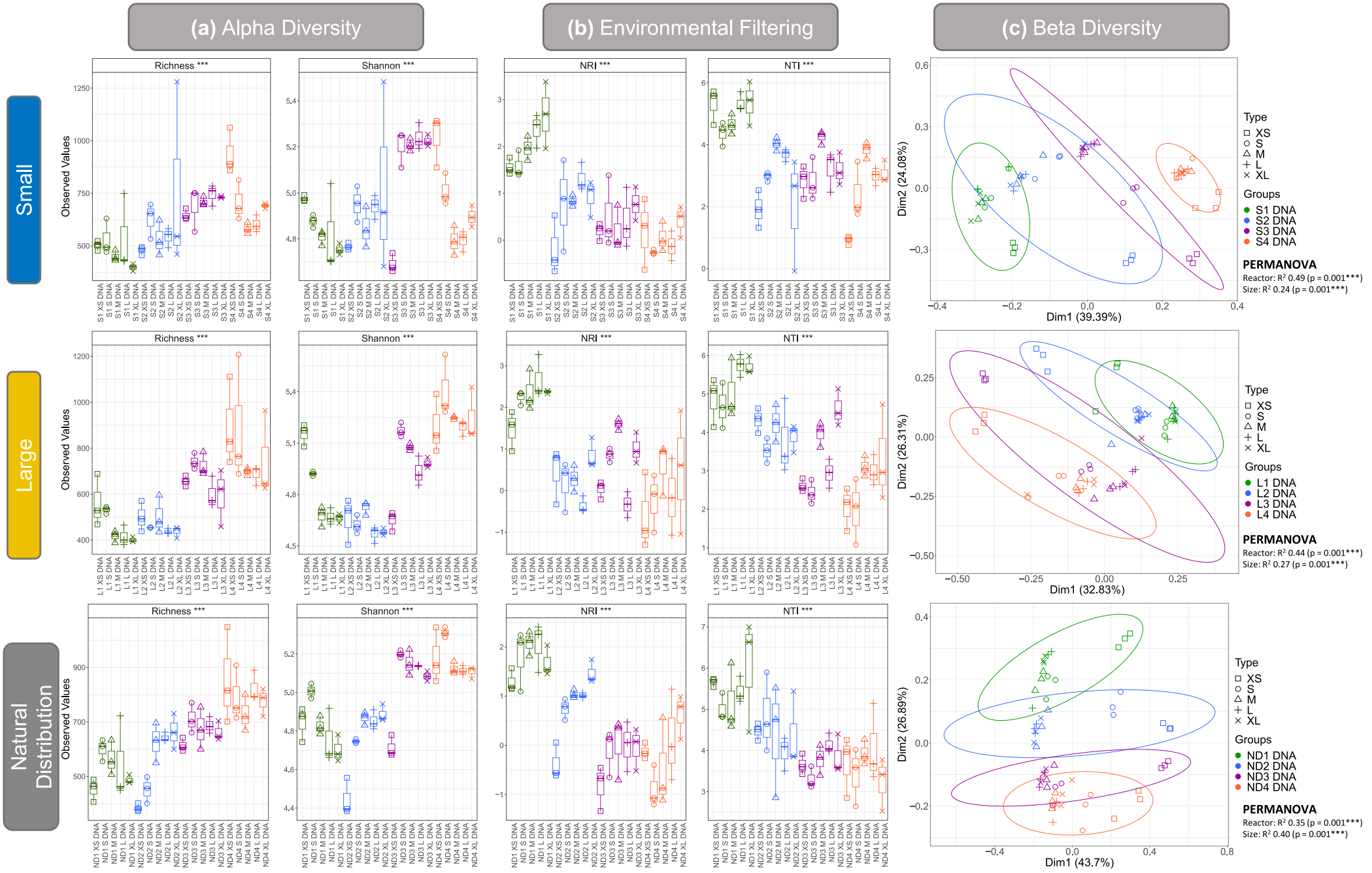
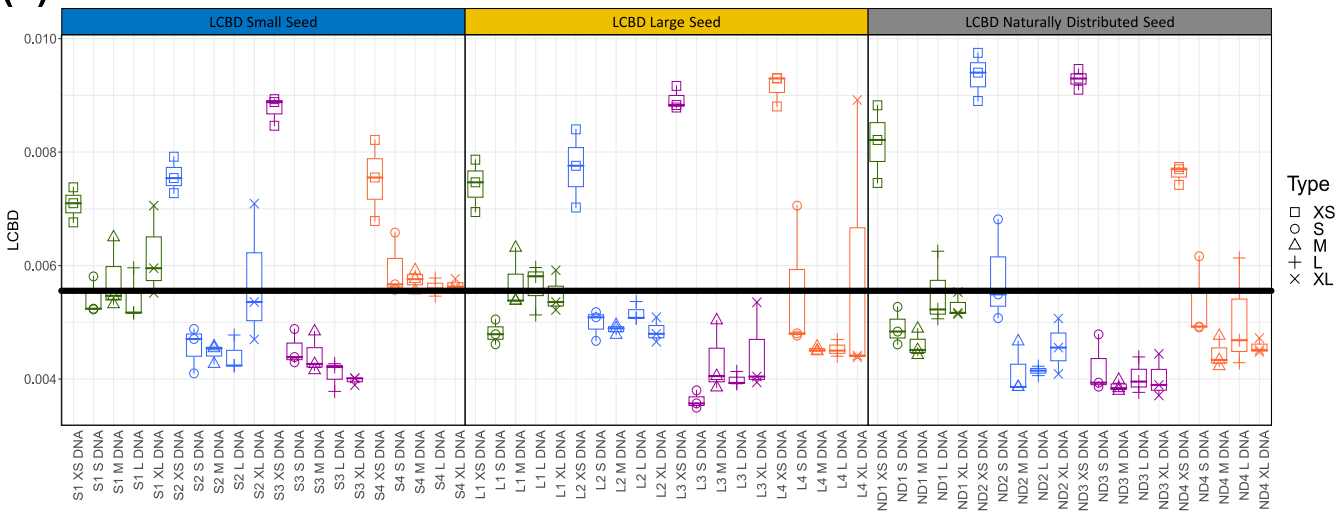


Figure 4. (a) Alpha diversity indices; Rarefied Richness and Shannon Diversity for all biomass sizes recovered from S, L and ND reactors. **(b)** Environmental Filtering Analysis; NRI and NTI values for all biomass sizes recovered from S, L and ND reactors. **(c)** Principal Component Analysis with samples grouped by reactor and biomass size, where ellipses were drawn using 95% confidence intervals basedon standard deviations

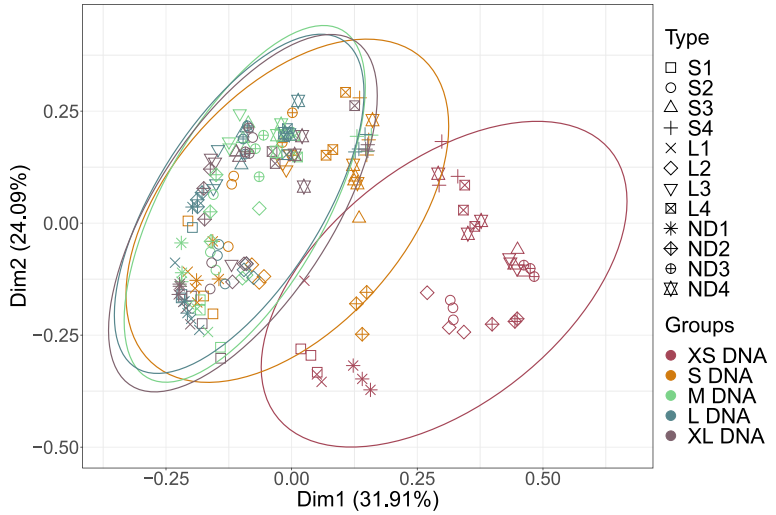
3.3 XS Biomass

XS biomass generally consisted of small particulates and flocculent biomass, and was observed on the top of the sludge bed throughout the trial (Chapter 3, Fig 2). The total community (DNA) of the XS biomass, from all reactors was clearly different from the rest of the granule sizes based on PCoA (Fig. 5). Of the other sizes, S granules were the most similar to XS indicating that they may indeed have originated from growth of XS biomass. Local Contribution to Beta Diversity (LCBD) analysis showed that XS granules contributed the most to mean beta diversity (Fig. 5). XS biomass made a significant positive contribution to total beta diversity and a significant negative contribution to total levels of environmental pressure (NRI & NTI), based on subset regression analysis (Fig. 5). This indicates that XS biomass was quite different in community composition from other size fractions and less influenced by the environment. S granules also had a negative contribution to overall levels of environmental pressure, whereas, M, L and XL granules generally had a positive contribution.

(a)



(b)



(c)

Subset Regression Summary		Alpha Diversity		Beta Diversity			Environmental Filtering	
		Richness	Shannon Diversity	LCBD (Bray-Curtis)	LCBD (Unweighted - Unifrac)	LCBD (Weighted - Unifrac)	NRI	NTI
Granule Size	Extra Small (XS)			+	+	+	-	-
	Small (S)		+				-	-
	Medium (M)				-			+
	Large (L)							+
	Extra Large (XL)				+			+
Performance Data	CH_4/Hr (ml)	-	-			-	+	
	sCOD Removal (%)			-	-	-	+	+
	pCOD Washout	-	-			-	+	

Figure 5. (a) Local Contribution to Beta Diversity (LCBD) of all DNA samples using the Bray -Curtis distance metric. **(b)** Principle Component Analysis (PCoA) using the Bray-Curtis distance metric of all DNA samples, grouped by biomass size, where ellipses were drawn using 95% confidence intervals based on standard deviation. **(c)** Subset Regression Analysis assessing the contribution of different sample types to different diversity metrics. + (Orange) boxes indicate a positive contribution by a particular sample type to a particular diversity metric. – (Blue) boxes indicate a negative contribution by a parti cular sample type to a particular diversity metric. Summar statistics which were used for subset regression analysis are available in Table 1

The community of the granular biomass (S, M, L, XL) had a higher relative abundance of archaea than the XS biomass. *Methanosaeta* (the only acetoclastic methanogen in the top 25 taxa), *Methanobacterium* and *Methanospirillum* sp were lower in relative abundance in XS biomass than the other granule sizes, whereas *Methanocorpusculum sp*. were more abundant in the XS biomass (Fig. 6). Several bacterial families appeared to be more abundant in the XS biomass, including *Streptococcaceae* and *Eubacteriaceae,* leading to a much higher abundance of Firmicutes at phylum level (Fig. 6). The candidate phylum FCPU426 and candidate genus Aegiribacteria (classified at phylum level in SILVA132 database) have higher in abundances in granular communities, as do *Geobacter sp*.

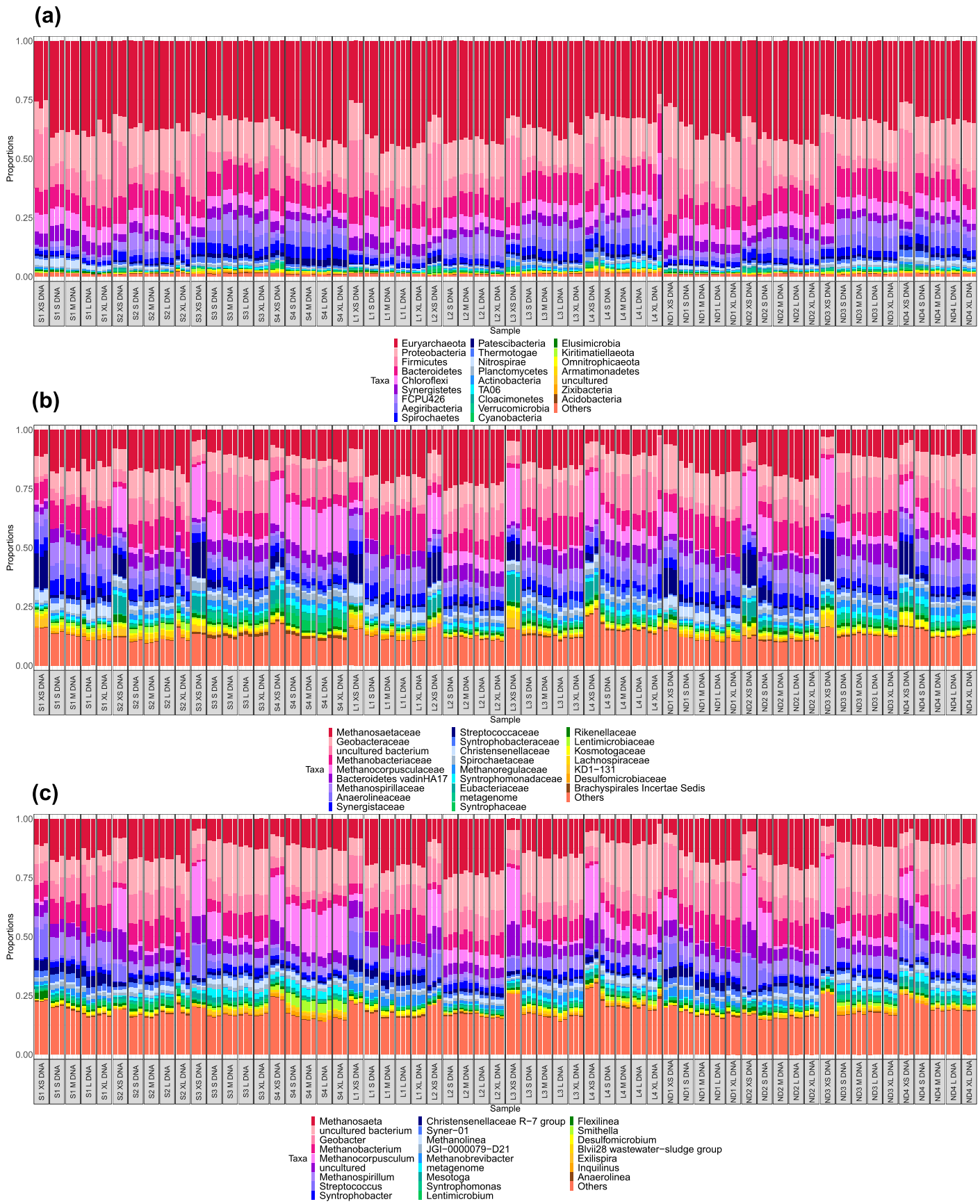


Figure. 6. Bar charts depicting the top 25 most abundant taxa at a) Phylum level b) Family and c) Genus, where others represents taxa outside of the top 25 most abundant organisms.

3.4 Discriminant Analysis

40 discriminant taxa which had a major influence in determining beta diversity were identified in the total microbial community (DNA samples only) at genus level (Fig. 7) by MINT analyisis. Two blocks (Block 1 & Block 2) were identified which show clear clustering of Genera within the XS biomass. Within block 1 *Streptococcus* and *Methanocorpusculum sp* were generally more abundant in XS biomass. Several taxa which were more abundant in XS biomass are also highlighted in block 2, including: *Lactococcus, Ignavibacterium, Spaerochaeta* and the families *Pedosphaeraceae* and *Rikenellaceae*. Taxa which appeared to be lower in abundance in XS biomass included; *Desulfomicrobium, Lentimicrobium, Longilinea, Anaerolinea, Syntrophorhabdus, Syntrophobacter, Methanosaeta, Methanobacterium, Methanospirillum* and *Aegribacteria*.

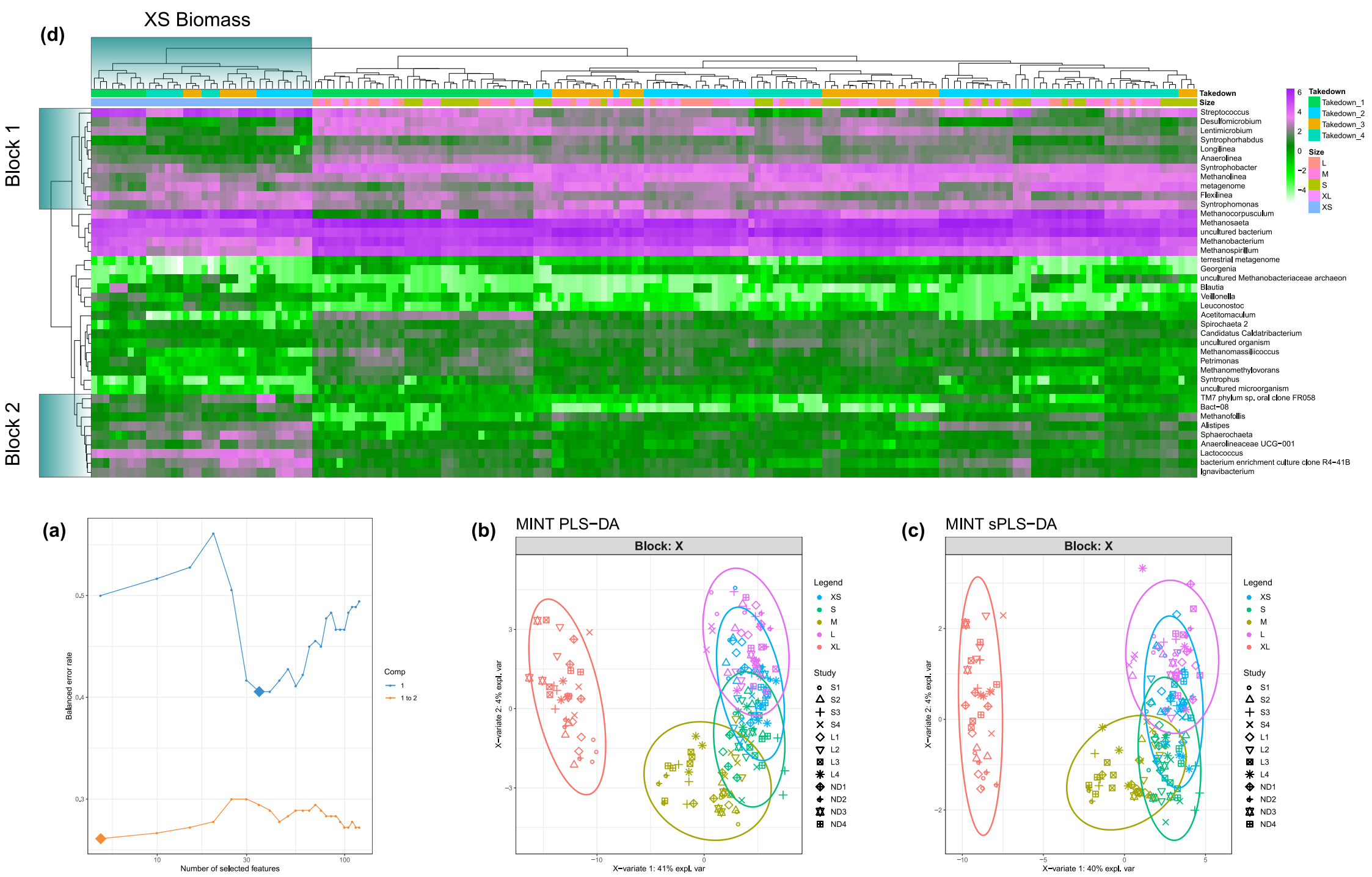


Figure. 7 (a) Classification error rates over the components and the numbers of optimal features (genera) in each component, included in the model, chosen by the lowest error rates which are denoted by diamonds. (b) Ordination of whole ASV table (c) Ordination of discriminant Genera only (d) Heatmap depicting discriminant Genera. Rows and columns are ordered according to hierarchical (average linking) clustering to identify clusters of Genera among groups. Block 1 and 2 indicate clustering of discriminant genera within the XS biomass group.

3.5 Analysis of the Active Microbial Community

DNA and RNA samples clustered separately in PCoA using the Bray-Curtis distance metric (Fig. 8). Similar to analysis of the total community the active portion of XS biomass clustered separately from the rest of the sizes. Alpha diversity was significantly lower in the active community, for all granule sizes in the final takedown. Of the most abundant taxa the relative abundance of *Geobacter* increased the most in the active community of all size fractions, as did *Methanosaeta* and *Smithella* sp. Although, *Smithella* were much more abundant in the S and L active communities than the ND active community.

87 discriminant ASVs were identified between the total and active communities of TD4 (Fig. 9) by MINT analysis. Many of these ASVs were more strongly associated with the active community, and are indicated in Block 1, including the Genera: *Geobacter, Lactococcus* and *Desulfovibrio,* indicating that these genera may have been more active than is apparent at DNA level.

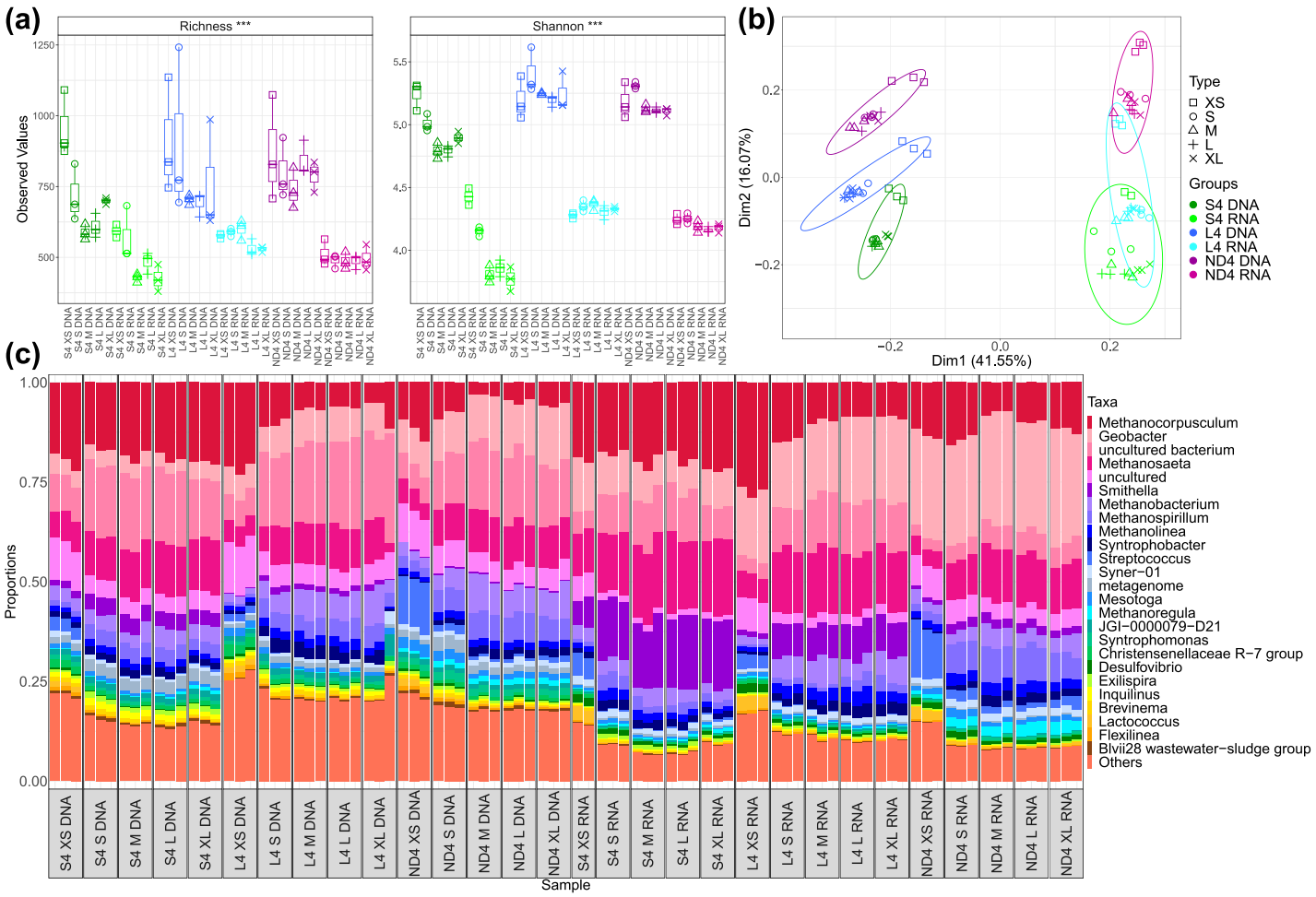


Figure. 8 (a) Alpha diversity indices; Rarefied Richness an Shannon Diversity for total (DNA) and active (RNA) communities in RS4, RL4 and RND4. (b) Principle Component Analysis (PCOA) of total (DNA) and active (RNA) communities in RS4, RL4 and RND4. (c) Bar charts depicting the top 25 most abundant genera in the total (DNA) and active (RNA) communities of RS4, RL4 and RND4 where others represents taxa outside of the top 25 most abundant organisms

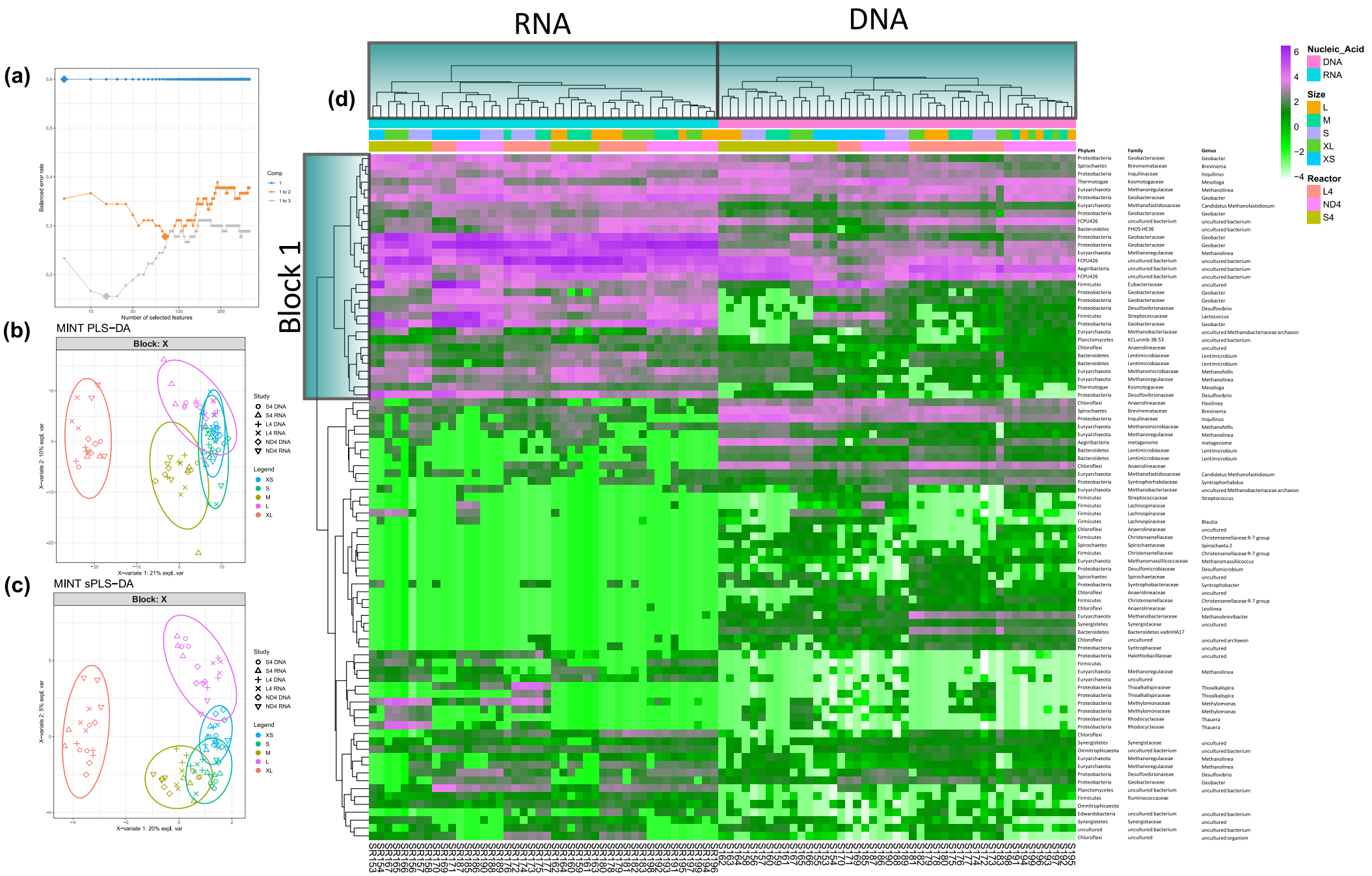


Figure. 9 (a) Classification error rates over the components and the numbers of optimal features (genera) in each component, included in the model, chosen by the lowest error rates which are denoted by diamonds. (b) Ordination of whole ASV table (c)Ordination of discriminant ASVs only (d) Heatmap depicting discriminant Genera. Rows and columns are ordered according to hierarchical (average linking) clustering to identify clusters of Genera among groups

3.5.1 Total Vs Active Communities of Each Recovered Size

The 87 ASVs identified as a result of total/active community MINT analysis (Fig. 9) were visualized as Heat Trees to allow for pairwise comparisons of phylogenetic differences between samples (Fig 10). Multiple members of the *Deltaproteobacteria* appeared to be universally more abundant in the active community of all granule sizes, including *Geobacter* and *Desufovibrio sp*. Several other members of the *Proteobacteria* including *Thauera* and *Methlyomonas* were also generally more relatively abundant in the active community, particularly in S granules. The *Streptococcaceae* family were also universally more abundant in the active community, across all sizes and reactors.

3.5.2 Differences in Active Communities of Different Sizes

Direct comparisons of the active communities only, of all granule sizes originating from RS4, RL4 and RND4 revealed distinct patterns in the active community (Fig 11). Active *Streptococaceae* appear more abundantly in XS biomass when compared to any other recovered size, in any reactor (Fig.11). *Geobacter* and *Desulfovibrio* were present in similar abundances between sizes. The other *Proteobacteria,* mentioned previously, including *Methylomonas* and *Thauera sp* were more abundant in S granules than any other granule size (Fig. 11)

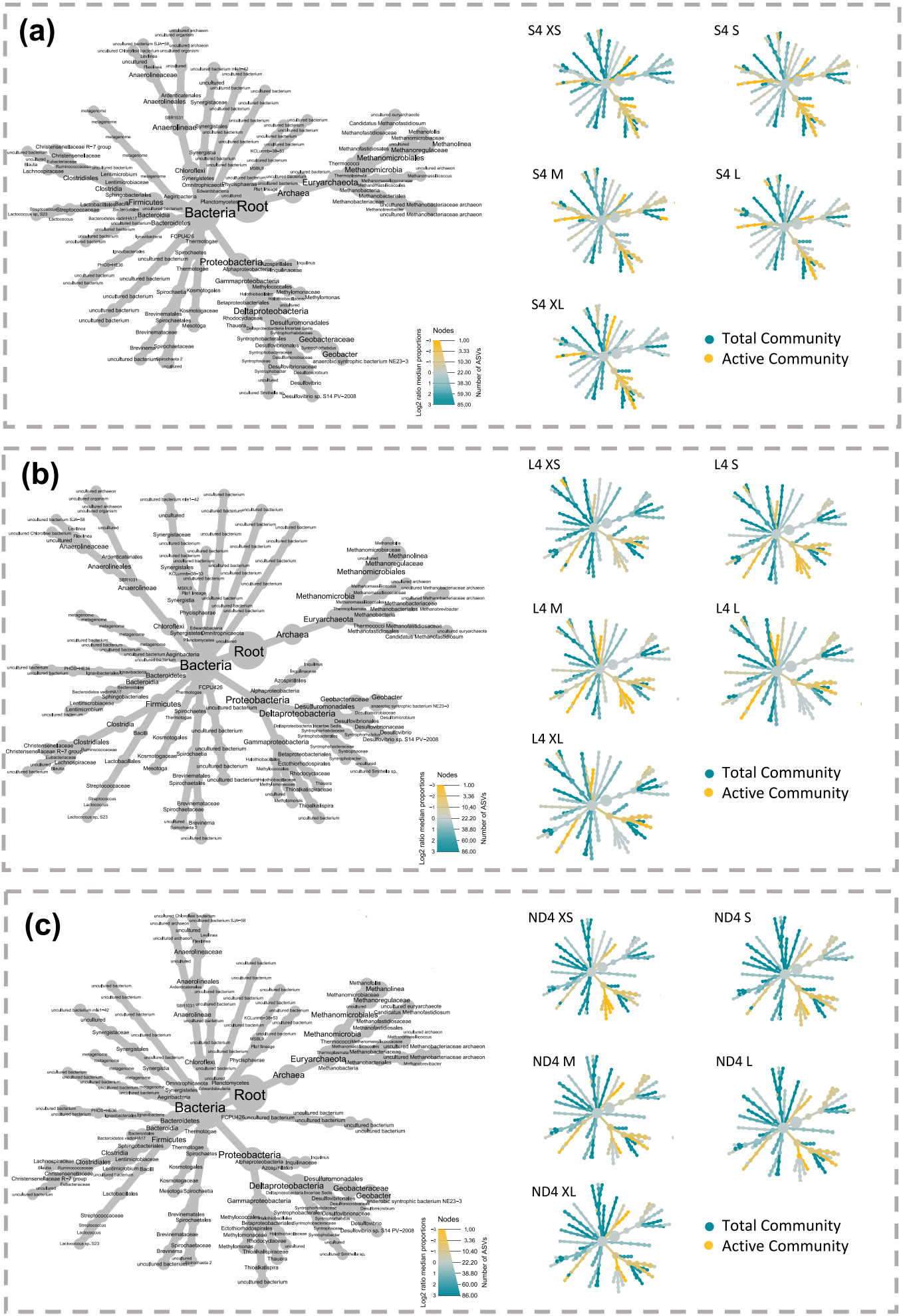


Figure. 10 Heat Trees depicting differential abundances of discriminant ASVs (identified by MINT analysis (Fig S5)) in the total (DNA) and active (RNA) community of all granule sizes from RS4, RL4 and RND4. The circle size and the colour intensity reflect the species abundance and the log2 median proportion between the two groups respectively

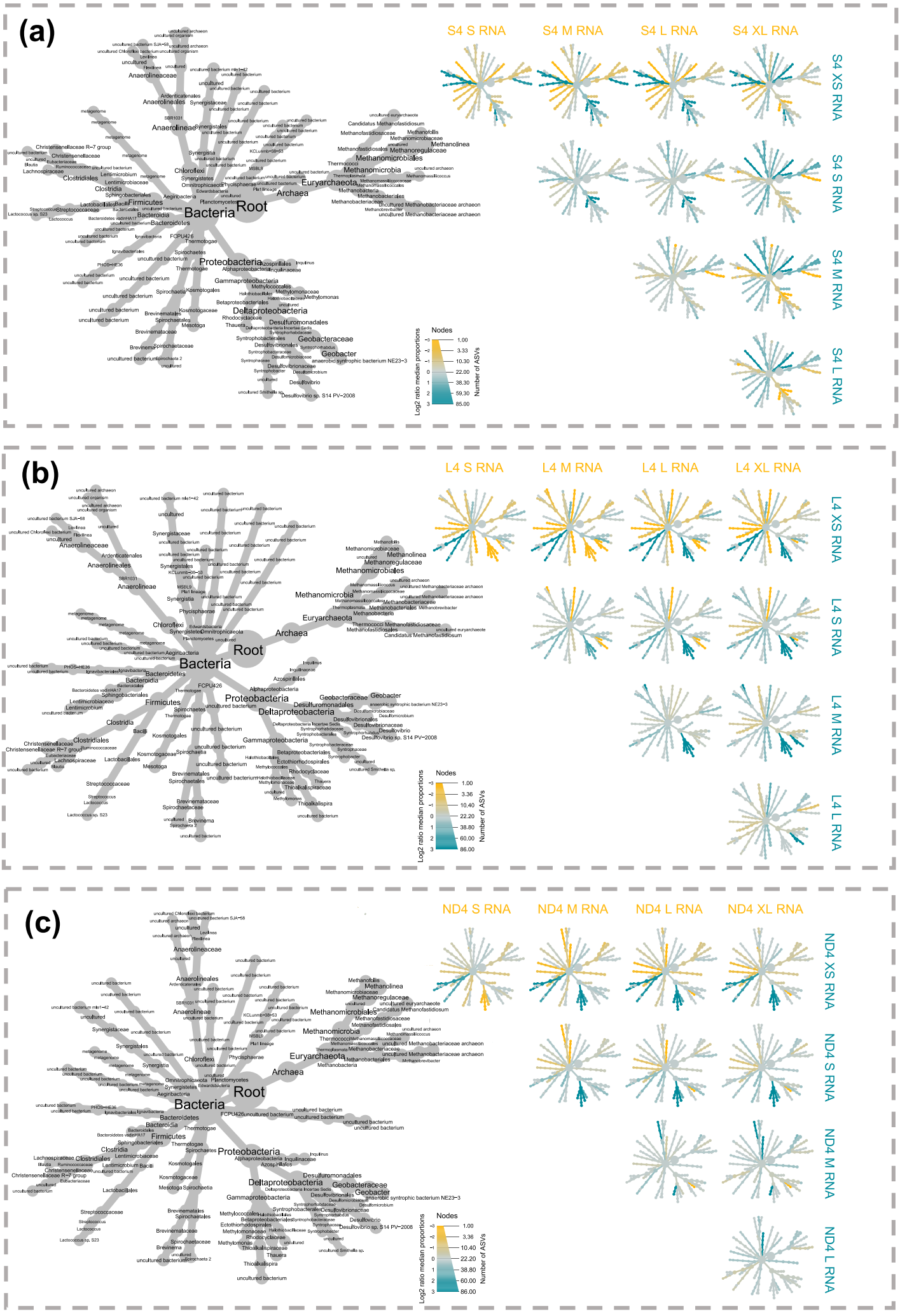


Figure. 11 Heat Trees depicting differential abundances of discriminant ASVs (identified by MINT analysis (Fig S5)) in the active (RNA) community of all granule sizes from RS4, RL4 and RND4. The circle size and the colour intensity reflect the species abundance and the log2 median proportion between the two groups respectively

4 Discussion

4.1 Influence of Granule Size-separation

ND1-4 performed better than either of the size constrained reactor sets (Chapter 3), which may have been more stable due to the presence of the XS biomass which was sieved out of the two size-restricted bioreactor sets prior to seeding. The XS community in the ND seed sludge had higher abundances of Firmicutes and Bacteroidetes (Fig. 2), including, *Prevotella, Petrimonas*, and *Bacteroides* sp which often have roles in the hydrolysis/fermentation steps of AD (Saha et al., 2020; Venkiteshwaran et al., 2016). Therefore, alteration of the seed by size separation, may have constrained the performance of RL1-4 and RS1-4 by inhibiting the initial steps of the AD pathway. The XS fraction of the seed was also the most diverse fraction (Fig.1). Lower levels of diversity in S and L seed could also explain the higher performance variability (Chapter 3, Fig. 6, Fig. 10) in those bioreactors, compared to RND1-4, as higher diversity is generally thought to increase stability in ecological systems (McCann, 2000). Beta diversity analysis showed that samples clearly clustered by reactor (Fig. 4) and takedown (Fig. 3) and it was apparent that takedown (incubation time), rather than seed size, appeared to have caused the most variation in samples (Fig. 3).

4.2 Granulation as a Driver of Diversity

Increased performance stability over time in the S and L bioreactors could have been due to development of a full complement of granule sizes (Chapter 3, Fig. 3) and resultant increases in microbial community diversity (Fig. 4). This, in turn may have led to more functional redundancy and flexibility in the microbial community which enabled more stable performance and could make the system more robust to shocks (De Vrieze et al., 2017). This poses the question of what was driving the diversification over time and could it be controlled to improve stability? Diversity generally increased in reactors which had lower biomass washout (pCOD), indicating that diversity and biomass dynamics may be linked (Fig. 5). Biomass washout

decreased over the course of the trial in RL1-4 and RND1-4 resulting in a net increase in biomass in these reactor sets (Chapter 3, Fig. 10). RS1-4 however, all had net decreases in sludge volume (Chapter 3 Fig. 10) and RS4 is the only reactor which had a decrease in diversity relative to the previously sampled reactor. RS4 also experienced a drop in sCOD removal towards the end of the trial (Chapter 3, Fig. 6). It may be the case that the prolonged sludge loss from RS4 led to this drop in diversity and performance which was not observed in any other reactor. High levels of phylogenetic clustering were observed in all samples, possibly caused by high levels of high levels of environmental pressure (>2 NTI), indicating deterministic influences on community assembly (Li et al., 2018; Meyerhof et al., 2016) (Fig.4). The degree of global clustering was generally lower in all samples (<2 NRI) indicating that environmental pressure was mainly having an effect in terminal clades, rather than at a deeper phylogenetic level. Environmental pressure was generally highest in the first takedown, indicating the strong influence seeding had on the microbial community. As environmental pressure decreased over time, alpha diversity increased, indicating that increased stochasticity during time of operation led to diversification in all reactors (Fig. 4). A general trend of environmental pressure increasing with granule size was also seen in all reactors (Fig. 4). XS and S granules also had a negative contribution to total levels of environmental pressure whereas M, L and XL granules had a net positive contribution to environmental pressure levels (Fig. 5). This perhaps shows that as granules grew their respective communities were more influenced by their environment, perhaps due to factors such as substrate limitation at the centre of the granules (Díaz et al., 2006b; Doloman et al., 2017a; Grotenhuis et al., 1991). Indeed, SEM analysis showed much higher incidences of granule damage in larger granules (Chapter 3, Fig. 4)

Soil aggregates have previously been proposed to act as evolutionary incubators for microbial communities, in which the internal microbial community evolves independently of other

aggregates and upon breakage, contributes to net diversity in the bulk soil (Rillig et al., 2017). The higher levels of environmental pressure in larger granules could indicate that diversification and deterministic community evolution occurred internally as they grew. Upon breakage, M, L or XL granules would then release their independently evolved, internal microbial communities. This continual release of previously isolated communities, facilitated by cycles of growth, could be what caused increased diversity with reactor operation time. Therefore, it might be possible to implement a bioreactor management strategy to promote diversification and thus stability, by increasing the rate of granule breakage and reformation, perhaps through mechanical or hydraulic disturbances.

4.3 Distinct Pre-Granular and Granular Communities

4.3.1 Pre-Granular Community

The pre-granular, XS biomass was morphologically different from the rest of the sizes (Chapter 3, Fig. 4) and had a clearly distinct microbial community (Fig.5), which may have had a role in initiating granule formation, or as a background planktonic community. At phylum level it was apparent that the pre-granular community had a much higher abundance of Firmicutes than the other size fractions (Fig. 6). Within the Firmicutes there were several taxa, including *Streptococcaceae* and *Eubacteriaceae* which are widely regarded to carry out functions such as acidogenesis in AD (Venkiteshwaran et al., 2016). *Methanocorpusculum* sp were the only methanogen, within the 25 most abundant taxa which were more prevalent in the XS community than the granular communities (Fig. 6). *Methanocorpusculum* are hydrogenotrophic methanogens (Zellner et al., 1989), but little else is known about their role in anaerobic digestion.

The XS biomass was generally present on top of the sludge bed, which can be explained by its very low settling velocity (Chapter 3, Fig. 5). This community is likely under-sampled in most lab scale studies as granules are generally the target biomass. Sampling through reactor ports,

in the middle of the sludge bed may also lead to under representation of flocculent biomass which is generally located higher in the reactor. However, in one study, the planktonic microbiome was also found to be significantly different from the granular community with a high proportion of Firmicutes (Zhu et al., 2017). This community was more susceptible to operational changes than the granular community and was potentially influenced by the breakup of granules and resultant release of the granular organisms (Zhu et al., 2017).

The abundance of acidogenic and fermentative bacteria such as *Streptococcaceae* in the XS biomass (Fig. 6 & 7) indicated that this size may have specifically carried out acidogenesis. Acidogenesis has previously been suggested to occur on the outermost layer of larger granules or as the predominant function of small granules and flocculent biomass in the bulk liquid (Arcand et al., 1994; Batstone et al., 2004; Guiot et al., 1992b; McHugh et al., 2003). Recently, *Lactococcus* species have been hypothesized to attach loosely to the outer layer of granules in lab scale reactors treating dairy wastewater (McAteer et al., 2020) and were washed after an increase in upflow velocity. Therefore, it may be the case that in the present study, this acidogenic community was not only present as a planktonic/flocculent community but may have been loosely attached to the surface of mature granules. *Streptococcus sp* have been suggested to be important for granulation in acidified conditions (Trego et al., 2020a) and at high salinity (Gagliano et al., 2020; Sudmalis et al., 2018). Their presence in the XS fraction here could indicate that the *Streptococcaceae* are more important in initiating granulation than was previously thought.

4.3.2 Granular Community

Proteobacteria generally had higher relative abundances in granular communities, as did Candidatus *Aegiribacteria* and candidate division FCPU426. The candidate genus Candidatus *Aegiribacteria*, was first identified in metagenomic samples taken from below the Chemocline in a Meromictic Lake in British Colombia (USA) (Hamilton et al., 2016) and is a member of

the Fibrobacteres, Chlorobi, Bacteroidetes (FCB) superphylum (Hamilton et al., 2016). Once the granular community formed in S granules the abundance of methanogens other than *Methanocorpusculum sp* increased. Notably, *Methanosaeta sp* were generally the most relatively abundant organism in granular communities. *Methanosaeta* are generally thought to play an important role in anaerobic granulation due to their filamentous morphology (Wiegant et al., 1988) and their increased abundance in granular communities here aligns with this hypothesis.

Several Acetogens, and Methanogens were identified as discriminant taxa, which were more abundant in the granular samples including; *Anaerolinea, Longilinea, Syntrophorhabdus Syntrophobacter, Methanospurillum* and *Methanosaeta* (Fig.7). This is in agreement with the hypothesis of Guiot et al (1992), who proposed that acetogens and methanogens form the core of granules, and therefore, as they grow, their abundance increases relative to other groups such as acidogens which are at the granule surface. The presence of more syntrophic organisms in the granular community is unsurprising as granulation has long been hypothesized to support increased syntrophy (Stams et al., 2012). *Geobacter sp* , known electroactive organisms and syntrophic partners of Methanogens (Rotaru et al., 2014), were also more abundant in the granular communities.

4.4 The Active Community

The active community was clearly separate from the total community in the final takedown (Fig. 8), indicating that a number of redundant organisms may have been present in total communities. While these inactive organisms might have been redundant at the time of sampling, they may also have been active at one point during granule growth and involved in granule formation. Their presence within the granule architecture, even if inactive, may be required for the structural integrity of granules. *Smithella sp* are a member of the order *Syntrophobacterales* and are often involved in syntrophic propionate oxidation in anaerobic

digestion (de Bok et al., 2001). Their increased relative abundance in the active community of S4 (Fig. 8) in particular, could have been due to poor performance of this reactor in response to increased propionate accumulation (Chapter 3, Table 6). Active *Smithella sp* were lowest in abundance in the ND bioreactor, which may be because of the lack of VFA accumulation and more stable operation of this bioreactor.

4.4.1 Total Vs Active Communities

Geobacter sp and *Methanosaeta sp* were both more relatively abundant in the active community than the total community (Fig. 6) and have previously been observed to carry out Direct Interspecies Electron Transport (DIET) (Holmes et al., 2017). DIET between non-surface attached microorganisms is generally considered to benefit from microbial aggregation (Summers et al., 2010). DIET was first proposed to occur in methanogenic granules in 2011 (Morita et al., 2011) and was subsequently confirmed in ethanol metabolizing granular communities where electron transfer between *Geobacter sp* and *Mehanosaeta sp*. was confirmed (Rotaru et al., 2014). As ethanol was one of the main components of the feedstock here, and both *Geobacter sp* and *Mehanosaeta sp*. were abundant in the active granular community it is likely that DIET occurred by this mechanism in the present study and may have enhanced granulation (Fig 10).

Desulfovibrio sp were also more abundant in the active community than the total community (Fig. 10). Sulfate reducing bacteria (SRB) such as *Desulfovibrio sp* often compete with methanogens in anaerobic environments where sulfate is present (Jing et al., 2013; Stefanie et al., 1994). However, in conditions of low sulfate concentrations such as the present study, SRBs often form a syntrophic partnership with hydrogenothrophic methanogens (Bryant et al., 1977; McInerney and Bryant, 1981; Walker et al., 2009). Bryant *et al* (1977) isolated a co-culture of a *Desulfovibrio* and *Methanobacterium sp* from an anaerobic digester. In this co-culture *Desulfovibrio* catabolized lactate and ethanol (the main components of feed in this study)

providing H_2 for *Methanobacterium sp.* The high activity levels of *Desulfovibrio* and high relative abundance of hydrogenothrophic methanogens in the granular community of this study could be the result of such a partnership.

4.4.2 Comparison of the Active Community Across Granule Sizes

Active *Streptococcaceae* were more abundant in XS biomass than any other size (Fig. 11), in all reactors. In the context of community succession and granulation this acidogenic community containing the *Streptococcaceae* may be acting as primary consumers, breaking down the most complex components of the feedstock (lactose). This would provide simpler substrates for other groups such as syntrophic acetogenic/methanogenic communities in granules. While they were still present and active in granular communities, this could be as an outer layer, as was previously hypothesized (McAteer et al., 2020).

The high abundance of active *Thauera sp* in S granules across all bioreactor sets may indicate that they could have a role in the initial formation of granules (Fig.11). *Thauera sp* are facultative anaerobes and are often found in aerobic granular bioreactors where they are generally thought to play an important role in granulation through high levels of EPS production (Lv et al., 2014b; Yang et al., 2014; Zhao et al., 2013; Zou et al., 2019). Aerobic organisms have been suggested to play a role in anaerobic granulation previously (Gupta and Gupta, 2005). It may be the case that *Thauera* are involved in the initial formation of an anaerobic environment on chunks of old granules and establishing a new EPS matrix on their surface. This, along with the metabolites arising from the acidogenic community of the XS fraction would provide the ideal environment for methanogenic granule formation.

4.5 Mechanism of Granule formation

The results of SEM analysis presented in Chapter 3 indicate that XS biomass is morphologically different from granules and is more flocculent in nature (Chapter 3, Fig 4).

This community may act as a precursor to granule formation and initiate granulation by entangling granule chunks in flocculent biomass. It is also apparent that the XS community was significantly different from the granular community. Since this community had higher abundances of acidogenic organisms it may have acted as primary consumers, which broke down larger organic molecules and provided simpler substrates for methanogenic communities in granule chunks, facilitating granule re-formation. The initial formation of more typical granular communities in S granules and accommodation of strictly anaerobic methanogenic communities may also be aided by facultative anaerobes such as *Thauera,* which could remove oxygen at a localized scale and aid in the production of an EPS matrix. It appeared that formation of granules led to increased levels of syntrophy including; traditional methanogenesis (acetogens and methanogens); SRBs and hydrogenotrophic methanogens; and DIET mediated methanogenesis. Once a stable syntrophic granule community was formed in S or M granules it persisted during growth, until, eventually increased environmental pressure began to influence the community. This environmental pressure may be caused by factors such as substrate limitation at the core of the granule or gas build up inside the granule leading to granule disintegration. Evidence of cracking, and breaking in larger granules corroborates this (Chapter 3, Fig. 4). Upon disintegration, this previously isolated community could then contribute to overall diversity in the reactor and facilitate the growth of new granules (Fig. 12).

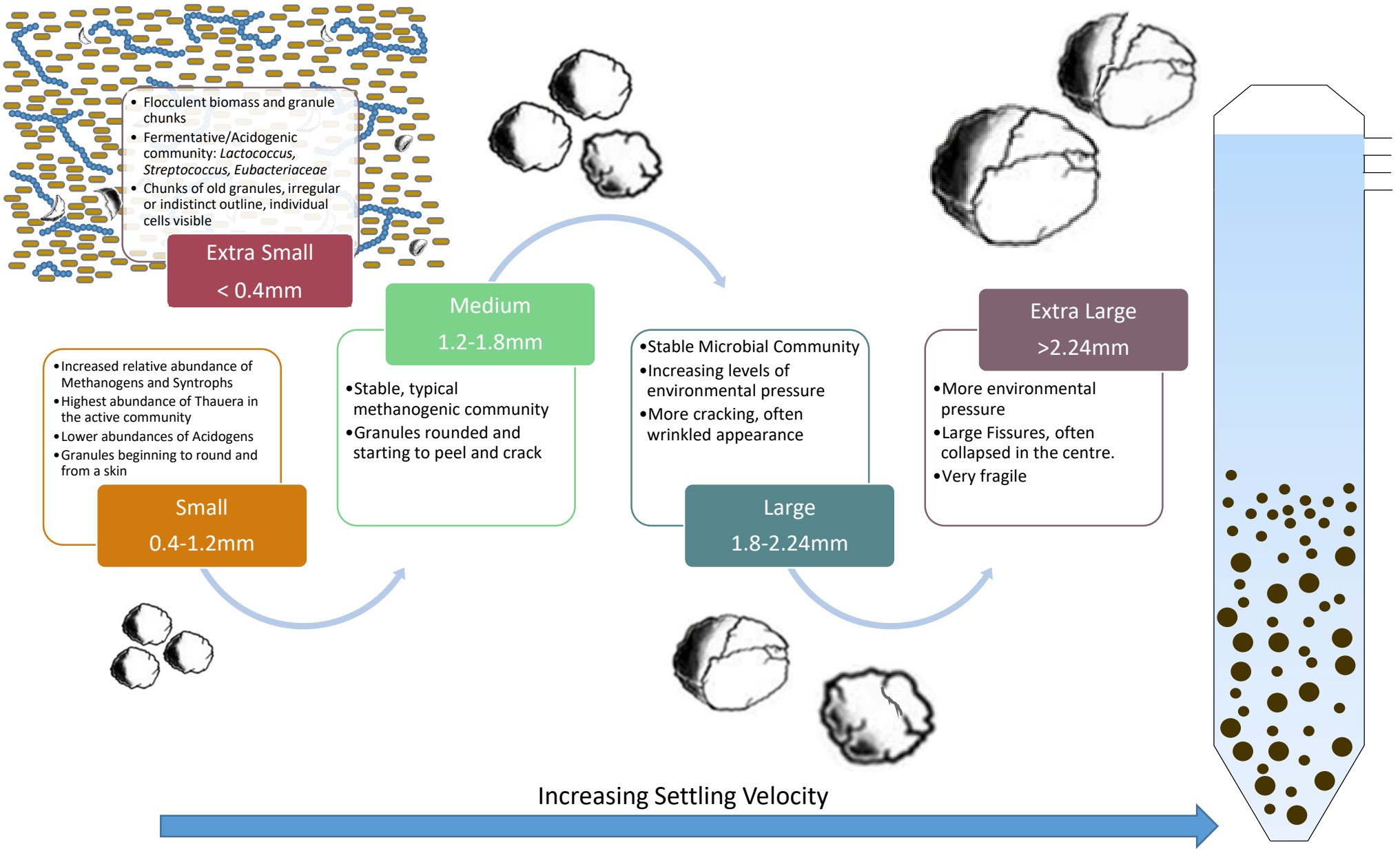


Figure 12. Summary of Microbial Community Analysis

5 Conclusion

Seed size had only a slight influence on the resultant microbial communities in each reactor with incubation time and recovered size causing most of the beta diversity between samples. Granule growth leads to increased environmental pressure on internal communities, possibly leading to divergent community evolution in individual granules. Subsequent cycles of granule disintegration and re-formation may have released these diversified internal communities and led to increases in community richness and diversity. Aerobic organisms such as *Thauera* sp may have a role in facilitating the establishment of highly syntrophic methanogenic communities, through oxygen depletion. Acidogenic organisms such as the *Streptococcaceae* were prominent in the XS biomass, which was considered pre-granular and may have a more important role in granulation than was previously thought. However, more research specifically targeting their role in granulation is required.

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CHAPTER 5

Differential Microbial Communities Evolve in Methanogenic Granular Sludge to Reduce Selenate and Selenite

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1 Introduction

Anaerobic granular sludge has been successfully applied for the treatment of wastewaters and production of valuable biogas since the 1980s (Lettinga et al., 1980). Granular sludge has also been used for removal and recovery of other macro nutrients, such as phosphorous and nitrogen (Nancharaiah and Reddy, 2018). In addition, more specialised applications are also being developed, such as the treatment of selenium contaminated wastewaters (Dessì et al., 2016).

Selenium (Se) exists in a range of different oxidation states (+6, +4, 0, -2), with varying chemical properties. The selenate (Se(VI)) and selenite (Se(IV)) oxyanions of selenium are soluble, bioavailable and toxic, and are known to be potent pollutants of surface waters (Ohlendorf et al., 2002). Sources of selenium contamination include agriculture, mining, metal-ore smelting, and energy generation (Lemly et al., 2004), and incidents of Se contamination in the environment have occurred with detrimental effects to wildfowl and fish stocks (Hamilton et al., 2004). Elemental selenium (Se(0)) is solid, and less toxic and bioavailable than either selenate or selenite (Tan et al., 2016). Thus, its formation is desirable in the treatment of selenium-containing wastewaters.

Selenium undergoes a global biogeochemical cycle similar to that of nitrogen and sulphur, which is heavily influenced by microbial activity. Microbial reduction of selenium oxyanions to elemental selenium Se(0) is thought to play a significant role in global selenium cycling and many different mechanisms of selenium reduction have been proposed (Eswayah et al., 2016). These mechanisms fall into two broad categories: assimilatory reduction and dissimilatory reduction (Lenz and Lens, 2009). Assimilatory reduction of selenium involves transport of selenate and selenite into cells with the incorporation of selenium into selenoproteins in the form of selenide, which is only thought to play a minor role in selenium cycling (Eswayah et al., 2016). Dissimilatory reduction of selenium, on the other hand, occurs through anaerobic

respiration or through various detoxification mechanisms, and results in the formation of Se(0) nano-particles (SeNPs) (Eswayah et al., 2016; Wadhwani et al., 2016). Anaerobic respiration of selenium involves the use of selenate and selenite as terminal electron acceptors. Specific respiratory selenate reductases have been identified in well studied species, such as *Thauera selenatis* (Rech and Macy, 1992; Schröder et al., 1997), but do not appear to be universally present in all selenate-respiring organisms (Wen et al., 2016). Selenite reduction appears to be even more complex, often involving various other respiratory reductases, such as nitrite reductase, sulphite reductase and fumarate reductase (DeMoll-decker and Macy, 1993; Li et al., 2014; Nancharaiah and Lens, 2015). Selenite reduction is also often attributed to detoxification mechanisms, which can occur aerobically or anaerobically and involves the non-respiratory reduction of selenite using various biological molecules such as reduced glutathione or siderophores. Reduction of selenium oxyanions to Se(0) is not only desirable for wastewater treatment but also allows for the recovery of selenium nanoparticles, which have a variety of applications. Their semiconductor and photoelectrical properties make them useful in electronics (Wadhwani et al., 2016) and SeNPs have even been used in biomedicine as cancer therapeutics (Khurana et al., 2019).

It is also unclear whether selenate and selenite reduction is carried out by taxonomically different microbial communities. To date, significantly more organisms have been identified which are capable of selenite reduction than selenate reduction, through both respiration and detoxification mechanisms (Tan et al., 2016). Reduction of selenate to Se(0) is a two-step process (selenate to selenite, and selenite to Se(0)). It is unknown whether selenate-reducing communities typically comprise of separate sub-communities that evolve to perform the respective steps of the process or include mostly organisms capable of complete reduction of selenate to Se(0). Therefore, further investigation of selenium-reducing microbial communities in granules used for wastewater treatment is required, so as to better understand and leverage

the process. For example, identification of separate selenate- or selenite-reducing clades of microorganisms could allow for improved screening of inoculum sources for bioreactors operated for selenium removal. Methanogenic granular sludge has previously been shown to be effective in reducing the concentration of selenium oxyanions in wastewaters (Lenz et al., 2008b; Soda et al., 2011). However, the removal rates achieved by different granular sludges varied greatly (Astratinei., et al 2006), and only a handful of studies examined the microbiology underpinning the process (Dessì et al., 2016; Gonzalez-Gil et al., 2016).

Microbial community analysis can be useful for understanding the phylogeny of selenate and selenite reduction. However, it does not provide any information on the respective mechanisms. Pure cultures, due to their relative simplicity, can be useful in gaining a mechanistic understanding of microbial processes and can be advantageous over mixed-culture 'omics' approaches in studying relatively poorly understood processes such as selenium reduction. To date, isolates of selenate- or selenite-respiring microorganisms have originated from disparate sources and were phylogenetically diverse (Narasingarao and Häggblom, 2007; Rauschenbach et al., 2011; Stolz et al., 1999). However, no isolates have yet been obtained from anaerobic granular sludge.

The aims of this study were to (i) investigate potential differences in the evolution of separate selenate and selenite reducing communities; (ii) determine whether selenite reducing community makes up a sub-population of the selenate reducing community; (iii) expand our understanding of the microbial ecology of selenium cycling by identifying numerous, potentially Se reducing taxa, and (iv) obtain selenium reducing isolates from anaerobic granular sludge which could be used for potential future studies.

2 Materials and Methods

2.1 Enrichment Cultures

Granular sludge was sourced from a mesophilic (37°C), anaerobic, 'internal circulation' (IC) bioreactor (650m^3) from a dairy-processing and ethanol-production plant in Ballineen, Co. Cork, Ireland. Sludge was crushed in a 15ml centrifuge tube using a glass rod and homogenised by mixing in order to allow for sub-culturing under anaerobic conditions using a 5ml syringe and needle. 1g of crushed sludge was added to each of duplicate 60-ml serum bottles along with 30 ml minimal media containing (g L^{-1}): NH_4Cl (0.3), NaCl (0.3), $CaCl_2*2H_2O$ (0.11), $MgCl_2*6H_2O$ (0.1), 1 mL L^{-1} trace metal solution and 2 mL L^{-1} of vitamin solution (Shelton and Tiedje, 1994). The bottles were crimp-sealed, and the headspace was evacuated and replaced with 1 atm N_2. Sodium selenite and sodium selenate were added separately to each enrichment to a final concentration of 10 mM as the only available electron acceptors (Fig. 1). Sodium DL Lactate and sodium acetate were added to final concentrations of 16 and 4 mM, respectively, to provide electron donors in excess. Abiotic controls were also set up as above which did not include any biomass in order to control for possible chemical reduction of selenate and selenite.

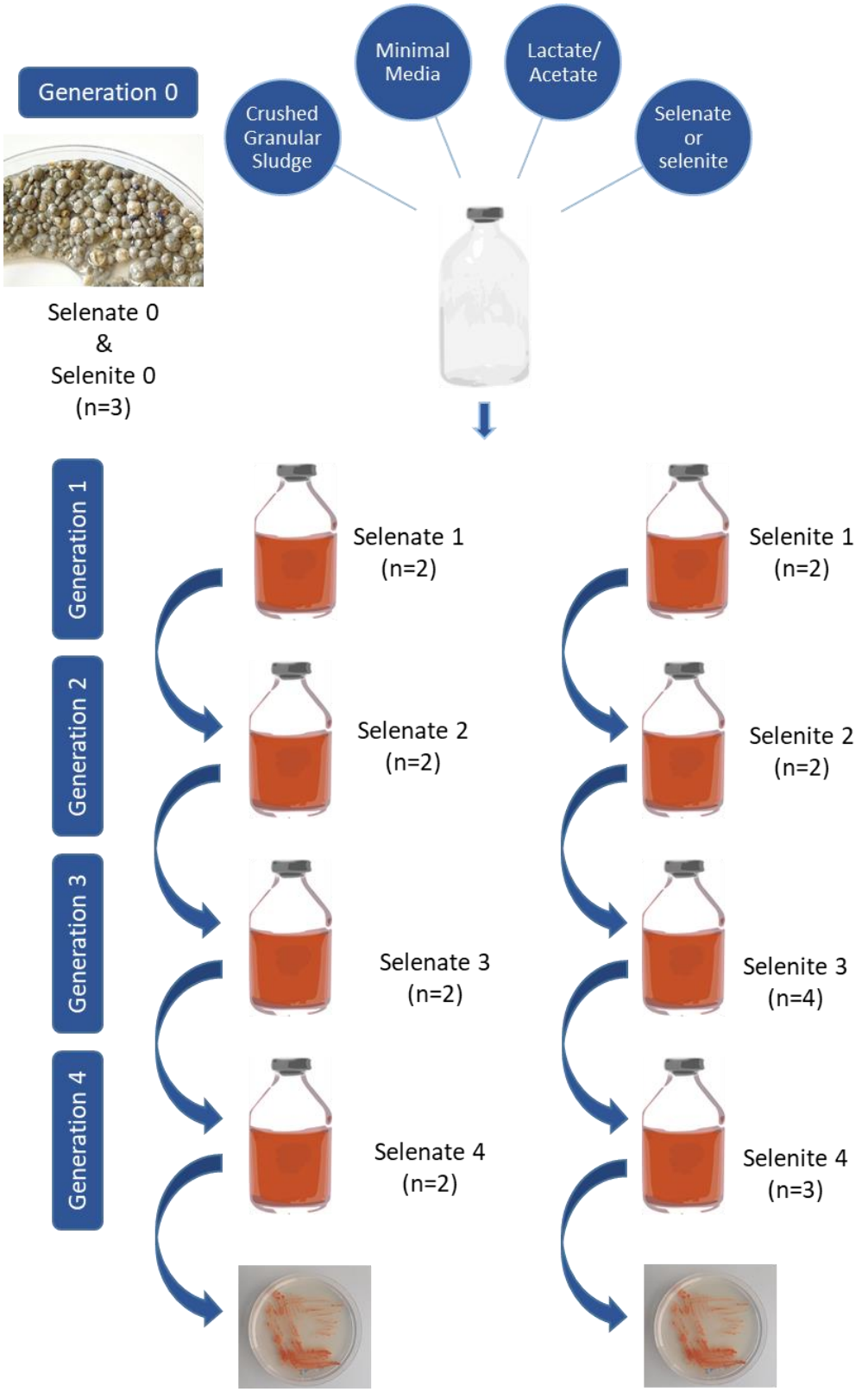

Figure. 1 Schematic depiction of the enrichment and isolation process. Sample groups are referred to as selenate 0-4 and selenite 0-4, corresponding to their respective sub-culture or 'generation'.

Enrichments were successively sub-cultured (10%) to fresh media once the culture media had become opaque (Fig. 2) due to production of red selenium nanoparticles (approx. 1-2 weeks). Sub-culturing was performed using standard anaerobic techniques at approximately 10% (v/v) (Fig. 1). Occasionally, sub-culturing was performed in duplicate to increase sample numbers e.g. selenite 3. Following sub-culture the remaining biomass in each enrichment was separated from the supernatant by centrifugation at 5000xg for 10 min. The resulting supernatant was then sampled for downstream chemical analysis. The pellet was re-suspended in 0.5 ml phosphate-buffered saline (PBS) solution and stored at -20°C for DNA extraction at a later date. However, selenate 1 and selenite 1 contained a large amount of sludge biomass and were therefore re-suspended in 5ml (PBS) and 0.5ml of this re-suspension was stored for DNA extraction.

Figure 2. Selenium nanoparticle production in enrichment cultures and abiotic control containing no biomass.

2.2 DNA Extraction and PCR Amplification

Biomass samples were thawed at room temperature and the entire re-suspension was used for DNA extraction. Cells were lysed by bead beating in a 1% cetyltrimethylammonium bromide (CTAB) lysis buffer and DNA was isolated using a phenol-chloroform method as described by Griffiths et al., (2000). PCR amplification of the V4 region of 16S rRNA genes was performed using the 515F 806R primer pair (Caporaso et al., 2011). PCR consisted of initial denaturation for 3 min at 95˚C followed by 25 cycles of annealing at 55˚C for 30 s and denaturation at 95˚C for 30 s. Extension was performed at 72˚C for 30 s. Library preparation and sequencing was carried out on the Illumina MiSeq platform by the FISABIO (Valencia, Spain).

2.3 Chemical and microscopic analyses

Total dissolved selenium was measured using inductively coupled plasma mass spectrometry (ICP-MS). Supernatant was obtained as described in Section 2.1 and further processed by centrifuging at 14,600xg and filtering using a 0.22 µm Sartorius Minisart single-use syringe filter to remove solid elemental selenium nano-particles and microbial cells. Samples were acidified to a final concentration of 2% nitric acid and diluted 330X in 1% nitric acid to achieve an appropriate concentration for measurement.

Aliquots of 0.5 ml were taken from enrichment cultures using sterile needles and syringes immediately prior to sub-culturing for transmission electron microscopy (TEM). Samples were centrifuged at 5000 xg for 10 min and the resultant pellet was re-suspended and fixed overnight in 2.5% (w/v) glutaraldehyde in phosphate buffer. Fixed samples were centrifuged again and the resultant pellet was washed and re-suspended in PBS. Whole mounts were prepared by air drying 20 µl of fixed samples on formvar coated 200 mesh copper grids (Agar Scientific). Samples were then imaged on a Hitachi 7000 transmission electron microscope.

2.4 Isolation of Selenium Reducing Microorganisms

Once advanced enrichments were obtained (after three or four generations) pure cultures were isolated using the Hungate method (Hungate, 1969). Colonies were picked from Hungate tubes and streaked on 1.5% (w/v) agar, supplemented with 0.5 g/l yeast extract, in petri dishes. Single colonies were picked from agar plates and re-streaked to ensure purity. All manipulations were carried out in an anaerobic glove box and all incubations were carried out under anaerobic conditions in anaerobic jars with oxoid AnaeroGen gas packs (Oxoid) at 30°C. Singular red colonies formed after approximately three days, and were picked and transferred back to the enrichment medium, which was supplemented with yeast extract, where the formation of red selenium nanoparticles was observed after approximately 2-3 days. Aliquots of 1 ml of this culture were centrifuged at 5000 xg and the resultant pellets were used for genomic DNA extraction as described previously (Section 2.2). The 16S rRNA genes were amplified by PCR using the 27F and 1492R primer pair. PCR consisted of initial denaturation for 10 min at 95˚C followed by 25 cycles of annealing at 55˚C for 45 s and denaturation at 95˚C for 60 s. Extension was performed at 72˚C for 60 s.

The forward and reverse compliment sequences were aligned using the MEGA-X software package and a consensus sequence was formed. The resultant 16S rRNA gene sequences were BLAST searched in the NCBI database for taxonomical identification. The top 10 hits, along with any other notable matches, were aligned using MUSCLE (Edgar, 2004) and a maximum likelihood phylogenetic tree was constructed using MegaX software (Kumar et al., 2018) according to the recommendations by Hall (2013).

2.4.1 Selenite Reduction Assay

Selenite reduction in isolated pure cultures was analysed according to the methodology described by Li et al (2014). Briefly, strains were cultured from glycerol stocks in LB medium and 1 ml of an overnight culture was used to inoculate 60-ml anaerobic serum vials containing

30 ml sterile minimal media as described in Section 2.1. Incubations were carried out at 30°C. Aliquots of 1 ml of culture were sampled daily for selenite quantification as described by Li et al (2014).

2.5 Bioinformatics and Statistical Analysis

2.5.1 Bioinformatics

OTU abundance tables were constructed as follows: trimming and filtering of paired-end reads was performed with Sickle v1.200 (Joshi and Fass, 2011) using a sliding window approach and trimming regions where the phred score drops below 20. Reads of fewer than 10 bp were then discarded. Error correction was carried out with BayesHammer (Nikolenko et al., 2013) from the Spades v2.5.0 assembler. Forward and reverse reads were assembled into a single sequence spanning the entire V3-V4 region using pandaseq v(2.4) with a minimum overlap of 20 bp. The above pipeline was previously shown to significantly reduce substitution rates (main form of error) (Schirmer et al., 2015, D'Amore et al., 2016). VSEARCH (v2.3.4) was subsequently used for OTU construction (all these steps are documented at https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline. In short, barcodes were added to reads from different samples, which were subsequently pooled. Reads were de-replicated and sorted by decreasing abundance, and singletons were removed. Reads were clustered at 97% similarity. Clusters with chimeric models built from more abundant reads were then removed (--uchime_denovo option in vsearch). Additional chimera removal was performed using a reference-based chimera filtering step (--uchime_ref option in vsearch) with a gold database (https://www.mothur.org/w/images/f/f1/Silva.gold.bacteria.zip). The original barcoded reads were matched to clean OTUs at 97% similarity to make an OTU table consisting of 1875 OTUs for n=25 samples. OTUs were classified using the SILVA SSU Ref NR database (v123) database with assign_taxonomy.py script from the Qiime (Caporaso et al., 2010) workflow. OTUs were then aligned against each other using Kalign v2.0.4 (Lassmann and

Sonnhammer, 2005) (using the options -gpo 11 -gpe 0.85) and FastTree v2.1.7 (Price et al., 2010) was used to generate a phylogenetic tree in NEWICK format. A biome file was generated by combining the abundance table with taxonomy information using the make_otu_table.py command from the Qiime workflow.

2.5.2 Statistical Analysis

Differential abundance analysis was used to identify OTUs that were significantly different between groups. This was performed with the DESeqDataSetFromMatrix() function from DESeq2 package(Love et al., 2014) with an adjusted p-value significance cut-off of 0.05 and log2 fold change cut-off of 2. DESeqDataSetFromMatrix() utilises negative binomial GLM to obtain maximum likelihood estimates for the log fold change of ASVs between two groups. Subsequently, Bayesian shrinkage was used to get shrunken log fold changes and then the Wald test was used for obtaining significances. All other statistical analyses were described previously (Chapter 4, Section 2.5).

3 Results

3.1 Chemical analysis, and Microscopy, of Enrichment Cultures

ICP-MS revealed approximately 50% conversion of selenate and selenite to Se(0) in each generation of both enrichments (Table 1).

Table 1. SeO_x conversion in each enrichment generation

SeO_x Conversion* (%)		
	Selenite Enrichment	Selenate Enrichment
Gen 1	59.1 (2.9)	58.4 (3.7)
Gen 2	48.7 (2.4)	50.0 (2.8)
Gen 3	46.3 (2.4)	47.0 (4.2)
Gen 4	47.5 (0.7)	51.1 (2.7)

*Mean (Standard Deviation) Value of Duplicate Enrichments with

Diverse cell types and morphologies were associated with selenium nano-particle production in selenate and selenite enrichments (Fig. 3). Nanoparticles appeared to occur both intracellularly and extracellularly. Extracellular nano-particles were generally between 100 and 300 nm, whereas intracellular nanoparticles appeared smaller. A possible site of nano-particle extrusion was observed (Fig. 3D).

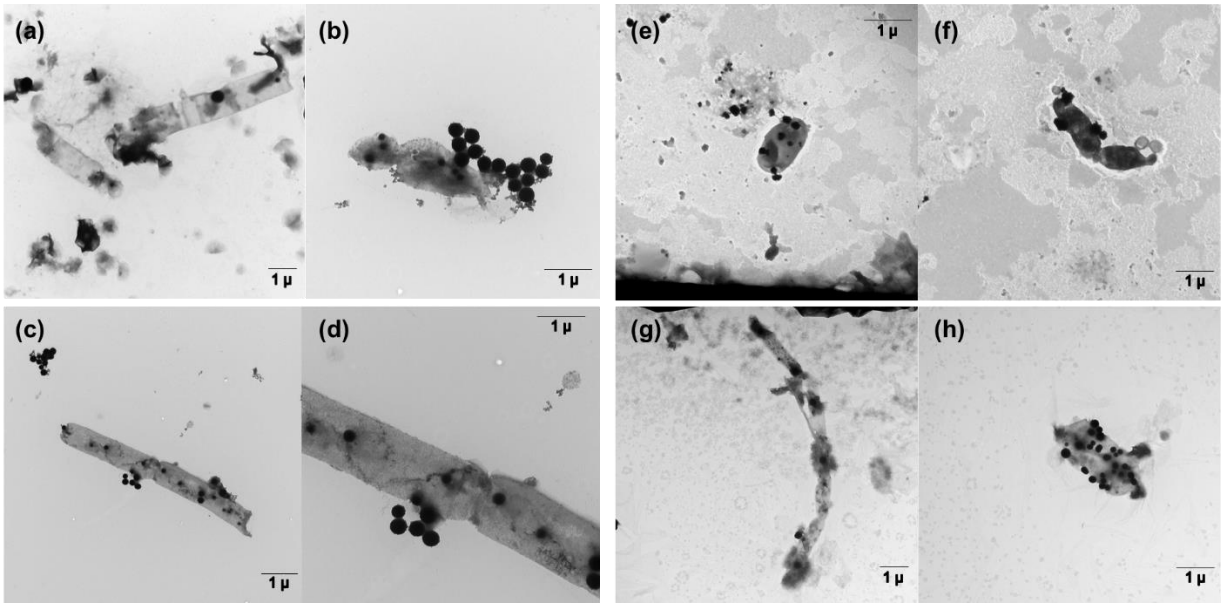

Figure 3. Transmission electron micrographs illustrating the diversity of cell types associated with nanoparticles in selenite enrichments (a-d) and selenate enrichments (e-h).

3.2 Microbial Community Analysis

3.2.1 Microbial Community Diversity

Microbial community composition was analysed in each enrichment following sub-culture to the next generation (Fig. 1). Significant decreases in diversity, evenness and richness of both enrichments were apparent from generation 1 to generation 4 (Fig. 4). However, some small differences in alpha diversity patterns were observed between enrichments for the initial and second generations. For example, alpha diversity increased in selenate 1, followed by subsequent decreases in each subsequent generation, whereas a steady decrease from selenite 0 to selenite 4 was observed in the selenite enrichment (Fig. 4).

Significant changes in microbial community beta diversity over successive generations of both enrichments were observed with the Bray-Curtis and Weighted Unifrac distance matrices (Fig. 4). Generation 1 in both enrichments clustered closely to generation 0 with both distance metrics. Generations 2-4 then clearly evolved from the original source. After inoculation it is clear that the microbial communities in each enrichment diverged and clear separation was

observed in the 4th generation, between the selenate and selenite enrichments (Fig. 4).

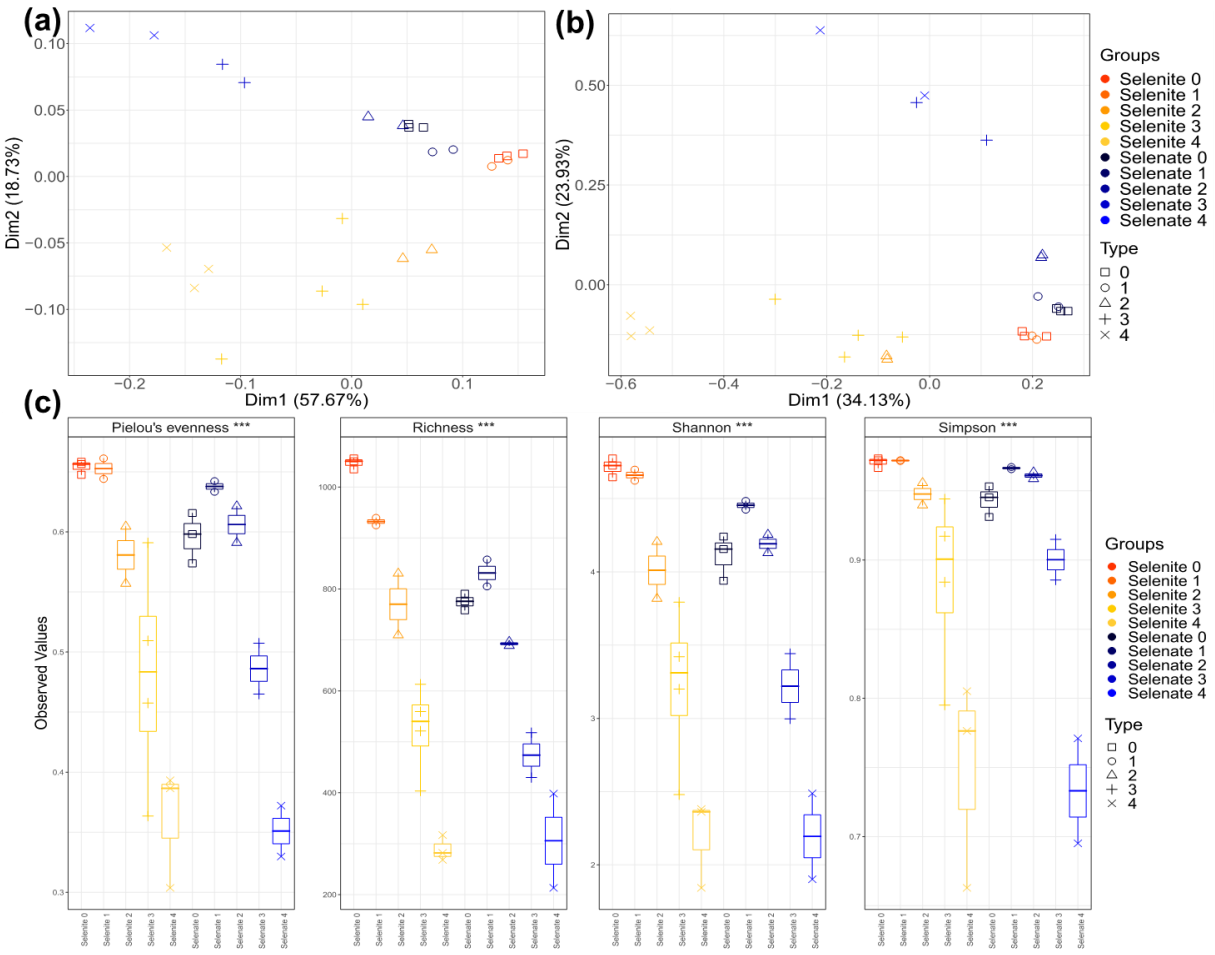

Figure 4. Principal Component Analysis with samples grouped by enrichment and generation, using the **(a)** Bray-Curtis and **(b)** Weighted Unifrac distance matrices, where ellipses were drawn using 95% confidence intervals based on standard deviation. **(c)** Alpha diversity indices; Pielou's Evenness, Rarefied Richness, Shannon Diversity and Simpson Diversity for all samples

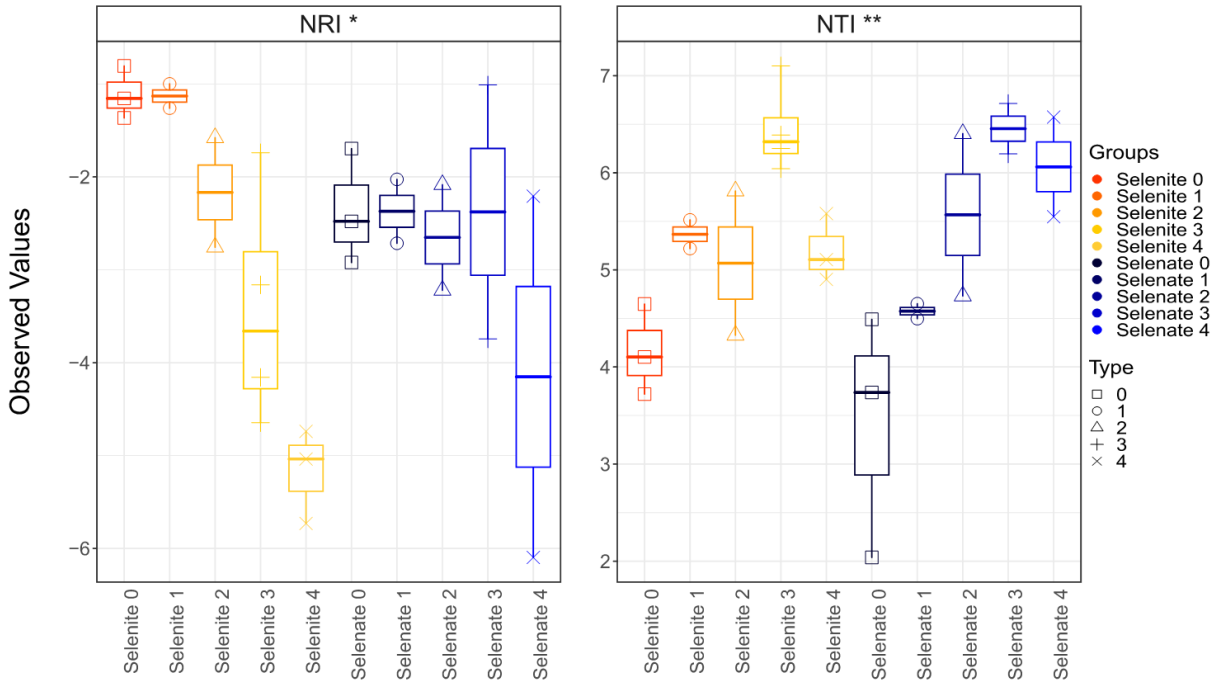

Figure 5. Environmental Filtering Analysis; NRI and NTI values for all selenate and selenite enrichments.

Environmental pressure on terminal clades was high in all samples (>2 NTI) and increased with each generation (Fig. 5). However, at a deeper phylogenetic level environmental pressure was lower (< -2 NRI) and decreased with each enrichment generation (Fig. 5).

3.2.2 Microbial Community Composition

At phylum level clear taxonomic differences, in the most abundant taxa, were observed based on electron acceptor. Actinobacteria and Proteobacteria became dominant in the selenite fed enrichments, whereas Firmicutes were more abundant in the selenate fed enrichments (Fig. 6). Euryarchaeota decreased from 50-60% in the seed to approximately 15-20% in selenite 4 and 5-10% in selenate 4 (Fig. 6). At family level, it is apparent that each enrichment became dominated by a few, key taxa. Selenite 3 and 4 were dominated by *Propionibacteriaceae* and *Shewanellaceae,* respectively (Fig. 6). *Methanobacteriaceae* persisted at a similar abundance throughout each generation whereas *Methanosaetaceae* decreased in abundance. The selenate enrichments became dominated by *Veillonellaceae*, *Campylobacteraceae*, *Clostridiaceae* and *Comamonadaceae.* As with the selenite enrichments, *Methanosaetaceae* decreased in

abundance from the selenate 0 to selenate 4, as did *Methanosarcinaceae* and *Methanoregulaceae* (Fig. 6).

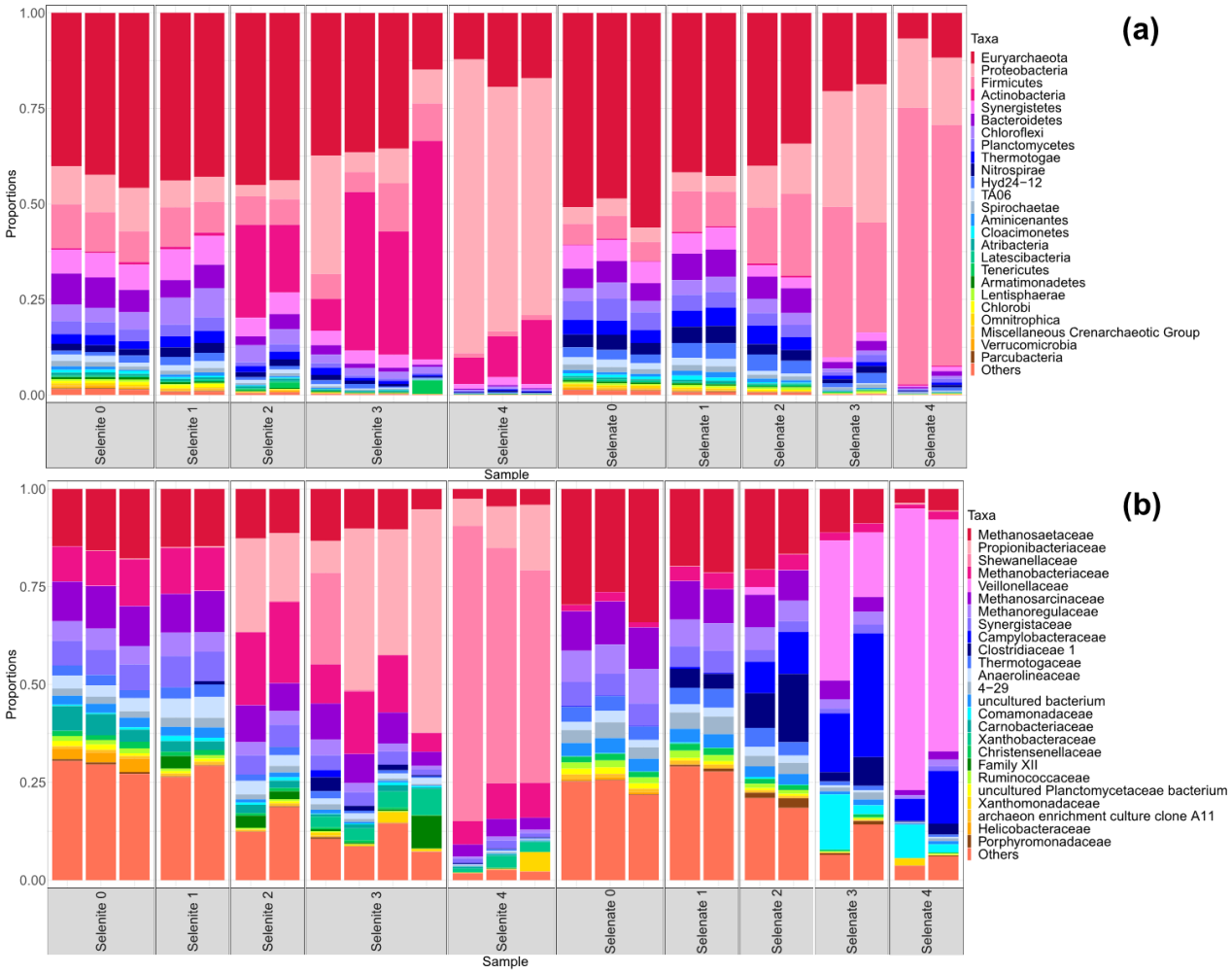

Figure 6. Top 25 most abundant taxa in each generation of selenate and selenite enrichments.

3.2.3 Differential Taxa Analysis

Differential taxa analysis identified 34 genera and 32 families which were significantly different in terms of abundances between selenite 4 and selenate 4. The most abundant of these, which were more prevalent in selenite 4, included *Shewanella*, *Propionibacteriaceae*, and *Xanthobacter* (Fig. 7). Taxa which were significantly more abundant in selenate 4 included OTUs belonging to *Veillonellaceae*, *Sulfurospirillum*, *Comamonas*, *Clostridium*, and *Rhizobium*.

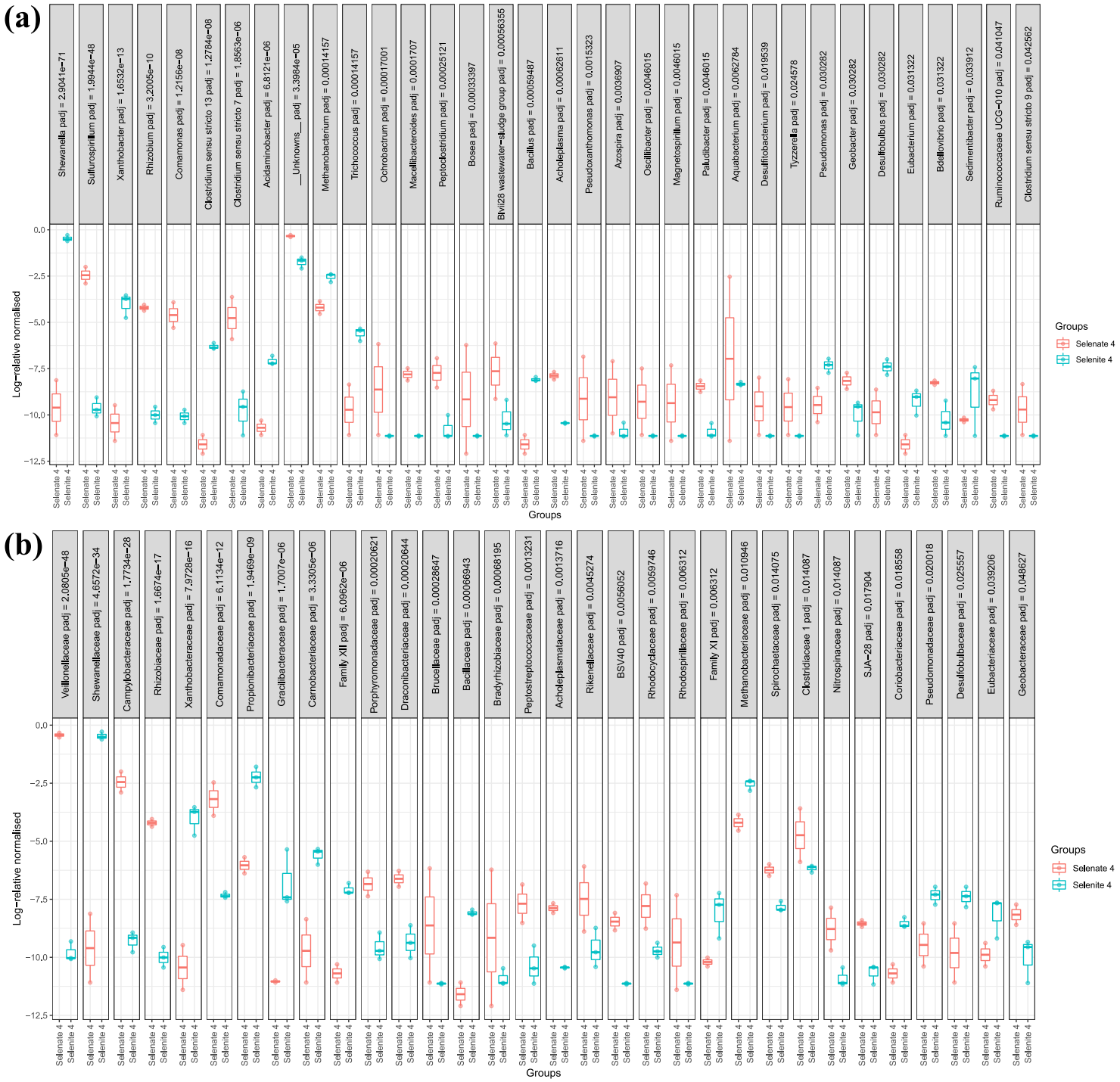

Figure 7. Differential analysis of **(a)** genera and **(b)** families that are significantly more abundant in either the 4th generation of selenate and selenite enrichments (Adjusted P values ≤0.05) with at least log2 fold change from the base mean abundances for the samples.

3.2.4 Discriminant Taxa

105 discriminant OTUs were identified by SPLS-DA, which accounted for much of the beta diversity between samples. These discriminant taxa accounted for much of the beta diversity across all samples (Fig.8). Several OTUs appear to be differentiating the selenate 2, 3 and 4 samples from the rest of the enrichments (Block 1, Fig.8). This block included OTUs classified as *Clostridia, Sulfurospirillum, Rhodocyclaceae, Dechloromonas* and *Veillonellaceae* among others. Block two differentiates the selenite 2,3 and 4 samples from the other enrichments (Fig. 8) and includes OTUs assigned to *Shewanella, Xanthobacter* sp, *Propionibacteriaceae* and *Methanobacterium*.

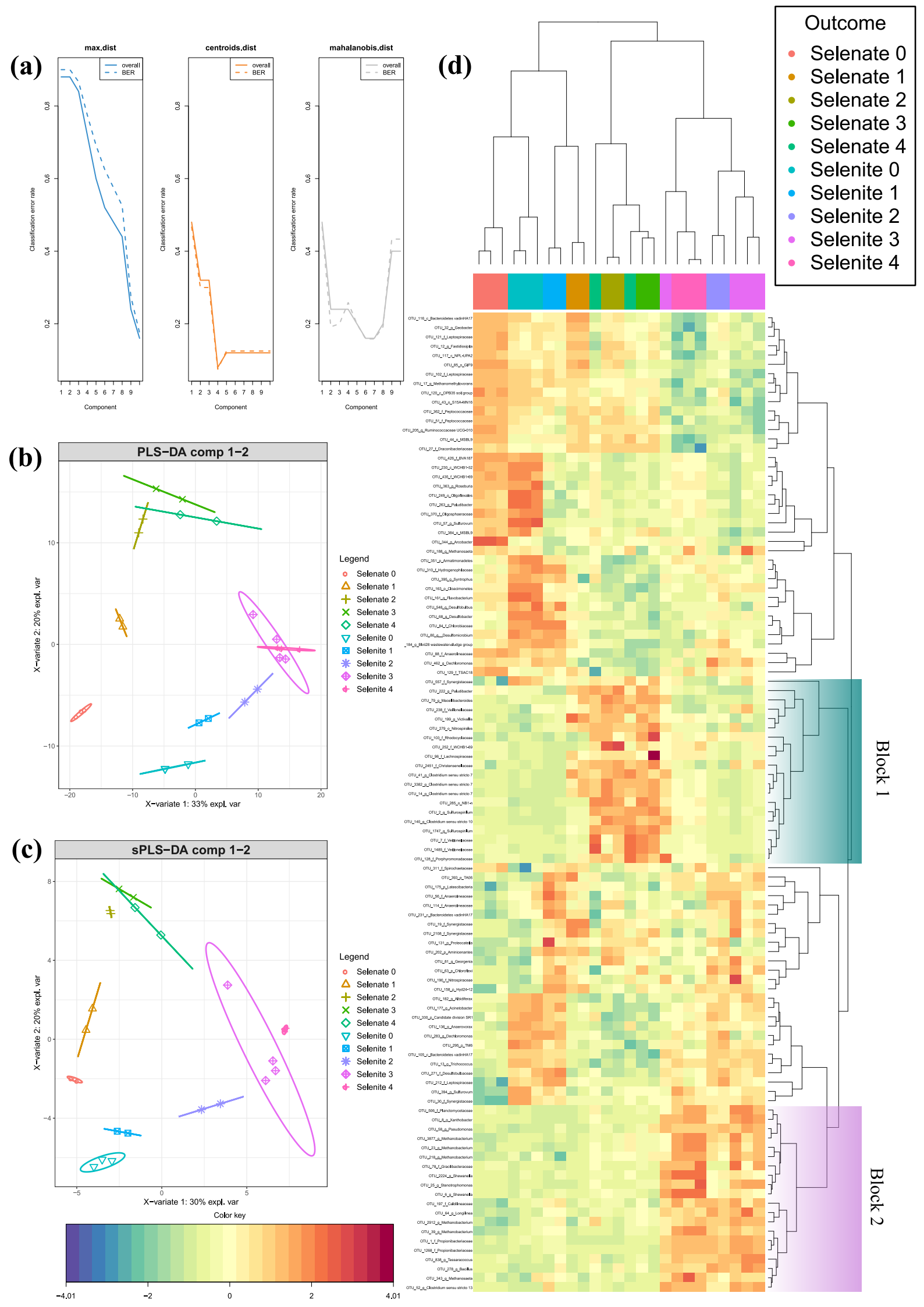
(a)
max.dist
centroids.dist
mahalanobis.dist
overall
BER
Classification error rate
Component
(b)
PLS−DA comp 1−2
X−variate 1: 33% expl. var
X−variate 2: 20% expl. var
(c)
sPLS−DA comp 1−2
X−variate 1: 30% expl. var
X−variate 2: 20% expl. var
Color key
−4.01
−2
0
2
4.01
(d)
Outcome
Selenate 0
Selenate 1
Selenate 2
Selenate 3
Selenate 4
Selenite 0
Selenite 1
Selenite 2
Selenite 3
Selenite 4
Legend
Block 1
Block 2

Figure. 8(a) Classification error rates over the components and the numbers of optimal features (OTUs) in each component, included in the model, chosen by the lowest error rates which are denoted by diamonds. (b) Ordination of all OUTs (c) Ordination of discriminant OTUs only (d) Heatmap based on sPLS-DA of amplicon sequencing data depicting discriminant genera. Rows and columns are order according to hierarchical (average linkage) clustering to identify clusters of genera among groups. Block 1 contains discriminant taxa more abundant in selenate 2, 3 and 4 and Block 1 contains taxa more abundant in selenite 2, 3 and 4

3.3 Isolation of Selenium Reducing Microorganisms

3.3.1 Phylogenetic Analysis

A single strain was isolated from each of the selenate and selenite fed enrichments. Isolate G4A2 was isolated from the selenate fed enrichment and shared approximately 93% similarity with several uncultured bacterial clones. The best classified match was a *Sulfurospirillum* sp, which also had a similarity of 93% and a query coverage of 93%. Isolate G4I1 was obtained from the selenite enrichment and had a high degree of similarity (>99%) to various *Shewanella* sp in the NCBI database. Several of these matches were identified to species level as *Shewanella xiamenensis*. The top 10 hits for each isolate, along with several other closely related sequences, which were identified to genus/species level, were aligned and are displayed as phylogenetic trees (Fig. 9). Unfortunately, isolate G4A2 was subsequently lost from culture due to difficulties in reviving a glycerol stock and therefore no further characterisation was carried out.

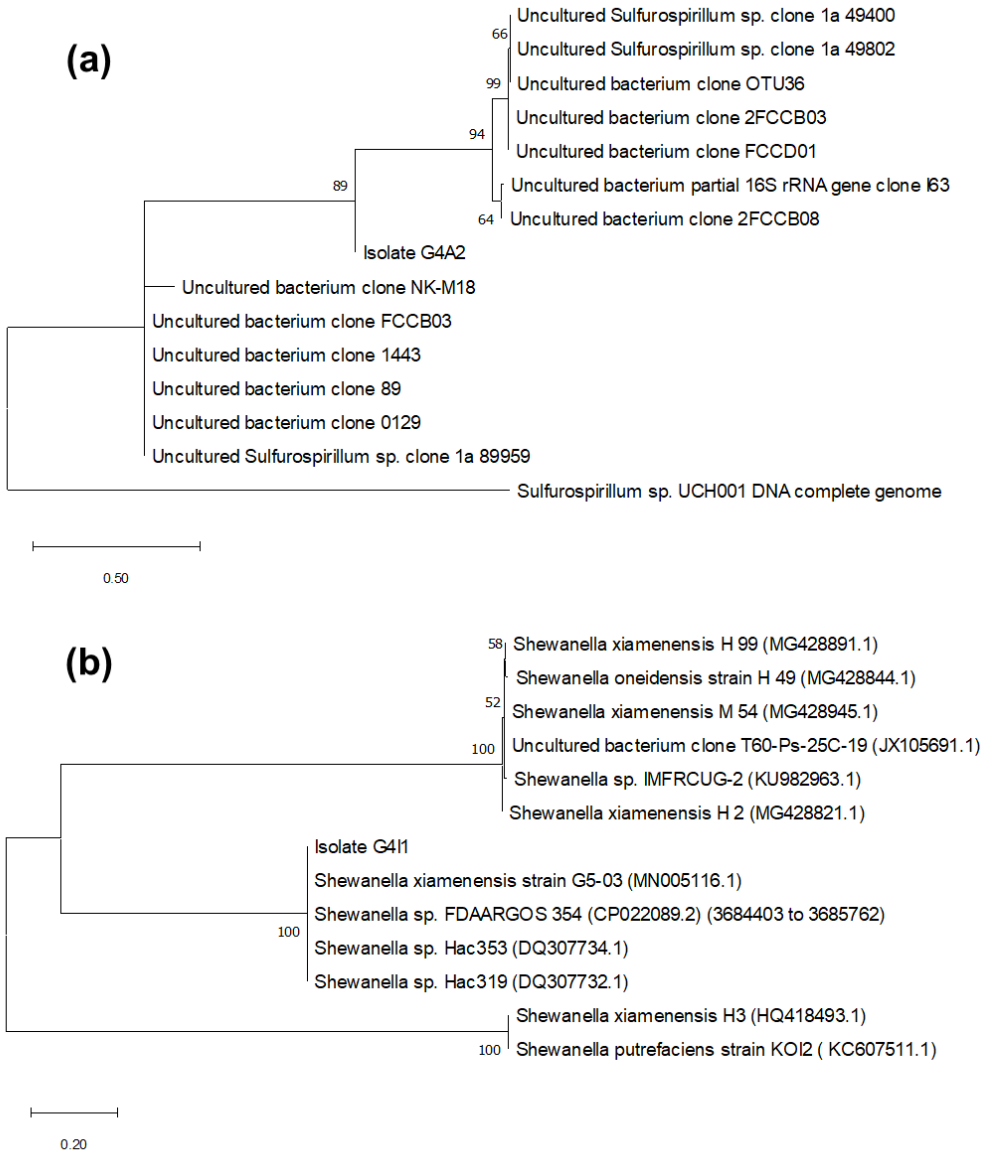

Figure 9. Phylogenetic trees inferred by using the Maximum Likelihood method with the (a) Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) and the (b). Jukes-Cantor model (Jukes and Cantor, 1969). Trees with the highest log likelihood (a: -4341.39, b: -1555.21) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option).

3.3.2 Selenite Reduction and Microscopic Observations

Selenite concentrations in incubations of isolate G4I1 decreased from approximately 11 mM to 3 mM after over 200 h, corresponding to an approximately 70% reduction (Fig. 10). The selenite reduction rate was calculated as 0.04 mM selenite h^{-1} based on the linear portion of the chart (69-208 h) which had an R^2 value of 0.9905. Selenium nanoparticles produced by G4I1 appeared to be located both intracellularly and extracellularly, and ranged in size from 50 to 300 nm (Fig. 10)

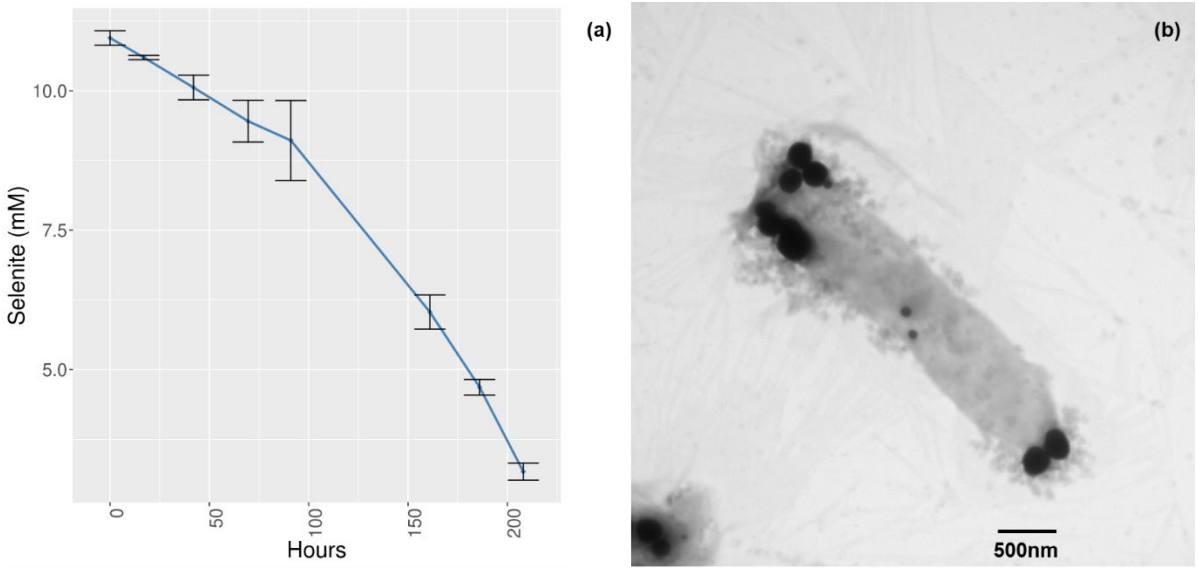

Figure 10. (a) Selenite reduction by Isolate G4I1 and **(b)** TEM micrograph of Isolate G4I1.

4 Discussion

4.1 Chemical Analysis and Microscopy

Total selenium in the supernatant of each enrichment culture at the time of sub-culture was approximately 50% of the initial concentration (Table. 1), indicating that selenate and selenite conversion to selenium nanoparticles had proceeded. The diverse cellular morphologies associated with nanoparticles, as observed by TEM, indicated that multiple organisms may be responsible for nanoparticle production in the mixed-culture enrichments (Fig. 3). The location (intracellular or extracellular) and size of nanoparticles also varied greatly. Both intracellular and extra-cellular nanoparticles have been observed previously (Wadhwani et al., 2016) and multiple hypothesised production mechanisms have been proposed to explain their location (Nancharaiah and Lens, 2015). In the case of intracellular nanoparticle production, the mechanism of nanoparticle extrusion is still unclear. Previous microscopic observations of large (average diameter of 313 nm) nanoparticles and high numbers of lysed cells were used to suggest that selenite reduction in granular sludge resulted in large intracellular selenium nanoparticles which stretched and eventually broke cells (Gonzalez-Gil et al., 2016). It is unlikely that this was the case in the present study as nanoparticles were generally associated with intact cells (Fig. 3). Further research is required in this area to identify the mechanism of nanoparticle production and expulsion.

4.2 Microbial Community Analysis

4.2.1 Diversity

The decrease in alpha diversity over enrichment generations indicates the evolution of highly specialised selenate and selenite reducing communities (Fig. 4). The 3rd and 4th generations of each enrichment had similar values for all diversity indices, however, there was more variation in the selenite fed enrichment, potentially indicating a higher diversity of organisms capable of reducing selenite, which has been reported previously (Eswayah et al., 2016). If a two-step

selenate reduction process occurred, in which separate communities reduced selenate and selenite, it might be expected that the diversity in the selenate enrichments would be higher than the selenite enrichments. This however, was not the case (Fig. 4), indicating that there may not have been separate selenate and selenite reducing communities in the selenate enrichment and complete selenate reduction to Se(0) was carried out by a distinct community.

Only minor changes in community beta diversity occurred in the first generation of each enrichment, both in terms of OTU abundances (Fig. 4) and phylogeny (Fig. 4). The communities of successive generations then diverged significantly for selenite and selenate fed enrichments. This indicates that continuous sub-culture in the presence of selenate or selenite had an influence on the community and there is potentially a phylogenetic difference between selenate and selenite reducing communities. The high levels of environmental pressure in terminal clades (>2 NTI) of each sample indicates that the enrichment process had a deterministic influence on community assembly (Fig. 5). In addition, the increasing levels of environmental pressure with each generation indicate that this influence grows stronger with each sub-culture. In contrast, environmental pressure based on global clustering was lower (approx. -2 NRI) and decreased with each generation, indicating that environmental pressure was influencing the community in terminal clades rather than on a global scale.

4.2.2 Divergent Microbial Communities

It might be expected that the community which evolved in the selenite fed enrichments constituted a subset of the selenate reducing community, since the product of selenate reduction is selenite. However, vastly different taxonomic compositions emerged in each enrichment, indicating that the selenate enrichment possibly became dominated by organisms capable of the complete reduction of selenate to Se(0) (Fig. 6). However, *Veillonellaceae sp* dominated the 3rd and 4th generations of the selenate enrichment but have previously only been associated with selenite reduction (Gonzalez-Gil et al., 2016). Their increased relative abundance may

indicate that they also have the ability to reduce selenate or that a two-step selenate reduction pathway occurred, whereby *Veillonellaceae* reduced selenite, which had originated from selenate reduction, to Se(0). Selenite reduction by *Veillonellaceae* species was previously demonstrated to be as a result of a detoxification mechanism rather than anaerobic respiration (Gonzalez-Gil et al., 2016; Pearce et al., 2009). Therefore, it is possible that in the current study *Veillonellaceae* were reducing selenite as a means of detoxification and were part of a cooperative selenate reducing community. *Sulfurospirillum* sp, a genus within the *Campylobacteraceae* family, were the second most abundant genus in the latter generations of the selenate enrichments (Fig. 6) and are known to carry out dissimilatory selenate reduction to selenite, linked to anaerobic respiration (Stolz et al., 1999). Therefore, in a mixed culture enrichment such as this, it may be the case that Se (0) was formed by multiple mechanisms.

The dominance of the *Shewanella* genus in selenite 4 (Fig. 6) indicates their role in selenite reduction. Shewanella sp are known to be capable of oxidising organic compounds using a diverse set of terminal electron acceptors, including selenite, making them important for carbon cycling and potentially useful in bioremediation (Fredrickson et al., 2008; Klonowska et al., 2005). *Propionibacteriacea*e were dominant in selenite 3 but their relative abundance decreased in selenite 4 and have not previously been associated with selenite reduction. *Propionibacteriaceae* are a family in the Actinobacteria consisting of 15 genera (Stackebrandt, 2014) but could only classified to family level in the current study. Two metagenome assembled genomes, related to the *Propionibacteriaceae* were previously assembled from shotgun metagenomics samples taken from a bioreactor treating selenate and nitrate containing mine-influenced wastewater, although its potential function was unclear (Baldwin et al., 2019).

4.2.3 Impact on Methanogens

Selenate and selenite are generally thought to be toxic to methanogens (Lenz et al., 2008c). Therefore, it is unsurprising that the relative abundance of *Methanosaeta* decreased over the

course of the enrichment process in the presence of both selenate and selenite (Fig. 6). *Methanobacterium* sp however, maintained a roughly similar relative abundance throughout each generation of the enrichment process (Fig. 6), potentially indicating their tolerance for toxic selenium compounds. This apparent tolerance to selenate and selenite concentrations may make them potentially important for continued methane production in methanogenic reactors treating wastewaters with elevated selenium concentrations.

4.2.4 Differential Taxa Analysis

Differential taxa analysis of the selenite 4 and selenate 4 cultures identified over 30 genera and families which were significantly different between the two groups (Fig. 7). Many of these taxa were of low abundance and not immediately apparent on viewing the most abundant organisms (Fig 6). While it is likely that in an enrichment culture, the most abundant organisms are carrying out the desired function, low abundance organisms may have important ecological roles in microbial community function and often contribute disproportionately to functionality (Jia et al., 2019; Shade et al., 2014). Therefore, some of the organisms identified by differential analysis may be of critical importance to selenate and selenite reduction. *Comamonas* sp were significantly more abundant in the selenate enrichments (Fig. 7), which is in line with previously reported results, albeit in soil enrichments (Navarro et al., 2015). *Comamonas testosteroni* S44 was previously found to aerobically reduce selenate using components of the sulphate reduction pathway and reduce selenite with a putative selenite reductase (SerT) (Tan et al., 2018). This further emphasises the potential for a number of selenium reduction mechanisms occurring side by side in mixed microbial communities. Several *Clostridium* sp were also more abundant in selenate 4 than selenite 4 (Fig. 7). Selenate reduction to Se(0) in *Clostridium sp* has been reported previously under aerobic and anaerobic conditions via multiple proposed mechanisms (Bao et al., 2013; Yanke et al., 1995). Therefore, in this case *Clostridium sp* and *Comamonas sp*. may be responsible for selenate and selenite reduction in

the selenate fed enrichments. *Rhizoium sp* were also significantly more abundant in Selenate 4 (Fig. 7). Unlike *Clostridum* sp and *Comamonas* sp, *Rhizobium* sp have only been associated with selenite reduction and not selenate reduction (Hunter, 2014; Hunter et al., 2007). *Rhizobium selenireducens* was originally isolated from a selenate reducing community in a lab-scale bioreactor (Hunter and Kuykendall, 2007) but it was proposed that selenate was reduced to Se (0) in a two-step process; 1) reduction of selenate to selenite and 2) reduction of selenite to Se(0) and that the isolated Rhizobium species was solely involved in step two (Hunter et al., 2007). This may also be the case in the present study. Xanthobacter sp were significantly more abundant in selenite 4 than selenate 4 (Fig. 7). *Xanthobacter* are a member of the Alphaproteobacteria and have not previously been associated with selenite reduction.

4.2.5 Discriminant Taxa

Several of the organisms identified by differential taxa analysis were also found to be discriminant taxa by sPLS-DA, which were responsible for most of the beta diversity (Fig. 8). For example, a cluster of OTUs labelled Block 1 contained OTUs assigned to *Veillonellaceae, Clostridium* and *Sulfurospirillum* sp and differentiated selenate 2,3 and 4 from the rest of the samples. A cluster of OTUs including: *Shewanella* and *Xanthobacter* sp, *Propionibacteriaceae* and *Methanobacterium sp* were more dominant in selenite 2, 3 and 4 samples once again emphasising their potential role in selenite reduction or their tolerance to selenite in the case of *Methanobacterium* (Fig. 8).

4.3 Diverse Mechanisms of Selenium Reduction

The potential diversity of selenium reducing organisms and the variety of mechanisms by which reduction takes place in mixed cultures makes it particularly difficult to study these organisms in the environment. For example, no single gene target is available for PCR based analysis, similar to the mcrA gene in methanogens (Luton et al., 2002; Morris et al., 2016) or the amo gene for ammonia oxidation (Prosser and Nicol, 2008; Tourna et al., 2008). Primers

have been designed to target the selenate reductase gene of *Thauera selenatis* (SerA) (Wen et al., 2016). However, successful amplification was only achieved using two out of the three selenate reducing organisms studied. Selenite reduction capabilities may be even more difficult to detect due to the involvement of both detoxification mechanisms (Ojeda et al., 2020; Tugarova and Kamnev, 2017) and anaerobic respiration-linked selenite reduction (DeMoll-decker and Macy, 1993; Harrison et al., 1984; Li et al., 2014; Ojeda et al., 2020) .

Since all of these reduction mechanisms result in the formation of selenium nanoparticles perhaps gene targets associated with nanoparticle assembly or extrusion would be more suitable targets than those involved in reduction. However, this would require significant further investigation. Pure culture studies utilising transcriptomic analysis and subsequent mutant based studies could be useful in elucidating such a mechanism. In addition, meta-genomic and meta-transcriptomic studies of mixed culture environmental samples would provide a more real-world approach, which could also be useful.

4.4 Isolation of Selenium Reducing Microorganisms

The best classified match to Isolate G4A2 was a *Sulfurospirillum* sp (Fig. 9) isolated from a bioelectrochemical system (Ueoka et al., 2018). *Sulfurospirillum* sp are known to reduce selenate to selenite via anaerobic respiration and form Se(0) nanoparticles by an unknown mechanism (Oremland et al., 2004). As G4A2 was lost form culture, no further characterisation could be carried out.

Isolate G4I1 had high sequence similarity (>99%) with several other sequences in the NCBI database assigned to *Shewanella xiamenensis* (Fig. 9). *S. xiamenensis* was first isolated from coastal sea sediments and was shown to anaerobically respire selenite with lactate as an electron donor (Huang et al., 2010). It has subsequently been studied in relation to bioremediation of metal contaminated wastewaters (Zinicovscaia, 2020). However, in-depth

studies of its selenite reducing abilities are lacking. Since *Shewanella* sp are generally thought to reduce selenite via anaerobic respiration and accounted for over 50% of the microbial community it is likely that this was the dominant mechanism of selenite reduction in selenite 4. Several other *Shewanella* sp also had high sequence similarity including *Shewanella onedensis*, which is well studied in relation to selenite reduction (Li et al., 2014; Pearce et al., 2009). To the best of my knowledge this is the first selenium respiring isolate from methanogenic granular sludge and therefore represents a promising option for further study.

Selenium nanoparticles were previously determined to have been produced intracellularly in *S. oneidensis* (Li et al., 2014). However, nanoparticles observed in cultures of isolate G4I1 were predominantly extracellular with some smaller nanoparticles which appeared to be intracellular (Fig. 10) indicating possible internal nanoparticle formation and subsequent extrusion. The rate of selenite reduction by G4I1 was much higher (Fig. 10) than previously reported for *Shewanella* sp (Li et al., 2014). However, selenite concentrations in the current study were considerably higher, which has previously been shown to increase the rate of selenite reduction in numerous studies (Lortie et al., 1992)

5 Conclusions

Diverse cell morphologies in mixed culture enrichments were associated with selenium nanoparticle production, which appeared to be both located intracellularly and extracellularly, indicating multiple mechanisms of nanoparticle production. Microbial community alpha diversity decreased with each generation in both enrichments resulting in highly specialised selenate and selenite reducing communities. Increasing environmental pressure on terminal clades caused by continuous sub-culture in each enrichment led to the divergence of each community from source. Vastly different microbial communities evolved in the presence of selenate and selenite, and, based on taxonomic composition and known selenium reduction mechanisms, it appears that multiple mechanisms of selenium reduction were likely occurring in enrichments. A single isolate was successfully obtained from the selenite enrichment for further study, which was classified as a *Shewanella* sp. A combination of pure culture studies and omics approaches, including transcriptomics, should be employed to determine the contribution of various mechanisms to selenium reduction in real-world scenarios.

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CHAPTER 6

Final Discussion, Conclusions, Perspectives and Future Research

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1 Final Discussion

The formation of dense, settleable granular aggregates in high rate anaerobic bioreactors such as the UASB and EGSB is essential for their operation and allows for good biomass retention and high liquid upflow velocities. Since the development of such bioreactors in the 1980s the mechanism of granule formation (granulation) has been questioned by researchers. However, a number of aspects of granulation have been overlooked. For example, granulation is often studied during start-up only, rather than as an on-going, dynamic process which occurs continually in a stable reactor. In addition, the microbial ecology of granulation is relatively understudied. Granules contain physio-chemical gradients (e.g. oxygen) and ecological niche spaces which are required to accommodate all of the microorganisms necessary for the complete degradation of organic compounds into methane and CO_2. Granulation is also thought to facilitate increased syntrophic interactions between microorganisms such as acetogens and methanogens. Therefore, the development of an ecological granulation model, based on continual granulation, rather than just during start-up could be a useful addition to the field of granulation research. Several unifying concepts for methanogenic, aerobic and anammox granulation were outlined in Chapter 2 and several research gaps relating to microbial ecology were identified. Chapters 3 and 4 of this thesis attempted to address these gaps in methanogenic granules, using a novel lab-scale bioreactor trial accompanied by in depth physical characterisation of granules coupled to microbial community analysis using 16S rRNA gene and gene transcript sequencing. The patterns in microbial community analysis observed in this thesis such as a distinct flocculent community, community succession in growing granules and increasing environmental pressure with granule size should be investigated in other granule types to see if they are common and could contribute to a unified theory of granulation.

The future of granular sludge technology may not be entirely reliant on established granule types such as aerobic, methanogenic and anammox granules. Photogranules and hydrogenic

granules also represent unique possibilities (Milferstedt et al., 2017) and granular biomass has also been used for the removal of toxic compounds such as selenium oxyanions selenate and selenite (Lenz et al., 2008a). However microbial ecology of selenium reducing organisms is not well understood. Therefore, Chapter 5 of this thesis attempted to explore the differences in selenate and selenite reducing microbial communities in granular sludge. Several novel findings arising from the literature review and experimental work outlined in this thesis are listed below.

2 Final Conclusions

1. Unifying concepts of granulation

Several unifying concepts relating to granulation theories were identified for anaerobic, aerobic and anammox granular sludge. These include the role of: selection pressure, shear forces, EPS production, divalent cations and inorganic nuclei in granulation. However, some differences were also identified. For example, concentric layers are not universal to all sludge types and a cell cluster like architecture is more common in anammox granules. Additionally, methanogenic granules appear to be more reliant on filamentous organisms such as *Methanosaeta* in the early stages of granule formation, rather than EPS, which is more important in aerobic and anammox granulation. The role of quorum sensing also appears to be different in methanogenic granules from aerobic or anammox granules.

2. Granule size distribution impacts bioreactor performance.

A full complement of granule sizes is required for optimal bioreactor performance

Chapter three examined the effect of granule size separation prior to seeding on bioreactor performance. Clear differences were observed between bioreactors seeded with size-constrained granules and bioreactors seeded with a full complement of naturally distributed granules. Biomass dynamics in the bioreactors and pCOD in the effluent were particularly affected by granule seed size. High levels of pCOD in the effluent of bioreactors seeded with small granules were thought to be caused by washout of the less settleable seed sludge, whereas the reactors seeded with large granules had high pCOD washout due to granule disintegration. As granule size distributions in size constrained bioreactors reverted to the natural distribution, bioreactor performance improved.

3. **An innate size distribution is associated with specific bioreactor conditions**

The granule size distribution in bioreactors seeded with large granules reverted to a distribution similar to that of the bioreactors seeded with a full complement of granules. The bioreactors seeded with small granules did not quite reach the same distribution but may have required more operation time or have been impaired by continual biomass washout. This indicates that a specific size distribution is associated with given bioreactor conditions and alteration of size distributions will only be temporary, before the innate size distribution associated with operational conditions is reinstated.

4. **Distinct morphological features are associated with different growth stages of methanogenic granules**

Clear associations between granule morphology and granule size were observed, which indicated that the smallest granules may be broken pieces of old granules or flocculent biomass, which had arisen from planktonic growth. Four stages of granule development were identified and a granule "aging" system was proposed which could be useful in assessing the overall health of the sludge bed.

5. **A distinct microbial community is associated with granule formation at full scale and lab scale**

XS biomass was predominantly acidogenic

XS biomass was not only different from other sizes morphologically but had a significantly different microbial community, both in the seed sludge, which originated from a full scale IC bioreactor and at lab scale. The microbial community of XS granules had a higher proportion of acidogenic organisms such as *Streptococcaceae* indicating that this size fraction may be responsible for providing simpler organic molecules such as VFA's to acetogenic or methanogenic communities. It was proposed that the XS biomass was present as a background flocculent or planktonic community in the bioreactor or as an outer layer of granules which, in

this case, could be thought of as primary consumers in a community succession model for methanogenic granulation.

6. **Granulation facilitated increased syntrophic interactions**

In contrast to the XS biomass, granular communities had higher proportions of syntrophs and methanogens and resembled a more typical methanogenic community. Several other organisms which are known to form syntrophic partnerships with methanogens were also more abundant in granular communities such as SRBs and *Geobacter sp.* Therefore, granulation appeared to facilitate increased syntrophic interactions.

7. **Aerobic organisms may be important in the establishment of an anaerobic environment in early stages of granule development**

Thauera sp have been identified as important for aerobic granulation previously and were prominent in the active community of S granules. Their role in granulation may be to initiate the formation of an anaerobic environment by removing dissolved oxygen through aerobic respiration, allowing for the establishment of strict anaerobes such as methanogens.

8. **Continual cycles of growth and breakage in anaerobic granules may contribute to overall diversity in anaerobic bioreactors**

Continual increases in species richness and diversity were observed during stable reactor operation, despite reasonably high levels of biomass washout. It is believed that these increases in diversity and richness were caused by independent evolution of microbial communities inside individual granules, in a similar manner to what has previously been proposed for soil aggregates (Rillig et al., 2017). Upon breakage the internal microbial communities of granules, which had evolved due to environmental pressures such as substrate limitation were released, leading to diversification in the bioreactor meta-community.

9. **Separate microbial communities evolved in the presence of selenate and selenite**

The results from Chapter 5 of this thesis indicate that exposure to selenate and selenite appeared to have differential impacts on a granular sludge microbial community, indicating that in mixed cultures, phylogenetically different organisms reduce selenate and selenite. It had been hypothesised that the selenite reducing community might be a sub-community of the selenate reducing community, given that selenate reduction to Se(0) is a two-step process with selenite as the intermediary. However, this does not appear to have been the case.

10. **Methanobacteriaceae appear to be more tolerant to selenate and selenite than other methanogens**

Selenate and selenite are generally considered to be toxic to methanogens and inhibit methanogenesis. However, Methanobacteriaceae appeared to persist throughout enrichment generations, making them potentially useful in simultaneous methane production and selenium removal from wastewaters.

11. **Multiple mechanisms of selenate and selenite reduction likely occur side by side in mixed microbial communities**

Based on existing literature, the taxa associated with selenate and selenite reduction in Chapter 5 do not appear to share common mechanisms of selenium reduction and it is probable that multiple different mechanisms occur side by side in mixed microbial communities.

3 Perspectives

Some key findings in this thesis could have direct application in real world scenarios. Firstly, the identification of a distinct flocculent community which may specifically carry out the acidogenesis step of AD could be useful in developing management strategies or novel bioreactor designs. The flocculent community in bioreactors may be easier to manipulate through routine bioreactor operation than the granular communities due to its lower settling velocities, which could be advantageous for bioreactor management. For example, VFA accumulation in failing bioreactors could be reduced by selectively washing out this flocculent VFA-producing community and allowing acetogenic and methanogenic communities to "catch up" by consuming residual VFAs. This community could also be enhanced, perhaps by employing some mechanical breakage of larger granules to promote more flocculent growth. In addition, the flocculent or planktonic community of bioreactors is often under sampled and based on the findings of this thesis, should be studied more closely from now on.

A number of findings from this thesis could also act as a starting point for further research. For example, the granule classification system proposed in Chapter 3 could be improved with more high throughput microscopic methods and automated image analysis. This could provide a useful tool for determining the general "health" of a sludge bed. The impact of on-going granulation on microbial community dynamics and knock on effects on bioreactor performance also warrants further investigation. It was proposed in Chapter 4 that cycles of granule growth ad breakage contribute to increasing diversity and thus stability. Therefore, it may be possible to improve bioreactor stability by accelerating these cycles, perhaps through mechanical granule breakage. Chapter 5 concluded that diverse communities employed various mechanisms of selenium reduction. Based on this work it is clear that further research is required to explore the phylogenetic range of selenium and perhaps more importantly the mechanisms by which it occurs. (meta-) Genomic and (meta-) transcriptomic methods could

identify genetic targets for more in depth studies and elucidate the evolutionary relationships of selenium reducing species.

4 Future Research

This thesis addressed some knowledge gaps associated with methanogenic sludge granulation, particularly in relation to microbial ecology. Several novel findings were presented which could have implications for bioreactor operation. However, further studies are required to determine if these findings are universal. For example, the synthetic wastewater in Chapter 3 was relatively simple, with the most complex organic molecule being lactose. Further trials with more complex synthetic or real wastewaters are required to determine if these results are replicable and assess the role of hydrolytic organisms in granule formation. Additionally, full scale bioreactor sampling and size separation is required to identify whether these observations are simply artefacts of lab-scale operation.

A granule classification system based on granule morphology was proposed in Chapter 3, which could be useful in assessing the health of a sludge bed. However, the methodology is too low throughput to be applied practically. Using un-fixed, wet granules in Environmental-SEM would speed up the process and reduce fixation artefacts. Additionally, computational image processing could standardise the process and make it more objective. Nonetheless, the concept may be worth exploring further

"Omics" technology including metagenomics and metatranscriptomics would also be useful in identifying functional changes during granule formation, growth and disintegration. 16S rRNA gene sequencing provides information on who is present and gene transcript sequencing provides information on who is active (De Vrieze et al., 2018) but omics technologies would reveal what these organisms are doing. This is necessary to get a conclusive picture of the granulation process. For example, identifying predominant EPS producers or revealing the role

of quorum sensing, or DIET in granulation could enable selective enrichment of key granule forming organisms.

Metagenomic sequencing not only allows functional information to be uncovered, but is now commonly used to reconstruct whole genomes from metagenomes (MAGs). In Chapter 4 it was proposed that communities in individual granules evolved during granule growth. MAGs from single granules (rather than multiple granules) actively undergoing growth would allow strain level diversification inside granules to be studied, indicating whether the granule life-cycle facilitates genetic evolution. This would provide an additional layer of depth to the hypothesis that granules act as evolutionary incubators, which was first proposed for soil aggregates (Rillig et al., 2017) and continual disintegration and reformation influences diversity in the bioreactor meta-community.

Chapter two identified several unifying concepts in granulation across three sludge types. Similar studies to the bioreactor trial in Chapter 3 and 4 of this thesis focusing on aerobic and anammox granules could help in the formation of a universal granulation theory.

With regards to selenium reduction future studies should have a more mechanistic than phylogenetic focus as phylogenetic information is not yet useful due to a lack of understanding of the mechanism of selenium reduction in different organisms. Pure culture studies utilising transcriptomic analysis of gene expression of various organisms performing selenate and selenite reduction in different conditions are required to get better insights into the diverse mechanism of selenium reduction. This could then be followed by a study of mixed cultures using omics techniques to determine the prevalence of the various mechanisms of selenium reduction are in natural or engineered environments

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