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Review

Granular biofilms: formation, function, application, and new trends as model microbial communities

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LIST OF ABBREVIATIONS

AD – Anaerobic digestion

AHL – N-acetyl-homoserine-lactone

Anammox – Anaerobic ammonium oxidation

AOB – Ammonia oxidising bacteria

cDNA – Complementary DNA

COD – Chemical oxygen demand

EGSB – Expanded granular sludge bed

EPS – Extracellular polymeric substances

FISH – Fluorescence *in situ* hybridization

HRT – Hydraulic retention time

HTS – High-throughput sequencing

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mRNA – Messenger RNA

MS – Mass spectrometry

NMR – Nuclear magnetic resonance

NOB – Nitrite-oxidising bacteria

OLR – Organic loading rate

OTU – Operational taxonomic unit

PAO – Polyphosphate-accumulating organism(s)

qPCR – Quantitative PCR

rRNA – Ribosomal RNA

SBR – Sequencing batch reactor

SIP – Stable isotope probing

SRT – Solids retention time

TCA – Tricarboxylic acid

T-RFLP – Terminal restriction fragment length polymorphism

UASB – Upflow anaerobic sludge bed

VFA – Volatile fatty acids

VSS – Volatile suspended solids

V_{up} – Upflow velocity

WWTP(s) – Wastewater treatment plant(s)

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Abstract

As the global demand for water increases, so does the quantity of wastewater requiring treatment. Due to a relatively low carbon footprint, compared with conventional wastewater treatment technologies, anaerobic digestion (AD) was identified in the 1970s as a forerunner in the push for sustainability, when interest in sustainable technologies and renewable energy sources was first sparked. AD technology development ultimately resulted in the discovery of the 'anaerobic granule'. It is a spontaneously-forming bio-aggregate of microbial cells capable of digesting pollutants and producing methane-rich biogas as a renewable source of bioenergy. The high settling velocity of such granules meant that AD systems could be operated as high-rate treatment processes, because the active, relatively-slow-growing, pollutant-removing biomass would be retained inside, and not washed out of, even bioreactors operated at extremely high volumetric loading rates. In the intervening years the emergence of the anaerobic ammonium oxidising (anammox) granule, aerobic granule, hydrogenic granule, oxygenic photogranule, and many other functionally-specialised granules, has opened new opportunities in wastewater treatment biotechnology. Whilst environmental engineering based around wastewater treatment is still a growing field of research and implementation, the granule (in all forms) is starting to catch the attention of microbial ecologists. It is a self-immobilised biofilm, with many of the properties of 'conventional' biofilms formed in Nature. However, as a single entity, a granule represents an entire community of microorganisms, competing or functioning cooperatively or in syntrophy. Together, inside a bioreactor, granules perform side-by-side arguably representing a meta-organism. Granules are gaining traction as the perfect samples for high-throughput studies on fundamental ecological concepts. Granular biofilms can be used to test hypotheses around drivers of diversity, community assembly, biofilm formation and maturation, community expansion and succession, community stress response, among others. This review outlines the history of three of the most influential types of granules: the anaerobic (methanogenic), aerobic and anammox granule. The main biochemical processes found in each type; their primary

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characteristics; and the typical makeup of the microbial community underpinning the processes are compared. Finally, the adoption of granules as the perfect 'playground' for experiments in microbial ecology is reviewed.

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1. Introduction

Increasing environmental awareness since the 1970s, along with the implementation of legislation promoting sustainable and renewable technologies, has resulted in growing interest in AD as a sustainable means of wastewater treatment. Compared to conventional activated sludge technologies, which are widespread throughout the industrialised world, AD systems are low-cost, energy-efficient, relatively low-technology and can meet essentially all criteria for water protection (McCarty, 1964; McKeown et al., 2012). AD is considered a renewable energy technology, producing a combustible biogas, composed primarily of methane, as an end-product. Furthermore, recent research has centred around manipulating the AD process to produce, and recover, value-added intermediates, such as VFA, including caproic acid (Kleerebezem et al., 2015; Nzeteu et al., 2018). Renewed interest, across the past 40 years, in AD for sustainable wastewater treatment resulted in the remarkable discovery of aerobic granules – the first documented self-immobilised, granular biofilms (van Lier et al., 2015).

The application of granular biofilms in engineered systems completely revolutionised AD, and wastewater treatment and valorisation, in the intervening decades. AD was transformed from a low-rate, low-profile process into a high-rate treatment technology suitable for high-strength, low-strength, psychrophilic, thermophilic, high-salinity, and many other types of waste treatment, and conversion, applications (Gagliano et al., 2017; Kato, 1994; Lettinga et al., 1980; McHugh et al., 2003; McKeown et al., 2009a). The tendency of granules to settle to the bottom of bioreactors, even under conditions of high-upflow-volumetric loading, meant that the active biomass supporting the treatment process was retained in, and did not get washed out of the, digester systems, thus supporting higher rates of hydraulic loading and faster operation. Additionally, the structure of sludge granules facilitated efficient mass transport of substrates between various microbial trophic groups making up the biofilms, and provided protection for

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more-sensitive species from micro-climatic changes (Hulshoff Pol et al., 2004). Over time, specialised granules were developed for various other processes, namely aerobic treatment, anaerobic ammonium oxidation (Anammox), and hydrogenic (hydrogen-producing) granules (Milferstedt et al., 2017a). The success of these technologies, centring around the granule, contributes to status of wastewater treatment as the most widely applied biotechnology in the world.

Whilst the past several decades of research and development in applying granule-based technologies has been exciting for biotechnology and environmental engineering, the discovery of the granule has also sparked the interest of microbial ecologists. In no other form in Nature are biofilms observed with such a definite microbial community boundary, having a clear beginning and end. Moreover, granules are complex, often compact, aggregates of microbial cells imparting architectural ingenuity and functional specialisation. In short, they provide the perfect playground for high-throughput studies on microbiome structure, community assembly, biofilm growth and disintegration, community stress response, and a myriad other compelling concepts in Microbial Ecology. The use of granules for more than just wastewater treatment is just gathering momentum, with landmark studies providing key insights into the mechanisms of biofilm formation (Tan et al., 2014) and drivers of diversity in complex microbial communities (Leventhal et al., 2018).

The aim of this paper is to provide a review for, on one hand, biotechnologists interested in the impact and applications of granular-based wastewater treatment processes, and, on the other, microbial ecologists interested in the potential of the granule as a useful tool to answer pressing questions on the nature of microbial communities.

2. History of, and primary biochemical conversions in, granular biofilms

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2.1 Conventional wastewater treatment processes

'Modern' wastewater treatment systems in the developed world are almost entirely reliant on technologies developed at the turn of the 20th century, and first implemented in any organised way in England. The (at that time) revolutionary technology, relies on a combination of physical and biological treatment to remove solids and nutrients from wastewater (Henze et al., 2008). The systems are usually referred to as conventional activated sludge treatment, and have changed very little over the past 100 years. The process is expensive and highly energy-intensive, requiring massive energy inputs for mixing, pumping and aerating. Such WWTPs require large areas for multiple 'process units' and, in essence, represent sloppy 'carbonomics' – in other words, the approach is linear and wasteful rather than circular and virtuous, and is not sustainable.

Conventional activated sludge treatment relies on aerobic respiration, and O_2 reduction by chemoheterotrophic bacteria during a (usually) three-stage treatment process. In the first stage – primary treatment – solid material is physically separated from bulk liquid, usually in sedimentation tanks (Tillman, 2017). The liquid phase, or primary effluent, is moved to secondary treatment: an aerobic, biological treatment phase, where microorganisms oxidise residual organic material, including suspended solids and soluble chemical oxygen demand (COD), primarily to carbon dioxide and water (Riffat, 2012). Secondary effluent, with a greatly reduced COD (up to 95%) may proceed to tertiary treatment: the final and most complete stage. There, various physical, biological or chemical techniques may be used to remove contaminating nutrients, including nitrogen and phosphorus. Tertiary biological treatment variously implies (1) nitrification – incorporating ammonium oxidation (nitritation) to nitrite by AOB, and nitrite oxidation (nitratation) to nitrate by NOB – with oxygen used as terminal electron acceptor, and (2) denitrification processes, either as separate unit processes or integrated into the secondary

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biological stage. Heterotrophic denitrifiers convert nitrate to N_2 under anoxic conditions (Kuai and Verstraete, 1998), but require organic substrates for carbon and reducing power, and, since secondary treatment removes most of the carbon it may be necessary for WWTP operators to provide fresh organic carbon, such as in the form of methanol, to fuel denitrification (delivering a curiosity and yet further inefficiency). More recently, applications based on autotrophic denitrification, in which the reducing power for denitrifying organisms, such as *Thiobacillus* sp., was supplied from pyrite (e.g. Yang et al., 2017) or biogenic sulfur (e.g. Kostrytsia et al., 2018) have been presented.

2.2 Discovery of anaerobic granules

The discovery of anaerobic granules marked a defining point in wastewater engineering. Sometimes referred to as methanogenic granules, they were first observed in the Dorr Oliver Clarigesters installed in South Africa in the 1950s, but were not reported until 1979 when detected in samples taken from these up-flow digesters (Lettinga, 2014). Indeed, these anaerobic granules are most frequently found in such up-flow digesters, where hydrodynamic conditions promote aggregation of microbial cells (Lettinga et al., 1980). Granules opened the door in AD engineering to a new generation of high-rate treatment systems, including expanding AD into new areas of wastewater treatment combined with other processes, such as sulphur cycling, micro-aerobic, anoxic, and conventional aerobic processes. Such was the promise of the technology to valorise wastes, Lettinga (2001) later described the field as his "faith, vision, hope and expectation" – the upending of old norms in sanitary engineering in favour of joined-up, closed-loop, sustainable cycles harnessing the full extent of the natural Carbon cycle.

High-rate treatment relies upon decoupling solids retention time (SRT) from hydraulic retention time (HRT). The settleability of anaerobic granules facilitates this, allowing retention of active

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biomass in 'retained-biomass' digesters, and allowing for larger volumes of high- or low-strength wastewater to be treated. Each granule comprises of a complex community of anaerobic microorganisms embedded in a matrix of EPS. The community spans several trophic groups including hydrolysers, fermenters, acetogens, sulfate reducers, syntrophic bacteria, and methanogens, among others (Vanwonterghem et al., 2014). The AD process is generally considered to consist of four stages: (i) hydrolysis; (ii) acidogenesis/fermentation; (iii) acetogenesis; and (iv) methanogenesis, with each stage facilitated by a different trophic group, and with substrates passed between successive groups. The collective allows for complete mineralisation of complex organic substances to methane along the AD pathway (Fig 1). In the 40 years following the discovery of anaerobic granules, the technology has transitioned from focused, laboratory-scale experiments to successful, full-scale implementation, treating a wide variety of wastewater types (van Lier et al., 2015).

2.3 Aerobic granules

While anaerobic granules were the first to be described, this was soon followed in the 1990s by the development, and application, of aerobic granules (Heijnen and van Loosdrecht, 1998), which marked the first revolutionary advancement in aerobic treatment since the first formal applications over 75 years earlier. As with the anaerobic granule, aerobic counterparts also comprise of a densely-packed, complex, microbial community and exhibit excellent settleability inside digester systems, thus facilitating extended SRTs. The most notable difference is that, whilst anaerobic granules are cultivated under strictly anaerobic conditions, aerobic granules are most often cultivated in sequencing batch reactors (SBRs) operated with an aeration phase as the primary mixing mechanism (Adav et al., 2008). Consequently, the microbial community contains aerobic bacteria in the outermost layers, but often with an anaerobic core (Lv et al., 2014a; Tay et

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al., 2002). Indeed, there are some disagreements among experts whether aerobic granules are truly 'aerobic' at all.

Respiratory rates of aerobic microorganisms exceed those of anaerobic communities. The pathway for microbially-mediated aerobic degradation of organic matter begins with an initial, intracellular, oxidative, enzymatic attack facilitated by oxygenases and peroxidases. Following this, glycolysis and the tricarboxylic acid (TCA) cycle degrade organic matter into a series of intermediates that can be further respired into carbon dioxide and water, or be used by the cell for biosynthesis (Fritsche and Hofrichter, 2000). In the AD pathway, the various trophic groups are each responsible for one step in the degradation pathway, passing substrates along the trophic chain, until methane is produced in the final step: the process is reliant upon an inclusive, comprehensive community. During aerobic respiration, a single aerobic cell is capable of the entire process, containing all the necessary enzymes. At WWTPs, aerobic metabolisms result in the removal of organic contaminants – measured as COD – and nutrients – mainly nitrogen and phosphorus. Reactors containing aerobic granules usually are operated to stimulate heterotrophic removal of organic pollutants and simultaneous nitrification-denitrification and/or enhanced biological phosphorus removal (He et al., 2017; Winkler et al., 2012).

2.4 Anammox granules

Whilst both anaerobic and aerobic granules support organic matter conversions along the carbon cycle, the third type of innovative granule harnesses natural conversion processes from the nitrogen cycle. Fixed nitrogen, in the form of ammonium and nitrate has been conventionally converted *via* nitrification and denitrification, both of which are energy-intensive processes requiring aeration and additional carbon supplementation, respectively. Anaerobic ammonium oxidation (anammox) bacteria, however, are able to take a short-cut right through the nitrogen

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cycle (Fig 1), and convert ammonium and nitrite directly to molecular nitrogen in one step (Kartal et al., 2010). In fact, it is suggested that annamox bacteria are responsible for as much as 50% of nitrogen transformations in marine environments (Kuenen, 2008). The anammox process was observed as early as the 1920s, but not fully understood until the mid-1990s (Mulder et al., 1995). It had been accepted that ammonium was chemically inert and required oxygen, and a mixed-function oxygenase, to be oxidized – hence the conventional focus on nitrification and denitrification processes – but it was not until simultaneous reductions of ammonium and nitrate were correlated with the production of nitrogen gas that the anammox process, and the implicated bacteria, were discovered and harnessed for wastewater treatment (Kuenen, 2008).

Early anammox applications were slow, two-phased (separate nitrification and anammox reactors), and experienced problems with biomass retention. However, the innovative cultivation of annamox granules, containing a rich community of both nitrifying and anammox bacteria, allowed the application of one-phase nitrogen removal processes with excellent biomass retention (Abma et al., 2010, 2007). Additionally, because anammox bacteria are autotrophs, carbon supplementation for denitrification was no longer required. The popular granular anammox systems have revolutionised an energy intensive, high-carbon-footprint process. Applications are usually operated as partial nitritation/anammox processes, where nitrification (facilitated by AOB and NOB) is halted following nitrite production of nitrite, which can be accumulated and used by anammox bacteria (Zhao et al., 2018). Therefore, NOB, and nitratation, must be inhibited so as to avoid competition with anammox activity.

2.5 Granular oddities

Anaerobic, aerobic, and anammox granules are the most widely utilised in built ecosystems, but the phenomenon of microbial aggregation into granular-type structures is not confined to such

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cases. Granular aggregation has been observed by the 'pink berry' communities involved in syntrophic sulfur cycling in salt marshes (Wilbanks et al., 2014), the large 'lake ball' communities formed by the rare green alga *Aegagropila linnaei* (Togashi et al., 2014), the colonies of cyanobacteria known as 'waterwarts' observed in warm lakes (Garcia-Pichel et al., 2002), and, more recently, novel photogranules formed under both static and mixed conditions (Milferstedt et al., 2017b). Equally, in engineered ecosystems, hydrogenic granules are used for the production of biohydrogen (Fang et al., 2002). Granulation is, thus, a relatively widespread, and very successful, survival strategy employed by a wide range of prokaryotic and eukaryotic organisms alike, and in a variety of natural and built ecosystems. It is a means for efficient transfer of electrons and substrates, whilst offering some degree of community protection from the challenges of microclimatic variances.

3. Granular sludge bioreactor designs

3.1 High-rate, upflow bioreactors

The most widespread examples of granular-based bioreactor technology are the UASB and EGSB bioreactor configurations. UASB bioreactors were developed in the late 1970s and originally used to treat sugar beet wastewater (Lettinga et al., 1980). Since then, UASB systems have been successful at demonstration-, pilot- and full-scale for denitrification and treatment of organic pollutants at high hydraulic, and organic, loading rates (Lettinga, 1995). Additionally, they have since been shown to adequately treat not only sugar beet wastes, but also raw sludge, and slaughterhouse and potato starch wastewaters (Koster and Lettinga, 1988; Lettinga et al., 1983, 1980; Sayed et al., 1988) among many others. The UASB design relies on an active sludge mass (sludge bed), which settles to the bottom of the bioreactor and is a collective of anaerobic granules (Lettinga and Pol, 1986). In these systems, wastewater enters the bioreactor through the

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bottom and is pumped up through the granular sludge bed. During this time the microorganisms in the granules encounter, and mineralise, organic pollutants in the wastewater. By the time the water has been pumped to the top, where it leaves the system, a large portion of the COD has been removed. The UASB design often includes a phase separator, which collects biogas evolving from the liquid phase (Van Haandel and Lettinga, 1994).

Developed in the 1990s, the EGSB design has gradually surpassed the popularity of the UASB due to its ability to accommodate organic loading rates 6-7 times higher than a typical UASB (Frankin, 2001; Riffat, 2012). EGSB configurations are similar to the UASB, but further include intense recycling of reactor liquor to effect mixing of the sludge bed, which contributes to higher conversion rates (Kato et al., 1998, 1997, 1994). EGSB bioreactors can be: started up (to full capacity) rapidly; applied to treat a broad scope of wastes; and operated successfully even under extreme environmental conditions (Lettinga, 1996). Over the years, many modifications have been made to the classical UASB and EGSB designs. Notably, membrane-coupled EGSB bioreactors have been reported with increased removal of COD (Chu et al., 2005). More recently the addition of a pumice stone filter to the EGSB has been documented (Keating et al., 2018). EGSB systems have been extensively tested under psychrophillic conditions, and biofilm robustness, and community and process stability, comparable with mesophilic treatment, have been extensively documented (Collins et al., 2005a, 2005b; Connaughton et al., 2006; Madden et al., 2014; McKeown et al., 2012, 2009a, 2009b, 2008). While the UASB and EGSB were originally designed and operated for anaerobic treatment, they are now used for anammox, biohydrogen production and several other applications (Mu and Yu, 2006; Tang et al., 2011). A key feature, however, of UASB and EGSB operation, is that the wastewater influent is typically supplied continuously.

3.2 Sequencing batch bioreactors

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The SBR is a commonly used adaptation of discontinuous treatment systems, to which influent is not supplied constantly, but pulse-wise. Aerobic granules are often formed in such systems (Morgenroth et al., 1997). Mixing is accomplished by periodically aerating the tank. Specific durations of aeration vary between cases, but in general, operation of an SBR with aerobic granules involves (i) the addition of wastewater to the tank, (ii) an aeration/treatment period, (iii) a settling period when the granules settle quickly to the bottom of the bioreactor, and (iv) removal of the treated effluent before (v) another batch of wastewater is added to the system and the cycle is repeated (Beun et al., 1999). The settling time can be used to select for fast-settling granules within the system, effectively removing any flocs (Beun et al., 2002). The discontinuous nature of operation can, in many cases, be advantageous if, for instance, a continuous supply of wastewater is unavailable.

4. Mechanisms of granule formation

4.1 General understanding of granulation

Granulation is the process by which a granule is formed. A seminal review on anaerobic granulation was published in 2004 (Hulshoff Pol et al., 2004) outlining numerous hypothesised granulation mechanisms and providing a conclusive summary of many granulation concepts. In the intervening period, research on the actual mechanisms of anaerobic granulation has declined. Several comprehensive reviews on aerobic, and anammox, granulation have also been published recently (Franca et al., 2018; Manonmani and Joseph, 2018; Nancharaiah and Kiran Kumar Reddy, 2018; Wilén et al., 2018), although predominantly in isolation rather than in conjunction with other types of granular sludge. Whilst no single model of granulation can yet be applied to all forms of granular sludge, several unifying concepts are common. These include: selection

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pressure; importance of EPS; bridging by filamentous microorganisms; structural organisation of microorganisms; influence of inoculum; cell surface hydrophobicity; electrostatic charge; and the influence of shear force.

4.2 Influence of selection pressure & shear force

The selection pressure theory states that dense, well-settling granules are continually selected for by liquid and gas upflow inside a bioreactor, while lighter granules are washed out (Hulshoff Pol et al., 1983; Liu et al., 2005). In anaerobic systems, selection pressure is a function of HRT and liquid upflow velocity (V_{up}). Increasing V_{up} and shortening HRT (i.e. increasing selection pressure) has led to improved granulation (Arcand et al., 1994; Noyola and Moreno, 1994; Wang et al., 2018; Xu et al., 2018) as well as the retention of well-settling granules (Hulshoff Pol et al., 1988). Increased selection pressure has also been shown to correlate with increased granule size, and stability, in anammox granules (Ma et al., 2013; Reino and Carrera, 2017).

High selection pressure, and organic loading rate (OLR), were shown by Liu and Tay (2015) to be key factors in achieving fast aerobic granulation, who also proposed a granulation mechanism whereby poorly settling flocs are washed out of the reactor quickly due to the high hydraulic selection pressure. The remaining, well-settling, aerobic granules then become overstressed by high OLR leading to increased EPS production and changes in cell surface hydrophobicity, thereby promoting granulation. Newly formed granules then grow quickly due to the high OLR. In contrast, Szabó et al. (2017) found that OLR had little effect on granulation. Liu and Tay (2002) emphasized the importance of shear force in granular sludge formation, proposing that hydrodynamic shear force not only influences the structural integrity of granules but also affects metabolic and kinetic aspects of bioreactor performance.

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4.3 Microbial-based theories, including filamentous bridging

Other granulation theories are based on the morphological characteristics of key microorganisms. The EPS matrix, common to all granule types, provides a web of polymers to which new cells can adsorb. One widely accepted theory of anaerobic (methanogenic) granulation, referred to as 'spaghetti theory,' centres around the importance of *Methanosaeta,* which are thought to provide a branched growth network, due to their filamentous morphology, within which other cells can embed (Wiegant et al., 1988). Subsequent growth of the aggregate then occurs *via* cell multiplication whilst shear forces act to shape the aggregate into a dense sphere (Hulshoff Pol et al., 2004; Liu et al., 2003).

Beun et al. (1999) suggested the involvement of fungi in the initial stages of aerobic granulation. The authors observed initial formation of large, fungal mycelial pellets, which subsequently fragmented. The fragmented pellets acted as an immobilisation matrix for bacterial populations, which proliferated, eventually forming dense well-settling granules. However, other, non-filamentous bacteria have been shown to be important: i) *Zoogloea* sp. were shown to be important for the formation of dense, well settling granules, whereas ii) *Burkholderiales* sp. were more predominant in "fluffy", slow-settling granules (Weissbrodt et al., 2012), and, finally, iii) *Candidatus* Accumulibacter and Competibacter were also identified as being important players during the process (Weissbrodt et al., 2013). Barr et al. (2010) proposed a mechanism whereby two separate types of aerobic granules form simultaneously. They found that one 'type' was dominated by *Candidatus* Accumulibacter while the second 'type' was made up of diverse micro-colonies, and proposed that these two types separately break apart and the broken 'bits' re-aggregate into new granules containing remnants of both original granule types. Whilst the specific mechanisms remain undecided, central to each of these theories is the presence of a crucial organism at the outset of granulation.

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4.4 Role of EPS in granulation

Almost all proposed mechanisms of sludge granulation broadly agree on the importance of EPS in initial stages of granule formation. In anaerobic systems, EPS production is generally thought to increase cell surface hydrophobicity, providing a 'sticky' matrix for additional cells/flocs to adsorb (Ding et al., 2015; Sheng et al., 2010). Xu et al. (2018) proposed that granulation relied on a continually decreasing increasing OLR and V_{up}, which initially stimulated EPS production, particularly proteins, resulting in greater cell surface hydrophobicity and, therefore, aggregation into granules. Similar observations were made by other studies (Torres et al., 2018; Wang et al., 2018) as well as for aerobic granulation (Gao et al., 2011; Kent et al., 2018). Cui et al. (2014) proposed an aerobic granulation mechanism based on simultaneous growth of autotrophic and heterotrophic microorganisms, in which the heterotrophs grew quickly and established an EPS matrix as the basis for granule formation.

Anammox granulation has been proposed in three steps. The first step involves initial cell-to-cell contact of anammox bacteria and aggregation within a thin layer of EPS. Growth occurs in the second step, and, finally, these aggregates fuse together along with other (heterotrophic) bacteria, forming an anammox granule. As with anaerobic and aerobic granules, proteins were thought to play an important structural role (Lin and Wang, 2017). Divalent cationic bridging was also identified as an important aspect of EPS bridging in anammox granular sludge, leading to dense, stable granules (Lin and Wang, 2017). Indeed, multiple studies have implicated the importance of cations, such as Ca^{2+} or Mg^{2+}, interacting with EPS (Caudan et al., 2014; Kończak et al., 2014). Whatever the precise mechanism, one unifying concept is common among all EPS-related granulation theories: EPS production by single cells or small aggregations of cells forms an initial matrix for the attachment of other cells, and protein content of EPS is beneficial for granulation.

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4.5 Granulation based upon a layered structure model

Many studies have described anaerobic, aerobic, and anammox granules as being made up of concentric layers. A layered structure for methanogenic granules was first suggested by MacLeod et al. (1990), and later by Ahn (2000), whereby aggregations of *Methanosaeta* formed a central core. Other functional groups, such as fermentative bacteria, acidogens and acetogens, are thought to then form concentric layers around the archaeal core (Hulshoff Pol et al., 2004; McHugh et al., 2003) – thus building a 'methanogenic' consortium capable of achieving the entire AD process. This theory is corroborated by studies using 16S rRNA-targeted fluorescence *in situ* hybridization (FISH) (Raskin et al., 1994; Collins et al., 2005; Sekiguchi et al., 1999; Tagawa et al., 2000). In aerobic granules, layers typically comprise an anaerobic core with aerobic organisms on the surface (Franca et al., 2018; Tay et al., 2002). Lv et al. (2014b) proposed that granules assemble according to a deterministic mechanism rather than a stochastic, aggregation/disintegration model. And, it was hypothesised that in anammox granules, aggregation of single cells leads to the formation of flocs, which grow and develop an outer rim of aerobic ammonium oxidisers leading to an anoxic core of anammox bacteria. A novel, 'budding' mechanism has also been proposed, whereby growth of anammox bacteria at the core of the granule causes protrusions at the outer surface, which are broken off by shear forces, forming the basis for new granules (Vlaeminck et al., 2010).

4.6 Formation around inorganic nuclei

Inert particulate matter has been proposed to act as an inorganic nucleus for growth during initial stages of anaerobic sludge granulation, even influencing the size distribution of granules in reactors (Pereboom, 1994). Some studies have also observed the beneficial effects for

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granulation of adding nucleating agents, such as activated carbon and zeolite (Fernández et al., 2008; Montalvo et al., 2014; Zhang et al., 2015; Zhou et al., 2015). Verawaty et al. (2011) used a fluorescent labeling approach to study the effect of adding crushed aerobic granular biomass to floccular biomass, finding considerable improvements in granulation and hypothesising that crushed granules acted as nuclei for the attachment of flocs and in preventing biomass washout. This is an idea that extends to both aerobic and anammox granules (Long et al., 2014; Ni et al., 2010).

5. Physico-chemical characterisation of granular sludge

Size-distribution, density, settleability, EPS composition, porosity, volatile solids concentrations (measured as VSS), and shear strength are common physico-chemical parameters measured for all types of granules. These parameters are inter-related and have strong implications for granule, and bioreactor, function.

5.1 Common characteristics of anaerobic granules

Size distribution of granules has long been an important characteristic. Coupled with the density of the aggregate, this determines the settleability, and retention, in AD systems (Bellouti et al., 1997; Jeison and Chamy, 1998). Many studies describe the effect of hydrodynamics on granule size (Arcand et al., 1994; Chang and Lin, 2004), but others (e.g., Batstone and Keller, 2001) found feed (wastewater) to be a stronger influence. Generally, the diameter of anaerobic granules ranges from 0.1 mm to 5 mm (Ahn, 2000; Shin et al., 2019; Trego et al., 2018). Batstone and Keller (2001) found that though the size distribution varied across several sludge sources, the diameter of most granules was between 0.5 and 2 mm. Methanogenic granules are usually dark in colour, spherical, and have settling velocities around 60 m/h (Hulshoff Pol et al., 2004;

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Milferstedt et al., 2017a). They are naturally porous, but porosity was found to increase with granule size, likely having strong effects on mass transfer, biogas diffusion and activity (Afridi et al., 2017; Jiang et al., 2016; Wu et al., 2016).

Indeed, size has also been found to have implications for granule activity. Bhunia and Ghangrekar (2007) found that as granule size increased, methanogenic activity also increased, though they propose that at an undetermined threshold size, activity would begin to decrease again. They argue, based on previous findings (Alphenaar et al., 1993; Batstone and Keller, 2001; Fukuzaki et al., 1995; Henze and Harremoës, 1983), that as size increases substrate diffusion decreases, and additionally, that as size increases additional layers of EPS inhibit substrate diffusion – thus decreasing overall activity. A study by Díaz et al. (2006) separated granules based on colour: black, grey, or brown. They noted differing structure in granules of different colour, where black granules were small and compact, grey granules were more layered, and brown granules were less compact and more porous. They suggested that the larger, brown granules corresponded to older aggregates, and, further, that the different colour 'types' were at different development stages – but this hypothesis was never further developed or tested.

5.2 Common characteristics of aerobic granules

Generally, aerobic granules are smaller than anaerobic granules, ranging from 0.2–2.0 mm in diameter, sometimes reaching 3 mm (Cydzik-Kwiatkowska et al., 2016; Jang et al., 2003; Tay et al., 2001; Zheng and Yu, 2007). The smaller size, compared to their anaerobic counterparts, has been attributed to the more rigorous mixing strategies used in aerated digesters (Dangcong et al., 1999). Aerobic granule size has been shown to vary with OLR with higher organic loading leading to smaller granules (López–Palau et al., 2009). Tay et al. (2001) concluded, however, that substrate type determined granule size, comparing granulation under glucose and acetate feeds,

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with glucose producing bigger granules. When one sludge sample was separated into a range of size fractions, it was found granule size varied from <0.5–4 mm, with a majority between 1 and 3 mm in diameter (Toh et al., 2003). The aggregates are usually described as round or spherical (Dangcong et al., 1999; Tay et al., 2001), but irregular shapes have been reported (Jang et al., 2003). In one case, uneven surface morphology was described and attributed to the easily-degradable nature of the wastewater and the high specific loading rate applied (Morgenroth et al., 1997), both of which can result in lower-density biofilms (van Loosdrecht et al., 1995).

The density of aerobic granules varies with depth and the surface (the outermost 600 μm) has been shown to contain the densest layers of the biofilm (Toh et al., 2003). Nonetheless, settling velocities between 25 and 35 m/h are repeatedly reported (Jang et al., 2003; Tay et al., 2002, 2001), and there is general agreement around the settling characteristics of aerobic granules. When Toh et al. (2003) separated granules according to size, they found settling velocities increased with size, until granule diameters reached 2-3 mm when the settling velocity decreased. The larger granules were found to be less solid, contain more cavities, and have lower biofilm strength, than their smaller counterparts. Furthermore, they observed the interior of larger granules had deteriorated, likely due to limited substrate transfer. Subsequently, high EPS concentrations were associated with increased settleability (Deng et al., 2016). EPS in aerobic granules is predominantly composed of protein (Adav et al., 2008; McSwain et al., 2005), although polysaccharides were relatively more abundant in outer layers (McSwain et al., 2005).

5.3 Common characteristics of anammox granules

Anammox granules are usually defined by a reddish, carmine colour, due to high concentrations of hemachrome (Tang et al., 2011), which is directly related to anammox activity (Song et al., 2017). The surface morphology is said to vary depending on bioreactor operating conditions,

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sometimes described as globular and rigid, planar and flexible, or cauliflower-shaped, with irregular and uneven characteristics (Lu et al., 2012; Song et al., 2017; Vlaeminck et al., 2010). Settling velocities are wide-ranging (35–160 m/h) but granule densities are close to 1.02 g /cm^3 (Lu et al., 2012; Song et al., 2017; Tang et al., 2011). Anammox granules are generally larger than aerobic granules, sometimes spanning over 6 mm in diameter (Lu et al., 2012; Tang et al., 2011). Mean diameters are generally 2.5–3 mm (Lu et al., 2012; Ni et al., 2011; Song et al., 2017; Tang et al., 2011), but can be <1.5 mm (Ma et al., 2013; Ni et al., 2015; Okabe et al., 2011). It was proposed that extra-large anammox granules form after smaller granules collide and then attach together (Song et al., 2017).

Granule size determines surface-to-volume ratio, which has important implications for mass transport, and, consequently, for both reactor performance and modelling (Volcke et al., 2012). Indeed, it was determined that larger anammox granules achieved higher conversion rates due to higher ammonium surface loads and lower oxygen penetration depths, making large granules more anoxic and conferring a competitive advantage on the annamox bacteria (Vlaeminck et al., 2010; Volcke et al., 2010). However, the bigger the granule gets, the more prone seems to floatation and washout. Gas pockets were observed in larger granules where EPS had obstructed interstitial channels used for gas diffusion. When the pockets fill with trapped gas the granule tends to float. Since this phenomenon was linked to larger granules, Lu et al. (2012) suggested controlling the size by limiting granular diameter to 2.20 mm to avoid washout.

In fact, anammox bacteria have been found to produce significantly more EPS in aggregates than has been observed in anaerobic or aerobic granules. For example, 111–127 mgVSS/g total EPS was found in anammox granules compared to about 60 mg/g in aerobic and anaerobic granules (Ni et al., 2015). Most studies found protein is the most common EPS component but that concentrations fluctuate with operating, or enrichment, conditions. However, the spatial

distribution of EPS components in anammox granules appears organised, and potentially stratified. Humic-like substances were predominant in loosely-bound EPS, whilst proteins dominated tightly-bound EPS (Jia et al., 2017). Moreover, α-polysaccharides and proteins were found at the centre of granules, and considered to be the structural backbone of the granule, whilst β-polysaccharides were concentrated in the outermost layers beneath which the anammox bacteria proliferated (Lin and Wang, 2017). This type of organized layering of EPS not only influences structural properties, but may suggest spatial organisation of the microbial community.

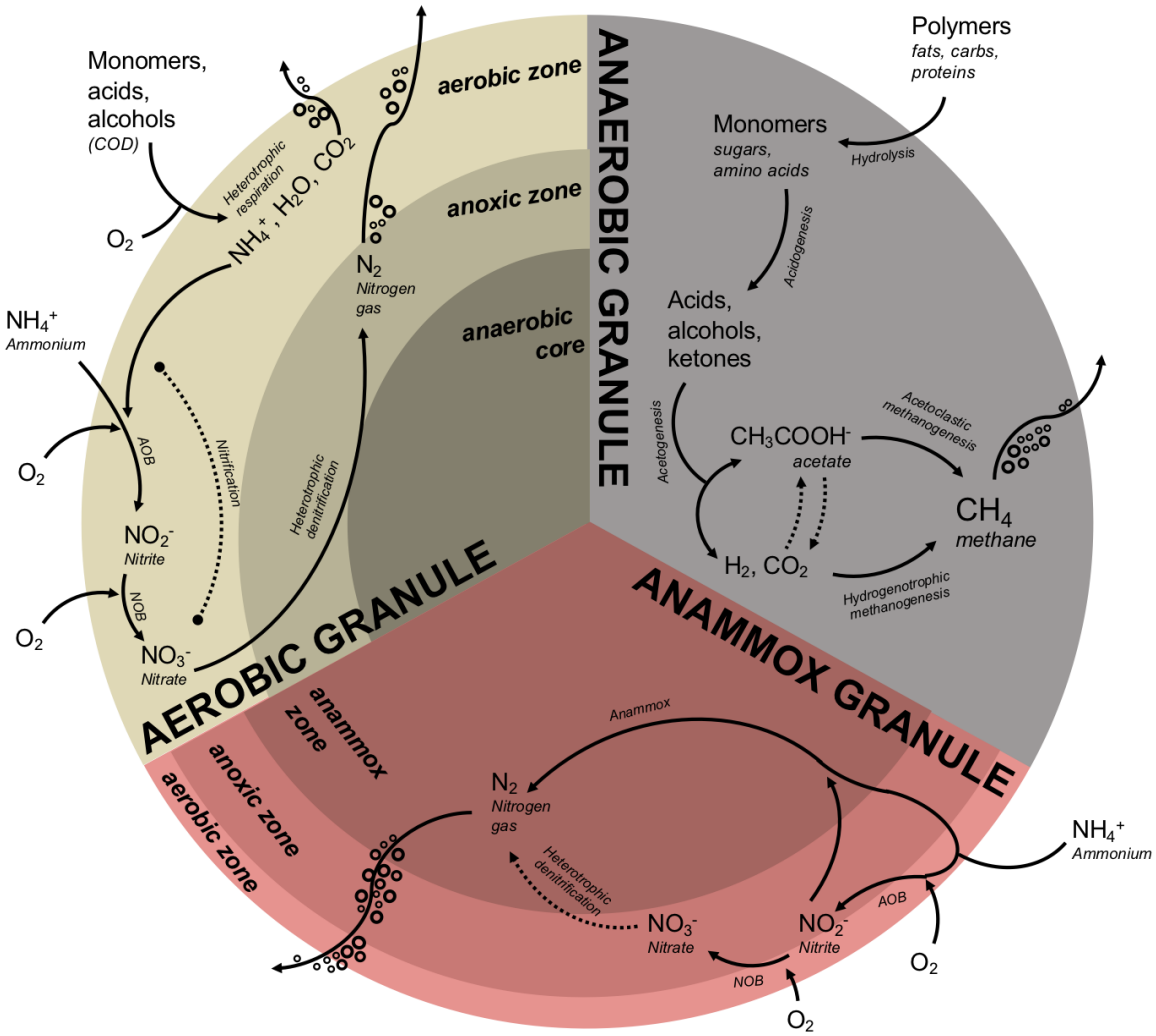

Figure 1. Combined schematic illustrating zones of activity, and biochemical conversions, characteristic of anaerobic, aerobic and anammox granules.

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6. Granular sludge microbiomes

Just as with many other similar types of microbial communities from across the built environment, granular biofilms have been widely studied using the tools of microbial ecology. The tools used include those based on targeted rRNA genes, but have evolved to include 'omics strategies focused on systems biology of whole microbial communities. Equally, however, granules represent unique forms of microbial communities, easily available from a range of environmental biotechnologies, and with no obvious or widely available equivalents in Nature, and so offer interesting model structures – tools themselves – for high-throughput studies in microbial ecology.

6.1 Evolution of techniques and practices in microbial ecology

Microbial ecology concerns diversity within microbial communities, and their interactions with each other and their environment. It has the goal to not only identify specific microorganisms in defined environments, but to also discover their functions and, thereby, assess their role(s) in the environment (Zoetendal et al., 2004). The concept of microbial diversity is at the heart of such studies and has been defined as the complexity and variation at different levels of taxonomic organisation (Torsvik and Øvreås, 2002). Traditionally, studies of microbial diversity were culture-dependent – largely based on the assumption that cultivation on an artificial medium would yield most of the organisms present in a sample – while, in fact, fewer than 1% of all microbial cells in a typical environmental sample will be culturable under laboratory conditions, a challenge known as 'The Great Plate-Count Anomaly' (Amann et al., 1995; Keller and Zengler, 2004; Staley and Konopka, 1985). The true microbial diversity of the environment cannot be determined using antiquated approaches that reply on cultivation alone. Indeed, prior to the

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development of modern, molecular microbial ecology, it was impossible to distinguish the evolutionary relationships that connect all of life (Pace, 1997). Moreover, prior to the use of comparative analyses of DNA sequences, there was no classification of the evolutionarily-distinct *Archaea* – key contributors to the AD process.

As such, 'molecular methods' have become the cornerstone of microbial ecology, underpinning the assessment of spatial and temporal fluctuations in microbial diversity (O'Flaherty et al., 2006). The molecular toolbox was founded on the identification of the 5S, 16S, and 18S ribosomal RNA (rRNA) marker gene sequences (Lane et al., 1985; Woese, 1987). Nearly 30 years since the taxonomic level of 'domain' was proposed by Woese et al. (1990), the 16S rRNA gene is central to prokaryotic species identification. It is highly conserved in all prokaryotic species, part of the ribosome and required by all organisms for the synthesis of proteins (Head et al., 1998). Several mainstay techniques in microbial ecology rely upon the 16S rRNA gene, including: (i) Fluorescence *in situ* hybridisation (FISH) – uses specifically designed, fluorescent rRNA probes that hybridise to phylogenetic markers and to detect, and determine the location, of target organisms. Historically used for genome mapping, FISH can be applied to study the spatial distribution of species in biofilms (O'Connor, 2008); (ii) terminal restriction fragment length polymorphism (T-RFLP) – assesses the diversity, and structure, of complex microbial communities for comparison across ecosystems by detecting small variations in [typically] the 16S rRNA gene (Moyer et al., 1994). More recently, and despite now numbering amongst "old-school" techniques, T-RFLP has been re-evaluated as an important tool to quickly screen AD communities (De Vrieze et al., 2018); (iii) stable isotope probing (SIP) – can be used to trace incorporation of substrates enriched in rare, stable isotopes, such as 13C-carbon, into DNA, rRNA, or mRNA and implicate microbial species involved in specific metabolic processes similar to those found *in situ* (Radajewski et al., 2000); (iv) quantitative polymerase chain reaction (qPCR) – considered a high-throughput method for quantification of target genes in a sample,

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indicating species abundance (Higuchi et al., 1992); and (v) high-throughput sequencing (HTS) – can be used to sequence PCR amplicons of 16S rRNA genes from community nucleic acids, providing deep and resolute taxa identification and community characterisation.

Combinations of these (and other) tools have helped uncover new insights to microbial diversity and function in the environment (Hall, 2007). HTS, in particular, is used beyond targeted rRNA studies and to support comprehensive analyses of whole genomes and transcriptomes (Shendure and Ji, 2008). HTS has contributed to achieving a whole-system approach in microbial ecology – whereby not only whole genomes and transcriptomes, but metagenomes and metatranscriptomes may be sequenced, representing entire communities (Hemme et al., 2010; Mason et al., 2012; Riesenfeld et al., 2004; Taş et al., 2014). The DNA sequence of a genome of a particular species will reveal all of the genes that can potentially be expressed by that organism – in essence, it uncovers the genomic potential – but when studying entire microbial communities, a metagenomic approach involves the random sequencing of the collective DNA of the whole community (Hugenholtz and Tyson, 2008). Metagenomics has transformed microbial ecology but DNA from dormant and dead cells will persist and can be picked up with the analysis (Bakken and Frostegård, 2006), so to further elucidate which genes are being expressed the metatranscriptome is sequenced (Sorek and Cossart, 2010). Using RNA – a single stranded compliment of encoded genomic material – is much more sensitive to environmental conditions, allowing analysis of the active genes in the environment. Metatranscriptomics involves reverse transcribing the extracted RNA to cDNA, a replicate of the gene that was expressed (Frias-Lopez et al., 2008).

The functional expression of microbiomes cannot be entirely captured by RNA-based studies as not all of the mRNA will be translated into proteins, and RNA-based studies are unable to capture processes such as proteolysis/protein turnover. To reveal finer detail on gene

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expression, metaproteomic studies can be designed (Hettich et al., 2013) to identify proteins extracted from environmental samples (Wilmes and Bond, 2006). Proteins are the final product of functional gene expression, and the basic molecular machinery – the molecules that carry out cellular metabolism (Hettich et al., 2013). Proteomics can provide insight to metabolic pathways (Abram et al., 2011) and identify specifically which proteins are associated with environmental stresses. The workflow generally comprises of extraction, separation and identification (usually using mass spectrometry) of the proteins from a sample (Tanca et al., 2014). Genome-centric studies combining metagenomics, metatranscriptomics and metaproteomics can be used to assess the contribution of microbial communities to entire ecosystem function (Maron et al., 2007) – one of the primary aims of microbial ecology.

However, to get beyond analyses of just metabolic potential, and to uncover community function under defined conditions, metabolomics may also be used. This is a developing field centring around the analysis of the metabolites produced by, and present in, biosystems (Smolinska et al., 2012). The aim is to characterise precise interactions between organisms and their environment on the molecular level, including the functional state and health of organisms or their responses to environmental stresses. Metabolites are analysed by a variety of procedures (Goodacre et al., 2004) but generally include nuclear magnetic resonance (NMR) or mass spectrometry (MS) as part of the main analytical platforms (Smolinska et al., 2012). Combining 'omics-derived datasets affords comprehensive insights to the genomic, and metabolic, potential and activity of whole ecosystems, allowing for improved management of engineered, and natural, environments based on interspecies interactions and key functional capacities (Vanwonterghem et al., 2014), but it also provides a framework for fundamental study of microbial biofilms.

6.2 Microbiome of anaerobic granules

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As yet, there is a striking absence of studies making use of metagenomics or metatranscriptomics to elucidate the whole-community structure of anaerobic granules. Other AD systems have been studied in detail, but not the communities which make up methanogenic granular aggregates. What we know about the community structure of anaerobic granules is instead limited to 16S rRNA-based studies, which are often used to determine how communities respond to shifts in environmental conditions. In particular, granular microbiomes are found to shift dramatically in response to temperature and substrate (Cerrillo et al., 2016; Keating et al., 2018; McKeown et al., 2009a; Na et al., 2016; Zhu et al., 2017). Keating et al. (2018) found the active community of low-temperature-adapted granules consisted primarily of *Proteobacteria*, *Synergistetes*, *Bacteroidetes*, *Chloroflexi* (particularly the genus *Anaerolinea*) and the methanogenic archaea *Methanosaeta*. High relative abundances of *Synergistetes* – usually rare in such systems – were thought important for low-temperature AD, whilst *Methanosaeta* and *Anaerolinea* – which are filamentous – were considered potentially important for structural integrity of the biofilms, especially during granulation. Other studies found *Methanosaeta* are indeed critical during granulation, even under high salinity conditions (Gagliano et al., 2017). In fact, under various conditions, *Methanosaeta* were reported to be abundant under, or to clearly response to, environmental stress (Cho et al., 2016; Connelly et al., 2017; Na et al., 2016; Zhu et al., 2017). Proteobacteria, many of which are found in syntrophy with a methanogenic partner, and *Chloroflexi* were abundant under various conditions (Bovio et al., 2019).

Datasets on methanogenic granules have not yet been published, but many studies have applied metagenomics to better understanding the AD process in samples of digestate, often from municipal wastewater treatment plants or biogas digesters. In such samples the community was comprised of 85-95% bacteria and 3-6% archaea, with bacterial populations dominated by *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* – regardless of operating conditions, and in agreement with many of the rRNA-focused studies (Guo et al., 2015; St-Pierre and Wright, 2014; Treu et

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al., 2016; Yang et al., 2014). Archaeal communities sampled comprised primarily of *Methanosaeta* and *Methanosarcina* (Yang et al., 2014), with functional enzyme-encoding genes, such as acetate kinase (*AckA*), phosphate acetyltransferase (*PTA*) and acetyl-CoA synthetase (*ACSS*), indicating acetoclastic methanogenesis as the dominant methane-generating pathway (Guo et al., 2015). Meanwhile, in biogas digesters treating cattle manure, the archaeal community was found to be less diverse and dominated by *Methanomicrobia* and *Methanobacteria*, suggesting a hydrogenotrophic pathway for methanogenesis (Treu et al., 2016).

Metagenomics has also been used to study the organisation of AD consortia, and has uncovered previously unknown lineages. In one case the entirety of the AD community could be grouped into two categories (Campanaro et al., 2016): i) organisms specialised in one primary function, such as central carbohydrate metabolism, or utilisation of amino-sugars; and ii) multi-functional organisms with the genetic capacity for multiple metabolic functions, such as sugar fermentation and methane production (Vanwonterghem et al., 2016). Campanaro et al. (2016) used this concept and the metagenomic data to propose a funnel-type model to describe organisation of the AD community, whereby each of the four stages of AD is catalysed by a more specialised trophic group of microorganisms, culminating in methanogenesis by a group of highly specialised archaea with a minimal range of substrates. Landmark studies providing metagenomic and/or metatranscriptomic analyses of anaerobic granules may well yield valuable insights to biofilm organisation, microbial stress responses, community assembly, and mass transport of substrates between trophic groups.

6.3 Microbiome of aerobic granules

Aerobic treatment relies on heterotrophic removal of organics, as well as of nitrogen and phosphorus by simultaneous nitrification-denitrification and biological phosphorus removal

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processes. Aerobic granules thus typically comprise of communities enriched with bacteria involved in nitrification, denitrification and phosphorus removal, such as the AOB, NOB, bacterial denitrifiers and polyphosphate-accumulating organisms (PAO) (Oehmen et al., 2010; Winkler et al., 2012). Many studies have analysed the microbiome of aerobic granules during granulation and under fluctuating conditions. Li et al. (2008) monitored the microbial community during granulation under different substrate loading rates, finding that loading resulted in reduced species diversity, whilst reduced loading rates produced granules with higher diversity. No trend was apparent in the key species dominating during granulation they suggested no one particular species is required for aerobic granulation. While that may be the case, the dominant microorganisms in aerobic granules across most studies are from the Proteobacteria, specifically Beta- and Gamma-proteobacteria (Adav et al., 2010; Aqeel et al., 2016; Chen et al., 2016; He et al., 2017; Li et al., 2008; Liu et al., 2017; Sun et al., 2017; Tan et al., 2014; Zhang et al., 2018).

When anaerobic granules were transitioned to aerobic granules, the relative abundance of Proteobacteria increased from 18% to 39% and included *Comamonadaceae*, *Xanthomonadaceae* and *Rhodocyclaceae* (Sun et al., 2017). *Comamonadaceae* is a large family belonging to the order Burkholderiales and phylum Betaproteobacteria. They are usually found in nitrifying/denitrifying sludge (Li et al., 2008) and comprise over 100 species with a wide variety of metabolic capabilities, including aerobic organotrophy, anaerobic denitrification and Fe^{3+} reduction (Willems, 2014). *Rhodocyclaceae* is a family of facultatively anaerobic Gammaproteobacteria detected in the core of aerobic granules (Lv et al., 2014a), capable of complete denitrification, observed during aerobic granulation, associated with increased protein in EPS (Aqeel et al., 2016), and possibly important for aggregation.

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Although used in engineered systems for wastewater treatment, aerobic granules have recently been used by microbial ecologists in several landmark studies to test fundamental ecological concepts. In aerobic granules enriched for enhanced phosphorus removal the abundance of *Candidatus* Accumulibacter, a known PAO from the Betaproteobacteria, increased (Henriet et al., 2016). Leventhal et al. (2018) used shotgun metagenomics of single aerobic granules from an enhanced biological phosphorus removal (EBPR) reactor to investigate strain-level diversity in biofilms. They found single granules were highly replicated at the genus level and could not be grouped into community types. However, community types for *Ca.* Accumulibacter – the most abundant organism – emerged at strain-level. They proposed strain-level diversity drives community assembly and overall biofilm diversity (Leventhal et al., 2018). Tan et al. (2014) used aerobic granules and cDNA-based analysis to assess the role of quorum sensing in community assembly. They found strong correlations between the N-acetyl-homoserine-lactone (AHL)-based quorum sensing system (a highly studied quorum sensing system) and granule formation. They further observed that biofilm disintegration was correlated with reduced AHL, and they proposed quorum sensing may be a key component of biofilm formation and the maintenance of structural integrity (Tan et al., 2014). Granules provide perfectly compact, yet complex, communities for ecological studies into community assembly, and expansion and succession of the microbiome.

6.4 Microbiome of anammox granules

Anammox bacteria are uniquely able to combine ammonium oxidation with reduction of either nitrite or nitrate to dinitrogen gas. The anammox bacteria are currently classified across six candidate genera within the phylum Planctomycetes and order Brocadiales (Guo et al., 2016; Jetten et al., 2010; Khramenkov et al., 2013; Kuenen, 2008). Many studies report *Candidatus* Brocadiacea as the most abundant anammox bacterium in anammox granules (Bagchi et al.,

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2016; Gonzalez-Gil et al., 2015; Lawson et al., 2017; Speth et al., 2016), although *Candidatus* Kuenenia (Guo et al., 2016) and *Candidatus* Jettenia (Hu et al., 2012) were reported as dominant too. What appears consistent is that anammox granules usually consist of one or two dominant anammox bacteria – for example, Gonzalez-Gil et al. (2015) found *Ca.* Brocadiacea made up 99% of the total anammox community in their samples. In 2006, the first anammox genome – of *Ca.* Kuenenia – was released, revealing the metabolic pathway underpinning the anammox process. Initially, nitrite is reduced to nitric oxide (NO) by cd_1 nitrite reductase (NirS). Further condensation of ammonium and NO by hydrazine synthase (HZS) produces the highly toxic intermediate, hydrazine. The oxidation of hydrazine by a hydroxylamine oxioreducatase (HAO)-like hydrazine dehydrogenase (HDH) produces the final end-product, N_2 (Strous et al., 2006). The entire catabolic process takes place inside an intracytoplasmic compartment, the anammoxosome (Jetten et al., 2009). It is speculated that this architectural feature evolved in anammox bacteria to protect the rest of the cellular organelles from hydrazine toxicity (van Niftrik et al., 2004).

While the process is autotrophic, some anammox bacteria are speculated to carry out heterotrophic, or potentially mixotrophic, ammonium oxidation, simultaneously removing carbon and nitrogen (Shu et al., 2015). What is universally recognised is that anammox granules support multiple reactions resulting in the proliferation of a complex, multi-trophic, cooperative (and sometimes competitive) microbial community aiding in nitrogen cycling. Anammox bacteria can co-occur with NOB and AOB, which can also be involved in anammox-based nitrogen removal. For example, in aerobic (outermost) regions of anammox granules AOB, such as *Nitrosomonas*, provide nitrite for NOB which compete with anammox bacteria for substrate (Guo et al., 2016; Speth et al., 2016). The granule community commonly includes members of the Proteobacteria, Chlorobi, Bacteroidetes, and Chloroflexi phyla (Chu et al., 2015; Guo et al., 2016; Lawson et al., 2017; Shu et al., 2015; Speth et al., 2016). Multiple studies have even detected

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methanogens – speculated to be using residual hydrogen gas to produce methane – as well as methanotrophs – capable of methanotrophic denitrification (Gonzalez-Gil et al., 2015; Hu et al., 2012). Whilst the microbiome of anammox granules supports multiple cooperative reactions, Gonzalez-Gil et al. (2015) found that they contained significantly fewer (about 350) operational taxonomic units than usually reported for anaerobic granules (888-1130). Reduced community richness may result in (i) functional specialisation of the anammox microbiome, (ii) selection by the environment, or operating process, for competitive microorganisms, or (iii) utilisation by anammox communities of a narrower substrate range than a typical anaerobic granule.

7. Outlook: granules as tools beyond waste conversion technologies

Granular biofilms have completely revolutionised sustainable wastewater treatment. The extraordinary discovery of the anaerobic granule in the 1970s launched an entire field of research into granular-based biotechnologies resulting in many other specialised types of granules. Notably, anammox and aerobic granule-based technologies have been successfully demonstrated at laboratory-, pilot-, and full-scale. Recently, however, and with the help of step-change advances in microbial ecology, including metagenomics, metatranscriptomics, metaproteomics and metabolomics, the granule is emerging as a perfect, high-throughput playground to test fundamental ecological concepts. Using holistic approaches, microbial ecologists piece together the complex interactions between key microoganisms and the mechanisms involved in community assembly, stress response, and the operation of biofilm life-cycles. A deeper understanding of those processes applies not only to granular biofilms, but to biofilm structures and microbial communities widespread elsewhere in the built environment and in Nature.

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