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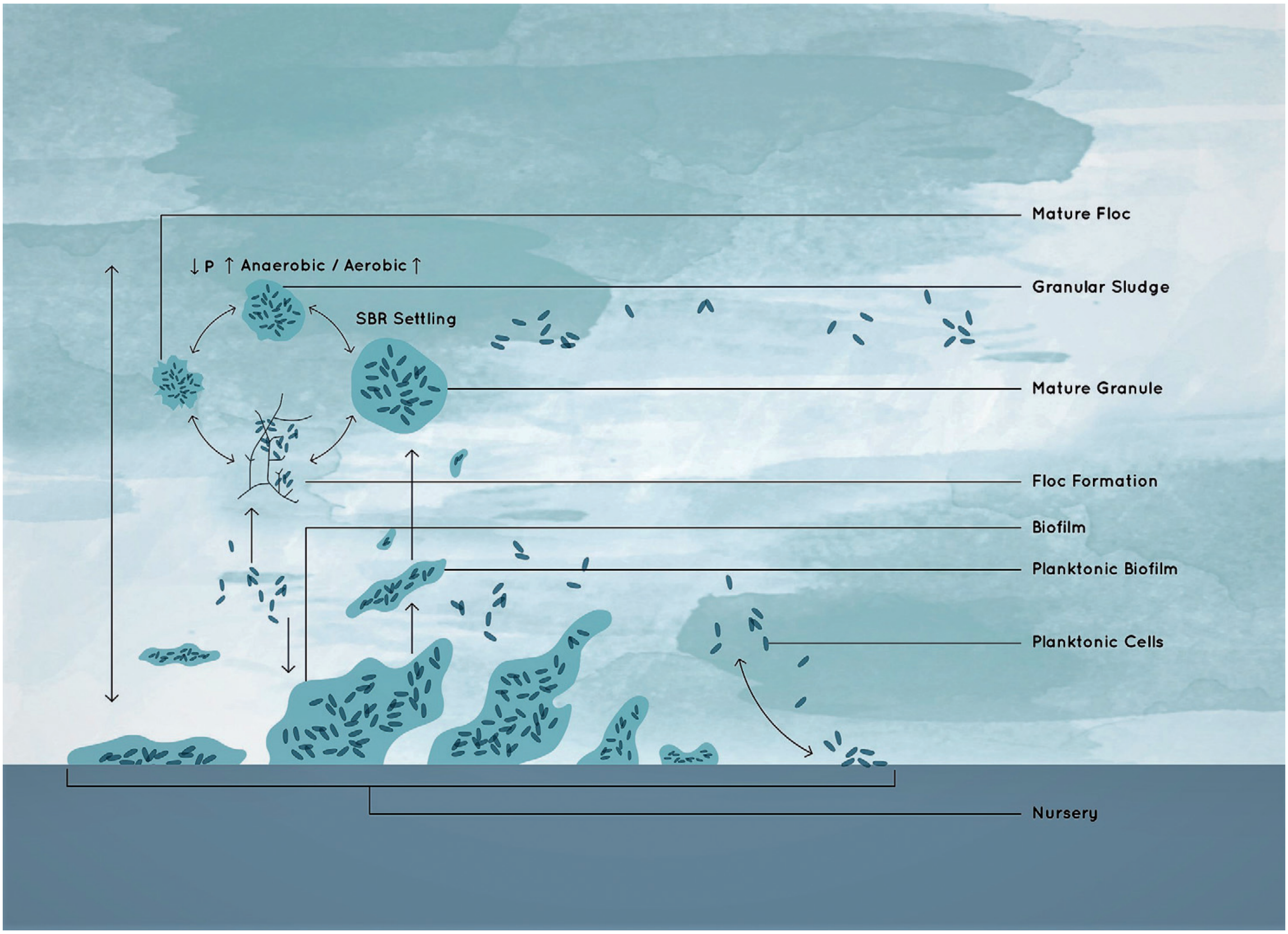

Featuring work from Prof. Steven N Liss, Chemistry and Biology, Faculty of Science, and Vice-President Research and Innovation, Ryerson University, Toronto, Canada.

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Microbial aggregation reflect dynamic processes; and, structures that form are not necessarily discrete, but fall along a continuum. This review focuses on the drivers affecting the formation and stability of flocs, granules, and biofilms, and conditions that support hybrid and integrated systems for biological wastewater treatment.

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Drivers of bioaggregation from flocs to biofilms and granular sludge

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Microorganisms in natural and engineered environments interact with surfaces and form aggregates consisting of cells and an extracellular matrix. The design of the process and appropriate operational conditions drive the formation of these biofilms, flocs, and granular structures. The application of granular sludge technologies for nutrient removal is relatively new. Although research and practice benefit from several decades of investigation of biofilm and anaerobic granular sludge systems, a thorough understanding of factors affecting granulation is only beginning to emerge from bench, pilot, and full-scale investigations. Challenges intrinsic to maintaining granular and biofilm structures include management of resistance to substrate transport, establishment of targeted microbial niches, role of extracellular polymeric substances, and impacts of toxic compounds, among others. There is increasing recognition of the potential value of hybrid process configurations that optimize interactions between flocs, granules, and/or biofilm features for process enhancement and robustness. While these structures appear distinct, it is not uncommon to find a mixture of these structures present in a single system and dynamics leading to a transition from one structure to another. The transitions are dependent on changes in the microbial community and properties of the extracellular matrix. This review focuses on the drivers affecting formation and stability of flocs, biofilms, and granules and conditions that support integrated technologies for biological wastewater treatment.

Water impact

The future of water/wastewater infrastructure must innovatively evolve in response to pressures (*e.g.* climate change, water quality deterioration, energy/resource costs, and urbanization) on legacy systems, and inadequate or ageing infrastructure, as well as opportunities created by the circular economy. Fixed-film and granule-based processes are increasingly important in wastewater treatment; therefore conditions that drive microbial communities that form these structures and contribute to their stability are key to successful implementation and operations.

1. Introduction

Environmental biotechnology processes such as those designed for biological nutrient removal (BNR) from wastewater rely on the proliferation and metabolic activity of specialist microorganisms present in the seeding incoming wastewater to form a microbial community engineered under deterministic and stochastic conditions. In such processes, microorganisms seldom function as single cells dispersed in suspension (such as commonly conceptualized in industrial biotechnology) but form aggregates of various architectures from suspended activated sludge flocs to biofilms attached on fixed or mobile carrier materials to advanced forms of mobile aggregates of biofilms, called "granules". Bioaggregation is the predominant mode of growth in nature as it protects the microorganisms from predation, environmental stresses, enhances access to nutrients and facilitates microbial syntrophy.[1] The key to mixed-culture processes is to harness this natural propensity for process design, stability, and resiliency. The microbial composition and activity play an important role in the removal of organic matter and nutrients from the wastewater, with bioaggregation facilitating overall process efficiency through improved separation of treated water and biomass retention.[2] Therefore, the aim is to create conditions that favor the development of compact and stable bioaggregates that readily separate from the bulk aqueous phase without leaving excessive concentrations of suspended solids in the effluent. Bioaggregation is a complex process that is influenced by

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several factors. The mechanisms involved in the formation of suspended bioaggregates into flocs, biofilms and granules are essentially the same, where environmental factors such as feeding regime and hydrodynamic conditions are decisive for the structure to form.[3] Biological aggregation phenomena should, therefore, be considered in such systems as a continuum from flocs to biofilms and granules (Fig. 1). A thorough understanding of biological, chemical, and physical mechanisms that govern microbial immobilization and structural dynamics is necessary to manage bioaggregation.[2]

The activated sludge process for treatment of wastewater relies on the ability of bacteria to form flocs which can be separated from the treated water. Activated sludge flocs are suspended aggregates of bacterial cells assembled in a matrix of extracellular polymeric substances (EPS).[1] Secretion of EPS promotes bacterial adhesion and embed the different floc components. Bacteria which lack the ability to aggregate are washed out from the secondary settlers (clarifiers) which causes poor effluent quality and inefficient recycling of return sludge to the activated sludge process. The settling and stability properties depend on the floc

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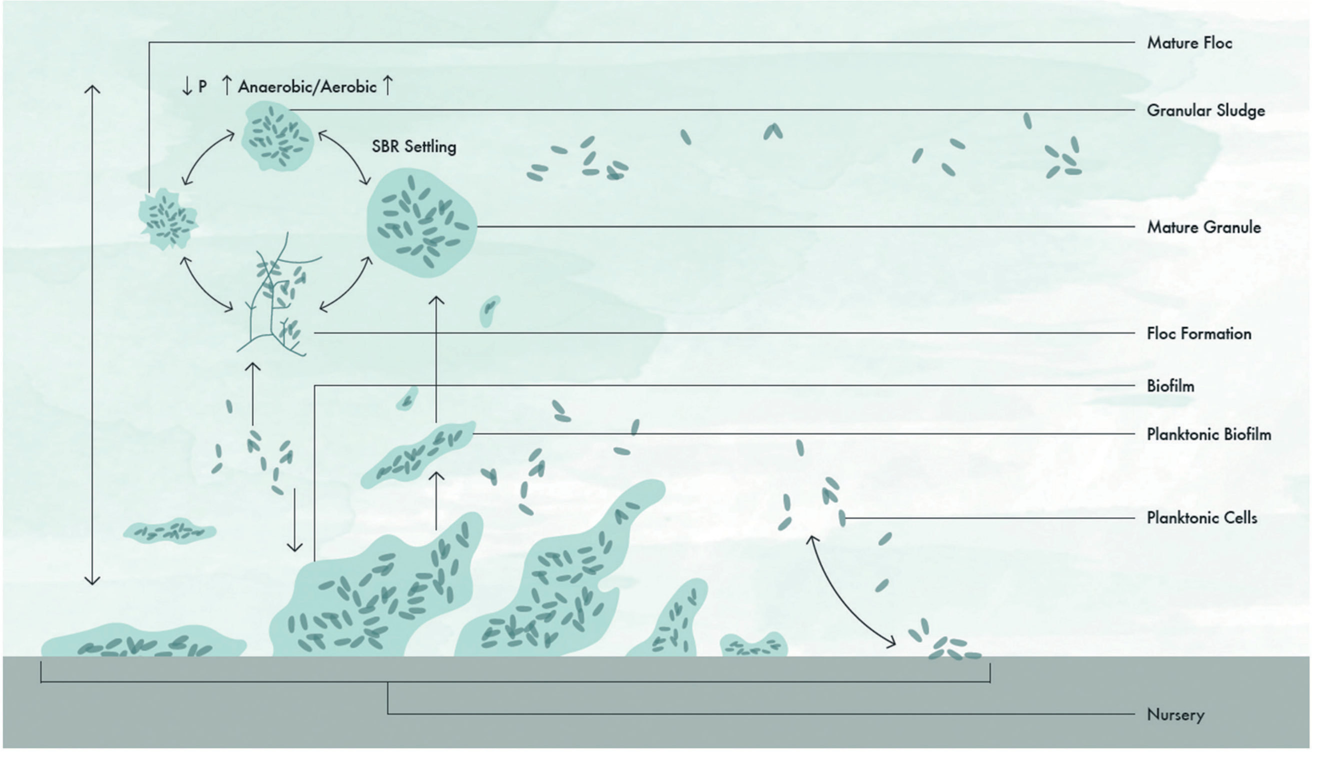

Fig. 1 Schematic representation of the dynamics in biofilm, flocs and granular structures. In a hybrid system, biofilms serve as a nursery where dispersed planktonic cells can attach to the substratum to initiate biofilm floc or to each other to initiate floc formation; and, dispersed planktonic biofilms can associate with flocs and develop into granular sludge or granules. Similarly, flocs and granules can disintegrate or change structure (*e.g.* filamentous outgrowth) or transition to intermediary forms (*e.g.* granular sludge).

structure, size, and intra-particle forces holding the floc components together. Most bacteria grow in colonies which contribute to the formation of compact and strong aggregates.[4] The filamentous bacteria often grow in flocs for which they may contribute to floc strength by providing backbones, but are definitely linked to poorer settling properties.[5] Enhancement of aggregation and controlling settling properties of biomass is less of a consideration for biomass retention in some BNR systems

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and lagoons with extended aeration and/or long hydraulic retention times. These systems can include quiescent zones that allow for settling that occurs naturally over longer periods; whereas, structural properties and settling behavior are important in other systems including sequencing batch reactors (SBR), to form BNR granules.

Flocs and granules can be viewed as suspended biofilms, without carrier material.[6,7] The landscape of the microbial population within a community of an attached biofilm, or suspended biofilm (floc or granule) depends on different internal gradients of nutritional and environmental condition. The size of a biofilm, floc and granule influence the diffusion of dissolved oxygen and nutrients and complexity of microbial community.[8,9] The flocs and biofilms are integrated structures. However, the physical, chemical and microbial structures of biofilms and flocs may vary and complement each other for BNR in a hybrid system with biofilms and flocs growing in the same bioreactor.[10]

The granular sludge process has premium BNR capacity in a more compact system compared to conventional activated sludge processes.[11] Nevertheless, the demonstrated instability of granular sludge has initially limited its widespread large-scale application.[12,13] Despite the discovery of so-called "aerobic" granules nearly two-decades ago for the aerobic removal of COD[14] there is little information on microbial community interactions and dynamics in relation to the operating conditions of the treatment process.[15] The application of anaerobic selectors do make the difference in stabilizing the granular biofilm architectures by selecting for slow-growing organisms.[15–17] The advent of advanced molecular and microscopy techniques allow for in-depth elucidation of microbial community composition and architectures of granules[18,19] and could, therefore, pave the way for new strategies to improve water treatment. The use of multivariate analyses in numerical ecology enables to screen for multi-scale correlations,[18,20,21] while in-depth examination of parameter effects[16] is needed toward process design, understanding, and control. Furthermore, there are inconsistent and conflicting reports on factors affecting granulation including dissolved oxygen and shear rate.[22–25] Unlike anaerobic granules, a wider diversity of aerobic (O_2 as terminal electron acceptor), anoxic (nitrite or nitrate as e-acceptor) and anaerobic (no terminal e-acceptor)† microorganisms can contribute to granulation in BNR systems, each of which may have different ecological requirements to establish across redox and substrate gradients within these aggregates (Table 1).

Recent analytical developments associated with the use of molecular methods and advanced microscopy have further increased our understanding of microbial interactions and structural features of the EPS found in granular biofilm structures.[15,26] In this review, we examine the drivers of the formation and stability of the microbial structures that are functional components of BNR in wastewater treatment. Flocs, granules and biofilms, distinct entities, in one respect, are relatively pleomorphic in another and these structures can be viewed as dynamic shifting from one structure to another (Fig. 1). It is not uncommon to find that systems designed and operated for one type is in fact a hybrid system. The structural characteristics of these bioaggregates are dependent on microbial community dynamics, activity, and the extracellular matrix. The tendency of mature bioaggregates to disperse demand implementation of strategies (such as hybrid systems) to enhance performance and increased resiliency and stability.

2. Formation of flocs, biofilms and granules in BNR systems

Granulation typically arises from microbial flocs with the same initial aggregation of cells involving a number of mechanisms supporting bioflocculation. For example, initial cell–cell contact (attractive forces between cells causing them to aggregate), formation of microbial aggregates embedded in EPS onto which cells can attach and multiply,[27–29] followed by the hydrodynamic shaping of the aggregate into spherically shaped aggregates.[23] The management of the microbial resource *via* engineering of operational conditions can therefore drive granule formation and stability.[15] Granules have stratified structures like mature biofilms, therefore granules are also called suspended biofilms.[15] The attached biofilm formation can be broadly described as a stepwise process, which includes three phases: (a) an initial reversible phase (biofilm initiation), (b) an irreversible attachment phase (biofilm growth and maturation), and (c) a dispersion phase to start a new biofilm.[9] The dispersed cells reattach and regrow to form new biofilms that result in overall stable biofilm systems.

Three basic strategies involving a feast/famine regime, shear force, and short settling time have been proposed to stimulate BNR granule formation (Fig. 2).

Nonetheless, the essence of granulation originates at the microbial level. The invention of granular sludge development was based on the observation that slow-growing microorganisms are capable to convert easily degradable organic substrates under feast conditions to form complex storage polymers such as polyhydroxyalkanoates (PHA), which they can subsequently utilize when easily degradable organic substrates are less available during famine conditions.[30] The continuous research and gradual optimization of operational

† In the wastewater engineer's lexicon, redox conditions are considered as "aerobic" in the presence of dissolved oxygen as terminal electron acceptor *via* aeration, as "anoxic" in the presence of nitrite and nitrate as e-acceptors, and "anaerobic" in the absence of terminal e-acceptors in the bulk. This is in relative mix up with microbiology terms of "aerobic" respiration (O_2 as terminal e-acceptor), "anaerobic" respiration (inorganics like nitrite and nitrate as terminal e-acceptors), and fermentative conditions. One typical illustration of this apparent wording opposition resides in the anaerobic ammonium oxidation (anammox) process that has been characterized by microbiologists (therefore "anaerobic") whilst engineers should call it "anoxic" ammonium oxidation according to their systematics (presence of nitrite as terminal e-acceptor). The engineer's lexicon that is applied in the wastewater sector is primarily used in this article.

Table 1 Predominant bacterial genera found in BNR granules

Genus	Bacterial class	Metabolic characteristic	Type of wastewater	Ref.
Rhodanobacter	*γ-Proteobacteria*	Complete DEN	Synthetic	Aqeel *et al.*[26]
Klebsiella	*γ-Proteobacteria*	Aerobic DEN	Synthetic	Taheri *et al.*[141]
Thauera	*β-Proteobacteria*	Aerobic DEN	Piggery wastewater	Zhao *et al.*[142]
Zooglea	*β-Proteobacteria*	DEN	Synthetic	Zhao *et al.*[143]
Competibacter	*γ-Proteobacteria*	GAO	Synthetic	Weissbrodt *et al.*[15]
Accumulibacter	*β-Proteobacteria*	PAO	Synthetic	Weissbrodt *et al.*[15]
Candidatus Jettenia	*Planctomycetes*	Anammox	Synthetic	Quan *et al.*[144]
Brocadiacea	*Planctomycetes*	Anammox	Full-scale wastewater	Gonzalez-Gil *et al.*[100]

DEN = denitrifying, PAO phosphorous accumulating bacteria, GAO glycogen accumulating bacteria.

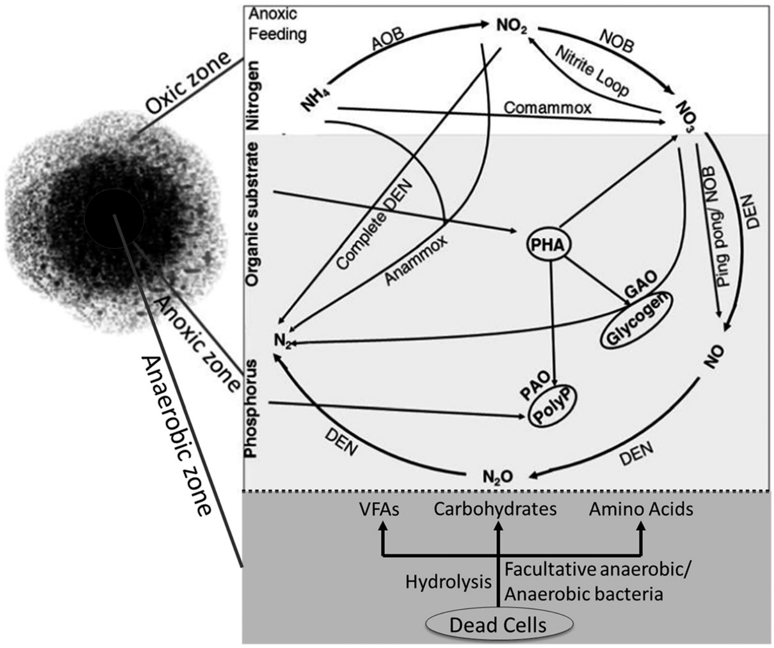

Fig. 2 Microenvironmental conditions in BNR granules. The microenvironment in oxic and anoxic zones of BNR granules helps the growth of specific bacteria (*e.g.* autotrophs and heterotrophs) to support simultaneous nutrient removal processes. There are several pathways for biological nutrient removal where bacteria compete for nutrients.

conditions such as anaerobic feeding (feast) and oxic/anoxic react time during famine conditions facilitate slow-growing bacteria and discourage fast-growing bacteria.[24] This can notably be achieved by designing efficient anaerobic selectors that select for preferential storage of organics by slow-growing bacteria such as polyphosphate-accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) prior to switching on aeration.

The use of shear force for BNR granules was adapted based on observations of anaerobic granulation in up-flow anaerobic sludge bioreactors (UASB), where the up-flow velocity of wastewater and gases (produced during fermentation) and the downward settling of the biomass supported anaerobic granule formation.[31] The high shear force results in erosion of surface particles, physically break filamentous outgrowth and facilitates mass transfer to the deep layers of the granules and biofilms. The erosion of surface particles results in short mean cell residence times of fast-growing heterotrophic bacteria located in the outer layers.[32–35] Slow-growing organisms such as autotrophic nitrifying and anammox bacteria, PAOs, and GAOs form strong microcolonies that remain intact at a high shear rate.[15,36]

The nitrifying bacteria form dense microcolonies that are selected when a short settling time for granulation is applied. The growth of these dense microcolonies results in granulation that in turn can support the growth of denitrifying bacteria in the core of the granules. Additionally, a model that proposes the clumping of different microcolonies with a filamentous backbone or adhesive bacteria to form large aggregate and support growth of denitrifying bacteria facilitates granulation.[37,38] Weissbrodt *et al.*[38] reported on the role of heterotrophic *Zoogloea* in the formation of granules that has adhesive properties to support granule formation. It was observed that after initial granulation, which was dependent on fast-growing bacteria, the slow-growing PAOs and GAOs out-compete the fast-growing heterotrophic bacteria. Therefore, a gradual shortening of the settling time (15 to 3 minutes) was adopted for granule maturation. Nevertheless, the presence of filamentous bacteria and of *Zoogloea*-like organisms are mainly a sign of a deficient anaerobic selector. The design of an efficient anaerobic selector is the key for a stable process.[39,40]

3. Extracellular matrix maintaining the cohesion of bioaggregates

The complex arrangement of EPS creates a distinct environment supporting both the diffusion of nutrients and retention of metabolites and a highly selective environment for the microbial community.[41] EPS are important constituents of flocs, granules, and biofilms contributing to both structural and functional features of as well protecting cells from harsh environmental conditions.[6,22] The proteins and polysaccharides (major components of EPS) form attractive and repulsive interactions within EPS supporting aggregation of cells and the formation of a compact structure.[42,43] In spite of decades of research, the knowledge about its production, composition, and function is limited. Hence, contradictory results can be found in the literature regarding the importance of EPS in granule formation, partly due to differences in extraction and analytical methods.[44] The protein to polysaccharide ratio is higher in granules as compared to flocs;[26,45] whereas the protein to polysaccharide ratio is higher in flocs as compared to biofilms, cultivated in an integrated fixed-film activated sludge system.[10] The protein content of the EPS contributes to a more hydrophobic surface and a more compact and tightly packed granular structure, compared to flocs.[46] In contrast, the polysaccharide content supports hydration and facilitates retention of nutrients and metabolites through interactions of the charged surface and sorption in granules.[22,47] Exopolysaccharides or glycosides have been found to be an important gelling agent in BNR granules, more adhesive than EPS extracted from activated sludge.[48,49]

Transmission electron microscopy observations have revealed cell to cell interaction with the help of thin fibers in the environmental biofilms and flocs.[27] Later, immunohistochemistry studies have shown that these fibers were the amyloid adhesins that are abundant in the environmental biofilms and flocs.[36,50] Bioinformatics analyses indicate that amyloid adhesins are widespread in bacteria related to phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Thermodesulfobacteria*.[51] Whole genome microarray analyses of flocs have shown that the expression of pili and lectins is upregulated under phosphorus limiting conditions that can also affect microbial cohesion.[52] Venter *et al.*[53] applied "whole-genome shotgun sequencing" to microbial population collected from the Sargasso Sea that revealed about 300 flagellar genes.

The extracellular adhesins (*e.g.*, flagella, pili, lectins and amyloid adhesins) are abundant in environmental, multi-species microbial communities of biofilms and flocs. However, the specific role of the extracellular adhesins in the environmental biofilms, flocs and granules is not well understood. Most of the information about the potential significance of extracellular adhesins is inferred from the pure culture and co-culture biofilm studies. The extracellular protein forms three types of specific interactions in the extracellular matrix: (a) protein to protein interaction (amyloid adhesins), (b) protein to polysaccharide interaction (lectins), and relatively less understood (c) protein to extracellular DNA interaction (type IV pili binds specifically to the extracellular DNA, where DNase treatment can inhibit the development of *Pseudomonas*[54] and *Acidovorax* biofilms[55]).

Amyloid adhesins are beta-sheet rich, fibrous proteins, which are insoluble and resistant to the proteolytic enzyme activity of proteases.[56] Amyloid fibers of neighboring bacteria cluster together and twist to form rope-like structures. The clustering and rope formation of amyloid adhesins at inter-bacterial surfaces pulls the neighboring bacteria close to each other. The amyloid-adhesin-expressing bacteria are tightly packed, 20-fold stiffer and relatively more hydrophobic compared to non-amyloid adhesin producing bacteria.[57,58] Amyloid adhesins mediate cell–cell interactions for microbial aggregation in flocculation and biofilm formation.[59] The inhibition of amyloid adhesin synthesis results in a thin biofilm, due to the loss of cell–cell interaction.[57]

Lectins, sugar-binding proteins (that are highly specific for their target polysaccharide) are produced by many microorganisms that facilitate cell–cell and cell-surface adhesions. Usually, these lectins are small binding sites present on the tip of the cellular appendages, which make these appendages adhesive for biofilm formation and development.[60] For example, the type 1 fimbrin D-mannose specific adhesin (FimH) present on the tip of type I pili binds specifically to mannose sugars.[61] FimH is very adaptive to the environmental stress and there are multiple wild type FimH mutants.[62] The binding force of the FimH to the target polysaccharide is positively correlated with shear stress. The FimH has a polysaccharide-recognizing domain, which can bind to mono-mannose. The FimH possess a second polysaccharide-binding domain, which cannot bind to the second polysaccharide in hydrostatic conditions unless there is a shear force. The shear stress in hydrodynamic conditions stretches the inter-domain space of FimH to bind the tri-mannose.[61,63]

Cellular appendages (flagella and type IV pili) help motile bacteria to move towards the favorable conditions (a) in an aquatic system for biofilm initiation, (b) within biofilm or (c) detach from the biofilm. In the (a) case, flagella (chemotactic swimming) and type IV pili (twitching or gliding) mediate initial bacterial attachment and spread of biofilm on a substrate. Flagellated bacteria can resist the hydrodynamic conditions (where non-flagellated bacteria are washed away) to anchor on a substrate for biofilm initiation.[64] In the (b) case, type IV pili enable bacteria to move vertically within biofilms for favorable conditions. Motile bacteria (with flagella and type IV pili) are present in the outer layer of biofilm, which is relatively rich in the nutrient source; whereas nonmotile bacteria are predominant in the core of the biofilms.[65,66] In the (c) case, bacterial attachment downregulates the flagellar synthesis and induces synthesis of exopolysaccharides and amyloid adhesins.[67] In contrast, flagellar synthesis is induced prior to biofilm sloughing. The motile bacteria (with flagella and type IV pili) mediate sloughing when conditions are not favorable in a biofilm. Additionally, it has been observed in *Bacillus subtilis* biofilms that D-amino acids are secreted during the late stage of biofilm development, before biofilm sloughing. D-Amino acids trigger the disassembly of amyloid fibers, which result in the disintegration of the biofilm.[68]

A two-layer model with respect to the arrangement of EPS as a tightly bound layer at the core and a loosely bound layer at the outer region of bioaggregates appears to be an important feature of formation and stability of granules and biofilms and their structure that requires greater attention.[42,69,70] Basuvaraj *et al.*[69] described the formation of a granular sludge composed of a predominantly tightly bound protein-rich EPS and a smaller proportion of loosely bound EPS. The loosely bound layer was more pronounced in the seeding activated sludge floc structure and was richer in polysaccharides. The autotrophic nitrifying biofilms and flocs show that these are predominantly composed of tightly bound EPS.[71]

EPS in flocs and granules may be hydrolyzed to form soluble microbial products (SMP).[72,73] The EPS hydrolysis for SMP production has been largely attributed to heterotrophs; whereas, autotrophs have been reported to account for <10% of the SMP, with 5% associated with ammonia oxidizing bacteria and 3% related to nitrite oxidizing bacteria.[73] Ni *et al.*[74] have reported for granular sludge that soluble microbial products generated can be used as an organic substrate by denitrifying bacteria. Therefore, the growth of autotrophic nitrifying bacteria in the outer layer helps to conserve the nutrient resources, by minimizing the loss of SMP to the aquatic column. Whereas, the SMP secreted by the heterotrophic bacteria (in the inner layer) can be utilized by denitrifying bacteria to budget the nutrient resources.

4. Microbial community architectures and selection phenomena in bioaggregates

Granules and biofilms are organized multicellular structures composed of mixed microbial populations, which cooperate for the optimum harvesting of resources from the surrounding environment and competitive advantage for the capture of limited resources (Fig. 3).[24,75,76] The cooperation in microbial community include: (a) production of EPS that can be hydrolyzed by other bacteria and synthesis of complex storage polymers that serve as nutrient source for some bacteria; (b) production of metabolic byproducts that can be used by other bacteria, *e.g.*, nitrite and nitrate resulting from the metabolism of nitrifying bacteria are used as electron acceptors by denitrifying bacteria. The effective cooperation between bacteria present in the outer oxic zone and deep anaerobic layer of the granules results in structural and functional stability of the granular sludge.

The spherical architecture of BNR granules with outer aerobic layer and core with anoxic/anaerobic is a widely accepted conceptual model of the granular structure, typically implemented in mathematical models. The stratified structure and the ability to support simultaneous nitrification and denitrification in granules are owing to the thickness of large size of granules (diameters > 200 μm) where the penetration of dissolved oxygen is restricted to the outer 50–100 μm.[77,78] However, there are few contradicting observations, where the distinction between communities across the depth is absent.[79] Barr *et al.*[79] hypothesized that granules with stratified structure form by the growth of single microcolony; whereas, granules with homogeneous microbial community form by aggregation of microcolonies. Weissbrodt *et al.*[15] highlighted with CLSM different types of internal architectures of granules (i) with smooth continuous biofilm matrices when dominated by fast-growing heterotrophs like *Zoogloea* spp., (ii) with conglomerates of microcolonies when slow-growing organisms like PAOs, GAOs, and nitrifiers populate across the granule cross-section, and (iii) unfavorably overgrown by filamentous bacteria when the organic matter was not adequately removed in an (anaerobic) selector. In contrast to Barr *et al.*,[79] Weissbrodt *et al.*[15] stated that the growth physiology of the predominant microorganisms do impact on the resulting internal structure of granules. This statement is valid across flocs, granules, and biofilms, and meets with previous modelling reports of Alpkvist *et al.*[80] Gonzalez-Gil and Holliger[78] recreated the three-dimensional image of the granules that revealed a "cavernlike" granular structure. The "cavernlike" channels in the granules improve the diffusion of nutrients and deviate the microbial growth, compared to the widely accepted model of a spherical granular structure. Additionally, the denitrifying bacteria could be observed in the outer layer because some bacteria are capable of aerobic denitrification.[81]

4.1. Microbial niches of the oxic zone of bioaggregates

The operational conditions for wastewater treatment are engineered to drive the selection of cooperative microbial communities that maintain granular sludge with optimum size and activity. For example, in the oxic zones of these granules, the metabolism and biomass assimilation of the nitrite-oxidizing bacteria (NOB) that convert nitrite to nitrate are dependent on ammonia oxidizing bacteria (AOB) that convert ammonium to nitrite. Furthermore, since NOB such

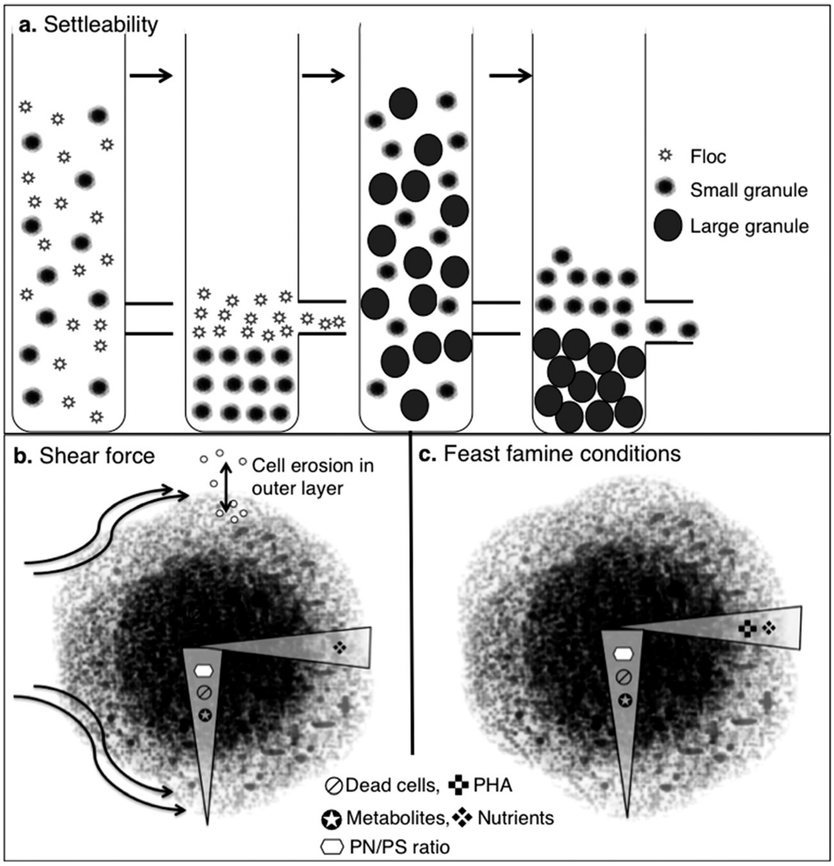

Fig. 3 Conditions selecting for BNR granule formation and retention (a) within sequencing batch reactors the settling phase facilitates selection of the larger, compact and fast settling granules and eradicate the relatively slow settling microbial aggregates. (b) Shear forces erode the fast-growing bacteria from the outer layer resulting in its low cell residence time, whereas within the core slow-growing cells are retained (until cell lysis due to aging) and out-compete fast-growing bacteria. Shear forces induce PS secretion resulting in a PS rich outer layer, which facilitates granule formation (c) "Feast famine" conditions facilitate the development of slow-growing bacteria and conversion of readily degrading organic matter to storage polymers including PHA. Gradients and stratification arise with respect to the diffusion of external nutrients and secretion of metabolic byproducts.

as *Nitrobacter* and *Nitrospira* compete for available nitrite resources in wastewater, the operational and nutritional conditions can favor the growth of one bacterial group over another. *Nitrospira* outcompetes *Nitrobacter* at microaerophilic and low nitrite concentrations.[82] Conversely, *Nitrobacter* outcompetes *Nitrospira* at higher nitrite and oxygen concentration.

The dependence of NOB on AOB is reflected by a low NOB/AOB ratio (0.5) in conventional activated sludge flocs. However, in granules (where *Nitrobacter* predominates) a higher NOB/AOB ratio (3) has been reported. The ping-pong theory and nitrite loop theory have been used to explain the abundance of NOB in granules.[79,83] According to the ping-pong theory: in the oxic region, NOB convert nitrite to nitrate autotrophically and then metabolize nitrates heterotrophically in the anoxic region to produce nitric oxide. The NOB (which typically grow autotrophically are also observed in deeper anoxic region) grow mixotrophically to outnumber the AOB. NOB are capable to use organic substrate present in the feed during the feast phase and convert these to storage polymers such as PHA.[79] During famine phase, NOB uses organic matter either stored as PHA or released by cell lysis. The heterotrophic growth of NOB (which is independent from the availability of nitrite) results in an increase in the NOB/AOB ratio in granular biomass.[79] Alternatively, a nitrite loop may develop whereby the nitrite is oxidized by NOB to nitrate, followed by reduction back to nitrite by denitrifying bacteria. The accumulated nitrite is now available again to be used by NOB.[79,83] The recent insights in *Nitrospira*-like organisms able a complete ammonium oxidation (comammox) into nitrate[84–86] can further explain the predominance of NOB at the expense of AOB in granules.

4.2. Microbial niches of the anoxic zone of bioaggregates

Heterotrophic bacteria typically prefer dissolved oxygen as an electron acceptor; however, denitrifying heterotrophs can use nitrate and nitrite as an electron acceptor under anoxic conditions. In conventional wastewater treatment (floccular sludge and biofilm-based systems) denitrification is usually performed in a separate tank under anoxic conditions. In the granular sludge that removes nutrients simultaneously, denitrification occurs in the anoxic region where denitrifying bacteria reduce nitrate and nitrite to nitrogen gas.

In BNR granules, non-denitrifying heterotrophs can outcompete denitrifying bacteria in the presence of limited organic substrate and extensive aeration.[87] Therefore, strategies to ensure optimum granulation involve conditions (*e.g.*, feast-famine) to discourage the growth of heterotrophs in the oxic zone. When this strategy does not efficiently inhibit the heterotrophic growth, usually higher organic substrate in the wastewater combined with improved biomass transport with shear force is required to support denitrifying heterotrophic growth in the anoxic zone. Denitrifying bacteria, which are able to utilize storage polymers (*e.g.*, PHA) as an organic substrate can outcompete fast growing heterotrophs and facilitate granule formation.[17,30] Furthermore, in the anoxic zone, PAOs and GAOs are also capable to use storage polymers (PHA) and thus compete with denitrifying microorganisms for the limited organic substrate.[88,89] However, PAOs (which are desired for enhanced biological phosphorous removal – EBPR) and GAOs (which are typically considered undesired in EBPR systems) are both capable of denitrification.[15,90–92] The competition between the microbial community for limited PHA can be manipulated by optimizing the operational conditions according to the requirement of the system.[93]

Conditions where substrates (external or stored) are limited, denitrifying bacteria prefer nitrate as an electron acceptor. Denitrifying bacteria reduce nitrite and nitrate sequentially, to nitric oxide, nitrous oxide, and dinitrogen gas, with the help of reductase enzymes. Reduction of nitrate is preferred over nitrite reduction (by nitrate reductase and nitrite reductase, respectively) during denitrification because nitrate can accept relatively more electrons than nitrite, which results in accumulation of nitrite at low organic loading, in anammox process granules. The accumulated nitrite thus favors the coexistence of anammox and denitrifying bacteria in granules.[74] Conversely, nitrous oxide reduction to nitrogen gas is halted at low organic loading to nitrogen loading ratios, to budget the organic loading. Consequently, nitrous oxide (N_2O, a greenhouse gas) emission is undesired in simultaneous nitrification and denitrification in granules.[94] Additionally, the activity of nitrous oxide reductase is inhibited at low dissolved oxygen concentrations, resulting in the unfavorable emission of N_2O.[95]

The microbial ecology of denitrifying bacteria varies with operational and nutritional conditions such as solid retention time, temperature, pH, carbon source, chemical oxygen demand and nitrogen ratios.[96] For example, most denitrifying bacteria require a pH of 6.5–7.5, for nitrogen removal. However, *Rhodanobacter* related to the class *Gammaproteobacteria* (complete denitrifying bacteria, which can reduce nitrate and nitrite to nitrogen gas) are capable of denitrification at a broader range of pH 4–8.[97,98] *Rhodanobacter* were predominant in granules where pH was not maintained during the react cycle (pH ranged 7–4) in sequencing batch reactor (SBR).[26]

The microbial community composition in the anoxic zone depends upon the concentration and composition of nutrients in the influent, activity of bacteria in the outer layer (that depends on operational conditions including temperature, pH, dissolved oxygen concentration) and architecture of nutrient transport channels in the bioaggregates. The anammox and denitrifying bacteria can coexist in the anoxic zone of the granules.[99,100] The growth rate of anammox bacteria is lower compared to their competitors: AOB and NOB in the outer layer and denitrifying bacteria in the inner layer. To support the growth of anammox bacteria: (1) the growth of AOB must be limited and NOB inhibited, for surplus ammonia and nitrite for anammox activity. The activity of AOB and NOB populations can be suppressed by several mechanisms, in bioreactors with simultaneous nitrification and denitrification activity. For example, by decreasing the temperature from 29 °C to 15 °C (ref. 101) and intermittent and low aeration rate.[102] Ma *et al.*[102] showed that the dissolved oxygen concentration during aeration ranged between 0.08–0.25 mg L^{-1}, which was enough to support ammonium oxidation. Aeration was interrupted when nitrite start to accumulate, to discourage nitrite oxidation and thereby facilitate anammox bacteria;[102] (2) the growth of denitrifying bacteria is discouraged by feeding high strength nitrogenous wastewater to the system, with relatively low organic substrate (300 mg L^{-1})[74] and a low carbon to nitrogen ration of two.

4.3. Microbial niches of the anaerobic zone of bioaggregates

Most of the environmental bacteria are opportunist that grow when conditions are favorable. With increase in the size of the granule or biofilm, anaerobic bacteria proliferate in the core of these bioaggregates.[103] In the anaerobic zone a number of bacteria related to different functional activities have been observed, including bacterial groups with anammox, denitrifying, hydrolyzing, and fermentative activities.[18,19,99] Hydrolyzing bacteria degrade the complex storage polymers, EPS, and dead cells to produce organic substrate to support the growth of nutrient-removing bacteria (denitrifiers, PAO and GAO).[18] As size of the granules further increases, mass transport of nutrients in and waste-products out of the core is reduced that suppress the growth of bacterial subpopulations in the inner parts of the bioaggregates. A core of dead cells has been observed at a depth of 900 μm.[31] However, the threshold size for mass transport may vary depending upon local operation condition and/or due to clogging of the mass transport channels by excessive EPS.[104] The clogging of mass transport channels reduces the metabolic activity of nutrient removing bacteria. The growth of fermentative bacteria in the core is favored when organic substrate starts to accumulate with hydrolytic activity.[105] Fermentative bacteria create acidic conditions that further inhibit the growth of competitive bacteria and promote cell lysis that results in granule disintegration. In large size granules with clogged channels,[104] hydrolysis of dead cells and EPS complemented with fermentation (that produce fermentative gases) creates a hollow core.[106] The granules with hollow core loose density, float on the surface and are washed out of the system.[107] Therefore, maintaining an optimal granule size is imperative for the stability of the system.

4.4. Microbial community dynamics and granule instability

The dominant populations in BNR granular sludge as revealed by various studies are summarized in Table 1. It has been suggested that operational or nutritional conditions that do not favor the growth of these dominant bacteria, result in changes in the relative abundance of unfavorable microbial populations, and consequently in granule instability.[26,108] Zou *et al.*[108] reported that changes in nitrogen concentration in influent wastewater resulted in an imbalance in nitrogen removal and phosphorous accumulating organisms. This shift in the microbial community structure resulted in functional instability and subsequent disintegration of the BNR granules. Isanta *et al.*[109] examined the sensitivity of the anammox process and the microbial community of granular sludge to temperature changes (temperature increased from 35–46 °C for eight days). An imbalance in the microbial community and functional instability of anammox processes was observed following this temperature change for eight days. The functional stability of the bioreactor recovered after 70 days of operation. In these two recent studies, the microbial diversity was further reduced in the recovered bioreactor following the transient instability and recovery period.[26,109]

5. Instability and control of floc, biofilms, and granular structures

Activated sludge floc instability can be caused by fluctuations in chemical composition and physical properties of incoming wastewater and dynamics in operational environmental conditions, such as temperature which affects microbial activity as well as the structural and functional properties of the EPS; pH and ionic composition which determine surface charge; divalent cations such as Ca^{2+} which contribute to the cation-bridging of EPS; and various toxins.[1] These parameters have a similar influence on biofilms and granules.[3,43]

Biofilm sloughing can be triggered by several physical, chemical or biological stressors that can be induced due to changes in environmental or nutritional conditions. The microcolonies growing in a biofilm have varied responses to nutritional and environmental stress.[110] Biofilm dispersion increases with an increase in surface area loading rate and hydraulic retention time in moving bed bioreactors treating wastewater.[111,112] In membrane aerated biofilm reactors, higher chemical oxygen demand favours biofilm stability; whereas higher oxygen partial pressure results in loss of EPS at the bottom of biofilm that triggers biofilm detachment.[113] The biomass in BNR system undergoes consistent hydraulic shear force. Biofilms and granules that are formed at higher shear force form dense, compact and stable biofilms and granules. The higher shear force improves EPS production and efficiency of mass transfer to improve the structural and functional stability of the biofilms and granules.[23]

Bacteria can relocate within biofilms towards a more favorable microenvironment or detach from a biofilm to search for better environmental and nutritional resources.[114] A subpopulation of bacteria may lyse when conditions are not favorable for bacteria in deeper layers of the biofilms. The organic matter released due to cell lysis serves as nutrition for the nearby surviving bacteria.[115] An increase in carbon source up-regulates the flagellum expression and down-regulates the adhesive extracellular pili.[116] The switch in the expression of extracellular adhesins results in sloughing, which leaves a hollow biofilm.[110] Overall, these phenomena demonstrate that biofilms are organized structures with the ability to reorganize with changes in environmental or nutritional conditions.[32,117]

In granular sludge, filamentous outgrowth, granule disintegration, presence of gas bubbles, and higher polysaccharide content in the extracellular matrix, can lead to poor settleability. Shorter settling time helps in the removal of these compromised structures; however, if instability events become dominant in the system, it results in biomass washout and system failure.[26,118] The application of short settling time is also difficult at full scale. A good management of hydraulic regimes during fill/draw phases is essential to maintain faster-settling aggregates in the reactor while washing-out slow-settling flocs.[40,119] Liu and Liu[120] reviewed and listed several operational and nutritional conditions causing granule instability, including long solid retention time and low dissolved oxygen concentration. Interestingly granules have been cultivated successfully under these operating conditions.[119] Additionally, filaments are usually considered to cause granule instability and biomass washout.[26,118] Conversely, it has been observed that filaments play a role in granule formation. The filaments serve as the backbone and immobilize the bacteria community to form large flocs, which can be converted into granular sludge by hydrodynamic shear forces. Furthermore, filaments can bridge the large flocs and enmesh them to form granules.[7,26,121,122] Nevertheless, the presence of filaments will mainly always remain a latent issue. A good anaerobic selector should specifically suppress the growth of filamentous organisms, protecting granules from a filamentous bulking outbreak.[15]

5.1. Practical considerations to manage structural and functional characteristics

Key issues in the development and scaling of biofilm- and granule-based wastewater treatment processes are presented in Table 2. Anaerobic feeding of a readily degradable organic substrate facilitates the conversion of organic substrates to complex storage polymers, to support slow-growing PAOs and GAOs. If the organic substrate is fed under oxic conditions, the substrate is consumed by fast-growing heterotrophic bacteria present in the outer layer which results in starvation

Table 2 Key issues in the development and scaling of biofilm and granular based wastewater treatment processes[a]

Formation and stability	Scaling and full-scale operations	Fundamental questions offering new insights
i) Feeding conditions & pulse *vs.* slow feed	i) Controlling hydrodynamics and mixing	i) How to better integrate or design BNR granular, and biofilm systems to support anammox processes
ii) Composition and variability of industrial wastewater	ii) Selective sludge removal and design criteria for height to diameter ratios moving from lab to full-scale	ii) How to selectively remove specific groups of bacteria (*e.g.* heterotrophs to enhance partial nitration and anammox (PN/A)) from granules
iii) Nutrient loading and stability (*e.g.* high strength organics, N & P)	iii) Operating at high salt and high solids concentrations	iii) What is the role of flocs in granular systems? Are hybrid systems of flocs-films, floc-granules and/or film-granules better path to stability, treatment, and operational efficiency outcomes?
iv) Sludge volume loading	iv) Applicability of current biofilm models for granular systems	iv) Increased understanding of the role of EPS in the formation and stability of granular sludge
v) Effect of metals		v) Resolving microbial interactions and functions related to formation, function and stability of films and granules

[a] Summary of key issues identified by wastewater engineering researchers and practitioners in industry, covering expertise in microbiology, civil and environmental engineering, and chemical engineering, in attendance at the *Drivers of Granular Sludge and Biofilm Stability Workshop* (March 18, 2018), "IWA Biofilms: Granular Sludge Conference 2018", IWA Biofilms Specialist Group, Delft University of Technology, Delft, Netherlands, March 18–21, 2018.

and lysis of slow-growing bacteria present in the core.[123] Polymeric substrates which are slowly degradable, and which are not consumed under anaerobic conditions in the core, contribute to conditions that enable fast-growing filamentous outgrowth at the surface of the granules where oxic conditions exist. The presence of polymeric organic substrates in wastewater is one of the limiting factors for stability of granular sludge in full-scale wastewater treatment plants.[124,125] Wagner *et al.*[126] proposed to treat wastewater with high polymeric substrates content by operating SBRs with longer anaerobic conditions for effective hydrolysis and to improve the granule stability. For optimum nutrient removal and structural stability of granules, a balance between aeration and non-aeration periods during the operating cycles of the reactor is therefore required.

Another example of the need to understand the relationships of these microbial systems is the formation of a relatively less dense and "fluffy" granular structure in the presence of a higher EPS polysaccharide content. While these structures retain many of the features of a functional granule including size, they settle slowly in the bioreactor (like flocs)[127,128] and are thus no longer granules by virtue of their settleability characteristics. If it is desirable to retain these granular structures in the reactor, an adjustment upward in settling time is required. Washing out slow-settling aggregates by gradually decreasing the settling time from 15 minutes to 3 minutes has been efficient to form dense granules from "fluffy" structures at lab scale.[15,39]

Simultaneous nitrogen removal is possible because BNR granules retain metabolic byproducts, which can be used by other bacteria,[24] however, it also results in retention of metabolites, which may be toxic to the microbial community.[26] A few studies have reported the presence of bacteria capable of producing toxic metabolites or enzymes in BNR granules, which can lead to the disintegration of the granular structure. Adav *et al.*[129] identified bacteria, which produce protease enzymes that were responsible for a decline in EPS protein content resulting in granule disintegration. The adhesive protein content can also decline due to loss of specific bacterial populations which contribute to the secretion of protein in EPS, resulting in granule instability.[26,130] *Janthinobacterium* (capable of producing an antibiotic pigment called violacein) have been observed in BNR granules that may contribute to bacterial cell lysis resulting in granule instability.[26,131] Incubation of BNR granules with enzymes degrading the α (1–4) glucans and proteins and exposure to shear stress resulted in aggregate instability.[132] Little is, however, known about the biosynthesis of these molecules, or their concentrations in full scale operations.

Overgrowth of a microbe that is producing less common non-toxic products of metabolism may contribute to microbial community dynamics that lead to granule instability. Aqeel *et al.*[26] reported on the outgrowth of *Auxenochlorella* in BNR granules that can accumulate lipid and chitin. Successional changes in the community resulted in an initial decline of *Gammaproteobacteria* followed by the growth of the bacteria *Janthinobacterium* and *Chitinophaga*, which can use these byproducts as nutrient substrates. In this instance, *Chitinophaga* was antagonistic towards *Auxenochlorella* resulting in the recovery of the granular structure and return to a granular microbial community dominated by the denitrifying genus *Rhodanobacter*.[26,133]

Most of the information on granulation is based on intensive research on laboratory scale SBRs, which are operated under well-controlled operational and nutritional conditions. Few reports have been published that describe structural and functional characteristics of granules in full-scale plants.[119,134,135] Pronk *et al.*[119] reported the detailed operational conditions for granulation in full-scale wastewater treatment, where feast-famine conditions and fast settling times were applied. The full-scale SBR was seeded with surplus biomass from an existing granular bioreactor that was operated with a long solid retention time (20–38 days) for selection of slow-growing bacteria. The full-scale SBR was fed anaerobically for 60 minutes, aerated for 300 minutes during the react phase, settled for 30 minutes, and the treated water was drained in 60 minutes, during dry weather conditions. During a period of rain the number of SBR cycles was increased to allow for a constant daily organic loading of the plant: the reactor was anaerobically fed for 90 minutes, aerated for 60 minutes during the react phase, and settled for 30 minutes. The length of anaerobically feeding and react phase were optimized during seasonal variations according to the fluctuation in environmental and nutritional conditions. In full-scale SBR, the stable granule formation was achieved after five months of startup period that was maintained by optimizing the operational conditions.

Current efforts further granulation in existing flow-through installations to retrofit and intensify them while keeping the original plant design and components. The key remains in the design of a procedure to select for fast-settling aggregate while separating slower-settling flocs.[136–138] While process engineering considerations are under elaboration world-wide, the underlying microbiology still remains a central element of the puzzle. Several full-scale plants operated for full BNR with well-operating anaerobic selectors have been observed to granulate "spontaneously". A good conjunction of process engineering and microbial community engineering should be kept in mind in order to manage the continuum from flocs to granules.

5.2. Hybrid systems to optimize granule structure–function relationship

In SBRs, the larger and dense granules settle to the bottom of the tank and relatively smaller and less dense structures are drained from the system. This results in a gradual development of granules with a large diameter. The large diameter limits the transport of nutrients in the granules which ultimately lead to cell lysis of the slow-growing bacteria in the anaerobic core and granule disintegration.[123,139] The large granules are also more prone to channel blockage which limits the mass transfer of nutrients even more. The blocked channels also result in entrapment of gas bubbles and lowering of granule density. Eventually, these structures float to the surface and are washed out of the system.[107]

Hybrid biomass with a wider range of bioaggregate sizes is suggested to support the stability of granular sludge. The hybrid biomass may reduce the overall settleability compared to uniform larger granules. However, hybrid biomass has advantages that the disintegration of larger granules has minimal effect on the overall settleability of the granular sludge; where the disintegrated structures can reform into granules. Pronk *et al.*[119] reported stable full-scale granular sludge system with granules ranging from 0.2 mm to larger than 1 mm, with 20% flocs smaller than 0.2 mm in size. Zhu *et al.*[140] suggested selective biomass removal to achieve hybrid granular sludge that improved the stability of the system. In addition to removal of floccular sludge (to keep an SRT of 2.7 days for flocs) from of the settled biomass, the aged larger granules were also removed (SRT of 9.9 days for aged granules) from the bottom of the tank, to promote a hybrid biomass in the system.[140] However, the selective removal of biomass is possible in bioreactors with higher height to diameter ratios (h/d), where the efficient separation of biomass is possible during settling time. Higher aeration rate is used to minimize the development of large size granules where h/d is lower, but only with limited success.

Granulation in integrated fixed-film activated sludge (a hybrid system) represents an interesting development to maintain hybrid biomass and minimize the development of large size granules. Granule formation is relatively reproducible, and the resulting granules are relatively stable in bioreactors operated under controlled conditions, as compared to full-scale wastewater treatment plants. The selective pressure applied for granule sludge production results in a decrease in overall microbial diversity. The dominant microbial population is usually distinct from one system to the other (Table 1) due to unique nutritional and environmental conditions for most wastewater systems. The loss of microbial diversity may contribute to granule instability due to the inability to adapt to changes in operational or nutritional/environmental conditions. Whereas, full-scale wastewater treatment plants are operated under diverse environmental and nutritional conditions, some of which is due to seasonal changes (winter) and precipitation events (rain). The seasonal variation also affects the efficiency of conventional full-scale wastewater treatment. In contrast, full-scale integrated fixed-film activated sludge (IFAS) systems show year-round stable BNR due to the presence of both attached and suspended biomass.[10] The biomass in hybrid reactors shows bioaggregates with a wider range in size and density. The wider range of this type of biomass has diverse redox properties. The wider size range of the hybrid biomass in IFAS systems conserves a more diverse microbial community, where different microbial groups are dominant in attached and suspended biomass.[10] This result in improved adaptability for structural and functional stability during a change in environmental and nutritional conditions. In an experiment where the attached and suspended biomasses were separated to understand the capability of hybrid biomass in BNR, it has been observed that the contribution from the attached and suspended biomass is not additive. Instead, it has been synergistic to improve the overall BNR in IFAS system. Additionally, it has been observed that biofilms in IFAS systems tend to slough and readily associate with the suspended biomass. The sloughed biomass forms the stable granular flocs that improve the overall settling properties of the biomass.

6. Conclusion

Microbial aggregation phenomena should be considered on a continuum from flocs to biofilms and granules. Biofilm and granule formation start with initial bioaggregation that grow in size and when it reaches a threshold limit, biofilms and granules disperse to produce floccular biomass and start a new cycle of biofilm and granule formation. The microbial aggregates are organized structures that can be engineered to achieve a target microbial niche for the desired function. Biofilm sloughing results in partial loss of biomass and transient decline in biological nutrient removal; whereas when granules disperse (in fast settling operations) it may result in a major biomass loss and system failure. Granular sludge management should, therefore, include a strategy that prepares for dispersion events, by keeping (a) a heterogeneous size range of granules that compensate for dispersion of a fraction of the granules and simultaneously favor regranulation of dispersed biomass; and (b) a hybrid microbial aggregates (flocs, granules, and biofilms) in the system. The hybrid biomass ensures microbial structures with diverse redox condition and diverse microbial population for enhanced performance and resilience.

Conflicts of interest

There are no conflicts of interest to declare.

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