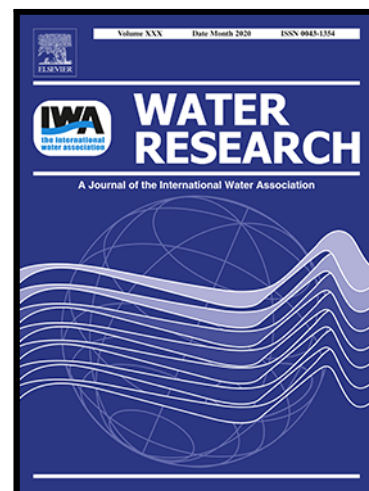# Journal Pre-proof

A review of filamentous sludge bulking controls from conventional methods to emerging quorum quenching strategies

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Highlights

- Classical bulking hypotheses limit the application of conventional bulking control.

- The microbial interactions are regulated through quorum sensing behaviors.

- Functional signal molecules regulate specifically filamentous microorganisms.

- Filamentous bulking has been verified to be related with quorum sensing.

- Quorum quenching is effective and precise to inhibit filamentous bulking.

A review of filamentous sludge bulking controls from conventional methods to emerging quorum quenching strategies

Xin Lu[a], Ge Yan[a], Liang Fu[a], Bin Cui[a], Jinfeng Wang[b], Dandan Zhou[a] *

[a] Jilin Engineering Lab for Water Pollution Control and Resources Recovery, Northeast Normal University, Changchun 130117, China.

[b] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210023, Jiangsu, PR China

Corresponding Authors

E-mail: zhoudandan415@163.com (Dandan Zhou*)

Abstract:

Filamentous bulking, which results from the overgrowth of filamentous microorganisms, is a common issue that frequently disrupts the stable operation of activated sludge processes. Recent literature has paid attention to the relationship between quorum sensing (QS) and filamentous bulking highlighting that the morphological transformations of filamentous microbes are regulated by functional signal molecules in the bulking sludge system. In response to this, a novel quorum quenching (QQ) technology has been developed to control sludge bulking effectively and precisely by disturbing QS-mediated filamentation behaviors. This paper presents a critical review on the limitations of classical bulking hypotheses and traditional control methods, and provides an overview of recent QS/QQ studies that aim to elucidate and control filamentous bulking, including the characterization of molecule structures, the elaboration of QS pathways, and the precise design of QQ molecules to mitigate filamentous bulking. Finally, suggestions for further research and development of QQ strategies for precise bulking control are put forward.

Keywords:

Contents

1. Introduction

The activated sludge process is a commonly used biologically engineered system in wastewater treatment plants (WWTPs) for treating both domestic and industrial wastewaters (Guo et al., 2012a; Zhang et al., 2017). However, its stable operation is frequently disrupted by filamentous bulking, which occurs due to the overgrowth of prokaryotic bacteria or eukaryotic fungi (Guo et al., 2014; Lu et al., 2022a; Wang et al., 2013). Filamentous bulking can lead to a multitude of problems, including poor settleability, sludge washout, the degradation of effluent quality, and in severe cases, even the failure of the entire system (Guo et al., 2012a, 2014; Wang et al., 2016). Despite the significant impact of filamentous bulking, the lack of understanding of its molecular biological mechanisms has limited the effectiveness of existing control approaches, which suffer from a variety of drawbacks and limitations.

Quorum sensing (QS), a novel sociomicrobiology theory, has recently emerged to explain how bacterial cells coordinate the collective behavior of multi-species biofilms formation in pure cultivation (Federle and Bassler, 2003; Whiteley et al., 2017). In complex activated sludge systems, QS mediates the synthesis and secretion of extracellular polymeric substances (EPS), promoting sludge flocs to form granulation and membrane biofouling in membrane bioreactors (MBRs) (Ding et al., 2015; Huang et al., 2019; Lee et al., 2018; Wang et al., 2020). This cell-to-cell communication theory has been observed in filamentous fungi as well, where they communicate through signaling molecules and make different morphological responses by regulating the expressions of specific genes in various microecosystems,

such as rhizosphere, human body, and food (Barriuso et al., 2018; Peleg et al., 2010; Venturi and Keel, 2016). Recently, researchers have focused on the relationship between QS-mediated morphological transformation of filaments and filamentous bulking (Dong et al., 2022; Feng et al., 2022; Lu et al., 2022a, 2023; Shi et al., 2022a, 2022b). N-acyl homoserine lactones (AHLs), common bacterial signal molecules, have been identified key players in the process of filamentation in both fungi and bacteria. These molecules are detected by the fungal or bacterial cells and are responsible for regulating genes associated with filamentation, ultimately leading to the occurrence of filamentous bulking (Feng et al., 2022; Lu et al., 2022a).

The observation of QS in natural environments has led to the development of quorum quenching (QQ) as a means of preventing changes in gene expression by disturbing or blocking QS behavior (Lee et al., 2018; Oh and Lee, 2018). For instance, the application of vanillin, a typical natural compound, has been shown to reduce the abundance of biofilm-associated genes (*bdlA*, *lasI*, *pgaC*) and prevent membrane biofouling (Pal et al., 2020). Moreover, vanillin has also been demonstrated to effectively prevent the occurrence of filamentous bulking by inhibiting the expressions of signal synthesis genes (Shi et al., 2022a, 2022b). Besides, certain inhibitory signal molecules, such as 3-oxo-C6-HSL, 3-oxo-C10-HSL, C12-HSL, and C14-HSL, have been found to have the potential to inhibit filamentous bacterial or fungal bulking, which decreases the relative abundance of dominant filaments and maintains excellent biological activity of sludge floc (Dong et al., 2022; Feng et al., 2022; Lu et al., 2022a). Thus, the application of QS/QQ to regulate filamentous

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microbes is a promising and environmentally friendly alternative for the precise control of filamentous bulking.

In this paper, the limitations classical bulking hypotheses and traditional control methods are reviewed, the intrinsic connections between QS and filamentous bulking are summarized, and the application of QQ technology for targeted bulking control is discussed. A novel QQ strategy for precise bulking control is put forward based on the current understanding of the molecular biological mechanisms of filamentous bulking as reviewed in this study.

2. Classical bulking hypotheses and conventional control methods

2.1. Classical bulking hypotheses and limitations

2.1.1. Classical bulking hypotheses

Several hypotheses have been proposed to elucidate the causes of sludge bulking, including diffusion-based selection theory, storage selection theory, kinetic selection theory, and nitric oxide (NO) hypothesis (Wang et al., 2013) (Fig. 1.). Diffusion selection theory suggests that filaments use a high surface area ratio to absorb low concentrations of substrate (Fig. 1a) (Sezgin et al., 1978). Storage selection theory presumes that non-filaments have a strong ability to selectively store energy or organics under adverse circumstances (Fig. 1b) (Flippin et al., 1985). Kinetic selection theory postulates a dynamical model to explain filamentous bulking in which filaments have lower maximum growth rates (μ_{max}) and affinity constants (K_s) than the floc-forming bacteria (Fig. 1c, 1d, 1e) (Chudoba et al., 1973a, 1973b). Finally,

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nitric oxide (NO) hypothesis proposes that filaments are unable to accumulate nitrites and NO (Fig. 1f), and thus survive in aerobic zones (Casey et al., 1992).

2.1.2. Limitations

While classical bulking hypotheses provide valuable insights into the dynamics of the sludge bulking process and the microscopic mechanisms underlying filamentous bulking, they were formulated over 30 years ago and may not fully capture the complexities of modern wastewater treatment systems. Therefore, while these theories have explanatory power, they may not be able to explain all the phenomena associated with sludge bulking, exhibiting some apparent inconsistencies and contradictions. For example, recent studies have shown that filamentous bacteria possess high storage capacity, challenging the conventional understanding that Type 0092 bacteria are unable to use nitrates as electron receptors (Casey et al., 1992). Furthermore, conclusive evidence filaments having low μ_{max} and K_s is currently lacking. So far, over 30 different filament morphotypes have been identified in bulking sludge systems, each exhibiting distinct and complex ecophysiologies (Wang et al., 2016). Therefore, classical bulking hypotheses remain subject to ongoing debate due to a lack of molecular biological recognition and technology, which limits our ability to fully understand the complex ecophysiologies of filamentous bacteria in sludge bulking systems.

2.2. Conventional bulking control methods and limitations

2.2.1 Conventional bulking control methods

Sludge bulking has conventionally been controlled by non-specific and specific

approaches (Fig. 2. and Table 1). Non-specific methods involve the use of chemical agents, such as chlorine (Logue et al., 1983), ozone (Leeuwen., 1988), hydrogen peroxide (Keller et al., 1973), metal salts (Finger, 1973), and even ammonium bromide (Nastro et al., 2006) to kill filamentous bacteria. Recently, a magnetic field is used to improve the settleability of sludge flocs through strengthening polymeric interactions and electrostatic forces (Syamim et al., 2019). Specific methods aim to prevent the proliferation of bulking filaments by implementing upstream aerobic, anaerobic, or anoxic reactors before aerobic tanks (Chudoba et al., 1973a, 1973b). Nano zero-valent iron is added to selectively killing filamentous organisms (Xu et al., 2014). In addition, natural predators of filamentous microbes, such as rotifers or bacteriophages, selectively eliminate excess filaments in domestic wastewater with low organic loads (Fiałkowska et al., 2008; Inamori et al., 1991; Kotay et al., 2011).

2.2.2. Limitations and inspirations

Although conventional bulking methods have shown effectiveness in the short term, their notable drawbacks cannot be ignored. Non-specific disinfectors not only reduce the total bacterial population but also damage the sludge floc structure, resulting in further complications (Wang et al., 2016). Biological selectors, on the other hand, are not always effective in controlling bulking and achieving satisfactory operational parameters (Zhang et al., 2017). Anaerobic selector inhibits the overgrowth of *Type 021N*, but this can trigger *Thiothrix* specie*s* or *Candidatus Microthrix parvicella*, which utilize high sulfide and long-chain fatty acids (LCFA) as substrates, respectively (Martins et al., 2003; Fan et al., 2020; Wágner et al., 2022).

For condition-based control, increasing aeration intensities can result in spreading smaller flocs and a significant amount of sludge washout (Syamim et al., 2022). At last, biological controls have not been applied in full-scale WWTPs, because of the failure of rotifer and bacteriophage to maintain in highly toxic industry and in municipal sewage containing heavy metals (Klimek et al., 2013; Syamimi et al., 2022). In conclusion, these conventional methods are not precisely selective towards targeted filaments, and they cause additional issues.

In a recent breakthrough, researchers have discovered a balanced relationship between different filamentous microorganisms and floc-forming bacteria, determined by bacterial AHLs, that can affect the occurrence of sludge bulking under adverse circumstances (Feng et al., 2022; Lu et al., 2022a, 2023). Specifically, C12-HSL and C14-HSL have been found to mediate transboundary cell-to-cell communication between fungal filaments and floc-formers (Feng et al., 2022; Lu et al., 2022a). Furthermore, the 3-OH-C10-HSL and 3-oxo-C10-HSL have been identified as crucial factors that drive cooperation and competitive interactions between filamentous *Sphaerotilus natans* and QS bacteria, respectively (Lu et al., 2023). These findings suggest that dominant filaments and floc-formers establish a complex web of interactions in which signals are sent and received. Therefore, interfering with their cell-to-cell communication by disrupting signaling pathways can effectively inhibit filamentous microorganisms.

3. Molecular biological mechanisms of filamentation

3.1. The species of filamentous microorganisms causing sludge bulking

Substantial studies have mainly focused on the ecophysiology of filamentous microbes associated with sludge bulking, which involve both the prokaryotic and eukaryotic ecospheres. As shown in Table 2, *M. parvicella*, a member of Actinobacteria, has often been found to cause sludge bulking in wastewater treatment plants (WWTPs) with low dissolved oxygen (DO), low temperatures, and nutrient limitations (Fan et al., 2020). Other Bacteria, including *S. natans*, *Thiothrix eikelboomii*, *Leucothrix*, *Beggiatoa*, and Type 021 N, all members of Proteobacteria, have been observed to dominate under conditions where DO concentrations are less than 2 mg/L or where the food-to microorganism (F/M) ratio is less than 0.2 kgBOD$_5$/kg MLSS·d (Guo and Zhang, 2012; Lu et al., 2023; Mielczarek et al., 2012; Wang et al., 2016; Wu et al., 2019). In addition to bacteria, fungal bulking is common under low pH (< 5.5) conditions, with species such as *Trichosporon asahii*, *Geotrichum* and *Penicillium* being frequently observed (Feng et al., 2022; Meyers and Jr, 1984; Zheng et al., 2011). *Galactomyces geotrichum* exhibited overgrowth tendencies at DO levels of less than 2 mg/L or high F/M rates of 4.3 g COD L^{-1} day (Lu et al., 2022a; Matos et al., 2012). Despite the significant amount of research conducted on filamentous microbes, the physiological habits of these bacterial and fungi remain poorly understood from the perspective of molecular biology, due to difficulties associated with cultivating and maintaining pure cultures.

3.2. Fungal filamentation pathway

The molecular mechanisms of fungal filamentation have been widely studied in the field of plants and human biology (Brand and Gow, 2009; Takeshita et al., 2014). As depicted in Fig. 3a, fungal growth is impelled by cell polarization through the extension of the cell wall and membrane, a process that relies on the continuous transport of secretion vesicles from the hyphal cell body to the filament end (Takeshita et al., 2014). The actin cytoskeleton, an essential component for maintaining and promoting hyphal elongation, is regulated by the polarisome that contains *Bni1*, *Spa2*, *Bud6*, and *Pea2* compounds (Moujaber and Stochaj, 2020; Wendland and Walther, 2005). Furthermore, two signal transduction pathways, namely Ras1-cAMP-PKA-Nrg1 and mitogen-activated protein kinase (MAPK), are crucial in regulating fungal filamentation. These pathways subsequently stimulate cell-wall biosynthesis and enhance cell-wall integrity by increasing the expression of the downstream gene *Bni1* (Wendland and Walther, 2005; Yoshimi et al., 2016). Thus, fungal filamentation is a positive feedback loop regulated by *Ras1* receptor activation, which consequently mediates the transcriptional regulation of genes associated with mycelium formation. This intricate molecular machinery highlights the importance of understanding fungal physiology and its potential applications in various fields.

3.3. Filamentous bacterial growth

To date, the molecular mechanisms of filamentous bacteria are only studied in three genera causing sludge bulking in WWTPs, including *M. varicella,* Type 0092*, and S. natans.* McIlroy et al. (2013) constructed a metabolic model of *M. varicella,*

using a genome-wide approach, and illustrated that this bacterium esterifies long-chain fatty acid (LCFA) to exocellular triacylglycerol (TAG) as carbon storage material at low oxygen tensions (Fig. 3b). Additionally, the microaerophilic and anaerobic lifestyles of *M. varicella* rely on a ferredoxin-dependent 2-oxoglutarate oxidoreductase (EC 1.2.7.3) that reduces 2-oxoglutarate to succinyl-CoA. In another study, Eikelbloom Type 0092, as a member of the phylum Chloroflexi, whose long filament morphotypes associated with bulking episodes, evolved the Calvin Benson Bassham (CBB) cycle to achieve lithotrophy and synthesize glycogen to store polysaccharides under oligotrophic conditions (Fig. 3c) (McIlroy et al., 2016). Moreover, Lu et al. (2023) reported that individual *S. natans* store polysaccharide polymers to form filamentous sheaths through the evolution of the reverse tricarboxylic acid cycle (rTCA), which enables them to striver under low oxygen conditions (Fig. 3d). These findings suggest that carbon fixation evolution, mediated by the expressions of specific genes, is associated with the growth of filamentous bacteria in WWTPs.

4. The roles of QS in filamentous bulking

4.1. Quorum sensing, signal molecules and signal transduction pathways

4.1.1 Quorum sensing

Microorganisms never grow individually in any microenvironment (Whiteley et al., 2017). Bacteria exploit cell-to-cell communication systems to sense their population density and make a coordinated response to the surrounding environment,

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a process known as QS (Sharma et al, 2020). QS regulates specific gene expressions to control a series of group phenotypes, including symbiosis, nutrient competition, dissemination, sporulation, EPS secretion, and biofilm formation (Federle and Bassler, 2003). Such intraspecific communications have also been studied widely in several dimorphic fungi (Padder et al., 2018). A remarkable discovery was that the pathogenic fungus *Candida albicans* forms filaments at densities $<10^6$ cells/mL and grows as a yeast form when inoculated at $>10^6$ cells/mL (Cho et al., 2010; Padder et al., 2018). This morphology transformation between budding yeast and mycelia corresponds to an adaptive response to environmental changes, which is critical for their survival (Albuquerque and Casadevall, 2012; Avbelj et al., 2016). Whether bacterial or fungal, species depend on QS to regulate the flexibility of their collective phenotypes in response to changing environments.

4.1.2 Quorum sensing molecules

In general, QS is a phenomenon initiated when small-molecule signal chemicals reach a threshold concentration in the surrounding environment (Sharma et al, 2020). These signals are widespread and diverse in microbial communities, and are actively or passively accepted by cells and result in the production of more mutual benefits and/or more signals themselves (Abisado et al., 2018). Bacterial signal molecules have been divided into three general classes based on different QS systems: i) AHL secreted by Gram-negative bacteria; ii) peptide secreted by Gram-negative bacteria; and iii) Autoinducer-2 (AI-2) shared by Gram-negative and Gram-positive bacteria (Oh and Lee, 2018). Compared to other signaling molecules, AHLs have been more

widely studied due to their diverse structures (Stephens and Bentley, 2020). The 50 characterized structures of AHLs contain a common homoserine lactone ring connected to an acyl side chain of varying length (4–18 carbon) and a branch structure modified by a 3-oxo substituent or a 3-hydroxy substituent (Hawver et al., 2016). These structures determine microbial ecological functions affecting cell growth, motility, biofilm formation, EPS production, virulence, and bacterial warfare (Liu et al., 2022). Similarly, farnesol and tyrosol are two common signal molecules in various fungi that inhibit and promote hyphal development, respectively (Cho et al., 2010; Padder et al., 2018). Besides, farnesoic acid, tryptophol, and 1-phenyl-ethanol have been discovered to inhibit hyphal growth in *C. albicans* (Barriuso et al., 2018; Padder et al., 2018). As volatile signal, carbon dioxide (CO_2) has been shown to trigger the yeast-to-mycelium morphological switch in *C. albicans* and to promote germination in *Aspergillus niger* conidiospores (Barriuso et al., 2018; Padder et al., 2018). Thus, understanding the microecological function of signal molecules is pivotal to precisely control cells' morphology and physiology.

4.1.3 Specific signal transduction pathway

QS regulates various bioprocesses through signal synthesis, signal reception, and phosphorylation/dephosphorylation cascades, ultimately influencing the transcription of target genes. The LuxR/LuxI feedback system is a classic paradigm in the Gram-negative bacteria (Haque et al., 2018), where AHL is produced by *LuxI* and freely diffuses in and out of cells to stimulate the transcriptional regulator *LuxR* (Miyamoto and Meighen, 2006). The expression of the luxCDABEG (*lux*) genes

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encoding the luciferase enzyme complex is activated through its signaling cascade pathway (Haque et al., 2018; Miyamoto and Meighen, 2006). In filamentous fungi, extracellular fungal signals simultaneously trigger Ras1-cAMP-PKA and MAPK pathways, which are typical and important signal transduction pathways (Padder et al., 2018; Barriuso et al., 2018; Nigg et al., 2016). Downstream specific genes associated with filamentation have down/up-regulated expressions to produce different signal responses (Padder et al., 2018; Barriuso et al., 2018;). Despite the numerous and various QS signal molecules that exist in microenvironment, the same species have exclusive signal reception, transduction, and transport pathways.

4.2. QS regulates transboundary interaction

Fungi and bacteria often coexist in proximity in microecological environments. The dynamic balance of fungal–bacterial interactions can alter the homeostasis of the microenvironment and lead to either competition or cooperation between microbes (Barriuso et al., 2018; Peleg et al., 2010; Venturi and Keel, 2016). Intriguingly, bacterial signal molecules can interfere with anticipated fungal–bacterial interactions. For instance, in the rhizosphere, fluorescent fungus *Fusarium oxysporum* and bacteria *Pseudomonas auruginous* have been observed to interfere with each other by releasing signal molecules, thereby affecting the occurrence, and spread of plant diseases (van Overbeek and Saikkonen, 2016; Venturi and Keel, 2016). In the lungs of cystic fibrosis (CF) patients, the presence of both *P. aeruginosa* and *C. albicans* has been found to lead to the secretion of 3-oxo-C12-HSL by *P. aeruginosa*, which inhibits fungal mycelial formation and biofilm development (Barriuso et al., 2018;

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Peleg et al., 2010). As shown in Table 3, some bacterial signal molecules also affect morphogenesis transformations from the yeast phase to the mycelial phase, including the diffusible signal factor (DSF) secreted by bacteria genus *Burkholderia* and *Stenotrophomonas*, AI-2 secreted by bacteria *Aggregatibacter actinomycetemcomitans*, and bacterial indole and C10-HSL, et al. (Bachtiar et al., 2014; Boon et al., 2008; Lu et al., 2022b; Raut et al., 2012). Transboundary communication can also affect the excessive growth and morphological changes of dominant fungal species in bulking sludge systems.

4.3. The roles of QS in filamentous bulking

4.3.1 The signaling molecules that regulate filamentations.

The occurrence of filamentous bulking under adverse conditions is driven by QS-mediated intraspecific, interspecific, and transboundary interactions, as shown in Table 3 (Dong et al., 2022; Feng et al., 2022; Lu et al., 2022a, 2023; Shi et al., 2022a, 2022b, 2023). Generally, these signal molecules were identified to play a key role in microbial responses to filamentation pressures, such as low DO and low pH (Shi et al., 2022a, 2022b). For example, increasing levels of C6-HSL have been shown to dramatically increase the abundance of filamentous bacteria *Thiothrix* from 0.39% to 27.30% during filamentous bulking in low DO stimuli (Shi et al., 2022a, 2022b); commercial 3-OH-C10-HSL at a concentration of 500 nM has been found to significantly increase *Sphaerotilus* biomass induced filamentous bulking in both sheath filaments and individual cells (Lu et al., 2023). In contrast, the levels of C12-HSL decreased with increasing abundance of dominant fungus *Galactomyces* at

low DO concentrations, accompanied with the reduction of spore germination and mycelial extension (Lu et al., 2022a). Under low pH stimuli, C12-HSL and C14-HSL were found to inhibit *Penicillium* bulking, whereas C7-HSL triggered the hyphal growth and exopolysaccharide synthesis (Feng et al., 2022). Taken together, these findings highlight the role of QS-mediated interactions in microbial evolution and adaptation to changing environments, with filamentous bulking being a manifestation of this dynamic process.

4.3.2 The signals synthesis, reception, and transduction

Recent studies have confirmed that QS-regulated signal synthesis, reception, and transduction have also been verified in the bulking sludge. The dominant *Thiothrix* has been shown to gradually evolve genes responsible for AHLs synthesis (*lasI*, *rpaI*, and *hdtS*) and genes involved in synthesizing AHL precursor substances (*fabD*, *fadD*, and *metK*) with the bulking sludge (Ahlgren et al., 2011; Gould et al., 2004; Shi et al., 2022a, 2022b). LasR and CciR signal receptor proteins, responsible for recognizing C6-HSL, had the highest abundance with increasing the relative abundance of *Thiothrix* (Lugo et al., 2017; Shi et al., 2022a, 2022b). For dominant *S. natans* bulking, a specific receptor protein LuxR and two-component system phoR were identified to be activated by combining with 3-OH-C10-HSL (Hawver et al., 2016; Liu et al., 2022; Lu et al., 2023). In the fungal bulking system, the increasing levels of C7-HSL, and decreasing levels of C12-HSL and C14-HSL act on the specific signal receptors, namely *Ras1* and *Rho1*, and then trigger the Ras1-cAMP-PKA-Nrg1 and MAPK signaling transduction pathways, leading to *Penicillium* or *Galactomyces* dominant

bulking (Barriuso et al., 2018; Lu et al., 2022a; Feng et al., 2022). While these pathways are similar to those involved in biofilm formation and microbial growth, the triggered performances and metabolic expressions are uniquely specific to environmental conditions such as DO, pH and hydraulic stress.

4.3.3 Microbial community regulations

Microbial interactions play a crucial role in shaping community composition, and QS-mediated signals are essential in driving the evolution of microbial communities towards dominant filamentous microbes in bulking sludge. Specifically, 3-oxo-C6-HSL has been shown to strengthen bacterial cooperation with the microbial community, with 7 QS-related keystones identified as key contributors to the high connectedness and dense interplays within the 3-oxo-C6-HSL network (Dong et al., 2022). Meanwhile, C12-HSL and C14-HSL have been found to regulate transboundary competition between dominant *Penicillium* and nitrogen-removing QS bacteria, such as *Nitrosomonas*, *Nitrosospira*, *Acidovorax*, and *Pseudomonas*, whereas C7-HSL contributed to transboundary cooperation between enriched QS bacteria, such as *Serratia*, *Aeromonas*, and *Penicillium* in fungal bulking (Feng et al., 2022). Notably, the directed network of *Sphaerotilus* bulking revealed that 3-OH-C10-HSL has a positive feedback regulatory role in the cooperation of *Sphaerotilus* with three QS floc-forming bacteria, including *Acidovorax*, *Aeromonax*, and *Pseudomonas* (Lu et al., 2023). Additionally, 3-oxo-C10-HSL stimulated social interactions between *Sphaerotilus* and its five competitors, namely *Defluviicoccus*, *Shinella*, *Iamia*, *Lautropia*, and *Rhodobacter* (Lu et al., 2023). Understanding the roles of

QS-mediated signals in the complex interactions between filamentous microbe and floc-forming bacteria has significant importance in manipulating their behavior through QQ.

4.3.4 Metabolic functions regulations

The proliferation of *Thiothrix* and the filamentation of *S. natans, Penicillium* or *G. geotrichum* are all regulated by the expressions of specific genes depending on the QS-AHL system. C6-HSL has shown to trigger the up-regulated expressions of succinate dehydrogenase (SDH) and NADH dehydrogenase (ND1) responsible for the activity of the electron transport chain, as well as the cyclin dependent kinase 2 (CDK2) associated with microbial proliferation through the G1/S transition, resulting in the proliferation of *Thiothrix* (Miyadera et al., 2003; Shi et al., 2022a, 2022a). In the process of fungal bulking (Fig. 4a), bacterial AHLs regulated the expression of hypha-associated genes (*Boi1*, *Boi2*, and *Bni1*), actin cytoskeleton, cell wall, mitochondria, vesicles synthesis, TCA cycle, oxidative phosphorylation, and EPS biosynthesis through activating signaling transduction pathways (Feng et al., 2022; Lu et al., 2022a). As shown in Fig. 4b, 3-OH-C10-HSL contributed to driving the reverse TCA cycle and gluconeogenesis. This process caused the individual *S. natans cells* to secrete glucose and galactose, which coated their surfaces and led to the formation of filamentous sheath (Ali and Stokes, 1971; Lu et al., 2023). Simultaneously, 3-OH-C10-HSL triggered the up-regulated expression of chemotaxis and flagellum assembly, accelerating individual cells to swim out of the sheath and form new filaments (Lu et al., 2023). Overall, the development on the QS-regulated metabolic

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pathways, provided valuable clues for precisely preventing filamentous bulking by proposing functional QQ molecules.

5. QQ strategies for targeted bulking control

5.1. Using QQ strategies to control sludge bulking

Some bacteria, such as *Serratia liquefaciens*, *Vibrio Fischer*, *Vibrio harveyi*, and *Serratia Ficaria*, can disrupt or quench cells' QS system by secreting signaling analogs including penicillin acid, patulin, halogenated furanose compounds, and vanillin, among others (Oh and Lee, 2018; Ponnusamy et al., 2009; Rasmussen et al., 2005). This process is known as quorum quenching (QQ) (Fig. 5a). In the field of wastewater treatment, vanillin has been used as a broad-spectrum QQ inhibitor to alleviate biofouling by blocking the biosynthesis and secretion of EPS mediated by QS (Pal et al., 2020;). The QQ technology can be a viable quenching strategy for filamentous bulking control, as it can disrupt QS-mediated filamentation (Fig. 5b). In this context, some inhibitory signal molecules have been preliminarily explored against filamentous microbes and natural quenchers to effectively prevent and control filamentous bulking based on the molecular biological mechanisms of QS-mediated filamentation (Dong et al., 2022; Feng et al., 2022; Lu et al., 2022a, 2023; Sam et al., 2022; Shi et al., 2022a, 2022b, 2023; Wang et al., 2021).

5.1.1. The effects of QQ on fungal sludge bulking

As mentioned above, long-chain AHLs, such as C12-HSL and C14-HSL, have been identified as ideal chemicals for suppressing fungal bulking by inhibiting the

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yeast-to-filaments morphologies transition. In pure *G. geotrichum*, 500 nM commercial C12-HSL significantly inhibited spore germination and mycelial growth by 20% and 15%, respectively (Lu et al., 2022a). Similarly, 50 nM C14-HSL supplementation in the *Penicillium* cultures led to a 30% decrease in mycelial length and a 26% decrease in Adenosine Triphosphate (ATP) production after 24 h (Feng et al., 2022). Notably, the addition of 5 µM C14-HSL significantly reduced fungal bulking sludge SVI values by 40%, resulting in only a small number of filaments on the biofloc surface (see Fig. 6a) (Feng et al., 2022). C12-HSL and C14-HSL have a similar skeleton structure to that of fungal signal farnesol, which inhibits filamentation by mimicking farnesol's action on inhibiting the Ras1-cAMP-PKA-Nrg1 and MAPK signaling transduction pathways (Barriuso et al., 2018; Feng et al., 2022; Lu et al., 2022a). Therefore, C12-HSL and C14-HSL can effectively prevent the recurrence of fungal bulking by inhibiting gene expression associated with fungal filamentation.

5.1.2. The effects of QQ on filamentous bacterial bulking

Inhibitory signals have shown promise in disrupting signal transmission and feedback among bacterial social networks, making them potential QQ inhibitors for bulking control. 3-oxo-C6-HSL and C6-HSL contribute to *Thauera* dominance and prevent *Comamonas* proliferation during community succession, thus improving phenol degradation (Valle et al., 2004). Analogously, 3-oxo-C6-HSL, a structurally similar molecule to C6-HSL, was found to decrease the total relative abundance of dominant Thiothrix by 2.37% and restore a complex bacterial community with intense

species interactions (Dong et al., 2022). This molecule also increased hydrophobic protein production and enhanced sludge hydrophobicity, fundamentally alleviating sludge bulking (Dong et al., 2022). Similarly, 5 μM 3-oxo-C10-HSL, an analogue of 3-OH-C10-HSL, was found to reduce the abundance of dominant *Sphaerotilus* to <0.27 % and protruding filaments to 0.01–0.03 $\mu m/\mu m^2$, effectively restoring the SVI below 150 mL/g (see Fig. 6b) (Lu et al. 2023). The treatment of bulking sludge with 5 μM 3-oxo-C10-HSL has been shown to maintain morphology features and surface properties of bioflocs, which results in improved bioactivity (Lu et al. 2023). In addition, it was revealed that *Caldilineaceae* in filamentous bulking exhibited a fast response to C14-HSL, which could also be an effective and efficient way to alleviate sludge bulking (Wang et al., 2021). These trial explorations of QQ using molecular structure modification of bacterial AHLs represent a more precise strategy for bulking control by targeting dominant filaments, while also maintaining floc stability and preserving excellent bioactivity.

Vanillin is a widely used QQ inhibitor with a better inhibitory effect on short-chain AHLs, reducing their availability to cells. Recently, 50 mg/L vanillin has been applied to control filamentous bulking, resulting in excellent sludge settleability (Shi et al., 2022a, 2022b, 2023). Further, it has been demonstrated that vanillin has the potential to inhibit the expression of the signal synthesis gene *hdtS*, leading to a decrease in the concentration of C6-HSL (Shi et al., 2022a, 2022b). This appropriate concentration of vanillin weakened the QS-AHL system of dominant *Thiothrix*, while strengthening QQ-AHL system, which was the main reason for successfully inhibiting

the proliferation of filamentous bacteria (Shi et al., 2022a, 2022b). More importantly, vanillin is an environmentally friendly material with negligible negative effects on microbial activity.

5.2. The techno-economic feasibility of QQ technology

The economic evaluation of QQ technology for controlling biofouling has demonstrated energy and cost savings compared to traditional aeration methods (Oh et al., 2018). Traditional control methods for high incidences of sludge bulking problems, such as the repeated dosing of chemical disinfectants or continuous optimization of biological selector parameters (Sam et al., 2022; Syamimi et al., 2022), are closely associated with high costs and energy consumption. While QQ inhibitors may seem more expensive than chemical disinfectants, a well-targeted quencher should effectively avoid the recurrence of sludge bulking (Lu et al., 2023; Shi et al., 2022a, 2022b). QQ technology offers a prominent advantage with excellent bioactivity and trace addition, which favors the saving of operating costs in the activated sludge process (Lu et al., 2023; Shi et al., 2022a, 2022b). Moreover, this environmentally viable technology reduces energy consumption of high aeration by setting an aerobic selector or increasing the DO condition (Liu et al., 2018; Oh et al., 2018). With more extensive research on QS-mediate sludge bulking, more types of quenchers will be developed and optimized for sludge control. Additionally, artificial intelligence can aid in research and development of chemical synthesis at a relatively low cost in the future.

5.3. Perspectives

Recent advancements in understanding the relationship between the QS-AHL system and filamentous bulking have opened a novel molecular biological perspective for effectively controlling filamentous bulking. The quenching technology based on QQ inhibitors has prominent advantages, including the use of trace amounts, high efficiency, environmental safety, and biomass harmlessness. However, further research is needed to address the following concerns: (1) The long-term stability of QQ needs to be investigated and monitored during practical applications; (2) the research on high-cost commercial chemicals must be leveraged to develop economically synthesized process for structural analogues; (3) the community stability and biotoxicity of QQ during long reaction times should be evaluated; (4) The broad structures of functional signal molecules corresponding to different analogues need to be characterized, and optimal combinations of multiple quenchers must be investigated. It is very important to identify multifunctional quenching analogues that offer broad-spectrum filamentous bulking control for effective sludge management. Continued research and development in this field have the potential to revolutionize sludge bulking control and enhance wastewater treatment processes.

6. Summary

This paper provides an overview of the transformation of bulking control approaches from traditional methods to precise and targeted prevention strategies based on QS theory. The QS behavior plays a crucial role in the formation of

filaments in fungi and filamentous bacteria, which is a key factor in the occurrence of sludge bulking. The QS-AHL system activates signal receptors that regulate the expression of genes involved in the morphological transformation and physiological metabolism of filamentous microorganisms. QQ-AHL systems have been developed to quench signal transmission in the bulking sludge by interfering with signaling molecule analogues. This innovative technology is a promising solution to sludge bulking problems, given its precise, effective, and environmentally friendly advantages in controlling the QS-AHL system and inducing a quorum quenching response.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
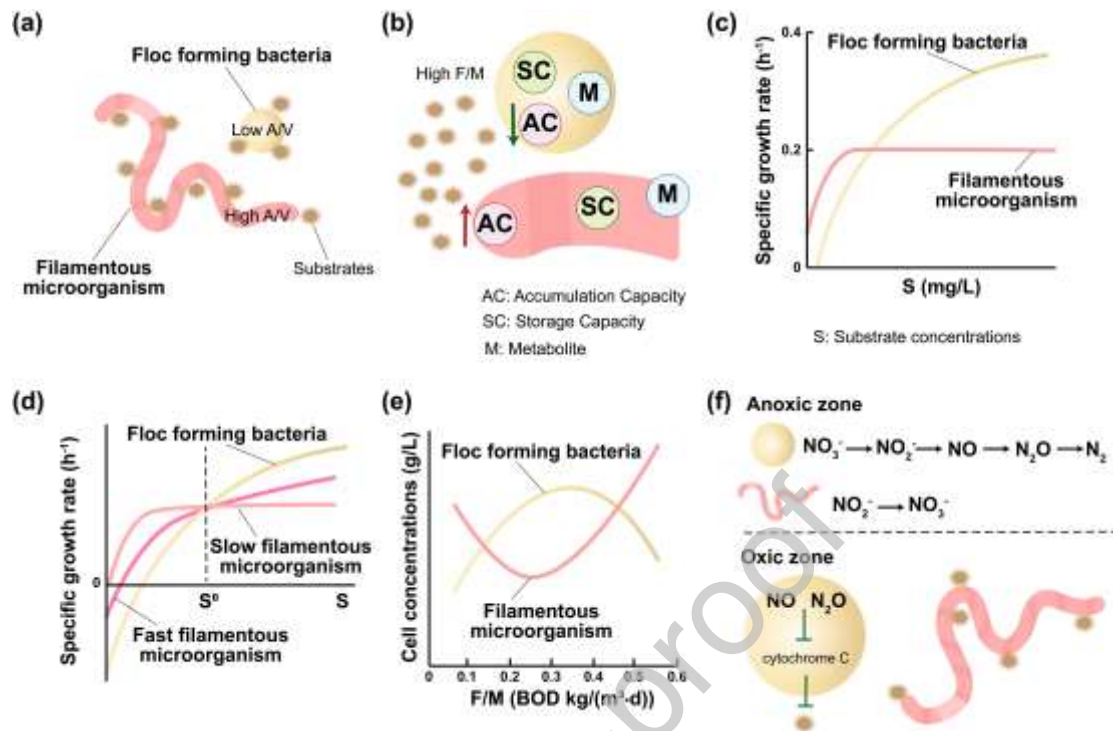
Fig. 1. Schematic illustration of classical bulking hypotheses. a) Aera/Volume (A/V) hypothesis; b) storage selection theory; c) kinetic selection theory; d) hunger hypothesis; e) unified theory; d) nitrogen oxide hypothesis.
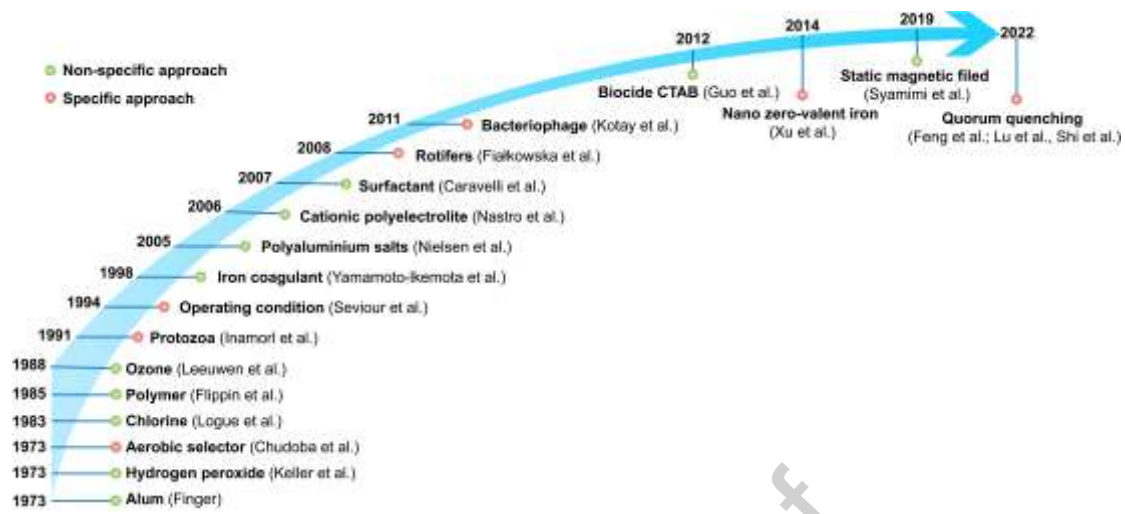
Fig. 2. Timeline of research and development in sludge bulking control.
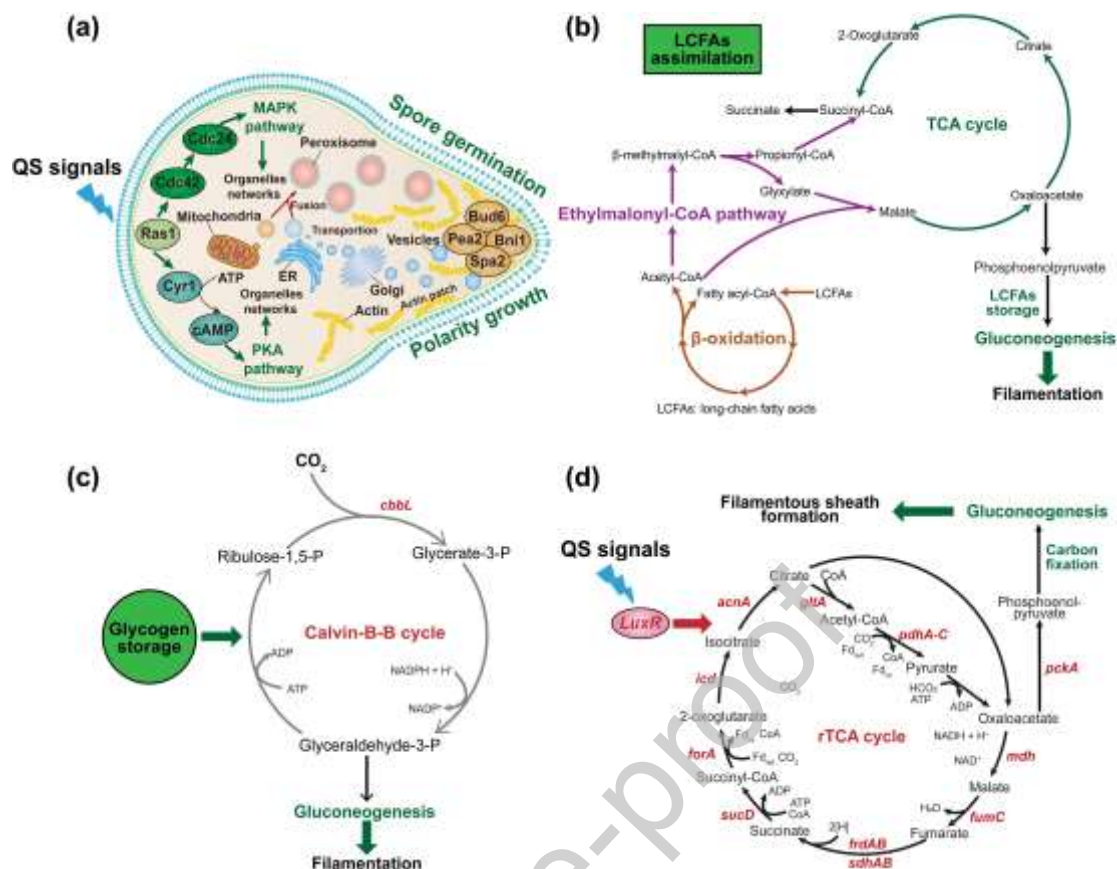
Fig. 3. The physiological metabolism model associated with filamentation based on the knowledge of molecular biology of filamentous microorganisms. (a) *Galactomyces* (Lu et al., 2022a). Copyright, 2022, Elsevier. (b) *Candidatus Microthrix parvicella*; (c) Eikelboom Type 0092; (d) *Sphaerotilus natans*.
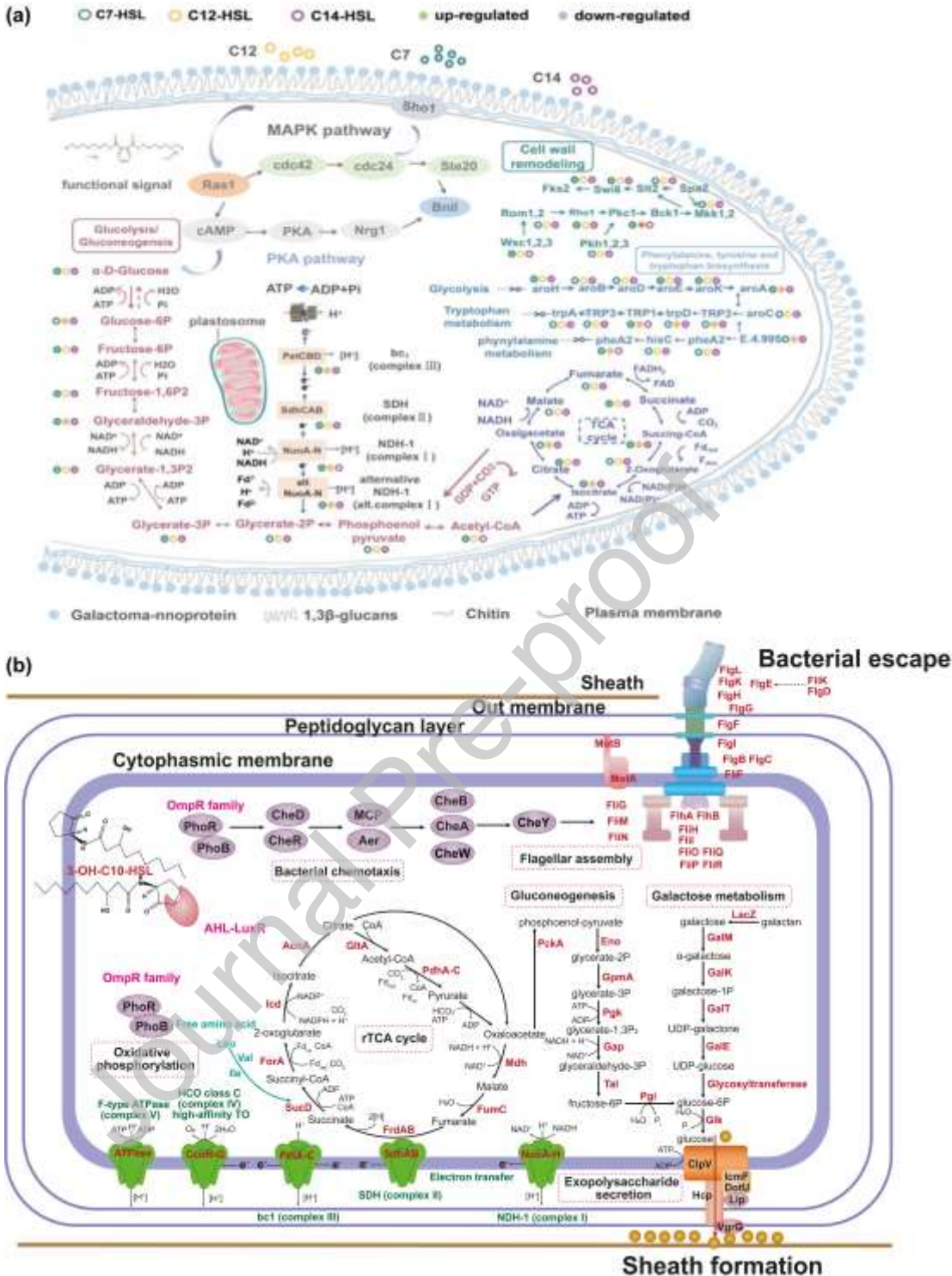
Fig. 4. QS mediated metabolic pathway in sludge bulking system. (a) *Penicillium* bulking; (b)

Sphaerotilus bulking. (Feng et al., 2022; Lu et al., 2023) Copyright, 2022 and 2023, Elsevier.
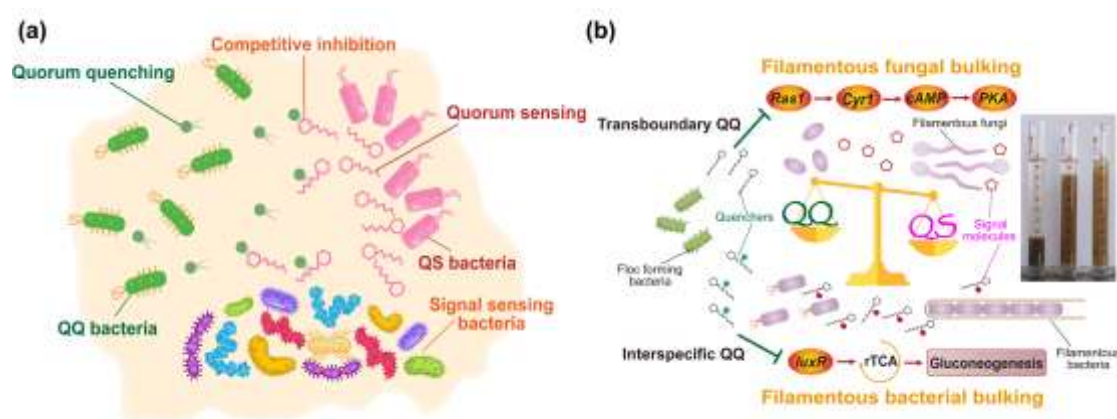
Fig. 5. (a) Schematic diagram of QS and QQ in activated sludge microecosystem; (b) concept of QS/QQ-based strategies for sludge bulking control (Feng et al., 2022; Lu et a., 2022a; Lu et al., 2023).
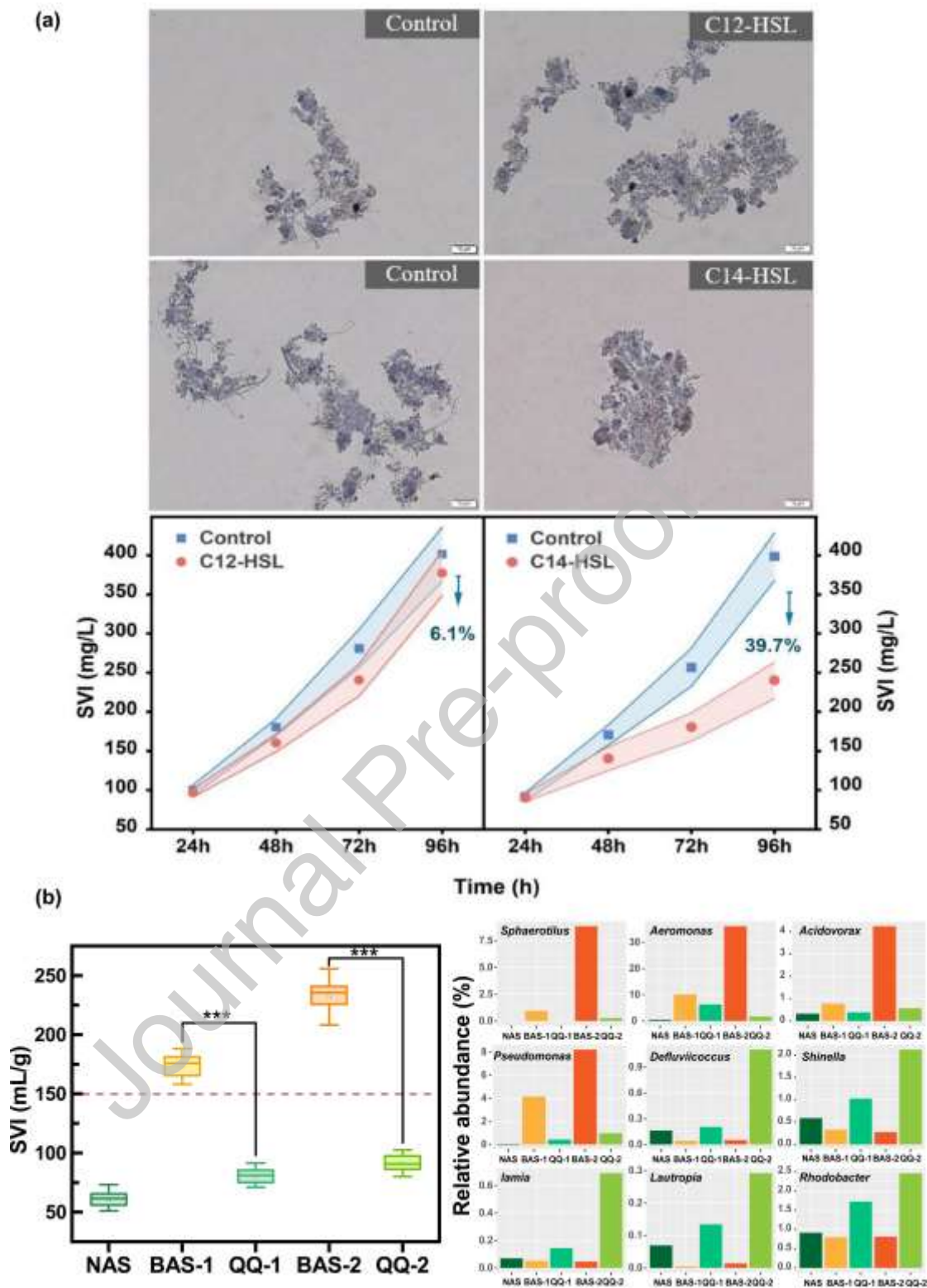
Fig. 6. The effect of QQ on targeted bulking control. (a) *Penicillium* bulking; (b) *Sphaerotilus*

bulking. (Feng et al., 2022; Lu et al., 2023) Copyright, 2022 and 2023, Elsevier.

Table 1 Advantages/disadvantages of some methods used to control filamentous bulking.

Bulking control methods		Advantages	Disadvantage	References
Non-Specific Methods	Chlorination	Cheap and simple	Non-selective nature; Undesirable by-products	Logue et al., 1983
	Hydrogen Peroxide	No toxic residual by-products	Expensive	Keller et al., 1973
	Ozone	No side effect on effluent quality	Not universal	Leeuwen., 1988
	Metal Application (Aluminium and Iron)	Improving floc density	Short-term solutions	Finger, 1973
	Synthetic Polymer	Adding flexibility	Adverse for floc-formers	Nastro et al., 2006
	Magnetic Field Application	Promoting cells' aggregation	Failure to apply at scale	Syamimi et al., 2019
	Selector Control	No chemicals	Complicated operations	Chudoba et al., 1973
Specific Methods	Condition Control	Conducive for floc-formers	Sludge washout at high DO; Nitrification eliminates at low SRT	Syamimi et al., 2022
	Nano zero-valent iron	Selectively killing filamentous organisms	Nitrification inhibition	Xu et al., 2014
	Quorum Sensing	Selective, precise, and effective	Long reaction time	Shi et al., 2022
	Rotifer Control	Universal filament consumers	Short-lived	Fiałkowska et al., 2008
	Bacteriophage Control	Targeting filamentous species	Short-lived	Kotay et al., 2011

Table 2 The dominant filamentous microbes causing sludge bulking under different conditions.

Environmental conditions / Type of filaments	Low DO	Low T	Low F/M	N and P absence	High sulfur sulfide	Low pH	References
Microthrix parvicella	√	√	√				Fan et al., 2020
Thiothrix eikelboomii	√			√	√		Wu et al., 2019
Sphaerotilus natans	√			√	√		Lu et al., 2023
Haliscomenobacter hydrossis	√		√				Kotay et al., 2011
Beggiatoa	√				√		Guo et al., 2012a
Type 021N	√			√	√		Guo et al., 2012b
Type 0092	√		√				Guo et al., 2014
Leucothrix mucor	√						Wang et al., 2016
Type 1851	√						Wang et al., 2013
Type 0041			√	√			Guo et al., 2013
Nostocoida limicola			√	√			Guo et al., 2014
Thiothrix nivea	√		√	√	√		Guo et al., 2014
Trichosporon asahii						√	Zheng et al., 2011
Galactomyces geotrichum	√					√	Matos et al., 2012
Geotrichum candidum						√	Meyers, 1984
Penicillium						√	Feng et al., 2022

Table 3 The effect of bacterial signaling molecules on the morphology of filamentous cells.

Bacterial signaling molecules	Species	Morphological transformation	References
C6-HSL	*Thiothrix*	Promoting filamentation	Shi et al., 2022a, 2022b
3-OXO-C6-HSL	*Thiothrix*	Inhibiting filamentation	Dong et al., 2022
C7-HSL	*Penicillium*	Promoting filamentation	Feng et al., 2022
C10-HSL	*Galactomyces*	Promoting filamentation	Lu et al., 2022b
3-OXO-C10-HSL	*Sphaerotilus*	Inhibiting filamentation	Lu et al., 2023
3-OH-C10-HSL	*Sphaerotilus*	Promoting filamentation	Lu et al., 2023
C12-HSL	*Galactomyces; Penicillium*	Inhibiting filamentation	Feng et al., 2022
3-OXO-C12 HSL	*Candida albicans*	Inhibiting filamentation	Barriuso et al., 2018
C14-HSL	*Penicillium; Caldilineaceae*	Inhibiting filamentation	Feng et al., 2022
AI-2	*Candida albicans*	Inhibiting filamentation	Bachtiar et al., 2014
Diffusible signal factor (DSF)	*Candida albicans*	Inhibiting filamentation	Boon et al., 2008
Indole	*Candida albicans*	Inhibiting filamentation	Raut et al., 2012

Graphical Abstract