Accepted Manuscript

Rapid start-up of denitrifying granular sludge by dosing with semi-starvation fluctuation C/N ratio strategy

Wenyu Niu, Jianbo Guo, Jing Lian, Yuanyuan Song, Caicai Lu, Haibo Li, Yi Han, Pengna Yin

PII: S0960-8524(17)30881-7
DOI: http://dx.doi.org/10.1016/j.biortech.2017.05.206
Reference: BITE 18232

To appear in: Bioresource Technology

Received Date: 9 April 2017
Revised Date: 27 May 2017
Accepted Date: 30 May 2017

Please cite this article as: Niu, W., Guo, J., Lian, J., Song, Y., Lu, C., Li, H., Han, Y., Yin, P., Rapid start-up of denitrifying granular sludge by dosing with semi-starvation fluctuation C/N ratio strategy, Bioresource Technology (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.05.206

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Rapid start-up of denitrifying granular sludge by dosing with semi-starvation fluctuation C/N ratio strategy

Wenyu Niu\textsuperscript{a}, Jianbo Guo\textsuperscript{a,b,*}, Jing Lian\textsuperscript{a}, Yuanyuan Song\textsuperscript{b}, Caicai Lu\textsuperscript{b}, Haibo Li\textsuperscript{b}, Yi Han\textsuperscript{b}, Pengna Yin\textsuperscript{b}.

\textsuperscript{a}. School of Environmental Science and Engineering & Pollution Prevention Biotechnology Laboratory of Hebei Province, Hebei University of Science and Technology, Yuhua East Road 70#, Shijiazhuang 050018, PR China

\textsuperscript{b}. Tianjin Key Laboratory of Aquatic Science and Technology, School of Environmental and Municipal Engineering, Tianjin Chengjian University, Jinjing Road 26#, Tianjin 300384, PR China

Abstract

This study cultivated denitrifying granular sludge in three UASB reactors by the semi-starvation fluctuation C/N ratio strategy (reactor 1 (R1): constant C/N ratio; R2: regular fluctuation C/N ratio; and R3: semi-starvation fluctuation C/N ratio (SSF)). Microbial aggregates appeared in R1, R2 and R3 on days 28, 14 and 6, respectively. Compared with the results in R1 and R2, the guanosine tetraphosphate (ppGpp) concentration was highest, the acyl homoserine lactones (AHLs) concentration quickly reached a certain threshold, and more protein (PN) of extracellular polymeric substances (EPS) secretion resulted in the rapid formation of denitrifying granular sludge in R3. The SSF strategy enhances microbial diversity, and denitrifying granular sludge has a better nitrogen removal performance. The result demonstrates that ppGpp,
AHLs, EPS and the denitrifying sludge granulation process are associated. A mechanism for denitrifying sludge granulation with SSF strategy was proposed from the aspect of quorum sensing (QS).

**Keywords**: semi-starvation fluctuation C/N ratio; ppGpp; AHLs; denitrifying sludge granulation; performance.

1. **Introduction**

Nitrate pollution is widely distributed in surface and ground water, which can cause eutrophication, carcinoma, malformation and mutation (Liu et al., 2012). Compared with a variety of physicochemical methods to remove nitrogen from water, biological denitrification processes have thus far shown broad application prospects in nitrate removal due to their low cost and environmentally friendly products (Xing et al., 2016). The overall development of biological wastewater treatment is directed towards high efficiency, energy conservation and equipment miniaturization.

Moreover, granular sludge is one of the important means of achieving these goals (Xue et al., 2016). Therefore, the denitrification process has been widely studied to optimize the factors that could accelerate the granulation process. Zhao et al. (2013) showed that the C/N ratio was the primary parameter significantly affecting the denitrification rate in many bioreactors. Wu et al. (2012) showed that different C/N ratios had different effects on the formation of granular sludge, when the influent C/N mass ratio was lower than 2 or greater than 8, the aerobic granules were unsuccessfully cultivated in the sequencing batch reactors. Theoretically, without
considering microbial growth (the carbon source content is just enough to degrade the nitrate, and the microbes are in a starvation state), 1 mg NO$_3^-$-N hypothetically demands 2.86 mg biodegradable chemical oxygen demand (COD) to accomplish the denitrification process (Zhang et al., 2016). Previous research results noted that if considering microbial growth (the carbon source content can be used to degrade nitrate, as well as can be utilized by microbes), the calculated optimal COD/NO$_3^-$-N (C/N) ratio is 3.7 (Chiu & Chung, 2003). Thus, the C/N ratio for the microbes in a semi-starvation state is 3.28:1. However, an intermittent feeding strategy had a critical role in granule formation (Yang et al., 2014). Still, few studies have investigated the effect of denitrification performance and granulation of denitrifying sludge under a semi-starvation fluctuation C/N ratio condition.

Besides, Sun et al. (2016) provided an intermittent feeding strategy that can seriously affect the quorum sensing (QS) system and comprehensive evidence that the QS system is closely associated with the granulation process. Another previous study showed that QS from activated sludge flocs could regulate the enzyme activity in specific cells through the response to changes in the concentration of acyl homoserine lactones (AHLs) such as lipase and cellulose (Chong et al., 2012). Additionally, recent studies indicated that guanosine tetraphosphate (ppGpp) is not only involved in the inhibition of RNA synthesis by cells during stringent stress (Traxler et al., 2008) but also in the synthesis of the flagellum and the effects of bacteria self-immobilization (Magnusson et al., 2007), which are closely relevant to denitrifying granulation.
Overall, these results suggest that QS plays a major role in the ecological environment of denitrifying granular sludge. While, we are well-informed on the signaling molecules behavior in denitrifying granular sludge, research on the relations between the mechanism of denitrifying granulation and signaling molecules are far from sufficient.

Accordingly, the objectives of this study were primarily (1) to investigate the effect of semi-starvation fluctuation C/N ratio strategy on the performance of the UASB in biological nitrate reduction using methanol as the carbon source; (2) to determine whether ppGpp, AHLs, EPS and the denitrifying granulation process are associated and explore the mechanism of denitrifying granular sludge; and (3) to identify and phylogenetically analyse the primary functional denitrifiers in the UASB operating under semi-starvation fluctuation C/N ratio conditions.

2. Materials and methods

2.1. Reactor setup and operation

Three identical Plexiglas UASB reactors were set up to cultivate the denitrifying granular sludge by different feeding methods (R1, constant C/N ratio; R2, regular fluctuation C/N ratio; and R3, semi-starvation fluctuation C/N ratio). The reactor was 60 cm in height with an internal diameter of 6.8 cm, and it was equipped with a three-phase separator with a working volume of 3.5 L. The influent was synthetic wastewater, which contained the following compounds (mg L$^{-1}$): chemical oxygen demand (COD) by methanol and NO$_3^-$-N by sodium nitrate, and 0.1mL microelement
solution. The microelement solution contained the following compounds (g L\(^{-1}\)):

- 0.43 ZnSO\(_4\)•7H\(_2\)O,
- 0.014 H\(_3\)BO\(_4\),
- 0.99 MnCl\(_2\)•4H\(_2\)O,
- 0.25 CuSO\(_4\)•5H\(_2\)O,
- 0.19 NiCl\(_2\)•6H\(_2\)O,
- 0.24 CoCl\(_2\)•6H\(_2\)O.

Flocculent seed sludge was collected from the return sludge-thickening tank at the XianYang Road Wastewater Treatment Plant, TianJin, China. The mixed liquor suspended solids (MLSS) and the mixed liquor volatile suspended solids (MLVSS) were 40.21±2.01 g L\(^{-1}\) and 25.64±1.28 g L\(^{-1}\), respectively.

The experimental process lasted 45 days, which were divided into two periods (i.e., the acclimatization (1-30 days) and examination periods (31-45 days)) according to the different feeding methods (Fig. 1). The hydraulic retention time (HRT) remained at 3.5 h throughout the whole operation. The reactors were operated at room temperature (16-25\(^\circ\)C).

2.2. Signal molecules analytical method

All samples were chromatographed by an Agilent Acquity UPLC (Agilent, USA) at a 0.3 mL min\(^{-1}\) flow rate. The column dimensions were 4.6×50 mm, and it was filled with BEH C18 packing material with 1.8 \(\mu\)m particle size. The column was thermostated at 35\(^\circ\)C; the sample system was at 4\(^\circ\)C. The mobile phase consisted of a linear gradient (70 – 90\%) of solvent B (methanol) and solvent A (water with 0.1% formic acid). The effluent was ionized by electrospray ionization in positive mode and was analysed using the multiple reactions monitoring approach by Agilent 6430B MS (Agilent, USA). The matrix-matched multiple reaction monitoring experiments were conducted for two standard AHLs.
In this study, HPLC (Agilent1100, Agilent Co. Ltd., USA) was performed with a C18 column and a diode array detector (DAD). The initial conditions of HPLC analysis for ppGpp were as follows: mixed solvent (95% solvent A as 20 mM Tris with pH 8.0 and 5% solvent B as 20 mM Tris + 1.5 M sodium formate with pH 8.0) at 1 mL min\(^{-1}\), detection at 260 nm, and a column temperature of 40°C. The level of sodium formate buffer was ramped up to 60%. The standards of AHLs and ppGpp were purchased from Biolog (America) and TriLink BioTechnologies (America), respectively.

2.3. Other analysis methods

The COD, NO\(_3^–\)-N, MLSS and MLVSS were measured using standard methods (APHA, 1998). The average particle size of denitrifying granular sludge was measured by sieve method. Heat EPS extraction procedures were used, and the extracellular polysaccharides (PS) and extracellular proteins (PN) were assayed by the anthrone-sulfuric acid method (Gaudy & Gaudy, 1962) and Coomassie brilliant blue assay (Fr/Olund et al., 1995), respectively.

3. Results and discussion

3.1. Effect of semi-starvation fluctuation C/N ratio on the characteristics of denitrifying granular sludge

3.1.1. Denitrifying sludge granulation

Initially, seed sludge in R1, R2 and R3 was black in color and flocculent in morphology. Nevertheless, sludge in the three reactors showed diverse appearances
after cultivation for 30 days when the acclimatization period finished. In the
acclimatization period, the sludge in R1 (constant C/N ratio) formed tiny aggregates
after operation for 28 days at the C/N ratio of 9:1. Comparably, the sludge in R2
(regular fluctuation C/N ratio) and R3 (semi-starvation fluctuation C/N ratio) formed
numerous tiny aggregates developed on day 14 and day 6, respectively. On day 20, the
sludge in R3 had achieved complete granulation, and on day 30, the sludge in R2 had
achieved complete granulation. The MLSS and MLVSS of R1, R2 and R3 were
19.86±0.9 g L\(^{-1}\), 12.19±0.6 g L\(^{-1}\) and 15.11±0.7 g L\(^{-1}\), 6.46±0.3 g
L\(^{-1}\) and 10.01±0.5 g L\(^{-1}\), respectively (Table. 1). The VSS/SS varied from 0.638 to
0.761, 0.531 and 0.663 for R1, R2 and R3, respectively. The average particle size
varied from 0.1±0.03mm to 0.57±0.09mm, 1.14±0.17mm and 1.42±0.22mm for R1,
R2 and R3, respectively. From days 31 to 45 (i.e. the examination period). During this
period, the sludge bed volume continued to increase. However, the diameters of the
mature granules in the R2 and R3 varied slightly. In the sludge in R1, the tiny
aggregates were developing at the C/N ratio was maintained at 5:1. However, the
sludge in R1 was still unable to achieve complete granulation. The MLSS and
MLVSS of the R1, R2 and R3 were 25.18±1.2 g L\(^{-1}\), 20.09±0.9 g L\(^{-1}\), 19.95±0.9 g L\(^{-1}\)
and 13.08±0.6 g L\(^{-1}\), 9.27±0.4 g L\(^{-1}\), 9.99±0.5 g L\(^{-1}\), respectively. The VSS/SS varied
as well, with values of 0.519, 0.461 and 0.501 for R1, R2 and R3, respectively. The
average particle size varied to 0.88±0.14mm, 1.49±0.23mm and 1.84±0.28mm for R1,
R2 and R3, respectively.
These results indicate that dosing with semi-starvation fluctuation C/N ratio strategy could promoted denitrifying granular sludge formation and this change is irreversible. This strategy was also able to inhibit the growth of microorganisms.

3.1.2. EPS characterization

Table. 1 shows the correlation between the different feeding methods and the variation of PN, PS, humic substances in R1, R2 and R3 during the whole operation. Although the total amount of EPS in R1 was higher than in R2 and R3, the content of PN in R2 and R3 exceeded that of R1, the PS contents in R2 and R3 were below that in R1. In R1, the PN content decreased from 3.38±0.17 to 2.5±0.12 mg g\(^{-1}\) MLVSS during the acclimatization period, and increased to 5.92±0.29 mg g\(^{-1}\) MLVSS at the end. In contrast, the PN concentration of R2 and R3 increased from 3.38±0.17 to 4.28±0.21 and 4.75±0.23 mg g\(^{-1}\) MLVSS, respectively, during the acclimatization period and finally increased to 8.79±0.44 and 10.19±0.5 mg g\(^{-1}\) MLVSS, respectively. Notably, the PN of EPS contents highest in R3 on day 6, the most likely explanation for this is that the semi-starvation fluctuation C/N ratio condition stimulated more PN secretion, but the microorganisms that could use there PNs as a carbon source was scarce. These results indicated that the semi-starvation fluctuation C/N ratio strategy was beneficial to PN production. Moreover, this change was irreversible.

Overall, the most likely explanation for the above phenomena is that there is extreme disturbance in the external environment during the process. The disturbance
provides more opportunities for microbial contact and stimulated more protein of EPS secretions due to the semi-starvation fluctuation C/N ratio strategy.

3.2. Effect of semi-starvation fluctuation C/N ratio on UASB reactor performances

The influent and effluent COD concentrations in R1, R2 and R3 are shown in Fig. 1. During the acclimatization period, after 9 days of acclimatization, the COD removal rates in R1 remained over 90% between days 10 and 30. The COD removal rates in R2 and R3 fluctuated between 70% and 95% from days 1 to 23, and between 65% and 100% from days 1 to 28, respectively, and remained over 90% for the remaining duration. In testing the denitrifying granular sludge performance phase, the COD removal rates of both R2 and R3 remained over 95% throughout the entire phase. Comparatively, when the COD concentration increased to 1000 mg L$^{-1}$ in R1, the removal rates first declined to 85% and continued to decline to 77% when the COD concentration increased to 1500 mg L$^{-1}$.

As shown in the acclimatization period (Fig. 1A), the R1 nitrogen removal rates fluctuated between 58% and 96% from days 1 to 8 at the C/N ratio of 9:1. After the 8 days, the microbial bioactivity gradually increased, and the nitrogen removal rates remained above 87% from days 9 to 30. The R2 wave curve is similar that of R1 (Fig. 1B). Comparatively, the R3 nitrogen removal rates fluctuated between 15% and 100% from days 1 to 24 in semi-starvation fluctuation C/N ratio conditions and remained above 93% throughout the rest of the days.
Notably, the nitrogen removal rates were quite different among R1, R2 and R3 in the examination period. The R1 nitrogen removal rates dramatically declined to 74% and 60% when the nitrate concentration increased to 200 and 300 mg L\(^{-1}\), respectively, between days 37 to 43. However, the R2 and R3 nitrogen removal rates remained over 97% throughout this period.

Overall, the denitrifying granular sludge of the UASB reactors dosing with the regular fluctuation C/N ratio and semi-starvation fluctuation C/N ratio strategy had excellent performances compared to the conventional strategy.

### 3.3. Effect of semi-starvation fluctuation C/N ratio on the AHLs and ppGpp produced by microorganisms

Table 2 shows the two types of AHLs (3-oxo-C8-HSL and C14-HSL) concentrations in samples collected from R1, R2 and R3. In the acclimatization period in R1, both AHLs concentrations increased, from 0.11±0.01 (3-oxo-C8-HSL) and 0 (C14-HSL) µg g\(^{-1}\) MLVSS to 0.35±0.01 and 0.24±0.01 µg g\(^{-1}\) MLVSS, respectively, after 6 days of operation. Afterwards, the AHLs were relatively stable from days 12 to 18. Compared to the initial concentration, the 3-oxo-C8-HSL concentration presented a rising trend, eventually increasing more than four-fold. The C14-HSL concentration increased to 0.34±0.01 µg g\(^{-1}\) MLVSS in the last days of this period. Compared to R1, both AHLs of R2 steadily increased, and both AHLs of R3 first increased and then decreased during this period. The 3-oxo-C8-HSL concentration of R2 increased from 0.11±0.01 to 0.44±0.02 µg g\(^{-1}\) MLVSS after 18 days of operation. Afterwards, the
AHLs increased to 0.84±0.04 µg g\(^{-1}\) MLVSS from days 18 to 30, and the C14-HSL concentration increased to 0.42±0.02 µg g\(^{-1}\) MLVSS just after 18 days of operation. The C14-HSL concentration increased to 0.58±0.02 µg g\(^{-1}\) MLVSS in the last days of this period. Indeed, the 3-oxo-C8-HSL concentration of R3 increased from 0.11±0.01 to 0.58±0.02 µg g\(^{-1}\) MLVSS just after 6 days of operation. The 3-oxo-C8-HSL concentration had decreased slightly on day 12, but afterwards, the AHLs were relatively stable from days 12 to 30. The C14-HSL concentration of R3 increased to 0.43±0.02 µg g\(^{-1}\) MLVSS just after 6 days of operation as well, but then the C14-HSL concentration of R3 decreased to 0.37±0.01 µg g\(^{-1}\) MLVSS on day 30. A detailed comparison of the AHLs concentrations (Table. 2) and granulation processes (Table. 1) shows that the increase in AHLs concentration in the three reactors was accompanied by the formation of denitrifying granules. This experimental observation indicates that the increase in AHLs concentration is a prerequisite for the formation of denitrifying granular sludge. Furthermore, while the granulation process was accomplished, the denitrifying granular sludge had a superior ability to adapt to the external environment, and then the microbial reduced AHLs secretion. During the examination period, the 3-oxo-C8-HSL concentrations in all three reactors showed a steady trend from low to high in the following order: R1< R3< R2, even though certain differences still existed. The C14-HSL concentration of R2 and R3 showed a steady trend, but no trend was found for C14-HSL in R1. The combined COD removal rates (Fig. 1), nitrogen removal rates (Fig. 1) and AHLs concentration (Table. 2) show that a higher AHLs
concentration in the reactor was beneficial to COD and nitrogen removed (Hu et al., 2016).

Table 2 shows the ppGpp concentrations in samples collected from R1, R2 and R3. In the acclimatization period in R1, the ppGpp concentration was lower than 2.37±0.11 µg g⁻¹ MLVSS because of sufficient carbon in this period. Comparatively, the ppGpp concentration of R2 fluctuated between 3.05±0.15 and 3.49±0.17 µg g⁻¹ MLVSS which was slightly higher than in R1 because of the low C/N ratio. The concentration of ppGpp in R3 was the highest of the three reactors at more than 6.44±0.32 µg g⁻¹ MLVSS. During the examination period, because of the excess carbon source and identical operating conditions during this period, the ppGpp concentration was low, and the values were same in the three reactors.

Combine with the denitrifying sludge granulation process, these results showed that there is a very close relationship between the ppGpp concentration and the formation of the denitrifying granular sludge. This relationship also explained why the semi-starvation fluctuation C/N ratio strategy accelerated the formation of the denitrifying granular sludge.

3.4. Mechanism of denitrifying sludge granulation dosing with semi-starvation fluctuation C/N ratio strategy

This study is the first comprehensive research on the relationship between ppGpp variation, AHLs variation, EPS characterization, the mechanism of granulation and performances of denitrifying granular sludge dosing with semi-starvation fluctuation
C/N ratio strategy. Given the results of ppGpp and AHLs concentration, it was easy to discover the consistency between these two indices. The ppGpp is a signal molecule used to synthesize flagellum, and the flagella are microbial motility organs. When the C/N ratio suddenly decrease and the carbon source is insufficient, the external environment stimulates the microbial secretion more ppGpp and AHLs. The ppGpp and AHLs, under the cooperative action of the signal molecules and flagellum, stimulate the search for food. The ppGpp and AHLs produced by sludge dosing with the semi-starvation fluctuation C/N ratio strategy were in higher proportion than with a constant C/N ratio. Likewise, the rate of granulation in R3 was faster than in R1 and R2. The ppGpp and AHLs results verified the previous conclusion that more ppGpp and AHLs are beneficial for the attachment of microbial individuals and promote the granulation of denitrifying sludge (Li et al., 2015).

The self-immobilization of bacteria into granules involves physical, chemical, and biological cell-to-cell interactions (Liu & Tay, 2004). Based on microscopic observations and the impacts of the operational parameters on the granulation processes in this work, a possible mechanism of denitrifying sludge granulation dosing with the semi-starvation fluctuation C/N ratio strategy in the perspective of QS could be elucidated as follows (Fig. 2) :1) the semi-starvation fluctuation C/N ratio condition provides an intensified selection pressure which promotes ppGpp and AHLs production; 2) the ppGpp and AHLs molecules switch on the key synthesis of microbial flagella and stimulates bacteria PN secretion, which changes cell motility
and biological cell-to-cell interactions to form small aggregates; 3) the bacteria continuously adhere onto the surface of the aggregates and become more compact; 4) microbial forces (e.g., more PN production and higher quantities of flagellum) further stimulate the integration and stabilization of granule formation to a mature state; and 5) matured granules are regulated and shaped by shear forces.

4. Conclusions

This study determined whether ppGpp, AHLs, EPS and the denitrifying sludge granulation process are associated. Compared to the constant C/N ratio, activated sludge cultivated by dosing with SSF strategy was observed to produce higher concentrations of ppGpp and AHLs, and a decrease in the total amount of EPS. The functional strains identified in three reactors revealed that the semi-starvation fluctuation C/N ratio condition was favorable to improving the performance of the reactor. Furthermore, these results showed a feasibility of cheap and fast ways to cultivate the denitrifying granular sludge for future implementation in treating actual wastewater.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 51678387), Key project of Tianjin Natural Science Foundation (17JCZDJC39300) and the Science Research Program of High Level Talents in Hebei Universities (Grant No. GCC2014045, China).
Fig. S1 shows the images of sludge morphology in R1. This images described the change of sludge morphology in R1 throughout the operation.

Fig. S2 shows the images of sludge morphology in R2. This images described the change of sludge morphology in R2 throughout the operation and compared the sludge morphology of three reactors at the end of acclimatization period.

Fig. S3 shows the images of sludge morphology in R3. This images described the change of sludge morphology in R3 throughout the operation and compared the sludge morphology of three reactors at the end of examination period.

Fig. S4 shows the relative abundances of the dominant genera in each sample. After 30 days’ operation, there are higher biodiversity in R2 and R3 than in R1. These results indicated that dosing with the regular fluctuation C/N ratio and semi-starvation fluctuation C/N ratio strategy were beneficial to microbial diversity compared to the conventional strategy.

References


Fig. 1. Performance of COD and NO$_3^-$-N removal rates in (a) R1, (b) R2 and (c) R3.
Fig. 2. Schematic of the mechanism of denitrifying sludge granulation dosing with semi-starvation fluctuation C/N ratio strategy controlled by quorum sensing (QS) system.
Table 1. Effect of semi-starvation fluctuation C/N ratio on the characteristics of denitrifying granular sludge

<table>
<thead>
<tr>
<th>Sludge characteristics</th>
<th>Reactor</th>
<th>Time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MLSS (g L(^{-1}))</td>
<td>R1</td>
<td>40.21±2.1</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>26.46±1.3</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>17.99±0.8</td>
</tr>
<tr>
<td>MLVSS (g L(^{-1}))</td>
<td>R1</td>
<td>25.64±1.2</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>16.9±0.8</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>11.78±0.5</td>
</tr>
<tr>
<td>VSS/SS</td>
<td>R1</td>
<td>0.638</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>0.638</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>0.638</td>
</tr>
<tr>
<td>EPS (mg g(^{-1})MLVSS)</td>
<td>R1</td>
<td>7.86±0.4</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>10.38±0.5</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>14.6±0.7</td>
</tr>
<tr>
<td>PN (mg g(^{-1})MLVSS)</td>
<td>R1</td>
<td>3.38±0.17</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>5.57±0.27</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>10.36±0.5</td>
</tr>
<tr>
<td>PS (mg g(^{-1})MLVSS)</td>
<td>R1</td>
<td>0.71±0.04</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>1.03±0.05</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>1.69±0.08</td>
</tr>
<tr>
<td>Average particle size</td>
<td>R1</td>
<td>0.1±0.03</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>0.1±0.03</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>0.1±0.03</td>
</tr>
</tbody>
</table>
Table. 2. The trend of AHLs and ppGpp produced by sludge samples of R1, R2 and R3.

<table>
<thead>
<tr>
<th>Signal molecule</th>
<th>Reactor</th>
<th>AHLs and ppGpp concentrations (µg g⁻¹ MLVSS)</th>
<th>Time(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3-oxo-C8-HSL</td>
<td>R1</td>
<td>0.11±0.01</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>0.11±0.01</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>0.11±0.01</td>
<td>0.58±0.02</td>
</tr>
<tr>
<td>C14-HSL</td>
<td>R1</td>
<td>0</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>0</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>0</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>ppGpp</td>
<td>R1</td>
<td>1.82±0.09</td>
<td>2.14±0.11</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>1.82±0.09</td>
<td>3.38±0.16</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>1.82±0.09</td>
<td>6.61±0.33</td>
</tr>
</tbody>
</table>
Graphical Abstracts

[Diagram showing the process of signal molecule formation and its effect on EPS production.]

- Signal molecule formation
- EPS production and its influence on microbial activity
- Graphs depicting changes in EPS over time under different conditions

Using EPS as a carbon source to enhance growth and metabolic activity.
Highlights

Semi-starvation fluctuation C/N ratio (SSF) could promote ppGpp and AHLs content.

The ppGpp, AHLs, EPS and denitrifying sludge granulation process are correlated.

The denitrifying granular sludge was rapid formation by dosing with SSF strategy.

A mechanism for denitrifying sludge granulation with SSF strategy was proposed.