Where are signal molecules likely to be located in anaerobic granular sludge?

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\textbf{A B S T R A C T}

Quorum sensing is a concentration-sensing mechanism that plays a vital role in sludge granulation. In this study, the regularities of distribution of different signal molecules, including intra- and interspecific signal molecules (diffusible signal factor, DSF), inter-specific signal molecules (autoinducer-2, AI-2) and intraspecific signal molecules (acyl-homoserine lactones, AHLs), from three types of anaerobic granular sludge were investigated. The results showed that 70–90\% of DSF was distributed in sludge, while AI-2 in the water phase accounted for over 80\% of the total content. Interestingly, there was a positive correlation between DSF and AI-2, which played opposite roles in granulation. Moreover, more than 55\% of short and medium acyl chain AHLs tended to spread in aqueous water, while the long acyl chain AHLs were closer to granular sludge than the short and medium acyl chain AHLs. With the exception of one type of sludge, the percentage of long acyl chain AHLs in the sludge phase was greater than 70\%. The different distributions of signal molecules were primarily determined based on their physicochemical properties, including molecular weight and solubility in water or organic solutions. In addition, the basic properties of sludge, such as the granular level or the production of EPS, were closely related to the diversity, distribution and concentration of signal molecules. As a medium in granulation, extracellular polymeric substances production was regulated by different signal molecules from different parts of anaerobic granular sludge. This study provides a foundation for investigation of quorum sensing in the system of anaerobic granular sludge.

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\section{Introduction}

Quorum sensing (QS), which is cell–cell communication among bacteria, has recently been intensively regulated. This process, which includes intra- and interspecies communication (Fuqua \textit{et al.}, 1994), is accomplished through the exchange of extracellular signalling molecules released by bacteria that are known as auto-inducers (AI). When the signalling molecules in their microenvironment reach a threshold, the signal is received and the molecules begin regulating particular gene expression. This enables bacterial populations to enhance the
effectiveness of community cooperation (Taga and Bassler, 2003). These processes include virulence factor expression, antibiotic (Chong et al., 2012) and extracellular enzyme production (Lazar, 2011), biofilm formation (Chen et al., 2002), degradation of organic pollutants (Jiang et al., 2006; Valle et al., 2004; Yong and Zhong, 2010) and plasmid transfer (Deng et al., 2011).

QS among bacterial cells has been a microbiological research focus in environmental sciences. Previous studies demonstrated that adding boron into sequencing batch reactor (SBR) would accelerate the growth of aerobic granular sludge. This occurs because boron is an important component of AI-2, which has a close relationship with the formation of aerobic granular sludge (Zhang et al., 2011). It has been suggested that AI-2 plays an important role in maintaining the stable structure and complete form of aerobic granular sludge, but that it may not be required at the beginning of aerobic granulation (Xiong and Liu, 2010). It has also been suggested that the production and expression of QS signal chemicals from granules and granule precursors induces the gene expression of bacteria in suspensions to enable attached growth rather than suspended growth (Ren et al., 2010).

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 cellulase (Li et al., 2004). It has been suggested that AHLs or functional molecules were present in activated sludge flocs, but not in the bulk aqueous phase. Moreover, Xiong and Liu (2012) showed that using samples of aerobic granulation homogenized by ultrasonication to determine AI-2 provided better results than ordinary supernatant. Therefore, we wondered which position the signal molecules preferred in anaerobic granular sludge. According to the mechanism of QS, the concentration of signal molecules sensed by bacteria determined whether QS would occur or not; therefore, it is critical to determine if the concentration of molecules present and those sensed by bacteria corresponded to each other. However, no conclusions about the distribution of signal molecules in the sludge system have been reached to date. Moreover, sludge systems, especially those composed of granular sludge, have characteristics that differ from other sludge. Accordingly, it is unclear which portion of the sludge system (Water phase, Washing water phase and Sludge phase) should be used to measure signal molecules, as different types of signal molecules have their own properties, such as solubility, diffusivity, stability and other properties (Decho et al., 2011), and whether the signal molecules played a role or not is related to the concentration of the specific portions in sludge. Therefore, this study is conducted to investigate the concentration distribution of signal molecules in the sludge system.

The formation of such granular sludge is often regarded as a special case of biofilm development. Additionally, these studies have suggested that signal molecules play an important role in the formation and maintenance of aerobic granular sludge (Ren et al., 2010; Valle et al., 2004; Xiong and Liu, 2010; Zhang et al., 2011), but there have been no such studies conducted to investigate anaerobic granular sludge. Therefore, this study was conducted to confirm the types, concentrations and distribution of signal molecules in anaerobic granular sludge, and to provide a reference for further research about the mechanism of QS in anaerobic granular sludge. For example, confirming the main conditions of different types of signal molecules in sludge system, or applying QS to regulate the rapid granulation of anaerobic sludge and guiding the addition of signal molecules in practical application.

2. Materials and methods

2.1. Seeding sludge

The anaerobic granular sludge used in this experiment was obtained from three typical industrial wastewater treatment plants, a pharmaceutical factory, brewery and paper-making factory (S1, S2 and S3, respectively). The sludge was cultivated in an expanded granular sludge bed for 3–5 days using cane sugar as a carbon source. The synthetic wastewater with a COD = 2500 ± 200 mg/L was replaced every 24 h and the temperature was maintained at 30 ± 2 °C in a greenhouse. The physicochemical properties of the sludge of the three types of sludge are shown in Table 1. The average granular diameters of sludge S1, S2 and S3 were 1.76, 0.91 and 1.64 mm, respectively. Overall, the characteristics suggested that there were differences among sludge and that the indicators including the biomass and biological activities, granular diameter distribution and the composition of extracellular polymeric substances (EPS) of the sludge were correlated with each other.

2.2. Extraction of signal molecules

The signal molecules of 100 mL of the mixture of sludge and water were divided into Water phase, Washing water phase and Sludge phase, and an aqueous phase which was produced from Water phase and the Washing water phase. The mixture of sludge and water was centrifuged at 22 091 × g for 5 min. For the Water phase, the supernatants were harvested and the volume was recorded, after which the pellet was resuspended to the initial volume in physiological saline (0.9% NaCl) and the supernatants were collected through centrifuging. These operations were repeated with the pellet three times, and this was noted as the Washing water phase. Subsequently, the remaining sludge was crushed in physiological saline, and this was recorded as the Sludge phase. At the same time, 1 mL of the above liquid from each process was collected, respectively, filtered through a 0.22 μm syringe filter and stored at −20 °C for the AI-2 assay. Next, 50 mL of sample were extracted with an equivalent volume of ethyl acetate, after which the mixture was shaken vigorously and the
phases were allowed to separate (Ravn et al., 2001). The entire extraction process was repeated three times, after which the combined ethyl acetate fractions were evaporated to dryness and re-dissolved in 2 mL of 50% acetonitrile for subsequent analysis (Wang et al., 2012). The above processes to extract the sample that was added to the standard of signal molecules showed that the efficiency of the extractions was about 80%.

2.3. Physicochemical properties of signal molecules

A list of signal molecules commonly mentioned in the wastewater system is provided in Table 2, which shows that the physicochemical properties of signal molecules such as molecular structure, molecular weight, solubility (log S) and lipo-hydro partition coefficient (log P) differ greatly among molecules.

2.4. Analytical method

2.4.1. High performance liquid chromatography (HPLC) determination of signal molecules

Diffusible signal factor (DSF) and part of the AHLs were analysed using a Waters HPLC (Yang et al., 2006) system with an X Bridge C-18 column (5 μm d, 4.6 × 250 mm). The samples were then passed through a 0.22-μm filter before HPLC analysis. The HPLC operation was as follows: AHLs were eluted with 40% acetonitrile in water applied at a flow rate of 0.7 mL min⁻¹ with an injection volume of 10 μL for separation (Wang et al., 2012). Detection was conducted at 210 nm wavelength. Synthetic AHLs (purchased from Sigma–Aldrich, Germany) were used as standards. The retention times of AHLs were 4.2, 6.3 min for C4-HSL, 3-oxo-C8-HSL, respectively. The HPLC operation to analyse DSF was the same with AHLs, except for the mobile phase (Deng et al., 2010; Wang et al., 2003), which was consisted of acetonitrile (80%) and water (20%).

2.4.2. Bioassay of AHLs

AHLs were estimated based on the reporter strains of QS. The violacein production by strain Chromobacterium violaceum CV026 was used to indicate the presence of short and medium acyl chain AHLs, while the inducement of β-galactosidase (β-Gal) by strain Agrobacterium tumefaciens NTL4 was used for long acyl chain AHLs (Wang et al., 2012). Both bioindicator methods consisted of semi-quantitative analysis. The violacein unit was calculated as \([A_{585}/A_{600}] \times 1000\), and the Miller unit was calculated as \([A_{420} - 1.75 \times A_{550}/A_{600} \times 0.1 \text{ mL } \times \text{ reaction time})] \times 1000\) (Blosser and Gray, 2000; Miller, 1972; Ponnusamy et al., 2009; Wang et al., 2012).

2.4.3. AI-2 determination

The AI-2 activity was measured by an indicator strain, Vibrio harveyi BB170, which produced light in response to AI-2 (Taga and Xavier, 2011). The reporter strain was grown in AB medium overnight at 30 °C. When the OD₆₀₀ of the culture was about 0.7–1.2, it was diluted into fresh medium as 1:5000. Next, 20 μL of the sample was added into wells containing 180 μL of this diluted BB170 culture. Wells without a sample were used as a negative control. The bioluminescence was measured using a multifunctional microplate reader (SpectraMax M5, USA) and the relative light units calculated as the intensity of the sample at 490 nm divided by the intensity of the control.

2.4.4. Other methods

COD was measured using a DR2800 spectrophotometer (Hach Company, Loveland, CO, USA). Heat EPS extraction procedures (Yang and Li, 2009) were used, after which the extracellular polysaccharides and extracellular protein were assayed by the phenol/sulphuric acid method (Dubois et al., 1956) and Coomassie brilliant blue assay (Bradford, 1976), respectively.

2.5. Calculations

The signal molecules of the anaerobic granular sludge system originate from one portion dissolved in water and another portion distributed in sludge. The following equation can be used to calculate the value determined by HPLC:
\[ Q_1 = (X \times C_1 + 50 \times C_2 + Y \times C_3)/0.1 \]  
(1)

where \( Q_1 \) (\( \mu g/L \)) is the total amount of one type of signal molecule in 1 L of the mixture of sludge and water, \( X \) (mL) is the volume of water after centrifuging the 100 mL mixture, “50” is the volume of Washing water phase and \( Y \) (g) is the dry weight of sludge in the 100 mL mixture. \( C_1 \) (\( \mu g/mL \)), \( C_2 \) (\( \mu g/mL \)) and \( C_3 \) (\( \mu g/g \) SS) are the concentrations of signal molecules from the water phase, washing phase and sludge phase, respectively.

The values of DSF in different phase measured by HPLC were calculated as follows:

\[ Q(\text{Water}) = Q_0 \times 2 \times V \times 10/50 \]  
(2)

\[ Q(\text{Washing}) = Q_0 \times 2 \times 10 \]  
(3)

\[ Q(\text{Sludge}) = Q_0 \times 2 \times 10 \]  
(4)

\[ Q(\text{Mixture}) = Q_0 \times 2 \times 20 \]  
(5)

where \( Q \) (Water or Washing or Sludge or Mixture) (\( \mu g/L \)) is the content in 1 L, \( Q_0 \) (\( \mu g/mL \)) is the value determined by HPLC and it was the average value from 5 parallel samples. “2” (mL) is the volume after extraction and “10” is the expanding factor of calculating system from 100 mL to 1 L. In Eq. (2), \( V \) (mL) is the volume of water after centrifuging the 100 mL mixture and “50” (mL) is the volume for extraction in Water phase. “20” (mL) in Eq. (5) is the expanding factor of calculating system from 50 mL to 1 L.

The specific calculating process of violacein or \( \beta \)-Gal as follows:

\[ Q(\text{Water}) = Q_0 \times V/50 \]  
(6)

\[ Q(\text{Washing}) = Q_0 \]  
(7)

\[ Q(\text{Sludge}) = Q_0 \]  
(8)

The results of \( \log S \) and \( \log P \) were predicted by ChemBioDraw Ultra 13.0. Clog \( P \) was the value of \( \log P \) calculated by Clog \( P \) Calculations (Kenny et al., 2013).

References for molecular structures were summarized from the following references: Deng et al., 2011; Galloway et al., 2011; Pereira et al., 2013; Decho et al., 2011.

### Table 2 - List of physicochemical properties of signal molecules.

<table>
<thead>
<tr>
<th>Signal type</th>
<th>Molecular structure</th>
<th>Molecular weight</th>
<th>( \log S )</th>
<th>( \log P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSF</td>
<td><img src="DSF.png" alt="Molecular structure" /></td>
<td>212.3</td>
<td>-3.451</td>
<td>4.41</td>
</tr>
<tr>
<td>AI-2</td>
<td><img src="AI-2.png" alt="Molecular structure" /></td>
<td>192.9</td>
<td>0.911</td>
<td>-1.93 (Clog ( P ))</td>
</tr>
<tr>
<td>C4-HSL</td>
<td><img src="C4-HSL.png" alt="Molecular structure" /></td>
<td>171.2</td>
<td>-0.616</td>
<td>-0.68</td>
</tr>
<tr>
<td>C6-HSL</td>
<td><img src="C6-HSL.png" alt="Molecular structure" /></td>
<td>199.3</td>
<td>-1.442</td>
<td>0.16</td>
</tr>
<tr>
<td>C8-HSL</td>
<td><img src="C8-HSL.png" alt="Molecular structure" /></td>
<td>227.3</td>
<td>-2.271</td>
<td>0.99</td>
</tr>
<tr>
<td>3-oxo-C6-HSL</td>
<td><img src="3-oxo-C6-HSL.png" alt="Molecular structure" /></td>
<td>213.2</td>
<td>-0.853</td>
<td>-1.11</td>
</tr>
<tr>
<td>3-oxo-C8-HSL</td>
<td><img src="3-oxo-C8-HSL.png" alt="Molecular structure" /></td>
<td>241.3</td>
<td>-1.68</td>
<td>-0.27</td>
</tr>
</tbody>
</table>

The specific calculating process of violacein or \( \beta \)-Gal as follows:

\[ Q(\text{Water}) = Q_0 \times V/50 \]  
(6)

\[ Q(\text{Washing}) = Q_0 \]  
(7)

\[ Q(\text{Sludge}) = Q_0 \]  
(8)

The results of \( \log S \) and \( \log P \) were predicted by ChemBioDraw Ultra 13.0. Clog \( P \) was the value of \( \log P \) calculated by Clog \( P \) Calculations (Kenny et al., 2013).

References for molecular structures were summarized from the following references: Deng et al., 2011; Galloway et al., 2011; Pereira et al., 2013; Decho et al., 2011.
\[ Q(\text{Mixture}) = Q_0 \times 2 \]  

(9)

where \( Q \) (Water or Washing or Sludge or Mixture) (units) is the content in 100 mL, \( Q_0 \) (units) is the value determined by reporter strain and it was the average value from 5 parallel samples. In Eq. (6), \( V \) (mL) is the volume of water after centrifuging the 100 mL mixture and “50” (mL) is the volume for extraction in Water phase. “2” (mL) in Eq. (9) is the expanding factor of calculating system from 50 mL to 100 mL.

### 3. Results and discussion

#### 3.1. Intraspecific signal molecules (AHLs)

AHLs are the most thoroughly studied and well-known type of signal molecules among Gram-negative bacteria (Manefield and Whiteley, 2007), and they consist of a homoserine lactone ring that contains various amide-linked side chains identified by a range of 4–18 carbons in length (Decho et al., 2011). The synthesis and secretion of EPS have been shown to be regulated by QS in most bacteria (Marketon et al., 2003).

#### 3.1.1. Short and medium acyl chain AHLs

C6-HSL is the primary medium acyl chain AHL (Steindler and Venturi, 2007); therefore, it is commonly used in the detection of this type of AHL. As shown in Fig. 1, the content of violacein represented the concentration of short and medium acyl chain AHLs. These results suggest that the contents in the water phase of S2 and S3 were about two to three times greater than in the sludge phase, and could represent the total content of the mixture. There were no differences in the two phases of short and medium acyl chain AHLs (p > 0.05). According to Decho et al. (2011), the aqueous solubility of AHLs was inversely proportional to their molecular weight. In other words, lower molecular weight AHLs tended to have better relative aqueous solubility. This might be the main reason responsible for the high content of short and medium acyl chain AHLs in the aqueous phase.

As shown in Fig. 1 and Table 1, the synthesis of EPS might be mutual affected by the content of short and medium acyl chain AHLs in water and the mixture. For example, the content of EPS in S3 was 27.28 ± 1.95 mg/g VSS, which was significantly higher than that in S1 and S2 (p < 0.01), and the content of AHLs in water has the same tendency. Additionally, the short and medium acyl chain AHLs in water of S1 were supplemented by AHLs from the sludge. Sludge with a larger average granular diameter perhaps appeared to have a tendency to secrete more AHLs.

#### 3.1.2. Long acyl chain AHLs

NTL4 was used to indicate the presence of long acyl chain AHLs by induction of \( \beta \)-Gal activities (Farrand et al., 2002). The results shown in Fig. 2 suggested that the content of long acyl chain AHLs in the Water phase was 12–33 Miller units, which was slightly higher than that in the Sludge phase. However, for long acyl chain AHLs, the ratio of the content in water to that in sludge was about 50% of that of short and medium acyl chain AHLs, except for S1. Moreover, as shown in Table 3, the ratio of long acyl chain AHLs in water to that in the mixture was lower than that of short and medium acyl chain AHLs, except for S1. These findings indicate that long acyl chain AHLs were preferentially distributed in the surface or the area near sludge than in aqueous water. The poor diffusivity of long-chain AHLs, which was caused by their low solubility (Decho et al., 2011), was directly responsible for the above results. Moreover, these properties caused cell–cell communication to occur over only a short range (Boyer and Wisniewski-Dye, 2009; Decho et al., 2011). For the abnormal results of S1, the reason may be described that the stability and distribution of AHLs was affected by a lot of factors, such as pH, temperature, salinity, other ions and the different resource of sludge (Decho et al., 2011). The distributions of different acyl chain AHL in anaerobic granular sludge were not consistent with the results of Chong et al. (2012; Valle et al., 2004).

As shown in Fig. 2, the long acyl chain AHLs in S2 were significantly higher than that in S1 and S3 (p < 0.01). This was likely because smaller sludge granules with high specific surface areas obtained more nutrients and had a more vigorous material metabolism and secreted more amount of long acyl chain AHLs than that of larger granules. The result

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**Fig. 1** — The content of short and medium acyl chain AHLs in different phases of the mixture of water and sludge. Black (■), grey (●) and white (□) represent S1, S2 and S3, respectively.

**Fig. 2** — The content of long acyl chain AHLs in different phases of the mixture of water and sludge. Black (■), grey (●) and white (□) represent S1, S2 and S3, respectively.
suggested that the content of AHLs in S2 was much higher than that in S1 and S3.

3.1.3. Specific AHLs

Table 4 shows two types of AHLs largely quantified by HPLC. The results suggested that more than 50% of C4-HSL, which is a type of short acyl chain AHL, was distributed in aqueous water, which was a result of its preferable solubility ($\log S = -0.616$) and lipo-hydro partition coefficient ($\log P = -0.68$). About 50–80% of 3-oxo-C8-HSL was present in the Sludge phase, which was similar to the trend observed for long acyl chain AHLs. These results were in accordance with its poor solubility ($\log S = -1.68$) and lipo-hydro partition coefficient ($\log P = -0.27$) (Decho et al., 2011). These findings indicate that AHLs with low molecular weight tended to spread to the water, while those with high molecular weight (long acyl chains or substituents) prefer to stay in the sludge phase (Decho et al., 2011). The different distribution of AHLs between S1 and S2, or S1 and S3 might have been caused by the different sludge activity.

### Table 4 – Distribution of AHLs in different phases.

<table>
<thead>
<tr>
<th>Sludge</th>
<th>Phase</th>
<th>C4-HSL (%)</th>
<th>3-oxo-C8-HSL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Water</td>
<td>49.77 ± 0.83</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Washing water</td>
<td>N.D.</td>
<td>7.45 ± 2.19</td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>43.65 ± 4.92</td>
<td>47.00 ± 4.10</td>
</tr>
<tr>
<td></td>
<td>Mixture</td>
<td>100 ± 31.36</td>
<td>100 ± 15.50</td>
</tr>
<tr>
<td>S2</td>
<td>Water</td>
<td>47.00 ± 1.12</td>
<td>35.75 ± 11.23</td>
</tr>
<tr>
<td></td>
<td>Washing water</td>
<td>7.89 ± 4.77</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>53.73 ± 0.03</td>
<td>73.06 ± 25.45</td>
</tr>
<tr>
<td></td>
<td>Mixture</td>
<td>100 ± 3.21</td>
<td>100 ± 0.26</td>
</tr>
<tr>
<td>S3</td>
<td>Water</td>
<td>55.99 ± 3.82</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Washing water</td>
<td>11.78 ± 2.59</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>42.97 ± 6.97</td>
<td>77.46 ± 22.63</td>
</tr>
<tr>
<td></td>
<td>Mixture</td>
<td>100 ± 3.85</td>
<td>100</td>
</tr>
</tbody>
</table>

N.D. = not detected.

The content of AHLs in the mixture of water and sludge was prescribed as 100 (%), and the content of AHLs in other phases was described as the percentage (%) in the mixture.

Moreover, interspecific QS has been shown to be associated with aerobic granular sludge formation (Jiang et al., 2006; Xiong and Liu, 2010, 2012; Zhang et al., 2011). Fig. 3 shows that the relative AI-2 content in the Water phase was higher than that in the Sludge phase, and AI-2 in water could represent the total content of the mixture. That was to say, the relative AI-2 content in Water phase was more than 80% of that in the mixture and it was similar to Valle’s results (Valle et al. 2004). These results were likely due to its favourable solubility ($\log S = 0.911$; $\log Clog P = -1.93$). Because of its unique properties, the content of AI-2 in the Washing water phase was similar to that in the Water phase. This phenomenon might be explained as follows: the concentration of AI-2 in the Water phase of the mixture was already saturated; however, when the water was removed and physiological saline was added, the AI-2 with excellent solubility and low log $P$ adsorbed onto the sludge was released into the fresh physiological saline.

Pearson’s correlation analysis demonstrated a negative correlation between the relative AI-2 content in the mixture and the average granular diameter with an $r$ value of $-0.859$ ($p < 0.01$). The mechanism responsible for this relationship was likely as follows: the granular sludge with a small diameter synthesized and secreted much more AI-2 to accelerate shortening of the gaps between large granules. When the granule reached its limiting diameter or its activity dropped, the content of AI-2 would be maintained at a relatively low level, and most of the granules in S1 would belong to the above situation. These results conformed to those of previous studies, in which AI-2 was found to play a positive role in regulation of the granulation of sludge (Ren et al., 2010; Xiong and Liu, 2010).

### 3.3. Intra- and interspecific signal molecules (DSF)

DSF is a type of intra- and interspecific signal molecule secreted by Xanthomonas. DSF is a long-chain fatty acid that regulates EPS production and biofilm formation. (Deng et al., 2011; Tao et al., 2010). As a type of signal molecule in Gram-negative bacteria, DSF decreases the production of EPS, especially polysaccharides (Tao et al., 2010), which was the matrix adhered to by aggregating bacteria. As an interspecific

![Fig. 3 – The relative AI-2 content in different phases of the mixture of water and sludge. Black (■), grey (○) and white (□) represent S1, S2 and S3, respectively.](image)
signal molecule, DSF regulates biofilm dispersal \cite{deng2011} and virulence factor production \cite{deng2010, he2008}.

As shown in Fig. 4 and Table 5, 70–90% of the DSF was concentrated in the sludge. Conversely, DSF almost could not be found in the Water phase and the percentage of DSF in the mixture was less than 10%. These results were observed for all three sludge investigated herein and reflect the poor solubility of DSF in water (log $S = -3.451$) and its good solubility in organics (log $S = 4.41$). Therefore, signal molecules with low diffusivity were almost completely adsorbed around organic granules.

The content of DSF in S1, S2 and S3 was $193.31 \pm 29.79 \mu$g/L, $1191.35 \pm 96.70 \mu$g/L and $916.80 \pm 90.22 \mu$g/L, respectively. Among the three types of sludge investigated, the content of DSF had a negative correlation with the average granular diameter with an $r$ value of $-0.782$ ($p < 0.01$). As for the effects of DSF on dispersal of the biofilm, the negative correlation between the concentration of DSF and the average granular diameter was extremely interesting. A possible mechanism for this phenomenon is as follows: the DSF synthesized by bacteria was used to resist the role of AI-2 in the sludge system. Granular sludge, especially that with the smaller diameter, undergoes granulation and disintegration during the metabolic process \cite{franco2006}. Currently available information indicates that granular sludge would disintegrate when it reached its limiting diameter because of a lack of nutrients among the internal bacteria of granules \cite{ni2009}. Previous studies focused on the influence of environmental conditions, but did not investigate the roles of QS. Therefore, it has been speculated that the secretion of DSF protects internal bacteria in the granule and maintains the existing granular diameter, but that the function and secretion of DSF decreases as the diameter of the sludge granule increases, which could be negative feedback regulation. These results suggest that the content of DSF had a positive correlation with the potential for sludge granulation.

The EPS production in S2 was lower than that in S3, while the content of DSF between them showed the opposite tendency. These findings were in accordance with those studies conducted by Deng et al. \cite{deng2011} and Tao et al. \cite{tao2010}, who suggested that another type of approach by which DSF inhibits cell aggregation was through reduction of the synthesis of EPS. Since EPS production was decided based on the content of AHLs and DSF, it was not surprising that the EPS production in S1 and S2 were not synchronized with their corresponding DSF.

As shown in Table 6, there were no significant differences ($p > 0.05$) between the total content of DSF calculated by Eq. (1) and that determined by HPLC, except for S3. In S3, the high EPS production, especially protein production, might discourage the communication among signal molecules and it likely caused a lower percentage of DSF to be transferred from the sludge phase to the mixture of water and sludge. As a result, the extraction process could accurately express the total content of DSF in the mixture and was suitable for determination of signal molecules in anaerobic granular sludge.

### Table 5 – Percentage of DSF from different phases in the mixture of water and sludge.

<table>
<thead>
<tr>
<th>Water phase (%)</th>
<th>Washing water phase (%)</th>
<th>Sludge phase (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 4.15 ± 1.19</td>
<td>1.66 ± 1.36</td>
<td>82.86 ± 16.29</td>
<td>11.33</td>
</tr>
<tr>
<td>S2 1.52 ± 0.17</td>
<td>0.97 ± 0.10</td>
<td>91.82 ± 4.96</td>
<td>5.69</td>
</tr>
<tr>
<td>S3 7.77 ± 1.08</td>
<td>4.41 ± 0.21</td>
<td>67.02 ± 2.25</td>
<td>23.54</td>
</tr>
</tbody>
</table>

### Table 6 – DSF content in the mixture determined by different calculating processes.

<table>
<thead>
<tr>
<th>Calculation 1 (µg/L)</th>
<th>Measured value (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 171.41 ± 32.37</td>
<td>193.31 ± 29.79</td>
</tr>
<tr>
<td>S2 1119.98 ± 63.45</td>
<td>1191.35 ± 96.70</td>
</tr>
<tr>
<td>S3 700.26 ± 55.77</td>
<td>915.80 ± 90.22*</td>
</tr>
</tbody>
</table>

* In the same row indicates significant differences ($p < 0.05$).

### 3.4 Interaction among signal molecules

Granular sludge, which is a microbial aggregate and complex system, contains a variety of signal molecules that are simultaneously present \cite{deng2010, shrout2012}. Accordingly, interactions among these molecules must play an essential role in regulating biofilm formation. However, this has seldom been investigated.

Short, medium and long acyl chain AHLs indirectly promoted the aggregation and granulation of sludge by regulating the secretion of EPS \cite{marketon2003, shrout2012}. Conversely, the presence of DSF decreased EPS production, prevented bacteria from adhering, gathering and growing in a biofilm \cite{deng2010, deng2011, he2008, tao2010}. Although they played negative roles in the biofilm, the results of DSF and AHLs in Figs. 1, 2 and 4 suggest that the content of DSF was positively correlated with short and medium acyl chain AHLs, but negatively correlated with long acyl chain AHLs. These signal molecules with different distributions would be a factor which should not be ignored. Since AHLs were assayed by a semi-quantitative method, the interaction between different AHLs...
with DSF was unclear. Accordingly, future studies are needed to explain this phenomenon.

Interspecific signal molecules such as AI-2 induce the expression of related genes to strengthen the communication among interspecific bacteria in the sludge system and guide sludge cells to switch from planktonic growth to attached growth (Ren et al., 2010; Xiong and Liu, 2010, 2012; Zhang et al., 2013), which is prerequisite for biofilm formation. Conversely, DSF would disperse the cells in biofilm (Deng et al., 2010, 2011; He and Zhang, 2008; Tao et al., 2010; Wang et al., 2003). Studies have shown that AI-2 promoted the granulation of sludge (Jiang et al., 2006; Xiong and Liu, 2010), which was nearly spread to the Water phase, while the concentration of DSF in the Sludge phase suppressed bacterial cell aggregation (Deng et al., 2011). Interestingly, two types of signal molecules with the opposite effects on granular sludge were highly positively correlated with an r value of 0.952 (p < 0.01) and opposing distribution. These findings indicated that there must be unknown and complex interactions, including restriction or promotion between AI-2 and DSF in the anaerobic granular sludge. The different distribution must be a crucial factor in the QS mechanism. Future studies investigating the different distribution of signal molecules should focus on the threshold concentration of signal molecules sensed by bacteria.

4. Conclusions

In this study, the distributions of different signal molecules in three types of anaerobic granular sludge were investigated. DSF was primarily distributed in sludge, but AI-2 was mostly presented in water. More interestingly, the content of DSF was perfectly correlated with that of AI-2. Moreover, short and medium acyl chain AHLs tended to spread in aqueous water, while the distribution of long acyl chain AHLs was closer to that of granular sludge than that of short acyl chain AHLs. The production of EPS or granular level of sludge was closely related to the diversity, distribution and concentration of signal molecules. As a medium in granulation, EPS production had a correlation with different signal molecules from different phases.

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