Deciphering bacterial interactions via DSF-regulated public goods in an anammox community

CURRENT STATUS: POSTED

Yongzhao Guo
Peking University

Yunpeng Zhao
Peking University

Xi Tang
Peking University

Tianxing Na
Peking University

Juejun Pan
Peking University

Huazhang Zhao
Qinghai University

Sitong Liu
Department of Environmental Engineering, Peking University, Beijing, China

liusitong@pku.edu.cn Corresponding Author
ORCiD: https://orcid.org/0000-0001-6849-8704

DOI: 10.21203/rs.2.17231/v1

SUBJECT AREAS
General Microbiology

KEYWORDS
Quorum Sensing, Diffusion Signal Factor (DSF), Bacterial Interaction, Public Good, Anammox Consortia
Abstract
Background: Bacterial interaction and communication via quorum sensing (QS) received extensively attention, as it can coordinate bacterial behavior and activity through the QS signal molecules in microbial community. Though the exchange of public goods regulated by QS have been explored in pure culture, how signal sense, transmit, and affect the social traits through regulating public goods in complex communities remains unclear.

Results: The levels of public goods (e.g. extracellular polymeric substances (EPS) and amino acids) changed significantly when exogenous diffusion signal factor (DSF), a kind of QS molecules, was added. Approaches involving meta-omics and hierarchical signalling network construction give insight into that anammox species (Jettenia caeni, AMX1) and Proteobacteria-affiliated bacterium (PRO1) can sense and transit DSF signals, thus directly regulating the production and exchange of public goods via the secondary messenger c-di-GMP regulator, Clp. In detail, these two kinds of species can supply more costly amino acids for DSF-Secretor species (like AMX2, CFX1, CFX3, and PRO4) after sensing DSF. Meanwhile, DSF-Secretor species encoded diverse genes involved in hydrolysis of extracellular protein and carbohydrate and genes involved in transportation of peptides and sugars. The exogenous DSF-inducement also leads to the high expression of these genes, which indicated DSF-Secretor species helped anammox bacteria scavenge extracellular detritus. This process can be considered as a feedback of public goods supply by anammox bacteria, as this process contributed to create a suitable environment for anammox bacteria growth. Namely, DSF can bridge bacterial interactions through regulating public goods. Furthermore, the trade-off induces discrepant metabolic loads of different microbial clusters and community succession. It illustrated the potential to artificially alleviate metabolic loads and thus increase proliferation rate for certain bacteria through QS.

Conclusions: DSF can bridge interactions of anammox bacteria and DSF-Secretor species through regulating production and exchange of public goods using Clp regulator. Deciphering microbial interactions via QS provides insights for understanding the molecular evolution of QS in microbial community.
Background

Quorum sensing (QS), a prevalent interaction or communication avenue in the microbial community, can coordinate bacterial behaviors at the population level via QS signal molecules. These molecules have been the focus of research on pathogenic bacteria [1–3]. Recently, the QS phenomenon has been found in wastewater bio-treatment and has attracted widespread attention [4, 5]. Wastewater treatment sludges usually are in consortia, confining bacteria with phylogenetically high diversity in a limited space, which greatly abridges the spatial distances and affects ecological interactions based on the exchange of small molecules or metabolites in the vicinity [6].

Of the exchanged molecules, public goods are substances that are produced at a cost to provide shared benefits for a local neighborhood of cells. Previous studies demonstrated that digestive enzymes [7,8], polymeric substances [9], chelating compounds [10], or amino acids [11] could be secreted into extracellular environments as public goods. Extracellular polymeric substances (EPS) are critical for wastewater treatment sludge because of their role in aggregation and biomass tolerance against inhibitors [5, 12]. Studies have suggested that amino acid auxotrophy potentially shapes the community composition [13, 14]. Therefore, EPS and amino acids as public goods are particularly attractive for wastewater treatment.

Interestingly, it has been suggested that the production or exchange of public goods can be regulated by QS [1, 2, 15]. Theoretical analysis has revealed that the secretion of public goods is more beneficial at higher cell densities [16, 17]. Empirical studies have further identified a positive density-dependent benefit for public goods, which might be involved in the QS-controlled objects. Examples of such benefits include extracellular digestive enzymes regulated by QS that are produced for the best cooperative investments in Pseudomonas aeruginosa [7, 15] and an antibiotic-degrading enzyme in a synthetic QS system [18]. These researches suggest that QS could be a key regulator for well-defined pure culture. However, how QS regulates the bacteria communication in complex communities via public goods is still unclear.

Recently, anammox (anaerobic ammonium oxidation) process that directly converts nitrite and ammonium to nitrogen gas have attracted widespread attention for its high nitrogen removal
capability and low energy consumption [19]. Anammox bacteria are not currently available as pure cultures [20] and are inclined to live, with other species with cross-feeding actions via public goods [21]. The existence of the genes coding for QS molecules in anammox community was first evidenced by Strous et al. [22] while deciphering the metagenome. Signal messenger bis-(3’–5’)-cyclic dimeric guanosine monophosphate (c-di-GMP) was also found in anammox culture [23], and it acts as an important regulator for anammox consortia against environmental stresses [12]. Importantly, according to a recent report, among the multiple QS signal systems, diffusible signal factor (DSF) signaling plays a key role in the anammox community during anammox reactor start-up [24]. Therefore, further work on DSF-regulated interactions, especially via public goods, within the anammox community is quite worthwhile. It would pave the path for understanding microbial communications and interactions within the consortia, and the potential regulation of whole consortia towards highly-efficient and low-metabolic energetic loads.

Here, we utilized the approach of exogenous DSF-addition to obtain conditions with coercive inducement of the DSF signal. The levels of public goods (EPS and free amino acids) were compared between the DSF-addition group and the control group. The community structure was determined along with the long-term reactor operation (95 days). The mechanism of DSF-regulated interactions within the complex community via public goods was further explored by combined metagenomes, metatranscriptomes, and hierarchical signaling network construction (c-di-GMP receptor Clp regulon). The results emphasized the role of public goods in bridging the microbial communication regulated by QS and provided a valuable avenue for understanding the molecular biology and evolution of QS in the microbial community.

Results
Community overview based on metagenomes and metatranscriptomes
DSF signals were confirmed in the anammox culture using DSF biosensor assays and HPLC-MS detection (Additional file 1: Figure S1). To decipher the mechanism of DSF regulation within the anammox community, meta-omics approaches were utilized. Assembly and binning of the metagenomic sequencing reads resulted in 12 high-quality draft metagenome-assembled genomes
(MAGs) (Fig. 1 and Additional file 1: Table S1). The 12 draft genomes accounted for 59.3% of the original sequencing data from the untreated samples (Additional file 1: Table S2). At least 87.2% of mRNA reads from the metatranscriptomic samples were mapped to the assembly above, indicating that the data to be analyzed were able to capture a majority of the transcriptomic activities (Additional file 1: Table S2).

The relative abundance and gene transcription for each MAG are shown in Additional file 1: Fig. S2 (Additional file 2). In all, two anammox strains were recovered, Jettenia (AMX1) and Brocadia (AMX2) (Fig. 1). The AMX1 strain dominated, with a relative abundance of 71.8% and gene transcription of over 95.9% in control and DSF-addition samples (Additional file 1: Table S2), respectively. The results indicated that AMX1 contributed almost all of the anammox activity in the anammox biomass. Other organisms, with an abundance of > 1%, were CYN1, PRO1, PRO2, CFX1, CFX2, and ATM1, affiliated to the phyla Cyanobacteria, Proteobacteria, Chloroflexi, and Armatimonadetes (Additional file 1: Table S1 and Figure S2).

Bioinformatic analysis of DSF signaling in the anammox community
Identification of the anammox community members having the ability to synthesize DSF molecule was the first step. Biosynthesis of the DSF family of signals is dependent on the synthase RpfF [25], which has amino acid sequence relatedness to enoyl-CoA hydratase [26]. After domain research against HMMER database, totally 36 putative enoyl-CoA genes were found that were allocated to 7 out of the total 12 MAGs in the anammox community. Further, multiple sequence alignment identified 13 (out of 36) putative RpfF genes in the 7 MAGs (AMX1, PRO1, CDK1, AMX2, CFX3, CFX2, and CFX1).

In a previous work [27], three categories of DSF-dependent QS systems have been identified. The first category was originally identified in Xcc consisting of RpfC/RpfG responsible for signal perception and transduction [28]. After conservative domain search and multiple sequence alignment, interestingly, the analogous RpfC/RpfG synthetic genes were found only in the AMX1 genome (Additional file 1: Figure S3). Here, it should be emphasized that RpfG protein contains a conservative HD-GYP domain, which has phosphodiesterase activity and is able to degrade c-di-GMP, a universal bacterial secondary messenger regulating a wide range of functions [29]. Thus, it indicates a potentially hierarchical DSF
signaling network via c-di-GMP for AMX1.

The second category of DSF-dependent signaling has only a novel sensor protein RpfR, which is able to perceive and transduce the DSF signal [30]. Interestingly, the RpfR protein contains a conservative EAL domain, which negatively controls the intracellular c-di-GMP level. It indicates that DSF signal is transduced downstream via c-di-GMP by the RpfR protein. Importantly, this type of DSF signal sensor was predicted to be found only in the PRO1 genome (Additional file 1: Figure S3).

The third category is a cluster of genes involved in the signaling of another diffusible signal factor-family molecule cis 2-decenoic acid (BDSF). No putative gene clusters were found in all the 12 MAGs in the metagenome. In this study, we have emphasized on the diffusible signal factor-family molecule cis-11-methyl-dodecennoic acid (DSF), and thus BDSF-type signaling will not be discussed in this study. Based on these results, we categorized the recovered MAGs according to their condition whether they had genes for DSF biosynthesis, or DSF perception, or DSF signal transduction (Additional file 1: Table S3). Since AMX1 and PRO1 had all the three functional genes, they were named as DSF-QS MAGs. Another group, including CDK1, AMX2, CFX3, CFX2, and CFX1, was only able to synthesize DSF and was categorized as DSF-Secretors. Finally, the other MAGs without any of these functions were named as DSF-Null species.

Significant changes of anammox community structure under long-term addition of DSF

The changes in anammox community structure along with the long-term reactor operation were determined using 16S rRNA gene sequencing (Fig. 2). We found that exogenous DSF had different impacts on different MAGs. Especially, when the three categories of MAGs were combined, we found an obvious decrease in relative abundance during the reactor operation for DSF-QS MAGs (AMX1 and PRO1); for most DSF-Secretor MAGs (like AMX2, CFX3, CFX1, and PRO4), relatively higher abundance was found during the reactor operation; for DSF-Null MAGs (e.g. PRO2, ATM1, and PRO3), the exogenous DSF-addition had little influence on their abundance (Fig. 2a).

We also found obvious clusters for the different samples using sparse partial least squares-discriminant analysis (sPLS-DA) (Additional file 1: Figure S4). There was no difference between control
and DSF-addition reactor until day 30. Interestingly, on days 55, 75, and 95, the samples from each reactor were clustered together, resulting in an obvious distinction between the two reactors. This indicated that DSF acts as a motor to make a difference in the community structure, and the effect was a relatively long-term process.

Altered levels of EPS and amino acids as public goods due to exogenous DSF addition

Extracellular polymeric substances (EPS) and free amino acids are important types of public goods because of their potential benefits for the population as a whole, which is emphasized here. First, the contents of EPS in the batch assays were determined (Figs. 3a and 3b). The level of extracellular proteins (PN) was significantly higher when exogenous DSF was added ($P = 0.0051$, Kruskal–Wallis ANOVA). On the contrary, exogenous DSF induced significantly lower levels of extracellular polysaccharides (PS, $P = 0.0045$, Kruskal–Wallis ANOVA). This reduction was coupled to a significant decline in the pools of intracellular c-di-GMP levels ($P = 0.019$, Kruskal–Wallis ANOVA) (Fig. 3c).

We also detected the EPS contents during the reactor development (Additional file 1: Figure S5). The extracellular PN contents increased in the two reactors, compared to the initial time point. Surprisingly, PN levels in DSF-addition reactor were significantly lower than those of control, which was contrary to the results of the short-term batch assays (Fig. 3a). The extracellular PS contents in the DSF-addition reactor presented an obvious decline during the reactor development, and showed significantly lower levels than those of control, which was consistent with the profiles of short-term batch assays (Fig. 3b).

To determine the levels of amino acids released and traded in the anammox culture, amino acid concentrations in the supernatants were also detected (Fig. 3d). We found a significant difference in the levels of the essential amino acids, aspartate and valine, in the DSF-addition and control reactors. Higher levels of asparagine were detected under DSF-addition condition (Fold change = 2.07, $P = 0.038$, Kruskal–Wallis ANOVA), whereas lower levels of valine were detected under DSF-addition condition (Fold change = 0.58, $P = 0.019$, Kruskal–Wallis ANOVA).

Genome scale search for DSF-dependent c-di-GMP effector Clp regulon

As reported previously, Clp was mediated by DSF cell–cell communication signal [31], and further
identified as a novel class of c-di-GMP effectors [32]. To ascertain the DSF-dependent downstream regulatory networks within the metagenome, especially for the DSF-QS MAGs, a search for the global regulation factor Clp was carried out here. From the results of re-designed computational algorithm, we found a total of 155 and 143 genes for AMX1 and PRO1, respectively, which were predicted to be directly regulated by Clp.

These genes encode proteins with multiple functions, including energy production and conversion, signal transition mechanisms, replication, recombination and repair, cell wall/membrane/envelope biogenesis, posttranslational modification, protein turnover, chaperones; and particularly mass transport and metabolism like amino acids, nucleotide, carbohydrate, coenzyme, lipid, and inorganic ion (COG database) (Additional file 1: Figure S6 and Additional file 3).

**Genes for EPS production and degradation regulated by DSF**

Notably, the genes for EPS production and degradation in the DSF-QS MAGs were found within the Clp regulon. In Fig. 4a, significantly higher levels of two peptidase genes (AMX1_186_4, Fold change = 1.29, P-value = 0.023; AMX1_276_20, Fold change = 1.17, P-value = 0.008; T-test) were observed for DSF-addition, which were directly regulated by DSF in AMX1 genome. For other MAGs, more genes with significantly higher expression levels (count: 26 vs 16) were also found under DSF-addition (Additional file 4).

Diverse genes involved in the hydrolysis of carbohydrate bonds were also expressed in the anammox community (CAZy database) (Fig. 4b). For DSF-QS MAGs (AMX1 and PRO1), no ORF was found to be extracellular, but 4 genes that were up-regulated (PRO1_223_31, PRO1_256_56, PRO1_83_4, and PRO1_13_152) in the PRO1 genome were predicted periplasmic localizations. On the contrary, many genes from the other MAGs were up-regulated (count: 32 vs 23) and predicted extracellular, outer membrane and periplasmic localizations. These genes were predicted to be responsible for the PS levels.

**Genes for amino acid production and exchange regulated by DSF**

Amino acid exchange is an important mechanism that shapes the microbial community [13]. We combined the metagenome and metatranscriptome data to figure out the amino acid auxotrophy
As expected, the pathways for the biosynthesis of certain amino acids were missing in almost all the MAGs. Surprisingly, we observed that PRO1 could biosynthesize all the essential amino acids. In addition, PRO1 genome has the highest number of genes for amino acid transporters, which indicated the critical role of PRO1 in the amino acid trade for the whole community. Under DSF-addition condition, the average gene expression of amino acid biosynthetic pathway encoded by each MAG was calculated. As seen in Fig. 5a and Additional file 1: Table S4, for DSF-QS MAGs, biosynthetic pathways of tryptophan and lysine for AMX1, and phenylalanine for PRO1 were significantly up-regulated, whereas the methionine biosynthetic pathway for PRO1 was significantly down-regulated; these pathways were within the Clp regulon and directly regulated by DSF. Interestingly, the synthesis of these four amino acids is expensive, as reported earlier [33]. The indirect impact of DSF on the amino acid synthetic pathways is also shown in Fig. 5a. Importantly, we found that almost all these DSF-indirectly affected amino acids were less costly to synthesize [33], e.g., serine for AMX1, CYN1 and PRO3, glycine for AMX2, PRO3, alanine for CDK1 and CFX3, and asparagaine for PRO2.

Combined with the amino acid transporter profiles, we found the up-regulated genes within the Clp regulon that were involved in transporting amino acids in AMX1 and PRO1 (Fig. 5b), suggesting that DSF directly regulates certain amino acid transporters. For other MAGs, except CDK1 and CFX1, DSF had little impact on the amino acid transporters.

Discussion
EPS and amino acids as public goods regulated by DSF in anammox community
One important type of bacterial cell-cell communication is referred to as QS. It controls gene expression, and thus regulates the behaviors of the microbial population by releasing chemical signal molecules termed autoinducers. Recent researches have proposed that bacterial interactions via public goods is regulated by QS, which impacts energetic and metabolic strategies, and the behaviors of the whole community [1, 2, 15]. Here, we utilized exogenous DSF-addition strategy and found significantly changes in levels of signal-induced public goods (EPS and amino acids) in the anammox community (Fig. 3). Therefore, the pathway and effect of DSF regulation of public goods was further
explored.

DSF signal transition pathways were confirmed within the anammox community, which provided the foundation for analysis of the DSF-regulation mechanism. Two typical DSF signal transition pathways were found in AMX1 (RpfC/RpfG-like DSF signal transition) and PRO1 (RpfR-like DSF signal transition) (Additional file 1: Figure S3). It should be noted that proteins RpfG and RpfR, predicted with HD-GYP domain and EAL domain, respectively, were able to degrade the second messenger c-di-GMP [30, 34], which was also coupled to the significantly lower levels of c-di-GMP under DSF-addition condition (Fig. 3c). Accordingly, we searched for the DSF-dependent c-di-GMP receptors, and thus the Clp protein was emphasized here. As reported earlier [31, 32], the degradation of c-di-GMP releases free Clp proteins, which further activates the downstream genes within the Clp regulon.

Based on the Clp regulon predicted by our re-designed algorithm, the genes for public goods production, utilization, and exchange were tracked. During short-term DSF-addition assays, the genes for peptidases and carbohydrate bonds hydrolases were up-regulated, which were regulated directly by DSF and predicted to be located closer to the extracellular environments (Fig. 4). The locations were also found to allow these molecules to be used extracellularly [21]. As a result, these proteins contribute to the total amount of extracellular proteins (PN) (Fig. 3a). The concentrations of PN were significantly lower under DSF-addition condition in the long-term reactors, compared to the control, which is contrary to the results of the short-term assays. It is likely that these peptidases were still active and further degraded the PN into small peptides and amino acids during relatively longer periods [8, 21], which is responsible for the lower levels of extracellular PN during long-term detection. Similarly, up-regulated levels of the carbohydrate bond hydrolases activate the hydrolysis of extracellular PS, which contributed to the lower levels of PS.

As for amino acids, besides the degradation of proteins by peptidases, we found that DSF directly induces the DSF-QS species (AMX1 and PRO1) to produce costly amino acids like tryptophan, lysine and phenylalanine (Fig. 5). Further, controlled the expression of transporters to exchange amino acids through cellular membrane into the outer environments, considering these genes have active binding sites within the Clp regulon. This process could be an important source of amino acids as extracellular...
DSF-inducement via public goods and its impact on community structure

Here, we proposed that the accumulated effect from different metabolic strategies of DSF-regulated public goods for anammox MAGs was an important cause for the changes in community structure. First, multiple pathways were found to be regulated by DSF, including public goods metabolism and transport (Additional file 1: Figure S6), which suggested that other factors could also be responsible for the changes in the community structure. However, previous studies have shown that amino acids, as public goods, used for cross-feeding within community were able to shape the community structure [13, 14], and EPS played an important role in community assembly regulated by QS [5]. Additionally, we found significant changes in the levels of EPS and amino acids (as public goods) during short-term assays (for several hours, Fig. 3), but the community structure did not alter until 30 days (Additional file 1: Figure S4). Anammox community structure changed in a DSF-coordinated manner under long-term artificial exogenous DSF inducement.

Overloaded metabolism for public goods under DSF regulation decreased the abundance of DSF-QS species (AMX1 and PRO1) during the development of reactors. Exogenous DSF directly induced AMX1 and PRO1 to produce higher levels of extracellular proteins (Fig. 4) and costly amino acids (Fig. 5) that needed substantial energy to biosynthesize [11, 33, 35]. The rate-yield trade-off in microbial metabolism emerges because some molecules are costly to manufacture, like proteins, and that the bacteria needs to make decisions about resource allocation [36]. In this study, exogenous DSF induced the DSF-QS MAGs to produce costly proteins and metabolites persistently, thus decreasing the fraction of metabolic resources for growth. Similar trade-offs in bacterial cellular economics, causing shifts in growth strategies were also discussed using models [36] and experiments (E. coli) [37].

Positive trade-offs in public goods for DSF-Secretor species contributed to the increase in their abundance under long-term exogenous DSF-addition. DSF-family molecules belong to short-chain fatty acids [27], with non-signaling properties [38], but few genes were found to have increased expression levels in the pathway of fatty acid degradation, predicted from KEGG database for the
DSF-Secretor species (Additional file 1: Figure S7). This indicated that exogenous DSF functioned as signals and not as nutrients during our experiment. As shown in Fig. 4, these MAGs were indirectly affected by DSF to up-regulate the expression of peptidases and carbohydrate bond hydrolases located in the periplasmic, outer membrane and extracellular environment, which were able to degrade macromolecules like proteins or polysaccharide into smaller molecules like peptides, amino acids, or monosaccharides. These small molecules were transported into the bacterial cells for energy; or as carbon (C) or nitrogen (N) source for metabolism and growth, especially for CDK1, CFX2 and CFX1, which is also supported by the relatively higher levels of carbohydrate metabolism (Additional file 1: Figure S6). The small metabolite utilization has been also discussed in anammox community [21]. Furthermore, only cheap amino acids, such as alanine and glycine [33] were influenced by DSF signal for CDK1, AMX2 and CFX3 (Fig. 5a), and the expression of amino acid transporters for CDK1 and CFX1 showed higher levels under DSF-addition condition. It can be deduced that these MAGs trade cheap amino acids for some costly amino acids without spending energy. Cheap amino acids, as an ideal source of protein synthesis and energy generation, were used for growth under DSF-addition. Similar phenomena have been found previously for pure cultures and natural environments [35, 39].

No obvious changes in the production or availability of DSF-regulated public goods for DSF-Null species led to neutral or even less advantageous existence within the complex community. Lower levels of gene cluster were found for ATM1, CYN1 and PRO3, which indicated the lower levels of metabolic status under DSF signal condition (Additional file 1: Figures S7 and S8). Down-regulation in the levels of peptidases and lack of activated amino acids transporters in the DSF-Null MAGs under DSF-addition implied their inability to produce or trade public goods.

**Interactions via DSF-regulated public goods among anammox community**

The exogenous addition of DSF provided an unnatural DSF signal inducement in the whole anammox community, and the information of endogenous DSF for anammox culture was still needed further discussion. Interestingly, categories of DSF-QS and DSF-Secretor species were predicted to biosynthesize DSF, but not the DSF-Null species (Fig. 2), and different categories of MAGs had
different responses towards public goods production and exchange under the DSF signal (Figs. 4 and 5a). The coupling suggested that different categories of MAGs use DSF as a signal for different purposes.

DSF, as a signal molecule, must mediate certain communication between species [40]. For DSF-QS species in medium, AMX1 uses DSF to communicate with other species in a coordinated manner, like synthesizing certain amino acids for each other; or AMX1 uses DSF for the purpose of trading amino acids, such as tyrosine, histidine and methionine, which were auxotrophic in AMX1 genome (Fig. 5a). Amino acid biosynthesis is an energy-intensive process and supplementation by bacteria might enable microbes to focus their metabolism on the uptake of reducing equivalents rather than biosynthesis [14, 33].

The DSF acts as an important messenger between DSF-QS and DSF-Secretor species, which would enhance their interactions and stabilize the community (Fig. 6). As reported previously [41], bacteria within a complex structured community, like the anammox community, did not distribute randomly, but spatially within the complex biofilm. DSF signal thus presents a spatial heterogeneity; it can act as a timer [42], which bacteria can be utilized by the bacteria to sense the signal and infer the relatedness to their neighbors. The bacteria thus tune their investment into costly and exploitable cooperation in a coordinated manner and also fight against the cheaters [7, 15, 43]. The DSF-Secretor species were also able to produce and secrete DSF, which was perceived by AMX1 and PRO1 (Fig. 6). As a result, costly amino acids and other auxotrophic amino acids were released as pubic goods. The supplementation of costly amino acids would ease the energetic burden of the MAGs [14]. In return, DSF-Secretor species might secrete certain peptidases and carbohydrate bond hydrolases, which would degrade the organic molecules within anammox community and create a more suitable environment for the anammox species [44]. It was shown that after long-term DSF-addition, the anammox activity per anammox bacterial cell was enhanced, because no significant differences in the nitrogen removal capability (NRR) were found between the two reactors (Additional file 1: Figures S9 and S10); but lower abundance of anammox bacteria was found in similar amount of anammox biomass after the operation of DSF-addition reactor (Additional file 1: Table S5). The DSF plays an
important ‘timer’ role in bridging the interactions among different members in the anammox community. Once the ‘timer’ is disrupted, like artificial addition of DSF to the anammox culture or even persistently for long period, its regulation would fail and then the balance of the community would be altered, similar to the changes in community structure after long-term DSF-addition.

Significance of this study and prospects for the future

QS has been an important research topic for microbial ecology. It is a prevalent avenue of cell-cell communication that coordinates gene expression in bacterial populations [45]. With the help of empirical data, meta-omics, and prediction of gene regulons, our study found that DSF, a QS signal, played a critical role in bridging the interactions via regulation of public goods in anammox community. The production and transport of EPS and free amino acids was regulated by DSF in the anammox community in this study, wherein their levels in the anammox biomass changed significantly through artificial short-/long-term addition of DSF to the culture. In addition, we found certain species within in anammox community that use DSF as messenger to communicate with their cooperators. The proper use of DSF would potentially ease the metabolic loads of the bacterial communities leading to efficient cooperation, like the enhanced anammox cell activity per anammox bacterial cell under DSF inducement, as illustrated in this study. Deciphering the pathways and effect of their regulation would enable us to artificially regulate the proliferation rates and activities of certain species of bacteria for better cooperation in enhancing the performance of the anammox community.

Bacteria coexist in nature with other microorganisms as consortia where interactions occur to build an advanced society [46]. In such microbial networks, microbes can coordinate different types of interactions, such as exchange of metabolites. These interactions allow the microorganisms to cooperate with the other members, broaden their metabolic capabilities, and improve robustness to environmental fluctuations [47]. This study investigated the mechanism of QS-regulated public goods in the anammox community through metagenomes, metatranscriptomes, and hierarchical signaling network construction. It provided useful information for further research on bacterial communication, especially in complex communities. Unlike previous reports focusing on the QS-controlled pathways or
functions, the present study emphasized the critical role of public goods in QS regulation of complex communities, which increases our current understanding of the molecular biology and evolution of QS in the microbial community.

Conclusions
The integrated multi-omics analysis gives insight into how DSF coordinate bacterial interaction through regulating public goods. The production and exchange of public goods and bacterial interaction in response to the DSF addition were investigated in anammox consortia. The results showed that the anammox bacteria and PRO1 produced more costly EPS and amino acids for DSF-Secretor species via Clp regulator after sensing DSF. In return, DSF-Secretor species help anammox bacteria degrade the extracellular detritus. Meanwhile, exogenous DSF addition induces different responses in different clusters of the anammox community, resulting in different metabolic and energetic loads for the production and exchange of public goods, which are responsible for the change in community structure. Deciphering the effect of their regulation would enable us to artificially alleviate metabolic loads and regulate the proliferation rates of certain species for better cooperation in enhancing the performance of the anammox community.

Methods
Batch assays
Batch assays were performed in 250-mL serum bottles. Each vial was inoculated with anammox consortia to a final concentration of 1.45 gVSS L⁻¹ [12]. The biomass was washed using anammox medium without nitrogen and re-suspended in the cultivation medium. The cultivation medium contained 100 mg NO₂⁻-N L⁻¹ and 100 mg NH₄⁺-N L⁻¹ along with the other components as shown in Additional file 1: Extended Materials and Methods. The vials were made anoxic by flushing with N₂ for 10 min, and the serum bottles were sealed tightly with rubber caps to avoid any influent of external oxygen.

To study the effects of DSF on anammox biomass, we conducted successive 5-cycle batch assays. Six vials were separated into two groups (each group with three replicates). DSF molecule (0.05 μM, the determined optimal concentration) was added to one group (called DSF-addition, details in Additional
file 1: Extended Materials and Methods and Figure S11); the same volume of medium without DSF was added to the other group (called control group). As mentioned above, once the NO$_2^-$ was degraded completely, it was considered as the end of each cycle of the batch assays. All the supernatants were collected for amino acid detection, and 1 mL of biomass (ca. 10 mgVSS L$^{-1}$) was harvested for EPS and c-di-GMP detection. The diagram of the batch assays is shown in Additional file 1: Figure S12.

After the final cycle of batch assays, the biomass was immediately frozen in liquid nitrogen for DNA and RNA (3 biological replicates) extraction for the purpose of metagenomic and metatranscriptomic analysis.

Reactor operation
Two identical sequencing batch reactors (SBRs) (R-control and R-DSF), with a working volume of 1.0 L were operated for 95 days. Both SBRs were inoculated with 0.29 gVSS L$^{-1}$ anammox consortia [12]. Stock solution of DSF was artificially added to the R-DSF reactor with syringes after influent feeding every hydraulic retention time (HRT) to a final concentration of 0.05 μM in the reactor medium, while the same volume of medium without DSF was added to the R-control reactor after every HRT. Other settings in the two reactors were same. Their hydraulic retention times (HRTs) were initially set as 24 h, with the influent nitrogen concentration at 50 mg NO$_2^-$-N L$^{-1}$ and 50 mg NH$_4^+$-N L$^{-1}$. When NO$_2^-$-N in effluent was below 10 mg-N L$^{-1}$, the nitrogen load was increased by increasing the influent ammonium (NH$_4^+$) and nitrite (NO$_2^-$) concentrations. The composition of media solution used in the reactors was the same as the batch assays. The pH of the SBRs was not controlled, but it remained within the range of 7.5 to 8.0, and the temperature was maintained at 37 ± 1°C. The influent medium of SBRs was flushed with a gas mixture of N$_2$/CO$_2$ (95/5%) and kept in a glass bottle with rubber stopper to maintain anaerobic conditions. The concentrations of NH$_4^+$-N, NO$_3^-$-N and NO$_2^-$-N were measured with UV-visible spectrophotometry (UV-1750, Shimadzu) according to the standard methods of the American Public Health Association [48]. Bacteria samples (3 biological replicates) were taken from the SBRs at days 0, 30, 55, 75 and 95 for the determination of extracellular proteins.
and polysaccharides and their sums (EPS) and extraction of DNA for 16S rRNA gene sequencing.

**DNA and RNA sequencing**

The anammox biomass harvested from the batch assays and SBRs was used for metagenomic and metatranscriptomic sequencing and from reactor for 16S rRNA sequencing, respectively. Total genomic DNA was isolated from each sample using the FastDNA® SPIN Kit for Soil (MP Biotechnology, Solon, OH, USA) according to the manufacturer’s protocol. For metagenome sequencing, the isolated DNA was fragmented, pair-end library was constructed, and adapters were prepared, following which the samples were sequenced on the Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, USA). For 16S rRNA sequencing, primers 338F and 806R targeting the V3–V4 region were used. Total RNAs were extracted using the E. Z. N. A.® Soil RNA Midi Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s protocols. After quality determination, rRNA in the total RNA of samples were removed, cDNA libraries were constructed, and samples were sequenced on the Illumina Hiseq 2500 platform (Illumina Inc., San Diego, CA, USA). All sequencing was performed at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Detailed information is given in Additional file 1: Extended Materials and Methods.

**Prediction of DSF perception, transition, and biosynthesis genes**

Two types of DSF perception and transition gene cluster or genes, *rpfC/rpfG* and *rpfR*, and biosynthesis gene *rpfF* were predicted based on the domain search against HMMER web server (2015 update) [49] using the metagenomic and metatranscriptomic data. The overall profile of the prediction is shown in Additional file 1: Figure S13. Briefly, *rpfC/rpfG* gene cluster was first predicted by HD domain. The HD-GYP domain was used to find *rpfG*, and accordingly *rpfC* was next to *rpfG* in the genome. The *rpfR* genes were predicted with intact PAS-GGDEF-EAL domains. Putative *rpfF* genes were searched for enoyl-CoA hydratase using HMMER website server, and then aligned to filter the ORFs with conservative sites (two well-conserved glutamate residues Glu141 and Glu161 for *Xanthomonas campestris pv. campestris* (Xcc) *rfpF* gene) [26]. Details are provided in Additional file 1: Extended Materials and Methods.

**Prediction of regulator Clp protein and genome scale searching of Clp binding sites**
Clp is an important DSF signal regulator, with a characteristic cNMP-binding domain [31]. The first work was to search cNMP-binding domain for ORFs (detailed information in Additional file 5). After that, sequence alignment was conducted to filter ORFs with conservative sites (a glutamic acid residue (E99) for Xcc Clp gene) as reported earlier [32]. The final ORFs were the homologs of Clp genes.

A PYTHON script was used to parse and match the promoter sequences of the Clp-regulated genes using the putative Clp-binding motif ‘TGTGA-N6-TCACA’ [50]. The promoter region sequence, which was arbitrarily defined as a 500 bp region upstream of an ORF, was obtained from a previous study [31]. A confidence of 60% match to the non-variable sequences of the putative Clp-binding motif is taken as the cut-off value. Only the best match was listed if more than one putative motif were located per promoter.

Declarations
Ethics approval and consent to participate
Not applicable.
Consent for publication
Not applicable.
Availability of data and material
All data associated with 16S rRNA can be found in NCBI under BioProject PRJNA483093, with SRA accession number SRP155452; and the raw data can be found under BioSample accession number SAMN09726107 - SAMN09726133.

All data associated with metagenome and metatranscriptome can be found in NCBI under BioProject PRJNA482907. Illumina HiSeq metagenomic data can be found under BioSample SAMN09714581, and Illumina HiSeq metatranscriptomic data can be found under BioSample SAMN09714594, SAMN09714596, SAMN09714598, SAMN09714599, SAMN09714604, and SAMN09714607. Annotated GenBank files for the whole genome sequences (MAGs) described in this study can be found under the accession numbers listed in Additional file 1: Table S6.

Competing interests
The authors declare that they have no competing interests.

Funding
This work was supported by the National Natural Science Foundations of China [Nos. 51721006, 51922016 and 51878008] for financial support.

Authors’ contributions
YG and SL conceived and designed the study. YG and YZ acquired the data. YG, YZ, XT, TN, and JP analyzed and interpreted the data. YG, YZ, HZ, and SL drafted or revised the article. All authors discussed the results and commented on the manuscript.

Acknowledgements
The authors are grateful to the laboratory of Yawen He (Shanghai Jiao Tong University, China) for kindly providing the DSF biosensor strain FE58.

Authors’ information
1 College of Environmental Sciences and Engineering, Peking University, Beijing 100871, China
2 Key Laboratory of Water and Sediment Sciences, Ministry of Education of China, Beijing 100871, China
3 State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining 810016, Qinghai, China

References
1. Fuqua WC, Winans SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol. 1994;176:269–75.
2. Keller L, Surette MG. Communication in bacteria: An ecological and evolutionary perspective. Nat Rev Microbiol. 2006;4:249–58.
3. Diggle SP, Gardner A, West SA, Griffin AS. Evolutionary theory of bacterial quorum sensing: When is a signal not a signal? Philos Trans R Soc B Biol Sci. 2007;362:1241–9.
4. Feng H, Ding Y, Wang M, Zhou G, Zheng X, He H, et al. Where are signal molecules likely to be located in anaerobic granular sludge? Water Res. 2014;50:1–9.
5. Tan CH, Koh KS, Xie C, Tay M, Zhou Y, Williams R, et al. The role of quorum sensing signalling in EPS production and the assembly of a sludge community into aerobic granules. ISME J. 2014;8:1186–97.
6. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2:95–108.
7. Darch SE, West SA, Winzer K, Diggle SP. Density-dependent fitness benefits in quorum-sensing bacterial populations. Proc Natl Acad Sci. 2012;109:8259–63.

8. Greig D, Travisano M. The Prisoner’s Dilemma and polymorphism in yeast SUC genes. Proc R Soc B Biol Sci. 2004;271:25–6.

9. Van Gestel J, Weissing FJ, Kuipers OP, Kovács ÁT. Density of founder cells affects spatial pattern formation and cooperation in Bacillus subtilis biofilms. ISME J. 2014;8:2069–79.

10. Luján AM, Gómez P, Buckling A. Siderophore cooperation of the bacterium Pseudomonas fluorescens in soil. Biol Lett. 2015;11: 20140934.

11. Pande S, Kaftan F, Lang S, Svato A, Germerodt S, Kost C. Privatization of cooperative benefits stabilizes mutualistic cross-feeding interactions in spatially structured environments. ISME J. 2016;10:1413–23.

12. Guo Y, Liu S, Tang X, Wang C, Niu Z, Feng Y. Insight into c-di-GMP Regulation in Anammox Aggregation in Response to Alternating Feed Loadings. Environ Sci Technol. 2017;51:9155–64.

13. Mee MT, Collins JJ, Church GM, Wang HH. Syntrophic exchange in synthetic microbial communities. Proc Natl Acad Sci. 2014;111:2149–56.

14. Embree M, Liu JK, Al-Bassam MM, Zengler K. Networks of energetic and metabolic interactions define dynamics in microbial communities. Proc Natl Acad Sci. 2015;112:15450–5.

15. Allen RC, McNally L, Popat R, Brown SP. Quorum sensing protects bacterial co-operation from exploitation by cheats. ISME J. 2016;10:1706–16.

16. Pai A, You L. Optimal tuning of bacterial sensing potential. Mol Syst Biol. 2009;5:1–11.

17. Heilmann S, Krishna S, Kerr B. Why do bacteria regulate public goods by quorum sensing?-How the shapes of cost and benefit functions determine the form of optimal regulation. Front Microbiol. 2015;6:767.

18. Pai A, Tanouchi Y, You L. Optimality and robustness in quorum sensing (QS)-mediated regulation of a costly public good enzyme. Proc Natl Acad Sci. 2012;109:19810–5.

19. Kartal B, Kuenen JG, Van Loosdrecht MCM. Sewage treatment with anammox. Science. 2010;328:702–3.
20. van Kessel MA, Stultiens K, Slegers MF, Guerrero Cruz S, Jetten MS, Kartal B, et al. Current perspectives on the application of N-damo and anammox in wastewater treatment. Curr Opin Biotechnol. 2018;50:222–7.

21. Lawson CE, Wu S, Bhattacharjee AS, Hamilton JJ, McMahon KD, Goel R, et al. Metabolic network analysis reveals microbial community interactions in anammox granules. Nat Commun. 2017;8:15416.

22. Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW, et al. Deciphering the evolution and metabolism of an anammox bacterium from a community genome. 2006;440:790–4.

23. Guo Y, Liu S, Tang X, Yang F. Role of c-di-GMP in anammox aggregation and systematic analysis of its turnover protein in Candidatus Jettenia caeni. Water Res. 2017;113:181–90.

24. Tang X, Guo Y, Jiang B, Liu S. Metagenomic approaches to understanding bacterial communication during the anammox reactor start-up. Water Res. 2018;136:95–103.

25. Barber CE, Tang JL, Feng JX, Pan MQ, Wilson TJG, Slater H, et al. A novel regulatory system required for pathogenicity of Xanthomonas campestris is mediated by a small diffusible signal molecule. Mol Microbiol. 1997;24:555–66.

26. Cheng Z, He YW, Lim SC, Qamra R, Walsh MA, Zhang LH, et al. Structural basis of the sensor-synthase interaction in autoinduction of the quorum sensing signal DSF biosynthesis. Structure. 2010;18:1199–209.

27. Zhou L, Zhang LH, Cámara M, He YW. The DSF Family of Quorum Sensing Signals: Diversity, Biosynthesis, and Turnover. Trends Microbiol. 2017;25:293–303.

28. He YW, Zhang LH. Quorum sensing and virulence regulation in Xanthomonas campestris. FEMS Microbiol Rev. 2008;32:842–57.

29. Hengge R. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol. 2009;7:263–73.

30. Deng Y, Schmid N, Wang C, Wang J, Pessi G, Wu D, et al. Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. Proc Natl Acad Sci. 2012;109:15479–84.

31. He Y-W, Ng AY-J, Xu M, Lin K, Wang L-H, Dong Y-H, et al. Xanthomonas campestris cell-cell
communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. Mol Microbiol. 2007;64:281–92.

32. Tao F, He Y-W, Wu D-H, Swarup S, Zhang L-H. The Cyclic Nucleotide Monophosphate Domain of Xanthomonas campestris Global Regulator Clp Defines a New Class of Cyclic Di-GMP Effectors. J Bacteriol. 2010;192:1020–9.

33. Akashi H, Gojobori T. Metabolic efficiency and amino acid composition in the proteomes of Escherichia coli and Bacillus subtilis. Proc Natl Acad Sci. 2002;99:3695–700.

34. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He Y-W, et al. Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc Natl Acad Sci. 2006;103:6712–7.

35. Polz MF, Cordero OX. Bacterial evolution: Genomics of metabolic trade-offs. Nat Microbiol. 2016;1:16181.

36. Molenaar D, Van Berlo R, De Ridder D, Teusink B. Shifts in growth strategies reflect tradeoffs in cellular economics. Mol Syst Biol. 2009;5:323.

37. Basan M, Hui S, Okano H, Zhang Z, Shen Y, Williamson JR, et al. Overflow metabolism in Escherichia coli results from efficient proteome allocation. Nature. 2015;528:99–104.

38. Schertzer JW, Boulette ML, Whiteley M. More than a signal: non-signaling properties of quorum sensing molecules. Trends Microbiol. 2009;17:189–95.

39. Roller BRK, Stoddard SF, Schmidt TM. Exploiting rRNA operon copy number to investigate bacterial reproductive strategies. Nat Microbiol. 2016;1:16160.

40. Ryan RP, Dow JM. Communication with a growing family: Diffusible signal factor (DSF) signaling in bacteria. Trends Microbiol. 2011;19:145–52.

41. Hense BA, Müller J, Kuttler C, Hartmann A. Spatial heterogeneity of autoinducer regulation systems. Sensors. 2012;12:4156–71.

42. Schluter J, Schoech AP, Foster KR, Mitri S. The Evolution of Quorum Sensing as a Mechanism to Infer Kinship. PLoS Comput Biol. 2016;12:1004848.

43. Nadell CD, Drescher K, Foster KR. Spatial structure, cooperation and competition in biofilms. Nat
Rev Microbiol. 2016;14:589–600.

44. Jin RC, Yang GF, Yu JJ, Zheng P. The inhibition of the Anammox process: A review. Chem Eng J. 2012;197:67–79.

45. Whiteley M, Diggle SP, Greenberg EP. Progress in and promise of bacterial quorum sensing research. Nature. 2017;551:313–20.

46. Haruta S, Kato S, Yamamoto K, Igarashi Y. Intertwined interspecies relationships: Approaches to untangle the microbial network. Environ Microbiol. 2009;11:2963–9.

47. Brenner K, You L, Arnold FH. Engineering microbial consortia: a new frontier in synthetic biology. Trends Biotechnol. 2008;26:483–9.

48. APHA. Standard Methods for the Examination of Water and Wastewater, 20th ed. Am Public Heal Assoc Washington, DC, USA. 1998.

49. Finn RD, Clements J, Arndt W, Miller BL, Wheeler TJ, Schreiber F, et al. HMMER web server: 2015 update. Nucleic Acids Res. 2015;43:30–8.

50. Dong Q, Ebright RH. DNA binding specificity and sequence of Xanthomonas campestris catabolite gene activator protein-like protein. J Bacteriol. 1992;174:5457–61.

Figures
Figure 1

Phylogenetic tree of all recovered draft genomes from the anammox bioreactor. Tree includes MAGs recovered from this study (red) and closely related genomes downloaded from the NCBI genome repository. GenBank accession numbers for each genome are provided in parentheses. Branch node numbers represent bootstrap support values.
(a) The changes of abundance for MAGs in anammox community from 16S rRNA sequencing during the reactor operation. Error bars are defined as s.e.m. (n = 3, biological replicates). Significant differences are indicated as follows: *P < 0.05 and **P < 0.01. (b) The rpff gene copies in each MAG genome.
Figure 3

The differences of PN contents (a), PS contents (b), levels of intracellular c-di-GMP (c) of anammox biomass, and levels of supernatant amino acids (d) responding to normal or exogenous DSF-addition conditions in batch assays. Error bars are defined as s.e.m. (for PN/PS/c-di-GMP, n = 15; for amino acids, n = 9; biological replicates). Significant differences are indicated as follows: *P < 0.05 and **P < 0.01.
Predicted peptidases and carbohydrate hydrolases recovered from the MAGs. Number (bubble diameter) of selected peptidases possibly involved in EPS matrix protein degradation (a) and carbohydrate hydrolases possibly involved in PS degradation (b). Peptidases were annotated against the MEROPS database. Carbohydrate hydrolases were annotated against the CAZy database. The subcellular location (extracellular, outer membrane or periplasm) of each peptidase was predicted using the subcellular localization predictor (CELLO).
(a) Specific amino acid auxotrophies present in each MAG for anammox community. Amino acids have been ranked according to biosynthetic cost (arrow). A colored square denotes that a species can synthesize an amino acid. The intensity of each color (based on the scale) represents the ratios of DSF-addition to control based on the relative expression of the synthesis pathways (Methods). Significant differences are indicated as follows: *P < 0.05 and **P < 0.01. (b) Number (bubble diameter) of amino acid and peptide transporters
predicted across the recovered genomes. Transporters were annotated against the transporter classification database (TCDB).

Figure 6

Proposed interactions via DSF-regulated public goods between DSF-QS and DSF-Secretor MAGs in the anammox community. Orange arrows indicate DSF signaling in the anammox community; purple arrows indicate amino acid production and exchange; light blue arrows indicate EPS production, degradation, and utilization. The presence of a periplasm has been ignored for clarify of the schematic.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

Additional file 1.doc
Additional file 5.xlsx
Additional file 3.xlsx
Additional file 4.xlsx
Additional file 2.xlsx