Evidence of differential adaptation to decreased temperature by anammox bacteria

Running title: The proteome response of anammox bacteria to low temperature

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Summary

Low temperature is recognized as one of the major barriers for the application of the anaerobic ammonium oxidation (anammox) process to treat mainstream wastewater. Studies are yet to reveal the underlying biological limitations and molecular mechanisms associated with the inhibition of low temperature on the anammox process. In this study, metaproteomics was used to examine proteome modulation patterns of the anammox community occurring at different temperatures. The anammox community remarkably altered their proteomes when the temperature decreased from 35 to 20°C. This was especially for proteins involved in energy conversion, transcription and translation, and inorganic ion transport. However, at 15^{o}C the anammox activities became distinctly inhibited, and there was evidence of energy limitations and severe stress in *Candidatus* Kuenenia and to a lesser degree in *Candidatus* Brocadia. *Candidatus* Jettenia exhibited more changes in its proteome at 15°C. From the proteomes, at the lower temperatures there was evidence of stress caused by toxic nitrogen compounds or reactive oxygen species in the anammox bacteria. Hydroxylamine oxidoreductase (HAO)-like proteins and an oxidative stress response protein (a catalase) were in high abundance to potentially ameliorate these inhibitory effects. This study offers metaproteomic insight into the anammox community-based physiological response to decreasing temperatures.

Originality-Significance Statement.

Low temperature is recognized as one of the major barriers for the application of the

anammox process to treat mainstream wastewater. However, the underlying molecular mechanisms are not well understood currently. In this study, metaproteomics was used to examine proteome modulation patterns of the anammox community occurring at different temperatures. This study is the first to identification of proteome modulation of anammox bacteria, which allows us to fill the important gap between macroscopic characterization of anammox bacteria and underlying proteome modulation mechanisms response to the thermal decreases. Also, the analysis framework developed herein could be extended to other functional cultures and environmental samples.

Introduction

The anaerobic ammonium-oxidizing (anammox) bacteria, discovered in the early 1990s (Mulder et al., 1995; Strous et al., 1999a), form a deep-branching, monophyletic group within the Planctomycetes phylum. These are reported to anaerobically oxidize ammonium to dinitrogen gas with nitrite as an electron acceptor (Kuenen 2008; Strous et al., 2006). For the anammox reactions, the first step is the reduction of nitrite to nitric oxide by nitrate reductase (NirS). Then ammonium reacts with nitric oxide, catalyzed by hydrazine synthase (HZS), to form hydrazine. Finally, hydrazine is oxidized into dinitrogen gas by hydrazine dehydrogenase (HDH) or hydroxylamine oxidoreductase (HAO) (Strous et al., 2006). Anammox bacteria redetected in various environments including wetlands, open oceans and marine sediments, and wastewater treatment plants (WWTPs) (Dale et al., 2009; Kuypers et al. 2003, Lam et al., 2007; Kuenen 2008; Lackner et al., 2014; Nicholls and Trimmer 2009; Penton et al., 2006; Zhu et al., 2011). The anammox process plays a significant role in global nitrogen cycling. Impressively, it is estimated that up to 67% of dinitrogen gas production is derived from the anammox process in marine oxygen-minimum zones (Dalsgaard et al., 2005; Francis et al., 2007). In addition to its significant ecological role, the application of anammox bacteria can provide an improved sustainable solution for nitrogen removal from wastewater. Compared with conventional nitrification-denitrification treatment processes, the anammox-based process has a number of operational and economic advantages that include less sludge production, no requirement of organic carbon, less aeration and lowered greenhouse

gas emissions (Kartal et al., 2010; Lackner et al., 2014).There are increasingly more WWTPs employing anammox to treat both sidestream and mainstream wastewater worldwide.

However, it is observed that anammox bacteria can be very sensitive to fluctuations of temperature in engineered ecosystems (Strous et al., 1999b), and this may limit application of the process at WWTPs in countries with cooler climates (Cao et al., 2017; Vlaeminck et al., 2012). For example, it is reported that the nitrogen removal performance of an anammox process was dramatically lowered ten times when the temperature decreased from 29 to 12.5^{o}C (Laureni et al., 2015). Although low temperature is recognized as a critical barrier for the application of anammox for mainstream wastewater treatment, little is known about the underlying molecular mechanisms behind this limitation. Studies of this temperature effect have mainly focused on characterizing the overall nitrogen removal performance, or developing control strategies to improve the process stability under low temperatures (Dosta et al., 2008; Gilbert et al.,2014; Lotti et al., 2014; Lotti et al., 2015). There are few studies attempting to elucidate the molecular details of anammox bacteria in relation to temperature (Rattray et al., 2010). Currently, there are no studies that are focused to understand the response of anammox bacteria to temperature changes at the proteomic level.

Recently, mass spectrometry based metaproteomic approaches are attracting particular attention for discovering microbial functions in mixed culture systems (Barr et al., 2015; Mosier et al., 2015; Salerno et al., 2016; de Almeida et al., 2016). mRNA levels can respond rapidly and sensitively to environmental conditions changes, while changes in protein abundances can be comparatively much slower (Zhu et al., 2017). The correlation between mRNA and protein inventories in environmental microbial communities can be generally week (Zhu et al., 2017). It can be observed that mRNA levels do not always coincide with bacterial activities (Wang et al., 2016). Whereas protein expression can reflect specific microbial activities in a given ecosystem (Wilmes and Bond, 2006). Metaproteome expression states can be used to infer the actual functionality of an ecosystem, and may be more directly related to the microbial activity in comparison to information based on metagenomics (functional gene detection) or metatranscriptomics (mRNA expression) (Wilmes and Bond, 2006). Proteomics using isobaric tags for relative and absolute quantification (iTRAQ) has advanced through the improvement of measurement precision, accuracy and reproducibility (Ross et al., 2004). The approach has proven to be effective for conducting quantitative proteomic comparisons within complex environmental samples. Recently, a quantitative proteomic approach (similar to iTRAQ) was applied to discover the impacts of elevated temperatures on the physiology of individual microbial groups in acid mine drainage biofilms (Mosier et al., 2015).

Mass spectrometry based protein identification relies upon an accurate database,

which is typically derived from the functional prediction of open reading frames (ORF) detected in genome sequence data. Currently, the genomes and corresponding protein sequences are available for anammox bacteria belonging to the genera of *Candidatus* Kuenenia (one genome, PRJNA16685) (Strous et al., 2006), *Candidatus* Brocadia (two genomes, PRJDB103 and PRJNA263557) (Ferousi et al., 2013; Oshiki et al., 2015), *Candidatus* Jettenia (one genome, PRJDB68) (Hira et al., 2012) and *Candidatus* Scalindua (one genome, PRJNA262561) (Speth et al., 2015). In mixed culture systems, an organism's response to changing conditions will be associated with its behavior within the microbial community. Thus, the understanding of functional and physiological changes needs to be determined within the ecosystem community. Consequently, the metaproteomics approach is most suitable to study the anammox microbial community and to gain insight for predicting the impact, resilience, and response of the anammox process at sub-optimal temperatures.

This study investigates the underlying molecular details of decreased temperature-induced physiological changes of bacteria directly in the anammox community. A lab-scale sequence batch reactor (SBR) was operated for over 450 days to obtain an enriched anammox culture (80% anammox bacteria). Multi-plexiTRAQ proteomic analysis was performed on six samples of an anammox culture that were exposed to temperatures ranging from the optimum of 35^{o}C (Van de Graaf et al., 1996) to lower temperatures of 20°C and 15°C where stress may occur (Cao et al., 2017). Our findings provide insight of the response of anammox bacteria to temperature

changes within the community ecosystem. Understanding of proteome modulation patterns of the anammox community occurring at different temperatures will lead to process optimization by calibrating operational parameters and by enhancing preferring anammox pathways, which will result in robust anammox reactions even at relatively low temperate.

Results and Discussion

Reactor characterization and biomass response under low temperature

A laboratory-scale SBR was operated for more than 450 days to enrich anammox bacteria. The operating temperature of the SBR was maintained at 35 ± 1°C. Quasi-steady state performance of the anammox system was confirmed by stable anaerobic nitrogen removal, which was maintained at approximately 0.22 ± 0.01 g N/g MLVSS/day. During this quasi-steady state, the influent contained around 360 mg/L NO_2^--N, and 300 mg/L NH_4^+-N, both of which were completely converted with $\triangle NO_2^-$-N: $\triangle NH_4^+$-N at around 1.26 (Figure S1, Supporting Information (SI)). Fluorescence *in situ* hybridization (FISH) indicated that anammox bacteria accounted for up to 80% of the total microbial community. Bacteria of the genera *Candidatus Kuenenia* and *Candidatus* Brocadia accounted for around 50% and 22% of the total community, respectively. Bacteria of the genus *Candidatus* Scalindua were not detected by FISH, indicating their low abundance or absence in the anammox enriched culture. The SBR performance and FISH results indicate a highly-enriched anammox culture was obtained. This enriched culture was suitable for the temperature

exposure experiments and the subsequent comparative investigations by quantitative proteomics.

The anammox activities of the enrichment culture were determined after being subjected to the temperatures of 15 °C, 20°C or 35 °C. These were conducted in duplicate in a total of six sequencing batch incubations that lasted 15 days. The specific ammonium oxidation rates were determined for each of the batch incubations. At 20°C this rate was approximately half of that at 35°C (1.74 ± 0.24 vs. 3.71 ± 0.52 mg N/g MLVSS/ h). At 15°C, the ammonium oxidation rate was much lower at about one-tenth of that observed at 35°C (Figure 1a). The corresponding specific nitrite reduction rates were also determined and were suppressed at the lower temperatures (Figure 1b). In these incubations, the observed nitrogen transformations were resulting from the activities of the mixed-culture anammox bacteria. To date, pure cultures of anammox organisms are yet to be isolated (Jetten et al., 2001; 2005). Consequently, application of molecular omic approaches are required to distinguish the nitrogen transformation pathways mediated by the different anammox species (Hu et al., 2012; Oshiki et al., 2015; Strous et al., 2006).

Metaproteomic overview

For the protein identifications, this study used a manually curated database constructed from the genome sequences of the anammox genera of *Candidatus* Kuenenia (Strous et al., 2006), *Candidatus* Brocadia (Ferousi et al., 2013; Oshiki et

al., 2015), *Candidatus* Jettenia (Hira et al., 2012) and *Candidatus* Scalindua (Speth et al., 2015). After the temperature exposure experiments of the batch incubations that were operated for 30 cycles (15 days), samples from the three temperature conditions (in total 6 samples) were collected for protein extraction and subsequent identification and quantification. Using the six-plexiTRAQ proteomic approach, we identified 1539 anammox proteins (Table S1, SI). This is comparable with a recent metaproteomic study that used a similar peptide labeling approach for investigating laboratory-grown acid-mine drainage biofilms. In that instance, a total of 1724–1916 proteins were identified, which included 1596 uniquely assigned to one organism (Mosier et al., 2015). It is worth to note that the abundance of proteins assigned to the different anammox genera within the three cultures was very similar (Table S2, SI). Similar to the FISH results, this indicated the cultures were dominated by *Candidatus* Kuenenia (~ 50%) and *Candidatus* Brocadia (~ 32%). Consequently, at the different enrichment temperatures, there seems to be little change regarding the main anammox community composition.

Principal component analysis of the 1539 protein abundance values detected in the sequence batch incubations operated at 15°C, 20°C and 35°C was performed (Figure 2). It can be seen, regarding protein abundance, that the 35 ˚C cultures were well separated from the 15 and 20 ˚C cultures, and this is along the axis showing the most differences, component 1 (Figure 2). These findings imply that the proteins produced by the enriched anammox bacteria are regulated by temperature changes and

potentially the different strains may be preferentially active at different temperatures.

The metaproteomic analysis showed that heme-containing proteins were among the top 10 most abundant protein species detected at 35°C (Table 1). Many of the abundant proteins detected were associated with the anammox central catabolism for nitrogen conversions. This included nitrite reductase, hydrazine synthase and hydrazine dehydrogenase (Table 1). The high levels of these proteins implicate their importance in the roles of growth and maintenance for anammox bacteria.

Proteome changes of the anammox enriched culture with decreasing temperature

The metaproteomes were compared to identify those proteins that were statistically enriched in either the low or high temperature. A large number of proteins were differentially abundant at 20°C relative to 35 °C, followed by those at 15°C relative to 35°C. There were fewer significant changes in protein abundance between cultures grown at 20°C and 15°C. There were 90 differentially abundant proteins detected between 35°C and 20°C, 71 were detected between 35°C and 15°C, and 13 between 20°C and 15°C.

The majority of proteins showing significant changes in abundance at lower temperatures belonged to the following Clusters of Orthologous Groups (COG) functional categories: energy production and conversion; translation, ribosomal

structure and biogenesis; inorganic ion transport and metabolism; replication, recombination and repair; and posttranslational modification, protein turnover and chaperones (Figure 3). In particular, at 20°C relative to 35°C, more than five times as many proteins involved in energy production and conversion (C), more than twice as many proteins involved in translation, ribosomal structure and biogenesis (J), and three times as many proteins involved in inorganic ion transport and metabolism (P), were found to have increased abundance in comparison to those with decreased abundance (Figure 3).

Three anammox genera (*Candidatus* Kuenenia, *Candidatus* Brocadia and *Candidatus* Jettenia) were selected for further analysis due to their overall abundances in the present enriched cultures. Protein abundance was determined by normalizing individual proteins to the total protein abundance for each specific anammox genus. This allowed for evaluation of protein abundance within the individual genus.

Overall, among the three anammox genera 70 proteins had significant changes in abundance between 35°C and 20°C (Figure 4a–c), 42 proteins had significant changes between 35°C and 15°C (Figure 4d–f), and only seven proteins showed significant changes in abundance between 20°C and 15°C. The proteins with altered abundances spanned a broad range of functional COG categories (Figure 4).

All proteins quantified for the genus *Candidatus* Kuenenia were affiliated to the species *K. stuttgartiensis*. At 20°C relative to 35°C, 39 proteins of *K. stuttgartiensis* displayed significant changes in abundance (Figure 4a), this included 32 with increased and 7 with decreased abundances. Proteins involved in energy production and conversion (C), translation/ribosomal structure and biogenesis (J), and inorganic ion transport and metabolism (P) outnumbered those from other processes (Figure 4a). Eight other proteins also showed significant alterations in abundance; however, their functions were unknown (Figure 4a). At 15°C relative to 35°C, only 9 *K. stuttgartiensis* proteins showed significant changes in abundance, this included 4 with increased and 5 with decreased abundances (Figure 4d). For *Candidatus* Brocadia, 15 proteins had significant changes in abundance at 20°C compared to 35°C, including 9 with increased and 6 with decreased abundances (Figure 4b). The majority of these proteins were involved in energy production and conversion (C), amino acid transport and metabolism (E), posttranslational modification, protein turnover, chaperones (O), and signal transduction mechanisms (T) (Figure 4b). Compared to proteins at 35 °C, 5 *Candidatus* Brocadia proteins had increased abundance, while 8 were decreased at 15 °C (Figure 4e). Energy production and conversion (C) was also the most significantly affected process at 15°C (Figure 4e). Regarding *Candidatus* Jettenia, in total 16 proteins had significantly changed abundance at 20°C in comparison to 35°C (Figure 4c). Among these, 14 had increased abundance, while only 2 proteins had decreased levels (Figure 4c). The majority of the proteins with significant changes in abundance were involved in energy production and conversion (C),

translation/ribosomal structure and biogenesis (J), and transcription (Figure 4c). At 15°C relative to 35°C, 20 proteins had significantly altered levels for *Candidatus* Jettenia (Figure 4f). Most of these proteins were associated in the categories of energy production/conversion (C) and signal transduction mechanisms (T) (Figure 4f).

The dominant anammox bacteria had altered proteome profiles at the different temperatures of 35°C and 20°C. Particularly, the levels of proteins involved in energy production/conversion (C), transcription (K) and translation (J) were significantly altered. The differences in protein abundances of *Candidatus* Kuenenia, *Candidatus* Brocadia and *Candidatus* Jettenia, at 20°C relative to 35°C, were generally similar. A large number of proteins were significantly modulated, among which, the proteins with increased levels overwhelmingly outnumbered those with lowered abundance at the lower temperatures (Figure 4a–c). The results suggest that the anammox bacteria were remarkably altering their molecular processes to enable them to adapt to the effects of the decreased temperature.

Adaptations of *Candidatus* Kuenenia to lower temperature

In the SBR enrichment culture *Candidatus* Kuenenia was the most abundant of the bacteria, and the largest number of quantified proteins were detected against this anammox organism in the batch Incubations (Table S1, SI). In addition, all proteins quantified for this genus came from one species, *K. stuttgartiensis*, which allowed a

more detailed investigation of the temperature related proteome changes at the species level.

For *K. stuttgartiensis*, no proteins associated with the central anammox reactions, i.e. NirS, HZS and HDH, were significantly altered during the batch incubations at 35 and 20°C. Consequently, the stable levels of these key enzymes might be an adaption strategy by anammox bacteria, to maintain constitutively high levels across a range of temperatures. This strategy could maximize relevant metabolic activities at sub-optimal temperatures. In contrast, proteins involved in downstream electron transport networks had increased levels at the lower temperature. Among them, protein kuste2877 had similarities to an undeca-heme-containing cytochrome c protein, while kustc0457 was identified as a hydroxylamine oxidoreductase (HAO)-like protein (Table 2). Increased levels of these electron transport proteins suggest that parts of their energy harvesting processes are sensitive to decreased temperature. It is reported that low temperatures can impede electron transfer to cause electron imbalance at certain points of the respiratory chain, leading to production of reactive oxygen species (ROS) in aerobic microorganisms (Moreno-Sánchez et al., 2013). Although *K. stuttgartiensis* are obligate anaerobes, the proteome expression profile at 20°C indicates an analogous scenario. It is possible that the downstream electron transfer proteins are more susceptible to decreased temperature than the anammox central enzymes, and this could cause in an electron transfer imbalance. Electrons of anaerobic reactions usually have low redox potential, and during

imbalanced conditions, these maybe prone to be adventitious transfers (Imlay, 2003). In addition, there is a potential for establishment of the Fenton reaction with iron released from damaged or digested Fe-S cluster proteins. This would contribute to production of hydroxyl radicals (Imlay, 2003). Such events could account for the increase of the protein kustd1301 (Table 2), which has high similarity to catalase, an oxidative stress response protein that decomposes H_2O_2 into H_2O and O_2 (catalytic activity), and oxidizes H donors with consumption of peroxide (peroxide activity) (Aebi, 1984). Consequently, kustd1301 is likely contributing to alleviate oxidative stress in conditions of lower temperature.

The *K. stuttgartiensis* genome encodes 10 different *hao*-like paralogs (de Almeida et al., 2011; Kartal et al., 2010) and these HAO-like proteins dominated the anammox bacterium proteome. Potentially these catalyze the three-electron oxidation of hydroxylamine to nitric oxide and nitrite (Maalcke et al., 2014; 2016). At 20°C, the reduced anammox reaction rates (when compared to that 35°C) may result in nitrite accumulation in both the cytoplasm and the anammoxsome. In the absence of corresponding upregulation of nitrite transporters (either uptake or efflux), the increased HAO activity may serve as a pathway for controlling nitrite levels in cell envelopes, performing the reversed reactions mentioned above, and thereby avoiding the potential damage induced by excessive nitrite. Consequently, this could explain the increased expression of HAO-like proteins at 20°C (Table 2). In addition, *hao*-like proteins are speculated to utilize hydrazine, although at lower catalytic rates than

hydroxylamine conversions (Maalcke et al., 2014; Kartal et al., 2011). This could be part of the organisms strategy for managing levels of inhibitory nitrogenous compounds (e.g. nitric oxide, nitrite and hydroxylamine) (Kartal et al., 2011). In addition to balancing these nitrogenous compound levels, the increased levels of HAOs are likely involved in providing nitric oxide for anammox reactions at the lower temperature of 20°C.

Candidatus Jettenia exhibits a greater proteome response at lower temperature, compared to _Candidatus_ Kuenenia and _Candidatus_ Brocadia

Decreasing the temperature to 15°C had severe adverse effects on the activities of the enriched anammox culture with much reduced ammonium oxidation rates detected (Figure 1). Generally, it may be expected that an increased stress condition, such as lower temperature, would induce a larger number of proteins with changes in abundance (Mosier et al., 2015). Surprisingly, the total number of significantly altered _Candidatus_ Kuenenia proteins at 15°C (Figure 4d) was much smaller than that at 20°C (9 and 39, respectively). Of these 9 proteins, kustc0824 and kustc0827 had significantly decreased levels and these have high similarity to subunits of the proton-translocating NADH dehydrogenase I, NuoC and NuoF. NADH dehydrogenase I is a key electron transfer unit that translocates two H^+/e^-(Friedrich et al., 1995), for generating a proton motive force (PMF). As anammox bacteria conserve energy by utilizing the PMF, decreased levels of this NADH dehydrogenase would result in reduced energetic capabilities and reduced cellular activities. Thus,

during low-temperature stress conditions, cells may shut down most physiological processes and enter into a stagnant state (Valledor et al., 2013). Additionally, this may result in the organism's proteome remaining largely unchanged and explain why fewer proteins had significantly altered levels at 15°C. The results suggest that *Candidatus* Kuenenia is energy limited at this low temperature, which could explain the much lower levels of the ammonium oxidation and nitrite reduction rates detected in the culture (Figure 1a).

The proteome profile of *Candidatus* Brocadia at 15°C relative to 35 ˚C was seen to feature more proteins with lowered abundance in comparison to proteins with increased levels (Figure 4e). However, two proteins associated with translation, the 50S ribosomal proteins L11 and L17, two HAO-like proteins and one aldehyde dehydrogenase had significantly higher levels at the lower temperature. Potentially, the increased HAO-like proteins may be serving to relieve the cells of inhibitory nitrite levels, such as was observed at 20°C. Aldehyde dehydrogenase belongs to a family of $NAD(P)^+$-dependent enzymes with a broad substrate specificity that catalyze the oxidation of various toxic aldehydes to carboxylic acids (Perozich et al., 1999). Thus, at the lower temperature the increased levels of the dehydrogenase may serve as a defense strategy for the detoxification of excessive aldehydes.

In contrast, the proteome profile for *Candidatus* Jettenia was very different to those of the other two anammox genera. Twenty proteins were significantly altered at 15 °C

relative to 35 °C, which is greater than the number (16) of significantly altered proteins at 20 °C relative to 35 °C (Figure 4c, f). Compared to 35°C, chaperonin GroEL was significantly more abundant at 15 °C (Table 3). The chaperonin is involved in restoring protein structure and stability (Hendrick and Hartl, 1993). Protein folding and stability are both temperature-dependent, and *in vitro* studies have characterized cold denaturation in addition to the better-known heat denaturation (Dill et al., 1989; Privalov, 1990). The increased levels of the chaperonin suggest that the low temperature denatured proteins, and that *Candidatus* Jettenia was responding to maintain accurate protein structure.

At low temperatures, it is possible that energy conservation processes are compromised. This is likely occurring here for *Candidatus* Jettenia, as we detected increased levels of five proteins from the energy production and conversion COG category (Figure 4f). These proteins included a pyruvate ferredoxin/flavodoxin oxidoreductase, a putative cytochrome c, an F_0F_1-ATP synthase B subunit, a dihydrolipoamide dehydrogenase, and a hydroxylamine oxidoreductase (Table 3). The high levels of pyruvate ferredoxin/flavodoxin oxidoreductase at low temperatures would support the metabolism of pyruvate, which is a central metabolite involved in various anabolic and catabolic reactions. This activity would provide acetyl-CoA and carbon for other physiological processes. The high levels of HAOs at 15°C would enable the detoxification of inhibitory nitrogenous compounds. These increased

activities are likely improving the energetic capacity of these anammox bacteria at the low temperature.

Low temperature can adversely affect transcription by impeding the unwinding of the DNA, making it difficult for RNA polymerase to bind (Feller and Gerday, 2003). Indeed, we identified increased levels of the RNA polymerase sigma 70 subunit (RpoD) at the lower temperature of 15°C (Table 3). RpoD is an indispensable component of the transcriptional machinery, binding to the DNA, and higher levels of this may ensure efficient transcription. Low temperature would also pose problems for translation (Jones and Inouye, 1994). High levels of translation initiation factor (IF-3) were observed in the current study in the culture maintained at 15°C (Table 3). These observed changes suggest that this anammox bacterium, *Candidatus* Jettenia, was altering its transcription and translational abilities to adapt to the low temperature.

The intention of the sequencing batch incubations at the different temperatures was to detect proteome wide changes from the different bacteria in the anammox enrichment. These were short term incubation experiments (15 days) and were not designed to cause microbial community changes. Indeed, our metaproteomic analysis indicates that the communities remained similar at the different temperatures, and this is logical given the potential slow growth of these organisms (Strous et al., 1999b). However, we did detect differences in the genus specific proteome profiles at the low temperature, in particular there was evidence that more changes of *Candidatus*

Jettenia proteome was observed at 15˚C in comparison to the other two anammox genera studied here. Consequently, different anammox bacteria may have particular advantages and be selected for at different temperatures. It is seen that anammox bacteria isolated from wastewater treatment reactors have an optimum temperature of about 35°C (Strous et al., 1999b), e.g. *Candidatus* Brocadia anammoxidans (Schmid et al., 2005) and *Kuenenia stuttgartiensis* (Schmid et al., 2000). However, anammox bacteria in natural ecosystems such as Northern European soils and marine sediments, thrive at low temperatures, for example *Candidatus* Scalindua spp., are detected in environments at<10 °C (Van De Vossenberg et al., 2008).

In summary, a metaproteomic analysis was used to detect the impact of different temperatures on the global protein expression profiles of an enriched anammox culture, which allows us to fill the important gap between macroscopic characterization of anammox bacteria and underlying proteome modulation mechanisms response to the thermal decreases. We detected the key responses of the anammox community when decreasing the temperate from 35°C (Figure 5). In particular, it was seen that at the lower temperature of 20°C the integrity of the proteins within anammox bacteria was maintained through degradation and recycling rather than through the repair of malfunctioning proteins. At the lower temperatures of 20°C and 15°C, the anammox bacteria maintained constant levels of most key enzymatic proteins involved in the central anammox reactions, this included nitrite reductase, hydrazine synthase, and hydrazine dehydrogenase. At the lower

temperatures, stress due to toxic nitrogen compounds and/or reactive oxygen species is likely occurring within the anammox bacteria. HAO-like proteins and an oxidative stress response protein (a catalase) were at significantly higher levels to compensate for the adverse effects. Additionally, many of the proteins with significantly altered levels at the lower temperatures had unknown functions, and these could be interesting targets for future characterization.

It is likely that anammox bacteria from distinct genera mutually benefit each other in mixed culture in natural or engineered ecosystems (Guo et al., 2016; Luo et al., 2017). Additionally, niche differentiation of anammox bacteria would allow for asynchronous responses to fluctuating conditions, which would assist to maintain function of the community across changing environments. Importantly, our study revealed that the different anammox genera reacted differently at the lowered temperature. This was especially at 15°C where limited proteome wide changes were detected for *Candidatus* Kuenenia and *Candidatus* Brocadia. In comparison, *Candidatus* Jettenia exhibited many changes of protein abundance at the low temperature. This supports the hypothesis that different anammox bacteria will be favored at different temperatures. This is particularly significant in the optimization of the operation and performance of WWTPs and for understanding anammox activities in natural environments.

Indeed, when regarding the operation performance of anammox bioreactors, many

studies report that when the temperature is lowered the reactor performance can deteriorate at first and then after time improve or at least stabilize. This is the case when the final lowered temperature was higher than 12°C (Dosta et al., 2008; Hu et al., 2013; Hendrickx et al., 2012). However, when the temperature is further decreased to be less than 10°C, the reactor performance is reported to deteriorate and finally result in failure of anammox (Lotti et al., 2014). It is not clear how anammox bacteria would respond the long-term exposure of low temperature at the protein level in those studies, but likely the culture would experience change to the anammox community composition as we suggest herein.

For the full-scale operation of anammox process, the strategy to avoid performance deterioration is to keep the temperature stable or above an appropriate level. However, this is not practically useful for anammox implementation in cold regions. In the next steps, we need to consider how to optimize the reactor operation under low temperature. This may be achieved by further understanding these molecular mechanisms with a view to manipulate those.

Experimental procedures

Reactor operation

Anammox bacteria enrichment cultures were generated within a laboratory-scale anammox sequencing batch reactor (SBR) (15 L). In the SBR the mixed liquid volatile suspended solid (MLVSS) concentration was maintained at approximately 5.5

g/L, the temperature was at 35 ± 1°C and the pH controlled at 7–8. The SBR was operated with three cycles per day (8 h for each cycle), that consisted of five consecutive phases of: filling (20 min), anoxic mixing (360 min), settling (60 min), effluent decanting (20 min), and an idle phase (20 min). The decanting volume was 50% of the working volume. The synthetic medium contained 640 mg/L of nitrogen, which was provided as NH_4^+ (nitrogen: 280 mg/L) and NO_2^- (nitrogen: 360 mg/L). The other mineral components of the medium were as described previously (Van de Graaf et al., 1996). The reactor was operated for more than 450 days.

Fluorescence *in situ* hybridization (FISH)

Fixation and hybridization of biomass samples were conducted as previously described (Joss et al., 2011; Nielsen et al., 2009). Prior to hybridization, the fixed samples were placed on ice and homogenized by ultrasonic dispersion to disrupt large aggregates. The FISH probes used were: EUB338 mix for the detection of all bacteria cells (Daims et al., 1999); Amx368, specific for the currently known anammox genera (Schmid et al., 2003); Amx820, specific for *Candidatus* Kuenenia and *Candidatus* Brocadia (Schmid et al., 2003); KST157, specific for *Candidatus* Kuenenia (Schmid et al., 2003); Ban162 (Schmid et al., 2001) and Bfu613 (Kartal et al., 2008), specific for *Candidatus* Brocadia; and Sca1309 (Schmid et al., 2003), specific to *Candidatus* Scalindua (Table S3, SI). FISH preparations were visualized with a Zeiss LSM 510 Meta confocal laser-scanning microscope (CLSM) using a Plan-Apochromat 63× oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The

percentage of anammox bacteria was determined via FISH image analysis using image-analyzing software (Image-Pro Plus, V6.0, Media Cybernetics). The standard error of the mean (SE_{mean}) was calculated as the standard deviation divided by the square root of the number of images. The detailed procedure of FISH is described in the Supporting Information.

Temperature treatments of the anammox enrichment

Anammox bacteria enrichment culture (2L) was taken from the parent SBR during one idle phase, and then washed with 1x Phosphate Buffered Saline to remove residual components. Fresh medium was introduced to make a total volume of 3.6 L, and the mixed culture was divided equally into six aliquots of 600ml into 1000ml serum bottles. Initially, all bottles were purged with an Ar/CO_2 (95/5%) gas mixture to establish anoxic conditions, the pH was adjusted to 7.2 and the bottles were then sealed with gas-tight rubber stoppers. Groups of two reactor bottles were incubated at either 35°C, 20°C or 15°C and shaken at 200 rpm. Media was added to the bottles to achieve nitrogen concentrations of 80 mg/L nitrogen from NO_2^-, and 110 mg/L nitrogen from NH_4^+ (other mineral components of the media were identical to those in the parent SBR). The reactor bottles were incubated for a total of 15 days during which two incubation cycles per day were performed. At the end of each cycle, half of the medium was carefully replaced with fresh medium. To do this, the reactor medium was forced out through a needle immerged in the liquid culture (internal diameter 2mm) by providing high purity dinitrogen gas through another needle (0.6 mm

internal diameter) positioned in the upper gas phase of the bottle reactor. Both needles were stainless steel, and fixed through the upper gastight rubber stopper. Following that fresh media was injected into the bottle reactors using a 500ml syringe connected to a needle immerged in the liquid phase, while gas was released through another needle inserted into the rubber stopper. Anoxic conditions were maintained in the reactor bottles by purging the media with Ar/CO_2 (95/5%) gas mixture and maintaining gastight conditions. The reactor pH was monitored at the end of each cycle and adjusted to around 7.2 with hydrochloric acid and sodium bicarbonate. Stable biomass concentrations were maintained in the bottle reactors during the incubation period as the MLVSS measured at the experiment start and end was approximately 2000 mg/L. Analysis of the nitrogen species and MLVSS of the cultures were conducted as described previously (Wang et al., 2016). At the 30^{th} cycle, sludge samples of approximately 0.5 g were taken from each bottle reactor maintained at the three temperatures (six reactors in total included biological duplicates) for the 6-plex iTRAQ proteomic analysis.

Protein extraction and preparation

Proteins were extracted from the temperature-treated anammox bacteria enrichment cultures (35°C, 20°C, 15°C) using an SDS protein extraction protocol based on a previously reported method (Chourey et al., 2010). Following protein digestion by trypsin, peptide-labeling using the iTRAQ 6-plex labeling reagents (AB SCIEX, Foster City, California, USA) was performed according to the manufacturer's

instructions. The labeled peptides were fractionated by ultra-performance liquid chromatography using a C18 column (Waters BEH, C18, 2.1x50mm,1.7μm) (Waters Corporation, Milford, Massachusetts, USA). The absorbance at 214 nm was monitored, and a total of 10 fractions were collected (the detailed protein extraction and preparation procedures are in the Supporting Information).

Protein identification and quantification

Each fraction was separated by nano-high performance liquid chromatography (Eksigent of AB Sciex, California, USA) on a secondary reversed-phase analytical column (Eksigent, C18, 3μm, 150mmx 75μm) according to the manufacturer's instructions. Subsequently, peptides were eluted using a 5–45% gradient of solvent B (98% ACN with 0.1% formic acid), over 5–100 min. The total flow rate was maintained at 300 nL/min. An electrospray voltage of 2.5 kV versus the inlet of the mass spectrometer was used. The Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) was operated in information-dependent data acquisition mode to switch automatically between mass spectrometry (MS) and tandem mass spectrometry (MS/MS) acquisition. MS spectra were acquired across the mass range of 350–1250 m/z. The 10 most intense precursors were selected for fragmentation per cycle, with a dynamic exclusion time of 30s. The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset accession numberPXD006032.

Protein database, mapping and analysis

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.0). Mascot was set up to search the concatenated database containing 27,827 protein sequences determined from the genome sequences of *Candidatus* Kuenenia, *Candidatus* Brocadia, *Candidatus* Jettenia, and *Candidatus* Scalindua. Mascot searches were conducted with a fragment ion mass tolerance of 0.05 Da, and a parent ion tolerance of 10.0 PPM. Carbamidomethyl cysteine and iTRAQ 6-plex labeling of lysine at the N-terminus were specified in Mascot as fixed modifications. Oxidation of methionine and iTRAQ 6-plex labeling of tyrosine were specified as variable modifications.

Scaffold (version Scaffold_4.4.5, Proteome Software Inc., Portland, OR, USA) was used to validate the MS/MS based peptide and protein identifications. Peptide identification was accepted at a false discovery rate of <1.0% as determined by the Scaffold Local FDR algorithm. Protein probabilities were assigned by the Protein Prophet algorithm (Searle, 2010). Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least 2 identified peptides. Functional categories of identified proteins were determined using the Clusters of Orthologous Groups (COG) database and the BLAST algorithm. Differentially abundant proteins were defined as those with normalized total intensity ratios that were either ≥ 1.2 or ≤ 0.8, and having a Rank Product P-value <0.05 (Mosier et al., 2015). In this study, we assumed that the total amount of protein per

cell is stable, in the cultures incubated at the different temperatures. Protein abundance was normalized at the community level to determine each protein's abundance relative to all proteins in the sample. This normalization just considers biomass differences between samples, rather than differences in each organism's abundance. When analyzing the changes of protein abundance at the genus level, protein abundance was determined by normalizing individual proteins to the total protein abundance from each specific anammox genus within a same sample.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1. The ten most abundant proteins quantified from three anammox genera within the sludge incubated at 35°C

Accession	Gene tags	Identified Proteins	MS signal intensity
***Candidatus* Kuenenia**			
gi|91202984	kustd1878	Porin	2.09E+08
gi|564731309	—	Chain A, Hydroxylamine Oxidoreductase	1.11E+08
gi|939186673	—	Chain A, Hydrazine Synthase	1.23E+08
gi|91200563	kuste2860	hypothetical (di heme) protein	1.08E+08
gi|939186674	—	Chain B, Hydrazine Synthase	8.64E+07
gi|91203321	kustd2215	strongly similar to 10 kDa chaperonin (GroES protein)	8.54E+07
gi|91202446	kustd1340	similar to hydroxylamine oxidoreductase hao	6.73E+07
gi|91203786	kustc0694	hydrazine dehydrogenase (HDH)	7.51E+07
gi|91203216	kustd2110	conserved hypothetical protein	5.74E+07
gi|91202620	kustd1514	S-layer protein	5.67E+07
***Candidatus* Brocadia**			
gi|816980804	BROFUL_00700	hypothetical protein BROFUL_00700	1.90E+08
gi|762180876	BROSI_A1433	hypothetical protein BROSI_A1433	1.67E+08
gi|762180077	BROSI_A0629	hydrazine synthase alpha subunit	1.11E+08
gi|816981233	BROFUL_00383	hydrazine synthase subunit B	9.46E+07
gi|816981561	BROFUL_00163	Chaperonin GroES	9.24E+07
gi|364505647	—	hydrazine synthase subunit A, partial	6.90E+07
gi|762181769	BROSI_A2345	hydrazine-oxidizing enzyme	5.07E+07
gi|816979735	BROFUL_01551	hydroxylamine oxidoreductase-like protein, partial	4.61E+07
gi|816979507	BROFUL_01720	Putative transposase, partial	4.17E+07
gi|762183013	BROSI_A3601	Chaperonin GroEL	3.54E+07
***Candidatus* Jettenia**			
gi|386406010	KSU1_B0536	conserved hypothetical protein	2.05E+08
gi|164605312	—	similar to hypothetical (di heme) protein	1.18E+08
gi|164605314	—	hydroxylamine oxidoreductase	1.17E+08
gi|164605313	—	similar to hypothetical (di heme) protein	1.08E+08
gi|386405673	KSU1_B0199	putative heme protein	9.41E+07
gi|118123413	—	hydrazine-oxidizing enzyme	8.50E+07
gi|386403142	KSU1_D0441	putative hydrazine hydrolase C subunit	4.44E+07
gi|386404695	KSU1_C0935	alkylhydroperoxide reductase	3.12E+07
gi|386403047	KSU1_D0346	RNA polymerase sigma 70 subunit RpoD	3.00E+07
gi|386405720	KSU1_B0246	Chaperonin GroEL	2.51E+07

Table 2. Differentially abundant proteins detected within the batch incubations at 20°C relative to 35°C

Accession	Gene tags	Identified Proteins	Fold change*
Candidatus Kuenenia			
gi|91200176	kuste2473	hypothetical protein kuste2473	1.5
gi|91203443	kustc0351	conserved hypothetical protein	1.4
gi|91203550	kustc0458	similar to hydroxylamine oxidoreductase	1.2
gi|91200659	kuste2956	strongly similar to 50S ribosomal protein L7/L12	1.2
gi|91203549	kustc0457	hydroxylamine oxidoreductase hao-like protein	1.3
gi|91200580	kuste2877	similar to undeca heme containing cytochrome c protein	1.2
gi|91202775	kustd1669	conserved hypothetical protein	1.2
gi|91204318	kustc1226	conserved hypothetical	1.2
gi|91200695	kuste2992	strongly similar to 50S ribosomal protein L17	1.3
gi|91200668	kuste2965	strongly similar to 50S ribosomal protein L4	1.3
gi|91204335	kustc1243	strongly similar to Methyl-accepting chemotaxis protein	1.7
gi|91200042	kuste2339	similar to pyruvate synthase alpha chain	1.2
gi|91204079	kustc0987	strongly similar to 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino) imidazole-4-carboxamid e isomerase	1.2
gi|91202378	kustd1272	conserved hypothetical protein	1.5
gi|91201300	kuste3597	conserved hypothetical protein	1.8
gi|91203635	kustc0543	Predicted orf	1.4
gi|91204285	kustc1193	similar to heme d1 synthesis protein nirH/nirL	1.2
gi|91200051	kuste2348	conserved hypothetical protein putative tatA/E	1.3
gi|91202407	kustd1301	strongly similar to catalase	1.4
gi|91202665	kustd1559	similar to 30S ribosomal protein RpsT	1.4
gi|91200676	kuste2973	strongly similar to 30S ribosomal protein S17	1.3
gi|91204599	kusta0082	strongly similar to nucleoside diphosphate kinase	1.3
gi|91200672	kuste2969	strongly similar to 50S ribosomal protein L22	1.3
gi|91200422	kuste2719	similar to octaprenyl diphosphate synthase	1.4
gi|91203548	kustc0456	hypothetical protein kustc0456	1.4

gi|91203213	kustd2107	hypothetical protein kustd2107	1.2
gi|91202431	kustd1325	unknown protein	1.4
gi|91202894	kustd1788	unknown protein	1.2
gi|91204453	kustb0208	unknown protein	1.4
gi|227248568	PRK02304	unnamed protein product	1.3
gi|91200177	kuste2474	unknown (diheme) protein	1.4
gi|91200757	kuste3054	unknown protein	1.3
gi|91201139	kuste3436	hypothetical protein kuste3436	0.8
gi|91202579	kustd1473	hypothetical protein kustd1473	0.8
gi|91201141	kuste3438	conserved hypothetical protein	0.8
gi|91204195	kustc1103	strongly similar to ribonuclease PH	0.8
gi|91200245	kuste2542	similar to acetolactate synthase	0.8
gi|91200488	kuste2785	unknown protein	0.8
gi|91201134	kuste3431	unknown protein	0.8
***Candidatus* Brocadia**			
gi|816979735	BROFUL_01551	hydroxylamine oxidoreductase-like protein, partial	1.6
gi|816978248	BROFUL_02744	putative heme protein small subunit NaxS	1.3
gi|816981232	BROFUL_00382	hydrazine synthase subunit C	1.2
gi|816980468	BROFUL_00960	Putative peptidyl-prolyl cis-trans isomerase	1.3
gi|816978241	BROFUL_02750	putative cytochrome c	1.4
gi|816978823	BROFUL_02271	translation elongation factor G	1.3
gi|816977992	BROFUL_02942	hypothetical protein BROFUL_02942	1.3
gi|816981195	BROFUL_00400	hypothetical protein BROFUL_00400	1.3
gi|816980082	BROFUL_01278	hypothetical protein BROFUL_01278	1.3
gi|762180583	BROSI_A1140	phosphomannomutase	0.8
gi|816980820	BROFUL_00716	nitrogen regulatory protein	0.8
gi|816978461	BROFUL_02592	hypothetical protein BROFUL_02592	0.7
gi|816978084	BROFUL_02869	hypothetical protein BROFUL_02869	0.7
gi|816979507	BROFUL_01720	Putative transposase, partial	0.6
gi|816979291	BROFUL_01865	putative heme protein	0.8
***Candidatus* Jettenia**			
gi|164605314	—	hydroxylamine oxidoreductase	1.3
gi|386403047	KSU1_D0346	RNA polymerase sigma 70 subunit RpoD	1.4
gi|386404247	KSU1_C0487	conserved hypothetical protein	1.2
gi|386405356	KSU1_C1596	conserved hypothetical protein	1.3
gi|386403132	KSU1_D0431	conserved hypothetical protein	1.4
gi|386403895	KSU1_C0135	hypothetical protein KSU1_C0135	1.6
gi|386406021	KSU1_B0547	putative 30S ribosomal protein S6	1.3
gi|386404637	KSU1_C0877	RNA-binding protein	1.3
gi|386402929	KSU1_D0228	cobyrinic acid a, c-diamide synthase	1.3
gi|386404089	KSU1_C0329	acetyl coenzyme A synthase alpha subunit	1.7
gi|386406136	KSU1_B0662	3-isopropylmalate dehydratase large subunit	1.3
gi|386405739	KSU1_B0265	putative cytochrome c	1.3

gi|386403880	KSU1_C0120	ATPase	1.4
gi|386404050	KSU1_C0290	two-component sensor kinase	1.4
gi|386402762	KSU1_D0061	conserved hypothetical protein	0.8
gi|386403147	KSU1_D0446	putative heme protein	0.7

*Fold change, is the ratio of the abundance of the protein identified from the 20^{o}C incubation culture to that from the 35^{o}C incubation culture. Differentially abundant proteins were those with normalized total intensity ratios of ≥ 1.2 or ≤ 0.8, combined with a Rank Product P-value <0.05.

Table 3. Proteins detected within the batch incubations with significantly altered levels at 15°C relative to 35°C

Accession	Gene tags	Identified Proteins	Fold change*
***Candidatus* Kuenenia**			
gi|91202276	kuste4574	similar to hydroxylamine oxidoreductase hao	1.2
gi|91203443	kustc0351	conserved hypothetical protein	1.2
gi|91202431	kustd1325	unknown protein	1.4
gi|91200757	kuste3054	unknown protein	1.3
gi|91201139	kuste3436	hypothetical protein kuste3436	0.8
gi|91203919	kustc0827	similar to proton-translocating NADH dehydrogenase I, 51 kDa subunit (NuoF)	0.8
gi|91201095	kuste3392	hypothetical protein kuste3392	0.7
gi|91203916	kustc0824	strongly similar to proton-translocating NADH dehydrogenase I chain C (NuoC)	0.8
gi|91200097	kuste2394	hypothetical protein kuste2394	0.8
***Candidatus* Brocadia**			
gi|816979735	BROFUL_01551	hydroxylamine oxidoreductase-like protein, partial	1.3
gi|816979724	BROFUL_01552	aldehyde dehydrogenase	1.9
gi|816979734	BROFUL_01550	hydroxylamine oxidoreductase-like protein	1.3
gi|816978815	BROFUL_02263	50S ribosomal protein L11	1.3
gi|816979249	BROFUL_01894	50S ribosomal protein L17	1.8
gi|762180589	BROSI_A1146	protein contains FOG domain	0.6
gi|762179911	BROSI_A0457	transaldolase	0.8
gi|762180583	BROSI_A1140	phosphomannomutase	0.8
gi|816980209	BROFUL_01178	formate dehydrogenase	0.7
gi|762181905	BROSI_A2482	site-specific tyrosine recombinase	0.5
gi|816979744	BROFUL_01545	putative peptidase	0.7
gi|762179705	BROSI_A0251	Dihydrolipoamide acetyltransferase	0.6
gi|816979887	BROFUL_01449	alcohol dehydrogenase	0.8
***Candidatus* Jettenia**			
gi|386404435	KSU1_C0675	Pyruvate ferredoxin/flavodoxin oxidoreductase	1.4
gi|386403129	KSU1_D0428	methenyl tetrahydrofolatecyclohydrolase	2.9
gi|164605314	—	hydroxylamine oxidoreductase	1.2
gi|386403047	KSU1_D0346	RNA polymerase sigma 70 subunit RpoD	1.5
gi|386405967	KSU1_B0493	putative cytochrome c	1.4
gi|386404247	KSU1_C0487	conserved hypothetical protein	1.3
gi|386406255	KSU1_A0041	conserved hypothetical protein	1.8
gi|386405720	KSU1_B0246	Chaperonin GroEL	1.4
gi|386402843	KSU1_D0142	glycoside hydrolase	1.3
gi|386405114	KSU1_C1354	two-component sensor kinase	1.3
gi|386405356	KSU1_C1596	conserved hypothetical protein	1.3

gi|386404677	KSU1_C0917	F0F1 ATP synthase B subunit	1.5
gi|386405097	KSU1_C1337	translation initiation factor IF-3	1.6
gi|386403132	KSU1_D0431	conserved hypothetical protein	1.3
gi|386406074	KSU1_B0600	conserved hypothetical protein	1.4
gi|386404633	KSU1_C0873	two-component sensor kinase	1.3
gi|386405443	KSU1_C1683	dihydrolipoamide dehydrogenase	1.4
gi|386406010	KSU1_B0536	conserved hypothetical protein	0.8
gi|164605312	—	similar to hypothetical (di heme) protein	0.8
gi|386405654	KSU1_B0180	conserved hypothetical protein	0.6

*Fold change, is the ratio of the abundance of the protein identified from the 15^{o}C incubation culture to that from the 35^{o}Cincubation culture. Differentially abundant proteins were those with normalized total intensity ratios of ≥ 1.2 or ≤ 0.8, combined with a Rank Product P-value <0.05.

FIGURE LEGENEDS

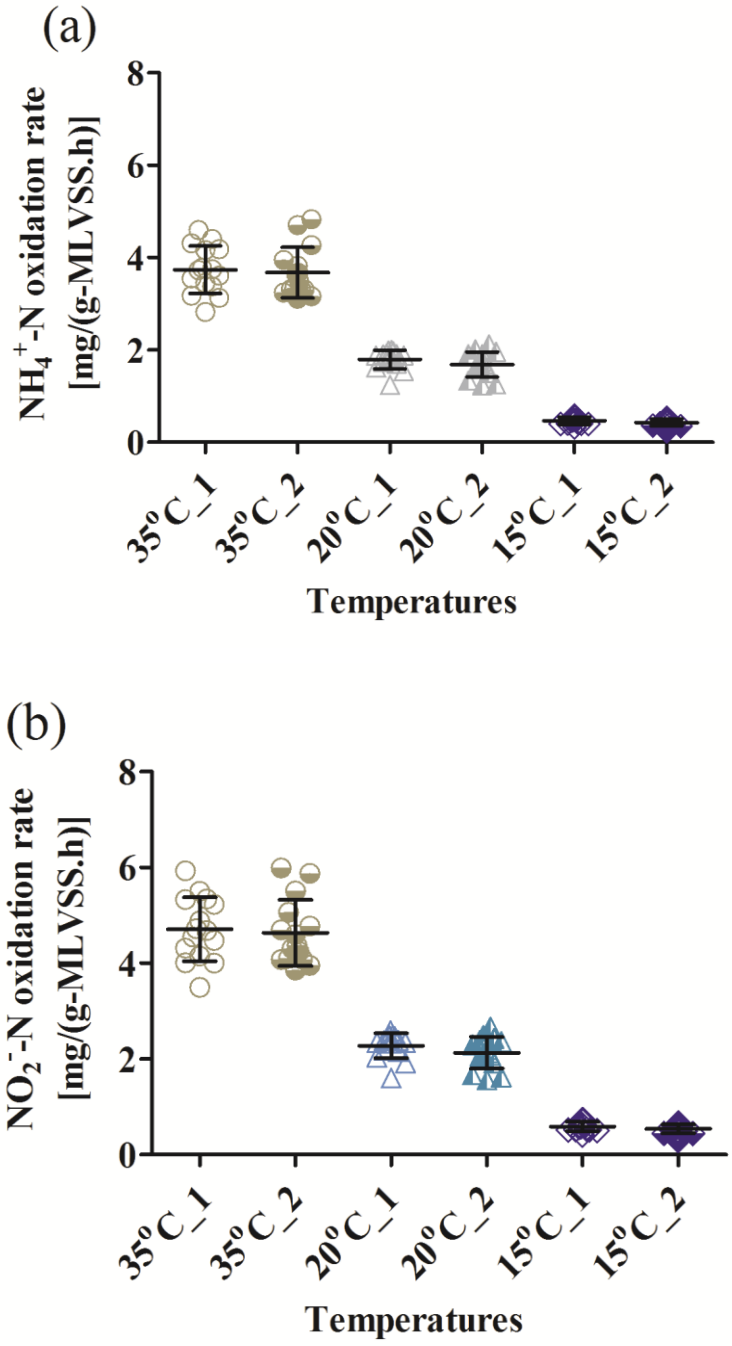
Figure 1 The ammonium oxidation (a) and nitrite reduction (b) rates detected in the six sequence batch incubations as the temperatures of 15°C, 20°C and 35°C. A decrease of both ammonium oxidation and nitrite reduction rates with decreasing temperature was evident.

Figure 2 Principal component analysis of the 1539 protein abundance values detected in the sequence batch incubations operated at 15°C, 20°C and 35°C

Figure 3 The number of proteins associated with all anammox genera assigned to categories of Clusters of Orthologous Groups (COG)with significantly different abundance at lower temperatures. The COG categories are J: Translation, ribosomal structure and biogenesis; K: Transcription; L: Replication, recombination and repair; D: Cell cycle control, cell division, chromosome partitioning; V: Defense mechanisms; T: Signal transduction mechanisms; M: Cell wall/membrane/envelope biogenesis; N: Cell motility; Z: Cytoskeleton; W: Extracellular structures; U: Intracellular trafficking, secretion and vesicular transport; O: Posttranslational modification, protein turnover, chaperones; C: Energy production and conversion; G: Carbohydrate transport and metabolism; E: Amino-acid transport and metabolism; F: Nucleotide transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; P: Inorganic ion transport and metabolism; and Q: Secondary metabolites biosynthesis, transport and catabolism.

Figure 4 The number of proteins associated with individual anammox genus assigned to Clusters of Orthologous Groups (COG) categories with significantly different abundances at lower temperatures. Protein abundance changes from *Candidatus* Kuenenia at 20 ˚C in comparison to 35 ˚C (a) and at 15 ˚C in comparison to 35 ˚C (d). Protein abundance changes from *Candidatus* Brocadia at 20 ˚C in comparison to 35 ˚C (b) and at 15 ˚C in comparison to 35 ˚C (e). Protein abundance changes from *Candidatus* Jettenia at 20 ˚C in comparison to 35 ˚C (c) and at 15 ˚C in comparison to 35 ˚C (f). The COG categories are those referred to in Figure 3.

Figure 5 Summary of metaproteomic insights into the physiological response of anammox bacteria to decreased temperature. Green shapes are proteins with nearly unchanged abundance; Yellow shapes are proteins with decreased abundance; and red: Proteins are those with increased abundance. Nar: Nitrate reductase; Nir: Nitrite reductase; HZS: Hydrazine synthase; HDH: Hydrazine dehydrogenase; HAO: Hydroxylamine oxidoreductase; NADH: Nicotinamide adenine dinucleotide; ROS: reactive oxygen species



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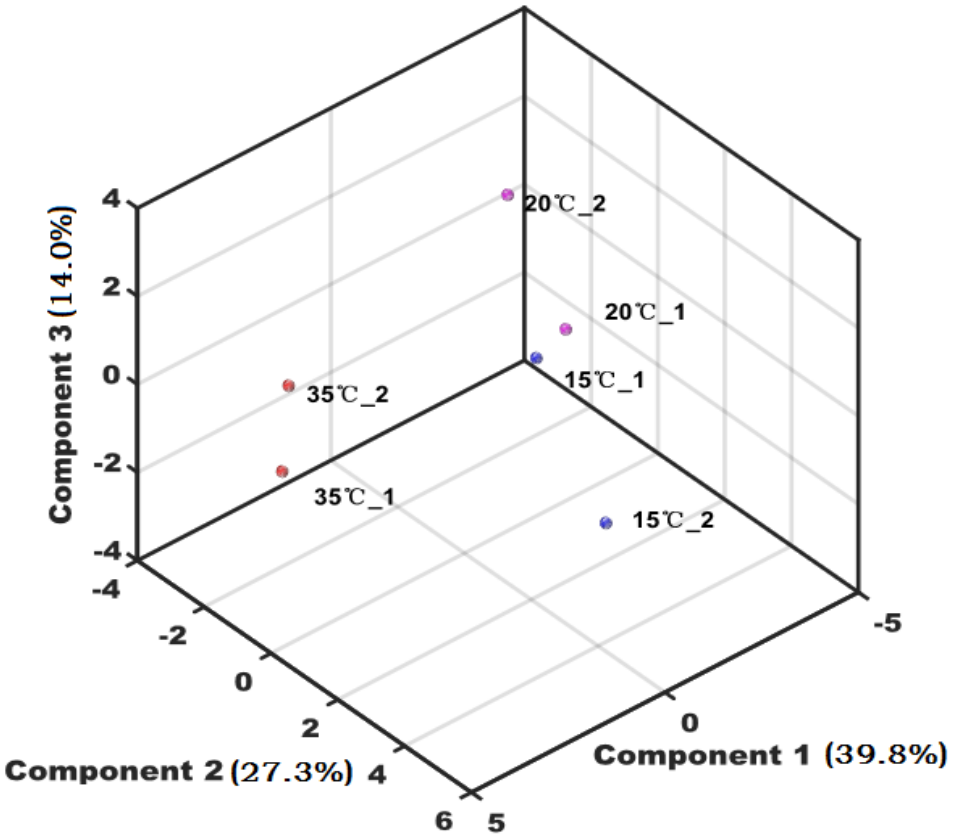
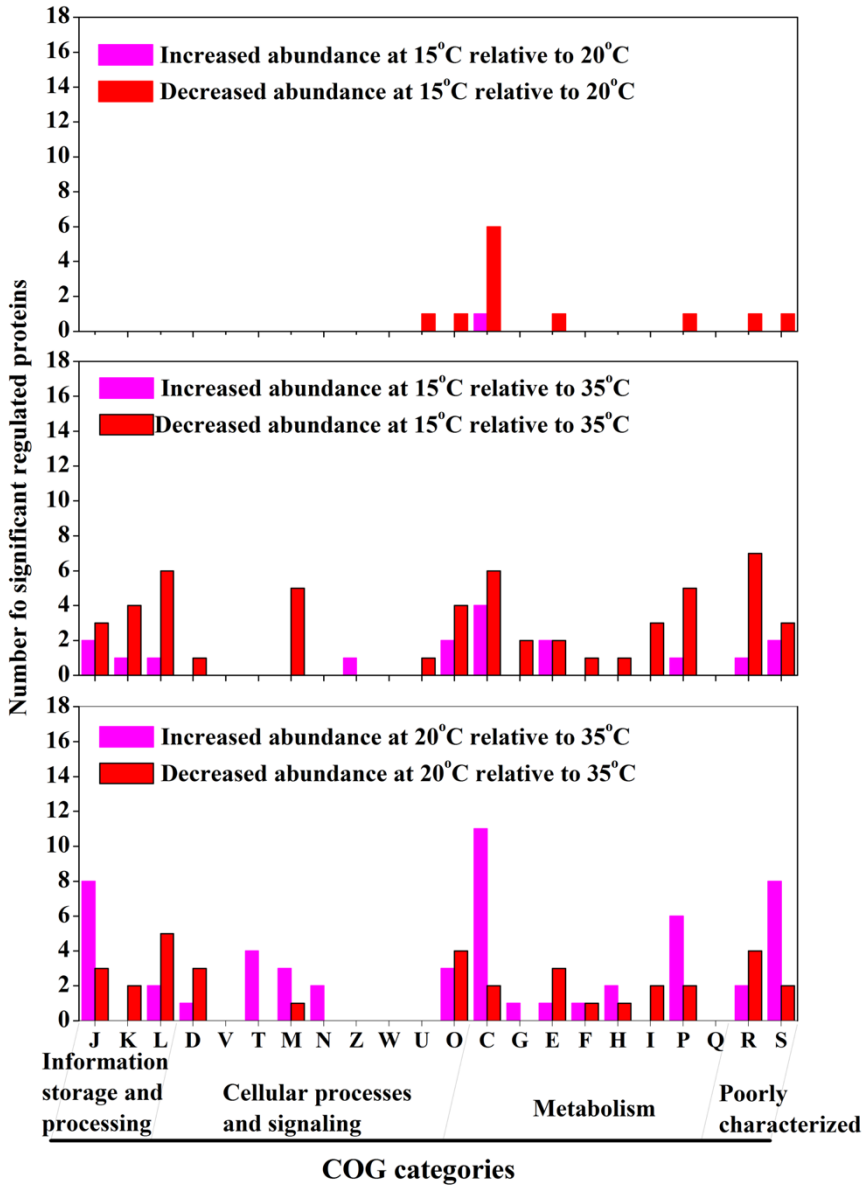


Figure 2 Principal component analysis of the 1539 protein abundance values detected in the sequence batch incubations operated at 15°C, 20°C and 35°C



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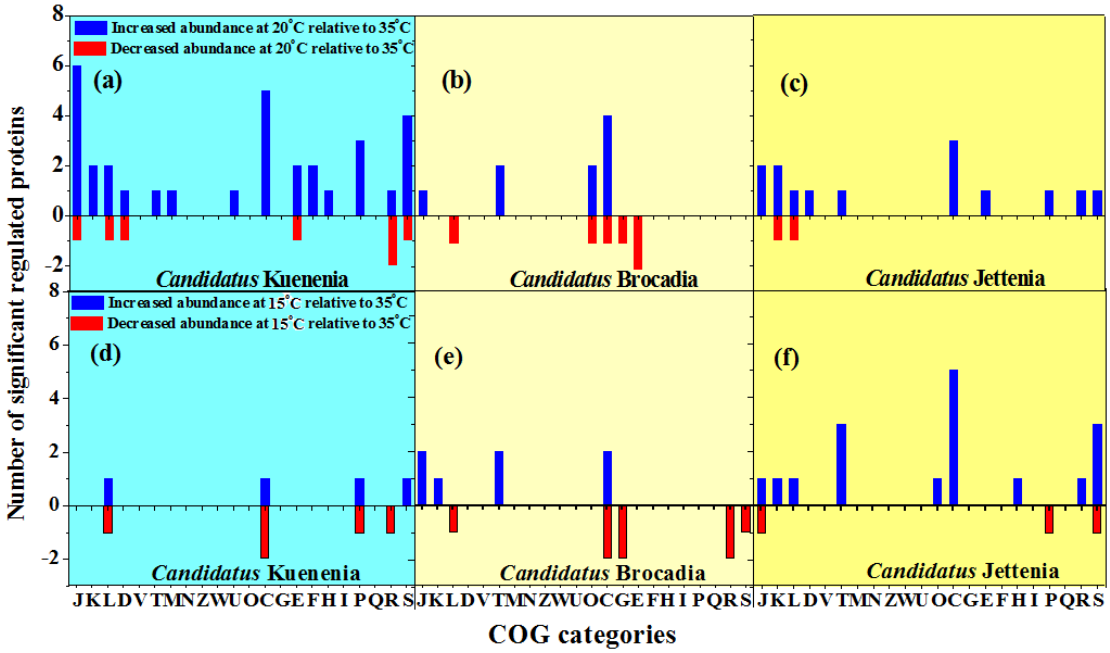
biosynthesis, transport and catabolism; R: General function prediction only; and S: Function unknown.



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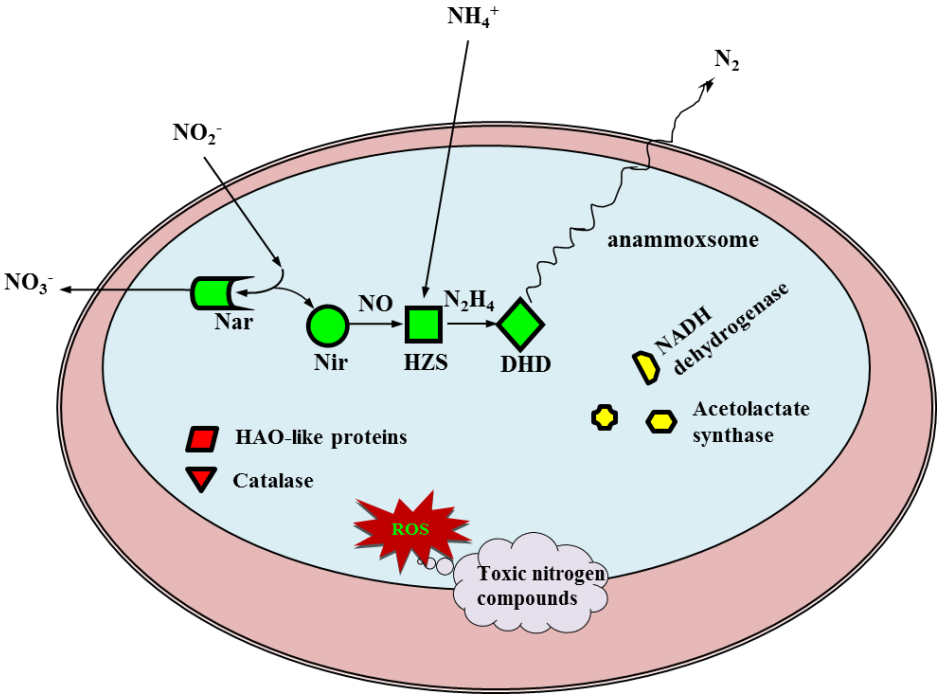

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