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MASTERARBEIT

Titel der Masterarbeit

„Transcript level dynamics in the archaeal ammonia-oxidizer *Nitrososphaera viennensis* in response to ammonia supply“

Verfasser

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1 ABSTRACT

Nitrification is a central process in the global nitrogen cycle and is to a large extent performed by bacteria and archaea. Ammonium starvation experiments in ammonia oxidizing bacteria (AOB) revealed interesting aspects of survival under energy-limiting conditions and of the regulation of ammonia oxidation. In order to get insights into transcriptional dynamics in an ammonia-oxidizing Archaeon (AOA) a starvation and recovery experiment was conducted with "*Candidatus* Nitrososphaera viennensis*"* EN 76*,* an isolate from soil. The organism was grown in liquid culture until the medium was depleted for ammonium and cells entered stationary phase. After starvation for 8 days, ammonium was re-added to the medium to initiate recovery. Shifts in mRNA levels of key genes in ammonia oxidation (AO), C-metabolism and information processing were followed by quantitative PCR. High levels of transcripts coding for subunits A, B and X of ammonia monooxygenase (AMO) were only detected when NH_4^+ was present. In contrast, high transcript levels of one of the multiple *amo*C genes was constitutively maintained even when all NH_4^+ was converted to NO_2^- (day 7) as well as when cultures were amended again with 1 mM NH_4^+ and recovered from 8 days of starvation. Expression of 16S rRNA, RNA polymerase subunit B (*rpo*B), 4-hydroxybutyryl-CoA dehydratase (*hcd)* and a copper-dependent nitrite reductase (*nir*K) showed distinct patterns compared to AO-related genes in terms of time and intensity. This study delivers first reports on transcription in AOA and shows similarities but also differences to comparable experiments in AOB. Although still preliminary, hints to the involvement of specific endoribonucleases regulating the shifts in transcript levels emerged from our study. In general this work demonstrates that the experimental approach of ammonia starvation and recovery is suitable for the study of transcript dynamics in AOA also on a genome-wide level.

2 INTRODUCTION

Nitrification and its global importance

Nitrification, the aerobic oxidation of ammonia (NH_3) to nitrate (NO_3^-) is a major biogeochemical process central to the global nitrogen cycle whose fluxes amount to 10^{12} to 10^{14} g N per year (Galloway et al. 2004; Gruber and Galloway 2008). In terrestrial ecosystems, nitrification can lead to a net loss of fixed-nitrogen from soils as nitrate is in general more susceptible to leaching and is additionally removed mostly anaerobically by denitrifiers (Jetten 2008; Prosser 2011). Nitrification and denitrification are accompanied by emissions of N_xO_x gases which contribute to the greenhouse effect (e.g.: N_2O) and lead to ozone depletion (eg.: NO, NO_2) (Galloway et al. 2003). The nitrification process therefore stands in competition with plants and heterotrophic microorganisms for the uptake of nitrogen (Verhagen et al. 1995) and is responsible for up to 70% loss of ammonium-based fertilizers from agricultural systems (Prosser 2011). As anthropogenic inputs of nitrogen to terrestrial ecosystems already exceed inputs from natural nitrogen-fixation (Gruber and Galloway 2008) it is crucial to understand processes involved in nitrogen turnover in order to predict the impact of human activities on the trophic status of ecosystems (Figure 1) and more generally global change (Ollivier et al. 2011; Cantarel et al. 2012)

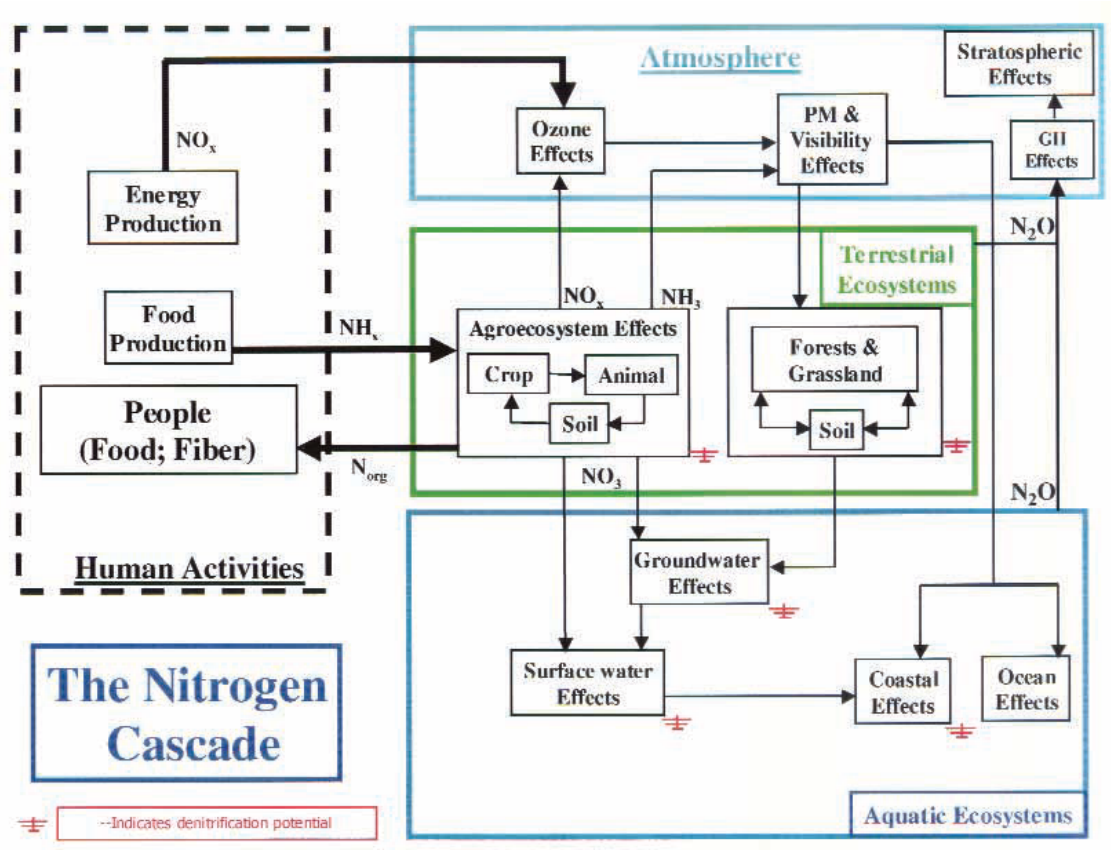

Figure 1: Interaction between human activities, nitrogen cycle and the biosphere (Galloway et al. 2003).

Microbial origin of nitrification

In a broader definition, nitrification includes oxidation of any reduced form of nitrogen and is performed by auto- and heterotrophic organisms from all 3 domains of life (Stein 2011). Classical nitrification is a biological process consisting of two consecutive steps catalyzed by two distinct microbial guilds which are so far only represented by prokaryotic organisms. The first step of the nitrification process yields nitrite (NO_2^-) from the oxidation of ammonia by "ammonia-oxidizers"

while the second step produces nitrate from the oxidation of nitrite by "nitrite-oxidizers" (Daims et al. 2011).

The isolation of autotrophic ammonia-oxidizing bacteria (AOB) was reported as early as 1890 by Sergei Winogradsky (Winogradsky 1890). Since this early work a lot more species were cultivated and well described (Koops and Pommerening-Röser 2001) of which most belong to β-proteobacteria except for representatives of the genus *Nitrosococcus* which are γ-proteobacteria. The latter group is exclusively found in saline environments in contrast to the ubiquitous distribution of both the *Nitrosomonas* and *Nitrosospira* cluster of β-proteobacteria.

AOB were thought to be solely responsible for most of the ammonia oxidation until the recent discovery of ammonia-oxidizing archaea (AOA). Metagenomic surveys conducted in marine (Venter et al. 2004) and terrestrial environments (Treusch et al. 2005) detected archaeal genes homologous to subunits of ammonia monoxygenase (AMO) one of the key enzymes of the bacterial ammonia oxidation pathway. These first evidences for archaeal involvement in AO were confirmed shortly after by cultivation of an aerobic autotrophic archaeal ammonia-oxidizer from a marine water tank (Könneke et al. 2005). Additionally AOA have been enriched from thermal springs (de la Torre et al. 2008; Hatzenpichler et al. 2008), sediments (Park et al. 2010) and acidic agricultural soils (Lehtovirta-Morleya et al. 2011). An isolate named *Nitrososphaera viennensis* has recently been obtained in pure culture from a viennese garden soil (Tourna et al. 2011). All AOA belong to the phylum of Thaumarchaeota (Brochier-Armanet et al. 2008; Spang et al. 2010; Pester et al. 2011) formerly known as mesophilic Crenarchaeota. AOA are widespread and abundant in soils (Leininger et al. 2006) and marine ecosystems (Francis 2005; Wuchter 2006) and they are able to grow autotrophically. AO could be linked to thaumarchaeal

activity and growth in soil microcosms (Offre et al. 2009; Zhang et al. 2010; Offre et al. 2011) but still their contribution to nitrification *in situ* is unclear.

The relative contribution of AOB and AOA in the ammonia oxidation process in soil

Overall the nitrification rate is limited by the initial step of the process in which ammonium is oxidized to nitrite and therefore ammonium oxidizing organisms are important determinants of global nitrogen fluxes. Thus, it is essential to understand differences between key-players of this process which might have varying relevance corresponding to different environments. As soon as AOA were discovered questions about their global importance in AO, if and how they co-occur with AOB and what shapes their niches (Erguder et al. 2009) arose. High constant loads of ammonium sustained a high nitrification potential co-occurring with high AOB abundance in Slovenian wetlands. But AOB were below the detection limit when ammonium concentrations were lower and in turn AOA were abundant (Höfferle et al. 2010). These findings are in line with microcosm studies where AOB responded significantly only on high ammonium inputs (Di et al. 2010; Verhamme et al. 2011) and all isolated AOA were inhibited by ammonium concentrations higher than 20mM (Tourna et al. 2011) in contrast to several hundreds mM in eutrophic AOBs. These distinct responses of AOB and AOA to ammonia concentrations were also found in other studies and suggest a general categorization of AOB and AOA as possible r- and K-strategists, respectively. Nevertheless, the theory of r/K-selection can be applied meaningfully only when accompanied by a conceptual framework including the function of the compared groups on a given ecosystem level and of course may also be applicable within AOB or AOA itself. r-strategists allow for fast growth and dispersion by high saturation levels for ammonium and concomitant high AO rates, whereas K-strategists grow more slowly but have high affinity to

ammonium which is an advantage in oligotrophic sites. High concentrations like in agricultural soils seem to be rather favorable for AOB (Jia and Conrad 2009) while AOA communities are also stable and gain even more importance when ammonium concentrations are low like in unfertilized soils (Leininger et al. 2006). Given these two extremes opposing each other there are of course still different "shades of grey" in between and contradictory results (Schauss et al. 2009; Kelly et al. 2011) may be related to a more complex set of factors selecting for AOB and/or AOA including soil-pH (Nicol et al. 2008), temperature (Tourna et al. 2008; Szukics et al. 2010), moisture (Höfferle et al. 2010; Szukics et al. 2010) and soil-vegetation interactions (Rasche et al. 2011).

Bacterial and Archaeal AO pathways

In Bacteria the first step of AO to nitrite (NO_2^-) via hydroxylamine (NH_2OH) (Hofman and Lees 1953) is catalyzed by a monooxygenase (Hollocher et al. 1981), termed ammonia monooxygenase (AMO) (Bedard and Knowles 1989). Despite its prediction as a membrane protein, so far surprisingly only soluble AMO was obtained from *N. europaea* cells (Gilch et al. 2009). Thus detailed investigation of structure and function of AMO is still hampered by the lack of a purified AMO. However, due to homology to particulate methane monooxygenase (*pMmo*) (Holmes et al. 1995; Klotz and Norton 1998; Norton et al. 2002) which consists of at least 3 subunits A, B and C (Zahn and DiSpirito 1996; Nguyen et al. 1998), a similar heteromultimeric structure has been assumed for AMO. Using acetylene as inhibitor of AO (Hynes and Knowles 1978) which irreversibly and covalently binds to subunit A of AMO showed that it contains the active site (Hyman and Arp 1992) where ammonia (Suzuki et al. 1974) and structurally similar substrates like methane (Hyman and Wood 1983) are bound. Binding of ammonia is followed by

integration of one atom of molecular oxygen to form hydroxylamine while the second oxygen atom is reduced by two electrons to water. A hydroxylamine oxidoreductase (HAO) oxidizes hydroxylamine further to nitrite which is the reaction where four electrons are gained. Two out of these four electrons most probably flow downstream the respiratory chain and the remaining two are cycled back against the electron transport chain to a higher redox potential and are used to form water in the AMO catalyzed reaction (Klotz and Stein 2011). Figure 2 depicts a model for AO and nitrous oxide formation in AOB adapted and simplified from Klotz and Stein (2011)

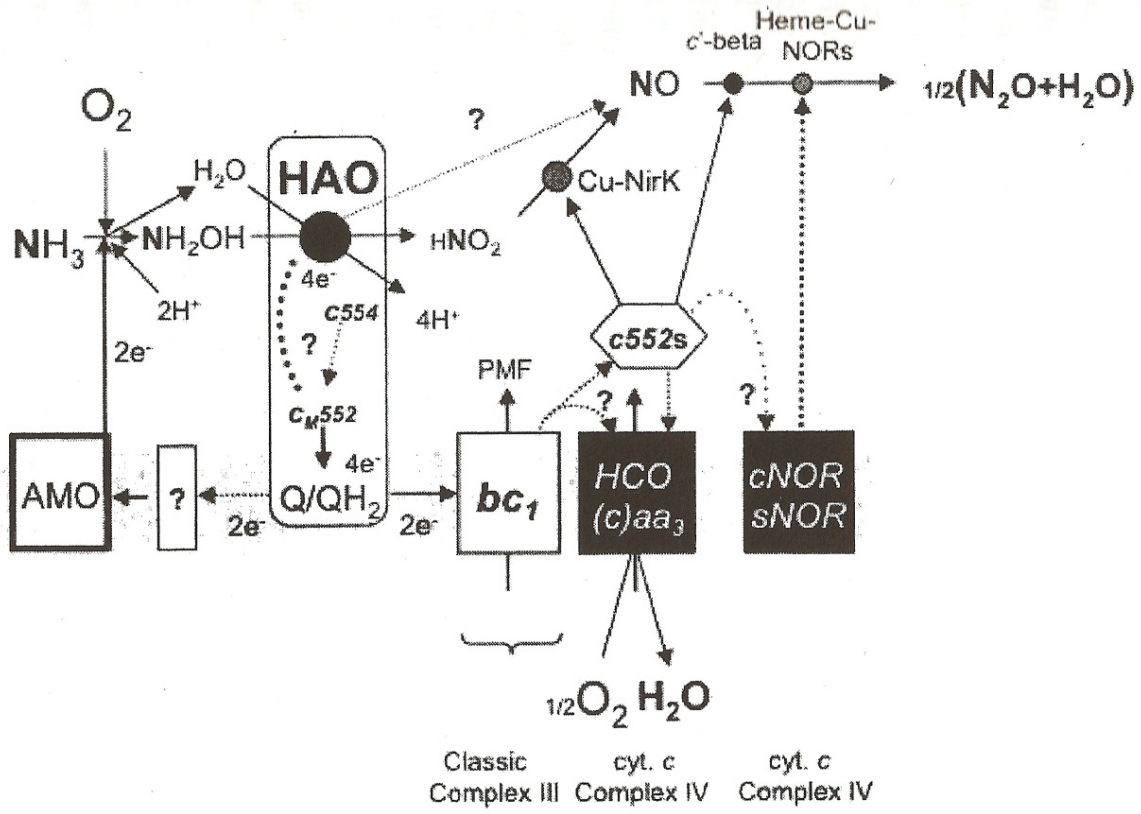

Figure 2: Proposed ammonium oxidation pathway for AOB from Klotz and Stein (2011). AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; Q/QH2 Ubiquinone oxidized/reduced; c554, c552, bc1 and HCO(c)aa$_3$, various cytochromes; NOR, nitric oxide reductase; Cu-NirK, Copper dependent nitrite reductase; PMF, proton motive force.

Despite its important function in AOB, a homolog of HAO has not been recognized in AOA genomes. Figure 3 depicts a putative model of how AOA accomplish AO based on genome predictions, assuming that either nitroxyl or hydroxylamine is the intermediate (Urakawa et al. 2011). However, very recently Vajrala et al. (2012) have published strong evidence for hydroxylamine as an intermediate product of AO in *Nitrosopumilus maritimus*.

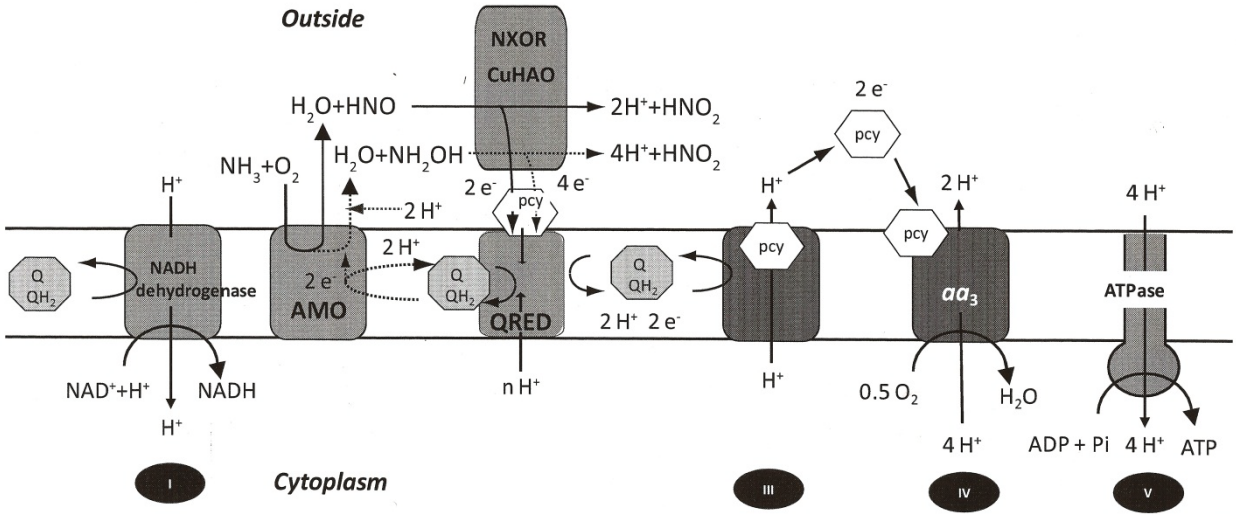

Figure 3: Proposed ammonium-oxidation pathway for AOA from Urakawa et al. (2011). AMO, ammonia monooxygenase; NXOR, putative nitroxyl oxidoreductase; CuHAO, copper hydroxylamine oxidoreductase; Q/QH2 quinone oxidized/reduced; pcy, plastocyanins.

Genomic organization and regulation of the expression of AMO-encoding genes

In AOB the *amo*-operon is organized in a C-A-B structure. In contrast to γ-proteobacteria the *amo*-operon occurs more than once in β-proteobacteria and additional single copies of *amoC* can be found (Norton et al. 2002).

All AOA genomes which are sequenced so far contain just one copy of the *amo*A and *amo*B genes (Spang and Bartossek personal communication) but *amo*C occurs in some AOA in multiple copies like in β-proteobacteria. There is no conserved C-A-B structure in AOA like in AOB but a putative *amo*X gene is always located directly next to *amoA* (Bartossek et al. 2012; Spang et al. 2012). The *amo*B gene of *N. viennensis* lies 115 ORFs upstream of *amo*X, while *amo*C is separated by ten more ORFs upstream of *amo*B. *N. viennensis* encodes five additional *amo*Cs out of which two are truncated.

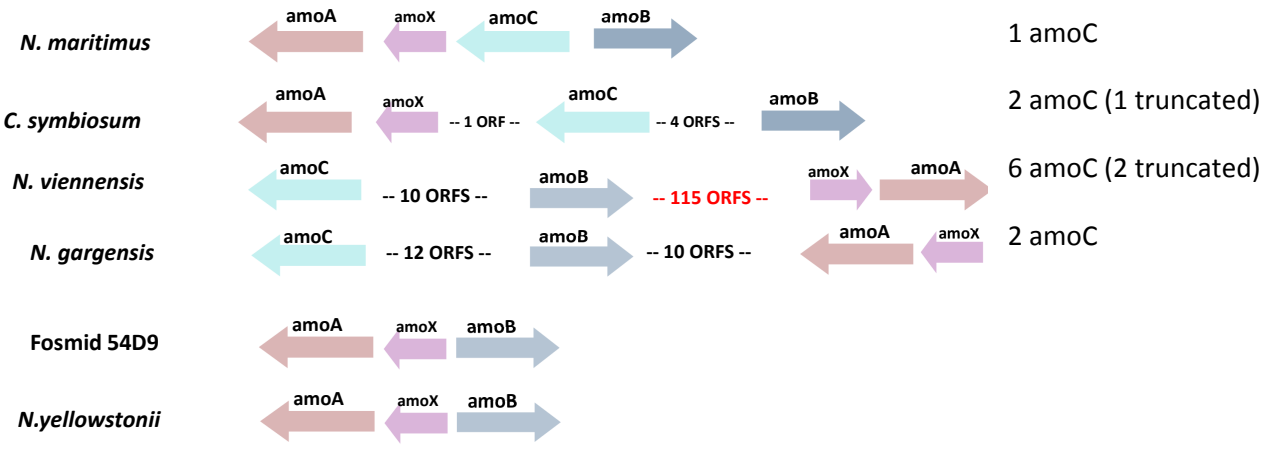

Figure 5: Genomic organization of *amo* genes in AOA; adapted from Spang et al. 2012

In AOB a polycistronic mRNA of the full *amo*CAB-operon is produced (Sayavedra-Soto et al. 1998; Hommes et al. 2001; El Sheikh and Klotz 2008). Nevertheless, individual genes are differentially regulated depending on external condititions like light, inhibitors or NH_4^+-availability and concentration (Hommes et al. 1998; Sayavedra-Soto et al. 1998; Stein et al. 2000; Hommes et al. 2001; Berube et al. 2007; El Sheikh and Klotz 2008). Our study presents for the first time transcriptional patterns of *amo* genes from an AOA isolate which allows comparison to responses of their bacterial counterparts.

Transcriptional responses of AOB upon ammonia starvation and recovery

Thriving in an environment like soil requires adaptation to fluctuating conditions where the most prominent factor is water which enables solubilization and thereby allowing uptake of substrates by microorganisms. AO communities are dispersed along steep gradients of aeration (which is mostly related to water content) and nutrients which shape a huge variety of ecological niches within micrometers. Regulation of different metabolic pathways determining the fate of nitrogen and energy is therefore essential and already present on the level of transcription.

Induction of *amo*A and *hao* gene expression in *N. europaea* was shown to be correlated with NH_4^+ availability (Sayavedra-Soto et al. 1996; Sayavedra-Soto et al. 1998; Stein and Arp 1998). By NH_4^+-starvation and after re-addition of NH_4^+ it was shown that AOB are able to recover and differentially express genes encoding for subunit C of AMO (Berube et al. 2007; Berube and Stahl 2012). Their ability to respond and the velocity of the response to restore AO and transcripts to the levels similar to exponential phase, are influenced by the duration of the starvation period (Bollmann et al. 2005) as well as cell density (Batchelor et al. 1997).

Transcriptional regulation in Archaea

The transcriptional responses of AOB and AOA share at least in part some similarities but it should be noted that the regulatory background of their gene expression is quite different. In general transcript pools are balanced by synthesis and decay of mRNA and unless either of the two processes is experimentally excluded (Miller et al. 2011), most approaches capture only steady-state or net levels without any information of their genesis. A complex regulatory network underlying transcription and transcript stability makes it difficult to dissect factors for direct

causal relationships but still knowledge of the basic machinery could already be gained in all 3 domains of life. In contrast to eukaryotes, transcription in archaea and bacteria is catalyzed by a single heteromultimeric RNA polymerase (RNAP). Although the large RNAP subunits are homologous in all domains of life, archaeal RNAP is structurally and functionally most similar to eucaryal representatives (Huet et al. 1983; Langer et al. 1995).

Transcription initiation

In bacteria the σ-factor recognizes and binds to the promotor region usually -35 and -10 bp upstream of the transcription start site (TSS) (Murakami et al. 2002),thereby initiating assembly of the RNAP-holoenzyme and subsequent transcription. The presence of different σ-factors enables variable promotor recognition thus different regulation of groups of genes (reviewed by Wösten (1998)). Archaeal and eucaryal promotors primary consist of a "TATA"-box which is recognized by the TATA-binding protein (TBP) (Qureshi et al. 1995). In archaea it is placed on average 25 bp upstream of the TSS and often followed by a second major promotor element, called the B recognition element (BRE), as it interacts with another protein called Transcription Factor B (TFB) (Hausner et al. 1996; Qureshi and Jackson 1998; Littlefield et al. 1999). TFB is homologous to Transcription Factor II B (TFIIB) from eukaryotes (Creti et al. 1993) and together with TBP is sufficient for reconstituting archaeal RNAP activity (Hethke et al. 1996) *in vitro*. *N. viennensis* encodes four similar but not identical TFBs which could allow basal variations in gene expression patterns like in *Halobacterium* NRC-1 (Facciotti et al. 2007). A third eucarya-like transcription factor, TFE, was predicted from genomic conservation (Kyrpides and Ouzounis 1999) and shown to strongly facilitate archaeal transcription (Bell et al. 2001). Specific TFs are predicted from archaeal genomes (Kummerfeld and Teichmann 2006; Wu et al. 2008) which can

either enhance or inhibit RNA synthesis (reviewed in Geiduschek & Ouhammouch (2005) and Bartlett (2005)).

Transcription termination

Transcription termination is another key process regulating RNA synthesis and is quite different between eucarya and bacteria (Gilmour & Fan, 2008). In archaea very little is known about transcriptional termination but it seems to be induced by oligo-T elements (Santangelo et al. 2009). Additionally to sequence based termination, riboswitches (Vitreschak et al. 2004) bind metabolites or substrates thereby inducing conformational changes in the nascent RNA which can in turn lead to termination or anti-termination (Santangelo and Artsimovitch 2011).

RNA stability and enzymatic degradation

After synthesis the fate of transcripts is further determined by their stability and decay rate. mRNA half-lives vary considerably between species and even within species depending on the growth phase (Jäger et al. 2002) but however they do not correlate with generation times (Evguenieva-Hackenberg and Klug 2011). In general endonucleases cut mRNA internally thus fragmenting it to smaller pieces and thereby allowing more effective degradation by exonucleases. So far, there is no direct evidence for endoribonucleolytic activity involved in mRNA degradation in archaea. The crystal structure of a protein in *Sulfolobus tokodaii* shows similarities to the YjgF protein family (Miyakawa et al. 2005) where among others (Volz 1999) endoribonucleolyticly active proteins are found (Morishita et al. 1999). However the YjgF family comprises as well proteins predicted for different catalytic processes, but share a deep catalytic

cleft which is proposed to bind and process a variety of substrates (Sinha et al. 1999). *N. viennensis* also encodes for two YjgF-family proteins termed Ern1 and Ern2. Additionally to these enzymes, immunological relationship to *E.coli* RNase E together with endoribonucleolytic activity was shown in protein extracts from halophilic archaea (Franzetti et al. 1997) but was not further specified. In genomes from this archaeal group and some methanogens no homologs of any subunit of the archaeal exosome can be found (Portnoy and Schuster 2006). This complex consists of multiple associated proteins with different functions (Evguenieva-Hackenberg et al. 2003) and was shown to be required for polyadenylation and subsequent exoribonucleolysis from 3' – 5' (Portnoy et al. 2005; Portnoy and Schuster 2006)(Portnoy et al., 2005; Portnoy & Schuster, 2006). Together with all exosome subunits other exoribonucleases which drive degradation as well from 5' – 3' direction (Hasenöhrl et al. 2011) are also encoded in the *N. viennensis* genome.

Goal of this study

The goal of this study was to get preliminary insights into transcript dynamics in *N. viennensis* as a reaction to varying levels of ammonium and thereby to provide a basis for experimental design of an RNA-seq project. Sampling strategies are crucial in order to obtain data which allows to draw statistically reliable conclusions. Thus, a test experiment should help to determine a proper sampling strategy. Cell counting and nitrite measurements should give a proxymation for growth and activity. Triplicate pure cultures of *N. viennensis* are sampled over time during normal growth and following ammonia depletion which results in a starvation period. Subsequently a recovery response after ammonium re-addition should be observable. By monitoring key genes of the AO pathway but also key genes involved in carbon metabolism and information processing

by RT-qPCR the goal was to explore shifts in gene expression levels at the transition between conditions when NH_4^+ just becomes available again. As NH_4^+ is the primary energy and nitrogen source in the media a high sampling rate in early recovery should dissect this transient shift from the starvation state to recovery and growth. Thus different transcriptional responses in the variety of metabolic key genes should allow to distinguish between the direct response to the substrate from the general response upon increasing energy availability. A basis for experimental design for an RNA-seq project should thereby be provided and hypotheses to transcriptional regulation and its underlying factors in thaumarchaeal protagonists of ammonia oxidation in soil can then be examined in a broader picture. This will extend our understanding of transcriptional responses to starvation in Thaumarchaeota and how quick this successful archaeal clade recovers and thus survives changing environmental conditions in soils.

Rationale for the choice of genes to monitor

In order to get preliminary insights into transcriptional dynamics in *N. viennensis* we monitored expression of genes involved in AO and additionally genes of carbon and information processing to cover a broad functional range and to have controls for those genes considered to be involved in nitrogen conversion.:

Genes potentially involved in ammonia oxidation

We monitored expression of genes encoding for AMO "subunits A,B,C, X" and for a putative copper dependent nitrite reductase (*nir*K).

Proteins encoded by *amoA* and *amoB* were shown to be associated together with cytochrome c1 in soluble fractions of *N. europaea* protein extracts (Gilch et al. 2009). The absence of subunit C

in the soluble form of "AMO" fits to *in silico* predictions for membrane spanning domains and its proposed role as membrane anchor and/or chaperone (Klotz et al. 1997). However the presence and function of *amo*C has never been directly shown and its existence was inferred from homology to *pmmo* and the proximity of the corresponding gene to *amo*A and B in AOB genomes (Klotz et al. 1997). Comparison of genomes from AOA revealed "*amo*X", an additional candidate which is present next to *amo*A, highly conserved in Thaumarchaea only, but the least conserved of all *amo* genes (Bartossek et al. 2012).

A gene for an additional enzyme homologous to copper-dependent nitrite-reductases (*nir*K) which is usually associated with denitrifying organisms is also found in AOA (Treusch et al. 2005; Bartossek et al. 2010) and AOB. N*ir*K seems to be involved in nitrite reduction in *N. europaea* (Beaumont et al. 2002; Beaumont et al. 2004; Schmidt 2004; Beaumont et al. 2005) and different expression patterns of *nirK* in response to nitrite occur even between different species of *Nitrosomonas* (Cua and Stein 2011). Considering its diversity the functional range could be much broader (Bartossek et al. 2010). As archaeal homologs to *nirK* have been found to be as highly expressed as archaeal *amo*ABC and ammonium transporters in various metatranscriptomes (Gifford et al. 2010; Hollibaugh et al. 2010; Shi et al. 2010; Radax et al. 2012), at least an indirect involvement of NirK in AO can be assumed. Thus novel patterns of transcript dynamics and functional roles of NirK might be revealed in Thaumarchaeota where the AO pathway, together with its associated intermediates and byproducts, is still not understood.

Carbon- and information processing related genes

The 4-hydroxbutyryl-CoA dehydratase (4-HCD) (Martins 2004) encoded by *hcd* has an irreplaceable role in the 3-hydroxypropionate/4-hydroxybutyrate pathway (Berg et al. 2007) where it converts 4-hydroxybutyryl-CoA to crotonyl-CoA. Representation of the whole pathway in genomic data of *Nitrosopumilus maritimus* (Walker et al. 2010) and *Cenarchaeum symbiosum* (Hallam et al. 2006) hints to the employment of the 3-hydroxypropionate/4-hydroxybutyrate pathway for C-assimilation in Thaumarchaeota. and can indeed be used Although the hydroxybutyryl-CoA dehydratase was initially described in anaerobic clostridia to be involved in fermentation (Scherf et al. 1994; Gerhardt et al. 2000) , the *hcd* gene encoding for 4-HCD is found as well in autotrophic archaea (Berg et al. 2007; Berg et al. 2010) and can be used as marker gene for autotrophic growth (Zhang et al. 2010; Offre et al. 2011). Anaerobic bacterial *hcd* genes always form a separate phylogenetic cluster additionally to two clusters assigned to hyperthermophilic crenarchaea and one cluster associated with thaumarchaea (Berg et al. 2007). Therefore we assume *hcd* as a key gene for C-assimilation in *N. viennensis* and its transcriptional patterns to be distinct from nitrogen related genes.

Among information processing or housekeeping genes the subunit B of RNA polymerase (*rpo*B) and the 16S rRNA gene were chosen and were assumed to produce a pattern distinct from AO-related genes.

Genes encoding putative RNA degrading enzymes or their regulators

After observation of the strong decrease in transcript numbers on the onset of stationary phase (see results), we wanted to search for hints of active degradation. Ern1 and ern2 were used to

monitor transcription of genes encoding for two similar proteins with homologies to endoribonucleolyticly active members of the YjgF family (see above). Directly adjacent to ern2 another gene annotated as padR-family like transcriptional regulator (Barthelmebs et al., 2000) was found in the *N. viennensis* genome. Due to its genomic linkage with ern2 it was another interesting candidate potentially involved in regulating mRNA levels. Subunit A of the archaeal thermosome (thsA) was shown to co-precipitate with the exosome complex in *S. solfataricus*, although only in very low amounts. Therefore partial association with the complex was suggested (Evguenieva-Hackenberg et al. 2003). As cells were harvested in mid-exponential phase only (OD_{600} = 0.5) and due to interactions with 16S rRNA (Ruggero et al., 1998), it cannot be ruled out that amounts of thsA associated with the exosome or the thermosome complex vary along culture development.

3 MATERIAL & METHODS

Organism

This study was performed on "*Candidatus* Nitrososphaera viennensis" strain EN 76 (Tourna et al. 2011). The name of the organism is still not validly described and is therefore still in the "*Candidatus"* status. For simplicity the full name and strain specification is omitted in the rest of this work.

Experimental setup

Two experiments termed "growth curve" and "recovery" were conducted. Media was 10% inoculated with pre-grown late exponential *N. viennensis* culture and grown in liquid Fresh Water medium (FWM) as previously described (Könneke et al. 2005; Tourna et al. 2011) FWM was amended with 1mM NH_4Cl, 0.05mM sodium pyruvate and 2mM sodium bicarbonate.

For the growth curve setup *N. viennensis* was grown in triplicate, in 400ml liquid FWM in 1l Schott bottles and incubated at 37 °C on a rotary shaker at 100rpm for 15 days. Samples were taken daily for cellcounts (50µl), nitrite-measurement (20µl) and RNA extractions (Replicate 1 & 2: 30ml; Replicate 3&4: 2x15ml).

In the recovery setup *N. viennensis* was grown in triplicate, in 200ml liquid FWM in 500ml Schott bottles and incubated at 37 °C, shaking was not necessary due to smaller volume. The starvation period was defined as the onset of stationary phase (when all ammonium was converted to nitrite). After 8 days of starvation (15 days after inoculation) 3 cultures were supplemented with NH_4Cl to 1mM and 3 cultures were left untreated as controls. 20 µl samples

for nitrite-measurements were taken on days 2, 6, 7, 15, 17, 19 and 21 after inoculation. RNA-extractions were performed on 15ml culture after 30 minutes, 2 hours, 4 hours, 1 day, 2 days, 4 days, and 6 days of recovery.

Nitrite measurements

Nitrite concentration of culture samples (20µl) was determined using a colorimetric assay developed by Johann Peter Griess (Griess; 1879). In principle sulfanilamide is diazotized by reacting with nitrite. Coupling this reaction with N-(1-naphthyl)-ethylenediamine dihydrochloride a pink azo dye is formed in correlation to nitrite concentration (Figure 6). Like every dye, this azo dye absorbs certain wavelengths of visible light and therefore, after applying the Griess reaction, absorbance was measured by a TECAN Sunrise spectrophotometer at 545 nm wavelength and absorption values were plotted on a standard curve (0-1000 µM NO_2^-). When concentrations above the highest standard (1000 µM) were expected, samples were diluted 1:2.

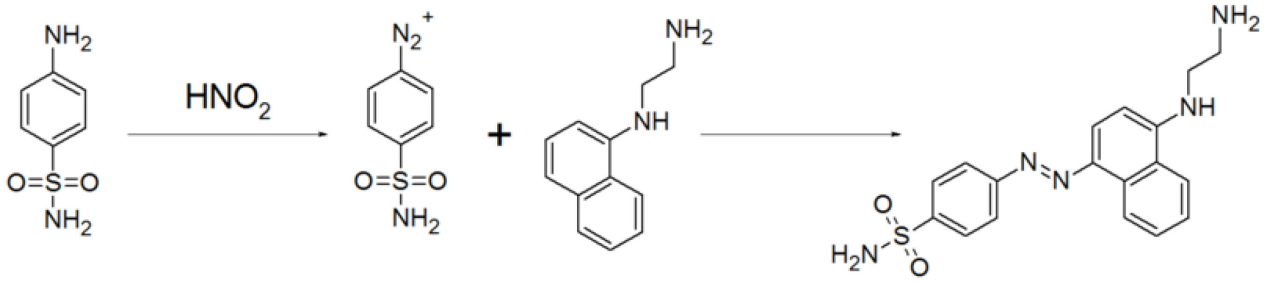

Figure 6: Reaction of nitrite with sulfanilamide followed by reaction with N-(1-naphthyl)-ethylenediamine dihydrochloride to form the pink azo dye

Cell counts

Cell counts were performed by phase contrast light microscopy of 10µl of culture samples. Cells present in 15 to 25 microscopic fields were enumerated at 1000x magnification in a Nikon phase contrast microscope and the average number of cells in a microscopic field was multiplied by a conversion factor ($2.5x10^6$) to get an estimate of the total amount of cells in a milliliter of culture.

RNA-extraction, purification and cDNA synthesis

Total nucleic acid extracts were prepared from varying volumes (see experimental setup) of culture samples using a modified version of the protocol by Griffiths et al (2000). Cells were immediately collected after sampling by centrifugation for 35 min at 48200 x g at 4 °C. Supernatant was discarded and cell pellets were re-suspended in 500µl of hot (65 °C) 1% SDS-extraction buffer before being transferred to lyzing matrix E bead-beating tubes (MPbio). After addition of 0.5ml phenol:chloroform:isoamylalkohol (25:24:1) pH 4.5, cells were lyzed using a FastPrep FP120 beadbeater (Qbiogene, Savant) for 30 sec at speed 4. Bead-beating tubes were then cooled on ice for 2 minutes and centrifuged for 10min at 16 000 x *g* and 4 °C. The aqueous phase containing the nucleic acids was transferred to a fresh 1.5 ml tube (Eppendorf) and phenol traces were removed by mixing with 500 µl of Chloroform:Isoamylalcohol (24:1). Emulsions were centrifuged for 10 min at 16 000 x *g* and 4 °C and the aqueous phase was transferred to a fresh tube. Nucleic acids were precipitated over night at 4° using isopropanol in presence of 0.2M NaCl and 20µg/ml glycogen (RNA grade, Fermentas). Precipitated nucleic acids were pelleted by 30 min of centrifugation at 16 000 x *g* and 4 °C and the supernatant was discarded. The pellet was washed with 500µl cold (-20 °C) 70% ethanol to remove the excess of salts and the ethanol

was discarded after centrifuging the tubes at 16 000 x *g* and 4 °C for 10 min. Nucleic acids were resuspended in 30µl nuclease free water (Qiagen) and stored at -80 °C before use.

Nucleic acid extracts were purified with an RNeasy purification mini kit (Qiagen) and were then treated with RQ1 DNase (Promega) following manufacturers instructions. Total RNA was purified with a second RNeasy purification mini kit. The presence of DNA traces was assessed by using an end-point PCR assay with 35 cycles on 2µl total RNA extract (and water or *amo*A qPCR standards as controls) in presence of 400nM of each *amo*A-qPCR primer, 2mM $MgCl_2$ and 0.25mM dNTPs. 5 µl of PCR reaction mix (containing green Flexibuffer) was loaded on a 1,5% agarose gel and exposed to electrophoresis followed by EtBr staining.

First strand cDNA synthesis was performed using random hexamers (2.5mM) and SuperScript III Reverse Transcriptase (SSIII RT)(Invitrogen) following manufacturer's instructions. In order to additionally test for DNA contaminations in each sample negative controls without SSIII RT were prepared. For testing SSIII RT or reaction mix contaminations, controls without template, and without both template and SSIII RT were additionally prepared. Reverse transcription reactions were incubated for 10min at 25 °C, 50 min at 42 °C and stopped by exposing to 70 °C for 15min.

Primer design

All primers were designed by using the Primer3 (Rozen and Skaletsky 2000) webserver (http://frodo.wi.mit.edu) with default parameters and *N. viennensis* target gene sequences as input sequences. Two primer pairs were designed for every gene analyzed in this study, one for generating standard-templates and the other one for qPCR reactions. Standard-templates were aimed to resemble full length transcripts in tradeoff with most optimal primer kinetics and nested

qPCR primer pairs were designed to retrieve amplicons containing around 200 nts in the optimum. Specificity of the primers was assessed first *in silico* by checking for other putative binding sites over the whole genome and in a second time by gradient (annealing temperatures 49 °C – 64 °C) PCR on genomic DNA from *N. viennensis*. Products retrieved by gradient PCRs were analyzed after ethidiumbromide staining in 1.5% agarose gel electrophoresis. Samples containing specific products (only one single sharp band on the gel) were purified using the NucleoSpin® Gel and PCR clean up kit and quantified fluorospectrometrically on a NanoDrop 1000 (NanoDrop Technologies, USA). Purified amplicons obtained from the primer pairs capturing the longer fragments were used as templates in a second gradient PCR to additionally test behavior of the nested primer pairs used for qPCR in order to obtain optimal annealing temperatures.

Table 1: Primers designed in this study for generating standard templates for qPCR. Position delineates the nucleotide matching to 5' end of the primer for each gene

gene	position	sequence	Ampliconsize (nts)	GC%	Tm (°C)
amoA	99	GCAGGAGACTACATCTTCTA	510	45	49,4
	609	TGGACATACAGATGGATGGC		50	59,3
hcd	224	AAACAGGTTCCTCCACATCG	583	50	60,0
	807	TACTCGCCGTCCAGAAAGAT		50	59,8
nirK	278	CGATACCGTGGAAATCACCT	721	50	59,8
	999	ACGTCGTCTGCGGTAGAGTT		55	59,9
rpoB	309	ATGCTAGAGTGCTCCGTCGT	2720	55	60,0
	3029	CCTCTGTAGGCTGCTTGGTC		60	60,0
padR	22	CGCGTTGGAAGCTCTATACC	267	55	59,9
	289	GCACGATGCCGTTTAGAGTT		50	60,3
thsA	745	GCGCTGGAGATTGAAAAGAC	361	50	60,0
	1106	CCTTCGATGAACACCCACTT		50	60,0
amoC4	139	ACGATTATTCGCGACTCTGC	320	50	60,4
	459	CGCATAGACCCTCACAAGGT		55	60,1
amoX	29	GTGGACGTAAGGGTGGTAGC	278	60	61,4
	307	AAGCCCTTACCGTCTTCTCG		55	59,4
amoB	146	TCTCCGACAATACCCTCCAG	397	55	59,4
	543	CGGTCTGGTAGCGAAGGTTA		55	59,4
amoC1	11	TGCCAGCACTGATACCAAAA	510	45	55,3
	521	TTGTACATTTCCGCCACGTA		45	55,3
Ern1	147	GGTGAAATTCAAAGGCAAGA	337	45	55,3
	484	CGGCGACAAACTCTACTTCC		55	59,4
Ern2	199	CAGTACGGAGCAACAATGGA	164	50	57,3
	363	AAGCTCGGGGAAAGCTAGAC		55	59,4

Table 2: qPCR primers designed for quantifying cDNA by real-time PCR assays

gene	position	sequence	Ampliconsize (nts)	GC%	Tm (°C)
amoA	376	ACAAGCACGCTGTCATCATC	195	50	59,9
	571	CAACCCAGTCATGGGTACTG		55	60,2
hcd	599	CTCGCACTGGATACTCGTCA	183	55	60,0
	782	CGTTTGGAATGAACACGTTG		45	60,0
nirK	492	TACGGTGCATTCGTAGTCCA	123	50	60,1
	615	ATGAGGTCAGGCTGCTCTGT		55	60,0
rpoB	2277	TACGAAGCAGAAGCAAAGCA	123	45	59,9
	2400	AGAATGTCGCCACCGTTTAC		50	60,0
padR	51	TTCCCGCCACTATATCCTGA	182	50	60,4
	233	ATTGAATACCTGCCGTCGTC		50	60,0
thsA	892	CAGAAGGGAATCGACGACAT	206	50	60,1
	1098	GAACACCCACTTGTCCGTCT		55	60,0
amoC4	160	TTCACTCCTGCAAACTGGTG	176	50	59,9
	336	CACTCCAATTGCAAACATCG		45	60,1
amoX	97	CGCAGAGTCGAAAACATGAA	158	45	55,3
	255	GGCTTCGATTACGGTCAGAA		50	57,3
amoB	148	TCCGACAATACCCTCCAGAC	180	55	59,4
	328	TTTGACCTGCTCTGATGTCG		50	57,3
amoC1	234	TGACAGGAAGGTCCCGATAC	184	55	59,4
	418	CCACTACGACACCCATGTTG		55	59,4
Ern1	230	CTGCGCGACTTTGTACCATA	138	50	57,3
	368	TTTATGACCTTGGGCTGGTC		50	57,3
Ern2	202	TACGGAGCAACAATGGACAG	110	50	57,3
	312	AGGGTTGCCAGAAAAGACCT		50	57,3

In order to differentiate at least between 3 out of 4 *amoC* encoding genes (2 truncated versions were neglected), 2 different primer pairs were designed. The 4 full length *amo*Cs are numbered in relation to their similarity (1=best blastp hit) to *Nitrosopumilus maritimus* (72-91% on AA-level) and are fairly similar to each other (73-96%). QPCR products from cDNA templates were cloned and sequenced. They contained only *amoC*2 amplicons (100% of reliable sequences) although they had 3 mismatches (MM) to the amoC1-F primer (Figure 7). Therefore no separate primers

for *amoC*2 were designed, which is the one closest (10 ORFs) to *amo*B. *amo*C3 perfectly fits the amoC1-418R primer, but the forward primer showed 5 MM, thereby inhibiting amplification of *amoC3* and making it the only copy of full length *amo*C genes not covered by our setup. The most distantly related *amo*C4 shows 6 MM to amoC1-F and should not be picked up by this primer pair, thus a primer pair specific for *amo*C4 was designed.

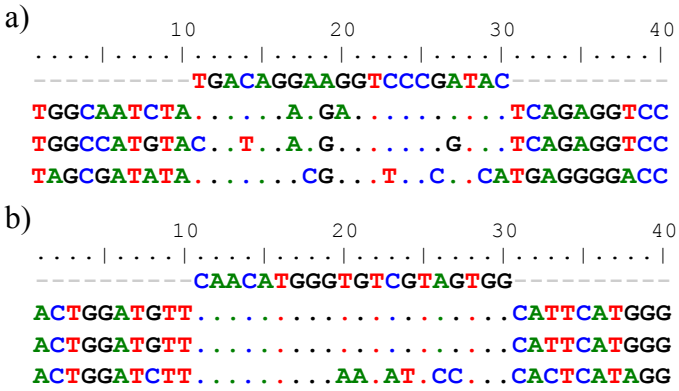

Figure 7: Alignments of qPCR primer pair for *amoC1* (a: 234F; b: 418R) to the other full length *amoC*s (2nd line, *amo*C2; 3rd line, *amo*C3; 4th line, *amo*C4) of *N. viennensis*. Dots indicate identical bases.

Quantitative real-time PCR

Quantification of cDNA was performed using a SybrGreen I approach. Dilution series (2x10-2x10^7 gene copies) of PCR products obtained by primers listed in table 1 were used as standard samples. Reactions were performed on 5µl template solution (cDNA, standards, controls) in 25µl total volume containing QuantiFast SYBR Green PCR Master Mix (QiaGen) and 1µM of each primer. All qPCR runs were performed on an Eppendorf mastercycler using Eppendorf realplex standard software and manufacturers protocols. Cycling conditions were set as described in the

QuantiFast handbook. Standards were amplified using primers listed in table 2 with efficiency and r^2 listed in table 3.

Table 3: Mean efficiency (E) of qPCR reactions and the coefficient of determination (r^2) for standardcurves

standard	E	r^2
amoA	0,87	0,9972
amoB	0,91	0,9957
amoC2	0,91	0,9978
amoC4	0,83	0,9937
amoX	0,98	0,9956
nirK	0,85	0,9980
hcd	0,85	0,9994
rpoB	0,86	0,9992
padR	0,85	0,9972
thsA	0,98	0,9992
Ern1	0,76	0,9992
Ern2	0,88	0,9962
16S	0,89	0,9983

Rapid amplification of cDNA ends (RACE)

To determine transcriptional start sites of *amo*A and *amo*C RACE was performed. RNA quantities from our extractions were around 500ng per sample which was too small for standard protocols, eg. Primer extension. Thus 5' RACE was tried like already described from (Tillett et al. 2000)(see Figure 8). In summary RNA was reverse transcribed using either a specific primer for *amoA,* amoA-prime-416R (5'-TTGAACAGCGGGAAGGATAG-3') or cDNA synthesis was primed by general random hexamers (Fermentas). RNA was either removed by alkaline hydrolysis cleavage and neutralized with tris-HCl or by RNase treatment. cDNA was precipitated in isopropanol in presence of sodium acetate and glycogen and re-suspended in nuclease free

water (QiaGen). Ligation of the 5' phosphorylated and 3' deoxyuridine blocked anchor oligonucleotide (5'- AGGCGAGAAGAGAACGTAAGCATGAG-3') to the single stranded cDNA was performed by T4 RNA ligase (Fermentas) following manufacturers protocol. Ligation-reactions were used in subsequent PCRs both directly without prior purification and also after anew washing and precipitation. As depicted in Figure 8 anchor ligated cDNA was amplified using an anchor specific primer (5'-CTCATGCTTACGTTCTCTTCTCGCCT-3') in 2 rounds of PCR with either primer amoA-295R (5'-CCACGATGAAGTCGTATGGA-3') or amoC1-521R given in Table 1 and subsequently in a final PCR with nested primers amoA-88R (5'-AGTCTCCTGCGTTGATGGTT-3') or amoC1-418R given in Table 2. After several unsuccessful trials (products were too short) with differing reverse transcription and PCR cycling temperatures ("touchdown" and "normal"), T4gp32 (MPBio) (up to 2,65 µM) and trehalose (up to 17,37%) were added to increase full length cDNA yield but still without proper products.

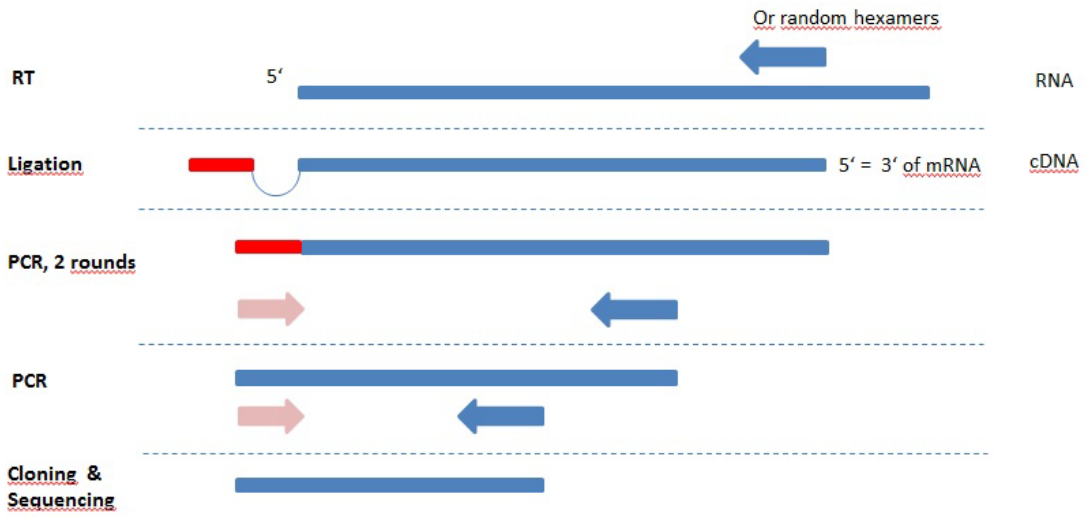

Figure 8: Graphical overview of workflow used for 5' RACE. Arrows indicate Primers (tip symbolizes 3' end of the primer). Red bar indicates the 5' phosphorylated and 3' end deoxyuridine blocked anchor.

Cloning & Sequencing

Cloning of RACE and *amo*C qPCR products was performed with the pGEM®-T Easy cloning system (PROMEGA) vectors and TOP10 competent *E.coli* cells (prepared in our own laboratory) following the protocol always using an insert:vector molar ratio of 3:1. Transformed *E.coli* cells were selected on LB agar plates with 100mg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranosid and 80µg/ml X-Gal to allow for blue/white screening. After colony-PCRs (colonies were picked with a pipette tip and resuspended in 48µl PCR reaction mix) with M13-primers, products were purified using NucleoSpin II PCR purification kit and eluted in 30µl of nuclease free water (QiaGen). By application of Primer SP6 samples were monodirectionally sequenced by the LGC genomics (www.agowa.de) sequencing service following the given instructions.

Statistical analysis

All statistical calculations and tests were performed by using SigmaPlot 12.0. The relatively high threshold for low statistical significance of the t-test between log transformed values of +30min samples to controls (table 3) was used to allow for biological variation between the 3 independent cultures as Culture 3 and its control behaved equal to the other 2 but delayed.

4 RESULTS

Growth

Cell-counts by phase contrast microscopy and nitrite-measurements were highly and significantly correlated (Pearson's r=0.973; $p<10^{-18}$) over the full growth curve and therefore in the recovery setup only nitrite measurements were used to monitor cell growth. Growth of *N. viennensis* followed a logarithmic curve showing 1 – 2 days of lag phase followed by exponential growth for 5 - 6 days. Cell numbers increased from $7.71x10^6$ cells/ml on day 2 to $4.86x10^7$ cells/ml on day 8, thus *N. viennensis* grew relatively slow with a generation time of approximately 79 h in exponential phase. Ammonium was converted to nitrite almost stoichiometrically with a speed of 172 ± 2.4µM NO_2^- produced per day or 7 µM NO_2^-/h. According to nitrite-measurements cultures reached stationary phase on day 7 (988.44 ± 8.62µM NO_2^-) and concomitantly cell counts did not further increase significantly after day 7 (Figure 9).

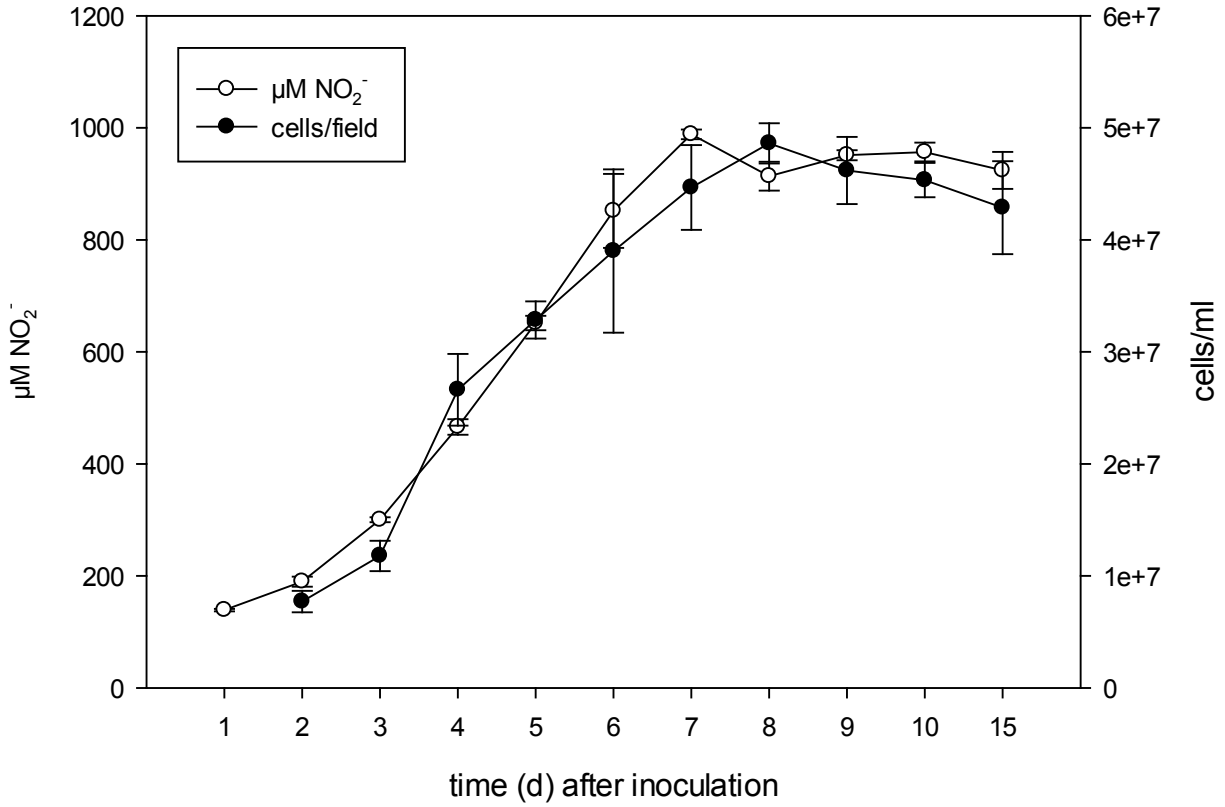

Figure 9: Growth curve and NO2- production of N. viennensis in FWM medium at 37 °C containing 1mM NH4+. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

After 8 days of starvation the re-addition of NH_4^+ to a concentration of 1mM was followed by NO_2^--production at comparable velocity to normal growth. After 6 days 749.1 ± 37.06 µM NO_2^- were produced (Figure 10) and controls stagnated. In the recovery setup no cell counts were performed.

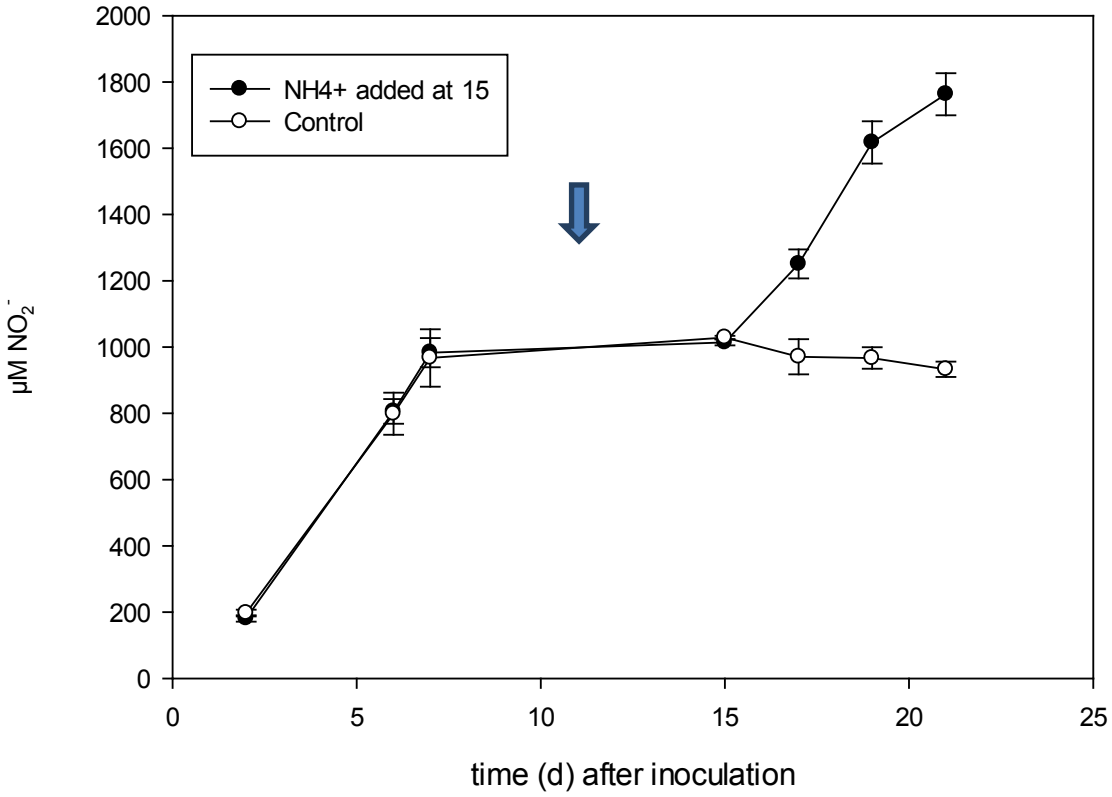

Figure 10: NO2- production of Candidatus Nitrososphaera viennensis at 37 °C in FWM medium containing initially 1mM NH4+. The blue arrow indicates re-addition of NH4+ to 1mM after 15 days (8 days of starvation) to initiate recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

Transcript levels of genes involved in ammonia oxidation

Transcript abundances of genes encoding for subunits A, B, C and X of ammonia monooxygenase and NirK were followed in *Nitrososphaera viennensis* growing in liquid culture, during 8 days starvation and recovery after re-supplement of ammonia. All in all copy numbers of *amo*A, B and X per cell were below 1 and ranged approximately from 1 copy in 10 to 10^5

cells. During exponential growth transcript abundances were stable around 1 copy in 10 to 100 cells. On the onset of stationary phase copy numbers decreased 13-39 fold from day 6 to day 7 (Figure 11).

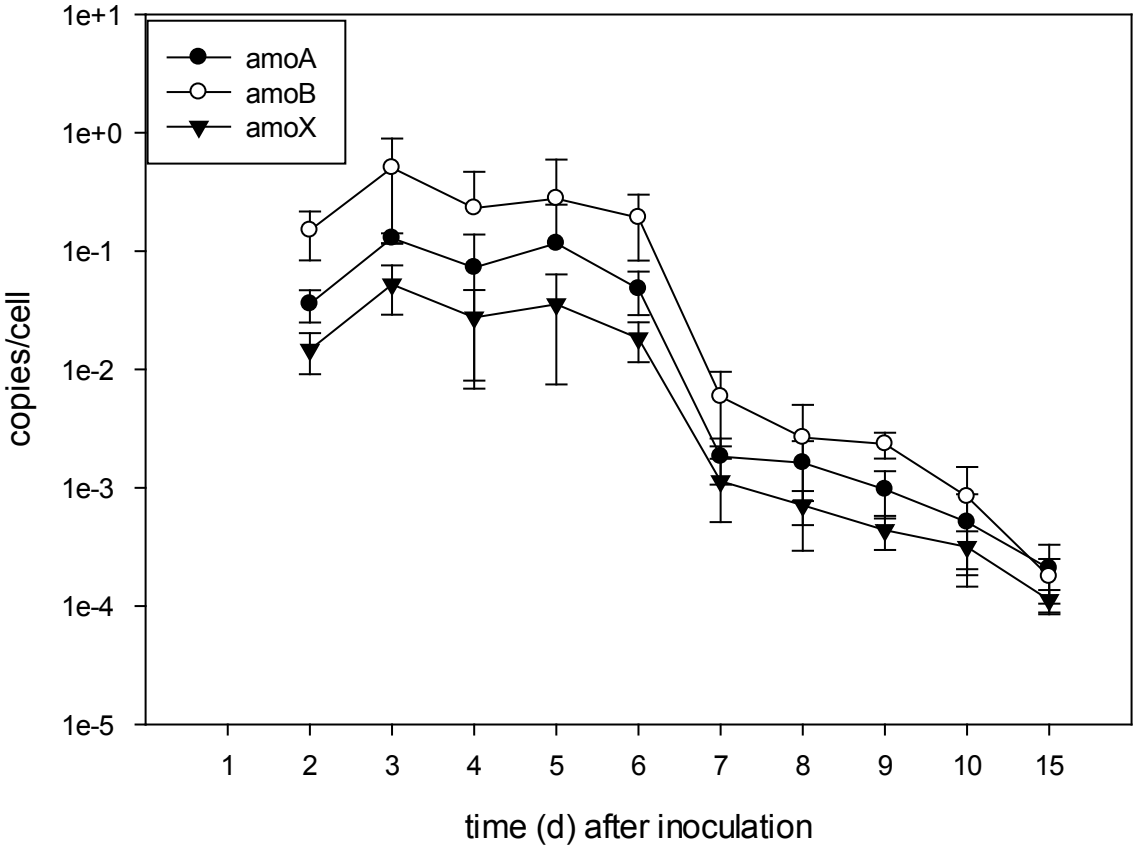

Figure 11: Copies of *amo*A, B and X per cell during growth and stationary phase. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

In contrast, more than 1 copy of *amo*C2 in 100 cells was kept also during starvation (Figure 12). Furthermore, in the recovery setup this difference was even more pronounced as *amo*C2-

transcripts showed a stable ratio of around one to control samples (no ammonium added) until day 17 (2 days after ammonium re-addition) and *amo*A mRNA was 100-1000 fold induced already after 1 day of recovery (Figure 13). The increase of *amoC*2 treatment/control ratio after 4 and 6 days of recovery (Figure 13) resulted from a decrease in controls.

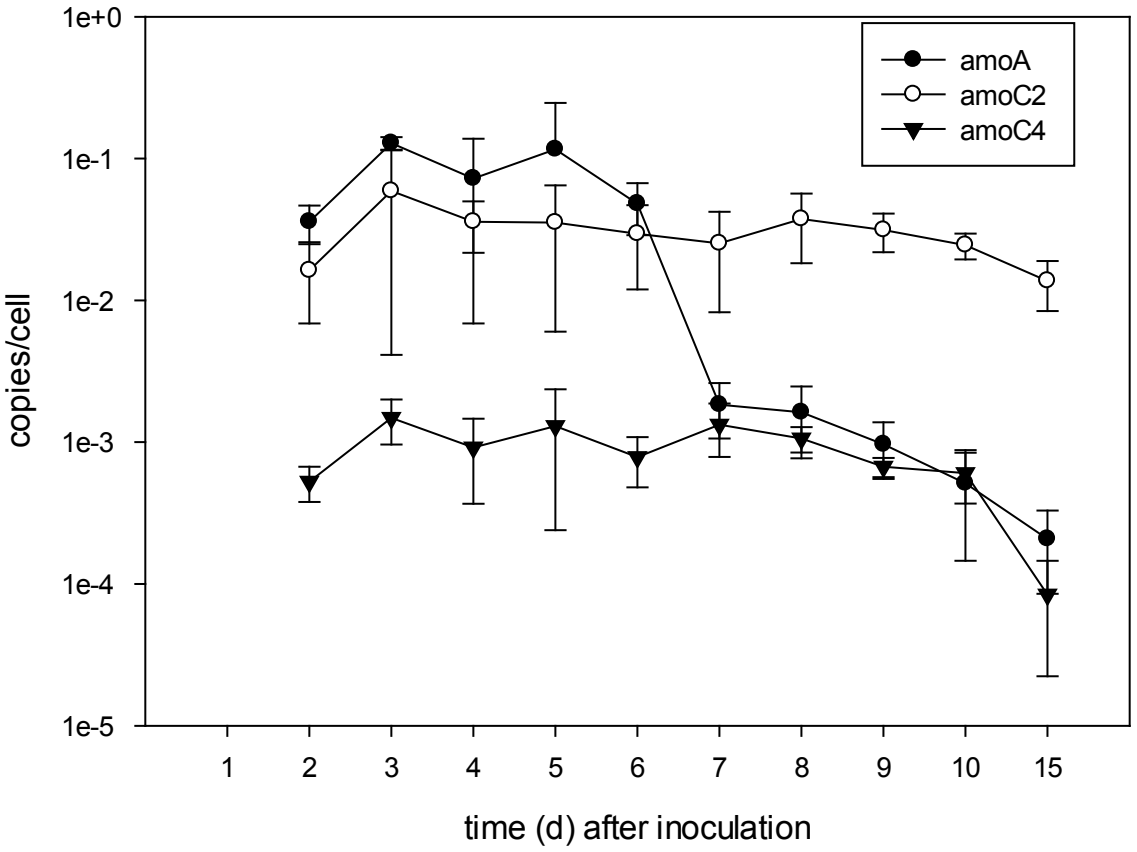

Figure 12: Copies per cell of *amo*A, C2 and C4 along growth and stationary phase. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

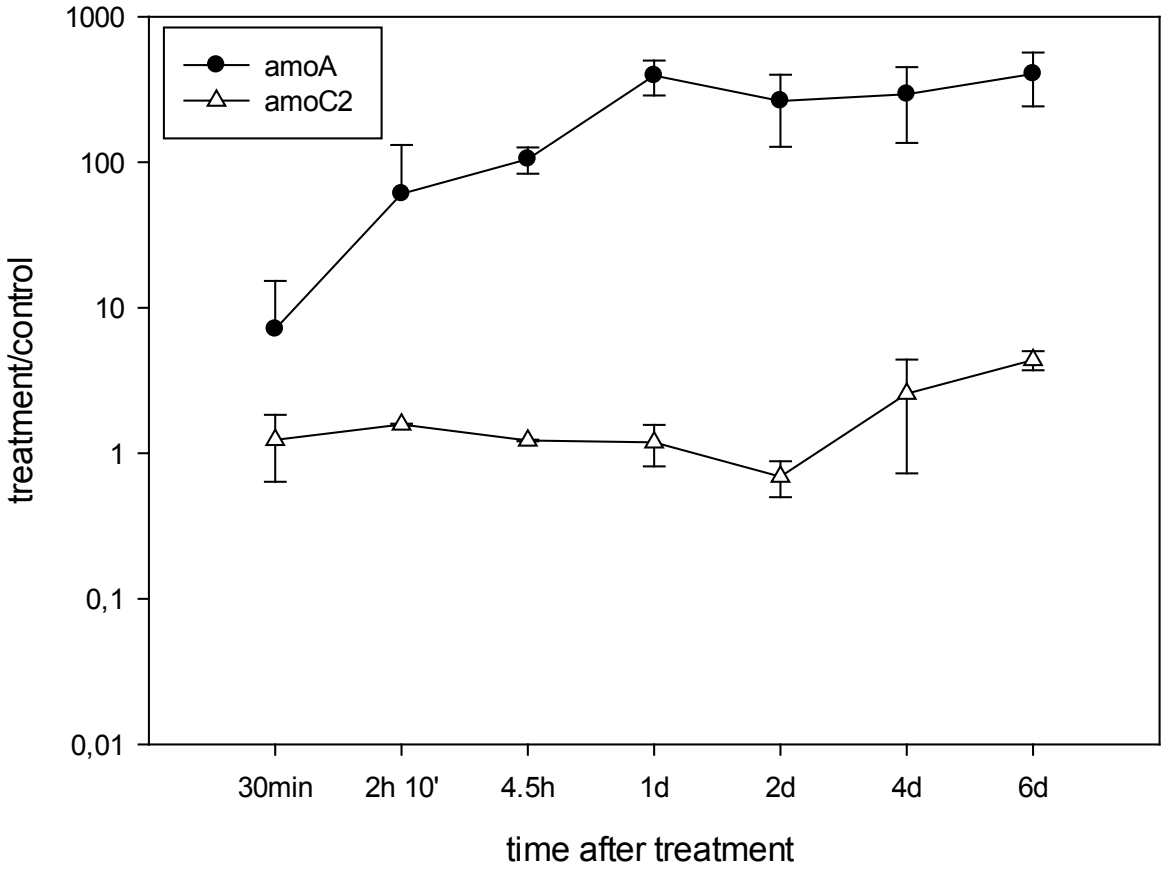

Figure 13: Transcript abundances of *amo*A and *amo*C2 divided by their own control during recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

However, this general insensitivity of *amoC*2 gene expression to ammonia in both setups made it an optimal standard to normalize all other transcript abundances. During growth and starvation *nirK* showed similar patterns to those of *amo*A, *B* and *X (*Figure 14 and 15) but in contrast was not different from controls after 30 minutes in recovery (Figure 16 and Table 4). *AmoC*4 transcript levels were detected in very low but stable amounts (around 1 copy in 1000 cells) until

day 10 but dropped almost to detection limit on day 15 (Figure 12). During recovery there was no clear pattern in *amoC*4 transcript levels with seemingly random induction or repression happening within each replicate over time and as well within one time point between triplicate samples (data not shown).

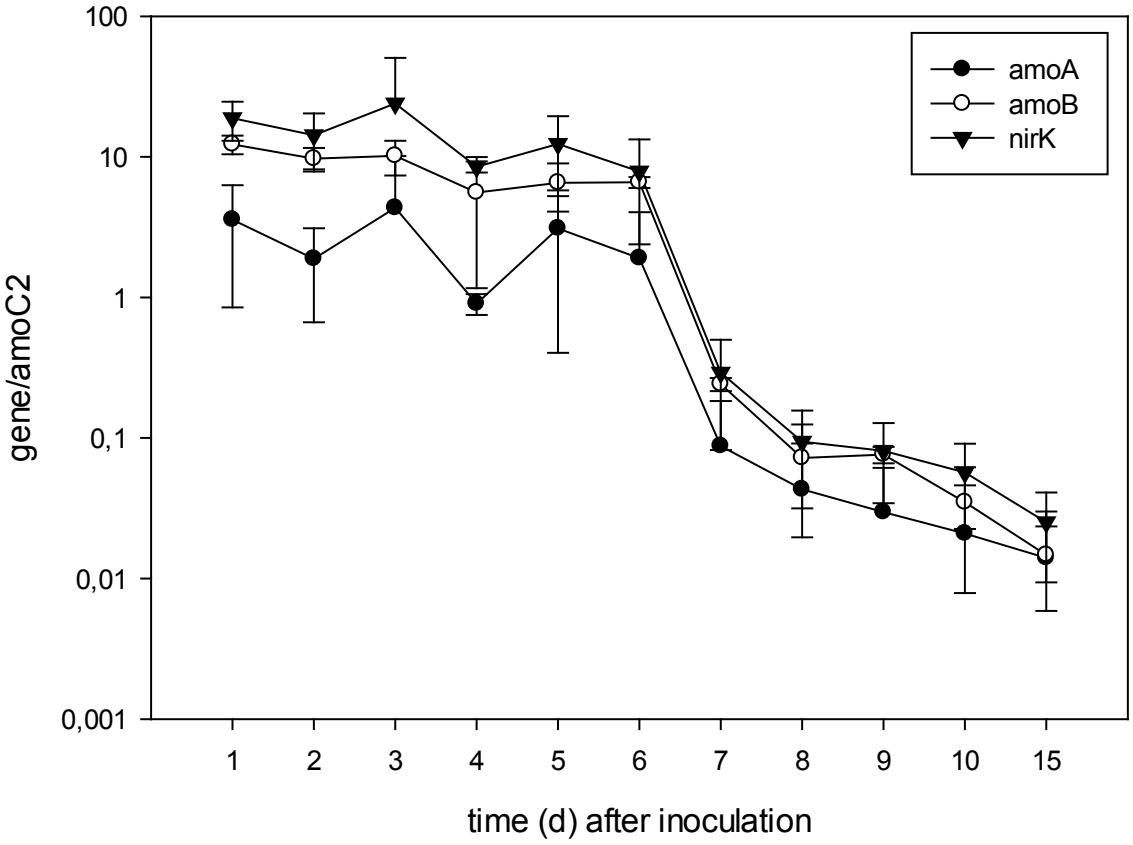

Figure 14: *amo*A,B and nirK copies normalized to *amo*C2 along normal culture development and starvation (starting between day 6 and 7). Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

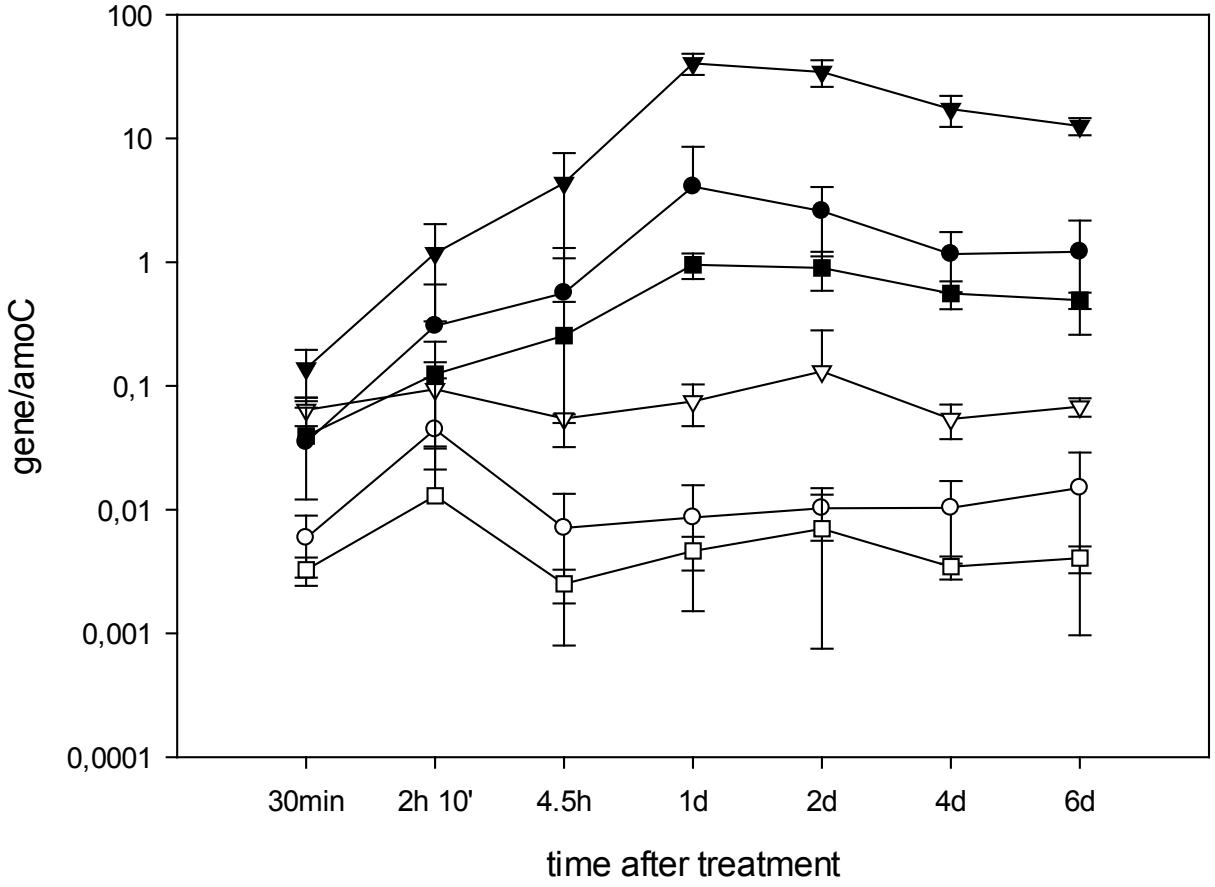

Figure 15: Ratios of *amo*A (circle), B (triangle), and X(square) to *amo*C2 in cultures supplemented with ammonium (black) to controls (white) during recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

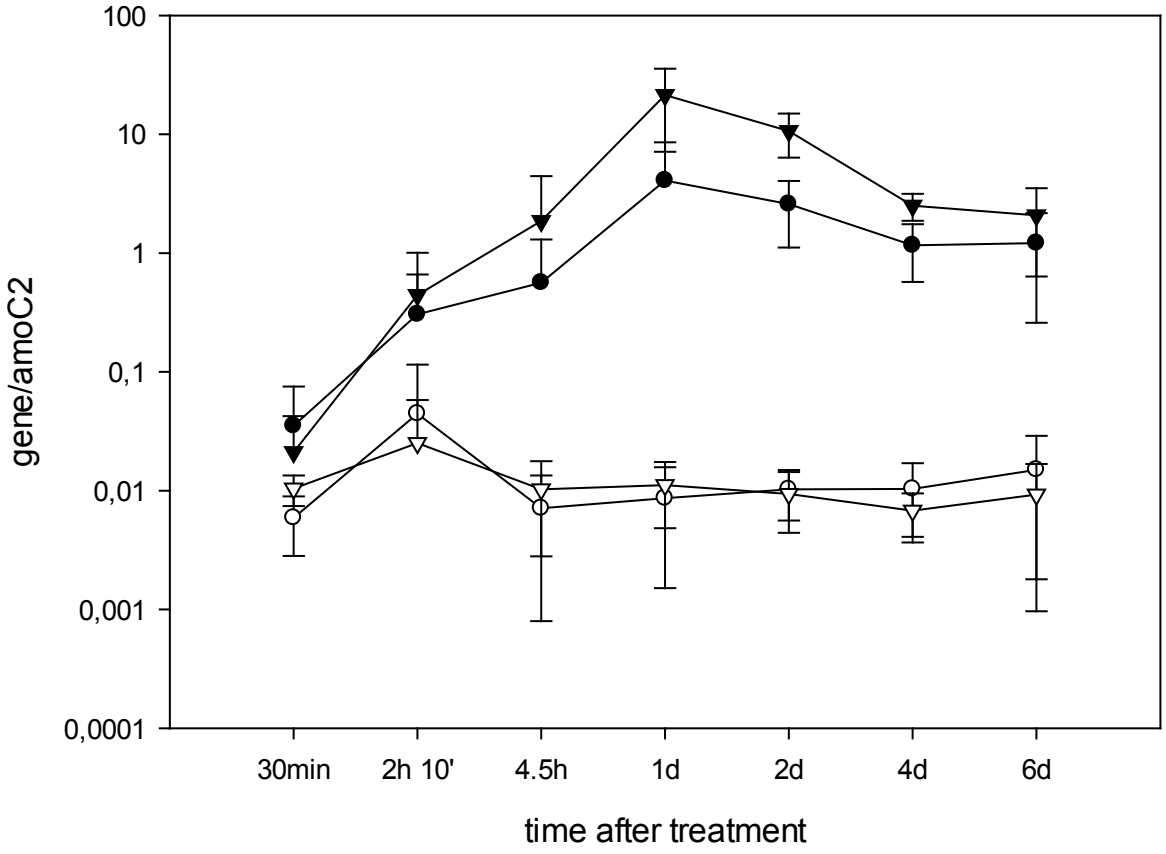

Figure 16: : Ratios of *amo*A (circle) and *nirK*(triangle) to *amo*C2 in cultures supplemented with ammonium (black) to controls (white) during recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

Table 4: p-values from t-tests between treatment and control transcript abundances at 30 min of recovery. One asterisk marks low but adequate (see methods) statistical significance (confidence >75%), two asterisks indicate high significance (confidence >98%)

	p	
amoA	0,222	*
amoB	0,141	**
amoX	0,017	**
nirK	0,465	
hcd	0,088	**
rpoB	0,661	

Transcript integrity

Primers used in qPCR for quantification of *amo*A (376F and 571R) capture only a fragment of *amo*A thereby allowing also detection of partly degraded *amo*A mRNA. Thus 35 cycles of normal PCR were done with qPCR and full-length primers (16F-609R) to check for complete *amo*A cDNA over the growth curve. Primers used in qPCR for *amo*A produced visible products from all samples and decreased until day 15 (Figure 17) like it was seen in qPCR results. No *amo*A was detectable after day 8 when the same amount of samples was used with full length primers.

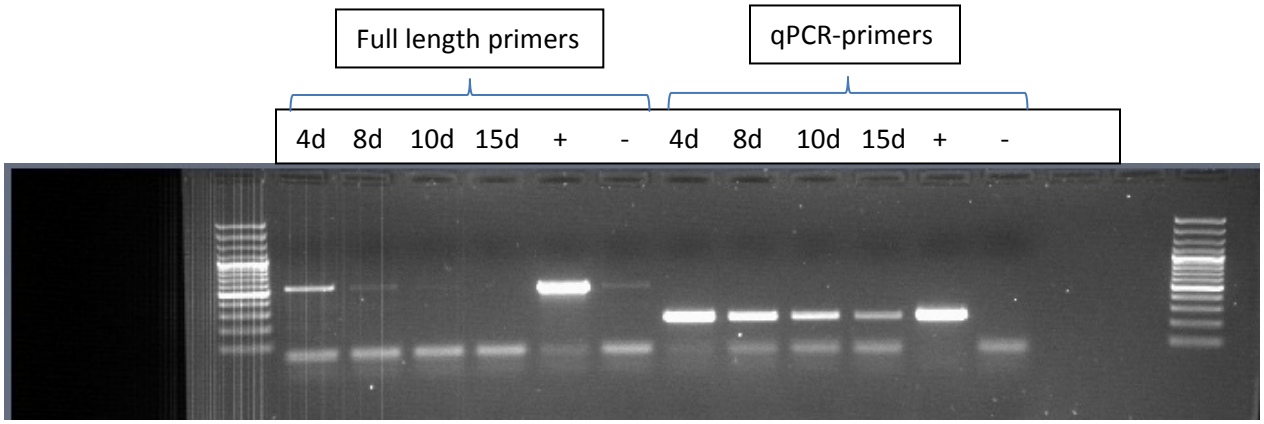

Figure 17: Picture of agarose gel loaded with products from 35 cycles PCR with *amo*A near full length primers (16F-609R) and qPCR primers.

Levels of carbon and information processing-related transcripts

As indirect control for AO-related genes and in order to get a broader picture of the dynamics in gene expression *hcd, rpoB* mRNA and 16S rRNA abundances were additionally monitored. Transcript abundances of *hcd* during exponential growth were around the same level as *amoC*2

(hcd/amoC2 = 1) but decreased on average 3-fold at the onset of stationary phase (Figure 18). Though *hcd* showed lowest values during exponential growth, when cultures reached stationary phase it was highest expressed among all other investigated genes (excluding *amoC2*).

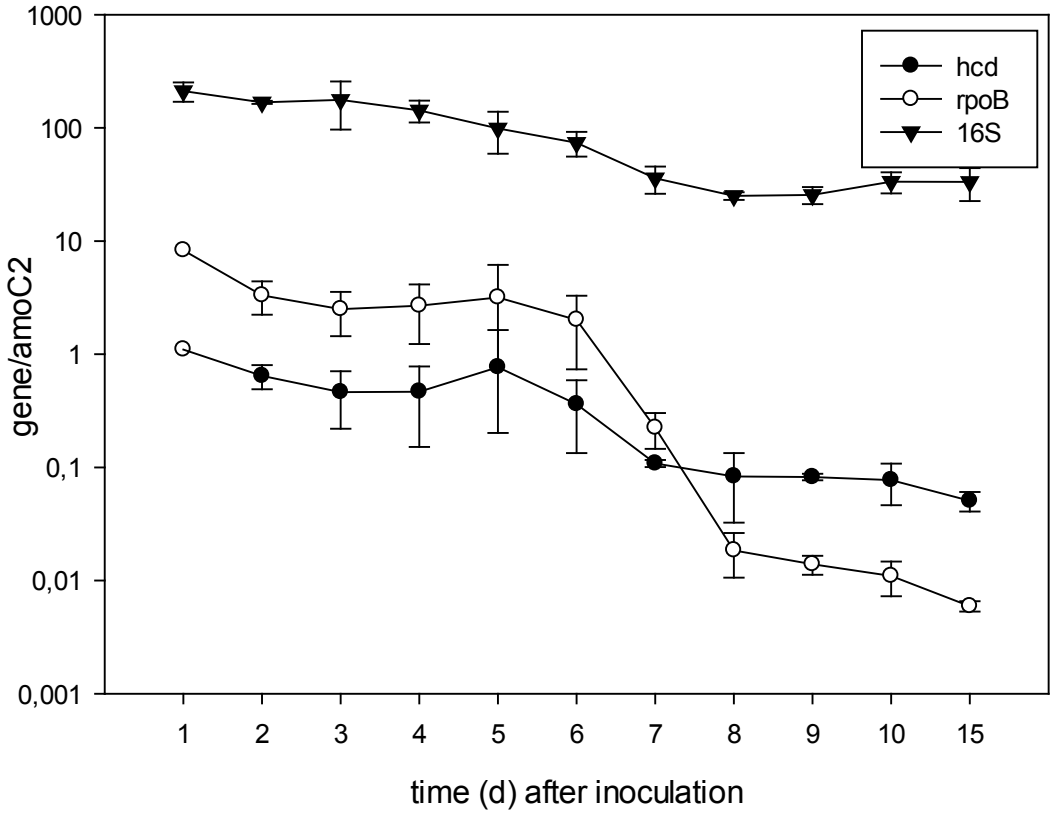

Figure 18: *hcd, rpoB* and 16S rRNA copies per *amoC*2 copies along normal culture development and starvation (starting between day 6 and 7). Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

Abundances of the 16S rRNA levels in exponentially growing cultures were initially at approximately 200 copies per *amo*C2 copy and constantly decreased along ammonia depletion (Figure 18). Once stationary phase was reached 16S rRNA levels stabilized at around one order

of magnitude lower levels than on day 1. After constant expression of *rpoB* during exponential growth, a strong decrease at day 7 was observed again but in contrast to the other genes, *rpoB* abundances continued to drop steeply until day 8 (Figure 18). Like *nir*K, *rpo*B expression in contrast to *amoA,B X* and *hcd* was not significantly different from controls at 30 min of recovery but started to increase afterwards (Figure 19).

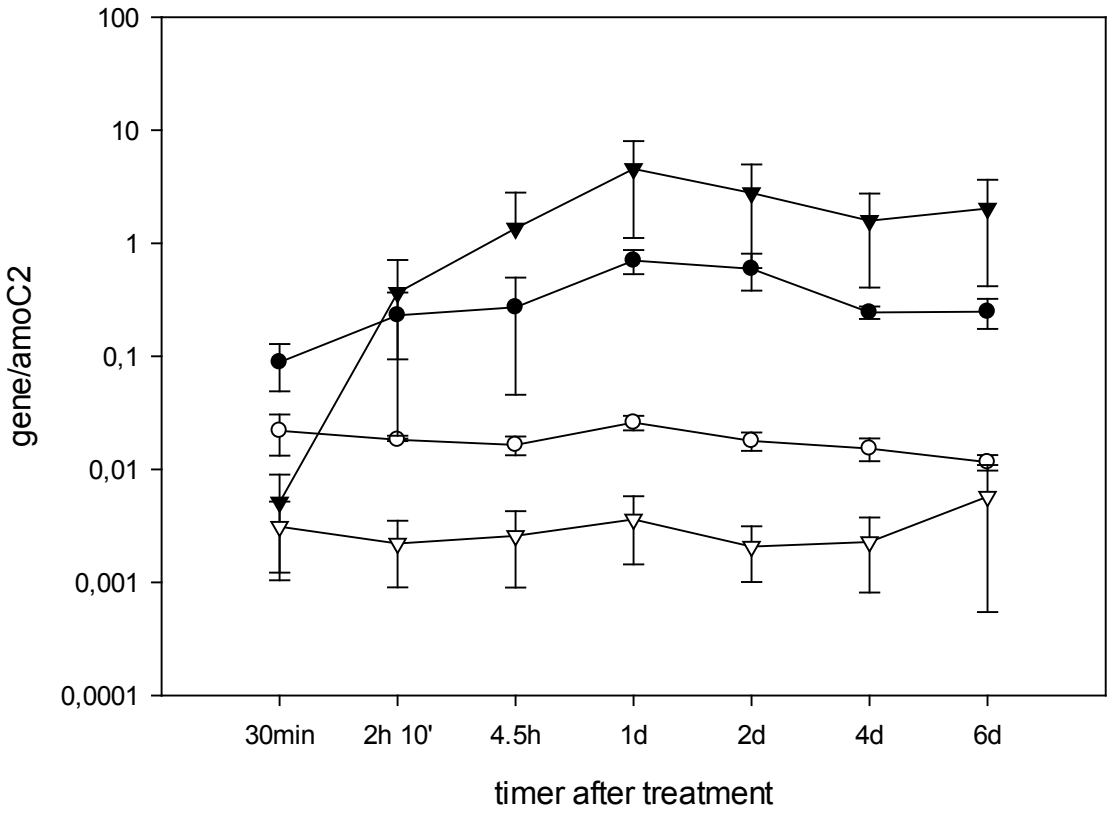

Figure 19: Ratios of *hcd* (circle) and *rpoB* (triangle) to *amoC2* in cultures supplemented with ammonium (black) to controls (white) during recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

Transcript dynamics of genes encoding putative RNA degrading enzymes or their regulators

Transcripts of genes encoding for putative endoribonucleases Ern1 and Ern2, as well as padR, a putative transcriptional repressor encoded directly adjacent to Ern2 and subunit A of the archaeal thermosome were monitored. After more or less stable expression of all these 4 genes during growth (Figure 20), Ern2 transcript abundance increased which stood in clear contrast to all other investigated genes. A strong increase during starvation was also observed for padR on day 10 and day 15 which again was unique among all genes monitored in this study.

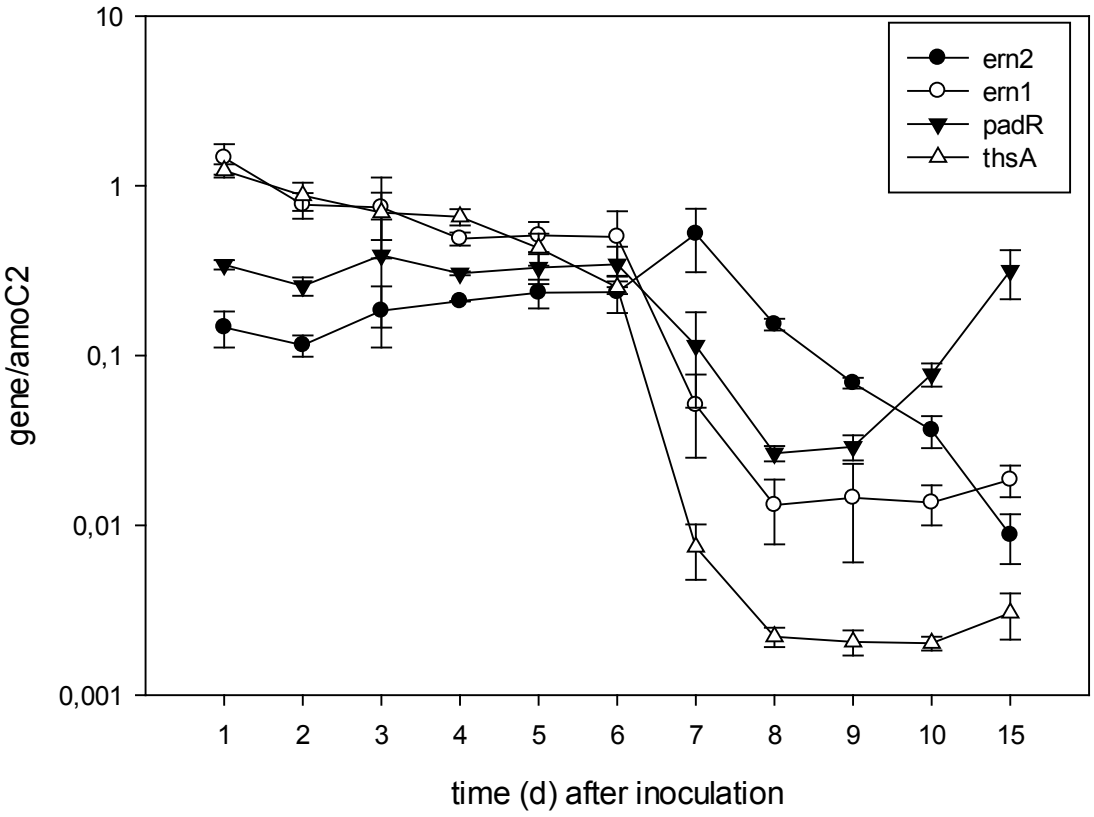

Figure 20: *Ern2, Ern1, padR* and thsA copies per *amoC*2 copies along normal culture development and starvation (starting between day 6 and 7). Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

During recovery Ern2 and padR were monitored only where Ern2 slightly increased during recovery but not significantly before 1 day of recovery (Figure 21). In contrast padR showed a significant decrease after 4.5 hours and stayed at stable levels also in untreated samples.

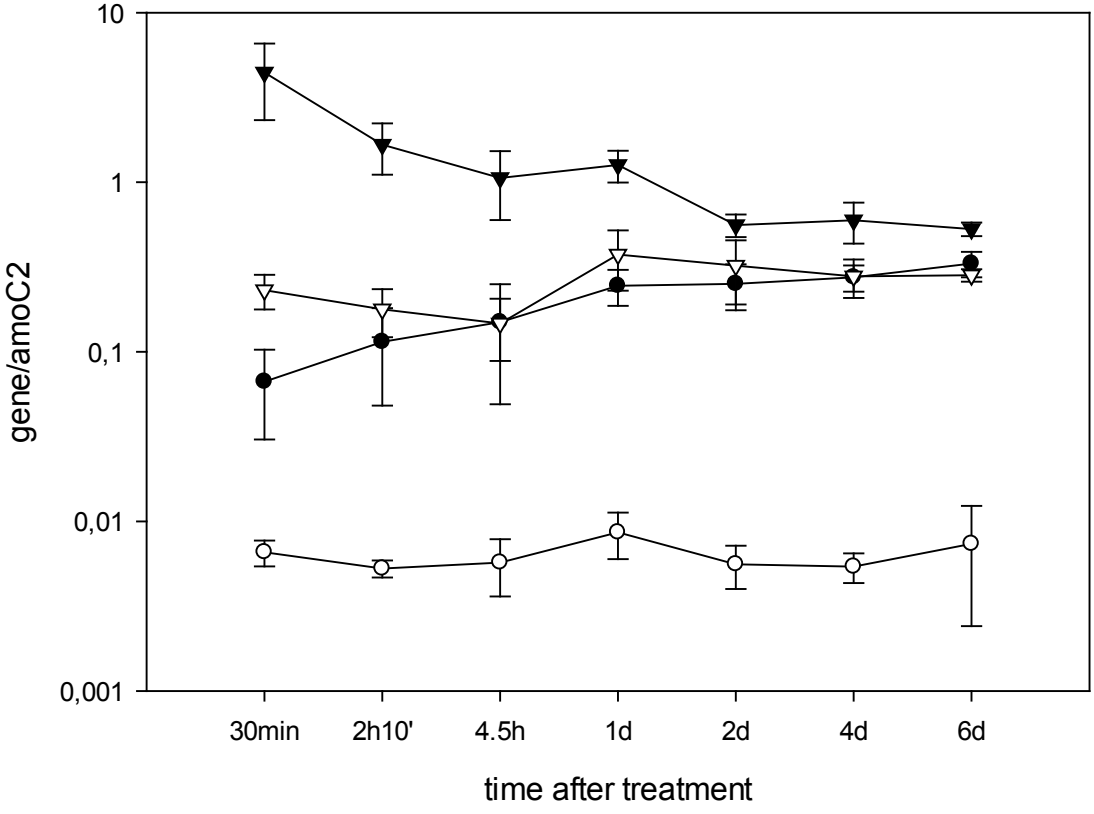

Figure 21: Ratios of *Ern2* (circle) and *padR* (triangle) over *amoC2* in cultures supplemented with ammonium (black) compared to controls (white) during recovery. Data points represent means of measurements from triplicate cultures and error bars depict standard deviations.

5' RACE

To identify transcriptional start sites, and from this to further infer common promotor patterns or to confirm absence of a 5' UTR (Bartossek et al. 2012), 5' RACE of *amo*A and *amo*C was attempted. All trials produced inconsistent results with reads of 10 to several hundreds of nts missing to full length ORFs as predicted by MAGE (Figure 21, 22).

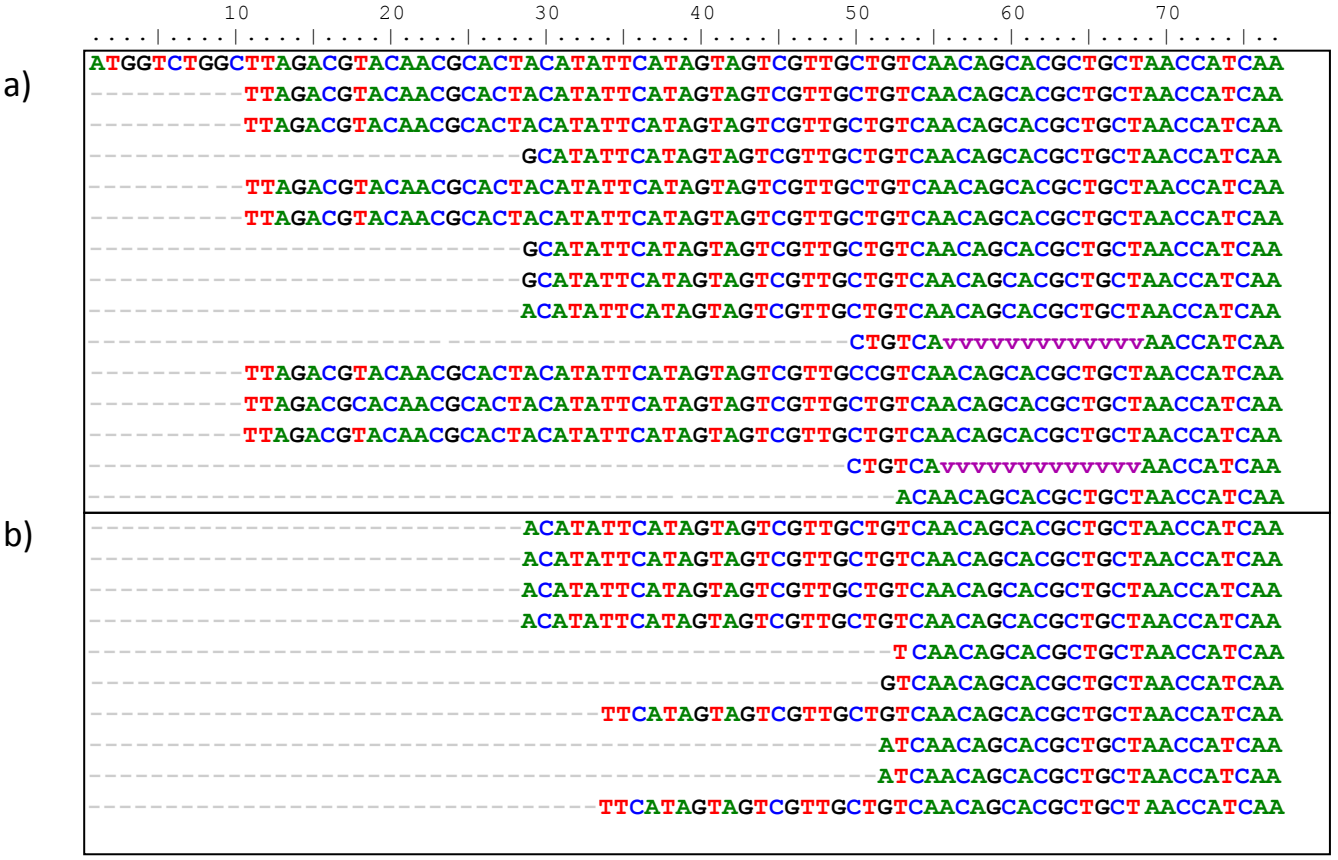

Figure: 22: Alignment of *amo*A (first line) with sequences retrieved from *amo*A 5' RACE after *amo*A-primed (a) and random hexamers-primed (b) cDNA synthesis. Deletions are indicated by "v".

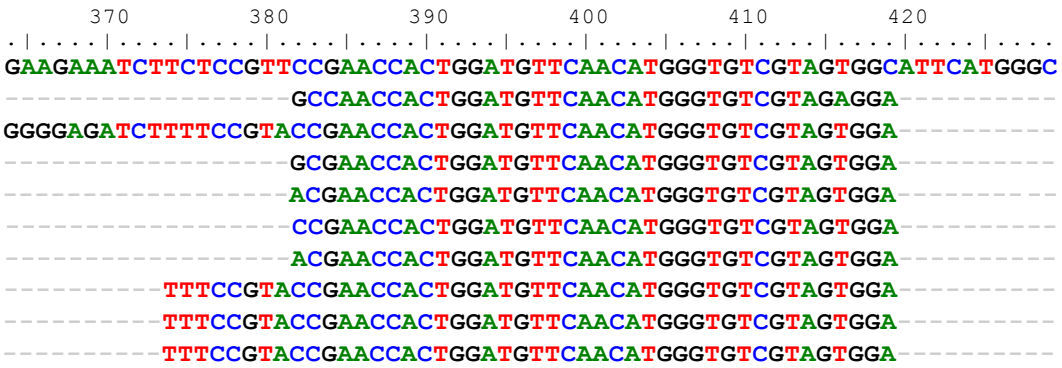

Figure 23: Alignment of *amo*C2 (first line) with sequences retrieved from *amo*C2 5' RACE after random hexamer primed cDNA synthesis.

5 DISCUSSION

In AOA our knowledge of transcriptional regulation, stress responses but also basics of their energy generation pathway is still at the beginning to develop. With this study we aimed at getting first insights into the "black box" of AO related gene expression in archaea. For this we monitored mRNA levels in a pure culture of our model AOA from soil, "*Candidatus* Nitrososphaera viennensis" upon depletion and supply of ammonia.

Growth

We could confirm that starvation responses and strategies to survive in and recover from energy-limited conditions are present in *N. viennensis*. This stands in clear contrast to *N. maritimus* not recovering already after 72 hours of ammonium starvation (Martins-Habbena personal communication) which might reflect the terrestrial and marine origin of *N. viennensis* and *N. maritimus*, respectively. However, the inability of *N. maritimus* to recover is unique and surprising as also all investigated AOB survive starvation (Geets et al. 2006). Further general comparisons to AOB are difficult as they are already different within itself (Tappe et al. 1999; Bollmann et al. 2002) and the velocity of the recovery response at least in terms of NO_2^- production also strongly depends on the duration of the starvation period (Bollmann et al. 2002; Berube et al. 2007). Whether *N. viennensis* would also show a quick recovery of AO within minutes or hours after NH_4^+ addition or if it responds differently after a longer starvation periods remains to be investigated in further studies.

Transcript patterns

Procedural limitations

When new fields are explored, first attempts may prove difficult and evaluating proper experimental designs for bigger future work is more economic in terms of time and money when preliminarily a smaller but still reliable method is chosen. Therefore qPCR measurements were conducted as a pilot project but some small drawbacks of this method should be noted.

Absolute amounts of mRNA molecules per cell below 1 are also found in active *E. coli* cell with single cell sensitivity for genes encoding for highly expressed proteins (Taniguchi et al. 2010). Thus our copy/cell ratios measured by qPCR after reverse transcription ranging from approximately 10^{-4} during starvation to 1 copy per cell in exponential phase are in agreement with the results of Yaniguchi et al. although our results might additionally underestimate mRNA abundances due to losses during cDNA synthesis (which were not tested).

Relative amounts between different transcripts should be taken with care when they rely on different standards (Love et al. 2006)(Feigl, Großpraktikumsprotokoll 2011) but still the variations in transcript abundances over time within one gene should be reliable as values are obtained in the same measurement with the same standard curve templates.

As qPCR primers are capturing only a fragment of the full length transcripts the integrity of cDNA measured in our qPCR assays may be questioned. Therefore abundances of PCR amplicons retrieved from qPCR primers and primers covering near full range of the transcript (Figure 17) were compared and our results may hint to an overestimation of *amo*A transcripts during starvation. However, as a faint band is visible in the negative control but nothing in lanes >8d and additionally only a weak signal is detected at day 4 compared to positive control,

samples may contain inhibitors for the full length primer pair and thus results may be misleading. Nevertheless, the possibility is very low that all transcripts detected during starvation are degraded to fragments containing the full region covered by the qPCR primers and therefore our results should indeed reflect biological truth.

Starvation

AO-related transcripts except *amo*C responded to depletion of ammonia whereas non AO-related gene expression responded either to a smaller extent and/or in different patterns. In general, a state-transition pattern (Yosef and Regev 2011; Bar-Joseph et al. 2012) is observable after all ammonium is consumed. Expression of *amo*A,B,X, *nirK* and *rpo*B decrease in similar proportions while *hcd* and 16S showed a relatively small decrease. Contrastingly, *amo*C2-transcripts are kept at high abundance after all NH_4^+ is consumed. Maintenance of certain *amo*C transcript levels during starvation was also shown for *amo*C1,2 in *N. europaea* after 3 days at 4° without NH_4^+ (Sayavedra-Soto et al. 1998; Hommes et al. 2001). Same results were shown by Berube et al. (2007) even after 6 days of starvation at 4° but not after 6 days at 20° which points towards temperature dependent differences caused by mRNA stability. In this study, we conducted starvation up to 8 days at 37° where mRNA is probably even more unstable and yet, high *amo*C2 as well as low but still detectable pools of *amo*A,B,X and *nir*K are found in *N. viennensis*. In contrast to this and other AOBs (Bollmann et al. 2005; El Sheikh and Klotz 2008), *amo*A mRNA is quickly undetectable in starved *N. europaea* cultures (Sayavedra-Soto et al. 1998; Stein and Arp 1998). However, this comparison must be taken with care as the latter two studies approximated mRNA abundances by Northern blotting without prior PCR steps. Together with this our results somehow strengthen the advice of Bollmann et al. (2005) that assumptions

on activity drawn from transcript abundances must be taken with care, nevertheless it might work for certain organisms and genes (Helbling et al. 2012). However, by sampling over time in absence and presence of NH_4^+ it became clear that high *amo*A,B,X and *nir*K transcript abundances co-occur with presence of NH_4^+ and AO activity but low transcript pools are also still detectable during starvation. Thus correlations of activity and transcript levels only make sense by comparing transcript pools in truly active states to starved conditions of the same community, as e.g. transcripts may be just stabilized and not actively synthesized. Active transcription in Archaea often requires a TATA-box which is conservatively found upstream of *amo*A,B,C and X among Thaumarchaeota (Bartossek et al. 2012). An additional *cis*-regulatory element, BRE, is only found and conserved upstream of *amo*A, B and X but not *amo*C2 (Bartossek et al. 2012) and may be one point of differential regulation among others which could explain the pattern of *amo*C2 being distinct from the other *amo* genes. Another possible explanation for the strong decrease of *amo*A, B, and X in contrast to *amo*C2 might be related to varying susceptibility to degradation of each transcript. Transcriptional start sites for the 4 *amo* genes were predicted to the same positions as the translational start sites (Bartossek et al. 2012) and therefore absence of a 5' UTR influencing mRNA stability can be assumed. To confirm this 5'RACE for *amo*A and *amo*C2 was conducted but unfortunately failed to produce full length reads and further studies (by primer-extensions) with higher RNA quantities have to be conducted to retrieve reliable results. The transcript pattern of a putative endoribonuclease termed Ern2 (Figure 21) hints to involvement of this enzyme in mRNA degradation and seems to be repressed by padR but these data are too preliminary to draw any solid conclusions. Further work regarding degradation and/or stabilization of mRNA is needed and only helpful when followed by whole transcriptome and proteome analysis and direct proof in archaea of the *in silico* annotated functions. Transcription of the predicted ORF *amo*X (Bartossek et al. 2012) is confirmed by our study for

the first time and followed *amo*A patterns, thus *amo*X may indeed be involved in AO but its presence and role on protein level also remains to be investigated.

Recovery response

In general, soil ammonia oxidizers seem to remain in a kind of "stand-by state" in order to manage quick responses to NH_4^+ pulses in their environment, even after almost one year of starvation (Wilhelm et al. 1998). After 8 days of starvation N. viennensis increased *amo*A,B and X mRNA abundances upon NH_4^+ re-supply within 30 minutes and other trials in bigger volumes confirmed this even within 10 min for *amo*A (Offre in personal communication) when samples were quickly cooled before centrifugation. In these early samples a time dependent difference in the mode of regulation (Yosef and Regev 2011) to AO related genes except *amo*C emerged for *rpo*B and surprisingly also *nir*K which showed a significant response not before 2 hours. After 1 day of recovery mRNA levels reached comparable abundances as during exponential growth and seemed to be saturated as they did not further increase. *N. briensis* was starved for 7 days and reached similar *amo*A transcript abundances as in late-exponential phase already after 4 hours (Bollmann et al. 2005). In comparison to that, *N. viennensis* did not reach exponential phase levels as fast as after 4 hours but unfortunately the time frame of sampling in this study was too coarse to determine saturation before 1 day of recovery. The recovery response in *N. europaea* involves induction of a divergent monocistronic copy of *amo*C (*amo*C3) (Berube et al. 2007) which is also associated with recovery from other stressors (Berube and Stahl 2012). The presence of multiple copies of *amo*C could allow for different responses to varying environmental conditions also in *N. viennensis*.

Hcd and the enigma of pyruvate dependence and storage of C-compounds

By Raman-spectra it was shown that *N. viennensis* produces and stocks huge amounts of poly-hydroxybutyrate (PHB)(Spang et al. 2012). According to the genomic repertoire of *N. viennensis* this PHB could stem from 3-hydroxybutyryl-CoA (Spang et al. 2012) an intermediate in the 3-hydroxypropionate/4-hydroxybutyrate cycle ((Berg et al. 2007) and may be used to regenerate acetyl-CoA and NADH+H pools. Acetyl-CoA can additionally be gained by decarboxylation of pyruvate which plays an important role in C-metabolism also in Thaumarchaeota (Spang et al. 2012). This decarboxylation may indeed be happening in *N. viennensis* as $^{13}CO_2$ is found in the headspace of cultures incubated with ^{13}C-labeled pyruvate (Tourna et al. 2011). Considering *hcd*-transcript patterns and its place in the 3-hydroxypropionate/4-hydroxybutyrate cycle, the presence of PHB in *N. viennensis* in the context of starvation may be interesting and it may be assumed that PHB is used during starvation. When *N. viennensis* is inoculated into media containing no pyruvate, it resumed growth until stationary phase at normal velocity. After a second transfer to new media lacking pyruvate, *N. viennensis* grew extremely slowly and did not reach stationary phase even after 2 months of growth (data not shown). If all pyruvate (0.05mM) was left after pre-growth of the inoculum then pyruvate concentration would be diluted to 0.005 mM after 10% inoculation. At this concentration generation times should already be much longer (Tourna et al. 2011) than what was actually observed in the first round of growth without pyruvate. Therefore it is likely that *N. viennensis* produces PHB while energy and organics are available in excess and makes use of this storage compound when conditions are limiting to maintain acetyl-CoA pools and regenerate reduction equivalents. This hypothesis may be further strengthened by the intermediate transcript abundances of *hcd* in stationary phase and recovery controls but unfortunately we could not measure pyruvate and/or PHB concentrations. By centrifugation it was impossible to spin down cells after pyruvate starvation, thus harvesting of the cells and

subsequent RNA extractions could not be conducted. However, an additional interesting setup for follow-up studies which emerged from our results would be to starve the cells for 14 d and follow transcripts in combination with measurements of pyruvate and PHB concentrations. Given the huge physiological impact of pyruvate (Tourna et al. 2011) and PHB (probably responsible for change of buoyancy) and their possible absence in the recovery setup, it is likely that the shift observed in the treated and untreated samples of late recovery may be related to limitation of reduced C-compounds as this shift is even most pronounced in *hcd* transcripts (data not shown). Nevertheless, this hypothesis needs to be further tested by addition of pyruvate after 14 days in recovery which should significantly increase hcd transcripts again or by establishing PHB and/or pyruvate measurements along the time course of an RNA-seq ammonium starvation experiment.

6 CONCLUSION AND PERSPECTIVES

Ammonium depletion, starvation and recovery induced by re-addition of the primary energy source of *N. viennensis* has proven to be sufficient for inducing differential transcriptional responses within minutes. Gene expression of *amo*A,B and X always showed similar trends, whereas *nir*K and *rpo*B showed a time dependent shift in the recovery response and *hcd* and 16S responded in general to a lesser extent. With finer sampling resolution in early recovery, PHB- and pyruvate measurements followed by a whole transcriptome analysis and together with work also on protein level, this approach of ammonia depletion and supply will provide even more insights into transcriptional regulation and its connection to protein activities and environmental conditions. Application of RNA-seq to a time-series of samples from *N. viennensis* during exponential growth, starvation and recovery will reveal more genes of importance in- or at the transition between these phases and may even allow identification of new genes involved in the AO pathway of archaea and links to their physiology. Genome-wide time series gene expression data allow to reconstruct networks of co-expressed genes, which in turn are useful to infer functions for hypothetical protein-coding genes by application of different computational methods and hypotheses (reviewed in De Smet and Marchal (2010); Bar-Joseph et al. (2012)). By this, candidates for a second enzyme like HAO in AOB or even more participants with functional relevance in archaeal AO could be revealed among a high number of uncharacterized, hypothetical, ORFAN or wrongly annotated genes present in AOA genomes. Thereby we will expand our understanding of cell functioning in Thaumarchaeota and their prominent role in the global nitrogen cycle.

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ZUSAMMENFASSUNG

Nitrifikation ist ein zentraler Prozess im globalen Stickstoffzyklus und wird zu einem großen Anteil von Bakterien und Archaeen vollzogen. Ammonium-Hungerexperimente mit Ammoniak oxidierenden Bakterien (AOB) enthüllten interessante Aspekte des Überlebens während Energie-limitierten Umständen, und der Regulation der Ammoniak Oxidation. Um Einblicke in transkriptionelle Dynamiken in einem Ammoniak oxidierendem Archaeon (AOA) zu bekommen, wurde ein Hunger- und Erhohlungsexperiment mit „*Candidatus* Nitrososphaera viennensis“ EN 76, ein Isolat aus Boden, durchgeführt. Der Organismus wuchs in Flüssigkultur bis das Ammonium im Medium ausgebeutet war und die Zellen in die stationäre Phase eintraten. Nach einer Hungerphase von 8 Tagen, wurde dem Medium wieder Ammonium hinzugefügt, um die Erholung zu initiieren. Veränderungen in Boten-RNS (mRNA) Niveaus von Schlüsselgenen der Ammoniakoxidation (AO), des Kohlenstoffmetabolismus und der Informationsverarbeitung wurden durch quantitative Polymerasekettenreaktion (qPCR) verfolgt. Hohe Niveaus von Transkripten welche für die Untereinheiten A, B und X der Ammonia Monooxygenase (AMO) kodieren, wurden nur detektiert wenn NH_4^+ verfügbar war. Im Gegensatz dazu wurden hohe Transkriptniveaus von einem der *amo*C gene konstitutiv erhalten, auch wenn das gesamte NH_4^+ schon zu NO_2^- konvertiert wurde und ebenso wenn die Kulturen wieder auf 1mM NH_4^+ ergänzt wurden und sich von der 8-tägigen Hungerphase erholten. Die Expression von 16S rRNS, der Untereinheit B der RNS Polymerase (*rpo*B), der 4-hydroxybutyryl-CoA Dehydratase (*hcd)* und einer Kupfer-abhängigen Nitrit Reduktase (*nir*K) zeigte unterschiedliche Muster verglichen mit den AO-assoziierten Genen in Bezug auf Zeit und Intensität. Diese Studie liefert erste Berichte über Transkription in AOA und zeigt Gemeinsamkeiten aber auch Unterschiede zu

vergleichbaren Experimenten in AOB. Obwohl noch immer vorläufig, Hinweise auf die Beteiligung einer spezifischen Endoribonuklease, welche die Veränderungen der Transkriptniveaus reguliert, können aus dieser Studie abgeleitet werden. Generell zeigt diese Arbeit dass der experimentelle Ansatz des Ammoniakhungerns und die darauf folgende Erholung, für die Erforschung von Transkriptdynamiken in AOA auch über das gesamte Genom brauchbar ist.

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