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INVESTIGATION OF ACYL HOMOSERINE LACTONE QUORUM SENSING IN *NITROSPIRA INOPINATA*, A COMPLETE AMMONIA OXIDIZER

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INVESTIGATION OF ACYL HOMOSERINE LACTONE QUORUM SENSING IN *NITROSPIRA INOPINATA*, A COMPLETE AMMONIA OXIDIZER

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Abstract

Quorum sensing is an important system of regulation in microorganisms. However, quorum sensing has been better studied in heterotroph model organisms compared to chemolithoautotrophs, such as nitrifying microorganisms (nitrifiers). In *Nitrospira inopinata* ENR4, a complete ammonia oxidizer (comammox), only the presence of putative quorum sensing gene homologs to *lasI* and *lasR* which encode for acyl-homoserine lactone synthase and transcriptional regulator LasR , respectively, have been discovered. We sought to confirm the function of the *lasI* and *lasR* homologs in *N. inopinata* through heterogeneous expression of these genes in *Pseudomonas putida* F1. Three acyl homoserine lactones (3-OH-C10-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL) were found to be produced by the putative AHL synthase in *P. putida* F1. Only two of the AHLs (3-OH-C10-HSL and 3-OH-C14-HSL) were detected in *N. inopinata* cultures. The addition of these three acyl homoserine lactones into *N. inopinata* cultures resulted in slower growth and utilization of ammonia and nitrite. Whole-cell transcriptomics analysis of 3-OH-C12-HSL addition cultures revealed downregulation of energy production pathways and the fatty acid biosynthesis pathway compared to no-AHL addition controls. Our study confirms a functional quorum sensing system in *N. inopinata* that is mediated via three acyl homoserine lactones and opens future research pathways into the quorum sensing regulation of comammox *Nitrospira* and other nitrifiers.

Introduction

Nitrification is an essential step in the global nitrogen cycle, which involves the oxidation of ammonia to nitrite and the oxidation of nitrite to nitrate by specific groups of microorganisms. Ammonia-oxidizing bacteria (AOB) and archaea (AOA) oxidize ammonia to nitrite to produce energy for their metabolic functions. Nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate as their primary source of energy. Complete ammonia oxidizers (commamox) can fully oxidize ammonia to nitrate for their energy requirements.

Commamox was the most recent to be discovered out of the other nitrifier groups with the isolation of *Nitrospira inopinata* in 2015 and belong to the genus *Nitrospira* (1). *Nitrospira inopinata* was initially isolated from a biofilm found on the surface of a pipe in a warm freshwater environment (1). *N. inopinata* does contain homologs for ammonia oxidation genes when compared to other ammonia oxidizing microbes (AOM), however, the *amoA* gene is different to other known *amoA* genes in other AOM (1). Another difference between *N. inopinata* and other *Nitrospira* NOB is that *N. inopinata* cannot grow with only nitrite as the sole energy source. *N. inopinata* lacks genes to assimilate nitrite as a nitrogen source for biosynthesis (1).

Bacteria employ a few different methods to detect and respond to other bacteria cells. Quorum sensing is one of these methods that bacteria can use (2). Quorum sensing allows bacterial cells to determine the relative size of a recognized bacterial population and regulate behaviors in response to high-cell density or low-cell density environmental conditions. Quorum sensing relies on bacterial populations producing and detecting extracellular signaling molecules termed autoinducers. Generally, high concentrations of autoinducers due to high-cell density populations cause cells that recognize that autoinducer to increase autoinducer production and

induce quorum-regulated behaviors such as changes in metabolism, production of virulence factors, and biofilm formation (2). Both Gram-positive and Gram-negative bacteria can use quorum sensing to regulate their own population behaviors, but they produce distinct types of autoinducers. Gram-positive bacteria produce oligopeptides as autoinducers for quorum sensing regulation, while Gram-negative bacteria produce acyl-homoserine lactones as autoinducers for the same purpose. In *Nitrospira winogradsky*, a Gram negative nitrite-oxidizing bacterium, induction of quorum sensing behaviors included the inhibition of nitrogen assimilation, which resulted in slowed cellular growth (3).

The biosynthesis of acyl groups with differing sizes of the carbon chain of acyl-homoserine lactones was found to be driven by the competition of two enzymes in *Pseudomonas aeruginosa* (4). This biosynthetic pathway would be important to highlight since *Nitrospira inopinata* was hypothesized to produce three acyl-homoserine lactone compounds with different carbon chain sizes in the acyl group. *P. aeruginosa* was known to produce a 3-oxo-C12-homoserine lactone as a signaling molecule for quorum sensing regulation under normal conditions at high concentrations of cells (4). However, *P. aeruginosa* was also observed to produce AHLs with smaller acyl carbon chains such as AHLs with C6-C10 acyl carbon chains (4). 3-oxo-C12-homoserine lactone was known to be synthesized by acyl-homoserine lactone synthase (LasI) with 3-oxo-C12-Acyl Carrier Protein (ACP)as the intermediate needed from the fatty acid biosynthesis pathway. LasI could also synthesize the AHLs with small acyl groups using 3-oxo-acyl-ACP of the appropriate acyl carbon chain size before undergoing further elongation of the acyl chain. LasI must compete with 3-oxoacyl-ACP reductase (FabG), a protein that continues the process of elongating the acyl chain of the 3-oxo-acyl-ACP intermediate required by LasI. FabG could outcompete LasI for 3-oxo-acyl-ACPs with acyl carbon chains

smaller than 12 carbons long, however, LasI could outcompete FabG for 3-oxo-C12-ACP. This results in more 3-oxo-C12-homoserine lactones to be produced than 3-oxo-acyl-homoserine lactones with smaller acyl carbon chains under normal conditions (4).

In this study, *N. inopinata* was examined to determine if it could produce acyl-homoserine lactone autoinducers from a putative *lasI* homolog and if the produced acyl-homoserine lactones influenced gene transcription. The production of acyl-homoserine lactones by *N. inopinata* was determined by extracting these quorum sensing molecules from *N. inopinata* cultures using ethyl acetate and detecting them via liquid chromatography mass spectrometry (LCMS). The three acyl-homoserine lactones produced by the putative NinI enzyme, as identified through heterogeneous expression experiments, were 3-OH-C10-HSL, 3-OH-C12-HSL, 3-OH-C14-HSL. The effect of the three acyl-homoserine lactones on global gene transcription was determined by analyzing the transcriptomes of *N. inopinata* cultures grown with added acyl-homoserine. We hypothesize that these putative AHL synthetase genes are responsible for AHL production in *N. inopinata*. In addition, *N. inopinata* can produce different forms of AHL compounds under distinct growth conditions, which are involved in regulating their physiology and metabolic processes.

Methods

Stock Solutions:

Ammonia chloride (NH_4Cl) Stock Solution was prepared by adding 26.75 g of NH_4Cl into 500 mL of MilliQ water. After mixing to dissolve NH_4Cl, the stock solution was then

autoclaved and left to cool at room temperature. The stock solution was then stored at 4°C until needed. The stock solution had a final concentration of 1 M NH_4Cl.

Sodium Bicarbonate ($NaHCO_3$) Stock Solution was prepared by adding 8.4 g of $NaHCO_3$ into 100 mL of MilliQ water in a serum bottle. After mixing to dissolve $NaHCO_3$, the serum bottle containing the stock solution was sealed with a rubber stopper and autoclaved. The stock solution was then stored at room temperature until needed and had a final concentration of 1 M $NaHCO_3$.

Potassium Phosphate Monobasic (KH_2PO_4) Stock Solution was prepared by adding 0.2 g of KH_2PO_4 into 500 mL of MilliQ water. After mixing to dissolve the KH_2PO_4, the stock solution was then autoclaved and left to cool at room temperature. The stock solution was then stored at 4°C until needed and had a final concentration of 2.9 mM KH_2PO_4.

Iron Sodium EDTA (FeNaEDTA) Stock Solution was prepared by adding 137.65 mg of FeNaEDTA into 50 mL of MilliQ water. After mixing to dissolve the FeNaEDTA, the bottle containing the stock solution was covered with aluminum foil to block light from reaching the stock solution. The stock solution was then autoclaved for 20 minutes and cooled at room temperature. The stock solution was then stored at 4°C under dark until needed and had a final concentration of 7.5 mM FeNaEDTA.

Trace Element Stock Solution was prepared by adding 8.3 mL of 12 M hydrochloric acid into 991.7 mL of MilliQ water. The following compounds were then added to this acidic solution: 30 mg of boric acid (H_3BO_3), 100 mg of manganese dichloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 190 mg of cobalt dichloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 24 mg of nickel dichloride hexahydrate ($NiCl_2 \cdot 6H_2O$), 2 mg of copper dichloride dihydrate ($CuCl_2 \cdot 2H_2O$), 144

mg of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), and 36 mg of sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$). After mixing to dissolve all added compounds, the stock solution was autoclaved and allowed to cool at room temperature. The stock solution was stored at 4°C until needed. The stock solution had final concentrations of 0.5 mM H_3BO_3, 0.5 mM $MnCl_2 \cdot 4H_2O$, 0.8 mM of $CoCl_2 \cdot 6H_2O$, 0.1 mM of $NiCl_2 \cdot 6H_2O$, 0.01 mM of $CuCl_2 \cdot 2H_2O$, 0.5 mM of $ZnSO_4 \cdot 7H_2O$, and 0.15 mM of $Na_2MoO_4 \cdot 2H_2O$.

HEPES Buffer Stock Solution was prepared by adding a total of 119.2 g of HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) and 12 g of sodium hydroxide (NaCl) to 300 mL of MilliQ water over several steps that involve adding smaller amounts of each compound: Add 30 g of HEPES and dissolve completely, and add 3 g of NaCl and dissolve. Repeat with 30 g of HEPES and 3 g of NaCl, then finally add 29.2 g of HEPES and 3g of NaCl, dissolving thoroughly after each step. After all amounts of the two compounds were added and dissolved, the solution was transferred to a graduated cylinder, and the total volume was fixed to a total volume of 500 mL by adding additional volume of MilliQ water. The bottle containing the stock solution was then covered with aluminum to keep light from reaching the stock solution. The stock solution was then autoclaved and cooled at room temperature. The stock solution was stored at 4°C until needed. The stock solution contains final concentrations of 1 M HEPES and 0.6 M NaCl.

Nitrite Stock Solution was prepared by adding 34.5 g of sodium nitrite ($NaNO_2$) into 500 mL of MilliQ water. After mixing to dissolve the $NaNO_2$, the stock solution was then autoclaved and cooled at room temperature. The stock solution was stored at 4°C until needed. The stock solution had a final concentration of 1 M $NaNO_2$.

Commamox Media:

Commamox Media was used for the growth of *Nitrospira inopinata* in batch cultures. Commamox media with 1mM ammonia was prepared by first adding 2.0 g of NaCl, 0.8 g of magnesium dichloride hexahydrate $MgCl_2*6H_2O$, 0.2 g of $CaCl_2*2H_2O$, and 1.0 g of KCl into 2 L of MilliQ water. The resulting solution was then autoclaved. A day after autoclaving, 10 mL of KH_2PO_4 stock solution, 4 mL of NaH_2PO_4 stock solution, 20 mL of HEPES buffer stock solution, 2 mL of iron EDTA stock solution, 2 mL of trace elements stock solution, and 2 mL of NH_4Cl stock solution were ascetically added to the autoclaved 2 L salt solution through a Whatman Uniflow syringe filter that contains a PVDF membrane with 0.22 µm pores. The medium had a pH of 7.5 and was stored at 4°C until needed.

Nitrite-Oxidizing Bacteria Media:

Nitrite-Oxidizing Bacteria Media was primarily used for the growth of *Nitrobacter winogradsky* in batch cultures. Nitrite-Oxidizing media with 30mM NO_2 was prepared by first adding 2.0 g of NaCl, 0.8 g of $MgCl_2*6H_2O$, 0.2 g of $CaCl_2*2H_2O$, and 1.0 g of KCl into 2 L of MilliQ water. The resulting solution was then autoclaved. A day after autoclaving, 10 mL of KH_2PO_4 stock solution, 4 mL of NaH_2PO_4 stock solution, 2 mL of iron EDTA stock solution, 2 mL of trace elements stock solution, and 60mL of $NaNO_2$ stock solution were ascetically added to the autoclaved 2 L solution through a Whatman Uniflow syringe filter that contains a PVDF membrane with 0.22 µm pores. The media's pH was changed to 6.8 with HCl and NaOH solutions. After the pH was adjusted to 6.8, the media was filter-sterilized through vacuum filtration using a Fisher Scientific 250mL filter unit with a 50mm diameter and 0.2µm aPES membrane to remove contamination introduced by the pH adjustment and pH monitoring. The medium was stored at 4°C until needed.

Urea Media:

Urea Media was primarily used for the growth of *Nitrospira inopinata* in one AHL Extraction Experiment. Urea media with 2.5 mM urea was prepared by first adding 2.0 g of NaCl, 0.8 g of $MgCl_2*6H_2O$, 0.2 g of $CaCl_2*2H_2O$, and 1.0 g of KCl into 2 L of MilliQ water. The resulting solution was then autoclaved. A day after autoclaving, 10 mL of KH_2PO_4 stock solution, 4mL of NaH_2PO_4 stock solution, 20 mL of HEPES buffer stock solution, 2 mL of iron EDTA stock solution, 2 mL of trace elements stock solution, and 5 mL of 1 M urea stock solution were ascetically added to the autoclaved 2 L solution through a Whatman Uniflow syringe filter that contains a PVDF membrane with 0.22 μm pores. The medium had a pH of 7.5 and was stored at 4°C until needed.

Stock Culture Maintenance

Stock cultures of *N. inopinata* were maintained in 100 mL of Commamox media supplemented with either 1 mM or 5 mM ammonia at 37°C. The concentrations of ammonia, nitrite, and nitrate were regularly monitored to determine when a stock culture was ready for transfer into fresh media. Stock cultures were ready for transfer when almost all the ammonia substrate was depleted in the stock culture. For transfers into new stock cultures, 2 mL of the depleted stock culture was aseptically transferred into 100 mL of new Commamox media (2% transfer).

Plasmid and Strain Construction for Heterogeneous Expression

Two plasmid fragments were combined by using E. coli *DH5α*-mediated assembly to construct our pNinR plasmid (5). The first fragment was the NinR ORF and the downstream 216-bp intergenic region containing the ninI promoter which was synthesized by Sangon Biotech Co., Ltd. The second fragment was the PCR-linearized pBBRgdh-mCherry (PCR amplified by using the primers pBBR-ninR-F and pBBR-ninR-R and pLL1 (6) as template). Our primers contained a 25 bp of homologous overlap. This was required for the *E. coli DH5α*-mediated assembly. To create pNinR-NO we used the pBBR-mcherry-R and pBBR-mcherry-F as primers for PCR amplification a PCR fragment lacking the *ninR* gene from pNinR and assembled it in *E. coli DH5α*. To create pNinI, we combined a synthetic *ninI* DNA fragment (Synthesis by Sangon Biotech Co., Ltd.) with a 4,207-bp fragment of pBBRgdh (PCR amplified by using the primers pBBR-ninI-F and pBBR-ninI-R). To construct pNinI, we used *E. coli*-mediated assembly as described above. Transformants were selected on LB with gentamicin agar plates and plasmid DNA sequences were confirmed by DNA sequencing. The sequence-confirmed plasmids were then used to electrotransform *Pseudomonas putida* F1 (7).

Acyl-HSL Bioassays for NinR Heterogeneous Expression

AHLs were extracted from broth culture samples with two equal volumes of ethyl acetate with 0.01% acetic acid. AHL concentrations from ethyl acetate with 0.01% acidic acid extracts or HPLC fractions were measured by using the bio-reporter *P. putida* (pNinR) culture volumes. Cell culture extracts, HPLC fractions, and AHLs used as standards were added to 16 mm glass tubes. The solvent was evaporated under N_2 gas flow. One milliliter of *P. putida* (pNinR) culture grown in LB media with OD600 of 0.01 was added to each tube. The culture with added sample precipitate was incubated for 16 hours at 30 °C with shaking. To measure fluorescence, 150 μL

of each incubated sample was transferred to separate wells of 96 well black microtiter plates. mCherry fluorescence at 587 nm excitation and 610 nm emission was measured by using a Biotek H1 plate reader. Standard curves were generated by measuring the fluorescence response to different concentrations of 3-OH-C12-HSL standard samples by *P. putida* (*pNinR*) cultures.

Purification and Identification of Acyl-HSL during NinI Heterogeneous Expression

We purified the *P. putida* (pNinI) AHL from 1 L of culture after cells had been removed by centrifugation. The method for purification was modified based on a previously described study (6). The flow rate was adjusted to 1 mL/min. We used the 30% and 50% methanol fractions from the C18 Sep-Pak cartridge step, and the second isocratic HPLC was performed with 60% methanol. Purified material and synthetic acyl-HSL were analyzed by LC-MS/MS in a Waters Acquity UPLC C18 RP column with a Thermo Linear Trap Quadrupole Orbitrap MS System.

Culture Preparations for Acyl-homoserine Lactone Detection Experiments

The purpose of this experiment was to determine if AHL compounds were able to be extracted and detected from *N. inopinata* cultures. *Nitrobacter winogradskyi* was used as a positive control organism, which was known to produce an AHL during growth. Two replicate incubations were prepared in this experiment. Two culture replicates were inoculated with a 2% transfer of *N. inopinata* and contained 100 mL of HEPES-buffered Commamox media with 1 mM ammonia. Two culture replicates were inoculated with a 2% transfer of *N. winogradskyi* and contained 200 mL of *Nitrobacter* media with 30 mM nitrite. Daily samples were taken from each

N. inopinata culture replicate to determine the concentrations of ammonia, nitrite, and nitrate, tracking the progress of growth. Daily samples were also taken from each *N. winogradskyi* culture to determine nitrite consumption and nitrate accumulation, tracking growth progress. Samples for AHL measurements were taken from each culture replicate at two time points: the same day after bacterial inoculation (Time 0) and when all nutrients were exhausted in each culture replicate (Time 1). For *N. inopinata* culture replicates, both ammonia and nitrite were considered as nutrients. For *N. winogradskyi* culture replicates, nitrite was considered as the nutrient. Two samples were taken from each culture replicate at T0 and T1 and subjected to cell removal by either filtration or centrifucation.

The purpose of this experiment was to determine if AHLs can be detected at different time points during the growth of *N. inopinata* cultures. Four replicate incubations were prepared in this experiment. Each replicate contained 100 mL of HEPES-buffered Commamox media with 5 mM ammonia. The replicates were inoculated with a 2% transfer from *N. inopinata* stock culture grown in HEPES-buffered Commamox media with 5 mM ammonia. Ammonia, nitrite, and nitrate concentrations were measured to track the growth of each culture replicate. Samples were taken from four time points for AHL detection and quantification in each culture replicate. The time points were taken shortly after inoculation on the first day of growth (Time 0), when ammonia concentrations began to be rapidly decreased (Time 1), when ammonia concentration reached close to 1 mM (Time 2), and when both ammonia and nitrite were exhausted (Time 3). At each time point, 5 mL of culture were taken from each culture replicate using a sterile syringe and needle. After retrieving the culture volume, the needle was detached from the syringe and a 0.2 μm syringe filter was attached to the syringe currently containing the culture volume. The culture volume was then filtered through the attached syringe filter and collected in separate 15

mL Falcon tubes in sterile conditions in a biosafety hood. The samples were then stored at -20°C until processed for AHL extraction for AHL detection and quantification.

The purpose of this experiment was to repeat the results of Experiment 2 if AHLs could be detected in cultures grown in Commamox media with 5 mM ammonia and an initial pH of 7.5. Four culture replicates were used in this experiment. Four replicates contained 100 mL of Commamox media with 5mM ammonia and 20 mM of HEPES buffer. The replicates were inoculated with a 2% transfer from *Nitrospira inopinata* stock culture grown in Commamox media with 5 mM ammonia and 20 mM HEPES buffer. Ammonia, nitrite, and nitrate concentrations were analyzed and determined to track the growth of each culture replicate using Commamox media. Samples were taken from four time points for AHL detection and quantification of AHLs produced by *Nitrospira inopinata* in each culture replicate. The time points were taken shortly after inoculation on the first day of growth (Time 0), when ammonia concentrations began to be rapidly decreased (Time 1), when ammonia concentration reached close to 1 mM (Time 2), and when both ammonia and nitrite were exhausted (Time 3). At each time point, 5 mL of culture were taken from each culture replicate using a sterile syringe and needle. After retrieving the culture volume, the needle was detached from the syringe and a 0.2 μm syringe filter was attached to the syringe currently containing the culture volume. The culture volume was then filtered through the attached syringe filter and collected in separate 15 mL Falcon tubes in sterile conditions in a biosafety hood. The samples were then stored at -20°C until processed for AHL extraction for AHL detection and quantification.

The purpose of this experiment was to determine if AHLs could be detected in cultures grown in Urea media with 2.5 mM urea and an initial pH of 7.5. 4 culture replicates were used in this experiment. Four replicates contained 100 mL of Urea media with 2.5 mM urea and 20 mM

of HEPES buffer. The replicates were inoculated with a 2% transfer from *Nitrospira inopinata* stock culture grown Urea media with 2.5 mM urea and 20 mM HEPES buffer. Nitrite and nitrate concentrations were analyzed and determined to track the growth of each culture replicate using Urea media. Samples were taken from four time points for AHL detection and quantification of AHLs produced by *Nitrospira inopinata* in each culture replicate. The time points were taken shortly after inoculation on the first day of growth (Time 0), when nitrite+nitrate concentrations began to be rapidly increased (Time 1), when nitrite+nitrate concentrations reached close to 4 mM (Time 2), and when nitrite was exhausted (Time 3). At each time point, 5 mL of culture were taken from each culture replicate using a sterile syringe and needle. After retrieving the culture volume, the needle was detached from the syringe and a 0.2 μm syringe filter was attached to the syringe currently containing the culture volume. The culture volume was then filtered through the attached syringe filter and collected in separate 15 mL Falcon tubes in sterile conditions in a biosafety hood. The samples were then stored at -20°C until processed for AHL extraction for AHL detection and quantification.

The purpose of this experiment was to modify the AHL extraction methods to more reliably detect AHL from *N. inopinata* cultures grown in Commamox media with 5 mM ammonia at pH 7.5. Four culture replicates were used in this experiment. Four replicates contained 100 mL of Commamox media with 5 mM ammonia and 20 mM of HEPES buffer. The replicates were inoculated with a 2% transfer from *Nitrospira inopinata* stock culture grown in Commamox media with 5 mM ammonia and 20 mM HEPES buffer. Ammonia, nitrite, and nitrate concentrations were analyzed and determined to track the growth of each culture replicate using Commamox media. Samples were taken from four time points for AHL detection and quantification of AHLs produced by *Nitrospira inopinata* in each culture replicate. The time

points were taken shortly after inoculation on the first day of growth (Time 0), when ammonia concentration began to be rapidly decreased (Time 1), when ammonia concentrations reached close to 1 mM (Time 2), and when ammonia and nitrite were exhausted (Time 3). At each time point, 10 mL of culture were taken from each culture replicate using a sterile syringe and needle. After retrieving the culture volume, the needle was detached from the syringe and a 0.2 μm syringe filter was attached to the syringe currently containing the culture volume. The culture volume was then filtered through the attached syringe filter and collected in separate 15 mL Falcon tubes in sterile conditions in a biosafety hood. The samples were then stored at -20°C until processed for AHL extraction for AHL detection and quantification. Three different AHL extractions with different methods were done with each of the samples taken from each of the culture replicates.

The purpose of this experiment was to determine if extracting AHLs from large volumes of samples would improve the reliability of AHL extractions. One culture replicate inoculated with *Nitrospira inopinata* was used for this experiment. 200 mL of Commamox media with 5 mM ammonia and 20mM HEPES buffer was inoculated with a 2% inoculation from a *Nitrospira inopinata* stock culture grown using Commamox media with 1 mM ammonia and 10 mM HEPES buffer. Ammonia, nitrite, and nitrate concentrations were monitored to determine the optimal time to retrieve samples for large volume AHL extraction. Samples for large volume AHL extraction were taken when ammonia and nitrite concentrations were exhausted completely. 60 mL of culture supernatant was retrieved as the AHL extraction sample with a syringe and filtered with an attached 0.2 μm syringe filter to remove cells. The 60 mL of filtered supernatant was collected in two 50 mL Falcon tubes. The sample was stored at -20°C until needed for AHL extraction.

The purpose of this experiment was to repeat the extraction of AHLs from *N. inopinata* culture samples taken from cultures at four time points and detect AHLs from those samples. Four *Nitrospira inopinata* culture replicates were used for this experiment. 100 mL of Commamox media with 5 mM ammonia and 20 mM HEPES buffer was inoculated with a 2% inoculation from a *Nitrospira inopinata* stock culture grown using Commamox media with 5 mM ammonia and 20 mM HEPES buffer. Ammonia, nitrite, and nitrate concentrations were monitored to determine the optimal time points to retrieve samples for AHL extractions. The time points were shortly after inoculation on the first day of growth (Time 0), when ammonia concentrations began to be rapidly decreased (Time 1), when ammonia concentration reached close to 1 mM (Time 2), and when both ammonia and nitrite were exhausted (Time 3). At each time point, 10 mL of culture were taken from each culture replicate using a sterile syringe and needle. After retrieving the culture volume, the needle was detached from the syringe and a 0.2 μm syringe filter was attached to the syringe currently containing the culture volume. The culture volume was then filtered through the attached syringe filter and collected in separate 15 mL Falcon tubes in sterile conditions in a biosafety hood. The samples were then stored at -20°C until processed for AHL extraction for AHL detection and quantification.

Acyl-homoserine Lactone Extraction for LCMS Analysis

Acyl-homoserine lactones (AHLs) needed to be extracted from the aqueous samples that were taken from microbial cultures using organic solvents. Dichloromethane (DCM), ethyl acetate, and hexane were three organic solvents that were initially tested to determine which was the best

solvent to extract the AHLs studied in this paper. Ethyl acetate and later ethyl acetate with 0.1% acetic acid were found to be ideal solvents to extract acyl-homoserine lactones.

The initial acyl-homoserine lactone extraction method uses the following steps. A 2 mL volume of each sample was dispensed into separate 4 mL glass vials after the 1 mL metal syringe was cleaned between transfers of different samples. The 1 mL metal syringe was cleaned between transfers by first rinsing the needle of the syringe with a stream of methanol. The inside of the syringe was then rinsed by up taking and expelling 1 mL of methanol twice. 1 mL of ethyl acetate was transferred into each 4 mL vial with 2 mL of each separate sample with a 1 mL metal syringe. Each 4 mL vial was sealed with a solvent-proof plastic screw cap. Each vial was then vortexed for a few seconds and sonicated in a water bath for 10 minutes. The samples were vortexed for a few seconds and centrifuged at 3000 rpm for 5 minutes to separate the aqueous (sample) and organic (ethyl acetate) phases in the vials. As much of the organic phase as possible was transferred to a 2 mL vial with a metal syringe from above the aqueous phase without transferring aqueous phase contamination. After each transfer of the organic phase into the new 2 mL vials, the needle of the syringe was washed with a stream of pure methanol and the inside of the syringe was washed with 1 mL of pure ethyl acetate twice. A small volume of the organic phase may be visibly left on top of the aqueous phase. After each organic phase was transferred to their respective 2 mL vials, 1 mL of new ethyl acetate solvent was transferred to each 4 mL vial. The 2 mL vials with the transferred ethyl acetate organic phase were then placed under tubes that expelled nitrogen gas into the 2 mL vials with heat applied to the vials from a hotplate. The nitrogen gas flow and heat should cause the evaporation of the ethyl acetate to evaporate at a more rapid pace and leave the dissolved compounds as precipitate in the bottom of each vial. The vials containing both the aqueous samples and the newly added ethyl acetate solvent were then

vortexed individually for a few seconds. The vials were then sonicated in a water bath for 10 minutes. After sonication the vials were dried from water and vortexed individually for a few seconds. The vials were then centrifuged at 3000 rpm for 5 minutes to separate the aqueous phase and organic phase. The 2 mL vials were taken away from the nitrogen gas supply and heat source. The organic phase was transferred without aqueous phase contamination from each 4 mL vial to their respective 2 mL vial that contained the previous transfer of ethyl acetate organic phase. After each transfer of the organic phase into the new 2 mL vials, the needle of the syringe was washed with a stream of pure methanol and the inside of the syringe was washed with 1 mL of pure ethyl acetate twice. A small volume of organic phase remained on top of the aqueous phase due to difficulty in extracting the entire volume of organic phase without introducing aqueous phase contamination. After transferring the ethyl acetate organic phases to their respective 2 mL vials, the 2 mL vials were placed back under the nitrogen gas source for the complete evaporation of the ethyl acetate contained in each vial. After complete evaporation of all liquid in each 2 mL vial, 500 µL of pure methanol was added to each 2 mL vial. After addition of methanol, the vials were vortexed for a few seconds to dissolve any precipitate left over from the evaporation of the ethyl acetate organic phases and mix the mixture. A sample with added AHLs was prepared as a standard sample. The vials were then loaded into LC-MS for analysis.

Experiments 1-4 used the initial AHL extraction methods without modifications. For the AHL extractions for Experiment 5, modifications were made to the initial AHL extraction methods to potentially improve the reliability of AHL extractions and detection from samples. Experiment 5 employed three different AHL extraction groups with each having a different AHL extraction method to extract from the samples retrieved from the *N. inopinata* cultures during

their incubation and growth at Times 0-3. One AHL extraction group used the initial, unmodified AHL extraction method on the retrieved samples. A second extraction group used a modified AHL extraction method that reduced the time spent for water bath sonication of the 2 mL aqueous sample + the added 1 mL of ethyl acetate solvent from 10 minutes to 5 minutes. The third extraction group used a modified AHL extraction method that reduced the time spent for water bath sonication of the 2 mL aqueous sample + the added 1 mL of ethyl acetate solvent from 10 minutes to 5 minutes and after the complete evaporation of the 2 mL of extracted ethyl acetate by nitrogen gas flow, 250 µL of pure methanol and 250 µL of pure de-ionized water was added to each 2mL vial to dissolve the extracted precipitate before LC-MS analysis.

Experiment 6 used a modified AHL extraction method to adapt to the larger volume of sample extracted from. In this extraction method, there were four different volumes of the retrieved sample extracted from: a 20 mL volume, a 10 mL volume, and two 2 mL volumes. Each volume was separated for individual extractions. The extraction method used to extract AHLs from the two 2 mL volumes was similar to the initial AHL extraction method with a few modifications. The extraction method used to extract from the 20 mL and 10 mL volume samples was different from the initial AHL extraction method that was originally used to extract from 2 mL sample volumes.

Large volume AHL extraction methods: In a clean 100 mL graduated cylinder, the volume of filtered culture supernatant sample was measured and poured into a clean 100 mL separating funnel with the bottom spigot closed. Half the volume of ethyl acetate with 0.1% acetic acid solvent solution compared to the sample volume was then measured with in the 100 mL graduated cylinder and poured into the separating funnel already containing the sample. With the bottom spigot and top cap closed on the separating funnel, the separating funnel was

manually shaken to mix the aqueous sample and organic phase ethyl acetate + 0.1% acetic acid solutions together for approximately 30-60 seconds. Periodically every 10 seconds, the shaking was stopped, and the top cap was removed to off gas any evaporated solvent to prevent any internal pressure increase. After agitation, the separating funnel was vertically secured so the bottom spigot was pointing downwards, and the top cap was removed from the separating funnel. The solution in the separating funnel was allowed to separate completely from each other to allow the collection of each phase with little contamination from the other phase. The bottom layer should be the aqueous phase containing the filtered sample, and the top layer should be the organic phase containing the ethyl acetate + 0.1% acetic acid solution. In a clean 50 mL beaker, the bottom layer of solution was collected by slightly opening the bottom spigot on the separating funnel to allow the bottom layer to slowly leave the funnel. Once all of the bottom layer was collected, a small volume of the top, organic layer was allowed to pour out of the funnel to wash the aqueous solution from the spigot. The rest of the organic phase solution was collected in a clean, 60 mL tube. After the collection of the organic phase solution, the bottom spigot was closed, and the collected aqueous solution was poured back into the separating funnel. Half the volume of new ethyl acetate + 0.1% acetic acid compared to the original volume of the aqueous sample was then measured in a graduated cylinder and poured into the separating funnel with the aqueous sample. The top cap was placed to close the separating funnel and the solution contained was mixed with manual agitation for approximately 30-60 seconds. After 10 seconds, the top cap was removed to off gas any evaporated solvent and normalize the pressure in the separating funnel. After agitation, the top cap was removed from the separating funnel and the solution could separate into aqueous and organic phase layers without agitation. The bottom aqueous layer was collected in a 50 mL beaker by slowly draining the bottom layer solution

through the bottom spigot. The organic phase solution was collected in the 60 mL tube that contains the previous organic phase solvent by draining the organic phase solution through the bottom spigot. The graduated cylinder was rinsed with a methanol wash on its walls and disposed of as waste solvent. A secondary wash of the graduated cylinder was done with DCM, and the wash solvent was disposed of. The graduated cylinder was then allowed to dry to evaporate any remaining waste solvent. The separating funnel and its top cap was also washed with two washes consisting of methanol and DCM, respectively, and the separating funnel was allowed to dry. Once dried, this large volume extraction process was repeated with the next of the two volumes with new beakers and a new, separate 60 mL tube. Once all large volume samples were extracted from, the 60 mL tubes containing the ethyl acetate + 0.1% acetic acid used in the extractions were placed under nitrogen gas flow and low heat on a hot plate to evaporate the ethyl acetate + 0.1% acetic acid. Once the solution was completely evaporated, the 60 mL tubes were taken from the heat and nitrogen gas sources, and 1 mL of methanol was added to each of the 60 mL tubes to dissolve the precipitate left in each tube. The 60 mL tubes were then vortexed for several seconds to dissolve all the precipitate left at the bottom and on the walls of the tubes. After dissolving all the precipitate, the 1 mL of methanol from each tube was transferred with a clean, metal syringe into separate 2 mL vials. The vials were then placed under nitrogen gas flow to evaporate the methanol solution contained in the 2 mL vials. After evaporation, 500 µL of 1:1 methanol:water solution was added to each 2 mL vial to dissolve the precipitate deposited in the bottom of the vials, and the vials were vortexed for several seconds. The vials were then analyzed with LC-MS.

The final acyl-homoserine lactone extraction method uses the following steps. A 2 mL volume of each sample was dispensed into separate 4 mL glass vials after the 1 mL metal syringe was

cleaned between transfers of different samples. The 1 mL metal syringe was cleaned between transfers by first rinsing the needle of the syringe with a stream of methanol. The inside of the syringe was then rinsed by up taking and expelling 1 mL of methanol twice. 1 mL of ethyl acetate with 0.1% acetic acid was transferred into each 4 mL vial with 2 mL of each separate sample with a 1 mL metal syringe. Each 4 mL vial was sealed with a solvent-proof plastic screw cap. Each vial was then vortexed for a few seconds and sonicated in a water bath for 5 minutes. The samples were vortexed for a few seconds and centrifuged at 3000 rpm for 5 minutes to separate the aqueous (sample) and organic (ethyl acetate) phases in the vials. As much of the organic phase as possible was transferred to a 2 mL vial with a metal syringe from above the aqueous phase without transferring aqueous phase contamination. After each transfer of the organic phase into the new 2 mL vials, the needle of the syringe was washed with a stream of pure methanol and the inside of the syringe was washed with 1 mL of pure ethyl acetate with 0.1% acetic acid twice. A small volume of the organic phase may be visibly left on top of the aqueous phase. After each organic phase was transferred to their respective 2 mL vials, 1 mL of new ethyl acetate with 0.1% acetic acid solution was transferred to each 4 mL vial. The 2 mL vials with the transferred ethyl acetate organic phase were then placed under tubes that expelled nitrogen gas into the 2mL vials. This caused the evaporation of the ethyl acetate with 0.1% acetic acid to evaporate at a more rapid pace and leave the dissolved compounds as precipitate in the bottom of each vial. The vials containing both the aqueous samples and the newly added ethyl acetate with 0.1% acetic acid solution were then vortexed individually for a few seconds. The vials were then sonicated in a water bath for 5 minutes. After sonication the vials were dried from water and vortexed individually for a few seconds. The vials were then centrifuged at 3000 rpm for 5 minutes to separate the aqueous phase and organic phase. The 2 mL vials were taken

away from the nitrogen gas supply. The organic phase was transferred without aqueous phase contamination from each 4 mL vial to their respective 2 mL vial that contained the previous transfer of ethyl acetate organic phase. After each transfer of the organic phase into the new 2 mL vials, the needle of the syringe was washed with a stream of pure methanol and the inside of the syringe was washed with 1 mL of pure ethyl acetate with 0.1% acetic acid twice. A small volume of organic phase remained on top of the aqueous phase due to difficulty in extracting the entire volume of organic phase without introducing aqueous phase contamination. After transferring the ethyl acetate organic phases to their respective 2 mL vials, the 2 mL vials were placed back under the nitrogen gas source for the complete evaporation of the ethyl acetate with 0.1% acetic acid contained in each vial. After complete evaporation of all liquid in each 2 mL vial, 250 µL of pure deionized water was added to each vial. 250 µL of pure methanol was also added to each 2 mL vial. After addition of methanol and deionized water, the vials were vortexed for a few seconds to dissolve any precipitate left over from the evaporation of the ethyl acetate organic phases and mix the mixture. The final concentration of methanol to deionized water should be 1:1. The vials were then loaded into LC-MS for analysis.

AHL-addition Growth Experiment

The purpose of this experiment was to determine if the addition of a single AHL into culture media would affect the growth of *N. inopinata* after inoculation. This experiment consists of 3 experimental replicate groups and 1 control replicate group. The three experimental groups are the 3-OH-C10-HSL addition group, the 3-OH-C12-HSL addition group, and the 3-OH-C14-HSL addition group. Each treatment group consists of four culture replicates with 100 mL of Commamox media that contains 5mM ammonia and 20mM HEPES buffer at a pH of 7.5. Before

inoculation and addition of the Commamox media into the 250 mL glass bottles, 0.1 mL of 1 mM AHL dissolved in methanol was added to the four 250 mL glass bottles to be used for the culture replicates of each respective AHL treatment group. The methanol was completely evaporated under aseptic conditions. For the control treatment group, 0.1 mL of pure methanol was added to the four bottles and completely evaporated. After the evaporation of methanol, 100 mL of Commamox media was added to each of the glass bottles and swirled to dissolve any deposited AHL. Each culture replicate was then inoculated with a 2% transfer (2mL) of culture volume from a *N. inopinata* stock culture. The culture replicates were incubated at 37°C. Daily culture samples (1mL) were retrieved from each culture replicate for ammonia, nitrite, and nitrate concentration determination. The concentrations of ammonia, nitrite, and nitrate were used to analyze the growth of each culture replicate group. Samples were taken from culture replicates and analyzed until the culture replicates in the control treatment group were under stationary phase conditions (15 days).

Culture Preparation for RNA Extraction and Purification

Sixteen *Nitrospira inopinata* were inoculated and grown to harvest cells for future RNA extraction and purification. The purified RNA would then be sequenced and analyzed to discover significant changes in the transcriptomes of each experimental group when compared to one another. The sixteen cultures were divided into four experimental treatment groups with four cultures included in each group. Each culture contained 100 mL of Commamox media with 5 mM ammonia concentration that was inoculated with 2 mL from a *Nitrospira inopinata* stock culture. This stock culture was grown with Commamox media containing 1 mM ammonia concentration. The inoculated cultures were incubated at 37°C. Once cultures exhausted 80% of ammonia or began to slow their ammonia oxidation rate from their exponential phase ammonia

oxidation rate, cells were harvested for RNA extraction and purification. Cells from each culture were filtered from the culture media with a Sterlitech PES membrane filter with a 47 mm diameter and 0.2 μm pores and captured on the filter pad. The filter pads containing the cells from each separate culture were then wrapped in sterile aluminum foil and stored at -80°C until RNA was extracted and purified from each filter pad. Samples were taken from the cultures for cell count quantification by flow cytometry analysis immediately after inoculation and before cells were harvested for later RNA extraction and purification.

RNA Extraction and Purification

The filter pads with the collected cells were processed to extract and purify the RNA contained in the cells. For each culture's filter pad with cells, 1 mL of 100% phenol solution was heated in a water bath at 80°C. The filter pads were thawed at room temperature for 5-10 minutes and two 2 mL autoclaved screw-cap tubes and one 1.7 mL microcentrifuge tube were prepared for each sample's filter pad. The filter pad was cut into small strips with sterilized scissors and placed into the first 2 mL screw cap tube with (the bead apparatus) in the 2 mL screw cap tube. 350 μL of RNA extraction buffer solution, 50 μL of 10% SDS solution, and 425 μL of heated phenol solution into the 2 mL screw cap tube containing the filter cap strips. The screw cap tube was then disrupted at speed 4.0 in the bead beater for 30 seconds. The screw cap tube was then centrifuged at 16,000 xg for 5 minutes at 4°C. The aqueous phase at the bottom of the 2 mL screw cap tube was then transferred to a new 2 mL screw cap tube. 450 μL of 25:24:1 phenol/chloroform/isoamyl alcohol was then added to this 2 mL screw cap tube. The screw cap tube was then disrupted at speed 4.0 in bead beater for 15 seconds. The screw cap tube was centrifuged at 16,000 xg for 5 minutes at 4°C. The aqueous phase was transferred to a new 2 mL screw cap tube and 400 μL of chloroform solution was added with the aqueous phase. The

solution in the 2 mL screw cap was disrupted in bead beater at speed 4.0 for 15 seconds. The screw cap tube was centrifuged at 16,000 xg for 5 minutes at 4°C. The aqueous phase was transferred to a 1.7 mL micro centrifuge tube. 90 µL of 4 M lithium chloride solution was added to the aqueous phase to bring the concentration of lithium chloride in the aqueous phase to 0.8 M. 3.5 µL of Invitrogen™ GlycoBlue™ Coprecipitate solution (15 mg/mL) was added to the extract solution and mixed. An equal volume of ice-cold 100% isopropanol solution was added to the extract solution and mixed. The extract solution was incubated at -80°C overnight to precipitate RNA.

After overnight incubation at -80°C for nucleotide precipitation, the RNA samples were thawed and centrifuged at 21,000 xg for 15 minutes at 4°C. A blue pellet should form at the bottom of the tube for each sample. The supernatant was removed from the tube without disturbing the blue pellet. 1 mL of ice-cold 75% ethanol was added into the tube with the blue pellet. The tube was inverted twice and centrifuged at 21,000 xg for 5 minutes at 4°C. The supernatant was then removed as completely as possible without disturbing the blue pellet. The tubes were opened and dried for 1 minute, but before the blue pellet was completely dried. The blue pellet was then dissolved with 90 µL of RNase and DNase free water and 10 µL of DNase free buffer. The solution was pipetted up and down to dissolve the blue pellet. After dissolving the blue pellet, the solution was transferred to a clean 650 µL microcentrifuge tube for DNase treatments. 2 µL of DNase solution was added to the RNA extract solution and mixed completely. The mixed solution was then incubated at 37°C for 30 minutes. After incubation, 10 µL of DNase inactivation reagent was added to the extract solution and mixed by pipetting the solution up and down. The solution was incubated for 2 minutes with regular mixing of the solution to keep the inactivation reagent suspended. The solution was then centrifuged after

incubation at 10,000 xg for 1.5 minutes. The supernatant was transferred to a new 650 µL tube. 10 µL of DNase free buffer and 2 µL of DNase solution was added to the transferred supernatant. The solution was mixed completely and incubated at 37°C for 30 minutes. After incubation, 10 µL of DNase inactivation reagent was added to the extract solution and mixed by pipetting the solution up and down. The solution was incubated for 2 minutes with regular mixing of the solution to keep the inactivation reagent suspended. The solution was then centrifuged after incubation at 10,000 xg for 1.5 minutes. The supernatant was transferred to a new 1.7 mL RNase-free microcentrifuge tube. 25 µL of 4 M lithium chloride was added to this transferred supernatant to a final concentration of 0.8 M lithium chloride. 375 µL of ice-cold 100% ethanol solution was added to the supernatant solution. The RNA extract solution was then incubated at -80°C for 1 hour to precipitate RNA. After incubation, the RNA extract solution was thawed, and the solution was pipetted up and down to mix and resuspend RNA. The entire volume of the mixture was transferred to an RNase free MP spin column, and the spin column was centrifuged at 12,000 xg for 1 minute. The liquid in the bottom of the collection tube was discarded. 400 µL of 75% ice-cold ethanol was added to the top of the MP spin column. The spin column was centrifuged at 12,000 xg for 1 minute, and the liquid at the bottom of the collection tube was discarded. 400 µL of 75% ice-cold ethanol was added to the top of the spin column a second time. The column was centrifuged at 12,000 xg for 1 minute, and the liquid at the bottom of the collection tube was discarded. The empty spin column was then centrifuged at 12,000 xg for 2 minutes. The collection tube holding the MP spin column was then replaced with a new RNase-free 2mL microcentrifuge tube for final RNA sample collection. The spin column was then allowed to air dry in sterile conditions in a biosafety hood for 1 minute at room temperature without being completely dried. 50 µL of RNase-free water was added to the spin column and

incubated at room temperature for 1 minute. The spin column was then centrifuged at 10,000 xg for 1 minute. 50uL of RNA sample was collected at the bottom of the collection tube. The spin column was then discarded and 5 µL of the RNA sample volume was transferred into two separate RNase-free 1.7 mL microcentrifuge tubes. The aliquots in one of the two additional tubes were used to measure RNA concentration and DNA contamination while the aliquot in the other tube was used to measure RNA quality. All three tubes with RNA samples and aliquots were stored at -80°C.

RNA Quantification of RNA Extract Samples

This experiment was done to determine the concentration of RNA in our extracted RNA samples. For each RNA sample, the aliquot containing 5 µL of the RNA sample assigned for RNA concentration and DNA contamination analysis was thawed. 2 µL of RNase-free water was pipetted onto the Nanopore sensor and used as a blank measurement before analyzing the RNA samples. The 2 µL of RNase-free water was gently wiped away from the Nanopore sensor with a Kim-wipe. 2 µL of RNA sample was then loaded onto the Nanopore sensor for RNA concentration readings.

RNA Quality Analysis

DNA contamination left in RNA samples after purification was determined by qPCR analysis. The *amoA* gene in *N. inopinata* was the amplification target of the primers used in this experiment. The primers comA-659R and comA-244F were employed in this qPCR analysis (8).

The quality of RNA in purified samples was analyzed by determining the RIN (RNA Integrity Number) values with an Agilent 2100 Bioanalyzer. Samples with RIN values greater than 7 were deemed of high quality for RNA sequencing.

RNAseq Analysis

After RNA sequencing, the RNA reads of sample were trimmed using the Trimmomatic program (v.0.36) (9). The RNA reads were then aligned using the Bowtie program (v.2.3.4) (10). These alignments were then processed through a Samtools program (v.0.1.19) and sorted into BAM files (11). The htseq tool of the HTseq 2.0 program was used to assign RNA reads to open reading frames (12). The equation to calculate TPM values of each transcribed gene was as follows (13):

$$TPM = \frac{(rg \cdot rl \cdot 10^6)}{(cl \cdot t)}$$

In the equation, rg was the number of reads assigned to a gene, rl was the length of the read, cl was the length of the coding sequence of a gene, and *t* was the sum of $rg \cdot rl \cdot cl^{-1}$ for all genes. The DESeq2 program (v.1.2.10) was used to undergo further differential transcription calculations on genes between AHL-addition cultures and no-addition cultures (14).

Substrate Concentration Determination

Ammonia, nitrite, and nitrate concentrations were determined from daily samples to track the growth of inoculated culture replicates. Ammonia concentrations were determined by analyzing fluorescence levels after the addition of o-phthaldialdehyde reagent (OPA) to samples (15). Nitrite concentrations were determined by analyzing the absorbance of samples with a spectrophotometer after the addition of sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride solutions (EPA Method 354.1). Nitrate concentrations were determined by first reducing nitrate to nitrite with the addition of vanadium chloride solution in samples. The

absorbances of the total oxidized nitrogen concentrations (NO3+NO2) and the nitrite concentrations (no vanadium chloride addition to sample) were quantified using the spectrophotometer method described above. Nitrate was determined at this point by subtracting the nitrite concentration from the total oxidized nitrogen (NO2+NO3) concentration. Urea concentrations were determined by first converting urea to ammonia with the addition of urease solution into samples. The fluorescence of the total ammonia (urea + ammonia) concentration and the ammonia concentration (no urease addition to sample) would then be measured in each sample after OPA reagent addition. The fluorescence of the urease-treated sample (urea-N+ ammonia) would be subtracted by the fluorescence of the untreated (ammonia only) sample to determine urea-N in the original sample.

Cell Counts

Cell count samples were diluted with a 1:100 dilution with the addition of TE buffer to generate cell densities in between 10^4 and 10^6 cells per mL. Cells in a 990 µL of the diluted samples were stained with the addition of 10 µL prepared SYBR Green I solution for 15 minutes in the dark. The SYBR Green I solution was prepared by adding 10 µL of SYBR Green I stock solution (Invitrogen 10,000 x stock solution) into 1 mL of dimethyl sulfoxide (DMSO). This prepared solution was stored in the dark. These stained cells were then counted with a Becton Dickinson Accuri C6 flow cytometer as previously described (16, 17). TE buffer was used as a blank control to determine background. Twenty microliters of the stained sample was analyzed with the FL-1 channel (excitation wavelength = 485 nm, emission wavelength = 535 nm) under slow mode to quantify the stained cells. The SSC threshold was set as 10,000 for the FL-1 channel. Gating was used to separate background from positive cell count readings in samples.

Results

Heterogeneous Expression of Putatative *Nitrospira inopinata* LasI and LasR genes in *Pseudomonas putida* F1

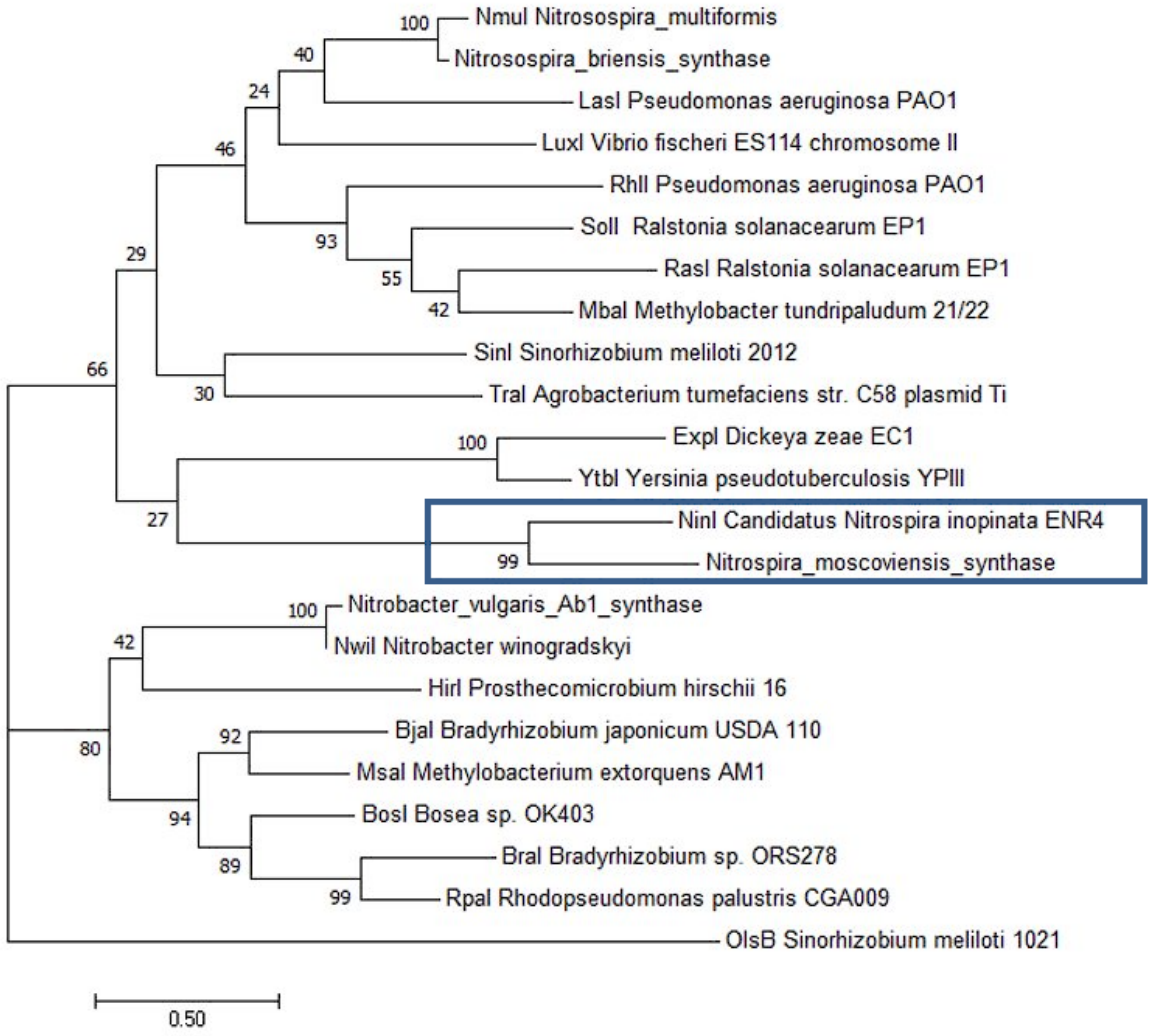


Figure 1. Phylogenetic tree of AHL synthase gene homologs among heterotroph and nitrifier bacteria using Maximum Likelihood method. Encased in the blue box, the AHL synthase gene homolog contained in the genome of *Nitrospira inopinata*, *ninI*, is in the same clade as the AHL synthase gene homolog contained in the genome of *Nitrospira moscoviensis*.

Nitrospira inopinata encodes *ninI* and *ninR* which are homologous to *luxI* and *luxR*. *LuxI* and *luxR* are known to be involved in the synthesis and detection of acyl-homoserine lactones, respectively, in Gram-negative bacteria. To confirm the function of the *ninI* gene, it was cloned into *Pseudomonas putida* F1 which does not contain its own AHL-mediated quorum sensing system. A plasmid (pNinI) including the *ninI* gene was constructed to carry the *ninI* gene into the *P. putida* clones. Three different acyl-homoserine lactones were detected from *P. putida* F1 cultures (pNinI). The acyl-homoserine lactones identified from mass spectra analysis were 3-OH-C10-HSL (272.1856 m/z), 3-OH-C12-HSL (300.2169 m/z), and 3-OH-C14-HSL (328.2482 m/z). Major identifying mass spectra peaks for all three AHLs are the protonated, unbroken molecule (stated with each AHL previously) and a peak with m/z ratio equaling 102.055. This peak represents the homoserine head portion of the molecule with the acyl tail cleaved away. To test the function of *ninR*, a plasmid (NinR-pNinI-mcherry) was constructed contained the *ninR* gene, a promoter regulating *ninI* gene transcription (pNinI), and an mcherry gene. The mcherry gene encodes for a fluorescent protein and its expression is regulated by the pNinI promoter and *ninR* gene. Significant fluorescence levels were detected after incubation of *P. putida* (Ninr-pNinI-mcherry) in media containing 1µM 3-OH-C10-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL. The *ninR* gene was able to recognize the added AHLs and allowed the production of the mcherry fluorescent genes. Significant fluorescent levels were also detected after P. putida incubation in media containing added AHLs other than the three AHLs produced by *ninI*. These AHLs include 3-OH-C8-HSL, 3-O-C12-HSL, and 3-O-C6-HSL.

Detection and Quantification of Acyl-homoserine lactones in N. inopinata

We sought to determine if *Nitrospira inopinata* could produce three different acyl-homoserine lactones and if our method of AHL extraction would successfully extract AHLs. *Nitrobacter winogradskyi* was a bacteria species that was known to produce an acyl-homoserine lactone. Supernatant retrieved from *Nitrobacter winogradskyi* cultures were extracted from to be a positive control group besides the standard mix sample. This was done to determine if our AHL extraction method could extract detectable amounts of AHLs from culture supernatant with similar cell density to *Nitrospira inopinata* cultures. Two acyl-homoserine lactones were detected in the sample extracts in this experiment. 3-hydroxy-C10-homoserine lactone (3-OH-C12-HSL) was detected in *Nitrospira inopinata* sample extracts. For the *Nitrospira inopinata* samples, only the sample extracts that contained detectable 3-hydroxy-homoserine lactone were derived from samples that employed filtration as the method to remove cells from the sample supernatant obtained from the culture replicates. The N*itrospira inopinata* sample extracts that were derived from samples that employed centrifugation as the cell removal method did not contain detectable amounts of acyl-homoserine lactones. Detectable amounts of an acyl-homoserine lactone were found in the *Nitrobacter winogradskyi* sample extracts. 3-C10-homoserine lactone (3-C10-HSL) was detected. This acyl-homoserine lactone was different than the acyl-homoserine lactones that were sought in this extraction experiment. 3-C10-HSL did not contain a hydroxyl group on the third carbon of the ten-carbon acyl group. The three acyl-homoserines that were sought in this experiment should contain a hydroxyl group on the third carbon of the acyl group. 3-C10-HSL that was detected in the *Nitrobacter winogradskyi* samples did contain an unsaturated acyl carbon chain. One double bond was attributed to the acyl carbon chain and was between the third and fourth carbon on the acyl carbon chain. 3-C10-HSL was detected in *Nitrobacter winogradskyi* sample extracts that were derived from samples that

employed either filtration or centrifugation as cell removal methods. For *Nitrobacter winogradskyi* samples, the cell removal method did not seem to have an effect on the detection of acyl-homoserine lactones. However, the detection of AHLs in *Nitrospira inopinata* samples that used filtration as a cell removal method and absence of detectable AHLs in samples that used centrifugation as a cell removal method was not anticipated. To remove any possible effect cell removal had on AHL extraction, future AHL detection experiments only employed filtration as the cell removal method when obtaining samples for AHL extraction from culture replicates. Only one acyl-homoserine lactone was detected in the *Nitrospira inopinata* samples. Samples for AHL extractions were taken from *Nitrospira inopinata* culture replicates when the ammonia and nitrite concentrations were simultaneously near zero.

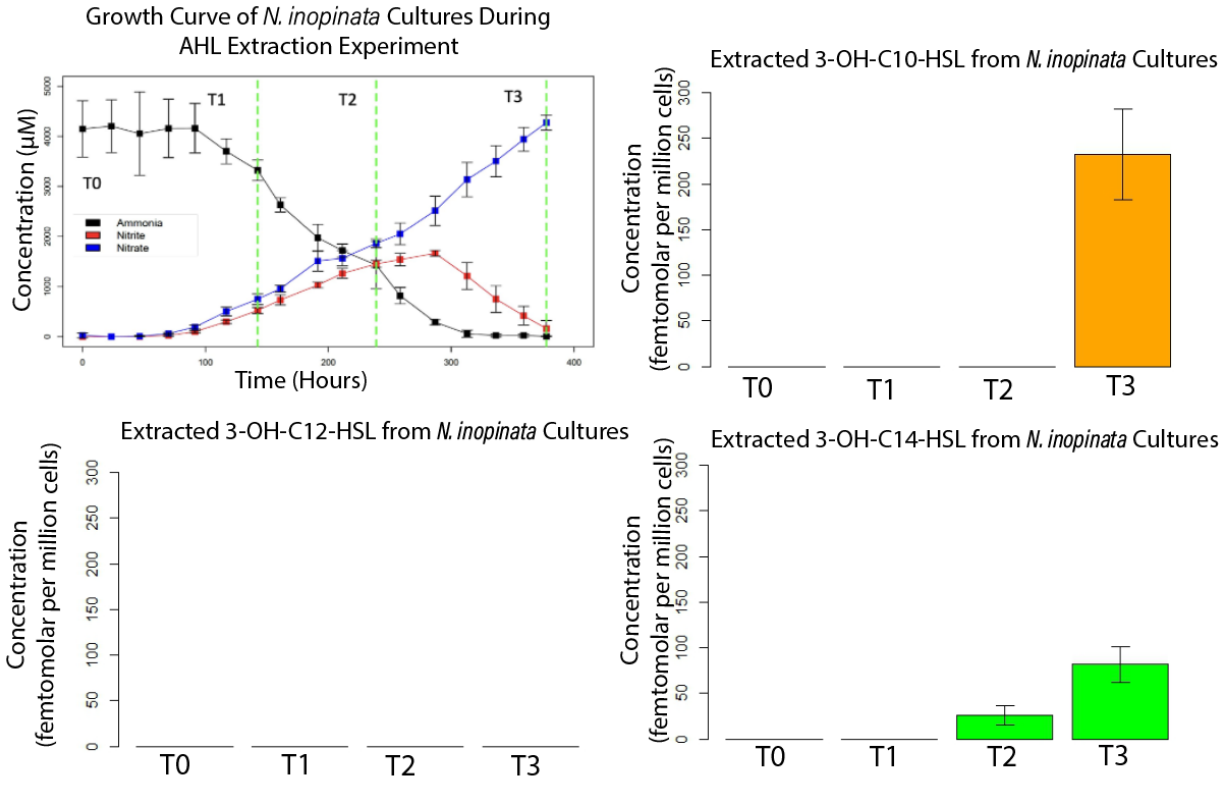


Figure 2. The average growth curve of the *N. inopinata* culture replicates grown for the extraction of acyl-homoserine lactones (AHLs) in Experiment 2. Samples for AHL extraction

were taken from Time Point 0 (T0), Time Point 1 (T1), Time Point 2 (T2), and Time Point 3 (T3). Time Point 0 was retrieved immediately after initial inoculation (Topleft). The average concentrations of each extracted AHL at the four timepoints (T0, T1, T2, T3) from culture media samples (Topright, Bottomleft, Bottomright).

Since an acyl-homoserine lactone was detected in *Nitrospira inopinata* cultures, we carried out an experiment to extract AHLs from samples taken from multiple time points along the growth curve of *Nitrospira inopinata* cultures. This was done to determine if the other AHLs were detectable during different times and conditions during the growth cycle of *Nitrospira* culture replicates. Two acyl-homoserine lactones were detected in the samples in two of the time points when samples were collected. C10-homoserine lactone and 3-hydroxy-C14 homoserine lactone were detected among the samples that were extracted from in this experiment. 3-hydroxy-C14 homoserine lactone was detected in samples taken from Time Point 2 (T2) and Time Point 3 (T3). C10-homoserine lactone was detected in samples taken from Time Point 3 (T3). Time Point 2 samples were taken when the *Nitrospira inopinata* cultures were under exponential growth conditions. Ammonia concentrations in the culture replicates at this Time Point were approximately 1mM. Time Point 3 samples were taken from culture replicates under late exponential growth conditions. Ammonia and nitrite concentrations at this Time Point were near zero. The C10-homoserine lactone that was detected in this experiment was different than the 3-hydroxy-homoserine lactone detected in the previous experiment. The AHL detected in this experiment does not contain a hydroxyl group on the acyl group. In a repeat of this experiment, only two of the sixteen samples that were extracted from contained detectable AHLs. The two samples were taken from two separate culture replicates taken from Time Point 3 when ammonia

and nitrite concentrations were near zero. The two detected AHLs were 3-OH-C10-HSL and 3-OH-C12-HSL.

Table 1. Acyl-homoserine lactones produced in nitrifier bacteria and one *Pseudomonas* species.

Species	AHL	Chemical Formula	Molecular Mass
Nitrospira inopina	3-OH-C10-HSL	$C_{14}H_{25}NO_4$	271.4
Nitrospira inopinata	3-OH-C12-HSL	$C_{16}H_{29}NO_4$	299.4
Nitropira inopinata	3-OH-C14-HSL	$C_{18}H_{34}NO_4$	327.5
Nitrospira moscoviensis	C8-HSL	$C_{12}H_{21}NO_3$	227.3
Nitrosospira multiformis	C10-HSL	$C_{14}H_{25}NO_3$	255.2
Nitrosospira briensis	3-OH-C14-HSL	$C_{18}H_{34}NO_4$	271.4
Nitrobacter vulgaris	C10:1-HSL	$C_{14}H_{24}NO_3$	254.4
Pseudomonas aeruginosa	C4-HSL	$C_8H_{13}NO_3$	171.2
	3-oxo-C12-HSL	$C_{16}H_{27}NO_4$	297.4

Another experiment that was similar to the previous experiment was conducted and sought to determine if detectable AHLs could be extracted from *N. inopinata* cultures grown with urea as the source of ammonia. Only one sample out of sixteen contained detectable AHLs. This sample was taken from one culture replicate at Time 0 which was right after the inoculation of *N. inopinata* into the culture media. 3-OH-C10-HSL and 3-OH-C12-HSL were detected in this sample.

Since the detection of acyl-homoserine lactones in *N. inopinata* cultures was not repeatable compared to our initial detections, modified AHL extraction methods were tested to give more reproducible results. Three modified AHL extraction methods were tested to

determine which method was the best for AHL extraction. Hydrolyzed 3-OH-C14-HSL was detected in all extracted samples from Time Point 3. 3-OH-C10-HSL was detected in only one sample in a very low concentration. 3-OH-C12-HSL was detected in ten of the twelve samples extracted from in this experiment at very low concentrations. It was determined in this experiment that the AHL extraction with 5 min sonication and 1:1 ratio of MeOH:H2O was more optimal for future AHL extractions. Another modified AHL extraction method that could extract from larger volumes of sample supernatant than 2 mL of sample supernatant was also tested. This was done to discover if extracting from larger volumes would allow for better detection of extracted AHLs. In all five of the extracted samples, 3-OH-C10-HSL was detected. The peak area of this AHL also increased as the volume of the extracted sample increased.

Effect of Acyl-homoserine lactone Addition on *N. inopinata* growth.

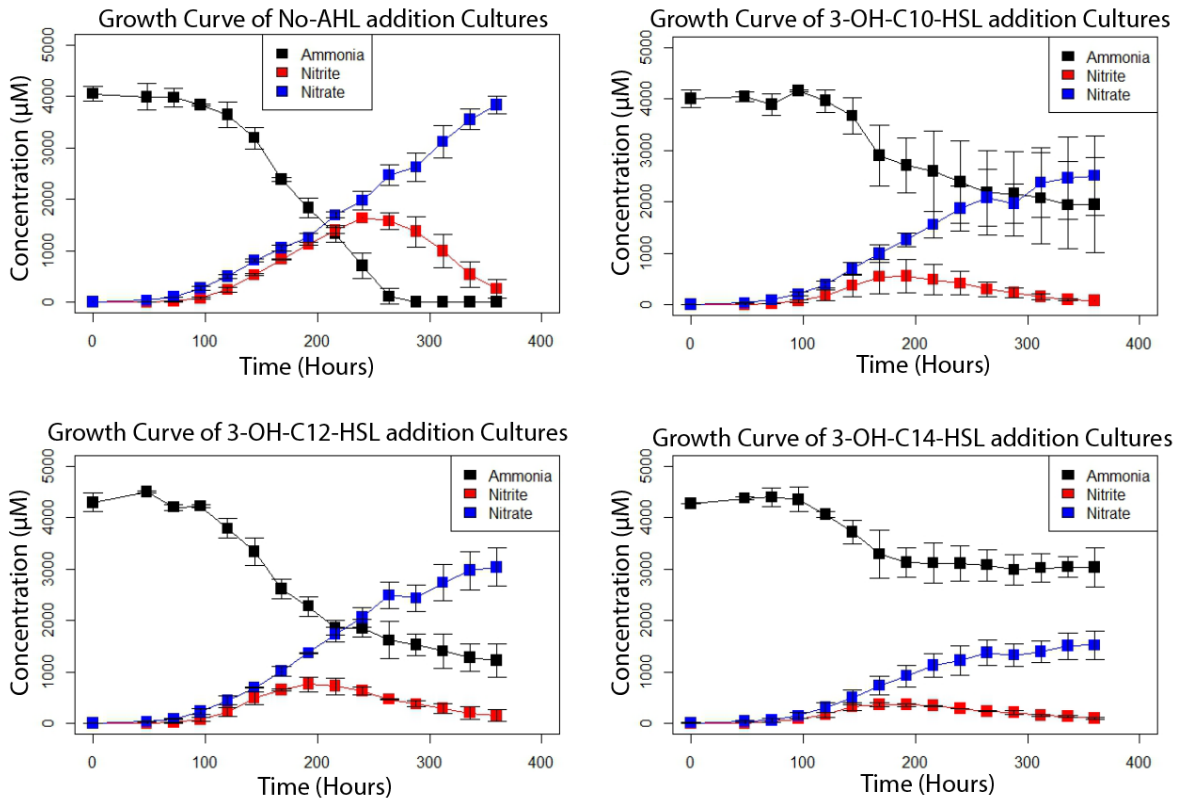


Figure 3. Average growth curves of the no-AHL addition culture group (Topleft), 3-OH-C10-HSL addition culture group (Topright), 3-OH-C12-HSL addition culture group (Bottomleft), and 3-OH-C14-HSL addition culture group (Bottomright). Ammonia concentrations were denoted as black lines and points. Nitrite concentrations were denoted as red lines and points. Nitrate concentrations were denoted as blue lines and points. Error bars for each nutrient concentration data point denote the standard deviation between the nutrient concentrations across the four cultures within the experimental group.

The growth of *Nitrospira inopinata* was shown to be affected by the addition of a single AHL into the culture media. The average doubling time for *N. inopinata* in the control group was 14.9 hours. In all three of the AHL-addition groups, the doubling times of *Nitrospira inopinata* in the cultures were slower than the control group cultures. The average doubling times in the AHL-addition culture groups were 20.4 hours (3-OH-C10-HSL), 18.1 hours (3-OH-C12-HSL),

and 22.4 hours (3-OH-C14-HSL). Ammonia concentrations decreased at a slower rate in all three of the AHL-addition culture groups, and ammonia concentrations in the AHL-addition cultures did not reach zero in the time when samples were taken. Nitrite accumulation in the AHL-addition cultures was lower at its highest concentration points compared to the control group cultures. Average maximum nitrite accumulation reached over 1.5 mM in the control culture group, and average maximum nitrite accumulation in all three AHL-addition cultures was under 1.0 mM.

The 3-OH-C14-HSL addition culture group was shown to have the slowest growth rate in this experiment and showed a growth pattern different from the control group. The control group behaved in this experiment as *N. inopinata* was expected to grow as in a batch culture: Ammonia concentrations decreased first as ammonia was oxidized to nitrite. Nitrite then accumulated as ammonia was oxidized to nitrite at a faster rate than nitrite oxidation to nitrate. Nitrite accumulation then stopped as ammonia concentrations reached near zero. Nitrite concentrations then decreased as nitrite was oxidized to nitrate as the sole energy source in the culture. Ammonia concentrations decreased for the first seven days after inoculation then stayed relatively constant for the rest of the experiment which suggests that the culture group had entered a stationary phase of growth. Nitrite concentrations were seen to slowly decrease during this stationary phase. The 3-OH-C10-HSL and the 3-OH-C12-HSL addition group cultures showed similar average growth patterns as the 3-OH-C14-HSL addition cultures. However, these two AHL addition groups did show a slow, constant decrease in ammonia after Day 7, and in total, more ammonia was oxidized to nitrite than the 3-OH-C14-HSL culture groups. Out of the three AHL addition culture groups, 3-OH-C12-HSL addition cultures used the most ammonia on

average and had the average doubling time (18.1 hours) that was closest to the average doubling time of the control group (14.9).

Transcription Response of Acyl-homoserine Lactone Addition in *N. inopinata*

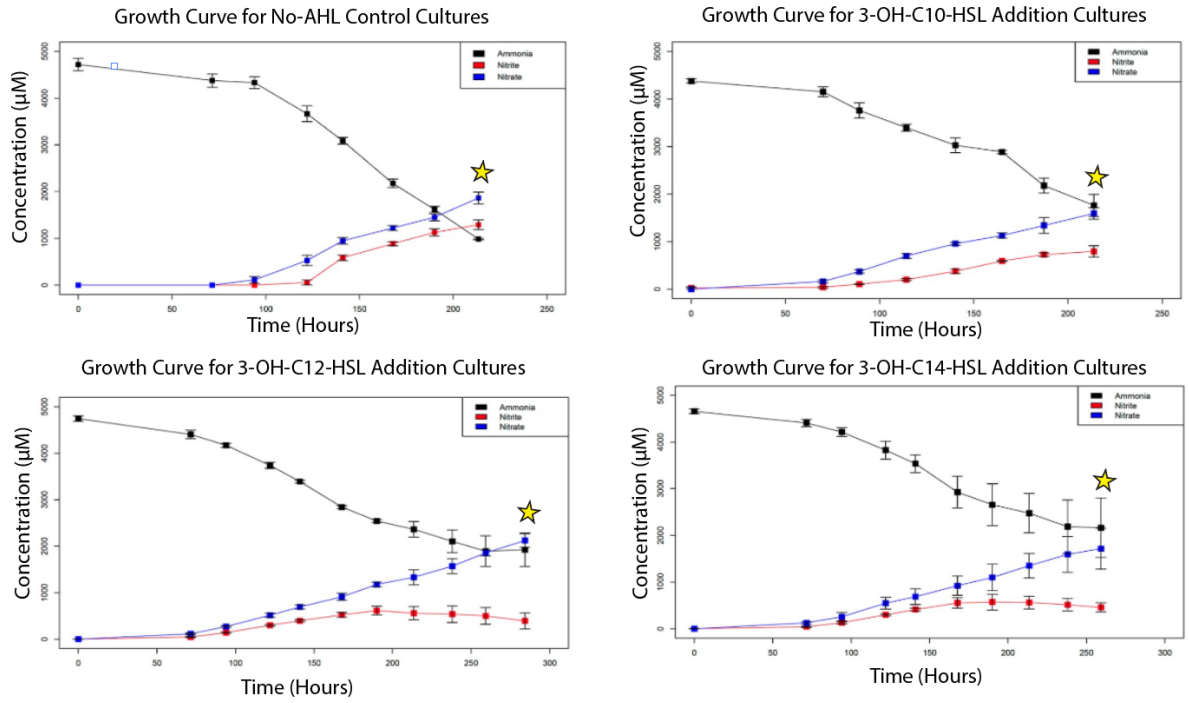


Figure 4. Average growth curves of the no-AHL addtion culture group (Topleft), 3-OH-C10-HSL addition culture group (Topright), 3-OH-C12-HSL addition culture group (Bottomleft), and 3-OH-C14-HSL addition culture group (Bottomright) before cell harvesting for RNAseq analysis. Ammonia concentrations were denoted as black lines and points. Nitrite concentrations were denoted as red lines and points. Nitrate concentrations were denoted as blue lines and points. A gold star denotes the timepoint the cells in the cultures were harvested before RNA extraction and purification for RNAseq analysis. Error bars for each nutrient concentration data

point denote the standard deviation between the nutrient concentrations across the four cultures within the experimental group.

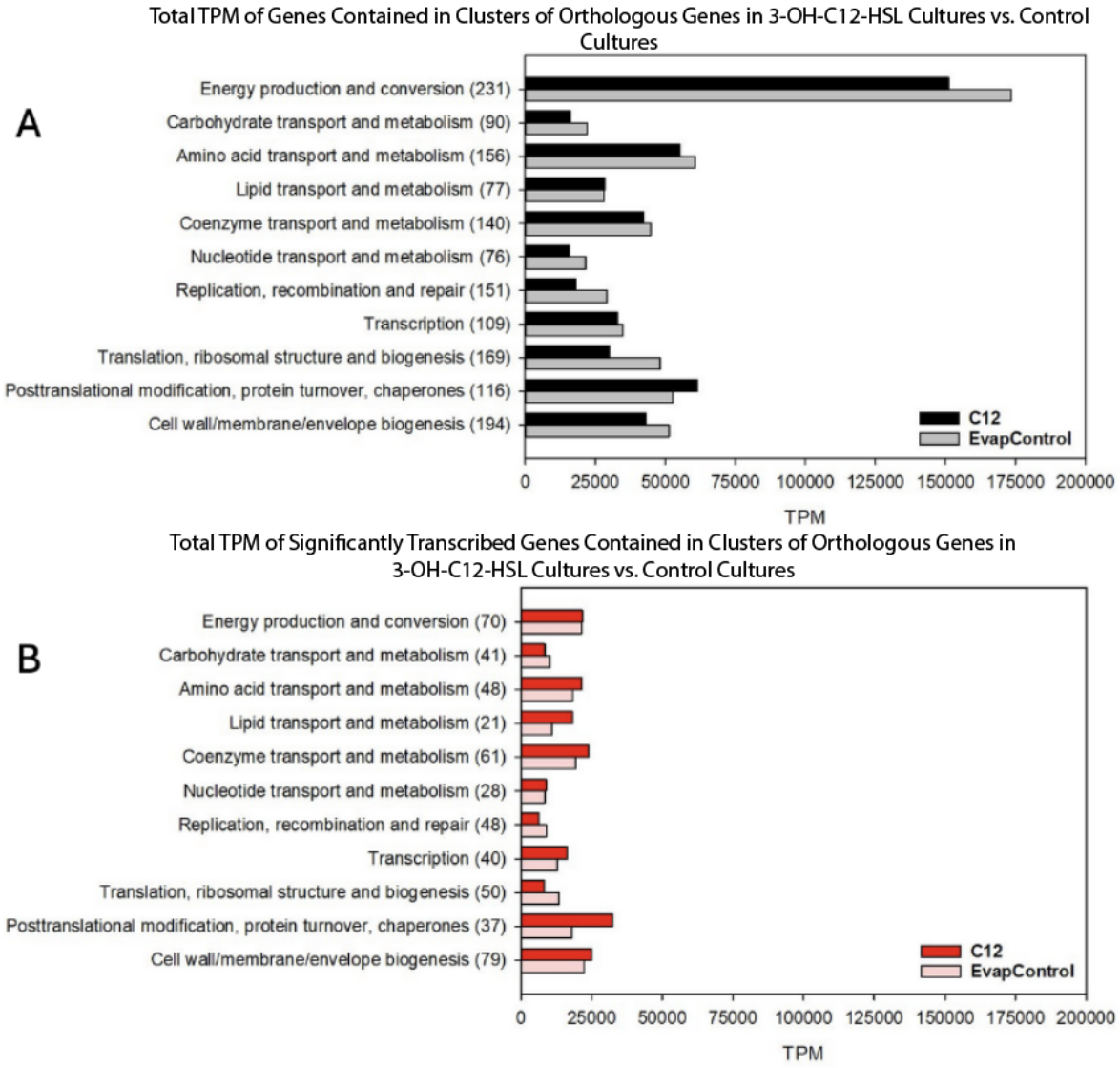


Figure 5. This figure shows the sum of transcripts per million (TPM) for a given cluster of orthologous genes (COG) for all transcribed, protein-coding genes detected in *Nitrospira inopinata* cultures under normal control conditions (EvapControl) and 1 µM 3-OH-C12-HSL

addition conditions (C12) (A). The sum of transcripts per million (TPM) for a given cluster of orthologous genes (COG) for differentially transcribed protein-coding genes (B). The number of transcribed, protein-coding genes included in each cluster of orthologous genes (COG) was included in parentheses next to the name of the cluster of orthologous genes (COG).

The global transcriptome of *Nitrospira inopinata* obtained from the 1 μM AHL-addition (C10, C12, or C14) experimental groups were compared to that of the control group. Only the 3-OH-C12-HSL-addition experimental group portrayed RNA transcript sequences that were differentially transcribed. The 3-OH-C10-HSL addition and 3-OH-C14-HSL addition groups did not contain differentially transcribed genes ($p > 0.05$) compared to the control group. Growth in media with 3-OH-C12-HSL addition significantly affected the transcription levels of genes belonging to clusters of orthologous genes (COGs) pertaining to posttranslational modification and turnover of proteins and protein chaperones, lipid metabolism and transport, and energy production and conversion.

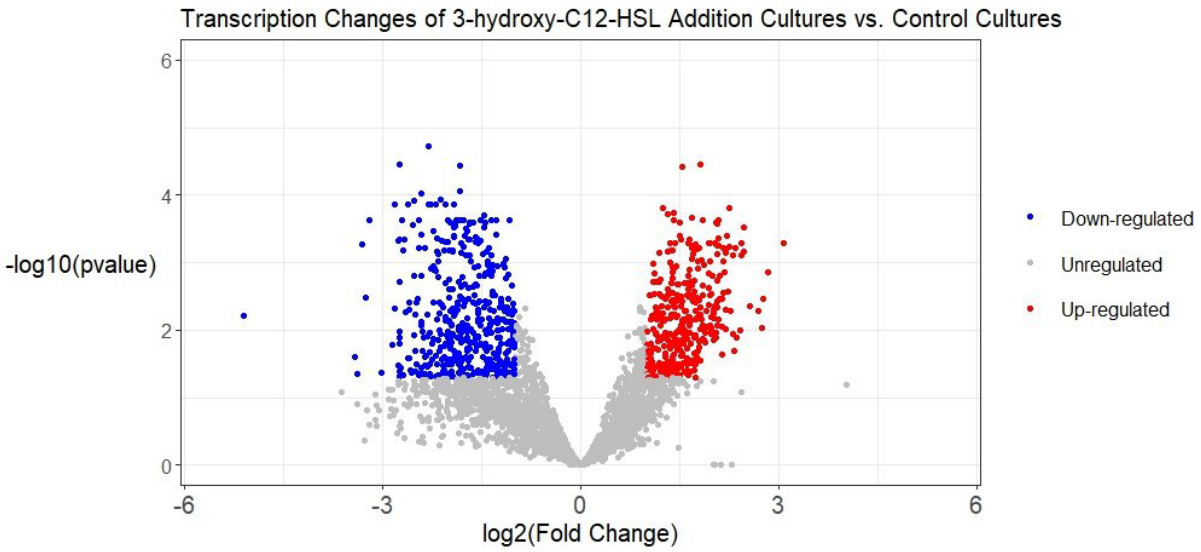
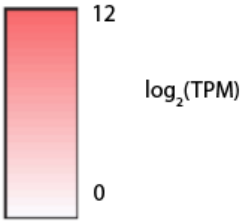


Figure 6. A volcano plot depicting the relative change in the average transcription of genes in N. inopinata grown in 3-OH-C12-HSL addition cultures compared to the average transcription of genes in no AHL addition cultures. The log2(Fold Change) values of each gene were calculated by comparing the average TPM values of that gene between the 3-OH-C12-HSL addition cultures and the no AHL addition cultures. Negative log2(Fold Change) values denote that the gene was transcribed in lower numbers in the 3-OH-C12-HSL addition cultures compared to the no addition cultures. Positive values denote that the gene was transcribed in higher numbers in the addition cultures compared to the no addition cultures. The –log10(pvalue) values for each gene rate the significance of the change in transcription of that particular gene between the 3-OH-C12-HSL addition cultures and the no addition cultures. Higher values denote smaller p-values. Red points denote genes that are significantly transcribed in higher levels in the 3-OH-C12-HSL cultures. Blue points denote genes that are significantly transcribed in lower levels in the 3-OH-C12-HSL cultures compared to the no addition cultures.

Gene Tag	Annotation	log2(Control TPM)	log2(C12 TPM)	p-value
NITINOP_1206		8.16	8.86	3.55E-05
NITINOP_1821		7.22	7.67	3.84E-05
NITINOP_2112	*clpB*	8.41	9.61	1.55E-04
NITINOP_0413	*wecB*	8.19	8.40	1.55E-04
NITINOP_1364		7.32	7.60	1.82E-04
NITINOP_0888		7.53	7.76	1.90E-04
NITINOP_0809		8.17	8.82	2.20E-04
NITINOP_0483		8.05	8.89	2.35E-04
NITINOP_2024	*lon*	8.07	9.05	2.35E-04
NITINOP_1169		7.50	7.92	2.35E-04
NITINOP_0972	*rarA*	8.53	8.98	2.51E-04
NITINOP_1994	*nrdZ*	8.35	9.37	2.60E-04
NITINOP_0560		8.61	9.64	2.67E-04
NITINOP_1463		8.32	9.80	3.02E-04
NITINOP_2506		7.59	8.05	3.98E-04
NITINOP_1307		8.99	10.22	4.05E-04
NITINOP_2660		8.65	9.75	4.32E-04
NITINOP_2240	*putA*	8.01	8.40	4.46E-04
NITINOP_2632		7.97	8.66	4.46E-04
NITINOP_3249	*ftsX*	8.57	9.49	5.08E-04
NITINOP_0797		6.80	7.32	5.08E-04
NITINOP_0673	*cydA*	8.57	9.49	5.08E-04
NITINOP_1159		9.37	10.83	5.08E-04
NITINOP_1467		8.44	9.42	5.12E-04
NITINOP_1219		8.47	8.82	5.15E-04
NITINOP_1448	*lpxC*	8.46	10.46	5.15E-04
NITINOP_0036		6.85	7.18	5.23E-04
NITINOP_0921		8.91	9.68	5.39E-04
NITINOP_1600	*ubiA*	8.18	9.29	5.51E-04
NITINOP_2659		6.91	8.05	5.64E-04
NITINOP_1365		8.47	9.23	5.64E-04
NITINOP_1366		8.78	9.91	5.86E-04
NITINOP_2714	*cooE*	7.58	8.30	5.97E-04
NITINOP_1657	*tdh*	8.47	9.29	6.08E-04
NITINOP_1981	*ecfE*	9.08	10.25	6.08E-04
NITINOP_3177		8.08	8.78	6.08E-04
NITINOP_0390		9.30	10.82	6.09E-04
NITINOP_0210		5.68	6.42	6.42E-04
NITINOP_0808		7.94	8.23	6.53E-04
NITINOP_1228		9.11	10.32	6.54E-04
NITINOP_1411		7.62	7.93	6.69E-04
NITINOP_0184		10.32	11.89	6.82E-04
NITINOP_1506	*kdpD*	8.08	8.36	6.91E-04
NITINOP_0950		9.23	10.52	7.14E-04
NITINOP_0761		7.71	7.83	7.19E-04
NITINOP_1554		8.35	9.01	7.49E-04
NITINOP_1260	*fmt*	8.81	9.88	7.49E-04
NITINOP_3376		8.06	9.56	7.69E-04
NITINOP_2646		9.59	11.01	7.95E-04
NITINOP_0672	*cydB*	8.12	8.65	8.65E-04

Gene Tag	Annotation	log2(Control TPM)	log2(C12 TPM)	p-value
NITINOP_0296	*murG*	7.72	4.37	1.92E-05
NITINOP_0290	*ftsZ*	8.01	4.34	3.55E-05
NITINOP_2374	*eno*	7.54	4.69	3.65E-05
NITINOP_2179		7.54	4.61	8.67E-05
NITINOP_0289		7.71	4.33	9.33E-05
NITINOP_1116	*nuoM*	7.94	4.86	1.17E-04
NITINOP_1117	*nuoM*	7.95	4.51	1.24E-04
NITINOP_0292		7.37	3.52	1.36E-04
NITINOP_1598		7.40	4.35	1.36E-04
NITINOP_0297	*ftsW*	8.07	5.05	1.36E-04
NITINOP_2551	*yvqK*	6.84	3.61	1.36E-04
NITINOP_1595		7.28	3.71	1.36E-04
NITINOP_1022		8.33	5.00	1.36E-04
NITINOP_3131		7.25	4.05	1.36E-04
NITINOP_0298	*murD*	7.78	5.20	1.96E-04
NITINOP_1355		7.63	4.24	2.32E-04
NITINOP_1504	*kdpB*	8.59	4.67	2.32E-04
NITINOP_2377	*mgtE*	7.29	4.73	2.35E-04
NITINOP_0649		6.22	2.47	2.35E-04
NITINOP_1617	*dop*	7.88	5.53	2.35E-04
NITINOP_2073	*lolC*	7.31	3.94	2.35E-04
NITINOP_3080	*manB_pgm*	7.55	5.06	2.35E-04
NITINOP_2553	*cobT*	7.16	4.18	2.35E-04
NITINOP_2740		7.12	4.15	2.35E-04
NITINOP_0491		7.91	5.74	2.35E-04
NITINOP_2180	*ppdK*	7.81	4.89	2.51E-04
NITINOP_1291		7.44	4.70	2.51E-04
NITINOP_2330	*czcA*	7.56	5.06	2.51E-04
NITINOP_0198		8.36	5.69	2.55E-04
NITINOP_1233	*panC*	7.38	4.38	2.59E-04
NITINOP_2397	*nadE*	7.04	4.30	2.62E-04
NITINOP_0295	*murC*	7.83	4.34	2.82E-04
NITINOP_3132		7.65	4.66	2.89E-04
NITINOP_1173		8.01	5.51	3.02E-04
NITINOP_2878	*carB*	7.62	4.65	3.02E-04
NITINOP_0406		7.68	4.83	3.15E-04
NITINOP_1118	*nuoN*	7.89	4.74	3.40E-04
NITINOP_2069		7.23	4.48	3.40E-04
NITINOP_1596		7.72	4.35	3.91E-04
NITINOP_2009	*zwf*	7.59	5.26	3.91E-04
NITINOP_2376	*era*	7.01	4.21	3.95E-04
NITINOP_3328	*secF*	7.59	4.70	3.98E-04
NITINOP_0652		7.19	4.57	4.18E-04
NITINOP_2847		7.35	4.33	4.19E-04
NITINOP_2923		7.12	3.97	4.43E-04
NITINOP_1831	*aroC*	7.44	3.63	4.46E-04
NITINOP_0291	*ftsA*	7.48	3.78	4.46E-04
NITINOP_2416		7.86	5.22	4.46E-04
NITINOP_1953	*miaA*	7.28	4.55	4.46E-04
NITINOP_1044	*cmr3*	7.78	5.06	4.46E-04



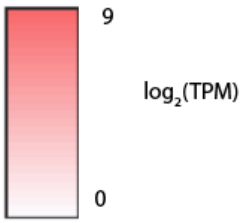


Figure 7. A heatmap of the fifty genes that were most significantly transcribed in higher numbers in the 3-OH-C12-HSL addition cultures compared to the no addition controls appears on the left half of the figure. A heatmap of the fifty genes that were most significantly transcribed in lower numbers in the 3-OH-C12-HSL addition cultures compared to the no addition controls appears

on the right half of the figure. In each heatmap, the gene tag of each gene and its annotation are in the left most columns. Genes without annotations were putative or unknown genes. The log2(Control TPM) values and the log2(C12 TPM) values for each gene were calculated by using the average TPM values of the gene from each respective culture group (C12 TPM = Average TPM of 3-OH-C12-HSL addition cultures; Control TPM = Average TPM of no AHL addition cultures). Genes with higher transcriptions appear more red while genes with lower transcription values appear more white. White denotes no transcription of that gene. The identity of the top 50 up-transcribed genes are as follows: The gene, NITINOP_1206, encoded for a conserved protein of unknown function. The gene, NITINOP_1821, encoded for a putative copper protein of the cupredoxin family. The gene, *clpB*, encoded for a protein disaggregation chaperone. The *wecB* gene encoded for a UDP-N-acytylglucosamine 2-epimerase. The gene, NITINOP_1364, encoded for a putative N-acetyltransferase. The gene, NITINOP_0888, encoded for a putative transporter subunit of the ABC superfamily. The genes, NITINOP_0809 and NITINOP_0483 encoded for conserved proteins of unknown functions. The *lon* gene encoded for a Lon protease. The gene, NITINOP_1169, encoded for a conserved exported protein of unknown function. The *rarA* gene encoded for a replication-associated recombination protein A. The *nrdZ* gene encoded for a ribonucleoside-diphosphate reductase, NrdZ. The gene, NITINOP_0560, encoded for a putative efflux transporter. The genes, NITINOP_1463 and NITINOP_2506, encoded for conserved proteins of unknown functions. The gene, NITINOP_1307, encoded for a putative membrane-bound serine protease. The gene, NITINOP_2660, encoded for a putative DegP-like periplasmic serine endoprotease. The *putA* gene encoded for the bifunctional protein PutA. The gene, NITINOP_3249, encoded for a purative Slp outer membrane lipoprotein. The gene *ftsX* encoded for a FtsX cell division protein.

The gene, NITINOP_0797, encoded for a protein of unknown function. The *cydA* gene encoded for a cytochrome bd oxidase subunit I. The gene, NITINOP_1159, encoded for a putative enzyme with a nucleoside triphosphate hydrolase domain. The gene, NITINOP_1467, encoded for a putative peptidylprolyl isomerase. The gene, NITINOP_1219, encoded for a conserved protein of unknown function. The *lpxC* gene encoded for a UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase. The gene, NITINOP_0036, encoded for a putative Hsp20 heat shock protein. The gene, NITINOP_0921, encoded for a flavin-containing amine oxidase. The *ubiA* gene encoded for a 4-hydroxybenzoate octaprenyltransferase. The gene, NITINOP_2659, encoded for a conserved protein of unknown function. The gene, NITINOP_1365, encoded for a putative polysaccharide deacetylase. The gene, NITINOP_1366, encoded for a putative membrane-associated zinc metallopeptidase. The *coaE* gene encoded for a dephospho-CoA kinase. The *tdh* gene encoded for a NAD(P)-binding threonine 3-dehydrogenase. The *ecfE* gene encoded for a M50 peptidase. The gene, NITINOP_3177, encoded for a conserved exported protein of unknown function. The gene, NITINOP_0390, encoded for a putative Slp outer membrane lipoprotein. The gene, NITINOP_0210, encoded for a putative acyl carrier protein. The gene, NITINOP_0808, encoded for a putative TldE-like peptidase. The gene, NITINOP_1228, encoded for a conserved protein of unknown function. The gene, NITINOP_1411, encoded for a putative HAD-like hydrolase. The gene, NITINOP_0184, encoded for a conserved protein of unknown function. The *kdpD* gene encoded for a sensory histidine kinase. The gene, NITINOP_0950, encoded for a putative orotate phosphoribosyltransferase. The genes, NITINOP_0761 and NITINOP_1554, encoded for conserved proteins of unknown functions. The *fmt* gene encoded for a 10-fromyltetrahydrofolate:L-methionyl-tRNA N-formyltransferase. The genes, NITINOP_3376 and

NITINOP_2646, encoded for conserved proteins of unknown functions. The *cydB* gene encoded for a cytochrome bd oxidase subunit II. The identity of the top 50 most down-transcribed genes are as follows: The *murG* gene encoded for a UDP-N-acytylglucosamine—N-acetylmuramyl-(pentapeptide) pyrosphosphoryl-undecapenol N- acetylglucosamine transferase. The *ftsZ* gene encoded for a GTP-binding tubulin-like cell division protein. The *eno* gene encoded for an enolase. The gene, NITINOP_2179, encoded for a Glycine—tRNA ligase beta subunit. The gene, NITINOP_0289, encoded for a conserved protein of unknown function. The two copies of the *nuoM* gene, NITINOP_1116 and NITINOP_1117, encoded for NAHH-quinone oxidoreductase membrane subunit M. The gene, NITINOP_0292, encoded for a putative FtsQ cell division protein. The gene, NITINOP_1598, encoded for a putative nitrite oxidoreducatse membrane subunit. The ftsW gene encoded for a integral membrane protein involved in stabilizing FtsZ ring during cell division. The yvqK gene encoded for the Cob(I)yrinic acid diamide adenosyltransferase. The genes, NITINOP_1595 and NITINOP_1022, encoded for proteins of unknown functions. The gene, NITINOP_3131, encoded for a putative oxidoreductase of the Gfo/Idh/MocA family. The murD gene encoded for a UDP-N-acetylmuramoylalanine—D-glutamate ligase. The gene, NITINOP_1355, encoded for a protein of unknown function. The gene kdpB encoded for a potassium translocating ATPase subunit B. The gene mgtE encoded for a MgtE magnesium transporter. The gene, NITINOP_0649, encoded for a putative OsmC peroxiredoxin. The dop gene encoded for a depupylase. The lolC gene encoded for a lipoprotein-releasing system LolC transmembrane protein. The manB_pgm gene encoded for a bifunctional phosphoglucomutase/phosphomannomutase. The cobT gene encoded for a nicotinate-nucleotide dimethylbenzimidazole-P phophoribosyl transferase. The gene, NITINOP_2740, encoded for a putative ABC-type sugar transport system permease component.

The gene, NITINOP_0491, encoded for a putative outer membrane efflux protein. The ppdK gene encoded for a pyruvate phosphate dikinase. The gene, NITINOP_1291, encoded for a putative acyltransferase. The czcA gene encoded for a CzcA cation efflux system protein. The gene, NITINOP_0198, encoded for a protein of unknown function. The panC gene encoded for a pantothenate synthetase. The nadE gene encoded for a NH(3)-dependent NAD(+) synthetase. The murC gene encoded for a UDP-N-acetylmuramate:L-alanine ligase. The gene, NITINOP_3132, encoded for a conserved protein of unknown function. The gene, NITINOP_1173, encoded for a putative agmatinase. The carB gene encoded for a carbamoyl-phosphate synthase large subunit. The gene, NITINOP_0406, encoded for a putative exopolysaccharide biosynthesis related tyrosine-protein kinase. The nuoN gene encoded for a NADH-quinone oxidoreductase subunit N. The genes, NITINOP_2069 and NITINOP_1596, encoded for conserved proteins of unknown functions. The zwf gene encoded for glucose-6-phoshate 1-dehydrogenase. The era gene encoded for a Era GTPase. The secF gene encoded for a SecF protein translocase subunit. The gene, NITINOP_0652, encoded for a putative ABC-type transport system periplasmic binding component. The genes, NITINOP_2847 and NITINOP_2923, encoded for conserved proteins of unknown functions. The aroC gene encoded for a chorismate synthase. The ftsA gene encoded for a ATP-binding cell division protein involved in recruitment of FtsK to Z ring. The gene, NITINOP_2416, encoded for a conserved protein of unknown function. The miaA gene encoded for a tRNA dimethylallyltransferase. The cmr3 gene encoded for a CRISPR-associated protein.

The fifty most downregulated genes of the 3-OH-C12-HSL addition transcriptome when compared to the no AHL addition control transcriptome were initially analyzed to determine function and if multiple genes were a part of a regulated operon. The transcription of eight genes

from an operon region (NITINOP_0289-0298) were found to be upregulated, and the functions of all these genes governed cell division and cell wall biosynthesis. Another pair of genes that were upregulated (NITINOP_1116 and NITINOP_1117) encoded for the M subunit of the NADH-quinone oxidoreductase that forms Complex I in the Electron Transport Chain (ETC) in *N. inopinata*. Another pair of genes (NITINOP_2551 and NITINOP_2553 encoded for proteins associated with cobalamin biosynthesis. Another pair of genes (NITINOP_2374 and NITINOP_2376) are transcribed in the same direction near each other. These genes encode for enolase, which was involved in gluconeogenesis, and GTPase ERA, which encodes for a protein involved in ribosome assembly in *N. inopinata*.

The fifty most upregulated genes of the 3-OH-C12-HSL addition transcriptome were initially analyzed to determine function and if multiple genes were a part of a regulated operon. Two genes (NITINOP_0672 and NITINOP_0673) encoded for subunits of cytochrome bd oxidase which is Complex IV in the Electron Transport Chain. Two genes were involved in posttranslational modification and encoded for a protein disaggregation chaperone (NITINOP_2112) and putative petidylpropyl isomerase (NITINOP_1467). A DNA replication gene was also found to be upregulated. This gene (NITINOP_0972) encoded for recombination protein A. The gene (NITINOP_3249) encodes for cell division protein FtsX which is involved in the cell division cycle and building cell walls in *N. inopinata*. Three genes encoding for proteases and protein degradation were also found to be included in the fifty most upregulated genes. One gene (NITINOP_2024) encodes for a Lon protease. Another gene (NITINOP_2660) encodes for a putative DegP-like periplasmic serine endoprotease. A third gene (NITINOP_1981) encodes for a peptidase M50.

Posttranslational Modification

Genes from the COG that have functions relating to posttranslational modifications of proteins, protein turnover, and protein chaperones had a higher TPM sum under 3-OH-C12-HSL addition (32436 transcripts per million) compared to the control group (18002 transcripts per million). It was found that the transcription of 37 genes in this COG were significantly affected by addition of 3-OH-C12-HSL for *N. inopinata* cultures. The transcription of genes encoding for proteases were found to be increased significantly in these AHL addition cultures. A gene involved in thioredoxin activities was found to be transcribed in smaller numbers in AHL addition cultures. A gene encoding a Pup ligase was transcribed in significantly higher amounts in the AHL addition cultures.

Pup ligase was a protein involved in pupylation, a posttranslational protein modification pathway found in certain groups of prokaryotes. This system tags the C-terminal of target proteins with the ligation of the prokaryotic ubiquitin-like protein (Pup). Pupylation found in prokaryotes was also analogous to the eukaryotic protein modification pathway that employs ubiquitin to tag proteins. The pupylation pathway was found in bacteria species belonging to groups such as Actinobacteria and *Nitrospira* (18). It may be possible that pupylation performs a similar regulatory function in *Nitrospira inopinata.* However, the function of pupylation could be explored in *Nitrospira inopinata* in the future.

Thioredoxin was involved in antioxidant activities in prokaryotic and eukaryotic cells. Thioredoxin was found to be able to reduce hydrogen peroxide and help combat other oxygen radicals as part of the oxidative stress responses of E. coli. Thioredoxin had the ability to reduce certain proteins in the cytoplasm of bacteria. Thioredoxin was also discovered to be an important subunit in the assembly of T7 phages in bacteria (20).

Lon and HtpX were both proteases involved in the degradation of targeted proteins. HtpX was a cytoplasmic membrane-bound protease that was found in bacteria including species in *Pseudomonas* and *Escherichia*. HtpX was discovered to contribute to the resistance of the aminoglycoside antibiotic tobramycin and toxic membrane proteins in *Pseudomonas aeruginosa* (21). Lon was an important protease found in many bacteria and can regulate cellular functions through the degradation of other target proteins. The downregulation and degradation of Lon in the pathogens, *Escherichia coli* and *Pseudomonas*, allows for increased expression of virulence factor genes which could permit *Pseudomonas* and *E. coli* to resist phagocytosis by host cells and certain antibiotics, respectively (22). *Salmonella enterica* controls the presence of Lon to regulate the expression of certain virulence genes. A decrease in Lon allows for an increase in the expression of virulence genes that allow for adherence in host while at the same time decreasing the expression of other genes associated with motility to promote intracellular growth in host macrophages (22).

Nitrogen Oxidation and Assimilation

The addition of 3-oxo-C12-HSL had a significant effect on the transcription of one gene involved in the nitrification pathway in *N. inopinata*. The gene, *amoC*, encodes for one of the subunits of ammonia monooxygenase. This gene was found to be transcribed in significantly higher amounts in the experimental cultures with a nearly three-fold change in transcription compared to the control ($P<0.05$). The other two subunits of ammonia monooxygenase, *amoA* and *amoB*, did not show significant changes ($P=0.44$ and $P=0.28$, respectively) in transcription between the control and experimental culture groups. Ammonia monooxygenase begins the nitrification reaction process in *N. inopinata* with the oxidation of ammonia to hydroxylamine.

Nitrospira inopinata contains three different homologous *amoC* genes that encode for a subunit of ammonia monooxygenase. These subunits were found to be associated with stress responses such as ammonia starvation in *Nitromonas europaea* since these conditions influenced the transcription of these genes in *N. europaea*. It was discovered that the *amoC* subunits were able to act as chaperone proteins to the ammonia monooxygenase complex and improve the integrity of the protein complex (23, 24) All genes involved in the other steps of nitrification (*hao*, *haoB*, *nxrA*, *nxrB*) did not portray significant changes in transcription levels under 3-oxo-C12-HSL addition. The gene, *hao*, codes for hydroxylamine dehydrogenase which catalyzes the oxidation of hydroxylamine to nitrite. The genes, *nxrA* and *nxrB*, code for two subunits that form nitrite oxidoreductase. Nitrite oxidoreductase catalyzes the oxidation of nitrite to nitrate.

The transcription of the two genes coding for cytochrome bd was found to be affected by 3-OH-C12-HSL addition. The two genes, *cydA* and *cydB*, encode the two subunits that make up the cytochrome bd protein complex. Both genes were transcribed in significantly higher amounts under 3-OH-C12-HSL addition ($P<0.001$; $P<0.001$). Cytochrome bd was an important part of the electron transfer chain (ETC) in *N. inopinata*. This enzyme serves as the Complex IV in the ETC and reduces oxygen gas to water using electrons obtained from ammonia and nitrite oxidation to generate protons. These protons could then be utilized in ATP synthesis. The gene, *cytB*, encodes for a putative quinol-cytochrome c reductase that potentially forms Complex III in the ETC of *N. inopinata*. This gene was significantly transcribed in higher concentrations under AHL-addition conditions with over a two-fold change in transcription compared to the controls ($P<0.05$). This complex shuttles electrons to Complex IV during the ammonia oxidation pathway.

The transcription of genes involved with nitrogen assimilation in *N. inopinata* were not significantly differentially transcribed under AHL addition compared to controls. The nitrogen

assimilation genes analyzed in *N. inopinata* code for glutamine synthase (*glnA*), glutamate synthase (*gltD; gltB*), and glutamate dehydrogenase (*gudB*) and are involved in two different nitrogen assimilation pathways. Glutamine synthase and glutamate synthase are a part of the GOGAT pathway of nitrogen assimilation. In the GOGAT pathway, Glutamine synthase catalyzes the synthesis of glutamine from ammonia and glutamate. Secondly, Glutamate synthase catalyzes the synthesis of glutamate from the transfer of one amine group from glutamine to α-ketoglutarate resulting in two glutamate molecules. Glutamate dehydrogenase conducts the activity of the second nitrogen assimilation pathway in *N. inopinat*a and can do perform two different reactions. Glutamate dehydrogenase could catalyze the degradation of glutamate into ammonia and α-ketoglutarate or could catalyze the formation of glutamate using ammonia and α-ketoglutarate.

The transcription of genes involved in urea metabolism were found to be affected by 3-OH-C12-HSL addition. The one of the genes that code for urease (*ureA, ureB; ureC*) and its accessory proteins (*ureF; ureG*) was found to be affected by 3-OH-C12-HSL addition. This gene, *ureD*, was found to be transcribed in lower amounts under 3-OH-C12-HSL addition by a three-fold change in transcription compared to the controls ($P<0.01$) and codes for an accessory protein associated with urease. Urease catalyzes the breakdown of urease into carbamate and ammonia. This ammonia then could be assimilated or utilized for energy production by *N. inopinata*. All but one of the genes that code for subunits of a urea ABC transporter (*urtA, urtB, urtC, urtD; urtE*) were found to be transcribed in higher amounts under 3-OH-C12-HSL addition. The gene that was not found to have a significant difference in transcription was *urtE*. This urea transporter protein imports urea into *N. inopinata* cells for urease activity to occur.

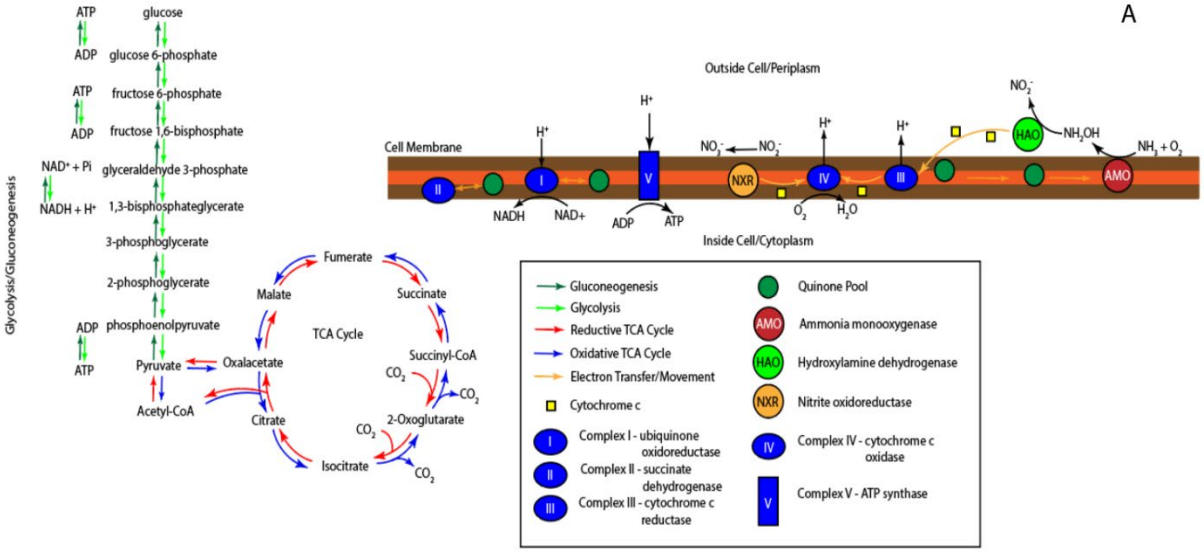
Translation

Most genes that encode ribosomal proteins did not have significant differential transcription levels between AHL-addition cultures and control cultures. Only 9 of the 43 genes involved in ribosomal subunit proteins displayed significantly differential transcription under 3-OH-C12-HSL addition conditions compared to the control.

Genes involved in the synthesis and conversion of different aminoacyl-tRNAs were found to be significantly differentially transcribed between 3-OH-C12-HSL addition and control conditions. Eleven out of 25 involved in aminoacyl-tRNA synthesis and conversion were shown to be significantly differentially transcribed. Four of these genes (*gltX, alaS, fmt; leuS*) were found to have increased levels of transcription in the AHL-addition cultures compared to the control cultures. The genes, *gltX, alaS*, and *leuS*, encoded for glutamate-tRNA ligase, alanyl-tRNA synthase, and leucyl-tRNA synthase in *N. inopinata* respectively.

Gluconeogenesis and TCA Cycle

A



B

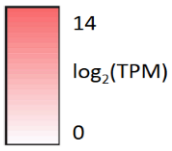
Electron Transport Chain

Gene Tag	Annotation	log2(Control TPM)	log2(C12 TPM)	
NITINOP_1107	*nuoA*	9.76	9.77	
NITINOP_1379	*nuoA*	7.34	7.87	*
NITINOP_1108	*nuoB*	9.83	9.58	
NITINOP_1378	*nuoB*	8.52	8.99	
NITINOP_1109	*nuoC*	8.63	8.64	
NITINOP_1377	*nuoC*	8.54	7.60	
NITINOP_1110	*nuoD*	9.91	9.78	
NITINOP_1376	*nuoE*	7.47	5.93	
NITINOP_0978	*nuoF*	9.47	9.46	
NITINOP_1375	*nuoF*	8.22	6.73	
NITINOP_1373	*nuoH*	8.91	8.74	
NITINOP_1112	*nuoI*	9.43	7.43	
NITINOP_1372	*nuoI*	8.96	7.89	
NITINOP_1113	*nuoJ*	7.89	6.09	
NITINOP_1371	*nuoJ*	7.35	4.72	***
NITINOP_1114	*nuoK*	5.70	2.41	
NITINOP_1370	*nuoK*	4.69	2.26	
NITINOP_1115	*nuoL*	8.49	5.49	**
NITINOP_1369	*nuoL*	7.69	4.66	***
NITINOP_1116	*nuoM*	7.94	4.86	***
NITINOP_1117	*nuoM*	7.95	4.51	***
NITINOP_1368	*nuoM*	7.69	4.84	**
NITINOP_1118	*nuoN*	7.89	4.74	***
NITINOP_1367	*nuoN*	7.74	6.28	

Gluconeogenesis

Gene Tag	Annotatiion	log2(Control TPM)	log2(C12 TPM)	
NITINOP_2245	*pgm*	7.55	5.44	*
NITINOP_3080	*manB_pgm*	7.55	5.06	***
NITINOP_0914	*glk*	8.88	8.66	
NITINOP_0912		9.14	9.11	
NITINOP_0538	*pfkA*	7.70	6.23	
NITINOP_3078	*fbp*	8.25	8.24	
NITINOP_3077	*fbaB*	9.23	9.50	
NITINOP_3159	*tpiA*	7.16	4.43	***
NITINOP_3157	*gapA*	9.85	9.57	
NITINOP_3158	*pgk*	8.31	6.92	
NITINOP_1254	*gpmA*	7.39	6.19	
NITINOP_0768	*apgM*	8.18	7.12	
NITINOP_2374	*eno*	7.54	4.69	***
NITINOP_2630	*prkA*	7.42	6.86	
NITINOP_2180	*ppdK*	7.81	4.89	***
NITINOP_2186	*acsA*	8.98	8.75	
NITINOP_0615		7.59	6.77	
NITINOP_2493	*lpd*	7.18	5.72	
NITINOP_1653	*adhA*	7.93	7.43	
NITINOP_0505	*adhC*	8.73	8.36	
NITINOP_0238	*forA*	12.27	12.07	
NITINOP_0241	*forC*	11.36	11.44	
NITINOP_0242	*forB*	10.97	10.78	
NITINOP_1627	*porE*	7.72	7.37	
NITINOP_1628	*porC*	11.22	10.87	
NITINOP_1629	*porB*	12.44	12.18	
NITINOP_1630	*porA*	11.99	12.00	

P<0.05 *
P<0.01 **
P<0.001 ***



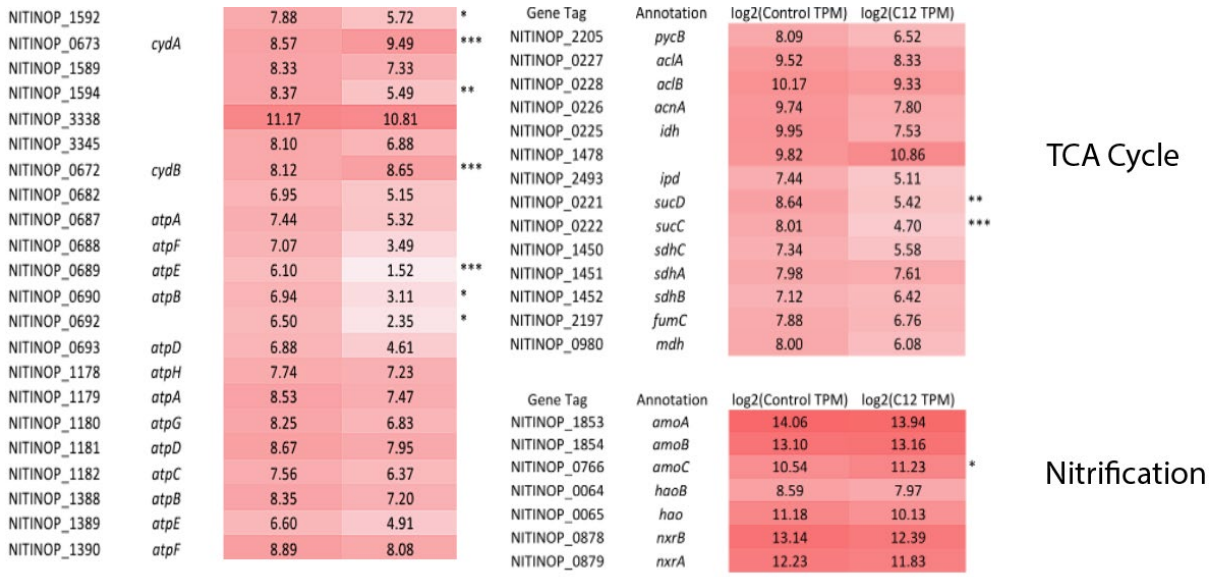


Figure 8. The energy production and carbon fixation pathways utilized by *Nitrospira inopinata*. These pathways include the TCA cycle and Gluconeogenesis for carbon fixation. The energy production pathways include the nitrification pathway that completely oxidizes ammonia to nitrate and the electron transport chain (A). Heatmap portraying calculated log2(TPM) values of pathway genes obtained from RNAseq analysis of 3-OH-C12-HSL addition cultures compared to no-AHL addition control cultures. In the gluconeogenesis pathway, the gene, *pgm*, encoded for a phosphoglucomutase protein. The *manB_pgm* gene encoded for a bifunctional phosphoglucomutase and phosphomannomutase protein. The *glk* gene encoded for the glucokinase protein. The gene, NITINOP_0912, encoded for a putative glucose-6-phosphate isomerase. The *pfkA* gene encoded for a 6-phophofructokinase protein. The *fbp* gene encoded for a fructose-6-bisphosphatase protein. The *fbaB* gene encoded for a fructose-bisphosphophosphate aldolase protein. The *tpiA* gene encoded for a triosephosphate isomerase protein. The *gapA* gene encoded for a glyceraldehyde-3-phosphate dehydrogenase protein. The pgk gene encoded a phosphoglycerate kinase. The *gpmA* gene encoded for a bisphosphoglycerate-dependent phosphoglycerate mutase. The *apgM* gene encoded for a bisphosphoglycerate-independent

phosphoglycerate mutase. The *eno* gene encoded for an enolase protein. The *prkA* gene encoded for a pyruvate kinase. The *ppdK* gene encoded for a pyruvate phosphate dikinase. The *acsA* gene encoded for an acetyl-CoA synthase. The gene, NITINOP_0615, encoded for a putative acyl-CoA synthase. The *lpd* gene encoded for a dihydrolipoyl dehydrogenase. The *adhA* gene encoded for a putative alcohol dehydrogenase and the gene *adhC* encoded for an alcohol dehydrogenase protein. The genes, *forA, forB; forC*, encode for subunits of a 2-oxoglutarate:ferredoxin oxidoreductase protein. The genes, *porA, porB, porC; porE*, encoded for subunits of the pyruvate:ferredoxin oxidoreductase protein. In the TCA cycle, the *pycB* gene encoded for a subunit of the pyruvate carboxylase protein. The *aclA* and *aclB* genes encoded for subunits of the ATP-citrate lyase protein. The *acnA* gene encoded for the aconitate hydratase protein. The *idh* and NITINOP_1478 genes encoded for the isocitrate dehydrogenase protein. The *ipd* gene encoded for the dihydrolipoyl dehydrogenase protein. The *sucC* and *sucD* genes encoded for subunits of succinyl-CoA synthase. The *sdhA, sdhB*, and *sdhC* genes encoded for subunits of succinate dehydrogenase/fumerate reductase which serves as Complex II in the ETC. The *fumC* gene encoded for a fumerate hydratase protein. The *mdh* gene encoded for malate dehydrogenase. In the commamox nitrification pathway, the *amoA, amoB; amoC* genes encoded for subunits of ammonia monooxygenase. The hao gene encoded for a hydroxylamine dehydrogenase. The *haoB* gene encoded for a hydroxylamine oxidation protein HaoB. The *nxrA* and *nxrB* genes encoded for subunits of nitrite oxidoreductase. In the ETC, the genes, *nuoA* (NITINOP_1107; NITINOP_1379), *nuoB* (NITINOP_1108; NITINOP_1378), *nuoC* (NITINOP_1109; NITINOP_1377), *nuoD, nuoE, nuoF* (NITINOP_0978; NITINOP_1375), *nuoH, nuoI* (NITINOP_1112; NITINOP_1372), *nuoJ* (NITINOP_1113; NITINOP_1371), *nuoK* (NITINOP_1114; NITINOP_1370), *nuoL* (NITINOP_1115; NITINOP_1369), *nuoM*

(NITINOP_1116, NITINOP_1117; NITINOP_1368), *nuoN* (NITINOP_1118; NITINOP_1367) all encoded for subunits of ubiquinone oxidoreductase which serves as Complex I. The gene, NITINOP_1592, encoded for a putative quinol-cytochrome c reductase which serves as Complex III. The genes, *cydA, cydB*, NITINOP_1589, NITINOP_1594, NITINOP_3338; NITINOP_3345, encoded for a cytochrome bd which serves as Complex IV. The genes, *atpA* (NITINOP_0687; NITINOP_1179), *atpB* (NITINOP_0690; NITINOP_1388), *atpC, atpD* (NITINOP_0693; NITINOP_1181), *atpE* (NITINOP_0689; NITINOP_1389), *atpF* (NITINOP_0688; NITINOP_1390), *atpG, atpH*, NITINOP_0682 and NITINOP_0692, encoded for subunits for ATP synthase which would serve as Complex V (B).

The transcription of some genes involved in glycolysis in *N. inopinata* were found to be significantly differentially transcribed under AHL-addition conditions compared to controls. Ultimately, the gluconeogenesis pathway and TCA cycle seem to be downregulated. Five genes (*pgm, manB_pgm, tpiA, eno; ppdk*) out of 27 genes encoding for functional proteins within the gluconeogenesis and TCA cycle pathways were found to be significantly differentially transcribed in lower levels in AHL-addition cultures compared to controls. Phosphoglucomutase, encoded by *pgm,* was found to portray a two fold decrease in transcription compared to the controls ($P<0.01$), and was an enzyme associated with gluconeogenesis in *N. inopinata* and performed the interconversion of glucose-1-phosphate and glucose-6-phosphate. Enolase, encoded by the gene *eno,* portrayed a nearly four-fold decrease in transcription levels compared to the controls ($P<0.001$), and was an enzyme associated with gluconeogenesis and performed the interconversion of glycerate-2-phosphate and phosphoenolpyruvate. Pyruvate phosphate dikinase, encoded by *ppdk,* was shown to have a four-fold decrease in transcription

levels compared to the controls (P<0.001), and was an enzyme associated with the transfer of a phosphate group from phosphoenolpyruvate to AMP forming ATP and pyruvate. This enzyme was also capable of the reverse reaction.

Fatty Acid and Acyl Homoserine Lactone Biosynthesis

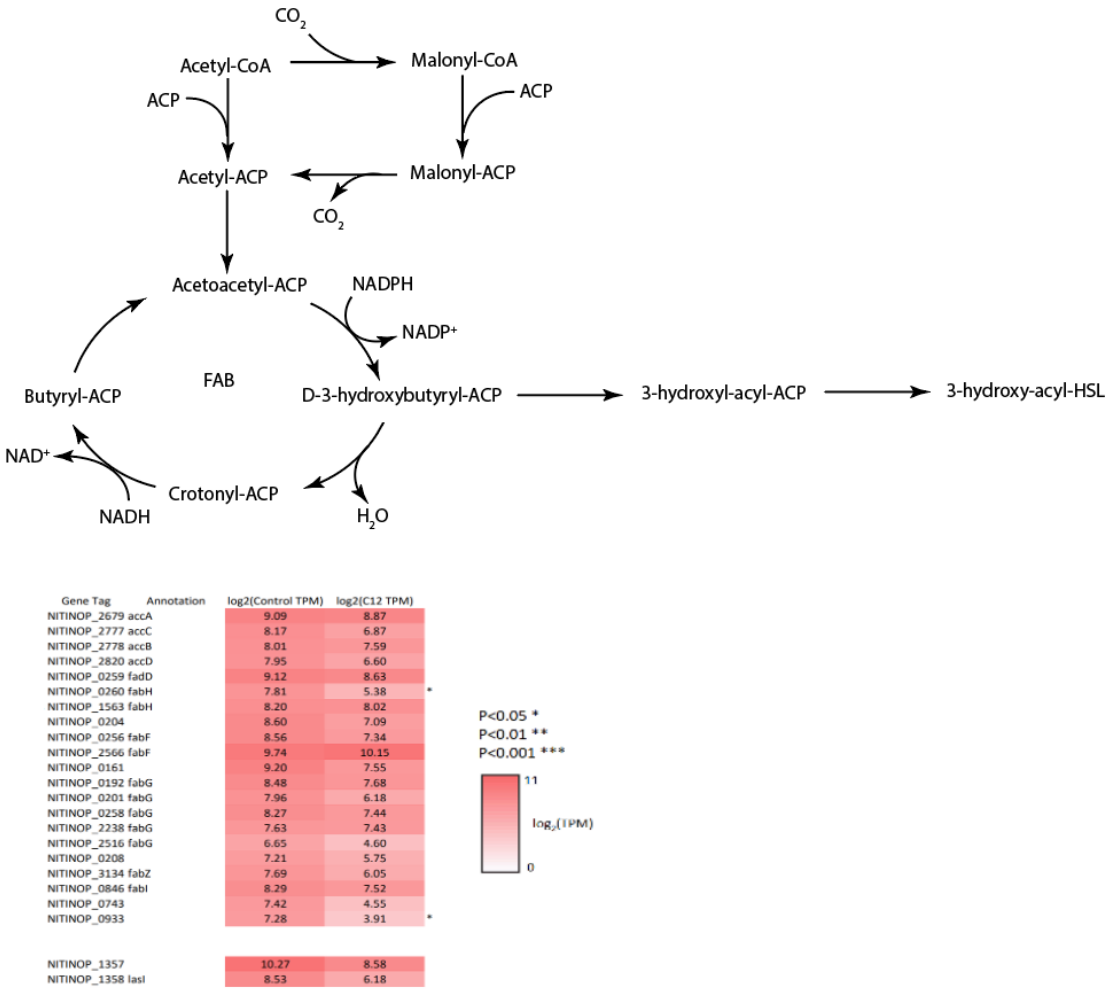


Figure 9. The fatty acid biosynthesis and acyl-homoserine lactone synthesis pathway utilized by *Nitrospira inopinata* and a heatmap portraying calculated log2(TPM) values of pathway genes

obtained from RNAseq analysis of 3-OH-C12-HSL addition cultures compared to no-AHL addition control cultures. The genes, *accA, accB, accC; accD*, encode subunits of the protein acetyl-CoA carboxylase 2. The gene *fadH* encodes for the protein malonyl-CoA-ACP transacylase. The two *fadH* genes, NITINOP_0260 and NITINOP_1563, encode for the protein 3-oxoacyl-ACP synthase 3. The gene, NITINOP_0204, encodes for a beta ketoacyl synthase protein. The two *fabF* genes, NITINOP_0256 and NITINOP_2566, encode for the 3-oxoacyl-ACP synthase 2 protein. The gene, NITINOP_0161, encodes for an uncharacterized oxidoreductase. The five *fabG* genes, NITINOP_0192, NITINOP_0201, NITINOP_0258, NITINOP_2238; NITINOP_2516, encode for the 3-oxoacyl-ACP reductase protein. The gene, NITINOP_0208, encodes for a protein of unknown function. The *fabZ* gene encodes for a hydroxymyristol ACP dehydratase protein. The fabI gene encodes for an enoyl-ACP reductase protein. The gene, NITINOP_0743, encodes for an AMP-dependent synthetase and ligase protein. The gene, NITINOP_0933, encodes for a putative 2-succinylbenzoate—CoA ligase protein. The gene, NITINOP_1357, encodes for a putative LasR receptor protein. The gene, *lasI*, encodes for a N-acyl-L-homoserine lactone synthase protein.

The addition of 3-OH-C12-HSL did have an effect on the transcription of genes involved in fatty acid biosynthesis in *N. inopinata* when compared to controls. The transcription of two genes out of 21 genes associated with the fatty acid biosynthesis pathway in *N. inopinata* were found to be significantly differentially transcribed under AHL-addition. The first gene (*fabH)* encoded for 3-oxoacyl-acyl carrier protein synthase III and was found to be transcribed in significantly lower levels in AHL-addition cultures compared to the control ($P<0.01$). The second gene was found to encode for a putative 2-succinylbenzoate—CoA ligase and was found

to be transcribed in significantly lower levels in AHL-addition cultures compared to the control ($P<0.01$).

The addition of 3-OH-C12-HSL to *N. inopinata* cultures did not have a significant impact on two key genes involved in acyl-homoserine lactone synthesis and acyl-homoserine lactone signaling. The transcription of the gene, *lasI*, was found to be marginally impacted by the addition of 3-OH-C12-HSL in experimental cultures compared to the controls ($P=0.062$). This gene encodes for LasI, which was a protein that could synthesize acyl-homoserine lactones. A gene that encodes for a putative LasR signaling protein in *N. inopinata* was found to not be significantly differentially transcribed ($P=0.366$) when 3-OH-C12-HSL addition cultures were compared to controls.

Phospholipid Biosynthesis

The transcription of genes associated with phospholipid biosynthesis in N. inopinata were found to be significantly differentially transcribed in 3-OH-C12-HSL addition cultures when compared to controls. Five out of the 14 genes associated with the lipid biosynthesis pathway (*plsY*, *cdsA*, *pgsA*, and 2 putative genes) were found to significantly differentially transcribed in lower levels in the AHL-addition cultures compared to controls. The gene, *plsY*, was found to encode a glycerol-3-phosphate acyltransferase protein. The gene, *cdsA*, was found to encode a phosphitidate cytidylytransferase protein. The gene, *pgsA*, was found to encode a CDP-diacylglycerol – glycerol-3-phosphate 3-phosphatidyltransferase protein.

Discussion

Overall, the study of quorum sensing systems in nitrifier prokaryotes was currently lacking compared to the study of quorum sensing in heterotrophs. Genetic surveys of putative quorum sensing genes in nitrifiers have revealed that many different species of AOB, NOB, and commamox species belonging to the genus *Nitrospira* contain different putative AHL synthase and receptor gene homolog that were phylogenetically organized into five clades (25). Acyl-homoserine lactone screening studies in these nitrifiers have found many different types of AHLs produced by numerous species within four of the five clades. The other clade contains commamox *Nitrospira* species which have not displayed AHL production and are distinct from other nitrite-oxidizing *Nitrospira* species. We determined if the putative AHL genes found in the commamox species, *Nitrospira inopinata*, were functional with heterogeneous expression experiments and would induce quorum sensing transcription behaviors when grown in cultures.

Nitrospira inopinata was found to naturally produce two acyl-homoserine lactones when grown in culture media. Our heterogeneous expression work found that the *lasI* and *lasR* genes found in *N. inopinata* were functional when heterologously expressed in *Pseudomonas putida*. The mutant *P. putida* containing this *lasI* gene was found to produce 3-OH-C10-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL. Two of these acyl-homoserine lactones, 3-OH-C10-HSL and 3-OH-C14-HSL, were extracted from *N. inopinata* culture media at different time points during incubation. Unlike the study done by Melbye 2017, we decided to include heterologous expression experiments in our AHL screening of *N. inopinata* to further connect the production of AHLs to their putative AHL synthase gene homolog. Previous screens for AHL production in these nitrifiers have yielded mixed results on the relationship between known putative AHL QS genes and observed AHL production. *Nitrosomonas europaea* was observed to produce three AHL compounds (C6-HSL, C8-HSL; C10-HSL), however, no homolog to known AHL

synthases was found in its genome (26). AHL synthase and receptor genes were found *Nitrosospira multiformis*, but AHL production was not observed in culture studies. Typically, *lasI* and *luxI* type AHL synthases produce AHLs with a ketone group on the acyl chain such as 3-oxo-C12-HSL produced in *Pseudomonas aeruginosa*. However, *Pseudomonas fluorescens* was found to produce several different AHLs with a *lasI* homolog that contain a hydroxyl group on the acyl chain, similar to the AHLs extracted from *N. inopinata* cultures, and AHLs that contain no additional functional groups on the acyl chain (27, 28, 29). The production of 3-OH-Acyl-HSLs in *N. inopinata* cultures was proved possible in our heterologous expression and AHL extraction experiments, and thus our results were not anomalies. Additionally in other AHL screening studies, the presence of putative AHL QS genes led to the observation of AHL production in cultures of a few nitrifier species. The AOB species, *Nitrosospira multiformis* and *Nitrosopira briensis*, were observed to produce a single AHL, C10-HSL and 3-OH-C14-HSL, respectively. The same study also found that the NOB species, *Nitrobacter vulgaris* and *Nitrospira moscoviensis*, also produced a single AHL in pure cultures, C10:1-HSL and C8-HSL, respectively (25). Generally, nitrifier species seem to be able to produce a wide range of AHL products in the few species that have been studied and grown in pure cultures. However, the presence or absence of known AHL QS homologs in a few nitrifier species have shaken the certainty that AHL production wholly relies on known AHL QS genes. Since the putative *lasR* and *lasI* genes were found to be functional in *Nitrospira inopinata*, the effect of the addition of the three AHLs synthesized and recognized by these genes affected the growth of *N. inopinata* cultures.

The addition of a single acyl-homoserine lactone (3-OH-C10-HSL, 3-OH-C12-HSL; 3-OH-C14-HSL) into *N. inopinata* cultures was found to slow the growth rate of these cultures

compared to no-addition controls. The 3-OH-C10-HSL addition, 3-OH-C12-HSL addition, and 3-OH-C14-HSL addition cultures had doubling times (20.4 hours, 18.1 hours, and 22.4 hours) that were longer than no-addition controls (14.9 hours). The AHL-addition cultures also had slower ammonia consumption that eventually stalled in later samples while no-addition control cultures rapidly consumed ammonia to exhaustion. Nitrite was accumulated in lower concentrations in AHL-addition cultures and was slowly consumed compared to the no-addition control cultures that rapidly consumed nitrite after ammonia concentrations were low. However, the addition of other AHLs (C6-HSL, C8-HSL, and C12-HSL) to anoxic sludge samples from wastewater treatment plants was found to aid in the enrichment of commamox *Nitrospira* in those samples and increase the growth rate of the enriched commamox *Nitrospira*. (30). The AHLs used in that study are different than the AHLs used in our growth experiments. The growth conditions used in that study also differed in growth conditions compared to our cultures. C6-HSL, C8-HSL, and C12-HSL do not contain hydroxyl functional groups in their acyl chain group. The three AHLs that were found to be produced by *lasI* in *N. inopinata*, 3-OH-C10-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL, do contain a hydroxyl functional group in their acyl chain group. Our data shows that *N. inopinata* can naturally produce 3-OH-C10-HSL and 3-OH-C14-HSL in cultures, so these two AHLs may be utilized by *Nitrospira inopinata* to regulate quorum sensing behaviors on the species level. 3-OH-C14-HSL could act as the cell concentration indicator for the *N. inopinata* population, and if the levels of this AHL are high enough, the *N. inopinata* population would undergo quorum sensing behaviors that slow growth due to too many local *N. inopinata* cells to possibly conserve resources due to increased competition. The 3-OH-C10-HSL likely was also a quorum sensing signal to *N. inopinata* cells to slow their growth as well. This was likely since 3-OH-C10-HSL was extracted from *N. inopinata* cultures

at a time point when ammonia was exhausted, and nitrite levels were also low after nitrite was utilized as the sole substrate for energy production. Further, *Nitrospira inopinata* can struggle to oxidize nitrite to nitrate without external sources of reductants and can only utilize ammonia for nitrogen assimilation for cellular growth and biosynthesis. *Nitrobacter winogradskyi*, a nitrite oxidizing bacteria, quorum sensing induction with AHL addition slowed growth as well (3). This supports that the addition of recognized AHLs could lead to a quorum sensing response that slows growth once activated. AHL quenching and AHL addition experiments with nitrifiers have shown growth responses to these signaling molecules. *Nitrobacter winogradskyi*, a NOB, was found to regulate growth rate through a decrease in transcription of nitrogen assimilation and energy production genes after AHL addition and AHL quenching experiments (3). The addition of the following AHLs with unhydrolyzed acyl chains, C6-HSL, C8-HSL; C12-HSL, into wastewater treatment plant sludge samples was observed to enrich and increase the growth rate of commamox *Nitrospira* species contained within the sludge samples (30). Although the production of AHLs with unhydrolyzed acyl chains have not been confirmed in commamox *Nitrospira* species, comammox *Nitrospira* seem to be boosted by the presence of a few of these unhydrolyzed AHLs. This reaction was different from other studied nitrifiers such as *Nitrobacter winogradskyi* which slowed growth in the presence of the unhydrolyzed AHLs.

Nitrospira inopinata was affected by the addition of one of the three acyl-homoserine lactones discovered to be produced by the putative AHL synthase encoded within its genome. The addition of 3-OH-C12-HSL into *N. inopinata* culture media caused numerous genes within several pathways to be significantly transcribed compared to the genes in no-addition control cultures. The reductive TCA cycle, fatty acid biosynthesis, and gluconeogenesis pathways were downregulated in no-addition controls due to decreases in gene transcription. The electron

transport chain was also downregulated, but the commamox nitrification pathway was not found to be up or downregulated. The downregulation of these pathways did support the observations seen in the AHL-addition growth experiments where the growth of the AHL-addition cultures portrayed slower growth rates. However, the lack of significant differential transcription in genes in the nitrification pathway does not support the observed slow growth rate in AHL-addition cultures. Also, the GOGAT pathway that was utilized by *N. inopinata* to assimilate ammonia for biological nitrogen was not significantly affected. In *Nitrobacter winogradskyi*, AHL addition was shown to arrest growth and genes associated with nitrogen assimilation were significantly downregulated in AHL addition cultures (3). Posttranslational modification and protein cycling could be a level of regulation that affects the arrest of ammonia and nitrite utilization in the AHL-addition conditions. Putative pup ligase genes were found to be upregulated in 3-OH-C12-HSL addition cultures. The pup ligase system tags the C-terminal of target proteins with the ligation of the prokaryotic ubiquitin-like protein (Pup). Pupylation found in prokaryotes was also analogous to the eukaryotic protein modification pathway that employs ubiquitin to tag proteins. The pupylation pathway was found in bacteria species belonging to groups such as Actinobacteria and *Nitrospira* (18). Typically, in Actinobacteria such as *Mycobacterium*, pupylation tags a protein to be degraded by proteases which could allow for the recycling and regulation of targeted proteins (18). It was previously discovered that pupylation in *Mycobacterium smegmatis* had a significant impact on the concentration of certain groups of proteins under prolonged nitrogen starvation such as proteins involved in nitrogen assimilation (19). It may be possible that quorum sensing inducement allows for the activation of pup ligase protein regulation. This protein-level regulation would not be observable with transcriptomic analysis. 3-OH-C10-HSL addition conditions and 3-OH-C14-HSL addition conditions did not

have significant effects on the transcription levels of any gene in *N. inopinata* compared to the no-addition controls. However, the notion that the 3-OH-C12-HSL addition was the only condition to significantly affect the transcription of genes in *N. inopinata* could be explained. In the AHL extraction and quantification experiment performed on no-AHL-addition *Nitrospira inopinata* cultures, 3-OH-C10-HSL and 3-OH-C14-HSL were extracted from *N. inopinata* culture samples taken at instances later in the growth phase pattern of *N. inopinata*. The instance in the growth phase pattern that the *N. inopinata* cells were harvested from the no-AHL-addition control cultures for the RNAseq experiment was similar to the "T2" sampling instance for the AHL extraction experiment. It was possible that in the RNAseq experiment the no-addition control cultures contained naturally produced 3-OH-C14-HSL and potentially some level of 3-OH-C10-HSL since it was not precisely known when *N. inopinata* began to produce the 3-OH-C10-HSL extracted in AHL extraction samples at the "T3" instance. Naturally produced levels of these two AHLs could mean that the no-addition control *N. inopinata* cells were already recognizing and responding to these AHL signals with changes in their transcription levels. This would lead to the no-addition control cultures and the two naturally produced AHL-addition cultures to have more similar transcription behaviors and less significant changes in transcription compared to the 3-OH-C12-HSL addition cultures since 3-OH-C12-HSL was yet to be seen produced in *N. inopinata* cultures.

The AHL-mediated quorum sensing system in *N. inopinata* that was studied with our experiments does seem to be functional. The three AHLs that were studied in our experiments did have significant effects on growth rate although RNAseq data was not significantly different between 3-OH-C10-HSL addition, 3-OH-C14-HSL addition, and no-addition control cultures. The two AHLs, 3-OH-C10-HSL and 3-OH-C14-HSL, most likely were utilized by *Nitrospira*

inopinata to regulate species-level quorum sensing behaviors. 3-OH-C14-HSL most likely served as the typical QS signal that was always produced and increased as cell populations increased. 3-OH-C10-HSL would likely serve as a QS signal that alerts *N. inopinata* cells to depleted ammonia concentrations and/or to adapt their energy metabolism to nitrite only conditions. The role of 3-OH-C12-HSL to *N. inopinata* quorum sensing seems to be unknown. 3-OH-C12-HSL was not found to be produced by *N. inopinata* cultures, yet the addition of this AHL slowed the growth of *N. inopinata*. However, our understanding of acyl homoserine lactone quorum sensing relied on controlled laboratory experiments with single species cultures. In the environment, *Nitrospira inopinata* exists amongst numerous other bacterial species with wide variation in metabolic strategies and acyl homoserine lactones produced as signaling molecules with constantly changing growth conditions. Therefore, quorum sensing responses in *Nitrospira inopinata* were likely also constantly changing, especially as cross-talk could have the potential to occur as AHLs produced by other bacteria could affect *Nitrospira inopinata*. The importance of quorum sensing in *Nitrospira inopinata* may be higher within and around biofilms. Bacterial populations encased in biofilm matrixes would be more isolated from the outer environment in that new substrate would be slower to reach encased biofilm pockets. Cells, waste products, and AHLs would also be slower to leave the pocket and diffuse in the environment. These conditions would allow the bacterial population to build up AHL concentrations and respond to their growing population size and dwindling substrate.

Conclusion

We have demonstrated that *Nitrospira inopinata* contains a functional quorum sensing system that relies on produced 3-OH-acyl-HSLs. More importantly, our heterogeneous expression and AHL extraction experiments have linked the production of three extracted acyl

homoserine lactones (3-OH-C10-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL) with the putative *lasI* synthase gene previously discovered in the genome of *Nitrospira inopinata*. However, only two of the three AHLs (3-OH-C10-HSL and 3-OH-C14-HSL) were observed in the growth conditions used in our *Nitrospira inopinata* cultures. We found that all three AHLs decreased the growth rate of *Nitrospira inopinata* when one AHL was added to culture media while only the addition of 3-OH-C12-HSL had any effect on the transcription of genes compared to no-addition controls. While 3-OH-C12-HSL was not observed in our cultures, all three of these AHLs are important for the inducement of quorum sensing behaviors in *Nitrospira inopinata*. AHL studies in nitrifiers such as *Nitrospira inopinata* will benefit from experiments utilizing chemostat cultures since cell concentrations can be better regulated in chemostat reactors compared to static cultures. Cell concentrations need to be high to better induce AHL production in the culture which we attempted to do by increasing the concentration of ammonia in our media from 0.5-1mM previously used by us to upkeep *Nitrospira inopinata* cultures to 5mM in these experiments. Media used in chemostat cultures can employ even higher concentrations of ammonia to accommodate higher cell populations. Further, studies on acyl homoserine lactone production and quorum sensing in nitrifiers will benefit from heterogeneous expression of AHL-related genes in non-QS heterotroph organisms. Nitrifiers grow slower than model heterotroph organisms and competence for plasmid uptake may not be easily achieved in nitrifiers. Quorum sensing in nitrifiers may generally rely on self-produced types of AHLs to negatively impact their growth to prepare for stressful conditions induced by increased competition from the high cellular population of nitrifier group. However, it was previously observed that comammox *Nitrospira* could react to positively hasten their growth when exposed to non-hydroxylated acyl group AHLs which *Nitrospira inopinata* could not produce. Therefore, comammox *Nitrospira*

AHL studies can use co-cultures of comammox *Nitrospira* paired with another bacterium that could produce other types of AHLs such as non-hydroxylated acyl group AHLs to better gauge the effects that microbial community quorum sensing signaling molecules have on comammox *Nitrospira*.

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