Quantitative PCR assay development for monitoring genes suspected of contributing to PFAS degradation by *Gordonia* sp. strain NB4-1Y

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April 2025

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BIOL 4480

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Abstract:

This study investigates the ability of *Gordonia* sp. strain NB4-1Y to degrade environmentally concerning perfluoroalkyl and polyfluoroalkyl substances, PFAS. Six genes of interest were selected as targets for reverse transcriptase quantitative digital polymerase chain reaction (dPCR) assay development. These selections were based on published transcriptome data (Bottos et al., 2020) and recently acquired proteome data (McAmmond et al., unpublished). Using the most recent, and complete, version of the NB4-1Y genome (Ony et al. 2024, GenBank: CP132196.1), forward and reverse primer pairs and dPCR probes were designed using Geneious Prime (Dotmatics, 2024) and Primer Express (Thermo Fisher Scientific, 2019) software for the six genes of interest. The primer pairs were tested and validated using a combination of end-point PCR to optimize PCR conditions and Sanger sequencing to confirm PCR amplicon identity. Once the primers were proven to amplify the correct gene regions, a time course experiment with NB4-1Y provided with PFAS (6:2 fluorotelomer sulfonic acid; 6:2 FTSA), $MgSO_4$, or no sulfur source was conducted. mRNA was extracted from cell pellets harvested during the time course experiment and purified prior to using reverse transcriptase to generate cDNA which was used for dPCR quantification. Once validated, the assays can be used to quantify mRNA levels over time when NB4-1Y is provided with various sulfur sources. If mRNA levels are higher for genes of interest in samples provided with PFAS, the hypothesis that these genes of interest contribute to the ability of NB4-1Y to metabolize PFAS will be supported.

Introduction:

Perfluoroalkyl and polyfluoroalkyl substances, PFAS, are a large group of synthetic compounds characterized by long fluorinated carbon chains, making them incredibly stable and able to easily accumulate in the environment (Bottos et al., 2020). The sulfonated PFAS, 6:2 fluorotelomer (6:2 FTSA), has been used as an ingredient in aqueous film-forming foams (AFFFs) used for hydrocarbon fire suppression (Yang et al., 2022). The prevalence of 6:2 FTSA in the environment can be attributed to the long-term use of AFFFs in firefighting and firefighting training or from the biotransformation of PFAS precursors (Yang et al., 2022). Due to their toxicity and bioaccumulation potential, researching potential strategies for PFAS degradation is incredibly important.

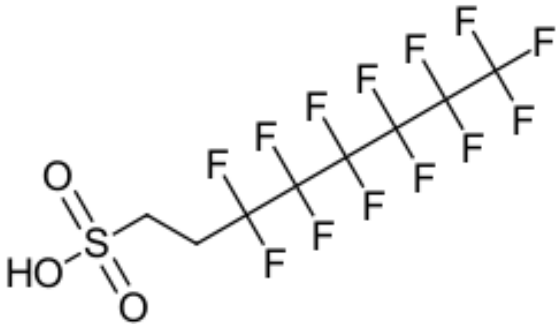


Figure 1. Chemical structure of 6:2 fluorotelomer sulfonate (6:2 FTSA), a sulfonated PFAS.

Gordonia sp. strain NB4-1Y, referred to as NB4-1Y, is an aerobic, gram-negative bacterium, originally isolated from vermicompost. NB4-1Y has been previously reported to degrade PFAS into numerous breakdown products (Shaw et al., 2019). Specifically, NB4-1Y has been reported to use 6:2 FTSA as a sulfur source for growth. Proteins that may be involved in this metabolism have been identified, (Bottos et al., 2020), and, if they are involved, the genes coding for them should be expressed more when NB4-1Y is exposed to 6:2 FTSA compared to $MgSO_4$.

In the current study six genes of interest, thought to be involved in the metabolism of 6:2 FTSA by NB4-1Y, have been selected as targets for reverse transcriptase quantitative digital polymerase chain reaction (dPCR) assay development. Target gene selections were based on previous transcriptome (Bottos et al., 2020) and proteome (McAmmond et al.,

unpublished) data. Specifically, dimethylsulfone monooxygenase *sfnG* was selected as a gene target because it may desulfonate 6:2 FTSA. Four LLM class flavin-dependent oxidoreductase genes were selected because these versatile enzymes are known to catalyze a variety of reactions including hydroxylations, epoxidations, and Baeyer–Villiger rearrangements (Maier et al., 2015). One gene coding for an acyl-CoA dehydrogenase family protein is also a target, because there has been some evidence acetyl-CoA adducts could be formed during PFAS metabolism (Mothersole et al., 2024a; Mothersole et al., 2024b). Once validated, the developed assays will be used to quantify mRNA levels over time when NB4-1Y is provided with PFAS, $MgSO_4$, and 1-octanesulfonate, a non-fluorinated structural analogue of 6:2 FTSA, as sole sources of sulfur. If the gene targets are expressed more in samples exposed to PFAS then the hypothesis that these genes of interest contribute to the ability of NB4-1Y to metabolize PFAS will be supported.

Methodology:

Primer and Probe Design:

Using the most recent, and complete, version of the NB4-1Y genome (Ony et al. 2024, GenBank: CP132196.1), forward and reverse primer pairs and dPCR probes were designed using Geneious Prime (Dotmatics, 2024) and Primer Express (Thermo Fisher Scientific, 2019) software for six genes of interest. These genes and the proteins they code for (Table 1) were chosen for primer and probe design based on previous transcriptome (Bottos et al., 2020) and proteome (McAmmond et al., unpublished) data. Primers and probes (Table 2) were designed following primer design guidelines from Thermo Fisher Scientific (Thermo Fisher Scientific, 2022; Behind The Bench, 2019). Forward and reverse primer GC content was kept between 30 % - 80 %. Primer length was kept around 20 base pairs, and melting temperature (Tm) between 58 °C - 60 °C. dPCR probes were designed to have 30 % - 80 % GC content, 13 – 25 bp lengths, and melting temperatures between 68 °C - 70 °C. For both primers and probes, a G residue on the 5’ end was avoided because having a G residue adjacent to the reporter dye may quench the reporter fluorescence (Thermo Fisher Scientific, 2022). Additionally, runs of identical nucleotides, consecutive A residues, G

residues on the 3' end, and CC dinucleotides were avoided when possible (Thermo Fisher Scientific, 2022). The amplicon lengths for all primer probe sets were between 100 and 160 bp as per design recommendations (Thermo Fisher Scientific, 2022).

Table 1. Proteins coded for by genes of interest and their corresponding accension number selected for primer and probe design.

Protein	Accension number
dimethylsulfone monooxygenase (SfnG)	Q9K23_RS05625
LLM class flavin-dependent oxidoreductase	Q9K23_RS08775
LLM class flavin-dependent oxidoreductase	Q9K23_RS08755
acyl-CoA dehydrogenase family protein	Q9K23_RS21130
LLM class flavin-dependent oxidoreductase	Q9K23_RS08670
LLM class flavin-dependent oxidoreductase	Q9K23_RS08675

Table 2. Forward and reverse primer and probe sequences with their corresponding % GC, Tm, and length.

Accession Number	Name	Primer or Probe Sequence	Tm (°C)	%GC	Length
Q9K23_RS05625	1,317,500 R	TCAACGGGTTCATCATCGC	59.0	53	19
	1,317,351 F	ACATCCCCTTCTTGTCGCC	58.5	58	19
	1,317,416 P	ACGATTCGCCTTCTCGAT	70.0	50	18
	1,317,434 R	AGAAGGCGAATCGTCCTGC	59.0	58	19
	1,317,310 F	GCACCAGGTCCTCGAAGCT	59.0	63	19
	1,317,386 P	TGCTGAACTGCGCCGC	70.0	69	16
Q9K23_RS08775	2,058,865 R	ACCTCGGTAGGGTTTTCGAAC	58.1	52	21
	2,058,716 F	GACGACAGGAACGACGAAGC	59.4	60	20
	2,058,788 P	TTGCGGTAGCTGGCA	69.0	60	15
Q9K23_RS08755	2,053,849 R	CTGAACAGATCGCCGATGC	58.9	58	19
	2,053,750 F	TGGTCGACGAACAACTCGAG	58.5	55	20
	2,053,801 P	CCCGCAACGAACCA	68.0	64	14
Q9K23_RS21130	4,878,910 R	ATCACCTGCTGCACCAACG	59.3	58	19
	4,878,758 F	GTCGTATCTGCAGCTCGTCCA	60.0	57	21
	4,878,805 P	CACTCGACGATGTCGC	70.0	63	16
Q9K23_RS08670	2,035,659 R	GAAGTTCCTGGTCGCCTTCC	59.6	60	20
	2.035, 509 F	GGTGAAGTCGCCGTACATGG	60.0	60	20
	2,035,586 P	AAGGTGCCGGCCAT	68.0	64	14
Q9K23_RS08675	2,036,528 R	GGACTGCTGATCGGCACCT	59.8	63	19
	2,036,370 F	ATGGTCTCCCGGGATTCAC	58.2	58	19
	2,036,487 P	TCGTGCAGCGCATG	69.0	64	14

Primer Validation:

Once primers and probes were designed, forward and reverse primers were ordered and received June 2024. Dehydrated primers were resuspended with PCR water to make 100 μM solutions. To make 10 μM working solutions, 1 in 10 dilutions were prepared for all primers. Primer pairs were tested and validated using a combination of end-point PCR to optimize PCR conditions and Sanger sequencing to confirm PCR amplicon identity. Trial PCRs were performed using various temperatures to determine the best melting temperatures (Tm) for each primer pair. After trial PCRs, samples were visualized using gel electrophoresis. The template DNA used was NB4-1Y genomic DNA extracted from cell pellets grown in YEME media. DNA was extracted using the DNeasy Powersoil Pro DNA Extraction Kit and quantified using a Qubit Fluorometer Broad Range DNA Kit to have a concentration of 270 ng/μL. The sample showed high molecular weight DNA when visualized using gel electrophoresis.

Primer pairs were validated by Sanger sequencing using the BigDye Terminator v 1.1 cycle sequencing kit according to manufacturers instructions (Thero Fisher Scientific, 2024). For cycle sequencing, forward primers were diluted to be 3.2 μM by mixing 3.2 μL of primer with 6.8 μL of PCR H_2O. The diluted primers were vortexed and briefly centrifuged before use. Cycle sequencing reaction components and their respective volumes are listed in Table 3. For the control treatment, control DNA, provided in the kit, was used instead of diluted DNA sample.

Table 3. Cycle sequencing reaction components and the required volumes for standard 10 μL reactions.

Component	Quantity per reaction (μL)
BigDye Terminator v1.1 Master Mix	4
Forward or reverse primer	1
PCR H_2O	4
Diluted DNA (~ 1-3 ng/μL)	1

Following cycle sequencing, the samples were purified by ethanol/EDTA precipitation as outlined in the Big Dye Terminator v1.1 Cycle Sequencing Kit User Guide (Thermo Fisher Scientific, 2024). Samples were resuspended in 10 μL of HiDye Formamide and immediately proceeded to Sanger sequencing. Sequencing results obtained were compared to the NB4-1Y genome using NCBI BLAST and Genious software.

Time Course Experiment:

Pure NB4-1Y culture stocks were maintained on Microbank beads (Pro-Lab Diagnostics, Inc., Richmond Hill, ON, Canada) and stored at - 80 °C. A single bead was streaked onto a nutrient agar plate and incubated at 30 °C until isolated colonies grew. Single isolated colonies from the nutrient agar plate were used to prepare inoculum cultures in autoclaved 50 mL glass tubes containing 20 mL sulfur-free acetate (SFA) medium, prepared according to Bottos et al., 2020, and 20 μL of 60 mM $MgSO_4$ stock solution. A sterile control was also prepared. Starter cultures were incubated on a tissue culture roller drum (New Brunswick Scientific, Enfield, CT, USA) set to 150 rotations per minute at 30 °C. Experimental cultures were prepared with 200 mL SFA media in autoclaved 500-mL Erlenmeyer flasks. All experimental treatments, 160 μM 6:2 FTSA sterile control, no sulfur source control, 160 μM 6:2 FTSA, and 160 μM $MgSO_4$, were prepared in triplicate. For experimental treatments, 533 μL of 60 mM $MgSO_4$ stock solution and 8000 μL of 4 mM 6:2 FTSA stock solution was added to respective treatment flasks to reach a final concentration of 160 μM. Experimental cultures were inoculated with 2 mL of starter cultures for a 1 % (v/v) inoculum. All flasks were plugged with foam stoppers, covered with aluminum foil, and incubated in an orbital shaker at 30 °C with 150 rotations per minute.

Optical density (OD), fluoride ion concentration, and biomass readings were taken for all samples at 12 time points over a period of 17 days. The OD readings used 200 μL of culture and were measured using a VARIOSKAN LUX at 600 nm. SFA medium was used as a blank. For fluoride readings, 2 mL of culture was centrifuged at 8200 x g for 5 min, and supernatant was mixed with 2 mL low level TISAB buffer (Thermo Fisher Scientific, 2016) for

readings, as per manufacturers instructions. The cell pellets were discarded. Fluoride readings were taken using a calibrated Thermo Scientific Orion Fluoride Ion Selective Electrode. Cell pellets were harvested following the biomass collection and RNA stabilization procedure described in Bottos et al., 2020. For biomass readings, the wet weight of NB4-1Y cell pellets were obtained using an analytical balance.

RNA Extractions:

RNA extractions were performed using NB4-1Y pellets, stored at – 80 °C, obtained from the time course experiment. One cell pellet was chosen from no sulfur, 160 μM FTSA, and 160 μM $MgSO_4$ treatments from time point 12 (07/12/24). Additionally, a 160 μM $MgSO_4$ pellet from time point 3 (06/28/24) was chosen to compare mRNA concentrations from two time points. During the time course experiment, the biomass of $MgSO_4$ pellets peaked earlier in the experiment than 6:2 FTSA samples, so it was hypothesized more mRNA could be extracted from the $MgSO_4$ pellet taken from the earlier point. RNA extractions were performed following the RNA extraction protocol outlined in Bottos et al., 2020 and the Qiagen RNeasy Minikit Protocol (RNeasy Mini Kit. ID: 74104). To minimize genomic DNA contamination two optional on-column DNase treatments were performed, one during RNA extraction and one immediately following RNA extraction. Multiple aseptic strategies were used during the extraction procedure to try and minimize DNA contamination such as performing most of the protocol in a biosafety cabinet and spraying the workspace down with RNAse zap spray. Qubit High Sensitivity RNA and DNA kits were used to determine DNA and RNA concentrations after RNA extraction.

To confirm the absence of DNA contamination, two end-point PCR experiments were carried out. One with 16S rRNA gene primers (27F and 1492R) and a second with *snfG* primers (1,317,310F and 1,317,434R). Each experiment had a set of six samples: no sulfur source RNA, 160 μM FTSA RNA, 160 μM $MgSO_4$ RNA (from timepoint 3), negative control, positive control with NB4-1Y genomic DNA, and another positive control with 160 μM FTSA RNA spiked with NB4-1Y genomic DNA. All reactions had a total volume of 20 μL. For the

16S rRNA gene PCR the cycle sequencing conditions were as follows: (1) polymerase activation 95 °C for 4 min, (2) 35 cycles of denaturation 95 °C for 30 s, annealing 53.4 °C for 45 s, and extension 72 °C for 2 min, (3) 1 cycle of extension 72 °C for 5 min and a 10 °C hold. For the *snfG* PCR, the same cycle sequencing conditions were used, but the annealing temperature was adjusted to 60 °C. Following PCR, amplicons were separated on a 2% agarose gel with a 0.75 cm thickness at 70 volts for 70 minutes.

cDNA Synthesis with Reverse Transcriptase:

To synthesize cDNA from extracted RNA samples, the reverse transcription protocol for SuperScript IV VILO Master Mix with ezDNase enzyme was followed (Invitrogen, 2016). RNA samples were taken from no sulfur source, 160 μM 6:2 FTSA, and 160 μM $MgSO_4$ treatments to produce cDNA. A no reverse transcriptase (NRT) control was also prepared using the SuperScript IV VILO No RT Control mix (Invitrogen, 2016). Quantification and visualization of cDNA samples was performed to confirm successful cDNA synthesis using Qubit Fluorometer High Sensitivity DNA kit and gel electrophoresis, respectively.

Quantitative PCR (qPCR):

Multiple real-time quantitative PCR (qPCR) experiments were performed to test the performance of individual and duplexed custom TaqMan Gene Expression assays using TaqMan Fast Advanced Master Mix and following real-time PCR protocol from TaqMan Fast Advanced Master Mix User Guide (Thermo Fisher Scientific, 2021). The qPCR thermocycling conditions were as follows: (1) polymerase activation 95 °C for 20 s, (2) 40 cycles of denaturation 95 °C for 1 s and annealing 60 °C for 20 s.

Digital PCR (dPCR):

A quantitative digital polymerase chain reaction (dPCR) experiment was carried out with cDNA and genomic DNA samples. This experiment was done to test the performance of individual and duplexed custom assays, and to investigate whether initial quantitative data

would support the hypothesis that genes of interest would be more expressed in NB4-1Y samples provided with 6:2 FTSA. The dPCR experiment was performed following QuantStudio Absolute Q DNA Digital PCR Master Mix quick start guide procedure (Thermo Fisher Scientific, 2020). The dPCR thermocycling conditions were as follows: (1) PCR preheat 96 °C for 10 min, (2) 40 cycles of denaturation 96 °C for 5 s and annealing 60 °C for 15 s.

Results:

Primer Validation:

End-point PCR was used to determine optimal melting temperatures (Tm) for each primer pair of interest (Table 4). Following that, amplicons from each reaction were sequenced using Sanger chemistry, and the sequences (Table 4) were compared to the NB4-1Y genome. Alignments, (e.g., Figure 2), showed all seven primer pairs successfully amplified the proper gene product.

Table 4. Sanger sequencing results and optimal PCR melting temperatures (Tm) for each primer pair targeting a specific gene identified by accession number.

Accession Number	Sanger Sequencing Result	Optimal Tm (°C)
Q9K23_RS05625	TTCTCGATGATCTCGCGGRGGGKGTCGSGGGCCTCGGCCTCGGKGTCGCGGGCGATGATRAACCCGTTG	61
Q9K23_RS05625	CCGGTGGCACTGCCCGCCTGCTGAACTGCGCCGCGGAACCCCTCCACCGCAGGACGATTCGCCTT	60
Q9K23_RS08775	GCGGGTCACGAATATCGTTGCGGTAGCTGGCAGGRGTCGCARAATTGTCGGCAAARAARACRAAGTCCAWTTTCGSSCGTTCRAAAMCCCTA	62
Q9K23_RS08755	GAACCAGTCCTCGAKGGCATCGGSGATYKGTTMAG	56
Q9K23_RS21130	GATGTCGCCGGCTGGGKGCGGGCCCGCACCCGCACCTTCACCCACGCCGCAGCCGACCTGCCGCGCGAGGATCCGTTGGTGCAGCAGGTGATA	61
Q9K23_RS08670	CGTTGAGCAGCAGCCGCCCGCCGGACAGG	62

	TTCTGGAAGGTGCCGGCCATYTGCGCGGCG ACGAACGGCGCGGTCATGCCGGGACGGAA GGCGACCAGGAACTTA	
Q9K23_RS08675	GCCGGCCGGTCTGCGACCGGGTGCGTTCC CATTCGGCCCGGTAGGCATCGATCTTGGCCC GCTGGTCGTGCAGCGCATGGTCGGCGAAGG TGCCGATCAGCAGT	61

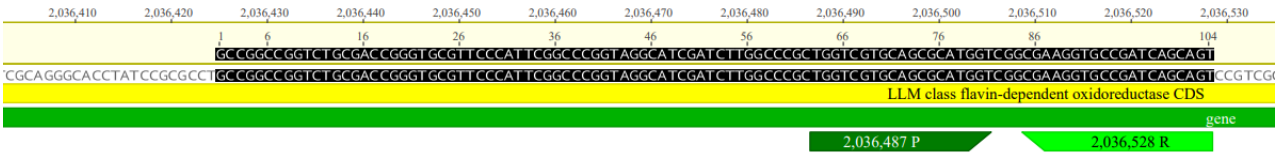
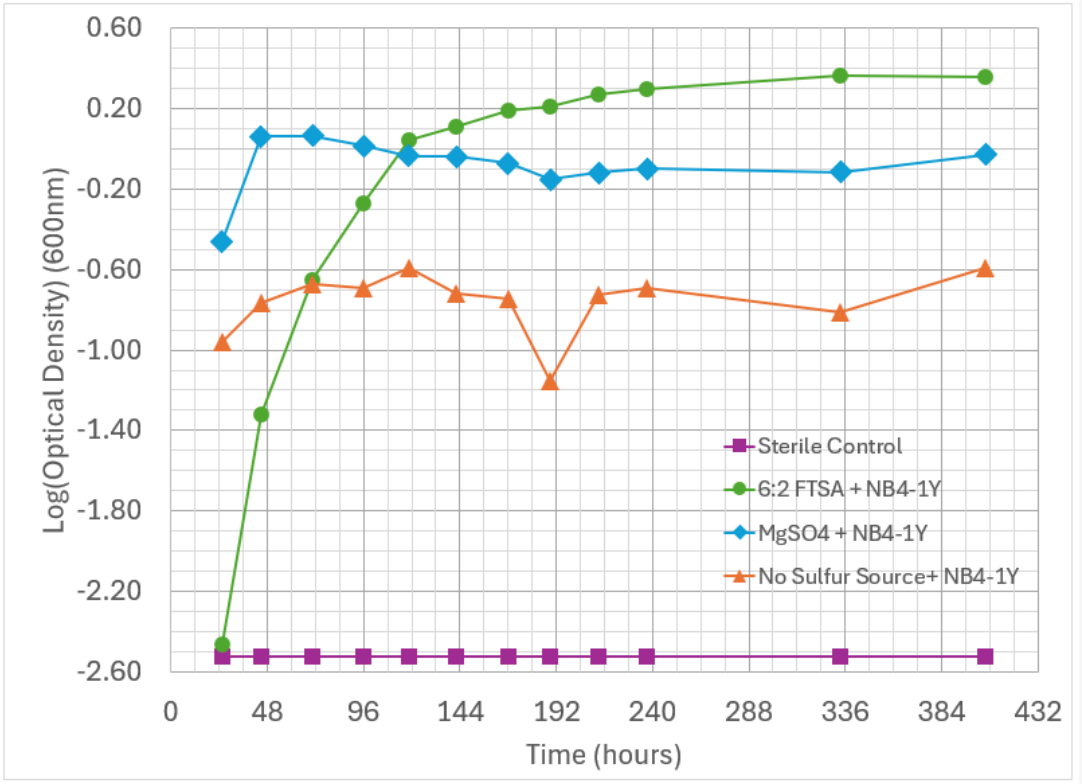


Figure 2. Alignment of Sanger sequencing results (top) with NB4-1Y genome (bottom) displaying 100 % PCR amplicon identity, indicating successful amplification of target gene coding for LLM class flavin-dependent oxidoreductase, Q9K23_RS08675 (Dotmatics, 2024).

Time Course Experiment:

To observe the kinetics of NB4-1Y growth using 6:2 FSTA or $MgSO_4$ as sole sulfur source, a time course experiment was carried out in triplicate. At each time point, OD 600 nm, fluoride, and biomass wet weight was measured (Figures 3-5). To observe bacterial growth of NB4-1Y cultures, optical density was measured using a VARIOSKAN LUX at 600 nm. All three experimental treatments, 6:2 FTSA, $MgSO_4$, and no sulfur, were in exponential phase between 24 and 48 hours. Sterile controls exhibited no increase in OD 600 nm throughout the experiment and the no sulfur source control reached a maximum OD 600 nm of 0.26. The 6:2 FTSA treatment reached a maximum OD 600 nm of 2.31 after 333 hrs. The $MgSO_4$ treatment reached a maximum OD 600 nm of 1.16 much sooner after 70 hrs.



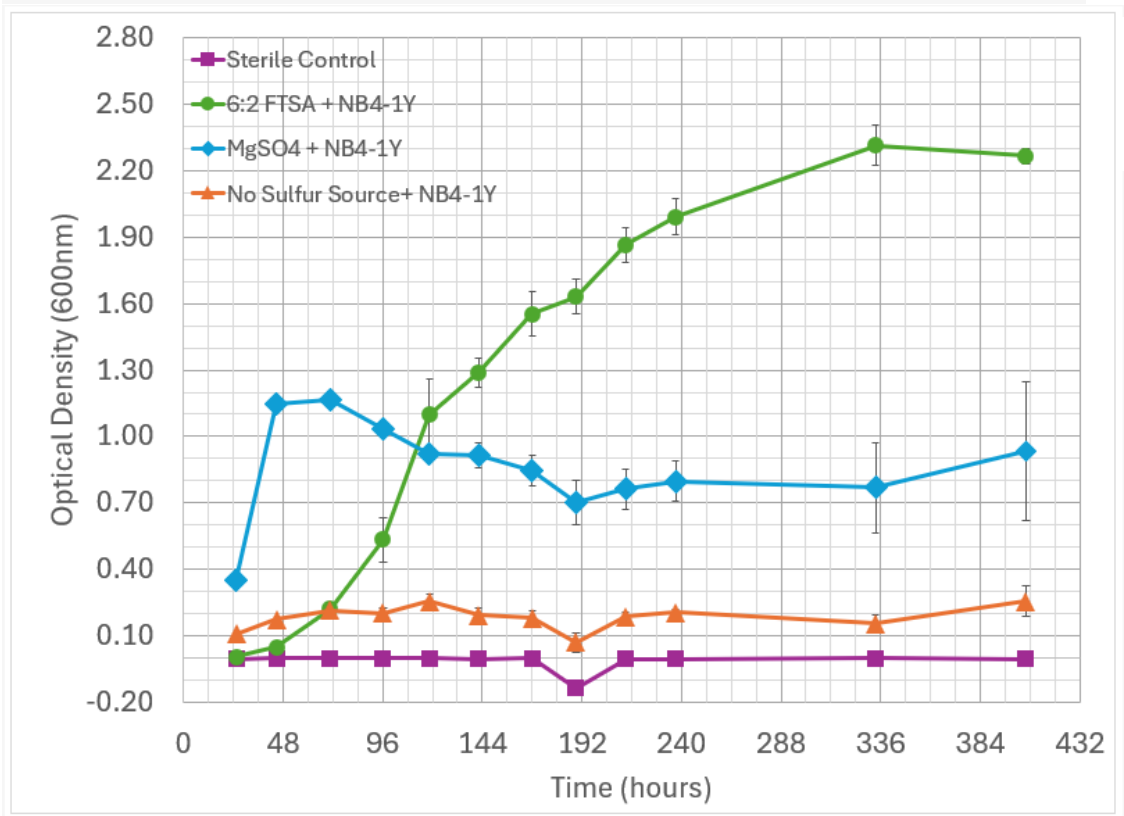


Figure 3. Growth, monitored using OD 600 nm, of NB4-1Y provided with 6:2 FTSA or $MgSO_4$ as sulfur sources. Graph A represents OD data using log scale and graph B represents OD data using linear scale (n = 3; error bars represent standard deviation).

Fluoride readings were taken using a fluoride ion selective electrode to observe free fluoride ion concentration increase as NB4-1Y metabolized 6:2 FTSA. Free fluoride ion concentrations were plotted over time as averages of each sample condition in triplicate (Figure 4). Only NB4-1Y samples provided with 6:2 FTSA as a source of sulfur showed an increase in free fluoride ion concentration over time. All other treatments maintained low free fluoride ion concentration.

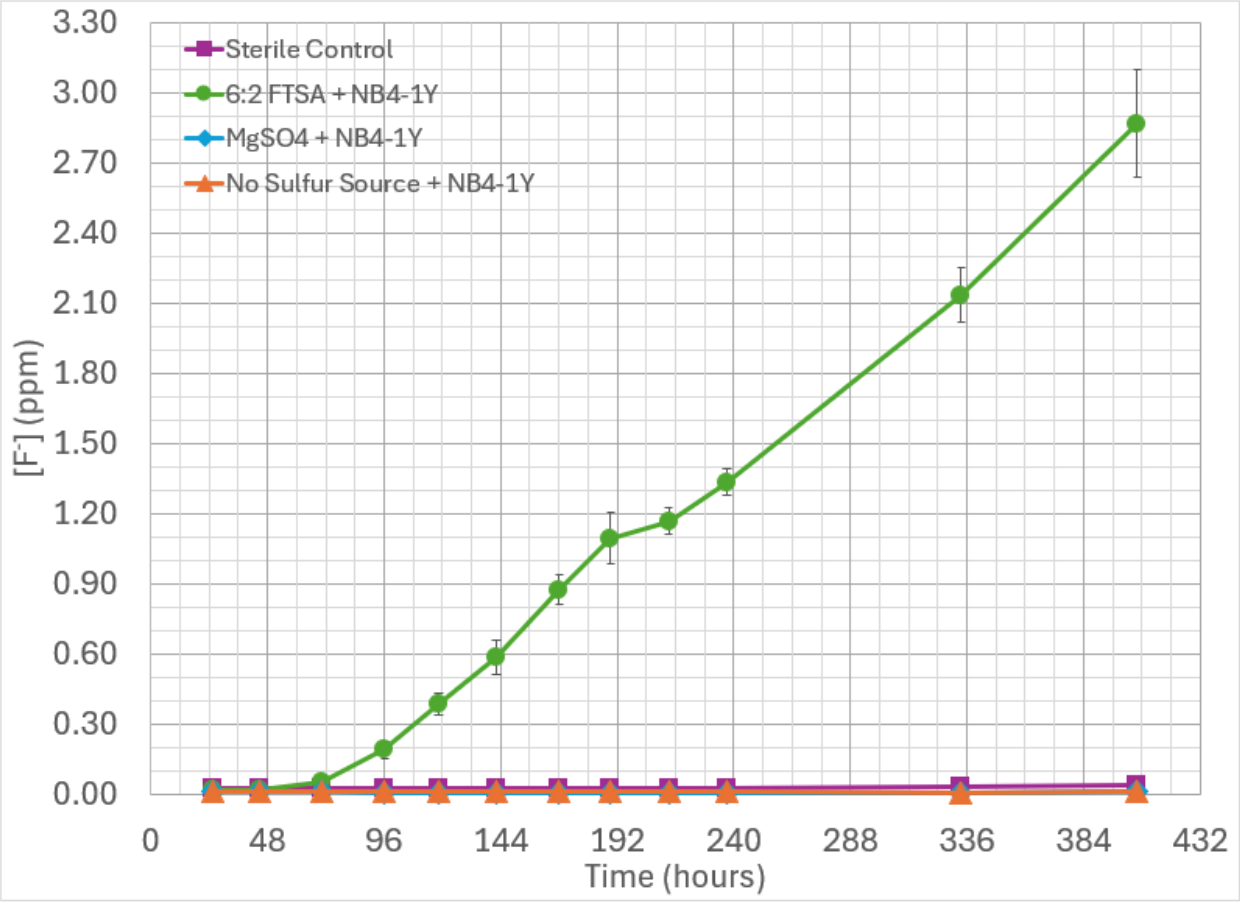


Figure 4. Fluoride release in NB4-1Y cultures provided with 6:2 FTSA or $MgSO_4$ as sulfur source (n = 3; error bars represent standard deviation).

To monitor bacterial growth, wet weight was measured using an analytical balance to quickly assess the biomass, in g/L, for all experimental treatments. Biomass values were plotted over time as averages of each sample condition in triplicate (Figure 5). NB4-1Y samples provided with 6:2 FTSA and $MgSO_4$ reached a maximum biomass after 213 hrs of

43.56 g/L and 29.11 g/L respectively. Both sterile and no sulfur source controls reached a maximum biomass of 17.11 g/L after 189 hrs. Throughout the experiment, wet weight measurements for all treatments were irregular with higher error than OD 600 nm and fluoride measurements.

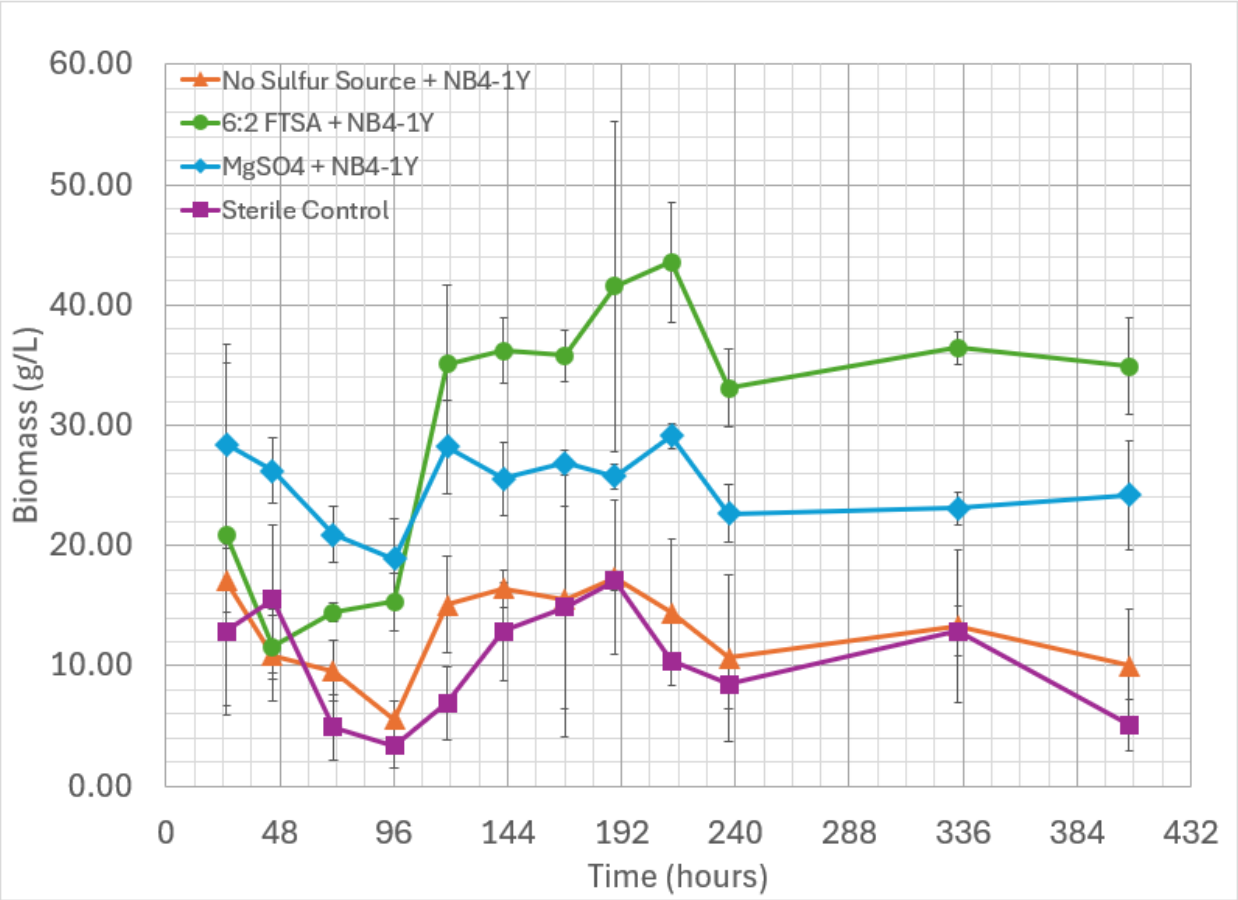


Figure 5. Growth, monitored using biomass, of NB4-1Y provided with 6:2 FTSA or $MgSO_4$ as sulfur sources (n = 3; error bars represent standard deviation).

RNA Extractions:

To obtain sufficient RNA samples for downstream qPCR and dPCR applications, RNA was extracted from NB4-1Y pellets from the time course experiment. Two DNase treatments and an RNA cleanup were performed to minimize genomic DNA contamination. DNA and RNA was quantified for all samples (Table 5). To confirm absence of genomic DNA contamination, RNA samples were visualized using gel electrophoresis after end-point PCR

using primers designed to amplify the *snfG* gene of interest and a reference 16S rRNA gene (Figure 6). As expected, in the positive controls for the 16S rRNA gene and *snfG* gene, amplicons were observed at 1500 bp and 150 bp respectively. No DNA contamination was observed in RNA samples as confirmed by the absence of 16S rRNA gene and *snfG* gene amplicons. There was primer dimer present in RNA samples shorter than 100 bp as indicated in Figure 6.

Table 5. DNA and RNA concentrations of RNA extracted from NB4-1Y samples provided with no sulfur source, 6:2 FTSA, or $MgSO_4$ quantified using Qubit high sensitivity DNA and RNA kits. Samples A, B, and C were taken from time point 12 (07/12/24), and sample D was taken from time point 3 (06/28/24)

Sample	**RNA concentration (ng/μL)**	**DNA Concentration (ng/μL)**
(A) No sulfur	< 0.02	<0.0005
(B) 160 uM FTSA	47.4	6.17
(C) 160 uM $MgSO_4$	< 0.02	<0.0005
(D) 160 uM $MgSO_4$	22.8	0.859

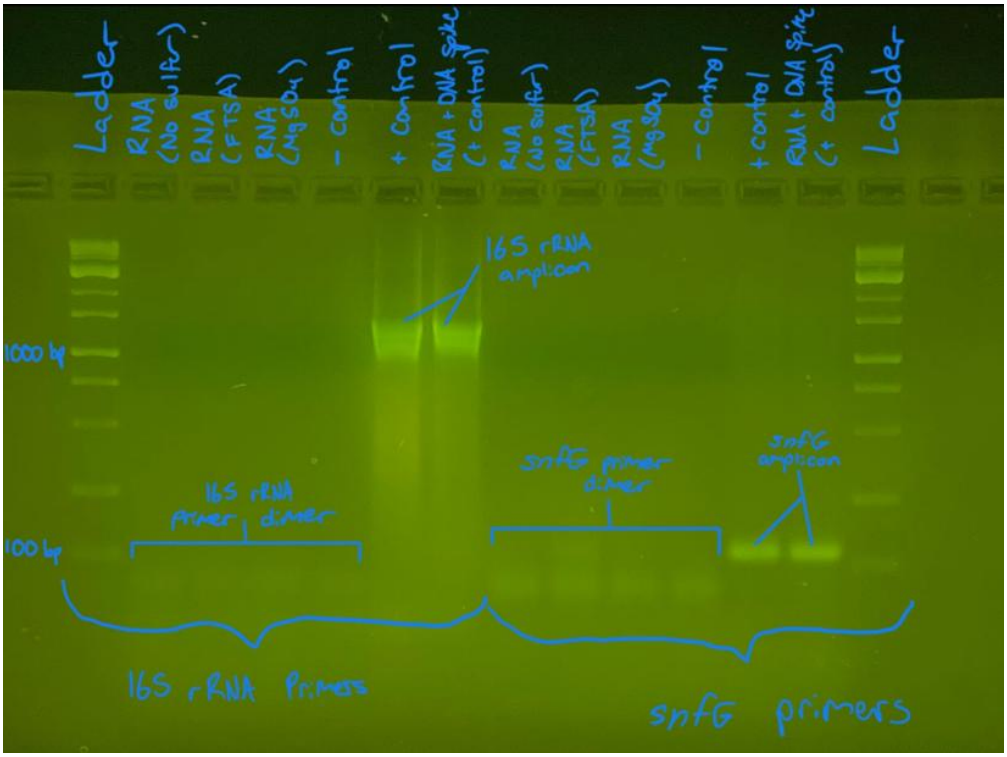


Figure 6. Verification of 16S rRNA gene and *snfG* gene absence, using end-point PCR, in NB4-1Y RNA samples visualized using 2 % agarose gel electrophoresis (70 volts for 70 min).

cDNA Synthesis with Reverse Transcriptase:

To quantify gene expression using qPCR and dPCR, RNA samples were converted to cDNA with reverse transcriptase. Using the RNA samples described above, cDNA was generated using SuperScript IV VILO master mix with ezDNase enzyme. To confirm successful cDNA synthesis, end-point PCR using 16S rRNA gene and *snfG* primers was carried out. Both primer sets amplified their targets as determined by examining amplicon sizes (1500 and 150 bp, respectively) using agarose gel electrophoresis (Figure 7). No cDNA was present in negative and no reverse transcriptase (NRT) controls, however there was some expected *snfG* and 16S rRNA primer dimer.

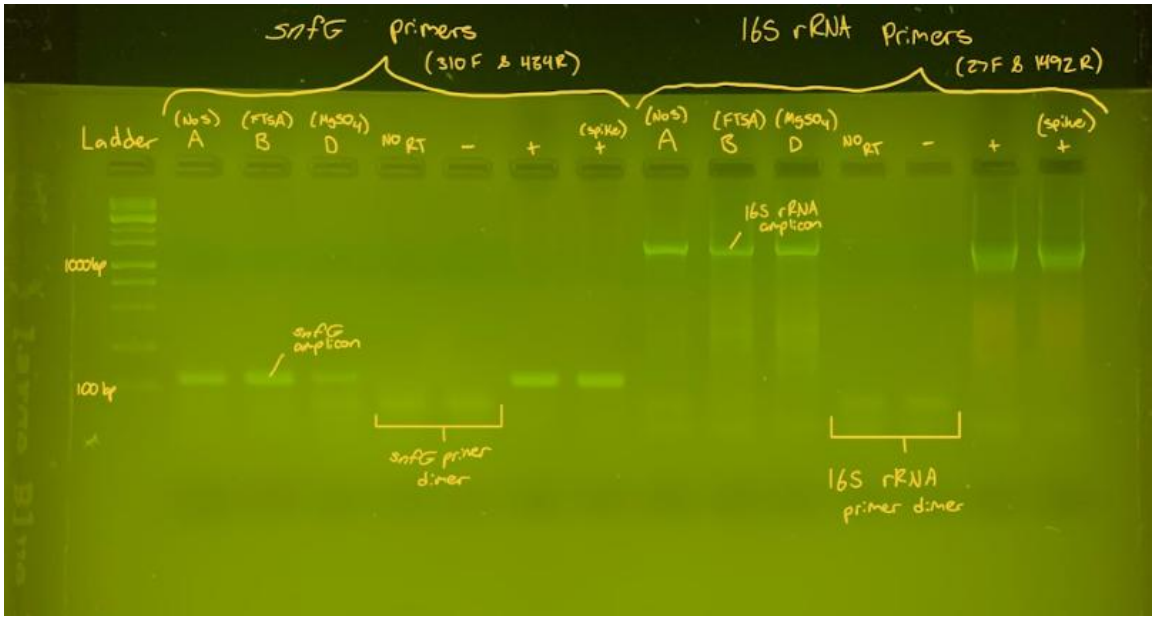


Figure 7: Amplification of *snfG* and 16S rRNA gene fragments from NB4-1Y cDNA using PCR visualized using 2 % agarose gel electrophoresis (70 volts for 70 min).

Quantitative PCR (qPCR):

To evaluate the performance of custom TaqMan assays, quantitative PCR (qPCR) experiments were carried out using both singleplex and duplex reactions. To validate duplex reactions, diluted NB4-1Y genomic DNA was used and the Ct values of singleplex and duplex reactions were compared for each assay. A difference of 0.5 to 1 Ct cycle was considered insignificant and indicated a successful duplex reaction (Bustin et al., 2009). The dimethylsulfonne monooxygenase (*snfG*) assay and the LLM flavin-dependent oxidoreductase (RS08775) assay had a difference in Ct values between singleplex and duplex reactions of 0.533 or less. When the two LLM flavin-dependent oxidoreductase assays were duplexed, the difference between singleplex and duplex Ct values was 0.557 or less. Therefore, the custom TaqMan assays were successfully duplexed without significant change to the Ct values. To quantify the expression of target genes in NB4-1Y cDNA samples, a standard curve was produced using NB4-1Y genomic DNA (Figure 8). 1/Ct values were calculated and plotted against increasing log(DNA content) in ng to produce the standard curve. The R^2 values for each assay ranged from 0.9748 to 0.9818 for

methylsulfone monooxygenase (*snfG*) and LLM class flavin-dependent oxidoreductase (RS08755) targets, respectively. Amplicon content in ng in NB4-1Y cDNA samples was then interpolated using the standard curve and equations of the lines. The calculated amplicon content (Table 6) indicated an increase in the expression of genes of interest in NB4-1Y samples provided with 6:2 FTSA as its sole sulfur source which supported the hypothesis that these genes of interest are involved in PFAS metabolism.

Table 6. cDNA content in NB4-1Y samples exposed to different sulfur sources calculated using qPCR standard curves.

	dimethylsulfone monooxygenase (*snfG*) (RS05625)			LLM class flavin-dependent oxidoreductase (RS08755)			LLM class flavin-dependent oxidoreductase (RS08775)		
Sulfur Source	no sulfur	6:2 FTSA	$MgSO_4$	no sulfur	6:2 FTSA	$MgSO_4$	no sulfur	6:2 FTSA	$MgSO_4$
Ct value	28.985	26.523	34.604	28.399	25.102	29.273	25.206	21.497	27.935
Amplicon content (ng)	0.0017	0.0076	0.0001	0.0061	0.0618	0.0036	0.1751	9.0060	0.0188

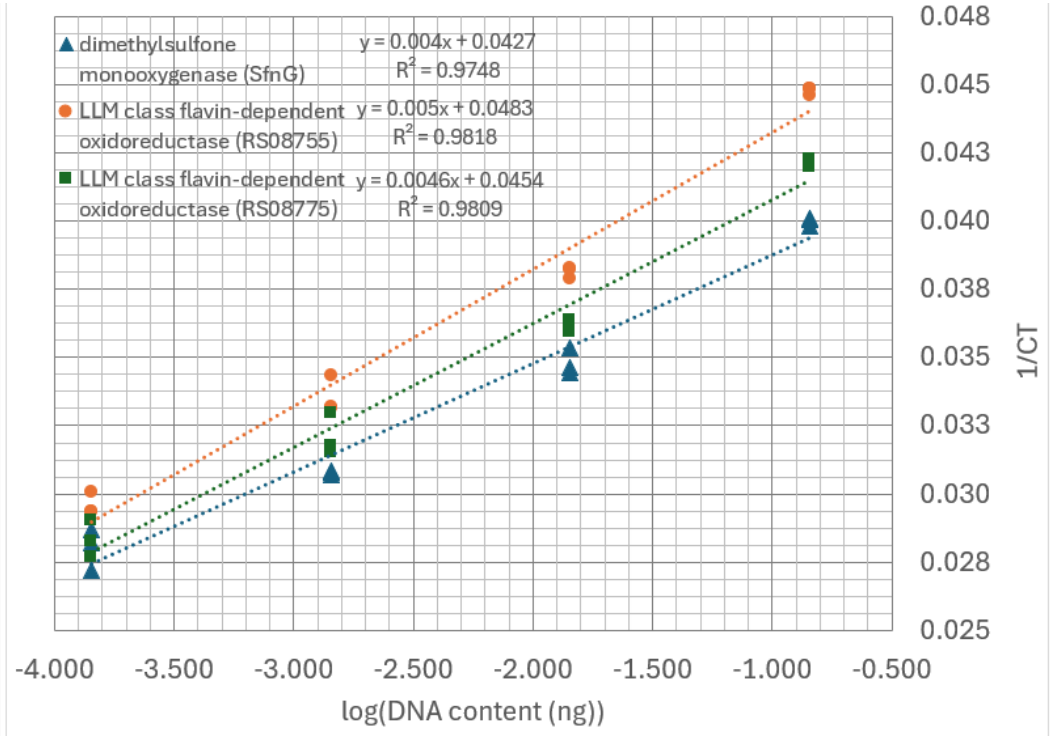


Figure 8. qPCR standard curves generated using independent (dimethylsulfone monooxygenases (*snfG*) and duplexed (LLM class flavin-dependent oxidoreductases) assays displaying 1/CT values as log(DNA content in ng) of NB4-1Y genomic DNA increases.

Digital PCR (dPCR):

To quantify gene expression of target genes and to test the performance of individual and duplexed custom assays, a quantitative digital polymerase chain reaction (dPCR) experiment was carried out with NB4-1Y cDNA and genomic DNA samples. PCR amplification of 9 μL of reaction mix spread across 20,480 fixed array microchambers resulted in numbers of positive wells and negative wells calculated by dPCR software. The software set a threshold of fluorescence that determined whether a well was considered positive or not (Figure 9). Digital PCR instrument output, presented as gene copies/μL, was used to plot gene expression levels (Figure 10) for three genes of interest coding for dimethylsulfone monooxygenase (*snfG*) and two LLM class flavin-dependent oxidoreductases, RS08755 and RS08775. As was observed in qPCR assays, expression of these genes was higher in NB4-1Y samples exposed to 6:2 FTSA compared to $MgSO_4$ and no sulfur source controls. These data further support the successful development of a quantification method for mRNA levels using qPCR and dPCR. These results also support the hypothesis that these genes, suspected of being involved in 6:2 FTSA metabolism, may be upregulated in NB4-1Y samples using 6:2 FTSA for sulfur.

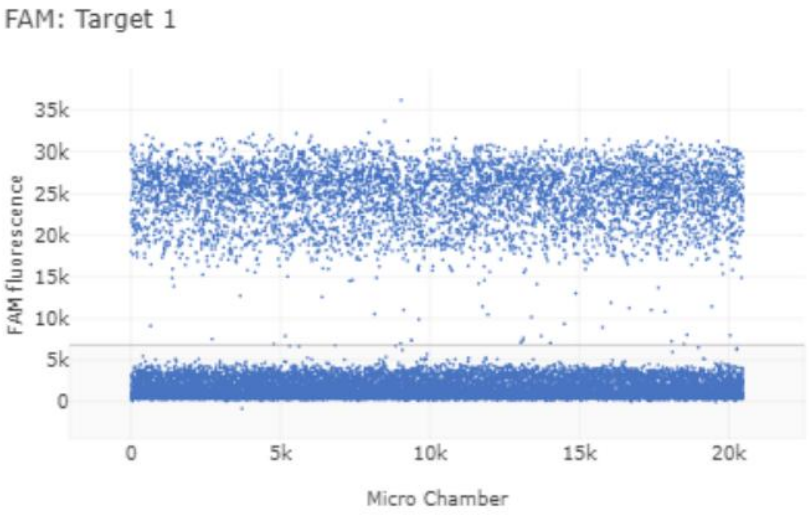


Figure 9. Cluster plot showing positive and negative reads of 6:2 FTSA NB4-1Y sample for LLM class flavin-dependent oxidoreductase gene target, RS08755. Grey line indicates a threashhold set by dPCR sofware defining the fluorecence level required to be considered positive.

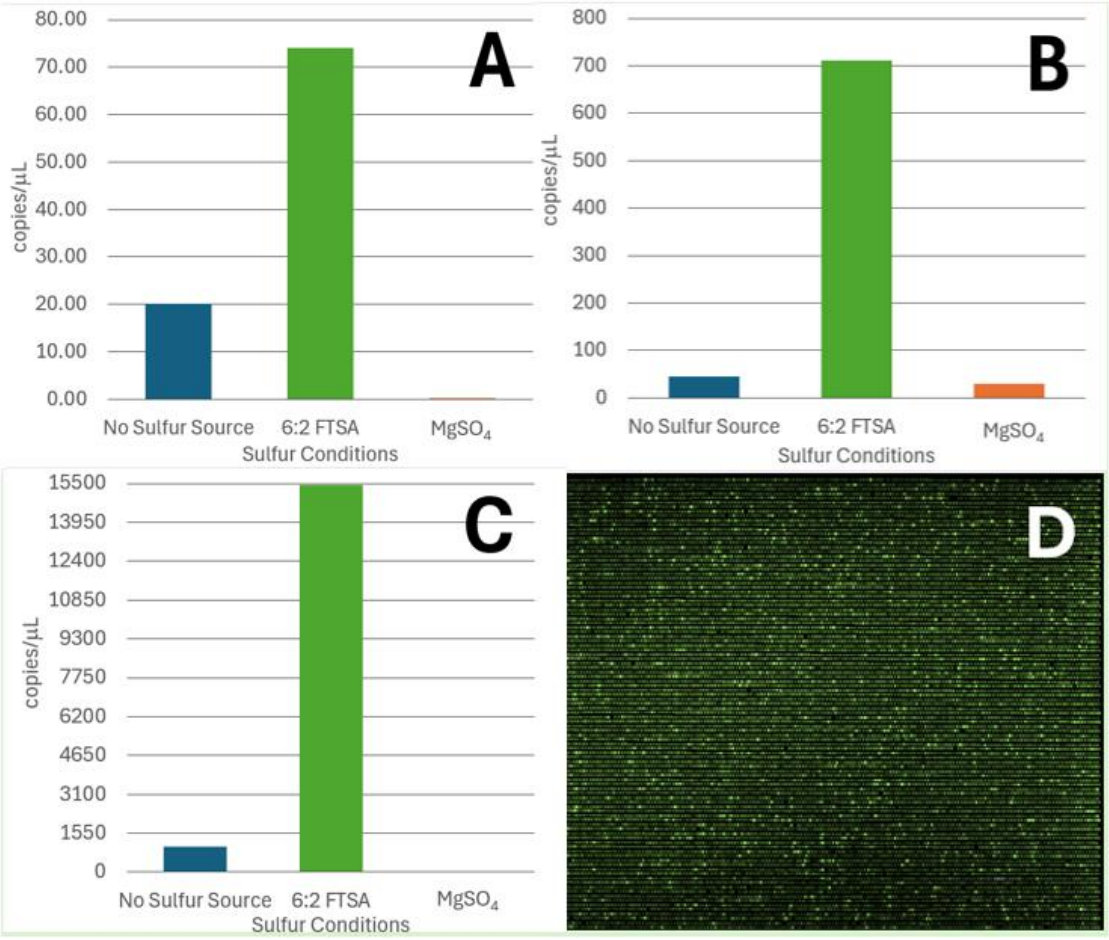


Figure 10. Quantification of target genes (A) dimethylsulfone monooxygenase (*snfG*), (B) LLM class flavin-dependent oxidoreductase (RS08755), and (C) LLM class flavin-dependent oxidoreductase (RS08775) from cDNA generated from NB4-1Y mRNA using chip digital PCR. (D) dPCR reaction unit with 20,480 fixed array microchambers with a total volume of 9 µL. Green spots indicate presence of target gene.

Discussion:

In the current study, the development of a method to quantify mRNA levels for genes of interest using real time quantitative PCR (qPCR) and chip digital PCR (dPCR) was investigated. For quantifying RNA, reverse transcriptase real-time quantitative and chip digital PCR have become established techniques for gene expression analysis due to their ability to quantify cDNA quickly and accurately (Dheda et al., 2005; Niu et al., 2021). In this study, PCR, Sanger sequencing, qPCR, and dPCR results confirmed the successful

development of a method for the quantification of mRNA levels for genes thought to be involved in 6:2 FTSA metabolism in NB4-1Y. Higher gene expression levels in NB4-1Y samples given 6:2 FTSA as their sole source of sulfur were observed compared to $MgSO_4$ and no sulfur source controls. These findings support the hypothesis that these genes coding for the enzymes dimethylsulfone monooxygenase (*sfnG*) and LLM class flavin-dependent oxidoreductases may be involved in 6:2 FTSA metabolism by NB4-1Y.

Desulfonation is thought to be a rate limiting step in the biotransformation of 6:2 FTSA (Yang et al., 2022). Some genes of interest in this study, coding for dimethylsulfone monooxygenase (*sfnG*) and LLM class flavin-dependent oxidoreductases, were chosen based on their suspected role in the desulfonation of 6:2 FTSA by NB4-1Y. An acyl-CoA dehydrogenase family protein, although not thought to be involved in desulfonation, was also picked as a gene target because it is thought to be involved in later steps of 6:2 FTSA metabolism. These genes are thought to be involved based on previously acquired transcriptome data (Bottos et al., 2020), and proteome data (McAmmond et al., unpublished; Van Hamme et al., 2013). Previous studies have also suspected monooxygenases to be involved in the biotransformation of ether PFAS through oxidation pathways (Jin et al., 2023). Another study involving the breakdown of 6:2 FTSA by *Dietzia aurantiaca* sp. strain J3 showed similar breakdown products being produced when compared to PFAS degradation by NB4-1Y (Mendez et al. 2022). Through proteomic analysis, Mendez et al. (2022) observed alkanesulfonate monooxygenase *ssuABCD* upregulation in *Dietzia aurantiaca* sp. strain J3 when exposed to 6:2 FTSA as a sole sulfur source. Higher expression levels of alkanesulfonate monooxygenase has been observed in *Rhodococcus jostii* RH1 grown with 6:2 FTSA as a sole sulfur source (Yang et al., 2022). It's possible the desulfonation of 6:2 FTSA by NB4-1Y could be catalyzed by many different enzymes such as an alkane monooxygenase, LLM class flavin oxidoreductase, and dimethylsulfone monooxygenase.

Throughout the development of this gene expression quantification method a few challenges arose. The high GC content of the NB4-1Y genome, 68%, presented some challenges during primer and probe design, particularly, when trying to avoid repeating bases or dinucleotides. During the time course experiment, wet biomass measurements were inconsistent and had a relatively large standard error. This was likely due to inconsistent pipetting when removing supernatant which would have introduced variability in wet weight measurements. To get more accurate biomass measurements and reduce error, cell pellets could be dried prior to weighing them. Initial qPCR and dPCR results were promising and confirmed the successful development of the proposed mRNA quantification method. However, qPCR and dPCR results were not normalized because the focus of this work was on method development. Normalizing qPCR and dPCR data is essential to reduce experimental error that can arise from variability in total RNA content, RNA stability, enzymatic efficiencies, or sample loading variation (Hruz et al., 2011). Data normalization can be done in several ways including standardizing the amount and quality of RNA sample into each reverse transcriptase reaction or using reference genes as an internal standard (Dheda et al. 2005). Ideally, reference gene expression levels do not vary significantly with varying experimental conditions, so they are representative of the total amount of mRNA present in a sample (Hruz et al., 2011). There is a limited amount of information in the literature regarding which reference genes to use in bacterial systems. However, one study normalized their data to the 16S rRNA gene and reported dPCR data as relative abundance values (Ferraro et al., 2024). Studies in eukaryotic systems have normalized dPCR data by using reference genes that code for enzymes involved in glycolysis like GAPDH (Hruz et al., 2011) and pyruvate kinase family protein (Zmienko et al. 2015). Therefore, the normalization of qPCR and dPCR data using reference genes such as the 16S rRNA gene should be investigated and incorporated into the design of future experiments to measure gene expression in NB4-1Y.

The primers and probes designed and validated in this study can now be used to quantify mRNA levels over time when NB4-1Y is provided with 6:2 FTSA, $MgSO_4$, or 1-

octanesulfonate, a non-fluorinated analog of 6:2 FTSA, as sole sources of sulfur. The resulting data will be compared using multivariate analysis of variance (MANOVA). Comparisons of these data to transcriptome and proteome data, will inform recommendations made to guide future protein purification and characterization experiments.

Conclusion:

In this study, primers and probes were designed and successfully amplified gene regions of interest, coding for a dimethylsulfone monooxygenase (*snfG*), several LLM class flavin-dependent oxidoreductases, and an acyl-CoA dehydrogenase family protein, as confirmed by end-point PCR and Sanger sequencing. A method was developed to quantify the expression levels for these genes of interest using reverse transcriptase quantitative digital polymerase chain reaction (dPCR). Initial qPCR and dPCR results showed expression levels of three genes of interest in NB4-1Y samples grown in 6:2 FTSA were higher compared to $MgSO_4$ and no sulfur source controls, thus supporting the stated hypothesis. This method can now be applied to quantifying mRNA levels over time when NB4-1Y is provided with various sulfur sources, with the end goal of describing the molecular basis of PFAS metabolism by NB4-1Y.

Acknowledgements:

This work was supported through funding from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Strategic Environmental Research and Development Program (SERDP) ER20-1023. I would also like to thank my supervisor Dr. Jon Van Hamme, co-supervisor Dr. Eric Bottos, and fellow lab mates for all their help and support throughout this project.

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