Mechanisms of persistence of the ammonia oxidising bacteria- *Nitrosomonas* to the biocide Free Nitrous Acid (FNA).

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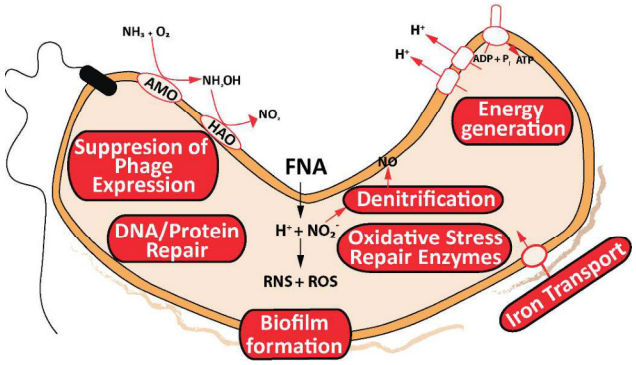
Abstract Art



ABSTRACT

Free Nitrous Acid (FNA) exerts a broad range of antimicrobial effects on bacteria although susceptibility varies considerably amongst microorganisms. Among nitrifiers found in activated sludge of wastewater treatment processes (WWTP), nitrite oxidising bacteria (NOB) are more susceptible to FNA compared to ammonia oxidising bacteria (AOB). This selective inhibition of NOB over AOB in WWTP bypasses nitrate production and improves the efficiency and costs of the nitrogen removal process in both the activated sludge and anaerobic ammonium oxidation (Anammox) system. However, the molecular mechanisms governing this atypical tolerance of AOB to FNA have yet to be understood. Herein we investigate the varying effects of the antimicrobial FNA on activated sludge containing AOB and NOB using an integrated metagenomics and label free quantitative sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) metaproteomic approach. The *Nitrosomonas* genus of AOB on exposure to FNA maintains internal homeostasis by upregulating a number of known oxidative stress enzymes such as pteridine reductase and dihydrolipoyl dehydrogenase. Denitrifying enzymes were upregulated on exposure to FNA suggesting the detoxification of nitrite to nitric oxide. Interestingly proteins involved in stress response mechanisms such as DNA and protein repair enzymes, phage prevention proteins as well as iron transport proteins were upregulated on exposure to FNA. Interestingly enzymes involved in energy generation were upregulated on exposure to FNA. The total proteins specifically derived from the NOB genus *Nitrobacter* was low and as such did not allow for the elucidation of the response mechanism to FNA exposure. These findings give us an understanding of the adaptive mechanisms of tolerance within the AOB *Nitrosomonas* to the biocidal agent FNA.

1. INTRODUCTION:

Studies on bacteria have shown that the protonated form of nitrite i.e. HNO_2 also known as free nitrous acid (FNA), is a strong biocide [1]. Additionally, FNA was seen to have broad bacteriocidal and biocidal effects on an array of microorganisms, which have led to its use in a range of applications for wastewater treatment processes (WWTP). This includes the control of microbial induced sewer corrosion; the enhanced biodegradability of microbes in activated sludge to achieving reduced N_2O production in the activated sludge process [2-8]. It has been hypothesised that once inside the cell, FNA dissociates to form various reactive nitrogen species (RNS) and reactive oxygen species (ROS) speculated to enhance the toxicity of FNA, but the chemistry of this reaction is not well characterised [9]. These reactive species can cause direct oxidative damage to cellular proteins, cell membrane and cell wall components as well as nucleic acids. It is hypothesised that FNA can act as a protonophore by collapsing the proton membrane potential and thereby inhibiting ATP production [10]. Further application of FNA for control of microbial growth and activity would benefit from improved understanding of how it causes toxicity to various microorganisms.

Transcriptomic based investigations have been conducted on *Pseudomonas aeruginosa* (PAO1) and *Desulfovibrio vulgaris* (Hildenborough) to determine the toxic mechanisms of FNA. At 0.1 mg/L FNA-N caused inhibition of cellular respiration that led to PAO1 re-routing its carbon metabolic pathway from the tricarboxylic acid (TCA) cycle to the pyruvate fermentation pathway. Inhibition of protein synthesis and inactivation of ribosome components was also evident [5]. Studies on PAO1 showed that FNA at 5.0 mg/L FNA-N caused cell death [11]. For the bacterium *D. vulgaris*, FNA caused increased expression of

genes coding for oxidative stress enzymes indicating that FNA caused oxidative stress as well as decreased anaerobic respiration and a shut down of protein synthesis[4].

In wastewater treatment processes (WWTP), the removal of nitrogen is carried out via the use of the activated sludge process. This is achieved by the actions of nitrifying and denitrifying bacteria in a multi-step process. Ammonium is converted to nitrite, by the ammonia-oxidising bacteria (AOB), and then converted to nitrate by the nitrite-oxidising bacteria (NOB). Denitrifying bacteria consequently reduces nitrite and nitrate to nitrogen gas [12-14]. The most commonly found nitrifying bacteria in activated sludge belong to the *Nitrosomonas* genus for the AOB and the *Nitrobacter* and *Nitrospira* genera for the NOB [15]. Interestingly, studies on activated sludge systems show that NOB species are more sensitive to FNA than AOB [10, 16, 17]. FNA concentrations of greater than 1.5 mg/L FNA-N are found to selectively inhibit the NOB population [16]. In a recent study the growth of NOB was selectively inhibited whereas the AOB population remained high when the sludge was treated with FNA at 1.8 mg/L FNA-N [15]. Fortuitously, this phenomenon could benefit nitrogen removal in WWTP as the suppression of NOB results in 'partial nitritation' where nitrite is formed instead of nitrate in both conventional nitrogen removal using activated sludge system and the anaerobic ammonium oxidation (Anammox) [15]. This can lead to high economic and operational benefits for WWTP due to the decreased oxygen demand for nitrification, less organic carbon required for denitrification and potentially reduced N_2O emissions [6].

Despite the range of applications of FNA in WWTP, there is limited understanding of the increased tolerance of AOB over NOB to the biocide. NOB, in general have two additional pathways to remove toxic nitrite build up compared to AOB (Figure S1). AOB and

NOB both have the nitrite detoxifying gene nitrite reductase (*nirK)* that converts nitrite to nitric oxide (NO) [18]. The nitrite reductase (*nirBD)* and nitrite oxidoreductase (*norA/B)* genes, present exclusively in NOB convert nitrite to ammonia and nitrate respectively thereby detoxifying toxic nitrite. The limited numbers of detoxifying pathways in AOB is contrary to its observed tolerance. To date there is no clear understanding of the underlying mechanisms that govern this tolerance to FNA.

Studies investigating the global responses of AOB to various stress conditions are sparse and have been limited to responses to iron stress, toxic zinc exposure, oxidative stress induced by hydrogen peroxide and starvation [19-22]. In this study we reveal the reasons for FNA tolerance in AOB compared to NOB using a combined metagenomic and a quantitative sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) metaproteomic approach. SWATH-MS is a label free proteomics approach that allows for the unbiased, reproducible quantification of essentially any protein of interest without the use of expensive labelling approaches [23]. Additionally SWATH-MS requires low amounts of proteins (1 µg) for analysis along with a 5 µg aliquot of pooled samples for the creation of a spectral library using information dependent acquisition (IDA) [23]. Of the reports on microbial metaproteomics, to our knowledge this study describes the first use SWATH-MS on an environmental microbiome sample.

2. MATERIALS AND METHODS

2.1. Reactor set up, side stream treatments and sampling:

An 11 L sequencing batch reactor (SBR) originally seeded with activated sludge from a domestic wastewater treatment plant in Brisbane, Australia was operated in a temperature controlled room (22±1 °C) supplied with an ammonium based synthetic feed sans organic

carbon for the selection of nitrifying bacteria. The synthetic wastewater composition per liter was: 0.2949 g of NH_4HCO_3 (57 mg NH_4^+-N), 0.33 g $NaHCO_3$, 0.184 g of NaCl, 0.072 g of $NaH_2PO_4 \cdot H_2O$, 0.035 g $MgSO_4 \cdot 7H_2O$, 0.029 g KCl and 0.3 mL of a trace element stock solution prepared as previously described [24]. The reactor was operated with four cycles daily (6 hours each), which consisted of a 90 min aerobic feed period wherein 5L of synthetic wastewater was pumped into the reactor, a 210 min aerobic mixing period, a 50 min settling stage and a 10 min decanting period. No sludge was wasted during the study. During aeration periods, a dissolved oxygen (DO) concentration of 2.5-3.0 mg/L and a pH of 7.5 were maintained in the reactor using programmed logic controllers. The hydraulic retention time (HRT) of the reactor was 13.2 hours. At different SBR operational stages, a side stream treatment of FNA was applied to the sludge that was removed and then returned to the reactor (Figure 1(A)). In Stage 1 of the SBR operation, there was no FNA side stream treatment. In Stage 2, 2750 ml (25%) of the mixed liquor was withdrawn every day (second daily cycle) from the main SBR at the end of the aerobic stage (before settling) and the sludge was thickened to 130 ml. The sludge was then treated for 24 hours with an initial concentration of 3.64 mg/L FNA-N (Stage 2) while maintaining a pH of 6.0 in a side stream FNA treatment reactor using a programmed logic controller. Following FNA treatment, the sludge was then returned to the main SBR. At the end of the second daily cycle, the amount of treated wastewater decanted was altered so that the 13.2 h HRT was maintained. Similarly, in Stage 3 the same sludge treatment was carried out every day with a changed side stream FNA treatment of 1.82 mg/L FNA-N. The nitrogen species of the side stream reactor were measured at 0 and 24 hours following treatment.

For the metagenomic investigation, samples of mixed liquor were collected at the end of each stage of operation from the main reactor. The samples once collected were

centrifuged at 14,000 x g for 2 mins (4 ℃) and the supernatants removed. The remaining pellets were snap frozen in liquid nitrogen (-196 °C) to eliminate enzymatic activity and then stored at -80 °C until DNA extractions were performed. For the metaproteomic studies, samples were collected at the end of Stage 3, from where the thickened sludge (130 ml) was equally distributed to an FNA treatment reactor at 1.82 mg/L FNA-N and a control reactor without FNA treatment i.e. 0 mg/L FNA-N. Triplicate samples for metaproteomics were taken from both side stream reactors at the treatment times of 0 min, 20 min, 2 hours, 12 hours and 24 hours for 3 consecutive days. Following centrifugation, supernatant was removed and pellets were snap frozen in liquid nitrogen and stored at -80 °C until protein extraction was carried out.

2.2. Analytical methods and DNA extraction:

The ammonium, nitrite and nitrate concentrations in the SBR effluent were measured 2-4 times every week using a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee, Wisconsin, USA). Microbial DNA was extracted from sludge samples for metagenomics using the PowerSoil® DNA isolation Kit (MO BIO Laboratories) as per the manufacturer's instructions. Metagenomic DNA was sequenced using the Illumina NextSeq 500 platform using the Nextera library protocol (Illumina) at the Australian Centre for Ecogenomics, University of Queensland.

2.3. Metagenomic assembly and analyses:

Raw DNA sequences were added to the Metagenomics Rapid Annotation (MG-RAST) server (v4) for rapid annotation and the determination of microbial community composition at the three different stages of the reactor operation. The sequence datasets for the stages 1, 2 and 3 were loaded into MG-RAST with identifications of mgm4688234.3,

mgm4688240.3 and mgm4688237.3 respectively [25]. The raw Illumina sequence reads were also processed using two different bioinformatic pipelines to create a robust and tailored metagenome database. Briefly, in the first pipeline (Pipeline 1), adaptors of the forward and reverse reads were clipped and quality trimmed with trimmomatic using a minimum quality score of 3 for leading and trailing bases, along with a minimum average quality score for 4 bp as 15 limiting the minimum required length to 50bp [26]. Additionally microbial community analysis was carried out using the GraftM tool wherein reads were parsed through GraftM to identify those containing 16S reads using the May, 2015 Greengenes database 97% OTUs (operational taxonomic units) as a reference with default parameters [27]. The quality controlled reads were then merged with BBmerge [28]. The quality controlled paired reads were assembled using CLC Genomics Cell assembler v8.0. The assembled contigs were then binned using GroopM and Metabat [29, 30]. In the second bioinformatic pipeline (Pipeline 2) the raw reads were processed using an automated pipeline called IMP (Integrated Meta-omic Pipeline) that involved iterative co-assembly and mapping [31]. The metagenome bins were generated through this pipeline using a VizBin-based workflow [32, 33]. The quality of the bins generated from both these bioinformatic pipelines were then estimated using CheckM. Taxonomies of the bins were inferred using the genome taxonomy database (GTDB) [34]. Nitrifying population genome bins with greater than 75% completeness and contamination less than 10 % were annotated using Prokka and concatenated to create a custom tailored database for metaproteomics [35, 36].

2.4. Protein extraction and digestion:

Protein extraction was carried out on all triplicate samples collected from the side stream treatment reactor under 1.82 mg/L FNA-N and a 0 mg/L FNA-N as a control. A 10ml protein extraction buffer was prepared using 10 ml of B-PER Bacterial Protein Extraction

Reagent mix (Thermo Fisher Scientific), 7.7 mg of dithiothereitol and 1 tablet of Complete mini EDTA-free Protease Inhibitor Cocktail (Roche). 1 ml of this buffer was added to each thawed sludge pellet (Section 2.1) for extraction and left at room temperature for 30 minutes with periodic vortexing to solubilise the protein. The cell debris was removed after centrifugation at 15,000 g for 15 mins following which the protein supernatant was incubated overnight with 10% Trichloroacetic acid (TCA) at 4 °C. The protein was recovered by centrifugation at 18,000 g for 15 mins following which the pellets were washed twice with cold acetone and subsequently dried at room temperature. Once dried the pellets were re-suspended in 100 µl of buffer containing 2 M thiourea, 7 M urea and 100 mM ammonium bicarbonate. Protein quantification was carried out through the 2-D Quant Kit (GE Healthcare). Subsequently, reduction of proteins was carried out with 5 mM dithiothereitol for 30 mins at 56 °C. Alkylation of the protein was carried out by incubation in the dark for 30 mins at room temperature with 25 mM iodoacetamide (Sigma-Aldrich). Additional 50 mM dithiothereitol was added to quench the alkylation reaction once the incubation was complete. Samples were diluted with 50 mM ammonium bicarbonate to reduce the urea concentration to 2 M. Digestion with 1:50 trypsin:protein ratio was performed at 37 °C for 4 hours. Following that a second digestion was performed with a 1:25 trypsin:protein ratio at 37 °C overnight [37]. Peptides were further concentrated and purified using the C-18 ZipTip® Pipette Tips (Merck Millipore) using 5% acetonitrile/0.1% trifluoroacetic acid for washing and then elution with 80% acetonitrile/0.1% trifluoroacetic acid. The samples were dried using a Speed-Vac to remove acetonitrile and peptides were re-suspended in 0.1% formic acid into vials used for mass spectrometry (Agilent Technologies) [38].

2.5. Metaproteomic analysis:

247 Following purification, 1 μg of digested protein was used for SWATH acquisitions
248 and 5 μg aliquots of pooled samples were used to create a spectral library using information
249 dependent acquisition (IDA) mode. Peptides were directly analysed on a LC-ESI-MS/MS
250 with a Prominence nanoLC system (Shimadzu) and a Triple-ToF 5600 instrument (ABSciex)
251 equipped with a Nanospray III interface as previously described [39]. Mass spectrometry
252 (MS) data of pooled IDA samples was searched using ProteinPilot™ software (ABSciex,
253 Forster City CA) against a custom tailored database containing the population genome bins of
254 nitrifiers generated from both the metagenomic pipeline analysis (Table 1) and the genomes
255 of known nitrifiers downloaded publically from Uniprot Swiss-Prot database (June 2016
256 release). The search settings included enzyme digestion set to trypsin, cysteine alkylation set
257 to iodoacetamide, and global false discovery rate (FDR) set at 1%. The quantified proteome
258 SWATH files were generated using the PeakView with 5 peptides per protein and 3
259 transitions per peptide. The MSstats package in R was used for statistical analysis of the
260 spectral information and the p-value stringency was set to ≤ 0.05 across triplicate samples
261 [40]. To determine the $\log_2$(FC) ($\log_2$ Fold change) of proteins we compared the 0 minutes
262 time point to the other time points wherein samples were collected i.e. 20 minutes, 2 hours,
263 12 hours and 24 hours at the two FNA concentrations using the 'groupComparison' function
264 of MStats. The sequences, molecular functions and biological processes of the proteins were
265 queried and verified against the curated UNIPROT and NCBI databases [41]. Additional
266 statistical analysis was carried out at each sample time-point between the control and the
267 FNA treated sample to verify the statistical significance of $\log_2$(FC) protein expression.
268

269 **3. RESULTS AND DISCUSSION:**

270 3.1. Reactor performance:

271 The SBR was operated for nitrification activity in three main stages. In Stage 1 there
272 was no side stream treatment of the activated sludge and near complete conversion of
273 ammonium to nitrate occurred (97.0±0.7 %; Figure 1A). This was due to the high activity of
274 both AOB and NOB (Figure 1B). During Stage 2, when part of the sludge was treated at 3.64
275 mg N/L FNA-N in the side stream treatment reactor, the levels of nitrate in the effluent
276 decreased sharply (Figure 1B), indicating that the NOB population was severely inhibited.
277 This coincided with high levels of nitrite in the effluent, manifested by the activity of AOB at
278 the high FNA treatment. An increase in ammonium was detected in the effluent after day 55
279 of operation, indicating there was some inhibition of AOB activity by the high FNA sludge
280 treatment. At this point the side stream FNA treatment was terminated for a 10-day period to
281 recover AOB activity. In Stage 3 a side stream treatment of FNA at 1.82 mg N/L was applied
282 to the SBR sludge for 24 hours at a pH of 6.0. The measured nitrogen species, including VSS
283 and other parameters including the activity of the AOB and NOB populations at the end of
284 both stages 1 and 3 have been summarized in Table S1. Additionally nitrogen species
285 measurements of the side stream FNA reactor at stage 3 is represented in Table S2 During
286 this treatment ammonium in the mainstream SBR reactor was nearly completely converted to
287 a mixture of nitrite (58.5±0.3% of the total effluent nitrogen) and nitrate (40.2±0.7% of the
288 total effluent nitrogen) (Figure 1B). Thus, at this level of treatment AOB were active and
289 there was some activity of NOB. The results of the SBR operation are in agreement with
290 previous studies showing that AOB are less sensitive to FNA toxicity in comparison to NOB
291 [6, 15]. Consequently, FNA could be used to effectively control the NOB and AOB
292 population and thereby the levels of nitrogen species produced, which can practically be
293 beneficial to achieve the more economically favorable partial nitritation compared to the
294 conventional nitrification and denitrification of WWTP. This control could also allow for the

295 effluent to be directly used for the treatment of a low organic carbon stream operating for
296 nitrogen removal in a two-stage anaerobic ammonia oxidation (ANAMOX) system [42].
297
298 (Position for Figure 1)
299
300 3.2. Microbial community composition of the reactor:
301 As expected the synthetic wastewater feed containing ammonium and no organic
302 carbon provided an amiable environment for the growth of autotrophic nitrifiers [43].
303 Microbial community composition generated from MG-RAST were extracted and
304 represented as percentages (Figure 1C). Nitrifiers dominated the microbial community in the
305 SBR, however, a variety of low abundance heterotrophic bacteria were also detected. Among
306 the nitrifiers, the 3 genera of AOB in the reactor included *Nitrosomonas*, *Nitrosospira*,
307 *Nitrosococcus* and 3 genera of NOB detected included *Nitrobacter*, *Nitrospira* and
308 *Nitrococcus* (Table S2). The dominant AOB genus *Nitrosomonas* represented 20.48%,
309 11.92% and 21.94% of the microbial communities within the reactor operation Stages 1, 2
310 and 3 respectively. It is worth noting the drop in the *Nitrosomonas* populations during Stage
311 2, suggesting that the high FNA treatment caused killing of some of these more resilient
312 nitrifiers. This is in agreement with the increase in ammonium detected in the effluent, at
313 nearly 20 mg/L around day 55 of the SBR operation (Figure 1B). After the sludge FNA
314 treatment was stopped, the activity of the AOB population recovered as can be seen on day
315 71 of the SBR operation (Figure 1B). For NOB in the SBR, the dominant genus *Nitrobacter*
316 constituted 1.56%, 2.95% and 3.18% of the microbial communities in Stages 1, 2 and 3
317 respectively (Table S2). Additionally, relative abundance of the microorganisms in these
318 Stages was determined by GraftM, and this showed similar results to those obtained through
319 MG-RAST (Table S2(b)). The AOB family *Nitrosomonadaceae* represented 30.23 %, 8.33 %

and 35.26 % of Stage 1, 2 and 3 respectively. The NOB genus *Nitrobacter* represented 0.05 %, 0.16 % and 0.06 % of the microbial community from Stage 1, 2 and 3 (Table S3). Despite relatively low proportions of the NOB community the NOB activity was high in Stage 1 as evidenced by the nitrate detected (Figure 1B).

3.3. Nitirifier genomes detected in the reactor

The population genome bins of nitrifiers generated using the 2 bioinformatic pipelines with completeness cut-off of greater than 75% and a contamination less than 10 were obtained from the SBR. They included the NOB genus *Nitrobacter* and the AOB genus *Nitrosomonas* as represented in Table 1. The genera of the detected population genome bins are in agreement with the dominant nitrifiers detected from the MG-RAST analysis (Figure 1C). A custom sequence database was generated using the annotated population genomes of nitrifiers obtained from the SBR together with annotated genomes of publically available *Nitrobacter* and *Nitrosomonas* species [44]. This custom database was used to maximise the detection of proteins for our metaproteomic analyses.

(Position for Table 1)

3.4. Metaproteomic responses of nitrifiers to FNA:

The SBR operation towards the end of Stage 2 i.e. 3.64 mg/L FNA-N was characterized by fluctuations in the nitrogen species (Figure 1B). This suggests that the FNA concentration was high enough to even suppress growth of AOB. The metaproteomic investigation of nitrifiers was carried out at Stage 3 i.e. 1.82 mg/L FNA-N as the operation of the SBR reactor was stable as evidenced from the nitrogen species of the effluent (Figure 1B). Metaproteomic investigation was also carried out on a control sample i.e. 0 mg/L FNA-

345 N to compare the responses to nitrifiers without FNA treatment. Using the ProteinPilot™
346 software a total of 419 proteins were identified against the custom database at a global FDR
347 of 1%. The SWATH files generated from the Peak View software were analysed using the R-
348 package MSstats. A total of 359 proteins met the p-value stringency of ≤ 0.05 across
349 triplicates of which 344 originated from the *Nitrosomonas* genera and 15 from the
350 *Nitrobacter* genera (Figure S2). The quantitative SWATH-MS analysis on the *Nitrosomonas*
351 genera showed changed expression of various proteins, which have been discussed below (for
352 detailed description see Supplementary Table 1). The mass spectrometry data has been
353 deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the
354 accession no: PXD007514 [45]. Additionally statistical analysis summarising the change in
355 protein expression between the control (0 mg/L FNA-N) and 1.82 mg/L FNA-N (p-value
356 stringency of ≤ 0.05 across triplicates) at 20 minutes, 2 hours, 12 hours and 24 hours were
357 also investigated. The results of this analysis have been represented in Supplementary table 2.
358
359 3.4.1. Metaproteomic response
360 3.4.1.1. *Nitrosomonas* genus
361 The change in the regulation and expression of proteins is likely due to the change in
362 expression of the gene. As such changed protein expression levels reveal the responses of
363 microorganism to a perturbed condition. In our study we compared the change in protein
364 expression over time without FNA (control) treatment to the perturbed 1.82 mg/L FNA-N
365 samples. The quantitative metaproteomic approach revealed the upregulation and
366 downregulation of a number of cellular proteins by *Nitrosomonas* in response to the
367 antimicrobial action of FNA. Functionally a majority of these proteins have not been well
368 studied in bacterial systems, however they are expected to fulfil similar roles within bacteria.
369

Oxidative stress enzymes:

Nitrosomonas like other aerobic bacteria, experiences oxidative and nitrosative stress from a variety of sources in its natural environment. The few oxidative stress studies on AOB have been limited to catalase and superoxide dismutase (SOD) [22, 46, 47]. A number of proteins involved in oxidative stress were upregulated after a period of exposure to FNA. These included pteridine reductase and S-adenosylmethionine synthase (Figure 2(A)). The oxidative stress enzyme cytochrome c_{551} peroxidase was however upregulated in both the control and perturbed condition (Figure 2(A)). Statistical analysis between the control and perturbed condition at 24 hours showed that cytochrome c_{551} peroxidase (Ns76) was upregulated by 0.42 $\log_2$(FC) (Supplementary Table 2). An upregulation of these enzymes shows evidence that FNA induces oxidative damage on *Nitrosomonas.* This stress reaction is thought to primarily occur due to the presence of reactive oxygen and nitrogen species (ROS/RNS) [48]. The enzyme pteridine reductase is widespread in proteobacteria, and has been well studied in the protozoan parasite *Leishmania* where it is known to reduce the susceptibility of the protozoan to ROS and RNS [49]. A $\log_2$(FC) of S-adenosylmethionine synthase from 0.54 to 1.44 at 12 hours and 24 hours respectively on exposure to 1.82 mg/L FNA-N was seen (Supplementary Table 1). S-adenosylmethionine synthase is an enzyme known to produce S-adenosylmethionine, an important methyl donor for methylation of DNA, RNA, proteins and other macromolecules essential for normal gene regulation [50]. S-adenosylmethionine is known for preventing oxidative stress and has also been implicated in functioning as a neuroprotective agent in mice [51]. Furthermore, S-adenosylmethionine has been associated with attenuating oxidative stress in ethanol-LPS-Induced fibrotic rat models [52]. Increased abundance of cytochrome c_{551} peroxidase was detected for several *Nitrosomonas* population bins in both the control and FNA exposed conditions (Figure 2(A)). Cytochrome c_{551} peroxidase has known antioxidant activity that catalyses the reduction of

toxic H_2O_2 [53]. Cytochrome *c* apart from being a part of the electron transport chain can also suppress ROS [54]. Previous studies have shown strong evidence in support of its protective function on deoxyribose against oxidative damage in vivo [55]. The antioxidant protein thioredoxin and its associated domain proteins were also upregulated on exposure to FNA [56]. Thioredoxin is a key protein involved in the oxidative stress response in plants [57]. Thioredoxin, in mammalian endothelial cells has also been known to be in involved in a regenerative machinery to regenerate proteins inactivated by oxidative stress [58]. Additionally, thioredoxin in *Streptococcus pneumoniae* were also found to resist oxidative stress conditions [59]. Dihydrolipoyl dehydrogenase enzyme is known to be an active nitric oxide scavenger by reducing ubiquinone to uniquinol, thus providing strong evidence for the action of RNS formed from FNA [60]. Peptide methionine sulfoxide reductase (msrA) is a repair enzyme that repairs protein inactivated by oxidation [61, 62]. The msrA derived from Ns85 was seen to be upregulated at 24 hours after exposure to 1.82 mg/L FNA-N. SOD is known to act as a strong antioxidant wherein it converts two molecules of superoxide to oxygen and hydrogen peroxide. Hydrogen peroxide is removed by catalase and peroxidase enzymes [63]. Whilst we did not detect the expression of catalase enzyme we did see a slight decrease in expression of the protein SOD (Ns76) over 24 hours after exposure to 1.82 mg/L FNA-N (Supplementary Table 1). However, according to the ProteinPilot analysis this SOD had high spectral coverage (66%) suggesting this is a highly abundant protein in the cell. Studies by Wood *et al.* (2001) describes the SOD as constitutively expressed [22]. This slight change in expression we detected supports the suggestion that SOD is constitutively expressed and that the *Nitrosomonas* has a high innate ability to deal with oxidative stress from SOD as well as from other proteins that are discussed here. Overall there is clear evidence showing that FNA mediates an oxidative stress response on *Nitrosomonas* possibly through ROS and RNS [64].

420

421 *Enzymes involved in nitrogen metabolism and energy conversion.*

422 Denitrifying enzymes are thought to protect AOB from the negative effects of nitrite
423 [65]. An increased expression level of the denitrifying enzyme nitrite reductase in
424 *Nitrosomonas* was detected during FNA treatment (Figure 2(B)). Nitrite reductase is known
425 to reduce toxic nitrite to nitric oxide (NO), a free radical in a process known as nitrifier
426 denitrification [66]. Interstingly nitrite reductase is thought to confer tolerance against nitrite
427 as seen in a pure culture of *Nitrosomonas europaea* [67]. Oxygen sensitive hydroxylamine
428 reductase, which catalyses the reduction of hydroxylamine to ammonia and water was
429 upregulated in both the control and perturbed conditions [68]. However, at 1.82 mg/L FNA-
430 N the protein was upregulated by 1.48 $\log_2$(FC) at 24 hours (Supplementary Table 2),
431 signifying that perhaps the minor increase in the ammonia concentration in the FNA reactor
432 could possibly be explained by the action of this enzyme (Table S2).

433

434 The key nitrifying and energy generation enzymes in AOB, ammonia monoxygenase
435 (AMO) and hydroxylamine oxidoreductase (HAO) were detected in multiple *Nitrosomonas*
436 genomes of the SBR sludge (Figure 2(B)). The detected increased abundance of these
437 proteins on exposure to FNA is of interest as the side stream FNA treatment reactor has
438 limited availability of ammonia, high concentration of nitrite and low availability of
439 dissolved oxygen (Table S2). The stress induced by FNA and the need to maintain internal
440 homeostasis causes the *Nitrosomonas* genera to use energy dependent mechanisms. This
441 results in the upregulation of enzymes such as AMO and HAO, in anticipation of available
442 ammonia for energy generation. This is in agreement with a previous study showing
443 consistently high levels of AMO and HAO enzymes in *Nitrosomonas* for long periods
444 (months) even in the absence of ammonia [69]. A previous study showed that nitrite inhibited

445 the AMO enzyme activity but did not investigate the expression levels of this enzyme [70].
446 Of interest to note is the variation of protein expression of the same protein within different
447 *Nitrosomonas* populations of the same genus (Figure 2(B)). A number of proteins involved in
448 the energy generation pathways including ATP synthase subunits and cytochrome *c* oxidase
449 subunits were seen to be upregulated on exposure to FNA (Figure 2(B)) [71]. The observed
450 upregulation of these proteins in exposure to FNA strongly suggests that FNA initiates the
451 generation of more ATP possibly used up in the energy dependent mechanisms of internal
452 homeostasis.
453
454
455 (Position for Figure 2)
456
457 *DNA/Protein repair*
458 FNA has also been postulated to directly act on protein and DNA through ROS and
459 RNS intermediates [72]. As such FNA exposure would result in the upregulation of a number
460 of enzymes involved in DNA and protein repair [64]. Among the DNA repair enzymes,
461 single stranded DNA-binding protein was upregulated on exposure to FNA (Figure S3(A)).
462 This protein is known to be involved in DNA mismatch, recombinational damage repair
463 mechanisms as well as SOS response [73]. The histone like DNA-binding protein HU-beta
464 protein that is known to prevent denaturation of DNA by wrapping itself around it was
465 upregulated on exposure to 1.82 mg/L FNA-N [74]. In contrast, the DNA helicase RecQ, a
466 DNA repair enzyme in both human and bacteria was however observed to be downregulated
467 with and without FNA exposure [75].
468

Expression of a number of proteins involved in protein repair mechanisms also changed on exposure to FNA. Protein-L-isoaspartate *O*-methyltransferase in humans is known to recognise damaged proteins and is involved in repairing them [76, 77]. Peptide methionine sulfoxide reductase (MsrA), an important repair enzyme for proteins that have been damaged on oxidation, again was upregulated, thus shedding evidence to the oxidative damage induced by FNA exposure [61, 62, 78]. The chaperone protein ClpB in bacteria is known to be part of the stress induced multi-chaperon system and it is known to help in the refolding of denatured stress-damaged protein [79]. This protein was however undetected in the control. Previous studies carried out on *Nitrosomonas europaea* showed that the response of the oxidation of chloroform increased the expression of ClpB, 6 to 10 fold in response to oxidation caused by chloroform [80]. The evidence of ClpB and MsrA being upregulated strongly supports the evidence that FNA causes oxidative damage to proteins. Other proteins found to be associated with protein repair include the 60 kDa and 10 kDa chaperonins. These chaperonins are essential for the folding of proteins in bacteria but little can be drawn from their expression in response to FNA [81].

Other stress responses:

Evidence from the metaproteomic analysis shows that FNA activated a number of other stress responses within the *Nitrosomonas* genus as evidenced by the change in protein expression as shown in Figure S3(B). The protein "AAA domain/putative AbiEii toxin/Type IV TA system" is an altruistic cell death system that is activated by phage infection thereby limiting viral replication [82]. This suggests that FNA caused the activation of the temperate phage and that *Nitrosomonas* suppresses the expression of this phage. Studies carried out in *Pseudomonas aeruginosa* on being exposed to nitric oxide (NO), a free radical that could possibly be formed from FNA showed that NO exposure caused bacteriophage genes to be

494 upregulated [83]. The modulator of FtsH protease HflK is known to govern the
495 lysogenization frequency of phage lamda in the bacteria *E. coli* [84]. In our study there was a
496 slight decrease in the expression of this protein. The bleomycin resistance protein, known to
497 repair DNA breakage and lesions was upregulated on exposure to FNA (Figure S3(B)) [85,
498 86].
499
500 Biofilm formation in the *Nitrosomonas* genus of AOB is not thoroughly understood
501 but biofilm formation has been shown to be enhanced by the growth of other heterotrophic
502 bacteria [87]. It is of interest to note that biofilm formation has been previously induced in
503 *Nitrosomonas* on exposure to higher concentrations of the RNS/ROS nitric oxide [88]. Beta-
504 lactamase hydrolase-like protein and alginate export proteins play an important role in the
505 formation of biofilms [89, 90]. The protein alginate export is known to export alginate, a
506 model extracellular polysaccharide (EPS) externally to aid in the formation of a protective
507 biofilm. The expression of beta-lactamase hydrolase-like protein and alginate export protein
508 increased after FNA exposure suggesting that *Nitrosomonas* is inducing growth of biofilm to
509 protect itself from the biocidal action of FNA (Figure S3(B)). Alginate export expression was
510 seen to change positively after exposure to FNA. On the other hand
511 phosphomannomutase/phosphoglucomutase, a protein associated with the production of
512 alginate and lipopolysaccharide (LPS) biosynthesis, was severely downregulated on exposure
513 to FNA [91] .
514
515 Of the detected proteases involved in proteolysis, the protease HtpX was the only one
516 seen to be upregulated in the FNA treated sample. Protease HtpX, is a membrane bound
517 metalloprotease that is known to be a stress-controlled protease possibly degrading damaged

518 oxidised proteins [64, 92]. This evidence lends further support to the fact that FNA causes
519 oxidative damage to the proteins.
520
521 FNA exposure is seen to alter iron bioavailability within the cell through the RNS and
522 ROS that are formed. A number of enzymes contain iron as a co-factor and as such plays a
523 critical role in maintaining cellular homeostasis within the microorganisms [93]. Iron storage
524 proteins, such as bacterioferritin derived from two different population genomes of
525 *Nitrosomonas* were both downregulated after exposure to FNA (Figure S3(B)) [94]. Iron
526 transport proteins such as haemoglobin, haemoglobin-haptoglobin binding protein and the
527 *Nitrosomonas* ABC iron transporter as well as the catecholate siderophore receptor Flu, were
528 all upregulated after exposure to FNA as shown in Figure S3(B) [95-98]. The haemoglobin
529 and haemoglobin-haptoglobin-binding protein which form part of a receptor required for
530 heme uptake is upregulated on exposure to FNA [95]. Catecholate siderophore receptor Flu
531 are known to transport siderophore which are low molecular weight ferric ion specific
532 chelating agents used by microorganisms to scavenge iron from the environment [99].
533 Siderophore transport proteins were seen to be marginally upregulated on exposure to FNA
534 as shown in Figure S3(B). As evidenced, iron transport across the membrane into the cell was
535 facilitated signifying the change in iron levels internal of the *Nitrosomonas* genera. A
536 decrease in expression levels of Fe-S enzymes was detected post FNA treatment (Figure
537 S3(B)). Thus it can be clearly seen that FNA disrupts the bioavailability of cellular iron
538 within the *Nitrosomonas* population.
539
540 The regulation of other proteins involved in key metabolic processes such as carbon
541 dioxide fixation, respiration, TCA cycle, glycolysis, DNA replication, RNA transcription and
542 protein translation were also studied and have been discussed in the Supplementary section

543 S6. An overview of the amino acid, fatty acid biosynthesis and breakdown and carbohydrate
544 metabolism has also been discussed in the supplementary section S6.
545
546 3.4.1.2. *Nitrobacter*
547 This study also intended to look at the response of NOB particularly *Nitrobacter*
548 genera to the FNA. However, only 15 proteins were detected to have a $\log_2$(FC) within the
549 *Nitrobacter* genera (Figure S8). The low detection of *Nitrobacter* proteins by our
550 metaproteomic approach very likely reflects the lower abundance of these microorganisms in
551 the mixed culture community (Table S2 and S3). As such it was difficult to draw any
552 conclusions of changes in the metabolic pathways from the limited proteins we detected. An
553 enriched NOB culture from activated sludge would be beneficial in investigating the
554 mechanism of NOB susceptibility to FNA.
555
556 3.5. *Persistence to FNA in Nitrosomonas reveals multipronged mechanisms*
557 The tolerance of the *Nitrosomonas* can be attributed to an upregulation of oxidative
558 stress enzymes, denitrification, DNA and protein repair mechanisms as well as other defence
559 pathways such as the inhibition of phage formation. There was also evidence that FNA alters
560 the cellular iron bioavailability within a cell leading to an upregulation of enzymes involved
561 in iron transport across the membrane although the mechanism through which this occurs
562 remains unclear. The *Nitrosomonas* population shows an upregulation of the energy
563 producing nitrification pathway enzymes i.e. AMO and HAO despite the low ammonia and
564 dissolved oxygen in the FNA treatment reactor. We hypothesise that this upregulation is due
565 to a need for internal energy generation in anticipation of available ammonia. Overall
566 *Nitrosomonas* exerts a strong response to deal with oxidative stress caused by FNA.
567

(Position for Figure 3)

There was strong evidence to suggest that FNA caused oxidative stress on the *Nitrosomonas* population possibly through its ROS and RNS intermediates. We developed an overview to diagrammatically represent the effects of FNA and the responses of *Nitrosomonas* to the biocide (Figure 3). This study provides a fundamental understanding of the molecular mechanisms involved in the tolerance of *Nitrosomonas* to FNA. The findings made here are relevant to applications that are based on the suppression of NOB over AOB. Use of FNA can allow for better utilisation of energy resources such in the activated sludge process and for the development of better lines of feed suited for the Anammox process.

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ASSOCIATED CONTENT

Supplementary Information Available

Supplementary data associated with this article can be found free of charge on the ACS Publication website at http://pubs.acs.org/.

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910 Table 1: The population genome bins from metagenomic pipeline 1 and 2, of known nitrifiers
911 generated from the reactor and the publically available genomes that were combined and
912 added to the custom database for metaproteomic analyses.

	Genomes	Code names	Completeness (%)	Contamination	Average fold coverage (X)	Scaffolds with coverage (%)	Bin bases covered (%)
Metagenome Population genome bins	***Nitrosomonas genus***						
	Pipeline 1						
	Nitrosomonas_99	Ns99	99.52	1.46	3051.57	100	99.96
	Nitrosomonas_89	Ns89	89.13	2.45	1164.01	100	100
	Nitrosomonas_82	Ns82	82.31	4.23	165.11	100	99.99
	Pipeline 2						
	Nitrosomonas_01321	Ns85	85.2	9.78	1233.78	100	100
	Nitrosomonas_L1322	Ns79	76.52	1.13	3057.37	100	100
	Nitrobacter genus						
	Pipeline 1						
	Nitrobacter_95	Nb95	95.99	1.42	219.73	100	100
	Nitrobacter_88	Nb88	88.88	1.69	221.64	100	99.98
	Nitrobacter_79	Nb79	79.96	2.38	116.9	100	99.97
	Pipeline 2						
	Nitrobacter_G121121	Nb83	83.33	7.71	212.39	100	100
Publically available nitrifier genomes (NCBI)	***Nitrosomonas genus***						
	Nitrosomonas Ureae	NsU					
	Nitrosomonas sp AL212	NsA					
	Nitrosomonas Eutropha C91	NsEt					
	Nitrosomonas Europaea	NsEr					
	Nitrosomonas Cryotolerans	NsCr					
	Nitrosomonas Communis	NsC					
	Nitrobacter genus						
	Nitrobacter Winogradskyi	NbW					
	Nitrobacter Hamburgensis	NbH					
	Nitrobacter sp Nb-311A	NbN					

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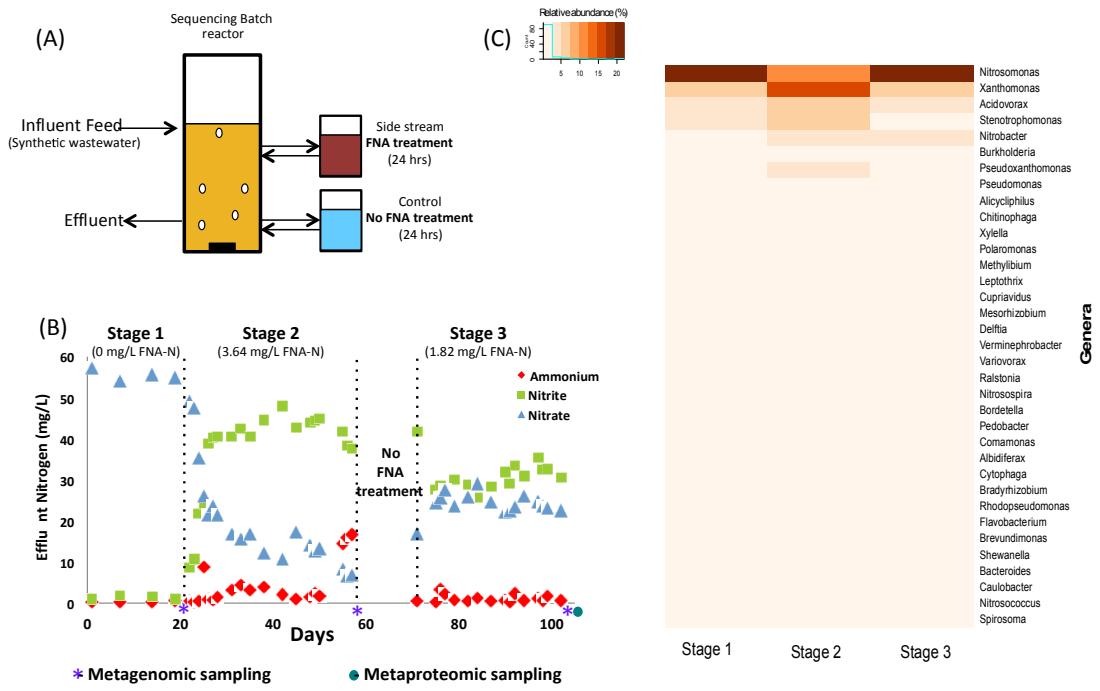
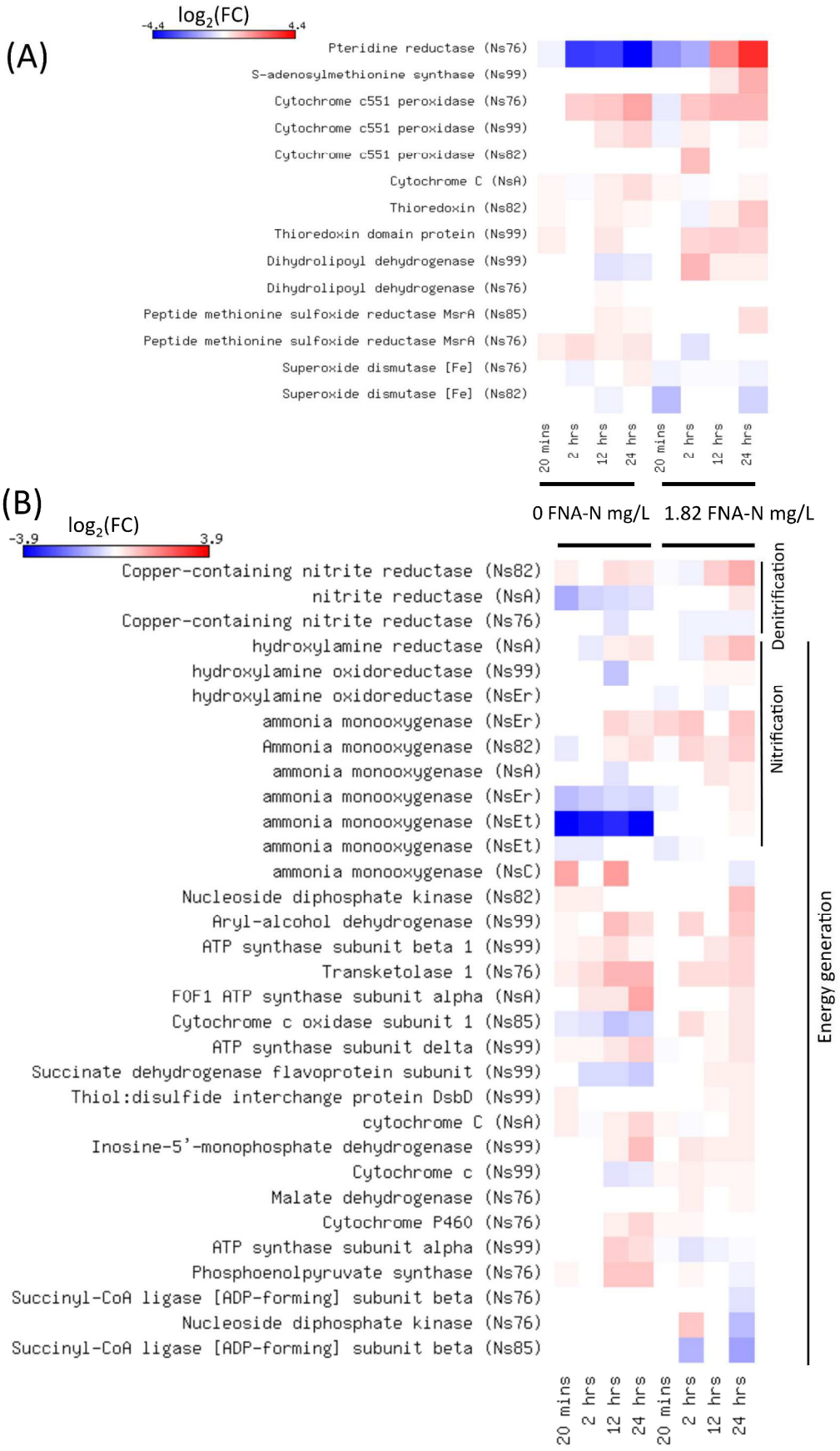
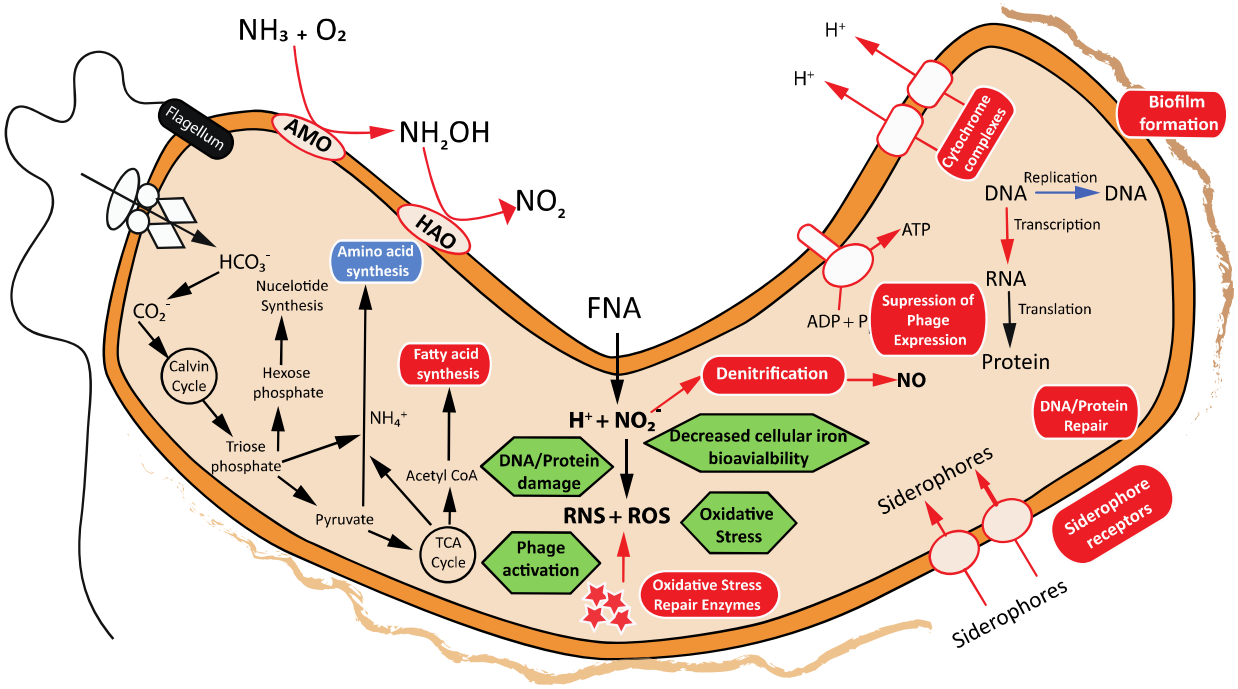


Figure 1. (A) A schematic representation of the configuration of the SBR reactor and the side stream reactor wherein FNA treatment was carried out. (B) Levels of ammonium, nitrate and nitrite detected in the effluent of the SBR reactor at the 3 different operational stages (Stages 1, 2, and 3) (C) The microbial community compositions of the SBR reactor during the 3 operational stages showing the dominant *Nitrosomonas* genus as derived from MG-RAST.



930 Figure 2: Heatmap showing the regulation of (A) Oxidative stress proteins and (B) enzymes

involved in the nitrogen metabolism and energy generation represented as $\log_2$(FC) across both the 0 and 1.82 mg/L FNA-N conditions. The names within the brackets indicate the population genomes from where the respective genes were derived as shown in Table 1. White sections within the heatmap represent data wherein triplicates did not meet the stringency levels of p-value $p \leq 0.05$. Blue represent a negative $\log_2$(FC) and red represents a positive $\log_2$(FC).



945 Figure 3: An overview model of the responses of *Nitrosomonas* on exposure to FNA. The red
946 arrows and boxes (▬) represent metabolic pathways that are upregulated, the blue arrows
947 and boxes (▬) represent metabolic pathways that are downregulated and the green boxes (▬)
948 indicate the main mechanisms of biocide action of FNA within the *Nitrosomonas* genus.
949