CHEMICAL INHIBITION OF NITRIFICATION: EVALUATING METHODS TO DETECT AND CHARACTERIZE INHIBITION AND THE ROLE OF SELECTED STRESS RESPONSES UPON EXPOSURE TO OXIDATIVE AND HYDROPHOBIC TOXINS

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ABSTRACT

This research first examined nitrification inhibition caused by different classes of industrially relevant chemicals on activated sludge and found that conventional aerobic nitrification was inhibited by single pulse inputs of every chemical tested, with 1-chloro-2,4-dinitrobenzene (oxidant) having the most severe impact, followed by alkaline pH 11, cadmium (heavy metal), cyanide, octanol (hydrophobic) and 2,4-dinitrophenol (respiratory uncoupler). Of the different chemicals tested, the oxidative and hydrophobic chemicals showed severe nitrification inhibition relative to other treatment processes and therefore deserved further investigation. For oxidative chemicals, we hypothesized that the more severe inhibition was because nitrifying bacteria lack one or more of the microbial stress response mechanisms used to mediate the toxic effect of oxidative chemicals. During these experiments, we showed that a rapid (minutes) antioxidant potassium efflux mechanism does not exist in two nitrifying bacteria, *Nitrosomonas europaea* and *Nitrospira moscoviensis*. Furthermore, we showed that another important antioxidant molecule, glutathione, was not oxidized as readily as in a non-nitrifying bacterium. Furthermore, we hypothesized that hydrophobic chemical-induced nitrification inhibition recovered more quickly because of the presence of membrane modification stress response mechanisms. While testing this hypothesis, we showed that *N. europaea* modified its cell membrane in response to hydrophobic chemicals using a long-term (hours) membrane modification mechanism that required the synthesis of new fatty acids, but it did not contain a short-term (minutes) response mechanism involving a *cis/trans* isomerase. Therefore, investigating these nitrifier stress responses showed that nitrifiers lack short-term stress responses that may be used to rapidly detect inhibition, indicating that conventional methods of detecting nitrification inhibition, like differential respirometry and nitrate generation rate (NGR), are still the fastest and easiest methods to use. Because several conventional methods exist, we also investigated differences between differential respirometry and a UV method we developed to measure NGR. During these tests, we showed that the UV NGR method provided a more reliable measure of nitrification inhibition than differential respirometry, and that the time to maximum nitrification inhibition depended on the properties of the chemical toxin, which implies that longer exposure times may be needed to accurately predict nitrification inhibition.

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
AMO	Ammonia Monooxygenase
ANAMMOX	Anaerobic Ammonia Oxidation
AOB	Ammonia Oxidizing Bacteria
ATU	Allylthiourea
BOD	Biochemical Oxygen Demand
CDNB	1-Chloro-2-4-Dinitrobenzene
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
DNP	2,4-Dinitrophenol
GGKE	Glutathione-Gated Potassium Efflux
GSH	Glutathione
HAO	Hydroxylamine Oxidoreductase
HRT	Hydraulic Residence Time
IC	Ion Chromatography
LPS	Lipopolysaccharide
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
NEM	N-ethylmaleimide
NGR	Nitrate Generation Rate
NOB	Nitrite Oxidizing Bacteria
NOR	Nitrite Oxidoreductase
NPR	Nitrite Production Rate
nSOUR	Nitrification Specific Oxygen Uptake Rate
OUR	Oxygen Uptake Rate
RND	Resistance-Nodulation-Cell Division
SBR	Sequencing Batch Reactor
SNGR	Specific Nitrate Generation Rate
SOUR	Specific Oxygen Uptake Rate
SRT	Solids Residence Time
TCMP	2-Chloro-6(Trichloromethyl)Pyridine
TKN	Total Kjeldahl Nitrogen
UV	Ultraviolet

1 EXECUTIVE SUMMARY

1.1 Introduction

Although several different methods can be used to treat wastewaters, biological wastewater treatment is the most commonly used. Because wastewater influents have a variable composition and can have both domestic and industrial sources, wastewater treatment facilities can occasionally receive shock loads of toxic chemicals that will upset the treatment process. Such upset events will disrupt different treatment processes, including BOD removal efficiency, nitrification, settleability and deflocculation. Unfortunately, few studies have been conducted under controlled conditions that examine the process upset effects induced by a toxic source (Love and Bott, 2000). Additionally, very little work has been done to try and understand the cellular level mechanistic cause of the upset effects induced by the toxicant (source). By studying the source-cause-effect relationships of different toxins, it is possible to better understand the nature of upset events and develop improved methods to mediate upset events or even to detect upset events before they occur. The initial goal of this research was to determine the source-effect relationships that are evoked in activated sludge treatment processes by several different chemical classes from common industrial sources. From the results of the source-effect studies, three hypothesis were developed and tested that examine detection methods and the molecular level mechanisms that may be responsible for the increased levels of inhibition that were found for one particular activated sludge treatment process: nitrification.

1.2 Phase 1 Research: Source-Effect Studies-Chapter 2

The initial phase of this research examined the process upset effects caused by six different industrial chemicals: an electrophilic solvent (1-chloro-2,4-dinitrobenzene, CDNB), a heavy metal (cadmium), a hydrophobic chemical (1-octanol), an uncoupling agent (2,4-dinitrophenol, DNP), alkaline and acidic pH, and cyanide in its weak metal complexed form. Respiratory inhibition testing was performed to determine the amount of chemical to add for each shock event. The concentrations that were found to inhibit respiration by 15, 25 and 50% of normal levels were used for these tests.

During these source-effect studies, a range of process upset effects were observed and a summary of these are listed on Table 1.1. The results presented in this table showed that the only

Table 1.1 Summary of Process Effects for Seven Source Chemicals for the 10-Day SRT Biomass Using a Pseudo-Quantitative Scale[a].

Measurement	Observed Problematic Process Effect Relative to Control	Cadmium	CDNB	Cyanide	DNP	Octanol	pH 11
Effluent Total Suspended Solids (TSS)/Volatile Suspended Solids(VSS)	Increase in effluent TSS/VSS	↓↓↓↓	↓↓↓	0	↓↓	+	↓↓↓↓
Effluent Chemical Oxygen Demand (COD)	Decrease in COD removal	↓↓↓↓	↓↓↓	↓	↓↓	+	↓↓↓↓
Specific Oxygen Uptake Rate (SOUR)	Decrease in SOUR	↓↓↓↓	↓↓↓	↓↓	↓↓/++[b]	↓↓	↓↓↓
Soluble Potassium	Increase in soluble K^+ concentration	↓↓	↓↓↓	0	0	0	X
Inorganic Nitrogen effluent concentrations and Nitrate Generation Rate (NGR)	Nitrification inhibition	↓↓	↓↓↓	↓↓↓	↓↓	↓↓	↓↓↓↓
Sludge Volume Index (SVI)	Increase in SVI	++	+	↓	0	0	+
Capillary Suction Time (CST)	Increase in CST	0	0	0	0	0	0

[a] The qualitative scale reflects the intensity of the effect for the IC_{50}-shocked reactors and the indicated NH_3 and pH shock level, in comparison to a negative control. The intensity scale ranges from ↓↓↓↓ (most intense process deterioration effect), 0 (no effect), and ++++ (most intense process improvement effect). X means inconclusive results.

[b] For DNP, there was an inhibition of respiration for the first 2 days, followed by a stimulation of respiration after 2 days

treatment process that was negatively impacted by every contaminant tested was nitrification. Furthermore, nitrification showed extremely long recovery times (Table 1.2). Together, this suggested that nitrification is the most sensitive of the wastewater treatment processes tested. The high sensitivity of nitrification to inhibition by chemical toxins has been found by others (Daigger and Sadick, 1998; Blum and Speece, 1991; Hockenbury and Grady, 1977), but these results present the first controlled test that examined nitrification inhibition relative to other wastewater treatment plant processes.

Table 1.2 Nitrate generation rate (NGR) inhibition levels relative to the control for the first sample collected after each toxin was added (6 hours after addition) and recovery times to control levels based on NGR and effluent nitrate.

NGR Percent Inhibition (7 Hours After Shock[a])			
	Low Concentration[b] (15% respiratory inhibition)	Mid Concentration[b] (25% respiratory inhibition)	High Concentration[b] (50% respiratory inhibition)
Cadmium	-	-	-
CDNB	100	98	100
Cyanide	70	95	100
DNP[c]	18 (-25)	65 (-30)	42 (-40)
Octanol	35	40	63
pH[d]	25	42	81

Time to Recovery (Days)						
	NGR	Effluent NO_3^-	NGR	Effluent NO_3^-	NGR	Effluent NO_3^-
Cadmium	-	2	-	6	-	11
CDNB	17	13	21	17	No Recovery	19
Cyanide	10	1	13	1	13	3
DNP	10	0	10	0	10	0
Octanol	4	No Recovery	4	No Recovery	No Recovery	No Recovery
pH	1	0	1	0	21	19

[a] Negative values indicate stimulation of NGR, positive values reflect decrease of NGR
[b] Dash (-) indicates no data available
[c] Values in parentheses indicate the maximum stimulation after recover from inhibition
[d] For pH inhibition, low concentration = pH 5, mid concentration = pH 9, high concentration = pH 11

Given these results, we more closely examined the nitrification inhibition noted during these experiments and found that the inhibition predicted using NGR was significantly higher than what was predicted using respirometry. During the initial phase of the study, nitrification-specific respiration was not examined, and we felt that the differences noted between the two

inhibition detection methods deserved further investigation. Furthermore, we observed that maximum nitrification inhibition did not occur immediately after a chemical was added, but rather several hours or days after it was added. Together, these suggested that the type of inhibition detection method and the exposure time used for detecting inhibition is very important in obtaining an accurate prediction of inhibition. If the method used to predict nitrification inhibition or the contact time used during the test did not accurately predict the full extent of inhibition, then operators using these methods could underestimate the extent of the effect and, consequently, make poor decisions regarding the steps required to prevent or mediate nitrification upset events. Therefore, we chose to investigate differences in nitrification inhibition detection methods and the time-dependence of inhibition as the next phase of research.

The source-effect experiments also revealed that two contaminants showed very high inhibition levels for nitrification relative to the other process parameters tested. These chemicals were the oxidative/electrophilic chemical CDNB and the hydrophobic chemical 1-octanol. CDNB showed the highest inhibition for nitrification and the longest recovery times (Table 1.2). Octanol showed high levels of inhibition relative to other process effects, but also showed relatively fast recovery times. We felt that the interesting results for nitrification inhibition observed for these two chemicals deserved further investigation. After an extensive literature review to determine possible causal mechanisms and stress responses for nitrification inhibition caused by oxidative and hydrophobic chemical inhibition, experiments investigating a few possible mechanisms were completed for each of these chemical classes.

1.3 Phase 2 Research: Inhibition Method Comparisons – Chapters 3 & 4

The source-effect experiments showed that respirometry and nitrate generation rate (NGR) tests did not predict the same level of nitrification inhibition and that maximum inhibition did not occur immediately. Given that the whole cell NGR method showed that tests lasting hours to days might be needed to predict the degree of nitrification inhibition, we pondered whether understanding and detecting the activation of stress mechanisms might provide a faster means to obtain a warning of nitrification inhibition. After an extensive literature review (presented in Chapter 2), the following hypothesis was developed:

Hypothesis: *The severity of nitrification inhibition by industrial toxins that is predicted using inhibition detection methods is time-dependent. In addition, the most accurate methods that are used to detect nitrification inhibition are based on a direct measure of the nitrification rate.*

In order to test this hypothesis, inhibition induced by CDNB, cadmium and chlorine bleach was measured using a differential respirometric technique and NGR. In addition, time dependence of the inhibition was examined by running both differential respirometry and NGR tests over a long term experiment (48-72 hrs) using 4L sequencing batch reactors (SBRs). NGR examines the rate of nitrate production and provides a direct measure of the nitrification rate. Differential respirometry examines a nitrification-inhibited oxygen uptake rate, or OUR, (conducted with nitrifier specific chemical inhibitors) relative to the total OUR to yield a nitrification OUR. Although it does not directly measure a product of nitrification, this is accepted as a measure of nitrification rate because the OUR of nitrifying bacteria stoichiometrically relates to the rate of nitrate generation (Grady et al., 1999).

When performing these experiments, we found that current methods for analyzing nitrite and conducting NGR tests were very time consuming. Therefore, we first set out to develop a more rapid method for performing NGR. A method using ultraviolet spectrophotometry at wavelengths between 225 and 240 nm without chemical manipulation was developed and verified against ion chromatography. The method was shown to quickly and accurately measure nitrate concentrations after correcting for nitrite interference. Additionally, cadmium, chlorine and CDNB were tested for nitrification inhibition using this method. Cadmium presented no interference with this method and CDNB was found to cause a correctable interference with the test. Only chlorine provided an uncorrectable interference for this method; therefore, tests using chlorine as an inhibitor relied upon time-consuming ion chromatography for measuring nitrate from NGR assays.

The UV-based NGR method was used to test differences between NGR and differential respirometry. The differential respirometry approach yield highly variable results versus NGR-based inhibition measurements, which were much more stable. These results suggest that a direct measure of nitrification, like NGR, provides a better measure of nitrification inhibition. The unreliable measurements provided by differential respirometry may be partly explained by reactions between the external nitrification inhibitor and the shock chemicals or components of the mixed liquor matrix. Some commercially available devices used to measure nitrification

inhibition are based on differential respirometric techniques and these results indicate that such devices may not accurately depict nitrification inhibition induced by chemical toxins.

Longer-term inhibition experiments showed that maximum nitrification inhibition did not occur immediately for cadmium and CDNB. These chemicals are not quickly removed from the wastewater stream and did not cause maximum inhibition until between 6 and 24 hours after the chemical shock was applied. However, chlorine, a chemical that reacts and dissipates very quickly, caused maximum inhibition almost immediately. These results suggest that inhibition is only time dependent if the inhibitory chemical is not quickly removed from the wastewater system and imply that measurement techniques should be performed over a longer time period to determine maximum inhibition.

1.4 Phase 3 Research: Oxidative Stress Responses in Nitrifying Bacteria – Chapter 5

The objective of the next phase of research was to determine the reason why oxidative chemicals like CDNB inhibited nitrification to such a significant extent. To do this, a thorough literature review was conducted to determine both the inhibitory mechanisms of oxidative chemicals and the stress response mechanisms bacteria have to combat oxidative stress (presented in Chapter 1). Results of this survey showed that oxidative chemicals cause inhibition in bacteria by damaging proteins and DNA (Ferguson et al., 1996; McLaggan et al., 2000). Several oxidative stress response mechanisms were also found that appear to be highly conserved in many different Gram-negative bacteria. Because the genome of the ammonia oxidizing bacterium *Nitrosomonas europaea* was recently sequenced (Chain et al., 2003), we were able to search the genome for the presence of these mechanisms (genes were present if $e \leq 0.001$). The results of this search are shown in Table 1.3 below. Examining this table, it appears that the genes encoding a majority of the protective enzymes are present in *N. europaea*, even though the regulatory mechanisms are absent or different from those found in other bacteria. The one mechanism that did not appear to contain the enzymes required for proper function was the glutathione gated potassium efflux (GGKE) mechanism first described by Kroll and Booth (1981). This mechanism induces cytoplasmic acidification which protects proteins and DNA and activates other protective enzymes. It is thought to be highly conserved, as it was found in several Gram-negative heterotrophic species(Booth et al., 1993). Taken together, this

information led us to develop the following hypothesis to explain why nitrifying bacteria appear to be more sensitive to oxidative chemicals than other bacteria:

Hypothesis: *Autotrophic nitrifying bacteria are more sensitive to shock loads of oxidative chemical toxins because they lack a potassium efflux mechanism to help protect against oxidative shock.*

To address this hypothesis, we monitored nitrifying bacteria for potassium efflux in response to the oxidative chemicals N-ethylmaleimide and chlorine bleach. We also examined if glutathione is produced and oxidized in nitrifying bacteria exposed to electrophiles. Glutathione is a small tripeptide molecule that is used to regulate the known GGKE mechanism. In addition, glutathione itself works to help mediate oxidative chemical shock by acting as a sacrificial nucleophile which gets oxidized, instead of allowing proteins and DNA to be damaged by electrophilic stressors.

Experiments were performed using a nitrifying enrichment culture and pure cultures of the ammonia oxidizing bacterium (AOB) *N. europaea* and the nitrite oxidizing bacterium (NOB) *Nitrospira moscoviensis.* *Pseudomonas aeruginosa*, a heterotrophic bacterium with a sequenced genome, was used as a positive control organism for all tests. For these experiments, soluble potassium levels were monitored to observe increases associated with the GGKE mechanism, or a surrogate potassium efflux mechanism. Results using the enrichment culture suggested that nitrifiers did not efflux potassium in response to oxidative toxins. Because of this, further studies were planned using pure cultures of nitrifying bacteria to address the hypothesis more clearly. Results using the pure cultures of nitrifying bacteria suggest that neither *N. europaea* nor *Ni. moscoviensis* efflux potassium in response to the oxidative chemical N-ethylmaleimide. Both released potassium in response to the ionophore nigericin, which suggested that potassium was present in the cells and could be release without lysing the cells. These results indicated that nitrifying bacteria do not contain a GGKE mechanism or a surrogate potassium efflux mechanism to respond to oxidative stressors, which helps to explain why nitrification is more sensitive to oxidative chemical upset than other treatment processes. Furthermore, it presents the first evidence for a Gram-negative bacterium that does not contain the GGKE mechanism. These organisms may not contain a GGKE mechanism because the induced cytoplasmic acidification would be detrimental to electron transport and energy generation in nitrifying bacteria. In addition, it has been thought that GGKE originally evolved as a protection mechanism for methyglyoxal, which is an oxidative byproduct of glucose metabolism (Ferguson

et al., 2000; Ferguson and Booth, 1998). *N. europaea* has historically been classified as an obligate chemolithoautotroph that uses inorganic ammonia as its energy source and fixes carbon dioxide as its carbon source. Recently, it was found to be a facultative chemolithoorganotroph that can also use selected organic compounds, such as pyruvate and fructose but not glucose, as a carbon source (Hommes et al., 2003). Consequently, it has been well established that *N. europaea* does not metabolize glucose, which means it does not produce methylglyoxal and would have no need to develop the GGKE response. Therefore, the GGKE mechanism may not have evolved in, or was removed from nitrifying bacteria because it may interfere with the metabolism of ammonia and nitrite.

Table 1.3 Summary of selected genes involved in oxidative stress response mechanisms and their presence in the *N. europaea* genome.

Gene Name	Function	Presence in *N. europaea*
Genes involved in the GGKE mechanism		
gshB	glutathione synthetase	Yes
gst	glutathione S-transferase	Yes
gorA	glutathione oxidoreductase	No
kefB	Glutathione regulated potassium efflux system protein KefB	No[a]
kefC	Glutathione regulated potassium efflux system protein KefC	No[a]
dps	DNA binding protein Dps	No
Genes involved in the *oxyR* system		
oxyR	*oxyR* system regulatory protein OxyR	No
katG	hydroperoxidase/catalase	No[b]
aphCF	alkyl hydroperoxide reductase	No[b]
grxA	glutaredoxin	No[b]
trxC	thioredoxin	No[b]
oxyS	regulatory RNA	No
Genes involved in the *soxRS* system		
soxR	*soxRS* system regulatory protein SoxR	No
soxS	*soxRS* system regulatory protein Soxs	No
sodA	Manganese superoxide dismutase	No[b]
tolC	outer membrane efflux protein TolC	Yes[c]
arcAB	multidrug resistance efflux system arcAB	No[d]
***rpoS* regulated oxidative stress response genes**		
rpoS	sigma factor S	No
katE	hydroperoxidase/catalase	No[b]
nth	endoonuclease	No
sodC	copper-zinc superoxide dismutase	No
xthA	Exonuclease III	Yes[c]
topA	topoisomerase I	No[b]

[a] pH adaptation potassium efflux system protein F and D found, but not glutathione regulated
[b] homologous gene with unknown regulation mechanism found
[c] BLAST search yielded protein with similar coding strand
[d] *arcAB* not found but numerous multidrug efflux pumps located in genome

Concentrations of the total and oxidized forms of glutathione were also monitored to determine the behavior of glutathione in response to varying concentrations of chlorine bleach. These results showed that glutathione was oxidized in both *N. europaea* and *P. aeruginosa*, but

that the amount of glutathione oxidized per mg of chlorine added was less in *N. europaea*. This may be due to the large membrane structures of nitrifying bacteria, which may prevent oxidative chemicals from entering cells and reacting with glutathione quickly. Coupled with known genomic information, the results of this study provide greater insight into why nitrification is one of the most susceptible processes in biological wastewater treatment.

1.5 Phase 4 Research: Hydrophobic Stress Responses in Nitrifying Bacteria – Chapter 6

The objective of the final phase of the research was to examine why nitrification was inhibited by the hydrophobic chemical 1-octanol, but recovered more quickly from the shock event. After performing a review of the available literature (Chapter 1), we found that hydrophobic chemicals cause inhibition mainly by interacting with the cell membrane of bacteria (Sikkema et al., 1995). This has been found to affect the membrane fluidity (Sikkema et al., 1994) and cause swelling of the membrane bilayer (Aono et al., 1994). The membrane swelling and fluidity alterations have been associated with leakage of macromolecules and ions out of the cells (Aono et al., 1994; Heipieper et al., 1991), which causes a disruption of the proton gradient and membrane potential (Sikkema et al., 1994). Recovery from hydrophobic stress events has been associated with several response mechanisms, and search of the *N. europaea* genome revealed that genes coding for these enzymes exist (Table 1.4). As Table 1.4 shows, several multidrug efflux systems exist in *N. europaea* to remove the toxins from the cells and membranes. In addition, enzymes that modify the cell membrane to combat the fluidity changes associated with hydrophobic shock were found. Given that several mechanisms were found, we developed the following hypothesis:

Hypothesis: *Hydrophobic organic chemicals are toxic to nitrifying organisms due to the insertion of these chemicals into the cell membranes, and recovery is mediated by altering the membrane structure.*

To test this hypothesis, the research objective was to determine if the membrane fatty acid content is altered in response to hydrophobic chemicals. This was used to both confirm insertion of the chemicals into the membrane as well as confirm that nitrifiers contain stress mechanisms to alter membrane fatty acid composition in response to changes in membrane fluidity induced by hydrophobic chemicals.

Experiments were performed using pure cultures of the AOB *N. europaea.* *P. aeruginosa* was used as a positive control organism for all tests. For these experiments, changes in the fatty

acid content of the cell membrane were measured in response to shock loads of 1-octanol. *P. aeruginosa* showed an increase in the amount of saturated fatty acids and an increase in the relative amounts of cis isomers to trans isomers of C18 unsaturated fatty acids. Results obtained with *N. europaea*showed that the cells modified the saturated-to-unsaturated fatty acid ratio in response to the hydrophobic chemical 1-octanol, and the modification of this ratio corresponded with recovery of the nitrite production rate, indicating that modifying the membrane may contribute to recovery of the ammonia oxidizing capabilities of *N. europaea.* This agrees with our hypothesis that membrane modifications occur in response to hydrophobic chemical shock that allow for nitrification recovery, but the lack of any cis-to-trans ratio modifications contradicts the genomic information that indicates this organism should be capable of modifying the cis-to-trans ratio as a short-term stress response. Although this study provides evidence that some membrane modification mechanisms exist, other stress response mechanisms may exist and more research is needed in this area to determine exactly what mechanisms are activated in the presence of hydrophobic contaminants.

Table 1.4 Summary of select genes involved in hydrophobic stress response mechanisms and their presence in the *N. europaea* genome.

Gene/Protein Name	Function	Presence in *N. europaea*
Membrane Modification Mechanisms		
β-ketoacyl-ACP synthase II	modification of membrane fatty acid composition	Probable Homolog Found
cti	fatty acid *cis-trans* isomerase	Probable Homolog Found
Efflux Systems		
AcrA-AcrB-TolC	multidrug/solvent efflux system	Probable Homologs Found
AcrR	*acrAB* system regulator	No
MexA-MexB-OprM	multidrug/solvent efflux system	Probable Homologs Found
MexC-MexD-OprJ	multidrug/solvent efflux system	Probable Homologs Found
MexE-MexF-OprN	multidrug/solvent efflux system	Probable Homologs Found
MexR	*mex* systems regulator	No
SrpA-SrpB-SrpC	multidrug/solvent efflux system	Probable Homologs Found[a]
TtgA-TtgB-TtgC	multidrug/solvent efflux system	Probable Homologs Found[a]
MepA-MepB-MepC	multidrug/solvent efflux system	Unknown[b]
MepR	*mepABC* system regulator	Unknown[b]

[a] Homologous SrpB sequence in *N. europaea* is the same sequence homologous to TtgB
[b] *mepABC* and *mepR* could not be checked against *N. europaea* genome as gene sequences could not be located

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2 LITERATURE REVIEW ON FACTORS THAT INFLUENCE NITRIFICATION INHIBITION IN ACTIVATED SLUDGE CULTURES

2.1 Nitrification Inhibition

2.1.1 Overview of Nitrification Inhibition

Ammonia toxicity to aquatic organisms has been well documented using both invertebrate organisms like cladocerans (Sarma et al., 2003) and vertebrates like fish (Hillaby and Randall, 1979; Wicks et al., 2002). Due to this toxicity, it is important to remove ammonia or convert it to another form before discharging ammonia-containing wastewaters into rivers, lakes and streams. To this end, numerous processes have been developed using both chemical and biological methods of ammonia removal. Of these processes, the most common biological process for ammonia removal is conventional aerobic nitrification, which involves the conversion of ammonia to nitrate through biological oxidation. This conversion is accomplished using two genera of bacteria, the ammonia oxidizing bacteria (AOB) that oxidize ammonia to nitrite, and the nitrite oxidizing bacteria (NOB) that convert the nitrite produced by the AOB to nitrate. Aerobic nitrification is used not only to convert ammonia to a less toxic form (nitrate) before discharge, but it is also commonly used for total nitrogen removal at wastewater plants. Nitrification is important in total nitrogen removal because many wastewater treatment facilities that are required to remove total nitrogen must convert the ammonia to nitrate during nitrification before the nitrate can be converted to gaseous nitrogen during the anoxic process of denitrification. As regulations governing ammonia and total nitrogen discharge into receiving waters become more strict, the extent to which nitrification is utilized will grow. Thus, it becomes very clear that aerobic nitrification remains a very important process in conventional wastewater treatment.

In an ideal world, nitrification of wastewaters would occur without any difficulties under a variety of operational conditions. Unfortunately, this is not the case and nitrification can be disrupted, or "upset" very easily. These upset events can be caused by operational and design problems or induced by shock loads of a variety of industrial chemical toxins. In fact, the process of nitrification has been found to be more sensitive to such upset events than processes such as BOD removal. In a study performed by Blum and Speece (1991), the inhibition of ammonia oxidation by *Nitrosomonas europaea*, a common ammonia oxidizing bacterium, was

examined with relation to the respiration inhibition of activated sludge aerobic heterotrophs. During the study, they found that the concentration causing 50% inhibition of ammonia oxidation was substantially lower than the concentration causing 50% inhibition of respiration in the heterotrophs for nearly all of the chemicals tested. In fact, of 67 chemicals tested, only 7 were found to have similar or lower impact on the nitrifying organism than the heterotrophs. In a similar study performed by Wood et al. (1981), a comparison was made between the nitrification efficiency and BOD removal efficiency of an activated sludge exposed to different chemical toxins. Their results were in agreement with those found by Blum and Speece in that many compounds appeared to be less toxic to carbonaceous BOD removal (performed by heterotrophic bacteria) than to ammonia oxidation and nitrate production. In addition to results found by others, studies performed in our laboratory have also found the nitrification process to be especially susceptible to inhibition when compared with other processes like COD removal, respiration and effluent suspended solids removal (Love et al., 2002a; Love et al., 2002b; Love et al., 2003). Table 2.1 summarizes the effects from six different chemicals tested in our lab on different wastewater treatment processes. The scale presented is pseudo-quantitative and based on the relative impact of each process effect and comparisons of impact between contaminants.

Table 2.1 Summary of process effects for nitrifying biomass exposed to five chemical toxins using a pseudo-quantitative scale[1].

Measurement	Observed Problematic Process Effect Relative to Control	Cadmium	CDNB	Cyanide	DNP	Octanol	pH 11
Effluent Total Suspended Solids (TSS)/Volatile Suspended Solids(VSS)	Increase in effluent TSS/VSS	↓↓↓↓	↓↓↓	0	↓↓	+	↓↓↓↓
Effluent Chemical Oxygen Demand (COD)	Decrease in COD removal	↓↓↓↓	↓↓↓	↓	↓↓	+	↓↓↓↓
Specific Oxygen Uptake Rate (SOUR)	Decrease in SOUR	↓↓↓↓	↓↓↓	↓↓	↓↓/++[b]	↓↓	↓↓↓
Soluble Potassium	Increase in soluble K^+ concentration	↓↓	↓↓↓	0	0	0	X
Inorganic Nitrogen effluent concentrations and Nitrate Generation Rate (NGR)	Nitrification inhibition	↓↓	↓↓↓	↓↓↓	↓↓	↓↓	↓↓↓↓
Sludge Volume Index (SVI)	Increase in SVI	++	+	↓	0	0	+
Capillary Suction Time (CST)	Increase in CST	0	0	0	0	0	0

[a] The qualitative scale reflects the intensity of the effect for the IC_{50}-shocked reactors and the indicated NH_3 and pH shock level, in comparison to a negative control. The intensity scale ranges from ↓↓↓↓ (most intense process deterioration effect), 0 (no effect), and ++++ (most intense process improvement effect).
X means inconclusive results.
[b] For DNP, there was an inhibition of respiration for the first 2 days, followed by a stimulation of respiration after 2 days.

2.1.2 Methods Used to Detect Nitrification Inhibition

Detecting nitrification inhibition is important for identifying and preventing nitrification upset from occurring. Several methods exist for determining nitrification inhibition in activated sludge treatment systems by comparing nitrification rates. One of the most common of the rate detection methods is respirometry (Ren, 2004). Respirometry is a rapid test (often < 20 minutes) and is accepted as a measure of nitrification rate because the oxygen uptake rate (OUR) of nitrifying bacteria stoichiometrically relates to the rate of nitrate generation (White, 2000; Grady et al., 1999). Unfortunately, it measures the total oxygen uptake rate of all biomass, not just the

nitrifiers. To determine the OUR of only the nitrifiers in mixed liquor, two different techniques have been applied. The first technique involves aerating the biomass for several hours without any substrate so that the biomass is in an endogenous state. The OUR of the total endogenous biomass is measured and compared with the OUR of biomass to which ammonia was added so that only the nitrifying bacteria are respiring above the endogenous state. The difference between these rates gives the OUR of the nitrifying bacteria, which provides a surrogate measure of the rate of nitrification (Hu et al., 2003a; Hu et al., 2003b; Chandran and Smets, 2000).

The other respirometric technique commonly employed uses selective inhibitors of nitrification to determine the OUR of the nitrifying bacteria. This technique, referred to as differential respirometry, can be used on actively growing biomass because it does not require the endogenous OUR. In this technique, the OUR of the total biomass is measured and compared to the OUR of a biomass in which a chemical inhibitor specific to nitrification was added so that only the nitrifying bacteria are not respiring. The nitrification OUR is determined by subtracting the nitrification-inhibited OUR from the total OUR (Ginestet et al., 1998, Chandran and Smets, 2000). The nitrification specific inhibitor used in differential respirometry is typically either allylthiourea or 2-chloro-6-(trichloromethyl)pyridine (APHA, 1998; Benes et al., 2002; Reuschenbach et al., 2003).

Another method that is used to determine nitrification rates for inhibition determination is a base titration method. This test is also very rapid and similar to respirometric tests, the validity of this method is based on the stoichiometric relationship between the acid produced and nitrate generated during the nitrification process (White, 2000; Grady et al., 1999). During this test, a known concentration of a strong base is used to maintain a constant pH within the biomass over the time period of the test. By monitoring the exact rate of base addition, the nitrification rate can be determined from stoichiometry (Ficara and Rozzi, 2001).

The last method for measuring nitrification rates is the nitrate generation rate (NGR) test. During this test, nitrate production is measured over time and the rate of nitrate production gives the rate of nitrification. Unlike respirometric or titrimetric techniques, NGR detects a unique product of nitrification and, therefore, provides a direct measure of the nitrification rate. Although it does not require addition of any chemicals like differential respirometry or base titration, it does generally take longer to perform than either of these methods because

measurements of nitrate are not able to be made continuously (Grunditz and Dalhammar, 2001; Hooper and Terry, 1973; Lee et al., 1997).

2.1.3 Influence of Operational Practices on Nitrification

From an operational standpoint, nitrification has been found to be very unstable at low solids retention times (SRT). This effect was described by Poduska and Andrews (1974), who noted that the percent nitrification of wastewaters occurring at activated sludge plants increased with increasing SRT. This increase was very drastic, going from almost no nitrification to nearly 100% nitrification over an SRT range of less than one day. These results suggest that a minimum retention time is required for stable nitrification to occur. Results observed by McClintock et al. (1993) also support this idea. In their study, the removal of ammonia nitrogen increased with increasing SRT for a given temperature. In addition, McClintock also examined changes in the rate of nitrification with changes in SRT and found that the rate of nitrification was faster at shorter SRTs than longer SRTs, but incomplete nitrification still occurred.

Together, these results suggest that the instability of nitrification at short SRTs values is not due to inhibition, but rather is likely due to the slow growth of the autotrophic nitrifying bacteria. At short SRTs, nitrifying organisms would be washed out of a system because they are growing too slowly to multiply and remain within the system. The washout was confirmed by Abeysinghe and coworkers when they studied the effects of bioaugmentation on nitrifying systems at short SRT values (Abeysinghe et al., 2002). During this study, the authors showed that nitrification did not occur at a 2 day SRT but could be operated if nitrifying bacteria were added to the wastewater. After a one-time addition of the organisms, it was noted that ammonia oxidation to nitrate decreased with time and an analysis of the population of nitrifying organisms showed a concurrent decrease, suggesting washout was responsible for the loss of nitrification. Overall, this suggests that washout of nitrifying organisms at short SRT is the reason for the observed loss of nitrification.

Loss of nitrification due to washout of the biomass can occur through mechanisms other than short SRT. For instance, the growth rate of nitrifying organisms is known to be affected by temperature (Abeysinghe et al., 2002; Grady et al., 1999; Hooper and Terry, 1973; McClintock et al.,1993). The effect of temperature on the rate of nitrification was evaluated by measuring the ammonia nitrogen remaining in various bioreactors using the same biomass but exposed to

different temperatures, ranging from 25ºC to 10ºC. Results observed by Hooper and Terry (1973) and McClintock et al. (1993) show that lower temperatures yield a reduced nitrification efficiency. A study performed by Neufeld et al. (1986) found the same reduced efficiency for temperatures lower than 15ºC, but also found that temperatures above 30ºC also decreased the rate of oxidation and efficiency of nitrification. Abeysinghe and coworkers (2002) used the information gained about decreased rates of nitrification due to temperature and evaluated the washout of nitrifying bacteria at low temperatures during bioaugmentation. During their study, a 5 day SRT reactor was operated at 22ºC and full nitrification occurred. However, when the reactor temperature was dropped to 4ºC, it was noted that nitrification did not occur to any great extent. Upon direct addition of nitrifying organisms, ammonia oxidation began to occur but could not be maintained without constant addition of new organisms. To test if this was due to washout, the concentration of nitrifying organisms was monitored after a one-time addition to the low temperature reactor. Immediately, the nitrifier population began to decrease, which coincided with the observed reduced nitrification efficiency. Together, their data suggest that the loss of nitrification at low temperatures is due to a depressed growth rate in the nitrifying organisms that results in their washout from the reactor. This is supported by results presented by Grady et al. (1999) and McClintock et al. (1993), where increasing the SRT of low temperature inhibited nitrifying reactors can increase the nitrification efficiency by increasing the detainment of nitrifying bacteria. Thus, it appears that a major mechanism for loss of nitrification may be due to the washout of nitrifying organisms, which could not only be due to a decreased retention time for the biomass, but also due to inhibition of growth that can be caused by a number of sources, including temperature deviations and inhibition by chemical toxins.

2.1.4 Influence of Chemicals on Nitrification

Much of the research on nitrification inhibition by chemical toxins has been focused on identifying which chemicals affect the nitrification process. Several comprehensive studies on the impact of chemicals on nitrification inhibition have been performed. Possibly the earliest and most extensive study was performed by Tomlinson and coworkers (1966) who found that nitrification in activated sludge is adversely affected by a very wide range of industrial chemicals from cyanides and azides to sulfur compounds, phenolic compounds, alcohols, heavy metals, metal binding agents and antibiotic drugs like hydroxyquinoline and streptomycin. Since then,

several other studies have been performed, including the study by Blum and Speece (1991) that compared organism sensitivities. During their study, they found that ammonia oxidizing bacteria are adversely affected by many organic hydrophobic chemical compounds, including aromatics like xylene and toluene, chlorinated aromatics, alcohols like octanol, many respiratory uncouplers, many aliphatic and halogenated aliphatics, ethers, ketones and nitrile compounds. Hooper and Terry (1973) also performed an extensive study confirming much of what was previously known about inhibition of ammonia oxidizing bacteria by metal binding compounds, respiratory uncouplers and alcohols, but they also found that many enzyme and heme-protein binding compounds also inhibited pure cultures of ammonia oxidizing bacteria. A more detailed description of some of the more come commonly studied inhibitors of nitrifying bacteria follows.

Cyanide is one of the more often studied chemical inhibitors of nitrification. Cyanide can be widely present in wastewaters due to its use in metal-plating and its presence in flue-scrubber gas wash waters. The presence of even small amounts of cyanide has been found to be extremely detrimental to the nitrification process, with concentrations of free cyanide (HCN or CN^-) as low as 0.1 mg/L inhibiting nitrification in activated sludge (Neufeld et al., 1986). Neufeld et al. (1986) also showed that metal-cyanide complexes and thiocyanates were toxic, but to a much lesser extent. The influence of cyanide on activated sludge has not only been studied on laboratory-scale activated sludge and enrichment culture systems (Gaudy et al., 1982; Gernaey et al., 1997; Ludzack and Schaffer, 1962; Neufeld et al., 1986; Tomlinson et al., 1966), but also at full-scale treatment facilities (Daigger and Sadick, 1998).

Heavy metals are another group of widely studied and important nitrification inhibitors . Heavy metals like cadmium, chromium, copper, lead, mercury and zinc are widely used in industry and commonly end up in wastewater streams. Because heavy metals are prevalent in wastewater, numerous studies have been performed to examine the effects of heavy metals on activated sludge (Braam and Klapwijk, 1981; Gernaey et al., 1997; Lee et al., 1997) and pure cultures (Sato et al., 1988; Tomlinson et al., 1966). In all cases, heavy metals were been found to be toxic to nitrification, but to varying degrees.

Copper is known to be inhibitory to nitrification (Gernaey et al., 1997; Braam and Klapwijk, 1981; Tomlinson et al., 1966). However, the results reported by Tomlinson et al. for copper yield interesting results, as low concentrations were found to stimulate nitrification in pure cultures of AOB. Copper has also been found to increase the ammonia oxidizing activity of

cell-free extracts of AOB (Ensign et al., 1993; Juliette et al., 1995). Because of these studies, it has been hypothesized that an essential enzyme (ammonia monooxygenase) used in ammonia oxidation contains a copper active site (Bedard and Knowles, 1989). (The enzymatic pathways for both ammonia oxidation and nitrite oxidation are described in sections 2.2.1 and 2.2.2, respectively) This has been supported by data showing the extreme sensitivity of nitrification to metal binding compounds like allylthiourea (Hooper and Terry, 1973; Tomlinson et al., 1966). These metal binding compounds seem to only selectively inhibit the ammonia oxidizing bacteria and not the NOBs or heterotrophic bacteria, as reported by Ginestet et al. (1998).

Many organic metal chelating compounds that are found to be inhibitory to nitrification contain sulfur groups (Hockenbury and Grady, 1977). Not only are metal chelating sulfur compounds inhibitory, but a wide range of these chemicals have been found to be toxic to nitrifiers (Hockenbury and Grady, 1977; Hooper and Terry, 1973; Tomlinson et al., 1966). Juliette and coworkers (1993) found that thioethers were inhibitory to AOBs because they acted as a competitive substrate and are broken down to sulfoxides. In addition, even the sulfur containing amino acids cysteine and methionine have been found to inhibit nitrification (Apontoweil and Berends, 1975; Ferguson et al., 1995; Wagner and Loy, 2002). Together, these results show the extreme toxicity of sulfur containing compounds to nitrification, and AOB in particular.

Upon further examination of the different chemical upsets that occur, it appears that solution pH plays a very important role in the stability of nitrification, as pH extremes can be inhibitory to the bacteria that carry out this process. It has been reported that AOB grow optimally between pH 5.8 and 8.5, while NOB grow best from pH 6.5 to 8.5 (Princic et al., 1998). Princic and coworkers also found that nitrification was inhibited at pH values below 5.8 for a nitrifying mixed culture enriched from activated sludge. However, when examining pH effects on nitrifying organisms it is important to remember the speciation of ammonia and nitrite, the two substrates used for energy production in AOB and NOB. It had previously been known that ammonia and nitrite were actually toxic to the organisms that utilize and produce them. In a revolutionary study by Anthonisen and coworkers (Anthonisen et al., 1976), it was determined that inhibition was due to free ammonia (NH_3) and nitrous acid (HNO_2) rather than ammonium (NH_4^+) or nitrite (NO_2^-). The speciation of these, and therefore the toxicity of each, is dependent upon pH and concentration, where significant portions of the total nitrite is in the form of nitrous

acid at low pH values and a significant percentage of the total ammonia is in the free ammonia form at high pH values. In addition, Anthonisen et al. found that AOB were less sensitive to free ammonia than the NOB, but no significant difference was noted between the organisms for nitrite inhibition. Thus, when pH is considered as an inhibiting agent of nitrification, it is important to consider the effects of speciation on other chemicals in the solution.

Research done in our laboratory also examined the effects of selected chemicals on nitrification in activated sludge (Kelly et al., 2004). For our experiments, six different chemicals were tested from six different chemicals classes (defined by their toxic mode of action) in order to determine which chemical classes may inhibit nitrification and to what extent. The chemical classes (and the representative chemical studied from that class) used in these experiments were: heavy metal (cadmium), cyanide, pH (pH 11), electrophile/oxidative chemical (1-chloro 2,4-dinitrobenzene/CDNB), organic respiratory uncoupler (2,4-dinitrobenzene/DNP) and a hydrophobic organic chemical (octanol). During our study, a one-time shock of the chemical was added to sequencing batch reactors containing a nitrifying activated sludge, and both nitrate generation rates (NGRs) and effluent nitrogen species were monitored over time. In addition, time to full recovery of nitrification was determined during this experiment. The results of this study are summarized on Table 2.2 and reflect the impact of low, mid and high concentrations of each chemical (i.e., concentrations that caused 15, 25 and 50% respiratory inhibition in activated sludge, respectively) on both NGR and the time to recover from nitrification inhibition.

When examining this data in conjunction with the data presented in Table 2.1 above, two things become very apparent. First, every chemical we tested impacted nitrification to some extent, which is consistent with the sensitivity of nitrification and nitrifying bacteria found by others. Second, the inhibition caused by each chemical and the time to it took to recover from that inhibition varied substantially with the type and class of chemical tested. From this data, it appears that the most severe inhibition and longest recovery times for nitrification was due to the electrophilic chemical CDNB. Very little is known about these chemicals as far as their impact on nitrification as few detailed studies have targeted them. To date, no literature has been found that has specifically examined the effects of CDNB on nitrifying biomasses, making CDNB and other electrophiles prime candidates for further study.

Table 2.2 NGR inhibition levels relative to the control for the first sample after each toxin was added, and recovery times to control levels based on NGR and effluent nitrate.

NGR Percent Inhibition (7 Hours After Shock[a])			
	Low Concentration[b]	Mid Concentration[b]	High Concentration[b]
Cadmium	-	-	-
CDNB	100	98	100
Cyanide	70	95	100
DNP[c]	18 (-25)	65 (-30)	42 (-40)
Octanol	35	40	63
pH[d]	25	42	81

Time to Recovery (Days)						
	NGR	Effluent NO_3^-	NGR	Effluent NO_3^-	NGR	Effluent NO_3^-
Cadmium	-	2	-	6	-	11
CDNB	17	13	21	17	No Recovery	19
Cyanide	10	1	13	1	13	3
DNP	10	0	10	0	10	0
Octanol	4	No Recovery	4	No Recovery	No Recovery	No Recovery
pH	1	0	1	0	21	19

[a] Negative values indicate stimulation of NGR, whereas positive values reflect decrease of NGR relative to the control
[b] Dash (-) indicates no data available
[c] Values in parentheses indicate the maximum stimulation after recover from inhibition
[d] low concentration = pH 5, mid concentration = pH 9, high concentration = pH 11

Another chemical that showed significant inhibition was the organic hydrophobic chemical, 1-octanol. Although octanol and other hydrophobic chemicals and alcohols have been found to inhibit nitrifying bacteria (Blum and Speece, 1991), little work has been done to examine the causes of such inhibition by these compounds. In one study performed by Keener and Arp (1994), the effects of the aromatic compounds benzene, toluene and xylene on the AOB *N. europaea* were examined. Ammonia oxidation was found to be significantly inhibited; however, the authors also found that the toxins were oxidized to phenolic products and that ring cleavage and complete mineralization did not occur for these products. It was suggested by the authors that the inhibition of nitrification by these compounds was due to interference with ammonia monooxygenase (AMO), which catalyzed the oxidation of these compounds. Although direct interference with AMO availability through competition is one possible inhibition

mechanism for hydrophobic organic contaminants, there may be other causes of inhibition that have not yet been investigated.

AMO is just one enzyme involved in the ammonia oxidation pathway. The other major enzyme, described in more detail below, is hydroxylamine oxidoreductase (HAO). Several studies have been performed to investigate the differential inhibition of these two molecules. The general consensus of those studies is that, for a given chemical inhibitor, AMO is the more sensitive enzyme (Hooper and Terry, 1973; Tomlinson et al., 1966). These conclusions were drawn through experiments where hydroxylamine was added to ammonia oxidizing cultures. Hydroxylamine is the product of AMO-controlled ammonia oxidation and the substrate used by HAO to produce nitrite. Upon addition of hydroxylamine with a toxicant, the authors found that the rate of nitrite formation increased when compared to addition of the toxicant alone, suggesting that AMO was the more sensitive enzyme in the ammonia oxidation pathway. Thus, it is most likely that any chemical causing inactivation/inhibition of the enzymes in the ammonia oxidation pathway would be directed against AMO and not due to inhibition of HAO. For NOB, one major enzyme controls the oxidation of nitrite to nitrate, and therefore differential inhibition of the enzymes in the pathway is not an issue.

From the vast amount of information available regarding nitrification inhibition, it is clear that nitrification is a biological process that is very sensitive to upset from a wide variety of sources, both physical and chemical. Due to the importance of the nitrification process to wastewater treatment and the increased sensitivity of autotrophic nitrifying bacteria to toxic shock loads of chemical contaminants, it becomes important to study the effects of chemical toxins on the nitrification process, and extensive screening studies have occurred that examine which chemicals cause nitrification. However, to date very few studies have been performed that examine the molecular level stress responses elicited by nitrifying bacteria in response to toxic chemicals. One objective of this study will be to conduct an initial evaluation of the role that stress response mechanisms in nitrifying bacteria play to counteract the adverse effects of oxidative chemicals and organic hydrophobic chemicals.

2.2 Cell Biology and Energy Production in Nitrifying Bacteria

In order to understand more exact mechanisms of inhibition in nitrifying bacteria, it is important to understand their physiology. As discussed previously, the process of aerobic nitrification is carried out by the AOB and NOB, which encompass two distinct classes of

bacteria. With very few exceptions, both AOB and NOB are obligate autotrophic bacteria (Matin, 1978). This means that they require inorganic carbon (CO_2) as the major carbon source. Though many strains can assimilate some organic carbon, this amount is very small when compared with inorganic carbon utilization (Matin, 1978). Carbon assimilation is a very energy demanding process, with an estimated 80% of the energy produced by an autotroph going to carbon fixation (Forrest and Walker, 1971; Kelly, 1978). Because of the high energy demand for carbon fixation, the very slow growth rates observed for both genera of nitrifying bacteria are due to their autotrophic mode of life (Wood, 1986).

2.2.1 Ammonia Oxidizing Bacteria

AOBs are all Gram-negative members of the β and γ subdivisions of proteobacteria (Hooper et al., 1997). These organisms are autotrophic, with carbon dioxide as the main carbon source. They use ammonia via aerobic oxidation as their main source of energy. Five major genera of AOB have been identified. The first genus to be described was *Nitrosomonas* by Sergej Winogradsky (1892). These bacteria, of which *N. europaea* was the first discovered and most studied, are rod-shaped, motile bacteria with very large intracytoplasmic membranes that are arranged around the edges of the bacteria. The *Nitrosococcus* were first described by Winogradsky (1892) and further investigated by Watson (1965). These bacteria are cocci in shape, motile and also have very large intracytoplasmic membrane areas. The *Nitrosolobus*, first described by Watson and coworkers (Watson et al., 1971), are irregular in shape, motile and partially compartmentalized by invaginations of the plasma membrane and other segments of the cell envelope. The last two genera lack the extensive intracytoplasmic membranes observed in the other members of the AOB. The *Nitrosovibrio* are motile, curved rods (Harms et al., 1976) and the genus *Nitrosospira* are tightly wound, motile spirochetes (Winogradsky and Winogradsky, 1933).

The sole source of energy for AOB comes through the aerobic oxidation of ammonia to nitrite. Although several different genera of AOB exist, the major components of energy generation and the electron transport chain appear to be conserved (Bock et al., 1986). In the first step of the process, ammonia (NH_3) is oxidized to hydroxylamine (NH_2OH) and water using oxygen. This reaction is catalyzed by the inner membrane-bound enzyme ammonia monooxygenase (AMO) and requires two electrons (Andersson and Hooper, 1983; Hollocher et

al., 1981; Hooper et al., 1997). AMO is a multimeric protein containing two distinct subunits (Hooper et al., 1997) and possibly containing copper (Bedard and Knowles, 1989; Ensign et al., 1993) and iron (Keener and Arp, 1994; Zahn et al., 1996) at the active site.

After the hydroxylamine is produced from the reaction of ammonia with oxygen, it is converted to nitric acid (HNO_2) and 4 protons (H^+) by the periplasmic enzyme hydroxylamine oxidoreductase (HAO) (Wood, 1986). HAO is a monomeric protein and contains 8 heme-binding peptide sequences (Sayavedra-Soto et al., 1994). During this oxidation of hydroxylamine to nitric acid, 4 electrons are released and transferred to cytochrome 554 (cyt_{554}) (Arciero et al., 1991). From here, 2 electrons are passed to a ubiquinone (UQ) where they can be used to maintain the operation of AMO or catalyze the formation of reduced compounds, like NADH (Hooper et al., 1997; White, 2000). The remaining two electrons will pass through cyctochrome c-552 (cyt_{552}) to a cytochrome aa_3 oxidase, which will transfer the electrons to the terminal electron acceptor oxygen and use the energy to maintain the proton gradient across the inner membrane (DiSpirito et al., 1986). The energy of the proton gradient can then be used by an ATPase to produce ATP, which is the major energy storage molecule of all cells. This entire process is shown schematically in Figure 2.1. From this schematic, it can be seen that the oxygen requirements of ammonia oxidizing bacteria are very high due to the need of oxygen both as the terminal electron acceptor in the electron transport chain, and as an electron acceptor during the AMO catalyzed conversion of ammonia to hydroxylamine.

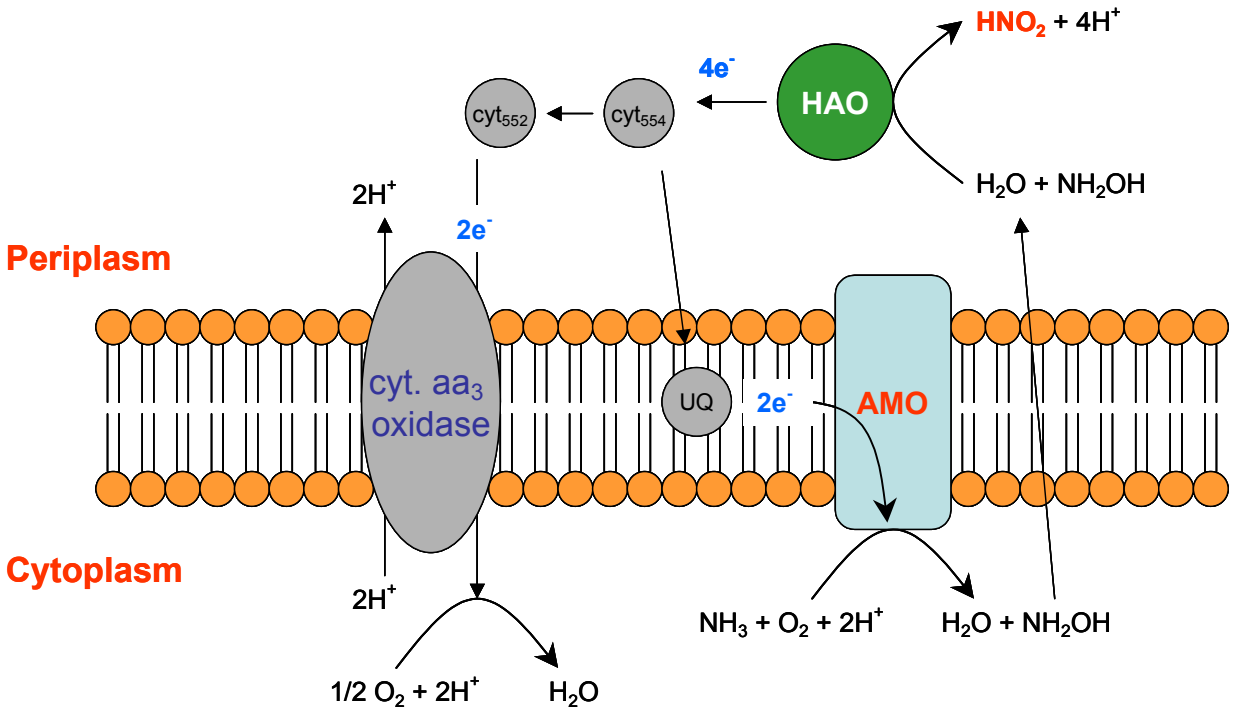

Figure 2.1 Components of the ammonia oxidation system and electron transport in *N. europaea*. Figure adapted from White (2000).

2.2.2 Nitrite Oxidizing Bacteria

Some of the NOB are closely related to the AOB. Many NOB are members of the α, δ and γ subdivisions of proteobacteria; however, a distantly related phylum of NOB known as *Nitrospira* were identified more recently (Burrell et al.,1998). NOB are autotrophic, like the AOB, and derive their energy from the aerobic oxidation of nitrite to nitrate.

There have been four genera of NOB described to date. The first isolated and best described is *Nitrobacter winogradsky* (Winogradsky, 1892). These organisms are motile and have been found to be pleomorphic, meaning that they can be found as rods, cocci or pear-shaped. As with many of the AOB, *Nitrobacter* have large intracytoplasmic membranes. The second genus is *Nitrococcus*, first described by Watson and Waterbury (Watson and Waterbury, 1971). They are coccoid shaped, motile cells with large intracytoplasmic membranes that live in marine environments. In the same paper, Watson and Waterbury first describe the third genus of nitrite oxidizers, *Nitrospina*. These organisms also grow in marine environments but are rod-shaped and do not have extensive intracytoplasmic membranes. The last and most recently

discovered genus is *Nitrospira* (Watson et al., 1986). These organisms are tightly or loosely wound spirochetes with a very wide periplasmic space and no intracytoplasmic membranes.

NOB convert nitrite to nitrate and, as with the ammonia oxidizing bacteria, it appears that the oxidizing systems are conserved across the different genera. The conversion of nitrite to nitrate is catalyzed by a membrane bound enzyme known as nitrite oxidoreductase (NOR) (Wood, 1986), as shown schematically in Figure 2.2 (O'Kelly et al., 1970; Sundermeyer-Klinger et al., 1984; Tanaka et al., 1983). Like AMO, NOR is a multimeric protein with two subunits and is known to have redox centers (active sites) of molybdenum, copper, manganese and an iron-sulfur (Meincke et al., 1992). During this process, two electrons are released and transferred to a cytochrome c (cyt. c) (Sundermeyer-Klinger et al., 1984). It is suspected that the transfer from NOR to the cytochrome is driven by the membrane potential (Nicholls and Ferguson, 1992). Once at the cytochome c, the electrons can then be transferred for use in production of reduced compounds like NADH or shifted to a cytochrome aa_3 oxidase (Wood, 1986). Here, as with most aerobic bacteria, the cytochrome aa_3 oxidase transfers the two electrons to oxygen, the terminal electron acceptor, and uses the energy gained to maintain a proton gradient across the inner membrane. Again, it is this proton gradient that provides most of the energy to the cell.

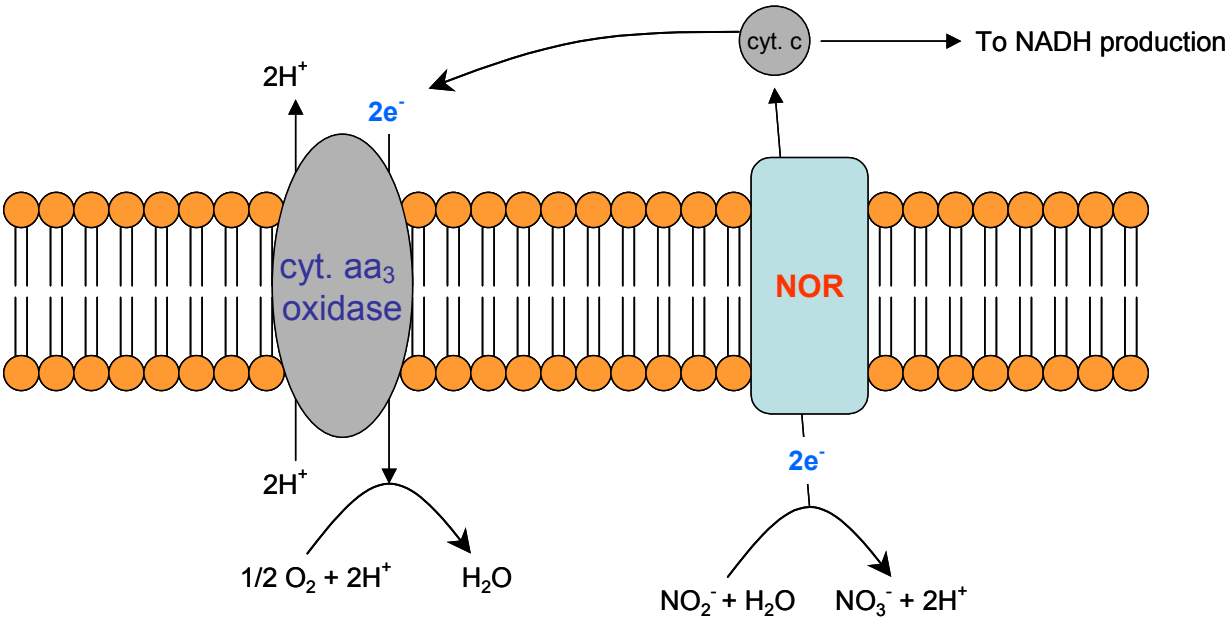

Figure 2.2 Components of the nitrite oxidation system electron transport in *Nitrobacter*. Figure adapted from White (2000).

2.3 Oxidative Chemical Inhibition

As stated previously, results found in our lab showed that the most severe inhibition of nitrification was due to the uncharged organic oxidative/electrophilic chemical 1-chloro-2,4-dinitrobenzene (CDNB). A discussion of the toxic nature of CDNB and other oxidative chemicals is given below. In addition, known stress response mechanisms for oxidative chemical inhibition and possible reasons for the increased susceptibility of nitrifying organisms are discussed.

2.3.1 Toxic Modes of Action

Electrophilic chemicals act as oxidizing agents by accepting electrons from other compounds and are commonly used in industrial manufacturing processes. Despite the apparent widespread use of uncharged organic electrophilic chemicals in industry, very few studies have been performed that look at its effects on activated sludge bacteria. Instead, most studies on the effects of electrophiles on bacteria have been performed using pure cultures of Gram-negative bacteria like *Escherichia coli*. Although *E. coli* is not a prevalent organism in activated sludge cultures, it is Gram-negative and most organisms that have been phylogenetically characterized in activated sludge systems (including nitrifiers) are Gram-negative (Wagner and Loy, 2002). Therefore, it is expected that chemical effects observed from pure cultures of these bacteria can be used to draw inferences regarding the effects of electrophiles on activated sludge bacteria.

Through studies with pure cultures, several mechanisms of action have been associated with oxidative/electrophilic chemicals. One of the two major modes of action is damage to deoxyribonucleic acid (DNA) (Ferguson et al., 1996; Ferguson et al., 1995). More specifically, it has been found that the uncharged electrophile methylglyoxal reacts *in vitro* with the nucleotide bases guanine, adenine and cytosine (Papoulis et al., 1995). The effect of electrophiles on DNA was elucidated by Summer and Göggelmann (1980) and Gupta and coworkers (1997), who showed that CDNB and homologous compounds were mutagenic to the Gram-negative organism *Salmonella typhimurium.* Furthermore, they showed that the degree of mutagenicity increased with increased electronegativity (or increasing degree of electrophilicity) of the halogen. When thinking about this toxicity mechanism with relation to nitrifying bacteria, it can be seen that damage inflicted on the DNA of the organisms may cause inhibition through a wide variety of means, including death and lysis of the cells, production of inactive essential

proteins, or failure to produce essential proteins. Any one or a combination of these may occur and inhibit nitrification due to the presence of an oxidative chemical, resulting in growth impairment or death.

The other major mechanism by which oxidative chemicals appear to damage or inhibit bacteria is by directly damaging proteins (Apontoweil and Berends, 1975; Ferguson et al., 1995; Ferguson et al., 1997; McLaggan et al., 2000). More specifically, electrophilic compounds are thought to act by directly oxidizing thiol bonds and attacking nucleophilic protein centers (Ferguson et al., 1995; Ferguson et al., 1997; McLaggan et al., 2000). In a study performed by Lo and coworkers, one electrophilic compound, methylglyoxal, was found to directly attack the side chains of the amino acids arginine, lysine and cystine (Lo et al., 1994). Extrapolating these results to both classes of nitrifying bacteria, it can be seen that inhibition of the nitrification process by electrophiles may also be due to action against essential proteins.

Two very important proteins from the two different classes of nitrifying organisms that may be affected were presented above. Both AMO and NOR contain sulfur groups and thiol bonds to maintain their three-dimensional shape and active confirmations. Reactions with these bonds can likely alter the shape or activity of these proteins, resulting in inhibition of ammonia or nitrite oxidation. Another possible mechanism of inhibition for AMO is interference with the copper active site of this enzyme. It has been proposed that the copper cycles between its oxidized and reduced states when the enzyme is active (Wood, 1986). If an oxidizing agent is present, it would prevent reduction of the copper and thus prevent AMO from functioning properly. An additional inhibition mechanism for NOR involves the iron-sulfur group, which was previously identified to be the active site of the protein. The disassembly of iron-sulfur clusters by oxidative chemicals has been documented (Carmel-Harel and Storz, 2000) and this site may be dramatically affected by electrophilic chemicals that attack sulfur groups and thus remove the nitrite oxidizing ability of the molecule. In addition, if redox cycling of specific metals is required for NOR activity, which appears to be a likely possibility (Wood, 1986), then oxidation of these metals by an electrophile would lead to inactivation of the enzyme and inhibition of nitrite oxidation.

These molecular level toxic mechanisms can manifest into macro-scale process upset events in activated sludge both due to the toxic mechanism and the stress-response of the bacteria to the mechanism. Of the process performance parameters that have been monitored for

oxidative chemical shock, a few studies have been conducted where the effects on activated sludge deflocculation were investigated. One study examined the effects of two electrophilic chemicals, CDNB and N-ethylmaleimide (NEM), and found that both induced rapid deflocculation (Bott and Love, 2002). The same deflocculation was noted when examining the effects of cadmium, which is electrophilic, on activated sludge during the experiments of Bott and Love and those performed by Weber and Sherrard (1980). As cadmium is a heavy metal, the exact mechanism for the cause of the deflocculation event may not be the same as the organic uncharged electrophiles CDNB and NEM, but it does lend weight to the argument that oxidative chemicals can induce deflocculation in activated sludge. It has been hypothesized that this deflocculation is not a direct result of the electrophilic toxins themselves but, rather, one resulting from a protective stress-response mechanism of the bacteria to electrophilic chemicals (Bott and Love, 2002). A detailed analysis of this and other oxidative stress-response mechanisms found in Gram-negative bacteria is given below.

In addition to the deflocculation event noted for activated sludge biomass exposed to electrophilic chemicals, our lab has also found that CDNB severely inhibits nitrification and that recovery of this process takes much longer than recovery from the deflocculation event (3 days for deflocculation versus no recovery after 28 days for nitrification) (Kelly et al., 2004; Love et al., 2002b). From effluent nitrogen species data that was monitored over time, it was noted that nitrite oxidation took longer to recover than did ammonia oxidation, suggesting an increased sensitivity of NOB to electrophiles. This may be due to the iron-sulfur active site in NOR, which is a more significant target for oxidation than the active site in AMO. It may also be due to differences between stress response mechanisms in AOB and NOB, which will be discussed below.

Whatever the reason for the differences in nitrifying organism inhibition, it does not explain the increased recovery time of nitrification versus other process parameters. One possible explanation is washout of the bacteria. As the deflocculation event occurred at the same time nitrification was lost, the extra biomass lost due to deflocculation may account for the nitrification loss. This is because the SRT was effectively lowered by the excess loss of biomass in the effluent and washout of the slower growing nitrifiers may have occurred (Abeysinghe, et al., 2002). This explanation is not likely, however, as the excess biomass lost from the system only decreased the sustained solids retention time to 5 days, which is still above the minimum

SRT of 3 days for stable nitrification without further inhibition suggested by Grady et al. (1999). In addition, nitrification inhibition occurred before any solids were wasted from the system, as was noted by a reduced nitrate generation rate for the CDNB shocked biomass. A more likely explanation for the increased inhibition would be washout due to the CDNB inhibition decreasing the growth rate. This slowed growth rate may lead to washout of the biomass from the system as described by Abeysinghe and coworkers for temperature inhibition of nitrification (Abeysinghe et al., 2002). As long as inhibitory concentrations of the chemical remained in the system, the growth rate would not increase to the point of allowing re-growth of the bacteria to reestablish stable nitrification.

2.3.2 Oxidative Stress-Response Mechanisms

Glutathione, Glutathione-Gated Potassium Efflux and Cytoplasmic Acidification

It has been proposed that the deflocculation event noted during our experiments for activated sludge exposed to electrophilic chemical toxins was caused by a rapid efflux of potassium from the cytoplasm of the cells into the environment. The potassium efflux reduced floc strength by increasing the intrafloc monovalent: divalent (M:D) cation ratio (Bott and Love, 2002), which has been found by others to be an important factor in controlling the strength of activated sludge flocs (Higgins and Novak, 1997a; Higgins and Novak, 1997b; Novak et al., 1998). It has been suggested that the potassium efflux event noted for the electrophile-shocked activated sludge is analogous to a similar potassium efflux event noted in pure cultures of heterotrophic Gram-negative bacteria (Bott and Love, 2002). From these pure culture experiments, which mainly used *E. coli*, it appears that the observed efflux of potassium is the direct result of a stress-response mechanism elicited by the bacteria in response to electrophilic shock events (Booth et al., 1993; Ferguson et al., 2000; Ferguson and Booth, 1998; Ferguson, 1999). This stress response mechanism has been dubbed the glutathione gated potassium efflux (GGKE) system (Bott and Love, 2002).

Glutathione. As the name implies, a very important molecule for activation of this stress response mechanism is glutathione. Reduced glutathione (GSH) is a tripeptide that is found in both eukaryotes and prokaryotes and is the major soluble non-protein thiol found in cells (Booth et al., 1993; Elmore et al., 1990). It also appears that glutathione provides a major cellular defense against electrophilic compounds by acting as a “sacrificial nucleophile”. As

electrophilic compounds act against sulfur groups in cells, glutathione "sacrifices" itself and is oxidized by the electrophile to protect proteins and DNA and promote survival of the cell. This has been found for both *E .coli* cells exposed to methyglyoxal (Carmel-Harel and Storz, 2000; Ferguson et al., 2000; Ferguson and Booth, 1998), and chlorine bleach (Chesney et al., 1996), for *Pseudomonas fluorescens* exposed to cadmium (Hultberg, 1998) and for *Pseudomonas aeruginosa* exposed to reactive oxygen species (Park et al., 1993). The glutathione is then either oxidized by the electrophile (forming GSSG) or reacted to form a glutathione-S-conjugate with the compound (GSX) (Booth et al., 1993). In the case of CDNB, the electrophile studied in our labs, it forms a conjugate called 2,4-dinitrobenzyl-S-glutathione (DNBSG), which is likely exported from the cell and degraded by a transpeptidase (Ness et al., 1997; Vuilleumier, 1997). Many times, the conjugation reactions are catalyzed by another essential molecule known as glutathione-S-transferase (Booth et al., 1993). In addition, once molecules of glutathione are oxidized, in most organisms they are not simply discarded, but rather returned to their reduced state by another important NADPH-dependent molecule known as glutathione reductase (Abordo et al., 1999; Carmel-Harel and Storz, 2000), which allows for glutathione to be recycled within cells.

Interestingly, previous research has shown that glutathione production is induced by oxidative chemicals such as cadmium in both human cells (Stohs and Bagchi, 1995, Ercal et al., 2001) and in yeast cells (Vido et al., 2001) but the results for bacteria are mixed. In one study by Park and coworkers (1993), glutathione levels increased in *P. aeruginosa* cells exposed to oxygen radicals while a separate study done by Malin Hutberg (1998) showed a decrease in glutathione levels in *P. fluorescens* cells exposed to cadmium. A stimulation of glutathione production would be expected as it would provide a higher level of protection to cells in reponse to an oxidative chemical shock event. The drop in levels seen in the Hutberg study was not explained, but is likely because the method was only reading the reduced glutathione levels, which would have been oxidized upon exposure to cadmium.

Glutathione Gated Potassium Efflux and Cytoplasmic Acidification. Not only does glutathione play an important role in protecting cellular components from electrophiles through self-sacrifice, but it also is important in regulation of the potassium efflux response. It has been found that the reduced form of glutathione (GSH) negatively regulates (turns off) the potassium efflux channels located in the cell membranes (Elmore et al., 1990; Miller et al., 1997).

Furthermore, the oxidized (GSSG) and conjugate (GSX) forms of glutathione positively regulate (turn on) the potassium efflux channels (Elmore et al., 1990; Ferguson et al., 1993; Ferguson et al., 1997). From the studies done to date, it appears that two potassium efflux channels exist that are regulated, or "gated" by glutathione. Originally described in the early 1970's as protein channels TrkB and TrkC, they were renamed KefB and KefC by Bakker and coworkers after it was determined that these proteins controlled the efflux of potassium from cells (Bakker et al., 1987). These efflux pumps, which are turned on in response to oxidative stress, are potassium-proton antiporters, meaning they influx protons as they efflux potassium cations. This results in the acidification of the cytoplasm, which has been found to confer oxidative stress resistance to the cells (Ferguson et al., 1996; Ferguson et al., 1998; Ferguson et al., 1997; Ness et al., 1997). Furthermore, it appears that these two proteins do not have sulfhydryl groups that are essential for their activity, or such groups are extremely well protected (Bakker et al., 1987) and, therefore, potassium efflux can occur without inhibition from the electrophiles they are meant to protect against. In addition, this system is thought to be conserved in Gram-negative bacteria as it has been found in several different species tested, including *E. coli, S. typhimurium, A. aerogenes, P. aeruginosa and R. sphaeroides* (Booth et al., 1993).

In addition to protection from electrophiles afforded by glutathione, it appears that the acidification of the cytoplasm by the GGKE response turns on DNA repair mechanisms (Ferguson et al., 1997), as DNA is a target for oxidative chemicals. One of these has been found to be increased production of Dps, a protein that non-specifically binds to DNA within cells (Hengge-Aronis, 1993). Dps is believed to bind to DNA without any sequence specificity and afford protection to cells against oxidative damage (Hengge-Aronis, 1993; Martinez and Kolter, 1997). During oxidative challenge, Ferguson and co-workers found that levels of Dps increased in *E. coli*, and the overall viability of wild type cultures increased as well over mutants deficient in Dps (Ferguson et al., 1998). Interestingly, the stationary phase stress response regulator RpoS, which has been found to be important in regulation of Dps transcription (Hengge-Aronis, 1993), was not required to up-regulate Dps in response to oxidative stress, suggesting that some other regulation mechanism may be required to trigger the acidification of the cytoplasm by the GGKE response (Ferguson et al., 1998). Several other oxidative stress-response proteins controlled by RpoS are discussed below.

Recently, the complete sequence of the ammonia oxidizing bacteria *Nitrosomonas europaea* was completed and analyzed (Chain et al., 2003). Availability of this genome allows for a complete search of genes related to stress response and protection within an AOB strain, and may give some insight into the increased sensitivity noted for these bacteria to many chemical stressors. The known protein coding genes for *N. europaea* are located at the Protein Information Resource NREF Database (http://pir.georgetown.edu) and the *N. europaea* genome can be searched at the Oak Ridge National Laboratory *Nitrosomonas* genome homepage (http://genome.ornl.gov/microbial/neur/embl/). A list of several known oxidative stress response genes and their presence (or absence) in *N. europaea* is given in Table 2.3. Genes were considered present at an $e \leq 0.001$.

Table 2.3 Summary of selected genes involved in oxidative stress response mechanisms and their presence in the *N. europaea* genome.

Gene Name	Function	Presence in *N. europaea*
Genes involved in the GGKE mechanism		
gshB	glutathione synthetase	Yes
gst	glutathione S-transferase	Yes
gorA	glutathione oxidoreductase	No
kefB	Glutathione regulated potassium efflux system protein KefB	No[a]
kefC	Glutathione regulated potassium efflux system protein KefC	No[a]
dps	DNA binding protein Dps	No
Genes involved in the *oxyR* system		
oxyR	*oxyR* system regulatory protein OxyR	No
katG	hydroperoxidase/catalase	No[b]
aphCF	alkyl hydroperoxide reductase	No[c]
grxA	glutaredoxin	No[d]
trxC	thioredoxin	No[e]
oxyS	regulatory RNA	No
Genes involved in the *soxRS* system		
soxR	*soxRS* system regulatory protein SoxR	No
soxS	*soxRS* system regulatory protein Soxs	No
sodA	Manganese superoxide dismutase	No[f]
tolC	outer membrane efflux protein TolC	Yes[g]
arcAB	multidrug resistance efflux system arcAB	No[h]
***rpoS* regulated oxidative stress response genes**		
rpoS	sigma factor S	No
katE	hydroperoxidase/catalase	No[b]
nth	endoonuclease	No
sodC	copper-zinc superoxide dismutase	No
xthA	Exonuclease III	Yes[g]
topA	topoisomerase I	No[i]

[a] pH adaptation potassium efflux system protein F and D found, but not glutathione regulated
[b] catalase gene with unknown regulation mechanism found
[c] alkyl hydroperoxide reductase gene with unknown regulation mechanism found
[d] glutaredoxin gene with unknown regulation mechanism found
[e] Six thioredoxin genes with unknown regulation mechanisms found
[f] manganese iron superoxide dismutase with unknown regulation mechanism found
[g] BLAST search yielded protein with similar coding strand
[h] *arcAB* not found but numerous multidrug efflux pumps located in genome
[i] three distinct topoisomerase genes with unknown regulation found

After searching the genome of *N. europaea* for the genes that encode the proteins involved in the GGKE mechanism, some very interesting results were noted. First, a gene coding for glutathione synthetase (TrEMBL # Q82V16) was present in the genome; indicating that AOB have the ability to produce glutathione. In addition, a gene for glutathione S-transferase (TrEMBL # Q82TE5) was also present; indicating that conjugates between glutathione and the oxidative stressor could be produced. However, a gene coding for glutathione reductase was not found (Chain et al., 2003), indicating that once the glutathione is oxidized it cannot be reduced back to GSH and reused to confer protection against oxidative chemicals. Although a genome sequence for a NOB has not yet been completed, it is possible that many of the genes are conserved between AOB and NOB as both groups of organisms are closely related and autotrophic. The absence of a glutathione reductase may indicate one possible mechanism by which nitrifiers are more sensitive to oxidative stress than heterotrophic bacteria. In addition, just because a gene is present in an organism's genome does not mean that the gene is expressed. Therefore, it is possible that the genes for glutathione synthetase are not expressed and glutathione is not produced, leaving *N. europaea* and possibly other AOBs without a critical oxidative stress defense mechanism.

A search was also performed to examine the presence of other genes critical to the GGKE response mechanism, which is thought to be conserved in all Gram-negative bacteria. The results found two different pH adaptation potassium efflux system proteins. The first, pH adaptation potassium efflux system protein F (TrEMBL # Q82TI2), is an integral membrane protein that is part of a pH regulation system but is not related to either KefB or KefC. The second, PhaD (TrEMBL # Q82TI4), is a transmembrane protein with function similar to NADH:ubiquinone oxidoreductase (complex I) ,which catalyses the transfer of two electrons from NADH to ubiquinone and is associated with proton translocation across the membrane. This protein was also not closely related to either of the GGKE Kef proteins. When the *N. europaea* genome was searched for the presence of these genes, no positive matches were found, indicating that the well characterized glutathione-gated potassium channels do not exist in *N. europaea*. The lack of such a mechanism may mean that acidification of the cytoplasm and activation of DNA protection proteins is not possible, or that acidification is controlled by some other unknown mechanism. In addition, the DNA binding protein, Dps was also absent from the

N. europaea genome, further lending weight to the argument that acidification of the cytoplasm does not occur in AOBs. The lack of this DNA protection mechanism may indicate why AOBs are sensitive to electrophilic chemicals. Interestingly, it is believed that this is the first evidence of a Gram-negative bacterium that lacks the conserved GGKE mechanism, perhaps because it is the first autotrophic organism examined.

OxyR and SoxRS Antioxidant Systems

Oxidative stress to cells can also be induced by metabolic byproducts that are oxidative in nature. An increase in the production of such byproducts has been found when external oxidative chemicals like CDNB were added to pure cultures of cells (Abordo et al., 1999). Two of the more potent oxidative chemicals produced in cells are superoxide ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2), both of which are produced during the autooxidation of components of the respiratory chain in cells (Gonzalez-Flecha and Demple, 1995). Two different stress-response systems have been identified that work to protect cells against superoxide and hydrogen peroxide produced in cells. These systems have been identified as the OxyR and SoxRS systems, and some genes from these systems have also been found to afford protection to cells from external sources of oxidation, like NEM (Vattanaviboon et al., 2001).

The OxyR System. OxyR was described through a series of mutations that increased resistance to hydrogen peroxide and lead to an increase in the expression of hydrogen peroxide inducible proteins (Storz and Zheng, 2000). OxyR is a regulator of many genes that are expressed upon exposure to peroxide stress. These genes include *katG*, which codes for a hydroperoxidase, and *aphCF*, which codes for an alkyl hydroperoxide reductase. Both of these proteins help to mediate toxic effects of peroxides by eliminating the oxidants (Christman et al., 1985). In addition, the genes, *gorA* (glutathione reductase – discussed above), *grxA* (glutaredoxin) and *trxC* (thioredoxin) are all controlled by OxyR and help to maintain the thiol-disulfide levels in cells by reversing damage to proteins caused by oxidizing agents (Storz and Zheng, 2000). Interestingly, OxyR also induces expression of the non-specific DNA binding protein Dps, discussed above (Martinez and Kolter, 1997). Finally, OxyR activates the expression of a small RNA molecule known as OxyS, which protects cells against mutation through an unknown mechanism (Altuvia et al., 1997). Several of the genes regulated by OxyR

have also been found to be regulated by the alternative sigma factor RpoS, as will be discussed later.

Upon examining the genome of *N. europaea* for the presence of these genes, no matches were found. In fact, the sequence for the regulatory gene *oxyR* is entirely absent from the genome (Chain et al., 2003). The absence of this regulatory protein means that if the genes for peroxide protection are present, then they are either controlled by some other universal regulator or are independently regulated. In addition to the absence of *oxyR*, the genes encoding for OxyS and glutathione reductase are also missing from the genome. If *oxyS* is missing, then AOB and possibly NOB are missing an RNA that provides protection against mutations that can be caused by oxidative chemicals, and may also lead to an increased sensitivity to those chemicals. The implications for the lack of glutathione reductase are discussed above.

While matches for the OxyR regulated *katG*, *ahpCF*, *grxA* and *trxC* were not found, genes coding proteins with the same function were noted for all of these. A gene encoding an alkyl hydroperoxide reductase (TrEMBL # Q820H3) was found, as was a gene encoding a generic catalase (TrEMBL # 82TK1). A catalase protein was previously found in the AOB *N. europaea* and *Nitrosospira multiformis* during a study performed by Wood and Sorensen (Wood and Sorensen, 2001), suggesting that genes involved in oxidative stress response might be conserved across the different AOB species. In addition a glutaredoxin gene was found (TrEMBL # Q82SU3) and 5 different sequences were located that code for thioredoxin-like proteins. This means that genes are present that are capable of activating glutaredoxin and thioredoxin, which are proteins that can repair oxidative chemical-induced damage to proteins. Furthermore, these proteins assist in detoxifying peroxides. However, it is unclear how the genes for these proteins are regulated in response to such oxidative stress events, and it is possible that there is no regulatory mechanism. If this is true, then these genes could not be expressed at a higher rate when nitrifiers are exposed to shock loads of electrophilic/oxidative chemicals, which would leave these organisms more susceptible to inhibition by such compounds. The study conducted by Wood and Sorensen helps to confirm this idea as the catalase activity in both *Nitrosomonas* and *Nitrosospira* did not increase after the cells were exposed to H_2O_2 (Wood and Sorensen, 2001).

The SoxRS System. The second system, SoxRS, has been found to increase protection against superoxide and increase transcription of a number of superoxide inducible genes (Storz and Zheng, 2000). Regulation of the *soxRS* inducible genes occurs in a two step process. The first involves conversion of SoxR to an active state by oxidation of an iron-sulfur cluster. SoxR in turn enhances transcription of *soxS* and the resulting protein, SoxS, induces expression of the *soxRS* regulon genes (Wu and Weiss, 1992). One of the most important genes controlled by the SoxRS system is *sodA*, which codes for a manganese superoxide dismutase that can convert superoxide back to oxygen. In addition, this system governs expression of the DNA repair enzyme endonuclease IV, which is important to repair any oxidative damage caused to DNA. Interestingly, SoxS also increases expression of genes that code for outer membrane proteins known to control multidrug efflux, *tolC* and *acrAB*. It is thought that expression of these genes in conjunction with the noted upregulation of MicF, which represses the production of an outer membrane porin called OmpF, will result in the exclusion of compounds that might generate superoxide (Storz and Zheng, 2000). It is also important to note that the *soxRS* regulon has some overlap with the multiple antibiotic resistance regulon controlled by the protein MarA (Storz and Zheng, 2000). This may also explain why the genes *tolC*, *acrAB* and *micF* are induced by SoxS.

Examining the *N. europaea* genome for the presence of the genes controlled in the *soxRS* regulon yields interesting results. As was found for the OxyR system, neither the gene coding for SoxR nor the gene coding for SoxS were found in the genome (Chain et al., 2003). In addition, a search for the multiple antibiotic resistance regulator MarA yielded negative results. As before, this suggests that genes involved in resistance and repair of oxidative damage caused by superoxide and other electrophiles may be regulated independently or by another universal regulator, or may be unregulated entirely. Although the regulators of these superoxide responses were not found, a manganese and iron superoxide dismutase (SOD) does exist in the genome (TrEMBL # Q83W28) and has been detected in response to oxidative stress in both *Nitrosomonas* and *Nitrosospira* (Wood and Sorensen, 2001). Many different multidrug efflux proteins have been found in the genome of *N. europaea*, including a possible match for the outer membrane protein TolC. As with peroxide resistance, the results of this search suggest that protection of AOB from superoxide and other oxidative stress events may occur because of the presence of enzymes like SOD, which can help in detoxifying the cells, and multidrug efflux proteins, which can remove damaging contaminants from the cells. However, any regulatory

mechanisms for these systems are unknown at this time. As with catalase, results presented by Wood and Sorensen indicate that superoxide dismutase activity is unaffected by introduction of oxidative chemicals (Wood and Sorensen, 2001), which indicates that a regulatory mechanism may not exist.

RpoS Controlled Oxidative Stress Responses

RpoS is a sigma subunit of RNA polymerase, which carries out transcription of genes encoded by DNA. RpoS, or sigma S, is considered a primary sigma factor because it recognizes similar promoter sequences to the vegetative sigma factor, sigma 70 (Lonetto et al., 1992). Expression of RpoS is very low during the exponential phase of cell growth (active growth with sufficient nutrients) but is high during the stationary phase of growth when cells are exposed to a variety of stressed conditions, including high osmolarity or acidic pH (Hengge-Aronis, 2000). RpoS is thought of as a master regulator of the general stress response (Hengge-Aronis, 2002) and, although first discovered in *E. coli*, RpoS has been identified in numerous Gram-negative bacteria but has not yet been found in members of the α proteobacteria, to which some members of the NOB belong (Hengge-Aronis, 2000). Mutants lacking the gene that encodes RpoS have increased sensitivity to a variety of stressors that induce the expression of that gene, including high temperatures (Hengge-Aronis, 1993) and even oxidative chemical shock (Ferguson et al., 1998). The increased sensitivity of *rpoS* mutants suggests that RpoS induces expression of numerous genes involved in stress tolerance. Below is a discussion of critical genes regulated by sigma S involved in oxidative stress tolerance and repair, and the implications they may have on the oxidative stress tolerance of nitrifying bacteria. A review of many of the genes involved in oxidative stress and other stress responses that are associated with RpoS is given by Eisenstark et al. (Eisenstark et al., 1996).

Many of the oxidative stress genes previously discussed in conjunction with other response mechanisms are also regulated by sigma S. The first of these is glutathione reductase (Becker-Hapak and Eisenstark, 1995), which is involved in the reduction of glutathione and is also known to be regulated by OxyR. Other genes in the *oxyR* regulon that are also regulated by RpoS include *katG*, and *dps* (Michan et al., 1999). Interestingly, the study by Michan et al. showed that RpoS may also control expression of *oxyR* itself and, thus, exert regulatory control over the entire OxyR response mechanism. Double regulation of these genes means that they

may be turned on in response to other stressors, like pH or osmotic shock, and may help with DNA repair and protection under these other stress conditions as well.

In addition to the genes already discussed, several others exist that are controlled by RpoS and help protect cells from electrophilic shock or mediate its effects. One of these genes is *katE*, which encodes a catalase that is not regulated by OxyR (Hengge-Aronis, 1993). Another gene of importance to oxidative stress response is *xthA*, which codes for exonuclease III and is involved with excising damaged sections of DNA for repair. Expression of *xthA* increased as cell growth enters into the late exponential and stationary phases, and it was inferred that this gene was regulated by RpoS (Hengge-Aronis, 1993; Sak et al., 1989). Another gene that has more recently been found to be regulated by RpoS is *sodC*, which encodes a copper-zinc superoxide dismutase that performs the same function as the iron-manganese superoxide dismutase discussed above, except that it is exported to the periplasmic space rather than remaining in the cytoplasm (Gort et al., 1999). Tse-Dinh found that another enzyme, topoisomerase I, is regulated by RpoS. This enzyme was found to provide significant protection against electrophile attack, specifically against hydrogen peroxide and NEM (Tse-Dinh, 2000). Topoisomerase I has two possible actions that can afford protection to the DNA. One action regulates DNA supercoiling. In its second action, topoisomerase I prevents R-loop formation at DNA sites where high transcription rates occur as cells mount their defense against stress.

Several of these genes were notably absent from the genome of *N. europaea*. Although there is a catalase gene in the genome and catalase activity has been observed, the catalase gene, *katE*, was not found. In addition, no genes encoding a copper zinc superoxide dismutase were discovered. This would suggest that the cells have no protective mechanism against superoxide in the periplasm, and that protection only occurs in the cytoplasm where a manganese SOD enzyme is present. Genes were found that encode for at least at least two different exonucleases in *N. europaea;* however, no known match to exonuclease III was found in the protein databank. Upon searching the *N. europaea* genome specifically for exonuclease III from *E. coli*, it appears that a possible match does exist that has not yet been confirmed. The presence of such enzymes is not surprising, as exonuclease activity is required for general maintenance of cells and during replication of DNA to repair errors. A search was also performed to look for the presence of a topoisomerase and four distinct topoisomerases were found in the genome of *N. europaea*. Again, this is not surprising as topoisomerases are essential for supercoiling the DNA of bacteria

(as discussed above). The most interesting absence was that of the gene *rpoS*, which encodes for the master general stress response regulator RpoS (Chain et al., 2003). Although RpoS is not found in every bacterium, it is still conserved across many different genera. The absence of such a regulatory system would mean that responses to different stressors cannot be linked, which would mean that damage caused to proteins or DNA from one chemical stressor may go undetected and unrepaired.

The results from our evaluation of the *N. europaea* genome for known oxidative/electrophilic stress response mechanisms show that some genes that protect against this type of stress are present, like glutathione synthetase. Although several mechanisms have recently been identified in the genome of *N. europaea*, there is no guarantee that these genes are expressed. To date, only Wood and Sorensen (2001) have shown that genes responsible for oxidative stress mediation are expressed in AOB. Even though some genes and proteins important to oxidative stress responses exist, several notable genes were missing from the genome, including *dps* and the genes encoding the glutathione-gated potassium hydrogen antiporters KefB and KefC, which were originally thought to be conserved in all Gram-negative bacteria. More importantly, investigations into the genome of *N. europaea* have shown that the known regulatory mechanisms for such stress response mechanisms are absent. As these mechanisms have only been looked at in the *N. europaea* genome, it is possible that they are present in other AOB and NOB. Because of the close geneology and autotrophic nature of the nitrifying bacteria, it is more likely that their physiologies are similar and thus they may lack the same mechanisms. Taken together, this investigation into the existence of oxidative stress response mechanisms provides insight into possible reasons for the susceptibility of nitrifying bacteria to electrophilic chemicals.

2.4 Organic Hydrophobic Chemical Inhibition

1-Octanol is a second chemical that showed significant inhibition of nitrification relative to carbon removal and flocculation during our preliminary experiments. Octanol is a hydrophobic organic chemical with solvent-like properties. A discussion of the toxic nature of ocantol and other organic hydrophobic chemicals follows. In addition, known stress response mechanisms for inhibition due to hydrophobic chemicals and possible reasons for the increased susceptibility of nitrifying organisms to such chemicals are discussed.

2.4.1 Toxic Modes of Action

Hydrophobic organic chemicals are used widely in industrial practices, mainly as fuels and industrial solvents. For this reason, many different hydrophobic organic chemicals have been found in the influent and effluent waters of wastewater treatment plants (Paxeus, 1996). Because of the widespread use of hydrophobic organic chemicals, much work has been done examining the effects of these chemicals on bacteria; however, relatively little work has been done using nitrifying bacteria. As with CDNB, the majority of the work has been done using Gram-negative bacteria like *E. coli* and various Pseudomonad species. Again, as nitrifying bacteria are also Gram-negative and have both outer and cytoplasmic membranes, the toxic modes of action of hydrophobic organic solvents are likely to be the same for both.

Many hydrophobic industrial chemicals are thought to induce toxic effects through narcosis, a mode of action marked by a slowing of cellular activity (Bradbury and Lipnick, 1990). During experiments run in our lab, 1-octanol was used as the hydrophobic organic chemical. 1-Octanol is amphipathic, meaning that it has both polar properties (due to the hydroxyl group on the first carbon) and nonpolar properties (due to the long hydrophobic carbon chain). Based on previous work, 1-octanol has been said to function in a manner similar to narcotics (Bearden et al., 1999; McKim et al., 1987; Veith et al., 1983) and the site of action of these chemicals on organisms is thought to be the cytoplasmic membrane (Sikkema et al., 1995). Pure culture work has shown that the main target within cells for most hydrophobic (lipophilic) chemicals is the membrane of cells (Aono et al., 1994; Ingram, 1977; Seeman and Roth, 1972; Sikkema et al., 1994). This is likely due to the hydrophobic nature of these molecules, which causes the molecules to "seek out" similar molecules. When examining the physiology of nitrifying bacteria, the majority of these bacteria contain very large cytoplasmic/inner membrane surface areas. Because of this, they are an ideal target for hydrophobic chemical contaminants and likely incorporate more individual hydrophobic chemical molecules per cell than other Gram-negative bacteria with less membrane area.

The lipid bilayer that forms the cytoplasmic membrane of all living cells contains a hydrophilic phosphate head group and a hydrophobic center that provides an ideal environment for hydrophobic organic chemicals to locate (Cooper, 2000). The manner in which hydrophobic solvents partition into cell membranes depends on the structure and properties of the solvent. Short chain alkanols interact more with the phospholipid head groups, whereas completely

hydrophobic alkanes associate more with the hydrophobic fatty acid acyl chains. Longer chain alkanols have the hydroxyl group associate with the phosphate head group and the carbon chain will orient with the fatty acid tails (Weber and de Bont, 1996). The exact position of other types of molecules is not well predicted based on structure. Less hydrophobic but non-amphipathic molecules like toluene are expected to accumulate more between the fatty acid chains than near the polar head groups (Lohner, 1991; Weber and de Bont, 1996). This was confirmed in a study by Sikkema and coworkers, who showed that many different aromatic and cyclic hydrocarbons tended to accumulate more around the fatty acid side chains than by the polar head groups of lipid molecules (Sikkema et al., 1994).

When examining the effects of organic molecules on the membrane structures of Gram-negative bacteria, it is important to remember that two membranes exist for these organisms, an outer membrane and a cytoplasmic membrane. In a study done by de Smet et al., it was found that the outer membrane of *E. coli* was nearly unaffected upon exposure to toluene, while considerable damage was done to the cytoplasmic membrane (de Smet et al., 1978). Several effects can result from the insertion of hydrophobic chemicals into the cytoplasmic membrane, one of which is an alternation in the membrane fluidity. The membrane fluidity is an indirect measure of the ordering of lipids in the bilayer. The more fluid the membrane, the less ordered the lipids become. The way the membrane fluidity and stability is altered depends upon the type and structure of the hydrophobic compound. Lohner (1991) found that for short carbon chain alcohols (chain length, $C < 10$), the transition temperature of the cytoplasmic membrane decreased and thus the fluidity increased. For longer chain ($C > 10$) alcohols, the opposite was found in that the fluidity of the membrane decreased. It has been proposed that this is due to interactions between the carbon chain of the alcohol and the fatty acid side chains of the lipids. The longer the side chain of the alcohol, the more it can interact with the fatty acids and increase ordering in the membrane. The shorter chain alcohols do not efficiently interact with the fatty acids, only the phosphate head group, and therefore decrease ordering within the membrane (Lohner, 1991; Weber and de Bont, 1996). During the same study, Lohner found that alkanes have a similar effect on membrane fluidity, where long chain alkanes ($C \geq 12$) decrease fluidity while membranes exposed to short chain alkanes ($C < 12$) showed an increase in membrane fluidity (Lohner, 1991). Again, the effects of aromatic compounds are more difficult to predict, but a study done by Sikkema and coworkers found that the addition many different cyclic and

aromatic hydrocarbons caused an increase in the fluidity of the membranes (Sikkema et al., 1994).

In parallel to the increase in fluidity, the accumulation of hydrophobic organic chemicals in the membrane resulted in a swelling of the membrane bilayer. This effect was observed by Seeman and Roth (1972) when examining the effects of anesthetics on human erythrocytes. Similar results were found by Sikkema and coworkers on liposomes derived from *E. coli* membranes (Sikkema et al., 1994) and by Aono et al. for *E. coli* K-12 intact cells (Aono et al., 1994). The swelling of the membranes caused by accumulation of lipophilic contaminants also causes a deterioration of the membrane integrity. This has been noted by several researchers who have observed leakage of macromolecules like RNA, phospholipids, and proteins from the cells into the environment and the area between the two lipid layers (Aono et al., 1994; Jackson and de Moss, 1965; Woldringh, 1973). Even more importantly, leakage of ATP and ions like sodium, potassium and protons across the cytoplasmic membrane has also been found in several studies (da Silveria et al., 2002; Heipieper et al., 1991; Sikkema et al., 1994; Sikkema et al., 1992). Furthermore, by increasing the permeability of the membrane and allowing for leakage of protons and ions, a disruption of the proton gradient and membrane potential occurs due to introduction of hydrophobic compounds (Sikkema et al., 1994; Sikkema et al., 1992). The disruption of the proton gradient and membrane potential means that cells cannot gain energy for metabolism and maintenance. Although this has not been specifically examined using nitrifying bacteria, the effects would likely be the same. The implication of proton motive force disruption on the process of nitrification would be devastating. For ammonia oxidation, disruption of the proton motive force may occur due to the dependence of ammonia monooxygenase on electrons gained in the electron transport chain (see Figure 2.1 above). If the proton motive force is disrupted in AOB, it may cause an increase in the flow of electrons from hydroxylamine oxidation to the terminal oxidase of the system. This would deprive the ammonia monooxygenase of electrons required to oxidize ammonia and thereby inhibit ammonia oxidation. This idea is similar to a theory proposed by Wood (1986) for ammonia oxidation inhibition due to respiratory uncouplers, which also disrupt the proton motive force. When examining inhibition to NOB through the disruption of the proton motive force, two possibilities exist. The first is that inhibition of the AOB by hydrophobic compounds removes production of nitrite, the electron donor for NOB, and therefore would inhibit the NOB by removing their

"food". The other possibility stems from the energy barrier that is lowered by the proton motive force that allows oxidation of nitrite (Wood, 1986). If the ability to lower this energy barrier is removed, then nitrite will not be oxidized to nitrate as rapidly. Again, this theory is similar to one first presented by Wood (1986) for observations into the inhibition of nitrite oxidation by respiratory uncouplers.

In addition to disruption of the proton motive force and energy generation in cells, alteration of membrane structure may also affect protein function. Hydrophobic chemicals are known to adversely affect membrane bound proteins, such as transport proteins (In't Veld et al., 1991) and cytochromes (Sikkema et al., 1994). In't Veld postulated that the disruption of transmembrane transport protein function was due to expansion of the membrane and increase in the lipid bilayer thickness caused by insertion of lipophilic compounds. This increase in bilayer thickness did not allow the proteins to function properly because they contain transmembrane domains that are very specific to the thickness of a membrane (In't Veld et al., 1991). This may also be a reason for the inhibition of nitrification. In AOB, the enzyme AMO is made up of two subunits. Within each of these subunits, there are several transmembrane domains (Hooper et al., 1997) that would not fit properly into a membrane expanded by lipophilic chemicals. This expansion may pull the subunits apart and inactivate the enzyme.

Proteins and enzymes may also be affected directly by hydrophobic compounds. In a study performed by Franks and Lieb, the effects of hydrophobic general anesthetics (halothane, hexanol, decanol) on pure luciferease enzyme were examined. By removing the lipids, the direct effects of the compound on the protein could be examined. The study showed that the luciferase activity decreased with an increasing concentration of the anesthetic (Franks and Leib, 1984). This suggests that the toxic mode of action of the anesthetics tested is against the enzyme directly. The authors further hypothesized that as luciferin, the activating molecule for luciferase, is hydrophobic and binds in a hydrophobic pocket in luciferase, then hydrophobic chemicals may act by competitively binding in the hydrophobic pocket of the luciferase enzyme. This may also be the case for nitrifiers. Results by Suzuki and coworkers have found that the substrate for AMO is ammonia, not ammonium (Suzuki et al., 1974). As ammonia is an uncharged molecule, the active site in AMO may be a hydrophobic pocket. Therefore, hydrophobic chemicals may inhibit ammonia oxidation by competitively binding in the hydrophobic pocket of AMO and preventing the ammonia substrate from entering the enzyme to

be oxidized. This is not as clear for NOB, as it is yet unknown if the substrate for nitrite oxidoreductase is nitrite or nitrous acid.

2.4.2 Hydrophobic Stress Response Mechanisms

Membrane Modifications

Several different mechanisms exist for bacteria to cope with hydrophobic chemical stress. The first mechanisms discovered were those that modify the membrane structure. These modifications are meant to counteract the changes in membrane fluidity experienced upon insertion of different hydrophobic chemicals into the cytoplasmic membrane. As was found with the alternation in membrane fluidity, the changes of membrane composition are related to the type of hydrophobic contaminant (Weber and de Bont, 1996). For alcohols, Ingram showed that the fatty acid composition of the *E. coli* membrane was altered by the presence of alcohols and that the composition was highly dependent on the chain length of the alcohol (Ingram, 1976). What he found was that alcohols with a carbon chain length of 1 to 4 showed a decrease in the 16:0 fatty acid composition (palmitic acid) and an increase in the 18:1 (vaccenic acid) fatty acid composition. As short chain alcohols are known to decrease the membrane fluidity, the increase in unsaturated fatty acid composition would counteract this affect by increasing the membrane fluidity. The opposite was found for alcohols with 5 or more carbons in the chain, where the amount of 16:0 saturated fatty acids increased and the 18:1 unsaturated fatty acids decreased. Again, these results show that the alteration in the membrane structure is meant to counteract the increased fluidity caused by the longer chain alcohols by increasing the saturated fatty acid content to decreased the membrane fluidity. Ingram also found that growth of *E. coli* ceased upon exposure to the alcohols until these modifications to the saturated:unsaturated fatty acid content of the cells was completed, after which growth again occurred (Ingram, 1976). Other studies have shown that, in general, for compounds that decrease the membrane fluidity, an increase in the amount of unsaturated fatty acids is observed and for compounds that increase the membrane fluidity, an increase in the amount of saturated fatty acids is seen (Chen et al., 1995; Heipieper and DeBont, 1994; Ingram, 1977).

The identification and characterization of several organic solvent tolerant mutants of both *E. coli* and various *Pseudomonas* species also showed another important aspect of the membrane that aids in solvent tolerance. In a study by Aono and coworkers, the cell surface properties of

several solvent tolerant *E. coli* K-12 mutants were examined with respect to the parent strain (Aono and Kobayashi, 1997). They found that the cell surface of the mutant strains were less hydrophobic than that of the parent strain. This would mean that less solvent would partition into the less hydrophobic membrane of the solvent tolerant mutants. Although no differences were found in the fatty acid content of the mutants, the mutants did produce more lipopolysaccharide (LPS) than the parent strain, which resulted in the increased hydrophilicity of the cells and possibly provided a barrier to the entrance of hydrophobic solvents into the cell membranes (Aono and Kobayashi, 1997). Pinkart et al. examined the same properties for a solvent tolerant and solvent sensitive strain of *Pseudomonas putida* (Pinkart et al., 1996). When the fatty acid content of the two strains was examined, no significant differences were found; however, significant differences did exist in the LPS content of the two strains. The solvent tolerant strain exhibited a typical rough-type LPS when grown both in the presence and absence of *o*-xylene while the solvent sensitive strain exhibited a smooth-type LPS. The solvent tolerant strain was found to be less permeable to *o*-xylene and it was hypothesized that this was due to the difference in LPS structure, not fatty acid content. Upon exposure to *o*-xylene, there was no change in the LPS content of the sensitive strain while there appeared to be a shift from a higher molecular weight LPS to a lower molecular weight LPS for the tolerant strain (Pinkart et al., 1996). Interestingly, the permeability of the tolerant strain did not change with the changing LPS, suggesting that the type of LPS was more important than the alteration in LPS content.

A third alteration to the membrane content has also been identified in *Pseudomonas* species and occurs more rapidly than the synthesis of new fatty acids or LPS alterations. This mechanism is the conversion of unsaturated fatty acids from the *cis* form to *trans*. This mechanism was first described by Heipieper and coworkers as a response of *Pseudomonas putida* P8 to substrate toxicity caused by phenol (Heipieper et al., 1992). During these experiments, cells exposed to increasing amounts of phenol, which was used as the substrate, were found to convert the 16:1 and 18:1 *cis* unsaturated fatty acids to 16:1 and 18:1 *trans*. It was found that this conversion conferred some resistance to the bacteria against the phenol shock. Later studies found that this conversion occurred for a number of hydrophobic solvents and also in the *Vibrio* genus (Heipieper and DeBont, 1994; Junker and Ramos, 1999; Okuyama et al., 1991; Pinkart et al., 1996). This conversion from *cis* to *trans* decreases the membrane fluidity by increasing the ordering in the fatty acids, much the same way as the conversion from unsaturated

to saturated fatty acids. As this conversion occurred in seconds, and did not correspond with the synthesis of new proteins or lipids, it was proposed that a novel enzyme performed the conversion from *cis* to *trans*. Because this isomerization of the unsaturated fatty acids occurred in response to 4-chlorophenol and chloramphenicol, it was suggested that the enzyme is non-specifically regulated and likely associated with the cell membrane to rapidly respond to any hydrophobic chemical (Heipieper et al., 1992). The gene encoding the enzyme responsible for this conversion was later sequenced by Holtwick et al. and labeled *cti* for *cis-trans* isomerase (Holtwick et al., 1997). This *cis-trans* isomerization is considered to be a short-term response that allows cells to adapt almost immediately to solvents and provide the resistance and time necessary to synthesize new membrane components like saturated fatty acids (Junker and Ramos, 1999).

Several genes have been identified that are responsible for these adaptation stress response mechanisms. It has been suggested that the synthesis of new fatty acids in response to solvent stress is controlled by two transacylase enzymes, sn-glycerophosphate transacylase and monoacyl glycerophosphate transacylase, which were originally thought to incorporate a greater amount of unsaturated fatty acids into the phospholipids of *E. coli* in response to lowered temperature (Ingram, 1976). It is now known that only one enzyme, β-ketoacyl-ACP synthase II, is responsible for temperature-induced alterations in the ratio of saturated to unsaturated fatty acids in *E. coli* (Magnuson et al., 1993). It is, therefore, logical that this same enzyme is responsible for altering the ratio of saturated to unsaturated fatty acids in the membrane with response to hydrophobic chemical shock because the same chemical alteration occurs as with temperature shifts. This enzyme appears to be conserved in numerous bacteria, including Gram-negative (Heidelberg et al., 2000) and Gram-positive species (Ferretti et al., 2001). After searching the *N. europaea* genome for this enzyme, no exact match was found. A comparative search of the genome against the gene sequence from *E. coli* yielded a probable match, indicating that *N. europaea*, and likely nitrifying bacteria in general, have the ability to alter their fatty acid composition in response to temperature and possibly hydrophobic stress. A summary of the important stress response mechanisms and their presence in the genome of *N. europaea* is listed in Table 2.4.

Table 2.4 Summary of select genes involved in hydrophobic stress response mechanisms and their presence in the *N. europaea* genome.

Gene/Protein Name	Function	Presence in *N. europaea*
Membrane Modification Mechanisms		
β-ketoacyl-ACP synthase II	modification of membrane fatty acid composition	Probable Homolog Found
cti	fatty acid *cis-trans* isomerase	Probable Homolog Found
Efflux Systems		
AcrA-AcrB-TolC	multidrug/solvent efflux system	Probable Homologs Found
AcrR	*acrAB* system regulator	No
MexA-MexB-OprM	multidrug/solvent efflux system	Probable Homologs Found
MexC-MexD-OprJ	multidrug/solvent efflux system	Probable Homologs Found
MexE-MexF-OprN	multidrug/solvent efflux system	Probable Homologs Found
MexR	*mex* systems regulator	No
SrpA-SrpB-SrpC	multidrug/solvent efflux system	Probable Homologs Found[a]
TtgA-TtgB-TtgC	multidrug/solvent efflux system	Probable Homologs Found[a]
MepA-MepB-MepC	multidrug/solvent efflux system	Unknown[b]
MepR	*mepABC* system regulator	Unknown[b]

[a] Homologous SrpB sequence in *N. europaea* is the same sequence homologous to TtgB
[b] *mepABC* and *mepR* could not be checked against *N. europaea* genome as gene sequences could not be located

The *cis-trans* isomerase enzyme, Cti, must be regulated, as the isomerization mechanism occurs rapidly in response to solvent stress and in the presence of the translation blocker chloramphenicol (Heipieper, et al., 1992); however, a regulation mechanism for this gene has not been identified to date. As stated previously, this gene has been identified in several *Pseudomonas* species, including *P. aeruginosa* (Stover et al., 2000), and the isomerase activity has been noted in numerous species (Sikkema et al., 1995). Upon examination of the *N. europaea* genome, no identified *cis-trans* isomerase gene was present. We also compared the gene sequence of Cti in *P. aeruginosa* against the *N. europaea* genome and a probable match was found, indicating that *N. europaea* likely has the ability to rapidly isomerize its unsaturated fatty acids in response to hydrophobic stressors. The regulatory mechanism for the *cis-trans* fatty acid isomerase activity is unknown, and it is not possible to examine the *N. europaea* genome for its presence. However, if the regulatory mechanism does not exist, then its activity could not be up-regulated in response to a hydrophobic chemical shock event and may indicate why nitrifying bacteria are more sensitive to such an event.

Active Efflux Mechanisms

Another mechanism many bacteria have in response to hydrophobic/lipophilic chemical stress is to actively efflux the chemical from the cell cytoplasm and membrane. Efflux systems were originally identified for their role in effluxing a wide range of antibiotics and, therefore, are collectively referred to as multidrug efflux systems (Kieboom and de Bont, 2000; Sikkema et al., 1995; Weber and de Bont, 1996). These systems have all been identified as members of the resistance-nodulation-cell division (RND) family of transporters (Kieboom and de Bont, 2000; Paulsen et al., 1996). There are many different protein channels that have been identified as multidrug efflux systems and all have been found to be active transporters. In other words, all require the involvement of the proton motive force as they are either proton dependent or ATP dependent transporters (Paulsen et al., 1996).

Initially it was unknown if these systems played a role in solvent efflux, as only the active efflux of antibiotics had been observed. Isken and de Bont first found that ^{14}C-labeled toluene was removed from a strain of *P. putida* that cannot metabolize or transform the solvent (Isken and de Bont, 1996). Further investigation using the respiratory chain inhibitor potassium cyanide or the proton conductor carbonyl cyanide *m*-chlorphenylhydrazone showed that significant accumulation of toluene occurred in cells exposed to either inhibitor. Together, this suggested that an efflux mechanism existed for lipophilic chemicals and that the system was actively regulated, rather then relying upon passive transport across the membrane; this mechanism parallels the multidrug efflux systems previously mentioned (Isken and de Bont, 1996). A similar study performed by Ramos and coworkers confirmed this response by using a similar compound, 1,2,4-trichlorobenzene (Ramos et al., 1997).

Upon discovery of an organic solvent efflux mechanism similar to the multidrug efflux systems, investigations into the roles of the known multidrug efflux systems in organic solvent efflux were performed. Several have been found to play an important role in removing organic solvents from bacteria, and most have been identified in *Pseudomonas* species (Kieboom and de Bont, 2000). One particular RND efflux system, called the AcrAB system, has been identified in *E. coli* and is believed to play an important role in the tolerance of the organism to hexane, cyclohexane (White et al., 1997) and toluene (Aono et al., 1998). This system, like all RND-type efflux systems, is made up of three proteins working in conjunction; an inner membrane

transporter protein (AcrB), an outer membrane protein (TolC) and a membrane fusion protein (AcrA) that links the two membrane proteins (Kieboom and de Bont, 2000; Paulsen et al,. 1996; White et al., 1997). When the gene sequences for each component of the AcrAB system were examined against the genome of *N. europaea*, a low percent homology match was found for each, although a high percent homology match was not found. This suggests that increased susceptibility of *N. europaea* to hydrophobic shock is not due to the lack of such a system, but some other possibility.

Because a system similar to AcrAB was present, the regulation mechanism for this system needs to be examined for its presence and its possible role in enhancing the susceptibility of nitrifiers to hydrophobic chemicals. TolC was previously discussed with the *sox*RS oxidative stress response mechanism. As discussed before, this protein has been found to be regulated by MarA and SoxS. As both of these proteins are absent from the genome, it is possible that the similar gene found in the *N. europaea* genome is not regulated. For the proteins AcrA and AcrB, two possible regulatory mechanisms have been identified. One is the previously discussed positive regulator protein MarA (Ma et al., 1995) that is notably absent from *N. europaea*. Another is the protein AcrR, which mediates over expression of the *acrAB* gene locus (Ma et al., 1996). This gene was also not found in the *N. europaea* genome. In the same study, the *acrAB* system's transcriptional activity increased as the cells entered stationary phase, suggesting that the general stress response regulator RpoS may also play a role in the positive regulation of this efflux system (Ma et al., 1996). The absence of RpoS from the genome for *N. europaea* suggests that expression of the multidrug efflux systems in this organism and possibly all nitrifying bacteria is constitutive and cannot be up or down regulated in response to a hydrophobic chemical shock. Again, this lack of regulation may be important in explaining why nitrifying bacteria are more susceptible to shock events.

All of the remaining identified solvent/multidrug efflux systems have been identified in *Pseudomonas* species. Of these, three are found in *P. aeruginosa*. The first is the MexA-MexB-OprM system; these three proteins are the membrane fusion protein, inner membrane transporter, and the outer membrane protein, respectively (Li et al., 1998). This system was found to convey the most resistance to *P. aeruginosa* exposed to the organic solvents *n*-hexane, *p*-xylene and toluene when compared with the other two systems. In addition, this system was found to be essential for growth in the presence of solvents based on experiments with a mutant strain

lacking the MexAB-OprM system, which failed to grow on any of the tested solvents (Li et al., 1998). The other two systems (presented in the same protein type order as above) were MexC-MexD-OprJ and MexE-MexF-OprN. Unlike the MexAB-OprM system, these two systems are not expressed during growth under laboratory conditions but, instead, are only expressed in multidrug resistant mutants of *P. aeruginosa*. These two systems were also found to confer some solvent resistance but were not essential for growth of the bacteria in the presence of the solvents (Li et al., 1998). Poole and coworkers found that these efflux systems are probably negatively regulated in response to solvent and antibiotics by the protein MexR (Poole et al., 1996).

Although not present in *E. coli*, these solvent/multidrug efflux systems may be conserved across different Gram-negative bacteria and were also examined for their presence in *N. europaea*. When examining the known proteins of *N. europaea*, MexB is present (TrEMBL # Q82XT4) but none of the other proteins involved in the MexAB-OprM system or either of the other two systems were found. When comparing the gene sequences for MexA and OprM against the *N. europaea* genome, probable matches were found for both genes, indicating that this system or a homologous system is present in the AOB. Probable matches were also found for each of the genes of the MexCD-OprJ and MexEF-OprN efflux systems. This suggests, as did the probable matches for the AcrAB-TolC system, that *N. europaea* contains several multidrug efflux systems that are homologous to the three Mex systems of *P. aeruginosa* and, therefore, would be able to survive in the presence of hydrophobic or lipophilic compounds. MexR, the negative regulator of the Mex systems, was not present in the genome of *N. europaea*, suggesting again that no regulation mechanism exists for the multidrug efflux systems within nitrifying bacteria. However, because MexR is a negative regulator, absence of the gene for this protein means that expression of these efflux systems may not be repressed. A lack of repression would confer higher resistance to solvents and not increased sensitivity; however, it the latter was the observed response. This may indicate that another regulator is present in nitrifying bacteria, or that the constitutive expression of the Mex-like efflux systems is not enough to overcome a high concentration shock of hydrophobic chemicals. It is also possible that this system does not confer resistance to alcohol solvents like octanol, as no previous experiments have shown an increased tolerance of *P. aeruginosa* to alcohols.

The remaining three RND family efflux systems were discovered in strains of *P. putida*. The first of these is the SprA-SprB-SprC system and has been found to confer resistance to the solvents toluene, *p*-xylene, octanol, ethylbenzene, propylbenzene, cyclohexane and hexane (Kieboom et al., 1998a). This system functions in much the same manner as the previously discussed systems, and contains a membrane fusion protein that links the inner membrane transporter to the outer membrane protein. However, unlike the previously discussed systems, SprABC is only induced by the presence of solvents, not antibiotics. It confers antibiotic resistance to *P. putida* only after cells were incubated in the presence of the solvent toluene, suggesting that antibiotics do not induce its expression (Kieboom et al., 1998a). The protein sequences for this system could not be found in the Protein Information Resource, but were previously determined by Kieboom et al. (1998b). By using the sequences presented in this paper to search the genome of *N. europaea*, probable matches were found for each of the components of the SrpABC system. The presence of such a system in nitrifying bacteria would indicate that they could tolerate levels of octanol as well as other solvent tolerant bacteria, but our results indicate otherwise. This brings up the question of regulation or even translation of such a system. If the system cannot be expressed at higher than constitutive levels, then increased concentrations of the solvents would not be tolerated. If the genes are not even expressed, then efflux of the contaminants would not occur and the bacteria would be sensitive.

A second system known to confer resistance to toluene, *p*-xylene and cyclohexane is the MepA-MepB-MepC system, first described by Fukumori et al. (1998). The authors found that this system was negatively regulated by the protein MepR, which contained a gene sequence very similar to the *E. coli* AcrAB system regulator AcrR. Unfortunately, the entire sequence of this system was not published by the authors and the listed GenBank accession number (AB008909) does not match any known sequence and thus no search of the *N. europaea* genome could be performed.

The third system identified in *P. putida* is the TtgA-TgtB-TgtC system and has been found to confer resistance to toluene, *m*-xylene and 1,2,4-trichlorobenzene (Kieboom and de Bont, 2000). When examining for the presence of this system in *N. europaea*, a probable match was found; however, the probable matching sequence for the inner membrane transporter protein TgtB in *N. europaea* was the same sequence that was found to overlap for SrpB. This indicates

that these two systems in *P. putida* are very closely related and it is likely that only one such system exists in *N. europaea*.

Unfortunately, hydrophobic chemical stress response mechanisms have not been as well characterized as those for oxidative stress response. This means that more is unknown about the regulatory mechanisms for the identified systems and it is likely that many systems have yet to be found. From the results presented here, it appears that *N. europaea*, and probably other nitrifying bacteria, do contain some of the stress response mechanisms for hydrophobic/lipophilic chemicals. Such responses found include the systems for fatty acid isomerization and solvent efflux. However, as with oxidative chemical stress, the existence of genes encoding these systems does not mean that the stress response mechanisms are expressed during episodes of hydrophobic chemical exposure. In addition, it appears that many of the known regulatory mechanisms for solvent response are either unknown or were not found in *N. europaea*, further indicating that the increases sensitivity may arise from the inability to regulate such responses. Overall, the genomic sequence of *N. europaea* does provide some insight into the nature of susceptibility to both oxidative and hydrophobic chemical shocks, but additional information is needed in order to gain a more complete understanding of nitrification inhibition.

2.5 References

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3 CHEMICAL INHIBITION OF NITRIFICATION IN ACTIVATED SLUDGE

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4 ULTRAVIOLET SPECTROPHOTOMETRIC DETERMINATION OF NITRATE: DETECTING NITRIFICATION RATES AND INHIBITION

4.1 Abstract

Nitrification is an important process in wastewater treatment that, unfortunately, can be easily upset by toxic industrial chemicals. Such upset events can lead to treatment facilities exceeding discharge permits and facing fines. It is therefore important to detect nitrification inhibition quickly so upset events can be prevented or controlled. The objective of this study was to develop a rapid method for measuring and detecting nitrification inhibition by using nitrate generation rates (NGRs). A method using ultraviolet spectrophotometry at wavelengths between 225 and 240 nm without chemical manipulation was developed to measure NGRs and was verified against ion chromatography. The method was shown to quickly and accurately measure nitrate concentrations after correcting for nitrite interference. Additionally, cadmium, chlorine and 1-chloro-2,4-dintrobenzene (CDNB) were tested for nitrification inhibition using this method. Of these, CDNB was found to cause a correctable interference with the test. Only chlorine provided an uncorrectable interference for this method.

Key Words: Nitrification Inhibition, Detection, Method Protocol, UV, Ultraviolet Spectrophotometry, Industrial Toxin

4.2 Introduction

Ammonia toxicity to aquatic systems has been well documented (Sarma et al., 2003; Hillaby and Randall, 1979; Wicks et al., 2002). Because of its toxicity, ammonia discharge into receiving waters is strictly regulated and regulatory agencies throughout the world are continually decreasing the amount of ammonia and total nitrogen that can be discharged into receiving waters. As discharge limits are lowered, ammonia conversion and nitrogen removal processes become increasingly important. Although new treatment processes for ammonia removal, such as anaerobic ammonia oxidation, are gaining in popularity, the most widely used processes for ammonia and total nitrogen removal in a broad range of wastewater applications is still conventional aerobic, autotrophic nitrification.

Nitrification has been found to be one of the most sensitive wastewater treatment processes prone to upset by industrial chemicals (Tomlinson et al., 1966; Kelly II et al., 2004;

Blum and Speece, 1991; Wood et al., 1981). As nitrification is inhibited by unexpected shock loads of industrial influents, increases in effluent discharges of ammonia and total nitrogen can occur and cause a treatment facility to exceed discharge limits. This can lead to heavy fines and penalties for the facility, and leave the downstream environment susceptible to damage. Clearly, it is very important to quickly and accurately detect nitrification inhibition in order to prevent upset of the process before it occurs.

Currently, nitrification inhibition in wastewater treatment can be determined using several methods. Of these, respirometry, which measures oxygen uptake rate (Hu et al., 2003; Tomlinson et al., 1966), and nitrate generation rate (NGR) (Gernaey et al., 1997; Kelly II et al., 2004; Neufeld et al., 1986), which determines the nitrate production rate, are most commonly used. Another method for determining nitrification rates and inhibition uses titrimetric techniques that examine nitrification rates based on the rate of base addition for pH stabilization (Ficara and Rozzi, 2001). Of these, only NGR provides a direct measure of the nitrification rate because it measures the production rate of nitrate, a unique product of nitrification.

One setback of using NGR versus respirometry or titrimetric techniques is that measurement of nitrate in wastewater is time consuming and difficult to perform with continuous monitoring. The most commonly accepted methods for measuring nitrate include colorimetric methods and ion chromatography (APHA, 1998). Analysis by ion chromatography can be slow, while colorimetry for nitrate involves its catalytic reduction to nitrite before detection, plus the catalysts generate hazardous waste that requires costly disposal. Nitrate ion selective electrodes show more promise because they provide quick measurements and have been applied successfully. Unfortunately concentration drift can occur when they are used in wastewater samples, which requires frequent re-calibration of the probes (Pedersen et al., 2002). Another technology that can be used for nitrate detection is direct ultraviolet (UV) spectrophotometry without chemical manipulation. This technique has been used to monitor nitrate in full-scale wastewater treatment facilities and provides rapid readings of nitrate concentration (Neu, 1995; Chevalier et al., 2002).

Because of strict time limitations for monitoring nitrification at full-scale facilities and during laboratory experiments, a rapid, accurate, and sensitive test for detecting nitrification inhibition using a direct measure of nitrification rates is needed. UV detection of nitrate has not been directly applied to determining NGRs, but we believed this analytical approach is

promising for applying to NGR analyses. Therefore, the objective of this study was to adapt UV spectrophotometric techniques for measuring nitrate into a new simple and rapid monitoring technique for NGR analysis so that the impact of wastewaters on nitrification can be rapidly detected.

4.3 Methodology

4.3.1 Mixed Liquor Source Description and Sample Treatment

Mixed liquor was obtained the morning of each experiment from the Blacksburg-VPI Wastewater Treatment Facility at Stroubles Creek. The treatment process at the Stroubles Creek facility consists of a nitrifying activated sludge treatment process with a 10-14 day solids retention time (SRT). Mixed liquor grab samples were taken directly from the aerated mixed liquor basins and transported to the laboratory within 20 minutes. The mixed liquor samples were aerated, magnetically mixed, and continuously fed a synthetic wastewater solution as previously described (Kelly et al., 2004) to mimic the average feed loading conditions at the Stroubles Creek facility and to maintain a constant biomass composition for experiments. The total volume of feed solution used was less than 2% of the volume of mixed liquor so suspended solid concentrations were not significantly changed throughout the experiment (8-10 hours). This was confirmed by measuring mixed liquor volatile suspended solids at various times during the experiment.

4.3.2 Nitrate Generation Rate Using UV spectrophotometry

To determine the NGR of the mixed liquor nitrifying community, 200 ml of mixed liquor was added to each of two 300 ml biochemical oxygen demand bottles to obtain duplicate measurements. The bottles were magnetically mixed and continuously aerated for the duration of the NGR test. These mixed liquor "reactors" were then spiked with $(NH_4)_2SO_4$ so that the ammonia concentration in each reactor was 20 mg/L-N. This was done to achieve an initial NH_4^+-N concentration well above the half saturation constant (K_{NH}) for nitrification, which is typically around 1 mg/L-N (Grady et al., 1999), in order to ensure measurement of the maximum NGR. Once the ammonia was added, ten ml samples were collected from each bottle every 10 minutes for 50 minutes. Samples were centrifuged for 3 minutes at 3400xg to pellet the solids. The supernatant was then removed and filtered through a 0.45 μm nitrocellulose filter for analysis. A Beckman DU 640 UV/Vis spectrophotometer (Beckman Coulter, Inc., Fullerton,

CA) was used for nitrate analysis. Absorbance readings (zeroed against distilled water) were taken in a quartz cuvette at a single optimum wavelength between 225 and 240 nm that was determined to be the optimum wavelength based on peak absorbance readings on the day of the experiment. The absorbance readings were then compared to a standard curve, which was prepared as described below.

Standard curves for nitrate were developed by making nitrate standards in mixed liquor filtered through a 0.45 μm filter on the day of the experiment (0-20 mg/L nitrate added). Standards were made in mixed liquor so that interferences in the matrix would be common, and therefore negated, between standards and samples. The baseline nitrate concentration of the mixed liquor was measured using ion chromatography as described below. The known amounts in the standards were calculated by adding the IC measured baseline nitrate concentration to the known amount of chemical added. Standards were then measured for UV absorbance as described above. A plot of nitrate concentration versus absorbance yielded a linear standard curve to which a linear regression was performed (best fit determined by least squares analysis, Sigma Plot 8.0®) and nitrate concentrations in unknowns were calculated from absorbance readings.

Because nitrite was found to interfere with UV determination of nitrate, nitrite concentrations were measured and subtracted from the UV-determined nitrate readings. Nitrite concentrations for each sample were determined colorimetrically according to method 4110 B as described in Standard Methods (APHA, 1998) and analyzed using the Beckman DU 640 UV/Vis spectrophotometer. These concentrations were then converted to absorbance by developing a UV nitrite standard curve in the same manner as described for nitrate below. The UV absorbance of nitrite was found to be linear with Sigma Plot 8.0® and independent of the nitrate concentration. At 229 nm, every 1 mg/L-N nitrite showed an absorbance of 0.10, while at 235 nm, the absorbance per 1 mg/L-N nitrite was 0.045. The absorbance associated with the measured nitrite concentration was calculated using this relationship and was subtracted from the total absorbance readings for each sample in order to get a nitrate absorbance reading. The nitrate absorbance readings were then converted to nitrate using the nitrate standard curve.

To determine the NGR, the calculated nitrate concentrations were plotted with time for a given reactor. The linear slope of this curve (best fit determined by least squares analysis, Sigma

Plot 8.0®) gave the nitrate concentration generated with time. This was normalized to the MLVSS concentration to give the NGR, in mg-N/min-g MLVSS.

4.3.3 Determining Nitrification Inhibition Using NGR

The UV spectrophotometric method was evaluated as a basis for determining nitrification inhibition using three different chemical inhibitors: cadmium, 1-chloro-2,4-dinitrobenzene (CDNB), and chlorine. Inhibited or "shocked" reactors were prepared by adding varying concentrations of the chemical contaminants to yield $NGR_{inhibited}$. Control reactors, to which no contaminant was added, were run simultaneously to generate $NGR_{control}$, and served as a basis for comparisons. NGR tests were then run in duplicate according to the method listed above. Equation 1 was used to calculate the percent inhibition caused by each concentration of a given contaminant.

$$\text{Percent Inhibition} = \frac{NGR_{control} - NGR_{inhibited}}{NGR_{control}} \times 100\% \quad \text{[Equation 1]}$$

4.3.4 Analytical Procedures

Mixed liquor total (MLSS) and volatile (MLVSS) suspended solids were measured in duplicate according to sections 2540 D and 2540 E of Standard Methods (APHA, 1998). Samples for nitrate and nitrite were analyzed using either UV spectrophotometry, as described above, or ion chromatography (IC). For IC analysis, samples were taken from the mixed liquor and centrifuged at 3400xg for 2 minutes. The supernatant was then filtered through a 0.45 μm nitrocellulose filter and stored at -20°C for no more than 28 days prior to analysis. Nitrate and nitrite were analyzed in duplicate using a Dionex DX120 suppressed conductivity ion chromatograph with an AS14 anion separation column and an AG14 guard column. Samples were carried in an eluent of 3.5 mM Na_3CO_3 and 1.0 mM $NaHCO_2$.

4.4 Results and Discussion

4.4.1 UV Method Precision and Bias

Precision and bias of this method were determined according to Standard Methods section 1040 B (APHA, 1998) using 7 replicates of several different standards (Table 4.1). For the method validation tests, standard curves were developed at 229 and 235 nm, which were the wavelengths used for all tests. At 229 nm, the precision did not exceed 0.33 mg/L-N and the bias did not exceed -0.3 mg/L-N for either 7.1 or 26.1 mg/L-N nitrate. For 235 nm tests, the

precision and bias for both concentrations did not exceed 0.10 and 0.09 mg/L-N, respectively. The lower precision and bias noted at 235 nm may be due to a higher correlation coefficient for the linearly fitted data. The higher correlation at 235 nm was due to the lower absorbance response for a given nitrate concentration, which meant that the response at 229 nm was only linear to a nitrate concentration of about 25-30 mg/L-N. Detection limits for UV determination of nitrate in water and wastewater solutions have been previously documented to range from 0.1 to 25.0 mg/L nitrate (Melchert and Rocha, 2005; Ferree and Shannon, 2001); however, because the wastewater matrix used as a solvent for standards in this protocol inherently contained nitrate above 3 mg/L, the method detection levels for this protocol could not be determined in the wastewater matrix. For the instrument used, standards made in distilled water had a linear detection range of 0.01-50 mg/L-N for nitrate measured at 229 nm, 0.01-100 mg/L-N for nitrate measured at 235 nm, and 0.01-50 mg/L-N for nitrite measured at both 229 and 235 nm.

Table 4.1 Precision and bias for the UV measured nitrate method for 7.1 and 26.1 mg/L-N nitrate measured at 229 and 235 nm.[a,b]

RESULTS AT 229 NM			
Total Absorbance = 0.0502*Nitrate Concentration (mg/L-N) + 0.4885, R^2=0.9981[c]			
7.1 mg/L NO_3^--N		26.1 mg/L NO_3^--N	
Precision	Bias	Precision	Bias
0.13 mg/L-N	**-0.12 mg/L-N**	**0.33 mg/L-N**	**-0.30 mg/L-N**
RESULTS AT 235 NM			
Total Absorbance = 0.0165*Nitrate Concentration (mg/L-N) + 0.2382, R^2=0.9997[c]			
7.1 mg/L NO_3^--N		26.1 mg/L NO_3^--N	
Precision	Bias	Precision	Bias
0.10 mg/L-N	**-0.09 mg/L-N**	**0.07 mg/L-N**	**0.06 mg/L-N**

[a]All numbers are based on statistical analysis of 7 replicate measurements.
[b]Equations given for the standard curves were developed using a minimum of 5 different standard concentrations.
[c]For each standard curve, duplicate absorbance measurements were made for each standard.

4.4.2 Optimum Wavelength Determination

Using UV wavelength scans, the optimum wavelength for detecting the nitrate concentration in most wastewater samples was found to be 229 nm. Scans were performed by reading absorbance every 1 nm between 200 and 300 nm using filtered (0.45 μm nitrocellulose filter) mixed liquor that was spiked with known, increasing concentrations of nitrate. Nitrate concentrations for these standards were also measured in the filtered mixed liquor using ion

chromatography so that the exact concentrations could be known for the scans. After examining the scans of several different mixed liquor samples and nitrate concentrations, it became obvious that excessive noise was present below 225 nm and absorbance response was too low above 240 nm (Figure 4.1). Therefore, 225 to 240 nm was chosen as the wavelength range to examine the linearity of nitrate absorbance. The standard curves developed for several wavelengths between 225 and 240 nm were compared for (i) goodness of fit, as measured with R^2 values, (ii) the ability to provide reproducible regressions over several days with freshly prepared standards, and (iii) the ability to maintain a high absorbance response and resolution for nitrate (Figure 4.2). 229 nm was chosen as the wavelength to use for most UV nitrate analysis because of the high goodness of fit, reproducibility, resolution, and response. All calibration curves developed for nitrate were developed using this wavelength, and curves look like those presented in Figure 4.2. Exceptions to using this wavelength for analysis are discussed below.

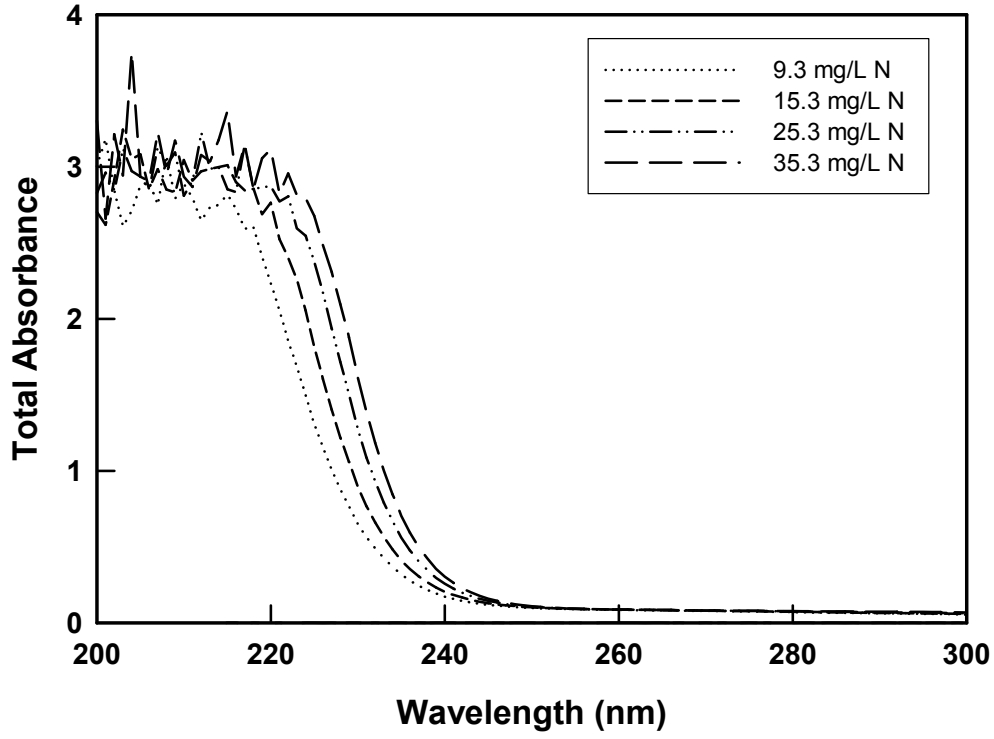

Figure 4.1 Wavelength scan of nitrate absorbance in filtered mixed liquor. Nitrate concentrations are given in mg/L as nitrogen. Distilled water scan showed 0 absorbance over the wavelength range.

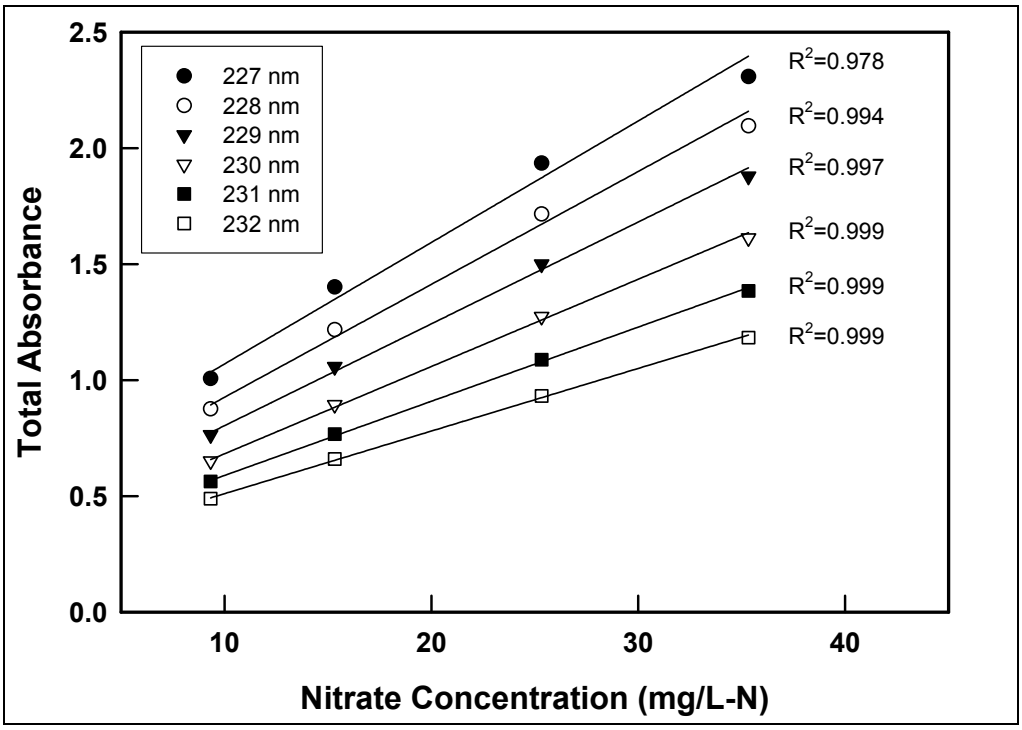

Figure 4.2 Linearity of UV absorbance with increasing nitrate concentration for wavelengths from 227 to 232 nm. R^2 values reflect the goodness of fit for the linear regressions shown.

4.4.3 Nitrite Interference

Nitrite in the mixed liquor provided an interference to UV nitrate measurements that could be corrected. The interference was discovered when the accuracy of the UV method for detecting nitrate was checked using ion chromatography. The check revealed that the UV method over-predicted the nitrate concentration by as much as 40% when nitrite was present at concentrations as low as 2 mg/L (data in Appendix B). As nitrite is also measured using UV spectrophotometry (Thomas et al., 1996; Ferree and Shannon, 2001; Chevalier et al., 2002), experiments were performed to determine if it was interfering with nitrate readings. Results showed that low concentrations of nitrite provided significant absorbance within the same wavelength range that nitrate was measured (Figure 4.3). In addition, the absorbance measurements showed that the slope describing nitrite absorbance did not change with increasing concentrations of nitrate; indicating nitrite absorbance is linear and additive in the same wavelength range as nitrate. Because of the linear, additive properties of nitrite absorbance, absorbance due to nitrite was subtracted from the total absorbance readings to give the net absorbance indicative of only the nitrate concentration. After correcting the absorbance readings

for nitrite, comparisons of UV measurements to IC measurements showed very similar results (Table 4.2), indicating that the nitrite corrected UV method provides accurate nitrate measurements.

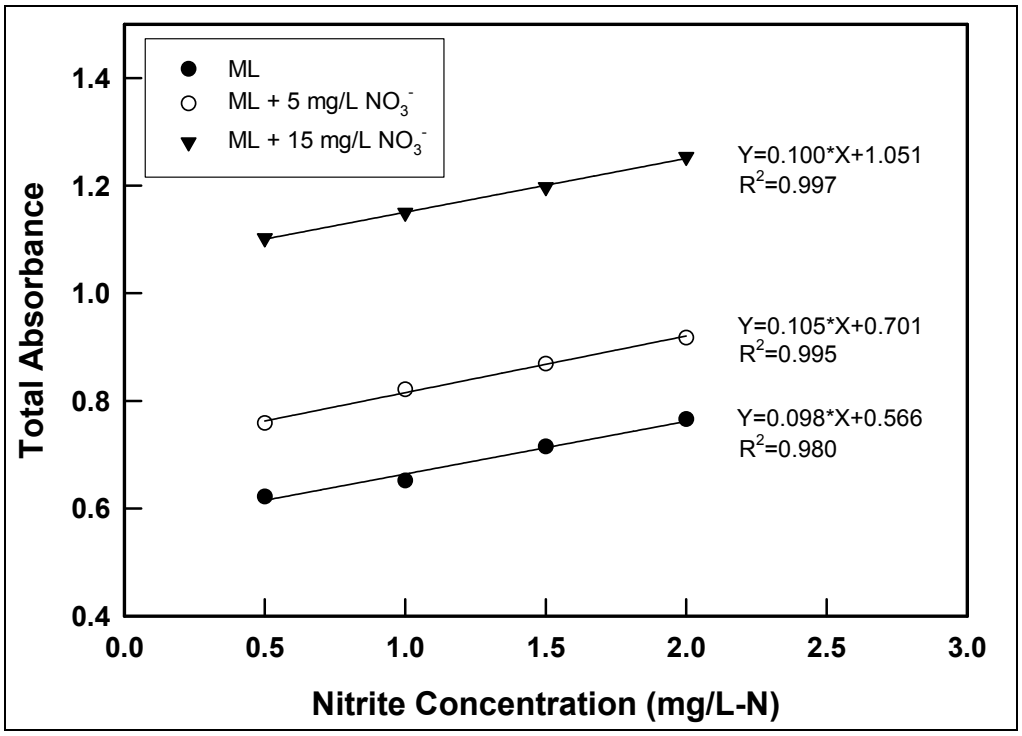

Figure 4.3 Linearity of nitrite UV absorbance in mixed liquor (ML) measured at 229 nm; ML contained 7.3 mg/L inherent NO_3^-. Different lines correspond to increasing concentrations of nitrate in the mixed liquor. R^2 values reflect the goodness of fit for the linear regressions shown.

Table 4.2 Comparison of nitrite corrected UV measured nitrate concentrations to ion chromatography (IC) measured nitrate concentrations. Number given is average of 4 measurements ± (standard deviation).

UV Measured Nitrate Concentration (mg/L-N)	IC Measured Nitrate Concentration (mg/L-N)
10.5 ± (0.3)	10.4 ± (0.3)
11.4 ± (0.5)	11.3 ± (0.4)
12.3 ± (0.6)	12.0 ± (0.4)[a]
12.5 ± (0.5)	12.6 ± (0.2)
13.6 ± (0.7)	13.6 ± (0.4)
14.1 ± (0.5)	14.2 ± (0.3)

[a]Average and standard deviation are of 3 measurements.

4.4.4 Inhibitory Compound Interference

When testing the UV NGR method using inhibitory compounds, CDNB caused a correctable interference within the wavelength range. The measured interference for CDNB was found when cadmium, chlorine and CDNB were each examined for absorbance in the UV range used to measure nitrate before performing complete inhibition tests using the UV method. Of the three compounds examined, only CDNB was found to absorb in the wavelength range for nitrate measurements as cadmium and chlorine were not found to directly interfere with nitrate detection. This was expected, as others have used UV spectrophotometry to analyze for the presence, type, and concentration of aromatic compounds (Touraud et al., 1998; Dixit and Ram, 1996). Although it interfered at the measured wavelengths, nitrate readings could be corrected by subtracting the absorbance due to the added concentration of CDNB. The only change to the original UV method was that the optimum wavelength for a linear response of nitrate readings was shifted from 229 to 235 nm due to the additional absorbance caused by CDNB (data in Appendix B).

Although chlorine was not found to directly interfere with UV nitrate determination, the IC measured results with mixed liquor did not match those found using the UV detection. Therefore, we concluded that toxins that react readily with organic matter, like chlorine, are problematic for the UV NGR method because the reactions/interactions affect the absorbance

readings (data in Appendix B). From these results, UV techniques were used to measure inhibition with cadmium and CDNB but IC analysis had to be used for chlorine.

4.4.5 Using UV for NGR Measurements

Once the UV method for measuring nitrate for NGR and inhibition detection was confirmed, experiments conducted using cadmium and CDNB showed that this method provided a fast and accurate method for determining nitrification inhibition. This can be seen in Figure 4.4, which shows a typical nitrate generation curve developed for cadmium. This figure shows the results for control mixed liquor (no cadmium) and mixed liquor shocked with 30 mg/L cadmium. Figure 4.4 also shows that nitrate data that was obtained using the UV technique yielded linear plots of nitrate generation rate with high correlation coefficients. Once NGRs were determined for the different chemical concentrations, inhibition was calculated to generate an inhibition curve. A typical curve that was developed using the UV NGR technique for cadmium is shown in Figure 4.5. As these results show, the UV NGR technique can be used to develop curves quickly for inhibition studies and prediction.

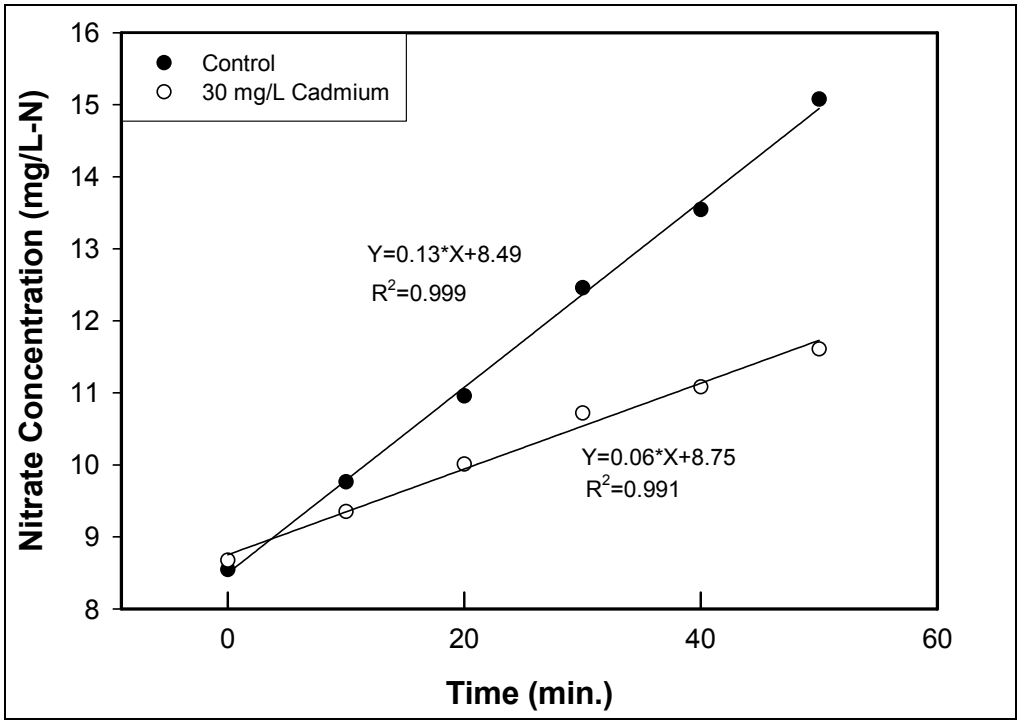

Figure 4.4 Nitrate generation curve for cadmium inhibited and control (0 Cd added) mixed liquor. R^2 values reflect the goodness of fit for the linear regressions shown.

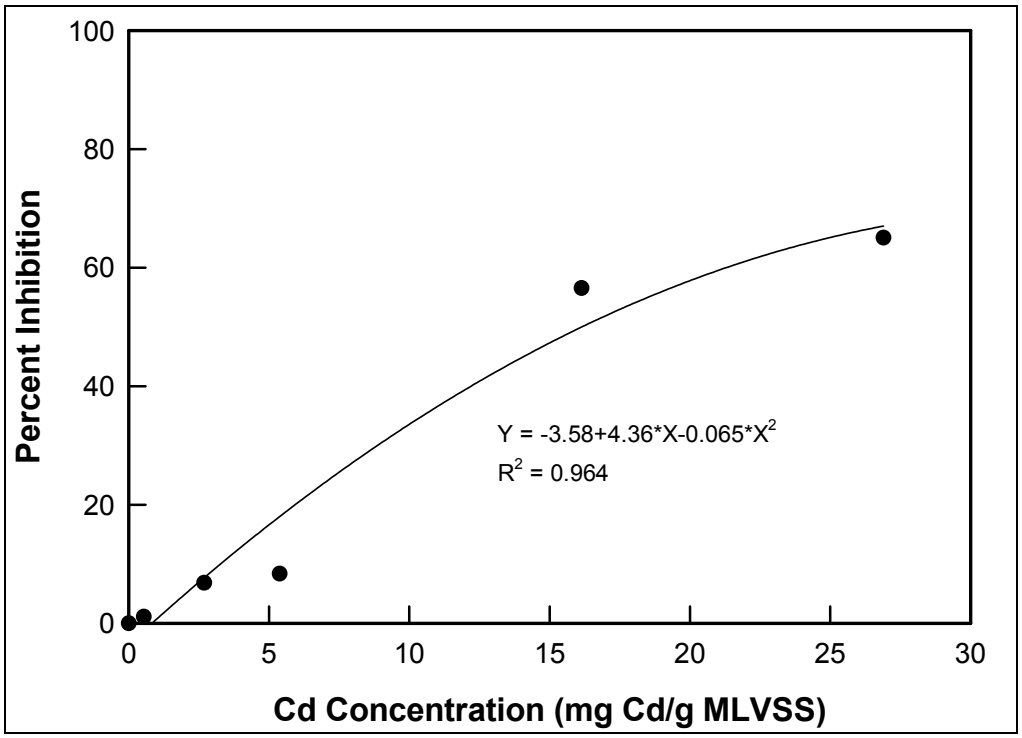

Figure 4.5 Nitrification inhibition curve developed using UV detection of NGR for cadmium induced inhibition. R^2 values reflect the goodness of fit for the linear regressions shown.

4.4.6 Significance of Work

The initial purpose of developing this method was to reduce the time required to collect and analyze data used to determine nitrification inhibition. However, when testing the method we found that it was also easy to implement and required less capital outlay in instrumentation because it used instrumentation that most wastewater analysis labs already have, which makes it more likely to work in wastewater treatment plant labs. Initially, nitrate probes were considered because they provide instant results, can be attached to a data acquisition unit for continuous operation, and require little to no sample processing for laboratory scale experiments. However, we encountered excessive drift in nitrate concentration readings when operated in mixed liquor during our initial experiments (data in Appendix B). Drift has been seen by others (Pedersen et al., 2002) who circumvented the problem by repeatedly re-standardizing the probes in mixed liquor using an automated system. Although re-standardization makes use of probes an option if one develops a re-standardization protocol, we decided to abandon this approach in favor of an operationally simpler method because of limitations of time and difficulty for wastewater treatment plant operators.

After ruling out the use of probes, we focused on demonstrating a method using UV spectrophotometry because it is has been proven to detect nitrate in activated sludge (Thomas et al., 1996; Ferree and Shannon, 2001; Chevalier et al., 2002) and commercially available UV spectrophotometric units exist for monitoring nitrate in mixed liquor basins at full-scale facilities. Although the developed method is simpler to set up at the laboratory scale, it does require increased sample processing, as the mixed liquor must be filtered before readings are taken. Additional disadvantages to this method include the fact that nitrate concentration results are not instantaneous (sample processing time required) and data collection cannot be automated or continuous. However, these setbacks can be overcome by using a robotic sample collection system to automate sampling or an in-line filter system to remove particulates prior to the spectrophotometric unit.

Regulations governing ammonia and nitrogen discharge clearly make it important to have techniques that can be used to detect nitrification inhibition quickly and accurately. Ion chromatography can take upwards of 20 minutes per nitrate sample for analysis, and respirometry systems do not directly measure nitrification rates in wastewater treatment facilities. Furthermore, differential respirometry, which relies on determining nitrification oxygen uptake rates from the difference of the total oxygen uptake rate and nitrification inhibited oxygen uptake rate, has been found to be a highly variable and unreliable means of measuring nitrification inhibition (Kelly and Love, In Review). The UV technique discussed here provides an accurate and rapid assessment of nitrification inhibition, as the total analysis time is less than 1.5 hours and uses simple analytical instrumentation found in most laboratories. Although this technique requires increased sample processing for nitrite correction, the colorimetric method used to measure nitrite is rapid and easy and does not significantly increase the analysis time. In addition, corrections can be made for interferences caused by chemicals that do not react with organic matter. Even though wastewater facility operators would not necessarily know what interfering chemicals would be present in the wastewater stream, the UV measured NGR test could be standardized using the filtered mixed liquor to be tested. By doing this, chemical interferences would be eliminated as the interfering chemicals would be measured as part of the matrix background.

Though the method as used here was not automated for on-line measurement of nitrification inhibition, on-line spectrophotometric monitoring devices are commercially

available for monitoring nitrate and nitrite in full-scale wastewater treatment facilities (Neu, 1995; Chevalier et al., 2002). We believe that with minor modifications to these technologies, this technique could be adapted for on-line detection of nitrification inhibition, or adapted for use as an upstream monitoring device for nitrification upset. By using this technique to monitor for nitrification inhibition, operators at wastewater treatment facilities can gain the ability to react to nitrification upset events and take steps to minimize or mitigate the effect.

4.5 Conclusions

The UV spectrophotometric technique described here for measuring nitrification inhibition is simple to perform and provides quick results. Nitrate measurements made using this technique are accurate, as confirmed using ion chromatography. Corrections for non-reacting chemical interferences could be made with this technique. It is the authors' opinion that this technique can be a powerful tool for monitoring nitrification rates and inhibition both in laboratory studies and eventually in full-scale treatment facilities.

4.6 References

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5 A Critical Comparison of Two Common Methods to Detect Nitrification Inhibition in Activated Sludge

5.1 Abstract

Nitrifying biological treatment systems are prone to upset by industrial toxins and, therefore, identifying inhibition is important in upset prevention. Several methods can be used to determine nitrification inhibition in wastewater treatment. The objective of this study was to compare two of these methods, differential respirometry and nitrate generation rate (NGR), and to determine if a short-term (<1 hour) test adequately describes the full extent of inhibition. Three industrially relevant toxins were used to test these objectives: cadmium, 1-chloro-2,4-dinitrobenzene (CDNB) and chlorine. The results indicate that differential respirometry yielded highly variable results that were different on average from NGR-based inhibition measurements, which were much more consistent. This suggests that a direct measure of nitrification, like NGR, provides a better measure of nitrification inhibition. In addition, cadmium and CDNB, chemicals that are not quickly removed from the wastewater stream, were found to cause maximum inhibition between 6 and 24 hours after the chemical shock was applied. However, chlorine, a chemical that reacts and dissipates very quickly, caused maximum inhibition almost immediately.

Key Words: Nitrification, Inhibition, Respirometry, Nitrate Generation, Toxin

5.2 Introduction

Regulations governing ammonia and total nitrogen discharge into receiving waters are continually tightening throughout the world. As discharge limits are lowered, ammonia conversion and nitrogen removal processes are growing in importance. Although new treatment processes for ammonia removal are gaining in popularity, the most widely used processes for ammonia and total nitrogen removal in wastewater treatment continue to rely on conventional aerobic, autotrophic nitrification. Conventional nitrification involves the two-step biological conversion of ammonia to nitrate. A common challenge with nitrification is the ease with which it is inhibited by chemicals (Tomlinson et al., 1966; Blum and Speece, 1991; Wood et al., 1981, Hockenbury and Grady, 1977). Studies using nitrifying mixed liquor have found that single shock loads of a wide variety of industrial chemicals can inhibit the nitrification process for

weeks, and significant recovery times are often needed to regain nitrification (Nowak and Svardal, 1993; Kelly II et al., 2004; Stasinakis et al., 2003). When inhibition occurs, treatment facilities can exceed discharge limits, incur heavy fines and cause damage to the downstream environment. Clearly, it is important to have methods or devices that quickly and accurately detect the presence of nitrification inhibiting chemicals so that corrective actions can be taken to minimize or prevent process upset.

Currently, nitrification inhibition in wastewater treatment can be determined using several methods. Two prevalent methods include respirometry based on oxygen uptake rates (Hu et al., 2003; Gernaey et al., 1997; Tomlinson et al., 1966) and nitrate generation rate (NGR) (Grunditz and Dalhammar, 2001; Neufeld et al., 1986) based on nitrate production rates. Respirometry is a rapid test (often < 20 minutes), however; it measures the total oxygen uptake rate of all biomass, not just the nitrifiers. To determine the oxygen uptake rate (OUR) of only the nitrifiers in mixed liquor, differential respirometry can be used whereby the nitrification-inhibited OUR is subtracted from the total OUR to yield a nitrification OUR. This is accepted as a measure of nitrification rate because the OUR of nitrifying bacteria stoichiometrically relates to the rate of nitrate generation (White, 2000; Grady et al., 1999). Because OUR methods are rapid and have a clear stoichiometric relationship to the nitrification rate, devices using differential respirometry to detect nitrification inhibition are commercially available for full-scale use. However, there are limitations in using this chemically-dependent technique, including possible impact of the nitrification-specific inhibitor on heterotrophs and reactions between the inhibitor and chemicals in the activated sludge matrix. These limitations would not apply to other respirometric methods; however, they are pertinent to this study, which focuses only on differential respirometry.

Unlike differential respirometric techniques, NGR detects a unique product of nitrification and, therefore, provides a direct measure of the nitrification rate. It does not require the addition of chemical inhibitors, but it does generally take longer to perform than respirometry. NGR has been used in both laboratory (Grunditz and Dalhammar, 2001; Hooper and Terry, 1973) and full-scale facilities (Grunditz and Dalhammar, 2001; Lee et al., 1997), but, to the authors' knowledge, commercially available full-scale NGR units do not exist. This could be because nitrate is typically measured by ion chromatography or nitrate-selective electrodes. Ion chromatography is not yet easily adapted for continuous nitrate measurement and electrodes

tend to show a false drift in nitrate concentrations when used in activated sludge (Pedersen et al., 2002), making both approaches difficult to adapt to full-scale continuous monitoring. A promising approach that has been adapted to full-scale continuous monitoring of nitrate is ultraviolet spectrophotometry, but to the authors' knowledge no UV spectrophotometry device has been adapted for measuring NGR real-time or by automated means.

Previous experiments conducted in our labs examined inhibition using both total OUR and NGR and suggested that the extent of nitrification inhibition was more accurately depicted using NGR (Kelly II et al., 2004). However, data obtained during our previous study did not include differential respirometry. Furthermore, our data showed that the full extent of nitrification inhibition did not occur until hours after a shock load of industrial toxin was introduced. The time dependence has been seen by others (Hu et al., 2003) and is a very important factor to consider when defining protocols for determining the degree of inhibition because the inhibition results could influence operating processes at a full-scale facility.

The objective of this study was to compare both differential respirometry and NGR as methods for detecting nitrification inhibition in order to show that a direct measure of the nitrification rate provides a better measure of inhibition. In addition, we examined the time dependence of the extent of inhibition to prove that the full extent of nitrification inhibition is time dependent. Three industrially relevant chemical toxins were chosen: cadmium, 1-chloro-2,4-dinitrobenzene (CDNB), and chlorine. Cadmium and CDNB are found in various industrial wastewaters (Weber and Sherard, 1980; Lee and Sax, 1987, Environmental Defense Scorecard, 2005; EPA, 2005) , and chlorine is used in biological wastewater treatment to control bulking and foaming problems (Neethling et al., 1987; Cotteux and Duchene, 2003; Marstaller et al., 1992). NGR was used to determine inhibition levels for several concentrations of the toxins, starting immediately after their addition to the mixed liquor. Several of these concentrations were also tested using differential respirometry in order to compare the two methods. To determine the time to maximum inhibition, NGR and differential respirometry were conducted over time after toxin addition using sequencing batch reactors (SBRs). Selected experiments were repeated in continuous-flow stirred tank reactors (CSTRs) to compare the results between the two reactor configurations.

5.3 Methods and Materials

5.3.1 Mixed Liquor Source Description and Sample Treatment

Mixed liquor was obtained the morning of each experiment from the Blacksburg-VPI Stroubles Creek Wastewater Treatment Facility (WWTF). The WWTF process consisted of a nitrifying activated sludge with a 10 to 14 day solids retention time (SRT). Grab samples were taken directly from the aerated mixed liquor basins and transported to the laboratory within 20 minutes. For the short-term method comparison experiments, the mixed liquor samples were aerated, magnetically mixed, and fed a small volume (<2% of total volume) of concentrated synthetic wastewater solution (Kelly II et al., 2004) used to mimic the average feed loading conditions at the WWTF. Feeding continued throughout the experimental period to maintain the same levels of biological activity. For the sequencing batch reactor (SBR) time dependence experiments, the mixed liquor was immediately added to the SBRs, which are described below.

5.3.2 Short-Term Inhibition Determination: Method Comparisons

Using differential respirometry or NGR for the short-term method comparison experiments, the degree of inhibition was examined immediately after addition of one of three chemical contaminants: cadmium ($CdCl_2$), CDNB, or bleach (NaOCl). NGR tests were conducted with mixed liquor exposed to varying contaminant concentrations to develop inhibition curves for each contaminant. Once an inhibition curve was developed using NGR, selected toxin concentrations were chosen to compare the NGR results to the specific oxygen uptake rate (SOUR) differential respirometry method.

NGR tests were conducted and analyzed as described in Kelly and Love (In Review). Briefly, mixed liquor was mixed, aerated, and spiked with $(NH_4)_2SO_4$ to a final concentration of 20 mg/L-N so that the maximum nitrate generation rate was measured. Samples were pulled at 10 minute intervals over a 50 minute period and processed for nitrate and nitrite analysis. Ultraviolet (UV) spectrophotometry was used to determine the nitrate concentration for cadmium- and CDNB-inhibited experiments at wavelengths between 225 and 240 nm (determined on the day of the test). Because nitrite interferes with UV nitrate readings, nitrite was determined colorimetrically according to method 4110 B in Standard Methods (APHA, 1998). Through use of a standard curve that related nitrite concentration to absorbance, nitrite-influenced absorbances could be determined and subtracted from absorbance readings for

nitrate+nitrite to generate a nitrate profile. To determine the specific NGR (SNGR,mg-N/min-g MLVSS) the slope of the nitrate profile was normalized to the MLVSS concentration (method 2540 E (APHA, 1998)). The percent inhibition was calculated according to Equation 1. $SNGR_{control}$ refers to the response by mixed liquor to which no chemical was added.

$$\frac{SNGR_{control} - SNGR_{inhibited}}{SNGR_{control}} \times 100\% \qquad \text{equation [1]}$$

An inhibition curve was developed using least squares regression analysis of the data (Sigma Plot 8.0®), and the chemical concentration that caused 50% inhibition of nitrification (nIC_{50}) was determined from the fitted model equation. Chlorine inhibited reactors could not be analyzed for nitrate using UV because of an interference at the test wavelengths. Samples collected from the chlorine SNGR assay were analyzed for nitrate using ion chromatography as described in Kelly et al. (2004).

SOURs were determined from aerated mixed liquor that was added to 300 ml BOD bottles and spiked with selected concentrations of chemical contaminants. Synthetic wastewater (previously described) was added to achieve an initial concentration of 100 mg/L COD so unrestricted respiration could occur during the short test and $(NH_4)_2SO_4$ was added to a concentration of 20 mg/L-N to achieve maximum nitrification rates. The oxygen uptake rate for the mixed liquor was determined in duplicate using Orion model 97-08 oxygen electrodes (Orion Research, Inc., Beverly, MA) connected to a data acquisition system. A minimum dissolved oxygen drop of 2.0 mg/L or a time of 20 minutes was required for the test. The OUR was performed and calculated according to section 2710 B of Standard Methods (APHA, 1998). SOUR was calculated by dividing the OUR by the MLVSS concentration. Nitrification inhibited SOUR was measured by adding 30 mg/L of 2-chloro-6-(trichloromethyl)pyridine (TCMP), a selective inhibitor of ammonia oxidation. We experimentally determined that this concentration inhibited the mixed liquor SNGR by >98% but it did not inhibit respiration rates in a pure culture of a heterotrophic activated sludge isolate, *Pseudomonas aeruginosa* (data in Appendix C). The SOUR of the nitrifying community (nSOUR) was then determined through differential respirometry by taking the difference between the total SOUR and the nitrification inhibited SOUR. Percent inhibition was calculated using a parallel equation to that for SNGR.

All SNGR and SOUR tests were conducted at room temperature (23-25°C). To minimize time dependent influences of data analysis and interpretation, the order of dosed toxicant

concentrations was randomized. All tests for SNGR and SOUR were conducted in duplicate and short-term experiments were repeated at least once.

5.3.3 Long-Term Time Dependence Experiments

SBRs were used as the operating reactors for each contaminant. The experimental setup consisted of four 4-L beakers maintained at a 12 day target SRT to ensure complete nitrification. Of these, one reactor was used as a control and received no contaminant and one reactor was shocked with the contaminant concentration that inhibited nitrification by 50% (IC_{50}) based on short-term SNGR measurements. One reactor was a nitrification inhibited control reactor to which TCMP (30 mg/L) was added, and one reactor contained the contaminant plus TCMP. The TCMP reactors were used to measure differential respirometry. Reactors were maintained with a 1 day nominal hydraulic retention time (HRT). Detailed operation of the SBRs is described in Kelly et al. (2004). SNGR and differential SOUR assays were conducted as previously described immediately after contaminant addition (0 hr) and after 3, 6, 12, 24 and 48 hours.

To compare the results of a completely mixed continuously fed system to a batch fed system, Eckenfelder style 10 L continuously stirred tank reactors (CSTRs) were used and shocked with cadmium only. These reactors used the same HRT and SRT as the batch system. All tests for SNGR and SOUR were conducted in duplicate and experiments for both the SBR and CSTR reactors were repeated at least once.

5.4 Results and Discussion

5.4.1 Inhibition Detection Method Comparisons

Initial results of the short-term inhibition tests for cadmium first showed that an interaction occurred between cadmium and the biomass. This was observed when calculating the IC_{50} concentrations from the two trials, which were found to be 29 and 41 mg/L. When the concentrations were normalized to biomass concentration (mg Cd/g MLVSS), the nIC_{50} concentrations for both experiments were 16 mg/g MLVSS. Since the nIC_{50} values are the same when normalized to the biomass concentration, it indicates a surface interaction is occurring between cadmium and the biomass. This pattern is consistent with what has been found by others for cadmium (Hu et al., 2003; Costley and Wallis, 2001).

Short-term cadmium results also indicated that the SNGR test provided a more precise and accurate description of nitrification inhibition than the differential SOUR test, which tended to predict lower nitrification inhibition levels than the SNGR test. For cadmium, nSOUR measurements were performed with cadmium concentrations of 1, 5, 10 and 50 mg/L. The actual (not model predicted) percent inhibition data for nSOUR and the SNGR tests at these concentrations are summarized on Table 5.1. The SNGR inhibition level determined for each of the concentrations shown on Table 5.1 was approximately the same for the two trials; therefore, SNGR was very stable and repeatable. In contrast, the nSOUR results varied more between trials, as seen by the increase from 4% to 17% inhibition for 10 mg/L cadmium. Also, the results for nSOUR were less likely to follow a logical pattern; for example, negative inhibition (indicating a lower nSOUR in the control than the cadmium shocked reactor) was observed at low concentrations. Furthermore, the percent inhibition levels based on SNGR and nSOUR differed for the same cadmium concentration. Assuming that SNGR provides an accurate measure of nitrification inhibition because it measures nitrification rate directly by measuring the rate at which a unique nitrification product is produced, these results indicate that differential respirometry using nSOUR under-predicted nitrification inhibition for cadmium. Differences in nitrification inhibition may be due to interactions between the nitrification specific inhibitor, TCMP, which was used for the nSOUR assays and the activated sludge matrix.

Table 5.1 Comparison of SNGR to nSOUR for prediction of nitrification inhibition percentages for different concentrations of cadmium, CDNB and chlorine.[a,b]

	Trial 1[c]		Trial 2[c]		Average IC_{50} Concentration
CADMIUM					
Concentration (mg/L-Cd)	**SNGR**	**nSOUR**	**SNGR**	**nSOUR**	16 (mg/g MLVSS)
1	1%	-68%	--[d]	--[d]	
5	7%	--[d]	6%	-21%	
10	8%	4%	8%	17%	
50	65%	21%	61%	24%	
CDNB					
Concentration (mg/L)	**SNGR**	**nSOUR**	**SNGR**	**nSOUR**	6.8 (mg/g MLVSS)
5	24%	-33%	21%	32%	
10	37%	-34%	31%	33%	
30	56%	--[d]	83%	37%	
60	100%	31%	--[d]	--[d]	
CHLORINE					
Concentration (mg/L-Cl_2)	**SNGR**	**nSOUR**	**SNGR**	**nSOUR**	1.7 (mg/g MLVSS)
5	45%	88%	62%	77%	
10	81%	99%	73%	95%	
30	82%	156%	85%	99%	

[a]Percentages are an average of two measurements.
[b]IC_{50} concentrations are the average of two trials.
[c] Trials are independent repeats of the experiments.
[d]--Denotes concentration was not tested during the trial.

The CDNB short-term experiments showed similar results to cadmium in that nSOUR was more variable and measured a lower percent inhibition than SNGR, again indicating that SNGR provides a better measure of nitrification inhibition. The nSOUR tests were performed for 5, 10, 30 and 60 mg/L of CDNB (Table 5.1). SNGR results were again reproducible between trials and inhibition curves predicted similar results for both CDNB trials. However, the nSOUR results varied significantly between trials and within a given experiment, and comparisons between SNGR and nSOUR inhibition results at each inhibitor concentration did not match. Just as with cadmium, the nSOUR inhibition was lower than the inhibition measured by SNGR, with a few exceptions. These results parallel the cadmium results and suggest that nSOUR does not provide an accurate, reproducible measure of nitrification inhibition for CDNB.

The nIC_{50} concentrations for CDNB were found to be 20 mg/L (7.8 mg/g MLVSS) and 14 mg/L (5.7 mg/g MLVSS) during repeated experiments using SNGR, although the degree of surface interaction that occurred during CDNB-induced inhibition is unclear. CDNB is only moderately soluble (~400 mg/L, unpublished data), which suggests that hydrophobic interactions

should occur with the biomass. Other data on CDNB and nitrification in activated sludge were not found in the literature and, therefore, this study serves as a first report on how this chemical interacts within activated sludge.

Results observed for bleach-induced nitrification inhibition suggest that curative chlorine dosing for filamentous bulking can inhibit nitrification. This is easily seen when examining the inhibition data obtained for chlorine bleach. For the two chlorine trials, the nIC_{50} concentrations predicted were found to be 3.6 and 2.5 mg/L as free chlorine (Cl_2). These values correspond to 2.0 and 1.4 mg/g MLVSS and are well within normal chlorine doses for curative bulking, which can range from 1 to upwards of 40 mg/g MLVSS when applied to the RAS stream (Neethling et al., 1987; Cotteux and Duchene, 2003; Marstaller et al., 1992). Although our additions were directly to the activated sludge mixed liquor, 2.0 mg/g is still lower than the typical chlorine dosage and it is obvious why some facilities experience nitrification upset when chlorinating for bulking control (Cotteux and Duchene, 2003; Marstaller et al., 1992).

Chlorine did not yield data that was as reproducible at low loads for either SNGR or nSOUR (Table 5.1). This is consistent with rapid changes in inhibition at low concentrations, seen in Figure 5.1. Despite the differences, the overall inhibition curves predict similar levels of inhibition for the two trials, indicating reproducibility of the results. nSOUR results were much more reproducible for chlorine shock than for CDNB or cadmium shock, but just like cadmium and CDNB, nSOUR did not predict the same levels of inhibition as SNGR. However, in contrast to the other compounds, the nSOUR test predicted higher levels of inhibition for every chlorine concentration tested when compared to SNGR. It is unclear why this difference occurred, and may suggest that an inhibitor with a different toxic mode of action was created by reactions between hypochlorite and TCMP. If that is the case, SNGR again is the better predictor of the true degree of inhibition in the presence of hypochlorite or hypochlorous acid.

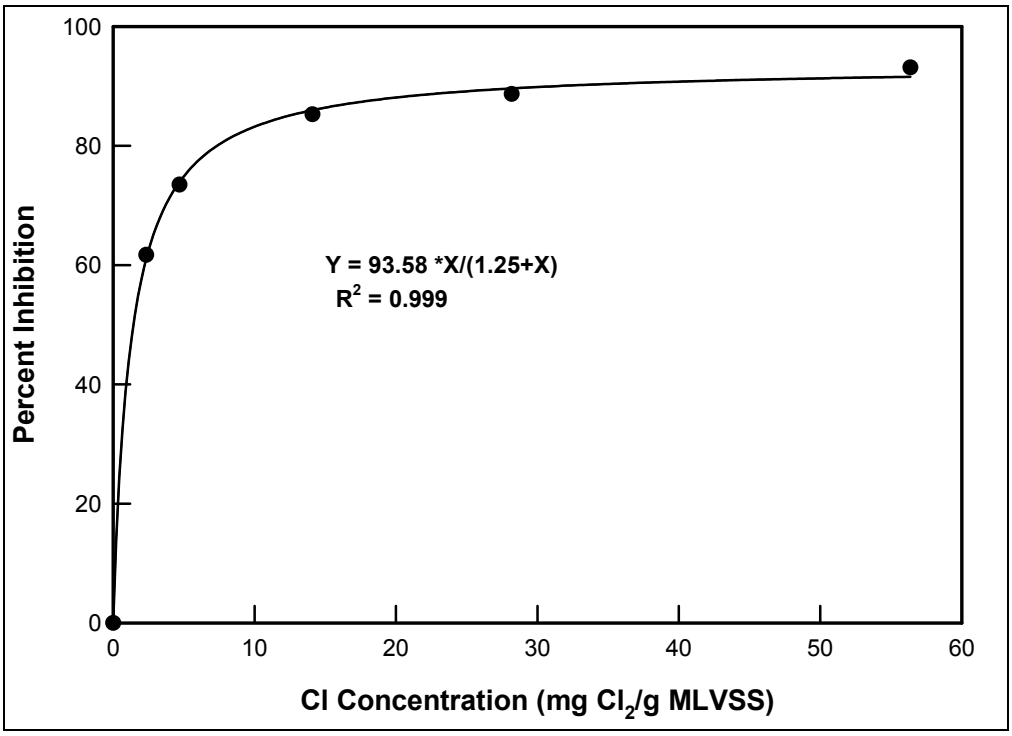

Figure 5.1 Nitrification inhibition curve developed using SNGR for chlorine bleach (concentration determined as Cl_2). Equation describes the best-fit curve shown.

Reactions between the external nitrification inhibitor used for the nSOUR tests and chemicals or components of the mixed liquor matrix may explain why differential respirometry did not provide a reliable measure of nitrification inhibition. Initially, allylthiourea (ATU) was used in our experiments to inhibit nitrification (Benes et al., 2002; Hooper and Terry, 1973), but we found that purported reactions between ATU and chlorine cancelled out effects of both chemicals (data in Appendix C). Because ATU contains a thiol group than can be easily oxidized by chlorine, and several amine groups that can undergo chlorine substitution, the canceling effects of these chemicals is very probable. It is thought that the thiol group in ATU acts as a metal chelator and binds copper to inhibit ammonia oxidation (Hooper and Terry, 1973; Wood et al., 1981) and oxidation of the thiol would prevent ATU from inhibiting nitrification. Because of this, we switched to TCMP to circumvent these interactions. TCMP, which is a chlorinated methyl pyridine, does not contain thiol or amine groups and is thought to inhibit nitrification by interfering with the cytochromes necessary for ammonia oxidation (Hooper and Terry, 1973). Switching to TCMP appeared to prevent the canceling effects observed for chorine and ATU, but it is still possible that interactions occurred between TCMP and other compounds

in the mixed liquor or even the added toxicants. Any reactions that occurred could produce a canceling, additive, or multiplicative effect on observed nitrification inhibition within the nSOUR test similar to what we found with ATU, and would result in the type of variable results that were observed.

In summary, the short-term method comparison experiments showed that the SNGR test was a more reproducible method for predicting nitrification inhibition. In contrast, nSOUR was generally found to be more variable and did not predict the same level of inhibition as SNGR. Assuming that SNGR provides an accurate measure of nitrification inhibition because it involves the direct measurement of a unique product of the metabolism being monitored, the comparison results suggest that differential respirometry does not provide an accurate or reproducible measure of nitrification inhibition in nitrifying mixed liquor. Furthermore, differential respirometry cannot be used to examine differences in inhibition patterns between the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB), because only the total respiration rate is measured. Because the NGR test measures nitrite and nitrate levels, a buildup of nitrite during the inhibition tests would indicate a differential inhibition of the NOB over the AOB. This shows that the NGR test can also be used to determine which group of nitrifying bacteria is more severely inhibited by a given toxin, further supporting the conclusion that the NGR test is superior to differential respirometry for determining nitrification inhibition.

5.4.2 Time Dependence of Inhibition

For all time-dependent inhibition tests, the nIC_{50} concentration determined by SNGR for each of the three contaminants during the short-term assays was added as a single dose shock to the SBRs at the beginning of each trial. These concentrations can be found on Table 5.1.

The results for the cadmium shocked SBR reactors suggest that the full extent of nitrification inhibition is delayed beyond the time period used for the short-term nitrification inhibition tests. This is seen for the cadmium experiment in Figure 5.2A, where the SNGR of the control reactor (no cadmium added) remained stable for the duration of the experiment while the cadmium inhibited reactor SNGR decreased for the first 6 hours after the cadmium shock. After 6 hours, the SNGR began to recover, suggesting the biomass was beginning to recover from the shock event. The same results are seen when examining the calculated percent inhibition (Figure 5.2B), where the maximum percent inhibition after 6 hours was 73%. This is significantly higher

than the 50% that was predicted by the short-term SNGR test for the contaminant dose used. When this experiment was repeated, the maximum inhibition measured by SNGR was again observed after approximately 6 hours. The delayed inhibition seen for cadmium may be due to continued exposure to the contaminant, as previous experiments conducted in our labs found that washout of 20 mg/L of total cadmium took over 15 days, even though soluble cadmium levels fell below 1 mg/L after 12 hours (unpublished data). These results support our hypothesis that maximum nitrification inhibition does not occur immediately after exposure to a toxin.

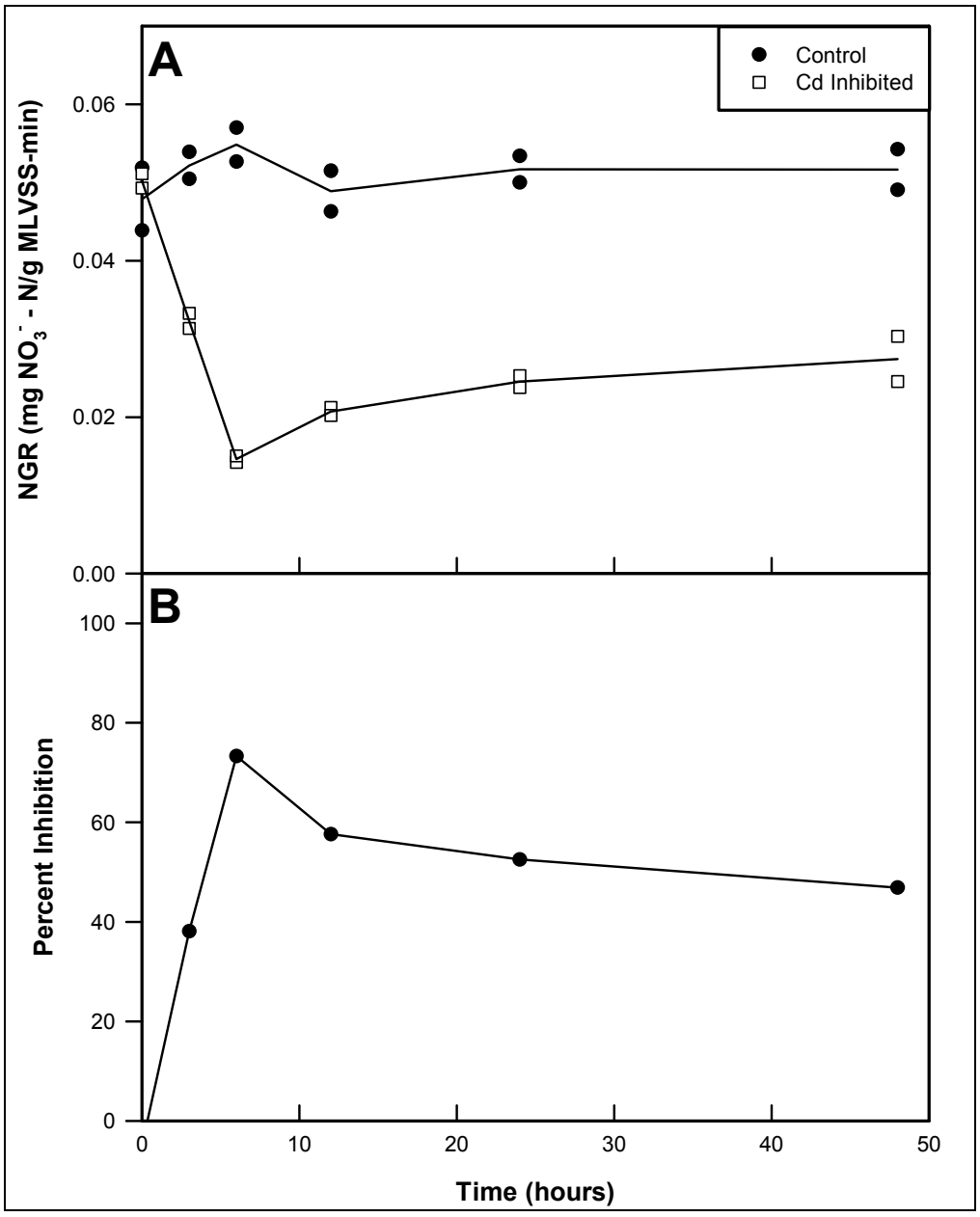

Figure 5.2 (A) Nitrate generation rate monitored over time for the control (no cadmium) and cadmium inhibited SBR. (B) Nitrification inhibition calculated from the SNGR results for the control and cadmium inhibited reactors. Data point shows average of two measurements.

Like the cadmium shock experiment, the CDNB shock also showed that inhibition was time dependent. This is seen in Figure 5.3, which shows that the initial inhibition increased from 42% until the maximum inhibition of 100% occurred 12 hours later. Also, the biomass did not begin to recover its nitrification capabilities over the experimental time of 48 hours. Because we

know from previous experience that CDNB either washes out of this SBR system or is transformed by the biomass after 48 hours (unpublished data), the results obtained here suggest that the continued inhibition is not due to continued exposure to CDNB. Additionally, recovery from such a severe shock event would likely require regrowth of the nitrifying community (Kelly II et al., 2004) and, given the long times required for growth of most nitrifying bacteria (Bock et al., 1986), could take several days or weeks. Similar nitrification inhibition results were obtained when the experiment was repeated (data in Appendix C), which also showed that maximum nitrification inhibition did not occur until 12 hours after the initial CDNB shock.

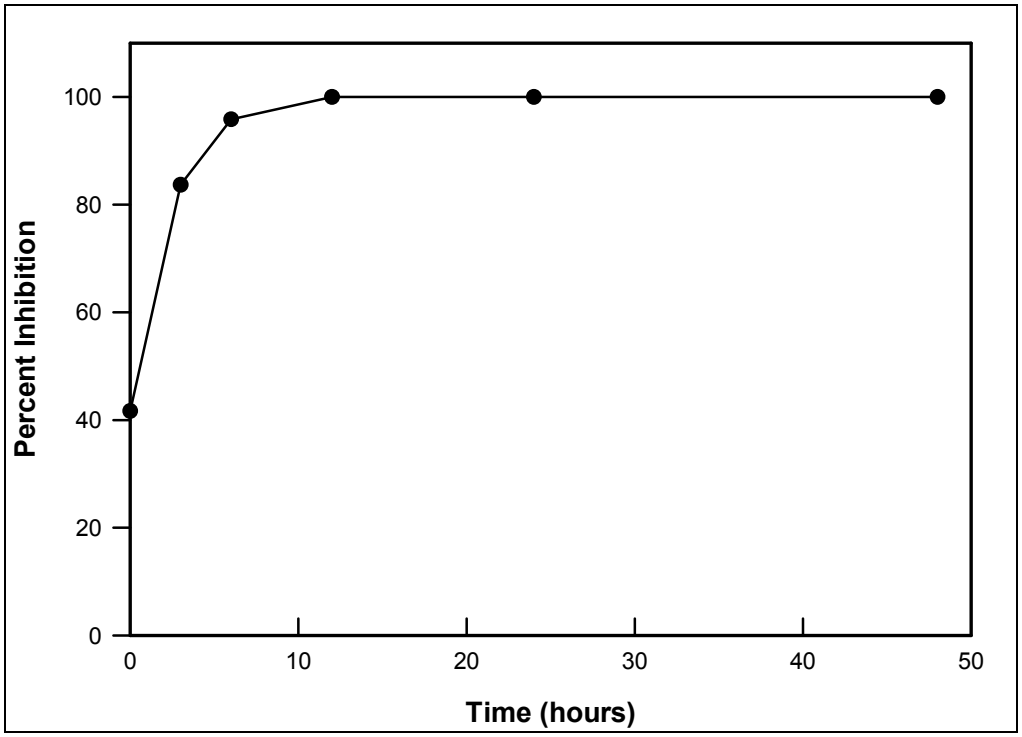

Figure 5.3 Nitrification inhibition caused by CDNB and calculated from the SNGR results for the control and CDNB inhibited reactors. Data point shows average of two measurements.

Chlorine inhibition did not appear to increase significantly with time, contradicting our time-dependence hypothesis and the results observed for cadmium and CDNB. The SNGR results for this contaminant showed only a slight increase (<2%) from the initial inhibition and provided a very close approximation to the maximum nitrification inhibition (Figure 5.4). The same results were seen for the repeated experiment (data in Appendix C). Although nitrification

inhibition did not increase with time, the inhibition persisted for 6 hours before recovery began; such a time delay is consistent with CDNB and cadmium induced inhibition.

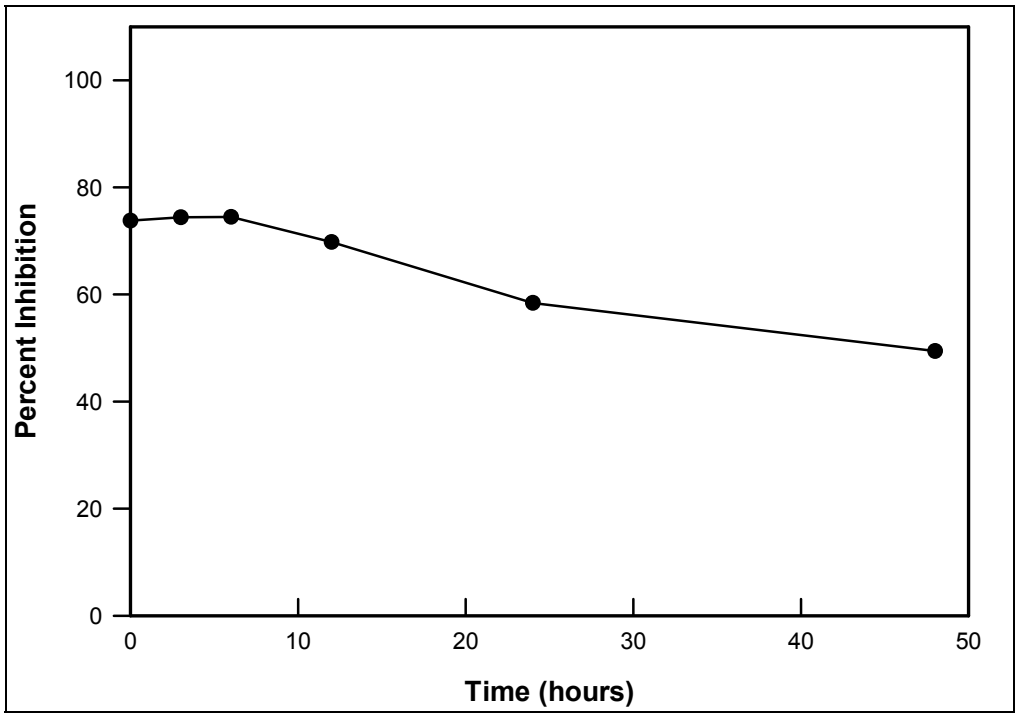

Figure 5.4 Nitrification inhibition caused by chlorine bleach and calculated from the SNGR results for the control and chlorine inhibited reactors. Data point shows average of two measurements.

Bleach-induced nitrification inhibition did not increase with time because of rapid reactions that occurred within the mixed liquor. In preliminary batch experiments, bleach concentrations up to 200 mg/L (as Cl_2) were added to mixed liquor, and after 10 minutes less than 0.2 mg/L residual remained (data in Appendix C). This is in line with what was found by Neethling and coworkers (1987), who reported that 20 mg/L of bleach (as Cl_2) added to mixed liquor for bulking control dissipated by 99% in less than 10 seconds. Assuming chlorine is reacting rapidly with the mixed liquor in the SBRs, then there is probably little free chlorine left to inhibit nitrification after 3 hours. Based on the known behavior of chlorine in mixed liquor, maximum nitrification inhibition for fast-reacting chemicals like chlorine should be observed soon after exposure to the contaminant, which is supported by the previous results.

Differential respirometry measurements were also conducted during the SBR experiments and again showed that the nSOUR tests were more variable and did not provide the

same measure of nitrification inhibition as the SNGR tests. This is easily seen in Figure 5.5, which shows a comparison of the percent nitrification inhibition predicted by SNGR and nSOUR for the cadmium shock event. In this figure, the percent nitrification inhibition that was determined using the SNGR test increased up to 6 hours before it began to recover. In contrast, the inhibition determined using the nSOUR test was more variable and showed a general decreasing trend from the initial inhibition. The percent inhibition became negative at one point, which indicated that the control nSOUR was lower than the cadmium inhibited nSOUR. These illogical results are similar to what was observed during the short-term experiments. Erratic results for nSOUR were also seen during the repeat of the cadmium experiments, and they did not match the first trial pattern. Therefore, as with the short-term experiments, nSOUR experiments for Cd in an SBR were not reproducible between trials. The differential respirometry results for both CDNB and chlorine were also erratic and showed no distinct patterns (data in Appendix C). The nSOUR inhibition results shown here are obviously contrary to what was found using the SNGR test and again show that SNGR provides a better measure of nitrification inhibition.

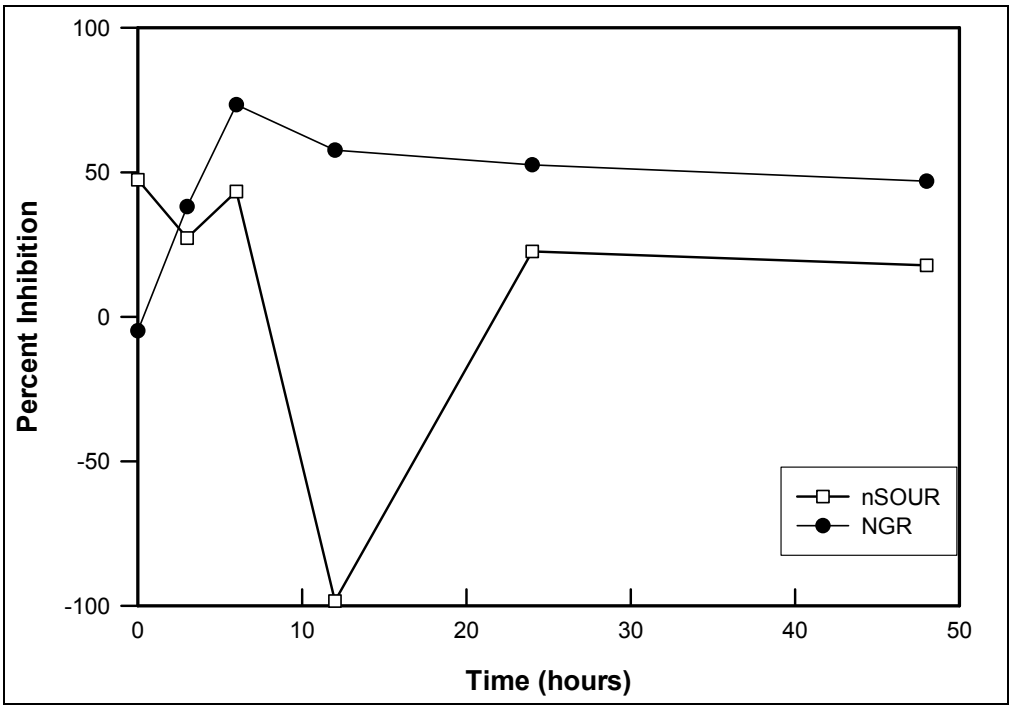

Figure 5.5 Comparison of nSOUR and SNGR nitrification percent inhibition for cadmium inhibited SBRs. SNGR inhibition is the same as presented in Figure 5.2B. Data points show an average of 2 measurements.

Taken together, the results from the long-term experiments lend weight to the conclusion from the short-term experiments that SNGR provides a better measure of nitrification inhibition than differential respirometry. However, it is more important to realize that the long-term results show that the maximum inhibition does not occur immediately for inhibitory chemicals that are not quickly eliminated from the mixed liquor, like cadmium and CDNB. Rather, maximum inhibition can occur hours after a shock load of the contaminant hits an activated sludge system. Current exposure time standards for respiratory inhibition measurements in activated sludge recommend a toxin exposure time of 2 hours before measuring inhibition (ASTM, 1995). However, the results presented here suggest that using an exposure time of 2 hours may under predict the full extent of nitrification inhibition, depending on the contaminant. The consequence of this is large, because an operator who relies upon inhibition test measurements taken within the first 2 hours of chemical exposure may conclude that no response is needed, but hours later the inhibition may increase to a point that causes the facility to exceed their nitrogen discharge limits. Once nitrification is inhibited, it can take days to regain capacity, possibly throwing the utility into noncompliance with its monthly average effluent permit guidelines. Thus, it is very important to realize longer inhibition monitoring times may be required to avoid such costly mistakes.

5.4.3 Continuous Flow Reactors

The results obtained for cadmium inhibited CSTRs (used to compare a continuous and batch system) also showed a time dependence for the maximum nitrification inhibition. Figure 5.6 shows that the nitrification inhibition, as measured by SNGR, increased with time until a maximum inhibition was reached after 6 hours; this result was observed during a repeat experiment (data in Appendix C). The CSTRs followed the same SNGR-derived inhibition pattern that was observed during the cadmium inhibition studies with the SBRs, which also achieved maximum nitrification inhibition at 6 hours (Figure 5.6). Although the extent of inhibition did not match between the two configurations, the time when maximum inhibition occurred and the time when nitrification recovery was initiated were the same, which suggests that there were no significant differences in the inhibition behavior for the CSTR and SBR systems. Interestingly, the average value of the cadmium inhibited SNGR after 6 hours was the

same for both the SBR and the CSTR systems (0.013 mg-N/g MLVSS-min). Unexpectedly, there was a consistent decrease in the control reactor SNGR that began after 6 hours during both independent experiments (data in Appendix C). Nevertheless, the percent inhibition as reported in Figure 5.6 reflects that the degree of inhibition in the chemically shocked reactor was more severe, and is consistent with what was expected based on the previous experiments.

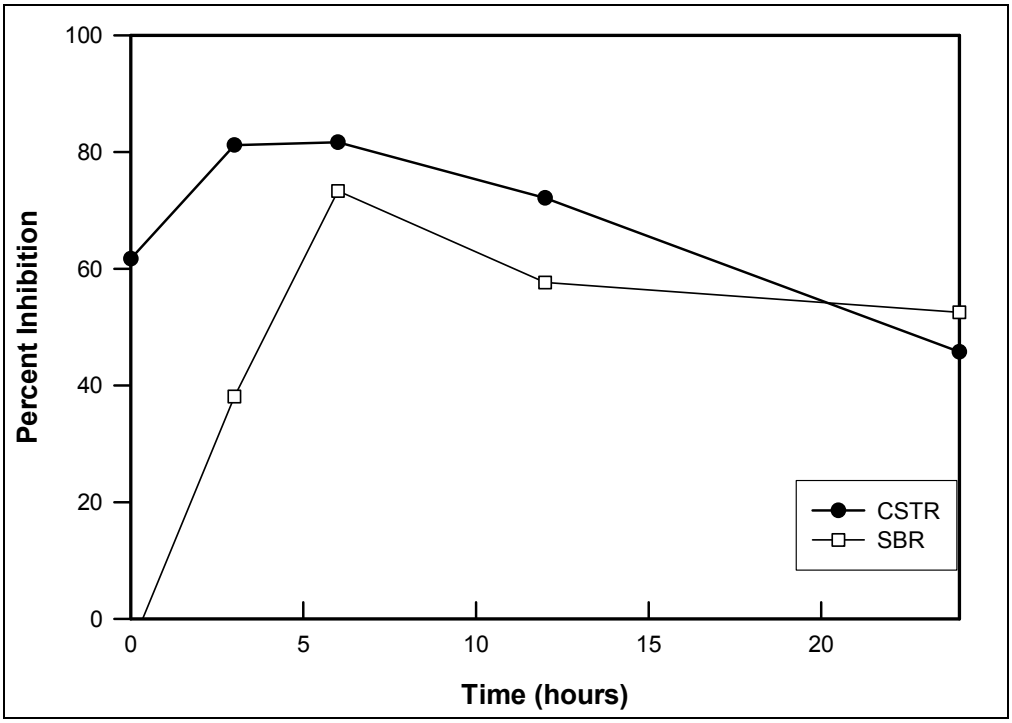

Figure 5.6 Comparison of cadmium induced nitrification percent inhibition for CSTR and SBR reactors as measured by SNGR. SBR inhibition is the same as that shown on Figure 5.2B. Data points show the average of 2 measurements.

The differential respirometry measurements taken for the CSTRs showed more stable oxygen uptake results than were observed using the batch system. From Figure 5.7, we see that the nSOUR measured nitrification inhibition results were more stable and had defined trends when compared with the SBR results. The nSOUR of the inhibited CSTR (Figure 5.7) also showed the same inhibitory trend as the SNGR for that reactor (Figure 5.6). The extreme negative inhibition observed for this reactor after 24 hours was due to an unexpected decrease in the control nSOUR that was similar to the decrease observed at the same time for the control SNGR results discussed above. Although the CSTR nSOUR results are more stable than those observed for the batch reactor experiments, the predicted inhibition still did match inhibition

measured by SNGR (Figure 5.6). Again, if we assume that the SNGR measurement shows correct inhibition, the differences between SNGR and nSOUR further emphasize that SNGR provides a better measure of nitrification inhibition, even in a continuous flow system. However, because of the higher stability of nSOUR measurement, this assumption may not be accurate and further testing would be required.

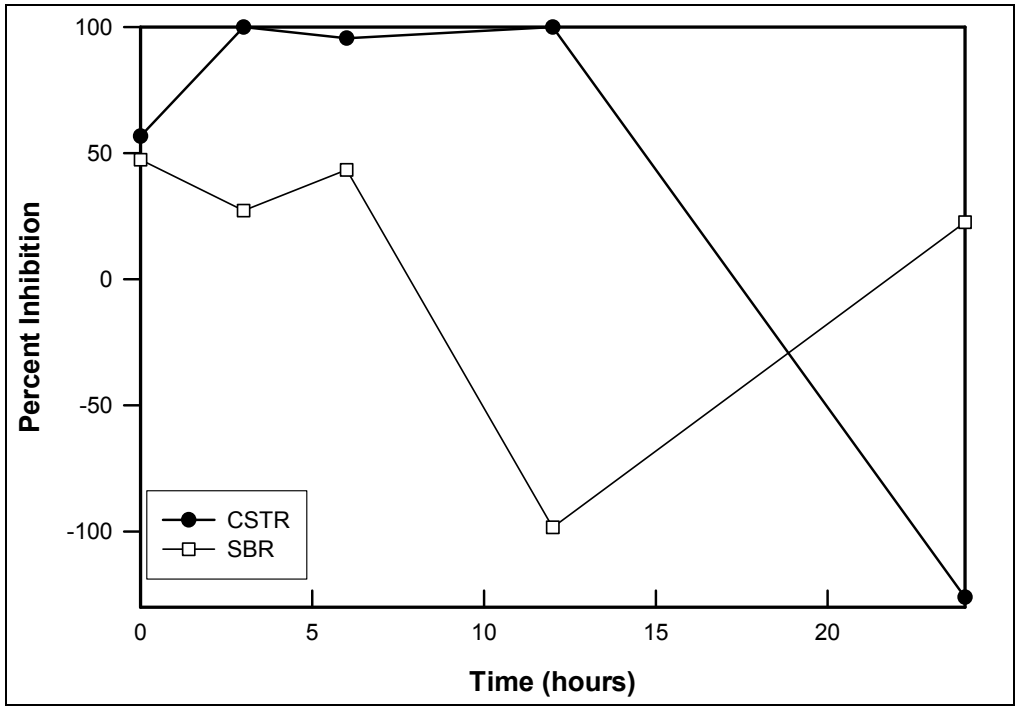

Figure 5.7 Comparison of cadmium induced nitrification percent inhibition for CSTR and SBR reactors as measured by nSOUR. SBR inhibition is the same as that shown on Figure 5.5. Data points show tha average of 2 measurements.

5.5 Conclusions

Based on the results of this study, we conclude that sNGR provides a more accurate measure of nitrification inhibition than differential respirometry. Although other respirometric techniques may also provide accurate inhibition results, only differential respirometry and sNGR were evaluated in this study.

These results also indicate that chemicals like cadmium and CDNB that are not quickly removed from the mixed liquor showed that maximum nitrification inhibition was delayed until several hours after the initial exposure to mixed liquor. This means that short-term tests can under-predict the full extent of nitrification for these chemicals.

In contrast, chemicals like hypochlorite that quickly react and are removed from the mixed liquor showed maximum nitrification inhibition almost immediately upon exposure to the mixed liquor. In addition, chlorine concentrations that are typically used for filamentous bulking control can inhibit nitrification in activated sludge mixed liquor. This means that by using hypochlorite to cure one problem (bulking), an operator may be creating another (increased effluent ammonia or total nitrogen).

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6 GLUTATHIONE MEDIATED OXIDATIVE STRESS RESPONSE MECHANISMS IN NITRIFYING BACTERIA

6.1 Abstract

Nitrification in activated sludge is especially susceptible to oxidative chemical toxins. Several oxidative stress response mechanisms exist in bacteria, and one highly conserved biomolecule involved with antioxidant activities is glutathione. In many Gram-negative heterotrophic bacteria, glutathione mediates the glutathione-gated potassium efflux (GGKE) response, which activates secondary stress responses that protect important intracellular components. A search of the genome of the Gram-negative ammonia oxidizing autotroph *Nitrosomonas europaea* revealed that glutathione synthase was present, while several key enzymes involved with glutathione reduction and GGKE are missing; however, other mechanisms that facilitate potassium efflux for oxidative stress protection may exist. Experiments were performed using a nitrifying enrichment culture, *N. europaea,* and the nitrite oxidizing bacterium *Nitrospira moscoviensis*. Mixed liquor and *Pseudomonas aeruginosa* PAO1 were used as positive controls. Concentrations of total and oxidized glutathione were measured in *N. europaea* after exposure to the oxidative chemical sodium hypochlorite. The data showed that glutathione was present but was oxidized to a lesser degree in *N. europaea* compared to *P. aeruginosa*. Additional batch experiments were performed and soluble potassium levels were monitored to observe increases associated with oxidant induced potassium efflux mechanisms. Results using *N. europaea* and *Ni. moscoviensis* suggest that neither bacteria efflux potassium in response to the oxidants N-ethylmaleimide or sodium hypochlorite, indicating that no oxidative stress-induced potassium efflux mechanism exists in these nitrifiers. Coupled with known genomic information, the results of this study provide greater insight into why nitrification is one of the most susceptible processes in biological wastewater treatment.

Key Words: Nitrification Inhibition, Glutathione, Potassium Efflux, Oxidative Chemical Shock, Stress Response

6.2 Introduction

Biological wastewater treatment systems are the most common way to treat municipal wastewaters. Because municipal wastewater systems can receive wastewater from both domestic and industrial sources, accidental releases of shock loads of industrial chemical toxins can

occasionally occur. These releases can upset critical treatment processes, like BOD removal and nitrification, and cause wastewater treatment facilities to violate discharge permits. Previous work completed by us (Kelly II et al, 2004) and others (Blum and Speece, 1991; Tomlinson et al., 1966) has shown that conventional aerobic nitrification was the biological wastewater treatment process that is most susceptible to chemical inhibition. Recently, we compared the treatment process upset potential of six chemicals comprising different chemical classes, and all inhibited nitrification (Love et al., in press). Among those chemicals tested, the representative oxidative toxin, 1-chloro-2,4-dinitrobenzene (CDNB) was the most detrimental to nitrification. From this information, we decided to investigate why oxidative chemical toxins inhibit nitrification more severely than other chemical classes.

The process of nitrification is carried out by two distinct classes of autotrophic Gram-negative bacteria (Matin, 1978). The ammonia oxidizing bacteria (AOB) convert ammonia to nitrite (Hooper et al., 1997), while the nitrite oxidizing bacteria (NOB) convert nitrite to nitrate (Sundermeyer-Klinger et al., 1984) to complete nitrification. Although oxidative chemicals are widely used in industry, few studies have been performed that examine the effects of these chemicals on activated sludge or nitrifying bacteria. Instead, previous work has primarily involved the use of pure cultures of Gram-negative heterotrophic bacteria. From these studies, two possible modes for oxidative chemical inhibition can be inferred for nitrifying bacteria. The first mechanism is DNA damage (Ferguson et al., 1996). If the DNA of nitrifying bacteria is damaged, cells may not be able to produce essential proteins, or may die if the damage is extensive. The other major mechanism involves oxidizing thiol bonds and nucleophilic centers in proteins (Ferguson et al., 1997; McLaggan et al., 2000). Two proteins essential to nitrifying organisms are ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR). Both contain thiol bonds and sulfur active sites (Meincke et al., 1992; Hooper et al.. 1997) that can be oxidized, rendering such proteins inactive (Carmel-Harel and Storz, 2000). In addition, it has been proposed that the copper cycles between its oxidized and reduced states in active AMO, and that metal redox cycling is also important in NOR activity (Wood, 1986). Thus, if an oxidizing agent is present, it would prevent redox cycling of the copper and proper function of AMO.

Unfortunately, only knowing how oxidative chemicals can inhibit nitrifying bacteria does not explain why they are more susceptible to inhibition by these chemicals. Because all bacteria have DNA that can be damaged and essential enzymes that contain oxidizable material, there

must be some other inhibition mechanism involved that makes them more susceptible. In order to determine what that is, it is important to examine the known bacterial stress response mechanisms that nitrifiers have to help protect against oxidative stress. Recently, the complete sequence of the AOB *Nitrosomonas europaea* was completed (Chain et al., 2003), allowing for a complete search of genes related to stress responses and protection within this species. The known protein coding genes for *N. europaea* are located at the Protein Information Resource NREF Database (National Biomedical Research Foundation, 2005) and the *N. europaea* genome can be searched at the ORNL *N. europaea* genome homepage (DOE Joint Genome Institute, 2005). Using these resources, we performed a detailed examination of the genome with relation to known oxidative stress response mechanisms in other bacteria. Results of this search are found in Table 6.1, which lists several known oxidative stress response genes and if these genes are present in *N. europaea*. Presence of a gene was indicated by an $e \leq 0.001$.

Looking at the list of stress response genes on Table 6.1, it appears that the genes encoding a majority of the protective enzymes are present in *N. europaea*, even though the protective enzymes that are present do not appear to be regulated by the same mechanisms found in other bacteria. Among these include the OxyR and SoxRS regulatory stress response mechanisms. For example, the genes encoding for the protective enzymes catalase and superoxide dismutase were found, but the known regulatory genes were not. In addition, a study by Wood and Sorenson (2001) showed both catalase and superoxide dismutase activity in different AOB. The one oxidative stress mechanism examined that does not appear to have the correct genes to function properly is the glutathione-gated potassium efflux (GGKE) mechanism first described by Kroll and Booth (1981). If this mechanism is missing, it may be one reason why nitrifying bacteria are more sensitive to oxidative chemical toxins. In this paper, we focus on the role of glutathione and potassium efflux mechanisms in protecting nitrifying bacteria that are exposed to oxidative chemical toxins.

The GGKE protection mechanism was first observed in pure cultures of the Gram-negative bacterium *Escherichia coli* (Kroll and Booth, 1981). This mechanism, which has been proposed to play a role in deflocculation events in activated sludge exposed to oxidative chemicals (Bott and Love, 2002; Bott and Love, 2004), appears to be highly conserved because it has been found in several Gram-negative heterotrophic species that have been experimentally tested (Booth et al., 1993). As the name implies, a very important molecule for activation of this

stress response mechanism is glutathione. Glutathione is the major soluble non-protein thiol found in both eukaryotic and prokaryotic cells (Apontoweil and Berends, 1975) and provides a major cellular defense against oxidative compounds (Carmel-Harel and Storz, 2000; Ferguson et al., 2000; Ferguson and Booth, 1998). Glutathione helps protect cells from enzyme and DNA oxidation by acting as a sacrificial nucleophile that scavenges oxidizing chemicals and is subsequently oxidized or conjugated with the chemical toxin (Vuilleumier, 1997). Once glutathione is oxidized it can be rereduced by a glutathione reductase (Carmel-Harel and Storz, 2000), which allows for glutathione to be recycled and reused within cells. Glutathione also protects cells by regulating the potassium efflux response in Gram-negative bacteria, as it activates the GGKE mechanism when it is oxidized. In studies with *E. coli,* it appears that only two potassium efflux channels, KefB and KefC, are regulated, or "gated," by glutathione (Ferguson et al., 2000; Ferguson and Booth, 1998). These efflux pumps are potassium-H^+ antiporters, which means that as potassium is released, the cytoplasm is acidified; this acidification response has been found to confer stress resistance to the cell (Ferguson et al., 2000; Ferguson and Booth, 1998; Ferguson et al., 1997; Ferguson et al., 1996).

After searching the genome of *N. europaea* for the genes that encode the proteins involved in the GGKE mechanism, some very interesting absences were noted. A gene coding for glutathione reductase was not found (Chain et al., 2003), indicating that once the glutathione is oxidized it cannot be reduced back to the GSH form and continue protection against oxidative chemicals. No matches were found for KefB and KefC, indicating that known GGKE-linked channels do not exist in *N. europaea* and the organism may not be able to acidify its cytoplasm in response to oxidative stress. In addition, Dps, a DNA protecting protein regulated by several systems including cytoplasmic pH (Ferguson et al., 1998), is absent from the *N. europaea* genome, further lending weight to the argument that acidification of the cytoplasm may not occur in nitrifying organisms. The absences of these mechanisms from the *N. europaea* genome indicate that the organism lacks a GGKE mechanism and other essential oxidative stress protection mechanisms related to glutathione and GGKE. The lack of these mechanisms may explain why nitrifying bacteria are sensitive to electrophilic chemicals.

Table 6.1 Summary of select genes involved in oxidative stress response mechanisms and their presence in the *N. europaea* genome.

Gene Name	Function	Presence in *N. europaea*
Genes involved in the GGKE[e] mechanism		
gshB	glutathione synthetase	Yes
Gst	glutathione S-transferase	Yes
gorA	glutathione oxidoreductase	No
kefB	GGKE protein KefB	No[a]
kefC	GGKE protein KefC	No[a]
Dps	DNA binding protein Dps	No
Genes involved in the *oxyR* system		
oxyR	*oxyR* system regulatory protein OxyR	No
katG	hydroperoxidase/catalase	No[b]
aphCF	alkyl hydroperoxide reductase	No[b]
gorA	glutathione oxidoreductase	No
grxA	glutaredoxin	No[b]
trxC	thioredoxin	No[c]
oxyS	regulatory RNA	No
Genes involved in the *soxRS* system		
soxR	*soxRS* system regulatory protein SoxR	No
soxS	*soxRS* system regulatory protein Soxs	No
sodA	Manganese superoxide dismutase	No[b]
tolC	outer membrane efflux protein TolC	Yes[d]
arcAB	multidrug resistance efflux system arcAB	No[h]

[a] pH adaptation potassium efflux system protein F and D found, but not glutathione regulated
[b] homologous gene with unknown regulation mechanism found
[c] BLAST search yielded protein with similar coding strand
[d] *arcAB* not found but numerous multidrug efflux pumps located in genome
[e] Glutathione Gated Potassium Efflux

From these genome searches, it is apparent that the known GGKE mechanism is not present in *N. europaea*; however, nitrifying bacteria may have a surrogate potassium efflux mechanism that is used to respond to oxidative compounds. Understanding why nitrification and nitrifying bacteria are sensitive to oxidative damage, and how these bacteria respond to oxidative stress may provide insight in determining means to detect and mediate such upset events before they occur. Therefore, the objective of this study was to determine, first, if glutathione is present and how it behaves during oxidative shock events in nitrifying bacteria and, second, to examine if nitrifiers contain a unique and previously unidentified mechanism to efflux potassium in response to oxidative chemicals. This was accomplished using both enriched and pure cultures of nitrifying bacteria and shocking them with the oxidative chemicals N-ethylmaleimide (NEM) and chlorine bleach (as NaOCl). NEM was used because it has previously been found to elicit the GGKE response in pure cultures (Ferguson et al., 2000) and activated sludge (Bott and Love, 2002), while NaOCl was chosen because it is a commonly used oxidative chemical that is used for activated sludge bulking control (Neethling et al., 1987). These cultures were monitored to

see if potassium was released into the solution, indicating that an alternative mechanism for potassium efflux exists, though GGKE may be occurring. For these experiments, mixed liquor was used as a positive control. In addition, total and oxidized glutathione levels were monitored to determine if glutathione is present and oxidized in response to increasing concentrations of chlorine bleach. Three pure cultures were chosen for these experiments: *Nitrosomonas europaea*, *Nitrospira moscoviensis*, and *Pseudomonas aeruginosa* PAO1. *N. europaea* was the AOB chosen for because it is the most commonly studied AOB (Schramm et al., 1998b) and it is the only autotrophic bacterium with a sequenced genome (Chain et al., 2003). *Ni. moscoviensis* was used as the representative NOB because several studies have found that *Nitrospira* species are the dominant NOB populations in activated sludge (Schramm et al., 1998a; Burrell et al., 1998). *P. aeruginosa* PAO1 was chosen for use as a positive control bacterium because it is known to contain the GGKE mechanism (Stover et al., 2000).

6.3 Methodology

6.3.1 Bacterial Cultures and Growth Conditions

Mixed Liquor Source Description and Sample Treatment

Mixed liquor was obtained the morning of each experiment from the Blacksburg-VPI Wastewater Treatment Facility at Stroubles Creek. The treatment process at the Stroubles Creek facility consists of a nitrifying activated sludge treatment process with a 10-14 day solids retention time (SRT). Mixed liquor grab samples were taken directly from the aerated mixed liquor basins and transported to the laboratory within 20 minutes. The mixed liquor samples were aerated, magnetically mixed, and continuously fed a synthetic wastewater solution as previously described (Kelly et al., 2004) to mimic the average feed loading conditions at the Stroubles Creek facility and to maintain a constant biomass composition for experiments. The total volume of feed solution used was less than 2% of the volume of mixed liquor; therefore, suspended solid concentrations were not significantly changed throughout the experiment (2-4 hours). This was confirmed by measuring mixed liquor volatile suspended solids (MLVSS) at various times during the experiment. Mixed liquor suspended solids (MLSS) and MLVSS were measured in triplicate according to sections 2540 D and 2540 E of Standard Methods (1998).

Nitrifying Enrichment Culture

A nitrifying enrichment culture was maintained in a 15 L chemostat operated at a 15 day HRT/SRT. The culture was initially seeded with mixed liquor from the Blacksburg VPI Stroubles Creek Wastewater Treatment Facility. The reactor was completely mixed using a 104 rpm paddle mixer and aerated to maintain a DO concentration above 4.0 mg/L. The reactor was batch fed (42 ml in 30 seconds every hour) approximately 1,350 mg/L-N (about 90 mg/L in the reactor) $(NH_4)_2CO_3$ in the *N. europaea* medium described below (without the phenol red or $(NH_4)_2SO_4$). Routine analysis of the reactor was performed once steady state was achieved and showed that total ammonia concentrations (measured according to methods 4500-NH_3 B and 4500-NH_3 C in Standard Methods (APHA, 1998) were undetectable in the effluent. Steady state solids concentrations were approximately 500 mg/L MLSS and 450 mg/L MLVSS.

Ammonia Oxidizing Bacterium: *Nitrosomonas europaea*

The *N. europaea* cells were grown under sterile conditions using medium composed of 0.2 g/L $MgSO_4 \cdot 7H_20$, 20 mg/L $CaCl_2 \cdot 2H_20$, 87 mg/L K_2HPO_4, 2.52 g/L EPPS, 10 µg/L $Na_2MoO_4 \cdot 2H_20$, 17.2 µg/L $MnSO_4 \cdot H_20$, 0.4 µg/L $CoCl_2 \cdot 7H_20$, 170 µg/L $CuCl_2 \cdot 2H_20$, 10 µg/L $ZnSO_4 \cdot 7H_20$, 100 µg/L chelated iron, 250 µg/L phenol red and 1.32 g/L $(NH_4)_2SO_4$ at 29ºC in the dark in 5 L batch cultures. Sterile aeration was provided and mixing was achieved with magnetic stirrers. Growth curves were developed by measuring nitrite and performing cell counts. Nitrite samples were stored at -20^{o}C for no more than 28 days prior to analysis and analyzed using a colorimetric procedure (method 4110 B) as described in Standard Methods (APHA, 1998). Cell counts were performed using a Helber bacteria single round cell counting chamber manufactured by Weber Scientific International (Middlesex, UK).

Nitrite Oxidizing Bacterium: *Nitrospira moscoviensis*

Ni. moscoviensis was cultured at 32ºC in 5 L batch flasks with sterile aeration and magnetic mixing. The medium used was described by Ehrich et al. (1995) with one modification; 10 mg/L EDTA was added to prevent precipitation of phosphate salts. Growth curves for this organism were developed by examining nitrate production and cell counts. Cell counts were performed as described for *N. europaea* above. Nitrate samples were stored at -20^{o}C for no more than 28 days prior to analysis and analyzed using ion chromatography (method 4110 C) as described in Standard Methods (APHA, 1998). Upon thawing the samples, 5 ml of each was loaded into a vial and placed into an AS40 autosampler. Samples were injected into a

Dionex DX120 suppressed conductivity ion chromatograph containing an AS14 anion separation column with an AG14 guard column for anion analysis (Dionex Corp., Sunnyvale, CA). Samples were carried in an eluent of 3.5 mM Na_3CO_3 and 1.0 mM $NaHCO_3$.

Bacterium Known to Contain GGKE Mechanism: *Pseudomonas aeruginosa* strain PAO1

P. aeruginosa PAO1 was grown at 20°C in the dark in 1 L batch flask with aeration and mixing provided by shaking. It was grown in full strength mineral salts (FSMS) medium containing 186 mg/L EDTA, 11 mg/L $FeCl_2 \cdot 2H_2O$, 150 mg/L $MgSO_4 \cdot 7H_2O$, 4.5 mg/L $MnSO_4 \cdot 4H_2O$, 500 μg/L $NaMoO_4 \cdot 2H_2O$, 150 μg/L H_3BO_3, 100 mg/L NH_4Cl, 1.64 g/L NaAcetate, 20 mg/L $CaCl_2$, 1.5 mg/L $ZnCl_2$, 500 μg/L $CuCl_2 \cdot 2H_2O$, 1.5 mg/L $CoCl_2 \cdot 6H_2O$, 1.5 g/L $NaH_2PO_4 \cdot 2H_2O$, 3 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.5 g/L NH_4Cl, 0.25 g/L NaCl) and approximately 5 mg/L potassium (added as KH_2PO_4) so that the soluble potassium concentration was sufficiently low to allow for better detection of the increase in soluble potassium associated with potassium efflux, but not growth limiting. Growth curves for this organism were developed by performing cell counts as described for *N. europaea* above.

6.3.2 Potassium Efflux Determination

Potassium efflux was determined in the enrichment culture using an experimental batch reactor that received chemical oxidative toxins, a negative control reactor (no chemical addition) and a positive control reactor (chemical addition to mixed liquor, which has been previously shown to efflux K^+; in response to oxidative chemical shock (Bott and Love, 2002). A minimum suspended solids concentration of 1,600 mg/L MLVSS was required to readily detect K^+ efflux. To achieve this, the enrichment culture was washed, concentrated to 1,600 mg/L MLVSS and resuspended in low K^+ media to avoid high background K^+ levels. For experiments, 100 ml of enrichment culture or mixed liquor was mixed, aerated, and fed with each culture's normal growth substrate (20 mg/L-N ammonium carbonate for the enrichment culture and 100 mg/L of COD solution for the mixed liquor (Kelly II et al., 2004)) to allow for normal aerobic respiration of the bacteria. At least two samples for soluble, total and cell/floc-associated (biomass-associated) K^+ were analyzed according to the method presented by Bott and Love (2002). A K^+ balance was performed by comparing the total K^+ in the samples to the soluble + cell/floc-associated fractions; if K^+ efflux occurred, the soluble fraction of K^+ increased while the cell/floc-associated fraction decreased. Triplicate samples were taken from each reactor before

adding 50 mg/L N-ethylmaleimide (NEM), and 2.5, 5, 7.5, 10, 15, and 20 minutes after addition. NEM is an oxidative chemical that has been previously found to elicit K^+ efflux in mixed liquor (Bott and Love, 2002) and Gram-negative bacteria (Ferguson et al., 2000). All experiments were run at least twice to confirm results. Sample processing is described below.

One ml samples for total potassium were removed from the reactors at designated times and digested according to a modified EPA method performed by Bott and Love (2002). For this, samples were acidified using nitric acid (to a 10% nitric acid solution) and digested in a closed reflux method by boiling at 100°C for 30 minutes. Digested samples were then diluted 1:10 and filtered through a 0.45 μm filter for K^+ analysis. Soluble and cell/floc-associated samples were fractionated using the following procedure. Samples (1.2 ml) were removed from the reactors and placed into a microcentrifuge tube containing 200 μl of non-toxic dimethyl-tetrachlorophenylsiloxane copolymer (ρ=1.019 g/cm^3). The samples were then centrifuged at 13,000 x g for 2 minutes to separate the cells from the liquid. The copolymer was used to separate the solid pellet (cell/floc-associated fraction) from the liquid supernatant (soluble-fraction). The copolymer also formed a non-permeable barrier so that potassium associated with the cells could not diffuse into the supernatant before the fractions were removed for analysis. After separation, 1 ml of the supernatant was removed and diluted 1:10 using 2% nitric acid, then storaged until it could be analyzed for K^+. For the cell/floc pellet, the copolymer was removed, the pellet was resuspended in one ml of 10% nitric acid and the suspension was digested and treated for K^+ analysis as described above for the total fraction. All K^+ samples were analyzed using a Perkin-Elmer flame atomic adsorption spectrophotometer (Perkin Elmer Inc., Wellesley, MA) according to method 3500-K B in Standard Methods (APHA, 1998).

For pure cultures, K^+ efflux was determined using an experimental batch reactor (NEM or NaOCl addition to either *N. europaea* or *Ni. moscoviensis*), a negative control reactor (no oxidant addition) and a positive control reactor (oxidant addition to *P. aeruginosa* PAO1). An additional positive control experiment was also performed using pure cultures of each organism exposed to nigericin, an antibiotic that causes bacteria to leak cytoplasmic K^+ into solution (Hofer, 1977). Previous work done in our labs with pure cultures found that a minimum cell concentration of 10^9 cells/ml was required to detect K^+ efflux using the methods employed in this study. Therefore, all pure cultures were concentrated (again in low K^+ media) to at least this concentration for experiments. Experiments were conducted when the pure cultures reached

late-log phase growth. All reactors were mixed, aerated, and fed with each culture's normal growth substrate (ammonium sulfate for *N. europaea*, sodium nitrite for *Ni. moscoviensis*, and sodium acetate for *P. aeruginosa* PAO1) to allow for normal aerobic respiration of the bacteria. As with the enrichment culture, at least two samples for soluble, total and cell-associated (biomass-associated) K^+ were analyzed according to the method presented by Bott and Love (2002). Samples were taken from each reactor before any oxidative chemical was added, and 15, 45 and 90 minutes after addition. Sample treatment was the same as described above. To confirm results, experiments were run at least twice. At the conclusion of pure culture experiments, Live/Dead staining was performed using the *Bac*Light™ Bacterial Viability Kit available from Molecular Probes© to determine if significant bacterial death occurred in response to the added chemicals.

6.3.3 Glutathione Determination in Pure Cultures

Glutathione concentrations were determined using experimental reactors (*N. europaea*), a negative control reactor (no chemical addition) and a positive control reactor (chemical addition to *P. aeruginosa* PAO1; an organism known to have glutathione). Mixed liquor was also tested, but high variability was observed for the total and oxidized glutathione data, which prevented use of the method within the mixed liquor matrix.

For experiments, pure cultures were concentrated in their growth media to a minimum of 10^{10} cells/ml, as previous work done in our labs with pure cultures found this to be adequate for examining glutathione. *Ni. moscoviensis* was not used in these experiments because it was difficult to achieve the cell concentrations that were required to detect glutathione using the method presented below. As with the K^+ efflux experiments, all experiments were conducted when the pure cultures reached the late-log growth phase and all reactors were mixed, aerated, and fed with each culture's normal growth substrate.

As with the efflux experiments, at least two samples for oxidized and total glutathione were analyzed colorimetrically using the Bioxytech® GSH/GSSG-412™ (oxidized) and GHS-420™ (total) glutathione analysis kits manufactured by Oxis Research ™ (Oxis International, Portland, OR). For these experiments, varying concentrations of sodium hypochlorite (bleach) were added and samples were taken after 20 minutes of exposure, because it was determined that near maximum K^+ efflux occurred after that time. In order to remove chemical interferences

caused by chlorine, 3 to 5 mL samples were washed by centrifugation at 3400 x g and resuspended in phosphate buffer (3.4 g/L KH_2PO_4 and 4.35 g K_2HPO_4, pH= 6.9). Cells were then ruptured for glutathione analysis by passing the samples through a SLM/AMINICO French® pressure cell press and 40,000 psi AMINICO pressure cell 3 times at 15,000 psi (AMINICO, Silver Spring, MD). Samples were then analyzed according to the method as suggested by Oxis Research ™ for the oxidized and total glutathione test kits. To confirm results, experiments were performed at least twice. NEM was tested but generated highly variable and inconclusive results, presumably because of a chemical interference with the method; therefore, results based on NEM stress are not presented here.

The total glutathione concentrations measured in each of the chlorine shocked systems were averaged. This was done after regression analysis of the data showed that there were no statistically significant differences between the total glutathione measured in the control cells and any of the chlorine shocked cells for either *N. europaea* ($p=0.86$) or *P. aeruginosa* ($p=0.17$) using a 95% confidence interval ($\alpha=0.05$).

6.4 Results and Discussion

6.4.1 Nitrifying Enrichment Culture Showed Low K^+ Efflux

Enrichment culture potassium efflux experiments did not conclusively reveal whether or not potassium efflux occurs in nitrifying bacteria. For the initial experiment, the enrichment culture was concentrated to yield a suspended solids concentration of 1,699 ± 42 mg/L TSS and 1,580 ± 42 mg/L VSS. For the mixed liquor, the suspended solids concentration tested was 1,633 ± 19 mg/L MLSS and 1,356 ± 16 mg/L MLVSS. These values were within the range that was previously determined to be sufficient to detect K^+ efflux (data in Appendix D). Figure 6.1 shows the soluble K^+ results for both the unshocked controls and NEM-shocked enrichment and mixed liquor cultures. This figure shows that for both the enrichment and mixed liquor NEM-shocked reactors, the soluble K^+ levels increased with time, which suggests that K^+ efflux was occurring in both cultures. As expected, the control reactors for both cultures did not significantly change over the duration of the experiment. Furthermore, the results show that the maximum increase in soluble K^+ for the positive control mixed liquor was about 2.2 mg/L while the enrichment culture, which had a similar suspended solids concentration, only increased by

1.4 mg/L. This suggests that the enrichment culture did not efflux as much potassium as the mixed liquor on a per-gram biomass basis.

In addition to testing soluble potassium during these experiments, total and floc-associated potassium measurements were performed in order to conduct a mass balance and ensure that the amount of total K^+ equaled the sum of the soluble and floc-associate K^+. The experiment was performed twice. Results showed that the mass balance closed when examining these K^+ amounts for all enrichment and mixed liquor reactors (data in Appendix D).

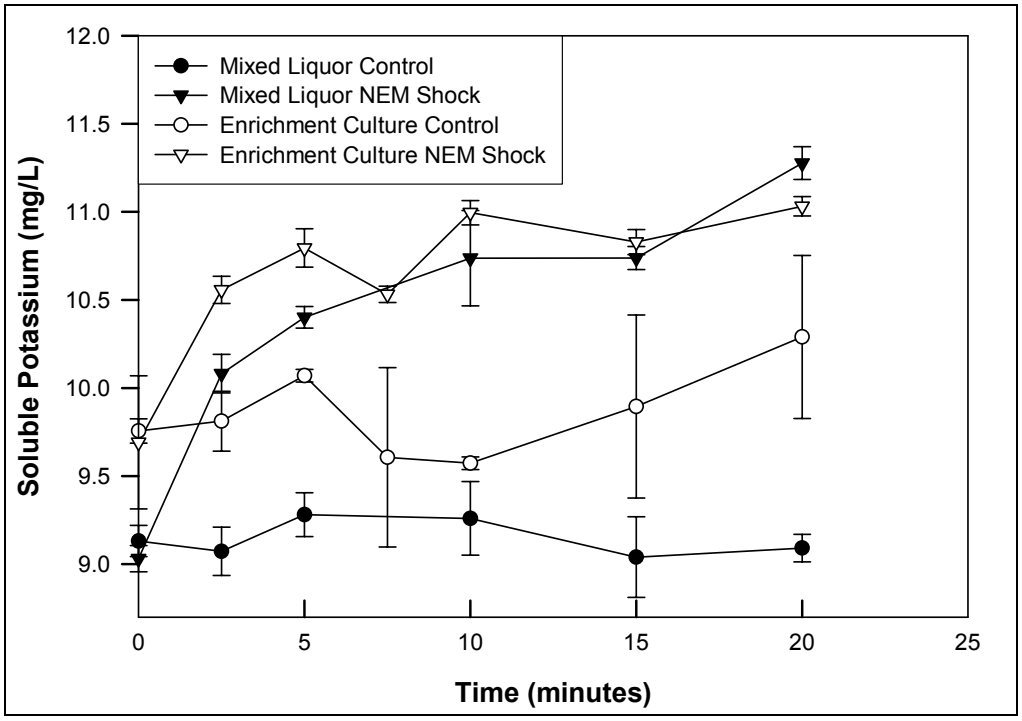

Figure 6.1 Soluble potassium levels for mixed liquor and nitrifying enrichment culture exposed to 50 mg/L NEM. Error bars indicate standard deviation of 3 samples.

The smaller increase in soluble K^+ observed for the enrichment culture may be due to an increased population of nitrifying bacteria present in the enrichment culture. If nitrifying bacteria do not efflux K^+, as we hypothesize, then an increase in the number of non-effluxing organisms (autotrophic nitrifiers) relative to organisms that can release potassium upon NEM exposure (Gram-negative heterotrophs) would decrease the total amount of potassium released per unit MLVSS. This would result in a lower concentration of soluble K^+ released to the bulk liquid phase, as observed. This result motivated the decision to use pure cultures of AOB and

NOB for further K^+ efflux experiments since they can be used to definitively determine if potassium efflux does or does not occur in nitrifying bacteria.

6.4.2 Nitrifying Pure Cultures Showed No K^+ Efflux

The pure culture experiments showed that potassium efflux did not occur in either *N. europaea* or *Ni. moscoviensis* cultures in response to either NEM or chlorine, but did occur for *P. aeruginosa* cultures used as a positive control. The soluble K^+ levels measured during the NEM experiments are shown in Figures 6.2, 6.3 and 6.4. When examining the results of the first experiment, no significant increase in soluble K^+ was observed in the 50 mg/L NEM shocked cells when compared to the unshocked control *N. europaea* culture (Figure 6.2). The *P. aeruginosa* positive control culture (Figure 6.4) shows a maximum increase of about $2.8x10^{-12}$ mg/cell soluble K^+ in the NEM shocked cells when compared to the unshocked control over the 90 minute experiment. For these two cultures, control soluble K^+ levels remained relatively constant for the duration of the experiment. However, for the *Ni. moscoviensis* culture, control soluble K^+ levels decreased by approximately $0.8x10^{-12}$ mg/cell over the course of the experiment (Figure 6.3). For this culture, NEM shocked cells also showed a modest $0.6x10^{-12}$ mg/cell maximum increase in soluble K^+ over the initial sample (Time 0) taken just prior to the addition of NEM. This increase was much smaller than the $2.8x10^{-12}$ mg/cell increase observed for the *P. aeruginosa* culture that is known to contain the GGKE mechanism, which suggests that the slight increase observed for *Ni. moscoviensis* was not significant. Subsequent experiments with *Ni. moscoviensis* confirmed that no potassium efflux occured, as the NEM-shocked reactors did not show elevated soluble K^+ levels relative to the control reactors during two subsequent experiments (data in Appendix D). Repeats of these experiments for the *N. europaea* and *P. aeruginosa* cultures also showed the same trends shown in Figures 6.2 and 6.4, respectively.

For all cultures, the potassium selective ionophore nigericin was also used as a positive control to test if K^+ was present in, and could be released from, the cytoplasm of the cells. The soluble K^+ levels in all cultures increased by 0.4, 1.3, and $0.8x10^{-12}$ mg/cell for *N. europaea*, *Ni. moscoviensis*, and *P. aeruginosa*, respectively (Figures 6.2, 6.3 and 6.4), which indicated that potassium was present and released In addition, Live/Dead staining showed that significant cell death did not occur in cells exposed to NEM or nigericin for any of the cultures (data in

Appendix D). This indicates that the concentrations of NEM and nigericin added did not kill the cells and that the K^+ efflux observed for *P. aeruginosa* was most likely due to activation of the GGKE mechanism and not cell lysis .

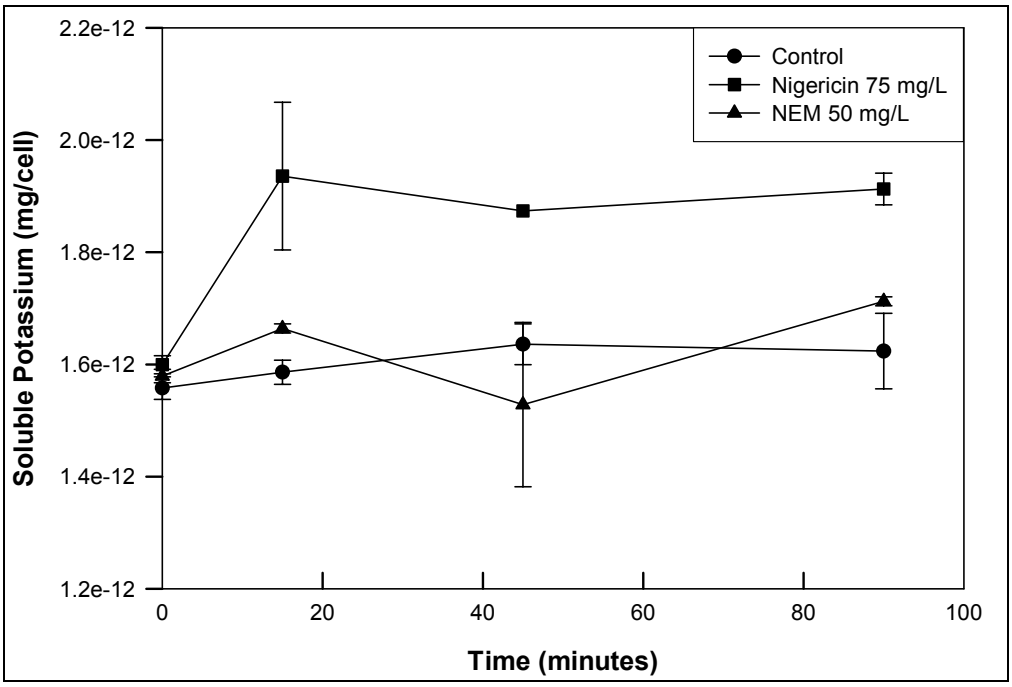

Figure 6.2 Soluble potassium for unshocked control, nigericin and NEM-shocked reactors of *N. europaea*. Cell concentration at the beginning of the experiment was 3.72×10^9 cells/ml. Error bars indicate the range of 2 samples.

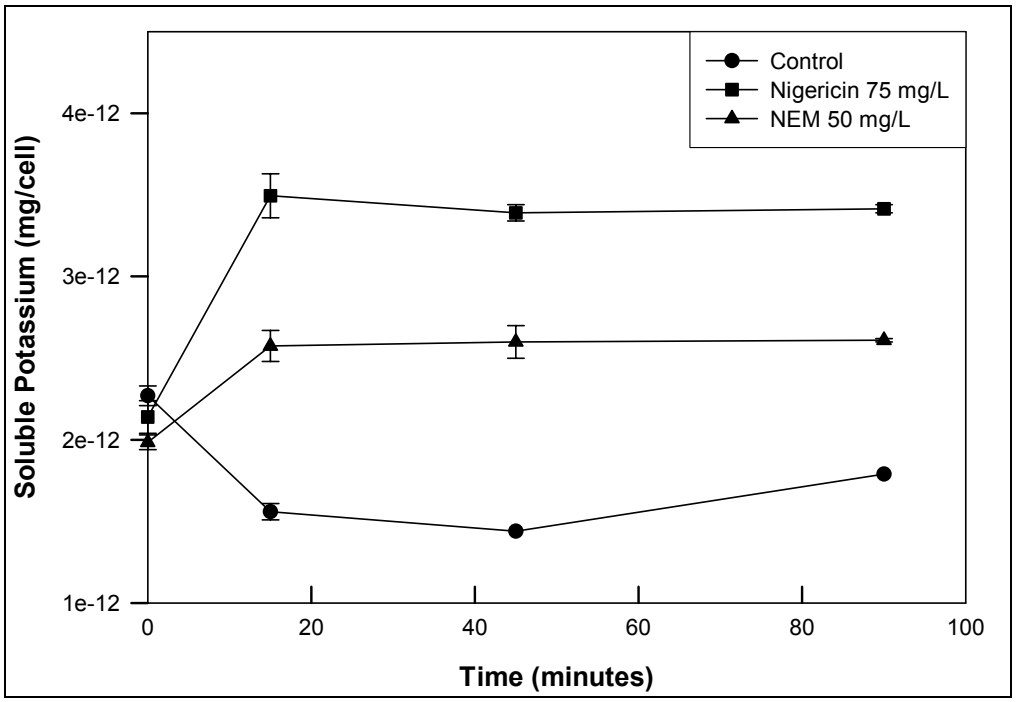

Figure 6.3 Soluble potassium for unshocked control, nigericin and NEM-shocked reactors of *Ni. moscoviensis*. Cell concentration at the beginning of the experiment was $>1x10^9$ cells/ml. Error bars indicate the range of 2 samples.

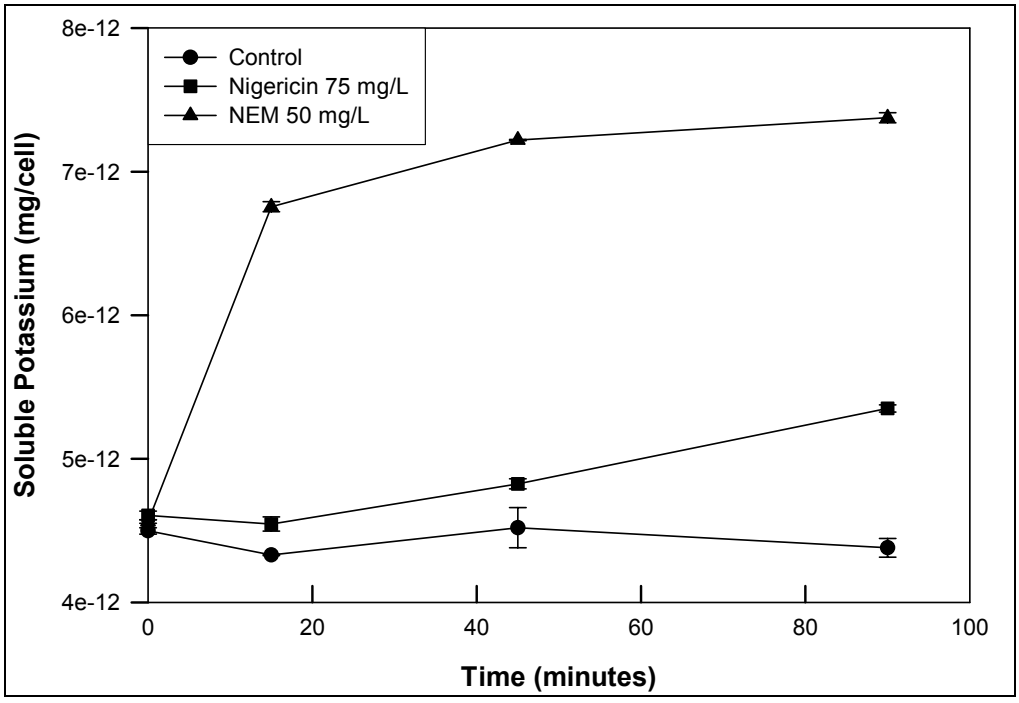

Figure 6.4 Soluble potassium for unshocked control, nigericin, and NEM-shocked reactors of *P. aeruginosa* PAO1. Cell concentration at the beginning of the experiment was $2.00x10^9$ cells/ml. Error bars indicate the range of 2 samples.

Chlorine bleach was also tested at concentrations of $4.72x10^{-10}$ and $23.6x10^{-10}$ mg/cell (1 and 5 mg/L as Cl_2) and showed no potassium efflux for the *N. europaea* culture (Figure 6.5). *P. aeruginosa* cultures did show efflux at both concentrations of added NaOCl (Figure 6.6). At $4.72x10^{-10}$ mg Cl_2/cell, maximum K^+ efflux was about $0.7x10^{-12}$ mg K^+/cell over the control reactor. For $23.6x10^{-10}$ mg Cl_2/cell, the efflux was $2.9x10^{-12}$ mg K^+/cell, which is very similar to what was observed for 50 mg/L of NEM. As with NEM, Live/Dead staining showed that no significant cell death occurred in cells exposed to $4.72x10^{-10}$ or $23.6x10^{-10}$ mg Cl_2/cell for any of the cultures (data in Appendix D). These results further support the previous findings that *N. europaea* does not efflux potassium in response to oxidative chemical toxins.

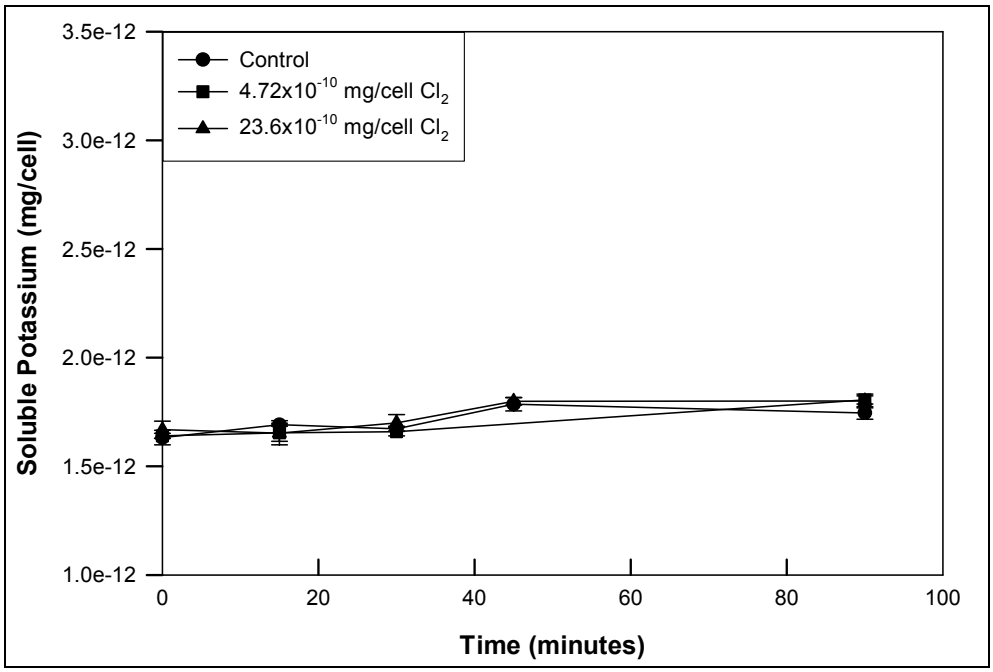

Figure 6.5 Average soluble potassium for unshocked control and chlorine-shocked reactors of *N. europaea*. Cell concentration at the beginning of the experiment was $2.12x10^9$ cells/ml. Error bars indicate the standard deviation of 3 independent reactor samples. Control only used 2 independent reactor samples

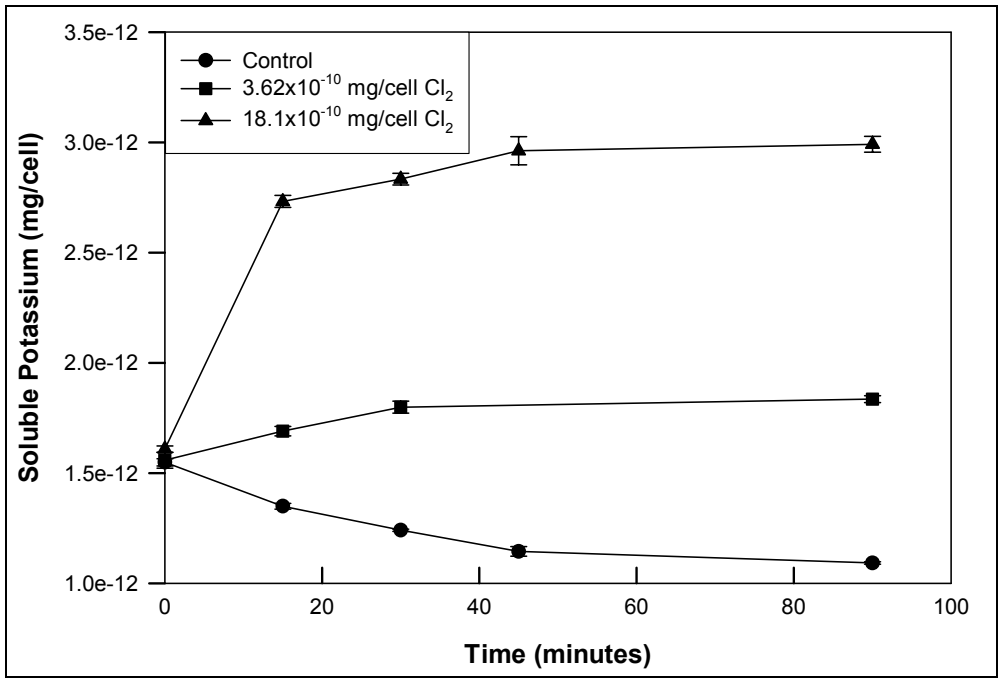

Figure 6.6 Average soluble potassium for unshocked control and chlorine-shocked reactors of *P. aeruginosa* PAO1. Cell concentration at the beginning of the experiment was 2.76×10^9 cells/ml. Error bars indicate the standard deviation of 3 independent reactor samples. Control only used 2 independent reactor samples.

Since potassium was not released from either of the selected AOB and NOB cultures that were exposed to NEM or chlorine bleach (NaOCl), no mechanism is present that releases intracellular potassium in response to oxidative chemical shocks. The potassium results confirm the genomic search results from *N. europaea*, which indicated that the genes coding for the glutathione-gated potassium/proton efflux channels required for potassium efflux are not present in the genome (Table 6.1). These results provide the first reported evidence of a Gram-negative bacteria that does not contain a potassium efflux mechanism for responding to oxidative stress, which was thought to be highly conserved in these bacteria (Booth et al., 1993). These are also the first autotrophic bacteria that have been examined for oxidant-induced potassium efflux, which may suggest that other autotrophic Gram-negative bacteria may not contain a potassium efflux oxidative stress response mechanism either.

Although these bacteria do not release potassium, they may contain some other means of acidifying their cytoplasm to protect against oxidative chemicals. Cytoplasmic acidification in response to oxidants was not tested during this study, but given that potassium efflux does not occur in response to oxidative chemicals, it seems unlikely that this type of protection

mechanism exists in these bacteria. Examining this protection mechanism on an evolutionary basis further lends weight to the argument that cytoplasmic acidification does not occur in nitrifiers. It has previously been shown that the growth substrate for AOB is NH_3, not NH_4^+ (Suzuki et al., 1974). At lower pH, ammonia is primarily in the protonated form (NH_4^+), and is not able to be used by the bacteria for generation of energy or reducing power. Therefore, any cytoplasmic acidification, at least in AOB, would be detrimental to energy production and production of reducing compounds that are needed to help mediate damage and control stress responses. The same may hold true for NOB, as lower pH would keep nitrite protonated as nitrous acid, which has been found to be highly toxic to nitrifying bacteria in activated sludge (Anthonisen et al., 1976). Low pH conditions have also caused nitrification inhibition in wastewater treatment facilities and pure cultures of AOB and NOB (Anthonisen et al., 1976; Princic et al., 1998; Grunditz and Dalhammar, 2001). In addition, it has been thought that GGKE originally evolved as a protection mechanism for methyglyoxal, which is an oxidative byproduct of glucose metabolism (Ferguson et al., 2000; Ferguson and Booth, 1998). As nitrifying bacteria are autotrophic and do not metabolize glucose, they would have had no need to develop the GGKE response. Therefore, the GGKE mechanism may not have evolved in nitrifying bacteria, or was removed from nitrifying bacteria because it may interfere with metabolism of ammonia and nitrite.

6.4.3 Glutathione in Nitrosomonas and Pseudomonas is Oxidized by Chlorine

Experiments testing the presence and oxidized state of glutathione in response to sodium hypochlorite shock showed that glutathione is present in both *N. europaea* and *P. aeruginosa*, and became oxidized in response to increasing concentrations of free chlorine. The oxidized glutathione concentrations are shown in Figure 6.7. This figure shows that the amount of oxidized glutathione increased as the amount of free chlorine in solution increased, which is in agreement with the results obtained by Chesney et al. (1996). An exception to this was observed for *P. aeruginosa* exposed to 25 mg/L of free chlorine. If a trend line is fit to the data, then the glutathione oxidized per mg chlorine added can be determined. For this experiment, the glutathione in *P. aeruginosa* was oxidized stoichiometrially at 9.80 nmoles/mg of added free Cl_2 (ignoring the 25 mg/L point). This compares to 4.48 nmoles/mg of added free Cl_2 observed for *N. europaea*, or stoichometric equivalent that is 2.2x higher. A repeat of the experiment yielded

slightly different stoichometric equivalents (6.19 and 2.58 nmoles/mg of added free Cl_2 for *P. aeruginosa* and *N. europaea*, respectively) but the equivalent for *Pseudomonas* was still 2.4x higher than that of the *Nitrosomonas*, which is consistent with the initial experiments. Therefore, although the stoichiometric oxidation equivalents were not the same, the overall trend observed for glutathione oxidation during the experiments was repeatable.

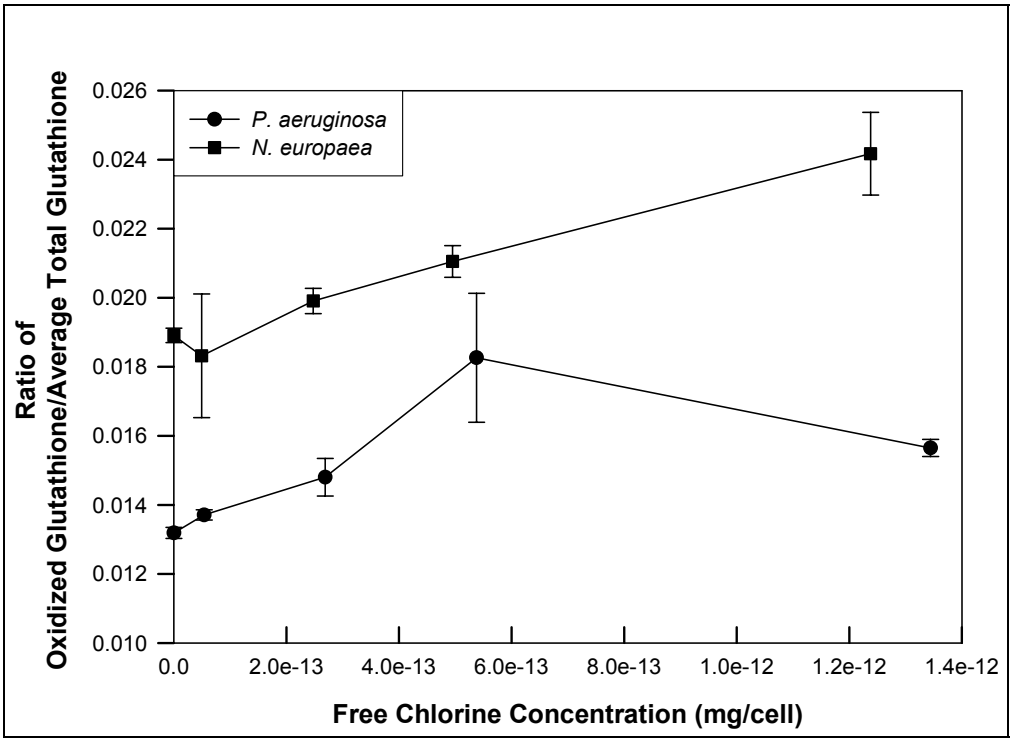

Figure 6.7 Ratio of oxidized glutathione to average total glutathione concentrations for *N. europaea* and *P. aeruginosa* cells exposed to sodium hypochlorite for 20 minutes. Cell concentrations at the beginning of the experiment were 2.02 x 10^{10} cells/ml for *N. europaea* and 1.86x10^{10} cells/ml for *P. aeruginosa*. Error bars indicate the range of 2 samples.

As stated above, the only point that did not follow an increasing trend for oxidized glutathione was the 25 mg/L chlorine concentration for *P. aeruginosa*. Although the exact reason for this decrease is unknown, it could be related to a sharp drop in total glutathione noted for the same time point. The drop in total and oxidized glutathione observed for *P. aeruginosa* may have been caused by chlorine induced cell lysis. Because all cells were washed prior to lysis on the French® press, washing the cells would have removed any glutathione in the bulk solution from chlorine induced lysis. Total glutathione levels during the repeats were at approximately the same concentration; however, data was more variable and no trends were

observed (data in Appendix D). For both cultures, the cellular glutathione concentrations ranged from $7x10^{-10}$ to $14x10^{-10}$ nmoles/cell. This is similar to a level of $7x10^{-10}$ nmole/cell reported by Fahey and coworkers (1978) for *P. fluorescens*. In addition, several studies have shown that the oxidized form of glutathione composes as little as 5% of the total glutathione during homeostasis (Apontoweil and Berends, 1975; Fahey et al., 1978), which is similar to what has been shown here for initial conditions (~2-3%). Taken together, the results obtained during this study confirm the concentrations and behavior of glutathione that have been found by others.

The results show that glutathione behaved similarly in both *P. aeruginosa* and *N. europaea* in that increasing concentrations of oxidized glutathione were observed when the cells were exposed to increasing concentrations of chlorine. The presence of glutathione in *N. europaea* was not surprising, knowing the highly conserved nature of glutathione and the important role glutathione has in protecting cells. However, the lower amount of glutathione oxidized per mg of chlorine was unexpected. Because the enzyme glutathione reductase was not found in the genome of *N. europaea,* we expected to see more oxidized glutathione per cell because it cannot be re-reduced during the oxidation event. Therefore, it appears that the lack of glutathione reductase is not detrimental to glutathione behavior during an oxidation event.

Glutathione is not only important in the GGKE mechanism, but also as a reductant for other important enzymatic systems. One such enzyme that has been found in both *P. aeruginosa* (Stover et al., 2000) and *N. europaea* (Chain et al., 2003) is glutathione peroxidase, which helps reduce toxic oxidative chemicals using glutathione as a reductant (Carmel-Harel and Storz, 2000). Thus, glutathione is important for oxidative stress response in nitrifiers, even if a glutathione mediated potassium efflux mechanism does not exist. However, the question remains: why are nitrifiers more susceptible to oxidative stressors? This may be explained in part by the lack of a potassium efflux mechanism for cytoplasmic acidification. It may also be partially explained by the lower rate of glutathione oxidation that was observed for *N. europaea* when compared with *P. aeruginosa*. This lower rate may be caused by the inability of the oxidant (chlorine) to penetrate the cells and make contact with the glutathione in the cytoplasm. Since nitrifying bacteria have very large cytoplasmic membrane invaginations, oxidants may take longer to reach the cytoplasm and have more time to react with membrane lipids and membrane associated proteins like ammonia monooxygenase and nitrite oxidoreductase. This would lead to a smaller amount of oxidant reaching the cytoplasm, less glutathione being

oxidized, and more damage to important metabolic enzymes. Therefore, the increased inhibition that has been observed for nitrifiers in response to oxidative chemicals in wastewater treatment processes (Kelly II et al., 2004) is likely caused not only by a lack of protection mechanisms like GGKE, but also to large membranes that hinder oxidant movement to the cytoplasm and contain important metabolic enzymes, like AMO and NOR, that can be easily oxidized by these chemicals.

6.5 Conclusions

We conclude that *N. europaea* and *Ni. moscoviensis* do not contain a surrogate of the glutathione gated potassium efflux mechanism that is found in other Gram-negative bacteria, or any oxidant responsive potassium efflux mechanism. This work provides the first evidence of Gram-negative bacteria that do not contain the GGKE mechanism and the first work where an autotrophic organism was studied. The reason these bacteria lack a GGKE mechanism for cytoplasmic acidification may be evolutionary, as acidification is detrimental to metabolic processes within the cells.

These results also indicate that glutathione in *N. aeruginosa* and *P. aeruginosa* is oxidized in response to chlorine bleach but the amount of glutathione oxidized per mg of chlorine was lower in *N. europaea* than in *P. aeruginosa*. This may be due do the large membrane invaginations in nitrifying bacteria that hinder the passage of oxidative chemicals into the cytoplasm and allow the oxidative chemicals to react more easily with important membrane bound enzymes like ammonia monooxygenase and nitrite oxidoreductase. Together, the lack of a K^+ efflux oxidative stress response mechanism and the possible high reactivity with membrane bound enzymes may explain why oxidative chemicals easily inhibit nitrifiers.

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7 CELL MEMBRANE MODIFICATIONS IN *NITROSOMONAS EUROPAEA* EXPOSED TO THE HYDROPHOBIC CHEMICAL 1-OCTANOL

7.1 Abstract

Previous experiments have shown that nitrification in activated sludge is upset by the hydrophobic chemical 1-octanol, but that this upset event recovered more quickly than upsets caused by other chemical classes. Therefore, the role of membrane modification stress response mechanisms in pure cultures of nitrifying bacteria were investigated in order to determine if they occur and are helping to protect against inhibition by hydrophobic contaminants. Genomic information for the ammonia oxidizing bacterium *Nitrosomonas europaea* indicated that the organism has the genes that enable cell membrane modifications in response to hydrophobic chemicals. Therefore, tests were conducted to confirm that these genes were activated in response to hydrophobic shock. Experiments were performed using pure cultures of *N. europaea* exposed to 1-octanol shocks. *Pseudomonas aeruginosa* was used as a positive control organism for all tests. Results showed that changes in the membrane content of *N. europaea* were different from those observed for P. aeruginosa. *P. aeruginosa* showed an increase in the amount of saturated fatty acids and an increase in cis isomers of unsaturated fatty acids, while *N. europaea* showed changes to the unsaturated to saturated fatty acid ratios that varied based on the amount of octanol added. These results suggest that, although some membrane modifications occur in *N. europaea,* not all of the mechanisms coded by the genome are used to respond to octanol shock events.

Key Words: Nitrification Inhibition, Cell Membrane, Hydrophobic Chemical Shock, Stress Response, 1-Octanol

7.2 Introduction

It is well known that nitrification is easily inhibited by a wide range of industrial chemicals (Kelly II et al., 2004; Blum and Speece, 1991; Tomlinson et al., 1966) and that nitrification is more sensitive to chemical toxins than other processes in wastewater treatment, like BOD removal (Kelly II et al., 2004, Blum and Speece, 1991). One chemical tested in our previous laboratory studies that significantly inhibited nitrification while not severely affecting other processes was the hydrophobic chemical 1-octanol (Love et al., In Press). Although 1-octanol inhibited nitrification, recovery from this inhibition event was also found to occur more

rapidly than for other chemical classes tested. Based on these results, we decided to further investigate why 1-octanol, and possibly other hydrophobic chemical toxins, inhibited nitrification but allowed nitrifying bacteria to quickly recover from such inhibition events.

The cause of hydrophobic chemical induced inhibition in bacteria is related to the site of action of these chemicals: the cell membrane (Ingram, 1977; Sikkema et al., 1994; Aono et al., 1994). Since nitrifying bacteria have very large membrane surface areas, they are an ideal target for hydrophobic chemical contaminants. When hydrophobic chemicals insert themselves into cell membranes, several effects on the membrane can occur and vary based on the structure of the hydrophobic solvent. First, membrane fluidity has been found to either increase (short-chain alcohols and alkanes, cyclic and aromatic compounds) or decrease (long-chain alcohols and alkanes) (Lohner, 1991; Sikkema et al., 1994) in response to selected chemicals. Swelling of the membrane bilayer has also been found in *Escherichia coli* cells exposed to toluene (Aono et al., 1994). Membrane swelling and membrane fluidity alterations have both been associated with leakage of macromolecules and ions (like proteins, ATP and potassium) out of the cytoplasm and into the environment (Jackson and de Moss, 1965; Woldringh, 1973; Aono et al., 1994; Heipieper et al., 1991). This increased membrane permeability can disrupt the proton gradient and membrane potential (Sikkema et al., 1994), which, given the dependence of ammonia and nitrite oxidation on the proton motive force (Hooper et al., 1997; Wood, 1986), would result in disruption of ammonia and nitrite oxidation and inhibition of the nitrification process. It has also been postulated that altering the membrane structure may affect the function of membrane bound proteins by expanding the cell membrane and preventing the individual subunits of transmembrane proteins from interacting properly, thus inactivating them (In't Veld et al., 1991). Though no particular studies have examined the effects of hydrophobic chemicals on nitrifying ammonia oxidizing bacteria (AOB), ammonia monooxygenase (AMO) is an important metabolic enzyme that catalyzes the oxidation of ammonia and is a multi-subunit transmembrane protein (Hooper et al., 1997). Given the hypothesis proposed by In't Veld et al., AMO would not fit properly into a membrane expanded by lipophilic chemicals, thus inactivating the enzyme and inhibiting nitrification.

Knowing how hydrophobic chemicals may inhibit nitrifying bacteria does not explain why they appear to recover from hydrophobic chemical inhibition more quickly than other chemically induced inhibition. Recently, the complete sequence of *N. europaea* was completed

(Chain et al., 2003), allowing for a search of genes related to stress response and protection within this species. The known protein coding genes for *N. europaea* are located at the Protein Information Resource NREF Database (National Biomedical Research Foundation, 2005) and the *N. europaea* genome can be searched at the ORNL *N. europaea* genome homepage (DOE Joint Genome Institute, 2005). Using these resources, we performed a detailed examination of the genome with relation to known membrane stress response mechanisms found in other Gram-negative bacteria that are closely genetically related. Results of this search in relation to *N. europaea* are found in Table 7.1.

Table 7.1 Summary of select genes involved in hydrophobic stress response mechanisms and their presence in the *N. europaea* genome.

Gene/Protein Name	Function	Presence in *N. europaea*
Membrane Modification Mechanisms		
β-ketoacyl-ACP synthase II	modification of membrane fatty acid composition	Probable Homolog Found
cti	fatty acid *cis-trans* isomerase	Probable Homolog Found
Efflux Systems		
AcrA-AcrB-TolC	multidrug/solvent efflux system	Probable Homologs Found
AcrR	*acrAB* system regulator	No
MexA-MexB-OprM	multidrug/solvent efflux system	Probable Homologs Found
MexC-MexD-OprJ	multidrug/solvent efflux system	Probable Homologs Found
MexE-MexF-OprN	multidrug/solvent efflux system	Probable Homologs Found
MexR	*Mex* systems regulator	No
SrpA-SrpB-SrpC	multidrug/solvent efflux system	Probable Homologs Found[a]
TtgA-TtgB-TtgC	multidrug/solvent efflux system	Probable Homologs Found[a]
MepA-MepB-MepC	multidrug/solvent efflux system	Unknown[b]
MepR	*mepABC* system regulator	Unknown[b]

[a] Homologous SrpB sequence in *N. europaea* is the same sequence homologous to TtgB
[b] *mepABC* and *mepR* could not be checked against *N. europaea* genome as gene sequences could not be located

As can be seen in Table 7.1, several different mechanisms exist for bacteria to cope with hydrophobic chemical stress. One type of mechanism that many bacteria have in responding to hydrophobic/lipophilic chemical stress is to actively efflux the chemical from the cell cytoplasm and membrane and thus remove the source of inhibition. These efflux systems are collectively referred to as multidrug efflux systems (Weber and de Bont, 1996; Kieboom and de Bont, 2000) and have all been identified as members of the resistance-nodulation-cell division (RND) family

of transporters (Kieboom and de Bont, 2000; Paulsen et al., 1996). There are many different protein channels that have been identified as multidrug efflux systems and all have been found to be active transporters (Paulsen et al., 1996). Several RND systems that have been identified in other Gram-negative organisms are listed on Table 7.1. The Acr system was first identified in *E. coli* (Aono et al., 1998), the Mex systems were identified in *P. aeruginosa* (Li et al., 1998), and the Srp, Ttg and Mep systems were all found in *P. putida* (Kieboom et al., 1998; Fukumori et al., 1998; Kieboom and de Bont, 2000). Although exact matches for these systems were not found in *N. europaea*, gene sequences encoding homologous systems were found, indicating that *N. europaea* contains efflux systems for responding to hydrophobic chemicals. Interestingly, although similar efflux systems were found in the genome, matches for the known regulatory mechanisms of these systems were not found. This indicates that such systems are either constitutively expressed or have some other unknown means of regulation in *N. europaea* and possibly other AOB.

Another type of response mechanism that was found involves enzymes that modify the cell membrane fatty acid composition in order to adapt to changes in the membrane fluidity. Two of the more important enzymes that have been identified are listed on Table 7.1. One of these is β-ketoacyl-ACP synthase II, which has been found to alter the ratio of saturated to unsaturated fatty acids in *E. coli* (Magnuson et al., 1993). By changing this ratio, the membrane can be made more (increase unsaturated fatty acids) or less (increase saturated fatty acids) fluid in order to counteract changes in the membrane fluidity caused by lipophilic chemicals. These modifications take several minutes to hours to occur, however, as new fatty acids need to be synthesized.

The other enzyme that is used to respond to changes in membrane fluidity is the cis/trans isomerase Cti, which converts cis unsaturated fatty acid isomers to a trans form during hydrophobic chemical shock (Holtwick et al., 1997; Heipieper et al., 1992). This response is much faster than synthesizing new fatty acids, and can occur within seconds of a cell being exposed to a hydrophobic chemical. The modification from cis to trans works in a manner similar to the switch from unsaturated to saturated fatty acids in that it helps to decrease the membrane fluidity in response to lipophilic shock. When the double bond in the cis fatty acid is rotated to a trans isomer position, the fatty acid tail becomes more straight. This structure is more similar to that of a saturated fatty acid, and the more straight structure increases ordering

and decreases fluidity of the cell membrane. Again, as with the efflux proteins, no exact matches for the genes coding the membrane modification enzymes β-ketoacyl-ACP synthase and Cti were found in the *N. europaea* genome, but probable matches exist. The presence of the genes coding these two enzymes indicates that *N. europaea* and likely other AOB should have the ability to mediate membrane fluidity changes caused by hydrophobic chemical shock events.

Although the presence of the genes coding these enzymes in *N. europaea* may explain why nitrification recovers from hydrophobic chemical shocks quickly, working forms of these enzymes may not exist. Determining if these mechanisms do exist in nitrifying bacteria may lead to the development of technologies that detect and/or mediate hydrophobic chemical upset events before they occur. From the information found in the literature and genomic searches, we hypothesize that the nitrification inhibition induced by 1-octanol in our previous studies was caused by the insertion of octanol into the cell membrane and that the rapid recovery of nitrification from the octanol shock event was due to the presence of membrane modification stress response mechanisms. Therefore, the objective of this study was to examine changes in the cell membrane of *N. europaea* in response to the hydrophobic chemical 1-octanol. Two pure cultures were chosen for these experiments: *N. europaea* and *Pseudomonas aeruginosa* PAO1. *N. europaea* was chosen because it is the most commonly studied AOB (Schramm et al., 1998) and it is the only autotrophic nitrifying bacteria with a sequenced genome (Chain et al., 2003) while *P. aeruginosa* PAO1 was chosen for use as a positive control bacterium because it is known to contain many of the above mentioned hydrophobic stress response mechanisms (Stover et al., 2000). Using these cultures, changes to the fatty acid content were examined in order to determine if short-term (cis/trans isomerization) and long-term (changes to saturated and unsaturated fatty acid composition) membrane modifications occurred in response to octanol shock.

7.3 Methodology

7.3.1 Bacterial Cultures and Growth Conditions

Ammonia Oxidizing Bacterium: *Nitrosomonas europaea*

The *N. europaea* cells were grown using a medium composed of 0.2 g/L $MgSO_4 \cdot 7H_20$, 20 mg/L $CaCl_2 \cdot 2H_2O$, 87 mg/L K_2HPO_4, 2.52 g/L EPPS, 10 μg/L $Na_2MoO_4 \cdot 2H_2O$, 17.2 μg/L $MnSO_4 \cdot H_2O$, 0.4 μg/L $CoCl_2 \cdot 7H_2O$, 170 μg/L $CuCl_2 \cdot 2H_2O$,10 μg/L $ZnSO_4 \cdot 7H_2O$, 100 μg/L

chelated iron, 250 μg/L phenol red and 1.32 g/L $(NH_4)_2SO_4$ at 29ºC in the dark in 5 L batch cultures. Sterile aeration was provided and mixing was achieved with magnetic stirrers. Growth curves were developed by measuring nitrite and performing cell counts. Nitrite samples were stored at -20°C for no more than 28 days prior to analysis and analyzed using a colorimetric procedure (method 4110 B) as described in Standard Methods (APHA, 1998). Cell counts were performed using a Helber bacteria single round cell counting chamber manufactured by Weber Scientific International (Middlesex, UK) on an Axioskop 2 plus phase contrast light microscope with a 40X objective lens (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Bacterium Known to Modify Cell Membrane: *Pseudomonas aeruginosa* strain PAO1

P. aeruginosa PAO1 was grown at 20°C in the dark in 1 L batch flask with aeration and mixing provided by shaking. It was grown in full strength mineral salts (FSMS) medium (186.12 mg/L EDTA, 11 mg/L $FeCl_2 \cdot 2H_2O$, 150 mg/L $MgSO_4 \cdot 7H_2O$, 4.5 mg/L $MnSO_4 \cdot 4H_2O$, 500 μg/L $NaMoO_4 \cdot 2H_2O$, 150 μg/L H_3BO_3, 100 mg/L NH_4Cl, 1.64 g/L NaAcetate, 20 mg/L $CaCl_2$, 1.5 mg/L $ZnCl_2$, 500 μg/L $CuCl_2 \cdot 2H_2O$, 1.5 mg/L $CoCl_2 \cdot 6H_2O$, 3.4 g/L KH_2PO_4, 4.35 g/L K_2HPO_4, 0.5 g/L NH_4Cl, 0.25 g/L NaCl). Growth curves for this organism were developed by performing cell counts as described for *N. europaea* above.

7.3.2 Determining Membrane Fatty Acid Content Changes

The fatty acid content of the bacterial cell membranes was determined using experimental reactors (octanol addition to *N. europaea*), a negative control reactor (no chemical addition) and a positive control reactor (octanol addition to *P. aeruginosa* PAO1). Pure cultures were concentrated to a minimum of 10^{10} cells/ml, as previous work done in our labs with pure cultures found this to be adequate for determining the cell membrane composition (data in Appendix E). Experiments were conducted when the pure cultures reached late-log phase growth. All reactors were mixed, aerated, and fed with each culture's normal growth substrate (ammonium sulfate for *N. europaea* and sodium acetate for *P. aeruginosa* PAO1) to keep the biomass in suspension and allow for normal aerobic respiration of the bacteria. 1-Octanol was chosen as the model hydrophobic chemical and was dosed at three concentrations (5, 50 and 200 mg/L) to simulate perturbation.

Duplicate samples were taken for fatty acid analysis. Samples were taken for analysis immediately prior to addition of 1-octanol and 2, 10, 30, 60 and 240 minutes after the addition of

the hydrophobic chemical 1-octanol. The 1 mL liquid samples were saponified in 1 mL of 3.75 N NaOH in methanol by digesting in a boiling water bath for 30 minutes. After the samples were saponified, 2 mL of 3.25 N HCl in methanol was added and the samples were heated to 80ºC for 30 minutes to derivatize the saponified fatty acids to fatty acid methyl esters (FAMEs). These FAMEs were extracted into 1 mL of hexane by vortex mixing for a minimum of 30 seconds. Samples were then stored at –20°C for no longer than 96 hours before analysis.

The extracted FAMEs in hexane were analyzed on a Hewlett Packard 5890A GC-FID (Hewlett Packard Inc., Rockville, MD) using a Supleco® S-2380 ™ capillary column (Supelco Co., Bellefonte, PA). The injector temperature was 250°C, the detector temperature was 260°C and the oven temperature was ramped from 100°C to 205°C at 7°C/minute, held at 205°C for 2 minutes, then ramped to 250°C at 4°C/minute and held at 250°C for 15 minutes. The fatty acids that were measured during these experiments were the saturated fatty acids C14:0, C16:0, C18:0, and C20:0 and the unsaturated fatty acids C18:1cis, C18:1trans, C18:2cis and C18:2trans.

7.3.3 Determining Nitrite Concentrations

Nitrite concentrations were measured for the *N. europaea* culture from samples collected at the same times that fatty acid samples were taken (listed above). Nitrite was measured to determine the degree of inhibition during the experiment and to determine if membrane changes correlated with recovery of ammonia metabolism. Nitrite was measured colorimetrically according to method 4110 B in Standard Methods (1998). Inhibition was calculated using the nitrite production rates (NPR) obtained from the slope of the nitrite produced over time using equation 1.

$$\text{Percent Inhibition} = \frac{NPR_{control} - NPR_{inhibited}}{NPR_{control}} \times 100\% \quad \text{[Equation 1]}$$

7.4 Results and Discussion

7.4.1 N. europaea Showed Inhibition and Recovery From Octanol Shock

N. europaea was inhibited by the addition of octanol, but began to recover by the end of the 4 hour experiment. As shown in Table 7.2, initial inhibition was observed for the first one hour of the experiment, where the addition of 5, 50 and 200 mg/L of 1-octanol inhibited the cells by 59, 42 and 81%, respectively. For the last 3 hours of the experiment, the 5, 50 and 200 mg/L

shocked cells were inhibited by -20, 46 and 46%, respectively. By the end of the experiment, the 5 mg/L shocked reactor had recovered to the same level as the control reactor, while the 200 mg/L shocked reactors had begun to recover based on nitrite production rate results. Because the experiments were conducted with batch cultures and the added octanol was not removed from the system, the recovery that was observed for these cells indicated that some adaptation was occurring within *N. europaea*. The mechanism that *N. europaea* used to adapt to the presence of octanol may be related to the membrane modification stress response mechanisms that were discussed previously. Since only the 5 and 200 mg/L shocked reactors showed recovery of inhibition relative to the control reactor, we focused the remaining analysis on the membrane modifications occurring in these reactors.

Table 7.2 Nitrite production rates and inhibition relative to the control for *N. europaea* shocked with 1-octanol.[a]

Reactor	Time 0-60 minutes		Time 60-240 minutes	
	Nitrite Production Rate (mg NO_2^--N/min)	Inhibition Relative to Control	Nitrite Production Rate (mg NO_2^--N/min)	Inhibition Relative to Control
Control	1.32		3.55	
5 mg/L Octanol	0.54	59.2%	4.25	-19.8%
50 mg/L Octanol	0.76	42.3%	1.90	46.4%
200 mg/L Octanol	0.25	81.4%	1.91	46.2%

[a]Error bars indicate the range of 2 samples.

7.4.2 N. europaea Alters Fatty Acid Composition in Response to Octanol

The results of the fatty acid experiments show that *Nitrosomonas europaea* alters the fatty acid content of its cell membrane in response to various concentrations of 1-octanol. The *P. aeruginosa* positive control cells also showed a change in the fatty acid composition in response to octanol shock. The summary results are shown in Table 7.3. The total percent increase from the initial time point (just prior to octanol addition) shows that, except for the C20 fatty acids, each fatty acid increases for *N. europaea* cells over the 4 hour experiment. The 5 and 200 mg/L shocked reactors did not show the same degree of change for the same fatty acids, indicating that there might be a unique dose-response relationship with the contaminant. For example, the unsaturated C18 fatty acid increased 49% in the control reactor, but increased only 30% in the 5

mg/L shocked reactor, while the 200 mg/L octanol shocked reactor increased 62%. In comparison, the *P. aeruginosa* cells had a similar response for both the 5 and 200 mg/L shocked reactors. Here, increasing trends in the fatty acid content were observed for both reactors, with a greater change seen for the higher concentration of octanol added. How these changes relate to the different membrane modification mechanisms is discussed in greater detail below.

Table 7.3 Percent increase in fatty acid concentration after 4 hours of exposure for *N. europaea* and *P. aeruginosa* cells in the presence or absence of 1-octanol.[a]

Fatty Acid Type	*N. europaea* Negative Control	*N. europaea* 5 mg/L Octanol	*N. europaea* 200 mg/L Octanol	*P. aeruginosa* Negative Control	*P. aeruginosa* 5 mg/L Octanol	*P. aeruginosa* 200 mg/L Octanol
Saturated						
C14:0	69.5	70.8	92.8	34.9	54.1	200.4
C16:0	82.4	41.5	66.3	-0.7	13.5	119.5
C18:0	48.8	30.0	61.6	-31.8	-18.1	62.1
C20:0	-7.7	-11.2	17.5	Not Available	Not Available	48.4
Unsaturated						
C18:1cis	54.9	23.6	27.3	96.3	122.3	296.1
C18:1trans	471.4	77.7	40.8	78.1	73.0	166.3
C18:2cis	45.8	19.0	15.6	7.7	33.5	149.9
C18:2trans	17.5	8.2	31.5	58.9	50.3	90.7

[a]Values listed are an average of duplicate samples.

7.4.3 N. europaea Does Not Use Short-Term Membrane Modifications

N. europaea cells did not change the cis-to-trans ratio of C18 unsaturated fatty acids relative to the negative control reactor at either of the octanol concentrations tested. Even though Table 7.3 shows that there are considerable differences in the individual C18 unsaturated fatty acids for all of the reactors, Figure 7.1 shows that the total ratio of the unsaturated fatty acids did not change considerably over the course of the experiment. In contrast, the positive control culture (*P. aeruginosa*) increased the ratio of cis-to-trans fatty acids over the course of the experiment. For *P. aeruginosa*, the negative control reactor showed a 7X increase in cis unsaturated fatty acids over the first hour, after which the levels drop about 12X. For all of the shocked *Pseudomonas* cells, the levels of measured unsaturated fatty acids increased about 15X over the course of the experiment, with most of this increase occurring within the first 30 minutes of exposure. Alterations in the cis-to-trans ratio of unsaturated fatty acids is thought to

be a short-term response mechanism, and a study by Heipieper and de Bont (1994) found that the shift in the cis/trans isomers took 30 minutes to reach the final cis-to-trans ratio. For our experiments, *P. aeruginosa* showed that the shift in the cis-to-trans ratio reached a maximum change after 30 minutes for the 5 mg/L shocked reactor, which agrees with the time found by Heipieper and de Bont. The 200 mg/L shocked reactor did not reach a maximum increase in the cis-to-trans ratio before the experiment was stopped at 4 hours, indicating that inhibition may have prevented the cis/trans isomerization from occurring more quickly at higher concentrations of 1-octanol.

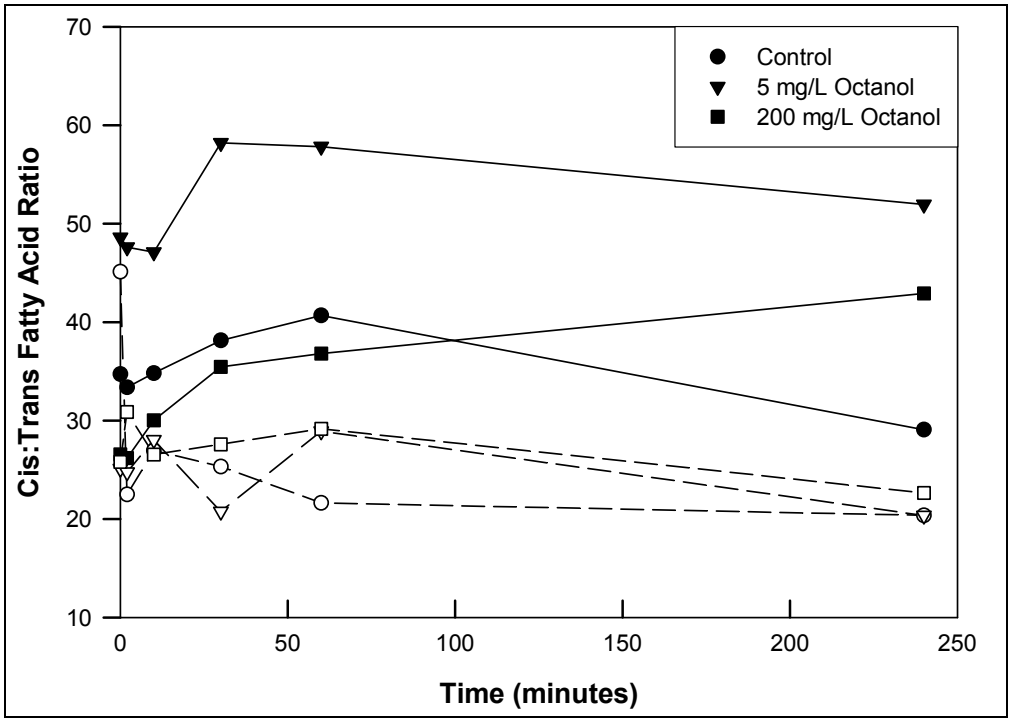

Figure 7.1 Change in the total measured cis:trans unsaturated fatty acids ratio over time for cells exposed to octanol. *P. aeruginosa* is shown as closed symbols and solid lines. *N. europaea* is shown as open symbols and dashed lines. Cell concentrations for *N. europaea* and *P. aeruginosa* were 1.26 and 1.64×10^{10} cells/ml, respectively. Points are the average of duplicate measurements.

The C18 unsaturated fatty acid data also revealed that, with the exception of the *P. aeruginosa* 5 mg/L shocked reactor, the initial (Time 0) cis-to-trans ratio of C18 unsaturated fatty acids was similar, between 25 and 35, for both cultures. This means that both organisms have about the same membrane fluidity, if only the ratio of C18 unsaturated fatty acids is examined and not the total composition of the unsaturated fatty acids in the cell membrane.

Several studies have shown that the relative amounts of the cis and trans isomers of C16 unsaturated fatty acids can also change significantly in response to hydrophobic chemicals and play an important role in membrane fluidity (Pinkart et al., 1996; Heipieper et al., 1992; Weber. and de Bont, 1996), but only the C18 unsaturated fatty acids were measured during this experiment.

Even though both *N. europaea* and *P. aeruginosa* changed the total amount of unsaturated C18 fatty acids in response to octanol, only *P. aeruginosa* showed modifications to the cis-to-trans C18 unsaturated fatty acid ratio. This means that *N. europaea* likely contains a cis/trans isomerase to alter the isomer form of unsaturated fatty acids, but it does not use it to change the relative cis/trans ratio and alter the membrane fluidity in response to 1-octanol. For *P. aeruginosa*, a shift toward cis unsaturated fatty acids indicates that the cells are trying to increase membrane fluidity to counteract a decrease in the fluidity that was presumably caused by octanol. There are many conflicting results in the literature examining changes in fatty acids in response to similar hydrophobic chemicals. Our results contradict a study done by Lohner (1991), who found that short carbon chain alcohols (chain length, $C < 10$) caused an increase in membrane fluidity, and required a shift towards trans unsaturated fatty acids. However, a study done by Heipieper and de Bont (1994) reported that the amount of cis fatty acids increased in *Pseudomonas putida* exposed to the short-chained alcohol ethanol, which supports our results. Another study from the same research group reported an increase in the relative amount of trans fatty acids in response to ethanol for the same organism (Weber and de Bont, 1996). Further conflicting results arose for a study involving *P. putida* exposed to o-xylene, which showed an increase in the cis isomers relative to trans isomers at 75 mg/L, while 200 mg/L showed an increase in the trans isomers relative to cis isomers (Pinkart et al., 1996). Since several studies provide conflicting results for the effects of chemicals on cis/trans isomers of unsaturated fatty acids, and this is the first study using octanol, the results presented here are cannot be verified against other work.

7.4.4 N. europaea Uses Long-Term Membrane Modifications in Response to Octanol

When examining the long-term membrane response in *N. europaea,* the results showed that the relative ratios of saturated to unsaturated fatty acids were unchanged for the first 60 minutes, after which significant changes occurred (Figure 7.2). The long time period observed

before the shift occurred is consistent with the fact that this is a long-term membrane modification mechanism. Between 60 minutes and 4 hours, the membranes in the 5 mg/L octanol shocked reactor decreased in saturated-to-unsaturated fatty acids relative to cells that were not shocked while, strangely, the 200 mg/L reactor showed the opposite effect. The reason for the different results is unknown, but seem to correspond with the previous conflicting results for cis/trans isomerization and suggest that high versus low concentrations of octanol elicit different responses. It is also important to note that the change in the saturated-to-unsaturated fatty acid ratios corresponded with recovery of the nitrite generation rate, which supports our hypothesis that membrane modifications lead to recovery of nitrification from hydrophobic chemical inhibition.

The results for the *P. aeruginosa* culture, however, were contrary to what was expected and showed no change relative to the negative control reactor. For this culture there is a slight increase in saturated-to-unsaturated fatty acid after 10 minutes of exposure, but this increase is small and may not be significant. The lack of any change may indicate that no long-term response is needed for octanol shock in these cells.

The results presented in this study that indicate no change in the saturated-to-unsaturated fatty acid ratio for *P. aeruginosa* and conflicting changes for the two concentrations of octanol tested on *N. europaea* are contrary to what was expected for both cultures. For *N. europaea,* a consistent increase or decrease would be expected. The cis/trans results for *P. aeruginosa* indicate that a decrease in membrane fluidity was presumably caused by octanol. Therefore a shift towards unsaturated fatty acids would have been expected. A decrease in membrane fluidity caused by octanol was also indicated by the decrease in the saturated-to-unsaturated fatty acids for *N. europaea* cells exposed to 5 mg/L octanol. However, the increase observed for *N. europaea* cells exposed to 200 mg/L octanol contradicts the decrese in fluidity and may be due to some inhibition mechanism that has not been characterized for *N. europaea*. In addition, since *P. aeruginosa* is known to modify the ratio of saturated-to-unsaturated fatty acids in response to hydrophobic chemicals, and it showed a shift in the cis-to-trans ratio in response to octanol, a shift in the ratio from saturated to unsaturated would have been expected. The lack of response may indicate that the modifications to the cis-to-trans ratio may have been adequate for responding to the induced octanol stress.

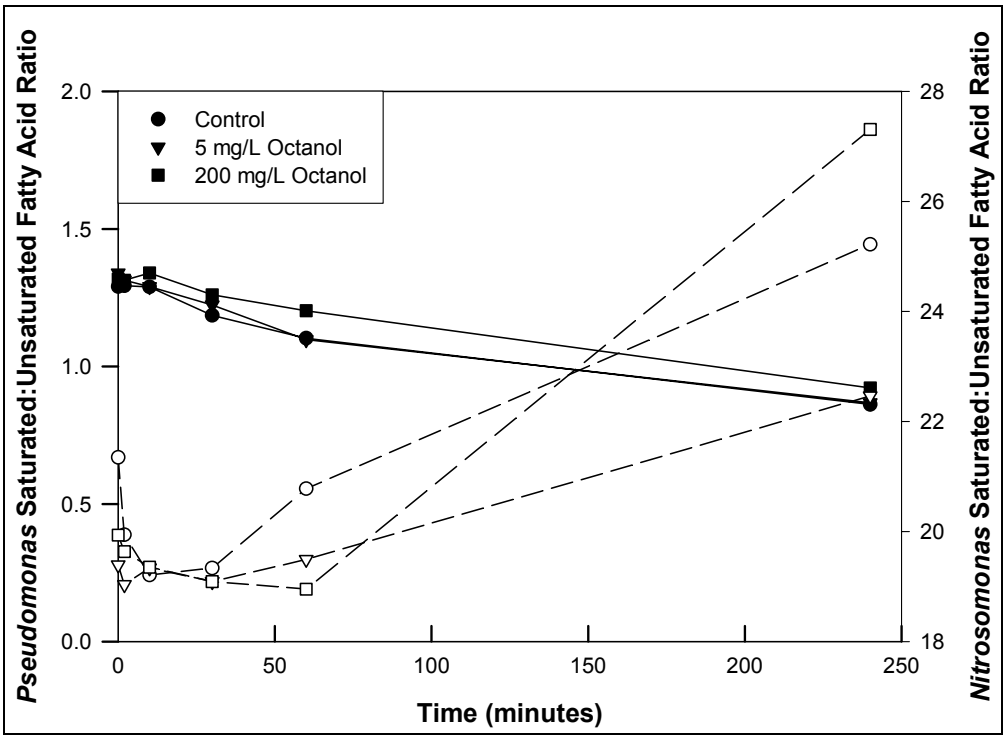

Figure 7.2 Change in the total saturated:unsaturated fatty acids ratio over time for cells exposed to octanol. *P. aeruginosa* is shown as closed symbols and solid lines. *N. europaea* is shown as open symbols and dashed lines. Cell concentrations for *N. europaea* and *P. aeruginosa* were 1.26 and 1.64×10^{10} cells/ml, respectively. Points are the average of duplicate measurements.

Interestingly, the fatty acid data also revealed that the relative amounts of saturated-to-unsaturated C18 fatty acids present in the two cultures were significantly different. The ratio is approximately 1.3 for *P. aeruginosa* while it is almost 20 times greater for *N. europaea*. This is contrary to the results found for the cis-to-trans C18 unsaturated fatty acid ratios, which were about the same for the two cultures. These differences are significant and may have something to do with the unique membrane structure observed in nitrifying bacteria. In addition to differences in the relative amounts of fatty acids, the actual quantity of extracted fatty acids was significantly different between the cultures. The total amount of saturated fatty acids measured was about 2X higher for the *P. aeruginosa* culture, while the C18 unsaturated fatty acids was about 30X higher in *P. aeruginosa* than it is in *N. europaea* (data not shown). The higher ratio of the saturated fatty acids in *N. europaea* implies that the membrane of this organism is more rigid and less fluid than the membrane for *P. aeruginosa*. This decreased membrane fluidity would decrease the movement of membrane bound proteins. In *N. europaea,* AMO is a

membrane bound protein used for generating energy from ammonia oxidation, and decreased movement of this enzyme and others related to energy generation may be advantageous to the growth of the organism.

7.4.5 Future Work

Although this study provides evidence that some membrane modification mechanisms exist, other modes of inhibition and mechanisms of recovery may play a role in protecting nitrifying bacteria from hydrophobic chemical shocks. Several possibilities for this exist. For one, the genome codes for a large number of efflux systems (Table 7.1). If these efflux systems exist and are working, then the octanol and other hydrophobic chemicals would be removed from the membrane quickly and would help reduce the toxicity of the hydrophobic chemicals. It is also possible that hydrophobic chemical inhibition is not only directed against the cell membrane, but against proteins in the cells. A study performed by Franks and Lieb (1984) indicates that some hydrophobic chemicals directly affect some enzymes and proteins by competitively binding in the hydrophobic pocket of the enzymes. This may also be the case for *N. europaea,* where ammonia, the uncharged molecule NH_3, is the substrate for the enzyme AMO (Suzuki et al., 1974). This means that the active site in AMO may be a hydrophobic pocket, which is supported by the largely hydrophobic peptide sequence coded by the gene sequence for the subunits of this enzyme (McTavish et al., 1993). Therefore, hydrophobic chemicals may inhibit ammonia oxidation by competitively binding in the hydrophobic pocket of AMO and preventing oxidation of ammonia. From this information, further studies are needed to test the effects of other hydrophobic chemicals besides 1-octanol, and determine if other stress response mechanisms are occurring within nitrifying organisms in response to hydrophobic chemicals. This would provide a better understanding of hydrophobic nitrification inhibition and the responses that nitrifying bacteria have against hydrophobic stressors may lead to the development of systems that can predict upset events before they occur.

7.5 Conclusions

To the authors' knowledge, this work provides the first study of the cell membrane structure of any nitrifying bacteria and the first work examining the effects of 1-octanol on

bacterial cell membrane fatty acid composition. Based on the results of this study, we conclude that *N. europaea* can modify the saturated-to-unsaturated fatty acid content in response to the hydrophobic chemical 1-octanol and that the modification of this ratio was also found to correspond with recovery of the nitrite production rate, indicating that modifying the membrane may contribute to recovery of the ammonia oxidizing capabilities of *N. europaea*. The saturated-to-unsaturated fatty acid ratio results suggest that *N. europaea* does modify the fatty acid content of its cell membrane in response to the hydrophobic chemical contaminant 1-octanol, but that the change in the ratio also depends on the concentration of octanol added. This agrees with our hypothesis that membrane modifications occur in response to hydrophobic chemical shock that allow for recovery of nitrification. However, the lack of any cis-to-trans ratio modifications contradicts the genomic information that indicates this organism should be capable of modifying the cis-to-trans ratio as a short-term response. Furthermore, the conflicting responses in the saturated-to-unsaturated fatty acid ratios for different octanol concentrations also contradicts the idea that the response should be uniform for a given chemical stressor.

Although this study provides evidence that some membrane modification mechanisms exist, other stress response mechanisms may exist and more research is needed in this area to determine exactly what mechanisms are occurring. Knowing what stress response mechanisms are or are not present in nitrifying bacteria will lead to a better understanding of how these organisms react to chemical inhibitors, which may lead to the development of techniques to detect and/or mediate chemical upset events before they can occur at a full-scale treatment facility.

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8 ENGINEERING SIGNIFICANCE

Because many municipal wastewater treatment systems have industrial sources, the accidental release of industrial chemical toxins will occasionally occur. Such shock loads can cause significant upset of treatment processes. In particular, nitrification is one of the most susceptible treatment processes to chemical upset, which has been shown in this work and the work of others. A knowledge of the molecular level causal mechanisms of inhibition and the stress response mechanisms that nitrifying bacteria use to respond to toxicant stressors could provide valuable information that could be used to determine methods to mediate nitrification upset once it occurs or even detect such upset events before they can occur.

Currently, very little is known about stress responses in nitrifying bacteria, so this study set out to examine the role of selected stress responses in *N. europaea* and *Ni. moscoviensis*. The information gained from this study demonstrated the activation of one stress response mechanism in nitrifiers involving long-term membrane fatty acid synthesis, which occurred in response to hydrophobic chemicals after several hours of exposure. However, of the studied stress response mechanisms, nitrifiers did not demonstrate an ability to activate several short-term responses that were studied, including oxidant-activated potassium efflux and hydrophobic shock-activated *cis/trans* fatty acid isomerase. Because nitrification tends to be the most sensitive process to upset, warning devices that are based on activation of these conserved responses, which are found in other Gram negative bacteria, could be used indicate that nitrification upset will likely occur in addition to upset to heterotrophic-based processes. However, the lack of these short-term responses in nitrifiers means that such devices would not be specific to nitrification inhibition and also could not be calibrated to predict the extent of nitrification inhibition caused by a given toxin.

The major implications of this study are that, although stress responses may exist in nitrifying bacteria, the short-term responses studied here do not appear to be present or used. Therefore, the conventional methods currently used to detect nitrification inhibition remain the fastest and easiest ways to monitor the nitrification process. In this work we showed that nitrate generation rate (NGR) provided the most consistent measure of nitrification inhibition, which implies that commercial units using differential respirometry will not provide as accurate a measure of inhibition from chemical sources. Although respirometric techniques are currently easier to adapt for online monitoring, the UV method developed here for NGR shows significant

promise as a monitoring tool. The UV NGR technique could be easily implemented as an online full-scale monitoring technique if a robotic sample collection system is used to automate sampling, or an in-line filter system is used to remove particulates prior to sample entering a spectrophotometeric unit. In addition, by monitoring nitrite during this test, we can examine which of the different classes of nitrifiers is inhibited during an upset event, which cannot be done using respirometric techniques. As technology progresses, a warning device that uses UV NGR may be developed that will allow operators at wastewater treatment facilities to be more proactive and less reactive in responding to nitrification upset events.

Even though conventional methods were shown to be the best for detecting nitrification inhibition during this study, they may not truly be the best and cheapest nitrification upset warning device that can be developed. It is obvious that more work is needed to examine other possibilities for warning devices, and several potential areas for future work come from this study. First, it is clear that nitrifying bacteria do not regulate genes and proteins in the same manner as other bacteria. By studying regulation in nitrifying bacteria, not only can we gain a better knowledge of the nitrification process, but the short-term regulatory responses that nitrifiers have may be used to develop a cheap and efficient warning device. Second, nitrifying bacteria must have some stress response mechanisms and in studying these, a unique mechanism may be found that can be used to specifically detect nitrification inhibition. A third area of research that does not directly come from this study is metabolic fingerprinting, or a study of the metabolic products produced by nitrifying bacteria. It is thought that studying the metabolic products of nitrifying bacteria, patterns in the "fingerprints" they produce in response to toxic chemicals may be used to determine what type and possibly even what concentration of contaminant is present, which would make a very powerful tool for predicting toxin induced upset events.

Designing a robust wastewater treatment system that can react to or resist chemical upset events while continuously and successfully providing a clean effluent is the goal of every wastewater engineer. The ability to design such a robust system requires knowledge, not only of the basics of treatment design, but also of the physiological responses of the bacteria. This study provides a small piece of the picture of how stress responses in nitrifying bacteria affect their susceptibility to industrial chemical contaminants. However, in order to gain a better understanding of how these stress responses can be used to detect and/or mediate nitrification

upset at full-scale treatment facilities, future work should focus on how the lack of these stress response mechanisms can be overcome to prevent nitrification inhibition and should try to identify the stress responses that are present in nitrifying bacteria.

APPENDICES

Appendix A: Data for Chapter 3

Table A.1 MLSS data for CDNB inhibited nitrifying reactor experiments.

	Control MLSS			IC_{15} MLSS			IC_{25} MLSS			IC_{50} MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.5	1307	1247	1267	1447	1247	1313	1307	1240	1407	1133	1067	1347
1.25	1480	1553	1360	1253	1287	1313	1313	1393	1293	1280	1227	1207
2.25	1340	1367	1380	1333	1280	1307	1273	1347	1267	1153	1133	1153
3.25	1513	1553	1593	1600	1573	1527	1533	1527	1527	1213	1273	3247
4.25	1620	1613	1593	1733	1700	1780	1640	1680	1660	1267	1240	1240
5.25	1560	1553	1540	1820	1767	1847	1780	1747	1733	1353	1373	1360
6.25	1587	1580	1693	1847	1840	1827	1707	1760	1780	1413	1380	1353
7.25	1567	1600	1593	1900	1927	1907	1880	1833	1860	1593	1620	1640
8.25	1627	1613	1620	1960	1973	1947	1987	1993	1987	1780	1780	1780
9.25	1500	1493	1447	1913	1847	1947	1893	1827	1967	1667	1773	1700
11.25	1727	1707	1747	2100	2080	2060	2160	2187	2160	2033	2020	1927
13.25	2273	2187	2147	2820	2780	2887	2893	2940	2927	2887	2907	2880
15.25	1980	2047	1973	2413	2327	2320	2433	2387	2433	2553	2687	2673
17.25	2027	2033	2060	2213	2220	2267	2313	2400	2413	2647	2640	2587
19.25	2093	2147	2067	2253	2300	2267	2460	2500	2473	2433	2367	2413
21.25	2200	2113	2160	2320	2320	2280	2560	2613	2593	1893	1793	1787
23.25	2020	2027	2007	2187	2233	2240	2387	2380	2400	1273	1240	1227
25.25	2560	2493	2507	2993	3060	3007	3367	3340	3287	987	813	980
27.25	2220	2180	2233	2593	2687	2533	2767	2660	2733	1007	1093	1033
28.25	2333	2367	2333	2713	2740	2760	2907	2927	2873	1280	1347	1340

Table A.2 MLVSS data for CDNB inhibited nitrifying reactor experiments.

	Control MLVSS			IC_{15} MLVSS			IC_{25} MLVSS			IC_{50} MLVSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.5	1207	1080	1100	1293	1133	1207	1233	1127	1300	1033	973	1160
1.25	1260	1320	1140	1067	1060	1093	1100	1187	1107	1033	1027	1013
2.25	1260	1260	1267	1220	1140	1140	1180	1247	1207	1033	993	1027
3.25	1387	1360	1427	1480	1440	1387	1367	1367	1367	1093	1113	1127
4.25	1433	1453	1420	1540	1553	1567	1493	1487	1467	1093	1073	1087
5.25	1340	1327	1373	1573	1540	1553	1547	1533	1493		1160	1127
6.25	1393	1400	1467	1613	1573	1580	1520	1560	1533	1167	1233	1113
7.25	1327	1347	1333	1613	1607	1600	1573	1527	1540	1293	1333	1327
8.25	1340	1373	1380	1647	1667	1620	1653	1667	1667	1480	1473	1480
9.25	1273	1307	1240	1613	1600	1720	1633	1567	1680	1380	1500	1453
11.25	1447	1440	1440	1753	1727	1720	1827	1813	1820	1693	1667	1613
13.25	2000	1920	1880	2400	2413	2500	2547	2547	2480	2500	2487	2453
15.25	1707	1747	1673	2033	1960	1967	2053	1993	2040	2133	2220	2227
17.25	1647	1673	1707	1807	1780	1867	1920	2040	1967	2220	2187	2160
19.25	1753	1767	1693	1853	1920	1867	2040	2133	2027	2007	1987	2053
21.25	1870	1813	1853	1973	1960	1907	2180	2233	2240	1547	1533	1533
23.25	1713	1707	1700	1867	1887	1893	2033	2020	2047	1073	1060	1060
25.25	2180	2093	2153	2473	2547	2553	2827	2820	2800	853	713	847
27.25	1892	1887	1933	2213	2287	2120	2393	2240	2327	887	927	907
28.25	2020	2060	2047	2353	2387	2387	2520	2533	2480	1140	1207	1213

Table A.3 Effluent nitrate data for CDNB inhibited nitrifying reactor experiments.

	Control Nitrate		**IC_{15} Nitrate**		**IC_{25} Nitrate**		**IC_{50} Nitrate**	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	11.085	13.830	9.443	14.500	14.542	13.864	8.442	11.856
0.5	12.851	14.697	12.235	14.643	14.699	11.106	11.085	9.229
0.75	8.228	12.823	15.088	15.032	3.651	3.603	3.663	7.553
1.0	9.971	13.211	10.312	13.135		4.865	3.861	4.466
1.5	11.460	11.715	11.709	8.361	8.382	0.693	1.024	1.615
2.0	9.855	12.650	12.668	6.015	0.318	0.441	0.428	0.563
2.5		14.599	3.174	3.381	0.353	0.389	0.385	0.162
3	5.629	5.579	1.620	1.630	1.627	0.368	0.140	0.145
4	6.293	6.386	2.377	3.116	0.607	0.624	0.233	0.237
5		11.822	6.555	6.888	1.208	1.179	0.299	0.152
6	19.359	19.347	14.249	14.268	3.402	3.487	0.400	0.162
7	17.105	18.991	12.525	14.437		6.827	0.158	0.157
8	15.168	15.283	15.292	9.248	9.275	5.658	0.165	0.164
9	13.058	13.213	6.476	7.277	4.672	4.668	0.526	0.173
11	8.357	8.323	4.475	4.515	4.520	3.978	0.247	0.247
13	8.031	9.294	10.138	9.087	6.870	6.863		0.360
15	11.002	12.823	12.830	14.270	6.146	7.110		0.725
17	9.555	10.204	9.607	10.768	10.696	6.501	1.529	1.530
19	4.595	5.649	4.614	5.854	4.964	5.306	3.549	4.105
21	5.625	6.684	4.347	5.855	3.780	6.146	2.646	3.629
23	3.650	5.811	3.981	4.734	4.787	5.697	3.415	3.342
25	3.183	3.786	3.722	4.999	3.700	5.228	2.152	2.813
27	8.052	9.605	9.596	7.735	4.415	7.369	2.518	3.373
28	9.278	10.878	7.422	8.414	5.774	7.036	4.460	5.282

Table A.4 Effluent nitrite data for CDNB inhibited nitrifying reactor experiments.

Time	Control Nitrite		IC_{15} Nitrite		IC_{25} Nitrite		IC_{50} Nitrite	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	1.104	1.687	1.622	1.445	0.000	0.000
0.5	0.000	0.000	1.563	2.192	2.183	0.432	0.362	0.000
0.75	0.000	0.000	2.222	2.242	0.459	0.405	0.417	0.000
1.0	0.000	0.000	2.040	2.498	2.576	1.154	0.000	0.000
1.5	0.000	0.000		6.231	6.294	2.056	0.000	0.000
2.0	0.000	0.000	0.000	8.002	1.133	1.685	0.000	0.000
2.5	0.000	0.000	10.927	11.695	1.443	1.584	1.637	
3	0.000	0.000	6.667	6.646	6.617	1.320	0.000	0.000
4	0.000	0.000	1.182	1.491	3.302	3.467	0.000	0.000
5	0.000	0.000	1.029	1.144	4.995	5.040	0.920	0.000
6	0.000	0.000	0.000	0.000	6.389	6.377	0.800	0.000
7	0.000	0.000	0.000	0.000	0.000	1.367	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.507	0.498
9	0.000	0.000	0.000	0.000	0.000	0.000	0.639	0.666
11	0.000	0.000	0.000	0.000	0.000	0.000	0.963	0.957
13	0.000	0.000	0.000	0.000	0.000	0.000		1.753
15	0.000	0.000	0.000	0.000	0.000	0.000		4.788
17	0.000	0.000	0.000	0.000	0.000	0.000	4.163	4.074
19	0.000	0.000	0.000	0.000	0.000	0.000	0.760	0.961
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27	0.000	0.000	0.000	0.000	0.000	0.000	0.937	1.116
28	0.000	0.000	0.000	0.000	0.000	0.000	0.526	0.517

Table A.5 Effluent ammonia data for CDNB inhibited nitrifying reactor experiments.

Time	Control Ammonia		IC_{15} Ammonia		IC_{25} Ammonia		IC_{50} Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	0.000	0.000	0.000	2.364	6.574	6.295
0.5	0.000	0.000	0.348	0.695	7.230	8.773	10.933	11.725
0.75	0.000	0.000	0.000	0.000	7.923	9.269	8.677	8.172
1.0	0.000	0.000	0.000	0.000	8.643	10.151	11.834	7.546
1.5	0.000	0.000	0.000	0.000	8.020	11.049	8.556	10.520
2.0	0.000	0.000	0.000	0.000	8.396	11.447	11.974	14.666
2.5		0.000	0.000	0.000	11.367	12.845	15.800	21.794
3	0.609	0.000	0.000	0.000	11.598	15.142	15.163	25.698
4	1.046	0.000	0.574	0.000	5.056	6.166	18.145	32.163
5	0.828	0.000	0.000	0.000	2.727	4.321	18.619	26.507
6	0.127	0.106	0.106	0.158	0.000	0.630	24.091	26.788
7	0.570	0.000	0.000	0.000	0.000	0.000	20.457	18.954
8		0.639	0.000	0.000	0.000	0.000	22.459	22.342
9	1.537	0.000	0.000	0.000	0.000	0.000		23.578
11	0.000	0.000	0.000	0.000	0.000	0.000	14.102	15.215
13		0.000	0.000	0.000	0.000	0.000		15.885
15	1.554	1.169	0.000	0.000		10.627	13.472	
17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
28	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.6 Nitrate Generation Rate (NGR) data for CDNB inhibited nitrifying reactor experiments.

Time	Control NGR	IC_{15} NGR	IC_{25} NGR	IC_{50} NGR
days	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)
0.5	2.646	0.000	0.848	0.000
1.25	2.444	0.715	0.059	0.133
2.25	1.604	0.656	0.240	0.394
3.25	2.017	0.372	0.054	0.059
5.25	1.246	0.857	0.204	0.096
7.5	4.134	2.213	1.445	0.383
9.25	3.115	2.060	0.354	0.001
13.25	2.657	1.517	1.267	0.025
17.25	3.053	2.881	1.583	0.127
21.25	1.387	1.730	2.245	0.472
25.25	2.586	1.689	2.137	0.961
28.25	1.173	1.565	1.864	0.833

Table A.7 Effluent alkalinity data for CDNB inhibited nitrifying reactor experiments.

Time	Control $CaCO_3$		IC_{15} $CaCO_3$		IC_{25} $CaCO_3$		IC_{50} $CaCO_3$	
	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	144.6	143.9	142.4	140.1	154.4	151.4	176.2	177.0
0.5	151.4	154.4	150.6	149.9	195.1	195.8	203.4	201.9
1	166.5	165.7	158.9	158.2	223.7	222.2	237.3	238.0
2	167.2	168.7	169.5	165.7	249.3	252.3	271.2	272.7
3	237.3	238.0	241.0	242.5	308.8	309.6	364.5	372.1
5	165.7	178.5	186.0	186.8	204.9	206.4	312.6	313.3
7	145.4	146.9	159.7	158.9	177.8	177.8	291.5	290.7
9	166.5	184.5	198.1	198.1	207.1	205.6	293.7	346.5
11	214.7	211.6	229.7	229.7	232.7	230.5	291.5	297.5
15	165.7	170.2	168.0	167.2	192.1	193.6	226.7	230.5
19	210.1	211.6	210.9	210.9	211.6	210.9	226.0	226.0
23	199.6	200.4	198.8	199.6	195.8	197.3	214.7	215.4
27	180.8	184.5	195.8	193.6	192.8	195.8	213.9	212.4
28	180.8	185.3	196.6	197.3	199.6	199.6	206.4	207.1

Table A.8 Effluent pH data for CDNB inhibited nitrifying reactor experiments.

Time	Control pH		IC_{15} pH		IC_{25} pH		IC_{50} pH	
days	1	2	1	2	1	2	1	2
0.25	7.89	7.84	7.74	7.76	7.76	7.8	7.99	8
0.5	7.81	7.83	7.74	7.73	7.89	7.9	7.96	7.96
0.75	7.86		7.93		7.99		8.05	
1	7.88	7.88	7.92	7.95	8.04	8.1	8.12	8.11
1.5	7.83		7.91		8.03		8.11	
2	7.87	7.87	7.91	7.91	8.08	8.1	8.2	8.19
2.5	7.83		7.84		8.02		8.13	
3	7.78	7.8	7.98	8	8.08	8.1	8.09	8.08
4	7.95	7.94	8.04	8.05	8.01	8	8.06	8.07
5	8.07	8	7.97	8.08	7.94	8.2	8.11	8.13
6	7.78	7.79	7.9	7.9	7.91	7.9	8.07	
7	7.98	7.9	7.97	7.96	8.07	8.1	8.09	8.1
8	7.81		7.85		7.92		7.94	
9	7.94	7.95	7.91	7.91	8.02	8	8.01	8.01
11	7.88	7.99	7.97	7.96	7.98	8	7.94	7.93
15	7.59	7.9	7.8	7.82	7.83	7.9	7.84	7.8
17	7.96		7.8		7.74		7.59	
19	8.11	8.12	8.07	8.11	7.98	8	7.9	7.94
21	7.81		7.96		7.88		7.67	
23	8.15	8.19	8	8.02	8.01	8	7.78	7.88
25	7.59		7.92		7.67		7.55	
27	7.96	7.99	7.96	7.89	7.68	7.7	7.68	7.7
28	7.95	8.11	8.08	8.09	7.94	8	7.93	7.94

Table A.9 Effluent CDNB concentrations for CDNB inhibited nitrifying reactor experiments.

	Control		IC_{15}		IC_{25}		IC_{50}	
Time	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0	0	0						
0.25	0	0	0	0	5.463	5.763	65.233	64.923
0.5	0	0	0	0	0	0	41.255	41.391
0.75	0	0	0	0	0	0	23.55	23.778
1	0	0	0	0	0	0	8.629	8.744
1.5	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0

Table A.10 Actual calculated Solids Residence Time (SRT) for CDNB inhibited nitrifying reactor experiments.

Time days	**Control** SRT days	**IC_{15}** SRT days	**IC_{25}** SRT days	**IC_{50}** SRT days
1	8.7	8.0	7.7	6.4
2	9.3	8.8	8.7	7.1
3	8.5	8.5	8.4	6.4
4	4.7	9.0	9.0	6.6
5	6.5	8.8	8.7	7.2
6	8.8	9.0	9.4	8.0
7	7.9	9.1	9.1	8.7
8	8.6	9.2	9.0	9.0
10	9.0	9.4	9.5	9.0
12	8.3	8.8	8.4	8.2
14	8.7	8.6	9.6	9.5
16	8.9	9.2	9.4	9.3
18	9.3	8.9	9.3	5.6
20	8.3	8.3	9.0	2.4
22	9.2	9.4	9.4	3.4
24	8.5	8.3	9.7	2.7
26	7.8	8.4	7.9	8.8
28	9.5	9.8	6.6	10.0

Table A.11 MLSS data for cadmium inhibited nitrifying reactor experiments.

	Control MLSS			IC_{15} MLSS			IC_{25} MLSS			IC_{50} MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	1193	1147	1367	1253	1233	1407	1473	1413	1433	1187	1233	1327
0.75	1260	1200	1333	1233	1260	1253	1107	1060	1080	987	1020	1007
1.25	1233	1200	1187	1227	1233	1253	1213	1120	1140	1047	1007	
2.25	1233	1227	1193	1180	1273	1180	1020	1020	967	913	947	993
2.75	1252	1320	1324	1247	1240	1233	1220	1127	1160	1200	1153	1173
3.75	1287	1293	1247	1213	1260	1227	1153	1173	1093	933	960	940
5	1220	1220	1260	1267	1213	1227	1260	1553	1260	1140	1213	1160
5.75	1080	1053	1167	1047	1007	1027	1113	1087	1107	980	960	947
7.25	1093	1107	1133	1233	1187	1213	1160	1173	1087	1200	1133	1520
8	1160	1027	1107	1333	1173	1173	1100	1127	1080	947	980	947
8.75	1013	1020	1007	1027	1047	1040	1093	1280	1027	973	913	967
11.25	984	1036	1040	967	1020	992	987	1047	1000	1013	967	1113
13	1127	1247	1120	1093	1153	1140	1187	1347	1200	980	1027	1127
15	1100	1173	1067	1120	1133	1133	1067	1147	1207	1073	1053	1093
17	1196	1220	1156	1107	1180	1200	1227	1173	1100	1120	1087	1113
19	1307	1253	1253	1287	1313	1313	1260	1340	1253	1180	1213	1120
21	1420	1353	1360	1380	1353	1373	1347	1327	1373	1440	1287	1333
23	1580	1407	1427	1367	1447	1520	1353	1440	1320	1413	1307	1233
25	1447	1480	1453	1587	1507	1547	1547	1593	1567	1460	1307	1460
27	1507	1433	1460	1427	1427	1387	1553	1593	1487	1427	1480	1480
29.75	1567	1640	1647	1620	1560	1553	1540	1487	1487	1453	1427	1467

Table A.12 MLVSS data for cadmium inhibited nitrifying reactor experiments.

	Control MLVSS			IC_{15} MLVSS			IC_{25} MLVSS			IC_{50} MLVSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	953	900	1067	980	953	1093	1173	1107	1120	907	927	1000
0.75	980	953	1087	993	1013	993	860	867	887	747	787	787
1.25	980	960	953	947	993	993	947	880	913	773	760	
2.25	987	1000	980	893	953	973	800	820	747	727	747	820
2.75	1004	1072	1084	1000	1020	1027	1000	927	960	973	933	953
3.75	1047	1053	993	1007	1027	1007	960	947	900	760	753	773
5	980	987	1027	1033	953	987	1033	1273	1027	913	993	940
5.75	853	820	927	820	760	780	867	867	887	787	740	740
7.25	860	933	900	907	907	933	880	880	833	927	893	1113
8	947	827	907	1067	953	953	860	933	873	760	793	760
8.75	907	880	873	927	900	907	933	1140	907	827	760	773
11.25	824	860	860	787	820	808	807	840	793	813	793	900
13	980	1073	993	947	1000	993	993	1153	1020	840	893	993
15	947	1013	927	967	953	953	920	1007	1053	907	880	927
17	1020	1032	988	913	1007	1020	1040	1020	947	933	907	953
19	1127	1087	1093	1107	1127	1120	1107	1153	1087	1000	1027	933
21	1227	1153	1160	1173	1160	1160	1167	1100	1173	1227	1047	1093
23	1433	1273	1300	1207	1320	1353	1247	1313	1173	1280	1147	1107
25	1253	1287	1233	1360	1300	1313	1300	1367	1333	1233	1113	1247
27	1293	1193	1247	1227	1213	1173	1300	1360	1233	1200	1240	1253
29.75	1353	1400	1413	1393	1320	1333	1313	1273	1267	1240	1240	1260

Table A.13 Effluent nitrate data for cadmium inhibited nitrifying reactor experiments.

Time	Control Nitrate		IC_{15} Nitrate		IC_{25} Nitrate		IC_{50} Nitrate	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		23.434	16.717	16.666	15.489	15.451	15.581	15.577
0.5	18.113	18.105	13.211	13.209	11.205	11.175	12.113	12.139
0.75	17.446	17.457	11.626	11.603	8.843	8.901	7.181	7.169
1		14.595	12.470	12.450	8.197	8.163	5.917	5.909
1.25	16.559	16.563	15.003	14.985	0.512	0.512	4.939	4.842
1.75	15.162	15.220	13.885	13.882	10.315	10.120	4.106	4.079
2.25	14.433	14.411	12.854	12.871	11.438	11.577	5.384	5.393
2.75	0.512	0.512			8.788	8.849	6.938	6.953
3.75	12.135	12.439	11.193	11.228	7.910	7.972		8.464
4.75					13.007	12.998	10.619	10.652
5.75	19.952	19.909	17.760	17.738	18.260	18.245	12.578	12.498
6.75	20.407	20.443	19.156	19.150	18.233	18.255	13.152	13.173
7.75	20.622	20.971	20.057	20.063	19.252	19.242	15.245	15.193
8.75	18.118	18.129	17.157	17.153	12.921	12.918	13.987	13.987
10.75	18.666	18.684	17.750	17.747	18.358	18.369	19.036	19.039
12.75	27.536	27.589	19.303	19.311	21.828	21.849	21.543	21.539
14.75	17.145	17.140	23.044	23.043	22.222	22.214	23.750	23.720
16.75	26.954	26.972	23.700	23.706	26.642	26.602	26.192	26.178
18.75	24.202	24.489	21.902	21.751	24.040	23.989	24.613	24.578
20.75	20.861	20.940	20.183	20.426	22.718	22.628	20.533	20.486
22.75	20.035	20.006	16.301	16.347	26.496	26.596	21.147	21.171
24.75	24.182	24.162	26.786	27.072	24.060	24.163	28.404	28.077
26.75	23.111	23.084	25.418	25.308	22.646	22.700	23.468	23.604

Table A.14 Effluent nitrite data for cadmium inhibited nitrifying reactor experiments.

Time	Control Nitrite		IC_{15} Nitrite		IC_{25} Nitrite		IC_{50} Nitrite	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		5.34	0.214	0.207	0.238	0.232	0	0
0.5	0.000	0.210	0.219	0.250	0.000	0.000	0.000	0.000
0.75	0.257	0.243	0.323	0.329	0.241	0.287	0.000	0.000
1	0.000	0.220	0.590	0.584	0.269	0.285	0.000	0.000
1.25	0.259	0.255	0.438	0.428	0.000	0.000	0.233	0.216
1.75	0.299	0.292	0.000	0.000	0.432	0.414	0.229	0.230
2.25	0.305	0.241	0.000	0.000	0.437	0.458	0.330	0.370
2.75	0.000	0.000			0.000	0.000	0.460	0.497
3.75	0.223	0.204	0.000	0.000	0.000	0.000	0.000	0.398
4.75					0.319	0.332	0.631	0.733
5.75	0.313	0.283	0.237	0.280	0.238	0.297	0.639	0.611
6.75	0.301	0.299	0.239	0.274	0.275	0.275	0.727	0.760
7.75	0.261	0.273	0.234	0.216	0.254	0.262	1.130	1.085
8.75	0.206	0.200	0.170	0.171	0.185	0.189	0.220	0.219
10.75	0.180	0.189	0.158	0.157	0.166	0.169	0.203	0.202
12.75	0.223	0.224	0.000	0.168	0.163	0.163	0.207	0.204
14.75	2.583	2.584	0.000	0.000	0.000	0.000	0.000	0.000
16.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
18.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
24.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
26.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.15 Effluent ammonia data for cadmium inhibited nitrifying reactor experiments.

Time	Control Ammonia		IC_{15} Ammonia		IC_{25} Ammonia		IC_{50} Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.386	0.458	3.644	3.018	3.789	3.483	3.017	3.730
0.5	0.232	0.223		0.492	5.609	5.624	6.491	6.538
0.75	0.158	0.084	5.778	4.695	7.600	6.734		
1	0.192	0.148		2.728	6.005	6.332	8.567	10.523
1.25	0.247	0.242	0.768	0.789	6.586	6.719	5.988	8.620
1.75	0.619	0.130	0.046	0.050	2.862	3.339	9.012	8.867
2.25	0.375	0.303	0.255	0.320	0.782	0.629	7.317	6.834
2.75	3.105	1.697			0.245	0.355		2.128
3.75	0.223	0.198	0.140	0.190	0.098	0.094	0.119	0.149
4.75					0.740	0.625	0.361	0.305
5.75			0.739	0.536	0.629		2.950	2.919
6.75	0.577	0.377	0.185	0.151	0.284	0.230		2.881
7.75	0.353	0.225	0.201	0.262	0.288	0.266	1.512	1.530
8.75	0.359	0.175	0.135	0.182	0.227	0.259	0.152	0.170
10.75	0.399	0.282	0.181	0.212	0.289	0.291	0.310	0.130
12.75	0.281	0.178	0.185	0.209		0.209	0.206	0.197
14.75	2.822	2.109	0.232	0.343	0.281	0.401	0.222	0.483
16.75	0.438	0.293	0.245	0.270	0.416	0.132	0.106	0.209
18.75	0.236	0.177	0.265	0.184	0.135	0.150	0.185	0.392
20.75	0.582	0.184	0.143	0.144	0.336	0.228	0.440	0.421
22.75	0.151	0.283	0.251	0.332	0.424	0.480	0.214	0.227
24.75	0.537	0.408	0.419	0.428	0.187	0.185	0.242	0.296
26.75	0.166	0.146	0.038	0.149	0.246	0.253	0.129	0.131
29.75	0.164	0.264	0.269	0.278	0.235	0.238	0.113	0.261

Table A.16 Effluent alkalinity data for cadmium inhibited nitrifying reactor experiments.

	Control $CaCO_3$			IC_{15} $CaCO_3$			IC_{25} $CaCO_3$			IC_{50} $CaCO_3$		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	69.1	75.6	57.1	61.9	61.1	59.5	61.9	60.3	60.3	49.0	53.9	45.8
0.75	65.1	53.9	53.1	84.4	89.2	87.6	104.5	105.3	104.5	111.0	104.5	105.3
1.25	69.1	71.6	64.3	64.3	65.1	70.8	112.6	113.4	100.5	132.7	129.4	130.2
2.25	72.4	68.3	68.3	74.8	76.4	76.4	83.6	84.4	83.6	128.6	128.6	123.8
3.75	72.4	71.6	73.2	80.4	77.2	78.8	93.3	89.2	92.5	93.3	92.5	95.7
5.75	49.0	48.2	50.7	56.3	55.5	57.1	99.7	61.9	59.5	88.4	84.4	86.8
7.75	41.0	40.2	37.8	40.2	39.4	41.8	43.4	43.4	44.2	61.1	59.5	61.9
10.75	12.9	14.5	13.7	25.7	24.1	24.9	20.1	17.7	19.3	24.1	24.9	24.1
14.75	10.5	7.2	4.8	6.4	6.4	5.6	6.4	4.8	7.2	8.0	7.2	7.2
18.75	13.7	15.3	15.3	20.9	20.9	21.7	18.5	15.3	15.3	15.3	14.5	15.3
22.75	28.1	25.7	28.1	44.2	41.8	40.2	33.8	32.2	33.0	28.1	28.1	27.3
26.75	22.5	21.7	23.3	20.1	19.3	19.3	24.9	26.5	26.5	24.9	24.9	24.1
29.75	7.2	13.7	8.8	8.0	4.8	6.4	6.4	5.6	7.2	10.5	8.8	9.6

Table A.17 Effluent pH data for cadmium inhibited nitrifying reactor experiments.

Time	Control pH			IC_{15} pH			IC_{25} pH			IC_{50} pH		
days	1	2	3	1	2	3	1	2	3	1	2	3
0.25	7.60	7.85	7.70	7.57	7.70	7.86	7.83	7.60	7.84	7.65	7.90	7.71
0.50	7.62			7.89			7.93			7.97		
0.75	7.78	7.65	7.83	7.92	8.00	7.82	7.67	7.75	7.75	7.8	7.74	7.77
1	7.75			7.64			7.98			8.07		
1.25	7.92	7.97	7.92	7.75	7.7	7.65	7.8	7.7	7.7	7.78	7.77	7.75
1.75	7.8			7.82			7.92			8.13		
2.25	7.78	7.99	7.9	7.96	7.94	7.97	7.85	7.8	7.9	7.93	7.77	7.71
2.75	7.79						8.01			8.01		
3.75	7.79	7.84	7.98	7.95	7.92	7.95	8.1	7.8	7.7	7.78	7.96	7.72
5.75	7.94	7.72	7.85	7.76	7.65	7.74	7.89	7.7	7.7	7.89	8.14	7.83
7.75	7.59	7.41	7.6	7.45	7.53	7.55	7.66	7.6	7.7	7.69	7.74	7.97
10.75	7.19	7.09	7.03	7.22	7.37	7.27	7.03	7.1	7.2	7.37	7.3	7.38
14.75	6.4	6.41	6.3	6.1	6.17	6.17	6.21	6.1	6.2	6.4	6.4	6.44
18.75	6.92	7.23	7.03	7.22	7.11	7.2	6.96	7	7	7.26	7.08	7.05
22.75	7.26	7.23	7.2	7.36	7.45	7.76	7.43	7.5	7.4	7.16	7.2	7.24
26.75	7.18	7.18	7.19	7.05	7.06	7.08	7.4	7.3	7.2	7.3	7.34	7.42
29.75	6.71	6.66	6.64	6.34	6.39		6.4	6.4	6.4	6.63	6.65	6.59
29.75	7.60	7.85	7.70	7.57	7.70	7.86	7.83	7.60	7.84	7.65	7.90	7.71

Table A.18 Effluent cadmium concentrations for cadmium inhibited nitrifying reactor experiments.

	Control			IC_{15}			IC_{25}			IC_{50}		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	0.01	0.01	0.00	1.61	1.62	1.61	1.90	1.90	1.91	4.51	4.18	4.18
0.5	0.00	0.00	0.00	1.07	1.07	1.07	1.43	1.43	1.43	2.86	2.75	2.75
0.75	0.01	0.01	0.01	1.04	1.04	1.05	1.65	1.67	1.67	2.75	2.64	2.64
1	0.00	0.00	0.00	0.85	0.86	0.86	1.05	1.04	1.05	1.64	1.63	1.65
1.25	0.00	0.00	0.00	0.92	0.92	0.92	1.09	1.09	1.09	1.84	1.83	1.83
1.75	0.00	0.00	0.00	0.70	0.70	0.68	0.92	0.92	0.92	1.33	1.33	1.33
2.25	0.00	0.00	0.00	0.63	0.63	0.63	0.98	0.95	0.95	1.42	1.43	1.44
2.75	0.00	0.00	0.00				0.64	0.64	0.64	0.99	0.99	0.99
3.75	0.00	0.00	0.00	0.41	0.41	0.41	0.56	0.56	0.56	0.95	0.95	0.96
4.75							0.42	0.42	0.42	0.65	0.65	0.65
5.75	0.00	0.00	0.00	0.20	0.20	0.20	0.27	0.27	0.27	0.47	0.47	0.47
6.75	0.00	0.00	0.00	0.14	0.14	0.13	0.24	0.24	0.24	0.38	0.38	0.38
7.75	0.00	0.00	0.00	0.11	0.11	0.10	0.10	0.10	0.09	0.39	0.39	0.39
8.75	0.00	0.00	0.00	0.06	0.06	0.06	0.04	0.04	0.05	0.29	0.29	0.29
10.75	0.03	0.02	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.10	0.10	0.10
12.75	0.01	0.01	0.01	0.03	0.04	0.04	0.01	0.01	0.01	0.05	0.05	0.05
14.75	0.00	0.00	0.00	0.03	0.03	0.03	0.03	0.03	0.02	0.05	0.05	0.05
16.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01
18.75	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01
20.75	0.00	0.00	0.01	0.09	0.09	0.09	0.00	0.00	0.00	0.00	0.00	0.00
22.75	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.03	0.03	0.03
24.75	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27.75	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.00	0.02	0.01	0.01	0.02

Table A.19 Actual calculated Solids Residence Time (SRT) for cadmium inhibited nitrifying reactor experiments.

Time days	**Control** SRT days	**IC_{15}** SRT days	**IC_{25}** SRT days	**IC_{50}** SRT days
1	7.4	5.5	4.9	4.4
2	7.4	5.8	5.1	4.8
3	7.9	6.2	5.6	5.6
4	7.4	6.4	5.8	5.5
5	10.0	10.0	6.5	6.2
6	7.7	6.0	5.0	4.7
7	8.0	6.9	5.8	6.1
8	8.0	7.3	7.7	7.4
9	6.6	6.4	5.6	5.8
11	7.6	6.8	6.4	5.8
13	8.1	7.8	7.4	6.0
15	8.6	7.7	7.7	8.1
17	8.8	7.4	7.6	6.8
19	8.8	8.3	7.8	7.6
21	8.2	8.7	8.4	8.4
23	9.5	5.0	9.5	8.8
25	8.9	9.0	9.3	8.2
27	8.6	8.1	8.8	8.3
30	8.6	8.5	7.8	8.4

Table A.20 MLSS data for 1-octanol inhibited nitrifying reactor experiments.

	Control MLSS			IC_{15} MLSS			IC_{25} MLSS			IC_{50} MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.5	1224	1113	1100	1293	1260	1200	1333	1353	1353	1300	1273	1253
1.5	1473	1380	1387	1353	1347	1380	1533	1553	1567	1427	1473	1413
2	1380	1333	1400	1407	1447	1460	1460	1520	1553	1513	1573	1500
3	1347	1540	1433	1467	1520	1533	1533	1680	1573	1647	1573	1633
4	1527	1507	1493	1520	1547	1493	1540	1527	1540	1480	1560	1580
5.5	1740	1840	1740	1710	1810	1780	1690	1780	1760	1790	1790	1840
6.25	1520	1610	1530	1640	1710	1670	1640	1660	1590	1740	1680	1650
7	1500	1540	1513	1653	1647	1560	1633	1613	1573	1567	1653	1600
8	1547	1600	1633	1667	1567	1507	1513	1540	1533	1560	1647	1580
9	1647	1667	1633	1553	1593	1567	1540	1487	1533	1593	1547	1600
11	1567	1667	1567	1647	1553	1540	1440	1147	1420	1447	1473	1467

Table A.21 MLVSS data for 1-octanol inhibited nitrifying reactor experiments.

	Control MLVSS			IC_{15} MLVSS			IC_{25} MLVSS			IC_{50} MLVSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.5	1092	987	987	1160	1140	1073	1207	1213	1200	1160	1147	1107
1.5	1367	1273	1267	1253	1253	1307	1413	1467	1453	1340	1367	1307
2	1173	1140	1193	1213	1233	1260	1260	1320	1327	1307	1320	1287
3	1207	1333	1227	1273	1327	1333	1353	1367	1373	1440	1373	1427
4	1380	1327	1300	1320	1360	1287	1327	1353	1360	1320	1387	1387
5.5	1560	1620	1540	1540	1600	1590	1500	1550	1530	1560	1600	1600
6.25	1400	1470	1390	1430	1530	1480	1410	1480	1420	1530	1480	1490
7	1353	1387	1360	1473	1460	1400	1487	1447	1420	1413	1493	1453
8	1407	1433	1460	1493	1407	1367	1367	1373	1380	1393	1473	1420
9	1487	1487	1473	1400	1447	1427	1373	1340	1367	1427	1380	1440
11	1387	1493	1393	1460	1393	1387	1260	1013	1273	1320	1320	1320

Table A.22 Effluent nitrate data for 1-octanol inhibited nitrifying reactor experiments.

	Control Nitrate		IC_{15} Nitrate		IC_{25} Nitrate		IC_{50} Nitrate	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	3.265	3.854	3.095	3.348	2.272	3.387	3.408	2.687
0.5	3.059	3.159	0.383	0.382	0.397	0.395	0.723	0.912
0.75	1.659	1.892	0.371	0.370	0.375	0.374	0.391	0.390
1	0.898	0.844	0.415	0.398	0.411	0.429	0.392	0.393
1.25		0.726	0.140	0.118	0.044	0.045	0.042	0.048
1.75	0.755	0.746	0.304	0.283	0.261	0.045	0.062	0.062
2.25	3.785	3.714	3.216	3.248	2.480	2.512	0.095	0.094
2.75	3.765	4.744	4.086	4.057	4.170	4.204	3.256	3.204
3.75	4.385	7.150	4.576	5.166		5.162	3.406	4.308
5.25	11.486	12.863	7.412	9.114	7.144	7.689	5.057	5.881
6	11.342	13.243	8.662	9.482	7.797	8.366	5.138	5.761
6.75	3.434	3.964	2.553	2.945	2.180	2.864	2.487	3.002
7.75	6.768	7.155		5.840	4.882	5.158	5.285	5.488
8.75		11.553	0.193	0.192	5.735	6.017	6.035	6.062
10.75	14.900	14.872	8.035	8.012	8.431	8.450	6.467	6.483

Table A.23 Effluent nitrite data for 1-octanol inhibited nitrifying reactor experiments.

	Control Nitrite		IC_{15} Nitrite		IC_{25} Nitrite		IC_{50} Nitrite	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25			0.208	0.211	0.221	0.263	0.304	0.392
0.5	0.000	0.272	0.000	0.000	0.000	0.000	0.000	0.000
0.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.187	0.000	0.000	0.000	0.000	0.000	0.000
1.25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.25	0.000	0.000	0.152	0.135	0.000	0.000	0.000	0.000
2.75	0.149	0.151	0.198	0.168	0.149	0.170	0.277	0.279
3.75	0.286	0.296	0.303	0.323		0.350	0.523	0.588
5.25	0.292	0.287	0.267	0.313	0.301	0.288	0.409	0.418
6	0.246	0.296	0.259	0.266	0.267	0.320	0.387	0.384
6.75	0.237	0.243	0.214	0.254	0.224	0.243	0.295	0.259
7.75	0.240	0.273		0.241	0.243	0.233	0.348	0.399
8.75		0.234	0.212	0.219	0.261	0.255	0.258	0.331
10.75	0.257	0.250	0.265	0.232	0.236	0.240	0.276	0.284

Table A.24 Effluent ammonia data for 1-octanol inhibited nitrifying reactor experiments.

Time	Control Ammonia		IC_{15} Ammonia		IC_{25} Ammonia		IC_{50} Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	0.000	0.000	0.000	0.000	3.481	3.392
0.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000				0.000
1.25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.25		0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5.25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8.75	0.000	0.000	8.810	9.561	0.000	0.000	0.000	0.000
10.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.25 Nitrate Generation Rate (NGR) data for 1-octanol inhibited nitrifying reactor experiments.

Time	Control NGR	IC_{15} NGR	IC_{25} NGR	IC_{50} NGR
days	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)
0.5	2.664	1.735	1.608	0.972
1.5	2.266	1.231	1.956	0.752
2	2.237	1.396	1.385	0.801
4	1.767	1.642	1.590	0.917
6	2.602	2.504	2.658	1.622
8	3.354	2.396	2.312	1.759
11	2.374	2.218	2.627	2.196

Table A.26 Effluent alkalinity data for 1-octanol inhibited nitrifying reactor experiments.

Time	Control $CaCO_3$		IC_{15} $CaCO_3$		IC_{25} $CaCO_3$		IC_{50} $CaCO_3$	
	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	181.7	184.1	189.7	190.5	183.3	184.9	185.7	188.9
1	238.0	241.2	247.6	249.2	245.2	246.8	247.6	250.0
1.75					250.0	250.0	250.0	251.7
2.25	234.0	238.0	241.2	242.0				
2.75	239.2	234.8	241.2	240.8	238.8	238.0	242.4	245.2
3.75	231.6	234.0	241.2	234.8	237.2	237.2	236.4	236.4
6	198.6	197.8	213.1	213.1	217.1	216.3	225.9	225.9
7.75	225.1	222.7	236.4	234.0	236.4	236.4	229.9	
10.75	203.4	205.0	225.9	228.3	233.2	233.2	230.7	229.1

Table A.27 Effluent pH data for 1-octanol inhibited nitrifying reactor experiments.

Time	Control pH		IC_{15} pH		IC_{25} pH		IC_{50} pH	
days	1	2	1	2	1	2	1	2
0.25	8.04	8.03	7.91	7.90	8.11	7.95	7.57	7.40
1	8.12	8.10	8.12	8.21	8.14	8.11	8.12	8.13
1.75					8.10	8.18	8.26	8.24
2.25	8.30	8.17	8.13	8.15				
2.75	8.18	8.08	8.13	8.09	8.08	8.08	8.10	8.08
3.75	8.09	8.08	7.96	8.03	8.06	8.06	8.05	8.07
6	8.13	8.17	8.21	8.14	8.24	8.24	8.25	8.34
7.75	8.10	8.03	8.01	8.08	8.07	8.08	8.06	
10.75	8.22	8.16	8.19	8.18	8.21	8.20	8.16	8.22

Table A.28 Effluent octanol concentrations for 1-octanol inhibited nitrifying reactor experiments.

	Control		**IC_{15}**		**IC_{25}**		**IC_{50}**	
Time days	1 mg/L	2 mg/L	1 mg/L	2 mg/L	1 mg/L	2 mg/L	1 mg/L	2 mg/L
0	0.00	0.00						
0.25	0.00	0.00	0.00	0.00	0.00	0.00	11.5251	13.4167
0.5	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00
0.75	0.00	0.00	0.00	0.00	0.00	0.00	12.47	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table A.29 Actual calculated Solids Residence Time (SRT) for 1-octanol inhibited nitrifying reactor experiments.

Time days	**Control** SRT days	**IC_{15}** SRT days	**IC_{25}** SRT days	**IC_{50}** SRT days
1	7.9	7.7	8.1	7.1
2	7.1	7.4	7.9	7.7
3	8.1	7.8	7.9	8.3
4	8.1	7.1	7.8	8.4
6	8.8	8.3	8.2	8.8
7	9.6	8.7	8.4	8.9
8	7.9	8.1	7.8	8.4
9	8.9	7.9	8.6	9.2
11	9.0	9.5	9.4	9.4

Table A.30 MLSS data for pH inhibited nitrifying reactor experiments.

	Control MLSS			pH 5 MLSS			pH 9 MLSS			pH 11 MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	1380	1393	1407	1433	1420	1440	1453	1493	1460	1253	1240	1307
1.25	1524	1532	1552	1492	1504	1480	1640	1724	1660	1408	1340	1352
2.25	1340	1360	1340	1293	1280	1333	1360	1367	1427	1100	1127	1093
3.25	1293	1307	1280	1347	1367	1347	1387	1393	1453	1153	1193	1153
4.25	1328	1187	1233	1253	1240	1260	1247	1253	1207		1033	1033
5.25	1604	1720	1647	1707	1720	1767	1700	1773	1727	1573	1567	1587
6.25	1276	1308	1268	1332	1328	1348	1380	1368	1380	1312	1280	1304
7.25	1547	1600	1533	1707	1653	1633	1687	1673	1660	1633	1667	
8.25	1700	1767	1753	1773	1733	1827	1787	1847	1827	1887	1773	1807
9.25	1727	1747	1773	1913	1893	1967	1920	1880	1947	1907	1907	1893
11.25	1693	1680	1680	1840	1887	1960	1827	1740	1807	1887	1913	1847
13.25	1953	1880	1893	2120	2167	2067	2060	2020	2047	2133	2113	2160
15.25	2100	2207	2140	2453	2393	2453	2407	2400	2407	2380	2393	2373
17.25	1900	1940	1920	2220	2220	2173	2247	2207	2167	2333	2327	2267
19.25	1920	1920	1847	2280	2113	2267	2227	2207	2287	2407	2433	2460
21.25	1800	1740	1787	2033	2060	2107	2107	2107	2087	2260	2240	2300
23.25	1980	1880	1900	2420	2347	2373	2340	2300	2307	2513	2520	2460
25.25	1887	1980	1947	2107	2087	2120	2427	2407	2340	2587	2613	2620
30.25	2213	2193	2233	2393	2393	2440	2540	2487	2500	2253	2353	2327

Table A.31 MLVSS data for pH inhibited nitrifying reactor experiments.

	Control MLSS			pH 5 MLSS			pH 9 MLSS			pH 11 MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	1173	1200	1233	1273	1200	1287	1153	1240	1233	887	887	933
1.25	1208	1212	1236	1200	1224	1204	1268	1348	1292	1004	960	980
2.25	1133	1153	1153	1133	1127	1173	1153	1160	1200	887	887	873
3.25	1100	1100	1080	1153	1133	1147	1173	1180	1220	920	973	927
4.25	1076	920	980	987	987	1007	980	973	967	753	740	720
5.25	1324	1373	1313	1373	1413	1413	1347	1413	1400	1213	1227	1240
6.25	1116	1128	1104	1156	1156	1164	1200	1172	1184	1096	1072	1092
7.25	1293	1353	1253	1453	1373	1360	1333	1367	1313	1247	1273	1287
8.25	1453	1500	1493	1507	1467	1573	1500	1567	1553	1580	1493	1513
9.25	1473	1487	1527	1627	1613	1687	1613	1587	1673	1607	1593	1580
11.25	1447	1460	1453	1607	1620	1720	1580	1520	1547	1580	1620	1553
13.25	1673	1647	1660	1900	1880	1840	1807	1747	1753	1833	1840	1847
15.25	1773	1853	1807	2047	2013	2060	2013	1993	2033	1960	1993	1927
17.25	1647	1680	1660	1907	1927	1867	1920	1887	1873	1933	1947	1907
19.25	1700	1733	1613	1953	1820	2007	1900	1900	2013	2113	2053	2107
21.25	1587	1553	1567	1780	1793	1813	1800	1820	1813	1933	1920	1953
23.25	1733	1633	1680	2120	2053	2053	2040	2033	2000	2173	2167	2127
25.25	1573	1633	1640	1733	1733	1773	2020	1960	1920	2127	2153	2160
30.25	1887	1880	1913	2013	2027	2093	2160	2127	2127	1900	1987	1980

Table A.32 Effluent nitrate data for pH inhibited nitrifying reactor experiments.

Time	Control Nitrate 1	Control Nitrate 2	pH 5 Nitrate 1	pH 5 Nitrate 2	pH 9 Nitrate 1	pH 9 Nitrate 2	pH 11 Nitrate 1	pH 11 Nitrate 2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		21.944	16.231	17.905		24.318	24.032	15.729
0.5	21.684	24.059	19.673	24.641	24.949	24.531	11.223	10.860
0.75	11.970	17.580	17.582	23.718	13.375	18.749	5.365	8.135
1	8.120	19.212	18.707	20.899	17.484	20.533	20.505	5.767
1.5	17.564	23.828	16.402	20.287	16.117	21.072	2.973	2.968
2	12.516	16.257	16.361	16.185	12.019	17.251	17.273	1.364
2.5		20.916	13.753	17.706	14.258	18.325	0.804	0.833
3	17.327	19.546	14.396	18.617	16.944	18.713	0.000	0.000
4	15.025	15.817	10.842	13.283	10.343	11.754	0.822	0.893
5	12.297	14.700	9.227	13.070	7.709	9.551	0.646	0.000
6	9.482	13.722	13.645	12.445	6.289	7.377	0.000	0.000
7	21.262	46.868	20.292	34.098	33.036	20.891	0.000	0.000
8	13.187	16.114	12.467	13.768	5.346	8.022	0.872	1.005
9	6.010	9.026	4.182	5.445	4.218	4.704	0.000	0.305
11	10.092	13.365	7.573	11.279		4.454	7.717	8.891
13	14.692	15.648	12.651	12.724	7.678	7.882	5.018	4.727
15	25.424	25.364	21.141	12.914	17.201	17.105	13.985	13.884
17	12.354	12.185	12.527	15.297	14.118	9.743	9.382	11.455
19		12.340	9.704	12.899	12.738	14.154	16.255	18.464
21	24.540	24.606	19.204	19.316	14.781	14.837	19.915	19.927
23	7.686	9.491	7.990	6.836	6.388	7.024	7.692	10.187
25	11.635	15.440	11.491	13.277	10.724	11.522	11.439	16.011
27	10.141	12.872	8.527	10.682		8.297	13.078	14.634
30	5.935	7.040	7.050	6.725	5.202	6.812	14.113	14.296

Table A.33 Effluent nitrite data for pH inhibited nitrifying reactor experiments.

Time	Control Nitrite 1	Control Nitrite 2	pH 5 Nitrite 1	pH 5 Nitrite 2	pH 9 Nitrite 1	pH 9 Nitrite 2	pH 11 Nitrite 1	pH 11 Nitrite 2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		0.798	0.000	0.000		1.253	1.027	0.651
0.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.812	1.574
8	0.000	0.000	0.000	0.000	0.000	0.000	1.131	1.505
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.630
11	0.000	0.000	0.000	0.000	0.000		0.000	0.000
13	0.000	0.000	0.000	0.000	0.000	0.000	3.074	2.958
15	0.000	0.000	0.000	0.000	0.000	0.000	2.722	2.702
17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
30	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.34 Effluent ammonia data for pH inhibited nitrifying reactor experiments.

Time	Control Ammonia		pH 5 Ammonia		pH 9 Ammonia		pH 11 Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	5.060	6.301	0.000	0.000	5.652	10.701
0.5		0.000	1.605	1.451	0.000	0.000	11.292	17.078
0.75	3.745	0.000	0.300	0.000	0.006	0.000		13.328
1	0.000	0.000	0.000	0.000	0.000	0.000	14.401	17.712
1.5	0.000	0.000	0.000	0.000	0.000	0.000		17.262
2	4.706	0.000	0.000	0.000	0.000	0.000		13.329
2.5	0.000	0.000	0.000	0.000	0.000	0.000	19.381	23.879
3	0.000	0.000	0.000	0.000	0.000	0.000		19.093
4	0.000	0.000	0.000	0.000	0.000	0.000	17.293	22.616
5	0.000	0.000	0.000	0.000	0.000	0.000		23.401
6	1.720	2.748	0.000	0.000	0.000	0.000	19.086	21.943
7	1.602	0.000	0.000	0.000	0.000	0.000	22.482	25.355
8	0.000	0.000	0.000	0.000	0.000	0.000	22.249	26.270
9	0.000	0.000	0.000	0.000	0.000	0.000	14.985	17.297
11		0.000	0.000	0.000	0.000	0.000		
13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.35 Nitrate Generation Rate (NGR) data for pH inhibited nitrifying reactor experiments.

Time	Control NGR	pH 5 NGR	pH 9 NGR	pH 11 NGR
days	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)
0.5	2.952	2.221	1.722	0.551
1.25	2.112	2.455	2.354	0.404
2.25	3.443	3.321	1.913	0.258
3.25	1.508	1.803	2.305	0.092
5.25	2.445	1.843	1.494	0.028
7.5	2.804	2.266	2.171	0.074
9.25	1.002	1.207	0.825	0.063
13.25	2.640	2.491	1.903	0.346
17.25	1.992	2.083	2.086	1.667
21.25	2.747	3.534	2.776	3.884
25.25	2.284	2.945	2.133	2.205
30.25	1.760	1.460	2.034	3.069

Table A.36 Effluent alkalinity data for pH inhibited nitrifying reactor experiments.

Time	Control $CaCO_3$		pH 5 $CaCO_3$		pH 9 $CaCO_3$		pH 11 $CaCO_3$	
	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	190.6	188.3	21.8	20.7	263.6	259.9	542.3	504.6
0.5	177.8	180.8	33.9	30.9	232.0	236.5	558.1	561.1
1	179.3	175.5	82.9	80.6	203.4	205.6	438.4	436.9
2	82.1	82.1	52.7	50.5	97.2	97.2	278.7	271.9
3	110.0	109.2	106.2	106.2	125.8	126.5	299.8	298.3
5	165.7	162.7	168.7	168.0	182.3	180.0	298.3	300.5
7	161.9	163.4	166.5	166.5	185.3	184.5	323.9	321.6
9	204.9	205.6	218.4	219.2	221.4	218.4	308.8	308.8
11	195.8	195.8	207.1	207.1	213.9	211.6	199.6	200.4
15	123.5	125.0	134.1	130.3	147.6	149.1	150.6	152.1
19	138.6	131.8	149.9	149.9	167.2	166.5	146.5	168.7
23	192.1	192.1	204.1	203.4	205.6	205.6	193.6	192.8
27	150.6	154.4	169.5	173.2	175.5	177.8	180.8	169.5
30	195.1	196.6	207.1	206.4	213.2	210.9	192.8	192.1

Table A.37 Effluent pH data for pH inhibited nitrifying reactor experiments.

Time	Control pH		pH 5 pH		pH 9 pH		pH 11 pH	
days	1	2	1	2	1	2	1	2
0.25	7.89	7.89	6.56	6.54	8.11	8.1	9.82	9.82
0.5	8.02	8.02	6.71	6.71	8.12	8.1	8.8	8.78
0.75	7.96		7.23		8.05		8.31	
1	7.96	7.95	7.5	7.56	8.06	8.1	8.56	8.5
1.5	7.68		7.36		7.81		8.37	
2	7.74	7.77	7.55	7.44	7.84	7.9	8.29	8.23
2.5	7.38		7.35		7.54		8.18	
3	7.84	7.74	7.64	7.67	7.75	7.7	8.2	8.21
4	7.84		7.79		7.81		8.17	
5	7.89	7.95	7.89	7.9	7.94	7.9	8.21	8.24
6	7.96		7.92		7.87		8.12	
7	8.12	8.04	8.07	8.08	8.21	8.2	8.2	8.16
8	7.88		7.92		7.9		7.94	
9	8.11	8.11	8.14	8.17	8.12	8.1	8.07	8.11
11	8.06	8.05	8.05	8.08	8.09	8.1	8.09	8.11
15	7.92	7.88	7.94	7.54	7.86	7.8	8	8.08
17	8.03		7.89		7.82		8.01	
19	7.97	7.97	8	7.95	8	7.9	8	7.97
21	7.76		7.78		7.78		7.83	
23	7.83	7.99	8.03	7.99	8.39	8.2	8.15	8.14
25	8		7.91		7.88		7.92	
27	8.18	8.2	8.11	8.16	8.12	8.1	8.05	8.03
30	8.1	8.07	8	8.04	8.16	8.1	8.12	8.12

Table A.38 Actual calculated Solids Residence Time (SRT) for pH inhibited nitrifying reactor experiments.

Time days	**Control** SRT days	**pH 5** SRT days	**pH 9** SRT days	**pH 11** SRT Days
1	6.0	4.9	7.0	3.1
2	7.2	7.2	7.4	5.4
3	7.3	7.1	7.4	6.0
4	9.6	9.7	9.5	9.5
5	6.7	6.9	7.6	7.5
6	5.3	5.6	6.1	5.8
7	7.1	7.4	7.8	8.5
8	8.0	8.0	8.5	8.5
10	8.2	8.5	8.6	8.5
12	8.2	8.7	9.0	9.2
14	8.0	8.4	8.5	8.9
16	8.2	8.9	8.6	9.4
18	8.5	8.8	8.6	9.5
20	8.2	8.8	8.4	9.9
22	8.6	8.8	8.6	9.6
24	9.3	9.8	9.8	9.8
26	9.2	9.0	9.3	9.1
28	9.2	8.8	9.5	10.0

Table A.39 MLSS data for zinc-cyanide complex inhibited nitrifying reactor experiments.

	Control MLSS			IC_{15} MLSS			IC_{25} MLSS			IC_{50} MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	4950	5130	5300	5270	5270	5240	5390	5250	5280	5680	5330	5330
1.25	4650	5480	5310	5490	5530	5440	5540	5420	5520	5770	5830	5730
2.25	4510	4520	4370	4820	4570	4440	4670	4660	4620	4590	4640	4610
3.25	4260	4390	4440	4430	4360	4450	4560	4560	4550	4570	4500	4510
4.25	4040	4130	3910	4030	4150	4120	4180	4250	4260	4290	4250	4110
5.25	3720	3800	3850	3930	3950	3980	4000	4070	4030	4220	4150	4150
6.25	3790	3640	3630	3580	3280	3550	3600	3580	3590	3660	3720	3740
7.25	3430	3470	3510	3330	3250	3270	3530	3430	3500	3550	3610	3610
8.25	3940	3450	3360	3190	3230	3290	3370	3300	3330	3410	3530	3340
9.25	3380	3410	3450	3560	3530	3550	3650	3610	3680	3590	3530	3670
11.25	3240	3350	3410	3280	3220	3290	3380	3270	3400	3410	3330	3390
13.25	3220	3150	3180	2940	2880	3020	3210	3250	3060	3090	3120	3230

Table A.40 MLVSS data for zinc-cyanide complex inhibited nitrifying reactor experiments.

	Control MLVSS			IC_{15} MLVSS			IC_{25} MLVSS			IC_{50} MLVSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	3220	3380	3510	3460	3490	3420	3520	3470	3440	3770	3460	3460
1.25	3040	3560	3380	3530	3560	3460	3500	3450	3530	3690	3710	3670
2.25	2900	2900	2790	2910	3250	2870	3020	3010	2940	2950	2990	2990
3.25	2840	2910	2950	3070	2860	2930	2990	3000	2970	3020	2960	2950
4.25	2730	2760	2630	2650	2730	2710	2760	2810	2770	2780	2750	2700
5.25	2440	2460	2520	2550	2580	2570	2570	2650	2660	2770	2730	2740
6.25	2350	2370	2340	2320	2040	2320	2360	2310	2340	2380	2420	2440
7.25	2290	2310	2380	2210	2170	2190	2340	2280	2320	2360	2390	2390
8.25	2730	2280	2190	2070	2110	2180	2220	2190	2210	2240	2290	2190
9.25	2240	2280	2280	2330	2330	2350	2400	2390	2380	2350	2320	2410
11.25	2160	2240	2290	2200	2180	2230	2290	2220	2290	2280	2240	2290
13.25	2220	2110	2050	2020	1970	2030	2130	2190	2040	2070	2080	2200

Table A.41 Effluent nitrate data for zinc-cyanide complex inhibited nitrifying reactor experiments.

	Control Nitrate		**IC_{15} Nitrate**		**IC_{25} Nitrate**		**IC_{50} Nitrate**	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		13.726	2.591	2.882	0.961	1.132	0.000	0.000
0.5	17.654	17.303	12.581	12.869		2.526	0.730	0.721
0.75	21.010	19.980	16.715	16.551	16.521	9.849	1.834	1.941
1	17.847	19.940	14.291	17.427	10.391	12.036	11.291	8.336
1.5	20.092	25.011	17.112	21.419	11.230	15.669	10.030	13.456
2	13.457	25.521	15.380	23.332	11.912	18.983	16.299	18.394
2.5	23.685	23.659	26.137	26.347	18.943	18.948	19.659	19.592
3	14.127	17.376	24.612	23.726	14.460	17.263	14.790	24.742
4	6.270	7.402	14.011	15.031	7.735	8.081	7.103	8.222
5	13.065	15.676	18.203	21.956	14.534	18.519	17.831	16.905
6	15.189	19.806	22.576	26.203	19.347	23.275	18.391	22.566
7	15.601	20.836	26.872	29.103	17.241	22.874	20.370	22.158
8	11.892	15.261	17.486	24.229	17.371	18.473	16.620	20.588
9	14.553	19.669	8.737	11.433	12.742	15.569	12.985	16.633
11	6.484	7.971	13.770	15.856	9.710	11.357	10.331	11.221
13	17.306	18.276	23.058	23.550	18.258	20.637	20.273	20.694

Table A.42 Effluent nitrite data for zinc-cyanide complex inhibited nitrifying reactor experiments.

	Control Nitrite		**IC_{15} Nitrite**		**IC_{25} Nitrite**		**IC_{50} Nitrite**	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	8.336	9.303	8.392	10.044	0.000	0.000
0.5	0.000	0.000	0.000	0.000		6.816	3.443	3.187
0.75	0.000	0.000	0.000	0.000	0.000	0.000	14.980	15.923
1	0.000	0.000	0.000	0.000	0.000	0.000	5.547	4.505
1.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.43 Effluent ammonia data for zinc-cyanide complex inhibited nitrifying reactor experiments.

Time	Control Ammonia		IC_{15} Ammonia		IC_{25} Ammonia		IC_{50} Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	0.000	0.000	0.000	0.000	8.496	10.633
0.5	0.000	0.000	0.000	0.000	0.000	0.000	11.097	13.305
0.75	0.000	0.000	0.000	0.000	0.000	0.000		3.345
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.44 Nitrate Generation Rate (NGR) data for zinc-cyanide complex inhibited nitrifying reactor experiments.

Time	Control NGR	IC_{15} NGR	IC_{25} NGR	IC_{50} NGR
days	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)
0.5	3.526	1.073	0.166	0.005
1.25	1.866	0.000	0.088	0.043
2.25	2.026	1.665	1.689	1.389
3.25	2.480	2.103	2.129	1.946
5.25	1.953	1.347	1.512	1.141
7.25	1.205	0.993	0.934	0.548
9.25	1.592	1.759	1.499	1.193
13.25	1.424	2.075	1.455	2.630

Table A.45 Effluent alkalinity data for zinc-cyanide complex inhibited nitrifying reactor experiments.

Time	Control $CaCO_3$		IC_{15} $CaCO_3$		IC_{25} $CaCO_3$		IC_{50} $CaCO_3$	
	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	253.9	253.0	267.7	267.7	280.6	285.2	344.1	345.0
0.5	230.0	230.9	253.0	251.2	273.2	272.3	340.4	336.7
1	194.1	191.4	201.5	187.7	224.5	215.3	225.4	234.6
2	150.0	147.2	175.7	169.3	216.2	207.0	207.0	201.5
3	274.2	276.9	240.1	241.0	279.7	280.6	276.0	272.3
5	234.6	238.3	216.2	216.2	244.7	245.6	236.4	243.8
7	170.2	171.1	163.8	156.4	168.4	174.8	182.2	193.2
9	217.1	219.0	253.0	252.1	234.6	235.5	236.4	238.3
11	242.9	247.5	213.4	211.6	239.2	233.7	235.5	231.8

Table A.46 Effluent pH data for zinc-cyanide complex inhibited nitrifying reactor experiments.

Time	Control pH		IC_{15} pH		IC_{25} pH		IC_{50} pH	
days	1	2	1	2	1	2	1	2
0.25	7.96	7.98	8.04	8.04	8.00	7.97	8.08	8.14
0.5	7.86	7.87	7.89	7.94	7.98	7.96	7.96	7.94
0.75	7.88		7.96		7.94		7.74	
1	8.23	8.23	8.31	8.26	8.25	8.30	8.20	8.28
1.5	7.84		7.94		7.92		7.89	
2	8.00	8.02	8.14	8.13	8.07	8.04	8.05	8.01
2.5	8.00		8.04		8.02		8.05	
3	8.17	8.16	8.13	8.15	8.14	8.12	8.11	8.18
4	8.19		8.34		8.36		8.21	
5	8.04	8.01	8.08	8.04	8.05	8.13	8.07	8.06
6	7.89		7.92		7.87		7.83	
7	8.22	8.25	8.14	8.14	8.23	8.22	8.19	8.15
8	7.94		8.02		7.99		7.97	
9	8.11	8.13	8.19	8.19	8.10	8.15	8.08	8.18
11	8.09	8.09	8.11	8.18	8.23	8.23	8.14	8.16
13	7.81		8.04		8.16		8.07	

Table A.47 Actual calculated Solids Residence Time (SRT) for zinc-cyanide complex inhibited nitrifying reactor experiments.

Time days	**Control** SRT days	**IC_{15}** SRT days	**IC_{25}** SRT days	**IC_{50}** SRT Days
1	8.8	9.0	8.7	8.9
2	9.0	9.5	9.0	9.3
3	9.2	9.3	9.1	9.2
4	9.1	9.3	9.3	9.5
5	9.0	9.5	9.0	9.1
6	9.0	9.6	9.0	9.2
7	9.2	8.8	8.9	9.0
8	9.2	9.1	9.1	9.1
10	9.1	9.0	9.0	9.0
12	9.1	9.2	9.2	9.2

Table A.48 MLSS data for DNP inhibited nitrifying reactor experiments.

	Control MLSS			IC_{15} MLSS			IC_{25} MLSS			IC_{50} MLSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	2680	2727	2747	2620	2633	2600	2527	2580	2560	2567	2560	2567
0.75	2767	2780	2707	2527	2600	2453	2607	2580	2447	2447	2433	2413
1.25	2733	2760	2727	2387	2433	2440	2413	2387	2400	2387	2387	2400
1.75	2820	2840	2833	2407	2393	2380	2360	2367	2347	2207	2213	2220
2.25	2853	2853	2893	2313	2387	2353	2273	2413	2293	2253	2233	2253
2.75	2747	2760	2747	2160	2193	2133	2120	2233	2113	2007	1967	1960
3.25	2900	2773	2800	2147	2240	2140	2227	2260	2240	2140	2067	2093
4.25	2580	2653	2553	2100	2207	2167	2127	2140	2147	2073	2047	2013
5.25	2533	2460	2513	2027	2047	2053	2107	2067	2113	2067	2040	2040
6.25	2347	2367	2320	1973	1980	2020	2073	2040	2040	2027	1973	2040
7.25	2273	2227	2253	1893	1933	1913	1993	1933	1947	1940	1940	1967
8.25	1953	1900	1980	1787	1873	1787	1753	1840	1900	1880	1947	1907
9.25	1820	1940	1867	1787	1780	1847	1827	1827	1747	1840	1867	1873
11.25	1227	1200	1147	1628	1640	1593	1713	1660	1767	1733	1780	1767
13.25	727	733	720	1453	1467	1533	1633	1560	1533	1807	1740	1793
15.25	767	767		1467	1480	1660	1567	1707	1540	2027	1900	1960
17.25	1080	1313	1233	1687	1680	1647	1520	1540	1553	2213	2227	2240
19.25	672	676	688	916	924	956	924	960	956	1256	1272	1288
21.25	1067	1100		440		420	353	353	353	2120	2040	2147
23.25	1487	1460	1687							2613	2460	2653
25.25	587	860	927							1867	2140	2233
27.25	1287	1220	1253							2113	2133	2107
30.25	1964	1860	1900							2088	2020	2000

Table A.49 MLVSS data for DNP inhibited nitrifying reactor experiments.

	Control MLVSS			IC_{15} MLVSS			IC_{25} MLVSS			IC_{50} MLVSS		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.50	2080	2127	2140	2053	2060	2047	2013	2020	2013	1993	2000	2007
0.75	2107	2120	2087	1907	1980	1880	2027	1973	1867	1880	1873	1860
1.25	2140	2113	2120	1853	1900	1900	1900	1893	1880	1900	1873	1887
1.75	2220	2233	2220	1887	1880	1867	1847	1860	1860	1720	1747	1740
2.25	2240	2247	2273	1800	1873	1833	1800	1900	1813	1780	1767	1800
2.75	2300	2253	2253	1807	1813	1773	1860	1867	1747	1747	1660	1613
3.25	2280	2187	2207	1693	1740	1680	1767	1807	1767	1673	1613	1640
4.25	2033	2020	2007	1660	1727	1713	1673	1700	1687	1620	1593	1560
5.25	1907	1867	1927	1607	1593	1627	1667	1640	1693	1613	1607	1580
6.25	1853	1860	1840	1613	1593	1633	1713	1653	1667	1633	1593	1640
7.25	1740	1727	1780	1500	1547	1513	1580	1527	1573	1580	1573	1587
8.25	1500	1467	1493	1387	1453	1407	1387	1447	1480	1473	1507	1507
9.25	1440	1520	1480	1447	1427	1493	1473	1453	1400	1453	1500	1487
11.25	973	933	887	1296	1300	1260	1380	1353	1387	1387	1413	1407
13.25	613	613	607	1227	1207	1247	1353	1307	1300	1513	1473	1507
15.25	573	693	773	1280	1253	1247	1340	1340	1347	1640	1660	1653
17.25	973	1140	1040	1433	1407	1413	1333	1367	1387	1907	1940	1960
19.25	580	572	596	796	792	824	820	840	840	1072	1100	1108
21.25	907	927	927	400	353	367	340	327	340	1820	1760	1867
23.25	1227	1180	1347							2147	2060	2200
25.25	533	733	787							1600	1787	1873
27.25	1027	980	1027							1713	1740	1700
30.25	1568	1492	1500							1696	1628	1480

Table A.50 Effluent nitrate data for DNP inhibited nitrifying reactor experiments.

	Control Nitrate		IC_{15} Nitrate		IC_{25} Nitrate		IC_{50} Nitrate	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25		18.217	12.079	13.629	8.696	12.545	11.694	13.658
0.5	15.433	17.875	16.872	19.381	19.354	17.017	16.717	20.166
0.75	20.252	19.483	13.597	16.103	12.822	12.613	18.127	18.260
1	15.146	21.622	13.289	18.957	9.273	14.159	13.278	17.947
1.5	11.327	11.290	10.903	13.763	6.894	9.905		20.250
2	10.343	11.978	11.895	15.262	15.233	6.094	15.979	18.135
2.5	7.897	10.142	10.750	12.831	3.299	3.887		13.780
3	8.693	9.013	9.699	11.177	2.026	2.123	10.332	11.325
4	15.864	17.591	10.295	12.924	3.575	4.356	13.610	14.862
5	25.962	26.328	20.926	21.233	4.872	5.494		18.580
6	15.634	18.107	10.650	13.773		3.427	10.314	12.676
7	16.072	20.308	19.636	13.945	7.123	9.056	9.749	11.769
8	12.566	15.937	6.666	8.916	11.489	11.597	13.172	12.938
9	9.033	13.331	12.112	15.176	12.308	13.863	18.899	25.712

Table A.51 Effluent nitrite data for DNP inhibited nitrifying reactor experiments.

	Control Nitrite		IC_{15} Nitrite		IC_{25} Nitrite		IC_{50} Nitrite	
Time	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	0.000	0.000	0.000	0.000	2.485	2.829
0.5	0.000	0.000	0.000	0.000	0.000	0.000	6.347	7.628
0.75	0.000	0.000	0.000	0.000	0.000	0.000	8.558	8.595
1	0.000	0.000	0.000	0.000	0.000	0.000	5.229	6.930
1.5	0.000	0.000	0.000	0.000	0.000	0.000		0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000		0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.52 Effluent ammonia data for DNP inhibited nitrifying reactor experiments.

Time	Control Ammonia		IC_{15} Ammonia		IC_{25} Ammonia		IC_{50} Ammonia	
	1	2	1	2	1	2	1	2
days	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N	mg/L-N
0.25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A.51 Nitrate Generation Rate (NGR) data for DNP inhibited nitrifying reactor experiments.

Time	Control NGR	IC_{15} NGR	IC_{25} NGR	IC_{50} NGR
days	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)	(mg N/g MLVSS-hr)
0.5	2.285	1.862	0.804	1.333
1.25	2.529	2.814	1.878	1.335
2.25	1.973	2.433	2.210	1.443
3.25	2.030	2.390	2.637	1.730
5.25	2.036	2.538	2.750	2.567
7.25	2.097	2.235	2.135	2.871
9.25	2.000	1.764	1.433	1.881

Table A.52 Effluent alkalinity data for DNP inhibited nitrifying reactor experiments.

	Control $CaCO_3$		IC_{15} $CaCO_3$		IC_{25} $CaCO_3$		IC_{50} $CaCO_3$	
Time	1	2	1	2	1	2	1	2
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	215.0	218.1	212.0	212.7	222.7	220.4	198.9	201.2
0.5	161.3	165.1		174.3	206.6	188.2	154.4	160.5
1	155.9	160.5	156.7	155.1	175.1	178.2	145.9	143.6
2	198.1	202.8	186.6	184.3	222.7	226.6	176.6	177.4
3	188.2	195.8	192.0	193.5	226.6	227.3	192.0	192.8
5	154.4	154.4	172.8	172.0	230.4	229.6	176.6	177.4
7	177.4	180.5	207.4	206.6	217.3	212.7	203.5	205.8
9	149.8	150.5	186.6	188.9	172.8	172.8	180.5	181.2
11	135.9	136.7	174.3	173.6	150.5	152.1	169.0	169.7
15	133.6	144.4	176.6	175.9	152.8	154.4	176.6	176.6
19	107.5	107.5	188.9	190.5	169.0	169.0	177.4	182.0
23	144.4	145.9					230.4	232.7
27	235.0	217.3					226.6	234.2
30	152.8	156.7					142.8	145.9

Table A.53 Effluent pH data for DNP inhibited nitrifying reactor experiments.

Time	Control pH		IC_{15} pH		IC_{25} pH		IC_{50} pH	
days	1	2	1	2	1	2	1	2
0.25	8.22	8.20	8.04	8.00	7.93	7.97	7.91	7.92
0.5	8.36	8.35	7.98	7.98	8.08	8.20	8.09	8.11
0.75	7.66		7.65		7.65		7.53	
1	8.02	7.92	8.05	8.04	8.04	8.04	7.98	7.90
1.5	7.72		7.74		7.73		7.62	
2	7.95	8.14	8.10	8.13	8.27	8.29	8.18	8.16
2.5	8.33		8.37		8.34		8.16	
3	8.23	8.16	8.30	8.18	8.10	8.20	8.13	8.13
4	7.62		7.80		7.77		7.81	
5	7.85	7.90	7.89	7.98	8.17	7.93	8.00	8.04
6	7.67		7.82		7.73		7.80	
7	8.06	8.12	8.24	8.32	8.40	8.35	8.31	8.33
8	7.55		7.89		7.98		7.91	
11	7.54	7.31	7.74	7.69	7.91	7.96	7.93	7.82
13	7.56		7.75		7.86		7.89	
15	7.58	7.69	7.98	7.86	8.07	8.08	8.13	8.12
17	7.47		7.77		7.91		8.00	
19	7.42	7.54	7.93	7.96	7.98	7.99	8.09	8.10
21	7.39						7.81	
23	7.64	7.77					8.11	8.23
25	8.22						8.16	
27	8.08	8.21					8.25	8.24
30	7.96	7.99					7.96	7.97

Table A.54 Effluent DNP concentrations for DNP inhibited nitrifying reactor experiments.

	Control			IC_{15}			IC_{25}			IC_{50}		
Time	1	2	3	1	2	3	1	2	3	1	2	3
days	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0.25	0.00	0.00	0.00	17.86	20.12	20.17	41.11	41.51	41.52	101.46	102.53	102.52
0.5	0.00	0.00	0.00	14.83	14.92	14.83	30.78	31.25	30.88	79.26	79.80	80.19
0.75	0.00	0.00	0.00				22.69	23.07	22.97	60.26	61.29	61.55
1	0.00	0.81	0.88	8.34	8.49	8.49	17.34	17.24	17.16	46.52	41.58	38.92
1.5	0.00	0.00	0.00	5.06	5.12	5.14	9.70	9.75	9.82	28.16	28.39	28.57
2	0.00	0.00	0.00	3.12	3.14	3.08	5.46	5.51	5.44	16.46	14.56	13.92
2.5	0.00	0.00	0.00	1.41	1.48	1.41	2.86	2.88	2.89	9.55	9.66	9.69
3	0.00	0.00	0.00	1.07	0.79	0.76	1.48	1.48	1.51	5.58	5.54	5.57
4	0.00	0.00	0.00	0.27	0.30	0.29	0.54	0.54	0.55	2.04	1.47	1.68
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.37	0.47

Table A.55 Actual calculated Solids Residence Time (SRT) for CDNB inhibited nitrifying reactor experiments.

Time days	Control SRT days	IC_{15} SRT days	IC_{25} SRT days	IC_{50} SRT days
1	9.0	8.7	8.7	8.1
2	9.3	8.9	8.9	8.4
3	9.7	9.5	9.7	9.3
4	9.4	8.9	9.4	9.1
5	9.6	9.2	9.6	8.9
6	9.1	8.9	9.5	9.1

Appendix B: Data for Chapter 4

Table B.1 Raw data for Table 4.1. Values given are the absorbance at 229 and 235 nm for the listed nitrate concentrations.

Sample Replicate	7.1 mg/L NO_3^--N		7.11 NO_3^--N		7.15 NO_3^--N		7.25 NO_3^--N		7.6 NO_3^--N	
	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm
1	0.8387	0.3537	0.8372	0.3531	0.8202	0.3473	0.8343	0.3513	0.8258	0.3447
2	0.8387	0.3539	0.8374	0.3531	0.8199	0.3472	0.8338	0.3510	0.8252	0.3447
3	0.8385	0.3539	0.8372	0.3530	0.8197	0.3471	0.8338	0.3511	0.8252	0.3446
4	0.8387	0.3539	0.8377	0.3534	0.8197	0.3471	0.8335	0.3510	0.8255	0.3447
5	0.8387	0.3539	0.8374	0.3529	0.8199	0.3473	0.8335	0.3508	0.8252	0.3446
6	0.8387	0.3539	0.8372	0.3531	0.8194	0.3471	0.8332	0.3508	0.8258	0.3447
7	0.8387	0.3536	0.8369	0.3531	0.8197	0.3474	0.8335	0.3508	0.8258	0.3449

Sample Replicate	26.1 mg/L NO_3^--N		26.12 mg/L NO_3^--N		26.14 mg/L NO_3^--N		26.30 mg/L NO_3^--N		26.50 mg/L NO_3^--N	
	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm	229 nm	235 nm
1	1.7953	0.6720	1.7676	0.6620	1.7744	0.6575	1.7790	0.6664	1.7930	0.6660
2	1.8001	0.6711	1.7744	0.6615	1.7654	0.6576	1.7859	0.6664	1.7906	0.6655
3	1.7953	0.6708	1.7767	0.6620	1.7676	0.6567	1.7930	0.6667	1.7859	0.6649
4	1.7906	0.6710	1.7836	0.6618	1.7699	0.6571	1.7836	0.6667	1.7882	0.6654
5	1.7977	0.6720	1.7767	0.6615	1.7676	0.6570	1.7906	0.6667	1.7859	0.6652
6	1.8026	0.6725	1.7676	0.6613	1.7721	0.6571	1.7859	0.6662	1.7836	0.6657
7	1.7977	0.6720	1.7836	0.6620	1.7654	0.6573	1.7882	0.6665	1.7859	0.6651

Table B.2 Raw data for Figures 4.1 and 4.2. Values listed are absorbance at the different wavelengths.

Wavelength nm	Distilled H_2O	9.33 mg/L NO_3^--N	15.33 mg/L NO_3^--N	25.33 mg/L NO_3^--N	35.33 mg/L NO_3^--N	55.33 mg/L NO_3^--N
200	-0.0006	3.0028	2.7018	2.8267	3.3038	2.9059
201	-0.0002	3.1822	2.6180	2.9604	2.6593	3.0573
202	-0.0010	2.8456	2.9126	2.8456	3.2136	2.9126
203	-0.0007	2.6075	3.2444	3.1194	2.9433	3.1194
204	-0.0010	2.7092	3.0516	2.9724	3.7506	3.4496
205	-0.0012	2.8765	3.0806	2.9345	2.9345	3.0014
206	-0.0006	2.9038	2.8526	2.8526	2.9038	3.2048
207	-0.0007	2.7501	2.9841	3.1303	3.2272	3.1303
208	-0.0002	3.0729	2.8511	3.0060	2.9480	2.8968
209	-0.0006	2.7916	2.8294	3.0926	3.1718	3.0926
210	-0.0001	2.8891	3.0440	2.8099	2.9348	2.8891
211	-0.0002	2.7942	2.9081	2.9081	2.8667	2.9081
212	-0.0007	2.6449	2.9694	3.2247	3.0786	3.0786
213	-0.0001	2.7343	2.9842	2.9842	3.0353	3.0353
214	-0.0006	2.7479	2.9978	2.9978	3.1739	3.1739
215	0.0001	2.8193	3.0112	2.8515	3.3634	3.0112
216	0.0001	2.7231	2.8992	2.8323	2.9370	3.0753
217	-0.0005	2.5912	2.8430	3.1440	3.1440	3.1440
218	0.0002	2.6021	2.8539	2.8539	2.8539	3.0969
219	-0.0001	2.4072	2.6870	2.8631	3.0550	3.2311
220	-0.0001	2.2328	2.7629	2.8720	3.1151	3.0639
221	0.0001	2.0546	2.5226	2.7725	2.8236	3.2496
222	-0.0004	1.8534	2.4018	2.8048	2.9560	3.0810
223	-0.0002	1.6845	2.2571	2.5953	2.8120	2.8120
224	0.0002	1.4973	2.0543	2.5448	2.7947	2.9038
225	0.0001	1.3056	1.8122	2.3764	2.6775	2.7566
226	0.0000	1.1534	1.6036	2.1724	2.4847	2.7028
227	0.0005	1.0069	1.4015	1.9351	2.3083	2.6404
228	-0.0006	0.8762	1.2178	1.7164	2.0959	2.4983
229	0.0003	0.7639	1.0575	1.4998	1.8784	2.3951
230	0.0002	0.6511	0.8942	1.2737	1.6126	2.1481
231	-0.0004	0.5633	0.7678	1.0879	1.3843	1.8891
232	0.0003	0.4892	0.6602	0.9315	1.1830	1.6423
233	-0.0004	0.4230	0.5645	0.7892	1.0020	1.3969
234	0.0003	0.3670	0.4827	0.6689	0.8456	1.1802
235	0.0002	0.3161	0.4099	0.5604	0.7032	0.9789
236	-0.0001	0.2763	0.3525	0.4756	0.5920	0.8169
237	0.0001	0.2425	0.3042	0.4045	0.4983	0.6827
238	0.0000	0.2157	0.2659	0.3473	0.4238	0.5734
239	0.0001	0.1911	0.2307	0.2946	0.3544	0.4724
240	0.0001	0.1714	0.2034	0.2549	0.3021	0.3970
241	0.0001	0.1568	0.1823	0.2233	0.2611	0.3363
242	-0.0003	0.1434	0.1646	0.1975	0.2270	0.2878
243	0.0004	0.1333	0.1501	0.1760	0.1994	0.2466
244	0.0002	0.1238	0.1370	0.1571	0.1749	0.2113
245	-0.0003	0.1166	0.1280	0.1438	0.1574	0.1864
246	0.0003	0.1114	0.1201	0.1326	0.1430	0.1657
247	-0.0001	0.1065	0.1138	0.1232	0.1317	0.1490
248	0.0001	0.1023	0.1087	0.1157	0.1218	0.1352
249	0.0004	0.0994	0.1039	0.1099	0.1141	0.1249
250	0.0001	0.0966	0.1006	0.1047	0.1082	0.1160
251	-0.0001	0.0939	0.0972	0.1011	0.1031	0.1094

252	0.0001	0.0926	0.0956	0.0978	0.0998	0.1042
253	0.0003	0.0907	0.0935	0.0953	0.0965	0.1000
254	-0.0002	0.0892	0.0915	0.0935	0.0933	0.0967
255	0.0003	0.0885	0.0907	0.0914	0.0921	0.0943
256	-0.0002	0.0870	0.0893	0.0900	0.0897	0.0918
257	-0.0004	0.0862	0.0880	0.0889	0.0883	0.0902
258	0.0004	0.0860	0.0877	0.0876	0.0881	0.0891
259	0.0001	0.0851	0.0868	0.0873	0.0868	0.0878
260	0.0001	0.0845	0.0862	0.0864	0.0864	0.0874
261	0.0003	0.0836	0.0852	0.0854	0.0854	0.0863
262	0.0001	0.0830	0.0848	0.0851	0.0848	0.0856
263	0.0000	0.0822	0.0839	0.0846	0.0842	0.0852
264	0.0002	0.0818	0.0837	0.0839	0.0840	0.0842
265	0.0005	0.0817	0.0832	0.0837	0.0835	0.0845
266	-0.0001	0.0801	0.0819	0.0825	0.0826	0.0837
267	0.0004	0.0802	0.0816	0.0824	0.0825	0.0833
268	-0.0005	0.0784	0.0804	0.0814	0.0813	0.0830
269	-0.0002	0.0780	0.0801	0.0810	0.0810	0.0828
270	0.0007	0.0782	0.0797	0.0810	0.0809	0.0828
271	-0.0003	0.0764	0.0787	0.0798	0.0803	0.0822
272	0.0004	0.0767	0.0784	0.0796	0.0804	0.0823
273	-0.0007	0.0752	0.0775	0.0789	0.0796	0.0817
274	0.0000	0.0743	0.0768	0.0778	0.0790	0.0816
275	0.0009	0.0744	0.0760	0.0779	0.0790	0.0815
276	-0.0001	0.0727	0.0754	0.0770	0.0783	0.0808
277	0.0001	0.0720	0.0740	0.0765	0.0774	0.0807
278	-0.0001	0.0712	0.0731	0.0758	0.0769	0.0806
279	-0.0001	0.0702	0.0725	0.0752	0.0770	0.0798
280	0.0000	0.0693	0.0713	0.0749	0.0763	0.0804
281	0.0003	0.0687	0.0714	0.0740	0.0763	0.0799
282	0.0002	0.0674	0.0703	0.0727	0.0757	0.0793
283	-0.0002	0.0667	0.0690	0.0727	0.0745	0.0793
284	0.0003	0.0661	0.0688	0.0719	0.0746	0.0794
285	0.0005	0.0654	0.0680	0.0713	0.0747	0.0791
286	0.0000	0.0641	0.0670	0.0714	0.0735	0.0793
287	0.0004	0.0632	0.0667	0.0704	0.0736	0.0791
288	-0.0003	0.0625	0.0655	0.0698	0.0727	0.0788
289	-0.0001	0.0621	0.0651	0.0696	0.0727	0.0789
290	0.0004	0.0614	0.0645	0.0690	0.0727	0.0794
291	0.0004	0.0610	0.0640	0.0684	0.0723	0.0791
292	-0.0001	0.0596	0.0630	0.0680	0.0716	0.0788
293	0.0002	0.0592	0.0621	0.0672	0.0712	0.0791
294	0.0004	0.0587	0.0617	0.0667	0.0712	0.0785
295	-0.0001	0.0573	0.0606	0.0662	0.0700	0.0780
296	0.0002	0.0574	0.0604	0.0657	0.0699	0.0782
297	0.0003	0.0564	0.0600	0.0648	0.0696	0.0778
298	-0.0002	0.0554	0.0589	0.0645	0.0687	0.0775
299	0.0006	0.0554	0.0587	0.0639	0.0683	0.0770
300	-0.0005	0.0537	0.0578	0.0631	0.0676	0.0761
301	-0.0003	0.0536	0.0570	0.0622	0.0671	0.0757
302	0.0004	0.0533	0.0567	0.0619	0.0667	0.0752
303	-0.0006	0.0522	0.0554	0.0607	0.0656	0.0742
304	-0.0001	0.0517	0.0548	0.0600	0.0647	0.0735
305	0.0004	0.0513	0.0544	0.0599	0.0642	0.0726
306	-0.0002	0.0500	0.0531	0.0583	0.0629	0.0711
307	-0.0001	0.0493	0.0522	0.0574	0.0619	0.0699
308	0.0003	0.0487	0.0517	0.0564	0.0607	0.0683

309	0.0004	0.0487	0.0513	0.0557	0.0596	0.0672
310	-0.0002	0.0467	0.0494	0.0543	0.0580	0.0649
311	0.0005	0.0469	0.0497	0.0535	0.0577	0.0640
312	0.0001	0.0461	0.0483	0.0523	0.0561	0.0622
313	-0.0005	0.0441	0.0471	0.0512	0.0545	0.0600
314	0.0005	0.0446	0.0469	0.0502	0.0537	0.0595
315	0.0004	0.0437	0.0456	0.0486	0.0518	0.0572
316	-0.0001	0.0426	0.0444	0.0475	0.0507	0.0550
317	0.0003	0.0423	0.0438	0.0469	0.0497	0.0540
318	-0.0001	0.0417	0.0431	0.0457	0.0481	0.0512
319	0.0004	0.0407	0.0424	0.0451	0.0473	0.0504
320	0.0001	0.0402	0.0413	0.0439	0.0456	0.0490
321	-0.0003	0.0393	0.0408	0.0429	0.0452	0.0472
322	0.0001	0.0383	0.0396	0.0413	0.0426	0.0443
323	-0.0002	0.0371	0.0387	0.0403	0.0416	0.0432
324	0.0005	0.0372	0.0377	0.0394	0.0408	0.0415
325	0.0000	0.0358	0.0374	0.0381	0.0400	0.0402
326	-0.0001	0.0356	0.0363	0.0372	0.0383	0.0392
327	0.0001	0.0353	0.0357	0.0372	0.0375	0.0381
328	0.0001	0.0341	0.0353	0.0359	0.0365	0.0367
329	0.0000	0.0339	0.0346	0.0353	0.0363	0.0357
330	0.0007	0.0341	0.0339	0.0352	0.0345	0.0348
331	0.0002	0.0334	0.0338	0.0338	0.0347	0.0339
332	0.0003	0.0327	0.0327	0.0338	0.0335	0.0333
333	-0.0001	0.0319	0.0321	0.0329	0.0335	0.0320
334	0.0003	0.0316	0.0315	0.0320	0.0326	0.0319
335	0.0000	0.0314	0.0312	0.0321	0.0317	0.0311
336	0.0004	0.0308	0.0315	0.0315	0.0318	0.0310
337	-0.0001	0.0300	0.0299	0.0303	0.0307	0.0299
338	-0.0002	0.0301	0.0304	0.0309	0.0308	0.0296
339	0.0000	0.0294	0.0294	0.0298	0.0302	0.0291
340	-0.0001	0.0290	0.0289	0.0291	0.0295	0.0277
341	-0.0002	0.0288	0.0292	0.0290	0.0296	0.0282
342	0.0000	0.0286	0.0282	0.0286	0.0290	0.0276
343	0.0002	0.0276	0.0279	0.0279	0.0279	0.0270
344	-0.0005	0.0273	0.0276	0.0279	0.0280	0.0269
345	0.0006	0.0268	0.0271	0.0274	0.0273	0.0262
346	0.0006	0.0267	0.0268	0.0274	0.0267	0.0258
347	-0.0002	0.0262	0.0259	0.0261	0.0267	0.0250
348	0.0001	0.0256	0.0256	0.0259	0.0256	0.0245
349	0.0004	0.0254	0.0254	0.0262	0.0258	0.0245
350	0.0003	0.0247	0.0245	0.0249	0.0258	0.0242
351	0.0002	0.0248	0.0251	0.0254	0.0248	0.0239
352	0.0001	0.0249	0.0246	0.0251	0.0246	0.0239
353	0.0003	0.0238	0.0240	0.0237	0.0248	0.0236
354	0.0007	0.0240	0.0240	0.0243	0.0244	0.0232
355	0.0000	0.0233	0.0235	0.0241	0.0240	0.0228
356	0.0009	0.0235	0.0233	0.0235	0.0241	0.0222
357	0.0006	0.0230	0.0236	0.0238	0.0232	0.0220
358	-0.0004	0.0217	0.0227	0.0229	0.0217	0.0223
359	0.0000	0.0211	0.0231	0.0223	0.0233	0.0209
360	0.0004	0.0218	0.0226	0.0222	0.0218	0.0216
361	-0.0002	0.0224	0.0206	0.0224	0.0228	0.0210
362	0.0004	0.0207	0.0205	0.0209	0.0229	0.0209
363	0.0006	0.0202	0.0202	0.0202	0.0205	0.0194
364	-0.0013	0.0204	0.0191	0.0196	0.0198	0.0185
365	0.0005	0.0203	0.0203	0.0205	0.0205	0.0194

366	-0.0003	0.0191	0.0185	0.0194	0.0198	0.0189
367	-0.0002	0.0194	0.0194	0.0199	0.0207	0.0183
368	0.0005	0.0188	0.0204	0.0205	0.0198	0.0193
369	0.0005	0.0194	0.0177	0.0191	0.0200	0.0184
370	-0.0002	0.0181	0.0184	0.0177	0.0194	0.0177
371	0.0006	0.0177	0.0189	0.0182	0.0186	0.0174
372	0.0000	0.0176	0.0164	0.0169	0.0179	0.0164
373	-0.0002	0.0168	0.0173	0.0173	0.0188	0.0163
374	-0.0005	0.0172	0.0164	0.0166	0.0174	0.0157
375	0.0003	0.0168	0.0180	0.0177	0.0185	0.0171
376	0.0000	0.0166	0.0168	0.0169	0.0169	0.0166
377	0.0003	0.0163	0.0157	0.0161	0.0174	0.0147
378	0.0000	0.0161	0.0161	0.0161	0.0171	0.0157
379	-0.0001	0.0154	0.0156	0.0157	0.0159	0.0152
380	0.0001	0.0145	0.0148	0.0153	0.0153	0.0138
381	0.0004	0.0158	0.0158	0.0153	0.0157	0.0151
382	-0.0005	0.0147	0.0142	0.0149	0.0144	0.0137
383	0.0007	0.0143	0.0152	0.0153	0.0157	0.0139
384	0.0000	0.0142	0.0148	0.0148	0.0151	0.0142
385	0.0001	0.0141	0.0136	0.0145	0.0144	0.0135
386	0.0006	0.0145	0.0140	0.0142	0.0154	0.0137
387	0.0007	0.0142	0.0134	0.0147	0.0143	0.0135
388	0.0003	0.0132	0.0127	0.0133	0.0144	0.0126
389	0.0001	0.0133	0.0133	0.0133	0.0142	0.0128
390	-0.0006	0.0125	0.0127	0.0126	0.0128	0.0123
391	0.0000	0.0122	0.0124	0.0134	0.0136	0.0114
392	0.0001	0.0127	0.0131	0.0130	0.0133	0.0126
393	0.0001	0.0126	0.0126	0.0126	0.0132	0.0118
394	0.0002	0.0116	0.0121	0.0124	0.0132	0.0112
395	-0.0004	0.0120	0.0116	0.0121	0.0122	0.0117
396	-0.0002	0.0118	0.0107	0.0119	0.0121	0.0110
397	0.0004	0.0116	0.0117	0.0115	0.0124	0.0109
398	-0.0004	0.0117	0.0109	0.0115	0.0121	0.0109
399	-0.0004	0.0113	0.0109	0.0115	0.0116	0.0104
400	0.0006	0.0112	0.0120	0.0115	0.0123	0.0111

Table B.3 Nitrite interference data for Figure 4.3. Values listed are absorbance at 229 nm for varying concentrations of nitrite.

Nitrite (mg/L NO_2^--N)	Mixed Liquor	Mixed Liquor + 5 mg/L NO_3^--N	Mixed Liquor + 15 mg/L NO_3^--N
0.5	0.6219	0.7589	1.1022
1	0.6519	0.8213	1.1496
1.5	0.7150	0.8689	1.1970
2	0.7659	0.9173	1.2536

Table B.4 Raw data for Table 4.2. Values listed are absorbance for varying concentrations of nitrite.

Time (minutes)	UV Measured Nitrate Concentration (mg/L NO_3^--N)				IC Measured Nitrate Concentration (mg/L NO_3^--N)			
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 1	Sample 2	Sample 3	Sample 4
0	10.228	10.314	10.738	10.875	10.171	10.429	10.195	10.784
10	10.990	10.945	11.827	11.983	11.089	10.911	11.565	11.685
20	11.727	11.781	12.931	12.790	11.908	11.717	12.450	
30	12.116	11.953	13.045	12.694	12.500	12.426	12.927	12.557
40	13.070	12.839	14.195	14.128	13.185	13.478	13.818	14.046
50	13.973	13.524	14.378	14.538	14.056	13.862	14.319	14.530

Table B.5 Raw data for Figure 4.4. Values listed are nitrate concentrations over time for mixed liquor.

Time (minutes)	Control Nitrate (mg/L-N)	30 mg/L Cd Nitrate (mg/L-N)
0	8.54	8.68
10	9.76	9.35
20	10.95	10.01
30	12.46	10.72
40	13.54	11.08
50	15.08	11.61

Table B.6 Raw data for Figure 4.5. Values listed are nitrate generation rates for mixed liquor.

Cadmium Concentration (mg/L)	Slope (mg NO_3^--N/L-min)	NGR (mg NO_3^--N/min-MLVSS)
0	0.138	0.074
0	0.136	0.073
1	0.128	0.069
1	0.143	0.077
5		
5	0.128	0.069
10	0.125	0.067
10	0.126	0.068
30	0.059	0.032
30	0.060	0.032
50	0.048	0.026
50	0.048	0.026

Table B.7 Nitrate reading interference caused by CDNB. Values show absorbance reading increase with CDNB concentration.

Added CDNB (mg/L)	Absorbance at 229 nm	Absorbance at 235 nm
1	1.4941	0.6148
10	1.9712	1.1179
30	2.7740	2.0734
50	4.0750	3.0277

Table B.8 Nitrate reading interference caused by chlorine bleach (NaOCl). Values show increase in nitrate generation rate (decreased inhibition) with increasing chlorine concentration.

Added free Cl_2 (mg/L)	NGR (mg NO_3^--N/min-MLVSS)	% Inhibtion
0	0.041	0
2	0.046	-13.3
10	0.057	-40.2
30	0.065	-60.3
50	0.100	-145.1
75	0.100	-145.5

Table B.9 Nitrate probe drift for standards after 1 hour of use.

Initial Standard Nitrite Concentration (mg/L-N)	Standard Nitrite Concentration after 1 hr (mg/L-N)	Standard Nitrite Concentration after 1.5 hr (mg/L-N)
9.33	11.38	12.49
15.33	17.84	18.42
25.33	28.75	30.15
35.33	39.95	42.87
55.33	65.89	67.65

Table B.10 Wavelength scans for comparison of nitrate and nitrite absorbance readings at 20 mg/L-N nitrate and nitrite.

Wavelength	Nitrite Sample: 20 mg/L-N		Nitrate Sample: 20 mg/L-N	
nm	Abs 1	Abs 2	Abs 1	Abs 2
200.0	2.6130	2.4533	2.4533	2.6642
201.0	2.5431	2.6223	2.7192	2.7772
202.0	2.7513	2.6544	2.7001	2.7001
203.0	2.6850	2.7308	2.5089	2.5089
204.0	2.7606	2.7148	2.5687	2.5387
205.0	2.5968	2.5968	2.7887	2.6290
206.0	2.5661	2.7288	2.7288	2.9920
207.0	2.8894	2.5883	2.6164	2.5883
208.0	2.9112	2.7351	2.6102	2.6682
209.0	2.6879	2.9889	2.7926	2.9309
210.0	2.8979	2.7730	2.5511	2.6217
211.0	2.9682	2.6160	2.6952	2.7921
212.0	2.9328	2.8870	2.6829	2.6317
213.0	2.8223	2.9984	2.8223	2.7875
214.0	2.7113	2.7693	2.6602	2.6850
215.0	2.8870	2.7242	2.5859	2.7523
216.0	2.9867	2.6857	2.6622	2.6622
217.0	2.8385	2.9524	2.6302	2.8063
218.0	2.7866	3.0084	2.7074	2.6839
219.0	2.6300	2.7419	2.5574	2.5744
220.0	2.7768	2.6799	2.6385	2.5186
221.0	2.8443	2.8443	2.3993	2.4589
222.0	2.7430	2.8521	2.3962	2.3855
223.0	2.5739	2.7748	2.2649	2.2493
224.0	2.5503	2.5503	2.0349	2.0536
225.0	2.5153	2.5433	1.8109	1.8329
226.0	2.4475	2.4252	1.6140	1.6123
227.0	2.2631	2.3097	1.3955	1.3955
228.0	2.0772	2.1065	1.1928	1.1934
229.0	1.9400	1.9169	1.0146	1.0126
230.0	1.7200	1.7200	0.8351	0.8359
231.0	1.5282	1.5269	0.6979	0.6972
232.0	1.3493	1.3535	0.5806	0.5813
233.0	1.1818	1.1834	0.4782	0.4781
234.0	1.0293	1.0289	0.3910	0.3911
235.0	0.8721	0.8710	0.3141	0.3139
236.0	0.7383	0.7391	0.2555	0.2555
237.0	0.6207	0.6210	0.2057	0.2055
238.0	0.5230	0.5232	0.1668	0.1669
239.0	0.4288	0.4284	0.1312	0.1312
240.0	0.3536	0.3532	0.1049	0.1049
241.0	0.2901	0.2902	0.0843	0.0844
242.0	0.2349	0.2354	0.0676	0.0675

243.0	0.1878	0.1875	0.0536	0.0539
244.0	0.1472	0.1466	0.0419	0.0423
245.0	0.1180	0.1183	0.0342	0.0343
246.0	0.0932	0.0931	0.0268	0.0271
247.0	0.0738	0.0740	0.0220	0.0222
248.0	0.0567	0.0569	0.0174	0.0174
249.0	0.0447	0.0443	0.0135	0.0136
250.0	0.0353	0.0355	0.0116	0.0117
251.0	0.0283	0.0285	0.0095	0.0094
252.0	0.0231	0.0230	0.0076	0.0076
253.0	0.0187	0.0188	0.0064	0.0066
254.0	0.0162	0.0162	0.0054	0.0051
255.0	0.0141	0.0142	0.0050	0.0046
256.0	0.0128	0.0129	0.0045	0.0045
257.0	0.0119	0.0125	0.0043	0.0042
258.0	0.0112	0.0112	0.0035	0.0036
259.0	0.0111	0.0114	0.0039	0.0041
260.0	0.0110	0.0104	0.0036	0.0036
261.0	0.0104	0.0103	0.0033	0.0035
262.0	0.0102	0.0108	0.0038	0.0039
263.0	0.0103	0.0103	0.0030	0.0031
264.0	0.0106	0.0108	0.0037	0.0036
265.0	0.0104	0.0105	0.0035	0.0036
266.0	0.0102	0.0106	0.0034	0.0034
267.0	0.0103	0.0105	0.0033	0.0035
268.0	0.0109	0.0108	0.0039	0.0035
269.0	0.0114	0.0115	0.0039	0.0040
270.0	0.0116	0.0114	0.0046	0.0046
271.0	0.0113	0.0117	0.0045	0.0044
272.0	0.0116	0.0110	0.0042	0.0046
273.0	0.0118	0.0119	0.0051	0.0050
274.0	0.0119	0.0123	0.0055	0.0053
275.0	0.0117	0.0117	0.0051	0.0049
276.0	0.0119	0.0125	0.0060	0.0060
277.0	0.0124	0.0122	0.0054	0.0058
278.0	0.0124	0.0121	0.0052	0.0055
279.0	0.0125	0.0125	0.0064	0.0064
280.0	0.0122	0.0121	0.0066	0.0064
281.0	0.0122	0.0127	0.0069	0.0066
282.0	0.0129	0.0127	0.0068	0.0074
283.0	0.0132	0.0131	0.0078	0.0075
284.0	0.0124	0.0124	0.0075	0.0076
285.0	0.0126	0.0128	0.0081	0.0077
286.0	0.0129	0.0131	0.0080	0.0086
287.0	0.0130	0.0123	0.0079	0.0081
288.0	0.0130	0.0131	0.0089	0.0090
289.0	0.0134	0.0135	0.0098	0.0093
290.0	0.0126	0.0126	0.0091	0.0093

291.0	0.0128	0.0128	0.0095	0.0096
292.0	0.0131	0.0126	0.0095	0.0090
293.0	0.0134	0.0133	0.0101	0.0100
294.0	0.0133	0.0135	0.0104	0.0105
295.0	0.0129	0.0130	0.0101	0.0104
296.0	0.0133	0.0130	0.0106	0.0105
297.0	0.0132	0.0129	0.0104	0.0109
298.0	0.0135	0.0135	0.0111	0.0108
299.0	0.0130	0.0126	0.0104	0.0107
300.0	0.0131	0.0133	0.0110	0.0108
301.0	0.0129	0.0135	0.0109	0.0112
302.0	0.0134	0.0130	0.0108	0.0106
303.0	0.0133	0.0133	0.0108	0.0112
304.0	0.0136	0.0140	0.0112	0.0110
305.0	0.0134	0.0134	0.0107	0.0105
306.0	0.0135	0.0136	0.0109	0.0102
307.0	0.0135	0.0132	0.0101	0.0098
308.0	0.0136	0.0136	0.0094	0.0091
309.0	0.0134	0.0140	0.0096	0.0097
310.0	0.0137	0.0140	0.0089	0.0089
311.0	0.0140	0.0139	0.0085	0.0086
312.0	0.0148	0.0147	0.0086	0.0084
313.0	0.0147	0.0148	0.0083	0.0081
314.0	0.0143	0.0143	0.0070	0.0074
315.0	0.0151	0.0150	0.0068	0.0070
316.0	0.0152	0.0152	0.0064	0.0065
317.0	0.0159	0.0153	0.0062	0.0062
318.0	0.0161	0.0159	0.0066	0.0067
319.0	0.0163	0.0167	0.0053	0.0056
320.0	0.0164	0.0161	0.0041	0.0048
321.0	0.0166	0.0164	0.0049	0.0054
322.0	0.0173	0.0162	0.0027	0.0031
323.0	0.0172	0.0171	0.0025	0.0028
324.0	0.0179	0.0178	0.0027	0.0027
325.0	0.0181	0.0180	0.0024	0.0023
326.0	0.0191	0.0190	0.0022	0.0023
327.0	0.0191	0.0187	0.0006	0.0009
328.0	0.0198	0.0197	0.0016	0.0021
329.0	0.0202	0.0208	0.0017	0.0010
330.0	0.0210	0.0210	0.0008	0.0015
331.0	0.0215	0.0213	0.0011	0.0008
332.0	0.0223	0.0217	0.0002	0.0004
333.0	0.0223	0.0227	0.0001	-0.0004
334.0	0.0228	0.0232	-0.0001	0.0002
335.0	0.0239	0.0238	0.0005	0.0002
336.0	0.0251	0.0247	0.0006	0.0005
337.0	0.0254	0.0251	0.0001	0.0001
338.0	0.0261	0.0263	0.0003	0.0001

339.0	0.0270	0.0267	0.0003	0.0001
340.0	0.0273	0.0269	-0.0005	-0.0001
341.0	0.0282	0.0282	0.0000	0.0001
342.0	0.0282	0.0286	0.0001	0.0003
343.0	0.0293	0.0287	-0.0001	-0.0004
344.0	0.0299	0.0298	0.0002	0.0001
345.0	0.0300	0.0296	-0.0007	-0.0004
346.0	0.0297	0.0304	-0.0006	-0.0004
347.0	0.0306	0.0306	-0.0005	-0.0002
348.0	0.0319	0.0313	0.0001	-0.0004
349.0	0.0317	0.0314	-0.0007	-0.0007
350.0	0.0310	0.0313	-0.0007	-0.0008
351.0	0.0316	0.0316	-0.0007	-0.0005
352.0	0.0322	0.0323	-0.0004	-0.0005
353.0	0.0322	0.0319	-0.0007	-0.0011
354.0	0.0325	0.0327	0.0002	-0.0005
355.0	0.0322	0.0322	-0.0006	-0.0001
356.0	0.0322	0.0320	-0.0006	-0.0005
357.0	0.0316	0.0314	0.0012	-0.0003
358.0	0.0309	0.0306	-0.0012	-0.0013
359.0	0.0309	0.0308	0.0002	-0.0002
360.0	0.0306	0.0298	-0.0005	-0.0008
361.0	0.0304	0.0297	-0.0006	-0.0003
362.0	0.0291	0.0297	-0.0008	-0.0012
363.0	0.0284	0.0289	-0.0006	-0.0005
364.0	0.0266	0.0280	-0.0010	-0.0010
365.0	0.0260	0.0266	-0.0010	-0.0009
366.0	0.0258	0.0258	-0.0007	-0.0010
367.0	0.0255	0.0252	-0.0004	-0.0004
368.0	0.0245	0.0255	0.0001	-0.0005
369.0	0.0243	0.0245	-0.0005	0.0001
370.0	0.0229	0.0227	-0.0005	-0.0008
371.0	0.0219	0.0213	-0.0015	-0.0014
372.0	0.0210	0.0203	-0.0013	-0.0010
373.0	0.0193	0.0192	-0.0009	-0.0006
374.0	0.0182	0.0185	-0.0005	-0.0007
375.0	0.0168	0.0174	-0.0010	-0.0002
376.0	0.0160	0.0163	-0.0012	-0.0014
377.0	0.0148	0.0148	-0.0014	-0.0016
378.0	0.0134	0.0134	-0.0008	-0.0013
379.0	0.0128	0.0130	-0.0012	-0.0011
380.0	0.0122	0.0124	-0.0011	-0.0005
381.0	0.0111	0.0111	-0.0008	-0.0007
382.0	0.0094	0.0096	-0.0014	-0.0010
383.0	0.0093	0.0088	-0.0013	-0.0010
384.0	0.0087	0.0085	-0.0008	-0.0010
385.0	0.0078	0.0074	-0.0012	-0.0008
386.0	0.0067	0.0066	-0.0006	-0.0009

387.0	0.0058	0.0057	-0.0010	-0.0017
388.0	0.0055	0.0052	-0.0006	-0.0013
389.0	0.0045	0.0042	-0.0014	-0.0017
390.0	0.0041	0.0041	-0.0006	-0.0016
391.0	0.0032	0.0038	-0.0009	-0.0009
392.0	0.0033	0.0035	-0.0009	-0.0010
393.0	0.0028	0.0026	-0.0013	-0.0007
394.0	0.0025	0.0025	-0.0008	-0.0014
395.0	0.0018	0.0021	-0.0010	-0.0011
396.0	0.0021	0.0017	-0.0009	-0.0012
397.0	0.0010	0.0012	-0.0016	-0.0009
398.0	0.0014	0.0012	-0.0011	-0.0007
399.0	0.0006	0.0011	-0.0009	-0.0009
400.0	0.0008	0.0005	-0.0012	-0.0010

APPENDIX C: DATA FOR CHAPTER 5

Table C.1 MLSS and MLVSS values for the cadmium short-term experiment presented in Chapter 5 as Trial 1.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	2187	1807
Initial NGR	2247	1867
Final NGR	2280	1853
Final NGR	2253	1867
SOUR	2247	1907
SOUR	2200	1853

Table C.2 NGR values for the cadmium short-term experiment presented in Chapter 5 as Trial 1.

Reactor	Cadmium Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 2-1	0	0.1379	0.0742
Control 2-2	0	0.1364	0.0734
1 mg/L-1	1	0.1283	0.0690
1 mg/L-2	1	0.143	0.0769
5 mg/L-1	5		
5 mg/L-2	5	0.1278	0.0688
10 mg/L-1	10	0.1252	0.0674
10 mg/L-2	10	0.1262	0.0679
30 mg/L-1	30	0.0588	0.0316
30 mg/L-2	30	0.0604	0.0325
50 mg/L-1	50	0.048	0.0258
50 mg/L-2	50	0.0479	0.0258
Control 2-1	0	0.1379	0.0742
Control 2-2	0	0.1364	0.0734

Table C.3 SOUR values for the cadmium short-term experiment presented in Chapter 5 as Trial 1. TCMP reactors are inhibited for nitrification.

Reactor	Cadmium Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/g MLVSS-min)
Control 1-1	0	1.129	0.6074
Control 1-2	0	1.2755	0.6862
Control TCMP 1-1	0	0.9374	0.5043
Control TCMP 1-2	0	0.9743	0.5241
Control 2-1	0	1.2809	0.6891
Control 2-2	0	1.3142	0.7070
Control TCMP 2-1	0	1.0011	0.5385
Control TCMP 2-2	0	1.0178	0.5475
1 mg/L-1	1	1.2706	0.6835
1 mg/L-2	1	1.0918	0.5873
1 TCMP -1	1	0.8278	0.4453
1 TCMP -2	1	0.5658	0.3044
10 mg/L-1	10	1.1978	0.6444
10 mg/L-2	10	1.2953	0.6968
10 TCMP -1	10	0.9636	0.5184
10 TCMP -2	10	0.9744	0.5242
50 mg/L-1	50	1.0974	0.5904
50 mg/L-2	50	1.1043	0.5941
50 TCMP -1	50	0.8591	0.4622
50 TCMP -2	50	0.8896	0.4786

Table C.4 MLSS and MLVSS values for the cadmium short-term experiment Trial 2.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	3227	2653
Initial NGR	3240	2653
Final NGR	3260	2653
Final NGR	3200	2607
SOUR	3187	2607
SOUR	3253	2660

Table C.5 NGR values for the cadmium short-term experiment Trial 2.

Reactor	Cadmium Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 1-1	0	0.2027	0.0768
Control 1-2	0	0.2055	0.0779
Control 2-1	0	0.2039	0.0773
Control 2-2	0	0.2170	0.0822
1 mg/L-1	1	0.1904	0.0722
1 mg/L-2	1	0.1960	0.0743
5 mg/L-1	5	0.1917	0.0726
5 mg/L-2	5	0.1992	0.0755
10 mg/L-1	10	0.1908	0.0723
10 mg/L-2	10	0.1927	0.0730
30 mg/L-1	30	0.1319	0.0500
30 mg/L-2	30	0.1292	0.0490
50 mg/L-1	50	0.0829	0.0314
50 mg/L-2	50	0.0774	0.0293

Table C.6 SOUR values for the cadmium short-term experiment Trial 2. TCMP reactors are inhibited for nitrification.

Reactor	Cadmium Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/g MLVSS-min)
Control 1-1	0	1.1006	0.4171
Control 1-2	0	1.0995	0.4167
Control TCMP 1-1	0	0.8920	0.3380
Control TCMP 1-2	0	0.8770	0.3323
5 mg/L-1	1	1.0336	0.3917
5 mg/L-2	1	1.0783	0.4086
5 TCMP -1	1	0.8665	0.3284
5 TCMP -2	1	0.9088	0.3444
10 mg/L-1	10	1.0573	0.4007
10 mg/L-2	10	1.0086	0.3822
10 TCMP -1	10	0.9517	0.3606
10 TCMP -2	10	0.8835	0.3348
50 mg/L-1	50	0.8243	0.3124
50 mg/L-2	50	0.8855	0.3356
50 TCMP -1	50	0.7771	0.2945
50 TCMP -2	50	0.7193	0.2726
Control 2-1	0	0.9890	0.3748
Control 2-2	0	1.0379	0.3933
Control TCMP 2-1	0	0.9750	0.3695
Control TCMP 2-2	0	0.9247	0.3504

Table C.7 MLSS and MLVSS values for the CDNB short-term experiment presented in Chapter 5 as Trial 1.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	3010	2510
Initial NGR	2930	2460
Final NGR	3000	2490
Final NGR	2930	2430
SOUR	3000	2480
SOUR	2990	2470

Table C.8 NGR values for the CDNB short-term experiment presented in Chapter 5 as Trial 1.

Reactor	CDNB Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 1-1	0	0.1755	0.0710
Control 1-2	0	0.1783	0.0721
1 mg/L-1	1	0.1755	0.0710
1 mg/L-2	1	0.1868	0.0755
5 mg/L-1	5	0.1402	0.0574
5 mg/L-2	5	0.1373	0.0562
10 mg/L-1	10	0.1252	0.0519
10 mg/L-2	10	0.1113	0.0462
20 mg/L-1	20	0.0523	0.0223
20 mg/L-2	20	0.0495	0.0211
30 mg/L-1	30	0.0297	0.0130
30 mg/L-2	30	0.0277	0.0121
Control 1-1	0	0.1755	0.0710
Control 1-2	0	0.1783	0.0721

Table C.9 SOUR values for the CDNB short-term experiment presented in Chapter 5 as Trial 1. TCMP reactors are inhibited for nitrification.

Reactor	CDNB Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/g MLVSS-min)
Control 1-1	0	1.0521	0.4254
Control 1-2	0	1.0045	0.4061
Control TCMP 1-1	0	0.9089	0.3675
Control TCMP 1-2	0	0.8765	0.3544
5 mg/L-1	1	0.906	0.3663
5 mg/L-2	1	0.8547	0.3456
5 TCMP -1	1	0.8074	0.3264
5 TCMP -2	1	0.7646	0.3091
10 mg/L-1	10	0.8621	0.3486
10 mg/L-2	10	0.8061	0.3259
10 TCMP -1	10	0.7624	0.3082
10 TCMP -2	10	0.7179	0.2903
30 mg/L-1	30	0.6858	0.2773
30 mg/L-2	30	0.6612	0.2673
30 TCMP -1	30	0.6003	0.2427
30 TCMP -2	30	0.5709	0.2308
Control 2-1	0	1.059	0.4282
Control 2-2	0	1.0118	0.4091
Control TCMP 2-1	0	0.9421	0.3809
Control TCMP 2-2	0	0.8421	0.3405

Table C.10 MLSS and MLVSS values for the CDNB short-term experiment Trial 2.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	3120	2640
Initial NGR	3190	2700
Final NGR	3080	2550
Final NGR	3060	2570
SOUR	3140	2680
SOUR	3010	2650

Table C.11 NGR values for the CDNB short-term experiment Trial 2.

Reactor	CDNB Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 1-1	0	0.1885	0.0716
Control 1-2	0	0.1815	0.0690
1 mg/L-1	1	0.1928	0.0733
1 mg/L-2	1	0.183	0.0695
4.9 mg/L-1	4.9	0.1292	0.0497
4.9 mg/L-2	4.9	0.1483	0.0571
9.8 mg/L-1	9.8	0.1153	0.0449
9.8 mg/L-2	9.8	0.1136	0.0442
27.9 mg/L-1	27.9	0.0812	0.0332
27.9 mg/L-2	27.9	0.0694	0.0283
52.2 mg/L-1	52.2	0	0.0000
52.2 mg/L-2	52.2	0	0.0000

Table C.12 SOUR values for the CDNB short-term experiment Trial 2. TCMP reactors are inhibited for nitrification.

Reactor	CDNB Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/g MLVSS-min)
Control 1-1	0	1.144	0.4335
Control 1-2			
Control TCMP 1-1	0	1.0347	0.3921
Control TCMP 1-2	0	0.9839	0.3728
5 mg/L-1	1	0.9506	0.3602
5 mg/L-2	1	0.9106	0.3451
5 TCMP -1	1	0.8356	0.3166
5 TCMP -2	1	0.7858	0.2978
10 mg/L-1	10	0.88	0.3335
10 mg/L-2	10	0.8196	0.3106
10 TCMP -1	10	0.7589	0.2876
10 TCMP -2	10	0.6996	0.2651
60 mg/L-1	60	0.6173	0.2339
60 mg/L-2	60	0.5444	0.2063
60 TCMP -1	60	0.5664	0.2146
60 TCMP -2	60	0.4704	0.1783
Control 2-1	0	1.2511	0.4741
Control 2-2	0	1.0276	0.3894
Control TCMP 2-1	0	1.1105	0.4208
Control TCMP 2-2	0	1.0737	0.4069

Table C.13 MLSS and MLVSS values for the chlorine short-term experiment presented in Chapter 5 as Trial 1.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	2127	1727
Initial NGR	2093	1680
Final NGR	2160	1747
Final NGR	2147	1727
SOUR	2093	1680
SOUR	2153	1760

Table C.14 NGR values for the chlorine short-term experiment presented in Chapter 5 as Trial 1.

Reactor	Free Chlorine Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 1-1	0	0.0605	0.0352
Control 1-2	0	0.0515	0.0299
5 mg/L-1	5	0.0384	0.0223
5 mg/L-2	5	0.0045	0.0026
10 mg/L-1	10	0.0257	0.0149
10 mg/L-2	10	0.004	0.0023
30 mg/L-1	30	0.0098	0.0057
30 mg/L-2	30	0.0067	0.0039
60 mg/L-1	60	0.0045	0.0026
60 mg/L-2	60	0.0082	0.0048
120 mg/L-1	120	0.004	0.0023
120 mg/L-2	120	0.0037	0.0022

Table C.15 SOUR values for the chlorine short-term experiment presented in Chapter 5 as Trial 1. TCMP reactors are inhibited for nitrification.

Reactor	Free Chlorine Concentration (mg/L)	OUR ($mg\text{-}O_2/L\text{-}min$)	SOUR ($mg\text{-}O_2/g\ MLVSS\text{-}min$)
Control 1-1	0	1.1028	0.6412
Control 1-2	0	1.0475	0.6090
Control TCMP 1-1	0	0.805	0.4680
Control TCMP 1-2	0	0.7801	0.4535
Control 2-1	0	1.0991	0.6390
Control 2-2	0	1.1021	0.6408
Control TCMP 2-1	0	0.8597	0.4998
Control TCMP 2-2	0	0.8255	0.4799
5 mg/L-1	5	0.3494	0.2031
5 mg/L-2	5	0.2909	0.1691
5 TCMP -1	5	0.2291	0.1332
5 TCMP -2	5	0.2866	0.1666
10 mg/L-1	10	0.1244	0.0723
10 mg/L-2	10	0.1486	0.0864
10 TCMP -1	10	0.1229	0.0715
10 TCMP -2	10		
30 mg/L-1	30	0.0371	0.0216
30 mg/L-2	30	0.0365	0.0212
30 TCMP -1	30	0.0319	0.0185
30 TCMP -2	30	0.038	0.0221

Table C.16 MLSS and MLVSS values for the chlorine short-term experiment Trial 2.

Sample	MLSS (mg/L)	MLVSS (mg/L)
Initial NGR	2193	1807
Initial NGR	2227	1840
Final NGR	2227	1807
Final NGR	2320	1887
SOUR	2207	1807
SOUR	2193	1793

Table C.17 NGR values for the chlorine short-term experiment Trial 2.

Reactor	Free Chlorine Concentration (mg/L)	Slope of Nitrate Data (mg-N/L-min)	NGR (mg-N/g MLVSS-min)
Control 1-1	0	0.0796	0.0437
Control 1-2	0	0.0746	0.0409
Control 2-1	0	0.0752	0.0412
Control 2-2	0	0.0731	0.0401
5 mg/L-1	5	0.0399	0.0219
5 mg/L-2	5	0.0429	0.0235
10 mg/L-1	10	0.0131	0.0072
10 mg/L-2	10	0.0161	0.0088
30 mg/L-1	30		
30 mg/L-2	30	0.0134	0.0073
60 mg/L-1	60	0.0054	0.0030
60 mg/L-2	60	0.0091	0.0050
120 mg/L-1	120	0.0039	0.0021
120 mg/L-2	120	0.0044	0.0024

Table C.18 SOUR values for the chlorine short-term experiment Trial 2. TCMP reactors are inhibited for nitrification.

Reactor	Free Chlorine Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/g MLVSS-min)
Control 1-1	0	1.0582	0.5804
Control 1-2	0	1.1508	0.6312
Control TCMP 1-1	0	0.8661	0.4750
Control TCMP 1-2	0	0.8982	0.4926
Control 2-1	0	1.4045	0.7703
Control 2-2	0	1.3838	0.7589
Control TCMP 2-1	0	1.0794	0.5920
Control TCMP 2-2	0	1.0745	0.5893
5 mg/L-1	5	0.43	0.2358
5 mg/L-2	5	0.409	0.2243
5 TCMP -1	5	0.376	0.2062
5 TCMP -2	5	0.3608	0.1979
10 mg/L-1	10		
10 mg/L-2	10	0.2067	0.1134
10 TCMP -1	10	0.3555	0.1950
10 TCMP -2	10	0.3596	0.1972
30 mg/L-1	30	0.0685	0.0376
30 mg/L-2	30	0.0526	0.0288
30 TCMP -1	30	0.0614	0.0337
30 TCMP -2	30	0.0546	0.0299

Table C.19 Trial 1 MLSS and MLVSS values for the control cadmium long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2960	3040	3020	3120	2480	2540	2520	2600
3	3180	3240	3260	3180	2600	2680	2600	2540
6	2760	2720	2860	2820	2200	2220	2280	2320
12	2660	2760	2780	2840	2240	2300	2340	2400
24	2280	2300	2400	2440	1820	1900	1940	2020
48	2380	2280	2320	2280	1920	1900	1880	1860

Table C.20 Trial 1 MLSS and MLVSS values for the nitrification inibited cadmium long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2980	2920	2740	2800	2480	2500	2340	2380
3	2940	2980	2940	2860	2440	2460	2380	2320
6	2800	2820	2740	2860	2280	2320	2200	2300
12	2720	2760	2640	2660	2280	2320	2240	2260
24	2440	2500	2380	2340	2000	2100	1960	1940
48	2400	2440	2220	2200	1960	1980	1820	1900

Table C.21 Trial 1 NGR values for the cadmium long-term experiment presented in Chapter 5.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.0438	0.0518	0.0511	0.0493
3	0.0504	0.0539	0.0313	0.0332
6	0.0526	0.0570	0.0142	0.0150
12	0.0463	0.0514	0.0212	0.0202
24	0.0500	0.0530	0.0237	0.0253
48	0.0490	0.0540	0.0245	0.0303

Table C.22 Trial 1 Total SOUR values for the cadmium long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.516	0.490	0.188	0.176
3	0.491	0.421	0.156	0.146
6	0.428	0.387	0.130	0.120
12	0.363	0.366	0.162	0.163
24	0.312	0.305	0.172	0.179
48	0.326	0.327	0.221	0.213

Table C.23 Trial 1 Nitrification Inhibited SOUR values for the cadmium long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.428	0.396	0.140	0.128
3	0.397	0.342	0.095	0.081
6	0.320	0.303	0.070	0.071
12	0.327	0.327	0.085	0.091
24	0.238	0.240	0.122	0.121
48	0.231	0.229	0.138	0.137

Table C.24 Trial 2 MLSS and MLVSS values for the control cadmium long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2480	2500	2380	2440	2080	2080	2020	2020
3	2460	2980	2400	2420	2060	2580	2080	2120
6	2280	2200	2200	2200	1920	1920	1860	1840
12	2420	2320	2280	2180	2040	2000	1980	1880
24	2200	2140	2120	2160	1800	1820	1820	1860
48	1940	1960	1820	1820	1700	1760	1620	1600

Table C.25 Trial 2 MLSS and MLVSS values for the nitrification inhibited cadmium long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2320	2340	2400	2520	2040	2040	2040	2200
3	2320	2440	2520	2440	1980	2060	2140	2120
6	2140	2120	2040	2200	1780	1800	1740	1860
12	2140	2280	2220	2260	1860	1920	1940	1920
24	2180	2040	2080	1980	1860	1800	1860	1800
48	1820	1860	1840	1860	1660	1600	1540	1560

Table C.26 Trial 2 NGR values for the cadmium long-term experiment.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.062	0.053	0.038	0.035
3	0.026	0.027	0.010	0.011
6	0.027	0.025	0.011	0.010
12	0.041	0.040	0.015	0.016
24	0.042	0.044	0.021	0.021
48	0.045	0.052	0.029	0.021

Table C.27 Trial 2 Total SOUR values for the cadmium long-term experiment.

Time (hr)	Control-1 SOUR ($mg-O_2/g$ MLVSS-min)	Control-2 SOUR ($mg-O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg-O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg-O_2/g$ MLVSS-min)
0	0.555	0.552	0.290	0.287
3	0.319	0.308	0.120	0.122
6	0.458	0.423	0.154	0.159
12	0.229	0.247	0.186	0.191
24	0.237	0.210	0.213	0.213
48	0.223	0.269	0.370	0.366

Table C.28 Trial 2 Nitrification Inhibited SOUR values for the cadmium long-term experiment.

Time (hr)	Control-1 SOUR ($mg-O_2/g$ MLVSS-min)	Control-2 SOUR ($mg-O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg-O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg-O_2/g$ MLVSS-min)
0	0.361	0.370	0.127	0.139
3	0.395	0.394	0.075	0.077
6	0.423	0.419	0.084	0.086
12	0.401	0.417	0.086	0.089
24	0.318	0.308	0.099	0.100
48	0.329	0.366	0.135	0.137

Table C.29 Trial 1 MLSS and MLVSS values for the control CDNB long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2660	2720	2700	2680	2200	2180	2200	2140
3	2720	2700	2760	2640	2200	2180	2180	2200
6	2200	2160	2220	2160	1780	1740	1820	1800
12	2180	2060	2120	2140	1860	1820	1800	1820
24	2040	1920	2000	2060	1780	1720	1760	1800
48	2020	12118	2060	1960	1740	1600	1740	1720

Table C.30 Trial 1 MLSS and MLVSS values for the nitrification inhibited CDNB long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2800	2740	2780	2660	2240	2160	2200	2140
3	2680	2840	2740	2780	2200	2240	2140	2220
6	2340	2280	2360	2380	1840	1820	1940	1940
12	2180	2160	2200	2200	1840	1780	1820	1840
24	2000	2020	1920	2040	1800	1760	1760	1820
48	2040	2000	2080	2060	1720	1680	1820	1820

Table C.31 Trial 1 NGR values for the CDNB long-term experiment presented in Chapter 5.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.050	0.044	0.028	0.027
3	0.046	0.048	0.006	0.009
6	0.044	0.042	0.004	0.000
12	0.043	0.046	0.000	0.000
24	0.051	0.046	0.000	0.000
48	0.044	0.043	0.000	0.000

Table C.32 Trial 1 Total SOUR values for the CDNB long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.546	0.552	0.320	0.323
3	0.563	0.575	0.194	0.196
6	0.586		0.219	0.229
12	0.264	0.270	0.306	0.311
24	0.184	0.182	0.322	0.328
48	0.182	0.180	0.380	0.385

Table C.33 Trial 1 Nitrification Inhibited SOUR values for the CDNB long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.387	0.390	0.246	0.245
3	0.367	0.343	0.165	0.171
6	0.415	0.414	0.176	0.182
12	0.335	0.357	0.212	0.223
24	0.272	0.282	0.202	0.211
48	0.309	0.313	0.245	0.244

Table C.34 Trial 2 MLSS and MLVSS values for the control CDNB long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	3280	3340	3240	3220	2740	2800	2720	2780
3	3460	3440	3360	3360	2860	2820	2760	2820
6	3120	3180	3200	3280	2520	2640	2620	2740
12	3120	3100	3220	3060	2500	2540	2580	2560
24	2900	2880	2900	2940	2340	2340	2380	2400
48	2700	2640	2780	2800	2280	2220	2320	2360
72	2500	2480	2480	2620	2040	2080	2000	2180

Table C.35 Trial 2 MLSS and MLVSS values for the nitrification inhibited CDNB long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	3480	3500	3340	3400	2920	2980	2960	2920
3	3420	3480	3300	3260	2900	2880	2740	2720
6	3180	3220	3300	3000	2640	2660	2700	2500
12	3120	3140	3240	3180	2500	2580	2640	2640
24	3000	3040	3000	2960	2480	2480	2460	2420
48	2740	2740	2780	2740	2300	2300	2340	2340
72	2740	2740	2760	2740	2260	2300	2300	2260

Table C.36 Trial 2 NGR values for the CDNB long-term experiment.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.075	0.075	0.026	0.029
3	0.029	0.029	0.023	0.023
6	0.040	0.049	0.000	0.008
12	0.046	0.042	0.000	0.008
24	0.047	0.044	0.005	0.000
48	0.047	0.051	0.010	0.011
72	0.061	0.068	0.044	

Table C.37 Trial 2 Total SOUR values for the CDNB long-term experiment.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.800	0.801	0.442	0.431
3	0.262	0.260	0.179	0.178
6	0.289	0.285	0.239	0.235
12	0.383	0.380	0.305	0.307
24	0.443	0.425	0.384	0.374
48	0.571	0.542	0.417	0.407
72	0.307	0.278	0.149	0.142

Table C.38 Trial 2 Nitrification Inhibited SOUR values for the CDNB long-term experiment.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.382	0.371	0.240	0.224
3	0.325	0.318	0.174	0.172
6	0.356	0.336	0.199	0.196
12	0.313	0.312	0.209	0.208
24	0.299	0.296	0.256	0.249
48	0.323	0.315	0.294	0.292
72	0.309	0.298	0.297	0.294

Table C.39 Trial 1 MLSS and MLVSS values for the control free chlorine long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2040	2060	2460	1980	1680	1700	2160	1660
3	2000	2040	1900	1940	1660	1720	1640	1620
6	1920	1900	1840	1740	1620	1620	1540	1460
12	1880	1820	1800	1800	1600	1580	1540	1520
24	1740	1840	1720	1660	1460	1540	1420	1380
48	1700	1700	1640	1500	1460	1480	1420	1320

Table C.40 Trial 1 MLSS and MLVSS values for the nitrification inhibited free chlorine long-term experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2040	1940	2100	2200	1680	1580	1720	1840
3	2040	2060	2080	1960	1700	1700	1740	1660
6	1840	1860	1840	1780	1560	1600	1500	1520
12	1840	1740	1760	1820	1600	1500	1560	1600
24	1840	1820	1800	1720	1560	1520	1520	1460
48	1640	1700	1600	1620	1400	1440	1360	1420

Table C.41 Trial 1 NGR values for the free chlorine long-term experiment presented in Chapter 5.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.033	0.077	0.017	0.016
3	0.038	0.087	0.014	0.016
6	0.075	0.040	0.018	0.010
12	0.076	0.052	0.016	0.031
24	0.064	0.054	0.026	0.038
48	0.067	0.074	0.028	0.030

Table C.42 Trial 1 Total SOUR values for the free chlorine long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.517	0.321	0.072	0.075
3	0.493	0.311	0.118	0.127
6	0.558	0.327	0.138	0.146
12	0.362	0.306	0.170	0.169
24	0.379	0.318	0.239	0.250
48	0.321	0.302	0.267	0.271

Table C.43 Trial 1 Nitrification Inhibited SOUR values for the free chlorine long-term experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.320	0.072	0.065	0.067
3	0.322	0.118	0.081	0.086
6	0.339	0.138	0.093	0.096
12	0.305	0.170	0.113	0.115
24	0.326	0.239	0.175	0.184
48	0.304	0.267	0.187	0.194

Table C.44 Trial 2 MLSS and MLVSS values for the control free chlorine long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2500	2460	2640	2520	2000	1960	2120	2040
3	2340	2400	2500	2540	1960	1980	2080	2040
6	2300	2300	2380	2340	1860	1820	1960	1880
12	2100	2120	2200	2180	1820	1760	1900	1880
24	1900	1820	2080	2120	1520	1400	1660	1720
48	1740	1760	1920	1920	1520	1480	1640	1680
72	2500	2460	2640	2520	2000	1960	2120	2040

Table C.45 Trial 2 MLSS and MLVSS values for the nitrification inhibited free chlorine long-term experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2420	2340	2460	2460	1980	1920	2040	2000
3	2400	2440	2480	2380	1960	1980	2020	1960
6	2380	2340	2280	2300	1900	1880	1860	1880
12	2160	2260	2200	2120	1880	1960	2000	2000
24	2020	2100	1960	1960	1620	1640	1600	1580
48	1900	1860	1720	1740	1660	1680	1600	1620
72	2420	2340	2460	2460	1980	1920	2040	2000

Table C.46 Trial 2 NGR values for the free chlorine long-term experiment.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.070	0.071	0.016	0.021
3	0.100	0.095	0.023	0.027
6	0.089	0.073	0.019	0.022
12	0.070	0.092	0.025	0.024
24	0.075	0.089	0.034	0.034
48	0.070	0.072	0.035	0.037

Table C.47 Trial 2 Total SOUR values for the free chlorine long-term experiment.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.501	0.493	0.132	0.131
3	0.463	0.459	0.160	0.163
6	0.470	0.474	0.177	0.184
12	0.311	0.283	0.217	0.222
24		0.337	0.246	0.262
48	0.260	0.280	0.195	

Table C.48 Trial 2 Nitrification Inhibited SOUR values for the free chlorine long-term experiment.

Time (hr)	Control-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	Control-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-1 SOUR ($mg\text{-}O_2/g$ MLVSS-min)	IC_{50}-2 SOUR ($mg\text{-}O_2/g$ MLVSS-min)
0	0.286	0.298	0.084	0.086
3	0.299	0.304	0.101	0.104
6	0.305	0.314	0.113	0.116
12	0.293	0.287	0.128	0.131
24	0.307	0.329	0.191	0.200
48	0.293	0.266	0.191	0.196

Table C.49 Trial 1 MLSS and MLVSS values for the cadmium long-term Eckenfelder experiment reactors presented in Chapter 5.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2110	2180	2170	2170	1730	1770	1780	1750
3	2130	2150	2160	2170	1780	1790	1810	1790
6	2110	2060	2110	2100	1740	1710	1740	1720
12	2010	2020	2010	2030	1670	1620	1610	1610
24	1970	1950	1910	1850	1660	1610	1560	1520
48	1700	1670	1600	1610	1390	1370	1280	1300

Table C.50 Trial 1 NGR values for the cadmium long-term Eckenfelder experiment presented in Chapter 5.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.079	0.073	0.030	0.028
3	0.076	0.074	0.013	0.016
6	0.063	0.062	0.012	0.011
12	0.060	0.059	0.017	0.016
24	0.028	0.031	0.015	0.017
48	0.005	0.008	0.025	0.028

Table C.51 Trial 1 Total SOUR values for the cadmium long-term Eckenfelder experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR (mg-O_2/g MLVSS-min)	Control-2 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-1 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-2 SOUR (mg-O_2/g MLVSS-min)
0	0.495	0.500	0.231	0.232
3	0.479	0.478	0.083	0.086
6	0.488	0.495	0.087	0.085
12	0.410	0.393	0.098	0.096
24	0.115	0.107	0.140	0.134
48	0.054	0.056	0.176	0.179

Table C.52 Trial 1 Nitrification Inhibited SOUR values for the cadmium long-term Eckenfelder experiment presented in Chapter 5.

Time (hr)	Control-1 SOUR (mg-O_2/g MLVSS-min)	Control-2 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-1 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-2 SOUR (mg-O_2/g MLVSS-min)
0	0.322	0.332	0.156	0.159
3	0.309	0.312	0.089	0.091
6	0.321	0.344	0.082	0.076
12	0.243	0.203	0.098	0.099
24	0.094	0.104	0.109	0.112
48	0.061	0.068	0.128	0.124

Table C.53 Trial 2 MLSS and MLVSS values for the cadmium long-term Eckenfelder experiment reactors.

Time (hr)	Control		IC_{50}		Control		IC_{50}	
	MLSS-1 mg/L	MLSS-2 mg/L	MLSS-1 mg/L	MLSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L	MLVSS-1 mg/L	MLVSS-2 mg/L
0	2170	2150	2150	2160	1800	1780	1760	1770
3	2150	2150	2120	2140	1750	1760	1710	1740
6	2090	2120	2080	2070	1690	1710	1680	1710
12	1990	1980	1990	1980	1670	1630	1620	1650
24	1920	1910	1890	1830	1620	1570	1540	1490
48	1670	1680	1580	1610	1400	1410	1320	1330

Table C.54 Trial 2 NGR values for the cadmium long-term Eckenfelder experiment.

Time (hr)	Control-1 NGR (mg-N/g MLVSS-min)	Control-2 NGR (mg-N/g MLVSS-min)	IC_{50}-1 NGR (mg-N/g MLVSS-min)	IC_{50}-2 NGR (mg-N/g MLVSS-min)
0	0.078	0.088	0.028	0.032
3	0.069	0.070	0.015	0.015
6	0.072	0.076	0.014	0.015
12	0.062	0.062	0.017	0.012
24	0.027	0.033	0.026	0.026
48	0.007	0.008	0.027	0.030

Table C.55 Trial 2 Total SOUR values for the cadmium long-term Eckenfelder experiment.

Time (hr)	Control-1 SOUR (mg-O_2/g MLVSS-min)	Control-2 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-1 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-2 SOUR (mg-O_2/g MLVSS-min)
0	0.455	0.480	0.192	0.194
3	0.488	0.496	0.074	0.075
6	0.486	0.470	0.076	0.083
12	0.309	0.331	0.079	0.080
24	0.103	0.086	0.117	0.113
48	0.055	0.053	0.168	0.170

Table C.56 Trial 2 Nitrification Inhibited SOUR values for the cadmium long-term Eckenfelder experiment.

Time (hr)	Control-1 SOUR (mg-O_2/g MLVSS-min)	Control-2 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-1 SOUR (mg-O_2/g MLVSS-min)	IC_{50}-2 SOUR (mg-O_2/g MLVSS-min)
0	0.298	0.315	0.132	0.136
3	0.365	0.380	0.085	0.087
6	0.275	0.303	0.071	0.075
12	0.173	0.262	0.073	0.078
24	0.077	0.061	0.096	0.093
48	0.052	0.065	0.123	0.137

Table C.57 *Pseudomonas aeruginosa* PAO1 SOUR values for varying concentrations of the nitrification inhibitor TCMP. Cell counts were 3.56, 3.70, 4.16, and 3.62 x 10^8 cells/ml for the control 1, control 2, 3 mg/L TCMP and 30 mg/L TCMP reactors, respectively.

Reactor	TCMP Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/10^8 cells-day)
Control 1-1	0	0.3365	0.1361
Control 1-2	0	0.282	0.1141
Control 2-1	0	0.2115	0.0823
Control 2-2	0	0.2105	0.0819
3 mg/L TCMP -1	3	0.2393	0.0828
3 mg/L TCMP -2	3	0.2309	0.0799
30 mg/L TCMP -1	30	0.3306	0.1337
30 mg/L TCMP -2	30	0.321	0.1298

Table C.58 SOUR values for free chlorine exposed mixed liquor when nitrification was inhibited with allylthiourea (ATU).

Reactor	Free Chlorine Concentration (mg/L)	OUR (mg-O_2/L-min)	SOUR (mg-O_2/10^8 cells-day)
Control 1-1	0	1.5459	0.8834
Control 1-2	0	1.455	0.8314
Control ATU 1-1	0	1.0491	0.5995
Control ATU 1-2	0	1.069	0.6109
Control 2-1	0	1.2501	0.7143
Control 2-2	0	1.2107	0.6918
Control ATU 2-1	0	1.0406	0.5946
Control ATU 2-2	0	1.0219	0.5839
2 mg/L-1	2	1.2908	0.7376
2 mg/L-2	2	1.2372	0.7070
2 ATU -1	2	1.0225	0.5843
2 ATU - 2	2	1.0143	0.5796
30 mg/L-1	30	0.6701	0.3829
30 mg/L-2	30	0.6993	0.3996
30 ATU -1	30	1.0901	0.6229
30 ATU -2	30	1.0744	0.6139
50 mg/L-1	50	0.7205	0.4117
50 mg/L-2	50	0.7825	0.4471
50 ATU -1	50	1.0506	0.6003
50 ATU -2	50	1.0985	0.6277
150 mg/L-1	150	0.1644	0.0939
150 mg/L-2	150	0.1732	0.0990
150 ATU -1	150	0.9859	0.5634
150 ATU -2	150	1.1837	0.6764

Appendix D: Data from Chapter 6

Table D.1 MLSS and MLVSS values for enrichment and mixed liquor cultures exposed to NEM.

Culture	MLSS (mg/L)			MLVSS (mg/L)		
Enrichment Culture	1744	1692	1660	1648	1596	1564
Mixed Liquor	1640	1648	1612	1356	1372	1340

Table D.2 Soluble potassium data for enrichment culture. Values are for the enrichment cultures shocked with 50 mg/L NEM. Trial 1 is shown in Figure 6.1, Trial 2 is repeat of the experiment.

TRIAL 1						
Time (minutes)	Enrichment Control (mg/L)			Enrichment NEM Shock (mg/L)		
0	9.67	9.69	9.74	9.98	10.08	10.04
2.5	9.63	9.64	9.71	10.53	10.57	10.42
5	10.11	10.06	10.04	10.83	10.9	10.91
7.5	10.02	10.13	10.06	10.52	10.57	10.57
10	9.62	9.6	9.58	10.9	11.02	10.93
15	10.38	10.23	10.48	10.85	10.87	10.92
20	10.72	10.73	10.68	11.01	10.94	11.1
TRIAL 2						
Time (minutes)	Enrichment Control (mg/L)			Enrichment NEM Shock (mg/L)		
0	9.78	9.83	9.83	9.28	9.37	9.4
2.5	9.92	9.99	9.98	10.58	10.59	10.65
5	11.98	11.99	12.01	10.74	10.77	10.62
7.5	9.16	9.12	9.15	10.56	10.52	10.45
10	9.55	9.57	9.52	10.99	11.07	11.06
15	9.38	9.46	9.44	10.71	10.81	10.81
20	9.9	9.79	9.92	11.04	11.07	11.03

Table D.3 Soluble potassium data for mixed liquor culture. Values are for the mixed liquor cultures shocked with 50 mg/L NEM. Trial 1 is shown in Figure 6.1, Trial 2 is repeat of the experiment.

TRIAL 1						
Time (minutes)	**Mixed Liquor Control (mg/L)**			**Mixed Liquor NEM Shock (mg/L)**		
0	9.02	9.11	9.04	9.05	8.92	9.04
2.5	9.16	9.17	9.25	10.18	10.13	10.2
5	9.21	9.24	9.18	10.38	10.32	10.38
10	9.07	9.07	9.07	10.47	10.52	10.49
15	9.27	9.17	9.29	10.77	10.81	10.78
20	9.17	9.13	9.17	11.26	11.26	11.42
TRIAL 2						
Time (minutes)	**Mixed Liquor Control (mg/L)**			**Mixed Liquor NEM Shock (mg/L)**		
0	9.22	9.19	9.21	9.15	9.03	9
2.5	8.91	8.99	8.96	10.04	9.91	10.03
5	9.52	9.31	9.23	10.39	10.44	10.5
10	9.45	9.42	9.48	11.05	10.91	10.98
15	8.83	8.79	8.89	10.68	10.64	10.75
20	8.98	9.07	9.03	11.14	11.33	11.25

Table D.4 Floc-associated potassium data for enrichment culture. Values are for the enrichment cultures shocked with 50 mg/L NEM.

TRIAL 1						
Time (minutes)	Enrichment Control (mg/g MLVSS)			Enrichment NEM Shock (mg/g MLVSS)		
0	4.33	4.36	4.35	3.92	3.90	3.90
2.5	3.95	3.97	3.97	3.18	3.18	3.19
5	3.71	3.70	3.74	2.82	2.83	2.80
7.5	3.71	3.71	3.71	2.84	2.85	2.86
10	3.70	3.73	3.73	2.70	2.69	2.63
15	3.41	3.38	3.39	2.53	2.54	2.56
20	3.62	3.60	3.66	2.39	2.37	2.39
TRIAL 2						
Time (minutes)	Enrichment Control (mg/g MLVSS)			Enrichment NEM Shock (mg/g MLVSS)		
0	3.69	3.72	3.70	3.67	3.70	3.72
2.5	4.97	4.98	4.97	2.77	2.78	2.76
5	3.76	3.77	3.83	2.23	2.23	2.24
7.5	3.93	3.96	3.93	2.82	2.83	2.84
10	3.88	3.90	3.89	2.27	2.28	2.29
15	3.71	3.70	3.70	2.59	2.59	2.58
20	1.67	1.63	1.69	2.48	2.49	2.46

Table D.5 Floc associated potassium data for mixed liquor culture. Values are for the mixed liquor cultures shocked with 50 mg/L NEM.

TRIAL 1						
Time (minutes)	Mixed Liquor Control (mg/g MLVSS)			Mixed Liquor NEM Shock (mg/g MLVSS)		
0	7.06	6.97	7.01	6.98	6.96	6.98
2.5	7.00	7.00	7.05	6.16	6.14	6.17
5	7.09	7.15	7.09	6.16	6.06	6.06
10	7.11	7.15	7.07	5.76	5.66	5.70
15	6.70	6.71	6.72	5.72	5.71	5.67
20	7.28	7.27	7.31	7.10	7.08	7.09
TRIAL 2						
Time (minutes)	Mixed Liquor Control (mg/g MLVSS)			Mixed Liquor NEM Shock (mg/g MLVSS)		
0	6.90	6.93	6.86	6.95	7.02	7.03
2.5	7.16	7.15	7.13	5.98	5.98	5.88
5	7.11	7.15	7.18	5.79	5.80	5.75
10	7.05	7.07	7.10	5.54	5.57	5.49
15	6.78	6.81	6.74	5.46	5.51	5.47
20	7.24	7.31	7.35	7.54	7.51	7.45

Table D.6 Floc-associated potassium data for enrichment culture. Values are for the enrichment cultures shocked with 50 mg/L NEM.

TRIAL 1						
Time (minutes)	Enrichment Control (mg/L)			Enrichment NEM Shock (mg/L)		
0	15.14	15.18	15.21	14.91	14.85	15
2.5	14.59	14.75	14.71	14.36	14.2	14.39
5	14.45	14.56	14.55	14.52	14.58	14.64
7.5	14.61	14.61	14.61	13.77	13.77	13.74
10	14.65	14.61	14.65	15.44	15.54	15.65
15	14.38	14.62	14.42	14.1	14.09	14.21
20	14.83	14.98	15	14.54	14.48	14.48
TRIAL 2						
Time (minutes)	Enrichment Control (mg/L)			Enrichment NEM Shock (mg/L)		
0	14.51	14.65	14.65	14.74	14.88	14.78
2.5	14.53	14.67	14.64	15.22	15.19	15.25
5	14.91	14.91	15.07	14.41	14.42	14.42
7.5	15.76	15.7	16.03	14.93	14.97	14.93
10	14.91	14.94	14.86	13.96	13.9	13.91
15	14.89	15.11	15.13	14.9	14.85	14.81
20	14.92	14.77	14.81	14.06	14.06	14.07

Table D.7 Floc associated potassium data for mixed liquor culture. Values are for the mixed liquor cultures shocked with 50 mg/L NEM.

TRIAL 1						
Time (minutes)	Mixed Liquor Control (mg/L)			Mixed Liquor NEM Shock (mg/L)		
0	17.91	18.03	18.01	20.36	20.27	20.5
2.5	17.34	17.21	17.31	19.93	19.86	19.7
10	17.68	17.62	17.63	17.8	17.59	17.53
15				17.2	17.24	17.32
20	17.44	17.38	17.33			
TRIAL 2						
Time (minutes)	Mixed Liquor Control (mg/L)			Mixed Liquor NEM Shock (mg/L)		
0	17.98	17.86	17.8	16.52	16.73	16.61
2.5	18.87	18.76	18.69	17.36	17.37	17.53
10	17.39	17.48	17.53	17.28	17.28	17.45
15				17.46	17.33	17.36
20	17.52	17.53	17.63	17.24	17.32	17.29

Table D.8 Soluble potassium data for *N. europaea* NEM experiments presented in Figure 6.2.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control	5.87	5.72
15	Control	5.82	5.98
45	Control	5.95	6.22
90	Control	5.79	6.29
180	Control	6.46	6.01
0	NEM 50 mg/L	5.92	5.83
15	NEM 50 mg/L	6.16	6.22
45	NEM 50 mg/L	5.14	6.23
90	NEM 50 mg/L	6.34	6.4
180	NEM 50 mg/L	7.95	7.45
0	Nigericin 74.7 mg/L	6.01	5.89
15	Nigericin 74.7 mg/L	6.71	7.69
45	Nigericin 74.7 mg/L	6.97	6.97
90	Nigericin 74.7 mg/L	7.22	7.01
180	Nigericin 74.7 mg/L	8.31	8.24

Table D.9 Soluble potassium data for *N. europaea* NEM experiment repeat.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control	11.76	12.03
10	Control	11.59	11.98
30	Control	12.48	12.23
60	Control	12.36	12.38
90	Control	12.56	12.53
0	NEM 50 mg/L	11.5	11.43
10	NEM 50 mg/L	11.6	11.9
30	NEM 50 mg/L	12.02	12.16
60	NEM 50 mg/L	12.03	12.51
90	NEM 50 mg/L	12.62	12.34

Table D.10 Soluble potassium data for *Ni. moscoviensis* NEM experiments presented in Figure 6.3.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control	2.33	2.21
15	Control	1.61	1.51
45	Control	1.44	1.44
90	Control	1.79	
180	Control	2.2	1.94
0	NEM 50 mg/L	2.03	1.94
15	NEM 50 mg/L	2.48	2.67
45	NEM 50 mg/L	2.5	2.7
90	NEM 50 mg/L	2.62	2.6
180	NEM 50 mg/L	3.75	3.85
0	Nigericin 74.7 mg/L	2.04	2.24
15	Nigericin 74.7 mg/L	3.36	3.63
45	Nigericin 74.7 mg/L	3.44	3.34
90	Nigericin 74.7 mg/L	3.44	3.39
180	Nigericin 74.7 mg/L	4.01	4.45

Table D.11 Soluble potassium data for *Ni. moscoviensis* NEM experiment repeat.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control		4.53
15	Control	4.59	4.58
45	Control	4.6	4.62
90	Control	4.83	
180	Control	5.65	5.78
0	NEM 50 mg/L	4.55	
15	NEM 50 mg/L	4.52	4.54
45	NEM 50 mg/L	4.68	4.59
90	NEM 50 mg/L	4.82	4.88
180	NEM 50 mg/L	5.93	5.99
0	Nigericin 74.7 mg/L	4.57	
15	Nigericin 74.7 mg/L	4.76	4.77
45	Nigericin 74.7 mg/L	4.89	4.88
90	Nigericin 74.7 mg/L	4.95	5.01
180	Nigericin 74.7 mg/L	6.03	5.92

Table D.12 Soluble potassium data for *P. aeruginosa* NEM experiments presented in Figure 6.4.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control	9.04	8.95
15	Control	8.65	8.67
45	Control	8.76	9.32
90	Control	8.89	8.63
180	Control	8.39	8.32
0	NEM 50 mg/L	9.1	9.15
15	NEM 50 mg/L	13.44	13.58
45	NEM 50 mg/L	14.45	14.43
90	NEM 50 mg/L	14.82	14.68
180	NEM 50 mg/L	16.03	15.6
0	Nigericin 74.7 mg/L	9.15	9.27
15	Nigericin 74.7 mg/L	8.99	9.19
45	Nigericin 74.7 mg/L	9.58	9.72
90	Nigericin 74.7 mg/L	10.75	10.65
180	Nigericin 74.7 mg/L	16.89	16.45

Table D.13 Soluble potassium data for *P. aeruginosa* NEM experiment repeat.

Time (minutes)	Reactor	Soluble Potassium (mg/L)	Soluble Potassium (mg/L)
0	Control	0.9	0.78
10	Control	1.43	1.43
30	Control	1.45	1.29
60	Control	1.24	1.18
90	Control	0.91	0.84
0	NEM 50 mg/L	0.99	0.81
10	NEM 50 mg/L	3.57	3.31
30	NEM 50 mg/L	3.72	3.67
60	NEM 50 mg/L	3.95	3.83
90	NEM 50 mg/L	3.98	3.87
0	Nigericin 200 mg/L	0.81	0.85
10	Nigericin 200 mg/L	2.15	2.53
30	Nigericin 200 mg/L	2.57	2.82
60	Nigericin 200 mg/L	2.8	2.66
90	Nigericin 200 mg/L	2.73	2.81

Table D.14 Live/Dead stain results for *N. europaea, Ni. moscoviensis,* and *P. aeruginosa* NEM potassium efflux experiments. Numbers indicate the number of cells.

Reactor	*N. europaea*	*N. moscoviensis*	*P. aeruginosa*
Live Results			
Control 1	35	4	201
Control 2	36	3	159
Control 3	71	16	193
NEM 1	46	6	165
NEM 2	33	5	198
NEM 3	38	6	228
Nigericin 1	60	6	153
Nigericin 2	41	7	109
Nigericin 3	51	5	77
Dead Results			
Control 1	8	2	1
Control 2	2	1	4
Control 3	4	4	2
NEM 1	2	2	4
NEM 2	1	2	3
NEM 3	4	3	6
Nigericin 1	0	1	5
Nigericin 2	0	2	3
Nigericin 3	1	5	3
Total Percent Live			
Control	91.0	76.7	98.8
NEM	94.4	70.8	97.8
Nigericin	99.3	69.2	96.9

Table D.15 Soluble potassium data for *N. europaea* chlorine experiments presented in Figure 6.5.

Time (minutes)	Reactor	Reactor 1 (mg K+/L)	Reactor 2 (mg K+/L)	Reactor 3 (mg K+/L)
0	Control	3.53	3.39	
15	Control	3.6	3.57	
30	Control	3.57	3.52	
60	Control	3.72	3.85	
90	Control	3.76	3.64	
0	Cl_2 1 mg/L	3.48	3.45	3.5
15	Cl_2 1 mg/L	3.46	3.42	3.64
30	Cl_2 1 mg/L	3.47	3.54	3.54
60	Cl_2 1 mg/L	3.79	3.82	3.87
90	Cl_2 1 mg/L	3.68	3.69	3.68
0	Cl_2 5 mg/L	3.59	3.58	3.44
15	Cl_2 5 mg/L	3.58	3.51	3.42
30	Cl_2 5 mg/L	3.59	3.53	3.69
60	Cl_2 5 mg/L	3.84	3.77	3.83
90	Cl_2 5 mg/L	3.86	3.74	3.85

Table D.16 Soluble potassium data for *P. aeruginosa* chlorine experiments presented in Figure 6.6.

Time (minutes)	Reactor	Reactor 1 (mg K+/L)	Reactor 2 (mg K+/L)	Reactor 3 (mg K+/L)
0	Control	4.32	4.23	
15	Control	3.69	3.76	
30	Control	3.41	3.44	
60	Control	3.1	3.22	
90	Control	3.03	3	
0	Cl_2 1 mg/L	4.37	4.23	4.42
15	Cl_2 1 mg/L	4.71	4.62	4.73
30	Cl_2 1 mg/L	5.01	4.92	5.07
60	Cl_2 1 mg/L			
90	Cl_2 1 mg/L	5.03	5.1	5.11
0	Cl_2 5 mg/L	4.46	4.42	4.38
15	Cl_2 5 mg/L	7.5	7.58	7.43
30	Cl_2 5 mg/L	7.88	7.76	7.75
60	Cl_2 5 mg/L	8.35	8	8.19
90	Cl_2 5 mg/L	8.28	8.23	8.42

Table D.17 Live/Dead stain results for *N. europaea* and *P. aeruginosa* potassium efflux experiments with chlorine bleach. Numbers indicate the number of cells.

Reactor	*N. europaea*	*P. aeruginosa*
Live Cells		
Control 1	20	29
Control 2	14	26
Control 3	18	22
1 mg/L Cl_2 1	15	
1 mg/L Cl_2 2	18	28
1 mg/L Cl_2 3	16	37
5 mg/L Cl_2 1	24	15
5 mg/L Cl_2 2	33	22
5 mg/L Cl_2 3	25	23
Dead Cells		
Control 1	5	1
Control 2	4	1
Control 3	4	1
1 mg/L Cl_2 1	2	
1 mg/L Cl_2 2	5	2
1 mg/L Cl_2 3	3	1
5 mg/L Cl_2 1	4	2
5 mg/L Cl_2 2	10	1
5 mg/L Cl_2 3	8	2
Total Percent Live		
Control	79.9	96.2
1 mg/L Cl_2	83.6	95.4
5 mg/L Cl_2	79.4	92.0

Table D.18 Glutathione results for *N. europaea* experiments with chlorine bleach presented in Figures 6.7 and 6.8.

Free Chlorine (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione (nmoles/10^{10} cells)
0	0.188	0.184	7.00
1	0.163	0.198	12.55
5	0.192	0.200	13.43
10	0.203	0.212	9.82
25	0.226	0.250	6.44
200 mg/L Chloramphenicol	0.189	0.195	8.73

Table D.19 Glutathione results for *N. europaea* repeat experiments with chlorine bleach.

Free Chlorine (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione Sample 1 (nmoles/10^{10} cells)	Total Glutathione Sample 2 (nmoles/10^{10} cells)
0	0.100		8.42	13.44
1	0.106	0.142	7.07	7.54
5	0.106	0.152	6.11	12.74
10	0.117	0.165	5.07	13.91
25	0.138	0.174	13.38	3.92

Table D.20 Glutathione results for *N. europaea* experiments with NEM.

NEM (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione Sample 1 (nmoles/10^{10} cells)	Total Glutathione Sample 2 (nmoles/10^{10} cells)
0	0.100	0.160	8.42	13.44
10	0.061	0.135	6.66	4.27
50	0.051	0.135		6.88
100	0.071	0.137	2.58	4.05
200	0.083	0.123	4.30	8.01

Table D.21 Glutathione results for *P. aeruginosa* experiments with chlorine bleach presented in Figures 6.7 and 6.8.

Free Chlorine (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione (nmoles/10^{10} cells)
0	0.143	0.140	12.15
1	0.145	0.148	10.74
5	0.153	0.164	13.02
10	0.176	0.216	13.76
25	0.170	0.165	7.23
200 mg/L Chloramphenicol	0.170	0.178	10.14

Table D.22 Glutathione results for *P. aeruginosa* repeat experiments with chlorine bleach.

Free Chlorine (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione Sample 1 (nmoles/10^{10} cells)	Total Glutathione Sample 2 (nmoles/10^{10} cells)
0	0.079	0.143	6.10	7.12
1	0.080	0.175	8.33	6.99
5	0.104	0.179	12.94	13.32
10	0.110		4.50	7.91
25		0.258	17.96	8.48

Table D.23 Glutathione results for *P. aeruginosa* experiments with NEM.

NEM (mg/L)	Oxidized Glutathione Sample 1 (nmoles/10^{10} cells)	Oxidized Glutathione Sample 2 (nmoles/10^{10} cells)	Total Glutathione Sample 1 (nmoles/10^{10} cells)	Total Glutathione Sample 2 (nmoles/10^{10} cells)
0	0.079	0.143	6.10	7.12
10	0.083	0.123	7.46	9.47
50	0.085	0.238	5.73	7.59
100	0.089	0.138	5.49	3.10
200	0.123	0.139	4.77	6.52

Appendix E: Data from Chapter 7

Table E.1 Saturated fatty acids concentrations for *N. europaea* cells exposed to 1-octanol (Trial 1).

Reactor	Time (minutes)	C 14:0		C 16:0		C 18:0		C 20:0	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.102	0.084	24.248	25.445	0.366	0.318	0.011	0.026
	2	0.090	0.123	25.037	23.760	0.327	0.330	0.017	0.023
	10	0.094	0.099	24.999	24.762	0.312	0.316	0.016	0.018
	30	0.082	0.088	23.864	24.682	0.294	0.308	0.013	0.011
	60	0.095	0.088	27.210	25.099	0.332	0.315	0.018	0.012
	240	0.156	0.160	44.518	46.096	0.520	0.498	0.017	0.018
5 mg/L Octanol	0	0.095	0.081	24.749	25.790	0.350	0.318	0.016	0.030
	2	0.088	0.090	25.248	24.040	0.343	0.309	0.024	0.024
	10	0.085	0.087	26.074	25.532	0.321	0.323	0.017	0.009
	30	0.081	0.092	23.160	24.657	0.296	0.315	0.013	0.010
	60	0.090	0.101	24.875	26.457	0.321	0.338	0.012	0.018
	240	0.140	0.160	37.941	33.587	0.424	0.444	0.014	0.027
50 mg/L Octanol	0	0.082	0.093	25.033	23.309	0.302	0.403	0.012	0.013
	2	0.088	0.082	25.436	24.809	0.332	0.324	0.018	0.012
	10	0.091	0.092	24.261	24.750	0.295	0.319	0.012	0.013
	30	0.085	0.093	23.669	26.318	0.304	0.285	0.013	0.016
	60	0.097	0.098	27.267	26.571	0.348	0.327	0.010	0.009
	240	0.146	0.157	34.577	37.151	0.393	0.444	0.019	0.017
200 mg/L Octanol	0	0.085	0.086	23.001	23.874	0.294	0.296	0.012	0.022
	2	0.090	0.095	23.401	24.540	0.297	0.311	0.020	0.005
	10	0.087	0.076	24.892	21.957	0.325	0.270	0.008	0.006
	30	0.095	0.096	26.628	26.339	0.337	0.342	0.008	0.014
	60	0.110	0.090	27.479	24.140	0.347	0.314	0.010	0.008
	240	0.164	0.165	37.903	40.032	0.473	0.481	0.023	0.017

Table E.2 C18 Unsaturated fatty acids concentrations for *N. europaea* cells exposed to 1-octanol (Trial 1).

Reactor	Time (minutes)	C 18:1 trans		C 18:1 cis		C 18:2 trans		C 18:2 cis	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.009	0.015	0.498	0.559	0.008	0.019	0.532	0.729
	2	0.037	0.032	0.542	0.539	0.022	0.015	0.649	0.656
	10	0.036	0.034	0.589	0.548	0.012	0.013	0.710	0.693
	30	0.035	0.035	0.518	0.553	0.017	0.010	0.669	0.714
	60	0.041	0.036	0.575	0.511	0.020	0.016	0.738	0.621
	240	0.079	0.060	0.820	0.818	0.019	0.013	0.897	0.942
5 mg/L Octanol	0	0.028	0.033	0.550	0.578	0.021	0.020	0.671	0.751
	2	0.034	0.034	0.583	0.552	0.019	0.015	0.718	0.680
	10	0.028		0.583	0.545	0.022	0.015	0.755	0.736
	30	0.041	0.034	0.533	0.538	0.020	0.021	0.658	0.700
	60	0.027		0.542	0.585	0.017	0.018	0.704	0.759
	240	0.053		0.758	0.636	0.033	0.011	0.892	0.800
50 mg/L Octanol	0	0.026	0.043	0.561	0.638	0.013	0.005	0.718	0.730
	2	0.030	0.038	0.554	0.549	0.011	0.014	0.744	0.729
	10		0.022	0.531	0.545	0.025	0.011	0.678	0.685
	30	0.039	0.040	0.523	0.572	0.018	0.014	0.657	0.717
	60	0.033	0.031	0.573	0.563	0.018	0.011	0.764	0.725
	240	0.055	0.044	0.647	0.706	0.028	0.040	0.848	0.890
200 mg/L Octanol	0	0.032	0.027	0.512	0.503	0.015	0.016	0.649	0.637
	2	0.025	0.031	0.533	0.546	0.008	0.015	0.654	0.672
	10	0.034	0.030	0.568	0.524	0.012	0.014	0.687	0.593
	30	0.025	0.041	0.591	0.630	0.015	0.018	0.747	0.754
	60	0.036	0.033	0.690	0.564	0.012	0.010	0.770	0.655
	240	0.037	0.046	0.611	0.682	0.013	0.028	0.700	0.786

Table E.3 Nitrite concentrations over time for *N. europaea* cells exposed to 1-octanol (Trial 1).

Time (min.)	Control 1	Control 2	5 mg/L Octanol 1	5 mg/L Octanol 2	50 mg/L Octanol 1	50 mg/L Octanol 2	200 mg/L Octanol 1	200 mg/L Octanol 2
0	29.0	29.7	30.0	30.5	24.9	24.3	11.7	11.6
2	15.8	17.0	26.4	26.4	22.8	23.5	26.2	27.8
10	30.7	30.6	29.9	29.8	30.2	32.7	27.9	23.6
30	70.4	70.2	43.0	43.0	43.5	47.3	31.0	29.8
60	98.0	98.0	59.8	59.5	69.1	69.5		33.6
240	740.7	733.2	827.1	822.7	414.0	409.0	375.9	378.6

Table E.4 Saturated fatty acids concentrations for *P. aeruginosa* cells exposed to 1-octanol (Trial 1).

Reactor	Time (minutes)	C 14:0		C 16:0		C 18:0		C 20:0	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.798	0.869	43.170	46.152	1.557	1.623	0.013	
	2	0.842	0.861	44.581	45.407	1.549	1.257	0.026	
	10	0.910	0.822	47.084	41.893	1.625	1.437	0.013	
	30	0.976	0.954	46.802	45.682	1.569	1.510	0.057	
	60	1.025	1.015	46.016	45.750	1.392	1.407	0.053	
	240	1.145	1.104	45.186	43.509	1.135	1.033		
5 mg/L Octanol	0	0.779	0.836	42.552	45.259	1.499	1.609		
	2	0.878	0.708	46.589	37.538	1.624	1.334		
	10	0.891	0.896	46.182	46.221	1.611	1.631		
	30	0.983	0.996		48.321	1.625	1.602		
	60	1.017	1.013	46.320	45.943	1.080	1.430		
	240	1.212	1.277	48.522	51.162	1.237	1.307		
50 mg/L Octanol	0	0.846	0.879	45.147	46.889	1.569	1.668	0.156	
	2	0.926	0.927	48.174	48.013	1.713	1.715	0.159	0.167
	10	0.927		47.370	36.155	1.634	1.248	0.166	0.089
	30	1.091	0.974	53.432	47.632	1.777	1.573	0.071	0.110
	60	1.023	1.030	47.262	47.325	1.422	1.477	0.087	0.109
	240	1.258	1.271	49.673	50.088	1.274	1.300	0.098	0.099
200 mg/L Octanol	0	0.410	0.414	22.571	22.726	0.780	0.808	0.078	0.107
	2	0.660	0.557	44.720	45.722	1.622	1.690	0.102	0.085
	10	0.656	0.707	43.324	43.170	1.525	1.526	0.115	0.142
	30	0.887	0.852	45.285	42.812	1.558	1.480	0.116	0.143
	60	0.785	0.862	43.293	42.178	1.381	1.347	0.099	0.153
	240	1.235	1.242	49.422	49.991	1.290	1.285	0.133	0.142

Table E.5 C18 Unsaturated fatty acids concentrations for *P. aeruginosa* cells exposed to 1-octanol (Trial 1).

Reactor	Time (minutes)	C 18:1 trans		C 18:1 cis		C 18:2 trans		C 18:2 cis	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	1.058	0.725	15.135	16.485	0.087	0.175	19.898	19.448
	2	0.826	0.806	16.308	16.762	0.228	0.266	18.575	19.318
	10	0.727	0.683	18.284	16.207	0.299	0.323	20.019	16.234
	30	0.941	0.706	20.410	20.407	0.157	0.299	19.805	19.596
	60	0.904	0.732	23.402	23.262	0.262	0.207	19.622	19.336
	240	2.107	1.070	31.189	30.889	0.189	0.226	21.354	21.008
5 mg/L Octanol	0	0.530	0.286	15.138	16.362	0.246	0.331	17.286	18.922
	2	0.542	0.174	17.035	13.830	0.305	0.368	20.119	15.135
	10	0.573	0.215	17.414	17.792	0.331	0.450	19.802	18.941
	30	0.520	0.229	20.201	20.837	0.227	0.431	20.024	20.800
	60	0.476	0.241	22.606	22.773	0.335	0.448	20.852	20.474
	240	0.891	0.521	33.842	36.198	0.376	0.491	23.368	24.962
50 mg/L Octanol	0	0.125	0.084	17.492	17.743	0.608	0.697	16.418	19.297
	2	0.155	0.081	18.936	18.328	0.532	1.138	20.366	20.387
	10	0.102	0.069	19.265	14.482	0.507	0.651	19.807	13.845
	30	0.119	0.092	24.048	21.448	0.566	0.657	21.867	20.062
	60	0.120	0.109	24.465	24.198	0.579	0.696	19.978	21.119
	240	0.179	0.138	37.261	37.522	0.705	0.867	23.571	24.053
200 mg/L Octanol	0	0.043	0.054	8.022	8.050	0.562	0.660	9.524	9.433
	2	0.087	0.085	15.938	15.749	0.956	1.536	19.410	18.680
	10	0.084	0.075	15.515	15.476	0.962	1.071	17.896	16.957
	30	0.071	0.082	16.605	15.481	0.925	0.949	20.376	19.404
	60	0.079	0.076	17.230	16.504	0.841	0.986	19.878	19.319
	240	0.130	0.128	31.673	31.993	1.092	1.237	23.230	24.149

Table E.6 Live/Dead stain results for *N. europaea* and *P. aeruginosa* membrane experiments (Trial 1). Values indicate the number of cells.

Reactor	*N. europaea*	*P. aeruginosa*
Live Cells		
Control 1		
Control 2		
Control 3		
5 mg/L Octanol 1		
5 mg/L Octanol 2		
5 mg/L Octanol 3		
50 mg/L Octanol 1		
50 mg/L Octanol 2		
50 mg/L Octanol 3		
200 mg/L Octanol 1		
200 mg/L Octanol 2		
200 mg/L Octanol 3		
Dead Cells		
Control 1		
Control 2		
Control 3		
5 mg/L Octanol 1		
5 mg/L Octanol 2		
5 mg/L Octanol 3		
50 mg/L Octanol 1		
50 mg/L Octanol 2		
50 mg/L Octanol 3		
200 mg/L Octanol 1		
200 mg/L Octanol 2		
200 mg/L Octanol 3		
Total Percent Live		
Control		
5 mg/L Octanol		
50 mg/L Octanol		
200 mg/L Octanol		

Table E.7 Saturated fatty acids concentrations for *N. europaea* cells exposed to 1-octanol (Trial 2).

Reactor	Time (minutes)	C 14:0		C 16:0		C 18:0		C 20:0	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.030	0.034	1.076	1.258	0.168	0.114	0.050	0.038
	2	0.031	0.020	1.113	0.908	0.111	0.053	0.030	0.027
	10	0.022	0.023	0.809	0.952	0.049	0.085	0.024	0.021
	30	0.026	0.102	1.018	2.301	0.096	0.568	0.023	0.037
	60	0.024	0.031	1.042	1.167	0.105	0.115	0.014	0.019
	180	0.034	0.032	1.282	1.246	0.064	0.105	0.011	0.023
5 mg/L Octanol	0	0.062	0.032	1.216	1.004	0.213	0.092	0.017	0.005
	2	0.023	0.107	1.085	2.671	0.078	0.833	0.014	0.047
	10	0.036	0.033	1.334	1.039	0.220	0.190	0.028	0.019
	30	0.063	0.066	1.146	1.324	0.102	0.121	0.016	0.026
	60	0.033	0.025	1.016	0.976	0.088	0.077	0.024	0.021
	180	0.026	0.027	1.211	1.477	0.105	0.164	0.019	0.021
50 mg/L Octanol	0	0.022	0.019	1.089	0.922	0.055	0.136	0.000	0.002
	2	0.022	0.022	1.087	1.236	0.146	0.120	0.001	0.002
	10	0.021	0.017	1.087	0.752	0.088	0.066	0.000	0.001
	30	0.026	0.023	1.089	1.009	0.058	0.076	0.001	
	60	0.023	0.024	1.022	0.994	0.104	0.054		0.002
	180	0.029	0.043	1.428	1.630	0.130	0.155	0.000	0.002
200 mg/L Octanol	0	0.022	0.019	0.842	1.055	0.101	0.103	0.002	0.002
	2	0.044	0.026	1.476	1.154	0.222	0.093	0.001	
	10	0.023	0.024	0.893	1.106	0.087	0.079	0.000	
	30	0.030	0.026	1.258	1.314	0.152	0.103		
	60	0.028	0.024	1.015	0.938	0.111	0.152		0.002
	180	0.047	0.039	1.612	1.474	0.126	0.144	0.002	0.001

Table E.8 C18 Unsaturated fatty acids concentrations for *N. europaea* cells exposed to 1-octanol (Trial 2).

Reactor	Time (minutes)	C 18:1 trans mg/L-1	C 18:1 trans mg/L-2	C 18:1 cis mg/L-1	C 18:1 cis mg/L-2	C 18:2 trans mg/L-1	C 18:2 trans mg/L-2	C 18:2 cis mg/L-1	C 18:2 cis mg/L-2
Control	0	0.055	0.018	0.387	0.373	0.018	0.016	0.582	0.673
	2	0.059	0.023	0.371	0.283	0.016	0.012	0.596	0.523
	10	0.025	0.027	0.246	0.294	0.009	0.007	0.462	0.545
	30	0.031	0.200	0.337	0.464	0.011	0.017	0.578	0.690
	60	0.031	0.053	0.339	0.423	0.009	0.013	0.524	0.631
	180	0.050	0.043	0.541	0.537	0.007	0.011	0.727	0.658
5 mg/L Octanol	0	0.029	0.045	0.403	0.340	0.022	0.009	0.582	0.566
	2	0.054	0.290	0.344	0.483	0.013	0.013	0.615	0.474
	10	0.046	0.015	0.435	0.333	0.015	0.011	0.659	0.500
	30	0.027	0.031	0.397	0.462	0.014	0.017	0.539	0.631
	60	0.069		0.389	0.352	0.012	0.007	0.545	0.545
	180	0.031	0.043	0.539	0.732	0.018	0.017	0.654	0.891
50 mg/L Octanol	0	0.025	0.018	0.380	0.298	0.008	0.003	0.673	0.466
	2	0.031	0.019	0.353	0.389	0.009	0.007	0.565	0.684
	10	0.018	0.012	0.355	0.233	0.009	0.008	0.639	0.431
	30		0.045	0.364	0.342	0.013	0.006	0.583	0.593
	60	0.024	0.015	0.335	0.332	0.010	0.009	0.525	0.477
	180	0.047	0.027	0.594	0.676	0.015	0.014	0.757	0.892
200 mg/L Octanol	0	0.019	0.021	0.267	0.354		0.004	0.463	0.606
	2	0.073	0.023	0.431	0.369	0.006	0.007	0.640	0.666
	10	0.022	0.023	0.297	0.368	0.007	0.006	0.512	0.600
	30	0.017	0.039	0.406	0.435	0.012	0.012	0.715	0.770
	60	0.016	0.013	0.347	0.397	0.009	0.013	0.541	0.528
	180	0.021	0.018	0.618	0.570	0.010	0.013	0.838	0.741

Table E.9 Nitrite concentrations over time for *N. europaea* cells exposed to 1-octanol (Trial 2).

Time (min.)	Control 1	Control 2	5 mg/L Octanol 1	5 mg/L Octanol 2	50 mg/L Octanol 1	50 mg/L Octanol 2	200 mg/L Octanol 1	200 mg/L Octanol 2
0	0.23	0.22	0.20	0.21	0.20	0.22	0.20	0.22
2	0.28	0.21	0.20	0.23	0.20	0.24	0.20	0.22
10	0.25	0.20	0.20	0.23	0.20	0.23	0.20	0.22
30	0.23	0.21	0.21	0.23	0.22	0.22	0.21	0.24
60	0.30	0.22	0.20	0.24	0.21	0.25	0.23	0.25
180	0.33	0.35	0.29	0.27	0.26	0.32	0.33	0.37

Table E.10 Saturated fatty acids concentrations for *P. aeruginosa* cells exposed to 1-octanol (Trial 2).

Reactor	Time (minutes)	C 14:0		C 16:0		C 18:0		C 20:0	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.651	0.563	42.786	36.611	1.656	1.337	0.034	0.032
	2	0.437	0.460	28.744	29.997	1.118	1.174	0.031	0.045
	10	0.580	0.525	37.555	34.115	1.513	1.364	0.042	0.039
	30	0.533	0.487	34.023	31.581	1.402	1.349	0.053	0.051
	60	0.571	0.672	36.424	43.175	1.603	1.879	0.050	0.055
	180	0.645	0.586	39.488	36.717	1.854	1.762	0.083	0.089
5 mg/L Octanol	0	0.620	0.588	40.491	37.556	1.822	1.767	0.096	0.066
	2	0.496	0.631	32.438	41.131	1.475	1.895	0.064	0.099
	10	0.609	0.632	38.903	40.941	1.932	2.043	0.125	0.180
	30	0.541	0.648	34.915	41.570	1.797	2.082	0.138	0.129
	60	0.575	0.647	36.609	40.510	1.958	2.233	0.181	0.169
	180	0.733	0.719	45.274	44.676	2.678	2.639	0.282	0.289
50 mg/L Octanol	0								
	2								
	10	0.656	0.904	41.544	41.073	1.634	1.596	0.065	0.037
	30	0.659	0.642	42.908	41.778	1.684	1.544	0.055	0.056
	60	0.678	0.680	43.754	42.988	1.745	1.707		0.051
	180	0.774	0.758	47.298	47.306	2.176	2.094	0.089	0.088
200 mg/L Octanol	0								
	2								
	10	0.648	0.639	42.832	41.677	1.575	1.539	0.037	0.047
	30	0.638	0.637	42.700	40.954	1.782	1.695	0.057	0.118
	60	0.693	0.656	45.002	42.995	1.986	2.032	0.083	0.083
	180	0.802	0.813	49.466	50.413	2.576	2.429	0.142	0.141

Table E.11 C18 Unsaturated fatty acids concentrations for *P. aeruginosa* cells exposed to 1-octanol (Trial 2).

Reactor	Time (minutes)	C 18:1 trans		C 18:1 cis		C 18:2 trans		C 18:2 cis	
		mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2	mg/L-1	mg/L-2
Control	0	0.098	0.072	10.104	8.706	0.130	0.138	20.893	17.246
	2	0.089	0.066	6.915	7.129	0.115	0.105	14.182	14.650
	10	0.075	0.093	9.106	8.266	0.149	0.106	17.677	16.821
	30	0.064	0.056	8.587	7.966	0.119	0.101	16.773	16.244
	60	0.098	0.093	9.850	11.680	0.156	0.203	16.636	19.939
	180	0.066	0.065	13.340	12.546	0.136	0.244	19.640	15.131
5 mg/L Octanol	0	0.071	0.093	9.566	8.890	0.139	0.113	20.422	19.213
	2	0.077	0.054	7.708	9.771	0.117	0.179	16.192	19.475
	10	0.088	0.074	9.363	8.769	0.156	0.173	18.497	19.811
	30	0.096	0.080	9.858	10.433	0.148	0.148	16.464	20.183
	60	0.091	0.116	9.945	10.931	0.167	0.198	17.160	17.924
	180	0.102	0.094	15.580	15.393	0.205	0.199	20.930	20.622
50 mg/L Octanol	0								
	2								
	10	0.154	0.080	9.824	9.600	0.144	0.143	17.249	17.744
	30	0.067	0.062	10.342	10.102	0.139	0.146	19.326	18.884
	60	0.064	0.089	11.386	11.207	0.161	0.152	18.114	18.174
	180	0.101	0.105	16.333	16.456	0.204	0.233	19.983	18.558
200 mg/L Octanol	0								
	2								
	10	0.126	0.080	9.848	9.987	0.273	0.140	14.738	20.231
	30	0.086	0.099	10.013	9.530	0.203	0.129	18.140	19.636
	60	0.093	0.091	10.736	10.266	0.194	0.206	19.690	17.926
	180	0.136	0.129	16.243	16.265	0.189	0.246	22.456	21.023

Table E.12 Live/Dead stain results for *N. europaea* and *P. aeruginosa* membrane experiments (Trial 2). Values indicate the number of cells.

Reactor	*N. europaea*	*P. aeruginosa*
Live Cells		
Control 1	22	242
Control 2	15	279
Control 3	26	212
200 mg/L Octanol 1	32	224
200 mg/L Octanol 2	44	201
200 mg/L Octanol 3	55	241
Dead Cells		
Control 1	6	9
Control 2	2	12
Control 3	5	7
200 mg/L Octanol 1	4	12
200 mg/L Octanol 2	8	5
200 mg/L Octanol 3	5	9
Total Percent Live		
Control	82.9	96.3
200 mg/L Octanol	88.5	96.2

VITA

Richard T. Kelly II is the oldest son of Richard T. and Jane E. Kelly. He was born and raised in central Pennsylvania. For his undergraduate education, Richard studied civil engineering at Bucknell University in Lewisburg, PA. Richard worked for the Pennsylvania Department of Transportation as a roadway construction inspector and for Gannett Fleming, Inc. as an engineering intern during summer breaks. While completing his degree became interested in environmental engineering and after graduating with a BSCE in 2000 began attending Virginia Tech as a master's student in the Department of Civil and Environmental Engineering. Richard received his MSCE in 2002 and remained at Virginia Tech to complete a Ph.D. in civil engineering. Richard focused on nitrification inhibition in wastewater treatment during his doctoral research and defended his dissertation on June 21, 2005.