INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

University of Alberta

Fungal Based Nitrogen and Phosphorus Concentration Reduction in Wastewater

by

Rodney Keith Guest ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

in

Environmental Science

Department of Civil and Environmental Engineering

Edmonton, Alberta

Fall 2005

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

0-494-08649-1

Your file Votre référence
ISBN:
Our file Notre reference
ISBN:

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Dedication

For my wife Tashsa

and

my children

Jaeden and Salena

Abstract

This research project investigated the development of a fungal nitrogen and phosphorus wastewater treatment system. Fungi were isolated from a unit process at the Gold Bar Wastewater Treatment Plant. A subset of the isolates were tested using batch reactors to determine the initial kinetic description of nitrogen and phosphorus decrease from wastewater. Test conditions evaluated included: aerobic, microaerophilic and anoxic atmospheres; pH; dilute wastewater; mixed culture; and successive inoculations. Additional testing was performed using batch and fed-batch attached growth systems with surface to volume ratios of 25 and 50 m^2 m^{-3}. It was found that the fungi isolated were capable of a single-step direct ammonium (NH_4^+) concentration decrease pathway. This nitrogen decrease system was most active using a microaerophilic environment. A zero-order kinetic model best fit the NH_4^+ decrease data. Calculated NH_4^+ substrate utilisation rates ranged between 0.033 to 0.08 mg-N L^{-1} hr^{-1} for the five fungal species tests. However, with suspended growth conditions NH_4^+ decrease suffered from variable performance. The attached growth NH_4^+ decrease tests saw a 50% decrease in the variation of the NH_4^+ utilisation rates. NH_4^+ utilisation rates observed for the attached growth decrease were also in the range of 0.031 to 0.076 mg-N L^{-1} hr^{-1}. Fungi were shown to be capable of phosphate (o-PO_4^{3-}) concentration decrease from wastewater. It was also found that the oxygen tension has a clear role in fungal o-PO_4^{3-} decrease. Fungi will reduce the concentration of o-PO_4^{3-} under all three oxygen tensions tested. However, the mechanism of uptake and final biochemical destination of o-PO_4^{3-} cannot be stated with the data collected. However the data support different mechanisms for the

concentration decrease, especially for anoxic uptake and that energy requirements were substantially less than conventional bacterial o-PO_4^{3-} systems. The COD to o-PO_4^{3-} decrease ratio was estimated to be 6.5 g COD : 1 g of o-PO_4^{3-}. The major issue for further development of fungal nitrogen and phosphorus treatment technology, the slow substrate utilisation rates, may be addressed by increasing the microbial mass.

Acknowledgement

A doctoral dissertation is never a singularly individual accomplishment. There are a number of people I would like to acknowledge for their help and contributions during my studies.

First, I would like to thank Dr. Daniel W. Smith my supervisor. His drive for original research in the field of environmental engineering lead to the development of fungal based nitrogen decrease topic. While always being available and willing to provide guidance, support and discussion, he also allowed freedom to develop and execute the research. I will always be grateful for his mentorship and providing me the opportunity to work on many interesting projects.

Words cannot express my love and gratitude for my wife Tashsa. Without her support and hard work, I could have never had the dedicated time necessary to complete my research. She has been a "single parent" for the last three years raising our children; I only hope one day to repay her dedication. Although my children Jaeden and Salena are young, I hope they can one day understand and forgive my absentee parenting.

I would also like to thank my friends and colleagues Khosrow Farahbakhsh and Clark Svrcek for their support, encouragement and the departmental technical staff Nick Chernuka, Maria Demeter and Gary Solonynko for their time and assistance in the laboratory.

Without financial and material support from several institutions, this research would not have been possible. I would like to thank Alberta Ingenuity Centre for Water Research, Natural Sciences and Engineering Research Council of Canada by

way of a Discovery Grant provide to Dr. Daniel W. Smith, and City of Edmonton through the creative sentencing order of the Honourable Judge Caffaro, Assistant Chief Judge of the Provincial Court of Alberta, for their financial contributions; the City of Edmonton Gold Bar Wastewater Treatment Plant for their wastewater supply and Zenon Environmental for providing hollow fibre membrane material.

Table of Contents

Chapter One 1
- 1.0 Introduction 1
  - 1.1 Overview and Problem Statement 1
  - 1.2 Research Hypotheses and Objectives 3
  - 1.3 Research Approach 4

Chapter Two 6
- 2.0 Literature Review: Potential for Using Fungus for Ammonia Nitrogen Treatment in Wastewater 6
  - 2.1 Fungal Nitrification 6
  - 2.2 Potential Benefits of Fungal Nitrification 8
  - 2.3 Fungal Denitrification 10
  - 2.4 Fungal Denitrification Rates and Stoichiometry 15
  - 2.5 Key Points of Fungal Denitrification 16
  - 2.6 One Microorganism for Both Nitrification and Denitrification 16
  - 2.7 Fungi Used for Wastewater Treatment 17
  - 2.8 Causes of Filamentous Bulking of Activated Sludge 20
  - 2.9 Phosphorus Uptake and Utilization in Fungi 22
  - 2.10 Summary 26

Chapter Three 28
- 3.0 Materials and Methods 28
  - 3.1 Isolation and Screening of Fungi from Gold Bar WWTP 28
    - 3.1.1 Isolation and Storage of Fungi Isolates 28
    - 3.1.2 Screening for Fungal Nitrogen Concentration Decrease 28
      - 3.1.2.1 Batch Culture Apparatus 28
      - 3.1.2.2 Preparation of Primary Effluent Medium for Screening 30
      - 3.1.2.3 Inoculum Preparation and Batch Trials 30
    - 3.1.3 Fungal Identification 31
  - 3.2 Batch Kinetic Screening 31
    - 3.2.1 Reactor Setup and Apparatus 31
      - 3.2.1.1 Preparation of Primary Effluent Medium 32
      - 3.2.1.2 Inoculum Preparation and Batch Trials 33
      - 3.2.1.3 Inoculum Preparation for Recovered Fungal Isolates 33
      - 3.2.1.4 Inoculum Preparation for Mixed Culture Experiments 34
  - 3.3 Attached-growth and Fed-batch Attached-growth 34
    - 3.3.1 Reactor Setup and Apparatus 34
  - 3.4 Analytical Methods 35
    - 3.4.1 Ion Chromatography 35
    - 3.4.2 COD Measurements 36
    - 3.4.3 Nitrogen Analysis 36
      - 3.4.3.1 Ammonium measurements 36
      - 3.4.3.2 Total Kjeldahl Nitrogen (TKN) 37
    - 3.4.4 Total Suspended Solids (TSS) and Total Solids (TS) 37

Chapter Four 38

4.0 Experimental Results 38
  4.1 Isolation and Screening of Fungi to Determine Potential for Ammonia Nitrogen Concentration Decrease from Wastewaters 38
    4.1.1 Sampling 38
    4.1.2 Screening Isolates for Nitrogen and Phosphorus Concentration Decrease 39
  4.2 Batch Kinetic Ammonium Concentration Decrease Studies 45
    4.2.1 Suspended-growth 45
      4.2.1.1 Oxygen Effects on the Decrease of NH_4^+ Concentration in Wastewater 45
      4.2.1.2 Fate of Ammonium 53
      4.2.1.3 Estimation of pH Range for Fungal Ammonia Concentration Decrease 59
      4.2.1.4 Dilute Wastewater Effects 62
      4.2.1.5 Successive Inoculation Culture tests 67
      4.2.1.6 Mixed Culture 71
    4.2.2 Attached-growth 72
      4.2.2.1 Fate of Ammonium in Attached-Growth Reactor Systems 78
      4.2.2.2 Fed-batch Attached-Growth Systems 81
  4.3 Batch Kinetic Phosphate Concentration Decrease Studies 85
    4.3.1 Suspended-growth 85
      4.3.1.1 Oxygen Effects on the Concentration Decrease of Phosphate in Wastewater 88
      4.3.1.2 Dilute Wastewater Effects 98
      4.3.1.3 Successive Inoculation and Mixed Culture Tests 102
    4.3.2 Attached-Growth 105
Chapter Five 108
5.0 Discussion of Results 108
  5.1 Isolation and Screening Results 108
    5.1.1 Identified Isolates 108
    5.1.2 Fate and Decrease of Ammonium 109
    5.1.3 Concentration Decrease of Phosphorus 111
  5.2 Kinetics of Ammonium Concentration Decrease in Wastewater By Fungi 113
    5.2.1 Simultaneous Nitrification-Denitrification or Direct Ammonium Nitrogen Decrease 114
    5.2.2 Fungal Ammonium Concentration Decrease Kinetics and Stoichiometry 118
    5.2.3 Testing the Range of Fungal Denitrification 122
    5.2.4 Improving Fungal Ammonium Concentration Decrease 124
    5.2.5 Potential for Development of Fungal Nitrogen Treatment Technology 125
  5.3 Concentration Decrease of Phosphate From Wastewater By Fungi 127
    5.3.1 Correlation of Initial Phosphate Concentration with Observed Decrease 127
    5.3.2 Oxygen Tension Impacts on Phosphate Concentration Decrease 128

5.3.3 Potential Development of Fungal Phosphate Treatment Technology 131
Chapter Six 133
6.0 Conclusions and Recommendations 133
6.1 Conclusions 133
6.2 Recommendations 135
Chapter Seven 138
7.0 References 138
Appendix A Summary of Data 143

List of Tables

Table 2-1 Chemical distribution of phosphorus in actively growing fungal cells. 23
Table 4-1 Estimated yeast and fungi concentration found in Gold Bar WWTP. 39
Table 4-2 Average initial primary effluent wastewater medium parameters. 39
Table 4-3 Results of screening isolates from the pre-anoxic zone Gold Bar WWTP BNR. 40
Table 4-4 Result of screening isolates from the anaerobic zone Gold Bar WWTP BNR. 40
Table 4-5 Screening result of isolates from the anoxic zone Gold Bar WWTP BNR. 41
Table 4-6 Screening results of isolates from the aerobic zone Gold Bar WWTP BNR. 41
Table 4-7 Identified isolates and mean percent (%) concentration decrease of ammonia and o-phosphate. 44
Table 4-8 Observed NH_4^+ kinetic rate and percent decrease for various fungi under three oxygen tensions 50
Table 4-9 Suspended culture growth rates for three fungal species under three oxygen environments. 53
Table 4-10 Fungal growth rates for two pH conditions tested 62
Table 4-11 Mean parameters for undiluted and diluted primary wastewater medium. 63
Table 4-12 Observed growth rate for fungi growing on dilute wastewater. 64
Table 4-13 Percent ammonium decrease for two dilute sewage runs 64
Table 4-14 Ammonium utilization rates and percent decrease for successive inoculations of various fungi. 68
Table 4-15 Mixed culture ammonium degradation rate and percent decrease. 72
Table 4-16 Comparison of surface area impact on NH_4^+ r_{su} constant. 77
Table 4-17 Comparison of surface area impact on NH_4^+ percent decrease. 77

Table 4-18 Comparison of zero-order kinetic rate constants observed for suspended- or attached-growth systems 78

Table 4-19 Comparison NH_4^+ decrease observed for suspended- or attached-growth systems 78

Table 5-1 Elemental composition of bacteria, yeasts and fungi 111

Table 5-2 Estimated NH_4^+-nitrogen assimilated for growth of new biomass 111

Table 5-3 Gibbs free energy for DAND pathway scenarios at pH 7.0 118

Table 5-4 Estimated microaerophilic μ_{max} for fungal NH_4^+ decrease based on substrate utilization rate. 120

Table 7-1 Summary of suspended-growth data for *Geotrichum* sp. (2000) 144

Table 7-2 Summary of suspended-growth data for *Geotrichum* sp. (2007) 145

Table 7-3 Summary of suspended-growth data for yeast 146

Table 7-4 Summary of suspended-growth data for *Penicillium* sp 147

Table 7-5 Summary of suspended-growth data for *Phoma* sp 148

Table 7-6 Summary of suspended-growth data for Mixed Culture experiments 149

Table 7-7 Summary of attached-growth ammonium and phosphate concentration decrease. 150

List of Figures

Figure 2-1 Bacterial nitrification pathway. 8

Figure 2-2 Fungal nitrification. 9

Figure 2-3 Biochemical pathway for bacterial denitrification. 11

Figure 2-4 Fungal denitrificaiton biochemcial pathways. 13

Figure 3-1 Batch screening single reactor apparatus. 29

Figure 3-2 Reactor schematic for batch kinetic testing. 32

Figure 3-3 Attached-growth batch reactor schematic. 35

Figure 4-1 Ammonium concentration decrease by suspended *Geotrichum* sp. (2000) culture under three oxygen tension levels. 46

Figure 4-2 Ammonium concentration decreases by suspended *Geotrichum* sp. (2007) culture under three oxygen tension levels. 47

Figure 4-3 Ammonium concentration decreases by suspended *Penicillium sp.* (3004) culture under three oxygen tension levels. 47

Figure 4-4 Ammonium concentration decreases by suspended *Phoma* sp. (3013) culture under three oxygen tension levels. 48

Figure 4-5 Ammonium concentration decreases by suspended by yeast species (2008) culture under three oxygen tension levels. 48

Figure 4-6 Suspended microaerophilic culture nitrogen species plot for *Geotrichum* sp. (2000) 54

Figure 4-7 Suspended microaerophilic culture nitrogen species plot for *Geotrichum* sp. (2007) 55

Figure 4-8 Suspended microaerophilic culture nitrogen species plot for yeast species. 55

Figure 4-9 Suspended microaerophilic culture nitrogen species plot for *Penicillium* sp. 56

Figure 4-10 Suspended microaerophilic culture nitrogen species plot for *Phoma* sp. 56

Figure 4-11 Ammonium volatilization control data at various pH 58

Figure 4-12 Ammonium volatilization control pH change time profile. 58

Figure 4-13 Impact of initial pH change on ammonium decrease by *Geotrichum* sp. (2000) 60

Figure 4-14 Impact of initial pH change on ammonium decrease by *Geotrichum* sp. (2007) 60

Figure 4-15 Impact of initial pH change on ammonium decrease by yeast..... 61

Figure 4-16 Impact of initial pH change on ammonium decrease by *Penicillium* sp..... 61

Figure 4-17 Impact of initial pH change on ammonium decrease by *Phoma* sp..... 62

Figure 4-18 Ammonium concentration decrease in dilute sewage for *Geotrichum* sp. (2000) 64

Figure 4-19 Ammonium concentration decrease in dilute sewage for *Geotrichum* sp. (2007) 65

Figure 4-20 Ammonium concentration decrease in dilute sewage for Yeast. 65

Figure 4-21 Ammonium concentration decrease in dilute sewage for *Penicillium* sp..... 66

Figure 4-22 Ammonium concentration decrease in dilute sewage for *Phoma* sp..... 66

Figure 4-23 Ammonium concentration decrease for three successive inoculations of *Geotrichum* sp. (2000) 68

Figure 4-24 Ammonium concentration decrease for three successive inoculations of *Geotrichum* sp. (2007) 69

Figure 4-25 Ammonium concentration decrease for three successive inoculations a yeast species. 69

Figure 4-26 Ammonium concentration decrease for three successive inoculations of *Penicillium* sp..... 70

Figure 4-27 Ammonium concentration decrease for three successive inoculations of *Phoma* sp..... 70

Figure 4-28 Mixed fungal culture ammonium concentration decrease in wastewater. 72

Figure 4-29 Ammonium decrease in batch 25 m^2/m^3 fungal attached-growth systems. 73

Figure 4-30 NH_4^+ Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Geotrichum* sp. (2000) 75

Figure 4-31 NH_4^+ Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Penicillium* sp. 75

Figure 4-32 NH_4^+ Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Phoma* sp. 76

Figure 4-33. Nitrogen species observed during NH_4^+ decrease test using *Penicillium sp* 79

Figure 4-34. Nitrogen species observed during NH_4^+ decrease test using *Phoma sp* 80

Figure 4-35 Nitrogen species observed during NH_4^+ decrease test using *Geotrichum sp.* (2000) 80

Figure 4-36 Fed-batch 50 m^2/m^3 attached-growth system for *Penicillium* sp. 82

Figure 4-37 Fed-batch 50 m^2/m^3 attached-growth reactor initially anoxic for *Penicillium* sp 82

Figure 4-38 Fed-batch 50 m^2/m^3 attached-growth system for *Phoma* sp. 84

Figure 4-39 Fed-batch 50 m^2/m^3 attached-growth reactor initially anoxic for *Phoma* sp. 84

Figure 4-40 Amount of o-PO_4^{3-} decreased versus initial o-PO_4^{3-} concentration for all data. 87

Figure 4-41 Amount of o-PO_4^{3-} decreased versus initial o-PO_4^{3-} concentration for microaerophilic data only. 87

Figure 4-42 Aerobic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2000) 89

Figure 4-43 Aerobic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2007) 89

Figure 4-44 Aerobic o-PO_4^{3-} concentration decrease by yeast. 90

Figure 4-45 Aerobic o-PO_4^{3-} concentration decrease by *Penicillium* sp. 90

Figure 4-46 Aerobic o-PO_4^{3-} concentration decrease by *Phoma* sp. 91

Figure 4-47 Microaerophilic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2000) 92

Figure 4-48 Microaerophilic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2007) 93
Figure 4-49 Microaerophilic o-PO_4^{3-} concentration decrease by yeast. 93
Figure 4-50 Microaerophilic o-PO_4^{3-} concentration decrease by *Penicillium* sp. 94
Figure 4-51 Microaerophilic o-PO_4^{3-} concentration decrease by *Phoma* sp 94
Figure 4-52 Anoxic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2000) 96
Figure 4-53 Anoxic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2007) 96
Figure 4-54 Anoxic o-PO_4^{3-} concentration decrease by yeast 97
Figure 4-55 Anoxic o-PO_4^{3-} concentration decrease by *Penicillium* sp. 97
Figure 4-56 Anoxic o-PO_4^{3-} concentration decrease by *Phoma* sp. 98
Figure 4-57 o-PO_4^{3-} concentration decrease in dilute wastewater by *Geotrichum* sp. (2000) 99
Figure 4-58 o-PO_4^{3-} concentration decrease in dilute wastewater by *Geotrichum* sp. (2007) 100
Figure 4-59 o-PO_4^{3-} concentration decrease in dilute wastewater by a yeast. 100
Figure 4-60 o-PO_4^{3-} concentration decrease in dilute wastewater by *Penicillium* sp. 101
Figure 4-61 o-PO_4^{3-} concentration decrease in dilute wastewater by *Phoma* sp. 101
Figure 4-62 Concentration decrease of o-PO_4^{3-} for three successive generations of *Geotrichum* sp. (2000) 102
Figure 4-63 Concentration decrease of o-PO_4^{3-} for three successive generations of *Geotrichum* sp. (2007) 103
Figure 4-64 Concentration decrease of o-PO_4^{3-} for three successive generations of yeast 103
Figure 4-65 Concentration decrease of o-PO_4^{3-} for three successive generations of *Penicillium* sp. 104
Figure 4-66 Concentration decrease of o-PO_4^{3-} for three successive generations of *Phoma* sp. 104
Figure 4-67 Concentration decrease of o-PO_4^{3-} by a mixed culture. 105

Figure 4-68 Attached-growth concentration decrease of o-PO_4^{3-} by *Penicillium* sp. under two oxygen tensions... 107

Figure 4-69 Attached-growth concentration decrease of o-PO_4^{3-} by *Phoma* sp. under two oxygen tensions.. 107

List of Symbols

ATP	adenosine triphosphate
BAF	biological aerated filter
BNR	biological nutrient removal
BOD	biological oxygen demand
COD	chemical oxidation demand
DAND	direct ammonium nitrogen reduction
F/M	food to microorganism ratio
ΔG^o	Gibbs free energy
IC	ion chromatography
IFAS	integrated fixed-film activated sludge
k	zero-order rate constant
K_s	half saturation coefficient
μ	growth rate
μ_{max}	maximum specific growth rate
M	mycelia mass
MBBR	moving bed bioreactor
MBR	membrane bioreactor
MLSS	mixed liquour suspended solids
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Nar	nitrate reductase
Nir	nitrite reductase
Nor	nitric oxide reductase
N_2	nitrogen gas
N_3^-	azide
NH_4^+	ammonium
NO	nitric oxide
N_2O	nitrous oxide
NO_2^-	nitrite

NO_3^{2-}	nitrate
P	phosphorus
PBS	phosphate buffered saline
PDA	potato dextrose agar
PH_3	phosphine gas
o-PO_4^{3-}	ortho-phosphate
P450Nor	cytochrome P450 nitric oxide reductase
rpm	revolutions per minute
r_{su}	substrate utilization rate
SBR	sequencing batch reactor
SND	simultaneous nitrification-denitrification
S_s	substrate concentration
t	time
TKN	total kjeldahl nitrogen
TS	total solids
TSS	total suspended solids
WWTP	wastewater treatment plant
X	biomass
Y	yield factor

Chapter One

1.0 Introduction

1.1 Overview and Problem Statement

Increasingly stringent environmental regulations on the quality of the effluent from wastewater treatment plants (WWTPs) are necessitating the evaluation and development of treatment technologies to improve performance. Biological nutrient decrease (BNR) processes are of particular interest. The bacteria responsible for the decrease of nitrogen are susceptible to the influence of many unfavourable factors resulting in significantly reduced performance or complete failure of the system.

Bacterial nitrification-denitrification is a complicated process carried out by very sensitive bacteria. Bacterial nitrification is a two-stage process requiring two groups of fastidious microbes and relies on diffusion of an intermediate compound. The two groups of nitrifiers have different growth rates and sensitivities to inhibitory compounds (Downing et al. 1964; Tomlinson et al. 1966). Denitrifiers are also a very fastidious group of bacteria. Conventional nitrogen treatment systems, with variable flow and influent wastewater quality due to combined sewers, often have problems maintaining stable performance. Nitrifiers and denitrifiers require low food to microorganism ratios with minimal change, however, the diurnal cycle of raw sewage and combined sewers and storm events tend to lower the food to microorganism ratio. In WWTP, where toxic and inhibitory compounds periodically enter the system, bacterial nitrogen treatment systems can completely fail. When this type of problem occurs, it can take a WWTP three to six weeks to recover. During this time period, however, effluents from the WWTP may not meet discharge requirements resulting in damage to aquatic ecosystems and decrease in water quality for downstream users. The rapidly changing quality and flow of wastewater does not complement the sensitive nature of nitrifying and denitrifying bacteria.

Many modifications have been made to conventional nutrient treatment systems in order to overcome the limitations of the microbial populations. Sequencing batch reactors (SBRs), moving bed bioreactors (MBBRs), integrated fixed-film activated sludge systems (IFAS), biological aerated filters (BAFs) and membrane bioreactors (MBRs) are all examples of improvements to conventional BNR. These improvements centre on stabilising and or retaining the bacterial populations responsible for nutrient decrease in the reactors even under environmental conditions that limit their performance. However, the dominant feature of all these progressions in technology has been centred on bacterial nitrification and denitrification. This means there is likely a maximum achievable decrease because of the underlying limitations of the bacterial populations.

Nitrification and denitrification have long been considered solely a bacterial process. Consequently, biological wastewater treatment has focused on bacteria, excluding the effect of other microorganisms. In the early 1990s, Shoun (1991) reported the discovery of fungal denitrification. Since that time, Shoun has worked to determine the biochemical pathways for fungal denitrification. Based on the literature there are several potential advantages in development of a fungal nitrification-denitrification system. The advantages potentially address three major issues of conventional bacterial nitrogen treatment technology; 1) the fastidious nature of bacteria 2) improvement in the rate and stoichiometry and 3) process simplification. However, several basic questions must be answered prior to development of a new treatment technology. Primarily fungal denitrification has been exclusively worked on in very controlled laboratory systems with defined medium and laboratory strains. The fungi identified as capable of denitrification have been limited. They have not been usually found in wastewater treatment activated sludge systems. Initial research must be based around finding fungi from a wastewater treatment system and determining their ability to decrease wastewater parameters of interest.

Concurrent development of advanced wastewater treatment technology like MBR and MBBR (including IFAS) has reduced the need for, and limitations of, secondary clarifiers in WWTPs. MBR, MBBR and IFAS allow for longer solids retention times and increased mixed liquor suspended solids (MLSS), removing a barrier for exclusive use of bacteria for organic carbon and nutrient decrease. A fungi nitrogen treatment system has the potential to overcome issues associated with conventional biological nutrient systems. Combining fungal nitrogen concentration decrease with membrane bioreactors or hybrid activated sludge-attached-growth systems is an area for advanced research into new technologies.

1.2 Research Hypotheses and Objectives

It was hypothesized that fungi were not only capable of biological nitrogen concentration decrease but they also possess characteristics advantageous to significant improvements in wastewater treatment technology.

The primary objectives of this study were to explore the potential for creating a fungal nitrogen treatment system as well as investigating fungal characteristics of wastewater denitrification. The secondary objective of this study was also to assess fungal concentration decrease of phosphate to determine the potential for a combined nitrogen and phosphorus treatment system.

1.3 Research Approach

The approach taken to assess the potential of fungi was based on an applied development of technology. This means procedures were developed that used as many real world elements as possible in a controlled manner. This results in the data being close to a representation of real system working conditions. This approach is also more conservative, in that the test systems contain more variance, however, significant results provide stronger conclusions for technology development.

The research approach can be broken into three phases. Phase 1 was isolation, screening and identification of fungi from the Gold Bar WWTP. Isolation of fungi found in a wastewater treatment plant significantly improved the probability of success. The fungi isolated may have had an adaptive tolerance of the wastewater environment when compared to a laboratory or culture collection fungal strain.

Phase 2 was to investigate the fungi screened in Phase 1 for factors that impacted fungal nitrogen and phosphate concentration decrease from primary treated wastewater in a suspended culture system. The experiments in Phase 2 were designed to collect batch kinetic data and initially investigated the effects of aeration, pH, dilute sewage, mono and mixed culture and adaptive improvement. It was expected to optimize the nitrification cycle followed by a denitrification cycle. These initial experiments used either individual or combinations the fungi isolated from the WWTP.

The third phase of the experiments examined nitrogen and phosphate concentration decrease in an attached-growth and fed-batch attached-growth systems. These experiments were developed in response to data generated in Phase 2 that suggested an attached-growth system warranted testing. Attached-growth experiments aimed to investigate the impact of surface area, microaerophilic and anoxic conditions and to demonstrate continuity of fungal nitrogen treatment.

Chapter Two

2.0 Literature Review: Potential for Using Fungus for Ammonia Nitrogen Treatment in Wastewater[1]

2.1 Fungal Nitrification

Since the early 1930s it has been established that fungi have the ability to perform nitrification. Early studies indicated the ability in a narrow range of species with limited ability to use urea or ammonia as an energy source in the nitrification process. More recently, a much broader range of fungi have been identified to carry out nitrification as well as use urea and ammonia as an oxidizable nitrogen energy and nutrient source. Studies conducted to date are essentially limited to pure cultures in a defined medium. Studies report production of nitrite and nitrate but not the overall removal or rate of removal from the medium.

Eylar and Schmidt (1959) performed a comprehensive study that undeniably confirmed fungal nitrification. Some 978 heterotrophic microorganisms were isolated from various soil environments and grown in liquid culture to determine their potential for nitrification. Of 978 isolates, 415 were fungi and most of these were strains of *Aspergillus flavus*. The study found that fungi did not accumulate significant amounts of nitrite when compared to bacteria. However, the fungi tested did produce very high concentrations of nitrate although no comparison to nitrate production by autotrophic nitrifying bacteria was made by the authors. The study found that the isolates of *Aspergillus* spp. did not nitrify ammonia or urea.

---

[1] The literature review is an expanded adaptation of the article "A potential new role for fungi in a wastewater MBR biological nitrogen decrease system" by R.K. Guest and D.W. Smith (2002). *Journal of Environmental Engineering and Science* 1(6): 433-437.

Hirsch et al. (1961) studied the formation of nitrite and nitrate by actinomycetes (a group of Gram positive, mainly soil bacteria) and fungi under various conditions. This study also found that fungi had the ability to nitrify; however the main product again was nitrate. Several findings from this study are worth noting. First, when the pH of the medium dropped below 5.0, nitrification was inhibited; however, growth of the fungi was not affected. Second, fungi carried out oxidation of ammonia. The result suggested oxidation of ammonia might be strain related. Finally, the study noted a trend that greater quantities of carbon source and ammonia source in the medium resulted in higher amounts of nitrate being formed. However, this trend was not normalized for the fungal mass.

Falih and Wainwright (1995) tested the ability of a wide range of yeast and filamentous fungi to oxidize urea or ammonium. To facilitate direct comparison of results, tests were conducted on 17 different fungi grown in a standardized liquid medium; whereas, previous studies had used multiple different medium and mainly focused on species of *Aspergillus*, *Fusarium* and *Penicillium*. Their results showed that, with the exception of two species, all fungi oxidized ammonium or urea to nitrate. Fungi studied included: *Phanerochaete chrysosporium, Hymenoscyphus ericae, Pythium oligandrum, Rhizomucor pusillus, Aspergillus oryzae, Mucor strictus, Fusarium solani, Aspergillus niger, Cladosporium herbarum, Penicillium chrysogenum, Penicillium notatum, Penicillium expansum, Geotrichum candidum, Williopsis californica* and two unidentified soil yeasts.

Kurakov and Popov (1996) studied the nitrifying activity and phytotoxicity of 40 strains of 13 species of fungi. Forty-five percent of strains tested showed nitrifying activity; however a greater number of strains showed nitrification when grown on an organic nitrogen source. *A. flavus* strain y4 showed maximal activity producing 0.1 mg N-NO_3 mL^{-1} of medium. The culture was grown over 10 to 12 days under static conditions. The study found that fungi showed one to four orders of magnitude greater resistance to nitrification inhibitors and one to four orders of magnitude greater formation of nitrates and nitrites than autotrophic nitrifying bacteria. Inhibitory compounds tested on fungi were: nitrapyrin, carbamoylmethylpyrazole, dicyandiamide, and 4-amino-1,2,4 triazole.

2.2 Potential Benefits of Fungal Nitrification

There are multiple reasons to replace bacterial nitrifiers in a wastewater treatment system. These reasons focus on the fastidious nature of bacterial nitrifiers. Bacterial nitrification is a two-stage process requiring two groups of bacteria. The bacterial nitrification pathway is shown in Figure 2-1. The oxidation of ammonia to nitrite is mediated by *Nitrosomonas* spp. and the oxidation of nitrite to nitrate is carried out by *Nitrobacter* spp. The bacterial system relies on diffusion of the intermediate compound, nitrite, to the *Nitrobacter* spp.

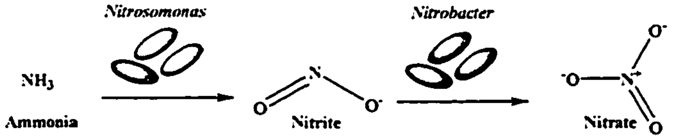

Figure 2-1 Bacterial nitrification pathway.

The two groups of bacteria responsible for nitrification also have different growth rates and show different sensitivities to inhibitory compounds (Downing et al. 1964; Tomlinson et al. 1966). In relation to a nitrification component of a wastewater treatment system, a bacterial system appear to be more complex and therefore more difficult to stabilize when a process upset occurs. Nitrifying bacteria also do not compete well in biological nitrogen treatment systems when the food to microorganism ratio is high. This deficiency is related to the efficiency of the bacterial substrate uptake rates as well as the range of substrates that can be used to generate energy. Filamentous microorganisms naturally take over the wastewater treatment systems due to having very efficient substrate uptake systems as well as a greater surface area due to filamentous growth (Wanner and Grau 1988). A low F/M ratio in wastewater occurs primarily when wastewater is diluted with storm water or runoff. This is a common occurrence for many WWTPs.

Fungal nitrification, as reported in the literature, has several advantages over bacterial nitrification. The first major advantage is that fungi complete nitrification in a single step (Eylar and Schmidt 1959; Kurakov and Popov 1996). A diagram of fungal nitrification is shown in Figure 2-2 but, simply stated, ammonia is oxidized to nitrate by various fungi.

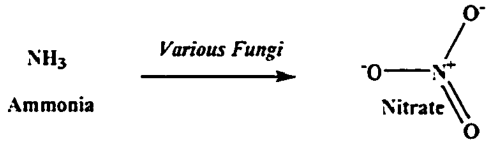

Figure 2-2 Fungal nitrification.

In theory, a single step nitrification process is advantageous for a WWTP, there are less options to correct a process upset compared to a bacterial nitrification system. A second advantage for using fungi, is greater resistance to inhibitory compounds and heavy metals (Arora et al. 1992). Soil fungal nitrifiers are reported to have greater resistance (one to four orders of magnitude) to inhibitory compounds than bacterial nitrifiers (Tu 1992; Kurakov and Popov 1996). For WWTPs receiving significant quantities of industrial effluents, increased resistance to both heavy metals and inhibitory compounds could lead to a more robust treatment process. Several explanations can be offered on the ability of fungi to resist inhibitory compounds. First, mycelial growth may provide greater protection to sensitive organelles of fungi. The larger surface area would act in the same manner as the extra polysaccharide matrix of a biofilm; a type of adsorption matrix. Second, fungi are eukaryotic cells, which contain significantly more genes than bacteria providing other methods for dealing with inhibitory compounds. These methods could be co-metabolic pathways or complete degradation pathways. In either case, the net result is the potential for improved nitrification process stability in the presence of inhibitory compounds.

The final major potential benefit of fungal nitrification is the production of greater end products. Kurakov and Popov (1996) reported one to four orders of magnitude greater production of nitrates by fungi over bacteria. However, what was not indicted in the study was the rate at which nitrification was taking place. The potential suggested by this study is that fungi should provide stoichiometric complete nitrification. In terms of a nitrogen treatment system, this could translate into a decrease in size of the nitrification components of treatment plants. This could be advantageous for two reasons, a decrease in treatment plant footprint and, second, a decrease in the aeration equipment and operating cost.

2.3 Fungal Denitrification

The first reports of fungal denitrification were by Malinowsky and Ottow (1984). However this information was only published in German and did not have wide circulation. Furthermore, Malinowsky and Ottow (1984) do not appear to have continued their research. This resulted in a research group in Japan in the early 1990s claiming the first reported observation of fungal denitrification (Shoun and Tanimoto 1991). Until these two reports, denitrification was considered solely a bacterial process. Bacterial denitrification involves the reduction of nitrate to a form of nitrogen gas. The classic biochemical pathway described in the literature is for *Paracoccus denitrificans* and is shown in Figure 2-3. The pathway involved four sequential reductions using nitrate reductase, nitrite reductase, nitric oxide reductase and finally nitrous oxide reductase. Bacterial denitrification is assumed to exist to allow bacteria to respire using nitrate as an alternate electron acceptor in the absence of oxygen. It is beyond the scope of this review to discuss in detail bacterial denitrification. Interested readers are directed to Berks et al. (1995) or Ferguson (1994).

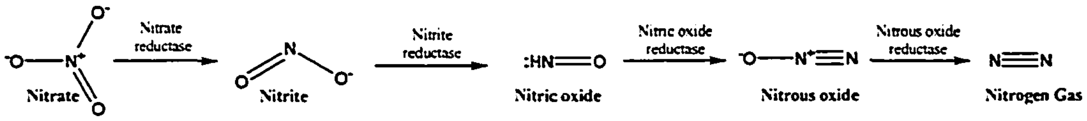

Figure 2-3 Biochemical pathway for bacterial denitrification.

To date, the major research into fungal denitrification has been confined to biochemical and molecular genetics studies. The current hypothesis for the origins of fungal denitrification is two fold. First is that the nitrate (Nar) and nitrite (Nir) reductase components originate from the protomitochondrion (bacteria that became mitochondria of eukaryotic cells) and second is that there was a horizontal gene transfer event from bacteria to fungi of the nitric oxide reductase (Nor) (Kobayashi et al. 1996). However, the nitric oxide reductase found in fungal denitrification has not been identified in bacteria. This means source and evolution of fungal denitrification is still very speculative and only based on detailed study of two fungal species *Fusarium oxysporum* and *Cylindrocarpon tonkinense.*

Nitrate decrease to nitrous oxide by fungi uses three reductases in sequence; Nar, Nir and Nor. The biochemical pathway is shown in Figure 2-4. The Nar and Nir reductase are associated with the respiratory chain and generate energy in the form of ATP (Kobayashi et al. 1996). Nor for fungal denitrification uses a distinct cytochrome (P450Nor) compared to bacterial Nor (cytochrome cb) (Takaya et al. 1999). In fungi, Cytochrome P450Nor catalyzes the conversion of nitric oxide to nitrous oxide. P450Nor is part of the cytochrome P450 super-family, a group of monooxygenases containing a protoheme prosthetic group (Takaya and Shoun 2000). However, P450Nor used by fungi shows a distinctive catalytic property when compared to other members of the P450 family. P450Nor receives its electrons directly from nicotinamide adenine dinucleotide (NADH) or possibly nicotinamide adenine dinucleotide phosphate (NADPH) without any other mediator proteins (Shimizu et al. 2000; Oshima et al. 2004; Umemura et al. 2004). This means P450Nor is not directly associated with the respiratory chain to generate cellular energy in the form of ATP. Studies have found that fungi produce two variants of P450Nor from the same gene, one is membrane bound and localized in the mitochondria and second is a free cytosol enzyme (Takaya et al. 2002). Two possible explanations for the distinctive catalytic property of P450Nor have been suggested in the literature; first, P450Nor acts as an electron acceptor to regenerate NAD^+ and second, P450Nor acts to detoxify nitric oxide and potentially peroxynitrite, both of which can significantly damage mitochondrion function (Mehl et al. 1999; Zou et al. 2000).

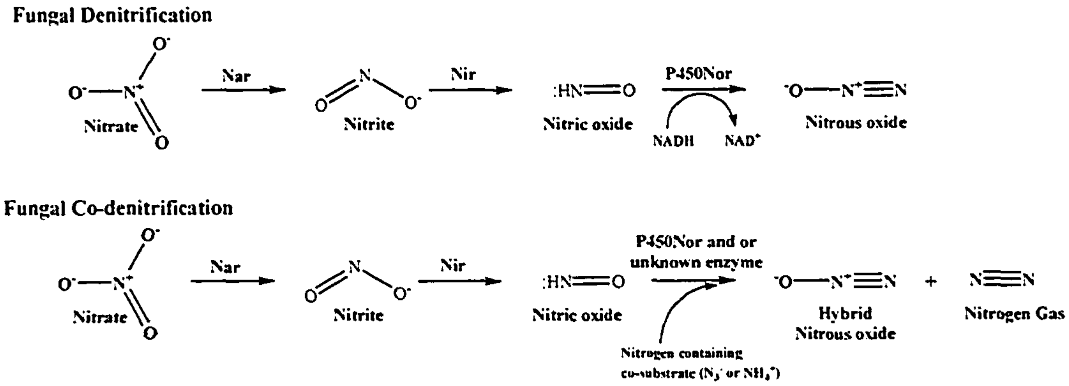
Fungal Denitrification
Nitrate
Nar
Nitrite
Nir
Nitric oxide
P450Nor
NADH
NAD+
Nitrous oxide
Fungal Co-denitrification
Nitrate
Nar
Nitrite
Nir
Nitric oxide
P450Nor and or unknown enzyme
Nitrogen containing co-substrate (N3- or NH4+)
Hybrid Nitrous oxide
Nitrogen Gas

Figure 2-4 Fungal denitrificaiton biochemcial pathways.

Fungal denitrification also shows a second unique characteristic labelled as co-denitrification (Tanimoto et al. 1992). In co-denitrification, fungi can utilize nitrogen compounds other than nitrate and nitrite in the production of nitrous oxide (N_2O) or dinitrogen gas (N_2). In experiments with [^{15}N]-azide and [^{14}N]-nitrite, it was found that the nitrous oxide produced was a mixture of $^{14}N^{14}NO$ and $^{15}N^{14}NO$ (hybrid N_2O) and nitrogen gas was $^{15}N^{15}N$ (Shoun 1992; Shoun et al. 1992). Both azide and nitrite nitrogen was converted to nitrogen gas. Azide and other nitrogen nucleophiles normally inhibit the bacterial denitrification processes. Hybrid N_2O is catalyzed by the P450Nor cytochrome and requires NO and nitrogen containing co-substrate; tested to date are azide (N_3^-) and ammonia (NH_4^+). The co-denitrification reaction by P450Nor does not receive electrons from NADH; the co-substrate of either N_3^- or NH_4^+ replaces NADH (Su et al. 2004). This is shown schematically in Figure 2-4. The cytochrome responsible for the production of N_2 and the range of co-substrates has yet to be determined, but the P450Nor cytochrome is potentially responsible. Co-denitrification provides an explanation for the greater resistance of fungi to inhibitory compounds.

A third unique characteristic of fungal denitrification is the operation of this pathway in the presence of low levels of oxygen (Zhou et al. 2001; Takaya et al. 2003). The regulation of the fungal denitrification gene has at least two transcription controls. The first is nitrate and the second is oxygen. Furthermore, nitrate assimilation and denitrification are controlled at the same time (Takaya et al. 2002). When oxygen is available in excess (aerobic), the denitrification gene is repressed and the nitrate assimilation pathway is active. When oxygen is limited to a low level, both the nitrate assimilation gene and nitrate denitrification pathway is active and therefore nitrate is reduced simultaneously by two pathways. In comparison, bacterial denitrification is completely repressed in the presence of oxygen.

F. oxysporum was the first fungus shown to have denitrification abilities (Shoun and Tanimoto 1991) and several fungi related to it, specifically members of the genera *Gibberella, Nectria, Calonectria,* and *Cylindrocarpon* (family Nectriaceae) show similar capabilities. *Trichoderma* and it relatives *Hypocrea* and *Hypomyces* (family Hypocreaceae) can also denitrify. Further research has shown that cytochrome P450Nor is widely distributed in fungi and yeast (Shoun et al. 1998). Prior to the discovery of fungal denitrification, all fungi were assumed to be obligate aerobes. This has led to the recent change of status for many fungi from obligate aerobes to facultative anaerobes.

The work by Shoun et al. (1992) found that, for a nitrate or nitrite substrate, the major product of fungal denitrification is nitrous oxide. Only 9 of 39 fungi tested in the study, exhibited complete denitrification to nitrogen gas and, in most cases, production of nitrogen gas was low. The biochemical pathway that has been determined for fungal denitrification supports that the major end product is N_2O. However, the biochemical pathway has really only been studied in detail for two fungi *F. oxysporum* and *C. tonkinense*. Combining this knowledge with the, as yet undetermined, biochemical pathway for the formation of N_2 in co-denitrification, further investigation is necessary to elucidate the complete fungal denitrification pathway. The fact that several fungal species exhibit production of N_2 via co-denitrification indicates a high probability that the current biochemical pathway for fungal denitrification is incomplete. Bacterial denitrifiers also commonly exhibit the trend of not having a complete denitrification pathway which leads to the major end-products of NO or N_2O.

2.4 Fungal Denitrification Rates and Stoichiometry

Fungal denitrification rates are difficult to characterize in terms of typical wastewater treatment units. Most of the published biochemical literature reports rates of fungal denitrification to be greater than bacterial denitrification (Shoun 1992; Tanimoto et al. 1992; Usuda et al. 1995). One exception is the study by Kurakov et al. (2000) who have reported that fungal denitrification rates are 3 to 6 times lower. However, neither the study by Kurakov et al. (2000) nor the work by the Shoun group (Shoun 1992; Tanimoto et al. 1992; Usuda et al. 1995) state clearly on what basis the comparison was made. It is also not clear from the literature that fungal denitrification growth conditions have been sufficiently examined to determine whether fungal rates are greater or less than bacterial systems.

The reported stoichiometry for fungal denitrification is close to 100 %; this represents complete conversion of nitrite to gaseous denitrification products (Tanimoto et al. 1992). In most biological systems, complete conversion usually does not occur. Many enzyme systems require minimum substrate concentration for induction as well as maintenance. Because of the dual operation of both nitrate assimilation and denitrification pathways being controlled by low levels of oxygen in the presence of nitrate, suggests stoichiometric conversion is possible. Furthermore the fact that cytochrome P450Nor is not associated with the energy generation of ATP further enhances the potential stoichiometric conversion.

2.5 Key Points of Fungal Denitrification

- P450Nor mediated, unique to fungi
- NO_2^- or NO_3^- is the starting molecule
- The major end-product is N_2O
- Fungal denitrification from NO_3^- results in rapid increase in free NO_2^- concentration in the medium
- Is only based in detail on two fungal species *F. oxysporum* and *C. tonkinense*
- Pathway is active in the presence of low concentrations of oxygen
- Co-denitrification unique to fungi
  - P450Nor mediated production of hybrid N_2O
  - NADH replaced by co-substrate that contains nitrogen (azide or ammonia)
  - Pathway for production of N_2 incomplete

2.6 One Microorganism for Both Nitrification and Denitrification

To date no single bacterial species has been identified that is capable of both nitrification and denitrification. Both processes have been distinctly separated along species lines. This has always necessitated that nitrogen wastewater treatment processes must involve at least two stages; one optimized for nitrifying bacteria and one for denitrifying bacteria. Noted in the review of the literature, *Fusarium solani* has been identified independently to perform nitrification (Falih and Wainwright 1995) and denitrification (Shoun et al. 1992). A single group of microorganisms that are capable of both nitrification and denitrification could lead to advancement in biological nitrogen treatment technology. With a single microorganism being able to carry out both functions, there is the potential of simplifying current treatment technology.

2.7 Fungi Used for Wastewater Treatment

During the early 1950s to early mid 1960s researchers started to recognize the potential for fungi to carry out wastewater treatment. Cooke (1976; 1986) carried out a number of surveys of receiving water bodies, a trickling filter, an activated sludge process, and an anaerobic digester for the various types of microorganisms found in the processes. The studies found fungi occurred in all the systems; however no quantitative enumerations were performed. Cooke (1976) identified ten major taxons of fungi in wastewater: *Phlyctochytrium* spp., *Rhizophidium* spp, *Achlya* spp., *Pythium* spp., *Mucor* spp., *Torulopsis* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. *and Trichoderma* spp. Cooke (1976) advocated the use of fungi in wastewater treatment because, fungi appeared to show higher rates of organic matter degradation, and because they showed a much greater ability to degrade cellulose, hemi-cellulose, and lignin materials. However, Cooke's research did not move beyond a survey of the populations to creating a wastewater treatment system based on fungi.

Thanh and Simard (1973) reported treatment of wastewater by various yeast species. The focus of the work was to produce food yeast (animal feed and single cell protein) on a low cost available medium (wastewater). The study screened 27 yeast strains for their ability to produce a high biomass while maximizing concentration decrease of phosphate, ammonia and organic matter. The studies were conducted in batch, 500 mL, baffled culture shake flasks containing 150 mL of sterile wastewater. Reported phosphate removal ranged from 12 to 100%; total nitrogen removal from 22 to 93%; ammonia nitrogen from 27 to 90%, and COD removal from 0 to 72%. The concentration decrease in total nitrogen from the system indicates denitrification was occurring in the yeast cultures although the authors did not recognize the result. The study found that glucose had a significant impact on the total concentration decrease of nitrogen from the cultures.

Hiremath et al. (1985) performed a similar study except they tested seven fungal species isolated from a wastewater stabilization pond. The major goal of the study was to maximize biomass production of fungi as a food source for animal or human consumption. The trials were conducted in 2 L, conical flasks containing 1.5 L of sterile fresh wastewater. Flasks were inoculated with pure cultures of fungi and incubated at room temperature for 10 days. The culture flasks were gently agitated twice a day. The study reported BOD_5 removal between 53 and 72%; phosphate removal from 34 to 77%, and ammonia nitrogen removal between 49 and 77%. The study did not measure any other forms of nitrogen and, therefore, there is no indication of possible fungal denitrification activity. However, the study also indicated, that adding a simple sugar enhanced the concentration decrease of ammonia and phosphate.

Both studies were performed under non-ideal condition with no process optimization of the parameters reported. Although the data show fungi can treat wastewater, they do not give any indication of the maximum removal efficiencies. However, both studies indicate promising results for removal of nitrogen and phosphorus. Additional evidence, although not conclusive, that fungi are capable of wastewater treatment was found during a study of onsite sphagnum peat wastewater treatment system. Brooks (1988) found excellent removal of BOD_5, organic and ammonia nitrogen (90 to 95% and 95 to 99%, respectively). Based on standard bacterial and fungi enumeration techniques, the ratio of fungi to bacteria was 8:1 during winter and 2.4:1 during the summer. This led to the hypothesis that fungi had a large role in removal of nitrogen from the wastewater. However, due to the use of standard enumeration techniques, it is very likely the populations were underestimated due to the fastidious nature of environmental microbes. Furthermore, a direct comparison ratio of fungi to bacterial colony forming units is not appropriate. This ratio employs the flawed assumption that each bacterium and fungus is equal in degradation rate and range (BOD, ammonia and organic nitrogen) of the wastewater. A different experimental design and enumeration technique is needed before comparisons can be made.

The three studies discussed above represent the extent of research performed to date on using fungi to treat domestic wastewater. However, fungi have been used in specialized industrial waste stream treatments. These applications typically have been focused on specific contaminate removal, such as colour, heavy metals and hydrocarbons, aromatics and xenobiotics (Aitken 1993; Nicell 2001; Ikehata et al. 2004). More recently, fungi have been evaluated for the treatment of wastewater sludge. The work has focused around using fungi to enhance the biodegradation, settleability and dewatering characteristic of sludge (Molla et al. 2002a; Molla et al. 2002b; Alam et al. 2004).

2.8 Causes of Filamentous Bulking of Activated Sludge

In order to develop a fungal nitrogen treatment system, the conditions needed for the selection of fungi in a competitive environment must be known. Currently these have not been fully studied. However, control of filamentous bulking in the activated sludge process has been studied due to its detrimental effect on the performance of conventional wastewater treatment systems. The literature on causes and control of filamentous bulking provides a road map to finding the correct conditions for selection of fungi in a mixed culture environment.

The majority of filamentous bulking has been reportedly caused by bacteria (Wanner and Grau 1988; Grady et al. 1999), the most common being *Nocardia* spp., type 1701, type 021N, type 0041, *Thiothrix* spp. and *Microthrix parvicella*. Fungi have been indicated as the dominant or secondary bulking filament in approximately 1% and 2% of wastewater treatment plants in the United States (Grady et al. 1999). Some researchers have argued that the numbers of fungi may be higher than reported due to the fact that analysts are not looking for fungi and that fungi have atypical forms in wastewater (Cooke 1976; Wanner and Grau 1988).

The hypotheses about the causes of filamentous bulking are multiple. The major supposition regarding the formation of bulking sludge focuses around limited substrate concentrations and the higher surface area to volume ratio for filamentous microorganisms (Wanner and Grau 1988; Grady et al. 1999; Martins et al. 2004). A combination of selection factors, when combined with the advantage of filamentous growth, allows competitive advantage for filamentous bacteria and fungi. Selection factors indicated as causes of bulking are: low dissolved oxygen; completely mixed reactors; septic wastewater; nutrient deficiency; and low pH. Low pH has been identified as a unique characteristic associated with fungal filamentous bulking (Wanner and Grau 1988; Grady et al. 1999). However, the other factors also provide additional selective pressure.

If fungi are used in a wastewater nitrogen treatment system, sludge bulking will likely occur. This means that development of fungal systems will be limited to using systems that can deal with bulking sludge such as membrane bioreactors or an attached-growth system. However, there are some potential benefits to encouraging fungal growth. Limiting substrate concentration, i.e. low F/M ratio, can be beneficial for design and operation of WWTPs. Wastewater characteristics vary substantially during the day and over the course of the week and year. For conventional activated sludge systems, performance decreases as the F/M ratio drops. Therefore, maintaining consistent performance during high or low flow periods is difficult. Prior solutions have included by-pass operation, flow equalization, or addition of a carbon source. The solutions are either unacceptable based on regulatory requirements in the case of by-pass operation, or involve significant capital and or operational costs.

Complete mix reactors also contribute to a low F/M ratio, but also provide dilution effects of toxic and inhibitory compounds. WWTPs receiving industrial effluents often have major upsets or complete failure of nutrient removal systems operated in a plug flow design.

Low dissolved oxygen used as a selection factor for filamentous growth can also be beneficial in the operation of a WWTP. Aeration systems could be designed with smaller foot prints or the use of lower cost, less efficient designs could be employed. Lower dissolved oxygen also translates into lower operational costs for aeration aspects, but it can also affect sludge handling. Typically, lower dissolved oxygen and substrates reduce the production of biomass, therefore reducing sludge handling requirements. The use of filamentous fungi to carry out nutrient treatment has the potential to lead to a design for a more robust treatment system able to tolerate the natural variable quality and quantity of domestic wastewater.

2.9 Phosphorus Uptake and Utilization in Fungi

Research concerning phosphorus uptake, storage and utilization has focused on the importance of phosphorus for living systems. Phosphorus in cells is found in many components, therefore research has evolved around how fungi (and all living organisms) manage and control this essential nutrient. The most comprehensive review on the subject of phosphorus in fungi was written by Beever and Burns (1980). Since the advent of modern biochemistry and molecular methods, a greater understanding of genetic control has been established. This has been recently reviewed by Persson et al.(2003). The focus of both reviews is inorganic phosphate (o-PO_4^{3-}). However, intricately linked to phosphorus management in fungi is polyphosphate storage and function. Fungal polyphosphates have been heavily researched by Kulaev (1983; 1999) and Kronberg (1999).

A limited number of species have been used to understand fungal phosphorus systems. *Saccharomyces cerevisiae*, *Neurospora crassa*, *A. niger* and *Candida* spp. have been used, but *S. cerevisiae* is the most common and well developed. Phosphorus content of fungi expressed as percent dry weight can range from 0.4 to 4.5% but there have been reports of as high as 20% dry weight for yeasts contained largely in the vacuoles (Kornberg et al. 1999). Table 2-1 adapted from Beever and Burns (1980) shows the distribution of phosphorus as a percentage for actively growing fungi. As can be seen, the amount and percentage of phosphorus in various cell components can vary dramatically across species. Furthermore the pools of phosphorus in cells vary dramatically based on physiological state. The values shown in Table 2-1 should only be viewed as a crude range.

Table 2-1 Chemical distribution of phosphorus in actively growing fungal cells†.

	N. crassa		*S. cerevisiae*		*Mucor racemosus*	
Phosphate Containing fraction	μmol P (g d.w.)$^{-1}$	Total P (%)	μmol P (g d.w.)$^{-1}$	Total P (%)	μmol P (g d.w.)$^{-1}$	Total P (%)
orthophosphate	20	4	116	13	148	8
Polyphosphate (soluble and insoluble)	115	23	310	34	1120	65
soluble organic P	80	16	39	4	86	5
Lipid P	75	15	48	5	62	3
RNA and DNA	220	42	404	44	320	19

†Adaptation after Beever and Burns (1980)
(g d.w.)$^{-1}$ =per gram dry weight.

Inorganic polyphosphates are chains of phosphate residues linked by high energy phosphoanhydride bonds. These polymers can be as short as four residues but have been known to contain several hundred residues. For decades, polyphosphates have been ignored as an active component in all life. However polyphosphates are considered to be pre-biotic, existing prior to cellular life development (Kornberg and Fraley 2000). Polyphosphates are believed to be one of the basic building blocks for life, providing support structures and energy for reactions prior to the existence of adenosine triphosphate (ATP), ribonucleic (RNA) and deoxyribonucleic acid (DNA). This is the reason that polyphosphates are found in all life, Eubacteria, Archaea and Eucarya (Kulaev et al. 1999).

Research has now shown polyphosphate has numerous and significant biological functions. Included in the list are: substitution for ATP kinase; o-PO_4^{3-} reservoir; internal pH buffering; chelation of metals as reservoirs and detoxification; mRNA processing; and genetic regulatory role in physiological response to environmental stimulus (Kornberg et al. 1999; Kulaev et al. 1999). It is beyond the scope of this review to discuss in detail the literature on polyphosphates. However, the key point about knowledge of polyphosphates is that it is still in its infancy, but initial research has indicated that it is fundamentally important in many crucial cellular functions. Readers are directed to reviews by Kulaev (1983), Kronburg (1999), Wood and Clark (1988) and Harold (1966).

Phosphate utilization in fungi has been extensively studied using *S. cerevisiae*, however much remains to be investigated. Early research by Beever and Burns (1980) indicated fungi had two phosphate uptake systems; low affinity and high affinity. The dual system appeared to coexist in yeast and *N. crassa* and operated based on external o-PO_4^{3-} concentrations. Since that time, the structure and regulation of phosphate have been extensively studied in *S. cerevisiae*. It now appears that, in addition to the low and high affinity, system there is a family of at least six phosphate transporters. A review by Persson et al. (2003)details the current knowledge and the reader is directed there for more information. What follows is a summary of key points known about phosphate systems in fungi.

Genetic identification of o-PO_4^{3-} transporters and their regulation is an area of very active research. The low affinity o-PO_4^{3-} uptake system has yet to be described genetically. Two of the six known transporters have been characterized in some detail; Pho84p and Pho89p (Persson et al. 2003). The Pho84p transporter is a o-PO_4^{3-} -H^+ symport regulated by extracellular o-PO_4^{3-} concentration and pH. Maximal activity has been observed at pH 5.0. Pho84p transporters are active when cells are grown in 200 to 300 μM o-PO_4^{3-} and is repressed when o-PO_4^{3-} concentrations are adequate. Maximal activity occurs between 50 to 70 μM o-PO_4^{3-} and the Pho84p is repressed when o-PO_4^{3-} concentration reaches 10 μM. Observed experimentally, the rate of Pho84p uptake increases with exponential growth in low o-PO_4^{3-} medium, reaches maximum activity at mid to late exponential growth phase prior to a rapid decline. The Pho89p transporter is Na^+ coupled and has maximal activity at pH 9.5. Pho89p is stimulated by increases in external Na^+ concentration and also induced by higher pH rather than low o-PO_4^{3-} concentrations alone (Persson et al. 2003). The other four identified transporters have not been characterized in any detail.

When *S. cerevisiae* is exposed to low o-PO_4^{3-} concentrations a minimum of 22 genes are activated (Persson et al. 2003). Many of these genes have not been characterized. Further complicating o-PO_4^{3-} uptake in fungi is the interaction of polyphosphate concentrations in the cell. The two branches of research, polyphosphate and o-PO_4^{3-} uptake, have long been studied in isolation and are only now being recognized as a whole integrated system. What is known about the relationship between the two systems is limited. However a significant number of genes (some know, some unknown) are actively regulating both polyphosphate and o-PO_4^{3-} concentrations in fungal cells. One conclusion that has been determined from research thus far is that polyphosphate synthesis governs intracellular o-PO_4^{3-} concentrations which exerts a negative feedback on the Pho84p transporter (Persson et al. 2003). Given the fundamental nature of polyphosphate and its multiple cellular roles, the relationship between o-PO_4^{3-} and polyphosphate is far from completely understood and extremely complicated.

There are very little environment related data for fungal uptake of phosphates. This is largely due to researchers using fungi to develop a model of phosphorus utilization that can be used in higher organisms. The studies delineated in Section 2.7 are the only known observations of o-PO_4^{3-} uptake by fungi in wastewater. o-PO_4^{3-} uptake was not the primary objective in the studies and was only presented as percent decrease. There has been no study of how operational factors (e.g., aeration) impacted o-PO_4^{3-} concentration decrease. Synthesizing the genetic, biochemical and environmental aspects of phosphates in fungi shows there is a tremendous amount of research to be conducted in all three areas. It also means there is a potential for developing phosphate treatment technology using fungi.

2.10 Summary

Development of advanced wastewater treatment technology like MBRs, MBBRs and IFASs has reduced the need for, and limitations of, secondary clarifiers in wastewater treatment plants. MBRs, MBBRs and IFAS allow for longer solids retention times and increased MLSS, removing a barrier to exclusive use of bacteria for organic carbon and nutrient removal. Microscopic fungi can perform nitrification and denitrification under laboratory conditions. Evidence has been presented to show fungi cannot only grow on wastewater, but also, under the correct selective conditions, dominate activated sludge systems.

A fungal nitrogen treatment system has the potential to overcome issues associated with conventional BNR systems and is an area for advanced research into new technologies. Specifically identified issues include: system complexity; resistance to inhibitory compounds; rate and range of degradation ability; system operation conditions; system footprint; and operational costs. However, several basic question need to answered prior to development of a new treatment technology. Primarily, fungal denitrification has been exclusively studied with very controlled laboratory systems with defined medium and laboratory strains. The fungi identified as capable of denitrification are limited and not usually found in wastewater treatment activated sludge systems. Initial research must be based around finding fungi from a wastewater treatment system and determining their ability to degrade wastewater parameters of interest. The remaining chapters in this thesis focus on determining the potential of, and identification of important factors in, development of a fungal nitrogen and phosphorus treatment technologies.

Chapter Three

3.0 Materials and Methods

3.1 Isolation and Screening of Fungi from Gold Bar WWTP

3.1.1 Isolation and Storage of Fungi Isolates

Grab samples from Gold Bar's BNR reactor number five from each of the four zones (anaerobic, pre-anoxic, anoxic and aerobic) were taken using sterile sampling cup and bottles. Samples were put on ice and transported to laboratories at the University of Alberta for analysis. The samples were serially diluted and spread plated in triplicate on Cooke's Rose Bengal Agar (Sigma Aldrich) with 70 mg chlortetracycline hydrochloride L^{-1} (Sigma Aldrich). Based on the differential and selective characteristics of the medium, fungal isolates were picked from the plates and streaked on Potato Dextrose Agar (PDA, Fisher Scientific) with 70 mg chlortetracycline hydrochloride L^{-1}. Isolates were checked for purity prior to storage on PDA agar slants.

3.1.2 Screening for Fungal Nitrogen Concentration Decrease

3.1.2.1 Batch Culture Apparatus

Each batch culture apparatus employed a 125-mL Corning spinner flask with double side arms. A single reactor schematic is shown in Figure 3-1. The side arms were fitted with Teflon coated septa penetrated with 6.35 mm OD glass tubing for air inlet and exhaust. Laboratory air was humidified by sequentially sparging air through four 2-L bottles fitted with three port caps and containing deionised water. The humidified air was sterile filtered through a Whatman HEPA-Vent filter (6723-5000) with a 0.3 μm particle retention. Once the air was sterile filtered, the airflow was divided by a five-way brass manifold to the reactors. The brass manifold allowed control of airflow to each reactor by five integrated needle valves. Air flow into the reactors was determined by measuring the air flow rate exiting the reactor and by adjusting the needle valves. A second air system supplied another five reactors. Spinner flasks were mixed using two, five-place stirrers (Type 45700 Cellgro S45725, Thermolyne) operated at 50 rpm..

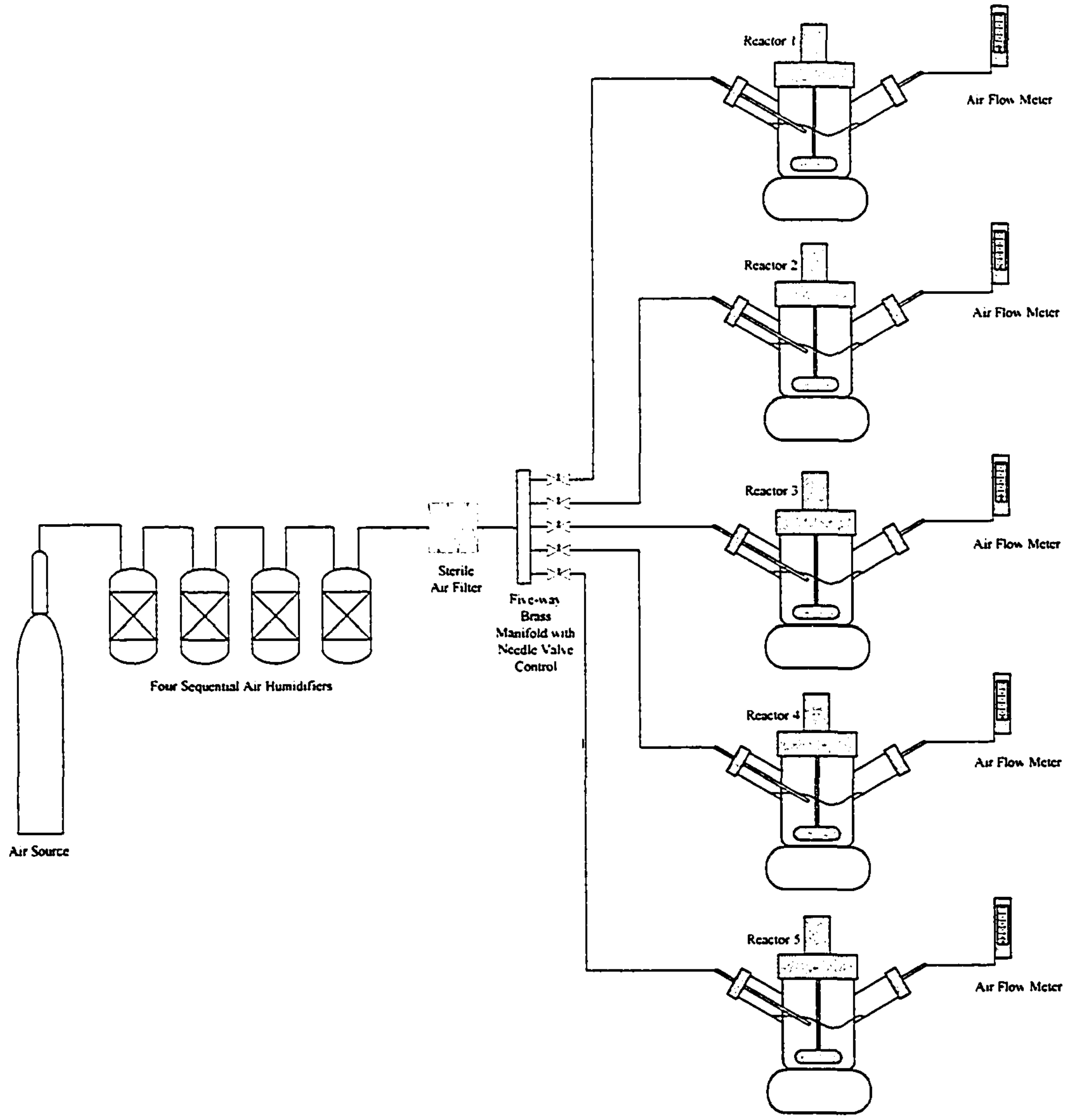
Reactor 1
Air Flow Meter
Reactor 2
Air Flow Meter
Reactor 3
Air Flow Meter
Sterile Air Filter
Five-way Brass Manifold with Needle Valve Control
Four Sequential Air Humidifiers
Reactor 4
Air Flow Meter
Air Source
Reactor 5
Air Flow Meter

Figure 3-1 Batch screening single reactor apparatus.

3.1.2.2 Preparation of Primary Effluent Medium for Screening

Approximately 20 L batches of primary effluent from Gold Bar WWTP were collected using a hand pump just upstream of the inlet to the nutrient removal process. A hollow fibre membrane unit (ZW-1, Zenon Environmental) (0.1 µm pore size) was used to pre-filter the effluent. The effluent was then 0.2 µm sterile filtered into 1 L sterile containers for use in batch trials. The sterile effluent was stored at 4 °C for up to a maximum of 3 weeks prior to use in batch trials. No growth was observed in the stored effluent.

3.1.2.3 Inoculum Preparation and Batch Trials

Fungal inoculum was prepared from stock culture stored on PDA slants. Fungal isolates were aseptically transferred to PDA plates using a swab soaked in phosphate buffered saline (PBS). Cultures were grown at 20 °C for 3 days. Five millilitres of sterile PBS was added to the plate and a sterile swab was used to scrape the surface of the agar. The fungal suspensions were aseptically transferred to 500-mL Erlenmeyer flasks containing 100-mL of sterile primary effluent and a Teflon coated magnetic stir bar. Cultures were incubated at room temperature (Approximately 21 °C) in a water bath and stirred at 50 rpm on five place stirrer (Type 45700 Cellgro S45725, Thermolyne). After 2 days of growth in the flasks, 30 mL of the inoculum was aseptically transferred to one of the batch reactors that contained 190 mL of fresh primary effluent medium. In each run, one fungal isolate was tested in duplicate in the reactors. Two reactors were retained as controls for each run. In total four fungi could be tested per run. Cultures were harvested after 3 days and the concentration decrease of wastewater parameters were measured as described in Section 3.4.

3.1.3 Fungal Identification

Unknown isolates identified with potential for nitrogen and or phosphorus concentration decrease were sent to the University of Alberta Microfungus Collection & Herbarium (UAMH) for identification. Standard procedures were used to determine the fungi to the genus level.

3.2 Batch Kinetic Screening

3.2.1 Reactor Setup and Apparatus

Ten sets of batch culture apparatus were designed and constructed. Each reactor employed 2 L HDPE (Nalgene, 21262000) containers with a three port cap. One port was for adding air, one for exhaust and the last for sampling. Laboratory air was sterile filtered through a Millipore Millex (SLFG85010) filter with a 0.2 µm particle retention. Aeration was measured and controlled for each reactor by direct-reading flow meters with integrated needle valves (Cole Parmer U-32047-12). The inlet air tube was extended through the cap to a glass Pasteur pipette to provide coarse bubbling air. For microaerophilic experimental conditions the Pasteur pipette was removed and the air was flushed only into the headspace of the reactor. Anoxic test conditions were created by continuously flushing the headspace with nitrogen gas. Each reactor had a working volume of approximately 1.9 L. Two five-place stirrers (Type 45700 Cellgro S45725, Thermolyne) operating at 100 RPM were used for mixing using 60 mm diameter egg shaped magnetic stir bars. Figure 3-2 shows a schematic of the reactors. Aseptic sampling was performed on the reactors by blocking the vent line. The positive pressure created, forced the medium out through the sample line of the reactor.

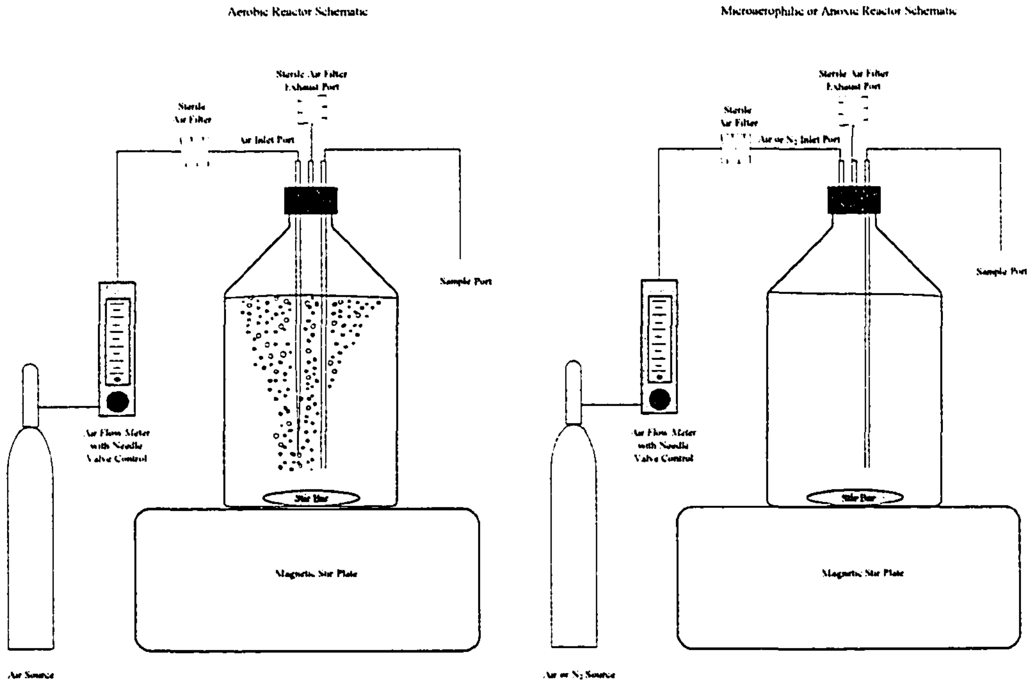
Aerobic Reactor Schematic
Sterile Air Filter
Exhaust Port
Sterile
Air Filter
Air Inlet Port
Sample Port
Air Flow Meter
with Needle
Valve Control
Stir Bar
Magnetic Stir Plate
Air Source
Microaerophilic or Anoxic Reactor Schematic
Sterile Air Filter
Exhaust Port
Sterile
Air Filter
Air or N_2 Inlet Port
Sample Port
Air Flow Meter
with Needle
Valve Control
Stir Bar
Magnetic Stir Plate
Air or N_2 Source

Figure 3-2 Reactor schematic for batch kinetic testing.

3.2.1.1 Preparation of Primary Effluent Medium

Due to the number of reactors and volume of medium required, primary treated wastewater was made sterile by autoclave at 121 °C. For trials that involved modified pH, the pH modification was made prior to autoclaving. The pH was modified by adding either ACS reagent grade concentrated hydrochloric acid or 10 N sodium hydroxide. For trials using anoxic conditions, the primary effluent was sparged with nitrogen gas for 15 min prior to autoclaving. For trials involving dilute sewage, the primary effluent was mixed 50% with deionized water.

3.2.1.2 Inoculum Preparation and Batch Trials

Fungal inocula were prepared from stock cultures stored on PDA slants. A sterile swab soaked in PBS was used to smear PDA plates. Cultures were grown at 20 °C for 3 days. Five millilitres of sterile PBS was added to the Petri plate and a sterile swab was used to scrape the surface of the agar. The fungal suspensions were aseptically transferred to 500 mL baffled shake flasks containing 300 mL of sterile primary effluent. Cultures were incubated at 25 °C on a bench top shaker table (New Brunswick, Model 4000) at 135 rpm. After 44 hr of growth in the flasks, 150 mL of the inoculum was aseptically transferred to each batch reactor that contained 1800 mL of fresh primary effluent medium. In each run, one fungal isolate was tested in duplicate in the reactors. All reactors were operated at 20 °C and were sampled either in 24 or 48 hr cycles. There was sufficient volume in each reactor for nine, 200 mL samples to be withdrawn for complete analysis.

3.2.1.3 Inoculum Preparation for Recovered Fungal Isolates

Trials from which fungi were recovered and re-introduced to new reactors were performed using an intermediate plating step. The intermediate plating step was necessary to allow time to reset the experimental apparatus. At the end of an experimental trial, a 1 mL sample was aseptically drawn from the reactor and spread plated on PDA agar in duplicate. Both cultures were incubated at 20 °C for 3 days. One plate was sealed with parafilm and stored at 4 °C while the second plate was used to create a new inoculum for the next experimental run. The inoculum procedure used was the same as described in Section 3.2.1.2.

3.2.1.4 Inoculum Preparation for Mixed Culture Experiments

Inoculum for mixed culture experiments was created by mixing equal portions with sufficient volume of each individually prepared fungal Inocula for the number of reactors testing the condition. The procedure for preparing the single inoculum was as previously described. The inocula mixed together for experiments were *Geotrichum* sp. 2000, *Geotrichum* sp. 2007, Yeast, *Penicillium* sp, and the *Phoma* sp.

3.3 Attached-growth and Fed-batch Attached-growth

3.3.1 Reactor Setup and Apparatus

Attached-growth reactors were developed from the 2 L reactors used in the kinetic screening. A reactor schematic is shown in Figure 3-3. The attachment medium was Nalgene 180 clear PVC tubing. Two tubing diameters of 3.2 mm I.D. and 4.8 mm I.D. were used. The tubing was cut into rings 2 to 3 mm in length. The estimated inside surface area/volume for the attachment medium was 24 m^2/m^3. Initial attached-growth reactors used 10% attachment medium to reactor volume. Secondary studies used 20% attachment medium to reactor volume. The percent of attachment medium to reactor volume was limited to 20% due to interference with mixing. Greater than 20% rings prevented the magnetic stirrer from operating stably. Fed-batch studies used the same experimental setup. However, once nitrogen was completely removed from the wastewater, the entire liquid content of the reactor was drained and replaced with 1.8 L of sterile primary effluent.

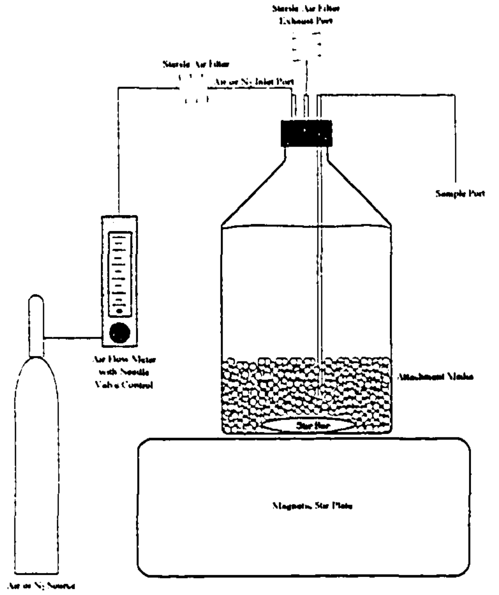
Sterile Air Filter
Exhaust Port
Sterile Air Filter
Air or N2 Inlet Port
Sample Port
Air Flow Meter
with Needle
Valve Control
Attachment Media
Stir Bar
Magnetic Stir Plate
Air or N2 Source

Figure 3-3 Attached-growth batch reactor schematic.

3.4 Analytical Methods

3.4.1 Ion Chromatography

Nitrate, nitrite, o-phosphate, sulfate, chloride and bromide were determined using ion chromatography (IC) in accordance with Standard Method 4110 B (American Public Health Association et al. 1995). The method was performed with a Dionex 300 IC unit fitted with a Dionex AS-9-SC 4mm X 250 mm analytical column coupled to a conductivity detector. The detection limit for the analytes listed above has been determined to be in the range of <0.1 mg L^{-1} but >0.01 mg L^{-1}. Post analysis of the IC peaks was performed with Dionex AI-450 Chromatography software.

Sample preparation for the IC was performed with 0.22 µm Millipore Millex (SLGV013NL) filters or with Whatman Uniprep Filter (UN113ENYL). The Whatman units are fitted with a glass fibre pre-filter and a 0.2 µm hydrophilic nylon membrane. Chloride interference was removed, when necessary, with Maxi-Clean

IC-Ag prep cartridges. The IC-Ag is a strong cation exchanger that removes chloride, iodide and bromide through formation of Ag-halide salts. All chemicals and standards used for the IC were ACS reagent grade purchased from Sigma Aldrich. Water used was Elga HPLC Type I reagent grade water with a resistivity of greater than 18.0 MΩ. IC regenerant, eluent and mixed standards solutions were filtered through 0.2 μm Whatman Nylon 47 mm diameter membranes (7402-004).

3.4.2 COD Measurements

Standard Method 5220 D (American Public Health Association et al. 1995) closed reflux colorimetric method for COD was employed. Borosilicate glass tubes (16 x 100 mm) with a polypropylene screw cap (Fisher 14-962-26F) were used in conjunction with a HACH COD Reactor. All chemicals were ACS reagent grade from Fisher Scientific. A Pharmacia Biotech Novaspec II spectrophotometer was used to measure absorbance at 600 nm.

3.4.3 Nitrogen Analysis

3.4.3.1 Ammonium measurements

An Accumet ammonia combination ion selective electrode (Fisher 13-620-505) was used for determination of ammonia according to Standard Method 4500-NH_3 D (American Public Health Association et al. 1995). A sodium hydroxide EDTA solution was used to ensure no interference from mercury or silver complexing. The Accument probe was used with an Accument Research AR50 pH/mV/ISE meter (Fisher 13-636-AR50).

3.4.3.2 Total Kjeldahl Nitrogen (TKN)

TKN analyses were performed on samples using block digestion, steam distillation and auto-titration. A Tecator 1015 Digester System 20 block and Tecator Kjeltec System 1026 distillation unit were used according to manufacture instructions to process samples. A Mettler Toledo DL53 Titrator was used with 0.05 N hydrochloric acid for titration. The titrant was calibrated with sodium carbonate with a methyl red indicator.

3.4.4 Total Suspended Solids (TSS) and Total Solids (TS)

TSS were used to determine biomass accumulation in the batch reactors. The tests were carried out according to Standard Method 2540 D (American Public Health Association et al. 1995). Tests were conducted with 40-mL porcelain Gooch crucibles fitted with 24 mm organic binder free borosilicate glass fiber filters (Fisherbrand G4 09-804-24C). Sample volumes were used to obtain between 10 and 200 mg of material on the filter. TS were conducted according to Standard Method 2540 B (American Public Health Association et al. 1995). Between 10 and 25 mL of the mixed liquor was pipetted directly into 40 mL crucibles.

Chapter Four

4.0 Experimental Results

4.1 Isolation and Screening of Fungi to Determine Potential for Ammonia Nitrogen Concentration Decrease from Wastewaters

4.1.1 Sampling

Table 4-1 shows the results of the enumeration of the grab samples taken from Gold Bar WWTP BNR Reactor #5. The data presented is from a single sampling event and therefore is of a highly speculative nature. A much more rigorous sampling program was needed in order to evaluate the range and predominance of fungi in the BNR reactor. The sampling techniques and enumeration only identified the non-fastidious yeast and fungi. In order to expand the range and improve the accuracy of data and fungi from the activated sludge system, biofilm surfaces needed to be sampled and baiting techniques used (Mueller et al. 2004). The sampling method could also not differentiate between viable fungi inhabiting the BNR reactor and fungal spores only passing through the reactors. The weaknesses in the data do not allow interpretation of or conclusions about the observable trends in Table 4-1. However, the purpose of the sampling was to obtain fungi from a wastewater environment. From the plates, a total of 39 isolates with visually distinguishable colony morphologies were labelled and stored for screening tests. Table 4-1 shows the number from each of the zones, with greater than two-thirds from the anoxic and anaerobic zones. Due to a loss of viability, only 22 isolates where screened for the concentration decrease of nitrogen and phosphorus from wastewater.

Table 4-1 Estimated yeast and fungi concentration found in Gold Bar WWTP.

BNR Reactor Zone	Reactor Zone Volume (m^3)	Reactor Zone Flow Order	Concentration (CFU/mL)		No. of Unique Isolates
			Yeast	Fungi	
Aerobic	8,160	4	17,200	200	5
Pre Anoxic	516	1	25,900	300	7
Anoxic	1,562	3	75,500	470	16
Anaerobic	642	2	33,800	530	11

4.1.2 Screening Isolates for Nitrogen and Phosphorus Concentration Decrease

The average primary effluent wastewater medium initial parameters are contained in Table 4-2. Mean initial COD, NH_4^+, and o-PO_4^{3-} were 183 mg L^{-1}, 21.6 mg-N L^{-1} and 17.1 mg L^{-1}, respectively. In a few batches of the wastewater medium, trace amounts of NO_3^- were detected. The initial o-PO_4^{3-} concentration was high in the wastewater, but this was found to be the result of natural variation in Gold Bar WWTP influent o-PO_4^{3-}. Typical o-PO_4^{3-} in the influent wastewater for many WWTP was between 1 and 8 mg L^{-1} (Metcalf & Eddy et al. 2003). The high concentration found in Gold Bar WWTP had the effect of masking the percent decrease calculation. Each isolate was screened at least in duplicate. Screening results for the isolates from pre-anoxic, anaerobic anoxic and aerobic zones are shown in Table 4-3, Table 4-4, Table 4-5 and Table 4-6, respectively.

Table 4-2 Average initial primary effluent wastewater medium parameters.

Parameter	COD mg/L	TSS mg/L	NH_4^+ mg-N/L	NO_3^- mg-N/L	NO_2^- mg-N/L	o-PO_4^{3-} mg/L	SO_4^{2-} mg/L	Br^- mg/L	Cl^- mg/L
Mean	180	1.2	21.6	0.1	0.0	17.1	103.3	0.4	83.3
95% C.I. (n=6)	7	0.3	0.7	0.1	0.0	0.5	3.6	0.3	3.1

Table 4-3 Results of screening isolates from the pre-anoxic zone Gold Bar WWTP BNR.

Isolate	Number of Trials (n)	COD Decrease (%)	NH_4^+ Decrease (%)	Final NH_4^+ (mg-N L^{-1})	o-PO_4^{3-} Decrease (%)	o-PO_4^{3-} Decrease (mg L^{-1})
1000	2	27	-38.1	23.9	NA	NA
1001	2	34	13.7	19.0	1.3	0.2
1002	2	31	-5.7	18.3	NA	NA
1003	2	13	1.7	17.0	NA	NA
1004	2	29	13.4	15.0	NA	NA
	Mean	27	-3.0	18.6	1.3	0.2
	95% C.I.	5	15.4	2.5	6.8	1.0

Table 4-4 Result of screening isolates from the anaerobic zone Gold Bar WWTP BNR.

Isolate	Number of Trial (n)	COD Decrease (%)	TSS (mg L^{-1})	NH_4^+ Decrease (%)	Final NH_4^+ (mg-N L^{-1})	Final Concentration NO_3^- (mg-N L^{-1})	Final Concentration NO_2^- (mg-N L^{-1})	o-PO_4^{3-} Decrease (%)	o-PO_4^{3-} Decrease (mg L^{-1})
2000	2	49	70.6	56.4	9.1	<0.01	<0.01	1.6	0.3
2002	2	56	51.8	-18.0	28.5	<0.01	<0.01	-8.3	-1.4
2004	3	48	65.5	15.6	17.6	<0.01	<0.01	-1.0	-0.2
2006	2	28	30.5	-2.5	22.5	<0.01	<0.01	-5.7	-0.8
2007	2	30	46.0	27.7	19.4	<0.01	<0.01	53.0	10.6
2008	2	33	61.5	42.9	12.4	<0.01	<0.01	27.5	5.2
	Mean	41	54.3	20.3	18.3	NA	NA	11.2	2.3
	95% C.I.	6	7.8	18.5	4.4	NA	NA	10.9	2.5

Table 4-5 Screening result of isolates from the anoxic zone Gold Bar WWTP BNR.

Isolate	Number of Trial (n)	COD Decrease (%)	TSS (mg L^{-1})	NH_4^+ Decrease (%)	Final NH_4^+ (mg-N L^{-1})	Final Concentration		o-PO_4^{3-} Decrease (%)	o-PO_4^{3-} Decrease (mg L^{-1})
						NO_3^- (mg-N L^{-1})	NO_2^- (mg-N L^{-1})		
3000	2	48	35.6	5.7	19.7	<0.01	<0.01	4.3	0.7
3002	2	50	53.0	18.9	17.6	<0.01	<0.01	12.6	2.4
3003	2	18	24.8	24.9	18.1	0.48	<0.02	-14.0	-2.4
3004	2	20	34.0	44.5	12.0	<0.01	<0.03	6.4	1.2
3005	2	29	32.5	26.1	15.5	<0.01	<0.04	39.2	7.3
3006	2	6	40.0	13.6	20.8	0.485	<0.05	-19.0	-3.2
3009	2	37	32.1	-0.2	24.2	0.52	<0.06	11.6	2.0
3011	2	14	61.5	26.5	15.4	<0.01	<0.01	8.0	1.5
3013	4	54	38.0	58.7	11.8	<0.01	<0.01	8.6	1.6
	Mean	31	39.0	24.3	17.2	NA	NA	6.4	1.2
	95% C.I.	11	8.0	15.4	3.8	NA	NA	10.5	1.9

Table 4-6 Screening results of isolates from the aerobic zone Gold Bar WWTP BNR.

Isolate	Number of Trial (n)	COD Decrease (%)	TSS (mg L^{-1})	NH_4^+ Decrease (%)	Final NH_4^+ (mg-N L^{-1})	Final Concentration		o-PO_4^{3-} Decrease (%)	o-PO_4^{3-} Decrease (mg L^{-1})
						NO_3^- (mg-N L^{-1})	NO_2^- (mg-N L^{-1})		
4001	2	11	19.0	36.5	13.3	<0.01	<0.01	8.3	1.6
4002	2	36	40.4	22.8	20.7	<0.01	<0.01	-21.0	-2.9
	Mean	24	29.7	29.6	17.0	NA	NA	-6.4	-0.7
	95% C.I.	15	13.4	8.1	4.2	NA	NA	35.4	5.0

The pre-anoxic zone isolates (Table 4-3) minimally decreased the NH_4^+ content in the medium. Isolates 1000 and 1005 actually showed an increase in NH_4^+ concentrations. The IC data which included the o-PO_4^{3-} measurement were lost for almost all the pre-anoxic zone isolates due to equipment failure that led to sample storage time well in excess of those recommended in Standard Methods (American Public Health Association et al. 1995).

Three anaerobic zone isolates (Table 4-4) were found to have high potential for NH_4^+ and o-PO_4^{3-} concentration decrease. Isolates 2000 and 2008 gave average NH_4^+ concentration decrease of 56 and 43 %, respectively. Where as isolate 2000 showed minimal decline of o-PO_4^{3-}, 2008 had a 27% concentration decrease which equals 5.2 mg L^{-1}. Although this is a small percentage, 5 mg L^{-1} concentration decrease for many WWTP would equate to complete removal based on average influent o-PO_4^{3-} concentrations (Metcalf & Eddy et al. 2003). Isolate 2007 only showed a 28% concentration decrease of NH_4^+, but decreased o-PO_4^{3-} by 53% or a total of 10 mg L^{-1}. The o-PO_4^{3-} concentration decrease was very noteworthy. Isolates 2000, 2007 and 2008 were selected to be identified and tested in kinetic studies.

The anoxic zone isolates comprised nine members and was the largest group to be screened. Isolates 3004 and 3013 both had high average NH_4^+ concentration decrease of 44.5 and 59%, respectively. Both isolates also showed some o-PO_4^{3-} concentration decrease around 1.2 mg L^{-1}. Isolate 3005, while showing 26% decline of NH_4^+, decreased o-PO_4^{3-} by 39% or 7.3 mg L^{-1}. Isolates 3004, 3013, and 3005 where selected for identification and further testing.

Only two isolates from the aerobic zones were screened for concentration decrease of NH_4^+ and o-PO_4^{3-}. Isolate 4001 had an average NH_4^+ decline of 37% and a decrease of o-PO_4^{3-} of 1.6 mg L^{-1} Isolate 4002 did show some concentration decrease of NH_4^+ but this was deemed not to warrant further testing. Isolate 4001 was selected for identification and further testing.

The aerobic conditions maintained through the 3-day incubation period were expected to produce significant amounts of nitrification products. As shown in Table 4-4, Table 4-5 and Table 4-6, NO_3^- or NO_2^- was not detected in the medium after the incubation period except with isolates 3003, 3006 and 3009. These three isolates did not actually accumulate NO_3^-, the final concentration in the medium was due to initial amounts of NO_3^- being present.

Concentration decrease of COD by all fungi ranged from as low as 11% to 56% with an overall average of 30%. A high decrease of COD did not always correlate with higher concentration decrease of NH_4^+ and o-PO_4^{3-} across the various species. This is likely due to the diversity of the isolates showing various ranges of degradative capacities, substrate affinities and energy utilization. Since there were only two replicates for most isolates, there was insufficient data to conduct meaningful correlation analyse for each isolate.

The selected isolates of interest were sent to the UAMH for identification. The results of the identification are shown in Table 4-7 along with key summary information concerning NH_4^+ and o-PO_4^{3-}concentration decreases. Identification was only to the genus level. Isolates 2000 and 2007 were two *Geotrichum* spp. (both likely *Geotrichum candidum*); isolates 3004 and 4001 were two non-identical

Penicillium spp.; isolate 3005 was a *Mucor* sp.; isolate 3013 was a *Phoma* sp.; and isolate 2008 was a yeast, very tentatively identified as a *Pichia* sp. *Geotrichum* sp (2000), yeast (2008), *Penicillium* spp. (3004, 4001) and *Phoma* sp. (3013) were selected for their NH_4^+ treatment potential. *Geotrichum* sp. (2007), *Mucor* sp. (3005), the yeast (2008) were selected for o-PO_4^{3-} treatment potential. Table 4-7 also shows the estimated average COD to combined NH_4^+ and o-PO_4^{3-} concentration decrease ratio. Because NH_4^+ and o-PO_4^{3-} decreased simultaneously in the medium it is not possible to separate and calculate the ratio for NH_4^+ and o-PO_4^{3-}, individually. However, shown in brackets next to the ratio is the percent of the ammonia contribution to the ratio. Table 4-7 indicates that the identified fungi have a range of concentration decrease ratios from as low as 2.6:1 to 8.2:1. For *Geotrichum* sp. (2000), *Phoma* sp. (3013), *Penicillium* spp. (3004) and (4001) the concentration decrease ratio was largely (>83%) due to NH_4^+. For *Geotrichum* sp. (2007), yeast (2008), and *Mucor* sp. (3005) concentration decrease ratio was due to a mix of NH_4^+ and o-PO_4^{3-} (40 to 60%).

Table 4-7 Identified isolates and mean percent (%) concentration decrease of ammonia and o-phosphate.

Isolate	Genus Identification	WWTP BNR Isolation Zone	Mean NH_4^+ Decrease (%)	Mean o-PO_4^{3-} Decrease (%)	Decrease Ratio (COD:NH_4^++o-PO_4^{3-})
2000	*Geotrichum* sp.	Anaerobic	56	2	7.3:1 (97% NH_4^+)
2007	*Geotrichum* sp.		28	53	2.7:1 (41% NH_4^+)
2008	Yeast*		43	28	4.8:1 (64% NH_4^+)
3004	*Penicillium* sp.	Anoxic	45	6	3.9:1 (89% NH_4^+)
3005	*Mucor* sp.		26	39	4.9:1 (43% NH_4^+)
3013	*Phoma* sp.		59	9	8.2:1 (89% NH_4^+)
4001	*Penicillium* sp.§	Aerobic	37	8	2.6:1 (83% NH_4^+)

*Tentative *Pichia* sp.
§Different from 3004

4.2 Batch Kinetic Ammonium Concentration Decrease Studies

4.2.1 Suspended-growth

4.2.1.1 Oxygen Effects on the Decrease of NH_4^+ Concentration in Wastewater

Initial batch testing of the fungal NH_4^+ concentration decrease rates focused on the effects of aeration. Based on the literature, it was believed fungi would require a two step process to decrease NH_4^+; aerobic nitrification followed by anoxic conditions to stimulate denitrification. The first studies tested submerged coarse bubble aeration at a rate 0.2 L min^{-1}. However, it was by accident that the importance of microaerophilic conditions necessary for the fungal system was discovered. During two runs, the tubing providing air inside the reactors became disconnected within the first 10 hr of operation. For the remaining run time, the two cultures received air only in the headspace of the reactor and therefore aeration was dependent on the mass transfer at the air-liquid interface (surface re-aeration). With mixing being provided by slow speed magnetic stirrer at 100 rpm, surface re-aeration was low. The aerobic effect on NH_4^+ concentration decrease was low for most fungal species tested. However, the two reactors with only headspace aeration (i.e. microaerophilic conditions) showed a rapid decrease in the NH_4^+ concentration without the production of nitrite or nitrate. Due to these results, microaerophilic and anoxic conditions were studied in detail. Figure 4-1 to Figure 4-5 show the effects of three oxygen tensions on the five fungi tested. The exact concentration of oxygen saturation in the reactors could not be determined for any of the reactors, however, the aerobic and anoxic cultures can be considered saturated and void of oxygen, respectively. In Figure 4-1 to Figure 4-5 the aerobic and microaerophilic data were obtained using a paired comparison experimental design. Five of the reactors were aerobic and the other five were microaerophilic. The wastewater medium was from the same batch and the inocula were split between aerobic and microaerophilic reactors. The only difference between the reactors was the aeration of the wastewater. The simplicity of the split comparison design highlights the significance of microaerophilic system for fungi to decrease NH_4^+ from wastewater. The response was similar for *Geotrichum* spp., the *Penicillium* sp. and the *Phoma* sp. The yeast (Figure 4-5), while showing that microaerophilic conditions decrease the NH_4^+ the most, exhibited little difference between the aerobic, microaerophilic and anoxic conditions.

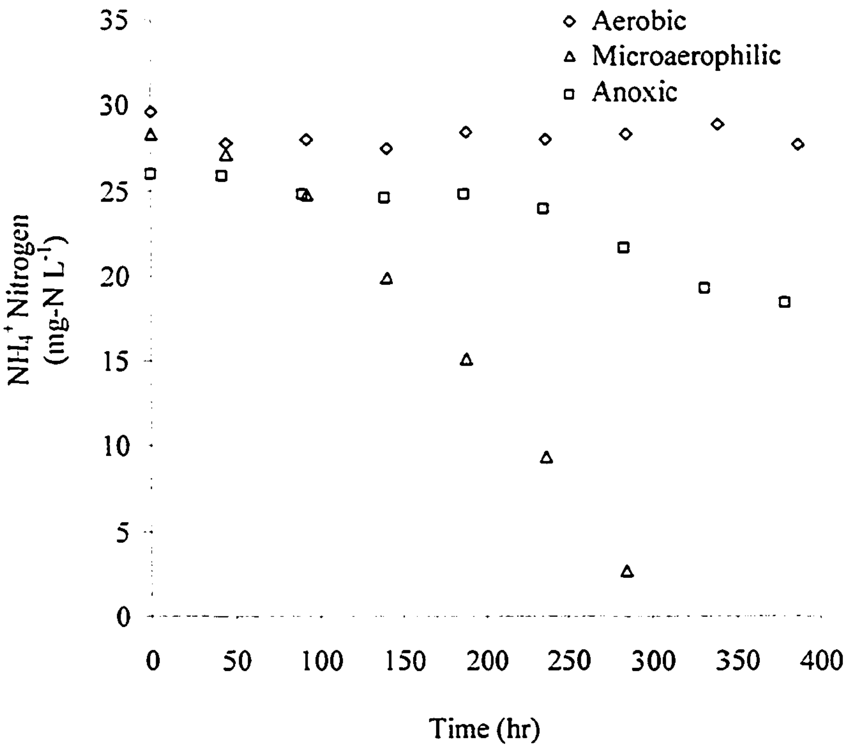

Figure 4-1 Ammonium concentration decrease by suspended *Geotrichum* sp. (2000) culture under three oxygen tension levels.

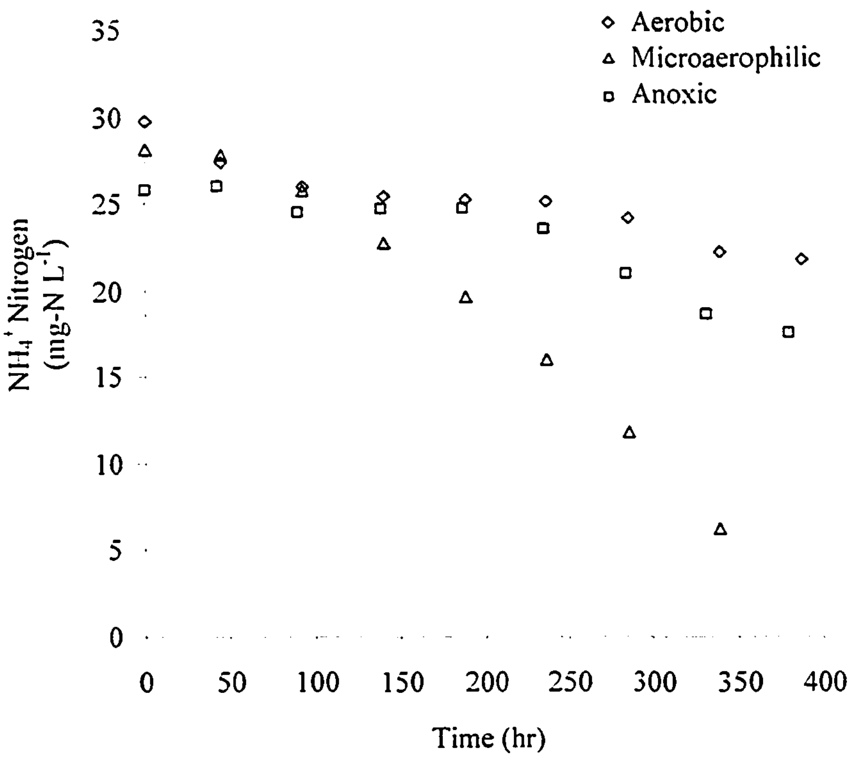

Figure 4-2 Ammonium concentration decreases by suspended *Geotrichum* sp. (2007) culture under three oxygen tension levels.

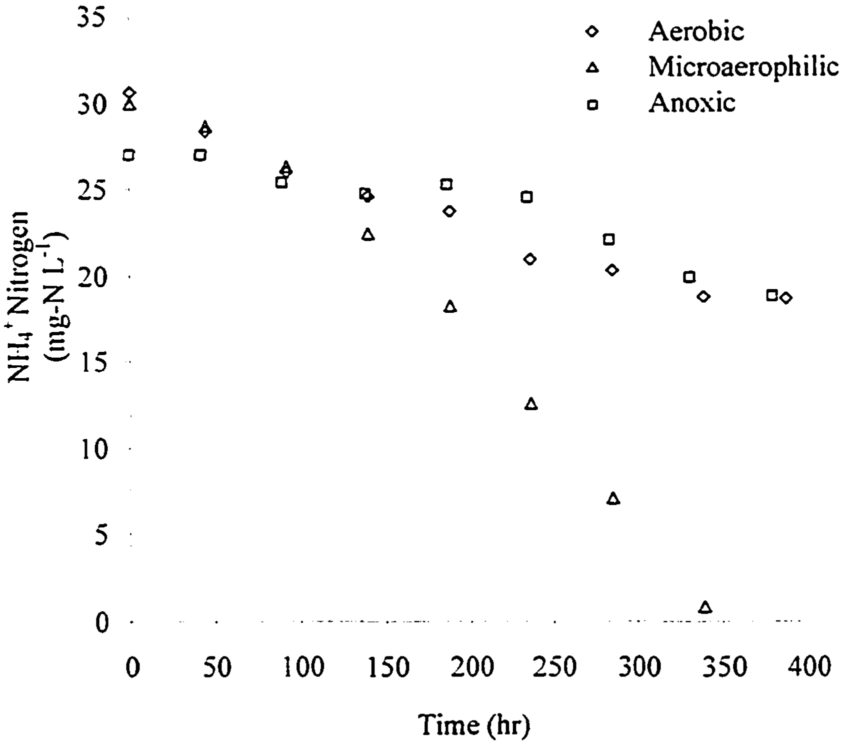

Figure 4-3 Ammonium concentration decreases by suspended *Penicillium sp.* (3004) culture under three oxygen tension levels.

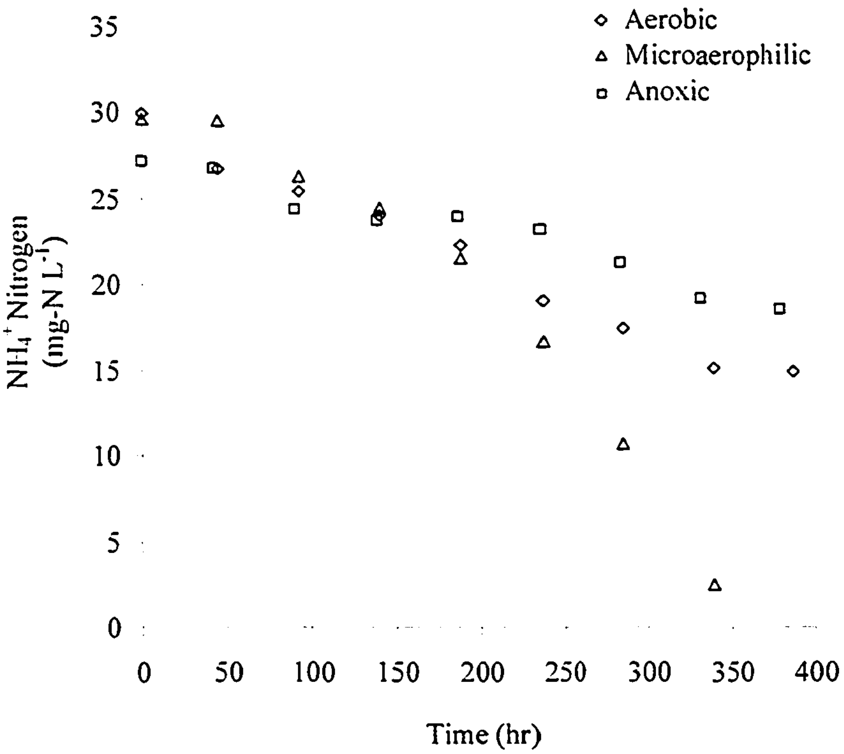

Figure 4-4 Ammonium concentration decreases by suspended *Phoma* sp. (3013) culture under three oxygen tension levels.

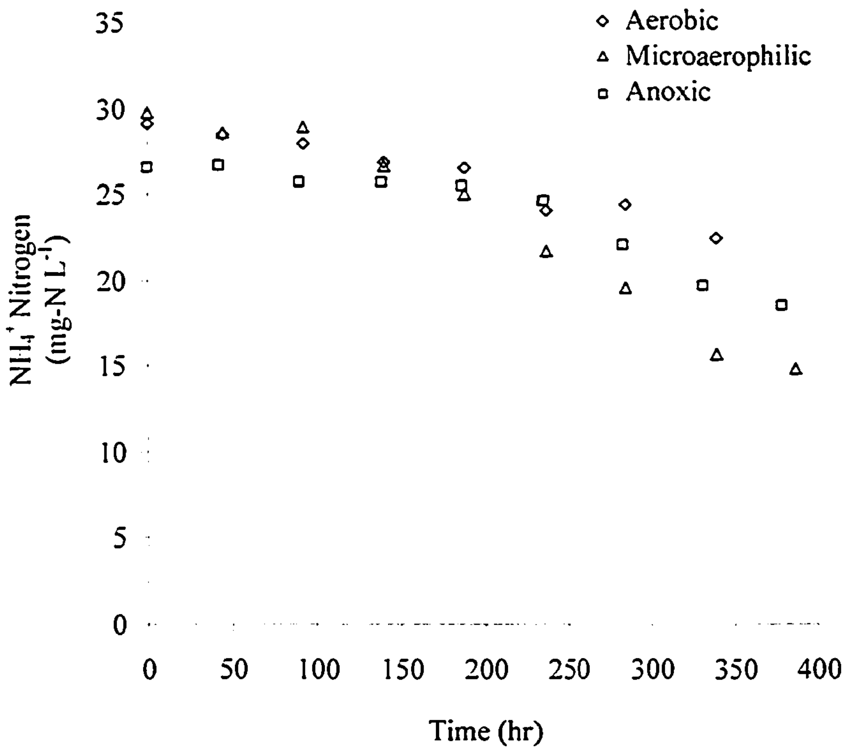

Figure 4-5 Ammonium concentration decreases by suspended by yeast species (2008) culture under three oxygen tension levels.

There are three important features in the results for the effect of oxygen on fungal treatment of NH_4^+. First, while not shown in Figure 4-1 to Figure 4-5, the failure to produce products of nitrification even under aerobic conditions; second, for most of the fungi tested, there was minimal aerobic and anoxic decrease of NH_4^+; and third, the microaerophilic condition was necessary for NH_4^+ concentration decrease. Anoxic treatment resulted in 6 to 8 mg-N/L of NH_4^+ decrease. Microaerophilic conditions resulted in a net decrease between 23 and 29 mg-N/L of NH_4^+ for *Geotrichum* spp., the *Penicillium* sp. and the *Phoma* sp.

Another key trend shown in Figure 4-1 to Figure 4-5 is the shape of the NH_4^+ decrease curve. The curve shows an initial lag followed by a linear decrease over time. Although the lag times varied from run to run, it was a consistent trend and likely the product of batch growth and variation in the wastewater medium. The lag times varied between zero and 150 hr with a typical value of 100 hr. The linear NH_4^+ decrease over time indicated a zero order-kinetic model best described the data. Table 4-8 shows the calculated average zero-order kinetic constant (k) and NH_4^+ percent decrease by fungi observed under the three oxygen tensions. The zero-order constant is the NH_4^+ substrate utilization rate (r_{su}). The utilization rates shown in Table 4-8 were calculated from the data points after the lag phase.

The r_{su} values in Table 4-8 included run to run variations due to changes in wastewater medium and inoculum development. Even including run to run variation, the trend in r_{su} values in Table 4-8 show that higher rates of NH_4^+ concentration decrease typically occurred under the microaerophilic condition for the fungi but not for the yeast. The NH_4^+ percent decrease mirrors the r_{su} trend, but shows greater separation between the three oxygen tensions. Excluding the yeast data, the average decrease observed was between 56 and 66 %, with the maximum observed between 78 and 97% for the microaerophilic runs. The variation presented in Table 4-8 for both the r_{su} and NH_4^+ concentration decrease percent was high. This was expected due to the experimental design and presentation of the data in this format versus presenting the data in a paired comparison format. The paired comparison format shown in Figure 4-1 to Figure 4-5 clearly demonstrates the importance of oxygen tension on NH_4^+ concentration decrease. However, presentation of the data in Table 4-8 was indicative of fungal performance under variable conditions that would be seen in a WWTP. The data in Table 4-8 show the highest variation for both r_{su} and NH_4^+ percent decrease occurred under microaerophilic condition. Due to the data collected on the impacts of oxygen on NH_4^+ concentration decrease almost all subsequent testing was performed using a microaerophilic environment.

Table 4-8 Observed NH_4^+ kinetic rate and percent decrease for various fungi under three oxygen tensions.

			r_{su} (mg-N L^{-1} hr^{-1})			NH_4^+ Decrease (%)		
Fungi	Oxygen State	n	Mean	Max*	SD†	Mean	Max*	SD†
Geotrichum sp. (2000)	Aerobic	3	0.013	0.019	0.010	17	23	9
	Microaerophilic	5	0.051	0.114	0.040	58	90	26
	Anoxic	3	0.017	0.034	0.014	25	33	8
Geotrichum sp. (2007)	Aerobic	2	0.028	0.038	0.014	28	29	2
	Microaerophilic	6	0.069	0.217	0.077	56	78	21
	Anoxic	3	0.012	0.023	0.010	22	35	14
Yeast	Aerobic	3	0.045	0.060	0.014	35	50	14
	Microaerophilic	5	0.033	0.067	0.022	47	68	24
	Anoxic	3	0.012	0.022	0.009	20	32	13
Penicillium sp.	Aerobic	2	0.053	0.076	0.031	39	39	0
	Microaerophilic	6	0.066	0.105	0.035	63	97	32
	Anoxic	3	0.011	0.020	0.009	18	28	12
Phoma sp.	Aerobic	2	0.048	0.057	0.012	40	50	15
	Microaerophilic	6	0.080	0.112	0.056	66	92	22
	Anoxic	3	0.012	0.021	0.009	19	30	13

*Maximum
†Standard Deviation

Growth rates for fungi were estimated from suspended solids analysis. Mycelial growth organisms display growth kinetics based on apical extension of the hyphal mass. Growth rates have been described by equation (1) (Stanbury et al. 1995):

$$M^{\frac{1}{3}} = kt + M_o^{\frac{1}{3}} \qquad (1)$$

where M_o and M are the fungal mass at time zero and time t, respectively. Plotting the cube root of fungal mass against time yields a straight line, the slope of which is the growth rate. Because of the complexity of mycelia growth, observed rates are typically between exponential and cubed root. Exponential growth is described by equation (2) (Stanbury et al. 1995):

$$X_t = X_o e^{\mu t} \qquad (2)$$

Where X_o and X_t are the initial biomass and biomass at time zero and t, respectively. Taking the natural logarithm of the equation yields:

$$\ln X_t = \ln X_o + \mu t \qquad (3)$$

The plot of the natural logarithm of biomass versus time should yield a straight line, the slope of which is the growth rate (μ). Both methods were used to estimate growth rates of fungi in the tests. The calculated rate constants from the two methods were averaged to better represent the growth rate from a run. Table 4-9 shows the estimated growth rates for the fungi tested using the three oxygen tensions. The general trend was that the aerobic conditions gave the highest growth rate followed by microaerophilic and finally anoxic. The microaerophilic growth rates ranged from 0.002 to 0.013 hr^{-1}.

Growth rates for nitrifying bacteria are typically 0.032 hr^{-1} (Rittmann 1984) and 0.0125 hr^{-1} for denitrifying bacteria (Metcalf & Eddy et al. 2003). Comparing these rates with Table 4-9, fungal rates are 75% less in aerobic environment and 57% lower in an microaerophilic environment. The slow growth rates exhibited by fungi are an issue in attempting to design an activated sludge system. Long residence times would be necessary to ensure washout would not occur. This suggests that a fungal based system would more likely employ membrane bioreactor or attached-growth technology.

Table 4-9 Suspended culture growth rates for three fungal species under three oxygen environments.

Oxygen State	Species	μ (hr^{-1})	Standard Deviation	n
Aerobic	*Geotrichum* sp. (2000)	0.017	0.004	3
	Geotrichum sp. (2007)	0.017	0.005	2
	Yeast	0.019	0.012	3
	Penicillium sp.	0.013	0.001	2
	Phoma sp.	0.009	0.006	2
Microaerophilic	*Geotrichum* sp. (2000)	0.009	0.005	5
	Geotrichum sp. (2007)	0.010	0.002	6
	Yeast	0.013	0.002	5
	Penicillium sp.	0.002	0.002	6
	Phoma sp.	0.009	0.004	5
Anoxic	*Geotrichum* sp. (2000)	0.004	0.001	2
	Geotrichum sp. (2007)	0.007	0.002	2
	Yeast	0.009	0.000	2
	Penicillium sp.	0.009	0.004	2
	Phoma sp.	0.004	0.003	2

4.2.1.2 Fate of Ammonium

As previously stated, the finding of NH_4^+ concentration decrease without the production of nitrate or nitrite under aerobic or microaerophilic conditions was not expected. The initial research performed only collected data on ammonium, nitrite and nitrate. To explore the fate of ammonium in suspended culture, a TKN analysis was added to allow determination of the organic nitrogen and total nitrogen in the system. There were two major, equally possible, explanations for the decrease of ammonium. First, nitrogen assimilation and, second, a type of fungal denitrification not previously described in the literature. Figure 4-6 to Figure 4-10 show the various forms of nitrogen for the five fungi tested under microaerophilic environments. All five fungi studied showed the same trend in terms of NH_4^+ concentration decrease and no detectable production of nitrite and nitrate. The key trend shown in all the figures is the organic nitrogen content in the system was essentially constant. If ammonium was being assimilated, the organic nitrogen should have increased by the corresponding amount of ammonium decrease. The data clearly indicate assimilation is not responsible for NH_4^+ decline in the reactor systems. This provides further evidence that fungi are performing some form of denitrification.

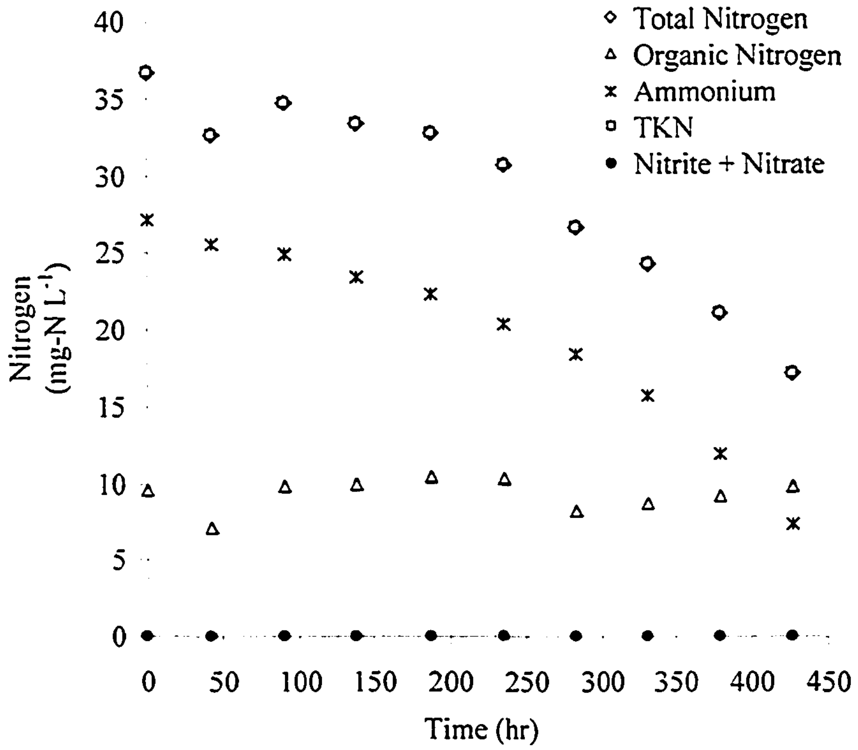

Figure 4-6 Suspended microaerophilic culture nitrogen species plot for *Geotrichum* sp. (2000)

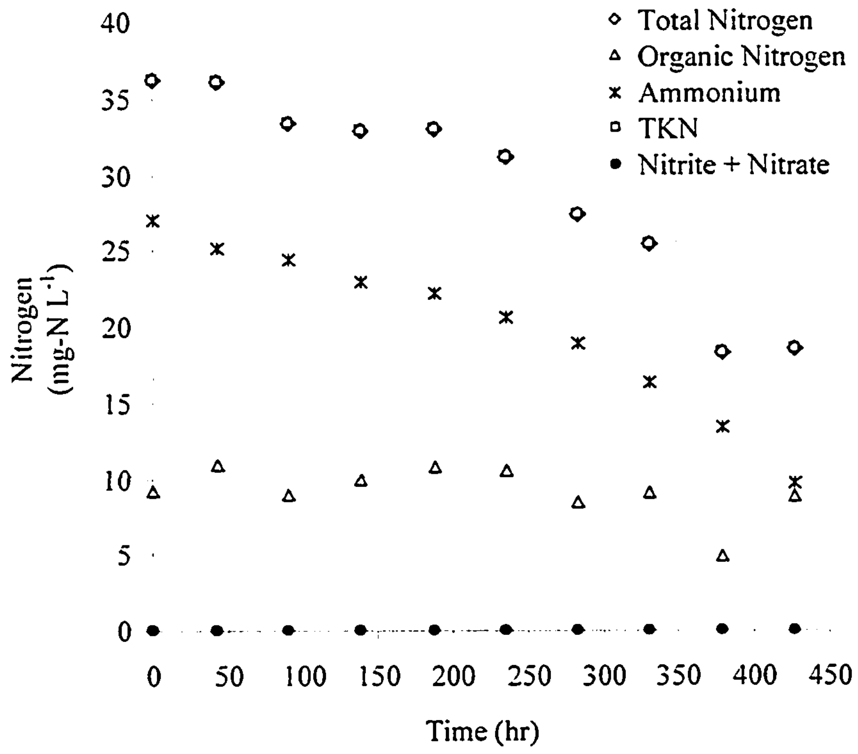

Figure 4-7 Suspended microaerophilic culture nitrogen species plot for *Geotrichum* sp. (2007)

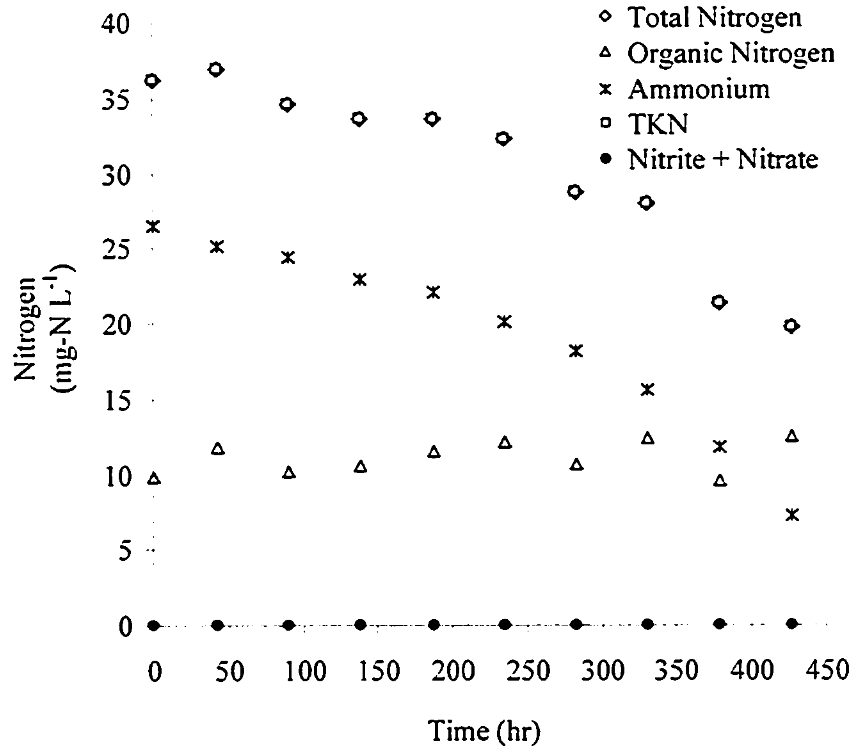

Figure 4-8 Suspended microaerophilic culture nitrogen species plot for yeast species.

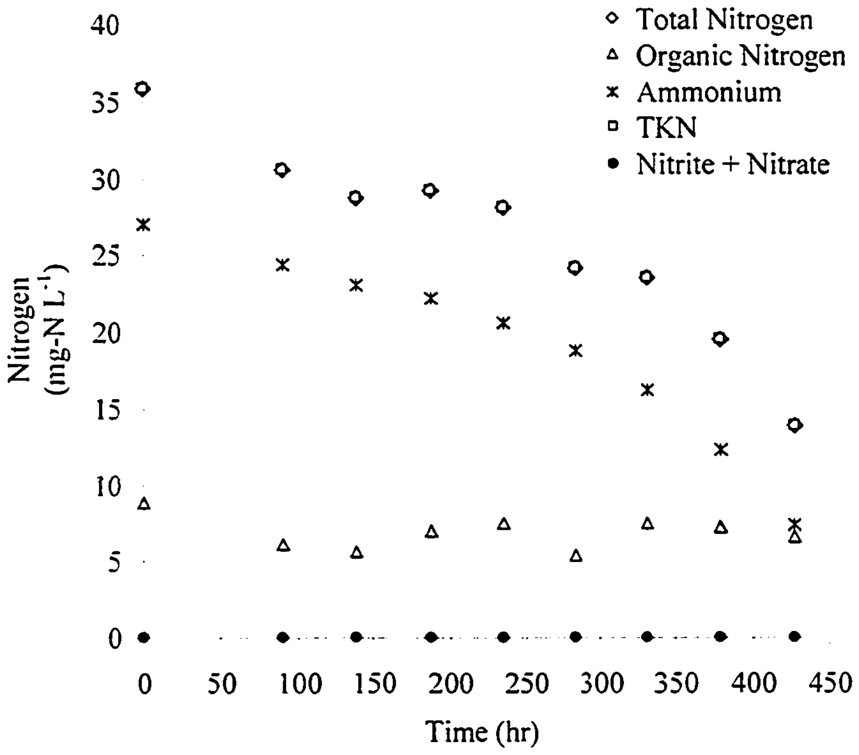

Figure 4-9 Suspended microaerophilic culture nitrogen species plot for *Penicillium* sp.

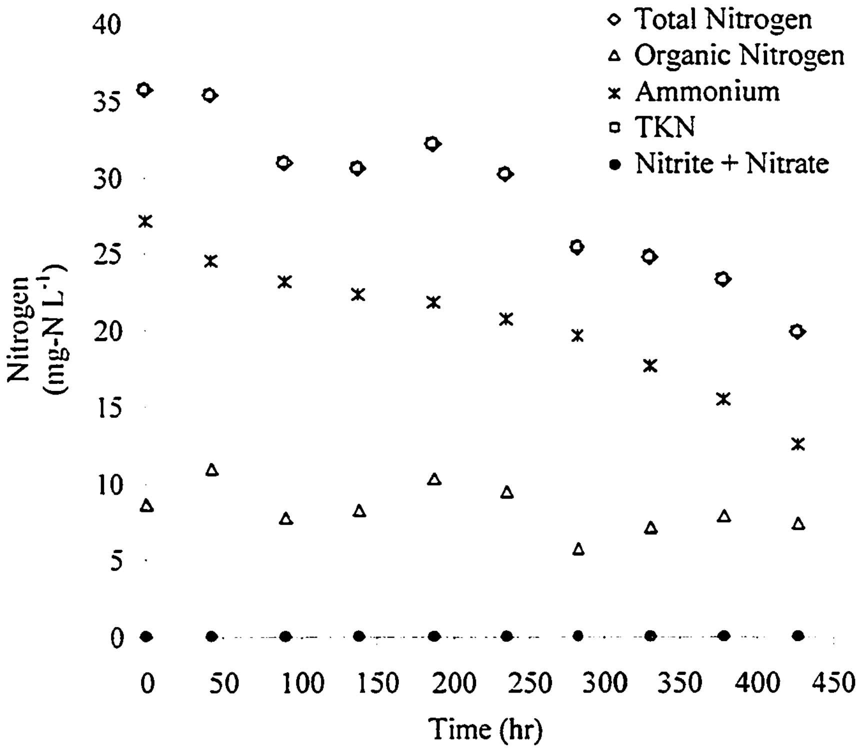

Figure 4-10 Suspended microaerophilic culture nitrogen species plot for *Phoma* sp.

One other minor possibility existed that could explain ammonia concentration decrease independently of the fungal action. Ammonia volatilization directly from the wastewater could have resulted in some losses due to the pH profile observed for all reactor runs. The initial pH in reactor runs was typically between 7.0 and 7.5. During the course of an experimental run pH would rise to between 8.0 and 8.7. Therefore, there was a correlation between pH and NH_4^+ concentration decrease. At pH 8.7 the theoretical percent of ammonia gas in the liquid is 22%. An ammonium volatilization control run of the same length of time and test conditions was performed using nine reactors at three initial pH values; 7.0, 8.0 and 9.0. The initial theoretical amount of ammonia gas in the reactors was 0.6, 5.3 and 36%, respectively. The control was to determine the extent of NH_4^+ decrease due to volatilization and provide data to model any nitrogen losses. Figure 4-11 and Figure 4-12 show the results of the control runs. The key point, shown in Figure 4-11, was that volatilization of NH_4^+ in the pH range observed in experimental runs involving fungi was negligible or within the error of the analytical method. Figure 4-12 shows the measured pH values over time in the control reactors. What is clearly seen is that, regardless of the initial pH, the pH in the reactors increased till it reached an stable value. The data in Figure 4-12 indicate that the observed increase in pH of the wastewater is a property of the wastewater and not due to fungal action. Furthermore, in the initial pH 9.0 reactors, the final pH reached 9.5 meaning the theoretical ammonia gas fraction in the liquid was 64%. Even with the large fraction, NH_4^+ loss due to volatilization was minimal.

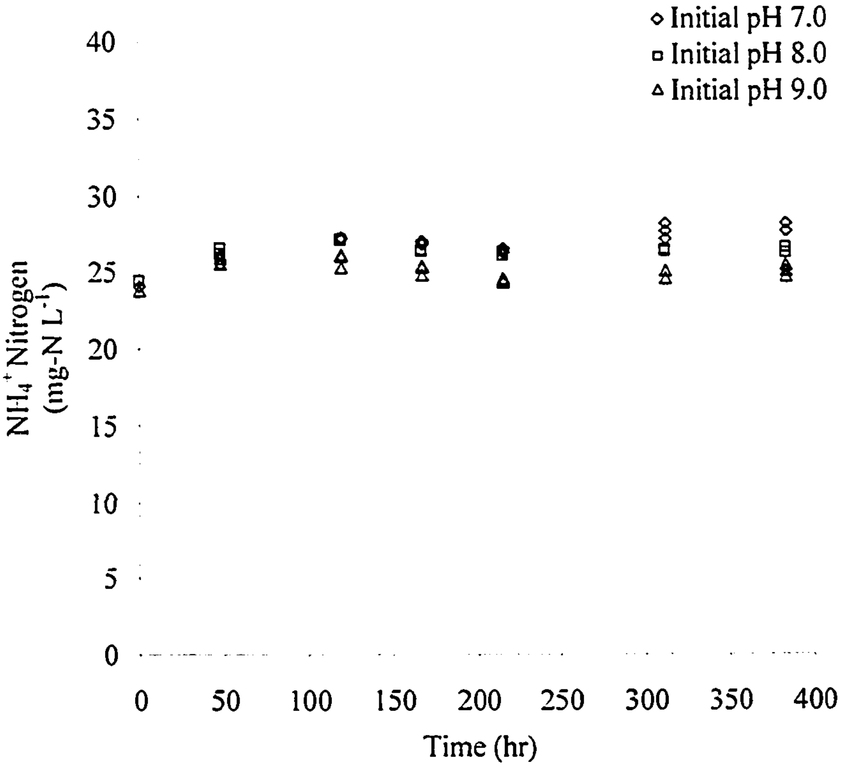

Figure 4-11 Ammonium volatilization control data at various pH

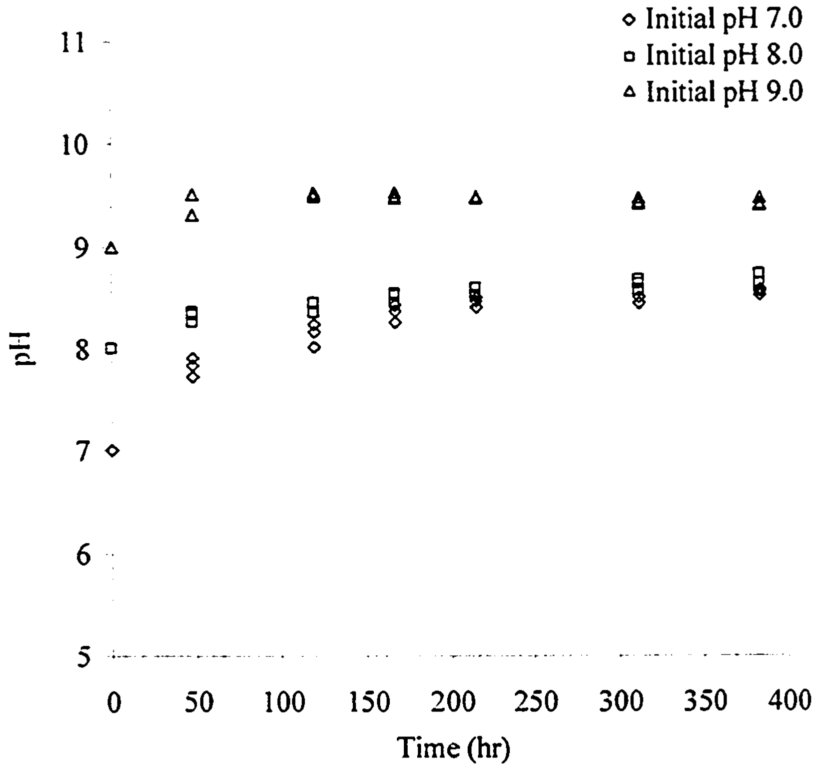

Figure 4-12 Ammonium volatilization control pH change time profile.

4.2.1.3 Estimation of pH Range for Fungal Ammonia Concentration Decrease

The effect of pH was investigated on fungal ammonium concentration decrease. The tests were to provide information on the range of pH over which fungi would perform NH_4^+ concentration decrease. Figure 4-13 to Figure 4-17 show the resulting NH_4^+ and pH profiles. The initial acidic pH 5.5 resulted in minimal NH_4^+ decrease. The pH profile showed an extremely rapid pH change in the first 48 hr, from 5.5 to 7.7. The NH_4^+ volatilization control also exhibited this trend but not nearly at the same rate. Although fungal NH_4^+ concentration decrease did not occur, the fungi were not growth-inhibited at a pH of 5.5. Table 4-10 shows that the observed growth rates at an initial pH of 5.5 were similar to values reported in Table 4-9. Adjusting the initial pH to 8.0 gave NH_4^+ concentration decrease between 35 and 59%. These values fall into the average concentration decreases based on the microaerophilic studies. Based on data collected, fungal NH_4^+ concentration decrease will occur over the pH range of 6.8 to 9.0. However these values are not hard boundaries, just the current pH values were fungal NH_4^+ concentration decrease has been observed.

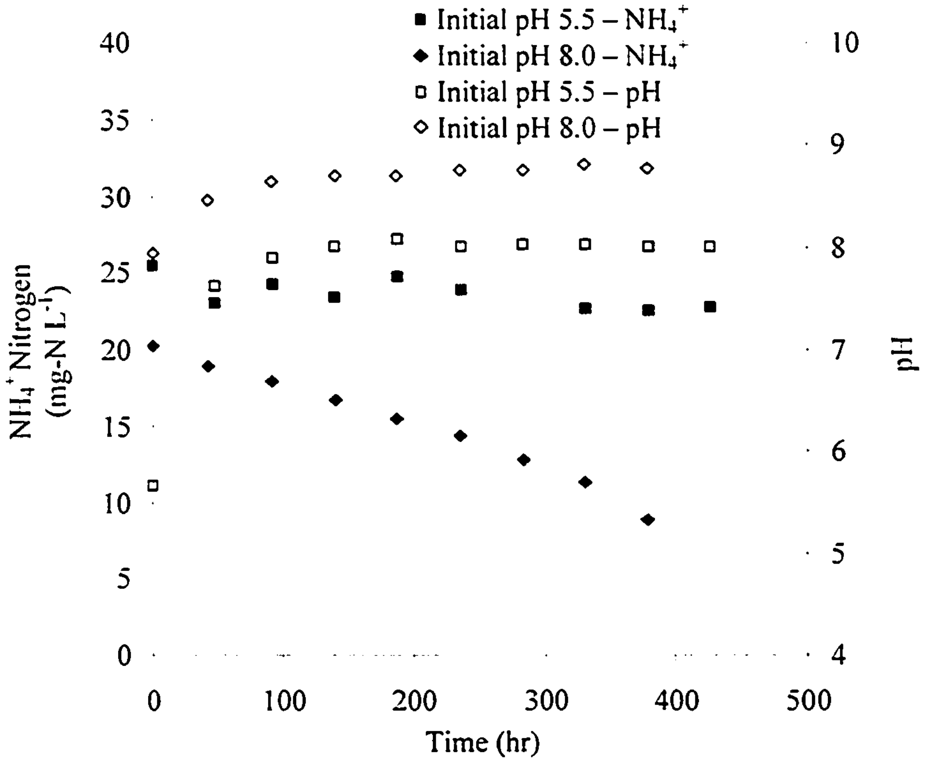

Figure 4-13 Impact of initial pH change on ammonium decrease by *Geotrichum* sp. (2000)

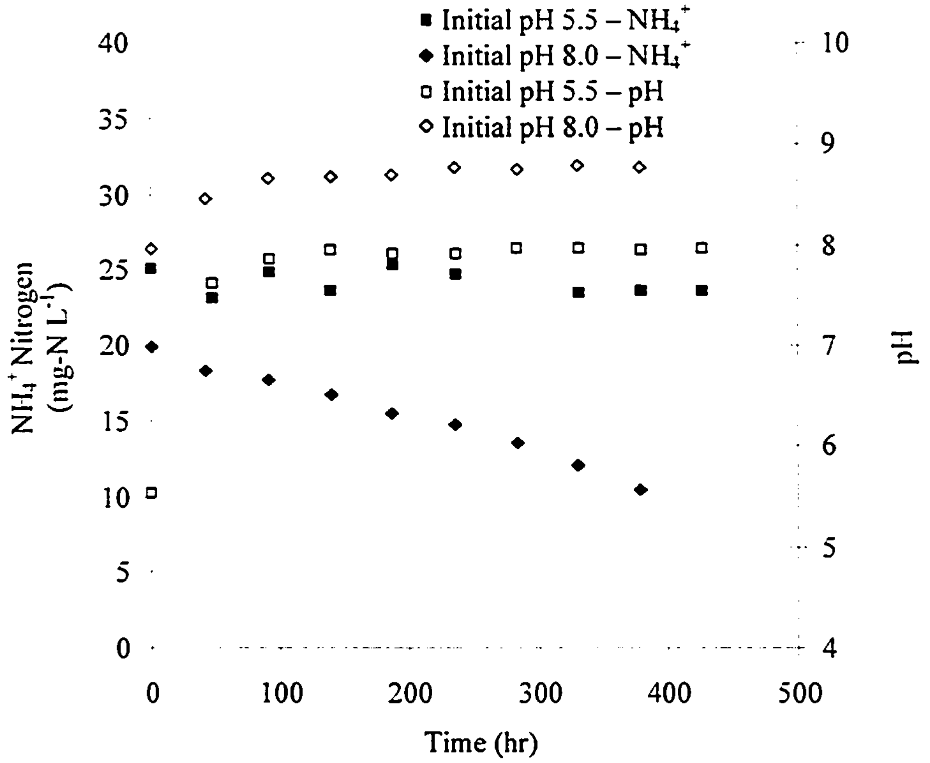

Figure 4-14 Impact of initial pH change on ammonium decrease by *Geotrichum* sp. (2007)

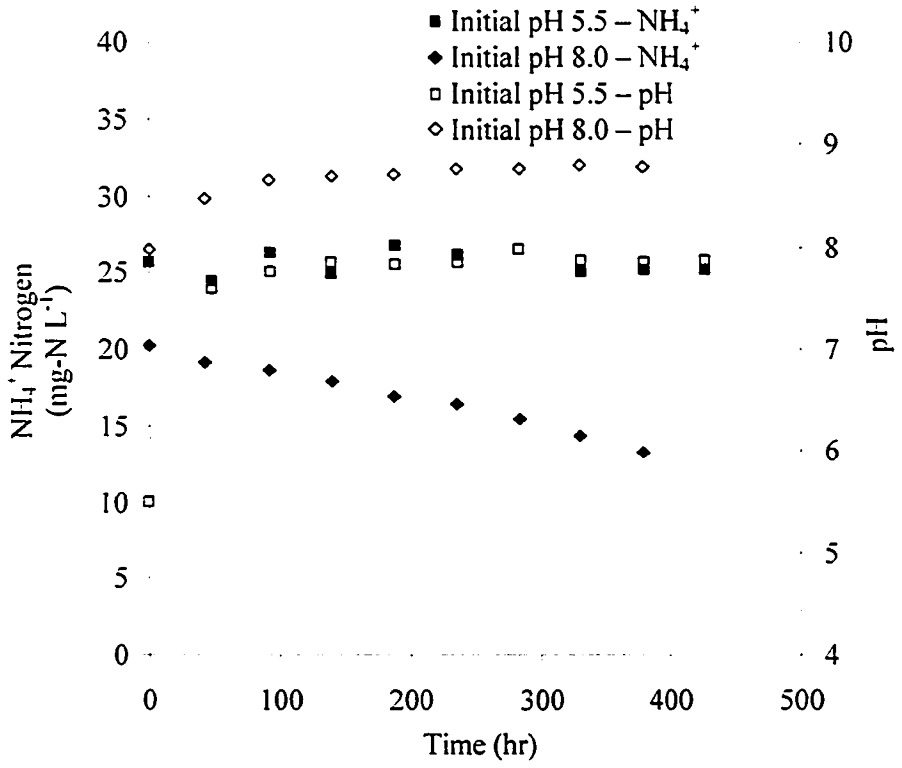

Figure 4-15 Impact of initial pH change on ammonium decrease by yeast.

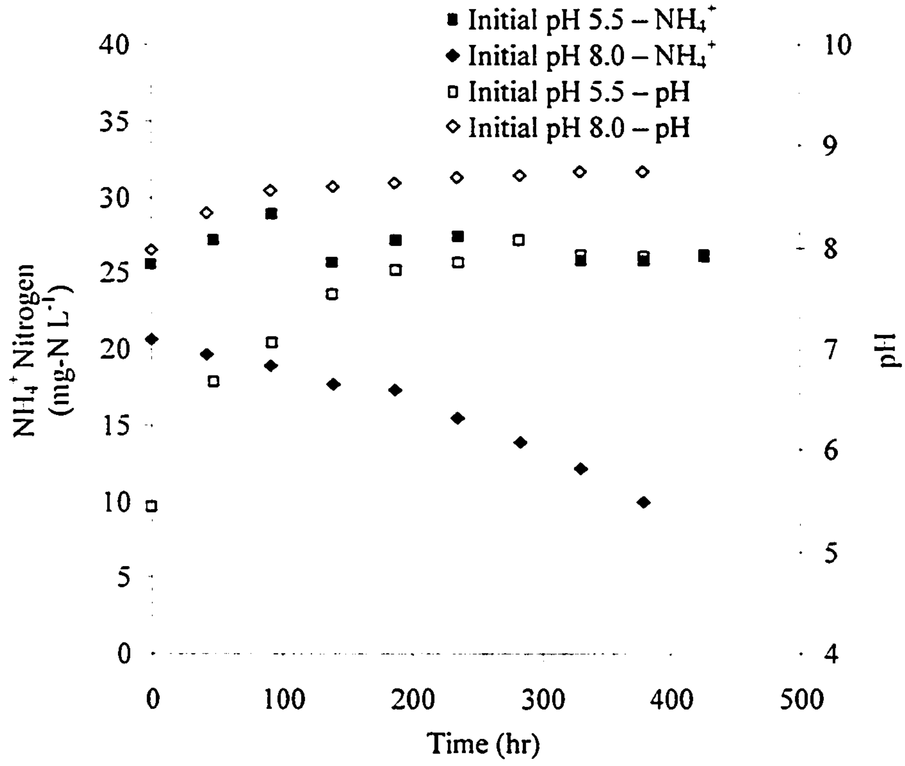

Figure 4-16 Impact of initial pH change on ammonium decrease by *Penicillium* sp.

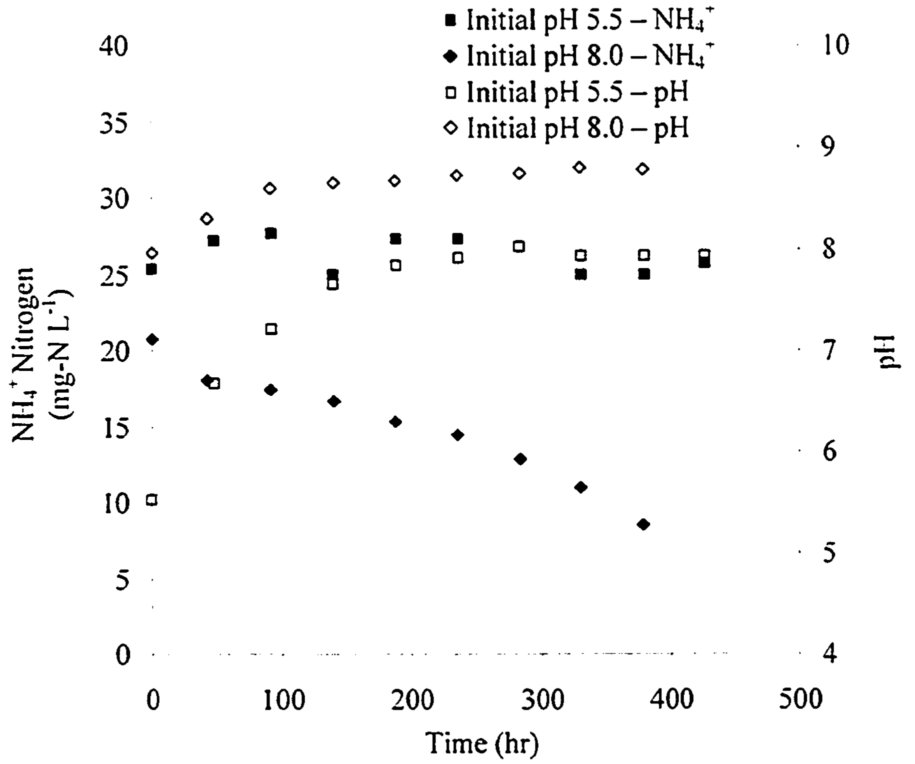

Figure 4-17 Impact of initial pH change on ammonium decrease by *Phoma* sp.

Table 4-10 Fungal growth rates for two pH conditions tested.

	μ (hr^{-1})	
	Initial pH 5.5	Initial pH 8.0
Geotrichum sp. (2000)	0.008	0.017
Geotrichum sp. (2007)	0.016	0.009
Yeast	0.010	0.014
Penicillium sp.	0.010	0.012
Phoma sp.	0.011	0.018

4.2.1.4 Dilute Wastewater Effects

Fungi were also screened for their performance in dilute sewage treatment. Table 4-11 shows the average wastewater parameters used for both dilute sewage testing and standard testing. The undiluted wastewater would be already categorized as low strength for most parameters (Metcalf & Eddy et al. 2003). The 50% dilution used provided a reasonable approximation of very weak wastewater. Growth rates of the fungi studied are contained in Table 4-12. Comparison of the rates in Table 4-12 with those in Table 4-9 for microaerophilic conditions shows only a negligible difference. Growth rates for the *Geotrichum* spp., the yeast were identical to those in Table 4-9. *Penicillium* sp. showed an increase whereas the *Phoma* sp. decreased; however, accounting for the variance and small sample size the result can be considered the same as in Table 4-9.

Figure 4-18 to Figure 4-22 displays the results of the ammonium concentration decrease in dilute sewage under microaerophilic conditions. Run 1 was of a shorter time than Run 2, however, the trend was very similar in both runs. It is likely that, if Run 1 had continued, NH_4^+ concentration decrease would have been more similar to those of Run 2. The percent NH_4^+ decreases are shown in Table 4-13. Run 1 showed decreases between 13 and 17% whereas for Run 2 between 34 and 43%. The percent decreases for Run 2, while lower, are within the standard deviation of the data reported in Table 4-8. This indicates that the fungi have some ability to tolerate a more dilute wastewater stream while still reducing the NH_4^+ concentration. Further testing is required to determine more precise parameter estimates.

Table 4-11 Mean parameters for undiluted and diluted primary wastewater medium.

	COD mg L^{-1}	TS mg L^{-1}	NH_4^+ mg-N L^{-1}	NO_3^- mg-N L^{-1}	NO_2^- mg-N L^{-1}	o-PO_4^{3-} mg L^{-1}
Undiluted Wastewater	275	775	28.1	<0.01	<0.01	18.0
Diluted Wastewater	140	490	12.7	<0.01	<0.01	8.1

Table 4-12 Observed growth rate for fungi growing on dilute wastewater.

Species	μ (hr^{-1})	Standard Deviation
Geotrichum sp. (2000)	0.009	0.001
Geotrichum sp. (2007)	0.010	0.000
Yeast	0.013	0.001
Penicillium sp.	0.009	0.005
Phoma sp.	0.004	0.005

Table 4-13 Percent ammonium decrease for two dilute sewage runs

	NH_4^+ Decrease (%)	
Species	Run 1	Run 2
Geotrichum sp. (2000)	13	43
Geotrichum sp. (2007)	16	42
Yeast	17	43
Penicillium sp.	16	39
Phoma sp.	17	34

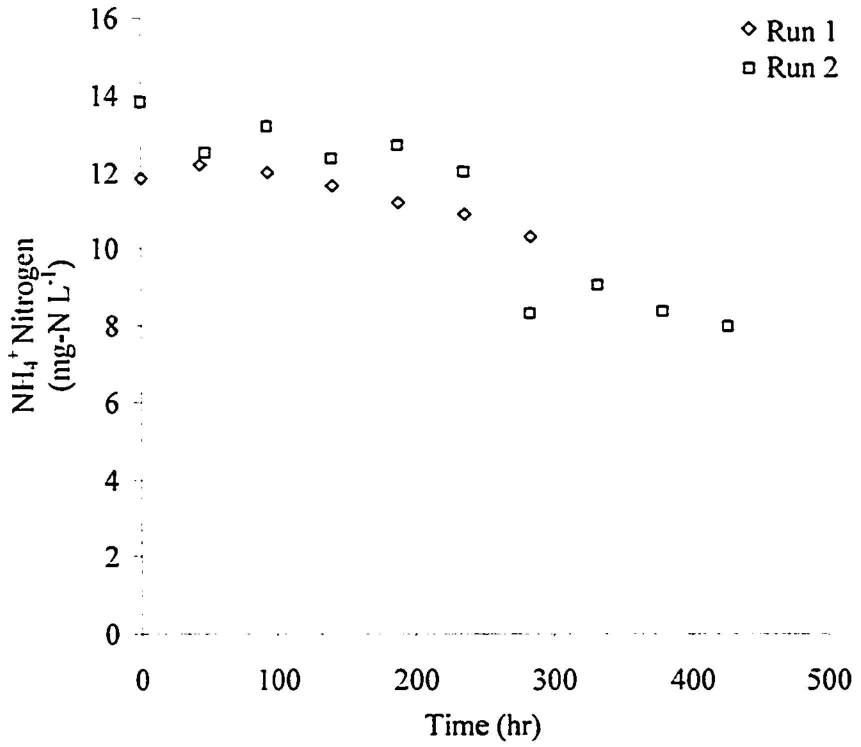

Figure 4-18 Ammonium concentration decrease in dilute sewage for *Geotrichum* sp. (2000)

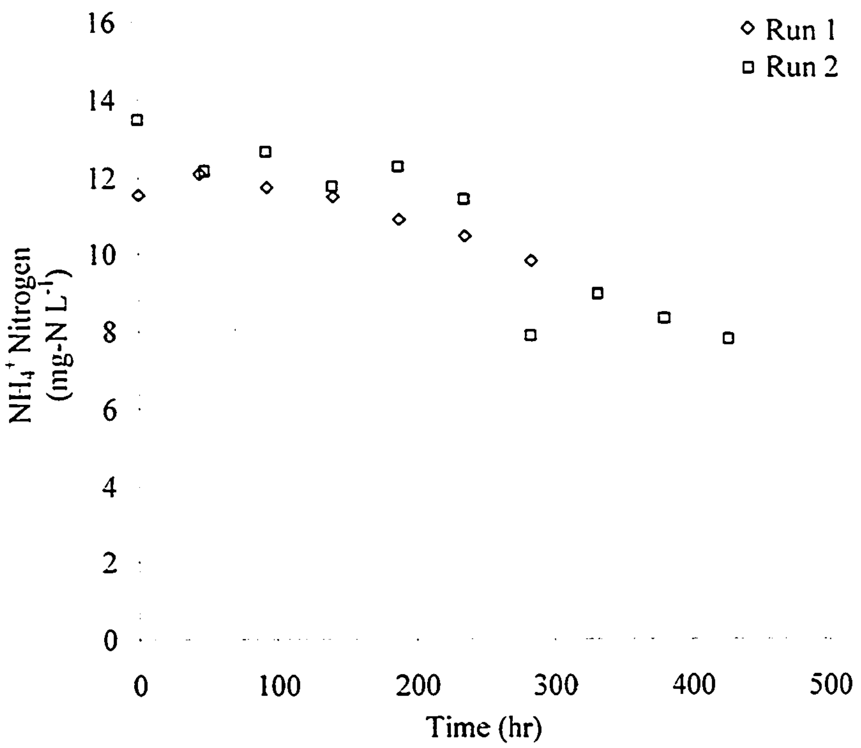

Figure 4-19 Ammonium concentration decrease in dilute sewage for *Geotrichum* sp. (2007)

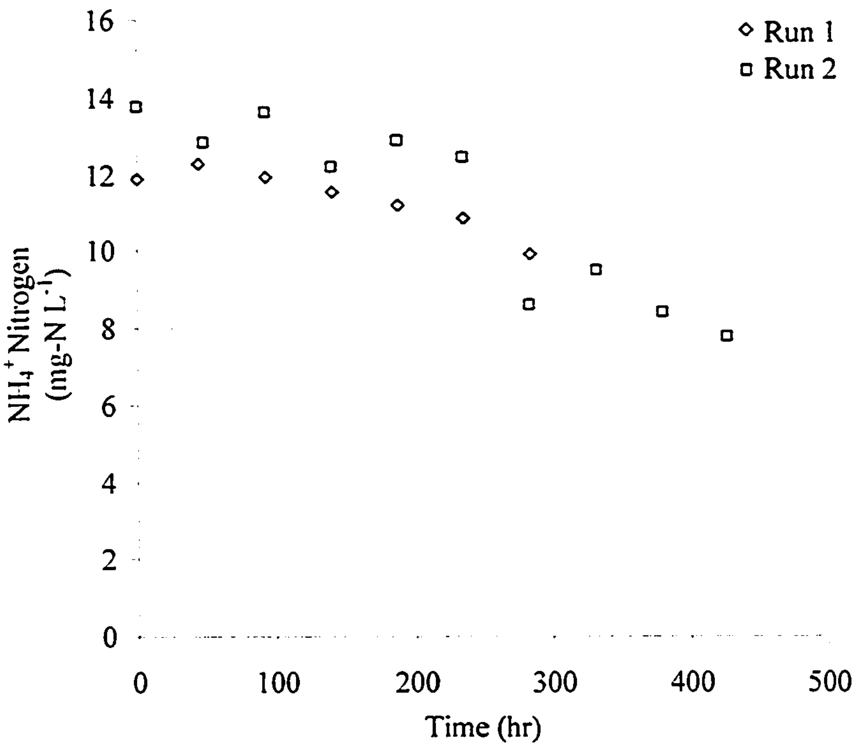

Figure 4-20 Ammonium concentration decrease in dilute sewage for Yeast.

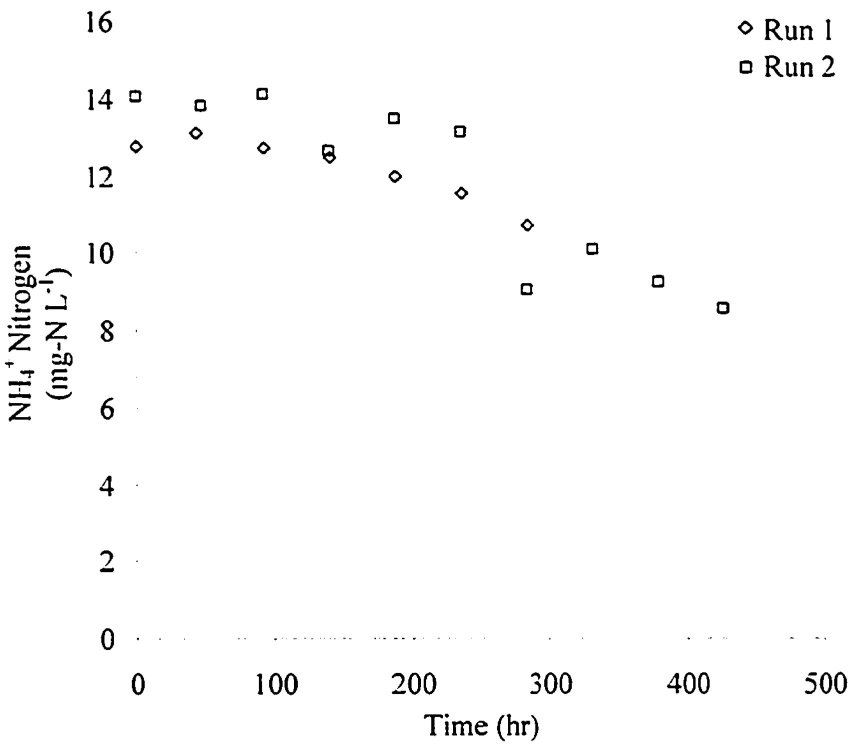

Figure 4-21 Ammonium concentration decrease in dilute sewage for ***Penicillium*** sp.

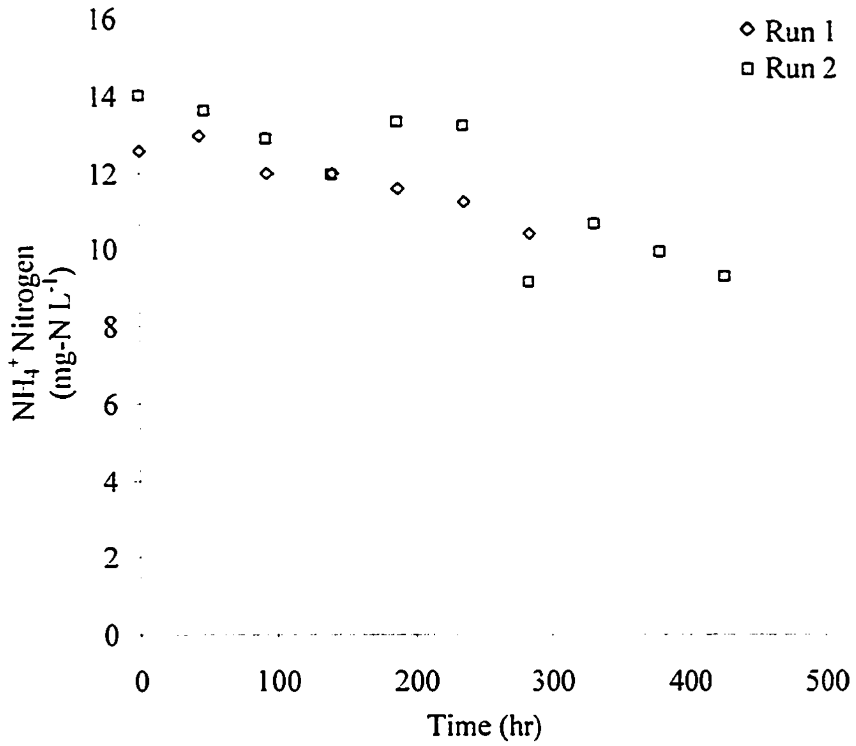

Figure 4-22 Ammonium concentration decrease in dilute sewage for ***Phoma*** sp.

4.2.1.5 Successive Inoculation Culture tests

For all experimental runs, the inocula were made from a stock culture of the original isolates from the WWTP. This was done to minimize variation in the experiments from changes in fungi over their storage time. Successive inoculation culture tests were conducted to observe if improvement in the rate and overall concentration decrease would occur as the fungi were repeatably exposed to the wastewater conditions. This type of testing looked for an adaptive response. Table 4-14 contains the NH_4^+ utilization rate and percent decrease for the five fungi tested over three successive inoculations. The NH_4^+ concentration decrease curves are seen in Figure 4-23 to Figure 4-27. Overall the data did not indicate an increase in performance over the three experimental cycles with the exception of one isolate, *Geotrichum* sp (2000). However, this was not likely a significant pattern given all the results. Comparison of the r_{su} and NH_4^+ concentration decrease data in Table 4-14 to those given in Table 4-8 indicates, statistically, that the data falls within the range. The batch to batch variation in the wastewater medium was the dominant factor. With no significant difference and no obvious data patterns these data points can be pooled with the result from the earlier microaerophilic studies.

Table 4-14 Ammonium utilization rates and percent decrease for successive inoculations of various fungi.

	r_{su} (mg-N L^{-1} hr^{-1})			NH_4^+ Decrease (%)		
	G1[†]	G2[*]	G3[§]	G1[†]	G2[*]	G3[§]
Geotrichum sp. (2000)	0.035	0.035	0.058	53	59	72
Geotrichum sp. (2007)	0.038	0.040	0.041	61	58	55
Yeast	0.050	0.029	0.025	68	57	41
Penicillium sp.	0.061	0.028	0.048	95	49	61
Phoma sp.	0.054	0.026	0.064	87	52	69

[†] Inoculation One
[*] Inoculation Two
[§] Inoculation Three

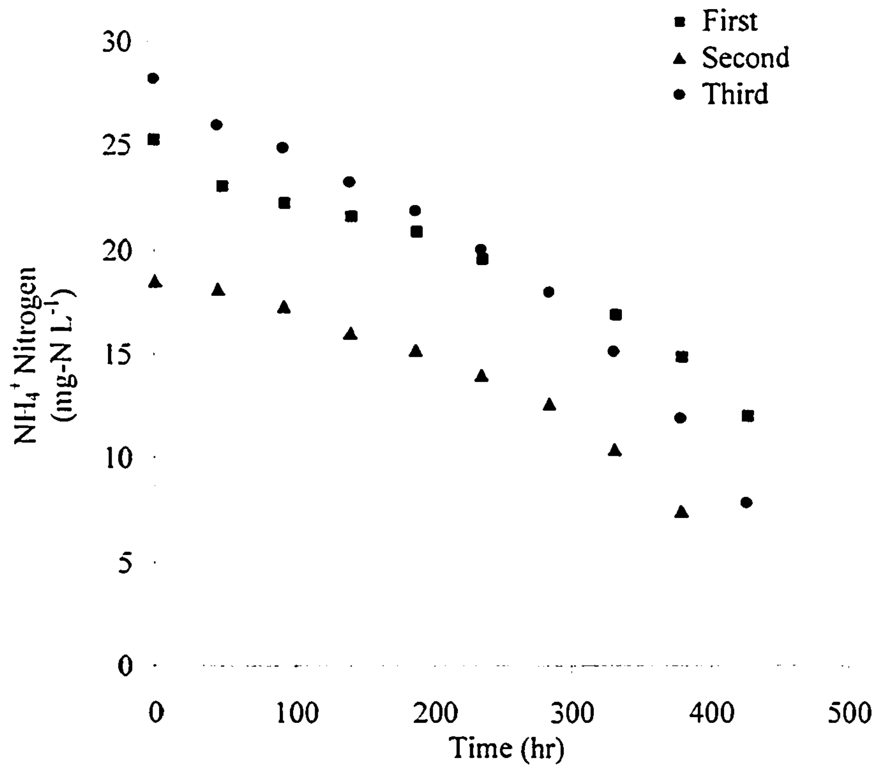

Figure 4-23 Ammonium concentration decrease for three successive inoculations of *Geotrichum* sp. (2000)

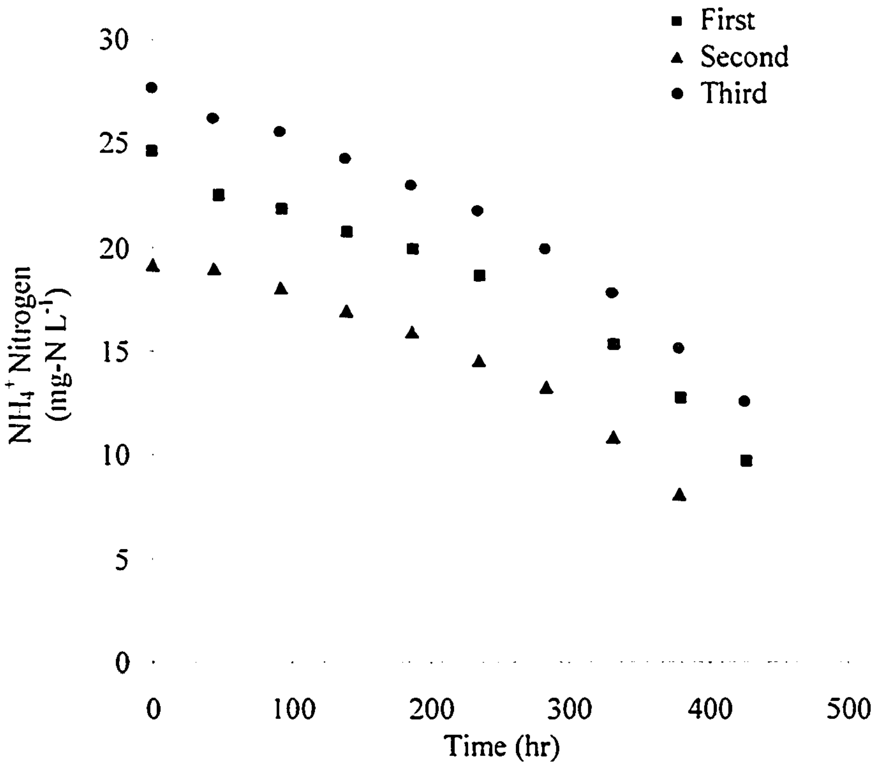

Figure 4-24 Ammonium concentration decrease for three successive inoculations of *Geotrichum* sp. (2007)

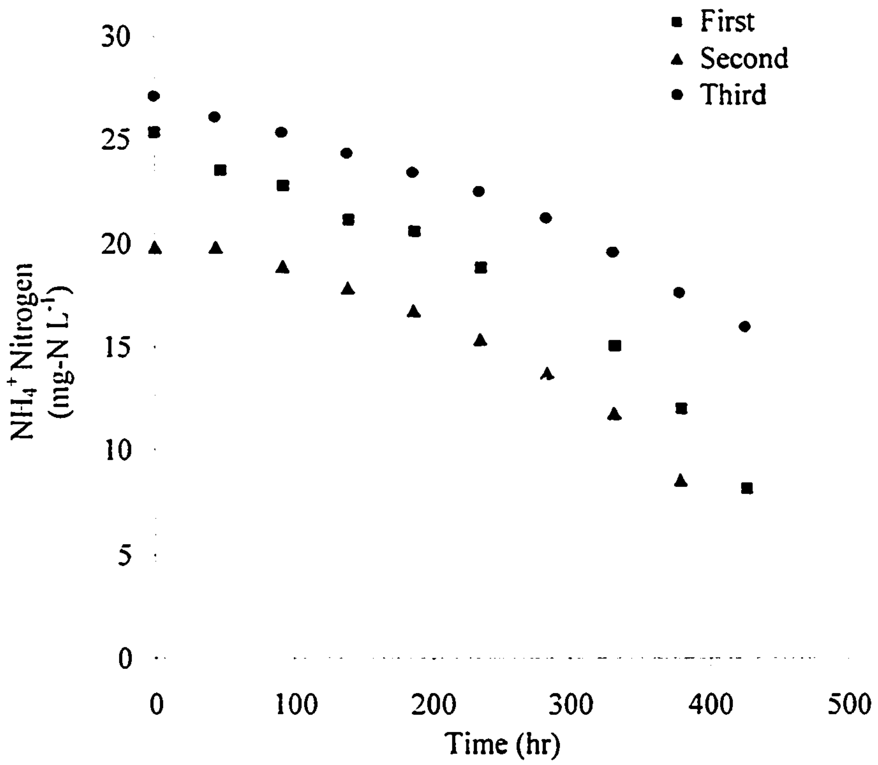

Figure 4-25 Ammonium concentration decrease for three successive inoculations a yeast species.

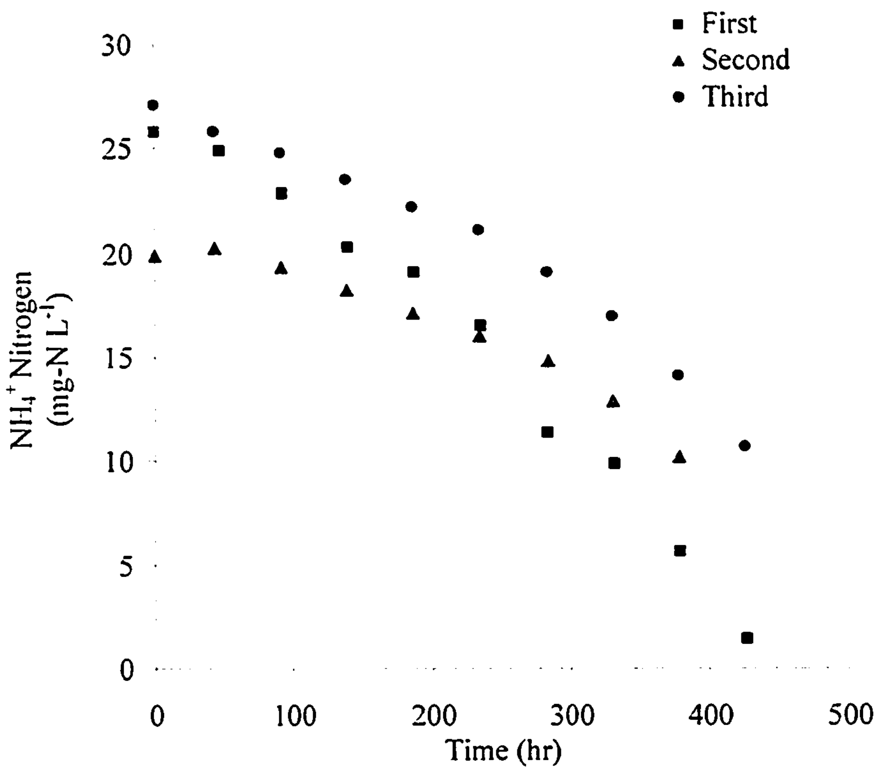

Figure 4-26 Ammonium concentration decrease for three successive inoculations of *Penicillium* sp.

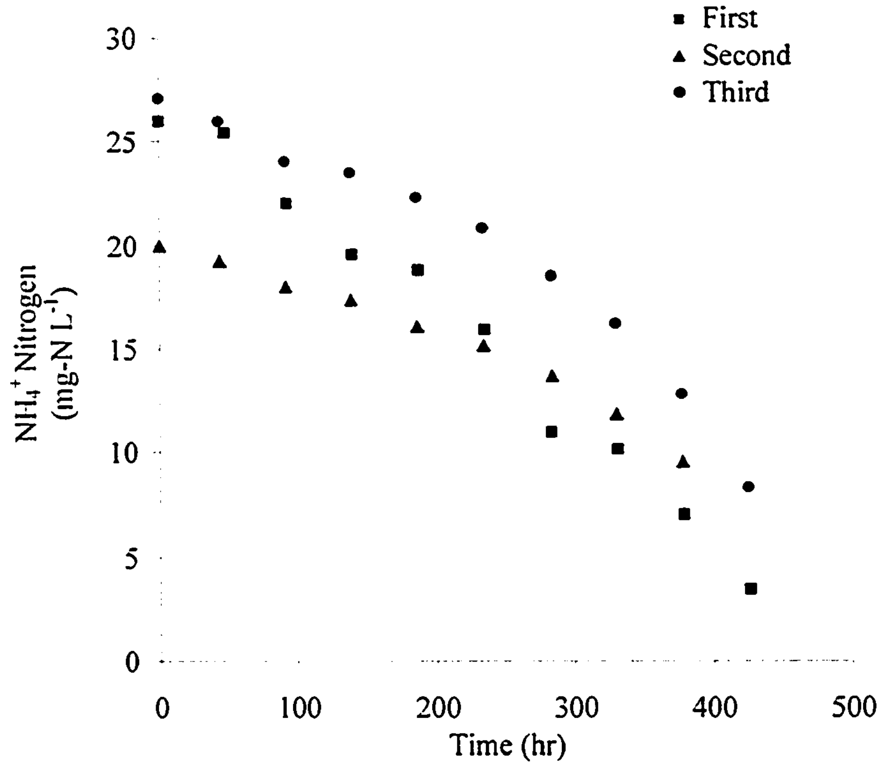

Figure 4-27 Ammonium concentration decrease for three successive inoculations of *Phoma* sp.

4.2.1.6 Mixed Culture

A mixed culture was evaluated in order to determine if NH_4^+ concentration decrease was improved or impeded. Six experimental runs involving the mixed culture were performed to establish the impact. The results are shown in Figure 4-28 and Table 4-15. Mixed culture NH_4^+ treatment rates and percent decrease all fell within the ranges reported in Table 4-8 established for the individual species. The average mixed culture decrease was 58% with a standard deviation of 9% and average r_{su} was 0.037 mg-N L^{-1} hr^{-1} with a standard deviation of 0.012. Although the mixed culture did not improve the amount decreased, there was an improvement in the variance. The standard deviation for both the r_{su} and NH_4^+ concentration decrease were, at the minimum, smaller by 46% and 58%, respectively. Given the observed variation in fungal NH_4^+ concentration decrease, factors improving stability are important.

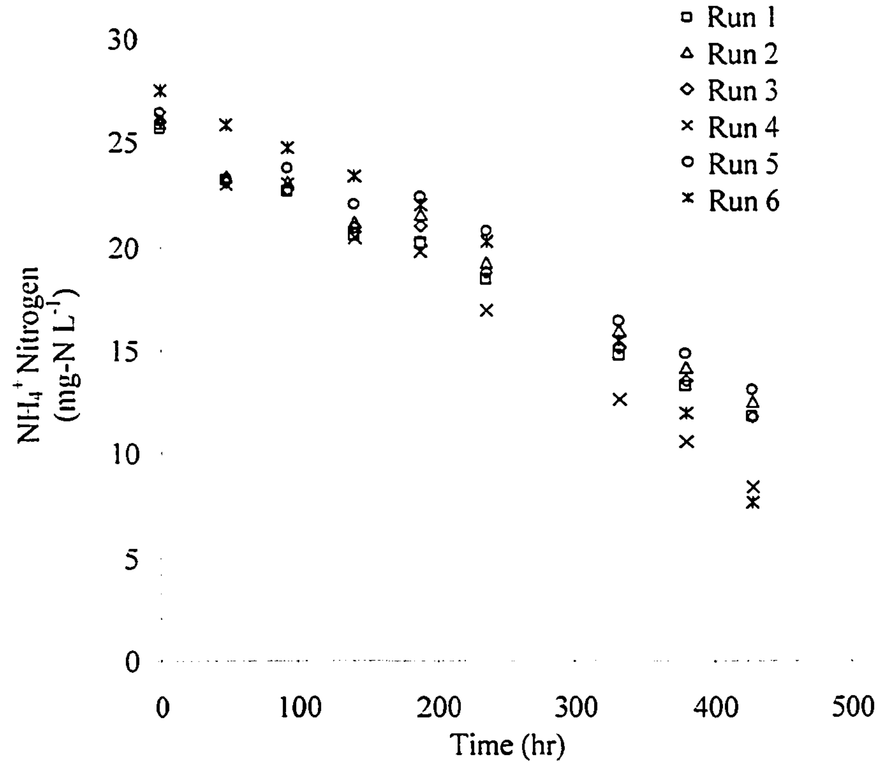

Figure 4-28 Mixed fungal culture ammonium concentration decrease in wastewater.

Table 4-15 Mixed culture ammonium degradation rate and percent decrease.

Run	r_{su} (mg-N L^{-1} hr^{-1})	NH_4^+ Decrease (%)
1	0.032	54
2	0.030	52
3	0.032	55
4	0.041	68
5	0.029	50
6	0.059	72
Mean	0.037	58
SD	0.012	9

4.2.2 Attached-growth

Attached-growth experiments were conducted to see if improvement in NH_4^+ concentration decrease could be achieved. Observations from the suspended-growth reactor runs indicated that, surface area to volume ratio could be a significant factor. Once the pH correlation was eliminated, NH_4^+ concentration decrease was correlated with the decrease in reactor volume as samples were withdrawn. This suggested that surface area to volume ratio maybe a factor that should be evaluated. Figure 4-29 displays the results from the first attached-growth reactor runs using an attachment medium with a surface area to reactor volume ratio of 25 m^2/m^3. although the *Geotrichum* spp. and the yeast achieved a decrease between 46 and 52%, the *Penicillium* sp. and *Phoma* sp. gave complete removal of NH_4^+ concentration in 330 hrs. The attached-growth experimental runs were the first time complete NH_4^+ concentration decrease occurred. In addition, the time for this decrease was, on average, 100 hrs less than the results observed for suspended-growth reactors which only achieved on average 50 to 60% decrease.

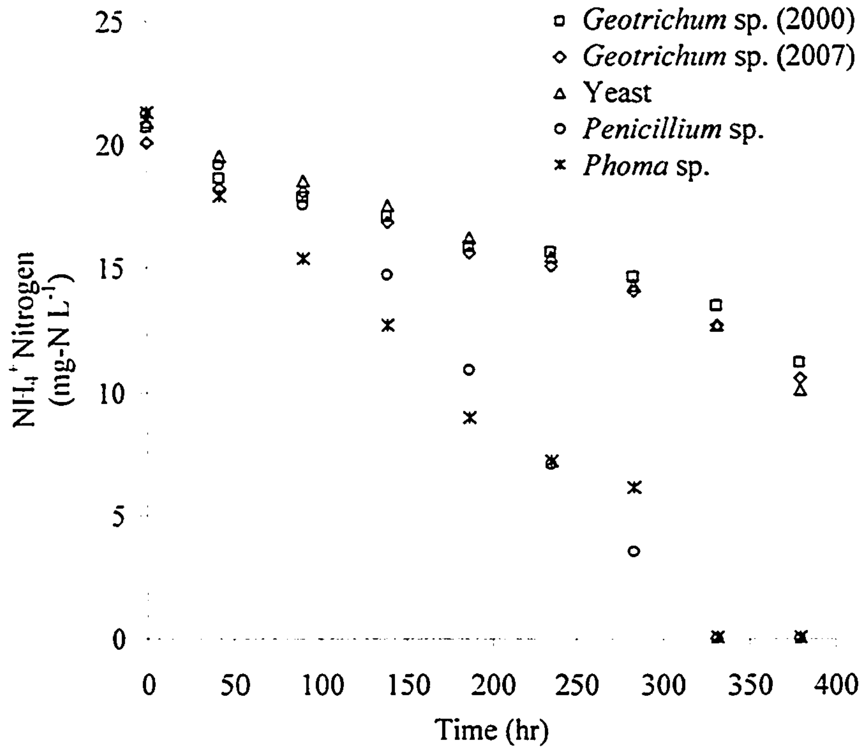

Figure 4-29 Ammonium decrease in batch 25 m^2/m^3 fungal attached-growth systems.

The results from the first attached-growth experiment prompted the investigation of available surface area to determine if further improvement in NH_4^+ concentration decrease could be achieved. Due to the poor improvement shown by the *Geotrichum* spp. and yeast, the remaining attached-growth experiments focused on *Penicillium* sp. and *Phoma* sp. but did include one *Geotrichum* sp. (2000). Figure 4-30 to Figure 4-32 show the NH_4^+ concentration decrease based on surface area for *Geotrichum* sp. *Penicillium* sp and *Phoma* sp. Consistent with the results presented in Figure 4-29, *Geotrichum* sp. (2000), showed that the attached-growth system did not dramatically improve the concentration decrease. Although Run 2 for 50 m^2/m^3 (Figure 4-30) did result in complete removal of NH_4^+, the results of the run appear to be part of the natural variation in the system and the lower initial NH_4^+ concentration.

For *Penicillium* sp. and *Phoma* sp., complete NH_4^+ concentration decrease was achieved for almost all runs. It should be noted that the slope on the NH_4^+ concentration decrease curves were all very similar (linear decline with respect to time), continuing to support the zero-order substrate utilization model.

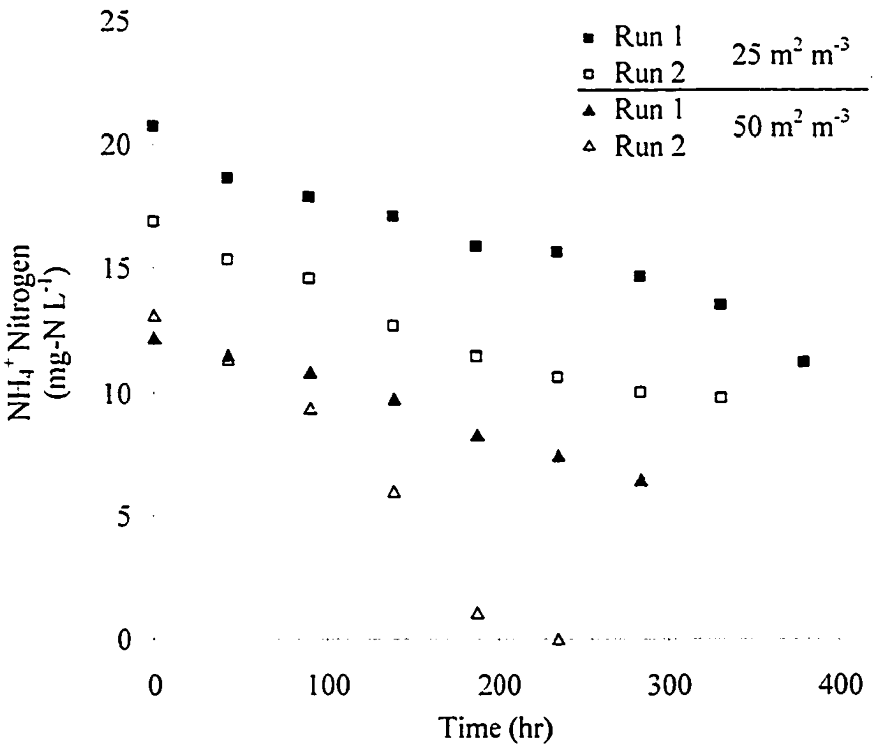

Figure 4-30 NH_4^+ **Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Geotrichum* sp. (2000)**

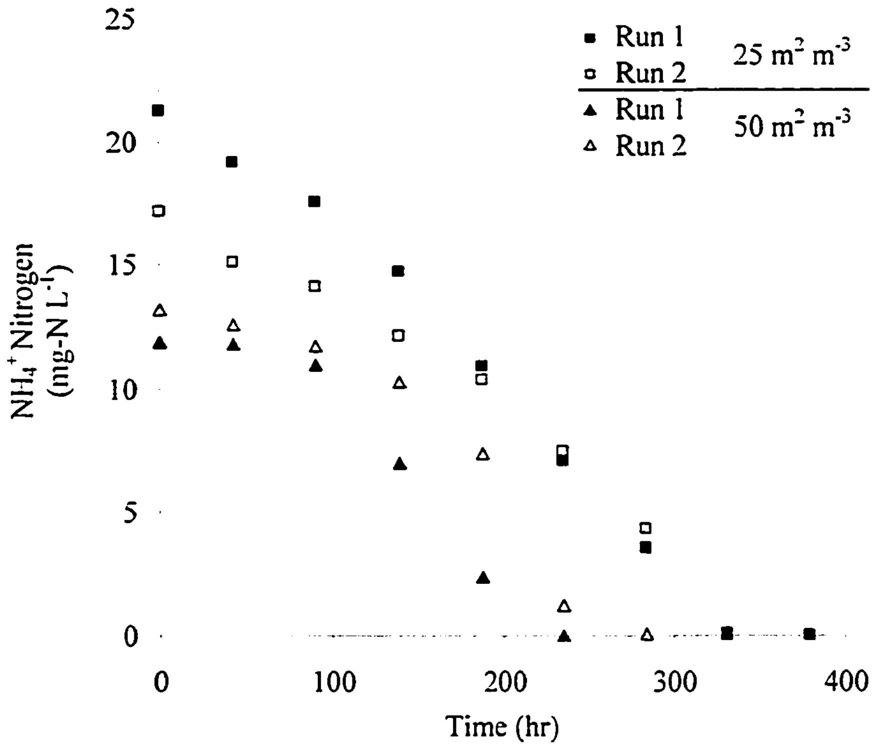

Figure 4-31 NH_4^+ **Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Penicillium* sp.**

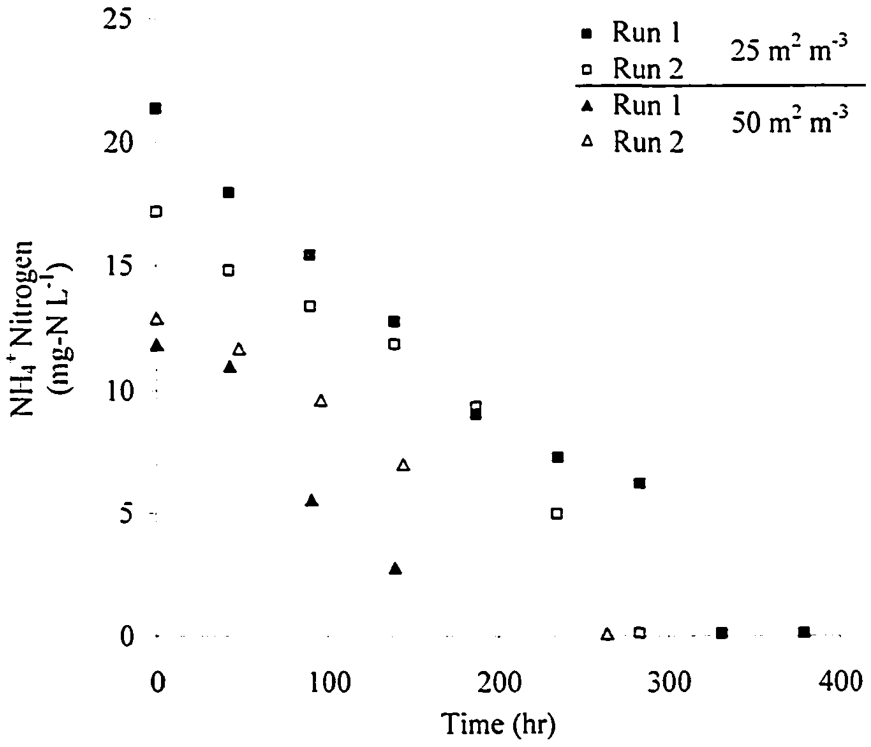

Figure 4-32 NH_4^+ Concentration decrease in batch 25 and 50 m^2/m^3 attached-growth systems for *Phoma* sp.

Table 4-16 and Table 4-17 show a comparison of the three fungal species and how the surface area to reactor volume ratio impacted the r_{su} and NH_4^+ concentration decrease. Based on the data presented in Table 4-16 and Table 4-17 there was no statistically significant difference between the rates or overall decrease due to the surface area available. This, however, does not mean surface area is not a significant factor. The surface area in the reactors was limited by the crude attachment medium used and the mixing. It is likely given the large experimental variance due to using wastewater medium and the small difference between the surface areas tested led to the perceived result of no difference. Larger surface area may see a statistically different result, meaning further testing is required. Given the results, the current surface area data can be pooled to provide better estimates of r_{su} and NH_4^+ concentration decrease.

Table 4-16 Comparison of surface area impact on NH_4^+ r_{su} constant.

Species	Surface Area to Reactor volume (m^2/m^3)	Number of Trials (n)	r_{su} (mg-N L^{-1} hr^{-1}) Mean	r_{su} (mg-N L^{-1} hr^{-1}) Standard Deviation	Statistical Difference (p)
Geotrichum sp. (2000)	25	2	0.022	0.001	0.22
	50	2	0.041	0.028	
Penicillium sp.	25	2	0.066	0.012	0.21
	50	4	0.081	0.021	
Phoma sp.	25	2	0.071	0.017	0.30
	50	6	0.061	0.022	

Table 4-17 Comparison of surface area impact on NH_4^+ percent decrease.

Species	Surface Area to Reactor volume	Number of Trials (n)	NH_4^+ Decrease (%) Mean	NH_4^+ Decrease (%) Standard Deviation	Statistical Difference (p)
Geotrichum sp. (2000)	25	2	44	2	0.19
	50	2	74	37	
Penicillium sp.	25	2	100	0	0.23
	50	4	88	19	
Phoma sp.	25	2	100	0	0.11
	50	6	75	24	

Comparison of r_{su} and NH_4^+ concentration decrease obtained from the suspended-growth and attached-growth systems are shown in Table 4-18 and Table 4-19. The kinetic rates were not statistically different (p=0.05) from each other. However, the attached-growth system dramatically decreased the observed variation in the r_{su}. The decrease was approximately 50% compared to suspended-growth reactors. Comparison of overall NH_4^+ concentration decrease, shown in Table 4-19, shows that statistically (p=0.05), *Penicillium* sp. and *Phoma* sp. performed better using the attached-growth medium. *Geotrichum* sp. (2000) did not show any difference compared to suspended-growth system. *Penicillium* sp. also showed a 50% decrease in the standard deviation. However, *Phoma* sp. as presented in Table 4-19 showed no improvement. This appearance of no difference is misleading because the standard deviation included two experimental runs that were terminated prior to complete removal occurring. If these data point are excluded the standard deviation would be 13.2%, or approximately a 50% decrease in the standard deviation compared to suspended-growth data.

Table 4-18 Comparison of zero-order kinetic rate constants observed for suspended- or attached-growth systems.

Species	Growth System	r_{su} (mg-N L^{-1} hr^{-1})	Standard Deviation	Number of Trials (n)	Statistical Difference (p)
Geotrichum sp. (2000)	Suspended	0.043	0.032	9	0.22
	Attached	0.031	0.019	4	
Penicillium sp.	Suspended	0.054	0.039	10	0.07
	Attached	0.076	0.019	6	
Phoma sp.	Suspended	0.061	0.048	9	0.44
	Attached	0.063	0.020	8	

Table 4-19 Comparison NH_4^+ decrease observed for suspended- or attached-growth systems.

Species	Growth System	Average NH_4^+ Decrease (%)	Standard Deviation (%)	Number of Trials (n)	Statistical Difference (p)
Geotrichum sp. (2000)	Suspended	54	25	9	0.40
	Attached	59	27	4	
Penicillium sp.	Suspended	60	30	10	0.01
	Attached	92	16	6	
Phoma sp.	Suspended	62	24	9	0.05
	Attached	81	24	8	

4.2.2.1 Fate of Ammonium in Attached-Growth Reactor Systems

Figure 4-33 to Figure 4-35 are plots of concentration of nitrogen species over time in the attached-growth reactors for *Penicillium* sp., *Phoma* sp. and *Geotrichum* sp. (2000), respectively. The *Penicillium* sp. and *Phoma* sp. showed the same results as the suspended culture data. Total nitrogen and NH_4^+ decreased, organic nitrogen was constant and there was no measurable production of nitrite or nitrate. However, the nitrogen species pattern for *Geotrichum* sp. (2000) changed when compared to all the data collected for the suspended-growth systems (Figure 4-6). Seen in Figure 4-35, organic nitrogen started to increase after 90 hrs of incubation. This result was consistent for all the attached-growth runs with *Geotrichum* sp. (2000). This indicated assimilation of NH_4^+ into organic matter was occurring and was responsible for 61% of the total NH_4^+ concentration decrease while 39% appears to have left the system. The reason for the change in the data trend was not obvious. Although the result could be attributed to attached-growth, it was difficult to support the hypothesis that, by simply adding small plastic rings, the *Geotrichum* sp. (2000) was stimulated to accumulate significant quantities of organic nitrogen. Further investigation is needed, but an alternative possible explanation is a mutation of *Geotrichum* sp. (2000) in the biochemical pathway used in this NH_4^+ concentration decrease process.

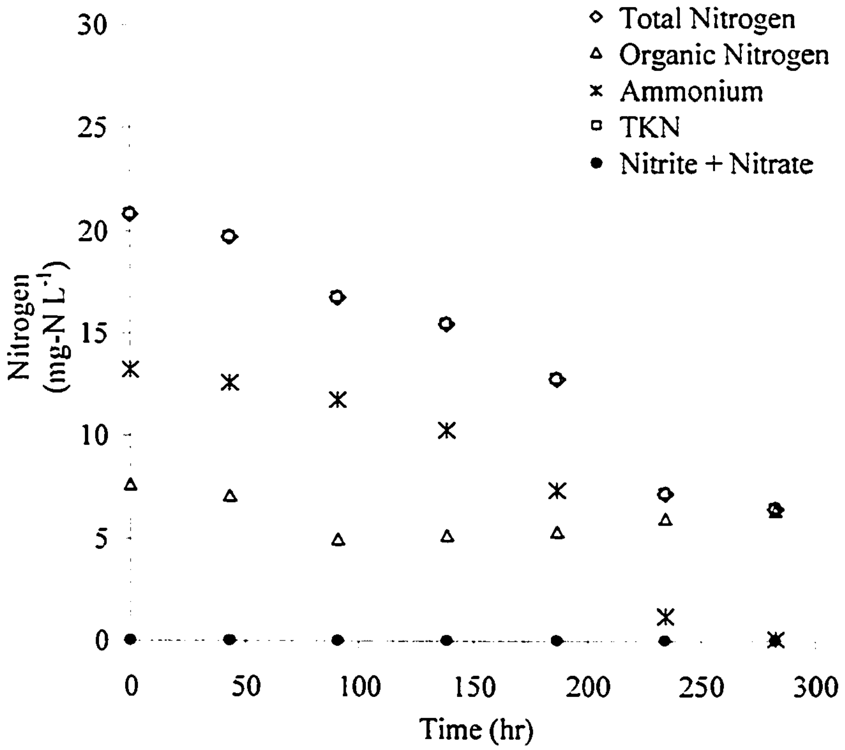

Figure 4-33. Nitrogen species observed during NH_4^+ decrease test using *Penicillium sp.*

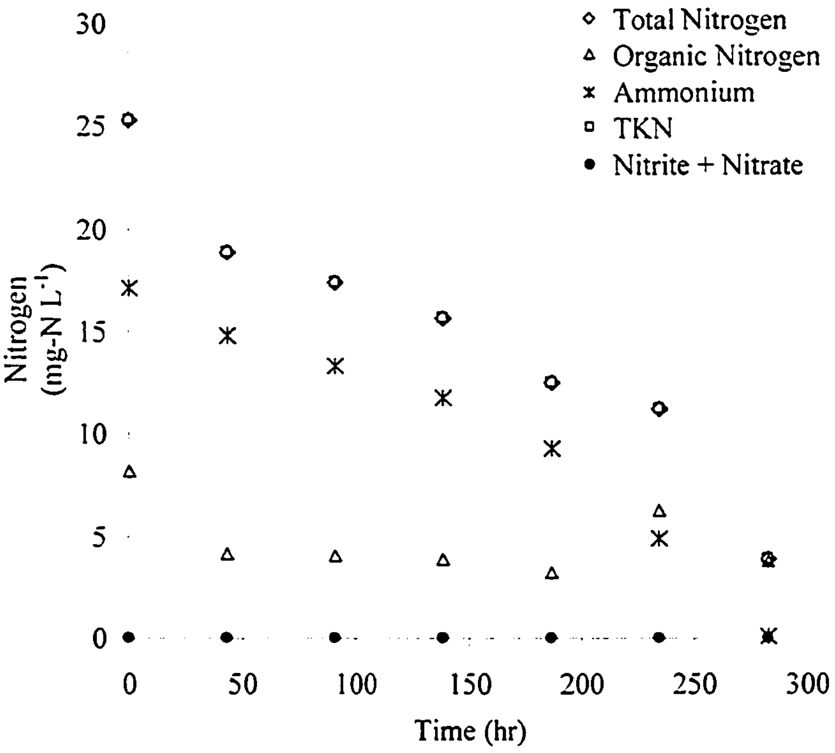

Figure 4-34. Nitrogen species observed during NH_4^+ decrease test using *Phoma sp.*

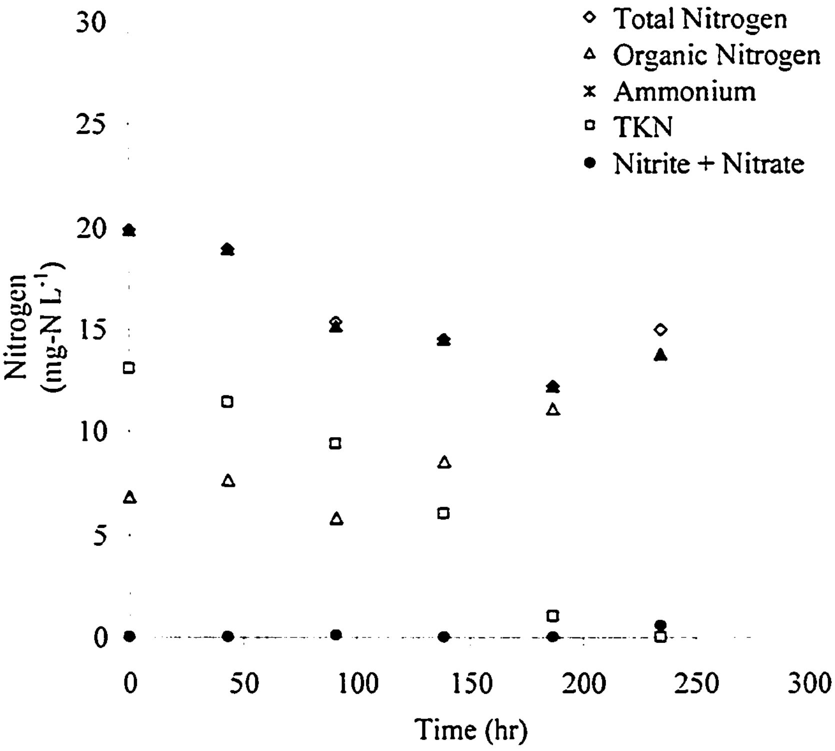

Figure 4-35 Nitrogen species observed during NH_4^+ decrease test using *Geotrichum sp.* (2000)

4.2.2.2 Fed-batch Attached-Growth Systems

Four fed-batch reactors were run in order to demonstrate continuity of the NH_4^+ concentration decrease for *Penicillium* sp. and *Phoma* sp. The results of the fed-batch runs are shown in Figure 4-36 to Figure 4-39. Figure 4-36 and Figure 4-37 display the *Penicillium* sp. results. The first fed-batch reactor was terminated early when it received a batch of wastewater that had been acidified to pH 2. However, two full cycles had occurred demonstrating some continuity of the process. For both *Penicillium* sp. and *Phoma* sp. the second reactor's first batch of wastewater was treated under anoxic condition followed by conversion to microaerophilic environment. In Figure 4-37, the first anoxic cycle for *Penicillium* sp. showed NH_4^+ concentration decrease of 37%. Upon conversion to microaerophilic conditions, complete removal of NH_4^+ was restored on the subsequent batch. The data reinforce three points for *Penicillium* sp.; first continuity of process, second the importance of the microaerophilic environment; and third restoration of NH_4^+ concentration decrease after returning from non-optimal operation conditions.

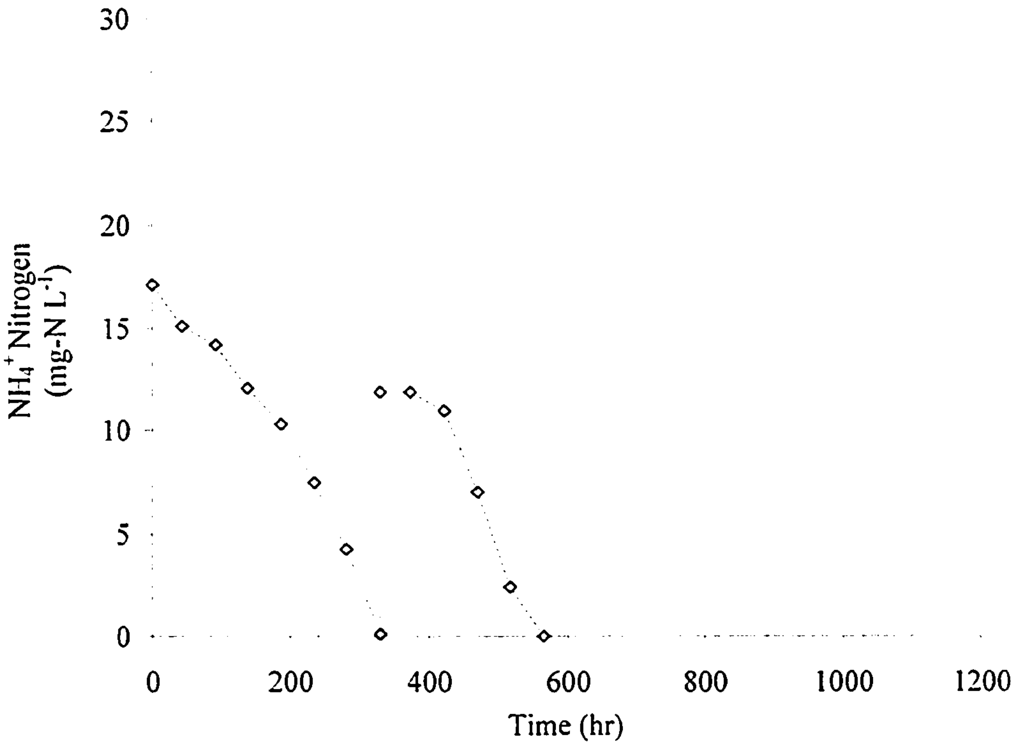

Figure 4-36 Fed-batch 50 m^2/m^3 attached-growth system for *Penicillium* sp.

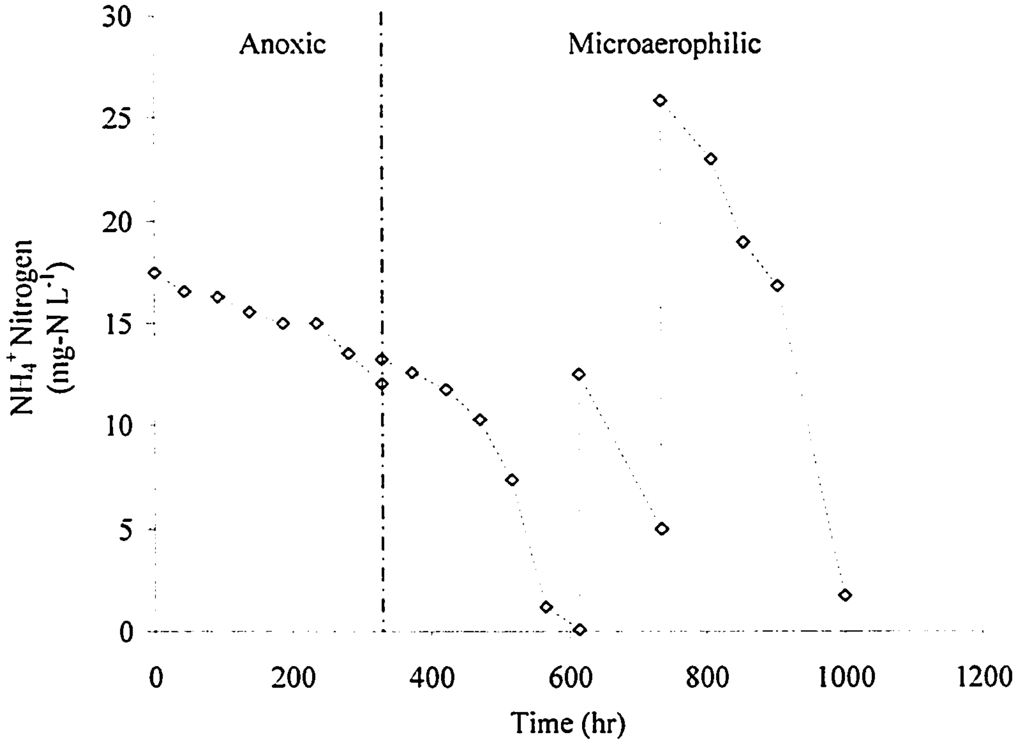

Figure 4-37 Fed-batch 50 m^2/m^3 attached-growth reactor initially anoxic for *Penicillium* sp.

Figure 4-38 and Figure 4-39 contain the fed-batch results for the *Phoma* sp. In the microaerophilic reactor, four cycles were completed. All cycles resulted in almost complete decrease of NH_4^+ concentration. The fourth cycle tailed only after 72% concentration decrease. In the second reactor (Figure 4-39), the same tailing event occurred. Both tailing events occurred for the same batch of wastewater, however the fourth cycle in Figure 4-37, for *Penicillium* sp., which also used the same batch of wastewater did not show the tailing effect. This may be an indication of a sensitivity of *Phoma* sp. to a component in the wastewater medium. Longer testing cycles are needed to further evaluate other possibilities.

The second *Phoma* sp. reactor with the first anoxic cycle displayed an identical recovery as shown by the *Penicillium* sp. when converted back to microaerophilic conditions. However, the amount of NH_4^+ decreased under the anoxic condition was 60%. The pattern of data was very similar to that of microaerophilic suspended-growth culture. The best NH_4^+ concentration decrease observed under suspended anoxic growth was 30%. These data may indicate that the *Phoma* sp. anoxic NH_4^+ concentration decrease was greater than previously observed. Further data are required to evaluate this potential.

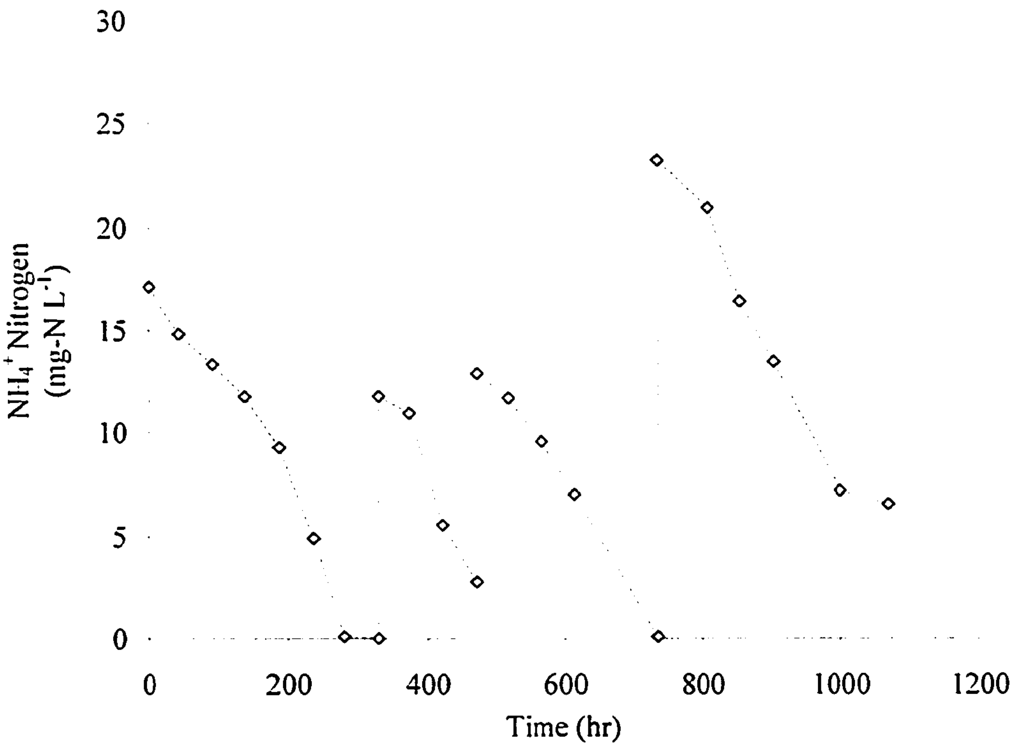

Figure 4-38 Fed-batch 50 m^2/m^3 attached-growth system for *Phoma* sp.

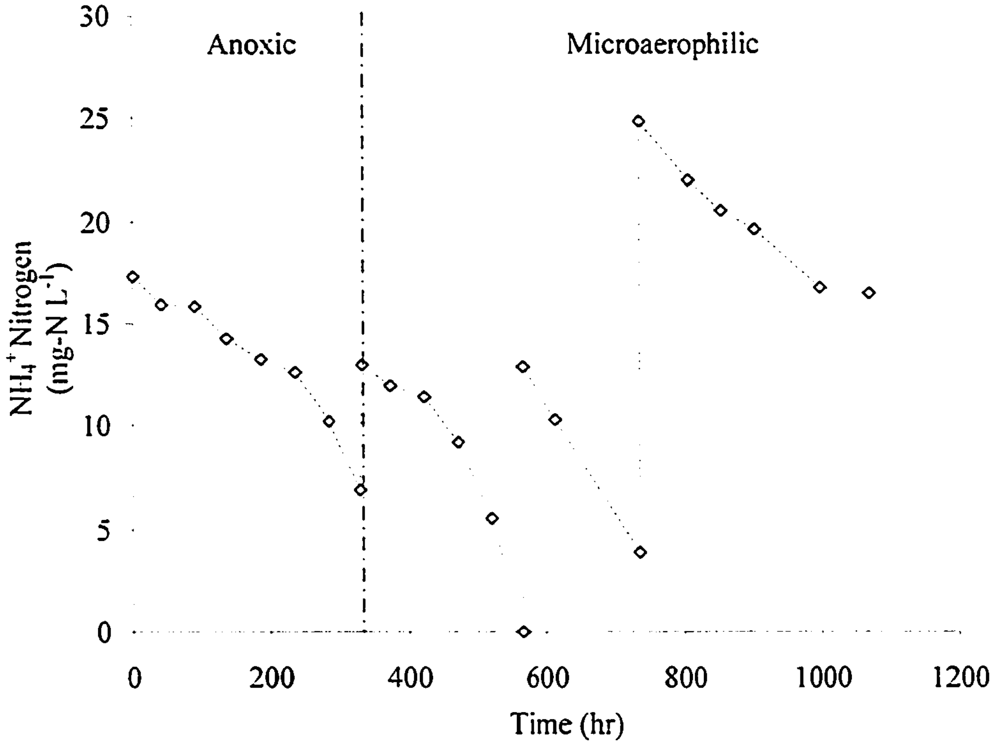

Figure 4-39 Fed-batch 50 m^2/m^3 attached-growth reactor initially anoxic for *Phoma* sp.

4.3 Batch Kinetic Phosphate Concentration Decrease Studies

4.3.1 Suspended-growth

Phosphate data were collected in conjunction with NH_4^+ batch kinetic runs presented in Section 4.2. Only o-PO_4^{3-} was monitored and not other parameters such as total phosphorus. This limits knowledge of the movement of phosphorus through the fungal system. However, the primary goal of this research was to develop fungal nitrogen treatment technology. The o-PO_4^{3-} data collected does give an indication of the potential for fungi to decrease o-PO_4^{3-} from wastewater and also allows examination of the potential for development of a combined NH_4^+ and o-PO_4^{3-} treatment system.

Analysis of the phosphate data was problematic. Undoubtedly, the use of wastewater as the growth medium contributed significantly to the variability observed in the data, however, there was a major observed correlation between the initial o-PO_4^{3-} concentration and the amount of o-PO_4^{3-} reduced from the medium. Higher initial concentrations of o-PO_4^{3-} resulted in a greater decrease. Figure 4-40 shows a scatter plot of initial o-PO_4^{3-} concentrations versus the amount of o-PO_4^{3-} reduced for the entire data set; suspended, attached, aerobic, microaerophilic, anoxic, successive generation, mixed culture and pH effects. The correlation coefficient for the entire data stet was 0.88 regardless of the experimental condition. Figure 4-41 shows the microaerophilic data subset which also had a correlation coefficient of 0.88. Although there is some variability in the both scatter plots, the data trend is very clear: a strong positive correlation exists indicating there is an overriding trend in the phosphate data set that in general supersedes test conditions.

The second key point that can be seen in Figure 4-40 and Figure 4-41 is that, with low initial o-PO_4^{3-} concentrations of around 1 mg L^{-1}, o-PO_4^{3-} concentration in the bulk solution actually increased to between 2 to 4 mg L^{-1}. The results are an indication that phosphate concentration decrease by fungi in the tested systems is complicated and will not likely be described with a single reaction kinetic equation. The data collected with the range of initial o-PO_4^{3-} concentrations indicate at least two systems for control of o-PO_4^{3-} uptake and a transition area. The data collected are not of sufficient resolution nor do they contain the static conditions needed (due to the factors tested and batch variation of the wastewater) to build a successful model of fungi o-PO_4^{3-} concentration decrease. It is also difficult to use these data to provide boundary conditions for o-PO_4^{3-} concentration decrease due to the happenstance nature. o-PO_4^{3-} was monitored as a secondary objective and tests were not designed to fully elucidate its patterns of decrease. This means there are large gaps in the data, making conclusions difficult and potentially misleading. However, specific experimental test conditions did reveal data patterns for fungal concentration decrease of o-PO_4^{3-} and were extremely valuable due to their unique nature in the literature and as a guide to future research.

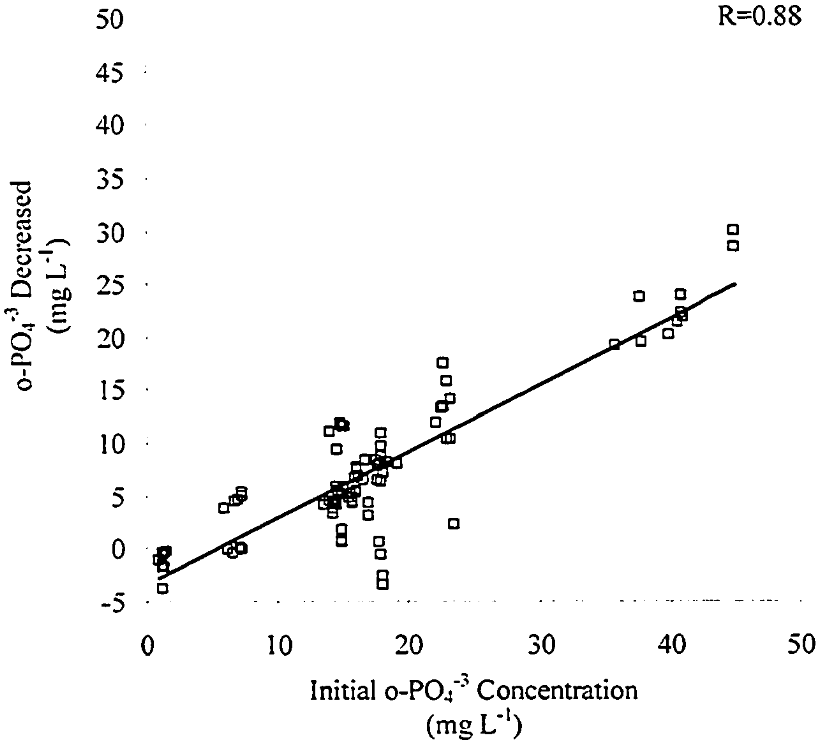

Figure 4-40 Amount of o-PO$_4^{3-}$ decreased versus initial o-PO$_4^{3-}$ concentration for all data.

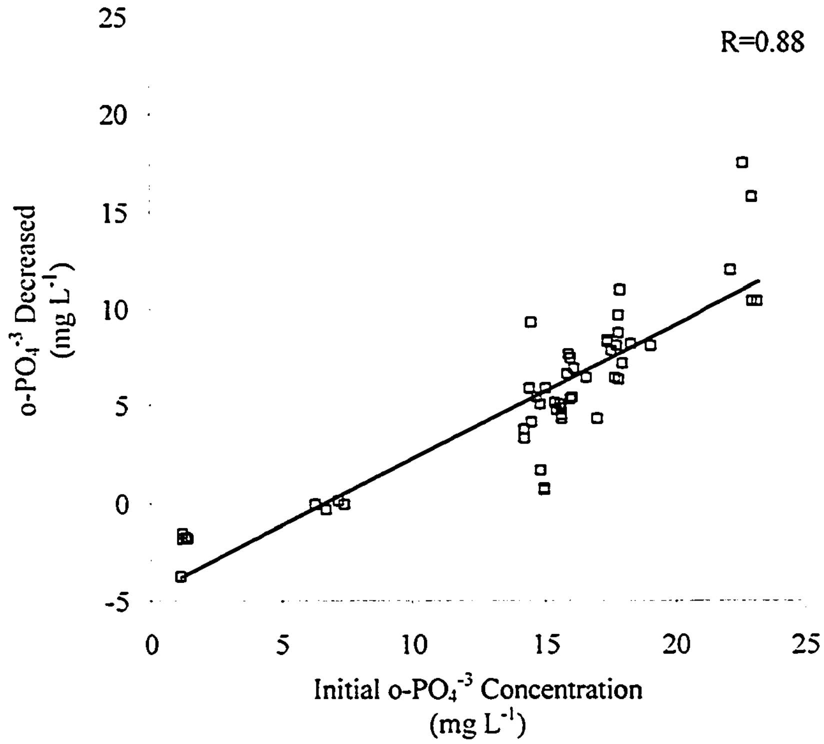

Figure 4-41 Amount of o-PO$_4^{3-}$ decreased versus initial o-PO$_4^{3-}$ concentration for microaerophilic data only.

4.3.1.1 Oxygen Effects on the Concentration Decrease of Phosphate in Wastewater

Figure 4-42 to Figure 4-46 show the aerobic concentration decrease of o-PO_4^{3-} by the five fungal species tested. The time course plots were best fit with a second-order kinetic model. As can be seen for the majority of the runs and species tested, o-PO_4^{3-} has a rapid initial decline followed by a declining rate. Two notable exceptions can be seen in Figure 4-42 and Figure 4-43. In Figure 4-42 Run #3 by the *Geotrichum* sp. (2000) showed negligible decrease of o-PO_4^{3-}. Comparison to Run #3 for the four other species shows it was the only species to have this trend for that batch of wastewater and the anomaly must, therefore be attributed to a species effect. The second notable exception to the second order fit of the data is shown in Run #2, Figure 4-43, for the *Geotrichum* sp. (2007) As seen in the figure there was a sharp decline followed by a slightly increasing plateau followed by a second rapid decline. This reactor was the one that lost its sparging aeration system very early in the run and, therefore, only received headspace aeration. Comparison of the data pattern with the microaerophilic runs in Figure 4-47 to Figure 4-51 revealed a very similar trend.

The overriding trend of higher initial o-PO_4^{3-} concentrations leading to greater decrease was also seen in the aerobic figures, but the small data set made it impossible to say if this trend was definitive for this subset. Furthermore, the range of o-PO_4^{3-} concentrations was too small for a reliable analysis. The trend seen could simply be the effect of the batches of wastewater used in the aerobic trials.

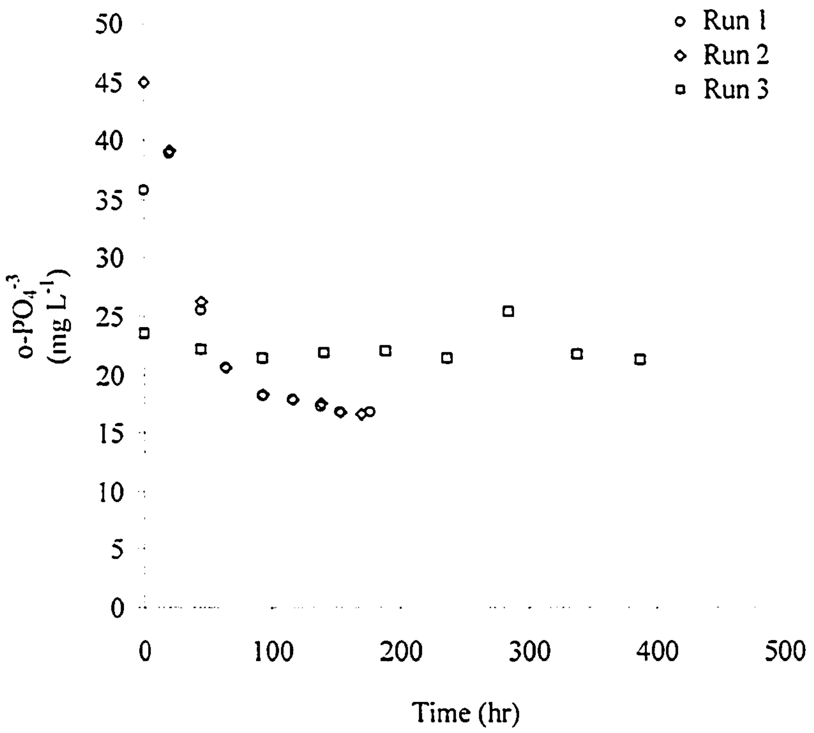

Figure 4-42 Aerobic o-PO$_4^{3-}$ concentration decrease by *Geotrichum* sp. (2000)

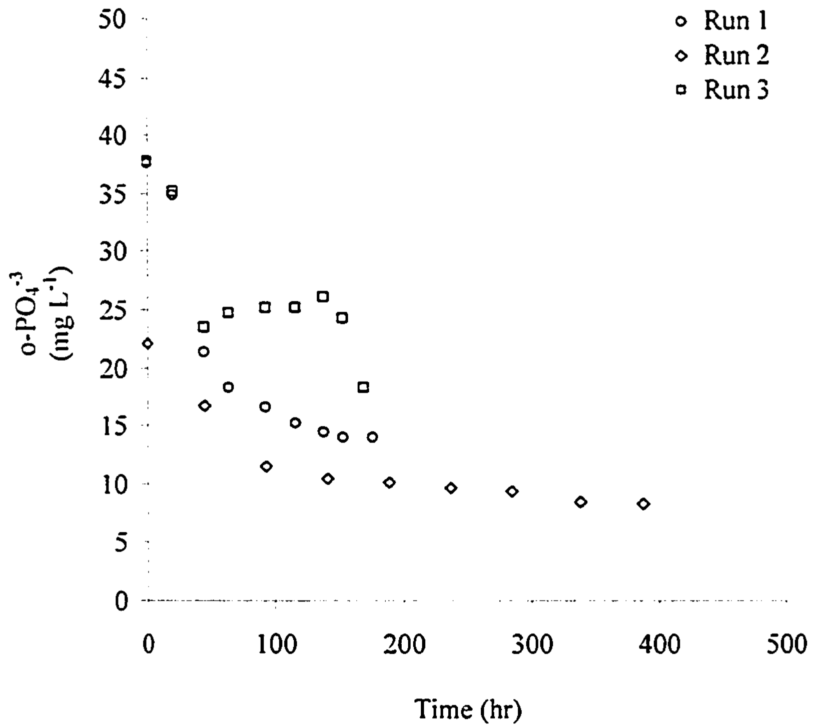

Figure 4-43 Aerobic o-PO$_4^{3-}$ concentration decrease by *Geotrichum* sp. (2007)

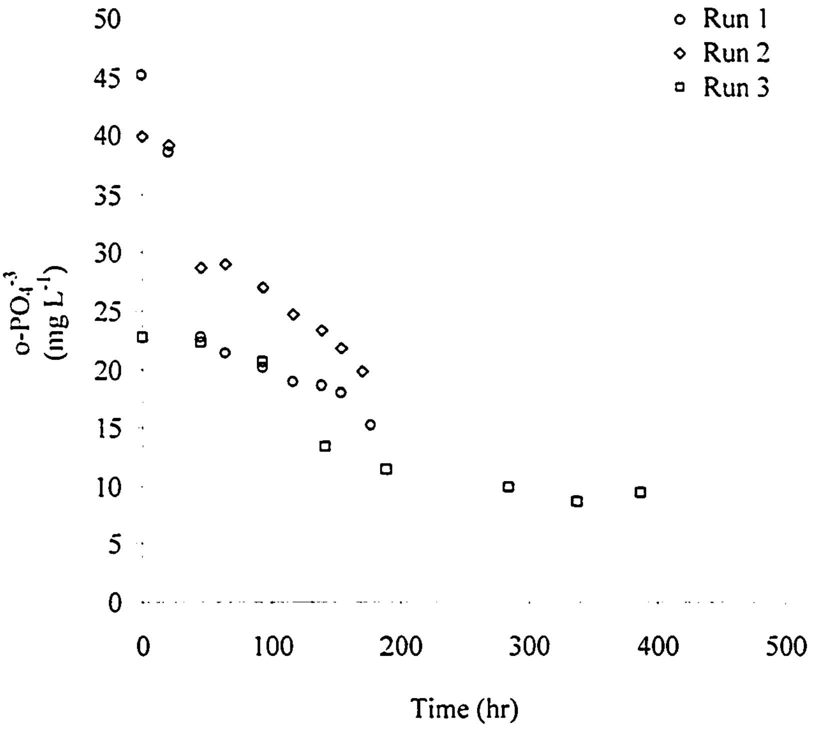

Figure 4-44 Aerobic o-PO$_4^{3-}$ concentration decrease by yeast.

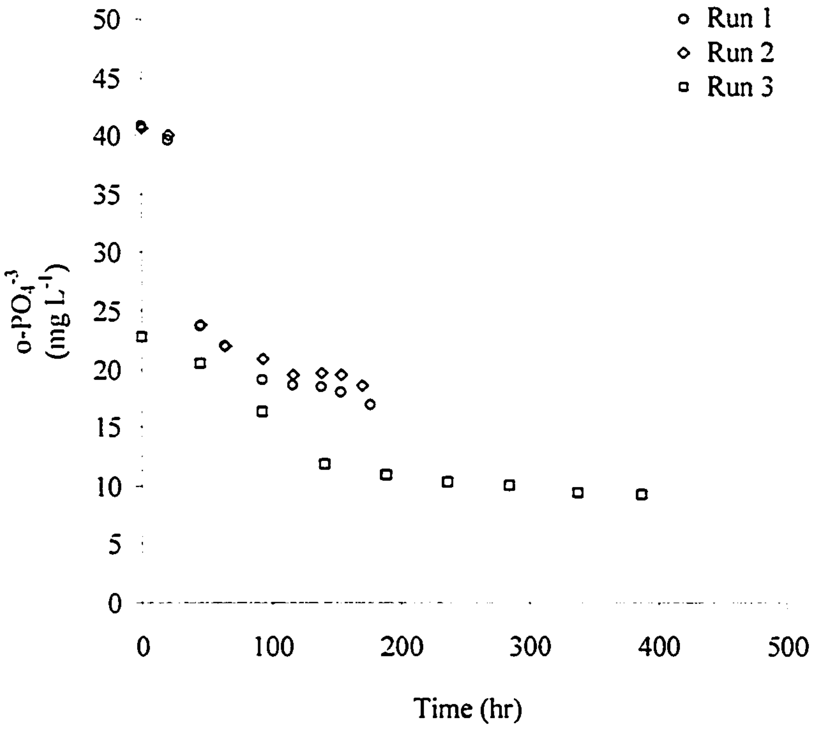

Figure 4-45 Aerobic o-PO$_4^{3-}$ concentration decrease by *Penicillium* sp.

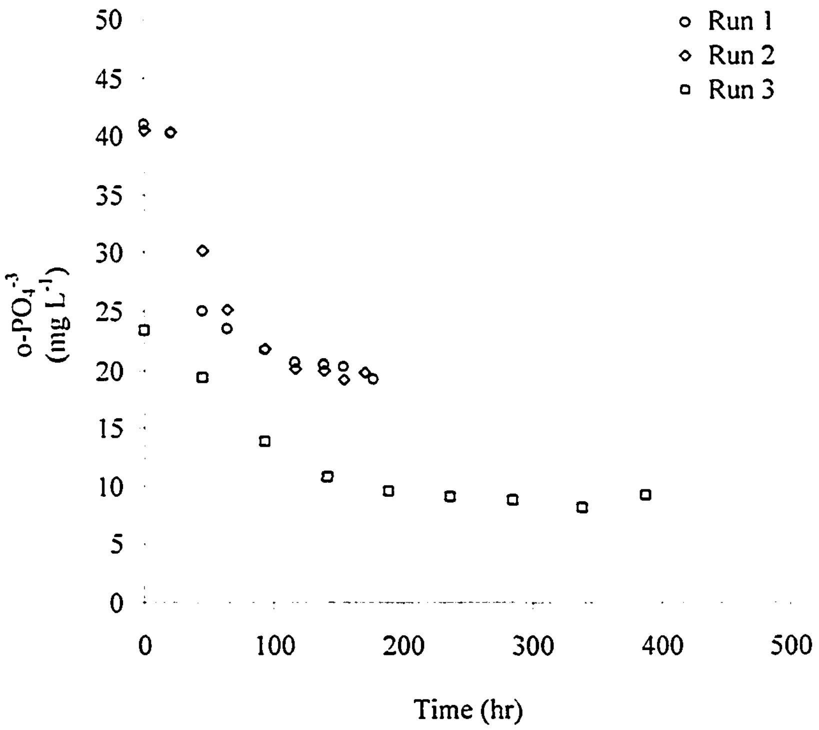

Figure 4-46 Aerobic o-PO$_4^{3-}$ concentration decrease by *Phoma* sp.

Effects of Microaerophilic environment on o-PO$_4^{3-}$ concentration decrease are shown in Figure 4-47 to Figure 4-51. Although there was considerable variation in the lag times and amounts of decrease, there was a clear data pattern of a small initial decline followed by a small increase (hump) then transition to a linear decline in o-PO$_4^{3-}$ concentration over time. The data were not conducive to modelling with a standard rate equation due to the irregular pattern, variation in lag time and the overriding trend of initial o-PO$_4^{3-}$ concentration. Due to the fairly linear trend in the data after the transition, a zero order kinetic equation was fit to the tail of the data. However, the calculated rate constants were misleading due to the complex nature of fungal o-PO$_4^{3-}$ concentration decrease and are not provided. However, the overall amount of o-PO$_4^{3-}$ reduced from the wastewater medium ranged between -3.8 and

17.5 mg L^{-1} depending on the initial o-PO_4^{3-} concentration in the medium across all five species tested.

There were two exceptions to the data pattern described above. In Figure 4-48 Run #2, o-PO_4^{3-} concentration increased after the linear decline then appears to sharply resume a linear decrease. This could have been analytical error but the pattern was based on a two-point trend. This was the only case of the data pattern being observed and, therefore, was likely an insignificant trend. Run #2 in Figure 4-50 for the *Penicillium* sp. showed a sharp increase in o-PO_4^{3-} concentrations. This was a one-point trend and, therefore, likely not significant.

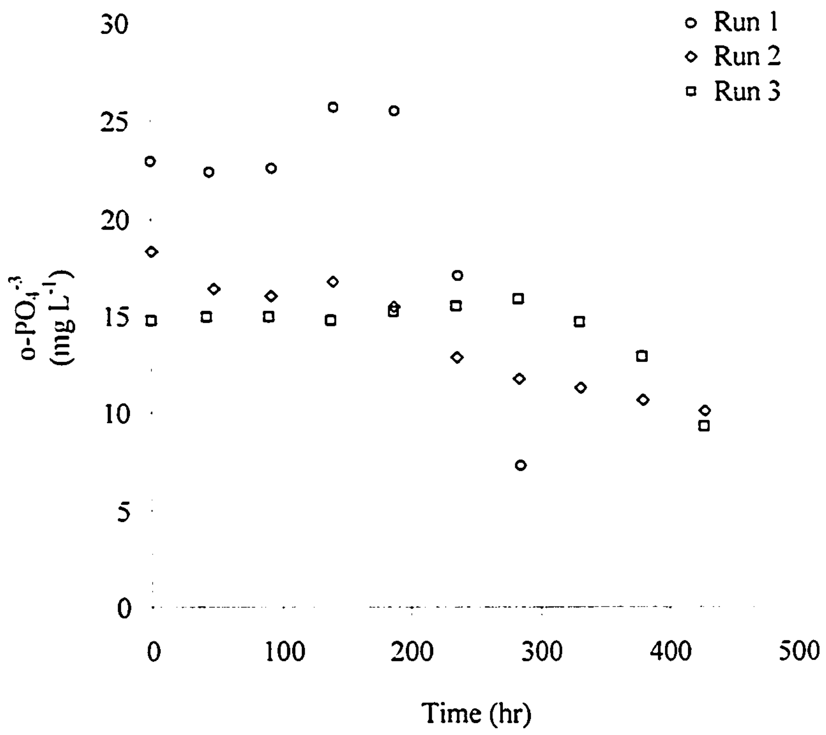

Figure 4-47 Microaerophilic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2000)

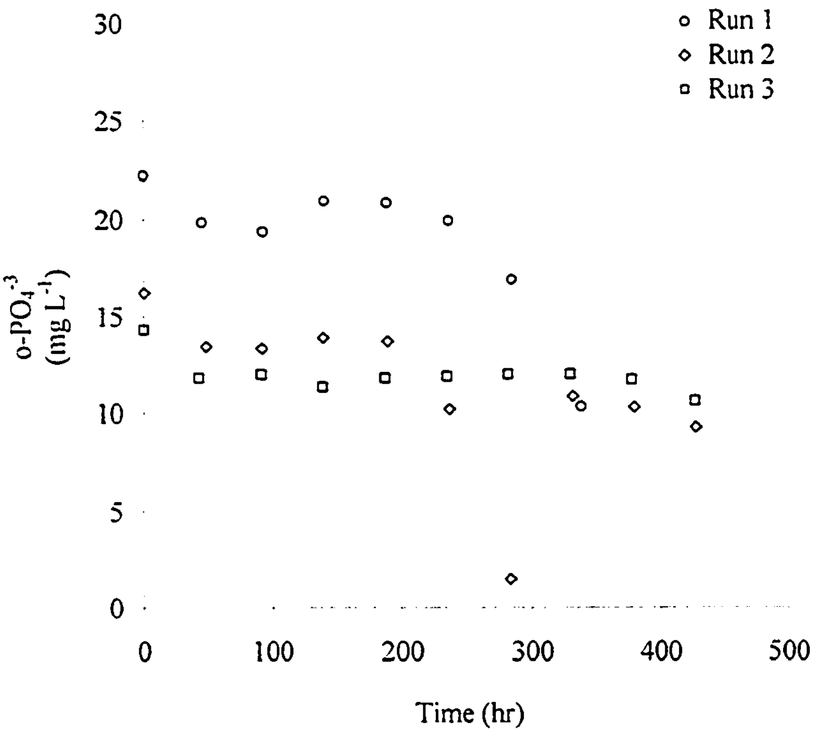

Figure 4-48 Microaerophilic o-PO$_4^{3-}$ concentration decrease by *Geotrichum* sp. (2007)

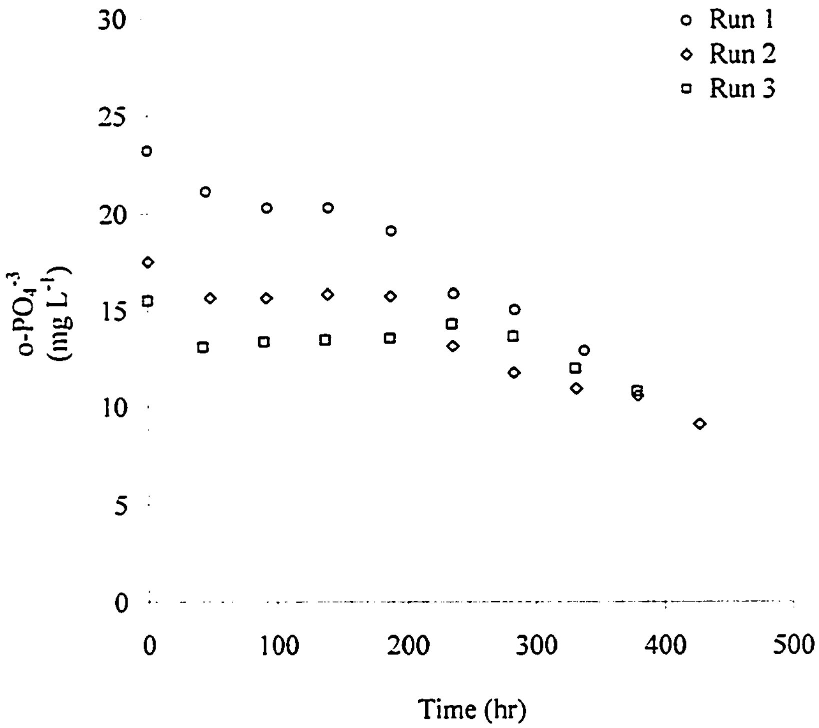

Figure 4-49 Microaerophilic o-PO$_4^{3-}$ concentration decrease by yeast.

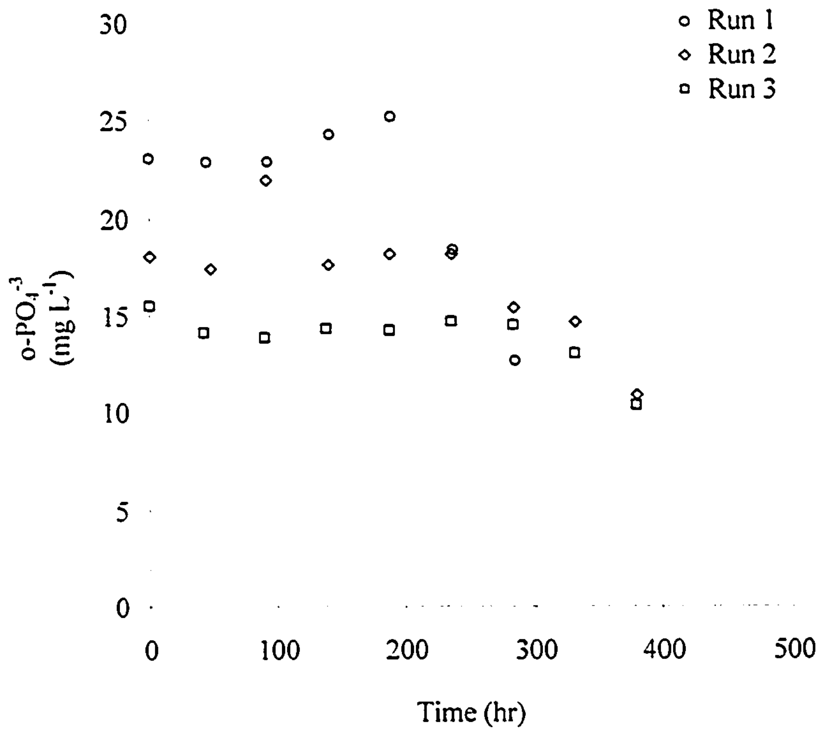

Figure 4-50 Microaerophilic o-PO$_4^{3-}$ concentration decrease by *Penicillium* sp.

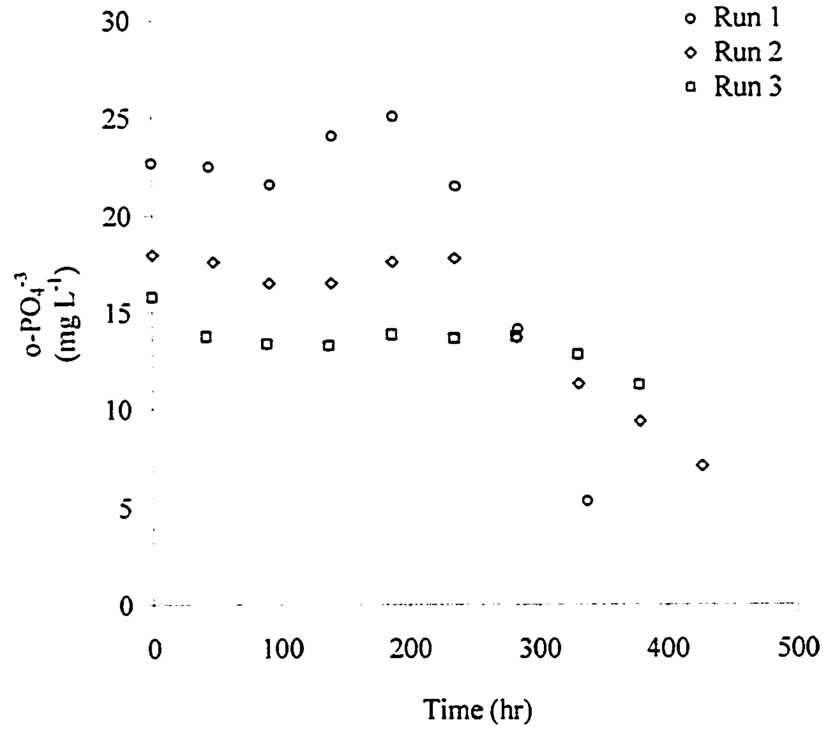

Figure 4-51 Microaerophilic o-PO$_4^{3-}$ concentration decrease by *Phoma* sp.

Figure 4-52 to Figure 4-56 illustrate the effect of anoxic environment on o-PO_4^{3-} concentration decrease. Two trends were clearly observable. The first trend was the pattern of o-PO_4^{3-} concentration decrease; variable lag times followed by a declining rate decrease. The second trend was the effect of the initial o-PO_4^{3-} concentration on the overall decrease. The data in Figure 4-52 to Figure 4-56 shows the pattern most clearly due to the range of initial o-PO_4^{3-} concentrations shown on the graphs. For the anoxic environment, o-PO_4^{3-} concentration decrease ranged between -0.7 to 11.7 mg L^{-1} o-PO_4^{3-} depending on initial o-PO_4^{3-} concentration.

Collectively the effect of oxygen tension on o-PO_4^{3-} decrease indicated two important trends. First, the impact of three oxygen tensions on the concentration decrease pattern of o-PO_4^{3-} potentially suggests different mechanisms of uptake and usage within fungi. The data patterns change considerably from aerobic to microaerophilic and lastly to anoxic. This maybe indicative of a change in regulatory pathway in the fungi tested. Without data on the other forms of phosphorus in the wastewater medium and in fungal biomass, confirmation of different mechanisms cannot be further developed. However, it can be clearly stated that oxygen tension does impact o-PO_4^{3-} decrease. The second trend to be noted was the similarity of the data patterns observed for o-PO_4^{3-} decrease across the five species tested. The three oxygen tensions produced similar patterns regardless of the species tested. This was a clear indication that o-PO_4^{3-} decrease systems response to oxygen tension in fungi are wide spread.

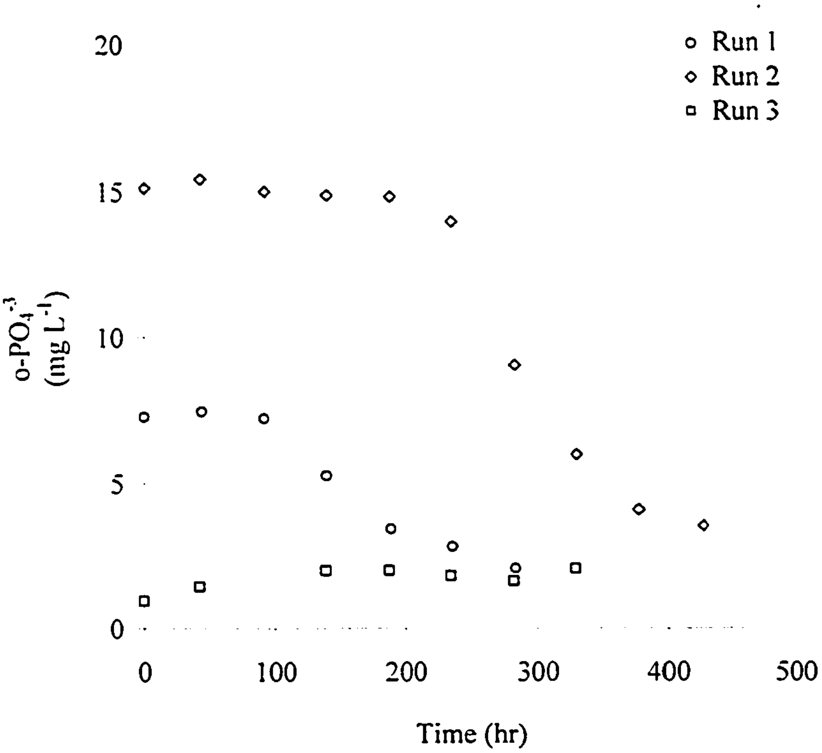

Figure 4-52 Anoxic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2000)

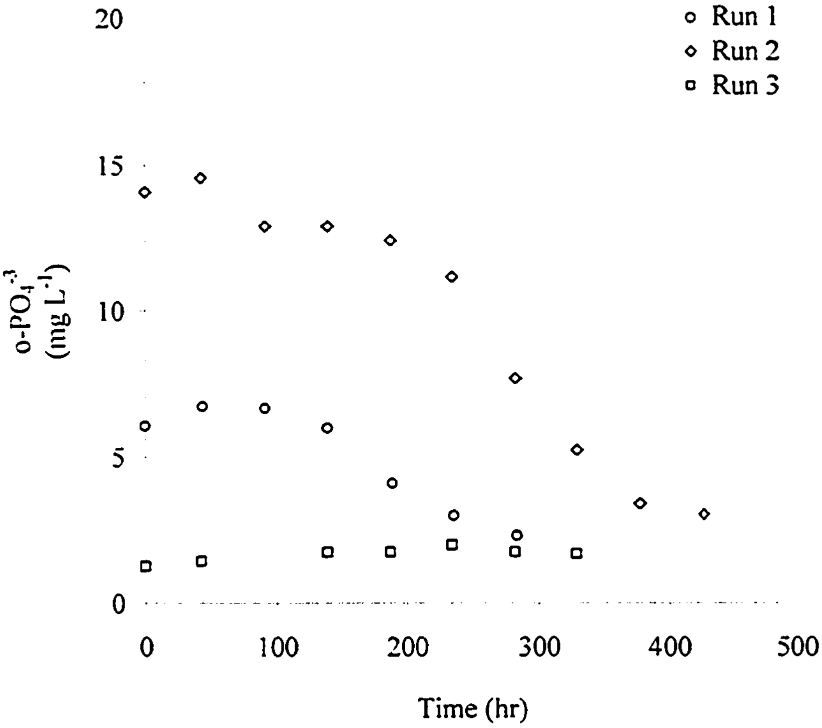

Figure 4-53 Anoxic o-PO_4^{3-} concentration decrease by *Geotrichum* sp. (2007)

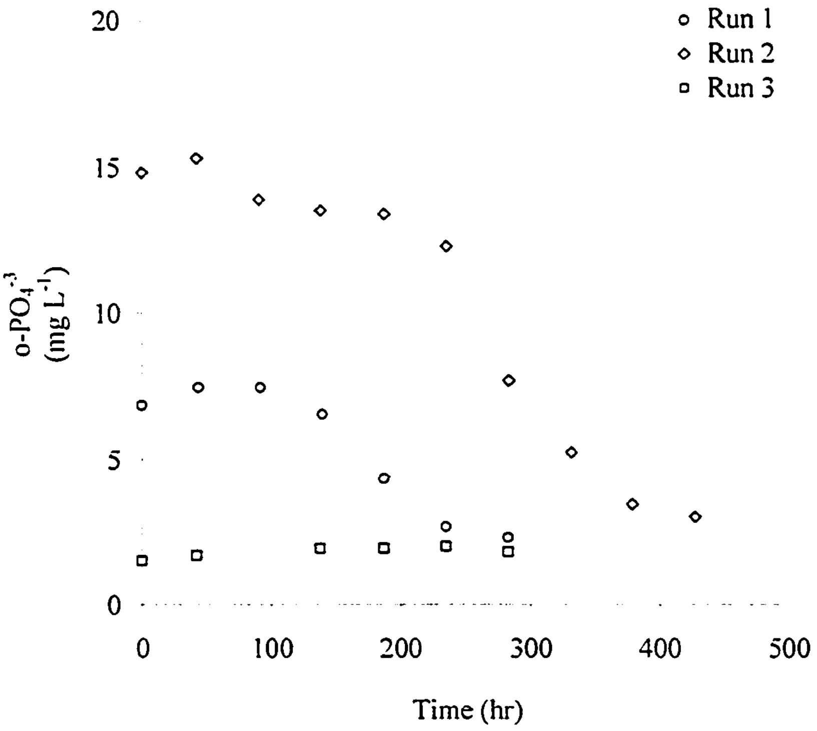

Figure 4-54 Anoxic o-PO$_4^{3-}$ concentration decrease by yeast

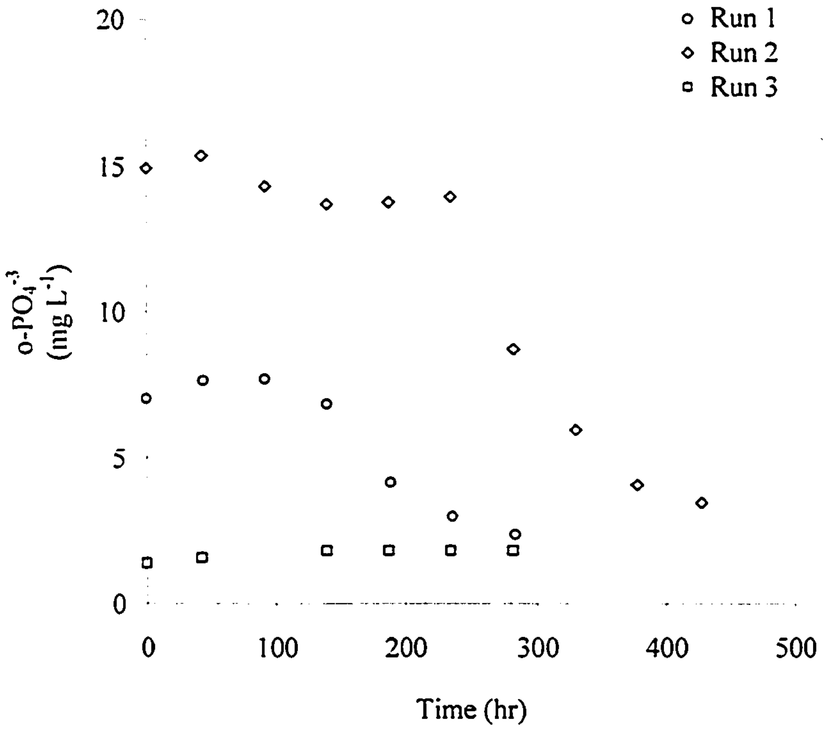

Figure 4-55 Anoxic o-PO$_4^{3-}$ concentration decrease by *Penicillium* sp.

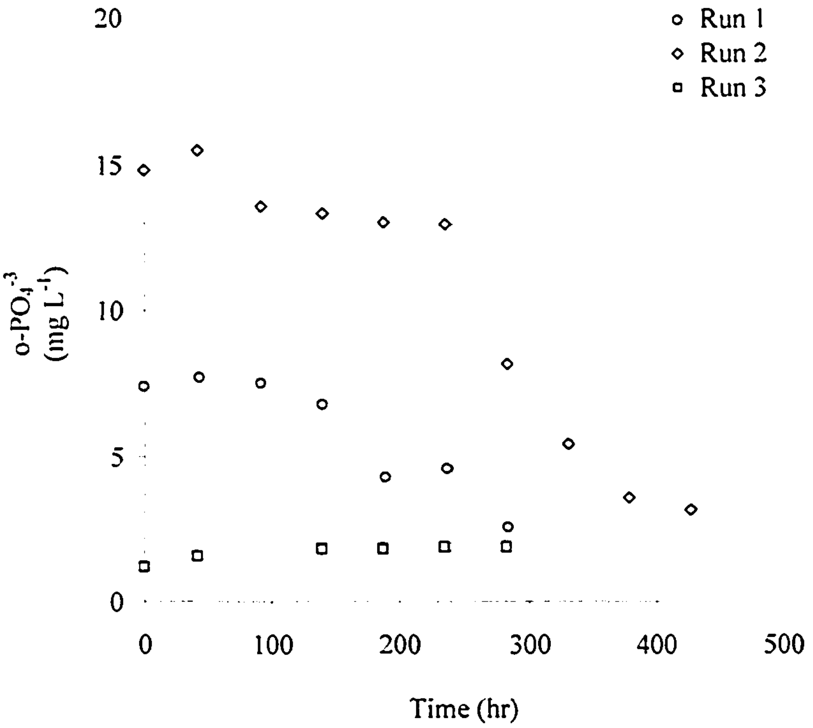

Figure 4-56 Anoxic o-PO$_4^{3-}$ concentration decrease by *Phoma* sp.

4.3.1.2 Dilute Wastewater Effects

Phosphate concentration decrease in dilute wastewater was observed under two oxygen tensions and is shown in Figure 4-57 and Figure 4-58. Two microaerophilic runs failed to show a decrease in o-PO$_4^{3-}$ concentration or showed a slight increasing trend. However, the most important trend was observed for the anoxic run; after a lag of approximately 100 hrs, o-PO$_4^{3-}$ concentration dropped. This was the result of a single trial, but, it was a significant result because it was part of a paired comparison with the first microaerophilic trial. The result was identical for all five of the fungi tested leading to between 3.7 to 4.9 mg L^{-1} of o-PO$_4^{3-}$ decrease. The results give support to the idea that oxygen tension was a determining factor for different phosphate pathways or usage in the cell. The paired comparison in dilute wastewater gave a strong indication that anoxically, o-PO_4^{3-} had a definitive biochemical purpose.

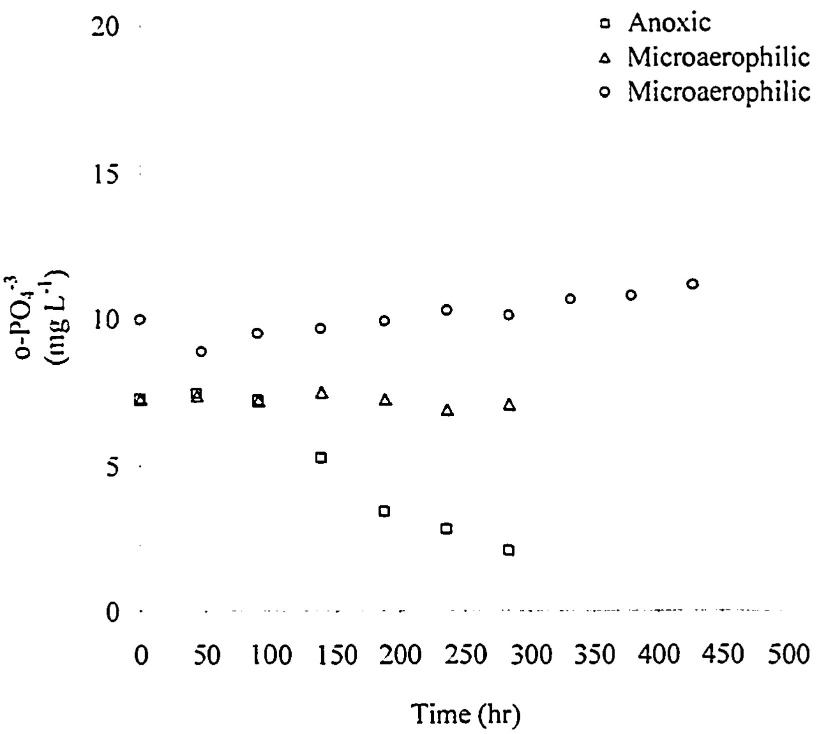

Figure 4-57 o-PO_4^{3-} concentration decrease in dilute wastewater by *Geotrichum* sp. (2000)

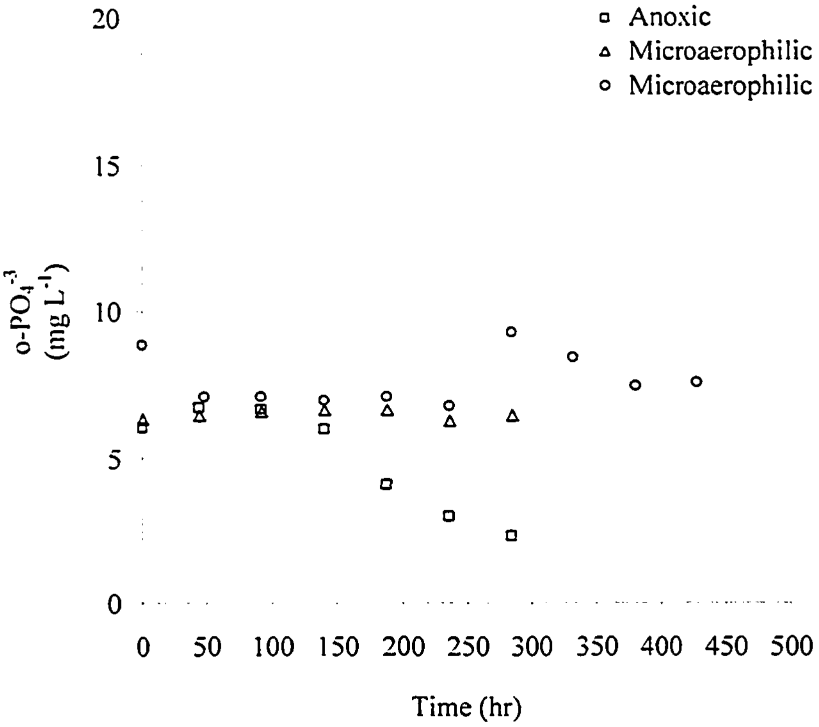

Figure 4-58 o-PO_4^{3-} concentration decrease in dilute wastewater by *Geotrichum* sp. (2007)

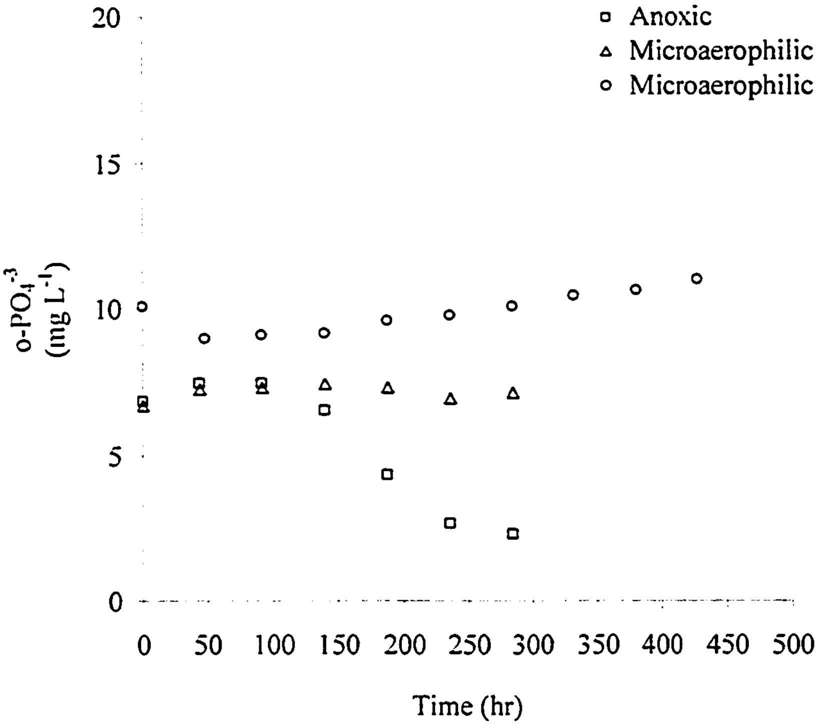

Figure 4-59 o-PO_4^{3-} concentration decrease in dilute wastewater by a yeast.

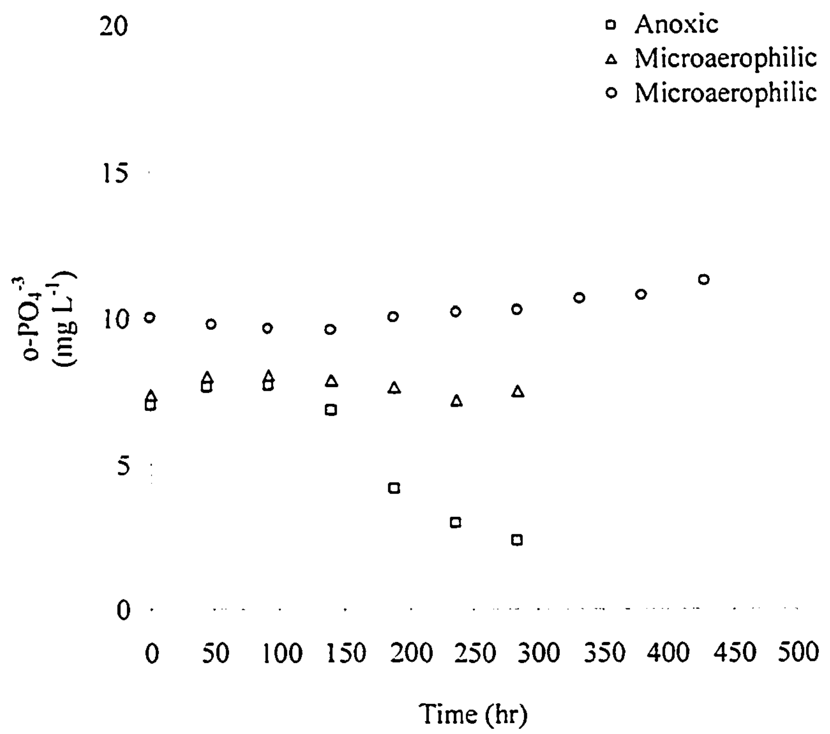

Figure 4-60 o-PO$_4^{3-}$ concentration decrease in dilute wastewater by *Penicillium* sp.

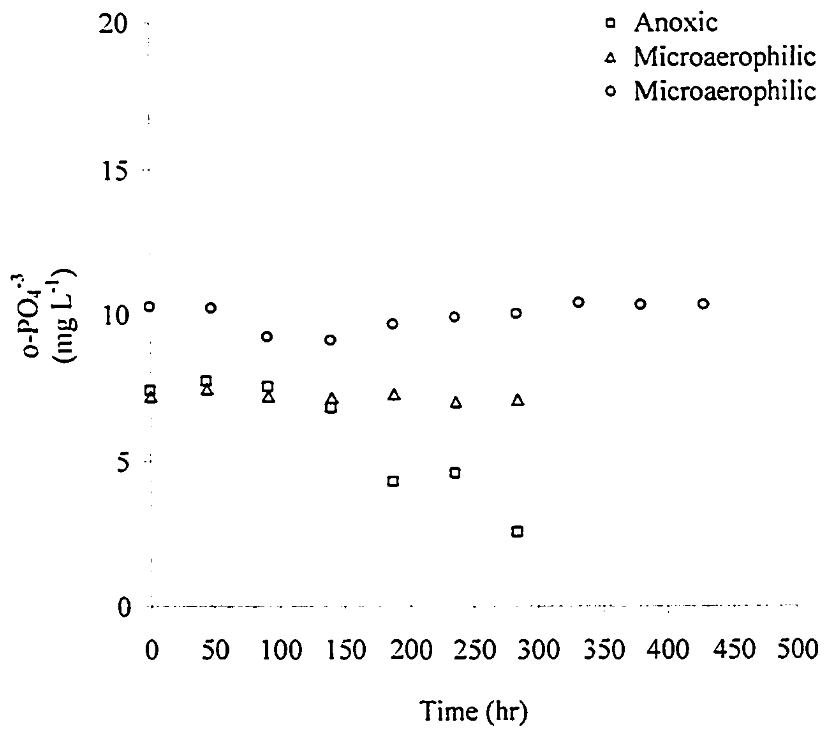

Figure 4-61 o-PO$_4^{3-}$ concentration decrease in dilute wastewater by *Phoma* sp.

4.3.1.3 *Successive Inoculation and Mixed Culture Tests*

Successive inoculation and mixed culture testing under microaerophilic environment showed no improvement in o-PO_4^{3-} concentration decrease. Successive inoculation data in Figure 4-62 to Figure 4-66, shows o-PO_4^{3-} concentration decrease was clearly being driven by the microaerophilic environment. The data pattern was the same as was describe in Section 4.3.1.1. The initial concentration decrease lag time and appearance of a hump varied in distinction across the five species but was observable. o-PO_4^{3-} concentration decrease was between 8 to 10 mg L^{-1} with an initial concentration between 16 to 18 mg L^{-1}. Mixed culture results are shown in Figure 4-67 and again demonstrate the microaerophilic data pattern for o-PO_4^{3-} concentration decrease.

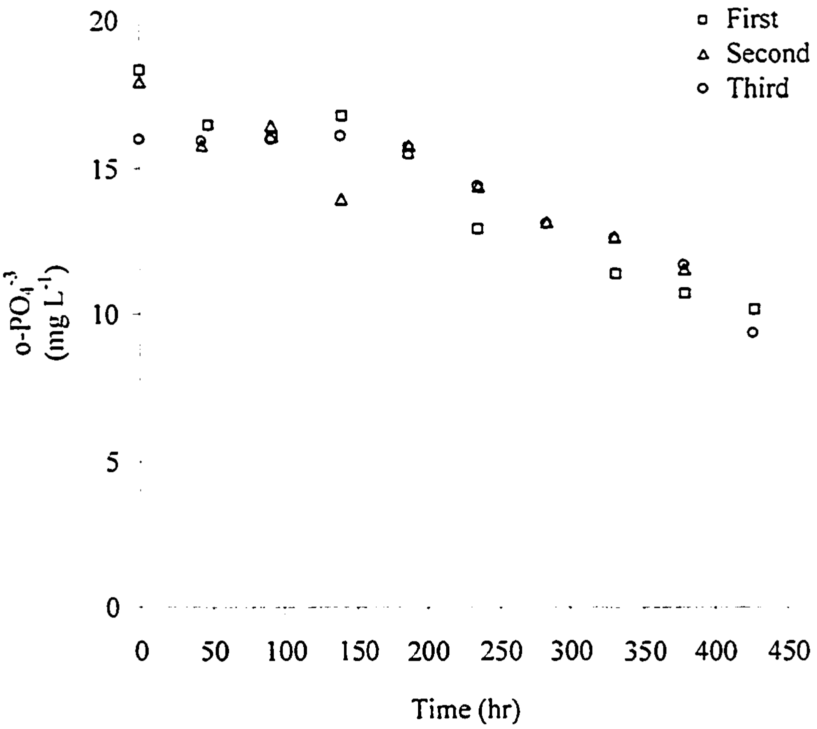

Figure 4-62 Concentration decrease of o-PO_4^{3-} for three successive generations of *Geotrichum* sp. (2000)

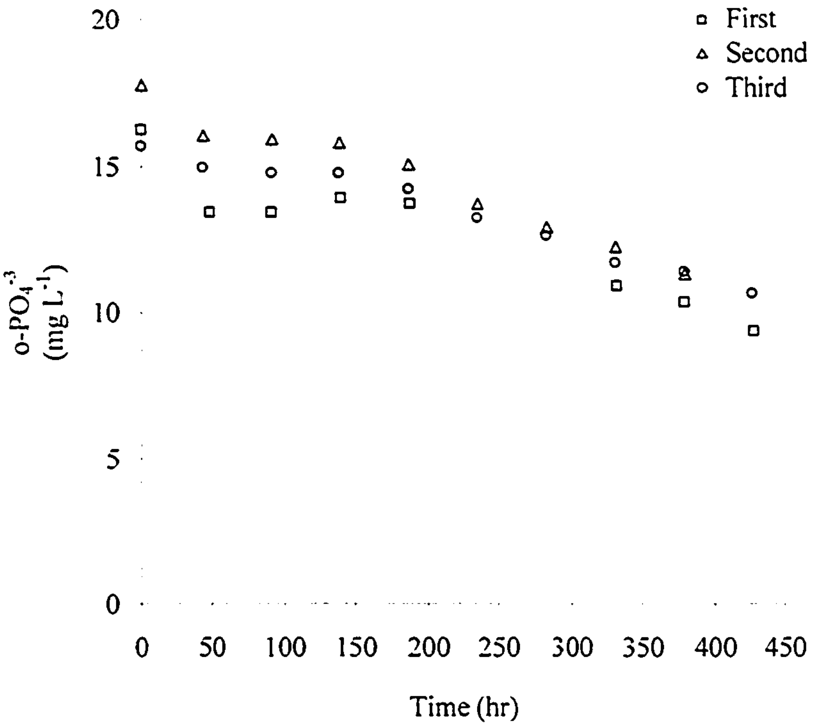

Figure 4-63 Concentration decrease of o-PO_4^{3-} for three successive generations of *Geotrichum* sp. (2007)

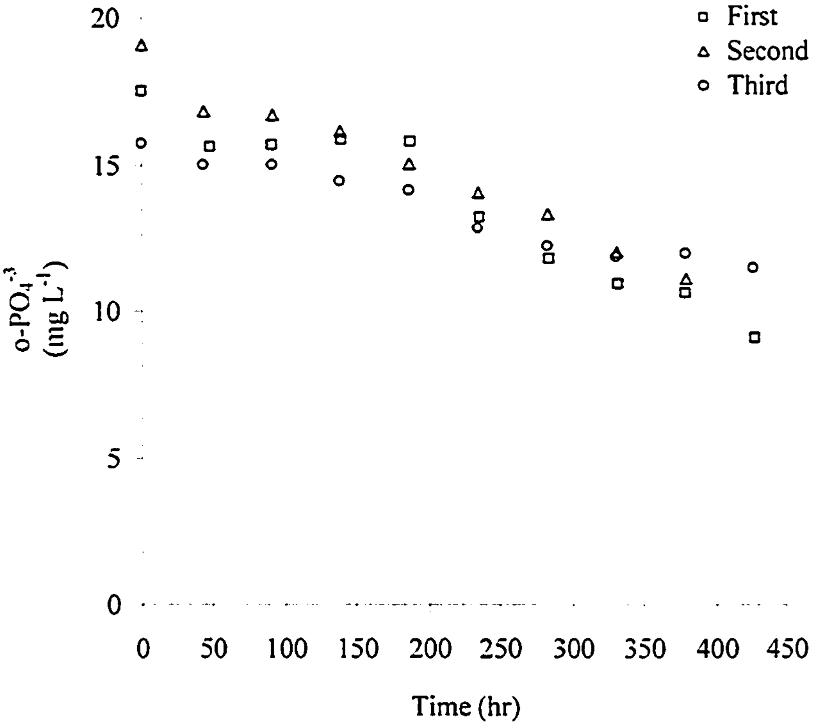

Figure 4-64 Concentration decrease of o-PO_4^{3-} for three successive generations of yeast.

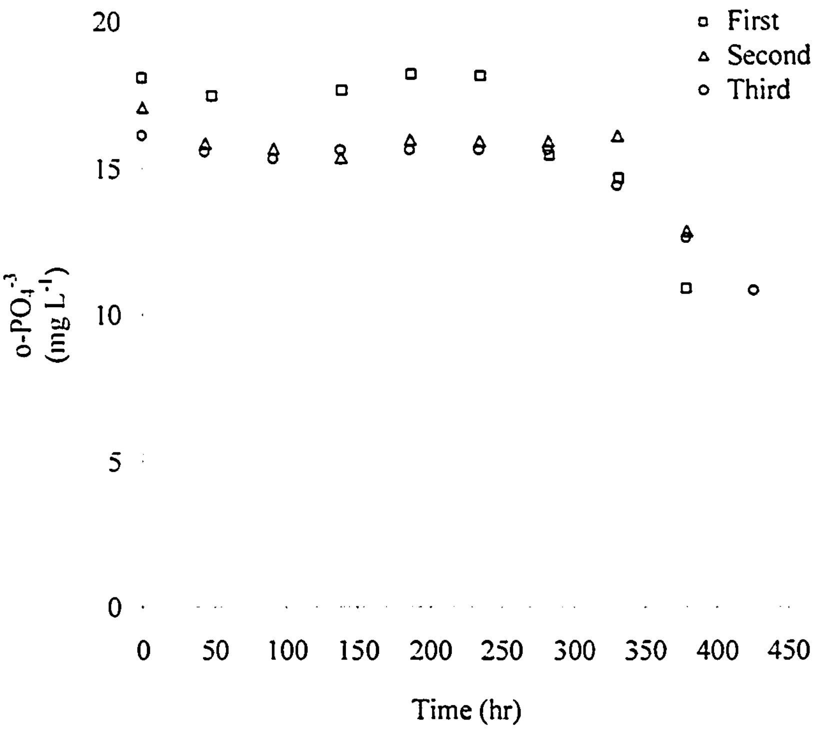

Figure 4-65 Concentration decrease of o-PO$_4^{3-}$ for three successive generations of *Penicillium* sp.

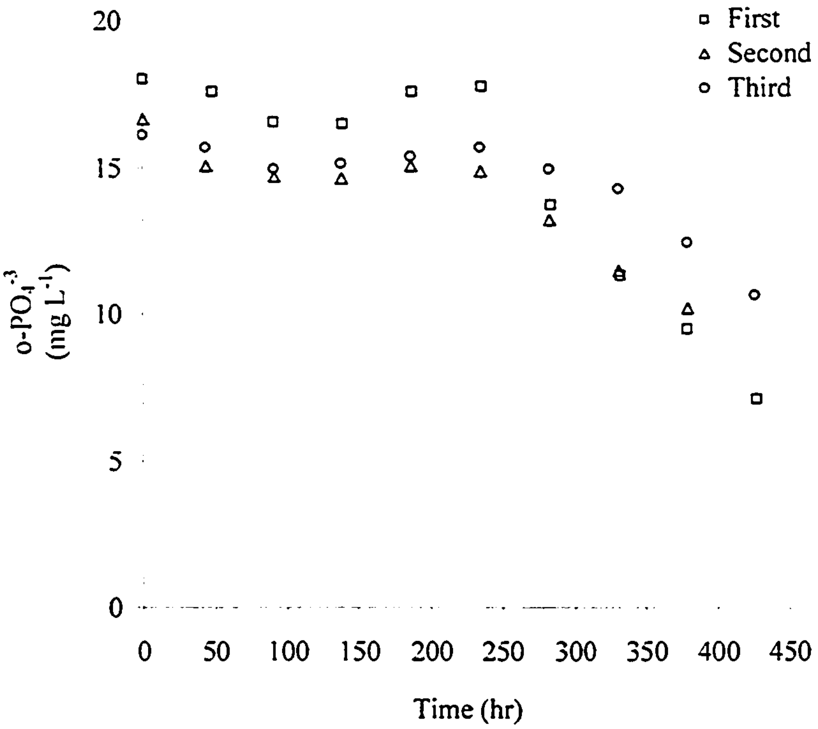

Figure 4-66 Concentration decrease of o-PO$_4^{3-}$ for three successive generations of *Phoma* sp.

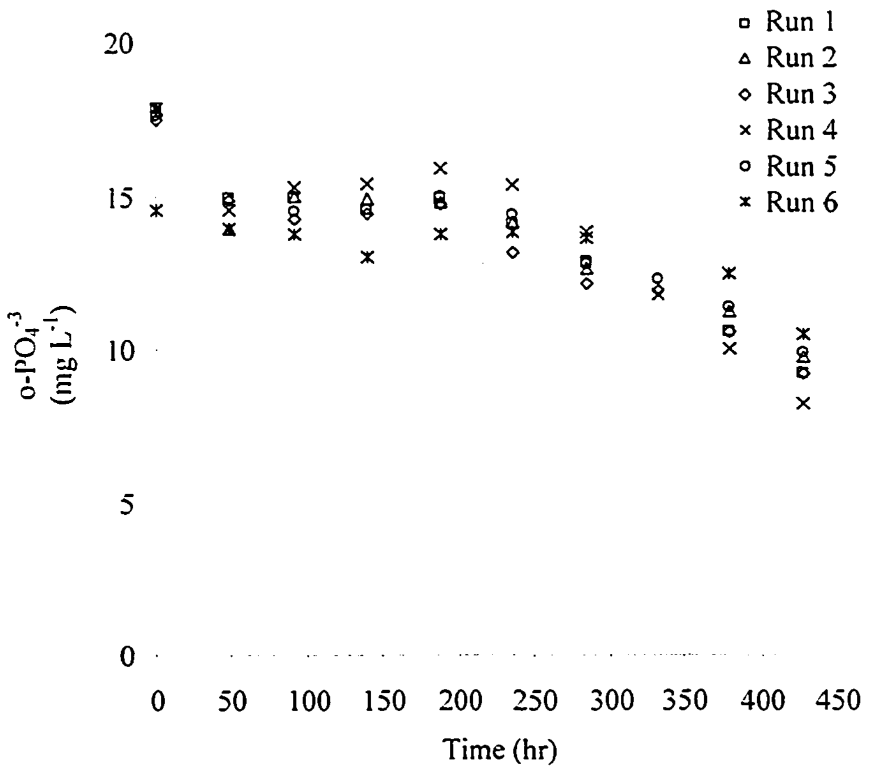

Figure 4-67 Concentration decrease of o-PO$_4^{3-}$ by a mixed culture.

4.3.2 Attached-Growth

Attached-growth concentration decrease of o-PO$_4^{3-}$ curves are presented in Figure 4-68 and Figure 4-69 for the *Penicillium* sp. and *Phoma* sp., respectively. The data were shown for two oxygen tensions: microaerophilic and anoxic. The batches of wastewater used in the attached-growth systems contained low concentrations of o-PO$_4^{3-}$ (around 1 mg L^{-1}) except for the first batch which contained approximately 15 mg L^{-1} The result shown in Figure 4-68 and Figure 4-69 were typical of batch run tests that only had 1 mg L^{-1} o-PO$_4^{3-}$. o-PO$_4^{3-}$ would slowly increase to between 2 and 3 mg L^{-1}. The effect was identical for both microaerophilic and anoxic conditions and corresponded to results presented in Section 4.3.1.1 for suspended-growth systems.

For the one run with initial o-PO_4^{3-} concentration of approximately 15 mg L^{-1}, concentration decrease was approximately between 7 and 10 mg L^{-1} which again is similar to previous observations (Section 4.3.1.1). The data pattern for the *Penicillium* sp. appeared to be different from the previous results under microaerophilic environment. There was no observed lag and the concentration decrease proceeded linearly and then abruptly tailed. With the limited data, due to the small range of o-PO_4^{3-} concentration in the wastewater, it was impossible to say if the pattern was consistent or was just part of the observable variation. The *Phoma* sp. showed the classic data pattern for microaerophilic growth. Further testing with a greater range of o-PO_4^{3-} concentrations was needed. However, the overriding trend seen in suspended-growth was also observable in attached-growth. Higher initial o-PO_4^{3-} concentration was a predictor of overall concentration decrease.

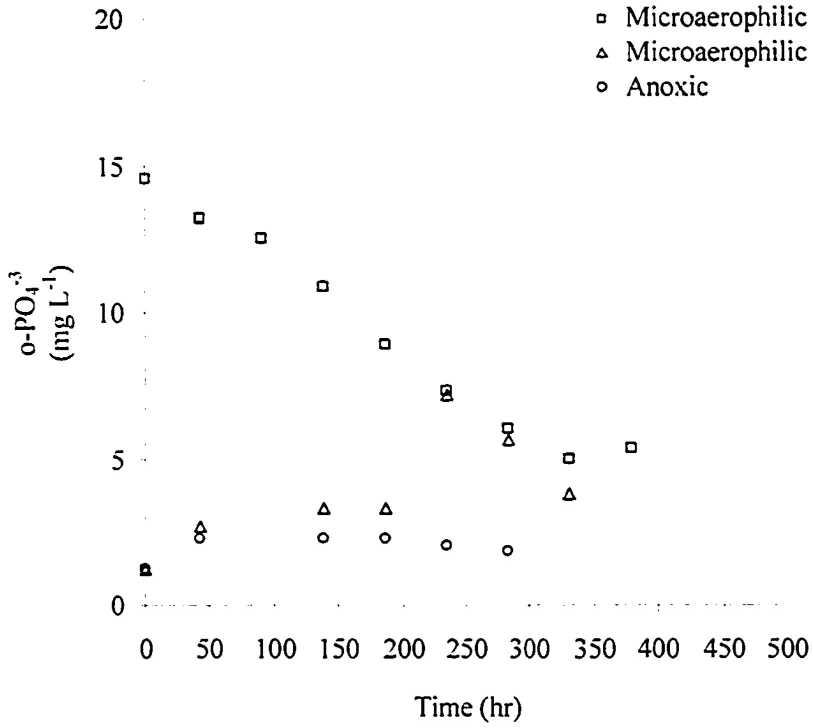

Figure 4-68 Attached-growth concentration decrease of o-PO$_4^{3-}$ by *Penicillium* sp. under two oxygen tensions.

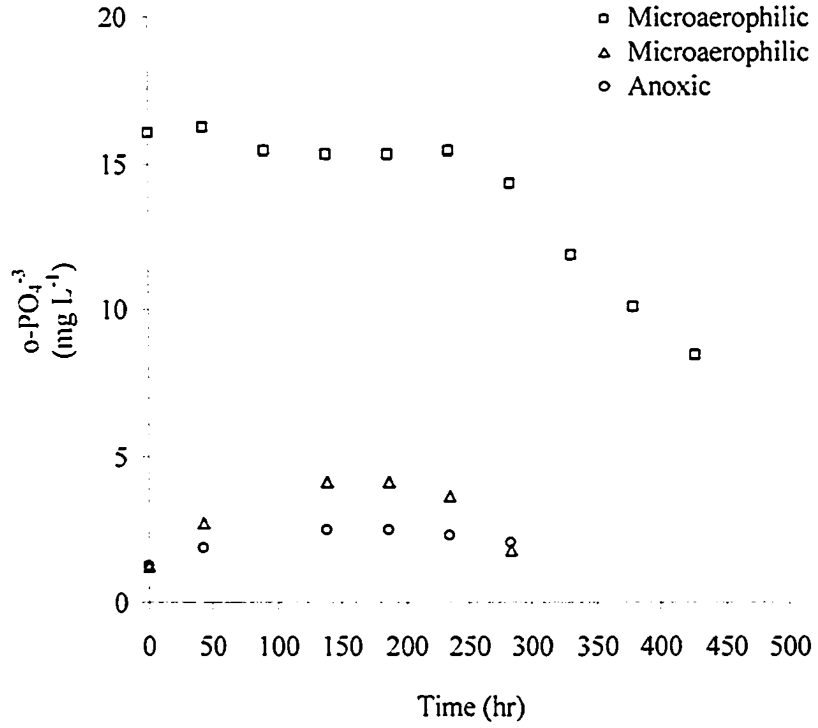

Figure 4-69 Attached-growth concentration decrease of o-PO$_4^{3-}$ by *Phoma* sp. under two oxygen tensions.

Chapter Five

5.0 Discussion of Results

5.1 Isolation and Screening Results

5.1.1 Identified Isolates

Cooke's (1976; 1986) survey of fungi in wastewater identified ten major genera of fungi; *Phlyctochytrium* spp., *Rhizophidium* spp, *Achlya* spp., *Pythium* spp., *Mucor* spp., *Torulopsis* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. *and Trichoderma* spp. In more recent survey, Fakhru'l-Razi et al (2002) identified five major genera of fungi in wastewater; *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp., *Spicaria* spp., and *Hyaloflorae* spp. Common to the data collected in this study are the *Penicillium* spp. and the *Mucor* spp. The *Geotrichum* spp., the *Phoma* spp. had not been noted in the literature as being a common member of the wastewater micro-flora. The *Penicillium* spp. isolates have shown good potential for NH_4^+ treatment in wastewater and are likely going to be found in many other wastewater treatment plants. This is a necessity for successful development of fungal nitrogen treatment technology that promising fungi survive in the wastewater environment. The fact that *Phoma* spp. and *Geotrichum* spp. have not been previously found could simply be a function of the few surveys of wastewater treatment plants performed, or it could be an indication they are not common in the wastewater environment. Further work is needed to better assess the prevalence of the fungi identified in this study.

5.1.2 Fate and Decrease of Ammonium

Ammonium concentration decrease for the selected isolates was significant. However, the lack of nitrification end-products (NO_2^- and NO_3^-) raised questions as to the fate of NH_4^+. Fungi were reported to have greater rates and higher production of NO_2^- and NO_3^- than bacteria, but this was not observed (Eylar and Schmidt 1959; Kurakov and Popov 1996). However, this does not mean that nitrification was not occurring in the batch screening tests. The work by Hirsch et al. (1961), Eylar and Schmidt (1959) and Falih and Wainwright (1995) showed that accumulation of NO_2^- and NO_3^- in the medium was heavily influenced by the constituents of the medium. Hirsch et al. (1961) specifically found that increasing ammonium concentration with sucrose increased the accumulation of nitrate in the medium. Although the primary effluent wastewater medium used for this study, had relatively high in ammonium concentrations, it likely did not contain simple sugars. The concentration of simple sugars may have been reduced during primary treatment. It was possible that fungi were converting NH_4^+ to NO_2^- or NO_3^-, but maintaining the end-products intracellularly.

The other major possible explanation for the lack of nitrification end-products, was that NH_4^+ was being assimilated into the cell mass. Based on yeast and fungi elemental composition reported in Table 5-1, it is possible to estimate the amount of ammonium that was assimilated for new cellular material based on the suspended solids data collected to monitor fungi growth. Table 5-2 indicates there was strong evidence that a large percentage of the ammonium nitrogen decreased from the wastewater medium was unaccounted. The two *Penicillium* spp. and the *Phoma* sp. showed greater than 70% of the decreased NH_4^+ was not likely contained in the cell mass. Between 42 and 50% of NH_4^+ was not accounted for the remaining species. Table 5-2 estimate of assimilation suggests that the hypothesis that NO_2^- or NO_3^- could be stored intracellularly was possible. However, it also opened the possibility that fungi could be using NH_4^+ in a denitrification pathway. Assimilation was only estimated and, therefore, measurement of total nitrogen and organic nitrogen content of the systems must be confirmed to fully support the data presented in Table 5-2. Oxidation of NH_4^+ to NO_3^- or NO_2^- requires 4.57 g COD/g N or 3.43 g COD/g N (Grady et al. 1999), respectively. The COD concentration decrease ratios found for the identified fungi where greater than 80% of the ratio is assumed to be from NH_4^+ (Table 4-7) can be grouped; *Penicillium* spp 2.6 g COD/ g N, 3.9 g COD/g N and for *Geotrichum* spp. and *Phoma* spp. 7.3 and 8.2, respectively. The data from the two *Penicillium* spp. support the hypothesis that NH_4^+ was possibly being converted to NO_2^- or NO_3^- and stored intracellularly, however, this was inconclusive. The ratio for the *Geotrichum* spp. and *Phoma* spp. was approximately double suggesting a further reaction or alternative biochemical pathway. Again, this was inconclusive without knowing the true total nitrogen and organic nitrogen in the system, however, the data indicated a lack of fit with theory.

Table 5-1 Elemental composition of bacteria, yeasts and fungi (% by dry weight)[£]

Element	Bacteria	Yeasts	Fungi
Carbon	50 to 53	45 to 50	40 to 63
Hydrogen	7	7	—
Nitrogen	12 to 15	7.5 to 11	7 to 10
Phosphorus	2.0 to 3.0	0.8 to 2.6	0.4 to 4.5
Sulphur	0.2 to 1.0	0.01 to 0.24	0.1 to 0.5
Potassium	1.0 to 4.5	1.0 to 4.0	0.2 to 2.5
Sodium	0.5 to 1.0	0.01 to 0.1	0.02 to 0.5
Calcium	0.01 to 1.1	0.1 to 0.3	0.1 to 1.4
Magnesium	0.1 to 0.5	0.1 to 0.5	0.1 to 0.5
Chloride	0.5	—	—
Iron	0.02 to 0.2	0.01 to 0.5	0.1 to 0.2

[£] Adapted from Stanbury et al. (1995)

Table 5-2 Estimated NH_4^+-nitrogen assimilated for growth of new biomass

Isolate	Genus Identification	TSS mg L^{-1}	Estimated NH_4^+ for new cell mass mg-N L^{-1}	NH_4^+ Decreased from media mg-N L^{-1}	Unaccounted for nitrogen mg-N L^{-1}	Unaccounted nitrogen %
2000	*Geotrichum* spp.	70.6	6.0	11.8	5.8	49
2007	*Geotrichum* spp.	46.0	3.9	7.4	3.5	47
2008	Yeast	61.5	5.4	9.3	3.9	42
3004	*Penicillium* spp.	34.0	2.9	9.7	6.8	70
3005	*Mucor* spp.	32.5	2.8	5.5	2.7	50
3013	*Phoma* spp.	38.0	3.2	12.6	9.4	74
4001	*Penicillium* spp.	19.0	1.6	7.7	6.0	79

5.1.3 Concentration Decrease of Phosphorus

For some of the fungal isolates tested, the concentration decrease of phosphate clearly suggested that the mechanism and energy requirements were different when compared to bacterial based phosphate decrease systems currently used in WWTPs. The minimum COD to phosphorus decrease ratio is 35 g COD/g P for most of the common biological phosphorus treatment systems (Metcalf & Eddy et al. 2003). The COD decrease ratios presented in Table 4-7 were for combined nitrogen and phosphorus concentration decrease. For isolate 2007 (*Geotrichum* spp.), a total of 10.6 mg-P/L was decreased from the medium. This should have required 370 mg L^{-1} of COD plus the COD for decreasing the concentration of NH_4^+. For isolate 2007, 50 mg L^{-1} COD was used for a total concentration decrease of 18 mg L^{-1} of nitrogen (7.4 mg L^{-1}) and phosphorus (10.6 mg L^{-1}) giving a COD decrease ratio of 2.7 g COD/ g N+P. This is an order of magnitude lower compared to published bacterial system values. There is very little published literature with which to compare the observed phosphate decrease in wastewater results. Hiremath et al (1985) only reported the percent decrease of phosphate (51 to 82 %) for the various species tested, except for one species which was used as an example of the decrease curves obtained in the study. From the graph, the initial o-PO_4^{3-} concentration was approximately 5 mg L^{-1} and the overall decrease was approximately 4 mg L^{-1}. Thanh and Simard (1973) characterized the action of yeast on decreasing phosphate in wastewater also in percentages. Without addition of any of sugar, they reported between 20 and 47% decrease in phosphates with an average initial concentration of 12.8 mg L^{-1}. Based on the average initial o-PO_4^{3-} concentration Thanh and Simard (1973) observed approximately 6 mg L^{-1} (based on 47% decrease) overall decrease and a COD to o-PO_4^{3-} decrease ratio of 13 g COD:1 g P. Given the results from this study and the small data set available in the literature, fungi appear to have a lower substrate to phosphate concentration decrease ratio than the phosphate accumulating bacteria used in wastewater treatment. For phosphate concentration decrease in wastewater treatment, reducing the amount of carbon necessary could simplify the treatment process. Currently, for many phosphorus treatment technologies, the addition of short chain organics is necessary to stimulate luxuriant uptake of phosphate (Metcalf & Eddy et al. 2003). Production of the short chain organics is typically carried out by fermentation of primary sludge or is purchased from local suppliers. Both solutions are costly. Fungi may have a role to play in phosphate concentration decrease in wastewater treatment.

5.2 Kinetics of Ammonium Concentration Decrease in Wastewater By Fungi

The research presented in Section 4.2 demonstrates the potential for development of fungal ammonium treatment technology. This entire research project is unique in the field of nutrient removal technology, and therefore, there is no basis for comparison. This limits the extent of discussion in terms of comparison to other research in the field. However, the results of the experiments have several significant findings; direct use of NH_4^+ by fungi; a possible form of simultaneous nitrification-denitrification; and expansion of the fungi capable of NH_4^+ concentration decrease by apparent denitrification.

The results of all the batch kinetic tests showed no detectable production of nitrite or nitrate. Nitrogen species analyses showed that NH_4^+ was not being assimilated and volatilization of NH_4^+ was minimal; but the mass balance for the reactor systems showed a net loss of NH_4^+ from the systems. These data strongly supports the hypothesis that fungi are using a biochemical pathway to convert NH_4^+ to a gas containing nitrogen (NO, N_2O, N_2). Fungal nitrogen content expressed as percent dry weight in Table 5-1 ranges from 7 to 10%. If fungi were only using nitrogen for growth via assimilation, then nitrogen, should follow a first-order relationship and lowest concentration of ammonium decrease that would have been observed was approximately 7 mg-N L^{-1}. However, *Geotrichum* sp. (2000), *Penicillium* sp. and *Phoma* sp. all decreased NH_4^+ to less than 0.1 mg-N L^{-1}.

Regardless, what is lacking from the data to fully support the denitrification conclusion is reactor off gas analysis for nitrogen, nitrous oxide and nitric oxide (i.e., the products of denitrification). Without these data, the possibility exist that NH_4^+ is being converted into a compound that is not measured by the analytical techniques used. Although the probability of this is small, it must be acknowledged. However, it is assumed for the rest of the discussion that NH_4^+ is undergoing a transformation and leaving the system via gaseous products.

5.2.1 Simultaneous Nitrification-Denitrification or Direct Ammonium Nitrogen Decrease

The apparent lack of nitrification products and evidence supporting direct use of NH_4^+ in denitrification is a significant finding. All fungal denitrification literature to date maintains that NO_2^- is the required nitrogen species for fungal denitrification pathway or the co-denitrification pathway (Shoun et al. 1989; Shoun and Tanimoto 1991; Shoun 1992; Shoun et al. 1992; Tanimoto et al. 1992; Usuda et al. 1995). The data collected and presented, raises at least two distinct possibilities for NH_4^+ decrease observed in this research; 1) the fungi are performing an internal nitrification step as part of a simultaneous nitrification-denitrification (SND) pathway, or 2) NH_4^+ is undergoing direct ammonium nitrogen decrease (DAND) via a pathway not described in the literature. The SND hypothesis is supported more by the data and literature on fungal denitrification; however there are inconsistencies that support the DAND hypothesis.

The data and literature that support the SND hypothesis includes research by Zhou et al. (2001) and the aerobic and microaerophilic reactor data. The fact that some amount of oxygen is required and results in the best NH_4^+ decrease is indicative that oxygen could be used for nitrification. It is well documented in the literature that fungi can perform nitrification (see sections 2.0 and 5.1.2) and, therefore, it is entirely conceivable that fungi can directly link their nitrification pathway to a denitrification pathway. Zhou et al. (2001) reported that the fungus *F. oxysporum*, required a minimal amount of oxygen for induction of denitrification. They also reported that excessive oxygen repressed the enzyme system. The study by Zhou et al. (2001) provides partial corroboration of the significance of the microaerophilic and aerobic data collected in this research. The majority of the data collected showed that aerobic and anoxic conditions limited the decrease of NH_4^+ whereas microaerophilic conditions saw maximal NH_4^+ concentration decrease. The analogous effect of oxygen, found in this research as in the Zhou et al. (2001) data, suggest the control of the pathway is similar. This lends support to fungi performing denitrification. The study by Zhou et al. (2001), however, was performed in defined medium using nitrate or nitrite as the nitrogen source-again raising questions about the biochemical pathways involved. What is supported by both the literature and data collected, is that oxygen is a significant regulator of the fungal NH_4^+ concentration decrease system.

The literature on fungal denitrification unequivocally states that NO_2^- and NO_3^- are the nitrogen species required for fungal denitrification. The data presented here clearly shows that NH_4^+ is being used directly. The fact that Shoun (Shoun et al. 1992; Su et al. 2004) and his research group have not observed the ability of fungi to use NH_4^+ directly in denitrification is significant. Shoun's work has focused on two fungi; *F. oxysporum* and *C. tonkinense* (Su et al. 2004). This means, the range of fungal denitrification known in the literature is limited. These two fungi may not be capable of directly coupling their nitrification and denitrification pathways for a SND system. Support for this explanation can come from the work of (Shoun 1992; Shoun et al. 1992) on co-denitirification pathways. Testing with NH_4^+ as the secondary nitrogen source found co-denitrification would not occur without NO_2^- or NO_3^- present (Su et al. 2004). Su (2004)have tried using NH_4^+ as the primary nitrogen species for denitrification and failed with *F. oxysporum* and *C. tonkinense*. The fungi isolated and used in this research have not been previously examined for their denitrification ability. This means the data collected and presented expands the fungal denitrification pathway and the scope of fungi capable of performing it.

Support for an alternative biochemical pathway utilizing a DAND with NH_4^+ is supported by inconsistencies in the data generated and the need for oxygen in order to perform nitrification. The SND hypothesis means oxygen is required for an internal nitrification step coupled to the denitrification pathway. However, if an internal nitrification step is occurring that requires oxygen, then anoxic conditions should have completely suppressed NH_4^+ concentration decrease in the experiments performed in this research. For the most part, NH_4^+ concentration decrease under anoxic atmosphere was low, but averaged around 20% and reached as high as 35% for the suspended cultures. In addition, the one anoxic run in the attached-growth system with the *Phoma* sp. had NH_4^+ concentration decrease of 60%. Although some of the NH_4^+ concentration decrease would be from assimilation, the amount required for assimilation was much less than the amount of NH_4^+ used from the medium. This suggests that NH_4^+ concentration decrease can be performed independently of oxygen, therefore, lending support to the DAND pathway hypothesis. However, even though the reactors were purged with nitrogen and maintained a nitrogen headspace, it was not possible to insert a dissolved oxygen probe in the reactors to ensure all the oxygen had been voided from the system. Without precise dissolved oxygen measurements there is a possibility that some oxygen entered back into the reactors. This could have occurred after the medium was purged, prior to and during the autoclave cycle. The limited number of ports on the reactors did not allow a secondary air line to purge with a secondary gas such as nitrogen.

A DAND pathway is also supported by thermodynamics. Calculation of Gibbs free energy change for three scenarios is shown in Table 5-3. The direct use of NH_4^+ to the three end-products of denitrification (NO, N_2O and N_2) is very favourable for NO and N_2O and unfavourable for N_2. All three of the proposed DAND reactions, result in the formation of H^+, which was not observed during the course of experiments. This, however, does not mean the DAND reactions for NO and N_2O are not occurring, it would mean that the H^+ must be coupled with another reaction. The free energy changes in Table 5-3 indicate that N_2O and NO should be the main end-products of a DAND pathway. This is useful information for future research. The direct use of NH_4^+ resulting in N_2 is not a thermodynamically favourable reaction, however, the pathway indicates the formation of hydrogen gas (H_2). H_2 can be used to generate cellular energy in a coupled reaction with carbon dioxide (CO_2) or sulfate (SO_4^{2-}) as occurs in anoxic digestion during methanogenesis. In the case of CO_2, the free energy change is -131 kJ, which is sufficient to overcome the energy requirements to produce the H_2. For SO_4^{2-} the free energy change is -155 kJ and also is sufficient, but IC analyses of SO_4^{2-} data showed no decrease in concentration during any experiments (Data not shown). Other reaction couplings could be possible that would yield higher energy.

Table 5-3 Gibbs free energy for DAND pathway scenarios at pH 7.0

Balanced Decrease-Oxidation Reaction	ΔG^o (kJ)
$NH_4^+ + \frac{5}{4}O_2 \rightarrow NO + \frac{3}{2}H_2O + H^+$	-229
$2NH_4^+ + 2O_2 \rightarrow N_2O + 3H_2O + 2H^+$	-528
$2NH_4^+ \rightarrow N_2 + 3H_2 + 2H^+$	79

Either the SND or the DAND pathway or a combination of the two pathways could be valid but, in both cases, the reaction pathways must be balanced due to the lack of effect on pH. During conventional nutrient removal, the bacteria responsible for nitrification cause the destruction of alkalinity and in denitrification the bacteria responsible produce alkalinity. The data presented show that fungi are not having an impact on pH. This may be a reflection of a SND pathway, or it could indicate an alternative DAND pathway. The data contained in this thesis warrant further exploration of fungal nitrogen biochemistry. Although fungal denitrification has been well studied in two fungi, the published literature must be considered incomplete.

5.2.2 Fungal Ammonium Concentration Decrease Kinetics and Stoichiometry

There are two methods to approach the calculation of maximum specific growth rate (μ_{max}) for a particular substrate, one based on growth of the microorganism and the second is based on the substrate utilization rate. The apparent zero-order fit of the data allows a simplification of the Monod equation (4) for determination of μ_{max}:

$$\mu = \mu_{\max} \frac{S_s}{K_s + S_s} \tag{4}$$

The data presented strongly suggest that ammonium substrate (S_s) is much greater than the half saturation coefficient (K_s). Therefore, the Monod equation simplifies to:

$$\mu \approx \mu_{\max} \tag{5}$$

Therefore the values presented in Table 4-9 (Estimated Growth Rates) were an approximation of μ_{max}. However, given complexities of fungal mycelia type growth, calculation of μ_{max} is better described by the second approach using the rate of substrate utilization (r_{su}).

The r_{su} is a function of μ_{max}, the fungal mass (X), the substrate yield factor (Y), S_s and K_s. The relationship is show in Equation (6)

$$r_{su} = -\frac{\mu_{\max} X S_s}{Y(K_s + S_s)} \tag{6}$$

Rearranging the equation to solve for μ_{max} and assuming that S_s is much greater than K_s (zero order supported data) yields Equation (7):

$$\mu_{\max} = -\frac{(Y)(r_{su})}{X} \tag{7}$$

Substitution of $Y = \frac{\Delta X}{\Delta S}$ and $r_{su} = \frac{\Delta S}{\Delta t}$ into Equation (7) yields Equation (8):

$$\mu_{\max} = \frac{\left(\frac{\Delta X}{\Delta S}\right)\left(\frac{\Delta S}{\Delta t}\right)}{X} \tag{8}$$

Equation (8) simplifies to Equation (9):

$$\mu_{\max} = \frac{1}{\Delta t} \tag{9}$$

Using Equation 9, μ_{max} was calculated for the fungi tested under microaerophilic conditions for both suspended and attached-growth cultures. The estimated μ_{max} values are shown in Table 5-4. These are the first reported values of μ_{max} for fungal NH_4^+ concentration decrease from wastewater. The half saturation coefficient, K_s, could not be directly estimated with the data collected. However, the data supports that K_s must be very small given the *Geotrichum* sp. (2000), *Penicillium* sp. and *Phoma* sp. lowered the NH_4^+ concentration to less than 0.1 mg L^{-1} without an obvious change in the zero-order substrate utilization rate.

Table 5-4 Estimated microaerophilic μ_{max} for fungal NH_4^+ decrease based on substrate utilization rate.

Species	Average μ_{max} (hr^{-1})	Standard Deviation (hr^{-1})
	Suspended-growth Culture	
Geotrichum sp. (2000)	0.0041	0.0008
Geotrichum sp. (2007)	0.0049	0.0040
Yeast	0.0032	0.0012
Penicillium sp.	0.0035	0.0007
Phoma sp.	0.0062	0.0040
	Attached-growth Culture	
Geotrichum sp. (2000)	0.0034	0.0007
Penicillium sp.	0.0051	0.0011
Phoma sp.	0.0054	0.0017

The largest two limiting factors for using fungal NH_4^+ treatment technology is the rate of degradation and the variability in a suspended-growth application. The zero-order substrate utilization rate model used best describes the data. The slow rate of fungal NH_4^+ concentration decrease could be a function of several factors. First, fungal denitrification is not a primary energy generation pathway (See Section 2.3).

This means there could be a limit to the ammonium concentration decrease rate dependent upon production of peroxynitrite or another compound. This, again, is supported by the need for some oxygen to induce a fungal denitrification pathway. Optimizing conditions that lead to the production of this compound could see further increase in fungal NH_4^+ substrate utilization rate. The second factor impacting the slow rate of fungal denitrification is that the amount of fungal biomass reported for the batch kinetic studies rarely rose above 150 mg L^{-1}. When the biomass in the fungal systems is compared to conventional biological nutrient removal systems that typically have MLSS of approximately 3000 mg L^{-1}, there is a considerable difference. Boosting fungal biomass should improve the rate of NH_4^+ decrease.

Suspended-growth culture of filamentous fungi may be limiting the biomass. Although the mixing in the reactor was very gentle, the shear force could break filaments once they extended beyond a certain length. It is possible this constant damage could impact NH_4^+ concentration decrease by first limiting the biomass and second constantly damaging the cellular structure requiring constant repair. This is partially corroborated by the improvement in process stability of at least 50% for *Penicillium* sp. and *Phoma* sp. when switched to the attached-growth system where fungi grow protected on the inside surface area of the ring medium used. For the *Penicillium* sp., attached-growth did result in an increased rate of NH_4^+ concentration decrease, however, a similar improvement did not occur for the *Phoma* sp. However, there is reason to expect further improvement in the attached-growth system comparable to the range observed for bacteria. The attached system used in this research had a very low surface area compared to commercially available medium.

For example Hydroxly Pac (Hydroxyl Systems Inc.) has a surface area to volume ratio of 402 m^2/m^3 or AGAR (USFilter) process medium has 500 m^2/m^3, therefore, in comparison to the tested medium surface area to volume ratio was 0.1 of the commercial products. Although there is no guarantee a larger surface area will increase the rate, some improvement should be seen. As mentioned before, the lack of improvement observed for the 25 and 50 m^2/m^3 tested in this research was likely due to the experimental variation overriding the small difference between the tested surface area to volume ratios.

5.2.3 Testing the Range of Fungal Denitrification

The experiments involving manipulation of pH and treatment of dilute sewage were conducted to establish an initial range of conditions for fungal denitrification that could be expected in a wastewater treatment application. The range for pH was from 6.8 to 9.0; however these were not hard boundaries. The data presented in Section 4.2.1.3 only show that the NH_4^+ concentration decrease was inhibited at pH 5.0; somewhere between pH 5 and 6.8 was the true boundary. The significance of the pH range for fungi was not so much the range but, rather, the fact that fungal growth was not inhibited. However, the pH profile for the initially pH 5.0 cultures showed a rapid rise in pH within the first 48 hr. The actual amount of time the fungi were exposed to low pH was at best 0.1 of the total culture time which raises questions about growth inhibition at lower pH. It is well documented the low pH growth preference for most fungi when competing with bacteria. Low pH is one of the primary selective factors for isolation of fungi and has been noted as the dominant factor that induces fungal activated sludge bulking (Section 2.8).

The lower pH preference of fungi could be a useful trait for treatment of wastewater with low alkalinity. Often with low alkalinity wastewater undergoing bacteriological nitrogen concentration decrease, alkalinity must be added and actively controlled to prevent acidification of the wastewater during nitrification (Metcalf & Eddy et al. 2003). A loss in pH control for conventional systems can result in complete loss of the activities of the bacterial population. Fungal tolerance of low pH is also the primary factor that could be used to establish a fungal treatment system in a competitive wastewater environment. Finally, the pH boundary condition established with this research used the suspended-growth system which does have a higher variance in the concentration decrease of NH_4^+. Although the data clearly showed that the initially low pH completely inhibited the NH_4^+ decrease, an attached-growth system may show a more robust pH range. This is a topic for further research, not only due to the limited data, but the importance in further development of the technology to pilot scale.

The dilute sewage studies and the entire batch kinetic studies give an indication that fungi are capable of NH_4^+ concentration decrease under adverse conditions. First, the primary treated effluent used in this study would be considered weak wastewater in terms of most parameters (Metcalf & Eddy et al. 2003). A further 50% dilution was a good representation of wastewater diluted via a storm event. The results showing fungi were able to achieve complete NH_4^+ decrease of weak wastewater without supplemental carbon sources demonstrates fungal potential. The dilute sewage trials further expanded the potential of fungi providing a more robust treatment technology. Although the removal of NH_4^+ was lower with dilute sewage, the data were collected using the variable suspended-growth system. Accounting for the variance it is difficult to state that the dilute sewage trials were significantly different from other suspended-growth trials. Further testing with attached-growth fungal systems and dilute sewage would continue to further elucidate the range of treatment conditions fungi can process.

5.2.4 Improving Fungal Ammonium Concentration Decrease

Two sets of experiments were designed to see how fungal NH_4^+ concentration decrease could be improved; successive inoculations and a mixed culture. The goal of the successive generation tests was to observe improvement in fungal NH_4^+ concentration decrease due to an adaptive response to the wastewater. The results in Section 4.2.1.5 failed to show a consistent data pattern over the three generations. This failure is more likely due to the experimental procedure used rather than the fungi tested. Due to a lack of resources, at the end of each reactor run the fungi were re-plated back onto PDA. Time was needed to clean and reset the reactor apparatus prior to the next run. This meant the fungi were moving from a nutrient rich environment (PDA) to the dilute environment of wastewater. This was not the ideal selective pressure to use for determining an adaptive response. Directly using the fungi at the end of one run to inoculate the next run would have been more likely to establish a pattern. Furthermore, only three successive generations was a low number to start observing adaptive changes in a microorganism without a strong stimulus. Support for using a strong stimulus for an adaptive responses can be observed in the fed-batch attached-growth data in Section 4.2.2.2. Four cycles of wastewater being added to the same reactor for *Penicillium* sp. and the *Phoma* sp. also did indicate an adaptive response. The data for the *Phoma* sp. actually showed a decline on the last cycle. This was attributed to the sensitivity of the *Phoma* sp. to a component in the wastewater. Using a strong adaptive pressure (classical strain improvement) by addition of a compound that would stimulate fungal denitrification (peroxynitrite if it can be stably produced) is an avenue for further research.

Mixed culture results in Section 4.2.1.6 showed that the combination of five microorganisms had reduced the variance of NH_4^+ concentration decrease. This result is not surprising, because the various fungi would have shared the concentration decrease of NH_4^+. If one was inhibited or performed less than optimally, it would be masked by the other four fungi. Combining mixed culture with attached-growth may further stabilize NH_4^+ decrease. Aside from the stabilization of NH_4^+ decrease variance, the data do not indicate any other advantages. However, that does not mean they do not exist. Although true synergistic or antagonistic effects are unlikely, symbiotic interaction between the fungi will occur especially as the complexity of the community grows (greater numbers of species). Fungi have been noted for increased degradation range in mixed culture (Arora et al. 1992). Further research in this area is needed to continue the documentation of how mixed cultures of fungi will reduce NH_4^+ concentrations from wastewater.

5.2.5 Potential for Development of Fungal Nitrogen Treatment Technology

This research is fundamental in nature and represents the developmental stages. The data have supported some clear benefits, single-step denitrification and dilute sewage, while highlighting further challenges; the low rate of NH_4^+ utilization. The data clearly show that attached-growth systems have a higher potential for success with fungi. The increasing prevalence of attached-growth systems used for wastewater treatment bolsters development of the process in this direction.

The most significant finding of the research is the single-step denitrification like process under microaerophilic conditions. This is clearly a simplification over current conventional design for NH_4^+ treatment. A fungal NH_4^+ treatment system supported by the data is a single reactor design with quiescent mixing and only surface re-aeration required for optimal decrease of NH_4^+. This is a major change to conventional design, which could see significant cost savings in design, construction and operation of biological nitrogen treatment processes. However, there are serious challenges that must be overcome.

The largest obstacle for moving this research out of the development stage is the rate of NH_4^+ degradation. Clearly, a 300 hrs hydraulic retention time is not possible in a large scale wastewater treatment plant. The required size of the treatment basins and land space would be too expensive. The NH_4^+ treatment time necessary must be decreased to no more than 10 hrs. This requires a much larger fungal biomass. Moving to the commercially available attached medium will increase the surface area to volume ratio by a factor of ten. The impact of more surface area is not clear at this time and requires additional investigation.

The second challenging obstacle is selection of fungi in a competitive environment. Mentioned at various points in this dissertation, low pH can be used to select fungi over bacteria. The data indicate that a low pH will inhibit fungal NH_4^+ concentration decrease. The pH range has to be further investigated to know if this is a permanent effect. Assuming it is, this means low pH can only be used as a selective pressure to establish the fungi in the reactor. Once the population has been established, the fungi will have to dominant the system and resist bacterial succession. Using the attached-growth reactor system may facilitate this by providing fungi with the ability to form a strong biofilm structure, which will keep their numbers high and allow them to use the substrate effectively. This strategy will require a specialized reactor start-up procedure and some cost of chemicals unless the influent wastewater is naturally acidic. Although the challenges to further development of fungal nitrogen treatment technology are significant, the potential benefits in simplified treatment and more robust treatment, strongly support further study.

5.3 Concentration Decrease of Phosphate From Wastewater By Fungi

5.3.1 Correlation of Initial Phosphate Concentration with Observed Decrease

The correlation of the initial o-PO_4^{3-} concentration with the amount of o-PO_4^{3-} removed can be partially supported by the literature on o-PO_4^{3-} uptake. The range of wastewater o-PO_4^{3-} concentrations studied falls within the activity range of the Pho84p transporter (Persson et al. 2003). Maximal activity should have been observed at 5 mg o-PO_4^{3-} L^{-1}. The maximum activity observed in the data set occurred aerobically at 45 mg L^{-1} (highest initial concentration resulting in the largest amount of o-PO_4^{3-} reduced from the medium). The trend that o-PO_4^{3-} was no longer decreased at 1 mg L^{-1} corresponded to the published literature value for the Pho84p transporter. The data collected indicate maximal activity of the transporter is at a much higher level than reported by the literature. This suggests the literature value is incorrect or the observed uptake of o-PO_4^{3-} was not through the Pho84p transporter. Evidence for the former is supported by the pH profiles observed in the culture which were initially pH 7 to 7.5 and would rise to pH 8 to 8.7. Pho84p is a proton symport transporter and has maximal activity at pH of approximately 5.0 (Persson et al. 2003). This would suggest Pho89p may be the transporter involved due to its maximal activity occurring at pH 9.0 and external sodium concentration of 15 to 25 mM. The literature does give the activity range for Pho89p. However, it does state Pho89p maximal activity is 100 times lower than the Pho84p transporter (Persson et al. 2003). Given the number of unknown o-PO_4^{3-} transporters, neither Pho84p nor Pho89p may be responsible for the observed data. With the test conditions being vastly different compared to biochemical preparations used in the literature, the complexity of the o-PO_4^{3-} polyphosphate system and the described transporters are from *S. cerevisiae*, it is not surprising the data generated in this research does not match with published literature. However the data generated does point to factors that influence or even regulate o-PO_4^{3-} polyphosphate system.

5.3.2 Oxygen Tension Impacts on Phosphate Concentration Decrease

The data patterns from the three oxygen tensions tested are indicative of two or three o-PO_4^{3-} uptake scenarios. Oxygen has not been described in the literature as a regulator of o-PO_4^{3-} or polyphosphate systems in fungi (Kulaev and Vagabov 1983; Kornberg et al. 1999; Persson et al. 2003). This may be due to simply having not been tested before or oxygen may not actually be a direct regulator but is influencing a physiological state which is known to impact o-PO_4^{3-} uptake. What can be stated, based on the data produced, is that aerobic uptake is controlled separately compared to microaerophilic and anoxic systems. This is based on the distinct o-PO_4^{3-}

concentration decrease curves observed for aerobic conditions and the apparent second order rate.

Data from microaerophilic tests appears to show a transition in o-PO_4^{3-} uptake. The pattern of a small initial decline followed by a plateau with hump prior to a linear decline could be the result in changes in the oxygen profile in the reactor over time. Oxygen could not be monitored in the reactors, but it would be expected that oxygen concentration would drop during the maximum growth period for the fungi due to the limited mixing driving oxygen transfer into the wastewater. If the oxygen concentration dropped low enough to induce a physiological response, this could have caused a transition in o-PO_4^{3-} uptake. The transition could also have been in response to pH changes in the culture. If the o-PO_4^{3-} transporter was Pho84p and as the pH rose the transporter was switched to Pho89p, this could have caused the transition observed in the data. However, if this were the cause of the transition it should have been observed in all the reactors due to the very similar pH profiles seen. This suggests oxygen is directly impact o-PO_4^{3-} uptake.

The anoxic condition concentration decrease data is also partially supported by the literature in the same way as the aerobic data. However, the anoxic data points to a third system of o-PO_4^{3-} uptake. Optimal activity of the o-PO_4^{3-} uptake was higher than suggested in the literature but the lower concentration limit for o-PO_4^{3-} uptake is very clearly close to 1 mg L^{-1}, regardless of the initial starting concentration. The anoxic data raise the question: what is o-PO_4^{3-} being used for in the cell? To convert o-PO_4^{3-} into polyphosphate is energy expensive for cells and in bacteria requires ATP through polyphosphate kinase. However, this enzyme has not been found in yeast or fungi and therefore a novel pathway is possible, but the energy is still likely to come from ATP. Under anoxic conditions, fungal energy storage (ATP) is generated via glycolysis. Glycolysis, however, only has a small net energy yield of 2 ATP per molecule of glucose compared to a maximum of 38 ATP per molecule of glucose under aerobic conditions (via the tricarboxylic acid cycle) (White 1995). With limited energy supplies it would seem unlikely that o-PO_4^{3-} would be transported into the cell and converted to polyphosphate. The paired comparison trial of the dilute wastewater under microaerophilic and anoxic atmosphere contributes to the speculation. The fact that under microaerophilic condition, o-PO_4^{3-} was not used compared to the anoxic system, suggests that o-PO_4^{3-} was being used for energy generation especially under the limited substrate conditions imposed by the dilute wastewater. Theoretically, it is possible for o-PO_4^{3-} to be an electron acceptor, reducing o-PO_4^{3-} to phosphine gas (PH_3). Glindemann et al. (1996) detected PH_3 in landfills, wastewater treatment plants, composting facilities and river sediments. However, the existence of a biological o-PO_4^{3-} reduction pathway has been disputed in the literature from a thermodynamic and metabolic point of view (Hanrahan et al. 2005). There is great interest in reduced phosphorus compounds in evolutionary microbiology due to their presence in prebiotic times on earth (Glindemann et al. 1999). Regardless, definitive proof of microbial action producing of PH_3 has not been observed but, based on the literature on the subject, microorganisms (in particular bacteria) are believed to be the source (Hanrahan et al. 2005). Because there are no reports in the literature of anoxic studies of fungal uptake of o-PO_4^{3-}, there can be no corroboration of the data generated. The data generated are also limited in nature and was not the primary objective of the study. The lack of information about the fate of o-PO_4^{3-} in the reactors further limits knowledge and the strength of any conclusions. What can be said is that a more through investigation of anoxic o-PO_4^{3-} uptake is needed.

5.3.3 Potential Development of Fungal Phosphate Treatment Technology

Based on the observed data and limited literature, fungal o-PO_4^{3-} concentration decrease in wastewater treatment technology will be limited to concentrations above 1 mg L^{-1} due to the affinity of the o-PO_4^{3-} transporter system. This lower boundary would likely limit the development of fungal o-PO_4^{3-} treatment technology due to current treatment standards requiring discharged wastewater from communities with populations over 20,000 to be 1 mg L^{-1} total phosphorus based on monthly averages (Alberta Environmental Protection 1997). However, the data and knowledge of o-PO_4^{3-} concentration decrease in fungi is incomplete and other transporter systems may exist that operate at lower concentrations. The interaction of cellular polyphosphates must also be accounted for in current limitations. The anoxic uptake of o-PO_4^{3-} by fungi is an interesting phenomena for two reasons; first the potential for development of a dephosphation system and, second, under anoxic conditions with dilute wastewater, o-PO_4^{3-} concentration decrease was not inhibited. Regardless of the fate of o-PO_4^{3-} in the fungi under anoxic conditions, the former observation is extremely significant when compared to current bacterial phosphate treatment systems where the needed COD to phosphorus ratio is in the order of 35 to 1. In the anoxic runs of COD to phosphorus and nitrogen concentration decrease ratio had an average of 6.5 to 1 with ammonia nitrogen concentration decrease being almost negligible. This is a vastly different requirement that would aid wastewater treatment processes.

Development of a single step anoxic o-PO_4^{3-} treatment system, even is the maximum effluent quality if only 1 mg L^{-1}, could be more cost effective than current bacterial based systems. If a fungal system was paired with chemical phosphorus system to polish final effluent, it could be more cost effective than having to provide volatile fatty acids in large quantities. A long term cost analysis would need to be performed, but if the majority of phosphorus can be removed for "free", chemically reducing the remaining amount could be cost effective.

The second issue that hampers development of fungal phosphate treatment was also noted for nitrogen-the slow rate of decrease. The rate of phosphorus decrease is very similar to the rates found for nitrogen treatment. However, accounting for the very low active mass, full assessment of the potential cannot be completed.

Chapter Six

6.0 Conclusions and Recommendations

6.1 Conclusions

The data collected in this study are the first reported attempt to use microorganisms other than bacteria to reduce the concentrations of NH_4^+ and o-PO_4^{3-} from wastewater. Using fungi isolated from a wastewater environment, experiments were conducted to determine the feasibility and identify factors which impact any observed concentration decrease in NH_4^+ and o-PO_4^{3-}. The unique nature of the data serves as the first benchmark in fungal wastewater treatment of NH_4^+ and o-PO_4^{3-}. The following is list of conclusions generated from the data and analysis.

Fungi are capable of NH_4^+ concentration decrease from wastewater resulting in the loss of nitrogen from the system. Although showing similarities with the reported fungal denitrification system, there is a significant difference: the direct use of NH_4^+. At this time, with the data collected, neither a simultaneous nitrification-denitrification system nor a direct use of NH_4^+ system can be absolutely concluded. However, the data strongly support that the NH_4^+ used was not assimilated and leaves the system as a gas. Gas chromatography for denitrification end products is required for confirmation.

Fungal NH_4^+ concentration decrease occurs best in a microaerophilic environment. Maximum NH_4^+ concentration decrease occurred in this state compared to fully aerobic and anoxic conditions. This identifies oxygen as a control parameter in technology development and also as a biochemical regulator.

The current range of pH over which fungal NH_4^+ concentration decrease was successfully observed is 6.8 to 8.9. However, the growth range of the fungi covers the entire span of the range tested (5.0 to 9.0). NH_4^+ concentration decrease of dilute wastewater will occur as shown in suspended cultures. However, the extent and stability of the NH_4^+ concentration decrease requires further study.

Suspended-growth NH_4^+ concentration decrease by fungi resulted in variable performance. The proportion attributed to changes in wastewater and natural species variation cannot be resolved from the data set. It was found that mixed culture resulted in an improvement in stability for suspended-growth conditions.

Fungal NH_4^+ treatment stability and decrease profile was improved in an attached-growth matrix. The data for the attached-growth reactors were very clear in demonstrating an improvement in NH_4^+ decrease for *Phoma* sp. and *Penicillium* sp. Surface area increased in the range tested in the attached-growth reactors did not improve NH_4^+ decrease.

The attached-growth system tested showed fungal NH_4^+ concentration decrease can be maintained in a continuous fashion. Fed-batch attached-growth experiments for *Penicillium* sp. and *Phoma* sp. were maintained over four NH_4^+ decrease cycles demonstrating continuity.

The range of fungal species that show characteristics similar to fungal denitrification has been expanded. All species tested; *Geotrichum* spp., *Penicillium* sp., *Phoma* sp., and the yeast displayed some level of activity. The *Phoma* sp. and *Penicillium* sp. showed the highest decreases and, once used in attached-growth systems, had very good stability.

Fungi are capable of o-PO_4^{3-} concentration decrease from wastewater. The decrease appears to be limited to a final o-PO_4^{3-} concentration of 1 mg L^{-1} based on the affinity of o-PO_4^{3-} transporters in fungi. o-PO_4^{3-} uptake activity found in the results is different than the values published for the two characterized o-PO_4^{3-} transporters.

Oxygen tension has a clear role in fungal o-PO_4^{3-} decrease. How oxygen interacts with o-PO_4^{3-} uptake and polyphosphate systems has not been reported before. Fungi will decrease o-PO_4^{3-} under all three oxygen tensions tested. The mechanism of uptake and final biochemical destination of o-PO_4^{3-} cannot be stated with the data collected. However, the data support different mechanisms for the decrease, especially for anoxic uptake. The range of o-PO_4^{3-} concentrations in the wastewater used in attached-growth systems prevented any conclusions regarding attached-growth o-PO_4^{3-} decrease.

In closing, the data collected clearly suggest that fungal nitrogen and phosphate treatment technology is an area for advanced research and has the potential for significant advancement in wastewater treatment nutrient removal technology.

6.2 Recommendations

A number of recommendations can be made for future research on this topic. The majority of the recommendations reflect the need to expand and further develop knowledge in this emerging research area of fungal ammonium and phosphate treatment in wastewater.

There is a serious need for confirmation of fungal ammonium nitrogen decrease mechanism from wastewater. Although total nitrogen is being reduced in suspended and attached-growth, detection of denitrification end-products would be the ultimate confirmation. In addition, off-gas analysis would provide information on the fungal denitrification pathway being used by the fungi in this research.

The direct use of NH_4^+ by fungi in this research must be investigated. The question is NH_4^+ undergoing an internal nitrification step to NO_2^- or NO_3^- prior to denitrification or is NH_4^+ acting directly in a DAND pathway, as it can in co-denitrification. Because NH_4^+ has not been noted for direct use in fungal denitrification, this is an important avenue of research.

Attached-growth systems show good potential for further development. Future work should include revisiting some of the test conditions used in suspended cultures. The pH range, dilute sewage are two factor worth investigation but also temperature. All experiments in this research were conducted at room temperature. Determination of fungal performance at temperatures between 5 and 20 °C is important for cold climate application of this research. The attached-growth systems must also be expanded to use a commercially available medium so that the surface area is comparable to what is used in industry.

Major effort needs to be expended to determine if fungal NH_4^+ concentration decrease rates can be improved to be at least equivalent to bacterial rates. Two approaches are necessary: first, increasing the active fungal mass by an order of magnitude; and, second, a more precise examination of factors that influence fungal denitrification, in particular oxygen. Detailed oxygen profiles would significantly improve knowledge on the conditions needed for maximum NH_4^+ concentration decrease.

Phosphate decrease by fungi warrants further examination on several fronts, regardless of the likely 1 mg L^{-1} lower limit, for two reasons; anoxic o-PO_4^{3-} decrease, and the low COD to P decrease ratios observed. Anoxic concentration decrease of o-PO_4^{3-} must be studied in detail to determine the fate of o-PO_4^{3-}. Although unlikely, the data generated suggest that o-PO_4^{3-} may be used for energy storage anoxically. Expanding the analysis for the various forms of o-PO_4^{3-} in the wastewater and off-gas would provide the necessary information.

The low COD to phosphate concentration decrease ratio also makes further investigation of fungal phosphate treatment important. The large difference been the ratio observed for fungi in this research compared to the ratios needed for conventional bacterial based systems could make fungal o-PO_4^{3-} treatment economically interesting for further study.

The final recommendation is that research must begin on development of a protocol for selection of fungi in a competitive wastewater environment. These studies, while investigating a protocol, must also look at short term and long term stability of the fungal populations and their ability to resist bacterial competition.

Chapter Seven

7.0 References

Aitken, M. D. (1993). Waste Treatment Applications of Enzymes - Opportunities and Obstacles. Chemical Engineering Journal and the Biochemical Engineering Journal 52(2): B49-B58.

Alam, M. Z., A. Fakhru'l-Razi and A. H. Molla (2004). Treatment and Biodegradation Kinetics of Microbially Treated Domestic Wastewater Sludge. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 39(8): 2059-2070.

Alberta Environmental Protection (1997). Standards and Guidelines for Municipal Waterworks, Wastewater and Storm Drainage Systems. Edmonton, AB, Alberta Environment Protection.

American Public Health Association, American Water Works Association and Water Environment Federation (1995). Standard Methods for the Examination of Water and Wastewater. Washington DC, USA.

Arora, D. K., R. P. Elander and K. G. Mukerji (1992). Fungal Biotechnology. Handbook of Applied Mycology. D. K. Arora. New York, Marcel Dekker Inc.: 931-961.

Beever, R. E. and Burns D. J. W. (1980). Phosphorus Uptake, Storage and Utilitization by Fungi. Advances in Botanical Research 8: 127-219.

Berks, B. C., S. J. Ferguson, J. W. B. Moir and D. J. Richardson (1995). Enzymes and Associated Electron Transport Systems That Catalyse the Respiratory Reduction of Nitrogen Oxides and Oxyanions. Biochimica et Biophysica Acta 1232: 97-173.

Brooks, J. L. (1988). The Role of Fungi in the Sphagnum Peat Wastewater Treatment System. PhD Dissertation. University of Maine. Orono.

Cooke, W. B. (1976). Fungi in Sewage. Recent Advances in Aquatic Mycology. E. B. G. Jones. London, Elek Science.

Cooke, W. B. (1986). The Fungi of Our Mouldy Earth. Berlin, J. Cramer.

Downing, A. L., T. G. Tomlinson and G. A. Truesdale (1964). Effect of Inhibitors on Nitrification in the Activated-Sludge Process. Journal of the Institute of Sewage Purification: 537-554.

Eylar, O. R. J. and E. L. Schmidt (1959). A Survey of Heterotrophic Microorganisms from Soil for Ability to Form Nitrite and Nitrate. Journal of General Microbiology 20: 473-481.

Fakhru'l-Razi, A., M. Z. Alam, A. Idris, S. Abd-Aziz and A. H. Molla (2002). Filamentous Fungi in Indah Water Konsortium (Iwk) Sewage Treatment Plant for Biological Treatment of Domestic Wastewater Sludge. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering A37(3): 309-320.

Falih, A. M. K. and M. Wainwright (1995). Nitrification in Vitro by a Range of Filamentous Fungi and Yeasts. Letters in Applied Microbiology 21(1): 18-19.

Ferguson, S. J. (1994). Denitrification and Its Control. Antonie van Leeuwenhoek 66: 89-110.

Glindemann, D., A. Bergmann, U. Stottmeister and G. Gassmann (1996). Phosphine in the Lower Terrestrial Troposphere. Naturwissenschaften 83(3): 131-133.

Glindemann, D., R. M. de Graaf and A. W. Schwartz (1999). Chemical Reduction of Phosphate on the Primitive Earth. Origins of Life and Evolution of the Biosphere 29(6): 555-561.

Grady, C. P. L. J., G. T. Daigger and H. C. Lim (1999). Biological Wastewater Treatment. New York, Marcel Dekker Inc.

Guest, R. K. and D. W. Smith (2002). A Potential New Role for Fungi in a Wastewater Mbr Bilogical Nitrogen Reduction System. Journal of Environmental Engineering and Science 1(6): 433-437.

Hanrahan, G., T. M. Salmassi, C. S. Khachikian and K. L. Foster (2005). Reduced Inorganic Phosphorus in the Natural Environment: Significance, Speciation and Determination. Talanta 66(2): 435-444.

Harold, F. M. (1966). Inorganic Polyphosphates in Biology: Structure, Metabolism, and Function. Bacteriological Reviews 30(4): 772-794.

Hiremath, A. B., P. M. Nimbargi and Y. M. Jayaray (1985). Domestic Sewage Treatment by Fungi and Biomass Production. Environment and Ecology 3: 568-571.

Hirsch, P., L. Overrein and M. Alexander (1961). Formation of Nitrite and Nitrate by Actinomycetes and Fungi. Journal of Bacteriology 82: 442-448.

Ikehata, K., I. D. Buchanan and D. W. Smith (2004). Recent Developments in the Production of Extracellular Fungal Peroxidases and Laccases for Waste Treatment. Journal of Environmental Engineering and Science 3(1): 1-19.

Kobayashi, M., Y. Matsuo, A. Takimoto, S. Suzuki, F. Maruo and H. Shoun (1996). Denitrification, a Novel Type of Respiratory Metabolism in Fungal Mitochondrion. Journal of Biological Chemistry 271(27): 16263-16267.

Kornberg, A. and C. D. Fraley (2000). Inorganic Polyphosphate: A Molecular Fossil Come to Life. ASM News 66(5): 275-280.

Kornberg, A., N. N. Rao and D. Ault-Riche (1999). Inorganic Polyphosphate: A Molecule of Many Functions. Annual Review of Biochemistry 68: 89-125.

Kulaev, I. S. and V. M. Vagabov (1983). Polyphosphate Metabolism in Microorganisms. Advances in Microbial Physiology 24: 83-171.

Kulaev, I. S., V. M. Vagabov, T. V. Kulakovskaya, L. P. Lichko, N. A. Andreeva and L. V. Trilisenko (1999). The Development of A. N. Belozersky's Ideas in Polyphosphate Biochemistry. Biochemistry (Moscow) 65(3): 271-278.

Kurakov, A. V., A. N. Nosikov, E. V. Skrynnikova and L. v. NP (2000). Nitrate Reductase and Nitrous Oxide Production by *Fusarium oxysporum* 11dn1 under Aerobic and Anaerobic Conditions. Current Microbiology 41(2): 114-119.

Kurakov, A. V. and A. I. Popov (1996). Nitrifying Activity and Phytotoxicity of Microscopic Soil Fungi. Eurasian Soil Science 28: 73-84.

Malinowsky, P. and J. C. G. Ottow (1984). Conditions of the N_2O Release (Denitrification) by Fungi in Batch Cultures. Third European Congress on

Biotechnology, Munich, West Germany, European Federation of Biotechnology.

Martins, A. M. P., K. Pagilla, J. J. Heijnen and M. C. M. van Loosdrecht (2004). Filamentous Bulking Sludge - a Critical Review. Water Research 38(4): 793-817.

Mehl, M., A. Daiber, S. Herold, H. Shoun and V. Ullrich (1999). Peroxynitrite Reaction with Heme Proteins. Nitric Oxide-Biology and Chemistry 3(2): 142-152.

Metcalf & Eddy, I., G. Tchobanoglous and F. Burton (2003). Wastewater Engineering, Treatment and Reuse. Boston, McGraw-Hill.

Molla, A. H., A. Fakhru'l-Razi, S. Abd-Aziz, M. M. Hanafi, P. K. Roychoudhury and M. Z. Alam (2002a). A Potential Resource for Bioconversion of Domestic Wastewater Sludge. Bioresource Technology 85(3): 263-272.

Molla, A. H., A. Fakhru'l-Razi, M. M. Hanafi, S. Abd-Aziz and M. Z. Alam (2002b). Potential Non-Phytopathogenic Filamentous Fungi for Bioconversion of Domestic Wastewater Sludge. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 37(8): 1495-1507.

Mueller, G. M., G. F. Bills and M. S. Foster (2004). Biodiversity of Fungi: Inventory and Monitoring Methods, Elsevier Academic Press.

Nicell, J. A. (2001). Environmental Applications of Enzymes. Interdisciplinary Environmental Review 3(1): 14-41.

Oshima, R., S. Fushinobu, F. Su, L. Zhang, N. Takaya and H. Shoun (2004). Structural Evidence for Direct Hydride Transfer from Nadh to Cytochrome P450nor. Journal of Molecular Biology 342(1): 207-217.

Persson, B. L., J. O. Lagerstedt, J. R. Pratt, J. Pattison-Granberg, K. Lundh, S. Shokrollahzadeh and F. Lundh (2003). Regulation of Phosphate Acquisition in *Saccharomyces cerevisiae*. Current Genetics 43(4): 225-244.

Rittmann, B. E. (1984). Achieving Biologically Stable Drinking Water. Journal American Water Works Association 76(10): 106-114.

Shimizu, H., E. Obayashi, Y. Gomi, H. Arakawa, S. Y. Park, H. Nakamura, S. Adachi, H. Shoun and Y. Shiro (2000). Proton Delivery in No Reduction by Fungal Nitric-Oxide Reductase - Cryogenic Crystallography, Spectroscopy, and Kinetics of Ferric-No Complexes of Wild-Type and Mutant Enzymes. Journal of Biological Chemistry 275(7): 4816-4826.

Shoun, H. (1992). Fungal Denitrification and Cytochrome-P-450. Nippon Nogeikagaku Kaishi-Journal of the Japan Society for Bioscience Biotechnology and Agrochemistry 66(2): 154-157.

Shoun, H., M. Kano, I. Baba, N. Takaya and M. Matsuo (1998). Denitrificaiton by Actinomycetes and Purification of Dissimilatory Nitrite Reductase and Azurin from Streptomyces Thioluteus. Journal of Bacteriology 180(17): 4413-4415.

Shoun, H., D. H. Kim, H. Uchiyama and J. Sugiyama (1992). Denitrification by Fungi. FEMS Microbiology Letters 94(3): 277-281.

Shoun, H., Y. Sudo, K. I. Sato and T. Beppu (1989). Components of the Cytochrome P-450 Monooxygenase System of the Fungus, *Fusarium oxysporum*:

Conditions for Induction and Isolation of Cytochrome B-5. Agricultural and Biological Chemistry 53(8): 2153-2162.

Shoun, H. and T. Tanimoto (1991). Denitrification by the Fungus *Fusarium oxysporum* and Involvement of Cytochrome P-450 in the Respiratory Nitrite Reduction. Journal of Biological Chemistry 266(17): 11078-11082.

Stanbury, P. F., A. Whitaker and S. J. Hall (1995). Principles of Fermentation Technology. Oxford, Pergamon Press.

Su, F., N. Takaya and H. Shoun (2004). Nitrous Oxide-Forming Codenitrification Catalyzed by Cytochrome P450nor. Bioscience Biotechnology and Biochemistry 68(2): 473-475.

Takaya, N., S. Kuwazaki, Y. Adachi, S. Suzuki, T. Kikuchi, H. Nakamura, Y. Shiro and H. Shoun (2003). Hybrid Respiration in the Denitrifying Mitochondria of *Fusarium oxysporum*. Journal of Biochemistry 133(4): 461-465.

Takaya, N. and H. Shoun (2000). Nitric Oxide Reduction, the Last Step in Denitrification by *Fusarium oxysporum*, Is Obligatorily Mediated by Cytochrome P450nor. Molecular and General Genetics 263(2): 342-348.

Takaya, N., S. Suzuki, S. Kuwazaki, H. Shoun, F. Maruo, M. Yamaguchi and K. Takeo (1999). Cytochrome P450nor, a Novel Class of Mitochondrial Cytochrome P450 Involved in Nitrate Respiration in the Fungus *Fusarium oxysporum*. Archives of Biochemistry and Biophysics 372(2): 340-346.

Takaya, N., H. Uchimura, Y. P. Lai and H. Shoun (2002). Transcriptional Control of Nitric Oxide Reductase Gene (Cyp55) in the Fungal Denitrifier *Fusarium oxysporum*. Bioscience Biotechnology and Biochemistry 66(5): 1039-1045.

Tanimoto, T., K. I. Hatano, D. H. Kim, H. Uchiyama and H. Shoun (1992). Co-Denitrification by the Denitrifying System of the Fungus *Fusarium oxysporum*. FEMS Microbiology Letters 93(2): 177-180.

Thanh, N. C. and R. E. Simard (1973). Biological Treatment of Wastewater by Yeasts. Journal of the Water Pollution Control Federation 45: 675-680.

Tomlinson, T. G., A. G. Boon and G. N. A. Trotman (1966). Inhibition of Nitrification in the Activated Sludge Process of Sewage Disposal. Journal of Applied Bacteriology 29(2): 266-291.

Tu, C. M. (1992). Effect of Some Herbicides on Activities of Microorganisms and Enzymes in Soil. Journal of Environmental Science and Health Part B Pesticides Food Contaminants and Agricultural Wastes 27(6): 695-709.

Umemura, M., F. Su, N. Takaya, Y. Shiro and H. Shoun (2004). D88a Mutant of Cytochrome P450nor Provides Kinetic Evidence for Direct Complex Formation with Electron Donor Nadh. European Journal of Biochemistry 271(14): 2887-2894.

Usuda, K., N. Toritsuka, Y. Matsuo, D. H. Kim and H. Shoun (1995). Denitrification by the Fungus *Cylindrocarpon tonkinense*: Anaerobic Cell Growth and Two Isozyme Forms of Cytochrome P-450nor. Applied and Environmental Microbiology 61(3): 883-889.

Wanner, J. and P. Grau (1988). Filamentous Bulking in Nutrient Removal Activated-Sludge Systems. Water Science and Technology 20(4-5): 1-8.

White, D. (1995). The Physiology and Biochemistry of Prokaryotes. Oxford, Oxford University Press Inc.

Wood, H. G. and J. E. Clark (1988). Biological Aspects of Inorganic Polyphosphates. Annual Reviews 57: 235-260.

Zhou, Z. M., N. Takaya, M. A. C. Sakairi and H. Shoun (2001). Oxygen Requirement for Denitrification by the Fungus *Fusarium oxysporum*. Archives of Microbiology 175(1): 19-25.

Zou, M. H., A. Daiber, J. A. Peterson, H. Shoun and V. Ullrich (2000). Rapid Reactions of Peroxynitrite with Heme-Thiolate Proteins as the Basis for Protection of Prostacyclin Synthase from Inactivation by Nitration. Archives of Biochemistry and Biophysics 376(1): 149-155.

Appendix A
Summary of Data

Table 7-1 Summary of suspended-growth data for *Geotrichum* sp. (2000)

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg $L^{-1}hr^{-1}$	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Aerobic	1	-0.019	35	27	23	36	17	53	250	89	65	6	19
Aerobic	2	-0.019	35	28	20	45	17	63	277	92	67	5	26
Aerobic	3	-0.001	30	28	7	24	21	10	254	130	49	29	62
Microaerophilic	4	-0.114	28	3	90	23	7	68	256	126	51	3	5
Microaerophilic	5	-0.035	25	12	53	18	10	45	292	215	26	4	6
Microaerophilic	6	-0.034	20	9	57	16	11	31	222	182	18	2	3
Microaerophilic	7	-0.061	27	7	73	15	9	37	296	179	40	5	6
Microaerophilic	8	-0.009	19	15	19	1	3	0	166	92	45	42	21
Anoxic	9	-0.034	26	17	33	15	4	77	286	178	38	5	13
Anoxic	10	-0.010	15	12	23	1	2	0	183	165	10	7	5
Initial pH 5.5	11	-0.005	25	23	11	18	18	0	295	206	30	42	33
Initial pH 8.0	12	-0.028	20	9	56	14	10	30	218	190	13	2	2
Dilute WW Microaerophilic	13	-0.006	12	10	13	7	7	1	109	63	42	28	29
Dilute WW Anoxic	14	-0.008	12	10	18	7	2	73	111	63	43	6	21
Dilute WW Microaerophilic	15	-0.015	14	8	43	10	11	0	202	155	23	12	12
Inoculation 1	16	-0.035	25	12	53	18	10	45	292	215	26	4	6
Inoculation 2	17	-0.035	18	7	59	18	12	35	240	162	33	5	7
Inocualtion 3	18	-0.058	28	8	72	16	9	42	302	246	19	2	3

Table 7-2 Summary of suspended-growth data for *Geotrichum* sp. (2007)

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg L^{-1}hr^{-1}	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Aerobic	19	-0.038	37	26	29	38	14	63	239	74	69	5	16
Aerobic	20	-0.018	30	22	27	38	18	52	258	78	70	7	23
Microaerophilic	21	-0.217	35	10	71	17	8	50	243	84	65	5	6
Microaerophilic	22	-0.079	28	6	78	22	10	54	263	132	50	4	6
Microaerophilic	23	-0.033	25	10	61	16	9	43	298	208	30	4	6
Microaerophilic	24	-0.023	20	10	47	14	11	23	231	191	17	3	4
Microaerophilic	25	-0.052	25	10	62	14	11	26	289	192	34	5	6
Microaerophilic	26	-0.008	18	15	19	1	3	0	165	104	37	40	18
Anoxic	27	-0.023	26	17	35	14	3	79	303	236	22	3	7
Anoxic	28	-0.011	18	14	22	1	2	-37	185	165	11	6	5
Initial pH 5.5	29	-0.002	25	24	6	17	14	19	305	231	24	16	53
Initial pH 8.0	30	-0.024	20	10	48	14	10	30	230	193	16	3	4
Dilute WW Microaerophilic	31	-0.007	12	11	8	6	6	-2	108	65	40	51	46
Dilute WW Anoxic	32	-0.003	12	11	8	6	2	62	105	65	38	9	44
Dilute WW Microaerophilic	33	-0.018	13	8	42	9	8	14	205	149	27	10	13
Inoculation 1	34	-0.035	25	12	53	16	9	43	298	208	30	4	6
Inoculation 2	35	-0.035	18	7	59	18	11	36	265	225	15	2	4
Inocualtion 3	36	-0.058	28	8	72	16	11	32	311	214	31	5	6

Table 7-3 Summary of suspended-growth data for yeast

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg L^{-1}hr^{-1}	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Aerobic	37	-0.060	36	24	34	45	15	66	240	130	46	3	9
Aerobic	38	-0.042	30	15	50	40	20	51	266	112	58	4	10
Aerobic	39	-0.033	36	29	22	23	9	59	243	122	50	6	15
Microaerophilic	40	-0.020	29	22	23	23	13	45	261	139	47	7	18
Microaerophilic	41	-0.067	25	8	68	17	9	48	301	227	25	3	4
Microaerophilic	42	-0.027	20	10	52	15	11	30	239	197	18	3	4
Microaerophilic	43	-0.042	26	7	73	15	13	11	302	262	13	2	2
Microaerophilic	44	-0.010	18	14	22	1	3	-134	166	101	39	31	16
Anoxic	45	-0.022	26	18	32	15	3	80	290	245	16	2	5
Anoxic	46	-0.011	18	14	22	1	2	-25	196	166	15	8	8
Initial pH 5.5	47	-0.001	26	25	2	18	17	3	304	249	18	48	92
Initial pH 8.0	48	-0.018	20	13	35	15	10	30	225	175	22	4	7
Dilute WW Microaerophilic	49	-0.007	12	10	17	7	7	-6	111	69	37	26	21
Dilute WW Anoxic	50	-0.003	12	11	7	7	2	66	106	71	33	7	44
Dilute WW Microaerophilic	51	-0.022	14	8	44	10	11	0	212	134	37	15	13
Inoculation 1	52	-0.050	25	8	68	17	9	48	301	227	25	3	4
Inoculation 2	53	-0.029	20	9	57	19	11	42	259	230	11	2	3
Inocualtion 3	54	-0.025	27	16	41	16	11	27	306	235	23	5	6

Table 7-4 Summary of suspended-growth data for *Penicillium* sp.

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg $L^{-1}hr^{-1}$	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Aerobic	55	-0.076	37	23	38	41	17	59	253	89	65	4	11
Aerobic	56	-0.031	31	19	39	23	9	59	258	78	70	7	15
Microaerophilic	57	-0.128	37	25	34	41	19	54	250	161	35	3	7
Microaerophilic	58	-0.105	30	1	97	23	13	45	265	140	47	3	4
Microaerophilic	59	-0.061	26	1	95	18	11	40	293	188	36	3	4
Microaerophilic	60	-0.034	21	9	58	15	10	33	222	165	26	3	5
Microaerophilic	61	-0.061	27	7	73	15	14	4	286	144	50	7	7
Microaerophilic	62	-0.010	19	15	20	1	3	-127	176	134	24	19	11
Anoxic	63	-0.020	27	19	28	15	3	77	295	139	53	8	21
Anoxic	64	-0.010	19	15	20	1	2	-31	198	183	8	4	4
Initial pH 5.5	65	-0.002	26	26	-2	18	21	-15	307	158	49	NA	NA
Initial pH 8.0	66	-0.027	21	10	52	14	10	31	222	137	38	5.6	7.9
Dilute WW Microaerophilic	67	-0.008	13	11	16	7	8	-1	124	80	36	22	21
Dilute WW Anoxic	68	-0.003	13	12	5	7	2	67	115	78	32	7	58
Dilute WW Microaerophilic	69	-0.021	14	9	39	10	11	0	203	130	36	17	13
Inoculation 1	70	-0.061	26	1	95	18	11	40	293	182	38	4	5
Inoculation 2	71	-0.028	20	10	49	17	13	25	219	125	43	7	10
Inocualtion 3	72	-0.048	27	11	61	16	11	33	292	159	46	6	8

Table 7-5 Summary of suspended-growth data for *Phoma* sp.

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg $L^{-1}hr^{-1}$	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Aerobic	73	-0.057	37	26	29	41	19	53	246	81	67	5	15
Aerobic	74	-0.040	30	15	50	23	9	61	258	78	70	6	12
Microaerophilic	75	-0.163	38	19	48	41	19	53	249	87	65	4	9
Microaerophilic	76	-0.112	30	2	92	23	5	77	258	117	55	3	5
Microaerophilic	77	-0.054	26	3	87	18	7	61	289	119	59	5	8
Microaerophilic	78	-0.029	21	11	48	16	11	28	225	157	30	5	7
Microaerophilic	79	-0.043	27	13	54	15	14	5	287	153	47	9	9
Anoxic	80	-0.021	27	19	30	7	3	66	284	153	46	10	16
Anoxic	81	-0.011	19	15	21	15	3	79	198	183	8	1	4
Initial pH 5.5	82	-0.003	25	26	-2	18	22	-19	298	143	52	NA	NA
Initial pH 8.0	83	-0.029	21	8	59	14	10	30	227	168	26	4	5
Dilute WW Microaerophilic	84	-0.008	13	10	17	7	7	1	127	81	36	20	21
Dilute WW Anoxic	85	-0.002	13	12	4	7	3	66	119	99	17	4	40
Dilute WW Microaerophilic	86	-0.017	14	9	34	10	10	0	202	101	50	22	21
Inoculation 1	87	-0.054	26	3	87	18	7	61	289	119	59	5.1	7.5
Inoculation 2	88	-0.026	20	10	52	17	10	39	215	86	60	7.6	12.4
Inocualtion 3	89	-0.064	27	8	69	16	11	34	314	137	56	7.3	9.4

Table 7-6 Summary of suspended-growth data for mixed culture experiments

		Ammonium Reduction				Phosphate Reduction			COD Reduction				
Condition	Run	r_{su} mg $L^{-1}hr^{-1}$	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	COD:N+P Ratio	COD:N Ratio
Microaerophilic	90	-0.032	26	12	54	18	9	49	296	140	53	7	11
Microaerophilic	91	-0.030	26	13	52	18	10	45	298	140	53	7	12
Microaerophilic	92	-0.032	26	12	55	17	9	47	296	134	55	7	11
Microaerophilic	93	-0.041	26	8	68	18	8	54	295	145	51	5	8
Microaerophilic	94	-0.029	26	13	50	18	10	44	302	137	55	8	12
Microaerophilic	95	-0.059	28	8	72	15	10	28	295	141	52	6	8

Table 7-7 Summary of attached-growth ammonium and phosphate concentration decrease.

				Ammonium Reduction				Phosphate Reduction		
Species	Oxygen Tension	SA: Volume ratio $m^2 m^{-3}$	Run	r_{su} mg L^{-1} hr^{-1}	Initial mg L^{-1}	Final mg L^{-1}	Reduction %	Initial mg L^{-1}	Final mg L^{-1}	Reduction %
2000	Microaerophilic	25	1	-0.022	21	11	46	15	14	5
	Microaerophilic	25	2	-0.023	17	10	43	1	7	-417
	Anoxic	25	3	-0.018	18	11	37	1	2	-72
	Microaerophilic	50	4	-0.021	12	6	47	2	6	-309
	Microaerophilic	50	5	-0.060	13	0	100	1	4	-192
3004	Microaerophilic	25	6	-0.075	21	0	100	15	5	64
	Microaerophilic	25	7	-0.058	17	0	99	1	4	-218
	Microaerophilic	50	8	-0.078	12	0	100	1	6	-435
	Microaerophilic	50	9	-0.072	13	0	100	1	4	-184
	Microaerophilic	50	10	-0.063	12	5	60	NA	NA	NA
	Microaerophilic	50	11	-0.110	26	2	93	NA	NA	NA
	Anoxic	25	12	-0.014	17	12	31	1	2	-53
3013	Microaerophilic	25	13	-0.059	21	0	100	14	9	40
	Microaerophilic	25	14	-0.083	17	0	100	1	2	-47
	Microaerophilic	50	15	-0.086	12	3	77	1	2	-35
	Microaerophilic	50	16	-0.055	13	0	100	NA	NA	NA
	Microaerophilic	50	17	-0.069	23	7	72	NA	NA	NA
	Microaerophilic	50	18	-0.079	13	0	100	1	6	-314
	Microaerophilic	50	19	-0.054	13	4	70	NA	NA	NA
	Microaerophilic	50	20	-0.025	25	17	33	NA	NA	NA
	Anoxic	25	21	-0.044	17	7	60	1	2	-63
2008	L-Aerobic	25	22	-0.026	21	10	52	14	9	36
2007	L-Aerobic	25	23	-0.023	20	11	47	13	8	38