Phylogenetic characterization of the nitrifying populations in municipal wastewaters and in biological treatment systems to improve modeling practices

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

AMO	Ammonia monooxygenase
ANO	Autotrophic Nitrifying Organisms
AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
ASMs	Activated Sludge Models
BABE	BioAugmentation Batch Enhanced
BAR	Bioaugmentation Regeneration
BNR	Biological Nitrogen Removal
BOD	Biochemical Oxygen Demand
CAS	Conventional Activated Sludge
COD	Chemical Oxygen Demand
COD_{Inf}	Influent Chemical Oxygen Demand
CS	Combined Sewer
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
EP	Endogenous Phase
GMP	Good Modeling Practices
H_0	Null hypothesis
H_1	Alternative hypothesis

HAO	Hydroxylamine oxidoreductase
HNO	Nitroxyl hydride
HRT	Hydraulic Retention Time
IFAS	Integrated Fixed Film Activated Sludge
INF	Influent
InNITRI	Inexpensive Nitrification
IWA	International Water Association
MANOVA	Multivariate Analysis of Variance
MAR	Major Axis Regression
MID	Multiplex identifier
ML	Mixed liquor
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
NC	Negative Control
NH_2OH	Hydroxylamine
NOB	Nitrite Oxidizing Bacteria
$N_{shared\ reads,inf}$	Number of reads of shared OTUs from influent
$N_{shared\ reads,ml}$	Number of reads of shared OTUs from mixed liquor
$N_{T,\ ml}$	Total number of reads from mixed liquor
$N_{T,inf}$	Total number of reads from influent
OTU	Operational Taxonomic Unit

OUR	Oxygen Uptake Rate
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PCS	Partially combined sewer
RAS	Return Activated Sludge
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
$R_{i,inf}$	Rank of i^{th} OTU in influent
$R_{i,ml}$	Rank of i^{th} OTU in mixed liquor
SBRs	Sequencing Batch Reactors
sCOD	Soluble Chemical Oxygen Demand
SF	Safety Factor
SR	Shared Reads
SRT	Solids Retention Time
$[SRT_{min}]_{\lim}$	Absolute Minimum Solids Retention Time
$SRT_{operation}$	Operational Solids Retention Time
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
TR	Test Reactor
VSS	Volatile Suspended Solids

WRRF	Water Resource Recovery Facility
WWTP	Wastewater Treatment Plant
W_i	Weight component of OTU

Symbols	**Modeling parameters**	**Units**
fp	Fraction of biomass leading to particulate product	g-COD$_p$/g-tCOD
f_{SB}	Fraction of soluble biodegradable organics	g-COD$_{SB}$/g-tCOD
f_{XU}	Fraction of particulate undegradable organics	g-COD$_{XU}$/g-tCOD
f_{SU}	Fraction of soluble undegradable organics	g-COD$_{SU}$/g-tCOD
f_{XCB}	Fraction of slowly biodegradable substrates	g-COD$_{XCB}$/ g-tCOD
f_{XOHO}	Fraction of ordinary heterotrophic biomass	g-COD_X$_{Bio}$/g-tCOD
f_{XANO}	Fraction of nitrifying biomass	g-COD_X$_{Bio}$/g-tCOD
X_{ANO}	Autotrophic nitrifying organisms	g-X_{ANO}/m^3
$X_{ANO,Inf}$	Influent nitrifier seed level	g-COD$_{XANO,Inf}$/m^3
$X_{ANO,ML}$	Autotrophic nitrifying biomass in Reactor	g-COD$_{XANO,ML}$/m^3
X_U	Particulate undegradable	g-COD$_{XU}$/m^3
XC$_B$	Slowly biodegradable substrates	g-COD$_{XCB}$/m^3
X_{ND}	Slowly biodegradable organic nitrogen	g-COD$_{XND}$/m^3
SRT$_{min}$	Minimum solids retention time	days
TKN_{Inf}	Influent Total Kjeldahl Nitrogen	mg-N/L
$S_{s,min}$	Minimum substrate concentration attainable	mg-COD/L
S_{O2}	Dissolved oxygen	mg-O_2/L
S_{NOx}	Effluent nitrate plus nitrite	mg-N/L
S_{NHx}	Effluent ammonium	mg-N/L
$S_{NHx,\ Inf}$	Influent ammonium	mg-N/L
$S_{NHx,\ consumed\ by\ OHO}$	Ammonium consumed by heterotrophic biomass	mg-N/L
$S_{NHx,Inf,nitrifiable}$	Nitrifiable influent ammonium concentration	mg-N/L

S_{Alk}	Alkalinity	mg-$CaCO_3$/L
i_{N_XBio}	N- content of active biomass	g-N/g-COD
i_{P_XBio}	P-content of active biomass	g-P/g-COD
Y_{OHO}	Yield of ordinary heterotrophic biomass	g-COD/g-COD
Y_{ANO}	Yield of nitrifying biomass	g-COD/g-N
$\mu_{OHO,max}$	Heterotrophic max. specific growth rate	d^{-1}
$\mu_{ANO,max}$	Nitrifiers' max. specific growth rate	d^{-1}
$\mu_{ANO,max,T}$	Maximum growth rate at temperature T	d^{-1}
$\mu_{ANO,max,20°C}$	Maximum growth rate at 20 °C	d^{-1}
$K_{SB,OHO}$	Half-saturation constant for S_B	mg-COD/L
$K_{O2,OHO}$	Half-saturation constant for S_{O2}	mg-O_2/L
$K_{O2,ANO}$	Half-saturation constant for S_{O2}	mg-O_2/L
$K_{NHx,OHO}$	Half-saturation constant for S_{NHx}	mg-N/L
$K_{NHx,ANO}$	Half-saturation constant for S_{NHx}	mg-N/L
$K_{NOx,OHO}$	Half-saturation constant for S_{NOx}	mg-N/L
$\eta\mu_{OHO,Ax}$	Reduction factor for anoxic growth of X_{OHO}	--
b_{OHO}	Heterotrophic decay rate	d^{-1}
b_{ANO}	Autotrophic decay rate	d^{-1}
$b_{ANO,T}$	Decay rate at temperature T	d^{-1}
$b_{ANO,20°C}$	Decay rate at 20 °C	d^{-1}
c	Constant (ratio of ammonium concentration and the half salutation constant for ammonium)	--
q_{am}	Ammonification rate	g-COD/m^3/d
$q_{XCB\_XB,hyd}$	Max. specific hydrolysis rate	g-X_{CB}/g-X_{OHO}/d
q_{add}	Specific addition rate of nitrifiers	g-COD$_{XANO}$/m^3/d
θ	Temperature coefficient	--
$\theta\mu_{OHO,Max}$	Temperature coefficient for $\mu_{XOHO,Max}$	--
$\theta\mu_{ANO,Max}$	Temperature coefficient for $\mu_{XANO,Max}$	--

θ_{bOHO}	Temperature coefficient for b_{OHO}	--
θ_{bANO}	Temperature coefficient for b_{ANO}	--
$\theta_{KSB,OHO}$	Temperature coefficient for $K_{SB,OHO}$	--
$\theta_{KNHx,ANO}$	Temperature coefficient for $K_{NHx,ANO}$	--
Q_{Inf}	Flow of influent wastewater	m^3/d
COD_{Inf}	Total COD in the influent	mg-COD/L
OUR_{max}	Maximum oxygen uptake rate	mg-O_2/L
$OUR_{max,T}$	Maximum oxygen uptake rate at temperature T	mg-O_2/L
$OUR_{max,20^oC}$	Maximum oxygen uptake rate at 20 ºC	mg-O_2/L
$X_{ANO,Inf}$	Influent nitrifier seed level	mg-COD/L
$X_{ANO,MLfromSnhx,Inf}$	Autotrophic nitrifying biomass in the reactor	mg-COD/L
θ_h	Hydraulic retention time	days
θ_x	Solids retention time	days
$\theta_{x,design}$	Design solids retention time	days
$\theta_{x,min}$	Minimum solids retention time	days
$[\theta_{x,min}]_{lim}$	Absolute minimum solids retention times	days
$\theta_{x\ min,\text{no seeding}}$	Minimum solids retention time without seeding	days
$\theta_{x\ min,\text{with seeding}}$	Minimum solids retention time with seeding	days
$[\theta_{x,min,\text{with seeding}}]_{lim}$	Absolute minimum solids retention time with seeding	days
V_{aer}	Volume of the aeration basin	m^3

ABSTRACT

Nitrification is a very important process in wastewater treatment systems performing biological nitrogen removal. The size, footprint and energy consumption of nitrifying activated sludge systems are governed by the requirement of the system to remove ammonia. Ammonia is an important wastewater quality parameter to consider because of its toxicity to aquatic life in receiving water bodies; hence environmental regulations regarding discharge requirements for ammonia are becoming more stringent. Research in this field is critical in order to better understand the treatment process so as to fine-tune the existing suite of treatment technologies and to develop novel engineering solutions to mitigate levels of ammonia in wastewater discharges. Although the complexity of nitrification in activated sludge systems is still being demystified, the possible seeding of biological reactors by raw sewage which contains nitrifying bacteria, has been overlooked so far. Even current best modeling practices such as the International Water Association (IWA) Good Modeling Practices (GMP) for biological wastewater treatment, assume that there is no biomass in raw municipal wastewaters. Process-engineered bioaugmentation of activated sludge by addition of indigenous or allochthonous consortia of nitrifiers has proved to be a powerful strategy to enhance biological nitrification in stressful conditions. This study explores the potential of a natural seeding of nitrifiers at a full-scale wastewater treatment level.

Through the application of high-throughput DNA sequencing, we have shown that raw sewage was indeed supplementing full-scale bioreactors with active nitrifiers. The phylogenetic profiles of the nitrifiers identified in raw wastewaters were strikingly similar to the nitrifying bacterial populations in the bioreactors. Respirometric assays showed that nitrifying biomass in the studied influents was alive and active, and was capable of reaching full metabolic induction within a few hours. We showed that this natural seeding phenomenon results from a stochastic process of immigration of bacterial species. Based on these findings, we expanded the metacommunity concept of species to include immigrant communities (raw sewage, in this case) linked to local communities (bioreactors). In order to validate the concept of influent nitrifier-seeding observed in full-scale Water Resource Recovery Facilities (WRRFs), we replicated this phenomenon in the laboratory by adding influent biomass harvested from a full-scale wastewater treatment facility to laboratory-scale sequencing batch reactors (SBRs) operated at washout conditions (low temperature and solids retention time). Addition of influent solids restored nitrification and stabilized the SBR systems. Lastly, we examined the impact of such natural influent nitrifier

seeding on the performance of activated sludge models. Activated sludge models are essential tools in process design, operation and optimization of biological wastewater treatment systems. However, they do not consider active biomass in influents because there is little knowledge regarding its impact on models and no standardized protocols to detect and quantify its level. Incorporating the natural seeding of nitrifiers, at a level pre-determined by respirometric assays, in the nitrification model, significantly enhanced the performance of the model and its predictive capacity. However, if natural seeding of nitrifiers is not considered, it is still possible to obtain reasonable fits between measured and predicted datasets by arbitrarily adjusting the maximum growth rate of nitrifiers and its temperature dependency coefficient. Our findings, thus, point to a potential calibration bias between approaches involving natural seeding of nitrifiers as determined by respirometric assays, or adjusting the maximum growth rate of nitrifiers and its temperature coefficient to fit measured and predicted data. This may explain the variability observed in maximum growth rates when calibrating systems which receive wastewaters from different sources - such as municipal vs. industrial sources - or from wastewater collection systems with smaller sewer networks. Nonetheless, for all the simulations performed, only the scenarios incorporating natural seeding of nitrifiers, resulted in the most accurate and precise predictions of the effluent ammonium and nitrate concentrations. It is, therefore, recommended that Good Modeling Practices be amended to include the quantification of the level of nitrifiers in influents during wastewater characterization. Adopting such approach, will stabilize the model parameters and improve the accuracy of the modeling process.

RÉSUMÉ

La nitrification est un processus très important dans les systèmes de traitement des eaux usées qui sont conçus à l'élimination biologique de l'azote. La taille, l'empreinte et la consommation d'énergie des systèmes de boues activées nitrifiantes sont régis par l'exigence du système à éliminer l'ammoniac. L'ammoniac est un important paramètre de qualité des eaux usées en raison de sa toxicité pour la vie aquatique dans les eaux réceptrices; donc, les règlements environnementaux concernant les exigences de rejet pour l'ammoniac, sont de plus en plus stricts. La recherche dans ce domaine est essentielle pour mieux comprendre le processus de traitement de manière à affiner l'éventail de technologies de traitement existantes et de développer des solutions d'ingénierie novatrices pour atténuer les niveaux d'ammoniac dans les rejets d'eaux usées. Bien que la complexité de la nitrification dans les systèmes de boues activées soit toujours en train d'être démystifiée, l'ensemencement possible des réacteurs biologiques par les eaux usées qui contiennent des bactéries nitrifiantes, a été négligé jusqu'à présent. Même les meilleures pratiques actuelle de modélisation, tels que les bonnes pratiques de modélisation de l'International Water Association (IWA) pour le traitement biologique des eaux usées, assument qu'il n'y a pas de biomasse dans les eaux usées municipales. La bioaugmentation artificielle des boues activées par l'addition de consortiums autochtones ou allochtones de nitrifiants s'est montrée être une stratégie efficace pour améliorer la nitrification biologique dans des conditions stressantes. Cette étude explore le potentiel d'un ensemencement naturel de nitrifiant dans des usines de traitement des eaux usées à grande échelle.

Grâce à l'application de séquençage de l'ADN à haut débit, nous avons démontré que les eaux usées fournissaient en effet des nitrifiants actifs à des bioréacteurs à grande échelle. Les profils phylogénétiques des nitrifiants identifiés dans les eaux usées étaient étonnamment semblables aux populations de nitrifiants dans les bioréacteurs. Des analyses respirométriques ont montré que la biomasse nitrifiante dans les affluents était vivante et active, et était capable d'atteindre une induction complète de leur métabolisme dans quelques heures. Nous avons montré que ce phénomène d'ensemencement naturel résulte d'un processus stochastique d'immigration d'espèces bactériennes. Basé sur ces résultats, nous avons élargi le concept de la métacommunauté des espèces en incluant des communautés immigrantes (eaux usées, dans ce cas) liées aux communautés locales (bioréacteurs). Afin de valider le concept d'ensemencement induit par

l'affluent observé à grande échelle dans les usines de traitement des eaux usées, nous avons reproduit ce phénomène dans le laboratoire en rajoutant des solides d'affluent échantillonnés à partir d'une installation de traitement des eaux usées à grande échelle, à des réacteurs biologiques séquentiels (RBS) à l'échelle du laboratoire opérant dans des conditions proches de lessivage (basse température et temps de rétention des solides courts). L'addition des solides d'affluent restaurait la nitrification et stabilisait les systèmes des RBS. Enfin, nous avons examiné l'impact de tel ensemencement naturel sur la performance des modèles de boues activées. Les modèles de boues activées sont des outils essentiels dans la conception des processus, d'exploitation et d'optimisation des systèmes de traitement biologique des eaux usées. Cependant, ils ne considèrent pas la biomasse active dans les affluents parce qu'il y a peu de connaissance quant à son impact sur les modèles et il n'y a pas de protocoles standards afin de détecter et quantifier son niveau. L'intégration de l'ensemencement naturel des nitrifiants, à un niveau prédéterminé par des tests respirométriques, dans le modèle de nitrification, a amélioré de manière significative, la performance du modèle et sa capacité prédictive. Toutefois, si l'ensemencement naturel des nitrifiants n'est pas considéré, il est toujours possible d'obtenir des ajustements raisonnables entre les ensembles de données mesurées et simulées en ajustant arbitrairement le taux de croissance maximum des nitrifiants et son coefficient de température. Nos résultats indiquent ainsi un biais potentiel de calibration entre les approches impliquant l'ensemencement naturel des nitrifiants tel que déterminé par des tests respirométriques, ou l'ajustement du taux de croissance maximum des nitrifiants et de son coefficient de température pour caler les données mesurées et prédites. Cela peut expliquer la variabilité observée dans le taux de croissance maximum utilisé lors de la calibration des systèmes qui reçoivent des eaux usées provenant de différentes sources - telles que les sources industrielles vs municipales - ou des systèmes de collecte des eaux usées avec des réseaux d'égout plus petits. Néanmoins, pour toutes les simulations effectuées, seuls les scénarios incorporant l'ensemencement naturel des nitrifiants, ont abouti à des prédictions les plus exactes et précises des concentrations d'ammoniac et de nitrate dans l'effluent. Il est, par conséquent, recommandé que les bonnes pratiques actuelles de modélisation soient modifiées pour inclure la quantification du niveau de nitrifiant dans les affluents lors de la caractérisation des eaux usées. L'adoption de cette approche, permettra de stabiliser les paramètres du modèle et d'améliorer la précision du processus de modélisation.

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PREFACE

In accordance with the "*Guidelines for Thesis Preparation*", this thesis is presented in a manuscript-based format. A general introduction and literature review are presented in Chapter 1 and 2. Chapter 3-6 comprise of one published article, and three research papers which will be submitted for publication to the Journal of International Society for Microbial Ecology (ISME), Water Research, and Environmental Science and Technology journals, respectively. The author of this thesis is the primary author of all the manuscripts. In the final Chapter, a General Discussion and Conclusions are presented. Below is a detailed description of the efforts of each contributing author.

Shameem Jauffur, Siavash Isazadeh, and Dominic Frigon, (2014). Should activated sludge models consider influent seeding of nitrifiers? Field characterization of nitrifying bacteria. *Water Science and Technology*, 70: 1526-1532.

Authors' contributions:

Shameem Jauffur: Designed the study, conducted the experimental procedures, analysed the results, and wrote the manuscript.

Siavash Isazadeh: Helped with the study design, and revised the manuscript.

Dominic Frigon: Supervised the research, helped with the study design, and revised the manuscript.

Shameem Jauffur, David Stephens, Zeinab Bakhshi and Dominic Frigon. Activated sludge wastewater treatment nitrifying populations determined by natural influent seeding. To submit to *The ISME Journal-Nature* (in preparation).

Authors' contributions:

Shameem Jauffur: Designed the study, conducted the experimental procedures, analysed the results, and wrote the manuscript.

David Stephens: Performed statistical tests of correspondence between community structures, and revised part of the manuscript.

Zeinab Bakhshi: Assisted with the collection of samples and molecular DNA analyses.

Dominic Frigon: Supervised the research, helped with the study design, and revised the manuscript.

Shameem Jauffur, Zeinab Bakhshi and Dominic Frigon. Influent seeding of wastewater treatment plants: implications for low temperature nitrification and heterotrophic population structures. To submit to *Water Research* (in preparation).

Authors' contributions:

Shameem Jauffur: Designed the study, conducted the experimental procedures, operated the reactors, analysed the results, and wrote the manuscript.

Zeinab Bakhshi: Assisted with the collection of samples and molecular DNA analyses.

Dominic Frigon: Supervised the research, helped with the study design, and revised the manuscript.

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Authors' contributions:

Shameem Jauffur: Designed the study, conducted the experimental procedures, analysed the results, performed the modeling, and wrote the manuscript.

Zeinab Bakhshi: Assisted with the collection of samples from WRRFs and performing of respirometric assays.

Dominic Frigon: Supervised the research, helped with the study design, and revised the manuscript.

CHAPTER 1

Introduction

1.1 INTRODUCTION

The new Canadian federal *Wastewater Systems Effluent Regulations* adopted in 2012, amending the Fisheries Act, have imposed year-round limit (1.25 mg-N/L at 15 °C ± 1 °C) to the discharge of unionized ammonia (NH_3) in treated municipal wastewater effluents (CanadaFisheriesAct 2012, U.S.AFederalRegister 2013). Considered as an acutely toxic compound, NH_3 is a significant threat to natural aquatic ecosystems (Nguyen and Tanner 1998). The release of NH_3/NH_4^+ in natural water bodies increases the biochemical oxygen demand (BOD) load causing oxygen depletion leading to anoxic zones in water systems such as estuaries (Eddy 2005). Effluents rich in NH_4^+ support local primary productivity and can cause eutrophication and development of harmful algal blooms (Aníbal et al. 2014). Opportunistic bloom-forming algae have been shown to have higher uptake rates and growth response for NH_4^+ than for nitrate (NO_3^-) given that NH_4^+ is already reduced and can be directly incorporated into amino acids intracellularly (Cohen and Fong 2006).

The most widely applied process worldwide to remove NH_3 from municipal wastewater is nitrification because of its effectiveness and relatively low cost as compared to other technologies (Mahvi et al. 2008). Nitrification is the biological conversion of NH_3 to nitrate (NO_3^-) via nitrite (NO_2^-) as a secreted intermediate (Peng et al. 2015). This conversion reduces the toxicity of NH_3 and the BOD associated with it. Nitrification is the first step in conventional biological nitrogen removal (BNR) systems where NH_3 is first completely oxidized to NO_3^-, which is then reduced during denitrification to dinitrogen gas (Ahn et al. 2008). Nitrifying bacteria involved in nitrification are considered to be highly specialized chemolithoautotrophs that use NH_4^+ or NO_2^- to generate energy and reductant for growth, and CO_2 as a carbon source (Palatinszky et al. 2015). They are obligate aerobes and grow exclusively in aeration basins of biological wastewater treatment systems (Satoh et al. 2003).

Microorganisms mediating nitrification have slow growth rates (Rittmann and McCarty 2001). In addition, they are susceptible to various environmental conditions such as pH (Shanahan and Semmens 2015), dissolved oxygen (Fitzgerald et al. 2015), NH_3 and NO_2^- concentrations (Yang et al. 2010) and the presence of inhibitory compounds (Kim et al. 2008). They are also sensitive to temperature; at temperatures below 10 °C, nitrification can be compromised (Zhang et al.

2014). This is why nitrifiers dictate the aerobic solids retention time (SRT) to be adopted for the design of Water Resource Recovery Facilities (WRRFs) (Tang and Chen 2015).

Proper design of activated sludge plants requires long enough aerobic SRTs in order to maintain nitrification for NH_3 removal. Given the slow growth rates of nitrifiers, their susceptibility to various environmental conditions and the impact of low temperatures on nitrification, designers have the tendency to increase the SRT, leading to an increase in the volume of aeration basins and the aeration demands. However, such an expansion of treatment infrastructures is costly. Only an accurate approach to model wastewater treatment systems performing nitrification can help to reduce this oversizing of the treatment plants. So far, studies have been focused on nitrifiers in the aeration basin. The possible natural seeding of activated sludge bioreactors with nitrifiers from municipal wastewaters has been overlooked. Even current best practices for biological wastewater treatment modeling, such as the International Water Association (IWA) consensus Activated Sludge Models (ASMs), assume no active nitrifying biomass in municipal wastewaters at the entrance of treatment facilities. No studies have yet systematically reported the possible presence of nitrifiers in raw sewage and their potential impacts on activated sludge systems. Possible occurrence of a natural influent nitrifier seeding may actually cause models to underestimate nitrification in extreme situations and lead to oversizing of aerated bioreactors. If significant influent nitrifier seeding is evidenced, this will trigger important reviews of wastewater treatment system design practices especially for cold climate.

1.2 RESEARCH OBJECTIVES

The overall objective of this study was to explore the presence of nitrifying bacteria in raw influent wastewaters reaching full-scale wastewater treatment facilities in order to unravel the possible natural seeding of activated sludge bioreactors with nitrifiers from influent wastewaters. The sub-objectives of this research project were as follows.

1. To compare the phylogenetic diversity of nitrifying populations in full-scale municipal WRRFs' influent and activated sludge biological reactors. *Do nitrifying species entering full-scale WRRFs get established in activated sludge bacterial communities?*
2. To characterize the metabolic activity of nitrifying populations in wastewater influent and determine the time needed to reach full induction of their metabolic potential. *How active are the seeding nitrifiers and how long does it take for them to reach full activity?*

3. To investigate the impact of seeding the influent of laboratory-scale sequencing batch reactors (SBRs) with harvested nitrifiers from real influent sampled at full-scale WRRFs, and with enriched cold-adapted nitrifying biomass. *Can we demonstrate seeding in laboratory-scale activated sludge reactors?*
4. To evaluate the impact of the presence of a seeding nitrifying population on the quality of mathematical models' predictions. *Can modelers inadvertently capture the natural seeding of nitrifiers during model calibration?*

The above objectives were answered by using DNA-targeted molecular approaches and next-generation sequencing technology, operating laboratory-scale sequencing batch reactors (SBRs) and performing mathematical modeling studies.

1.3 THESIS ORGANIZATION

This thesis is structured into four chapters describing novel research followed by a chapter of general discussion and conclusions.

Chapter 3: lays the foundation of this research project where an initial hypothesis was formulated and eventually verified as the research unfolded. Three research questions were asked: a) Are nitrifiers present in municipal wastewaters reaching full-scale treatment systems, and if yes are they alive? b) Are the nitrifiers detected in influent wastewaters the same as those present in activated sludge in bioreactors? c) What impacts may such contribution of nitrifiers by raw sewage have on activated sludge modeling? In order to provide preliminary answers to these questions, influent and mixed liquor samples were collected from three full-scale WRRFs located near the region of Montreal. The presence of nitrifiers was studied by genomic DNA extraction, PCR amplification of the *amoA* (detecting ammonia oxidizing bacteria, AOB) and *nxrB* (detecting nitrite oxidizing bacteria, NOB) genes, and sequencing of the genetic materials by the GX FLS Titanium 454-sequencing technology. The viability of nitrifiers in influent wastewaters at one plant was assessed by respirometric assays. Based on the observed level of seeding as determined by activity tests, a simple simulation was conducted to evaluate the potential impact of the presence of nitrifiers in raw sewage on the absolute minimum solids retention times ($[SRT_{min}]_{lim}$) of an activated sludge system.

Chapter 4: builds on the findings of Chapter 3. Therein, we further examined the hypothesis that nitrifiers in influent wastewaters are playing a key role in seeding and structuring nitrifying bacterial communities in activated sludge bioreactors. We studied the metagenomic profiles of nitrifying bacteria in influent and mixed liquor samples from eight full-scale activated sludge WRRFs bearing different layouts and configurations by massively parallel pyrosequencing. Diversity and biotic similarity analyses were performed to study the nitrifying bacterial community compositions of the influent and mixed liquor in order to substantiate our hypothesis on the natural seeding of nitrifiers at full-scale wastewater treatment systems. A probabilistic rank-based statistical model was developed to test the similarity between the operational taxonomic unit (OTU) structures of the influent and mixed liquor. We showed that the structure of nitrifying populations in the activated sludge was determined by incoming nitrifying communities from raw wastewater. The immigrant communities detected in sewage were found to be intimately linked to local communities in activated sludge through dispersal and immigration.

Chapter 5: validates the hypothesis of influent-induced nitrifier seeding observed at full-scale wastewater treatment systems. The natural nitrifier seeding phenomenon was replicated at laboratory-scale level using sequencing batch reactors (SBRs) operated at washout conditions (temperature at 5 ºC and SRT at 7 days) in order to find out whether addition of influent solids can restore nitrification. We evaluated the nitrification activity during the seeding experiments by measuring the effluent ammonium (NH_4^+) and nitrate (NO_3^-) concentrations. In addition, the autotrophic nitrifying and heterotrophic bacterial communities in the seeded and non-seeded SBRs were assessed to find out the impact of seeding sewage biomass on the microbial community structures of the SBR systems. Previous studies posited that the contribution of raw wastewater in shaping the microbiome of activated sludge was negligible. In this case, we showed the reverse by describing the influence of sewage autotrophic nitrifying and heterotrophic biomass on structuring the microbial communities in activated sludge bioreactors.

Chapter 6: integrates the findings from the above chapters and examines the potential impacts of natural nitrifier seeding on activated sludge model performance. Current best modeling practices assume no biomass during influent characterization since the potential seeding of nitrifiers by incoming sewage was unknown, and no standardized approach was available to detect and quantify its level. We overcame this limitation by appropriately

quantifying the level of natural influent nitrifier seeding at various full-scale WRRFs, and used it to assess the impact of seeding on nitrification modeling. Nitrification at a full-scale activated sludge WRRF was modeled using an advanced simulation software based on one year of operational data. The modeling was performed as a single-step process under a no-seeding and an influent nitrifier seeding scenario, and the resulting simulations were assessed by Major Axis Regression (MAR). In addition, we describe how modelers can unknowingly capture the phenomenon of natural seeding in their models by adjusting other biokinetic parameters during model calibration *in lieu* of considering the actual seed level for a given activated sludge system.

Chapter 7: provides a general discussion of the salient findings of the thesis, and presents general conclusions of this doctoral research.

1.4 CONTRIBUTION

Activated sludge is the most widely applied process in engineered systems to treat wastewaters (Bagheri et al. 2015, Hreiz et al. 2015). Significant amount of studies have been undertaken to understand the physical, chemical, biological and ecological aspects of such complex system (Friedrich et al. 2015). However, interest has mainly been focussed on the bioreactor and clarifier. Even nitrification has extensively been studied as a transformation process at the level of the bioreactor, and strategies developed to enhance it especially under unfavorable conditions, no particular attention has been given to the possible natural seeding of bioreactors with nitrifiers from raw sewage. In this study, we expanded the conventional analysis of nitrification to consider the potential seeding of nitrifiers that may be occurring upstream of aeration basins. By so doing, we showed that wastewaters contribute to their own treatment by supplying bioreactor systems with valuable nitrifiers which are essential for nitrification. The specific contributions of this work are highlighted below.

1. **Revealed the presence of nitrifiers (both AOB and NOB) in municipal wastewaters reaching full-scale wastewater treatment systems.** The presence of this important group of bacteria in municipal raw sewage is systematically shown in this study.

2. **Evidenced the existence of a natural seeding of nitrifiers in full-scale activated sludge bioreactors.** The direct correspondence of AOB and NOB in influent and mixed liquor is

demonstrated for the first time thereby supporting the existence of natural seeding of nitrifiers in wastewater treatment systems. The nitrifying community structure in activated sludge is shown to be determined by the nitrifying community profile of influent wastewaters.

3. **Showed that nitrifiers present in influent wastewaters are alive and require a few hours to reach full metabolic induction in presence of their substrates and optimum growth conditions.** This is important since nitrifiers conveyed by raw sewage can be adsorbed on activated sludge flocs and contribute to nitrification in bioreactors.

4. **Demonstrated that natural influent nitrifier seeding can support nitrification under unfavorable conditions (low temperature and SRT).** This was an important step to validate the concept of natural influent-induced nitrifier seeding, and show its contribution in failing systems where nitrification should be possible metabolically. Moreover, by inducing nitrification through addition of influent solids to laboratory-scale bioreactors operated at washout conditions, and causing their failure by interrupting the supply of influent solids, show that some level of control can be exercised with regard to natural seeding of bioreactors. This opens potential avenues for sewage work designers to engineer redundancy in nitrifying bacterial communities by increasing nitrifier fractions in sewer systems to enable natural bioaugmentation in activated sludge bioreactors.

5. **Evidenced the contribution of raw sewage in shaping heterotrophic bacterial communities in activated sludge.** Previous studies advocated that the contribution of influent in shaping the heterotrophic bacterial communities of activated sludge was negligible. In this thesis, it is shown that raw sewage influences the heterotrophic bacterial community structure in activated sludge.

6. **Demonstrated the impact of natural influent nitrifier seeding on the calibration of mathematical model parameters.** Current best modeling practices assume no biomass in influent wastewaters due to a lack of knowledge on the existence of a natural nitrifier seeding at full-scale wastewater treatment facilities, and no standardized experimental protocol to determine its intensity. In this study, the level of influent nitrifier seeding was

quantified at various WRRFs, and used to assess its potential impact on the predictive capacity of nitrification model. Integrating natural nitrifier seeding in activated sludge model can significantly enhance the fit between measured and simulated data. It is also shown how modelers can unknowingly capture the phenomenon of natural seeding by adjusting the maximum growth rate of nitrifiers and/or the temperature correction factor for nitrifiers' growth rate during model calibration.

Some of the work presented in this thesis have already been published while the remaining findings will be submitted for publication.

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Jauffur, S., Bakhshi, Z., Frigon, D. Influent seeding of wastewater treatment plants: implications for low temperature nitrification and heterotrophic population structures. To submit to ***Water Research*** (in preparation).

Jauffur, S., Bakhshi, Z., Frigon, D. Importance of influent nitrifiers to accurately model biological wastewater treatment systems. To submit to ***Environmental Science and Technology*** (in preparation).

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CHAPTER 2

Literature review

2.1 NITRIFICATION IN WASTEWATER TREATMENT

The presence of ammonia nitrogen in wastewater is toxic to receiving water bodies and aquatic life (Wajsbrot et al. 1991). Domestic wastewater comprises of fecal matter and urine, with the latter representing the main contributor of nutrients with 85% nitrogen (Beler-Baykal et al. 2004). Wastewater discharges containing an elevated concentration of NH_3 can increase the BOD load of natural waters leading to oxygen depletion and formation of anoxic zones which are harmful to aerobic aquatic organisms. Ammonia entering water bodies has also been reported to cause proliferation of harmful algal blooms capable of producing toxins which can affect functioning of natural ecosystems and human health (Hallegraeff 1993).

Elaborating proper engineering solutions for ammonia removal from wastewaters is, therefore, imperative to protect the surrounding environment. Biological nitrogen removal (BNR) has been a widely preferred option to physicochemical processes due to its higher effectiveness and lower cost (Chai et al. 2015). In conventional BNR, ammonia removal occurs through two main processes: ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) with oxygen as electron acceptor in an aerobic nitrification reaction, and subsequent reduction of the NO_3^- to molecular nitrogen gas (N_2) with organic matter as electron donor in an anoxic denitrification reaction (Shen 2014). The nitrification reaction actually involves two sequential oxidation steps: nitritation (also referred to as partial nitrification) where NH_4^+ is converted to NO_2^- (Eq. 2.1), and nitratation where the NO_2^- produced from the first step is oxidized to NO_3^- (Eq. 2.2) (Henze 2008). Nitrification, thus, transforms the most reduced form of nitrogen (ammonium: −3) into the most oxidized form of nitrogen (nitrate: +5) in the natural nitrogen cycle (Ward et al. 2011). The two stoichiometric redox reactions involved in nitrification are shown below (equations are normalized to 1 electron equivalent transferred).

$$\frac{1}{6}NH_4^+ + \frac{1}{4}O_2 \rightarrow \frac{1}{6}NO_2^- + \frac{1}{3}H^+ + \frac{1}{6}H_2O \qquad \Delta G^{0\prime} = -45.79\frac{kJ}{\bar{e}\ eq} \quad (Eq.\ 2.1)$$

$$\frac{1}{2}NO_2^- + \frac{1}{4}O_2 \rightarrow \frac{1}{2}NO_3^- \qquad \Delta G^{0\prime} = -37.07\frac{kJ}{\bar{e}\ eq} \quad (Eq.\ 2.2)$$

The two consecutive steps in nitrification are performed by chemolithoautotrophic bacteria (Purkhold et al. 2000). Ammonia Oxidizing Bacteria (AOB) mediate the oxidation of NH_3 to hydroxylamine (NH_2OH) using ammonia monooxygenase (*AMO*) and to NO_2^- using hydroxylamine oxidoreductase (*HAO*). *AMO* is a heteromultimeric enzyme encoded by the *amoCAB* operon while *HAO* is a homotrimer encoded by the *hao* gene, both of which are present in all AOB species (Hommes et al. 1998). On the other hand, Nitrite Oxidizing Bacteria (NOB) converts NO_2^- into NO_3^- through the action of the nitrite-oxidizing enzyme (*NXR*) which is a membrane-bound heterodimeric protein consisting of a large α subunit (*nxrA* gene) and a small β subunit (*nxrB* gene) (Meincke et al. 1992). The metabolic pathway for the conversion of NH_3 to NO_3^- is shown in Figure 2.1

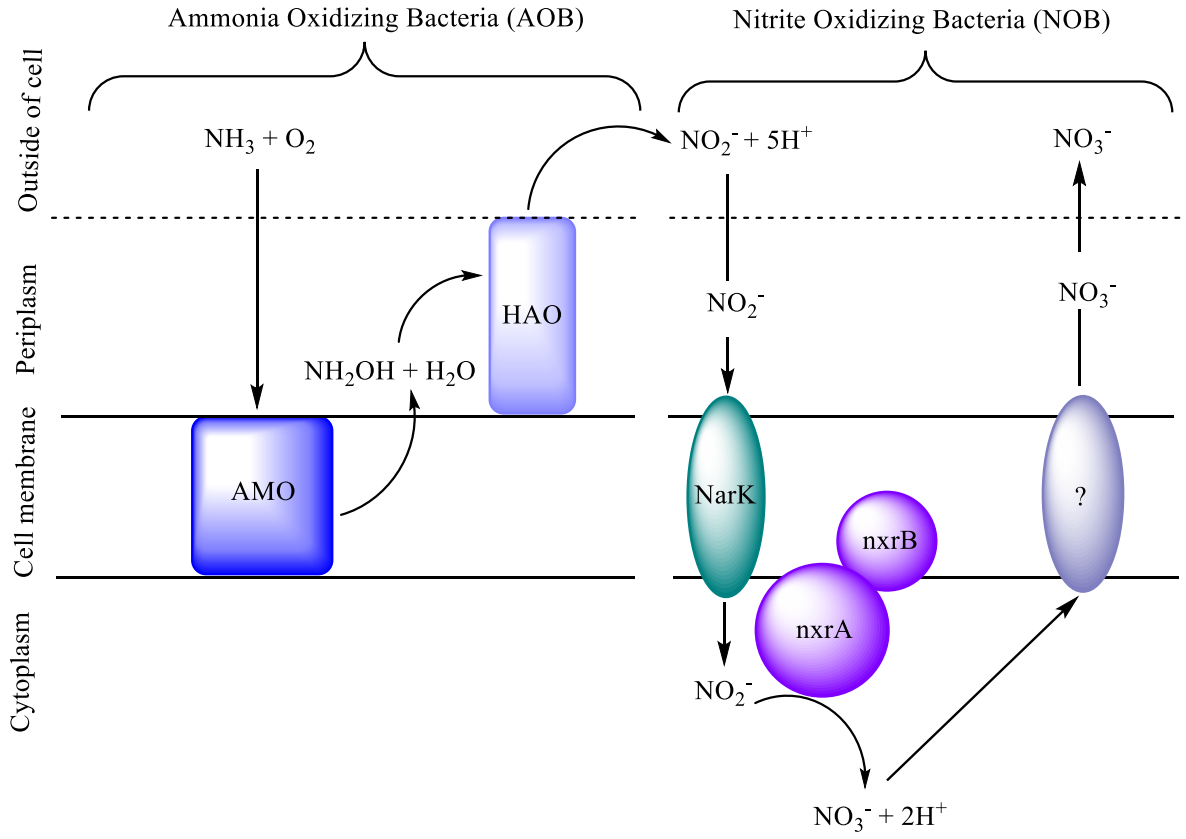

Figure 2.1. Flow of substrates in nitrification process through pertinent catabolic modules in ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Descriptors: *AMO*-ammonia monooxygenase; *HAO*-hydroxylamine oxidoreductase; NH_2OH-hydroxylamine; *nxr*-nitrite-oxidizing enzyme; *nxrA*-large α subunit of *nxr* enzyme; *nxrB*-small β subunit of *nxr* enzyme; *narK*-NO_2^-/NO_3^- transporter. The specific mechanism of NO_3^- transport from the cytoplasm to periplasm is unknown. Adapted from Klotz and Stein (2008).

The oxidation of NH_3 to NO_2^- by autotrophic nitrifying microorganisms was long thought to be mediated by only bacteria. However, Ammonia Oxidizing Archaea (AOA), which are ubiquitously distributed in marine and terrestrial environments, have also been shown capable of NH_3 oxidation (Prosser and Nicol 2008). The biochemical pathways for NH_3 oxidation in AOA are different than in AOB. Other than coding for an evolutionarily different ammonia monooxygenase (*AMO*), it has no central cytochrome network or homolog for hydroxylamine oxidoreductase (*HAO*) (Hatzenpichler 2012). Ammonia oxidation is believed to be mediated by the *AMO* enzyme to produce nitroxyl hydride (HNO) which is then oxidized to NO_2^- by nitroxyl oxidoreductase (*NXOR*) (Miranda 2005).

Both *amoA* and *nxrB* genes have been used as functional markers for the molecular fine-scale analysis of natural AOB and NOB populations in environmental samples through PCR assays using specific primers (Rotthauwe et al. 1997, Starkenburg et al. 2006). Positive PCR amplification of *amoA* and *nxrB* genes is specific for the presence of AOB and NOB, providing a rapid and sensitive method to assess the presence of nitrifiers.

From a phylogenetic point of view, nitrifying microorganisms are clustered in a few evolutionary lineages within the prokaryotic and archaeal domains. The AOB comprise of bacteria from the family *Nitrosomonadaceae* falling under the *Betaproteobacteria,* and *Nitrosococcus* of the *Gammaproteobacteria*. Based on 16S rRNA sequences, the *Nitrosomonadaceae* family is divided into four genera, *Nitrosomonas*, *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio*. The AOA stem from three major archaeal lineages based on 16S rRNA gene analysis, namely Group I affiliated with the kingdom *Crenarchaeota*, and the remaining two groups (Group II and III) belonging to the kingdom *Euryarchaeota*. Increasing amount of genomic data has recently revealed a new AOA lineage belonging to the *Thaumarchaeota*, the members of which are also present in marine and terrestrial environments (Stahl and de la Torre 2012). The NOB comprise of bacteria from the following groups: *Nitrobacter* of the class *Alphaproteobacteria*, *Nitrospina* of the class *Deltaproteobacteria*, *Nitrococcus* of the class *Gammaproteobacteria*, *Nitrospira* having their own phylum (Brenner and Staley 2005), *Nitrotoga* of the class *Betaproteobacteria* (Alawi et al. 2007) and *Nitrolancetus* of the phylum *Chloroflexi* (Sorokin et al. 2012).

Recently, complete oxidation of NH_4^+ to NO_3^- by one microorganism (complete ammonia oxidation: comammox) was shown to be possible with the isolation of two *Nitrospira* species that

encode all the enzymes necessary for NH_4^+ oxidation to NO_3^- via NO_2^- (van Kessel et al. 2015). However, their ammonia monooxygenase (*AMO*) enzyme was found to be phylogenetically distinct from the *AMO* from AOB and AOA, rendering recent acquisition by horizontal gene transfer from known ammonia-oxidizing microorganisms unlikely.

To complete the picture of the known microbial diversity involved in NH_4^+ conversion, it is necessary to mention the anammox bacteria. The bacteria combine the metabolism of NH_4^+ oxidation and NO_2^- reduction to produce dinitrogen gas (N_2) under anaerobic condition. They are at the center of the most innovative technological advances in the removal of ammonia nitrogen from wastewater (Strous et al. 1999). The known species exhibiting the anammox metabolism belong to the *Planctomycetes* phylum.

AOB and NOB share a close symbiotic relationship with each other forming densely packed microcolonies and cell clusters, since the product of ammonia oxidation by AOB, which is NO_2^-, is the substrate for NOB, and whose accumulation would be inhibitory to AOB (Daims et al. 2006). In biological wastewater treatment, AOB from the *Betaproteobacteria* class primarily predominates in activated sludge bioreactors and mainly include species of the *Nitrosomonas* and *Nitrosospira* genera (Juretschko et al. 2002). For NOB, *Nitrospira* has been reported to be the most dominant group in wastewater treatment plants (Vanparys et al. 2006).

2.2 SENSITIVITY OF NITRIFICATION TO COLD TEMPERATURE

Nitrification is sensitive to low temperature, and maintaining it is of considerable concern during winter in northern climates (Delatolla et al. 2009). The limitation of nitrification at low temperature is intimately linked to the chemolithoautotrophic metabolism of nitrifiers in which nitrogen acts as electron donor and releases low amount of energy which is translated into a low specific growth rate (μ_{ANO}) and biomass yield (Y_{ANO}) (Rittmann and McCarty 2001). As compared to the growth rates of heterotrophs (4-13 d^{-1}), nitrifiers are very slow growers (0.62-0.92 d^{-1}) (Ward et al. 2011). The minimum generation time for nitrifying bacteria at 30 °C has been shown to be around 15 h and this can increase up to 200 h at 5 °C (Kos 1998). Previous reports indicate that both nitrite and nitrate formation are strongly inhibited at temperatures less than 10 °C (Yao et al. 2013). McCartney and Oleszkiewicz (1990) reported that nitrification is severely compromised below 6 °C in reactors under different operational conditions. Experiments conducted on suspended biomass growth systems receiving municipal wastewaters have shown a

reduced or no nitrifier growth at temperatures below 4 °C (Van Dyke et al. 2003). The inconsistency in the ability to maintain nitrification at low temperatures has mainly been related to the capacity of the system to grow and maintain nitrifiers for long SRTs (Delatolla et al. 2009).

Considering previous observations, it is not surprising why many biological wastewater treatment systems carrying out nitrogen removal have encountered failures during winter seasons (Ilies and Mavinic 2001, Kim et al. 2006). In treatment systems with low level of biomass such as aerated lagoons the problem of maintaining nitrification during winter is exacerbated by the long hydraulic retention time (HRT) which significantly reduces the temperature and causes nitrification failure. Subsequently, ammonia removal in aerated lagoons occurs mainly during summer and fall seasons (Houweling et al. 2008). For activated sludge Wastewater Treatment Plants (WWTPs), now more appropriately termed as Water Resource Recovery Facilities (WRRFs) (Vanrolleghem and Vaneeckhaute 2014), this forces designers to increase the SRT during cold temperature as shown in Figure 2.2 (Grady 1999) and apply an appropriate safety factor to prevent loss of nitrification activity (Rittmann and McCarty 2001). As a consequence, the footprint of these plants and the necessary capital investment are higher than for warmer climates.

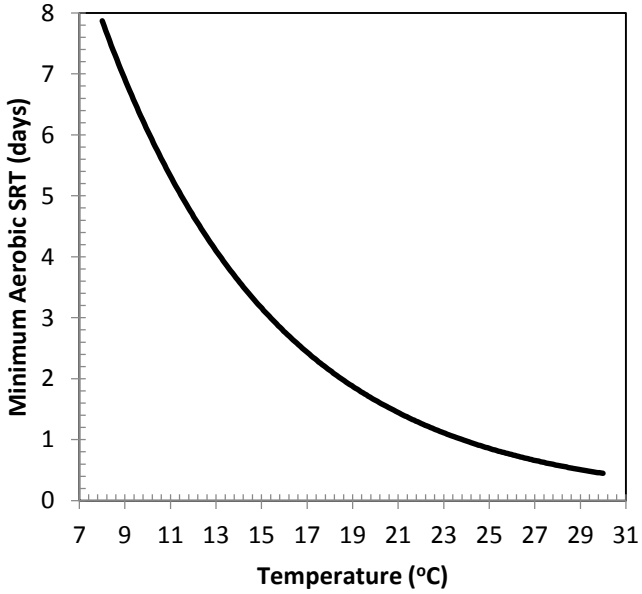

Figure 2.2. Variation of SRT with temperature (Grady et al., 1999).

With the new Canadian federal *Wastewater Systems Effluent Regulations* adopted in 2012, amending the Fisheries Act, a year-round limit to the discharge of unionized ammonia (NH_3) has

been imposed for treated municipal wastewater effluents. The maximum permissible concentration limit of unionized NH_3 in effluent according to the Wastewater Systems Effluent Regulations (2012) is 1.25 mg/L, expressed as nitrogen (N) at 15 °C ± 1°C. Further to the enforcement of a stricter regulation, plant operators are compelled to find ways to improve nitrification in their system. Several strategies have been developed to enhance nitrification, and include extending the SRT through application of membrane separation instead of gravity sedimentation (Kishino et al. 1996), integrating fixed film media in BNR activated sludge processes to form an Integrated Fixed Film Activated Sludge (IFAS) system supporting attached growth of nitrifiers (Sriwiriyarat and Randall 2005), and applying granular sludge biomass after acclimatizing it to low temperature (de Kreuk et al. 2005). In other cases, the aeration period can be extended to achieve complete nitrification at low temperature (Guo et al. 2010). Another successful approach is the supplemental addition of nitrifying biomass grown in a side-stream aerated tank for cultivating backup biomass of nitrifiers to balance washout from the system (Kos 1998).

2.3 ARTIFICIAL VS. NATURAL SEEDING OF NITRIFIERS

Process-induced nitrification through the artificial addition of nitrifying biomass grown in a side-stream reactor, referred to as *bioaugmentation*, has proved to be a cost-effective strategy to achieve high nitrification efficiencies at relatively low SRTs (Tang and Chen 2015, Wett et al. 2011). Warm ammonium-rich liquids originating from supernatants (e.g. anaerobic digesters operated at 35 °C) and sludge dewatering liquors (centrate) are used to concentrate nitrifying biomass in a side-stream activated sludge tank and is used as seed source for bioaugmentation of the main-stream bioreactors (Gujer 2010, Peng et al. 2015). Studies have shown that bioaugmentation using enriched nitrifying biomass can remove up to 25% of the NH_4^+ entering a wastewater treatment system (Berends et al. 2005, Janus and Van Der Roest 1997). Kos (1998) showed that adding nitrifying sludge from a side-stream process treating reject wastewater (30-35 °C; 0.3-0.9 g NH_4^+-N/L) reduced the apparent aerobic SRT by 60%. This strategy is also useful when treating wastewater at low temperature. Nitrifying bacteria (both AOB and NOB) selected at low temperature have been used by Cui et al. (2014) to enrich activated sludge treating municipal wastewater at 10 °C. This allowed an overall ammonia removal of 85% in the mainstream bioreactor.

Different technologies integrating the process of bioaugmentation have been patented. The InNITRI® (Inexpensive Nitrification) process consists of constantly adding supplemental nitrifiers from a side-stream reactor (the InNITRI® reactor) fed with reject wastewater from an anaerobic digester (Figure 2.3.a) (Kos 1998). It allows nitrification at low SRTs and even in small aeration tanks. It was mainly developed as an inexpensive option for wastewater treatment facilities in northern climate for year-round ammonia removal.

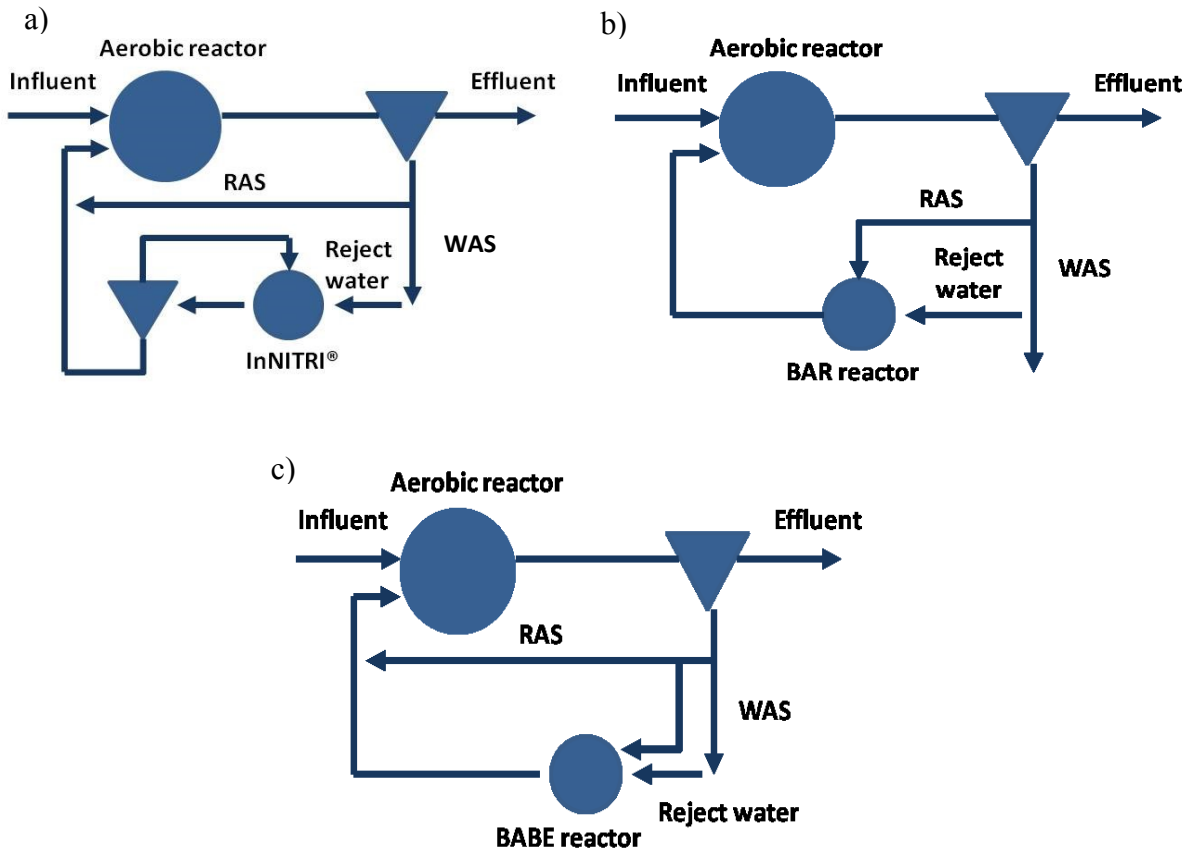

Figure 2.3. Bioaugmentation processes: a) InNITRI®, b) BAR®, and c) BABE®. Warm reject water rich in ammonium comes from anaerobic digesters. Descriptors: RAS-Return Activated Sludge; WAS-Waste Activated Sludge.

The BAR® (Bioaugmentation Regeneration) technology was proposed by Novák et al. (2003) in which return activated sludge (RAS) and reject anaerobic digester effluent are both mixed in the aerated BAR reactor to enrich nitrifiers which are then redirected to the main-stream treatment (Figure 2.3.b). Another strategy is the BABE® (BioAugmentation Batch Enhanced) process in which side-stream reject anaerobic digester effluent high in ammonium concentration and part of

the RAS from the main biological treatment unit are combined in a batch reactor. The RAS is used to augment the nitrifying biomass in the settled sludge (Fig. 2.3.c). A model-based evaluation for upgrading the Walcheren WRRF (140,000 Population Equivalent) in the Netherlands has shown that beside achieving an enhanced ammonia removal efficiency, the BABE technology has allowed to reduce the area requirement by 50% and enabled cost saving of up to $130,000 per year (Salem et al. 2003).

Although the impact of process-induced bioaugmentation on nitrifying activated sludge systems has been established, it is not known whether a natural bioaugmentation through the possible flow of nitrifiers in raw sewage occurs at full-scale wastewater treatment facilities. Considering that urine, which is the main contributor of macronutrients to domestic wastewater, contains a high level of nitrogen (up to 8,000-10,000 mg-N/L in the form of urea) (Jin et al. 2014), it may be possible that nitrogen-transforming microorganisms such as nitrifiers are present as well. In an early study, cells of *Nitrobacter* sp. were detected in influent wastewaters reaching a full-scale Water Resource Recovery Facility (WRRF) in Sweden using monoclonal antibodies in ELISA assays (Sandén et al. 1996). This indicates the presence of potential nitrifiers in raw sewage. However, it is not known whether this is a localized observation or if the influent nitrifiers can be metabolically induced. The absence of favorable environmental conditions in sewers (e.g. low oxygen concentration) may render these bacteria dormant and metabolically inactive. Potentially, the activity and growth of sewer microorganisms can resume if appropriate environmental conditions are re-established (Raunkjær et al. 1995).

Moreover, if natural seeding of nitrifiers is occurring at full-scale wastewater treatment level, it is still not known whether they establish themselves among the activated sludge communities in aeration basins. The success of bioaugmentation largely depends on the effective establishment and metabolic adaptation of the seeded biomass in the mainstream reactor (Mannucci et al. 2015). There are cases where the seeded nitrifiers did not adapt to the bioreactor conditions and suffered a reduction in nitrifying activity (Parker and Wanner 2007). This is why it is highly desirable to add endogenous populations rather than non-representative nitrifiers to mainstream bioreactors (Bouchez et al. 2000).

2.4 MODELING PRACTICES AND NATURAL SEEDING

Mathematical models have proved to be extremely useful in understanding the stoichiometry and kinetics of nitrification in activated sludge systems (Sin et al. 2009). These models have been at the centre for selecting operational strategies to improve process stability, effluent quality, and minimize operational costs (Grady 1999, Sahlstedt et al. 2004). The International Water Association (IWA) consensus Activated Sludge Models (ASMs) have been widely used to model and predict nitrogen transformations in wastewater treatment. Since the publication of ASM1 (Henze 1987), several extensions have been proposed to fix shortcomings of ASM1 and include new processes (Hauduc et al. 2013).

Building an activated sludge model incorporating nitrogen removal is a step-wise, bottom-up process, relating the stoichiometry of biological reactions and their corresponding kinetics. Nitrification models have been based on different types of approach such as the "black-box" approach where only the influent and effluent characteristics are considered, ignoring whatever is happening inside the biological reactor, and the "grey-box" models (e.g. ASM1, Henze (1987); ASM2, Henze et al. (1995); ASM2d, Henze et al. (1999)), in which the flow of COD in the system is partitioned into relevant fractions. However, in all cases, the model input consists mainly of a list of measurable raw wastewater parameters transformed to an influent vector with concentrations (Meijer 2004). The bioconversion of influent ammonium (S_{NH}) can be modeled through a one-step nitrification (Gujer et al. 1999) since nitritation is considered as the rate-limiting step in nitrification (Lijklema 1973) or through a two-step nitrification with nitrite (S_{NO}) as an intermediate (Sin et al. 2008) since NO_2^- accumulation has been reported to occur in specific conditions (Kaelin et al. 2009).

Setting up a nitrification model involves three main steps: identifying the relevant components (i) of the model, identifying the biological processes (j) occurring in the system i.e. conversions or transformations which affect the model components, and deriving the kinetic expressions or rate equations (ρ_j) for each process. These three steps can be represented in a Gujer matrix as shown in Table 2.1.

Table 2.1. Gujer matrix showing process kinetics and stoichiometry for nitrification (ASM1).

Components (i) → / Process (j) ↓	X_{ANO}	X_{CB}	X_U	X_{ND}	S_{O2}	S_{NOx}	S_{NHx}	S_{Alk}	Process rate (ρ_j)
Aerobic growth of autotrophic nitrifiers	1				$\frac{4.57-Y_{ANO}}{Y_{ANO}}$	$\frac{1}{Y_{ANO}}$	$-i_{N\_XANO}-\frac{1}{Y_{ANO}}$	$\frac{i_{N\_XANO}}{14}-\frac{1}{7Y_{ANO}}$	$\mu_{ANO,Max}(\frac{S_{NHx}}{K_{NH,ANO}+S_{NHx}})(\frac{S_{O2}}{K_{O2,ANO}+S_{O2}})X_{ANO}$
Decay of autotrophic nitrifiers	-1	$1-f_P$	f_P	$-i_{N\_XANO}-f_P-i_{XP}$					$b_{ANO}X_{ANO}$

Notations: X_{ANO} – Autotrophic nitrifying organisms; X_{CB} – Slowly biodegradable substrates; X_U –Particulate undegradable organics; X_{ND} – Slowly biodegradable organic nitrogen; S_{O2} – Dissolved oxygen; S_{NOx} – Nitrate plus nitrite; S_{NHx} – Ammonium plus ammonia nitrogen; S_{Alk} – Alkalinity; Y_{ANO} – Yield for autotrophic nitrifying organisms; b_{ANO} – Decay rate for autotrophic nitrifying organisms; f_P – Fraction of biomass leading to particulate product; i_{N_XANO} – Mass of nitrogen per mass of COD in biomass; i_{xp} – Mass of nitrogen per mass of COD in products from biomass. Standard notations based on Corominas et al. (2010) are presented.

Based on the above matrix, only the autotrophic nitrifying biomass (X_{ANO}) in the activated sludge reactor is considered during the modeling of nitrification. According to Henze et al. (2000), the concentration of biomass in the influent is assumed to be negligible because there is no standardized protocol to detect and quantify its level. If natural nitrifier seeding is prevalent at full-scale WRRFs, then modelers, by ignoring such aspect, may actually be underestimating the level of nitrification. This can lead to the design of oversized bioreactors. Or, modelers may be unknowingly capturing this natural phenomenon during model calibration. According to Salem et al. (2003), process-induced bioaugmentation has a direct impact on the minimum SRT (SRT_{min}) of an activated sludge system. Based on mass balance, and the assumption that the system is at steady state, and no nitrifying biomass is supplied to the bioreactor, the SRT_{min} can be calculated using Eq. 2.3.

$$\mathrm{SRT_{min}} = \frac{S_{\mathrm{NHx}} + K_{\mathrm{NHx,ANO}}}{S_{\mathrm{NHx}}\mu_{\mathrm{max}} - b_{\mathrm{ANO}}(S_{\mathrm{NHx}} + K_{\mathrm{NHx,ANO}})} \qquad \text{Eq. 2.3}$$

Where SRT_{min} is the minimum solids retention time, S_{NHx} is the effluent ammonium concentration, $K_{NHx,ANO}$ is the half-saturation constant for nitrifiers, μ_{max} is the maximum growth rate of nitrifiers and b_{ANO} is the decay rate for nitrifiers. If nitrifiers from a side-stream reactor are added to the main bioreactor as is the case for bioaugmentation, the SRT_{min} is then given by Eq. 2.4.

$$\mathrm{SRT_{min}} = \frac{S_{\mathrm{NHx}} + K_{\mathrm{NHx,ANO}}}{S_{\mathrm{NHx}}\mu_{\mathrm{max}} - (b_{\mathrm{ANO}} - q_{\mathrm{add}})(S_{\mathrm{NHx}} + K_{\mathrm{NHx,ANO}})} \qquad \text{Eq. 2.4}$$

Where q_{add} is the specific addition rate of nitrifiers. The concept of natural nitrifier seeding by raw sewage involves the same steady-state mathematical equations, and its occurrence at full-scale wastewater treatment systems will entail a reduction in the SRT_{min}. If the actual SRT is less than the SRT_{min}, nitrification stops due to washout. With natural influent nitrifier seeding, nitrification can, thus, be achieved at smaller SRTs.

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CHAPTER 3

Should activated sludge models consider influent seeding of nitrifiers? Field characterization of nitrifying bacteria

Connecting text: The possible natural seeding of full-scale bioreactors with nitrifiers has not been systematically studied yet. In this chapter, we formulated the hypothesis that raw sewage contributes to natural seeding of full-scale wastewater treatment bioreactors with nitrifiers. We present initial results demonstrating the presence of nitrifying bacteria in municipal wastewaters reaching full-scale WRRFs and their potential to be metabolically induced. Based on the findings, we investigated the possible impacts of natural influent-induced nitrifier seeding on activated sludge modeling through a simple simulation of the absolute minimum solids retention times ($[SRT_{min}]_{\lim}$) at low temperature where nitrification is sensitive. This initial study was the justification for the expanded work that followed in the other chapters.

The results of this research have been published in the following paper:

Shameem Jauffur, Siavash Isazadeh and Dominic Frigon. Should activated sludge models consider influent seeding of nitrifiers? Field characterization of nitrifying bacteria. *Water Science and Technology* (2014). 70: 1526-1532.

3.1 INTRODUCTION

Ammonia (NH_3) represents a serious environmental hazard and a deadly threat to fish and other aquatic life due to its toxicity (Campos et al. 2008). The most widely applied process worldwide for ammonia removal from municipal wastewater is nitrification: the aerobic biological conversion of NH_3 to nitrate (NO_3^-) via nitrite (NO_2^-) as a secreted intermediate (Mahvi et al. 2008). This microbially catalyzed oxidation is a key process in wastewater treatment and effectively reduces the toxicity associated with NH_3 and the biochemical oxygen demand (BOD) of NH_3/NH_4^+. However, nitrification is a fragile temperature sensitive process (Van Dyke et al. 2003). It is, therefore, not surprising why many biological wastewater treatment systems carrying out nitrification have encountered failures during winter season (Ilies and Mavinic 2001). Yet, since NH_3 is toxic to aquatic life during all seasons, stricter effluent standards are being implemented and enforced in North American jurisdictions to regulate throughout the year the discharge of total ammonia nitrogen (Canada Fisheries Act 2012, U.S.A Federal Register 2013). Consequently, the conversion of NH_3 into NO_3^- is a requirement even during the winter season, which drives the increase of the design solids retention time (SRT) to prevent nitrifier washout (Rittmann and McCarty 2001). As a consequence, the footprints of these Water Resource Recovery Facilities (WRRFs) and the necessary capital investment are higher than for warmer climates.

Nitrification is carried out by microorganisms clustered in a few evolutionary lineages within the bacterial and archeal domains. In activated sludge wastewater treatment systems, however, nitrification is mainly performed by bacterial nitrifiers (Shen 2014). The bacterial nitrifying populations are functionally classified as (1) Ammonia Oxidizing *Bacteria* (AOB), which oxidize NH_3 to NO_2^-, and (2) Nitrite Oxidizing *Bacteria* (NOB), which convert NO_2^- to NO_3^- (Schramm 2003). Because of the high toxicity of NO_2^-, ammonia and nitrite oxidizers share a close symbiotic relationship with each other forming densely packed microcolonies in wastewater treatment systems (Daims et al. 2006).

So far, the possible seeding of nitrifiers in terms of AOB and NOB by influent wastewaters to activated sludge bioreactors has not been considered to be significant. Even current best practices for biological wastewater treatment modelling, such as the International Water Association (IWA) consensus Activated Sludge Models (ASMs), assume no active biomass in municipal wastewater

at the entrance of treatment facilities. However, neglecting the presence of nitrifiers in municipal wastewaters may cause the models to underestimate the extent of nitrification in wastewater treatment plants, and lead to over-sizing of aerated bioreactors. This is especially true in cold temperature conditions where nitrifiers are less active. Therefore, evidencing the existence of significant influent nitrifier seeding may trigger important reviews of wastewater treatment system design practices for cold climate.

To our knowledge, no studies have been conducted on the presence of nitrifiers in municipal wastewaters reaching full-scale activated sludge wastewater treatment facilities, and on the extent of the correspondence between species present in the influent and mixed liquor. In the current study, we provide answers to the following questions, which we believe are necessary steps to quantify the extent of seeding of mixed liquor by the influent and the practical importance of this seeding. (1) Are autotrophic nitrifiers present and active in influents of full-scale municipal WRRFs? (2) If present, are the influent nitrifying populations the same as those present in mixed liquors (i.e., is seeding possible and observed)? (3) Given the level of observed potential seeding, what type of gains in design SRT could be made if seeding is considered during modelling?

3.2 MATERIALS AND METHODS

3.2.1 Site description and sample collection

An influent sample (composed of 24 1-L samples collected every hour over a 24-hour period) and a mixed liquor suspended solids (MLSS) sample (composed of 4 grab samples collected at different locations in one aeration basin) were collected at each of 3 full-scale activated sludge WRRFs located near Montréal (Québec, Canada) in February 2013 (winter season) (Table 3.1).

Table 3.1. Description of the activated sludge wastewater treatment plants included in this study.

WRRFs	Geographic location		Sewer type[a]	Plant process[b]	Flow rate (m^3/day)	SRT (day)	HRT (hour)	Residential: industrial COD ratio in influent
	Latitude N	Longitude W						
Cowansville	45°13'16.55"	72°46'30.41"	CS	CAS	14,000	10	18	90:10
LaPrairie	45°24'16.48"	73°33'22.06"	CS/PCS	CAS	65,000	7	15	45:55
Vaudreuil	45°23'25.30"	74° 1'37.34"	CS	SBR	18,000	5	3	50:50

a: CS-combined sewer; PCS-partially combined sewer

b:CAS-conventional activated sludge; SBR-sequencing batch reactor

The biomass was spun by micro-centrifugation in 1.5 ml eppendorf tubes and frozen at −20 °C until time of analysis.

3.2.2 DNA extraction and PCR amplification of *amoA* and *nxrB* genes

Genomic DNA was extracted from 0.25 g of centrifuged wet solids (influent or mixed liquor) using the *MO BIO* UltraClean™ Fecal DNA Kit (Carlsbad, CA). The presence of nitrifiers was determined by PCR amplification of specific functional genes using barcoded primers of approximately 50 bp. The AOB population was analyzed by targeting the *amoA* functional gene using the forward primer *amoA-1F* GGGGTTTCTACTGGTGGT and reverse primer *amoA-2R* CCCCTCTGCAAAGCCTTCTTC (Rotthauwe et al. 1997); while the phylum *Nitrospira* of the NOB population was studied as the dominant nitrite-oxidizing population in wastewater treatment systems (Hovanec et al. 1998) by targeting the *nxrB* gene using the forward primer *nxrB-F169* TACATGTGGTGGAACA and reverse primer *nxrB-638R* CGGTTCTGGTCRATCA (Maixner 2009). Each 50 µl PCR reaction mixture contained 2.5 µl of 0.5 M forward primer, 2.5 µl of 0.5 M reverse primer, 10 µl of 1× Bioline PCR colorless buffer (Taunton, MA, USA), 2.75 µl of 2.75 mM $MgCl_2$, 0.5 µl of 250 µM dNTP mixture, 2 µl of 12 ng/mL DNA template, 0.5 µl of 2.5 units/ µl Taq DNA Polymerase (Bioline, Taunton, MA, USA) and 29.25 µl of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, USA). The PCR thermocycling conditions for *amoA* gene fragment amplification were as follows: 95 °C for 4 min, 35 cycles of 95 °C for 40 s, 56 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min. The thermal profiles used for the amplification of *nxrB* gene target sequence were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 40 s, 62 °C for 40 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min. The PCR amplicons were purified using the *MO BIO* UltraClean™ PCR Clean-Up Kit (Carlsbad, CA).

3.2.3 GX FLS Titanium 454-pyrosequencing and sequence data analysis

The amplicon concentration of each sample was determined using the Quant-iT™ PicoGreen kit (Invitrogen, Carlsbad, USA) and normalized to a concentration of 30 ng/µl. The quality of the PCR products was assessed by the Bioanalyzer 2100 (Agilent Technologies). Purified amplicons were subjected to emulsion PCR based on Roche-454 Life Science Protocol and pyrosequenced

by the GS FLX Titanium Sequencing machine. The sequencing run was performed at the McGill University and Genome Quebec Innovation Centre (Montreal, QC) on ¼-PicoTiter plate. The *amoA* and *nxrB* gene sequences generated were trimmed and filtered using the QIIME Pipeline (Caporaso et al. 2010) to retain only good quality sequences devoid of primers and barcodes. Quality filtered sequences (minimum read length of 200 bp, quality score >25, and without ambiguous bases and mismatches) were clustered at 97% sequence similarity and assigned to operational taxonomic units (OTUs) using the RDP classifier (FunGene Pipeline and repository) (Wang et al. 2007). Bacterial diversity analyses were performed using the *BiodiversityR* package of the R-software, version 3.0.1, based on normalized OTU abundance data.

3.2.4 Respirometric-response assessment of influent nitrifying biomass

In order to assess the metabolic status of nitrifying biomass in the influent wastewater, approximately 8-10 L of influent were collected from the LaPrairie WRRF and the biosolids were concentrated to about 2,500 mg/L by centrifugation at 4000×g using the International Equipment Company (IEC) model Centra-8 centrifuge (USA). The concentrated biosolids were used to perform batch respirometric assays, at 20 ºC using a sample volume of 500 mL, by the Challenge Technology ™ AER-208 (US) Respirometer System, to stimulate oxygen uptake rate (OUR) of AOB and NOB populations. A volume of 1mL of NH_4^+ and NO_2^- was added each at a concentration of 31.5 mg/mL and 27.1 mg/mL respectively as electron donors and the resulting OUR profiles were measured (Moussa et al. 2003). The pH was maintained at around 8.3 using 0.05 M HEPES buffer.

3.2.5 Steady-state modelling

The required absolute minimum solids retention time ($[SRT_{min}]_{\lim}$) was calculated at different temperatures under nitrifier seeding and non-seeding conditions. The two temperature-dependent parameters, decay rate (b_{ANO}), and maximum growth rate ($\mu_{\mathrm{ANO,max}}$) were determined using Eq. (3.1) and (3.2), respectively. Steady-state equations based on mass balances on the control volume (reactor and settling tank) were used to calculate the $[SRT_{min}]_{\lim}$ required to maintain nitrifiers in the bioreactors (Rittmann and McCarty 2001, Salem et al. 2003). The $[SRT_{min}]_{\lim}$ resulting from treatment of a given bioreactor under non-seeding and seeding conditions was computed using Eq. (3.3) and (3.4), respectively.

Default stoichiometric, kinetic and composition model parameters were adopted from ASM3 to perform the calculations (Henze et al. 2000).

$$b_{\mathrm{ANO}} = \left(b_{\mathrm{ANO,20\,^{\circ}C}}\right)[e^{0.098(T-20^{\circ}C)}] \quad (3.1)$$

$$\mu_{\mathrm{ANO,max}} = \mu_{\mathrm{ANO,max,20^{\circ}C}}[e^{0.069(T-20^{\circ}C)}] \quad (3.2)$$

$$[SRT_{min}]_{lim,normal} = \frac{1}{\mu_{\mathrm{ANO,max}} - b_{\mathrm{ANO}}} \quad (3.3)$$

$$[SRT_{min}]_{lim,with\ seeding} = \frac{1}{\mu_{\mathrm{ANO,max}} - b_{\mathrm{ANO}} + q_{\mathrm{add}}} \quad (3.4)$$

Where, b_{ANO} is the endogenous decay rate of autotrophic nitrifying organisms ($b_{\mathrm{ANO,20\,^{\circ}C}}$ = 0.15 d^{-1}), $\mu_{\mathrm{ANO,max}}$ is the maximum growth rate of nitrifiers ($\mu_{\mathrm{ANO,max,20\,^{\circ}C}}$ = 0.76 d^{-1}), q_{add} is the specific nitrifier addition rate per nitrifying biomass already present (d^{-1}), $[SRT_{min}]_{\mathrm{lim}}$ is the absolute minimum solids retention time (d) and T is the temperature (ºC). The equation derived for the $[SRT_{min}]_{lim}$ with seeding assumes that the amount of biomass is constant, and independent of the SRT and NH_4^+/TKN loading. However, the biomass inventory can actually change with SRT and NH_4^+/TKN loading (*statement added after publication in Water Science & Technology, 2014*).

3.3 RESULTS AND DISCUSSION

3.3.1 Presence of nitrifiers in influent and mixed liquor

Nitrifiers (both AOB and NOB) were detected by PCR in all municipal influent samples collected from the three WRRFs. Defining OTUs as sequences with 97% similarity resulted in an average of 371 distinct OTUs in the influent samples and 236 OTUs in the mixed liquor samples for the AOB sequences. Less OTUs were detected for the NOB sequences with an average of 99 OTUs in the influent and 83 OTUs in the mixed liquor samples (Table 3.2). Similarly, the evenness of the OTU distributions was also generally lower in the mixed liquor than in the influent samples (Table 3.2). For both groups, the lower diversity for the mixed liquor than for the influent samples suggests that the activated sludge process operates some sort of selection or that the number of niches in the treatment process is lower than in the sewer system where the OTUs entering the plants originated.

Explicit comparison of the sequence reads showed a high level of sharing between the nitrifying AOB and NOB OTUs in the influent and mixed liquor samples (Table 3.2), indicating that the dominant OTUs in the influent and the mixed liquor were the same. The percentage of reads belonging to OTUs that appeared in both influent and mixed liquor of the same WRRF averaged 78% for AOB and 86% for NOB. The most abundant AOB and NOB OTUs in the influent also occurred as the most abundant OTUs in the mixed liquor at all 3 WRRFs. The identity of these most abundant OTUs changed between plants, suggesting variations in the seeding population. These findings strongly support the hypothesis that influent nitrifiers seed and structure the nitrifying bacterial populations in the aerated activated sludge mixed liquor.

The geographic location of the treatment facilities, the source of the wastewaters (e.g., residential or industrial), and the type of sewer system (seperated domestic and stormwater or combined) or the infiltration rate from the soil, may be factors determining the seeding populations. We are currently assembling samples from a larger pool of treatment facilities from around Montréal and different parts of the world to ascertain the importance of these factors and generalize the observations reported herein. Early results suggest that the presence of nitrifiers in the wastewater can be generalized.

Neutral models describing bacterial community dynamics suggested that immigration and/or random birth events dominate the bacterial community assembly in activated sludge treatment systems (Ofiţeru et al. 2010). The present study suggests that the main driver of the species assembly of the nitrifier population is in fact immigration through seeding of autotrophic nitrifying bacteria from influent. The scale of bacterial immigration from the source community is likely to be dependent on the size of the source bacterial reservoir with the immigration rate being higher when the source community size is small (Curtis and Sloan 2006). Hence, seeding of nitrifiers in wastewater treatment systems may be more significant as compared to seeding of heterotrophic bacterial populations since nitrifiers are much less diverse than heterotrophs. According to Curtis et al. (2006), AOB have a low diversity in WRRFs with only 100-200 species growing in WRRFs in a global community of 10^{27} individual bacteria. This is in line with the data presented herein with the AOB population comprising 99-394 OTUs and the NOB population comprising 77-92 OTUs.

Table 3.2. Diversity of the ammonia and nitrite oxidizing populations in influent and mixed liquor samples of the three activated sludge wastewater treatment plants.

WRRFs	Ammonia oxidizing *Bacteria* (AOB)							Nitrite oxidizing *Bacteria* (NOB) from phylum *Nitrospira*[c]						
	Number of OTUs		Shannon's Evenness		Overall shared reads (%)[a]	3 most abundant OTUs[b]		Number of OTUs		Shannon's Evenness		Overall shared reads (%)[a]	3 most abundant OTUs[b]	
	Influent	Mixed Liquor	Influent	Mixed Liquor		Shared reads (%)[a]	Identity	Influent	Mixed Liquor	Influent	Mixed Liquor		Shared reads (%)[a]	Identity
Cowansville	454	394	0.72	0.70	90	50	OTU2 OTU5 OTU141	131	92	0.36	0.23	95	85	OTU188 OTU441 OTU469
LaPrairie	396	99	0.73	0.59	64	53	OTU2 OTU5 OTU141	83	77	0.54	0.33	83	75	OTU225 OTU441 OTU501
Vaudreuil	263	215	0.61	0.53	80	79	OTU56 OTU765 OTU1393	82	81	0.56	0.55	80	62	OTU225 OTU374 OTU441

$$a\text{: \% } shared\ reads\ = \frac{\frac{No.of\ reads\ of\ shared\ OTUs\ from\ influent}{Total\ no.of\ reads\ from\ influent} + \frac{No.of\ reads\ of\ shared\ OTUs\ from\ mixed\ liquor}{Total\ no.ofreads\ from\ mixed\ liquor}}{2} \times 100\%$$

[b]: These OTUs were the 3 most abundant in both the influent and mixed liquor samples.

[c]: The NOB PCR primers detect only the *Nitrospira* phylum. It is, however, the dominant nitrite-oxidizing population in wastewater treatment systems.

3.3.2 Activity of nitrifiers present in influent wastewater

In the previous section, the presence of nitrifiers in the influent was detected using a PCR assay; and the pyrosequencing results showed that the most abundant OTUs in the mixed liquor corresponded to the ones in the influent. The data support the hypothesis of a significant seeding of the mixed liquor nitrifiers by the influent. The next question to ask is: are the nitrifiers in the influent active? To evaluate this question, respirometric assays were performed and showed that the influent AOB and NOB populations responded immediately to the addition of their respective electron donors. However, it took 6.5 h and 4.5 h, respectively, for the AOB and NOB to attain full metabolic activity (Figure 3.1). Therefore, nitrifiers entering WRRFs are alive, but they require a few hours to reach full metabolic induction. Because the induction time is much lower than the SRT (at least a few days), the influent nitrifiers should reach full-induction upon adsorbtion to the activated sludge flocs, thus, effectively seeding the activated sludge systems continuously. Based on the maximum activity data, the level of nitrifiers in the influent of LaPrairie WRRF was estimated at 5 mg-$COD_{biomass}$/L, which corresponded to a seeding level at this site of approximately 0.3 g of nitrifiers per day per gram of nitrifiers already present (as estimated by NO_3^- productions and N-balances). The level of biomass observed in the influent of LaPrairie WRRF justifies further considering potential seeding for modelling and design purposes.

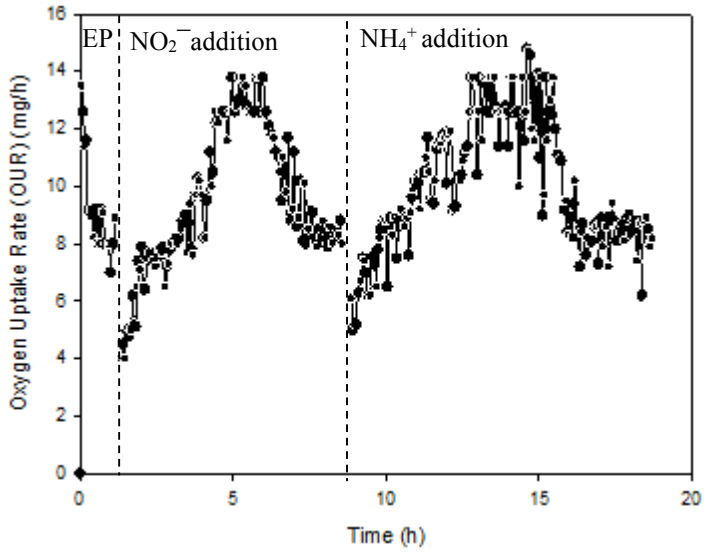

Figure 3.1. OUR profile obtained from respirometric analysis. NO_2^- and NH_4^+ were added as substrate at 1.25 h and 8.50 h, respectively. EP-Endogenous Phase.

3.3.3 Impact of nitrifier seeding on design SRT

The impact of the seeding of nitrifiers observed at LaPrairie WRRF was estimated by comparing the absolute minimum solids retention times ($[SRT_{min}]_{lim}$) without and with seeding (~0.3 g of nitrifiers per day per gram of nitrifiers already present). The specific nitrifier addition rate was for both AOB and NOB combined and was not partitioned into these two groups during the simulation study. Seeding did not make much of a difference on the $[SRT_{min}]_{lim}$ at 20 °C (Figure 3.2). However, as the temperature decreased, the effect on the $[SRT_{min}]_{lim}$ became more important, and the $[SRT_{min}]_{lim}$ was reduced by approximately 56% at 5 °C as compared to non-seeding condition (arrow in Figure 3.2).

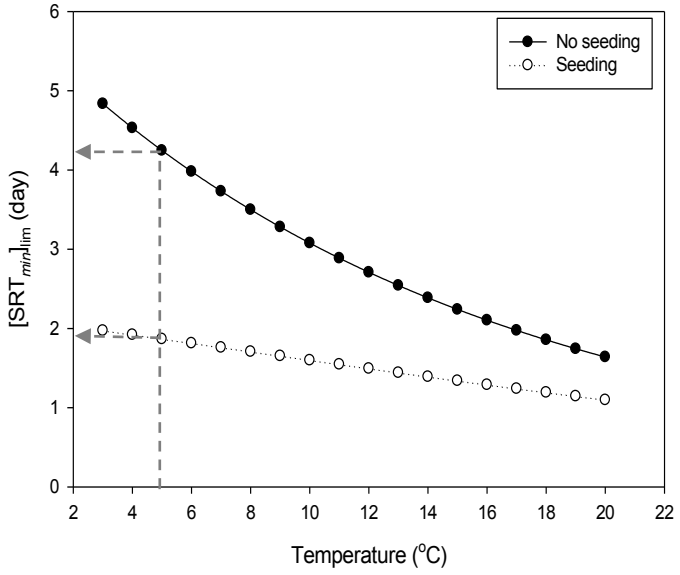

Figure 3.2. Effect of influent nitrifier seeding (0.3 g/d/g of nitrifiers already present) on the $[SRT_{min}]_{lim}$ of an activated sludge system at different temperatures.

These findings demonstrate the gains that can be made in design if seeding of nitrifiers by the influent stream is considered. The significant reduction in $[SRT_{min}]_{lim}$ can be achieved by considering influent nitrifier seeding. This reduction in $[SRT_{min}]_{lim}$ has direct impact on the sizing of bioreactors in cold regions. Illustrating this fact, let us consider the heterotrophic parameter values and influent composition given by Henze (2000) along with the nitrification parameters listed in materials and methods. Let us assume that a safety factor of 5 is applied to the operation SRT during the design (i.e., $SRT_{operation} = 5 \times [SRT_{min}]_{lim}$). Assuming that the designer aims at

keeping the same MLVSS for the two designs at 5 °C, the reduction of 56% of the $[SRT_{min}]_{lim}$ achieved by considering the natural seeding of the plant translates in a reduction of 30% of the reactor volume. This explicitly shows that considering nitrifier seeding from the influent stream can significantly reduce the size of aeration tanks at the design stage. Alleviating over-sizing of reactors would translate in reduction in capital expenditure for the construction of wastewater treatment infrastructures. Potentially, this would also reduce the costs of operation because lower oxygen demands would follow as the oxygen demand by the plant is proportional to the SRT (Rittmann and McCarty 2001). Finally, although our study shows the existence of potential seeding from influent municipal wastewaters to activated sludge bioreactors, the extent and efficiency of such seeding still needs to be assessed.

3.4 CONCLUSIONS

It is the first time that the correspondence of AOB and NOB OTUs in the influent and mixed liquor of activated sludge WRRFs is demonstrated. Our findings support the existence of a natural seeding of nitrifiers from influent streams to full-scale activated sludge reactors that may be contributing to the nitrification process in these systems. Consideration of influent nitrifier seeding showed that the required $[SRT_{min}]_{lim}$ is significantly reduced at cold temperature. This may be of importance for the design and sizing of bioreactors operating in cold climate. Finally, these findings may help improve operation optimality with the aim of sustaining nitrification all year-round, including extreme winter seasons.

3.5 ACKNOWLEDGEMENT

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CHAPTER 4

Influent wastewater nitrifiers determine the population structure in activated sludge treatment systems

Connecting text: Developing the findings of Chapter 3, we further explored the natural seeding phenomenon of full-scale bioreactors with nitrifiers from raw municipal wastewaters. In Chapter 4, findings on the microbial community compositions of influent wastewaters and activated sludge bioreactors from five additional wastewater treatment plants are presented. Also, the similarity between the influent wastewater and activated sludge mixed liquor population structures was statistically tested using a newly developed permutation procedure. All data strongly supported the hypothesis that the influent and mixed liquor community structures were similar. Besides providing new insights for modeling activated sludge wastewater treatment systems, the work has significant ecological implications on the impacts of influent nitrifying populations in structuring activated sludge nitrifying communities. The assembly of bacterial communities in open biological systems such as activated sludge has long been considered chaotic and unpredictable. Debate is still ongoing regarding the two main opposing principles involved in bacterial community assembly in natural and engineered ecological systems: the neutral theory in which immigration is a key component, and the niche theory which involves forces of selection and species sorting, to structure microbial communities. In the current study, it is showed that immigrant nitrifying communities in raw sewage determined the structure of nitrifying bacterial communities in activated sludge.

The results of this research will be submitted in this paper:

Shameem Jauffur, David Stephens, Zeinab Bakhshi and Dominic Frigon. Activated sludge wastewater treatment nitrifying populations determined by natural influent seeding. To submit to *The ISME Journal-Nature* (in preparation).

4.1 INTRODUCTION

Understanding the mechanisms shaping and modulating the structure and composition of microbial communities in ecosystems is an underpinning theme in ecology, and a variety of these mechanisms remains to be examined in greater details (Dini-Andreote et al., 2014). Such aspect acquires accrued importance in engineered systems where understanding and predicting microbial behavior can lead to the control and optimization of microbiologically-mediated transformations. When it comes to describing processes shaping local microbial communities in an ecosystem, two main opposing but not mutually exclusive, ecological theories clash namely the niche principle and the neutral theory of biodiversity (Curtis et al., 2006). According to the niche principle, the structures of microbial communities are exclusively shaped by deterministic elements such as environment, competition and niche differentiation (Ramette and Tiedje, 2007). Anathematic to the niche principle, the neutral theory of biodiversity is based on innate stochastic factors, relying on the provocative assumption that species performing the same ecological function are competitively neutral, and considers random processes of birth, death and limited dispersal through immigration to explain patterns of species abundances in communities (Bell, 2000, Hubbell, 2006). Recently, neutral community models and concepts have attracted attention in the field of wastewater treatment, and their contribution in the assembly of bacterial communities in activated sludge systems has gained more and more recognition (Sloan et al., 2006). However, the exact mechanisms by which the activated sludge communities are assembled and the role of the influent in influencing their structures remain elusive.

Wastewater treatment plants, now more appropriately termed as Water Resource Recovery Facilities (WRRFs) (Vanrolleghem and Vaneeckhaute, 2014), are inherent open systems relying on a rich and complex microbiome comprising of an assembly of active microorganisms to treat a wide array of contaminants in wastewater (Ibarbalz et al., 2013). They are considered as the most important biotechnological application worldwide (Wagner et al., 2006). Besides their important role in modern sanitation, biological WRRFs are uniquely suited systems for whole-scale ecosystem experimentation due to the level of physical, chemical and biological controls exerted on the processes (Daims et al., 2006, Vuono et al., 2015). Attempts to study neutral models involving microbial communities in activated sludge systems have mainly been made using functionally similar groups of bacteria such as nitrifiers, which are restricted to only a few

prokaryotic lineages and have specific ecological functions (Könneke et al., 2005). Considering the very large breadth of possible heterotrophic functions in a WRRF and the difficulties to experimentally ascertain them to given populations (Quince et al., 2008), it is impractical to study neutral models using heterotrophic bacterial populations if one wants to set study boundaries to populations performing ecologically similar functions (Cui et al., 2014). The alternative is to study populations with clear functional and phylogenetic assignments such as autotrophic ammonia oxidizing bacteria (AOB) (Koops and Pommerening-Röser, 2001). This is why neutral community model investigations have focused on AOB in the past (Sloan et al., 2006). In addition, there is considerable interest in understanding the ecology of nitrifiers since nitrification is the Achilles heel of many wastewater treatment systems, and the causes for nitrification failures are not always obvious (Wagner and Loy, 2002).

Neutral community models are based on the metacommunity concept which involves a pool of individuals from which local communities are assembled at random. The source communities are spatially linked to the sink communities through dispersal and immigration (Nemergut et al., 2013) as illustrated in Figure 4.1a. In this way, the environment is assumed to be homogenous and individuals have equal abilities of establishing themselves in the local communities (Pandit et al., 2009).

Neutral dynamics follow a process of stochastic drift with microorganisms migrating from the metacommunity to the local community. The resulting distribution in species abundances in local communities can be statistically modeled to determine the probability of migration within the relative locations of the communities (Pender et al., 2004, Rowan et al., 2003). By developing a model of taxa abundance under the assumption of neutral community assemblage, Sloan et al. (2006) showed that the expected relative abundance of a given taxon is equal to its average relative abundance in the metacommunity, and that the dispersions of observations between plants for a given taxon follow a beta distribution with the variance decreasing with increasing immigration rate.

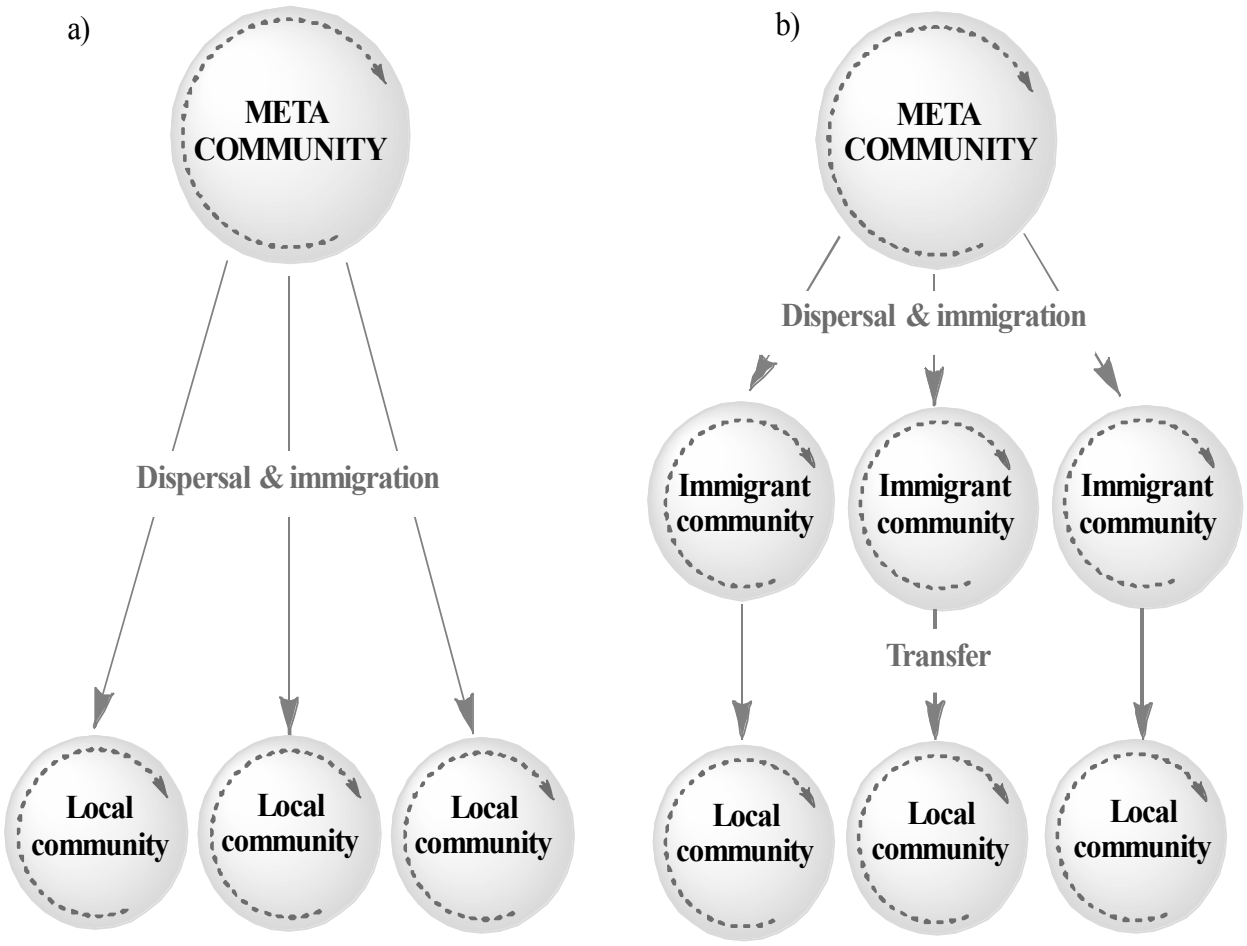

Figure 4.1. Metacommunity frameworks. a) Basic metacommunity concept involving sets of local communities spatially connected to a metacommunity through dispersal and immigration of potentially interacting species. This concept forms the basis of neutral community models which disregard differences between species at same trophic levels, and consider birth, death, immigration and limited dispersal to explain community composition and structure. b) Explicit elaboration of metacommunity framework to show that assembly of local communities (activated sludge communities herein) can occur in an external setting (the sewer system herein), and the assembled transitory immigrant community is neutrally transferred to the local community. Solid arrows represent direction of dispersal and immigration of species; circular dashed arrows represent selective pressures such as environment acting at the level of the metacommunity, immigrant and local communities to further structure community composition and diversity.

Although metacommunities at one end and local communities at the other have been considered

in neutral ecological models, the mechanisms linking the two have rarely been scrutinized. Specifically for activated sludge WRRFs, the influent represents a relatively large transferring community that could have profound impact on structuring the activated sludge community. Alternatively, the influent community may be shaped by strong seeding from human waste and groundwater infiltration, and selection in sewers with usually relatively low or no dissolved oxygen; thus the influent community may be quite different from the one found in activated sludge reactors. Consequently, the question on the system boundary where the community assemblage takes places arises, and it becomes critical to study the links between the local community found in the reactor and the transferred communities found in the influent wastewater (Figure 4.1b). If the sewer environment plays a different role than the activated sludge in selecting microbial community members, one would predict that the local communities would be better predicted by the average activated sludge community than by the influent community. On the other hand, if neutral community assemblage takes place in the sewer system under limited dispersal followed by transfer to the activated sludge, one would expect that the influent community structure would be a better predictor of the activated sludge community with the geographic distance between plants also playing an important role. Finally, specific interactions between different groups of microorganism may also be important in structuring the microbial community. These represent a set of testable alternative hypotheses to answer the question regarding the location of the community assembly (sewer system vs. activated sludge reactor) and the role of site location in structuring activated sludge communities.

In this study, we examined the composition of nitrifying bacterial populations (Ammonia Oxidizing Bacteria, AOB and *Nitrospira*-related Nitrite Oxidizing Bacteria, NOB) in raw wastewater as they are conveyed to full-scale wastewater treatment systems, and assess their potential impacts on the structure of nitrifying bacterial communities in wastewater treatment bioreactors. We modeled the contribution of the population structures of the influent community, physical distance between plants, and co-structure of the two guilds. In the long run, understanding the impact of seeding from wastewater on the resulting nitrifying activity and population assembly in activated sludge systems is crucial for full-scale bioreactor design as it determines the size of the reactor and the associated costs.

4.2 MATERIALS AND METHODS

4.2.1 Study sites and sample collection

Municipal influent (24-hour composite) samples were collected at the entrance of 8 full-scale biological Water Resource Recovery Facilities (WRRFs) located near Montreal (Quebec, Canada). Grab activated sludge was correspondingly sampled from the aerated bioreactors of each facility during the winter season (February-March 2013). The different WRRFs received wastewater flows ranging from 5,000 to 65,000 m^3/day and use the activated sludge type process bearing different layouts and configurations. The geographic locations of the sampling sites, main operational conditions, sources (residential or industrial) and characteristics of wastewaters are provided in the Supplementary Material (Supplementary Figure S4.1 and Table S4.1). Collected samples were immediately transported to the laboratory on ice with a travel time of 2-4 hours. Upon arrival at the laboratory, the samples were centrifuged (Microcentrifuge, Thermo Scientific, Sorvall Legend Micro 21R, USA) in 1.5 mL tubes and frozen at −80 °C until analysis.

4.2.2 Molecular techniques

Genomic DNA was extracted from 0.25 g of wet centrifuged solids using the *MO BIO* UltraClean™ Fecal DNA Kit (Carlsbad, CA) based on the manufacturer's protocol. The extracted DNA samples were diluted to 12 ng/mL and used as templates to amplify nitrification genes using primers targeting the *amoA* functional gene from AOB (Rotthauwe et al. 1997), *amoA* gene from Ammonia-Oxidizing *Archaea* (AOA) (Pester et al. 2012), *nxrB* gene from *Nitrospira*-related NOB, and *nxrB* gene from *Nitrobacter*-related NOB (Maixner 2009). The primer sequences and PCR thermocycling programs are shown in Table S2 of the Supplementary Materials. Each 50µl of PCR reaction mixture contained 0.5 µM of forward and reverse primer each, 1×5X Bioline PCR colorless buffer (Taunton, MA, USA), 2.75 mM $MgCl_2$, 250 µM dNTP (each), 12 ng/mL DNA template and 2.5 units Bioline Taq DNA Polymerase (Taunton, MA, USA) in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, USA). The PCR amplicons were purified using the *MO BIO* UltraClean™ PCR Clean-Up Kit (Carlsbad, CA) to remove primers, enzyme, buffer and primer-dimers.

For 454-pyrosequencing, only the primers targetting the *amoA* gene from AOB and *nxrB* gene from *Nitrospira*-related NOB were used because initial screening studies showed that *amoA* from

AOA and *nxrB* from *Nitrobacter*-related NOB were at low abundances such that their amplification resulted in very low concentration of PCR products or no product at all. Hence, focus was oriented towards the study of *amoA* from AOB and *nxrB* from *Nitrospira*-related NOB since they have been identified in previous studies as the most dominant populations in wastewater treatment systems (Wittebolle et al. 2008). The gene- and population-specific primers were incorporated in fusion primers to amplify community DNA by PCR for 454-pyrosequencing. Forward primer sequences were tagged with a 26-base primer A key, a 4-base library key, and a 10-base unique multiplex identifier (MID) barcode (Liu and Jansson 2010). All primers were checked *in silico* for the formation of stable hairpins, homodimers and heterodimers using the IDT OligoAnalyzer 3.1, and were synthesized by Integrated DNA Technologies (IDT), Inc. (USA).

4.2.3 GS FLX Titanium 454-pyrosequencing

The quality of the PCR amplicons was assessed using the DNA 1000 microfluidic chip (Agilent Technologies, USA) and the Bioanalyzer 2100 (Agilent Technologies, USA). Any amplicon contaminated with traces of unspecific fragments was further purified using the Agencourt AMPure^{TM} PCR purification kit (Beckman Coulter, Beverly, MA, USA). The concentration of each PCR amplicon was determined using the Quant-iT™ PicoGreen kit (Invitrogen, Carlsbad, USA) and normalized to a concentration of 30 ng/µl. Amplicon mixtures were subjected to emulsion PCR based on Roche-454 Life Science Protocol and pyrosequenced by the GS FLX Titanium Sequencing system using the unidirectional sequencing Lib-L chemistry. Sequencing was performed at the McGill University and Génome Québec Innovation Centre (Montreal, QC).

4.2.4 Analysis of pyrosequenced data

The *amoA* and *nxrB* fasta sequence data, flow and quality files were retrieved from raw Standard Flowgram Format (sff) files, de-multiplexed, trimmed and filtered using the QIIME software (Caporaso et al. 2010) to retain only good quality sequences devoid of primers and barcodes. USEARCH was used for sequence denoising and chimera removal (both *de novo* and reference-based). Additionally, chimeric sequences were analyzed with UCHIME using default parameters, and removed. Quality filtered sequences (minimum read length of 200 bp, quality score > 25, and without ambiguous bases and mismatches) were clustered into operational

taxonomic units (OTUs) at 97% sequence similarity using the Uclust algorithm (Edgar 2010), which is considered to be related to species level (Islam et al. 2015). Phylogenetic trees displaying the position of the most abundant AOB and NOB species were constructed using FastTree (Price et al. 2009) and visualized using FigTree v1.4.2. Taxonomic identification of dominant nitrifying bacterial OTUs was performed using the Functional Gene Pipeline and Repository of the Ribosomal Database Project (Fish et al. 2013) and BLASTN DNA query search (Altschul et al. 1990).

4.2.5 Population diversity and structural similarity between influent and mixed liquor

Bacterial diversity measures (Shannon entropy, Shannon evenness and Simpson diversity index) of the nitrifying populations in the influent and activated sludge samples were performed using PAleontological STatistics v3.0.8 (Hammer et al., 2001), based on standardized OTU abundance data. In order to assess the similarity between the influent and activated sludge nitrifying bacterial profiles, the Bhattacharya coefficient ($\boldsymbol{BC_{(p,q)}}$) of similarity was computed using the probability distributions of the OTUs from the two sample matrices using Eq. 4.1 (Sohn et al., 2015). The Bhattacharya coefficient provided a measure of the extent of overlapping between the OTU probability distributions of the influent and mixed liquor.

$$BC_{(p,q)} = \sum_i \sqrt{p_{Inf,i} q_{Ml,i}} \qquad \text{(Eq. 4.1)}$$

Where $\boldsymbol{p_{Inf}}$ and $\boldsymbol{q_{Ml}}$ are the relative abundances of the i^{th} OTU of the influent and activated sludge mixed liquor of the same WRRF, respectively.

The relationships between the population structures of the influent and respective activated sludge samples were tested using a non-parametric weighted rank-based statistic developed in this study. The null hypothesis (H_0) of different abundance-ranked distributions of OTUs was tested against the alternative hypothesis (H_1) of identical abundance-ranked distributions in the influent and activated sludge samples. The weighted rank-based test statistic was computed as shown in Eq. 4.2. The test statistics were tested for significance against probability distributions generated by permutation of observed abundances between OTUs 10,000 times, providing a minimum α-level of 0.0001.

$$\text{Test Statistic}_{\text{rank}} = \frac{1}{N(N-1)} \sum_{i=1}^{N} W_i (R_{i,inf} - R_{i,ml})^2 \qquad \text{(Eq. 4.2)}$$

Where N is the number of OTUs, $R_{i,inf}$ is the rank of the i^{th} OTU in the influent and $R_{i,ml}$ is rank of the i^{th} OTU in the mixed liquor. The weight component of each OTU (W_i) was incorporated in the above developed expression in order to standardize the dataset so that,

$$W_i = \frac{\max\{A_{i,inf}, A_{i,ml}\}}{\sum_{i=1}^{N} \{A_{i,inf}, A_{i,ml}\}} \qquad \text{(Eq. 4.3)}$$

Where $A_{i,inf}$ is the abundance of the i^{th} OTU in the influent and $A_{i,ml}$ is the i^{th} OTU in the mixed liquor. The test metric in Eq. 4.2 is sensitive to shift in OTU dominance between populations from different samples. Thus, it recognizes two ranked abundance OTU distributions as different if they are characterized by high dominance of a few but different OTUs. All scripts for the probability testing were written and processed in R v3.2.1 (RCoreTeam, 2015).

4.2.6 Variance partitioning of nitrifying communities

To further understand the basic predictors involved in shaping the activated sludge nitrifying bacterial communities, we analyzed the explanatory power of the following variables related to specific hypotheses (see Results section): influent AOB (or NOB) population structures (main hypothesis), site location (Alternative Hypothesis 1), activated sludge *Nitrospira*-related NOB (or AOB, respectively) population structures (Alternative Hypothesis 2), and average activated sludge AOB (or *Nitrospira*-related NOB, respectively) population structure for all WRRFs (Alternative Hypothesis 3). These explanatory matrices were used to partition the variance in AOB or *Nitrospira*-related NOB activated sludge population structures among sites (Legendre and Legendre, 2012). The variance partitioning with the variables representing the first two alternative hypotheses was obtained by redundancy analysis (RDA) on population structure datasets transformed to the Hellinger distance ($H_{(p,q)}$), which is related to the Bhattacharya coefficient ($BC_{(p,q)}$) of similarity by Eq. 4.4.

$$H_{(p,q)} = \sqrt{1 - BC_{(p,q)}} \qquad \text{(Eq. 4.4)}$$

In this analysis, site locations were represented by latitude-longitude coordinates which were converted to principal coordinates of neighbor matrices (PCNM) eigenfunctions (Prevost-Boure et al., 2014)**.** Variance partitioning was performed using the *varpart* function of the vegan library in the R software (Oksanen et al., 2013). Partitioning the activated sludge population structure variances between influent population structures and average activated sludge population structures (Alternative Hypothesis 3) were performed by simple ordinary least square regressions. The relative OTU abundances for this analysis were not transformed. Calculations were performed in Microsoft Excel 2010.

4.3 RESULTS

4.3.1 Diversity of nitrifying bacterial populations

The high-quality pyrotag sequences revealed the presence of both AOB and *Nitrospira*-related NOB in all influent samples of the 8 WRRFs. All sequence reads were processed at 97% similarity in order to generate unique sets of non-redundant OTUs from each sample (Table 4.1). On average, 337 and 221 AOB OTUs were obtained for the influent and mixed liquor samples, respectively, while averages of 96 and 74 *Nitrospira*-related NOB OTUs were obtained from the same respective samples. Although the OTU richness was generally much higher in the influent than in the activated sludge samples, the Hill diversity (based on the Shannon index) and Simpson diversity numbers did not show similar trends (Table 4.1). This was due to the presence of 1-4 markedly dominant OTUs in each sample as can be seen in the ranked-abundance OTU distributions (Supplementary Figure S4.2 and S4.3). Rarefaction curves depicting OTU richness reached the plateau level for some influent and mixed liquor samples indicating that almost complete diversity had been sampled, and any further sampling would have likely yielded only a few rare species (Supplementary Figure S4.4).

Since the assembly of bacterial communities in conventional activated sludge bioreactors is largely embodied in the bacterial diversity of the systems (Valentín-Vargas et al. 2012), the correlation between the diversities of nitrifying bacterial populations of the influent and mixed liquor samples was evaluated. Based on the Shannon diversity index, the influent and mixed liquor diversities were found to be highly correlated. The correlation coefficients of the Shannon diversity were 0.91 and 0.90 for the AOB and *Nitrospira*-NOB, respectively (Figure 4.2). This

suggests that the diversity of nitrifiers in the influent somehow determined the diversity of nitrifiers in the mixed liquor.

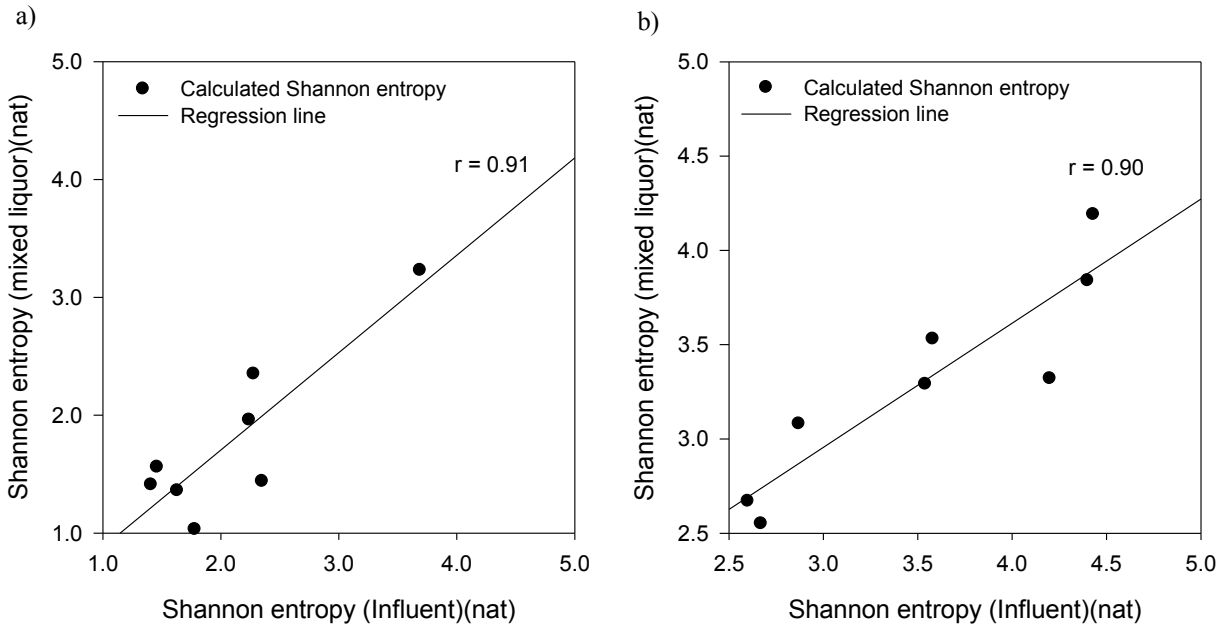

Figure 4.2. Correlation between Shannon entropies of influent and mixed liquor samples for AOB (a) and *Nitrospira*-related NOB (b) populations.

Table 4.1. Number of denoised sequences and OTUs, and diversity indices for AOB and NOB populations.

WRRFs	Ammonia Oxidizing *Bacteria* (AOB)									Nitrite Oxidizing *Bacteria* (NOB)								
	No. of denoised reads	No. of OTUs		Shannon diversity				Simpson diversity no.		No. of denoised reads	No. of OTUs		Shannon diversity				Simpson diversity no.	
				Entropy (nat) / Hill no.[c]		Evenness							Entropy (nat) / Hill no.		Evenness			
		INF[a]	ML[b]	INF	ML	INF	ML	INF	ML		INF	ML	INF	ML	INF	ML	INF	ML
Pooled all	30762	1380	1037	4.48 / 88	4.25 / 70	0.62	0.61	15	16	30991	450	352	2.73 / 15	2.49 / 12	0.45	0.42	5	4
Site by site																		
Cowansville	3818	454	394	4.43 / 84	4.19 / 66	0.72	0.70	14	11	3770	131	92	1.78 / 6	1.03 / 3	0.36	0.23	2	2
Farnham	4025	287	265	3.58 / 36	3.53 / 34	0.79	0.69	9	8	3896	202	51	3.69 / 40	3.23 / 25	0.70	0.67	15	15
Granby	3568	369	102	4.40 / 81	3.84 / 47	0.75	0.67	14	17	4015	55	72	1.46 / 4	1.56 / 5	0.36	0.37	3	3
LaPrairie	3876	396	99	4.20 / 67	3.32 / 28	0.73	0.59	11	11	3768	83	77	2.35 / 10	1.44 / 4	0.54	0.33	4	2
Marieville	4032	398	195	2.87 / 18	3.08 / 22	0.66	0.58	5	12	3943	64	96	1.41 / 4	1.41 / 4	0.52	0.31	2	2
Pincourt	3950	163	321	2.67 / 14	2.55 / 13	0.67	0.72	5	7	4016	87	52	2.24 / 9	1.96 / 7	0.50	0.53	5	4
Salaberry	3843	363	180	3.54 / 34	3.29 / 27	0.66	0.78	7	8	3866	62	69	1.63 / 5	1.36 / 4	0.39	0.32	3	2
Vaudreuil	3650	263	215	2.60 / 13	2.67 / 14	0.61	0.53	6	6	3717	82	81	2.28 / 10	2.35 / 10	0.56	0.55	6	6

Abbreviations: [a] INF-Influent; [b] ML-Mixed Liquor

[c] Hill no. = exp [Shannon entropy]

4.3.2 Correspondence of influent and mixed liquor nitrifying populations

By comparing the 10 most abundant OTUs of each sample, a discernible pattern emerged with the influent and activated sludge samples sharing the same AOB and *Nitrospira*-related NOB OTUs (Figure 4.3). This is especially true for the first 1-4 most abundant OTUs (depending on the sample and population) which were exactly the same in all the influent and mixed liquor samples from the same site. Based on the Bhattacharya coefficient, 60% to 81% of the relative distributions of the *amoA* sequence reads for the AOB populations were found to overlap between the influent and mixed liquor samples from the same site, while 64% to 96% of the *nxrB* sequence reads were found to overlap for the *Nitrospira*-related NOB populations between the two sample matrices (Figure 4.3). These findings point to a strong seeding of the activated sludge bioreactors by nitrifying bacteria from incoming raw wastewaters.

The relationships between the nitrifying bacterial populations of the influent and mixed liquor samples were statistically tested using a rank-based metric (Eq. 4.2) and produced probability distributions by random permutation of OTU abundances within a sample (Supplementary Figure S4.5 and S4.6). Equation 4.2 represents the difference in the abundance rank of the various OTUs in the influent and mixed liquor samples from the same site weighted to the maximum abundance of the OTUs in either samples from that site. The null hypothesis (H_0) of no specific relationship in the ranks of OTUs between the influent and mixed liquor from the same site was tested against the left-handed alternative hypothesis (H_1) of less or no difference. Systematically for each site and population, the alternative hypothesis was accepted over the null hypothesis; therefore, there is a statistically significant relationship between the dominant AOB and *Nitrospira*-NOB OTUs of the activated sludge mixed liquor and the incoming raw wastewater. The relationship was found to be non-random, supporting the interpretation that the influent nitrifying population structure actually determined the activated sludge nitrifying population structure at each site. In ecological terms, the transfer of nitrifying biomass was, thus, neutral in that further competitive selection that would have affected the population structure did not take place.

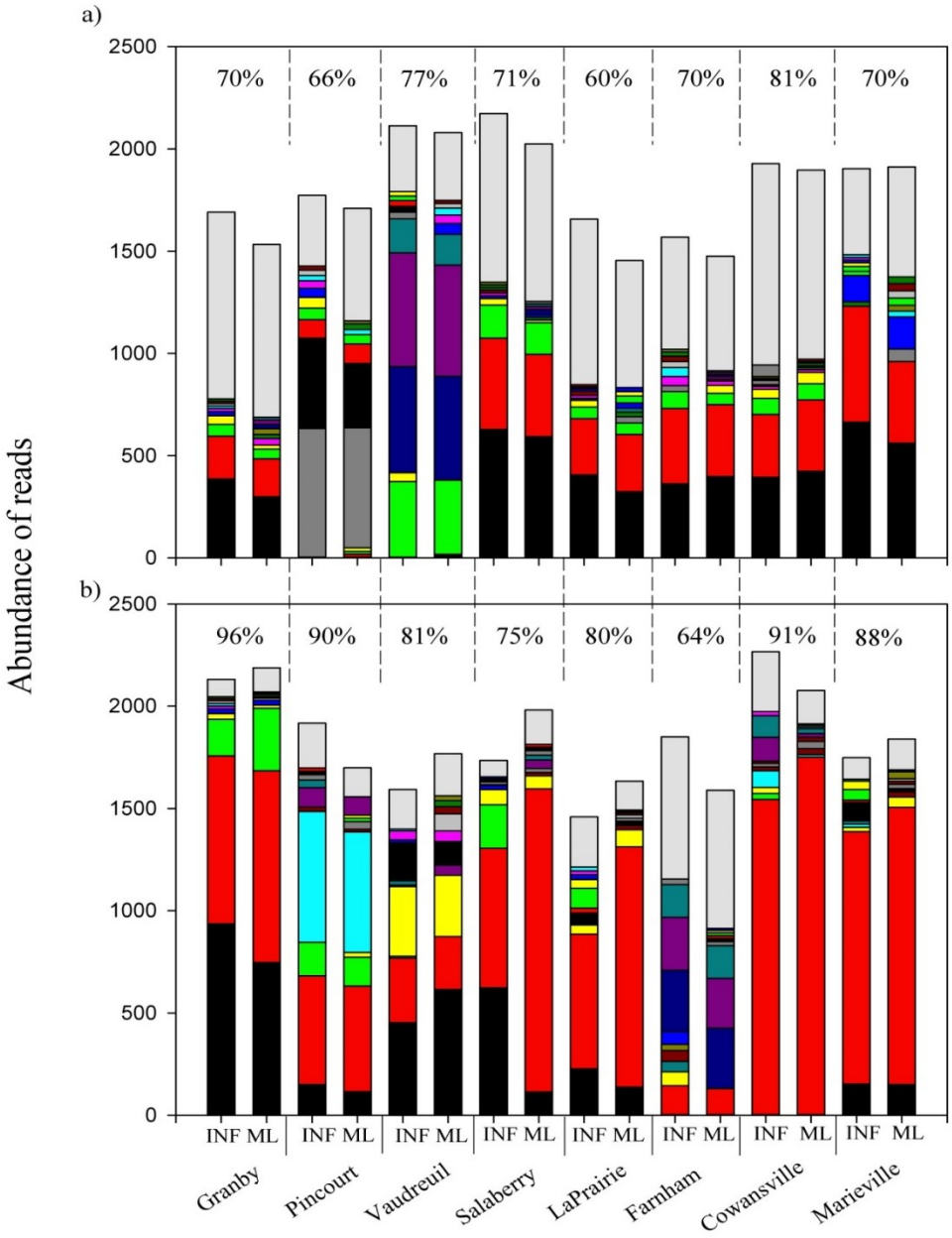

Figure 4.3. Distribution of 10 most abundant AOB (a) and Nitrospira-related NOB (b) OTUs in influent and mixed liquor at 8 full-scale activated sludge WRRFs. OTUs were generated using QIIME at a 3% cutoff level. Each OTU is coded with a unique color for all stacked bars, and the height of each bar represents the abundance of the OTU (for colored figure, see the web version of the article). The grey-colored portion at the top of each bar shows the rest of the OTUs. The percentage above each pair of bars is based on the Bhattacharya coefficient (BC), and indicates the extent of overlapping of the sequence reads from OTUs present in both influent and mixed liquor of a given WRRF (Eq. 4.1). Upper case sample descriptors: INF - influent; ML - mixed liquor.

4.3.3 Neutral transfer of nitrifying populations

To ascertain the interpretation of neutral transfer of nitrifier populations from influent wastewaters to activated sludge, three other hypotheses were considered. They were:

1. Determination of the population structures based on relative location of the WRRFs (i.e., limited dispersal between systems).
2. Co-selection of the AOB and *Nitrospira*-NOB species (i.e., correlation between the abundances of AOB and *Nitrospira*-NOB OTUs).
3. Determination of the population structure by selection in activated sludge reactors (i.e., better prediction of mixed liquor abundance of OTUs by average abundances across mixed liquor samples than by influent OTU abundances).

Neutral transfer of nitrifying populations (predicting a higher explanatory power for the influent population structure than other explanatory variables) was contrasted to the first two alternative hypotheses by partitioning the variance across sites of the mixed liquor population structures using RDA. This analysis found no correlation between the AOB and *Nitrospira*-NOB mixed liquor population, thus, rejecting the Alternative Hypothesis 2. The nitrifying population structures in the influent and mixed liquor samples were found to be spatially organized; however, the spatial organization (referred to as site location in Figure 4.4) explained only an additional 13% of the variance in mixed liquor population structures, while the influent population structure accounted for more than 65% of the variance (with 28-44% explanation only attributable to the influent nitrifying community structure; Figure 4.4). This result suggests that the influent nitrifying population structure is the main factor determining the activated sludge nitrifying population structure (i.e., rejecting Alternative Hypothesis 1). Finally, the neutral transfer of nitrifying populations was contrasted to Alternative Hypothesis 3 by variance partitioning of the activated sludge population structures using regression over the site-specific influent and average activated sludge population structures (Table 4.2). The average activated sludge structures alone explained less than 1.5% of the site-specific activated sludge structures, while the site-specific influent population structures alone explained 20-43%; thus, rejecting Alternative Hypothesis 3. Consequently, the studied sites were found to exhibit a neutral transfer of nitrifying populations from the influent to the activated sludge communities.

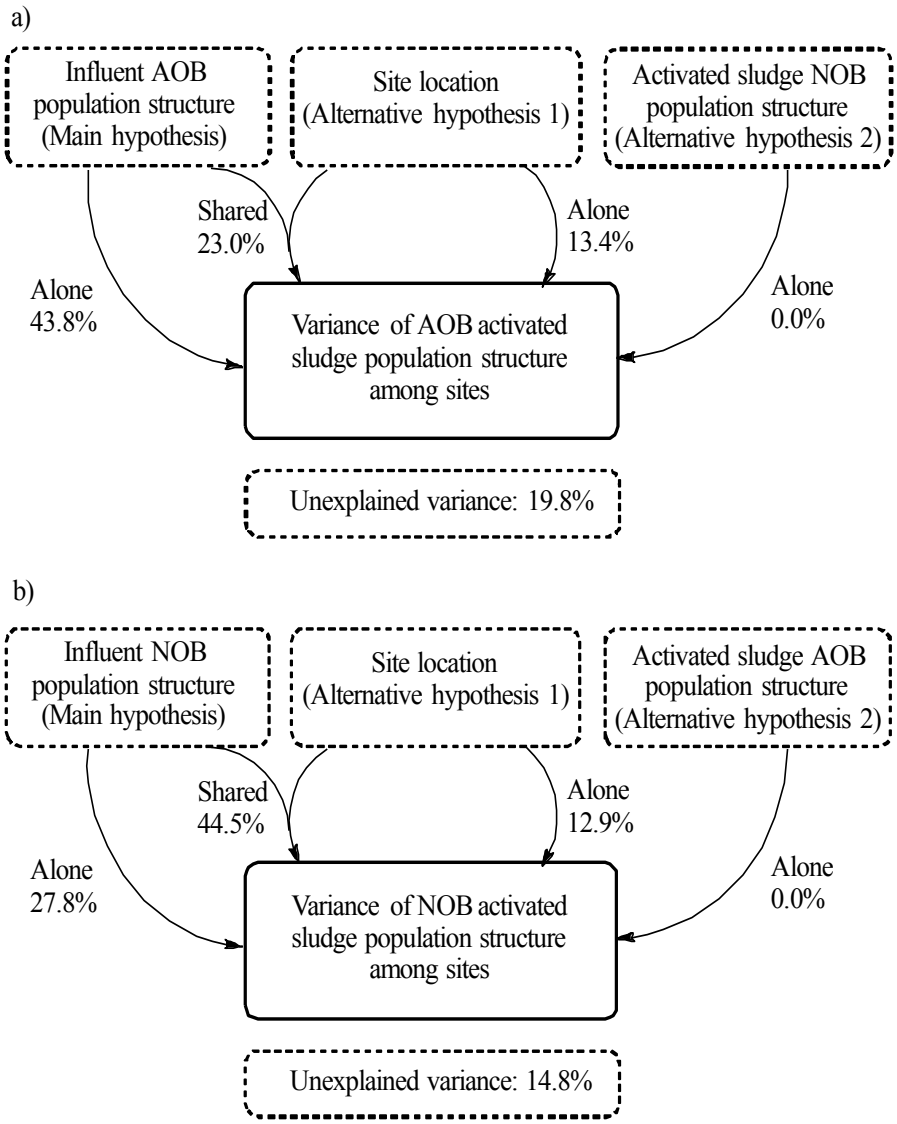

Figure 4.4. Evaluating the main hypothesis against Alternative Hypotheses 1 and 2 by partitioning the variance in nitrifying bacterial population structures among activated sludge sites between explanatory variables representing the various hypotheses (see text) for AOB (a) and *Nitrospira*-related NOB (b) populations using redundancy analysis.

Table 4.2. Evaluating the main hypothesis against Alternative Hypothesis 3 by partitioning the variance in nitrifying bacterial population structures among activated sludge sites between site-specific influent structures and average activated sludge structure.

	% variance	
Explanatory variables	**AOB**	***Nitrospira*-related NOB**
Site-specific influent population structure alone	43.0%	20.4%
Shared variance between influent population structure and site location	52.4%	67.3%
Average activated sludge population structure alone	0.2%	1.5%
Unexplained	4.4%	10.8%

4.3.4 Identity of dominant influent and mixed liquor taxa

Co-occurrence patterns have been observed between taxonomically related microorganisms derived from taxa sharing similar ecological niches (Ju and Zhang 2015). The taxonomic identity of the most abundant AOB and *Nitrospira*-related NOB OTUs in the influent and mixed liquor sample matrices was investigated in view of elucidating the phylogenetic relationships between them. Based on the Simpson diversity number (Table 4.1), which quantifies the number of dominant OTUs in a bacterial community (Jost 2010), the 16 most abundant AOB and *Nitrospira*-related NOB OTUs were identified to determine their phylogenetic affiliations (Figure 4.5).

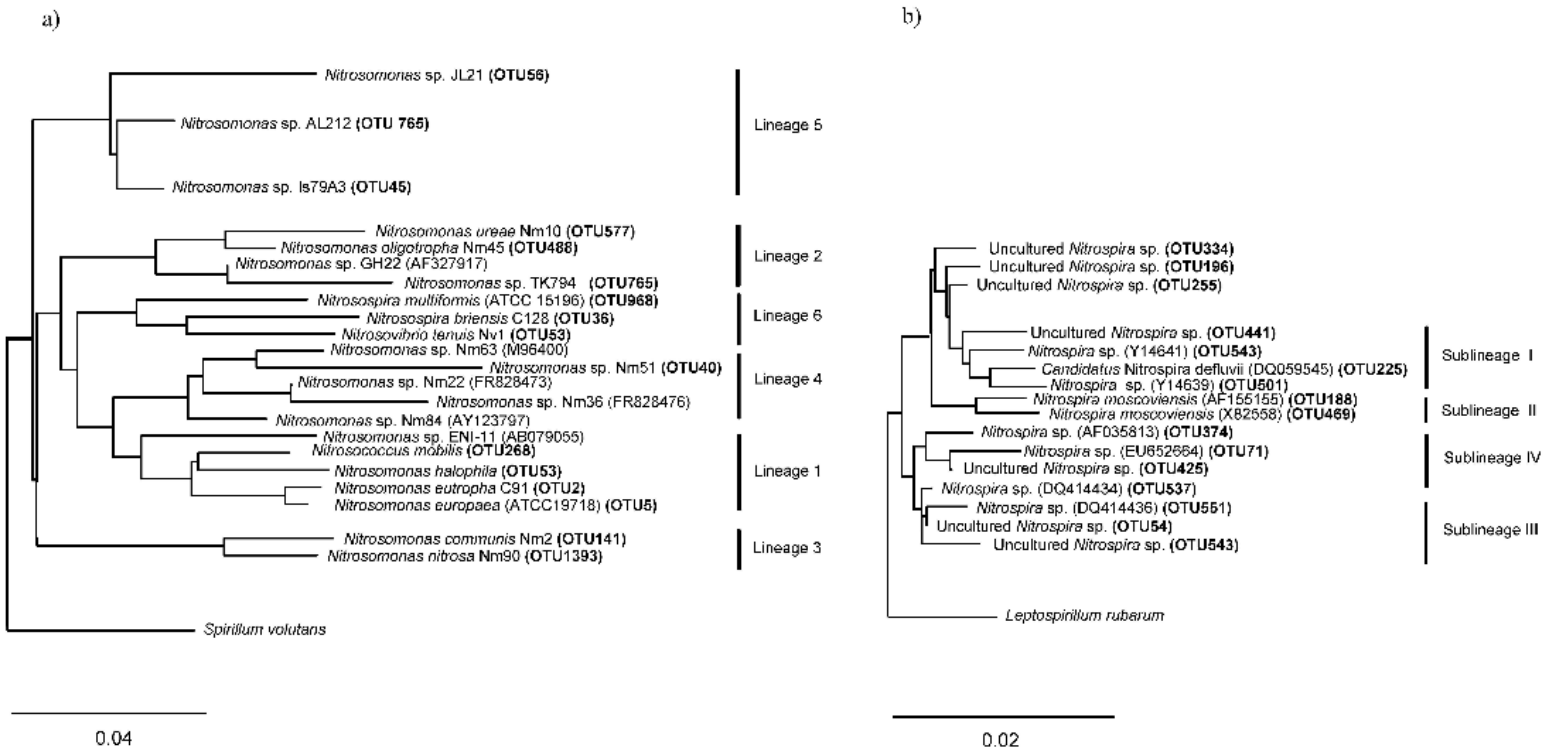

Figure 4.5. Phylogenetic trees showing the position of the most abundant AOB (a) and *Nitrospira*-related NOB (b) OTUs constructed at 3% cutoff level based on sequences from amplified *amoA* and *nxrB* genes. The species names correspond to entries in the database with identical sequence match as the ones for the OTUs identified in brackets. Entries without species designations are reported by their GenBank accession numbers. AOB lineages were defined by Brenner and Staley (2005) and Pommerening-Röser and Koops (2005), while NOB sublineages were defined by Lipski et al. (2001) and Ward et al. (2011). The scale bars represent 0.04 and 0.02 substitution per nucleotide position for the AOB and NOB phylogenetic trees, respectively.

The AOB populations were classified under lineages defined by Brenner and Staley (2005) and Pommerening-Röser and Koops (2005), while the *Nitrospira*-related NOB populations were grouped under sublineages defined by Lipski et al. (2001) and Ward et al. (2011). Among the AOB populations, three species/OTUs were found dominant at six WRRFs (Cowansville, Farnham, Granby, LaPrairie, Marieville and Salaberry) (Figure S4.2): *Nitrosomonas europaea* (OTU5) and *Nitrosomonas eutropha* (OTU2) from Lineage 1, and *Nitrosomonas communis* (OTU141) from Lineage 3. The influent and mixed liquor at the two other WRRFs (Pincourt and Vaudreuil) had a different and unique set of most abundant AOB species/OTUs: *Nitrosomonas*

oligotropha (OTU488) and *Nitrosomonas ureae* (OTU577) from Lineage 2 and *Nitrosospira multiformis* (OTU968) from Lineage 6 at Pincourt WRRF; and *Nitrosomonas nitrosa* (OTU1393) from Lineage 3, *Nitrosomonas* sp. JL21 (OTU56) and *Nitrosomonas* sp. AL212 (OTU765) from Lineage 5 were found dominant at Vaudreuil WRRF. Among the *Nitrospira*-related NOB populations, dominant OTUs from four distinct lineages could be recognized. Mostly, two Sublineage I OTUs (OTU441: uncultured *Nitrospira* sp., and OTU225: *Candidatus* Nitrospira defluvii) were common to the top most abundant OTUs in the influent and activated sludge of five of the WRRFs. At Farnham WRRF, OTUs from Sublineage II and III were found to be dominant.

4.4 DISCUSSION

4.4.1 Ecological link through neutral transfer of nitrifying communities

Untreated wastewater has been recognized to harbor a complex array of microbial taxa with a high level of diversity and richness (McLellan et al. 2010). In the current study, we show the presence of nitrifiers (AOB and *Nitrospira*-related NOB) in raw municipal wastewaters reaching the eight full-scale biological WRRFs surveyed. Attempts to detect *amoA* gene from AOA and *nxrB* gene from *Nitrobacter*-related NOB resulted in low or no amplification during the PCR assays indicating low abundance of these nitrifying microorganisms at the studied sites. Previous studies have shown that mostly AOB are responsible for ammonia oxidation in activated sludge systems especially members of the betaproteobacterial genera *Nitrosomonas* and *Nitrosospira* (Park et al. 2006, Purkhold et al. 2000). For NOB, evidence in literature suggests that *Nitrospira* is the most dominant NOB in activated sludge systems, and not *Nitrobacter* as previously thought (Altmann et al. 2004, Edwards et al. 2013, Schramm et al. 1999). These may explain why a strong amplification signal was detected for AOB and *Nitrospira*-related NOB in the analyzed samples. The nitrifiers detected in the influent of a particular WRRF in a recent study were shown to be alive and capable of attaining full metabolic induction within a few hours (Jauffur et al. 2014). The level of nitrifiers in the influent of that treatment facility was estimated at 5 mg/L of biomass chemical oxygen demand (COD) based on respirometric assays.

Analysis of the bacterial community profiles showed that the identities of the 1-4 most abundant AOB and *Nitrospira*-related NOB OTUs were the same in the activated sludge and in the

corresponding influent wastewaters. The population dominance profiles of the activated sludge mixed liquor nitrifiers appear to be determined by the structure of the influent nitrifying bacterial populations. Based on variance partitioning, it was found that the influent nitrifying communities (for both AOB and *Nitrospira*-related NOB) were the main factor determining the activated sludge nitrifying population structure by explaining more than 65% of the observed variance in the activated sludge nitrifying communities. This is consistent with neutral community theory where the influent AOB and NOB population structures mirrored those of the activated sludge nitrifying populations, and their transfer to the activated sludge systems was ecologically neutral. Hence, the influent nitrifying community is the main predictor driving community assembly in these systems.

4.4.2 Assembly of activated sludge nitrifying communities: Where and how?

The findings of the current study indicate that the assembly of activated sludge nitrifying communities does not primarily start in the bioreactors, but in the sewer infrastructures conveying the wastewaters to the treatment facilities. The hydraulic transport of these already structured communities to the bioreactors then drives the assembly of nitrifying communities in activated sludge. In a previous study, Ofiţeru et al. (2010) showed that immigration was the dominant driver in shaping the relative abundance and diversity of AOB in wastewater treatment systems. Here, we show the non-chaotic nature of the nitrifying bacterial community assembly process in activated sludge systems, which is essentially determined by immigration. Thus, selection does not appear to be the major factor at the studied sites to influence the nitrifying population structures in the activated sludge; although it may have been a factor in the initial assembly in the sewer system. These results have a direct theoretical impact on the model proposed by Curtis et al. (2006), which is based on the neutral theory of community assembly where stochastic dispersal of nitrifying species dictates the impact of the metacommunity on the structure of local activated sludge nitrifying populations. The data presented here suggest that the proposed model is at best incomplete, in the sense that the metacommunity of nitrifiers does not stochastically influence the nitrifying activated sludge communities, but it rather influences the population assembly in the sewer system which is then transferred to the activated sludge reactors. Thus, it appears that influent wastewaters convey already assembled nitrifying guilds which colonize aerated bioreactors and contribute to nitrification.

The central problem to understand the ecology of nitrifiers in these systems, however, seems to be the physical source of the nitrifying populations entering the sewers and ultimately seeding wastewater treatment facilities. Studies have shown that microbial sewage communities originate from residential and industrial premises, rainwater inputs and surface runoffs (Shanks et al. 2013). These microbial communities can colonize strategic locations in sewers as biofilms on pipe surfaces or are deposited in the sediments along the pipe system (Chen et al. 2003). Although the source of nitrifiers in influent wastewaters has not been investigated in the current study, it is believed that they originate primarily from soils and enter combined sewers through surface runoffs adhered to soil particles. While molecular surveys have shown the wide abundance of AOB in terrestrial environments (Fierer et al. 2009, Norton et al. 2008), Ammonia Oxidizing *Archaea* (AOA) have been shown to be numerically dominant in soils over AOB (He et al. 2007, Leininger et al. 2006, Nicol et al. 2008). It was, therefore, surprising to find AOA at low abundance in raw wastewater such that their PCR amplification was difficult to achieve. The dominance of AOB over AOA in wastewater systems observed in the current study, does not seem to be an artefact as it has been reported in earlier studies (Park et al. 2006, Zhang et al. 2009). Rather, this may be an indication of the possible selection process on ammonia oxidizers in the sewer environments. The selection may not operate at the species level, but at the AOB vs. AOA level. Several studies have shown that AOA are mainly found in environments with low NH_4^+ substrate availability, while AOB are more abundant in environments with high NH_4^+ concentrations (Beman et al. 2008, Francis et al. 2005, Jia and Conrad 2009). Since wastewaters contain high levels of NH_4^+, this may explain the selection of AOB over AOA in sewer systems.

At all studied sites, seeding of nitrifiers appeared to mediate a level of synchrony between the most dominant nitrifiers of the immigrant communities in influents and local communities in activated sludge. Although the direct link between the immigrant and local communities is hereby evidenced, the immigrant communities may be relayed to a larger-sized metacommunity upstream (Figure 4.1b). In the current case, we only measured the immediate immigrant communities in influent wastewaters dispersing to local communities in activated sludge WRRFs. It is believed that the composition and diversity of the immigrant communities are themselves modulated by species dispersal from the larger metacommunity. However, this is only hypothetical and has yet to be proven.

4.4.3 Influence of sewage-derived nitrifiers on activated sludge taxa

The most dominant AOB OTUs in the influent and activated sludge identified at six of the WRRFs corresponded to *Nitrosomonas europaea*, *Nitrosomonas eutropha* and *Nitrosomonas communis*. Previous studies have reported their wide abundance in activated sludge systems (Purkhold et al. 2000, Shimaya and Hashimoto 2011). The dominant nitrifying bacterial species identified at the other two WRRFs (Pincourt and Vaudreuil) namely *Nitrosomonas oligotropha, Nitrosomonas ureae*, *Nitrosospira multiformis* and *Nitrosomonas nitrosa* are also common AOB present in activated sludge (Wells et al. 2009, Zhang et al. 2011). According to previous reports the genera *Nitrosomonas* and *Nitrosospira* are the most important genera in activated sludge (Park and Noguera 2004). Predominance of these genera can mainly be attributed to their relatively higher growth rates than other AOB (Ward et al. 2011). This is consistent with our observation where *Nitrosomonas* and *Nitrosospira* species dominated the activated sludge systems investigated in this study. However, besides showing their dominance in activated sludge, this study also unravels their predominance in influent wastewater. The dominant wastewater-derived AOB has a strong influence on the identity of the dominant ammonia oxidizers in the mainstream reactor. This underlies the important contribution of influent wastewaters in supplying valuable AOB populations to bioreactors by natural seeding.

The most dominant *Nitrospira*-related NOB in the influent and activated sludge samples were mainly found to be uncultured *Nitrospira* species such as OTU441 belonging to Sublineage I. *Candidatus* Nitrospira defluvii was also identified as a dominant species in the influent and activated sludge samples. This species belongs to *Nitrospira* Sublineage I and has been reported to be an important NOB species in sewage treatment (Daims 2001). According to Lücker et al. (2010), the immense ecological significance of this particular group of bacteria contrasts with our limited knowledge about them since most of the activated sludge inhabiting members are still uncultured and unidentified. Similar to the AOB community profiles, the dominant *Nitrospira*-related NOB identified in the mixed liquors, corresponded to the same ones detected in the influent wastewaters. This again shows that the community structure of the dominant nitrifying species in the activated sludge is under the direct influence of nitrifying bacterial species from raw sewage conveyed by sewer infrastructures. The dominance of these identified nitrifiers may result from a selective process occurring in the sewer system. This is in agreement with Saunders

et al. (2015) who detected relatively high abundance of *Nitrospira* and *Nitrotoga* in influent and activated sludge of Danish wastewater treatment facilities. According to them, such dominance may involve a selection process which already starts in the sewer system itself.

4.4.4 Impact on wastewater treatment engineering

The findings of this study open up avenues for sewage works designers to engineer redundancy in AOB and NOB populations by seeding influent communities to increase nitrifier fractions in sewer systems which will result in natural bioaugmentation of nitrifying bacterial populations in activated sludge bioreactors. Such strategy may prove useful especially in cold climate where it has been shown through a modeling conjecture that natural nitrifier seeding can effectively reduce the minimum solids retention time and improve nitrification at cold temperature (Jauffur et al. 2014). According to Schroeder et al. (2015), the seeding success rate is dependent on the number of times the seeded taxa remain in the system above a certain relative abundance rather than die out. By providing enough SRT, this will allow incoming nitrifiers to adhere to activated sludge flocs and contribute to nitrification in the bioreactor. Nitrifiers have been shown to exhibit strong adhesion properties forming dense microcolonies in activated sludge flocs (Larsen et al. 2008). Understanding the elemental role played by natural influent nitrifier seeding in open biological systems can thus help for coherent development of operational and design strategies to improve process efficiency and stability.

4.5 ACKNOWLEDGEMENTS

We express our deepest gratitude to operators of the WRRFs for granting us permission to collect samples and providing us with operational data. We would also like to thank Siavash Isazadeh for his support during the initial phase of this project. This research was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada (grants CRDPJ 417654–11 and CRDPJ 459259-13), Air Liquide Canada and the Régie d'Assainissement des Eaux du Bassin LaPrairie.

4.6 SUPPLEMENTARY MATERIALS

Figure S4.1. Locations of Water Resource Recovery Facilities (WRRFs). Abbreviations: CAS-Conventional Activated Sludge; SBR-Sequencing Batch Reactor

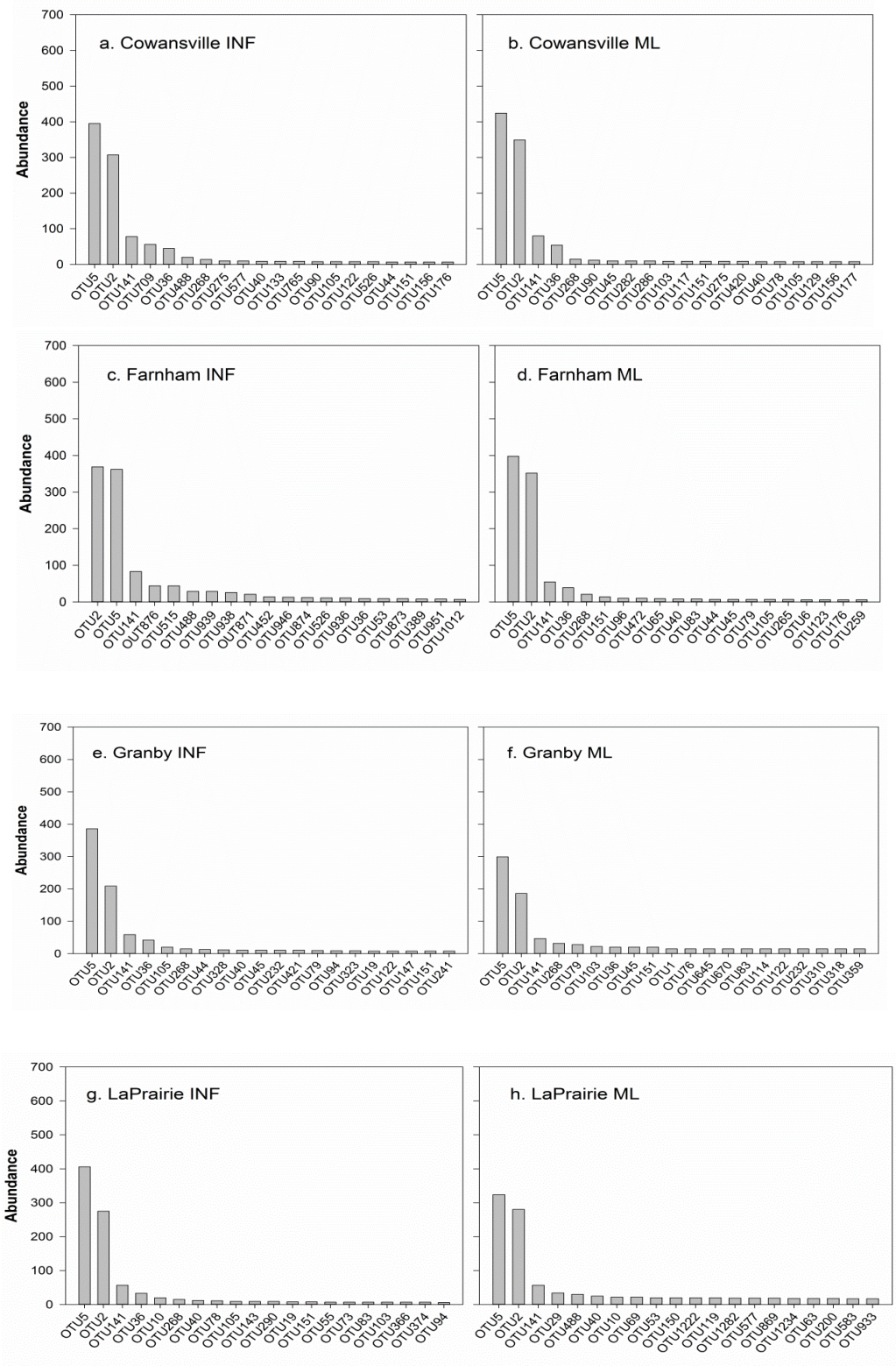

Figure S4.2. Rank-abundance charts showing 20 most abundant AOB OTUs defined at a cutoff level of 3% in influent and mixed liquor samples of Cowansville (a, b), Farnham (c, d), Granby (e, f), and LaPrairie (g, h). INF means Influent and ML means mixed liquor.

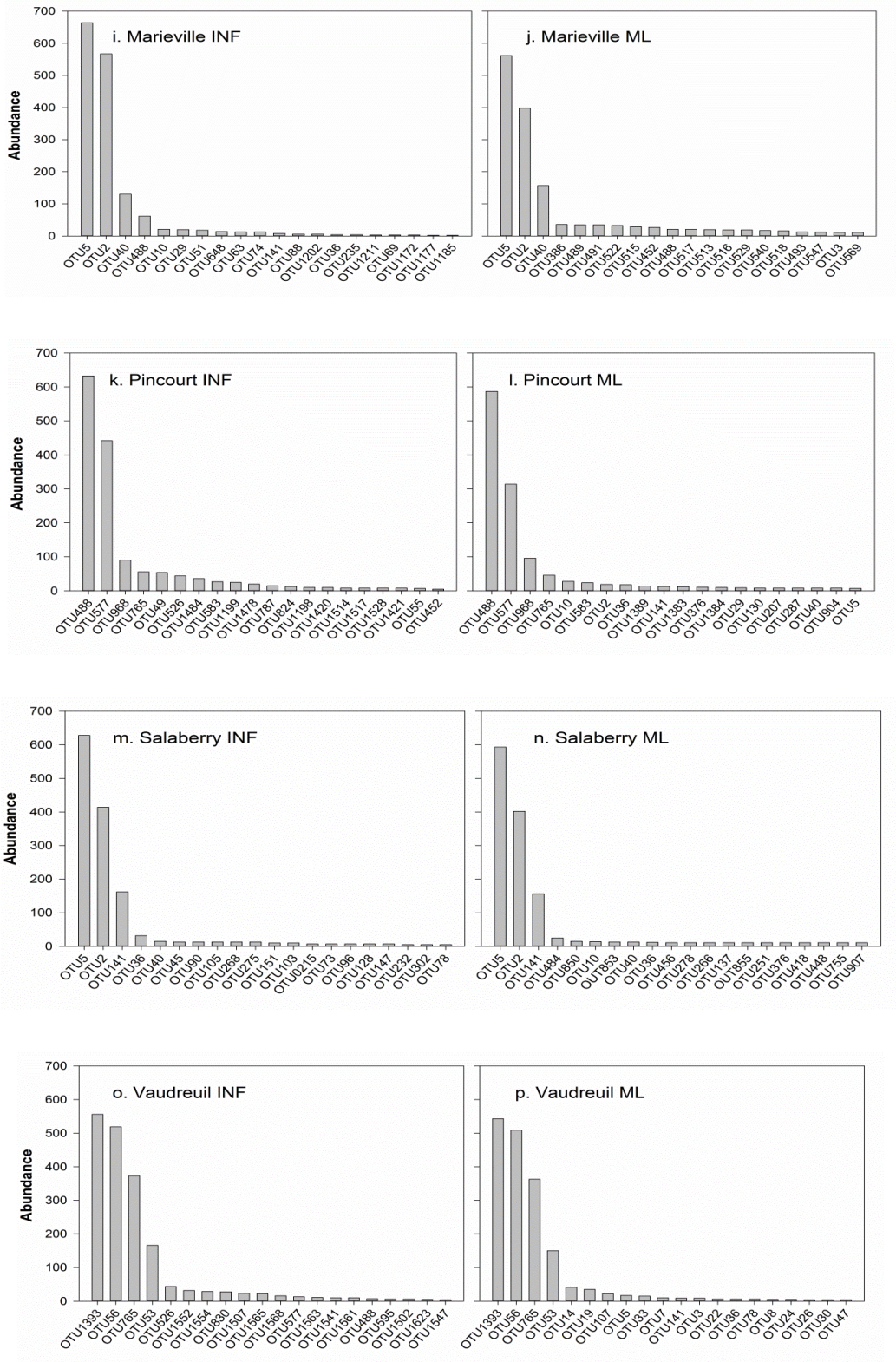

Figure S4.2. (continued) Rank-abundance charts showing 20 most abundant AOB OTUs defined at a cutoff level of 3% in influent and mixed liquor samples of Marieville (i, j), Pincourt (k, l), Salaberry (m, n), and Vaudreuil (o, p) . INF means Influent and ML means mixed liquor.

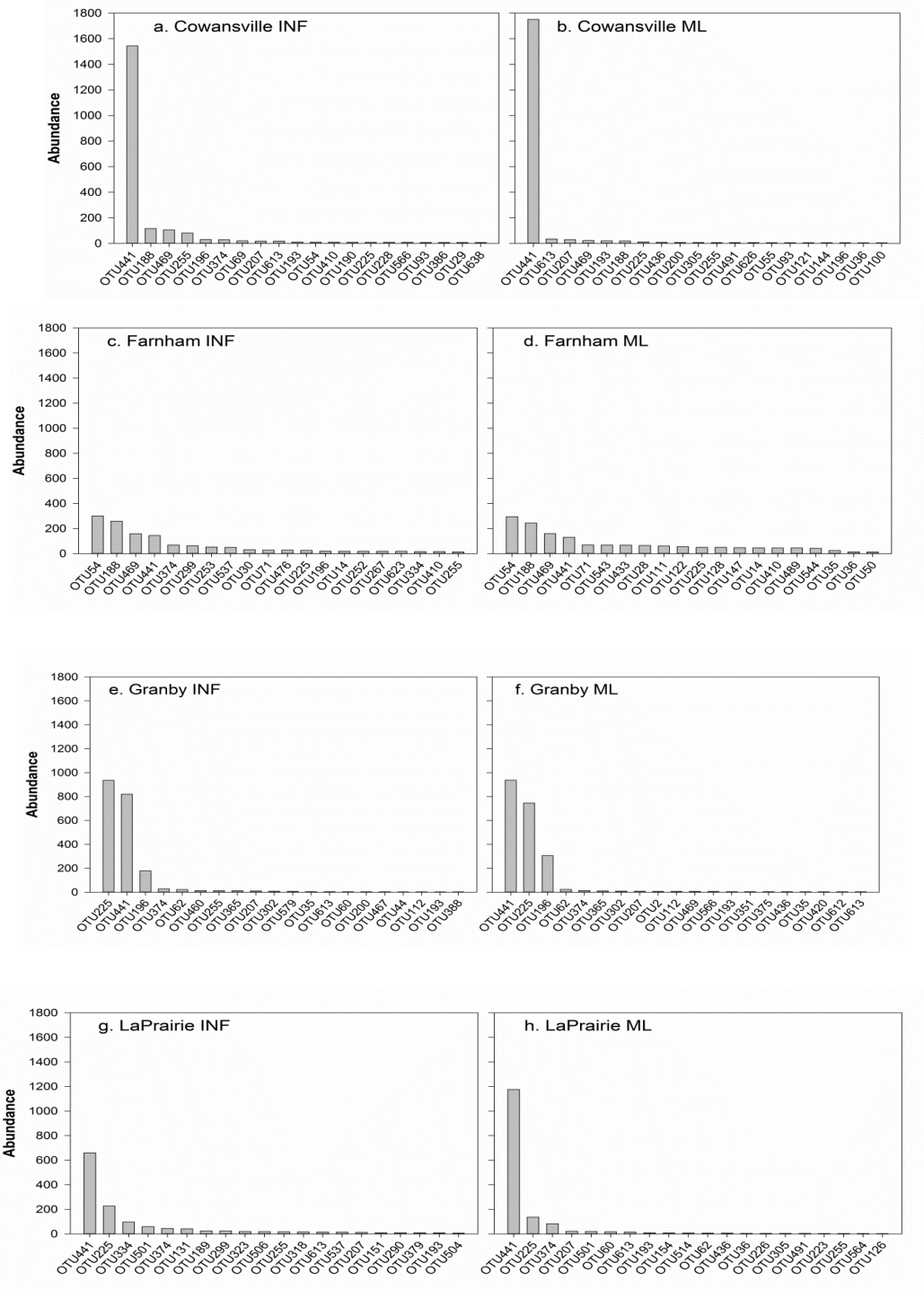

Figure S4.3. Rank-abundance charts showing 20 most abundant *Nitrospira*-related NOB OTUs defined at a cutoff level of 3% in influent and mixed liquor samples of Cowansville (a, b), Farnham (c, d), Granby (e, f), and LaPrairie (g, h). INF means Influent and ML means mixed liquor.

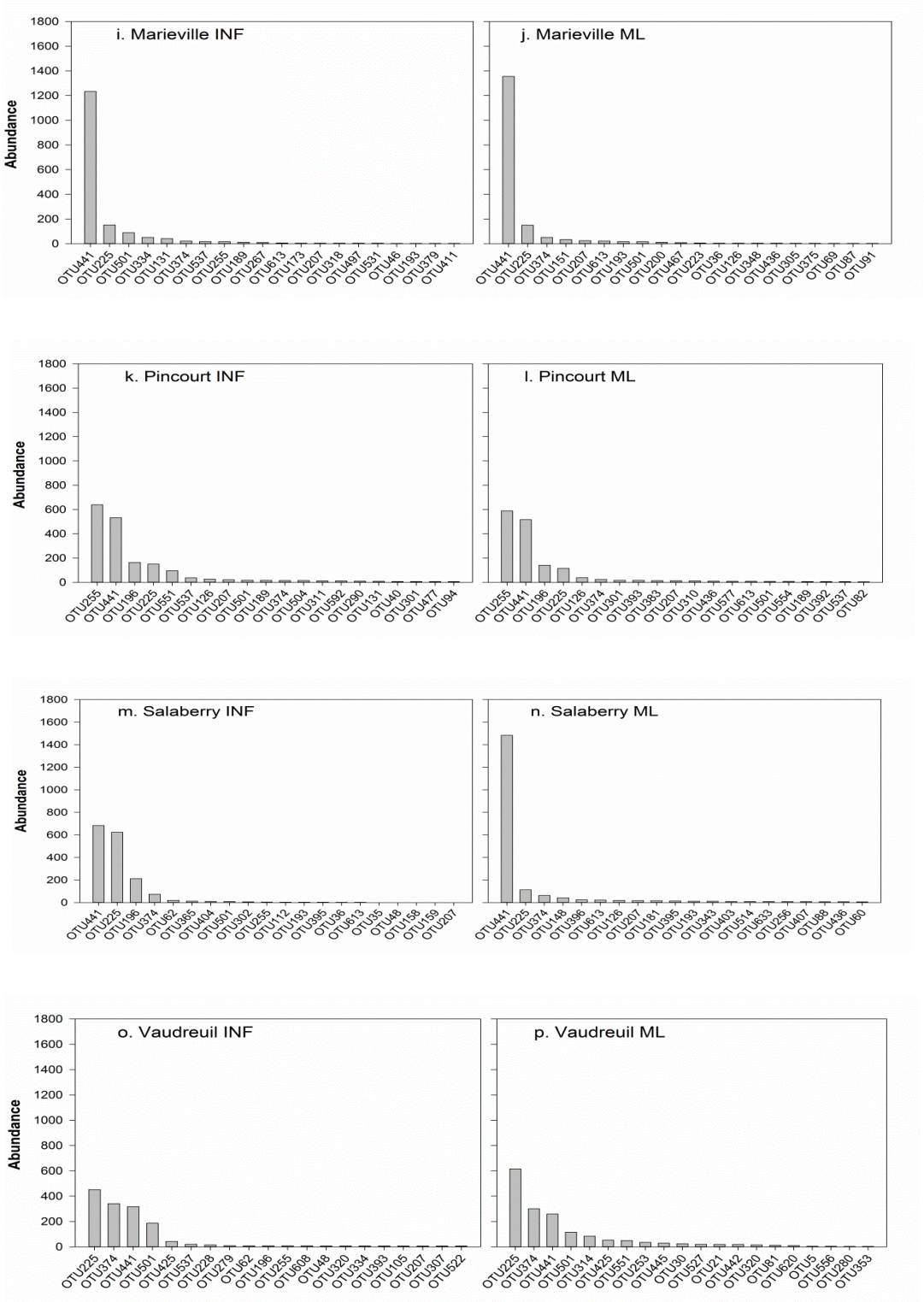

Figure S4.3. (continued) Rank-abundance charts showing 20 most abundant *Nitrospira*-related NOB OTUs defined at a cutoff level of 3% in influent and mixed liquor samples of Marieville (i, j), Pincourt (k, l), Salaberry (m, n), and Vaudreuil (o, p) . INF means Influent and ML means mixed liquor.

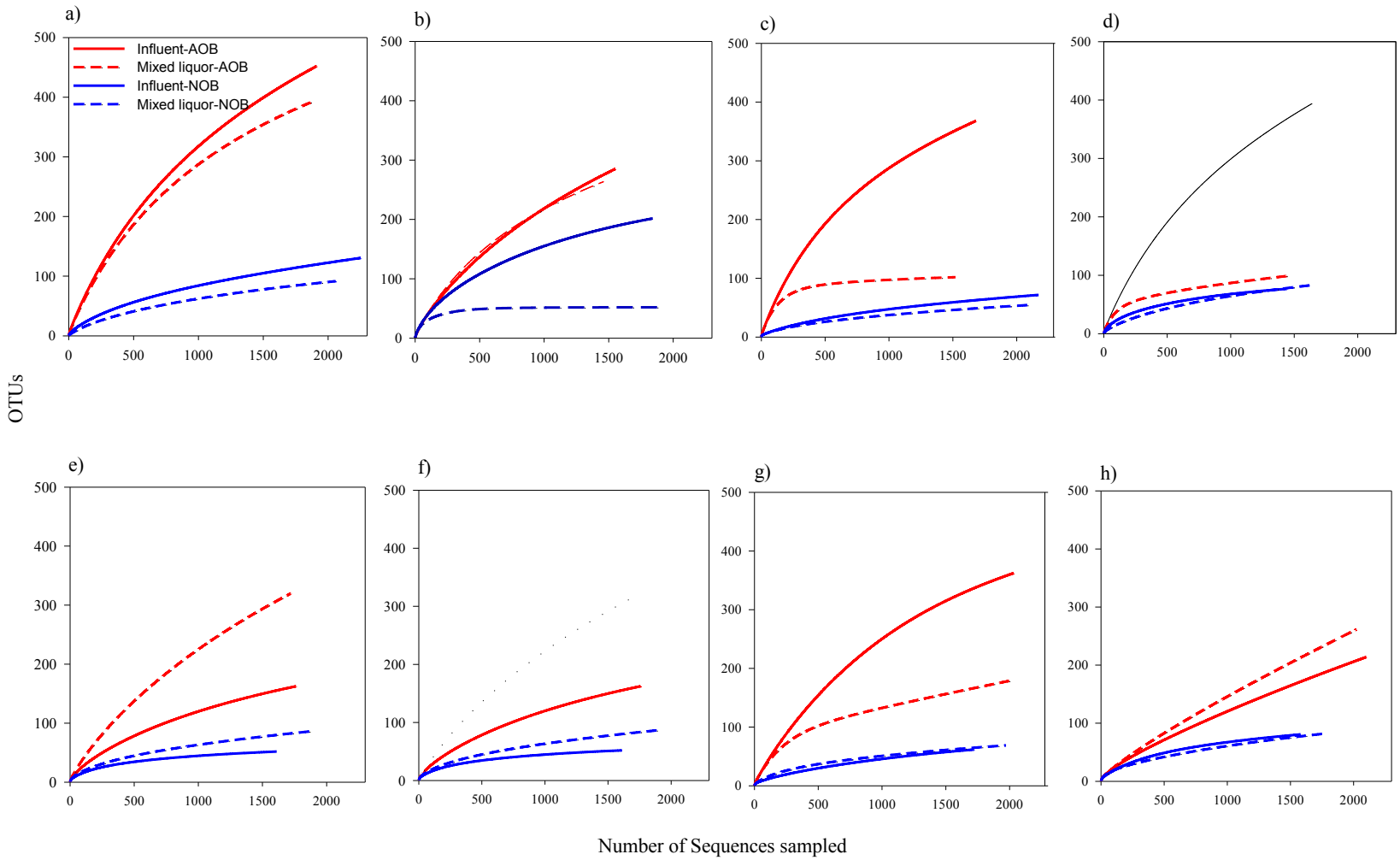

Figure S4.4. Rarefaction curves of AOB and *Nitrospira*-related NOB OTUs defined by 3% sequence variations in influent and mixed liquor samples for Cowansville (a), Farnham (b), Granby (c), LaPrairie (d), Marieville (e), Pincourt (f), Salaberry (g) and Vaudreuil (h). Legend in panel a) applies to all other panels.

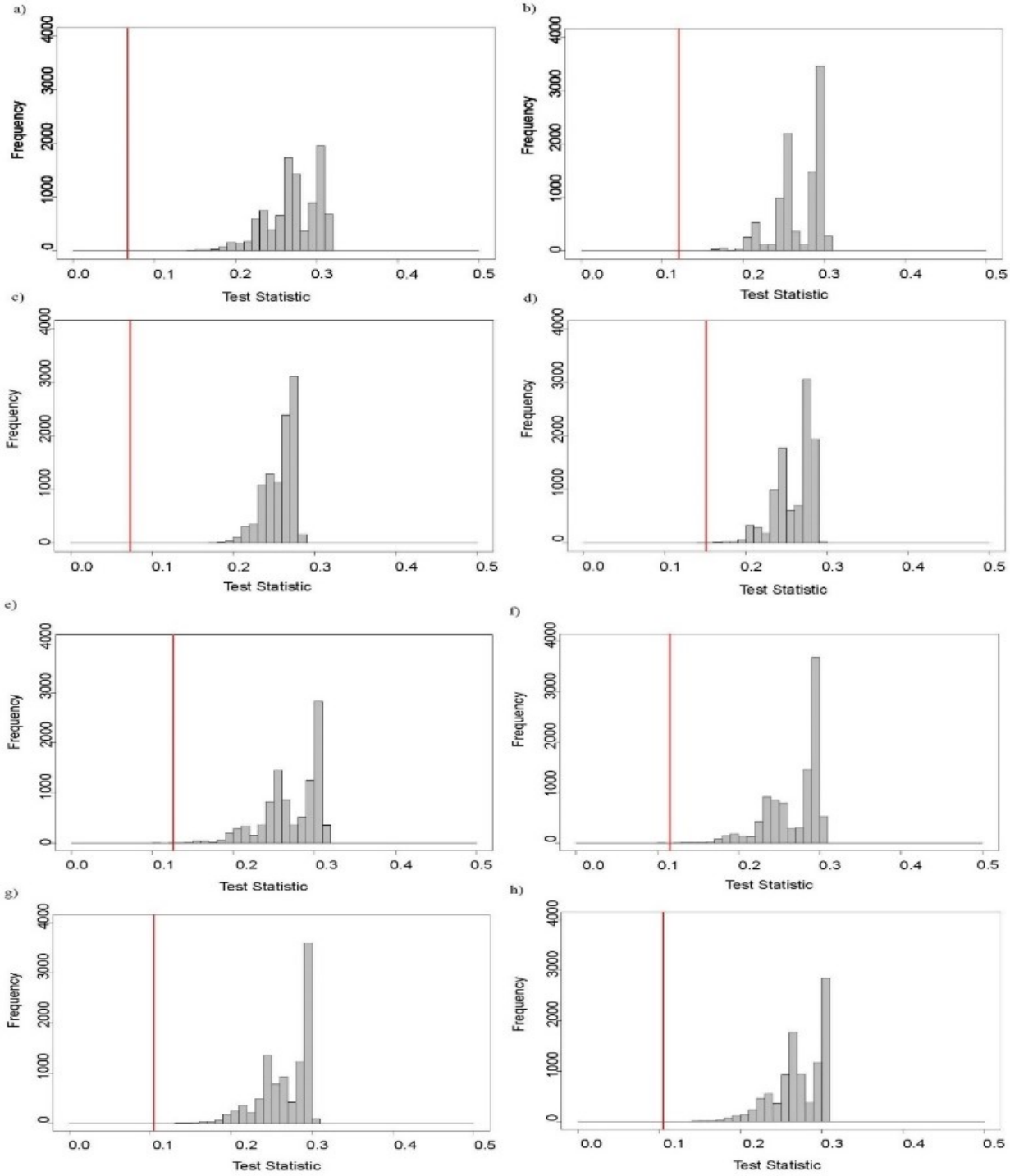

Figure S4.5. Permutated probability distributions of AOB OTUs generated using a weighted rank-based test statistic (eq. 2) for Cowansville (a), Farnham (b), Granby (c), LaPrairie (d), Marieville (e), Pincourt (f), Salaberry (g), and Vaudreuil (h). Number of permutations performed was 10,000. The red line is the test statistics for the population profiles obtained with the samples.

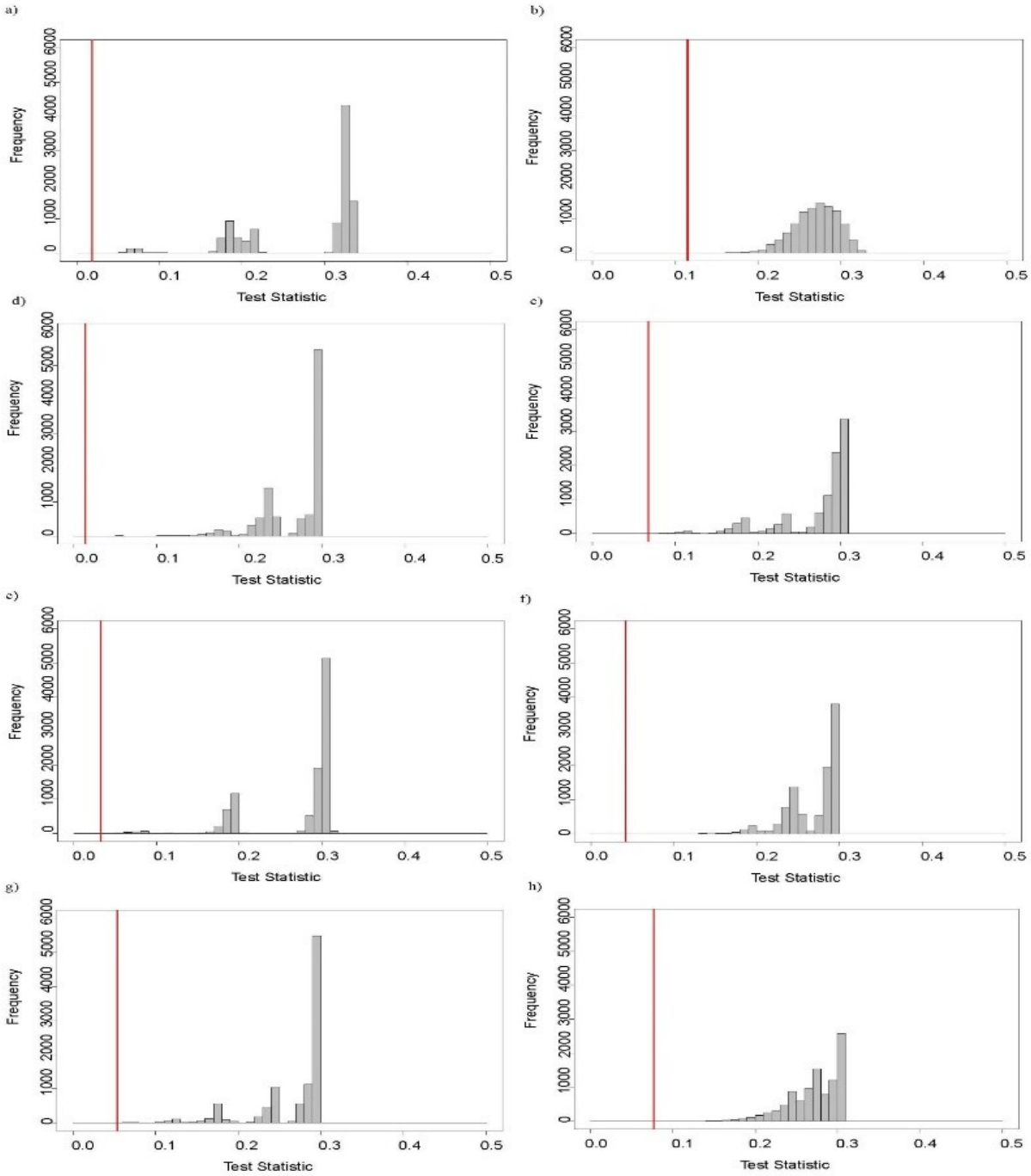

Figure S4.6. Probability frequency distributions of *Nitrospira*-related NOB OTUs generated using a weighted rank-based test statistic for Cowansville (a), Farnham (b), Granby (c), LaPrairie (d), Marieville (e), Pincourt (f), Salaberry (g), and Vaudreuil (h). Number of permutations performed was 10,000. The red line is the test statistics for the population profiles obtained with the samples.

Table S4.1. Study sites with description of treatment configurations, operating conditions, and characteristics of incoming municipal influent wastewater.

WRRFs	Geographic location		Average operation conditions					Average influent characteristics					
	Latitude N	Longitude W	Plant process[a]	Flow rate (m^3/day)	SRT (day)	HRT (h)	MLVSS (mg/L)	Source (%)[b]	COD (mg/L)	BOD_5 (mg/L)	NH_4-N (mg/L)	Total P (mg/L)	Temp. (ºC)[c]
Cowansville	45°13'16.55"	72°46'30.41"	CA	14 000	10	18	4910	90:10	233	146	6.2	4.1	7.5
Farnham	45°17'21.90"	72°59'35.05"	CA	6 000	80	48	6080	80:20	206	130	5.4	0.9	8.2
Granby	45°22'17.45"	72°46'23.98"	CA	55 000	7	20	3116	50:50	468	231	6.8	3.9	11.4
LaPrairie	45°24'16.48"	73°33'22.06"	CA	65 000	7	15	1850	45:55	333	143	13.2	9.8	10.5
Marieville	45°26'20.28"	73° 9'51.40"	CA	5 000	25	12	3200	80:20	308	129	7.2	3.8	8.6
Pincourt	45°23'25.30"	74° 1'37.34"	CA	6 000	15	8	2121	90:10	316	102	7.5	3.2	7.2
Salaberry	45°13'34.61"	74° 4'20.44"	CA	57 000	25	12	2500	82:18	245	195	4.0	2.4	7.5
Vaudreuil	45°23'25.30"	74° 1'37.34"	SBR	18 000	5	3	3000	50:50	322	186	6.9	3.5	7.9

[a]: CA-conventional aeration, SBR-Sequencing Batch Reactor.

[b]: Source of wastewater in % - Residential:Industrial.

Table S4.2. Primers and PCR thermocycling programs used for amplification of *amoA* and *nxrB* genes.

Target population	Primer	Sequence (5'-3')	PCR thermocycling program	Reference
AOB	*amoA-1F*	GGGGTTTCTACTGGTGGT	95 °C for 4 min, 35 cycles of 95 °C for 40 s, 56 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	Rotthauwe et al. 1997
	amoA-2R	CCCCTCTGCAAAGCCTTCTTC		
AOA	*amoA-19F*	ATGGTCTGGYTWAGACG	95 °C for 5 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 5 min	Pester et al. 2012
	amoA-616R	GCCATCCABCKRTANGTCCA		
NOB (*Nitrospira-related*)	*nxrB-F169*	TACATGTGGTGGAACA	95 °C for 5 min, 35 cycles of 95 °C for 40 s, 62 °C for 40 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	Maixner 2009
	nxrB-638R	CGGTTCTGGTCRATCA		
NOB (*Nitrobacter-related*)	*nxrB-F706*	AAGACCTAYTTCAACTGGTC	95 °C for 4 min, 35 cycles of 95 °C for 40 s, 56 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	Maixner 2009
	nxrB-R1431	CGCTCCATCGGYGGAACMAC		

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CHAPTER 5

Influent seeding of wastewater treatment plants: implications for low temperature nitrification and heterotrophic population structures

Connecting text: After observing the natural seeding of nitrifiers at full-scale wastewater treatment facilities (Chapter 4), the importance of raw wastewater seeding to sustain nitrification under washout conditions was demonstrated at the laboratory-scale level. This was performed by operating laboratory-scale sequencing batch reactors (SBRs) fed with sterile synthetic medium (i.e. absolutely no seeding) at low temperature and SRT. The natural seeding phenomenon was reproduced in the laboratory by adding solids collected from the influent of a full-scale wastewater treatment plant, to the synthetic feed supplied to the SBRs. This re-established nitrification under the unfavorable conditions, showing the necessity to consider influent nitrifier seeding to properly model activated sludge wastewater treatment plants.

In Chapter 4, the importance of wastewater seeding in determining the nitrifying population structures in the mixed liquor was observed at full-scale level. This result was further substantiated in Chapter 5. The data also extend the observation to heterotrophic bacterial populations. Studies by other groups argued that the contribution of influent municipal wastewaters in shaping the activated sludge microbiome was negligible, while the results of Chapter 5 suggest that it is not the case by showing that some heterotrophic populations change significantly and reproducibly during natural seeding.

The results of this research will be submitted for publication in this journal:

Shameem Jauffur, Zeinab Bakhshi and Dominic Frigon. Influent seeding of wastewater treatment plants: implications for low temperature nitrification and heterotrophic population structures. To submit to *Water Research* (in preparation).

5.1 INTRODUCTION

Although bacterial biomass is commonly quantified in activated sludge for wastewater treatment process design and operation (Ekama 1984, Henze 2000), it is rarely quantified in raw wastewater, and is generally neglected in mass balances due to low concentrations (Foladori et al. 2010). Even best modeling practices adopted by the International Water Association (IWA) consensus Activated Sludge Models (ASMs) assume no active biomass in influent wastewaters. According to Grady et al. (2011), two significant effects may result if active biomass is present in influent wastewaters. It will reduce the minimum substrate concentration attainable ($S_{s,min}$), and prevent washout even at low solids retention time (SRT). This underlies the concept of bioaugmentation of biomass in activated sludge systems that enhances process performance.

Recently, the presence of nitrifiers has been reported in raw wastewaters reaching full-scale wastewater treatment facilities (Jauffur et al. 2014, Saunders et al. 2015). The nitrifiers detected in the wastewater were shown to be alive with the potential of being fully metabolically induced within a few hours (Jauffur et al. 2014). Nitrifiers from influent wastewaters may be adsorbed to activated flocs and be retained in the bioreactor to provide additional nitrification activity. Moreover, it has been observed that dominant nitrifying operational taxonomic units (OTUs) identified in municipal influent wastewaters and corresponding activated sludge mixed liquors of full-scale Water Resource Recovery Facilities (WRRFs) were the same, suggesting that natural seeding of nitrifiers does indeed occur at full-scale wastewater treatment facilities (Chapter 4) (Jauffur et al. 2015, In preparation). This may be of significant importance especially in cold conditions where nitrification may be compromised (Van Dyke et al. 2003). Further research is necessary to understand the importance of influent wastewater in supplying indigenous nitrifying bacteria to activated sludge systems.

Influent wastewaters also contain a wide diversity of heterotrophic bacterial communities resulting from inputs of human fecal matter and water infiltration, or from growth of specific microbes in the sewer system (McLellan et al. 2010). Sewers are unique environments where a vast diversity of microorganisms can reside. A fresh sewage usually contains a limited supply of oxygen and most of the internal sewer system can be considered to be under sulphate-reducing anaerobic conditions (Guisasola et al. 2008), thereby favoring the growth of anaerobic fermenters (Nielsen 1991). However, it is possible to achieve partial oxidation of organic substrates by resident microorganisms during sewage conveyance. Gravity sewers have been

shown to abate the concentration of organic matter when the DO level in the water phase is greater than 1 mg/L with active bacteria growing in the sediments and as biofilms on the side walls of sewers (Chen et al. 2003). According to Lemmer et al. (1994), sewer biofilms developed on the inner wall of sewer pipes can have activities one or two magnitudes higher than that of activated sludge microorganisms. Erosion, sloughing and sewer conveyance of biomass by wastewaters lead to immigration of heterotrophic bacteria, which contribute to observed bacterial diversities in activated sludge. Thirty-five percent of heterotrophic bacterial OTUs identified in activated sludge was found to come from influent wastewaters in Danish WRRFs (Saunders et al. 2015).

The aim of this study was to reproduce in the laboratory, the natural seeding of nitrifiers by influent wastewaters observed at full-scale wastewater treatment plants. The laboratory reactors fed with sterile culture medium did not support nitrification under conditions of low operational temperature and SRT. However, ASM model simulations with typical default parameters suggest that nitrification should have occurred under these conditions. Thus, this study evaluated the importance of influent nitrifiers to support nitrification in activated sludge bioreactors. In addition, the impact of influent wastewater seeding on heterotrophic microbial community composition in activated sludge was also assessed.

5.2 MATERIALS AND METHODS

5.2.1 Sequencing batch reactor (SBR) set-up and operation

Three 4-L cylindrical double-wall jacketed laboratory-scale sequencing batch reactors (SBRs) made up of non-reactive Plexiglas were set-up with an actual working volume of 2 L. A schematic layout of the SBRs is shown in Figure 5.1. The temperature of the reactors was controlled using thermostatic water circulators (IsoTherm model 250LC, Fisher Scientific, MA). Influent, effluent and waste activated sludge (WAS) were pumped by means of Masterflex peristaltic pumps (Model SI-77911-20, Cole Parmer, USA). The operation of the SBR systems was automated using the Apex AquaController (model APEXLSYS, Neptune Systems, San Jose, CA), which also controlled the pH to 7.45 by titrating 0.25 M NaOH. The SBRs were aerated at an airflow rate of 2 L/min using compressed air, which was filtered and introduced through diffusion plates at the bottom of the reactors. During start-up, the reactors were inoculated with activated sludge mixed liquor suspended solids collected from the Régie d'Assainissement des

Eaux du Bassin La Prairie (RAEBL), a full-scale biological WRRF located near Montreal (Canada). The reactors were operated with a 6-h cycle. At the beginning of the cycle, aeration was started for 10 min prior to pumping 1 L of feed into each reactor for 5 min, followed by an aeration phase of 5 h. Prior to the end of the aeration phase, a specific volume of activated sludge was wasted to keep the SRT at a constant level. This was followed by a settling phase of 0.75 h after which 1 L of supernatant was removed resulting in a hydraulic retention time (HRT) of 0.5 day.

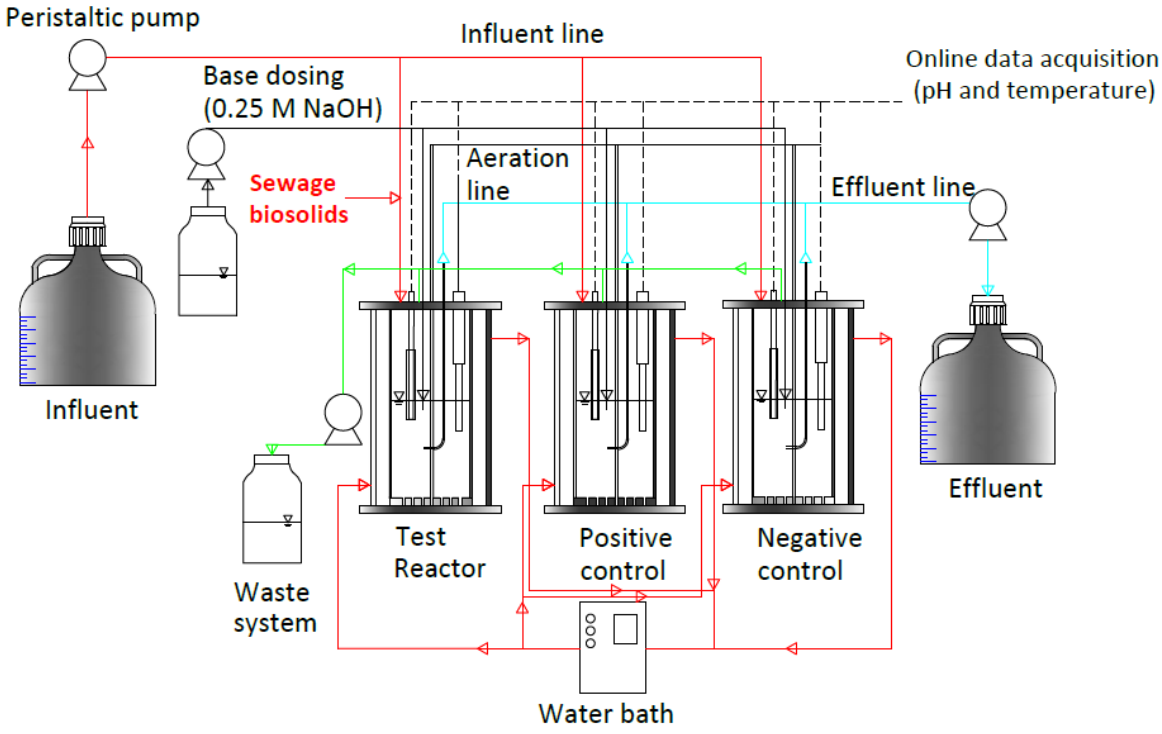

Figure 5.1. Schematic overview of sequencing batch reactors (SBRs). Set-up comprised of an influent line feeding synthetic wastewater (*Syntho*) to three SBRs (Test Reactor, Positive Control and Negative Control reactors) and an effluent line drawing treated effluent out of the system. Each treatment cycle (fill, react, settle, decant, idle) lasted for 6 h.

The three SBRs were fed with "*Syntho*", a synthetic feed developed by Boeije et al. (1999), which mimics the average composition and quality of domestic wastewater. The composition of 1 L of synthetic feed was as follows: 15 mg peptone, 120 mg CH_3COONa, 15 mg dry meat extract, 40 mL glycerol, 50 mg starch, 120 mg low fat milk powder, 75 mg urea, 9 mg uric acid,

11 mg NH_4Cl, 25 mg $MgHPO_4.3H_2O$, 20 mg $K_3PO_4.H_2O$, 10 mg sodium dodecyl sulfate (SDS), 10 mL Synapol and 10 mg diatomaceous earth. A mixture of trace elements based on typical concentrations found in sewage was added to the "*Syntho*" (Bollmann et al. 2011) and comprised of the following in 1 L: 4,292 mg NaEDTA, 2,780 mg $FeSO_4.7H_2O$, 99 mg $MnCl_2.4H_2O$, 24 mg $NiCl_2.6H_2O$, 24 mg $CoCl_2.6H_2O$, 13.4 mg $CuCl_2$, 143 mg $ZnSO_4.7H_2O$, 24 mg $Na_2MoO_4.2H_2O$, 23.2 mg WO_3 and 62 mg H_3BO_3. The feed (without the trace element solution) was autoclaved at 121 °C for 1 h and allowed to cool down prior to feeding the reactors. The COD and TKN of the formulated influent recipe was approximately 550 mg/L and 35 mg-N/L, respectively.

An additional SBR operated at a SRT of 15 days and HRT of 0.5 day, was used to enrich nitrifying biomass at 5 °C by feeding it with a recipe based on Bollmann et al. (2011). The mineral medium consisted of the following in 1 L: 660.7 mg $(NH_4)_2SO_4$, 840 mg $NaHCO_3$, 585 mg NaCl, 75 mg KCl, 147 mg $CaCl_2.2H_2O$, 49 mg $MgSO_4.7H_2O$, 1360.9 mg KH_2PO_4, 57.2 g HEPES buffer, 240 mg diatomaceous earth and trace element with the same chemical composition used to prepare the "*Syntho*" organic feed. Stock solutions were autoclaved at 121 °C for 1 h before use to prevent bacterial or fungal growth during storage. The pH of the mineral medium was adjusted to 7.8 using 1 M NaOH. The reactor was aerated with filtered compressed air enriched with 0.4% (v/v) CO_2 gas to support the autotrophic growth of nitrifiers (Denecke and Liebig 2003). The pH of the reactor was maintained at 7.45 by adding 0.25 M NaOH using an automatic base dosing system.

5.2.2 Natural influent seeding experiments

The SBRs were operated at a temperature of 8 °C and a SRT of 20 days for the initial 130 days (Phase I in Figure 5.2). Prior to the start of the seeding experiment, the temperature of the three SBRs was lowered to 5 °C, and the SRT was decreased to 7 days to cause nitrification failure in all the three reactors (Phase II in Figure 5.2). Following nitrifier washout and failure of the reactors to nitrify ammonium, the seeding experiments were started to find out whether seeding with municipal influent solids could re-establish nitrification and stabilize the bioreactors. One of the SBRs (Test Reactor) was seeded with influent solids harvested from the influent of the RAEBL full-scale WRRF by weekly centrifuging 10 L of a 24-h composite influent sample at 4,000×g using the IEC-Centra-8 centrifuge (Thermo Scientific, USA). The solids concentration was adjusted to seed the Test Reactor at a nitrifier seeding rate of 5 mg-$COD_{biomass}$/L (representing ~1.5% of the total COD), a level similar to what was observed at the full-scale

RAEBL WRRF based on respirometric assays (Jauffur et al. 2014). Another SBR, referred to as the "Positive Control" was seeded with cold-adapted enriched nitrifying culture at a similar rate. The third SBR was used as a "Negative Control" reactor and was not seeded.

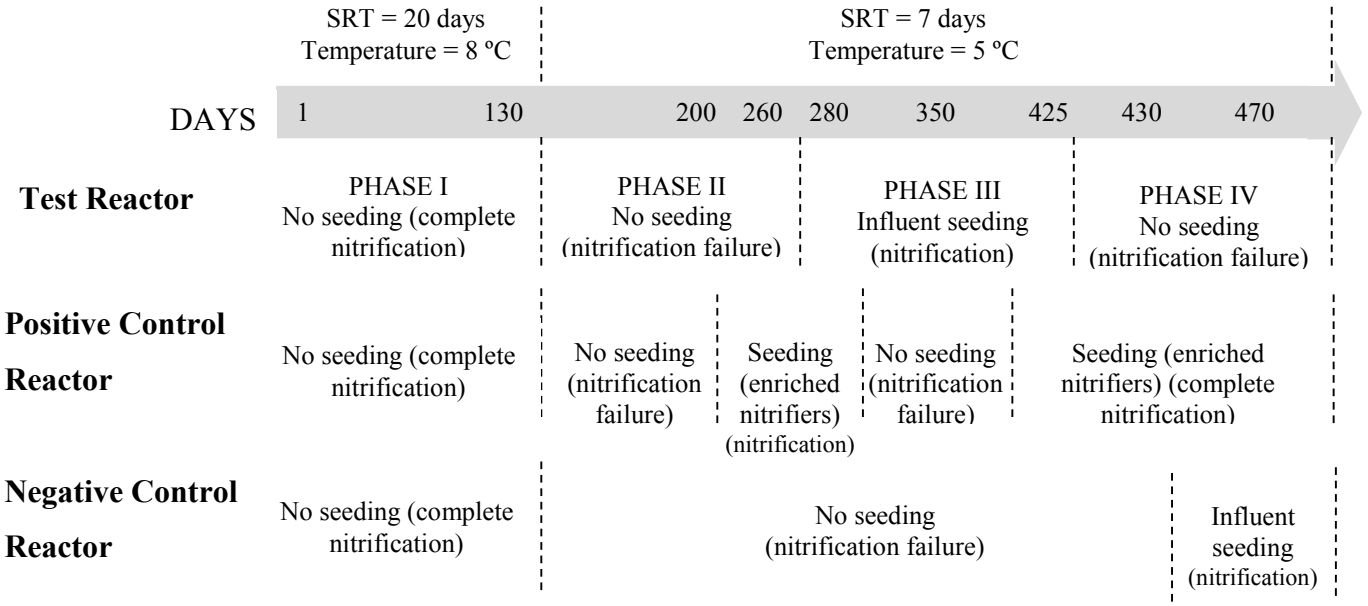

Figure 5.2. Timeline for seeding of SBRs (from Day 1 to Day 470). The SBRs were operated at 8 ºC and at a SRT of 20 days for the initial 130 days. The temperature and SRT were subsequently reduced to 5 ºC and 7 days, respectively for the rest of the operation period. Key days are identified on the timeline arrow to indicate start or halt in seeding. Seeding experiments on the Test Reactor were performed in 4 phases as indicated below the timeline. The seeding schedule for the control reactors are also displayed under the timeline bar. Biomass samples were collected from the SBRs on day 40, 70, 150, 200, 230, 270, 312, 354, 400, 430, 450 and 470 for bacterial community analysis. Respective seed biomass samples were also collected in parallel.

As shown in Figure 5.2, the seeding experiments for the Test Reactor were conducted in four phases: (i) Phase I - no seeding (complete nitrification at beginning of the experiment), (ii) Phase II - induction of nitrification failure, (iii) Phase III - seeding of reactor with municipal influent solids, and (iv) Phase IV - reversing the seeding regime between the Test and Negative Control reactors in order to show reproducibility of the influent seeding effect. The timeline for the seeding experiment on the control reactors is also shown in Figure 5.2. The nitrification performance was assessed by monitoring the concentration of ammonium (NH_4^+) and nitrite

(NO_2^-)/Nitrate (NO_3^-) in the influent and effluent. NH_4^+ was determined based on the Berthelot method involving the reaction of NH_4^+ as monochloramine with hypochlorite and phenol at a pH of 13 (Rhine et al. 1998). The concentration of NO_2^-/NO_3^- was measured based on azo dye formation and reduction of NO_3^- using hydrazine sulphate (Shand et al. 2008). Both tests were performed by microplate assays using the SPECTRAmax® microplate spectrophotometer (CA, USA). The spectrophotometer was operated using the SOFTmax® PRO software. The level of the mixed liquor volatile suspended solids (MLVSS) was monitored according to Standard method 2540 E (APHA 2005).

5.2.3 Bacterial community composition

Thirty-six activated sludge samples were collected across the duration of the study to assess the impact of seeding on the bacterial community composition of the SBRs. Seed samples (influent and cold-adapted nitrifier-enriched biomass) were also collected for analysis. Samples were centrifuged at 10,000×g in eppendorf tubes for 30 minutes using a microcentrifuge (Thermo Scientific, Sorvall Legend Micro 21R, USA) and preserved at −80 °C until time of analysis. A quantity of 0.25 g of wet centrifuged sample was used to extract genomic DNA using the PowerSoil® DNA Isolation Kit (*MO BIO* Laboratories, Inc., Carlsbad, CA). Diluted gDNA (12 ng/mL) was used to amplify the hypervariable V3-V4 region of the *16S rRNA* gene to assess the overall bacterial community composition of the SBRs and their respective seeds. A set of 3 barcoded forward primers targeting the V3 region (*E. coli* position: 338) (Pinto and Raskin 2012) and 1 reverse primer targeting the V4 region (*E. coli* position: 802) (Claesson et al. 2010) were used. Functional genes involved in nitrification were also amplified to identify the nitrifying bacterial profiles of the SBRs and their corresponding seeds. Ammonia oxidizing *bacteria* (AOB) were studied by targeting the *amoA* gene using the barcoded forward primer *amoA-1F* and reverse primer *amoA-2R* (Rotthauwe et al. 1997). Nitrite oxidizing bacteria (NOB) were studied by targeting the *nxrB* gene of the *Nitrospira* genus, considered as the dominant NOB population in activated sludge systems (Hovanec et al. 1998), using the barcoded forward primer *nxrB-F169* and reverse primer *nxrB-638R* (Maixner 2009b). The primer sequences and thermal cycles are provided in Table S1 of the Supplementary Data sheet. Each 50µl of PCR reaction mixture contained 0.5 µM of forward and reverse primer each, 1× 5X Bioline PCR colorless buffer (Taunton, MA, USA), 2.75 mM $MgCl_2$, 250 µM dNTP (each), 12 ng/mL DNA template and 2.5 units Bioline Taq DNA Polymerase (Taunton, MA, USA) in UltraPure™ DNase/RNase-

Free Distilled Water (Invitrogen, Carlsbad, USA).The PCR amplicons were purified using the *MO BIO* UltraClean™ PCR Clean-Up Kit (Carlsbad, CA). The quality of the PCR amplicons was assessed using the DNA 1000 microfluidic chip (Agilent Technologies, USA) and the Bioanalyzer 2100 (Agilent Technologies, USA). Any amplicon contaminated with traces of non-specific fragments was further purified using the Agencourt AMPure™ PCR purification kit (Beckman Coulter, Beverly, MA, USA). The concentration of each PCR amplicon was determined using the Quant-iT™ PicoGreen kit (Invitrogen, Carlsbad, USA) and normalized to a concentration of 30 ng/µl. Amplicon mixtures were subjected to emulsion PCR based on Roche-454 Life Science Protocol and pyrosequenced by the GS FLX Titanium Sequencing system using the unidirectional sequencing Lib-L chemistry. Sequencing was performed at the McGill University and Génome Québec Innovation Centre (Montreal, QC).

The *16S rRNA*, *amoA* and *nxrB* fasta sequence data, flow and quality files were retrieved from raw Standard Flowgram Format (sff) files, de-multiplexed, trimmed and filtered using the QIIME software (Caporaso et al. 2010) to retain only good quality sequences devoid of primers and barcodes. USEARCH was used for sequence denoising and chimera removal (both *de novo* and reference-based). Additionally, chimeric sequences were analyzed with UCHIME using default parameters, and were removed. Quality filtered sequences (minimum read length of 200 bp, quality score > 25, and without ambiguous bases and mismatches) were clustered at 97% sequence similarity, considered to be related to species level (Islam et al. 2015), into operational taxonomic units (OTUs) using the Uclust algorithm (Edgar 2010). Resulting clusters were subjected to *alpha* and *beta* diversity analyses using default scripts in QIIME and *BiodiversityR* package of the R-software, version 3.2.1. Phylogenetic trees displaying the position of the most abundant AOB and *Nitrospira*-related NOB species were constructed using FastTree (Price et al. 2009) and visualized using FigTree v1.4.2. Taxonomic identification of dominant nitrifying bacterial OTUs was performed using the Functional Gene Pipeline and Repository of the Ribosomal Database Project (Fish et al. 2013), and BLASTN DNA query search (Altschul et al. 1990).

In order to assess the similarity between the influent and mixed liquor nitrifying bacterial profiles, the percentage of sequence reads shared between the two sample matrices was computed using the equation below.

$$\% \text{ shared reads} = \frac{\frac{N_{shared\ reads,inf}}{N_{T,inf}} + \frac{N_{shared\ reads,ml}}{N_{T,ml}}}{2} \times 100\% \qquad \text{(Eq. 5.1)}$$

Where $N_{shared\ reads,inf}$ is the no. of reads of shared OTUs from the influent, $N_{shared\ reads,ml}$ is the no. of shared OTUs from the mixed liquor, $N_{T,inf}$ is the total no. of reads from the influent and $N_{T,\ ml}$ is the total no. of reads from the mixed liquor.

5.3 RESULTS

5.3.1 Impact of influent seeding on nitrification

During the period Day 1-130, all reactors were operated at 8 °C and a SRT of 20 days without seeding, and nitrification of ammonia was almost complete. During Day 131-260, the temperature was decreased to 5 °C, and the SRT was gradually reduced to 7 days, which caused the washout of nitrifiers and the increase in effluent NH_4^+ concentration to an average of 25 mg/L (Figure 5.3) in all reactors. At the same time, the effluent NO_2^-/NO_3^- concentration dropped to almost 0 mg-N/L. During Day 261-425, seeding the Test Reactor with approximately 5 mg-COD/L of nitrifying biomass by adding influent solids collected at the LaPrairie full-scale WRRF decreased the effluent NH_4^+ concentration to about 3 mg-N/L and increased NO_2^-/NO_3^- concentration to around 20 mg/L, while the Negative Control reactor remained under a state of nitrification failure with high levels of NH_4^+ and almost no production of NO_2^-/NO_3^- (Figure 5.3). This level of influent nitrifier seeding (5 mg-COD/L of nitrifying biomass) was previously determined in the influent of LaPrairie WRRF by respirometry (Jauffur et al. 2014). The constant seeding of the Test Reactor with influent solids maintained nitrification at 5 °C and a SRT of 7 days. During phase IV (Day 426-470), a halt in the supply of influent solids led the effluent NH_4^+ concentration to increase rapidly, indicating that nitrifying bacteria were being washed out of the SBR system. Seeding the Negative Control reactor during Day 430-470 with influent solids from the full-scale WRRF effectively caused the effluent NH_4^+ level to drop with a corresponding increase in effluent NO_2^-/NO_3^- concentration (Figure 5.3). These observations provide evidence of the presence of nitrifiers in raw wastewater which supported the nitrifying activity of the reactors.

Seeding the Positive Control reactor at Day 200, with the same rate of 5 mg-COD/L of nitrifying biomass as for the Test Reactor, with cold-adapted nitrifying culture resulted in the re-establishment of near complete nitrification in this reactor. However, the drop in NH_4^+

concentration in the effluent occurred within 17 days, while the NH_4^+ drop occurred within 75 days for the Test Reactor that received real municipal wastewater solids (Figure 5.3). Stopping the supply of nitrifying enrichment at Day 280 led to a gradual increase in effluent NH_4^+ concentration, while resuming seeding as from Day 350 restored nitrification. This shows that the supply of external seed was necessary to maintain nitrification in this reactor under unfavorable operational conditions (low temperature and SRT).

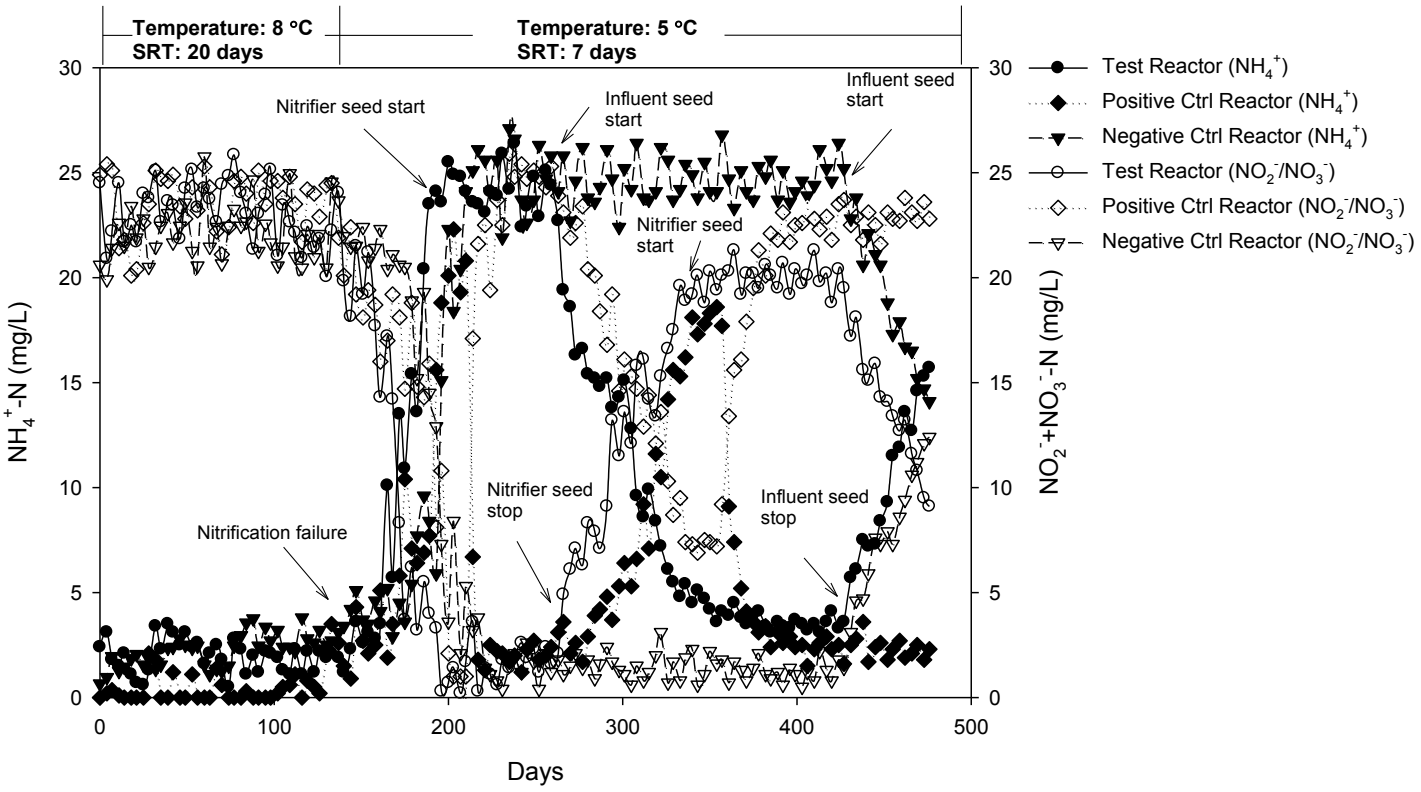

Figure 5.3. Nitrification performance of SBRs showing effluent NH_4^+ and NO_2^-/NO_3^- concentrations. Nitrification failure was induced at Day 131 by reducing the operational temperature from 8 ºC to 5 ºC and SRT from 20 to 7 days. The Test Reactor (Days 261-425) and the Negative Control reactor (Days 430-470) received influent solids as nitrifier seed (Influent seed). The Positive Control reactor (Days 200-279 and Days 350-470) received cold-adapted enriched nitrifying biomass as seed (Nitrifier seed). Other operational and performance parameters are provided in Figure S5.1 of the Supplementary Data Sheet.

5.3.2 Impact of seeding on nitrifying populations

Pyrosequencing of *amoA* and *nxrB* PCR amplicons from reactor and seed samples yielded 121,765 and 121,498 effective sequence tags for AOB and *Nitrospira*-related NOB, respectively. The sequence reads were clustered at 3% cutoff level to generate distinct species-level OTU phylotypes (Keijser et al. 2008). The number of sequence reads, OTU richness and diversity indices for each sample are shown in Table S5.2. AOB populations showed higher richness as compared to *Nitrospira*-related NOB populations, considering that only the *Nitrospira* genus was studied as NOB. The diversity, as measured by the Hill number (based on Shannon entropy), was fairly low in all reactor samples ranging from 2.08-9.32, and 1.73-4.85 effective number of species for AOB and *Nitrospira*-related NOB populations, respectively. This was due to the extreme dominance of 1-4 OTUs (Figure 5.4, S5.2 and S5.3).

During the first experimental period (Days 1-130) when nitrification was sustained within the SBRs because of the 20-d SRT, the most abundant nitrifying populations remained mostly the same for all samples (Figure 5.4, S5.2 and S5.3). A certain dynamics was observed in the population structures with the rank-order of OTU abundance, which sometimes varied between samples. When seeding was applied, the nitrifying bacterial population structures of the mixed liquors were very close to the nitrifying population structures of the corresponding seeds (Figure 5.4, S5.2 and S5.3). This remain true even if the structures of the seed samples varied over time, suggesting that the dynamics of the nitrifying bacterial structure in the mixed liquor were determined by the dynamics of the nitrifying population structure of the seed during the experimental period. Quantitative analyses (based on Eq. 5.1) showed that 86-98% of the *amoA* sequences were from AOB OTUs shared between the mixed liquor and seed samples collected on the same day (Figure 5.4, S5.2 and S5.3). For *Nitrospira*-related NOB, 93-99% of *nxrB* sequences were shared between matched mixed liquor and seed samples. Therefore, seeding had a strong impact in shaping the nitrifying population structure of the mixed liquors for all the reactors.

The taxonomic affiliations of the most abundant AOB and *Nitrospira*-related NOB OTUs were identified (Figure 5.5). For the AOB populations, species from five distinct lineages (lineage 1, 2, 3, 5 and 6) could be identified on the phylogenetic tree. Prior to seeding (Day 1-130), *Nitrosomonas oligotropha* (OTU409) was the most abundant AOB species in the Test Reactor. At the time of seeding, *Nitrosomonas europaea* (OTU195) and *Nitrosomonas eutropha*

(OTU603) emerged as the most dominant AOB species in the mixed liquor of the Test Reactor. Concurrently, they were also the most abundant AOB species in the influent seed. The same dynamics were observed when seeding the Negative Control reactor at the end of the experimental period. Seeding it with influent solids caused the sewage-derived AOB species, *Nitrosomonas europaea* (OTU195) and *Nitrosomonas eutropha* (OTU603), to emerge as the most dominant AOB species.

Similar observations were made for *Nitrospira*-related NOB. Prior to seeding, *Ca*. Nitrospira defluvii (OTU28) and uncultured *Nitrospira* sp. (OTU39) were the most dominant *Nitrospira*-related NOB in the Test Reactor. However, seeding the reactor with influent solids caused a shift in the population structure with uncultured *Nitrospira* sp. (OTU252 and OTU191) emerging as the most abundant *Nitrospira*-related NOB. This reflected the *Nitrospira*-NOB population structure of the influent seeds which were also dominated by the same species. Similar observation was made for the Negative Control reactor where the most abundant species namely uncultured *Nitrospira* sp. (OTU39) and *Ca.* Nitrospira defluvii (OTU28) were superseded by the dominant sewage-derived *Nitrospira*-related NOB namely uncultured *Nitrospira* sp. (OTU252 and OTU191).

Comparable dynamics were observed with the Positive Control reactor where the same nitrifying AOB species (*Nitrosomonas oligotropha*, OTU409) dominated the mixed liquor during the initial phase of the experiment. Supplementing the reactor with cold-adapted enriched nitrifying biomass caused *Nitrosomonas europaea* (OTU195) and *Nitrosomonas eutropha* (OTU603), to become the most abundant AOB species in the reactor. The seed also induced dominance of sewage-derived *Nitrospira*-related NOB in the mixed liquor of the Positive Control reactor.

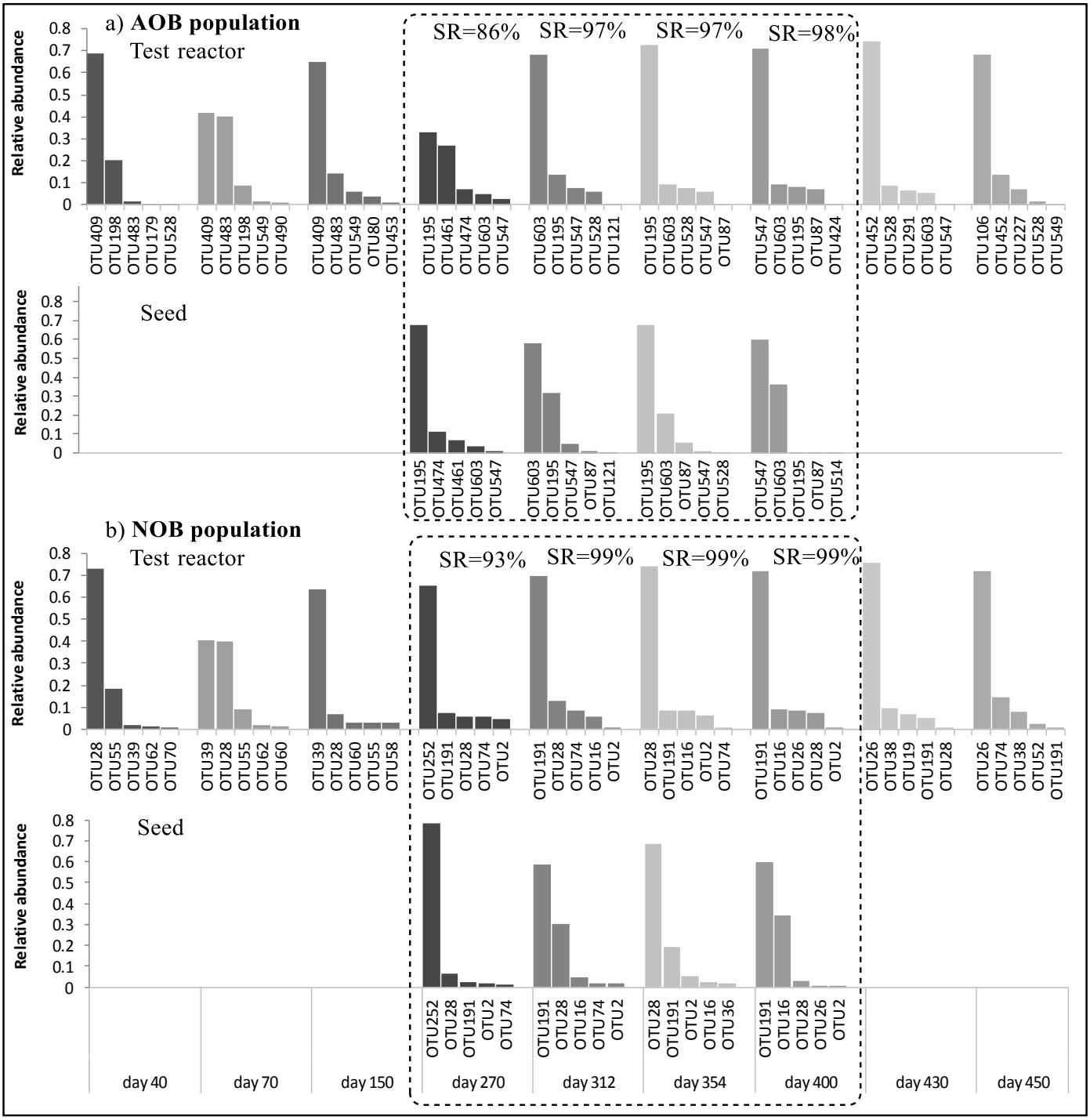

Figure 5.4. Bar charts showing the 5 most abundant OTUs for AOB (a) and NOB (b) populations in the Test Reactor and its corresponding influent seeds. Seeded activated sludge samples were collected on day 270, 312, 354 and 400, respectively for bacterial community analysis (indicated by the dashed-line box). SR – Shared Reads (Eq.5.1).

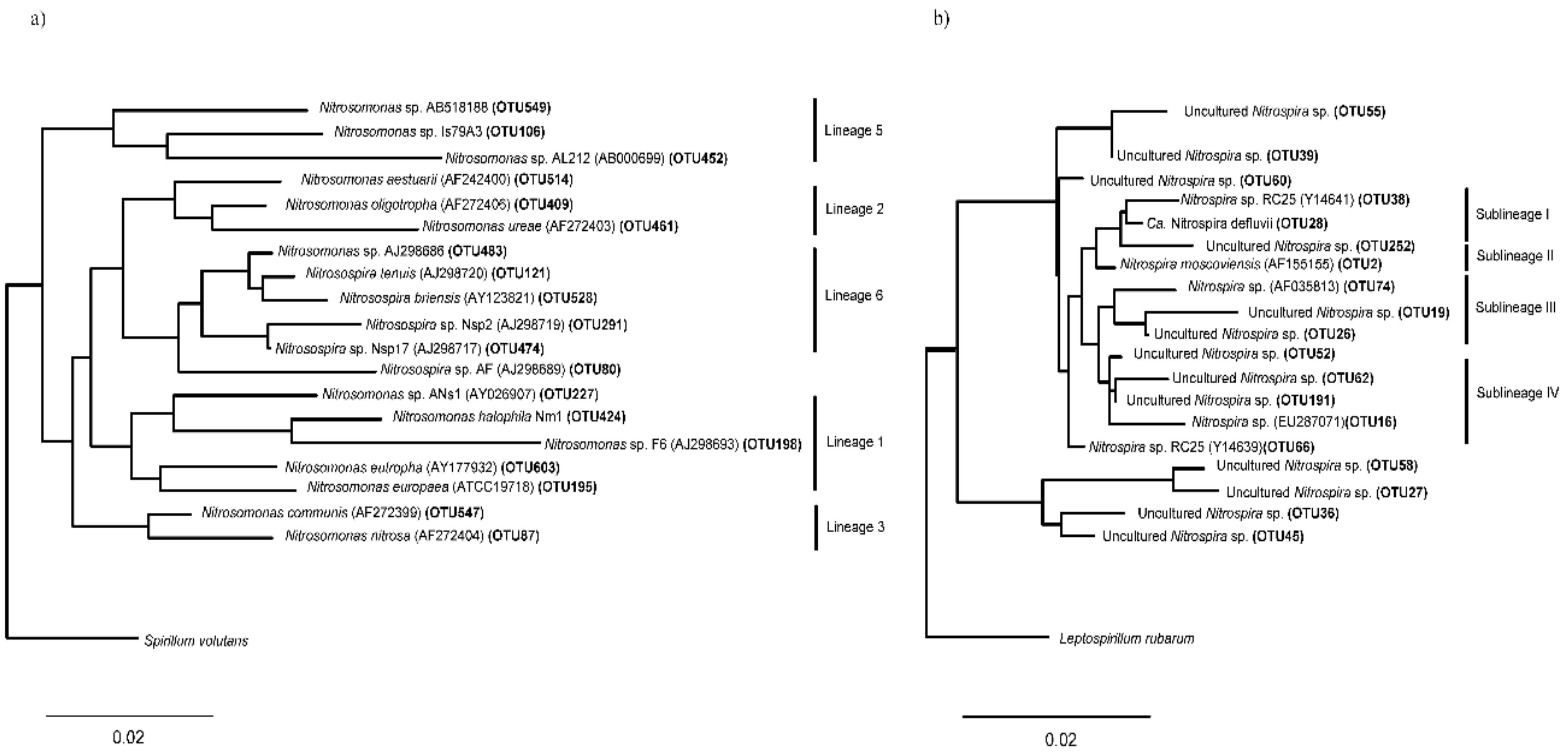

Figure 5.5. Dendograms showing phylogenetic positions of most dominant nitrifiers at species level based on sequences obtained after amplification of *amoA* and *Nitrospira*-related *nxrB* genes in activated sludge of Test Reactor and influent seed. Phylogenetic trees for most dominant AOB (a) and NOB (b) populations, identified based on computed Simpson diversity numbers representing number of dominant species in communities. Bracketed OTU numbers highlighted in bold belong to the most dominant AOB and *Nitrospira*-related NOB species detected in the samples. Entries without species designations are reported by their GenBank accession numbers. The scale bars represent 0.02 substitution per nucleotide position for the AOB and NOB phylogenetic trees.

5.3.3 Microbial community structures by *16S rRNA* gene sequence analysis

Addition of sewage solids to the Test Reactor influenced the heterotrophic bacterial community structures of the mixed liquor. The influent seeding increased the species richness and diversity of the activated sludge communities as shown in Table S5.3 of the Supplementary Data Sheet. A halt in the supply of influent seed decreased the richness and diversity of the reactor communities. Similarly, the species richness and diversity of the Negative Control reactor increased at the end of the experimental period when influent seed was supplied to the reactor (Table S5.3).

Principal Coordinate Analysis (PCoA) was used to visualize overall patterns of dispersion of bacterial communities of the seed and reactor samples (Figure 5.6). The mixed liquor samples from the three reactors obtained during the first experimental period (D1-D130) clustered in the top-right quadrant, while the influent and nitrifying enrichment seed samples clustered in the bottom- and top-left quadrants, respectively. This indicates that there were clear differences in the composition of the microbial communities. For the Test and Negative Control reactors, the onset of influent seeding induced gradual vectorial drifts of the activated sludge communities towards the seed communities (Figure 5.6). In the case of the Test Reactor, the drift seemed to be reversible as the community structure moved back towards the initial location on the PCoA plot for the period when supply of influent seed was halted (D430-470). The seed-induced drift was also apparent for the Positive Control reactor which displayed clear gradients following successive addition of enriched nitrifying biomass. When seeding was stopped between Day 312 to 354, the mixed liquor community structure drifted back toward the original cluster, but resumed its movement towards the nitrifying enrichment when seeding was restored.

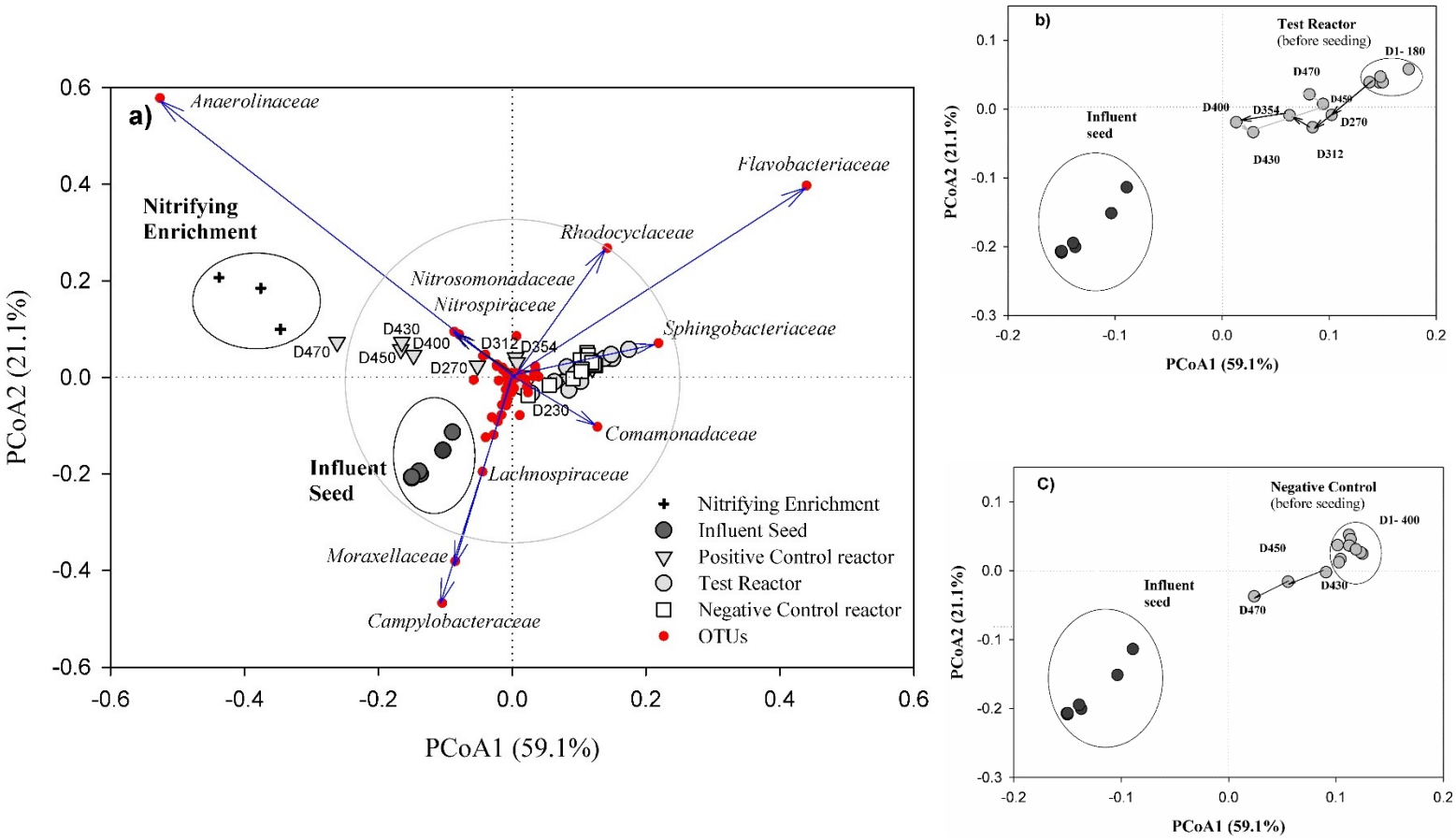

Figure 5.6. Principal coordinate analysis (PCoA) of *16S rRNA* gene amplicon pyrosequencing data for samples of mixed liquors from Test, Positive Control and Negative Control reactors, and influent and nitrifying enrichment seeds (a). Ordination of samples was based on Bray-Curtis distance at 3% cutoff level. The families to which the identified species belong to are projected on the PCoA plot as red dots. Samples are represented by different symbols. The grey circle corresponds to the equilibrium circle (Legendre and Legendre 1998). Zoomed in portion of PCoA for Test Reactor (b) Negative Control reactor (c) are presented. Numbers starting with "D" indicate the days on which the samples were collected.

The families which contributed significantly to the ordination have vectors outside the equilibrium circle as shown in Fig. 5.6. In the bottom-left quadrant, the *Moraxellaceae* and *Campylobacteraceae* families were found particularly imposing on the ordination pattern and predominantly present in influent samples. Lee et al. (2015) also observed their dominance in raw sewage. Opposite to these two vectors were the *Rhodocyclaceae* and *Flavobacteriaceae* families which mapped in the top-right quadrant close to the reactor samples. These families commonly occur in activated sludge (Zhang et al. 2011). The occurrence of *Rhodocyclaceae* and *Flavobacteriaceae* vectors at nearly 180º from the *Moraxellaceae* and *Campylobacteraceae* indicates a negative correlation between them. Seeding of the Test and Negative Control reactors with influent solids decreased the abundance of *Rhodocyclaceae* and to a lesser extent the abundance of *Flavobacteriaceae*, while increasing the abundance of *Moraxellaceae* and *Campylobacteraceae* (Figure S5.4). A halt in the supply of influent seed induced the reverse trend by increasing the abundance of *Rhodocyclaceae* and *Flavobacteriaceae*, and decreasing the level of *Moraxellaceae* and *Campylobacteraceae* in the mixed liquor. This shows the impact of the influent seeds on structuring the bacterial communities in the activated sludge. Even at the phylum level, this effect was somewhat noticeable where prior to seeding, the mixed liquor of the Test Reactor comprised of *Bacteroidetes* as the predominant phylum consisting of 62.7% of the detected OTUs, followed by *Proteobacteria* (25.6%). Supplementing influent solids, which consisted of predominantly *Proteobacteria* (50.1%) followed by *Bacteroidetes* (18.4%), reduced the abundance of *Bacteroidetes* in the Test Reactor by 15% and increased the level of *Proteobacteria* by 34%. Similar observation was made with the Negative Control reactor where the addition of influent seed reduced the abundance of the *Bacteroidetes* by 25% and increased the level of *Proteobacteria* by 5% in the mixed liquor.

The relationship between the influent, sludge of the Test Reactor during seeding, and Negative Control in the absence of seeding during the period Day 270-400 was further explored to determine the number of bacterial genera shared between them. Based on 168 identified genera, 48 were shared among the influent, Test Reactor and Negative Control reactor (Figure S5.5). Although the influent had distinct microbial community composition, it imparted 22% of the number of genera to the Test Reactor including *Nitrosomonas* and *Nitrospira* which were key members in re-establishing nitrification during the failure period. This shows that despite their marked differences, bacterial communities of the activated sludge reactor were not totally independent from influent wastewater communities.

5.4 DISCUSSION

5.4.1 Influent-induced nitrification

Addition of influent solids to the Test Reactor operated at near washout conditions (temperature of 5 °C and SRT of 7 days) restored nitrification causing a decrease in effluent NH_4^+ level and a concurrent increase in effluent NO_2^-/NO_3^- concentration. Interrupting the supply of influent seed to the Test Reactor caused the effluent NH_4^+ level to increase and the effluent NO_2^-/NO_3^- level to decrease indicating that a constant supply of influent seed was necessary to maintain nitrification in the Test Reactor. Reversing the seeding regime by supplementing the Negative Control reactor with influent seed restored nitrification showing that the influent seeding effect was reproducible and a certain level of operational control could be exerted on the process.

This provides strong evidence that incoming raw wastewater contains nitrifying bacteria. It also demonstrates that the phenomenon of natural nitrifier seeding is playing a key role in sustaining nitrification at full-scale level in extreme conditions such as low temperature and SRT. Since the metabolic induction time of influent nitrifiers have been shown to be only a few hours (Jauffur et al. 2014), and considering that wastewater treatment systems are normally operated at a few day SRT, the seeded nitrifiers could have been adsorbed on activated sludge flocs and bioaugment the nitrifying populations. Such adsorption is likely since nitrifiers have been shown to possess strong adhesion properties. Larsen et al. (2008) studied the adsorption of *N. oligotropha* and *Nitrospira* sp. on flocs and found that activated sludge flocs consisted of an easily detachable fraction (5-15%), a fraction resistant to deflocculation (15-40%), and a strong, non-detachable

fraction (50-75%). Nitrifiers presumably belong to the non-detachable fraction of activated sludge flocs.

Supplementing influent seed was found to be necessary in order to sustain NH_4^+ removal under conditions which were not conducive to nitrification. Such influent-induced regain in nitrification activity may be important in modeling nitrifying activated systems and points to an important limitation of ASM models which currently assume no autotrophic nitrifying biomass in influent. Integrating the natural seeding of nitrifiers by influent wastewaters may render nitrification model predictions more accurate especially for systems operating under unfavorable conditions such as cold climate or at sub-optimal SRT. In design of nitrifying activated sludge systems, a safety factor of 2-3 is usually applied to account for fluctuations in nitrifiers' growth rates resulting from specific local conditions (load variation, temperature, pH, inhibition and dissolved oxygen) (Rittmann and McCarty 2001). The supply of a natural nitrifier seed by the influent flow can increase this safety factor without increasing reactor footprint considering that any increase in SRT will increase the solids inventory, which must be offset by increasing the size of the bioreactor and clarifier. In addition, supply of influent seed can help a nitrifying system on the verge of washout to recover by re-establishing its nitrifying population.

Bacterial community analyses showed that the diversity of the bench-scale reactors was lower than the diversity observed at full-scale systems. For instance, the Hill diversity number for AOB populations in the seeded Test Reactor ranged between 3.18-3.71 as compared to full-scale studies where the observed AOB diversity ranged between 6.69-10.07 (Baek et al. 2010) and 13.0-66.0 (chapter 4) (Jauffur et al. 2015, In preparation). This is due to the presence of only a few dominant species (1-4) in the samples analyzed followed by long tails of rare taxa as shown by the OTU rank-abundance analyses. Moreover, discrepancies in bacterial diversities at lab- and full-scale systems have also been reported in other studies and may result from differences in operational and environmental factors (Briones and Raskin 2003).

Most importantly, the patterns of nitrifying bacterial population in the activated sludge of the Test Reactor, and the Negative Control reactor at the end of the experimental period, were similar to the ones of the corresponding influent seeds. The most abundant AOB and *Nitrospira*-related NOB OTUs observed in the influent seeds also occurred as the dominant ones in the activated sludge. This shows that seeding the Test Reactor or Negative Control reactor with influent solids restructured the community of the reactors with *Nitrosomonas europaea* and

Nitrosomonas eutropha emerging as dominant species in the activated sludge. Previous studies have reported the wide dominance of these species in full-scale WRRFs receiving municipal wastewaters (Figuerola and Erijman 2010, Zhang et al. 2011). Stopping the supply of influent seed to the Test Reactor incurred a restructuring of the most dominant AOB species, a period coinciding with a failure in nitrification. According to Wittebolle et al. 2005, nitrification failures at full-scale WRRFs are intimately linked to shifts in microbial community structures. For NOB populations, mostly uncultured *Nitrospira* species were found dominant in the activated sludge and influent seeds. Abundant identified species included *Ca.* Nitrospira defluvii and *Nitrospira moscoviensis,* which are characteristic NOB species inhabiting activated sludge (Koch et al. 2015, Kruse et al. 2013). In this case as well, the dominant *Nitrospira*-related NOB species identified in the activated sludge were found to also occur as dominant species in the influent seed. The same dynamics occurred in the Positive Control reactor where addition of cold-adapted enriched nitrifying biomass restructured the nitrifying communities such that the most abundant species in the seed became dominant in the mixed liquor of the reactor.

Based on the above observations, raw wastewater is found to be a key factor shaping nitrifying communities in full-scale wastewater treatment systems. The source of nitrifying bacteria in the influent wastewaters was not investigated in this study. However, it is believed that they enter sewer networks bound to soil particles through surface runoffs. Once in the sewer, they may colonize strategic places conducive for them to grow. Erosion and sloughing of sewer biofilms are potential means of biomass transportation to full-scale WRRFs (Jahn and Nielsen 1998). Considering that influent streams and full-scale bioreactors are physically connected systems, it is not surprising that bacterial species, including nitrifiers, are continuously supplied to aeration basins by influent wastewaters. In this case, we show that the influent is a key source for maintaining activity and community structure of nitrifiers in activated sludge.

5.4.2 Seeding effect on heterotrophic bacterial populations

Unlike nitrifying bacterial populations, heterotrophic communities have a large breadth of ecological functions (Quince et al. 2008). According to Saunders et al. (2015), these different functional entities in activated sludge ecosystems can mainly be classified as abundant core or transient populations. In this context, it would be legitimate to question the role of immigrant microorganisms conveyed by influent wastewaters and assess their impacts on the selection of

bacterial populations or on the niche occupation of bacterial communities in activated sludge systems.

The cluster analysis showed that activated sludge and influent seeds had distinct bacterial populations and mapped apart on the PCoA bi-plot. This difference between influent and sludge community compositions has been reported in earlier studies (Hashimoto et al. 2014, Lee et al. 2015, Liu et al. 2007). The influent communities comprised of families such as *Moraxellaceae, Campylobacteraceae* and *Lachnospiraceae* which are typically present in untreated wastewaters as also observed in previous studies (McLellan et al. 2010, Ye and Zhang 2013). The inability of certain influent bacteria to grow and persist in environmental conditions prevalent in activated sludge may explain this dissimilarity. Activated sludge microorganisms have broad carbon-utilization profiles with an enriched community of degraders acclimated to specific bioreactor environments, which would impose a selective growth of specific populations. Based on our observations, it is clear that the bioreactors imposed a selective pressure on the incoming immigrant heterotrophic populations leading to distinct populations as shown on the PCoA plot. Nonetheless, the immigrant communities also appear to modulate the structure of the activated sludge communities. For instance, seeding the Test Reactor with influent solids decreased the abundance of *Rhodocyclaceae* and *Flavobacteriaceae* while increasing the abundance of *Moraxellaceae* and *Campylobacteraceae*. Such negative correlation between these families was also observed when seeding the Negative Control reactor with influent solids. This effect can be visualized on the ordination plot where seeding of the reactors with influent solids induced vectorial drifts of the communities towards their respective seeds but regressed to their initial positions when seeding was halted. This shows that influent microbiome has an impact on shaping activated sludge communities, and argues against Hashimoto et al. (2014) who opined that influent communities have almost no influence on reactor communities. About 22% of the genera identified in the seeded Test Reactor appeared to come from the influent biomass indicative that raw sewage contributed in structuring the microbial community of the reactor. According to Verberk (2012), these overlapping taxa may be generalists capable of surviving in both sewer and reactor environments. In subsequence, activated sludge communities are not completely independent from influent communities as recognized by Lee et al. (2015).

At full-scale level, raw sewage communities may, thus, be contributing to determine bacterial community compositions and structures of activated sludge systems. Curtis and Craine (1998)

found a large number of microorganisms from sewage in mixed liquor samples even though according to them some may not have major functional significance in the treatment process. The supply of microorganisms to full-scale activated sludge systems may be constantly occurring through immigration as observed by Leibold et al. (2004). Our findings provide evidence that influent wastewater influences activated sludge communities by supplying consortia of bacteria, which may be contributing to the treatment of the incoming wastewater.

5.5 CONCLUSIONS

- Seeding sequencing batch reactors operated above washout conditions (low temperature and SRT) with real wastewater solids restored nitrification indicating the presence of live nitrifiers in raw sewage. The latter stabilized the treatment systems and maintained nitrification in an otherwise non-conducive condition.
- Phylogenetic identity of the most abundant nitrifiers in influent occurred as the most abundant nitrifiers in activated sludge.
- Results demonstrate the existence of natural seeding of nitrifiers at full-scale wastewater treatment level by incoming raw sewage.
- Heterotrophic bacterial community structures were distinct in influents and activated sludge mixed liquors. Nonetheless, seeding the reactors with wastewater solids modified the mixed liquor communities, making their structures more similar to the seed.

5.6 ACKNOWLEDGMENTS

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5.7 SUPPLEMENTARY MATERIALS

Table S5.1. Primers and PCR thermocycling programs used for amplification of *16S rRNA*, *amoA* and *nxrB* genes.

Target population	Primer	Sequence (5'-3')	PCR Thermocycling program	Reference
16S rRNA	*Bact-338F1*	CCTACGGGRGGCAGCAG	95 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1.5 min followed by a final extended elongation at 72 °C for 8.5 min	(Pinto and Raskin 2012)
	Bact-338F2	ACWYCTACGGRWGGCTGC		
	Bact-338F3	CACCTACGGGTGGCAGC		
	R802/18	TACNVGGGTHTCTAATCC		(Claesson et al. 2010)
AOB	*amoA-1F*	GGGGTTTCTACTGGTGGT	95 °C for 4 min, 35 cycles of 95 °C for 40 s, 56 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	(Rotthauwe et al. 1997)
	amoA-2R	CCCCTCTGCAAAGCCTTCTTC		
NOB (*Nitrospira-related*)	*nxrB-F169*	TACATGTGGTGGAACA	95 °C for 5 min, 35 cycles of 95 °C for 40 s, 62 °C for 40 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	(Maixner 2009a)
	nxrB-638R	CGGTTCTGGTCRATCA		

Table S5.2. Number of reads, OTU richness and diversity indices for autotrophic nitrifying bacterial populations in enriched nitrifier seed, municipal influent and SBR mixed liquor samples determined by *amoA* and *nxrB* PCR amplicon pyrosequencing.

Samples	Ammonia Oxidizing Bacteria (AOB) using *amoA*						Nitrite Oxidizing Bacteria (NOB) using *nxrB*					
	No. of reads (denoised)	OTU richness	Shannon diversity			Simpson diversity no.	No. of reads (denoised)	OTU richness	Shannon diversity			Simpson diversity no.
			Entropy (nat)	Hill no.	Equitability				Entropy (nat)	Hill no.	Equitability	
Enriched nitrifier seed												
Day 70	3915	98	1.36	3.90	0.30	2.40	3837	29	1.24	3.46	0.37	2.35
Day 270	3321	107	1.51	4.53	0.32	2.13	3001	35	1.35	3.86	0.38	2.55
Day 400	3363	98	1.01	2.75	0.22	2.11	3259	33	0.97	2.63	0.28	2.13
Influent seed												
Day 270	3033	86	1.48	4.40	0.33	2.11	3466	35	1.17	3.21	0.33	0.93
Day 312	3465	119	1.19	3.28	0.25	2.27	3354	33	1.12	3.07	0.32	2.27
Day 354	3423	116	1.18	3.24	0.25	1.99	3312	32	1.07	2.92	0.31	1.96
Day 400	3347	98	0.97	2.63	0.21	2.05	3270	33	0.97	2.63	0.28	2.09
Day 430	3745	123	1.33	3.79	0.28	2.42	3550	42	1.26	3.54	0.34	2.58
Day 450	3076	90	1.00	2.72	0.22	2.16	3000	33	1.00	2.72	0.29	2.23
Day 470	3441	101	1.09	2.99	0.23	2.24	3323	39	1.05	2.87	0.29	2.32
Test reactor												
Day 40	3231	104	1.28	3.60	0.28	1.96	4089	34	0.99	2.69	0.28	1.76
Day 70	3130	86	1.51	4.54	0.34	2.91	3045	30	1.46	4.29	0.43	3.01
Day 150	3974	79	1.48	4.39	0.35	2.25	3347	32	1.35	3.86	0.39	2.42
Day 270	3409	95	1.31	3.71	0.58	5.14	3011	74	1.51	4.51	0.35	2.26
Day 312	3083	114	1.24	3.46	0.27	2.03	3965	32	1.08	2.94	0.31	1.95
Day 354	3823	114	1.16	3.18	0.24	1.83	3695	36	1.25	3.49	0.35	2.15
Day 400	3011	102	1.25	3.49	0.25	1.90	3897	32	1.04	2.82	0.30	1.86
Day 430	3237	81	0.91	2.48	0.56	1.02	3128	26	0.81	2.24	0.24	1.67
Day 450	3422	62	0.73	2.08	0.50	0.81	3426	19	0.55	1.73	0.18	0.78
Positive Control												
Day 40	3521	93	1.76	5.82	0.39	2.98	3413	31	1.49	4.43	0.43	2.75
Day 70	3142	83	1.55	4.71	0.35	2.82	3077	36	1.44	4.22	0.40	2.31
Day 150	3137	104	2.23	9.32	0.48	3.29	3612	35	1.53	4.64	0.43	2.18
Day 230	3356	102	1.86	6.42	0.25	3.17	3809	43	1.58	4.85	0.42	2.93
Day 270	3296	118	1.92	6.82	0.40	3.24	3151	36	1.49	4.44	0.42	2.32
Day 312	3871	70	1.14	3.12	0.26	1.57	4313	28	1.08	2.94	0.32	1.51
Day 400	3847	98	1.78	5.92	0.39	1.95	3756	40	1.42	4.14	0.38	2.23
Day 430	3400	107	1.64	5.16	0.35	1.93	3302	46	1.52	4.57	0.40	2.67
Day 450	3473	118	1.76	5.81	0.37	2.06	3354	41	1.49	4.44	0.40	2.51
Day 470	3802	95	2.03	7.61	0.45	2.02	3707	48	1.55	4.71	0.41	2.85
Negative Control												

Day 40	3738	76	1.50	4.46	0.35	2.93	3637	22	1.38	3.98	0.45	2.94
Day 70	4467	96	1.53	4.64	0.34	2.88	3380	28	1.42	4.14	0.43	2.89
Day 150	3465	74	1.39	4.03	0.32	2.01	3523	33	1.22	3.38	0.35	1.95
Day 430	3820	70	0.98	2.66	0.23	1.73	3718	58	0.93	2.53	0.23	1.68
Day 450	3820	70	1.13	3.10	0.23	1.75	3754	20	1.02	2.45	0.30	1.70
Day 470	3161	80	1.25	3.50	0.25	2.05	3018	25	1.10	2.56	0.29	1.94

Table S5.3. Number of reads, OTU richness and diversity indices for the entire microbial communities from enriched nitrifier seed, wastewater influent and SBR mixed liquor as determined by *16S rRNA* gene amplicon pyrosequencing.

Samples	No. of reads (denoised)	OTU richness	Shannon diversity			Simpson diversity no.[b]
			Entropy (nat)	Hill no.[a]	Equitability	
Enriched nitrifier seed						
Day 70	6480	114	1.84	6.28	0.39	2.43
Day 270	5544	107	2.56	12.9	0.55	5.02
Day 400	6246	131	2.31	10.05	0.47	3.14
Influent seed						
Day 270	5599	242	3.69	40.05	0.67	18.09
Day 312	5731	237	3.71	40.89	0.68	18.60
Day 354	6566	217	3.74	42.25	0.69	19.39
Day 400	5930	319	4.00	54.82	0.69	24.11
Day 430	4040	205	3.69	39.86	0.69	17.82
Day 450	5761	213	3.76	42.81	0.70	20.06
Day 470	5478	225	3.70	40.49	0.68	18.24
Test reactor						
Day 40	5583	143	2.66	14.32	0.54	6.48
Day 70	5943	151	2.59	13.28	0.52	6.98
Day 150	5530	165	3.03	20.73	0.59	9.51
Day 200	5010	177	2.95	19.19	0.57	8.18
Day 230	5530	174	3.18	23.98	0.62	9.41
Day 270	5321	214	3.36	28.79	0.63	11.38
Day 312	5942	227	3.42	30.57	0.65	12.45
Day 354	6611	225	3.39	29.55	0.64	12.39
Day 400	6826	210	3.35	28.50	0.63	11.52
Day 430	6843	189	3.04	20.91	0.58	8.31
Day 450	6671	164	3.02	20.49	0.59	9.52
Day 470	6033	160	2.96	19.25	0.58	8.26
Positive Control						
Day 40	5788	151	3.08	21.83	0.61	9.80
Day 70	5016	139	2.71	14.98	0.55	7.05
Day 150	5652	143	2.81	16.62	0.57	6.32
Day 200	6579	178	3.39	29.66	0.65	10.58
Day 230	5536	204	3.56	35.10	0.67	15.14
Day 270	5945	210	3.62	37.34	0.68	15.23
Day 312	6794	170	3.07	21.58	0.59	8.65
Day 354	5454	144	2.54	12.68	0.51	4.75
Day 400	5871	208	3.62	37.34	0.68	15.35
Day 430	5245	211	3.71	40.85	0.69	15.42

Day 450	5303	214	3.85	46.99	0.72	15.73
Day 470	5880	217	3.88	48.42	0.69	15.81
Negative Control						
Day 40	5541	95	2.73	15.40	0.60	8.85
Day 70	5448	125	2.69	14.88	0.56	8.32
Day 150	5677	127	2.62	13.79	0.54	7.19
Day 200	5010	128	2.98	19.62	0.61	10.42
Day 230	5309	133	2.94	18.87	0.60	10.12
Day 270	5900	129	2.88	17.74	0.59	8.02
Day 312	5968	151	3.12	22.73	0.62	10.84
Day 354	7024	148	3.27	26.22	0.65	13.81
Day 400	5097	144	3.27	26.24	0.66	14.72
Day 430	5076	239	3.58	35.89	0.65	16.18
Day 450	5075	231	3.74	42.06	0.69	19.37
Day 470	5182	241	3.49	32.96	0.64	19.42

[a] Hill no. = exp [Shannon entropy]

[b] Simpson diversity no. = $1/\sum_{i=1}^{S} p_i^2$, where p_i is the proportion of the i^{th} OTU

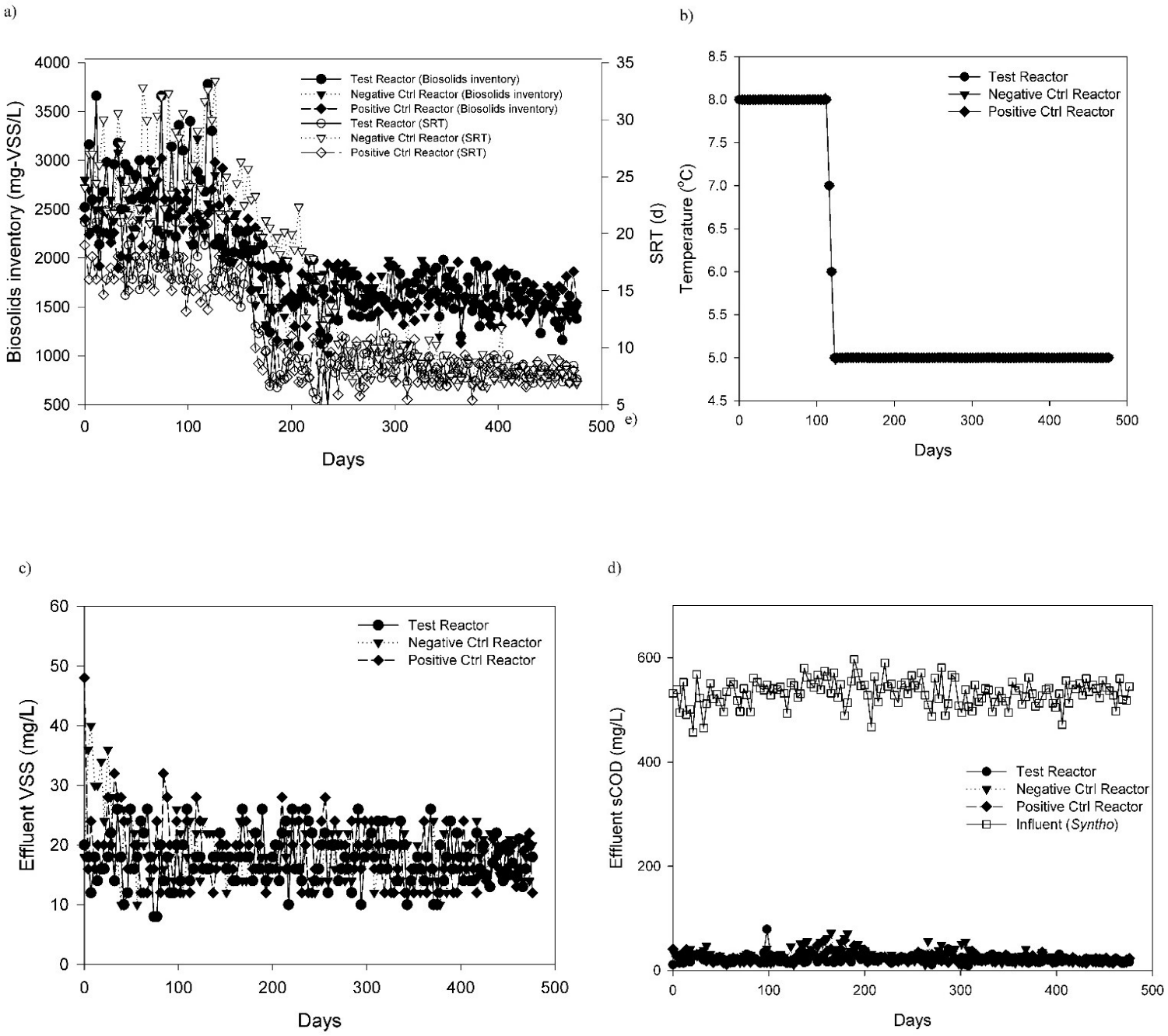

Figure S5.1. Operational and performance parameters for SBRs. Variation of biosolids inventory and SRT (a), temperature (b), effluent VSS (c), and effluent sCOD (d) over time.

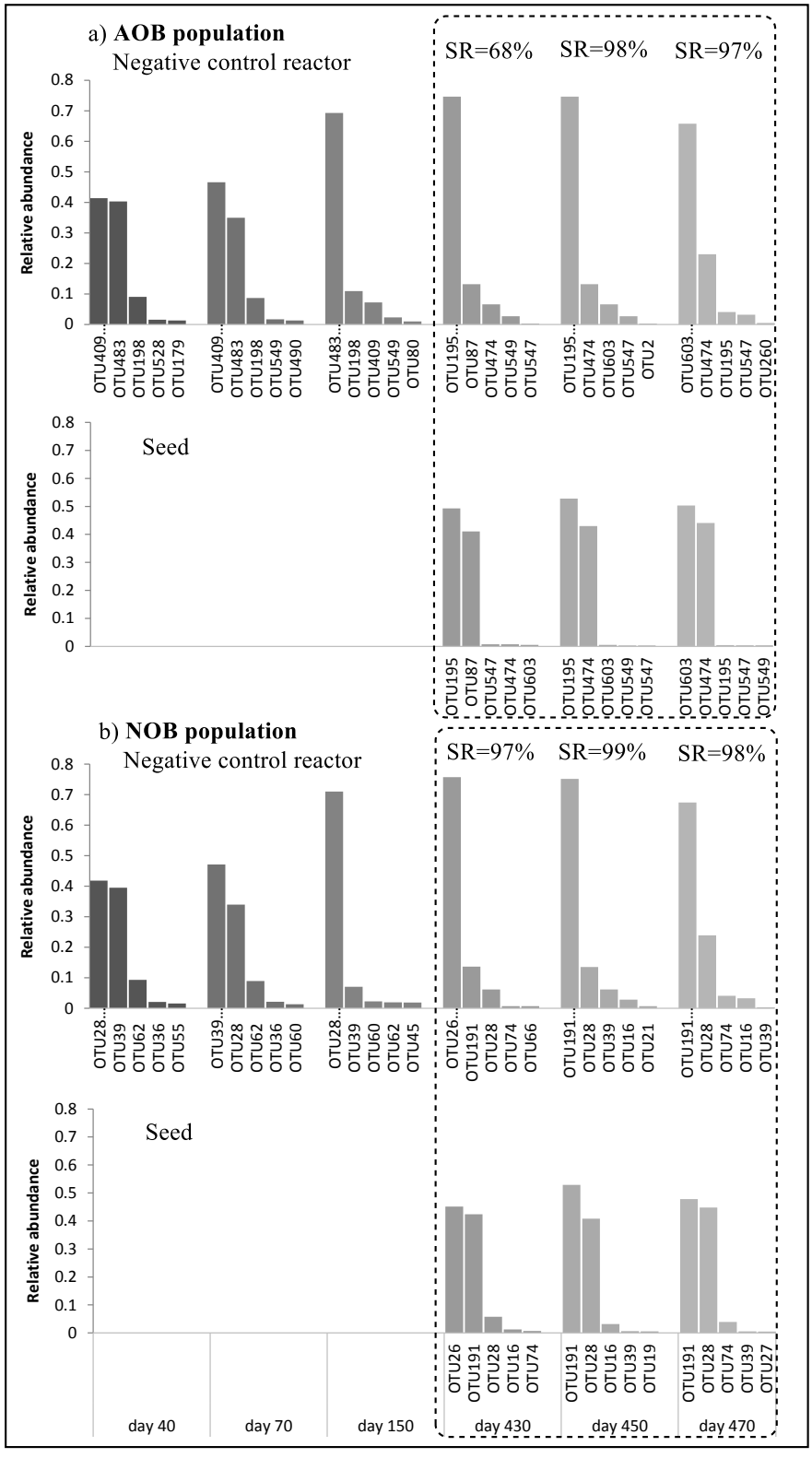

Figure S5.2. Bar charts showing the 5 most abundant OTUs for AOB (a) and NOB (b) populations in the Negative Control reactor and its corresponding seed. Seeding of the reactor with influent biomass was performed on day 430, 450 and 470, respectively (indicated by the dashed-line box). SR – Shared Reads (Eq. 5.1).

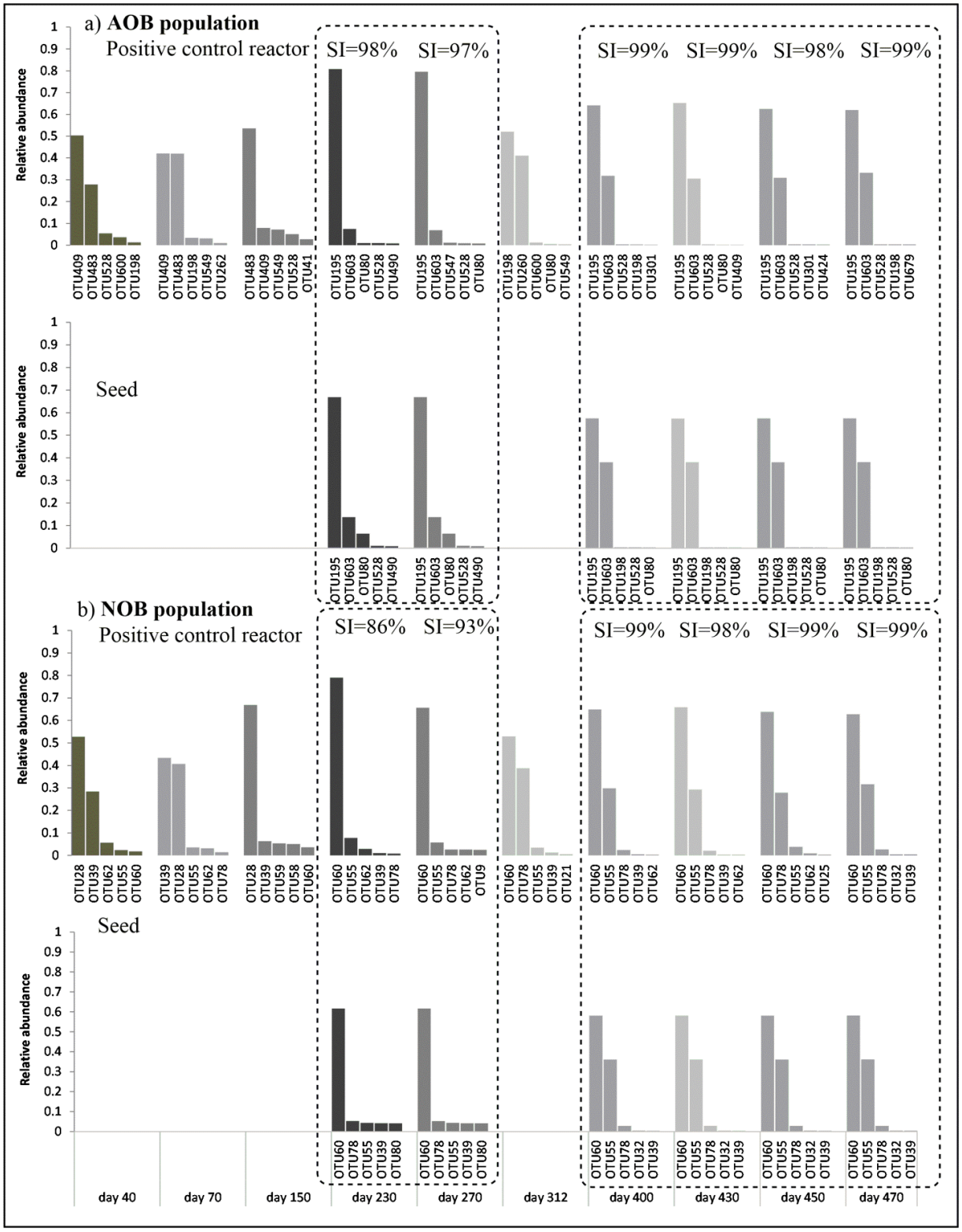

Figure S5.3. Bar charts showing the 5 most abundant OTUs for AOB (a) and NOB (b) populations in the Positive Control reactor and its corresponding seed. Seeding of the reactor with enriched cold-adapted nitrifying biomass was performed on day 230, 270, 400, 430, 450 and 470, respectively (indicated by the dashed-line box). SR - Shared Reads (Eq. 5.1).

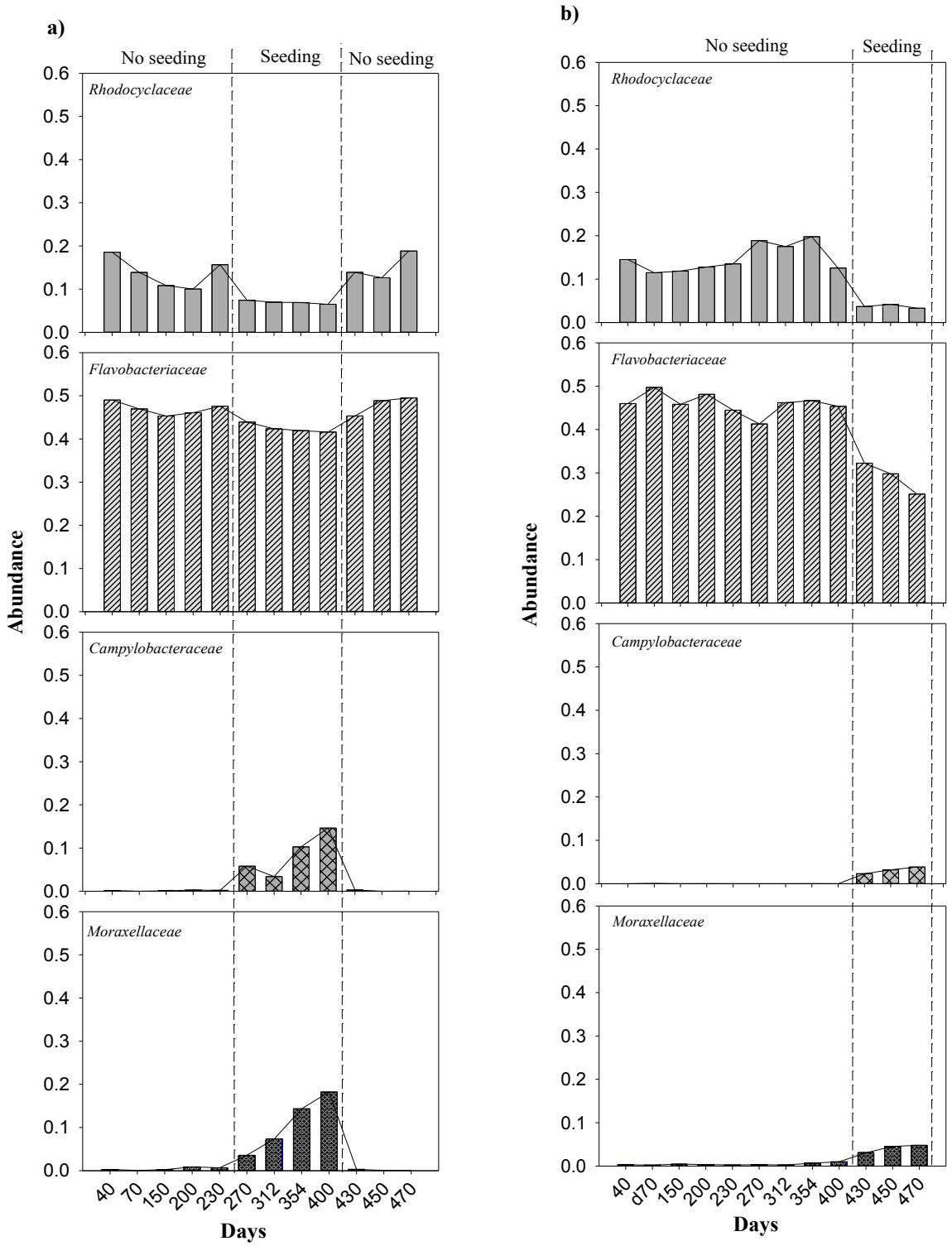

Figure S5.4. Abundance charts for the family *Rhodocyclaceae, Flavobacteriaceae, Campylobacteraceae* and *Moraxellaceae* for the Test Reactor (a) and Negative Control reactor (b). The seeding/no seeding periods are demarcated by the dashed lines.

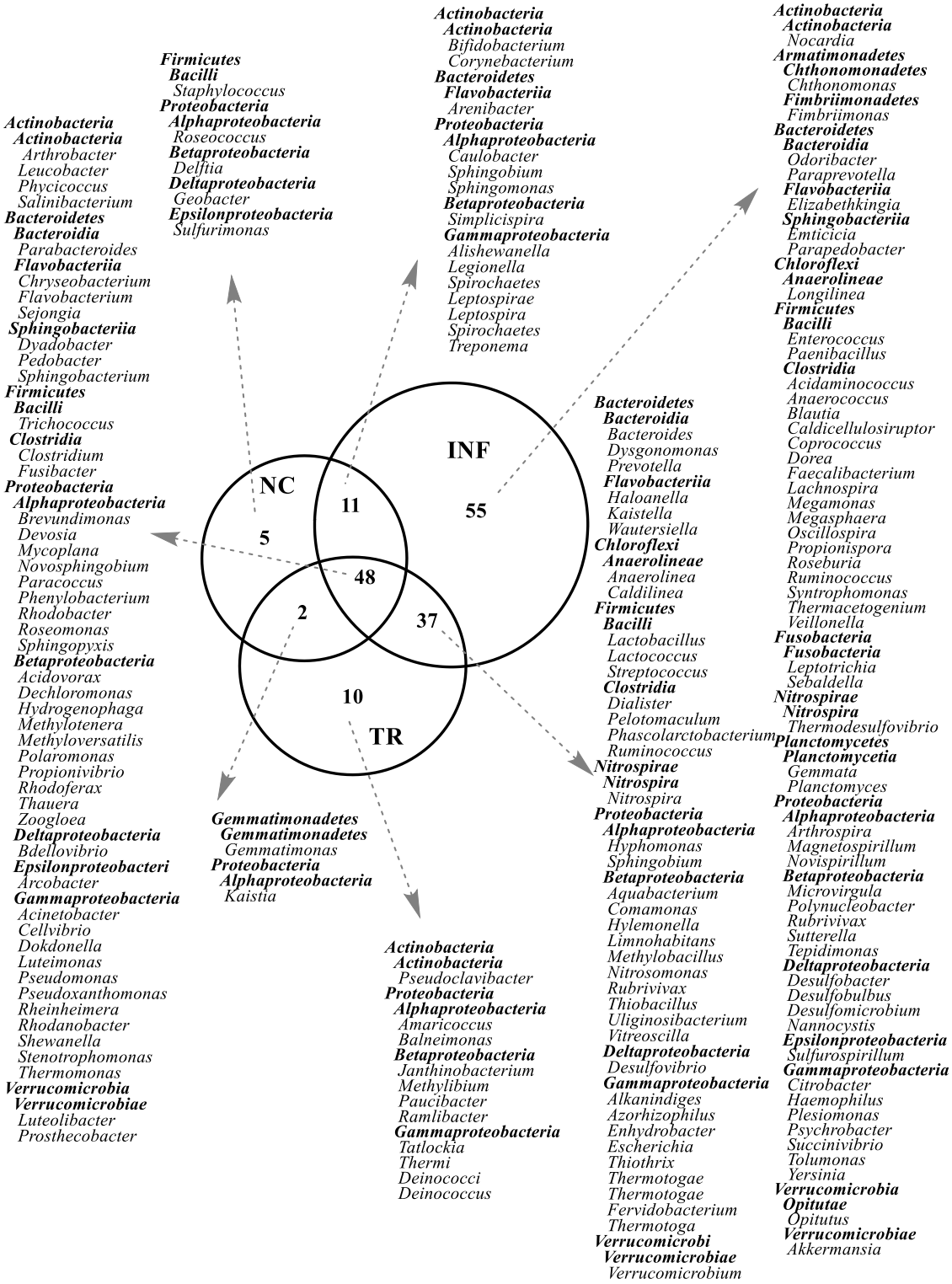

Figure S5.5. Venn diagram showing shared genera (indented with light font) between influent (INF), Test Reactor (TR) and Negative Control (NC) during period Day 270-400. Respective phyla (bold and not indented) and classes (bold and indented) to which the identified genera belong are also indicated.

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CHAPTER 6

Importance of influent nitrifiers to accurately model biological wastewater treatment systems

Connecting text: Chapter 4 and 5 evidenced the importance of natural influent nitrifier seeding of bioreactors in determining the nitrifying population structures in activated sludge, and in sustaining nitrification activity under unfavourable operational conditions. In Chapter 6, a modeling exercise using one year of operational data of a full-scale WRRF, was performed. Scenarios with or without influent nitrifier seeding were considered. The results suggest that various sets of model parameter values may have been derived to compensate for the assumption that nitrifiers are not present in influent wastewaters reaching full-scale WRRFs. Given that the influent nitrifier seed can vary between locations and types of sewer system, this study suggests that current best modeling practices should be amended to include nitrifying biomass in municipal wastewaters during influent characterization. However, standardized methodologies to detect and quantify influent nitrifiers have yet to be established.

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6.1 INTRODUCTION

Mathematical models are essential tools in process design, operation and optimization of wastewater treatment systems (Hauduc et al. 2013). To date, the International Water Association (IWA) Good Modeling Practices (GMP) for activated sludge wastewater treatment, do not consider active biomass in influent wastewaters (Rieger 2013). This assumption comes primarily from the fact that there is little knowledge regarding the impact of influent biomass on models and no standardized protocol to detect and quantify its level. Disposal of excrements, infiltration of soil particles, sloughing of sewer-line biofilms and resuspension of sewer sediments, contribute to the conveyance of microbial biomass to full-scale Water Resource Recovery Facilities (WRRFs) (Shanks et al. 2013). The seeding of heterotrophic biomass does not affect treatment processes because the growth rate is high and washout never occurs in practice (Henze 2000). However, this is not the case for nitrifiers, which are sensitive to environmental conditions and take longer times to recover once they have been washed-out of the system because of their low growth rates and biomass yields (Yu et al. 2012). Although Activated Sludge Models (ASM) do not consider influent nitrifying biomass, its presence in municipal wastewaters reaching full-scale WRRFs, especially without primary settling, may be important to keep sufficient nitrification in the system in cases where the sludge retention times (SRTs) are low (Petersen et al. 2002). According to Houweling et al. (2008), nitrification modeling outputs can be sensitive to influent nitrifying biomass seed, and a concentration as low as 10^{-4} mg-$COD_{biomass}$/L of nitrifiers, can help maintain nitrification. Yet, these speculations have not been thoroughly investigated, and the impacts of current modeling assumptions on calibration and simulation results, are not well described.

The presence of active nitrifiers has been reported in influent municipal wastewaters reaching full-scale WRRFs (Jauffur et al. 2014, Saunders et al. 2015), and the same dominant nitrifying species were observed in the influent and mixed liquor communities (Chapter 4) (Jauffur et al. 2015, In preparation -a). This supports the existence of a natural seeding of full-scale bioreactors with nitrifiers from raw sewage. Such natural phenomenon was successfully replicated by seeding laboratory-scale sequencing batch reactors (SBRs), which demonstrated that seeding by influent wastewater, can maintain nitrification under washout operational conditions (5 ºC and a 7-day SRT) (Chapter 5) (Jauffur et al. 2015, In preparation -b).

Since the publication of ASM1, several activated sludge models and model extensions have been proposed to fix shortcomings of ASM1 and include new process insights (Hauduc et al. 2013). As a consequence, significant historical variabilities in model parameter values can be observed when comparing different studies. Potential sources contributing to these variabilities include process characteristics such as types and strengths of wastewaters, different process configurations, and techniques used to estimate parameter values such as dedicated laboratory experiments (e.g., respirometry) or fitting of reactor operation data. In addition, variabilities in parameter values are intimately linked to the model structure where values for specific parameters can be hidden or lumped in other physiological, ecological or biokinetic processes. Sin et al. (2008) refer to this caveat as the identifiability problem where a model can reproduce a similar dynamic behaviour by adjusting another variable to fit predicted and observed data. Thus, a wrong assumption of a value can be compensated by increasing or decreasing another parameter value. This is why Choubert et al. (2009), suggested that parameter values proposed 20 years ago were inadequate and, demonstrated the need to update certain original ASM1 default parameter values to better predict nitrification models for activated sludge systems.

Since the presence of nitrifiers in influent wastewaters and their seeding effect have been demonstrated (Chapter 5) (Jauffur et al. 2015, In preparation -b), its disregard in modeling practices raises questions about the accuracy of nitrification model parameters and the resulting predictions from simulations. It is possible that natural seeding is hidden in the model structure during calibration by adjustment of model parameters. The current study aims at highlighting the parameters that may be modified to take into account the natural seeding of nitrifiers by influent wastewater. It also attempts at providing initial recommendations to include the measurement of nitrifying biomass in the Good Modeling Practices for wastewater characterization.

6.2 MATERIALS AND METHODS

6.2.1 Quantification of nitrifiers in wastewater

The level of nitrifiers was determined by respirometric assays in the influent wastewaters of three full-scale WRRFs: LaPrairie, Pincourt and Vaudreuil, which are located near Montreal (Quebec, Canada). Details on the geographic locations and operational configurations of the

WRRFs are provided in Table S6.1 (Supporting Information). Influent wastewater samples (composed of 24-1L samples collected every hour) were collected during the Summer 2014. The solids were concentrated to 2,500 mg/L by centrifugation at 4,000×g using the Sorvall™ ST 16 Centrifuge (Thermo Fisher, USA). The concentrated solids were transferred to 500-mL bottles to perform respirometric assays at 20 ºC using the Challenge Technology ^{TM} AER-208 (US) Respirometer System. Mixing of the respirometric bottle contents was performed by magnetic stirrers. The activities of ammonia and nitrite oxidizers were determined by measuring the oxygen uptake rate (OUR) after consecutive injections of $NaNO_2$ and NH_4Cl. The endogenous OUR was measured during the first 20 h of the assay followed by the addition of NO_2^- (100 mg-N/L) and $NaHCO_3$ (150 mg/L) to measure the activity of nitrite oxidizers, and NH_4^+ (100 mg-N/L) to determine the activity of ammonia oxidizers (Moussa et al. 2003). HEPES buffer (0.05M) was used to maintain the pH at around 8. All assays were performed in duplicate. Based on the OUR profiles, the nitrifying seed level in the influent was determined using Eq. 6.1.

$$X_{\mathrm{ANO,Inf}} = \frac{(\mathrm{OUR})(Y_{ANO})}{(1 - Y_{\mathrm{ANO}})(\mu_{\mathrm{ANO,max}})} \qquad \text{Eq. 6.1}$$

Where, $X_{\mathrm{ANO,Inf}}$ is the influent nitrifier seed level, Y_{ANO} is the yield for autotrophic nitrifying biomass, OUR is the oxygen uptake rate and $\mu_{\mathrm{ANO,max}}$ is the maximum growth rate of nitrifiers.

6.2.2 Seasonal nitrifying community composition

Influent (24-hour composite) samples were collected at the entrance of LaPrairie, Pincourt and Vaudreuil WRRFs during the winter and summer seasons. Grab mixed liquor samples were obtained at the same time from the corresponding aerated bioreactors of each treatment plant. Collected samples were immediately transported to the laboratory on ice with a travel time of 2-4 hours. The influent and mixed liquor biomass samples were spun by micro-centrifugation (microcentrifuge, Thermo Scientific, Sorvall Legend Micro 21R, USA) in 1.5 mL tubes and frozen at −80 °C until time of analysis. A quantity of 0.25 g of wet centrifuged biomass sample was used to extract genomic DNA using the PowerSoil® DNA Isolation Kit (*MO BIO* Laboratories, Inc., Carlsbad, CA). Diluted gDNA (12 ng/mL) was used to amplify functional genes involved in nitrification. Ammonia oxidizing bacteria (AOB) were studied by targeting the *amoA* gene using the barcoded forward primer *amoA-1F* and reverse primer *amoA-2R*

(Rotthauwe et al. 1997). *Nitrospira*-related Nitrite oxidizing bacteria (NOB) were studied by targeting the *nxrB* gene of the *Nitrospira* genus, which is considered as the most dominant NOB population in activated sludge systems (Daims 2001), using the barcoded forward primer *nxrB-F169* and reverse primer *nxrB-R638* (Maixner 2009). The primer sequences and PCR thermal cycles are provided in Table S6.2 (Supporting Information). Each 50µl of PCR reaction mixture contained 0.5 µM of forward and reverse primer each, 1× 5X Bioline PCR colorless buffer (Taunton, MA, USA), 2.75 mM $MgCl_2$, 250 µM dNTP (each), 12 ng/mL DNA template and 2.5 units Bioline Taq DNA Polymerase (Taunton, MA, USA) in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, USA). The PCR amplicons were purified using the *MO BIO* UltraClean™ PCR Clean-Up Kit (Carlsbad, CA) to remove primers, enzyme, buffer and primer-dimers. The purified PCR amplicons were sequenced using the GS FLX Titanium Sequencer based on the Roche-454 Life Science Protocol at the McGill University and Génome Québec Innovation Centre (Montreal, QC).

The *amoA* and *nxrB* fasta sequence data, flow and quality files were retrieved from raw Standard Flowgram Format (sff) files, de-multiplexed, trimmed and filtered using the QIIME software (Caporaso et al. 2010) to retain only good quality sequences devoid of primers and barcodes. USEARCH was used for sequence denoising and chimera removal (both *de novo* and reference-based). Chimeric sequences were also analyzed with UCHIME using default parameters, and removed. Quality filtered sequences (minimum read length of 200 bp, quality score > 25, and without ambiguous bases and mismatches) were clustered at 97% sequence similarity which is considered to be related to species level (Islam et al. 2015), and binned into operational taxonomic units (OTUs). Bacterial diversity measures (Shannon entropy and evenness) of the nitrifying populations in the influent and mixed liquor samples were performed using PAleontological STatistics v3.0.8 (Hammer et al. 2001). The average proportions of reads from OTUs found in both the influent and mixed liquor collected at a given WRRF during the winter or summer seasons were assessed using Eq. 6.2.

$$\%\ \text{shared reads} = \frac{\frac{N_{Shared\ reads,Inf}}{N_{T,Inf}} + \frac{N_{Shared\ reads,Ml}}{N_{T,Ml}}}{2} \times 100\% \qquad \text{Eq. 6.2}$$

Where $N_{Shared\ reads,Inf,}$ is the no. of reads of shared OTUs from the influent , $N_{Shared\ reads,Ml}$ is the no. of shared OTUs from the mixed liquor, $N_{T,Inf}$ is the total no. of reads from the influent sample and $N_{T,\ Ml}$ is the total no. of reads from the mixed liquor sample. The variations in nitrifying populations of the influent and mixed liquor samples between the winter and summer seasons were assessed by performing multivariate analysis of variance (MANOVA) (Witt et al. 2012). Taxonomic identification of the most dominant nitrifying bacterial OTUs was performed using the Functional Gene Pipeline and Repository of the Ribosomal Database Project (Fish et al. 2013) and BLASTN DNA query search (Altschul et al. 1990).

6.2.3 Temperature coefficients and half-saturation constants

Respirometric assays were performed to determine the temperature coefficients of enriched nitrifying biomass grown in laboratory-scale sequencing batch reactors (SBRs). The SBRs were fed with a recipe based on Bollmann et al. (2011), the composition of which, is provided in Table S6.3 (Supporting Information). Two types of nitrifying enrichments were cultivated: a cold-adapted biomass enriched at 5 ºC and a warm-adapted biomass enriched at 20 ºC at an SRT of 20 days. Both enrichments were subjected to respirometric assays at 5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC and 30 ºC, to determine their respective maximum oxygen uptake rates (OUR_{max}). The endogenous OUR was measured during the first initial hours of the assay followed by the injection of NO_2^- (100 mg-N/L) and $NaHCO_3$ (150 mg/L) to measure the activity of nitrite oxidizers, and NH_4^+ (100 mg-N/L) to determine the activity of ammonia oxidizers (Moussa et al. 2003). The OUR_{max}, which was only related to substrate consumption, was determined by subtracting the endogenous respiration rate. The temperature dependency factor (θ) was determined for the two enrichments (Sözen et al. 1998).

$$\mathrm{OUR_{max,T}} = \mathrm{OUR_{max,20°C}}\theta^{(T-20°C)} \qquad \text{Eq. 6.3}$$

Where $OUR_{max,T}$ is the maximum oxygen uptake rate at temperature T, $OUR_{max,20°C}$ is the maximum oxygen uptake rate at 20 ºC, and θ is the temperature dependence coefficient. The temperature coefficient (θ) was determined by the slope of the regression line obtained by plotting Ln $OUR_{max,T}$ of the nitrifying enrichments against temperature. This also represented the temperature coefficient ($\theta_{\mu ANO}$) for the autotrophic nitrifying growth rate.

The respirometric profiles of the 5 °C- and 20 °C-adapted nitrifying enrichments were also used to determine the half-saturation constants (K_{NH4} or K_{NO2}) based on the method proposed by van Haandel and Van Der Lubbe (2007). In accordance to Monod kinetics, the K_{NH4} and K_{NO2} were calculated as the NH_4^+ and NO_2^- concentrations, respectively, when the OUR was equal to half of the maximum OUR of the respirometric profiles.

6.2.4 Model description

The LaPrairie full-scale activated sludge WRRF was modeled using GPS-X v6.4 simulation package (Hydromantis 2015). Nitrification simulations were performed at steady-state using the comprehensive Mantis model based on a one year (2014) dataset. The different influent COD fractions used in the model are shown in Table S6.4 (Supporting Information). Standardized parameter notations based on Corominas et al. (2010) were used. The consistency of the influent data was validated using the built-in Influent Advisor tool in GPS-X prior to the simulation studies. The actual measured temperature of the wastewater for the whole year was considered during the simulation. Factors such as the yield (Y_{ANO}) was considered constant while parameters such as the maximum growth rate ($\mu_{ANO,max}$) and decay rate (b_{ANO}) were considered temperature-dependent like in ASM1 (Eq. 6.4 and 6.5) (Henze 2000, Salem et al. 2003).

$$\mu_{\mathrm{ANO,max,T}} = \mu_{\mathrm{ANO,max,20°C}}\theta_{\mu\mathrm{ANO}}^{(T-20°\mathrm{C})} \qquad \text{Eq. 6.4}$$

$$b_{\mathrm{ANO,T}} = b_{\mathrm{ANO,20°C}}\theta_{\mathrm{bANO}}^{(T-20°\mathrm{C})} \qquad \text{Eq. 6.5}$$

Where, $\mu_{ANO,max,T}$ is the maximum growth rate at temperature T, $\mu_{ANO,max,20°C}$ is the maximum growth rate at 20 °C, $b_{ANO,T}$ is the decay rate at temperature T, and $b_{ANO,20°C}$ is the decay rate at 20 °C, $\theta_{\mu ANO}$ is the temperature coefficient for growth rate, and θ_{bANO} is the temperature coefficient for decay rate, of autotrophic nitrifying organisms (ANO).

The actual variations of the influent loads and plant operational conditions were used as input files for the model simulations. Since the treatment facility has 4 aeration tanks (8400 m^3 each), the model was built using a plug-flow design with 4 compartments. The actual aeration system setup was reproduced in the model comprising of moderate bubble diffusers (2 ramps with 20 diffusers per basin). The oxygen transfer from the actual airflow data was calibrated under a

seeding scenario with a $\mu_{ANO,max}$ of 0.87 d^{-1} and a $\theta_{\mu ANO}$ of 1.072, using the effluent nitrate concentrations since anoxic zones were present at the beginning of the aeration tanks, which allowed denitrification to occur. Nitrification was modeled as a single-step process under two scenarios: without influent nitrifier seeding ($f_{XANO,\ Inf} = 0$) and with influent nitrifier seeding ($f_{XANO,Inf} = Q_{Inf}.X_{ANO,Inf}$/Total COD_{Inf}, where Q_{Inf} is the flow of influent wastewater, $X_{ANO,Inf}$ is the level of nitrifier seed in the influent and Total COD_{Inf} is the Total COD in the influent). The level of influent nitrifier seeding was determined by respirometric assays as described above. The seeding rate was assumed to be constant based on respirometric and metagenomic data. Model values were adopted from Hauduc et al. (2011) or determined by parameter estimation using GPS-X v6.4. Under both scenarios, the influent COD fractionations (estimated from historical data of the LaPrairie WRRF), compositions, and biokinetic coefficients (e.g. oxygen transfer coefficient) were kept the same, except the maximum growth rate and temperature coefficient for autotrophic nitrifying growth, which were calibrated to fit the measured and predicted data. The goodness-of-fit of the simulations was assessed by Major Axis Regression (MAR) (i.e., model II regression) to evaluate errors associated with the measured and predicted concentrations (Mesplé et al. 1996).

6.3 RESULTS AND DISCUSSION

6.3.1 Influent nitrifier seed level

Injection of NH_4^+ and NO_2^- substrates during respirometric assays induced positive oxygen uptake responses from the concentrated influent solids, which indicated the presence of active AOB and NOB populations, respectively, in the influent wastewaters (Figure 6.1).

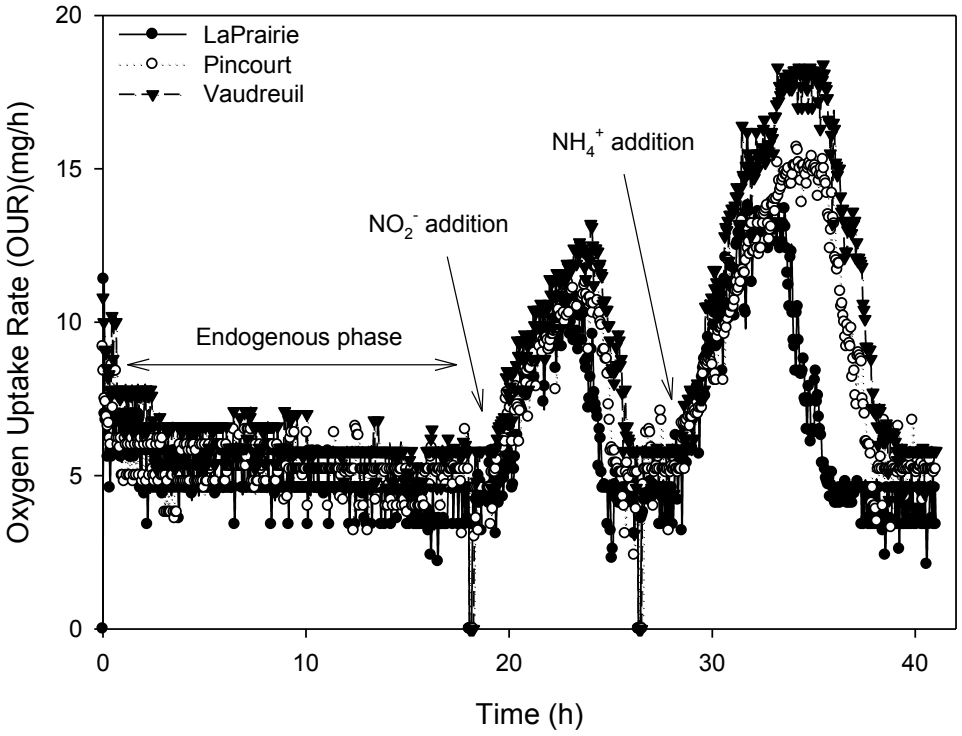

Figure 6.1. Respirometric OUR profiles of concentrated influent biomass of 3 WRRFs. Endogenous phase was measured during the first 20 h followed by sequential addition of NO_2^- and NH_4^+.

Based on the maximum oxygen uptake rate (OUR_{max}), the estimated levels of nitrifier seed in the influent solids (Eq. 6.1) at the studied sites, were found to range between 4.97-7.32 mg-$COD_{biomass}$/L (Table 6.1). The measured seed levels in the influent wastewater samples from LaPrairie WRRF, were 4.97 and 5.01 mg-$COD_{biomass}$/L for the summer and winter, respectively. This implies that there may not be significant difference in the nitrifying seeding intensity of the influent at this particular treatment facility.

Table 6.1. Concentrations of nitrifying biomass in influents of three full-scale WRRFs, and the time taken to reach complete metabolic induction after addition of substrates.

WRRFs	**LaPrairie**	**Pincourt**	**Vaudreuil**
Level of nitrifiers (mg-$COD_{biomass}$/L)	4.97	5.45	7.32
Metabolic induction time (h)			
AOB	6.55	7.80	9.10
NOB	4.71	6.27	5.97

It took the influent nitrifying biomass a few hours to become active and attain full metabolic induction following addition of substrates. Since the induction time is much lower than the SRT of the treatment plants (which is at least a few days), influent nitrifiers can be adsorbed on activated sludge flocs and get metabolically induced, thus contributing to nitrification in the activated sludge bioreactors. The presence of live nitrifiers in raw wastewater and their conveyance to full-scale bioreactors raise questions about their possible impacts on nitrification models.

6.3.2 Nitrifying population patterns across seasons

Analysis of the metagenomic DNA sequence data showed the presence of AOB and *Nitrospira*-related NOB in both winter and summer influent and mixed liquor samples of LaPrairie, Pincourt and Vaudreuil WRRFs. High-quality reads were assigned to specific OTUs at a 97% similarity level, considered to be the threshold for bacterial species (Pester et al. 2012). Averages of 274 and 114 AOB OTUs were identified for the winter and summer influent samples, respectively, while averages of 212 and 70 AOB OTUs were detected in the winter and summer mixed liquor samples, respectively (Table 6.2). A lesser number of *Nitrospira*-related NOB OTUs was identified, with averages of 84 and 68 influent OTUs, and 70 and 46 mixed liquor OTUs identified for the winter and summer, respectively. Interestingly, a higher diversity was observed in both influent and mixed liquor samples collected during winter as compared to summer (Table 6.2). The evenness was also higher for winter than for summer samples.

Distinct and consistent profiles were found between the influent and mixed liquor nitrifying populations of each WRRF. Equally important, the nitrifying population profiles were comparable between winter and summer influents, and winter and summer mixed liquors (Figure 6.2). A high percentage of sequence reads were from OTUs found in the influent and mixed liquor of the same WRRF, indicating strong seeding of the mixed liquor in both summer and winter.

Table 6.2. Number of denoised sequences, OTU richness, and Shannon diversity indices for AOB and *Nitrospira*-related NOB populations.

Bacterial group	WRRFs	No. of denoised reads	No. of OTUs				Shannon's diversity (H_α)							
							Entropy (nat)/Hill no*				Evenness			
			Influent		Mixed Liquor		Influent		Mixed Liquor		Influent		Mixed Liquor	
			Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
AOB	LaPrairie	7612	396	117	99	42	4.20/67	1.81/6	3.32/28	0.88/2	0.73	0.38	0.59	0.23
	Pincourt	7835	163	113	321	87	2.67/14	1.30/4	2.55/13	1.19/3	0.67	0.28	0.72	0.27
	Vaudreuil	7342	263	112	215	82	2.60/13	1.61/5	2.67/14	1.34/4	0.61	0.53	0.48	0.30
Nitrospira-NOB	LaPrairie	7445	83	74	77	52	2.35/10	2.02/8	1.44/4	1.22/3	0.54	0.47	0.33	0.30
	Pincourt	7815	87	72	52	36	2.24/9	1.89/7	1.96/7	1.87/6	0.50	0.44	0.53	0.50
	Vaudreuil	7380	82	59	81	49	2.28/10	2.20/9	2.35/10	1.87/6	0.56	0.50	0.55	0.48

* Hill no. = exp [Shannon entropy]

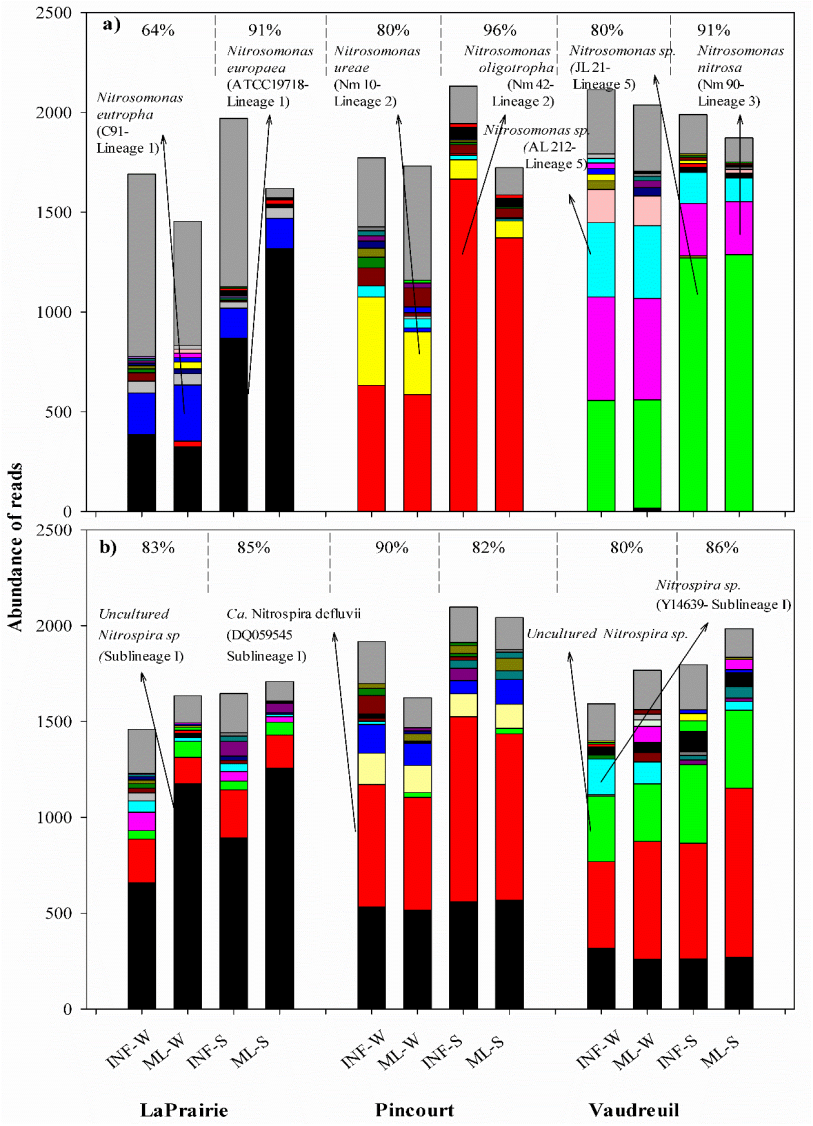

Figure 6.2. Distribution of 10 most abundant AOB (a) and *Nitrospira*-related NOB (b) OTUs in influent and mixed liquor samples from 3 full-scale activated sludge WRRFs. OTUs were generated using a 3% cutoff level. Each OTU is coded with a unique color for all stacked bars, and the height of each bar represents the abundance of the OTU (for a colored figure see the web version of the article). The grey-colored portion at the top of each bar shows the rest of the OTUs. The percentage above each pair of bars indicates the percentage of shared sequence reads from OTUs present in influent and mixed liquor, for both winter and summer, at a given WRRF. The most dominant nitrifying species are identified on chart. For AOB, the identified dominant species were found to belong to lineage 1, 2, 3 and 5 (Brenner and Staley 2005); For NOB, the identified dominant species were found to belong to sublineage I (Lipski et al. 2001, Ward et al. 2011).Upper case sample descriptors: INF-W: Influent Winter; INF-S: Influent Summer; ML-W: Mixed Liquor Winter; ML-S: Mixed Liquor Summer.

Principal Coordinate Analysis (PCoA) was used to visualize the ordination patterns of the influent and mixed liquor samples during both winter and summer. Three separate clusters corresponding to samples from the three WRRFs were identified for both AOB and *Nitrospira*-related NOB on the PCoA plots (Figure 6.3). The influent and mixed liquor samples of the same WRRF clustered together showing close resemblance in nitrifying community compositions. Moreover, the influent or mixed liquor samples from both seasons also clustered together indicating that there were relatively little differences between the nitrifying community compositions in winter and summer. The variations of the nitrifying communities between seasons were examined based on MANOVA on the community data using the season (winter/summer) and location factors, which revealed no significant differences for samples collected at the same WRRF between seasons ($P > 0.83$).

The seasonal similarity observed for nitrifying community compositions of the influent or mixed liquor at each WRRF, and the similar nitrifying seeding intensities measured in the influent of LaPrairie WRRF during winter and summer, imply that nitrifying communities in raw sewage and activated sludge may be stable across the year. Similar observations were made by Ju et al. (2014), who studied the seasonal microbial variations in activated sludge of a full-scale WRRF over 4 years. They found that functional categories, including nitrifiers in activated sludge, do not display significant difference between winter and summer. According to them, the same fundamental nitrification metabolism is shared in both winter and summer. In addition, gradients of influential factors may not be strong enough to induce significant changes in functional genes.

This is also in agreement with the findings of Wijffels et al. (1995), who reported that nitrifiers in a particular bioreactor system do not undergo population shifts like heterotrophic microbial communities, and that the same nitrifying microorganisms nitrifying ammonia at 30 ºC will be same ones nitrifying at 5 ºC. All these data agree with the current modeling practice of assuming model parameters constant throughout the year except for the rate constants which are adjusted for temperature variations based on Eq. 6.4 and 6.5.

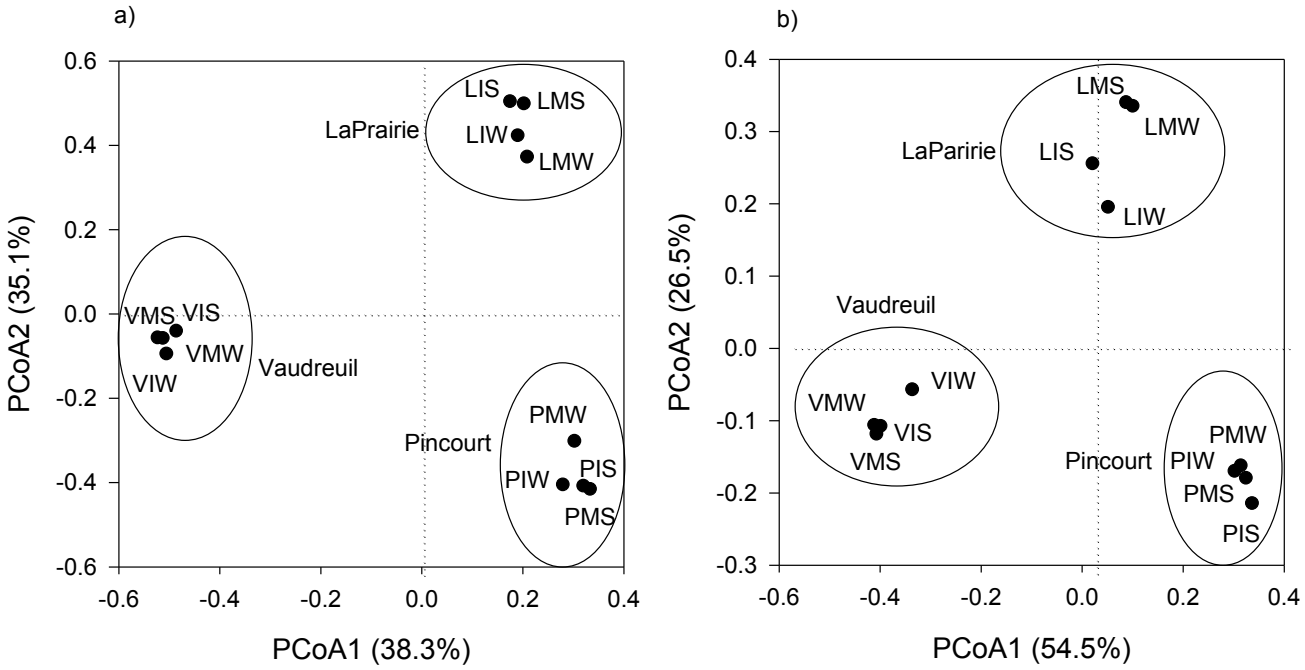

Figure 6.3. Principal Coordinate Analysis (PCoA) of AOB (a) and NOB (b) population compositions for influent wastewater and mixed liquor samples from LaPrairie, Pincourt and Vaudreuil, using Bray-Curtis distance metric. Samples are identified as follows: LIS-LaPrairie Influent Summer, LIW-LaPrairie Influent Winter, LMS-LaPrairie Mixed Liquor Summer, LMW-LaPrairie Mixed Liquor Winter, PIS-Pincourt Influent Summer, PIW-Pincourt Influent Winter, PMS-Pincourt Mixed Liquor Summer, PMW-Pincourt Mixed Liquor Winter, VIS-Vaudreuil Influent Summer, VIW-Vaudreuil Influent Winter, VMS-Vaudreuil Mixed Liquor Summer, and VMW-Vaudreuil Mixed Liquor Winter.

6.3.3 Temperature dependency of maximum growth rates and half-saturation constants

Temperature impacts significantly on nitrification activity, and the maximum growth rate of nitrifiers is intimately related to the temperature dependency coefficient ($\theta_{\mu ANO}$) (Eq. 6.4). In combination with the decay rate constant and its own temperature dependency coefficient (θ_{bANO}), these parameters determine the lowest SRT at which nitrification can be maintained at a given temperature. However, the presence of a natural seeding of nitrifiers from influent wastewaters will sustain nitrification at temperatures below the minimum temperature allowed by metabolic constraints (Chapter 5) (Jauffur et al. 2015, In preparation -b). Assuming the decay parameters to be constant, there may be a possible interplay during the calibration process between values of the maximum growth rate of nitrifiers, its temperature dependency coefficient, and the assumption of a prevailing influent-induced nitrifier seeding. Before specifically studying this interplay in the next section, the current section aims at ascertaining the type of temperature dependency coefficient that should be used to account for the effects of temperature on nitrification metabolism.

Several values for the temperature dependency coefficients have been published for nitrification in activated sludge, and are normally quoted in the range of 1.05-1.12 (Görgün et al. 2007, Salvetti et al. 2006, Sözen et al. 1998). ASM1, in its first publication, proposed a value of 1.103 (Henze 1987), while ASM2 adopted a value of 1.120 (Henze 2000). These ASM values were derived from activity studies (e.g., measurement of oxygen uptake rates under short-term temperature variations) with pure cultures or activated sludge mixed liquor suspended solids from reactors operated between 5 and 15 °C (Jones et al. 2007). An in-depth calibration study conducted in mid 2000s, on nitrification modeling parameters derived from pilot- and full-scale activated sludge reactors treating real municipal wastewaters, suggested that a value of 1.072 may be more appropriate (WERF 2003). This value, has since then, been widely accepted for designing wastewater treatment installations performing nitrification (Oleszkiewicz and Berquist 1988, Painter and Loveless 1983, WERF 2003), and is used by most commercially available wastewater process simulators such as GPS-X v6.4 (Hydromantis 2015) and Biowin (Wett et al. 2011). However, Choubert et al. (2009) had difficulty fitting OUR nitrification data at different temperatures for mixed liquor suspended solids obtained from full-scale wastewater treatment plants. They found that the value of 1.072 underestimated nitrification activities at low

temperatures, and proposed the alternative value of 1.059. Moreover, Hwang and Oleszkiewicz (2007) observed that the current default value of 1.072 could correctly predict nitrification when change in temperature is gradual, but could not accurately model the true metabolic nitrification rate when temperature fluctuates sharply, which affected nitrification much more than predicted. According to them this default value of 1.072 can model a gradual temperature change scenario, but may not be applicable for rapid temperature drops as is the case during Canadian winters or during rapid snow-melt in combined sewer systems. Under such circumstances, a value of 1.116 was found to better predict nitrification.

Given that none of the previous studies have considered the natural seeding of nitrifiers by influent wastewaters, it is likely that published parameter values for maximum growth rates and temperature coefficients (Hauduc et al. 2011) result from a combination of the effects of temperature on metabolism and the selection of microbial populations, as well as from the effects of seeding on the nitrifying biomass levels. To understand this relationship, the temperature dependency coefficient for the maximum autotrophic nitrifying growth rate was determined. A series of respirometric assays was conducted on nitrifying enrichment cultures from SBRs operated at 5 or 20 °C and a SRT of 20 days (Figure 6.4). For each nitrifying enrichment, two slopes could be identified, indicating that the temperature coefficients of the enriched biomass were different for the low (5-20 °C) and high (20-30 °C) temperature ranges, with the 5-20 °C range yielding higher temperature coefficients (Figure 6.4). Guo et al. (2010) studying enriched nitrifying biomass, also observed higher temperature coefficients over the 5-20 °C range (θ = 1.172) than the 20-35 °C range (θ = 1.062). Thus, temperature seems to have a more pronounced effect on the ammonia and nitrite oxidation rates at temperatures below 20 °C than above.

Over the 5-20 °C range, the 20 °C-adapted nitrifiers showed higher temperature sensitivities (θ = 1.088 and 1.083 for AOB and NOB, respectively) than the 5 °C-adapted nitrifiers (θ = 1.055 and 1.044 for AOB and NOB, respectively) (Figure 6.4). Therefore, it seems that there could be a temperature-induced selection of nitrifying populations. However, since the nitrifying populations observed in the summer and winter samples were found to be the same (Figure 6.2), it appears that the selection could occur at a longer time-scale than the one of the temperature dynamics observed at the plant. Furthermore, the 5 °C-adapted nitrifiers seem to have higher growth rates than the 20 °C-adapted nitrifiers below 11-14 °C. Given that the decay rates are

higher at 20 °C than at 5 °C, the density of nitrifiers per volatile suspended solids (VSS) was likely to be higher in the 5 °C-enrichment. Thus, the temperature dependency coefficient estimated for the 5 °C-adapted nitrifiers below 11-14 °C, is likely to an overestimate. Altogether, these observations suggest that the temperature dependency of the 20 °C-adapted nitrifiers over the 5-20 °C range, is most appropriate for modeling purposes.

The temperature coefficients of the 20 °C-adapted nitrifiers observed over the 5-20 °C range, are among the higher set of values reported in the literature (1.076-1.127) (WERF 2003). Most of these high values were obtained by performing activity assays over short-term temperature variations similar to the ones presented herein. Thus, the higher temperature dependency coefficients are more likely to capture the effects of temperature on nitrification metabolism. However, the adaptation to cold temperature, and the lower temperature sensitivity measured above 20 °C, suggest that a true metabolically-relevant temperature dependency coefficient for maximum nitrifying growth rate, could be region-specific and not applicable universally. This observation supports recent recommendation that temperature coefficient should be determined for specific influent wastewaters over the range of temperature likely to be encountered during operation (Guo et al. 2010). This specificity of temperature coefficient may be linked to the species composition of the nitrifying populations in the wastewater.

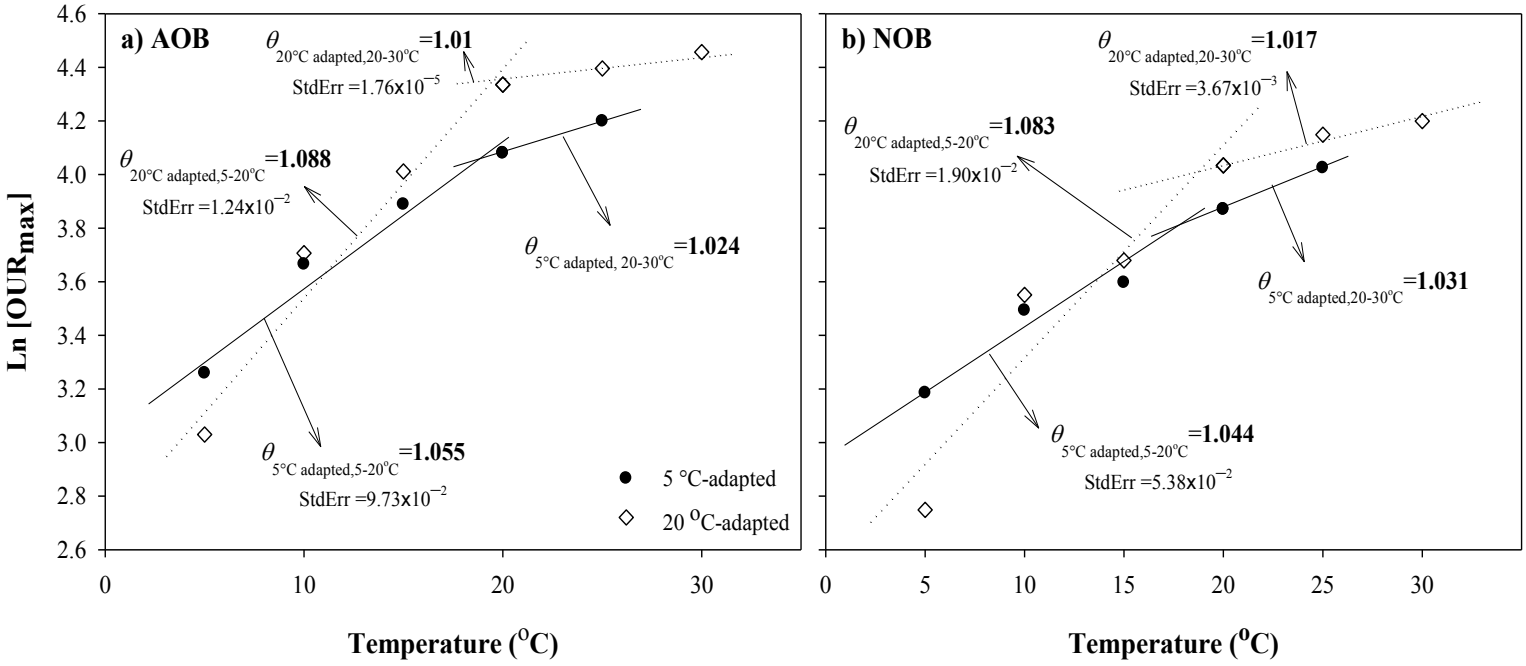

Figure 6.4. Effect of short-term variations in temperature on maximum oxygen uptake rate (OUR_{max}) of 5 °C- and 20 °C-adapted enrichment of nitrifiers after NH_4^+ addition for AOB (a) and NO_2^- addition for NOB (b) activities. The biomass samples were from SBRs receiving NH_4^+ as the only electron donor and operated at a SRT of 20 days; all assays had the same VSS concentrations. The slopes of the regression lines represent the Ln of the temperature coefficients for the autotrophic nitrifying growth rates.

Finally, the half-saturation constants of the 5 °C- and 20 °C-adapted nitrifying enrichments were determined at different temperatures based on respirometric assays. It was observed that the half-saturation constants were also temperature-dependent, and increased with increasing temperatures (Figure S6.1 – Supporting Information). This is in agreement with previous literature descriptions (Jorgensen and Gromiec 2013). The default values for the half-saturation constants were set at 0.7 for AOB and 0.1 for NOB in GPS-X v6.4 with a temperature coefficient of 1.072 (same as for the autotrophic nitrifying growth rate). These values were judged appropriate for simulating the full-scale plant data, and were kept constant for all simulations.

6.3.4 Modeling parameters describing nitrification in full-scale activated sludge reactor

In order to assess the interplay between the maximum growth rate, its temperature dependency coefficient and natural influent seeding, nitrification at the LaPrairie WRRF was modeled using one year of operational data. The simulation was carried out under an influent nitrifier seeding (4.97 mg-$COD_{biomass}$/L as determined by respirometry) and a non-seeding scenario. Since anoxic zones leading to denitrification were present in the aeration basins, the oxygen transfer from the air supplied by the blowers was calibrated using the measured effluent NO_3^- concentrations by considering a temperature dependency factor equal to 1.072 under seeding condition, while simultaneously calibrating the maximum growth rate ($\mu_{ANO,max}$=0.87 d^{-1} after calibration). The oxygen transfer coefficient was assumed to be the same for the other calibrated simulations.

The maximum growth rate ($\mu_{ANO,max}$) was determined by parameter estimation using GPS-X, by fitting the effluent NH_4^+ and NO_3^- concentrations, under influent nitrifier seeding and non-seeding assumptions (Figure 6.5 and 6.6). When the default value of the temperature dependency coefficient for the $\mu_{ANO,max}$ was used (1.072) under an influent nitrifier seeding scenario, a calibrated $\mu_{ANO,max}$ value of 0.87 d^{-1} was obtained (Table 6.3). Using the same $\mu_{ANO,max}$ value without considering seeding, the model was found to overestimate the residual NH_4^+ concentrations and underestimate the effluent NO_3^- concentrations (Figure 6.5 and 6.6), deviating the slope of the regression lines from 1 (for NH_4^+ simulation) and shifting the *y*-intercept away from 0 (for both NH_4^+ and NO_3^- simulations) (Table 6.3). The overestimation of effluent NH_4^+ or underestimation of effluent NO_3^- levels were more apparent during the cold seasons (day 0-150, and 330-360). Recalibrating the maximum growth rate for the scenario with no natural seeding resulted in predictions for effluent NH_4^+ and NO_3^- concentrations that were more comparable to the seeding scenario; however, the biases observed by the slope (should be 1 for no bias) and the intercept (should be 0 for no bias) were still worse for the calibration with no-seeding than for the calibration with seeding. The new calibrated $\mu_{ANO,max}$ under the no-seeding scenario was 1.24 d^{-1}, which is 43% higher than the scenario with natural seeding.

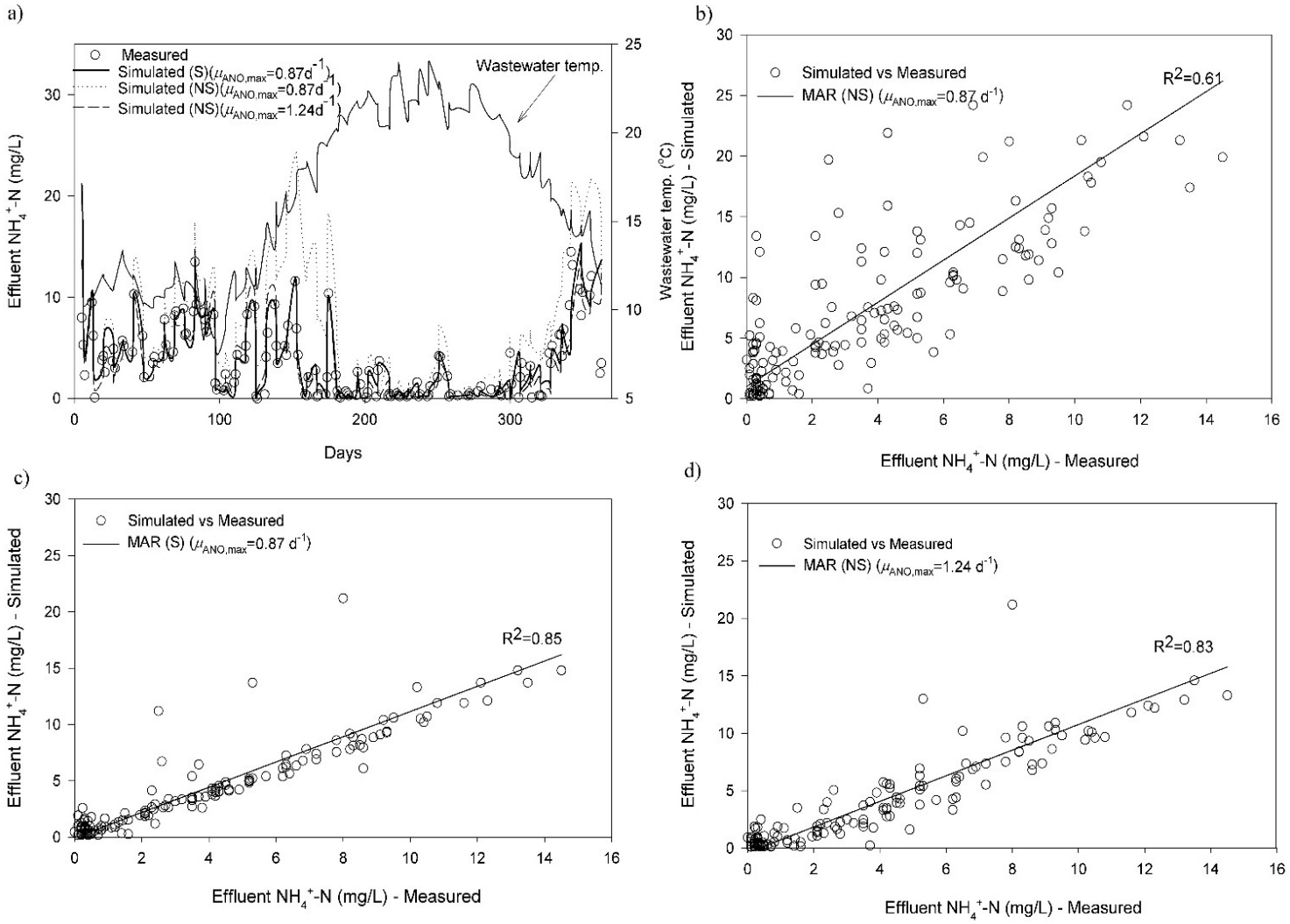

Figure 6.5. a) Simulation of effluent NH_4^+ concentration of LaPrairie WRRF under an influent seeding level of 4.97 mg-$COD_{biomass}$/L of nitrifiers (corresponding to~1.5% of total influent COD) at a $\mu_{ANO,max}$ of 0.87 d^{-1}, and non-seeding conditions at a $\mu_{ANO,max}$ of 0.87 d^{-1} and 1.24 d^{-1}, respectively. Simulation was performed using one year of operational data from Jan 1st (Day 1) to Dec 31st (Day 365). Major axis regression (MAR) of measured and predicted values for residual NH_4^+ with **b)** no seeding ($\mu_{ANO,max}$=0.87 d^{-1}), **c)** seeding ($\mu_{ANO,max}$=0.87 d^{-1}), and **d)** no seeding ($\mu_{ANO,max}$=1.24 d^{-1}) conditions. Descriptors: S-seeding; NS-no seeding; MAR-Major Axis Regression.

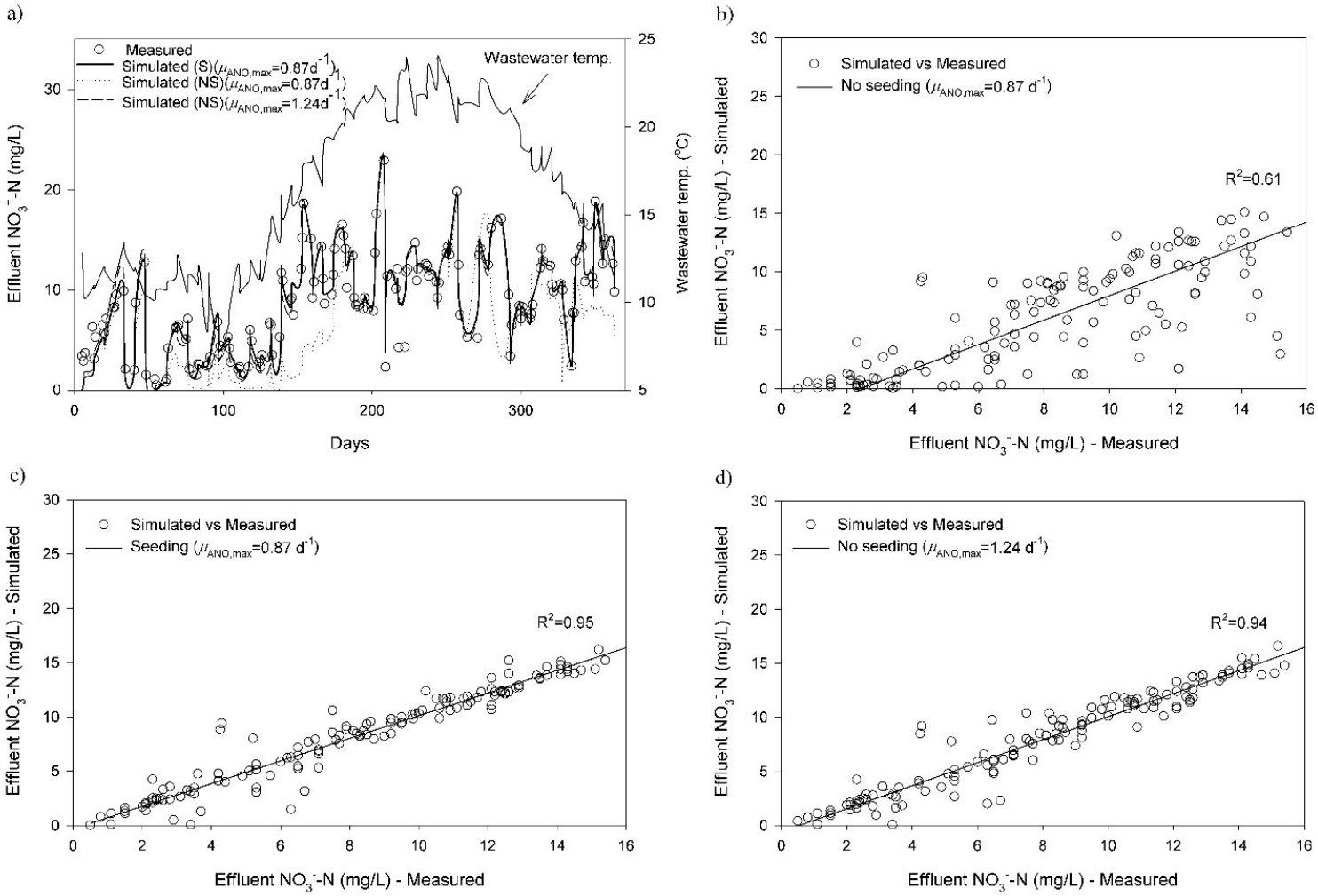

Figure 6.6. a) Simulation of effluent NO_3^- concentration of LaPrairie WRRF under an influent seeding level of 4.97 mg-$COD_{biomass}$/L of nitrifiers (corresponding to~1.5% of total influent COD) using a $\mu_{ANO,max}$ of 0.87 d^{-1}, and non-seeding conditions using a $\mu_{ANO,max}$ of 0.87 d^{-1} and 1.24 d^{-1}, respectively. Major axis regression (MAR) of measured and predicted values for residual NO_3^- with **b)** no seeding ($\mu_{ANO,max}$=0.87 d^{-1}), **c)** seeding ($\mu_{ANO,max}$=0.87 d^{-1}), and d) no seeding ($\mu_{ANO,max}$=1.24 d^{-1}) conditions. Descriptors: S-seeding; NS-no seeding; MAR-Major Axis Regression.

Table 6.3. Major Axis Regression of measured and simulated data using different temperature coefficients and calibration scenarios.

Temperature coefficient for autotrophic nitrifying growth (θ)	Calibration Scenario	Assumed nitrifier level in influent (mg-$COD_{biomass}$/L)	Calibrated max. growth rate of nitrifiers ($\mu_{ANO,max}$) (d^{-1})	Major Axis Regression (MAR) of Predictions (y-axis) in function of Observations (x-axis)					
				Effluent NH_4^+ simulation			Effluent NO_3^- simulation		
				Slope (± 95% C.I.*)	Intercept (± 95% C.I.*)	R^2	Slope (± 95% C.I.*)	Intercept (± 95% C.I.*)	R^2
1.059	Seeding	4.97	0.92	1.09 ± 0.08	−0.25 ± 0.41	0.81	1.10 ± 0.55	−0.91 ± 0.54	0.91
1.059	Seeding	0.00	0.92	1.65 ± 0.16	0.78 ± 0.81	0.67	1.07 ± 0.09	−2.50 ± 0.95	0.69
1.059	No Seeding	0.00	1.20	1.02 ± 0.08	−0.63 ± 0.43	0.78	1.17 ± 0.07	−1.39 ± 0.68	0.87
1.072	Seeding	4.97	0.87	1.01 ± 0.04	0.41 ± 0.08	0.85	1.04 ± 0.04	−0.33 ± 0.39	0.95
1.072	Seeding	0.00	0.87	1.34 ± 0.97	1.78 ± 0.19	0.61	1.04 ± 0.11	−2.82 ± 1.10	0.61
1.072	No Seeding	0.00	1.24	1.13 ± 0.09	−0.38 ± 0.44	0.83	1.07 ± 0.04	−0.59 ± 0.43	0.94
1.103	Seeding	4.97	0.85	1.02 ± 0.05	0.04 ± 0.48	0.80	1.01 ± 0.15	−0.16 ± 0.49	0.91
1.103	Seeding	0.00	0.85	1.80 ± 0.19	1.59 ± 0.99	0.58	0.99 ± 0.11	−2.36 ± 1.09	0.53
1.103	No Seeding	0.00	1.26	0.97 ± 2.02	−0.89 ± 0.47	0.76	1.13 ± 0.07	−0.58 ± 0.73	0.84

* C.I – refers to Confidence Interval

Simulation using different temperature sensitivity coefficients ($\theta_{\mu ANO}$) resulted in different calibrated $\mu_{ANO,max}$, which also changed depending on the calibration scenario (seeding or no seeding). Under the seeding scenario, increasing the temperature sensitivity coefficients, decreased the calibrated $\mu_{ANO,max}$ required to fit the data; while under the no-seeding scenario, the $\mu_{ANO,max}$ increased with increasing temperature dependency coefficients to compensate for the contribution of influent nitrifiers to sustain nitrification in the system at cold temperature (Table 6.3). This demonstrates the interplay between the studied variables ($\mu_{ANO,max}$, $\theta_{\mu ANO}$ and seeding), and how adjustment of model parameters can mask the contribution of influent nitrifiers in sustaining nitrification in activated sludge systems.

Previous authors have established a consensus on which parameters to keep fixed (yield coefficient and decay rate) and which parameters to change for different model applications (Gernaey et al. 2004). For nitrification models, the $\mu_{ANO,max}$ is usually the parameter to adjust in order to fit simulated and measured datasets. Early surveys of values used in the literature for $\mu_{ANO,max}$ found a very wide range of 0.25-3.0 d^{-1} or ± 169% from the midpoint value (Henze 1987, Metcalf and Eddy 2003). Later, this range seems to have subsided, and a more recent survey from practitioners and academic literature found a much narrower range of 0.8-1.8 d^{-1} for ASM1 (i.e., ± 77% from the midpoint value; Hauduc et al. 2011). This reduction was in part brought about by a large Water Environment Research (WERF) study reviewing the values of modeling parameters and showing that the $\mu_{ANO,max}$ and $b_{ANO,max}$ (decay rate) were highly correlated, and that stabilizing the decay rate also stabilized the growth rate (WERF 2003). Nonetheless, the WERF study still found a range of $\mu_{ANO,max}$ 0.75-1.17 d^{-1} (or ± 22% from the midpoint value), which is similar to the range of $\mu_{ANO,max}$ obtained in the current study for a single system by varying the seeding assumption from the influent. Therefore, it seems that the variability in $\mu_{ANO,max}$ comes from variations in seeding intensities from the influent, and modeling practices would be improved by considering the abundance of nitrifiers in influent wastewater.

To a smaller extent, the variation in $\mu_{ANO,max}$ may also depend on the variations in nitrifier species between regions, and arbitrary selection of temperature coefficients by modelers. The current study found lower temperature adjustment coefficients ($\theta_{\mu AN0}$) for nitrifier enrichments at 5 °C than at 20 °C (Figure 6.4). The average temperature of a given region may lead to specific endogenous nitrifying populations with specific temperature adjustment factors. Consequently, adopting the

wrong temperature adjustment factor for a region may lead to increased variability in $\mu_{ANO,max}$. This variability in $\mu_{ANO,max}$ values has been partly observed in the value for temperature coefficients (Kampschreur et al. 2009, Volcke et al. 2006, Wett and Rauch 2003). Therefore, it seems that considering natural nitrifier seeding in modeling and adopting metabolically-relevant and region-specific temperature adjustment factors ($\theta_{\mu ANO}$) would together stabilize the $\mu_{ANO,max}$ value.

The final argument strongly supporting the introduction of measurement of nitrifiers in the influent wastewater for modeling purposes is the increase in accuracy and precision. In the current study, despite that the adjustment of the maximum nitrifying growth rate can compensate for not considering natural seeding of nitrifiers, the simulations of effluent NH_4^+ and NO_3^- concentrations were always more accurate (MAR slopes closer to 1 and intercepts closer to 0) and precise (higher R^2) when seeding was considered (Table 6.3). This argues for the adoption of standardized protocols to quantify the level of nitrifiers in influents during wastewater characterization. These standardized assays could either be based on molecular techniques or respirometry, both of which are relatively inexpensive and rapid, two important considerations for practitioners (Vanrolleghem et al. 2003). The data presented here provide evidence that such an approach will make the modeling process more accurate, and render model parameters more stable. It can also increase the model accuracy for sites where seeding is expected to be low such as industrial premises or wastewater collection systems with small sewer networks.

In summary, this study shows that the presence of active nitrifiers in municipal wastewaters reaching full-scale wastewater treatment facilities, has a significant impact on nitrification model performances. Incorporating natural seeding of nitrifiers in the modeling framework enhances the accuracy and precision of predictions. However, when this natural seeding of nitrifiers is not considered, as is the case for current modeling practices, it is still possible to capture this natural phenomenon by adjusting other model parameters. The calibration bias observed in this study between the approaches adopted using either the observed natural seeding level as determined by respirometric assays or adjusting model parameters arbitrarily, shows the need to fine-tune modeling approaches. It also points to the potential variability in model parameters such as the maximum growth rate or temperature sensitivity coefficients that may be encountered between geographic regions or for systems receiving wastewater from different sources such as municipal vs. industrial or from wastewater collection systems with small sewer networks.

6.4 ACKNOWLEDGEMENT

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6.5 SUPPORTING INFORMATION

Table S6.1. Description of Water Resource Recovery Facilities (WRRFs).

WRRFs	Geographic location		Sampling date	Sewer type[a]	Plant process[b]	Flow rate (m^3/day)	SRT (day)	HRT (hour)	Residential : industrial COD ratio in influent	Influent temperature (°C)	
	Latitude N	Longitude W								Summer	Winter
LaPrairie	45°24'16.48"	73°33'22.06"	25.06.2014	CS/PCS	CAS	65,000	7	15	45:55	20.3	10.5
Pincourt	45°23'25.30"	74° 1'37.34"	26.06.2014	CS	CAS	6,000	15	8	90:10	21.1	7.2
Vaudreuil	45°23'25.30"	74° 1'37.34"	26.06.2014	CS	SBR	18,000	5	3	50:50	20.8	7.9

[a]: CS-combined sewer; PCS-partially combined sewer

[b]: CAS-conventional activated sludge; SBR-sequencing batch reactor

Table S6.2 Primers and PCR thermocycling programs used for amplification of *amoA* and *nxrB* genes.

Target population	Primer	Sequence (5'-3')	PCR thermocycling program	Reference
AOB	*amoA-1F*	GGGGTTTCTACTGGTGGT	95 °C for 4 min, 35 cycles of 95 °C for 40 s, 56 °C for 30 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	(Rotthauwe et al. 1997)
	amoA-2R	CCCCTCTGCAAAGCCTTCTTC		
NOB (*Nitrospira-related*)	*nxrB-F169*	TACATGTGGTGGAACA	95 °C for 5 min, 35 cycles of 95 °C for 40 s, 62 °C for 40 s, 72 °C for 1 min followed by a final extended elongation at 72 °C for 10 min	(Maixner 2009)
	nxrB-R638	CGGTTCTGGTCRATCA		

Table S6.3. Composition of mineral medium for nitrifying enrichments.

Chemical components	Unit (mg/L)
$(NH_4)_2SO_4$	660.7
$NaHCO_3$	840.0
NaCl	585.0
KCl	75.0
$CaCl_2.2H_2O$	147.0
$MgSO_4.7H_2O$	49.0
KH_2PO_4	1360.9
HEPES buffer	57.2
Diatomaceous earth	240.0
Trace element	
NaEDTA	4292.0
$FeSO_4.7H_2O$	2780.0
$MnCl_2.4H_2O$	99.0
$NiCl_2.6H_2O$	24.0
$CoCl_2.6H_2O$	24.0
$CuCl_2$	13.4.0
$ZnSO_4.7H_2O$	143.0
$Na_2MoO_4.2H_2O$	24.0
WO_3	23.2
H_3BO_3	62.0

Table S6.4. Influent fractions and model parameters used for simulation study.

Parameters	Units	Value
Influent COD organic fractions		
Soluble biodegradable organics (f_{SB})	g-COD_{SB} / g-Total COD	0.200[a]
Particulate undegradable organics (f_{XU})	g-COD_{XU} / g-Total COD	0.550[a]
Soluble undegradable organics (f_{SU})	g-COD_{SU} / g-Total COD	0.080[a]
Slowly biodegradable substrates (f_{XCB})	g-COD_{XCB} / g-Total COD	0.170[a]
Ordinary heterotrophic biomass (f_{XOHO})	g-$COD_{XOHO}.m^{-3}$	0.020[e]
Nitrifying biomass (f_{XANO})	g-COD_{XANO} / g-Total COD	0.00 or estimated value[d]
Influent fractions		
Particulate undegradable (X_U)/VSS ratio	g-COD/g-VSS	1.630[a]
$BOD_5/BOD_{ultimate}$ ratio	--	1.150[a]
VSS/TSS ratio	g-VSS/g-TSS	0.840[a]
Composition coefficients		
N- content of active biomass (i_{N_XBio})	g-N/g-COD	0.068
P-content of active biomass (i_{P_XBio})	g-P/g-COD	0.021
Biological parameters		
Yield of ordinary heterotrophic biomass (Y_{OHO})	g-COD/g-COD	0.670[b]
Yield of nitrifying biomass (Y_{ANO})	g-COD/g-N	0.240[b]
Heterotrophic max. specific growth rate ($\mu_{OHO,Max}$)	d^{-1}	3.200[b]
Nitrifiers' max. specific growth rate ($\mu_{ANO,Max}$)	d^{-1}	Varying
Half-saturation constant for S_B ($K_{SB,OHO}$)	mg-COD/L	5.000[c]
Half-saturation constant for S_{O2} ($K_{O2,OHO}$)	mg-O_2/L	0.200[c]
Half-saturation constant for S_{O2} ($K_{O2,ANO}$)	mg-O_2/L	0.250[c]
Half-saturation constant for S_{NHx} ($K_{NHx,OHO}$)	mg-N/L	0.050[c]
Half-saturation constant for S_{NHx} ($K_{NHx,ANO}$)	mg-N/L	0.700[c]
Half-saturation constant for S_{NOx} ($K_{NOx,OHO}$)	mg-N/L	0.100[c]
Reduction factor for anoxic growth of X_{OHO} ($\eta\mu_{OHO,Ax}$)	--	0.500[c]
Heterotrophic decay rate (b_{OHO})	d^{-1}	0.620[c]
Autotrophic decay rate (b_{ANO})	d^{-1}	0.170[c]
Ammonification rate (q_{am})	m^3/g-COD/d	0.080[c]
Max. specific hydrolysis rate ($q_{XCB\_XB,hyd}$)	g-X_{CB}/g-X_{OHO}/d	3.000[c]
Temperature coefficient for $\mu_{XOHO,Max}$ ($\theta\mu_{OHO,Max}$)	--	1.072[c]
Temperature coefficient for $\mu_{XANO,Max}$ ($\theta\mu_{ANO,Max}$)	--	Varying
Temperature coefficient for b_{OHO} (θ_{bOHO})	--	1.029[c]
Temperature coefficient for b_{ANO} (θ_{bANO})	--	1.029[c]
Temperature coefficient for $K_{SB,OHO}$ ($\theta_{KSB,OHO}$)	--	1.072[c]
Temperature coefficient for $K_{NHx,ANO}$ ($\theta_{KNHx,ANO}$)	--	1.072[c]

[a]: Estimated from historical data.
[b]: Model value adopted from (Hauduc et al. 2011).
[c]: Default value from GPS-X v6.4 (Hydromantis 2015).
[d]: Under no seeding scenario, f_{XANO} was assumed to be zero; under seeding scenario, f_{XANO} was computed as the fraction of the influent nitrifier addition to the total COD ($Q_{Inf}.X_{ANO,Inf}$/Total COD_{Inf}); the seed level was assumed constant (4.97 g-COD_{XANO} / g-Total) although it may be varying in the field on a day to day basis.
[e]: Represents default value set for influent Ordinary Heterotrophic Organisms (OHO) in BioWin v4.0.

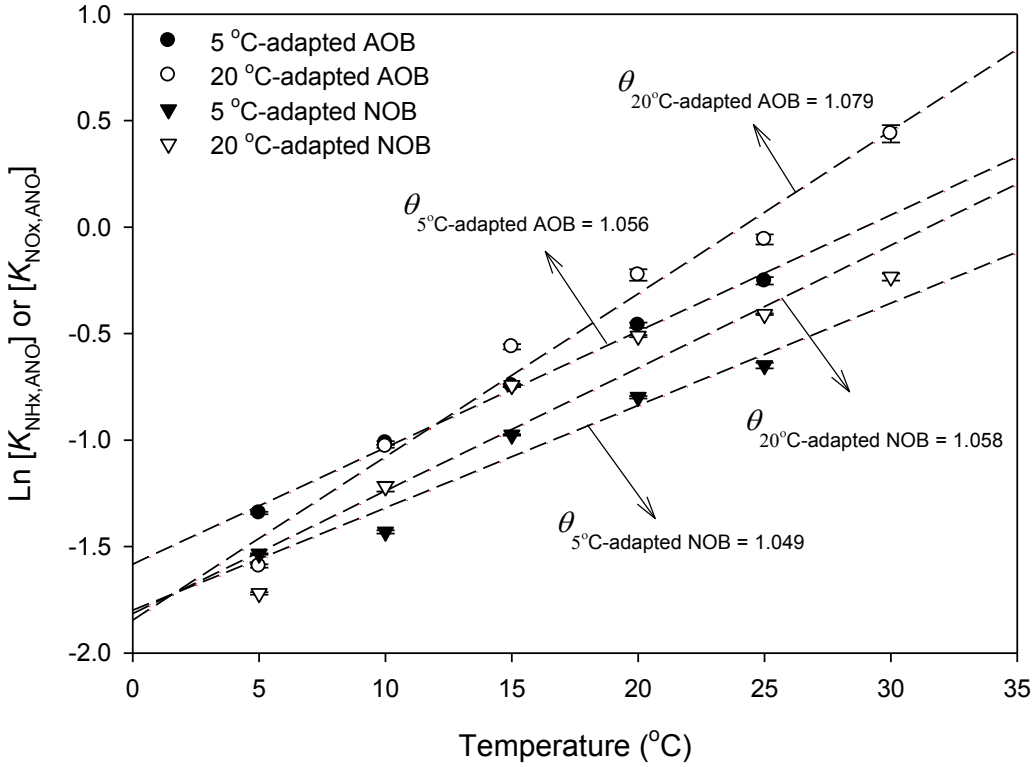

Figure S6.1. Effect of short-term variations in temperature on half-saturation constants of S_{NHx} and S_{NOx} for 5 °C- and 20 °C-adapted enrichment of nitrifiers after NH_4^+ addition for AOB and NO_2^- addition for NOB activities. The biomass samples were from SBRs receiving NH_4^+ as the only electron donor and operated at a SRT of 20 days; the same VSS concentrations were in all assays. The slopes of the regression lines represent the Ln of the temperature coefficients for the half-saturation constants.

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CHAPTER 7

General discussion and conclusions

The overall goal of this thesis was to explore the possible seeding of biological wastewater treatment systems with nitrifiers from raw municipal wastewaters. Unraveling the existence of such natural influent nitrifier seeding triggered a series of questions including its potential impacts on wastewater treatment facilities and on current wastewater modeling practices, which were answered during the course of this study.

7.1 MAIN OBSERVATIONS PRESENTED IN THIS THESIS

We revealed the presence of nitrifying bacteria (both AOB and *Nitrospira*-related NOB) in influent municipal wastewaters using high-throughput DNA sequencing. All the data obtained in the various experiments presented in this dissertation showed that the dominant nitrifying operational taxonomic units (OTUs) in the influent wastewater and the corresponding mixed liquor samples were the same, irrespective of the temperature or scale of operation. Respirometric assays on concentrated wastewater solids harvested at full-scale wastewater treatment facilities showed that the influent nitrifiers were alive and could reach full metabolic activity within a few hours after addition of substrates. This provided evidence supporting the existence of a natural seeding of nitrifiers at full-scale wastewater treatment plants. The influent nitrifying populations were found similar in composition and concentration during both winter and summer, indicating that the quality and level of influent nitrifier seed may remain constant across the year. This process of natural seeding of nitrifiers was successfully replicated in the laboratory where addition of influent solids harvested from a full-scale Water Resource Recovery Facility (WRRF) restored nitrification in bench-top reactors operated at washout conditions (low temperature and solids retention time [SRT]).

7.2 IMPLICATIONS FOR ECOPHYSIOLOGY OF NITRIFIERS IN ACTIVATED SLUDGE WASTEWATER TREATMENT AND SEWER SYSTEMS

7.2.1 Potential sources of nitrifiers in raw wastewaters

It has been shown that microbial sewage communities consist of a combination of human fecal microorganisms and non-fecal microorganisms originating from gray and black wastewater from residential and industrial units, rainwater inputs and surface runoffs (Shanks et al. 2013). These microorganisms can colonize strategic locations in sewers as biofilms on pipe surfaces or are deposited in the sediments along the pipe system (Chen et al. 2003). Although the sources of

nitrifiers in influent wastewaters have not been investigated in this study, it is believed that they originate primarily from soils and enter combined sewers through surface runoffs adhered to soil particles. Nitrifiers are abundant in soils where they are responsible for nitrogen cycling and regulating N-availability to vegetation (Tsiknia et al. 2015). The nitrifying community composition and distribution in soils have been shown to correlate with several geochemical variables such as pH (Dandie et al. 2011), TN and TOC content (Petersen et al. 2012, Wessén and Hallin 2011), C:N ratio (Wessén and Hallin 2011), NH_4^+ concentration (Verhamme et al. 2011) as well as the soil structure including its compactness, texture and drainage (Bru et al. 2011). Wastewaters from agricultural activities and urban development are shown to exert a significant N-loading in sewer systems (Castro et al. 2003, Fitch and Crowe 2010). These factors are likely to have an impact on determining the type and intensity of seeding nitrifying populations to the sewer systems. In addition to conveyance of these nitrifiers to combined sewer systems by wastewaters and rainwater inputs, it is believed that infiltration from soil water into the sewer pipes is also a major contributor of the seeding nitrifying populations. Sewer infiltration has been shown to be particularly prevalent in ageing and leaky infrastructures (Kuroda et al. 2012). Such infiltration fluxes can increase during precipitation events thereby increasing the water levels and hydraulic potential of sewer systems (Musolff et al. 2010).

Once inside the sewers, the nitrifying populations may be structured based on selective processes impacting on them. This is further discussed in the section below. Sewers have been shown to behave as active bioreactors where microbially-mediated transformations of compounds take place dynamically in the bulk water phase and biofilms (Tanaka and Hvitved-Jacobsen 1998). These processes proceed under aerobic as well as under anaerobic conditions. Hence, factors such as the type of wastewater (residential vs. industrial), its physicochemical characteristics (temperature, pH, alkalinity, DO level, NH_4^+ and organic substrate concentrations), flow and velocity as well as the physical structure of the sewer system (length, surface area and slope) are believed to influence the nitrifying seeding composition and intensity. Hydraulic shock loads especially during rain events may increase sloughing of nitrifying biofilms and resuspension of sediments, and their conveyance to WRRFs. The potential sources of nitrifying bacteria in sewers and the factors determining their composition and concentration are summarized in Figure 7.1.

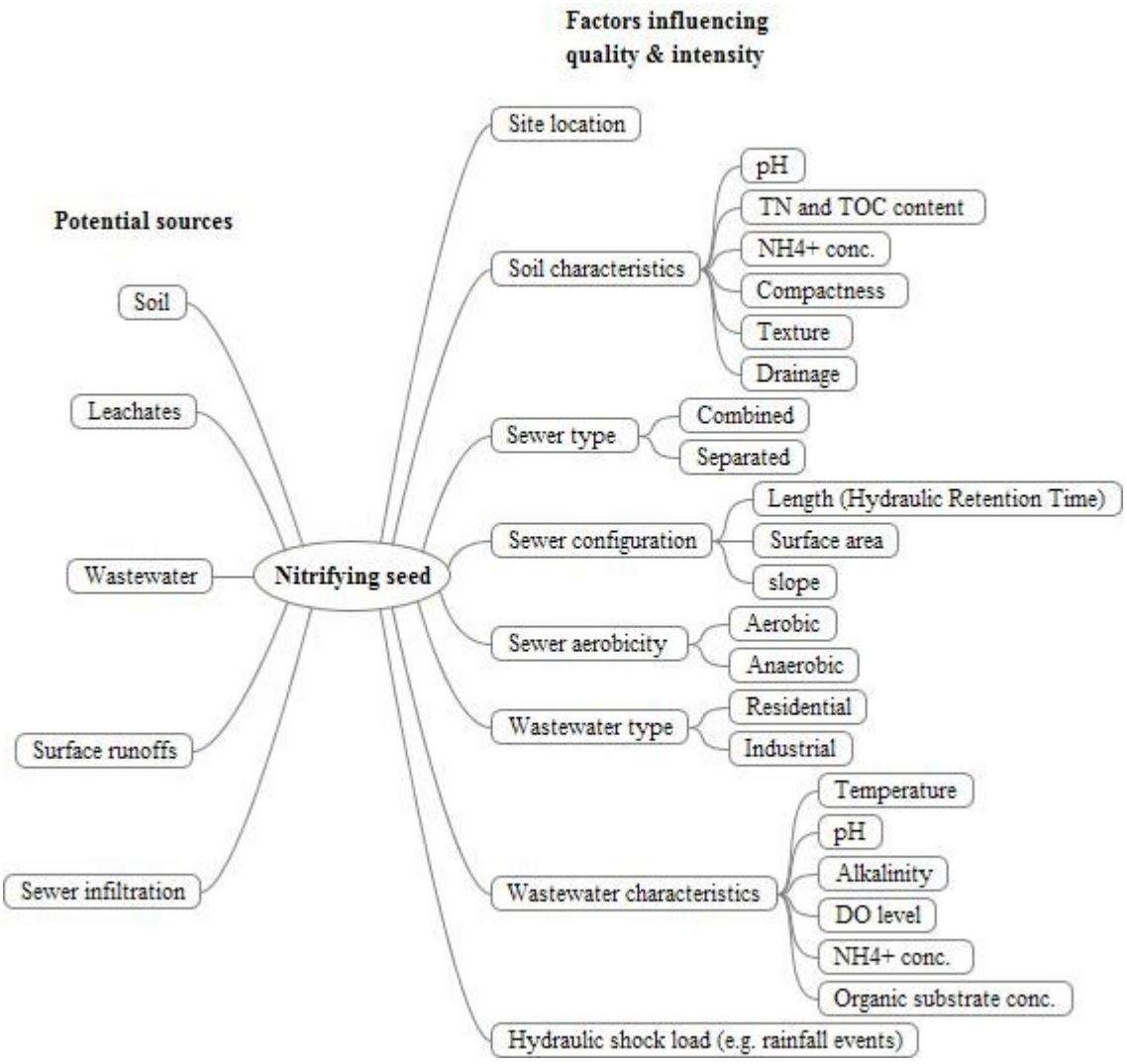

Figure 7.1. Schematic representation of the potential sources of nitrifying microorganisms in sewer systems and the factors determining their composition and intensity.

7.2.2 Immigration vs. selection of nitrifying species

We have shown that the assembly of nitrifying communities in full-scale activated sludge bioreactors, is not unpredictable or chaotic as hypothesized by previous authors (Ramette and Tiedje 2007), but is essentially determined by an immigration process. Thus, selection does not appear to be operating at the studied sites to impact on the nitrifying population structure. Another study has recently reported similar results for full-scale bioreactors in Denmark

(Saunders et al. 2015). These results have a direct theoretical impact on the model proposed by Curtis et al. (2006), which is based on the neutral theory of community assembly. This model infers that stochastic geographic dispersal of nitrifying species dictates the impact of the metacommunity on the structure of local activated sludge nitrifying populations. The data presented in this dissertation suggest that the proposed model, is at best incomplete, in the sense that the metacommunity of nitrifiers does not stochastically influence the nitrifying activated sludge communities, but it rather influences the populations immigrating into a given activated sludge system.

Although common dominant nitrifying species were detected in the influent wastewaters reaching the full-scale WRRFs, the raw wastewater at certain sites harboured dominant species that were different than at the other sites (Chapter 4). The reasons for such variability in dominant species conveyed by the sewer systems to particular WRRFs are not clear at this point. First, it may be that the process is also completely neutral, and that species present at a given site result from stochastic immigration, which is itself influenced by the structure of the metacommunity as stated above. Second, it may indicate a selection process operating in the sewer environment, a speculation which has been expressed in the literature (Saunders et al. 2015). Previous studies have shown that sewer systems favor the colonization of specific microorganisms depending on factors such as surface area, wastewater flow velocity and nutrient availability (Hvitved-Jacobsen et al. 2013). Hence, besides acting as hydraulic transport systems for sewage, sewer pipes are infrastructures where microbial processes occur; these may also include the selection of specific taxa for nitrifying microorganisms. Third, it may be possible that a selection process operates at the level of the soil environment surrounding the sewer pipes or at the scale of a watershed. Incidentally, the two sites (Pincourt and Vaudreuil), which harbored different sets of AOB populations from the rest of the studied WRRFs (Chapter 4) are located on the north shore of the St-Lawrence River, while the other sites are located on the south shore, thereby hinting at the possible impact of the surrounding environment on the sewer systems.

Based on the previous discussion, the physical source of the nitrifying populations entering the sewers and ultimately the wastewater treatment facilities, seems to be the central problem to understand the ecology of nitrifiers in these systems. It is likely that soil particles entering sewer lines by infiltration, are the main source of nitrifiers in municipal wastewaters. While molecular surveys have shown the wide abundance of AOB in terrestrial environments (Fierer et al. 2009,

Norton et al. 2008), Ammonia Oxidizing Archaea (AOA) have been shown to be numerically dominant in soils over AOB (He et al. 2007, Leininger et al. 2006, Nicol et al. 2008). It was, therefore, surprising to find AOA at low abundance in raw wastewater such that their PCR amplification was difficult to achieve. The dominance of AOB over AOA in wastewater systems observed in the current study, does not seem to be an artefact as it has been reported in early studies (Park et al. 2006, Zhang et al. 2009). Rather, this may be an indication of the possible selection process on ammonia oxidizers that was hypothesized above. The selection may not operate at the species level, but at the AOB vs. AOA level. Several studies have shown that AOA are mainly found in environments with low NH_4^+ substrate availability, while AOB are more abundant in environments with high NH_4^+ concentrations (Beman et al. 2008, Francis et al. 2005, Jia and Conrad 2009). Since wastewaters contain high levels of NH_4^+, this may explain the selection of AOB in sewer environments rather than AOA. To address this issue, it may require tracking down the nitrifying populations along the sewer lines up to the source, and compare their compositions and abundances using culture-independent methods such as high-throughput DNA sequencing and qPCR. This will help to shed light on the role of selection in the whole process.

7.2.3 Laboratory-scale vs. full-scale bioreactors and rare taxa

Seeding bioreactors with influent wastewaters affected reactor dynamics at both laboratory- and full-scale levels. However, the diversity was fairly low in the laboratory-scale reactors as compared to the full-scale plants even after seeding with influent wastewaters. The richness of nitrifying species was also higher in the full-scale systems as compared to the benchtop sequencing batch reactors (SBRs). This can be mainly explained by the presence of only a few dominant (1-4) species in the samples analyzed. Moreover, according to Van Der Gast et al. (2006), such disparity may result from the size of the bioreactor. Larger bioreactors have greater volume space for bacterial colonization as compared to small reactors which have less vacant niche space. The rate of species accumulation and taxonomic diversification may not only be restricted to the physical size of a reactor but may also depend on the time-scale over which the reactor receives wastewater flow, as well as the supply mode of the wastewater (continuous or discontinuous flow). Difference in diversity between the scales of operation may also be due to the feed composition (*Syntho* for laboratory-scale SBRs, and real wastewater for full-scale systems) as well as the operational conditions. These factors, altogether, may explain the limited

diversity of nitrifying populations in the laboratory–scale reactors as compared to the full-scale systems.

In both laboratory- and full-scale reactor systems, only a few dominant species were observed. Therefore, emphasis was mainly laid on these dominant species identified in both the influent seed and corresponding mixed liquor samples. The rest of the nitrifying bacterial communities were found to comprise mostly of rare species with low abundances. It appears that rare-biosphere taxa distribute according to Baas Becking's dictum "everything is everywhere, the *milieu* selects" (De Wit and Bouvier 2006). Studies have shown that dominant taxa perform most ecosystem functions while rare taxa are regarded as seed banks and play minor roles (Besemer et al. 2012, Pedrós-Alió 2006). However, this may not be always true. The long tail of taxa identified in the current study may have important ecological functions as well, and can serve as reservoirs of genetic and functional diversity. Studies have shown that rare species may colonize natural or bioengineered systems under optimal conditions (Pedrós-Alió 2012, Sogin et al. 2006). It has also been shown that rare taxa may become disproportionately active in some ecosystems (Campbell et al. 2011). But above all, the rare taxa observed in the current study, contributed to the *alpha* and *beta* diversities of the nitrifying communities. According to Gilbert et al. (2009), rare species occupying narrow niches, can contribute significantly to observed diversities in ecosystems. Moreover, according to Jones and Lennon (2010), rare biosphere can act as a functional cache or resource pool for responding to disturbance events, thereby contributing to community resilience. Based on these arguments, it will not be appropriate to discredit the contribution of rare taxa in our samples. The study of rare taxa in the influent wastewaters and activated sludge systems will require time-series analyses since these microorganisms are likely to be on their way to local extinction or are transient taxa that appear in the system for a brief period of time. In addition, they may be more sensitive to environmental or operational fluctuations. This is why their contribution in such systems may necessitate their study through time. Also, since their abundance is very low, it may be necessary to have increased sequencing depth by generating more DNA reads during metagenomics analyses.

7.3 IMPLICATIONS FOR ECOPHYSIOLOGY OF HETEROTROPHS IN ACTIVATED SLUDGE WASTEWATER TREATMENT

Heterotrophic bacterial communities have a wider range of ecological functions as compared to the nitrifiers. Since they are also conveyed by raw sewage to full-scale WRRFs, it was legitimate to question the role of immigrant heterotrophic populations in structuring the local communities in the bioreactors. The heterotrophic bacterial community structures of the influent wastewaters were found to be distinct from those of the activated sludge. Based on our analyses it was evident that the bioreactors were imposing a selective pressure on the incoming immigrants, leading to distinct communities. Several studies have highlighted the impact of environmental factors in exerting selective pressures on activated sludge communities (Collins et al. 2006, Falk et al. 2009, McGuinness et al. 2006). In some cases, such niche-based selection may result from the presence of specific substrate in activated sludge, which will potentially exert selective pressure causing populations to diverge from neutrality. For instance, the presence of high methanol concentration in the influent of the LaPrairie WRRF, was shown to correlate with the high abundance of *Methylotenera*, a genus which grows in the presence of methanol and nitrate (Isazadeh et al. 2014). Nonetheless, the heterotrophic immigrant communities also appeared to modulate the local heterotrophic communities in the activated sludge. The addition of influent solids to the SRBs decreased the abundance of *Rhodocyclaceae* and *Flavobacteriaceae*, while increasing the abundance of *Moraxellaceae* and *Campylobacteraceae*. The negative correlation observed between these families may result from competition of these taxa in the local communities. Quantifying the contribution of immigration or selection in structuring microbial communities in natural and bioengineered systems, still represents a challenge to the scientific community.

7.4 IMPLICATIONS FOR MODELING NITRIFICATION PROCESSES

We systematically evaluated the impacts of natural seeding of nitrifiers on the performance of activated sludge models. Based on the influent seed level measured at the LaPrairie WRRF, the nitrification process was modeled using one year of operational data. Simulations under an influent nitrifier seeding scenario resulted in a good agreement between the measured and predicted data. When seeding was not considered, the recalibrated model with higher maximum growth rates for nitrifiers, predicted effluent ammonium and nitrate concentrations that were less

accurate and precise. The actual value obtained for the maximum growth rate of autotrophic nitrifiers was found to be dependent on the temperature sensitivity coefficient. It seems that published values for the maximum growth rates of nitrifiers and temperature coefficients are based on the temperature effect on nitrification metabolism and on the natural influent seeding of nitrifiers. If yearly variations in nitrifying populations are ascertained, as we and others observed (Chapter 6), it is then, highly desirable to use temperature coefficient values, which capture the metabolic effect of temperatures. These can be obtained by short-term temperature variation experiments to study the activity of biomass using assays such as respirometry. This will render the values of maximum growth rate and temperature dependency coefficient more stable, and will improve the predictive ability of the model.

Based on the determination of the temperature dependence for autotrophic nitrifying growth of enriched biomass, it was found that different coefficients may be applicable over different temperature ranges, and for cultures adapted to different temperatures. Therefore, we subscribe to previous recommendations that the temperature coefficient for a specific system should be determined on local nitrifying biomass from mixed liquor or influent solids over the range of temperature likely to be encountered during the operation periods (Guo et al. 2010). Additionally, it may be useful to the modeling community, to systematically investigate the temperature dependency of nitrifying maximum growth rates across regional scales to capture the long time-scale selection of nitrifiers for different temperature averages and various wastewater treatment systems. The data presented in this thesis would suggest that the metabolically relevant temperature dependence factor could be different for warm and cold regions in general.

Above all, it is recommended that the levels of nitrifying biomass in influent wastewaters be determined during wastewater characterization. Although modelers may prefer to arbitrarily adjust the maximum growth rate based on time and cost considerations (Vanrolleghem et al. 2003), such an approach increases the uncertainty and bias associated with the model as observed in this study (Chapter 6) and as shown by other groups (Sin et al. 2008). Additionally, since the seeding intensity is likely to vary from site to site, measurement of nitrifying biomass in the influent wastewater may be necessary to calibrate the model for each site.

One of the most reliable and commonly used techniques to determine biokinetic parameters for activated sludge modeling is batch respirometry, which is the approach adopted in this thesis. Batch respirometry is a fast and reliable means for determining nitrifier seed levels in influent municipal wastewater. The only drawback is that the influent solids need to be concentrated by approximately 10 times. Since we found that the concentration of nitrifying seed level did not change at the LaPrairie wastewater treatment facility across the year, it may not be necessary to perform the measurements over a prolonged period of time. Alternatively, the respirometric assays could be replaced by quantitative PCR of functional genes involved in nitrification. This method has proved to be a fast and accurate tool for quantifying the level of nitrifiers in both natural and engineered systems (Geets et al. 2007). However, it would be important for the standardized assay to measure all the possibly relevant groups of nitrifiers, which may imply doing several PCR assays in parallel.

7.5 IMPLICATIONS FOR SIZING ACTIVATED SLUDGE BIOREACTORS

The capacity of influent nitrifiers to sustain nitrification under unfavorable conditions has been demonstrated in this study (Chapter 5), where supplementing laboratory-scale reactors operated at washout conditions with influent solids, restored nitrification. Analysis of the activated sludge from the seeded reactors showed that the added influent nitrifiers were successfully established in the mixed liquor bacterial communities to contribute to nitrification. This confirmed our observation that nitrifiers in raw wastewater act as potential seeds. Above, we recommended that the measurement of nitrifying biomass in influent wastewaters be incorporated in wastewater characterization and activated sludge modeling. Consequently, this will have an impact on the basic equations used to design activated sludge wastewater treatment plants. We derive here, some of the most critical equations and analyse their implications on the sizing of activated sludge bioreactors.

7.5.1 Use of minimum solids retention time (SRT) in design analysis

The design of an activated sludge system involves the following steps (Rittmann and McCarty 2001): defining the influent characteristics (flow and COD fractions), identifying the kinetic and stoichiometric coefficients (substrate uptake rate, growth rate, half-saturation constants, yield and decay rate), determining the design SRT ($\theta_{x,design}$), and finally the volume of the aeration basin (V_{aer}) such as to adjust the solid flux of the clarifier and the aeration capacity of the

bioreactor to proper ranges. Thus, the first degree of freedom in design practices, as suggested by Rittmann and McCarty (2001), is the determination of the θ_x (Eq. 7.1) by considering an appropriate safety factor to ensure an economical design, which can also provide a high-quality effluent despite sudden variations in influent characteristics and process conditions.

$$\theta_x = SF.[\theta_{x,min}]_{lim} \quad \text{Eq. 7.1}$$

Where, *SF* is the Safety Factor.

This is also based on the absolute minimum solids retention time ($[\theta_{x,min}]_{lim}$), which assumes that the effluent ammonium concentration (S_{NHx}) tends to infinity (Eq. 7.2)

$$[\theta_{x,min}]_{lim} = \frac{1}{\mu_{\mathrm{ANO,max}} - b_{\mathrm{ANO}}} \quad \text{Eq. 7.2}$$

Where, $\mu_{\mathrm{ANO,max}}$ is the maximum growth rate and b_{ANO} is the decay rate of nitrifiers.

Others prefer the simple definition of minimum SRT $[\theta_{x,min}]$ (Grady et al. 2011, Rittmann and McCarty 2001) (Eq. 7.3).

$$\theta_{x,min,\text{no seeding}} = \frac{1}{\frac{\mu_{\mathrm{ANO,max}} S_{\mathrm{NHx}}}{(K_{\mathrm{NHx}} + S_{\mathrm{NHx}})} - b_{\mathrm{ANO}}} \quad \text{Eq. 7.3}$$

Where S_{NHx} is the effluent ammonium concentration and K_{NHx} is the half-saturation constant for S_{NHx}. Both Eq. 7.2 and 7.3 were derived assuming a completely mixed reactor (Grady et al. 2011, Rittmann and McCarty 2001). However, these definitions are also valid for plug-flow reactors (Rittmann and McCarty 2001). The above derivations are for conditions where there is no nitrifier seeding. If one is to consider seeding with new general model parameter values, these equations would not be valid, and new expressions are required. In Chapter 3 (Jauffur et al. 2014), the following definitions for $\theta_{x,min,\text{with seeding}}$ and $[\theta_{x,min,\text{with seeding}}]_{lim}$ were used (Eq. 7.4 and 7.5). These definitions, however, assume that the biomass inventory is constant.

$$\theta_{x,min,\text{with seeding}} = \frac{1}{\frac{\mu_{\mathrm{ANO,max}} S_{NHx}}{(K_{\mathrm{NHx}} + S_{NHx})} - b_{\mathrm{ANO}} + q_{\mathrm{add}}} \quad \text{Eq. 7.4}$$

$$[\theta_{x,min,\text{with seeding}}]_{lim} = \frac{1}{\mu_{\text{ANO,max}} - b_{\text{ANO}} + q_{\text{add}}} \quad \text{Eq. 7.5}$$

Where, q_{add} is the nitrifier seeding rate, which is given by:

$$q_{\text{add}} = \frac{Q_{\text{Inf}} X_{\text{ANO,Inf}}}{V_{aer} \cdot X_{\text{ANO,ML}}} \quad \text{Eq. 7.6}$$

Both Eq. 7.4 and 7.5 assume that q_{add} is constant. Mass balances on substrate and biomass considering the influent nitrifier concentration ($X_{\text{ANO,Inf}}$) yield Eq. 7.7 for the autotrophic nitrifying biomass in the reactor ($X_{\text{ANO,ML}}$).

$$X_{\text{ANO,ML}} = \frac{\theta_x}{\theta_h} \cdot \frac{[Y_{\text{ANO}}(S_{\text{NHx,Inf}} - S_{\text{NHx}}) + X_{\text{ANO,Inf}}]}{(1 + b_{\text{ANO}}\theta_x)} \quad \text{Eq. 7.7}$$

The seeding level was shown to be relatively constant across the year by the determination of the seed level at LaPrairie WRRF during winter and summer (Chapter 6). However, the nitrifying biomass inventory will vary with changing solids retention time (θ_x) and Total Kjeldahl Nitrogen (TKN) available for nitrification (i.e., ammonifiable TKN minus the heterotrophic consumption of N, which is equivalent to $S_{\text{NHx,Inf}}$ in Eq. 7.7). Thus, q_{add} is not independent of the SRT and the nitrifiable nitrogen concentration. In this context, the $\theta_{x,min,\text{with seeding}}$ can be rewritten in the form of Eq. 7.8 to account for the variations in biomass inventory and the assumption that $S_{\text{NHx,Inf}} \gg S_{\text{NHx}}$.

$$\theta_{x,min,\text{with seeding}} = \frac{1}{\frac{\mu_{\text{ANO,max}} S_{\text{NHx}}}{(K_{\text{NHx}} + S_{\text{NHx}})} - b_{\text{ANO}} + \frac{X_{\text{ANO,Inf}}(1 + b_{\text{ANO}}\theta_x^{\min})}{\theta_x^{\min}(Y_{\text{ANO}} S_{\text{NHx,Inf}} + X_{\text{ANO,Inf}})}} \quad \text{Eq. 7.8}$$

If $S_{\text{NHx}} = cK_{\text{NHx}}$, then,

$$\theta_{x,min,\text{with seeding}} = \frac{1}{\left(1 + \frac{X_{\text{ANO,Inf}}}{Y_{\text{ANO}} S_{\text{NHx,Inf,nitrifiable}}}\right) \frac{c\,\mu_{ANO,\text{max}}}{(1+c)} - b_{\text{ANO}}} \quad \text{Eq. 7.9}$$

Where

$$S_{\text{NHx,Inf,nitrifiable}} = TKN_{\text{Inf}} - S_{\text{NHx,consumed by OHO}} \quad \text{Eq. 7.10}$$

Using the same notation, the $\theta_{x,min,\text{no seeding}}$ (i.e. SRT without seeding), is given by Eq. 7.11:

$$\theta_{x,\text{min,no seeding}} = \frac{1}{\frac{c\,\mu_{\text{ANO,max}}}{(1+c)} - b_{\text{ANO}}} \quad \text{Eq. 7.11}$$

Finally, a ratio between the minimum SRT with and without seeding can be defined as follows (Eq. 7.12):

$$\frac{\theta_{x,min,\text{with seeding}}}{\theta_{x,min,\text{no seeding}}} = \frac{c\,\mu_{\text{ANO,max}} - (1+c)b_{\text{ANO}}}{\left(1 + \frac{X_{\text{ANO,Inf}}}{Y_{\text{ANO}} S_{\text{NHx,Inf,nitrifiable}}}\right) c\,\mu_{\text{ANO,max}} - (1+c)b_{\text{ANO}}} \quad \text{Eq. 7.12}$$

7.5.2 Quantitative variation of solids retention time (SRT) with temperature

As shown above, the solids retention time (SRT) is a key parameter which dictates the size of activated sludge bioreactors. A short SRT will imply a small reactor size, while long SRTs will lead to reactors with bigger footprints and longer residence times for microorganisms. This is why important consideration is given to this parameter by designers so as to ensure the required effluent quality as specified by regulations. In addition, determination of the SRT is highly dependent on the temperature since its selection should always reflect the lowest sustainable temperature expected during reactor operation. This aspect is of prime importance for nitrifying systems because of the extreme sensitivity to temperature exhibited by the maximum specific growth rate coefficient for nitrifiers (Eq. 6.4, Chapter 6). The required SRT increases with decreasing temperatures to allow nitrifiers to grow, and prevent washout (Grady et al. 2011).

Based on Figure 7.2a, it is found that increasing the fraction of the influent nitrifier seed level reduces the ratio of the SRT with seeding to the SRT with no seeding "$\theta_{x,min,\text{with seeding}}/\theta_{x,min,\text{no seeding}}$" . This is further explored in Figure 7.1b, where the effect of seeding is found to be more pronounced at low than at high temperatures. With a higher seeding

level (0.8 as compared to 0.001 g-$X_{ANO,Inf}$/g-$Y_{ANO}S_{NHx,Inf,nitrifiable}$), the SRT ratio can attain very low levels at low temperatures. Influent nitrifier seeding can, thus, effectively reduce the minimum SRT and enable nitrification at low temperatures. This supports the interpretation that considering natural seeding of nitrifiers is most desirable to design nitrifying wastewater treatment systems for low temperature operations.

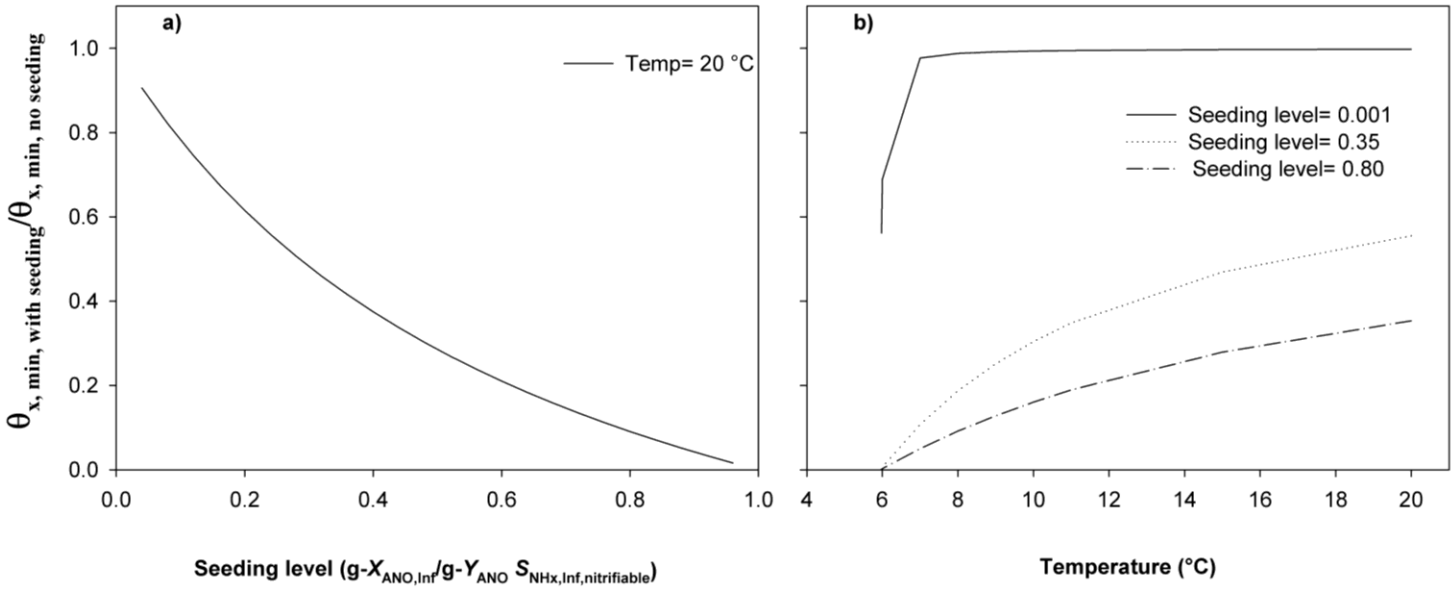

Figure 7.2. Effect of influent nitrifier seeding level (a) and temperature (b) on the ratio of the SRT with and without seeding. The unit of the seeding level in panel (b) is g-$X_{ANO,Inf}$/g-$Y_{ANO}S_{NHx,Inf,nitrifiable}$. For the simulation, the constant c was assumed to be 0.5, max. growth rate ($\mu_{ANO,max}$) 0.8 d^{-1}, decay rate (b_{ANO}) 0.15 d^{-1}, yield (Y_{ANO}) 0.25 g-COD/g-N, temp. coefficient for decay rate (θ_{bANO}) 1.029 and temp. coefficient for max. growth rate ($\theta_{\mu ANO}$) 1.072.

7.5.3 Estimation of influent-derived nitrifying biomass in mixed liquor

Based on Eq. 7.7, we estimated the amount of nitrifying biomass in the mixed liquor of the LaPrairie WRRF which is derived from influent solids using one year of operational data (2014). It was found that an average of 26% of the nitrifying biomass present in the mixed liquor actually came from influent solids conveyed to the treatment facility by the raw wastewater. Expressed differently, the ratio "$X_{ANO,Inf}/Y_{ANO}S_{NHx,Inf,nitrifiable}$" was found to be around 0.35, which is significant. Establishment of incoming nitrifiers among the mixed liquor communities, and their eventual metabolic induction may contribute to significant nitrification in this system

especially at cold temperature as demonstrated in Chapter 5. Based on Figure 7.2b, it is found that at 10 °C, the SRT is reduced by approximately 69.4% under a seeding level of 0.35 g-$X_{ANO,Inf}$/g-$Y_{ANO}S_{NHx,Inf,nitrifiable}$ as compared to a seeding level of 0.001 g-$X_{ANO,Inf}$/g-$Y_{ANO}S_{NHx,Inf,nitrifiable}$. From a design point of view, assuming that the MLVSS is kept constant for the two scenarios, the 69.4 % reduction in the SRT induced by natural seeding of nitrifiers is equivalent to a reduction of approximately 37 % of the reactor volume, which is significant. Such reduction in reactor volume would translate into a reduction in capital expenditure during construction phase as well as a reduction in operational cost since a smaller reactor will require a lower oxygen demand.

7.6 FINAL WORDS

This study showed the direct correspondence of nitrifying bacteria in raw sewage and activated sludge in bioengineered systems. Besides unraveling the existence of a natural seeding of nitrifiers at full-scale wastewater treatment level, we described the mechanism driving it and its potential impacts on the assembly of microbial communities in activated sludge systems, and on the performance of activated sludge models. This transdisciplinary work provides elements of answers to environmental engineers, plant operators and modelers, to better understand nitrification and its ecophysiology. The knowledge generated is of high importance in the conception, design and operation of wastewater treatment facilities considering that nitrification highly influences the determination of the size and energy requirement of activated sludge wastewater treatment systems performing nitrification. In addition, pragmatic approaches to subjectively adjust model parameters tend to add more noise to modeling data, and mask uncertainties. Determining the actual nitrifier seed level impacting on a particular bioengineered system is the correct approach in developing accurate models for eventual engineering applications.

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