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**Ecology of nitrification in oil refinery wastewater treatment
systems**

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Abstract

The diversity and abundance of ammonia oxidizing bacteria (AOB) and Archaea (AOA) were investigated in five oil refinery wastewater treatment plants in the UK using culture-independent molecular techniques. The AOB found in the plants were phylogenetically associated with the *Nitrosomonas oligotropha* and *Nitrosomonas marina* lineages and clones related to the *Nitrosomonas oligotropha* lineage may represent novel AOB species. Putative AOA were found in a trickling filter system and in two activated sludge systems and were phylogenetically placed within marine group 1.1a and terrestrial group 1.1b, *Crenarchaeota*. Evidence suggests that these are most closely related to AOA from other wastewater treatment systems.

Only three of the refinery wastewater treatment plants were nitrifying. AOB numbers quantified through real-time PCR of *amoA* ranged from 1.54×10^5 cells/mL to 9.49×10^5 cells/mL in the trickling filter system. In contrast, AOB numbers in one of the activated sludge systems ranged from 1.24×10^4 cells/mL to 1.68×10^6 cells/mL, while in the other, AOB numbers ranged from 7.14×10^3 cells/mL to 9.56×10^4 cells/mL, despite showing good nitrification. Conversely, AOA were detected in the trickling filter system at 1.54×10^7 cells/mL. In the activated sludge systems AOA were more abundant than AOB. In the activated sludge system with the higher AOB numbers, AOA abundance ranged from 4.32×10^6 cells/mL to 1.77×10^8 cells/mL, and in the activated sludge system with the lower AOB numbers, AOA abundance ranged from 6.15×10^6 cells/mL to 2.69×10^8 cells/mL. The high abundance of AOA in the nitrifying refinery wastewater treatment systems suggested that AOA may play an important role in nitrification in these reactors. However microautoradiography coupled with CARD-FISH (conducted by Dr Marc Mußmann, University of Vienna) indicated that the AOA were not chemoautotrophic ammonia-oxidizers. Assessment of AOA and AOB abundance in relation to ammonia removal in the treatment plants indicated that ammonia removal was consistent with the AOB numbers detected and thus, AOB are probably responsible for most of the nitrification observed and the overall contribution of putative AOA to nitrification in these wastewater treatment plants appears to be relatively minor.

Keywords: Ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), nitrite oxidizing bacteria (NOB), diversity, phylogeny, abundance, real-time PCR, CSAOR (cell specific ammonia oxidizing rate or rates), fluorescence *in situ* hybridization (FISH), oil refinery wastewater treatment plants.

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Declaration

I hereby certify that this work is my own, except where otherwise acknowledged, and that it has not been submitted for a degree at this or any other University.

Ivana Regina Couto de Brito

Table of Contents

	Page No
Title page.....	I
Abstract.....	II
Acknowledgements.....	III
Declaration.....	V
Table of Contents.....	VI
List of Figures.....	XI
List of Tables.....	XVI
Abbreviations.....	XVIII
Chapter 1 Introduction.....	1
1.1 Biological wastewater treatment systems.....	1
1.1.1 Activated sludge	6
1.1.2 The trickling filter.....	9
1.2 The nitrogen cycle.....	10
1.3 Nitrogen removal processes in wastewater treatment systems.....	13
1.3.1 Nitrification.....	14
1.3.2 Heterotrophic nitrification.....	17
1.3.3 Denitrification and aerobic denitrification.....	18
1.3.4 Anaerobic ammonia oxidation (ANAMMOX) and new processes for nitrogen removal.....	20
1.4 Culture independent techniques in the investigation of wastewater treatment systems.....	24
14.1 PCR, DGGE, cloning, sequencing and phylogenetic analyses.....	27
1.4.2 Real-time PCR.....	31
1.4.3 Whole cell fluorescence <i>in situ</i> hybridization (FISH).....	35
1.5 Oil refinery wastewaters.....	39
1.5.1 Oil refining and oil refinery processes.....	39
1.6 Aims and objectives.....	43

Chapter 2 Methodology	45
2.1 Wastewater treatment plants (WWTPs) location, bioreactor configurations and sampling procedures.....	45
2.1.1 Trickling filter sampling.....	49
2.1.2 Activated sludge sampling.....	50
2.1.3 Large scale screening of WWTPs for the presence of putative archaeal ammonia oxidizers.....	55
2.2 Measurements in the field.....	57
2.3 Wet chemistry analysis.....	57
2.3.1 Chemical oxygen demand (COD).....	57
2.3.2 Biochemical oxygen demand (BOD).....	59
2.3.3 Organic Kjeldahl nitrogen (TKN).....	61
2.3.4 Ammonia.....	62
2.3.5 Chloride, nitrite and nitrate determinations.....	62
2.3.6 Salinity.....	63
2.3.7 Suspended solids (SS), and volatile suspended solids (VSS).....	63
2.4 DNA extraction.....	65
2.5 Polymerase chain reaction (PCR).....	66
2.5.1 Amplification of 16S rRNA gene fragments of ammonia oxidizing bacteria (AOB).....	66
2.5.2 Amplification of ammonia monooxygenase genes (<i>amoA</i>) from Bacteria and Archaea.....	68
2.5.3 Agarose gel electrophoresis of PCR products.....	68
2.6 Denaturing gradient gel electrophoresis (DGGE).....	69
2.6.1 DGGE analysis of 16S rRNA gene fragments from ammonia oxidizing bacteria.....	69
2.6.2 DGGE analysis of <i>amoA</i> gene fragments from putative ammonia oxidizing Archaea.....	69
2.6.3 Numerical analysis of DGGE profiles.....	70
2.7 Cloning PCR-amplified betaproteobacterial ammonia oxidizer 16S rRNA and archaeal ammonia monooxygenase (<i>amoA</i>) gene fragments.....	70

2.8	DNA sequencing.....	72
2.9	Analysis of AOB 16S rRNA and AOA <i>amoA</i> gene clone libraries.....	73
2.10	Statistical comparisons between clone libraries.....	76
2.11	Phylogenetic analysis.....	78
2.12	Real-time PCR.....	80
	2.12.1 Preparation of standards.....	82
2.13	Calculation of cell specific ammonia oxidation rates (CSAOR).....	85
2.14	Whole-cell fluorescence <i>in situ</i> hybridization (FISH).....	86
	2.14.1 Fixation of samples in paraformaldehyde (PFA).....	86
	2.14.2 Hybridization procedure.....	86
	2.14.3 Preparation of gelatin coated slides.....	91
	2.14.4 Image analysis and cell counting procedure.....	91
	2.14.5 <i>In situ</i> detection and quantification of putative AOA through catalyzed reporter deposition-fluorescence <i>in situ</i> hybridization (CARD- FISH).....	94
2.15	<i>In silico</i> specificity evaluation of the primers and probes used or compared in this study, in real-time PCR, FISH and CARD-FISH assays.....	95
	2.15.1 <i>In silico</i> specificity evaluation of the primers and probes used for quantification of the AOB, <i>Crenarchaeota</i> and NOB 16S rRNA abundances.....	95
	2.15.2 <i>In silico</i> analysis of the primers used in this study for the quantification of AOB and AOA <i>amoA</i> gene abundances.....	99
2.16	Nitrification modelling.....	102
2.17	Statistics.....	103
Chapter 3 Diversity of AOB in oil refinery wastewater treatment systems.....		104
3.1	Introduction.....	104
3.2	Methods.....	107
3.3	Results and Discussion.....	109
	3.3.1 Differences in wastewater parameters among the oil refinery wastewater treatment systems.....	109
	3.3.2 PCR-amplification of 16S rRNA genes.....	117
	3.3.3 Interpretation and statistical analysis of DGGE profiles.....	119

3.3.4	Comparison of AOB communities based on 16S rRNA gene clone libraries.....	123
3.3.5	Composition of betaproteobacterial AOB 16S rRNA gene clone libraries from oil refinery WWTPs.....	134
3.3.6	Statistical comparisons between AOB 16S rRNA gene clone libraries.....	139
3.3.6.1	Phylogenetic analysis of betaproteobacterial AOB 16S rRNA sequences.....	141
3.4	Conclusions.....	152
Chapter 4 Diversity of AOA in oil refinery wastewater treatment systems.....		
4.1	Introduction.....	154
4.2	Methods.....	158
4.3	Results and Discussion.....	159
4.3.1	Occurrence of archaeal <i>amoA</i> in WWTPs.....	159
4.3.2	DGGE analysis of archaeal <i>amoA</i> genes.....	163
4.3.3	Analysis of archaeal <i>amoA</i> gene clone libraries.....	165
4.3.4	Statistical comparisons between clone libraries.....	172
4.3.5	Phylogenetic analysis of archaeal <i>amoA</i> gene sequences.....	173
4.4	Conclusions.....	179
Chapter 5 Ammonia oxidizer abundance and role in nitrification in oil refinery wastewater treatment systems.....		
5.1	Introduction.....	180
5.2	Methods.....	185
5.3	Results and Discussion.....	187
5.3.1	WWTP performance with respect to BOD, COD, TKN and ammonia removal.....	187
5.3.2	Quantification of total bacteria, AOB, marine and soil <i>Crenarchaeota</i> 16S rRNA gene abundance.....	196
5.3.3	Quantification of bacterial and archaeal <i>amoA</i> gene abundance.....	210
5.3.4	AOA alternative metabolism.....	223
5.3.5	AOB and AOA cell numbers and specific ammonia oxidizing activities.....	225

5.3.6 Relationships between physical-chemical parameters and AOB and AOA abundance.....	236
5.3.7 Is measured AOB biomass consistent with theoretical yields of AOB based on ammonia removal?.....	241
5.4 Conclusions.....	247
Chapter 6 Ammonia oxidizer and nitrite oxidizer abundance measured through FISH and real-time PCR.....	250
6.1 Introduction.....	250
6.2 Methods.....	255
6.3 Results and Discussion.....	258
6.3.1 Quantification of <i>Nitrospira</i> type I, <i>Nitrospira</i> type II and <i>Nitrobacter</i> 16S rRNA gene abundance.....	258
6.3.2 Relationships between physical-chemical parameters and NOB abundance.....	272
6.3.3 <i>In situ</i> detection, abundance and morphology of AOB, <i>Nitrospira</i> , and <i>Nitrobacter</i> investigated through fluorescence <i>in situ</i> hybridization (FISH).....	274
6.3.4 Comparison between real-time PCR and FISH.....	286
6.3.5 <i>In situ</i> detection, abundance and morphology of 16S rRNA crenarchaeotal groups investigated through DAPI and catalyzed-reporter deposition-fluorescence <i>in situ</i> hybridization (CARD-FISH).....	289
6.4 Conclusions	295
Chapter 7 General Discussion.....	296
Chapter 8 Conclusions.....	306
Chapter 9 Future Research.....	308
References.....	309
Appendix.....	343

List of Figures

	Page No.
Figure 1.1 Flow diagrams of a typical a) activated sludge system and b) trickling filter system	6
Figure 1.2 Microbial process reactions in the nitrogen cycle.....	11
Figure 1.3 Flux diagrams of nitrogen removal processes.....	22
Figure 1.4 Molecular toolbox applied in this study.....	26
Figure 1.5 FISH (fluorescence <i>in situ</i> hybridization) steps procedure.....	36
Figure 2.1 Oil refineries sampled in this study.....	46
Figure 2.2 Lindsey refinery wastewater treatment plant layout.....	48
Figure 2.3 Lindsey refinery aerial view and filter medium.....	49
Figure 2.4 Activated sludge systems from the Eastham, Humber and Grangemouth refineries sampled in this study, illustrating their specific designs.....	53
Figure 2.5 Humber refinery wastewater treatment plant layout.....	54
Figure 2.6 Number of mismatches and mismatch positions found in the targeted binding sites of the primers <i>amo</i> 196F, <i>amo</i> 277R and the <i>Taq</i> probe <i>amo</i> 247 used for the quantification of the AOA <i>amoA</i> gene abundance when aligned with 178 archaeal <i>amoA</i> clone libraries sequences obtained in this study.....	101
Figure 3.1 Agarose gel showing PCR products amplified from DNA extracted from samples collected in five oil refinery wastewater treatment plants in the UK with primers CTO 189f and CTO 654r.....	117
Figure 3.2 Comparison between DGGE profiles of 16S rRNA gene fragments from AOB communities from samples collected in five oil refinery wastewater treatment plants in UK	118
Figure 3.3 UPGMA cluster analysis of DGGE profiles of uncultured betaproteobacterial AOB 16S rRNA gene fragments obtained from samples collected from five oil refinery wastewater treatment plants in the UK	120
Figure 3.4 DGGE profiles obtained from replicate samples collected from reactors A and B from the trickling filters system from the Lindsey refinery on two sampling occasions.....	125

Figure 3.5	DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge systems from the Eastham and Pembroke refineries.....	126
Figure 3.6	DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge system from the Humber refinery on two sampling occasions	127
Figure 3.7	DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge system from the Grangemouth refinery.....	128
Figure 3.8	Abundance-based estimate curves obtained for eight AOB 16S rRNA gene clone libraries constructed in this study.....	133
Figure 3.9	Clone frequencies in eight AOB 16S rRNA gene clone libraries constructed in this study related to AOB <i>N. oligotropha</i> , <i>N. marina</i> and <i>N. communis</i> lineages, and non AOB <i>Betaproteobacteria</i>	134
Figure 3.10	Phylogenetic tree of betaproteobacterial ammonia oxidizing bacteria inferred from comparative analysis of 16S rRNA sequences.....	144
Figure 4.1	Agarose gel showing PCR products amplified with archaeal <i>amoA</i> primers Arch- <i>amoAF</i> and Arch- <i>amoAR</i> from DNA extracted from replicate samples collected in a survey in 23 full scale municipal WWTPs, and one pilot reactor treating wastewater from a leather processing plant from the British Leather Corporation (BLC) in UK.....	161
Figure 4.2	Agarose gel showing PCR products from DNA extracted from the Grangemouth, Lindsey, and BLC wastewater treatment reactors amplified with archaeal <i>amoA</i> primers Arch- <i>amoAF</i> and Arch- <i>amoAR</i> (Francis <i>et al.</i> , 2005).....	162
Figure 4.3	Comparison between DGGE profiles of archaeal <i>amoA</i> genes from DNA replicate samples collected from the Grangemouth, Lindsey and BLC wastewater treatment reactors.....	163
Figure 4.4	UPGMA cluster analysis of DGGE profiles of uncultured crenarchaeotal <i>amoA</i> gene fragments obtained from samples collected from the Grangemouth, Lindsey and BLC wastewater treatment reactors after Pearson correlation of whole-track densitometric curves of the profiles.....	164

Figure 4.5	Crenarchaeotal <i>amoA</i> gene clone library rank abundance plots derived from the Grangemouth reactors A and B.....	167
Figure 4.6	Crenarchaeotal <i>amoA</i> gene clone library rank abundance plots derived from the Lindsey reactor A and the BLC pilot reactor.....	168
Figure 4.7	Abundance-based estimate curves obtained for four AOA <i>amoA</i> gene clone libraries constructed in this study.....	171
Figure 4.8	Phylogenetic relationships among ammonia oxidizing archaeal <i>amoA</i> sequences.....	175
Figure 5.1	The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group <i>Crenarchaeota</i> in wastewater treatment reactors A and B from the Lindsey, Eastham, and Pembroke refineries.....	197
Figure 5.2	The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group <i>Crenarchaeota</i> in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions.....	198
Figure 5.3	The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group <i>Crenarchaeota</i> in wastewater treatment reactors A and B from the Grangemouth refinery sampled on three different occasions.....	199
Figure 5.4	The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group <i>Crenarchaeota</i> in one single replicate sample from the BLC pilot reactor.....	205
Figure 5.5	Phylogenetic reconstruction of crenarchaeotal 16S rRNA sequences recovered from the Lindsey, Humber, Grangemouth, and BLC wastewater treatment reactors (Mußmann <i>et al.</i> , 2008).....	207
Figure 5.6	The <i>amoA</i> gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Lindsey, Eastham and Pembroke refineries.....	211
Figure 5.7	The <i>amoA</i> gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions.....	212

Figure 5.8	The <i>amoA</i> gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Grangemouth refinery sampled on three different occasions.....	213
Figure 5.9	The <i>amoA</i> gene abundance for AOB and AOA in one single replicate sample from the BLC pilot reactor.....	215
Figure 5.10	PCA diagram. Biplot representation of first and second components of physical-chemical and qPCR abundance data considered in this study.....	238
Figure 5.11	Relationship between the theoretical biomass predicted in the Humber plus Grangemouth reactors using a single nitrification model (Rittmann <i>et al.</i> , 1999) and measured biomass obtained from (a) AOB <i>amoA</i> and (b) AOA <i>amoA</i> gene quantification through qPCR.....	243
Figure 6.1	The 16S rRNA gene abundance for <i>Nitrospira</i> type I, <i>Nitrospira</i> type II and <i>Nitrobacter</i> in wastewater treatment reactors A and B from the Lindsey, Eastham and Pembroke refineries.....	259
Figure 6.2	The 16S rRNA gene abundance for <i>Nitrospira</i> type I, <i>Nitrospira</i> type II and <i>Nitrobacter</i> in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions.....	260
Figure 6.3	The 16S rRNA gene abundance for <i>Nitrospira</i> type I, <i>Nitrospira</i> type II and <i>Nitrobacter</i> in wastewater treatment reactors A and B from the Grangemouth refinery sampled on three different occasions.....	261
Figure 6.4	The 16S rRNA gene abundance for <i>Nitrospira</i> type I, <i>Nitrospira</i> type II and <i>Nitrobacter</i> in one single replicate sample from the BLC pilot reactor.....	263
Figure 6.5	PCA diagram. Biplot representation of first and second components of physical-chemical and qPCR abundance data considered in this study.....	273
Figure 6.6	Comparison and relationship between AOB cell numbers obtained through real-time PCR of <i>amoA</i> genes and FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study.....	275

Figure 6.7	Comparison and relationship between <i>Nitrospira</i> type I plus type II cell numbers obtained through real-time PCR of 16S rRNA genes and the cell numbers of the genus <i>Nitrospira</i> obtained through FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study.....	276
Figure 6.8	Comparison and relationship between <i>Nitrobacter</i> cell numbers cell numbers for obtained through real-time PCR of 16S rRNA genes and FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study.....	277
Figure 6.9	Relationship between the theoretical biomass predicted in the Humber and Grangemouth reactors using a single nitrification model (Rittmann <i>et al.</i> , 1999) and measured AOB biomass obtained through FISH quantification.....	281
Figure 6.10	Confocal laser scanning Z-stack images of simultaneous <i>in situ</i> fluorescence hybridization of AOB and <i>Nitrospira</i> , AOB and <i>Nitrobacter</i> and <i>Nitrospira</i> and <i>Nitrobacter</i> cells targeted respectively by oligonucleotide probes labelled by 6-FAM and CY3 stains	283
Figure 6.11	Simultaneous <i>in situ</i> staining of the Grangemouth refinery sludge with the general DNA DAPI and clone specific probe Cren 1162 specifically targeting 16S rRNA sequences from the soil group 1.1b <i>Crenarchaeota</i> recovered from the Grangemouth reactor.....	289
Figure 6.12	CARD-FISH (catalyzed reporter deposition- fluorescence <i>in situ</i> hybridization) of crenarchaeotal cells with horse-radish peroxidase labelled probes combined with fluorescently labelled tyramides.....	293

List of Tables

	Page No.
Table 2.1 Sampling of oil refinery wastewater treatment plants in the UK.....	52
Table 2.2 Process descriptions of 23 municipal wastewater treatment plants (Pickering, 2008) and one pilot reactor treating tannery wastes surveyed in this study for the presence of putative AOA.....	56
Table 2.3 Primers and PCR conditions used in this study.....	67
Table 2.4 Real-time PCR primers and conditions used in this study.....	81
Table 2.5 Standards used in the real-time PCR assays performed in this study.....	82
Table 2.6 Oligonucleotide probes and hybridization conditions used in this study.....	88
Table 2.7 CARD-FISH probes and hybridization conditions used by Mußmann <i>et al.</i> , 2009, unpublished.....	95
Table 2.8 <i>In silico</i> specificity evaluation and overlap of the primers and probes used in this study for the quantification of the AOB 16S rRNA abundance.....	96
Table 2.9 <i>In silico</i> specificity evaluation and overlap of the primers and probes used in this study for the quantification of the Crenarchaeota 16S rRNA abundance.....	97
Table 2.10 <i>In silico</i> specificity evaluation and overlap of the primers and probes used in this study for the quantification of the NOB 16S rRNA abundance.....	98
Table 3.1 Process parameters measured <i>in situ</i> in five oil refinery wastewater treatment plants in the UK.....	111
Table 3.2 Wet chemistry data determined in five oil refinery wastewater treatment plants in the UK.....	113
Table 3.3 Description of eight AOB 16S rRNA gene clone libraries constructed in this study.....	124
Table 3.4 Coverage and diversity indices measured for eight AOB 16S rRNA gene clone libraries constructed in this study.....	129

Table 3.5	∫- LIBSHUFF population p values determined for comparisons of six AOB 16S rRNA gene clone libraries constructed in this study.....	139
Table 4.1	MLSS and influent ammonia concentrations reported in 29 wastewater treatment reactors surveyed in this study for the presence of putative AOA.....	160
Table 4.2	Description of four crenarchaeotal <i>amoA</i> gene clone libraries constructed in this study.....	166
Table 4.3	Coverage and diversity indices measured for four AOA <i>amoA</i> gene clone libraries constructed in this study.....	169
Table 4.4	∫- LIBSHUFF population p values determined for comparisons of four AOA <i>amoA</i> gene clone libraries constructed in this study.....	172
Table 5.1	BOD and COD ratios and removal efficiencies across the wastewater treatment plants investigated in this study.....	189
Table 5.2	TKN/BOD ratios, TKN and ammonia removal efficiencies and ammonia-nitrate conversion across the wastewater treatment plants investigated in this study.....	191
Table 5.3	Cell numbers and cell specific ammonia oxidation rates (CSAOR) calculated for AOB and AOA in three oil refinery WWTPs that were nitrifying in this study.....	227
Table 5.4	Theoretical nitrifier biomass predicted using a single nitrification model (Rittmann <i>et al.</i> , 1999) and measured biomass of AOB and AOA obtained <i>amoA</i> genes quantification through qPCR in Humber and Grangemouth reactors.....	242
Table 6.1	Theoretical nitrifier biomass predicted using a single nitrification model (Rittmann <i>et al.</i> , 1999) and measured AOB biomass obtained through FISH quantification in Humber and Grangemouth reactors	281
Table 6.2	AOA detected in the refinery sludges and in the BLC sludge by the probes used in the CARD-FISH hybridizations (Mußmann <i>et al.</i> , 2009, unpublished) and comparison to 16S rRNA genes quantification through qPCR in this study for marine group 1.1a and soil group 1.1b <i>Crenarchaeota</i>	291

Abbreviations

6-FAM	6-carboxyfluorescein
ABIL	Suspension of nitrifying cells (named ammonia binding inoculum liquid)
ACE	Abundance-based coverage estimator
AMO	Ammonia monooxygenase enzyme
<i>amoA</i> , <i>amoB</i> , <i>amoC</i>	Ammonia monooxygenase genes
ANAMMOX	Anaerobic ammonia oxidation
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
API	American Petroleum Institute
ATU	Allylthiourea
BOD	Biochemical oxygen demand
BODr	Biochemical oxygen demand removal
BSA	Bovine serum albumin
CANON	Completely autotrophic nitrogen removal over nitrite
CARD-FISH	Catalyzed reporter deposition fluorescence <i>in situ</i> hybridization
cDNA	Reverse copy product of rRNA
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
CODr	Chemical oxygen demand removal
CSAOR	Cell specific ammonia oxidation rate or rates
CONCAWE	Conservation of clean air and water in Europe
CY3	Tetramethyl isothiocyanate
CY5	Indocarbocyanine
daim	Digital image analysis for microbial ecology
DAPI	4', 6' diamino-2-phenylindole
Deamox	Denitrifying ammonium oxidation
DGGE	Denaturant gradient gel electrophoresis

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNB	Denitrifying nitrifying bioreactor
dNTP	Deoxynucleoside triphosphate
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
EDTA	Ethylenediamine tetraacetic acid
FA	Deionised formamide
FAS	Ferrous ammonium sulphate
FISH	Fluorescence <i>in situ</i> hybridization
FOV	Field of view
FRET	Fluorescence resonance energy transfer
HAO	Hydroxylamine oxidoreductase enzyme
HRP	Horseradish-peroxidase labelled probes
HRT	Hydraulic retention time
ISR	Intergenic spacer region
LB	Luria Bertani
MAR-FISH	Microautoradiography combined with Fluorescence <i>in situ</i> hybridization
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MRR	Mark-release-recapture methods
NOB	Nitrite oxidizing bacteria
NOR	Nitrite oxidoreductase enzyme
OLAND	Oxygen-limited nitrification and denitrification
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
qPCR	Real-time quantitative PCR or real-time PCR
RBC	Rotating biological contactor

RDP	Ribosomal database project
RNA	Ribonucleic acid
<i>rnn</i> operon	ribosomal 16S rRNA gene encoding region
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
SBR	Sequencing batch reactor
SBBR	Sequence batch biofilm reactor
SDS	Sodium dodecyl sulphate
SHARON	Single reactor system for high rate ammonia removal over nitrite
SRT	Sludge retention time or sludge age
SS	Suspended solids
TAE	Tris-acetate EDTA buffer
TAMRA	Tetramethyl-6-carboxyrhodamine
TCA	Oxidative tricarboxylic acid cycle
TKN	Organic Kjeldahl nitrogen
TKNr	Organic Kjeldahl nitrogen removal
TRIS	tris (hydroxymethyl) aminomethane
TSA	Tiramide signal amplification
UPGMA	Unweighted pair-group method with arithmetic averages clustering algorithm
v/v	Volume per volume
VLR	Vertical loop reactor
VSS	Volatile suspended solids
w/v	Weight per volume
WWTP	Wastewater treatment plant

CHAPTER 1

Introduction

1.1 Biological wastewater treatment systems

Wastewater treatment plants are designed to remove organic and inorganic aqueous pollutants considered to be harmful or that pose a risk to human health and natural aquatic resources. Permissible limits for a range of substances classified as toxic or dangerous are imposed by environmental agencies in order to protect the water bodies that directly receive effluent from wastewater treatment plants. For instance, the current discharge standards imposed by the IPPC directive 91/271 of the European Community for nitrogen are 10 mg N/L in treated urban and industrial water.

Different wastewater sources harbour different types and amounts of organic and inorganic pollutants, depending on their origin. For treatment purposes, they can be considered according to their source: domestic sewage only; sewage mixed with different industrial wastewaters; or wastewater of industrial origin only. For example, in municipal wastewater treatment systems, wastes are primarily domestic sewage, but also receive industrial wastewater contributions in lesser and variable proportions. The chemical composition of industrial wastewaters can vary significantly according to the type of industry and associated raw materials, products and processes involved. Different industrial process types, including food processing, chemicals and petrochemicals, paper, oil refining, textiles, tanneries, pharmaceuticals, among many other kinds of industrial activities, can produce a variety of problematic wastewaters.

In municipal wastewater treatment plants, about 20 to 50% of the degradable organic material present in the wastewater enters the plant in the form of soluble compounds; while in some specific types of industrial wastewaters, such as chemical, textile, pharmaceutical, petrochemical, cokery, the soluble organic materials may represent only a low to moderate fraction of the total degradable organic substrates (Metcalf and Eddy, 2003). Therefore, depending on the type of wastewater being treated, as well as its level of biodegradability and toxicity, a series of physical, chemical and biological processes are combined, in order to remove different forms of carbon, nitrogen and phosphorus present in the water, among other elements, in order to achieve the maximum possible efficiency in the treatment. Compared with physical and chemical processes, biological processes are considered to be efficient and cost effective; in most cases they will be the treatment of preference for a diverse range of wastes.

There are two forms in which microorganisms may grow: in suspension or attached to solid surfaces, like the ones typically found in activated sludge and trickling filters respectively. Aerobic heterotrophic bacteria are also able to produce extracellular biopolymers that result in the formation of biological flocs or biofilms for attached growth processes (Metcalf and Eddy, 2003). As microorganisms obtain their energy for growth and maintenance from oxidation-reduction reactions, the aim of any biological wastewater treatment plant is to deplete the electron donor which serves as a substrate for their growth and reproduction, that is, organic compounds in the case of heterotrophic bacteria, and inorganic compounds such as ammonia and nitrite in the case of autotrophic nitrifiers (Rittmann and McCarthy, 2001).

Within these biological systems, soluble organic matter can be immediately utilized by microbial cells while colloidal and particulate organic matter adsorbed on flocs and biofilms are firstly broken down through the action of microbial extracellular enzymes before becoming available for microbial oxidation. In aerobic processes, more than half of the complex organic waste represented by the empirical formula COHNSP is oxidized to simple end products such as CO₂, H₂O, NH₃, NO₃⁻, NO₂⁻, SO₄²⁻, PO₄²⁻ (mineralization), while the remainder is converted into new cellular material through assimilation, which may further be also oxidized by endogenous respiration (Metcalf and Eddy, 2003).

All biological treatment reactors are designed using mass balance calculations. These are calculated for each of the “electron donor” constituents of interest that are entering or leaving the system, along with their rates of depletion or production within the system, as well as the flow rates crossing a defined volume, which are measured as mass per unit volume per unit time (Rittmann and McCarty, 2001). Reactors are designed to operate with high microorganism concentrations; but their overall rate of metabolism is controlled by the limiting substrate concentration.

Monod kinetics (Monod, 1949) are most commonly used to model the relationship between the residual concentration of substrate that limits the microbial specific growth rate and the maximum specific growth rate of biomass obtained as a function of the substrate utilization. This is given by the equation:

$$\mu = \mu_{\max} \frac{S}{K_s + S} \quad (1.1)$$

where:

μ = specific growth rate (d⁻¹)

μ_{\max} = maximum growth rate at saturation concentration of growth limiting substrate (d⁻¹)

S = substrate concentration (mg/L)

K_s = half saturation constant (mg/L) which is the concentration of limiting substrate at which the specific growth rate equals one-half of the maximum specific growth rate ($\mu = \mu_{\max}/2$)

The specific growth rate μ corresponds to the change in biomass per day (related to the amount of biomass present) and is a function of the substrate concentration. The maximum bacterial specific growth rate relates to the maximum specific substrate utilization rate and occurs at high substrate concentrations. Hence when cells grow rapidly in the presence of non-limiting substrate concentrations they make their maximum investment of energy in cell synthesis. However, when the substrate concentration (electron donor) is limited, a larger portion of the energy obtained from the substrate oxidation must be used for cell maintenance (Gray, 1990).

A loss term not explicitly included in the standard Monod equation (Eq. 1.1) is endogenous decay. Endogenous decay includes cell mass losses derived from the oxidation of internal storage products for energy used for cell maintenance, cell death and lysis, and predation by organisms higher in the food chain (Gray, 1990). As the endogenous decay may also affect the specific growth rate μ , in practice the endogenous decay is also commonly considered by engineers in the Monod equation as follows:

$$\mu = \mu_{\max} \frac{S}{K_s + S} - K_d \quad (1.2)$$

where:

μ = specific growth rate (d^{-1})

μ_{\max} = maximum growth rate at saturation concentration of growth limiting substrate (d^{-1})

S = substrate concentration (mg/L)

K_s = half saturation constant (mg/L) which is the concentration of limiting substrate at which the specific growth rate equals one-half of the maximum specific growth rate ($\mu = \mu_{\max}/2$)

K_d = endogenous decay

The ratio of the amount of biomass produced to the amount of substrate (electron donor) consumed is defined as the biomass yield coefficient Y and is expressed as mass (or mole) of organism produced per mass (or mole) of substrate consumed. As a definitive stoichiometric relationship exists between the substrate removed and the observed biomass yield, the expression of yield can also be combined with the Monod equation to give the rate of substrate utilization as follows:

$$\frac{ds}{dt} = - \frac{\mu}{Y} X \quad (1.3)$$

where:

s = concentration of substrate (mg/L)

t = time (d)

μ = specific growth rate (d^{-1})

Y = yield coefficient

X = concentration of microorganisms (mg/L)

In summary, the performance and efficiency of biological processes are directly linked to the dynamics of substrate utilization and microbial growth. Therefore the effective operation of biological systems requires an understanding of the types of microorganisms that are involved, the specific reactions that they perform and their nutritional needs and kinetics. In addition, knowledge and control of environmental conditions such as temperature, pH, dissolved oxygen, and other relevant factors that affect organisms of interest, is also essential. By taking into account all these factors, organic and inorganic chemicals may then be effectively removed from water and thus the best treatment possible may be implemented.

1.1.1 Activated sludge

The activated sludge process (See Figure 1.1.a) is one of the most popular and versatile systems used for the treatment of a range of wastewaters.

The process consists of at least one aeration tank and one clarifier. In the aeration tank, wastes are aerated and kept in contact for sufficient time with a large concentration of microorganisms, referred to as mixed liquor suspended solids (MLSS) or mixed liquor volatile suspended solids (MLVSS) and composed mainly of bacteria grown as floc-forming aggregates, typically ranging from 50 to 200 μm in size (Metcalf and Eddy, 2003). After wastewater treatment, the mixed liquor flows to a clarifier, where the suspended microbial biomass is settled and thickened. The settled biomass is then either returned to the aeration tank to continue biodegradation of the incoming influent or removed from the activated sludge tank. Usually a portion of the settled biomass is removed daily or periodically as the process produces excess biomass that would accumulate along with the nonbiodegradable solids contained in the influent. In activated sludge systems, the typical liquid hydraulic retention time (HRT) required for organic oxidation is approximately six hours for heterotrophic bacteria and a minimum of 48 to 72 hours for nitrification (Gerardi, 2002; Gray, 1990).

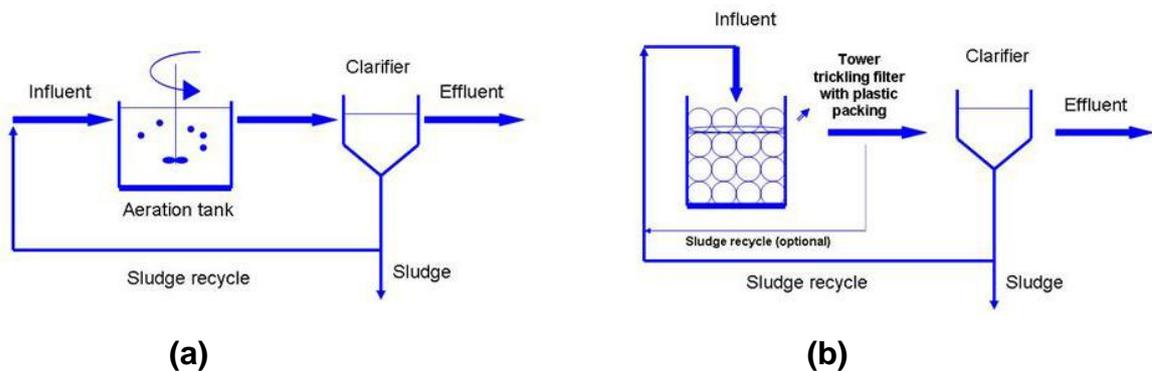


Figure 1.1 Flow diagrams of a typical (a) activated sludge system and (b) trickling filter system. Modified from Metcalf and Eddy, 2003.

The MLSS or MLVSS are mixtures of solids that result from the combination of recycled sludge with influent wastewater in the aeration tank. These solids are comprised of biodegradable volatile suspended solids or biomass, nonbiodegradable volatile suspended solids and inert inorganic total suspended solids. The nonbiodegradable solids fraction is derived from the influent wastewater and is also produced as cell debris from endogenous respiration, while the inert inorganic solids fraction originates in the influent wastewater (Metcalf and Eddy, 2003).

The MLSS is also used as a process parameter to measure the biomass production in the activated sludge tank, also referred to as the active biomass concentration. This is calculated by considering the ratio between the sum of the yield plus decay terms, and the total MLVSS within reactor. The MLSS concentration can be controlled by altering the sludge wastage rate. In theory, the higher the MLSS in the aeration tank, the greater is the efficiency of the process, because of the increased biomass able to utilize the available food. However, high operating values of MLSS are limited by the availability of oxygen in the aeration tank and the capacity of the sedimentation unit to separate and recycle the activated sludge (Gray, 1990).

From the MLSS two other important process parameters may be calculated and are also critical for monitoring and controlling nitrification and denitrification in the activated sludge process: the food-to-microorganism ratio (F/M) and the solids retention time (SRT) or sludge age.

The F/M ratio refers to the quantity (g) of biochemical oxygen demand (BOD) applied per day, per quantity of bacteria or microorganisms (g MLSS) in the aeration tank. This controls both the rate of biological oxidation and the volume of microbial biomass produced in the aeration tank through the microbial growth phases in the reactor. These can be either the log, decline or endogenous stages, with each phase characterizing a different type of activated sludge defined as either high-rate, conventional, or extended aeration. In conventional activated sludge units, normal MLSS concentrations may range from 1500 to 3500 mg/L, while in a high rate system MLSS concentrations may reach to up 8000 mg/L (Gray, 1990).

A high rate system is characterized by an excess of substrate and a maximum rate of metabolism. Once food becomes limited the rate of metabolism will rapidly decline until the microorganisms are in the endogenous respiration phase, where cell lysis and resynthesis take place. Under low sludge loadings there is almost complete oxidation of organics, resulting in a high quality effluent with the microorganisms flocculating and settling rapidly. Therefore the lower the F/M ratio, the greater the BOD removal efficiency that can be expected.

The solids retention time (SRT) or sludge age (Θ_x) is the average time the activated sludge solids are in the system, and is determined by dividing the mass of solids removed daily through the effluent, by the solids wasted from the process control. Sludge age is also one of the most important process controls of the activated sludge process because it relates directly to the growth rate of the active microorganisms, which in practice also controls the concentration of the growth-rate-limiting substrate in the reactor. Therefore the higher the Θ_x , the lower the microbial growth rate and the older the bacteria in the MLSS. The sludge age is increased in the activated sludge process by decreasing the quantity of solids wasted and vice versa.

A high sludge age is required for nitrifying bacteria which are retained in the aeration tank only if their reproductive rate is greater than their removal rate through sludge wasting and discharge in the final effluent. In operational terms nitrification can only be expected below a critical sludge loading F/M of 0.15 Kg/Kg/d, or a sludge age greater than four days (Metcalf and Eddy, 2003). The BOD/TKN ratio of the influent wastewater indirectly indicates the fraction of nitrifying bacteria in the MLVSS. Therefore, the lower the BOD/TKN ratio in the influent wastewater the greater the fraction of nitrifiers present in the MLVSS and the higher the degree of nitrification that can be expected.

1.1.2 The trickling filter

In attached growth processes, the wastewater flows through a packing medium sustaining the growth of a biofilm of attached microorganisms. Aerobic trickling filters (See Figure 1.1.b) are the most commonly used biofilm system, whereby the influent wastewater is distributed at the top of a filter containing non submerged packing material. Most modern trickling filters vary from 5 to 10 m in height and are filled with plastic packing material designed to create a 90-95% void space inside the reactor (Metcalf and Eddy, 2003).

The solids and excess biomass that sloughs periodically from the packed medium flows into a clarifier for solid/liquid separation before the sludge is collected at the bottom of the clarifier for waste sludge processing. Part of the sludge biomass may also be returned to the filter. In attached growth reactors, nitrification usually occurs in a separate tank from the tank used to remove most of the BOD from the wastewater, because heterotrophic bacteria have higher biomass yields than nitrifiers and quickly populate the surface area of fixed-film systems over nitrifying bacteria. Moreover, the BOD/TKN ratio of the effluent, resulting from a primary reactor which feeds a secondary reactor for being much lower, also favours the nitrifiers.

1.2 The nitrogen cycle

The element nitrogen is one of the constituents of amino acids and proteins. After carbon, which represents 50% of cellular dry weight, nitrogen represents about 15% of cellular dry weight content. Many forms of nitrogen exist in the environment, ranging from the most reduced form (-3) as organic proteinaceous material and ammonia (NH_3), to the most oxidized forms: nitrite (NO_2 ; +3) and nitrate (NO_3 ; +5). Nitrogen is the most abundant gas in the Earth's atmosphere (about 78%) present in its neutral state as dinitrogen gas (N_2). In order to become part of, and to be used along the global food chain; dinitrogen gas has first to be fixed (Sedlak, 1991; Barnes and Bliss, 1983).

The fixation of dinitrogen gas (Figure 1.2) may occur physically through lightning; be chemically induced through the Haber process, used to produce ammonia, which is ultimately the feedstock for other industrial processes (e.g. the fabrication of fertilizers, explosives and plastics); or be biologically mediated by nitrogen-fixing bacteria found associated with the root nodules of plants. Most prokaryotes use nitrogen in the form of ammonia or ammonium.

Ammonia is naturally produced through mineralization of organic nitrogen present in organic matter (ammonification) by extracellular biochemical action on dead plants, animal tissue, and animal faecal matter; and from dead and lysed cells (Barnes and Bliss, 1983).

Ammonia may be oxidized aerobically or anaerobically. In the aerobic process, known as nitrification, ammonia is firstly oxidized to nitrite, by two groups of prokaryotes: ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). Subsequently, nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB); see Figure 1.2 via steps 2 and 3. Ammonia may also be oxidized by other organisms (heterotrophic nitrification), including fungi, actinomycetes and other types of bacteria (Hirsch *et al.*, 1961; Verstraete and Alexander, 1972; Stroo *et al.*, 1986; Hayatsu *et al.*, 2008).

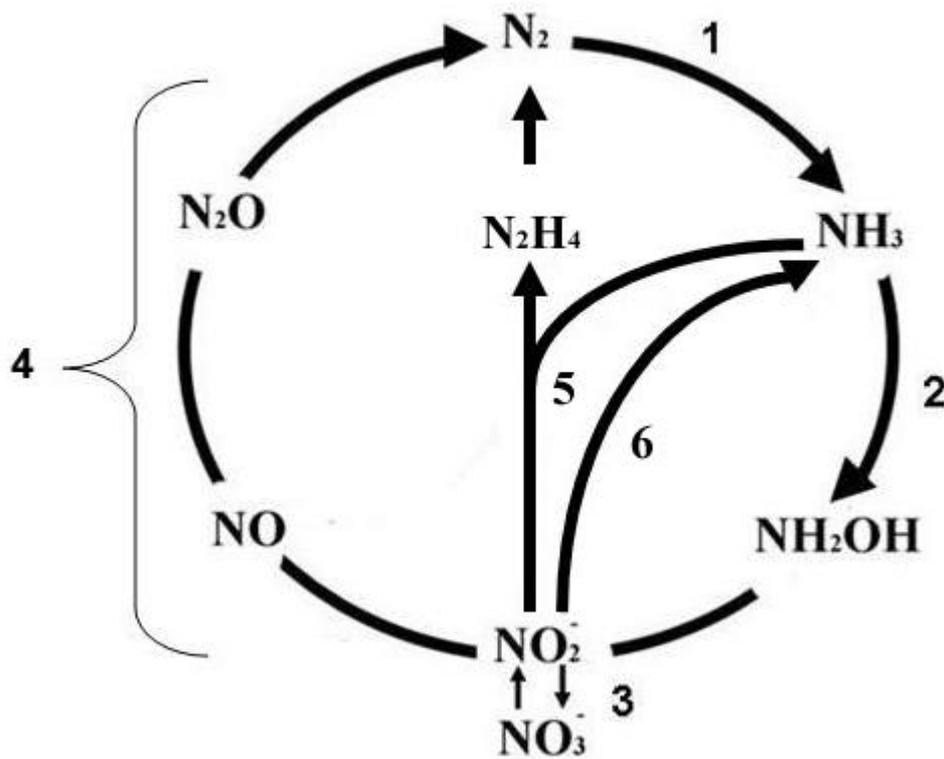


Figure 1.2 Microbial process reactions in the nitrogen cycle (1) Dinitrogen gas fixation (2) aerobic ammonium oxidation to hydroxylamine by bacteria and archaea (?) (3) aerobic nitrite oxidation (4) denitrification (5) anaerobic ammonia oxidation and (6) dissimilatory nitrate and nitrite reduction to ammonia. Adapted from Jetten, 2008.

Anaerobic oxidation of ammonia occurs through the action of chemolithotrophic bacteria, which were initially classified as a deep branch within the *Planctomycetes* phylum (Strous *et al.*, 1999b). Later, based on the genome studies of the uncultured anammox bacterium *Kuenenia stuttgartiensis* (Strous *et al.*, 2006) their phylogenetic position was revised and they were reclassified within the *Planctomycetes- Chlamydiae* superphylum.

In the anammox reaction (Kuenen, 2008), as shown on Figure 1.2, step 5, ammonia and nitrite are combined to form hydrazine, an important intermediate product, which is further converted to dinitrogen gas as follows:



Through denitrification, nitrate or nitrite are converted into dinitrogen gas (Figure 1.2, via step 4), with intermediates nitric oxide (NO) and nitrous oxide (N₂O), by several bacterial groups, archaea such as *Pyrobaculum aerophilum* and *Haloferax denitrificans* (Cabello *et al.*, 2004), and fungi such as *Fusarium oxysporum* and *Fusarium solani* (Bothe *et al.*, 2000; Hayatsu *et al.*, 2008; Fuji and Takaya, 2008). Recently denitrification was demonstrated to occur entirely in the benthic foraminifer *Globobulimina pseudospinescens* (Risgaard-Petersen *et al.*, 2006).

Dissimilatory nitrate and nitrite reduction to ammonia (Figure 1.2, via step 6) are performed by several bacteria in oxygen-limited conditions and dissipate excess reducing power, generate ammonia for assimilation or support anaerobic growth with nitrate or nitrite as electron acceptors. For example *Bacillus subtilis* has an aerobic metabolism, but can also sustain an anaerobic metabolism, reducing nitrate via nitrite to ammonia (Ye and Thomas, 2001).

1.3 Nitrogen removal processes in wastewater treatment systems

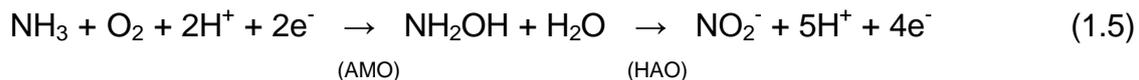
Nitrogen in domestic wastewater originates from protein metabolism in the human body, and thus organic compounds such as amino acids, proteins, and urea represent approximately 60% of nitrogen in fresh domestic wastewater, while ammonium ions represent around 40%. In the sewer system, ammonia is quickly converted to ammonium ions and urea is rapidly hydrolysed into ammonia and carbon dioxide; while proteins are firstly broken down into their amino acid constituents, and amino acids are deaminated with the formation of ammonia. By the time raw domestic sewage enters the treatment plant, 90% of the nitrogen is either present as ammonia or as unstable organic compounds that are readily transformed to ammonia. Overall organic and ammonia nitrogen concentration in municipal wastewater is typically found in the range of 25 to 45 mg/L (Metcalf and Eddy, 2003).

The removal of nitrogen in wastewater treatment plants is important because of water quality concerns in respect of the effect of ammonia in the receiving water, resulting from the depletion of dissolved oxygen (DO) concentrations and toxicity caused to fish and other aquatic organisms. In agricultural systems, nitrification stimulated by application of fertilizers based on inorganic ammonium or an organic nitrogen such as urea, may cause eutrophication from run-off and ground water pollution due to nitrate which is mobile in the soil and may be readily lost through leaching (Prosser, 1989). Furthermore, malfunction of septic tanks and the discharge of high levels of nitrate ions in the effluent of wastewater treatment plants may also contribute to nitrate pollution.

1.3.1 Nitrification

Nitrification is an aerobic microbial process that occurs in two steps. It is generally accepted that ammonia (NH_3) and not ammonium (NH_4^+) is the preferred substrate oxidized by ammonia-oxidizing bacteria (AOB). The concentrations of ammonium ions and ammonia in the treatment plant are dependent on the pH and temperature; and, for example, in the temperature range of 10° to 20° and the pH range of 7 to 8.5, which are typical of most reactors, 95% of ammoniacal nitrogen is present as ammonia (Prosser, 1989; Metcalfe and Eddy, 2003).

In the first step of nitrification (Eq. 1.5), ammonia is oxidized to hydroxylamine, an intermediate metabolic product, through the action of a membrane-bound, multisubunit ammonia monooxygenase enzyme (AMO) present in ammonia oxidizing bacteria (AOB) (McTavish *et al.*, 1993); and also in archaeal *Crenarchaeota* (AOA) (Hallam *et al.*, 2006b). Subsequently, hydroxylamine is oxidized to nitrite in the presence of a soluble enzyme located in the periplasm, hydroxylamine oxidoreductase (HAO) (Sayavedra-Soto *et al.*, 1994) as follows:



The conversion of ammonia to hydroxylamine is an endergonic reaction. The oxidation of hydroxylamine releases four electrons and two of these electrons are used to compensate for the electron input of the first reaction, whereas the other two are passed down an electron transport chain, generating a proton motive force.

In the second step of nitrification (Eq. 1.6), nitrite is oxidized to nitrate catalyzed by the enzyme nitrite oxidoreductase (NOR) present in nitrite-oxidizing bacteria (NOB) and uses oxygen supplied by water in the reaction:



The NOR enzyme is reversible and may carry out nitrate reduction (Prosser, 1989).

Nitrifying bacteria obtain their carbon from the fixation of inorganic carbon dioxide (CO₂) through the Calvin cycle. As little energy is obtained through the oxidation of inorganic chemical compounds, specific growth rates and growth yields of these bacteria are low in comparison to heterotrophic bacteria which obtain high energy gains through the oxidation of organic compounds and consequently higher growth rates and yields (Prosser, 1989). Both AOB and NOB growth rates and yields resulting respectively from ammonia and nitrite oxidation are low, and during stable nitrification conditions the rate of nitrite utilization is higher than the rate of ammonia oxidation by AOB, and consequently nitrite rarely accumulates.

The population size of nitrifying bacteria in wastewater treatment plants and bioreactors typically comprises only a very small proportion (1-10%) of the total microbial biomass. For instance, the rate of ammonia oxidation in activated sludge is approximately 0.5-3.0 mg/g/h, and 250 mg/g/h in pure culture (Prosser, 1989). Maximum specific growth rate values for both ammonia and nitrite oxidizers lies within the range 0.014-0.0064/h (equivalent to a doubling time of 50-11 hours; Prosser, 1989). Because nitrifiers grow much more slowly than heterotrophic bacteria, systems designed for nitrification require larger hydraulic and solids retention times than those systems designed only for BOD removal (Metcalf and Eddy, 2003).

Nitrifying bacteria are also sensitive to a number of environmental factors that may result in their being washed out of wastewater treatment systems. These factors include: toxicity due to the formation of free ammonia at high pH (9.4) or free nitrous acid generated from nitrite at pH lower than 6.0 (Anthonisen *et al.*, 1976); low dissolved oxygen concentrations; low temperature; organic matter; chemical inhibition by organic and inorganic compounds (e.g. heavy metals, phenol, sulphide and cyanide) and competition for ammonia and oxygen by heterotrophs; shorter hydraulic retention time; sludge age; BOD/TKN ratios; and deficiencies of key nutrients, for example, Ca, Mg and Cu for *Nitrosomonas* and Mo for *Nitrobacter* (Metcalf and Eddy, 2003; Gerardi, 2002).

Nitrification and nitrogen removal processes in municipal and industrial wastewaters (including practical examples) are discussed in detail by Sharma and Ahlert, 1977; Prosser, 1989; and Wiesmann, 1994; these authors provide a thorough review of the processes involved.

Nitrification has been investigated in a number of reactors of different configuration, both in lab scale and full scale treatment plants, to evaluate their performance and efficiency with respect to the removal of nitrogen compounds. Reactors with different configurations have been found to harbour different populations of AOB and NOB. Ammonia-oxidizing bacteria related to the genus *Nitrosomonas* (Juretschko *et al.*, 1998; Wagner *et al.*, 1998) from the *Betaproteobacteria*, have been observed as the most dominant AOB populations in most of the activated sludge plants and biofilms investigated. AOB typically form clusters or microcolonies inside flocs or biofilms (Wagner *et al.*, 1996) and, in general, are surrounded by clusters of NOB, in most cases from the phylum *Nitrospira*.

The participation of ammonia-oxidizing archaea (AOA) in the nitrification process was recently discovered (Könneke *et al.*, 2005); their role in nitrification seems to be significant in both oceans (Wuchter *et al.* 2006) and soils (Leininger *et al.*, 2006). However, several relevant aspects of their activity, metabolism, ecology and quantitative contribution to nitrification need to be elucidated in these environments (Nicol and Schleper, 2006; Francis *et al.*, 2007) as well as in wastewater treatment systems (You *et al.*, 2009) in ongoing research, and as the theme is new, they are just “beginning” to be understood.

1.3.2 Heterotrophic nitrification

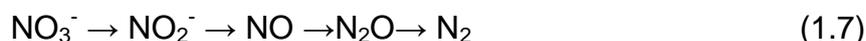
Heterotrophic nitrifiers include a wide range of fungi, actinomycetes and bacteria, and among these groups, fungi are the most efficient and most numerous, mainly occurring in acid forest soils (Prosser, 1989; de Boer and Kowalchuk, 2001; Hayatsu *et al.*, 2008).

Substrates for heterotrophic nitrification include ammonium, hydroxylamine, hydroxamic acids, amino or oxime nitrogen, nitrite, aliphatic and aromatic nitro compounds; and the products are nitrite, nitrate and a wide range of nitrogenous organic compounds. However, in contrast to chemoautotrophic nitrification, this process is not associated with energy production or heterotrophic growth and is considered as endogenous or secondary metabolism (Prosser, 1989).

Thiosphaera pantotropha (now renamed *Paracoccus denitrificans*) is the organism in which heterotrophic nitrification has been best studied for simultaneous ammonia oxidation and nitrate reduction (Robertson *et al.*, 1990) since these bacteria are both heterotrophic nitrifiers and aerobic denitrifiers. In competition experiments between *Nitrosomonas europaea* and *T. pantotropha* conducted at a range of dissolved oxygen concentrations and C: N ratios, *T. pantotropha* was able to out-perform *N. europaea* for ammonium at low dissolved oxygen concentrations and at high C: N ratios (>10). However, although a substantial amount of the ammonium was removed by the heterotroph; more ammonium was assimilated, reflecting the fact that heterotrophic biomass yields are much higher than autotrophic yields (Van Niel *et al.*, 1993; Jetten, 1997; Van Loosdrecht and Jetten, 1998). Therefore the rates of nitrification by heterotrophic organisms are low in comparison to those of autotrophic organisms. For example, reported values found for some heterotrophs (Jetten *et al.*, 1997) were as follows: *Pseudomonas aeruginosa* (12-28 nmol hydroxylamine /min.mg/dry weight); *Pseudomonas* sp. (24 nmol ammonia /min.mg/dry weight); *Alcaligenes* sp. (33 nmol pyruvic oxime/min.mg/dry weight); *Alcaligenes faecalis* (12-22 nmol ammonia/min.mg/dry weight); *Thiosphaera pantotropha* (35 nmol ammonia/min.mg/dry weight); and in contrast *Nitrosomonas* sp. oxidizing ammonia and hydroxylamine (130-1200 nmol N/min.mg/dry weight).

1.3.3 Denitrification and aerobic denitrification

Typically, processes used in wastewater treatment systems to remove nitrogen are based on nitrification, followed by denitrification. Biological denitrification or dissimilatory nitrate reduction is coupled to a respiratory electron transport chain, and nitrate or nitrite is used as an electron acceptor for the oxidation of a variety of organic and inorganic electron donors:



In the absence of DO or under limiting DO concentrations, the nitrate reductase enzyme is induced, and helps to transfer electrons to nitrate as a terminal electron acceptor. As shown in the equation 1.7, nitrate reduction to dinitrogen occurs in four sequential steps, mediated by four enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Schmidt *et al.*, 2003).

Denitrification requires an electron donor which can be organic material (e.g. biodegradable soluble COD in the influent; biodegradable soluble COD produced during endogenous decay; or from an exogenous source such as methanol or acetate) or reduced inorganic chemicals such as sulphide or hydrogen. Under electron donor limitation, intermediates produced during denitrification can be readily formed. One point of concern is the release of the gaseous intermediates, nitric- and nitrous oxide, from the treatment process into the environment, because they are recognized to increase the greenhouse effect as well as affecting stratospheric ozone (Gerardi, 2002). Zumft (1997) provides a detailed and complete review describing the cell biology and molecular basis of denitrification.

Denitrification may also occur under aerobic conditions, and the range of oxygen concentrations which allow this is variable and differs from one organism to another. For example, when *Thiosphaera pantotropha* (*Paracoccus denitrificans*) carries out aerobic denitrification, the majority of nitrite is directly converted to gaseous nitrogen products (Robertson and Kuenen, 1990; Schmidt *et al.*, 2003).

Nitrosomonas strains are able to denitrify and produce trace amounts of gaseous nitrogen compounds (N_2O , NO , or N_2) (Zart and Bock, 1998). Ammonia, hydrogen and hydroxylamine are also used as electron donors for nitrite reduction (Boettcher & Koops, 1994; Bock *et al.*, 1995). Although, N_2 has been measured as an end product in a number of studies (Poth, 1986; Beaumont *et al.*, 2004; Schmidt *et al.*, 2004), no genes encoding the enzyme nitrous oxide reductase were identified in any of the AOB genomes published up to date. Similarly, a clear defined metabolic pathway supporting the use of hydrogen as electron donor still remains to be determined since no hydrogenase genes were identified in the AOB genomes (Stein *et al.*, 2007; Arp *et al.*, 2007).

Nitrosomonas eutropha was shown to nitrify and denitrify simultaneously under fully oxic conditions. The denitrification activity of *N. eutropha* was induced and regulated by the presence of mixtures of the nitrogen oxides NO_2 and NO under oxic conditions (Zart and Bock, 1998; Schmidt *et al.*, 2001). At ratios of NO and NO_2 between 1000:1 and 5000:1, up to 50% ammonia conversion to NO_x compounds using nitrite as an electron acceptor was observed, while in their absence, denitrifying activity was reduced to only about 15% (Schmidt *et al.*, 2003).

1.3.4 Anaerobic ammonia oxidation (ANAMMOX) and new processes for nitrogen removal

Under anaerobic conditions ammonia is oxidized by these chemolithotrophic bacteria phylogenetically placed within the *Planctomycetes-Clamydiae* superphylum (Strous *et al.*, 2006) which use nitrite as an electron acceptor to produce nitrogen gas and a small amount of nitrate (about 1.3 moles of nitrite are used per mole of ammonia):



Anammox activity was first discovered in a denitrifying fluidized bed reactor treating methanogenic wastewater with sulphide as (limiting) electron donor (Mulder *et al.*, 1995; Van de Graaf *et al.*, 1995).

The “*Candidatus Brocadia anammoxidans*” was the first dominant anammox related bacterium to be physically isolated from enrichment cultures via Percoll density-gradient centrifugation (Strous *et al.*, 1999a), although because of their very low growth rate (doubling time 11 days) and growth yield (0.11 g VSS/ g NH₃-N), these bacteria have not yet been isolated in pure culture. Subsequently, molecular analysis surveys revealed that not only significant populations existed in wastewater treatment plants, mainly related to the genera *Brocadia*, *Kuenenia*, and *Scalindula* (Kuenen, 2008), but also that these bacteria are ubiquitously distributed in freshwater and in marine systems, where the ANAMMOX activity in the latter contributes for 30-50% of all marine N loss (Francis *et al.*, 2007).

The discovery that ammonia may be oxidized anaerobically, and that AOB may denitrify when in the presence of NO and NO₂ gases - has contributed to the design of novel biological wastewater treatment reactors for nitrogen removal from wastewaters containing high levels of ammonia and very low levels of organic carbon, such as, for example, concentrated anaerobic digester sludge liquor, landfill leachates, animal wastes, and composting sludge.

The new nitrogen removal processes (See Figure 1.3) known as ANAMMOX (anaerobic ammonia oxidation), SHARON (single reactor system for high rate ammonia removal over nitrite), CANON (completely autotrophic nitrogen removal over nitrite), OLAND (oxygen-limited nitrification and denitrification), and DEAMOX (denitrifying ammonium oxidation); basically involve the coupling of aerobic and anaerobic ammonia oxidation, and partial nitrification. Partial nitrification is reached when the nitrite oxidation step to nitrate is completely inhibited through a combination of operational factors that are used to selectively inhibit the growth of the nitrite oxidizers such as low oxygen, high temperature, sludge age control, and high pH.

ANAMMOX can occur in biofilm systems with very long retention times and no organic substrate is needed in the nitrogen removal process. Sequencing batch reactor (SBR) systems with granular growth have been successfully used to enrich efficient high biomass retention, and the very high affinity of these bacteria for ammonia and nitrite ($K_s < 0.1$ mg N/L) allows high maximum specific nitrogen consumption rate of 0.82 gN/g VSS.day. The highest observed capacity of ANAMMOX is 8.9 Kg N removed m³ reactor/day, (Kartal *et al.*, 2004).

In the SHARON (single reactor system for high rate ammonia removal over nitrite) process (Mulder *et al.*, 2001; Dongen *et al.*, 2001), ammonia is reduced by 50% through partial nitrification in one single aerated reactor without biomass retention, mainly conducted by *N. eutropha* (Logemann *et al.*, 1998); and the resultant products (50:50 ammonia and nitrite) are used to feed an ANAMMOX reactor linked in series to the SHARON reactor.

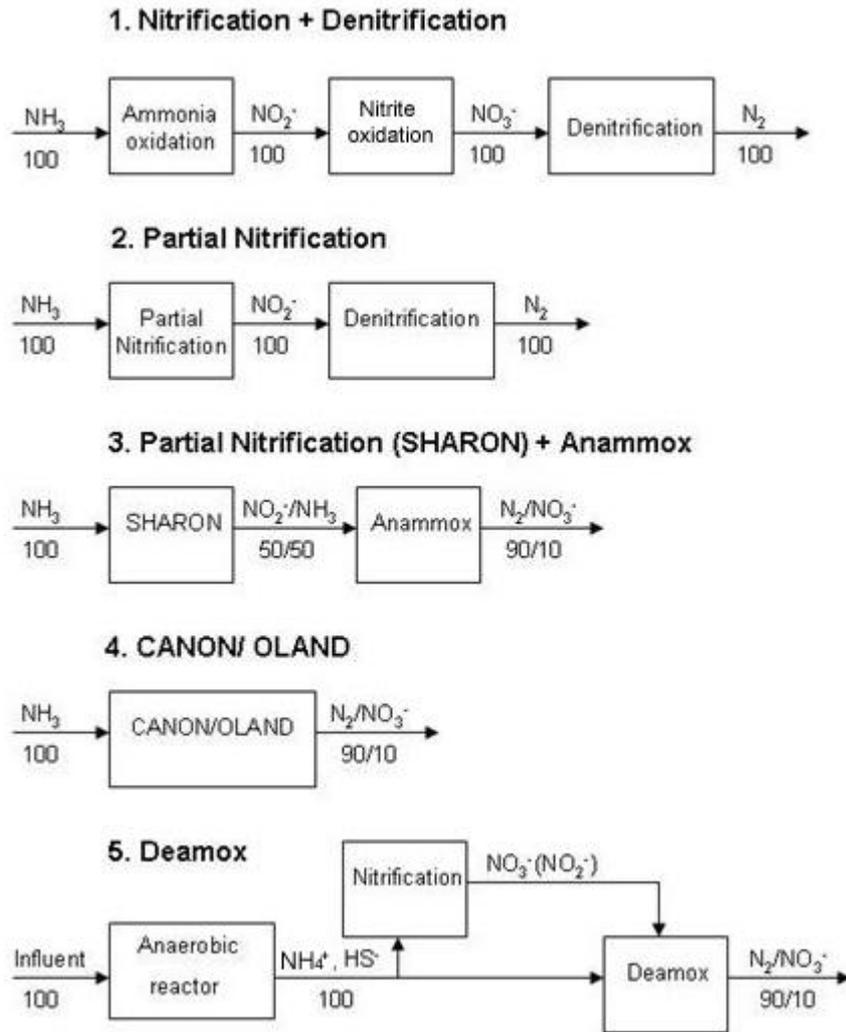


Figure 1.3 Flux diagrams of nitrogen removal processes: (1) conventional nitrification-denitrification; (2) partial nitrification (3) SHARON; (4) CANON and OLAND and (5) Deamox nitrogen removal processes. Values below arrows represent proportions of inorganic compounds. Modified from Schmidt *et al.*, 2003; and from Paredes *et al.*, 2007.

In the CANON (completely autotrophic nitrogen removal over nitrite; Third *et al.*, 2001; Sliemers *et al.*, 2003; Third *et al.*, 2005) and OLAND (oxygen limited autotrophic nitrification- denitrification system; Kuai and Verstraete, 1998) processes, partial nitrification, denitrification by *Nitrosomonas eutropha*, and anammox occur simultaneously within biofilm structures in a single reactor, under oxygen limited conditions.

Deamox (denitrifying ammonium oxidation) (Kalyuzhnyi *et al.*, 2006) is a new variation of the ANAMMOX process (Figure 1.3) in which denitrification of nitrate to nitrite (using sulphide as electron donor) and ANAMMOX are combined in the same reactor (Kalyuzhnyi *et al.* 2006). In short, an effluent rich in ammonia and sulphide is firstly produced in a pre-treatment step, which can be performed in various types of anaerobic reactors. Subsequently, the effluent is split into two flows, one partially fed to a nitrifying reactor to generate mainly nitrate and the other directly fed to the Deamox reactor, containing anaerobic ammonia oxidizing bacteria. Inside the Deamox reactor, sulphide reacts with nitrate and forms nitrite, and thus through anammox reaction, nitrite reacts with ammonia producing nitrogen gas and water. The theoretical $\text{H}_2\text{S}/\text{NO}_3^-$ ratio required in the Deamox reactor is 1:4 (on a molar basis) or 0.57 mg S- $\text{H}_2\text{S}/\text{mgN}-\text{NO}_3^-$. This type of process may be applicable to the treatment of food wastewaters, manure flushing, baker's yeast effluent, and sludge liquors.

In the literature these nitrogen removal processes have been extensively reviewed and some of them, for example, ANAMMOX or ANAMMOX combined with SHARON processes, have been successfully operated in full scale reactors (Jetten *et al.*, 2002; Schmidt *et al.*, 2003; Khin and Annachatre, 2004; Paredes *et al.*, 2007; Philips *et al.*, 2002; Peng and Zhu, 2006; Sinha and Annachatre, 2007). Furthermore, these processes are also considered more cost-effective and sustainable since they contribute to energy and resources savings, do not produce undesirable nitric and nitrous oxide gases into the atmosphere, respectively an ozone layer depleter and a potential greenhouse agent, and also generate much less sludge. For example, the use of the Anammox process would replace the conventional denitrification step completely, reducing operational costs of up 90%, and also would save half of the nitrification aeration costs.

1.4 Culture independent techniques in the investigation of wastewater treatment systems

Historically, the polymerase chain reaction (PCR) (Saiki, 1988) and the use of 16S rRNA ribosomal RNA (rRNA) as a molecular marker to infer phylogenetic relationships between microorganisms (Woese, 1987), were two remarkable scientific findings that revolutionized microbial ecology.

The 16S rRNA constitutes part of the ribosomes which are required by all organisms to synthesize proteins. The 16S rRNA gene contains approximately 1500 nucleotides, is well conserved across broad phylogenetic distances, there is limited evidence for horizontal transfer, and it has sequence domains that have evolved at different rates, that is, domains that have remained almost universally conserved across broad phylogenetic distances interspersed by more variable regions specific to a phylum up to subspecies. These characteristics give rise to 16S rRNA being used as a molecular marker to infer phylogenetic relationships between organisms, where these have homologous nucleotide positions which may be unambiguously aligned and compared (Röling and Head, 2005). By comparison with the more variable regions of the 16S rRNA genes, it is possible to design oligonucleotide primers and probes of distinct phylogenetic resolution from phylum to subspecies.

Comparative sequence analysis based on the functional *amoA* gene, encoding the alpha subunit of ammonia monooxygenase, has also been used as a molecular marker to investigate diversity and activity of ammonia oxidizing bacteria (Rotthauwe *et al.*, 1997; Aoi *et al.*, 2004). In a comparative study of all recognized species of AOB, it was demonstrated that there is a very good correlation between the evolutionary relationships inferred from comparative analysis of 16S rRNA and *amoA* sequences from AOB (Purkhold *et al.*, 2000).

More recently the *amoA* gene has also been used to access the diversity and functional activity of AOA (Treusch *et al.*, 2005; Francis *et al.*, 2005; Prosser and Nicol, 2008).

Culture independent techniques are important and fundamental tools in the investigation of wastewater systems. The combination between different molecular techniques has rendered important and valuable information in the understanding of a number of key organisms, which thus contributes to advancing knowledge about the ecology of biological wastewater treatment reactors.

To date a range of culture independent techniques have been applied to the investigation of wastewater treatment systems and bioreactors. In the literature there have been a number of comprehensive reviews regarding the state of the art in a range of relevant molecular techniques, as well as their drawbacks; some good examples are Amann *et al.*, 1995; Head *et al.*, 1998; Von Wintzingerode *et al.*, 1997; Røling and Head, 2005, Huang *et al.*, 2007; Wagner *et al.*, 2006; Neufeld *et al.*, 2007; Amann and Fuchs, 2008.

The following section provides a summary of the principles underlying the tool box of molecular techniques used in this study, and also introduces fundamental techniques commonly used for the identification, and quantification of target microorganisms in microbial studies (Figure 1.4).

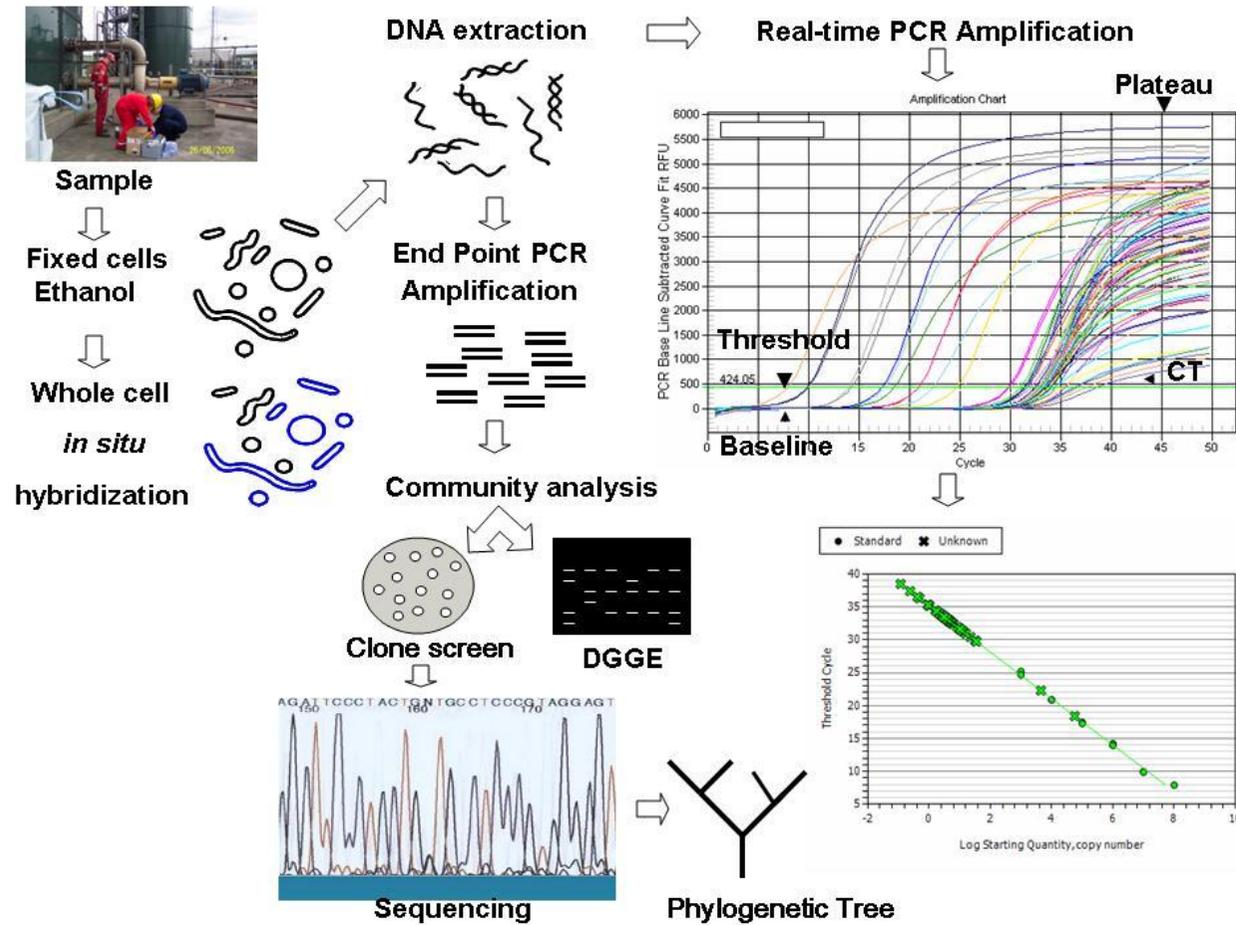


Figure 1.4 Molecular toolbox applied in this study. Modified from Head *et al.* 1998.

1.4.1 PCR, DGGE, cloning, sequencing and phylogenetic analyses

The polymerase chain reaction (PCR) and PCR-based analyses (Figure 1.4) of microbial communities start with a nucleic acid template preparation extracted from an environmental sample.

The PCR reaction occurs in an automated programmable thermal cycler in three phases: firstly the target DNA template is denatured at (94-96°C) to convert double-stranded DNA into single-stranded DNA; secondly oligonucleotide primers are annealed to the complementary priming sites in the target DNA (the temperature used for annealing the primers depends on the melting temperature of the primers and is determined empirically); and thirdly, the DNA is extended (68°-72°C) from the primers by the addition of the nucleotides through DNA polymerase activity, resulting in double-stranded DNA products. The repetitive cycling through these three steps results in an exponential increase in the DNA fragments being targeted (Röling and Head, 2005).

The PCR reaction occurs in three kinetic phases:

1- Exponential phase: corresponds to the early stages of the PCR reaction when all reagents are available and PCR products are being amplified at maximum reaction efficiency, and the doubling of PCR products (amplicon) occurs at every cycle.

2- Linear phase: occurs when some of the reagents in the PCR reaction are starting to be depleted, the reaction starts to slow down, and template replication efficiency on each cycle no longer renders exactly 2 amplicons, but rather amplicon degrades to 1.95 and gradually declines until it reaches the plateau phase.

3- Plateau phase or end point reaction: is reached when the reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.

One of the major limitations of PCR and PCR-based methods is that because PCR products are detected only in the plateau phase or the end-point of the PCR reaction, this includes variability introduced over the course of the overall reaction, and thus does not allow reliable quantification. For instance, even replicate samples starting out at the same quantity of DNA at the beginning of the reaction may end up with different PCR product yields. Therefore they can be used only as a qualitative or semi-quantitative method.

Some potential sources of bias to which PCR may be subjected include: preferential amplification of some templates, resulting from sub-optimal binding of the primer; primer specificity and primer efficiency; and competition between primer annealing and template reannealing during PCR amplification, particularly when primer annealing is at high stringency; contamination and inhibition of the PCR by substances co-extracted with the nucleic acids (humic acids); misincorporation of nucleotides by the Taq polymerase which increases with the number of PCR cycles and the length of the amplified fragment; and formation of chimeras (Head *et al.*, 1998; Von Wintzingerode *et al.*, 1997; Röling and Head, 2005).

A chimera is an rRNA gene fragment that results from the incomplete synthesis of a PCR fragment that for example might have annealed to a homologous rRNA gene fragment and formed a heteroduplex (the result of reannealing of denatured PCR fragments) sequence that was extended to full length and thus represents a complete rRNA sequence; however it is a false sequence that does not exist in any living organism.

Other problems that relate to the template and also may interfere are the template concentration, the different G+C contents, since the DNA of different microorganisms differ considerably in G+C content, and amplification of high % G+C templates may be discriminated against due to their lower efficiency of strand separation during the denaturation step of the PCR reaction. The number of rRNA operons in the microorganisms' genomes and genome sizes also vary as does rRNA sequence heterogeneity occurring within a single organism which can lead to an overestimation of diversity (Head *et al.*, 1998; Röling and Head, 2005).

PCR products obtained from environmental nucleic acids contain a mixture of rRNA genes from different organisms and they have to be separated before community analysis and diversity studies can be conducted. The separation of PCR-amplified DNA fragments is done through the use of molecular fingerprint techniques, such as, for example, DGGE (used in this study) and through cloning. DGGE is a method that allows a heterogeneous mixture of PCR amplified genes of the same length, but having different sequences to be separated by electrophoresis (Muyzer *et al.*, 1993). In this method samples are run vertically on a polyacrylamide gel containing a mixture of increasing concentrations of denaturants (formamide and urea). Because double stranded DNA fragments differing in their composition have different melting properties, they will stop at different positions on the gel. For instance sequences with high G+C content will be migrate further down the gel than low G+C content sequences. When the DNA stops on the gel a band is detected, and the sum of all bands on a sample represents a particular community structure profile.

One of the major limitations of DGGE is that it assigns a particular band to a particular organism when multiple gene fragments migrate to the same position but originate from different species. Other limitations are that the maximum length of the DNA that can be separated is low (500 bp) and although bands can be directly excised from DGGE gels for sequencing, they render less information in comparison, for example, with the sequence of cloned near full-length rRNA gene fragments, due to their limited length. Furthermore, only the most abundant species present in a sample will be represented on a DGGE gel (c.a. $\geq 1\%$ of the population). DGGE is relatively rapid to perform and one advantage is that many samples can be run simultaneously and compared. This method is also useful for studying the temporal variation of microbial populations when subjected, for example, to environmental perturbations (Head *et al.*, 1998).

Cloning and sequencing of PCR products, and subsequent comparative sequence analysis to determine the relationship of environmental sequences to cultured isolates and other sequences recovered from the environment, allows the heterogeneous population of rRNA PCR fragments in a sample to be characterized. Through cloning, single sequences are separated into individual *E. coli* clones in a clone library.

In short, PCR products are firstly mixed with a specially constructed vector. The TOPO- 4 TA commercial cloning vector kit for sequencing used in this study (Invitrogen) is a T-vector plasmid that when linearized has single deoxythymidine (T) residues at the 3' ends, suitable for cloning *Taq* amplified PCR products, because they have a single deoxyadenosine (A) at their 3' ends. The plasmid vector is supplied by the manufacturer already linearized and covalently bound to the tyrosyl residue (Tyr-274) of the Topoisomerase I enzyme on their phosphate groups attached to the 3' thymidine. The linearization of the plasmid is obtained by the cleavage of one of its DNA strands- on its phosphodiester backbone after the 5'-CCCTT motif- by the Topoisomerase I enzyme, and the energy resulting from the broken phosphodiester is conserved through the formation of a covalent bond between the DNA phosphate groups and the tyrosyl residue (Tyr-274) of the Topoisomerase I enzyme. During ligation with PCR products, the phospho-tyrosyl bond between the vector and the Topoisomerase is reversed and the Topoisomerase I enzyme relegates the ends of the cleaved vector strand to the *Taq* amplified PCR products and releases itself from the DNA. The constructed plasmid contains the lethal *E.coli* gene, *ccdB*, fused to the C-terminus of the *lacZ α* gene fragment, which encodes a potent cytotoxic protein. When a PCR product is ligated to the vector it disrupts the expression of the *lacZ α ccdB* gene fusion, and thus only the recombinants, i.e., those cells containing the vector and the inserted PCR product in the plasmid are able to grow upon transformation, whereas the cells containing the vector but not inserted PCR product are killed by the expression of the lethal *ccdB* gene.

Rapid screening of clones may be performed through colony PCR with primers that target plasmid-encoded priming sites flanking the cloned DNA and the cloned rRNA gene fragments.

Clones are then sequenced and the resultant sequences are compared to other 16S rRNA gene sequences available in a public database, to determine the identity of the organism and its phylogenetic relationship to known organisms. Through phylogenetic analyses, a phylogenetic tree may be constructed showing the evolutionary relationships of the sequences retrieved from the sample in relation to other sequences from cultured and uncultured organisms. As the most costly element of phylogenetic analyses is sequencing, this step also constrains the number of clones that are sequenced.

1.4.2 Real-time PCR

The real-time PCR or quantitative real-time PCR (qPCR) refers to DNA amplification which is monitored through the detection and quantification of a fluorescent reporter molecule signal, which is emitted at each PCR cycle and that accumulates in direct proportion to the number of PCR products formed through out the amplification reaction.

In a real-time PCR system, the PCR product (amplicon) is detected during the early exponential phase of the PCR reaction, when each molecule of DNA template being amplified produces exactly two amplicons; thus it allows for the initial DNA template concentration in a sample to be calculated with precision and accuracy. The increase in fluorescence is directly proportional to the increase in the amplified product during the PCR.

Fluorescence detection of DNA amplification is basically achieved through two types of assay: either by incorporation of a free dye, such as the SYBR[®] Green I, into the newly formed double- stranded DNA product; or by using a fluorescence probe detection system, whereby a probe is designed to anneal to a specific sequence of the DNA template between the forward and reverse primers, as for example in the 5' nuclease assay with the *Taq* Man probe.

The *TaqMan* probe is designed with a high energy dye, termed a reporter dye, at its 5' end; and a low-energy molecule, termed a quencher, at its 3' end. In the intact probe, as the two fluorescent dyes are kept in close proximity one from the other, they interact through electron excitation states transference, also termed fluorescence resonance energy transfer (FRET), in which the fluorescent emission from the reporter dye (the donor) in the 5' end of the probe is transferred to the quencher (the receptor) in the 3' end of the probe, and thus shifted to be released as light or as heat when excited by a light source. During DNA amplification, the probe is cleaved on its 5' end through the 5' exo-nuclease activity of the DNA polymerase enzyme, releasing the reporter dye into solution and stopping the FRET-related quenching to occur. When the fluorescent signal reporter increases to a detectable level it can be captured by the optical system of the thermo cycler instrument and displayed by the software as an amplification plot (See Figure 1.4). An amplification plot or amplification curve is defined as the plot of cycle number versus fluorescence signal which correlates with the initial amount of target nucleic acid during the exponential phase of PCR (Dorak, 2007).

The most important terms used in real-time PCR terminology, and fundamental to understanding how the real-time method works, are defined as follows (see also Figure 1.4 for illustration of these terms):

Baseline: refers to the initial cycles of PCR in which there is little change in the fluorescence signal (usually from cycles 3 to 15).

Threshold: is the numerical value or point of detection assigned for each run to calculate the Ct value (defined below) for each amplification plot. The threshold line is set in the exponential phase of the amplification for the most accurate reading. For this study the threshold was defined as 10 times the standard deviation around the average intensity of background fluorescence from no template control reactions. Therefore the greater the amount of initial template DNA, the earlier the fluorescence crosses the threshold and the smaller the Ct value will be.

C_T (threshold cycle): is the cycle number at which the sample fluorescence crosses the chosen threshold above the background fluorescence within the logarithmic increase phase, and it is inversely correlated to the logarithm of the initial copy number. The C_t is determined by both the chosen baseline setting and the chosen threshold setting (see graph in Figure 1.4).

The use of a standard curve based on known concentrations of DNA makes it theoretically possible to quantify DNA from any source. Hence a standard curve is obtained by plotting C_t values against log-transformed concentrations of serial tenfold dilutions of the target nucleic acid sample of known concentration (standard).

A standard curve using a defined amount of template should result in a slope, coefficient of determination (r^2) and y-intercept that demonstrate good efficiency, accuracy and sensitivity. This slope is used for efficiency calculation. Ideally, the slope should be 3.3 (3.1 to 3.6), which corresponds to 100% efficiency (precisely 1.0092) or twofold (precisely 2.0092) amplification at each cycle (Dorak, 2007).

In the literature, there is a lack of consensus regarding reporting of real-time experimental data. Recently, MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) (Bustin *et al.*, 2009; Bustin, 2010) have been published that recommend the reporting of sufficient experimental data and minimal standardized criteria for the assessment of quality in real-time PCR assays, which allows objective interpretation of results. Examples of experimental details to be included are: the coefficient of determination (R^2), used to assess the fit of the standard curve to the data points plotted; the PCR efficiency; the linear dynamic range, including the interval for the target template being quantified; primers specificities; the limit of detection (LOD) and the precision of the assay, besides other also relevant information regarding the samples themselves.

The determination of the limit of detection (LOD) of real-time assays is also a subject of debate and research. In most studies, no information is included about detection limits of real time assays or when they are included, it is common place to set up detection limits for the assay on the basis of negative controls or non template controls (NTC). However, the major constraint related to this approach is that when negative controls have “zero” concentration, Ct is undefined because of the logarithmic nature of the Ct values. Other examples include the use of the lowest Ct value resulting from a template of known concentration, originating from an organism of reference or from an environmental sample (Okano *et al.*, 2004; Leininger *et al.*, 2006); the use of the lowest template concentration, which might result in a Ct value significantly less than the total number of cycles performed ($\alpha = 0.05$); (Baldwin *et al.*, 2003), or even though the use of more sophisticated methods on the basis of computer simulation and modelling of Ct values versus template concentrations (Burns and Valdivia, 2008).

1.4.3 Whole cell fluorescence *in situ* hybridization (FISH)

Whole cell *in situ* hybridization techniques have been used for identification and absolute quantification, and spatial localization of specific microorganisms in environmental samples. In short, the FISH procedure involves five steps (Figure 1.5). First cells are fixed with denaturants and/or aldehydes to preserve the nucleic acids and cell morphology. In this study, samples initially fixed in ethanol by the time of sampling were subsequently fixed in paraformaldehyde. Following fixation, in a second step, cells are immersed in a hybridization buffer in suspension (the method used in this study) or attached to gelatine coated slides containing added fluorescently-labeled oligonucleotide probes that diffuse into the cells to bind to their complementary rRNA sequences on the ribosomes. Following the hybridization step, cells are washed with buffer to remove unbound probes.

Afterwards cells are mounted on gelatine-coated microscope slides for visualization by microscopy, for example, using a confocal laser scanning microscope (CLSM); see Figure 1.5. Microbial cells containing probes bound to their ribosomal 16S RNA fluoresce when excited with light of an appropriate wavelength, thus allowing the cells to be identified and quantified.

The optimal temperature and hybridization conditions for a newly designed probe must be determined empirically, by testing different concentrations of formamide in the hybridization buffer, conducted at a single temperature. The *daime* software specifically designed for image analyses (Daims *et al.*, 2006a) contains tools which allow images to be taken during assays of probe optimization, and may be easily and quickly analysed for selecting the best formamide concentration and temperature, that which produces the highest-intensity fluorescence signal when the probe fully matches to the complementary target rRNA, while not giving a signal with non-target cells. The probeBase website (Loy *et al.*, 2003) is a useful online database containing a complete description and relevant information about all published rRNA-targeted oligonucleotide probes (<http://www.probebase.net>).

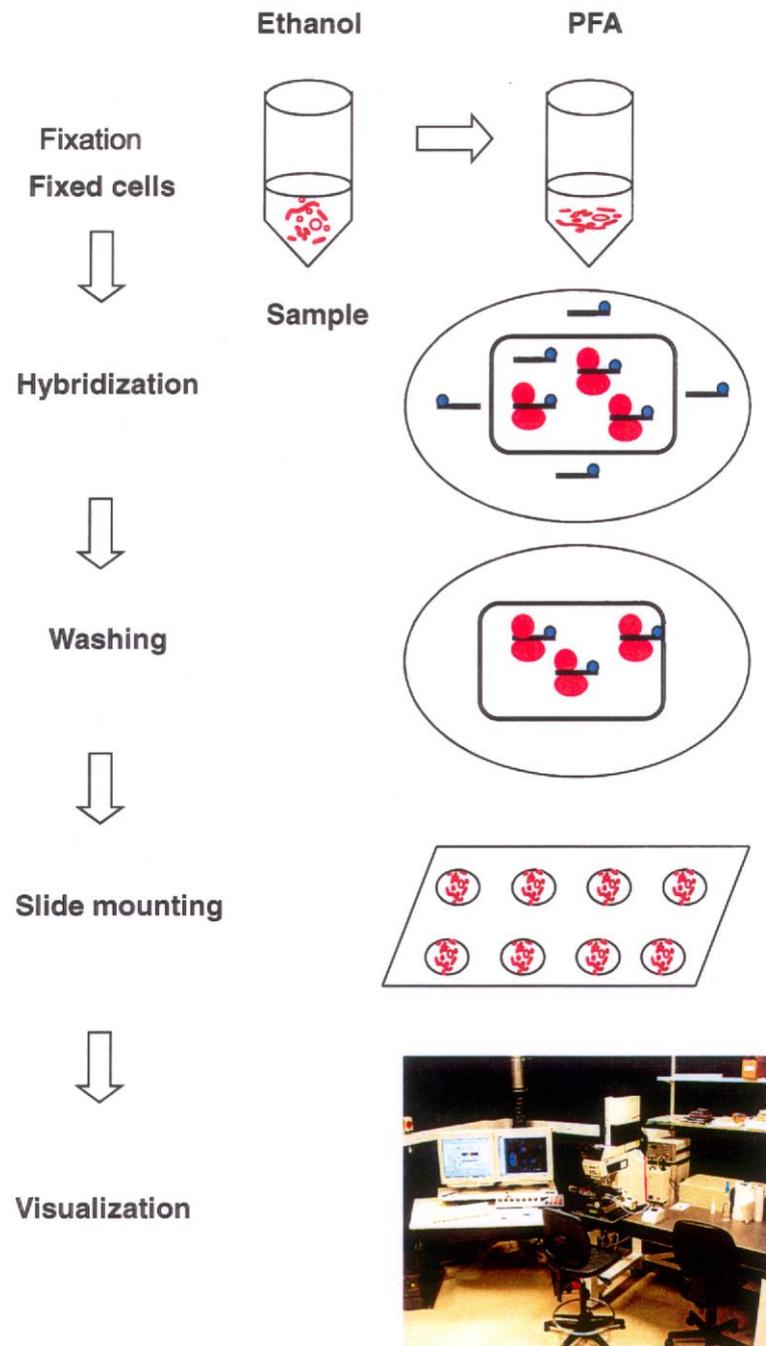


Figure 1. 5 Steps in the FISH (fluorescence *in situ* hybridization) procedure

There are four major limitations to which FISH is subject: cell permeability, target site accessibility, target site specificity and target site sensitivity. Cell permeability is generally achieved during fixation with alcohols (denaturants) or paraformaldehyde (cross-linking reagents). However, some cells may be impeded in their hybridization because the probes could not penetrate them. For example, some Gram-positive bacteria which have rigid cell wall structures need additional treatment with acid, solvents or enzymes (Davenport *et al.*, 2008). Once inside the cell, probes may be impeded in binding to their targets because of difficulties in accessing certain regions on the 16S rRNA ribosome, which may have highly stable secondary structure within the rRNA itself or be strongly linked to ribosomal proteins (Fuchs *et al.*, 1998). Probes may also bind to non-targeted organisms if they have poor specificity (several mismatches) in relation to their genuine target organisms, or if hybridization conditions are suboptimal (for example, too low a binding temperature). In general, probes containing a single labeled molecule give a strong signal only if cells are metabolically active and, hence, contain large numbers of ribosomes and target rRNA (Amann *et al.*, 1995; Head *et al.*, 1998; Moter and Göbel; 2000).

CARD-FISH (catalysed reporter deposition-fluorescence *in situ* hybridization) is a method that was developed to increase FISH sensitivity. It allows smaller; and less brightly-fluorescing cells to be detected. In the CARD-FISH method, single-cell identification by FISH using horseradish-peroxidase (HRP)-labelled oligonucleotide probes are combined with catalyzed reported deposition (CARD) of fluorescently labelled tyramides. When the probe linked to the enzyme HRP binds to its target within the cell, it catalyzes the oxidation of the fluorochrome labelled tyramide substrate, provoking its deposition in the vicinity of the enzyme (due to its covalent binding to the electron-rich proteins moieties within the target cells) and as a result, numerous fluorescent molecules are radicalized, producing a strong tyramide signal amplification (TSA) on the probe-target bindings sites (Amann and Fuchs, 2008).

A number of improvements have been made to the FISH technique since it was first applied for cell identification (DeLong, 1999). Improvements include the use of permeabilization treatments, the development of different types of probes, and probes combined with enzymes as in the CARD-FISH (Wagner *et al.*, 2003), in addition to numerous combinations of FISH with a range of other advanced techniques aiming to link phylogeny to activity and function in complex microbial communities. Some examples are the combination of FISH with analysis using microelectrodes to link identity and microbial activity in biofilms (Schramm *et al.* 1998; Schramm *et al.*, 1999; Okabe *et al.*, 1999), CLSM (confocal laser scanning microscopy) and digital image analyses (Wagner *et al.*, 1994; Daims *et al.*, 2006a) which allow not only cell identification and counting, e.g. in activated sludge flocs and biofilm structures, but also allow their morphology, spatial localization, arrangement and distribution pattern within flocs and biofilms may be visualized and analysed using digital image analysis.

FISH combined with microautoradiography (MAR-FISH) allows the measurement of radiolabelled substrate uptake from the medium on a single cell level (Lee *et al.*, 1999). More recently, the scope of MAR-FISH was improved to render it quantitative (Wagner *et al.*, 2006) and the method was combined with microelectrodes (Gieseke *et al.*, 2005) and stable isotope probing (Ginige *et al.*, 2004). The developments in FISH and more advanced stable isotope probing techniques such as Raman spectroscopy (RAMAN-FISH (Huang *et al.*, 2007) and other isotope-based techniques (Wagner, 2004; Wagner *et al.*, 2006; Neufeld *et al.*, 2007) have enabled multiple environments, substrates, and target organisms to be simultaneously monitored in complex microbial communities.

Therefore independent molecular techniques are fundamental and powerful tools for the study of the microbial communities in wastewater treatment plants and will continue to greatly advance knowledge about the function and ecophysiology of a number of important key microorganisms in biological wastewater treatment systems. In a broader sense, they will also contribute to the generation of more quantitative data that might be linked to process stability, which will further result in better process management and control of wastewater treatment plants.

1.5 Oil refinery wastewaters

1.5.1 Oil refining and oil refinery processes

Oil refineries may vary in size, age and complexity and consume water for several purposes: for example, cooling, steam generation, direct processing such as stripping, the dilution of chemicals, washing, sanitary, domestic and miscellaneous uses, as well as fire fighting. Due to the availability of large quantities of salt water, most UK refineries are found in coastal locations, often on estuaries (Cobb, 1980).

An oil refinery performs the “process” of converting crude oil into more readily usable energy packages, sometimes together with petrochemical feedstock and non-energy products such as sulphur, bitumen and solvents. Basically, NSO components such as pyridines, thiols and phenols are extracted from crude oil, which is composed mainly of hydrocarbons, but also includes other complex organic compounds. This is followed by the separation of the pure hydrocarbons into a number of fractions within a distillation unit, in order to produce by-products such as solvents, gasoline, diesel oil, heating oil, lubricants and asphalt (Atlas, 1984).

The need to provide a full range of oil products, in the proportion and quantities that the market demands, dictates the level of complexity of an oil refinery on the basis of the number and types of processes of separation, conversion, and the treatments that are being used. For example, in more complex refineries, besides distillation, catalytic cracker units which change the size and structure of the hydrocarbon molecules are required, in order to break down heavy oil fractions and increase the proportion of short chain alkanes for gasoline production (CONCAWE, 1979; CONCAWE, 1987).

Oil pollution from coastal refineries and offshore installations is controlled by the directive on Integrated Pollution Prevention and Control IPPC, 96/61/EC. This directive targets integrated pollution control of emissions into air, water and land, arising from a number of industrial activities, via the use of the best available technology and environmental practices. Hence modern refineries are designed with the goal of achieving minimal water consumption by applying air and recirculating water for cooling purposes, selecting processes which generate minimal quantities of wastewater, and reduce as much as possible, or even eliminate, contact of water with hydrocarbon streams.

Wastewater in oil refineries originates from several sources and varies according to the quantity and degree of contamination. The wastewater contains oil, the major contaminant in the wastewater being composed of a range of different hydrocarbons and other organic compounds present in the crude oil and chemical substances produced by the refinery process such as sulphides, mercaptans, cyanides, ammonia, phenols, inorganic salts, and traces of some heavy metals. Other contaminants that are not generated in the petroleum refining operations but also can be present in the wastewater include additives used for blending, conditioning agents for raising steam and cooling water, and products used in the control of equipment corrosion.

The major water flow contributions to the wastewater treatment plant units within oil refineries may arise from the follow sources (Blokker, 1971; CONCAWE, 1979; Cobb, 1980; CONCAWE, 1987):

- Ballast water from ships and product tankers, arriving at the refinery in ballast. Ballast water may represent a significant contribution for a coastal/estuarine refinery and may indeed have to be handled as a separate facility;
- Water originating from the process units such as: drainage of the bottom of crude and product tanks, crude oil washing, oily condensates from steam stripping, steam products from the distillation and chemical conversion units, and sour waters;

- Cooling water. Cooling water is not normally contaminated with oil, except in the event of heat exchanger tube failure. However, the wastewater of a cooling tower may contain low concentrations of conditioning chemicals;
- Rainwater run-off from paved areas in the processing units or the product loading stations, due to small spillages;
- Sanitary sewage.

Therefore the performance and degree of complexity of the wastewater treatment plant designed to treat refinery wastewaters will depend on the quantity and the degree of contamination of the wastewater, and even on the location of the discharge, that is, whether it is to a river, estuary or the sea.

Sour waters in particular represent the worst source of chemical contamination to the wastewater, and in most cases they are separated from the other sources of water and their release into the biological unit are controlled by dilution. Sour waters are the by-product of conversion processes such as cracking and hydrogen treatment of oils containing significant amounts of sulphur and nitrogen compounds, one part of which ends up as hydrogen sulphide and ammonia, and the other part of which leaves the plant as a water solution. Sour waters also may contain variable amounts of oil (in dissolved, suspended or emulsified state) dissolved inorganic salts and dissolved organic compounds, such as light hydrocarbons, phenols, thiophenols and other oxygenated products like organic acids (Blokker *et al.*, 1971). A significant amount of the wastewater is contributed by the desalters, vessels in which oil coming from the field is washed, in order to remove dispersed inorganic salts.

Physicochemical treatments of the wastewater in refineries include gravity separation of the oil from the water, followed by flocculation or flotation of emulsified oil prior to the biological treatment. Gravity separators are designed to remove floating oil by skimming, and yield oil that is recycled into the refining process, as well as water free of floating oil but still containing emulsified oil, which is then removed by flotation enhanced by chemical coagulants in dissolved air flotation units. Activated sludge is the most commonly used system to biologically treat refinery wastewaters.

The ecology of nitrification in activated sludge treating synthetic oil refinery wastewaters was investigated for the first time through culture-independent molecular techniques (Ballinger *et al.*, 1998) in a denitrifying-nitrifying laboratory reactor (DNB). This study clearly showed a high diversity of rRNA gene sequences related to *Nitrosomonas* spp. and also, a small number of sequences related to *Nitrospira* spp. However, molecular techniques have not to date been applied to investigate the composition and numbers of ammonia oxidizers and nitrite oxidizers in full scale oil refinery wastewater treatment plants. It is expected that this investigation can bring new insights into this type of wastewater and the bacterial processes involved, aiming to bring new knowledge and understanding to the process of nitrification.

1.6 Aims and objectives

Culture-independent techniques have been successfully applied in the investigation of wastewater treatment systems, and consequently better understanding and knowledge has been gained about the microbial ecology of those systems. Furthermore the use of molecular tools has resulted in the accumulation of qualitative and quantitative data on important key organisms, with the potential for contributing to better control of wastewater treatment systems. These data may be related to process parameters and integrated into mathematical models.

While considerable knowledge of the microbial ecology of wastewater treatment systems in general has been accrued in recent years, biological systems for the treatment of refinery wastewaters are largely unexplored and little is known about the key microorganisms responsible for several important processes in refinery wastewater treatment systems. There is therefore a gap in our knowledge of the microbial ecology of the biological treatment of oil refinery wastewaters. The application of culture-independent techniques to study nitrification in these systems is therefore likely to yield a new understanding of the organisms responsible and their abundance in relation to their nitrification performance. This may ultimately lead to better understanding of nitrification and improve nitrification efficiency in refinery wastewater treatment systems.

Recently, ammonia oxidizing Archaea (AOA) were demonstrated to take part in nitrification (Könneke *et al.*, 2005), and to contribute to nitrification in the marine environment and soils (Wuchter *et al.*, 2006; Leininger *et al.*, 2006). The first evidence of AOA in wastewater treatment plants was given by Park *et al.*, 2006, and subsequently they were shown to have a minor presence in a highly aerated full-scale activated sludge plant (Wells *et al.*, 2009).

It is therefore of interest to know whether putative AOA are present in oil refinery wastewater treatment systems and the extent of their contribution to nitrification in refinery wastewater treatment systems. On the other hand, little is known about which NOB are present in oil refinery systems, and in what abundance.

For instance, recent investigations of nitrite oxidizing bacteria (NOB) through culture independent techniques showed *Nitrospira* (Daims *et al.*, 2000) and not *Nitrobacter* to be the predominant nitrite oxidizer in wastewater treatment plants, whereas it was long thought that *Nitrobacter* was the most significant NOB-like organism carrying on nitrite oxidation in those systems. Therefore in face of the lack of knowledge in the literature in regard to nitrification and the main nitrifiers involved in the nitrification occurring in oil refinery wastewater treatment plants, the aim of this study was to investigate the nitrification process in full-scale oil refinery biological wastewater treatment plants using culture independent techniques, with the following specific objectives: to

- ❖ identify and quantify AOB present in oil refinery wastewater treatment plants;
- ❖ investigate the presence and abundance of putative AOA in refinery wastewater systems;
- ❖ investigate whether AOB and AOA numbers relate to nitrification performance; and whether they can be integrated in a model for nitrogen removal (Rittmann *et al.*, 1999);
- ❖ investigate the presence and abundance of nitrite-oxidizing bacteria (NOB) in oil refinery wastewater treatment plants.

Chapter 2

Methodology

2.1 Wastewater treatment plants (WWTPs) location, bioreactor configurations and sampling procedures

Five oil refinery wastewater treatment plants, one trickling filter and four activated sludge plants, located respectively in South Humberside, Cheshire, Pembroke and Grangemouth (Figure 2.1) were sampled.



Figure 2.1 Oil refineries sampled in this study identified by a circle
 Source: Petroleum Institute

Oil refinery wastewaters contain substantial amounts of residual oil and chemicals from the distillation and cracking process involved in oil refining. Oil refinery wastewater treatment systems include a primary treatment for oil removal through API type separators, which are physical systems designed by the American Petroleum Institute to remove oil and gross suspended solids from the water surface by density. Following this step, the wastewater is subjected to chemical flocculation or air flotation, and then proceeds to an equalization tank, in which homogenization and buffering of shock loading influent flows are promoted before proceeding for biological treatment, which is also defined as secondary treatment. The biological treatment basically comprises two reactors, in general operating in parallel, each one connected to one clarifier. Clarifiers are sedimentation tank units, used to separate and settle the suspended solids present in the effluent stream coming from the biological treatment, before it is released to receiving waters, as well as to recycle solids back to the bioreactor tank.

Samples for this study were collected from the equalisation tank, (considered as influent) and from inside the bioreactors, as well as from the outlet (effluent) of their respective clarifiers.

To illustrate how a wastewater treatment system in an oil refinery operates, two diagrams are presented, representing the two oil refineries sampled for this study: respectively the trickling filter wastewater treatment system of the Lindsey refinery (Figure 2.2) and the activated sludge wastewater treatment system of the Humber refinery (Figure 2.5).

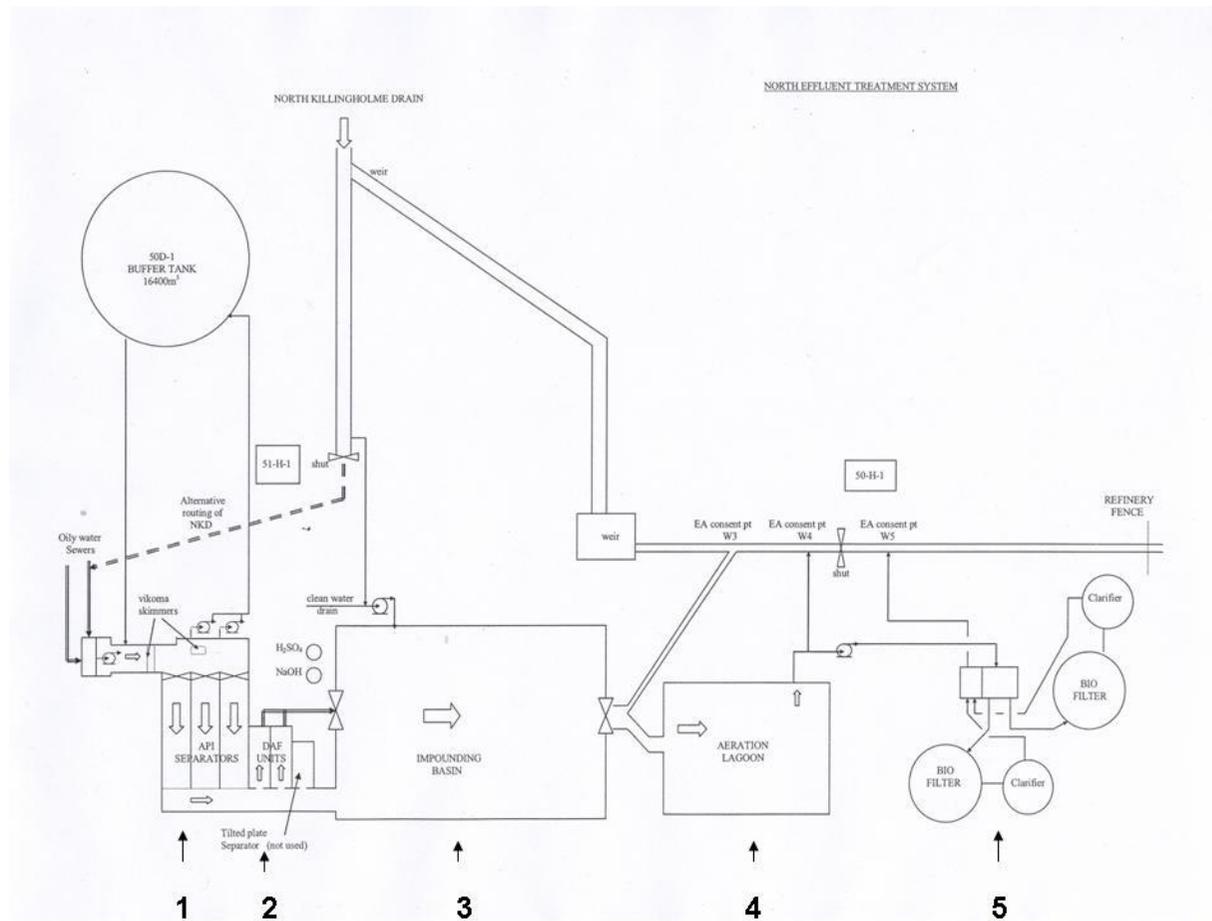


Figure 2.2 Lindsey refinery wastewater treatment plant layout, showing the operation units through which the waste stream is routed until its treatment is complete: 1- API separators used to remove oil from water surface by density; 2- dissolved air flotation unit in which the pollutants are removed from the wastewater by coagulation and precipitation chemical reactions; 3 – equalisation tank in which wastewater flows are homogenized and pH is corrected; 4– aeration tank where extensive aeration is promoted to reduce high levels of organic carbon loading; 5- biological treatment unit composed of two trickling filters operating in parallel, and their respective clarifiers.

2.1.1 Trickling filter sampling

In all plants sampled, two bioreactors were run in parallel and triplicate samples were collected from each of the reactors. In the trickling filter system, in the Lindsey refinery, samples were collected from the top of each reactor (Figure 2.3 a).



Webpage: <http://www.energyinst.org.uk>

(a)

Pack Media



(b)

Figure 2.3 Lindsey refinery aerial view and filter medium: a. trickling filter reactors (indicated by arrows); b. pack medium. The X symbol in the Figure shows the packed medium surfaces from which the biofilm was scraped.

Each filter was filled with a packing medium (Figure 2.3b) composed of plastic polyurethane structures designed to favour the growth of biofilm on their surface. The procedure adopted to collect the biofilm is described as follows:

Three replicate pieces of plastic packing medium were collected from each bioreactor using a hook, and the biofilm attached to the external surface of the medium was scraped off into a sterile Petri dish using sterile toothbrushes, and transferred to 50 ml sterile Universal plastic bottles with polypropylene screw caps (Bibby- Sterilin Ltd., Staffordshire, UK). The bottles contained 20 mL of ethanol solution 50 % (v/v).

Two sampling visits were carried out at this plant in September and October 2005, respectively.

2.1.2 Activated sludge sampling

Besides the trickling filter system described in the section 2.1.1, four activated sludge plants were sampled in this study and each one had a different design and size (Figure 2.4). For example, activated sludge plant 1, located in Cheshire (Figure 2.4 a) is a small plant treating wastewater from a refinery processing very heavy oils with a high bitumen and asphalt content. The bioreactor configurations allowed both reactors to be fed in parallel, or working in series, depending on the organic loading present in the influent to be treated. At the time of sampling, the bioreactors were working in series. The second activated sludge plant, located in Pembroke, Wales (not illustrated) also contained two reactors which operated in parallel, but instead of tanks, they comprised open activated sludge lagoons. When this plant was sampled, only one of the clarifiers was in operation, due to operational problems. Thus, at this plant, samples were collected from influent, from inside each bioreactor, and from the clarifier outlet (considered as final effluent). The third activated sludge plant was located in South Humberside, and samples were collected from influent, from inside the two bioreactors operating in parallel and from the outlet from both clarifiers (See Figure 2.4 b).

At this plant, the effluent from clarifiers proceeds to a large lagoon that, in addition to effluent from the treatment plant, receives all surface water and rain water from the plant, before being released into the adjacent river.

In the fourth activated sludge plant, located in Grangemouth, samples were also collected from influent, from inside each bioreactor running in parallel and after having passed through each clarifier (Figure 2.4 c). At this plant, the wastewater flow from each clarifier outlet was combined and the effluent was released into the river.

Grab samples were collected in triplicate for both culture-independent microbial community analysis and wet chemistry analysis. For culture-independent microbial community analysis, a 20 ml sample was collected from inside each reactor and transferred to 50 ml sterile Universal plastic bottles with polypropylene screw caps (Bibby- Sterilin Ltd., Staffordshire, UK) containing 20 ml of absolute ethanol. Samples for DNA extraction were collected in separate tubes from samples collected for fluorescence *in situ* hybridisation (FISH). For wet chemistry analysis, polyethylene bottles with a 500 ml capacity (Nalgene®, VWR International, Leicestershire, UK) were filled with sample. Samples collected from inside each reactor were also used for analysis of suspended solids and volatile suspended solids. Samples were kept at 4°C during transportation to the lab and samples collected for FISH analysis were fixed in 4% paraformaldehyde solution immediately on return to the laboratory. Samples for culture-independent microbial community analysis were stored at -20°C.

Each WWTP, including the one trickling filter plant and the four activated sludge plants, is referred to in the text by the name of its location as follows: Lindsey, Eastham, Pembroke, Humber and Grangemouth. In some Figures they are referred to by the first letter of their names, and in cases in which samples were collected from the same plant, but different period of times are being compared, they are either referred by their dates or referred to by the numbers 1, 2, 3 or 4, representing the order in which the sampling occasions occurred. The oil refinery wastewater plants sampled and the number of samplings carried out in this study are summarised in Table 2.1.

Table 2.1 Sampling of oil refinery wastewater treatment plants in the UK

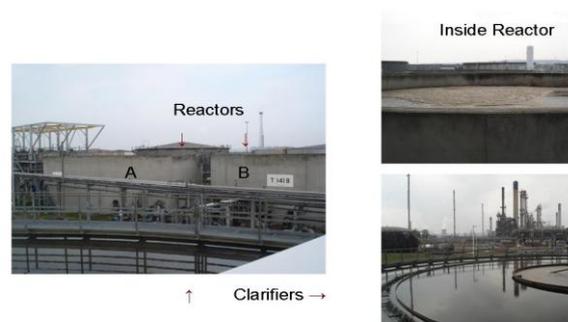
Plant	Points sampled	Number of sampling visits	Sampling dates
Lindsey	Influent, After Filters A and B	2	07.09.2005 07.10.2005
Eastham	Influent, Inside reactors A and B and after sedimentation tank (clarifier)	1	01.06.2006
Pembroke	Influent, Inside reactors A and B, after clarifiers A and B, and final effluent	1	09.06.2006
Humber	Influent, Inside reactors A and B, after clarifiers A and B and final effluent	4	21.06.2006 16.10.2006 16.11.2006 08.01.07
Grangemouth	Influent, inside reactors A and B, after clarifiers A and B, and final effluent	3	26.07.2006 28.02.2007 12.04.2007



(a)



(b)



(c)

Figure 2.4 Activated sludge systems from the (a) Eastham (b) Humber, and (c) Grangemouth refineries sampled in this study, illustrating their specific designs. Through the system in (a) reactors can operate in series, or in parallel.

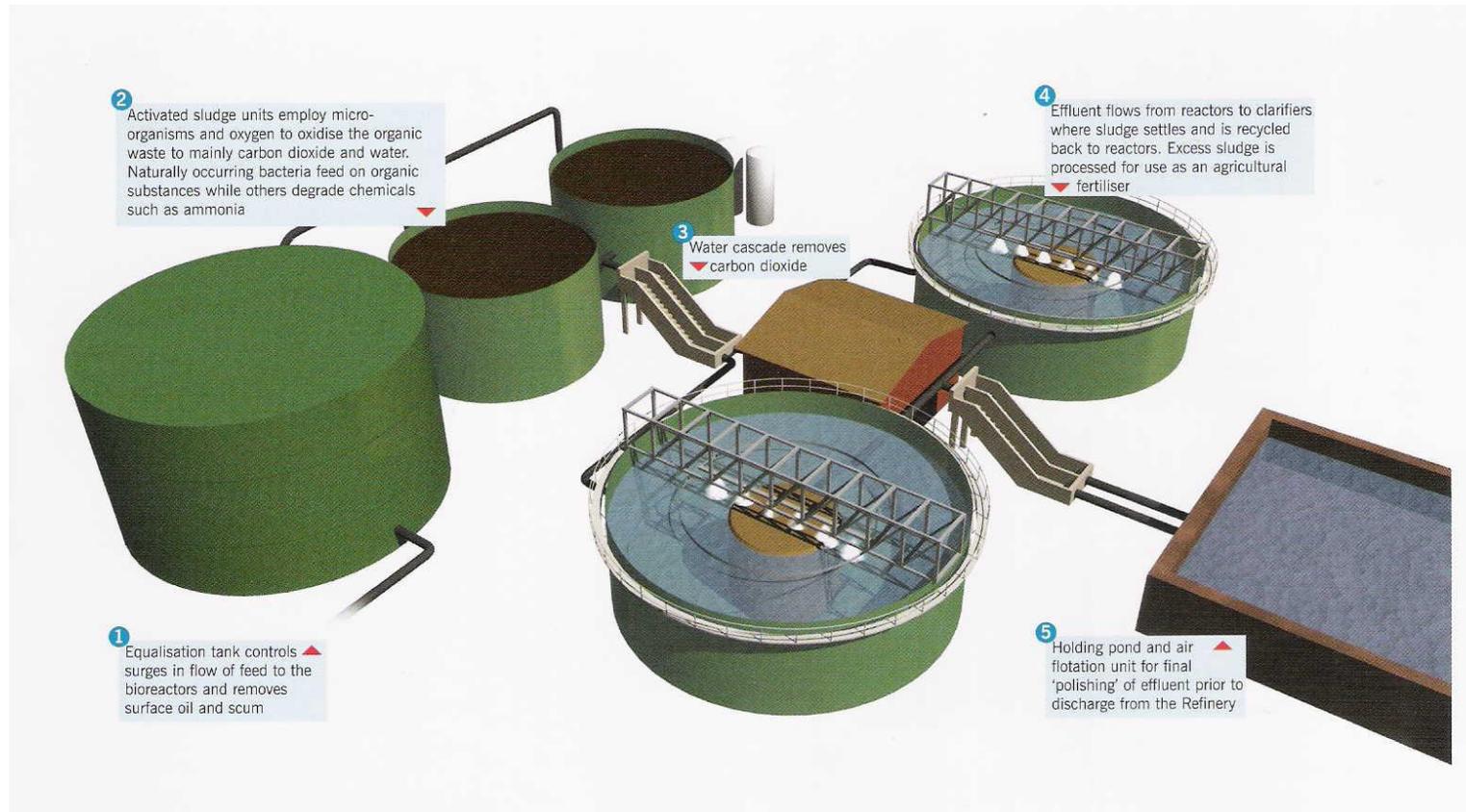


Figure 2.5 Humber refinery wastewater treatment plant layout. Source: Humber oil refinery

2.1.3 Large scale screening of WWTPs for the presence of putative archaeal ammonia oxidizers

The finding of Archaea that participate in nitrifying processes is relatively recent (Könneke *et al.*, 2005; Francis *et al.*, 2005).

The occurrence of ammonia oxidizing Archaea (AOA) in wastewater treatment systems was reported for the first time in five municipal wastewater treatment plants in the USA by Park and colleagues (2006).

In order to investigate if AOA were present in oil refinery wastewater treatment systems as well as in other wastewater treatment plants in the UK, all samples from this study and another 34 samples collected from 23 municipal WWTPs including a pilot reactor from a tannery (sampled for other studies by Rheanne Pickering and Joana Baptista) were screened by PCR with archaeal *amoA* primers developed by Francis *et al.* (2005) (see section 2.5.2) and for all samples where AOA were detected, *amoA* gene fragments were cloned and analysed by real-time PCR.

Table 2.2 Process descriptions of 23 municipal wastewater treatment plants (Pickering, 2008) and one pilot reactor treating tannery wastes surveyed in this study for the presence of putative AOA

Plants	Sampling date	Reactor description	Reactor Volume (m ³)
Atherstone	16.09.2004	Small nitrifying activated sludge	1655
Barnhurst	29.09.2004	Large nitrifying activated sludge	12633
Shardlow (Biffa)	22.11.2004	Nitrifying SBR ¹ for landfill leachate	628
Burton	16.09.2004	Pilot plant intermittent brewery waste	1.9
Burton	22.11.2004	Pilot plant intermittent brewery waste	1.9
Coalport	28.09.2004	Medium nitrifying activated sludge	10820
Coleshill	07.10.2004	Large nitrifying activated sludge	65076
Derby	16.09.2004	Large nitrifying EBPR ²	60000
Eccleshall	29.09.2004	Nitrifying oxidation ditch	2030
Finham	16.09.2004	Large nitrifying activated sludge	56304
Goscote	07.10.2004	Large nitrifying activated sludge	15200
Leek	07.10.2004	Non-nitrifying Vitox [®] activated sludge	4327
Loughborough	09.09.2004	Medium nitrifying activated sludge	11888
Minworth	07.10.2004	Large nitrifying activated sludge	260300
Newark	22.11.2004	Large nitrifying activated sludge	260300
New Wanlip	09.09.2004	Large nitrifying activated sludge	25132
Old Wanlip	09.09.2004	Large non-nitrifying activated sludge	49817
Packington	16.09.2004	Nitrifying oxidation ditch	13671
Rushmore	28.09.2004	Medium nitrifying activated sludge	13090
Stoke Bardolph (new)	09.09.2004	Large nitrifying activated sludge	4844
Stoke Bardolph (north)	09.09.2004	Large nitrifying activated sludge	9688
Stoke Bardolph (south)	09.09.2004	Large nitrifying activated sludge	9688
Stoke Bardolph (south)	28.09.2004	Large nitrifying activated sludge	44361
Stoke Bardolph (south)	28.09.2004	Nitrifying oxidation ditch	962
Strongford	04.12.2004	Pilot activated sludge treating tannery wastes	2
Wheaton Aston			
Northampton BLC			

¹SBR= Sequencing Batch Reactor

²EBPR= Enhanced Biological Phosphate Removal

2.2 Measurements in the field

During sampling, pH, conductivity, temperature and dissolved oxygen were measured *in situ* in samples removed from reactors using handmeters. An Ultrameter II model 6P pH meter from Myron L Company (Camlab Ltd, Cambridge, England) was used to measure pH, conductivity, and temperature. Before sampling, the meter was calibrated for pH and conductivity using commercially available standard solutions from Fisher Scientific UK Ltd (Leicestershire, England). Standard solutions of pH values 4, 7 and 10 were used to calibrate pH and a standard solution of value 1413 micro siemens/cm was used to calibrate conductivity.

Dissolved oxygen was measured using a dissolved oxygen (DO) meter YSI 550A from Hydrodata Ltd (Letchworth, UK). The meter was calibrated by using an automatic push-button option in which oxygen was measured in mg/L saturation, and both salinity and altitude were set to zero.

2.3 Wet chemistry analysis

Chemical analyses were performed in the laboratory immediately after sampling (less than 24 hours) following the standard methods, 20th edition (APHA, 1998).

2.3.1 Chemical oxygen demand (COD)

The method of closed reflux in HACH COD reactor vials was conducted.

With this method the quantity of organic carbon present in samples is measured through chemical reaction in which a strong oxidant (potassium dichromate) is used in excess to digest the sample at high temperature (150°C) in the presence of an acidic medium (sulphuric acid) and catalyser (silver sulphate).

The oxidant reacts with organic matter that can be oxidized, transforming it into CO₂ and H₂O. After sample digestion, the excess oxidant is titrated with ferrous ammonium sulphate in order to calculate the total quantity of oxygen consumed during oxidation of the organic matter to carbon dioxide and water, which is measured as equivalent amount of oxygen proportional to the quantity of potassium dichromate consumed.

To each reaction, a volume of 2 mL of potassium dichromate (0.075 N) containing mercuric sulphate (33.3 g/L) and 3.5 mL of a silver sulphate/sulphuric acid solution (5.5 g of silver sulphate/kg of sulphuric acid) were added to 2 mL of samples contained in a HACH COD vial (VWR International, Leicestershire, UK). Two blanks containing 2 ml distilled water were also included in the assay. The tubes were tightly capped, mixed several times and placed in a heated reactor block (Grant Instruments Ltd., Cambridge, UK) for two hours at 150°C. After that, samples were removed and allowed to cool to room temperature and afterwards, titrated with 0.025 N ferrous ammonium sulphate (FAS). The titration end point reaction was determined by using ferroin indicator (ferrous 1, 10 - phenanthroline sulphate) which produces a sharp change of colour from blue-green to reddish-brown when all the dichromate is reduced by ferrous ion. One mL of 0.25N solution of dichromate is equivalent to 2 mg of oxygen.

The COD calculation is expressed in mg/L and is given by the following equation:

$$\text{mg/L COD} = \frac{(A - B) \times C \times 8000}{\text{mL sample}} \quad (2.1)$$

where:

a= mL FAS used for blank

b= mL FAS used for sample

c= normality of ferrous ammonium sulphate

8000= milliequivalent weight of oxygen x 1000 ml/L

2.3.2 Biochemical oxygen demand (BOD)

Through BOD, the biodegradable organic carbon present in samples is determined by measuring the oxygen consumed during organic carbon oxidation through the action of microorganisms (the seed) inoculated into the samples to be measured.

Seeded dilution water was prepared by adding 20 mL/L each of the following solutions: phosphate buffer solution (8.5g KH_2PO_4 ; 21.75 g K_2HPO_4 ; 33.4 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 1.7 g NH_4Cl /L distilled water; pH 7.2), magnesium sulphate (22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /L distilled water), calcium chloride (36.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ /L distilled water), ferric chloride (0.25 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ /L distilled water), ATU, nitrification inhibitor (10g 2-chloro-6-pyridine/L distilled water), and 2 mL of settled sewage (the seed) to 20 L of distilled water that was saturated with oxygen, by aeration at room temperature for at least 3 hours before initiating the assay.

Samples were diluted with the seeded water in 2 litre volumetric flasks and transferred to four BOD bottles (VWR International, Leicestershire, UK).

The dilution required for preparing each sample was calculated on the basis of their COD concentrations by considering that the expected BOD concentration would be approximately half the COD concentration.

The oxygen concentration present in one of the four bottles from each sample was quantified to determine the initial oxygen concentration just after preparation of samples for the BOD assay while the other three remaining bottles were kept incubated at 20°C for 5 days.

The oxygen concentration in the samples was measured by titration using the Winkler method. Through this method 1 mL of manganous sulphate solution (480 g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ /L distilled water) followed by 1 mL of alkaline iodide azide solution (composed by 500 g NaOH, 150 g potassium iodide, and 10g sodium azide/L distilled water) were added to each sample. Bottles were then shaken and left to settle, followed by the addition of 1 mL concentrated H_2SO_4 , shaken more one time and left to settle. Using a measuring cylinder, 100 mL of sample were transferred into a conical flask and titrated with N/80 sodium thiosulphate until a pale straw colour was obtained. Then, a few drops of starch indicator solution (containing 10 g starch and 1 g salicylic acid/500 mL distilled water) were added to the flask and titration was continued until the blue colour disappeared. Each 1 mL of N/80 sodium thiosulphate is equivalent to 1 mg DO/L.

The remaining oxygen in the incubated bottles was determined at the end of the incubation period, and the difference between the initial quantity of oxygen and the oxygen quantity after 5 days of incubation was calculated as follows:

$$\text{BOD (mg/L)} = \frac{(D1 - D2) - (B1 - B2) f}{P} \quad (2.2)$$

where:

D1 = DO of diluted sample just after preparation

D2 = DO of diluted sample after incubation

P = decimal fraction of dilution of sample

B1 = DO of seeded blank just before incubation

B2 = DO of seeded blank after incubation

f = Ratio of seed in diluted sample to seed in seeded blank

2.3.3 Organic Kjeldahl nitrogen (TKN)

The Total Kjeldahl Nitrogen (TKN) method was used to determine the total nitrogen present in samples given by the sum of total organic nitrogen and ammonia nitrogen forms.

Samples used for TKN determination were first digested using sulphuric acid as oxidant in excess at high temperature (340°C) in order to degrade all the organic nitrogen and release ammonia in solution in the form of $(\text{NH}_4)_2\text{SO}_4$.

Volumes of 10 mL from each sample were placed in individual digestion tubes to which were added 14 mL concentrated sulphuric acid and two Kjeltabs (1000- Kjeltabs CK neutralization tablets; Thompson & Cooper Ltd., Cheshire, UK). The Kjeldahl tablet (of composition 3.5g K_2SO_4 / 0.4g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) is a catalyst added to facilitate the digestion reaction of the organic nitrogen. A reagent blank was also included in the assay.

Samples were digested using a digestion system (Turbotherm TTG25, Gerhardt, Bonn, Germany) coupled to a Turbosog Tur/TUK Scrubber Unit (Gerhardt, Bonn, Germany) for removal of aggressive acid fumes.

After digestion the tubes were left to cool and placed in a distillation system (Vapodest -30 Gerhardt, Bonn, Germany) together with a 250 mL conical flask, containing 50 mL containing borate buffer (9.5 g di-sodium tetraborate plus 8.8 mL 1 N NaOH/L distilled water).

The distillate concentration was determined by titration with N/50 H_2SO_4 up to a pale lavender colour endpoint, as follows:

$$\text{mg/L TKN} = \frac{(A - B) \times 0.28 \times 1000}{\text{mL sample}} \quad (2.3)$$

where:

A= volume H_2SO_4 titrated for sample

B= volume H_2SO_4 titrated for blank

0.28= conversion factor required when using N/50 H_2SO_4 acid solution to 1.0 mg of nitrogen equivalent

2.3.4 Ammonia

Ammonia concentrations in samples were determined as follows: volumes of 50 mL sample or reagent blank were firstly placed in digestion tubes. A few drops of phenolphthalein indicator and 3 mL borate buffer solution (9.5 g di-sodium tetraborate plus 8.8 mL 1 N NaOH/L distilled water) were added and pH was adjusted to above 8.3 using N/50 or N/10 NaOH solution.

The same procedures described in Section 2.3.3 for distillation, titration and ammonia calculation were performed for ammonia determination.

2.3.5 Chloride, nitrite and nitrate determinations

Anion analysis of nitrite (NO_2^-), nitrate (NO_3^-) and chloride (Cl^-) were performed by Ion Chromatography using a Dionex DX-100 ion Chromatograph (Dionex Corp., Sunnyvale, USA).

Anion standard solution (containing 5mg/L fluoride; 10mg/L chloride; 15 mg/L nitrite; 25 mg/L nitrate; 40 mg/L phosphate, and 30 mg/L sulphate) along with 5 mL filtered and ten fold diluted samples were transferred to autosampler vials. Samples were injected through an AS 40 automated sampler into the mobile phase eluent (of composition 1 mmol/L NaHCO_3 - 0.8 mmol/L Na_2CO_3) carrying the samples through the system at 1mL/min flow rate. In this system, anions are separated after passing through an ion exchange analytical column (Pac AS14 A); after passing through a conductivity suppressor, a semi-permeable membrane which reduces the background conductivity from the eluent and allows the sensitive detection of anions in the sample, their conductivity was detected through a conductivity cell detector. The conductivity cell transmits the signal produced by each anion to a data collection system (Chromeleon) which identifies the ions based on their retention time. Ion concentrations are automatically determined by integrating their peak area or peak height, and the sample peaks are compared to the peaks produced by the standard solution. Anion values were reported in ppm.

2.3.6 Salinity

Salinity was calculated based on chloride anion concentrations and expressed as a percentage by using the follow equation (Wooster *et al.*, 1969):

$$S = \text{Cl (ppt)} \times 1.80655 \quad (2.4)$$

where:

S = salinity
ppt = part per thousand

2.3.7 Suspended solids (SS), and volatile suspended solids (VSS)

Samples for solids determination were taken from influent, from inside reactors and after passing through the clarifiers. The term 'mixed liquor' is specifically used to refer to samples taken from inside activated sludge bioreactors and their suspended solids determination is known as 'mixed liquor suspended solids' (MLSS).

Suspended solids refer to the fraction of solids retained on specified glass fibre filters, prepared before analyses as follows: GF/A filter papers (Whatman filter, VWR International, Leicestershire, UK) were dried in an oven (Gallenkamp Hotbox Oven Size 2, Fisher Scientific, UK) at 104°C for 15 minutes, cooled in a desiccator, dried to constant weight at 550°C in a muffle furnace (Carbolite Furnaces, Sheffield, UK) for 10 minutes, and after cooling filters were weighed to the nearest 0.1 mg using an analytical balance (Mettler AJ150, VWR International, Leicestershire, UK) and their initial weights recorded.

Samples volumes of 10 mL were placed over the pre-prepared GF/A filter papers and filtered through a system of filtration set up with a suction flask, membrane filter and funnel (VWR International, Leicestershire, UK). After that filters were removed and left to dry for one hour at 104°C, cooled in a desiccator and their weight reported in mg/L.

Volatile suspended solids refer to weight loss on ignition. Thus to calculate the volatile solids fraction of samples, the difference between the weight of filter plus residue before ignition and after ignition was determined by igniting the weighed filters with dried residue in a muffle furnace at 550°C for 15 minutes.

The following calculation was performed to determine the solids concentration of samples submitted to either suspended solids or volatile suspended solids determination:

$$\text{mg/ L} = \frac{(A - B) \times 1000}{\text{Sample volume, mL}} \quad (2.5)$$

where:

A = weight of filter + dried residue, mg, and
B = weight of filter, mg

2.4 DNA extraction

With the exception of the Lindsey WWTP, in which a volume of 200 μl of biofilm sample was extracted, different volumes of samples were taken from each activated sludge plant in order to standardize the MLSS for all activated sludge plant samples (around 4000- 5000 mg/L) prior to DNA extraction. Samples were then centrifuged and resuspended in a volume of 250 μl of molecular grade water prior to DNA extraction.

Volumes of 978 μl sodium phosphate buffer and 122 μl ML (detergent) buffer solutions were added to samples and after that samples were physically lysed using a Ribolyser (Hybaid Ltd, Middlesex, UK) for 30 s at speed 6.5 m/s. DNA samples were extracted using the BIO 101 FastDNA spin kit for soil (Q-Biogene, UK), following the manufacturer's instructions. Basically, the FastDNA spin kit contains a lysing matrix, a mixture of ceramic and silica particles, designed to efficiently extract DNA of all bacteria, yeast, algae, nematodes and fungi, including cells that are hard to break, for example spores, with minimum shearing of the nucleic acids. The kit also includes detergents and salt solutions specifically used for DNA extraction, as well as reagents used for DNA purification and elution.

2.5 Polymerase chain reaction (PCR)

PCR was carried out in a thermal cycler (G-Storm GS1; GRI Ltd, Essex, UK). Reaction volumes of 50 μ l were set up in 0.2 ml PCR tubes containing: 39.3 μ l of sterile molecular biology-grade water, 5 μ l of 1 x NH_4^+ Buffer (160 mM $(\text{NH}_4)_2\text{SO}_4$, 670mM Tris-HCl (pH 8.8)), 0.1% Tween-20 and no Mg^{2+} , Bioline, London, UK); 1.5 μ l of 50 mM MgCl_2 (Bioline, London, UK), 1 μ l of a mixture of 10 mM deoxyribonucleoside triphosphates (dNTPs), 1 μ l forward and reverse primers (each 10 pmoles/ μ l), 0.2 μ l of BiotaqTM DNA polymerase, and 1 μ l of template DNA. A positive control, using DNA template from an environmental sample in which the target DNA had previously been found or from a cloned PCR-amplified gene from the target microorganism and a negative control, containing only the PCR reagents mentioned above, but no DNA template, were also included in each set of PCR reactions.

2.5.1 Amplification of 16S rRNA gene fragments of ammonia oxidizing bacteria (AOB)

Broad specificity bacterial primers pA and pH (Edwards *et al.*, 1989) were firstly used to amplify bacterial 16S rRNA genes, giving a PCR fragment of ca. 1500 bp. Following that, a nested PCR reaction was set up using specific primers CTO189f and CTO654r (Kowalchuck *et al.* 1997) to amplify the 16S rRNA gene of betaproteobacterial ammonia oxidizing bacteria from Lindsey, Humber and Grangemouth refinery WWTPs. Samples from the Eastham and Pembroke refinery WWTPs, by contrast, only required a single round of PCR with CTO primers to obtain a visible product by agarose gel electrophoresis. These primers amplify a fragment size of 465 bp. The forward primer CTO189f refers to an equimolar mixture of three different primers, named respectively CTO189fA, CTO189fB and CTO189fC. All primers and PCR conditions used in this study are summarised in Table 2.3.

Table 2.3 Primers and PCR conditions used in this study

Primers	Sequence (5'- 3')	PCR conditions	Purpose	Reference
pA	AGA GTT TGA TCC TGG CTC AG	10 min 95°C	General primers used to amplify bacterial 16S rRNA genes	Edwards <i>et al.</i> , 1989
pH	AAG GAG GTG ATC CAG CCG CA	30 cycles: 1 min 95°C, 1 min 55°C, 1min 72°C 10 min 72°C		
CTO189f:	GGA GRA AAG CAG GGG ATC G	3 min 95°C	Nested primers used to amplify betaproteobacterial ammonia oxidizers 16S rRNA genes after PCR amplification with primers pA- pH	Kowalchuck <i>et al.</i> , 1997
CTO189A-B	GGA GGA AAG TAG GGG GAT CG	30 cycles: 1 min 95°C, 1 min 57°C, 1 min 72°C		
+ CTO189C	CCG CCG CGC GGC GGG CGG GGC GGG	10 min 72°C		
CTO189f-clamp(underlined)	GGC ACG GGG GGA <u>GRA</u> AAG YAG GGG ATC G			
CTO654r	CTA GCY TTG TAG TTT CAA ACG C			
Arch- <i>amoA</i> F	STA ATG GTC TGG CTT AGA CG	5 min 95°C	Archaeal monooxygenase <i>amoA</i> gene amplification	Park <i>et al.</i> , 2006
Arch- <i>amoA</i> R	GCG GCC ATC CAT CTG TAT GT	30 cycles: 45 s 94°C, 1 min 53°C, 1 min 72°C 15 min 72°C		
pUCf	GTT TTC CCA GTC ACG AC	10 min 95°C	Primers used to check cloned PCR insert products in <i>E.coli</i> plasmid DNA	
pUCr	CAG GAA ACA GCT ATG AC	30 cycles: 1 min 95°C, 1 min 57°C, 1 min 72°C 10 min 72°C		

R= G or A; Y = C or T; K= G or T; S= G or C

2.5.2 Amplification of ammonia monooxygenase genes (*amoA*) from Bacteria and Archaea

Primers *amoA* F1 and *amoA* F2 (Rotthauwe *et al.*, 1997) were used to amplify ammonia monooxygenase genes from bacterial ammonia-oxidizers (Table 2.3). Primers Arch-*amoAF* and Arch-*amoAR* (Park *et al.*, 2006) were used to amplify ammonia monooxygenase genes from putative crenarchaeotal ammonia-oxidizers (Table 2.3).

2.5.3 Agarose gel electrophoresis of PCR products

PCR products were analysed on agarose gels (1 % w/v agarose in 1xTAE buffer (40 mM Tris acetate, 2mM EDTA pH 8.0) containing 2 µl of ethidium bromide (0.15µg/ml)). Lanes on the gel were loaded with 5 µl of PCR product mixed with 2 µl of loading buffer (0.25% (w/v) bromophenol blue; 40% (w/v) sucrose in filter sterilized water) and electrophoresis was conducted at 100 volts for 50 minutes. After electrophoresis, the gels were visualized by UV transillumination using a Fluor- S ® Multilmager (Bio-RAD, Hercules, CA, USA). PCR product size was estimated in comparison with a PCR molecular weight 0.5-2.0 kb marker (Sigma, UK), loaded on the same gel in order to check if a fragment of the expected size was amplified.

2.6 Denaturing gradient gel electrophoresis (DGGE)

2.6.1 DGGE analysis of 16S rRNA gene fragments from ammonia oxidizing bacteria

Volumes of 11 μ L of PCR products obtained with CTO primers, 1:1 with loading buffer (0.25% (w/v) bromophenol blue; 40% (w/v) sucrose in filter sterilized water), were loaded on 5 % (w/v) polyacrylamide gels (37:1 acrylamide: bisacrylamide), prepared with denaturant solutions ranging from 40% to 60% (100% denaturant is 7 M urea, 40% v/v formamide in 1 x TAE Buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0)). Electrophoresis was carried out on a D-Gene Denaturing Gradient Gel Electrophoresis System (Biorad, Hercules, CA, USA) filled with 1xTAE buffer at 60°C at a constant voltage of 200 V for 4.5 hours. After electrophoresis, the gels were stained with SYBR green I (Sigma, Poole, UK; diluted 1:10000 in 1xTAE, 30 μ l/300 ml buffer) for 30 minutes, and the DNA bands were visualised by UV transillumination using a Fluor- S[®] Multimager (Bio-RAD, Hercules, CA, USA).

2.6.2 DGGE analysis of *amoA* gene fragments from putative ammonia oxidizing Archaea

The PCR products amplified by primers Arch-*amoA*F and Arch-*amoA*R were run on 8% polyacrylamide gels prepared with denaturant gradient solutions ranging from 15% to 55% (100% denaturant is 7 M urea, 40 % formamide in 1 x TAE Buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0)) and electrophoresis was conducted at 75 V for 16 hours. These DGGE conditions were developed by Graeme Nicol (personal communication) from the University of Aberdeen.

After electrophoresis, the gels were stained with SYBR[®] green I (Sigma, Poole, UK; diluted 1:10000 in 1xTAE, 30 μ l/300 ml buffer) for 30 minutes and the DNA bands visualised by UV illumination using a Fluor- S[®] Multimager (Bio-RAD, Hercules, CA, USA).

2.6.3 Numerical analysis of DGGE profiles

DGGE gel images were converted, normalized and analyzed, using the gel analysis software Bionumerics (Applied Mathematics, St. Martens-Latem, Belgium).

Densitometric curve similarities between all pairs of DGGE profiles on gel were numerically compared by using the Pearson Product Moment Coefficient and resemblance values were calculated to generate a resemblance matrix. The matrix was then subjected to clustering using the unweighted pair-group method with arithmetical averages (UPGMA) clustering algorithm and a dendrogram showing the relationships among DGGE profiles was generated.

2.7 Cloning PCR-amplified betaproteobacterial ammonia oxidizer 16S rRNA and archaeal ammonia monooxygenase (*amoA*) gene fragments

Fresh PCR products obtained with CTO 189f, and CTO 654r primers (Table 2.2) were purified with QIAquick[®] Spin Columns (Qiagen, Crawley, UK) following the manufacturer's instructions, and cloned using a TOPO TA cloning kit (Invitrogen[™] Ltd, Paisley, UK).

The cloning procedure was performed in two sequential steps: (1) ligation and (2) transformation, respectively. For ligation, 0.5 to 4 µl of fresh PCR product (less than 24 hours old) was mixed with 1 µl of salt solution (containing 1.2 M NaCl and 0.06 M MgCl₂) and 1 µl of cloning vector pCR[®]4-TOPO and incubated at room temperature for 20-30 minutes. For transformation, 2 µl of the ligation reaction was transferred to vials containing 50 µl of One Shot[®] TOP10 chemically competent *E. coli* cells (previously thawed on ice) and left on ice for 30 minutes.

The cells were heat shocked for 30 s at 42°C in a water bath, and replaced on ice for 5 minutes. A volume of 250 µl of commercially prepared SOC medium (2%(w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added to cells and incubated at 37°C under shaking conditions of 150 rpm for one hour. After that, 50 µl and 75µl of the suspension of transformed cells were spread on plates containing LB-Luria Bertani Agar Medium (1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1.0 % NaCl, 1.5% (w/v) Agar pH 7.0), and 50 µg/ml of ampicillin. Plates were incubated overnight in an incubator at 37°C. Colonies were selected randomly and checked for inserts of the expected size using PCR amplification with the vector-specific primers pUCf and pUCr (see Table 2.3 on Section 2.5).

PCR products obtained from samples in which ammonia oxidizing archaea were detected with primers Arch-*amoAF* and Arch-*amoAR* were also cloned following exactly the same procedures described above.

2.8 DNA sequencing

A comparative analysis of all sequences was conducted to determine the occurrence of different operational taxonomic units (OTUs) within the clone library and when necessary full length sequences were obtained for representatives of each OTU identified.

Insert DNA from clones containing inserts of the correct size were amplified using primers pUCf and pUCr (Table 2.3 on Section 2.6). The PCR products were treated with EXOSAP-IT[®] (Cleveland, USA), to remove primer residues and dephosphorylate nucleotides. EXOSAP-IT[®] treated PCR products were diluted tenfold with molecular grade water and 48 clones from each clone library were sequenced with a single primer. Cloned 16S rRNA gene fragments from the Lindsey WWTP trickling filter system were sent for sequencing with pUCf primer (Table 2.2) while cloned 16S rRNA gene fragments from the other WWTPs with activated sludge systems (Eastham, Pembroke, Humber and Grangemouth activated sludge) were sequenced with primer CTO654r (Table 2.3). Lindsey was the first WWTP to be analysed and the sequencing primers used differed from those in other WWTP to make it easier to find the start point of the inserted PCR product from the occurrence of the primer sequence. This is because when using pUCf primer, the DNA fragment can be inserted in two different orientations; either starting from the 5' end of the cloned fragment or from the 3' end of the fragment and thus all sequences can be compared only when the whole inserted sequence is recognized. Archaeal *amoA* cloned gene fragments were sent for partial sequencing with primer Arch-*amoA*R (Table 2.3). Sequencing was conducted by the Genomics Sequencing Services (IRES-Devonshire Building <http://ires-genomics.ncl.ac.uk/>) Newcastle University using the BigDye chain terminator cycle sequencing protocol and ABI 3730 xl automated DNA sequencer (Applied Biosystems, Warrington, UK).

2.9 Analysis of AOB 16S rRNA and AOA *amoA* gene clone libraries

Coverage (Good, 1953) and diversity captured by each clone library were analyzed on the basis of rank abundance data.

Coverage gives an estimation of how well the sample represents the environment from which it was taken from, and correlates inversely to diversity. The higher diversity is in the library, the lower the coverage, for a given number of sequences.

- Coverage is calculated by the formula:

$$C = 1 - (s/n) \quad (2.6)$$

where:

s = is the number of OTUs appearing only once in the library
n = is the library size

Diversity comprises two components: richness (number of OTU types present in a library) and evenness (OTU type's abundance distribution in a library). Diversity indices summarize both components in a number to express diversity. There are several diversity indices and they differ on the basis of how they weight each component into one number.

Two diversity indices (Hill *et al.*, 2003) were considered to measure diversity from clone library data: Shannon (H') and Simpson's Dominance Index (1-D).

- Shannon index is given by the formula:

$$H' = -\sum p \ln p \quad (2.7)$$

where:

p = is the proportional abundance of clones in the OTU (estimated using n/N)

The Shannon index is influenced by both richness and evenness, and is more sensitive to increases in the number of OTUs appearing only once in the library.

- Simpson's Dominance Index is calculated by the formula:

$$D = \sum \frac{n(n-1)}{N(N-1)} \quad (2.8)$$

where:

n = is the proportion of clones in the OTU
 N = total number of individuals

Simpson's index is affected by the abundance of the most common OTUs in a library. As diversity increases D values decreases, and therefore Simpson's index is commonly expressed as $1 - D$.

Non parametric Chao 1 estimator (Chao, 1984; Chao, 1987) and abundance-based coverage estimator (ACE), were applied to determine if libraries were sampled sufficiently (Chao *et al.*, 1993).

Chao 1 and ACE estimators are adaptations of mark-release-recapture (MRR) methods; previously developed for estimation of animal populations. While Chao1 gives more weight to singletons (OTUs represented by only one individual in the library) and doubletons (two individuals) ACE also includes data from species represented by up to 10 individuals.

Chao 1 and ACE estimators are expressed respectively by the following formulae (Hughes *et al.*, 2001):

- $Chao\ 1 = S_{obs} + \frac{n_1^2}{2n_2}$ (2.9)

where:

S_{obs} = number of observed species
 n_1 = number of singletons
 n_2 = number of doubletons

$$\bullet \text{ ACE} = S_{\text{abund}} + \frac{S_{\text{rare}}}{C_{\text{ACE}}} + \frac{F_1}{C_{\text{ACE}}} Y_{\text{ACE}}^2 \quad (2.10)$$

where:

F_1 = number of singletons

S_{rare} = number of clones occurring ≤ 10 times

S_{abund} = number of clones occurring more than 10 times

C_{ACE} = a sample coverage estimate defined as the proportion of clones in relatively rare phylotypes (<10 clones) occurring more than once in the library

Y_{ACE}^2 = coefficient of variation of the F_1

When a clone library is sampled enough, Chao 1 and ACE estimators render stable asymptote curves.

Simpson's Dominance Index and Coverage estimator were calculated by hand. The Estimate S version 8.0 (<http://viceroy.eeb.uconn.edu/EstimateS>), statistical software for estimation of species richness and species shared between samples was used to calculate the Shannon index (H') and the non-parametric abundance based estimators Chao 1 and ACE, after 100 randomizations of sampling, without replacement.

Five input format files are accepted by Estimate S saved in tab-delimited text files. The input file used to perform these analyses was "Format 3", following an example used by Hughes and Bohannan, 2004, for clone data, where each clone represents the sampling of one individual. The data were entered in a spreadsheet in three columns: the first column contained numbers (1, 2, etc...) attributed to each OTU in the library, the second column contained the number of individual clones corresponding to each OTU, and the third column contained the value "1", indicating a sample size of "1". The title of the file is placed on the first line, and the total number of OTU types followed by the total number of clones in the library are placed on the second line; and the final line after the data must be filled with the values "-1, -1, -1", which indicate the end of the input.

2.10 Statistical comparisons between clone libraries

Clone libraries were statistically compared to one another on the basis of their composition by using the computer programme J- LIBSHUFF (Schloss *et al.* 2004). J- LIBSHUFF determines if two or more libraries were sampled from the same or from different populations.

The method is an adaptation of the Coverage formula (See Section 2.9) created by Good (1953), in which coverage values for a library are plotted as a function of evolutionary distances between sequences. The test begins by first constructing curves within each library (homologous coverage curves) and then comparing the homologous curves with heterologous coverage curves constructed from sequences obtained from both libraries.

J- LIBSHUFF is an implementation of LIBSHUFF (Singleton *et al.*, 2001) and uses the integral form of the Cramér- von- Mises statistic that is used to calculate the differences (ΔC) between homologous and heterologous coverage curves.

The exact formula of the statistic is given by:

$$\Delta C_{XY} = \int_0^{\infty} [C_X(D) - C_{XY}(D)]^2 dD \quad (2.11)$$

where:

$C_X(D)$ and $C_{XY}(D)$ = are measures of library coverage
 D = size of distance window used to determine the level of coverage

A Monte Carlo probability test is then applied to test the significance between the ΔC values calculated between both coverage curves. Thus sequences between two libraries (X) and (Y) that are being compared are randomly shuffled between libraries 10,000 times and after each shuffle a ΔC value is calculated. After shuffling, ΔC values are ranked from the largest to the lowest value, and ΔC values from the original libraries are compared against the ΔC values obtained from the randomizations. If the difference is greater than the 95% confidence interval, a significant p-value (≤ 0.05) is determined.

When significant p-values are obtained for both libraries this indicates that both libraries are statistically different. By contrast, when a significant small p-value is obtained for one library and a large p-value for the other, it implies that one library is a subset of the other and therefore that they were derived from the same population.

J- LIBSHUFF also calculates the experiment-wise error rate for multiple clone libraries comparisons and thus reduces the probability that a significant p- value occurred by chance and not due to actual biological differences.

The input file used for J-LIBSHUFF analysis consisted of a single distance matrix containing 16S rRNA sequence distance data from all libraries. Matrices with all sequences were built using the BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>), in which the sequences were aligned with Clustal W (Thompson *et al.*, 1994), and the output file was saved in PHYLIP format and imported into PHYLIP. In PHYLIP, a distance matrix was built using the Jukes and Cantor (1969) correction for multiple substitutions.

2.11 Phylogenetic analysis

The sequence data were initially analysed for quality according to the high, medium or poor quality of the reads. The high quality sequences were clear reads defined by one peak at each position for the full length of the sequence. Medium quality reads were sequences readable in large part but not in all of their length, becoming unclear due to more than one peak appearing at the same positions in the sequence. When the frequency of these ambiguous peaks increased, making the sequence readable only over shorter length size, they were considered as poor quality sequences.

The sequences were imported into BioEdit (Biological Sequence Alignment Editor for Windows 95/98/NT/XP v 7.0.5:

<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Sequences were trimmed at their 5' and 3' to remove vector derived and primer-derived sequence, and then aligned using ClustalW (Thompson *et al.*, 1994) and the alignment was saved in FASTA format. Where sequence reads were short they were allocated to OTUs identified on the basis of an analysis of full-length sequences by constructing an uncorrected distance matrix of all sequences trimmed to the length of the shortest sequence. This was done using TREECON for Windows (Van de Peer and De Wachter, 1994).

The FASTA files were imported into FastGroupII (Yu *et al.*, 2006). FastGroup II (<http://biome.sdsu.edu/fastgroup/>) is a programme which allows the dereplication of identical sequences and therefore, similar sequences are clustered in the same group. Through this tool, different OTUs and the number of clones found in each OTU were used to plot clone rank abundance curves, showing the total number of clones and their frequencies for each library. OTUs defined by 97% sequence identity were considered.

Representative sequences from each OTU identified from analysis with FastGroupII were selected for phylogenetic analysis. They were screened against GenBank and EMBL databases to identify the most closely related sequences. This was done using FASTA3- Nucleotide Similarity Search (Pearson and Lipman, 1988) (<http://www.ebi.ac.uk/fasta33>), and BLAST –Basic Local Alignment and Search Tool in Genbank Entrez nucleotide (Altschul *et al.*, 1990) (<http://www.ncbi.nlm.nih.gov/>). Only high quality sequences were used for subsequent phylogenetic analysis.

Phylogenetic trees were constructed using the ARB software (Ludwig *et al.*, 2004) with representative sequences from each OTU and sequences retrieved from GenBank. Parsimony phylogenetic trees were constructed in ARB using the DNA parsimony method, whereas the alignments obtained in ARB were imported into PHYLIP (Phylogeny Inference Package version 3.6) to construct distance phylogenetic trees using the neighbour-joining method (Saitou and Nei, 1987) with the Jukes and Cantor correction for multiple substitutions. Bootstrap analyses with 100 resamplings were conducted (Felsenstein, 1985) on both parsimony and distance trees to verify statistical confidence between branches on phylogenetic trees.

2.12 Real-time PCR

PCR assays were performed in 96 well plates using a iQ™5 multicolour real-time PCR thermocycler with an icycler iQ fluorescence detection system from BIO-RAD (Hercules, CA, USA) and software version 2.0 (BIO-RAD). Nine PCR assays were set up as follows: total 16S rRNA genes bacteria, 16S rRNA genes AOB, 16S rRNA genes *Nitrobacter*, 16 S rRNA genes *Nitrospira* type I and type II, ssu rRNA genes Marine *Crenarchaeota*, 16S rRNA genes Group I soil *Crenarchaeota*, AOB *amoA* and AOA *amoA*.

Reactions were performed in a total volume of 20 µl and contained, per reaction: DNA template (3 µl), 1 µl primers and TaqMan probes (from Thermo Electron GmbH (Ulm, Germany) or Genosys, USA) labelled with fluorescent dyes (6-FAM or HEX) and TAMRA dye as a quencher (or instead of probes, doubled stranded DNA binding fluorescent dye SYBR® Green I (Sigma, Poole, UK; 10000 x conc. in DMSO) further diluted 100fold and used at 1% by volume of the final mixture reaction volume; 10 µl iQ Supermix PCR reagent (from BIO-RAD, Hercules, CA, USA) and 6 µl Molecular Grade Water. All primers and probes used in this study as well as PCR conditions for each assay are summarized in Table 2.4.

Melting curves were analyzed for some assays using SYBR Green I in order to check reaction quality and the generation of non-specific products and primer dimer.

The iQ Supermix reagent is a PCR mixture containing the following: 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dNTPS, hot start enzyme *iTaq*™ DNA polymerase (activated after initial three minutes at 95°C), 50 units/ml, 6 mM MgCl₂, and stabilizers. In total, 64 tenfold diluted samples were analysed in triplicate for each PCR assay performed. This corresponded to all oil refinery wastewater plants sampled plus the sample obtained from a pilot reactor that treated leather-processing wastewater. Samples were run along with 10 fold serially diluted standards (see section 2.12.1) added to the assays in duplicate. Two negative controls containing no template were included and PCR inhibition was checked by including two samples randomly chosen, spiked with one of the standards.

Table 2.4 Real -time PCR primers and conditions used in this study

Primers/Probe	Sequence (5' – 3')	Primers/ Taq Probe Concentration/ reaction (pMol)	PCR conditions	Target gene	Target microorganism	Reference
1055f 1392r 16S Taq 1115	ATG GCT GTC GTC AGC T ACG GGC CGG TGT GTA C HEX- CAA CGA GCG CAA CCC- TAMRA	15 6.25	7 min 95°C 40 cycles: 20s at 95°C, 1 min 50°C, 20s 72°C		Total Bacteria	Harms <i>et al.</i> , 2003
CTO 189f A/B CTO 189f C RT1	GGA GRA AAG CAG GGG ATCG GGA GGA AAG TAG GGG ATCG CGT CCT CTC AGA CCA RCT ACTG	15	7 min 95°C 40 cycles: 10s 95°C, 1 min 60°C, 20s 72°C		AOB	Kowalchuck <i>et al.</i> , 1997 Kowalchuck <i>et al.</i> , 1997 Hermansson and Lindgren, 2001
Nitro- 1198f Nitro- 1423r Nitro- 1374 Taq	ACC CCT AGC AAA TCT CAA AAA ACCG CTT CAC CCC AGT CGC TGA CC 6FAM- AAC CCG CAA GGA GGC AGC CGACC- TAMRA	15 6.25	7 min 95°C, 40 cycles: 10s 95°C, 1 min 58°C, 20s 72°C	16S rRNA	<i>Nitrobacter</i> spp	Knapp and Graham, 2007
Ntspra- I-f Ntspra- 1431	GAA AGG ACT GCC CAG GAT AAC GGG TTG GCT TGG GCG ACT TCA	15	7 min 95°C, 40 cycles: 10s 95°C, 45s 58°C, 1 min 72°C		<i>Nitrospira</i> Type I	Modified from Ntspra-1158r (Maixner <i>et al.</i> , 2006) Maixner <i>et al.</i> , 2006
Ntspra- II-f Ntspra- 1150	TCT GGA ACA TTT CTG ACG CTGA GTT CTC CTG GGC AGT CTC TC	15			<i>Nitrospira</i> Type II	Modified from Ntspra-746r (Maixner <i>et al.</i> , 2006) Maixner <i>et al.</i> , 2006
GI_751F GI_956R	GTC TAC CAG AAC AYG TTC HGG CGT TGA CTC CAA TTG	12.5	7 min 95°C 50 cycles: 15s 94°C, 30s 58°C, 30s 72°C, 10s 78°C		Marine Group I archaeal SSU rRNA	Mincer <i>et al.</i> , 2007
771F 957R	ACG GTG AGG GAT GAA AGCT CGG CGT TGA CTC CAA TTG	10	7 min 95°C 55 cycles: 30s 95°C, 45s 54°C, 45s 72°C		Group I <i>Crenarchaeota</i>	Ochsenreiter <i>et al.</i> , 2003
<i>AmoA</i> 1-F <i>AmoA</i> 2-R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	6	7 min 95°C 40 cycles: 20s 95°C, 1.5 min 60°C, 1.5 min 72°C	<i>amoA</i>	AOB	Rotthauwe <i>et al.</i> , 1997
Amo196F Amo 277R Amo 247	GGW GTK CCR GGR ACW GCM AC CRA TGA AGT CRT AHG GRT ADC C 6FAM-CAA ACC AWG CWC CYT TKG CDA CCCA- TAMRA	20 10	7 min at 95°C 40 cycles : 15s at 95°C, 40 s at 55°C, 20s 72°C		AOA	Treusch <i>et al.</i> , 2005

D= A or G or T; H= A or C or T; K= G or T; M= C or A; R= G or A; S= G or C; W= A or T; Y= C or T

2.12.1 Preparation of standards

The abundance of the target gene in samples was determined by running the samples with standards from cloned PCR amplified target genes. Most of the standards used in the qPCR assays (See Table 2.5) were prepared from extracted plasmids. Standards used for the AOB 16S rRNA genes and AOA *amoA* assays respectively, were obtained from clones amplified with pUC primers, purified with EXOSAP-IT[®] (Section 2.8) and their DNA concentration measured using a DNA spectrophotometer NanoDrop[®]ND-1000 (Wilmington, USA). The concentration of DNA of plasmids containing inserted DNA used on *Crenarchaeota* 16S RNA gene assays (obtained from Dr. Mußmann, University of Vienna) was also measured using the same equipment. The concentration of DNA standards used specifically in the qPCR assays targeting the 16S rRNA gene of AOB, *Nitrospira* I, *Nitrospira* II and *Nitrobacter* was previously determined by Charles Knapp.

Table 2.5 Standards used in the real-time PCR assays performed in this study

Standards	Type	Reference
<i>Nitrobacter</i> sp. (DQ388518) <i>Nitrospira defluvii</i> (type I) (DQ059545)	Plasmids containing cloned 16S rRNA gene	Hawkins <i>et al.</i> , 2006 Spieck <i>et al.</i> , 2006
<i>Nitrospira moscoviensis</i> (type II) (X82558)		Maixner <i>et al.</i> , 2006
Unspecific AOB clone	AOB 16S rRNA gene	Courtesy of Rheanne Pickering
Marine group 1a and soil group 1b <i>Crenarchaeota</i>	Plasmid containing cloned 16S rRNA gene	Courtesy of Marc Mußmann
AOA Clone AGA51	Plasmid containing fragment of cloned <i>amoA</i> recovered from the Grangemouth WWTP	This study
AOB <i>amoA</i>	Plasmid containing fragment of cloned <i>amoA</i> from <i>Nitrosomonas europaea</i>	Courtesy of Michael Maguire

The total weight in grams per copy of either extracted plasmid containing the inserted target DNA size fragment or clones used as DNA standards was determined on the basis of the Avogadro's number (6.23×10^{23} molecules/ mole) as follows:

$$\bullet \text{ Weight (g)/ target gene} = 1.021 \times 10^{-21} \times (\text{plasmid} + \text{insert base size}) \times 1000 \text{ kb/kb} \quad (2.12)$$

where:

1.021×10^{-21} = average weight equivalent to a single base/ MW (g/mole), and MW (molecular weight) = number of base pairs \times 660 Da

After calculating the total weight of each standard, the exact number of target molecules/ μ l contained in each undiluted standard was determined using the formula:

$$\bullet \text{ Gene abundance}/\mu\text{l} = (\text{ng DNA}/\mu\text{l})/10^9 / \text{weight (g)/copy} \quad (2.13)$$

where:

ng DNA/ μ l = concentration of plasmids based on the nano-drop readings
 10^9 = conversion factor used to convert grams to nano-grams

After determining the DNA standard gene abundance/ μ l, standards were serially diluted in molecular grade water to give a range of concentration from 10^9 to 10^2 target sequence copies / μ l; and standards from 10^8 to 10^3 were run in the assays along with the environmental samples. A linear regression standard curve was generated automatically by the software used, through plotting the threshold cycle number at which the fluorescence generated within a reaction crosses the threshold (crossing point or C_T value) against the logarithm of gene abundance from each known standard concentration (DNA starting quantity).

The gene abundance in samples was then determined by interpolating the C_T values for samples on the respective standard curve obtained for each assay. The number of gene copies/mL was reported as follows: gene abundance/mL original sample = SQ x 10 μ l (sample dilution factor from eluted DNA) x 50 μ l (total eluted DNA volume from sample extraction)/ sample volume taken for extraction x 2 (sample original volume dilution factor: 20 mL sample: 20 mL ethanol), where SQ corresponds to the DNA starting quantity in gene copies per μ l and is automatically calculated by the software used together with the iQTM5 thermocycler.

Detection limits for the real-time PCR assays were set up as the mean of the C_t values obtained from two blank reactions or no template control (NTC) that were included in each assay, plus three standard deviations.

2.13 Calculation of cell specific ammonia oxidation rates (CSAOR)

To calculate specific ammonia oxidation rates per cell per hour (CSAOR) AOB *amoA* and AOA abundances were first converted into cell numbers. It was assumed that on average two bacterial *amoA* copies exist per AOB cell, based on the number of copies reported for *Nitrosomonas europaea* (MacTavish *et al.*, 1993); and one *amoA* gene copy was found in the genome of *Cenarchaeum symbiosum* (Hallam *et al.*, 2006a).

The following formulae (modified from Daims *et al.*, 2001c) were considered as follows:

$$1. \text{ Total number of cells inside reactor (calculated for AOB or AOA) =} \quad (2.14)$$

$$\text{cells/mL} \times 1,000,000 \times \text{reactor volume (m}^3\text{)}$$

2. Ammonia consumed in mg per hour was calculated as follows:

$$\text{Ammonia consumed (mg/h)} = \frac{[(\text{Ammonia in} - \text{Ammonia out}) \times (\text{Flow rate}/24) \times 1000000]}{\text{HRT}} \quad (2.15)$$

where:

Ammonia in = ammonia concentration in the influent, mg/L

Ammonia out = ammonia concentration in the effluent, mg/L

Flow rate given in megalitre (ML) per day

HRT = hydraulic retention time expressed in hours

3. Ammonia consumed in mg per hour per cell was obtained by dividing Formula 2 by Formula 1

4. CSAOR given in femtomoles per hour per cell was calculated by converting ammonia consumed (mg per hour per cell) into femtogram per hour per cell first, and then dividing by the molecular weight of ammonia (17).

2.14 Whole-cell fluorescence *in situ* hybridization (FISH)

2.14.1 Fixation of samples in paraformaldehyde (PFA)

Before hybridization, samples previously fixed in ethanol at a 1:1 ratio by volume, were centrifuged at 13,000 x g for 3 minutes, using a microcentrifuge (Hettich EBA 12 with a Universal 30F rotor model; DJB Labcare Ltd, Newport Pagnell, England), re-suspended in 1 mL of Phosphate Buffered Saline (PBS) [10 mM potassium phosphate, 150 mM sodium chloride, pH 7.2], centrifuged and re-suspended in 0.25 mL of PBS and 0.75 mL of freshly prepared and filter sterilized fixation solution of 4 % paraformaldehyde [in 44.5 ml double sterilized distilled water (autoclaved and filtered in 0.2µm filter pore membrane), 5 mL 10 x PBS, one drop 10 mM NaOH, 2g paraformaldehyde, pH of solution adjusted to 7.2, and subsequently kept for three hours at 4°C. After fixation, samples were washed once in PBS, centrifuged and resuspended in PBS: ethanol 1:1 ratio by volume, before being processed through the hybridization steps.

2.14.2 Hybridization procedure

Hybridization was conducted in suspension using 0.5 mL microcentrifuge tubes, following a protocol elaborated by Russell Davenport, adapted from Amann *et al.*, 1990a.

A set of four aliquots of a known sample volume (based on samples MLSS concentration i.e., 100 µl, 200 µl or 1 mL) was taken and placed in separate microcentrifuge tubes, each receiving one of the following treatments: a) no probe addition, b) AOB and Eubacteria target probe addition, c) addition of Eubacteria probe only, d) addition of nonsense (Anti-eub) Eubacteria probe only.

Samples stored in PBS: ethanol at 1:1 ratio by volume, were centrifuged at 13,000 x g for 3 minutes, resuspended in 500 µl PBS twice, and serially dehydrated in 500 µl of increasing ethanol concentrations of 60% (v/v), 80% (v/v) and 100% (v/v) for three minutes each, followed by centrifugation after each ethanol addition.

After the dehydration step, each sample was supplemented with a hybridization buffer solution to a final volume of 40 μl . The volume of hybridization buffer added per tube was complementary to the volume of each probe added (2 μl).

The samples analyzed in this study presented issues related to non-specific probe binding, and this problem was overcome by changing the initial pre-hybridization time from 15 to 45 minutes and doubling the quantity of Poly A and Denhardt's (blocking reagents) in the hybridization buffer. A volume of 1 mL of freshly prepared hybridization buffer contained: (200 μl) 4.5 M NaCl; (100 μl) 200 mM NaH_2PO_4 , pH 7; (400 μl) 50 x Denhardt's solution [a mixture composed of 40mg/ml of each blocking reagent: Ficoll, polyvinyl pyrrolidone (PVP) and bovine serum albumin (BSA)]; (100 μl) poly A; 0.5 M EDTA, (10 μl) 10% (w/v) sodium dodecyl sulphate (SDS); (X)% (v/v) fresh deionised formamide (FA), and (Y) μl of water that was firstly autoclaved and then filter sterilized (double sterilized water). The volume of water added to the buffer was complementary to the volume of FA optimal for the probes used and the sum of both volumes (X + Y) was equivalent to 450 μl .

Two FA concentrations were used for probes in this study, 35% for all probes targeting AOB and the genus *Nitrospira*; and 40% FA for probes targeting the genus *Nitrobacter* respectively. The probes used in this study and respective hybridization and wash conditions are summarized in Table 2.6.

Samples containing hybridization buffer were pre-hybridized for 45 minutes at 46°C in a heating block. Afterwards, 2 μl of each probe (concentration 50 ng/ μl) were added to the solution and subsequently incubated for 2.5 hours at 46°C.

Fluorescently labelled oligonucleotide probes were obtained from Thermo Electron GmbH (Ulm, Germany) 5'-monolabelled with cyanine dyes CY5, CY3, or 6-carboxyfluorescein (6 FAM dye).

Table 2.6 Oligonucleotide probes and hybridization conditions used in this study

Probe	Sequence (5' – 3')	Label	Formamide (%)	Target Microorganism	NaCl (mM)	Reference
EUB338	GCT GCC TCC CGT AGG AGT	CY5	35	Most Bacteria	84	Amann <i>et al.</i> ,1990b
EUB338-II	GCA GCC ACC CGT AGG TGT	CY5	35	Bacteria (<i>Planctomycetales</i> order)	84	Daims <i>et al.</i> ,1999
EUB338-III	GCT GCC ACC CGT AGG TGT	CY5	35	Bacteria (<i>Verrucomicrobiales</i> order)	84	Daims <i>et al.</i> ,1999
AntiEUB	CGA CGG AGG GCA TCC TCA	6 FAM	35	Bacteria(nonsense)-complementary to Eub probes	84	Manz <i>et al.</i> , 1992
Nso1225	CGC CAT TGT ATT ACG TGT GA	6 FAM	35	All known AOB, except <i>Nitrosococcus mobilis</i>	84	Mobarry <i>et al.</i> , 1996
NEU653	CCC CTC TGC TGC ACT CTA	6 FAM	35	Most halophilic and tolerant <i>Nitrosomonas</i> sp	84	Wagner <i>et al.</i> , 1995
CTE659	TTC CAT CCC CCT CTG CCG		35	AOB (competitor to NEU653)	84	Wagner <i>et al.</i> , 1995
6a192	CTT TCG ATC CCC TAC TTT CC	6 FAM	35	<i>Nitrosomonas oligotropha</i> lineage	84	Adamczyk <i>et al.</i> , 2003
C6a192	CTT TCG ATC CCC GAC TTT CC		35	AOB (competitor to 6a192)	84	Adamczyk <i>et al.</i> , 2003
Ntspa662	GGA ATT CCG CGC TCC TCT	CY3	35	<i>Nitrospira</i>	84	Daims <i>et al.</i> , 2001a
CNtspa662	GGA ATT CCG CTC TCC TCT		35	<i>Nitrospira</i> (competitor to Ntspa662)	84	Daims <i>et al.</i> , 2001a
NIT3	CCT GTG CTC CAT GCT CCG	6 FAM or CY3	40	<i>Nitrobacter</i>	56	Wagner <i>et al.</i> ,1996
CNIt3	CCT GTG CTC CAG GCT CCG	6 FAM	40	<i>Nitrobacter</i> (competitor to NIT3)	56	Wagner <i>et al.</i> ,1996

Total Eubacteria were targeted by the EUB338 probes which comprise a mixture of I, II and III probes (See Table 2.6) labeled with the fluorochrome CY5. AOB were targeted by a mixture of four probes labeled with 6-FAM, including the general probe for AOB Nso 1225, the specific probes NEU and 6a192, and competitor probes CTE659 and C6a192, used to target respectively the most halophilic and halotolerant AOB and the *Nitrosomonas oligotropha* lineage. All AOB probes and respective competitors were hybridized together to ensure that all AOB lineages were covered. Probes Ntspa662 and competitor CNTspa662 were used to target the genus *Nitrospira* while the probe NIT3 and competitor CNIT3 were used to target the genus *Nitrobacter*. Probes targeting the genus *Nitrospira* were labeled with the fluorochrome CY3, when mixed with probes targeting either AOB, or the genus *Nitrobacter* labeled with 6-FAM. Probes used to target the genus *Nitrobacter* were labelled with CY3 dye when mixed with probes targeting AOB. Oligonucleotide probes as well as hybridization and washing conditions used in this study are summarized on Table 2.6.

After hybridization, samples were washed twice for 15 minutes in wash buffer at 46°C, in order to remove non bound probe.

Wash buffer made up to a final volume of 20 ml contained: (370 or 250 µl) 4.5 M NaCl [the volume of NaCl salt matches the FA concentration used in the hybridization buffer; see Table 2.5]; (200 µl) 0.5 M EDTA, (2 ml) 200mM Tris-HCl, (200 µl) 10% (w/v) SDS and double sterilized water. As probes for *Nitrobacter* have an optimal stringency at 40% formamide concentration, and it is higher than the optimal stringency required for the probes targeting AOB or *Nitrospira* (at 35% formamide concentration), hybridization with probes targeting the *Nitrobacter* genus and a subsequent washing step were conducted before hybridization with specific probes for AOB or *Nitrospira* (sequential hybridization).

After the washing step, samples were washed with 500 µl ice-cold water, centrifuged and the resultant pellet re-suspended in 100 µl of molecular grade water. From that volume, an aliquot of 10 µl was taken and placed into all four wells of each gelatin-coated slide (See section 2.13.1).

A series of three slides per sample were mounted, each slide containing four treatments, as follows:

- Slide 1: a) no probe; b) AOB probes + *Nitrospira* probes; c) Eub probe, d) Anti-eub probe;
- Slide 2: a) no probe, b) AOB + *Nitrobacter* probes, c) Eub probe, d) Anti-eub probe;
- Slide 3: a) no probe, b) *Nitrospira* + *Nitrobacter* probes, c) Eub probe, and d) Anti-eub probe.

The slides were left to dry inside an incubator oven at 30°C. After that, each well on the slide was mounted in a small drop of antifadent-Citifluor (AF1, Canterbury, UK). The slides were covered with cover glasses and their edges sealed with nail- varnish and kept at 4°C protected from the light prior to analysis.

2.14.3 Preparation of gelatin coated slides

Teflon coated glass microscope slides (C.A. Hendley Essex Ltd, UK) were horizontally placed in a rack and immersed in a plastic container containing 10% solution of KOH (w/v) in 95% (V/V) ethanol for one hour. The solution was then replaced by distilled water and the slides were immersed in fresh distilled water for 3 s. This step was repeated more three times, and the slides were left to air dry.

A gelatin coating solution containing 0.1% (w/v) gelatin, and 0.01% (w/v) $\text{CrK}(\text{SO}_4)_2$, was prepared in hot distilled water contained in a plastic box heated to 70°C in a water bath. The slides were kept immersed in the gelatin solution for 30 s and then left to air dry for 5 minutes. This procedure was repeated three more times, and after that, the slides were left to dry and kept at 4°C before being used.

2.14.4 Image analysis and cell counting procedure

Digital images of the slides were recorded in three separate channels: red, green and blue (RGB colour system), using a Confocal Scanning Laser Microscopy (CLSM) - Leica TCS SP2UV from Leica Microsystems Ltd., Milton Keynes, UK, and LCS software version 2.61 Build 1537 (Leica Microsystems, Heidelberg, GmbH).

The different fluorescently labelled probe dyes were excited at different wavelengths by an Argon ion laser beam crossing the pinhole of an x 63 magnification oil immersion lens, which scanned the sample through different focal points (10 sections) at 1µm thickness intervals to give optical sections from top to bottom. The emitted fluorescence from each particular dye was collected in a photomultiplier detector of the microscope through its corresponding wavelength emission filter range.

The nominated spotted wells (a) and (d) on each slide (Section 2.13.3) containing respectively no probes and anti-eub probe were set up as negative controls accounting respectively for background autofluorescence, and non-specific binding of probes. Both negative controls were taken into account to set up the confocal microscope settings for fluorescence threshold levels for each slide analyzed individually and subtracted from the fluorescence signal levels conferred by the target probes added to well (b).

Ten Field of Views (FOV), from each well (b), from each slide sample, were randomly selected through a device on the screen of the computer which allowed for a randomly automated movement of the microscope stage.

Since each of the three slides analyzed from each particular plant contained two samples analysed for the same target microorganisms, in total two slides were analyzed for each target bacterial group.

The total number of cells hybridized simultaneously, with probes targeting most bacteria and specific probes targeting AOB, *Nitrospira* or *Nitrobacter* respectively, were counted in each optical section and recorded. Z-stack images obtained by CLSM were analysed using the Daim software version 2.1 (Daims, *et al.*, 2006a) for microbial ecology digital image analysis, and/or using the software Microsoft[®] Paint Version 5.1. Cell patterns as well as spatial distribution between AOB, *Nitrospira* and *Nitrobacter* were also analysed.

The average number of cells in one mL of sample was calculated as follows:

$$K = \frac{(N \times 2 \times A1)}{(A2 \times 0.01 \times DF)} \quad (2.16)$$

where:

N = Average number of cells per field of view (FOV)

2 = Dilution factor (original sample volume (20 mL) diluted in 20 mL of ethanol)

A1 (Area of sample spot) = 19.63 mm² (diameter= 5 mm)

A2 (Area of one FOV) = 0.056644 mm²

0.01 = Volume of sample (mL) applied to microscopic slide

DF = Dilution factor = Original volume of fixed sample taken for FISH hybridisation (in this study: 0.1, 0.2 or 1 mL)/ 100 µl (final volume of molecular grade water in which the final sample pellet was re-suspended).

Theoretical detection limits ranging from 6.9×10^2 to 6.9×10^3 cells/mL for the FISH method, were determined from the formula used to calculate the cell numbers (Eq. 2.16), assuming that only one cell was detected in all of the 10 fields of view examined, and thus the average number of cells per field of view being equivalent to 0.1 cells per field of view.

2.14.5 *In situ* detection and quantification of putative AOA through catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH)

The detection and quantification of putative AOA in samples from this study were also analyzed through CARD-FISH by Marc Mußmann, from the Department of Microbial Ecology, University of Vienna, who kindly allowed the inclusion of CARD-FISH pictures in this study (Chapter 6).

In the CARD-FISH method, a single oligonucleotide probe covalently crosslinked to a horseradish-peroxidase enzyme (HRP) is combined with fluorescently-labelled tyramides. When the enzyme HRP is present in the target cell it catalyzes the deposition of fluorescently-labelled tyramides and through the radicalization of multiple tyramide molecules a strong and stable fluorescent tyramide signal amplification (TSA) is produced between the probe- target binding sites (Amman and Fuchs, 2008). As the HRP-enzyme label is bigger than the normal fluorescent dyes commonly used for FISH, it requires that cells should be firstly treated to enhance cell wall permeabilization.

For quantification, samples were sonicated for 30 s on ice, filtered on polycarbonate membranes (0.2 µm), and filters were sectioned in two halves. The first half was used as a control and was stained with DAPI (4', 6' diamino-2-phenylindole) at 1 µg/mL concentration for the quantification of total cell numbers. The second half of the filter was mounted in 0.1% agarose and used for CARD-FISH as described by Ishii *et al.*, 2004, where cells were permeabilized by incubation with proteinase K (15 µg/mL), in 0.1 M Tris, 0.01 EDTA, pH 8.0 for 5-8 minutes at room temperature, and then submitted to the hybridization treatment and tyramide signal amplification as described by Pernthaler *et al.*, 2002.

Dual hybridizations were carried out by using a general probe targeting most *Crenarchaeota* and a specific probe targeting either marine group 1.1a or soil group 1.1b *Crenarchaeota*. The probes used in the CARD-FISH procedure are summarized on Table 2.7. Putative AOA cell numbers and proportional area occupied by putative AOA were calculated in relation to the total number of cells counted on the filter sections stained by DAPI.

Table 2.7 CARD-FISH probes and hybridization conditions used by Mußmann *et al.*, 2009, unpublished

Probe	Sequence (5' – 3')	Label	Formamide (%)	Target Microorganism	Reference
Cren 512	CGG CGG CTG ACA CCA G	HRP	5	Most <i>Crenarchaeota</i>	Jurgens <i>et al.</i> , 2000
Comp Cren512a	CGG CGG CTG GCA CCA G		5		Mußmann <i>et al.</i> , 2009 (unpublished)
Comp Cren 512b	CGG CGG CTG GCA CCC G		5		
Cren 537	TGA CCA CTT GAG GTG CTG	HRP	20	Marine group 1.1a <i>Crenarchaeota</i>	Teira <i>et al.</i> , 2004
Cren 1162	TTC CTC CGT CTC AGC GAC	HRP	20	Sub-cluster of terrestrial group 1.1b	Mußmann <i>et al.</i> , 2009 (unpublished)
Comp Cren1162	TTTC CTC TGC TGC ACT CTA		20	<i>Crenarchaeota</i> Competitor	

2.15 *In silico* specificity evaluation of the primers and probes used or compared in this study, in real-time PCR, FISH and CARD-FISH assays

2.15.1 *In silico* specificity evaluation of the primers and probes used for the quantification of the AOB, *Crenarchaeota* and NOB 16S rRNA abundances

The specificity of the primers and probes targeting the AOB, *Crenarchaeota* and NOB 16S rRNA abundances were evaluated *in silico* through the web server probeCheck (<http://www.microbial-ecology.net/probecheck>); (Loy *et al.*, 2008). The probeCheck employs an online version of the ARB probe match tool (Ludwig *et al.*, 2004), and is linked to 24 selected publicly online database collections such as the SILVA database (Pruesse *et al.*, 2007) and the Ribosomal Database Project (RDP) II (Cole *et al.*, 2009), containing high quality checked and aligned sequences.

The specificity of each primer and probe as well as their overlap when being compared in different assays, e.g. real-time PCR and FISH or CARD-FISH, are summarized in Tables 2.8, 2.9 and 2.10.

Table 2.8 *In silico* specificity evaluation and overlap of the primers and probes used in this study for the quantification of the AOB 16S rRNA abundance

Primers/probes	Assay	Intended target group	Hits (SILVA database)*		Non target groups description	Number of target hits also showed by respective FISH probes
			Target	Non target		
CTO 189f A/B/C	qPCR	AOB	202	38	<i>Bacteroidetes/Chloroflexii/Proteobacteria: Gammaproteobacteria/Alphaproteobacteria/Betaproteobacteria: Neisseriales/ Burkholderiales/Rhodocyclales/Thiobacillus/Dechloromonas</i>	N 195
RT1 reverse		AOB	336	8415	<i>Proteobacteria: Alphaproteobacteria/Betaproteobacteria/Deltaproteobacteria/Epsilonproteobacteria/Gammaproteobacteria</i>	328
Nso1225	FISH	All known AOB except <i>Nitrosococcus mobilis</i>	467	112	<i>Acidobacteria/Bacteroidetes/Chloroflexii/Nitrospirae/Firmicutes/Fibrobacteres/Gemmatimonadetes/Planctomycetes/Verrucomicrobia/Proteobacteria: Alphaproteobacteria: Nitrobacter/ Gammaproteobacteria/Deltaproteobacteria/ Betaproteobacteria: Methylophilales/Rhodocyclales: Azospira/Burkholderiales/ Gallionella/ Thiobacillus</i>	
NEU653		Most halophilic and tolerant <i>Nitrosomonas</i> sp	59	2	<i>Chloroflexi</i>	
6a192		<i>Nitrosomonas oligotropha</i> lineage	20	3	<i>Bacteroidetes/Betaproteobacteria: Thauera</i>	

*16S/18S rRNA- SILVA database version 102 (containing 460,783 sequences): accessed through the probeCheck (Loy *et al.*, 2008) online resource for evaluating probe and primer specificity. The number of hits refers to the number of records to sequences with no mismatch to the primers/probes being evaluated.

Table 2.9 *In silico* specificity evaluation and overlap of the primers and probes used in this study for the quantification of the *Crenarchaeota* 16S rRNA abundance

Primers/probes	Assay	Intended target group	Hits (SILVA database)*				Non target groups description	Number of target hits also showed by respective FISH probes	
			Crenarchaeal groups 1.1a	1.1b	Other ** Groups	Other Archaea		Crenarchaeal groups 1.1a N	1.1b N
GI_751F	qPCR	Marine Group I archaeal SSU rRNA	674					605	n/a***
GI_956R		Marine Group I archaeal SSU rRNA	1558	451	1151	43	Other crenarchaeal groups/ <i>Euryarchaeota</i> / <i>Korarchaeota</i>	1180	116
771F		Group I <i>Crenarchaeota</i>	1891	534	1198	21	Other crenarchaeal groups/ <i>Euryarchaeota</i>	1723	127
957R		Group I <i>Crenarchaeota</i>	401	451	745	41	Other crenarchaeal groups/ <i>Euryarchaeota</i> / <i>Korarchaeota</i>	360	115
Cren 537	FISH	Marine group 1.1a** <i>Crenarchaeota</i>	1767	0	0	0			
Cren 1162		Soil group 1.b** <i>Crenarchaeota</i>	0	131	0	0			

*16S/18S rRNA- SILVA database version 102 (containing 460,783 sequences): accessed through the probeCheck (Loy *et al.*, 2008) online resource for evaluating probe and primer specificity. The number of hits refers to the number of records to sequences with no mismatch to the primers/probes being evaluated.

** Other 23 crenarchaeal groups within the phylum *Crenarchaeota* in the SILVA database include: class *Thermoprotei*, marine benthic group A, marine benthic group B, miscellaneous crenarchaeotic group, terrestrial group, terrestrial hot spring group, South African Gold Mine GP1, PSL12, Group C3, '*Candidatus Nitrosocaldus yellowstonii*', and groups AK31, AK56, AK59, AK8, DF10, FS213A-60, OPPD003, Papm3AA3, pMC2A209, TOTO-AG-15 and Z273FAH8.

** Groups 1.1a and 1.1b represent the two crenarchaeal groups in which putative AOA were found, with the exception of *N. yellowstonii*. However, it should be mentioned also that, on the basis of comparative genomic studies of *Cenarchaeum symbiosum*, *Nitrosopumilus maritimus* and the draft genome of *Nitrosocaldus gargensis*, these organisms are distinct from *Crenarchaeota* and thus their phylogenetic reclassification is being proposed within a third novel phylum, named *Taumarchaeota* (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010).

***n/a: not applicable

Table 2.10 *In silico* specificity evaluation and overlap of the primers and probes used in this study for the quantification of the NOB 16S rRNA abundance

Primers/probes	Assay	Intended target group	Hits (SILVA database)*		Non target groups description	Number of target hits also showed by respective FISH probes N
Ntspra-I-f	qPCR	<i>Nitrospira</i> Type I	53	1	<i>Proteobacteria</i>	52
Ntspra-1431			47	3	<i>Proteoobacteria/Firmicutes</i>	45
Ntspra II-f		<i>Nitrospira</i> Type II	71	0		67
Ntspra-1150			119	8	<i>Bacteroidetes/Verrucomicrobia/Planctomycetes/Acidobacteria/ Nitrospiraceae: Leptospirillum</i>	114
Ntspra662	FISH		283	5	<i>Chloroflexil/Proteobacteria TA18</i>	
Nitro-1198f	qPCR	<i>Nitrobacter</i>	76	0		73
Nitro-1423r			63	7722	<i>Acidobacteria/Firmicutes/Bacteroidetes/Actinobacteria/Chloroflexil/Nitrospira/Planctomycetes/Proteobacteria/Tenericutes/Verrucomicrobia Bacteria_incertae_sedis/Cyanobacteria/TM7</i>	63
Nitro-1374 Taq			4	0		4
NIT3	FISH		80	3	<i>Bradyrhizobiaceae: Afipia</i>	

*16S/18S rRNA- SILVA database version 102 (containing 460,783 sequences): accessed through the probeCheck (Loy *et al.*, 2008) online resource for evaluating probe and primer specificity. The number of hits refers to the number of records to sequences with no mismatch to the primers/probes being evaluated.

2.15.2 *In silico* analysis of the primers used in this study for the quantification of AOB and AOA *amoA* gene abundances

The primers *amoA*-1F and *amoA*-2R (Rotthauwe *et al.*, 1997), used for the quantification of AOB *amoA* gene abundance, target a partial stretch of 491 bp of the ammonia monooxygenase gene.

In silico analysis of these primers conducted in some studies and comparisons with other primers published to date have shown these primers as one of the most preferable primer pair choice adopted in several environmental studies due to their good sensitivity, specificity and reliable performance in targeting the AOB *amoA* gene (Purkhold *et al.*, 2000; Junier *et al.*, 2008).

The forward primer *amoA*-1F targets a region of the gene which is highly conserved in all betaproteobacterial AOB, and it is matched perfectly or with one or two mismatches to sequences from betaproteobacterial AOB, but does not match sequences from gammaproteobacterial AOB, while the reverse primer *amoA*-2R matches only sequences from betaproteobacterial AOB. Primer *amoA*-1F has one to two mismatches to *Nitrosomonas europaea* and *Nitrosococcus mobilis*, one mismatch to some members from *Nitrosomonas marina* lineage, for example to TA-921-I-NH₄ and Nm51 strains and one to two mismatches to *Nitrosomonas oligotropha* lineage strains AL212 and JL21 and one to two mismatches to some members within the *Nitrosospira* group. In contrast, the reverse primer *amoA*-2R has one mismatch to some members within the *N. europaea* lineage as for example GH22 and TK794 strains (AB031869), three mismatches to *Nitrosomonas oligotropha* AL212 and JL21 strains, two mismatches to *Nitrosomonas cryotolerans*, and one to two mismatches to some members within the *Nitrosospira* group (Junier *et al.*, 2008).

The primers *Amo196F* and *Amo 277R* (Treusch *et al.*, 2005), used for the quantification of the AOA *amoA* gene abundance in the real-time PCR assay, target a partial stretch of 103bp within the archaeal monooxygenase gene. These primers were designed based on alignments of 200 amino acid sequence positions of the Sargasso Sea, the soil fosmid 54d9 and RT-PCR or direct PCR sequences from soil samples (Treusch *et al.*, 2005).

The specificities of the *amo196F*, *amo277R* primers and the TaqMan probe *amo247* were verified against the archaeal clone libraries sequences obtained in this study from the end-point PCR-amplification of the AOA *amoA* gene, using the primers Arch-*amoAF* and Arch-*amoAR* (Francis *et al.*, 2005) (Figure 2.6). The primers Arch-*amoAF* and Arch-*amoAR* (Francis *et al.*, 2005) target a 635 bp DNA fragment length, what almost corresponds to the entire archaeal *amoA* gene (657 bp) and were designed based on an alignment of 200 amino acid sequence positions of the Sargasso Sea and the soil fosmid 54d9 sequence.

Overall, primers *amoA196* and *amoA277R* had from one to three mismatches; and the probe *amoA247* had from zero to one mismatch against the 178 clone library sequences retrieved from this study and the 10 clone library sequences retrieved from the Humber WWTP by Marc Mußmann. The number of mismatches, their frequencies and positions for each primer and the probe differed among libraries, and are shown in detail in Figure 2.6.

Library	Sequences (N)	Primers/ Probe	N	Mismatches Sequences (%)				
				0	1	2	3	
Grangemouth A	43	<i>amo196F</i>	3	0	0	0	100	
		<i>amo277R</i>	2	0	0	100	0	
		<i>Amo247</i>	0	0	0	0	0	
Grangemouth B	43	<i>amo196F</i>	2-3	0	0	95	5	
		<i>amo277R</i>	0/2	2	0	98	0	
		<i>Amo247</i>	0-1	95	5	0	0	
Lindsey	37	<i>amo196F</i>	0-3	3	32	60	5	
		<i>amo277R</i>	0-1	84	16	0	0	
		<i>amo247</i>	0-1	73	27	0	0	
Humber	10	<i>amo196F</i>	2-3	0	0	80	20	
		<i>amo277R</i>	3	0	0	0	100	
		<i>amo247</i>	0-1	90	10	0	0	
BLC	45	<i>amo196F</i>	1	0	100	0	0	
		<i>amo277R</i>	0	100	0	0	0	
		<i>amo247</i>	0	100	0	0	0	

Mismatches positions		
<i>amo196F</i>	196 5'-GGWGTKCCRGGRACWVGCMAAC-3' 215 Primer	W=A/T
	GGCGTTCCGGTACTGCTAC	AGA51 (Standard) Grangemouth A
	GGCGTTCCAGGAACAGCGAC	AGB47 Grangemouth B
	GGTGTTCAGGCCTACTGCTAC	AGB50 Grangemouth B
	GGCGTTCCGGTACTGCTAC	AGB48 Grangemouth B
	GGAGTTCAGGAACAGCAAC	ALA31 Lindsey
	GGAGTTCAGGAACCGCAAC	ALA15 Lindsey
	GGCGTTCCAGGAACAGCGAC	ALA64 Lindsey
	GGCGTTCCAGGAACAGAGAC	ALA35 Lindsey
	GGCGTTCCAGGAACAACGAC	ALA54 Lindsey
	GGAGTTCAGGTACAGCTAC	amoA_H1_20 Humber
	GGAGCTCCAGGTACAGCTAC	amoA_H2_20 Humber
	GGTGTTCAGGAACAGCGAC	L7 BLC
	GGTGTTCAGGAACAACGAC	L75 BLC
<i>amoA277R</i>	298 5'-CRATGAAGTCRTAHGGRTADCC-3' 277 Primer	
	CAACAAGTCGTATGGATATCC	AGA51 (Standard) Grangemouth A
	CAATGAAGTCATATGGATAACC	AGB47 Grangemouth B
	CGACAAGTCGTATGGGTATCC	AGB50 Grangemouth B
	CAATGAAGTCATATGGGTATCC	ALA31 Lindsey
	CAACGAAGTCATATGGGTAAACC	ALA58 Lindsey
	CTACAAGTCATATGGATATCC	amoA_H1_20 Humber
	CAATGAAGTCATATGGATAACC	L7 BLC
<i>amoA247</i>	271 5'-CAAACCAWGWCCYTTKGCDAACCA3' 247 Probe	
	CAAACCATGCACCTTTGGCAACCCA	AGA51 (Standard) Grangemouth A
	CAAACCATGCACCTTTTGCTATCCA	AGB47 Grangemouth B
	CAAACCAAGGCTCCTTTTGCAACCCA	AGB50 Grangemouth B
	CAAACCATGCACCTTTTGCTATCCA	ALA64 Lindsey
	CGAACCATGCACCTTTTGCGATCCA	ALA15 Lindsey
	CAAACCATGCACCTTTTGCAACCCA	amoA_H1_20 Humber
	CAAACCAAGCCTTTTGCAACCCA	amoA_H12_2 Humber
	CAAACCATGCACCTTTTGCTATCCA	L7 BLC

Figure 2.6 Number of mismatches and mismatch positions found in the targeted binding sites of the primers *amo196F* and *amo277R* and the *Taq*probe *amo247* (Treich *et al.*, 2005), used for the quantification of the AOA *amoA* gene abundance, when aligned with 178 archaeal *amoA* clone libraries sequences, generated from PCR products amplified using the primers Arch-*amoAF* and Arch-*amoAR* (Francis *et al.*, 2005) from three refinery WWTPs and one pilot reactor (BLC) investigated in this study.

2.16 Nitrification modelling

The fraction of biomass represented by AOB in the sludge was theoretically estimated using a single nitrification model (Rittmann *et al.*, 1999), described through the following equation:

$$X_{AOB} = \frac{\Theta_x}{\Theta} \times \left[Y_{AOB} \times \frac{1 + (1-f_d) \times b_{AOB} \times \Theta_x}{1 + b_{AOB} \times \Theta_x} \right] \times \Delta_{ammonia} \quad (2.17)$$

Where:

X_{AOB} = AOB biomass in the sludge, mg/L
 Θ_x = sludge age or biomass retention time, mg/L
 Θ = Hydraulic retention time (HRT)
 Y_{AOB} = 0.34 Kg VSS/Kg N (AOB yield)
 f_d = 0.8 (fraction of new synthesized AOB biomass degradable by endogenous decay)
 b_{AOB} = 0.15 day⁻¹ (AOB endogenous decay coefficient)
 $\Delta_{ammonia}$ = ammonia removal
 Values adopted for Y_{AOB} , f_d and b_{AOB} were taken from the literature (Furumai and Rittmann, 1994).

AOB biomass was determined experimentally by converting AOB *amoA* abundance obtained through real-time PCR into cell numbers, and assuming that a single cell has a total density of about 620 femtogram dry weight/ μm^3 , based on a consensus value of 310 fg of carbon per μm^3 of biomass (Fry, 1990), which accounts for 50% of cellular biomass material, and a mean cell volume of 0.5 μm^3 . This approach was adopted by Coskuner *et al.*, 2005; and also taking into account the AOB yield obtained as a function of ammonia consumed in reactors.

The estimated AOB biomass (X_{aob}) was then compared to both the biomass determined from the real-time PCR method and the biomass determined from the FISH method to verify whether the theory and experimental measurements were in agreement with one another.

Similarly, AOA biomass was determined experimentally by converting AOA *amoA* abundance obtained through real-time PCR into cell numbers, assuming that a single cell has a total density of about 176 fg dry weight/ μm^3 , based on a consensus value of 310 fg of carbon per μm^3 of biomass (Fry, 1990), accounting for 50% of cellular biomass material, and a mean cell volume of 1.76 μm^3 . This volume was calculated from the AOA CARD-FISH images taken by Marc Mußmann from the putative AOA cell morphotype cells found in the Humber and Grangemouth reactors (See Figure 6.12, Panels A and B). As the cell diameters ranged between 1 μm and 2 μm , a mean diameter value of 1.5 μm was taken into account to calculate the geometric mean radius of the cell in the equation $4/3 \pi r^3$, assuming that cells are spherical, to convert cell biovolume to cell biomass. The measured AOA biomass was then compared to the estimated AOA biomass (X_{aoa}) that had been predicted by the model to verify whether or not the theory and experimental measurements were in agreement with one another.

2.17 Statistics

General statistics such as data normality checking using the Anderson-Darling test, descriptive statistics, Coefficient of variance (CV), Pearson correlation and regression analyses, Kruskal-Wallis non-parametric ANOVA test, and multivariate statistical analysis using principal component analyses (PCA), were applied to specific datasets using the statistical software package Minitab version 14 (Minitab Inc., State College, PA, USA).

Abundance data were log transformed and all the data submitted to PCA analyses were standardized in order to reduce the variability caused by variables being measured in different dimensional scales, applying the Z-score data reduction, where the mean was subtracted and divided by the standard deviation of each individual measured value to give a mean value of zero and a standard deviation of 1.

Residuals from significant regressions were checked for normality using the Anderson-Darling test and residual p values are also included in the description of regression results. Residuals are normally distributed when p values are higher than 0.05.

CHAPTER 3

Diversity of AOB in oil refinery wastewater treatment systems

3.1 Introduction

An extensive taxonomic revision of ammonia oxidizing bacterial phylogeny based on studies of 16S rRNA sequences reclassified these bacteria into two distinct groups: one group within the *Gammaproteobacteria* including the genus *Nitrosococcus* with two described species, *Nitrosococcus oceani* and *Nitrosococcus halophilus*, isolated respectively from marine and salt lake environments; and the second group, within the *Betaproteobacteria*, encompassing two genera, *Nitrosomonas* and *Nitrospira*, respectively (Head *et al.*, 1993; Purkhold *et al.*, 2000; Purkhold *et al.*, 2003).

Members of the *Nitrosomonas* group are classified into six lineages and one yet undefined AOB lineage known as *Nitrosomonas* cluster 5. The *Nitrosomonas oligotropha*, *Nitrosomonas marina*, *Nitrosomonas europaea*/*Nitrosococcus mobilis*, *Nitrosomonas communis*, *Nitrosomonas* sp. Nm143, and *Nitrosomonas cryotolerans* lineages include 16 cultured representatives (Stephen *et al.*, 1996; Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Koops and Pommerening-Röser, 2001; Purkhold *et al.*, 2003; Koops *et al.*, 2003). To some degree these lineages relate to differences in the ecology and physiological properties of their cultured representatives, such as salt requirements, affinity for ammonia (K_s) and urease activity (Koops and Pommerening-Röser, 2001; Koops *et al.*, 2003). *Nitrosomonas* cluster 5 is unusual in that it contains no known cultured ammonia oxidizing bacteria but only sequences recovered directly from the environment. The *Nitrosomonas* cluster 5 is formed by sequences from polluted marine environments, coastal sand dunes and freshwater environments (Stephen *et al.*, 1996; McCaig *et al.*, 1999; Speksnijder *et al.*, 1998; Kowalchuck *et al.*, 1997; Urakawa *et al.*, 2006).

The *Nitrosomonas oligotropha* lineage comprises two species, *Nitrosomonas ureae* and *Nitrosomonas oligotropha*, and many taxa only known from 16S rRNA sequences recovered from natural environments. The *Nitrosomonas marina* lineage includes *Nitrosomonas marina* and *Nitrosomonas aestuarii*. The *Nitrosomonas communis* lineage is divided into two sublineages, represented respectively by *Nitrosomonas communis*, and *Nitrosomonas nitrosa*. The *Nitrosomonas europaea/Nitrosomonas mobilis* lineage comprises four cultured species: *N. europaea*, *N. eutropha*, *N. mobilis* and *N. halophila*. The *Nitrosomonas* Nm143 lineage includes the estuarine isolate *Nitrosomonas* sp. Nm143 together with other three marine isolates, and the the *N. cryotolerans* lineage is represented by the *Nitrosomonas cryotolerans* sp. Nm55.

The genus *Nitrospira* was formed from the genera *Nitrospira*, *Nitrosolobus* and *Nitrosovibrio* (Head *et al.*, 1993). Within the genus *Nitrospira*, five clusters have been defined; 0, 1, 2, 3 and 4 (Stephen *et al.*, 1996; Purkhold *et al.*, 2000).

AOB which are members of the *Betaproteobacteria* in general have been reported as the most abundant ammonia oxidizing bacteria found in both domestic and industrial wastewater treatment plants (Snaidr *et al.*, 1997; Juretschko *et al.*, 2002; Bramucci *et al.*, 2003; Kapley *et al.*, 2007; Figuerola and Erijman, 2007). With respect to betaproteobacterial ammonia oxidizers, the genus *Nitrosomonas* is particularly prevalent in nitrifying wastewater treatment plants (Purkhold *et al.*, 2000). However, with the exception of *Nitrosomonas* cluster 5 and *Nitrosomonas* Nm143, members of all defined lineages within the genus *Nitrosomonas* have been detected in wastewater treatment plants: for example, *N. europaea*, *N. mobilis*, *N. eutropha* and *N. halophila* (Juretschko *et al.*, 1998; Rowan *et al.*, 2003a); *N. oligotropha*, *N. cryotolerans*, *N. communis* and *N. nitrosa* (Gieseke *et al.*, 2001; Limpiyakorn *et al.*, 2005; Layton *et al.*, 2005; Figuerola and Erijman, 2010); and *N. marina* (Purkhold *et al.*, 2000; Nicolaisen and Ramsing, 2002). However, from the data available it is not possible to say if these actual species were detected, but only that the species detected belonged to each of these lineages.

Members of the genus *Nitrosospira* have rarely been reported from WWTPs, but have been detected in a denitrifying-nitrifying-batch reactor treating refinery wastewater (Ballinger *et al.*, 1998) and in a membrane-bound biofilm (Schramm *et al.*, 2000). In one case they were the predominant AOB population in a laboratory fluidized bed reactor (Schramm *et al.*, 1998). *Nitrosospira* spp. is more commonly found in soil (Stephen *et al.*, 1998) and rhizosphere environments (Kowalchuk *et al.*, 1998; El Haleem *et al.*, 2000). *Nitrosospira* spp. also appear to be important AOB in marine environments (Freitag and Prosser, 2004; Ward *et al.*, 2007).

Different reactors or wastewater treatment plants (WWTPs) can harbour one abundant AOB population or several AOB populations. The diversity of AOB communities in WWTPs may be a function of environmental conditions or their variability, leading to the occurrence of different organisms best able to cope with the prevalent conditions at any point in time (Geets *et al.*, 2006; Wittebolle *et al.*, 2008). For instance, environmental parameters such as pH, ammonia levels, salinity, and dissolved oxygen are important factors affecting AOB and their performance in wastewater treatment systems (Prosser, 1989; Suwa *et al.*, 1994; Prinčič *et al.*, 1998; Kowalchuk and Stephen, 2001; Lydmark *et al.*, 2007). Furthermore while members of the *N. europaea*/*N. mobilis*/*N. eutropha* lineage have been reported most frequently in reactors treating wastes containing higher ammonia and salinity levels (Juretschko *et al.*, 1998; Rowan *et al.*, 2003a), *Nitrosomonas oligotropha* has been more commonly detected in plants treating wastewater with lower ammonia levels, and is also believed to tolerate low oxygen conditions (Limpiyakorn *et al.*, 2005; Otawa *et al.*, 2006; Park and Noguera, 2007).

There have been few studies of the ammonia oxidizing bacterial populations in oil refinery wastewater treatment systems (Ballinger *et al.*, 1998; Figuerola and Erijman, 2007, Figuerola and Erijman, 2010). This gap in our knowledge has been addressed in the present study where the AOB populations present in five full- scale WWTPs (one trickling filter and four activated sludge plants) treating oil refinery wastewater were characterized. The different communities are compared and discussed with respect to their diversity and function in relation to the conditions in each plant.

3.2 Methods

AOB populations in five oil refinery wastewater treatment plants - one trickling filter and four activated sludge systems - were investigated and compared.

Several chemical analyses were performed *in situ* at the time of sampling. pH, temperature, dissolved oxygen (DO), conductivity and temperature were measured on site, and chemical oxygen demand (COD), biochemical oxygen demand (BOD), total Kjeldahl nitrogen (TKN), Ammonia, nitrate, chloride, salinity, and mixed liquor volatile suspended solids (MLVSS) were measured in the laboratory shortly after sampling. These methods are described in more detail in Chapter 2.

AOB 16S rRNA gene sequences were detected by PCR using CTO primers, 189f/654r (Kowalchuck *et al.*, 1997). Nested amplification was required after a first round of PCR amplification in the case of some sludge samples, using general bacterial primers pA and pH (Edwards *et al.*, 1989).

The amplified 16S rRNA gene products were analyzed by both DGGE and by cloning and sequencing. Three replicate samples were collected from each reactor on every sampling occasion. DNA was extracted independently from all replicate samples and subject to DGGE analysis of amplified AOB 16S rRNA gene fragments to determine the degree of variation in the AOB communities in the replicate samples. If the DGGE profiles were highly similar a single sample was selected as representative of all replicates and used for construction of 16S rRNA gene clone libraries.

Replicate samples from all plants were subject to DGGE on a single gel and the gel was analyzed using Bionumerics (Applied Maths, St. Martens-Latem, Belgium). DGGE profiles were numerically compared using the Pearson product moment correlation coefficient, a method directly applied to the array of densitometric values (curve) forming the profile.

The resulting matrix of similarity values was then used to generate dendograms using the unweighted pair-group method with arithmetic averages (UPGMA). Standard statistics were applied to data extracted from the matrix of similarity values to assess the significance between different groups of community profiles.

The following metrics were calculated from rank abundance data that were used to summarize the composition of 16S rRNA gene clone libraries: Coverage (C) Shannon index (H'), Simpson's index (D), and non parametric abundance-based coverage estimators Chao1 and ACE.

To test if the 16S rRNA genes in clone libraries are likely to be sampled from the same community, libraries were compared by using the improved β -LIBSHUFF (Schloss *et al.*, 2004) algorithm. The β -LIBSHUFF algorithm and all the metrics mentioned above are described in more detail in Chapter 2.

The 16S rRNA gene sequences from each clone library were de-replicated using FastGroupII. Sequences were assigned to operational taxonomic units (OTU) on the basis of a cut off of 97% identity between sequences, and representative sequences from each OTU identified from analysis with FastGroupII were selected for phylogenetic analysis. Only high quality sequences were used for subsequent phylogenetic analysis.

A phylogenetic tree was constructed, using the ARB software (Ludwig *et al.*, 2004) with 44 representative sequences from all recognized betaproteobacterial AOB lineages; these also included the nearest neighbours of the refinery WWTP sequences from this study found in Genbank. Sequences with > 1000 bp, were imported into ARB, and aligned using the ARB automated sequence aligner, and manually checked according to the rRNA secondary structure model. After that, a total of 126 selected sequences recovered with CTO primer in this study were added to the constructed tree using the quick parsimony tool in ARB for adding shorter sequences, without interfering with the overall topology of the tree built with long sequences.

Parsimony trees were built in ARB and sequences from ARB were exported to build distance trees in PHYLIP. For both parsimony and distance trees 100 bootstrap resamplings were done and consensus trees were constructed. Bootstrap values above 50% supported by either or both neighbour-joining and parsimony were shown on trees.

The bootstrap analyses were applied to small datasets of long sequences (>1000 bp) only and small datasets including reference sequences and partial sequences from this study. For example, resampling analyses from sequences within the AOB tree built in ARB (Figure 3.10) were carried out separately for each of three AOB lineages, respectively *N. oligotropha* (Appendix A, Figures A3 and A4) *N. marina* (Appendix A, Figures A5 and A6) and *N. communis* (Appendix A, Figures A7 and A8).

3.3 Results and Discussion

3.3.1 Differences in wastewater parameters among the oil refinery wastewater treatment systems

Several process parameters were measured in five wastewater treatments plants in this study: Lindsey (trickling filter system), Eastham, Pembroke, Humber and Grangemouth (activated sludge systems).

The Lindsey treatment plant was sampled twice in September and October of 2005 respectively; while the Eastham and Pembroke plants were sampled only once, in June of 2006. The Humber plant was sampled four times, in June, October and November of 2006 and January of 2007 respectively, and Grangemouth three times, in July of 2006, February and April of 2007.

The process parameters measured in the field (Table 3.1) and the wet chemistry determinations made in the laboratory (Table 3.2) from samples collected from the wastewater treatment plants are described and discussed below. In this chapter only parameters measured in the influent and effluent from the wastewater treatment plants are reported. The performance of each WWTP in terms of organic loading reduction and nitrification efficiencies are presented and discussed in more detail in Chapter 5.

In relation to the overall variability of the parameters measured in the WWTPs investigated in this study, some differences were observed between plants:

In contrast to the other plants, pH levels recorded in Lindsey (7.56 and 7.76; Table 3.1) and Grangemouth (8.17, 8.40 and 7.14; Table 3.1) respectively were higher than the pH in Eastham (6.29), Pembroke (6.20) and Humber plants (ranging from 4.65 to 6.93; Table 3.1).

In general, the temperature of influent across plants ranged between 22.3 and 34.9° C, apart from lower values found respectively at Eastham (18.5° C) and Grangemouth (16.4°C) sampled for the second time in February of 2007 (Table 3.1). Coincidentally, these plants had been flooded by rainwater one day before sampling, and by the time of sampling the wastewater treatment system was not operating within normal process limits. This can also explain the lowest values of conductivity found in Eastham (474 µS) and Grangemouth (492 µS) in contrast to the very high values found in Lindsey, Pembroke and Humber (ranging from 2340 to 2716 µS). Despite this low value in Grangemouth, as shown on other sampling occasions, the influent to the Grangemouth plant generally had low conductivity (594 and 921µS, at other sampling times) in relation to the other plants.

Dissolved oxygen (DO) values were in the range of 0.7 to 4.5 mg/L. Values close to both extremes were recorded on different sampling dates in Lindsey (respectively 4.5 and 1.0 mg/L) and Humber (ranging from 0.7- 4.3 mg/L) while in Grangemouth, DO was more consistent and in the range of 3.0- 4.0. Values recorded in Eastham (1.8 mg/L) and Pembroke (2.8 mg/L) were lower than the range of values found in Grangemouth, but still higher in relation to the minimum values found in Lindsey and Humber. See Table 3.1.

Table 3.1 Process parameters measured *in situ* in five oil refinery wastewater treatment plants in the UK

WWTP	Sampling dates	pH		Temperature ° C		Conductivity (µS)		DO (mg/L)	
		Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Lindsey	07.09.2005	7.56	7.45	29.7	26.6	2360	2333	4.5	3.82
	07.10.2005	7.76	7.38	22.3	20.0	3263	3423	1	1.01
Eastham	01.06.2006	6.29	6.84	18.5	19.2	474	850	1.8	6.3
Pembroke	09.06.2006	6.20	7.21	31.5	30.7	2340	2360	2.8	2.0
Humber	21.06.2006	5.37	7.71	28.7	27.1	2280	2483	0.7	6.7
	6.10.2006	6.74	7.57	28.2	28.5	2553	2883	4.3	4.6
	16.11.2006	4.65	4.50	29.0	27.1	2756	2764	2.7	3.7
	8.01.2007	6.93	7.58	24.5	23.2	2716	3044	3.1	4.3
Grangemouth	26.07.2006	8.17	7.35	34.9	35.8	594	1079	3.0	3.2
	28.02.2007	8.40	7.57	16.4	18.3	492	1000	4.0	5.4
	2.04.2007	7.14	7.00	31.1	29.9	921	944	3.7	2.7

COD (ranging from 139 to 1456 mg/L; Table 3.2) was higher than BOD (ranging from 7 to 419 mg/L; Table 3.2) in all plants.

BOD/COD ratios across the refinery wastewater treatment plants ranged from 0.04 to 0.86. Typical BOD/COD ratios in municipal wastewater are ranged between 0.3 and 0.8, and when around 0.5 or above (Metcalf and Eddy, 2003) these wastes are easily treatable biologically. In contrast, however, much lower BOD/COD ratios found in the refinery wastewaters showed their higher recalcitrance, in that only a small fraction of the organic carbon is readily biodegradable.

In comparison to the other plants, influent to Lindsey and Grangemouth had the lowest COD and BOD values. In Lindsey, values of COD on both sampling dates were respectively 270 mg/L and 190 mg/L; and those of BOD were 36 and 7 mg/L (Table 3.2). In Grangemouth, COD ranged from 139 to 311 mg/L; and BOD from 70 to 120 mg/L (Table 3.2). Values of COD (352 mg/L) and BOD (36 mg/L) found in the influent to Eastham were also similar to values in Lindsey and Grangemouth. In contrast, influent to Humber had higher COD (610 -952 mg/L) and BOD (246- 419 mg/L).

The highest COD (1456 mg/L) and BOD (233 mg/L) values were reported in influent to the Pembroke plant. However, these can be considered outliers and may have occurred as the result of some uncontrolled chemical waste stream lines that were flowing into the influent to be treated, due to problems with one of the controlling valves reported by the plant operator at the time of sampling.

Table 3.2 Wet chemistry data determined in five oil refinery wastewater treatment plants in the UK

WWTP	Sampling dates	COD (mg/L)		BOD (mg/L)		TKN (mM)		NH ₃ (mM)		NO ₃ ⁻ (mM)		Salinity (%)		MLSS (mg/L)
		Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	From Inside reactors
Lindsey	07.09.2005	270	131	36	7	2.33	1.63	0.73	0.24	0.00	0.73	0.67	0.83	
	07.10.2005	190	127	7	0	1.06	0.30	0.51	0.00	0.00	0.55	1.09	1.17	
Eastham	01.06.2006	352	36	178	24	1.95	2.85	1.60	2.34	0.00	0.0003	0.08	0.10	710 (A)* 110 (B)
Pembroke	09.06.2006	1456	233	102	46	2.00	4.30	1.16	1.14	0.00	0.0032	0.72	0.81	906
Humber	21.06.2006	952	120	344	22	2.33	0.27	1.39	0.00	0.00	0.25	0.31	0.27	5406
	6.10.2006	834	149	419	6	2.93	0.00	1.27	0.00	0.008	1.32	0.45	0.47	10092
	16.11.2006	610	112	246	2	3.40	0.40	1.15	0.00	0.00	1.52	1.01	0.91	8813
	8.01.2007	700	99	378	4	2.80	0.00	0.93	0.00	0.00	1.56	0.80	0.85	9828
Grangemouth	26.07.2006	311	28	113	5	1.13	0.00	0.73	0.00	0.00	0.56	0.11	0.13	4758
	28.02.2007	177	49	70	3	0.00	0.00	0.06	0.00	0.04	0.001	0.13	0.17	6128
	02.04.2007	139	24	120	6	0.00	0.00	0.25	0.00	0.009	0.22	0.46	0.18	6051

* Refer to reactors A and B working in series

Ammonia concentrations (Table 3.2) in influent across the plants ranged from 0.25 to 1.60 mM; excluding a very low value (0.06 mM) corresponding to influent to Grangemouth sampled for the second time in February of 2007. This corresponded to the time when the wastewater system in Grangemouth had been flooded with rainwater as a result of a strong storm one day before sampling, and the plant was therefore not operating under normal conditions. This undoubtedly contributed to the low ammonia concentrations as well the low values for other parameters measured in the influent at this time. Similar ammonia concentrations (0.73 mM; Table 3.2) were recorded respectively in the influent to Lindsey (September of 2005) and Grangemouth (July of 2006) when the first samples were taken from these plants. In contrast, higher ammonia concentrations were found in the Humber plant (0.93-1.39 mM; Table 3.2). Ammonia in influent to Pembroke (1.16 mM; Table 3.2) was also similar to values found in Humber, and the highest ammonia concentration was found in influent to Eastham (1.60 mM; Table 3.2).

TKN concentrations across plants ranged from 0 to 3.40 mM (Table 3.2) with lower values being recorded in the influent to Grangemouth in contrast to higher values recorded in the influent to the Lindsey (2.33 and 1.06 mM; Table 3.2) and Humber (2.33- 3.40 mM: Table 3.2) reactors respectively. TKN concentrations in the influent to Eastham (1.95 mM; Table 3.2) and Pembroke (2.00 mM; Table 3.2) were relatively similar. Typical ammonia and TKN concentrations in municipal wastewater based on a flowrate of 450L/capita.d are generally found in the range from 1.78 mM to 3.21 mM as nitrogen (Metcalf and Eddy, 2003).

In oil refinery wastewater treatment systems TKN, in contrast to ammonia, is not a parameter commonly measured in influent, however, some data provided by the Humber plant operators indicated a period of time between September 2002 to August 2006 when TKN concentrations in the influent ranged from 0.39 to 8.1 mM (average 2.06 mM).

In contrast to the other plants, Pembroke and Eastham were not nitrifying. Ammonia concentrations in the effluent of these plants were respectively 2.34 and 1.14 mM; and TKN, respectively, was 2.85 and 4.30 mM (Table 3.2); while nitrate concentrations found in the effluent from these plants were close to zero. In effluent from plants that were nitrifying, that is Lindsey, Grangemouth and Humber (See Table 3.2) nitrate concentrations ranged from 0.22 up to 1.56 mM; higher concentrations of nitrate were recorded in effluent from the Humber plant (1.32-1.56 mM) compared to the Lindsey (0.55 and 0.73 mM) and Grangemouth (0.22 and 0.56 mM) plants. Pembroke and Eastham reactors also had the lowest values of MLSS at 906 mg/L in Pembroke; and in the case of Eastham, in which reactors were working in series and not in parallel as in the other activated sludge systems sampled, MLSS values were respectively 710 and 110 mg/L in the first and second reactor; Table 3.2.

In activated sludge systems that were nitrifying, high MLSS were reported; in Grangemouth reactors' values ranged from 4758 to 6128 mg/L; and the Humber reactors ranged from 5406 to 10092 mg/mL (Table 3.2). These high MLSS values also coincided with better performances in BOD removal in Humber and Grangemouth. Similarly, the low MLSS values in the Eastham and Pembroke reactors also matched up to the worst performance on BOD removal in these plants (See Section 5.3.1, Chapter 5).

The MLSS concentrations found in oil refineries' wastewater can be considered relatively high in comparison to other types of wastewater reported in the literature. For instance, MLSS ranged between 1550 -3678 mg/L in some municipal wastewater treatment plants (Coskuner *et al.*, 2005; Pickering, 2008; Limpiyakorn *et al.*, 2005) while they ranged from 1942 to 4242 mg/L in a full scale plant treating dairy wastewater (Ottawa *et al.*, 2006).

The overall salinity recorded across plants was large and ranged from 0.08 to 1.09% (Table 3.2). Salinity was low in influent to Grangemouth (0.11-0.43%) and Eastham (0.08; the lowest value found) while high in Lindsey (0.67 and 1.09%) and Pembroke (0.72%). In Humber, salinity variation was large including both low and high values (0.31- 1.01%; Table 3.2).

Interestingly, one of the potential sources contributing to salinity in oil refinery wastewater systems comes from the desalter effluent water used during the crude oil washing process, used to remove inorganic salts and suspended solids contained in the oil before it can be fractionated; and this wastewater also goes for biological treatment.

In general, analysing the data as a whole, a few characteristic features of each wastewater treatment system could be discerned:

- The Humber, Grangemouth and Lindsey plants were actively nitrifying while the Pembroke and Eastham plants were not nitrifying;
- Eastham and Grangemouth received influent with lower salinity while Lindsey, and Pembroke received influent with higher salinity;
- Grangemouth, Lindsey and Eastham received influent with lower organic loading, in contrast to Humber and Pembroke which received influent with higher organic loading;
- Lindsey and Grangemouth received influent with lower ammonia concentrations and Eastham, Pembroke and Humber received influent with higher ammonia concentrations;
- TKN concentrations in influent to Grangemouth were very low in relation to the normal range found on the other systems.
- Influent pH to Grangemouth and Lindsey was alkaline, in contrast with the slightly acid pH in the influent to Humber, Eastham and Pembroke;
- Eastham and Pembroke activated sludge systems that were not nitrifying had low MLSS, in contrast to Humber and Grangemouth activated sludge systems that were nitrifying with high MLSS.
- Humber influent could also be distinguish from the others on the basis of higher organic carbon, nitrogen, and MLSS concentrations, as well as higher nitrate concentrations in the effluent, and large variations in dissolved oxygen and salinity.

3.3.2 PCR- amplification of 16S rRNA genes

DNA extracted from the suspended solids from each plant was first amplified with general bacterial primers (See Chapter 2) and the PCR products obtained were subject to nested amplification with CTO primers selective for betaproteobacterial AOB (Kowalchuck *et al.*, 1997). DNA extracted from Eastham and Pembroke MLSS, which was previously concentrated before DNA extraction due to their low MLSS, was directly amplified with CTO primers. Figure 3.1 illustrates the results of PCR from all plants.

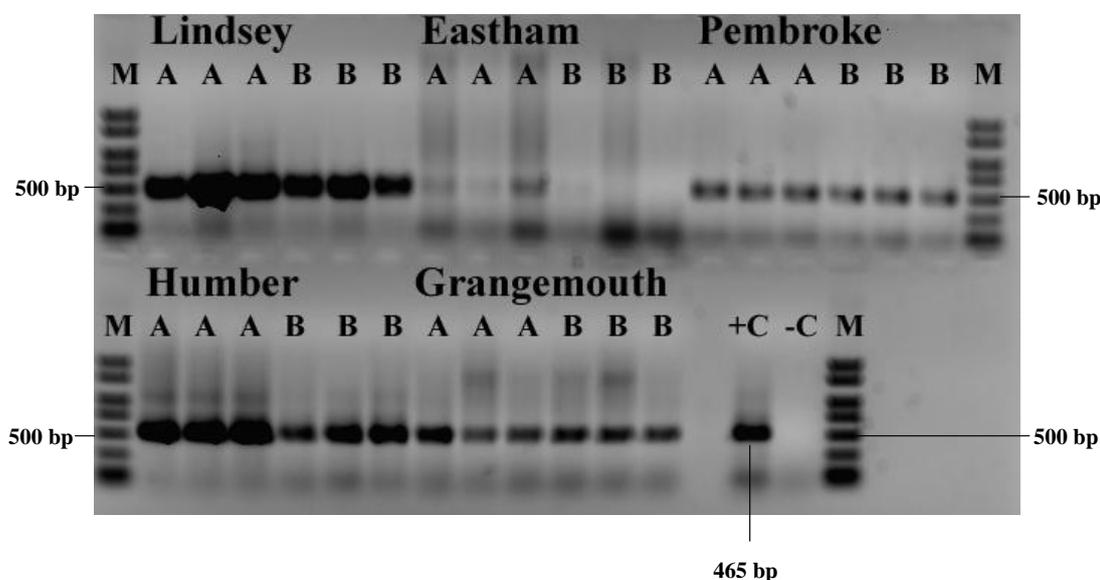


Figure 3.1 Agarose gel showing PCR products amplified from DNA extracted from samples collected in five oil refinery wastewater treatment plants in the UK with primers CTO189f and CTO 654r: one trickling filter (Lindsey), and four activated sludge systems (Eastham, Pembroke, Humber and Grangemouth). PCR products from Eastham and Pembroke originate from direct amplification with CTO primers, and PCR products from Lindsey, Humber and Grangemouth originate from nested PCR amplification of PCR products obtained firstly from amplification with general eubacterial primers pA-pH. M= DNA molecular weight marker (2000 bp- 50 bp from top to bottom); A= Reactor A; B= Reactor B; +C= environment sample positive for the expected fragment size of 465 bp; -C= only PCR reagents mixture containing no DNA template. Samples refer to the following sampling dates: Lindsey= 07.09.2005; Eastham= 01.06.2006; Pembroke= 09.06.2006; Humber= 21.06.2006 and Grangemouth= 26.07.2006.

The 16S rRNA gene PCR products obtained from replicate samples from all plants were analysed using denaturing gradient gel electrophoresis (DGGE). The DGGE profiles obtained are shown in Figure 3.2 where AOB communities in each plant were compared to one another.

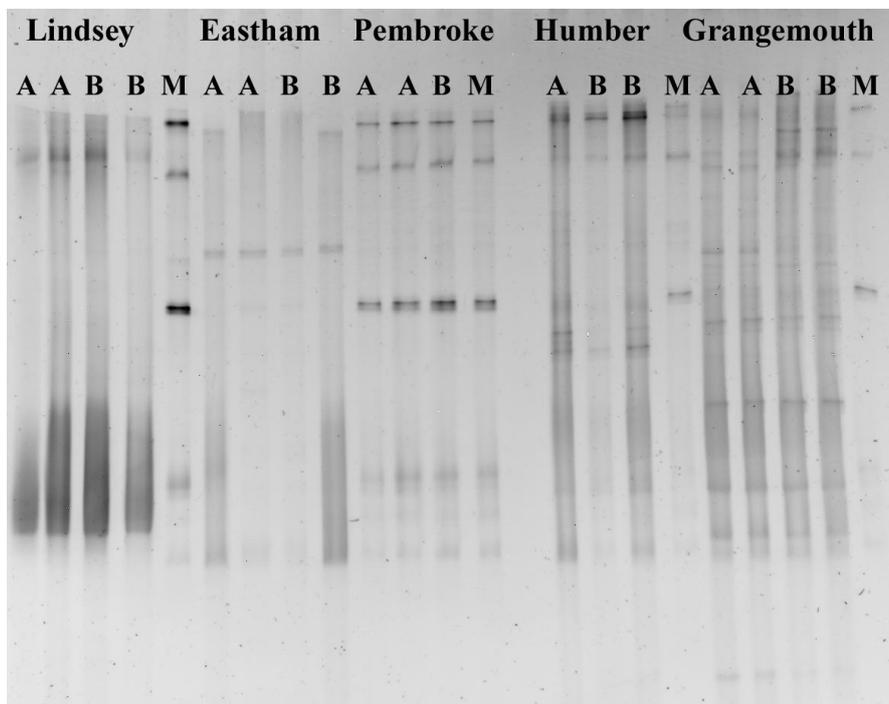


Figure 3.2 Comparison between DGGE profiles of 16S rRNA gene fragments from AOB communities (PCR products amplified with CTO189f and CTO654r primers) from samples collect in five oil refinery wastewater treatment plants in UK: one trickling filter (Lindsey) and four activated sludge systems (Eastham, Pembroke, Humber and Grangemouth). A= reactor A; B= reactor B; M= one replicate sample from Pembroke used as marker on the gel. Samples refer to the following sampling dates: Lindsey= 07.09.2005; Eastham= 01.06.2006; Pembroke= 09.06.2006; Humber= 21.06.2006 and Grangemouth= 26.07.2006.

3.3.3 Interpretation and statistical analysis of DGGE profiles

DGGE profiles were compared using the band-independent Pearson product moment correlation coefficient and the unweighted pair group clustering method with arithmetic averages (UPGMA).

The Pearson product moment coefficient is considered as more suitable for analysis of DNA fingerprints than band-based methods (Rademaker and Bruijn, 2004) since analysis of the whole densitometric curve profiles are more objective than the more subjective assignment of bands; it also takes into account differences in band intensity.

Cluster analysis was performed to analyse the relationships within and between wastewater treatment plants. The resultant dendrogram showed that AOB community profiles were distinct in different plants. Replicates from the same WWTP (Figure 3.3), with the exception of one replicate from Lindsey reactor B, grouped together in five distinct clusters at resemblance levels above 0.5 (Figure 3.3). This clustering also demonstrated that analysis of replicate samples from each plant was reproducible. The profiles from the Lindsey reactor clustered with those from the Humber reactor and Grangemouth profiles clustered with Eastham profiles, and both groups clustered to each other at a similarity level of 0.1 (Figure 3.3). Samples from the Pembroke plant appeared in a separated cluster unrelated to the others (Figure 3.3).

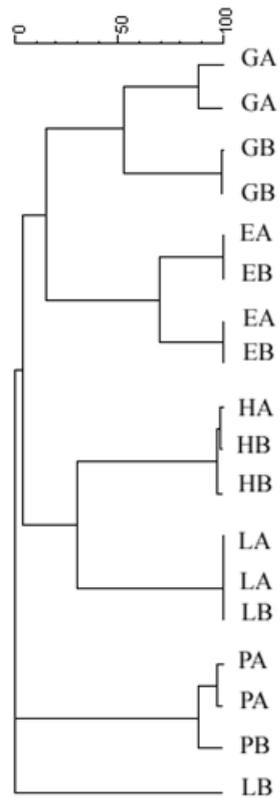


Figure 3.3 UPGMA cluster analysis of DGGE profiles of uncultured betaproteobacterial AOB 16S rRNA gene fragments obtained from samples collected from five oil refinery wastewater treatment plants in the UK: one trickling filter (L= Lindsey) and four activated sludge (E= Eastham, P= Pembroke, H= Humber and G= Grangemouth) after Pearson correlation of whole-track densitometric curves of the profiles. Letters A and B after plant designations refer to replicates from reactors A and B from their respective wastewater treatment plants. Samples correspond to the following sampling dates: Lindsey= 07.09.2005; Eastham= 01.06.2006; Pembroke= 09.06.2006; Humber= 21.06.2006 and Grangemouth= 26.07.2006.

Although cluster analysis is a powerful tool for reducing data complexity and thus revealing patterns underlying molecular datasets (Van Verseveld and Röling, 2004; Ramette, 2007), conclusions can not be drawn from cluster analysis regarding the statistical significance of the clusters recovered.

Therefore analysis of variance was conducted on the matrix containing the resemblance values obtained through the Pearson correlation, to test if within- and between- plants similarities differed significantly from one another with respect to their AOB communities.

The data were not normally distributed and were arcsin transformed (the best transformation for proportion data). However, after transformation the data still were not normally distributed, and thus the Kruskal-Wallis non parametric ANOVA was performed. Kruskal-Wallis tests the equality of medians between two or more populations based on data from independent, random samples.

Similarity coefficient values from a data matrix measure the strength of association between objects. While a value of zero indicates that two profiles compared are totally dissimilar, a value of one indicates that they are identical. Zero similarity values were found between profiles from Eastham in relation to Lindsey, Humber and Pembroke; as well as Pembroke in relation to the rest. Therefore Eastham was considered distinct from Lindsey as well as Pembroke distinct from the rest. In addition, the replicate from Lindsey reactor B that was recovered on its own in the UPGMA dendrogram was considered as an outlier. Although this sample (Figure 3.2) seems to be visually similar to the other replicates from Lindsey, it is possible that differences in loading or the large smear at the bottom of the Lindsey profiles influenced the analysis and this anomaly might perhaps be removed by repeating the analysis with different parameters for extracting the densitometric curve data or to exclude the smear at the bottom of the gel from the analysis.

According to the Kruskal-Wallis test replicate profiles from the same plant were not statistically significantly different (p values ≥ 0.05). However, Lindsey and Humber were statistically different ($p= 0.018$), as well as Grangemouth in relation to Humber ($p= 0.023$) and Lindsey ($p= 0.012$), but not in relation to Eastham ($p= 0.153$).

Therefore Lindsey, Eastham, Pembroke, Humber and Grangemouth represented distinct plants, harbouring a different AOB community structure in relation to one another, with the exception of the AOB community in Eastham, which had a degree of similarity with that in the Grangemouth plant.

As discussed before in Section 3.3, in general, the wastewaters from the different systems were relatively distinct and to a certain degree they may explain the differences observed in relation to their AOB communities.

Although not statistically different, comparison of the AOB community profiles from Eastham and Grangemouth should be interpreted with caution. If one examines the gel, fewer bands (4) were present in the Eastham DGGE profiles (Figure 3.2) compared to the number of bands (12-14) present in the Grangemouth DGGE profiles. Moreover, although similarity coefficients between DGGE profiles from the different reactors were low (from 0 to 0.51; Figure 3.3) both Eastham and Grangemouth profiles also clustered together at a low similarity (around 0.1) in comparison to, for example Humber and Lindsey DGGE profiles, that in contrast clustered at higher similarity (0.3), but were statistically different from each other. Moreover, there is also a large smear in the two of the profiles from Eastham and this might have adversely affected the outcome of the analysis. Ideally, if the analysis had been reconducted omitting the smears at the bottom of the gel, perhaps it would have been helpful in resolving these incongruences between both DGGE profiles.

Although the result of the Kruskal-Wallis test indicates no significant difference in the profiles, the mean similarity within replicate samples from the same plant is higher than the similarity between the profiles from the Eastham and Grangemouth plants.

3.3.4 Comparison of AOB communities based on 16S rRNA gene clone libraries

DGGE data were obtained from several replicate MLSS samples. Profiles from replicate samples were reproducible and on this basis, eight 16S rRNA gene clone libraries were constructed from individual samples taken on two sampling occasions from the Lindsey reactor (September and October 2005 (Figure 3.4b and 3.4d respectively) one sample from Eastham (Figure 3.5b) one from Pembroke (Figure 3.5d) and samples from the Humber reactor taken in June 2006 (Figure 3.6b) and January 2007 (Figure 3.6d). The 16S rRNA gene clone libraries from the Grangemouth plant were derived from two reactors (A and B; Figures 3.7b and 3.7d respectively) sampled in July of 2006. In total 297 16S rRNA sequences were considered in all analyses carried out and the structure of each library is summarized in Table 3.3.

Rank abundance plots using an OTU cut off of 97% sequence identity showing the patterns of each clone library are presented on Figures 3.4, 3.5, 3.6 and 3.7, respectively.

While some libraries were dominated by a few abundant clones, for instance, Lindsey's first sampling occasion (Figure 3.4b), Eastham (Figure 3.5b), Pembroke (Figure 3.5d), Humber's fourth sampling occasion (Figure 3.6d) and Grangemouth reactor A (Figure 3.7b), others exhibited a more even clone distribution, for example, Lindsey's second sampling date (Figure 3.4d) Humber's first sampling date (Figure 3.6b) and Grangemouth reactor B (Figure 3.7d).

Table 3.3 Description of eight AOB 16S rRNA gene clone libraries constructed in this study

Libraries	Sampling Dates	Clones in library (N)	Number of OTUs/library	Most abundant OTU (clone numbers)	Number of Doubletons (2 OTUs)	Number of Singletons (1 OTU)
Lindsey	07.09.2005	24	7	17	1	5
Lindsey	07.10.2005	21	5	4	1	10
Eastham	01.06.2006	42	10	30	1	7
Pembroke	09.06.2006	42	9	27	None	6
Humber	21.06.2006	41	32	3	3	26
Humber	08.01.2007	46	19	11	3	13
Grangemouth A	26.07.2006	38	12	24	1	9
Grangemouth B		43	21	12	5	13

A= Reactor A; B= Reactor B

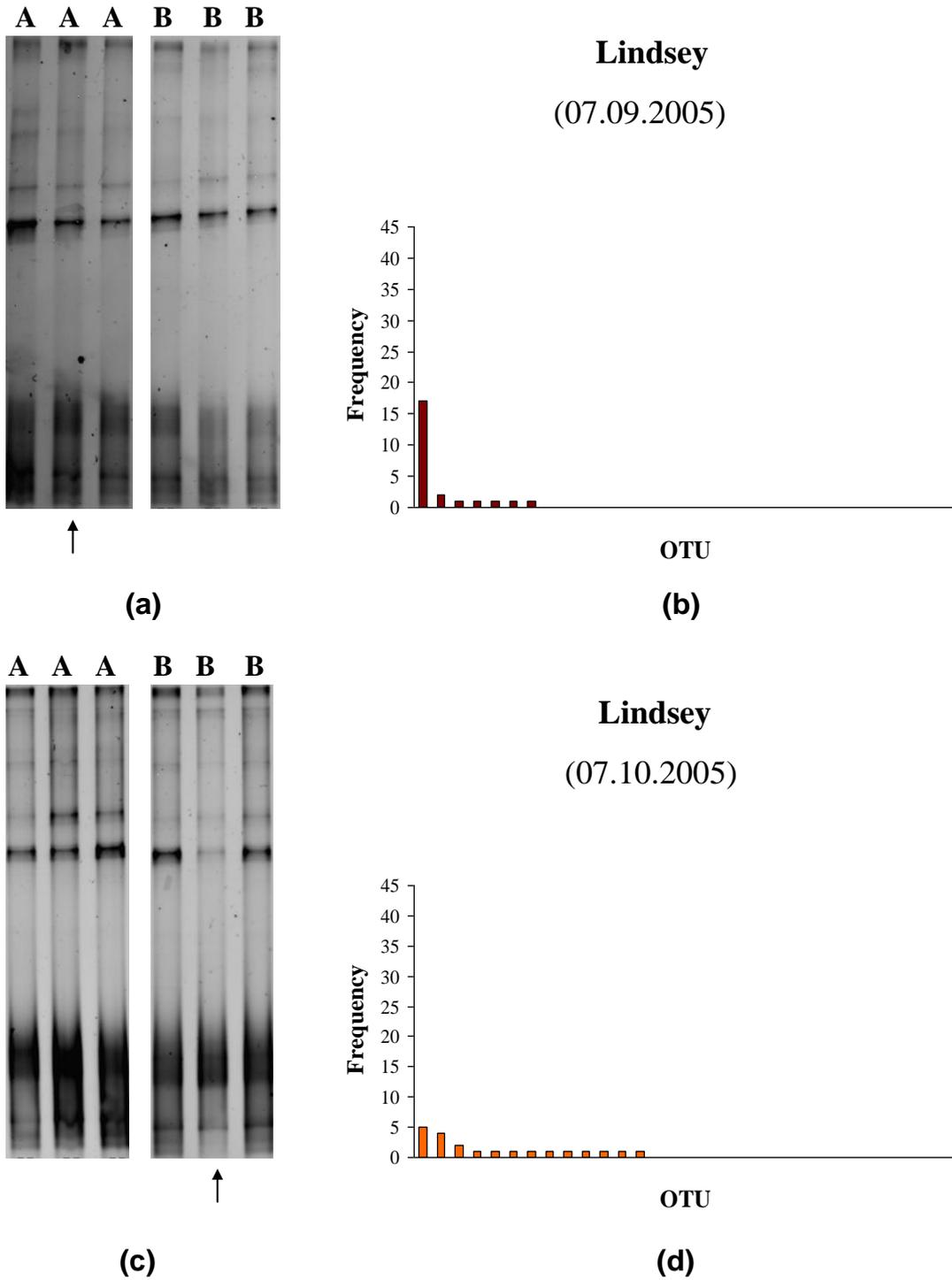


Figure 3.4 (a) and (c): DGGE profiles obtained from replicate samples collected from reactors A and B from the trickling filters system from the Lindsey refinery on two sampling occasions. Arrows on (a) and (c) indicate the replicates from which clone libraries were constructed and are represented by the rank abundance plots in (b) and (d).

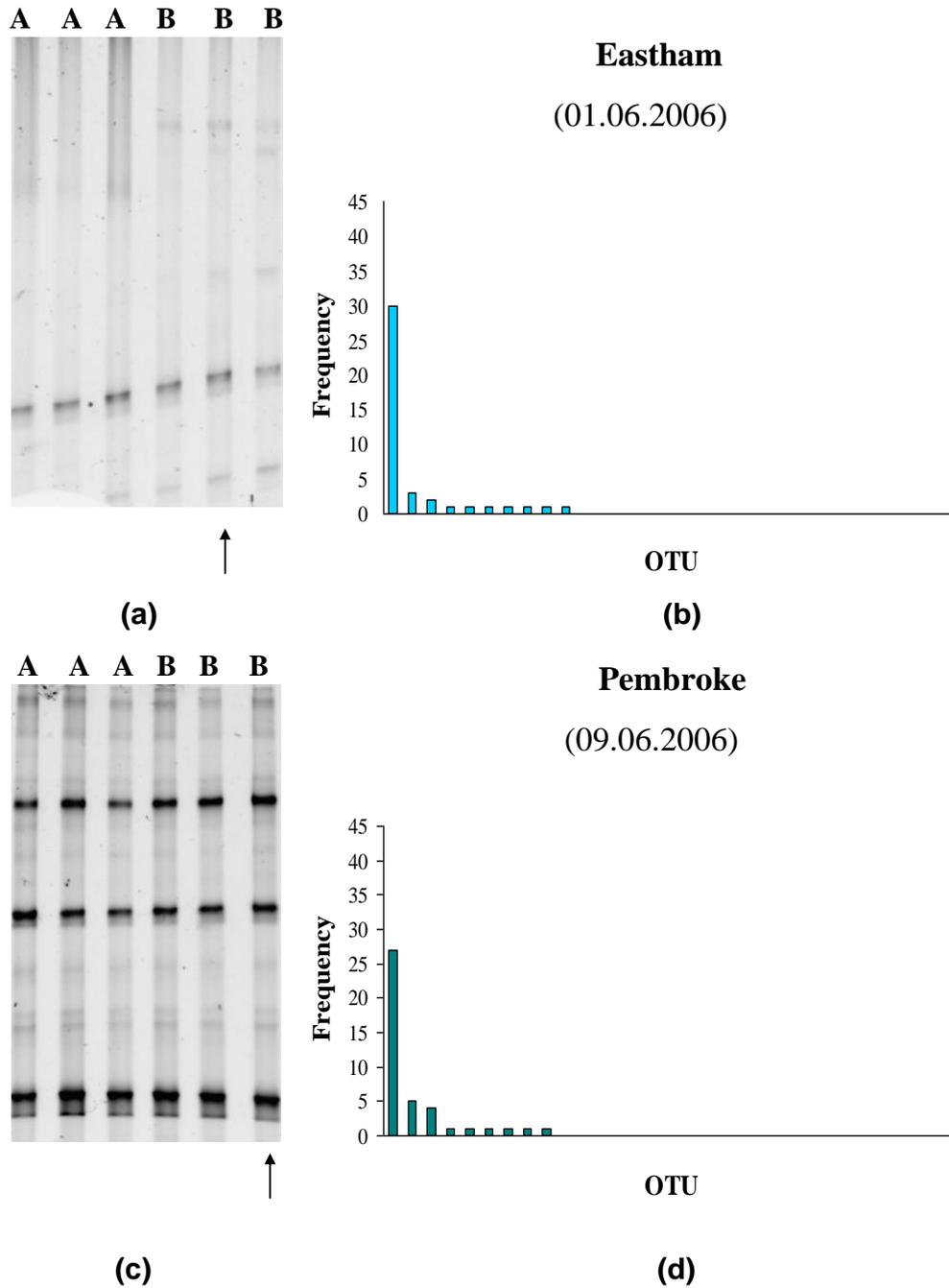


Figure 3.5 (a) and (c): DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge systems from the Eastham and Pembroke refineries. Arrows on (a) and (c) indicate the replicates from which clone libraries were constructed and are represented by the rank abundance plots in (b) and (d).

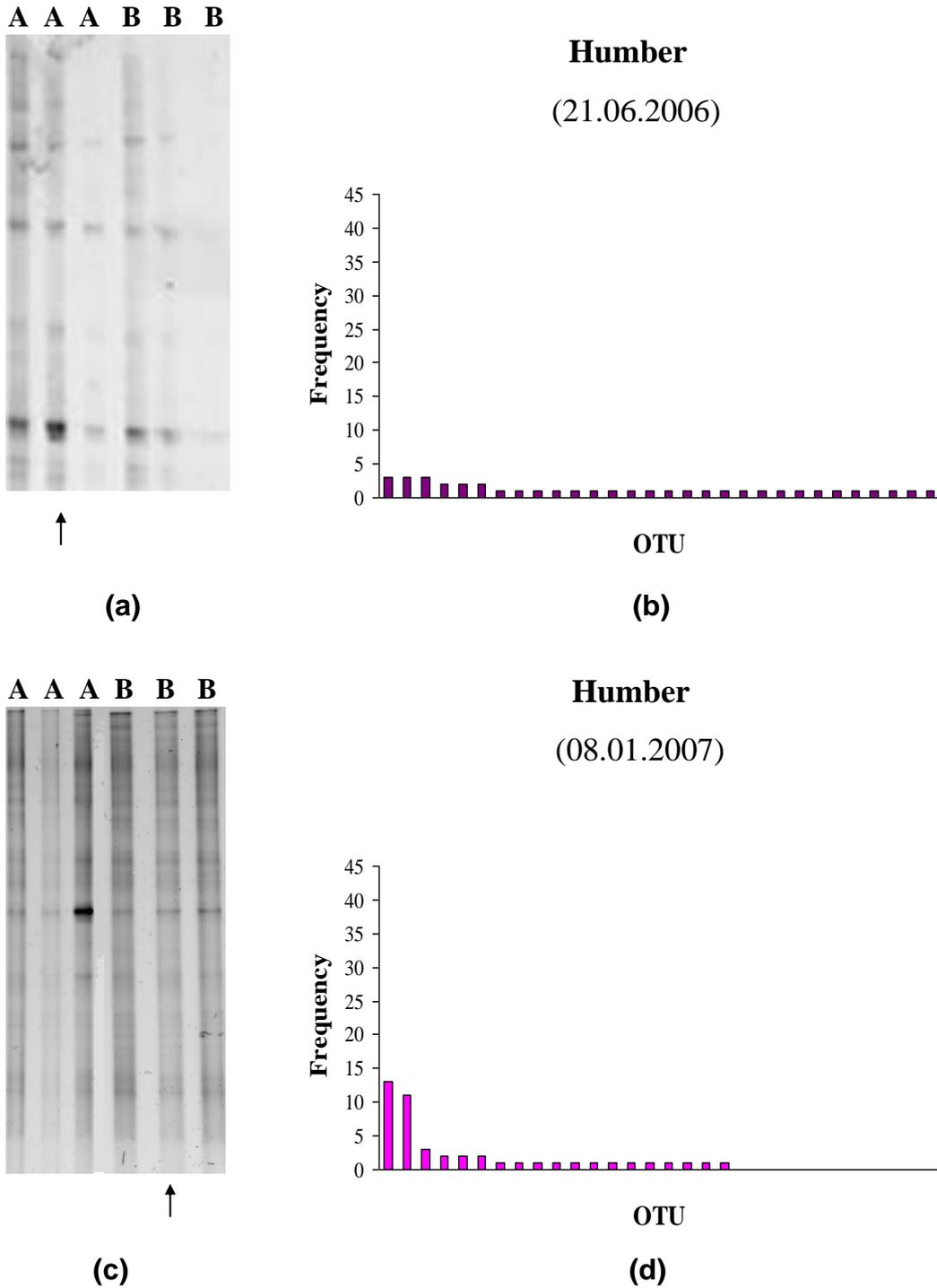


Figure 3.6 (a) and (c): DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge system from the Humber refinery on two sampling occasions. Arrows on (a) and (c) indicate the replicates from which clone libraries were constructed and are represented by the rank abundance plots in (b) and (d).

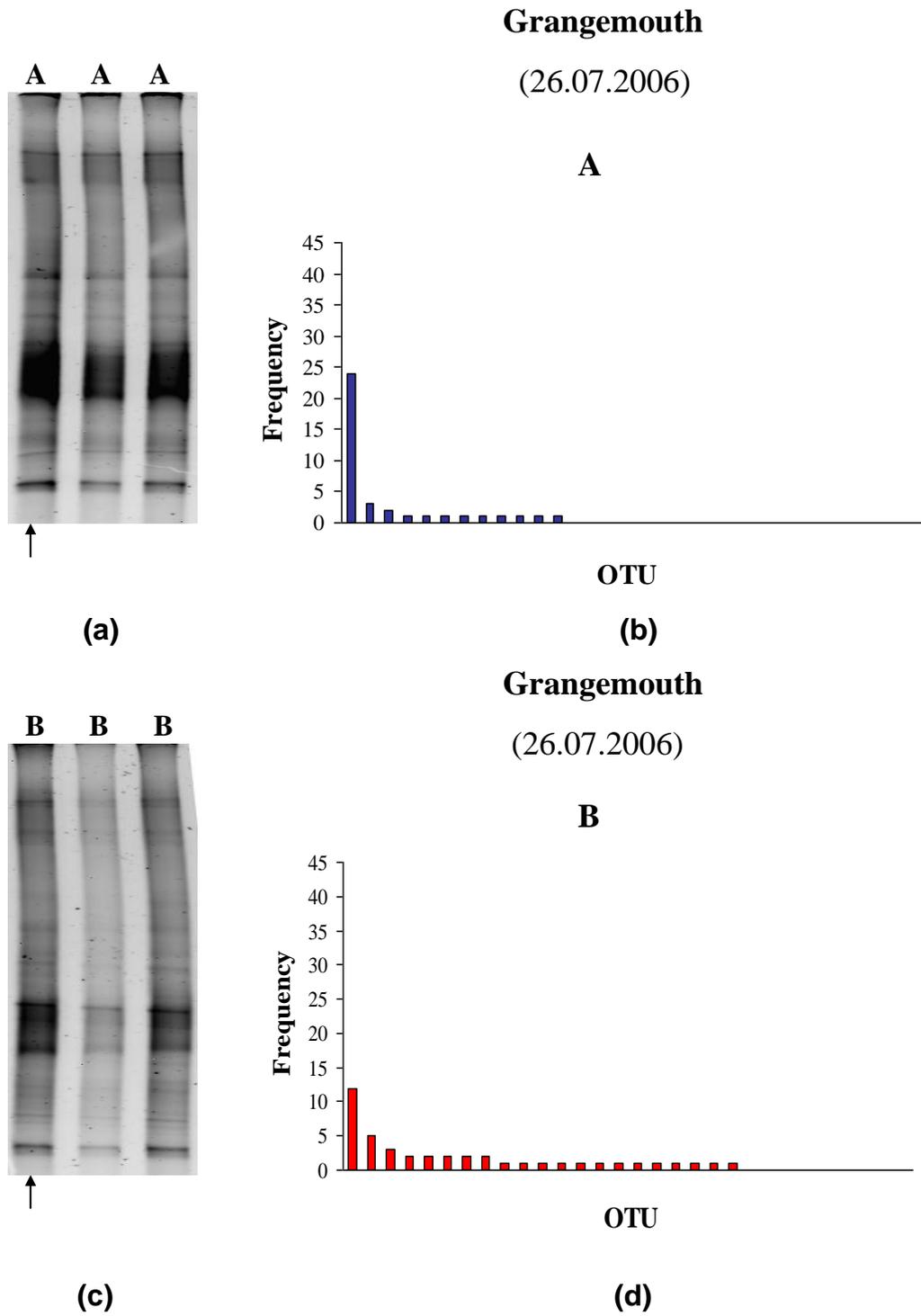


Figure 3.7 (a) and (c): DGGE profiles obtained from replicate samples collected from reactors A and B from the activated sludge system from the Grangemouth refinery. Arrows on (a) and (c) indicate the replicates from which clone libraries were constructed and are represented by the rank abundance plots in (b) and (d).

The Good's coverage, non parametric diversity estimators, and diversity indices, using both the Shannon index (H') and Simpson's index of diversity ($1-D$) were calculated for each library (Table 3.4).

The lower the coverage in a library, the higher is the diversity.

The overall coverage in libraries was high (ranging from 69.7% in Grangemouth reactor B to 85.7% in Pembroke). The two exceptions related to the first date on which the Humber treatment plant was sampled (36.6%: Table 3.4) and Lindsey's second sampling date (52.38%; Table 3.4).

Table 3.4 Coverage and diversity indices measured for eight AOB 16S rRNA gene clone libraries constructed in this study

WWTP	Sampling dates	Coverage (%)	Shannon	Simpson (1-D)
Lindsey	0.7.09.2005	79.16	0.86	0.51
Lindsey	07.10.2005	52.38	1.74	0.92
Eastham	01.06.2006	83.3	0.94	0.49
Pembroke	09.06.2006	85.7	1.08	0.57
Humber	21.06.2006	36.6	2.05	0.98
Humber	08.01.2007	71.7	1.95	0.90
Grangemouth A	26.07.2006	76.3	1.20	0.60
Grangemouth B	26.07.2006	69.7	2.14	0.91

Diversity is often expressed in the form of two measures: the number of OTUs present in a sample (richness) and their abundance distribution (evenness/dominance) in a sample (Hughes and Bohannon, 2004).

Diversity indices differ in the weight they give to richness or abundance respectively. While the Shannon index (H') is more affected by the number of rare types present in the library, Simpson's index of diversity ($1-D$) is more influenced by the most abundant OTUs in a library. All such indices have their strengths and weaknesses and it is therefore prudent to apply more than one measure to assess the diversity represented in a clone library.

Diversity indices summarize the diversity of a library in one single value. Therefore the higher the value the higher the diversity. The absolute values of diversity indices have little meaning when microbial communities are poorly sampled in clone libraries; however, they are useful in providing some indicative comparative numbers.

The lowest coverage of the clone library from Humber's first sampling date also corresponded to the highest Simpson's value found in the between-libraries comparison (0.98; Table 3.4). However, when the same reactor was sampled almost seven months later (Table 3.4) the clone library generated from the sample had coverage of 71.7% and diversity was slightly reduced (0.90; Table 3.4). The community composition changed completely between the two sampling dates, and this change was also in agreement with the apparent differences seen between the DGGE profiles that respectively relate to these libraries (Figures 3.6a and 3.6c).

Comparing reactors A and B from Grangemouth, the diversity expressed through the Shannon Index in reactor B (2.14; Table 3.4), which was the highest Shannon value reported between plants, was almost double the value found in reactor A (1.20; Table 3.4). While reactor A was dominated by an OTU type representing 63% of the library (Figures 3.7b and 3.7d) the most abundant OTU in reactor B represented 32% of the library and both richness and abundance distribution of clones between different OTU types changed in relation to reactor A. These results showed that the reactors were different from each other; the fact that they were operated at different sludge ages, respectively 25 days in reactor A and 14 days in reactor B, may also contribute to the differences seen in the composition of the libraries analyzed, where reactor A, with the higher sludge age, was less diverse.

DGGE profiles looked slightly different, and in the clustering dendrogram in Figure 3.3, replicates from reactor A clustered with replicates from reactor B at a level of similarity close to 0.5, that is, the lowest value in relation to the other replicates from other plants, which clustered at higher similarity (from 0.7 to 1.0; Figure 3.3). In spite of this, differences on the basis of DGGE profiles as shown by Kruskal-Wallis analysis were not statistically significant, and in this case the single clone libraries and DGGE data showed complementary results, in that the clone libraries confirmed that the composition of the AOB communities was different. On the basis of DGGE fingerprints, only the most abundant populations are detected on the gels. Clone libraries may provide more detailed information in relation to the richness and evenness present in the samples than DGGE, since there is a higher probability that less abundant and rare species may be captured through cloning than through DGGE. The same was observed in relation to the Lindsey reactor sampled at different times, in which diversity was higher in the sample taken in October (Shannon index=1.74 and Simpson index = 0.92; Table 3.4) compared to the sample taken in September 2005 (0.86 and 0.51; Table 3.4), although DGGE profiles from samples taken at different sampling times at the Lindsey treatment plant looked superficially similar (Figures 3.4a and 3.4c). Differences seen in DGGE profiles versus clone libraries may also derive from potential artifacts such as the formation of a heteroduplex molecule during PCR on the DGGE gel (in principle, 16S rRNA sequences from two organisms can give rise to four bands: two bands deriving from the source organisms, and two different heteroduplexes formed by hybridization of the complementary DNA strands from the two different organisms) or the comigration of fragments with different sequences. As a result, these may lead respectively to overestimations and underestimations of species richness.

In order to correctly infer the true diversity present in a sample and compare its diversity to other samples, it is important to determine if sufficient sampling of clones was performed. It has been established that when a sample is not sampled sufficiently, indices of diversity underestimate the real diversity present in a sample (Kemp and Aller, 2004a; Kemp and Aller, 2004b).

Two non-parametric richness estimators, Chao 1 and ACE, were applied to the clone library data in order to infer how well they were sampled. When a sample has been sampled sufficiently, both estimators reach a stable asymptote when an increasing number of OTUs is used in the analysis.

Libraries from the Grangemouth reactors A and B, Eastham, and from Humber's fourth sampling occasion reached a stable asymptote with both Chao1 and ACE estimators. The clone library from the Lindsey treatment plant on the first sampling date stabilized with Chao1 but not with ACE (Figures 3.8a and 3.8b).

No stable asymptote was reached for both estimators in the case of the Humber plant on the first sampling date and the Lindsey plant on the second sampling date. These observations correspond with the highest Simpson's index recorded for all of the libraries analysed (0.98 and 0.92 respectively; Table 3.4).

Although these estimators can indicate whether a library was sufficiently sampled, they cannot predict the size that an inadequate library would need to be for the estimators to give a stable asymptote (Bohannan and Hughes, 2003); therefore they tend to grossly underestimate diversity in a sample when the library is not sampled sufficiently. This is due to the fact that they rely only on the number of clones sampled in a library and do not assume any clone abundance distribution model. Therefore, they provide only a lower bound of diversity present in a sample and information related to rarer OTU classes that would be statistically unlikely to be detected (unseen diversity) cannot be estimated by these non-parametric estimators.

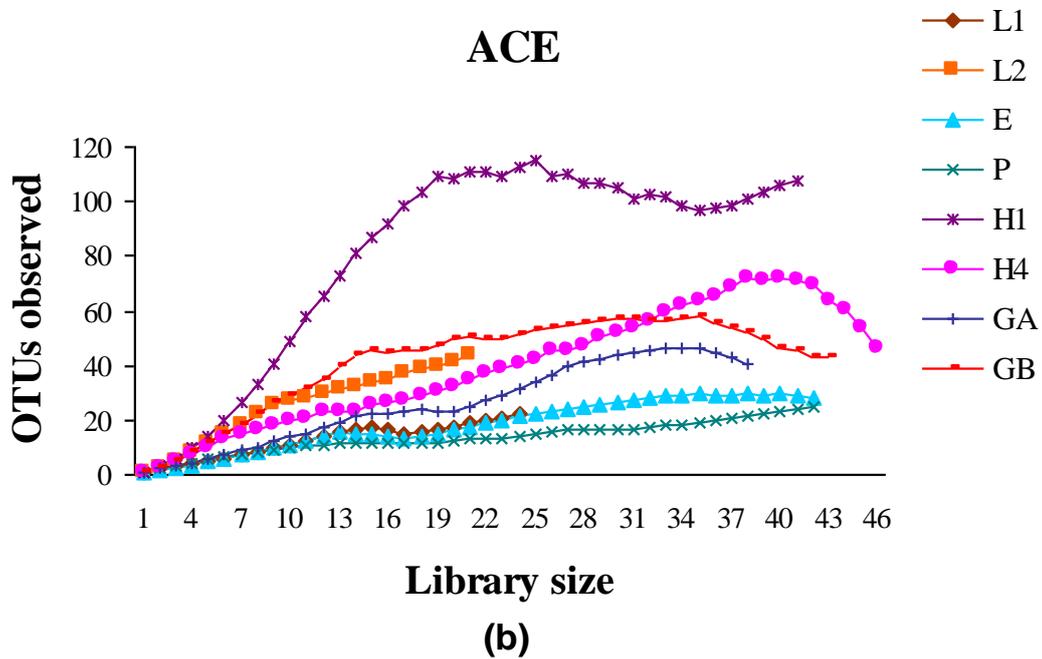
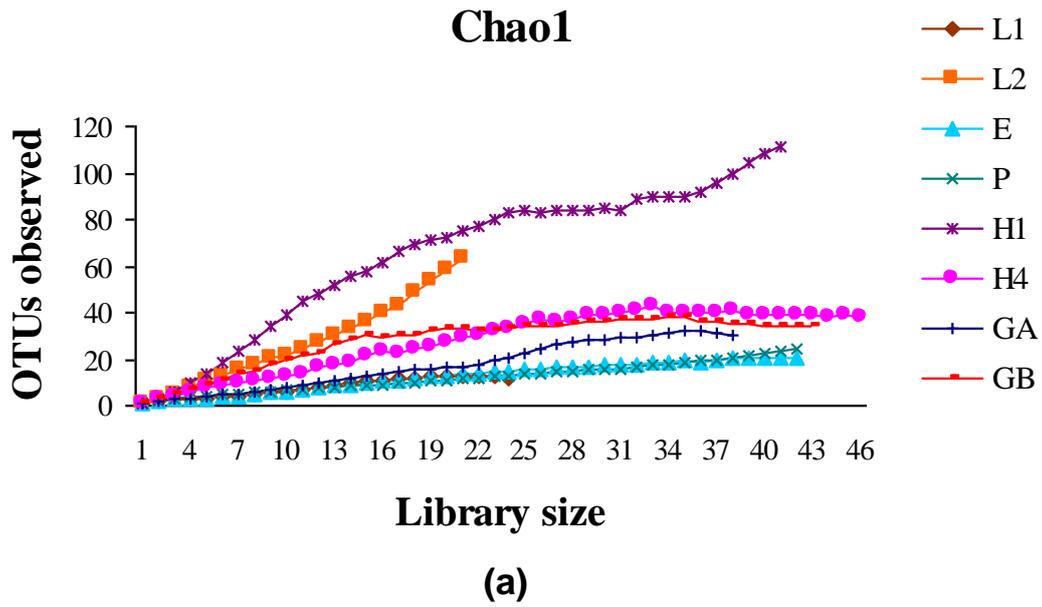


Figure 3.8 Abundance-based estimate curves obtained for eight AOB 16S rRNA gene clone libraries constructed in this study: a) Chao 1; b) ACE. From top to bottom on legend: GA and GB = refer respectively to reactors A and B from Grangemouth; H1 and H4= refer respectively to the first (21.06.2006) and fourth (08.01.2007) sampling occasions in Humber; E= Eastham; P= Pembroke; and L1 and L2= refer respectively to first (07.09.2005) and second (07.10.2005) sampling occasions in Lindsey.

3.3.5 Composition of betaproteobacterial AOB 16S rRNA gene clone libraries from oil refinery WWTPs

Sequences related to AOB and *Betaproteobacteria* which were not AOB were found in clone libraries (Figure 3.9).

Apart from the Humber plant, AOB were detected in all reactors. AOB related to *N. oligotropha*, and *N. marina* were detected in Lindsey (both samplings), Eastham, Pembroke and reactor B from Grangemouth. AOB from the *N. communis* lineage were detected at low relative abundance of only 9% in the Lindsey reactor on the second sampling date (Figure 3.9).

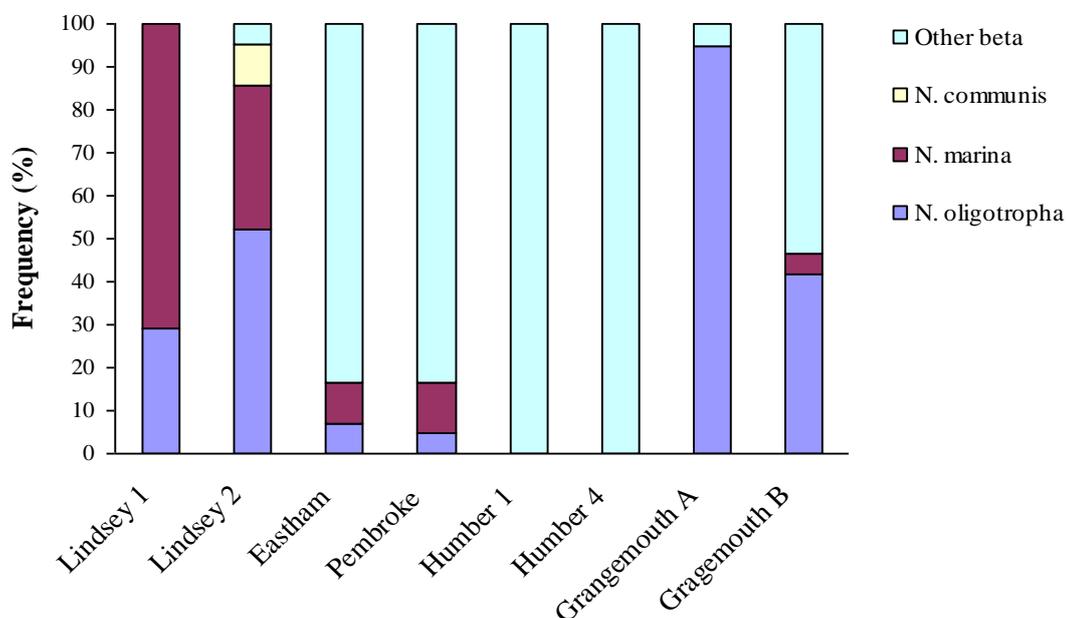


Figure 3.9 Clone frequencies in eight AOB 16S rRNA gene clone libraries constructed in this study related to AOB *N. oligotropha*, *N. marina* and *N. communis* lineages, and non AOB *Betaproteobacteria*. Libraries refer to the following sampling dates: Grangemouth reactors A and B= 26.07.2006; Humber 1= 21.06.2006; Humber 4= 08.01.2007; Eastham= 01.06.2006; Pembroke= 09.06.2006; Lindsey 1= 07.09.2005; Lindsey 2= 07.10.2005.

The composition of the AOB communities varied between reactors within the same plant and between different plants (Figure 3.9). *N. marina*-like sequences predominated in the Lindsey plant on the first sampling occasion (71% of clones) with *N. oligotropha*-like sequences being less abundant (29% of clones). In the Lindsey reactor on the second sampling occasion, *N. oligotropha*-like sequences (52% of clones) were slightly more abundant than *N. marina*-like sequences (33% of clones).

However the apparent temporal variation observed, showing a reduction in the frequency of *N. marina*-like-sequences and an increase in *N. oligotropha*-like-sequences is not reliable since the library was not sampled enough on the second occasion (none of the Chao1 and ACE estimators tested for this library reached a stable asymptote; Figures 3.8a and 3.8b). If more clones had been sampled in this library, a different picture might have been obtained in relation to the frequency between both *Nitrosomonas marina*-like-sequences and *N. oligotropha*-like-sequences. By contrast DGGE profiles from both sampling occasions in the Lindsey reactor (Figures 3.4a and 3.4c) appeared to be very similar.

Few AOB sequences were detected in samples from Eastham and Pembroke and the majority of clones (83% of clones) found in these libraries related to betaproteobacterial sequences from non-AOB. *N. oligotropha*-like sequences comprised 7% of the clones in Eastham and 5% of the clones in Pembroke; while *N. marina*-like sequences comprised 9.5% of the clones in Eastham and 12% of the clones in Pembroke. Eastham and Pembroke in contrast to the other plants were not nitrifying (almost zero nitrate in the effluent; Table 3.2).

In the Humber reactor, 100% of the clones were related to betaproteobacterial sequences from non-AOB on both sampling dates. Nevertheless, nitrification was occurring in this WWTP (levels of nitrate were found in the effluent of 0.25 and 1.56 mM respectively for the first and fourth sampling occasion; Table 3.2).

N. oligotropha-like sequences were the predominant AOB sequences in reactor A from Grangemouth (95% of clones). In contrast, AOB 16S rRNA gene clone libraries from reactor B only had 42% *N. oligotropha*-like sequences, with around 5% of the sequences related to *N. marina* and 53% of the sequences from other *Betaproteobacteria* (Figure 3.9). In Grangemouth, the sludge age in the two reactors sampled was different: 25 days in reactor A and 14 days in reactor B. The lower sludge age in reactor B was thus not good for slow growing organisms such as AOB and might result in lower AOB populations. When AOB populations are low, non-AOB sequences tend to be detected with CTO primers (Rowan *et al.*, 2003b).

Several primers that selectively amplify 16S rRNA genes from AOB have been described in the literature, yet none of the primers available are 100% sensitive, targeting all known AOB, nor is any 100% specific, excluding all non AOB (Purkhold *et al.*, 2000; Koops *et al.*, 2003).

Amplification of sequences from *Betaproteobacteria* which are not AOB using CTO primers (Kowalchuck *et al.*, 1997) has been reported before (Nicolaisen and Ramsing, 2002; Rowan *et al.*, 2003a; Rowan *et al.*, 2003b; Cébron *et al.*, 2004) and this seems to occur when the relative abundance of AOB in a sample is low in relation to the abundance of other Bacteria; in this case CTO primers target similar sequences from related *Betaproteobacteria* (Mahmood *et al.*, 2006). CTO primers (Kowalchuck *et al.*, 1997) are considered specific primers for all known betaproteobacterial AOB, though both CTO 189f and CTO654r primers have one to two mismatches to several cultured members of the *N. europaea*-*Nitrosococcus mobilis*, *N. oligotropha* and *N. marina* lineages, whereas the CTO654r primer has three mismatches to several cultured members of the *N. communis* lineage (Purkhold *et al.*, 2000).

In most libraries from this study, the sequences recovered specifically related to not only betaproteobacterial AOB, but also *Betaproteobacteria* which were not AOB. Thus, the diversity index values found in the refinery reactors would be better interpreted if they were considered as the sum of both of them. Therefore, the AOB diversity would be greater than diversity of *Betaproteobacteria* which are not AOB in the Lindsey and Grangemouth reactor A; and conversely, the diversity of *Betaproteobacteria* which are not AOB would be greater than the AOB diversity in the Eastham, Pembroke, Humber and Grangemouth reactor B.

AOB diversity investigated in activated sludge samples taken from 15 WWTPs fell into four categories according to the influent wastewater type (Boon *et al.*, 2002). The groups related to domestic type wastewater (group A); carbohydrate rich wastewater from paper and starch-related industries (group B); protein and fat rich wastewater from food and meat related industries (group C); and wastewater from the textile industry (group D). These were compared using the Shannon diversity index and the evaluation of DGGE densitometric curve patterns (Boon *et al.*, 2002). The Shannon index showed AOB diversity in group A to be similar to group B, respectively 0.45 ± 0.09 and 0.45 ± 0.16 and slightly higher in group C (0.58 ± 0.14) and group D (0.76 ± 0.09); but on average AOB diversity in all plants was 0.54 ± 0.17 .

Making a comparison between the Shannon indices from the refinery wastewater treatment plants, considering that AOB were 100% of the clones present in the Lindsey reactor on the first sampling date in September 2005, an equivalent Shannon index of 0.86 was found, similar to the Shannon index for the textile wastewater; while in the Eastham and Pembroke plants, diversity would be very low, corresponding respectively to 0.15 and 0.18, since only 17% of the clones were AOB clones; and in the Grangemouth reactors A and B, AOB diversity would be approximately the same, respectively 1.14 and 1.0. In comparison to the 2002 Boon *et al.* study, AOB diversity in the oil refineries was higher than the diversity found in domestic, paper, food and textile-related industrial wastewaters, but varied within a similar range. Therefore the diversity of AOB found in oil refinery WWTPs, as in other types of wastewater, appeared to be restricted and the sampling effort was able to capture a large fraction of the diversity specifically related to AOB found in the WWTPs sampled in this study. Most clones found between the 4 plants with detectable AOB were not exactly the same but belonged to the same *Nitrosomonas* lineages, that is, *N. oligotropha* and *N. marina* (Figure 3.9).

3.3.6 Statistical comparisons between AOB 16S rRNA gene clone libraries

To determine if the composition of the clone libraries differed significantly they were compared by using β -LIBSHUFF (Schloss *et al.*, 2004). This test compares two or more clone libraries and determines if they derive from the same or distinct communities.

Six clone libraries were compared: Lindsey, Eastham, Pembroke, Grangemouth, Humber sampled on the first occasion and Humber sampled on the fourth occasion.

Comparisons between reactors A and B from Grangemouth as well as comparisons between different sampling times in Lindsey using β -LIBSHUFF showed that they were not statistically significant (homologous and heterologous p-values higher than minimum p value 0.0003) for example, communities were sampled from the same community, and thus all sequences related to Grangemouth and Lindsey respectively were grouped and considered as single libraries (Table 3.5).

Table 3.5 β -LIBSHUFF population p values determined for comparisons of six AOB 16S rRNA gene clone libraries constructed in this study

Homologous library (X)	Heterologous library (Y)*					
	Lindsey	Eastham	Pembroke	Humber 1	Humber 4	Grangemouth
Lindsey		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Eastham	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Pembroke	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001
Humber 1	<0.0001	<0.0001	<0.0001		<0.0001	<0.0001
Humber 4	<0.0001	<0.0001	<0.0001	<0.0001		<0.0001
Grangemouth	<0.0001	<0.0001	<0.0003	<0.0001	<0.0001	

*Libraries are distinct from one another if both comparisons (X versus Y; lower triangle and Y versus X upper triangle) are significant at $p \leq 0.0003$ (after pair wise correction). Libraries presented in the table refer to the following sampling dates: Grangemouth= 26.07.2006; Humber 1= 21.06.2006; Humber 4= 08.01.2007; Eastham= 01.06.2006; Pembroke= 09.06.2006 and Lindsey both samplings occasions (07.09.2005 and 07.10.2005) considered as a single library.

Comparisons between the different plants were statistically significant, thus showing that they represent different communities ($p < 0.0003$; Table 3.5). It is important to point out that not only sequences related to AOB, but also sequences related to non AOB *Betaproteobacteria* were present in these libraries and in the case of both libraries from the Humber reactor, no AOB sequences were present.

Interestingly, community composition in the Humber reactor in June 2006 was significantly different from community composition in January 2007; that is, the communities had changed completely over time. This also was in agreement with the differences observed in relation to their DGGE profiles (Figures 3.6a and 3.6c).

In general the data obtained from DGGE and clone libraries were in agreement. Both techniques detect the most abundant organisms presented in samples. There is a threshold in DGGE analysis in which the most abundant species representing 1% or more of the sample are detected (Muyzer *et al.*, 1993) and therefore very rare and less abundant species under this threshold will probably not be detected on gels. In contrast, there is a greater chance that the more rare clones present in a sample may be observed by random sampling of sequences in clone libraries.

Using 16S rRNA gene clone libraries it was possible to detect variations related to the relative abundance of different species. Observed differences were not always significant, as was the case with the libraries from the Lindsey reactor samples taken on different dates and between the Grangemouth reactors operated at different sludge ages. Overall, the combination of both DGGE and 16S rRNA gene clone libraries in this study was able to provide a complementary picture of the AOB populations.

3.3.6.1 Phylogenetic analysis of betaproteobacterial AOB 16S rRNA sequences

Two methods were applied to analyse the 16S rRNA sequences, neighbour-joining distance analysis and maximum parsimony. The two methods differ in that they have different underlying assumptions and use different algorithms to calculate tree topologies; while the first one builds a distance matrix taking into account the overall divergence occurring between pairs of sequences, the second considers all characters at every position in the sequence and calculates the minimum number of evolutionary changes required to convert one sequence into the other in order to calculate the shortest or most parsimonious tree (Röling and Head, 2005). As each method is different from the other, they can also produce different results.

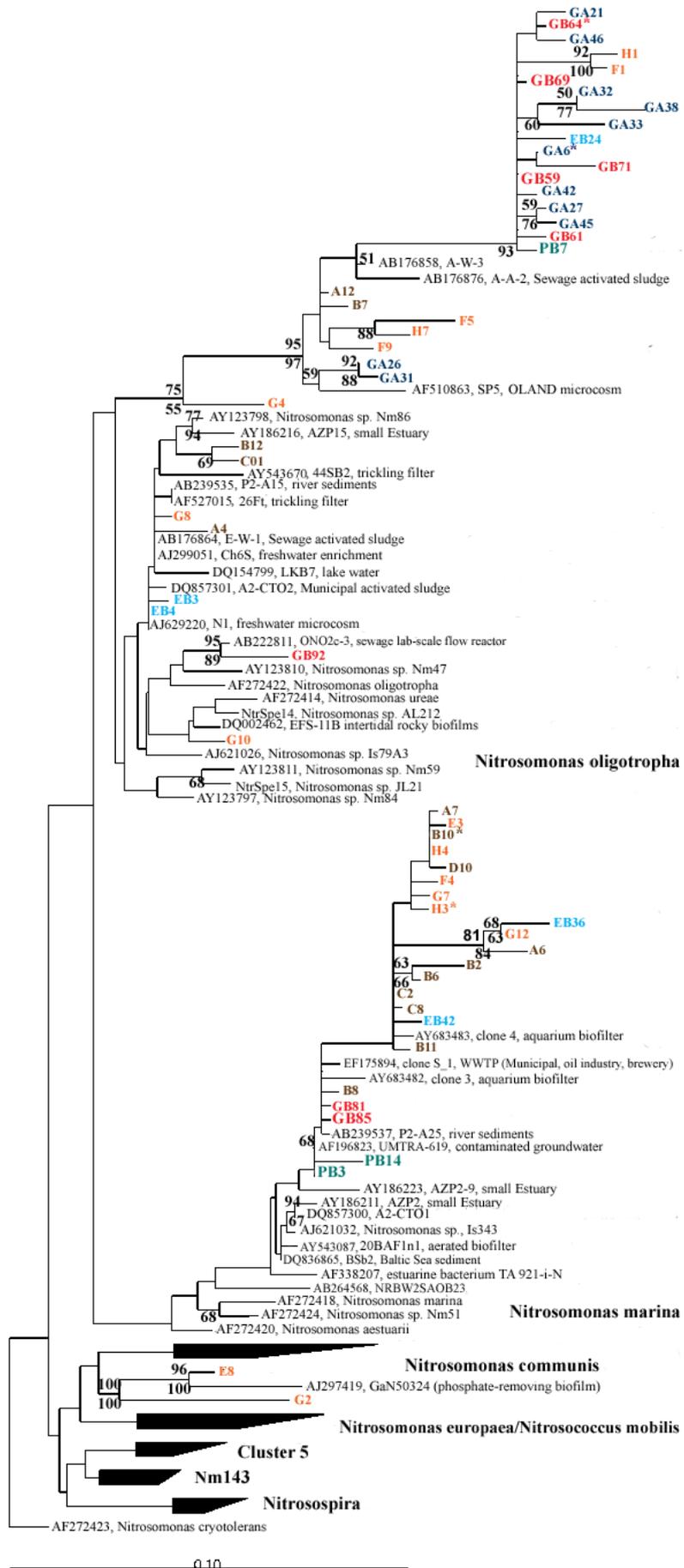
High bootstrap values were obtained for parsimony and distance analysis of 16S rRNA long sequences (>1000 bp) as can be seen in Figure 3.10. The phylogenetic tree AOB (Figures A1 and A2) of betaproteobacterial sequences greater than 1000 bp, used as the framework for the phylogenetic analyses reported here, can be found in Appendix A. The bootstrap values presented on the tree from Figure 3.10 correspond to values obtained from parsimony and neighbour-joining distance analyses of subsets of sequence data from the tree in Appendix A (See Methods Section 3.2) and included long sequences (>1000 bp) and partial sequences from this study. However, as bootstrap values on a tree are a function of the specific dataset analysed, it is important to emphasize that bootstrap values obtained from different datasets cannot be directly compared.

Bootstrapping (Felsenstein, 1985) is considered a very reliable statistical tool that is used to test the reliability of inferences made from phylogenetic trees. Through this method, the original data set is resampled with replacement and the probability that the same branch position will appear again is expressed as a percentage value. Therefore high bootstrap values on a tree show higher support for a particular group of sequences occurring together.

However, another point that should be taken into account is that high overall similarity between partial sequences may also occur as a result of more conservative regions of a molecule being compared and thus it reflects a lack of informative positions affecting the bootstrap confidence (Stephen *et al.*, 1996). This may be the case with many sequences from this study and other very closely-related sequences, where very low bootstrap values were obtained, but with very short branches connecting the sequences.

All AOB clones found in the Lindsey, Eastham, Pembroke and Grangemouth plants were related to either the *Nitrosomonas oligotropha* or *Nitrosomonas marina* lineages; apart from two clones retrieved from the Lindsey reactor on the second sampling date, which were related to the *Nitrosomonas communis* lineage (Figure 3.10). While the majority of clones (54 of 81 clones) from Grangemouth clustered within the *N. oligotropha* lineage, the majority of clones from Lindsey (24 of 45 clones) clustered within the *Nitrosomonas marina* lineage (Figure 3.10).

Within *N. oligotropha*, most of the clones retrieved from Grangemouth reactors A and B represented by clones GA6 and GB64 grouped very closely to each other in a distinct cluster (Figure 3.10) including two singletons from the Lindsey clone library sampled in October 2005 (H1 and F1) one singleton from Eastham (clone EB24) and one singleton from Pembroke (clone PB27) clone libraries (Figure 3.10). This group of sequences was quite distinct from the rest of the sequences and well-supported by parsimony analysis (93% bootstrap support) but not in a distance analysis; it occupied a long branch most closely related to clones A-W-3 and A-A-2 (Figure 3.10) which were retrieved from an anaerobic/anoxic/aerobic sewage-activated sludge system (Limpiyakorn *et al.*, 2005). For instance sequence identities between sequences within this unknown cluster (Figure 3.10) and clone A-W-3 were about 97%; *Nitrosomonas* sp. JL 21 was the closest sequence from a cultured organism related to this group of sequences with only 93% sequence identity. *Nitrosomonas* JL21 (Suwa *et al.*, 1997) was isolated from an activated sludge plant and with respect to ammonia tolerance is considered to be a sensitive strain. When in culture, strain JL21 was able to grow in ammonium sulphate concentrations up to 3.57 mM; but was completely inhibited in media containing 10.7 mM or above; they also are urease positive and have good flocculation capacity. These are two other important characteristics commonly found in organisms within the *Nitrosomonas oligotropha* lineage that distinguish them from the other *Nitrosomonas* lineages. Therefore all sequences together within this clade (Figure 3.10) may form a novel AOB lineage, first identified by Limpiyakorn *et al.*, 2005. The distinct cluster also contains a limited number of sequences from uncultured AOB that have only been identified in WWTP reactors and not in any other environments.



LEGEND

- Lindsey 1 (clones A, B, C and D)
- Lindsey 2 (clones E, F G, and H)
- Grangemouth A (clones GA)
- Grangemouth B (clones GB)
- Eastham (clones EB)
- Pembroke (clones PB)

Figure 3.10 Phylogenetic tree of betaproteobacterial ammonia oxidizing bacteria inferred from comparative analysis of 16S rRNA sequences. The tree is a neighbour-joining tree built in ARB with sequences > 1000 nucleotides. Bootstrap values above and below nodes derive respectively from distance and parsimony analyses. (*) indicates the most abundant clone in libraries. Sequences in the tree are from: Limpiyakorn *et al.*, 2005; Philips *et al.*, 2002; Purkhold *et al.*, 2003; Caffrey *et al.*, 2003; Rowan *et al.*, 2003a; Rowan *et al.*, 2003b; Nakamura *et al.*, 2006; Bollmann and Laanbroek, 2001; Hadas and Witzel, 2005 (unpublished); Geets *et al.*, 2007; Coci *et al.*, 2005; Limpiyakorn *et al.*, 2007; Purkhold *et al.*, 2000; Magalhães *et al.*, 2007; Nielsen *et al.*, 2004 (unpublished); Grommen *et al.*, 2005; Hornek *et al.*, 2007 (unpublished); Ivanova *et al.*, 2000; Voytek, 1996; Hardeman and Sjoling, 2006 (unpublished); Satoh *et al.*, 2007; Gieseke *et al.*, 2001; Stephen *et al.*, 1996; Urakawa *et al.*, 2006. Scale bar represents 10 % sequence divergence.

The current concept accepted for bacterial species definition (Stackbrandt and Goebel, 1994) is based on DNA-DNA homology between different cultured species and it has been demonstrated that different bacterial species having DNA similarity lower than 70% constitute distinct species.

The 16S rRNA is considered a good molecular marker for phylogenetic analysis (Ludwig and Schleifer, 1994; Ludwig *et al.*, 1998), providing better resolution for example, than the functional *amoA* molecular marker. (Rotthauwe *et al.*, 1997; Purkhold *et al.*, 2000; Purkhold *et al.*, 2003). However there are some limitations to 16S rRNA based analyses. It can be difficult to discriminate different species as different species because distinguishable on the basis of very low DNA-DNA homologies may have very high 16S rRNA sequence identity (Fox *et al.*, 1992).

The relationship between 16S rRNA sequence identity and DNA-DNA homology indicated that 16S rRNA identity values below 97% correspond to less than 70% DNA similarity (Stackbrandt and Goebel, 1994) and therefore may represent different species. This value was recently revised by Stackbrandt and Ebers (2006) who after analysing a dataset of 16S rRNA sequences with identities from 98.7 to 99% demonstrated that the organisms from which the sequences came had DNA-DNA association values below 70% and thus also represented distinct species.

Phylogenetic analyses in this study were done with partial 16S rRNA sequences, the sequencing of the entire 16S rRNA gene is highly recommended in order to produce better phylogenetic analyses (Ludwig and Schleifer, 1994; Ludwig *et al.*, 1998; Ludwig and Klenk, 2001). These would be essential for determining with greater reliability whether the distinct cluster (Figure 3.10) related to *N. oligotropha* found in the refinery wastewater treatment systems are indeed novel AOB species.

Other sequences related to the main AOB cluster within the *N. oligotropha* lineage retrieved from the Lindsey refinery WWTP (including clone F9, the second most abundant clone retrieved in the Lindsey clone library from October 2005) formed a larger clade (Figure 3.10) which included two other clones from Grangemouth reactor A (clones GA26 and GA31). This grouping was also strongly supported by bootstrap analysis (95% support in distance analysis and 97% in parsimony analysis; Figure 3.10).

Five other clones retrieved from the Lindsey reactor, clone GB92 from the Grangemouth reactor B and clones EB3 and EB4 from Eastham were found distributed in different sub-clusters within the *Nitrosomonas oligotropha* (Figure 3.10) and may represent AOB having different substrate affinities (K_s) and tolerance to ammonia concentrations (Suwa *et al.*, 1994; Bollmann and Laanbroek, 2001; Stehr *et al.*, 1995; Limpiyakorn *et al.*, 2006).

Ammonia concentrations found in the influent to the plants from this study were on the whole quite low, and ranged from 0.51 mM to 1.60 mM; Table 3.2. Similar values of ammonia were found at the Lindsey site on both sampling dates (0.73 and 0.51 mM; respectively) and Grangemouth (0.73 mM). In contrast, higher values of ammonia were found in Eastham (1.16 mM) and Pembroke (1.60 mM). Furthermore, these ammonia concentration values were also comparable to other municipal wastewater reported values consistent with the occurrence of *N. oligotropha*-like organisms, for example presenting ammonia concentrations in the range of 0.53 to 2.96 (Limpiyakorn *et al.*, 2005; Pickering, 2008).

Among the different AOB lineages, members of the *Nitrosomonas oligotropha* lineage are characterized by high affinities for ammonia with K_s values 0.0019- 0.0042 mM (Koops and Pommerening-Röser, 2001) and thus thrive better in low ammonium environments. This contrasts with AOB species with higher K_s values (e.g. 0.030- 0.061 mM, for *N. europaea* and *N. eutropha*) which are more often found dominating wastewater plants with higher ammonia inputs (Suwa *et al.*, 1994). For example, *N. oligotropha* was found to predominate in lab-scale sewage enrichments fed with 2 mM, 5 mM and 10 mM ammonium concentrations, while *N. europaea* was the only AOB selected in reactors fed with 30 mM ammonium (Limpiyakorn *et al.*, 2007).

Three groups of AOB sequences from the refinery WWTPs were identified within the *N. marina* lineage (Figure 3.10). These were related to Clone AZP2-9, a clone retrieved from Lower Azevedo Pond from the Elkhorn Slough estuary (Caffrey *et al.*, 2003). The first and the second groups (Figure 3.10) comprised the majority of the clones retrieved from the Lindsey reactors which had the highest salinities (0.67% and 1.09%) recorded for the WWTPs analysed in this study. Sequence identities between the most abundant clones retrieved from Lindsey reactors, clones B10 and H3 (Figure 3.10), and their closest cultured relative strain Is343 isolated from the brackish part of the river Schelde estuary (Coci *et al.*, 2005), were respectively 97.8 and 97.1%.

All sequences included in the second and in the third groupings within *N. marina* lineage (Figure 3.10), come from low salinity environments such as river sediments (Nakamura *et al.*, 2006); a freshwater aquarium biofilter (Grommen *et al.*, 2005); a municipal WWTP receiving influent inputs from sewage, brewery and the oil industry (unpublished); or contaminated groundwater (Ivanova *et al.*, 2000). The second group of sequences (Figure 3.10) also contained other singletons from Lindsey, two singletons from the Eastham library (clones EB36 and EB 42), which had the lowest salinity wastewater (0.08%), and clone 4 (Grommen *et al.*, 2005) that was retrieved from a freshwater aquarium biofilter.

The third group (Figure 3.10) included two singletons from Grangemouth (clones GB81 and GB85), a plant also treating lower salinity wastewater (0.11%), which were closely related to clone PA25 retrieved from river sediments (Nakamura *et al.*, 2006) receiving untreated sewage from a wastewater treatment plant with zero salinity, other sequences from a freshwater aquarium biofilter (Grommen *et al.*, 2005) and two singletons from the Pembroke library (0.72% salinity; Table 3.2), clones PB3 and PB14, which were related to clone UMTRA-619 retrieved from a polluted groundwater plume contaminated by a uranium mill disposal site (Ivanova *et al.*, 2000). Salinity was not measured in the plume, but the cluster containing this sequence as well other five sequences, was found to be related to clone WS26, also retrieved from an estuarine lake (Speksnijder *et al.*, 1998).

The 16S rRNA gene sequences found here within the *N. marina* group were not supported by bootstrap resampling, and this seems to be a result of the combination of high sequence identity and the low proportion of variable positions in the partial 16S rRNA gene sequences being compared.

Furthermore it would be important to sequence the entire 16S rRNA gene to produce more reliable phylogenetic inferences about these sequences and therefore to better characterize their phylogenetic position within *N. marina*.

Cultured strains of *N. marina* were originally isolated from marine environments; however, culture-independent methods have demonstrated that sequences related to the *N. marina* lineage were present in a nitrifying freshwater aquarium (Burrell *et al.*, 2001), adapted to low ammonia concentrations (0.29- 0.6 mM), and potentially representing novel AOB, since only 95% sequence identity was found between near-full length 16S rRNA sequences from this novel cluster (Figure 3.10) and cultured *N. marina*.

Therefore within the *N. marina* lineage, sequences from both saline and non-saline environments are found and, as in the case of *N. oligotropha*, niche differentiation of species in different sub-groups may occur with *N. marina*-like AOB and this may relate to differences in salinity optima and ammonia concentrations. However, the relationship of the *N. marina* lineage to other freshwater environments as well as estuaries characterized by similar salinities suggests that the AOB sequences in these three groups (Figure 3.10) represent species having lower salt tolerance than cultured *N. marina*. Furthermore the most similar AOB sequences (environmental and isolates) to the sequences identified in this study came from environments that are on the whole of relatively low salinity and this is consistent with what is known about related cultured AOB and the conditions found in the reactors investigated in this study.

The low salinity conditions found in the refinery wastewater treatment reactors were also similar to conditions found in the river Schelde estuary, characterized as a eutrophic estuary system receiving nitrogen input from treated and untreated sewage and industrial sources as well as from agricultural runoff (de Bie *et al.*, 2001). Shifts in AOB populations in the Schelde estuary in relation to gradients of salinity, ammonia and dissolved oxygen, measured at eight stations along a freshwater to brackish transect ranging from 0.05 to 1.6‰ salinity, showed AOB related to *N. oligotropha* to predominate in the freshwater part of this estuary. As in this study, they were also subjected to lower ammonium concentrations (0.25- 0.3 mM; though the ammonia concentrations were higher in relation to the other parts of the estuary), lower salinities (about 0.07- 0.49 ‰), and lower oxygen (below 20%). This was also the site of one of the major sources of untreated sewage into the estuary. In the most brackish parts of the estuary, AOB related to *N. oligotropha* were replaced by unidentified *Nitrosomonas*-like sequences and AOB related to *N. marina* appeared in the brackish parts of the estuary. In contrast, *Nitrospira*-like sequences appeared at all sampling sites, but only as a minor proportion of sequences. Interestingly, three sequences detected from some freshwater sites, as in this study, were also related to *N. marina*, but in a distinct clade and also more closely related to clone Ws26 from estuarine lake water (Speksnijder *et al.*, 1998).

The same pattern of *N. oligotropha*-like AOB predominating in the freshwater parts of other estuaries was also observed in the river Seine estuary (Cébron *et al.*, 2004) and was related to sewage wastewater pollution. Similar observations were made in the Ythan estuary on the east coast of Scotland (Freitag *et al.*, 2006) where *N. oligotropha* and *N. marina* were found to coexist in several sampling sites from the most freshwater regions of the Ythan estuary, in which conditions were not dissimilar to conditions in reactors from this study, at least with respect to ammonia and salt concentrations.

The influence of salinity and oxygen on AOB communities observed in the river Schelde estuary, were tested in enrichment culture studies (Bollmann and Laanbroek, 2002). These revealed that salinity rather than oxygen was the factor causing the shifts in AOB populations, while oxygen altered AOB activity only. AOB populations responded quickly to salt addition and distinct AOB were selected respectively in freshwater (0.13% salinity) and in brackish (1.63% salinity) enrichments. However, the highest nitrifying activities were reached when freshwater samples were enriched without any salt addition and when brackish samples were enriched under freshwater conditions. Interestingly salinity in the brackish condition was also much higher than the range of salinities (0.1-1%; Table 3.2) found in the refinery wastewater treatment systems and the selected AOB performed better nitrification when salinity in the culture medium was reduced or even suspended, thus showing their salt sensitivity.

Therefore in a succinct evaluation of the AOB communities in this study, *N. oligotropha* and *N. marina*-like sequences were found to coexist in reactors having low ammonia and low salinity conditions; these results are in line with what is known for the AOB found in this study as well as those in the environments in which they have been reported in relation to ammonia and salinity, such as estuaries and other wastewater treatment plants.

3.4 Conclusions

The refinery wastewater treatments systems investigated in this study were dominated by sequences related to AOB believed to be adapted to low ammonia concentrations and low salinity and this is consistent with the conditions in the reactors. Surprisingly, two clone libraries derived from two surveys conducted at the Humber plant with a period of seven months between sampling had no AOB sequences, although at the same time nitrification was occurring in the Humber plant. Although the occurrence of nitrification was poor at the time of the first survey, at the time of the second survey the plant was nitrifying at its maximum efficiency. Therefore the broader implication of these findings is that ammonia oxidizing archaea or other non-classical ammonia oxidizers may have a relevant role in nitrification in the Humber plant.

To sum up, the main conclusions from this chapter were as follows:

- The refinery WWTPs investigated had distinct chemistry, ammonia-oxidizing bacterial diversity and species composition;
- AOB diversity found in the refinery WWTPs was low;
- All AOB sequences detected in reactors were related to *Nitrosomonas*; Populations related to *N. oligotropha* and *N. marina* were found in almost all the plants investigated, and *N. communis*-like AOB were detected in the Lindsey plant;
- Other *Betaproteobacteria* were found in all oil refinery WWTPs, however AOB were the main populations occurring in two plants that were nitrifying: the Lindsey and Grangemouth plants;
- Most of the clones in the Lindsey plant were related to *Nitrosomonas marina*, while most of the clones in the Grangemouth plant were related to the *Nitrosomonas oligotropha* lineage and this was consistent with the salinity of the influent at these plants;

- Although Eastham and Pembroke were not nitrifying, AOB related to the same lineages as found in nitrifying reactors, *Nitrosomonas oligotropha* and *Nitrosomonas marina*, were detected;
- One distinct cluster within *Nitrosomonas oligotropha* may represent a novel AOB species associated with WWTPs.
- In the Humber reactor, nitrification was occurring despite the fact that betaproteobacterial AOB were not detected, suggesting that alternative organisms may be responsible for nitrification in this system, possibly, AOA, Anammox or gammaproteobacterial AOB.

Chapter 4

Diversity of AOA in oil refinery wastewater treatment systems

4.1 Introduction

The *Archaea* is an intriguing prokaryotic group which is always surprising microbial ecologists with its biochemistry, physiology, genetics, ecology and its puzzling evolutionary story.

Techniques based on the phylogenetic analysis of 16S rRNA gene sequences provoked a profound revolution in the classification of life into three distinct Domains (Woese *et al.*, 1990). One of these, the *Archaea*, was identified as a line of evolutionary descent that was distinct from *Bacteria* and *Eukarya*. However, the development of culture-independent analyses a little while after the initial 16S rRNA gene based phylogeny, which was carried out with cultured organisms and uncovered the *Archaea*, has revealed many new aspects of the ecology of *Archaea*.

Archaea encompass two major phyla, *Crenarchaeota* and *Euryarchaeota*, and were thought to be restricted to environments with extreme conditions of pH, salinity, temperature, and anoxia because cultured archaea originated from these extreme environments. However, culture-independent methods have revealed that they are common and widespread in a range of non-extreme environments. Uncultured mesophilic members of the *Crenarchaeota* (the so called Group I *Crenarchaeota*) and *Euryarchaeota* (The Group II *Euryarchaeota*) were revealed for the first time in coastal marine environments (Delong *et al.*, 1992; Fuhrman *et al.*, 1992) as abundant members of the planktonic communities. While Group II predominated in marine surface waters, Group I often occurred at different depths in the water column (Massana *et al.*, 1997; Massana *et al.*, 2000).

Furthermore, uncultured *Crenarchaeota* were shown to be widely distributed and more abundant than Group II *Euryarchaeota* in the oceans, comprising about 20% of the total bacterioplankton (Massana *et al.*, 1997; Delong *et al.*, 1999; Massana *et al.*, 2000; Lin *et al.*, 2006; Karner *et al.*, 2001).

The ubiquity of uncultured *Crenarchaeota* was further extended to a vast range of other 'ordinary environments' for instance, estuaries (Abreu *et al.*, 2001); anoxic marine salt marsh and continental shelf sediments (Munson *et al.*, 1997; Vetriciani *et al.*, 1999); freshwater sediments and associated ferromanganous micronodules (Schleper *et al.*, 1997; Macgregor *et al.*, 1997; Stein *et al.*, 2001); forest (Borneman and Triplett, 1997); agricultural (Buckley *et al.*, 1998); and sewage sludge polluted soils containing heavy metals (Sandaa *et al.*, 1999); rhizosphere systems (Simon *et al.*, 2000); in symbiosis with sponges (Preston *et al.*, 1996); in deep South African gold mine waters (Takai *et al.*, 2001); subsurface palaeosol (Chandler *et al.*, 1998); and even anaerobic fluidized-bed reactors and granular biofilms (Godon *et al.*, 1997; Collins *et al.*, 2005).

The absence of cultured representatives of these mesophilic prokaryotes historically prompted metagenomic analysis of uncultured *Archaea*. As a result, considerable progress and advances in understanding the physiology and ecology of these organisms has been gained through metagenomic studies in recent years (Stein *et al.*, 1996; Schleper *et al.*, 2005; Cavicchioli *et al.*, 2006; Hallam *et al.*, 2006a; Hallam *et al.*, 2006b).

New findings about the biochemistry and physiology of uncultured archaea are provoking a revolution in scientific knowledge of this group. One such discovery of particular significance relates to the participation of members of the *Crenarchaeota* in nitrification.

The involvement of Archaea in nitrification was first suggested when during the whole-genome shotgun sequencing of microbial populations from the Sargasso Sea (Venter *et al.*, 2004) an archaea-associated scaffold was found which contained a sequence homologous to the gene encoding the protein that hosts the active site of the monooxygenase responsible for the oxidation of ammonia to hydroxylamine (*amoA*), that is, the first step of ammonia oxidation. Until then, the oxidation of ammonia was thought to be carried out only by the autotrophic ammonia oxidizing bacteria of the *Beta*- and *Gammaproteobacteria* (McTavish *et al.*, 1993; Norton *et al.*, 2002). Concomitant to this event, during soil metagenomic analysis, a crenarchaeotal DNA fragment containing a ribosomal RNA operon linked to genes homologous to *amoA* and *amoB* was identified (Treusch *et al.*, 2005), further suggesting their participation in nitrification in soils.

Subsequently, the chemoautotrophic metabolism of *Archaea* oxidizing ammonia to nitrite was demonstrated for the first time when a mesophilic member of the *Crenarchaeota* “*Candidatus Nitrosopumilus maritimus*” (Könneke *et al.*, 2005) isolated from the rock substratum of a marine aquarium in Seattle, was successfully cultivated.

The broad capacity of *Crenarchaeota* as nitrifiers able to thrive in a range of habitats was extended when a thermophilic uncultured archaeon “*Candidatus Nitrosocaldus yellowstonii*” (de la Torre *et al.*, 2008) isolated from a terrestrial hot spring was demonstrated to be able to oxidize ammonia at temperatures up to 74°C in an enrichment culture. In addition another moderately thermophilic ammonia oxidizing crenarchaeota belonging to the soil group 1.1b of *Crenarchaeota*, “*Candidatus Nitrososphaera gargensis*” (Hatzenpichler *et al.*, 2008), was enriched from a hot spring at 46°C.

Recently, the complete genome of “*Candidatus Nitrosopumilus maritimus*” (Walker *et al.*, 2010) has been published, and the draft genome of the “*Candidatus Nitrososphaera gargensis*” has also been completed. Comparative genomic studies of *Cenarchaeum symbiosum*, *Nitrosopumilus maritimus* and the draft genome of *Nitrosocaldus gargensis* showed these organisms are actually different from *Crenarchaeota* and their phylogenetic reclassification within a third novel phylum, the phylum *Taumarchaeota* is being proposed (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010).

The widespread distribution of putative ammonia oxidizing archaea (AOA) has since been reported in a range of environments on the basis of PCR amplification of archaeal *amoA* genes. Putative AOA have now been identified in oxic and suboxic water column samples and sediments from different oceans (Francis *et al.*, 2005; Wuchter *et al.*, 2006), estuaries (Beman and Francis, 2006), soils (Leininger *et al.*, 2006), wastewater treatment plants (Park *et al.*, 2006), subsurface geothermal and radioactive hot spring environments (Spear *et al.*, 2007; Weidler *et al.*, 2007), as well as associated with corals (Beman *et al.*, 2007).

The occurrence of ammonia oxidizing archaea in wastewater treatment systems was first reported in 2006 by Park *et al.*, in five wastewater treatment plants in the USA in which nitrification was actively occurring.

The presence and diversity of AOA was investigated in the oil refinery wastewater treatment systems examined in this study, using the primers developed by Francis *et al.* (2005) in order to discover whether AOA occur in oil refinery wastewater treatment systems, and thus if they contribute to nitrification in these systems.

4.2 Methods

The presence of AOA was investigated in all samples from this study. DNA extracted from the samples was amplified using the archaeal *amoA* primers Arch-*amoAF* and Arch-*amoAR*, developed by Francis *et al.* (2005).

The presence of AOA was also investigated in samples originating from 23 full-scale wastewater treatment plants in the UK and in a pilot plant treating wastewater from a leather processing plant (British Leather Corporation; BLC). See description of all Plants in Chapter 2.

PCR products amplified with primers Arch-*amoAF* and Arch-*amoAR* were analyzed by DGGE and cloned and sequenced. As with analysis of AOB communities DGGE analysis of AOA communities was conducted on replicate samples from all of the refinery WWTPs in which AOA were detected. The replicate DGGE profiles were used to determine the variation in the replicate samples and single representative samples were selected for cloning and sequencing of *amoA* genes. The methodology adopted for the analysis of DGGE profiles, clone libraries analysis, and the phylogeny of AOA sequences are described in detail in Chapter 2.

Initially PCR of *amoA* sequences from the Humber refinery WWTP using the archaeal *amoA* primers was unsuccessful. However, the samples from the Humber refinery WWTP sampled on 8 January 2007 (the fourth sampling time) were provided to Marc Mußmann at the University of Vienna for a range of analyses and he successfully recovered archaeal *amoA* sequences and these were included in the statistical comparisons of clone libraries using J-LIBSHUFF and in the phylogenetic analysis in this study (See Section 4.3.4 for details).

A phylogenetic tree was built in ARB with archaeal *amoA* sequences retrieved from Genbank, and sequences from this study. From the resultant tree containing in total 1411 sequences, 136 sequences between 595 and 634 bp in length, representing each of the currently recognized archaeal *amoA* sequence clusters were chosen for phylogenetic analyses. Distance and parsimony analysis were conducted including bootstrap resampling. Bootstrap values $\geq 60\%$ were placed on the tree. The resultant distance and parsimony trees from these analyses are shown in Appendix B, Figures B1 and B2, respectively.

4.3 Results and Discussion

4.3.1 Occurrence of archaeal *amoA* in WWTPs

A survey of putative AOA was conducted in 29 wastewater treatment reactors including 23 municipal wastewater treatment plants, one pilot reactor treating tannery wastes and the five refinery wastewater treatment plants with different reactor configurations MLSS and ammonia concentrations in their influents, investigated in this study. Description of the 23 municipal wastewater plants and the pilot reactor are presented in Table 2.2 (Chapter 2, Section 2.1.3), and the MLSS and ammonia removal for all 29 reactors surveyed in this study are summarized in Table 4.1.

Table 4.1 MLSS and influent ammonia concentrations reported in 29 wastewater treatment reactors surveyed in this study for the presence of putative AOA

WWTP	Sample ⁺	Sampling date	Ammonia (mM)		MLSS (mg/L)
Municipal*					
Atherstone OD T1	1	16.09.2004	Influent	Effluent	
Barnhurst da 1	2	28.09.2004	1.55	0.12	2240
Barnhurst sa 6	3	28.09.2004	0.66	0.00	1972
Biffa SBR 1	4	22.11.2004	0.66	0.00	1972
Burton Sep	5	16.09.2004	n.d.	n.d.	n.d.
Burton Nov	6	22.11.2004	1.12	0.00	2390
Coalport 2	7	28.09.2004	0.92	0.05	2450
Coalport 4	8	28.09.2004	1.77	0.00	1730
Coalport 6	9	28.09.2004	1.77	0.00	1730
Coleshill lane 8	10	07.10.2004	1.77	0.00	1730
Coleshill lane 9	11	07.10.2004	1.94	0.00	1550
Derby 1 T3	12	16.09.2004	1.94	0.00	1550
Derby 2 T3	13	16.09.2004	0.53	0.00	3560
Derby 3 T1	14	16.09.2004	0.53	0.00	3560
Eccleshall	15	28.09.2004	0.53	0.00	3560
Finham 1.2	16	16.09.2004	0.98	0.00	1840
Goscote lane 1	17	07.10.2004	1.35	0.10	3530
Leek vitox	18	07.10.2004	1.71	0.00	2180
Loughborough 4	19	09.09.2004	2.96	0.00	2090
Minworth lane 5.2	20	07.10.2004	1.35	0.00	2380
Newark	21	22.11.2004	1.65	0.00	3070
Northampton BLC	22	04.12.2004	1.94	0.00	2600
New Wanlip 1 sa	23	09.09.2004	n.d.	n.d.	985
N	24	09.09.2004	2.24	0.00	2460
ew Wanlip 1 da	25	09.09.2004	2.24	0.00	2460
Old Wanlip 2 6	26	16.09.2004	1.35	0.88	1900
Packington OD1	27	28.09.2004	0.92	0.00	2470
Rushmore 2 new	28	28.09.2004	1.32	0.00	1680
Rushmore old	29	09.09.2004	1.32	0.00	1680
Stoke Bardolph New 1	30	09.09.2004	1.61	0.00	2170
Stoke Bardolph South 4	31	09.09.2004	1.61	0.00	2200
Stoke Bardolph North 6	32	28.09.2004	1.61	0.00	2510
Strongford Old 1A	33	28.09.2004	1.57	0.02	2580
Strongford Old 1B	34	28.09.2004	1.57	0.02	2580
Wheaton Aston OD			1.32	0.00	2070
Oil refineries					
Lindsey 1		07.09.2005	0.73	0.24	
Lindsey 2		07.10.2005	0.51	0.00	
Eastham		01.06.2006	1.60	2.34	160
Pembroke		09.06.2006	1.16	1.14	770
Humber 1		21.06.2006	1.39	0.00	4830
Humber 2	35	16.10.2006	1.27	0.00	9017
Humber 3 replicate 1	36	16.11.2006	1.15	0.00	7863
Humber 3 replicate 2	37	16.11.2006	1.15	0.00	7863
Humber 3 replicate 3	38	16.11.2006	1.15	0.00	7863
Humber 3 replicate 4	39	16.11.2006	1.15	0.00	7863
Humber 3 replicate 5	40	16.11.2006	1.15	0.00	7863
Humber 3 replicate 6		08.01.2007	0.93	0.00	8585
Humber 4		26.07.2006	0.73	0.00	4300
Grangemouth 1		28.02.2007	0.06	0.00	5288
Grangemouth 2		12.04.2007	0.25	0.00	5543
Grangemouth 3					

*Municipal WWTPs data source: from Pickering, 2008.

+Sample numbers correspond to the lanes in Figure 4.1

Of the 29 reactors analysed, archaeal *amoA* genes were detected in only three: the Lindsey refinery WWTP, the Grangemouth refinery WWTP and the pilot plant treating leather processing waste. Figure 4.1 shows the results of PCR analysis of archaeal *amoA* from 23 municipal WWTPs in the UK, the pilot reactor treating tannery wastes, and samples from the third sampling date at the Humber refinery WWTP in November 2006. Figure 4.2 shows positive PCR products obtained from samples surveyed from the Lindsey and Grangemouth refineries.

The sample from the pilot plant treating leather processing wastewater rendered a clear positive PCR product with primers Arch-*amoA*AF and Arch-*amoA*AR (lane 22 on Figure 4.1). However a very faint band was also be detected in one of the six replicate samples from the Humber refinery WWTP (lane 37).

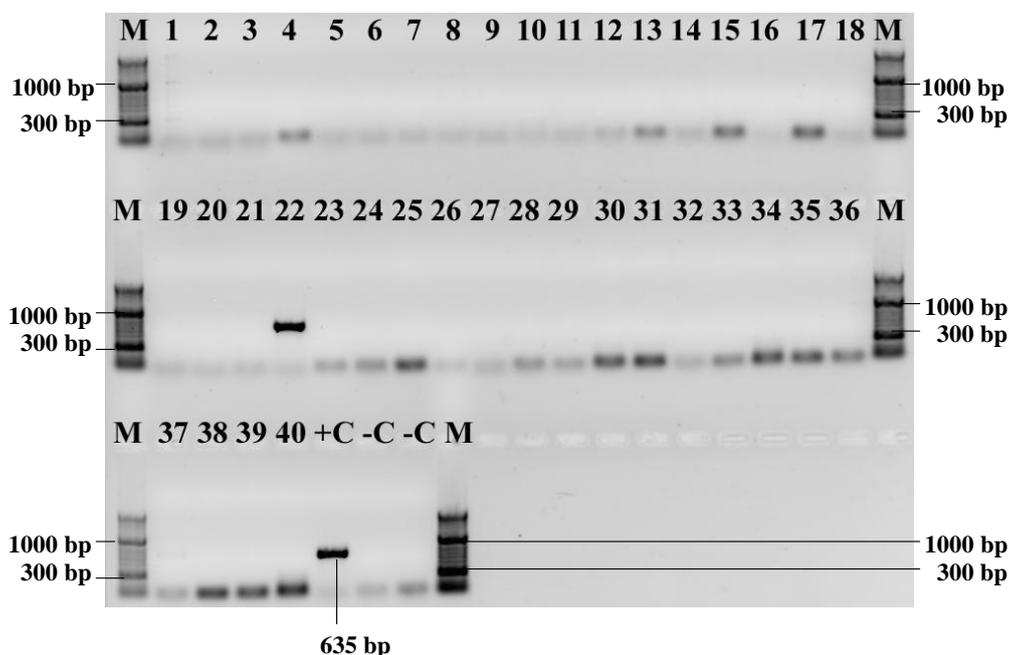


Figure 4.1 Agarose gel showing PCR products amplified with archaeal *amoA* primers Arch-*amoA*AF and Arch-*amoA*AR from DNA extracted from samples collected in a survey in 23 full scale municipal WWTPs, and one pilot reactor treating wastewater from a leather processing plant from the British Leather Corporation (BLC) in the UK. Lanes from 1 to 21 and from 23 to 34 refer to samples collected from 23 full scale WWTPs. Lane 22 refers to the BLC pilot plant. Lanes 35 to 40 included replicate samples from the Humber refinery WWTP third sampling on 16.11.2006). See Table 4.1 for details of each reactor. M= DNA molecular weight marker (2000 bp- 50 bp from top to bottom); +C = cloned archaeal *amoA* gene used as a positive control (expected fragment size of 635 bp); -C = negative control containing no DNA template.

Positive PCR products were obtained with primers Arch-*amoA*F and Arch-*amoA*R in samples from two oil refineries investigated in this study, Lindsey and Grangemouth (Figure 4.2). Putative AOA were detected in one replicate from reactor A from the Lindsey plant at the second sampling time (07.10.2005), and all replicates from Grangemouth, as well as in reactors A and B from the first (26.07.2006) and third sampling time (12.04.2007).

The band intensities from PCR products obtained from the replicate samples from the Lindsey treatment reactor and the BLC leather sample were strong in comparison to the PCR products obtained from samples from the Grangemouth treatment plant (Figures 4.2).

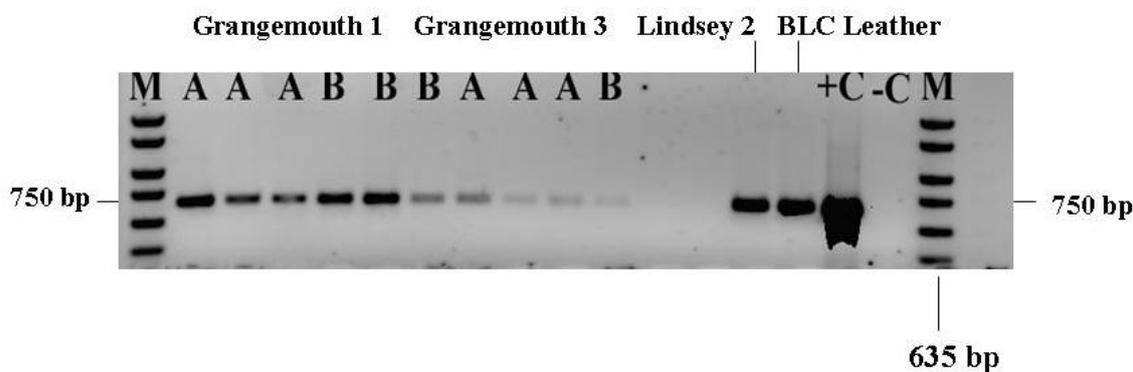


Figure 4.2 Agarose gel showing PCR products from DNA extracted from the Grangemouth, Lindsey and BLC wastewater treatment reactors amplified with archaeal *amoA* primers Arch-*amoA*F and Arch-*amoA*R (Francis *et al.*, 2005). M= DNA molecular weight marker (2000 bp-50 bp from top to bottom); A= Reactor A; B= Reactor B; +C = cloned archaeal *amoA* gene used as a positive control (expected fragment size of 635 bp); -C = negative control containing no DNA template. Samples refer to the following sampling dates: Lindsey 2 = 07.10.2005; Grangemouth1 = 26.07.2006; Grangemouth 3 = 12.04.2007; BLC leather = 04.12.2004.

4.3.2 DGGE analysis of archaeal *amoA* genes

The *amoA* genes amplified with primers Arch-*amoAF* and Arch-*amoAR* were analysed using DGGE (Figure 4.3).

Interestingly, DGGE profiles from the Grangemouth plant were identical, even when sampled nine months apart, on 26 July 2006 and 12 April 2007. DGGE profiles from the Lindsey and BLC reactors were distinct from the Grangemouth DGGE profile and more bands were detected in the Lindsey reactor (Figure 4.3).

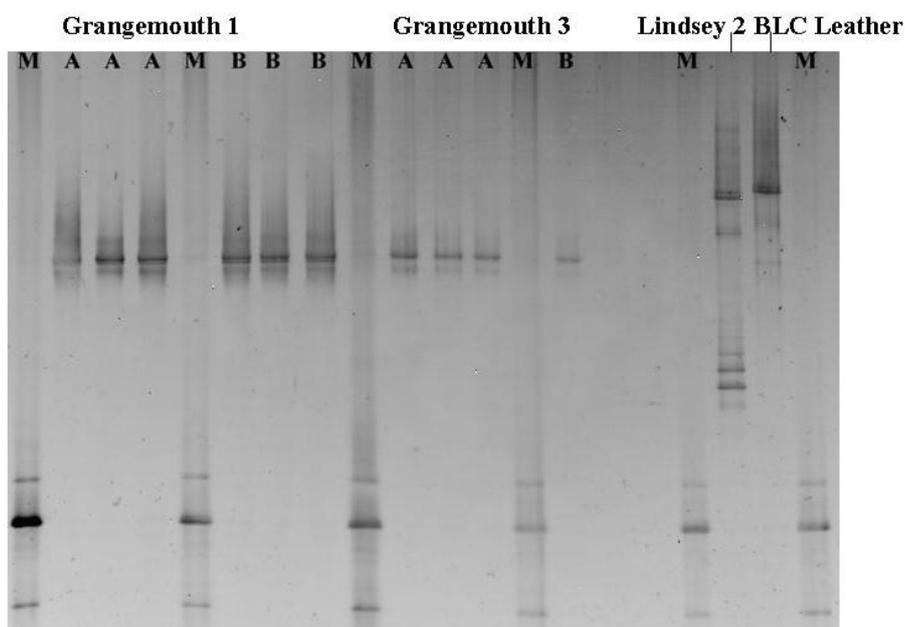


Figure 4.3 Comparison between DGGE profiles of archaeal *amoA* genes (PCR products amplified with Arch-*amoAF* and Arch-*amoAR* primers) from DNA samples from Grangemouth, Lindsey and BLC wastewater treatment reactors. A = reactor A; B = reactor B; M = bacterial DNA clone mixture used as a marker. Samples refer to the following sampling dates: Lindsey= 07.10.2005; Grangemouth1= 26.07.2006; Grangemouth 3= 12.04.2007; BLC leather= 04.12.2004.

Cluster analysis demonstrated that the Grangemouth AOA communities were distinct from the Lindsey and BLC reactor communities.

Although representing distinct industrial wastewater types, the BLC wastewater also had much higher COD (990 mg/L; Table 4.1) and BOD (549 mg/L; Table 4.1) in comparison to the Lindsey reactor, with COD 190 mg/L and BOD mg/L (Table 3.2); but interestingly their AOA communities were more similar to one another than the Grangemouth AOA communities.

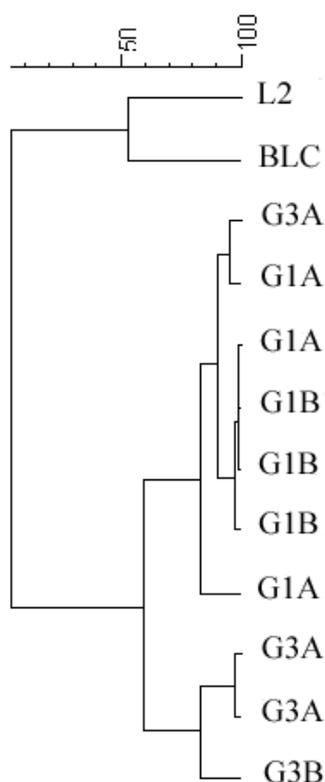


Figure 4.4 UPGMA cluster analysis of DGGE profiles of uncultured crenarchaeotal *amoA* gene fragments obtained from samples collected from the Grangemouth (G1 and G3), Lindsey (L2) and BLC wastewater treatment reactors after Pearson correlation of whole-track densitometric curves of the profiles. Letters A and B after the plant codes refer to replicates from reactors A and B from their respective wastewater treatment plants. Samples refer to the following sampling dates: Lindsey L2 = 07.10.2005; GrangemouthG1 = 26.07.2006; Grangemouth G3 = 12.04.2007; BLC = 04.12.2004.

The data were not normally distributed and Kruskal-Wallis non parametric ANOVA was performed. There were no statistically significant differences between replicates, reactors or different sampling occasions for the Grangemouth plant (p values ≥ 0.05).

As only a single replicate from the Lindsey and BLC reactors gave a positive archaeal *amoA* PCR, the test could not be applied between different WWTPs, but similarity values between the Lindsey, Grangemouth and BLC plants were low (0-0.3). The similarity between the Lindsey and BLC reactor was 0.53.

4.3.3 Analysis of archaeal *amoA* gene clone libraries

Four clone libraries were built in this study from DNA samples that yielded PCR products for AOA *amoA* genes: one replicate from reactor A from the second sampling time (07.10.2005) in the Lindsey plant; the BLC pilot plant (07.12.2004), reactor A and B from the Grangemouth plant sampled on 26 July 2006, the same samples that were used to derive the AOB 16S rRNA clone libraries from Grangemouth (26.07.2006). These samples were also used to generate AOB 16S rRNA gene clone libraries from the Grangemouth plant (Chapter 3).

In total 168 archaeal *amoA* sequences were considered in the clone library analyses.

Table 4.2 summarizes the information from each library, and the patterns observed are shown by rank abundance plots of OTUs grouped at the 97% sequence identity level (Figures 4.5 and 4.6). Grangemouth, reactors A and B, as well BLC leather clone libraries, appeared dominated by a single abundant clone type, while the sample from the Lindsey reactors had a more even distribution of OTUs with two abundant phylotypes.

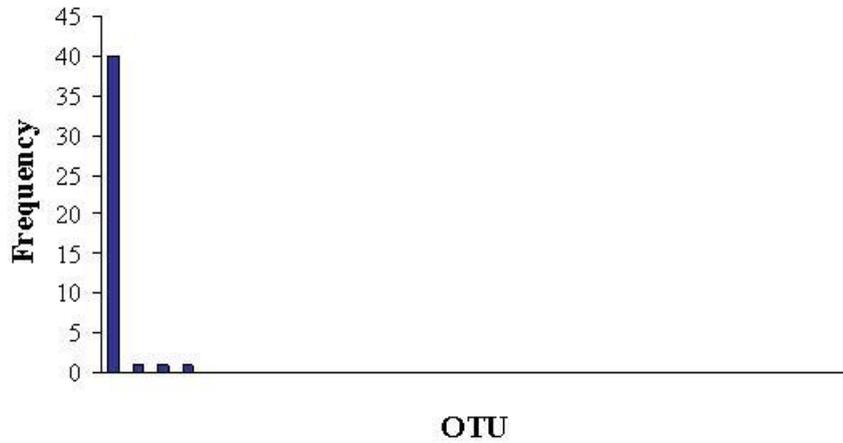
Table 4.2 Description of four crenarchaeotal *amoA* gene clone libraries constructed in this study

Libraries	Sampling Dates	Sequences in libraries (N)	Number of OTUs/Library	Most abundant OTU (clone numbers)	Number of Doubletons (2 OTUs)	Number of Singletons (1 OTU)
Lindsey	07.10.2005	37	7	22	None	5
Grangemouth A	26.07.2006	43	4	40	None	3
Grangemouth B	26.07.2006	43	8	34	2	5
BLC	04.12.2004	45	4	42	None	3

A= Reactor A; B= Reactor B

Grangemouth A

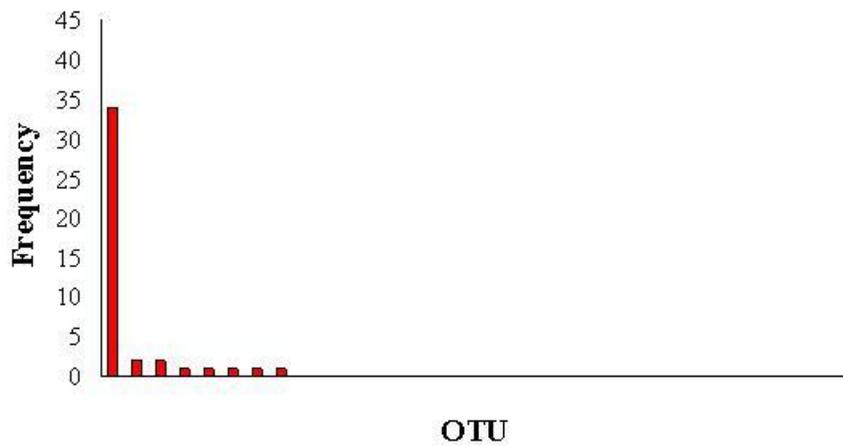
(26.07.2006)



(a)

Grangemouth B

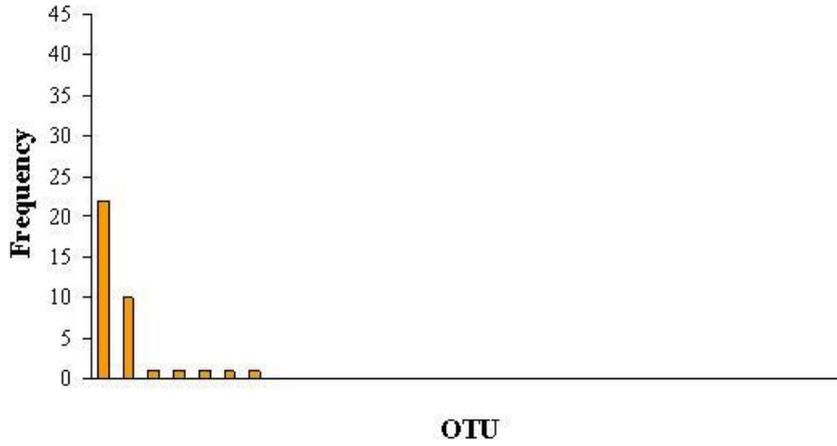
(26.07.2006)



(b)

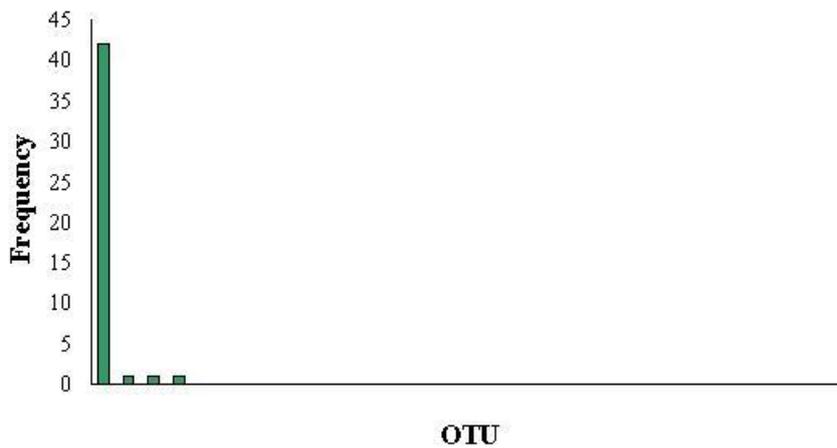
Figure 4.5 Crenarchaeotal *amoA* gene clone library rank abundance plots derived from the Grangemouth reactors (a) A and (b) B.

Lindsey
(07.10.2005)



(a)

BLC Leather
(04.12.2004)



(b)

Figure 4.6 Crenarchaeotal *amoA* gene clone library rank abundance plots derived from the (a) Lindsey reactor A and the (b) BLC pilot reactor.

As shown in Table 4.3, the coverage of the libraries was high (ranging from 77 % in Grangemouth reactor A to 93 % in the BLC reactor B).

The highest AOA diversity was found in the Lindsey plant (Shannon = 0.98; and Simpson = 0.59 respectively; Table 4.3). Conversely the lowest diversity was found in Grangemouth reactor A and BLC leather (Shannon = 0.26 and 0.27 respectively; Simpson = 0.13 and 0.13 respectively; Table 4.3).

Table 4.3 Coverage and diversity indices measured for four AOA *amoA* gene clone libraries constructed in this study

WWTP	Sampling dates	Coverage (%)	Shannon	Simpson (1-D)
Lindsey	07.09.2005	86.00	0.98	0.59
Grangemouth A	26.07.2006	77.08	0.26	0.13
Grangemouth B	26.07.2006	88.00	0.76	0.38
BLC	04.12.2004	93.33	0.27	0.13

Archaeal *amoA* gene diversity in reactor B from the Grangemouth plant was higher (Shannon= 0.76; Simpson= 0.38; Table 4.3) compared to *amoA* diversity in reactor A. (Shannon= 0.26; Simpson= 0.13; Table 4.3).

These reactors were operated at different sludge age; 25 days in reactor A and 14 days in reactor B, and this may explain the differences found in AOA diversity. Although the diversity was greater in reactor B, all the *amoA* sequences detected in reactor A were also found in reactor B. This difference was not uncovered in the DGGE analysis (Figure 4.3) and as seen in relation to the AOB libraries, this may be explained by the fact that in a DGGE gel only the most abundant species present at 1% or above in a sample may be visualized as bands on a gel (Muyzer *et al.*, 1993). On the other hand, one single band may also represent several different genes which may comigrate to the same position on the gel.

Both Chao1 and ACE estimators reached a stable asymptote with data from all libraries except the library obtained from the Lindsey reactor (Figure 4.7). It is also interesting that in contrast to what was found for the AOB libraries, the estimators Chao1 and ACE for all libraries were the same and estimated a lower number of OTUs, for example for Grangemouth reactor A and BLC less than 10 clones and for Grangemouth reactor B approximately 18 clones. Thus all the libraries, except for the library from the Lindsey plant were sufficiently sampled. Although coverage was high in Lindsey (86%: Table 4.3), neither Chao1 and ACE (Figure 4.7) reached an asymptote, showing that the sampling effort (37 clones; Table 4.2) for this library was not sufficient to recover the total *amoA* diversity present in this library.

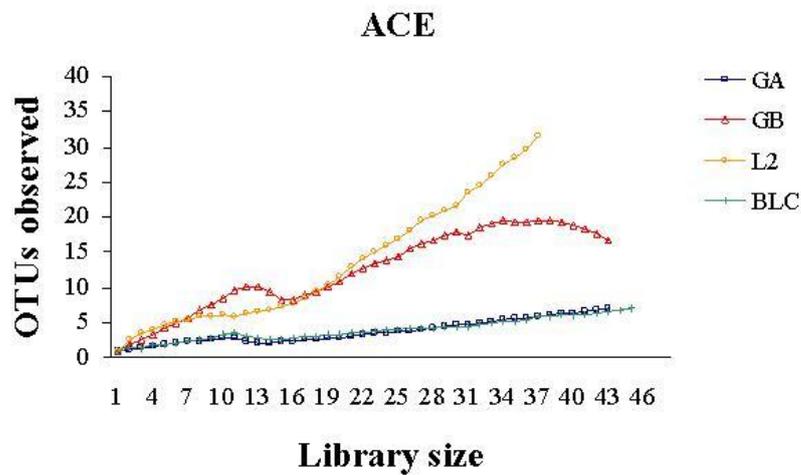
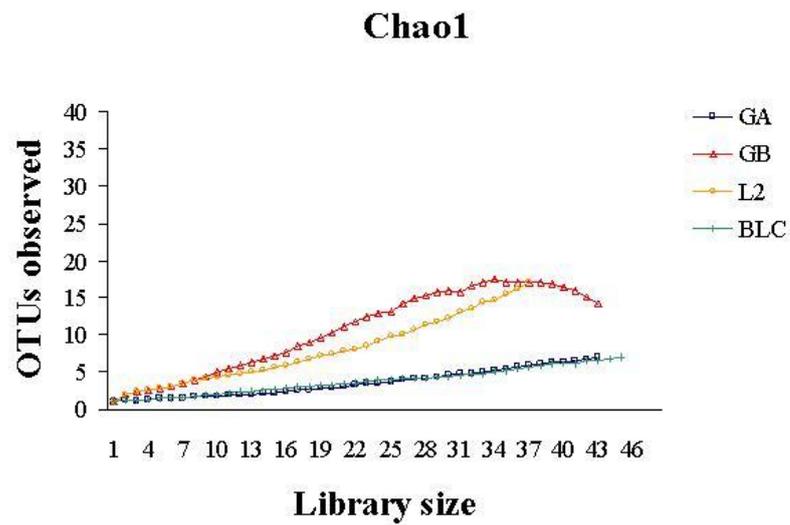


Figure 4.7 Abundance-based estimate curves obtained for four AOA *amoA* gene clone libraries constructed in this study: a) Chao 1; b) ACE. From top to bottom on legend: GA and GB refer respectively to reactors A and B from Grangemouth (26.07.2008); L2 = clone library from the Lindsey reactor (07.10.2005) and BLC = BLC pilot WWTP (04.12.2004).

4.3.4 Statistical comparisons between clone libraries

It is important to mention here that *amoA* genes from AOA were not detected through endpoint PCR reactions in the Humber refinery, with the exception of a faint band detected in 1 sample (Figure 4.1, lane 37). However, subsequent work conducted in collaboration with the University of Vienna detected AOA in the Humber treatment plant sampled on 8 January 2007. The analyses at the University of Vienna used identical PCR conditions to this study (See Chapter 2, Section 2.6), but bovine serum albumin (BSA) was added to the PCR reaction (Marc Mußmann, personal communication).

The diversity found in the resultant clone library was very low (Marc Mußmann, personal communication) and ten sequences provided from this library were also considered in the statistical comparisons between clone libraries, as well as in the phylogenetic analysis in this study.

According to J-LIBSHUFF analysis, reactors A and B from the Grangemouth plant were not statistically different (homologous and heterologous p-values higher than minimum p-value 0.0008); with the reactor B and reactor A communities were likely sampled from the same parent community. They were therefore grouped and considered as one single library among library comparisons (Table 4.4).

The *amoA* genes from the Grangemouth, Lindsey, BLC and Humber plants were all distinct ($p = 0.0000$) indicating that each has different AOA communities.

Table 4.4 J-LIBSHUFF population p values determined for comparisons of four AOA *amoA* gene clone libraries constructed in this study

Homologous library (X)	Heterologous library (Y)*			
	Lindsey 2	Grangemouth	BLC leather	Humber 4
Lindsey 2		<0.0001	<0.0001	<0.0001
Grangemouth	<0.0001		<0.0001	<0.0001
BLC	<0.0001	<0.0001		<0.0001
Humber 4	<0.0001	<0.0001	<0.0001	

*Libraries are distinct from one another if both comparisons (X versus Y and Y versus X are significant at $p \leq 0.0008$ (after pair-wise correction). Libraries presented on table refer to the following sampling dates: Lindsey= 07.10.2005; Grangemouth= 26.07.2006; BLC leather= 04.12.2004; Humber 4= 08.01.2007.

4.3.5 Phylogenetic analysis of archaeal *amoA* gene sequences

To date there have been few reports of archaeal *amoA* gene analysis in relation to the occurrence of putative AOA in WWTPs (Park *et al.*, 2006; Wells *et al.*, 2009; Zhang *et al.*, 2009). In the 2006 Park *et al.* work, most sequences were found to fall within four clusters designated A, B, C and D, retrieved from five municipal WWTPs operating aerated anoxic processes (Orbal and Vertical Loop Reactor (VLR[®]) systems) where extremely low oxygen conditions are applied to favour simultaneous nitrification and denitrification. In these WWTPs, active nitrification was occurring, and they were operating with influent ammonia concentrations ranging from 1.21 to 2.63 mM, low oxygen concentrations (0-2.5 mg/L) and high SRT (15-22 days).

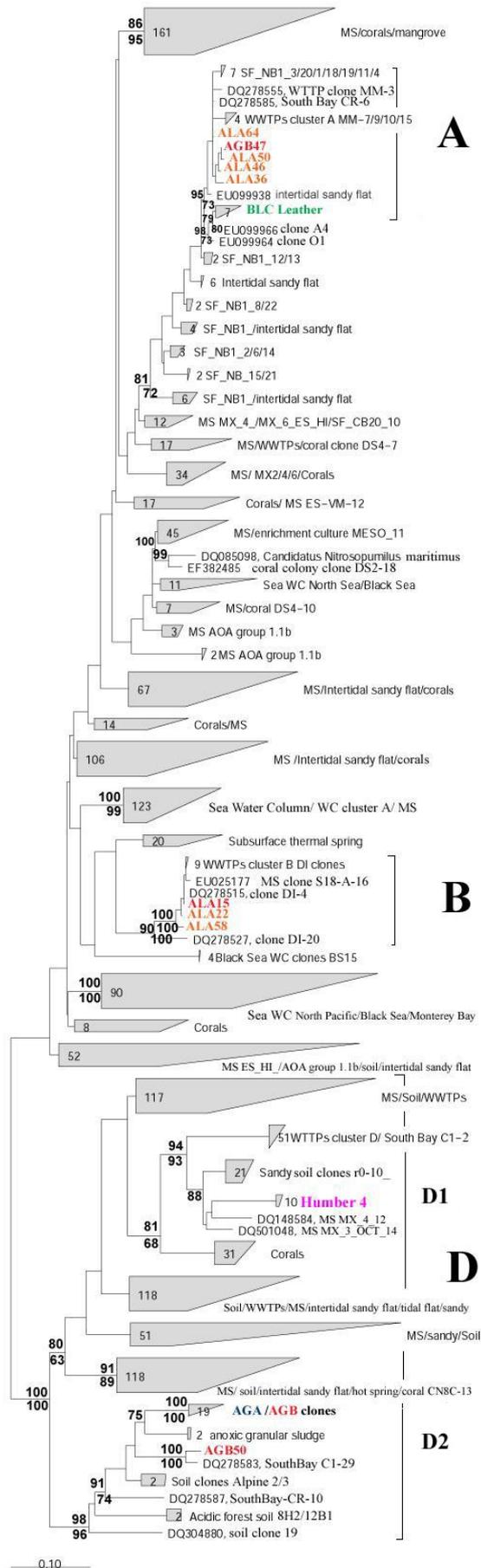
In this study, most of the AOA sequences retrieved from the oil refinery wastewater treatment systems related to clusters A, B and D from the Park *et al.* (2006) study; and to other sequences retrieved from marine and estuarine sediments, and soils (Figure 4.8).

The most abundant clone from the Lindsey plant (ALA64) representing 59.5% of the clones, as well as other singletons from the same library, one clone from Grangemouth reactor B and all clones retrieved from the BLC pilot reactor, were recovered in cluster A, as defined by Park *et al.* 2006. Within cluster A (Figure 4.8) the clone MM-3 was derived from one of the plants investigated by Park *et al.*, 2006, operating an aerated-anoxic VLR process (a variation of the Orbal process) as well as South Bay clone CR-6, a sequence retrieved from South San Francisco Bay close to the outfall from one of the WWTPs analysed, though it was not directly affected by the treatment plant outfall. In contrast, the second most abundant clone retrieved from the Lindsey reactor (clone ALA15) representing 27% of the clones (Figure 4.8) appeared related to sequences from cluster B (clones DI) and clone S18-A-16 retrieved from marine sediments of a Chinese estuary (Dang *et al.*, 2008). Cluster B was well supported by bootstrap resampling (100%).

Subcluster D1 within Cluster D contained all the sequences retrieved from the Humber reactor (Figure 4.8). These were distinct from, but related to, clones MX-4_12 and MX_3_OCT_14, retrieved from sediments of the Bahía del Tóbari estuary (Beman and Francis, 2006). Sequences within Cluster D1 were also related to other WWTP sequences from cluster D and clone South Bay C1-2, described by Park *et al.* (2006) as well as to sequences retrieved from sandy soil ecosystem (Leininger *et al.*, 2006) and from corals (Beman *et al.*, 2007).

Cluster D had 100% bootstrap support and also contained 100% of the sequences retrieved from Grangemouth reactor A and 98% sequences retrieved from reactor B. The sequences retrieved from Grangemouth reactors formed another distinct subgroup of WWTP-associated archaeal *amoA* sequences within cluster D, which was different from the subcluster containing the sequences retrieved from the Humber reactor, and therefore designed as subcluster D2 (Figure 4.8). Sequences retrieved from the Grangemouth plant were also related to two sequences retrieved from the anoxic granular sludge from an anaerobic ammonium oxidation system (unpublished) from where several soil *Crenarchaeota* 16S rRNA sequences were previously retrieved (Collins *et al.*, 2005). The entire cluster was closely related to clone AGB50, from Grangemouth reactor B, which grouped with clone South Bay CI-29, with high (100%) bootstrap support (Park *et al.*, 2006).

The majority of sequences from the Grangemouth reactor (cluster D) formed a distinct subcluster within cluster D, including sequence South Bay C1-29, which in the study of Park *et al.* (2006) referred to a sediment site located near to the plant outfall. Furthermore in line with the results found by Park *et al.* (2006), in which 67% of the sequences from the five activated sludge WWTPs investigated associated within cluster D, and represented an AOA type specific to wastewater treatment plants, the sequences retrieved from the Humber and Grangemouth treatment plants also related to cluster D. Park *et al.* (2006) reinforce the idea that cluster D *amoA* sequences might represent specific AOA which associated with wastewater treatment systems.



LEGEND

- Grangemouth A (clones AGA)
- Grangemouth B (clones AGB)
- Lindsey 2 (clones ALA)
- Humber 4
- BLC Leather

Figure 4.8 Phylogenetic relationships among ammonia oxidizing archaeal *amoA* sequences. MS = marine sediments; WC = water column. Bootstrap values (> 60%) shown at branch nodes above and below the lines are derived from distance and parsimony analyses respectively.

Neighbour-joining tree, built in ARB using the Jukes and Cantor DNA distance correction with sequences from this study and other sequences from: Francis *et al.*, 2005; Beman and Francis, 2006; Park *et al.*, 2006; Dang *et al.*, 2008; Beman *et al.*, 2007; Könneke *et al.*, 2005; Wuchter *et al.*, 2006; Coolen *et al.*, 2007; Mincer *et al.*, 2007; Spear *et al.*, 2007; Weidler *et al.*, 2007; He *et al.*, 2007; Leininger *et al.*, 2006; Hallam *et al.*, 2006b; Park *et al.*, 2008; Bae *et al.*, 2010. Sequences considered from other unpublished work: Shipin, 2007 (EF687846- EF687850); Magalhães *et al.*, 2007 (EU099927- EU099966); Ye *et al.*, 2006 (EF32877- EF32878); Boyle *et al.*, 2007 (EF530109- EF530126); Wakelin and Stephen, 2006 (DQ304862-DQ304894, DQ304896); Avrahami *et al.*, 2007 (DQ312267- DQ312293); Nicol and Prosser, 2006 (DQ534697- DQ534704); Jia and Conrad, 2007 (AB353501- AB353450); Samples refer to the following sampling dates: Lindsey2 = 07.10.2005; Grangemouth= 26.07.2006; Humber 4 =08.01.2007; BLC = 04.12.2004. Scale bar represents 10% sequence divergence.

No clones related to cluster C (Park *et al.*, 2006) were found in this study. It is important to note that AOA *amoA* genes could not be detected from the Humber reactor using the standard PCR protocol used in this study. However, they were detected by the PCR performed by Marc Mußmann at the University of Vienna; the only difference being that Bovine Serum Albumin (BSA) was added to the PCR.

BSA is known to improve the efficiency of PCR reactions (Kreader, 1996) and reduce inhibition caused by substances co-extracted with the DNA, such as phenolic compounds or heavy metals. Phenols and metals are pollutants commonly found in oil refineries wastewaters and it makes sense that BSA may have a positive effect on PCR amplification of DNA obtained from such environments. Despite the addition of BSA, the bands obtained by Mußmann were still very weak (personal communication).

Park *et al.* (2006) mentioned that AOA *amoA* sequences were not detected in some WWTPs, but despite this, they could detect archaeal *amoA* in sediments located in the vicinity of the wastewater outfall from such plants, for example South Bay C1 clones. On this basis they inferred that AOA were absent from these plants, or the archaeal *amoA* genes were below the detection limits of the PCR assay used, or alternatively, that other distinct types of archaeal *amoA* were present which did not contain the target sequences for the primers used in their study.

The diversity of AOA in all plants where they were detected was low. However, the hypothesis that the efficiency of the archaeal primers developed by Francis *et al.* (2005) might explain why weak bands were obtained with PCR from DNA from the Humber reactor, is supported by qPCR analysis of the same sample using a different set of primers (Treusch *et al.*, 2005), which targeted a nested PCR fragment of 103bp within the *amoA* gene fragment targeted by the primers designed by Francis *et al.*, 2005. This demonstrated that AOA were relatively abundant in the Humber sample (10^7 - 10^8 gene copies/mL; See Chapter 5).

Furthermore as pointed out by Park *et al.* (2006) AOA were detected in systems with very low oxygen, as well as subjected to transient anoxic and aerated conditions, and long retention times. Dissolved oxygen (DO) concentration in plants from this study ranged from 0.7 to 4.5 mg/L (Table 3.1; Chapter 3) and they were higher than the DO concentrations (in general ranging from 0.0 to 2.5 mg/l) reported for the plants investigated by Park *et al.* (2006) in which AOA were detected. However, in this study, clones retrieved from the Grangemouth refinery WWTP were found to be related to sequences retrieved from an anoxic granular sludge system for anaerobic ammonium oxidation (Bae *et al.*, 2010), therefore representing an environment of limited oxygen concentrations. This also suggests that oxygen may have had some influence in the Grangemouth reactors, specifically on the first sampling occasion at the Grangemouth plant in July 2006, from which the clone libraries constructed in this study were obtained.

As with the plants investigated by Park *et al.* (2006) which had long sludge retention times (15-22 days) and long hydraulic retention times (>24 hours), the activated sludge systems of the oil refinery WWTPs were also operated with long sludge ages. The Grangemouth plant operated reactors with sludge ages of 25 (reactor A) and 14 days (reactor B). In the Humber plant, although the aim was to keep a sludge age at 25 days on average, sludge ages much higher than this value were usually found in the wastewater treatment reactors, as well as longer hydraulic retention times (min.11 and max.33 hours).

It is also noteworthy that in the Grangemouth plant both AOB and AOA were detected, but by contrast, AOA but not AOB were detected in the Humber plant. This may indicate a possible role for AOA in nitrification in the Humber plant.

4.4 Conclusions

Putative AOA were found in three of the five refinery WWTPs investigated in this study, in which nitrification was occurring, in the Lindsey, Grangemouth, and Humber WWTPs. The fact that putative AOA were also found in the Humber WWTP contributed to reinforce the idea that AOA might have an important role in the refinery WWTPs, as no AOB related sequences were recovered from the two clone libraries derived from the Humber WWTP (See Chapter 3).

In relation to the AOA diversity found in the refinery wastewater treatment plants investigated in this study, the main conclusions from this Chapter are summarized as follows:

- As with AOB, AOA diversity found in some oil refinery wastewater treatment systems was low.
- Based on the AOA clone libraries analysed here, the Lindsey plant, which was a trickling filter system, harboured a greater diversity of AOA than the Grangemouth, BLC and Humber activated sludge systems.
- Most AOA in refinery wastewater treatment plants were typically dominated by a single OTU type, or in the case of the Lindsey plant, by two OTU types.
- While similar AOB OTU types were found in all plants investigated in this study, distinct AOA OTU types were found in each refinery WWTP.
- AOA found in the oil refinery WWTPs investigated here were related to AOA from low salinity environments – estuarine sediments, soil and other WWTPs.

Chapter 5

Ammonia oxidizer abundance and role in nitrification in oil refinery wastewater treatment systems

5.1 Introduction

Nitrification is a biological process that occurs in two consecutive reactions.

Ammonia oxidation is the first, and usually rate-limiting, step of the process of nitrification in which ammonia is converted to hydroxylamine and then nitrite through the action of ammonia monooxygenase and hydroxylamine oxidoreductase. The ammonia monooxygenase (AMO) enzyme is composed of three subunits: α , β and γ , encoded by the genes *amoA*, *amoB* and *amoC*. The gene encoding sub-unit A has been most commonly used as a molecular marker in ecological studies as it is well conserved, encodes the active site of the enzyme and occurs in all AOB (McTavish *et al.*, 1993).

The AMO enzyme was thought to occur only in ammonia oxidizing betaproteobacteria (AOB). However, recently, it was discovered that an ammonia monooxygenase homologue is also present in *Archaea* (Venter *et al.* 2004; Treusch *et al.*, 2005). The capacity of some *Archaea* to oxidize ammonia to nitrite was confirmed when the first member from the mesophilic *Crenarchaeota*, *Nitrosopumilus maritimus* (Könneke *et al.*, 2005) was isolated from a marine aquarium and successfully cultivated.

Following the revolutionary finding that *Archaea* have a role in the nitrification processes, abundance and activity of AOB and AOA, as well as their relative contributions to nitrification began to be investigated and both AOB and AOA involvement in nitrification were compared in a range of environments.

AOA have been demonstrated to be widespread, outnumbering AOB in several marine (Wuchter *et al.*, 2006; Lam *et al.*, 2007) and soil systems (Leininger *et al.*, 2006), as well as in rhizosphere systems. In rice paddy soils (Chen *et al.*, 2008) and freshwater macrophytes (Herrmann *et al.*, 2008) AOA numbers are also positively correlated with peaks in the potential nitrification rate. However, in some estuarine systems, AOB have been reported to outnumber AOA (Caffrey *et al.*, 2007; Mosier and Francis, 2008; Santoro *et al.*, 2008) and niche specialization between AOA and AOB has been suggested to occur as a function of salinity. In most cases, AOA were found to predominate in the regions of these estuaries characterised by greater amounts of freshwater.

The first demonstration of ammonia oxidation in the meso-thermophilic *Crenarchaeota* from soil group 1.1b came with the characterization of "*Candidatus Nitrososphaera gargensis*" (Hatzenpichler *et al.*, 2008); and a further thermophilic *Crenarchaeota*, "*Candidatus Nitrosocaldus yellowstonii*" (de la Torre *et al.*, 2008) capable of ammonia oxidation, has also been cultivated. Both organisms originated from hot springs and actively oxidize ammonia to nitrite. The enrichment cultures of these organisms contained no detectable AOB. In addition, *in situ* ammonia oxidizing activity and detection of AOA in several terrestrial hot spring environments, where no bacterial *amoA* genes were recovered, have been reported (Reigstad *et al.*, 2008). These findings reinforce the significant role of AOA in nitrification in these extreme environments, compared with AOB.

The abundance of AOB has been extensively investigated and they have been quantified in several aquatic and terrestrial systems as well as in wastewater treatment systems (Hermansson and Lindgren, 2001; Okano *et al.*, 2004; Limpiyakorn *et al.*, 2005; Layton *et al.*, 2005; Harms *et al.* 2003; Coskuner *et al.*; 2005, Pickering 2008) through culture-based methods, immunofluorescence and culture-independent techniques (Konuma *et al.*, 2001).

As only less than one percent of the total bacteria in the environment can be cultured, when culture-independent methods such as fluorescence *in situ* hybridization (FISH) and real-time PCR were developed, they provoked a great revolution in the quantification of uncultured microorganisms, significantly overcoming the bias and limitations of culture-methods (Wagner *et al.*, 1993) and since then they have been applied quantitatively to investigate nitrification in engineered systems (Daims *et al.*, 2001c; Harms *et al.*, 2003; Limpiyakorn *et al.*, 2005; Coskuner *et al.*, 2005; Pickering, 2008).

AOA abundance has been extensively quantified through real-time PCR and catalyzed reported deposition (CARD-FISH), a more sensitive method than conventional FISH. CARD-FISH is often applied when the cells being investigated are small, have low ribosome content and samples have high background fluorescence (for a good review of the CARD-FISH technique see Amann and Fuchs, 2008).

Although abundance of the functional gene *amoA* in environmental samples may indicate potential function, demonstration of the gene expression can render much more convincing evidence that activity is occurring.

The activity of AOB and AOA in the nitrification process has been investigated through reverse-transcription of the 16S rRNA and *amoA* mRNA (Nicol *et al.*, 2008) as well as through CARD-FISH coupled with microautoradiography (MAR-FISH; Neufeld *et al.*, 2007).

Reverse-transcriptase coupled with real-time PCR (RT-PCR) or combined with isotope methods (Leininger *et al.*, 2006) has been applied to measure gene expression in AOA and AOB and assess their relative contributions to nitrification in several soil systems (See Prosser and Nicol, 2008, for a good review).

Through RT-PCR, it is possible to measure the expression of transcribed 16S rRNA or *amoA* genes of AOA and AOB as a function of available ammonia concentrations or ammonia added to environmental samples, thus allowing AOB and AOA responses to ammonia to be specifically determined. RT-PCR generates double-stranded complementary DNA (cDNA) *in vitro* from single stranded RNA templates, and after reverse transcription the cDNA copies in the sample are quantified through real-time PCR.

AOB *amoA* expression has been investigated in some activated sludge systems through RT-PCR (Aoi *et al.*, 2002; Aoi *et al.*, 2004; Araki *et al.*, 2004; Ebie *et al.*, 2004) and AOA *amoA* gene expression was first demonstrated when AOA were discovered in soil (Treusch *et al.*, 2005).

Recently CARD-FISH has been successfully combined with microautoradiography (known as MAR-FISH - see Okabe *et al.*, 2004 for a good review of the MAR-FISH technique) and applied to demonstrate that during nitrification, "*Candidatus Nitrososphaera gargensis*" enrichments were most active at ammonia concentrations of 0.14 and 0.79 mM; but partially inhibited at higher ammonia concentrations (3.08 mM) (Hatzenpichler *et al.*, 2008).

Despite at least some *Crenarchaeota* being able to oxidize ammonia, their capacity for alternative mixotrophic or heterotrophic metabolism is currently under debate. Metagenomic studies of the marine *Crenarchaeota* known as *Cenarchaeum symbiosum* (Hallam *et al.*, 2006a; Hallam *et al.*, 2006b), as well as identifying the AMO genes encoding the enzymes involved in ammonia oxidation, also identified genes that encode the urease and urea transporters, and genes encoding an almost complete oxidative tricarboxylic acid cycle (TCA) characteristic of heterotrophic metabolism. This suggested that they can potentially metabolize reduced nitrogen forms as well oxidizing organic compounds.

The presence of a TCA cycle, although in this case complete, has also been identified for the four AOB genomes that have so far been completely sequenced, that is: *Nitrosomonas europaea* (Chain *et al.*, 2003), *Nitrosococcus oceani* (Klotz *et al.*, 2006), *Nitrosomonas eutropha* (Stein *et al.*, 2007) and *Nitrospira multiformis* (Norton *et al.*, 2008). Furthermore, although mainly autotrophic, AOB may also use carbon from pyruvate and fructose (Hommes *et al.*, 2003), and since AOB preserve ammonia oxidation as their unique source of energy they are able to grow facultatively as chemolithoheterotrophs (Arp *et al.*, 2007).

While in some terrestrial and marine environments, AOA have been inferred to be of greater quantitative significance for nitrification, to date there is only one report in the literature where such a comparison has been made in wastewater treatment systems (Wells *et al.*, 2009); this latter, however suggests the AOA contribution to nitrification to be minor. As shown by analysis of *amoA* clone libraries, in addition to AOB, AOA were found in the oil refinery wastewater treatment plants investigated in this study (See Chapter 4). To date AOA have not been quantified in WWTPs, and their relevance in the nitrification process in relation to AOB has not yet been investigated in these systems.

The aim of this study was therefore to assess the relative abundance of putative AOA relative to AOB in relation to nitrogen removal in wastewater treatment systems where putative AOA were detected.

5.2 Methods

BOD, COD, TKN, and ammonia removal efficiencies from the wastewater treatment plants at the Lindsey, Eastham, Pembroke, Humber and Grangemouth refineries were calculated by considering the difference between initial and final concentrations found respectively in influent and effluent samples and converted into percentage removal. Analysis of variance (ANOVA) was applied to effluent data from the refinery WWTPs that were nitrifying to determine if there were any differences in performance.

BOD/COD, TKN/BOD ratios in the influent to each wastewater treatment plant were determined as well as the relationship between ammonia consumption from the influent and nitrate production in the effluent. This information was used to calculate the percentage of ammonia removed by nitrification.

All the methods used in this study to determine the chemical parameters mentioned in this chapter are described in more detail in Chapter 2.

Some chemical parameters provided by the operators of the Grangemouth and Humber oil refineries were analysed and compared to values obtained in this study.

All samples collected from the five oil refinery wastewater treatment plants investigated in this study (12 from Lindsey, 3 from Eastham, 6 from Pembroke, 24 from Humber, 18 from Grangemouth, and the single replicate sample collected from the BLC wastewater treatment pilot reactor) gave a total of 64 samples where *Crenarchaeota*, AOA and AOB were quantified using real-time PCR.

DNA extracted from the 64 samples was diluted 10-fold and six real-time PCR assays were carried out in 96 well-plates to quantify the 16S rRNA gene abundances of total bacteria (Harms *et al.*, 2003); AOB (Hermansson and Lindgren, 2001); soil group *Crenarchaeota* (Ochsenreiter *et al.*, 2003); marine group *Crenarchaeota* (Mincer *et al.*, 2007); and the *amoA* gene abundances of AOB (Rotthauwe *et al.*, 1997) and AOA (Treusch *et al.*, 2005), respectively. The real-time PCR assays conducted in this study are described in more detail in Section 2.12, Chapter 2.

The real-time PCR results are reported as either 16S rRNA and *amoA* gene copies per mL of sample, or as cells/mL, after converting both AOB *amoA* and AOA abundances into cell numbers. To convert gene abundance into cell numbers it was assumed that on average two bacterial *amoA* copies exist per AOB cell, based on the number of copies reported for *Nitrosomonas europaea* (MacTavish *et al.*, 1993); and one *amoA* gene copy was found in the genome of *Cenarchaeum symbiosum* (Hallam *et al.*, 2006a). Specific ammonia oxidation rates per cell per hour were calculated according to the procedure described in Chapter 2, Section 2.13, adapted from Daims *et al.* (2001c). Detection limits for the real-time PCR assays were set up as the mean of the Ct values obtained from two blank reactions or no template control (NTC) that were included in each assay, plus three standard deviations.

The relationships between the gene abundance of the different groups of organisms measured through real-time PCR and the physicochemical parameters obtained from all oil refineries wastewater treatments plants were analysed through multivariate statistical analysis using principal component analysis (PCA), Pearson correlation and linear regression analyses.

The AOB biomass in the sludge from the Humber and Grangemouth refineries was theoretically estimated using the nitrification model developed by Rittmann *et al.*, 1999. Estimated AOB biomass predicted by the model was compared to biomass values determined from the real-time PCR data. Both the theoretical AOB biomass (X_{aob}) predicted by the model and the AOB biomass (X_{aob}) determined from qPCR data were related to one another to test if they were in agreement. See Chapter 2 (Section 2.16) for details of the calculations and the nitrification model.

5.3 Results and Discussion

5.3.1 WWTP performance with respect to BOD, COD, TKN and ammonia removal

In this study, as previously described in Chapter 2 (See Section 2.1), WWTPs at the Eastham, Pembroke, Humber and Grangemouth oil refineries are activated sludge systems where solids are kept in suspension and fully mixed with the wastewater being treated. In these systems, carbon removal and nitrification take place inside the same reactor. Lindsey WWTP is a trickling filters system sustaining the growth of attached biofilms through which the wastewater to be treated percolates from the top to bottom of the filters.

By contrast with the activated sludge systems, in the Lindsey WWTP, most of the organic load in the wastewater is first removed in an aerated lagoon prior to the filters (See Section 2.1, Chapter 2) and thus nitrification is favoured in the filters.

The Lindsey, Eastham, Pembroke, Humber and Grangemouth wastewater treatment plants were compared to one another in relation to their efficiency in the removal of organic carbon, Kjeldahl nitrogen and ammonia (See Tables 5.1 and 5.2).

Overall BOD removal efficiencies across reactors were high: 86% in Eastham, from 93 to 99% in Humber, and from 93 to 97% in the Grangemouth reactors (Table 5.1). In contrast, the Pembroke reactors had the poorest BOD removal efficiency (55%; Table 5.1). Likewise, COD removal efficiencies were 90% in Eastham, and ranged from 81 to 89% in Humber, from 62 to 92% in Grangemouth and reached 84% in the Pembroke reactors (Table 5.1).

The BOD and COD removal efficiencies were also high in the BLC pilot reactor, which is also an activated sludge system and were respectively 97 and 92% (Table 5.1). The BLC reactor was included here in order to compare its performance with the oil refinery wastewater treatment plants.

The trickling filters from the Lindsey refinery exhibited good BOD removal efficiencies (ranging from 75% to 100%) but the poorest efficiency of COD removal (ranged from 28 to 54%; Table 5.1).

BOD removal efficiencies among Lindsey, Humber and Grangemouth were not significantly different (ANOVA, $p= 0.127$), but COD removal was significantly different among the three plants (ANOVA, $p= 0.000$). Lindsey reactors had the worst COD removal in comparison to the Humber and Grangemouth reactors which were not significantly different from each other (ANOVA, $p= 0.615$).

As most of the organic carbon in the influent to the Lindsey reactors has been removed before it reaches the filters, as expected, much lower BOD/COD ratios (0.13 and 0.04; Table 5.1) were found in the influent to the Lindsey reactors in comparison to the BOD/COD ratios found in the activated sludge systems from Eastham, Humber and Grangemouth refineries, as well as the BOD/COD ratios found in the influent to the BLC pilot reactor (on average, 0.5; Table 5.1).

The much lower BOD/COD ratios in Lindsey reactors also explain the low COD removal efficiencies in Lindsey, since most of the COD left is recalcitrant COD whereas within high BOD systems a larger proportion of the COD is contributed by the BOD.

A very low BOD/COD ratio was found in the influent to the Pembroke reactors (0.07; Table 5.1); this was due to a very high value of COD (1456 mg/L) reflecting the chemical processes being operated in the refinery at the time the WWTP was sampled (June of 2006).

Although BOD/COD ratios found in the influent to the activated sludge systems were approximately similar, BOD and COD concentrations in the influent to the Eastham (178 and 352 mg/L respectively; Table 5.1) and Grangemouth reactors (BOD ranging from 70- 120 mg/L and COD from 139 to 311 mg/L; Table 5.1) were significantly lower than the values of BOD (246- 419 mg/L; Table 5.1; ANOVA, $p= 0.002$, and COD (610- 952 mg/L); ANOVA, $p= 0.001$, found in the influent to the Humber reactors.

Table 5.1 BOD and COD ratios and removal efficiencies across the wastewater treatment plants investigated in this study

WWTP	Sampling dates	BOD/COD ratio	BOD (mg/L)	BOD Removal efficiency (%)		COD (mg/L)	COD Removal efficiency (%)	
		Influent	Influent	Effluent A	Effluent B	Influent	Effluent A	Effluent B
Lindsey	07.09.2005	0.13	36	75	83	270	49	54
Lindsey	07.10.2005	0.04	7	100	100	190	28	38
Eastham	01.06.2006	0.5	178	86 *		352	90 *	
Pembroke	09.06.2006	0.07	102	55 *		1456	84 *	
Humber	21.06.2006	0.4	344	93	94	952	87	87
Humber	16.10.2006	0.5	419	98	99	834	84	81
Humber	16.11.2006	0.4	246	99	99	610	82	82
Humber	08.02.2007	0.54	378	99	98	700	89	83
Grangemouth	26.07.2006	0.4	113	96	96	311	92	90
Grangemouth	28.02.2007	0.4	70	93	97	177	62	83
Grangemouth	12.04.2007	0.86	120	94	97	139	91	75
BLC Leather	04.12.2004	0.55	549	97 *		990	92 *	
Process data provided by Humber	From Sep/2002 to Aug/2006	0.62 ±0.16	346 ±149 (n=20)	97 ± 2		573 ±199 (n= 200)	83 ± 9	
Process data provided by Grangemouth	From Nov/2004 to March/2007	0.4 ± 0.6	124 ±74 (n=139)	95 ± 7		314 ± 141 (n=505)	87 ± 10	

A= Rector A; B= Reactor B

*Correspond to final effluent after joining the effluent flows from both reactors A and B

The highest TKN/BOD ratios (0.91 and 2.13; Table 5.2) were also found in the influent to the Lindsey reactors (again reflecting the carbon removal in the aeration lagoon prior to the filters), in contrast to the TKN/BOD ratios found in the influent to the activated sludge systems from the Eastham, Pembroke, Humber and Grangemouth refineries, which ranged from 0 to 0.27 (Table 5.2).

Nitrification was occurring in the Lindsey, Humber and Grangemouth reactors (Table 5.2), and total ammonia removal was achieved on the second sampling occasion in the Lindsey refinery (October of 2005), as well as on all sampling occasions in the Humber and Grangemouth refineries (Table 5.2). Nitrate concentrations found in the effluent from the Lindsey, Humber and Grangemouth reactors ranged from 0.19 up to 1.54 mM (Table 5.2). However, nitrate production found among Lindsey, Humber and Grangemouth reactors were not statistically different (ANOVA, $p=0.377$).

By contrast, ammonia and TKN were not removed from the influent in the Eastham and Pembroke reactors, and no nitrate was detected in their final effluents, Table 5.2).

TKN removal efficiencies in the Humber reactors ranged from 86 to 100%.

Although 100% TKN removal was achieved in the Grangemouth reactors on the first sampling occasion in July 2006, no TKN was detected in the second and third samplings conducted respectively in February and April 2007. In comparison to the TKN concentrations found in the other refineries (from 1.06 in the Eastham influent up to 3.40 mM in the Humber influent; Table 5.2), the single value found in the influent to Grangemouth (1.13 mM; Table 5.2) was low, indicating that TKN contribution in the influent to Grangemouth in relation to the other plants investigated in this study was low. Moreover, TKN was not a chemical parameter routinely measured at this refinery.

Table 5.2 TKN/BOD ratios, TKN and ammonia removal efficiencies and ammonia-nitrate conversion across the wastewater treatment plants investigated in this study

WWTP	Sampling dates	TKN/BOD Ratio	TKN (mM)	TKN Removal efficiency (%)		Ammonia (mM)			NO ₃ Effluent (mM)		NH ₃ -NO ₃ * Conversion (%)		TKN- NO ₃ Conversion (%)	
				Influent	Influent	Influent	Effluent A	Effluent B	Effluent A	Effluent B	Effluent A	Effluent B	Effluent A	Effluent B
Lindsey	07.09.2005	0.91	2.33	26	34	0.73	64	70	0.69	0.75	147	145	115	94
Lindsey	07.10.2005	2.13	1.06	69	74	0.51	100	100	0.54	0.56	106	109	32	32
Eastham	01.06.2006	0.16	1.95	0		1.60	0		0		0		0	
Pembroke	09.06.2006	0.27	2.00	0		1.16	0		0		0		0	
Humber	21.06.2006	0.10	2.33	86	91	1.39	100	100	0.31	0.19	22	14	15	9
Humber	16.10.2006	0.10	2.93	100	100	1.27	100	100	1.28	1.35	101	106	43	46
Humber	16.11.2006	0.19	3.40	88	88	1.15	100	100	1.54	1.49	134	129	51	50
Humber	08.02.2007	0.10	2.80	100	100	0.93	100	100	1.55	1.55	167	167	55	55
Grangemouth	26.07.2006	0.14	1.13	100	100	0.73	100	100	0.51	0.61	70	83	45	54
Grangemouth	28.02.2007	0	0.00	0.00	0.00	0.06	100	100	0.0071	0.0014	1	2	0	0
Grangemouth	12.04.2007	0	0.00	0.00	0.00	0.25	100	100	0.20	0.23	81	92	0	0
Humber (process data)	Sep/2002 to Aug/2006					0.9 ± 2.1 (n=1103)	92 ± 10.3							
Grangemouth (process data)	Nov/2004 to March/2007					0.6 ± 0.55 (n=375)	95 ± 10							

A= Reactor A; B= reactor B

*Considering the simplest nitrification stoichiometric reaction in that each 1 mol of Ammonia is converted to 1 mol of NO₃

Note: In the conversion of ammonia to nitrate some of the values were higher than 100%, presumably because a proportion of the TKN is also converted to nitrate

Although better TKN removal efficiencies (69 and 74%) were achieved in the Lindsey reactors on the second sampling occasion conducted in October 2005 (Table 5.2), the trickling filter system's performance in removing TKN was inferior to that of the activated sludge systems.

Nitrite (0.20 mM) and nitrate (1.40 mM) were measured in the effluent sample from the BLC pilot reactor, thus showing that nitrification was also occurring when the plant was sampled in December 2004.

Nitrate production due to ammonia being consumed was higher than 100% in the Lindsey reactors as well as in the Humber reactors (Table 5.2), on the second, third and fourth sampling occasions conducted in the refinery in October and November 2006, and February 2007, respectively. The finding of higher nitrate concentrations produced in the effluent from the Lindsey and Humber reactors than could be accounted for by the quantity of ammonia removed (greater than 100% conversion efficiencies in Table 5.2), is likely to result from the conversion of a proportion of TKN to nitrate (Table 5.2).

It should be also noted that the Humber reactors were dosed with nutrients from a product called Nutromex containing 10% ammonium nitrate and 15% phosphate salts. Although this may exert some influence over the absolute nitrate concentrations found in the effluent from the Humber reactors, the nitrate concentrations found in the effluent from the Lindsey reactors were also higher than 100%, and by contrast with the Humber reactor, these reactors were dosed only with 75% phosphoric acid and no source of nitrogen. This raises the question of whether the nutrient dosage in the Humber reactors could be significantly contributing to the increase in total nitrate concentrations in the effluent.

The Nutromex injection rate in the Humber reactors is varied in order to ensure that a small residual amount of phosphate > 0.1 mg/L might be kept in the effluent from the clarifiers. Thus taking into account that Nutromex is added to the bioreactors to give a C:N:P mass ratio of 200:5:1, in equivalent proportion to P, at least a minimal residual nitrate concentration of about 0.55 mg/L should be expected in the effluent. Furthermore, considering that the amount of Nutromex to be added to reactors would give a nitrate concentration equivalent to $1/40^{\text{th}}$ of the concentration of organic carbon, represented by the COD concentration in the influent, and that the Humber reactors have a volume capacity of 4347 m^3 , expected nitrate concentrations given by the added nutrients on the four sampling occasions at Humber would correspond respectively to 0.25, 0.18, 0.14 and 0.14 mM. With the exception of the first sampling occasion in June 2006, when poor ammonia and TKN conversion to nitrate occurred in the Humber reactors (less than 20%; Table 5.2) these amounts of nitrate would not account for the concentrations of nitrate seen in the effluent, which were much higher, that is, 1.28, 1.35, 1.54, 1.49, 1.55, and 1.55 mM (Table 5.2). Thus the contribution of nitrate from the nutrients added as Nutromex might only have been significant on the first sampling occasion on 21.06.2006, in relation to the nitrate concentrations measured in reactors A and B, where the nitrate concentration in the effluent was 0.31 mM and 0.19 mM in reactors A and B respectively (Table 5.2). For instance, on the last sampling occasion in February 2007 (Table 5.2), all the ammonia (0.93 mM) and 55% of the TKN in the influent was converted to nitrate, resulting in 1.55 mM nitrate in the effluent from reactors, while expected nitrate concentration coming only from nutrient addition would be about 0.14 mM, that is, about 9% of the actual nitrate concentrations determined in the Humber reactor effluent.

Apart from the plants that were not nitrifying, in this study ammonia was efficiently removed in the Lindsey, Humber and Grangemouth reactors. Furthermore, although BOD removals were satisfactory in both trickling filters and the activated sludge systems, the activated sludge systems were apparently much more efficient in COD and TKN removal.

The MLVSS, ammonia, BOD, and COD concentrations measured in this study in the Humber and Grangemouth reactors were compared to process data provided by operators of the Humber reactor (from September/2002 to August/2006) and the Grangemouth reactor (from November/2004 to March/2007) (see Tables 5.1 and 5.2 and below) and the measurements from this study are consistent with the longer term operational data from the Humber and Grangemouth refineries.

The MLVSS (7236 ± 2283 mg/L; $n= 40$) concentrations found in the Humber reactors were often significantly higher than the MLVSS (4070 ± 865 mg/L; $n= 123$) concentrations found in the Grangemouth reactors (ANOVA, $p= 0.000$). Similarly, BOD (346 ± 149 mg/L; Table 5.1; $n=4$) and COD (573 ± 199 mg/L; $n=4$; Table 5.1) concentrations in the influent to the Humber WWTP were also significantly higher in comparison to the BOD (124 ± 74 mg/L; $n=3$; Table 5.1); ANOVA, $p= 0.003$; and COD (314 ± 141 mg/L; $n=3$; Table 5.1); ANOVA, $p= 0.002$.

In this study, pH measured in the influent to the Humber wastewater treatment reactors was slightly acid (around 6.5) in comparison with the slightly more alkaline pH (around 7.5) measured in the influent to the Grangemouth wastewater treatment reactors. These values were in agreement with the pH values measured over a longer operational period with values provided by the plant operators for the Humber and Grangemouth wastewater treatment plants being 6.5 ± 0.2 , ($n=1451$), and 7.3 ± 0.5 ($n=313$), respectively.

It is also important to point out that in contrast to the other activated sludge systems investigated in this study, the activated sludge system from the Humber refinery operated with very high MLVSS concentrations, long hydraulic retention times (HRT) and long sludge retention times (sludge age). According to the manager of the Humber wastewater treatment system, the normal HRT in the reactors is 33 hours and the minimum HRT is 11 hours.

Moreover, although their aim is to keep the sludge age in the range of 20-35 days, according to the data provided by the operator, sludge age values much higher than this are commonly used at the Humber wastewater treatment plant.

5.3.2 Quantification of total bacteria, AOB, marine and soil

***Crenarchaeota* 16S rRNA gene abundance**

PCR assays were conducted to quantify 16S RNA gene abundance of total bacteria, AOB, *Crenarchaeota* marine group 1.1a and soil group 1.1b. PCR efficiencies, regression data and detection limits of each real-time PCR assay are summarized in Appendix C.

Overall 16S rRNA gene abundance for total bacteria across the five oil refinery wastewater treatment plants investigated in this study ranged from 10^8 to 10^{10} gene copies/mL. The highest abundance was found in the Lindsey reactors (ranging from 7.53×10^9 to 3.29×10^{10} gene copies/mL; Figure 5.1), followed by Grangemouth (4.34×10^9 to 2.65×10^{10} gene copies/mL; Figure 5.3), Humber (2.64×10^8 to 4.77×10^9 gene copies/mL; Figure 5.2), Eastham (7.66×10^8 gene copies/mL), and Pembroke (2.27 to 3.22×10^8 gene copies/mL) reactors respectively (Figure 5.1).

AOB 16S rRNA gene abundance ranged from 10^7 to 10^9 gene copies/mL (See Figures 5.1, 5.2 and 5.3) and was also higher in Lindsey (1.53 to 2.33×10^9 gene copies/mL; Figure 5.1) while ranging from 6.21×10^7 to 2.62×10^9 gene copies/mL in the Grangemouth reactors (Figure 5.3), and from 2.35×10^7 to 2.39×10^9 gene copies/mL in the Humber reactors (Figure 5.2).

The AOB 16S rRNA gene abundance found in the Eastham reactor was 2.27×10^8 gene copies/mL, and in the two reactors from the Pembroke refinery 1.05 and 1.56×10^7 gene copies/mL respectively (Figure 5.1). These two activated sludge systems were not nitrifying (Table 5.1).

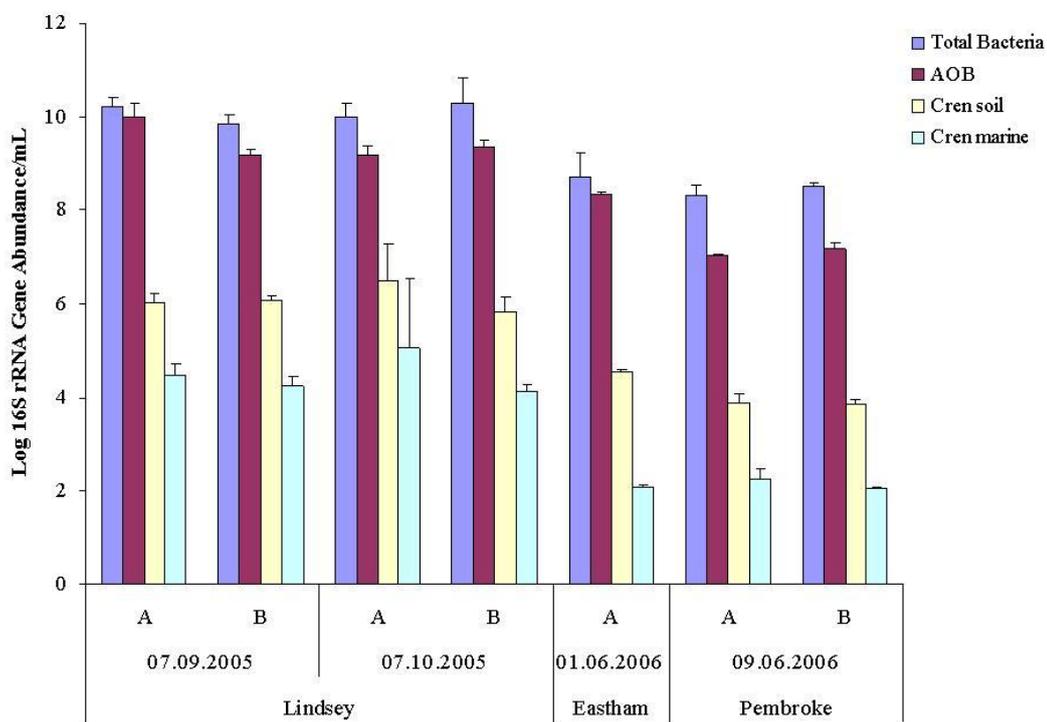


Figure 5.1 The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group *Crenarchaeota* in wastewater treatment reactors A and B from the Lindsey, Eastham and Pembroke refineries. Letters A and B refer to reactors A and B from each wastewater treatment plant. Error bars correspond to standard deviation of two or three replicate samples.

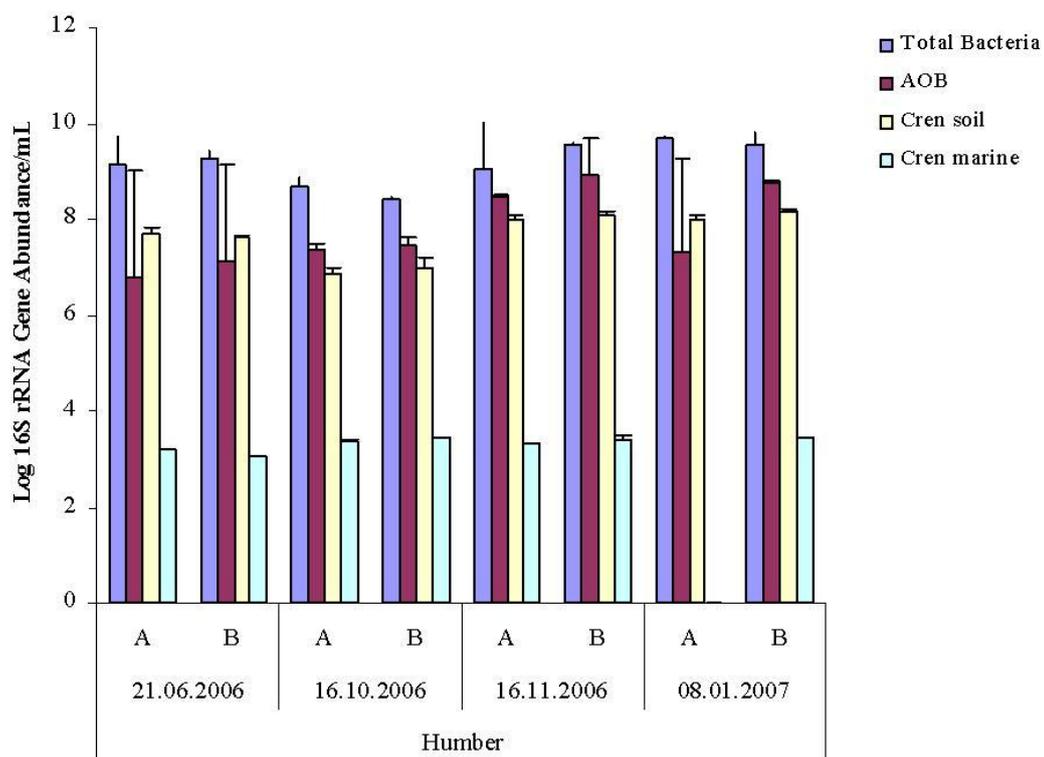


Figure 5.2 The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group *Crenarchaeota* in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions. Error bars correspond to standard deviation of the mean of two or three replicate samples.

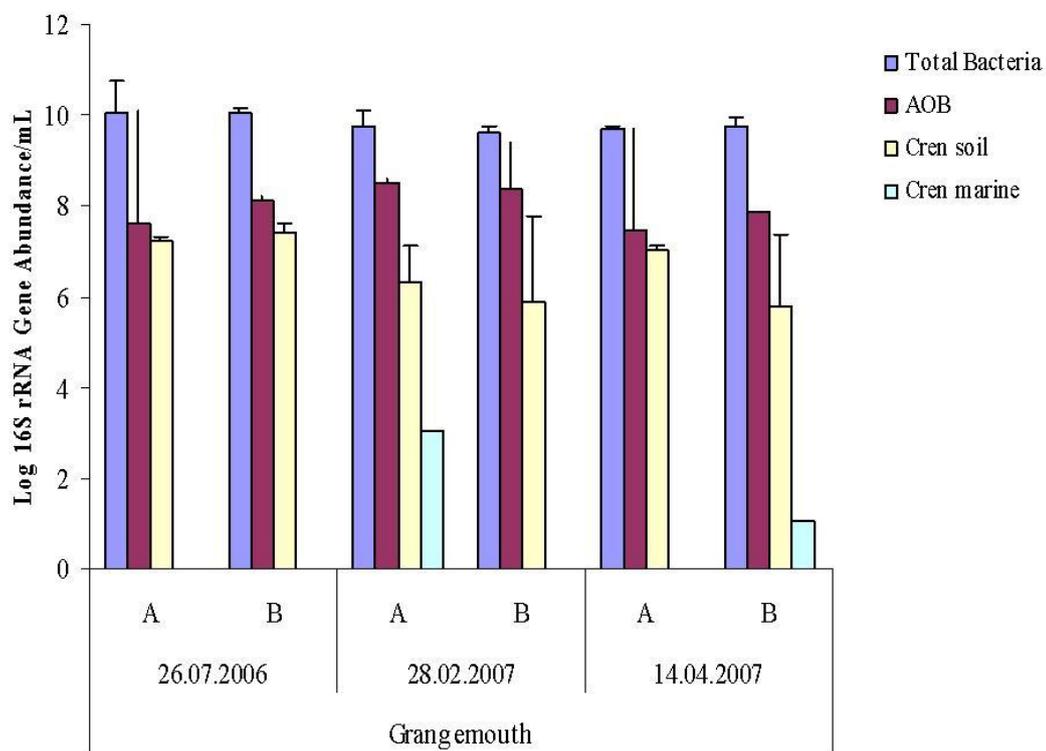


Figure 5.3 The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group *Crenarchaeota* in wastewater treatment reactors A and B from the Grangemouth refinery sampled on three different occasions. Error bars correspond to standard deviation of the mean of two or three replicate samples.

The 16S rRNA gene abundance for total bacteria in this study is in agreement with other values reported in the literature. For example, in some municipal wastewater treatment plants, total bacterial numbers of $4.3 \pm 2.0 \times 10^8$ cells/ mL (Harms *et al.*, 2003) and 1.6×10^9 - 2.4×10^{10} cells/ mL (Limpiyakorn *et al.*, 2005) were reported, while in an industrial wastewater treatment plant, total bacterial abundance was found to be $1.33 \pm 2.0 \times 10^9$ gene cells/mL (Layton *et al.*, 2005), assuming an average of 3.6 16S rRNA operons per genome of bacterial cell (Klappenbach *et al.*, 2001).

However, AOB 16S rRNA gene abundance in this study (ranging from 10^7 to 10^9 gene copies/mL; Figures 5.1, 5.2 and 5.3) was up to one or two orders of magnitude higher than the AOB 16S rRNA gene abundance found in the studies mentioned above. These were $1.2 \pm 0.9 \times 10^7$ cells/ mL (Harms *et al.*, 2003), 1.0×10^6 - 9.2×10^7 cells/ mL (Limpiyakorn *et al.*, 2005), and $1.37 \pm 1.56 \times 10^8$ cells/mL (Layton *et al.*, 2005). Furthermore AOB 16S rRNA gene abundance in seven wastewater treatment plants, treating municipal and several other industrial wastewater treatment reactors, was found to range from 10^4 to 10^6 cells/mL (Geets *et al.*, 2007), assuming one 16S rRNA operon per AOB cell (Aakra *et al.*, 1999).

In another study, investigating AOB abundance in 23 full scale municipal wastewater treatment plants in the UK (Pickering, 2008), the same plants that were screened in this study for the presence of putative AOA and AOB numbers were quantified through FISH and real-time PCR and both methods were compared. AOB numbers quantified through FISH were one order of magnitude higher than numbers obtained through real-time PCR, ranging from 2.48×10^5 to 7.72×10^6 cells/mL. Interestingly, while the AOB numbers obtained through FISH were consistent with nitrification performance, the 16S rRNA gene abundances obtained through real-time PCR were not significantly different among the wastewater treatment plants, even though they differed in their nitrification performance (Pickering, 2008).

Similar observations could be extended to this study where irrespective of whether the plants were nitrifying or not, AOB 16S rRNA gene abundances were at the same order of magnitude. For example, the abundance of AOB 16S rRNA genes measured at Eastham (non nitrifying) and Grangemouth (nitrifying) reactors was similar (ca. 10^8 gene copies/mL; Figures 5.1 and 5.3); and a similar pattern was observed in the Pembroke (non nitrifying) and Humber (nitrifying) reactors (ca. 10^7 gene copies/mL; Figures 5.1 and 5.2).

The high AOB 16S rRNA gene abundances found in this study could be explained by the lower specificity of the real-time assay performed to quantify the AOB 16S rRNA genes. The assay was conducted with the forward primers CTO 189f A/B and C, and the reverse primer RT1r as described by Hermansson and Lindgren, 2000. In this study, for the qPCR assay, instead of using the same *Taq* Man probe TPM1 used by Hermansson and Lindgren (2001), amplification was conducted using SYBR[®] Green I detection. Therefore it is likely that *Betaproteobacteria* closely related to AOB would have contributed to the real-time PCR signal obtained with the AOB-selective primers due to non specific binding of the primers.

The high AOB 16S rRNA gene abundance found in this study also did not always correspond with the AOB 16S rRNA clone libraries (see Section 3.3.5; Chapter 3). For example in 16S rRNA gene clone libraries generated with AOB selective primers from the Lindsey reactors, 100% of the sequences in the clone library obtained from reactor A and 85% of the sequences from reactor B were from AOB. In addition, 95% of the 16S rRNA gene sequences from Grangemouth reactor A and 74% of the sequences recovered from reactor B were from AOB. However, in the clone library from the Eastham plant only 16.5% of the clones were from AOB and for the Pembroke plant only 17% of the sequences recovered were from AOB. The situation was even more marked with samples from the Humber refinery, where no AOB sequences were identified in 16S rRNA gene clone libraries.

These data suggest that the apparently high AOB abundances measured in the Eastham (10^8 gene copies/mL; Figure 5.1), Pembroke (10^7 gene copies/mL; Figure 5.1) and Humber (from 10^7 to 10^9 gene copies/mL; Figure 5.2) reactors are likely to represent overestimates of AOB abundance. The AOB 16S rRNA gene abundance measured in the reactors at Lindsey (10^9 gene copies/mL; Figure 5.1) and Grangemouth (10^7 - 10^9 gene copies/mL; Figure 5.3) are likely to represent true AOB, as the AOB 16S rRNA gene clone libraries from these samples contained a high proportion of AOB 16S rRNA gene sequences (up to 100% of clones).

Furthermore, the target sequence for primer RT1r occurred in 86-92% of the AOB sequences recovered from the Lindsey reactor and 35% and 74% of sequences from Grangemouth reactors B and A, respectively. By contrast, the target for RT1r was found in only 17% of sequences from the Eastham clone library, 12% of sequences from the Pembroke clone library and 0-4.9% of sequences from the Humber clone libraries. The degree of mismatch between the primer and target site in sequences from the refinery wastewater treatment plants varied from 1-2 mismatches in sequences from Lindsey, 1-3 mismatches in the sequences from Grangemouth, 3 mismatches in sequences from Eastham and Pembroke and 2 to more than 3 mismatches in sequences from the Humber clone libraries. *In silico* specificity evaluation of the primer rRT1 showed that it targets a great number of other non AOB within the *Betaproteobacteria*, as, for example, members of the genera *Ralstonia*, *Thauera*, *Azoarcus*, and *Ferribacterium* as well as from other bacterial groups within the phylum *Proteobacteria* (Table 2.8; Section 2.15.1; Chapter 2). Although the use of a *TaqMan* probe would have contributed to increase the specificity of the assay, a quick search for the sequence of the probe TMP1 in the RDP and in the SILVA databases showed that, similarly to the primer rRT1, the probe also targets a great number of other non AOB.

In conclusion, the data on AOB abundance in the Eastham, Pembroke and Humber treatment plants are therefore likely to be incorrect, and actual AOB abundance is likely to be considerably lower than these values suggest. Furthermore, in line with the low number of AOB clones found in the Eastham and Pembroke clone libraries, nitrification was not occurring in the Eastham and Pembroke treatment plants.

In contrast, although no AOB sequences were detected in the two clone libraries derived from the Humber treatment plant, in the clone library derived from the first sampling date at the Humber plant, in October 2006, nitrification was poor (on average, 12% of the ammonia present was being converted to nitrate; Table 5.2); and more interestingly, in the second clone library derived from the last sampling date in February 2007, the Humber plant was nitrifying completely (100% ammonia conversion to nitrate in the Humber reactors; Table 5.2).

16S rRNA genes from soil group 1.1b *Crenarchaeota* were detected in all refinery wastewater treatment plants (Figures 5.1, 5.2, and 5.3), and were highest in the Humber reactors (7.43×10^6 to 1.48×10^8 gene copies/mL; Figure 5.2) followed by the treatment plants at Grangemouth (3.33×10^6 to 2.94×10^7 gene copies/mL; Figure 5.3) and Lindsey (8.01×10^5 to 9.48×10^6 gene copies/mL; Figure 5.1). The abundance of these *Crenarchaeota* was lower in the Eastham (3.60×10^4 gene copies/mL) and Pembroke reactors (8.03 and 7.30×10^4 gene copies/mL; Figure 5.1). The 16S rRNA genes from marine group 1.1a *Crenarchaeota* were also detected in all refinery wastewater treatment plants (Figures 5.1, 5.2, and 5.3), however in lower abundance. They were highest in Lindsey (ranging from 1.45×10^4 to up to 2.81×10^6 gene copies/mL, value found in one replicate sample; Figure 5.1), while in the other refinery plants they ranged from below the detection limits of the qPCR method (3.93 ± 0.8 ; Appendix C; Table C1) up to 2.78×10^3 gene copies/mL, the highest value being found in the Humber reactors (Figure 5.2).

In the BLC leather processing wastewater pilot reactor (Figure 5.4) 16S rRNA gene abundances for total bacteria and AOB were respectively 5.08×10^9 gene copies/mL and 1.11×10^8 gene copies/mL, which was within the same range as those found in the oil refinery systems. Interestingly, contrary to what was found in the refinery plants, 16S rRNA genes from soil group 1.1b *Crenarchaeota* were detected at low abundance in the BLC pilot reactor (2.68×10^3 gene copies/mL), while 16S rRNA genes from marine group 1.1a *Crenarchaeota* were much more abundant (1.43×10^7 gene copies/mL; Figure 5.4).

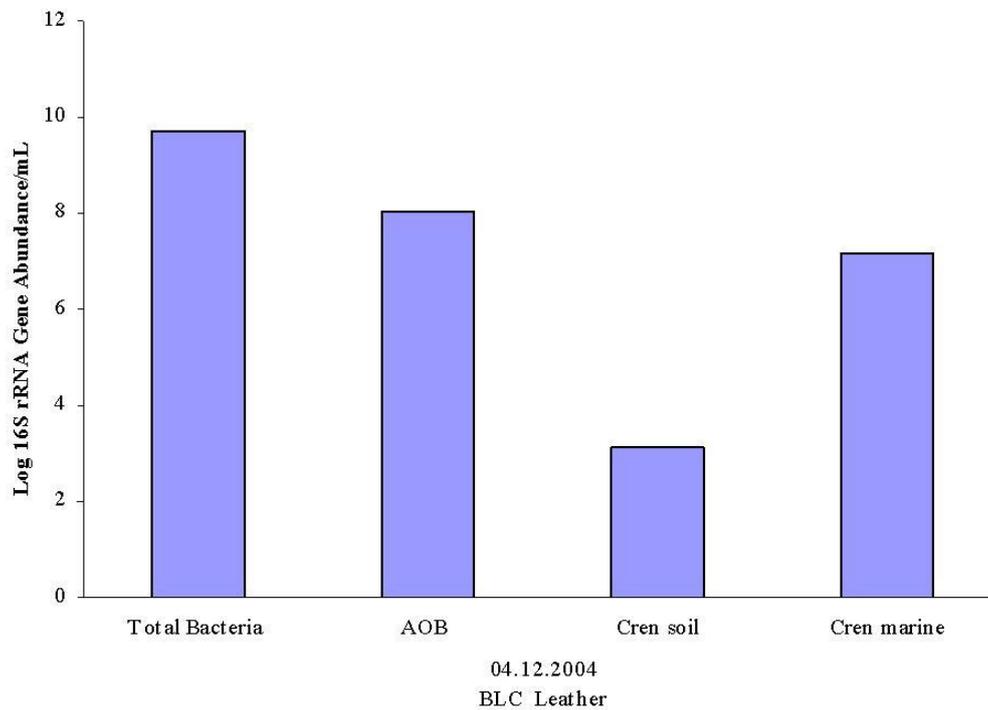


Figure 5.4 The 16S rRNA gene abundance for total bacteria, AOB, marine and soil group *Crenarchaeota* in a single sample from the BLC pilot reactor. No replicates were included in this analysis as it relied on a single archived sample provided from another study.

Mußmann *et al.* (2008) conducted a phylogenetic tree reconstruction for crenarchaeotal sequences originated from cloned PCR-amplified archaeal 16S rRNA gene fragments, by using the primers Arch21f (DeLong, 1992) and Univ 1392R (Pace *et al.*, 1986). The reconstruction included sequences retrieved from the Humber, Grangemouth and Lindsey treatment plants, and the BLC pilot reactor (see Figure 5.5 and the ISME conference poster in Appendix C). The result was a certain number of clones falling within the soil group 1.1b *Crenarchaeota*, forming a monophyletic group including clones retrieved from the tomato rhizosphere (Simon *et al.*, 2005) and other soil systems. This group included all clones retrieved from the Humber reactor (33 clones) and the Grangemouth reactor (24 clones), which revealed only one phylotype each; three clones from Lindsey reactor, and one clone from the BLC pilot reactor treating leather processing wastewater. By contrast 12 clones from Lindsey and most clones from the BLC pilot reactor (29 of 32 clones) fell within the marine group 1.1a *Crenarchaeota*, closely related to a uranium mill tailing clone (Figure 5.5).

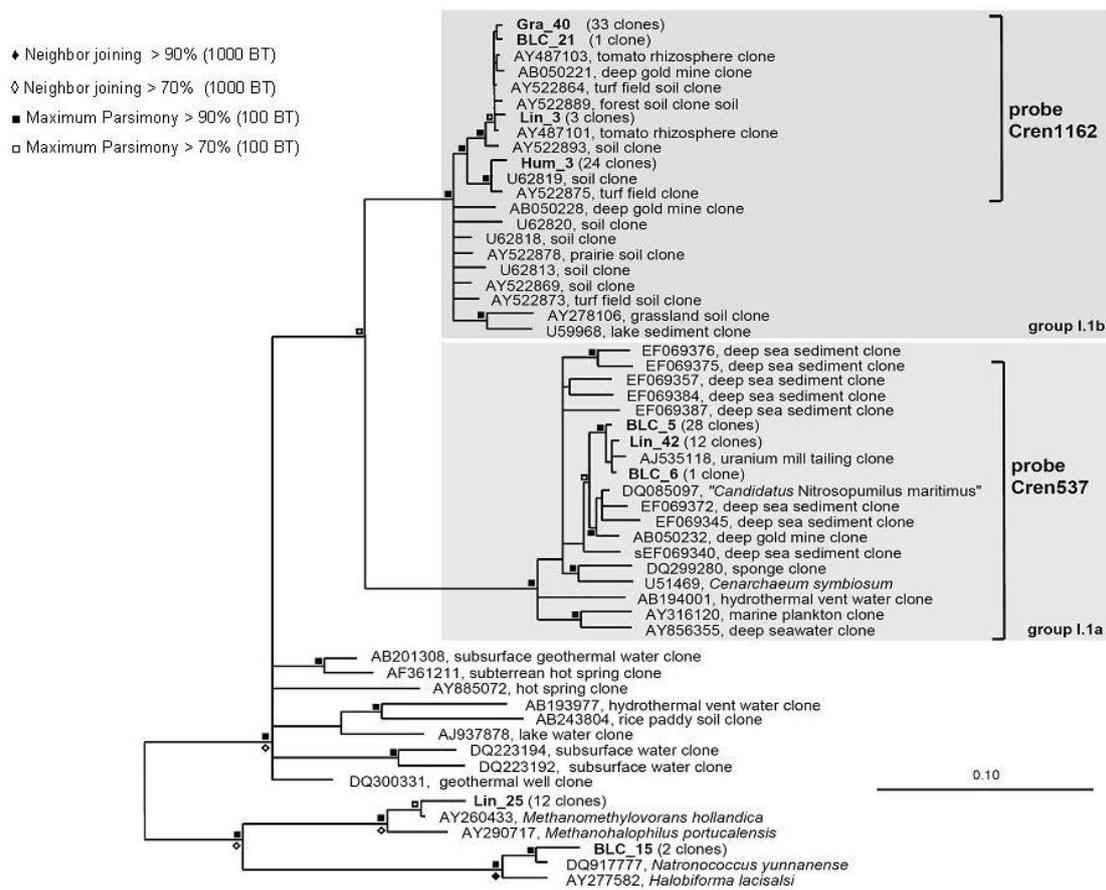


Figure 5.5 Phylogenetic reconstruction of crenarchaeotal 16S rRNA sequences recovered from the Lindsey, Humber, Grangemouth, and BLC wastewater treatment reactors (Mußmann *et al.*, 2008; ISME conference poster; see also Appendix C). Sequences were grouped based on a sequence identity cut-off > 99%. A consensus tree was constructed in ARB using the Maximum Parsimony, neighbour-joining with the Jukes and Cantor DNA distance correction, and the Maximum-likelihood treeing methods and 50% conservation filter for the tree calculation. Closed diamonds on the branches nodes refer to bootstrap values > 90%, and open diamonds refer to bootstrap values > 70% for Neighbour Joining using 1000 resampling iterations. Closed squares on branches nodes refers to bootstrap values > 90%, and open squares refer to bootstrap values > 70% for Maximum Parsimony using 100 resampling iterations. Gra = Grangemouth reactor; Hum = Humber reactor; Lin = Lindsey reactor and BLC = pilot reactor treating tannery wastes. Scale bar represents 10% sequence divergence.

Comparing the 16S rRNA gene abundance results for soil group 1.1b *Crenarchaeota* found in this study to the 16S rRNA *Crenarchaeota* tree (Figure 5.5), the abundance of 16S rRNA genes related to the soil group 1.1b *Crenarchaeota* was also comparatively higher in samples from the Humber and Grangemouth plants and matched well with the results shown on the 16S rRNA *Crenarchaeota* tree, where all clones recovered from these plants were also from this group of Archaea (Figure 5.5). In contrast, however, no soil group 1.1b *Crenarchaeota* 16S rRNA genes were detected in the BLC pilot reactor by real-time PCR (Figure 5.4), while one clone retrieved from this sample fell within the soil group 1.1b on the *Crenarchaeota* phylogenetic tree (Figure 5.5). Likewise, although relatively low numbers of 16S rRNA genes from marine group 1.1a *Crenarchaeota* were detected in the Lindsey reactor by real-time PCR and a much higher abundance of soil group 1.1b *Crenarchaeota* 16S rRNA genes was determined (Figure 5.1), the opposite was observed in the archaeal 16S rRNA gene libraries, in which 44% of the recovered clones were related to the marine group 1.1a *Crenarchaeota* and only 11% of the clones were related to the soil group 1.1b *Crenarchaeota* (Figure 5.5).

The discrepancy observed in the Lindsey treatment plant between *Crenarchaeota* 16S rRNA gene abundances and clone frequency of crenarchaeotal 16S rRNA sequences related to marine group 1.1a and soil group 1.1b in the clone libraries (Figure 5.5), might also likely result from the different specificities of the different set of primers used respectively for the quantification of marine group *Crenarchaeota* 16S rRNA from group 1.1a (primers GI_751F and GI_956R: Mincer *et al.*, 2007) and groups 1.1b (primers 771F and 957R: Ochsenreiter *et al.*, 2003) in the qPCR assays, and the more general primers used for the clone libraries construction, primers Arch21f (DeLong, 1992) and Univ 1392 (Pace *et al.*, 1986).

For instance, in the SILVA database (release version 102) there are 1953 sequences related to the crenarchaeal marine group 1.1a, and 614 sequences related to the crenarchaeal soil group 1.1b. The set of primers used to target the crenarchaeal group 1.1a, primers GI_751F and GI_956R, targeted respectively 674 and 1558 sequences within the crenarchaeal group 1.1a, while the set of primers used to target the crenarchaeal group 1.1b, primers 771F and 957R, targeted respectively 534 and 451 sequences within the group 1.1b (See Table 2.9; Section 2.15.1; Chapter 2). In contrast, the general archaeal primer Arch 21f targeted 3356 sequences in the SILVA database, from which 589 sequences within the group 1.1a and 95 sequences within the group 1.1b. The overlaps between both sets of primers and the general primer Arch21F were as follows: GI_751F/Arch21F: 220/589 (37%); and GI_956R/ Arch21F: 94/589 (67%) for the crenarchaeal group 1.1a; and 771F/ Arch21F: 92/95 (97%) and 957R/ Arch21F: 52/95 (55%) for the crenarchaeal group 1.1b, respectively.

When considering the lowest and the highest overlap between the primers, the overlap between the forward primer GI_751F and the general primer Arch21F was very low (37%), and, conversely, the overlap between the forward primer 771F and the primer Arch21F was very high (97%). Thus, it makes sense that, comparatively, a much lower congruence will be expected between results obtained from primers having a much lower overlap, as in the case of the crenarchaeal group 1.1a, than results obtained from primers having a much higher overlap, as in the case of to the crenarchaeal group 1.1b. Thus, also not surprisingly, these differences might have been reflected in the results obtained from the two different assays. Furthermore, it should be considered as well that numerically dominant genes amplified by end point PCR do not necessarily represent the numerically dominant genes in the environment, due to the intrinsic biases related to the end point PCR reactions, which are overcome by real-time PCR.

5.3.3 Quantification of bacterial and archaeal *amoA* gene abundance

Overall AOB *amoA* gene abundance across the oil refinery wastewater treatment plants ranged from 10^3 gene copies/mL in Eastham and Pembroke reactors, to up 10^6 gene copies/mL in Lindsey and Grangemouth reactors (Figures 5.6, 5.7 and 5.8). Likewise, AOA *amoA* gene abundance ranged from below the detection limits of the real-time PCR assay (34 gene copies/mL sample; Appendix C) in the Pembroke reactors to up 10^8 gene copies/mL in the Humber and Grangemouth reactors (Figures 5.6, 5.7 and 5.8). PCR efficiencies, regression data and detection limits of each real-time PCR assay are summarized in Appendix C.

AOB *amoA* gene abundance in the Lindsey reactors ranged from 3.08×10^5 to 1.90×10^6 gene copies/mL (Figure 5.6), while in the Grangemouth reactors it ranged from 2.48×10^4 to 3.35×10^6 gene copies/mL (Figure 5.8), and in the Humber reactors it ranged from 1.31×10^4 to 1.91×10^5 gene copies/mL (Figure 5.7). AOB *amoA* gene abundance in the Eastham reactor was 1.51×10^3 gene copies/mL and in the Pembroke reactors 1.86 and 2.39×10^3 gene copies/mL, respectively.

The numbers found in the treatment plants were also consistent with the process measurements, that is, no nitrification was occurring in the Eastham and Pembroke reactors, while good nitrification was occurring in the Lindsey, Grangemouth and in Humber reactors after the first sampling occasion (Table 5.2); however, in the Humber reactor, putative AOA rather than AOB were the dominant organisms. AOA abundance was higher in the Humber reactors (from 6.15×10^6 to 2.69×10^8 gene copies/mL; Figure 5.7) and Grangemouth (from 4.32×10^6 to 1.77×10^8 gene copies/mL (Figure 5.8), while they were detected only once in two replicate samples from the Lindsey reactor A (1.54×10^7 gene copies/mL) on the second sampling occasion conducted in October 2005. AOA *amoA* gene abundance was very low in the Eastham reactor (3.42×10^3 gene copies/mL), and AOA *amoA* was not detected in the Pembroke reactors (Figure 5.6).

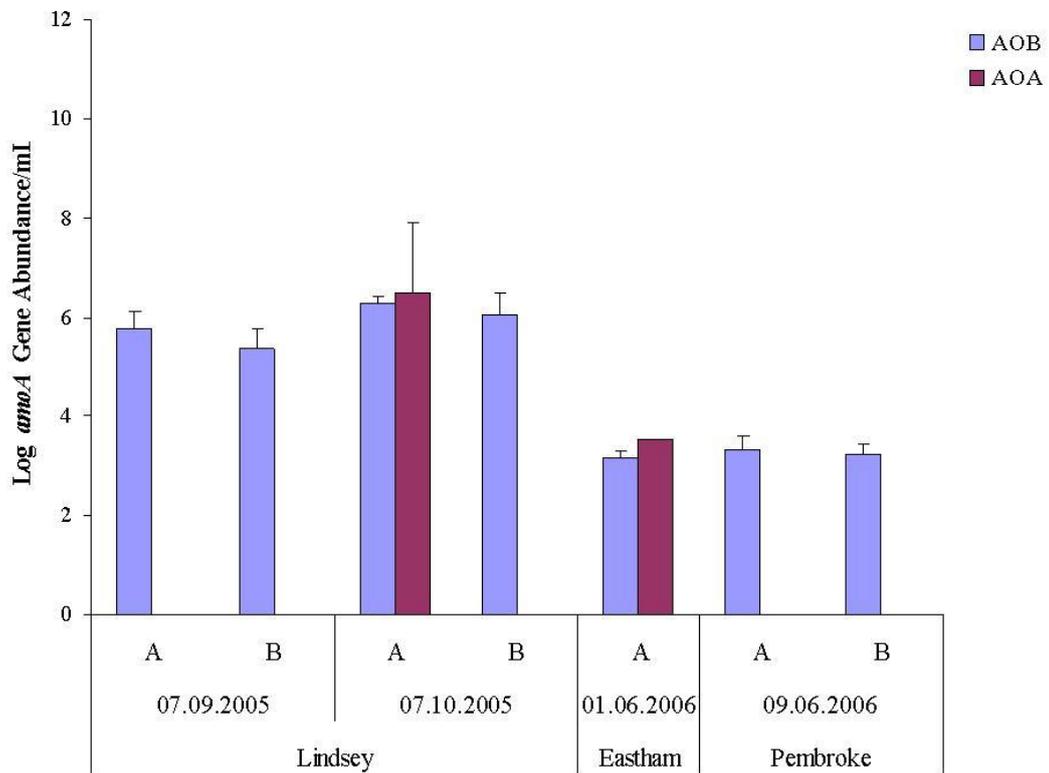


Figure 5.6 The *amoA* gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Lindsey, Eastham and Pembroke refineries. Error bars correspond to standard deviation of the mean of two or three replicate samples.

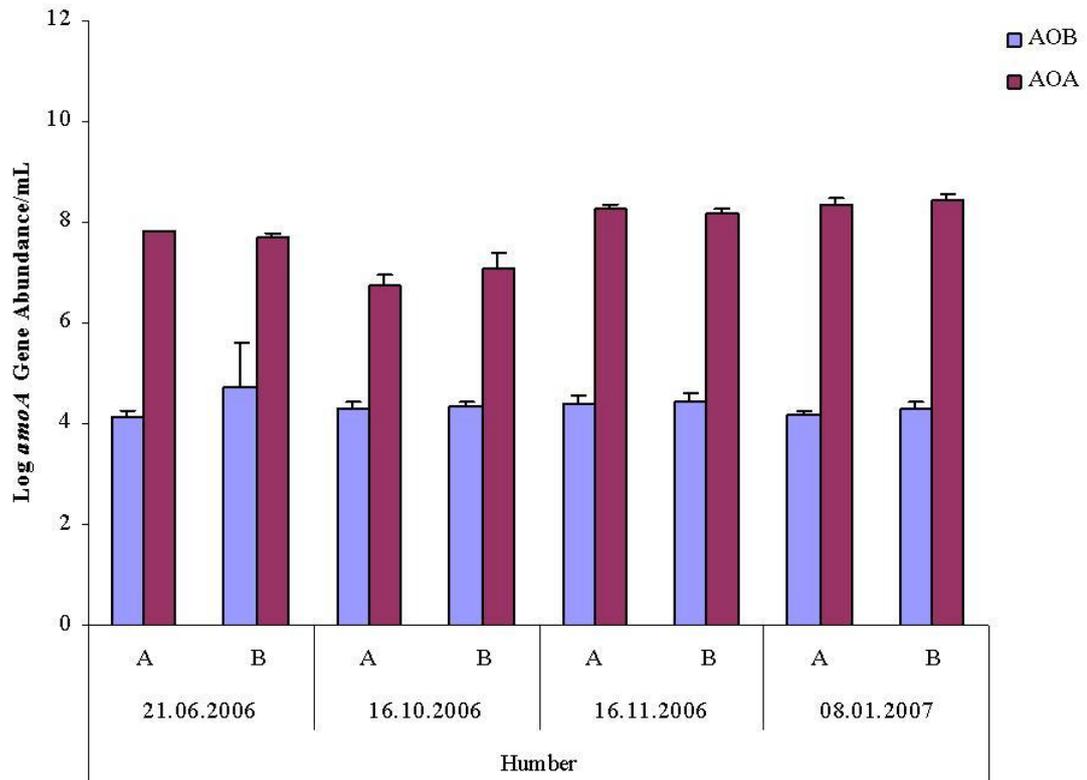


Figure 5.7 The *amoA* gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions. Error bars correspond to standard deviation of the mean of two or three replicate samples.

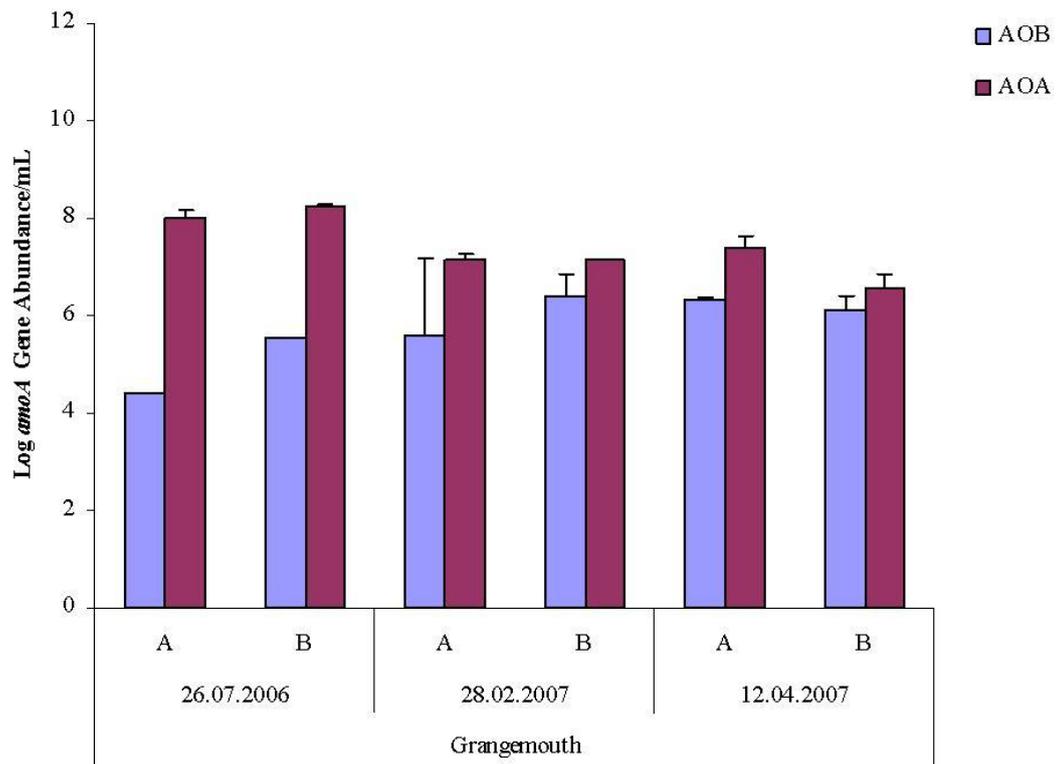


Figure 5.8 The *amoA* gene abundance for AOB and AOA in wastewater treatment reactors A and B from the Grangemouth refinery sampled on three different occasions. Error bars correspond to standard deviation of the mean of two or three replicate samples.

In the Lindsey, Humber and Grangemouth reactors in all cases AOA *amoA* genes outnumbered AOB *amoA* genes. For example, in the Lindsey reactor AOA *amoA* genes were detected only once in reactor A on the second sampling occasion (October 2005) at 1.54×10^7 gene copies/mL; while AOB *amoA* abundance in the same reactor was at 1.90×10^6 gene copies/mL. In Grangemouth reactors, the highest AOA *amoA* abundance occurred in reactors A (1.08×10^8 gene copies/mL) and B (1.77×10^8 gene copies/mL) on the first sampling occasion in July 2006, and also coincided with the lowest AOB *amoA* gene abundances in reactor A (2.48×10^4 gene copies/mL) and in reactor B (3.53×10^5 gene copies/mL). Likewise the highest AOA *amoA* abundances (from 1.48 to 2.69×10^8 gene copies/mL; Figure 5.7) in the oil refinery systems were found on the third (November 2006) and fourth (February 2007) sampling occasions at the Humber plant, and this also coincided with AOB being detected at low abundances (1.31 to 2.94×10^4 gene copies/mL; Figure 5.7).

In the BLC pilot reactor, AOA *amoA* gene abundance (1.04×10^8 gene copies/mL; Figure 5.9) was also three orders of magnitude higher than AOB *amoA* abundance (7.35×10^5 gene copies/mL).

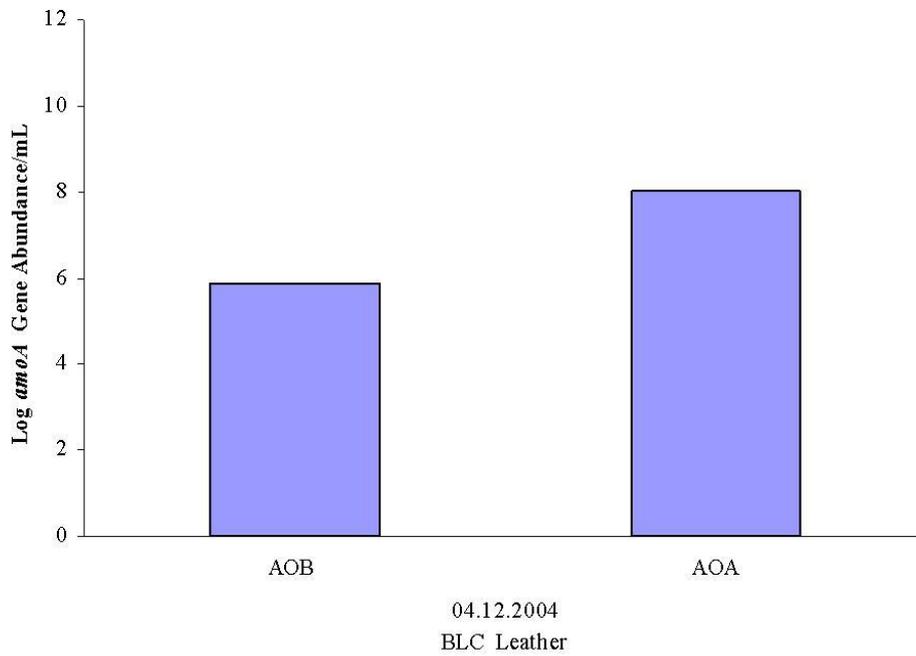


Figure 5.9 The *amoA* gene abundance for AOB and AOA in a single sample from the BLC pilot reactor (no replicates were included in this analysis as it relied on a single archived sample provided from another study).

AOB *amoA* gene abundance in the Lindsey and Grangemouth reactors and in the BLC pilot reactor was within the same range of values reported for municipal WWTPs of about $7.5 \pm 6.0 \times 10^6$ cells/mL (Harms *et al.*, 2003), as well as industrial WWTPs (ranging from 10^4 to 10^6 gene copies/mL) treating a range of wastewaters, such as paper, sewage, food and chemical related wastes (Geets *et al.*, 2007). The lowest abundances (7.6×10^4 gene copies/mL) were found in two WWTPs; one of them treating methylamine derivative wastes, that was close to failure and also had the lowest treatment efficiency (11%); and the other, treating paper wastewater, that had already failed (1.1×10^4 gene copies/mL) (Geets *et al.*, 2007). These values were lower than the highest value found in Humber reactor B (1.91×10^5 gene copies/mL; Figure 5.7) on the first sampling occasion conducted at the plant in October 2006, but nitrification was poor, based on low conversions of ammonia and TKN to nitrate production, respectively 15 and 9% efficiency (Table 5.2).

Interestingly, on subsequent sampling occasions at the Humber plant in October and November 2006 and February 2007, even though AOB *amoA* abundance was lower (ranged from 1.31 to 2.63×10^4 gene copies/mL), nitrification was good in the Humber reactors, with 100% efficiency on ammonia conversion to nitrate and 50% efficiency on TKN conversion to nitrate (Table 5.2).

Comparing the AOB abundances obtained using real-time PCR of 16S rRNA and *amoA* genes in this study, the AOB *amoA* gene quantification rendered much more plausible numbers, and was consistent with the data from the AOB clone library composition (See Chapter 3). In addition, the AOB *amoA* gene quantification was also comparable with other studies where AOB 16S rRNA gene abundance was quantified (Harms *et al.*, 2003; Limpiyakorn *et al.*, 2005; Limpiyakorn *et al.*, 2006; Geets *et al.*, 2007; Pickering *et al.*, 2008).

By contrast with AOB, this is the first time that AOA have been quantified in wastewater treatment systems, and studies in other WWTPs relating AOB and AOA numbers and dynamics have not yet been published.

The AOA *amoA* abundances found in this study were in line with AOA *amoA* gene abundances reported from other environments. For example AOA *amoA* genes ranged from 10^4 gene copies/mL in marine systems (Mincer *et al.*, 2007; Lam *et al.*; 2007 Beman *et al.*, 2008) and to ca. 10^8 gene copies/g in soil systems (Leininger *et al.*, 2006). This is also consistent with the maximum cell numbers (10^7 cells/mL) reached in culture by *Nitrosopumilus maritimus* (Könneke *et al.*, 2005) and *Nitrosocaldus yellowstonii* (de la Torre *et al.*, 2008).

In this study, the relative *amoA* gene abundances found for AOA (most cases 10^7 - 10^8 gene copies/mL) and AOB (10^4 - 10^6 gene copies/mL) in the oil refinery wastewater treatment systems (Figures 5.6, 5.7 and 5.8) and in the BLC pilot reactor (Figure 5.9) were also comparable to AOA (estimated around 10^7 cells/mL) and AOB (estimated around of 10^4 cells/mL) *amoA* abundances from the rhizosphere of a freshwater macrophyte (Hermann *et al.*, 2008). Likewise, they were comparable with other studies, where AOA (ranging from 10^6 to 10^8 gene copies/g soil) were found to outnumber AOB by 1-3 orders of magnitude in several types of soil systems (Leininger *et al.*, 2006; He *et al.*, 2007; Shen *et al.*, 2008). AOB abundance in soil has been shown to range from 10^4 - 10^7 gene copies/g soil (Hermansson and Lindgren, 2001; Okano *et al.*, 2004).

The screening of the primers *Amo196F* and *Amo 277R* targeted sites, i.e., the primers used for the archaeal *amoA* gene quantification in the real-time PCR assay, against the clone libraries sequences generated from the PCR products amplified using the primers Arch-*amoAF* and Arch-*amoAR*, showed that they had a very low overlap (0.6%). Of 178 archaeal *amoA* sequences recovered from all the refinery treatment plants, the primers *Amo196F* and *Amo 277R* matched perfectly to only one sequence in the clone library generated from the trickling filter sample, whereas the reverse primer *amoA 277R* matched perfectly to nine sequences, all of them also from the Lindsey WWTP. *In silico* analysis of the primers also showed they had from one to three mismatches to the 178 archaeal *amoA* sequences recovered in the clone libraries (See Figure 2.6, Section 2.15.2; Chapter 2). Furthermore, the search for the primers targeted sequences against sequences retrieved from the Genbank database (currently containing about 9000 archaeal *amoA* sequences; July, 2010), revealed that the primers *amoA196F* and *amo277R* targeted only 6% of the sequences. The number of sequences in studies in the literature in which the primers Arch-*amoAF* and Arch-*amoAR* were used for PCR amplification represented about 53% (4900 sequences) of the total number of sequences deposited in the database. Taking into account those sequences, the forward primer *amoA196F* targeted 474 (9.67%) sequences while the reverse primer *amoA277R* targeted 2604 (56%) sequences, and the overlap between both sets of primers occurred in 382 sequences, i.e., in 6% of the total number of sequences. In addition, the primers *amoA196F* and *amoA277R* also targeted 96 more sequences, but from other studies, which used other primers for PCR amplification.

In the literature, primers Arch-*amoAF* and Arch-*amoAR* have also been used for the AOA *amoA* gene quantification; for example, in some soils and rhizosphere of freshwater macrophyte, estuarine, and groundwater studies (He *et al.*, 2007; Shen *et al.* 2008; Hermann *et al.*, 2008; Mosier and Francis, 2008; Reed *et al.*, 2010); or only the reverse Arch-*amoAR* instead, whereas the forward was modified or adopted from other studies, such as in some marine water column and sediments, wastewater treatment plants and bioreactors studies (Beman *et al.*, 2008; Park *et al.*, 2008; Wells *et al.*, 2009; Jin *et al.*, 2010).

In conclusion, as the overlap between both set of primers used in this study was low, the primers used in the AOA *amoA* quantification would potentially be targeting different organisms in their majority, and thus underestimating the results. However, the standard used in the assay (AGA 51), a clone from the Grangemouth reactor A; which had three mismatches with the forward primer *amo196F*, two mismatches with the reverse primer *amo277R*, and zero mismatch with the probe; was reliably amplified with determination coefficient (R^2) of 0.992 and PCR efficiency of 82.9% (Table C1, Appendix.) Furthermore, AOA and crenarchaeal soil group 1.1b abundances also positively correlated (Pearson's correlation coefficient $r=0,746$; $p=0,000$) and ratios between AOA and crenarchaeal group 1.1b approximately equated to one (mean= 1.07 ± 0.25 ; $n=15$).

In contrast to the similar ratios found between AOA and crenarchaeal group 1.1b, and their positive relationship; ratios between the AOA and the crenarchaeal marine group 1.1a abundances, in the refineries WWTPs, ranged from 1.19 to 7.35, and not only varied largely (mean= 3.77 ± 3.64), but also correlated negatively ($r=-0.56$, $p=0.016$; $n=10$). In the BLC pilot reactor sample, AOA abundance was 10 times higher than the crenarchaeal group 1.1a abundance.

Similarly to this study, in another study, using the same set of primers for the AOA *amoA* gene quantification, and clones with standards generated from a clone library constructed from PCR amplification using the primers Arch-*amoAF* and arch-*amoAR*, putative AOA detected in samples from the deep-sea hydrothermal vent chimneys of the Juan the Fuca Ridge could be quantified at maximum numbers of $7.36 \pm 0.37 \times 10^4$ gene copies/g of chimney (Wang *et al.*, 2009).

The 16S rRNA gene-defined crenarchaeal groups 1.1a and 1.1b; in the Grangemouth and Humber treatment plants, were also quantified using CARD-FISH (Mußmann *et al.*, 2008) (10^7 - 10^8 cells/mL; ISME conference poster; See Appendix C). The values obtained with CARD-FISH, also broadly agreed with the real-time PCR data reported here (Humber, 10^7 - 10^8 gene copies/mL; Grangemouth, 10^6 - 10^8 gene copies/mL).

A high phylogenetic congruence between the 16S-rRNA defined archaeal diversity and the diversity associated with the functional archaeal *amoA* gene has been demonstrated in a diverse range of environments; however, the 16S rRNA defined is greater than the diversity associated to the functional *amoA* gene, and thus, it also contains organisms that are not AOA.

Putative AOA that have been found in a range of diverse environments up to date, with exception of *Nitrosocaldus yellowstonii* fall within *Crenarchaeota* groups 1.1a and 1.1b (Prosser and Nicol, 2008).

In silico specificity and coverage evaluation of the primer sets used in the real-time assays to target the crenarchaeal groups 1.1a (GI_751F and GI_956R; Mincer *et al.*, 2007) and 1.1b (771F and 957R; Ochsenreiter *et al.*, 2003) in this study, and the probes used in the CARD-FISH assays revealed that they are potentially targeting similar organisms.

In the SILVA database (release version 102) there are 1953 sequences within the crenarchaeal group 1.1a and 614 sequences within the crenarchaeal group 1.1b. The CARD-FISH probes are more specific than the primers used in qPCR, and they target a much smaller subset of sequences than the primers (See Table 2.9, Chapter 2). However, within the crenarchaeal groups 1.1a and 1.1b, in which putative AOA representatives might occur, the overlap between the probes and the primers was relatively high for the targeted crenarchaeal group 1.1a and very high for the targeted crenarchaeal group 1.1b (See Table 2.9, Chapter 2).

The probe Cren 537, designed to target the crenarchaeal marine group 1.1a, targeted 1767 sequences, all of which within the group 1.1a, representing 90.5% of the total number of sequences related to the crenarchaeal group 1.1a in the database. Likewise, the forward primer GI_751F targeted 674 sequences, all of them within the crenarchaeal marine group 1.1a, and overlapped with the probe Cren 537 in 89.7% of the sequences within the crenarchaeal group 1.1a. The reverse primer GI_956R; although less specific than the forward primer, also targeted without mismatches to other groups within the *Crenarchaeota* phylum and other phyla within the domain *Archaea*, targeted a much higher number of sequences within the crenarchaeal marine group 1.1a (1558 sequences) than the forward primer (674 sequences), and overlapped with the probe Cren 537 in 76% of the sequences (1180); (See Table 2.9, Chapter 2.)

The probe 1162, designed to target the crenarchaeal soil group 1.1b, targeted 131 sequences in the SILVA database, all of them within the soil group 1.1b. The probe 1162 overlapped in 127 sequences (97% of the sequences) with the primer 771F, and in 115 sequences (88% of the sequences) with the reverse primer 957R. Primer 771F targeted 534 sequences within the crenarchaeal soil group 1.1b, while the reverse primer 957R targeted 451 sequences within the crenarchaeal soil group 1.1b. Both primers also targeted other groups within *Crenarchaeota* and other archaeal phyla as *Euryarchaeota* and *Korarchaeota* (Table 2.9, Chapter 2).

The overlap between probe 1162 and the primers 771F and 957R was very high and this is also supported by the broad agreement obtained for the quantification of the crenarchaeal soil group 1.1b between the two independent methods.

In conclusion, the abundances and ratios reported here for AOA and the crenarchaeal group 1.1b were very consistent, as well as the overlap between the CARD-FISH probes and the primers used in the qPCR assays, at least to target the crenarchaeal group 1.1b in this study.

5.3.4 AOA alternative metabolism

The activity of AOA and AOB in the Humber and Grangemouth reactors was investigated through reverse transcriptase-PCR of *amoA* mRNA and MAR-FISH experiments conducted by Marc Mußmann at the University of Vienna (Mußmann *et al.*, 2008 ISME Conference poster in the Appendix C). Interestingly archaeal *amoA* expression was demonstrated to occur in the presence or absence of 2 mM ammonium; and in MAR-FISH experiments AOA did not assimilate ¹⁴C-labelled bicarbonate, in contrast to AOB in the same samples which assimilated bicarbonate even when present at low abundance. This was true even for the Humber reactor where very few AOB microcolonies were detected by FISH (Mußmann *et al.*, 2008 ISME Conference poster in the Appendix C). This suggests that if the AOA are indeed involved in nitrification, they may not use inorganic carbon as their primary carbon source and instead use organic carbon sources.

Genes encoding the AMO enzymes responsible for ammonia oxidation were found in metagenomic studies from the symbiont *Cenarchaeum symbiosum* (Hallam *et al.* 2006); however, urease and urea transporter enzymes, as well as genes encoding the almost complete oxidative tricarboxylic acid cycle (TCA) linked to the organic carbon oxidation path were also found, suggesting that they may metabolize reduced forms of nitrogen as well as potentially using both bicarbonate or organic carbon as a carbon source. In contrast a complete TCA cycle is present in AOB, although they preferentially use CO₂ as a carbon source (Chain *et al.*, 2003). Nevertheless this allows them to take some benefit from the use of pyruvate and fructose as a carbon source (Hommes *et al.*, 2003) when growing facultatively as chemolithoheterotrophs (Arp *et al.*, 2007).

AOA abundance in the rhizosphere of a freshwater macrophyte (Herrmann *et al.*, 2008) was estimated to be 10^7 cells/mL, with estimated CSAOR of 0.5 fmol/cell/h, and these correlated with potential rates of nitrification. Nevertheless the authors postulated an alternative metabolism for the AOA in relation to the exudates coming from the macrophyte roots, reinforcing the idea of a possible mixotrophic metabolism of AOA. The capacity of marine *Crenarchaeota* to take up amino acids has been demonstrated in water samples from 200 m depth in the northwest Mediterranean Sea and Pacific Ocean (Ouverney and Fuhrman, 2000). This suggests that heterotrophic metabolism may be possible with some marine *Crenarchaeota* which may not all therefore be AOA. The presence of different marine *Crenarchaeota* at different depths in the subtropical North Pacific Gyre also led to the suggestion that two distinct *Crenarchaeota* groups might exist in marine systems, one of which is autotrophic and another heterotrophic (Ingalls *et al.*, 2006). More recent findings in the subtropical and tropical North Atlantic deep sea (Agogu e *et al.*, 2008; see also Schleper, 2008, for a critical review of this study) showed that some *Crenarchaeota* lacked genes consistent with autotrophic metabolism. The full metabolic repertoire of *Crenarchaeota* including putative AOA remains to be elucidated and further studies are required to better clarify the potential metabolic diversity of *Crenarchaeota* related to putative AOA. Therefore future research into the biochemistry of AOA in Humber and Grangemouth will be essential to answer specific questions regarding whether AOA in the oil refinery wastewater treatment systems may switch between mixotrophic and heterotrophic metabolism when AOB are the primary ammonia oxidizers and what is the extent of the AOA contribution to nitrification in the Humber and Grangemouth reactors.

5.3.5 AOB and AOA cell numbers and specific ammonia oxidizing activities

Cell-specific ammonia oxidation rates (CSAOR) by AOB have been demonstrated as an important process parameter to assess how well AOB are oxidizing ammonia and consequently how well nitrification performance is occurring in WWTPs (Daims *et al.*, 2001c; Coskuner *et al.*, 2005; Pickering 2008).

AOB cell specific ammonia oxidation rates were investigated in five WWTPs and one lab-scale reactor (Coskuner *et al.*, 2005) and they were reported to vary by three orders of magnitude. In the study of Coskuner *et al.* (2005) the lowest CSAOR (0.03 fmol/cell/h) was found in a WWTP harbouring the highest AOB numbers, quantified through FISH (10^8 cells/mL), and conversely, the highest CSAOR (43 fmol/cell/h) was found in one WWTP that was close to the point of failing in nitrification and also harboured the lowest AOB numbers (10^5 cell/mL). Based on these results, the authors postulated that:

AOB in some plants would be working 1,000 times harder than AOB in other plants and therefore there should be a CSAOR threshold value, below which stable nitrification performance could be achieved and above which risk of failure could be expected (Coskuner *et al.*, 2005).

Subsequently, this hypothesis was investigated in 23 municipal WWTPs in the UK (Pickering, 2008), the same WWTPs that were screened in this study for the presence of putative AOA. A significant negative linear relationship was found between the number of AOB cells in the 23 WWTPs and their CSAOR. Lower CSAOR positively correlated with nitrification stability and higher AOB numbers. Conversely high CSAOR were found in the WWTPs with lower AOB cell numbers and exhibiting unreliable nitrification or nitrification failure.

As discussed in section 5.3.2, after careful data analysis it was concluded that the 16S rRNA gene-based quantification overestimated AOB abundance due to the lack of specificity of the primers used which amplified the target sequence from related non AOB *Betaproteobacteria*. Therefore these data are likely not to be reliable in comparison to the quantification of AOB based on the *amoA* gene. Furthermore the functional *amoA* gene was more specific than the 16S rRNA gene for AOB quantification and best reflected AOB abundance in situations of unstable or stable nitrification in the oil refinery wastewater treatment systems. Analysis of *amoA* genes also allowed a more defensible comparison to be made with AOA, and thus all subsequent assessments were based on quantification of *amoA* genes.

To verify whether AOB and AOA in the refinery wastewater treatment reactors would be following the trends demonstrated for municipal sewage treatment systems (Pickering, 2008), the gene abundances obtained for AOB *amoA* and AOA in Lindsey, Grangemouth and Humber were converted to cell numbers; and by assuming that AOA and AOB cells were equally active, the CSAOR in the refinery wastewater treatment reactors were estimated and related to both AOB and AOA cell numbers either individually or together, and compared with the nitrification performance in the Lindsey, Humber and Grangemouth wastewater treatment systems (Table 5.3).

Overall, estimated CSAOR in the oil refinery WWTPs, based on AOB *amoA* plus AOA *amoA* gene abundances, varied by up to four orders of magnitude and ranged from 0.0034 to 93.3 fmol/cell/h; Table 5.3.

As pointed out by Coskuner *et al.* (2005) the large variation found among ammonia oxidation rates in a WWTP may result from the effect of several chemical parameters affecting the AOB cells. For example, oxygen, temperature and ammonia concentrations may all potentially affect cell specific ammonia oxidation rates.

Table 5.3 Cell numbers and cell specific ammonia oxidation rates (CSAOR) calculated for AOB and AOA in three oil refinery WWTPs that were nitrifying in this study

Sampling dates	WWTP	AOB plus AOA responsible for all the nitrification						CSAOR (fmol/cell/h)*			
		A			B			A		B	
		AOB <i>amoA</i> (cells/mL)	AOA <i>amoA</i> (cells/mL)	CSAOR (fmol/cell/h)	AOB <i>amoA</i> (cells/mL)	AOA <i>amoA</i> (cells/mL)	CSAOR (fmol/cell/h)	If only AOB	If only AOA	If only AOB	If only AOA
07.09.2005	Lindsey	3.58×10^5	N.D.**	36.6	1.54×10^5	N.D.	93.3	36.6	n.d.***	93.3	n.d.
07.10.2005	Lindsey	9.49×10^5	1.54×10^7	0.87	7.79×10^5	N.D.	18.41	15.1	0.93	18.4	n.d.
21.06.2006	Humber	7.14×10^3	6.49×10^7	0.14	9.56×10^4	5.18×10^7	0.087	632	0.070	47.26	0.087
16.10.2006	Humber	1.05×10^4	6.15×10^6	0.36	1.12×10^4	1.43×10^7	0.088	120.2	0.21	112.4	0.088
16.11.2006	Humber	1.32×10^4	1.90×10^8	0.0042	1.47×10^4	1.48×10^8	0.0037	42.5	0.003	38.03	0.0037
08.02.2007	Humber	6.56×10^3	2.23×10^8	0.0034	1.08×10^4	2.69×10^8	0.0046	189.5	0.0055	115.18	0.0046
26.07.2006	Grangemouth	1.24×10^4	1.08×10^8	0.016	1.76×10^5	1.77×10^8	0.015	213.3	0.024	15.03	0.015
28.02.2007	Grangemouth	1.06×10^6	1.41×10^7	0.015	1.68×10^6	9.24×10^6	0.022	0.22	0.017	0.14	0.026
12.04.2007	Grangemouth	1.05×10^6	2.78×10^7	0.050	7.53×10^5	4.32×10^6	0.45	2.17	0.081	3.02	0.53

* Calculated CSAOR assuming that either the AOB or the AOA are responsible for all of the nitrification

** N.D., not detected (below detection limit of the assay. See Appendix C)

***n.d., not determined

The highest and lowest CSAOR in the refinery wastewater treatment plants coincided with the first sampling occasion conducted in the Lindsey refinery in September 2005 and the fourth sampling occasion in the Humber refinery in February 2007. The highest CSAOR (93.3 fmol/cell/hour; Table 5.3) was found on the first sampling occasion at the Lindsey reactor B, when AOB were detected at their lowest numbers (1.54×10^5 cells/mL; Table 5.3). On this occasion only a relatively small proportion of the total ammonia and TKN in the influent was removed (70% removal of ammonia and 34% removal of TKN) however the ammonia and TKN which were removed were efficiently converted to nitrate (c.a. 100% efficiency; Table 5.2). In contrast, on the fourth sampling occasion at Humber, the best nitrification occurred when minimum CSAOR were found in reactor A (0.0034 fmol/cell/h) and in reactor B (0.0046 fmol/cell/h), respectively (Table 5.3). This is consistent with the suggestion of Coskuner *et al.* (2005) that high CSAOR are associated with poorer nitrification performance.

In the literature, estimated CSAOR found for the AOB based on 16S rRNA genes quantified in municipal WWTPs were in most cases within the same range found in this study based on *amoA* gene quantification; and for example in some studies ranged from zero to 49.6 fmol/cell/h (Limpiyakorn *et al.*, 2005); 0.03 to 43 fmol/cell/h (Coskuner *et al.*, 2005), 0.2 to 74.7 to fmol/cell/h (Pickering, 2008). In another study, also in municipal wastewater treatment plants (Harms *et al.*, 2003) AOB *amoA* CSAOR found for *Nitrosomonas oligotropha* ranged from 3.5 to 56 fmol/ cell/ hour. Interestingly in the study of Harms *et al.* (2003) the highest CSAOR (56 fmol/cell/h) was removed from the calculations of the mean CSAOR because it was more than double the standard deviation and thus the mean CSAOR were recalculated at 12.4 ± 7.3 fmol/cell/hour. However, as demonstrated in other wastewater treatment systems by Pickering (2008), this outlier would be a critical CSAOR value, reflecting a real event in a plant possibly approaching failure.

In practice it is not physically feasible to separate the relative contributions made by AOB and AOA to ammonia oxidation. However, if it is assumed that in the Humber reactors (n=8) all ammonia removal was conducted by AOA rather than AOB (See Table 5.3), AOA would be oxidizing more ammonia and TKN in the third (November 2006) and fourth (February 2007) sampling occasions respectively when at their highest cell numbers (10^8 cells/mL; Table 5.3) and minimum CSAOR (0.0030 – 0.0055 fmol/cell/h). These events also matched the lowest ammonia concentrations (0.93 and 0.73 mM; Table 5.2), and higher TKN (3.40 and 2.80 mM; Table 5.2) measured in the influent to the Humber reactors, as well as to the highest conversions found between consumption of ammonia (129- 167%; Table 5.2), and TKN (55%; Table 5.2), versus nitrate production, in the effluent from the Humber reactors.

Conversely, assuming that AOB alone were responsible for all the nitrification in the refinery reactors, some CSAOR values would be excessively high in contrast to values reported for AOB in the literature, as in the case of Humber reactor A. At the first sampling date in June 2006 (632 fmol/cell/h; Table 5.3) and the fourth sampling in February 2007, although to a much lesser extent (189.5 fmol/cell/h; Table 5.3).

All samples from Humber had similar AOB population sizes and though all have high CSAOR, only three values out of eight were exceptionally high. Although most CSAOR in Humber were high, they are only 2 to 3 fold higher than the highest the value of 72 fmol/cell/h reported previously (Pickering, 2008). Nevertheless, they are probably indicative of a nitrification system that is operating close to failure.

In the Humber reactors AOB *amoA* numbers were low (c.a. 10^4 cells/mL; Table 5.3). In reactor B, on the first sampling occasion (June 2006), when AOB numbers were at their highest (9.56×10^4 cells/mL; Table 5.3) - and although this also coincided with the highest ammonia concentration in the influent (1.39 mM; Table 5.2) - this reactor had the poorest nitrification, with the lowest $\text{NH}_3\text{-NO}_3$ conversion value found in the Humber plant (14%; Table 5.2). This low value also might be because of denitrification, which could have removed some of the nitrate generated by nitrification.

Conversely, on subsequent sampling occasions, good nitrification occurred in terms of ammonia and TKN removal at the Humber plant, although AOB *amoA* cell abundances were lower (ranged from 6.56×10^3 to 1.47×10^4 cells/mL; Table 5.3), and also coincided with lower ammonia concentrations in the influent (from 1.27 to 0.93 mM, Table 5.2). Therefore although process data indicated that on the first sampling occasion at Humber in October 2006, reactors were poorly nitrifying, by contrast, on the fourth sampling at Humber in February 2007, process data indicated that good nitrification was occurring.

Furthermore in comparison to Lindsey and Grangemouth, in the Humber reactors, AOB were apparently also subjected to more inhibitory conditions, for example, high C/N ratios and low pH; and besides the lower ammonia concentrations these factors may also have contributed to low AOB numbers found in the Humber reactor.

Among the 23 municipal WWTPs investigated by Pickering (2008), 16 WWTPs were classified as stable, four WWTPs as unstable and three WWTPs as failing. While the stable WWTPs exhibited lower CSAOR and higher AOB abundance, the unstable and failing WWTPs had lower AOB abundance and high CSAOR. In the work of Pickering (2008), the number of cells quantified through FISH ranged from 2.67×10^7 to 4.5×10^8 cells/mL; and CSAOR from 0.2 to 74.7 fmol/cell/h. Based on those results, useful guidelines were suggested, including ranges for AOB numbers and CSAOR, within which stable nitrification would be expected. Thus, within an approximate CSAOR range of 4 – 10 fmol/cell/h (Pickering, 2008), WWTPs operating with AOB numbers from 2.0×10^6 cells/mL or above would be closer to the lower CSAOR limit, but would still have the potential to work harder, whereas WWTPs operating with AOB numbers between 6.9×10^5 to 1×10^6 cells/mL would be closer to the higher CSAOR limit. In contrast, WWTPs operating with AOB numbers of 2.0×10^5 cells/mL or lower would be operating in more unstable conditions and out of the stable CSAOR range, and therefore would have a much higher risk of failure.

In order to test whether the relationships between CSAOR, ammonia oxidizer cell numbers demonstrated in the municipal wastewater treatment systems (Pickering, 2008) also held in the oil refinery wastewater systems, an analysis of all the data from samples where where AOB *amoA* and AOA *amoA* were quantified was conducted. For AOB this represent samples from the Lindsey, Humber, and Grangemouth reactors (n= 18), and for AOA data from the Humber and Grangemouth reactors (n= 14).

In line with Pickering (2008) and Coskuner *et al.* (2005), the log of AOB plus AOA cell abundances and CSAOR were negatively correlated ($r = -0.743$, $p = 0.000$) in the oil refinery wastewater treatment systems. Interestingly, the same relationships do not hold if one considers separately just the AOA abundance or just the AOB abundance. However, when only AOB abundance is considered, even if the highest CSAOR value (632 fmol/cell/h; See Table 5.3) is excluded from the analysis, it continues looking much better in comparison to the AOA abundance analysis only, which is consistently poorer, even if the highest CSAOR value (93.3 fmol/cell/h; Table 5.3) is excluded from the analysis, and this is also an interesting observation in relation to the possible involvement of AOA in the nitrification within the refinery plants.

Comparing both AOB cell numbers and CSAOR in the Lindsey, Humber and Grangemouth reactors to the range of values suggested for the municipal sewage treatment systems (Pickering, 2008), situations of stable nitrification for AOB would thus be occurring in the Lindsey reactors (0.87 and 18.41 fmol/cell/h) in the plant's second sampling conducted in October 2005 and in the Grangemouth reactors (0.050 and 0.45 fmol/cell/h) on the third sampling occasion of April 2007. These conditions also coincided with the highest AOB cell numbers (from 7.53×10^5 to 1.68×10^6 cells/mL) found in these reactors (See Table 5.3); furthermore, CSAOR and cell numbers also corresponded to the best nitrification reached in the Lindsey and Grangemouth reactors (Table 5.2).

In contrast, situations of unstable nitrification or even failure would be occurring at the high CSAOR found in the Lindsey reactor B (93.3 fmol/cell/h) on the first sampling occasion at the Lindsey refinery in September 2005. This high CSAOR value also matched the lowest AOB cell numbers (1.54×10^5 in the Lindsey plant, suggesting that AOB in this reactor would be working too hard, and exhibiting lower than optimal nitrification; Table 5.3). This interpretation based on CSAOR and cell numbers also coincided with the poorest nitrification performance in the Lindsey reactor, and although higher ammonia (145%) and TKN conversions to nitrate (94%) were observed in the effluent, lower TKN (34%) and ammonia (70%) removal efficiencies occurred in the Lindsey reactor (Table 5.2).

In contrast to AOB, AOA were detected only once at 1.54×10^7 cells/mL (See Table 5.3) in Lindsey reactor A on the second sampling occasion at the plant in October 2005 (Table 5.2), when the reactor's AOB abundance was greatest (9.49×10^5 cell/mL; Table 5.3), and the CSAOR in the reactor was 0.87 fmol/cell/h.

In Grangemouth reactor B on the first sampling date when the CSAOR was 0.015 fmol/cell/h (Table 5.3), AOA were at their maximum numbers (1.77×10^8 cells/mL) and AOB at lower prevalence (1.76×10^5 cell/mL). Conversely, at CSAOR of 0.45 fmol/cell/h, AOA were at their minimum cell numbers (4.13×10^6 cells/mL; Table 5.3) and AOB were at much higher cell numbers (7.53×10^5 cells/mL). This also coincided with conditions of stable nitrification in the Grangemouth reactor.

Interestingly, in the Grangemouth reactors (n=6), a positive linear relationship was also found between AOA and COD ($r^2= 81$; $p= 0.014$). The highest AOA abundance in the Grangemouth reactors (ca. 10^8 cells/mL; Table 5.3) coincided with the highest COD concentration found in the influent to the Grangemouth reactor (311 mg/mL; Table 5.2), thus suggesting the involvement of AOA in the carbon metabolism.

In the Humber reactors AOA also reached their highest numbers (c.a. 10^8 cells/mL; Table 5.3) when AOB were at minimum numbers (c.a. $\leq 10^4$ cells/mL; table 5.3); however CSAOR were much lower (ranged from 0.0034 to 0.0046 fmol/cell/h; Table 5.3) than the values found in the Grangemouth reactor.

In the Eastham reactor, although ammonia and TKN were not being removed and nitrification was not occurring, AOB and AOA *amoA* genes were detected at a very low abundance (c.a. 10^3 gene copies/mL; Figure 5.5). This also matched the highest value of ammonia (1.60 mM; 1.95 mM TKN; Table 5.2) found in the influent to the refineries wastewater treatment plants.

Therefore, based on the observations of the reactors in this study, that suggest a possible link between AOA and organic carbon, a question is raised: do AOA have a role in ammonia oxidation in the refinery systems or are they mainly involved in carbon metabolism?

In the samples taken from the 23 municipal wastewater treatment systems and one pilot reactor treating tannery wastes from a leather processing plant (BLC) screened for archaeal *amoA* genes, putative AOA were found only in the BLC leather pilot reactor. Therefore it was evident that AOB made the major contribution to nitrification in the municipal systems, and AOA play at most a minor role in nitrification in these systems.

In the refinery systems, although it appears that AOB involvement in nitrification is greater than the involvement of AOA in the Lindsey and Grangemouth reactors, on the basis of AOB cell numbers and CSAOR, in the Humber reactors, process data indicated that nitrification was actively occurring, although AOB numbers were low (about 0.4% of the abundance of the AOA; Table 5.3). Therefore the Humber reactor is a special and interesting case where there is apparent nitrification in spite of low AOB numbers.

In the Humber reactors, given that either AOB plus AOA were contributing to ammonia oxidation, or that AOA only were responsible for all the nitrification occurring, it is notable that in both situations some estimated CSAOR for AOA (Table 5.3) found in this study were similar to other estimated CSAOR for AOA found in the literature, for example for *Nitrosopumilus maritimus* in pure culture (0.16 fmol/cell/hour; Könneke *et al.*, 2005); for marine *Crenarchaeota* enrichments from the North Atlantic, which ranged between of 0.083 and 0.16 fmol/cell/hour (Wuchter *et al.*, 2006); and for AOA in the rhizosphere of a freshwater macrophyte (0.5 fmol/cell/h; Herrmann *et al.*, 2008). On the other hand, some estimated CSAOR values for AOA in the Humber reactors, were considerably lower (ranged from 0.003 to 0.005 fmol/cell/h; Table 5.3) than those values.

In relation to ammonia concentrations, studies relating AOA to ammonia affinity or inhibition by ammonia are scarce; however, in a recent study testing the sensitivity of “*Candidatus Nitrososphaera gargensis*” (Hatzenpichler *et al.*, 2008) to ammonium concentration, “*Candidatus N. gargensis*” was shown to be very active at 0.14 and 0.79 mM ammonium, and partially inhibited at 3.08 mM ammonium. These levels are low in comparison to the ammonia concentrations that inhibit different types of AOB within the *N. oligotropha* lineage, (the most sensitive AOB with K_s values ranging from 0.030 to 0.061 ;Koops and Pommerening-Röser, 2001), which may tolerate up to 50 mM ammonia.

Therefore whether AOA, at least in the Humber reactor, are oxidizing ammonia at significant levels is still open to debate. However, the data from this study might suggest that AOA would be more active at ammonium concentrations between 0.73 to 0.93 mM, that is, when they were detected at their highest numbers (10^8 cells/mL); but, robust microcosm experiments testing the effect on nitrification of different ammonia concentrations in this AOA-dominated system would need to be set up in order to test this hypothesis.

5.3.6 Relationships between physical-chemical parameters and AOB and AOA abundance

In order to gain insight into the interrelations among physicochemical conditions and the abundance of AOB and AOA based on qPCR data, the data from the wastewater treatment reactors from the Humber, Grangemouth and Lindsey refineries were analysed using principal component analysis (PCA).

PCA cannot be used to test hypotheses, but rather as an exploratory tool to identify potential relationships between different parameters. In this study PCA was used to identify possible relationships between physical-chemical parameters, and bacterial and archaeal abundances.

PCA is a powerful technique for revealing hidden patterns in large and complex datasets, allowing the dimensionality of the data to be reduced and the maximal variance contained in the data depicted. This allows internal data structure to be viewed more clearly. The principles underlying PCA are based on linear algebra where a number of variables contained in a matrix are transformed to a new matrix of a few new orthogonal and mutually independent variables (multiple regression equations) known as principal components (Ramette *et al.*, 2007). The first principal component (PC) accounts for the greatest variance in the data, the second for the largest remaining variance in the data and so on.

PCA interpretation is to some degree subjective, but some obvious patterns in the data sets emerge. In the PCA diagrams the similarities between the projected variables (eigenvectors) on the space dimension are interpreted as a function of the angle between them and their signs on each PC. The cosine of the angle formed between two variable vectors is equal to the reproduced correlation between the variables; and therefore two variables with the same sign (direction) on a PC plot imply a positive correlation between the variables on that PC, while opposite signs imply a negative correlation. When the angle between two variables is 90° , they are independent from one another.

Although nitrite-oxidizing bacteria (NOB) 16S rRNA gene abundance was included in the PCA analysis, NOB abundance results are discussed in Chapter 6. As shown in the PCA graph (Figure 5.10), AOB *amoA* abundance in the refinery reactors inversely correlated with the BOD/TKN and COD/TKN ratios. At high C/N ratios heterotrophs are better competitors for ammonia than AOB (Verhagen *et al.*, 1992; Verhagen & Laanbroek, 1991), thus at high BOD heterotrophs are expected to outcompete AOB, and thus AOB numbers should be depressed and therefore a negative correlation between C/N ratios and AOB numbers is expected.

By contrast, AOA positively correlated to the soil group *Crenarchaeota* and negatively correlated with the marine group *Crenarchaeota* (both crenarchaeotal groups are referred on Figure 5.10 as Cren Soil and Cren marine respectively). Both AOA and Cren soil positively correlated to BOD/TKN and COD/TKN ratios, BOD removal (BODr) and COD removal (CODr), ammonia, TKN, NH₃-NO₃, TKN-NO₃ and TKNr removal (TKNr), indicating they might also have been removing ammonia and TKN through heterotrophic nitrification. Thus when BOD removal was higher, nitrification was higher as well. As shown by Mußmann *et al.* (2008) AOA in the Humber and Grangemouth reactors did not take up radiolabelled CO₂ during MAR-FISH experiments and thus they were shown not be autotrophic; thus, unlike AOB, they do not fix inorganic carbon. The AOA detected in the refinery wastewater treatment plants may therefore exhibit lithoheterotrophic metabolism if they are able to oxidize ammonia; or alternatively they may have no role in ammonia oxidation at all.

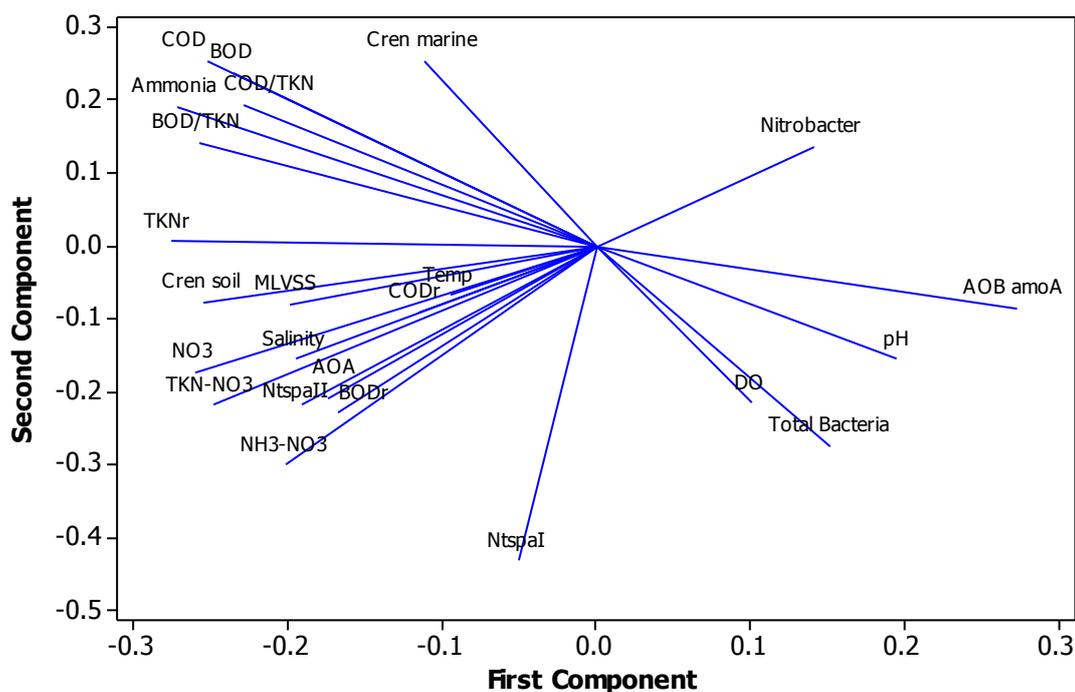


Figure 5.10 PCA diagram. Biplot representation of first and second components of physical-chemical and qPCR abundance data considered in this study. PC1 (45.3%) and PC2 (17.9%) refer to data from Lindsey, Humber and Grangemouth reactors. The projected parameters (eigenvectors) point in the direction of maximum variation. The length of each parameter is equivalent to their rate of change in the dataset. Parameters projected in opposite directions imply negative correlations while positive correlations are implied by parameters projected in the same direction. Oblique angles between two vectors imply in a certain degree of relatedness between vectors. Right angles between vectors show they are independent from one another. Labeled parameters on the biplot: Temperature, DO, pH, MLVSS, Ammonia, TKN, COD, BOD, NH₃-NO₃ = ammonia conversion to nitrate, Nitrobacter, NtspaI= Nitrospira type I, NtspaII= Nitrospira type II, TKNr=TKN removal, CODr= COD removal, BODr= BOD removal, NO₃, AOB *amoA*, total bacteria, AOA, Cren marine= Crenarchaeota from marine group 1.1.a and Cren soil= Crenarchaeota from soil group 1.1.b.

In all wastewater treatment reactors carbonaceous wastes greatly exceed nitrogenous wastes, and thus more substrate is available to heterotrophs which have a faster growth rate than nitrifiers. Moreover, nitrifiers, besides depending on the amount of ammonia available, are also affected by several operational factors such as temperature, oxygen, organic carbon, competition with heterotrophs for resources, toxic chemical substances and pH (Gerardi, 2002).

AOB *amoA* abundance correlated positively with pH and DO and negatively with ammonia, TKN, $\text{NH}_3\text{-NO}_3$, and temperature (Figure 5.10).

Situations where AOB *amoA* abundance was greater, in this case in the Lindsey and Grangemouth reactors, also coincided with neutral to alkaline pH conditions (7.4- 8.4; See Table 3.1, Chapter 3). Higher pH is a factor favouring AOB (Stein *et al.*, 1997). Moreover the influent to reactors where AOB *amoA* was not detected (Eastham, Pembroke and Humber refineries) had more acid pH (6.3, 6.2 in Eastham and Pembroke respectively; and between 4.6 and 6.9 in the Humber reactors; Table 3.1; Chapter 3).

Nitrification by AOB is generally accepted to occur optimally at a neutral to slightly alkaline pH (Prosser, 1989). Other studies testing the effects of pH in lab scale nitrifying reactors inoculated from a WWTP (Princic *et al.* 1998) have shown very low nitrification rates at pH 6, whereas nitrification rates were greater at pH 7 and 8. This is because AMO acts on NH_3 not NH_4^+ and at a higher pH the equilibrium is pushed in the direction of NH_3 . AOB do not have ammonia/ammonium transport proteins and at a lower pH the charged NH_4^+ does not easily cross the membrane, while at a higher pH the NH_3 can pass across the membrane (Stein *et al.*, 1997). In addition, while soil AOA have been shown to respond more quickly to fertilizer treatments in acid soils, AOB responded more quickly to fertilizer treatments in neutral to alkaline soils (He *et al.*, 2007; Shen *et al.*, 2008; Nicol *et al.*, 2008).

In this study, a clear trend linking AOA abundance to pH was not observed, since AOA abundance was high with both higher and lower pH. For instance in Lindsey, AOA were detected in samples of 7.76 but not pH 7.5 and in the Humber samples when pH was near to neutral (6.93), they were also at their highest numbers (10^8 cells/mL) at the minimum pH (4.65) determined on the Humber plant's third sampling occasion (see Chapter 3; Table 3.1). They also occurred with a high pH (8.17) in the Grangemouth reactor on the first sampling occasion. However, by contrast, Cren soil related negatively to pH.

The PCA analysis clearly demonstrates that several factors may be affecting, and thus contributing to, the reduced AOB *amoA* abundance in the refinery reactors, compared for example to municipal sewage treatment systems. However, when C/N ratios, ammonia and TKN are low, pH is more alkaline and oxygen levels are higher within reactors, they seem to perform better nitrification based on ammonia and TKN conversions to nitrate. Thus nitrification was most effective in the Lindsey and Grangemouth plants where these criteria were met.

5.3.7 Is measured AOB biomass consistent with theoretical yields of AOB based on ammonia removal?

Using process parameters such as sludge age, hydraulic retention time (HRT) and ammonia removal, Rittmann *et al.* (1999) have developed a nitrification model which allows the percentage of nitrifier biomass to be predicted in relation to the total biomass in an activated sludge reactor by measuring the MLVSS in the reactor. The equation which synthesizes this model is described in detail in Chapter 2.

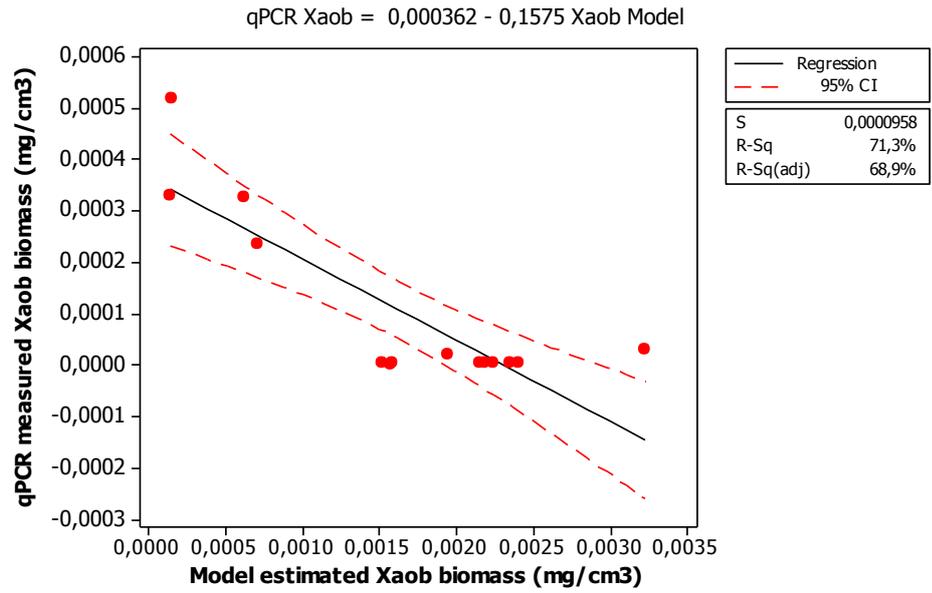
Four years of data from the Humber reactors A and B (n= 38) applied to the model showed the expected AOB biomass in the Humber sludge to be very low (ranged from 0.01 to 0.06%) in relation to estimated MLVSS (X_v), measured in mg/L, with mean values of 0.02% in reactor A and 0.03% in reactor B, respectively. If this AOB biomass was converted to AOB cell numbers they would be equivalent to an approximate range of cell numbers between 4.2×10^5 and 7.0×10^6 cells/mL.

If theoretical and empirical data are in perfect agreement, an R^2 of 100%, a slope of 1 and an intercept of zero between the model and experimental data would be expected (Coskuner *et al.*, 2005). The intercept of the line on the Y axis also gives us information about whether the model overestimated the measured values (intercept value lower than zero) or underestimated the measured values (intercept value higher than zero).

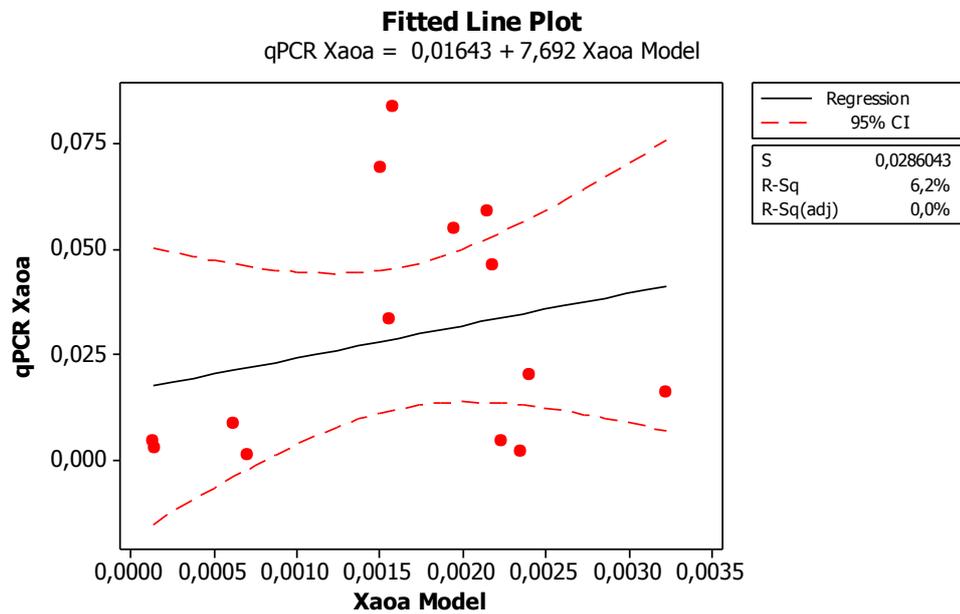
In this study, predicted and measured AOB *amoA* biomass were negatively correlated, that is, the model predicted high numbers, when the actual numbers were lower in all cases. The slope of the line was too low (-0.157) and $r^2 = 71.3\%$ (Figure 5.11.a). This was far from a perfect agreement with the model (Figure 5.11.a), both measurements systematically disagreed, and in this case, the model overestimated actual AOB biomass (Table 5.4).

Table 5.4 Theoretical biomass predicted using a single nitrification model (Rittmann *et al.*, 1999) and measured biomass of AOB and AOA obtained from *amoA* genes quantification through qPCR in the Humber and Grangemouth reactors

WWTP/Reactor	Sampling date	Sludge age (Θ_x) Days	AOB biomass (X_{aob}) Predicted by model (mg/cm^3)	AOB <i>amoA</i> cells converted into biomass (mg/cm^3)	AOA <i>amoA</i> cells converted into biomass (mg/cm^3)
Humber A	21.06.2006	48	2.4×10^{-3}	2.21×10^{-6}	2.0×10^{-2}
Humber B	21.06.2006	20	3.2×10^{-3}	2.96×10^{-5}	1.6×10^{-2}
Humber A	16.10.2006	38	2.4×10^{-3}	3.25×10^{-6}	2.0×10^{-3}
Humber B	16.10.2006	45	2.2×10^{-3}	3.47×10^{-6}	4.0×10^{-3}
Humber A	16.11.2006	37	2.1×10^{-3}	4.07×10^{-6}	6.0×10^{-2}
Humber B	16.11.2006	35	2.2×10^{-3}	4.55×10^{-6}	4.6×10^{-2}
Humber A	08.01.2007	60	1.5×10^{-3}	2.03×10^{-6}	6.9×10^{-2}
Humber B	08.01.2007	51	1.6×10^{-3}	3.34×10^{-6}	8.3×10^{-2}
Grangemouth A	26.07.2006	25	1.6×10^{-3}	1.28×10^{-6}	3.3×10^{-2}
Grangemouth B	26.07.2006	14	1.9×10^{-4}	1.82×10^{-5}	5.5×10^{-2}
Grangemouth A	28.02.2007	29	1.3×10^{-4}	3.29×10^{-4}	4.4×10^{-3}
Grangemouth B	28.02.2007	22	1.5×10^{-4}	5.19×10^{-4}	2.8×10^{-3}
Grangemouth A	12.04.2007	17	6.2×10^{-4}	3.25×10^{-4}	8.6×10^{-3}
Grangemouth B	12.04.2007	12	7.1×10^{-4}	2.33×10^{-4}	1.3×10^{-3}



(a)



(b)

Figure 5.11 Relationship between the theoretical biomass predicted in the Humber plus Grangemouth reactors using a single nitrification model (Rittmann *et al.*, 1999) and measured biomass obtained from (a) AOB *amoA* and (b) AOA *amoA* gene quantification through qPCR. Xaob = AOB biomass; Xaaa= AOA biomass.

In contrast to this study, Coskuner *et al.* (2005) found good agreement between model-estimated AOB biomass and experimentally measured AOB biomass obtained through FISH in samples from five municipal wastewater treatment plants and one lab-scale reactor. In the plants investigated by Coskuner *et al.* (2005), AOB numbers were much higher (10^5 to 10^8 cells/mL) than the AOB numbers in the refinery WWTPs (10^3 - 10^6 cells/mL; Table 5.3). Additionally the majority of AOB cells (>96% of AOB biomass) were found to be predominantly arranged as microcolonies, while in the refinery systems the majority of the AOB cells occurred as single cells dispersed within the flocs or ultimately as scarce and very small colonies (see Figure 6.10; Chapter 6). Furthermore measured AOB biomass values through FISH were all above 2% (2-7%) and therefore the measured values in the Coskuner *et al.* (2005) study are probably much more reliable than the very low abundances measured in this study. It may not therefore not be particularly surprising that the strong relationship seen by Coskuner *et al.* (2005) was not found in this study, as it is very difficult to determine a relationship with such low AOB numbers, due to the fact that there is a greater degree of error innately associated with measuring low values and thus greater difficulty in finding any agreement between model and measured data.

In a recent study, the model (Rittmann *et al.*, 1999) was also tested in a modified Ludzack-Ettinger (MLE) activated sludge process receiving pretreated wastewater from an oil refinery industry (Figuerola and Erijman, 2010), and also in line with this study, no agreement was found between model-estimated AOB biomass and experimentally measured AOB biomass. Similarly to some refinery WWTPs in this study, the plant was being operated with long hydraulic retention time (36 hours), sludge age (49 days), and low BOD (146 ± 78 mg/L); however, in contrast to the low ammonia concentrations found in this study, the WWTP was receiving higher ammonia concentrations in between 2.82 to 6.71 mM (mean= 4.76 mM). The measured AOB *amoA* abundance in cell numbers ($9.2 \pm 3.2 \times 10^7$ cells/mL), also obtained through real-time PCR quantification, converted into biomass, represented 1% of the sludge biomass, and systematically disagreed with the AOB biomass estimated by the model (8%). Interestingly, although ammonia concentrations were much higher than the ammonia concentrations found in this study, and comensurately also supported higher AOB *amoA* abundance, the AOB fraction measured in the WWTP also became below the range (2-7%) found in the municipal WWTPs (Coskuner *et al.*, 2005).

In this study, although the model did not accurately predict the measured AOB biomass, it did show that AOB were expected to represent a very small fraction of the biomass in the refinery wastewater treatment plants in this study, especially in the Humber reactors, which exhibited very low AOB abundance. This might therefore also be a good indication that the amount of nitrification seen would be too little to support the population size of AOA observed in the Humber reactor (10^7 to 10^8 cells/mL; Table 5.3), even assuming that all the ammonia was being oxidized by AOA rather than AOB.

In order to verify whether AOA population size would be consistent with the levels of ammonia removal in Humber and Grangemouth, estimated growth yields for AOA were calculated on the basis of the specific growth yield of *Nitrosopumilus maritimus* in pure culture (Könneke *et al.*, 2005). Considering that 1.4×10^7 cells/mL were produced from 0.3 mM ammonium being consumed (Könneke *et al.*, 2005), the conversion of these units values, e.g. cells per mL per mole of ammonia unit values into gram dry weight of biomass per mole of ammonia unit values, the estimated growth yield for AOA would be equivalent to 1.15 gram dry weight of biomass per mole of ammonia having been consumed. Further comparing the obtained estimated growth yield value for AOA to reported growth yield values for AOB in the literature, which are in the range of 0.4 to 1.4 of gram dry weight of biomass per mole of ammonia (Prosser, 1989), both values showed to be similar, and then the size of the AOA population that would be expected, assuming that all AOA cells were oxidizing ammonia, was calculated by using the Rittmann *et al.* (1999) model with the same parameters used for AOB. As a result, the AOA biomass predicted by the model found in Humber and Grangemouth has not correlated with the measured AOA biomass (Figure 5.11.b).

In this context, the levels of ammonia being oxidized would not support the AOA population size in the Humber and Grangemouth reactors which is too high (10^7 to 10^8 cells/mL) to be accounted for by the low quantity of ammonia removal measured. This therefore suggests they might not be ammonia oxidizers, but rather heterotrophs. Furthermore the assumption of the same growth yields and endogenous decay rates for AOB and AOA is also probably reasonable and valid.

5.4 Conclusions

23 municipal WWTPs in UK, five refinery wastewater treatment systems and one pilot reactor treating tannery wastewater, which were all screened for the presence of putative AOA. Of these, only four of the five refinery treatment reactors and the pilot reactor harboured AOA at detectable levels, and in these cases AOA (at an abundance of ca. 10^7 gene copies/mL) coexisted with AOB.

Of the five refinery wastewater treatment plants investigated in this study, Lindsey, Humber and Grangemouth were performing nitrification while nitrification was not detected in the Eastham and Pembroke reactors.

Ammonia was being removed efficiently in the Lindsey, Grangemouth and Humber reactors; however, the best performance in nitrification was achieved in the Grangemouth and Humber reactors (activated sludge) in comparison to the Lindsey reactors (trickling filters).

Likewise, the best BOD removal was achieved in the Lindsey, Humber and Grangemouth reactors; however, COD and TKN removal efficiencies were greater in the Humber and Grangemouth reactors.

AOB abundance in this study was measured by the amplification of 16S rRNA and the *amoA* genes. The functional *amoA* gene was a better marker than the 16S rRNA gene for the quantification of AOB in the oil refinery wastewater treatment plants. The *amoA* gene was more specific in the quantification of AOB and the abundance of AOB based on quantification of *amoA* also better reflected the environmental conditions in reactors.

AOB *amoA* abundance in the refinery wastewater treatment systems ranged from 3.07×10^5 to 1.90×10^6 gene copies/mL in the Lindsey reactors, and ranged from 2.48×10^4 to 3.35×10^6 gene copies/mL in the Grangemouth reactors. AOB *amoA* abundance in the Humber reactor ranged from 1.31×10^4 to 1.91×10^5 gene copies/mL. Likewise, AOA *amoA* was detected only once in the Lindsey reactor at 1.54×10^7 gene copies/mL, while the abundance ranged from 6.15×10^7 to 2.69×10^8 gene copies/mL in the Humber reactors and from 4.32×10^6 to 1.77×10^8 gene copies/mL in the Grangemouth reactors. AOB *amoA* was detected at very low abundances in the Eastham and Pembroke reactors (ca. 10^3 gene copies/mL).

The highest AOB *amoA* abundance in cell numbers (ca. 10^6 cells/mL), on the basis of AOA plus AOB cell numbers, in the Lindsey and Grangemouth reactors, coincided with lower cell specific ammonia oxidation rates of 0.015 and 0.87 fmol/cell/h and stable nitrification. Conversely the lowest AOB *amoA* abundance in cell numbers (1.54×10^5 cells/mL), on the basis of AOA plus AOB cell numbers, in the Lindsey reactors, coincided with the highest cell specific ammonia oxidation rates (93.3 fmol/cell/h). At this sampling time, the Lindsey reactor exhibited the lowest ammonia (70%) and TKN (34%) removal efficiencies observed for this plant.

The highest AOA *amoA* abundance in cell numbers (10^8 cells/mL), on the basis of AOA plus AOB cell numbers in the Humber reactors, coincided with the lowest CSAOR (0.034- 0.0046 fmol/cell/h), as well as the highest ammonia and TKN removal and the highest conversion between ammonia and TKN consumption and nitrate production (167% and 55%).

AOB biomass fraction in the Humber reactor estimated using a nitrification model was very low (0.01-0.06%), which would correspond to a range of cells in between of 4.2×10^5 and 7.0×10^6 cells/mL. Although model and measured AOB biomass measured through qPCR disagreed, measured AOB biomass was much lower (c.a. 10^4 cells/mL) than predicted AOB biomass in the Humber and Grangemouth WWTPs.

The nitrification model was tested with AOA *amoA* numbers; however they systematically disagreed. It seems unlikely that the high population size of AOA (10^7 - 10^8 cells/mL) observed here could be supported by autotrophic nitrification, further suggesting that they may have had an alternative role in wastewater treatment systems.

Chapter 6

Ammonia oxidizer and nitrite oxidizer abundance measured through FISH and real-time PCR

6.1 Introduction

In the second step of the nitrification process, nitrite is oxidized to nitrate mediated by the action of nitrite oxidizing bacteria (NOB). Nitrite oxidizers belong to four distinct genera *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*.

To date there are only eight cultured species of nitrite oxidizer; however, through several culture independent *in situ* investigations using, for example, FISH (fluorescence *in situ* hybridization) coupled to other techniques such as microsensor measurements (Schramm *et al.*, 1999), autoradiography (Lee *et al.*, 1999), CLSM (confocal scanning laser microscopy), and digital image analysis (Wagner *et al.*, 1994; Daims *et al.*, 2006a), it has been demonstrated that many other species exist, and in the future these may be successfully cultivated and isolated (Koops and Pommerening-Röser, 2001).

The genus *Nitrobacter* encompasses four described species within the *Alphaproteobacteria*: *Nitrobacter winogradskyi*, *Nitrobacter hamburgensis*, *Nitrobacter vulgaris* and *Nitrobacter alkalicus*. *Nitrobacter* spp. are widely distributed in soils and freshwater (Koops and Pommerening-Röser, 2001) but have also been isolated from wastewater treatment plants, building stones, brackish and marine water samples, and *Nitrobacter alkalicus* has been isolated from soda lake sediments (Sorokin *et al.*, 1998). *Nitrococcus* and *Nitrospina* are marine genera placed respectively within the *Gammaproteobacteria* and *Deltaproteobacteria* (Teske *et al.*, 1994).

However the phylogenetic placement of *Nitrospina* is somewhat ambiguous and a more detailed analysis may reveal that it does not belong in the *Deltaproteobacteria*. The genus *Nitrospira* forms a distinct phylum with iron-oxidizing bacteria from the genus *Leptospirillum*, and includes the cultured species *Nitrospira moscoviensis*, isolated from a corroding pipe from a heating system in Moscow (Ehrich *et al.*, 1995) and a marine species *Nitrospira marina* (Watson *et al.*, 1986).

As *Nitrobacter* has been isolated from many terrestrial environments, and is the most common genus of nitrite-oxidizer isolated in culture, *Nitrobacter* was considered to be the main NOB population driving nitrite oxidation, while *Nitrospira* was thought to be restricted only to marine environments, until *Nitrospira moscoviensis*, the first terrestrial member of the genus *Nitrospira* was isolated (Ehrich *et al.*, 1995). More recently, two novel *Nitrospira* species have also been proposed. These are: 'Candidatus *Nitrospira defluvii*', enriched from a municipal activated sludge plant (86% of cells in the enrichment culture; Spieck *et al.*, 2006); and 'Candidatus *Nitrospira bockiana*', also isolated from corroded steel pipes from heating systems in Moscow, Russia (Lebedeva *et al.*, 2008).

The nitrite oxidizers are considered very fastidious, slow-growers and very difficult to cultivate, and as *Nitrospira* has lower growth rates than *Nitrobacter* (Prosser, 1989) this makes their isolation and cultivation a difficult and challenging task. For example, the recently proposed species 'Candidatus *Nitrospira bockiana*' (Lebedeva *et al.*, 2008) was obtained only after 12 years of intensive and dedicated work where many techniques were combined to monitor the enrichment, including culture-independent molecular methods, immunofluorescence, electron-microscopy and fatty acids profiling. Although still very laborious, these new enrichment strategies represent a significant step towards achieving feasible cultivation and isolation, not only of fastidious uncultured NOB, but also numerous other ecologically important microorganisms.

Like the AOB, NOB can be distinguished based on some peculiar ecophysiological properties (Koops and Pommerening-Röser, 2001). For example, all *Nitrobacter* species, besides having the ability to use nitrite as their only energy source, can also take up different organic substrates including acetate, pyruvate and butyrate, among others, under both aerobic and anaerobic conditions (Prosser, 1989). *Nitrobacter* spp. can thus grow mixotrophically or heterotrophically in the presence of these substrates, while, to date, *Nitrospira*-like NOB were shown to assimilate pyruvate only under aerobic conditions (Daims *et al.*, 2001a). Pure cultures of *Nitrospira moscoviensis* were also demonstrated to have hydrogenase activity in the presence of nitrate as an electron acceptor (Ehrich *et al.*, 1995). Furthermore, *Nitrococcus*, *Nitrospina* and *Nitrospira marina* are obligate halophilic species; while in contrast, *Nitrospira moscoviensis*, in common with *Nitrobacter* species, has no obligate requirement for salt.

The genus *Nitrospira* was demonstrated for the first time as the main NOB population driving nitrite oxidation in WWTPs when no *Nitrobacter*-like NOB could be detected in eight municipal wastewater plants and one industrial intermittently nitrifying-denitrifying wastewater treatment plant receiving animal wastes (Wagner *et al.*, 1996). The NOB in these German wastewater treatment plants were investigated through *in situ* hybridization with specific probes targeting the genus *Nitrobacter*. Since then, the predominance of *Nitrospira* over *Nitrobacter* has been consistently shown in a range of wastewater treatment plants and reactors, where *Nitrobacter* in general is absent or coexists in low numbers with *Nitrospira* (Juretschko *et al.*, 1998; Juretschko *et al.*, 2002; Schramm *et al.*, 1998; Burrell *et al.*, 1998; Schramm *et al.*, 1999, Okabe *et al.*, 1999; Schramm *et al.*, 2000; Daims *et al.*, 2000, Gieseke *et al.*, 2001; Daims *et al.*, 2001b; Coskuner and Curtis, 2002; Gieseke *et al.*, 2003).

The appearance of *Nitrospira* sp., instead of *Nitrobacter* sp., as the most important and abundant NOB driving nitrite oxidation in several wastewater treatment reactors was an unexpected finding, since *Nitrobacter* were considered for a long time to be the most important bacteria responsible for nitrite oxidation in most environments. This in part reflects the relative ease with which *Nitrobacter* spp. have been isolated using culture-dependent methods, whereas prior to the culture-independent characterization of nitrite-oxidizing bacteria in wastewater treatment systems, cultured *Nitrospira* were primarily isolated from marine environments (Abeliovich, 2003).

Nitrospira have been reported in numerous other environments for example, heating systems (Ehrich *et al.*, 1995; Lebedeva *et al.*, 2008), freshwater aquaria (Hovanec *et al.*, 1998); groundwater contaminated by livestock waste (Cho and Kim, 2000); soils (Bartosch *et al.*, 2002; Freitag *et al.*, 2005); the rhizosphere (Marilley and Aragno, 1999); freshwater sediments (Altmann *et al.*, 2003); marine deep Sea sediments (Li *et al.*, 1999); rivers and estuaries (Cébron and Garnier, 2005); Australian caves (Holmes *et al.*, 2001); marine sponge tissue (Hentschel *et al.*, 2002); and even hot springs (Kanokratana *et al.*, 2004; Lebedeva *et al.*, 2005). This demonstrates that *Nitrospira* spp. are also ubiquitously distributed and exhibit considerable diversity.

Within the phylum *Nitrospira*, four sublineages (I to IV) have been defined (Daims *et al.*, 2001a). Sublineage I includes the recently enriched 'Candidatus *Nitrospira defluvii*', as well as other sequences that have been retrieved only from wastewater treatment systems. Sublineage II encompasses the cultured *Nitrosospira moscoviensis*, sequences retrieved from wastewater systems, and also from more diverse environments, such as soils, the rhizosphere, caves, lake water and freshwater aquaria. Sublineage III includes uncultured representatives specifically found in Australian caves, while Sublineage IV includes *Nitrospira marina*, as well as a related organism found in the deep sea.

Therefore, sublineage or type I represented by 'Candidatus *Nitrospira defluvi*', and sublineage or type II, phylogenetically related to *Nitrospira moscoviensis*, are the two most commonly found types of *Nitrospira* found in wastewater treatment plants and are able to coexist as a function of their different preferences for nitrite concentrations (Daims *et al.*, 2006b; Maixner *et al.*, 2006).

In wastewater treatment systems, active nitrification occurs in the outermost parts of activated sludge flocs or in the upper layers of biofilms (i.e., 50-150 μm depth), where AOB populations are typically arranged in dense and tightly packed microcolonies containing thousands of cells. These tend to occupy the external regions of flocs and biofilms where oxygen concentrations are higher. The NOB form smaller and looser aggregates and tend to occupy the internal parts of activated sludge flocs and biofilms, in close vicinity to AOB microcolonies (Wagner *et al.*, 1995; Schramm *et al.*, 1996; Okabe *et al.*, 1999; Schramm *et al.* 1999).

In this study, the process data showed that ammonia was converted to nitrate in the refinery WWTPs. To date, little is known, for example, about which NOB populations are involved in nitrite oxidation in refinery wastewaters or in other WWTPs. *Nitrospira* like bacteria are also responsible for nitrite oxidation in refinery WWTPs. Therefore, in order to answer these questions the detection and abundance of *Nitrospira* and *Nitrobacter* like bacteria in refinery wastewater treatment systems were investigated through real-time PCR and fluorescence *in situ* hybridization (FISH) coupled with CLSM and digital image analyses. In addition AOB abundance was investigated through FISH and compared to AOB numbers obtained through real-time PCR (See Chapter 5).

Numbers and morphology of AOA found in samples obtained from this study, were also investigated through CARD-FISH by Marc Mußmann from Vienna University, and with the author's permission, the data were included in this chapter for comparison and discussion in relation to the AOA numbers obtained in this study through real-time PCR.

6.2 Methods

DNA was extracted from 64 samples collected from the five oil refinery wastewater treatment plants investigated in this study, 12 from the Lindsey reactors, three from the Eastham reactor, six from the Pembroke reactor, 24 from the Humber reactors, 18 from the Grangemouth reactors, and one sample collected from the BLC pilot reactor treating leather processing wastewater. DNA extracts were diluted tenfold and the NOB were quantified by real-time PCR. Three real-time PCR assays were conducted to quantify the 16S rRNA genes of the nitrite oxidizing bacteria (NOB) *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* (Maixner *et al.*, 2006; Knapp and Graham, 2007). Detection limits for the real-time PCR assays were set up as the mean of the Ct values obtained from two blank reactions or no template control (NTC) that were included in each assay, plus three standard deviations.

The relationships between the NOB gene abundances measured through real-time PCR and the physical-chemical parameters obtained from all refinery wastewater treatments plants were analysed through Pearson correlation, linear regression and multivariate statistical analysis using principal component analysis (PCA).

The real-time PCR assays are described in detail in Chapter 2, Section 2.12. Ten replicate samples, representing all the refineries investigated in this study, and one sample from the BLC pilot reactor, were selected (based on the higher NOB 16S rRNA abundances obtained through real-time PCR) for quantification through fluorescence *in situ* hybridization (FISH) targeting respectively AOB, and the genera *Nitrospira* and *Nitrobacter*. The data from qPCR and FISH were compared.

The fluorescence *in situ* hybridization method used in this study is fully described in Chapter 2, Section 2.13.

During the FISH procedure, total bacterial numbers were determined by hybridization with the EUB338 mix probe, labelled with the fluorochrome CY5; while AOB were detected by hybridization with a mixture containing four probes labelled with 6-FAM, the general probe for AOB Nso 1225, and the specific probes NEU and 6a192, with unlabelled competitor probes CTE659 and C6a192, used to target respectively the most halophilic and halotolerant AOB and the *Nitrosomonas oligotropha* lineage.

Probes Ntspa662 and unlabelled competitor CNTspa662 were used to target the genus *Nitrospira* and the probe NIT3 and unlabelled competitor CNIT3 were used to target the genus *Nitrobacter*. Probes targeting the genus *Nitrospira* were labelled with the fluorochrome CY3, when mixed with probes targeting either AOB, or the genus *Nitrobacter* labelled with 6-FAM, while probes targeting the genus *Nitrobacter* were labelled with CY3 when mixed with probes targeting AOB labelled with 6-FAM.

Each mounted slide also included one positive control, hybridized with the general Eub338mix probe, targeting all Eubacteria; and two negative controls, one containing no probe in order to account for autofluorescence in the sample, and the other containing a non-sense antiEub probe, to account for non specific probe binding. The negative controls with no probe and hybridized with the probe antiEub were used to set up average thresholds to be subtracted from the signal obtained in hybridization reactions with the Eub338 mix, before the collection of images from the fluorescence conferred by the group-specific probes.

Ten fields of view (FOV) were randomly selected to be counted on each slide and images were recorded in three separate channels: red, green and blue (RGB colour system), using a Confocal Scanning Laser Microscopy (CLSM). Z-stack images obtained by CLSM were analysed using the daime digital image analysis software (Daims *et al.*, 2006a) using the visualizer tool, which allowed the red and blue or green and blue channels to be combined to count cells stained by both the general EUB338 mix probe and cells binding the specific probes used in this study, that is, targeting all AOB, *Nitrospira* or *Nitrobacter*. Cells were directly counted on the visualizer screen tool in daime, and/or also using the software Microsoft[®] Paint Version 5.1.

The total number of cells/mL was calculated according to the formula described in Chapter 2, and theoretical detection limits for FISH were estimated around 10^3 cells/mL. The cell counts obtained through FISH were compared to the real-time PCR results obtained for the corresponding replicate samples. To do this AOB *amoA* gene abundance and NOB 16S rRNA gene abundance were converted into cell numbers, assuming that on average two bacterial *amoA* copies exist per AOB cell, based on the number of copies reported for *Nitrosomonas europaea* (MacTavish *et al.*, 1993), and only one rRNA operon per cell in *Nitrospira* and *Nitrobacter*-like bacteria, respectively (Klappenbach *et al.*, 2001).

The AOB biomass in the sludge from the Humber and Grangemouth refineries were theoretically estimated using a nitrification model (Rittmann *et al.*, 1999) and compared to biomass values determined from the FISH data. Both the theoretical AOB biomass (X_{aob}) predicted by the model and the AOB biomass (X_{aob}) determined from FISH data were related to one another to test if they were in agreement. See Chapter 2 (Section 2.16) for details of the calculations and the nitrification model.

6.3 Results and Discussion

6.3.1 Quantification of *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* 16S rRNA gene abundance

Three real-time PCR assays were conducted to quantify 16S RNA gene abundance of the nitrite oxidizing bacteria (NOB) *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter*. PCR efficiencies and regression data of each real-time PCR assay are summarized in a Table in Appendix C.

All the three NOB, *Nitrospira* type I and type II and *Nitrobacter* were found in the five refinery wastewater treatment plants investigated in this study.

Overall 16S rRNA gene abundance for *Nitrospira* type I across the refinery wastewater treatment plants ranged from 10^4 to 10^{10} gene copies/mL, while *Nitrospira* type II gene abundance ranged from 10^4 to 10^8 gene copies/mL, and *Nitrobacter* gene abundance which varied most from below the detection limit of the qPCR assay (10^3 cells/mL; See Table C1; Appendix C) up to 10^7 gene copies/mL (see Figures 6.1, 6.2 and 6.3).

The highest 16S rRNA gene abundance for *Nitrospira* type I was found in the Lindsey reactors (ranging from 8.66×10^8 to 1.03×10^{10} gene copies/mL; Figure 6.1), followed by Grangemouth (from 6.75×10^7 to 1.86×10^9 gene copies/mL; Figure 6.3), Humber (from 2.02×10^7 to 2.34×10^9 gene copies/mL; Figure 6.2), Pembroke (1.61 and 1.89×10^5 gene copies/mL in reactors A and B, respectively; Figure 6.1) and lastly, the Eastham reactor (1.38×10^4 gene copies/mL; Figure 6.1).

Similarly, *Nitrospira* type II 16S rRNA gene abundance was also higher in the Lindsey plant (1.91 to 7.83×10^7 gene copies/mL; Figure 6.1), followed by the Humber plant (from 2.76×10^6 to 1.29×10^8 gene copies/mL; Figure 6.2), and ranged from 2.15×10^6 to 8.85×10^6 gene copies/mL in the Grangemouth reactors (Figure 6.3). Their abundance was lower in the reactors in Eastham (6.01×10^4 gene copies/mL; Figure 6.1) and Pembroke (1.83×10^5 and 2.46×10^5 gene copies/mL; Figure 6.1).

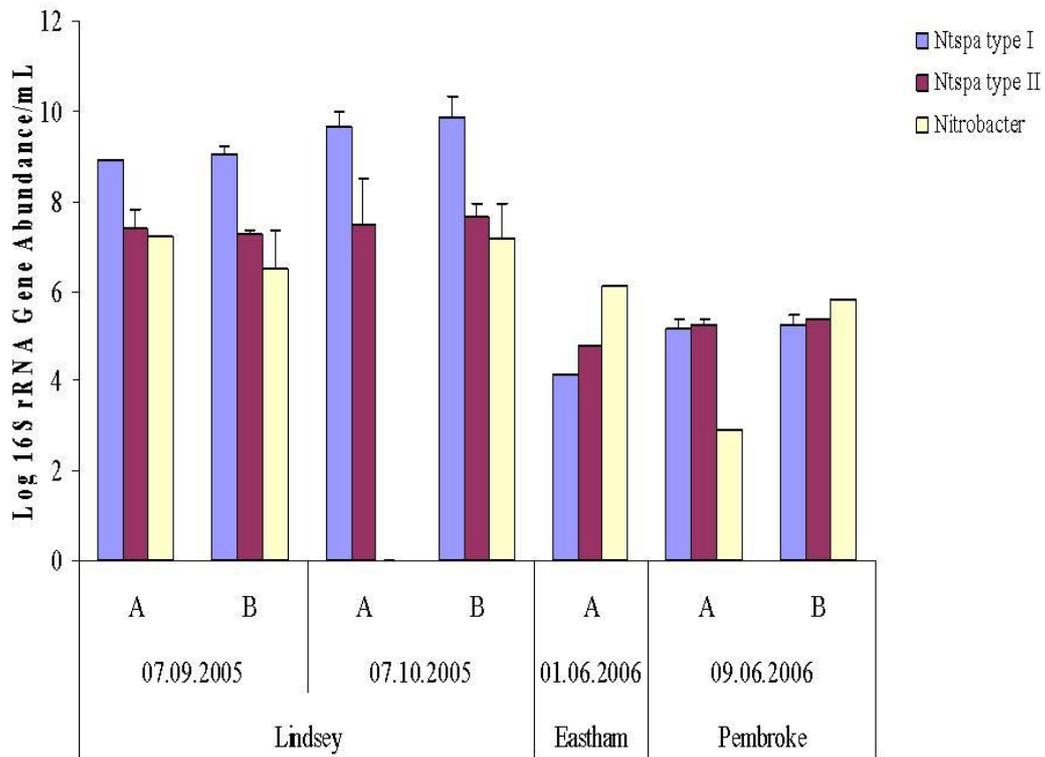


Figure 6.1 The 16S rRNA gene abundance for *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* in wastewater treatment reactors A and B from the Lindsey, Eastham and Pembroke refineries. Error bars correspond to standard deviation of the mean of two or three replicate samples.

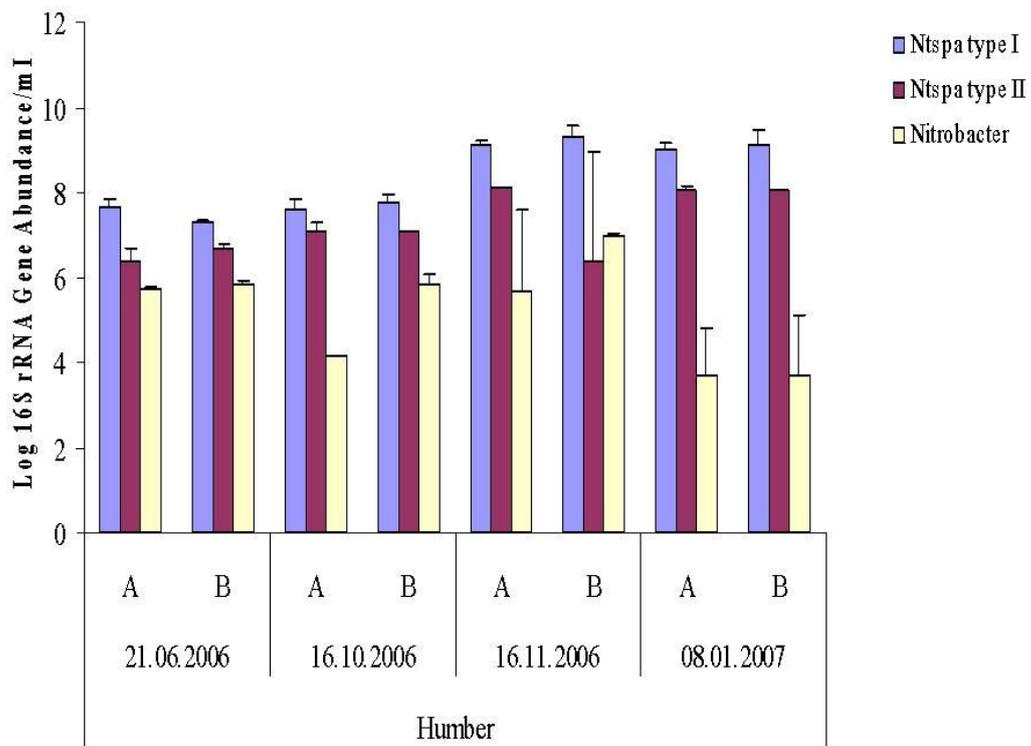


Figure 6.2 The 16S rRNA gene abundance for *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* in wastewater treatment reactors A and B from the Humber refinery sampled on four different occasions. Error bars correspond to standard deviation of the mean of two or three replicate samples.

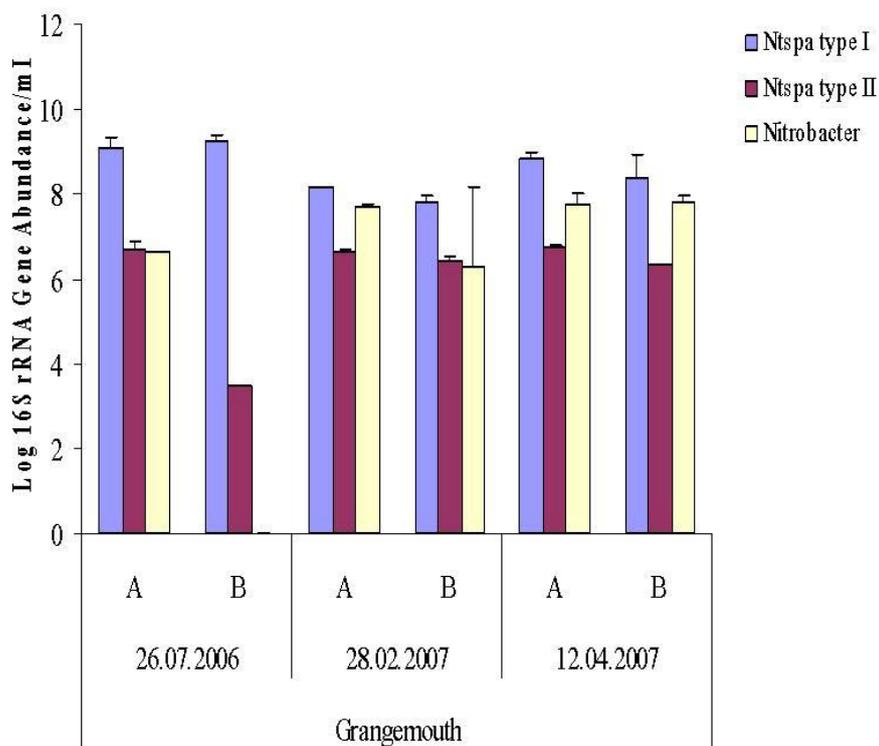


Figure 6.3 The 16S rRNA gene abundance for *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* in wastewater treatment reactors A and B from the Grangemouth refinery, sampled on three different occasions. Error bars correspond to the standard deviation of the mean of two or three replicate samples.

In contrast, between two sampling dates in September and October 2005, *Nitrobacter* gene abundance in the Lindsey reactors was around 10^6 gene copies/mL in reactor A and variable in reactor B (ranging from below detection limits (10^3 ; Table C1, Appendix C) to up around 10^7 gene copies/mL). In the Eastham and Pembroke reactors, which were not nitrifying, numbers were lower and also more variable, ranging between 10^2 to 10^5 gene copies/mL, and between 10^2 up to 10^6 gene copies/mL in the Humber reactor. The highest *Nitrobacter* abundance was found in Grangemouth (around 10^7 gene copies/mL; Figures 6.1, 6.2 and 6.3), with the exception of reactor B sampled in July of 2006, where no *Nitrobacter* were detected.

Overall 16S rRNA gene abundance of *Nitrospira* type II was one or two orders of magnitude lower than *Nitrospira* type I (see Figures 6.1, 6.2 and 6.3). In most cases, *Nitrobacter* gene abundance was lower than *Nitrospira* types I and II gene abundance. *Nitrobacter* gene abundance was higher than *Nitrospira* type I (1.34×10^4 gene copies/mL) and type II (6.01×10^4 gene copies/mL) in the Eastham (8.77×10^5 gene copies/mL; Figure 6.1) and Pembroke reactors B (3.23×10^5 gene copies/mL versus 1.89×10^5 for type I and 8.23×10^4 for type II, respectively), and also higher than *Nitrospira* type II, in Grangemouth sampled on February and April of 2007 (Figure 6.3).

In contrast to the NOB 16S rRNA abundance found in the refinery reactors, *Nitrobacter* predominated (2.06×10^6 gene copies/mL) over *Nitrospira* types I and II in the BLC pilot reactor sample (1.27×10^4 and 8.24×10^3 gene copies/mL respectively; Figure 6.4).

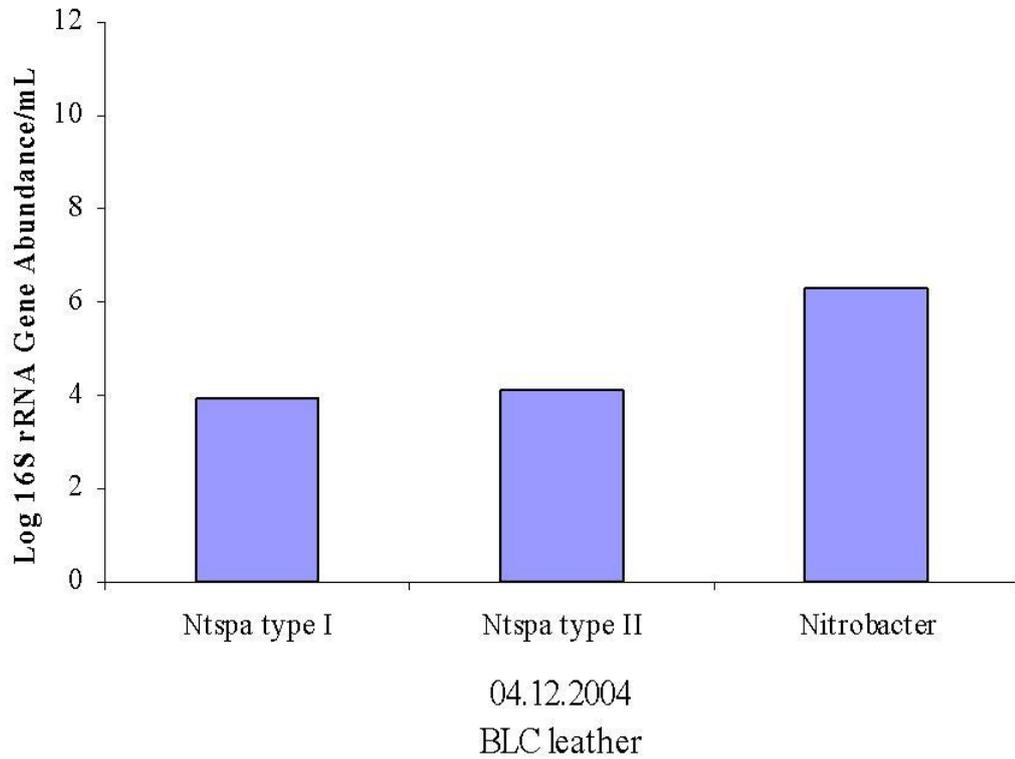


Figure 6.4 The 16S rRNA gene abundance for *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* in one single replicate sample from the BLC pilot reactor

The 16S rRNA gene abundance for *Nitrospira* seemed to be very high in some cases compared to other values reported in the literature for other municipal and industrial wastewater treatment plants. For example, *Nitrospira* abundance was $7 \pm 3.2 \times 10^7$ cells/mL in mixed liquor collected from 12 municipal WWTPs (Harms *et al.*, 2003). In 12 samples from another municipal wastewater treatment plant and three samples from an industrial chemical manufacturing plant (Dionisi *et al.*, 2002) *Nitrospira*-like bacteria determined through competitive PCR were found in similar numbers. In the municipal plants they ranged from 5.82×10^6 to 5.47×10^7 gene copies/mL and in the industrial wastewater treatment plant samples they ranged from 8.4×10^6 to 4.29×10^7 gene copies/mL.

In samples taken from seven WWTPs (Geets *et al.*, 2007) including hospital and municipal sewage, paper, pharmaceutical, chemical, food, and two specialized bench reactors, an OLAND and an ABIL (ammonia binding inoculum liquid), a mixed enrichment of nitrifying cells in suspension, *Nitrospira* abundance ranged from $1.1 \pm 5.6 \times 10^1$ gene copies/mL in the WWTPs treating pharmaceutical wastes, to up 10^7 gene copies/mL in the municipal WWTP, and in two other industrial WWTPs treating respectively paper and food wastes. Higher *Nitrospira* abundances also were found in the WWTP treating hospital sewage and in the ABIL inoculum (in between of 4.7 and 6.6×10^5 gene copies/mL). In contrast, lower *Nitrospira* abundances were found in the OLAND reactor ($5.4 \pm 3.7 \times 10^2$ gene copies/mL) and in another WWTP also treating paper wastes ($4.8 \pm 3.1 \times 10^3$ gene copies/mL). In the same study, *Nitrobacter* abundance was higher than *Nitrospira* abundance in the hospital sewage ($3.6 \pm 1.6 \times 10^6$ gene copies/mL), and in the ABIL inoculum ($1.0 \pm 1.1 \times 10^6$ gene copies/mL), while it was very low in the WWTP treating pharmaceutical wastes ($1.1 \pm 0.2 \times 10^3$ gene copies/mL), and it was not detected in the rest of the WWTPs (Geets *et al.*, 2007).

The primer sets used in this study to quantify *Nitrospira* type I and type II abundances (See Table 2.10; Section 2.15.1, Chapter 2) may target other non-target groups containing no mismatches to the primers sequences; however, this is a very low percentage (0.5%) compared to the high number of sequences related to *Nitrospira* which are targeted by these set of primers. The forward primers used to target respectively *Nitrospira* type I and *Nitrospira* type II targeted 98% of the sequences related to *Nitrospira* in the SILVA database, while their reverse primer pairs target 94% of the sequences related to the target *Nitrospira* group.

When evaluating the specificity of the primers for *Nitrospira* quantification in the studies mentioned above (Harms *et al.*, 2003; Dionisi *et al.*, 2002; Geets *et al.*, 2006), which also have used the same primer sets, the data showed that the forward primer NSR-113F also had high specificity: of 58 hits (98% of the sequences), only one non target organism from the genus *Rhodoflex* was hit by the primer. In contrast, the reverse primer NSR-1264R had low specificity: of 307 sequences, only 77 sequences (25% of the sequences) within the targeted *Nitrospira*-like group were hit by the primer, while the other 207 sequences related to other 14 non target bacterial groups, from which *Acidobacteria* represented 76% of the sequences.

In mixed liquor samples from five other municipal activated sludge WWTPs (Hall *et al.*, 2002), *Nitrospira* abundance ranged between 10^7 and 10^{10} gene copies/ mg MLVSS in three of the plants that were achieving 98.8-99.8% ammonia removal, while the lowest *Nitrospira* abundance (4.4×10^3 gene copies/ mg MLVSS) was found in one plant that was nitrifying poorly (48.1 % ammonia removal). However, there was one plant which was nitrifying efficiently (99.7% ammonia removal) but had low *Nitrospira* abundance (6.0×10^3 gene copies/ mg MLVSS). The authors suggested that either nitrite was removed through other processes such as denitrification, or that NOB numbers had been underestimated due to the specificity of the probes used. However, it could also be possible that *Nitrobacter*-like bacteria were responsible for nitrification, rather than *Nitrospira*, since in that study the authors did not look for other nitrite oxidizers like *Nitrobacter*.

The forward primer NSPTMf, used in the Hall *et al.* (2002) study, targeted 232 sequences in the SILVA database, from which 54% of the sequences were related to *Nitrospira*, while 46% of the sequences were related to other 12 other non target groups (106 sequences), mainly related to *Deltaproteobacteria* (34-36% of the sequences). In contrast, the NSPTMr reverse primer targeted 131 sequences, from which 94% sequences were related to *Nitrospira*, and 6% of the sequences were related to seven other non target bacterial groups. The *TaqMan* probe used in the method was also not so much specific either, and it targeted only 67 sequences related to *Nitrospira* (36%), whereas 121 sequences were related to other non target bacterial groups, also mainly related to *Deltaproteobacteria* (64% of the non target sequences).

When converting the *Nitrospira* abundance obtained in this study into gene copies per mg MLVSS, numbers were slightly lower; however, they were still more comparable with the values reported in the Hall *et al.* (2002) study. *Nitrospira* type I ranged from 4.1×10^6 to 2.8×10^8 gene copies/ mg MLVSS in the Humber reactors and from 6.7×10^7 to 4.3×10^8 in the Grangemouth reactors; while *Nitrospira* type II ranged from 4.2×10^5 to 1.3×10^7 gene copies/ mg MLVSS in Humber and from 4.1×10^5 to 1.2×10^6 gene copies/ mg MLVSS in the Grangemouth reactors. The abundance of *Nitrospira* types I and II abundances was also lower in the plants that were not nitrifying, that is, Eastham and Pembroke (ca. 10^3 to 10^4 gene copies/ mg MLVSS).

In conclusion, although overall *Nitrospira* numbers reported in the studies mentioned above were lower than the *Nitrospira* numbers obtained in this study, which were more comparable to the numbers obtained in Hall *et al.* (2002), the primers used in this study were much more specific than those used in those studies, and thus it is unlikely that these primers would be targeting other non-*Nitrospira* genes and giving a high false positive signal due to the overestimation of the *Nitrospira* abundance results. Therefore, the specificity of the primers used to target *Nitrospira* does not justify the high *Nitrospira* abundance obtained in this study.

In contrast to the results obtained in this study, *Nitrobacter* numbers ($1.8 \pm 0.7 \times 10^8$ cells/mL) were recently found predominating over *Nitrospira* numbers (1.4 ± 10^6 cells/mL) in the aerated basins of a modified Ludzack-Ettinger activated sludge process which received pre-treated wastewater from an oil refinery (Figuerola and Erijman, 2010). Also, overall *Nitrospira* abundance was lower by four orders of magnitude, whereas overall *Nitrobacter* abundance was higher by one order of magnitude than the *Nitrospira* and the *Nitrobacter* abundances found in this study.

In silico specificity evaluation of the primers used for *Nitrobacter* quantification in the studies mentioned before; i.e. Geets *et al.* (2006) and Figuerola and Erijman (2010), which also used the same set of primers; showed that the specificity of the forward primer was high. Of 82 hits, 77 sequences were related to *Nitrobacter* (94%), and only 5 sequences (6%) related to non target bacterial groups. In contrast, the reverse primer targeted 38884 sequences in the SILVA database, and none related to *Nitrobacter*.

Comparison of *in silico* specificity evaluation of the primers with other those studies, showed that the forward primer is more specific for *Nitrobacter* (100%); whereas the reverse primer, despite having a low specificity, targeted 63 sequences (0.8%) of 7785 sequences related to the target *Nitrobacter*. The reverse primer also targeted a large number of other nontarget organisms from other bacterial groups such as *Firmicutes*, *Acidobacteria*, and *Chloroflexi* among many others, having no mismatch to the reverse primer sequence (Table 2.10; Chapter 2). However, in contrast to the other studies, a *Taqman* probe was used in the assay, very specific (100% coverage) for the genus *Nitrobacter*, and this also contributed to increase the specificity of the assay. Therefore, the primers used in this study were more specific than the primers used in the studies mentioned before. An important observation that could be drawn from these comparisons is that, at least in refinery wastewaters, overall *Nitrobacter* abundance seems to be higher than in other types of wastewaters, and perhaps it might be also the case for the *Nitrospira* abundance found in this study, predominating over *Nitrobacter* in a low ammonia system.

The population sizes of NOB found in this study were also higher than the AOB *amoA* population size. *Nitrospira* type I abundance across the refinery WWTP reactors was one to five orders of magnitude higher than AOB abundance. *Nitrospira* type I abundance was three to four orders of magnitude higher than the AOB *amoA* abundance in the Lindsey reactors; one to five orders of magnitude higher in the Humber and Grangemouth reactors; and one to two orders of magnitude higher in the Eastham and Pembroke reactors. *Nitrospira* type II and *Nitrobacter* abundances were also higher than AOB *amoA* abundance by one to two orders of magnitude in all reactors.

In the literature, NOB abundance varied, and it has been found to be either lower or higher than AOB abundance. For instance, in some studies, NOB/AOB ratios ranged from 0.75 (Daims *et al.*, 2001b) to up 4.86 (Harms *et al.*, 2003), whereas in others, *Nitrospira* abundance has been found to be higher than AOB abundance by one to three orders of magnitude, while *Nitrobacter* abundance has been found to be higher than AOB abundance by one to two orders of magnitude (Schramm *et al.*, 2000; Geets *et al.*, 2007; Gieseke *et al.*, 2001).

In the Figuerola and Erijman (2010) study, AOB and NOB abundances were investigated during periods of unstable and fully nitrification. Interestingly, the AOB/NOB ratio was higher than 1 during unstable nitrification, whereas it shifted up to 3.6 at nitrification failure conditions in the WWTP ; and shifted down to 0.5 during full nitrification, i.e. when NOB abundance was twice the AOB abundance. In addition, *Nitrobacter* also predominated in all periods investigated, whereas *Nitrospira* appeared only during periods of fully nitrification; however, always in much lower numbers than *Nitrobacter*.

On the basis of the model (Rittmann *et al.*, 1999) used in this study (see Chapter 5) the predicted AOB numbers are low and this would imply that NOB numbers would also be low. Taking into account the AOB yields assumed by the model - that for each 1 mg of ammonia that has been consumed, the equivalent of 340 μg of new AOB cells and 40 μg of new NOB cells is produced (Rittmann and McCarty, 2001) - if all the nitrite being produced had been oxidized to nitrate by the NOB population, that would be equivalent to the NOB biomass predicted by the model, and the expected population size of NOB would range from 10^2 to 10^4 cells/mL in the Humber, and from 10^2 to 10^5 cells/mL in the Grangemouth reactors. Therefore the expected NOB numbers would be much lower than the actual NOB numbers found in this study.

However, both *Nitrospira* and *Nitrobacter* are able to grow mixotrophically, and thus the higher NOB abundance found in this study could also be explained by the fact that NOB are doing something other than oxidizing nitrite, and possibly growing mixotrophically.

Mixotrophic growth of *Nitrobacter* (Steinmuller and Bock, 1976; Bock *et al.*, 1983) and *Nitrospira* (Erlich *et al.*, 1995; Daims *et al.*, 2001a) were demonstrated in some studies, and they are also supported on the basis of the complete genome sequencing of *Nitrobacter winogradskyi* (Starkenburger *et al.*, 2006); *Nitrobacter hamburgensis* X14 and *Nitrobacter* sp. strain Nb-311A (Starkenburger *et al.*, 2008); and the “*candidatus Nitrospira defluvii*” (Lücker *et al.*, 2010). Furthermore, maximum specific growth rate and cell yields in NOB can be stimulated by mixotrophic growth (Prosser, 1989). For example, increased biomass yields (by a maximum of 48%) were obtained in *Nitrobacter* strains cultures growing in the presence of filtrates from a number of heterotrophs grown in yeast extract-peptone solution (Steinmuller and Bock, 1976).

The different metabolic properties of *Nitrobacter* and *Nitrospira* may also contribute to their coexistence in the refinery wastewater treatment reactors studied here. The coexistence of *Nitrospira* type I, *Nitrospira* type II and *Nitrobacter* in the reactors investigated in this study may be explained on the basis of the ecological K-r strategy model (Andrews and Harris, 1986), which postulates that there are two types of kinetic strategies that can be taken by organisms competing for the same substrate, where one of the organisms acting as an r-strategist grows rapidly in the presence of high substrate concentrations, while the other population, the K-strategist, is a slower grower, but has higher affinity for the substrate when present in lower concentrations.

The spatial distribution and coexistence of uncultured members of sublineages I and II of the genus *Nitrospira* (Maixner *et al.*, 2006) in biofilms and activated sludge, were also demonstrated to occur as a function of their preference for different nitrite concentrations. At higher nitrite concentrations *Nitrospira* sublineage I was selected and occurred in the immediate vicinity of AOB. In contrast, *Nitrospira* sublineage II, were more abundant in regions where nitrite concentrations were lower, at a greater distance from AOB (no less than 6 μm). As with *Nitrospira* and *Nitrobacter*, *Nitrospira* lineage I could also be classified as an r-strategist growing faster than *Nitrospira* lineage II and with lower affinity for nitrite. In contrast *Nitrospira* lineage II can be considered as a K-strategist, with slower growth and higher affinity for nitrite and thus better adapted to lower nitrite concentrations.

In a sequencing batch biofilm reactor (SBBR) where *Nitrobacter* was found to coexist with *Nitrospira*, although in lower numbers, it was hypothesized that temporarily higher peaks in nitrite concentrations may also favor *Nitrobacter* that outcompete *Nitrospira* when present above a certain abundance (Daims *et al.*, 2001b). This was demonstrated in two parallel chemostats fed at different nitrite concentrations (Nogueira and Melo, 2006). In the beginning of the experiment, the two chemostats were inoculated with a nitrifying activated sludge containing 8.7% *Nitrospira* (measured by FISH as relative cellular area in relation to total bacterial area) and no detectable *Nitrobacter*, and operated under the same conditions for 12 days. Afterwards, a mixed culture inoculum from a laboratory chemostat fed with a mineral medium and containing 53% *Nitrobacter* relative cellular area and no detectable *Nitrospira* was added twice in both chemostats. While one of the chemostats was kept at low initial and constant nitrite concentrations (0.0026 mM), the other chemostat was operated at increasing, high and transient nitrite concentrations (up to 1.73 mM). As a result, in the control chemostat, *Nitrospira* remained the dominant population, whereas in the chemostat operated at high nitrite concentrations, *Nitrobacter* became dominant and excluded *Nitrospira*, which could not be recovered even when nitrite levels were returned to the initial nitrite concentrations.

6.3.2 Relationships between physical-chemical parameters and NOB abundance

In the refinery treatment systems, the abundance of *Nitrospira* type I positively correlated to the abundance of *Nitrospira* type II, total bacteria, *Crenarchaeota* soil group 1.1b (Cren soil) and AOA. See Figure 6.5. Interestingly, *Nitrospira* types I/II and AOA/Cren soil rates also correlated positively; and the highest *Nitrospira* abundance also coincided with the highest AOA and Cren soil abundances in Humber and Grangemouth (ca. 10^8 gene copies/mL), thus suggesting they might also be using other substrates besides oxidizing nitrite. As seen in Chapter 5, Cren soil positively correlated with BOD and COD removal.

In contrast, *Nitrobacter* abundance positively correlated with AOB *amoA* abundance. This may indicate *Nitrobacter* is oxidizing nitrite which has been produced by AOB in close proximity with AOB cells, since at higher AOB abundance, overall levels of ammonia oxidation are higher and thus it is likely that more nitrite is being delivered to NOB.

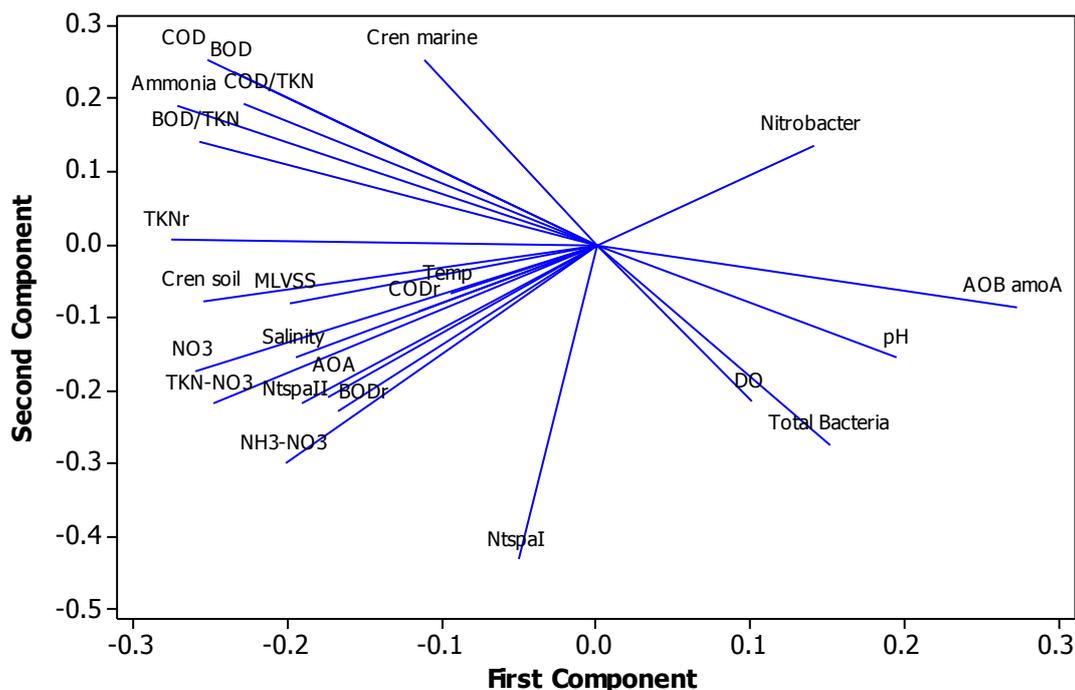


Figure 6.5 PCA diagram. Biplot representation of first and second components of physical-chemical and qPCR abundance data considered in this study. PC1 (45.3%) and PC2 (17.9%) refer to data from Lindsey, Humber and Grangemouth reactors. The projected parameters (eigenvectors) point in the direction of maximum variation. The length of each parameter is equivalent to their rate of change in the dataset. Parameters projected in opposite directions imply negative correlations while positive correlations are implied by parameters projected in the same direction. Oblique angles between two vectors imply in a certain degree of relatedness between vectors. Right angles between vectors show they are independent from one another. Labeled parameters on the biplot: Temperature, DO, pH, MLVSS, Ammonia, TKN, COD, BOD, NH₃-NO₃ = ammonia conversion to nitrate, Nitrobacter, NtspaI= Nitrospira type I, NtspaII= Nitrospira type II, TKNr=TKN removal, CODr= COD removal, BODr= BOD removal, NO₃, AOB *amoA*, total bacteria, AOA, Cren marine= Crenarchaeota from marine group 1.1.a and Cren soil= Crenarchaeota from soil group 1.1b.

6.3.3 *In situ* detection, abundance and morphology of AOB, *Nitrospira*, and *Nitrobacter* investigated through fluorescence *in situ* hybridization (FISH)

Numbers obtained for AOB using FISH found in the selected replicate samples from Eastham and Pembroke refineries were 6.1×10^4 and 1.04×10^3 cells/mL respectively. In samples from the Humber refinery AOB numbers ranged between 5.13×10^5 cells/mL in October 2006 to 2.57×10^6 cells/mL in January 2007. In the Grangemouth refinery AOB were present at 2.15×10^5 cells/mL and 3.05×10^5 cells/mL, on two sampling occasions, July 2006 and April 2007 (See Figure 6.6.a). FISH counts for the genus *Nitrospira* (Figure 6.7.a) were 1.97×10^4 cells/mL and 5.2×10^3 cells/mL in the Eastham and Humber reactors respectively; they ranged from 2.03×10^5 cells/mL in the Humber reactor in June 2006 to 5.70×10^6 cells/mL in November 2006, and in the Grangemouth refinery samples, they were present at 1×10^7 cells/mL in July 2006 and 3.57×10^6 cells/mL in April 2007, respectively. In contrast, *Nitrobacter* (Figure 6.8.a) reached 8.9×10^4 cells/mL in Eastham, 1.04×10^3 cells/mL in Pembroke; and ranged from 7.44×10^5 cells/mL to 4.60×10^6 in the Humber reactor, and around 10^5 cells/mL in the Grangemouth reactor. See also Table summarizing the FISH count data in Appendix C.

Depending on the complexity of environmental samples, as well as the distribution and arrangement of target microbial cells in flocs and biofilms, it can sometimes be difficult to count cells using FISH (Daims and Wagner, 2007). In this study, as the number of the target cells per field of view counted using FISH were low and their spatial arrangement and distribution was relatively simple (i.e. mostly individual cells or very small aggregates randomly spread over flocs), it was relatively easy to visualize and count the target cells in the refinery wastewater samples using the visualizer tool in the daime software, in which images of the total bacterial population and the specific populations of interest could be split or combined in different channels and then the cells directly counted on the computer screen either with visualizer and/or using the software Microsoft[®] Paint Version 5.1.

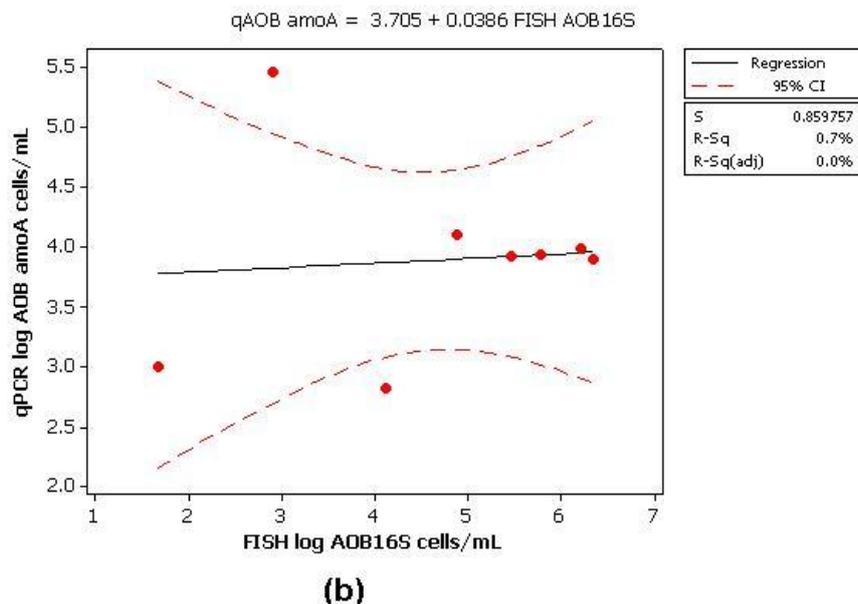
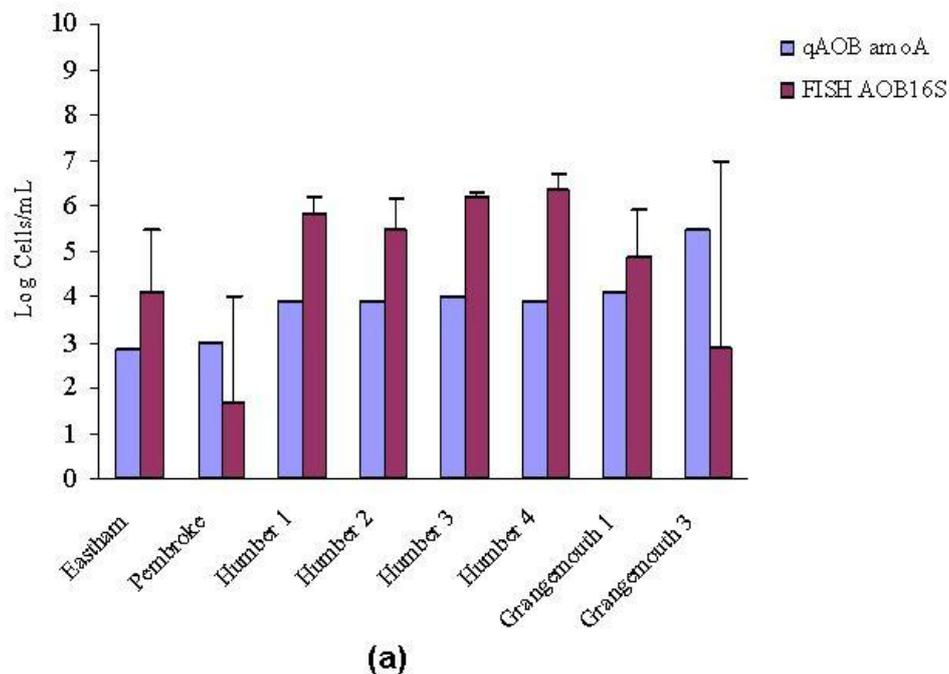
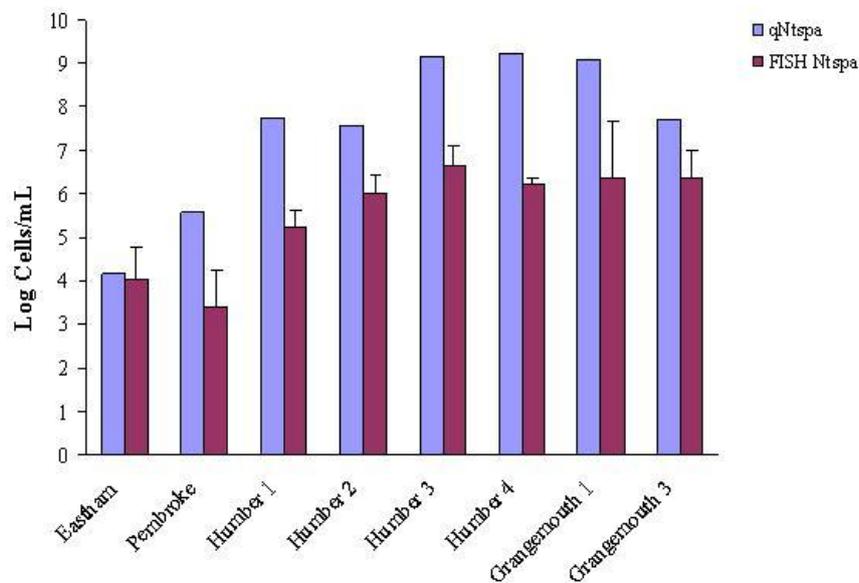
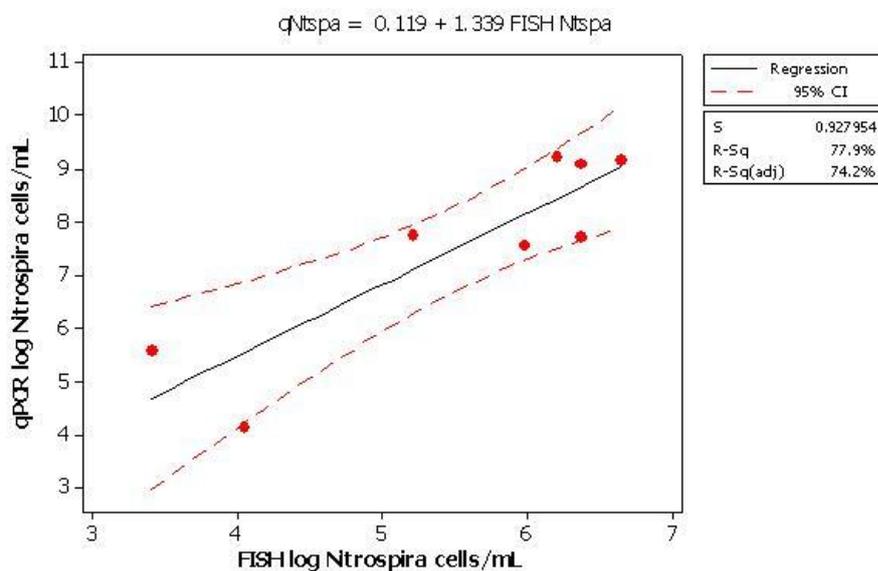


Figure 6.6 a) Comparison and b) relationship between AOB cell numbers obtained through real-time PCR of *amoA* genes and FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study. Error bars correspond to standard deviation of the mean of two replicate samples.

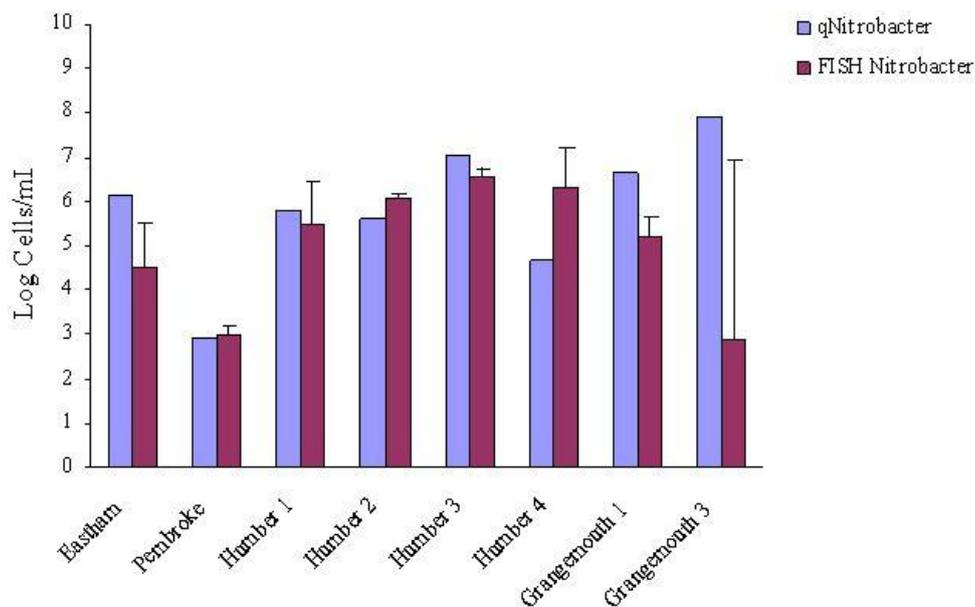


(a)

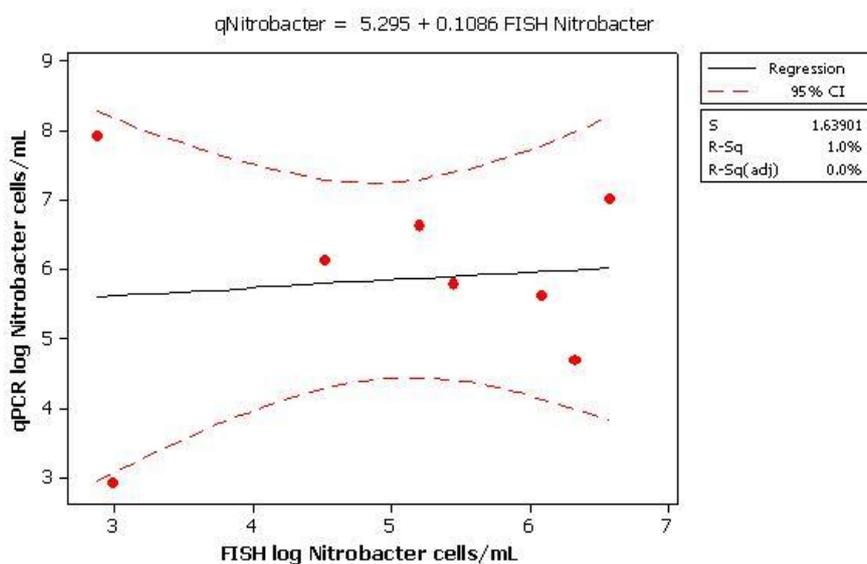


(b)

Figure 6.7 (a) Comparison and (b) relationship between cell numbers of *Nitrospira* types I plus type II obtained through real-time PCR of 16S rRNA genes and the cell numbers of the genus *Nitrospira* obtained through FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study. Error bars correspond to standard deviation of the mean of two replicate samples.



(a)



(b)

Figure 6.8 a) Comparison and b) relationship between *Nitrobacter* cell numbers obtained through real-time PCR of 16S rRNA genes and FISH in eight replicate samples selected from the activated sludge systems of four refinery wastewater treatment plants investigated in this study. Error bars correspond to standard deviation of the mean of two replicate samples.

FISH analysis was not possible with samples collected from the Lindsey refinery due to an excessive autofluorescence caused by microalgae present in the biofilms at the surface of the trickling filter; therefore cell numbers could not be obtained through FISH for the Lindsey reactors. In the sample from the BLC pilot reactor, cell numbers were below the detection limit of FISH (10^2 - 10^3 cells/mL). However, it is important to point out that the sample from the BLC reactor quantified through qPCR was different from the sample that was analysed using FISH, and this also may in part explain the discrepancy for the NOB abundance obtained through real-time PCR (10^3 and 10^4 gene copies/mL for *Nitrospira* types I and II respectively and 10^6 gene copies/mL for *Nitrobacter*; Figure 6.4). Another factor that also may have interfered was the high autofluorescence found in these samples. When the average fluorescence threshold from the negative control was subtracted from the positive control, target total bacterial numbers were very low, which contrasts with what was found for the analysis of 16S rRNA genes from total bacteria obtained through real-time PCR (10^9 gene copies/mL; See Figure 5.4, Chapter 5. In the Bio-Rad instruments, the fluorescence signal variation from well to well in the reaction plate is normalized using a second plate with a fluorescein dye prior to the real-time run, and thus it is not necessary the use of a internal reference dye in each master mix reaction on the experimental plate. However, if a sample or a replicate DNA standard that had been previously exposed to UV light, which would cause the DNA degradation and the leaving in the reaction solution of any compound that might fluoresce, had also been included in the assays and run along with the samples and the standards; it would had been one more control factor to be compared with the non template control sample (NTC), samples and the standards, also contributing to monitor autofluorescence levels intra-assay.

In the refinery activated sludge samples, AOB and NOB cell numbers obtained through FISH were detected at 10^3 and 10^4 cells/mL, respectively, in the Eastham and Pembroke refineries which had poor nitrification; and ranged from 10^5 to 10^6 cells/mL in Humber. In the Grangemouth reactor *Nitrospira* were one to two orders of magnitude higher than AOB while *Nitrobacter* were the same order of magnitude as AOB (10^5 cell/mL); see Figures 6.6.a, 6.7.a and 6.8.a. AOB numbers also positively correlated with *Nitrospira* ($r= 0.882$; $p= 0.004$) and *Nitrobacter* numbers ($r= 0.959$; 0.000).

This is also in line with other studies reflecting the syntrophic relationship that exists between these two nitrifying groups. (Schramm *et al.*, 1996; Mobarry *et al.*, 1996; Schramm *et al.*, 1998; Juretschko *et al.*, 1998; Schramm *et al.*, 1999; Okabe *et al.*, 1999; Daims *et al.*, 2000; Kowalchuck and Stephen, 2001; Coskuner and Curtis, 2002; Gieseke *et al.*, 2003). In some cases, AOB as well as *Nitrospira*-like NOB populations are also found in smaller numbers within deeper, anoxic layers in some biofilms.

In the literature AOB abundance in WWTPs or bioreactors occurs at levels around 10^7 cells/mL, but may range between 10^5 and 10^8 cells/mL, in flocs and biofilms (Daims *et al.*, 2001c; Coskuner *et al.*; Pickering, 2008; Kindaichi *et al.*, 2006), and in one phosphate-removing biofilm reactor was found to reach up to 10^9 cells/cm³ (Gieseke *et al.*, 2001).

Overall *Nitrospira*/AOB ratios across reactors in this study were respectively 0.32 in Eastham; 5 in Pembroke, and ranged from 0.27 to 3.47 in Humber; while *Nitrobacter*/AOB ratios were 1.46 in Eastham, 1 in Pembroke, ranged from 0.28 to 2.36 in Humber and were about 1 in Grangemouth. In comparison to the NOB/AOB ratios obtained based on qPCR quantification, NOB/AOB ratios on the basis of FISH quantification were much lower and also were similar to other NOB/AOB abundance ratios reported in the literature. In contrast, AOB numbers obtained through FISH were also one to three orders of magnitude higher than AOB *amoA* numbers obtained through qPCR (Chapter 5).

Numbers obtained through FISH for AOB were applied to the nitrification model to see if there was any agreement between theoretical estimated biomass and AOB biomass obtained through FISH (Table 6.1 and Figure 6.9). However, both measurements systematically disagree, and the model also overestimated the measured AOB biomass obtained through FISH.

However, an important conclusion that may be inferred from these results is that the model predicts low numbers of AOB, which AOB numbers measured by qPCR and FISH broadly agree with. Furthermore the low AOB numbers are also consistent with the low ammonia levels found in the refinery treatment systems, and therefore nitrifying populations tend to be rather low.

Table 6.1 Theoretical biomass predicted using a single nitrification model (Rittmann *et al.*, 1999) and measured AOB biomass obtained through FISH quantification in the Humber and Grangemouth reactors

WWTP/Reactor	Sampling date	Sludge age (Θ_x) Days	AOB biomass Predicted by model (X_{aob}) (mg/cm^3)	AOB cells measured through FISH converted into biomass (mg/cm^3)
Humber A	21.06.2006	48	2.4×10^{-3}	2.8×10^{-4}
Humber A	16.10.2006	38	2.4×10^{-3}	1.6×10^{-4}
Humber A	16.11.2006	37	2.1×10^{-3}	7.3×10^{-4}
Humber A	08.01.2007	60	1.5×10^{-3}	1.0×10^{-3}
Grangemouth A	26.07.2006	25	1.6×10^{-3}	1.3×10^{-4}
Grangemouth A	12.04.2007	17	6.2×10^{-4}	1.9×10^{-4}

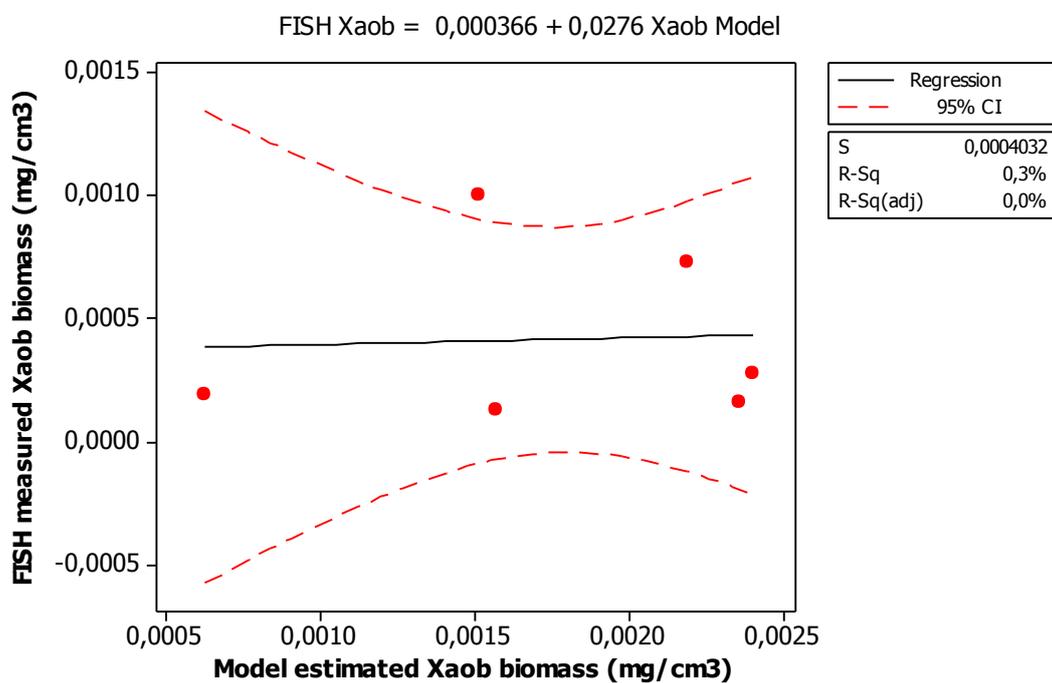


Figure 6.9 Relationship between the theoretical biomass predicted in the Humber and Grangemouth reactors using a single nitrification model (Rittmann *et al.*, 1999) and measured AOB biomass obtained through FISH quantification. X_{aob} = AOB biomass

Typically in WWTPs or bioreactors, AOB are found arranged within colonies, forming dense layers of large spherical ball-shaped microcolonies, reaching up to 50 μm in diameter, surrounded by and in close proximity to smaller, and more irregular and less dense clusters of *Nitrospira* or *Nitrobacter*-like NOB. In stable nitrification reactors with high nitrification efficiency, colonies may also contain thousands of AOB cells. For instance, in an industrial plant treating animal wastes, up to 3×10^3 rod-shaped cells densely packed within AOB colonies could be found in large colonies of 20 μm diameters (Wagner *et al.*, 1995).

Differences also were observed in relation to the *Nitrospira* microcolony sizes and shapes between biofilm and the activated sludge plants. For instance the smallest and the largest microcolony diameter of active *Nitrospira* investigated in four wastewater treatment plants (two biofilm plants and two activated sludge plants; Daims *et al.*, 2001a) reached respectively 4.9 and 38.1 μm and 1.6 and 31.7 μm in the two biofilm plants; while in contrast, in the activated sludge plants the average diameter of *Nitrospira* microcolonies was respectively $2.8 \pm 1.6 \mu\text{m}$ (with a minimum of 0.9 μm and a maximum of 7.5 μm); and $3.9 \pm 2.3 \mu\text{m}$ (with a minimum of 1.0 μm and a maximum of 13.6 μm). *Nitrospira* microcolonies within the biofilms also had a complex and irregular morphology with internal cavities and a network of cell-free channels, while in the activated sludge, cells were more tightly packed and the aggregates were significantly smaller and were more spherical in shape.

However, in contrast to those studies, typical AOB and NOB microcolonies were not observed in any of the sludges from the refinery wastewater treatment plants analyzed in this study, which instead noted dispersed individual AOB cells or very small aggregates overlain by *Nitrospira* or *Nitrobacter* cells or aggregates (see Figure 6.10). Hence the target cells appear in only a very few fields of view. Moreover despite nitrification occurring in the Humber and Grangemouth plants, the modelling indicated that the nitrifier populations are rather low, and thus although detectable by FISH they do not form the dense aggregates and microcolonies that others have observed in WWTPs (Figure 6.10).

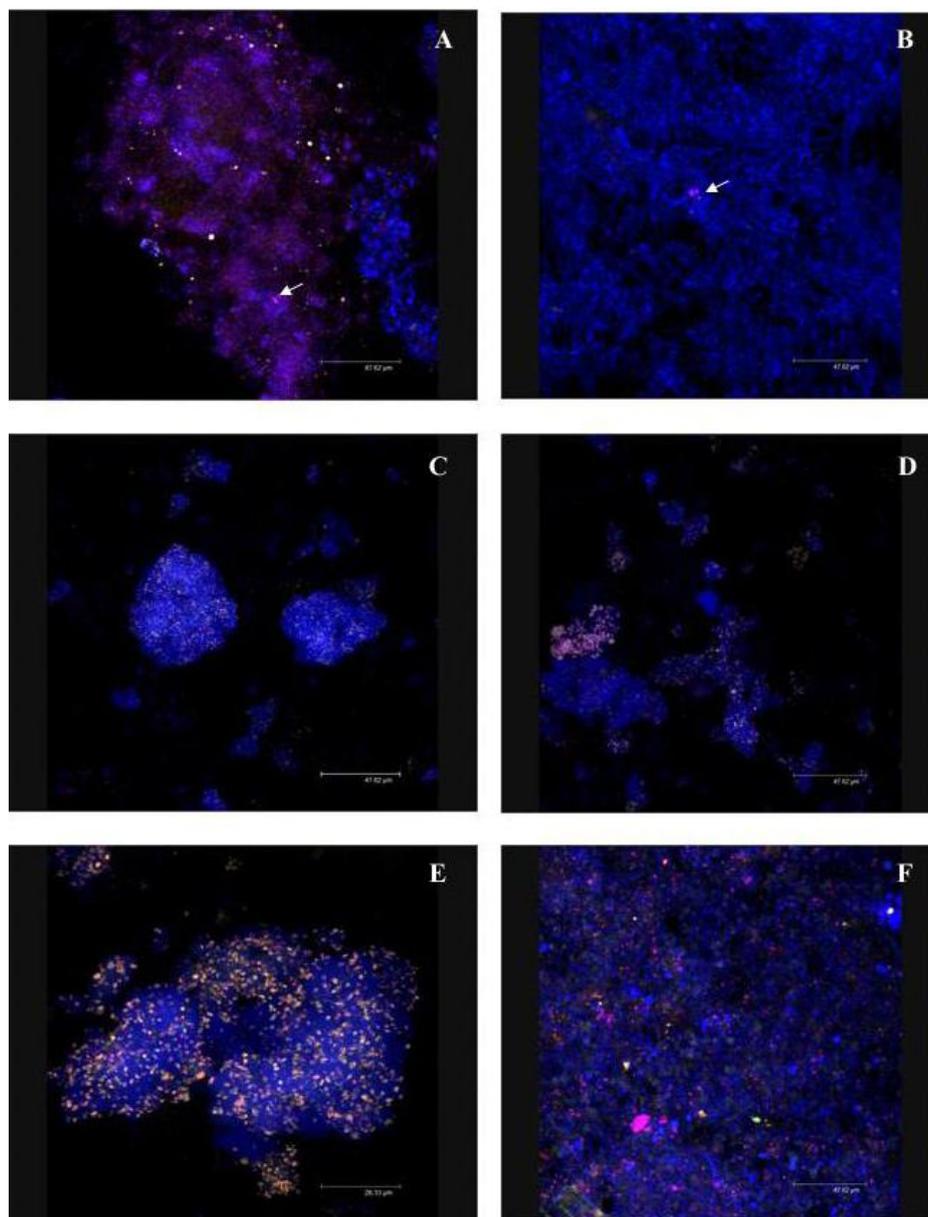


Figure 6.10 Confocal laser scanning Z-stack images (3D projected images composed of 10 optical section images of 0.1 μm thickness, spatially connected along the 2D axis) of simultaneous fluorescence *in situ* hybridization of AOB and *Nitrospira*, AOB and *Nitrobacter* and *Nitrospira* and *Nitrobacter* cells targeted respectively by oligonucleotide probes labelled with 6-FAM and CY3 stains.

Panels A to F, total bacteria targeted exclusively by EUB338 mix probes I, II, III labelled with CY5 are blue. Bacterial cells targeted by both CY5 and CY3 labelled probes are magenta, because of the overlapping of the labels. Similarly, cells stained simultaneously by CY5 and 6-FAM appeared as cyan and cells stained simultaneously by the three stains, CY5, 6-FAM and CY3 appear white.

A. Eastham refinery: AOB (cyan) and *Nitrobacter* cells (magenta) stained respectively by 6-FAM and CY3 pointed out by an arrow. Bar 47.62 μm .

B. Pembroke refinery: AOB (cyan) and *Nitrospira* cells (magenta) stained respectively by 6-FAM and CY3 pointed out by an arrow. Bar 47.62 μm .

C, D, E Humber refinery: AOB (cyan) and *Nitrospira* (magenta) cells (C); AOB (cyan) and *Nitrobacter* (magenta) (D); *Nitrospira* (cyan) and *Nitrobacter* (magenta) (E) stained respectively by 6-FAM and CY3. Bar on panels C and D, 47.62 μm and on Panel E, 28.33 μm .

F. Grangemouth refinery: AOB (cyan) and *Nitrospira* cells (magenta) stained respectively by 6-FAM and CY3. Bar 47.62 μm .

Therefore as expected in the Eastham and Pembroke reactors, that is, the two plants that were not nitrifying, there were very few nitrifying aggregates.

In the Eastham refinery treatment reactor (Figure 6.10.A), *Nitrobacter* was detected forming very small spherical microcolonies. In Figure 6.10.A, the overlap of AOB and *Nitrobacter* cells appears as white areas. *Nitrobacter* cells not associated with AOB appear magenta and are indicated by an arrow. AOB cells not associated with *Nitrobacter* cells would appear cyan but they were not detected in the field of view shown (Figure 6.10.A).

In the Pembroke reactors (Figure 6.10.B), the number of target cells was very low. However, in Figure 6.10.B two single *Nitrospira* cells can be seen coloured magenta due to the overlap of the CY5 label staining total bacteria and the CY3 label staining *Nitrospira* cells; these are indicated by an arrow (Figure 6.10.B).

Real-time PCR analysis indicates that *Nitrospira* types I and II were present in all samples investigated, with type I being predominant in all plants. In FISH analysis *Nitrospira* cells were visualized using a general probe that targets both *Nitrospira* Type I and Type II cells.

Type I or *Nitrospira defluvii* (Spieck *et al.*, 2006) are characterized by short, slightly curved cells or spiral-shaped cells, as well as having a strong tendency for aggregation. They are smaller (0.2-0.4 x 0.7- 1.7 μm) than *Nitrospira moscoviensis* which forms irregularly shaped cells or spiral-shaped rods (0.2-0.4 μm x 0.9- 2.2 μm) and also has the tendency to aggregate, however this much less pronounced than with *Nitrospira defluvii* (Spieck *et al.*, 2006). Likewise, in wastewater treatment systems *Nitrobacter* cells are typically found as single planktonic cells that may aggregate in spherical or irregular aggregates (Nogueira and Melo, 2006).

In the Humber reactors, AOB and *Nitrospira* cells formed irregular or spherical aggregates with *Nitrobacter* and *Nitrospira* cells overlapping. AOB and *Nitrospira*, AOB and *Nitrobacter* and *Nitrobacter* and *Nitrospira* cells found in Humber reactors are illustrated respectively in Figures 6.10.C, 6.10.D and 6.10.E. In Figure 6.10.C, magenta cells represent *Nitrospira*; while cyan cells represent AOB, as a result of the dual label overlap of CY5 stain for total bacteria and 6-FAM for AOB. In Figure 6.10.D, *Nitrobacter* cell aggregates are irregular in form and appear magenta-coloured, while AOB cells stained simultaneously by CY5 and 6-FAM are cyan. Areas that appear white indicate AOB and NOB cells overlaying each other on the Z-stack image.

In the Grangemouth reactors *Nitrospira* appeared as very small individual cells dispersed within flocs or forming very small cell aggregates or even small irregular microcolonies (of 1-20 cells), and AOB cells overlapped with *Nitrospira* cells (Figure 6.10.F). In contrast, *Nitrobacter* appeared as isolated planktonic cells and were not apparently associated with AOB cells. In the Grangemouth reactors (Figure 6.10.F), *Nitrospira* cell aggregates appeared magenta and where AOB and *Nitrospira* cells overlapped they appear white as a result of CY5, CY3 and 6-FAM labels overlapping in the signal of total bacteria, *Nitrospira* and AOB cells. In the Grangemouth reactors in July 2006, real-time PCR demonstrated that *Nitrospira* I was present in high numbers (10^9 cells/mL; Figure 6.3), while *Nitrospira* type II was not detected.

The aggregates of cells detected by FISH were consistent with the description of *Nitrospira defluvii* cells given by Spieck *et al.* (2006). The small rod-shaped cells dispersed in the sludge or clustering in spherical small aggregates (Figure 6.10.F) are therefore likely to represent *Nitrospira* type I cells. It has been suggested that *Nitrospira* sublineage I are adapted to higher nitrite concentrations than *Nitrospira* type II (Maixner *et al.*, 2006). Thus in biofilms and flocs, they occur close to AOB microcolonies with a maximum abundance of 5 μm (Maixner *et al.*, 2006). *Nitrospira* sublineage II, by contrast was most abundant at approximately 30 μm distant from AOB microcolonies (Maixner *et al.*, 2006).

6.3.4 Comparison between real-time PCR and FISH

The two methods used in this study for the quantification of AOB and NOB, that is, qPCR and FISH, were compared to verify if they were in agreement; and thus AOB *amoA* gene copies for AOB and 16S rRNA gene copies for NOB were converted to cells/mL. To convert gene abundance into cell numbers it was assumed that on average two bacterial *amoA* copies exist per AOB cell, based on the number of copies reported for *Nitrosomonas europaea* (MacTavish *et al.*, 1993); and only one 16S rRNA gene operon exists per cell in NOB (Klappenbach *et al.*, 2001).

Both qPCR and FISH have advantages and disadvantages. For example, a higher number of cells estimated using real-time PCR may be explained by the fact that all DNA present on samples may be amplified, independently of whether it has come from active or dead cells; while when using FISH, the most abundant active cells are targeted as a reflection of the number of ribosomes present within cells (Daims *et al.*, 2001b), which in general is higher when cells are active. However, this relationship is not always true for AOB which can maintain high ribosome numbers even after a period of prolonged exposure to starvation or inhibition, and thus indeed their numbers may not reflect active cells (Wagner *et al.*, 1995).

FISH is recognized as the most accurate culture-independent technique applied to the quantification of specific target cells (Coskuner *et al.* 2005, Wagner *et al.*, 2003); however, real-time PCR is faster and easier than FISH and is also sensitive and represents an alternative method to FISH when cell numbers are low, and thus below the FISH detection limits. In addition, real-time PCR may be useful when FISH is hampered by high background fluorescence and non-specific probe binding.

When comparing two methods that perform similar functions (i.e., FISH and qPCR are both used to determine the abundance of particular organisms), if they are comparable one should expect a perfect one to one correspondence between them, that is, if there is an agreement between the two methods, the slope of the line should equate to one and the intercept of the line should be zero.

In this study, the FISH and qPCR data did not agree for any of the target organisms (Figures 6.6.b, 6.7.b and 6.8.b). There was a systematic overestimation by qPCR in relation to *Nitrospira* quantification (Figure 6.7.b), although both measurements were highly correlated, while qPCR and FISH did not relate to each other in relation to either quantification of AOB or *Nitrobacter* cell numbers (Figures 6.6.b and 6.8.b). However, when the replicate sample from the third sampling date in April 2007 (Grangemouth 3) which presented a very high standard deviation value on the FISH data set (See table C3 on Appendix C), is removed from the analysis, the linear relationship between qPCR and FISH used for both AOB and *Nitrobacter* quantification increased, although they still did not agree. The slope of the line of the equation for AOB changed to 0.2462 and r^2 to 59.2%; while for *Nitrobacter* the slope of the line changed to 0.7136 and r^2 to 41.5%. The graphs showing these relationships are included in Appendix C.

In the literature, results comparing real-time PCR and FISH are contradictory. Some studies reported that they were comparable, while others reported the opposite. For example, Kindaichi *et al.* (2006) estimated *Nitrospira* numbers in autotrophic nitrifying biofilms in a rotating disk bench-scale reactor. *Nitrospira* numbers based on real-time PCR were estimated at 3.5×10^8 cells/cm³. Although the actual data were not given, *Nitrospira* cell numbers counted through FISH were reported to be slightly lower; however in the same order of magnitude at 10^8 cells/cm³ (Kindaichi *et al.*, 2006).

Pickering, 2008, also compared both methods for AOB quantification and found no correlation between them (Pearson's correlation coefficient $r=0.054$). In that study AOB measured using FISH in 23 municipal wastewater treatment plants in the UK ranged from 2.67×10^7 cells/mL to 4.5×10^8 cells/mL and were higher than numbers measured through real-time PCR which ranged from 2.48×10^5 to 7.72×10^6 cells/mL, and thus they were up to two orders of magnitude higher than real-time PCR-based cell counts. The discrepancy was attributed to the DNA extraction method adopted, which were not efficient in breaking up the tightly packed AOB microcolonies in the samples (Pickering, 2008).

In respect to the quantification of *Nitrospira*, if numbers obtained through qPCR quantification were overestimated, at least this was not related to the specificity of the primers used in the assay. In the assay, SYBR Green I was used; however, no apparent problems appeared related to the melting curve analysis either. However, to check other factors; for example, related to the quality of the standard; the assay would have to be repeated. In contrast, numbers obtained through FISH for *Nitrospira*, at least appeared to be more sensible and in line with the range of values found in the literature, ranging in most cases between of 10^5 - 10^7 cells/mL for *Nitrospira* and *Nitrobacter*. FISH probes used for the *Nitrospira* and *Nitrobacter* quantification were also very specific (Table 2.8, Chapter 2).

In respect of AOB quantification, AOB numbers obtained through real-time PCR quantification seemed to be more reasonable than numbers obtained through FISH quantification. Comparing AOB biomass measured through both methods to the AOB biomass estimated by the model, although none of them agreed with the model, at least a negative correlation was found between the measured biomass obtained through real-time PCR, whereas, none relationship was found between the AOB biomass measured through FISH and AOB biomass estimated by the model. Another factor that might also have some effect over the quantification by FISH would be the specificity of the general probe Nso1225. Although it is high for the target AOB (80%), the probe also has no mismatches to several other non AOB targets (Table 2.8, Chapter 2).

Despite the fact that the theoretical detection limit range was tentatively estimated for FISH (6.9×10^2 to 6.9×10^3 cells/mL) in three situations, i.e. two counts related to Granegmouth 3 sample for AOB and *Nitrobacter* and one count for AOB in the Pembroke sample, the detection limit was above than the true detection limit value (Table C2, Appendix C) because no cell was found in a total of 10 counted FOV, and thus counting more FOV would be the only way to truly lower the detection limit for counting small numbers of cells.

6.3.5 *In situ* detection, abundance and morphology of 16S rRNA crenarchaeotal groups investigated through DAPI and catalyzed-reporter deposition-fluorescence *in situ* hybridization (CARD-FISH)

The five oil refinery sludges from this study were analyzed by DAPI (4', 6'-diamidino-2-phenylindole) staining and by CARD-FISH by Marc Mußmann at the University of Vienna. Marc Mußmann has kindly allowed his CARD-FISH images (Figure 6.11 and 6.12) to be reproduced here in order to illustrate the 16S rRNA crenarchaeotal morphotypes found in the refinery sludges.

DAPI counts were performed to determine the total number of cells/mL in each of the sludges. Dual staining with DAPI and the probe Cren 1162, specifically targeting the terrestrial group 1.1b from *Crenarchaeota*, which was designed based on the clone sequences retrieved from this study, showed overall crenarchaeotal group 1.1b abundance to be low in relation to total biomass (Figure 6.11).

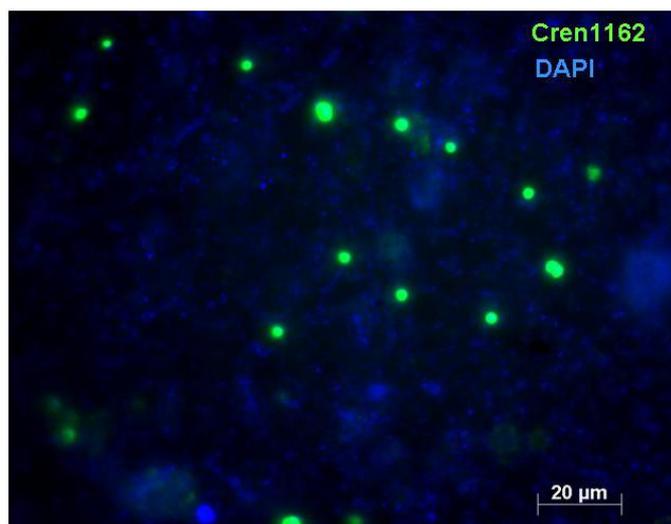


Figure 6.11 Simultaneous *in situ* staining of the Grangemouth refinery sludge with the general DNA stain DAPI and clone specific probe Cren 1162 specifically targeting 16S rRNA sequences from the soil group 1.1b *Crenarchaeota* recovered from the Grangemouth reactor. Cells stained by the probe Cren 1162 appear green, while cells stained by the DAPI appear blue.

Dual hybridizations were also carried out with a general probe targeting most *Crenarchaeota* and a specific probe targeting either marine group 1.1a or soil group 1.1b *Crenarchaeota*. The CARD-FISH procedure and all probes used in the CARD-FISH hybridizations are described in Chapter 2.

In CARD-FISH hybridizations, cells were also stained with probe Cren 512 (Jurgens *et al.*, 2000) which targets most *Crenarchaeota* except extremophilic lineages. This was used in combination with either probe Cren 537 (Teira *et al.*, 2004) which specifically targets the marine group 1.1a *Crenarchaeota*; or probe Cren 1162, which was recently designed specifically to target the soil group 1.1b *Crenarchaeota* (Table 6.2).

The 16S rRNA crenarchaeotal numbers in the oil refinery wastewater treatment reactors and in the sample from the BLC pilot reactor quantified using CARD-FISH were also of the same order of magnitude as AOA numbers obtained using real-time PCR (10^7 - 10^8 cells/mL; see Mußmann *et al.*, 2008 ISME Conference poster in Appendix C).

Both the 16S rRNA sequence data (Mußmann *et al.*, 2008) and the qPCR data showed that the *Crenarchaeota* in some of the reactors were dominated by soil *Crenarchaeota* and in others by marine *Crenarchaeota*.

Both *Crenarchaeota* groups were detected in all reactors through qPCR, and the crenarchaeal soil group 1.1b was shown to predominate in relation to the crenarchaeal marine group 1.1a, except for the BLC pilot reactor. In contrast, through CARD-FISH, both crenarchaeal types were detected in Lindsey, whereas only the crenarchaeal soil group 1.1b was detected and shown to predominate in the Humber and Grangemouth reactors. Only the crenarchaeal marine group 1.1.a was detected in the BLC reactor, and none of the crenarchaeal types were detected in Eastham and Pembroke (Table 6.2). These differences may be related to the sensitivity of the methods. Although marine group 1.1a *Crenarchaeota* abundance was low in most reactors (c.a. $\leq 10^4$ cells/mL; Figures 5.1, 5.2, 5.3, and 5.4 in Chapter 5) they could be detected through qPCR, but not through CARD-FISH.

Table 6.2 The 16S rRNA crenarchaeotal groups detected in the refinery sludges and in the BLC sludge by the probes used in the CARD-FISH hybridizations (Mußmann *et al.*, 2009, unpublished) and comparison to 16S rRNA gene quantification through qPCR in this study for marine group 1.1a and soil group 1.1b *Crenarchaeota*

Plant	Probe Cren 512	Probe Cren 537	Probe Cren 1162	qPCR data	
	Most Crenarchaeota	Marine Cren 1.1a	Soil Cren 1.1b	Marine Cren 1.1a	Soil Cren 1.1b
Lindsey	+	+	+	+	+
Eastham	-	-	-	+	+
Pembroke	-	-	-	+	+
Humber	+	-	+	+	+
Grangemouth	+	-	+	+	+
BLC	+	+	-	+	+

As shown before in Chapter 5, the different primers and probes used to target the crenarchaeal groups 1.1a and 1.1b had a high overlap (Table 2.9; Section 2.15.1; Chapter 2), and thus a much higher agreement would be expected in relation to the most abundant organisms being detected by both methods, as for example in the case of the crenarchaeal group 1.1a in the BLC reactor, and the crenarchaeal group 1.1b in Humber and Grangemouth WWTPs. However, although the overlap between primers and the probes was high, they also differed in their number of targeted sequences. For instance, the number of sequences related to the crenarchaeal group 1.1b was higher than if detected by qPCR, and conversely, higher than the number of sequences related to the crenarchaeal group 1.1a if detected through CARD-FISH. These differences might have been reflected in non detection of the less abundant organisms present in the samples by CARD-FISH, such as the crenarchaeal group 1.1b in Lindsey WWTP and the crenarchaeal group 1.1a, in most reactors when at very low abundances.

In the Humber and Grangemouth samples, CARD-FISH with probe Cren1162, specific to the soil *Crenarchaeota* and probe Cren 512, specific to most *Crenarchaeota*, revealed coccus-shaped cells that mainly occurred in aggregates (Figures 6.12.A and 6.12.B, see arrows on figures). In the Humber sludge, putative soil group 1.1b *Crenarchaeota* reached up to 10^8 cells/L, and represented approximately 11% of the total population stained by DAPI, while in the Grangemouth sludge they accounted for around 1% of the DAPI count (1×10^8 cells/mL).

Two types of *Crenarchaeota* cells were found in the Lindsey reactor. These hybridized either with probe Cren 1162 (green in Figure 6.12.E) or probe Cren 537a (also green in Figure 6.12.F) and belonged respectively to the soil group 1.1b *Crenarchaeota* or marine group 1.1a *Crenarchaeota* (Figures 6.12.E and 6.12.F). These resembled the AOA cells/colonies found in Humber and Grangemouth (Figures 6.12.A and 6.12.B) and BLC (Figures 6.12.C and 6.12.D).

In the Lindsey reactor, the crenarchaeotal abundance of marine and terrestrial clusters was less than 0.1% of total cells (10^7 cells/mL) and in the Lindsey sludge only very few DAPI-stained colonies allowed the discrimination of single cells.

CARD-FISH with probe Cren 512 in the BLC reactor showed two different morphologies: a predominate colony-like morphotype of 5-8 μm in diameter (pointed out by arrows on Figures 6.12.C and 6.12.D) with an abundance of 2×10^7 cells/mL, related to group 1.1a *Crenarchaeota*, and that was stained by both Cren 512 and Cren 537 probes, and accounted for less than 0.5% of total cell numbers; and a second morphotype which also appeared in the BLC sludge and comprised either single or paired cocci like those seen in the Humber samples (Figures 6.12.A and 6.12.B).

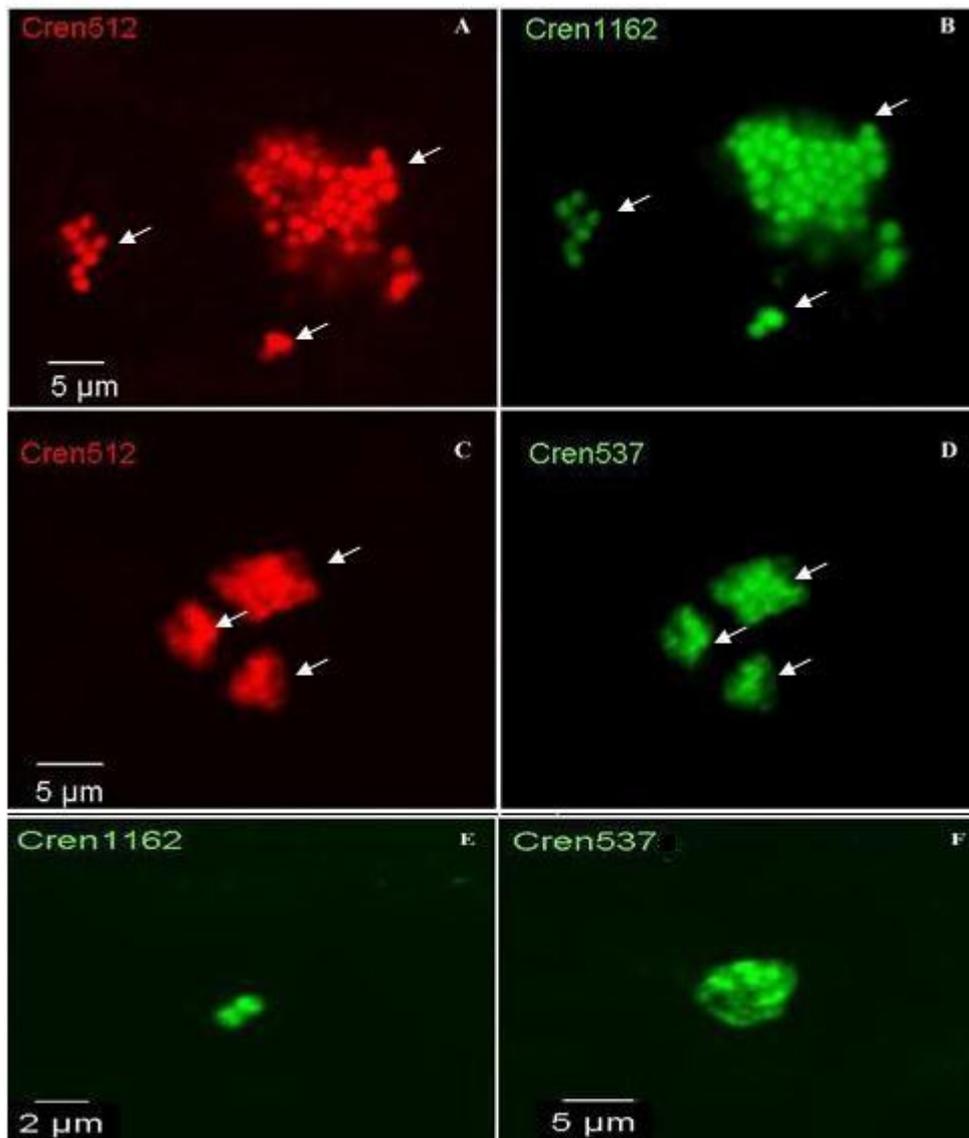


Figure 6.12 CARD-FISH (catalyzed reporter deposition-fluorescence *in situ* hybridization) of crenarchaeotal cells with horse-radish peroxidase labelled probes combined with fluorescently labelled tyramides. Panels A and B illustrate the crenarchaeotal morphotype that was identified in both Humber and Grangemouth reactors respectively by the general probe Cren 512 for *Crenarchaeota* (red) and the specific probe Cren1162 (green) targeting the soil group 1.1b *Crenarchaeota*. Panels C and D show the crenarchaeotal morphotypes found in the sample from the BLC pilot reactor targeted respectively by the general crenarchaeotal probe Cren512 (red) and probe Cren 537 (green) targeting the marine group 1.1a *Crenarchaeota*. Panels E and F show the crenarchaeotal morphotypes found in Lindsey reactors targeting the soil group 1.1b and the marine group 1.1a *Crenarchaeota* using probes Cren 1162 and probe Cren 537 respectively.

It is also interesting that the *Crenarchaeota* detected through CARD-FISH by Marc Mußmann showed the cells grouping into small colonies in samples from the Humber, Grangemouth and BLC reactors (See Figure 6.9).

For instance, the ability of AOB and NOB to grow in clusters and form microcolonies, as is commonly seen in sewage treatment systems, may be an important adaptation contributing to their superior nitrification performance in those treatment systems. Furthermore the microcolony morphology may also help to buffer the bacteria against organic shocks and inhibitors.

A good example of the morphological organization of cells may also be given by the ability of *Nitrospira* to form colonies when resources are abundant and to switch to a planktonic lifestyle when resources are limited. This ability may also represent an important strategy of *Nitrospira* for escaping from unfavourable conditions, thus increasing their chances of survival by enabling them to colonize new habitats, as pointed out by Spieck *et al.* (2008).

Therefore in this context, the arrangement of *Crenarchaeota* in microcolonies may also contribute to their success, for example, in the Humber and Grangemouth refineries (Figure 6.12.A and B) where they were found in higher abundance. Furthermore the absence of AOB colony structures in oil refineries may also contribute to increasing the vulnerability of AOB cells to exposure to chemical pollutants inside reactors, thus affecting the cells' activity as well as their abundance.

6.4 Conclusions

Nitrospira types I and II and *Nitrobacter* were found in all the plants investigated, and their abundance was higher in the plants that were nitrifying, (respectively 10^7 - 10^{10} , 10^6 - 10^8 and 10^6 - 10^7 gene copies/mL) compared to plants that were not nitrifying, such as the Eastham and Pembroke refinery WWTPs (10^2 - 10^5 gene copies/mL).

Nitrospira type I was the most abundant *Nitrospira* in all the plants that were nitrifying, followed by *Nitrospira* type II and *Nitrobacter*.

Nitrospira type I abundance was higher in the Lindsey plant (10^{10} gene copies/mL) in contrast to the plants at Humber (10^7 - 10^9 gene copies/mL) and Grangemouth (10^7 - 10^9 gene copies/mL); and the highest *Nitrospira* type I abundances coincided with the most stable nitrification in the Lindsey, Grangemouth and Humber reactors on the third and fourth sampling occasions in November 2006 and February 2007.

In this study qPCR and FISH were not comparable.

AOB abundances investigated through real-time PCR of *amoA* genes, through modelling and through FISH were all low; this was consistent with the amount of ammonia in refinery WWTPs which is also low as suggested by process data in this study. Interestingly, nitrifying bacteria appear not to form microcolonies effectively. Instead AOB were detected as individual cells dispersed within flocs.

CHAPTER 7

7 General Discussion

The main aim of this study was to investigate which microorganisms are involved in nitrification in full-scale refinery wastewater treatment plants, and in what abundance. Microbial ecology studies of this type of wastewater treatment plant using molecular techniques are very scarce and several valuable findings came from this investigation.

Distinct AOB lineages differ in their physiological properties and these differences directly reflect their ecology and distribution patterns in nature (Koops and Pommerening-Röser, 2001; Stehr *et al.*, 1995; Koops and Pommerening-Röser, 2001). All AOB species oxidize ammonia as their energy source; however, different AOB species have different affinities for ammonia as well different degrees of tolerance to ammonia, since ammonia is also toxic for AOB. On the basis of sensitivities to ammonia there are two types of AOB: one type that is sensitive to high ammonia concentrations and thus thrives better in low ammonia environments; and another type that is insensitive to ammonia and thus is able to thrive in environments with high ammonia concentrations, as well as to tolerate much higher ammonia concentrations (Suwa *et al.*, 1994; Suwa *et al.*, 1997). Different requirements for salt and salt tolerances also exist between different AOB species, as well as the presence or absence of urease activity (Koops *et al.*, 2003).

The refinery wastewater systems had low ammonia concentration (from 0.20 to 1.60 mM) and low salinity (0.1-1%) and thus selected for AOB related to *Nitrosomonas oligotropha* and *Nitrosomonas marina* lineages which are adapted to low ammonia and low salinity and have been detected in a number of low ammonia and low salinity environments (Stehr *et al.*, 1995; Limpiyakorn *et al.*, 2005; Grommen *et al.*, 2005). The majority of sequences retrieved from the Grangemouth reactors were recovered as a group of sequences within the *N. oligotropha* lineage. These sequences were related to sequences specifically retrieved from other wastewater treatment plants and may represent a novel AOB species (Chapter 3).

In contrast to the low ammonia and low salinity conditions found in the refinery wastewater treatment systems in this study, wastewater treatment plants receiving wastes with high ammonia loadings and much higher salinities tend to select for AOB from the *N. europaea-Nitrosococcus mobilis* lineage such as *N. eutropha*, *N. europaea*, *N. mobilis* and *N. halophila* (Juretschko *et al.*, 1998; Daims *et al.*, 2001b; Rowan *et al.*, 2003b; Moussa, 2004; Figuerola and Erijman, 2010). These AOB species have high K_s values (0.030 to 0.061 mM), low affinity for ammonia and tolerance of higher ammonia concentrations. For instance, *N. eutropha* can tolerate the highest ammonia concentrations (600 mM), followed by *N. europaea* and *N. halophila* (400 mM) and *Nc. mobilis* (250 mM); (Koops *et al.*, 2003). The ability of *N. eutropha* to thrive in high ammonia systems has been successfully exploited in reactors sustaining partial nitrification processes (Zart and Bock, 1998; Schmidt *et al.*, 2003).

The AOB species within the *N. europaea-Nitrosococcus mobilis* lineage are also able to tolerate high salt concentrations similar to or even higher than seawater salinities (3.5%). *N. europaea* and *N. eutropha* do not have a salt requirement, but can tolerate high salt concentrations up to 400 mM (2.3%); and in contrast *Nc. mobilis* and *N. halophila* have an obligate, but moderate, salt requirement and can tolerate up to 500 mM (2.9%) and 900 mM (5.2%) NaCl respectively (Koops *et al.*, 2003).

Comparing the ammonia concentrations in the influent to the refinery systems to other low ammonia systems such as some municipal wastewater treatment plants (0.53 to 2.96 mM; Limpiyakorn *et al.*, 2005; Pickering, 2008), ammonia concentrations in the latter seem to be greater than the range of ammonia concentrations found in the refinery WWTPs (in between of 0.06 and 1.60 mM). Although other environmental factors, e.g., pH, oxygen, C/N ratios, temperature, may also influence the overall available ammonia in wastewater treatment reactors, in theory, it is expected that higher available ammonia concentrations will sustain higher AOB numbers.

For example, in several municipal plants AOB numbers measured through either FISH (2.67×10^7 to 4.5×10^8 cells/mL; Pickering, 2008); or qPCR (1.0×10^6 to 9.2×10^7 cells/mL; Limpiyakorn *et al.*, 2005), were much higher than AOB numbers in the refinery wastewater systems (4.13×10^3 to 1.66×10^6 cells/mL; Chapter 5). In addition, AOB diversity in industrial WWTPs also appears to be reduced in comparison to municipal WWTPs. For example, nine AOB types were found in the study of Limpiyakorn *et al.* (2005) including, one type related to the *Nitrospira* group, one type related to *Nitrosomonas europaea-Nitrosococcus mobilis* lineage, one type related to *N. communis* lineage, one type related to *N. cryotolerans* lineage, four sub-types within the *N. oligotropha* lineage, and one unknown type related to the *Nitrosomonas* group.

Different wastewater treatment plants differ significantly in regard to species richness and abundance of AOB. While some plants are dominated by single AOB species (Jurestchko *et al.*, 1998; Rowan *et al.*, 2003b) other plants harbour a higher diversity of AOB species (Daims *et al.*, 2001b; Gieseke *et al.*, 2001; Gieseke *et al.*, 2003). Based on this, a single-species-dominated AOB system in a plant might render its nitrification more vulnerable, while the presence of several different AOB will increase the resistance to perturbation of nitrification, as well as rendering the system more stable, since as conditions change, other species best able to perform the same function will appear and thrive (Wittebole *et al.*, 2005; Siripong and Rittmann, 2007; Wittebole *et al.*, 2008).

In this study, the Grangemouth plant on the first sampling occasion was dominated by a single AOB taxon from the *N. oligotropha* lineage. This coincided with the lowest AOB abundance measured in this reactor.

It was hypothesized and further demonstrated by Siripong and Rittmann (2007) that activated sludge processes having stable and complete nitrification harbour similar diversity and functional redundancy among their ammonia and nitrite oxidizing bacteria. In that study, the authors found the same AOB and NOB like *Nitrospira* and *Nitrobacter* coexisting in seven municipal wastewater treatment plants, despite differences in operational conditions, such as types of influent, size, water temperature and SRT.

Similar AOB, related to *N. oligotropha* and *N. marina*, were found in all the refinery wastewater treatment plants investigated in this study, with the exception of the Humber plant. Because AOB abundance in the Humber reactor was too low in relation to the abundance of other non AOB *Betaproteobacteria*, no AOB sequences could be recovered in the two clone libraries from the Humber plant. All reactors investigated also harboured *Nitrospira* types I and II and *Nitrobacter*, implying functional redundancy in the AOB and NOB in the refinery WWTPs.

The trickling filter at the Lindsey WWTP had higher AOB (Simpson index = 0.92) and AOA (Simpson index = 0.59) diversity than the activated sludge systems (Simpson indices for AOB ranged from 0.49 to 0.91; and for AOA from 0.13 to 0.38). However, the trickling filter did not always perform better. Although diversity of AOA was much lower than diversity of AOB, AOA diversity in the trickling filter was also higher than AOA diversity in the activated sludge systems. Two dominant and five rare OTUs were found in the trickling filters while only one dominant and three rare OTUs predominated in the activated sludge reactors. Also interesting was that, when the *Crenarchaeota* selected in the Grangemouth and Humber reactors were analysed through CARD-FISH by Marc Mußmann (Chapter 6) they had the same morphology. Although both crenarchaeotal types related to the soil group 1.1b *Crenarchaeota*, they are likely to represent distinct ecophysiological types, since in the clone libraries using archaeal *amoA* primers (Francis *et al.*, 2005) they appeared related to two distinct sub-clusters D1 and D2, within cluster D, as defined by Park *et al.*, (2006) (see Chapter 4).

Despite the fact that AOB may be vulnerable to a number of operational and environmental factors, for instance, dissolved oxygen, pH, temperature, C: N ratio, inhibition by organic chemicals, and competition with heterotrophs for ammonia, oxygen and space in flocs or biofilms; they are also able to display strategic mechanisms to survive and thrive during unfavourable conditions, for example, the one caused by limiting or oscillating nutrient and oxygen conditions (Geets *et al.*, 2006).

The AOB are aerobes. AOB possess nitrifying activity not only at oxygen saturation, but also at extremely low oxygen concentrations (Bodelier *et al.*, 1996; Park and Noguera, 2007). Furthermore different substrate affinities for oxygen may also influence AOB activity and distribution in space or time, as seen in several biofilm studies. Most AOB selected in this study were related to *N. oligotropha* and *N. marina* lineages. AOB species within *N. oligotropha* are able to function at very low oxygen concentrations, and are also resistant to some heavy metals (Koops *et al.*, 2003). In addition, both *N. oligotropha* and *N. marina* are urease positive (Pommerening-Röser and Koops, 2005). These physiological traits might have also conferred to these AOB some kind of advantage in persisting and thriving in the refinery systems, mainly in the Humber plant which in contrast to Lindsey and Grangemouth plants, apparently presented more unfavourable environmental conditions to AOB, such as low pH, higher C: N ratios, oxygen and salinity fluctuations (See Chapter 3).

The capacity of AOB to respond to operational and environmental perturbations and/or be selected by them is an important factor behind their manipulation within wastewater treatment reactors and allows their growth to be stimulated. Increasing sludge age or aeration conditions in reactors are among the process parameters that might directly affect AOB, but at the same time might be controlled, and as a result optimal nitrification might be achieved (Ballinger *et al.*, 2002; Geets *et al.*, 2006).

Recently, 20 operational and environmental parameters directly driving AOB dynamics in two full scale municipal wastewater treatment plants were investigated (Wells *et al.*, 2009). Temperature, followed by oxygen, which was strongly and significantly linked to AOB dynamics, were reported as the most important operational parameters affecting AOB. Interestingly, lower temperature correlated positively with the selection of *Nitrosospira* over other AOB within these plants, which was also observed by Siripong and Rittmann (2007). Also in line with those studies, in the refinery reactors PCA analyses (Chapter 5) indicated that besides pH, temperature and DO positively correlated with AOB abundance.

For instance at the first sampling occasion in the Grangemouth plant in July 2006, it is likely that the lowest oxygen levels recorded in reactor A (3.0 mg/L; Table 3.1, Chapter 3) might have affected AOB abundance. This reactor also had the highest temperature recorded in this study (35°C; Table 3.1, Chapter 3). The high temperature might have reduced the oxygen levels in the reactor by favouring enhanced microbial activity, and consequently increased oxygen consumption by heterotrophs, in the degradation of the organic chemicals present in the wastewater.

Furthermore although the Grangemouth wastewater treatment plant is a conventional activated sludge system, the bulk of oxygen concentration measured with a DO probe is likely to be higher than oxygen concentration within flocs and in practice anoxic microenvironments are likely to be present due to deficits in oxygen diffusion. In addition, this could also allow organisms with different oxygen requirements to thrive in the same wastewater treatment reactor.

In soil microcosm experiments (Tourna *et al.*, 2008), community structure changes and activity of archaeal ammonia oxidizers related to marine group 1.a *Crenarchaeota* were demonstrated to occur in response to temperatures between 10° and 30°C, and both were greatest at 30°C. This highest temperature is near to the temperature found in the Grangemouth reactor, which also might suggest that temperature may have been a factor favouring not only AOB, but also AOA. In the Grangemouth reactor, AOB abundance was lowest (c.a. 10³ cell/mL) when on the contrary, AOA and soil group 1.b *Crenarchaeota* abundances were highest (c.a. 10⁸ cells/mL).

Furthermore only one type of AOB sequence from the *N. oligotropha* lineage was recovered in the 16S rRNA gene clone library derived from this reactor. This was related to 16S rRNA gene sequences from wastewater treatment plants having alternating oxygen (anaerobic/anoxic/aerobic) or low oxygen systems (Limpiyakorn *et al.*, 2005) and these sequences too may also represent a novel AOB species since they have less than 97% sequence identity with other described AOB or AOB sequences. Likewise AOA *amoA* sequences found in this reactor (Chapter 4) were closely related to two sequences retrieved from an anoxic granular sludge system for anaerobic ammonium oxidation (Bae *et al.*, 2010) which also represents an environment with limited oxygen concentrations.

As seen in Chapter 6, nitrifying bacteria appear not to form microcolonies effectively in the refinery WWTPs. In this study, AOB were detected mostly as individual cells dispersed within flocs, or ultimately in scarce and very small colonies, while NOB appeared as small aggregates. Interestingly, *Nitrobacter* and *Nitrospira* in the Humber plants appeared in close proximity to each other (Chapter 6) which differed, for example, from patterns of distribution observed in some biofilms, where they tend to be segregated as a function of oxygen and nitrite concentrations (Schramm *et al.*, 2000).

The type of distribution and aggregation found in this study is also in line with some studies investigating the distribution of AOB in flocs and biofilms, as a function of C: N ratios (Aoi *et al.*, 2000; Wu *et al.*, 2008). While AOB grouped together in larger and dense colonies in reactors receiving synthetic inorganic substrate, in reactors receiving organic substrates, they were sparsely distributed and were present in smaller microcolonies and/or as individual cells.

This study also represents the first time that AOA have been quantified in a wastewater treatment plant. In some cases, AOA were up to three times more abundant than AOB. In situations where AOA were found at their highest abundance (c.a. 10^8 cells/mL) AOB were at their lowest abundance (c.a. 10^3 - 10^4 cells/mL) (as in the third and fourth sampling occasions in the Humber plant, and in the first sampling occasion in the Grangemouth plant). This could suggest a role for AOA in ammonia oxidation with AOA taking over when AOB are less prevalent. However some evidence in this study indicated that this is not the case and that AOA might be mainly involved in organic carbon metabolism, and at least some of the AOA may not even be AOA at all, but are instead heterotrophs. First of all, modelling indicated that the amount of ammonia in the refinery reactors is too low to support the high population size of AOA observed even if it is assumed that all the ammonia was being oxidized by the AOA present in reactors.

Secondly, PCA analysis (Chapter 5) also suggests the involvement of AOA in organic carbon oxidation. In the Humber reactor a positive relationship was found between AOA abundance and BOD/TKN ratios. Likewise in the Grangemouth plant, a positive relationship was found between AOA and COD.

Thirdly, as demonstrated through MAR-FISH by Mußmann *et al.* (2008) the AOA in the Humber and Grangemouth reactors did not assimilate radiolabelled CO_2 , and thus they are not autotrophs. Other studies in the literature have also reported marine planktonic Archaea growing mixotrophically or heterotrophically (Hallam *et al.*, 2006; Ouverney and Fuhrman, 2000; Ingalls *et al.*, 2006; Agogu e *et al.*, 2008).

Wells *et al.* 2009 investigated the presence of AOA in highly aerated activated sludges and found that AOA represented only 15% of the ammonia oxidizers and most of the time could not be detected in these plants. This was in agreement with the minor relevance of AOA for autotrophic ammonia oxidation in refinery WWTPs inferred from this study and the failure to detect AOA in 23 municipal plants screened for the presence of putative AOA.

Therefore AOB are the main organisms supporting nitrification in the refinery wastewater treatment reactors investigated in this study. This was the case even in the Humber plant, which supported lower AOB abundance than the Lindsey and Grangemouth plants. This was also supported by chemical measurements that demonstrated that nitrification was occurring in the Humber plant. Furthermore although the model used in this study did not accurately predict the measured AOB biomass, it did show that the AOB were expected to represent a very small fraction of the biomass in the refinery wastewater treatment plants, especially in the Humber reactors which exhibited very low AOB abundance (Chapter 5).

While the majority of evidence suggests that AOA in the Humber and Grangemouth reactors do not play a major role in nitrification in these plants, this is not completely unequivocal and AOA may contribute to nitrification, either through mixotrophic growth or heterotrophic nitrification. Thus it would be useful to investigate the metabolic capabilities of the AOA populations in these plants and whether they do make some contribution to ammonia oxidation and how this compares with ammonia oxidation by AOB.

For example when AOB are at low abundance, might mixotrophic or heterotrophic ammonia oxidation by *Archaea* be more significant? The contribution made by bacteria other than non AOB to nitrification is considered negligible. However, heterotrophic nitrification may have a role at high C: N ratios and low pH. For instance, inhibition experiments have indicated that AOB are not the major contributors to nitrification in acid forest soils, where it has been speculated that undefined heterotroph nitrifiers are primarily responsible for ammonia oxidation such as fungi and some bacteria, such as *Arthrobacter* species (De Boer and Kowalchuk, 2001; Nicol and Schleper, 2006; Hayatsu *et al.*, 2008).

In this study the fact that AOA were also abundant under acid conditions in the Humber reactors may suggest that heterotrophic nitrification by *Archaea* is also possible. For instance in some soil studies, when fertilizer treatments were applied to acid soils, this stimulated AOA rather than AOB (He *et al.*, 2007; Nicol *et al.*, 2008). In contrast, fertilizer treatments applied to alkaline soils, stimulated AOB rather than AOA.

Therefore the degree to which oxidation of ammonia by AOA occurs in the Humber and Grangemouth reactors, if indeed AOA are oxidizing ammonia, seem to be minor, as is traditionally considered to be the case for bacterial heterotrophic nitrifiers.

The main aim of this study was to investigate which microorganisms are involved in nitrification occurring in full-scale refinery wastewater treatment plants, and what was their abundance. It is believed that an important and relevant contribution to the understanding of this process has been gained through this study and in that sense the present study has fulfilled the main aims and objectives initially proposed. While the study has developed the understanding of the nitrification process in these systems, it has also posed a number of important new questions. For example, are putative AOA identified on the basis of *amoA* sequences actually autotrophic ammonia oxidizers, as might be inferred with reference to the only cultured isolates of mesophilic *Crenarchaeota*? Do they oxidize ammonia at all? And if not, what are their metabolic capabilities and ecological role?

In this respect genomic and biochemical data will be required to help elucidate, for example, if the AOA in the Humber and Grangemouth reactors derive their energy primarily by mixotrophy or if they are heterotrophs, and also whether they represent only one mixotrophic population or perhaps two different populations, that is, one that is mixotrophic while the other is heterotrophic.

The refinery wastewaters studied here are complex in their chemical composition as well as in their microbial-chemical inter-relationships and thus represent a rich and vast subject to be explored in greater depth in the future.

Furthermore, finding the “optimal numbers” and “microbial diversity” of ammonia oxidizers consistent with stable nitrification, and the correlation of these with important operational parameter data, will allow the production of quantitative data and the biological component of the refinery systems to be included in quantitative models that will assist in the revision and/or elaboration of more reliable models. In a broader sense, valuable information will be gained with respect to nitrification process operation and control in the refinery systems.

CHAPTER 8

8 Conclusions

- ❖ AOB in the refinery wastewater treatment plants investigated in this study were phylogenetically related to *Nitrosomonas oligotropha* and *Nitrosomonas marina*-like bacteria; clones related to *Nitrosomonas oligotropha* may represent a novel AOB species.

- ❖ Putative AOA were detected in the Lindsey refinery trickling filters and in Grangemouth and in Humber activated sludge systems that were nitrifying satisfactorily. Cloned *amoA* genes retrieved from the Humber and Grangemouth reactors were related to *amoA* genes recovered from soil and were placed in two distinct sub-clusters within cluster D *amoA* sequences as defined by Park *et al.* (2006); while clones retrieved from the Lindsey trickling filter and the BLC pilot reactor were related to marine group 1.1a *Crenarchaeota* and were placed within cluster A, as defined by Park *et al.* (2006).

- ❖ AOB play the main role in nitrification in wastewater treatment plants and AOB *amoA* numbers around 10^6 cells/mL, in Lindsey and Grangemouth wastewater treatment plants, coincided with stable nitrification conditions; while AOB numbers in between 10^5 cells/mL or below were related to unstable and failed nitrification. In the Humber reactors, with the exception of the first sampling occasion, good nitrification was occurring, although lower AOB numbers were detected using real-time PCR. However, a nitrification model used to predict the AOB biomass fraction present in the Humber reactors suggested that AOB numbers, even below the detection limit of the qPCR, may be sufficient to explain the nitrification observed in Humber reactor.

- ❖ AOA numbers found in Lindsey, Humber and Grangemouth were around 10^7 - to 10^8 cells/mL and were one to three orders of magnitude higher than AOB numbers. However, the high AOA found in Lindsey and Grangemouth may not be oxidizing ammonia at all, but may instead be involved in carbon metabolism.
- ❖ The relative contribution of AOA to nitrification in these wastewater treatment plants is small. The fact that AOA were the predominant population of putative ammonia oxidizers in the Humber reactor at first glance suggested that they had a major role in nitrification. However, as demonstrated by Mußmann *et al.* (2008) they are not autotrophs, but at least chemolithoheterotrophs; and may even not have been oxidizing ammonia at all. Instead they may be heterotrophs and therefore their specific metabolic capabilities need to be better clarified and more thoroughly investigated in future studies.
- ❖ The nitrification model tested predicted low AOB biomass in the Humber and Grangemouth wastewater treatment plants. No agreement between theory and empirical data was found, as predicted numbers and measured numbers were low and towards the bottom end of the sensitivity range of the methods used. It may be difficult to determine a clear relationship between the very low levels of nitrification observed and the AOB numbers measured.
- ❖ AOB are the main organisms driving nitrification in refinery wastewater systems and the specific role of AOA in the Humber plant should be investigated in further studies.
- ❖ NOB detected through real-time PCR in the oil refinery wastewater treatment plants were related to *Nitrospira* types I and II and *Nitrobacter*, with predominance of *Nitrospira* type I in most reactors.

CHAPTER 9

9 Future Research

Great potential exists for future investigations of oil refinery wastewater treatment systems. For example, to better clarify the role of AOA in the Humber plant, it would be necessary to study this plant over a longer period of time and try to develop enrichments through which one was able to facilitate a further environmental genomic studies of AOA and even work on their culturability.

It would also be important to enrich and culture AOB from the Grangemouth reactors, which appear to represent novel species. A first step in this direction would be to sequence the entire 16S rRNA gene of some AOB clones and conduct a more rigorous phylogenetic analysis in order to prove that they may indeed be novel species.

Also it would be useful to apply a full cycle 16S rRNA based gene analysis in order to identify other potentially important bacterial groups in the Humber refinery which could be tested in experimental work and tested under the new hypothesis. For example, because the Humber reactors are operated with very long sludge ages, problems with foaming are also relatively frequent in the Humber WWTP. So, it would be interesting to investigate the microorganisms causing foaming in the plant. In relation to AOA, clones from Grangemouth appeared to be related to AOA identified in anoxic granular sludge carrying out anaerobic ammonium oxidation. The ecophysiology of *Crenarchaeota* has been recently investigated in anaerobic reactors and it would therefore be relevant to investigate the participation of AOA in these processes.

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Appendix A Phylogenetic trees

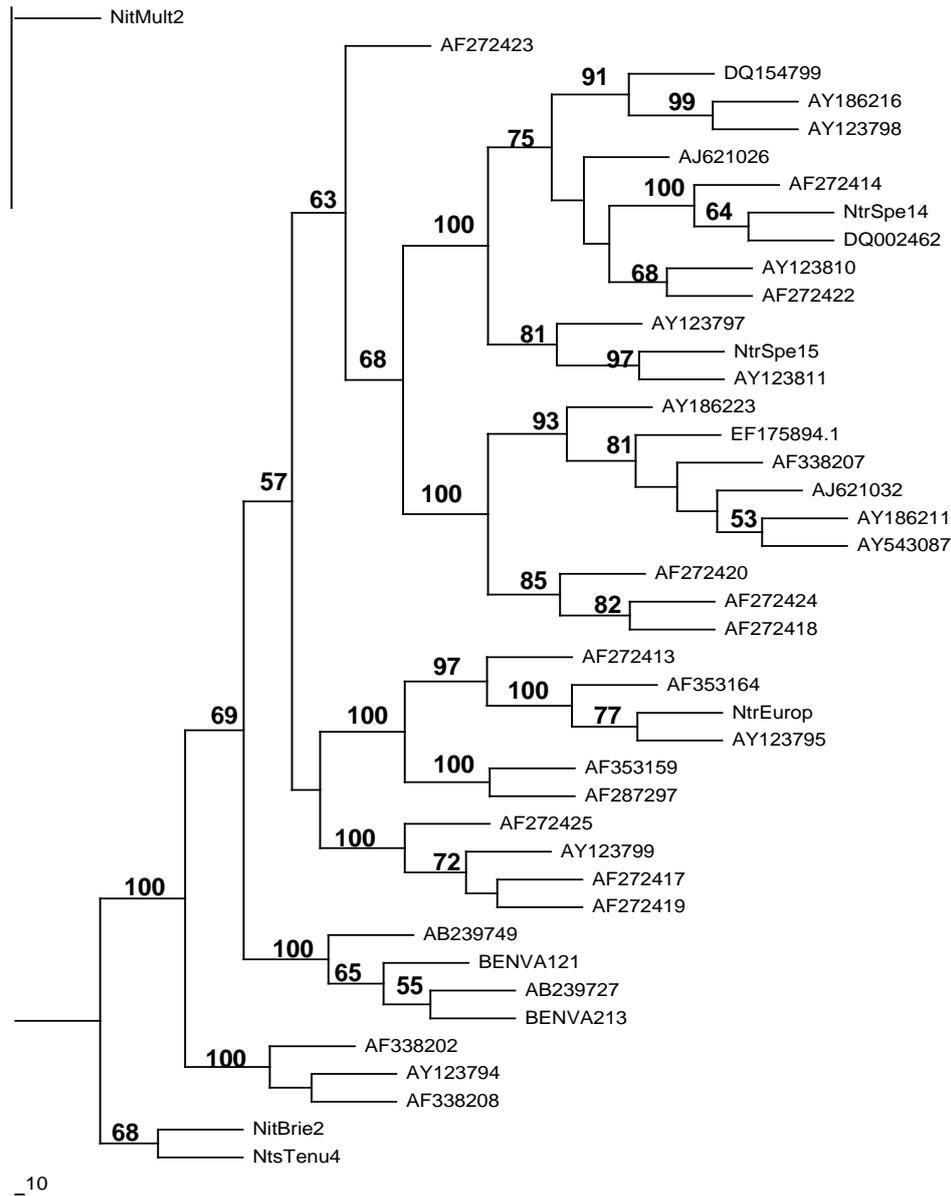


Figure A1 Neighbour-joining 16S rRNA phylogenetic distance tree of AOB (sequences ≥ 1000 nucleotides). Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

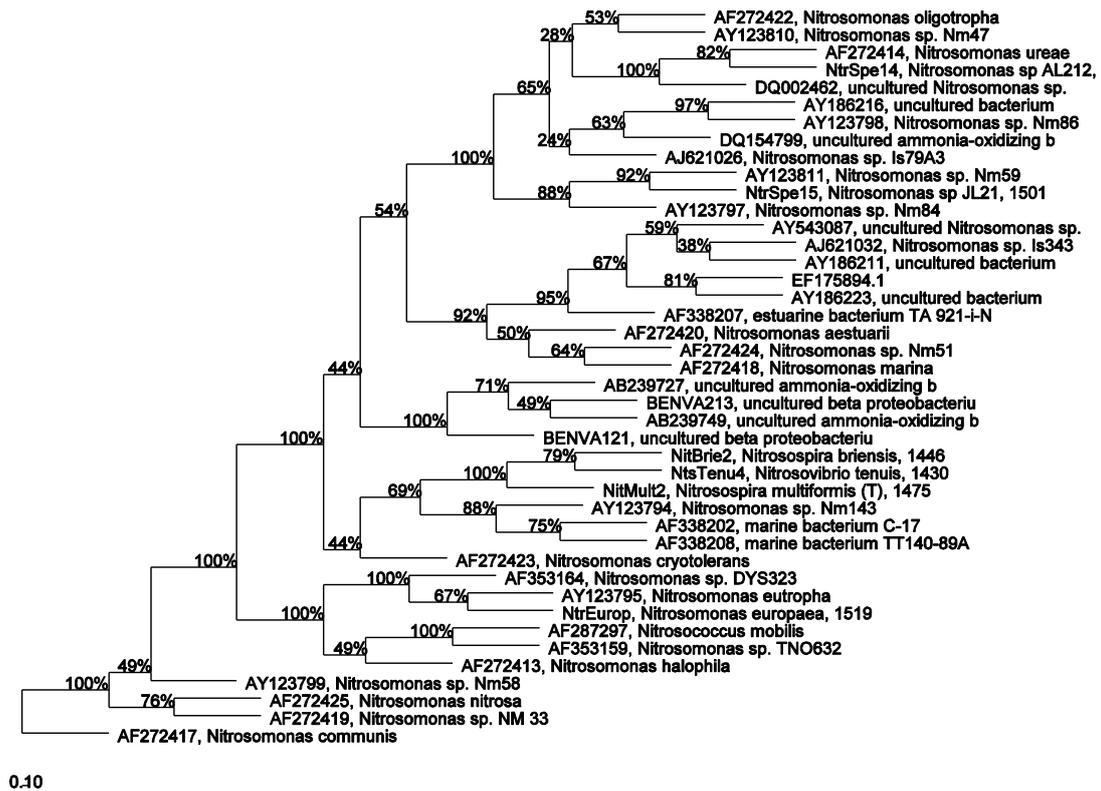


Figure A2 Maximum parsimony 16S rDNA phylogenetic tree of AOB (≥ 1000 nucleotides). Bootstrap values (100 replicates) ≥ 50 are shown at branch nodes.

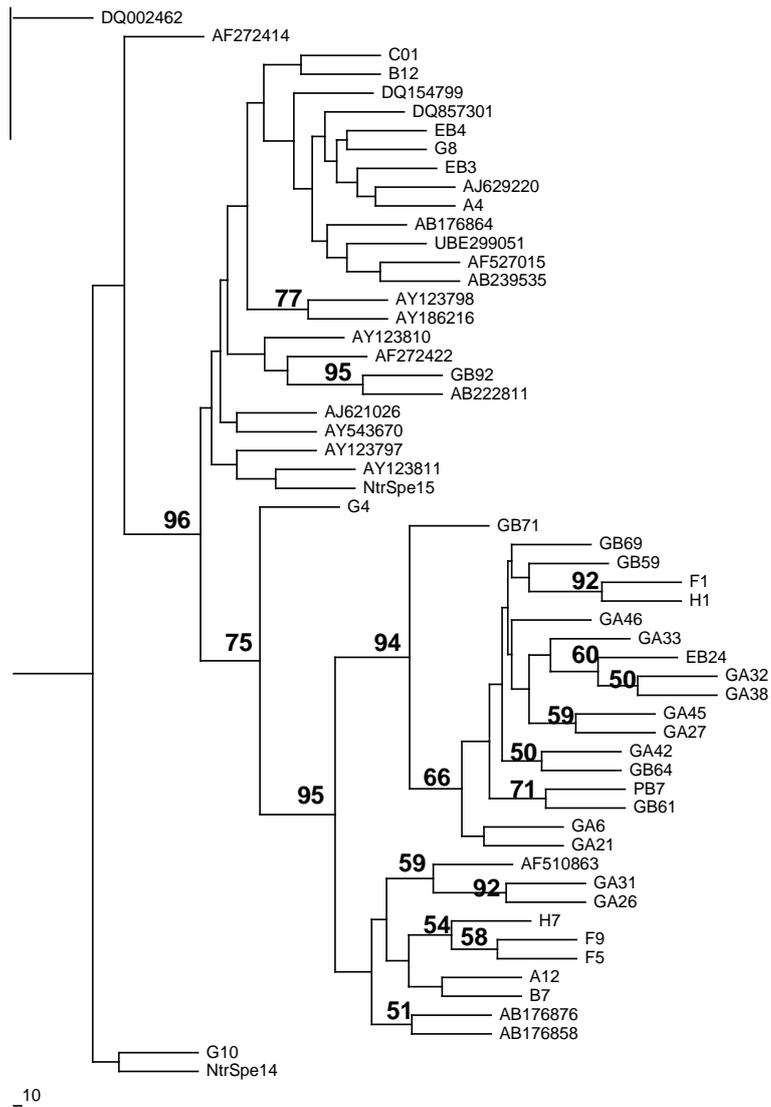
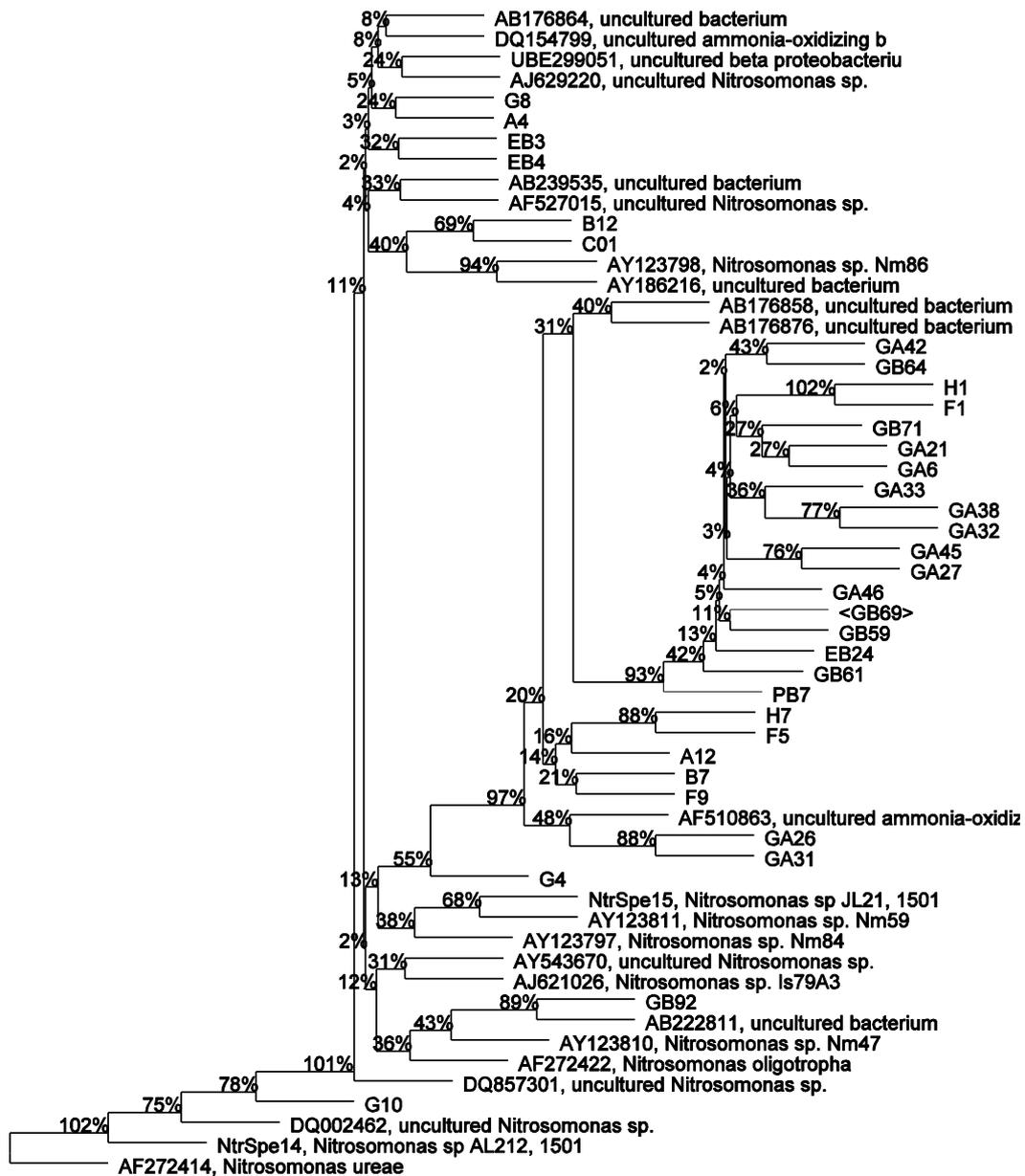
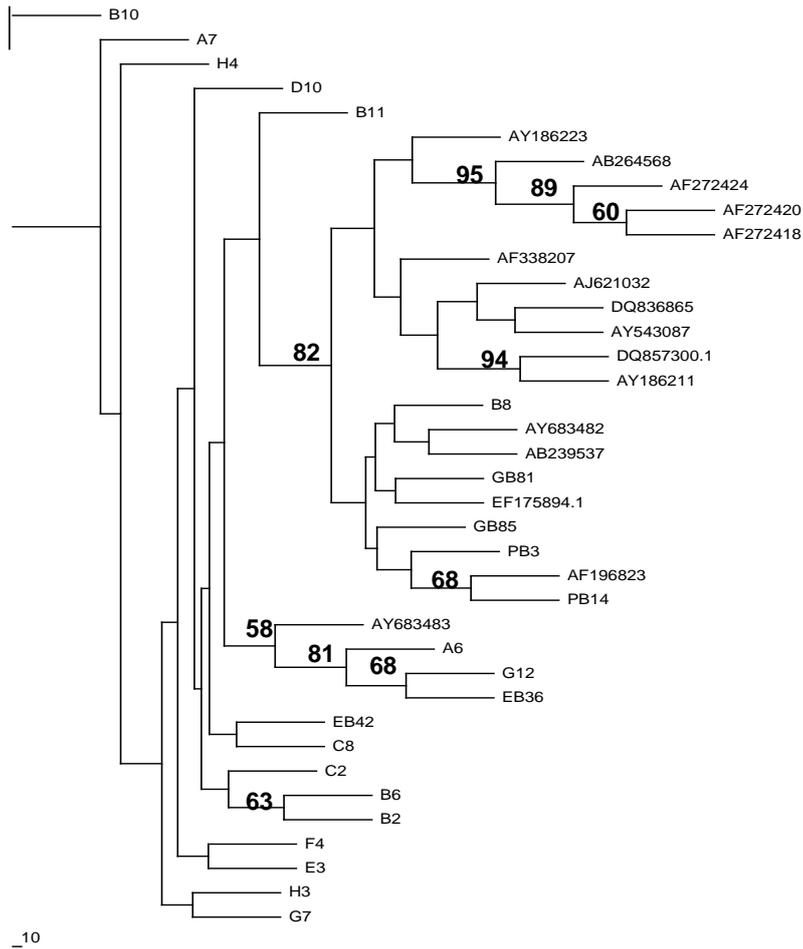


Figure A3 Neighbour-joining 16S rRNA phylogenetic distance tree related to *Nitrosomonas oligotropha* lineage (Figure 3.10). AOB sequences (≥ 1000 nucleotides, plus partial sequences from this study (~400-450 nucleotides) and other closer environmental sequences). Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.



0.10

Figure A4 Maximum parsimony 16S rRNA phylogenetic tree (related to *Nitrosomonas oligotropha* lineage). AOB sequences (≥ 1000 nucleotides; partial sequences from this study (~ 400 - 450 nucleotides), and other closer environmental sequences. Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.



_10

Figure A5 Neighbour-joining 16S rRNA phylogenetic distance tree (related to *Nitrosomonas marina* lineage (Figure 3.10). AOB sequences (≥ 1000 nucleotides plus partial sequences from this study (~ 400 -450 nucleotides) and other closer environmental sequences. Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

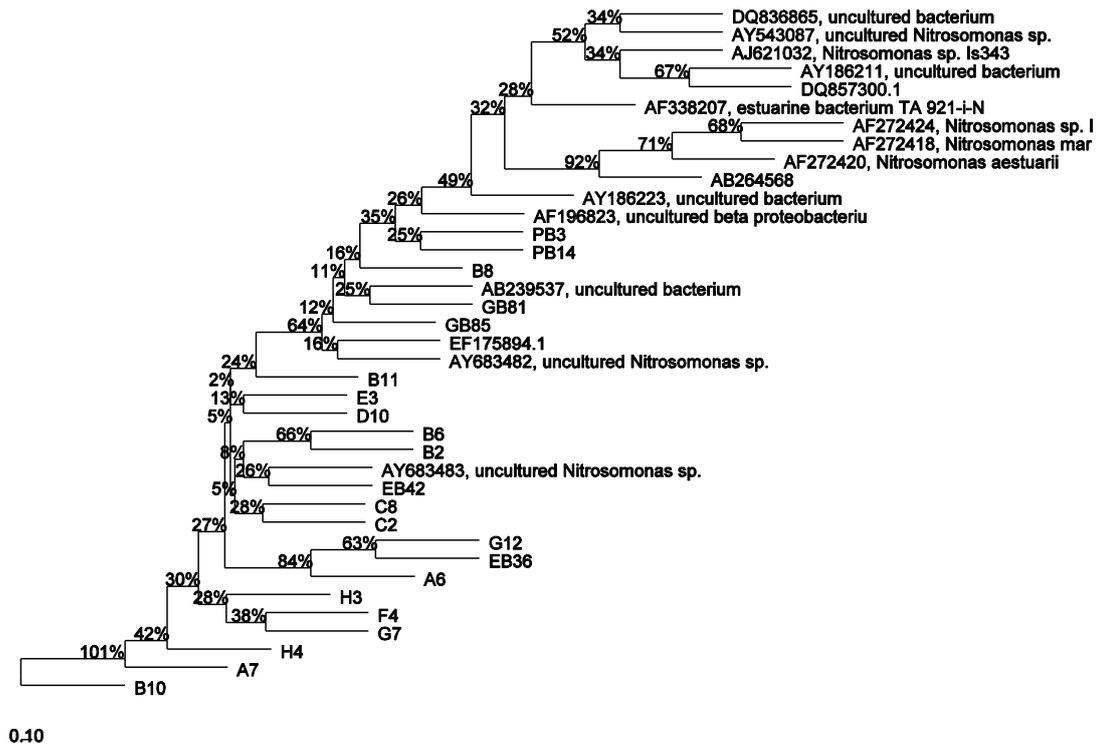


Figure A6 Maximum parsimony 16S rRNA phylogenetic tree related to *Nitrosomonas marina* lineage (Figure 3.10). AOB sequences (≥ 1000 nucleotides plus partial sequences from this study (~ 400 -450 nucleotides) and other closer environmental sequences). Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

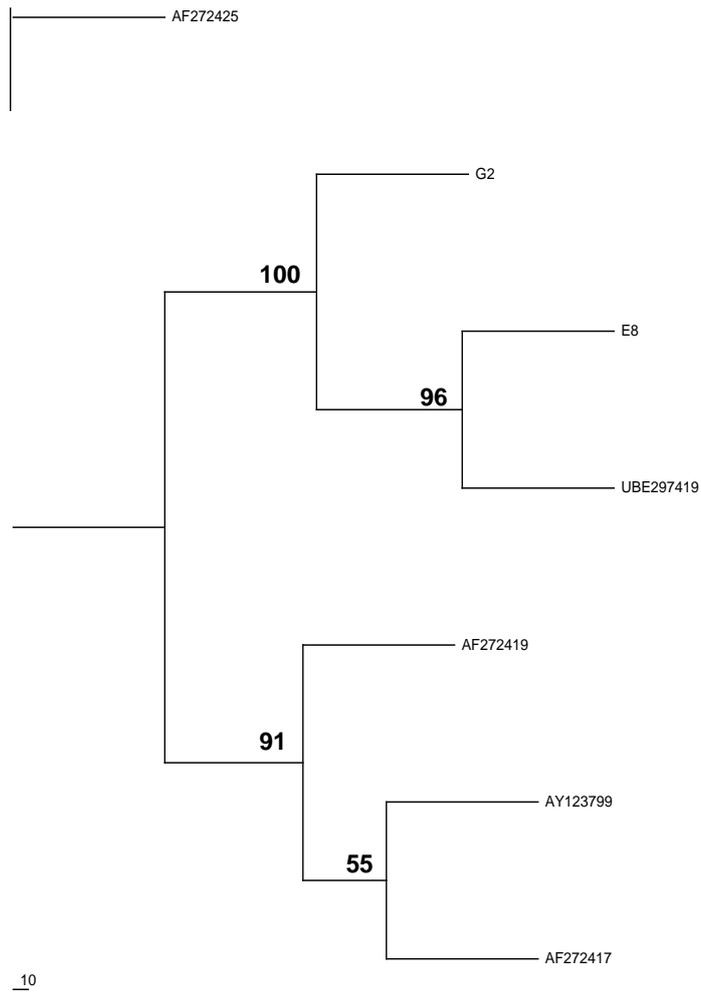


Figure A7 Neighbour-joining 16S rRNA phylogenetic distance tree related to *Nitrosomonas communis* (Figure 3.10). AOB sequences (≥ 1000 nucleotides plus partial sequences from this study (~400-450 nucleotides) and other closer environmental sequences. Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

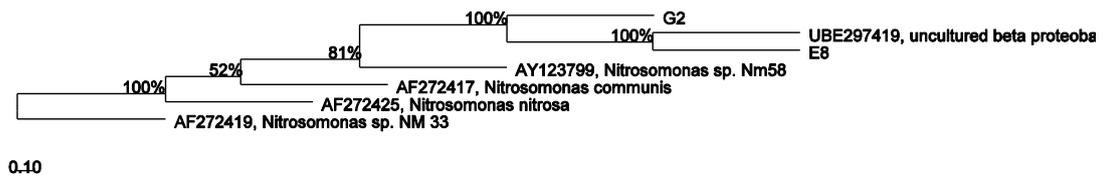


Figure A8 Maximum parsimony 16S rRNA phylogenetic tree related to *Nitrosomonas communis* lineage (Figure 3.10). AOB sequences (≥ 1000 nucleotides plus partial sequences from this study (~400-450 nucleotides) and other closer environmental sequences). Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

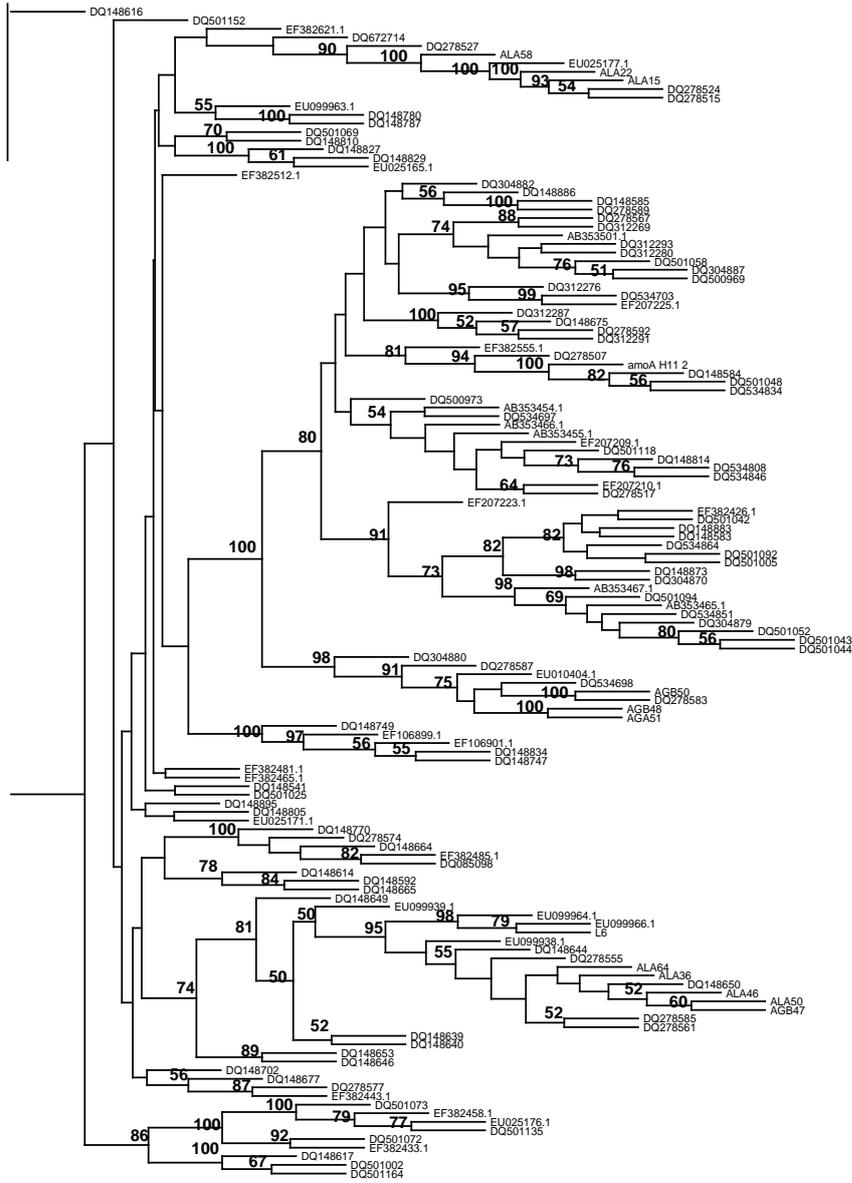


Figure B1 Neighbour-joining phylogenetic distance tree of 136 archaeal *amoA* gene sequences (595-634 bp). Bootstrap values (100 replicates) $\geq 50\%$ are shown at branch nodes.

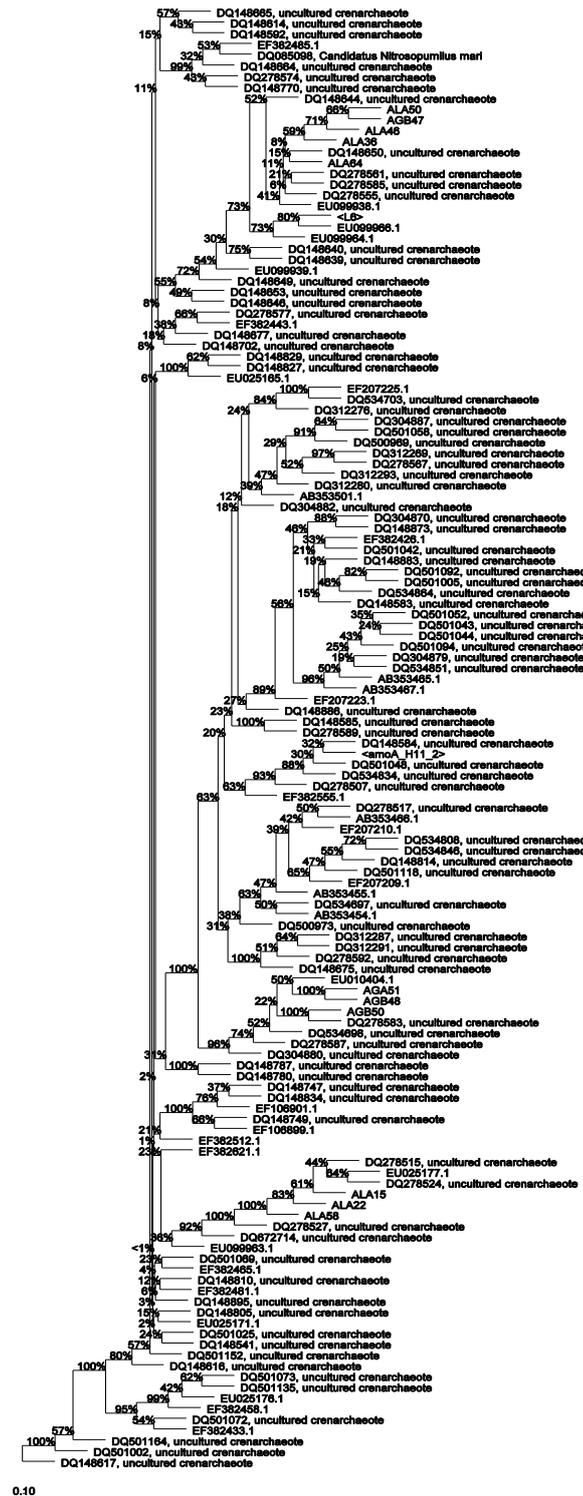


Figure B2 Maximum parsimony phylogenetic tree of 136 archaeal *amoA* gene sequences (595-634 bp).

Appendix C

Table C.1 Regression curves of nine quantitative real-time PCR assay conducted in this study to quantify abundance of 16S rRNA gene for total bacteria, AOB, marine and soil *Crenarchaeota* and *amoA* gene abundance for of *amoA* gene for AOB and AOA

Assay	Target gene	PCR Efficiency (%)	R ²	Slope	Y-intercept	Detection limits of the assay (gene copy numbers/mL)
Total <i>Bacteria</i>	16S rRNA	82.2	0.982	-3.837	41.419	813 + 2400
AOB	16S rRNA	71.7	0.799	-4.262	43.970	643 + 270
<i>Crenarchaeota</i> marine group 1a	16S rRNA	91.9	0.994	-3.533	35.208	3.93 + 0.8
<i>Crenarchaeota</i> soil group 1b	16S rRNA	68.9	0.988	-4.395	40.897	12.4 +7.86
AOB <i>amoA</i>	<i>amoA</i>	100.2	0.958	-3.317	35.848	21.5 +51.54
AOA <i>amoA</i>		82.90	0.992	-3.5928	48.64	0*
<i>Nitrospira</i> Type I	16S rRNA	99.5	0.862	-3.333	37.688	0*
<i>Nitrospira</i> Type II	16S rRNA	74.7	0.980	-4.126	40.674	0*
<i>Nitrobacter</i>	16S rRNA	102.5	0.955	-3.264	36.502	0*

* Note: Detection limits for the real-time PCR assays were set up as the mean of the Ct values obtained from two blank reactions or no template control (NTC) that were included in each assay, plus three standard deviations. However, in cases where the true positive Ct value for the NTC could not be determined, partly because the lowest DNA standard included in the assay was on 10³, a higher conservative value for the minimum detection limit for the assay was considered as the mean Ct value of the lowest standard included in the assay (10³) minus three standard deviations.

		Slide1		Slide2		Slide3	
		AOB	Ntspa	AOB	Nitro	Ntspa	Nitro
Eastham	1	1	1	1	2	7	4
	2	0	0	20	29	8	0
	3	0	0	29	36	5	0
	4	0	2	43	53	6	2
	5	0	0	20	30	3	0
	6	0	1	8	8	2	1
	7	0	0	1	1	7	2
	8	0	0	15	27	13	0
	9	0	0	17	26	0	0
	10	1	1	20	37	1	0
Pembroke	1	0	4	0	0	0	0
	2	0	3	0	0	1	1
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	1	1	0	0	0	0
	6	0	4	0	0	0	0
	7	1	1	0	2	0	0
	8	1	1	0	0	0	0
	9	0	0	0	0	0	0
	10	0	0	0	0	0	0
Humber1	1	10	21	27	42	10	1
	2	30	46	10	11	0	0
	3	8	16	15	27	0	0
	4	5	5	59	18	21	4
	5	0	0	2	2	4	1
	6	14	18	n.d	n.d	9	2
	7	6	7	4	4	0	0
	8	2	2	19	41	0	0
	9	8	9	16	20	0	0
	10	1	1	0	21	2	0
Humber2	1	1	2	13	13	3	1
	2	0	0	9	25	0	0
	3	0	0	55	85	4	4
	4	0	5	7	10	3	4
	5	0	0	0	5	5	40
	6	2	0	10	10	3	24
	7	12	25	20	20	26	6
	8	5	17	2	7	83	17
	9	0	5	3	15	35	25
	10	5	11	4	9	132	30
Humber3	1	0	0	35	45	56	20
	2	2	2	8	25	69	36
	3	5	6	11	39	60	32
	4	0	10	4	16	128	69
	5	4	9	17	39	63	40
	6	0	14	76	82	73	24
	7	0	0	19	21	84	40
	8	86	96	23	45	157	53
	9	94	153	58	80	99	74
	10	14	34	18	40	533	302

		Slide1		Slide2		Slide3	
		AOB	Ntspa	AOB	Nitro	Ntspa	Nitro
Humber4	1	14	23	7	92	50	40
	2	54	67	27	78	40	6
	3	13	13	21	50	33	0
	4	5	19	79	218	19	0
	5	18	18	156	189	48	7
	6	21	3	68	129	10	0
	7	12	10	69	104	n.d.	n.d.
	8	15	27	30	42	6	0
	9	14	4	59	189	n.d	n.d.
	10	18	0	44	165	32	5
Grange1	1	4	301	0	0	4	4
	2	1	293	0	0	5	5
	3	4	255	0	0	0	5
	4	5	336	0	0	0	0
	5	8	202	0	9	0	0
	6	5	307	1	1	11	11
	7	7	321	1	1	0	0
	8	9	299	0	0	2	2
	9	6	238	0	0	20	20
	10	11	289	0	0	0	0
Grange3	1	12	29	0	0	164	4
	2	12	14	0	0	66	5
	3	14	20	0	0	67	6
	4	8	11	0	0	158	23
	5	10	10	0	0	29	4
	6	8	20	0	0	33	25
	7	0	0	0	0	40	4
	8	8	8	0	0	4	4
	9	10	10	0	0	205	4
	10	6	6	0	0	136	3

Table C.3 Numbers of AOB, *Nitrospira* and *Nitrobacter* quantified through FISH in eight samples from four oil refinery activated sludge systems investigated in this study

Refineries	Mean cells/FOV		Cells/mL		Log Cells/mL		Mean	STDEV	CV
	Slide 1	Slide 2	Slide 1	Slide 2	Slide 1	Slide 2			
<i>AOB</i>									
Eastham	0.2	17.4	1386.202	120599.5	3.14214	5.081349	4.111744	1.371228	33.34906
Pembroke	0.3	0	2079.302	0	3.318126	0	1.659063	2.34627	141.4214
Humber1	8.4	16.88	315274.3	1169954	5.49869	6.068169	5.78343	0.402683	6.962696
Humber2	2.5	12.3	93831.65	852513.9	4.972354	5.930702	5.451528	0.677654	12.43054
Humber3	20.5	26.9	1420857	1864441	6.152551	6.270549	6.21155	0.083437	1.343263
Humber4	18.4	56	1275305	3881364	6.105615	6.588985	6.3473	0.341794	5.384876
Grange1	6	0.2	415860.5	13862.02	5.618949	4.141858	4.880403	1.044461	21.40112
Grange3	4.4	0	609928.7	0	5.78528	0	2.89264	4.090811	141.4214
<i>Nitrospira</i>									
Eastham	0.5	5.2	3465.504	36041.24	3.539892	4.556812	4.048352	0.719071	17.76207
Pembroke	1.4	0.1	9703.411	693.1008	3.986969	2.841423	3.414196	0.810024	23.72517
Humber1	12.5	4.6	86637.6	318826.4	4.937711	5.503556	5.220633	0.400112	7.664056
Humber2	6.5	29.4	450515.5	2037716	5.653711	6.309144	5.981427	0.463461	7.748339
Humber3	32.4	132.2	2245646	9162792	6.351342	6.962028	6.656685	0.43182	6.487019
Humber4	18.4	29.75	1275305	2061975	6.105615	6.314284	6.209949	0.147551	2.376046
Grange1	284.1	4.2	19690993	291102.3	7.294268	5.464047	6.379157	1.294161	20.28734
Grange3	12.8	90.2	887169	6251769	5.948007	6.796003	6.372005	0.599624	9.410285
<i>Nitrobacter</i>									
Eastham	24.9	0.9	172582.1	6237.907	5.236998	3.795109	4.516053	1.01957	22.57657
Pembroke	0.2	0.1	1386.202	693.1008	3.14214	2.841423	2.991781	0.212639	7.10744
Humber1	20.66	0.8	1431946	55448.06	6.155927	4.743894	5.449911	0.998458	18.32063
Humber2	19.9	15.1	1379271	1046582	6.13965	6.019774	6.079712	0.084765	1.39423
Humber3	43.2	69	2994195	4782395	6.47628	6.679646	6.577963	0.143801	2.186102
Humber4	125.6	7.25	8705346	502498.1	6.939786	5.701135	6.320461	0.875858	13.85751
Grange1	1.1	4.7	76241.08	325757.4	4.882195	5.512896	5.197545	0.445973	8.580451
Grange3	4.1	8.2	0	568342.6	0	5.754611	2.877305	4.069124	141.4214

