MICROBIAL AMMONIA OXIDATION IN DEEP-SEA HYDROTHERMAL PLUMES

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ABSTRACT

Autotrophic ammonia oxidation has been documented for the first time in deep-sea hydrothermal plumes – along the Endeavour Segment, Juan de Fuca Ridge, and in the Guaymas Basin, Gulf of California. Ammonium concentrations as high as 341 nM have been detected in the Endeavour plume, which supports autotrophic ammonia oxidation at \( \leq 91 \text{ nM d}^{-1} \), and potentially produces \textit{de novo} organic carbon at a rate (0.6-13 mg C m\(^{-2}\) d\(^{-1}\)) equivalent to 1300\% particulate organic carbon flux from the surface ocean. The thick organic-rich sediment cover in the Guaymas Basin endows an even higher ammonium concentration (\( \leq 2.9 \mu\text{M} \)) to the overlying hydrothermal plume, which fuels potential ammonia oxidation at \( \leq 517 \text{ nM d}^{-1} \), but its relative contribution to organic carbon is perhaps small compared to the large amounts of organic carbon coming from the surface ocean and hydrothermal fluid discharges. In fact, the abundance of organic matter and other reduced chemicals in the Guaymas Basin water might have stimulated more heterotrophic activities and associated ammonium assimilation, or acted as natural inhibitors, thus preventing higher ammonia oxidation rates or larger populations of ammonia-oxidizing bacteria (AOB). Assimilation of ammonium occurs at rates comparable to ammonia oxidation. Hence, assimilation is an equally important ammonium uptake pathway in both water columns.

AOB in both \( \beta \)- and \( \gamma \)-proteobacterial subgroups are present in the deep-sea hydrothermal plumes and background deep water at both locations, as well as in the hydrothermal fluids and sediments in the Guaymas Basin. They are often associated with
particles greater than 3 μm in diameter (32-95%). The total abundance of AOB in the Endeavour plume (≤ 16 ± 1.8 × 10^3 cells ml⁻¹) is up to ten-fold that in the above-plume background (1.6 ± 0.7 × 10^3 cells ml⁻¹). A less dramatic increase is observed in the Guaymas Basin plume (8.0 ± 0.9 × 10^3 cells ml⁻¹) relative to its overlying deep water (1.5-3.5 × 10^3 cells ml⁻¹). Sequence analyses of amplified partial 16S rRNA genes and the genes encoding ammonia monooxygenase subunit A (amoA) suggest that a novel lineage of β-Proteobacterial AOB might be present in both water columns.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................ iv

ABSTRACT......................................................................................... vi

LIST OF TABLES.................................................................................. x

LIST OF FIGURES............................................................................... xii

**CHAPTER ONE:** An Introduction: Potential Ammonia Oxidation in Deep-Sea Hydrothermal Plumes

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>9</td>
</tr>
</tbody>
</table>

**CHAPTER TWO:** Processing Deep-Sea Particle-Rich Water Samples for Fluorescence *In Situ* Hybridization: Consideration of Storage Effects, Preservation, and Sonication. (Co-author: James P. Cowen).

Published in: *Applied and Environmental Microbiology*, 70 (1), 25-33

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>17</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>18</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>22</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>28</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>31</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>36</td>
</tr>
</tbody>
</table>

**CHAPTER THREE:** Autotrophic Ammonia Oxidation in a Deep-Sea Hydrothermal Plume. (Co-authors: James P. Cowen and Ronald D. Jones).

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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>51</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>52</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>53</td>
</tr>
<tr>
<td>RESULTS</td>
<td>56</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>62</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>69</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>77</td>
</tr>
</tbody>
</table>

**CHAPTER FOUR:** Microbial Ammonia Oxidation and Enhanced Nitrogen Cycling in the Endeavour Hydrothermal Plume

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>102</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>103</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>104</td>
</tr>
<tr>
<td>RESULTS</td>
<td>107</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>117</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>128</td>
</tr>
</tbody>
</table>
CHAPTER FIVE: Ammonium Cycling in the Hydrothermally Influenced Water Column of the Guaymas Basin

5.0. ABSTRACT
5.1. INTRODUCTION
5.2. MATERIALS AND METHODS
5.3. RESULTS
5.4. DISCUSSION
5.5. CONCLUSIONS
5.6. REFERENCES

CHAPTER SIX: Molecular Analyses of Autotrophic Ammonia-Oxidizing Bacteria in Deep-Sea Hydrothermal Environments

6.0. ABSTRACT
6.1. INTRODUCTION
6.2. MATERIALS AND METHODS
6.3. RESULTS
6.4. DISCUSSION
6.5. REFERENCES

CHAPTER SEVEN: Conclusions and Future Perspectives

REFERENCES
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>List of the 16S rRNA-targeted oligonucleotide probes used in this study (Chapter 2)</td>
</tr>
<tr>
<td>2.2</td>
<td>Summary of results using different preservation methods in saltwater aquarium RS2</td>
</tr>
<tr>
<td>2.3</td>
<td>Effects of size-fractionation and sonication on total microbial abundance estimates by DAPI-staining and FISH with UNI oligonucleotide probes</td>
</tr>
<tr>
<td>3.1</td>
<td>List of the various 16S rRNA-targeted oligonucleotide probes used in this study (Chapter 3)</td>
</tr>
<tr>
<td>3.2</td>
<td>Specific ammonia oxidation rates ($k_{oxid}$) and turnover times for ammonium removals</td>
</tr>
<tr>
<td>3.3</td>
<td>Percentages of β-proteobacterial ammonia-oxidizing bacteria (β-AOB) and Type I methanotrophs relative to the whole microbial community in various size-fractions in various depths of the neutrally buoyant plume at Main Endeavour Field (MEF)</td>
</tr>
<tr>
<td>4.1</td>
<td>List of the various 16S rRNA-targeted oligonucleotide probes used in this study (Chapter 4)</td>
</tr>
<tr>
<td>4.2</td>
<td>Specific rate constants ($k_{tot}$ and $k_{oxid}$) and turnover times ($T_{tot}$ and $T_{oxid}$) with respect to total net ammonium removal and ammonia oxidation respectively</td>
</tr>
<tr>
<td>4.3</td>
<td>Comparison of total net NH$_4^+$ removal rates and ammonia oxidation rates obtained from incubations at 200 atm (repressurized) and those at atmospheric pressure</td>
</tr>
<tr>
<td>4.4</td>
<td>Concentrations and carbon stable isotopic values ($\delta^{13}C$) of particulate organic carbon (POC), concentrations of particulate nitrogen (PN), particulate C/N ratios, as well as the corresponding total net removal rates, ammonia oxidation rates, $^{15}N$-ammonia oxidation rates and assimilation rates</td>
</tr>
<tr>
<td>4.5</td>
<td>Distribution of total ammonia-oxidizing bacteria (AOB) as the β-proteobacterial subgroups of <em>Nitrosospira</em> spp. and <em>Nitrosomonas</em> spp., and the γ-proteobacterial subgroup of <em>Nitrosococcus</em> spp., in the free-living (0.2-3.0 µm-diameter) and particle-associated (3.0-10 µm and ≥ 10 µm) communities at Main Endeavour Field (MEF) and High Rise vent fields</td>
</tr>
</tbody>
</table>
5.1 List of the various 16S rRNA-targeted oligonucleotide probes used in this study (Chapter 5).

5.2 Concentrations of ammonium measured in warm diffuse fluids and hot focused vent fluids sampled by titanium major samplers operated by the submersible DSV2 ALVIN.

5.3 Specific rate constants ($k_{tot}$, $k_{oxid}$ and $k_{15N}$) and turnover times ($T_{tot}$, $T_{oxid}$ and $T_{15N}$) with respect to total net ammonium removal rates, potential ammonia oxidation rates and ammonia oxidation rates measured via the $^{15}$N-tracer method respectively.

5.4 Comparison of total net ammonium removal rates with and without the removal of larger particles greater than 3 µm- and 10 µm- diameter in size.

5.5 Concentrations and stable isotopic values of particulate organic carbon (POC) and particulate nitrogen (PN), particulate C/N ratios, as well as assimilation rates along with the corresponding total net ammonium removal rates, ammonia oxidation rates and assimilation rates.

5.6 Distribution of total ammonia-oxidizing bacteria (AOB) as the β-proteobacterial subgroups of *Nitrosospira* spp. and *Nitrosomonas* spp., and the γ-proteobacterial subgroup of *Nitrosococcus* spp., in the free-living (0.2-3.0 µm-diameter) and particle-associated (3.0-10 µm and ≥ 10 µm) communities at the South Site and the North Site.

6.1 List of the various 16S rRNA-targeted oligonucleotide probes used in this study (Chapter 6).

6.2 List of the primers used in this study, along with their sequences, targeted sites, targeted genes and thermal cycling conditions.

6.3 Construction of clone libraries and the number of chimera-checked full sequences.

6.4 Results of the PCR on various samples from the Guaymas Basin and the Endeavour Segment.
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Effects of storage without preservatives: temporal changes in total microbial abundance estimated by DAPI-staining and Eubacterial abundance by EUB338; and temporal changes in β-AOB abundance by NS0190 and NS01225.</td>
<td>46</td>
</tr>
<tr>
<td>2.2</td>
<td>Combined microbial abundance estimates of different size-fractions with post-filtration paraformaldehyde and pre-filtration overnight formaldehyde fixation in sample WA.</td>
<td>47</td>
</tr>
<tr>
<td>2.3</td>
<td>Total microbial abundance estimated by DAPI-staining and Eubacterial abundance by EUB338 in sample series RS2 preserved with five different methods.</td>
<td>48</td>
</tr>
<tr>
<td>2.4</td>
<td>Abundance of β-Proteobacterial ammonia-oxidizing bacteria (β-AOB) detected by NS0190 and by NS01225 in RS2 subsamples preserved with five different methods.</td>
<td>49</td>
</tr>
<tr>
<td>2.5</td>
<td>Increases in total microbial abundance estimated by DAPI-staining and UNI-hybridization after sonication, with an optimal sonication time at around 15-20 seconds in both (a) 5.0-10 μm and (b) &gt;10 μm size fractions.</td>
<td>50</td>
</tr>
<tr>
<td>3.1</td>
<td>Location of the Endeavour Segment and Juan de Fuca Ridge. The inset shows an enlarged view of the Endeavour Segment with the sampling stations and the five known vent fields.</td>
<td>93</td>
</tr>
<tr>
<td>3.2</td>
<td>Vertical profiles of temperature anomalies (Δθ) and particle anomalies (Δc) at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02.</td>
<td>94</td>
</tr>
<tr>
<td>3.3</td>
<td>Vertical profiles of mean NH₄⁺ concentrations, total net removal rates and ammonia oxidation rates at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02.</td>
<td>95</td>
</tr>
<tr>
<td>3.4</td>
<td>Vertical profiles of total microbial abundance detected by DAPI-staining and FISH with UNI oligonucleotide probe, along with the profiles of Eubacterial abundance detected by FISH with EUB338 oligonucleotide probe at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02.</td>
<td>96</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Vertical profiles of β-proteobacterial ammonia-oxidizing bacterial abundance detected by FISH with the NSO190 oligonucleotide probe at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02</td>
<td>97</td>
</tr>
<tr>
<td>3.6</td>
<td>Vertical profiles of the abundance of <em>Nitrosomonas</em> spp. detected by FISH with Nsm156 oligonucleotide probe, and of <em>Nitrosospira</em> spp. detected by FISH with Nsv443 oligonucleotide probe at stations (a) V00T-01 and (b) V00T-02</td>
<td>98</td>
</tr>
<tr>
<td>3.7</td>
<td>Vertical profiles of the abundance of type I (filled squares) and type II (open squares) methanotroph-like cells at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02</td>
<td>99</td>
</tr>
<tr>
<td>3.8</td>
<td>FISH micrographs showing β-AOB cells hybridized with the Cy3-labelled NSO190 oligonucleotide probes (red) versus type I methanotrophs hybridized with 6-FAM-labelled 10-γ probes (green). The corresponding views of DAPI-stained cells (blue) are shown in the lower panels</td>
<td>100</td>
</tr>
<tr>
<td>3.9</td>
<td>Microbial abundance in different size-fractions at various depths at station V00T-01: (a) DAPI-stained cells, (b) Eubacteria detected by EUB338, (c) β-AOB detected by NSO190 (d) <em>Nitrosomonas</em>-like cells detected by Nsm156, (e) <em>Nitrosospira</em>-like cells detected by Nsv443, (f) Type I Methanotrophs detected by 10-γ and (g) Type II Methanotrophs by 9-α</td>
<td>101</td>
</tr>
<tr>
<td>4.1</td>
<td>A bathymetry map showing the locations of the five known active vent fields (SQ = Sasquatch, SD = Salty Dawg, HR = High Rise, MEF = Main Endeavour Field, MO = Mothra), tow-yo tracks (Tows A to F; dark blue) and vertical cast stations</td>
<td>158</td>
</tr>
<tr>
<td>4.2</td>
<td>Neutrally-buoyant hydrothermal plume signals indicated by potential temperature anomalies (Δθ)</td>
<td>159</td>
</tr>
<tr>
<td>4.3</td>
<td>(a) Distribution of ammonium concentrations (mean of duplicates) at the neutrally buoyant plume depths (1800-2150 m), superimposed on a bathymetry map of the Endeavour Segment; (b) Vertical profiles of ammonium concentrations at stations A to F</td>
<td>160</td>
</tr>
<tr>
<td>4.4</td>
<td>Plot of ammonium concentrations versus potential temperature anomalies (Δθ)</td>
<td>162</td>
</tr>
<tr>
<td>4.5</td>
<td>Depth-profiles of total net ammonium removal rates and autotrophic ammonia oxidation rates at stations A to F</td>
<td>163</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Results from substrate limitation experiments on total net ammonium removal rates and autotrophic ammonia oxidation rates.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>Effects on total net ammonium removal rates and autotrophic ammonia oxidation rates after filtration through 3-μm-pore-size membrane filters and 10-μm-pore-size Nitex screens.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>166</td>
<td></td>
</tr>
<tr>
<td>(a) Methane concentrations (nM) at the depth range of 1800-2150 m in the vicinity of the Endeavour Segment, and (b) the carbon stable isotopic values of methane (‰) in the same water subsamples, overlaid on a bathymetry map of the region.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Depth-distribution of total microbial abundance estimated by DAPI-cell counts, and eubacterial abundance estimated by EUB338-hybridized cell counts at Stations A, B, C, E and F.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>Abundance of β-proteobacterial ammonia-oxidizing bacteria estimated by NSO190- and NSO1225- cell counts, and the abundance of γ-proteobacterial ammonia-oxidizing bacteria estimated by Nscoc128 cell counts at Stations A, B, C, E and F.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.11</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>Abundance of specific groups of β-proteobacterial ammonia-oxidizing bacteria: <em>Nitrosomonas</em> spp. estimated by Nsm156-hybridized cell counts, and <em>Nitrosospira</em> spp. estimated by Nsv443-hybridized cell counts at Stations A, B, C, E and F.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.12</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>Abundance of Type I methanotrophs estimated by My705-hybridized cell counts, and Type II methanotrophs estimated by Ma450-hybridized cell counts at Stations A, B, C, E and F.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.13</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>Distribution of microbial abundance in different size-fractions in five selected samples from Stations A, B and C: (a) DAPI-stained cells, (b) Eubacteria detected by EUB338, (c) βAOB detected by NSO190, (d) βAOB detected by NSO1225, (e) <em>Nitrosopira</em>-like cells, (f) <em>Nitrosomonas</em>-like cells, (g) γAOB, (h) Type I methanotrophs and (i) Type II methanotrophs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.14</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td>A sigmoidal relationship was shown between ammonia oxidation rates and ammonium concentrations in the Endeavour neutrally buoyant plume, combining data from 1999, 2000 and 2002.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure Page

5.1 (a) A bathymetry map of the Gulf of California, indicating the locations of the Guaymas Basin and other adjacent basins within the Gulf. (b) A bathymetry map of the hydrothermally active Southern Trough in the Guaymas Basin, showing the locations of the two major sampling sites: South Site (casts V1-V6) and North Site (cast V7, the last upward tow in T3, T4 and T5) and the two tow-yo tracks (T2 and T3) in red................................................................. 237

5.2 Composite vertical profiles of (a) potential temperature or $\theta$, (b) salinity, (c) potential density or $\sigma_{b}$, (d) apparent potential temperature anomalies or $\Delta\theta^*$, (e) particle anomalies or $\Delta c$, (f) dissolved oxygen content and (g) oxygen deficit or $\Delta O_2$, at the South Site, measured during sampling casts V1 to V6.... 238

5.3 Composite vertical profiles of (a) potential temperature or $\theta$, (b) salinity, (c) potential density or $\sigma_{b}$, (d) apparent potential temperature anomalies or $\Delta\theta^*$, (e) particle anomalies or $\Delta c$, (f) dissolved oxygen content and (g) oxygen deficit or $\Delta O_2$, at the North Site, measured during sampling casts V7, T3, T4 and T5................................................................. 239

5.4 Sample plots of potential temperature ($\theta$) versus salinity at (a) the South Site (sampling cast V5), and (b) the North Site (sampling cast T5), illustrating the distinct discontinuity in $\theta$-$S$ signatures observed at $\sim$1750 m in all samples within the Guaymas Basin.................................................. 240

5.5 (a) Vertical profiles of ammonium concentrations, in logarithmic scale, at the South Site. (b) Vertical profiles of total net ammonium removal rates, potential autotrophic ammonia oxidation rates and ammonia oxidation rates measured in the $^{15}$N-labelled experiments at the South Site. (c) Vertical profiles of ammonium concentrations, in logarithmic scale, at the North Site. (b) Vertical profiles of total net ammonium removal rates, potential autotrophic ammonia oxidation rates and the ammonia oxidation rates measured in the $^{15}$N-labelled experiments at the North Site............................................ 241

5.6 Vertical profiles of (a) methane concentrations and (b) carbon stable isotopic composition of methane at the South Site. Vertical profiles of (c) methane concentrations and (d) carbon stable isotopic values at the North Site........... 242

5.7 Vertical distribution of microbial abundance at the South Site: (a) total microbial and Eubacterial abundance, (b) abundance of $\beta$-proteobacterial ammonia-oxidizing bacteria ($\beta$AOB) and $\gamma$-proteobacterial ammonia-oxidizing bacteria ($\gamma$AOB), (c) total abundance of ammonia-oxidizing bacteria as the sum of $\beta$AOB and $\gamma$AOB, (d) Nitrosospira spp. and Nitrosomonas spp., and (e) Type-I and Type-II methanotrophs................. 243
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8 Vertical distribution of microbial abundance at the North Site: (a) total microbial and Eubacterial abundance, (b) abundance of β-proteobacterial ammonia-oxidizing bacteria (βAOB) and γ-proteobacterial ammonia-oxidizing bacteria (γAOB), (c) total abundance of ammonia-oxidizing bacteria as the sum of βAOB and γAOB, (d) <em>Nitrosospira</em> spp. and <em>Nitrosomonas</em> spp., and (e) Type-I and Type-II methanotrophs</td>
<td>244</td>
</tr>
<tr>
<td>5.9 Distribution of microbial abundance in free-living fraction versus particle-associated size-fractions in two selected samples from the South Site and two from the North Site: (a) DAPI-stained cells, (b) Eubacteria hybridized with oligonucleotide probe EUB338, (c) βAOB hybridized with NSO190, (d) βAOB hybridized with NSO1225, (e) <em>Nitrosospira</em>-like cells, (f) <em>Nitrosomonas</em>-like cells, (g) γAOB, (h) Type I methanotrophs and (i) Type II methanotrophs</td>
<td>245</td>
</tr>
<tr>
<td>5.10 Total net ammonium removal rates, potential ammonia oxidation rates, ammonia oxidation rates measured by the $^{15}$N-tracer technique, together with the assimilation rates are plotted as a function of ammonium concentrations in logarithmic scale</td>
<td>246</td>
</tr>
<tr>
<td>5.11 The total abundance of ammonia-oxidizing bacteria (AOB), as the sum of the β- and γ- Proteobacteria subgroups, in relation to (a) NH$_4^+$ concentrations and (b) ammonia oxidation rates measured by the $^{15}$N-tracer technique ($^{15}$NH$_3$ oxidation rates)</td>
<td>247</td>
</tr>
<tr>
<td>6.1 A bathymetry map showing the locations of the five known active vent fields (SQ = Sasquatch, SD = Salty Dawg, HR = High Rise, MEF = Main Endeavour Field, MO = Mothra), tow-yo tracks and vertical cast stations</td>
<td>303</td>
</tr>
<tr>
<td>6.2 A bathymetry map showing the location of sampling stations, North Site and South Site, in the Guaymas Basin, Gulf of California</td>
<td>304</td>
</tr>
<tr>
<td>6.3 Abundance of β-proteobacterial ammonia-oxidizing bacteria estimated by NSO190- and NSO1225- cell counts, and the abundance of γ-proteobacterial ammonia-oxidizing bacteria at Stations A, B, C, E and F</td>
<td>305</td>
</tr>
<tr>
<td>6.4 Abundance of specific groups of β-proteobacterial ammonia-oxidizing bacteria, <em>Nitrosomonas</em> spp. and <em>Nitrosospira</em> spp. at Stations A, B, C, E and F</td>
<td>306</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>6.5 Abundance of autotrophic ammonia-oxidizing bacteria in the Guaymas Basin water column at the South Site: (a) β-proteobacterial ammonia-oxidizing bacteria (βAOB) and γ-proteobacterial ammonia-oxidizing bacteria (γAOB), (b) Total abundance of ammonia-oxidizing bacteria as the sum of the βAOB and γAOB, and (c) abundance of the two main groups of βAOB, <em>Nitrosospira</em> and <em>Nitrosomonas</em>. Abundance data at the North Site: (d) βAOB and γAOB (e) Total abundance of ammonia-oxidizing bacteria, and (f) abundance of <em>Nitrosospira</em> and <em>Nitrosomonas</em></td>
<td>307</td>
</tr>
<tr>
<td>6.6 Bootstrapped (1000 resamplings) neighbor-joining distance tree based on the 16S rRNA gene sequenced from clone libraries constructed from samples collected the water column of Guaymas Basin and Endeavour Segment</td>
<td>308</td>
</tr>
<tr>
<td>6.7 Bootstrapped (1000 resamplings) neighbor-joining distance tree of βAOB based on the <em>amoA</em> gene sequenced from clone libraries constructed from the water column samples of the Guaymas Basin and the Endeavour Segment, as well as the Guaymas Basin hydrothermal sediment samples</td>
<td>309</td>
</tr>
<tr>
<td>6.8 Bootstrapped (1000 resamplings) neighbor-joining distance tree constructed from partial sequences of the 16S rRNA gene amplified from Guaymas Basin hydrothermal sediment samples and closely related sequences obtained from BLAST searches</td>
<td>310</td>
</tr>
<tr>
<td>6.9 Neighbor-joining phylogenetic tree based on the <em>amoA</em> gene of γ-proteobacterial ammonia-oxidizing bacteria sequenced from clone libraries constructed from the supernatant water overlying hydrothermal sediment samples (Core C) of the Guaymas Basin</td>
<td>311</td>
</tr>
</tbody>
</table>
CHAPTER ONE

An Introduction:

Potential Ammonia Oxidation in Deep-Sea Hydrothermal Plumes
All life on Earth requires nitrogen. Since there are only certain amounts of nitrogen on the planet, nitrogen has to be cycled through various systems and the subsystems therein. In the oceans, nitrogen exists in a variety of molecular forms, redox states and phases, but only some, collectively known as 'fixed nitrogen', can be utilized by marine organisms. They include ammonium, nitrite, nitrate and organic nitrogen. Nitrate is scarce in the surface waters of oligotrophic open ocean, not abundant in suboxic and anoxic waters, but plentiful in eutrophic coastal and upwelling regions, as well as in the deep-sea water column below the permanent thermocline. Altogether nitrate forms the biggest fixed nitrogen pool in the global oceans (677 Tg N) (Capone, 1991, 2000). Organic nitrogen, particularly in the dissolved forms, is the second largest pool (554 Tg N) (Capone, 1991), whereas the ammonium and nitrite pools are considered much smaller (7-8 Tg N and unknown respectively) (Capone, 1991, 2000).

The transformations of one form of nitrogen to another are primarily governed by microbially mediated redox reactions. Nitrification is the two-step oxidation of ammonium ($\text{NH}_4^+$) to nitrite ($\text{NO}_2^-$) and then nitrate ($\text{NO}_3^-$). It connects the recycling of organic nitrogen (ammonification) to denitrification, which is the major nitrogen loss term in the oceans. Nitrification may also contribute to the loss of fixed nitrogen via the gaseous by-products nitrous oxide and nitric oxide (Yoshida, 1988; Capone, 1991). The current paradigm of marine nitrogen cycling implies that the degradation of organic matter sinking from the euphotic zone releases $\text{NH}_4^+$, which will eventually be completely nitrified to $\text{NO}_3^-$ in the deep sea, and recharge the euphotic zone through regional upwellings or upward eddy diffusion. However, most marine nitrification studies to date have focused on the surface or coastal ocean (Ward et al., 1984; Ward, 1987; Ward et al., 1989; Lipschultz et al., 1990; Dore and Karl, 1996; Bianchi et al.,
Little is known about nitrification in the deep-sea water column (Lipschultz et al., 1996; Cowen et al., 1998). Although most deep-sea nitrification is considered to take place not far below the thermocline (Ward, 2000), particulate organic matter continues to sink and decompose along the way to the seafloor such that at least some degree of nitrification should continue at depth.

High NH$_4^+$ concentrations are introduced into the deep sea from all sedimented deep-sea hydrothermal systems, such as the Guaymas Basin (10.3-15.6 mM) (Von Damm et al., 1985), Okinawa Trough Backarc Basin (5 mM) (Sakai et al., 1990), as well as the unsedimented Endeavour Segment, Juan de Fuca Ridge (0.6-0.95 mM) (Lilley et al., 1993). The source of NH$_4^+$ in the Endeavour hydrothermal fluids remains undetermined, though it most likely come from the decomposition of subseafloor organic matter associated with sediments buried at an early stage of the ridge’s evolution (Lilley et al., 1993). Despite intensive fluid mixing and $>10^4$ dilution of the hydrothermal fluids with ambient deep water, up to 400 nM of NH$_4^+$ still remains in the Endeavour neutrally-buoyant hydrothermal plume, compared to $\leq 50$ nM in background deep waters and most hydrothermal plumes over unsedimented ridges (Cowen et al., 1998). NH$_4^+$ enrichment is foreseeably even greater in sedimented ridges. If nitrification were indeed a ubiquitous, though slow, process in the deep ocean, the injection of this ‘hydrothermal’ NH$_4^+$ would likely stimulate or enhance this nitrogen conversion process.

NH$_4^+$ in the Endeavour plume is removed at an estimated turnover time of 8-28 days (Cowen et al., 1998), comparable to that calculated for methane oxidation (De Angelis et al., 1993). However, the exact fate of this NH$_4^+$ is unknown. NH$_4^+$ can be removed via four pathways: aerobic autotrophic ammonia oxidation to nitrite as the first
step of nitrification, heterotrophic oxidation to remove the excessive intracellular \( \text{NH}_4^+ \) that would otherwise become toxic (Robertson and Kuenen, 1990; Wehrfritz et al., 1993), anaerobic ammonia oxidation to dinitrogen gas in strictly anoxic conditions, or assimilation into organic matter. The primary difference between autotrophic and heterotrophic ammonia-oxidation is that the energy evolved from the former reaction is harnessed for CO\(_2\) fixation. If half of the \( \text{NH}_4^+ \) removal reported in Cowen et al. (1998) were channeled to autotrophic oxidation, it could have accounted for 4-49% of total surface organic carbon flux to these plume depths (Cowen et al., 2001). The fate of this ammonium is thus important not only to the marine nitrogen cycle, but also potentially to the marine carbon cycle, particularly in the deep-sea where organic carbon production is limited.

The neutrally-buoyant hydrothermal plumes offer favorable growth conditions for aerobic chemolithoautotrophic ammonia-oxidizing bacteria (AOB): intermediate to well oxygenated waters (Ward, 1987; Bodelier et al., 1996), nearly neutral pH (Frijlink et al., 1992; Princic et al., 1998), high ammonium (Princic et al., 1998), high suspended particle concentration (Stehr et al., 1995a; Stehr et al., 1995b; Hagopian and Riley, 1998) and low (or no) light (Herrigan and Springer, 1990; Guerrero and Jones, 1996a; Guerrero and Jones, 1996b). AOB have great adaptability and are able to maintain constant cellular levels of DNA, RNA and proteins under starvation-survival state (Johnstone and Jones, 1988). They can conserve nitrifying capacity and have quick resuscitation capabilities (Bodelier et al., 1996; Tappe et al., 1999). They also have the versatility to temporarily utilize alternative substrates like methane (Jones and Morita, 1983b) and urea (Burton and Prosser, 2001), and to denitrify NO, NO\(_2\) and NO\(_2^-\) under suboxic or anoxic conditions (Poth, 1986; Wrage et al., 2001; Schmidt et al., 2002). Therefore, any spatial
and temporal variability potentially associated with hydrothermal plumes (Kadko et al., 1990) should not deter AOB from proliferating in these waters.

Ammonia oxidation is regulated by a combination of interdependent environmental factors, among which the most important are dissolved oxygen content, temperature, salinity, light, pH, substrate (NH$_4^+$/NH$_3$) availability, molar ratios of total organic carbon to total nitrogen (TOC: TN) (Verhagen et al., 1992; van Niel et al., 1993; Strauss and Lamberti, 2000), lability of organic carbon (Butturini et al., 2000; Zhu and Chen, 2001), and the metabolic capacity of the resident microbial communities. Within a deep-sea hydrothermal plume, the first five parameters stay relatively constant, yet the remaining ones vary with the position or age of the plume. While substrate availability (i.e. NH$_4^+$) would certainly be an important controlling factor on ammonia oxidation rates (Suzuki et al., 1974; Ward, 1985), it is perhaps the TOC: TN and lability of organic carbon that regulate the partitioning between autotrophic and heterotrophic N$^+$ uptakes (Butturini et al., 2000; Zhu and Chen, 2001), and so in part the resident microbial community structures. When the TOC: TN ratio is high and the organic carbon is labile, the faster-growing heterotrophs may thrive on the available organic carbon and outcompete the slower-growing autotrophic nitrifiers (Prosser, 1989) for NH$_4^+$ (Verhagen et al., 1992; van Niel et al., 1993; Strauss and Lamberti, 2000). However, the potential role of organic carbon in deep-sea nitrification remains poorly known.

Unsedimented and sedimented deep-sea hydrothermal systems contrast in some of the aforementioned regulating parameters. For instance, the NH$_4^+$ discharged from the sedimented Guaymas Basin is an order of magnitude higher than that from the unsedimented Endeavour system. Seasonal upwelling in the Guaymas Basin gives rise to high seasonal surface production, high sedimentation rate of organic carbon (Altabet et
al., 1999) and thus thick organic-rich sediment cover on the seafloor (Curray et al., 1982). Consequently, the hydrothermal fluids passing through the sediments and the subsequent hydrothermal plumes are also enriched in organic carbon and its degradation products (Welhan and Lupton, 1987; Karl et al., 1988; Martens, 1990). Some of the organic carbon may be refractory, some may inhibit nitrification (e.g. micromolar concentrations of CH₄ and CO (Hyman and Wood, 1983; Jones and Morita, 1983a, b), while some may yet be decomposing and releasing more NH₄⁺. In contrast, the Endeavour Segment is located 200 km offshore, where surface production and sedimentation rates are much lower (Roth and Dymond, 1989). A relatively large fraction of organic carbon might be produced in situ via autotrophic ammonia oxidation and other autotrophic processes within the Endeavour plume, unlike the already organic-rich Guaymas Basin water that likely favors heterotrophic activities. As a result, AOB in the Endeavour plume might form a larger proportion of the total microbial community than their counterparts in the Guaymas Basin.

The primary objective of this research project is to compare the fate and implications of the elevated NH₄⁺ in the deep-sea hydrothermal plumes at the unsedimented Endeavour Segment and the sedimented Guaymas Basin, in both geochemical and microbiological perspectives. A holistic approach involving chemical analyses, stable isotopic analyses, combined with molecular biological analyses, was employed. The tested hypotheses are as follows:

1. Autotrophic ammonia-oxidizing bacteria are ubiquitous in the deep sea, but their abundance, normally limited by the low NH₄⁺ availability, is enhanced in the NH₄⁺-rich deep-sea hydrothermal plumes at the Endeavour Segment and Guaymas Basin.
2. Ammonia oxidation rates in the hydrothermal plumes of both the Endeavour Segment and the Guaymas Basin are significantly higher than that in ambient background deep water.

3. Autotrophic ammonia oxidation is the major sink of the hydrothermally discharged NH$_4^+$ in the Endeavour hydrothermal plume, but less so in the Guaymas Basin hydrothermal plume owing to the higher heterotrophic activities of the latter.

4. Autotrophic ammonia oxidation produces at least a comparable amount of organic carbon in situ in the Endeavour plume compared to the particulate organic carbon flux from the surface ocean, yet their contribution is much less in the Guaymas Basin plume where higher organic carbon content might be present.

5. Ammonia oxidation is enhanced in particle-associated microbial communities relative to free-living microbial communities, since ammonia-oxidizing bacteria are inclined to be particle-associated.

Autotrophic ammonia-oxidizing bacteria (AOB) cluster in two major groups based on the 16S rRNA gene analyses (Head et al., 1993; Teske et al., 1994). Their unique phylogenetic characteristics have allowed the use of sensitive molecular biological techniques to detect and analyze the community structures of these microbes without cultivation, such as polymerase chain reaction (PCR) with AOB-specific primers (McCaig et al., 1994; Voytek and Ward, 1995) and fluorescence in situ hybridization (FISH) with AOB-specific 16S rRNA-targeted probes (Mobarry et al., 1996). However, hydrothermal plumes are often laden with particles (Baker et al., 1985), which potentially cause clogging during filtration and interference on fluorescence signals, thus resulting in inaccurate abundance estimates if the latter technique is used. Therefore, some sampling
and sample processing considerations for applying FISH in particle-rich seawater such as hydrothermal plumes, is explored in Chapter 2. Different combinations of existing preservation protocols, size-fractionation sequential filtration, as well as sonication are tested.

Subsequently, optimized protocols detailed in Chapter 2 were integrated and applied to detect and quantify autotrophic ammonia-oxidizing bacteria for the first time in a deep-sea hydrothermal plume. Parallel measurements of ammonia oxidation rates are related to the microbial abundance data in Chapter 3, as a preliminary testing of the presence of ammonia oxidation and ammonia-oxidizing bacteria in the Endeavour hydrothermal plume.

More detailed examination of the distribution and dynamics of ammonium and microbial ammonia oxidation rates in various parts of the Endeavour neutrally buoyant hydrothermal plume is presented in Chapter 4. This chapter also addresses the partitioning of the ammonium uptake between ammonia oxidation and assimilation, as well as their implications on nitrogen and carbon cycling.

Chapter 5 investigates the cycling of ammonium in the deep water column of the sedimented Guaymas Basin, that is influenced by a combination of productive surface water, organic-rich sediment cover, hydrothermal discharges and unique hydrography. Experiments and analyses similar to those in Chapter 4 have been performed, and the findings are compared with those from the Endeavour study.

Molecular surveys on the ammonia-oxidizing bacteria from both the Endeavour Segment and the Guaymas Basin are described in Chapter 6. Partial sequences of the 16S rRNA gene and the functional gene coding for ammonia monooxygenase subunit A (amoA) targeting specifically these bacteria are analyzed to infer their phylogenies.
Finally, Chapter 7 summarizes the important findings from earlier chapters and discusses how these results correspond to the hypotheses being tested. The potential implications and future research perspectives of ammonia oxidation in deep-sea hydrothermal plumes is further addressed.

REFERENCES


denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* **33**: 
1723-1732.


CHAPTER TWO

Processing Deep-Sea Particle-Rich Water Samples for

Fluorescence In Situ Hybridization: Considerations of Storage Effects,

Preservation and Sonication

By

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2.0. Abstract

Particles are often regarded as microniches of enhanced microbial production and activities in the pelagic ocean, and are vehicles of vertical material transport from the euphotic zone to the deep-sea. Fluorescence \textit{in situ} hybridization (FISH) can be a useful tool to study the microbial community structures associated with these particles, and thus their ecological significance; yet an appropriate protocol for processing deep-sea particle-rich water samples is lacking. Some sample processing considerations were discussed in the present study, and different combinations of existing procedures for preservation, size-fractionation sequential filtration and sonication were tested in conjunction with FISH. Results from this study showed that water samples should be filtered and processed within no more than ten to twelve hours after collection, or else preservation was necessary. The commonly used pre-filtration formaldehyde fixation was shown to be inadequate for the rRNA targeted by FISH. However, pre-filtration formaldehyde fixation followed by immediate freezing and post-filtration paraformaldehyde fixation, yielded highly consistent cell abundance estimates even after 96 days or potentially longer storage. Size-fractionation sequential filtration and sonication together enhanced cell abundance estimates by several folds. Size-fractionation sequential filtration effectively separated particle-associated microbial communities from their free-living counterparts, while sonication detached cells from particles or aggregates for more accurate cell counting using epifluorescence microscopy. Optimization in sonication time is recommended for different specific types of samples. These tested and optimized procedures can be incorporated into a FISH protocol for sampling in deep-sea particle-rich waters.
2.1. INTRODUCTION

Particles play an important role in the biogeochemical cycling of global oceans. Ranging from submicrometer colloids to transparent exopolymer particles, large mineral flocs and marine snow, particles serve as vehicles of material transport enhancing fluxes from the euphotic zone to the deep-sea (Pilskaln et al., 1995; Berelson, 2002; Jackson and Burd, 2002). The general paradigm of marine nutrient cycling is that remineralization occurs during the sinking of particulate organic matter out of the euphotic zone through the water column, and that the recycled nutrients will eventually recharge surface biological production via upwelling. Most of these remineralization processes are microbially mediated, yet little is known of the actual microbial communities and activities on particles in the deep-sea water column. In surface and coastal ocean environments, particles are considered the nutrient-rich microscale hotspots for microbial production compared to ambient seawater (Caron et al., 1986; Alldredge and Silver, 1988; Long and Azam, 2001). Such enhanced microbial production on particles is more significant in the deep-sea water column where primary production is even more limited.

Qualitative phylogenetic studies have revealed disparate microbial communities between particle-associated assemblages and their free-living counterparts in aquatic environments (DeLong et al., 1993; Acinas et al., 1999; Crump et al., 1999; Phillips et al., 1999). However, more quantitative measurements are required to elucidate the ecological significance of particle-associated microbes. Fluorescence in situ hybridization (FISH) has recently become a very useful tool to enumerate microbes in mixed communities specific at domain to species levels (Amann et al., 1995). FISH can
reveal microbial community structures, and relate microbial communities to geochemical
cycling, especially in the case of particular functional groups like nitrifiers (Mobarry et
al., 1996; Schramm et al., 1998; Gieseke et al., 2001) or methanotrophs (Murrell et al.,
1998; Bourne et al., 2000). However, a working protocol is lacking that differentiates
particle-associated microbes from free-living microbes in particle-rich seawater.

Water from the deep-sea is commonly sampled using 10- to 30-liter Niskin™
bottles mounted on a CTD-rosette package. Three major challenges have to be met for
FISH sub-sampling from these Niskin™ bottles. First, the abundance of cells and
particles in the deep-sea are relatively low, so that a large volume of water has to be
concentrated to yield sufficient working materials. This is conventionally done either by
centrifugation (Amann et al., 1990), or filtration onto filter membranes followed by cell
transfers to gelatin-coated slides for FISH (Kamer and Fuhrman, 1997). Both methods
cause several-fold greater cell losses than a protocol in which FISH is directly performed
on the same filter membranes (Lemke et al., 1997). Consequently, a direct filtration-to-
FISH method (Glöckner et al., 1996) was chosen as the basic protocol in this study.

The second challenge is concerned with sample preservation. It usually takes at
least an hour from water collection at depth to on-deck sub-sampling, and then up to
another 24 hours until the water is filtered for FISH. Therefore, it is essential to test for
any storage effects both with and without preservatives. An efficient preservation
method is imperative to ensure sample integrity until the actual hybridization procedure is
performed in a shore-based laboratory. The two most commonly used preservation
methods are post-filtration, 30-minute 4% paraformaldehyde fixation (Glöckner et al.,
1996; Pernthaler et al., 2002) and pre-filtration formaldehyde (2% final concentration)
overnight fixation (DeLong et al., 1999; Karner et al., 2001). Another option is to preserve the whole water sample with formaldehyde (2% final concentration) followed by immediate freezing (Karner et al., 2001). However, no systematic studies have been published to date that compare the effectiveness of these various preservation methods for FISH through time.

The third challenge is the differentiation between particle-associated and free-living microbial assemblages. Size-fractionation sequential filtration approaches have been successfully applied in a few phylogenetic studies to separate these two microbial assemblages in aquatic environments (Crump et al., 1999; Phillips et al., 1999); and sonication has been used to detach cells from particles for FISH (Weiss et al., 1996; Grossart and Simon, 1998; Böckelmann et al., 2002). The sonication times employed in published studies range from two seconds to thirty minutes. While too short a sonication time is obviously insufficient to detach cells from aggregates or particles, prolonged sonication likely disrupts the cells. Other available methods of microbe-particle separation include homogenization and chemical treatments with surfactants like tetrasodium pyrophosphate (Velji and Albright, 1986; Schallenberg et al., 1989), Tween 20 or 80 (Yoon and Rosson, 1990; Chevaldonne and Godfroy, 1997; dos Santos Furtado and Casper, 2000), and Triton X-100 (Nebe-von-Caron et al., 2000; Proctor and Souza, 2001), among which only tetrasodium pyrophosphate has been used with FISH thus far (Zarda et al., 1997; Grossart and Simon, 1998; Battin et al., 2001). Only sonication was tested in the present study.

The objectives of this study are to evaluate the effects of storage and various preservation methods over time, as well as the efficiency of combined size-fractionation
sequential filtration and sonication in differentiating and enumerating particle-associated and free-living microbes in marine samples. The ultimate goal is to establish a sampling protocol for FISH that is optimized for studying particle-associated versus free-living microbial assemblages in the deep-sea.

2.2. MATERIALS AND METHODS

Water samples for the various experiments were taken from three salt-water aquaria RS1, RS2 and WA, and a coastal site at Hawaii Kai on the island of Oahu, Hawaii (HK). Samples WA and HK were particularly loaded with visible particles. A direct-filtration-to-FISH method with post-filtration paraformaldehyde fixation (Glöckner et al., 1996) was used as the basic protocol. Briefly, water samples were filtered onto 0.2 μm-pore-size, 25 mm-diameter white polycarbonate membrane filters (Osmonics, Inc.), supported by 0.45 μm-pore-size nitrocellulose membrane filters (Whatman, Inc.) under a low vacuum pressure (5 in. Hg). While still on the glass filtering tower with the vacuum broken, each sample filter was fixed with 3 ml cold freshly prepared and 0.2 μm-filtered 4% paraformaldehyde in phosphate buffered saline (PBS, 1x, pH 7.2). After a 30-minute fixation, the fixative was drawn off by again applying low vacuum. Each filter was then rinsed once with 3 ml PBS (1x) and once with 3 ml deionized water; both solutions were drawn off by vacuum. An additional 1-minute cell permeabilization treatment was performed with 1 ml of 50% ethanol and 2% w/v sodium chloride. The filters were removed from the filtering towers, air-dried in the dark, and stored in sterile petri-dishes at -20 °C until hybridization. Modifications made in the various experiments are described in the following sections.
2.2.1. Experiments on Storage Effects without Preservatives

Water from RS1 and RS2 was sub-sampled within two hours after collection, and processed according to the basic protocol. The remaining water samples were refrigerated at 4°C until the next sub-sampling time, when the same procedures of filtration and fixation were repeated. The sub-sampling time intervals were 2, 3, 4, 5, 18, 21, 24 and 27.5 hours in series RS1, and were 1, 3, 7, 13.8, 21, 36, 52 hours in series RS2. Replicate samples were collected at each time interval.

2.2.2. Experiments on Preservation Methods

Two preservation methods were tested using the WA sample: (1) half of the water sample was immediately filtered and preserved with 4% paraformaldehyde as in the basic protocol; and (2) the remaining water sample was immediately preserved with formaldehyde (2% final concentration), and refrigerated (4°C) overnight (16 hours) before filtering onto 0.2 μm-pore-size white polycarbonate membrane filters.

In the RS2 sample series, five preservation methods were tested: (1) \textit{pfa}: post-filtration 4% paraformaldehyde fixation as in the basic protocol; (2) \textit{F}: pre-filtration fixation with formaldehyde (2% final concentration) for at least 8 hours; (3) \textit{F+pfa}: pre-filtration formaldehyde fixation (2% final concentration, ≥8 hours) followed by an additional post-filtration 4% paraformaldehyde fixation; (4) \textit{FF}: pre-filtration formaldehyde fixation (2% final concentration) and immediate freezing at -20°C; and (5) \textit{FF+pfa}: pre-filtration formaldehyde fixation with immediate freezing and post-filtration 4% paraformaldehyde fixation. One large acid-washed bottle was used to collect the original aquarium water, which was then divided into 1-liter and 2-liter acid-washed
polyethylene bottles for treatments (1) and (2)+(3) respectively. For treatments (4) and (5), 125-ml acid-washed bottles were filled with the original water sample, fixed with 2% formaldehyde (final concentration) and frozen immediately. At the designated time intervals, replicate bottles were taken out to thaw at room temperature two hours prior to filtration. The sub-samples for different time intervals of treatments (4) and (5) were drawn from different smaller bottles instead of one single large bottle, in order to eliminate potential degradation effects of repeated freezing and thawing. Caution was taken to ensure complete thawing before filtration. Sub-sampling time intervals were approximately 1, 3, 7, 14, 21, 36, 52 hours and 4 days for treatments (1) and (3), while treatment (2) was additionally sub-sampled after 37 days. Owing to the time required for freezing and thawing, and the limited availability of water samples, treatments (4) and (5) targeted longer time intervals of ~1, 4, 37 and 96 days. All resulting 0.2 \( \mu \text{m} \)-pore-size membrane filters were stored frozen until hybridization.

2.2.3. Experiments on Particle Separation & Sonication

The WA and HK samples were used in the particle separation and sonication experiments. The water samples were preserved with formaldehyde (2% final concentration) immediately upon sample collection. The purpose of this fixation procedure is to strengthen cellular structures and to help preserve cellular rRNAs targeted by FISH during sonication (e.g. (Weiss et al., 1996; Grossart and Simon, 1998; Boetius et al., 2000; Battin et al., 2001)). Three size-fractions were investigated in this study: \( \geq 10 \) \( \mu \text{m} \), 5.0-10 \( \mu \text{m} \) and 0.2-5.0 \( \mu \text{m} \). The water samples were first gravity-filtered through sterilized 10 \( \mu \text{m} \)-pore-size Nitex™ screens mounted on sterilized polypropylene beakers.
without bottoms, then through 5.0-μm-pore-size polycarbonate membrane filters in sterilized polycarbonate filtration units. The 5.0-μm-filtrates were subsequently filtered onto 0.2 μm polycarbonate membrane filters as the ‘free-living’ (0.2-5.0 μm) fractions. The 10 μm Nitex™ screens and the 5.0 μm membrane filters were thoroughly rinsed with 0.2 μm-double-filtered seawater into separate sterile graduated beakers. The rinsates, together with the corresponding Nitex™ screens or filters, were sonicated for 0 or 5 seconds in the WA series, and 0, 5, 10, 15, 20, 25 and 30 seconds in the HK series. An ultrasonic bath with 130 W output (Branson Ultrasonics Corp.) and 6-7 cm water depth was used. Then the rinsates were filtered onto separate 0.2 μm polycarbonate membrane filters, which represented the ‘particle-associated’ fractions (>10 μm and 5.0-10 μm). All 0.2 μm membrane filters were preserved with 4% paraformaldehyde and stored at −20°C until hybridization. Replicates were taken for each size-fraction, and whole-water samples were also processed without size-fractionation nor sonication.

2.2.4. Fluorescence In Situ Hybridization (FISH)

The hybridization procedures followed the basic protocol described in (Glöckner et al., 1996). Each membrane filter was cut into four sections for hybridization with different oligonucleotide probes. The filter sections were placed into a pre-warmed sterile 24-well microtiter plate (Nunc), one filter section per well, and overlain by 20 μl of pre-warmed sterile hybridization solution. The hybridization solutions contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS), 2.5 ng μl\(^{-1}\) fluorescently-labelled oligonucleotide probes and 20-55% formamide according to the stringencies used with the corresponding probes (Table 1). The microtiter plate was
placed in a pre-warmed equilibrated chamber saturated with an atmosphere of hybridization solution minus the probes, and incubated at 46°C for two hours. Each filter section was next transferred to a pre-warmed (48°C), sterile 20-ml glass vial filled with pre-warmed sterile washing solution. These solutions contain 56-225 mM NaCl (Table 1), 20 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.01% SDS. The filter sections were left freely floating within the vials without agitation at 48°C for 15 minutes, whereupon the washing solutions were replaced with fresh solutions. After another 15-minute incubation, the filter sections were air-dried in the dark. In case of a second hybridization, the dried filter sections were returned to the microtiter plate, and the same hybridization and washing procedures were repeated. Each filter section was then stained with 30 μl of DAPI solution (50 μg ml⁻¹) for 8 minutes in the dark, rinsed with double-deionized water and air-dried. Finally, the filter sections were mounted on to glass microscope slides with FluoroGuard™ Antifade Reagent (Bio-Rad Laboratories, Inc.), and stored at −20°C before viewing. Fixed samples from a pure culture of Nitrosomonas cryotolerans were used as positive controls during each run of FISH.

The slides were viewed under an Eclipse E400 microscope equipped with Y-FL Epi-fluorescence attachments (Nikon, Inc.) and filter sets specific for each fluorochrome used (Chroma Technology Co.). At least 20 fields or 1000 cells were counted for each hybridization. The counting arithmetic means of all replicates were averaged and reported with standard errors (± SE). All reported FISH cell counts have been corrected for any non-specific binding to non-targeted cells or non-cellular particles, and for any autofluorescence under the same wavelengths, by subtracting the counts of the negative control NON338 of the same fluorochrome used in the probes.
2.2.5. Oligonucleotide Probes

The sequences of the oligonucleotide probes employed in this study are listed in Table 2.1, together with the respective targeted microorganisms, and the stringencies used in hybridization and washing solutions. A universal oligonucleotide probe (UNI) (Giovannoni et al., 1988) was used in the sonication experiments; while EUB338, a probe specific for the Domain Bacteria (referred to as Eubacteria in the following sections) (Amann et al., 1995), and two probes specific for β-proteobacterial ammonia-oxidizing bacteria (NSO190 and NSO1225) (Mobarry et al., 1996) were used in the storage effects and preservation experiments. A negative control oligonucleotide probe NON338, (Stahl and Amann, 1991), which does not target any organisms, was used in all samples. All oligonucleotide probes were purchased custom-made with the fluorochromes Cy3 or 6FAM attached at the 5’-ends of the oligonucleotide probes (Integrated DNA Technologies, Inc.).

2.2.6. Statistical Analyses

All statistical analyses were performed using Statistica 6.0 (StatSoft®). The normality of data sets has been checked prior to any parametric tests; non-parametric tests were used for non-normal data sets. The significance of all parameters in the regression analyses presented has been verified (p<0.05) by one-way ANOVA. The overall goodness of fit and significance of the regression analyses are indicated by the $R^2$ and p-values from one-way ANOVA. In each sample series, the first data point of any type of cell counts acquired with the basic protocol is taken as the reference value, which is assumed to be the closest to the in situ abundance.
2.3. RESULTS

2.3.1. Storage Effects without Preservatives

In sample RS1, although the sample means of DAPI and Eubacterial cell counts were significantly different from their reference values (p<0.05 and p<0.01 respectively, t-tests) (Fig. 2.1a), no systematic temporal trends are discernable by one-way ANOVA or any trend analyses (p>0.05). β-Proteobacterial ammonia-oxidizing bacterial (β-AOB) abundance, as enumerated by both NS0190 and NS01225, were not significantly different from reference values (p>0.05, t-tests) and no temporal trends were evident either (p>0.05, ANOVA) (Fig. 2.1b).

However, longer storage time in RS2 resulted in reduced microbial abundance for both DAPI and FISH (Fig. 1c, d). The DAPI cell counts decreased by 26% after 52 hours, and the mean was significantly different from the reference value (p<0.05, t-test), but there was no statistically significant temporal trend. All FISH cell counts were significantly different from their respective reference values (p<0.05, t-tests). Eubacterial abundance decreased exponentially with time ($R^2=0.72$, p<0.05 ANOVA). β-AOB abundance enumerated by NS01225 also decreased (log-linear: $R^2=0.53$, p<0.05 ANOVA), and so did that enumerated by NS0190, though not significantly (linear: $R^2=0.61$, p=0.07 ANOVA).

2.3.2. Comparison of Preservation Methods

Overnight formaldehyde fixation in the WA sample yielded significantly lower UNI-hybridized cell counts than paraformaldehyde-fixation (p<0.01, Wilcoxin Matched Pairs Test), yet no significant difference could be observed in DAPI cell counts obtained
from the two preservation methods (p>0.05, Wilcoxin Matched Pairs Test) (Fig. 2.2). These results imply that formaldehyde is not as effective a fixative as paraformaldehyde for rRNAs which FISH targets, even though it may be an effective fixative for DNA to which DAPI binds.

In the RS2 sample series, five preservation methods were tested. The results for paraformaldehyde-fixation (pfa) were the same as those for the RS2 storage effect experiment, in which all FISH cell counts showed significantly different means from reference values (p<0.05, t-tests) with systematic declining trends (Fig. 2.3 a, 2.4 a). Unlike the WA sample, formaldehyde-fixation (F) in the RS2 series did cause highly significantly lower cell counts in both DAPI and EUB with respect to reference values (p<0.005 and p<0.0005 respectively, t-tests), probably due to the longer preservation time. There was a systematic decline in NSO190-detected β-AOB counts (natural-logarithmic, $R^2$=0.69, p<0.05 ANOVA) (Fig. 2.4 b), but not in other cell counts (p>0.05 ANOVA) (Fig. 2.3 b, 2.4b). In comparison, the formaldehyde-paraformaldehyde fixation method (F+pfa) resulted in no significant difference in any cell counts from the respective reference values (p>0.05, t-tests). However, if the anomalously high values at 22 h were excluded (Fig. 2.4 c), the remaining data showed an exponential decrease in β-AOB abundance as detected by both NSO190 ($R^2$=0.95, p<0.05, one-way ANOVA) and NSO1225 ($R^2$=0.76, p<0.05 ANOVA). The anomalies at 22 h might be a result of heterogeneity introduced from particle-associations common for β-AOB (Stehr et al., 1995; Phillips et al., 1999). There were no noticeable temporal trends in DAPI and EUB, but the data variability was quite large (Fig. 2.3c).
The fourth preservation method, formaldehyde-freezing (FF), resulted in significantly different DAPI and EUB cell counts from their reference values (p<0.05, t-tests), but no particular declining temporal trends were discernable in the data (Fig. 2.3 d, 2.4 d). Lastly, the combined formaldehyde-freezing-paraformaldehyde method (FF+pfa) caused neither significantly different means from the respective references (p>0.05, t-tests), nor any declining temporal trends in any of the cell counts (p>0.05 ANOVA), even after 96 days of storage. In fact, most of the measured values lie within the 95% confidence intervals of the respective original values (Fig. 2.3 e, 2.4 e). These results were summarized in Table 2.2.

2.3.3. Particle Separation & Sonication Effects

Substantial amounts of cell aggregates and exopolymeric substances (EPS) were present in both the WA and HK whole-water samples, making cell counting under the microscope difficult. Size-fractionation sequential filtration clearly facilitated cell counting. In the WA sample, the sums of all size-fractions in DAPI and UNI-hybridized cell counts yielded total microbial abundances that were 220% and 148%, respectively, of the non-fractionated whole-sample (Table 2.3). Similarly, 239% total DAPI counts and 167% total UNI counts were obtained after size-fractionation sequential filtration in the HK sample (Table 2.3).

Brief sonication (5 seconds) in the WA sample increased the detected DAPI cell counts in all individual size-fractions by 0.3-2.2 fold (p<0.05, one-tailed pair t-test) and, similarly, the UNI cell counts by 1.5-10 fold (p=0.09, one-tailed pair t-test). Consequently, the sums of all sonicated size-fractions yielded 291% DAPI and 326% UNI total microbial abundance relative to the non-sonicated whole sample (Table 2.3).
Longer sonication in the HK sample significantly increased both the DAPI and UNI cell counts, especially after >15-20 seconds in both 5.0-10 μm (p<0.01 t-tests for both DAPI and UNI) and >10 μm size-fractions (DAPI: p<0.005; UNI: p<0.05, t-tests) (Fig. 2.5). Trend analyses indicate significant third-order relationship between cell counts and sonication time in the 5.0-10 μm fraction (R²=0.996, p<0.001 for DAPI and R²=0.945, p<0.05 for UNI), but not in the >10 μm size-fraction (R²=0.842, p=0.35 for DAPI and R²=0.729, p=0.22 for UNI). The total microbial abundance as the sum of the two sonicated large size-fractions and the non-sonicated 0.2-5.0 μm size-fraction (no sonication was performed in this size-fraction), is equivalent to 370% and 272% of the non-sonicated whole-sample estimates by DAPI and UNI respectively (Table 2.3). Further sonication (>20-25 seconds), however, started to reduce the cell counts in both the 5.0-10 μm and >10 μm size-fractions. In general, more even cell distribution was achieved on filters, and cell counting became much easier with less flocculent material present after sonication.

2.4. DISCUSSIONS

2.4.1. Sample Storage and Preservation

Prolonged storage of unpreserved water samples in a container may result in either increases or decreases in observed microbial abundance. Examples of these ‘bottle effects’ include biofilm formation on the interior walls of bottles, cell aggregation, unusually fast microbial growth due to exclusion of some large natural predators during sampling, decreases due to cell deaths or substrate depletion, and changes in microbial community structure due to protistan predation. In the RS1 sample, the storage time of
27.5 hours without preservation induced only minor positive changes to the microbial abundance estimates. However, after approximately 10-12 hours, all abundance estimates in the RS2 sample decreased systematically falling below the 95% confidence intervals (95% CI). Although the exact temporal trends observed may not necessarily apply to all samples from various environments, these results strongly imply that unpreserved water samples can only be stored for a limited time without significant changes in microbial abundance; in this case no more than 10-12 hours. If longer processing time is required, preservation is imperative.

Pre-filtration 2% formaldehyde fixation is the most common alternative to post-filtration paraformaldehyde fixation (e.g. (DeLong et al., 1999; Karner et al., 2001; Rudolph et al., 2001)), and is also a routine fixation method in DAPI-staining for total microbial abundance (Porter and Feig, 1980). Good sample preservation for FISH should retain all cellular ribosomal RNA content, protect cell integrity and morphology, and allow good probe penetration during hybridization. Paraformaldehyde (O-CH₂-O-CH₂-O-CH₂) is a trimer of formaldehyde (HCHO), and both are cross-linking fixatives which form DNA-protein cross-links within the cells. Due to its monomer structure, formaldehyde is the least cross-linking, takes longer to form sufficient irreversible cross-links within the cells, and is thus the least stabilizing among all aldehydes. Thus, the required fixation time was usually at least overnight or 16 hours in the above studies. In both the WA and RS2 samples, DAPI and most FISH cell counts obtained using formaldehyde fixation alone were significantly lower than the reference values. In RS2, the 46% reduction in DAPI cell counts after 37 days of preservation in formaldehyde, is similar to the 39% reduction reported in glutaraldehyde-fixed samples over 40 days in
another study (Turley and Hughes, 1992), but slower than the 30-40% reduction observed in viral abundance in formaldehyde-fixed samples after 7 days (Danovaro et al., 2001).

Since RNA is less stable than DNA, such ineffectiveness in formaldehyde-fixation is even more pronounced in FISH cell counts. The EUB338-detected cell abundance in the same RS2 sample was reduced by 53% after 37 days, NSO190-detected β-AOB by 71% and NSO1225-detected β-AOB by 48%. Although all cell counts at 2 and 4 days seemed to be close to the reference values, judging from the overall temporal trends these data points at 2 and 4 days appeared to be anomalies rather than representative measurements of the sample. The anomalies could have resulted from sample heterogeneity due to particle associations. On the whole, formaldehyde is an inadequate preservative for both rRNA in FISH, and DNA in DAPI-staining after prolonged storage.

In some studies, formaldehyde has also been used in conjunction with another aldehyde or precipitating preservatives like methanol or ethanol (e.g. (DeLong et al., 1989; Battin et al., 2001)). The addition of 4% paraformaldehyde after filtration (F+pfa) in sample series RS2 appeared to have stabilized the nucleic acids in the first few hours. Most DAPI and EUB cell estimates in F+pfa treated samples lay within the 95% CI, yet they also showed the largest variability among all tested methods (standard deviation = 22% and 23% of the means). This preservation method may have been adequate only for the first ten hours, after which both β-AOB estimates decreased exponentially, falling well below the 95% CI.

For long-term storage, immediate freezing with formaldehyde (FF), or even better the combined formaldehyde-freezing-paraformaldehyde fixation (FF+pfa), seem to be
the most effective preservation methods. Both methods resulted in the smallest variability among the five methods tested (e.g. standard deviation = 16% and 14% of the means of EUB), except for an outlier at 4 days for β-AOB. In fact, most abundance estimates for FF+pfa treated samples lay within the 95% CI of the reference values even after 96 days. Early cryopreservation evidently helped the retention of cellular rRNA. The FF and especially FF+pfa preservation methods are particularly useful for deep-sea sampling where time is often limited.

2.4.2. Particle Separation and Sonication

Quantifying microbial abundance in particle-rich waters using epifluorescence microscopy can be challenging. For example, flocculent materials often introduce background fluorescence, and the three-dimensional structures of cell aggregates hinder accurate cell counting. In addition, some mineral particles autofluoresce in the same emission wavelengths. Separation of microbes from particles should result in more accurate abundance estimates. In this study, size-fractionation sequential filtration removed the large particles (>10 μm) from smaller particles (<10 μm) or free-living cells, that helped reduce clogging in subsequent filtrations. The division into various size-fractions also provides an opportunity to examine microbial association with various particle-sizes, while revealing the characteristics of the particles. In the WA and HK samples, significantly higher microbial abundance estimates from DAPI and FISH were obtained after size-fractionation, and there seemed to be effective separation between the particle-associated and free-living microbial assemblages. Size-fractionation sequential filtration has been commonly applied in various aquatic ecological studies (Mousseau et al., 1996; Crump et al., 1999; Riemann et al., 2000; Simek et al., 2001).
Sonication has been widely applied to separate microbes from particles in soil (Ramsay, 1984; Sessitsch et al., 2001), sediments (Ravenschlag et al., 2001; Danovaro et al., 2002), biofilms (Battin et al., 2001), water samples (Velji and Albright, 1986), limnetic snow (Weiss et al., 1996; Grossart and Simon, 1998) and a hydrothermal vent chimney (Chevaldonne and Godfroy, 1997). Sonication invariably increased microbial abundance estimates. In this study, very few aggregates remained after sonication, and cells could be counted with greater ease on one plane of view under the microscope. The optimal sonication time in the HK sample was determined to be 15 to 20 seconds for both particle size-fractions, resulting in over 270% of the reference cell counts when combined with the use of size-fractionation sequential filtration. The optimal sonication time likely depends on the surface characteristics of cells and associated particles, or the presence of any chemical surfactants. For instance, out of the few reported studies, the optimum sonication times were 60 seconds for bacteria on leaf litter (Shelley and Perry, 2000), and 3 minutes for viruses in marine sediments (Danovaro et al., 2001). Moreover, since further sonication after the optimal time started to reduce cell counts, optimization tests for sonication time are highly recommended for specific applications.

2.5. CONCLUSIONS

This study illustrates the importance of validating different combinations of existing procedures for FISH sampling in specific environments. In this study, the commonly used pre-filtration formaldehyde fixation was inferior to post-filtration paraformaldehyde fixation for FISH processing, and was inadequate for prolonged storage. If long-term storage is required, the pre-filtration formaldehyde fixation followed by immediate freezing and post-filtration paraformaldehyde fixation is the
optimal preservation method, and is particularly useful for intensive deep-sea sampling. Size-fractionation sequential filtration is effective in separating particles into various size-classes, and sonication is effective in detaching microbes from particles or aggregates. It is necessary to optimize the sonication time for specific sample types. Together, size-fractionation sequential filtration and sonication can significantly increase cell abundance estimates compared to non-fractionated and non-sonicated whole-samples in particle-rich waters. These optimized procedures can be incorporated into one effective FISH sampling protocol to study particle-associated microbes versus free-living microbes in the deep-sea water column.

2.6. REFERENCES


Heterogeneity of Sediment Biofilms along Environmental Gradients in a Glacial Stream.

Berelson, W. M. (2002). Particle settling rates increase with depth in the ocean. *Deep-Sea

aggregates by a combined technique of fluorescent in situ hybridization and lectin-

Boetius, A., Ravenschlag, K., Schubert, C. J., D., R., Widdel, F., Gieseke, A., Amann, R.,

rDNA probes for specific detection methane oxidising bacteria. *FEMS Microbiology

populations in macroaggregates (marine snow) from surface waters of the North Atlantic.
*Journal of Marine Research* 44: 543-565.


attached and free-living bacterial communities in the Columbia River, its estuary, and the

Virus Abundance in Marine Sediments. *Applied and Environmental Microbiology* 67(3):
1384-1387.


Table 2.1. List of the 16S rRNA-targeted oligonucleotide probes used in this study, together with the target organisms, target sites on the 16S rRNA gene with respect to \textit{E. coli} positions, formamide concentrations (\%FA) in hybridization solutions and NaCl concentrations in the stringent washing solutions. ‘N/A’ means ‘not applicable’. W = A or T, K = G or T.

<table>
<thead>
<tr>
<th>ProbeName</th>
<th>Sequences</th>
<th>Target Organisms</th>
<th>Target site (\textit{E. coli} Positions)</th>
<th>%FA</th>
<th>NaCl (mM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNI</td>
<td>5'-GWA TTA CCG CGG CKG CTG -3'</td>
<td>Universal</td>
<td>519-536</td>
<td>20</td>
<td>70</td>
<td>(Giovannoni \textit{et al.}, 1988)</td>
</tr>
<tr>
<td>EUB 338</td>
<td>5'-GCT GCC TCC CGT AGG AGT -3'</td>
<td>Domain Bacteria</td>
<td>338-355</td>
<td>20</td>
<td>225</td>
<td>(Amann \textit{et al.}, 1995; Schramm \textit{et al.}, 1998)</td>
</tr>
<tr>
<td>NON 338</td>
<td>5'-ACT CCT ACG GGA GGC AGC -3'</td>
<td>Negative control</td>
<td>N/A</td>
<td>20</td>
<td>225</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>NSO 190</td>
<td>5'-CGA TCC CCT GCT TTT CTC C -3'</td>
<td>(\beta)-Proteobacterial AOB</td>
<td>190-208</td>
<td>55</td>
<td>20</td>
<td>(Mobarry \textit{et al.}, 1996; Schramm \textit{et al.}, 1998)</td>
</tr>
<tr>
<td>NSO1225</td>
<td>5'-CGC GAT TGT ATT ACG TGT GA -3'</td>
<td>(\beta)-Proteobacterial AOB</td>
<td>1225-1244</td>
<td>35</td>
<td>191</td>
<td>(Mobarry \textit{et al.}, 1996; Schramm \textit{et al.}, 1998)</td>
</tr>
</tbody>
</table>
Table 2.2. Summary of results using different preservation methods in saltwater aquarium RS2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>All Microbes (DAPI)</th>
<th>Eubacteria (EUB338)</th>
<th>(\beta)-AOB (NSO190)</th>
<th>(\beta)-AOB (NSO1225)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Different Means(^1)</td>
<td>Declining Trend(^2)</td>
<td>Different Means(^1)</td>
<td>Declining Trend(^2)</td>
</tr>
<tr>
<td>pfa</td>
<td>Y</td>
<td>NS</td>
<td>Y</td>
<td>Exponential</td>
</tr>
<tr>
<td>F</td>
<td>Y</td>
<td>NS</td>
<td>Y</td>
<td>NS</td>
</tr>
<tr>
<td>F+pfa</td>
<td>N</td>
<td>NS</td>
<td>N</td>
<td>NS</td>
</tr>
<tr>
<td>FF</td>
<td>Y</td>
<td>NS</td>
<td>Y</td>
<td>NS</td>
</tr>
<tr>
<td>FF+pfa</td>
<td>N</td>
<td>NS</td>
<td>N</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) Significance of difference between experimental treatments and the corresponding reference values was determined by t-test with \(p<0.05\).

\(^2\) Significance of declining time trends was verified by one-way ANOVA with \(p<0.05\).

\(^3\) These two exponential declining trends were observed after excluding the data points at ~22 hours.

Y=Yes, N=No, NS=Not significant.
Table 2.3. Effects of size-fractionation and sonication on total microbial abundance estimates by DAPI-staining and FISH with UNI oligonucleotide probes. Cells abundances are expressed as Mean ± SE x 10^4 cells ml⁻¹ for the WA sample and x 10^5 cells ml⁻¹ for the HK sample. Σ_{all fractions} represent the sums of all size-fractions, and %WS indicates the percentage relative to the non-fractionated, non-sonicated whole-sample. Since no sonicated data are available for the 0.2-5.0 μm size-fraction in the HK sample, the non-sonicated cell counts were used to calculate the sonicated Σ_{all fractions}.

<table>
<thead>
<tr>
<th>Sample</th>
<th>WA</th>
<th></th>
<th>HK</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-sonicated</td>
<td>Sonicated</td>
<td>Non-sonicated</td>
<td>Sonicated</td>
</tr>
<tr>
<td>Size Class</td>
<td>DAPI %WS</td>
<td>UNI %WS</td>
<td>DAPI %WS</td>
<td>UNI %WS</td>
</tr>
<tr>
<td>Free-living</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2-5.0μm</td>
<td>19.9 ± 1.8</td>
<td>10.8 ± 1.0</td>
<td>38.2 ± 2.3</td>
<td>19.5 ± 2.9</td>
</tr>
<tr>
<td>Particle-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Associated</td>
<td>1.9 ± 0.7</td>
<td>0.5 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>&gt;10μm</td>
<td>3.3 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>Σ_{all fractions}</td>
<td>25.1 ± 4.9</td>
<td>2.20</td>
<td>12.7 ± 2.8</td>
<td>148</td>
</tr>
<tr>
<td>Whole-sample (WS)</td>
<td>11.4 ± 1.1</td>
<td>100</td>
<td>8.6 ± 0.9</td>
<td>100</td>
</tr>
</tbody>
</table>

45
Figure 2.1. Effects of storage without preservatives: Temporal changes in total microbial abundance estimated by DAPI-staining (●) and Eubacterial abundance by EUB338 (▽) in (a) RS1 and (c) RS2; and temporal changes in β-AOB abundance by NSO190 (●) and by NSO1225 (◇) in (b) RS1 and (d) RS2. The dotted and dashed lines in (a) and (c) indicate the 95% confidence intervals (95%CI) of DAPI and EUB reference values respectively. In (b) and (d), the dotted and dashed lines are the 95% CI of the NSO190 and NSO1225 reference values respectively. Please note the longer time scale used for RS2 in panels c and d. Error bars, standard errors.
Figure 2.2. Combined microbial abundance estimates of different size-fractions with post-filtration paraformaldehyde and pre-filtration overnight formaldehyde fixation in sample WA: total microbial abundance estimated by DAPI-staining and FISH with the universal oligonucleotide probe (UNI). The mid-lines represent median values, the boxes and bars are the 50- and 75-percentiles, and the ‘+’s indicate the outliers.
Figure 2.3. Total microbial abundance estimated by DAPI-staining (●) and Eubacterial abundance by EUB338 (▽) in sample series RS2 preserved with (a) paraformaldehyde (pfa), (b) formaldehyde (F), (c) combined formaldehyde-paraformaldehyde (F+pfa), (d) formaldehyde-freezing (FF) and (e) combined formaldehyde-freezing-paraformaldehyde (FF+pfa). Dashed lines show the 95% CI of the EUB reference, and the dotted lines show the 95% CI of the DAPI reference. Note the linear time scale in (a) but logarithmic time scale in (b)-(e). Error bars, standard errors.
Figure 2.4. Abundance of β-Proteobacterial ammonia-oxidizing bacteria (β-AOB) detected by NS0190 (●) and by NS01225 (○) in RS2 subsamples preserved with (a) pfa, (b) F, (c) F+pfa, (d) FF and (e) FF+pfa. Dashed lines show the 95% CI of the NS01225 reference and the dotted lines show the 95% CI of the NS0190 reference. Note the linear time scale in (a) but logarithmic time scale in (b)-(e). Error bars, standard errors.
Figure 2.5. Increases in total microbial abundance estimated by DAPI-staining (●) and UNI-hybridization (○) after sonication, with an optimal sonication time at around 15-20 seconds in both (a) 5.0-10 μm and (b) >10 μm size fractions. Error bars, standard errors.
CHAPTER THREE

Autotrophic Ammonia Oxidation in a Deep-Sea Hydrothermal Plume

By

Phyllis Lam

James P. Cowen and Ronald D. Jones (co-authors)

3.0. ABSTRACT

Direct evidence for autotrophic ammonia oxidation was documented for the first time in a deep-sea hydrothermal plume. Elevated NH$_4^+$ concentrations of up to 341 ± 136 nM were recorded in the plume core at Main Endeavour Field (MEF), Juan de Fuca Ridge. This fueled autotrophic ammonia oxidation rates as rapid as 91 nM d$^{-1}$, or 92% of the total net NH$_4^+$ removal. High abundance of ammonia-oxidizing bacteria (AOB) was detected using fluorescence in situ hybridization (FISH). AOB within the plume core (1.0 to 1.4 x 10$^4$ cells ml$^{-1}$) accounted for 7.0-7.5% of total microbial community, and were at least as abundant as methanotrophs. AOB were a substantial component of the particle-associated communities (up to 51%), with a predominance of the r-strategists *Nitrosomonas*-like cells. *In situ* chemolithoautotrophic organic carbon production via ammonia oxidation may yield 3.9-18 mg C m$^{-2}$ d$^{-1}$ organic carbon within the plume directly over MEF. This rate was comparable to that determined for methane oxidation in a previous study, or at least four-fold greater than the flux of photosynthetic carbon reaching plume depths measured in another study. Hence, autotrophic ammonia oxidation in the neutrally-buoyant hydrothermal plume is significant to both carbon and nitrogen cycling in the deep-sea water column at Endeavour, and represents another important link between seafloor hydrothermal systems to deep-sea biogeochemistry.
3.1. INTRODUCTION

Anomously high ammonium concentrations are found in the end-member fluids of the unsedimented deep-sea hydrothermal system of Endeavour Segment, Juan de Fuca Ridge (0.6-0.95 mM), relative to other unsedimented deep-sea hydrothermal systems (<0.01 mM) (Lilley et al., 1993). In spite of intensive fluid mixing and ≥10⁴ dilution with ambient deep water, ammonium concentrations of up to 400 nM still remain in the neutrally-buoyant hydrothermal plume over Endeavour compared to ≤50 nM in background deep waters and in typical hydrothermal plumes over unsedimented ridges (Cowen et al., 1998). Ammonium in the Endeavour plume is rapidly removed with an estimated turnover time of 8 to 28 days (Cowen et al., 1998), comparable to that observed for methane oxidation (De Angelis et al., 1993).

Ammonium can be removed from seawater via four pathways: aerobic autotrophic oxidation to nitrite as the first step of nitrification, heterotrophic oxidation as a likely mechanism to remove excess intracellular reductants (Robertson and Kuenen, 1990; Wehrfritz et al., 1993), anaerobic ammonia oxidation to dinitrogen gas, or assimilation into organic matter. The primary difference between aerobic autotrophic and heterotrophic ammonia oxidation is that the energy evolved from the former reaction is used to fix CO₂. Heterotrophic ammonia oxidation is usually thought to be much slower than and out-competed by the autotrophic counterparts in most aerobic environments (Focht and Verstraete, 1977), but recent studies showed that it could prevail under high C: N, high organic carbon or low dissolved oxygen conditions where coupling with aerobic denitrification took place (Robertson and Kuenen, 1990; Verhagen and
Laanbroek, 1991; van Niel et al., 1993). The third pathway, anaerobic ammonia oxidation (or Anammox), only occurs under anoxic conditions (e.g. Dalsgaard et al., 2003; Kuypers et al., 2003), so it is possible only in association with macro-aggregate particles within the well-oxygenated neutrally-buoyant plume, or within the underlying sediments. If half of the ammonium removal previously reported in the Endeavour neutrally-buoyant plume (Cowen et al., 1998) was due to aerobic autotrophic oxidation (with the other half due to assimilation), it would be equivalent to 4-49% of surface derived organic carbon flux to these plume depths (Cowen et al., 2001). The fate of this ammonium is thus important not only to the marine nitrogen cycle, but also potentially to the deep-sea marine carbon cycle.

The deep-sea hydrothermal plume at Endeavour offers favorable growth conditions for aerobic chemolithoautotrophic ammonia-oxidizing bacteria (AOB): intermediate to well oxygenated waters (Ward, 1987b; Bodelier et al., 1996), nearly neutral pH (Frijlink et al., 1992; Princic et al., 1998), high but not exceedingly high ammonium (Princic et al., 1998), high suspended particle concentrations (Stehr et al., 1995a; Stehr et al., 1995b; Hagopian and Riley, 1998) and no light (Horrigan and Springer, 1990; Guerrero and Jones, 1996a; Guerrero and Jones, 1996b). AOB have great adaptability and are able to maintain cellular levels of DNA, RNA and proteins under starvation-survival state (Johnstone and Jones, 1988). They can conserve nitrifying capacity with quick resuscitation capabilities (Bodelier et al., 1996; Tappe et al., 1999). They also have the versatility to temporarily utilize alternative substrates like methane (Jones and Morita, 1983) and urea (Burton and Prosser, 2001), and to denitrify NO, NO₂ and NO₃⁻ under suboxic or anoxic conditions (Poth, 1986; Wrage et al., 2001; Schmidt et
Therefore, any spatial and temporal variability potentially associated with hydrothermal plumes (Kadko et al., 1990) should not deter AOB from proliferating in these waters.

Based on phylogenetic analyses of the 16S rRNA gene, AOB cluster in the β- and γ-subgroups of Proteobacteria. The majority, including Nitrosomonas spp. and Nitrosospira spp., belong to β-Proteobacteria; whereas to date only three species have been classified into γ-Proteobacteria, namely Nitrosococcus oceani, Nitrosococcus halophilus and Nitrosococcus sp. C113 (Teske et al., 1994; Purkhold et al., 2000). Such monophyletic characteristics allow partial sequences specific for these AOB at subclass to species levels to be used as oligonucleotide probes in whole-cell fluorescence in situ hybridization (FISH) (e.g. (Mobarry et al., 1996) for identification and enumeration of AOB in mixed microbial communities.

The objective of the present study was to test whether microbial autotrophic ammonia oxidation occurs in the neutrally-buoyant plume of Endeavour, using a combination of molecular (FISH) and geochemical approaches. Population sizes and distributions of active AOB in the Endeavour hydrothermal plume were evaluated in conjunction with autotrophic ammonia oxidation rate measurements. The importance of these nitrifiers to the overall microbial community, and their relative contributions to the particle-associated versus free-living assemblages were also examined.
3.2. MATERIALS AND METHODS

3.2.1. Site Description and Sample Collection

Endeavour is the northern-most segment of Juan de Fuca Ridge (Fig. 3.1). It is a 300 km long 10 km wide, north-south trending crustal feature, with a 100-150 m-deep axial valley reaching ~2200 m depth on the valley floor (Thomson et al., 1992). There are five known active vent fields with ~2-3 km spacing, and active venting of hot focused (317-400°C) and warm diffuse hydrothermal fluids (Delaney et al., 1984; Lilley et al., 1993; Butterfield et al., 1994). The vigorous venting produces a hydrothermal plume in the water-column marked by distinct temperature and particle anomalies (Thomson et al., 1992), elevated concentrations of volatiles (H₂, CH₄ and NH₄⁺) (De Angelis et al., 1993; Cowen et al., 1998; Cowen et al., 2002), and various dissolved and particulate trace metals and organic matter (Dymond and Roth, 1988; Roth and Dymond, 1989; Cowen et al., 2001).

Water-column sampling was conducted using a CTD-transmissometer-Niskin™ bottle rosette package from the R/V Thomas G. Thompson. The neutrally-buoyant hydrothermal plume over the Main Endeavour vent Field (MEF) was sampled in the summers of 1999 and 2000, and an intended background station was included in each year (Fig. 1). In 2000, the remotely operated vehicle (ROV) JASON was used to sample the buoyant plume at 20 m above bottom (mab) with 1-liter Niskin™ bottles over Easter Island, a diffuse venting area within the MEF (JAS286-2), and Clam Bed, a diffuse venting area at 650 m to the south of the High Rise vent field (JAS287-2). Warm discharge fluids were also collected at Clam Bed (JAS287-1) in the same manner.
3.2.2. Ammonia Oxidation Experiments

Subsamples for NH$_4^+$ were drawn through a clean 202 μm Nitex™ screen into acid-cleaned 125 ml polyethylene bottles. Duplicate time-zero subsamples were frozen (-20°C) immediately. NH$_4^+$ removal rates were determined by single-end-point experiments, in which duplicates from selected depths were incubated at in situ temperature (2°C) and 1 atm in the dark for approximately 24 hours. This incubation time was within the linear phase of total net NH$_4^+$ removal according to preliminary time-series measurements (Cowen et al., 1998). Parallel duplicate subsamples were incubated after treatment with 86 μM (final concentration) of allylthiourea (ATU), a selective inhibitor to autotrophic ammonia oxidation (Ginestet et al., 1998) that has been successfully applied in aquatic nitrification studies (Feliatra and Bianchi, 1993; Bianchi et al., 1997; de Bie et al., 2002). To a lesser extent, methane oxidation by methanotrophs is also inhibited by ATU (Bédard and Knowles, 1989). This might lead to a reduction in methanotroph metabolic activities and hence possibly a reduction both in methanotroph assimilation of NH$_4^+$, and in their input of recycled NH$_4^+$ back to the seawater. The net effect of ATU-to-methanotroph inhibition on NH$_4^+$ net loss rate measurements was assumed to be negligible in this study. Although methanotrophs are also capable of oxidizing ammonia, it occurs at a much slower rate with a much higher (micromolar level) NH$_4^+$ saturation coefficient ($K_m$) (Bédard and Knowles, 1989). The NH$_4^+$ concentrations in the Endeavour plume were too low to stimulate this process at a significant level. All incubation experiments were terminated via freezing at -20°C, and the samples were kept frozen until further analyses.
Analyses for NH$_4^+$ followed the fluorescence method (Jones, 1991), and duplicate measurements were made for each of the duplicate subsample. The NH$_4^+$ concentration of each sample is reported as the mean of the four measurements followed by the standard error. Total net NH$_4^+$ removal rates ($R_{\text{total}}$) were calculated as total net NH$_4^+$ loss divided by incubation time, and the remineralization of NH$_4^+$ was not controlled. Autotrophic ammonia oxidation rates ($R_{\text{oxid}}$) were calculated as the difference between the net NH$_4^+$ removal rates with and without the presence of ATD. If $N_0$, $N_i$ and $N_{\text{ATU}}$ are the measured NH$_4^+$ concentrations of time-zero subsamples, untreated incubated subsamples and ATU-treated incubated subsamples, respectively, and $t$ is the incubation time, then:

$$R_{\text{oxid}} = \left[\frac{(N_0 - N_i)}{t}\right] - \left[\frac{(N_0 - N_{\text{ATU}})}{t}\right],$$

or simply,

$$R_{\text{oxid}} = \frac{(N_{\text{ATU}} - N_i)}{t}.$$

Standard errors of total net removal and ammonia oxidation rates were computed using propagation of errors from the variances in the $N_0$, $N_i$ and $N_{\text{ATU}}$ terms involved. Specific ammonia oxidation rate constants ($k_{\text{oxid}}$) were taken as the first-order forward reaction rate constants, where $R_{\text{oxid}}$ was normalized by $N_0$ (i.e. $k_{\text{oxid}} = R_{\text{oxid}}/N_0$). NH$_4^+$ turnover times with respect to ammonia oxidation were the reciprocals of $k_{\text{oxid}}$.

3.2.3. Oligonucleotide Probes

Using fluorescence in situ hybridization (FISH), $\beta$-proteobacterial ammonia-oxidizing bacteria ($\beta$-AOB) were detected by the 16S rRNA-targeted oligonucleotide probe NSO190 (Mobarry et al., 1996). This probe is highly specific for $\beta$-AOBs,
although it may miss some known \(\beta\)-AOB species (Utäker and Nes, 1998; Purkhold et al., 2000). Thus, the detected abundances should be treated as conservative estimates. NSO1225, which has a wider coverage but less specificity than NSO190 (Mobarry et al., 1996), was applied in conjunction with NSO190 in 2000, in an attempt to obtain a range of values that likely include the true \(\beta\)-AOB abundance. However, NSO1225 resulted in inconsistent cell counts in which the signal-to-noise ratios were noticeably lower compared to NSO190, perhaps due to the inferior fluorochrome used (6-FAM) versus Cy3 (of NSO190). Therefore, the results from NSO1225 are not reported here. In 2000, *Nitrosomonas* and *Nitrosospira* were further distinguished by the genera-specific probes Nsm156 and Nsv443 respectively (Mobarry et al., 1996). Type I and Type II methanotrophs were identified by the probes 10\(\gamma\) and 9\(a\) (Tsien et al., 1990). Cell counts using these two probes may overestimate methanotroph abundance, since the target sequences include some non-methanotrophs according to the current database (Cole et al., 2003). A universal probe (UNI) (Giovannoni et al., 1988) and a eubacterial probe (EUB338) (Amann et al., 1990) were also applied. Although EUB338 has been commonly used in various environmental studies, it apparently does not hybridize with *Plantomycetales* and *Verrucomicrobia* (Daims et al., 1999), so the resulting eubacterial abundance estimates may be conservative. The sequences of all oligonucleotide probes employed are listed in Table 3.1. These oligonucleotide probes were purchased custom-made with the fluorochromes Cy3 or 6-FAM attached at the 5'-ends (Integrated DNA Technologies, Inc.).
3.2.4. *Fluorescence In Situ Hybridization (FISH)*

Subsamples for FISH were collected in acid-cleaned 1 liter-polyethylene bottles, stored at 2°C in the dark and filtered within 24 hours. The sampling, fixing and hybridization procedures for FISH followed the protocol described by (Glöckner et al., 1996), with an additional post-fixation cell permeabilization step (1-minute-incubation with 1 ml 50% ethanol and 2% w/v sodium chloride). This protocol was performed directly on membrane filters and included a post-hybridization DAPI-staining. Depending on the particle loading of the water samples, 50-300 ml aliquots of the 1-liter water subsample were filtered in triplicates. Each membrane filter was cut into sections for hybridization with different oligonucleotide probes. FISH with each oligonucleotide probe were replicated on at least two of the triplicate subsample filters. The hybridization stringencies used for each oligonucleotide probe are listed in Table 3.1.

The processed filters were mounted on microscope slides with FluoroGuard™ Antifade Reagent (Bio-Rad Laboratories). They were viewed under an Eclipse E400 microscope equipped with Y-FL Epi-fluorescence attachments (Nikon, Inc.) and filter sets specific for viewing each fluorochrome used (Chroma Technology Co.). The microscope was also equipped with a SPOT RT Monochrome digital camera system (Diagnostic Instruments, Inc.) for photodocumentation. At least 20 fields were counted for each oligonucleotide probe on each of the replicate subsample filters. All cell counts (except for DAPI) have been corrected for any non-specific binding to non-targeted cells or non-cellular particles, and for any autofluorescence under the same emission wavelengths, by subtracting the counts of the negative control NON338 of the same fluorochrome used in the probes. This amounts to a median corrections of 5.7 % (2% and 15% lower and
upper quartiles respectively) relative to uncorrected counts for UNI and EUB, and of 62% (30% and 93% lower and upper quartiles) for other oligonucleotide probes. As a general observation, the percentage of this correction increases as the amount of particle-loading or cell aggregates increase. The mean values of replicate subsample counts are presented here with their standard errors, calculated by propagation of errors from variances in each replicate subsample counts.

3.2.5. Sampling for Particle-Associated and Free-living Microbial Communities

In 2000, size-fractionation sequential filtration was performed to distinguish between particle-associated and free-living communities (Lam and Cowen, submitted). One-liter water samples were immediately treated with 0.2 μm-filter-sterilized formaldehyde (3.7% final concentration) prior to filtration. Then samples were sequentially filtered through sterilized 10 μm Nitex™ screens, followed by 3.0-μm polycarbonate membrane filters. Aliquots of the final filtrates were filtered onto 0.2 μm polycarbonate membrane filters as the ‘free-living’ (0.2-3.0 μm) fractions. The 10 μm Nitex™ screens and the 3.0 μm membrane filters were thoroughly rinsed with 0.2 μm-filter-sterilized seawater into separate sterile graduated beakers. The rinsates, together with the corresponding Nitex™ screens or filters were sonicated for 25 seconds, and each rinsate were filtered onto 0.2 μm polycarbonate membrane filters. These represented the ‘particle-associated’ (3.0-10 μm and >10 μm) fractions. All 0.2 μm membrane filters were preserved with 4% paraformaldehyde, and were subjected to FISH processing as previously described. Triplicates were taken for each size-fraction.
3.2.6. Statistical Analyses

Statistical analyses were performed using the software Statistica 6.0 (StatSoft, Inc.) whenever possible. However, the limited data sets often precluded vigorous statistical testing. To examine the relationship between two measured parameters, the Product-Moment Correlation Coefficients (r) were computed along with independent one-way ANOVA (results shown as p-values). The normality of each data set was checked before performing these parametric tests. The degrees of similarity between certain microbial population sizes were assessed with Wilcoxin-Matched-Pairs Tests.

3.3. RESULTS

The neutrally buoyant hydrothermal plumes at Endeavour extended from 1850 m to 2150 m in depth, as revealed by temperature and particle anomalies (Δθ and Δc) (Fig. 3.2). Δθ is defined as the deviation from the predicted potential temperature based on the background linear mixing relationship between potential temperature, θ, and potential density (Baker, 1998). Δc is the excess light backscatter over the background. Strong plume signals were detected over the Main Endeavour Field (MEF) in both years (stations 99BC-02 and V00T-01, Fig. 3.2 a, d), and a moderate plume was found ~1 km to the northeast of MEF in 1999 (99BC-04, Fig. 3.2 c). No plumes were apparent at the two background stations (99BC-03 and V00T-02, Fig. 3.2 b, c).

3.3.1. Ammonium Concentrations and Ammonia Oxidation Rates

NH₄⁺ concentrations were generally elevated within the neutrally-buoyant plume over the MEF in both years. In 1999, the maximum NH₄⁺ concentration (341 ± 136 nM) was found at 2002 m within the intense plume (99BC-02) (Fig. 3.3 a). In spite of distinct
plume signals observed at station 99BC-04, only low \( \text{NH}_4^+ \) concentrations (36-55 nM) were observed. However, concentrations reached 446 ± 134 nM immediately above bottom at this same station (Fig. 3.3 c), which probably resulted from nearby discharge of diffuse fluids. Measurements at the background station (99BC-03) (50-67 nM) were consistent with epi-plume background concentrations (22-61 nM) (Fig. 3.3 b). Excluding the samples potentially under the influence of diffuse fluid discharges, \( \text{NH}_4^+ \) concentration showed a highly significant positive relationship with \( \Delta \theta \) (\( r=0.81, \) \( p<0.001 \)); or a significant positive relationship including all data in 1999 (\( r=0.58, \) \( p<0.05 \)). In 2000, the measured maximum \( \text{NH}_4^+ \) concentrations within the plume were smaller (\( \leq 185 \pm 12 \) nM) (Fig. 3.3 d). However, values for the epi-plume background were high (104-248 nM), as were those of the background station 10 km to the east of MEF (65-247 nM) (Fig. 3.3 e). In this year \( \text{NH}_4^+ \) concentration did not correlate with either \( \Delta \theta \) (\( r=0.10, p>0.05 \)) or \( \Delta c \) (\( r=0.12, p>0.05 \)).

In 1999, the measured total net \( \text{NH}_4^+ \) removal rates were 11-259 nM d\(^{-1}\) in the neutrally buoyant plume at MEF, corresponding to turnover times of 13 to 0.5 days. Autotrophic ammonia oxidation rates accounted for 3-91 nM d\(^{-1}\) (Fig. 3.3 a). At 99BC-04, only slow total net removal rate (3 ± 3 nM d\(^{-1}\)) was measured within the plume, yet ammonia oxidation rate was high (87 nM d\(^{-1}\)), which yielded the highest specific ammonia oxidation rate (\( k_{\text{oxid}} = 2.15 \) d\(^{-1}\)) reported in this study. The slow total net removal rate but high oxidation rate suggests rapid remineralization rates by the microbial communities which returns \( \text{NH}_4^+ \) to the system. The rapid oxidation rates likely maintained the low levels (36-55 nM) of the substrate \( \text{NH}_4^+ \) measured at this depth. In
contrast, a very high total net removal rate \((256 \pm 186 \text{ nM d}^{-1})\) but a lower ammonia oxidation rate \((72 \text{ nM d}^{-1})\) was measured at 5 mab at the same station (Fig. 3.3 c), implying high potential heterotrophic uptakes (heterotrophic oxidation plus assimilation). Little or no net removal was detected above the plume or at the background station (Fig. 3.3 b).

In 2000, total net removal rates measured in the plume dropped to \(22-93 \text{ nM d}^{-1}\), and autotrophic ammonia oxidation rates were \(28-63 \text{ nM d}^{-1}\) (Fig. 3 d). Despite rapid total net removal \((58 \pm 12.5 \text{ nM d}^{-1})\) in mid-water at the background station, ammonia oxidation rates remained low at all depths \((\leq 18 \text{ nM d}^{-1})\) (Fig. 3.3 e).

The values of \(k_{\text{oxid}}\) were generally elevated within the plume (0.27-2.15 d\(^{-1}\) in 1999 and 0.23-0.34 d\(^{-1}\) in 2000) compared to (0-0.28 d\(^{-1}\)) in epi-plume and background samples. The turnover times of \(\text{NH}_4^+\) due to ammonia oxidation in the plume were 0.5-3.8 days and 2.9-4.4 days in 1999 and 2000 respectively, compared to 3.6-59.8 days in background samples (Table 3.2).

The warm discharge fluids sampled at Clam Bed (JAS287-1) consisted of \(1,075 \pm 54.9 \text{ nM NH}_4^+\), the highest recorded in this study. Total net removal rate reached \(670 \pm 59.9 \text{ nM d}^{-1}\), of which \(350 \pm 89.9 \text{ nM d}^{-1}\) could be contributed to autotrophic oxidation. The corresponding \(k_{\text{oxid}}\) had a value of 0.33 d\(^{-1}\), with a turnover time of 3.1 days. In the buoyant plume 20 mab above this diffuse vent (sample JAS287-2), \(\text{NH}_4^+\) concentration \((155 \pm 25.0 \text{ nM})\) and the total net removal rate \((53 \pm 27.1 \text{ nM d}^{-1})\) were an order of magnitude lower. Up to \(115 \pm 1.1 \text{ nM NH}_4^+\) was measured in the buoyant plume (20 mab) over the Easter Island structure (JAS286-2). A negative total net removal rate (-12
± 21.1 nM d⁻¹) was measured here suggesting that remineralization of NH₄⁺ was more rapid than its removal. No autotrophic ammonia oxidation was detected in the latter two cases (JAS287-2 and JAS286-2).

3.3.2. General Microbial Abundance

Total microbial abundance, measured as the number of DAPI-stained cells, was the highest within the neutrally-buoyant plume over MEF for both years (25.1 ± 1.5 x 10⁴ cells ml⁻¹ in 1999 and 29.7 ± 7.5 x 10⁴ cells ml⁻¹ in 2000), at least five-times greater than that in background deep water (0.5-4.1 x 10⁴ cells ml⁻¹). Microbial abundance was positively correlated with both Δc (r=0.89, p=0.001 in 1999, r=0.72, p<0.05 in 2000) and Δθ (r=0.72, p<0.05 in 1999, r=0.89, p<0.001 in 2000). FISH cell counts with the universal oligonucleotide probe (UNI) showed the same depth-related trends as the DAPI cell counts (Fig. 3.4 a-e). At MEF, more than 90% of DAPI-stained cells in the neutrally-buoyant plume were simultaneously hybridized with UNI. These proportions dropped to 39-50% at epi-plume background depths, and the minimum of 8% was observed at the background stations. Since the basis of FISH lies on the binding of fluorescently-labelled oligonucleotides to the 16S rRNAs within a whole-cell, the fluorescence intensity increases with cellular rRNA content or cellular activities, and the detected cells should reflect the ecologically more important active populations (Kerkhof and Ward, 1993; Ravenschlag et al., 2000). Hence, although in theory both UNI and DAPI should enumerate all living cells, a decrease in UNI as a percentage of DAPI suggests lower average cellular rRNA contents or less active microbial populations in non-plume settings. In general, there was clear eubacterial dominance in all samples (54-159%
DAPI), and their abundance increased with plume intensity (Fig. 3.4 a-e). The observed Eubacterial abundance estimates were higher than the DAPI cell counts in the two bottom-most samples at station V00T-01. The most probable explanation is that the DAPI counts underestimated cell abundance due to the heavier interference from the slime-like exopolymeric substances at DAPI emission wavelengths than at Cy3- or 6-FAM wavelengths. This is supported by the observation that UNI-hybridized cells showed higher abundance than EUB338-hybridized cells.

Elevated microbial abundance relative to the deep-sea background was also found in the buoyant plume over Clam Bed (9.4 ± 0.9 x 10^4 cells ml\(^{-1}\) at 0 mab and 8.2 ± 0.9 x 10^4 cells ml\(^{-1}\) at 20 mab), of which 94% and 79% were Eubacteria. At 20 mab over the Easter Island structure, a total of 13.4 ± 0.7 x 10^4 cells ml\(^{-1}\) were present; 47% were identified as Eubacteria.

### 3.3.3. Abundance of \(\beta\)-Proteobacterial Ammonia-Oxidizing Bacteria

\(\beta\)-proteobacterial ammonia-oxidizing bacteria (\(\beta\)-AOB) were up to 20-times more abundant within the neutrally buoyant plume, than in the deep-sea background samples (Fig. 3.5 a-e). In 1999, their highest abundance (1.0 × 10^4 ± 0.2 × 10^4 cells ml\(^{-1}\), 7.5% DAPI) was measured at the depth of the \(\text{NH}_4^+\) concentration and ammonia oxidation rate maxima (Fig. 3.5 a). A similarly large \(\beta\)-AOB population of 1.4 × 10^4 ± 0.3 × 10^4 cells ml\(^{-1}\) (7.0% DAPI) was present in the 2000 plume (Fig. 3.5 d), versus ≤0.07 ×10^4 cells ml\(^{-1}\) in epi-plume and background samples. Although the highest \(\text{NH}_4^+\) concentration and ammonia oxidation rates were found in the warm discharge fluids sampled at Clam Bed, only a small \(\beta\)-AOB population of 0.04 ± 0.03 ×10^4 cells ml\(^{-1}\)
(1.8% DAPI) resided there. In comparison, a moderate β-AOB population was found at 20 mab (0.3 ± 0.08 ×10^4 cells ml^{-1}, 3.1% DAPI), although ammonia oxidation was undetectable. Fewer β-AOB (0.07 ± 0.05 ×10^4 cells ml^{-1}; 0.5% DAPI) were detected 20 mab over Easter Island. From the limited data set, *Nitrosomonas*-like bacteria seemed to predominate over *Nitrosospira*-like bacteria within the plume, but were similar in abundance in background deep water (Fig. 3.6 a, b). These β-AOB are termed *Nitrosomonas*-like and *Nitrosospira*-like bacteria here because they have not been sequenced and phylogenetically analyzed in detail. However, high specificity were found according to the Probe Match analyses on the RDP-II website (Cole *et al.*, 2003) (Table 3.1), that the Nsm156- and Nsv443- detected cells were most likely *Nitrosomonas* spp. and *Nitrosospira* spp. respectively.

### 3.3.4. Abundance of Methane-Oxidizing Bacteria (Type I and Type II Methanotrophs)

The vertical distribution profiles of type I methanotroph-like cells resembled the plume intensity profiles at most stations (Fig. 3.7 a-e). Their abundance within the plume maximum was similar in both years of study: 0.26-0.57 ×10^4 cells ml^{-1} in 1999 and 0.25-0.68 ×10^4 cells ml^{-1} in 2000. Apparently fewer methanotrophs were present above the plumes or at background stations (0-0.18×10^4 cells ml^{-1}), except for the near-bottom depths at the two background stations 99BC-03 and V00T-02. Type II methanotroph-like cells were only quantified in 2000. They were comparable in numbers to Type I methanotroph-like cells in the plume, but were less abundant than Type I methanotroph-like cells in most other cases (Fig. 3.7 d,e).
3.3.5. Particle-Associated Versus Free-Living Microbial Communities

Abundant cell aggregates and exopolymeric substances were often observed under the microscope in plume samples (Fig. 3.8 a, b), but not much in background samples. In general, particle-associated cells were slightly elongated, larger in size (≥ 2 μm long) and gave brighter signals relative to the smaller and dimmer free-living cells (<1 μm). When size-fractionation was performed, the detected total microbial and eubacterial abundance in individual size-classes showed similar vertical distributions to those of non-size-fractionated whole-samples (Fig. 3.9 a, b). Free-living microbes (0.2-3.0 μm) predominated at all depths, and constituted 82-93% of the total microbial populations throughout the neutrally-buoyant plume, compared to 69% at epi-plume depths. Microbe-particle association appears to gain proportional importance above the plume (Fig. 3.9), yet insufficient data precluded statistical analyses. Within the particle-associated communities, more cells were found in the ≥ 10 μm size-fraction (3-19% ∑ all size-fractions) than the 3.0-10 μm size-fraction (2-12% ∑ all size-fractions).

β-AOB formed a considerably larger component in the total particle-associated (≥3.0 μm) microbial community (up to 51% DAPI or 85% Eubacteria), than in the free-living community (5% DAPI and 31% Eubacteria) (Table 3.4). Approximately 21-62% of total β-AOB within the plume are particle-associated; this particle-association increases toward the plume periphery and epi-plume (Fig. 3.9 c). In general, more particle-associated β-AOB inhabited the ≥10 μm size-fraction (20-80% total β-AOB) than the 3.0-10 μm size-fraction (0-26% total β-AOB), except for the upper plume.
boundary (0-3% versus 23-46%, respectively). *Nitrosomonas*-like AOB were more abundant than *Nitrosospira*-like AOB in all size-fractions (Fig. 3.9 d, e).

Within the plume, 56-98% Type I methanotroph-like cells were found to be free-living, and their numbers were at least double that of Type II methanotroph-like cells. However, Type II methanotroph-like cells were equally or more abundant in the particle-associated communities (Fig. 3.9 f, g), and seemed to be more evenly distributed among the three size-fractions within the plume than Type I methanotroph-like cells. Like β-AOB, Type I methanotroph-like cells constituted a larger fraction of the total particle-associated community (≤25% DAPI, ≤129% Eubacteria), than of the free-living community (≤4% DAPI, ≤5% Eubacteria). At epi-plume depths, both Type I and Type II methanotroph-like cells were undetectable after size-fractionation.

### 3.4. DISCUSSION

#### 3.4.1. Ammonia Oxidation

The NH$_4^+$ concentrations of 36-446 nM measured within the Endeavour hydrothermal plume include the highest values ever reported in the deep-sea water column ((Cowen *et al.*, 1998) and this study). The few published values for comparable depths are ≤25 nM in the Sargasso Sea (Lipschultz *et al.*, 1996) and the eastern tropical Pacific (Ward and Zafiriou, 1988). The current paradigm of nitrogen cycling implies that the degradation of organic matter sinking from the euphotic zone releases recycled NH$_4^+$, which is eventually reoxidized to NO$_3^−$. Hence NH$_4^+$ concentration and its oxidation rates are expected to decrease exponentially with ocean depth. The injection of ‘new’
hydrothermal NH$_4^+$ from the seafloor to the water column thus provides an exceptional potential for elevated nitrification in the deep sea.

Autotrophic ammonia oxidation rates measured within the neutrally-buoyant plume at Endeavour (28-91 nM d$^{-1}$) are at least double the preliminary total net removal rates (5-15 nM d$^{-1}$) previously measured (Cowen et al., 1998), and are one to two orders of magnitude higher than other deep-sea values ($\leq$0.5 nM d$^{-1}$) (Ward and Zafiriou, 1988). The plume oxidation rates are more comparable to or greater than those measured in the euphotic zone of the North Pacific subtropical gyre (median 21.8 nM d$^{-1}$ to maximum 137 nM d$^{-1}$) (Dore and Karl, 1996), certain coastal ocean surface waters (19-240 nM d$^{-1}$) (Ward, 1985, 1987b) and parts of the subsurface oxygen minimum zone of the Eastern Tropical South Pacific (0--600 nM d$^{-1}$) (Ward et al., 1989). In fact, the actual in situ ammonia oxidation rates at depth may be even higher than reported here due to potential pressure effects. A preliminary experiment showed higher NH$_4^+$ removal rates in samples re-pressurized to in situ pressure of $\sim$200 atm than those incubated at atmospheric pressure (Cowen et al., 1998).

A small increase of $\sim$0.03-0.1 d$^{-1}$ in $k_{\text{oxid}}$ was found at the plume boundaries as well as within the moderate plume (Table 3.2), similar to the observations made for microbial methane oxidation (De Angelis et al., 1993) and manganese removal (Cowen et al., 1990). This is not surprising as ammonia-oxidizing bacteria are slow-growing, and tend to show a lag time in their response to enhanced substrate concentration (Belser, 1984; Hagopian and Riley, 1998). The turnover times for NH$_4^+$ within the plume relative to autotrophic removal (2.9-3.9 days) were at the lower end of CH$_4$ turnover times measured within the Endeavour plume (4-42 days) (De Angelis et al., 1993).
3.4.2. Regulation of Ammonia Oxidation

Ammonia oxidation is regulated by a combination of interdependent environmental factors. Among the most important are dissolved oxygen, temperature, salinity, light, pH, substrate availability, molar ratios of organic carbon to nitrogen (Butturini et al., 2000; Zhu and Chen, 2001), lability of organic carbon (Strauss and Lamberti, 2000; Wheatley et al., 2001), and the resident microbial communities. In the Endeavour neutrally-buoyant plume, dissolved oxygen, temperature, salinity, light and pH are essentially constant. While the total net NH$_4^+$ removal rates show strong positive correlation with NH$_4^+$ concentration ($r=0.92$, $p<0.001$ in 1999 and $r=0.81$, $p<0.005$ in 2000), autotrophic ammonia oxidation correlates only with NH$_4^+$ within the plume in 2000 ($r=0.95$, $p<0.05$), but not in 1999 ($r=0.51$, $p>0.05$). This ‘inconsistent’ correlation could be attributed to varying sources and rates of substrate supply. Two months prior to the 1999 sampling, a tectonic (Johnson et al., 2000) or magmatic event (Bohnenstiehl et al., 2002) near Endeavour significantly altered the heat and fluid fluxes near Endeavour vent fields, and subsequently the biogeochemistry of hydrothermal fluids and biological communities in the vicinity of the vent field (Johnson et al., 2000; Dziak and Johnson, 2002), including NH$_4^+$ in the end-member fluid discharge (Seewald, 2001). Furthermore, although the total microbial abundance within the plume was similar in 1999 and 2000 (1.3 to $2.5 \times 10^5$ and 0.9 to $3.0 \times 10^5$ cells ml$^{-1}$, respectively), EUB338-detected Eubacteria accounted for a much lower percentage in 1999 (54-67%) than in 2000 (94-100%), indicating a shift in microbial community structure. The degree of competition for NH$_4^+$ with AOB from other microbes might have shifted as well.
Despite high total net \( \text{NH}_4^+ \) removal rates, the anomalously high epi-plume concentrations of \( \text{NH}_4^+ \) over MEF and at the background station in 2000 did not induce high autotrophic ammonia oxidation, while heterotrophic uptake was apparently active. The partitioning between autotrophic and heterotrophic \( \text{NH}_4^+ \) uptakes is currently not well understood, but may be linked to the molar ratios of total organic carbon to total nitrogen in the environment, or to the lability of organic matter (Strauss and Lamberti, 2000; Strauss et al., 2002). When the C: N ratio is high, and/or the organic carbon is labile, the faster-growing heterotrophic microbes thrive on the available organic carbon, become N-limited and are likely able to outcompete the slower-growing autotrophic nitrifiers (Prosser, 1989) for \( \text{NH}_4^+ \) (Verhagen et al., 1992; van Niel et al., 1993; Strauss and Lamberti, 2000). However, more data are needed to verify this. Heterotrophic \( \text{NH}_4^+ \) uptake also predominated near the seafloor at 99BC-04. The presumed nearby diffuse discharge fluids there might harbor high concentrations of subseafloor organic matter and its thermal degradation products, which may include inhibitors of autotrophic ammonia oxidation, such as micromolar level of methane and carbon monoxide (Ward, 1987a; Roy and Knowles, 1994).

Another contributing factor to the partitioning between autotrophic and heterotrophic \( \text{NH}_4^+ \) uptakes might be the relatively low populations of AOB in the background and 99BC-04 near-bottom samples, or the lack of seed AOB populations needed to perform autotrophic ammonia oxidation. In contrast, the relatively larger AOB population present in the moderate plume center at station 99BC-04 could explain the observed high autotrophic ammonia oxidation but low total net removal rates. However,
AOB abundance was only positively correlated with NH$_4^+$ concentrations ($r=0.88$, $p<0.001$) and possibly ammonia oxidation rates ($r=0.70$, $p=0.18$) in 1999, but not in 2000 ($r=0.27$, $p>0.5$ and $r=0.16$, $p>0.5$). Different regulating factors for ammonia oxidation might become relatively more important in different situations, and their interdependence likely preclude generalizations of relationships between AOB abundance and ammonia oxidation. For instance, the medium-sized AOB population with no ammonia oxidation measured in the buoyant plume over Clam Bed could be a result of active remineralization that continued to add NH$_4^+$ to the water, or the slow response of AOB to intermittently available NH$_4^+$ in the variable buoyant plume.

3.4.3. Populations of Ammonia-Oxidizing Bacteria

These data represent the first published population numbers for autotrophic ammonia-oxidizing bacteria from deep-ocean depths. AOB abundance within the Endeavour plume (to $1.4 \times 10^4$ cells ml$^{-1}$) was up to 20-times greater than in the background deep-sea, although they were from one sixth to one half those found in one coastal ocean study ($2.9$ to $8.0 \times 10^4$ cells ml$^{-1}$) (Ward et al., 1982), and were considerably smaller than those detected in the oxygen minimum zone in the Eastern Tropical South Pacific ($6$ to $110 \times 10^4$ cells ml$^{-1}$) (Ward et al., 1989) or in some other coastal regimes ($15$ to $400 \times 10^4$ cells ml$^{-1}$) (Ward et al., 1984; Ward, 1987b). The measured abundances of AOB and methanotrophs (Types I and II combined) were similar within the Endeavour hydrothermal plume ($p>0.05$, Wilcoxin matched-pair tests for difference). On the other hand, the actual AOB abundance could be even greater than observed since the NSO190 oligonucleotide probe likely missed certain AOB species
(Utäker and Nes, 1998; Purkhold et al., 2000); while the 10-γ and 9-α probes overestimate methanotrophs (Table 3.1).

*Nitrosospira* spp. are generally regarded as ‘K-strategists’, those that are more abundant in low NH₄⁺ conditions; while *Nitrosomonas* spp. are ‘r-strategists’, those that proliferate under eutrophic or particle-rich conditions (Hiorns *et al.*, 1995; Stephen *et al.*, 1996; Kowalchuk *et al.*, 1997; Phillips *et al.*, 1999). These generalizations are consistent with the results of the present study. The predominance of *Nitrosomonas*-like bacteria is especially apparent in the plume core where high autotrophic ammonia oxidation rates were measured, and in particle-associated size-fractions. *Nitrosospira*-like bacteria were about equal in number with *Nitrosomonas*-like bacteria in free-living communities and in lower NH₄⁺ regimes like epi-plume depths or background stations, consistent with the theory that *Nitrosospira* are ‘K-strategists’. The NH₄⁺ concentrations in these regimes may not be low enough (minimum at 65 nM) to induce a more apparent community shift, or *Nitrosospira*-like bacteria may have been underestimated due to the less bright fluorochrome (6-FAM) that was used with the *Nitrosospira*-specific probe (Nsv443). Besides, γ-Proteobacterial AOB were not enumerated in this study and their contribution to the nitrifying communities could also be significant in oceanic regimes (Ward and O'Mullan, 2002). Moreover, novel species of AOB could also be present that were not detectable with the existing 16S rRNA-targeted probes designed on the basis of known isolated and cultured species. In particular, this could have been the case for the warm discharge fluids at Clam Bed (JAS287-1) where high ammonia oxidation rates but low AOB abundance were measured.
3.4.4. Particle-Associated versus Free-Living Microbial Communities

A larger proportion of AOB within the plume are associated with particles than the general population, a tendency that is enhanced at the plume boundaries or above the neutrally buoyant plume. This association is consistent with the observations that AOB often excrete exopolymeric substances (EPS) and form aggregates as a possible adaptation to low substrate or low pH conditions (de Boer et al., 1991; Stehr et al., 1995a; Stehr et al., 1995b; Hagopian and Riley, 1998). In fact, ammonia-oxidizers constituted a substantial portion of the total particle-associated microbial communities (Table 3), and their relative importance increased from the plume core (≥10 μm fraction: 7-12% DAPI and 14-38% Eubacteria) toward the lower plume boundary (≥10 μm fraction: 14-16% DAPI and 69-84% Eubacteria). Although nitrifying activities of particle-associated versus free-living communities were not measured, the bigger cell size and greater fluorescence signals of particle-associated cells indicated higher rRNA contents hence higher potential cellular growth rates (Kerkhof and Ward, 1993; Ravenschlag et al., 2000). Assuming that the AOB were not in a ‘survival state’, higher cellular growth rates might be a result of higher cellular nitrifying activities. The potential of enhanced nitrifying activities with particles is supported by the fact that the particle-associated AOB were dominated by the r-strategists Nitrosomonas-like cells, as was also found in a Mediterranean Sea study (Phillips et al., 1999). Similar observations of elevated nitrification activities associated with sinking particles have been documented in the Eastern Tropical North Pacific (Karl et al., 1984). Almost equally substantial proportions of the particle-associated communities were identified as methanotrophs,
which was in good agreement with previous speculations of methanotroph particle-associations (De Angelis et al., 1993).

3.4.5. Potential In Situ Organic Carbon Production via Ammonia Oxidation

Although no simultaneous carbon fixation measurements were made in this study, potential organic carbon production derived from autotrophic ammonia oxidation can be calculated assuming a yield of 0.3-1.4 g fixed C per mol N oxidized (Belser, 1984; Feliatra and Bianchi, 1993). Accordingly, ammonia oxidation rates of 28-91 nM d\(^{-1}\) could yield carbon fixation rates of 8-127 µg C m\(^{-3}\) d\(^{-1}\) within the Endeavour neutrally-buoyant plume. The integrated carbon fixation rates for the whole plume core (1850-2150 m) over the MEF would range from 3.9 to 26 mg C m\(^{-2}\) d\(^{-1}\). These values are at least four-fold higher than the surface-derived particulate organic carbon (POC) flux reaching plume depths at or near MEF (1-4 mg C m\(^{-2}\) d\(^{-1}\)) (Roth and Dymond, 1989; Cowen et al., 2001). They are also considerably higher than previous integrated rates of carbon fixation rates (0.2-0.9 mg C m\(^{-2}\) d\(^{-1}\)) derived from preliminary NH\(_4^+\) removal rates (Cowen et al., 1998), due largely to the much lower range of measured total net NH\(_4^+\) removal rates (5-15 nM d\(^{-1}\)) in this previous study. In comparison, measured organic carbon production from methanotrophy in the Endeavour plume was 0.4-6 mg C m\(^{-2}\) d\(^{-1}\) (De Angelis et al., 1993) and 1-2 mg C m\(^{-2}\) d\(^{-1}\) for hydrogen oxidation (McLaughlin, 1998). This implies that ammonia oxidation may contribute similar or even greater organic carbon within the neutrally-buoyant plume than either methanotrophy or hydrogen oxidation, at least in this part of the plume. In addition, since the abundance of ammonia-oxidizing bacteria (0.2-1.4 cells ml\(^{-1}\)) was similar to that of methanotrophs (Type I plus Type II) (0.2-1.3 cells ml\(^{-1}\)), and given similar organic carbon production
rates, their potential biomass may also be comparable assuming similar mortality rates and growth efficiencies.

Combining estimates of ammonia-oxidation and methanotrophy, organic carbon production via these two chemosynthetic pathways alone reached 4.3-32 mg C m\(^{-2}\) d\(^{-1}\) within the Endeavour neutrally-buoyant plume. This is a substantial organic carbon source for the deep-sea, equivalent to 133 to 3,400 \% of the surface-derived organic carbon flux reaching those depths. It is an order of magnitude greater than that of the chemosynthetic carbon production measured in the mid-water redox transition zone of the Cariaco Basin (10-333 \% of surface-derived organic carbon flux) despite a greater absolute production of the latter (324-1,908 mg C m\(^{-2}\) d\(^{-1}\)) (Taylor et al., 2001). Furthermore, only the organic carbon production in the plume core directly above the MEF has been integrated here. The proportional contribution of ammonia oxidation and methanotrophy to organic carbon production may be even greater in an aging plume. Although hydrogen and sulfur oxidation have been considered as potentially important carbon fixation processes in a hydrothermal plume, they probably occur in the early stages of hydrothermal plume formation (McCollom, 2000). Hence, the slower ammonia oxidation and methanotrophy are likely relatively more important carbon fixation pathways in the neutrally-buoyant phase of the Endeavour plume.

3.5. CONCLUSIONS

Autotrophic ammonia oxidation is documented for the first time in the Endeavour neutrally-buoyant hydrothermal plume, as supported by two complementary lines of evidence. Positive autotrophic ammonia oxidation rates were measured, including the highest rates ever reported in the deep sea. Populations of chemolithoautotrophic
ammonia-oxidizing bacteria (AOB) corresponding to the elevated ammonia oxidation rates, were 20-fold greater within the plume than in the deep-sea background. AOB populations formed a large component in the total microbial communities, and were even more prevalent in the particle-associated assemblages. The particle-associated AOB were dominated by an active group of r-strategists Nitrosomonas-like cells, which could imply enhanced nitrification activity associated with particles. This preliminary study indicates that the injection of NH$_4^+$ from some seafloor hydrothermal systems provides a unique opportunity for nitrification in the deep-sea water column. Autotrophic ammonia oxidation is likely a major process for in situ carbon fixation within the Endeavour neutrally-buoyant plume, that potentially exceeds the organic carbon flux from the surface ocean to this depth. Hence, nitrification in the neutrally-buoyant, laterally advected hydrothermal plume is important to the deep-sea carbon and nitrogen cycles over the Endeavour Segment.
3.6. REFERENCES


Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and


Table 3.1. List of the various 16S rRNA-targeted oligonucleotide probes used in this study, together with their respective targeting organisms, target sites with respect to *E. coli*, formamide concentrations (%FA) in hybridization solutions and NaCl concentrations in the stringent washing solutions.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequences</th>
<th>Target Organisms</th>
<th>Target site</th>
<th>%FA</th>
<th>NaCl (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNI</td>
<td>5'-GWA TTA CCG CGG CKG CTG -3'</td>
<td>Universal</td>
<td>519-536</td>
<td>20</td>
<td>70</td>
<td>(Giovannoni et al., 1988)</td>
</tr>
<tr>
<td>EUB 338</td>
<td>5'-GCT GCC TCC CGT AGG AGT -3'</td>
<td>Eubacteria a</td>
<td>338-355</td>
<td>20</td>
<td>225</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>NON 338</td>
<td>5'-ACT CCT ACG GGA GCC AGC -3'</td>
<td>Negative control</td>
<td>N/A</td>
<td>20</td>
<td>225*</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>NSO 190</td>
<td>5'-CGA TCC CCT GCT TTT CTC C -3'</td>
<td>β-Proteobacterial AOB b</td>
<td>190-208</td>
<td>55</td>
<td>20*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsm 156</td>
<td>5'-TAT TAG CGC ATC TTT CGA T -3'</td>
<td><em>Nitrosomonas</em> spp. c</td>
<td>156-174</td>
<td>5</td>
<td>56*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsv 443</td>
<td>5'-CCG TGA CCG TTT CGT TCC G -3'</td>
<td><em>Nitrosospira</em> spp.</td>
<td>444-462</td>
<td>30</td>
<td>112*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>9-α</td>
<td>5'-CCC TGA GTT ATT CGG AAC -3'</td>
<td>Type II Methanotrophs d</td>
<td>142-159</td>
<td>20</td>
<td>225</td>
<td>(Tsien et al., 1990)</td>
</tr>
<tr>
<td>10-γ</td>
<td>5'-GGT CCG AAG ATC CCC CGC TT -3'</td>
<td>Type I Methanotrophs e</td>
<td>197-216</td>
<td>20</td>
<td>225</td>
<td>(Tsien et al., 1990)</td>
</tr>
</tbody>
</table>

* Information on the NaCl concentrations in these washing solutions came from (Schramm et al., 1998).

a This excludes *Plantomycetales* and *Verrucomicrobia* (Daims et al., 1999).

b NSO190 has 3 mismatches with *Nitrosomonas ureae*, and 1 mismatch in a few *Nitrosomonas* spp. (Utaker and Nes, 1998; Purkhold et al., 2000).

c Three possible non-AOB targets are *Thauera linaloelentis* str. 47, *Pseudomonas butanovora* IAM 12574 and *Aquaspirillum sinuosum* LMG 4393 (Results from Probe Match (Cole et al., 2003)).

d Non-target species include some species in *Caulobacter* and *Belindica* (Results from Probe Match (Cole et al., 2003)).

e Non-target species include some species in *Neisseria, Azooarcus, Bordetella, Acidovorax, Burkholderia, Ectothiorhodospira, Thiobacillus, Achromatium, Dichelobacter, Legionella, Oceanospirillum* and a few relatives of *Pseudomonas* (Results from Probe Match (Cole et al., 2003)).
Table 3.2. Specific ammonia oxidation rates ($k_{oxid}$) and turnover times for ammonium removals.

<table>
<thead>
<tr>
<th>Station</th>
<th>Depth (m)</th>
<th>Specific Ammonia Oxidation Rates ($k_{oxid}$)</th>
<th>Turnover time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99BC-02</td>
<td>2180</td>
<td>0.442</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2115</td>
<td>0.024</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>2002</td>
<td>0.265</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>0.386</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>1917</td>
<td>0.404</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1775</td>
<td>1.028</td>
<td>1.0</td>
</tr>
<tr>
<td>99BC-04</td>
<td>2178</td>
<td>0.162</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>1997</td>
<td>2.147</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1759</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>V00T-01</td>
<td>2173</td>
<td>0.259</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2126</td>
<td>0.230</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>2006</td>
<td>0.227</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>1906</td>
<td>0.341</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1806</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1705</td>
<td>0.051</td>
<td>19.4</td>
</tr>
<tr>
<td>V00T-02</td>
<td>2207</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2106</td>
<td>0.058</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>2006</td>
<td>0.017</td>
<td>59.8</td>
</tr>
<tr>
<td></td>
<td>1906</td>
<td>0.280</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>1706</td>
<td>0.186</td>
<td>5.4</td>
</tr>
<tr>
<td>JAS286</td>
<td>20 mab</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>JAS287</td>
<td>0 mab</td>
<td>0.326</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>20 mab</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. Percentages of β-proteobacterial ammonia-oxidizing bacteria (β-AOB) and Type I methanotrophs relative to the whole microbial community in various size-fractions in various depths of the neutrally buoyant plume at Main Endeavour Field (MEF).

<table>
<thead>
<tr>
<th></th>
<th>Lower Plume Boundary</th>
<th>Plume Core</th>
<th>Above Plume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DAPI % EUB % DAPI % EUB % DAPI % EUB</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>β-AOB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-size-fractionated</td>
<td>0.5-0.7 0.3-0.5</td>
<td>0.9-7.0 1.0-7.5</td>
<td>7.4-8.4 12-15</td>
</tr>
<tr>
<td>Free-living</td>
<td>0.6-3.4 0.9-4.8</td>
<td>1.0-1.9 1.1-2.1</td>
<td>2.2-4.9 6.3-11.4</td>
</tr>
<tr>
<td>Particle-Associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0-10 μm</td>
<td>n.d. n.d. 10-11</td>
<td>16-22 0-20 0-31</td>
<td></td>
</tr>
<tr>
<td>≥ 10 μm</td>
<td>14-16 69-85 6.9-12</td>
<td>14-38 0-31 0-24</td>
<td></td>
</tr>
<tr>
<td><strong>Type I Methanotrophs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-size-fractionated</td>
<td>2.1-2.6 1.3-1.8</td>
<td>1.3-3.3 1.4-3.5</td>
<td>0-4.7 0-7.9</td>
</tr>
<tr>
<td>Free-living</td>
<td>1.7-2.1 2.5-3.0</td>
<td>3.8-4.1 4.2-4.5</td>
<td>n.d. n.d.</td>
</tr>
<tr>
<td>Particle-Associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0-10 μm</td>
<td>n.d. n.d. 5.5-9.6</td>
<td>11.5-13.4 n.d. n.d.</td>
<td></td>
</tr>
<tr>
<td>≥ 10 μm</td>
<td>0.7-25 3.4-129 0.9-7.0</td>
<td>4.9-7.9 0-5.3 0-4.0</td>
<td></td>
</tr>
</tbody>
</table>

'n.d.' = non-detectable

‘%EUB’ = abundance as a percentage of EUB338-detected Eubacteria. This % would be greater than % of total Eubacteria since EUB338 fails to hybridize to *Plantomycetales* and *Verrumicrobiota* (Daims et al., 1999).
Figure 3.1. Location of the Endeavour Segment and Juan de Fuca Ridge. The inset shows an enlarged view of the Endeavour Segment with the sampling stations and the five known vent fields (hatched) – from north to south: Sasquatch (SQ), Salty Dawg (SD), High Rise (HR), Main Endeavour Field (MEF) and Mothra (MO).
Figure 3.2. Vertical profiles of temperature anomalies (Δθ, thick solid lines) and particle anomalies (Δc, dash-dot lines) at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02. Calculations of Δθ and Δc are in text.
Figure 3.3. Vertical profiles of mean NH$_4^+$ concentrations (solid lines), total net removal rates (filled circles, dash lines) and ammonia oxidation rates (open circles, dash lines) at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02. The points represent mean values while the error bars are standard errors. The standard errors for total net removal rates and ammonia oxidation rates are computed by propagation of errors.
Figure 3.4. Vertical profiles of total microbial abundance detected by DAPI-staining (filled circles) and FISH with UNI oligonucleotide probe (open circles), along with the profiles of Eubacterial abundance detected by FISH with EUB338 oligonucleotide probe (filled triangles) at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02. The points represent mean values from duplicate subsamples, while error bars are standard errors computed using propagation of errors from the variances in subsample counts (20 fields per subsample count).
Figure 3.5. Vertical profiles of β-proteobacterial ammonia-oxidizing bacterial abundance detected by FISH with the NSO190 oligonucleotide probe at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02. The filled circles represent mean values from duplicate subsamples while error bars are standard errors computed using propagation of errors from the variances in subsample counts (20 fields per subsample count).
Abundance of *Nitrosomonas*-like and *Nitrosospira*-like bacteria (x 10^4 cells ml⁻¹)

![Graph showing vertical profiles of abundance](image)

**Figure 3.6.** Vertical profiles of the abundance of *Nitrosomonas* spp. detected by FISH with Nsm156 oligonucleotide probe (filled diamonds), and of *Nitrosospira* spp. detected by FISH with Nsv443 oligonucleotide probe (open inverted triangles) at stations (a) VOOT-01 and (b) VOOT-02. The diamonds and triangles represent mean values from duplicate subsamples while error bars are standard errors computed using propagation of errors from the variances in subsample counts (20 fields per subsample count).
Figure 3.7. Vertical profiles of the abundance of type I (filled squares) and type II (open squares) methanotroph-like cells at stations (a) 99BC-02, (b) 99BC-04, (c) 99BC-03, (d) V00T-01 and (e) V00T-02. The squares represent mean values from duplicate subsamples while error bars are standard errors computed using propagation of errors from the variances in subsample counts (20 fields per subsample count).
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Figure 3.8. FISH micrographs showing β-AOB cells hybridized with the Cy3-labelled NSO190 oligonucleotide probes (red) versus type I methanotrophs hybridized with 6-FAM-labelled 10-γ probes (green). The corresponding views of DAPI-stained cells (blue) are shown in the lower panels. At MEF, a lot of large bright β-AOB cells were embedded in slime-matrices or exopolymeric substances (a), but free-living β-AOB have also been observed (c). β-AOB outnumbered Type I methanotrophs within the plume over MEF, but not necessarily so in the moderate part of the plume (e).
Figure 3.9. Microbial abundance in different size-fractions at various depths at station V00T-01: (a) DAPI-stained cells, (b) Eubacteria detected by EUB338, (c) β-AOB detected by NSO190 (d) *Nitrosomonas*-like cells detected by Nsm156 , (e) *Nitrososira*-like cells detected by Nsv443, (f) Type I Methanotrophs detected by 10-γ and (g) Type II Methanotrophs by 9-α. Black solid bars represent the abundance of free-living microbes (0.2-3.0 μm), light gray represent the smaller particle-associated communities (3.0-10 μm), and dark gray represent the larger particle-associated communities (≥10 μm). Triangles indicate the abundance estimates in corresponding non-size-fractionated whole-samples as presented in Figs. 4-7, which are usually lower than the sums of all three size-fractions.
CHAPTER FOUR

Microbial Ammonia Oxidation and Enhanced Nitrogen Cycling

in the Endeavour Hydrothermal Plume
4.0. ABSTRACT

The fate of the ammonium injected from the seafloor hydrothermal system into the deep-sea water column was investigated at the Endeavour Segment, Juan de Fuca Ridge. Elevated NH$_4^+$ (≤ 177 nM) was confined within the axial valley and the hydrothermal plume, and it was quickly removed by both autotrophic ammonia oxidation and assimilation. Autotrophic ammonia oxidation rates accounted for at least 93% of the total net NH$_4^+$ removal rates and were highly elevated within the neutrally buoyant plume (≤ 53 nM d$^{-1}$) compared to < 5 nM d$^{-1}$ measured in above-plume background deep water. Ammonia oxidation rates showed a sigmoidal relationship with NH$_4^+$ concentrations using data collected over three years. The resulting empirical half-saturation constant (153 nM) was at least an order of magnitude lower than those measured in cultured studies. This high substrate affinity implies that the natural oceanic ammonia-oxidizing bacteria are highly adapted to the generally limited NH$_4^+$ in the deep sea. Ammonia oxidation in this plume potentially added 26-130 mg N m$^{-2}$ d$^{-1}$ of nitrate into the deep-sea water column. This oxidation process was heavily influenced by the presence of organic-rich particles, with which ammonia-oxidizing bacteria were often associated (40-68%). Ammonia-oxidizing bacteria, including those in both the β- and γ-Proteobacteria subgroups, contributed up to 10.8% of total microbial communities within the plume. NH$_4^+$ assimilation rates were also substantially enhanced within the neutrally buoyant plume (up to 26.4 nM d$^{-1}$) and accounted for at least 47% of total net NH$_4^+$ removal rates. The two NH$_4^+$ uptake rates together always exceeded the total net removal rates, suggesting active in situ regeneration of NH$_4^+$ (1.5-29.6 nM d$^{-1}$ or 8-89 mg N m$^{-2}$ d$^{-1}$ integrated over plume depths) that was at least an order of magnitude higher than what could be provided by the particulate export flux from the euphotic zone. Ammonia oxidation is responsible for NH$_4^+$ turnover in 0.7-13 days and is probably the predominant in situ organic carbon production process (0.6-13 mg C m$^{-2}$ d$^{-1}$) at an early stage of the Endeavour neutrally buoyant plume, while methanotrophy gains dominance as the plume ages.
4.1. INTRODUCTION

Nitrification is the two-step microbially mediated conversion of NH$_4^+$, the reduced form of inorganic nitrogen, to the oxidized forms of NO$_2^-$ and then NO$_3^-$. Nitrification links the recycling of organic nitrogen to the ultimate loss of fixed nitrogen mainly via denitrification. The current paradigm of marine nitrogen cycling implies that particulate organic matter sinks from the euphotic zone and is gradually remineralized to release NH$_4^+$, which in time is nitrified to contribute to the deep-sea NO$_3^-$ reservoir. Deep-sea NO$_3^-$ recharges the often nitrogen-limited euphotic zone via upwelling or eddy diffusion to support ‘new production’ (Dugdale and Goering, 1967). Most nitrification studies to date have focused on the near-surface or coastal ocean (Ward, 1987b; Lipschultz et al., 1990; Dore and Karl, 1996; Bianchi et al., 1999; de Bie et al., 2002), or sediments (Rysgaard et al., 1994; Bodelier et al., 1996; Laima et al., 2002). Although most deep-sea nitrification is considered to take place not far below the thermocline (Ward, 2000), particulate organic matter continues to sink and decompose along the way to the seafloor. Hence, some degree of nitrification should continue at depth. However, little is actually known about nitrification in the deep-sea water column (Lipschultz et al., 1996), at least in part because its rates are usually too slow to be measured (Ward and Zafiriou, 1988).

NH$_4^+$ concentrations are notably elevated in the end-member fluids of all sedimented deep-sea hydrothermal systems. For example, they reach millimolar levels in the fluids from the Guaymas Basin (10.3-15.6 mM) (Von Damm et al., 1985) and the Okinawa Trough Backarc Basin (5 mM) (Sakai et al., 1990), as well as in the fluids from the anomalous unsedimented Endeavour Segment, Juan de Fuca Ridge (0.6-1.0 mM) (Lilley et al., 1993). Although the exact source of NH$_4^+$ in the Endeavour hydrothermal
fluids remains undetermined, it is likely attributed to the decomposition of subseafloor organic matter associated with sediments buried at an early stage of the ridge’s evolution (Lilley et al., 1993). After at least a $10^4$ dilution of the hydrothermal fluids by the entrained ambient deep water (Lupton et al., 1985), up to 400 nM of NH$_4^+$ remains in the Endeavour neutrally buoyant hydrothermal plume, compared to $\leq 50$ nM in background deep waters and most hydrothermal plumes over unsedimented ridges (Cowen et al., 1998). This elevated NH$_4^+$ provides a unique opportunity to test the hypothesis that nitrifiers are ubiquitous but substrate-limited in the deep sea. Direct evidence for substrate stimulation of autotrophic ammonia oxidation, the first step in nitrification, has already been reported for the Endeavour neutrally buoyant plume (Lam et al., 2004; Chapter 3). The maximum ammonia oxidation rate of 91 nM d$^{-1}$ in the plume core accounted for 92% of total net NH$_4^+$ removal and is the highest rate ever reported in the deep-sea water column. In addition, this autotrophic ammonia oxidation rate in the plume core may yield *de novo* organic carbon as fast as 3.9-18 mg C m$^{-2}$ d$^{-1}$, which is four-fold greater than the flux of photosynthetic carbon reaching plume depths (Cowen et al., 2001). Hence, the fate of this hydrothermally discharged NH$_4^+$ is not only important to the deep-sea nitrogen cycle, but also potentially to the deep-sea carbon cycle, as organic carbon production is limited at these depths.

Nevertheless, it remains uncertain how NH$_4^+$ enrichment and autotrophic ammonia oxidation vary spatially within the axial valley, or as the plume ages while being advected away from hydrothermal vent sources. The time or age of a hydrothermal plume can be characterized by the various biogeochemical reactions occurring within the plume at different time-scales, with sulfide and iron being removed the fastest, closely followed by hydrogen, then particle anomalies, radon-222, methane and much later,
manganese (Kadko et al., 1990). Alternatively, the relative age of a plume might be revealed by how much less negative (or more positive) the carbon stable isotopic composition of methane is compared to that in the source fluids (Cowen et al., 2002). NH$_4^+$ seems to be removed via ammonia oxidation on a time-scale comparable to methane in the young plume core according to Lam et al. (2004) (Chapter 3), yet more measurements in other parts of the plume are necessary to verify the NH$_4^+$ turnover times in the overall plume environments. The potential roles of assimilation and regeneration in NH$_4^+$ fluxes are also unknown, but they may be important in the metabolically active biological communities likely found in the organic-rich plumes (Roth and Dymond, 1989; Winn et al., 1995; Cowen and German, 2001). In the presence of active NH$_4^+$ regeneration, high rates of assimilation and ammonia oxidation may co-occur and result in even faster turnover of NH$_4^+$ than that suggested by autotrophic ammonia oxidation rate measurements alone.

Hence, the objective of this study was to investigate the distribution and fate of the hydrothermally injected NH$_4^+$ in the aging hydrothermal plume over Endeavour Segment. Particular emphasis is placed on the partitioning between autotrophic ammonia oxidation and assimilation in various parts of the plume, the potential controlling factors and the implications of enhanced nitrogen cycling in the deep-sea water column. The significance of autotrophic ammonia oxidation (henceforth ammonia oxidation) as a form of in situ chemolithoautotrophy is compared with methane oxidation (or methanotrophy), a major form of in situ chemotrophy observed in the neutrally buoyant plume (De Angelis et al., 1993).
4.2. MATERIALS AND METHODS

4.2.1. Site Description and Water Sampling

The Endeavour Segment of Juan de Fuca Ridge is located approximately 300 km off the coasts of British Columbia and Washington State in the northeast Pacific. It is an intermediate-rate spreading center, with hydrothermal venting concentrated within a 1 km wide, 10 km long and 100-150 m deep axial rift valley situated along the ridge crest. The valley floor shoals from 2300 m in the south to 2170 m in the north (Thomson et al., 2003). There are five known active vent fields spaced about 2-3 km apart (Fig. 4.1) (Kelley et al., 2001a), hosting high-temperature (317-400°C) focused venting regions and extensive areas of diffuse flow (Delaney et al., 1984; Robigou et al., 1993; Butterfield, 1995; Lilley et al., 1995; Veirs et al., 1999; Kelley et al., 2001b). Hydrothermal plumes rise to neutral buoyancy at 50-350 m above bottom. Deeper portions of the plumes may remain trapped within the valley for a period of time and are steered by slow along-axis oscillatory currents, while those rising above the ridge crest may drift away with the ambient cross-axis flow (Thomson et al., 2003). The Endeavour plumes can be traced more than 10 km away from the axial valley and are characterized by distinct temperature and particle anomalies (Thomson et al., 1992), elevated concentrations of volatiles (H₂, CH₄ and NH₄⁺) (De Angelis et al., 1993; Cowen et al., 1998; McLaughlin, 1998), as well as various dissolved and particulate trace metals and organic matter (Dymond and Roth, 1988; Roth and Dymond, 1989; Cowen et al., 2001).

The water-column in the vicinity of the Endeavour axial valley was surveyed by a CTD-transmissometer-Niskin bottle rosette package from the R/V Atlantis. In order to map the neutrally buoyant plume, tow-yo surveys (Baker et al., 1985) were conducted along the entire axial valley (Tow A), along-axis over the west valley wall (Tow B),
across-axis at the northern end (Tow C), across Main Endeavour Field (Tow D), across Mothra vent field (Tow E) and at the southern end of the valley (Tow F). Tow F also extended to 7 km to the southwest of Main Endeavour Field (Fig. 4.1). More detailed water sampling of plume structures was achieved via vertical casts at six stations (Fig. 4.1). Two stations were located directly above active vent fields: Station A at Main Endeavour Field (or MEF; casts V1, V5, V8) and Station B at High Rise vent field (casts V2, V3). Station C (cast V6) was situated at 1 km to the west of MEF over the valley wall. Station D lay at the southern end of the axial valley (cast V4), while Station E was chosen outside the valley at a gap in the west valley wall, 4 km to the south-southwest of MEF (sampling cast V10). The most distal station (Station F) was located at 5.5 km southwest of MEF, away from the axial valley (cast T7-13). Seawater samples were collected from the plume core, below-plume deep water and above-plume background whenever possible. Hydrothermal plume signals were defined by anomalies in potential temperature ($\Delta \theta$) and particle concentration ($\Delta c$) (Baker, 1998).

Warm diffuse fluids (< 30 °C), usually over beds of tubeworms in MEF, were concentrated with a dome-like inverted funnel and sampled at the funnel top with titanium major samplers, operated by the submersible DSV2 ALVIN (Dives 3817-3820). Hot vent fluids (> 310 °C) discharged from black smoker chimneys at the Cathedral and S&M structures in the MEF were collected directly with titanium major samplers. These fluid samples were analyzed for $\text{NH}_4^+$, along with the concentrations and carbon stable isotopic composition of methane.

4.2.2. Sample Collection and Analysis for Ammonium

Water samples for $\text{NH}_4^+$ analyses were drawn through clean 202 μm Nitex screens into acid-cleaned 125 ml polyethylene bottles. Duplicate subsamples were stored
frozen (–20 °C) except they were analyzed immediately. All samples were analyzed on
shipboard usually within 24 hours, following the fluorescence method developed by
Jones (1991). The NH$_4^+$ concentration of each sample is reported here as the mean ±
standard deviation of duplicate subsamples.

4.2.3. Ammonia Oxidation Experiments

4.2.3.i. Basic Incubation Experiments

Incubation experiments were set up to measure total net NH$_4^+$ removal and
ammonia oxidation rates at the six sampling stations (Fig. 4.1). Single-end-point
experiments were conducted, in which duplicate or triplicate water subsamples, collected
in the same manner as for NH$_4^+$ analyses, were incubated at *in situ* temperature (2°C) and
1 atm in the dark for approximately 48 hours, in the presence and absence of nitrapyrin
(100 mg l$^{-1}$ final concentration; stock solution dissolved in dimethyl sulfoxide). The final
concentration of the solvent dimethyl sulfoxide (DMSO) in each incubation was 1 ml l$^{-1}$,
which has been reported to be innocuous to ammonia-oxidizing bacteria (Jones and
Morita, 1984). All incubations were terminated via freezing at –20 °C.

Nitrapyrin, or N-serve, is a specific inhibitor for autotrophic ammonia oxidation
that has been commonly applied in nitrification studies (Billen, 1976; Jones *et al.*, 1984).
Nitrapyrin is chosen over allylthiourea that was used in Lam *et al.* (2004) (Chapter 3),
because allylthiourea may inhibit other respiratory activities (Benes *et al.*, 2002), and
nitrapyrin seems to show greater inhibition to ammonia oxidation than allylthiourea (Roy
and Knowles, 1995). Nitrapyrin also appears to be more consistent in its inhibitory
effects on various species of ammonia-oxidizing bacteria and environmental samples
compared to other common specific inhibitors (Jones and Morita, 1984); R. D. Jones,
personal communications). Although nitrapyrin also inhibits methane oxidation (Topp
and Knowles, 1982), it should not affect the measurements of NH$_4^+$ removal here. The extent of inhibition to methane oxidation itself was actually examined in some simultaneous $^{14}$C-methane oxidation and $^{14}$C-carbon monoxide experiments (Jones et al., 1984), which will be reported in a separate study.

Total net NH$_4^+$ removal rates ($R_{tot}$) in the single end-point incubations were calculated as the total net NH$_4^+$ loss divided by incubation time. Ammonia oxidation rates ($R_{oxid}$) were calculated as the difference between the net NH$_4^+$ removal rates with and without the presence of nitrapyrin. If $N_0$, $N_t$ and $N_{NS}$ are the measured NH$_4^+$ concentrations in time-zero subsamples, untreated incubated subsamples and nitrapyrin-treated incubated subsamples respectively, and $t$ is the incubation time,

$$R_{oxid} = \frac{[(N_0 - N_t)/t] - [(N_0 - N_{NS})/t]} = \frac{(N_{NS} - N_t)/t}{t}.$$

In five selected samples, time-series incubations with four time-intervals (approximately 0, 24, 48, 72 hours) were also conducted with and without nitrapyrin, to check whether the 48-hour end-point lies within the linear phase of NH$_4^+$ removal. This was in fact found to be the case in all samples. The total net NH$_4^+$ removal rates ($R_{tot}$) for these time-series incubations were taken as the slopes of the respective linear regression of NH$_4^+$ concentration versus time (in hours) during the linear phase of the incubations without nitrapyrin ($m_T$). Autotrophic ammonia oxidation rates ($R_{oxid}$) were calculated as the difference between the slope of the regression without nitrapyrin ($m_T$) and that with nitrapyrin ($m_{NS}$), i.e.

$$R_{oxid} (nM \, d^{-1}) = (m_{NS} - m_T) \times 24 \, hours \, d^{-1}$$

where $m_T$ and $m_{NS}$ have units in nM h$^{-1}$. Assuming that total net NH$_4^+$ removal and ammonia oxidation are first-order reactions (Ward, 2000), the corresponding specific rate
constants \( (k_{\text{tot}} \text{ and } k_{\text{oxid}}) \) were computed by normalizing \( R_{\text{tot}} \) and \( R_{\text{oxid}} \) to the initial \( NH_4^+ \) concentrations, \( N_0 \), i.e.

\[
\begin{align*}
  k_{\text{tot}} &= \frac{R_{\text{tot}}}{N_0} \\
  k_{\text{oxid}} &= \frac{R_{\text{oxid}}}{N_0}
\end{align*}
\]

\( NH_4^+ \) turnover times were the reciprocals of specific rate constants.

4.2.3.ii. High Pressure Incubation Experiments

In a preliminary study, total net \( NH_4^+ \) removal rates were postulated to be enhanced by \textit{in situ} high pressure (~200 atm) (Cowen et al., 1998). Thus, parallel duplicate subsamples in six experimental series were repressurized to 200 atm after sample treatments (with and without nitrapyrin), for a 48-hour incubation at 2°C (Cowen et al., 1998). All incubations were terminated via freezing at -20°C, and were analyzed for \( NH_4^+ \) as described for the basic incubation experiments.

4.2.3.iii. Substrate Limitation Experiments

The hypothesis of substrate limitation at low \( NH_4^+ \) levels was tested on seven seawater subsamples from Stations A, C, D, E and F, tentatively representative of various parts of an aging plume and the above-plume background seawater. Triplicate subsamples were amended with 68-109 nM of \( NH_4^+ \), with and without the inhibitor nitrapyrin, and were incubated for approximately 48 hours at 2°C and 1 atm. Samples were stored frozen (-20°C) and were analyzed in the same manner as in the basic incubation experiments.

4.2.3.iv. Particle-Filtered Incubations

Some, if not all, ammonia-oxidizing bacteria are inclined to be associated with particles (Stehr et al., 1995). As a result, three experimental series were chosen from the particle-rich plumes (Stations A and B), and particles larger than 3 μm and 10 μm in
diameter were removed in parallel subsamples prior to incubation. Water samples were first filtered through 10 μm-pore-size Nitex screens. Half of the filtrate was subsampled for incubation with and without nitrapyrin in triplicates. The remaining half of the filtrate was further filtered through 3 μm-pore-size membrane filters, and the final 3 μm-filtrate was subsampled for triplicate incubations with and without nitrapyrin. All incubations were terminated via freezing at -20°C, and were analyzed for NH₄⁺ as described for the basic incubation experiments.

4.2.4. ¹⁵N-Ammonia Oxidation Rate Measurements

Ammonia oxidation rates were also measured via ¹⁵N-labelled experiments in selected samples. Seawater subsamples, in duplicate or triplicate, were collected in the same manner as for the total net NH₄⁺ removal experiments. Additions of ¹⁵N-ammonium chloride (99 atom%, Isotec, Inc.) were targeted to achieve levels of ≤ 10 % of initial NH₄⁺ concentrations. ¹⁵N-label additions were determined after NH₄⁺ analyses of the same untreated water samples immediately upon sample collection. The seawater subsamples were incubated at 2°C and 1-atm pressure for 0, 24 and 48 hours. Incubations were terminated by freezing at -20°C, and were stored frozen until further analyses.

In a shore-based laboratory, stable isotopic analyses of nitrite plus nitrate (NOₓ⁻) were performed using a bioassay method, in which NOₓ⁻ was reduced by pure bacterial cultures of Pseudomonas chlororaphis to nitrous oxide (Sigman et al., 2001). Then the samples were analyzed for δ¹⁵N-N₂O as previously described (Dore et al., 1998), except that the N₂O gas was injected directly into the gas-chromatography-mass-spectrometer system from the headspace of the sample vials without the need of prior gas stripping.
(Popp et al., 1995). The apparent $^{15}$N-ammonia oxidation rates ($R_{15N}^*$) are calculated as:

$$R_{15N}^* = \frac{(n_t - n_0)}{(n_{NH4^+} - n_0)} \times \frac{C}{t},$$

where $n_0$ and $n_t$ are the atom% of $^{15}$N in the NO$_x^-$ pool initially and at time $t$, while $n_{NH4^+}$ represents the atom% of $^{15}$N in the initial NH$_4^+$ pool. The NO$_x^-$ concentration was represented by $C$, which was assumed to be relatively constant at a typical deep-sea value of 40 $\mu$M. Correction for isotope dilution effects were performed according to the formulae derived by (Kanda et al., 1987). Briefly, let

$$b = \frac{\rho_A \times t}{(N_0 + N_{15N})}$$

where $N_0$ and $N_{15N}$ are the concentrations of ambient NH$_4^+$ and the added $^{15}$N tracers respectively, while $t$ is the incubation time and $\rho_A$ is the apparent NH$_4^+$ uptake rate. The values of $\rho_A$ include both the apparent ammonia oxidation rates and assimilation rates (see section 4.2.6 for the latter measurements). Then the correction factor, $x = \rho / \rho_A$, is calculated as:

$$x = -1 + \frac{(1-b)^{1-a}}{(a-1) b}$$

where $\rho$ is the true total uptake rate (ammonia oxidation + assimilation) and $a$ is the ratio of regeneration rate to $\rho$. If regeneration rates are calculated to be the difference between the total net ammonium removal rates (or $R_{tot}$, see section 4.2.3.i) and $\rho$, the values of $a$ can be first estimated using the apparent rate measurements and further iterated with the isotope dilution corrected rates. The resulting correcting factors were 1.02 to 3.21 (median = 1.31).
4.2.5. Particulate Organic Carbon and Particulate Nitrogen

Particulate organic carbon (POC) and nitrogen (PN) samples were collected by filtering eight to ten liters of seawater through 25-mm-diameter combusted glass fiber filters (GF/F; Whatman, Inc.) under a positive pressure (~10 psi) of 0.2-μm-filtered nitrogen gas. The filters were carefully folded, wrapped in combusted aluminum foil and frozen at -20 or -70 °C until further analyses. In a shore-based laboratory, the filters were dried briefly in a drying oven (60 °C), each acidified with a few drops of sulfurous acid (6 %) to eliminate inorganic carbon, and then dried again at 60 °C. The filters were carefully wrapped in 9 x 10 mm tin boats and compressed to small pellets. The samples were simultaneously analyzed for POC and PN contents, and the natural carbon (δ\(^{13}\)C-POC) and nitrogen (δ\(^{15}\)N-PN) stable isotopic compositions, using a Carlo Erba NC2500 elemental analyzer coupled with an on-line Finnigan MAT Delta S isotope ratio mass spectrometer via a Finnigan ConFlo II split interface. The δ notation is defined as

\[
\delta = \left( \frac{r_{\text{sample}} - r_{\text{std}}}{r_{\text{std}} - 1} \right) \times 1000 \text{‰}
\]

where \(r_{\text{sample}}\) and \(r_{\text{std}}\) are the atomic abundance ratios of the heavier to lighter stable isotopes in the sample and in the standard respectively. The stable isotopic ratio of carbon refers to \(^{13}\)C/\(^{12}\)C and the carbon standard is the Pee Dee Belemite. The corresponding ratio for nitrogen is \(^{15}\)N/\(^{14}\)N, and the standard is the dinitrogen gas in the air.

4.2.6. Assimilation Rate Measurements

A \(^{15}\)N-tracer technique was employed to measure assimilation rates. About 18 liters of seawater from two 10-liter Niskin bottles tripped at the same depth and location, were pooled into an acid-cleaned polyethylene cubitainer. \(^{15}\)N-ammonium chloride (99 atom%, Isotec, Inc.) was added at approximately tracer levels (≤ 10 % of initial NH\(_4^+\))
concentrations) and incubated at 2°C and 1 atm for approximately 48 hours. At the end of each experiment, the entire 18 liters of seawater sample were filtered through a combusted GF/F glass fiber filter and analyzed for POC and PN contents, $\delta^{13}$C-POC and $\delta^{15}$N-PN, as described above for the natural abundance samples. Assimilation rates ($R_{asm}$) over incubation time $t$ can be calculated as:

$$R_{asm} = \frac{(n_t - n_o)}{(n_{NH4+} - n_o)} \times \frac{C}{t}$$

where $n_o$ and $n_t$ are the atom% of $^{15}$N in the PN samples at time-zero and at time $t$ respectively, while $n_{NH4+}$ is the estimated initial NH$_4^+$ pool and $C$ is the concentration of PN. Corrections for isotope dilution effects are performed as described in section 4.2.4. However, due to possible contamination with nitrapyrin in the $^{15}$N-ammonia oxidation experiments (see Results), the ammonia oxidation rates derived from concentration differences in section 4.2.3.i ($R_{oxid}$) are used for the isotope dilution corrections instead.

4.2.7. Concentration and Carbon Stable Isotope Measurements of Methane

Water samples were collected for methane analyses in 260 ml glass serum bottles. Seawater was allowed to overflow for about three times the bottle volume. Then the bottles were filled to the top, avoiding bubbles. Saturated mercuric chloride solution (1 ml) was added to each sample prior to sealing with gray butyl rubber stoppers and aluminium crimp-seals. In a shore-based laboratory, these samples were analyzed simultaneously for methane concentrations and carbon stable isotopic compositions as previously described (Sansone et al., 1997).

4.2.8. Fluorescence In Situ Hybridization

Eubacteria, $\beta$-proteobacterial ammonia-oxidizing bacteria ($\beta$-AOB), $\gamma$-proteobacterial ammonia-oxidizing bacteria ($\gamma$-AOB), *Nitrosomonas* spp. and
Nitrosospira spp. (two major groups of β-AOB), as well as methanotrophs (Type I and Type II) were enumerated by fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes (Table 4.1). The detailed procedures have been described earlier in Lam et al. (2004) (Chapter 3). Ammonia-oxidizing bacteria in the β-Proteobacteria subgroup (βAOB) were detected by two oligonucleotide probes, NSO190 and NSO1225 (Mobarry et al., 1996). NSO190 is regarded as more specific for βAOB but likely misses some Nitrosomonas spp., whereas NSO1225 has a wider coverage, but it may also hybridize with some non-βAOB targets (see Table 4.1 and Utaker and Nes(1998), Purkhold et al. (2000). However, searches by Probe Match (Cole et al., 2003) in the Ribosomal Database Project II (Maidak et al., 1999) found more matching βAOB or βAOB-like sequences with NSO190 than with NSO1225 (384 versus 140). Hence, neither NSO190 nor NSO1225 gives a perfectly accurate estimate of the βAOB abundance. The βAOB abundance in this study is reported henceforth as a range between the hybridized-cell-counts of the two probes, and perhaps they should be regarded as potential underestimates. Hybridization with NSO1225 yielded slightly higher abundance estimates than that with NSO190 in most but not all cases. Total microbial abundance was obtained via DAPI-counter-staining after FISH processing on the same filter sections.

Size-fractionation sequential filtration through 10-μm-pore-size Nitex screens, 3.0- and 0.2-μm-pore-size membrane filters, was performed to differentiate between particle-associated and free-living communities (Lam and Cowen, 2004; Lam et al., 2004; Chapters 2 & 3) in five selected samples. They represent the above-plume
background and within-plume environments over the MEF (Station A) and High Rise (Station B) vent fields, as well as the aging plume at Station C.

4.3. RESULTS

4.3.1. Neutrally Buoyant Plume Signals

The neutrally buoyant plume along the Endeavour Segment extended from 50 m to 350 m above the seafloor, with the core (maximum $\Delta \theta = 0.12$ °C, $\Delta c = 0.65$) centered at 1900-2000 m within the axial valley. Stronger plume signals were observed directly above the vent fields Main Endeavour Field (MEF) and High Rise, followed by Mothra (Fig. 4.2). Plume signals remained high over the west wall of the axial valley, particularly in the southern half of the west along-axis Tow B (Fig. 4.2). The neutrally buoyant plume appeared to flow in a south to southwest direction away from the axial valley, presumably driven by local dominant currents (Thomson et al., 2003), and was still detectable at 7 km to the southwest of MEF ($\Delta \theta = 0.08$ °C, $\Delta c = 0.043$ at 1929 m). Station D showed the weakest plume signals (maximum $\Delta \theta = 0.005$ °C, $\Delta c = 0.043$ at 2224 m) amongst all the vertical sampling stations, where $\Delta \theta$ was $\geq 0.07$ °C within the plume.

4.3.2. Ammonium Distribution and Ammonia Oxidation Rates

Ammonium concentrations generally followed the spatial trend of hydrothermal plume signals ($\Delta \theta$ and $\Delta c$), with the $\text{NH}_4^+$ maximum observed in the plume core above High Rise (177 ± 3 nM) (Fig. 4.3b). Concentrations above MEF (87 ± 2 nM) and Salty Dawg (117 ± 4.5 nM) were also high, but the concentration above Mothra (28 ± 1.2 nM) was only moderate and that above Sasquatch (10 ± 0.8 nM) was close to background levels ($\leq 10$ nM) (Fig. 4.3 a, b). Slightly elevated $\text{NH}_4^+$ (up to 27 nM) was detected
directly across from High Rise and Salty Dawg over the west valley wall, yet low \( \text{NH}_4^+ \)
levels approaching background levels were found along the rest of Tow B towards the
south until Station E (Fig. 4.3 a). The \( \text{NH}_4^+ \) level was fairly high \(( \leq 79 \pm 2.8 \text{ nM} )\) at
Station E, where there was a gap in the valley wall, compared to 13-14 nM measured
within the axial valley at its southern end (Station D) (Fig. 4.3b). \( \text{NH}_4^+ \) concentrations
dropped quickly to background level with increasing distance from the axial valley, apart
from a slight elevation \((34 \text{ nM})\) at 6.5 km to the southwest of MEF within the aged plume
core.

The diffuse fluids collected over the tubeworm beds in MEF contained \( \text{NH}_4^+ \) in
the range of 1.1-8.1 \( \mu \text{M} \), while the hot black smoker fluids from the Cathedral and S&M
structures in MEF contained 3.5-24.0 \( \mu \text{M} \) \( \text{NH}_4^+ \). Although the concentrations in the hot
focused and warm diffuse fluids were two to four orders of magnitude higher than the
plume values, the former were an order of magnitude lower than those reported in the
end-member hydrothermal fluids in other studies (Lilley et al., 1993; Seewald et al.,
2003). Since the titanium major samplers are not perfectly gas-tight to the volatile \( \text{NH}_3 \),
the predominant form present in hot and warm hydrothermal fluids instead of \( \text{NH}_4^+ \), the
\( \text{NH}_4^+ \) measurements for the hot focused and warm diffuse fluids in this study are likely
underestimates.

4.3.2.i. Basic Incubation Experiments

The \( \text{NH}_4^+ \) level did not drop linearly with \( \Delta \theta \) within the neutrally buoyant plumes
\((r^2 = 0.288, p<0.0001, \text{linear regression analysis})\). Although the exact relationship
between \( \text{NH}_4^+ \) and \( \Delta \theta \) is unclear, the data points with both high \( \Delta \theta \) and high \( \text{NH}_4^+ \) formed
two clusters that represented the plume maxima directly above the High Rise and MEF
vent fields respectively (Fig. 4.4). If the NH$_4^+$ in the discharge fluids or plume maxima from these two vent fields mix conservatively with NH$_4^+$-poor ambient seawater, NH$_4^+$ from the two clusters should follow two linear mixing lines with decreasing $\Delta \theta$ to the $\sim$10 nM background at $\Delta \theta = 0.00 \, ^\circ \text{C}$. This is clearly not the case here. NH$_4^+$ decreased non-linearly with decreasing $\Delta \theta$, indicating rapid NH$_4^+$ uptake as the plume signals weakened. The mixing of NH$_4^+$ from various sources (e.g. other vent fields) and with the ambient seawater at different locations may contribute to the observed data scatter.

Total net NH$_4^+$ removal rates and ammonia oxidation rates were significantly correlated with NH$_4^+$ concentrations ($R=0.75$, $p<0.001$ and $R=0.71$, $p<0.005$ respectively; Spearman rank-order correlation) and roughly followed first-order reaction kinetics (see Discussion). Ammonia oxidation rates accounted for 93-280 % of total net NH$_4^+$ removal rates. The maximum total net NH$_4^+$ removal rate ($57 \pm 7.5$ nM d$^{-1}$) and ammonia oxidation rate ($53 \pm 8.7$ nM d$^{-1}$) were measured in the plume core over High Rise, whereas those in the plume core over MEF were lower (total net removal rate = $25 \pm 2.0$ nM d$^{-1}$; ammonia oxidation rate = $23 \pm 2.4$ nM d$^{-1}$) (Fig. 4.5 a, b). Both rates declined rapidly as the plume signals weakened with increasing distance away from these two plume cores, to $< 5$ nM d$^{-1}$ in the above-plume deep water ($< 1800 \, \text{m}$) and at Stations C, D and F (Fig. 4.5 c, d, f). Slightly elevated total net removal rates ($6 \pm 0.5$ nM d$^{-1}$) and ammonia oxidation rates ($8 \pm 1.0$ nM d$^{-1}$) were observed at Station E (Fig. 4.5 e), but both rates were close to zero at the distal off-axis Station F (Fig. 4.5 f).

Some total net NH$_4^+$ removal rates were negative at above-plume depths or within an aged plume in spite of positive ammonia oxidation rates. This suggests active regeneration of NH$_4^+$ in excess of uptake via ammonia oxidation and assimilation. If a
least-square linear regression were performed between ammonia oxidation rates and 
NH$_4^+$ concentrations despite the lack of normality in the data, the resulting intercept is 
10.7 nM of NH$_4^+$, which closely matches the observed background NH$_4^+$ concentration. 
This result suggests a threshold of ~10 nM of substrate for ammonia oxidation to 
become noticeable in this environment.

The specific rate constants calculated for total net removal and ammonia 
oxidation generally lay within the ranges of -0.20 to 0.43 d$^{-1}$ and -0.24 to 0.52 d$^{-1}$ 
respectively, except for the considerably higher values of 1.63 d$^{-1}$ (total net removal) and 
1.53 d$^{-1}$ (ammonia oxidation) found at the plume core over MEF. The latter two values 
corresponded to turnover times of 0.6 d and 0.7 d, while the remaining data suggested 
turnover times of up to 21 d and 13 d with respect to total net removal and ammonia 
oxidation respectively (Table 4.2). No systematic patterns in specific rate constants can 
be discerned.

4.3.2.ii. High-Pressure Incubation Experiments

The total net NH$_4^+$ removal or ammonia oxidation rates measured in the high-
pressure incubations were not significantly different from those measured in 1-atm 
incubations (p > 0.05, two-tailed t-tests) (Table 4.3), suggesting an absence of pressure-
enhancement effects. Nevertheless, it cannot be ruled out that the microbial communities 
did not fully return to their in situ activity levels after re-pressurization to 200 atm in the 
high-pressure incubations, or certain microbes that were more resilient to pressure 
changes might be selected for in the repressurized incubations.

4.3.2.iii. Substrate-Limitation Experiments

When additional NH$_4^+$ was introduced, total net NH$_4^+$ removal and ammonia 
oxidation rates were significantly enhanced in the samples tested (p<0.05, one-tailed t-
test) (Fig. 4.6). The largest stimulation (10-fold increase) of ammonia oxidation occurred at Station C (from the control at 2 nM d\(^{-1}\) to 20 ± 8.1 nM d\(^{-1}\)), while the ammonia oxidation rate was doubled at Station E. At the upper plume boundary over MEF (Station A) and at Station F, total net removal and ammonia oxidation rates increased from negative values to 7 ± 3.8 nM d\(^{-1}\) and 6 ± 2.7 nM d\(^{-1}\) respectively. However, no enhancement was observed at the lower plume boundary and above-plume depths at Station D.

4.3.2.iv. Particle-Filtered Incubation Experiments

The influence of particles on total net NH\(_4^+\) removal and ammonia oxidation rates varied among the three samples tested (Fig. 4.7). A dramatic reduction (99%) in total net NH\(_4^+\) removal was observed in the plume core sample over High Rise (Station B) after removing particles in 3.0-10 µm-diameter size range, yet particles larger than 10 µm-diameter did not appear to have much an effect. Ammonia oxidation in the same sample was reduced by 69% and 49% after 3.0-10-µm- and 10-µm- filtrations respectively. In the upper plume boundary sample from Station B, no noticeable changes were found in either total net removal or ammonia oxidation rates after either particle filtrations. Lastly, the 10-µm-filtration induced a 53% reduction in the total net NH\(_4^+\) removal rates in the plume sample over MEF (Station A), but no changes were observed after the 3.0-µm-filtration. There were also no apparent effects of particle filtration on ammonia oxidation rates in this plume sample.

4.3.3. \(^{15}\)N-Ammonia Oxidation Rates

Ammonia oxidation rates measured with the \(^{15}\)N-tracer method yielded values from −0.38 to 1.57 nM d\(^{-1}\) (Table 4.4), without any systematic changes or significant
correlation ($p>0.05$, Spearman rank-order correlation) with $\Delta \theta$, $\Delta e$, ammonium concentrations and total net removal rates. These rates are generally an order of magnitude lower than those measured with the concentration-difference method ($-5.3$ to $53.0$ nM d$^{-1}$) as described earlier, and these two parallel rate measurements are significantly different from each other ($p<0.005$, Wilcoxon matched pairs test). The low values derived from the $^{15}$N-tracer method are in fact not far from zero. Owing to the unfortunate loss of multiple sets of pipettors during pre-cruise shipment, the addition of $^{15}$N-tracers and that of the nitrapyrin in experiment 4.2.3.i were resorted to share the same pipettor. Despite the use of separate pipette tips, it is suspected that some residual nitrapyrin in the volatile solvent DMSO might have been trapped in the pipettor and contaminated the $^{15}$N-tracer incubations. Therefore, such low $^{15}$N-ammonia oxidation rates likely reflect nitrapyrin inhibition rather than the actual ammonia oxidation rates. Henceforth, discussions with ammonia oxidation rates will be referring to the measurements made in section 4.2.3.i only.

4.3.4. Concentrations and Stable Isotope Analyses of POC and PN

Particulate organic carbon (POC) and particulate nitrogen (PN) concentrations were elevated within plume depths over MEF and High Rise ($7.1-10.9$ μg C l$^{-1}$ and $1.6-2.1$ μg N l$^{-1}$ respectively), relative to above-plume background values ($3.3-6.2$ μg C l$^{-1}$ and $0.7-1.4$ μg N l$^{-1}$) (Table 4.4). The POC and PN concentrations in the plumes at Stations D and F ($4.6-6.4$ μg C l$^{-1}$ and $0.8-1.1$ μg N l$^{-1}$) were similar to above-plume values, yet higher concentrations were measured over the valley wall at Station C ($10.9$ μg C l$^{-1}$ and $2.1$ μg N l$^{-1}$). The particulate C/N ratios ranged between 5.2 and 7.0 in most samples (Table 4.4). Only five samples yielded ratios greater than 7.0, with the
maximum (8.4) obtained from above the plume. Lower $\delta^{13}$C-POC values were detected within or below the plume (< -32.6‰), compared to above-plume background (-31.0 to -26.4‰) (Table 4.4). Unfortunately, since these sample collections shared the same filtering apparatus with the assimilation rate experiments, likely contamination with heavy nitrogen isotopes precluded the measurements of the natural abundance of $\delta^{15}$N-PN. However, archived data did show lower values within or below the plume (3-5‰) relative to background deep water (6-13‰) along the Endeavour Segment (Lam and Cowen, unpublished data).

4.3.5. Assimilation Rates

$\text{NH}_4^+$ assimilation rates were significantly correlated with $\text{NH}_4^+$ concentrations (R=0.61, p<0.05, Spearman rank-order correlation), but not with hydrothermal plume $\Delta\theta$ or $\Delta c$ signals (p>0.05, Spearman rank-order correlation). Assimilation accounted for at least 47% of total net $\text{NH}_4^+$ removal. Like ammonia oxidation, assimilation rates were elevated within the neutrally buoyant plumes, reaching a maximum of 26.4 nM d$^{-1}$ at High Rise (Station B), seconded by 20.6 nM d$^{-1}$ at MEF (Station A), while they were 1.5-5.0 nM d$^{-1}$ in above-plume background samples (Table 4.4). At the other stations, assimilation rates were usually below 5 nM d$^{-1}$, except for that detected at Station E (16.3 nM d$^{-1}$). A very low assimilation rate (0.6 nM d$^{-1}$) was measured at the most distal off-axis Station F. The specific rate constants for $\text{NH}_4^+$ assimilation varied from 0.04 d$^{-1}$ to 0.63 d$^{-1}$ (mean 0.12 d$^{-1}$), without any systematic changes with $\Delta\theta$ or $\Delta c$ (p>0.05, Spearman rank-order correlation). The corresponding turnover times of ammonium due to assimilation are 1.6-25 days.
4.3.6. Concentrations and Carbon Stable Isotope Analyses for Methane

The distribution of methane along the Endeavour Segment closely resembled that of NH$_4^+$ (R=0.824, p<0.000001, Spearman rank-order correlation), decreasing non-linearly with hydrothermal δθ or Δc signals. The highest methane concentrations were usually obtained directly above vent fields within the axial valley (Fig. 4.8a): 861 nM at High Rise, 309 nM at Main Endeavour Field, 320 nM at Mothra and 532 nM at Salty Dawg, yet only background levels (2-3 nM) were detected near Sasquatch. CH$_4$ elevation could also be detected over the west valley wall across from MEF (78 nM, Station C), and it was even higher towards the southern end of Tow B (≤ 217 nM). CH$_4$ concentrations dropped to 2-4 nM at the far end of Tow F, 7 km southwest of the MEF (Fig. 4.8a).

The hot vent fluids (>310 °C) emanating from black smoker chimneys in MEF (Cathedral and S&M structures) possessed 201-268 μM of CH$_4$, compared to 2.8-17.3 μM measured in diffuse fluids rising up through beds of tubeworms. However, since the titanium major samplers are not perfectly gas-tight, these concentration measurements in hot and warm fluids should be treated as underestimates.

Carbon stable isotopic values of methane in the Endeavour neutrally buoyant plumes were previously found to show an inverse logarithmic relationship with concentrations due to the preferential uptake of isotopically lighter methane during methane oxidation (Cowen et al., 2002). The δ$^{13}$C-CH$_4$ values measured in hot vent fluids were −47.4 to −46.9 ‰, whereas a wider range of values were measured in the diffuse fluids (−47.3 to −36.0 ‰) that might imply some degrees of methane oxidation in certain samples. Low δ$^{13}$C-CH$_4$ values were maintained in the neutrally buoyant plume.
within the axial valley (< -30.0 °/oo). The lowest isotopic values were found directly above High Rise and Salty Dawg (-50.4 to -48.9 °/oo), or near Station E (-73.0 °/oo). The δ¹³C-CH₄ values increased considerably over the west valley wall, and reached +10.7 °/oo directly across from MEF (Station C), implying the greatest extent of methane oxidation (Fig. 4.8b). High values of δ¹³C-CH₄ were also detected away from the axial valley, up to +5.5 °/oo at 7 km to the southwest of MEF.

4.3.7. Microbial Community Structure

The total microbial abundance, estimated by counts of DAPI-stained cells, and Eubacterial abundance, estimated by EUB338-hybridized cell counts, were correlated with the plume signals Δθ (R=0.58, p<0.01 and R=0.66, p<0.005 respectively, Spearman rank-order correlation) and Δc (R=0.66, p<0.001 and R=0.67, p<0.0005, Spearman rank-order correlation). Substantially elevated total microbial abundance was observed within the plume maxima at MEF (1.51 ± 0.05 × 10⁵ cells ml⁻¹) and High Rise (1.45 ± 0.06 × 10⁵ cells ml⁻¹), as well as over the west valley wall at Station C (1.67 ± 0.07 × 10⁵ cells ml⁻¹), relative to above-plume communities (0.6-0.7 × 10⁵ cells ml⁻¹) (Fig. 4.9). An intermediate-sized microbial population remained in the far-field plume 5.5 km away from MEF (1.14 ± 0.04 × 10⁵ cells ml⁻¹; Station F), but that at the southern end of the valley fell close to above-plume levels (0.54 ± 0.04 × 10⁵ cells ml⁻¹; Station E).

Eubacterial abundance followed the same general trends as total microbial abundance at all stations examined (Fig. 4.9). Eubacteria usually predominated the microbial communities (47-110 % DAPI cell counts). The only two samples where Eubacteria contributed less than 50% were collected from High Rise (1700m and 2000 m), in which there might be a greater contribution from some Archaea, Eukaryota or the Eubacteria.
that were not hybridized by EUB338, including *Plantomycetales* and *Verrucomicrobia* (Daims et al., 1999).

The abundance of ammonia-oxidizing bacteria in the β-Proteobacteria subgroup (βAOB) was only moderately correlated with Δθ (R=0.52, p<0.05, Spearman rank-order correlation) and Δc (R=0.56, p<0.01, Spearman rank-order correlation), but not at all with NH₄⁺ concentrations or ammonia oxidation rates (p>0.05, Spearman rank-order correlation). βAOB abundance increased from the above-plume background of 0.6-1.3 × 10³ cells ml⁻¹ (1.2-3.2% DAPI) to a maximum of 6.5-11.1 × 10³ cells ml⁻¹ (4.5-7.6% DAPI) within the plume over High Rise (Fig. 4.10). A large βAOB population was also found at MEF (6.0-9.0 × 10³ cells ml⁻¹; 4.0-5.9% DAPI), but it was much smaller across the west valley wall at Station C (1.4-2.1 × 10³ cells ml⁻¹; 0.9-1.2% DAPI). The lowest βAOB abundance was found at Station E (0.1-0.6 × 10³ cells ml⁻¹; 0.2-1.1% DAPI) and Station F (0.5-1.3 × 10³ cells ml⁻¹; 0.4-1.1% DAPI). Among the βAOB, *Nitrosospira*-like cells appeared more abundant than the *Nitrosomonas*-like cells in most cases (Fig. 4.11).

The population of γ-proteobacterial ammonia-oxidizing bacteria (γAOB) was usually smaller than that of βAOB, except for the plume core above MEF, where a similarly large γAOB population was present (8.8 ± 0.6 × 10³ cells ml⁻¹; 5.8% DAPI) (Fig. 4.10). The depth distribution of γAOB relative to plume Δθ and Δc conformed to a pattern similar to that of βAOB. However, the γAOB abundance was only moderately correlated with Δc (R=0.47, p<0.05, Spearman rank-order correlation), but not with Δθ, NH₄⁺ concentrations or ammonia oxidation rates (p>0.05, Spearman rank-order correlation).
Methanotroph abundance was significantly correlated with plume signals $\Delta \theta$
(Type I: $R=0.70$, $p<0.01$; Type II: $R=0.57$, $p<0.01$, Spearman rank-order correlation)
and $\Delta c$ (Type I: $R=0.70$, $p<0.0005$; Type II: $R=0.73$, $p<0.0001$, Spearman rank-order
 correlation). High methanotroph abundance was especially observed close to the seafloor
in both MEF and High Rise vent fields (Fig. 4.12). There was no correlation between
methanotroph abundance and CH$_4$ concentrations or carbon stable isotopic compositions
of methane ($p>0.05$, Spearman-rank order correlation). All samples seemed to be more
populated with Type I methanotrophs than with Type II methanotrophs (Fig. 4.12). Their
abundance as individual groups (Type I: $2.2-5.1 \times 10^3$ cells ml$^{-1}$; Type II: $1.5-3.0 \times 10^3$
cells ml$^{-1}$) in the plume cores over MEF and High Rise was not as high as that of $\beta$AOB
(or $\gamma$AOB at MEF); nor was the total methanotroph abundance (Type I + Type II: $4.1-8.1$
$\times 10^3$ cells ml$^{-1}$) as high as the total AOB abundance ($\beta$AOB + $\gamma$AOB: $10.7-16.3 \times 10^3$
cells ml$^{-1}$). Conversely, the methanotrophs (total: $2.5-3.3 \times 10^3$ cells ml$^{-1}$) were more
abundant than AOB (total: $0.4-1.6 \times 10^3$ cells ml$^{-1}$) within the moderate plumes at
Stations E and F.

Most microbial cells (DAPI-stained cells) were found to be free-living (0.2-3.0
$\mu$m size-class) in most size-fractionation-sequential-filtration samples (Fig. 4.13 a).
Exceptions were found at High Rise, where the above-plume community was fairly
evenly distributed among the three size-fractions, and the in-plume free-living microbes
represented only 48% of the total community. Such phenomena became more apparent
in Eubacterial abundance, in which 83% and 52% of Eubacteria were particle-associated
in the High Rise above-plume and in-plume samples respectively (Fig. 4.13 b). On the
other hand, $\beta$AOB were found to be primarily particle-associated (46-98%) in almost all
samples investigated (Fig. 4.13 c, d). Within the particle-associated communities, βAOB showed greater association with the 10+ μm size-fraction (27-91%) than with the 3.0-10 μm fraction (0-67%). Both βAOB subgroups, *Nitrosospira* spp. and *Nitrosomonas* spp., had a high affinity for particle-association, though the former also had a strong free-living component within the two young plume cores (62% free-living at MEF, 59% free-living at High Rise) (Fig. 4.13 e, f). In contrast with βAOB, γAOB were mostly free-living (31-99.7%) (Fig. 4.13 g), as were both types of methanotrophs (Fig. 4.13 h, i).

### 4.4. DISCUSSION

The introduction of NH$_4^+$ from seafloor hydrothermal systems to the deep-sea water column clearly influences the cycling of nitrogen in the vicinity of the Endeavour Segment. The observed elevated NH$_4^+$ concentrations were highly confined within the Endeavour neutrally buoyant plume and above the axial valley and were rapidly dissipated as the plume aged. The major sinks for NH$_4^+$ in this environment include microbial autotrophic ammonia oxidation and assimilation. Apart from the external source of hydrothermal discharges, NH$_4^+$ may also be actively regenerated by local biological communities.

#### 4.4.1. Ammonia Oxidation and the Controlling Factors

Despite the lower ammonia oxidation rates in the Endeavour plume reported here for 2002 (3.5-53 nM d$^{-1}$) compared to those detected in 1999 and 2000 (28-91 nM d$^{-1}$) (Lam *et al.*, 2004; Chapter 3), these 2002 values are still higher than the only other deep-sea values ever reported for similar depths, below the oxygen minimum zone in the Eastern Tropical North Pacific (ETNP) (≤ 0.5 nM d$^{-1}$) (Ward and Zafiriou, 1988). In fact, the rates within the Endeavour plume were comparable to those measured in the
euphotic zone of the ETNP (5-20 nM d⁻¹) (Ward and Zafiriou, 1988), some coastal surface ocean regimes (<0.5-45 nM d⁻¹) (Ward, 1987b), part of the northwestern Mediterranean (10-30 nM d⁻¹) (Diaz and Raimbault, 2000), the subtropical North Pacific (1-137 nM d⁻¹) (Dore and Karl, 1996) and parts of the Southern Ocean (6-84 nM d⁻¹) (Bianchi et al., 1997). Even at above-plume depths near vent fields, ammonia oxidation was still detectable (1.7-4.7 nM d⁻¹), although it was close to the detection limit at the most distal station. The turnover times with respect to ammonia oxidation within the plume were relatively short (0.7-12.8 d). This suggests that NH₄⁺ removal via ammonia oxidation is faster than CH₄ oxidation (average turnover time 17 d) (De Angelis et al., 1993).

While temperature, salinity, oxygen, light and pH can be assumed constant for the deep-sea neutrally buoyant hydrothermal plume at Endeavour Segment, ammonia oxidation is regulated by a combination of interdependent factors such as substrate availability, microbial communities and the presence of particulate organic matter.

4.4.1.i. Substrate Availability

Based on culture studies, ammonia oxidation is commonly considered to show first-order reaction kinetics (Ward, 2000), that is, reaction rates increase linearly with NH₄⁺ concentrations. If this is the case for hydrothermal plume environments, ammonia oxidation should be susceptible to the temporal and spatial variability of the NH₄⁺ supply from hydrothermal discharges. Spatial variability of the NH₄⁺ supply within the axial valley is a function of the local circulation pattern, the proximity to each of the five known vent fields, the respective chemistry and discharge rates of vent fluids, as well as the degree of mixing among the various NH₄⁺ sources. The geologically younger vent fields, MEF and High Rise, apparently have more vigorous venting than the other three.
vent fields (Kelley et al., 2001a), which might explain the higher NH$_4^+$ levels and ammonia oxidation rates measured in the overlying plumes above the former two vent fields. Away from the axial valley, the observed NH$_4^+$ elevation is confined to the neutrally buoyant plume. The advection path of the neutrally buoyant plume is subject to local tidal oscillations and deep inertial currents. Thus the location of the plume is not temporally fixed. Temporal variability of both hot focused and diffuse fluid discharges may be introduced by tidal variations (Schultz et al., 1992; Schultz and Elderfield, 1997; Tivey et al., 2002), whereas episodic magmatic events have also been reported to alter the hydrothermal circulation and fluid chemistry (Lilley et al., 2003). The reduction of ammonia oxidation rates observed within the neutrally buoyant plume in 2002 relative to 1999 and 2000, corresponded to a lower NH$_4^+$ elevation measured within the plume in 2002 (up to 177 nM) than in 1999 and 2000 (up to over 400 nM) (Lam et al., 2004; Chapter 3). This is consistent with the decrease in NH$_4^+$ concentrations observed in the end-member hydrothermal fluids after the magmatic event recorded in 1999 (Seewald et al., 2003).

Ammonia oxidation rates were undoubtedly influenced by NH$_4^+$ availability, yet first-order kinetics do not always apply. A positive linear correlation between oxidation rates and substrate concentrations has been demonstrated in some studies (Ward, 1985), but not in others (Olson, 1981; Ward and Kilpatrick, 1990). Ammonia oxidation rates were significantly and linearly correlated with NH$_4^+$ concentrations within the Endeavour plume in 2002 (this study) and in 2000, but not in 1999 (Lam et al., 2004; Chapter 3). However, if the three data sets are combined, a sigmoidal relationship is revealed ($r^2=0.771$, p<0.0001) (Fig. 4.14). This trend is not surprising because ammonia oxidation is a microbially mediated process involving enzymes. Normally, Michaelis-Menten
kinetics would be expected for any enzymatic reactions. However, autotrophic ammonia-oxidizing bacteria are known to enter a dormant state during low NH$_4^+$ conditions (Jones and Morita, 1985; Johnstone and Jones, 1988). A certain substrate threshold is thus required before these ammonia-oxidizing bacteria fully respond to substrate stimulation. The substrate threshold was observed to be ~10 nM of NH$_4^+$ in 2002, though it might be slightly but not significantly higher combining data from all three years (Fig. 4.14). Substrate limitation occurred below this threshold, which is supported by the results of our substrate limitation experiments (Fig. 4.6). Only two out of seven samples did not show an increase in oxidation rates after substrate addition. These two samples were collected from the lower plume boundary and above the plume at Station D, where ammonia oxidation was likely limited by other regulating factors such as small populations of active autotrophic ammonia-oxidizing bacteria. Above the substrate threshold, ammonia oxidation rates increases linearly with NH$_4^+$ concentrations, as illustrated by some data collected from 2002 and those from 2000 (Fig. 4.14). It is only during this linear phase, or the substrate-regulating phase, where first-order reaction kinetics can be assumed. When NH$_4^+$ level exceeds approximately 250-300 nM, ammonia oxidation rates level off at a saturation oxidation rate of 87 nM d$^{-1}$ at an NH$_4^+$ concentration of ≥ 300 nM.

A half-saturation constant (K$_s$) can be empirically determined by finding the NH$_4^+$ concentration that corresponds to half of the saturation oxidation rate, i.e. 87 nM d$^{-1}$ × 0.5 = 43.5 nM d$^{-1}$, which is found to be 153 nM of NH$_4^+$ (Fig. 4.14). This is essentially the same as the only reported field-determined K$_s$ of 150 nM in the marine environment (Hashimoto et al., 1983), but much lower than the K$_s$'s at micromolar levels measured in cultures (Ward, 1987a; Frijlink et al., 1992). The lower field-determined K$_s$ implies that
the assemblages of autotrophic ammonia-oxidizing bacteria in natural oceanic environments have a much higher affinity for NH$_4^+$ than their cultured counterparts, and their saturation phase may begin at as low as $\leq 500$ nM NH$_4^+$. It might also explain why some previous attempts to detect first-order reaction kinetics in marine water columns were unsuccessful, when at least hundreds of nanomolar of NH$_4^+$ were added (Olson, 1981; Ward and Kilpatrick, 1990), which placed the NH$_4^+$ levels well into the saturation phase. However, it is also possible that ammonia-oxidizing communities that inhabit consistently more NH$_4^+$-enriched regimes (e.g. estuaries, sediments and cultures where there are tens of $\mu$M of NH$_4^+$) may adapt to higher K$_s$’s, which better match the higher ambient NH$_4^+$ levels.

4.4.1.ii. Ammonia-Oxidizing Bacteria

Ammonia-oxidizing bacteria (AOB) are ubiquitous in the deep-sea, as they were detected in all samples including those free of hydrothermal influence. This suggests that the potential for nitrification is also ubiquitous throughout the deep-sea. The abundance of AOB were clearly elevated in the Endeavour neutrally buoyant plume, but the relationships between AOB abundance and environmental variables or oxidation rates, are not always straightforward. Although the depth profiles of AOB abundance ($\beta$AOB and $\gamma$AOB individually and together) reflected those of ammonia oxidation rates, and the maximum AOB abundance coincided with the highest specific rate constant with respect to ammonia oxidation; AOB abundance was poorly correlated with either NH$_4^+$ concentrations or ammonia oxidation rates ($p>0.05$, Spearman rank-order correlation). The highest AOB abundance was found at intermediate NH$_4^+$ levels (70-90 nM) and intermediate oxidation rates (20-30 nM d$^{-1}$). Likewise, there might be at most a weak correlation between AOB abundance and ammonia oxidation rates in 1999 ($r = 0.70$, $p <$
0.18) but not at all in 2000 (r = 0.16, P > 0.5) (Lam et al., 2004; Chapter 3), neither in the Southern California Bight (Ward, 1987b) nor the Eastern Tropical South Pacific (Ward et al., 1989). The most probable explanation for such lack of correlation is that AOB population growth and ammonia oxidation rates respond to environmental changes on different time-scales. AOB may start increasing their cellular oxidation rates and other cellular activities as initial or short-term responses to increased substrate availability. If the increase in substrate availability is sustained, cellular oxidation rates and cellular yield of organic carbon continue to rise and pass their thresholds permitting cell growth and then cell multiplication. Larger cell volumes without population growth have been noted shortly after substrate addition (Tappe et al., 1999); while higher cellular oxidation rates and organic carbon yields during late exponential growth phase compared to other growth phases have also been documented (Glover, 1985). Hence, ammonia oxidation rates by the entire ammonia-oxidizing community may be expected to show a fast response to NH₄⁺ addition if NH₄⁺ concentration is the limiting or regulating factor (Johnstone and Jones, 1988; Tappe et al., 1999), whereas a longer period may be required for AOB abundance to change.

In summary, a relatively large AOB population may reflect long-term availability of NH₄⁺, hence sustainability for ammonia oxidation, in spite of some small fluctuations in supply rates. An example would be the MEF plume core where the AOB abundance remained fairly constant and large in the three years of sampling (~1.0 x 10⁴ cells ml⁻¹) despite variations in NH₄⁺ concentrations and ammonia oxidation rates. In contrast, a relatively small AOB population with high ammonia oxidation rates, and consequently high cellular ammonia oxidation rates, may represent a situation of newly available NH₄⁺ before the AOB have begun to multiply. Exceptionally high cellular ammonia oxidation
rates (oxidation rates normalized to the sum of βAOB and γAOB abundance) were computed for the young plume core over High Rise (21.2 × 10^{-12} \text{ mol N cell}^{-1} \text{ d}^{-1}) and at Station E (22.4 × 10^{-12} \text{ mol cell}^{-1} \text{ d}^{-1}), relative to 0.7-7 × 10^{-12} \text{ mol N cell}^{-1} \text{ d}^{-1} (mean: 1.9 × 10^{-12} \text{ mol cell}^{-1} \text{ d}^{-1}) in other samples in 2002. The \text{NH}_4^+ concentrations at the former two locations might have increased shortly before sampling, due to changes in venting, tidal variations, or other potential variability of substrate supply common in the plume environments as discussed earlier. If the elevated \text{NH}_4^+ supply is maintained, AOB abundance would likely start to increase. However, if the \text{NH}_4^+ increase is only temporary, then the AOB abundance would likely remain small.

4.4.1.iii. The Influence of Particulate Organic Matter

The Endeavour neutrally buoyant plume was characterized by almost double the particulate organic carbon and nitrogen contents as in the background deep-water, about 1.0 lower C/N ratios and as much as 6 \% lower δ^{13}C-POC and δ^{15}N-PN. These observations point to an abundance of probably more labile \textit{de novo} organic carbon and nitrogen, likely produced chemosynthetically at depth due to hydrothermal activities (Roth and Dymond, 1989), than the above-plume background deep water. Ammonia-oxidizing bacteria (AOB) have been observed to actively produce exopolymeric substances for particle-attachment (Stehr \textit{et al}., 1995; de Bie \textit{et al}., 2002) and may do so for a number of reasons. Firstly, remineralization of organic particles releases \text{NH}_4^+ as an additional source of substrate. Secondly, a low particulate C/N ratio implies the release of DOM with a lower C/N, which might reduce the competition for \text{NH}_4^+ from heterotrophic uptake (Strauss \textit{et al}., 2002). This is supported by the negative correlation between ammonia oxidation rates and particulate C/N ratios in this study (R = -0.54,
p<0.05, Spearman rank-order correlation), although no correlation could be found between assimilation rates and particulate C/N ratios (p>0.05, Spearman rank-order correlation). Therefore, the abundance of particles in deep-sea hydrothermal plumes should favor autotrophic ammonia oxidation. This expectation is consistent with the association of 40-68 % of total AOB (sum of βAOB and γAOB in all size-fractions) with particles greater than 3.0 μm in diameter in all samples examined (Table 4.5). The degree of particle-association of total AOB increased at above-plume depths (Table 4.5), which is consistent with the observations made in 2000 for β-proteobacterial AOB (βAOB) (Lam et al., 2004; Chapter 3). As the NH$_4^+$ concentrations decreased away from the plume core, more NH$_4^+$ might be available from the decomposing organic-rich particles than from the ambient seawater, that AOB would have an advantage of being particle-associated. The importance of particles for ammonia oxidation was also evidenced by the significant reduction in oxidation rates (49-69 %) after particle-removal in the High Rise plume core, though such reduction did not occur in the MEF plume core. The opposing observations in these two plumes might be attributed to their different AOB community structures, as various AOB species have different physiological responses to environmental factors, including their affinity for NH$_4^+$ and cellular ammonia oxidation rates, (Koops and Pommerening-Roser, 2001). In the MEF plume core, there was an exceptionally large component of Nitrosococcus spp. (γ-proteobacterial AOB) in the total microbial community (5.8 % of DAPI), while the βAOB subgroups, Nitrosomonas spp. and Nitrosospira spp., accounted for only 0.2 % of DAPI and 1.3 % of DAPI respectively. Nitrosococcus spp. contributed only 0-1.8 % of total microbial abundance in all other samples, including the High Rise plume. The
contribution from the free-living *Nitrosococcus* spp. alone to the total AOB population from all size-fractions was much higher in the MEF plume (24 %) than in the High Rise plume (5 %). On the other hand, the contribution from the free-living *Nitrosospira* spp. was similar to both plumes (35 % and 40 % respectively). In fact, the sum of all free-living AOB accounted for 60 % of total AOB in the MEF plume, compared to only 48 % in the High Rise plume (Table 4.5). Hence, particle-removal in the former would have a smaller impact on ammonia oxidation rates than the latter.

4.4.2. Assimilation of Ammonium

Unlike the euphotic zone, where most assimilation studies have been conducted, NH$_4^+$ assimilation in the deep sea does not involve phytoplankton. However, the assimilation rates in the Endeavour plume (1.5-26 nM d$^{-1}$) were comparable to those measured at the base of the euphotic zone in the Southern California Bight and off the Washington coast (<20 nM d$^{-1}$) (Ward, 1985), in the oxygen minimum zone of the Eastern Tropical South Pacific (5-10 nM d$^{-1}$) (Lipschultz *et al.*, 1990), and in portions of the Southern Ocean (0.75-1.4 nM d$^{-1}$) (Bianchi *et al.*, 1997). Assimilation rates were highly comparable with ammonia oxidation rates where parallel measurements were made. They were not significantly different from each other (p>0.05, Wilcoxon matched pairs test), suggesting that both processes are equally important uptake pathways for the elevated ammonium within the Endeavour hydrothermal plume.

4.4.3. Enhanced Nitrogen Cycling in the Endeavour Neutrally Buoyant Plume

The two uptake rate processes, ammonia oxidation and assimilation, combined are obviously greater in magnitude than the total net NH$_4^+$ removal rates in all the cases examined. This means that additional sources of NH$_4^+$ had to be available, most likely from the regeneration of NH$_4^+$ (or ammonification). Assuming that ammonia oxidation
and assimilation were the only two uptake terms, and that there were no external NH$_4^+$ inputs apart from the hydrothermal inputs, the resulting NH$_4^+$ regeneration rates within the Endeavour axial valley are about 1.5-29.6 nM d$^{-1}$, or 8-89 mg N m$^{-2}$ d$^{-1}$ integrated over the ~250-m thick neutrally buoyant plume. In comparison, the potential NH$_4^+$ regeneration from sinking particles can be estimated from the integrated loss of particulate nitrogen from just 1200 m to plume depths (1950 m), using sediment trap data from the vicinity of the Endeavour Segment (Roth and Dymond, 1989). This potential nitrogen flux was estimated to be 0.31 mg N m$^{-2}$ d$^{-1}$. Alternatively, 0.18 mg N m$^{-2}$ d$^{-1}$ was integrated over a narrower depth range (1637 m to 1986 m) with a more recent particle flux data set (Cowen et al., 2001). These two estimates of particulate nitrogen flux are equivalent to 0.2-4 % of the NH$_4^+$ regeneration flux calculated for the neutrally buoyant plume alone in this study. Hence, the majority of the required NH$_4^+$ regeneration had to take place locally within the plume. Apart from the increased microbial abundance (Lam et al., 2004; Chapter 3), zooplankton biomass in the immediately overlying water of the Endeavour plume was two- to three-fold that in the deep-sea background, and was likely associated with the organic-rich Endeavour plume (Burd and Thomson, 1994). This large zooplankton population could likely enhance NH$_4^+$ regeneration directly through excretion and indirectly by the decomposition of their carbon- and nitrogen- rich fecal pellets or the materials released during sloppy feeding (Antia et al., 1991).

The ammonia oxidation rates, integrated over plume depths within the Endeavour axial valley, were 26-130 mg N m$^{-2}$ d$^{-1}$ at individual stations, or approximately 72 mg N m$^{-2}$ d$^{-1}$ over the area (~10 km$^2$) of the axial valley. If ammonia oxidation is assumed to be the rate-limiting step of nitrification (Kowalchuk and Stephen, 2001), and that all

137
resultant nitrite is eventually oxidized to nitrate, then the nitrogen flux from ammonia oxidation would be equivalent to that from nitrate formation. In other words, hydrothermal venting of NH$_4^+$ induces a flux of 26-130 mg N m$^{-2}$ d$^{-1}$ to the deep-sea nitrate reservoir at Endeavour, which will be advected and eventually used to fuel 'new production' in the euphotic zone via upwellings or eddy diffusion. A crude estimate of the export flux of particulate nitrogen from 1200 m to plume depths in this region (Roth and Dymond, 1989) was calculated to be at most 0.6 mg N m$^{-2}$ d$^{-1}$. The much larger nitrogen flux (43 to 216 times) from nitrification within the plume over the particle export flux from the upper water column, revealed an important source of nitrate fueled indirectly by seafloor hydrothermal system to the deep-sea water column along the Endeavour Segment.

4.4.4. Ammonia Oxidation as an In Situ Organic Carbon Production Pathway

The principal pathways of chemotrophy in hydrothermal plumes are considered to be the oxidation of sulfur, hydrogen and methane (Winn et al., 1995; McCollom, 2000). The first two are probably important only at the buoyant or early neutrally-buoyant phases of plume formation, while methanotrophy is likely more important in the neutrally buoyant phase (Winn et al., 1995; McCollom, 2000). Ammonia oxidation has not received much attention, mainly because NH$_4^+$ is not much elevated in the hydrothermal fluids emanating from most sediment-starved mid-ocean ridge systems except for Endeavour (Lilley et al., 1993). Using a conversion factor of 0.3-1.4 g C mol$^{-1}$ N (Feliattra and Bianchi, 1993; Butturini et al., 2000) and the ammonia oxidation rates integrated plume depths within the Endeavour axial valley (1.9-9.3 mmol N m$^{-2}$ d$^{-1}$), ammonia oxidation potentially produce organic carbon at a rate of 0.6-13 mg C m$^{-2}$ d$^{-1}$ within the Endeavour plume. This is equivalent to as much as 1300 % of the
photosynthetically-derived particulate organic carbon reaching plume depths (Roth and Dymond, 1989; Cowen et al., 2001). Meanwhile, methanotrophic production within the plume accounted for 0.4-6 mg C m$^{-2}$ d$^{-1}$, as determined by DeAngelis et al. (1993), comparable to or lower than the organic carbon production by ammonia oxidation.

Ammonia-oxidizing bacteria (AOB) and methanotrophs share quite a number of physiological, biochemical and ecological similarities. Both groups of bacteria often co-occur in the same environmental settings (Bédard and Knowles, 1989). Their evolutionarily related monooxygenases (Holmes et al., 1995), the enzymes responsible for their respective oxidation reactions, are similarly structured. Hence, both enzymes can use either NH$_4^+$ or CH$_4$ as electron donors for energy production, though not necessarily promoting cell growth when the alternate is used (Bédard and Knowles, 1989). This capability also means that one substrate may act as a competitive inhibitor to the oxidation of another. Since the concentrations of CH$_4$ were usually more than double those of NH$_4^+$ in the Endeavour plume (CH$_4$: NH$_4^+$ \approx 1.0 in 71% of all samples; mean = 2.71), methanotrophs were unlikely to be using NH$_4^+$ as their energy substrate, as in other marine studies (Ward and Kilpatrick, 1990). Although it is likely that methanotrophs were competing with AOB for NH$_4^+$ assimilation to meet their nitrogen requirement (Carini et al., 2003), such competition should not affect our ammonia oxidation rate measurements using nitrapyrin, because assimilation does not directly involve the enzyme methane monooxygenase. As discussed earlier, NH$_4^+$ was removed faster than methane. The maximum ammonia oxidation rate was found in close proximity to vent fields, in samples with intermediate methane concentrations and carbon stable isotopic values. Therefore, ammonia oxidation is likely the predominant in situ organic carbon production process at an early stage of the neutrally buoyant plume, but after the
oxidation of sulfur and hydrogen. Methanotrophy becomes a more important chemotrophic pathway as the plume ages, when NH$_4^+$ levels drop. This suggestion is supported by the relative distributions of AOB and methanotrophs: AOB were more abundant than methanotrophs in close proximity to the axial valley, but less abundant at the more distal stations.

### 4.5. CONCLUSIONS

Ammonia-oxidizing bacteria are found to be ubiquitous in the deep sea according to the results of this study. They are highly adapted to the usually low NH$_4^+$ levels with an exceptionally low half-saturation constant and are able to swiftly remove the hydrothermally injected NH$_4^+$, within two weeks or much less. This hydrothermal input of NH$_4^+$ also fulfills the nitrogen requirements of an active biological community residing in the neutrally buoyant plume, with assimilation rates comparable to ammonia oxidation rates. Active in situ recycling of NH$_4^+$ within the plume sustains the high rates of both uptake processes. Hence, the hydrothermal NH$_4^+$ input is not only providing the reaction substrate for nitrification, but is also directly or indirectly accelerating nitrogen cycling in the deep-sea water column along the Endeavour Segment. Furthermore, ammonia oxidation is a significant pathway for in situ production of deep-sea organic carbon within the Endeavour plume. Given the even higher NH$_4^+$ emanating from the sedimented mid-ocean-ridges and back-arc hydrothermal systems, the occurrence of deep-sea nitrification and enhanced deep-sea nitrogen and carbon cycling may be more widespread than previously considered.
4.6. REFERENCES


*Microbiology of Deep-Sea Hydrothermal Vent Habitats (Ed. D. M. Karl)*, CRC Press,

Boca Raton, pp. 255-274.
Table 4.1. List of the various 16S rRNA-targeted oligonucleotide probes used in this study, together with their respective targeting organisms, target sites with respect to E. coli, formamide concentrations (%FA) in hybridization solutions and NaCl concentrations in the stringent washing solutions. The oligonucleotide probes were labeled with the fluorochromes Cy3 or Oregon Green (Molecular Probes, Inc.), and were custom-made by Integrated DNA Technologies, Inc.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequences</th>
<th>Target Organisms</th>
<th>Target site (E. coli Positions)</th>
<th>%FA</th>
<th>NaCl (mM)</th>
<th>Reference</th>
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<tr>
<td>EUB338</td>
<td>5'-GCT GCC TCC CGT AGG AGT -3'</td>
<td>Eubacteria a</td>
<td>338-355</td>
<td>20</td>
<td>225+</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>NON338</td>
<td>5'-ACT CCT ACG GGA GGC AGC -3'</td>
<td>Negative control</td>
<td>N/A</td>
<td>20</td>
<td>225+</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>NSO190</td>
<td>5'-CGA TCC CCT GCT TTT CTC C -3'</td>
<td>β-Proteobacterial AOB b</td>
<td>190-208</td>
<td>55</td>
<td>20+</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>NSO1225</td>
<td>5'-CGG GAT TGT ATT ACG TGT GA -3'</td>
<td>β-Proteobacterial AOB c</td>
<td>1225-1244</td>
<td>35</td>
<td>80+</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsm156</td>
<td>5'-TAT TAG CGC ATC TTT CGT G -3'</td>
<td>Nitrosomonas spp. d</td>
<td>156-174</td>
<td>5</td>
<td>56+</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsv443</td>
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<td>Nitrosospira spp.</td>
<td>444-462</td>
<td>30</td>
<td>112+</td>
<td>(Mobarry et al., 1996)</td>
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<tr>
<td>Nsoc128</td>
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<td>γ-Proteobacterial AOB</td>
<td>128-146</td>
<td>35</td>
<td>80</td>
<td>(Juretschko, 2000; Loy et al., 2003)</td>
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<td>My705</td>
<td>5'-CTG GTG TTC TTC CAG ATC -3'</td>
<td>Type I Methanotrophs</td>
<td>705-724</td>
<td>20</td>
<td>225</td>
<td>(Eller et al., 2001)</td>
</tr>
<tr>
<td>Mα450</td>
<td>5'-ATC CAG GTA CCG TCA TTA TC -3'</td>
<td>Type II Methanotrophs</td>
<td>450-470</td>
<td>20</td>
<td>225</td>
<td>(Eller et al., 2001)</td>
</tr>
</tbody>
</table>

a Information on the NaCl concentrations in these washing solutions came from (Schramm et al., 1998).
+ NaCl concentration for washing solution is adopted from (Gieseke et al., 2001).
This excludes Planomycetales and Verrucomicrobia (Daims et al., 1999).
b NSO190 has 3 mismatches with Nitrosomonas ureae, and 1 mismatch in a few Nitrosomonas spp. (Utäker and Nes, 1998; Purkhold et al., 2000)
c NSO1225 has 1 mismatch with Nitrosococcus mobilis (ARB Difference Alignment function ,probeBase (Loy et al., 2003), and may hybridize with Gallionella ferruginea (Results from Probe Match (Cole et al., 2003))
d Three possible non-AOB targets are Thauera linaloolentis str. 47, Pseudomonas butanovora IAM 12574 and Aquaspirillum sinusum LMG 4393 (Results from Probe Match (Cole et al., 2003)).
e There is one non-TypeII methanotroph target - str. TM19 (X97077) in the Belindica subgroup (Results from Probe Match (Cole et al., 2003)).
Table 4.2. Specific rate constants ($k_{\text{tot}}$ and $k_{\text{oxid}}$) and turnover times ($T_{\text{tot}}$ and $T_{\text{oxid}}$) with respect to total net ammonium removal and ammonia oxidation respectively. Units for $k_{\text{tot}}$ and $k_{\text{oxid}}$ are d$^{-1}$, and that for turnover time is days (or d).

<table>
<thead>
<tr>
<th>Station</th>
<th>Casts</th>
<th>Depth (m)</th>
<th>Total Net Removal</th>
<th>Ammonia Oxidation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$k_{\text{tot}}$</td>
<td>$T_{\text{tot}}$</td>
</tr>
<tr>
<td>A</td>
<td>V5</td>
<td>2010</td>
<td>1.63</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>V8</td>
<td>2006</td>
<td>0.19</td>
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</tr>
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<td>V8</td>
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<td></td>
<td>V2</td>
<td>1950</td>
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<td>2037</td>
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154
Table 4.3. Comparison of total net NH$_4^+$ removal rates and ammonia oxidation rates obtained from incubations at 200 atm (repressurized) and those at atmospheric pressure. Rates are shown as mean ± standard deviations. ‘n.m.’ denotes ‘not measured’.

<table>
<thead>
<tr>
<th>Station</th>
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<th>200 atm Incubations</th>
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<td>Total Net Loss nM d$^{-1}$</td>
<td>Ammonia Oxidation nM d$^{-1}$</td>
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<tr>
<td>A</td>
<td>9 ± 4.0</td>
<td>9 ± 4.5</td>
<td>6 ± 3.6</td>
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<tr>
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<td>-5 ± 1.2</td>
<td>-5 ± 0.3</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>25 ± 2.0</td>
<td>23 ± 2.4</td>
<td>21 ± 1.9</td>
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<tr>
<td>B</td>
<td>57 ± 7.5</td>
<td>53 ± 8.7</td>
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<tr>
<td>C</td>
<td>0.8 ± 2.6</td>
<td>2 ± 0</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>E</td>
<td>6 ± 0.5</td>
<td>8 ± 1.0</td>
<td>14 ± 0.4</td>
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Table 4.4. Concentrations and carbon stable isotopic values ($\delta^{13}$C) of particulate organic carbon (POC), concentrations of particulate nitrogen (PN), particulate C/N ratios, as well as the corresponding total net removal rates, ammonia oxidation rates, $^{15}$N-ammonia oxidation rates and assimilation rates (mean ± standard deviation). Blanks indicate parameters not measured.

<table>
<thead>
<tr>
<th>Station</th>
<th>Cast</th>
<th>Depth $m$</th>
<th>POC conc. $\mu g$ l$^{-1}$</th>
<th>$\delta^{13}$C %</th>
<th>PN conc. $\mu g$ l$^{-1}$</th>
<th>C/N</th>
<th>Total Net Removal Rates nM d$^{-1}$</th>
<th>Ammonia Oxidation Rates nM d$^{-1}$</th>
<th>$^{15}$NH$_3$ Oxidation Rates nM d$^{-1}$</th>
<th>Assimilation Rates nM d$^{-1}$</th>
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<td>5.35</td>
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<td>4.1 ± 0.3</td>
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<td>9.5 ± 4.0</td>
<td>9.2 ± 4.5</td>
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<td></td>
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<tr>
<td>V1</td>
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<td>4.1 ± 0.3</td>
<td>4.65 ± 1.4</td>
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<td>1.69</td>
<td>6.07</td>
<td>1.4 ± 0.8</td>
<td>3.5 ± 1.3</td>
<td>-0.24 ± 0.19</td>
<td>4.20</td>
</tr>
<tr>
<td>V2</td>
<td>2148</td>
<td>5.78</td>
<td>-33.10</td>
<td>1.30</td>
<td>5.19</td>
<td></td>
<td>10.4 ± 1.8</td>
<td>12.4 ± 1.8</td>
<td></td>
<td></td>
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<tr>
<td>V2</td>
<td>2041</td>
<td>7.56</td>
<td>-35.28</td>
<td>1.64</td>
<td>5.38</td>
<td></td>
<td>56.7 ± 7.5</td>
<td>53.0 ± 8.7</td>
<td>0.37</td>
<td></td>
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<tr>
<td>V2</td>
<td>1950</td>
<td>8.18</td>
<td>-34.44</td>
<td>1.64</td>
<td>5.82</td>
<td></td>
<td>5.4 ± 0.9</td>
<td>8.6 ± 2.0</td>
<td>-0.38 ± 1.62</td>
<td>26.39</td>
</tr>
<tr>
<td>V3</td>
<td>1849</td>
<td>6.75</td>
<td>-34.50</td>
<td>1.50</td>
<td>5.25</td>
<td></td>
<td>3.7 ± 1.7</td>
<td>4.7 ± 1.1</td>
<td>0.26</td>
<td>1.49</td>
</tr>
<tr>
<td>V2</td>
<td>1802</td>
<td>3.34</td>
<td>-28.09</td>
<td>0.73</td>
<td>5.34</td>
<td></td>
<td>3.2 ± 2.4</td>
<td>3.2 ± 1.4</td>
<td>0.55</td>
<td>4.88</td>
</tr>
<tr>
<td>V3</td>
<td>1693</td>
<td>7.07</td>
<td>-29.91</td>
<td>1.02</td>
<td>8.09</td>
<td></td>
<td>1.7 ± 5.1</td>
<td>1.33 ± 0.03</td>
<td>5.02</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>V6</td>
<td>1905</td>
<td>10.88</td>
<td>-32.67</td>
<td>2.07</td>
<td>6.13</td>
<td>0.8 ± 2.6</td>
<td>2.3 ± 0.0</td>
<td>-0.016</td>
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<tr>
<td>D</td>
<td>V4</td>
<td>2224</td>
<td>6.39</td>
<td>-28.86</td>
<td>1.07</td>
<td>6.97</td>
<td>3.7 ± 1.7</td>
<td>4.7 ± 1.1</td>
<td>0.26</td>
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</tr>
<tr>
<td>V4</td>
<td>1907</td>
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<td>-30.58</td>
<td>1.00</td>
<td>6.63</td>
<td></td>
<td>3.2 ± 2.4</td>
<td>3.2 ± 1.4</td>
<td>0.55</td>
<td>4.88</td>
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<tr>
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<td>-27.36</td>
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<td>1.7 ± 0.8</td>
<td>2.9 ± 2.5</td>
<td>1.57</td>
<td></td>
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<tr>
<td>E</td>
<td>V10</td>
<td>1974</td>
<td>14.05</td>
<td>-32.19</td>
<td>2.02</td>
<td>8.11</td>
<td>6.1 ± 0.5</td>
<td>8.1 ± 1.0</td>
<td>0.41</td>
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<tr>
<td>F</td>
<td>T7-13</td>
<td>2036</td>
<td>5.27</td>
<td>-34.32</td>
<td>0.77</td>
<td>7.99</td>
<td>-0.4 ± 1.5</td>
<td>-3.7 ± 1.5</td>
<td>0.83</td>
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Table 4.5. Distribution of total ammonia-oxidizing bacteria (AOB) as the β-proteobacterial subgroups of *Nitrosospira* spp. and *Nitrosomonas* spp., and the γ-proteobacterial subgroup of *Nitrosococcus* spp., in the free-living (0.2-3.0 μm-diameter) and particle-associated (3.0-10 μm and ≥ 10 μm) communities at Main Endeavour Field (MEF) and High Rise vent fields. The numbers in individual size-fractions are presented as percentages of the total AOB (sum of *Nitrosospira* spp., *Nitrosomonas* spp. and *Nitrosococcus* spp.) in all size-fractions. ‘FL’ denotes ‘free-living’, and ‘PA’ denotes ‘particle-associated’.

<table>
<thead>
<tr>
<th></th>
<th>Main Endeavour Field</th>
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<th>High Rise</th>
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<td></td>
<td>Plume core</td>
<td>Above plume</td>
<td></td>
<td>Plume core</td>
<td>Above plume</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>PA</td>
<td>FL</td>
<td>PA</td>
<td>FL</td>
</tr>
<tr>
<td></td>
<td>0.2-3.0 μm</td>
<td>3.0-10 μm</td>
<td>≥10 μm</td>
<td>0.2-3.0 μm</td>
<td>3.0-10 μm</td>
</tr>
<tr>
<td><em>Nitrosospira</em> spp.</td>
<td>35</td>
<td>13</td>
<td>8</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td><em>Nitrosomonas</em> spp.</td>
<td>1</td>
<td>8</td>
<td>10</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td><em>Nitrosococcus</em> spp.</td>
<td>24</td>
<td>0.1</td>
<td>0</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Total AOB</td>
<td>60</td>
<td>40</td>
<td>40</td>
<td>60</td>
<td>48</td>
</tr>
</tbody>
</table>

157
Figure 4.1. A bathymetry map showing the locations of the five known active vent fields (gray stars) (SQ = Sasquatch, SD = Salty Dawg, HR = High Rise, MEF = Main Endeavour Field, MO = Mothra), tow-yo tracks (Tows A to F; dark blue) and vertical cast stations (black circles). Stations A and B are located at MEF and HR respectively. 'Sta.' stands for 'Station'.
Figure 4.2. Neutrally-buoyant hydrothermal plume signals indicated by potential temperature anomalies ($\Delta \theta$). Negative values of longitude indicate degrees West, and the units of latitude are in degrees North.
Figure 4.3. (a) Distribution of ammonium concentrations (mean of duplicates) at the neutrally buoyant plume depths (1800-2150 m), superimposed on a bathymetry map of the Endeavour Segment. Please note that the contour intervals are not always even — 100 m intervals are used for the depth range of 2300-2600 m, but 50 m intervals are used for the depth range of 2000-2300 m. This is done in order to reveal the relief of the Endeavour axial valley. The bathymetry data are extracted from the CD-Rom of the RIDGE Multibeam Synthesis (Ryan et al., 1996).
Figure 4.3. (b) Vertical profiles of ammonium concentrations at stations A to F. Symbols represent mean values; error bars represent standard deviations which are often too small and hidden behind symbols. Different symbols within one profile indicate measurements made during different sampling casts.
Figure 4.4. Plot of ammonium concentrations versus potential temperature anomalies (Δθ). Circled were the measurements made inside the young plume cores directly above Main Endeavour Field (MEF) and High Rise vent fields respectively, while the dashed lines indicate the likely non-linear relationships with Δθ, suggesting non-conservative mixing of ammonium. Error bars represent standard deviations.
Figure 4.5. Depth-profiles of total net ammonium removal rates (circles) and autotrophic ammonia oxidation rates (inverted triangles) at stations A to F. Shown are the mean values with standard deviations as error bars, whose values are often too small to be seen.
Figure 4.6. Results from substrate limitation experiments on total net ammonium removal rates (shaded bars, ‘Total Net Loss’) and autotrophic ammonia oxidation rates (non-shaded bars, ‘Autotrophic’). Controls are shown in solid colors, while parallel incubations with substrate additions (Addition) are shown in hatched bars. The heights of bars represent mean values while the error bars are standard deviations. ‘Sta’ stands for ‘Station’.

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</tr>
</thead>
<tbody>
<tr>
<td>Initial NH₄⁺ (nM)</td>
<td>24.7</td>
<td>15.0</td>
<td>13.5</td>
<td>12.5</td>
<td>14.5</td>
<td>54.5</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺ Added (nM)</td>
<td>93.3</td>
<td>67.5</td>
<td>73.5</td>
<td>78.0</td>
<td>68.0</td>
<td>108.5</td>
<td>77.0</td>
<td></td>
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**Figure 4.7.** Effects on total net ammonium removal rates (Total) and autotrophic ammonia oxidation rates (Autotrophic) after filtration through 3-μm-pore-size membrane filters (3 μm) and 10-μm-pore-size Nitex screens (10 μm). Shaded bars represent total net removal rates, and non-colored bars represent ammonia oxidation rates. Downward-diagonal patterns indicate 3-μm filtrations, while upward-diagonal patterns indicate 10-μm filtrations. Non-filtered rates are shown in solid bars. Error bars represent standard deviations of the rate measurements.
Figure 4.8. (a) Methane concentrations (nM) at the depth range of 1800-2150 m in the vicinity of the Endeavour Segment, and (b) the stable isotopic values of methane (\(\delta^{13}C\)) in the same water subsamples, overlayed on a bathymetry map of the region. Please note that the contour intervals are not always even – 100 m intervals are used for the depth range of 2300-2600 m, but 50 m intervals are used for the depth range of 2000-2300 m. This is done in order to reveal the relief of the Endeavour axial valley. The bathymetry data are extracted from the CD-Rom of the RIDGE Multibeam Synthesis (Ryan et al., 1996).
Figure 4.9. Depth-distribution of total microbial abundance estimated by DAPI-cell counts (black circles), and eubacterial abundance estimated by EUB338-hybridized cell counts (white inverted triangles) at Stations A, B, C, E and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 4.10. Abundance of β-(NSO190, NSO1225) and γ-(Nscoc128) Proteobacterial Ammonia-Oxidizing Bacteria estimated by NSO190- (black circles) and NSO1225- (red circles) cell counts, and the abundance of γ-proteobacterial ammonia-oxidizing bacteria estimated by Nscoc128 cell counts (green inverted triangles) at Stations A, B, C, E, and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 4.11. Abundance of specific groups of β-proteobacterial ammonia-oxidizing bacteria: *Nitrosomonas* spp. estimated by Nsm156-hybridized cell counts (black diamonds), and *Nitrosospira* spp. estimated by Nsv443-hybridized cell counts (white diamonds) at Stations A, B, C, E and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 4.12. Abundance of Type I methanotrophs estimated by My705-hybridized cell counts (black squares), and Type II methanotrophs estimated by Mα450-hybridized cell counts (white squares) at Stations A, B, C, E and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 4.13. Distribution of microbial abundance in different size-fractions in five selected samples from Stations A, B and C: (a) DAPI-stained cells, (b) Eubacteria detected by EUB338, (c) βAOB detected by NS0190, (d) βAOB detected by NS01225, (e) Nitrosopira-like cells detected by Nsv443, (f) Nitrosomonas-like cells detected by Nsm156, (g) γAOB detected by Nscoc128, (h) Type I methanotrophs detected by My705 and (i) Type II methanotrophs by Mo450. Black solid bars represent the abundance of free-living microbes (0.2-3.0 μm), red represents the smaller particle-associated size-fraction (3.0-10 μm) and green represents the bigger particle-associated size-fraction (10+ μm). The abundance estimates for the corresponding non-size-fractionated whole samples are shown as triangles. All abundances were reported in 10^4 cells ml⁻¹. Please note the different scales in each plot.
Figure 4.14. A sigmoidal relationship was shown between ammonia oxidation rates and ammonium concentrations in the Endeavour neutrally buoyant plume, combining data from 1999 (squares), 2000 (triangles) and 2002 (circles). The curve shows the best-fit regression line. The regression equation is displayed with $R_{\text{oxid}}$ standing for ammonia oxidation rates and $N$ for ammonium concentrations. The horizontal dashed line refers to the empirical half-saturation ammonia oxidation rates (43.5 nM d$^{-1}$), while the vertical dashed line drops down from the intersection between the half-saturation oxidation rate and the regression curve, indicating a half-saturation $\text{NH}_4^+$ concentration of $\sim$153 nM. Data for 1999 and 2000 come from (Lam et al., 2004; Chapter 3).
CHAPTER FIVE

Ammonium Cycling in the Hydrothermally Influenced

Water Column of the Guaymas Basin
5.0 ABSTRACT

Autotrophic ammonia oxidation and the cycling of ammonium were investigated in the water column overlying the sedimented mid-ocean ridge spreading center in the Guaymas Basin, Gulf of California. Hydrothermal plumes were trapped below the sill depth (1560 m) inside the semi-enclosed basin and could not be detected by the conventional potential temperature anomalies. An apparent potential temperature anomaly (Δθ*) was derived as an alternative, which showed the water column from 1750 m downwards to be 0.01-0.03 °C warmer than it would be without hydrothermal inputs, while being occasionally interspersed with buoyant plumes of Δθ* up to 0.08 °C. NH₄⁺ concentrations were elevated to a maximum of 2945 ± 167 nM in the buoyant plumes and 588 ± 37 nM in the neutrally buoyant plumes, yet that at above-sill depths without direct hydrothermal influence could also be quite high (160 ± 40 nM) relative to typical deep-sea values. Ammonia oxidation was stimulated to as fast as 517 nM d⁻¹ in a buoyant plume, the highest rate ever reported in a deep-sea water column, and up to 142 nM d⁻¹ in the rest of the water column examined. The ubiquitous autotrophic ammonia-oxidizing bacteria were especially associated with organic-rich particles, either from the upper water column or from the hydrothermal sources at sub-sill depths, where degradation of organic matter or remineralization continually released additional NH₄⁺. Ammonia oxidation was responsible for at least 21% of the total net NH₄⁺ removal, but its rates did not increase further at higher NH₄⁺ concentrations, implying inhibition or greater importance of other uptake processes at high substrate levels. The concurrently high organic contents in the hydrothermal fluid discharges have likely enhanced heterotrophic activities and assimilation rates, the latter of which accounted for at least 57% of total net NH₄⁺ removal. Ammonia oxidation, along with assimilation and remineralization, played important roles in the active cycling of NH₄⁺ in the water column of the Guaymas Basin, yet the former might not be a significant source of organic carbon compared to the substantial sedimentary reservoir.
5.1. INTRODUCTION

In sedimented hydrothermal systems such as the Guaymas Basin and the Okinawa Trough Back-arc Basin, hot hydrothermal fluids pass through the sediments before entering the water column, unless there are direct basaltic conduits leading to the sediment-water interface. The additional reactions between hot fluids and sediments give the final hydrothermal fluid discharges distinctly different chemistry from those in sediment-starved hydrothermal systems. For example, these fluids emanating from sedimented hydrothermal systems possess lower concentrations of ore-forming metals, higher pH, higher total inorganic carbon content, higher alkalinity, as well as higher levels of methane and ammonium (Von Damm et al., 1985; Sakai et al., 1990) than the sediment-starved counterparts. Ammonium concentrations in the Guaymas Basin end-member fluids (10.3-15.6 mM) (Von Damm et al., 1985) are several orders of magnitude higher than those in most unsedimented ridge discharge fluids (≤ 0.01 mM) (Lilley et al., 1993). One exception is the unsedimented Endeavour Segment, Juan de Fuca Ridge, where a moderate amount of ammonium (0.64-0.95 mM) is present in the fluid discharges (Lilley et al., 1993) and persists in the neutrally buoyant plume (up to 341 ± 136 nM) after at least 10^4 dilution (Lam et al., 2004; Chapter 3). This hydrothermally injected ammonium could fuel a significant amount of autotrophic ammonia oxidation (up to 91 nM d^{-1}) within the deep-sea hydrothermal plumes (Lam et al., 2004; Chapter 3).

Autotrophic ammonia oxidation is the first step in nitrification, which returns the recycled nitrogen from its reduced form (NH_4^+) to its oxidized forms (NO_2^- and NO_3^-), and is responsible for replenishing the deep-sea nitrate reservoir in the marine nitrogen
cycle. Autotrophic ammonia-oxidizing bacteria have been found to be ubiquitous in the deep-sea water column, at least in the vicinity of the Endeavour Segment (Chapters 3 & 4). These autotrophic bacteria are highly adapted to the commonly low NH$_4^+$ levels, with unusually low half-saturation coefficients (153 nM), and are able to take full advantage of the hydrothermally discharged NH$_4^+$ to potentially form an amount of nitrate two to three orders of magnitude greater than the particulate nitrogen export from the surface ocean reaching these deep-ocean depths (Chapter 4).

Since the Guaymas hydrothermal system is injecting about ten to fifteen times more NH$_4^+$ in the discharge fluids than the Endeavour hydrothermal system, ammonia oxidation might be expected to be more vigorous in the deep-sea water column of the Guaymas Basin than that of the Endeavour Segment. Nevertheless, this would only be true if NH$_4^+$ is removed in the same proportions among the various pathways as in the Endeavour hydrothermal plume. Beside autotrophic ammonia oxidation, NH$_4^+$ can also be removed by assimilation, heterotrophic nitrification and anaerobic ammonia oxidation. Anaerobic ammonia oxidation is unlikely to be important in the well-oxygenated water column except for within macroaggregates. Although assimilation was found to be occurring at rates comparable to those of autotrophic ammonia oxidation in the Endeavour plume (Chapter 4), the unique chemical composition of the Guaymas Basin hydrothermal fluids (Von Damm et al., 1985) might favor metabolic pathways other than ammonia oxidation, leading to greater importance of assimilation. In addition, the thick sediment cover in the Guaymas Basin contains rich organic matter (Simoneit et al., 1979), which undergoes diagenesis and petroleum genesis under hydrothermal influence, resulting in organic-rich pore water and hydrothermal fluid discharges that bear a wide
variety of organic compounds (Simoneit, 1991). Such abundance of organic matter may favor heterotrophic activities, and so uptakes via assimilation as well as heterotrophic nitrification (Strauss et al., 2002).

Therefore, the objectives of this study were to examine the extent of ammonia oxidation and NH$_4^+$ cycling within the water column overlying a sedimented hydrothermal system in the Guaymas Basin, where the NH$_4^+$ as well as organic carbon contents are expected to be high. These results would be compared with those acquired in Chapter 4 from the unsedimented hydrothermal system along the Endeavour Segment.

5.2. MATERIALS AND METHODS

5.2.1. Geological Settings and Known Hydrothermal Activities

The Guaymas Basin lies in the central part of the Gulf of California, Mexico. It is a semi-enclosed, 2100 m-deep basin that is bounded by shallow sills and archipelagoes to the northwest and is connected to the rest of the Pacific via four deeper basins to the southeast (Fig. 5.1 a). The suboxic Pacific Intermediate Water enters the basin at 500-1000 m, as a continuum from the oxygen-minimum zone of the Eastern Tropical North Pacific. Below circa 1000 m, the Guaymas Basin is filled with Pacific Deep Water that flows in over the 1560-m deep sill demarcating the entrance to the adjacent Carmen Basin to the southeast (Robles and Marinone, 1987). The productive surface water (mean net primary production in 2001-2002 = 530 g C m$^{-2}$ y$^{-1}$) (Kahru et al., 2004), along with the semi-enclosed basin nature, gives rise to a 300-500-m thick sediment cover on the seafloor (Curray et al., 1982), which is actively accumulating at a fast rate of 1-4 m per thousand years (van Andel, 1964), compared to typical pelagic rates of 5-50 mm per thousand years over mature sea-floor spreading centers (Fisher and Becker, 1991). The
thick sediment cover in the Guaymas Basin contains 1-4 wt % of organic matter of predominantly marine autochthonous origin (Simoneit et al., 1979; Simoneit and Bode, 1982), and the organic carbon-to-nitrogen ratios (C/N) in the sediments range widely between 11 and 89 (Simoneit and Bode, 1982; de la Lanza-Espino and Soto, 1999).

Linking the East Pacific Rise spreading centers to the south and the San Andreas Fault system to the northwest, the young Guaymas Basin is spreading at a rate of 49-58 mm y\(^{-1}\) (Larson et al., 1968; DeMets et al., 1987). Hydrothermal activities within the basin are concentrated along the spreading axes marked by two parallel northeast-southwest trending troughs, the northern and southern troughs, which are 50-150 m deeper than the surrounding seafloor, and 20 km offset from each other (Curray et al., 1982). Two operative hydrothermal systems have been postulated in the Guaymas Basin: (1) dolerite sill intrusion into the highly porous hemipelagic sediments thermally altered the sediments and expelled the hydrothermally altered pore water, and (2) large magmatic intrusion at greater depths. The first system is generally of shorter duration and is associated with temperatures <200 °C, while the second system is more sustained and involves recharge of basin bottom waters that would be heated to >300 °C (Gieskes et al., 1982; Kastner, 1982). Hydrothermal activities and heat flows are greater in the southern trough than in the northern trough (Lonsdale and Becker, 1985; Fisher and Becker, 1991), and it was in the former where our study was conducted.

The southern trough is about 30 km long and 2-4 km wide. The seafloor is broken by a series of 0.1-1 km\(^2\) intra-rift hills rising ≤100 m above the surroundings, while side-scan sonar surveys have revealed subsurface sill complexes (Fig. 5.1 b) and buried mounds (Lonsdale and Becker, 1985). Hydrothermal discharges were found
usually within 500 m of the rift valley axis, either over sill complexes or mainly on their peripheries, as the sills act like ‘sealing caps’ to the deeper subsurface hydrothermal circulation (Lonsdale and Becker, 1985). The organic-rich pore water and hydrothermal fluid discharges bear a wide variety of lipids and hydrocarbons such as volatiles and oil droplets of hydrocarbons C\textsubscript{1} to C\textsubscript{40} (Simoneit, 1991). Fluid discharges are also imprinted with distinct anomalies like those found in dissolved silica, \textsuperscript{3}He, beryllium, barium, particulate manganese, methane and ammonium (Von Damm et al., 1985), which are still detectable in the bottom water of the basin (Campbell and Gieskes, 1984).

5.2.2. Water Sampling

The water column in the southern trough of the Guaymas Basin was surveyed by a CTD-transmissionmeter-Niskin bottle rosette package on board the R/V Atlantis in April and May 2002, when winter upwelling has ceased and the water column has become stratified again. Detailed water sampling at various depths was conducted at two sites: (1) South Site (111\textdegree 24.4′W, 27°0.48′N) (casts V1 to V6) – at about 300 m from the periphery of the subsurface South Sill (Lonsdale and Becker, 1985) and 150 m offset from plumes previously located by Lonsdale and Becker (1985) (Fig. 5.1 b); (2) North Site (111°24.0′W, 27°1.8′N) (cast V7, and the last upward tows of casts T3 to T5) – over the Central Sill between the DSDP Holes 477 and 477A, where hydrothermal plume signals have been previously reported (Lonsdale and Becker, 1985) (Fig. 5.1 b). In order to meet the water volume requirements for all analyses and experiments, multiple sampling casts had to be repeated at each site at different times or days. One tow-yo survey (T3) was performed from the South Site to the North Site, and one (T2) from 3.3 km northwest of South Site to 1 km west-southwest of South Site (Fig. 5.1 b).
yo surveys T4 and T5 circled in the vicinity of the North Site. Water samples were only taken in the last upward tow in each of T3, T4 and T5 after the ship had stopped at the North Site. The parameters measured and calculated include potential temperature (θ), salinity, potential density (σθ), particle anomaly (Δc), oxygen content and oxygen deficit (ΔO₂). Δc was calculated as the deviation from the background turbidity at above-sill depths (1200-1500 m) averaged across the basin. Since oxygen always showed a linear relationship with σθ down to about sill depth (r² > 0.99, linear regression) in all profiles examined in this study, ΔO₂ was calculated for below sill depths as the deviation of the oxygen content predicted from a linear oxygen-σθ regression above sill depth and was usually negative in value. Unfortunately, technical problems with the Niskin-bottle-rosette system later on during the research cruise precluded more detailed sampling and experimentation at the North Site.

Some warm diffuse fluids (< 60 °C) that were visible shimmering out of microbial mats were concentrated by a dome-like inverted funnel and sampled at the top with titanium major samplers and gas-tight samplers (the latter provided by M. Lilley, University of Washington), both operated by the submersible DSV2 ALVIN during dives numbers 3777-3783. Hot discharge fluids (up to 306°C) emanating from black-smoker chimneys were also collected. Both hot and warm fluid samples were analyzed for NH₄⁺, H₂, TCO₂, CH₄ and δ¹³C-CH₄ as described in the following sections. Some hydrothermal plume water at the South Site was sampled during the submersible dives with one-liter Niskin bottles on board of DSV2 ALVIN.
5.2.3. *Ammonia Oxidation Incubation Experiments*

5.2.3.i. Ammonium Concentration Measurements

Water samples for the NH$_4^+$ analyses were drawn through clean 202 µm Nitex screens into acid-cleaned 125 ml polyethylene bottles. Duplicate or triplicate subsamples were stored frozen (-20 °C) unless analyzed immediately. The subsamples were analyzed for NH$_4^+$ usually within 24 hours on shipboard following the fluorescence method (Jones, 1991). The NH$_4^+$ concentration of each sample is reported as the mean ± standard deviation of replicate subsample.

5.2.3.ii. Total Net Ammonium Removal & Ammonia Oxidation Rate Measurements

Duplicate or triplicate water subsamples were collected in the same manner as for NH$_4^+$ concentration measurements and were incubated at *in situ* temperature (2 °C) and 1-atm pressure in the dark for approximately 12, 24, 48 and 72 hours, in the presence and absence of dicyandiamide (2 mM final concentration; stock solution dissolved in dimethyl sulfoxide). The final concentration of the solvent dimethyl sulfoxide (DMSO) in each incubation was 1 ml l$^{-1}$, which has been reported to be innocuous to ammonia-oxidizing bacteria (Jones and Morita, 1984). Dicyandiamide (DCD) is a specific inhibitor for ammonia oxidation without significant inhibition of methane oxidation at the chosen concentration, unlike most other nitrification inhibitors (Roy and Knowles, 1995). Although allylsulfide may be similarly specific and has an advantage of the smaller dosage required than DCD (Roy and Knowles, 1995), allylsulfide was found to suppress other oxygen respiring activities by ~30% in the first 21 hours in preliminary experiments with seawater (data not shown) while DCD did not show such negative effects (data not shown). Unfortunately, perhaps due to contamination in the DCD stock solution, the
DCD treatments introduced an additional ~100 nM of NH$_4^+$ into the subsamples in the incubation experiments. Therefore, its use was aborted in the middle of the research expedition, and the rate measurements involving DCD for the South Site are considered here as potential ammonia oxidation rates only. None of the experiments conducted at the North Site involved the use of DCD. In three incubation experiments, parallel duplicate subsamples were repressurized back to 200 atm after sample manipulation, for a 24-hour incubation at 2°C to test for any pressure enhancement effects. All incubation experiments were terminated via freezing at -20°C.

Total net NH$_4^+$ removal rates ($R_{tot}$) were calculated as the slopes of the linear regression of NH$_4^+$ concentrations versus time in the incubations without DCD ($m_T$). Potential autotrophic ammonia oxidation rates ($R_{oxid}$) were calculated as the difference between the slopes of the linear regressions of NH$_4^+$ versus time with ($m_{DCD}$) and without ($m_T$) DCD, i.e. $R_{oxid}$ (nM d$^{-1}$) = ($m_{DCD}$ - $m_T$) x 24 hours d$^{-1}$. The relationship between NH$_4^+$ and time remained linear over 48 hours and also over 72 hours in most cases. Only the slopes over the linear phases were used in the above calculations. Assuming that total net NH$_4^+$ removal and potential ammonia oxidation are first-order reactions, the corresponding specific rate constants ($k_{tot}$ and $k_{oxid}$) were computed by normalizing $R_{tot}$ and $R_{oxid}$ by the initial NH$_4^+$ concentrations, $N_0$, i.e. $k_{tot} = R_{tot} / N_0$ and $k_{oxid} = R_{oxid} / N_0$ respectively. The NH$_4^+$ turnover times (units in days) were the reciprocals of specific rate constants (units in d$^{-1}$).

5.2.3.iii. Substrate Limitation Experiments

The hypothesis of substrate limitation at lower NH$_4^+$ levels was tested by adding 5 nM and 250 nM NH$_4^+$ into duplicate water subsamples collected at the core and the upper
boundary of the hydrothermal plume at the North Site. They were incubated for total net
\( \text{NH}_4^+ \) removal rate measurements and were analyzed for \( \text{NH}_4^+ \) in the same manner as for
the basic incubation experiments.

5.2.3.iv. Particle-Filtered Incubation Experiments

Comparison was made between total net \( \text{NH}_4^+ \) removal and ammonia oxidation
rates with and without prior removal of particles greater than 3 \( \mu \text{m} \)- or 10 \( \mu \text{m} \)-diameter
in size. Five water samples were selected to represent below-plume deep water, plume
core and above-plume background – two at the South Site and three at the North Site.
Water subsamples were first filtered through 10 \( \mu \text{m} \)-pore-size Nitex screens. Half of the
filtrate was subsampled for incubation with and without nitrpyrin, each in triplicates.
The rest of the filtrate was further filtered through 3 \( \mu \text{m} \)-pore-size membrane filters, and
the final 3 \( \mu \text{m} \)-filtrate was subsampled for triplicate incubations with and without
nitrpyrin. They were incubated for total net \( \text{NH}_4^+ \) removal rate measurements at 2\( ^\circ \)C and
1-atm pressure in the dark and analyzed for \( \text{NH}_4^+ \) in the same manner as in the basic
incubation experiments.

5.2.4. \( ^{15} \text{N} \)-Ammonia Oxidation Rate Measurements

Ammonia oxidation rates were also measured via \( ^{15} \text{N} \)-labelled experiments in
selected samples. Seawater subsamples, in duplicates or triplicates, were collected in the
same manner as for the total net \( \text{NH}_4^+ \) removal experiments. Additions of \( ^{15} \text{N} \)-
ammonium chloride (99 atom\%, Isotec, Inc.) were targeted to achieve levels of \( \leq 10 \% \) of
initial \( \text{NH}_4^+ \) concentrations. In the earlier experiments, the amounts of \( ^{15} \text{N} \)-label
additions were determined based on the \( \text{NH}_4^+ \) measurements made in a previous sampling
cast at the same depths and at the same site. However, due to the large temporal fluctuations in the water column, some $^{15}$N-label additions were found later to have exceeded the 10% tracer-levels to a maximum of a 46% increase. Those results were treated as potential oxidation rates. In later experiments, $^{15}$N-label additions were determined after NH$_4^+$ analyses of the same untreated water samples immediately upon sample collection. The seawater subsamples were incubated at 2°C and 1-atm pressure for 0, 24 and 48 hours. Incubations were terminated by freezing at −20°C, and were stored frozen until further analyses.

In a shore-based laboratory, stable isotopic analyses of nitrite plus nitrate (NO$_x^-$) were performed using a bioassay method, in which NO$_x^-$ was reduced by pure bacterial cultures of Pseudomonas chlororaphis to nitrous oxide (Sigman et al., 2001). Then the samples were analyzed for $\delta^{15}$N-N$_2$O as previously described (Dore et al., 1998), except that the N$_2$O gas was injected directly into the gas-chromatography-mass-spectrometer system from the headspace of the sample vials without the need of prior gas stripping (Popp et al., 1995). The apparent $^{15}$N-ammonia oxidation rates ($R_{^{15}N^*}$) are calculated as:

$$R_{^{15}N^*} = (n_t - n_o) / (n_{NH4^+} - n_o) \times C/t,$$

where $n_o$ and $n_t$ are the atom% of $^{15}$N in the NO$_x^-$ pool initially and at time t, while $n_{NH4^+}$ represents the atom% of $^{15}$N in the initial NH$_4^+$ pool. The NO$_x^-$ concentration was represented by C, which was assumed to be relatively constant at a typical deep-sea value of 40 µM and close to previous measurements made in the Guaymas Basin (Campbell and Gieskes, 1984; Karl et al., 1988). Correction for isotope dilution effects were performed according to the formulae derived by (Kanda et al., 1987). Briefly, let
\[ b = \frac{\rho_A \times t}{(N_0 + N_{15N})} \]

where \( N_0 \) and \( N_{15N} \) are the concentrations of ambient \( \text{NH}_4^+ \) and the added \(^{15}\text{N} \) tracers respectively, while \( t \) is the incubation time and \( \rho_A \) is the apparent \( \text{NH}_4^+ \) uptake rate. The values of \( \rho_A \) include both the apparent ammonia oxidation rates and assimilation rates (see section 5.2.6 for the latter measurements). Then the correction factor, \( x = \rho / \rho_A \), is calculated as:

\[ x = \frac{-1 + (1-b)^{-a}}{a-1} b \]

where \( \rho \) is the true total uptake rate (ammonia oxidation + assimilation) and \( a \) is the ratio of regeneration rate to \( \rho \). If regeneration rates are calculated to be the difference between the total net ammonium removal rates (or \( R_{\text{tot}} \), see section 5.2.3.ii) and \( \rho \), then the values of \( a \) can be first estimated using the apparent rate measurements and further iterated with the isotope dilution corrected rates. The resulting correction factors were 1.01 to 1.39.

5.2.5. Particulate Organic Carbon & Particulate Nitrogen

Eight to ten liters of each selected seawater sample was pressure-filtered (10 psi) through a 25-mm-diameter pre-combusted glass fiber filter (GF/F; Whatman, Inc.) under a positive pressure of 0.2-μm-filtered nitrogen gas, to collect for particulate organic carbon (POC) and nitrogen (PN). The filters were carefully folded, wrapped in pre-combusted aluminum foils and frozen at -20 or -70 °C until further analyses. In a shore-based laboratory, filters were dried briefly in a drying oven (60 °C), acidified with a few drops of sulfuric acid (6 %) to eliminate inorganic carbon, and then dried again at 60 °C. The dried filters were carefully wrapped in 9 × 10 mm tin boats and compressed to
small pellets. They were simultaneously analyzed for POC and PN contents, and for the natural stable isotopic abundance of carbon ($\delta^{13}$C-POC) and nitrogen ($\delta^{15}$N-PN), using a Carlo Erba NC2500 elemental analyzer coupled with an on-line Finnigan MAT Delta S isotope ratio mass spectrometer via a Finnigan ConFlo II split interface. The $\delta$ notation is defined as $[(r_{sample} - r_{std})/ r_{std} - 1] \times 1000/\%$, where $r_{sample}$ and $r_{std}$ are the atomic abundance ratios of the higher to lower stable isotopes in the sample and in the standard respectively. The atomic ratio for carbon stable isotopes refers to $^{13}$C/$^{12}$C, and the carbon standard is the Pee Dee Belemite. The atomic ratio for nitrogen stable isotopes is $^{15}$N/$^{14}$N, and the standard used is the dinitrogen gas in air.

5.2.6. Assimilation Rate Measurements

About eighteen liters of seawater were drawn from two 10-liter Niskin bottles, which sampled consecutively at the same depth and location, into an acid-cleaned polyethylene cubitainer. Tracer levels (≤ 10 % of initial $\text{NH}_4^+$ concentrations) of $^{15}$N-ammonium chloride (99 atom%, Isotec, Inc.) were added to the subsamples, which were then incubated in the dark at 2°C and 1 atm for approximately 48 hours. At the end of each experiment, the water sample was filtered through a pre-combusted GF/F glass fiber filter and analyzed simultaneously for POC and PN contents, as well as $\delta^{13}$C-POC and $\delta^{15}$N-PN, as described above for the natural abundance samples. Assimilation rates ($R_{asm}$) are calculated as: $R_{asm} = (N_p_t - N_p_o) / (N_{\text{NH}_4^+} - N_p_o) \times C_p / t$, where $N_p_o$ and $N_p_t$ are the atom% of $^{15}$N in PN at time-zero and at time $t$ respectively, while $N_{\text{NH}_4^+}$ represents the atom% of $^{15}$N in the initial $\text{NH}_4^+$ pool and $C_p$ is the concentration of PN. Corrections for isotope dilution effects are performed as described in section 5.2.4.
5.2.7. Analyses for Gases

Water samples were collected using 140 ml syringes for methane concentration analyses. They were analyzed on board ship using a modification of the headspace equilibration technique (McAuliffe, 1971). A headspace was created by expelling 70 ml of seawater sample while filling in with helium. The headspace and seawater sample in the syringe were allowed to equilibrate for ~1 hour before the headspace was analyzed for methane concentration with a gas chromatograph (SRI Instruments). Water samples were also collected for carbon stable isotopic analyses for methane in 160 ml glass serum bottles. Seawater was allowed to overflow for about three bottle volumes, and then the bottles were filled to the top while avoiding bubbles. Saturated mercuric chloride solution (1 ml) was added into each sample, before the bottles were capped with gray butyl rubber stoppers and crimp-sealed. Carbon stable isotopic analyses were performed in a shore-based laboratory as described previously (Sansone et al., 1997). The samples from the gas-tight samplers deployed during ALVIN dives were analyzed on shore for the gaseous contents of hydrogen, total inorganic carbon and methane (M. Lilley and E. J. Olson, University of Washington).

5.2.8. Fluorescence In Situ Hybridization

Subsamples for fluorescence in situ hybridization (FISH) were collected from casts V2 (2002 m, 1852 m and 1400 m) and V5 (1496 m) as representatives of the South Site, and from the last upward portions of casts T5 (1885 m, 1865 m and 1760 m) and T4 (1694 m) as representatives of the North Site. Eubacteria, β-proteobacterial ammonia-oxidizing bacteria (β-AOB), γ-proteobacterial ammonia-oxidizing bacteria (γ-AOB), *Nitrosomonas* spp. and *Nitrosospira* spp. (two major groups of β-AOB), as well as
methanotrophs (Type I and Type II) were enumerated using FISH with 16S rRNA-targeted oligonucleotide probes (Table 5.1) (Lam et al., 2004; Chapter 3). Ammonia-oxidizing bacteria in the β-Proteobacteria subgroup (βAOB) were detected by two oligonucleotide probes, NSO190 and NSO1225 (Mobarry et al., 1996). NSO190 is regarded as more specific for βAOB but likely misses some Nitrosomonas spp., whereas NSO1225 has a wider coverage but may also hybridize with some non-βAOB targets (see Table 4.1; Utäker and Nes (1998); Purkhold et al. (2000). However, searches by Probe Match in the Ribosomal Database Project II (Cole et al., 2003) found more matching βAOB or βAOB-like sequences with NSO190 than with NSO1225 (384 versus 140). Hence, neither NSO190 nor NSO1225 gives a perfectly accurate estimate of the βAOB abundance. The βAOB abundance estimates in this study are reported henceforth as the ranges between the hybridized-cell-counts of the two probes, and they should be regarded as potential underestimates. Hybridization with NSO1225 yielded sometimes higher and sometimes lower abundance estimates than did hybridization with NSO190. The total abundance of ammonia oxidizing bacteria (total AOB) is calculated as the sum of γAOB abundance and the mean of the βAOB abundance estimated from the NSO190- and NSO1225- hybridized cell counts. The total microbial abundance was obtained via DAPI-counter-staining after FISH processing on the same filter sections.

Size-fractionation sequential filtration through 10-μm-pore-size Nitex screens, 3.0- and 0.2-μm-pore-size membrane filters, was performed on four selected seawater samples to differentiate between particle-associated (3-10 μm and ≥10 μm) and free-living communities (0.2-3.0 μm). The detailed procedures have been described in
Chapters 2 and 3; Lam and Cowen, 2004; Lam et al., 2004). One in-plume sample and one above-plume background sample were each chosen from both the North and South Sites.

5.2.9. Statistical Analyses

Statistical analyses were performed using Statistica 6.0 (StatSoft) or Matlab 6.5 (The Mathworks, Inc.). All data were checked for normality before any statistical analyses. Parametric tests were used whenever possible, while non-parametric alternatives were used if the data under investigation were not normally distributed.

5.3. RESULTS

5.3.1. Physical Anomalies in the Water Column due to Hydrothermal Inputs

The water column in the Guaymas Basin had open exchanges with other basins only from the surface down to the sill depth at 1560 m. Below the sill depth, turbidity increased while oxygen content decreased (Fig. 5.2 e-f, 5.3 e-f). The vertical profiles of potential temperature (θ), salinity and potential density (σθ) appeared to maintain their curvatures for 100-150 m further downwards, indicating reasonable ventilation until about 1660-1710 m (Fig. 5.2 a-c, 5.3 a-c). There was a discontinuity in the θ-salinity plots at 1700-1750 m in all profiles examined (Fig. 5.4). The basin water was relatively homogenous with respect to salinity and σθ from about 1750 m to 30-90 m above bottom (mab). Within this depth range were found large peaks in Δc (up to 0.34) and prominent oxygen deficits (ΔO2 as low as -8 μmol kg⁻¹). The basin floor was lined with a thin layer (30-90 m thick) of colder, denser, less turbid but well-oxygenated water (Fig. 5.2, 5.3),
which was likely the Pacific Deep Water that replaced the ascending hydrothermal convection for mass conservation (Campbell and Gieskes, 1984).

Hydrothermal plumes were present in the water column between 15 mab and 200 mab, though the plume thickness varied from about 5 m to 180 m. Conventionally, hydrothermal plume signals are quantified by potential temperature anomalies (Δθ), which are calculated as the deviation from the potential temperature predicted by a linear regression with potential density (σθ), based on data that are free of hydrothermal influence (Lupton et al., 1985). These hydrothermally free background data are usually obtained from either a 100-m depth range directly above plume depths, or from a nearby water column of the same water mass without hydrothermal inputs. In the Guaymas Basin, however, the discontinuity in θ–S signatures at 1700-1750 m meant that the background data for regression had to be taken below this depth, yet this water layer was already under hydrothermal influence. Besides, some hydrothermal venting occurred within the thin cold water layer at the bottom of the water column. Therefore, no appropriate background data were available in the same vertical profiles. A complete background profile elsewhere in the basin was unattainable owing to the rapid across-basin horizontal mixing by strong tidal currents (≥ 12 cm s⁻¹) (Lonsdale and Becker, 1985), as exemplified by the vertical profiles of T2dn taken at the northwest rift wall supposedly remote from any known hydrothermal venting according to Lonsdale and Becker (1985). Although hydrographic data from other deeper and non-hydrothermally active basins within the Gulf of California may help to infer a background for the water column in the Guaymas Basin, such measurements were not obtainable during this study.
Hence, the hydrothermal plume signals described below, namely apparent potential temperature anomalies (Δθ*), were calculated as the deviations from a projected θ-depth profile presumably without hydrothermal influence.

From the vertical profiles in Fig. 5.2 and Fig. 5.3, it can be generalized that (1) θ decreased exponentially with depth, but the exact exponential relationship differed below sill depth, (2) the hydrothermal influence usually spanned between 1750 m and 30 mab, and (3) the thin cold water layer at the bottom was likely the Pacific Deep Water that flows into the basin. The data collected for 1500-1700 m from all sampling casts in the basin, and those collected for below 1980 m in selected casts that showed no obvious hydrothermal inputs, were used to fit an exponential decay model (based on the observed trends) with the least-square method (Curve-Fitting tool, MATLAB 6.5; The MathWorks, Inc.). The model takes the form of

\[ \theta_p = a \times \exp(-b(z-z_o)) + c, \]

where \( \theta_p \) is the predicted θ, \( z \) and \( z_o \) are the depth and reference depth (derived from the regression) respectively, and \( a, b \) and \( c \) are the constants (also derived from the regression analysis). The modeled fit has an \( R^2 \) value of 0.996, and a \( \theta_p \) value of 2.77 °C at 2010 m (Fig. 5.2a, 5.3a). A model using data from 1000 m to 1700 m depth has also been plotted in the same figures (Fig. 5.2a, 5.3a), which results in a \( \theta_p \) of 2.64 °C at 2000 m, similar to the 2.4 °C and 2.2 °C previously measured in the Carmen Basin and Farallon Basin respectively (Campbell and Gieskes, 1984). However, the latter model possibly represents values of θ without the sill effect, as opposed to the θ without hydrothermal influence within the Guaymas Basin. Thus, the results from the first model are used as the projected background θ here.
Hydrothermal plume signals or positive $\Delta \theta^*$ were consistently detected from 1750 m to about 1980 m at both the South Site and the North Site. They were even detected in the reference sampling cast T2dn (i.e. when the CTD was first lowered at the beginning of T2), which was located at 2.9 km to the west of the North Site, along the northwest rift wall where there were no observable hydrothermal activities on the seafloor in the vicinity (Lonsdale and Becker, 1985). Although both buoyant plumes and neutrally buoyant plumes showed positive $\Delta \theta^*$, the former are distinguished from the latter by having lower potential density ($\sigma_\theta$) than the immediately overlying water. The $\Delta \theta^*$ of neutrally buoyant plumes reached 0.03 °C, whereas the highest $\Delta \theta^*$ of buoyant plumes was almost 0.08 °C. There were large temporal variations in $\Delta \theta^*$ at both sites, which was not surprising given the fast reversing horizontal currents. For example, using the lower current speed estimate of 5 cm s$^{-1}$ (Salas-de-Leon et al., 2003), a water parcel would have already traveled 1.26 km in the seven hours between the sampling casts V1 and V2.

At the South Site, buoyant hydrothermal plumes were particularly noticeable intruding the bottom thin cold-water layer (1940-2010 m) in casts V1 and V6 (Fig. 5.2 c,d). The neutrally buoyant plume, with varying intensity, was consistently present from 1800 m to ≥1940 m in all casts at this site, although the overlying water in cast V4 was generally quite warm ($\Delta \theta^* ≤ 0.02$ °C). The strongest neutrally buoyant plume ($\Delta \theta^* ≤ 0.03$ °C) was observed in cast V5. It extended down towards the seafloor until a strong buoyant plume ($\Delta \theta^* ≤ 0.078$ °C) was found at ~1990 m (Fig. 5.2 d). The dissolved oxygen content detected in this cast was also the lowest ($\Delta O_2$ as low as ~8 μmol kg$^{-1}$) and
coincided with a dramatic increase in $\Delta c$ (Fig. 5.2 e, g). $\Delta O_2$ did not return to zero at the bottom ($-5 \mu$mol kg$^{-1}$), whereas it did in most other casts (Fig. 5.2 g).

At the North Site, the neutrally buoyant plume in cast T3 (the last upward portion of tow-yo cast T3 after the ship had stopped moving) was $\sim 0.01 ^{\circ}$C warmer than in other casts (Fig. 5.3 a). It was accompanied by very low $\Delta O_2 (\geq -8 \mu$mol kg$^{-1}$) (Fig. 5.3 g) and very large $\Delta c$ maxima ($\leq 0.28$) (Fig. 5.3 e). Buoyant plumes penetrated into the neutrally buoyant plumes during casts V7 ($\Delta \theta^* \leq 0.035 ^{\circ}$C) and T5 (the last upward portion of tow-yo T5) ($\Delta \theta^* \leq 0.078 ^{\circ}$C) (Fig. 5.3 d). The latter was 100-m thick and coincided with $\Delta c$ maxima ($\leq 0.18$) (Fig. 5.3 e). The neutrally buoyant plume was weak during cast T4 ($\Delta \theta^* \leq 0.01 ^{\circ}$C), though a strong $\Delta c$ maximum (0.2) occurred at the $\theta$–$S$ discontinuity at 1700 m without apparent plume signals.

### 5.3.2. Distribution of Ammonium

Ammonium concentrations in the water column were evidently influenced by the temporal variability of hydrothermal plume injections and were weakly but significantly correlated with both $\Delta \theta^*$ ($R=0.36$, $p<0.05$, Spearman rank-order correlation) and $\Delta c$ ($R=0.49$, $p<0.001$, Spearman rank-order correlation). At the South Site, $NH_4^+$ levels fluctuated from $40 \pm 6$ nM to $2945 \pm 167$ nM at depths close to the seafloor (1990 to 2000 m), where buoyant plume penetrations usually occurred (Fig. 5.5 a). The high $NH_4^+$ levels coincided with the presence of buoyant plumes. At 1800-1950 m under the influence of neutrally buoyant plumes, moderately high $NH_4^+$ levels were measured (~1850 m) in casts V2 ($588 \pm 37$ nM) and V6 ($132 \pm 1$ nM), but the measurement made in Dive 3780 was quite low ($30 \pm 6$ nM). The remaining water column possessed
generally low NH$_4^+$ concentrations (23-38 nM), except for an above-sill sample that contained 160 ± 40 nM of NH$_4^+$, consistent with the 151 ± 4 nM measured in a dive sample at a similar depth.

NH$_4^+$ concentrations approaching 2 μM were detected in the strong buoyant plumes at 1850-1950 m during casts V7 and T5 at the North Site (Fig. 5.5 c). The NH$_4^+$ levels below and above these plumes were only 5-20 nM, except for those detected (≤ 1844 nM) in the strong Δc maxima at ~1700 m during casts T3 and T4 when Δθ* was small (≤ 0.014 °C). NH$_4^+$ concentrations fluctuated around 82-96 nM at neutrally buoyant plume depths along the tow-yo cast T2 from the northwest towards the South Site. High NH$_4^+$ levels of 413-987 nM were recorded along the tow-yo cast T3 from the South Site to the North Site.

The concentrations of NH$_4^+$ in the warm diffuse fluids mostly ranged from 0.74 to 3.23 μM and appeared to increase with fluid temperature (26-58 °C) (Table 5.2). The sample with the maximum observed value (2.85 ± 0.23 mM) was collected from water vigorously shimmering out of microbial mats. The hot vent fluids (≤ 241 °C) emanating from chimneys contained millimolar levels of NH$_4^+$ up to 21.2 ± 0.18 mM.

5.3.3. Total Net Ammonium Removal and Ammonia Oxidation Rates

The majority of the incubation experiments (76 %) at the South Site showed zero or negative total net NH$_4^+$ removal rates, as low as -300 nM d$^{-1}$ at 1812 m in cast V1 (Fig. 5.5 b), implying active in situ remineralization of NH$_4^+$ (or ammonification). Among the only four positive total net removal rates measured, two were collected from the buoyant plume at 10-15 mab (155-278 nM d$^{-1}$), one from within the neutrally buoyant plume in
cast V6 (121 ± 6 nM d⁻¹) and one from an above-sill sample in cast V5 (74 ± 40 nM d⁻¹); all of which had greater than 100 nM of initial NH₄⁺ concentrations. The potential ammonia oxidation rates were always more positive than the total net removal rates (Fig. 5.5 b), again indicating active NH₄⁺ remineralization. The highest potential oxidation rate (517 ± 77 nM d⁻¹) was measured in the buoyant plume at 2000 m during a submersible dive, yet the potential ¹⁵NH₃ oxidation rates measured at similar depths in casts V3 and V6 were only 42.6-51.3 nM d⁻¹. A slightly lower range of potential ammonia oxidation rates (0-118 nM d⁻¹) was obtained in the overlying neutrally buoyant plumes (1800-1950 m), while the ¹⁵NH₃-tracer oxidation rate was 6.1 ± 0.3 nM d⁻¹ at 1854 m (cast V4). Above the sill, potential ammonia oxidation rates approached 140 ± 27 nM d⁻¹, consistent with the ¹⁵NH₃-tracer oxidation rate (144 ± 16 nM d⁻¹).

Fewer rate measurements were made at the North Site than at the South Site. The only positive total net removal rates measured at the North Site were found within the buoyant plume near 1900 m in cast T5 (23-60 nM d⁻¹), but the rates were negative in cast V7 (-77 ± 36 nM d⁻¹) (Fig. 5.5 d). The ammonia oxidation rates measured via the ¹⁵N-tracer method at similar depths were 12.9 ± 0.3 nM d⁻¹ in cast T5. Despite the high NH₄⁺ concentrations at 1694 m in cast T4, close to zero total net removal rate was recorded, but no ammonia oxidation rate measurements were made.

No correlation could be drawn between total net removal rates and NH₄⁺ concentrations (p>0.05, Spearman rank-order correlation), or between potential ammonia oxidation rates and NH₄⁺ concentrations (p>0.05, Spearman rank-order correlation). There were also no correlations between any of the measured rates with Δθ* or Δc.
The specific rate constants for the positive total net removal ($k_{\text{tot}}$) were 0.09-0.92 d$^{-1}$ at the South Site. Those for potential ammonia oxidation rates ($k_{\text{oxid}}$) were 0.02-5.06 d$^{-1}$, compared to 0.01-0.95 d$^{-1}$ calculated for $^{15}$NH$_3$ oxidation rates ($k_{15N}$), resulting in turnover times of 0.2-50 days and 1.1-96 days respectively. Smaller $k_{\text{tot}}$ (0.03-0.16 d$^{-1}$) and $k_{15N}$ (0.007-0.10 d$^{-1}$) were calculated for the North Site, and the subsequent turnover times were 6-33 days and 10-137 days. The values of $k_{\text{tot}}$ were significantly correlated with NH$_4^+$ concentration ($R=0.57$, $p<0.05$, Spearman rank-order correlation) but not with $\Delta c$ ($p>0.05$, Spearman rank-order correlation). Conversely, $k_{15N}$ was negatively correlated with $\Delta c$ ($R=-0.89$, $r<0.01$, Spearman rank-order correlation), $\Delta c$ ($R=-0.93$, $p<0.005$, Spearman rank-order correlation) and NH$_4^+$ concentration ($R=-0.82$, $p<0.05$, Spearman rank-order correlation).

The occurrence of pressure effects on total net removal or ammonia oxidation rates was inconclusive in our experiments. The high-pressure (200 atm) incubations yielded perhaps slightly positive total net removal rates ($8 \pm 54$ nM d$^{-1}$) at 2002 m at the South Site (cast V2), compared to $-219 \pm 46$ nM d$^{-1}$ measured in the 1-atm incubations. A very high pressure enhancement effect could be observed in the potential ammonia oxidation rate ($440 \pm 185$ nM d$^{-1}$) relative to a negative value at 1 atm ($-59 \pm 60$ nM d$^{-1}$). In contrast, more negative total net removal rates were detected after high-pressure incubations in cast V7 at the North Site ($-145 \pm 3$ nM d$^{-1}$ at 200 atm versus $-77 \pm 36$ nM d$^{-1}$ at 1 atm). More data are needed to clarify the effects of pressure on NH$_4^+$ removal.
cannot be ruled out that the microbial communities did not totally recover to their \textit{in situ} activity levels after re-pressurization back to 200 atm.

Substrate additions of 250 nM NH$_4^+$ resulted in an increase in total net removal rate from $-57 \pm 4$ nM d$^{-1}$ to $1 \pm 2$ nM d$^{-1}$ at the upper plume boundary at the North Site (1760 m, cast T5), yet a 5 nM NH$_4^+$ addition gave a more negative rate ($-104 \pm 4$ nM d$^{-1}$) than the unamended control. Similar substrate amendments in the plume core (1865 m, cast T5) also resulted in more negative rates ($-6 \pm 12$ nM d$^{-1}$ with 5 nM amendment, $-68 \pm 9$ nM d$^{-1}$ with 250 nM amendment) than the control ($23 \pm 6$ nM d$^{-1}$).

When larger particles ($\geq 3 \mu$m and $\geq 10 \mu$m) were removed, three samples showed very similar total net NH$_4^+$ removal rates as the non-filtered incubations, while two samples gave distinctly higher rates (Table 5.4). The elimination of larger particles in such experiments might have resulted in a reduction in NH$_4^+$ remineralization, thus a reduction in the local additional source for NH$_4^+$, instead of a true enhancement of NH$_4^+$ removal as suggested in the latter two experiments.

\textit{5.3.4. Particulate Organic Carbon and Particulate Nitrogen}

The concentrations of particulate organic carbon (POC) were in essence indistinguishable above (6.7-16.5 $\mu$g $\ell^{-1}$) and below (8.0-23.5 $\mu$g $\ell^{-1}$) the sill ($p > 0.05$, t-test). The corresponding carbon stable isotopic compositions ($\delta^{13}$C-POC) were $-30.9$ to $-27.0$ $\%_{oo}$ and $-30.9$ to $-26.3$ $\%_{oo}$ respectively. There were no significant relationships between POC concentrations and any of $\Delta$$\theta^*$, $\Delta$$c$ or $\Delta$$O_2$, and neither between $\delta^{13}$C-POC and any of the former parameters. In contrast, the concentrations of particulate nitrogen (PN) were significantly higher below the sill (1.1-2.5 $\mu$g $\ell^{-1}$) than above the sill (0.7-1.3
μg l⁻¹) (p<0.05, t-test) (Table 5.5), and were significantly correlated with both Δc (r =
0.86, p < 0.05, Product-moment correlation coefficient) and ΔO₂ (r = -0.94, p< 0.05,
Product-moment correlation coefficient). The nitrogen stable isotopic compositions were
7.2-12.0 ‰ below the sill and 10.5-17.6 ‰ above sill, but their difference was not
significant (p>0.05, t-test). The elemental carbon-to-nitrogen ratios (C/N) of particulate
organic matter were generally higher above the sill (8.7-16.1, median = 12.7) than below
sill (8.0-12.7, median = 8.7) (Table 5.5), though not statistically significant (p>0.05, t-
test).

5.3.5. Assimilation Rates

Assimilation rates accounted for at least 57 % of the total net NH₄⁺ removal rates
in the four samples examined, and such a percentage turned negative in one sample due
to the negative total net NH₄⁺ removal rates measured (i.e. there was total net NH₄⁺ gain).
Assimilation rates were not correlated with Δθ*, Δc, NH₄⁺ concentrations or any other
NH₄⁺ removal rate measurements (p>0.05, Spearman rank-order correlation). The
assimilation rates measured within the neutrally buoyant plume at the South Site were
15.5 nM d⁻¹ (2003 m) and 9.4 nM d⁻¹ (1854 m), very similar to that measured within the
buoyant plume (13.1 nM d⁻¹ at 1865 m) at the North Site, but were only a quarter of the
above-sill measurement (44.3 nM d⁻¹ at 1496 m). The corresponding specific rate
constants (kₐₕₐₜ) of the sub-sill samples ranged from 0.02 d⁻¹ to 0.029 d⁻¹, while that of the
above-sill sample (0.168 d⁻¹) were up to eight-fold greater. Assimilation rates were
equivalent to at least 31 % of the ¹⁵NH₃ oxidation rates and as much as 56 % greater in
the 1854-m sample at the South Site (Table 5.5). The rates of assimilation and ammonia
oxidation were not statistically different from each other based on the small data set available (p>0.05, Wilcoxon matched pairs test).

5.3.6. Analyses for Methane and Other Gases

The concentrations of methane (CH$_4$) in the water column were highly correlated with those of NH$_4^+$ (R=0.72, p<0.00001, Spearman rank-order correlation), and were on average almost ten times the NH$_4^+$ levels. CH$_4$ concentrations fluctuated with plume signals and varied with both $\Delta \theta^*$ (R=0.33, p<0.05, Spearman rank-order correlation) and $\Delta c$ (R=0.57, p<0.0001, Spearman rank-order correlation), but probably non-linearly. At the South Site, the maximum CH$_4$ concentration (11.2 $\mu$M) was detected in the neutrally buoyant plumes during casts V2 and V4, compared to only 16-65 nM in the overlying water (Fig. 5.6 a). CH$_4$ concentrations reached a maximum of 31.6 $\mu$M in a buoyant plume sample at the North Site (cast T5), followed by 26.0 $\mu$M at the $\Delta c$ maximum in the last upward portion of cast T3 (1667 m) (Fig. 5.6 c). There were consistently lower concentrations along the tow-yo cast T2 (17-38 nM) relative to those recorded during tow-yo cast T3 (5.5-26.0 $\mu$M), along the spreading axis between the South Site and North Site. The majority of seawater samples had carbon stable isotopic values from -44.6 to -42.0 $^\circ$/oo. Isotopically heavier methane were noted only in the neutrally buoyant plume depths at the South Site ($\delta^{13}C$-CH$_4$ $\leq$ -37.4 $^\circ$/oo) or at 1 km south-west of the South Site ($\delta^{13}C$-CH$_4$ $\leq$ -27.4 $^\circ$/oo), implying greater extent of methane oxidation or older residual methane (Cowen et al., 2002), while a very light value was measured at the buoyant plume core at around 1900 m (-51.6 $^\circ$/oo), indicating more recently formed methane (Fig. 5.6 b, d).
Methane constituted 35-49% of total gas contents in both the hot vent fluids (200-306 °C) and warm diffuse fluids (16-93 °C) sampled, which were equivalent to 3.3-24.1 mmol kg⁻¹ and 4.7-10.5 mmol kg⁻¹ respectively (Table 5.2), generally higher than the 2.0-6.8 mmol kg⁻¹ previously measured (Welhan and Lupton, 1987). Since Welhan and Lupton (1987) collected the hot hydrothermal fluids with titanium major samplers that are not totally gas-tight, our higher values of methane concentrations are believed to be more accurate. CH₄ concentrations were up to three orders of magnitudes greater than NH₄⁺ concentrations, where simultaneous NH₄⁺ measurements were available. Hydrogen contents were ≤ 1.1 μmol kg⁻¹ in all cases except for the 306 °C hot vent fluids (1.44 mmol kg⁻¹). Total inorganic carbon (TCO₂) concentrations were similar to those of methane (Table 5.2).

5.3.7. Microbial Community Structure

Total microbial abundance, estimated as counts of DAPI-stained cells, showed significant correlation with Δc (r = 0.67, p<0.05, Product-moment correlation coefficient) but not with Δθ* (p>0.05, Spearman rank-order correlation), while the abundance of Eubacteria as EUB338-hybridized cells exhibited moderate yet significant correlation with both Δθ* (R=0.54, p<0.05, Spearman rank-order correlation) and Δc (r=0.58, p<0.05, Product-moment correlation coefficient). The total microbial abundance at the South Site (Fig. 5.7 a) was clearly elevated within the neutrally buoyant plume around 1850 m (1.85 ± 0.07 × 10⁵ cells ml⁻¹) relative to the abundance at 2000 m (1.15 ± 0.06 × 10⁵ cells ml⁻¹) and above the sill (0.67-0.98 × 10⁵ cells ml⁻¹). Eubacteria made up 57-87% of the total microbial abundance, and followed a similar depth distribution (Fig. 5.7).
a). There were large total microbial \((1.55 \pm 0.04 \times 10^5 \text{ cells ml}^{-1})\) and Eubacterial populations \((0.91 \pm 0.05 \times 10^5 \text{ cells ml}^{-1})\) in the large buoyant plume (~1900 m) at the North Site (Fig. 5.8 a). However, even greater total microbial abundance \((1.77 \pm 0.07 \times 10^5 \text{ cells ml}^{-1})\) was recorded at 1694 m without any noticeable hydrothermal plume signals \((\Delta \theta^* \leq 0.0014 \, ^\circ \text{C})\), and Eubacteria contributed only 46% to this community. In comparison, much lower abundances were measured at 1760 m (total: \(0.45 \pm 0.03 \times 10^5 \text{ cells ml}^{-1}\); Eubacteria: \(0.27 \pm 0.02 \times 10^5 \text{ cells ml}^{-1}\)).

The abundance of \(\beta\)-Proteobacterial ammonia-oxidizing bacteria (\(\beta\)AOB) showed a significant relationship with \(\Delta c\) (NS0190: \(r=0.76, p<0.005\); NS01225, \(r=0.65, p<0.05\); Product-moment correlation coefficients), but not with \(\Delta \theta^*\) (\(p>0.05\) for both NS0190 and NS01225, Spearman rank-order correlation). \(\beta\)AOB seemed to be more populated at the plume depths \((3.3-5.1 \times 10^3 \text{ cells ml}^{-1}, 1.8-3.1\% \text{ DAPI})\) than above the sill \((1.1-3.2 \times 10^3 \text{ cells ml}^{-1}, 1.6-1.9\% \text{ DAPI})\) at the South Site (Fig. 5.7 b), while their abundance within and above the plume did not show much difference at the North Site \((2.0-3.4 \times 10^3 \text{ cells ml}^{-1}, 1.5-5.8\% \text{ DAPI})\) (Fig. 5.8 b). Among the \(\beta\)AOB, \textit{Nitrosomonas} spp. appeared to be slightly more dominant than \textit{Nitrosospira} spp. above the hydrothermally influenced water, whereas it might be the reverse within the neutrally buoyant or buoyant plumes (Fig. 5.7 d, 5.8 d).

The populations of \(\gamma\)-proteobacterial ammonia-oxidizing bacteria were usually smaller than those of \(\beta\)AOB \((0.3-4.5 \times 10^3 \text{ cells ml}^{-1}, 0.4-3.9\% \text{ DAPI})\), except at 1760 m at the North Site \((3.5 \pm 0.6 \times 10^3 \text{ cells ml}^{-1}, 8.0\% \text{ DAPI})\) and at 2000 m at the South Site \((4.5 \pm 0.7 \times 10^3 \text{ cells ml}^{-1}, 3.9\% \text{ DAPI})\) (Fig. 5.7 b, 5.8 b). The population distribution of
all AOB, as the sum of $\beta$AOB and $\gamma$AOB abundance, shared the same general pattern as the $\beta$AOB and $\gamma$AOB (Fig. 5.7 c, Fig. 5.8 c). It was significantly correlated with $\Delta c$ ($r=0.64$, $p<0.05$, Product-moment correlation coefficient) but not with $\Delta n^*$ ($p>0.05$, Spearman rank-order correlation), $NH_4^+$ concentration, total net $NH_4^+$ removal or ammonia oxidation rates ($p>0.05$, Product-moment correlation).

Type I methanotrophs ($3.1-7.8 \times 10^3$ cells ml$^{-1}$, 3.1-7.0% DAPI) appeared to predominate over their Type II counterparts ($0.6-6.2 \times 10^3$ cells ml$^{-1}$, 0.3-3.3% DAPI) except for one above-sill sample at the South Site (Fig. 5.7 e, Fig. 5.8 e). Type I methanotrophs showed a positive correlation with $\Delta n^*$ ($R=0.66$, $p<0.01$; Spearman rank-order correlation), $\Delta c$ ($r=0.85$, $p<0.001$; Product-moment correlation coefficient) and methane concentration ($r=0.96$, $p<0.01$, Product-moment correlation coefficient), while Type II methanotrophs did not. The combined methanotroph abundance was significantly greater (almost five-fold) than that of total AOB ($p<0.00001$, one-tailed t-test).

The majority of microbes were found to be free-living in the four samples tested (60-66% of total DAPI-stained cell counts) (Fig. 5.9 a), except for the above-sill sample at the South Site (48%). Eubacteria were mostly free-living at the North Site (60-71%), but not so at the South Site (44-46%) (Fig. 5.9 b). However, $\beta$AOB were generally associated with particles (40-92%), especially those greater than 10 $\mu$m-diameter in size. An exception was the plume sample at the South Site where $\beta$AOB were associated more with particles 3.0-10 $\mu$m-diameter in size (Fig. 5.9 c,d). Similarly, *Nitrosospira* spp. and *Nitrosomonas* spp. tended to be strongly particle-associated, except within the plume at
the South Site, where there were large free-living populations of both *Nitrosospira* spp. (54%) and *Nitrosomonas* spp. (78%) (Fig. 5.9 e, f). In contrast with βAOB, γAOB were mostly free-living (73-78%) except for above the sill at the South Site (22%) (Fig. 5.9 g). Both groups of methanotrophs were predominantly free-living (up to 96%) (Fig. 5.9 h, i).

5.4. DISCUSSION

5.4.1. Hydrothermal Plumes in the Guaymas Basin

In typical mid-ocean ridge spreading centers in the open ocean, the neutrally buoyant hydrothermal plumes are usually advected away from vent sources along isopycnals and can still be traced tens or hundreds of kilometers away by anomalies of potential temperatures (Thomson et al., 1992) or $\delta^3$He (Lupton, 1998). In the semi-enclosed Guaymas Basin, neutrally buoyant hydrothermal plumes are trapped within the basin below the sill depth (1560 m) until the bottom water is eventually ventilated in perhaps 3.7 years (Campbell and Gieskes, 1984). Hydrothermal fluids discharged into the water column are more prone to rapid horizontal homogenization across the entire basin by the strong reversing tidal currents (12 cm s$^{-1}$) (Lonsdale and Becker, 1985) rather than the slower vertical ventilation (Campbell and Gieskes, 1984). Hence, the seawater at sub-sill depths (below 1750 m) of the basin can be regarded as a time-integrated record of hydrothermal inputs and their reactions with the ambient Pacific Deep Water over the residence time of the basin bottom water. The microbes are given a long incubation time to interact with the substrates available in the surroundings.
5.4.2. Variations and Sources of Ammonium

The NH$_4^+$ concentrations in the sub-sill water column of the Guaymas Basin reached almost 3 μM (mean=408 nM, median=139 nM) in the hydrothermal plumes, which were on average higher than those detected in the plume core (≤ 341 ± 136 nM) over the Endeavour Segment, Juan de Fuca Ridge (Lam et al., 2004; Chapters 3 & 4), the only unsedimented hydrothermal system with a noticeable NH$_4^+$ anomaly in the water column. Other NH$_4^+$ measurements in the deep sea are less than 25 nM, as reported from the Sargasso Sea (Lipschultz et al., 1996) and the Eastern Tropical North Pacific (Ward and Zafiriou, 1988). The Guaymas Basin had NH$_4^+$ levels higher than some semi-enclosed yet shallower basins, such as the Gulf of Lions (<70 nM) (Diaz and Raimbault, 2000) and the Rhône River plume (0.2-2 μM) (Bianchi et al., 1994), both in the north-western Mediterranean; but lower than the more enclosed and anoxic central Baltic Sea (≤ 20 μM) (Voß et al., 1997), Cariaco Basin (<24 μM) (Taylor et al., 2001) and the Black Sea (<90 μM) (Konovalov et al., 2001).

Different depths of the water column within Guaymas Basin likely received different sources of NH$_4^+$. Three zones might be generalized: **Zone 1** – the hydrothermally influenced water below the θ-S discontinuity at ~1750 m; **Zone 2** – the water layer immediately below the sill (1560-1750 m) but above any direct hydrothermal influence; and **Zone 3** – the above-sill depths (<1560 m). Hydrothermal circulation accelerates the decomposition of sedimentary organic matter, which releases large amounts of NH$_4^+$ into the water column. Hydrothermal discharges are undoubtedly the major source of NH$_4^+$ in the bottom-most layer (**Zone 1**), where the water is only
ventilated every ~3.7 years (Campbell and Gieskes, 1984). The NH$_4^+$ is trapped and so has a higher chance to interact with microbes or other chemicals within this zone than in the more ventilated zones 2 and 3. Using silica anomalies, Campbell and Gieskes (1984) estimated a $10^3$ ($\pm$ 25%) dilution of the Guaymas hydrothermal fluids by the sub-sill basin water, which was consistent with the $^3$He observations (Lupton, 1983).

Consequently, a range of NH$_4^+$ concentrations in the end-member fluids from the lower limit of 10.5 mM measured by Von Damm et al. (1985) to the maximum value measured in the hot vent fluids in this study (21 mM), would predict an NH$_4^+$ range of 10.5-21 $\mu$M in the sub-sill water column. Nevertheless, only $< 3$ $\mu$M of NH$_4^+$ was measured in this study, with the higher values coming from the buoyant plumes. These discrepancies from the predicted values may suggest rapid biological uptake, fluctuations in NH$_4^+$ supply, or both; if the dilution ratio estimated by Campbell and Gieskes (1984) held. The biological uptake processes include ammonia oxidation and assimilation, which are discussed in details in the upcoming sections (sections 5.4.3 to 5.4.7). The observed fluctuations in NH$_4^+$ levels at the South Site, for instance, were up to three orders of magnitude over days or even hours, and could be caused by strong reversing tidal currents ($\sim$12 cm s$^{-1}$) (Lonsdale and Becker, 1985) and variations in fluid discharges. The likelihood of strong reversing horizontal advection was especially high at the South Site, since its closest known hydrothermal sources were probably about 150 m away, at the periphery of the South Sill (Lonsdale and Becker, 1985) (Fig. 5.1 b). In comparison, the hydrothermal plume signals from the North Site were much stronger and spanned a greater depth range, probably as a result of its being directly along a growth fault on the Central Sill and between the two drill holes, DSDP477 and DSDP477A (Fig. 5.1 b),
where strong plume signals have been reported in exactly the same location (Lonsdale and Becker, 1985). Large fluctuations in fluid discharges from a black smoker over the course of an hour have been reported previously (Karl et al., 1988), although some of these variations in fluid discharges, particularly diffuse venting, might also be tied to tidal variations (Tivey et al., 2002). A previous study was unsuccessful in detecting any NH$_4^+$ anomalies in the Guaymas Basin sub-sill water (Campbell and Gieskes, 1984), perhaps again because of fluid fluctuations, yet the sensitivity of their NH$_4^+$ analyses and storage artifacts might have also hindered the accuracy of their measurements.

The source of NH$_4^+$ for the above-sill water (Zone 3) was largely dependent on the productive surface ocean (Kahru et al., 2004), resulting in elevated NH$_4^+$ concentrations as high as 160 ± 40 nM, several fold greater than typical open ocean values at similar depths (<25 nM) (Lipschultz et al., 1996). This NH$_4^+$ was most likely delivered to this zone in the form of particulate organic matter that is continuously being decomposed to release NH$_4^+$. Indeed, NH$_4^+$ concentrations were more strongly correlated with Δc (R=0.49, p<0.001, Spearman rank-order correlation) than with Δθ* (R=0.36, p<0.05, Spearman rank-order correlation) in the Guaymas Basin water column.

The sub-sill water that was above direct hydrothermal influence (Zone 2) probably received NH$_4^+$ from both the upper water column and the hydrothermally influenced water below, while particulate organic matter likely acted as a vehicle of NH$_4^+$ transport into this zone. For example, almost 2 μM NH$_4^+$ were detected at ~1700 m at the North Site, where hydrothermal influence was essentially absent (Δθ* = 0.001°C). However, a Δc maximum (0.20) was present at this depth, suggesting the trapping of
particles at this 0-S discontinuity, or interface between two physically distinct water layers (Fig. 5.3e, 5.4). Subsequently, the particles decomposed and released more NH$_4^+$. This high NH$_4^+$ value also coincided with amongst the most abundant particulate nitrogen (2.4 $\mu$g l$^{-1}$), particulate organic carbon (23.5 $\mu$g l$^{-1}$) and total microbial populations (1.77 ± 0.82 $\times$ 10$^5$ cells ml$^{-1}$).

5.4.3. Autotrophic Ammonia Oxidation and Substrate Availability

Autotrophic ammonia oxidation was enhanced throughout the entire Guaymas Basin water column and was not solely dependent on the hydrothermally injected NH$_4^+$. Similar to NH$_4^+$ concentrations, higher autotrophic ammonia oxidation rates were usually detected in buoyant plumes (potential rates 37-517 nM d$^{-1}$) more than in the neutrally buoyant plumes (potential oxidation rates: 86-118 nM d$^{-1}$; $^{15}$N-tracer: 6-87 nM d$^{-1}$). The ammonia oxidation rates measured in the buoyant plumes were five times those in the Endeavour neutrally buoyant plume (3.5-91 nM d$^{-1}$) (Lam et al., 2004; Chapters 3 & 4) and were comparable to the oxidation rates reported for the oxygen minimum zone of the Eastern Tropical South Pacific (5-600 nM d$^{-1}$) (Ward et al., 1989). Nevertheless, the oxidation rates in the neutrally buoyant plumes in Guaymas Basin were only similar to those in Endeavour, whereas the higher rates (144 ± 27 nM d$^{-1}$, $^{15}$N-tracer) above the sill in Guaymas Basin were as much as the maximum rate measured in the North Pacific subtropical gyre (137 nM d$^{-1}$) (Dore and Karl, 1996). The productive surface waters of Guaymas Basin have apparently provided large amounts of NH$_4^+$ via downward fluxes of decomposing particulate organic matter to enhance ammonia oxidation in these non-hydrothermally influenced above-sill water.
Since temperature, light, salinity, oxygen and pH vary little in the sub-sill water column of the Guaymas Basin, autotrophic ammonia oxidation is largely a function of the interdependent factors such as ammonium availability, microbial communities, organic carbon and nitrogen contents. Ammonia oxidation did not follow first-order reaction kinetics in the Guaymas Basin, that is, ammonia oxidation rates did not show a linear relationship with NH$_4^+$ concentrations (p>0.05, Spearman rank-order correlation). This is consistent with the observations made in the Endeavour plume (Chapter 4) and in some other studies (Olson, 1981; Ward and Kilpatrick, 1990). Although large amounts of NH$_4^+$ were available in the Guaymas Basin water column, the highest ammonia oxidation rates did not occur at the maximum NH$_4^+$ concentration, but rather in the range of 100-200 nM (Fig. 5.10). If the additional amounts of NH$_4^+$ (~100 nM) inadvertently introduced in the DCD treatment are taken into account, the data points for the potential oxidation rates should be shifted +100 nM along the x-axis in Fig. 5.10, which would give a cluster of elevated rates at 100-300 nM NH$_4^+$. All specific rate constants with respect to the total net removal ($k_{TOT}$), potential oxidation ($k_{oxid}$) and $^{15}$NH$_3$-oxidation ($k_{15N}$) were also elevated within this NH$_4^+$ concentration range (Table 5.3). This range of NH$_4^+$ agreed with the empirical half-saturation constant ($K_s$) determined in the Endeavour plume (153 nM of NH$_4^+$), as well as the substrate(NH$_4^+$)-dependent phase of ammonia oxidation rates observed at 100-300 nM of NH$_4^+$ (Chapter 4). Moreover, the NH$_4^+$ concentration of the only sample showing positive results in the substrate limitation experiments also lay within this range after substrate amendments, but the NH$_4^+$ concentration of the other sample exceeded this range after the 250 nM amendments. The total net NH$_4^+$ removal rates increased linearly with NH$_4^+$ beyond 500 nM without
apparent increase in ammonia oxidation rates, which may imply greater importance of assimilation or heterotrophic uptake, or possibly inhibition of ammonia oxidation by the higher concentrations of certain organic compounds or trace metals in the less diluted hydrothermal discharges. Autotrophic ammonia oxidation rates accounted for ≥ 22% of total net NH₄⁺ removal in the Guaymas Basin water column, compared to ≥ 93% in the Endeavour plume (Chapter 4). Such a difference again suggests a more important role of assimilation or heterotrophic uptakes in the Guaymas Basin system. However, additional ammonia oxidation and assimilation rate measurements at micromolar levels of NH₄⁺ are necessary to further elucidate such reaction rate kinetics.

5.4.4. Ammonia Oxidation and Autotrophic Ammonia-Oxidizing Bacteria

Autotrophic ammonia-oxidizing bacteria (AOB) were detected in all samples in the Guaymas Basin water column regardless of hydrothermal influence, implying their ubiquity in the deep-sea water column. The abundance of AOB in the β- and γ-Proteobacteria subgroups, or the sum of the two, seemed to be elevated in the sub-sill water column (>1560 m) with a *Nitrosospira*-dominance, compared to above the sill (≤ 1560 m) where *Nitrosomonas* dominated. There was no clear relationship between the abundance of AOB (either as βAOB or γAOB) and NH₄⁺ concentrations or ammonia oxidation rates (p>0.05, Spearman rank-order correlation), similar to the observations made for the Endeavour plume (Chapter 4). The highest total AOB abundance (sum of βAOB and γAOB) coincided with intermediate NH₄⁺ concentrations (~500 nM) (Fig. 5.11 a) and intermediate ammonia oxidation rates (~40 nM d⁻¹) (Fig. 5.11 b). As discussed in Chapter 4 for the Endeavour plume, AOB have to increase their cellular
oxidation rates beyond a certain threshold before they can multiply, thus their abundance likely takes a longer time to respond to substrate increases than the overall ammonia oxidation rates. Consequently, a large AOB population with intermediate oxidation rates may indicate a longer-term sustainability for ammonia oxidation, compared to a small AOB population with higher oxidation rates.

Despite the higher \( \text{NH}_4^+ \) concentrations in the Guaymas hydrothermal plume than in the Endeavour plume, the maximum abundance of total AOB in the former \((7.9 \pm 0.9 \times 10^3 \text{ cells ml}^{-1})\) was less than half of that in the latter \((16.3 \pm 1.8 \times 10^3 \text{ cells ml}^{-1})\) (Chapter 4). In other words, the AOB were not fully utilizing the available \( \text{NH}_4^+ \) in the Guaymas system, consistent with the lower ratios of ammonia oxidation to total net \( \text{NH}_4^+ \) removal observed in the Guaymas water column \((\geq 22\%)\) than in the Endeavour plume \((\geq 93\%)\) (Chapter 4). One possible explanation is that the high \( \text{NH}_4^+ \) concentrations measured in the Guaymas Basin water column represented recent hydrothermal discharges, which AOB did not have a chance to fully colonize yet. Alternatively, it might be that the conditions in the Guaymas Basin somehow favored heterotrophic activities more than those in the Endeavour plume, such as the higher concentrations of particles and organic matter.

5.4.5. Particle-Associations of Ammonia-Oxidizing Bacteria

The potential influence of particulate organic matter on autotrophic ammonia oxidation can be both positive and negative. On the positive side, particulate organic matter, upon decomposition, can provide an additional source of \( \text{NH}_4^+ \) for the ammonia-oxidizing bacteria as mentioned earlier. On the negative side, particulate organic matter
may supply so much organic carbon that heterotrophic activities are favored more than
the autotrophic AOB. The potential positive influence is first discussed below, followed
by the discussion on the negative influence in Section 5.4.6.

The large amounts of organic-rich particles within the Guaymas Basin water
column arose from surface water productivity, hydrothermal inputs and in situ particle
production within the sub-sill basin. The release of NH$_4^+$ from these particles likely
attracts autotrophic ammonia-oxidizing bacteria (AOB), which are known to actively
produce exopolymeric substances for attachment with one another or with other particles,
especially during low NH$_4^+$ conditions (Stehr et al., 1995). Particle-association of AOB
in the Guaymas Basin water column was supported by the significant correlation between
the abundance of total AOB (sum of $\beta$AOB and $\gamma$AOB) and the particle anomalies ($\Delta c$
($r=0.64$, $p<0.05$, Product-moment correlation coefficient). In fact, AOB accounted for a
sizeable percentage (3-13% DAPI) of the overall particle-associated ($\geq 3$ $\mu$m diameter)
communities than of the free-living ($\leq 3$ $\mu$m diameter) communities (0.4-6% DAPI). The
lack of correlation between the total AOB abundance and $\Delta \theta^*$ ($p>0.05$, Spearman rank-
order correlation) also suggests that AOB abundance were more directly affected by
particle concentrations than recent hydrothermal inputs.

Among the AOB, mainly $\beta$AOB rather than $\gamma$AOB were responsible for the
observed particle-association (Table 5.6). Particle-association of AOB was reported to be
more prominent above and at the plume boundaries than within the plume core along the
Endeavour Segment, likely because particles released more NH$_4^+$ than what was available
in the ambient seawater in the former two cases (Lam et al., 2004; Chapter 3). The same
observation can be made at the South Site, where particle-association appeared much stronger above the sill (95%) than within the plumes (32%). The particle-associated AOB in the former was dominated by *Nitrosomonas*-like cells (42%), consistent with the greater particle-association of *Nitrosomonas* spp. than of *Nitrosospira* spp. in the northwestern Mediterranean (Phillips *et al.*, 1999). However, unlike the suggestion by Phillips *et al.* (1999) that *Nitrosospira* spp. were inclined to be free-living, a good proportion (>50%) of *Nitrosospira*-like cells were actually found associated with particles in three out of the four samples examined in this study (Fig. 5.9 e). These *Nitrosospira*-like cells, being 100% particle-associated themselves, contributed 58% to the overall AOB abundance in all size-fractions, which were 81% particle-associated in total (Table 5.6). Hence, the different extent of particle-association of *Nitrosomonas* spp. versus *Nitrosospira* spp. may not be as distinct as previously thought. On the other hand, this North Site sample was collected from a buoyant plume, while *Nitrosospira*-like 16S rDNA sequences have been recovered from the underlying hydrothermal sediments (Chapter 6). It is possible that these *Nitrosospira*-like cells had just been discharged along with the hydrothermal fluids into the buoyant hydrothermal plume, and these AOB might have an affinity towards particles different from the AOB already inhabiting the water column.

The strong particle-association of AOB has led to the proposition that the presence of particles enhanced ammonia oxidation rates, and it was tested in the particle-filtered versus control incubation experiments. Unfortunately, the removal of particles larger than 3 µm- and 10 µm- diameter in our particle-filtered incubations have almost certainly removed the still decomposing organic-rich particles, thus a continuous NH$_4^+$
supply for the microbial communities. The resulting particle-filtered total net removal rates would be unrealistically more positive than the controls. Hence, future investigations should include simultaneous rate measurements of NH$_4^+$ recycling to correct such experimental artifacts.

5.4.6. The Role of Organic Matter in Ammonia Oxidation

While the abundance of particulate organic matter may enhance ammonia oxidation, too high a ratio of total organic carbon to nitrogen contents (C/N) likely favors the growth of heterotrophs, including heterotrophic nitrifiers, more than the slower-growing autotrophic AOB (Strauss and Lamberti, 2000). In the extreme case, since so much carbon is available that nitrogen might run out before carbon (due to high C/N), then the heterotrophs would likely outcompete the slow-growing AOB for NH$_4^+$ (Strauss and Lamberti, 2000). The sediments in the Guaymas Basin contain petroleum products formed under hydrothermal influence and are composed of a variety of condensates, naphthenic and waxy organics with significant amounts of asphaltenes, sulfur and nitrogen compounds and polynuclear aromatic hydrocarbons (Simoneit, 1985). The lighter hydrocarbons (C$_1$-C$_{40}$), in particular, could rise up to 1 km above the seafloor, into the water column (Simoneit, 1991). Elevated concentrations of short-chain organic acids, especially those that can be easily broken down and favor heterotrophs, have also been reported in the porewaters (Martens, 1990) and would be highly likely in the hydrothermal discharges. As a consequence, heterotrophic activities are almost certain to be high in the Guaymas Basin water column. Perhaps because of the high levels and continuous production of NH$_4^+$ in the Guaymas water column, AOB were not totally outcompeted by heterotrophs based on the abundance estimates in this study.
Nevertheless, these slow-growing AOB did not achieve an abundance level as high as the heterotrophs or other faster-growing autotrophs (total AOB ≤ 11% DAPI). This might explain why heterotrophic, but not autotrophic, nitrifiers have been isolated from the Guaymas Basin hydrothermal environments (Mével et al., 1996), and that the AOB abundance here in the Guaymas Basin were at most half of the maximum AOB abundance in the Endeavour plume. The effective availability of NH$_4^+$ for the AOB would probably be reduced considerably by heterotrophic uptake and assimilation due to the faster growths of other microbes. This supposition agrees well with the fact that both the Guaymas and Endeavour systems share similar autotrophic ammonia oxidation rates with which the maximum total AOB abundance coincided (~40 nM d$^{-1}$ and 20-30 nM d$^{-1}$ respectively), while the corresponding NH$_4^+$ concentrations were several times higher in the Guaymas system (~500 nM) than in the Endeavour system (70-90 nM).

Furthermore, autotrophic ammonia oxidation could be inhibited by high levels of methane (Hyman and Wood, 1983; Jones and Morita, 1983b), carbon monoxide (Jones and Morita, 1983a), a variety of organic compounds, including acetylenic, heterocyclic and halogenated organic compounds, as well as a variety of sulfur-containing compounds (McCarty, 1999) and certain heavy metals such as cadmium, cobalt, cesium and strontium (Hu et al., 2002; Juliastuti et al., 2003). At least some of these compounds are quite abundant as thermal degradation products of sedimentary organic matter or petroleum products in the porewaters and hydrothermal discharges in the Guaymas Basin (Gieskes et al., 1991; Simoneit, 1991; Simoneit et al., 1992), and may be part of the reason why ammonia oxidation rates did not increase further than in the Endeavour hydrothermal plume as described earlier.
5.4.7. Assimilation of Ammonium & Enhanced Nitrogen Cycling

The assimilation of ammonium in the Guaymas water column (9.4-44.3 nM d⁻¹) was perhaps the fastest ever reported at these deep ocean depths. It was also similar to or faster than the measurements made in the Endeavour hydrothermal plume (0.5-20 nM d⁻¹) (Chapter 4), in the euphotic zone in the Southern California Bight (<20 nM d⁻¹) (Ward, 1985) and in the oxygen minimum zone of the Eastern Tropical South Pacific (5-10 nM d⁻¹) (Lipschultz et al., 1990), while it lay within the range measured in the euphotic zone of the Equatorial Pacific at 150°W (Raimbault et al., 1999). The assimilation rates were not statistically different from the ammonia oxidation rates measured by the ¹⁵N-tracer technique, at least based on the small data set available in this study (p>0.05, Wilcoxon matched pairs test). The highest assimilation rate measured was found above the sill at the South Site and coincided with amongst the highest ammonia oxidation rates measured (Fig. 5.11). This suggests that active cycling of nitrogen was not limited to the hydrothermally influenced sub-sill water. The large supply of nutrients and organic matter from the productive surface water apparently has also stimulated both autotrophic and heterotrophic NH₄⁺ uptakes.

Active in situ remineralization or regeneration of NH₄⁺ is indicated by the often-negative total net NH₄⁺ removal rates detected throughout the water column, and the fact that the sums of assimilation and autotrophic ammonia oxidation rates exceeded the total net removal rates. The negative total net removal rates mean that NH₄⁺ was being remineralized even faster than the autotrophic and heterotrophic uptakes combined. Assuming that ammonia oxidation and assimilation were the only NH₄⁺ uptake processes in the water column, the in situ regeneration rates of NH₄⁺ were estimated to be 3.5-114.3
nM d⁻¹. The maximum value was obtained from the above-sill sample at the South Site, again implying very active NH₄⁺ cycling above the sill depth independent of hydrothermal influence.

Given an estimated hydrothermal fluid flux of 10-12 m³ s⁻¹ at the Guaymas spreading center (Campbell and Gieskes, 1984), an NH₄⁺ range of 10.5-21 mM in end-member fluids would result in an NH₄⁺ input of 9.1 to 18.1 × 10⁶ mol N d⁻¹ into the water column, or 9-21 mg N m⁻² d⁻¹ assuming a basin area of 240 km × 60 km (Becker and Fisher, 1991). This crude NH₄⁺ flux estimate is similar to or greater than the export flux of particulate nitrogen (PN) from the euphotic zone (0.25-10 mg N m⁻² d⁻¹) (Thunell, 1998), thus the potential regeneration of NH₄⁺. If one assumes that ammonia oxidation is the rate-limiting step of nitrification (Kowalchuk and Stephen, 2001), and all resultant nitrite is eventually oxidized to nitrate with negligible nitrite or nitrate uptakes, then the nitrogen flux from ammonia oxidation would be equivalent to that from nitrate formation. Since ammonia oxidation accounted for at least 22% of the total net NH₄⁺ removal rates, the nitrogen flux from nitrate formation would be equal to at least 2.0-4.6 mg N m⁻² d⁻¹. Hence, the seafloor hydrothermal system accelerates the return of fixed nitrogen from sedimentary organic matter into the deep-sea nitrate reservoir to a rate of at least 2.0-4.6 mg N m⁻² d⁻¹. This nitrogen flux estimate is based only on the amount of NH₄⁺ discharged in the end-member fluids, yet the actual total flux could be even larger due to the continuous degradation of the organic matter discharged with the hydrothermal fluids, and the consequent remineralization of NH₄⁺ in the water column.
5.4.8. Ammonia Oxidation as an In Situ Organic Carbon Production Process

The relatively long residence time of the seawater below the sill-depth in the Guaymas Basin implies a long incubation time or greater opportunity for microbes to interact with various chemicals. For instance, slow microbial processes such as manganese oxidation occur in the Guaymas Basin on time-scales of days compared to perhaps months in other mid-ocean ridge hydrothermal systems (Campbell et al., 1988).

Taking the lower end of the ammonia oxidation rates measured with the $^{15}$N-tracer technique (6 nM d$^{-1}$), and a conversion factor of 0.3-1.4 g C mol$^{-1}$ N (Feliatra and Bianchi, 1993; Butturini et al., 2000), ammonia oxidation may be responsible for 5-25 mg C m$^{-2}$ d$^{-1}$ of in situ organic carbon production over the 250-m thick hydrothermally influenced water. This crude estimate is comparable to the particulate organic carbon flux from the surface ocean (2-50 mg C m$^{-2}$ d$^{-1}$) (Thunell, 1998).

Methane oxidation rates were not measured in this study. However, the $\sim$5-7 $\%_o$ higher $\delta^{13}$C-CH$_4$ values observed within the South Site neutrally buoyant plume relative to most of the remaining measurements ($\delta^{13}$C-CH$_4 = -42.0$ to -44.6 $\%_o$) suggest only small degree of methane oxidation at the two main sampling sites. Conversely, a much higher value ($\delta^{13}$C-CH$_4 = -27.4\%_o$) was measured in a single sample taken at 1 km southwest of the South Site, indicating a large extent of methane oxidation. Moreover, the abundance of total methanotrophs (Type I and Type II) was nearly always greater than that of total AOB (114-480% of total AOB), with only one exception at 1760 m at the North Site (total methanotrophs = 73% total AOB). This might indicate generally faster or greater extent of methanotrophy than ammonia oxidation in the Guaymas Basin.
water column, unlike the dominance of AOB over methanotrophs and of ammonia oxidation over methanotrophy close to hydrothermal vent sources along the Endeavour Segment (Chapter 4). Unfortunately, limited spatial and temporal coverage of both the $\delta^{13}$C-CH$_4$ and microbial abundance data preclude a more conclusive depiction of the actual extent of methane oxidation and the relative importance of ammonia oxidation versus methanotrophy.

There is currently no data available to compare the total in situ organic carbon production with the total organic carbon flux from the hydrothermal fluids and porewater discharges. However, the presence of a large organic carbon reservoir in the thick Guaymas Basin sediment cover suggests that the organic carbon discharge flux should be substantially greater.

5.5. CONCLUSIONS

The seafloor hydrothermal activities and the thick sediment cover of the Guaymas Basin endow the sub-sill water column with a unique hydrography and biogeochemistry. The elevated NH$_4^+$ concentrations were highly variable both temporally and spatially, due in part to the hydrography and in part to the active NH$_4^+$ production and consumption processes. Active NH$_4^+$ cycling probably took place throughout the entire water column, in the hydrothermally influenced water, as well as above the sill where plentiful NH$_4^+$ was derived from the particulate organic matter exported from the upper water column. Autotrophic ammonia oxidation was the most enhanced in the buoyant hydrothermal plume, but it was also quite active in the overlying particle-rich deep water without direct hydrothermal influence. Although the NH$_4^+$ concentrations were a few orders of
magnitude higher in the Guaymas water column than in the Endeavour hydrothermal plume, the concurrently higher organic content and possibly other chemical compounds in the Guaymas Basin water column have also stimulated more heterotrophic activities and associated assimilation, thus preventing autotrophic ammonia oxidation from achieving a rate as high as one would expect if this process were controlled by substrate availability alone. For the same reasons, autotrophic ammonia-oxidizing bacteria did not achieve a population any bigger than that in the Endeavour hydrothermal plume.
5.6. REFERENCES


227


**Table 5.1.** List of the various 16S rRNA-targeted oligonucleotide probes used in this study, together with their respective targeting organisms, target sites with respect to *E. coli*, formamide concentrations (%FA) in hybridization solutions and NaCl concentrations in the stringent washing solutions. The oligonucleotide probes were labeled with the fluorochromes Cy3 or Oregon Green (Molecular Probes, Inc.), and were custom-made by Integrated DNA Technologies, Inc.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequences</th>
<th>Target Organisms</th>
<th>Target site (E. coli Positions)</th>
<th>%FA</th>
<th>NaCl (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>5'-GCT GCC TCC CGT AGG AGT -3'</td>
<td>Eubacteria a</td>
<td>338-355</td>
<td>20</td>
<td>225*</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>NON338</td>
<td>5'-ACT CCT ACG GGA GCC AGC -3'</td>
<td>Negative control</td>
<td>N/A</td>
<td>20</td>
<td>225*</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>NSO190</td>
<td>5'-CAG TCC CCT GCT TTT TCT C -3'</td>
<td>β-Proteobacterial AOB b</td>
<td>190-208</td>
<td>55</td>
<td>20</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>NSO1225</td>
<td>5'-GCC GAT TGT ATT ACG TGT GA -3'</td>
<td>β-Proteobacterial AOB c</td>
<td>1225-1244</td>
<td>35</td>
<td>80+</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsm156</td>
<td>5'-TAT TAG CGC ATC TTT CGA T -3'</td>
<td>Nitrosomonas spp. d</td>
<td>156-174</td>
<td>5</td>
<td>56*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsv443</td>
<td>5'-CCG TGA CCG TTT CGT TCC G -3'</td>
<td>Nitrosospira spp.</td>
<td>444-462</td>
<td>30</td>
<td>112*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsoc128</td>
<td>5'-CCC CTC TAG AGG CCA GAT -3'</td>
<td>γ-Proteobacterial AOB</td>
<td>128-146</td>
<td>35</td>
<td>80</td>
<td>(Juretschko, 2000; Loy et al., 2003)</td>
</tr>
<tr>
<td>My705</td>
<td>5'-CTG GTG TTC CTT CAG ATC -3'</td>
<td>Type I Methanotrophs</td>
<td>705-724</td>
<td>20</td>
<td>225</td>
<td>(Eller et al., 2001)</td>
</tr>
<tr>
<td>Maα450</td>
<td>5'-ATC CAG GTA CCG TCA TTA TC -3'</td>
<td>Type II Methanotrophs e</td>
<td>450-470</td>
<td>20</td>
<td>225</td>
<td>(Eller et al., 2001)</td>
</tr>
</tbody>
</table>

* Information on the NaCl concentrations in these washing solutions came from (Schramm et al., 1998).

a NaCl concentration for washing solution is adopted from (Gieseke et al., 2001).

b This excludes *Plantomycetales* and *Verrucomicrobia* (Daims et al., 1999).

c NSO190 has 3 mismatches with *Nitrosomonas ureae*, and 1 mismatch in a few *Nitrosonomas* spp. (Utäker and Nes, 1998; Purkhold et al., 2000).

d NSO1225 has 1 mismatch with *Nitrosococcus mobilis* (ARB Difference Alignment function, probeBase (Loy et al., 2003), and may hybridize with *Gallionella ferruginea* (Results from Probe Match (Cole et al., 2003)).

e Three possible non-AOB targets are *Thauera linaloolentis* str. 47, *Pseudomonas butanovora* IAM 12574 and *Aquaspirillum sinuosum* LMG 4393 (Results from Probe Match (Cole et al., 2003)).

f There is one non-TypeII methanotroph target - str. TM19 (X97077) in the *Belindica* subgroup (Results from Probe Match (Cole et al., 2003)).
Table 5.2. Concentrations of ammonium measured in warm diffuse fluids and hot focused vent fluids sampled by titanium major samplers operated by the submersible DSV2 ALVIN. The numbers are expressed as mean ± standard deviation of the replicate subsamples. Gaseous concentrations of methane, hydrogen and total inorganic carbon (TCO$_2$) were sampled by gas-tight samplers on board the DSV2 ALVIN.

<table>
<thead>
<tr>
<th>Dive No.</th>
<th>Temp. (°C)</th>
<th>Ammonium (µM)</th>
<th>Methane (mmol kg$^{-1}$)</th>
<th>Hydrogen (mmol kg$^{-1}$)</th>
<th>TCO$_2$ (mmol kg$^{-1}$)</th>
<th>Total Gas Content (mmol kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Warm Diffuse Fluids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3777</td>
<td>38-40</td>
<td>2,850 ± 233</td>
<td>4.66</td>
<td>0.00019</td>
<td>6.85</td>
<td>13.03</td>
</tr>
<tr>
<td>3779</td>
<td>40</td>
<td>1.0 ± 0.05</td>
<td>7.01</td>
<td>0.00057</td>
<td>7.06</td>
<td>15.11</td>
</tr>
<tr>
<td>45-50</td>
<td>1.49 ± 0.03</td>
<td>8.64</td>
<td>0.00099</td>
<td>10.36</td>
<td>20.40</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>3.22 ± 0.44</td>
<td>10.5</td>
<td>0.00116</td>
<td>11.27</td>
<td>23.94</td>
<td></td>
</tr>
<tr>
<td>3780</td>
<td>26-27</td>
<td>0.74 ± 0.01</td>
<td>6.26</td>
<td>0.00105</td>
<td>7.82</td>
<td>15.49</td>
</tr>
<tr>
<td>58</td>
<td>3.22 ± 0.44</td>
<td>3.28</td>
<td>0.00032</td>
<td>5.22</td>
<td>9.20</td>
<td></td>
</tr>
<tr>
<td><strong>Hot Vent Fluids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3777</td>
<td>200</td>
<td>21,246</td>
<td>24.08</td>
<td>1.44190</td>
<td>22.25</td>
<td>49.72</td>
</tr>
<tr>
<td>3780</td>
<td>220</td>
<td>4,350</td>
<td>9.13</td>
<td>0.00038</td>
<td>13.13</td>
<td>25.95</td>
</tr>
</tbody>
</table>

232
Table 5.3. Specific rate constants ($k_{\text{tot}}$, $k_{\text{oxid}}$ and $k_{\text{15N}}$) and turnover times ($T_{\text{tot}}$, $T_{\text{oxid}}$ and $T_{\text{15N}}$) with respect to total net ammonium removal rates, potential ammonia oxidation rates and ammonia oxidation rates measured via the $^{15}$N-tracer method respectively.

Units for the specific rate constants are per day (or d$^{-1}$), and those for turnover time are days (or d).

<table>
<thead>
<tr>
<th>Cast or Dive</th>
<th>Depth</th>
<th>NH$_4^+$ Concentration (mean ± SD)</th>
<th>Total Net Removal</th>
<th>Potential Ammonia Oxidation</th>
<th>$^{15}$NH$_3$-Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(m)</td>
<td>(nM)</td>
<td>$k_{\text{tot}}$</td>
<td>$T_{\text{tot}}$</td>
</tr>
<tr>
<td><strong>South Site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dive 3779</td>
<td>2006</td>
<td>1486 ± 31</td>
<td>-0.01</td>
<td>-76.80</td>
<td></td>
</tr>
<tr>
<td>Dive 3780</td>
<td>2006</td>
<td>157 ± 4</td>
<td>-0.35</td>
<td>-2.85</td>
<td></td>
</tr>
<tr>
<td>Dive 3777</td>
<td>2004</td>
<td>2945 ± 167</td>
<td>0.09</td>
<td>10.61</td>
<td></td>
</tr>
<tr>
<td>Dive 3777</td>
<td>2003</td>
<td>190 ± 22</td>
<td>-0.16</td>
<td>-6.25</td>
<td>1.78</td>
</tr>
<tr>
<td>V3</td>
<td>2003</td>
<td>54 ± 9</td>
<td>-0.45</td>
<td>-2.22</td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td>2002</td>
<td>473 ± 57</td>
<td>-0.46</td>
<td>-2.17</td>
<td>-0.10</td>
</tr>
<tr>
<td>Dive 3783</td>
<td>2000</td>
<td>42,700</td>
<td>-0.44</td>
<td>-2.28</td>
<td></td>
</tr>
<tr>
<td>Dive 3783</td>
<td>2000</td>
<td>78,788 ± 5,104</td>
<td>0.58</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>V6</td>
<td>1996</td>
<td>175 ± 15</td>
<td>0.88</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>1934</td>
<td>23 ± 5</td>
<td>-0.58</td>
<td>-1.72</td>
<td>0.96</td>
</tr>
<tr>
<td>Dive 3780</td>
<td>1868</td>
<td>30 ± 7</td>
<td>0.22</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td>V6</td>
<td>1865</td>
<td>132 ± 1</td>
<td>0.92</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>1854</td>
<td>588 ± 37</td>
<td>0.92</td>
<td>3.9</td>
<td>0.26c</td>
</tr>
<tr>
<td>V1</td>
<td>1812</td>
<td>25 ± 5</td>
<td>-11.91</td>
<td>-0.08</td>
<td>-1.31</td>
</tr>
<tr>
<td>Dive 3781</td>
<td>1525</td>
<td>32 ± 2</td>
<td>0.18</td>
<td>5.63</td>
<td></td>
</tr>
<tr>
<td>Dive 3781</td>
<td>1522</td>
<td>30 ± 4</td>
<td>-0.35</td>
<td>-2.82</td>
<td></td>
</tr>
<tr>
<td>Dive 3779</td>
<td>1506</td>
<td>151 ± 4</td>
<td>-0.06</td>
<td>-16.36</td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td>1496</td>
<td>160 ± 40</td>
<td>0.46</td>
<td>2.17</td>
<td>0.95</td>
</tr>
<tr>
<td>V1</td>
<td>1486</td>
<td>31 ± 4</td>
<td>-1.03</td>
<td>-0.97</td>
<td>1.07</td>
</tr>
<tr>
<td>V1</td>
<td>1414</td>
<td>38 ± 4</td>
<td>-0.75</td>
<td>-1.33</td>
<td>0.84</td>
</tr>
<tr>
<td>V2</td>
<td>1400</td>
<td>33 ± 9</td>
<td>-0.99</td>
<td>-1.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**North Site**

<table>
<thead>
<tr>
<th>潜水或潜水</th>
<th>深度</th>
<th>NH$_4^+$浓度（平均±标准差）</th>
<th>总净氨氮解吸</th>
<th>潜在氨氮氧化</th>
<th>$^{15}$NH$_3$氧化</th>
</tr>
</thead>
<tbody>
<tr>
<td>T5</td>
<td>1885</td>
<td>1763 ± 12</td>
<td>0.03</td>
<td>33.3</td>
<td>0.01</td>
</tr>
<tr>
<td>V7</td>
<td>1875</td>
<td>1196 ± 31</td>
<td>-0.06</td>
<td>-16.7</td>
<td>0.10</td>
</tr>
<tr>
<td>T5</td>
<td>1865</td>
<td>140 ± 3</td>
<td>0.16</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>1760</td>
<td>9 ± 1</td>
<td>-3.16</td>
<td>-0.32</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1694</td>
<td>218 ± 8</td>
<td>-0.01</td>
<td>-100</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Addition of $^{15}$N-label resulted in a 302% increase in initial NH$_4^+$ concentration.

$^b$ Addition of $^{15}$N-label resulted in a 14% increase in initial NH$_4^+$ concentration.

$^c$ Addition of $^{15}$N-label resulted in a 46% increase in initial NH$_4^+$ concentration.

$^d$ Addition of $^{15}$N-label resulted in a 37% increase in initial NH$_4^+$ concentration.
Table 5.4. Comparison of total net ammonium removal rates with and without the removal of larger particles greater than 3 μm- and 10 μm- diameter in size. Rates are shown as mean ± standard deviations, and ‘n.m.’ denotes ‘not measured’.

<table>
<thead>
<tr>
<th>Cast</th>
<th>Depth m</th>
<th>Δc</th>
<th>Non-filtered nM d⁻¹</th>
<th>3 μm-filtered nM d⁻¹</th>
<th>10 μm-filtered nM d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>V6</td>
<td>1996</td>
<td>0.13</td>
<td>155 ± 15</td>
<td>135 ± 13</td>
<td>148 ± 6</td>
</tr>
<tr>
<td>V6</td>
<td>1865</td>
<td>0.00</td>
<td>121 ± 6</td>
<td>123 ± 3</td>
<td>122 ± 0</td>
</tr>
<tr>
<td>V7</td>
<td>1875</td>
<td>0.22</td>
<td>-77 ± 36</td>
<td>n.m.</td>
<td>-64 ± 15</td>
</tr>
<tr>
<td>T4</td>
<td>1694</td>
<td>0.17</td>
<td>-2 ± 9</td>
<td>17 ± 8</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>T5</td>
<td>1885</td>
<td>0.15</td>
<td>58 ± 8</td>
<td>72 ± 6</td>
<td>75 ± 12</td>
</tr>
</tbody>
</table>

234
Table 5.5. Concentrations and stable isotopic values of particulate organic carbon (POC) and particulate nitrogen (PN), particulate C/N ratios, as well as assimilation rates along with the corresponding total net ammonium removal rates, ammonia oxidation rates and assimilation rates (mean ± standard deviations). Blanks indicate parameters not measured.

<table>
<thead>
<tr>
<th>Cast</th>
<th>Depth</th>
<th>POC</th>
<th>δ^{13}C</th>
<th>PN</th>
<th>δ^{15}N</th>
<th>C/N</th>
<th>Total Net Removal Rates</th>
<th>Potential Ammonia Oxidation Rates</th>
<th>^{15}NH_{3} Oxidation Rates</th>
<th>Assimilation Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>m g l^{-1}</td>
<td>%o</td>
<td>μg l^{-1}</td>
<td>%o</td>
<td></td>
<td>nM d^{-1}</td>
<td>nM d^{-1}</td>
<td>nM d^{-1}</td>
<td>nM d^{-1}</td>
</tr>
<tr>
<td><strong>South Site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>2003</td>
<td>2003</td>
<td>32.01</td>
<td>-26.66</td>
<td>4.00</td>
<td>9.3</td>
<td>-24 ± 1</td>
<td>51 ± 2.3</td>
<td></td>
<td>15.4</td>
</tr>
<tr>
<td>V2</td>
<td>2002</td>
<td>32.00</td>
<td>13.80</td>
<td>-28.40</td>
<td>1.85</td>
<td>15.71</td>
<td>8.7</td>
<td>-219 ± 46</td>
<td>-59 ± 60</td>
<td></td>
</tr>
<tr>
<td>V6</td>
<td>1996</td>
<td>18.51</td>
<td>18.91</td>
<td>-26.63</td>
<td>2.51</td>
<td>7.4</td>
<td>155 ± 15</td>
<td>43 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>1934</td>
<td>18.91</td>
<td>18.91</td>
<td>-27.80</td>
<td>2.10</td>
<td>17.58</td>
<td>10.5</td>
<td>-14 ± 22</td>
<td>118 ± 23</td>
<td></td>
</tr>
<tr>
<td>V6</td>
<td>1865</td>
<td>12.46</td>
<td>12.46</td>
<td>-26.30</td>
<td>1.81</td>
<td>6.9</td>
<td>121 ± 6</td>
<td>87 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>1854</td>
<td>8.01</td>
<td>8.01</td>
<td>-27.72</td>
<td>1.10</td>
<td>8.5</td>
<td>6 ± 0.3</td>
<td>9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>1812</td>
<td>13.27</td>
<td>13.27</td>
<td>-30.92</td>
<td>1.22</td>
<td>10.46</td>
<td>12.7</td>
<td>-300 ± 1</td>
<td>-164 ± 5</td>
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</tr>
<tr>
<td>V5</td>
<td>1496</td>
<td>9.13</td>
<td>9.13</td>
<td>-26.96</td>
<td>1.23</td>
<td>8.7</td>
<td>74 ± 40</td>
<td>144 ± 16</td>
<td>44.3</td>
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</tr>
<tr>
<td>V1</td>
<td>1486</td>
<td>16.53</td>
<td>16.53</td>
<td>-30.86</td>
<td>1.31</td>
<td>12.02</td>
<td>14.7</td>
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<tr>
<td>V1</td>
<td>1414</td>
<td>9.86</td>
<td>9.86</td>
<td>-29.28</td>
<td>0.71</td>
<td>9.72</td>
<td>16.1</td>
<td>-29 ± 11</td>
<td>116 ± 31</td>
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</tr>
<tr>
<td>V2</td>
<td>1400</td>
<td>6.74</td>
<td>6.74</td>
<td>-28.13</td>
<td>0.74</td>
<td>7.16</td>
<td>10.6</td>
<td>-33 ± 7</td>
<td>1 ± 17</td>
<td></td>
</tr>
<tr>
<td><strong>North Site</strong></td>
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<tr>
<td>T4</td>
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<td>23.52</td>
<td>-30.46</td>
<td>2.36</td>
<td>11.6</td>
<td>-2 ± 9</td>
<td></td>
<td>13 ± 0.5</td>
<td>13.2</td>
</tr>
<tr>
<td>T5</td>
<td>1865</td>
<td>16.99</td>
<td>16.99</td>
<td>-29.37</td>
<td>2.35</td>
<td>8.4</td>
<td>23 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

235
Table 5.6. Distribution of total ammonia-oxidizing bacteria (AOB) as the β-proteobacterial subgroups of *Nitrosospira* spp. and *Nitrosomonas* spp., and the γ-proteobacterial subgroup of *Nitrosococcus* spp., in the free-living (0.2-3.0 μm-diameter) and particle-associated (3.0-10 μm and ≥ 10 μm) communities at the South Site and the North Site. The numbers in individual size-fractions are presented as percentages of the total AOB (sum of *Nitrosospira* spp., *Nitrosomonas* spp. and *Nitrosococcus* spp.) in all size-fractions. ‘FL’ denotes ‘free-living’, and ‘PA’ denotes ‘particle-associated’. Numbers in bold indicate the percentages of the predominant subgroups of the particular size-fractions in the samples.

<table>
<thead>
<tr>
<th>AOB Subgroups</th>
<th>South Site</th>
<th></th>
<th>North Site</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plume</td>
<td>Above sill</td>
<td>Plume</td>
<td>Above plume</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>PA</td>
<td>FL</td>
<td>PA</td>
</tr>
<tr>
<td></td>
<td>0.2-3.0 μm</td>
<td>3.0-10 μm ≥10 μm</td>
<td>0.2-3.0 μm</td>
<td>3.0-10 μm ≥10 μm</td>
</tr>
<tr>
<td><em>Nitrosospira</em> spp.</td>
<td>17.1</td>
<td>9.4</td>
<td>0</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>16.3</td>
<td>3.4</td>
<td>2.0</td>
<td>42.1</td>
</tr>
<tr>
<td><em>Nitrosococcus</em> spp.</td>
<td>34.4</td>
<td>8.7</td>
<td>2.6</td>
<td>9.4</td>
</tr>
<tr>
<td><em>Total AOB</em></td>
<td>68</td>
<td>32</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

236
Figure 5.1. (a) A bathymetry map of the Gulf of California (Lavín and Marinone, 2003), indicating the locations of the Guaymas Basin and other adjacent basins within the Gulf. The Gulf can be divided into the upper gulf (UGC), the northern gulf (NGC), the Archipelago region, the southern gulf (SGC) and the Entrance Zone leading to the Pacific Ocean. (b) A bathymetry map of the hydrothermally active Southern Trough in the Guaymas Basin (Koski et al., 1985), showing the locations of the two major sampling sites: South Site (casts V1-V6) and North Site (cast V7, the last upward tow in T3, T4 and T5) and the two tow-yo tracks (T2 and T3) in red. The red arrows denote the directions of the tows, and the shaded areas indicate subsurface sill systems reported in Lonsdale and Becker (1985).
Figure 5.2. Composite vertical profiles of (a) potential temperature or $\theta$, (b) salinity, (c) potential density or $\sigma_n$, (d) apparent potential temperature anomalies or $\Delta \theta^*$, (e) particle anomalies or $\Delta c$, (f) dissolved oxygen content and (g) oxygen deficit or $\Delta O_2$, at the South Site, measured during sampling casts V1 to V6. Also shown are the reference profiles of cast T2dn sampled along the northwest rift wall, relatively remote from known hydrothermal activities. The dashed line in (a) indicates the projected $\theta$ without hydrothermal influence, and the dot-dashed line indicates the projected $\theta$ without the sill effect (see text).
Figure 5.3. Composite vertical profiles of (a) potential temperature or $\theta$, (b) salinity, (c) potential density or $\sigma_\theta$, (d) apparent potential temperature anomalies or $\Delta \theta^*$, (e) particle anomalies or $\Delta c$, (f) dissolved oxygen content and (g) oxygen deficit or $\Delta O_2$, at the North Site, measured during sampling casts V7, T3, T4 and T5. Also shown are the reference profiles of cast T2dn sampled along the northwest rift wall, relatively remote from known hydrothermal activities. The dashed line in (a) indicates the projected $\theta$ without hydrothermal influence, and the dot-dashed line indicates the projected $\theta$ without the sill effect (see text).
Figure 5.4. Sample plots of potential temperature (θ) versus salinity at (a) the South Site (sampling cast V5), and (b) the North Site (sampling cast T5), illustrating the distinct discontinuity in θ-S signatures observed at ~1750 m in all samples within the Guaymas Basin. Buoyant plume signals are apparent at 1900-1950 m in (a) and at 1850-1900 m in (b), while the scatter is likely the results of turbulent mixing between recently injected hot buoyant hydrothermal discharges and the cold ambient deep water.
Figure 5.5. (a) Vertical profiles of ammonium concentrations, in logarithmic scale, at the South Site, including sampling casts V1 to V6 and DSV2 ALVIN dives 3777 and 3779-3781. (b) Vertical profiles of total net ammonium removal rates (circles), potential autotrophic ammonia oxidation rates measured by ammonium loss with and without dicyandiamide (inverted triangles), as well as the ammonia oxidation rates measured in the $^{15}$N-labelled experiments (squares) at the South Site. The color scheme indicating samples from various sampling casts or dives is the same as in (a). (c) Vertical profiles of ammonium concentrations, in logarithmic scale, at the North Site during sampling casts V7, T3, T4 and T5. (b) Vertical profiles of total net ammonium removal rates (circles), potential autotrophic ammonia oxidation rates (inverted triangles) and the ammonia oxidation rates measured in the $^{15}$N-labelled experiments (squares) at the North Site. The color scheme indicating various sampling casts or dives is the same as in (c). Symbols represent mean values and error bars are standard deviations. Horizontal dashed lines represent proposed range of generalized hydrothermal plume depths, while the light brown solid line at 1560 m represents the depth of the sill at the entrance from the Carmen Basin.
Figure 5.6. Vertical profiles of (a) methane concentrations and (b) carbon stable isotopic composition of methane at the South Site. Vertical profiles of (c) methane concentrations and (d) carbon stable isotopic values at the North Site. Different colored symbols represent data collected during different sampling casts or dives. The same color schemes in (a) and (c) also apply to the symbols in profiles (b) and (d) respectively. Horizontal dashed lines represent proposed range of generalized hydrothermal plume depths, while the light brown solid line at 1560 m represents the depth of the sill at the entrance from the Carmen Basin.
Figure 5.7. Vertical distribution of microbial abundance at the South Site: (a) total microbial abundance as DAPI-cell counts and Eubacteria hybridized with the oligonucleotide probe EUB338, (b) β-proteobacterial ammonia-oxidizing bacteria (βAOB) hybridized with the oligonucleotide probes NSO190 and NSO1225, along with γ-proteobacterial ammonia-oxidizing bacteria (γAOB) hybridized with Nscoc128, (c) total abundance of ammonia-oxidizing bacteria as the sum of βAOB and γAOB, (d) Nitrosospira spp. (Nsv443-hybridized cells) and Nitrosomonas spp. (Nsm156-hybridized cells), and (e) Type-I and Type-II methanotrophs hybridized with My705 and Ma450 oligonucleotide probes respectively. Symbols represent mean values and error bars are standard errors of forty replicate counts of duplicate subsamples.
Figure 5.8. Vertical distribution of microbial abundance at the North Site: (a) total microbial abundance as DAPI-cell counts and Eubacteria hybridized with the oligonucleotide probe EUB338, (b) $\beta$-proteobacterial ammonia-oxidizing bacteria ($\beta$AOB) hybridized with the oligonucleotide probes NSO190 and NSO1225, along with $\gamma$-proteobacterial ammonia-oxidizing bacteria ($\gamma$AOB) hybridized with Nscoc128, (c) total abundance of ammonia-oxidizing bacteria as the sum of $\beta$AOB and $\gamma$AOB, (d) *Nitrosospira* spp. (Nsv443-hybridized cells) and *Nitrosomonas* spp. (Nsm156-hybridized cells), and (e) Type-I and Type-II methanotrophs hybridized with My705 and Ma450 oligonucleotide probes respectively. Symbols represent mean values and error bars are standard errors of forty replicate counts of duplicate subsamples.
Figure 5.9. Distribution of microbial abundance in free-living fraction versus particle-associated size-fractions in two selected samples from the South Site and two from the North Site: (a) DAPI-stained cells, (b) Eubacteria hybridized with oligonucleotide probe EUB338, (c) βAOB hybridized with NSO190, (d) βAOB hybridized with NSO1225, (e) Nitrosospira-like cells hybridized with Nsv443, (f) Nitrosomonas-like cells hybridized with Nsm156, (g) γAOB hybridized with Nsoc128, (h) Type I methanotrophs hybridized with My705 and (i) Type II methanotrophs hybridized with Mo450. Black solid bars indicate abundances in the free-living size-fractions (0.2-3.0 μm), while the red bars represent the small particle-associated fractions (3.0-10 μm) and the green bars represent the larger particle-associated fractions (10+ μm). The corresponding abundance estimates for non-size-fractionated subsamples are shown as yellow triangles for comparison. Please note the different scales in the various plots.
Figure 5.10. Total net ammonium removal rates (dark circles), potential ammonia oxidation rates (white circles), ammonia oxidation rates measured by the $^{15}$N-tracer technique or $^{15}$NH$_3$ oxidation rates (red inverted triangles), together with the assimilation rates (yellow diamonds) are plotted as a function of ammonium concentrations in logarithmic scale. The highest $^{15}$NH$_3$ oxidation rates and elevated total net ammonium removal rates were concentrated at the 100-200 nM range of ammonium. If the ammonium (~100 nM) added with the dicyandiamide treatment was taken into account, the data points of potential ammonia oxidation rates should be shifted +100 nM along the x-axis, resulting in a clustering of high rates between 100-300 nM of ammonium which is consistent with the other two rate measurements. Assimilation rates might be slightly elevated within the same range, but more data are needed to verify this.
Figure 5.11. The total abundance of ammonia-oxidizing bacteria (AOB), as the sum of the β- and γ-Proteobacteria subgroups, in relation to (a) NH$_4^+$ concentrations and (b) ammonia oxidation rates measured by the $^{15}$N-tracer technique (15NH$_3$ oxidation rates). The error bars for AOB abundance represent standard errors from 40 replicate cell counts of duplicate subsamples, while the error bars for NH$_4^+$ and $^{15}$NH$_3$ oxidation rates are standard deviations of duplicate and triplicate measurements respectively.
CHAPTER SIX

Molecular Analyses of Autotrophic Ammonia-Oxidizing Bacteria
in Deep-Sea Hydrothermal Environments
6.0 ABSTRACT

Autotrophic ammonia-oxidizing bacteria (AOB), in β- and γ-proteobacterial subgroups, were detected in the deep-sea hydrothermal plumes and background seawater at the Endeavour Segment, Juan de Fuca Ridge, and the Guaymas Basin, Gulf of California. They were also detected in the diffuse hydrothermal fluids and hydrothermal sediments of the latter location. The maximum total abundance of AOB (16 ± 1.8 × 10³ cells ml⁻¹) within the Endeavour hydrothermal plume was about ten-fold the abundance in the overlying background deep water (1.6 ± 0.6 × 10³ cells ml⁻¹), estimated by fluorescence in situ hybridization (FISH) with 16S rRNA-targeted probes. In comparison, the more ammonium-rich Guaymas Basin hydrothermal plume harbored only a moderate AOB population (8.0 ± 0.9 × 10³ cells ml⁻¹), while that in the overlying background deep water was not much smaller (5.9 ± 0.7 × 10³ cells ml⁻¹). The β-proteobacterial AOB usually dominated over γ-proteobacterial AOB. Among the β-proteobacterial AOB, Nitrosospira-like cells were more abundant than Nitrosomonas-like cells in most cases. However, amplification of the 16S rRNA gene, as well as the gene coding for ammonia monooxygenase subunit A (amoA), detected sequences resembling recognized AOB only in the Guaymas Basin hydrothermal sediments, but not in the water column or hydrothermal fluids at either sites. The β-proteobacterial AOB-like sequences (both 16S rRNA and amoA) recovered from the Guaymas Basin sediments belong to Nitrosospira cluster 3, which have no previous marine representatives. The γ-proteobacterial AOB-like sequences (amoA only) recovered, also from Guaymas Basin sediments, are closely related to Nitrosococcus oceani. The sequences retrieved from the hydrothermal plumes and background seawater at the Endeavour Segment and Guaymas Basin potentially represented a novel lineage of β-proteobacterial AOB, as supported by sequence analyses of both the 16S rRNA and amoA genes that shared 87-93% and 76-77% similarity with known β-proteobacterial AOB.
6.1. INTRODUCTION

Hydrothermal fluids discharged from the mid-ocean ridge spreading centers in the sedimented Guaymas Basin, Gulf of California, and along the unsedimented Endeavour Segment, Juan de Fuca Ridge, contain millimolar levels of NH$_4^+$ (Von Damm et al., 1985; Lilley et al., 1993). Significantly elevated concentrations of NH$_4^+$ remain even after $10^3$-$10^4$ dilution with ambient seawater (Campbell and Gieskes, 1984; Lupton et al., 1985), which fuel ammonia oxidation at the highest rates ever reported in the deep-sea water column (Lam et al., 2004; Chapters 3-5). Ammonia oxidation, the conversion of NH$_4^+$ to nitrite, is the first-step in nitrification. It is important in the global cycling of nitrogen because this process connects the recycling of organic nitrogen to the major ultimate loss of fixed nitrogen via denitrification. The occurrence of nitrification in the deep sea also adds to the deep-sea nitrate reservoir, which will in turn recharge the often nitrogen-limited surface ocean for primary production, during upwelling or upward eddy diffusion.

Ammonia oxidation is mainly performed by chemolithoautotrophic bacteria that are capable of utilizing the energy evolved from this oxidation reaction to synthesize organic carbon from carbon dioxide (Frankland and Frankland, 1890; Winogradsky, 1890; Kowalchuk and Stephen, 2001). These ammonia-oxidizing bacteria (AOB) were originally grouped together with nitrite-oxidizing bacteria in the family *Nitrosobacteriaceae* based on their physiology and cell morphology (Watson et al., 1989); but sequence analyses of the 16S ribosomal RNA (16S rRNA) genes in the last decade placed these AOB into two monophyletic groups in *Proteobacteria*, one in the β-
subgroup and one in the \( \gamma \)-subgroup (Head et al., 1993; Teske et al., 1994). The majority of AOB species identified thus far belong to the \( \beta \)-subgroup, which can be further divided into two lineages, *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*); while only three species, namely *Nitrosococcus oceani*, *Nitrosococcus halophilus* and *Nitrosococcus* sp. C-113, are known to be in the \( \gamma \)-subgroup (Head et al., 1993; Teske et al., 1994). More recent analyses, incorporating many new 16S rRNA gene sequences from environmental libraries has led to the resolution of at least seven to nine clusters among the \( \beta \)-proteobacterial AOB – 4 to 5 within *Nitrosospira* and 3 to 4 within *Nitrosomonas* (Stephen et al., 1996; Purkhold et al., 2000; Purkhold et al., 2003). Comparative sequence analyses of the gene coding for the subunit A of ammonia monooxygenase (*amoA*), one of the key enzymes in ammonia oxidation, result in phylogeny that is largely congruent with the recognized species of AOB (Rotthauwe et al., 1997; Purkhold et al., 2000; Aakra et al., 2001). Therefore, this functional gene may be used as a surrogate phylogenetic marker for AOB.

Investigation of AOB diversity and community structure frequently employ gene amplification by polymerase chain reaction (PCR) with AOB specific-primers based on either 16S rRNA or *amoA* genes, followed by cloning and sequencing for phylogenetic analyses (McCaig et al., 1994; Kowalchuk et al., 1997; Sakano and Kerkhof, 1998; Phillips et al., 1999; Ward et al., 2000; de Bie et al., 2001; Hollibaugh et al., 2002; Ward and O'Mullan, 2002). In the year 2003 alone, 1253 new sequences of AOB have been deposited into the GenBank, out of which 487 sequences are coding for the 16S rRNA
gene. Alternatively, the amplified genes can be analyzed by fingerprinting techniques such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) (Muyzer and Smalla, 1998), or terminal restriction fragment length polymorphism (T-RFLP) (Horz et al., 2000) to differentiate community structures in various environmental settings (Stephen et al., 1998; Nicolaisen and Ramsing, 2002; Freitag and Prosser, 2003; Kreuzinger et al., 2003). Another frequently applied molecular tool is fluorescence in situ hybridization (FISH) with AOB specific, 16S rRNA-targeted oligonucleotide probes, which can detect and quantify active populations of AOB at the subclass level down to species level (Mobarry et al., 1996; Schramm et al., 1998; Gieseke et al., 2001). FISH was successfully applied to detect and enumerate AOB for the first time in the deep-sea water column, and populations of β-Proteobacterial AOB in the Endeavour hydrothermal plume were found to be elevated up to 20-fold relative to ambient deep water (Lam et al., 2004; Chapter 3).

Nevertheless, the application of FISH in frequently unexplored environments could lead to underestimates, because the design of the oligonucleotide probes used in FISH relies on known AOB 16S rRNA gene sequences. Any unknown species or strains of AOB that carry mismatches within the oligonucleotide probe sequence, usually about 20 bases in length, would not be detected. The AOB in deep-sea hydrothermal plumes can be introduced via hydrothermal discharges from the subseafloor biosphere, which are rather isolated habitats and might harbor unique communities of AOB. On the contrary, these AOB in the deep-sea hydrothermal plumes may also be phylogenetically similar to their counterparts in the rest of the oceans, since AOB are likely ubiquitous in the deep sea (Chapters 3-5). More sequence information and analyses are thus necessary to
examine the diversity of AOB in the oceans, in order to enable more accurate estimation of AOB abundance.

The current study aims to test the hypothesis that AOB are ubiquitous in the deep sea, through the use of the highly sensitive molecular biological techniques. The diversity and community structure of autotrophic ammonia-oxidizing bacteria in the unsedimented hydrothermal system of the Endeavour Segment is compared with those from the more organic-rich, sedimented hydrothermal system in the Guaymas Basin. AOB were detected and enumerated using FISH, while partial sequences of the 16S rRNA and amoA genes were PCR amplified using, in some cases, primers with degeneracies in attempt to embrace more possible AOB targets. These amplified gene fragments were subsequently sequenced and analyzed for their phylogenies.

6.2. MATERIALS AND METHODS

6.2.1. Site Descriptions

The Endeavour Segment of Juan de Fuca Ridge is located approximately 300 km off the coast of British Columbia in the northeast Pacific. Hydrothermal venting is concentrated within a 1 km wide, 10 km long and 100-150 m deep axial rift valley at a depth of 2170-2300 m. There are five known active vent fields spaced about 2-3 km apart (Kelley et al., 2001), hosting high-temperature (317-400°C) focused venting regions and extensive areas of diffuse flow (Delaney et al., 1984; Robigou et al., 1993; Butterfield, 1995; Lilley et al., 1995; Veirs et al., 1999; Kelley et al., 2001b). Hydrothermal plumes rise to neutral buoyancy at 50-350 m above the seafloor and can be traced to more than 10 km away by distinct temperature and particle anomalies (Thomson
et al., 1992), elevated concentrations of volatiles (H₂, CH₄ and NH₄⁺) (De Angelis et al., 1993; Cowen et al., 1998; McLaughlin, 1998), as well as various dissolved and particulate trace metals and organic matter (Dymond and Roth, 1988; Roth and Dymond, 1989; Cowen et al., 2001). Elevated NH₄⁺ concentrations (up to 341 ± 136 nM) and autotrophic ammonia oxidation rates (up to 91 nM d⁻¹) (Lam et al., 2004; Chapters 3) were focused within the axial valley and within the early stages of the predominantly southwest-bound advecting neutrally buoyant plume, where autotrophic ammonia oxidation accounted for at least 91% of total net NH₄⁺ removal (Chapter 4).

The Guaymas Basin is a 2100 m deep semi-enclosed basin in the central part of the Gulf of California. The water column below 1000 m is filled with the Pacific Deep Water that flows in via a 1560 m deep sill from the adjacent Carmen Basin. The basin floor is lined with a 300-500-m thick sediment cover (Curray et al., 1982), which is accumulating at a rate of 1-4 m per thousand years (van Andel, 1964), and contains 1-4 wt % of organic matter of predominantly marine autochthonous origin (Simoneit et al., 1979; Simoneit and Bode, 1982). Hydrothermal activities within the basin are concentrated along the spreading axes marked by two parallel northeast-southwest trending troughs (Curray et al., 1982). The current study is focused on the more hydrothermally active southern trough (Lonsdale and Becker, 1985; Fisher and Becker, 1991). The southern trough is about 30 km long, 2-4 km wide, and 50-150 m deeper than the surrounding seafloor. The seafloor is interrupted by a series of 0.1-1 km² intra-rift hills rising ≤100 m above the surroundings, and there are subsurface sill complexes and buried mounds (Lonsdale and Becker, 1985). Hydrothermal discharges were usually detected within 500 m of the rift valley axis, either over sill complexes or mainly on their
peripheries since the sills act as ‘sealing caps’ to the deeper subsurface hydrothermal circulation (Lonsdale and Becker, 1985). The organic compounds in the sediment undergo diagenesis and petroleum genesis due to hydrothermal circulation, resulting in organic-rich pore water and hydrothermal fluid discharges that bear a wide variety of lipids and hydrocarbons (volatiles and oil droplets of hydrocarbons C₁ to C₄₀+) (Simoneit, 1991). Fluid discharges are also imprinted with distinct anomalies such as those found in dissolved silica, ³He, beryllium, barium, particulate manganese, methane and ammonium (Von Damm et al., 1985), which are still detectable in the bottom water of the basin (Campbell and Gieskes, 1984; Chapter 5). Hydrothermal plumes rise to 10-150 m above bottom but are trapped below the sill inside the basin (Chapter 5). Elevated NH₄⁺ concentrations (maximum 2.9 ± 0.2 μM) and ammonia oxidation rates (maximum 517 nM d⁻¹) were found within the buoyant or neutrally buoyant hydrothermal plumes, with very large fluctuations (Chapter 5) due to strong horizontal currents at 12 cm s⁻¹ (Lonsdale and Becker, 1985). NH₄⁺ concentrations (up to 160 ± 40 nM) and ammonia oxidation rates (142 ± 16 nM d⁻¹) were also quite high in the above-sill deep water, likely as a consequence of the productive surface water (Chapter 5).

6.2.2. Fluorescence In Situ Hybridization for Water Column Samples

Water-column sampling at both the Endeavour Segment and the Guaymas Basin was conducted using a CTD-transmission-Niskin bottle rosette package deployed from the R/V Atlantis. Subsamples for fluorescence in situ hybridization (FISH) were collected in clean 1-liter polyethylene bottles. β-Proteobacterial ammonia-oxidizing bacteria (βAOB), Nitrosomonas spp. and Nitrosospira spp. (two major groups of β-
AOB), as well as γ-proteobacterial ammonia-oxidizing bacteria (γAOB) were enumerated using FISH with 16S rRNA-targeted oligonucleotide probes (Table 6.1) as described previously (Lam et al., 2004; Chapter 3). βAOB were detected using two oligonucleotide probes, NSO190 and NSO1225 (Mobarry et al., 1996). NSO190 is regarded as more specific for βAOB but likely misses some Nitrosomonas spp., whereas NSO1225 has a wider coverage but it may also hybridize with some non-βAOB targets (see Table 6.1; (Utaker and Nes, 1998; Purkhold et al., 2000). However, searches by Probe Match in the Ribosomal Database Project II (Cole et al., 2003) found more matching βAOB or βAOB-like sequences with NSO190 than with NSO1225 (384 versus 140). Hence, neither NSO190 nor NSO1225 gives a perfectly accurate estimate of the βAOB abundance. The βAOB abundance in this study is reported henceforth as a range between the hybridized-cell-counts by the two probes and perhaps should be regarded as a potential underestimate. The total abundance of ammonia oxidizing bacteria (total AOB) is calculated as the sum of γAOB abundance and the mean of the βAOB abundance estimated from the NSO190- and NSO1225- hybridized cell counts. The total microbial abundance was obtained via DAPI-counter-staining after FISH processing on the same filter sections. All data for microbial abundance have already been presented in detail in Chapters 4 and 5 for the hydrothermal plumes in the Endeavour Segment and Guaymas Basin respectively. The abundance data for ammonia-oxidizing bacteria are summarized here for comparison with the information obtained from the PCR-based techniques.
6.2.3. Water Column Sampling for DNA Extraction

Water subsamples were drawn from Niskin sample bottles with clean 18-liter polyethylene cubitainers for DNA extraction. Some water-column samples were collected by the Bag Samplers (General Oceanics) deployed by the submersible DSV2 ALVIN. Some warm diffuse discharged fluids (< 60 °C) that were visible shimmering over microbial mats were concentrated by a dome-like inverted funnel and sampled from the top with titanium major samplers onboard the DSV2 ALVIN. Using a peristaltic pump, each water sample was gently filtered through a 3.0 μm pore-size (47 mm diameter) polycarbonate membrane filter (Osmonics, Inc.) followed by a 0.22 μm pore-size cylindrical Sterivex-GS filter (Millipore, Inc.). Each of the 3.0 μm membrane filters was carefully folded and immersed in 1.8 ml SET buffer (0.75M sucrose, 40 mM EDTA, 50 mM Tris-HCl, pH 7.8, autoclaved and 0.2 μm-filtered) in sterile 2.0 ml cryogenic vials. The Sterivex filters were flooded with 1.8 ml SET buffer and sealed with ethanol-cleaned parafilm at both ends. Both types of filters were stored deep-frozen (-80°C) until DNA extraction in a shore-based laboratory. The microbes retained on the 3.0 μm membrane filters are considered particle-associated microbial communities (≥3.0 μm), while the microbes retained downstream on the 0.22 μm Sterivex filters represent the free-living microbial communities (0.22-3.0 μm). Selected non-size-fractionated subsamples were also collected by filtering sampled water directly through a 0.22 μm pore-size Sterivex filter without a 3.0 μm pore-size membrane filter.

In a shore-based laboratory, DNA extraction was performed with the AquaPure Genomic DNA Kit following manufacturer’s instructions (Bio-Rad Laboratories, Inc.),
with some modifications for the cell and protein lysates within Sterivex filters based on another protocol (Somerville et al., 1989). Briefly, the Sterivex filters were thawed on ice for 15 minutes. Cell lysates were accomplished within the Sterivex filter housings by first incubation with 40 µl of lysozyme (50 mg ml⁻¹) at 37°C for 1 hour, followed by a second incubation with 50 µl of Proteinase K (20 mg ml⁻¹) and 30 µl sodium deoxycholate (SDS, 20 w/v %) at 55°C for 2 hours on a rotator. The cell lysate of each sample was drawn from the Sterivex filter housing with a sterile 3-ml syringe and transferred to a sterile microcentrifuge tube. The Sterivex filter was rinsed with 1 ml Genomic DNA Lysis Solution (provided in AquaPure Genomic DNA Kit) and the rinse was combined with the cell lysate. The 3.0 µm polycarbonate membrane filters were thawed on ice, transferred with the SET buffer to 2-ml microcentrifuge tubes and centrifuged at 14,000 × g for 5 minutes. The supernatant was decanted and discarded, and cell lysis was performed with the AquaPure Genomic DNA Kit. Then the DNA from both types of filters was isolated, purified and rehydrated following manufacturer's recommendations.

6.2.4. Sample Collection from the Guaymas Sediments

Since autotrophic ammonia-oxidizing bacteria require oxygen for their chemolithoautotrophic activity, and the sediment cores from Guaymas Basin turn anoxic at relatively shallow depths, only the top centimeter of two sediment cores (Cores A and C) plus the supernatant water overlying one of them (Core C) were sampled for DNA extraction. Cores A and C were collected from the Everest Mound (27°00.89’N, 111°24.73’W) and the Orpheus Site (27°00.44’N, 111°24.61’W) respectively, both within the southern trough by the submersible DSV2 ALVIN in April 1998. The surface
of Core A was covered with an orange *Beggiatoa* spp. mat, while the sediments themselves consisted of soft olive-green ooze towards the top. The sediments were grayish below 7 cm with a petroleum-like sheen. Core C was not covered by any *Beggiatoa*, and the top 6 cm was essentially a black layer of liquid petroleum ooze, underlain by thick olive-colored sediments below.

6.2.5. Polymerase Chain Reaction (PCR)

Partial sequences of the 16S rRNA gene specific for ammonia-oxidizing bacteria (AOB) were amplified by polymerase chain reactions (PCR). A nested PCR approach was used, in which near-complete 16S rRNA genes were first amplified using Eubacteria-specific primer pair 8F-1492R (Table 6.2), the amplicons of which were further amplified with AOB-specific primer pairs βAMOf-NSO1225 (McCaig *et al.*, 1994; Mobarry *et al.*, 1996) and NOCl-NOC2 (Voytek *et al.*, 1998) to target the β- and γ-proteobacterial AOB respectively. Direct amplification with the AOB-specific primers was initially attempted but yielded no detectable amplicons.

Partial sequences (491-bp) of the functional gene, *amoA*, were amplified with the primers amoA-1F and amoA-2R specific for β-proteobacterial AOB (Rotthauwe *et al.*, 1997), whereas 507-bp long *amoA-amoB* fragments were amplified using primers amoA-3F and amoB-4R targeting γ-proteobacterial AOB (Purkhold *et al.*, 2000). Since no detectable *amoA* amplicons could be yielded in single direct amplification, 1 μl aliquots of amplicons from the original PCR reactions were re-amplified with the same *amoA*-targeted primers for both β- and γ-proteobacterial AOB. An attempt was made to amplify the *amoA* genes of β-proteobacterial AOB using a nested-PCR approach, with
the primer pair amoA-2F-amoA-5R in the first amplification (touchdown PCR), followed by amoA1F-amoA-2R (Webster et al., 2002), but without avail.

The reaction volume of all PCR reactions was 50 µl, and each reaction contained 200 µmol deoxynucleoside triphosphates (dNTPs), 50 pmol of each primer, 1× reaction buffer, 2-2.5 mM MgCl₂, 1-1.5 U Taq polymerase (Platinum Taq, Invitrogen Corp., or HotMaster Taq, Eppendorf) (Table 6.2) and 1-2 µl DNA sample. All primers were custom-ordered from Qiagen, Inc. Primer specificity and optimal primer annealing temperatures were tested for each primer set with nucleic acid extracts of pure Nitrosomonas europaea, Nitrosospira briensis, Nitrosococcus oceani, Nitrobacter hambergensis, Nitrospira marina and Nitrococcus mobilis (the former three kindly provided by M. A. Guerrero, Florida International University; and the latter three by A. Teske, University of North Carolina) prior to reactions with environmental samples. Some of these pure extracts were also used during each PCR run as positive and negative controls. PCR amplifications were performed mostly on a GeneAmp 9700 PCR Cycler (Applied Biosystems), or an Eppendorf gradient MasterCycler (Brinkman) for annealing temperature tests. Thermal cycling conditions with various primer sets are detailed in Table 6.2. The presence of PCR amplicons was checked by agarose gel electrophoresis (1.5 w/v%) with ethidium bromide staining.

6.2.6. Cloning of PCR Products and Plasmid Isolation

Positive PCR amplicons were purified with either the QIAquick 8 PCR Purification Kit (Qiagen, Inc.) with a QIAvac 6S vacuum manifold (Qiagen, Inc.), or the QIAquick 96 PCR BioRobot Kit with a BioRobot 9600 (Qiagen, Inc.) according to
manufacturer’s instructions. Purified PCR amplicons were stored frozen (-80°C) until cloning reactions. The sediment samples from the Guaymas Basin were further A-tailed by incubating 50 ng of each of the purified PCR products at 72 °C for 10 minutes in 5 μl PCR buffer (10x), 5 μl dNTPs (2 mM), 1 U Taq and distilled water in a total reaction volume of 50 μl. The A-tailed products were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), centrifuged for 5 minutes, and precipitated with 10% (v/v) 3 M sodium acetate and 2 volumes 100 % ethanol at −80°C overnight. Then they were centrifuged again for 20 minutes at 13,000 rpm at 4°C, washed in cold 70% ethanol, dried in a Speed-Vac and resuspended in 4 μl of distilled water. They were stored at −80°C until cloning.

The PCR amplicons from various water samples were pooled into twenty cloning reactions according to the geographic and hydrothermal plume (or fluid) characteristics, as well as the corresponding size-fractions, in order to reduce biases (Table 6.3). The different sediment samples from the Guaymas Basin were subjected to separate cloning reactions. The TOPO TA Cloning Kits with pCR2.1-TOPO® vector and TOP10F' Escherichia coli cells (Invitrogen Corp.) were used following manufacturer’s recommendations. Positive clones were selected via blue-white screening on Luria-Betrani (LB) agar plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 1.5% agar, 50 μg ml⁻¹ kanamycin, pH7.0) previously spread with 40 μl of 40 mg ml⁻¹ X-gal (5-bromo-4-chloro-3-indolyl-beta-D- galacto-(arentyougladyoutookorganic)-pyranoside) and 40 μl of 100 mM IPTG (isopropyl beta-D-thiogalactoside) and were incubated overnight at 37°C. Up to thirty positive (white) clones were randomly selected from each clone library for plasmid isolation, via overnight cultures in 4 ml LB Broth (1.0% tryptone,
0.5% yeast extract, 1.0% NaCl, 50 µg ml\(^{-1}\) kanamycin, pH7.0) at 37°C. The plasmids were then isolated and purified with the QIAprep 8 Miniprep Kit (Qiagen, Inc.) with a QIAvac 6S vacuum manifold (Qiagen, Inc.) following manufacturer’s instructions. The clones from the Guaymas sediment samples were picked and grown in overnight cultures with 1 ml of Super Broth (2.5% tryptone, 1.5% yeast extract, 1.0% NaCl, 50 µg ml\(^{-1}\) kanamycin, pH7.0) in a 96-well culture block. The plasmids were purified using QIAprep 96 Turbo BioRobot Kit (Qiagen) following manufacturer’s instructions (laboratory of M. L. Sogin, Marine Biological Laboratory). Purified plasmids were stored frozen (-80°C) until DNA sequencing.

6.2.7. DNA Sequencing

Sequencing reactions for all water column samples and the \textit{amoA} genes from the Guaymas sediments were performed with the CEQ\textsuperscript{TM} Dye Terminator Cycle Sequencing Kit (Beckman Coulter) following manufacturer’s instructions with some modifications. Since the maximum optimal sequence read-length of the sequencer was only about 760 bases, the 16S rRNA genes were sequenced once in the forward and once in the reverse direction, with the primers M13F (-20) and M13R (Qiagen, Inc.) respectively. Selected \textit{amoA} genes were also sequenced in both directions. The diversity within each clone library was first screened by sequencing the forward strands of up to thirty clones. The clones with unique forward sequences (<98% similarity) were selected for the sequencing in the reverse direction to acquire the full sequences. A reduced volume of 5 µl, instead of 20 µl, was used in the sequencing reactions presumably without loss of sensitivity and accuracy (Azadan \etal, 2002). Before the sequencing reactions, the DNA templates
were pre-heated to 96 °C for 1 minute and immediately cooled on ice. This treatment has been shown to improve sequencing signal strength and current stability (Dobbs, 1999).

The post-sequencing DNA purification was performed following manufacturer’s recommendations. Briefly, the DNA from the Guaymas Basin sediment sequencing reactions was precipitated by the addition of 50 μl of freezer (-20 °C)-cold 95-100% ethanol, and the plate was immediately centrifuged at 3,010 × g at 4°C for 10 minutes. The supernatant was removed by gently inverting the plate on a stack of clean paper towels. Each of the pellets was rinsed twice with 100 μl of freezer-cold 70% ethanol without vortex, and centrifuged at 14,000 rpm at 4°C for 5 minutes, followed by vacuum drying for about 45 minutes. Finally, each sample was resuspended with 30 μl of Sample Loading Solution (provided) and topped with a drop of light mineral oil (provided). The same procedures were applied to the water column samples, except that 1.5 ml microcentrifuge tubes were used instead of 96-well plates for the post-reaction clean-up, and that the supernatants were removed by aspiration and they were centrifuged only for 2 minutes after each ethanol rinse. Sequencing reads were performed on a CEQ™ 2000 XL automated capillary sequencer (Beckman Coulter, Inc.) according to the manufacturer’s protocol. Sequence reads were checked with the CEQ™ 2000 XL Sequence Analysis Software (version 4.3.9; Beckman Coulter, Inc.) and assembled using ContigExpress in VectorNTI 9.0.0 (InfoMax®, Invitrogen Corp.).

The 16S rRNA gene fragments from the Guaymas sediments were sequenced by a LI-COR 4200L automated sequencing apparatus using infrared dye-labeled T7F and M13R primers (LI-COR, Lincoln, NE), and a cycle sequencing protocol recommended
by the manufacturer (Sequitherm, Epicenter Inc., Madison, WI). The 3’- and 5’- ends of each sequence were read in one direction only, whereas the central 0.6-0.9 kb were read in both directions. The sequence data were first created as image files, which were then interpreted for base calls and PHD quality values by the eSeq DNA sequence analysis software (Li-COR). M13F (-20) and M13R primers (Operon, Inc.) were used in separate sequencing reactions for reads in both directions. The forward and reverse reads of each sequence were subsequently assembled using PHRAP ver. 0.960731 (Green, 1996).

All assembled 16S rDNA sequences were checked for possible chimeras using the CHIMERA_CHECK program on the Ribosomal Database Project II website (Cole et al., 2003). The 16S rDNA sequences were aligned using ARB (Ludwig et al., 2002) with the most closely related sequences (>1000 bp in length) acquired from BLAST searches (Altschul et al., 1997). The amoA gene sequences were also aligned in ARB with closely related sequences from BLAST, yet based on their coded amino acid sequences translated with the GCG Wisconsin package (Accelrys, Inc.) and aligned with Clustal X 1.81 (Higgins and Sharp, 1988). Sequence alignments were visually inspected and then subjected to phylogenetic analyses based on distance-matrix, maximum-parsimony and maximum-likelihood using PAUP 4.0b (Swofford, 2000). The AmoA amino acid sequences were also analyzed by parsimony and distance matrix methods with PAUP 4.0b, and by maximum likelihood with Phylip 3.6 (Felsenstein, 2004). These resultant AmoA phylogenetic trees had much less resolution among the closely related sequences as discussed in another study (Purkhold et al., 2000). Thus only the DNA-based trees were shown in this study. Bootstrap analyses with 1000 resamplings were used to provide confidence estimates for tree topologies. The 16S rRNA and amoA gene
sequences from this study have been deposited in the GenBank with accession numbers AY722807 to AY722843 (16S) and AY785999 to AY786052 (amoA) for Guaymas Basin sediment clones, AY786220 to AY786279 (16S), AY785963 to AY785988 and AY785997 to AY785998 (amoA) for Guaymas Basin water column clones, AY786280 to AY786294 (16S) and AY785989 to AY785996 (amoA) for Endeavour water column clones.

6.3. RESULTS

6.3.1. Abundance of Autotrophic Ammonia-Oxidizing Bacteria in the Water Column

6.3.1.i. Microbial Abundance in the Endeavour Hydrothermal Plume

The total microbial abundance measured by DAPI-stained cell counts was considerably elevated within the neutrally buoyant hydrothermal plume over the vent fields of Main Endeavour Field (MEF) (1.51 ± 0.05 × 10^5 cells ml⁻¹) and High Rise (1.45 ± 0.06 × 10^5 cells ml⁻¹), as well as over the west valley wall at Station C (1.67 ± 0.07 × 10^5 cells ml⁻¹), relative to above-plume abundance (0.6-0.7 × 10^5 cells ml⁻¹). Moderate microbial abundance (1.14 ± 0.04 × 10^5 cells ml⁻¹) was recorded in the far-field plume, 5.5 km away from MEF (Station F). Abundance (0.54 ± 0.04 × 10^5 cells ml⁻¹) close to above-plume background was found at the southern end of the axial valley (Station E). The ammonia-oxidizing bacteria in the β-Proteobacteria subgroup (βAOB) accounted for as much as 7.6 % of total microbial abundance (6.5-11.1 × 10³ cells ml⁻¹) within the neutrally buoyant plume over High Rise, or 4.0-5.9 % within the plume over MEF (6.0-9.0 × 10³ cells ml⁻¹), compared to only 1.2-3.2 % in above-plume background (0.6-1.3 × 10³ cells ml⁻¹) (Fig. 6.3 a-b). The abundance of βAOB was low over the west valley wall at Station C (1.4-2.1 × 10³ cells ml⁻¹; 0.9-1.2% DAPI) and decreased further to 0.1-0.6 ×
$10^3$ cells ml$^{-1}$ (0.2-1.1% DAPI) at Station E and $0.5-1.3 \times 10^3$ cells ml$^{-1}$ (0.4-1.1% DAPI) at the far-field Station F (Fig. 6.3 c-e). Among the βAOB, *Nitrosospira*-like cells were more abundant than the *Nitrosomonas*-like cells within the neutrally buoyant plumes, but the abundance of the latter was comparable to or even exceeded the former below and above the plumes (Fig. 6.4).

The γ-proteobacterial ammonia-oxidizing bacteria (γAOB) were also more abundant within the neutrally buoyant plumes than in the rest of the water column (Fig. 6.3) but were usually fewer in numbers than the βAOB, except for the plume core at MEF where the populations of γAOB and βAOB were similarly large (Fig. 6.3 a). The total AOB abundance, as the sum of γAOB and βAOB, was poorly correlated with NH$_4^+$ concentrations or ammonia oxidation rates ($p>0.05$, Spearman rank-order correlation). Total AOB abundance reached a maximum at intermediate NH$_4^+$ concentrations (70-90 nM) and intermediate ammonia oxidation rates (20-30 nM d$^{-1}$) (Chapter 4).

6.3.1.ii. Microbial Abundance in the Guaymas Basin Hydrothermal Plumes

Microbial abundance was measured at both the South Site and the North Site in the southern trough of the Guaymas Basin. High total microbial abundance ($1.85 \pm 0.07 \times 10^5$ cells ml$^{-1}$) was detected within the neutrally buoyant plume (~1850 m) at the South Site, relative to $0.67-0.98 \times 10^5$ cells ml$^{-1}$ above the sill depth (1560 m). A large microbial community ($1.55 \pm 0.04 \times 10^5$ cells ml$^{-1}$) was also present in the strong buoyant plume (~1900 m) at the North Site, but even greater total microbial abundance ($1.77 \pm 0.07 \times 10^5$ cells ml$^{-1}$) was recorded at 1694 m without any noticeable hydrothermal plume signals as apparent temperature anomalies ($\Delta \theta^* \leq 0.0014 \, ^\circ C$) (Chapter 5). The β-
proteobacterial ammonia-oxidizing bacteria ($\beta$AOB) seemed to be more populated at plume depths ($3.3-5.1 \times 10^3$ cells ml$^{-1}$, 1.8-3.1% DAPI) than above the sill ($1.1-3.2 \times 10^3$ cells ml$^{-1}$, 1.6-1.9% DAPI) at the South Site (Fig. 6.5 a), but there were no apparent trends in their vertical distribution at the North Site ($2.0-3.4 \times 10^3$ cells ml$^{-1}$, 1.5-5.8% DAPI) (Fig. 6.5 d). Among the $\beta$AOB, *Nitrosospira*-like cells seemed to predominate over *Nitrosomonas*-like cells within the neutrally buoyant or buoyant plumes, whereas it was the reverse at the depths above any hydrothermal influence (Fig. 6.5 c, f).

The abundance of $\gamma$-proteobacterial ammonia-oxidizing bacteria was usually lower than that of $\beta$AOB ($0.3-4.5 \times 10^3$ cells ml$^{-1}$, 0.4-3.9% DAPI), except for the sample from 1760 m at the North Site ($3.5 \pm 0.6 \times 10^3$ cells ml$^{-1}$, 8.0% DAPI). The total abundance of AOB shared the same general distribution patterns as that of $\beta$AOB or $\gamma$AOB alone (Fig. 6.5 b, e). Although noticeably higher within the plume, the total AOB abundance was significantly correlated with the particle anomalies, $\Delta c$ ($r=0.64$, $p<0.05$, Product-moment correlation coefficient) but not with the apparent potential temperature anomalies, $\Delta \theta^*$, ($p>0.05$, Spearman rank-order correlation), NH$_4^+$ concentration, total net or autotrophic NH$_4^+$ removal rates ($p>0.05$, Product-moment correlation) (Chapter 5).

### 6.3.2. Detection of Ammonia-Oxidizing Bacteria by PCR

The 16S rRNA genes of $\beta$-proteobacterial ammonia-oxidizing bacteria ($\beta$AOB) were successfully detected using the nested PCR approach in nearly all water column samples collected from the Endeavour Segment and the Guaymas Basin, as well as in the three Guaymas Basin sediment samples, in spite of the lack of products from direct amplification with the $\beta$AOB-specific primers (Table 6.4). $\beta$AOB were detected not only
in hydrothermal plumes of both the Endeavour Segment and Guaymas Basin, but also in above-plume background deep water free of direct hydrothermal influence, which was in agreement with the findings from FISH. Only in two subsamples were βAOB not found: one free-living (0.2-3.0 μm) size-fraction at 2010 m at Station A (MEF) of the Endeavour Segment and one non-size-fractionated subsample at 1885 m at the North Site of the Guaymas Basin. Interestingly, βAOB were in fact detected in the particle-associated size-fraction (≥ 3 μm) corresponding to the former exception, suggesting that βAOB had an affinity towards particle-association in that water sample, if similar efficiency in their respective DNA extraction is assumed. However, βAOB were found in both the parallel free-living and particle-associated (≥ 3 μm) size-fractions from a sample corresponding to the non-size-fractionated subsample at the North Site in Guaymas Basin (the latter exception noted above). Therefore, there must have been an artifact in either the amplification reaction or DNA extraction for this non-size-fractionated subsample, rather than a true absence of βAOB.

The results from the amoA gene detection were generally consistent with those from the 16S rRNA gene detection, that amoA genes of βAOB were amplified in most samples examined in both water columns and in all Guaymas Basin sediments samples (Table 6.4). Only eight subsamples showed negative results, out of which five came from free-living size-fractions (0.2-3.0 μm), while their particle-associated counterparts (≥ 3 μm) gave positive results. One example came from Station A (2010 m) at the Endeavour Segment, in which amoA genes were detected only in the particle-associated size-fraction but not in the free-living size-fraction, and the same results were obtained
from the βAOB-16S rRNA gene detection. On the other hand, the amoA genes of βAOB were detected in the non-size-fractionated subsample collected from the North Site (1885 m) of the Guaymas Basin, but parallel amplification of the 16S rRNA genes did not yield any detectable products. In this case, failure in DNA extraction of this non-size-fractionated subsample can be ruled out. The lack of detectable βAOB-16S rDNA amplicons from this subsample most likely resulted from an unsuccessful amplification reaction.

The 16S rRNA genes of γ-proteobacterial ammonia-oxidizing bacteria (γAOB) could not be detected in any samples, whether in the water column at either site or in the Guaymas Basin sediments (Table 6.4). However, faint bands of the γAOB amoA gene-targeted amplicons of the correct length (507 bp) could be visualized via gel electrophoresis in four samples in the Guaymas Basin water column, two at each of the North and South Sites. Strong positive signals were given by the Guaymas Basin sediment Core C sample and the overlying supernatant water (Table 6.4).

6.3.3. Clone Libraries and Phylogenetic Analyses

Fourteen out of twenty clone libraries constructed from the water column samples successfully yielded true positive target sequences: 9 libraries for the 16S rRNA genes and 5 libraries for the amoA genes, both specific for βAOB. If 2% and 5% sequence divergence of 16S rRNA and amoA genes were taken as thresholds to define operational taxonomic units (OTUs) (Purkhold et al., 2000; Francis et al., 2003), there were only 3 to 5 OTUs and 1 to 4 OTUs in each 16S rDNA and amoA clone library respectively. The goodness of representation by these OTUs has been checked by rarefaction analyses. The
numbers of OTUs reached asymptotic maxima if plotted against the numbers of clones sequenced. Despite the positive detection of PCR amplicons, no true targeted sequences could be found in the βAOB-amoA clone libraries from the free-living size-fractions of water column samples, βAOB-16S rRNA libraries from the Endeavour background sample, or the γAOB-amoA libraries from the Endeavour and Guaymas Basin water column samples (Table 6.3). All clone libraries of the Guaymas Basin sediment samples gave true targeted 16S rRNA and amoA gene sequences, except for the γAOB-amoA clone library of core C (Table 6.3). The phylogenetic trees based on the 16S rRNA and amoA gene sequences, as well as the AmoA amino acid sequences, have all been verified by maximum parsimony, distance matrix and maximum likelihood methods. Since nearly identical topologies were yielded from the different treeing methods, only the bootstrapped (1000 resamplings) neighbor-joining distance trees are shown (Fig. 6.6-6.9).

6.3.3.i. The Endeavour Hydrothermal Plume

All βAOB-targeted sequences from the Endeavour plume are only 87-92% similar to the closest known βAOB and fell into a separate large cluster. Their closest relatives (93% similar, represented by one clone in Fig. 6.6) found in the GenBank came from the Weser Estuary (Selje and Simon, 2003) where ammonium concentration is known to be high (De Jong et al., 1999), or from some equine fecal contamination (AY145571) (Simpson et al., 2004). The Endeavour plume free-living clones and particle-associated clones formed two disparate clusters (>3% divergence), except for the free-living clone 248EnFL8, which was more related to the particle-associated clone 274EnPA9 (Fig. 6.6). Similar to the 16S rRNA-based tree, all but one of the βAOB-
amoA gene sequences from the particle-associated size-fractions formed one cluster in the DNA-based phylogenetic tree (Fig. 6.7), together with one representative of the non-size-fractionated background deep-water subsample. The closest known (GenBank) relative to this cluster was a deep-sea sediment clone SAG-Sed(AMO)-2 (≥ 88% DNA similarity) (Elsaied and Naganuma, 2002) seconded by a Chesapeake Bay sediment clone CB2-37 (≥ 87% DNA similarity) (Francis et al., 2003). The other representative (548Enb18) of the Endeavour background deep-water clone library appeared more closely related to a Guaymas Basin clone (474GPA14) and some other uncultured amoA sequences from the sediments of Chesapeake Bay (CB3-27) (Francis et al., 2003) and Kysing Fjord (K1) (Nicolaisen and Ramsing, 2002) (Fig. 6.7). All Endeavour βAOB-amoA clones were 76-77% similar to the closest Nitrosospira cluster on the DNA level.

6.3.3.ii. The Guaymas Basin Hydrothermal Plumes

Most amplified fragments of the βAOB-like 16S rRNA gene from the Guaymas Basin water column were associated with the Endeavour free-living sequences but were quite distinct from the Endeavour particle-associated sequences (≥ 4% divergence) and other known βAOB (≥ 8% divergence) (Fig. 6.8). One exception came from the warm diffuse hydrothermal fluids (355GTPA3), which was grouped with the Endeavour particle-associated sequences. Two clones from the South Site plume (153GsFL4 and 346 GsFL4) were affiliated with some other β-Proteobacteria, such as Herbaspirillum spp. and an uncultured Nitrosomonas-like bacterium (UBA508162). Some particle-associated clones from warm diffuse fluids (10GTPA3 and 147GTPA3) and some free-living clones from the North Site plume (158GnFL6, 175GnFL6 and 177GnFL6) formed
two sub-clusters within the large cluster with most water column sequences from this study. The branching of these two sub-clusters was supported by moderately high % bootstrap values (77 and 63 respectively) (Fig. 6.8). The remaining sequences within the large cluster of new sequences vary little from each other (90-98 % similarity) regardless of their size-fractions or sites of collection. Phylogenetic analyses of the βAOB-amoA gene also suggested that the Guaymas Basin particle-associated clones, including both plume and warm diffuse fluids, were quite different from most Endeavour particle-associated clones (79-88 % DNA similarity), except for 520EnPA17 and 548Enb18. The closest sequences found were the same sequences from the sediments of Chesapeake Bay (CB3-27) (Francis et al., 2003) and Kysing Fjord (K1) (Nicolaisen and Ramsing, 2002) (Fig. 6.7).

6.3.3.iii. The Guaymas Basin Sediment Samples

Seventeen unique sequences of βAOB-16S rRNA genes and seven unique sequences of βAOB-amoA genes were obtained from the three Guaymas Basin sediment samples. All βAOB-16S rRNA gene sequences belonged to the cluster 3 of Nitrosospira spp. (≥ 90% similarity) and were closely related to the Nitrosospira sp. Nsp1, based on the 16S rRNA gene analyses (Fig. 6.6). Similar phylogenies were deduced from the amoA gene analyses, though the closest relative was found to be Nitrosospira briensis instead of Nitrosospira sp. Nsp1, in spite of the same DNA similarity values (97%) (Fig. 6.8). The γAOB-amoA genes were successfully sequenced only in the supernatant water of Core C. They were highly similar to each other (97-100% DNA similarity) or 98%
similar to *Nitrosococcus oceani* on the DNA level but were 22 % divergent from *Nitrosococcus halophilus* (Fig. 6.9)

### 6.4. DISCUSSION

The application of molecular techniques demonstrates the widespread distribution of ammonia-oxidizing bacteria in the deep-sea hydrothermal plumes and background seawater at the Endeavour Segment and Guaymas Basin, as well as in the warm diffuse hydrothermal fluids and hydrothermal sediments at the latter location. This observation is consistent with the hypothesis that nitrification occurs widely in the deep ocean and that it contributes to the deep-sea nitrate reservoir, which will in turn recharge the often nitrogen-limited surface ocean. Results from FISH with AOB-specific 16S rRNA-targeted oligonucleotide probes revealed the presence of both βAOB-like and γAOB-like cells in the deep-sea water column. However, the results from PCR and DNA sequencing found 16S rRNA and *amoA* gene sequences resembling recognized βAOB and γAOB (*amoA* genes only) in the Guaymas Basin sediments only. The sequences obtained from the water columns at both locations appeared to potentially form a novel lineage of βAOB, as supported by the sequence analyses of both 16S rDNA and *amoA* genes.

#### 6.4.1. Ammonia-Oxidizing Bacteria in Deep-Sea Water Columns

According to the data from FISH, AOB were apparently more abundant in the Endeavour hydrothermal plume compared to above-plume background seawater (as much as over ten-fold), but their abundance dropped dramatically as the plume moved away from the axial valley. AOB abundance is undoubtedly affected by the availability of ammonium and ammonia oxidation rates, but their relationships are rather complex
due to the interactions of various environmental factors, such as plume age, consistency of ammonium supply and organic carbon content (Chapter 4). In fact, the organic-rich conditions in the Guaymas Basin might have stimulated high heterotrophic activities, which would hinder further population growth of AOB even though provided with the much higher ammonium level ($\leq 2.9 \, \mu\text{M}$) (Chapter 5) than in the Endeavour plume ($\leq 177 \, \text{nM}$) (Chapter 4). In the Guaymas Basin water column, enriched ammonium is not only introduced by hydrothermal inputs, but also by the decomposition of particulate organic matter raining down from the productive surface ocean. As a result, the AOB abundance measured in the hydrothermal plume is not always higher than the overlying water column.

$\beta$-Proteobacterial AOB were found to dominate over $\gamma$-Proteobacterial AOB cells in most water column samples at both sampling sites, which contrasted with earlier general views of $\gamma$AOB-dominance in the marine environments (Ward, 1987; Ward et al., 1989); yet few studies to date have actually compared the abundance of $\beta$AOB and $\gamma$AOB simultaneously. Among the $\beta$AOB, *Nitrosospira*-like cells were usually more abundant than *Nitrosomonas*-like cells in the Endeavour plumes and to a certain extent in the Guaymas Basin plume, while the reverse was true for the background deep water at both Endeavour and Guaymas Basin. *Nitrosospira* and *Nitrosospira*-like species are often regarded as more widespread than *Nitrosomonas* and *Nitrosomonas*-like species in natural environments (Hiorns et al., 1995), as the latter seem to have a greater preference for ammonium-rich settings. For example, *Nitrosospira* and *Nitrosospira*-like species were readily detected in all environmental samples in some studies, but *Nitrosomonas*
and Nitrosomonas-like species were only found in enrichment cultures (Stephen et al., 1996; Hastings et al., 1998; Smith et al., 2001) or certain season when ammonium level was high (Whitby et al., 2000). Sometimes, the detected sequences belonged purely to the Nitrosospira and Nitrosospira-like clusters, such as in the polar oceans (Bano and Hollibaugh, 2000; Hollibaugh et al., 2002), soils (Stephen et al., 1996; Hastings et al., 2000; Kowalchuk et al., 2000), coastal sand dunes (Kowalchuk et al., 1997) as well as bioreactors (Schramm et al., 1998). Niche differentiation between these two major groups of βAOB became even more apparent in a study performed in the Mediterranean Sea, where Nitrosomonas dominated in the more ammonium-rich particle-associated size-fractions of sample seawater, while Nitrosospira dominated in the parallel free-living size-fractions (Phillips et al., 1999). Nitrosomonas spp. are known to produce exopolymeric substances to attach themselves to one another or on to other particles, thereby creating or taking advantage of existing microniches with relatively higher ammonium levels (Stehr et al., 1995; Hagopian and Riley, 1998). Hence, the similar or higher abundance of Nitrosomonas-like cells than Nitrosospira-like cells in the background deep waters of this study could reflect the affinity of AOB towards particles when hydrothermal ammonium input was low.

Although the 16S rRNA genes amplified from water column samples did not cluster with any recognized AOB, the analogous phylogenies inferred from sequence analysis of the functional gene amoA suggested that these water column sequences may belong to a novel lineage of βAOB. The amoA genes have not been detected in organisms other than AOB thus far. While the amoA genes of Nitrosospira spp. share a 65-79% DNA similarity with those of Nitrosomonas spp. (Purkhold et al., 2000), the 76-275
77% similarity shared between the amoA genes amplified in this study and known AOB species is also very close in comparison. The closest relatives to these newly sequenced clones, based on the amoA tree, are 87-92 % similar and come from the ammonium-rich (4-10 μM) Chesapeake Bay sediments (Francis et al., 2003). In fact, Francis et al. (2003) proposed that the Chesapeake Bay sediment clones, together with the Kysing Fjord sediment clones (Nicolaisen and Ramsing, 2002), should be recognized as a novel lineage of βAOB. In the present case, the sequences from the Endeavour and Guaymas Basin hydrothermal plumes should also be placed in this same novel lineage. Likewise, the amplified 16S rRNA genes are 87-93 % similar to recognized βAOB species, not too much lower than the 91.6-96.1 % similarity between the known Nitrosospira and Nitrosomonas clusters (Purkhold et al., 2000). The closest 16S rDNA relatives to the sequences acquired in this study (93% similar) also originated from ammonium-rich environments, such as the Weser estuary (Selje and Simon, 2003) and equine fecal contaminated samples (Simpson et al., 2004). Therefore, despite the fact that the two genes might not have come from the same organism or genome, the phylogenies inferred from both genes were strikingly consistent with each other. However, further research involving whole-genome sequencing or culture-based physiological studies coupled with simultaneous 16S rRNA and amoA gene sequence analyses, would be necessary to verify the ammonia oxidation activities and true identities of this potentially novel βAOB lineage.

The diversity of both the amplified 16S rRNA and amoA genes are relatively low in each clone library, but the phylogenetic tree based on the latter seems to show greater
resolution among phylogenetically similar species. It is noteworthy that the Endeavour particle-associated sequences of both genes formed a disparate group from the free-living counterparts, just like the phylogenetic-niche differentiation observed by Phillips et al. (1999), though not as dramatic. A similar but more subtle phenomenon is also observed in the Guaymas Basin water column, in which some of the free-living sequences from the North Site form a small sub-group within the main large group. Sequences from the warm diffuse hydrothermal fluids cluster into a sub-group within the same large cluster. They showed much greater affinity towards the water column sequenced clones than the sediment counterparts. This is logical since hydrothermal plumes evolved from continuous mixing and diluting of hydrothermal fluids by the ambient seawater.

6.4.2. β-Proteobacterial AOB in the Guaymas Basin Hydrothermal Sediments

High ammonium concentrations within sediment porewater are common due to the diagenesis of sedimentary organic matter, thus the presence of ammonia-oxidizing bacteria in the marine sediments is not surprising. Indeed, AOB especially in the β-proteobacterial subgroup, have been detected in a number of marine sediment studies (Stephen et al., 1996; McCaig et al., 1999; Bano and Hollibaugh, 2000; Nold et al., 2000; Ward et al., 2000; Koops and Pommerening-Roser, 2001; Hollibaugh et al., 2002). According to the analyses of both 16S rDNA and the amoA genes amplified from the Guaymas Basin hydrothermal sediments, all βAOB-like sequences fell into the Nitrosospira cluster 3. This is the first time that representatives of this cluster have been recovered from marine sediments or any marine environments, as all sequences previously reported belonged to Nitrosospira cluster 1 and Nitrosomonas clusters 5 and 6 (Koops and Pommerening-Roser, 2001; Kowalchuk and Stephen, 2001). This finding
contrasts with the absence of autotrophic nitrifiers reported for Guaymas Basin hydrothermal environments in another study (Mével et al., 1996), though their cultivation-dependent method might not have been sensitive enough to detect the slow-growing autotrophic AOB. Interestingly, heterotrophic nitrifiers were instead isolated during the study by Mével et al. (1996), which again implicates the organic-rich conditions of the sediments and possibly of the overlying seawater, which could support heterotrophic production. It might be the reason that AOB abundance and autotrophic ammonia oxidation rates are not more elevated in the Guaymas Basin hydrothermal plumes with all the available ammonium (Chapter 5).

6.4.3. γ-Proteobacterial AOB

Most AOB studies to date have been heavily biased towards βAOB while neglecting γAOB, partly because only three γAOB species have been recognized to date. Since so few species are known, the oligonucleotides designed to target this group are perhaps too specific for these three species, which may explain the unsuccessful priming of the primer set NOC1-NOC2 in the water column samples. Although this primer pair has been successfully applied in an Antarctic lake (Voytek et al., 1998) and in some parts of the Pacific Ocean (Ward and O'Mullan, 2002), it has also failed to yield any products in a hypersaline lake (Ward et al., 2000,) and a study of marine sediments (Freitag and Prosser, 2003). In comparison, γAOB were successfully detected in most water column samples from both Endeavour and Guaymas by FISH with the oligonucleotide probe Nscoc128 (Juretschko, 2000; Loy et al., 2003), and the estimated γAOB abundance could be comparable to that of βAOB at times. Searches by Probe Match (Cole et al., 2003)
showed that Nscoc128, the γAOB-specific oligonucleotide probe used in FISH, can match with all γAOB-like sequences found in the Ribosomal Database Project, including various strains of *Nitrosococcus oceani* and *Nitrosococcus halophilus*, yet the primer pair NOC1-NOC2 can only match with *Nitrosococcus oceani*. It is foreseeable that Nscoc128 may be used as a PCR primer to potentially yield targeted amplicons. Unfortunately, Nscoc128 was only published in ProBase (Loy et al., 2003) after the start of this sequencing work, so it was only applied in FISH. Given the fact that so little is known of this particular group of AOB, their distribution and abundance in the environment are probably greatly underestimated.

On the other hand, γAOB-like amoA genes were successfully amplified in the Guaymas Basin sediments, in spite of the lack of amplified γAOB-like 16S rRNA genes. This observation was similar to the detection of βAOB-like amoA genes but not βAOB-like 16S rRNA genes in the environmental samples of a freshwater lake study (Hastings et al., 1998). The primer design for the γAOB-specific amoA genes is perhaps more versatile than that for the γAOB-16S rDNA genes. Since we are interested in a particular functional process, there might be more ecological relevance and a higher chance of amplifying a key functional gene to that particular process, rather than the 16S rRNA genes unless the diversity of the 16S rRNA genes is well known.

6.4.4. FISH versus PCR-DNA Sequencing

The differences between the results from FISH and PCR-based DNA sequencing is rather prominent. Although consistent results between these two culture-independent molecular methods have been reported in some studies (Schramm et al., 1998; Gieseke et
al., 2001), different results have also been documented in others (Cottrell and Kirchman, 2000; Crocetti et al., 2000; Egli et al., 2003). Such differences might be attributed to the choice of primers or probes, and the various biases likely involved with the PCR-based methods.

The βAOB-specific oligonucleotide NS01225 was applied as both the reverse primer in PCR as well as a probe in FISH. Thus the abundance of the potentially novel group of AOB has been accounted for by the NS01225-hybridized cell counts, but not by the NSO190-hybridized cell counts, resulting in different βAOB abundance estimates for the same sample. In fact, both NSO190 and NSO1225 can match with some sequences that the other cannot (results from Probe Match (Cole et al., 2003); also see Table 6.1, Utåker and Nes (1998) and Purkhold et al. (2000). This points to the major limitation of FISH – its efficiency relies on probe design, which in turn is heavily dependent upon currently known sequence data. The application of FISH likely misses any novel species that carry mismatches with the probe sequence of choice. This limitation is also shared by PCR to a certain extent, if the chosen primers are overly specific to a small group of organisms, as discussed regarding the primer set NOC1-NOC2.

While the application of FISH can be quite objective given well-selected oligonucleotide probes and efficient hybridization procedures, more biases can be introduced in the various steps of the PCR-based DNA sequencing, including DNA extraction (Wintzingerode et al., 1997; Webster et al., 2003), PCR amplification (Suzuki and Giovannoni, 1996; Wintzingerode et al., 1997), clone library construction and clone selection for sequencing (Kemp and Aller, 2004). Since conventionally known βAOB-like sequences were amplified from the Guaymas Basin sediment samples using exactly
the same PCR conditions as the water column samples, bias against the amplification
reaction of βAOB-like sequences in the latter might not be very likely; although a higher
abundance of βAOB within the sediments could also allow easier amplification of βAOB
sequences in case of small degree of biases. The cloning procedures used for the two sets
of samples were almost identical except for an additional A-tailing step in the Guaymas
Basin sediments samples, and so should not be a large source of bias, especially because
Taq polymerase was used in all amplifications that would add an A-tail. Clone selection
could insert little bias in the sequencing results, due to the low diversity within each
library relative to the number of screened clones. Hence, biases in DNA extraction is
likely the major culprit. Firstly, insufficient or preferential disruption of cells is an
obvious source of bias, as the DNA still retained within cells would not be included in the
subsequent diversity analyses. The DNA extraction efficiencies on the same sample
differed according to the extraction protocol (Webster et al., 2003). In the meantime,
various sample types often require special treatments to optimize their DNA yields, yet
these optimizations might also select against other organisms. It may be acceptable if
one is interested only in that particular group, but it would be a problem if the community
diversity is of interest. For instance, more rigorous DNA extraction protocols are usually
needed for Gram-positive bacteria, but such treatment might break the nucleic acids of
Gram-negatives into fragments, possibly leading to the formation of chimeric PCR
products (Wintzingerode et al., 1997). The different DNA extraction protocols employed
for the particle-associated and free-living water samples versus the sediment samples
could have introduced different degrees of biases into each sample type. On the other
hand, if the amount of extracted DNA was too low, which is possible considering the fact
281
that direct amplification of AOB-specific genes did not yield detectable products, stochastic fluctuations in the interactions between PCR reagents and DNA templates (i.e. PCR drift) might ensue (Polz and Cavanaugh, 1998).

Our ability to detect and study AOB has increased tremendously owing to the recent advances in molecular biological techniques. However, studies to date have been skewed towards the use of these culture-independent molecular methods based on the sequence information of recognized AOB isolates whose ammonia-oxidizing activities have been confirmed. Such information must ultimately derive from the 25 species of ammonia-oxidizing bacteria in culture (Koops and Pomerening-Roser, 2001). It is convenient that these isolated AOB formed monophyletic clusters, and physiological studies of pure AOB cultures do suggest links between phylogenetic groups and physiological characteristics of ecological relevance (Prosser and Embley, 2002). Therefore, uncultured organisms bearing similar 16S rDNA sequences might well be AOB. Nonetheless, their capability of ammonia oxidation remains unknown. For example, *Nitrosospira* cluster 1 and *Nitrosomonas* cluter 5 do not have any cultured representatives to confirm their capability to oxidize ammonia (Koops and Pomerening-Roser, 2001; Kowalchuk and Stephen, 2001; Prosser and Embley, 2002). Furthermore, if genuine ammonia-oxidizing bacteria fall outside the recognized phylogenetic groups, they would not be detected using these culture-independent molecular techniques. Therefore, a polyphasic approach involving both culture-dependent and culture-independent methods to study microbial ammonia oxidation is appropriate.
6.6. REFERENCES


de Bie, M. J. M., Speksnijder, A. G. C. L., Kowalchuk, G. A., Schuurman, T., Zwart, G.,
dominant populations of ammonia-oxidating β-subclass Proteobacteria along the

Common Wadden Sea Secretariat, Trilateral QSR Group & Trilateral Monitoring
and Assessment Group, Wilhelmshaven. 259.

template preheating procedure. *CEQ™2000 - Application Information:* A-1872A.

record of settling flux from the Endeavour Ridge using moored sensors.
*Geochimica et Cosmochimica Acta* **52**: 2525-2536.

Egli, K., Bosshard, F., Werlen, C., Lais, P., Siegrist, H., Zehnder, A. J. B. and van, d. M.
contactor biofilm treating ammonium-rich wastewater without organic carbon.

monooxygenase and ammonia monooxygenase-encoding genes from different
deep-sea habitats, direct sequence submission to GenBank.

Felsenstein, J. (2004). PHYLIP (Phylogeny Inference Package) version 3.6, distributed
by the author. Department of Genome Sciences, University of Washington,
Seattle.

285


290


ammonia-oxidizing isolates: extension of the dataset and proposal of a new

Roth, S. E. and Dymond, J. (1989). Transport and settling of organic material in a deep-
sea hydrothermal plume: evidence from particle flux measurements. *Deep-Sea
Research* **36**: 1237-1254.

structural gene *amoA* as a functional marker: molecular fine-scale analysis of
natural ammonia-oxidizing populations. *Applied and Environmental
Microbiology* **63**: 4704-4712.

Structure during Operation of an Ammonia Biofilter with Molecular Tools.

Activities In Situ of Nitrosospira and Nitrospira spp. as Dominant Populations in
a Nitrifying Fluidized Bed Reactor. *Applied and Environmental Microbiology
64*(9): 3480-3485.

free-living bacterial communities in the Weser Estuary, Germany. *Aquatic

Guaymas Basin - generation, migration and deposition of petroleum. In: *The Gulf
and Peninsular Province of the Californias (Eds. J. P. Dauphin and B. R. T.
Simoneit)*, AAPG Memoir 47, pp. 793-825.

292


Table 6.1. List of the various 16S rRNA-targeted oligonucleotide probes used in this study, together with their respective targeting organisms, target sites with respect to *E. coli*, formamide concentrations (%FA) in hybridization solutions and NaCl concentrations in the stringent washing solutions. The oligonucleotide probes were labeled with the fluorochromes Cy3 or Oregon Green (Molecular Probes, Inc.), and were custom-made by Integrated DNA Technologies, Inc.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequences</th>
<th>Target Organisms</th>
<th>Target site <em>(E. coli Positions)</em></th>
<th>%FA</th>
<th>NaCl <em>(mM)</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON338</td>
<td>5'-ACT CCT ACG GGA GGC AGC -3'</td>
<td>Negative control</td>
<td>N/A</td>
<td>20</td>
<td>225*</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>NSO190</td>
<td>5'-CGA TCC CCT GCT TTT CTC C -3'</td>
<td>β-Proteobacterial AOB</td>
<td>190-208</td>
<td>55</td>
<td>20*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>NSO1225</td>
<td>5'-CGC GAT TGT ATT ACG TGT GA -3'</td>
<td>β-Proteobacterial AOB</td>
<td>1225-1244</td>
<td>35</td>
<td>80*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsm156</td>
<td>5'-TAT TAG CGC ATC TTT CGA T -3'</td>
<td><em>Nitrosomonas</em> spp.</td>
<td>156-174</td>
<td>5</td>
<td>56*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nsv443</td>
<td>5'-CCG TGA CCG TTT CGT TCC G -3'</td>
<td><em>Nitrosospira</em> spp.</td>
<td>444-462</td>
<td>30</td>
<td>112*</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nscoc128</td>
<td>5'-CCC CTC TAG AGG CCA GAT -3'</td>
<td>γ-Proteobacterial AOB</td>
<td>128-146</td>
<td>35</td>
<td>80</td>
<td>(Juretschko, 2000; Loy et al., 2003)</td>
</tr>
</tbody>
</table>

* Information on the NaCl concentrations in these washing solutions came from (Schramm et al., 1998).

* NaCl concentration for washing solution is adopted from (Gieseke et al., 2001).

*a* NSO190 has 3 mismatches with *Nitrosomonas ureae*, and 1 mismatch in a few *Nitrosomonas* spp. (Utaker and Nes, 1998; Purkhold et al., 2000). Searches by Probe Match in the Ribosomal Database Project II (Cole et al., 2003) found 384 matching sequences.

*b* NSO1225 has 1 mismatch with *Nitrosococcus mobilus* (ARB Difference Alignment function.probeBase (Loy et al., 2003)), and may hybridize with *Gallionella ferruginea* (Results from Probe Match). Searches by Probe Match in the Ribosomal Database Project II (Cole et al., 2003) found 140 matching sequences.

*c* Three possible non-AOB targets are *Thauera linaloolentis* str. 47, *Pseudomonas butanovora* IAM 12574 and *Aquaspirillum simusum* LMG 4393 (Results from Probe Match (Cole et al., 2003)).
Table 6.2. List of the primers used in this study, along with their sequences, targeted sites, targeted genes and thermal cycling conditions.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' – 3')*</th>
<th>E. coli (16S rRNA) or N. europaea (amoA) positions</th>
<th>Targets</th>
<th>Thermal Cycling Conditions</th>
<th>Taq (U)</th>
<th>MgCl (mM)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>TCC GGT TGA TCC TGC C</td>
<td>8-23</td>
<td>Eubacteria 16S rRNA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 52.5°C, 2min at 72°C); 10 min at 72°C</td>
<td>1</td>
<td>2</td>
<td>(Teske et al., 2002)</td>
</tr>
<tr>
<td>1492R</td>
<td>GGC TAC CTT GTT ACG ACT T</td>
<td>1492-1510</td>
<td>N. europaea amoA</td>
<td>3 min at 94°C; 30 x (40s at 94°C, 30s at 52°C, 2min at 72°C)</td>
<td>1</td>
<td>2</td>
<td>(Teske et al., 2002)</td>
</tr>
<tr>
<td>βAMOF</td>
<td>TGG GGR ATA ACG CAY CGA AAG</td>
<td>141-161</td>
<td>β-OB 16S rRNA</td>
<td>3 min at 94°C; 30 x (40s at 94°C, 30s at 52°C, 2min at 72°C)</td>
<td>1</td>
<td>2</td>
<td>(McCaig et al., 1994)</td>
</tr>
<tr>
<td>NSO1225</td>
<td>CGC GAT TGT ATT ACG TGT GA</td>
<td>1225-1244</td>
<td>amoA-16S rRNA</td>
<td>3 min at 94°C; 30 x (40s at 94°C, 30s at 52°C, 2min at 72°C)</td>
<td>1.5</td>
<td>2.5</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>NOC1</td>
<td>CGT GGG AAT CTG GCC TCT AGA</td>
<td>25-45</td>
<td>γ-OB 16S rRNA</td>
<td>3 min at 94°C; 30 x (40s at 94°C, 30s at 58.3°C, 2min at 72°C)</td>
<td>1.5</td>
<td>2.5</td>
<td>(Voytek et al., 1998)</td>
</tr>
<tr>
<td>NOC2</td>
<td>AGA TTA GCT CCG CAT CAG CT</td>
<td>1168-1188</td>
<td></td>
<td>5 min at 72°C</td>
<td>1.5</td>
<td>2.5</td>
<td>(Voytek et al., 1998)</td>
</tr>
<tr>
<td>amoA-1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
<td>332-349</td>
<td>β-OB amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 55°C, 1min at 72°C)</td>
<td>1.5</td>
<td>2</td>
<td>(Rothhauwe et al., 1997)</td>
</tr>
<tr>
<td>amoA-2R</td>
<td>CCC CTC KGS AAA GCC TTC TTC</td>
<td>802-822</td>
<td>amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 55°C, 1min at 72°C)</td>
<td>1.5</td>
<td>2</td>
<td>(Rothhauwe et al., 1997)</td>
</tr>
<tr>
<td>amoA-3F</td>
<td>GGT GAG TGG GTT AAC MG</td>
<td>295-310</td>
<td>γ-OB amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 48°C, 1min at 72°C)</td>
<td>1.5</td>
<td>2</td>
<td>(Purkhold et al., 2000)</td>
</tr>
<tr>
<td>amoB-4R</td>
<td>GCT AGC CAC TTT CTG G</td>
<td>30-44b</td>
<td>amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 48°C, 1min at 72°C)</td>
<td>1.5</td>
<td>2</td>
<td>(Purkhold et al., 2000)</td>
</tr>
<tr>
<td>amoA-2F</td>
<td>AAR GCC GCS AAG ATG CCG CC</td>
<td>279-298</td>
<td>β-OB amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 48°C, 1min at 72°C)</td>
<td>1</td>
<td>2</td>
<td>(Webster et al., 2002)</td>
</tr>
<tr>
<td>amoA-5R</td>
<td>TTA TTT GAT CCC CTC</td>
<td>1065-1079</td>
<td>amoA</td>
<td>3 min at 94°C; 30 x (20s at 94°C, 1min at 48°C, 1min at 72°C)</td>
<td>1</td>
<td>2</td>
<td>(Webster et al., 2002)</td>
</tr>
</tbody>
</table>

*Sequence degeneracies: K = G or T, M = A or C, R = A or G, S = G or C, Y = C or T.

*These applied only when Platinum Taq polymerase (Invitrogen, Corp.) was used, but not when HotMaster Taq polymerase (Eppendorff) was used, since the MgCl concentration in the latter was self-adjusting.  

bThese positions correspond to the amoB gene of Nitrosococcus oceani C-107.
Table 6.3. Construction of clone libraries and the number of chimera-checked full sequences. Note that clones were selected for sequencing in both directions (i.e. full sequences) after screening the forward strands of ~30 clones in each clone library. The numbers of full sequences are different from the numbers of OTUs.

<table>
<thead>
<tr>
<th>Clone Library</th>
<th>Size-Fraction</th>
<th>Water Column Samples (Purified PCR Amplicons)</th>
<th>No. of Clones</th>
<th>No. of Full Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 16S-Gb-FL</td>
<td>0.2-3.0 µm</td>
<td>G-V5</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>2 16S-Gb-PA</td>
<td>&gt;3.0 µm</td>
<td>G-V5</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>3 16S-GT-PA</td>
<td>&gt;3.0 µm</td>
<td>Dive3779-T</td>
<td>96</td>
<td>7</td>
</tr>
<tr>
<td>4 16S-GS-FL</td>
<td>0.2-3.0 G-V4</td>
<td>G-V6-1996m/1865m</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>5 16S-GS-PA</td>
<td>&gt;3.0 µm</td>
<td>G-V6-1996m/1865m</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>6 16S-GN-FL</td>
<td>0.2-3.0 G-T4</td>
<td>G-T5</td>
<td>55</td>
<td>6</td>
</tr>
<tr>
<td>7 16S-GN-PA</td>
<td>&gt;3.0 µm</td>
<td>G-T4</td>
<td>133</td>
<td>10</td>
</tr>
<tr>
<td>8 16S-En-FL</td>
<td>0.2-3.0 En-V1</td>
<td>En-V2 En-V5 En-V6</td>
<td>56</td>
<td>8</td>
</tr>
<tr>
<td>9 16S-En-PA</td>
<td>&gt;3.0 µm</td>
<td>En-V1 En-V2 En-V5 En-V6</td>
<td>83</td>
<td>10</td>
</tr>
<tr>
<td>10 16S-Enb-T</td>
<td>total</td>
<td>Dive3818-B4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>11 βamoA-Gb-FL</td>
<td>0.2-3.0 µm</td>
<td>G-V5</td>
<td>6</td>
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</tr>
<tr>
<td>12 βamoA-Gb-PA</td>
<td>&gt;3.0 µm</td>
<td>G-V5</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>13 βamoA-G-FL</td>
<td>0.2-3.0 µm</td>
<td>G-V4 G-V6-1996m/1865m G-T4 G-T5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>14 βamoA-G-PA</td>
<td>&gt;3.0 µm</td>
<td>G-V4 G-V6-1996m/1865m G-T4 G-T5</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>15 βamoA-GT-PA</td>
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<td>16</td>
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<td>17 βamoA-En-PA</td>
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<td>6</td>
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</tr>
<tr>
<td>19 γamoA-G</td>
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<td>G-V4 G-V6-1996m/1865m G-T4 G-T5</td>
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</tr>
<tr>
<td>20 γamoA-En</td>
<td>total</td>
<td>En-V1 En-V2 En-V5 En-V6</td>
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Guaymas Sediment Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Count</th>
<th>No. of Sequences</th>
</tr>
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<tr>
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<td>B</td>
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<td>C</td>
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<td>D</td>
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<td>21</td>
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<tr>
<td>E</td>
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<tr>
<td>F</td>
<td>100+</td>
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<td>0</td>
</tr>
<tr>
<td>H</td>
<td>100+</td>
<td>22</td>
</tr>
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</table>

\(^{16S} = \text{16S rRNA gene of } \beta\text{-proteobacterial AOB}\)

\(\betaamoA = \text{amoA gene of } \beta\text{-proteobacterial AOB}\)

\(\gammaamoA = \text{amoA gene of } \gamma\text{-proteobacterial AOB}\)

\(G = \text{Guaymas Basin water column sample}\)

\(En = \text{Endeavour Segment water column sample}\)

\(b = \text{background deep water}\)

\(S = \text{Guaymas Basin plume at the South Site}\)

\(N = \text{Guaymas Basin plume at the North Site}\)

\(FL = \text{free-living size-fraction (0.2-3.0 µm)}\)

\(PA = \text{particle-associated size-fraction (>3.0 µm)}\)

\(B = \text{water column sample collected by a Bag Sampler}\)

\(T = \text{warm diffuse fluids collected by titanium major samplers}\)
Table 6.4. Results of the PCR on various water column samples from the Guaymas Basin and the Endeavour Segment. The symbols ‘+’ and ‘−’ indicate the presence and absence of amplified products detected via agarose gel electrophoresis, while ‘(+)’ tends indicates relatively faint signals detected. The details for the primers used in various PCR amplification can be found in Table 6.2. ‘ND’= no data.

<table>
<thead>
<tr>
<th>Sampling Cast/ Dive No.</th>
<th>Depth (m)</th>
<th>Size Fraction (μm)</th>
<th>16S rRNA gene</th>
<th>amoA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct amplification</td>
<td>Nested PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eubacteria βAOB γAOB</td>
<td>βAOB γAOB</td>
</tr>
<tr>
<td><strong>Endeavour Segment water column</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Station A - MEF</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>1924</td>
<td>&gt;3.0</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>V1</td>
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<tr>
<td>V5</td>
<td>2010</td>
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<td>+</td>
<td>−</td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Dive3817-C</td>
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<td>Total</td>
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<td>−</td>
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<td>−</td>
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<td>−</td>
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<tr>
<td><strong>Station C - 1 km W to MEF</strong></td>
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</tr>
<tr>
<td>V6</td>
<td>1905</td>
<td>&gt;3.0</td>
<td>(+)</td>
<td>−</td>
</tr>
<tr>
<td>V6</td>
<td>1905</td>
<td>0.2-3.0</td>
<td>(+)</td>
<td>−</td>
</tr>
<tr>
<td>Sampling Cast/ Dive No.</td>
<td>Depth (m)</td>
<td>Size Fraction (μm)</td>
<td>16S rRNA gene</td>
<td>amoA gene</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
<td>--------------------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct amplification</td>
<td>Nested PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eubacteria</td>
<td>βAOB</td>
</tr>
<tr>
<td><strong>Guaymas Basin water column</strong></td>
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<td><strong>South Site</strong></td>
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</tr>
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<td>V4</td>
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<td>+</td>
<td>-</td>
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<td>0.2-3.0</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>V6</td>
<td>1996</td>
<td>&gt;3.0</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>1996</td>
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<td>(+)</td>
<td>-</td>
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<tr>
<td>V6</td>
<td>1865</td>
<td>&gt;3.0</td>
<td>+</td>
<td>-</td>
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<tr>
<td>V6</td>
<td>1865</td>
<td>0.2-3.0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>V6</td>
<td>1865</td>
<td>Total</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Dive3779-T 2007</strong></td>
<td>&gt;3.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>North Site</strong></td>
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<td></td>
</tr>
<tr>
<td>T4</td>
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<td>&gt;3.0</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>0.2-3.0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T4</td>
<td>1694</td>
<td>Total</td>
<td>+</td>
<td>-</td>
</tr>
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<td>T5</td>
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<td>-</td>
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<td>T5</td>
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<td>0.2-3.0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T5</td>
<td>1885</td>
<td>Total</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Guaymas Sediments</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Core A (top 1 cm)</td>
<td>Total</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Core C (top 1 cm)</td>
<td>Total</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Core C (supernatant)</td>
<td>Total</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

302
Figure 6.1. A bathymetry map showing the locations of the five known active vent fields (gray stars) (SQ = Sasquatch, SD = Salty Dawg, HR = High Rise, MEF = Main Endeavour Field, MO = Mothra), tow-yo tracks (black lines) and vertical cast stations (black circles). Stations A and B are located at MEF and HR respectively. 'Sta.' stands for 'Station'.
Figure 6.2. A bathymetry map showing the location of sampling stations, North Site and South Site, in the Guaymas Basin, Gulf of California (Koski et al., 1985).
Figure 6.3. Abundance of $\beta$-proteobacterial ammonia-oxidizing bacteria estimated by NSO190- (black circles) and NSO1225- (red circles) cell counts, and the abundance of $\gamma$-proteobacterial ammonia-oxidizing bacteria estimated by Nscoc128 cell counts (green inverted triangles) at Stations A, B, C, E and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 6.4. Abundance of specific groups of β-proteobacterial ammonia-oxidizing bacteria: *Nitrosomonas* spp. estimated by Nsm156-hybridized cell counts (black diamonds), and *Nitrosospira* spp. estimated by Nsv443-hybridized cell counts (white diamonds) at Stations A, B, C, E and F. Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 6.5. Abundance of autotrophic ammonia-oxidizing bacteria in the Guaymas Basin water column at the South Site: (a) β-proteobacterial ammonia-oxidizing bacteria (βAOB) estimated by NSO190- (black triangles) and NSO1225- (red triangles) cell counts, and the abundance of γ-proteobacterial ammonia-oxidizing bacteria (γAOB) estimated by Nscoc128 cell counts (green triangles), (b) Total abundance of ammonia-oxidizing bacteria as the sum of the βAOB and γAOB, and (c) abundance of the two main groups of βAOB, *Nitrosospira* (dark blue) and *Nitrosomonas* (light blue). Abundance data at the North Site: (d) βAOB estimated by NSO190- (black triangles) and NSO1225- (red triangles) cell counts, and the abundance of γAOB estimated by Nscoc128 cell counts (green triangles), (e) Total abundance of ammonia-oxidizing bacteria, and (f) abundance of *Nitrosospira* (dark blue) and *Nitrosomonas* (light blue). Symbols represent mean values from duplicate subsamples (20-field counts per subsample), while error bars indicate standard errors.
Figure 6.6. Bootstrapped (1000 resamplings) neighbor-joining distance tree based on the 16S rRNA gene sequenced from clone libraries constructed from samples collected from water column of Guaymas Basin and Endeavour Segment. The numbers on the branches show the % bootstrap support greater than 50%.

--- 0.01 substitutions/site

**Guaymas Hydrothermal Fluids**
- Guaymas North Site Plume FL
- Guaymas North Site Plume PA
- Guaymas South Site Plume FL
- Guaymas South Site Plume PA
- Guaymas Background FL
- Guaymas Background PA
- Endeavour Plume FL
- Endeavour Plume PA

**FL** = Free-living (0.2-3.0μm)
**PA** = Particle-associated (>3.0μm)

---

**Nitrososira sp. NsP12**
- Nitrososira sp. Ka4
- Nitrososira sp. NsP1
- Nitrososira briensis NsP1
- pHi4.2A112
- NBR298741

**Nitrosomonas europaen**
- Nitrosomonas marina
- Nitrosomonas cryotolerans

**Gallionella ferruginea**
- 346GsFL4
- 153GsFL4
- Ultramicrobacterium str. ND5
- Herbaspirillum lusitanum
- Glacier bacterium
- Uncultured Nitrosomonas-like bacterium

**Obligately oligotrophic bacteria**
- POCPN-S
- Uncultured freshwater bacterium

**Enderavour Plume**
- FL
- PA

**Weser estuarine bacterium**
- AY145571

**Uncultured bacterium in equine fecal contamination**
- AYZJZ674

**Uncultured methylotroph-like bacterium**
- AY4U8ZJ

**Uncultured ammonia-oxidizer-like bacterium in lake sediments**
- AFJ51570

**Methylobacillus pratensis**

**Methylophagamarina**

---

**0.01 substitutions/site**
Guaymas Sediment Core A Top 1 cm
Guaymas Sediment Core C Top 1 cm
Guaymas Sediment Core C Surface Water
Guaymas Hydrothermal Fluids PA
Guaymas Plume PA
Guaymas Background PA
Endeavour Plume PA
Endeavour Background

*PA = Particle-associated (>3.0 μm)

**Figure 6.7.** Bootstrapped (1000 resamplings) neighbor-joining distance tree of βAOB based on the amoA gene sequenced from clone libraries constructed from the water column samples of the Guaymas Basin and the Endeavour Segment, as well as the Guaymas Basin hydrothermal sediment samples. The numbers on the branches indicate the % bootstrap values (>50%).
Figure 6.8. Bootstrapped (1000 resamplings) neighbor-joining distance tree constructed from partial sequences of the 16S rRNA gene amplified from Guaymas Basin hydrothermal sediment samples and closely related sequences obtained from BLAST searches. The numbers on the branches indicate the % bootstrap support over 50%.
Figure 6.9. Bootstrapped neighbor-joining phylogenetic tree based on the amoA gene of γ-proteobacterial ammonia-oxidizing bacteria sequenced from clone libraries constructed from the supernatant water overlying hydrothermal sediment samples (Core C) of the Guaymas Basin. The numbers on branches show the % bootstrap values that are over 50% after 1000 resamplings.
CHAPTER SEVEN

Conclusions and Future Perspectives
This research project aimed to compare the fate and implications of the elevated NH$_4^+$ in the deep-sea hydrothermal plumes at the unsedimented Endeavour Segment, Juan de Fuca Ridge, and the sedimeted Guaymas Basin, Gulf of California. It has illustrated that autotrophic ammonia-oxidizing bacteria are likely widespread in the deep sea with or without hydrothermal influence, and how certain seafloor hydrothermal systems may influence nitrogen and carbon cycling in the deep-sea water column.

7.1. Ubiquity of Autotrophic Ammonia-Oxidizing Bacteria in the Deep Sea

The application of fluorescence in situ hybridization (FISH) successfully detected autotrophic ammonia-oxidizing bacteria (AOB) in the ammonium-enriched deep-sea hydrothermal plumes at the Endeavour Segment and Guaymas Basin, in the hydrothermal fluids and in the overlying background deep water (Chapter 3 to 6). Amplification of the genes encoding 16S rDNA and ammonia monoxygenase subunit A (amoA) also revealed AOB-like sequences within the Guaymas Basin sediments, as well as some perhaps novel groups of β-Proteobacterial AOB in the water columns at both locations. Hence, autotrophic ammonia-oxidizing bacteria, the key players responsible for the first and rate-limiting step of nitrification (Kowalchuk and Stephen, 2001), are most likely ubiquitous in the deep sea, with or without the influence from hydrothermal inputs (Hypothesis 1).

Although the 16S rRNA gene sequences amplified from the water columns form a group distinctly different from known AOB species, the similar phylogenies shown by the functional gene (amoA) sequence analyses support the possibility that these sequences may form a novel lineage of ammonia-oxidizing bacteria (Chapter 6). However, this possibility would require verification of their ammonia-oxidizing capabilities, incorporating simultaneous gene expression and phylogenetic analyses for multiple (functional and 16S rRNA) genes, as well as culture-dependent physiological studies.
Since the fluxes of particulate organic matter in the oceans decrease dramatically with depth (exponentially in the upper layers and perhaps linearly in the deeper layers) (Honjo, 1996), nitrification activities and AOB abundance, though present, are expected to be low in the deep pelagic. The injection of ammonium via hydrothermal plumes has provided an exception and has undoubtedly stimulated the growth of these AOB (Chapters 3 to 5), which are able to conserve nitrifying capacity and to quickly resuscitate (Bodelier et al., 1996; Tappe et al., 1999). The abundance of AOB reached a maximum of $16 \pm 1.8 \times 10^3$ cells ml$^{-1}$ in the Endeavour plume, or $8.0 \pm 0.9 \times 10^3$ cells ml$^{-1}$ in the Guaymas Basin plume, compared to $0.7-3.5 \times 10^3$ cells ml$^{-1}$ in the above-plume background deep water (Chapters 3 to 5). These quantitative data support the second part of Hypothesis 1, i.e. the abundance of AOB is enhanced in the Endeavour and Guaymas Basin hydrothermal plumes.

7.2. Ammonia Oxidation in Deep-Sea Hydrothermal Plumes

The high ammonium concentrations in deep-sea hydrothermal plumes have clearly stimulated autotrophic ammonia oxidation in the water column of both the Endeavour Segment (Chapters 3 and 4) and the Guaymas Basin (Chapter 5), to the highest rates ever reported at such ocean depths. Ammonia oxidation rates reached 91 nM d$^{-1}$ within the Endeavour plume (Chapters 3 and 4), and potentially 517 nM d$^{-1}$ within the Guaymas Basin plume (Chapter 5). These plume rates are up to twenty times greater than those measured in the background deep water at Endeavour, though the enhancement is less dramatic in the Guaymas Basin. The productive surface water in the Guaymas Basin confers to the underlying background deep water a relatively large supply of particulate organic matter, which is continuously being degraded and releasing ammonium. This results in ammonia oxidation rates up to $142 \pm 27$ nM d$^{-1}$, which is
obviously higher than that in the Endeavour background deep water, but still lower than the maximum rate measured within the Guaymas plume (Chapter 5). These observations are consistent with Hypothesis 2, that is, autotrophic ammonia oxidation is enhanced within the hydrothermal plumes at these two locations.

The most notable difference between the hydrothermal systems along the Endeavour Segment and in the Guaymas Basin is the presence of an organic-rich sediment cover in the latter. The hydrothermal fluid discharges must pass through these decomposing organic-rich sediments and carry with them remineralized ammonium along with the continuously degrading organics into the water column. Heterotrophic organisms had the opportunity to thrive in the organic-rich water, while autotrophic ammonia-oxidizing bacteria (AOB) no longer had the ecological advantage of making their own organic carbon as in the less organic-rich Endeavour hydrothermal plume. Since AOB are known to be slow-growing (Prosser, 1989), their ammonia oxidation rates accounted for a smaller percentage of total net ammonium loss in the Guaymas Basin plume (≥21%) than in the Endeavour plume (≥93%). This agrees with Hypothesis 3, which states that autotrophic ammonia oxidation is the major sink of the elevated ammonium in the Endeavour hydrothermal plume, but less so in the Guaymas Basin hydrothermal plume.

Ammonia oxidation within the Endeavour hydrothermal plume in the axial valley potentially produces an amount of organic carbon (0.6-13 mg C m\(^{-2}\) d\(^{-1}\)) up to 1300% of photosynthetic organic carbon reaching plume depths from the surface ocean, and is likely higher than methanotrophic production (0.4-6 mg C m\(^{-2}\) d\(^{-1}\)) (De Angelis et al., 1993). In the Guaymas Basin plume, ammonia oxidation may contribute to 5-25 mg C m\(^{-2}\) d\(^{-1}\) of in situ organic carbon production, comparable to particulate organic carbon flux.
from the surface ocean (2-50 mg C m\(^{-2}\) d\(^{-1}\)) (Thunell, 1998). However, large amounts of organic carbon, though not quantified in this study, are also available in the Guaymas Basin hydrothermal discharges and sediments. Therefore, while autotrophic ammonia oxidation seems to be a significant source of de novo organic carbon in the Endeavour hydrothermal plume, it seems to be less important in the Guaymas Basin hydrothermal plume in comparison (Hypothesis 4).

Lastly, Hypothesis 5 proposed that autotrophic ammonia oxidation would be enhanced in particle-associated size-fractions, because at least certain groups of AOB were known to have an affinity towards particle-associations (Stehr et al., 1995; Hagopian and Riley, 1998) and niche-differentiation has been previous reported in the marine environments (Phillips et al., 1999). The comparison between ammonia oxidation rates with and without removal of large particles (> 3 \(\mu\)m in diameter) did not yield very consistent results (Chapters 4 and 5). Some of these inconsistencies might be attributed to the potential artifacts of removing, from the incubations, particles that act as a continuous source of remineralized ammonium. The total net ammonium loss rates measured would thus be overestimated. Therefore, the test results for this hypothesis are inconclusive. However, different phylogenetic groups of AOB evidently show different degrees of particle-associations in both the Endeavour and Guaymas Basin water columns. \(\beta\)-Proteobacterial AOB are clearly inclined towards particle-association (> 3 \(\mu\)m diameter), while most \(\gamma\)-Proteobacterial AOB are free-living (< 3 \(\mu\)m diameter). Among the \(\beta\)-Proteobacterial AOB, \textit{Nitrosomonas} have a greater tendency of particle-association, but \textit{Nitrosospira} are important in both the particle-associated and free-living size-fractions.
7.3. Implications and Future Perspectives

The injection of ammonium from seafloor hydrothermal systems to the hydrothermal plumes at the Endeavour Segment and the Guaymas Basin undoubtedly stimulates autotrophic ammonia oxidation in the deep-sea water column. This ammonium also supports the nitrogen requirements of active biological communities (assimilation) in the water column, which consequently promotes active ammonium recycling. Hence, the overall nitrogen cycling in the deep-sea water column is accelerated. Although the amounts of ammonium being introduced from the seafloor hydrothermal systems do not appear significant compared to the massive deep-sea nitrate reservoir (677 Tg N) (Capone, 1991, 2000), they are significant at a local level in relation to particulate nitrogen exports from the surface ocean reaching these deep ocean depths (Chapters 4 and 5).

Nitrous oxide, a more effective greenhouse gas than carbon dioxide, is a by-product of ammonia oxidation. Recent findings suggest that nitrification (ammonia oxidation) is likely the primary source of deep-sea nitrous oxide (Bange and Andreae, 1999; Nevison et al., 2003). Bange and Andreae (1999) also predict an unaccounted source of nitrous oxide (3.8 Tg N₂O y⁻¹) in waters less than 2000 m deep. Since both the Endeavour and Guaymas Basin hydrothermal plumes lie within this depth range, they may also contribute, at least to some extent, to this missing source of nitrous oxide. The production of nitrous oxide within these and other hydrothermal systems can be an interesting area for future research.

Organic matter is the major independent variable that regulates between autotrophic ammonia oxidation versus heterotrophic ammonium uptake (Strauss and Lamberti, 2000; Strauss et al., 2002). The different degrees of organic richness between
the Endeavour and Guaymas Basin hydrothermal plumes, in addition to the different water residence time, likely have caused most of the differences observed in the respective ammonia oxidation rates and AOB abundance. As heterotrophic nitrifiers were reported to be present in the Guaymas Basin hydrothermal environments (Mével et al., 1996), their activities and interactions with autotrophic nitrifiers need to be better characterized. More detailed characterization of, and experimentation with, the organic compounds present, along with microbiological studies of both bacterial groups, may shed light on the regulation of such autotrophic versus heterotrophic activities.

Furthermore, intense degradation of organic matter may induce suboxic and anoxic conditions, which can lead to denitrification and anaerobic ammonia oxidation. Recent developments in molecular biological techniques such as microarrays have been shown to be efficient tools to simultaneously analyze the presence or expression of various functional genes involved in the nitrogen and carbon cycles (Taroncher-Oldenburg et al., 2003). As various nitrogen cycling processes are often coupled with one another, multidisciplinary approaches combining sensitive geochemical (such as stable isotopic tracers) and molecular biological techniques are necessary to promote our understanding of the complex antagonistic and synergetic relationships among the different nitrogen cycling processes. The ammonia oxidation and assimilation studies presented here only provide a glimpse of some very active nitrogen cycling processes occurring in two deep-sea hydrothermal plumes. Similar processes may also take place in other deep-sea ammonium-enriched settings like sediments or back-arc basins, to which more future research may be directed.
REFERENCES


