Organic Copper Binding Ligands and Thiol Compounds Produced by Bacteria and in the Elizabeth River, Virginia

Christina Louise Dryden
Old Dominion University

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ORGANIC COPPER BINDING LIGANDS AND THIOL COMPOUNDS PRODUCED BY BACTERIA AND IN THE ELIZABETH RIVER, VIRGINIA

by

Christina Louise Dryden
B.S. May 1996, Salisbury University

A Dissertation Submitted to the Faculty of Old Dominion University in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

OCEANOGRAPHY

OLD DOMINION UNIVERSITY
August 2004

Approved by:

Joh R. Donat (Co-Director)

Andrew S. Gordon (Co-Director)

Kenneth Mopper (Member)
ABSTRACT

ORGANIC COPPER BINDING LIGANDS AND THIOL COMPOUNDS PRODUCED BY BACTERIA AND IN THE ELIZABETH RIVER, VIRGINIA

Christina Louise Dryden
Old Dominion University, 2004
Co-Directors of Advisory Committee: Dr. John R. Donat
Dr. Andrew S. Gordon

This dissertation presents work focusing on copper and organic copper binding ligands in laboratory cultures and the Elizabeth River, Virginia. Laboratory cultures of the marine bacterium *Vibrio parahaemolyticus* were used to demonstrate the influence of elevated copper concentrations on copper-complexing ligand and thiol production. Copper-complexing ligands similar in binding strength to the strongest natural ligands were detected in *V. parahaemolyticus* cultures (log $K'_{\text{cul}} = 11.8-13.2$). A strong correlation ($r^2 = 0.973$) was found between total thiol and copper-complexing ligand concentrations at all copper concentrations examined.

A yearlong seasonal study was undertaken in a heavily polluted estuary to ascertain seasonal variations and correlations between dissolved thiols, copper-complexing ligands, and total dissolved copper. Copper-complexing ligands and thiol compounds were found to vary seasonally and corresponded to seasonal changes in abundance of bacterioplankton, autotrophic picoplankton, and chlorophyll $a$, suggesting a biological source. Data indicate thiol compounds contribute to the ligand pool with conditional stability constants (log $K'_{\text{cul}}$) between 11.7 -12.6.

A simple box model was developed for total copper in the Elizabeth River, Virginia using data from two estuarine transect cruises, the yearlong seasonal study, and limited
point source information. The two estuarine transect cruises recorded total dissolved copper concentrations increasing up the Elizabeth River from $6.6 \pm 1.0 \text{ nmol L}^{-1}$ to $50.7 \pm 0.9 \text{ nmol L}^{-1}$. Results revealed a net statistically significant input of total dissolved copper to the river as a whole. This result suggests that an important copper uptake process has not been considered since no evidence indicates total dissolved copper concentration is increasing with time.

A series of in situ experiments in the Elizabeth River, Virginia revealed that an intact estuarine microbial community responded to copper stress by production of extracellular high-affinity copper-complexing ligands. The rate of ligand production was dependent on copper concentration and resulted in a reduction of the concentration of free cupric ions, $\text{Cu}^{2+}$, by more than three orders of magnitude during a 2-week period in one experiment. This interactive response to copper stress may represent a feedback system through which microbial communities can potentially buffer dissolved $\text{Cu}^{2+}$ ion concentrations, thereby regulating copper bioavailability and toxicity.
ACKNOWLEDGMENTS

My deepest gratitude is for members of my dissertation committee who made this research possible and contributed significantly to my education at Old Dominion University: to Dr. John Donat for supplying enthusiasm and confidence, and challenging me to become a better analytical and environmental chemist; to Dr. Andy Gordon for providing encouragement, financial support, and teaching me about the realm of biology and microbiology; to Dr. Ken Mopper, for providing insight, creativity, and suggestions.

Sincere thanks to the other “Tinas”. Bettina - your steady friendship has helped me overcome many obstacles, thanks for the hours of study times, coffee breaks, and long talks. Martina – I’m inspired by your ambition and zest for life, thanks for listening.

I wish to thank present and past members of the ODU family for invaluable assistance, knowledge, and friendship: John Consolvo, Pete Morton, Senya Piraneo, Lisa Drake, Dave Burdige, Margie Mulholland, Skip Stiles, Gonzalo Carrasco, Brian Dyer, Duncan Byers, and Li Zhang. Thanks to the staff of the Departments of: Ocean, Earth, and Atmospheric Sciences; Chemistry and Biochemistry; and Biological Sciences.

I am especially grateful to my mother, Bonnie Bach, and grandmother, Jeune Rockenbaugh, for their unwavering support and love. My appreciation, admiration, and affection for you both can never be fully expressed in words. To my loving husband, James Dryden: No amount of thanks could ever repay your patience and continued support. Thank you for your generosity, love, and moral support! Funding provided by ODU’s Department of Ocean, Earth, and Atmospheric Sciences, Office of Naval Research’s Harbor Processes Program (N00014-99-1-0093 to A. S. Gordon and J. R. Donat and N00014-99-0386 to J.R. Donat and D.J. Burdige).
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>viii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ix</td>
</tr>
</tbody>
</table>

## Chapter

I. INTRODUCTION ..............................................................................................1

II. BACKGROUND ................................................................................................6

   - Study area ................................................................................................ 6
   - Copper toxicity .......................................................................................... 6
   - Organic complexation of copper ................................................................ 11
   - Sources/sinks of copper binding ligands ............................................... 13
   - Chemical characteristics of copper binding ligands ............................. 15
     - General chemical characteristics ...................................................... 15
     - Specific chemical nature .................................................................... 17
   - Hypothesis ............................................................................................... 21
   - Objectives and rationale ........................................................................ 21

III. PRODUCTION OF COPPER-COMPLEXING LIGANDS AND THIOLS
     BY THE HETEROTROPHIC BACTERIUM
     VIBRIO PARAHAEEMOLYTICUS ................................................................ 23

     - Introduction .......................................................................................... 23
     - Materials and methods .......................................................................... 25
       - Chemostat cultures of *V. parahaemolyticus* .................................. 25
       - Chemostat sampling and copper additions ........................................ 27
       - Total dissolved copper determination .............................................. 30
       - Copper complexation/speciation determination ................................ 31
       - Copper complexing ligand and conditional stability constant calculations ................................................................................. 32
       - Thiol analysis ..................................................................................... 34
     - Results and discussion .......................................................................... 34
     - Conclusions .......................................................................................... 46

IV. COPPER-COMPLEXING LIGANDS AND THIOL COMPOUNDS IN
    THE ELIZABETH RIVER, VIRGINIA:
    A SEASONAL SURVEY ............................................................................. 49

     - Introduction .......................................................................................... 49
     - Materials and methods .......................................................................... 52
       - Sample collection and handling ....................................................... 52
       - Reagents ......................................................................................... 54
V. SIMPLE BOX MODEL FOR TOTAL DISSOLVED COPPER IN THE ELIZABETH RIVER, VIRGINIA

Introduction..........................................................88
Methods.................................................................89
Sample collection...................................................89
Total dissolved copper analysis................................89
Elizabeth River box model.........................................91

Results........................................................................94
Measured copper in Elizabeth River.........................94
Simple box model of total dissolved copper in Elizabeth River .....................................................97

Discussion................................................................97

VI. INTERACTIVE REGULATION OF DISSOLVED COPPER TOXICITY BY AN ESTUARINE MICROBIAL COMMUNITY

Introduction................................................................102
Materials and Methods..............................................104
Study location..........................................................104
Sample collection, pre-equilibration, and incubation.....104
Total dissolved copper determination........................107
Copper complexation and speciation analysis............107
Copper-complexing ligand and conditional stability
constant calculations .................................................107
Enumeration of bacterioplankton and autotrophic
picoplankton............................................................108

Results and discussion..............................................108

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Chapter Page

Initial incubation conditions and total dissolved copper recoveries ...........................................108
Copper-complexing ligand production .................................................................110
Copper-complexing ligands and Cu²⁺ .....................................................................113
Population density and copper additions ..........................................................120
Copyright ........................................................................................................122

VII. SUMMARY AND CONCLUSIONS .................................................................123

REFERENCES .........................................................................................................................128

APPENDIX ................................................................................................................................146
LETTER OF PERMISSION FOR CHAPTER VI ...................................................147

VITA ...........................................................................................................................................148
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Inorganic copper speciation in seawater at salinity 35, temperature 25 °C, and carbonate alkalinity of 2.09 × 10^{-3} mol L^{-1} (adapted from Byrne et al., 1988)</td>
<td>8</td>
</tr>
<tr>
<td>2.</td>
<td>Copper speciation in <em>Vibrio parahaemolyticus</em> chemostat cultures (BDL = below detection limit: L = 1.3 nmol L^{-1} and thiol_{TD} = 130 nmol L^{-1})</td>
<td>37</td>
</tr>
<tr>
<td>3.</td>
<td>Study site data collected over one year</td>
<td>62</td>
</tr>
<tr>
<td>4.</td>
<td>Thiol Concentrations (nmol L^{-1}) determined via Vairavamurthy and Mopper (1990a) method</td>
<td>74</td>
</tr>
<tr>
<td>5.</td>
<td>CLE/CSV titration data of known thiol compounds</td>
<td>82</td>
</tr>
<tr>
<td>6.</td>
<td>Summary of initial in situ data during experiments performed in the Elizabeth River, Virginia</td>
<td>109</td>
</tr>
<tr>
<td>7.</td>
<td>Total dissolved copper concentration, ligand concentration (C_{l}), and conditional stability constants (K_{cul}) in incubation bottles with 100 nmol L^{-1} copper added (no azide added)</td>
<td>111</td>
</tr>
<tr>
<td>8.</td>
<td>Total dissolved copper concentration, ligand concentration (C_{l}), and conditional stability constants (K_{cul}) in incubation bottles with 200 nmol L^{-1} copper added (no azide added)</td>
<td>111</td>
</tr>
</tbody>
</table>
TABLE OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>This figure illustrates how increasing free cupric ion concentrations influences relative reproductive rates of four open ocean phytoplankton (adapted from Brand et al., 1986)</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Illustration of chemostat system</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>Flow diagram of experimental procedure used for sampling and adding copper to chemostat</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Influence of increasing copper concentration on bacterial concentration in chemostat</td>
<td>29</td>
</tr>
<tr>
<td>5.</td>
<td>Reaction of thiols with Ellman’s reagent (DTNB)</td>
<td>35</td>
</tr>
<tr>
<td>6.</td>
<td>Variation of the copper-complexing ligand (L) concentration produced in V. parahaemolyticus cultures (Trial 1 ▼, Trial 2 ■, Trial 3 ●, and Trial 4 △) with the total dissolved copper (CuTD) concentration (mean ± standard deviation; n = 3)</td>
<td>38</td>
</tr>
<tr>
<td>7.</td>
<td>Variation in free cupric concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ●, Trial 4 △, No ligand present ◊) with total dissolved copper (CuTD) concentration</td>
<td>40</td>
</tr>
<tr>
<td>8.</td>
<td>Total thiol concentration versus total dissolved copper concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ●, Trial 4 △) (mean ± standard deviation; n = 3)</td>
<td>44</td>
</tr>
<tr>
<td>9.</td>
<td>Total dissolved thiol versus copper-complexing ligand concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ●, Trial 4 △) (mean ± standard deviation; n = 3)</td>
<td>45</td>
</tr>
<tr>
<td>10.</td>
<td>Map of Elizabeth River, Virginia with study site indicated by black circle</td>
<td>53</td>
</tr>
<tr>
<td>11.</td>
<td>Chromatograms at 0.015 absorbance units full scale (AUFS)</td>
<td>58</td>
</tr>
<tr>
<td>12.</td>
<td>Temperature, salinity, and chlorophyll a conditions at the sampling site over the yearlong study</td>
<td>63</td>
</tr>
<tr>
<td>13.</td>
<td>Autotrophic picoplankton (□) and bacterioplankton (●) seasonality</td>
<td>65</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>14.</td>
<td>Concentrations of total dissolved copper ($C_{UD}$) in the Elizabeth River</td>
<td>66</td>
</tr>
<tr>
<td>15.</td>
<td>Concentrations of total dissolved copper ($C_{UD}$, □) and departure from normal precipitation (□) in the Elizabeth River</td>
<td>68</td>
</tr>
<tr>
<td>16.</td>
<td>Concentration of copper-complexing ligand (L) in the Elizabeth River</td>
<td>69</td>
</tr>
<tr>
<td>17.</td>
<td>Concentration of copper-complexing ligand (●) and surface water temperature (--) in Elizabeth River during October 2002 to September 2003</td>
<td>71</td>
</tr>
<tr>
<td>18.</td>
<td>Values of free cupric ion, $Cu^{2+}$, in the Elizabeth River</td>
<td>72</td>
</tr>
<tr>
<td>19.</td>
<td>Plot of the concentration of total thiol compounds as a function of copper-complexing ligand concentration in the Elizabeth River</td>
<td>76</td>
</tr>
<tr>
<td>20.</td>
<td>Concentration of mercaptosuccinic acid in surface water (n = 3) between October 2002 and September 2003</td>
<td>77</td>
</tr>
<tr>
<td>21.</td>
<td>Concentration of mercaptoethanol in surface water (n=3) between October 2002 and September 2003</td>
<td>79</td>
</tr>
<tr>
<td>22.</td>
<td>Reaction of iodoacetic acid thiol blocking agent with a thiol</td>
<td>85</td>
</tr>
<tr>
<td>23.</td>
<td>Map of Elizabeth River sampling sites for July 1999 and May 2000</td>
<td>90</td>
</tr>
<tr>
<td>24.</td>
<td>Box model with all quantifiable and non-quantifiable copper sources and sinks</td>
<td>92</td>
</tr>
<tr>
<td>25.</td>
<td>Concentration of total dissolved copper measured during July 1999 and May 2000</td>
<td>95</td>
</tr>
<tr>
<td>26.</td>
<td>Concentration of total dissolved copper at yearlong study site slightly north of station 6</td>
<td>96</td>
</tr>
<tr>
<td>27.</td>
<td>Seasonal changes in total dissolved copper concentration based on yearlong data and applied to four reservoirs of the box model</td>
<td>98</td>
</tr>
<tr>
<td>28.</td>
<td>Net flux of total dissolved copper concentration in Mid and lower river (Net = sum Cu In - sum Cu removed)</td>
<td>99</td>
</tr>
<tr>
<td>29.</td>
<td>Net input of total dissolved copper into whole Elizabeth River estuary</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure | Page
--- | ---
30. Map of Hampton Roads and the Elizabeth River | 105
31. Rates of ligand (L₁) production by intact microbial communities (solid symbols) and by azide killed controls (open symbols) as a function of copper concentration | 112
32. Rates of ligand (L₁) production by an intact microbial community incubated under ambient light exposure and in the dark (white bars are light bottles and black bars are dark bottles) | 114
33. Ligand (L₁) concentration as a function of time after A) 100 nmol L⁻¹ copper addition in three seasonal in situ incubations and B) corresponding free Cu²⁺ ion concentrations as a result of increased ligand concentrations in response to 100 nmol L⁻¹ copper addition | 115
34. Ligand (L₁) concentration as a function of time after A) 200 nmol L⁻¹ copper addition in three seasonal in situ incubations and B) corresponding free Cu²⁺ ion concentrations as a result of increased ligand concentrations in response to 200 nmol L⁻¹ copper addition | 118
35. A,B) Effect of copper addition on abundance of bacterioplankton and C,D) autotrophic picoplankton during in situ incubation of the intact microbial community | 121
CHAPTER I

INTRODUCTION

Like many trace metals, copper is released into coastal oceans, estuaries, and rivers from natural and anthropogenic sources. Copper is used in the manufacture of fungicides, pigments, water piping, and as a tributyltin (TBT) substitute in the formulation of marine antifouling paints. In addition, copper is released into the aquatic environment by natural sources such as volcanoes, decaying vegetation, and forest fires (Stumm and Morgan, 1996). In order to curtail anthropogenic inputs of chemicals (e.g., copper) into aquatic environments and minimize any potential adverse effects on the biology of rivers, lakes, and oceans, governments around the world have mandated regulatory controls on their release into domestic and industrial waste streams (USEPA, 1985, 1995).

Copper, although a micronutrient, at elevated concentrations can cause a toxic response in bacteria (Sunda and Ferguson, 1983), phytoplankton (Brand et al., 1986), crab larvae (Sanders et al., 1983), and copepod larvae (Sunda et al., 1987). With its known toxic effects, copper is routinely monitored in the evaluation of environmental risk (USEPA, 1985, 1995). Advances in analytical and sampling techniques have revealed that copper released in the environment undergoes a number of different chemical reactions thereby changing its chemical form or species. Dissolved copper may exist as free cupric ions (Cu$^{2+}$), inorganic complexes (e.g., with Cl$^{-}$, OH$^{-}$, CO$_3^{2-}$, and SO$_4^{2-}$), and complexes with various organic ligands (e.g. humic substances, phytoplankton metabolites, proteins, etc.). Studies have shown that dissolved copper is

This dissertation follows the format of the journal Marine Chemistry.
predominately organically complexed (90 to 99%) in most natural waters (van den Berg et al., 1987; Coale and Bruland, 1990; Moffett et al., 1990; Sunda et al., 1990; Donat et al., 1994; Moffett, 1995). By dominating copper speciation, organic complexation may control copper’s bioavailability (by controlling Cu$^{2+}$ activity the toxic form of copper), its reactivity with suspended particles, and ultimately govern its biogeochemical cycling in seawater (Donat et al., 1986; Sunda and Hanson, 1987; Moffett and Zika, 1987; Coale and Bruland, 1988, 1990; Moffett et al., 1990; Donat and van den Berg, 1992).

The source and chemical characteristics of these natural organic ligands controlling copper’s speciation are still under investigation. However, recent laboratory and field studies investigating the source of these natural organic ligands suggest a biological origin (Moffett et al., 1990; Bruland et al., 1991; Gordon et al., 1993; Moffett and Brand, 1996; Croot et al., 2000; Dryden et al., 2004). Sources of organic copper binding ligands not produced by marine organisms also exist in coastal environments. Estuarine sediment porewaters, sewage effluents, and estuarine and coastal humic substances have been examined as potential sources (Skrabal et al., 1997; Sedlak et al., 1997; Kogut and Voelker, 2001; Voelker and Kogut, 2001). Skrabal et al. (1997) and Shank et al. (2004a) found that ligands in estuarine sediment porewaters had binding characteristics similar to the strong copper binding ligands found in the water column. Suwannee River humic and fulvic acids (Xue and Sigg, 1999; Kogut and Voelker, 2001) as well as humic substances isolated from an organic-rich estuary (Shank et al., 2004b) have been used to demonstrate that terrestrial humic substances may make up a significant fraction of the strongest copper binding ligands in coastal systems. To date whether or not the strongest organic copper binding ligands are of planktonic or nonplanktonic origin is unknown.
Although the complete chemical nature of the copper-complexing ligands in natural waters remains unknown, preliminary investigations into the basic chemical characteristics of organic copper chelators have revealed common qualities. The main qualities found are that oceanic ligands are generally hydrophilic (>90%) (Mills et al., 1982; Hanson and Quinn, 1983; Donat et al., 1986) and of low molecular weight, <1000 Daltons (Da) (Gordon, 1992; Gordon et al., 1996; Wells et al., 1998; Midorikawa and Tanoue, 1998; Tang et al., 2001). Shank et al. (2004b) found that ligands isolated from an organic-rich estuary were hydrophobic suggesting chemical structural differences between oceanic and organic-rich estuarine ligands. Ross et al. (2003) and Vachet and Callaway (2003) explored the structural character of unknown copper-complexing ligands using immobilized metal affinity chromatography (IMAC) coupled with mass spectrometry. These two studies reveal isotopic patterns suggesting either nitrogen and oxygen functionality or sulfur, nitrogen, and oxygen functionality. Laglera and van den Berg (2003), Tang et al. (2001), Leal et al. (1999), and Leal and van den Berg (1998) investigated thiol compounds and found they might constitute a major part of the natural copper-complexing ligand pool. As these investigations demonstrate, the chemical characteristics and/or structure(s) of natural organic copper-complexing ligands remain an unidentified.

This dissertation is presented in the chronological order that I carried out the research. The subsequent paragraphs describe briefly each of three research chapters, which will be submitted to peer-reviewed journals for publication. Chapters III and IV are the principal chapters of my dissertation and the research is my independent work with guidance from dissertation committee. Chapters V and VI present research that I
carried out as a Graduate Research Assistant. These chapters present work that I performed under the direction of Dr. David Burdige (Chapter V) and Dr. Andrew Gordon (Chapter VI). Prior to the research chapters, Chapter II offers a background of the study area, copper, and copper-complexing ligands.

Leal et al. (1999) examined production organic copper-complexing ligands and thiol compounds in cultures of *Emiliania huxleyi* (Coccolithophores) finding similar production rates and concentrations with copper stress between copper-complexing ligands and thiol compounds. Recently there is increasing literature evidence that copper-complexing ligands might be thiol compounds or perhaps some other sulfur containing species (Leal and van den Berg, 1998; Rozan et al., 2000; Tang et al., 2001; Laglera and van den Berg, 2003). To support these recent findings, I undertook a laboratory study to expand the work of Leal et al. (1999) to another marine organism (a common heterotrophic marine bacterium, *Vibrio parahaemolyticus*), modify, and refine the thiol analysis to more definitively look at total thiol compounds. In Chapter III, I present the modifications I made to the Leal et al. (1999) laboratory study and my results.

After I began the laboratory study, I also started an analogous investigation of organic copper-complexing ligands and thiol compounds in a natural setting. Although existing investigations of organic copper-complexing ligands in the field have improved the understanding of concentrations in different marine environments, a seasonal perspective is lacking (Moffett et al., 1990; Moffett, 1995; Croot, 2003; Dryden et al., 2004). In Chapter IV, I describe a seasonal study in the Elizabeth River, Virginia along with an evaluation of the relationship between organic copper-complexing ligands and thiol compounds.
While working on the laboratory and field studies, a separate modeling effort began incorporating some of the fieldwork. Chapter V discusses the development of a simple box model to examine total dissolved copper in the Elizabeth River, Virginia. Data that were collected from the seasonal study in Chapter IV and a collaborative effort, funded by the Office of Naval Research Harbor Processes Program, studying the dynamics of copper in a coastal harbor were supplied to this model to determine the gaps in our knowledge about dissolved copper cycling in estuaries.

Finally, Chapter VI examines the dynamics of copper-complexing ligand production by natural microbial assemblages under realistic environmental conditions in situ, thus avoiding potential pitfalls inherent to extrapolation of laboratory studies to field conditions. Chapter VI has been published in *Limnology and Oceanography* and is reprinted here with permission (Appendix).
CHAPTER II

BACKGROUND

Study area

The Elizabeth River, a three-branched sub-estuary of the James River, is located in the Hampton Roads area of southeastern Virginia and is the Chesapeake Bay's southernmost tributary (Hargis et al., 1984). As a major deep-water port that is highly industrialized, the Elizabeth River has served for over 300 years as a site of civilian and military shipbuilding, shipping and shore side commerce, and associated manufacturing and processing. Due to pollution from heavy industry and shipping activities in the Elizabeth River, the Chesapeake Bay Program in 1983 identified the Elizabeth River system as one of the most heavily polluted regions of the Chesapeake Bay watershed (Chesapeake Bay Program, 1994; Huggett et al., 1984).

Copper toxicity

Copper is an essential trace metal, however, excess copper in a biologically available chemical form can have deleterious biological effects (Sunda and Ferguson, 1983; Brand et al., 1986; Donat and Bruland, 1995). The impact of copper on biological systems include changes in cell volume, inhibition of CO$_2$ fixation (Pandey and Singh, 1992), and inhibition of nitrate uptake and synthesis of nitrate reductase (Harrison et al., 1977; Morel and Price, 2003). Due to these effects, copper is one of the metals regularly investigated in the evaluation of environmental risk. Copper, like other metals, can enter the environment via natural processes as well as from anthropogenic sources, e.g. byproducts

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of industrial and agricultural activities. Sediment metal levels can show effects of industry and port activity, such as dredging, across an estuary. The necessity of dredging to maintain navigable harbors and waterways produces hundreds of millions of tons of spoil annually, some of which are enriched with metals. This waste must be maintained or disposed of in an environmentally sound manner. Separation of anthropogenic from natural source copper can be a difficult process. In many studies, discrete sources cannot be identified, particularly in heavily populated and industrialized, multi-use and highly complex areas such as estuaries.

Studies of nutrient/toxic effects of copper have generally focused on the dissolved fraction of the total pool, because this fraction interacts most directly with phytoplankton. Dissolved copper can be present in three general forms, expressed by the following mass balance equation:

\[
[Cu_T] = [Cu^{2+}] + [CuX] + [CuL]
\]  

(1)

where \([Cu^{2+}]\) is the concentration of the free, solvated copper ion \((Cu^{2+} \cdot (H_2O)_n)\), \([CuX]\) is the concentration of the copper bound in complexes with inorganic ligands (e.g., \(Cl^-\), \(OH^-\), \(CO_3^{2-}\), \(SO_4^{2-}\), etc.), and \([CuL]\) is the concentration of copper complexed by organic ligands (humic and fulvic substances, proteins, phytoplankton metabolites, various organic ligands). The inorganic speciation of copper (as represented by Eq. 2) in natural waters is well known (Byrne et al., 1988) (see Table 1).

\[
[Cu_{org}] = [Cu^{2+}] + [CuX]
\]  

(2)

Early studies with phytoplankton and other aquatic organisms demonstrated that the concentration of the free cupric \((Cu^{2+})\) ion, not the total copper concentration, controlled toxicity (Sunda and Guillard, 1976; Anderson and Morel, 1978; Brand et al., 1986). The
Table 1
Inorganic copper speciation in seawater at salinity 35, temperature 25 °C, and carbonate alkalinity of $2.09 \times 10^{-3}$ mol L$^{-1}$ (adapted from Byrne et al., 1988).

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride complex ($\text{CuCl}^+$)</td>
<td>2</td>
</tr>
<tr>
<td>Hydrated, free ion ($\text{Cu}^{2+} \cdot (\text{H}_2\text{O}_n)$)</td>
<td>5</td>
</tr>
<tr>
<td>Hydroxide complex ($\text{CuOH}^+$)</td>
<td>8</td>
</tr>
<tr>
<td>Carbonate complex ($\text{CuCO}_3$)</td>
<td>85</td>
</tr>
</tbody>
</table>
idea that the toxicity and bioavailability of copper could be controlled by the
concentration (or activity) of its ionic form and that organic copper complexes are
nonbioavailable (will not pass through microorganism cell membrane) are the
foundations of the "free ion model" (Sunda and Guillard, 1976; Anderson and Morel,
1978; Brand et al., 1986; Campbell, 1995). Lipophilic metal complexes, known to be
taken up by microorganisms by passive diffusion across the cell membrane (Tubbing et
al., 1994; Phinney and Bruland, 1994, 1997a, b), are the only organic complexes that
counter the "free ion model". Brand et al. (1986) illustrated how phytoplankton
reproductive rates can be influenced by the free cupric ($Cu^{2+}$) ion (Fig. 1). At a $[Cu^{2+}]$ of
$10^{-12}$ M (1 pmol L$^{-1}$), Brand et al. (1986) found reproductive rates beginning to decrease
in the cyanobacteria *Synechococcus bacillaris*, the most sensitive open ocean
phytoplankton species. While other *Synechococcus* species did not show a decrease in
reproductive rate until log $[Cu^{2+}]$ $\sim$ -11 (10 pmol L$^{-1}$), many of the phytoplankton species
examined by Brand et al. (1986) did not exhibit a toxic response to $Cu^{2+}$ until
concentrations reached the nanomolar (nmol L$^{-1}$; log $[Cu^{2+}]$ = -9) level. The
phytoplankton-$Cu^{2+}$ sensitivity study by Brand et al. (1986) revealed the following trend:
cyanobacteria were the most sensitive at $[Cu^{2+}]$ of 1 pmol L$^{-1}$, diatoms were the least
sensitive at $[Cu^{2+}]$ of 1 nmol L$^{-1}$, and coccolithophores and dinoflagellates showed
intermediate sensitivity. Thus, changes in free $Cu^{2+}$ activities could cause shifts in
species composition and succession within a phytoplankton community. Other marine
organisms such as bacteria (Sunda and Ferguson, 1983); crab larvae (Sanders et al.,
1983); and copepod larvae (Sunda et al., 1987) can be affected by even relatively low
$Cu^{2+}$ concentrations (15 to 30 pmol L$^{-1}$).
Fig. 1. This figure illustrates how increasing free cupric ion concentrations influences relative reproductive rates of four open ocean phytoplankton (adapted from Brand et al., 1986). The free cupric ion affects phytoplankton relative reproductive rates in the following order: cyanobacteria, dinoflagellates, coccolithophores, and diatoms.
Organic complexation of copper

Recent organic speciation work with copper shows that the organically complexed fraction dominates (94 to >99%) the dissolved speciation of copper in most natural waters, and is critically important in controlling the free cupric ion concentrations. By dominating copper speciation, organic complexation may control copper's bioavailability (by controlling Cu$^{2+}$ activity) and its reactivity with suspended particles. As a result, copper's biogeochemical cycling in seawater is influenced by organic complexation (Sunda and Ferguson, 1983; van den Berg, 1984; Buckley and van den Berg, 1986; Donat et al., 1986; Sunda and Hanson, 1987; Moffett and Zika, 1987; Coale and Bruland, 1988, 1990; Moffett et al., 1990; Donat and van den Berg, 1992; Donat et al., 1994). One biogeochemical cycling process affected by organic complexation is the adsorption of dissolved copper onto mineral surfaces. Laboratory studies have demonstrated that organic ligands can both increase copper adsorption, through coadsorption of copper and organics, and decrease copper adsorption, through competition between the dissolved organic ligands and surface binding sites for copper (Davis and Leckie, 1978; Davis, 1984; Zachara et al., 1995a, b).

Organic copper binding ligands have been operationally divided into strong ($L_1$ class - log $K'_{CuL.1} > 11$) and weaker ($L_2$ class - log $K'_{CuL.2} \sim 8-10$) ligand classes based on their conditional stability constants (see Eqs. 3 and 4) (Bruland et al., 1991; Donat et al., 1994; Donat and Bruland, 1995).

\[
Cu^{2+} + L_n \rightarrow CuL_n
\]  

(Eq. 3)

\[
K' = \frac{[CuL_n]}{[Cu'][L_n']}
\]  

(Eq. 4)

where $K'$ is the conditional stability constant, [Cu'] is the copper available for binding.
with the organic ligand and [L'] is organic ligand available to bind copper. When the \( L_1 \) concentration exceeds that of total copper, \( CuL_1 \) complexes dominate overall copper speciation. If the total copper concentration exceeds that of \( L_1 \), \( CuL_2 \) complexes can dominate copper speciation (e.g., South San Francisco Bay, Donat et al., 1994; several areas of Chesapeake Bay, Donat, 1994). Depending upon the concentration of these ligand classes relative to that of total dissolved copper and their relative binding strengths, either class may control copper speciation. In areas where anthropogenic inputs of copper exceed the concentration of the strong ligand class, a resulting increase in free copper may be observed (e.g., naval harbors) (Moffett et al., 1997; Blake et al., 2004). Sensitive phytoplankton species from the microbial community may be eliminated as free copper increases (Moffett et al., 1997).

Several researchers have argued that distinctions between \( L_1 \) and \( L_2 \) are arbitrary and this class division needs to be rethought (Mackey and Zirino, 1994; Zirino et al., 1998; Voelker and Kogut, 2001; Town and Filella, 2000). Specifically this distinction between \( L_1 \) and \( L_2 \) needs to change when examining waters (coastal and estuarine) where heterogeneous mixtures of compounds with a range of ligand properties exist (Voelker and Kogut, 2001). With no clear resolution for the arguments against operational division of the ligand classes, my dissertation research focused on the strongest ligand class \( (L_1) \) and no way implies insignificance of the weaker ligand \( (L_2) \) class. However, for data interpretation assuming a single ligand would bring simplicity to complicated results. More studies supporting the arguments against the \( L_1/L_2 \) classification need to be completed before the field of chemical oceanography discards this traditionally used classification.
Sources/sinks of organic copper ligands

The sources of organic copper binding ligands in seawater are not well known. However, compelling laboratory data indicate that marine organisms such as phytoplankton and bacteria can produce strong organic copper binding ligands. Laboratory studies with marine organisms have shown the synthesis and release of extracellular chelators, such as siderophores, to enhance metal (i.e., iron) assimilation (Davis and Byers, 1971; Lewis et al., 1995; Reid et al., 1993; Trick, 1989). Intracellular chelators, such as phytochelatins/metallothionein, to alleviate metal stress upon exposure to toxic metals have also been observed in the lab (Grill et al., 1985; Huckle et al., 1993; Ahner and Morel, 1995; Ahner et al., 1994, 1995, 1997). Another source of organic ligands could be from cell surface membrane complexation sites that facilitate metal uptake at cell surfaces. For example, iron studies have suggested that iron uptake is regulated by iron complexation at the cell membrane by specific, surface-associated sites (Anderson and Morel, 1982; Hudson and Morel, 1990). This type of metal uptake by ligands on a cell membrane surface has been demonstrated in diatoms and dinoflagellates by metal uptake experiments (Fisher and Wente, 1993).

Laboratory studies investigating copper-complexing ligand production by copper stressed aquatic organisms have focused on photoautotrophic microorganisms, i.e., *Emiliania huxleyi* (Leal et al., 1999; Vasconcelos et al., 2001), *Amphidinium carterae* (Croot et al., 2000), and the marine cyanobacterium *Synechococcus* (Moffett et al., 1990; Moffett and Brand, 1996; Gordon et al., 2000). The extracellular copper chelators produced during these laboratory experiments have binding characteristics comparable to the strong ligands, class L1, found in the water column (Moffett et al., 1990; Bruland et
The marine cyanobacterium *Synechococcus* is perhaps the most commonly studied component of the marine microbial population in these ligand production surveys with the most ignored component being heterotrophic bacteria. The ubiquitous heterotrophic marine bacterium, *Vibrio alginolyticus*, has been shown to produce extracellular chelators in response to copper (Schreiber et al., 1990; Gordon et al., 1993; Gordon et al., 2000). Hirose and Tanoue (2001) also investigated strong ligands produced by heterotrophic bacteria (*Alteromonas* sp., *Vibrio* sp., *Pseudomonas* sp., and *Flavobacterium* sp.) and found they could be potential iron chelators, and they proposed that the source for the strong ligands associated with particulate and dissolved organic matter in seawater could be microbial.

Other sources for organic copper binding ligands in coastal environments include estuarine sediment porewaters, sewage effluents, and terrestrial humic substances (Skrabal et al., 1997; Sedlak et al., 1997; Kogut and Voelker, 2001, Voelker and Kogut, 2001). Skrabal et al. (1997) found that ligands in estuarine sediment porewaters had binding characteristics similar to the water column strong copper binding ligands, L₁. Kogut and Voelker (2001) used Suwannee River humic acid and fulvic acid to demonstrate that terrestrial humic substances may make up a significant fraction of the L₁ strength copper binding ligands in coastal systems. Whether the strongest organic copper binding ligands, L₁, are of planktonic origin, nonplanktonic origin, or both is not precisely known.

Sinks for organic copper binding ligands have not been well defined by research or experimentation and discussion in the literature is limited. However, photochemical and...
microbial degradation processes known to break down other dissolved organic compounds in the marine environment, so these processes are likely candidates for the degradation of organic copper binding ligands (Miller and Moran, 1997).

Chemical characteristics of organic copper-complexing ligands

General chemical characteristics

The exact chemical nature and characteristics of naturally occurring and laboratory produced copper-complexing ligands is unknown and under investigation. Preliminary investigations into the basic chemical characteristics of organic copper chelators have revealed low molecular weight (<1000 Da) compounds (Gordon, 1992; Gordon et al., 1996; Wells et al., 1998; Midorikawa and Tanoue, 1998; Tang et al., 2001). Using immobilized metal affinity chromatography (IMAC), Gordon et al. (1996) found that UV-absorbing material with an affinity for copper isolated from estuarine waters has a nominal molecular weight <1000 Daltons (Da). In Narragansett Bay, Kozelka and Bruland (1998) discovered that most of the strongest copper binding chelators (class L1) would pass through 8000 nominal molecular weight cut-off (NMWCO) filters and ~ 50% pass through 1000 NMWCO filters. Midorikawa and Tanoue (1998) found the following distribution of molecular masses of oceanic copper-complexing organic ligands in Japanese coastal waters: low molecular masses (<1000 Da) were dominant (49-62%), while 26-33% of the total organic ligands were in the 1000-10,000 Da fraction, leaving 10-19% in the >10,000 Da fraction. Tang et al. (2001) size fractionated (0.45μm and <1000 Da) Galveston Bay samples and found that >99.9% of the dissolved Cu was bound by strong organic ligands in both size fractions. Exploring the molecular weight of
copper-complexing ligands by size fractionation provides indispensable information since little is known about exact chemical characteristics or structures.

Oceanic copper binding ligands have been found to be generally hydrophilic (>90%) (Mills et al., 1982; Hanson and Quinn, 1983; Donat et al., 1986). Shank et al. (2004b) investigated copper binding ligands from organic-rich estuaries and found that these ligands were hydrophobic (100%). Shank et al. (2004b) suggests that the hydrophilic and hydrophobic character discrepancy between oceanic and estuarine ligands indicates a structural difference.

Ross et al. (2003) and Vachet and Callaway (2003) combined the selective extraction ability of IMAC with mass spectrometry to explore the structural character of copper-complexing ligands from coastal and estuarine waters. Ross et al. (2003) used flow injection electrospray ionization mass spectrometry (FI-ESI-MS) to examine IMAC fractions from the coastal waters of British Columbia; their results suggest small peptides and/or other nitrogen-containing compounds, and the isotopic distribution rules out the presence of sulfur. Vachet and Callaway (2003) used high-performance size-exclusion chromatography (HPSEC) and ESI-MS to examine IMAC fractions. Vachet and Callaway (2003) analyzed Chesapeake Bay waters to find a heterogeneous mix of weakly binding compounds with molecular weights ranging from about 230 to >20,000 Da. The molecular weights suggest these ligands could be humic in origin as suggested by Kogut and Voelker (2001). However, the stronger binding ligands were less heterogeneous with molecular weights below 1600 Da and isotopic patterns suggesting sulfur, nitrogen, and oxygen functionality (Vachet and Callaway, 2003). Unfortunately, neither one of these studies performed a copper titration to determine conditional stability constants on the
IMAC isolate so the exact binding and ligand class \((L_1\) or \(L_2\)) is unknown. Yet, these techniques do present interesting findings that further our understanding of natural copper-complexing ligands and if used in coordination with methods to measure copper binding strengths perhaps exact chemical structures could be discovered.

*Specific chemical nature*

Suggestions of the existence of specific chelators accounting for copper binding ligands observed in natural waters have been offered by numerous authors (Hirose, 1994; Gordon, 1998). As discussed previously, a biological source (Moffett et al., 1990; Bruland et al., 1991; Moffett and Brand, 1996; Gordon and Dyer, 1994; Gordon et al., 1996, 2000) and nonbiological source (Sedlak et al., 1997; Kogut and Voelker, 2001, Voelker and Kogut, 2001) for these chelators has been suggested. Survey of recent literature reveals hypotheses about their chemical nature. One idea is that humic substances account for a major proportion of the copper binding ligands observed in estuarine and coastal waters (Moffett et al., 1997; Xue and Sunda, 1997; Xue and Sigg, 1999; Kogut and Voelker, 2001, 2003; Voelker and Kogut, 2001; Shank et al., 2004b). The other hypothesis is that thiol compounds are the major ligand for copper in natural waters because sulfhydryl groups are one of the strongest known complexing ligands for copper (Leal et al., 1998, 1999; Al-Farawati and van den Berg, 2001; Tang et al., 2001; Laglera and van den Berg, 2003). It is implied that each of these chemical compounds comes from a different source: humic substances derived from terrestrial detritus (Powell and Town, 1991) and thiol compounds resulting from biological production (Leal et al., 1999; Al-Farawati and van den Berg, 2001). The possibility exists that estuarine and coastal waters are composed of heterogeneous mixtures of compounds (Voelker and
Kogut, 2001) and both humic substances and thiol compounds as copper binding ligands could be observed. A critical look at humic substances and thiol compounds as copper-complexing ligands could unveil new information or shortcoming not yet considered.

Terrestrial humic substances have typically been thought of as a component of the weaker ligand class \((L_2 - \log K'_{CuL_2} \sim 8-10)\) in rivers and estuaries (Moffett et al., 1997; Sedlak et al., 1997; Xue and Sunda, 1997; Rozan et al., 1999). However, the latest laboratory and field evidence suggests that a portion of the terrestrial humic substances may exhibit a high affinity for copper typical of \(L_1\) class ligands. Xue and Sigg (1999) and Kogut and Voelker (2001) carried out copper speciation analyses on fulvic and humic acid solutions made from Suwannee River standards (SRFA and SRHA – International Humic Substances Society standards) and determined strong binding characteristics with concentrations typical of marine waters. In a freshwater matrix, the Suwannee River humic standards gave conditional stability constants \((K'_{CuL}) 10^{12.5-14.1}\) similar to the strongest binding class \((L_1)\) with concentrations \(-30\) nmol L\(^{-1}\) (Xue and Sigg, 1999). In a seawater matrix, SRFA and SRHA were also found to have conditional binding strengths similar to \(K'_{CuL_1} (10^{12-13.2})\) with concentrations ranging from 1.1 to 10.4 nmol L\(^{-1}\) (Kogut and Voelker, 2001). Shank et al. (2004b) examined strong copper complexation in an organic-rich estuary (Cape Fear River) and compared that data with the copper complexation of humic substances extracted from the same estuary. Shank et al. (2004b) found humic substances isolated from the Cape Fear River exhibited strong copper binding \((\log K'_{CuL} \sim 13.5)\) with a copper binding ligand \((L)\) concentration ranging from 6 to 143 nmol L\(^{-1}\). The isolated humic material’s copper-complexing ligand concentrations were linearly related to humic-derived dissolved organic carbon (DOC) \((r^2\)
Concentrations of copper binding ligands from the humic substance could account for all of the ligands in the Cape Fear (Shank et al., 2004b). It is necessary to evaluate this recent evidence for humic substances as strong copper binding ligands by reassessing analytical work in organic-rich environments. These results do not imply that SRFA and SRHA are only composed of the stronger ligand class ($L_1$). During the copper speciation analyses by Xue and Sigg (1999) and Kogut and Voelker (2001), a weaker ligand class ($L_2 \sim 137-250$ nmol L$^{-1}$) was also obtained indicating that the humic substances are complex materials composed of both the stronger and weak ligand classes.

Kogut and Voelker (2001) stated that a SRHA study by Hering and Morel (1988) using anodic stripping voltammetry (ASV) was unable to detect copper binding ligand concentrations lower than 50 nmol L$^{-1}$ thereby missing the lower concentration component that would contribute to the $L_1$ class in coastal waters. This statement is incorrect since an intercomparison of voltammetric techniques demonstrated that ASV gave similar copper binding ligand concentrations (13-16 nmol L$^{-1}$) as the CLE/ACSV (used by Kogut and Voelker, 2001). The main limitation in the works of Xue and Sigg (1999) and Kogut and Voelker (2001) was that they were only laboratory studies of SRHA and SRFA and did not directly determine if these humic substances contribute to the strong copper-complexing ligand pool of the Suwannee River. Shank et al. (2004b) isolated humic substances from the Cape Fear River and then determined their contribution to the strong copper binding ligand pool. However, Shank et al. (2004b) did not examine the original Cape Fear sample for potential artifacts in speciation measurements associated with the isolation and storage of collected humic substances because the original sample was discarded.
Thiols are widespread in coastal, estuarine, and oceanic waters (surface water concentrations ranging from 0.3 to 60 nmol L\(^{-1}\)) (Anderson et al., 1988; Matrai and Vetter, 1988; Vairavamurthy and Mopper, 1990a, b; Luther, et al., 1991; Al-Farawati and van den Berg, 2001; Laglera and van den Berg, 2003). Leal and van den Berg (1998) found conditional stability constants (\(\log K_{\text{CuL}} \approx 12\)) for cysteine and glutathione in the laboratory similar to the strong copper ligand class (\(L_1\)). The distribution of thiols in natural waters was related to strong copper-complexing ligands in Galveston Bay (Tang et al., 2001) and Scheldt estuary (Laglera and van den Berg, 2003). Tang et al. (2001) suggest that the thiols found in Galveston Bay could account for most of the copper-complexing ligands, whereas Laglera and van den Berg (2003) found that thiols could account for up to half of the total ligand concentration at low to intermediate salinities and for all of the ligands at high salinities. Al-Farawati and van den Berg (2001) studied thiols in coastal waters of the western North Sea and English Channel but did not analyze copper binding ligands at the same time. However, thiol concentrations were found to parallel those of chlorophyll and low thiol concentrations were found in low salinity waters (river water) entering the coastal systems demonstrating that thiols are produced by phytoplankton and not from riverine input. A direct biological connection and relationship between production of thiols and strong copper-complexing ligands by *Emiliania huxleyi* (Coccolithophores) was demonstrated by Leal et al. (1999).

The shortcoming in Leal et al. (1999), Tang et al. (2001), Al-Farawati and van den Berg (2001), and Laglera and van den Berg (2003) is use of a proxy in the identification of the "thiol-like" species. All of these studies use some variation of Le Gall and van den Berg (1993, 1998) and Al-Farawati and van den Berg (1997) CSV technique to identify
thiols in oxic waters. Tang et al. (2001) acknowledges this CSV technique has difficulties in identifying individual reduced sulfur compounds and used glutathione as the equivalent thiol species during titrations. Leal et al. (1999) and Laglera and van den Berg (2003) used a combination of glutathione and thioacetamide to identify the “thiol-like” peak. Al-Farawati and van den Berg (2001) used thiourea during the CSV analysis. They admit thiourea and thioacetamide are not candidates for the identity of the thiol peak but presently the thiol compound responsible for thiol (or thiol-like) peak is unknown so any thiol compound could be used. Although many of these studies relate the “thiol-like” peak to copper binding ligands no studies have been conducted to detect individual thiol compounds and relate these to copper binding ligands.

Hypothesis

Thiol compounds are important copper-complexing ligands in cultures of heterotrophic bacteria under copper stress and individual thiol compounds control the speciation of copper in estuarine waters of the Elizabeth River, Virginia.

Objectives and rationale

The primary goals of this research are to contribute to the understanding of the role individual thiol compounds and further strengthen the role that heterotrophic bacteria play in trace metal speciation. The objectives of this research are to:

1. determine the copper-complexing ligands in cultures of heterotrophic bacteria and the Elizabeth River, Virginia;
2. determine the concentration of individual thiol compounds;
3. evaluate the importance of individual thiol compounds in controlling the speciation of trace metals;

4. examine seasonality of individual thiol compounds and copper-complexing ligands in the Elizabeth River, Virginia;

5. assess possible sources of individual thiol compounds and copper-complexing ligands in the Elizabeth River, Virginia by correlation with biological parameters (chlorophyll $a$, autotrophic picoplankton and bacterioplankton abundance).

The rationale for this research was the major shortcoming in all the thiol work presented previously. Previous studies (Leal et al., 1999; Tang et al., 2001; Al-Farawati and van den Berg, 2001; Laglera and van den Berg, 2003) relate copper binding ligands to CSV “thiol-like” peak(s) but none of these studies investigates individual thiol compounds and their relationship to copper binding ligands. In addition, no studies have looked at seasonality of thiol compounds and copper-complexing ligands. Perhaps examination of individual thiol compounds plus seasonality could improve our lack of information about the dissolved concentrations of thiols in oxic waters and the role that they might play in controlling copper speciation in estuarine waters.

The lack of literature support for heterotrophic bacteria as strong copper binding ligand producers and no previous literature confirmation of thiol production by heterotrophic bacteria are the other research justifications. In heterotrophically dominated areas like the Chesapeake Bay and Elizabeth River, Virginia (Dorworth, 1989; Affronti, 1990; Affronti and Marshall, 1993), copper speciation might be controlled by heterotrophic bacteria rather than autotrophic phytoplankton.
CHAPTER III
PRODUCTION OF COPPER COMPLEXING LIGANDS AND THIOLS BY THE MARINE HETEROTROPHIC BACTERIUM

VIBRIO PARAHAEOMLYTICUS

Introduction

Copper is essential for marine organisms but can be toxic at elevated concentrations (Gillespie and Vaccaro, 1978). The bioavailability of copper is a function of its chemical species and the primarily toxic form is free cupric ion, \( \text{Cu}^{2+} \) (Anderson and Morel, 1978; Brand et al., 1986; Sunda, 1994). Complexation of copper by organic and inorganic ligands influences the concentration of \( \text{Cu}^{3+} \); \( \text{Cu}^{2+} \) concentrations decrease as the extent of complexation increases. In most surface waters, 90 to 99\% of the dissolved copper is strongly complexed by organic ligands (van den Berg et al., 1987; Sunda et al., 1990; Donat et al., 1994; Moffett, 1995). The source of these organic ligands is still under investigation but recent laboratory and field studies suggest a biological origin (Moffett et al., 1990; Bruland et al., 1991; Gordon et al., 1993; Moffett et al., 1997; Croot et al., 2000; Dryden et al., 2004).

Laboratory studies centered on biological ligand production have focused on photoautotrophic microorganisms, i.e., *Emiliania huxleyi* (Leal et al., 1999; Vasconcelos et al., 2001), *Amphidinium carterae* (Croot et al., 2000), and the marine cyanobacterium *Synechococcus* (Moffett et al., 1990; Moffett and Brand, 1996; Gordon et al., 2000). With the marine cyanobacterium, *Synechococcus* being the most commonly studied. A few ligand production surveys have investigated the heterotrophic component of the
aquatic microorganism population and one confirmed production of the strongest copper-complexing ligands found in natural waters (Schreiber et al., 1990; Gordon et al., 1993, 1998, 2000). Copper ligands derived from heterotrophic bacteria could play an important role in coastal and estuarine environments according to a study by Skrabal et al. (1997), which found a large source of benthic copper ligands fluxing out of sediment porewaters. Hirose and Tanoue (2001) also investigated strong ligands produced by heterotrophic bacteria (Alteromonas sp., Vibrio sp., Pseudomonas sp., and Flavobacterium sp.) and found they could be potential iron chelators. Hirose and Tanoue (2001) proposed that the source for the strong ligands associated with particulate and dissolved organic matter in seawater could be these marine microbially derived strong ligands.

Little is known about the composition of these naturally or laboratory produced ligands. The generally accepted basic chemical characteristics are that the copper-complexing ligands are generally hydrophilic and low molecular weight (Hanson and Quinn, 1983; Donat et al., 1986; Gordon et al., 1996; Wells et al., 1998; Midorikawa and Tanoue, 1998; Tang et al., 2001). Using the latest mass spectrometry techniques, Ross et al. (2003) and Vachet and Callaway (2003) speculated on ligand isotopic distributions as containing sulfur, nitrogen, and oxygen. Other studies by Leal et al. (1999), Leal and van den Berg (1998), Tang et al. (2001), and Laglera and van den Berg (2003) have focused their efforts on proving that natural copper-complexing ligands are thiol compounds. Clearly, the recent attention between thiol compounds and naturally produced copper-complexing ligands indicates a developing theme. This chapter describes my laboratory studies regarding a relationship between thiols and copper-complexing ligands in a culture of common heterotrophic marine bacteria.
Materials and methods

Chemostat cultures of *V. parahaemolyticus*

Axenic cultures of *V. parahaemolyticus* (clone BB22) were used to inoculate the experimental cultures (Fig. 2). Initially, bacteria were grown in small (20 ml) batch cultures until a sufficient cell density (10⁸ cell ml⁻¹) was reached. These batch cultures were then used to inoculate a one-liter chemostat (Bio-Flo III, New Brunswick Scientific). *V. parahaemolyticus* was cultured in artificial seawater medium (ASWM) with no added chelating ligand (e.g., EDTA or NTA) to avoid potential interference with the measurement of the microbially produced ligands (Gordon et al., 1993). Water used for reagent and culture preparation was Milli-Q reagent grade water with a resistivity equal to 18.2 MΩ cm⁻¹ (Millipore). ASWM medium was made with Instant Ocean (20 g per liter, Aquarium systems, Mentor, Ohio), amended with NH₄Cl (19 mmol L⁻¹), Na₂HPO₄ (0.15 mmol L⁻¹), and glucose (4 mmol L⁻¹, Sigma-Aldrich) as the only carbon source. The ASWM was filtered through a 0.22 μm filter (MSI, Fisher Scientific) and sterilized further by autoclaving. Copper standards were prepared by dilution from 1000 ppm atomic absorption standard (Fisher Scientific) and then acidified to pH ~2 with HCl (Trace Metal Grade, Fisher Scientific). To avoid precipitation during autoclaving, all nutrient and copper solutions were 0.22 μm filtered, autoclaved, and added separately. Sterile and trace metal clean techniques were used during the addition of nutrients and copper to the ASWM. Prior to inoculation of the chemostat, the entire chemostat vessel was sterilized via autoclaving. To maintain the sterile integrity of the chemostat, nutrients plus added copper and NaOH were added via the chemostat’s sterile pumping system.
Fig. 2. Illustration of chemostat system. Copper additions are made to the nutrient reservoir, which flows into the one-liter chemostat. The one-liter chemostat is continuously stirred at 100 rpm and sampled every two days during incubations.
Cultures were continuously regulated to a temperature of 25°C and pH of 7.5 by addition of NaOH. Air was bubbled through the culture at 1 liter per minute and stirring was set to 100 rpm. The cell density was maintained at 5-8 x 10⁸ cell ml⁻¹ with a dilution rate of 0.11 h⁻¹ (providing a doubling time of 6.3 hours) (Gordon et al., 1998). The dilution rate (0.11 h⁻¹) provided by the flow of the nutrient reservoir insured that every 24 hours the chemostat was exchanged 2.5 times. Cell densities and culture purity were monitored daily to observe growth and axenicity. Cell densities were measured by optical density and culture purity was monitored by streak plating on tryptic soy agar (Difco) (Gordon et al., 1998).

Four individual experiments (or trials) were run in the chemostat. With only one chemostat available the trials were run in sequence and thus are not true replicates.

**Chemostat sampling and copper additions**

Sub-samples (400 ml) of culture supernatants were collected from the chemostat using the chemostat’s peristaltic pumping system (Fig. 2). Prior to any copper additions, sub-samples of the chemostat were taken before and after *V. parahaemolyticus* inoculation. Collection of sub-samples occurred before copper was added and copper was added to the nutrient reservoir every two days (Fig. 3). The desired concentrations of total dissolved copper in the chemostat were 200, 500, 700, and 1000 nmol L⁻¹; in order to achieve these concentrations and overcome copper loss to the nutrient reservoir pumping system, µmolar additions were added to the nutrient reservoir (Fig. 4). Both trace metal clean and aseptic techniques were used during sample collection. Once samples were collected, they were centrifuged at 10,000 rpm for 15 minutes, and the particle free supernatant was gently vacuum filtered through a 0.22 µm filter (MSI, Fisher
Fig. 3. Flow diagram of experimental procedure used for sampling and adding copper to chemostat. Prior to inoculation of the chemostat, it is completely filled (1 liter) with ASWM from the nutrient reservoir.
Fig. 4. Influence of increasing copper concentration on bacterial concentration in chemostat. The dark circles represent typical bacterial concentrations observed in trials 1 through 3 (mean ± standard deviation; n = 3). The open triangles represent the number of cells found in trial 4. The # indicates where sampling only occurred and * specifies where sampling followed by copper addition took place.
Scientific) to insure complete removal of bacterial cells and particulate material. Sub-
samples for thiol analysis were promptly collected in acid-cleaned high density
polyethylene (HDPE, Nalgene) bottle and immediately derivatized and analyzed
following the Ellman (1959) method (see Thiol analysis section below). Sub-samples for
copper complexation and speciation analysis were collected in two 250-ml acid-cleaned
fluorinated high density polyethylene (FLPE, Nalgene) bottles and frozen until analyzed.
Sub-samples for total dissolved copper determinations were collected in two 30 ml acid-
cleaned HDPE bottles, acidified to pH 2 with HCl (Optima Grade, Fisher Scientific), and
stored at room temperature until analyzed. All samples were stored double-bagged in
plastic zip-lock bags to minimize contamination. All total dissolved copper and copper
speciation samples were analyzed within 2 to 4 weeks of initial sampling.

Subsequent handling and analysis of samples occurred in trace metal clean, positive
pressure laboratories supplied with HEPA-filtered air, on lab benches supplied with
recirculated, HEPA-filtered air. All plasticware was washed using standard trace metal
clean techniques. Samples and plasticware on the lab benches were handled with clean
disposable plastic gloves.

Total dissolved copper determination

Total dissolved copper concentrations were determined using cathodic stripping
voltammetry (CSV) as described by Campos and van den Berg (1994). Briefly, total
dissolved copper samples (acidified to pH 2) were UV-irradiated (1.2 kW Hg-arc lamp;
Ace Glass) for 12 hours just prior to total dissolved copper determination. A 10 ml
aliquot of the acidified, UV photooxidized sample was pipetted into a Teflon cup and pH
neutralized with aqueous ammonia (Optima Grade, Fisher Scientific). The pH of the
aliquot was then buffered to pH 8.0 by adding 100 μl of a 1.3 mol L\(^{-1}\) 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS, Sigma-Aldrich) buffer solution and then 25 μl of 0.01 mol L\(^{-1}\) salicylaldoxime (SA) solution were added. The EPPS buffer solution and SA solutions were freshly prepared every week in Milli-Q water (resistivity 18.2 MΩ cm\(^{-1}\), Millipore). The sample was mounted to the hanging mercury drop electrode (HMDE), purged for 8 minutes with filtered, ultrapure N\(_2\) while stirring (700 rpm: “fast”). The deposition and equilibration step parameters were “large” Hg drop; deposition potential of -0.15 V (vs. Ag/AgCl); “fast” stirring; varied deposition time (from 30 to 120 s depending on copper concentration); 30 s equilibration time. During the stripping step, the potential was ramped negative from -0.15 to -0.70 V (vs. Ag/AgCl) at 20 mV s\(^{-1}\) in the differential pulse mode (5 pulse s\(^{-1}\), 50 mV pulse amplitude) (Donat et al. 1994; Campos and van den Berg 1994). The copper peak height was recorded after standard additions of copper were made.

Measurements were made using a PC controlled TraceLab 50 system (Radiometer Analytical, Lyon, France) consisting of a POL 150 Polarographic Analyzer connected to a MDE150 Polarographic Stand equipped with a HMDE. Peak heights were collected and measured using TraceMaster 5 software (Radiometer Analytical, Lyon, France).

*Copper complexation/speciation determination*

Applying the method of Campos and van den Berg (1994), competitive ligand equilibration-adsorptive cathodic stripping voltammetry (CLE/ACSV) was used for the determination of copper-complexing ligand concentrations and the conditional stability constants of the copper complexes. These CLE/ACSV methods have been used successfully by others (Donat and van den Berg 1992; Donat et al. 1994; van den Berg...
and Donat 1992; Bruland et al. 2000). Approximately 125 ml of the sample were transferred to an acid-cleaned Teflon bottle (FEP, Nalgene), 600μl of a 1.3 mol L⁻¹ EPPS buffer, and 25μl of a 0.01 mol L⁻¹ SA solution were added and well shaken. Ten milliliters of sample were pipetted into 12 Teflon cell cups and additions of copper were made to these twelve cups, covered, and left to equilibrate overnight (minimum of 8 hours). The instrumental settings were similar to those used for total copper determinations and can be found in the previous section, Total dissolved copper determination (Campos and van den Berg, 1994).

**Copper-complexing ligand and conditional stability constant calculations**

Data from CLE/ACSV measurements and Ruzic/van den Berg linearization were used to obtain ligand concentrations (C_L) and conditional stability constants (K'_{CuL}) (Campos and van den Berg, 1994; Ruzic, 1982; van den Berg, 1982). A comprehensive description regarding the calculations and theory utilized are presented in Campos and van den Berg (1994), Rue and Bruland (1995), and references therein. The following relationship was used for the linearization:

\[
\frac{[Cu^{2+}]}{[CuL]} = \frac{[Cu^{2+}]}{C_L + 1/(K'_{CuL}C_L)}
\]  

where [Cu^{2+}] is the concentration of free cupric ion, [CuL] is the concentration of copper complexed by natural ligands L, C_L is total detected ligand concentration, and K'_{CuL} (with respect to Cu^{2+}) is the conditional stability constant. [Cu^{2+}] and [CuL] are calculated from the following equations:

\[
[Cu^{2+}] = [CuSA]/\alpha' = i_p/(S\alpha')
\]  

\[
[CuL] = Cu_r - [CuSA] = Cu_r - i_p/S
\]

where [CuSA] is the concentration of copper complexed by the added ligand
salicylaldoxime (SA), $i_p$ is the Cu$^{2+}$ reduction peak current measure during CLE/CSV analysis, $S$ is the slope of the linear portion of the titration curve at Cu_T concentrations exceeding $C_L$, Cu_T is the concentration of total dissolved copper, and $\alpha'$ is the overall side reaction coefficient for Cu. $\alpha'$ is defined as

$$\alpha' = (\alpha_{Cu^\prime} + \alpha_{CuSA})$$

where $\alpha_{Cu^\prime}$ is the inorganic side reaction coefficient for copper and $\alpha_{CuSA}$ is the side reaction coefficient for complexation of copper by SA (Campos and van den Berg, 1994). In CLE/ACSV, the strength of the complex formed by the added ligand (e.g., salicylaldoxime) is defined by the side reaction coefficient, $\alpha_{CuSA}$. This analytical competition strength, $\alpha_{CuSA} = 3.9$, was set to detect the strongest class of Cu-binding ligands, L1, during all analyses (Bruland et al., 2000). Values for $C_L$ and $K'_{CuL}$ were calculated from the least-squares linear regression of the Ruzic/van den Berg plot (Donat et al., 1994; Campos and van den Berg, 1994; Ruzic, 1982; van den Berg, 1982).

An ion pairing computer program (C.M.G. van den Berg, Oceanography, Liverpool University [http://www.liv.ac.uk/\%7Esn35/Documents/Speciation.xls]) was used to calculate the free concentrations of the major inorganic anions forming complexes with copper and the stability constants of their copper complexes (with respect to Cu$^{2+}$), as a function of salinity, pH, and temperature. Using ligand concentrations and conditional stability constants obtained by CLE/ACSV and the data obtained from the ion pairing program, the overall copper speciation and free Cu$^{2+}$ ion concentrations were calculated with the chemical equilibrium modeling program MINEQL+© (version 4.06, Environmental Research Software).

*Thiol analysis*
The total dissolved thiol content was determined using 5,5'-dithiobiis-(2-nitrobenzoic acid) (DTNB) following Wright and Viola (1998) which is a modified version of the Ellman (1959) method. Ellman’s reagent (DTNB) is a commonly used derivatization reagent (Fig. 5) for quantifying thiols in peptides and proteins. It readily forms a mixed disulfide with thiols liberating 2-nitro-5-thiobenzoic acid (TNB, visible absorption around 412 nm) (Ellman, 1959; Shimada and Mitamura, 1994; Wright and Viola, 1998).

Standards or samples were derivatized by addition of 50μl DTNB (10 μmol L⁻¹ solution in 250 mmol L⁻¹ Tris-HCl, pH 7.5) and 2.5 ml of 250 mmol L⁻¹ Tris-HCl (pH 7.5)/ 4 mol L⁻¹ guanidine-HCl solution to 250 μl sample or standard. The absorbance was recorded at 412 nm, where DTNB derivatives have a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ (Varian, Cary). Calculations of thiol concentration used the following formula:

\[ C_o = \frac{A}{\varepsilon} D \]  

where \( C_o \) is original thiol concentration, \( A \) is absorbance at 412 nm, \( \varepsilon \) is the molar extinction coefficient 13,600 M⁻¹ cm⁻¹, and \( D \) is dilution factor (Ellman, 1959).

Results and discussion

*V. parahaemolyticus* abundance (measured as cell numbers) found in the chemostat cultures typically ranged from 7 to 9 × 10⁸ cells ml⁻¹ except for trial 4 which ranged from 4 to 5.5 × 10⁸ cells ml⁻¹ (Fig. 4). The batch culture for trial 4 started with much lower cell abundances than the other three trials and the trial commenced to see if lower cell abundance would alter the results of the experiment. As trial 4 developed the cell
Fig. 5. Reaction of thiols with Ellman’s reagent (DTNB).
abundances were nearly half those observed for trial all other trials, however, no
difference in response to added copper, copper-complexing ligand production,
conditional stability constant (log $K'_{CuL}$), or thiol production was observed (Table 2).
The overall effect of increasing the copper concentration every two days in the chemostat
culture (Fig. 3, 4) was a decrease in cell abundance from day three until the end of the
experiment (average reduction in cell abundance $23 \pm 2\%$; $n = 4$). A gradual cell number
decrease with incremental copper increases, and recovery periods between copper
additions, was also observed in a chemostat culture of *Vibrio alginolyticus* (Gordon et al.,
1993). This gradual decrease in cell abundance, as opposed to complete death of the
chemostat cultures, could be due to the adaptation of the chemostat population to copper
as observed by Gordon et al. (1993), or due to the Cu$^{2+}$ buffering capability of the
copper-complexing ligands (L) produced in the chemostat (Table 2; Fig. 4).

During the culture experiments, no detectable amount of ligand was found at the
beginning of each trial (Time 0, Table 2) and ligand increased with increasing total
dissolved copper (Fig. 6). The ligand increase was closely coupled to total dissolved
copper concentration for all trials of the *V. parahaemolyticus* chemostat cultures ($[L] =
(0.77 \pm 0.03) [Cu]_{TD} + (23 \pm 17); r^2 = 0.98$). This relationship is similar to the 1:1
(L:Cu$_{TD}$) relationship found by Moffett and Brand (1996) for *Synechococcus* and Croot et
al. (2000) for *Amphidinium*. The results are very similar to a *Vibrio alginolyticus*
continuous culture (Gordon et al., 2000); however, ligand concentrations in the *V.
parahaemolyticus* cultures (this study) was found to sometime exceed Cu$_{TD}$ (Table 2),
unlike in the *V. alginolyticus* culture. The ligand increase with increasing total dissolved
copper suggests metal detoxification via ligand production and lowering copper ion
Table 2

Copper speciation in *Vibrio parahaemolyticus* chemostat cultures (BDL = below detection limit: L = 1.3 nmol L\(^{-1}\) and thiol\(_{TD}\) = 130 nmol L\(^{-1}\)). Total copper ([Cu\(_{TD}\)]\(^{\circ}\)) represents what cultures were exposed to, not what was added to the nutrient reservoir. Sub-samples were collected at incubation day 0 and then every two days, prior to the nutrient reservoir receiving a copper addition (see Fig. 3). The CuL (%) column corresponds to the percentage of the total copper speciation that exists as the copper ligand complex.

<table>
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<tr>
<th>Incubation time (days)</th>
<th>[Cu(_{TD})] (nmol L(^{-1}))</th>
<th>[L] (nmol L(^{-1}))</th>
<th>log K(_{CuL}^{\circ})</th>
<th>[Cu(_{L})] (mol L(^{-1}))</th>
<th>CuL (%)</th>
<th>[thiol](_{TD}) (nmol L(^{-1}))</th>
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<td>12.17 ± 0.32</td>
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Fig. 6. Variation of the copper-complexing ligand (L) concentration produced in *V. parahaemolyticus* cultures (Trial 1 ▼, Trial 2 ■, Trial 3 ●, and Trial 4 △) with the total dissolved copper ([Cu]TD) concentration (mean ± standard deviation; n = 3). Linear regression of all four trials produces [L] = (0.77 ± 0.03) [Cu]TD + (23 ± 17); $r^2 = 0.98$. 

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availability in the culture (Moffett and Brand, 1996; Dryden et al., 2004).

To test the possibility that ligand production is a detoxification response, log [Cu\(^{2+}\)] was plotted versus total dissolved copper concentration to investigate whether or not the free cupric ion was lowered (Fig. 7). All four trials began with relatively similar free cupric ion concentrations (ranging from log [Cu\(^{2+}\)] -9.2 to -8.8) (Fig. 7). A lowering of the [Cu\(^{2+}\)] (ranging from -10.9 to -12.4) was observed in three out of four trials at total dissolved copper concentrations below 300 nmol L\(^{-1}\). Free cupric concentrations in trial 1 never decrease. The lowering of [Cu\(^{2+}\)] in trials 2, 3, and 4 can be attributed to organic complexation domination (100%) of the copper speciation thereby maintaining the free cupric ion concentrations below 10\(^{-10.9}\) mol L\(^{-1}\) or 12 pmol L\(^{-1}\) (% CuL complex column, Table 2). However, in trial 1 organic copper complexation never dominates copper speciation because the CuL complex maximum is 81% of the total speciation and the free cupric ion concentration exceeds 10\(^{-9}\) mol L\(^{-1}\) or 1 nmol L\(^{-1}\). All four trials remained below free cupric concentrations observed with no copper-complexing ligands present (white diamonds, [Cu\(^{2+}\)] = 10\(^{-8.8}\) mol L\(^{-1}\) or 1.5 nmol L\(^{-1}\)). Results suggest that without copper-complexing ligand production, the free cupric ion concentration could have been 10 times higher (Fig. 7). All of the trials returned to their initial free cupric concentrations or slightly higher with only a slight decrease in the cell abundance was observed indicating tolerance of higher copper concentrations at the end of the incubation (Fig. 4; Table 2). The V. parahaemolyticus production of copper-complexing ligands and lowering of the free cupric ion below [Cu\(_{TD}\)] of 300 nmol L\(^{-1}\) demonstrate adaptation (or a shift in population) of the chemostat population to copper. This short-term adaptation observation is similar to studies by García-Villada et al. (2004), Baos et al. (2002), and
Fig. 7. Variation in free cupric concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ●, Trial 4 △, No ligand present ◊) with total dissolved copper (CuTD) concentration. Error bars smaller than symbols.

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Richau et al. (1997). The study by Richau et al. (1997) found bacterial cultures were able to adapt to high copper concentrations under two days using stress-induced mutation. This copper stress could have triggered accelerated mutation due to a direct alteration of the DNA molecule or by activating a genetic program that is induced under stress (Tenaillon et al., 2004; Sniegowski, 2004). On the other hand, García-Villada et al. (2004) and Baos et al. (2002) found pre-adaptive spontaneous mutation occurred within two to four days in microalgal cultures under copper stress. Pre-adaptive spontaneous mutation (e.g., copper-resistant cells) occurs randomly during replication of organisms prior to addition of any stresses and allows for short-term adaptation when stresses are encountered (Baos et al., 2002). Which adaptation (stress-induced or pre-adaptive) occurred in the \( V. \) *parahaemolyticus* cultures is unknown, however, adaptation on short time scales (within two days) is an accepted occurrence in both bacterial and microalgal cultures (Richau et al., 1997; Baos et al., 2002; García-Villada et al., 2004; Tenaillon et al., 2004; Sniegowski, 2004). Perhaps without production of copper binding ligands \( V. \) *parahaemolyticus* cultures might have been overwhelmed by the free cupric concentration and this adaptation could not have occurred. Even though active copper binding ligand production is assumed, cell lysis during filtration cannot be ruled out. To minimize a possible cell lysis artifact, gentle filtration and centrifugation techniques were employed.

The estimates of the conditional stability constants (log \( K'_{\text{CuL}} \)) found in the \( V. \) *parahaemolyticus* cultures during the four trials ranged from 11.79 to 13.19 (only two log units). These values are similar to the strong conditional stability constants found by Gordon et al. (2000) for *Vibrio alginolyticus* and encompass those observed for
*Synechococcus* (cyanobacteria), *Amphidinium carterae* (dinoflagellate), and *Skeletonema costatum* (diatom) (Croot et al., 2000; Moffett et al., 1990; Moffett and Brand, 1996). Croot et al. (2000) discovered differences in the copper-complexing ligands (with identical conditional stability constants) produced by *Amphidinium* and *Skeletonema* by studying pseudopolarograms of each culture which revealed a wide range of half-wave potentials. The pseudopolarogram technique of Croot et al. (1999) was not used during this experiment, however it could provide added detail about the unknown copper-complexing ligands for use in future identifications.

Peaks were observed at -0.5 and -0.65 V (vs. Ag/AgCl) for culture samples in the presence of added copper but not in the culture medium alone. These peaks have potentials similar to the “thiol-like” peaks identified by Al-Farawati and van den Berg (1997), Leal et al. (1999), and Laglera and van den Berg (2003). An attempt was made to identify these unknown peaks using glutathione, cysteine, and thioacetamide, following the method of Al-Farawati and van den Berg (1997) and Leal et al. (1999). In addition to glutathione, cysteine, and thioacetamide, several other thiol standards (including mercaptoacetic acid, 2-mercaptoethanesulfonic acid (coenzyme M), 2-mercaptoethanol, 2-mercaptopropionic acid, 3-mercaptopropionic acid, mercaptosuccinic acid, and monothioglycerol) were used in an attempt to identify the unknown “thiol-like” peaks. The CSV method of Al-Farawati and van den Berg (1997) was not specific enough to quantify the “thiol-like” peaks observed in the *V. parahaemolyticus* samples because several potentials of known thiol standards overlapped and more than one standard made the unknown “thiol-like” peak increase in peak height. In these culture samples, the reduction peak of cysteine overlapped and interfered with that of glutathione, which was
detected and documented by Le Gall and van den Berg (1993). Vasconcelos et al. (2002) were able to quantify cysteine and glutathione in their samples without the glutathione and cysteine peaks overlapping using the Al-Farawati and van den Berg (1997) CSV method. Perhaps *V. parahaemolyticus* samples differed significantly from the Vasconcelos et al. (2002) culture samples therefore excluding the use of the CSV technique for thiol quantification (Al-Farawati and van den Berg, 1997). Leal et al. (1999) used glutathione and thioacetamide (avoiding the possible glutathione and cysteine interference) to determine the concentration of two “thiol-like” peaks in their culture titrations but go on to state that the calibration using thioacetamide may not be a good analogue for the thiols since other compounds have a peak potential similar to thioacetamide (i.e., lumichrome, Morelli and Scarano, 1994). Consequently, since a specific thiol standard could not be found to identify the “thiol-like” peaks using cathodic stripping voltammetry (CSV) (Al-Farawati and van den Berg, 1997; Leal et al., 1999), total dissolved thiols (thiol\textsubscript{TD}) were quantified using the Ellman (1959) method. An effort was made to quantify dissolved thiols via the HPLC method of Vairavamurthy and Mopper (1990a, b), however, this method was ineffective with culture samples due to unknown interferences in the culture or perhaps inaccessible sulfhydryl groups.

Total dissolved thiol (thiol\textsubscript{TD}; Ellman, 1959) increased with increasing Cu\textsubscript{TD} and the correlation was 1.5:1 (thiol\textsubscript{TD}:Cu\textsubscript{TD}) ([thiol]\textsubscript{TD} = (1.50 \pm 0.05) [Cu]\textsubscript{TD} + (50 \pm 28), Fig. 8). There was good correlation ($r^2 = 0.99$) between thiol\textsubscript{TD} and Cu\textsubscript{TD}. The thiol production appeared to increase significantly (one order of magnitude) with Cu\textsubscript{TD}. Vasconcelos et al. (2002) and Leal et al. (1999) used the cathodic stripping voltammetric (CSV) method of Al-Farawati and van den Berg (1997) to quantify total thiol concentration but did not
Fig. 8. Total thiol concentration versus total dissolved copper concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ●, Trial 4 △) (mean ± standard deviation; n = 3). Linear regression gave the following equation: \([\text{thiol}]_{TD} = (1.50 \pm 0.05) [\text{Cu}]_{TD} + (50 \pm 28) \) \( r^2 = 0.99, n = 13 \).
Fig. 9. Total dissolved thiol versus copper-complexing ligand concentration (Trial 1 ▼, Trial 2 ■, Trial 3 ○, Trial 4 △) (mean ± standard deviation; n = 3). Linear regression gave the following equation: $[\text{thiol}]_{TD} = (1.89 \pm 0.09) [L] + (42 \pm 45) r^2 = 0.97, n = 15$. 

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include thiol\textsubscript{TD} versus Cu\textsubscript{TD} plots since copper stressed cultures were not the focus of their investigations.

The concentration of thiol\textsubscript{TD} produced is nearly double the concentration of ligand produced (Fig. 9; Table 2), however, it increased proportionately with \( L \) (linear regression: [thiol]\textsubscript{TD} = (1.89 \pm 0.09) [L] + (42 \pm 45); \( r^2 = 0.97 \)). Covariations between \( L \) and total thiol similar to this study were found by Vasconcelos et al. (2002) and Leal et al. (1999) using a CSV method to quantify thiol concentrations (Al-Farawati and van den Berg, 1997). However, Vasconcelos et al. (2002) saw a thiol\textsubscript{TD}:L relationship of 1:1, Leal et al. (1999) detected nearly a 1:2 relationship, and in this \textit{V. parahaemolyticus} study a 2:1 relationship was observed.

**Conclusions**

This study demonstrates that the marine bacterium \textit{V. parahaemolyticus} can be a source of the strongest organic copper chelators similar to those that control copper speciation in most natural waters. Although \textit{V. parahaemolyticus} is more copper tolerant than the cyanobacterium \textit{Synechococcus}, copper-complexing ligands were still produced in response to elevated copper concentrations (i.e., lowering the free cupric ion), effectively ameliorating potentially toxic copper. In natural waters where anthropogenic inputs of copper are prominent (e.g., Elizabeth River, Virginia) or where copper sensitive organisms are not prevalent, marine heterotrophs could be the major biological source of organic copper chelators. Even though strong copper binding ligand production has been demonstrated by heterotrophic bacteria (Schreiber et al., 1990; Gordon et al., 1993, 1998, 2000), this current research advances these findings by revealing that strong copper
binding ligands produced by *V. parahaemolyticus* have a similar behavior to and compelling relationship with thiol compounds. While this thiol and copper binding ligand behavior and relationship observed with the heterotrophic bacterium *V. parahaemolyticus* could be fortuitous, similar observations have been noticed in other marine organisms like *Emiliania huxleyi* (Leal et al., 1999; Vasconcelos et al., 2002). This research provides further evidence that heterotrophic bacteria produce strong copper-complexing ligands, which is deficient in the literature with the exception of the efforts by Schreiber et al. (1990) and Gordon et al. (1993, 2000).

Specific thiols (i.e., glutathione) have been found in ocean and estuarine waters with concentrations and stability constants similar to the strong class of organic copper chelators (Le Gall and van den Berg, 1998; Tang et al., 2001; Leal and van den Berg, 1998). Leal et al. (1999) implicated thiols as a major part of the copper binding ligands produced by a coccolithophore *Emiliania huxleyi* using a CSV technique and thiol standard to identify the thiols that they admit “may not be a good analogue for the thiols occurring in the cultures”. My research identified thiols produced by heterotrophic marine bacteria using a commonly used technique, the Ellman’s reagent (Ellman, 1959). By using the Ellman method, I was able to determine total thiols present in the culture not just the thiols that act like glutathione and thioacetamide using CSV (Leal et al., 1999). Perhaps the peaks thought to be thiols are other exudates released by algae that have peak potentials similar to glutathione and thioacetamide using CSV (such as phytochelatins or lumichrome) (Morelli and Scarano, 1994; Ahner and Morel, 1995; Scarano and Morelli, 1996; Leal et al., 1999). An effort was made to identify the specific thiol compounds present in the *V. parahaemolyticus* cultures using the Vairavamurthy and Mopper (1990a,
b) HPLC technique however no individual thiol compound(s) could be identified, probably due to interferences in the medium.

Since the concentrations of copper-complexing ligands and thiols were determined using separate methods, it has not been proven that the copper binding ligands detected are thiol compounds. However, strong correlations in concentration (Fig. 9) and their behavior in the presence of increasing copper (Figs. 6 and 8) support the hypothesis that thiol compound(s) may be an important part of the copper-complexing ligand pool (continuum). Because the identity of the copper-complexing ligands and thiols detected in culture are still unclear, it is not possible to make a direct link. Further research regarding the relationship of thiols and copper-complexing ligands, and the identity of the thiols that contribute to the ligand pool is therefore necessary. Consequently, the significance of this research is that heterotrophic bacteria can indeed produce strong copper binding ligands in the presence of copper stress and total thiol compounds produced by a culture of heterotrophic bacteria can be shown to have similar behavior as these strong copper-complexing ligands.
CHAPTER IV
COPPER-COMPLEXING LIGANDS AND THIOL COMPOUNDS IN
THE ELIZABETH RIVER, VIRGINIA: A SEASONAL SURVEY

Introduction

In some estuaries, contamination by metal pollution is a reason for concern because some metals are harmful to organisms at relatively low levels. Copper, although a nutrient metal, at elevated concentrations can cause a toxic response in bacteria (Sunda and Ferguson, 1983), phytoplankton (Brand et al., 1986), crab larvae (Sanders et al., 1983), and copepod larvae (Sunda et al., 1987). Copper speciation studies have shown that the dissolved speciation of copper in most natural waters is predominately organically complexed (Coale and Bruland, 1990; Moffett et al., 1990). By dominating copper speciation, organic complexation may control copper's bioavailability (by controlling Cu²⁺ activity), its reactivity with suspended particles, and ultimately govern its biogeochemical cycling in seawater (Donat et al., 1986; Sunda and Hanson, 1987; Moffett and Zika, 1987; Coale and Bruland, 1988, 1990; Moffett et al., 1990; Donat and van den Berg, 1992; Donat et al., 1994).

Sources of organic copper binding ligands in seawater are not well known, however, compelling laboratory and field data suggests that marine microorganisms produce strong organic metal binding ligands (Moffett et al., 1990; Gordon et al., 1996, 2000; Moffett and Brand, 1996; Croot, 2003; Dryden et al., 2004). Studies have shown that autotrophic picoplankton (the <2μm component of the phototrophic planktonic microflora), specifically the cyanobacteria Synechococcus spp., produce an extracellular copper
chelator with binding characteristics comparable to the strong ligands, L₁, in the water column (Moffett et al., 1990; Bruland et al., 1991; Moffett and Brand, 1994, 1996; Gordon et al., 1996, 2000). Other studies have shown that heterotrophic marine bacteria and fungi can produce dissolved, high-affinity copper ligands (Schreiber et al., 1990; Stein and Gessner, 1989; Gordon et al., 1996, 2000). Sources for organic copper binding ligands not produced by marine organisms also exist in coastal environments. Estuarine sediment porewaters, sewage effluents, and estuarine and coastal humic substances have been examined as potential sources of organic copper binding ligands (Skrabal et al., 1997; Sedlak et al., 1997; Kogut and Voelker, 2001, Voelker and Kogut, 2001). Skrabal et al. (1997) found that ligands in estuarine sediment porewaters had binding characteristics similar to the water column strong copper binding ligands, L₁. Kogut and Voelker (2001) used Suwannee River humic acid and fulvic acid to demonstrate that terrestrial humic substances may make up a significant fraction of the L₁ strength copper binding ligands in coastal systems. To date whether or not the majority of the strongest organic copper binding ligands, L₁, are of planktonic or nonplanktonic origin is unknown.

Although the chemical nature and complete chemical characteristics of the copper-complexing ligands in natural waters remains unknown, preliminary studies are investigating known copper-complexing compounds and copper-complexing functional groups. Laglera and van den Berg (2003), Tang et al. (2001), Leal et al. (1999), and Leal and van den Berg (1998) have investigated thiol compounds and suggested they might constitute a major part of the natural copper-complexing ligand pool.

Because questions still surround the origins of copper-complexing ligands and an exact chemical identities is still unknown it is difficult to investigate possible seasonal
changes these compounds might undergo. However, seasonal studies might shed light on the origins of the copper-complexing ligands if biological data is collected in the same study. Only recently, Croot (2003) collected seasonal ligand and biological data in a relatively pristine fjord off the coast of Sweden. Croot (2003) found that copper speciation appeared to be driven by the seasonal abundance of the cyanobacterium \textit{Synechococcus}. Unlike Croot (2003), the work presented in our study examined an anthropogenically impacted estuary and looked at seasonal changes in copper-complexing ligand concentrations and thiol concentrations over an annual cycle. The aim of this study was to ascertain whether any correlations exist between copper-complexing ligands and marine microorganism abundances as well as concentrations of thiol compounds and copper-complexing ligands. Results from this study advance our current lack of understanding about seasonal changes in concentrations and relationships between copper-complexing ligands and dissolved thiol compounds. The anthropogenically impacted study site is located in the Elizabeth River, Virginia bound by the cities of Chesapeake, Norfolk, and Portsmouth. Located in the Hampton Roads area of Virginia, the Elizabeth River is a three-branched estuary located on the southern shore of the James River and is the Chesapeake Bay's southernmost tributary. Pollution in the Elizabeth River is much higher than other waterways in southeastern Virginia resulting in one of the most polluted estuaries in the state (Huggett et al., 1984). For over 300 years, it has served as a site of shipping, civilian and military shipbuilding, and manufacturing (Hargis et al., 1984).
Materials and methods

Sample collection and handling

Surface water samples for this study were collected from the Elizabeth River at the Old Dominion University (ODU) sailing pier (Fig. 10). The ODU sailing pier is located slightly east of the main stem of the Elizabeth River at the mouth of the Lafayette River with depth of slightly more than two meters at high tide. To maximize the water column between the surface and the sediment all samples were collected during the peak of high tide and spring tide. Samples were collected monthly for one year to allow examination of temporal variability of the thiols and copper-complexing ligands in the Elizabeth River. During sample collection, the temperature, salinity, and pH were measured as well as noting any high wind conditions that might influence the shallow sampling site. The equipment and procedures used for sample collection and filtration without trace metal contamination have been described in detail previously (Bruland et al., 1979; Bruland, 1980; and Bruland et al., 1985). Briefly, using trace metal clean techniques, study site surface water was collected into acid-cleaned, fluorinated polyethylene (FLPE, Nalgene) bottles using acid-cleaned, Teflon tubing and a peristaltic pump (Bruland et al., 1979; Flegal et al., 1991; Donat et al., 1994). This bottle was immediately sub-sampled and derivatized for thiols (see Thiol procedure). The remainder of the sample was taken back to the labs at ODU and sub-sampled for total dissolved copper, copper speciation/complexation, and microbial enumeration under a laminar flow hood following trace metal clean techniques (Bruland et al., 1979; Flegal et al., 1991). Total dissolved copper and copper speciation sub-samples were filtered through 0.22 µm MSI polycarbonate cartridge filters (Fisher Scientific) prior to bottling. Total dissolved copper
Fig. 10. Map of Elizabeth River, Virginia with study site indicated by black circle.
sub-samples were put into high-density polyethylene (HDPE, Nalgene) bottles, acidified to pH 2 with HCl (Optima Grade, Fisher Scientific) and stored at room temperature. After filtration, copper speciation sub-samples were placed in FLPE bottles and immediately frozen unacidified. Unfiltered microbial enumeration sub-samples were fixed immediately with 2.5% glutaraldehyde and stored at 4°C (Porter and Feig, 1980).

Reagents

Water used for making standards and solutions was Milli-Q (Millipore, resistivity equal to 18 MΩ cm⁻¹). A fresh 0.01 mol L⁻¹ salicyladoxime (SA, Aldrich) stock solution was prepared weekly. The pH buffer contained 1.3 mol L⁻¹ 4-(2-hydroxyethyl) piperazine-1-propanesulfonic acid (HEPPS, Aldrich) and 0.55 mol L⁻¹ ammonium hydroxide (TraceMetal Grade, Fisher Scientific). The addition of 100 µl of the buffer solution to the samples yielded a pH of 8.0. Standard copper solutions were prepared by dilution of 1000 ppm atomic absorption standard solution (Fisher Scientific) with Milli-Q, and acidified to pH 2 using optima HCl (Fisher Scientific). LC grade solvents were used in the preparation of reagent solutions, standards and mobile phases. Stock solutions (5-20 mmol L⁻¹) of all the thiols (cysteine, glutathione, mercaptoacetic acid, 2-mercaptoethanesulfonic acid, 2-mercaptoethanol, 2-mercaptopropanionic acid, 3-mercaptopropanionic acid, mercaptosuccinic acid, and monothioglycerol) were prepared every 3 days and kept refrigerated. Working standard solutions (100 µmol L⁻¹) were prepared daily in N₂ sparged Milli-Q water (to minimize oxidation of the thiols). Tetrabutylammonium hydrogensulfate and 2, 2'-dithiobis(S-nitropyridine) (DTNP, purity > 97%) were obtained from Fluka. Thiols and a majority of the other chemicals used were obtained from Aldrich Chemical unless otherwise indicated.
Total dissolved copper determination

Dissolved copper concentrations in the acidified river samples were determined after UV photooxidation (12 h; 1.2 kW Hg-arc lamp; Ace Glass) by cathodic stripping voltammetry (CSV) (Campos and van den Berg, 1994). The voltammetric equipment consisted of a PC controlled TraceLab 50 system (Radiometer Analytical, Lyon, France) consisting of a POL 150 Polarographic Analyzer connected to a MDE150 Polarographic Stand equipped with a hanging mercury drop electrode (HMDE). The method program was stored and peak heights were collected on the PC via TraceMaster 5 software (Radiometer Analytical, Lyon, France). The reference electrode was Ag/saturated AgCl, KCl (3 mol L\(^{-1}\)), the counter electrode was a platinum wire, and the working electrode was a hanging mercury drop. A ten milliliter aliquot was pipetted into a Teflon cup and pH neutralized with aqueous ammonia (Optima Grade, Fisher Scientific). The pH of the aliquot was buffered to pH 8.0 by adding 100 \(\mu\)l of a 1.3 mol L\(^{-1}\) HEPPS buffer solution (final concentration of 0.01 mol L\(^{-1}\)). Next, 25 \(\mu\)l of a 0.01 mol L\(^{-1}\) SA solution was added for a final of concentration of 25 \(\mu\)mol L\(^{-1}\). Finally, the solution was mounted to the HMDE and purged for five minutes with filtered, ultrapure N\(_2\) while stirring (800 rpm). A sixty second deposition time and deposition potential of -0.15 V were used while stirring; followed by a 30 s non-stirring quiescent period. Finally, the potential was ramped negative from -0.15 to -0.70 V. The scanning parameters were: scan rate, 20 mV s\(^{-1}\); modulation amplitude, 50 mV and square-wave frequency, 50 Hz (Donat et al., 1994; Campos and van den Berg, 1994; Leal and van den Berg, 1998).

Copper-complexing ligand analysis

Copper-complexing ligand concentrations (C\(_L\)) and conditional stability constants
(K'_{Cu}) were determined by ligand equilibration-adsorptive cathodic stripping voltammetry (CLE/ACSV) (Campos and van den Berg, 1994). Approximately 125 ml of the sample was transferred to an acid-cleaned, Teflon bottle, 600 μl of a 1.3 mol L\(^{-1}\) HEPPS buffer (final concentration 0.01 mol L\(^{-1}\)), and 25 μl of a 0.01 mol L\(^{-1}\) SA solution was added and well shaken. The final 1.9 μmol L\(^{-1}\) SA concentration yielded an analytical competition strength, \(\alpha_{CuSA}\), of 3.6 (refer to Calculations of \(C_L\) and \(K'_{Cu}\) section) and was used for all titrations in order to detect the strongest class of Cu-binding ligands (Bruland et al. 2000). Ten milliliters of the buffer and SA spiked sample was pipetted into 12 Teflon cell cups and additions of copper were made to these twelve cups, covered, and left to equilibration overnight (minimum of 8 hours). The instrumental settings, deposition potential, and deposition time were similar to those used for total copper determinations and can be found in the Total dissolved copper determination section.

**Thiol determination**

Concentrations of individual thiol compounds were determined using a procedure described by Vairavamurthy and Mopper (1990a, b). Immediately after collection, samples for thiol determination (~ 20 ml) were derivatized by addition of 50 μl of a pH 6 sodium acetate buffer solution (0.5 mol L\(^{-1}\)) and 50 μl of DTNP solution (2 mmol L\(^{-1}\) in acetonitrile) per ml of sample and then set aside for five minutes at room temperature. Next, the derivatized samples were loaded onto Sep-Pak C\(_{18}\) cartridges (Waters), which were stored in tightly sealed vials at 4°C. The Sep-Pak C\(_{18}\) cartridges (Waters) had been preconditioned with 5 ml methanol followed by 5 ml Milli-Q before the sample was loaded. Prior to HPLC analysis, the derivatized samples were eluted from the Sep-Pak...
C₁₈ cartridges using 2 ml of methanol. A portion of this methanol eluate was mixed with an equal volume of Milli-Q water prior to injection. This methanol/Milli-Q mixture caused sample focusing at the top of the reverse phase HPLC column (Vairavamurthy and Mopper, 1990a, b).

Samples were analyzed by chromatographic separation by gradient elution using an aqueous solution of 0.05 mol L⁻¹ sodium acetate and 7.5 mmol L⁻¹ tetrabutylammonium hydrogensulfate at pH 3.5 (solvent A) and acetonitrile (solvent B, the stronger eluent). The flow rate used was 0.25 ml min⁻¹ with the following gradient: isocratic at 10% B for 1 min; 10 to 34% B in 8 min; 34 to 55% B in 14 min; 55 to 90% B in 5 min; isocratic at 90% B for 2 min; and back to 10% B in 2 min. A reversed-phase C₁₈ microbore column (150 mm x 2.1 mm) containing 4μm packing (Waters, Nova-Pak®, WAT023655) was used to obtain HPLC separations with a preceding C₁₈ guard column (OPTI-Guard). The HPLC instrument (Waters) consisted of a single piston pump with ternary gradient and UV detector. The detection wavelength was 320 nm and a 20 μl external loop was used for sample injection. The HPLC instrumental system was controlled by Waters Millennium³² software, which also recorded and stored all chromatographic data.

Individual thiol compounds were identified by co-injection and underivatized samples were also run to insure that no peaks were mistakenly identified as DTNP-derivatized peaks (Fig. 11C, shows standard addition spiking).

**Enumeration of bacterioplankton and autotrophic picoplankton**

Bacterioplankton and autotrophic picoplankton were enumerated using the DNA stain 4'6-diamidino-2-phenylindole (DAPI) following Porter and Feig (1980). Samples were immediately fixed with 2.5% glutaraldehyde, stored dark at 4°C, and enumerated.
Fig. 11. Chromatograms at 0.015 absorbance units full scale (AUFS). (A) Chromatogram of non-dervatized Elizabeth River sample. (B) Chromatogram of DTNP derivatives from Elizabeth River sample: (1) cysteine; (2) reagent peak (3) glutathione; (4) mercaptosuccinic acid; (5) mercaptoacetic acid; (6) 2-mercaptoethanol; (7) 3-mercaptopropionic acid; (8) reagent peak. (C) Chromatogram of DTNP derivatives from Elizabeth River sample plus standard addition of individual thiol standards (100 nmol L\(^{-1}\) of each standard present).

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within one week of collection. A sub-sample (0.1 to 1 ml, depending on cell density) was incubated with 100 µl of a 1 µg ml\(^{-1}\) DAPI solution for 5 minutes and then gently vacuum filtered through a 0.2 µm black filter (Poretics polycarbonate, Osmonics, Inc.). The DAPI stained samples were observed under UV excitation for total counts and blue light excitation for the chlorophyll autofluorescence of the autotrophic picoplankton (Affronti, 1990).

**Chlorophyll a determination**

Between 100 to 500 ml of the study site sample was filtered onto glass microfiber filters (GF/C 70 mm, Whatman) using gentle vacuum filtration under low light conditions. Next, the chlorophyll \(a\) was extracted in 90:10 acetone: water. Chlorophyll \(a\) was determined fluorometrically using a Turner 10-AU field fluorometer following the methods of Parsons et al. (1984) and Welschmeyer (1994).

**Calculations of \(C_L\) and \(K'_{CuL}\)**

To obtain ligand concentrations (\(C_L\)) and conditional stability constants (\(K'_{CuL}\)), the data from the CLE/ACSV measurements and Ruzic/van den Berg linearization were used (Campos and van den Berg, 1994; Ruzic, 1982; van den Berg, 1982). The following relationship was used for the linearization:

\[
\frac{[Cu^{2+}]}{[CuL]} = \frac{[Cu^{2+}]}{C_L} + \frac{1}{(K'_{CuL} C_L)}
\]

where \([Cu^{2+}]\) is the concentration of free cupric ion, \([CuL]\) is the concentration of copper complexed by natural ligands \(L\), \(C_L\) is total detected ligand concentration, and \(K'_{CuL}\) (with respect to \(Cu^{2+}\)) is the conditional stability constant. \([Cu^{2+}]\) and \([CuL]\) are calculated from the following equations:
where $[\text{CuSA}]$ is the concentration of copper complexed by the added ligand SA, $i_p$ is the
$\text{Cu}^{2+}$ reduction peak current measure during CLE/CSV analysis, $S$ is the slope of the
linear portion of the titration curve at $\text{Cu}_T$ concentrations exceeding $C_L$, $\text{Cu}_T$ is the
concentration of total dissolved copper, and $\alpha'$ is the overall side reaction coefficient for
Cu. $\alpha'$ is defined as

$$
\alpha' = (\alpha_{\text{Cu}} + \alpha_{\text{CuSA}})
$$

where $\alpha_{\text{Cu}}$ is the inorganic side reaction coefficient for copper and $\alpha_{\text{CuSA}}$ is the side
reaction coefficient for complexation of copper by SA (Campos and van den Berg, 1994).

Values for $C_L$ and $K'_{\text{CuL}}$ were calculated from the least-squares linear regression of the
Ruzic/van den Berg plot (Donat et al., 1994; Campos and van den Berg, 1994; Ruzic,
1982; van den Berg, 1982).

An ion pairing computer program (C.M.G. van den Berg, Oceanography, Liverpool
University [http://www.liv.ac.uk/%7Esn35/Documents/Speciation.xls]) was used to
calculate the free concentrations of the major inorganic anions forming complexes with
copper and the stability constants of their copper complexes (with respect to $\text{Cu}^{2+}$), as a
function of the collection site salinity, pH, and temperature. Using the ligand
concentration(s) and conditional stability constant(s) obtained by CLE/CSV plus the
information from the ion pairing model an overall copper speciation and free $\text{Cu}^{2+}$ ion
concentration was calculated with the chemical equilibrium-modeling program
MINEQL+©.
Results and discussion

Study site conditions

Surface water temperatures ranged from 4.9 to 27.6 °C, with a seasonal trend of high summer and fall temperatures and low winter temperatures (Fig. 12; Table 3). Salinities ranged from 8.7 to 22.3, with the highest occurring in August and lowest in April. Chlorophyll \(a\) concentrations ranged from 2.3 to 29.8 \(\mu\)g L\(^{-1}\), consistent with chlorophyll \(a\) values reported by Dorworth (1989) in the Hampton Roads area (Fig. 12). Peaks in chlorophyll \(a\) concentrations occurred in February, July, and December during the study. Dorworth (1989) reported high chlorophyll \(a\) values during spring and summer.

Bacterioplankton and autotrophic picoplankton

Bacterioplankton cell counts ranged from 1.2 to 7.8 \times 10^6 cells ml\(^{-1}\), averaging 4.4 \times 10^6 cells ml\(^{-1}\). An increase in the bacterioplankton cell numbers began in April and peaked in July, which corresponded to temperatures ranging from 14.5 to 29.4 °C (Fig. 13A). The bacteria counts and temperature were correlated, with bacteria cell numbers increasing with increasing temperature from January to July (Table 3; Fig. 13B). This temperature influence on heterotrophic bacteria has been previously observed in surface ocean waters (Li, 1998) and estuarine waters (Hoch and Kirchman, 1993; Shiah and Ducklow, 1994).

Autotrophic picoplankton ranged from 0.42 to 9.0 \times 10^8 L\(^{-1}\), averaging 2.4 \times 10^8 L\(^{-1}\). The autotrophic picoplankton concentration range and average value are comparable to studies by Dorworth (1989) and Affronti and Marshall (1993). August had the highest autotrophic picoplankton cell numbers (5.7 \times 10^8 L\(^{-1}\)), with an increase in cell numbers
Table 3  
Study site data collected over one year. Details about analysis described in text.

<table>
<thead>
<tr>
<th>Date</th>
<th>Temp (°C)</th>
<th>Salinity</th>
<th>[Cu]_\text{MB} (nmol L^{-1})</th>
<th>[L] (nmol L^{-1})</th>
<th>Log K_{\text{CuL}}</th>
<th>% Organic complexation</th>
<th>[Cu^{2+}] (pmol L^{-1})</th>
<th>Bacterioplankton (× 10^6 mL^{-1})</th>
<th>Autotrophic picoplankton (× 10^8 L^{-1})</th>
<th>Chl a (µg L^{-1})</th>
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</thead>
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<tr>
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<td>16.9</td>
<td>16.2</td>
<td>56.0</td>
<td>12.0</td>
<td>99.9</td>
<td>0.41</td>
<td>5.6</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
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<td>16.5</td>
<td>14.7</td>
<td>32.6</td>
<td>12.3</td>
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<td>4.0</td>
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<td>15.5</td>
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<td>99.9</td>
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<td>12.0</td>
<td>99.9</td>
<td>0.81</td>
<td>4.2</td>
<td>5.2</td>
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</tr>
</tbody>
</table>

Ave log K':
12.01 ± 0.24
Fig. 12. Temperature, salinity, and chlorophyll $a$ conditions at the sampling site over the yearlong study.
starting in May and decreasing sharply in September and October (Fig. 13A). From October to April, the autotrophic picoplankton numbers remained constant. Other studies of the lower Chesapeake Bay found similar seasonal patterns of autotrophic picoplankton abundance, with maximum abundance occurring during summer months (Dorworth, 1989; Affronti, 1990; Affronti and Marshall, 1993). The autotrophic picoplankton cell numbers remained below \(2.0 \times 10^8 \text{ L}^{-1}\) until reaching 25 °C when the numbers exponentially increased with increasing temperature (Fig. 13B).

*Total dissolved copper*

Total dissolved copper (\(Cu_{TD}\)) concentrations ranged from a low of 13.1 nmol L\(^{-1}\) in March 2003 to a high of 24.7 nmol L\(^{-1}\) during June 2003 (Fig. 14A; Table 3). The higher copper concentrations were recorded in the summer months (June to September), and the lower values during the winter months (November to January). Although the \(Cu_{TD}\) seasonal variation was only slightly more than 11 nmol L\(^{-1}\), a one-way analysis of variance (\(p<0.05\)) confirmed a statistical difference. Copper concentrations found during this yearlong study were similar to the few studies that have reported copper data for the Elizabeth River. Dryden et al. (2004) found copper concentrations ranging from 12.3 to 19.2 nmol L\(^{-1}\) and Sunda et al. (1990) found 14.7 to 28.2 nmol L\(^{-1}\) in the Elizabeth River, Virginia.

The salinity range (8.7 to 22.3) extended into the intermediate salinities where Laglera and van den Berg (2003) observed a noticeable mobilization of copper from the particulate phase and sediments (Knox et al., 1984; Baeyens et al., 1998; Paucot and Wollast, 1997). It was impossible to assess if any mobilization of copper occurred because the salinity range was too narrow with no salinities below 8 or above 22.
Fig. 13. Autotrophic picoplankton (□) and bacterioplankton (●) seasonality.
Fig. 14. Concentrations of total dissolved copper (CuTd) in the Elizabeth River.
observed. A linear regression using least squares fit of CuTD versus salinity reveals a low correlation coefficient of $r^2 = 0.31$ (Fig. 14B). From September to May, a direct relationship between rainfall and CuTD concentration was observed (Fig. 15) contrary to the inverse relationship reported by Sunda et al. (1990). However, June, July, and August do support the observation by Sunda et al. (1990) with higher CuTD associated with low precipitation. Overall, during this yearlong study above normal monthly precipitation resulted in high fresh water inputs and higher CuTD, which suggests that the source of copper to this study site could be local runoff (National Oceanic and Atmospheric Administration, 2003).

_Copper-complexing ligand_

A single copper binding ligand class with an average log $K'_{CuL}$ of $12.0 \pm 0.7$ was found during this seasonal study using CLE/CSV. This single ligand class corresponds to the strongest ligand class defined by conditional stability constants ($K'_{CuL}$) $10^{12-14}$. Dryden et al. (2004) also observed a single copper binding ligand class with a similar conditional stability constant in the Elizabeth River using CLE/CSV (same SA concentration and competition strength). Copper speciation was dominated (99.2 to 100%) by organic complexation (Table 3). Similar to the dissolved copper, concentrations of the copper-complexing ligand ranged from a low of 26 nmol L$^{-1}$ during April 2003 to a high of 56 nmol L$^{-1}$ in October 2002 (Fig. 16A). The higher ligand concentrations were recorded in the summer and fall months (June to October), and the lower values during the winter to spring months (November to May). This seasonal variation was confirmed by a one-way analysis of variance ($p<0.05$). A least squares regression revealed that ligand concentrations doubled as the salinity increased (slope =
Fig. 15. Concentrations of total dissolved copper (CuTD, •) and departure from normal precipitation (□) in the Elizabeth River.
Fig. 16. Concentration of copper-complexing ligand (L) in the Elizabeth River.
and might suggest copper binding ligands are linked to more saline waters flowing into the Elizabeth River.

Copper-complexing ligand concentrations increased as the water temperature increased (Fig. 17) during the spring and summer months, which could indicate a seasonal periodicity. Temperature increase preceded ligand concentration increases by over a month indicating a lead/lag time (Fig. 17). Perhaps copper binding ligand seasonal periodicity could be explained by a biological source since the highest ligand concentrations correspond to seasonal maximum abundance of bacterioplankton, autotrophic picoplankton, and chlorophyll $a$ maximum (Figs. 12, 13). Croot (2003) observed a similar association between ligand concentrations and *Synechococcus* abundance in the Gullmar Fjord. Culture and recent field evidence support the possibility that the detected ligand is biologically derived (Moffett et al., 1990; Bruland et al., 1991; Moffett and Brand, 1996; Gordon et al., 1996, 2000; Dryden et al., 2004). However, sediment porewaters, sewage effluents, and estuarine and coastal humic substances cannot be discounted as additional sources of L (Skrabal *et al.*, 1997; Sedlak *et al.*, 1997; Kogut and Voelker, 2001, Voelker and Kogut, 2001).

A seasonal dependence of the free cupric ion concentrations was observed (Fig. 18A) with free cupric ion concentrations remaining below 1.5 pmol L$^{-1}$ for a majority of the year. Only during March, April, and December did free cupric ion values reach the low pmol L$^{-1}$ range. The peak in April can be attributed to $\text{Cu}_{\text{TD}}$ approaching the copper binding ligand concentration and peaks in March and December are related to a slightly lower conditional stability constant ($\log K'_{\text{CuL}} = 11.8$). The $p\text{Cu}$ values remained around 12 (Fig. 18B) and influenced only the most copper sensitive species *Synechococcus*.
Fig. 17. Concentration of copper-complexing ligand (●) and surface water temperature (---) in Elizabeth River during October 2002 to September 2003.
Fig. 18. Values of free cupric ion, Cu$^{2+}$, in the Elizabeth River. The lines in panel B represent pCu (pCu = -log[Cu$^{2+}$]) causing reduced reproductive rates for diatoms, coccolithophores, dinoflagellates, *Synechococcus* sp., and *Synechococcus bacillaris* (Brand et al., 1986).
*bacillaris* for a majority of the year (as determined in laboratory studies by Brand et al., 1986).

**Thiol compounds**

The HPLC technique of Vairavamurthy and Mopper (1990a, b) revealed a number of discrete thiols present in the natural samples (Fig. 11). Six out of nine thiols investigated were present at different times during the yearlong study. By far the most prevalent of the six were mercaptosuccinic acid and 2-mercaptoethanol, found in every month but November through February. Thiols were primarily detected during the warmer more saline months from May to September (Table 4). No strong linear correlations were found when comparing correlation coefficients ($r^2$) of individual thiols and other measured parameters (temperature, salinity, and chlorophyll *a*). However, mercaptosuccinic and mercaptoacetic acid were both nonlinearly correlated with chlorophyll *a*. A majority of the thiols were detected during months with the highest concentration of chlorophyll *a*, and highest abundance of bacterioplankton and autotrophic picoplankton suggesting that in the Elizabeth River the source of these thiol compounds are the resident microorganisms. Al-Farawati and van den Berg (2001) suggested a similar source for the thiols found in coastal waters of the Western North Sea and English Channel. However, thiols derived from the sediments are also a potential source to the water column (Vairavamurthy et al., 1997; Vairavamurthy and Mopper, 1990a; Kiene and Taylor, 1988; Vairavamurthy and Mopper, 1987). Samples were taken of the sediment at the study site in order to quantify thiols in the pore water (data not shown). The pore waters of the study site sediment were rich in 3-mercaptopyropionic acid ($\mu$molar concentrations) with trace amounts of cysteine and mercaptoacetic acid
Table 4
Thiol Concentrations (nmol L\(^{-1}\)) determined via Vairavamurthy and Mopper (1990a) method. No values indicated thiol concentration was below detection limit of 5 nmol L\(^{-1}\).

<table>
<thead>
<tr>
<th>Date</th>
<th>Cysteine</th>
<th>Glutathione</th>
<th>Mercaptosuccinic</th>
<th>Mercaptoacetic</th>
<th>2-Mercaptoethanol</th>
<th>3-Mercaptopropionic</th>
<th>[thiol](_{TD})</th>
<th>[L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/4/02</td>
<td>28.8</td>
<td>131.3</td>
<td>20.3</td>
<td>167.8</td>
<td></td>
<td></td>
<td>348.2</td>
<td>56.0</td>
</tr>
<tr>
<td>11/15/02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>12/14/02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29.3</td>
<td></td>
</tr>
<tr>
<td>1/11/03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>2/8/03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td>3/10/03</td>
<td></td>
<td>10.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.0</td>
<td>26.4</td>
</tr>
<tr>
<td>4/23/03</td>
<td>21.1</td>
<td>14.7</td>
<td>10.3</td>
<td></td>
<td>13.7</td>
<td></td>
<td>59.8</td>
<td>25.9</td>
</tr>
<tr>
<td>5/16/03</td>
<td>23.5</td>
<td>15.2</td>
<td>17.0</td>
<td>19.0</td>
<td>15.9</td>
<td></td>
<td>90.6</td>
<td>31.8</td>
</tr>
<tr>
<td>6/13/03</td>
<td>34.7</td>
<td>14.8</td>
<td>118.3</td>
<td>36.3</td>
<td>155.7</td>
<td></td>
<td>359.8</td>
<td>51.7</td>
</tr>
<tr>
<td>7/29/03</td>
<td>25.6</td>
<td>18.4</td>
<td>58.8</td>
<td>19.9</td>
<td>126.8</td>
<td>38.7</td>
<td>288.2</td>
<td>47.6</td>
</tr>
<tr>
<td>8/12/03</td>
<td>35.0</td>
<td>25.3</td>
<td>70.9</td>
<td>39.2</td>
<td>148.0</td>
<td></td>
<td>318.4</td>
<td>49.8</td>
</tr>
<tr>
<td>9/10/03</td>
<td>21.4</td>
<td>24.5</td>
<td>82.5</td>
<td>43.3</td>
<td>168.5</td>
<td>28.7</td>
<td>368.9</td>
<td>46.2</td>
</tr>
</tbody>
</table>
(nanomolar concentrations; data not shown). It is possible that diffusion through the sediment into the overlying water column could be a source of thiol compounds at this shallow study site since all the thiols found in the pore waters were detected in the surface water; however, examining sediment pore waters was not the purpose of this work. In addition, two of the thiol compounds not detected in the sediment (mercaptosuccinic acid and 2-mercaptoethanol) and perhaps of biological origin play a more instrumental role in this research.

The sum of all the thiols \((\text{thiol}_{170})\) revealed a strong linear correlation with ligand concentration, where total thiol concentration increased with increasing ligand concentration \((r^2 = 0.95; \text{Fig. 19})\). This strong correlation suggests that the thiol compounds were a major part of the copper binding ligands detected during this yearlong study. In the Scheldt estuary, Laglera and van den Berg (2003) found thiol-like compound concentrations (analyzed via CSV method of Leal et al., 1999) correlated \((r^2 = 0.90)\) to copper binding ligand concentrations at salinities from 15 to 30. Tang et al. (2001) determined thiols by CSV (Le Gall and van den Berg, 1993, 1998) and found they were slightly correlated \((r^2 = 0.56)\) to the copper-complexing ligands in Galveston Bay. Copper binding ligands are present when the thiol concentrations are at zero (Fig. 19) and can be explained by discrepancies in the detection limits of the two methods used to determine the copper binding ligands and thiol compounds. The CLE/CSV detection limit for copper-complexing ligands was 0.3 nmol L\(^{-1}\) and HPLC thiol method detection limit was 5 nmol L\(^{-1}\), which exposes a 20-fold difference between techniques and provides evidence for the discrepancy (Campos and van den Berg, 1994; Vairavamurthy and Mopper, 1990a, b). If individual thiols determined via the Vairavamurthy and
Fig. 19. Plot of the concentration of total thiol compounds as a function of copper-complexing ligand concentration in the Elizabeth River. Notice the trend in the thiol compounds was similar to that of the copper-complexing ligands; however, the concentration of the copper-complexing ligands is less than the concentration of total thiol compounds.
Fig. 20. Concentration of mercaptosuccinic acid in surface water (n = 3) between October 2002 and September 2003.
Mopper (1990a, b) method were plotted against ligand concentration only two
(mercaptosuccinic acid and 2-mercaptoethanol) out of the six thiol compounds revealed a
strong correlation with copper-complexing ligand concentration.

The mercaptosuccinic acid concentration increase was tightly coupled to an increase
in ligand concentration ([mercaptosuccinic acid] = (3.9 ± 0.5) [L] – (99.2 ± 23.1); r² =
0.88; Fig. 20A). Initially, when ligand is present no mercaptosuccinic acid is found,
however once the mercaptosuccinic acid concentration reaches 40 nmol L⁻¹ it quickly
exceeds the copper-complexing ligand concentration. The mercaptosuccinic acid
concentration exceeded the concentration of copper binding ligand. This could be
explained by the HPLC mercaptosuccinic acid concentration equal to the truly dissolved
plus thiols adsorbed onto colloidal material (K. Mopper, personal communication). The
copper-complexing ligand concentration does not reflect colloidal adsorbed ligands that
will not be measured by CLE/CSV. From December 2002 to April 2003, concentrations
of mercaptosuccinic acid (Fig. 20B) were below 5 nmol L⁻¹, the detection limit for the
Vairavamurthy and Mopper (1990a) HPLC technique. A dual seasonal high was
observed during the fall and summer. In October 2002, the mercaptosuccinic acid was
131 nmol L⁻¹ and in June 2003, the concentration was 118 nmol L⁻¹. This seasonal
variation was confirmed by a one-way analysis of variance (p<0.05).

An increase in the concentration of 2-mercaptoethanol was coupled to an increase in
copper binding ligand concentration ([2-mercaptoethanol] = (5.6 ± 0.6) [L] – (126.6 ±
27.4); r² = 0.90; Fig. 21A). Once again a background of ligand is found without any 2-
mercaptoethanol present but once a 2-mercaptoethanol concentration of 30 nmol L⁻¹ was
reached the copper binding ligand concentration was exceeded. Similar to

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Fig. 21. Concentration of mercaptoethanol in surface water (n=3) between October 2002 and September 2003.
mercaptosuccinic acid, the concentration of 2-mercaptoethanol was below the 5 nmol L$^{-1}$ limit of detection for November and December 2002 and January 2003 (Fig. 21B). The highest concentrations were found in October 2002 and from June to September 2003 (169 nmol L$^{-1}$ high). The lowest detectable concentrations were observed from February to May 2003 (~14 nmol L$^{-1}$). This seasonal variation was confirmed by a one-way analysis of variance ($p<0.05$).

**Thiol compounds as copper-complexing ligands**

Six distinct thiols present in natural samples were investigated as possible copper binding ligands using CLE/CSV (Table 5). Trace-metal free UV-oxidized seawater (UV-SW; salinity 20) was prepared following the method of Donat and Bruland (1988) using trace metal free Chelex-100 (Price et al., 1989). Varying concentrations of thiol standards and copper were added to UV-SW and titrated using the earlier discussed CLE/ACSV method (see the Copper-complexing ligand analysis section). The total dissolved copper concentrations in these experiments ranged from 1 to 37 nmol L$^{-1}$ with thiol concentrations that ranged from 5 to 102 nmol L$^{-1}$. The CLE/CSV data was analyzed for the conditional stability constant (K'$_{CuL}$) and to determine the possible complex stoichiometry of any copper-thiol complexes (experimentally determined concentration compared to actual thiol standard amount added). Leal et al. (1999) used a similar procedure to compare synthetic thiols to copper-complexing ligands produced by *Emiliania huxleyi*. Results described in Table 5 are for each one of the thiol standards found and a mix of the two most commonly found thiols in the Elizabeth River (mercaptosuccinic acid and 2-mercaptoethanol).

The CLE/CSV determined conditional stability constants for each one of six thiol
standards (average log $K'_{CuL} \sim 12.1 \pm 0.5$) is statistically equivalent to the conditional stability constants found for naturally occurring copper-complexing ligands (average log $K'_{CuL} \sim 12.0 \pm 0.2$) found during this study. Therefore, the thiols present in the Elizabeth River samples are likely candidates as copper-complexing ligands. Laglera and van den Berg (2003) reported the determination of conditional stability constants for unknown copper-thiol complexes by CSV during titrations with copper in samples from the Scheldt estuary. The log $K'_{Cu-thio}$ values (12.3-14.1) reported by Laglera and van den Berg (2003) are statistically similar to the thiol standard conditional stability constants (12.1 ± 0.5) determined in this study. This evidence supports the finding that the individual thiols detected via Vairavamurthy and Mopper (1990a, b) HPLC method are likely candidates for the unknown strong copper-thiol complexes in natural waters.

The CLE/CSV titrations of the thiol standard revealed possible stoichiometries of copper-thiol complexes. The stoichiometry of copper-thiol complexes were investigated by varying the concentrations of thiol standards and copper added to trace metal free, organic free seawater (UV-SW) before concentrations of copper-complexing ligands were determined. It appears that the stoichiometry of copper-thiol complexes depends upon concentrations of each component (Table 5). A mixed stoichiometry was observed for the copper and thiol complexes and this observation is supported by the literature. Vairavamurthy et al. (2000) found a similar mixed stoichiometry for 3-mercaptopropionic acid binding with cadmium. In addition, when Leal and van den Berg (1998) were investigating the formation of a copper-glutathione they observed predominately a 2:1 (glutathione:Cu) complex but did observe formation of a 1:1 complex at higher copper concentrations when free glutathione was depleted.
Table 5
CLE/CSV titration data of known thiol compounds.

<table>
<thead>
<tr>
<th>Thiol Standard</th>
<th>[Cu]<em>{TD} compared to [thiol]</em>{TD}</th>
<th>% Recovery</th>
<th>Conditional stability constant (log K'_{cal.})</th>
<th>Measured stoichiometry (Cu_{TD} : thiol_{TD})</th>
<th>Possible stoichiometry (Cu_{TD} : thiol_{TD})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>12.4 ± 0.9</td>
<td>1 : 2.1 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>12.6 ± 0.8</td>
<td>1 : 2.1 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>Mercaptosuccinic acid</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>12.0 ± 0.3</td>
<td>1 : 2.1 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>Mercaptoacetic acid</td>
<td>Cu_{TD} ≥ thiol</td>
<td>100</td>
<td>12.1 ± 0.1</td>
<td>1 : 0.9 ± 0.1</td>
<td>1:1</td>
</tr>
<tr>
<td>Mercaptoacetic acid</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>11.9 ± 0.1</td>
<td>1 : 2.1 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Cu_{TD} ≥ thiol</td>
<td>100</td>
<td>12.0 ± 0.2</td>
<td>1 : 1.1 ± 0.1</td>
<td>1:1</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>12.0 ± 0.1</td>
<td>1 : 2.4 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>3-Mercaptopropionic acid</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>11.7 ± 0.1</td>
<td>1 : 2.1 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>3-Mercaptopropionic acid</td>
<td>Cu_{TD} ≥ thiol</td>
<td>100</td>
<td>12.1 ± 0.2</td>
<td>1 : 1.1 ± 0.2</td>
<td>1:1</td>
</tr>
<tr>
<td>Mix of 2-Mercaptoethanol/ Mercaptosuccinic acid</td>
<td>Cu_{TD} &lt; thiol</td>
<td>50</td>
<td>12.0 ± 0.4</td>
<td>1 : 1.6 ± 0.1</td>
<td>1:2</td>
</tr>
<tr>
<td>Mix of 2-Mercaptoethanol/ Mercaptosuccinic acid</td>
<td>Cu_{TD} ≥ thiol</td>
<td>100</td>
<td>12.2 ± 0.1</td>
<td>1 : 1.1 ± 0.1</td>
<td>1:1</td>
</tr>
</tbody>
</table>

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A discrepancy exists between concentration of the thiols and copper binding ligands since the total detected thiols exceeds ligand during most of the year perhaps because the HPLC technique measures truly dissolved thiols plus some thiols adsorbed onto colloidal material (K. Mopper, personal communication), while copper-complexing ligand concentration reflects only dissolved ligands. However, if only mercaptosuccinic acid and 2-mercaptoethanol are considered (the only thiols that were tightly correlated to ligand) then ligand concentration was exceeded from June to October but November to February ligand concentration exceeds thiol concentration. The possibility cannot be dismissed that parallels between copper-complexing ligand concentrations and mercaptosuccinic acid and 2-mercaptoethanol concentrations might be fortuitous because two different methods were used in the determination and no identification of the unknown ligands has been made. However, similarities in concentrations and stability constants between specific thiols (i.e., glutathione) found in ocean and estuarine waters and copper-complexing ligands have been reported elsewhere (Le Gall and van den Berg, 1998; Tang et al., 2000; Leal and van den Berg, 1998). Leal et al. (1999) also observed parallels between increasing L and thiols. Thiol concentrations in the study by Laglera and van den Berg (2003) could not accurately be determined from the CSV titrations. Some assumptions about possible upper limits of the thiol concentrations in their investigation were reported and these concentrations exceed the concentration of the copper-complexing ligand (reportedly weaker L_2 type ligands) at salinities similar to those found in the Elizabeth River. Vachet and Callaway (2003) and Ross et al. (2003) suggested possible functional groups and empirical formulas for the unknown compounds using immobilized-metal affinity chromatography (IMAC) and electrospray ionization.
mass spectrometry (ESI-MS). Thiol functionality was one of many groups suggested by both of these studies.

**Preliminary use of thiol blocking agent**

In an attempt to demonstrate that thiol compounds were detected as copper binding ligands during CLE/CSV analysis a thiol blocking agent was used to inactive the thiol group. First, DTNP (Vairavamurthy and Mopper, 1990a, b) was used as the thiol blocking agent, however it interfered with the CLE/CSV analysis hindering growth in the copper peak and a broad peak was observed between -0.4 to -0.6 V with addition of DTNP. Iodoacetic acid (IAA) was chosen over N-ethyl maleimide (NEM) as the thiol blocking agent due to a low molecular weight, simple structure, and ability to react with all thiols to form thioethers (Fig. 22, Konno, 1991; Hoffmann and van Mil, 1997). No interference was observed when IAA (35 µmol L⁻¹) was added during CLE/CSV analysis and no extraneous peaks were detected. In addition, IAA did not interfere with CLE/CSV of a well-characterized ligand (EDTA) indicating that would not block a non-thiol containing copper binding ligand. During a CLE/CSV analysis of various thiol standards (mercaptosuccinic acid, 2-mercaptoethanol, and cysteine) in trace metal free, organic free seawater (UVSW), addition of IAA gave varying results and was found to block between 53.79 ± 35.89 % (average ± standard deviation) of the thiol standard added. Samples from the Elizabeth River were also tested with the IAA thiol blocking agent and again a wide range of blocking was observed (38.23 ± 30.38 %). Although the thiol blocking agent results produced a wide range, thiol blocking was observed in the Elizabeth River sample which suggest that thiol compounds were being detected as copper binding ligands during CLE/CSV analysis. Perhaps, with further refinement of
Fig. 22. Reaction of iodoacetic acid thiol blocking agent with a thiol.
the thiol blocking agent and its concentration added during CLE/CSV analysis, the wide percentage range of thiols blocked will be improved. Also, the thiol blocked sample could also be analyzed via HPLC (DTNP, Vairavamurthy and Mopper, 1990a, b) to ensure that thiols detected using HPLC are blocked as well.

**Conclusions**

The results obtained from this yearlong observation of the Elizabeth River indicate that concentrations of total dissolved copper, copper-complexing ligands, 2-mercaptoethanol, and mercaptosuccinic acid varied seasonally. Variations in total dissolved copper concentrations appeared to be linked to above normal yearly precipitation (Fig. 15) counter to the inverse total dissolved copper-precipitation relationship observed by Sunda et al. (1990). The seasonal periodicity of copper-complexing ligand concentrations along with mercaptosuccinic acid and 2-mercaptoethanol correspond to the seasonal change in abundance of bacterioplankton, autotrophic picoplankton, and chlorophyll $a$, which suggests a biological source. Sedimentary sources of thiol compounds (Vairavamurthy and Mopper, 1987; Kiene and Taylor, 1988) and copper-complexing ligands (Skrabal et al., 1997) cannot be overlooked at such a shallow (water column 2.5 m at high tide) study site. Although these co-variations do not definitely identify sources of copper-complexing ligands and the thiol compounds, they are strong enough to suggest a source.

Questions about the stoichiometry of copper-thiol complexes still exist; however, this study revealed that the component concentrations (copper and specific thiol compound) must be considered. The ability of known thiol compounds (e.g.,
mercaptosuccinic acid and 2-mercaptoethanol) to act as copper-complexing ligands during a CLE-CSV titration designed to detect strong copper binding ligands suggests that these thiols (when present in the water column) could represent an important part of the natural copper-complexing ligand pool. The thiols in this study appear to contribute to the ligand pool with conditional stability constants (log $K'_{\text{CuL}}$) between 11.7 -12.6.
CHAPTER V

MODELING DISSOLVED COPPER IN ELIZABETH RIVER, VIRGINIA USING A BOX MODEL

Introduction

Although copper is an essential trace metal toxic, effects can be caused by excess copper in a biologically available chemical form (Sunda and Ferguson, 1983; Brand et al., 1986; Donat and Bruland, 1995). Copper is released into the environment from natural sources (e.g., forest fires and volcanic particles) and anthropogenic processes (i.e., mining, metal production, oil and coal combustion) (Salomons and Forstner, 1984). Because of its known toxic effects, copper is routinely monitored in the evaluation of environmental risk. Along with this collection of data, management of estuarine water quality necessitates predictive models to examine contaminant concentrations from growing populations in coastal areas.

In an effort to look at dissolved copper dynamics in a coastal harbor, a collaborative effort was funded by the Office of Naval Research to study a major U.S. Naval harbor, the Elizabeth River, Virginia. The ultimate goal of this effort is to develop a predictive biogeochemical model for estuarine systems. This effort would help us critically determine the gaps in our knowledge about dissolved copper cycling in estuaries, for future fieldwork and more detailed model development. In the work presented here we develop a simple two box model and use it to determine monthly dissolved copper concentrations over our two year study period. The model relies on limited copper point source data, two estuarine transect cruises, a yearlong seasonal study of dissolved copper,
salinity data, and rainfall data. The framework of this model can easily be manipulated to add further point sources, sinks, and new dissolved copper data as it becomes available.

Methods

Sample collection

In July 1999, surface water samples were collected along a 6-station estuarine transect from Hampton Roads up the Elizabeth River, and bottom water samples and sediment cores from 2 of these stations (Fig. 23). In May 2000, an additional Elizabeth River water column station (station 9) upstream of the Norfolk Naval Shipyard was added to this same estuarine transect. Using trace metal clean techniques, surface water was collected with Teflon tubing and a peristaltic pump and filtered through 0.22 µm MSI polycarbonate cartridge filters (Fisher Scientific) into acid-cleaned, HDPE bottles using acid-cleaned (Bruland et al., 1979; Flegal et al., 1991; Donat et al., 1994). Total dissolved copper samples were acidified to pH 2 with HCl (Optima Grade, Fisher Scientific) and stored at room temperature at least two-weeks before analysis. Benthic fluxes were determined using core incubation techniques at stations 5 and 8 (Burdige and Homstead, 1994; Skrabal et al., 1997; Burdige and Zheng, 1998).

Total dissolved copper analysis

Water column total dissolved copper concentrations were determined by graphite furnace atomic absorption after APDC/DDDC chelation/chloroform extraction (Bruland et al., 1979; Statham, 1985). In our benthic flux samples, total dissolved Cu concentrations were determined by chemiluminescence (Sunda and Huntsman, 1991).
Fig. 23. Map of Elizabeth River sampling sites for July 1999 and May 2000. Numbers indicate locations of stations. At stations 5 and 8 sediment cores were taken for benthic flux experiments.
Elizabeth River box model

To describe the transport of water and copper through the estuary a one-layer, segmented simple box model was developed (Fig. 24). By knowing the salinities in each box and the freshwater inputs, it is possible to calculate estuarine water transport because conservation of salt and volume must be satisfied. The following water balance and salt balance equations were used:

Mid-river water balance: \[ R + F_2 = F_i \] (9)

Lower river water balance: \[ F_i + F_4 = F_2 + F_3 \] (10)

Mid-river salt balance: \[ (R \times S_r) + (F_2 \times S_m) = (F_i \times S_m) \] (11)

Lower river salt balance: \[ (F_i \times S_m) + (F_4 \times S_b) = (F_2 \times S_i) + (F_3 \times S_i) \] (12)

where \( R \) is river discharge; \( F_1 \) and \( F_2 \) are exchanges of water from the mid-Elizabeth to the lower Elizabeth and vice versa; \( F_3 \) and \( F_4 \) are exchanges of water from the lower Elizabeth to the Bay and vice versa; \( S_x \) is salinity in the reservoirs \( x \), where \( x \) may be \( r \), \( m \), \( l \), or \( b \), representing the incoming river water, mid-river water, lower river water, and bay water.

In the model, a series of monthly mass balance calculations were assumed using the following input processes (corresponding to current quantifiable processes):

1. River input – Annual river discharge divided into monthly intervals (\( R \) in Fig. 24) based on monthly rainfall data (National Oceanic and Atmospheric Administration, 2003);

2. Water exchange between boxes – Monthly exchange fluxes (\( F_i \) in Fig. 24) were estimated using the water and salt balance equations above (eqns. 9-12) and salinity data from Old Dominion University’s Water Quality Lab (Chesapeake...
Fig. 24. Diagram of the simple box model with all quantifiable and non-quantifiable copper sources and sinks. The Elizabeth River has been divided into mid and lower boxes with major water exchange from the Chesapeake Bay and up river.
Bay program) collected during 1999 and 2000;

3. Total dissolved copper - Data for \( Cu_{TD} \) concentrations (\( Cu_x \) in Fig. 24) were collected during two river transects (late July 1999 and in early May 2000) and during a yearlong seasonal study at the Old Dominion University sailing dock close to station 6 (Chapter IV). These were combined to determine monthly \( Cu_{TD} \) values for the upper river, mid-river and Chesapeake Bay;

4. Copper point sources - Data from a report by Johnson et al. (1998) commissioned by the U.S. Navy was used to approximate copper point sources (\( P_x \) in Fig. 24) which were then partitioned to the mid and lower river according to Virginia Pollutant Discharge Elimination System (VPDES)-permitted facilities and vessel discharge locations (URI Consultants Inc., 1996);

5. Benthic fluxes of copper - Benthic fluxes of copper (\( B_x \) in Fig. 24) were based on results from benthic flux studies in July 1999 at sites in the mid-river (station 8) and lower river (station 5). To take into account seasonal variability these fluxes were divided in half for October thru April. All fluxes were converted to kg Cu/yr based on the dimensions of the mid or lower-river boxes.

Based on the above processes, and assuming that the river is in steady state on a monthly basis the following equations were used to estimate a dissolved copper balance:

Mid- river:

\[
RCu_r + F_2Cu_i + P_m + B_m = F_1Cu_m + X_m
\]  

Lower river:

\[
F_1Cu_m + F_2Cu_i + P_i + B_i = F_2Cu_i + F_2Cu_i + X_i
\]  

Entire river:

\[
RCu_r + P_m + P_u + B_m + B_u = F_2Cu_i + (X_m + X_i)
\]

where various \( X_i \) 's represent all fluxes we are unable to quantify (or the net input or removal of Cu from that box in that month required to achieve a monthly steady state).
Results

Measured copper in Elizabeth River

Little variation between the two seasonal estuarine transects was observed in the total dissolved copper (Cu\textsubscript{TD}) concentration (Fig. 25). Total dissolved (0.22\mu m-filtered) concentrations of copper generally increased along the transect from Hampton Roads Harbor (Stn 3) up the Elizabeth River during July 1999 and May 2000. Total dissolved copper increased upriver from 6.6 \pm 1.0 \text{n}\text{mol L}\textsuperscript{-1} at station 3 to 50.7 \pm 0.9 \text{n}\text{mol L}\textsuperscript{-1} at station 8, then decreased further upriver to 44 \text{n}\text{mol L}\textsuperscript{-1} at station 9. On both occasions, Cu\textsubscript{TD} increased nearly 3-fold between stations 5 and 6 indicating a major input of Cu\textsubscript{TD} into the river between the two stations. With current data, it is impossible to determine what the source(s) of copper might include. However, along this section of river numerous VPDES direct discharge sources exist. These include possible vessel discharge from Naval ship berths, marinas, marine terminal for commercial shipping and a large dredge spoil disposal site at Craney Island (URI Consultants, Inc., 1996; Johnson et al., 1998). Elevated copper levels were observed at the remaining stations up the river (stations 7, 8, and 9) indicating more potential copper sources that maintain Cu\textsubscript{TD} concentrations over those observed at the pristine down river stations.

The 2002-2003 study measured Cu\textsubscript{TD} concentrations ranging from a low of 13.1 \text{n}\text{mol L}\textsuperscript{-1} in March to a high of 24.7 \text{n}\text{mol L}\textsuperscript{-1} during June (Fig. 26). During the summer months (June to September), the highest Cu\textsubscript{TD} concentrations were recorded while the lowest Cu\textsubscript{TD} values were observed during the winter months (November to January). Dryden et al. (2004) found copper concentrations ranging from 12.3 to 19.2 \text{n}\text{mol L}\textsuperscript{-1} and Sunda et al. (1990) found 14.7 to 28.2 \text{n}\text{mol L}\textsuperscript{-1} in the Elizabeth River, Virginia similar to...
Fig. 25. Concentration of total dissolved copper measured during July 1999 (●) and May 2000 (□).
Fig. 26. Concentration of total dissolved copper at yearlong study site slightly north of station 6.
the yearlong study concentrations.

*Simple box model of total dissolved copper in Elizabeth River*

The 2002 to 2003 yearlong study revealed seasonal changes in Cu\textsubscript{TD} concentrations. Therefore this seasonality was applied to the Cu\textsubscript{TD} data collected during the two transect cruises (Fig. 27). This estimated seasonal data at stations 3-9 was then used in equations 13-14 to calculate monthly fluxes into or out of the mid and lower river boxes (Fig. 28). The calculations also predict that for all months there is a net input of Cu\textsubscript{TD} to the river as a whole (Fig. 29). Although seasonal imbalance in these fluxes is probable, these results clearly indicate an imbalance over an entire annual cycle. Since we have no evidence that the Cu\textsubscript{TD} concentration in the river is increasing with time, this result implies that we have clearly not accounted for an important copper uptake process in the Elizabeth River.

**Discussion**

This effort was undertaken to determine gaps in our knowledge about dissolved copper cycling in estuaries. It appears that the quantitative processes in the model do not adequately describe the biogeochemistry of copper in the river. Obviously future fieldwork and more detailed model development needs to account for this missing sink. There are several interesting aspects of the missing sink that this modeling effort has offered:

1. This sink is of the same magnitude or larger than point sources, benthic fluxes or Cu\textsubscript{TD} exchange by water movement;
2. There is a baseline level of removal (~4000 kg Cu/yr) that is elevated ~25% between approximately May and September/October.
Fig. 27. Seasonal changes in total dissolved copper (CuT$_D$) concentration based on yearlong data and applied to four reservoirs of the box model (bay ●; mid-river □; lower river △; river ○).
Fig. 28. Net flux of total dissolved copper concentration in mid (●) and lower river (△) (Net = sum Cu In - sum Cu removed).
Fig. 29. Net input of total dissolved copper into whole Elizabeth River estuary.
The seasonality of the removal from May to September/October strongly argues for a process that may be biologically driven, although at the present time abiotic seasonal processes cannot be overlooked. Another explanation for the missing sink results could be that the sink is net uptake of $\text{Cu}_{\text{TD}}$ onto particles (either by biotic or abiotic processes). Furthermore, these particles may then be transported out of the river or deposited (in a net sense) in the sediments. Future fieldwork studying the possibility of the missing sink being uptake onto particles needs to be addressed in fill in the gaps of the simple two-box model.
CHAPTER VI

INTERACTIVE REGULATION OF DISSOLVED COPPER TOXICITY BY AN ESTUARINE MICROBIAL COMMUNITY

Introduction

Copper is widely used in industrial applications, notably as the active agent in antifouling coatings on ship hulls, and meeting regulatory criteria is costly to industry. The current acute water quality criterion for copper in Virginia waters is 5.9 μg L\(^{-1}\) (92.8 nmol L\(^{-1}\)), based upon the National Ambient Water Quality Criterion for copper (U.S. EPA, 1999). Controlling the release of dissolved copper from commercial and military shipping activity is a major concern, particularly in industrialized estuaries, because copper can be toxic to marine organisms (such as phytoplankton) at free cupric ion concentrations of 0.01 nmol L\(^{-1}\) or above (Sanders et al., 1983; Sunda et al., 1987; Sunda and Ferguson, 1983; Brand et al., 1986). Therefore, understanding the dynamic ecological factors and feedbacks that affect the toxicity and bioavailability of dissolved copper is crucial for development of cost-effective management strategies for estuaries.

A major factor governing the toxicity and bioavailability of dissolved copper to marine organisms is its chemical speciation. Dissolved copper may exist in various forms (species): as free cupric ions (Cu\(^{2+}\)), inorganic complexes (e.g. with Cl\(^{-}\), OH\(^{-}\), CO\(_3^{2-}\), and SO\(_4^{2-}\)), and complexes with various organic ligands (e.g. humic substances, phytoplankton metabolites, proteins, etc.). The toxicity and nutrient availability of copper to marine organisms decrease as a result of complexation by natural organic ligands, indicating that the toxicity and availability of copper is controlled by the free
cupric ion, Cu^{2+} (Brand et al., 1986; Sunda and Guillard, 1976; Anderson and Morel, 1978). In marine and estuarine waters, many indigenous marine organisms are sufficiently sensitive to copper that they would be severely impacted by copper toxicity at ambient copper concentrations in the absence of organic complexation (Hering et al., 1987; Coale and Bruland, 1988). In most natural waters, the majority of dissolved copper (usually 95% or more) is complexed by strong organic ligands (usually termed L₁-class ligands) having conditional stability constants (K'_{CuL}) between 10^{11}-10^{13} (Coale and Bruland, 1990; Moffett et al., 1990).

While it has been known for some time that organic ligands control copper speciation in most natural waters, the sources of these ligands are not fully understood. In estuarine environments ligands may derive from terrestrial sources, sediment and, hypothetically, from water column processes (Sunda and Guillard, 1976; Brand et al., 1986; Moffett et al., 1990; Bruland et al., 1991; Sunda and Huntsman, 1995; Skrabal et al., 1997). Laboratory studies indicate that autotrophic picoplankton (the <2µm component of the phototrophic planktonic microflora) (Moffett et al., 1990; Bruland et al., 1991; Moffett and Brand, 1996; Gordon et al., 1996, 2000) and heterotrophic bacteria (Gordon et al., 2000) produce ligands having binding strengths similar to the L₁-class ligands observed in natural waters in response to elevated copper concentrations in culture. These culture observations have led to the hypothesis that a biological feedback system may regulate dissolved copper speciation in marine and estuarine waters (Bruland et al., 1991; Donat et al., 1994). While such a feedback system has been demonstrated in cultures of marine and estuarine microorganisms (Bruland et al., 1991; Moffett and Brand, 1996), it has not previously been demonstrated in natural microbial communities.
The objective of our study was to examine the dynamics of copper-complexing ligand production by natural microbial assemblages under realistic environmental conditions in situ, thus avoiding potential pitfalls inherent to extrapolation of laboratory studies to field conditions. This response would have important implications for our understanding of the fate and effects of copper in estuarine systems, since it would demonstrate that natural microbial communities could participate in a negative feedback loop that influences the bioavailability and toxicity of copper to themselves and to other estuarine biota.

Materials and methods

Study location

The study site was located at the mouth of the Elizabeth River, Virginia adjacent to Norfolk Naval Base, which is the home to the North Atlantic Fleet and is the largest naval base in the world. The Elizabeth River is an urban, industrialized sub-estuary of the Chesapeake Bay. The experimental station is well mixed and flushed due to its close proximity to the mouth of the Chesapeake Bay (Fig. 30).

Sample collection, pre-equilibration, and incubation

In May and November 2000, June 2001, and July 2002, estuarine seawater was collected from a depth of one meter at the mouth of the Elizabeth River, Virginia (Fig. 30). During sample collection, the temperature, salinity, and pH were measured. Using trace metal clean techniques, study site surface water was collected into acid-cleaned, polycarbonate carboys using acid-cleaned, Teflon tubing and a peristaltic pump (Bruland et al., 1979; Flegal et al., 1991; Donat et al., 1994). To minimize copper and ligand loss
Fig. 30. Map of Hampton Roads and the Elizabeth River. The Elizabeth River consists of a main stem and four branches (Lafayette River, Eastern Branch, Western Branch, and Southern Branch). The study site is indicated on map by a black circle.
to bottle walls by adsorption, each acid-cleaned, polycarbonate incubation bottle was pre-
equilibrated for one week with 0.22 μm filtered site water at 4°C prior to each
experiment. Water used for pre-equilibration had the same copper amendment that was
used in the experimental bottles.

Under a laminar flow hood, pre-equilibrated incubation bottles were filled with
unfiltered site water and received one of several experimental treatments or left unaltered.
These treatments included addition of copper (100 and 200 nmol L⁻¹) with and without
addition of the metabolic poison sodium azide (15 mmol L⁻¹). Dark incubation bottles
were used with selected treatments including the unaltered and addition of copper without
azide. Within ten hours after water collection, the incubation bottles were placed in a
moored array at the study site approximately 1 m below the water’s surface. After one or
two weeks of incubation, the bottles were retrieved for analysis. Each incubated bottle
was immediately sub-sampled for total dissolved copper, copper
speciation/complexation, and microbial enumeration under a laminar flow hood
following trace metal clean techniques (Bruland et al., 1979; Flegal et al., 1991). Total
dissolved copper and copper speciation sub-samples were filtered, using a peristaltic
pumping system, through 0.22 μm MSI polycarbonate cartridge filters (Fisher Scientific).
Total dissolved copper sub-samples were acidified to pH 2 with HCl (Optima Grade,
Fisher Scientific). Copper speciation sub-samples were not acidified but were
immediately frozen after collection until analyzed. Microbial enumeration sub-samples
were unfiltered, fixed immediately with 2.5% glutaraldehyde, stored at 4°C, and analyzed
within one week of collection (Porter and Feig, 1980).
Total dissolved copper determination

Total dissolved copper concentrations were determined using cathodic stripping voltammetry (CSV) as described by Campos and van den Berg (1994). The only modification made to the Campos and van den Berg (1994) CSV method included the use of a 4-(2-hydroxyethyl) piperazine-1-propanesulfonic acid (HEPPS) buffer solution (Sigma; final concentration 0.01 mol L\(^{-1}\)) rather than a borate buffer solution. The analytical system consisted of an EG&G PARC (Princeton, NJ) 264A polarographic analyzer interfaced with an EG&G PARC (Princeton, NJ) 303A hanging mercury drop electrode (HMDE).

Copper complexation and speciation analysis

Copper speciation was determined at natural pH using competitive ligand equilibration-adsorptive cathodic stripping voltammetry (CLE/ACSV) with salicylaldoxime (SA) as the competitive ligand (Campos and van den Berg, 1994). The theory, application, and limitations of CLE/ACSV methods for determining copper-complexing ligand concentrations are discussed in detail elsewhere (e.g., Campos and van den Berg, 1994; Donat and van den Berg, 1992; Donat et al., 1994; van den Berg and Donat, 1992; Bruland et al., 2000). The final concentrations of the HEPPS buffer and SA in a sample to be analyzed were 0.01 mol L\(^{-1}\) (resulting pH 8.2) and 2 μmol L\(^{-1}\), respectively. An analytical competition strength, log \(\alpha_{\text{Cu(2SA)}}\), of 3.8 (see Bruland et al., 2000 for description and definition) was used for all titrations to detect the strongest class of Cu-binding ligands.

Copper-complexing ligand and conditional stability constant calculations

To obtain ligand concentrations \((C_L)\) and conditional stability constants \((K'_{\text{CuL}})\), the
data from the CLE/ACSV measurements and Ruzic/van den Berg linearization were used (Campos and van den Berg, 1994; Ruzic, 1982; van den Berg, 1982). A detailed description of the theory behind the calculations used is presented in Campos and van den Berg (1994) and Rue and Bruland (1995). Using ligand concentrations and conditional stability constants obtained by CLE/ACSV, the overall copper speciation and free Cu\(^{2+}\) ion concentrations were calculated with the chemical equilibrium modeling program MINEQL+

Enumeration of bacterioplankton and autotrophic picoplankton

Epifluorescent direct counts of bacterioplankton and autotrophic picoplankton followed the method of Porter and Feig (1980) using the DNA stain 4’6-diamidino-2-phenylindole (DAPI). The DAPI stained samples were observed under UV excitation for total counts and blue light excitation for the chlorophyll autofluorescence of the autotrophic picoplankton (Affronti, 1990). The average counting error was 9% using the Porter and Feig (1980) method and counting twenty fields. Every field had a minimum of 30 individual cells of each type (i.e., bacterioplankton and autotrophic picoplankton).

Results and discussion

Initial incubation conditions and total dissolved copper recoveries

The initial conditions for all incubations are presented in Table 6. Study site salinities ranged from 13.2 to 18.2 on the sampling dates, and water temperature varied from 14.5 °C in November to 25.6 °C in July. Total dissolved copper concentrations ranged from 12.3 to 19.2 nmol L\(^{-1}\), 5 to 8 times lower than the current acute copper criterion in Virginia waters (93 nmol L\(^{-1}\)) (U.S. EPA, 1999). Initial bacterial
Table 6
Summary of initial in situ data during experiments performed in the Elizabeth River, Virginia.

<table>
<thead>
<tr>
<th>Date</th>
<th>Temp (°C)</th>
<th>Salinity</th>
<th>CuTD (nmol L⁻¹)</th>
<th>Autotrophic picoplankton numbers (10⁸ L⁻¹)</th>
<th>Bacterioplankton numbers (10⁶ ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2000</td>
<td>16.0</td>
<td>16.4</td>
<td>12.3</td>
<td>0.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>14.5</td>
<td>16.5</td>
<td>16.2</td>
<td>0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Jun 2001</td>
<td>21.8</td>
<td>18.2</td>
<td>19.2</td>
<td>1.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Jul 2002</td>
<td>25.6</td>
<td>13.2</td>
<td>15.5</td>
<td>1.4</td>
<td>5.3</td>
</tr>
</tbody>
</table>
concentrations varied 2-fold, from 2.3 to $5.3 \times 10^6$ ml$^{-1}$, and autotrophic picoplankton numbers varied threefold, from 0.5 to $1.4 \times 10^8$ L$^{-1}$. Peak bacterial and autotrophic picoplankton numbers were observed during July 2002, which also had the highest temperature (25.6 °C) and the lowest salinity (13.2).

When the bottle contents were analyzed after incubation, 41 to 120% of the copper added to experimental bottles was measured in the dissolved form (Tables 7 and 8). The balance was presumably partitioned between particulate matter, including cells, and the container walls (despite pre-conditioning of bottles with site water). Achterberg et al. (2003) observed similar total dissolved copper recoveries and removal by particles. Dissolved copper concentrations in these experiments ranged from 45 to 190 nmol L$^{-1}$ as compared to ambient concentrations of 10 to 50 nmol L$^{-1}$ throughout the Elizabeth River (J. R. Donat, unpublished data). The copper concentrations to which microbial communities were exposed in this study, then, were realistic relative to Elizabeth River ambient copper concentrations and to the acute copper criterion for Virginia waters (93 nmol L$^{-1}$) (U.S. EPA, 1999).

**Copper-complexing ligand production**

The ligand production rate (Fig. 31) increased with increasing copper additions in every experiment except November 2000 when 100 nmol L$^{-1}$ was added. This experimental data suggest that ligand production increased in response to above-ambient copper concentrations. A similar production of strong copper-complexing ligands in response to added copper has also been observed in cultures of the cyanobacterium *Synechococcus* (Moffett and Brand, 1996) and heterotrophic bacteria (Gordon et al., 2000; Schreiber et al., 1990). A net loss of ligands was observed in control incubations.
Table 7
Total dissolved copper concentration, ligand concentration ($C_L$), and conditional stability constants ($K'_{CuL}$) in incubation bottles with 100 nmol L$^{-1}$ copper added (no azide added).

<table>
<thead>
<tr>
<th>Date</th>
<th>Incubation time (weeks)</th>
<th>Total dissolved Cu (nmol L$^{-1}$)</th>
<th>$C_L$ (nmol L$^{-1}$)</th>
<th>$K'_{CuL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2000</td>
<td>0</td>
<td>46.9 ± 3.3</td>
<td>88.4 ± 3.0</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>51.2 ± 4.3</td>
<td>106.2 ± 3.8</td>
<td>12.7</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>0</td>
<td>45.3 ± 1.5</td>
<td>75.6 ± 3.7</td>
<td>12.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>54.8 ± 4.6</td>
<td>72.1 ± 7.7</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>52.7 ± 3.8</td>
<td>70.5 ± 7.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Jun 2001</td>
<td>0</td>
<td>93.1 ± 13.2</td>
<td>65.4 ± 6.9</td>
<td>12.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>120.1 ± 3.3</td>
<td>146.1 ± 5.1</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>80.9 ± 3.8</td>
<td>124.8 ± 9.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Jul 2002</td>
<td>0</td>
<td>76.9 ± 5.8</td>
<td>83.4 ± 5.3</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>104.4 ± 7.5</td>
<td>153.9 ± 7.7</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Table 8
Total dissolved copper concentration, ligand concentration ($C_L$), and conditional stability constants ($K'_{CuL}$) in incubation bottles with 200 nmol L$^{-1}$ copper added (no azide added).

<table>
<thead>
<tr>
<th>Date</th>
<th>Incubation time (weeks)</th>
<th>Total dissolved Cu (nmol L$^{-1}$)</th>
<th>$C_L$ (nmol L$^{-1}$)</th>
<th>$K'_{CuL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2000</td>
<td>0</td>
<td>81.9 ± 7.2</td>
<td>84.1 ± 1.1</td>
<td>12.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>93.9 ± 8.9</td>
<td>121.7 ± 17.1</td>
<td>12.7</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>0</td>
<td>99.4 ± 6.3</td>
<td>66.6 ± 2.5</td>
<td>12.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>161.0 ± 6.9</td>
<td>98.7 ± 1.4</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>190.1 ± 12.5</td>
<td>156.2 ± 4.0</td>
<td>12.9</td>
</tr>
<tr>
<td>Jun 2001</td>
<td>0</td>
<td>155.8 ± 10.6</td>
<td>107.6 ± 6.5</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>156.9 ± 2.8</td>
<td>160.7 ± 13.9</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>115.5 ± 8.4</td>
<td>124.9 ± 1.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

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Fig. 31. Rates of ligand ($L_1$) production by intact microbial communities (solid symbols) and by azide killed controls (open symbols) as a function of copper concentration. Ligand production rates were determined from the change in ligand concentration during the first week of incubation.
with no added copper and in copper treated samples containing the biological poison azide. Ligand loss could be due to photo-degradation, bio-degradation, or ligand adsorption onto container walls. Biologically mediated ligand production, and not leakage from cells or release due to cellular lysis, is the likely candidate because ligand concentrations showed either no change or a net decrease in the azide-killed controls.

Light/dark incubations were used to determine whether ligand production is linked to photosynthesis (Fig. 32). Light and dark production was not significantly different for the control and the 200 nmol L\(^{-1}\) Cu treatment in June 2001. However, incubations carried out in light and dark bottles (June 2001 and July 2002) showed a reduction in the rate of L\(_1\)-class ligand production in the dark bottles to which 100 nmol L\(^{-1}\) copper had been added. These findings suggest that different populations within the microbial community may be responsible for L\(_1\) class ligand production at different copper concentrations and that heterotrophic processes (probably bacterial) can significantly contribute to L\(_1\)-class ligand production under some conditions (i.e., copper stress). These results along with culture studies by Gordon et al. (2000) and Schreiber et al. (1990) supports the suggestion by Croot et al. (2000) that heterotrophic bacteria could be a major biological source of copper chelators.

* Copper-complexing ligands and Cu\(^{2+}\) *

With an addition of 100 nmol L\(^{-1}\) copper an increase in ligand concentrations was observed over the first incubation week for May 2000, June 2001, and July 2002 (Fig. 33A; Table 7). In November 2000, no increase in ligand concentration was detected over the two week incubation. The decrease in dissolved (i.e., filterable) ligand concentrations from week 1 to week 2 in the June 2001 incubation may be attributed to a loss of
Fig. 32. Rates of ligand (L₁) production by an intact microbial community incubated under ambient light exposure and in the dark (white bars are light bottles and black bars are dark bottles). July 2002 data only collected for unaltered and 100 nmol L⁻¹ copper treatment. ** indicates a significant difference (p<0.01; t-test) after 100 nmol L⁻¹ copper addition. Rates of ligand production in bottles with no copper addition and with a 200 nmol L⁻¹ addition were not significantly different. Error bars are standard deviations.
Fig. 33. Ligand (L₁) concentration as a function of time after A) 100 nmol L⁻¹ copper addition in three seasonal in situ incubations and B) corresponding free Cu²⁺ ion concentrations as a result of increased ligand concentrations in response to 100 nmol L⁻¹ copper addition. Error bars are standard deviations. The lines in panel B represent the free Cu²⁺ ion concentrations causing reduced reproductive rates for diatoms (dia), coccolithophores (cocco), dinoflagellates (dino), Synechoccocus sp. (Ssp), and Synechoccocus bacillaris (Sb) (Brand et al., 1986).
filterable bound copper species, either from adsorption onto container walls, particle surfaces, or coagulation of colloidal copper species (Wells et al., 1998).

A ligand concentration increase over the first week of incubation for May 2000, June 2001, and July 2002 resulted in a decrease in free Cu\(^{2+}\) ion concentration (Fig. 33B). In November 2000, the Cu\(^{2+}\) ion concentration did not significantly increase or decrease, which is expected since the ligand concentration remained constant. During June 2001, a decrease in the free Cu\(^{2+}\) ion was observed from week 1 to 2, however, as discussed previously the ligand concentration decreased instead of increasing as expected. Therefore, the decrease in the free Cu\(^{2+}\) ion was due to that 33% loss of total dissolved copper and not to increased complexation by ligands or changes in conditional stability constant.

The culture study by Brand et al. (1986) provides a useful reference for which phytoplankton might be impacted by the free Cu\(^{2+}\) ion concentrations found in our study. The free Cu\(^{2+}\) ion concentrations that have been reported to reduce the relative reproductive rates of several phytoplankton classes in that study are indicated as horizontal lines on Figure 33B. In the May 2000, November 2000 and July 2002 experiments, the free Cu\(^{2+}\) ion concentrations present at the beginning of the experiments after 100 nmol L\(^{-1}\) copper was added were high enough to reduce the relative reproductive rates of only the more copper sensitive cyanobacterium Synechococcus. In June 2001, at Time 0 all phytoplankton classes represented would have been impacted. The free Cu\(^{2+}\) ion concentration decreased for May 2000, June 2001, and July 2002 during the entire incubation. With no change in the ligand concentration in November 2000, the free Cu\(^{2+}\) ion concentration showed little reduction during the two week
incubation with only *Synechococcus* being affected.

An increase in ligand concentrations was observed over a one week period for May 2000, June 2001, and November 2000 incubations with an addition of 200 nmol L$^{-1}$ copper (Fig. 34A; Table 8). Ligand concentrations continued to increase in copper amended containers up to two weeks in November 2000 but in June 2001, ligand concentrations decreased after one week (Fig. 29A). This ligand decrease was similar to that observed with the addition of 100 nmol L$^{-1}$ Cu in June 2001. Again, the decrease may be attributed to a loss either from adsorption onto container walls, particle surfaces, or coagulation of colloidal copper species (Wells et al., 1998).

As the ligand concentration increased during May 2000 and June 2001, the concentration of free Cu$^{2+}$ ion decreased (Fig. 34B). In May 2000, the free Cu$^{2+}$ ion concentration decreased by approximately an order of magnitude one week after 200 nmol L$^{-1}$ copper was added to the incubation bottles, and in June 2001, the free Cu$^{2+}$ ion concentration decreased more than three orders of magnitude (Fig. 34B). Although the ligand concentration increased in November 2000, there was an equivalent increase in dissolved copper, resulting in no change in the free Cu$^{2+}$ ion. Additional ligand production would have been necessary to reduce the free Cu$^{2+}$ ion concentrations significantly in November 2000. The differences among initial free Cu$^{2+}$ ion concentrations in May 2000, June 2001, and November 2000 trials was largely caused by variations in dissolved copper owing to variable loss of copper from solution. At every sampling time and in each trial, the free Cu$^{2+}$ ion concentration was only a small fraction of the total copper (~ 2.5 to 0.005%), consistent with previously reports dissolved copper speciation in estuaries (Donat et al., 1994; Kozelka and Bruland, 1998).
Fig. 34. Ligand (L₁) concentration as a function of time after A) 200 nmol L⁻¹ copper addition in three seasonal in situ incubations and B) corresponding free Cu²⁺ ion concentrations as a result of increased ligand concentrations in response to 200 nmol L⁻¹ copper addition. Error bars are standard deviations. The lines in panel B represent the free Cu²⁺ ion concentrations causing reduced reproductive rates for diatoms (dia), coccolithophores (cocco), dinoflagellates (dino), *Synechoccus* sp. (Ssp), and *Synechoccocus bacillaris* (Sb) (Brand et al., 1986).
In a survey of algal species, Brand et al. (1986) found that cyanobacteria were the most sensitive to copper toxicity, dinoflagellates and coccolithophores had intermediate sensitivity, while diatoms were the least sensitive. In the November 2000 and June 2001 experiments, the free Cu$^{2+}$ ion concentrations present at the beginning of the experiments after 200 nmol L$^{-1}$ copper was added were high enough to reduce the relative reproductive rates of all the phytoplankton classes listed in Figure 34B. However, in the May 2000 experiment, the lower Cu$^{2+}$ ion concentration would cause a reduced reproductive rate in only the more copper sensitive cyanobacterium *Synechococcus*. In May 2000, the free Cu$^{2+}$ ion concentration decreased to a level that would affect the growth rate of only the most sensitive cyanobacterial species, *Synechococcus bacillaris*. Likewise, in June 2001, the decrease in the free Cu$^{2+}$ ion concentration observed over weeks 1 and 2 would be expected to affect only *Synechococcus*. Since the November 2000 free Cu$^{2+}$ ion concentration showed little reduction during the two week incubation, the free Cu$^{2+}$ ion concentration would have continued to affect all the phytoplankton presented in Figure 34B during the entire incubation.

Analogous to marine phytoplankton, Sunda and Ferguson (1983) found marine bacteria show toxic responses to relatively low Cu$^{2+}$ ion concentrations (15 to 30 pmol L$^{-1}$). Cultures of heterotrophic bacteria have also been reported to produce dissolved, high affinity copper ligands in response to elevated Cu$^{2+}$ concentrations (Gordon et al., 2000; Schreiber et al., 1990). Based on these culture studies and since the intact microbial community contained marine bacteria as well as marine phytoplankton, the marine bacteria could be producing copper-complexing ligands in response to elevated Cu$^{2+}$ ion concentrations.
Population density and copper additions

Copper additions of 100 and 200 nmol L\(^{-1}\) affected the population density of both autotrophic picoplankton and bacterioplankton (Fig. 35). Both the 100 and 200 nmol L\(^{-1}\) copper concentrations resulted in a reduction of the population density of autotrophic picoplankton and bacterioplankton with time. However, neither population was completely eliminated even by the 200 nmol L\(^{-1}\) copper addition. Several factors may explain why copper additions did not totally eliminate all the autotrophic picoplankton and bacterioplankton. One set of factors could be the amelioration of any copper toxicity by production of strong copper-complexing ligands, which results in a reduction of free Cu\(^{2+}\) ion concentration. Also, since a significant portion of the added copper was lost to adsorption on container walls or particles, an additional reduction in copper toxicity could occur resulting in remaining autotrophic picoplankton and bacterioplankton cells. Another plausible rationale could be that some remaining cells may be resistant to copper toxicity (Gordon et al., 1993). During all four trials, autotrophic picoplankton were somewhat more impacted by copper additions than bacterioplankton. This observation is consistent with laboratory studies of copper sensitivity in various bacterioplankton and autotrophic picoplankton species that generally show autotrophic picoplankton species, such as some *Synechococcus* sp., are among the most sensitive to copper (Brand et al., 1986).

Elevated copper concentrations, within realistic limits, induced the natural estuarine microbial communities of the Elizabeth River, Virginia to produce L\(_1\) class copper complexing ligands, which dramatically reduced the free Cu\(^{2+}\) ion concentrations in the water column. Our in situ data suggest that estuarine microbial communities have the
Fig. 35. A,B) Effect of copper addition on abundance of bacterioplankton and C,D) autotrophic picoplankton during in situ incubation of the intact microbial community. Abundances shown are the percent cells remaining in comparison to control bottles to which no copper was added (average counting error was 9%).
capacity to respond to copper stress and ameliorate copper toxicity by actively buffering
the free Cu\(^{2+}\) ion through organic ligand production. Production is not due simply to cell
death and lysis or it would have been observed in the azide-killed controls. Production of
these ligands will potentially affect the interaction of copper with all organisms in the
estuary as well as its biogeochemical cycling. This detoxification pathway is a negative
feedback loop that needs to be taken into account when predicting the impact of copper
discharge into estuarine systems. The results of this study indicate that both autotrophic
picoplankton and bacterioplankton can be important contributors to production of \(L_1\) class
ligands.

To our knowledge, this is the first study to show that in situ exposure of an intact
indigenous estuarine microbial community to copper stress led to production of \(L_1\) class
ligands, resulting in amelioration of copper toxicity. This information contributes to our
understanding of the complex processes taking place in copper polluted estuarine
environments. Clearly, additional measurements in different environments will be
required before generally applicable models of copper-responsive ligand production can
be developed. In addition, other sources of copper-complexing ligands such as sediment
pore waters (Skrabal et al., 1997), and the contribution by heterotrophic bacteria need to
be taken into account in any general models of copper biogeochemistry in estuaries.

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CHAPTER VII

SUMMARY AND CONCLUSIONS

The main goal was to explore the possible connection between naturally occurring copper-complexing ligands and thiol compounds. Laboratory investigations of copper-complexing ligand and thiol compound production by the marine bacterium, *Vibrio parahaemolyticus* and a yearlong seasonal study of copper complexation in an urbanized estuary are the results of my independent research. This dissertation also includes two research projects a box model describing copper in the Elizabeth River and research investigating exposure of an intact indigenous microbial community to copper stress in the same estuary.

Laboratory results from the *V. parahaemolyticus* chemostat cultures revealed that strong copper-complexing ligands (log $K'_{Cu^{II}}$ ranging from 11.79 to 13.19) with were closely coupled to increasing total dissolved copper. Increasing copper-complexing ligand concentrations with increasing total dissolved copper concentrations suggest metal detoxification via ligand production. This metal detoxification buffered the free cupric ion, $Cu^{2+}$, to a level no longer toxic to the bacteria. Results indicated that without copper-complexing ligand production, the free cupric ion concentration could have been as much as 10 times higher then with copper-complexing ligands present. If no copper buffering were required by *V. parahaemolyticus* cultures, increases in copper-complexing ligand concentrations would not be observed. This study demonstrated that marine bacteria could be a source of organic copper chelators that maybe similar to those that control copper speciation in marine waters. Although *V. parahaemolyticus* is more
copper tolerant than the cyanobacterium *Synechococcus*, copper-complexing ligands were still produced in response to elevated copper concentrations (i.e., lowering the free cupric ion), effectively ameliorating potentially toxic copper. In natural waters where copper concentrations are high or where copper sensitive organisms are not prevalent, marine heterotrophs could be the major biological source of organic copper chelators.

Analysis of *V. parahaemolyticus* cultures also indicated that concentrations of total dissolved thiol increased with increasing concentrations of total dissolved copper. Covariations between copper-complexing ligands and total dissolved thiols were found to exist with a thiol:Cu relationship of 2:1. Strong correlations (between thiols and copper binding ligands) in concentration and their behavior in the presence of increasing copper support the hypothesis that thiol compound(s) may be a part of the copper-complexing ligand pool. With the identity of the detected copper-complexing ligands and thiols in the *V. parahaemolyticus* cultures still unclear, it is not possible to make a direct link.

Results from the yearlong observation of the Elizabeth River indicate that concentrations of total dissolved copper, copper-complexing ligands, and two thiol compounds (2-mercaptoethanol and mercaptosuccinic acid) varied seasonally. Total dissolved copper concentrations seasonal variations show a correlation to above normal yearly precipitation. Variations in concentrations of copper-complexing ligands and two thiol compounds (mercaptosuccinic acid and 2-mercaptoethanol) corresponded to the seasonal change in abundance of bacterioplankton, autotrophic picoplankton, and chlorophyll *a*, which suggests a biological source. Although these co-variations do not definitely identify sources of copper-complexing ligands and thiol compounds.

The capability of the two thiol compounds (mercaptosuccinic acid and 2-
mercaptoethanol) found in the water column to act as copper-complexing ligands during a competitive ligand equilibration-cathodic stripping voltammetric titration was analyzed. The results revealed that the thiols in this study may contribute to the strong copper-complexing ligand pool with conditional stability constants (log $K'_{\text{CuL}}$) between 11.7 - 12.6. Similar to results from the *V. parahaemolyticus* cultures, the sum of all the thiols revealed a strong linear correlation with copper-complexing ligand concentration, where total thiol concentration increased with increasing copper-complexing ligand concentration. These two observed relationships coupled with the results that thiol compounds have conditional stability constants similar to strong copper-complexing ligands is more evidence in support of the hypothesis that thiol compound(s) may be a part of the copper-complexing ligand pool. A discrepancy exists between concentration of the thiols and copper binding ligands since the total detected thiols exceeds ligand during most of the year perhaps because the HPLC technique measures truly dissolved thiols plus some thiols adsorbed onto colloidal material (K. Mopper, personal communication), while copper-complexing ligand concentration reflects only dissolved ligands.

A statistically significant missing sink was revealed from results of a modeling effort to determine gaps in our knowledge about total dissolved copper cycling in estuaries. This modeling effort incorporated data collected during the yearlong seasonal study and during two transect cruises (July 1999 and May 2000) of the Elizabeth River. What was discovered about the sink was that it is of the same magnitude or larger than point sources, benthic fluxes or total dissolved copper exchange by water movement and there is a baseline level of removal (~4000 kg Cu/yr) that is elevated ~25% between...
approximately May and September/October. Removal appeared to be seasonal occurring from May to September/October, which strongly argues for a process that may be biologically driven. Still abiotic seasonal processes cannot be disregarded without further investigations. A second missing sink explanation could be that the sink is net uptake of copper onto particles via biotic and/or abiotic processes. Future fieldwork studying the possibility of the missing sink being uptake onto particles needs to be completed to fill in the gaps of the simple two-box model.

Natural estuarine microbial communities of the Elizabeth River, Virginia were induced to produce L₁ class copper complexing ligands, which dramatically reduced the free Cu²⁺ ion concentrations in the water column. In situ data revealed that estuarine microbial communities, under copper stress, can ameliorate copper toxicity by actively buffering the free Cu²⁺ ion through organic ligand production. Azide-killed controls proved production is not due simply to cell death and lysis. Production of these ligands will potentially affect the interaction of copper with all organisms in the estuary as well as its biogeochemical cycling. This detoxification pathway is a negative feedback loop that needs to be taken into account when predicting the impact of copper discharge into estuarine systems. The results of this study indicate that both autotrophic picoplankton and bacterioplankton can be important contributors to production of L₁ class ligands.

The objective and rationale for Chapters III and IV was the major shortcoming found in thiol work presented previously. Previous studies (Leal et al., 1999; Tang et al., 2001; Al-Farawati and van den Berg, 2001; Laglera and van den Berg, 2003) relate copper binding ligands to CSV “thiol-like” peak(s) but none of these studies investigates individual thiol compounds (using a technique similar to Vairavamurthy and Mopper...
(1990a,b)) and their relationship to copper-complexing ligands. In addition, no studies have looked at seasonality of thiol compounds and copper-complexing ligands. Perhaps examination of individual thiol compounds plus seasonality could improve our lack of information about the dissolved concentrations of thiols in oxic waters and the role that they might play in controlling copper speciation in estuarine waters. Definitely, the hypothesis has not been proven; however, evidence has been put forth in support of the hypothesis. The relationships found between thiol compounds and copper-complexing ligands and strong conditional stability constants of known thiol compounds has expanded our understanding and instigated new opportunities for research.
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APPENDIX

LETTER OF PERMISSION FOR CHAPTER VI
July 15, 2004

Christina L. Dryden
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