REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)* 30-06-2003	**2. REPORT TYPE** FINAL	**3. DATES COVERED** *(From - To)* 6/21/00 - 12/31/02

4. TITLE AND SUBTITLE Metal Ion Biosensor for Wastewater Discharge	**5a. CONTRACT NUMBER** N00014-00-1-0805
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER N/A
6. AUTHOR(S) Fierke, Carol A.	**5d. PROJECT NUMBER** N/A
	5e. TASK NUMBER N/A
	5f. WORK UNIT NUMBER N/A
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Michigan Chemistry Department 930 N. University Ann Arbor, MI 48109-1055	**8. PERFORMING ORGANIZATION REPORT NUMBER** N/A
9. SPONSORING / MONITORING AGENCY NAME (S) AND ADDRESS (ES) Office of Naval Research 800 North Quincy Street Arlington, VA 22217-5000	**10. SPONSOR/MONITOR'S ACRONYM (S)** ONR
	11. SPONSOR/MONITOR'S REPORT NUMBER (S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Unlimited

20031001 237

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A carbonic anhydrase-based metal ion biosensor was optimized for measuring copper in wastewater and seawater using molecular biology methods. CA variants were prepared that enhanced the copper ion specificity and/or decreased the copper affinity were incorporated into the sensor. We have also incorporated two additional, commercially available fluorescent labels (Alexa Fluor 660 and Oregon Green) into the transducer by covalently labeling a CAII mutant containing a single cysteine residue. Finally, we have used made fusions with fluorescent proteins (dsRed-CA) as a new method of transducing metal ion concentration. These methods will be useful in the development of sensors to determine the concentration of metal ions in wastewater and the ocean.

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

15. SUBJECT TERMS

Carbonic anhydrase, biosensor, copper, wastewater, seawater

16. SECURITY CLASSIFICATION OF: **Unclassified**			**17. LIMITATION OF ABSTRACT** Unclass	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** Carol A. Fierke
a. REPORT Unclass	**b. ABSTRACT** Unclass	**c. THIS PAGE** Unclass			**19b. TELEPHONE NUMBER** *(include area code)* 734-936-2678

FINAL REPORT

Grant#: N00014-00-1-0805

PRINCIPAL INVESTIGATOR: Dr. Carol Ann Fierke

INSTITUTION: University of Michigan

E-MAIL: fierke@umich.edu

GRANT TITLE: Metal Ion Biosensor for Wastewater Discharge

AWARD PERIOD: 21 June 2000 - 31 December 2002

OBJECTIVE: A sensor to measure the amount of copper(II) ion in treated wastewater effluent will be developed and evaluated. In particular, we propose to conduct field tests of our biosensor-based transducer to test its ability to detect copper levels in wastewater streams and seawater. To enhance the operation of this sensor under realistic conditions, we are: developing the use of CAII mutants with altered specificity and affinity for copper and zinc; exploring alternate fluorescent tags for transducing metal ion binding; and developing methods to enhance the stability of the sensor under realistic conditions. The ultimate goal is to demonstrate proof of principle in realistic scenarios, and develop performance criteria for production sensors.

APPROACH: A reliable sensor is needed to monitor copper in treated wastewater and seawater. Such a sensor must rapidly and accurately indicate copper levels. Furthermore, the sensor must have high selectivity so that it accurately reports the presence of copper only, and not interfering metal ions that are present in estuarine waters at concentrations much higher than copper.

This task is a product of an ONR 6.1 program focused on developing sensors that use biological molecules as transducers to measure metal ions in seawater. Carbonic anhydrase is a protein that can easily bind low concentrations of copper in the presence of the high concentrations of calcium and magnesium found in seawater. Therefore, we have chosen to use CAII as the recognition molecule in a metal ion biosensor. Several methods to transduce the recognition of the metal ion by CA II as a change in fluorescence intensity, wavelength, fluorescence lifetime, or polarization have been developed in collaboration with Professor Richard Thompson (University of Maryland Medical School) with ONR support. The binding of the metal ion is reversible and dependent upon the concentration of the free metal ion in solution; consequently the fluorescence signal continuously indicates the concentration of the metal ion at the transducer.

The affinity of carbonic anhydrase for metal ions is high, therefore the sensitivity for metal ions is large: Cu

(II) is detectable at picomolar (10^{-12} molar) concentrations. Moreover, the enzyme structure can be changed to significantly modify the selectivity and sensitivity of the transducer molecule. Additionally, fluorescent labels can be selectively attached to a specific position on the carbonic anhydrase protein by covalently labelling a single, novel cysteine residue. Alternatively, the fluorescent tag can be a fluorescent protein that is expressed as a fusion protein with carbonic anhydrase II. The ability to modify the properties of the metal ion transducer in this way is a unique feature of this ONR-developed technology. Here we propose to use molecular biology methods to enhance the performance of a carbonic anhydrase-based fluorescent biosensor to determine copper concentrations in wastewater and seawater under realistic conditions.

ACCOMPLISHMENTS: To begin with we prepared large quantities of fluorescently labeled N67C/C206A and N67C/C206A/E117A carbonic anhydrase variants to test the performance of the biosensor under realistic conditions. The asparagine-67→cysteine and cysteine-206→alanine substitutions create a unique cysteine near the active site of CAII that can be covalently labeled with iodoacetamido-fluorophores, allowing the detection of copper binding by changes in fluorescence. In collaboration with Richard Thompson (University of Maryland), we measured free copper levels (0.25 nM, 15 parts per trillion) using our method in samples of treated wastewater from a Navy facility, demonstrating that there were no overt interferents, and suggesting that low levels can be measured *in situ* using a fluorescence-based biosensor. Furthermore, we demonstrated that the detection limit for free Cu(II) in a seawater model is about 0.05 pM, and in actual sea water, it is about 0.1 pM (0.006 parts per trillion). We then compared this biosensor to voltammetric methods for measuring copper concentrations in seawater near Woods Hole, Massachusetts where the Cu concentration varies with time due to tidal flushing at an inlet in a well characterized manner. We measured an increase from ~ 0.1 pM to ~1.0 pM free Cu(II) over a period of approximately four hours *in situ* using a 25 meter optical fiber. The individual measurements took less than a minute, provided Cu(II) levels in real time, and were performed at 15 minute intervals; operation may be fully automated. The results by fluorescence intensity and modulation methods confirmed one another, and the increase was observed during two tidal cycles.

To further enhance biosensor performance we incorporated two additional, commercially available fluorescent labels (Alexa Fluor 660 and Oregon Green) into the transducer by covalently labeling a CAII mutant containing a single cysteine residue (either L198C/C206S or N67C/C206S). Both labels demonstrated excellent response. The Alexa Fluor could be excited by a red diode laser instead of an externally modulated Argon laser. The Oregon Green-labeled N67C-CAII displayed metal affinity identical to the original, unlabeled carbonic anhydrase. This is an exciting result as the lack of interference allows us to take complete advantage of the high metal affinity of CAII as well as using this labeled protein to measure the metal affinity of other CA variants. Finally, the Oregon Green labeled L198C-CA displayed significantly decreased problems with aggregation which should increase the lifetime of the sensor.

The affinity of the wild-type CA for Cu(II) is too large to be an effective equilibrium sensor for the desired maximal level of copper in the waste stream under many conditions. Furthermore, the zinc affinity is also high and zinc is a potential interferent in the waste stream. Therefore, we have incorporated CA mutants (H94N/L198C/C206S and H94D/L198C/C206S) with decreased affinity for copper (nanomolar to micromolar) but increased specificity for copper into the sensor. The efficacy of these new variants is being tested.

Finally, we have prepared fusion proteins between carbonic anhydrase and fluorescent proteins for use in this sensor. We successfully prepared the DNA construct for a fusion between CA and the fluorescent protein, DsRed2 as well as between CA and the yellow fluorescent protein (CA-YFP). We overexpressed and purified both fusion proteins. These fusion proteins allow ratiometric measurement of zinc concentrations by fluorescent energy transfer between a fluorescent sulfonamide ligand (that binds to zinc-CA but not apo-CA) and either DsRed2 or YFP. The CA-DsRed2 fusion protein displayed a larger FRET signal than the CA-YFP fusion and is useful for measuring low concentrations of Zn. Furthermore, the fluorescence intensity of the CA-DsRed2 fusion protein is quenched upon addition of pM concentrations of copper ions thus providing an exciting new pathway for development of copper sensors.

CONCLUSIONS: We have demonstrated that a CA-based fluorescent biosensor can successfully be used to measure low concentrations of copper in seawater. Furthermore, we have demonstrated that fusions of carbonic anhydrase with the fluorescent protein, DsRed2, can be used to measure both copper and zinc concentrations.

SIGNIFICANCE: These optimized fluorescent sensors can be used to rapidly measured metal concentrations in the ocean and in wastewater. Furthermore, the CA-DsRed2 fusion sensor can be expressed in cells to measure low concentrations of zinc and copper *in vivo*.

PATENT INFORMATION:
R. B. Thompson, M. Cramer, C. A. Fierke, H.-H. Zeng, and R. Bozym "Excitation Ratiometric Fluorescent Biosensor for Zionc Ion at Picomolar Levels," U. S. Provisional Patent Application 3645-0108P,

AWARD INFORMATION: NONE

PUBLICATIONS AND ABSTRACTS (for total period of the grant):

a. R. B. Thompson, H.-H. Zeng, M. Loetz, and C. A. Fierke, "Issues in enzyme-based metal ion biosensing in complex media," *Proc. SPIE Conference on In-vitro Diagnostic Instrumentation (Vol 3913)* (G. Cohn, Ed.) pp. 120 – 127 (2000).

b. J. A. Hunt, C. A. Lesburg, D. W. Christianson, R. B. Thompson, C. A. Fierke, "Active-site engineering of carbonic anhydrase and its application to biosensors," in *The Carbonic Anhydrases: New Horizons* (C. Chegwidden, Ed.) Basel: Birkhauser Verlag, pp. 221 – 240 (2000).

c. R. B. Thompson, H.-H. Zeng, B. P. Maliwal, and C. A. Fierke, "Real-time quantitation of Cu(II) by a fluorescence-based biosensing approach, " *Proc. SPIE Conf. on Advances in Fluorescence Spectroscopy V (Vol. 4252)* (R. B. Thompson and J. R. Lakowicz, Eds.), pp.12 – 19 (2001).

d. Fierke, C. A. and Thompson, R. B. (2001) Fluorescence-based biosensing of zinc using carbonic anhydrase, *Biometals* **14**, 205-22.

e. Thompson, R. B., Peterson, D., Mahoney, W., Cramer, M., Maliwal, B. P., Suh, S. W., Frederickson, C. J., Fierke, C. A. and Herman, P. (2002) Fluorescent zinc indicators for neurobiology, *J. Neurosci. Meth.* **118**, 63-75.

f. Thompson, R. B., Cramer, M. L., Bozym, R. and Fierke, C. A. (2002) Excitation ratiometric fluorescent biosensor for zinc ion at picomolar levels, *J. Biomed. Optics* **7**, 555-560.

g. Thompson, R. B., Zeng, H.-H., Maliwal, B. P. and Fierke, C. A. (2001) Real-time quantitation of Cu(II) by a fluorescence-based biosensing approach, *Proc. SPIE Conf. on Advances in Fluorescence Spectroscopy V*, **vol. 4252**, (R. B. Thompson and J. R. Lakowicz, Eds.), 12-19.

h. Thompson, R. B., Zeng, H.-H., Fierke, C. A., Fones, G. and Moffett, J. (2002) Real-time *in situ* determination of free Cu(II) at picomolar levels in sea water using a fluorescence lifetime-based fiber optic biosensor, *Proc. SPIE Conf. On Clinical Diagnostic Systems: Technologies and Instrumentation*, **vol. 4625** (G. E. Cohn, Ed.), in press.