REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
8-15-05	Final Report	Oct. 1, 2002 - Dec. 31, 2004

4. TITLE AND SUBTITLE
Metabolic Engineering of Seaweeds for the Detoxification of TNT-Contaminated Marine Waters

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-03-1-0081

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rorrer, Gregory L.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Chemical Engineering
Oregon State University
Corvallis, OR 97331

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
875 N. Randolph Street
Arlington, VA 22203-1995

10. SPONSOR/MONITOR'S ACRONYM(S)
ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project provided fundamental information on the ability of three representative marine macroalgae, commonly known as seaweed, to tolerate, take up, and metabolize the explosive compound 2,4,6-trinitrotoluene (TNT) dissolved in seawater. At biomass density of 1.2 g/L and initial TNT concentrations of 10 mg/L, TNT removal from seawater was 100% within 72 hours. TNT was not absorbed into the biomass, but was instead converted to products. Specific rates of TNT uptake were 0.018 L/g-hr for Acrosiphonia coalita filaments, 0.062 L/g-hr for Porphrya yezoensis blades, and 0.047 L/g-hr for Portieria hornemannii microplantlets. Two immediate products of TNT reduction, 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dintrotoluene (4-ADNT), were excreted to the liquid medium, but accounted less than 30% of the initial TNT. This study suggests that marine seaweed could help restore marine environments contaminated with TNT. However, since TNT contamination of the marine environment is not well documented, this project did not consider field studies to test or deploy this bioremediation technology.

15. SUBJECT TERMS
seaweed, macroalgae 2,4,6-tritritroluene, TNT, seawater, uptake, metabolism

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Gregory L. Rorrer
					19b. TELEPHONE NUMBER *(Include area code)* 541-737-3370

FINAL REPORT

GRANT #: N00014-03-1-0081
Office of Naval Research (ONR)

PRINCIPAL INVESTIGATOR: Dr. Gregory L. Rorrer
541-737-3370
rorrergl@engr.orst.edu

INSTITUTION: Oregon State University
Department of Chemical Engineering
Corvallis, OR 97331

GRANT TITLE: Metabolic Engineering of Seaweeds for the Detoxification of TNT-Contaminated Marine Waters

AWARD PERIOD: October 1, 2002 to December 31, 2004

OBJECTIVES

This project provided fundamental information on the ability of native marine macroalgae, commonly known as seaweed, to tolerate, take up, and metabolize the explosive compound 2,4,6-trinitrotoluene (TNT) dissolved in seawater.

Research Objectives: 1) Determine the intrinsic capacity of tissue cultures derived from three model marine macroalgae to remove TNT dissolved in seawater; 2) Measure the kinetics of TNT metabolite formation and elucidate the pathways for TNT biotransformation within these organisms; 3) Assess the viability of marine seaweeds following TNT exposure and uptake.

APPROACH

Tissue cultures of three representative species of marine macroalgae were developed. Axenic filamentous tissues of the temperate marine green alga *Acrosiphonia coalita* were established by isolation and culture of apical cell tips. Axenic plantlet (blade) cultures of the temperate red alga *Porphyra yezoensis* were established by isolation and culture of asexual monospores. Axenic microplantlets of the tropical marine red macroalga *Portieria hornemannii* were established by callus induction and shoot tissue regeneration techniques.

Macroalgal tissue cultures were cultivated in bubbler flasks containing 450 mL natural seawater medium and enriched nutrients at pH 8.2-8.5. After three weeks of growth, the culture was exchanged with fresh enriched seawater medium containing 2,4,6-trinitrotoluene (TNT) at initial concentrations ranging from 1.0 to 50 mg L^{-1}. Process conditions were set at temperature range of 13 to 23 °C, biomass loading of 1.0 g FW L^{-1}; aeration rate of 0.44-0.54 L air L^{-1} culture min^{-1}, illumination intensity of 0 (dark), 35, and 153 μE m^{-2} s^{-1}; and photoperiod of 14 hr light/10 hr dark.

Concentrations of unconsumed TNT, its immediate biological reduction products 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), and its abiotic, photolytic degradation products 1,3,5-trinitrobenzene (TNB) and 2,4-dintrotoluene (2,4-DNT) were

measured in both the liquid medium and within the biomass as a function of exposure time to TNT contaminated seawater. TNT and its metabolites in the liquid medium were isolated by solid-phase extraction on a Waters Sep-Pak/Porpax RDX cartridge, whereas metabolites within the biomass were isolated by solvent extraction with acetonitrile. TNT and its metabolites in these extracts were quantitatively assayed by HPLC on a Waters Nova-Pak C8 reverse-phase analytical column. The viability of microplantlets after exposure to TNT in seawater was assessed by photosynthetic oxygen evolution and respiration rate measurements.

ACCOMPLISHMENTS

The uptake and metabolism of the explosive compound 2,4,6-trinitrotolune (TNT) by three marine macroalgae was demonstrated: a filamentous tissue culture of temperate green alga *Acrosiphonia coalita*, a plantlet (blade) tissue culture of the temperate red alga *Porphyra yezoensis*, and a semi-differentiated shoot tissue culture (microplantlet culture) tropical red alga *Portieria hornemannii*. Suspension tissue cultures of each macroalga were grown up on enriched natural seawater medium within a bubble-aerated suspension to a fresh weight (FW) density of 1.2 g FW L^{-1} under illumination conditions (35-150 $\mu E\ m^{-2}\ s^{-1}$, photoperiod of 16:8 hr or 14:10 hr), and then challenged with seawater containing dissolved TNT at initial concentrations ranging from 1.0 to 50 mg L^{-1}. The uptake and transformation of TNT by each alga was similar. At initial TNT concentrations of 10 mg L^{-1} or less, TNT removal from seawater was 100% within 72 h for *Portieria hornemannii* and *Porphyra yezoensis*. Specific rates of TNT uptake were 0.016-0.018 L g^{-1} FW h^{-1} for *Acrosiphonia coalita* filaments, 0.047-0.062 L g^{-1} FW h^{-1} for *Porphrya yezoensis* blades, and 0.039-0.047 L g^{-1} FW h^{-1} for *Portieria hornemannii* microplantlets. These rates were at least 10 times higher than rates of abiotic TNT photo-degradation in the seawater control experiments. For the *Portieria hornemannii* microplantlets, specific rates of TNT uptake were the same under either illuminated or dark conditions. Specific rates of TNT uptake for marine macroalgae were 4-15 higher than those reported for aquatic vascular plants. In all culture systems, only trace amounts of TNT were found within the biomass, demonstrating that TNT was not simply absorbed into the plant tissue but was instead converted to products.

Two immediate products of TNT reduction, 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dintrotoluene (4-ADNT), were excreted to the liquid medium, but never accounted for more than 30% of the initial TNT. For the illuminated TNT uptake experiments, the abiotic TNT photo-oxidation product 1,3,5-trinitrobenzene (TNB) was also identified. Only trace amounts of 2-ADNT and 4-ADNT were found within acetonitrile extracts of the biomass.

Viability of seaweeds was sensitive to the initial concentration of TNT. Removal of TNT by *Portieria hornemannii* microplantlets at initial concentrations of 1.0 or 10 mg L^{-1} did not affect the respiration rate. At an initial TNT concentration of 10 mg L^{-1}, net photosynthesis decreased towards zero as TNT was consumed, whereas at an initial TNT concentration of 1.0 mg L^{-1}, the net photosynthesis rate was not affected. At initial TNT concentrations of 50 mg L^{-1}, TNT uptake was not sustained after 24 h, and plantlets died.

CONCLUSIONS

This study has demonstrated that three species of macrophytic marine algae -- one green alga and two red algae -- have an intrinsic capacity to remove TNT dissolved in seawater. These studies were performed using axenic tissue cultures, so that TNT metabolism by marine macroaglae could be investigated in the absence of other organisms. All three macroalgae initially transformed TNT to 2-ADNT and 4-ADNT, consistent with TNT biotransformation by terrestrial aquatic plants. Although the endpoints of TNT metabolism by these macroalgae were not chemically identified, it appears that the final products of TNT metabolism stay within the non-extractable portion of the plant tissue, a result consistent with TNT metabolism by terrestrial vascular plants. Future studies will work towards characterizing the physical location and chemical composition of the endpoints of TNT metabolism within the nonextractable algal biomass. Although three species of macroalgae were tested in this study, it is likely that the ability of marine macroalgae to metabolize TNT is widespread, if not generic. At initial TNT concentrations below 1.0 mg L^{-1}, photosynthesis was not inhibited and the organisms remained viable. Since potential applications will likely involve low TNT concentrations below 1 mg L^{-1}, the viability of marine macroalgae at low TNT concentration further demonstrates the utility of these organisms for treatment of seawater containing low concentrations of munitions constituents such as TNT.

SIGNIFICANCE

Contamination of the marine environment by munitions constituents is not well documented. However, several near-shore benthic marine environments are littered with unexploded ordnance (UXO) from past military operations. In general, there is a growing concern that UXO may have the potential to discharge munitions constituents to the surrounding sediment and seawater in the benthic marine environment. In particular, the munitions constituent 2,4,6-trinitrotoluene (TNT) is toxic to many benthic marine organisms. Clearly, there is a need to identify macrophytic, benthic marine organisms that are both tolerant to TNT and capable of TNT metabolism. The natural metabolic capacities of these organisms could be harnessed for use in engineered natural systems, e.g. bioremediation systems, for removal and detoxification of munitions constituents from the marine environment.

Marine macroalgae, commonly known as seaweed, are abundant in near-shore benthic environments. In controlled laboratory studies, this project has shown that marine seaweeds possess an intrinsic capacity to tolerate, take up, and metabolize the munitions constituent TNT. Consequently, marine seaweed could serve as natural remediators for the restoration of marine environments contaminated with TNT. However, since contamination of the marine environment by munitions constituents is not well documented, this project did not consider field studies to test or deploy this bioremediation technology.

PATENT INFORMATION

An Invention Disclosure Form will be filed with the Technology Transfer Office at Oregon State University. Title: Removal of TNT from Seawater by Marine Seaweed, co-inventors Gregory L. Rorrer (Professor) and Octavio Cruz-Uribe (Graduate Student), Department of Chemical Engineering, Oregon State University.

PUBLICATIONS AND ABSTRACTS

Peer Reviewed Journal Articles:

Cruz-Uribe, O., and Rorrer, G.L. Uptake and biotransformation of 2,4-trinitrotoluene (TNT) by microplantlet suspension cultures of the marine red macroalga *Portieria hornemannii.* Biotechnology & Bioengineering (in press 2005). Pre-print attached.

Cruz-Uribe, O., Cheney, D.P., and Rorrer, G.L. Comparison of TNT Removal from Seawater by Tissue Cultures of Three Marine Macroalgae. Submitted to *Marine Environmental Research* (in review 2005). Pre-print attached.

Peer Reviewed Book Chapters:

G. R. Lotufo, G.R., Rorrer, G.L., Cruz-Uribe, O, Cheney, D.P., Lydy, M.J., Steevens, J.A. Bioaccumulation, biotransformation and biochemical toxicology of explosives and related compounds in aquatic organisms. In: *Ecotoxicity of Explosives*, G. Sunahara, J. Hawari, G. Lotufo, R. Kuperman (Eds). CRC Press (in production).

Conference Presentations:

Rorrer, G.L. (*invited speaker*), Cruz-Uribe, O, and Cheney, D.P. "Detoxification of Organic Pollutants by Marine Seaweeds." *2005 Annual Meeting of the American Association for the Advancement of Science (AAAS)*, Symposium on Phytoremediation: New Solutions to Pollution Remediation on Land and in the Sea, Feb. 18, 2005, Washington, DC.

Cruz-Uribe, O., and Rorrer, G.L. (speaker). "Biotransformation of TNT in Seawater by Microplantlet Tissue Cultures of the Marine Red Alga *Portieria hornemannii.*" *Fall 2003 National Meeting of the American Institute of Chemical Engineers (AIChE),* Paper #411e, Session on Advances in Environmental Biotechnology I: Remediation, Nov. 19, 2003, San Francisco, CA.

Other Presentations:

Rorrer, G.L. (*invited speaker*), "Metabolic Engineering of Seaweeds for the Detoxification of TNT-Contaminated Marine Waters." *Joint Interagency Phytoremediation Research Program, Principal Investigators Meeting.* National Science Foundation, Jan. 20, 2004, Washington, DC.

Rorrer, G.L., Cruz-Uribe, O., Cheney, D.P. "Uptake and Metabolism of Trinitrotoluene from Seawater by Tissue Cultures of Native and Transgenic Marine Seaweeds." *Annual Technical Review, U.S. Navy Project on UXO/MC in the Marine Environment*, Feb. 6, 2004, US Army ERDC Environmental Research Laboratory, Vicksburg, MS.

Popular Scientific Press:

Chemical & Engineering News, Feb. 28, 2005, Louisa Dalton, Vol. 83(9), p. 14 with photo. Seaweeds have an Appetite for TNT.