The use of esterase activity as a measure of copper toxicity in marine microalgae.

Thesis submitted by

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in February 2006

for the degree of Master of Science
in Tropical Plant Sciences
within the School of Tropical Biology
James Cook University

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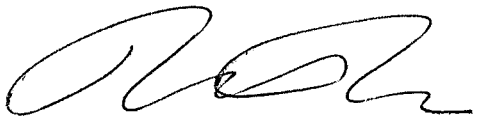

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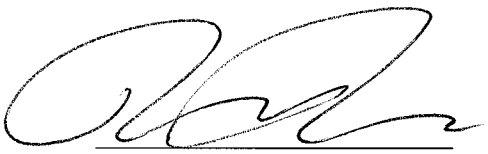

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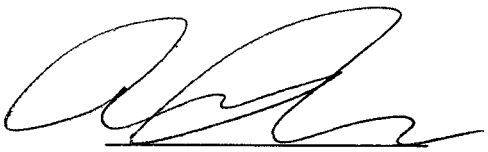
STATEMENT ON THE CONTRIBUTION OF OTHERS

I, the undersigned, acknowledge the following contributions to this thesis by others:

- Gathering of *Tetraselmis sp.* growth data by Leanne Sparrow, as seen and acknowledged in chapter 1.
- Analysis of bioavailable copper via inductively coupled plasma optical emission spectrometry by Stephen Boyle (Australian Institute of Marine Science) in chapter 1.
- Microphotographs of *Tetraselmis sp.* by Dr. Kirsten Heimann, as seen in chapter 3.
- Collaboration with Dr. K. Heimann on research design.
- Collaboration with Dr. K. Heimann, David Donald, Dr. Yvette Everingham, and Dr. Mike Steele on data analysis, as acknowledged throughout this thesis.
- Editorial assistance from Dr. K. Heimann.
- Financial help: CRC Reef in the form of an Augmentative postgraduate research grant, NQAIF (North Queensland Algal Culturing/ Identification Facility) waiving bench fees, and the James Cook University's School of Tropical Biology internal research allocation and competitive supplementary research allocation.



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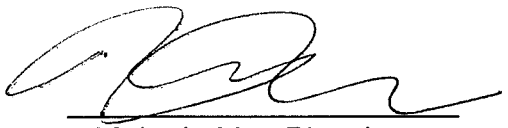
ACKNOWLEDGEMENTS

I, the author, would like to thank Dr. Kirsten Heimann, my research supervisor, for her assistance with accessing funding, especially regarding NQAIF, the North Queensland Algal Identification/ Culturing Facility, preparation for conferences, manuscript editing, and experimental design.

I would also like to express my thanks to CRC Reef, particularly Dr. Britta Schaffelke and Mr. Tim Harvey, for financial, administrative, and scholarly support of this thesis and its associated research.

Thanks to James Cook University's School of Tropical Biology for financial and administrative support.

I also thank the members of NQAIF for their conversations and support, and Matty Williams for his unwavering belief in my abilities.



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Abstract

Copper is necessary for normal cellular activity, but may become toxic at high levels. Copper is widely used in North Queensland as a component of agricultural chemicals and antifouling paints, and high levels of Cu have been measured in some near-shore marine environments. Because of this, there is a need to develop early warning systems of Cu pollution in marine microalgae. Fluorescein diacetate (FDA) is commonly used as a substrate in esterase activity assays as a measure of cellular activity. Intracellular cleavage of FDA by esterases results in free fluorescein, which can be quantified fluorometrically as a sublethal endpoint. The purpose of this research was to: 1) determine the effects of experimental design on esterase activity in *Tetraselmis* sp. (Chlorophyta) and *Chaetoceros gracilis* (Heterokontophyta), 2) evaluate the use of esterase activity as a bioassay endpoint for Cu toxicity in *Symbiodinium microadriaticum* (Dinophyta), and 3) evaluate the effects of pH, salinity, and temperature on esterase activity in *Tetraselmis* sp. employing two different experimental designs. Time was also devoted to standardisation of methods due to the lack of standardised protocols in the literature.

The effect of experimental design on esterase activity (fluorescence) in *Tetraselmis* sp. and *C. gracilis* was determined by two different protocols. In the first protocol, microalgae were incubated with Cu in culture flasks, followed by sample transfer and FDA-incubation in microtitre plates for analysis. The second protocol exposed microalgae directly to copper in microtitre plates, and Cu toxicity on esterase activity (percent inhibition of fluorescence) was quantified without sample transfer. The flask protocol showed high within-culture variability and was laborious, whereas the microtitre plate protocol displayed a significant and replicable effect of Cu on percent inhibition of fluorescence. The difference between the protocols is not due to binding of Cu ions to the Erlenmeyer flasks: analysis of bioavailable Cu by inductively coupled plasma optical emission spectrometry (ICP-OES) proved that there was no Cu binding of any nominal Cu concentrations over time. Therefore, differences are likely due to stagnation of esterase activity upon transfer from the flasks to the microtitre plates or differential adhesion of the organisms to the glassware, despite methodological uniformity.

Working with established cultures of zooxanthellae (*Symbiodinium microadriaticum*) is extremely challenging, and due to its strong adhesive properties, standardisation of initial inoculation density (a necessity in microalgal bioassays) is highly difficult. Adding to the challenge is a lack of published literature using established cultures of zooxanthellae, and the tendency of the authors to "pool" their data, burying the independent culture-specific dose-response relationships within large standard errors. The aim of this research section was to: quantify *S. microadriaticum* culture density using protein content and chlorophyll *a* autofluorescence as proxies, and direct cell count. The usefulness of these estimations in standardising initial inoculation density was evaluated in independent experiments using esterase activity and Cu toxicity on esterase activity in the previously standardised microtitre plate bioassay. The results of the bioassays showed irreproducible Cu dose-response curves and base esterase activities between independent cultures of *S. microadriaticum,* indicating that all three procedures for estimating culture density were unsuitable for standardising initial inoculation density. However, this research illustrated the effect of culture and data pooling on bioassay outcome, and recommended data handling protocols for future ecotoxicological research.

The third aim of this research was to quantify the effects of pH, salinity, and temperature on esterase activity and Cu toxicity in *Tetraselmis* sp. within the context of two different protocols: one that examined the effects of pH, salinity, and temperature within three independent cultures (WIC), and another that examined the effects of these parameters between 15 independent cultures (BIC) using the microtitre plate protocol. It is necessary to determine the effect of pH and salinity on metal toxicity because these parameters can affect metal speciation, which may alter overall toxicity. Temperature can influence cellular membrane permeability, which in turn may also affect toxicity. In general, pH and temperature had a significant effect on both esterase activity and Cu toxicity in both protocols, and salinity generally did not affect fluorescence or Cu toxicity. The effect of culture did not have a consistently significant effect on either fluorescence or Cu toxicity for all three environmental parameters in the WIC protocol. This research demonstrated that the microtitre plate bioassay for Cu toxicity should be performed at stable pH and temperature levels. It also

revealed that *Tetraselmis* sp. may be a suitable candidate for bioremediation of copper in marine and estuarine waters, due to its stable esterase activity at high levels of Cu (1.0mg Cu L^{-1}), and in changing pH, salinity, and temperature regimes.

In conclusion, experimental design significantly impacted esterase activity in *Tetraselmis* sp. and *C. gracilis*. The microtitre plate protocol is a rapid, cost-effective method to determine Cu toxicity on esterase activity in microalgae. However, some organisms (such as *S. microadriaticum*) are unsuitable for use in this bioassay due to their unique physical properties. The microtitre plate protocol is a useful tool to determine the effects of pH, salinity, and temperature on esterase activity and Cu toxicity in *Tetraselmis* sp. The ability of this organism to tolerate high levels of Cu and changing environmental parameters suggests that it may be a candidate for Cu bioremediation of marine and estuarine waters.

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