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

Thesis submitted by

Melanie Lise BLANCHETTE BSc(Hons) ***University of New Hampshire***

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James Cook University

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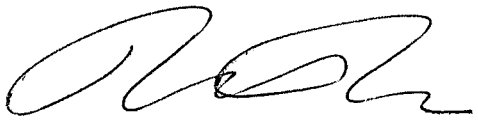

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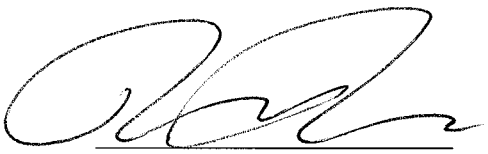

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STATEMENT ON THE CONTRIBUTION OF OTHERS

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.
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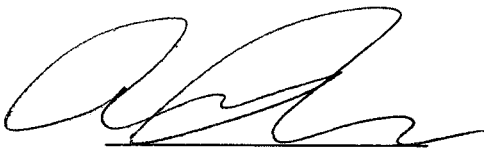

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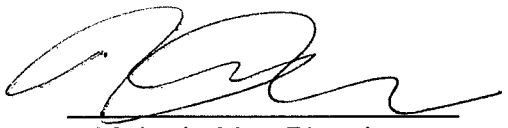

Melanie Lise Blanchette

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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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Chapter 1

The use of esterase activity as a measure of metal toxicity in marine microalgae- general introduction.

Increased levels of anthropogenic pollutants have contributed to the worldwide decline of marine environments (Patty et al. 1999, Edgar et al. 2000, Lytle and Lytle 2001). Contaminated terrestrial runoff becomes adsorbed by benthic sediments, and may leach back into the water column after rain or disturbance events (Lytle and Lytle 2001). Microalgae are important marine primary producers, and have the ability to accumulate pollutants such as heavy metals (Peterson et al. 1984, Lage et al. 1996, Chang and Reinfelder 2000). Metals readily transfer between marine trophic levels, with phytoplankton contributing to the initial uptake (Moreno-Garrido et al. 1999, Fernandez-Leborans and Herrero 2000, Chang and Reinfelder 2000, Ettajani et al. 2001, Wang 2002). Metals may bioaccumulate in the food web through predation by mollusks and other filter feeders as well as grazing by zooplankton (Smith et al. 1996, Chen and Folt 2000). The potential adverse effects of metal bioaccumulation on human and ecological health necessitate economically viable early warning systems to detect the presence of toxic levels of metals in the marine environment (Blaise et al. 1997).

Bioassays are toxicity tests that create “links” between a pollutant and its effects on an organism, and ideally utilise sublethal endpoints to provide an early warning detection of pollution (Blaise et al. 1997, Lytle and Lytle 2001). As critical marine and estuarine primary producers, microalgae have proved invaluable in toxicity bioassays. However, many toxicity tests do not account for the effects of environmental parameters such as pH, salinity, and temperature, which can profoundly affect bioassay outcome (Peterson et al. 1984, Latala and Surosz 1998, Craig et al. 2003). Knowledge of the chemistry, physiology, and morphology of test organisms is also incomplete, which may compromise the validity of some bioassays because the nature of metal uptake in microalgae appears to be species-specific (Romeo and Gnassia-Barelli 1985).

Metal accumulation in microalgae begins at the cellular armour-environment interface with metal adsorption to the cell wall (Romeo and Gnassia-Barelli 1995, Campbell et al. 2002). Metals are attracted to biotic ligands present on the cell’s surface, and quickly become adsorbed. The metals are then more slowly internalised by the cell via transport across the cell membrane (Knauer et al. 1997, Campbell et al. 2002). The biotic ligand model (BLM) is an interdisciplinary approach to quantifying the effects of metals on

aquatic organisms (Paquin et al. 2002), and states that the response of an organism to toxic levels of metals is quantifiable by the nature of metal binding to biotic ligands; the "sites of toxicity" of the organism (Paquin et al. 2002). Different chemical forms of metals with varying levels of toxicity exist in aquatic environments as: free ions, organically-complexed, and inorganically-complexed ([Schnoor et al. 1997], as cited in Paquin et al. 2002). The BLM suggests that the bioavailability of metals is largely due to the amount of free ions in the medium, which is considered the most toxic metal species because free ions are the most strongly attracted to biotic ligands (Paquin et al. 2002). To date, the BLM is the most integrated approach to quantify metal-organism interactions (Campbell et al. 2002, Paquin et al. 2002) but gaps exist in this hypothesis. Specifically, the BLM assumes that biotic ligands operate uniformly within a wide range of environmental conditions (such as pH, salinity, and temperature), and solution metal chemistry may not be an adequate predictor of metal toxicity in some organisms (Errecalde et al. 1998). The biotoxic effect of free ions and metal species in relation to changing environmental parameters is discussed in chapter 4 of this thesis.

Microalgae have a suite of defenses to cope with toxic levels of metals, and cell wall and membrane chemistry is crucial to negating these effects (Crist et al. 1981). When in contact with metals, phytoplankters may: bind the metals extracellularly (i.e. exclude the metals from intracellular sites) or produce phytochelatins (PC) that bind to free ions and decrease overall toxicity and shunt the toxins to inert intracellular sites (Morris 1980). Phytochelatins have a high affinity for metal ions, and toxic amounts of metals can induce the production of copious amounts of PCs in most phytoplankton species (Ahner et al. 1995). PCs may also bind trace metals for intracellular storage (Ahner et al. 1995). Inert extracellular ligands are also present on the surface of most microalgae which do not transport metals across the cell membrane, thereby preventing certain amounts of metals from entering the cell (Bates et al. 1982, Knauer et al. 1997). In contrast, active membrane-associated sites are involved in energy-consuming transport across the cell membrane. The amount of internalised metals correlates directly with the number of active ligands on a cell's surface (Bates et al. 1982). The mechanisms that phytoplankton species employ to address toxic levels of metals are diverse and likely species-specific,

which has enormous implications for cellular accumulation and trophic transfer of metals.

Despite the strategies employed to negate the effect of toxic levels of metals in microalgae, cell processes may still be negatively impacted. Esterases are enzymes essential to phospholipid turnover in cellular membranes (Dorsey et al. 1989), and their intracellular activity can be quantified *in vivo* by the conversion of fluorescein diacetate (FDA), a nonpolar, lipophilic, nonfluorescent compound that readily passes through the plasma membrane of cells (Rotman and Papermaster 1966, Bentley-Mowat 1982, Dorsey et al. 1989) to fluorescein (Dorsey et al. 1989, Snell et al. 1996). Copper has been shown to negatively affect the esterase activity of *Selenastrum capricornutum* (Chlorophyta), as measured by esterase-induced fluorescein fluorescence (Snell et al. 1996, Franklin et al. 2001, discussed in detail in chapter 2 of this thesis), though the exact toxic mechanism of copper on esterase activity remains unknown. Metals generally inhibit enzyme activity by binding to sulphydryl groups (integral to enzyme structure and catalytic action), or competing with essential trace elements for extracellular binding sites on the cellular surface (Van Assche and Clijsters 1990). However, it is unclear whether copper specifically affects esterases in either of the above manners. Toxic levels of copper may also negatively affect the growth of some microalgae by compromising membrane integrity (Franklin et al. 2001), interfering with photosynthesis (Baron et al. 1995), or cell division and cytokinesis (pers. obs., see chapter 4 of this thesis).

In small amounts, copper is essential for cellular function (Guillard and Ryther 1962). For example, Cu is a constituent of the primary electron donor in photosystem I; the Cu-protein plastocyanin (Baron et al. 1995). However, anthropogenic pollution often increases copper concentrations to toxic levels (Sadiq 1992). The use of sublethal endpoints, such as esterase activity (discussed in chapter 2), provides an early warning assessment of copper toxicity to marine microalgae. Esterase activity also elucidates the nature of copper toxicity, as the copper likely has to be transported intracellularly to affect esterase function. The nature of copper accumulation and toxicity is possibly modulated by species-specific extracellular morphological variation, which may have enormous implications for trophic transfer in marine communities.

The aim of this thesis was to evaluate assay conditions and the suitability of select marine microalgae in WET (whole-effluent) toxicity testing. Within the time constraints of an MSc, these analyses were restricted to one pollutant, copper, which is relevant to North Queensland, Australia. Using copper as the model toxicant, this thesis explored the logistics of laboratory-based esterase activity bioassays such as the suitability of specific microalgae as test species and established baseline data on which to build models of copper toxicity. Enzymatic analysis in biological matter is useful for diagnostic (toxicity) purposes, and many authors (Hoppe et al. 1988, Overbeck 1991, and Obst et al. 1998) state that the healthy function of an aquatic ecosystem cannot be comprehended without acknowledgement and characterisation of enzymatic activity. However, WET (whole-effluent) toxicity tests on microalgal enzymatic activity are logistically impossible to conduct in the field due to intraspecific biochemical differences in a natural population (Wood and Leatham 1992). Additionally, WET tests are designed to provide information to industries regarding the toxicity of their effluents to determine if they can be discharged safely into the environment, and are not field surveys of ecological contamination (Heimann, pers. comm.). As such, WET tests were never designed to assess the effect of pollutants on an ecosystem, and therefore are limited in scope to advise industries on the safe handling of their discharge. Despite the inability of laboratory-based bioassays to pinpoint the effects of a particular toxicant in nature, WET tests are advantageous in that they provide direct evidence of the effect of a pollutant on selected organisms.

In this thesis, evaluation of assay conditions and the suitability of select marine microalogae in WET testing was accomplished with the use of esterase activity as a measure of copper toxicity in: *Tetraselmis* sp. (Chlorophyta), *Chaetoceros gracilis* (Heterokontophyta), and *Symbiodinium microadriaticum* (Dinophyta). This thesis addressed the impact of experimental design on esterase activity in *Tetraselmis* sp., and *C. gracilis*, and highlighted the importance of considering species-specific biological characteristics when designing a bioassay with the technically challenging *S. microadriaticum*. Additionally, this thesis demonstrated the significant impact of pH and temperature on esterase activity in *Tetraselmis* sp., and provided evidence that

environmental parameters must be accounted for when performing bioassays for metal toxicity in marine microalgae.

Chapter 2

The impact of experimental design on growth and esterase activity as measures of copper toxicity in *Tetraselmis* sp. (CHLOROPHYTA) and *Chaetoceros gracilis* (HETEROKONTOPHYTA).

Introduction

In ecotoxicology, the use of sublethal bioassay endpoints provides an early warning detection system for pollution. Esterase activity is a sublethal endpoint that can be affected by pollutants in a dose-dependent manner (Snell et al. 1996). However, there is little standardisation within or between published esterase bioassay protocols, and therefore the results are applicable only to that particular study. This lack of standardisation in the literature presents difficulties when comparing toxicity to esterase activity among different species of microalgae, and constructing general conclusions regarding the effects of a specific pollutant.

Esterases are phylogenetically ubiquitous enzymes found in all living organisms (Snell et al. 1996), and are essential to phospholipid turnover in cellular membranes (Dorsey et al. 1989). Esterase activity is measured *in vivo* by the introduction of fluorescein diacetate (FDA, Orndorff and Hemmer 1927), a nonpolar, lipophilic, nonfluorescent compound that readily passes through the plasma membrane of cells (Bentley-Mowat 1982, Dorsey et al. 1989, Snell et al. 1996, Franklin et al. 2001a). Rotman and Papermaster (1966) were the first to recognise that FDA can be used as a test of membrane integrity, as intracellular FDA is cleaved by nonspecific esterases and converted to fluorescein, a fluorescing polar compound that only breaches a compromised plasma membrane. The fluorescent product created upon cleavage of FDA, fluorescein, can be quantified as a measure of cellular viability in a toxicity bioassay (Dorsey et al. 1989, Snell et al. 1996, Franklin et al. 2001a).

Advantages of using esterase activity as a bioassay endpoint are that bioassays can be conducted in many organisms, and sensitivities can be compared across phyla and kingdoms (Snell et al. 1996). In addition, esterase activity can be assessed using a wide variety of analytical tools. FDA has been used to visually assess fluorescence output in microalgae through epifluorescent microscopy as a measure of growth media composition and dark-survival in Dinophyta (Selvin et al. 1988/1989), and culture age in the Cyanophyta, Chlorophyta, and Xanthophyta (Heterokontophyta; Andersen 2004) (Pouneva 1997). Bentley-Mowatt (1982) first employed FDA for studying viability in microalgae as a response to copper toxicity using fluorescence

microscopy. More recently, flow cytometry has been used to assess esterase activity in microalgae using FDA (Arsenault et al. 1993, Gala and Giesy 1994, Lage et al. 2001, Franklin et al. 2001b), which is advantageous to probe the metabolic state of individual cells (Franklin et al. 2001a). Spectrofluorometry has also been used to assess esterase activity via fluorescein fluorescence in microalgae (Galgani et al. 1992, Worgan et al. 2003, Cronin et al. 2004), and the spectrofluorometers and associated protocols can range from rudimentary to highly technical.

Despite the practical advantages of having a wide variety of analytical tools to assess esterase-induced fluorescein fluorescence, standardisation between protocols becomes difficult and data often conflict. This is because expression of esterase activity (quantified by fluorescein fluorescence) depends upon environmental conditions such as temperature, salinity, and irradiance (Minier et al. 1993), substrate (FDA) incubation time (Heslop-Harrison and Heslop-Harrison 1970), and biological variables such as cell density and circadian rhythm (Gilbert et al. 1992). *Selenastrum capricornutum* (Chlorophyta) has been used extensively in published bioassays for copper toxicity to esterase activity, which facilitates comparison of bioassay outcome. Snell et al. (1996) quantified esterase-induced fluorescence of fluorescein in *S. capricornutum* using a microplate fluorometer after exposure to Cu (0.025-0.800mg L^{-1}) for one hour. After five minutes of FDA incubation, the one-hour esterase inhibition test yielded a no observed effect concentration (NOEC) of 0.200mg Cu L^{-1}, and a lowest observed effect concentration (LOEC) of 0.400mg Cu L^{-1}. In contrast, Franklin et al. (2001a) measured esterase-induced fluorescein fluorescence in *S. capricornutum* using a flow cytometer. They described a dose-dependent inhibition of esterase activity to Cu (0.005-1.0mg L^{-1}) after five minutes of FDA incubation. Time-course studies revealed a 3h IC_{50} (Cu concentration causing 50% inhibition of fluorescence) of 0.112mg Cu L^{-1}, and a 24h IC_{50} of 0.051mg Cu L^{-1}. Given that Snell et al. (1996) and Franklin et al. (2001a) used the same organisms and the same FDA incubation time but different analytical techniques to quantify esterase activity, the Franklin et al. (2001a) method appears to be substantially more sensitive. It is, however, unclear whether the difference in sensitivity of *S. capricornutum* is related to experimental conditions or method of quantification. It is also possible that cell

cycle, circadian rhythm, inoculation density, or *S. capricornutum* strain impacted on the outcome of these bioassays.

The purpose of the research presented in this chapter was to determine the effect of experimental design on esterase activity in *Tetraselmis* sp. (Chlorophyta) and *Chaetoceros gracilis* (Heterokontophyta). Two protocols were employed: one in which the microalgae were incubated with Cu in Erlenmeyer culture flasks, followed by sample transfer and FDA incubation in microtitre plates for analysis. The second protocol exposed microalgae directly to Cu in the microtitre plates, and Cu toxicity on esterase activity (percent inhibition of fluorescence) was quantified without sample transfer. Environmental and culture conditions between the two protocols were identical. The effect of Cu on culture growth of *Tetraselmis* sp. and *C. gracilis* was also examined, which was compared to the sublethal endpoint esterase activity.

Tetraselmis sp. and *C. gracilis* were chosen for this experiment because both are important aquaculture feed organisms (Duerr et al. 1998, Nelson et al. 2002, Nunez et al. 2002) for commercially lucrative organisms such as oysters and mussels (Ronquillo et al. 1997), and copper may bioaccumulate within the food web. Additionally, the presence of *Tetraselmis* in copper-contaminated environments has been shown to shield predatory rotifers from the toxic effects of Cu (Luna-Andradae et al. 2002). Toxicity to esterase activity has been examined in *Tetraselmis* sp. for mercury (Snell et al. 1996, Satoh et al. 2002), copper (Gilbert et al. 1992, Snell et al. 1996, Franklin et al. 2001a), and pesticides (Gilbert et al. 1992, Snell et al. 1996), which is useful when comparing the results presented in this chapter. However, to my knowledge there have been no studies of toxicity to esterase activity using *Chaetoceros*, and only one publication reviewing the effect of cell lysis on esterase activity in *Chaetoceros decipiens* (Agusti et al. 1998). The use of *C. gracilis* in this study expands the current knowledge base regarding copper toxicity to esterase activity in this organism.

Methods and Materials

Culture conditions and maintenance for *Tetraselmis* sp. and *Chaetoceros gracilis*

All culture handling and bioassay procedures were performed in a sterile laminar flow (AES Environmental Pty. Ltd.) using acid-washed (10% HCl) and autoclaved glassware and pipette tips. Guillard's f/2 growth medium (Guillard and Ryther 1962) was prepared from frozen 50x concentrated 20mL aliquots (Sigma, St. Louis, MO 63178 USA). Frozen aliquots were allowed to thaw at room temperature, and were mechanically mixed with 980mL of pre-filtered (0.45μm Whatman GFc filters) natural seawater (35ppt) for 30 minutes using a magnetic stirrer (200rpm, Schott laboratory stirrer SLR, Germany). Growth medium was then sterile-filtered (Millipore 47mm with Teflon® face, Bedford, MA, USA, Millipore Durapore membrane filters 0.45μm HV, Ireland) and stored in the dark at room temperature for a maximum of seven days. 1mL of six to eight-week old algal culture was inoculated into 99mL Guillard's f/2 growth medium and cultures were allowed to grow undisturbed (24°C, approximate irradiance of 37.68μmol photons x m^{-2} x s^{-1} (measured on a light meter: LI-COR LI-250, LI-COR Inc., Lincoln, Nebraska, USA) 12:12 light:dark cycle in an environmentally controlled growth chamber (Contherm Digital Series Cooled Incubator)) for 2-3 weeks (flask assay) or seven days (microtitre plate bioassay) before use.

Flask Bioassay

In this bioassay, algae were exposed to copper in 250mL Erlenmeyer flasks with subsequent sample transfer to a 96-well microtitre plate for fluorometric analysis.

Culture preparation and inoculation.

Algae were exposed to copper in 250mL Erlenmeyer flasks (acid-washed, autoclaved) with aluminium foil-covered cotton stoppers (Steristopper, Imbros, Hobart 7009, Australia). Algae (2-3 weeks old) from multiple independent culture flasks were combined in an acid-washed and autoclaved 2L Erlenmeyer flask; this was performed due to the large volume of culture

necessary for this experiment and to negate culture-specific variation. The 2L flask was gently swirled to homogenise cell density, and cell number was quantified on a Neubauer Improved Haemocytometer (Hirschman EM Techcolor haemocytometer, Leitz Wetzlab Dialux microscope with Leitz Wetzlab 40x objective, Germany). All 250mL Erlenmeyer flasks contained 149mL of inoculate standardised to $1.5x10^5$ cells mL^{-1} in Guillard's f/2 growth medium.

$1.5x10^5$ cells mL^{-1} is the standard initial inoculation density for all bioassays in this chapter based upon the esterase bioassay protocol of Snell et al. (1996). The control growth curves ($Ln(N_t/N_o)$, N_t=cells mL^{-1} at time point t (days), N_o=cells mL^{-1} in initial inoculation density, equation adapted from Fogg and Thake 1987) for *Tetraselmis* sp. (Fig. 2.1a) and *C. gracilis* (Fig. 2.1b) are presented below. During the bioassay, flasks were randomised for position in the culture chamber and exposed to 24°C on a 12:12 light:dark cycle. *Tetraselmis* sp. was exposed to an approximate irradiance of 37.68μmol photons x m^{-2} x s^{-1} (normal) light, whereas *C. gracilis* was exposed to two different approximate irradiances (37.68 and 11.50μmol photons x m^{-2} x s^{-1} (low) light) in two independent bioassays for Cu toxicity to culture growth.

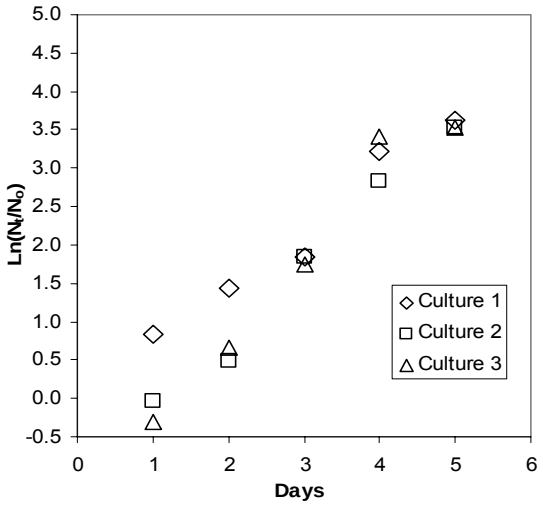


Figure 2.1a. Growth curves of *Tetraselmis* sp. n=3 with 3 replicates per count. Standard deviations are hidden by symbols. *Tetraselmis* sp. counts conducted by Leanne Sparrow; data used in this thesis with her permission.

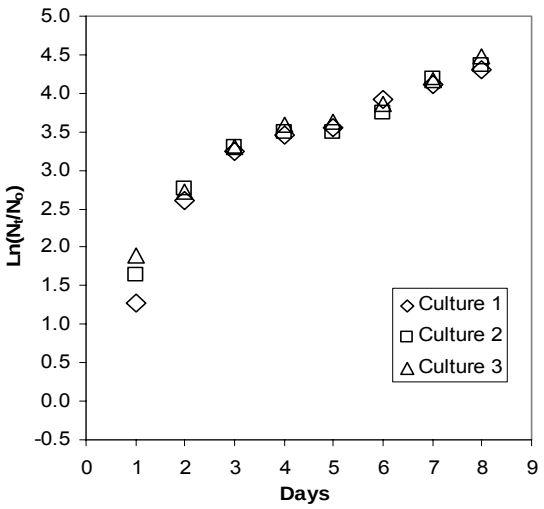


Figure 2.1b. Growth curves of *Chaetoceros gracilis*. n=3 with 3 replicates per count. Standard deviations are hidden by symbols

Copper stock and set-up of copper dose-response concentrations.

The copper stock ($CuCl_2 \bullet 2H_2O$, 150.0mg Cu L^{-1} (AJAX Chemicals, Auburn, NSW 2144)) was prepared in dI H_2O (Elix 5, Millipore filtration system equipped with a pre-filter and carbon trap) and autoclaved. $CuCl_2 \bullet 2H_2O$ was chosen as a pollutant rather than the frequently used $CuSO_4 \bullet 5H_2O$ because Cl^- is more prevalent in natural seawater (19.344 g kg^{-1} H_20) than SO_4^{2-} (2.712 g kg^{-1} H_20) (Libes 1992), and sulphates are readily taken up by microalgae as a nutrient (Mosulen et al. 2003), which may bias the treatment results. Nominal copper concentrations of trace (0.005 mg Cu L^{-1} in Guillard's f/2 medium), 0.125, 0.250, 0.500, and 1.00 mg Cu L^{-1} were used, with five randomly assigned replicates (flasks) per copper concentration. Nominal Cu stock concentrations were added to each flask; complementary amounts of autoclaved dI H_2O and copper stock were used to ensure all flasks had 150mL total volume and all flasks received a 1mL dI H_20 inoculate (Table 2.1).

Copper stock older than seven days was discarded according to James Cook University's occupational health and safety protocol (L. Winsor, pers. comm. 2005); this procedure was not performed in the NQAIF (North Queensland Algal Culturing/ Identification Facility) laboratory, which is a metal-free area. After each bioassay for Cu toxicity, copper waste was stored in plastic 2L screw-top containers (HDPE, ACI Australia) and NaOH pellets (Ajax Finechem Seven Hills, NSW 2147) were added to precipitate any dissolved copper. Post- precipitation, the copper-free seawater was decanted into the sink and the remaining precipitate was filtered using fluted filter paper (Whatman Qualitane 185mm, England) in a large plastic funnel. The filtrate was discarded into the sink, and the filter paper was allowed to dry in the funnel in a fume hood (Hamilton Australia, Brisbane, Qld) for 12 hours. Once dry, the filter papers were stored in a double layer of plastic bags, tied off with a twist tie. The bags containing the filters were sealed in a properly labelled cardboard box and disposed of by the James Cook University Central Services department.

Table 2.1. Cu Pipetting Scheme for the flask bioassay.

Nominal Cu (mg L^{-1})	Cu stock (mL)	DI H_2O (mL)
f/2 (trace Cu control)	0	1
0.125	0.125	0.875
0.250	0.250	0.750
0.500	0.500	0.500
1.000	1	0

Sample preparation for growth and fluorometric analysis.

Before collecting samples for growth and fluorometric analysis (at 6, 12, and 24 hours of Cu incubation), each Erlenmeyer flask containing *C. gracilis* or *Tetraselmis* sp. was gently agitated (swirled) to detach cells from the bottom of the flask. 275µL was transferred from each test flask into a microtitre plate under sterile conditions for quantification of esterase activity. An additional 1mL sample was also taken from each flask for growth analysis and transferred into a microcentrifuge tube with one drop of Lugol's iodine as a fixative. Cell densities (cells mL^{-1}) were quantified on a Neubauer Improved haemocytometer.

Quantification of esterase activity via fluorometric analysis.

The quantification of esterase activity via the Erlenmeyer flask method warranted transfer of cultures from the flasks to a white, flat-bottomed 96-well microtitre plate (Dynex Microlite 1) for fluorometric analysis. Sample transfer and analysis in the microtitre plates was performed at each time point (6, 12, and 24 hours) using a protocol adapted from Snell et al. (1996).

Esterase activity was quantified on a fluorescence spectrophotometer (Varian Cary Eclipse) with the settings as per Table 2.2.

Table 2.2. Spectrophotometric (Varian Cay Eclipse) settings for the microtitre plate bioassay for Cu toxicity to esterase activity.

Parameter	Setting
Software	Cary Eclipse
Operating Program	Kinetics
Excitation wavelength	485nm
Emission wavelength	530nm
Time (5 samples per group)	30sec
PMT detection	Medium

275µL of culture transfer was first added to each test well in a randomised design, followed by addition of the enzyme substrate fluorescein diacetate (FDA, $C_{24}H_{16}O_7$). Stock FDA was prepared fresh by dissolving 1mg FDA in 1mL acetone (Unilab LR). Solubilisation of FDA was aided by gently vortexing, and FDA stock was stored at -80°C until use. The stock FDA was diluted to a

"working FDA" solution prepared from 24μL of FDA stock in 760μL acetone in a microcentrifuge tube and mixed gently by inversion. Working FDA solutions were never older than 1 hour and originated from the same FDA stock aliquot to reduce variability. 37μL of working FDA solution (2.7μM final FDA concentration) was added to test wells containing 275μL of organism inoculate from each flask. Based upon pilot studies with *C. gracilis* (Figure 2.2), FDA was incubated for 30 minutes prior to reading, and addition of FDA was staggered so that each well had precisely 30 min of incubation with FDA. Staggering FDA addition is based upon the skill and speed of the researcher. In this bioassay, five wells received FDA every five minutes, with the difference between FDA additions from the first to the fifth well never exceeding 30s. In addition to the 25 wells containing algae and FDA, five wells contained 275μL of sample plus 37μL of sterile-filtered natural seawater with no copper or FDA to correct the data for the normal background fluorescence of the cells.

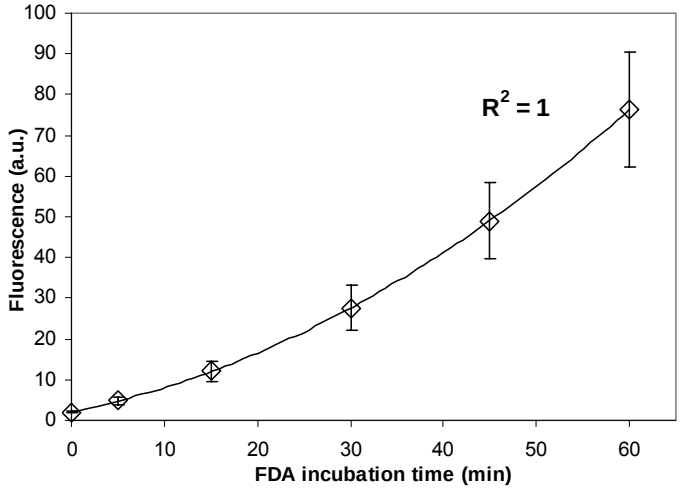


Figure 2.2. Time course of fluorescein diacetate (FDA) incubation on esterase-induced conversion of FDA to fluorescein (fluorescence (a.u.)) for *Chaetoceros gracilis*. n=5 replicates of one culture, standard error is shown, model is second-order polynomial, R^2 value calculated by Microsoft Excel®.

Five additional control wells contained 312μL Guillard's f/2 medium to monitor instrument drift. Only the middle wells of the cell plate were utilised for quantification of esterase activity to avoid variability in measurements due to "edge" effects associated with handling (Blaise et al. 1998). After addition of FDA to appropriate wells, samples were read on the spectrofluorometer according to the parameters in Table 2.2.

Microtitre plate bioassay

In this protocol, *Tetraselmis* sp. and *Chaetoceros gracilis* were exposed to Cu in the microtitre plate for 24h followed by direct fluorometric analysis. Unlike the flask assay, there was no sample transfer, and the 24h incubation of the cells in the plate necessitated an additional protocol to sterilise the plates.

Plate preparation

One white, flat-bottomed 96-well microtitre plate (Dynex Microlite, USA) was acid-washed with 10% HCl (24 hours) and rinsed thoroughly with dI H_2O. In the sterile laminar flow, the plate was submersed in 70% Ethanol (Recochem, Lytton, Qld 4178) for 20 minutes, rinsed thoroughly with dI H_2O and left to dry. The plate was then exposed to UV light within the laminar flow for 30 minutes.

Culture preparation, plate inoculation, and set-up of Cu dose-response concentrations.

Tetraselmis sp. and *C. gracilis* were exposed to the same environmental conditions as described for the flask bioassay. Each bioassay used an independent 7-day old algal culture standardised to $1.5x10^5$ cells mL^{-1} with Guillard's f/2 growth medium. 275µL of standardised cultures were added to each microtitre plate well, followed by addition of the nominal Cu concentrations: trace (0.005 mg Cu L^{-1} in Guillard's f/2 medium), 0.125, 0.250, 0.500, and 1.00 mg Cu L^{-1} with five replicates per Cu concentration in randomly assigned wells. Copper stock ($CuCl_2 \bullet 2H_2O$, 12.5mg Cu L^{-1}) was added with complementary amounts of dI H_2O to ensure each well received a total volume of 25µL of aqueous copper solution or dI H_2O (controls) (Table 2.3). Cu concentrations were added in a randomised, predetermined fashion, so as not to bias results due to location on the plate. The addition of 25µL dI H_2O to 275µL culture aliquot did not significantly alter the salinity of the test media, as determined on a salinometer (AO Special Scale, American Optical Corp., Keene, NH, USA). As mentioned above, copper stock solutions older than seven days were safely discarded.

Table 2.3. Cu pipetting scheme for the microtitre plate bioassay.

Cu (mg/L)	Cu stock (µL)	dI H_2O (µL)
f/2 (trace Cu control)	0	25
0.125	3.125	21.875
0.250	6.25	18.75
0.500	12.5	12.5
1.00	25	0

The controls consisted of five replicates (wells) of 275µL inoculate plus 25µL sterile filtered (0.45µm Whatman GFc filters) seawater to correct treatment results for the naturally occurring background fluorescence associated with the organisms. Five independent replicates of 300µL sterile-filtered Guillard's f/2 medium were also analysed, to ensure the accuracy of the fluorescence spectrophotometer (Varian Cary Eclipse).

After inoculation, plates were wrapped in plastic wrap (GLAD Wrap) and stored in the culture chamber (Contherm Digital Series cooled Incubator) in 24°C and an approximate irradiance of 37.68µmol photons x m^{-2} x s^{-1} on a 12:12 light:dark cycle for 24 hours. Plates remained undisturbed for the duration of exposure to copper. The plastic wrap allows for gas exchange while deterring bacteria (K. Heimann, pers. comm.), and an insignificant amount of evaporation within microtitre plate wells after 24 hours was documented by Blaise et al. (1998).

Quantification of esterase activity via fluorometric analysis.

After 24h of Cu exposure in the microtitre plate, esterase activity was quantified by measuring fluorescein fluorescence as stated in the flask bioassay. However, well contents were resuspended with a pipette six times prior to analysis to ensure a homogeneous distribution of cells within the wells (Hickey et al. 1991).

<u>Determination of medium-associated Cu in the flask bioassay.</u>

Experimental design

The amount of medium-associated Cu in the flask assay over time was determined by inoculating Erlenmeyer flasks (250mL, acid-washed, autoclaved, with aluminium-covered Steristoppers®) with 149mL sterile-filtered Guillard's f/2 medium (35ppt) and 1.0mL sterile nominal Cu concentration (trace: 0.005 mg

Cu L^{-1} in Guillard's f/2 medium, $CuCl_2 \bullet 2H_2O$: 0.250, 0.500, 1.00mg Cu L^{-1}). There were three independent replicates (flasks) per nominal Cu concentration. Flasks were treated as if they contained algae, and were randomly placed in the culture chamber (24°C, approximate irradiance: 37.68µmol photons x m^{-2} x s^{-1}, 12:12 light:dark cycle). 3mL samples were collected from each flask after 0, 3, 12, and 24h of incubation with Cu, and placed in a 10 mL acid-washed sample tube (gamma-sterile, V-bottom, Crown Scientific, NSW 2170, Australia). Samples were acidified with 1 drop of HNO_3 (70%, produced by the manufacturer APS Finechem, Seven Hills, NSW, Australia) to prevent the Cu from binding to the sample tubes and stored at room temperature for a maximum of 24h before analysis for Cu content.

Cu determination by inductively coupled plasma optical emission spectrometry (ICP-OES).

ICP-OES has been successfully used to quantify Cu in seawater (Bradford and Bakhtar 1991, Otero-Romani et al. 2005). The samples were processed at the Australian Institute of Marine Science (AIMS, Townsville, Australia) by analytical chemist Mr. Stephen Boyle using ICP-OES (Thermo IRIS). Cu standards were prepared by dilution of 1000mg L^{-1} Cu stock (Spectrosol™) in filtered seawater. Seawater for the preparation of standards was collected at the AIMS offshore pontoon, containing 3ppb background Cu and acidified with 0.1%HNO_3. The calibration blank was Milli-Q water (reverse osmosis filter with a carbon trap). Certified reference material NIST 1643C (National Institute of Standards & Technology) was analysed at the beginning and end of the sample analysis as a quality control check. Cu was analysed simultaneously on two emission wavelengths (324.7 and 327.3nm) to increase confidence in the results. Samples were analysed directly from the original sample tubes (10mL gamma-sterile, V-bottom, Crown Scientific, NSW 2170) with no additional treatment. Data are presented as Cu (mg L^{-1}) relative to nominal Cu (mg L^{-1}) at time points 0-24h.

Data Analysis

The effect of Cu on growth and esterase activity in the flask bioassay was analysed by one-way ANOVA (Statistica version 7, StatSoft Inc., Tulsa,

OK, USA) to test for significant ($p<0.05$) difference between the means of the Cu treatments. F-values are written as F(df effect, df error). To analyse the microtitre plate bioassay data, advice was obtained from Mr. David Donald of the James Cook University Mathematics and Physics Department, Townsville, Queensland, and analysis via ANCOVA was conducted using SPSS software (Version 12.0.1, SPSS Inc., USA). Analysis via ANCOVA was deemed appropriate due to the possibility of culture as a covariant, because independent cultures were used for the five bioassays, which may impact on the bioassay outcomes.

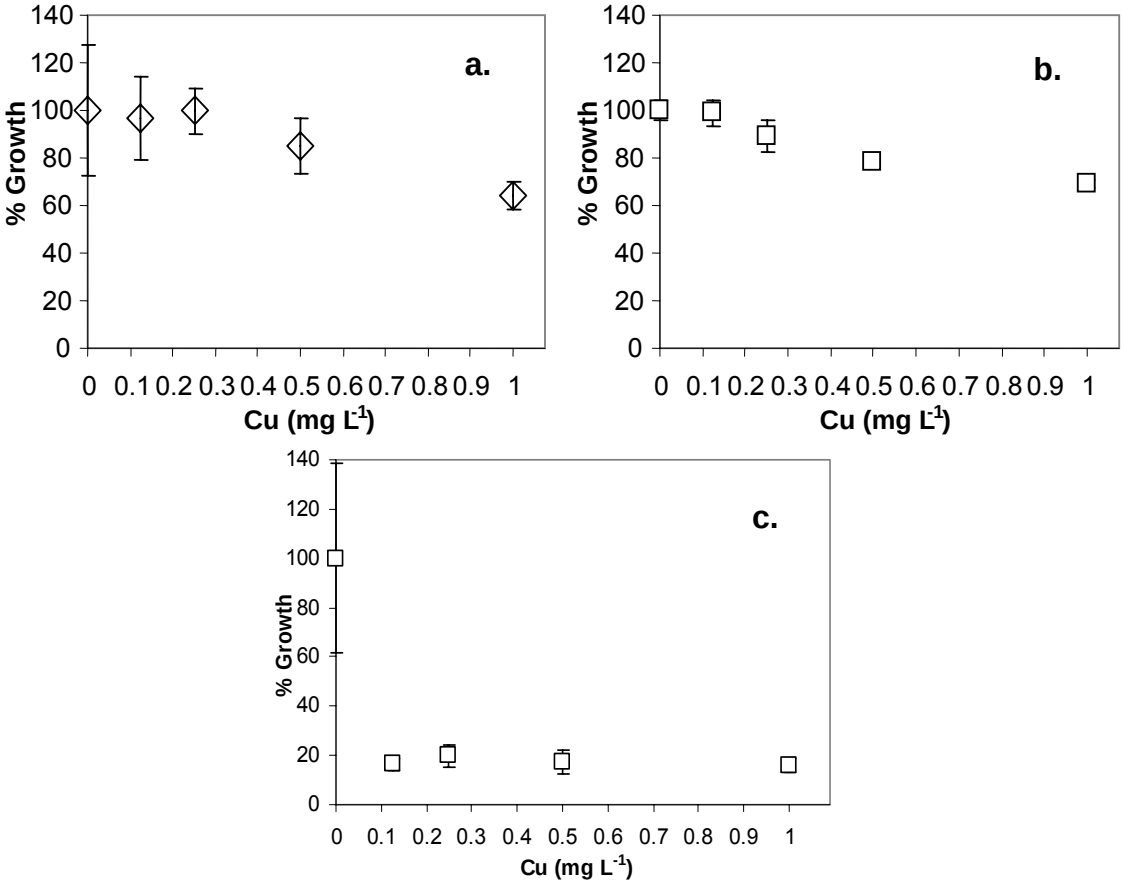
Data obtained for *C. gracilis* using the microtitre plate protocol conformed to the ANCOVA assumption of homogeneity of slopes between culture dose-response curves (Owen and Froman 1998), and an ANCOVA determined the effects of both Cu and culture on esterase activity (percent inhibition of fluorescence). Cu was the covariate and culture was assigned as a random factor. Measurements obtained for the *Tetraselmis* sp. data did not conform to the assumption of homogeneity of slopes, and a scatter plot of the unstandardised residuals versus known fluorescence (a.u.) revealed random scattering around zero, with only one substantial outlier. A sequence plot of the residuals versus culture showed no obvious pattern, therefore, Cu concentrations were log-transformed [Ln(1+Cu)], and plotted against percent inhibition of fluorescence. This approach yielded (to a reasonable visual approximation) homogeneous slopes between the independent bioassays. The ANCOVA for *Tetraselmis* sp. was performed with Ln(1+Cu) as the covariate, and culture as the random factor (see Results). F- and p-values are written as above.

Results

Dose-response to Cu using growth as an endpoint.

As shown by a one-way ANOVA, there was a statistically insignificant dose-response effect of 24h Cu treatment on culture growth in *Tetraselmis* sp. ($F_{(1,8)}$=4.98, p=0.089) at normal approximate irradiances (Figure 2.3a). However, graphical presentation of the data (Figure 2.3a) revealed a decline in percent growth at higher Cu concentrations; the lowest percent growth (63.83%) occurred at 1.0mg Cu L^{-1}.

In contrast, there was a significant ($F_{(1,8)}$=50.18, p<0.0001) dose-dependent effect of 24h Cu treatment on culture growth for *Chaetoceros gracilis*



Figures 2.3a, 2.3b, and 2.3c. 24h dose-response to Cu on percent growth of (2.3a) *Tetraselmis* sp. grown at an approximate irradiance of 37.68µmol photons x m^{-2} x s^{-1} (normal irradiance) (2.3b) *Chaetoceros gracilis* grown at normal irradiance (2.3c) *Chaetoceros gracilis* grown at an approximate irradiance of 11.50µmol photons x m^{-2} x s^{-1} (low irradiance).

grown at normal irradiances (approximately 37.68µmol photons x m^{-2} x s^{-1}, Figure 2.3b). As Cu concentrations increased, percent growth decreased, with the lowest percent growth (63.83%) occurring at 1.0mg Cu L^{-1}. At low irradiance (approximately 11.50µmol photons x m^{-2} x s^{-1}), the results of a one-way ANOVA ($F_{(1,8)}$=11.79, p=0.009) showed a significant dose-response to Cu on growth in *C. gracilis*, with percent growth declining as Cu concentrations increased (Figure 2.3c). Percent growth was reduced to between 15.95 and 19.75% of control cultures at all Cu treatment concentrations (0.125-1.00mg Cu L^{-1})- a far more severe inhibition by Cu at low concentrations compared to responses measured at normal irradiances (Figure 2.3b).

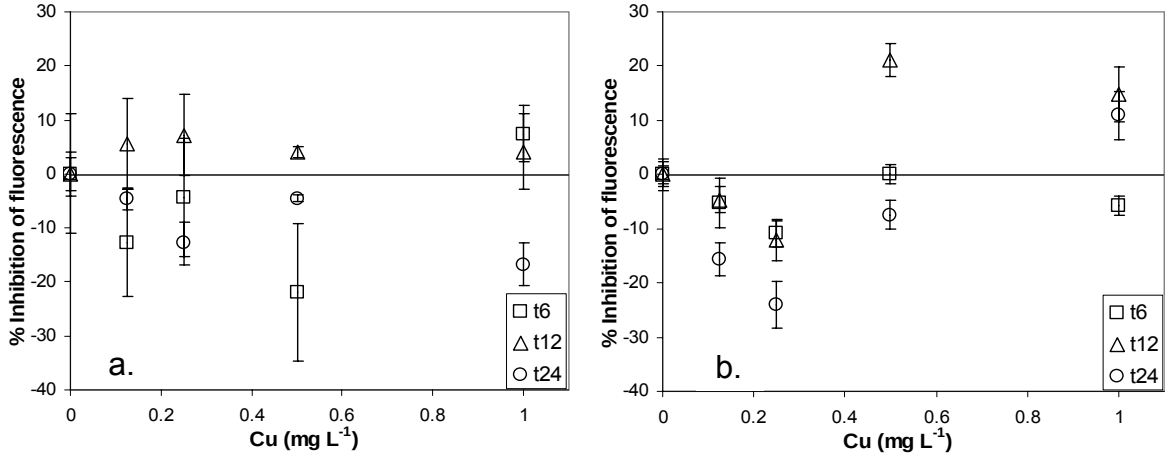
Dose-response to Cu and time-course studies using esterase activity as an endpoint in the flask bioassay protocol.

Using the flask protocol, a one-way ANOVA revealed no significant effect of Cu on esterase activity after 6 ($F_{(1,8)}$=0.224, p=0.649), 12 ($F_{(1,8)}$=0.231, p=0.643,), and 24 hours of exposure ($F_{(1,8)}$=3.46, p=0.100) in *Tetraselmis* sp. (Figure 2.4a). Over the three time points examined, no clear dose-response to Cu on esterase activity could be observed. Percent coefficient of variability (%CV) for each time point within each Cu concentration in dose-response treatments was generally low to moderate (Table 2.4).

Similar to the dose-response and time-course results for *Tetraselmis* sp. using the flask bioassay (Figure 2.4a), a one-way ANOVA also revealed no significant effect of Cu on esterase activity after 6 ($F_{(1,8)}$=0.826, p=0.390), 12 ($F_{(1,8)}$=0.988, p=0.349), and 24 hours of exposure ($F_{(1,8)}$=1.54, p=0.250) in *Chaetoceros gracilis* (Figure 4b), with no clear dose-response trend in the data. %CV's for each time point within each Cu concentration in dose-response experiments in *C. gracilis* were generally lower than those of *Tetraselmis* sp. (Table 2.4).

Table 2.4. Percent coefficient of variation (%CV) for each time point within each Cu concentration in the Erlenmeyer flask dose-response experiments for toxicity to esterase activity in *Chaetoceros gracilis* and *Tetraselmis* sp.

Cu incubation (h)	Trace (in f/2 medium)	0.125 mg Cu L^{-1}	0.250 mg Cu L^{-1}	0.500 mg Cu L^{-1}	1.00 mg Cu L^{-1}
***Tetraselmis* sp.**	*%CV*	*%CV*	*%CV*	*%CV*	*%CV*
6	22.18%	18.31%	21.24%	22.25%	11.02%
12	7.25%	15.68%	14.38%	1.77%	12.93%
24	8.56%	5.54%	9.97%	1.91%	9.50%
C. gracilis					
6	7.12%	10.83%	5.64%	4.49%	4.34%
12	6.67%	6.99%	10.67%	10.42%	16.34%
24	4.12%	7.56%	9.25%	6.42%	10.76%



Figures 2.4a and 2.4b. Dose-response to Cu in time-course experiments on esterase activity (percent inhibition of fluorescence) in (2.4a) *Tetraselmis* sp. and (2.4b) *Chaetoceros gracilis* using the flask bioassay.

<u>24h dose-response to Cu with esterase activity as an endpoint using the microtitre plate bioassay protocol.</u>

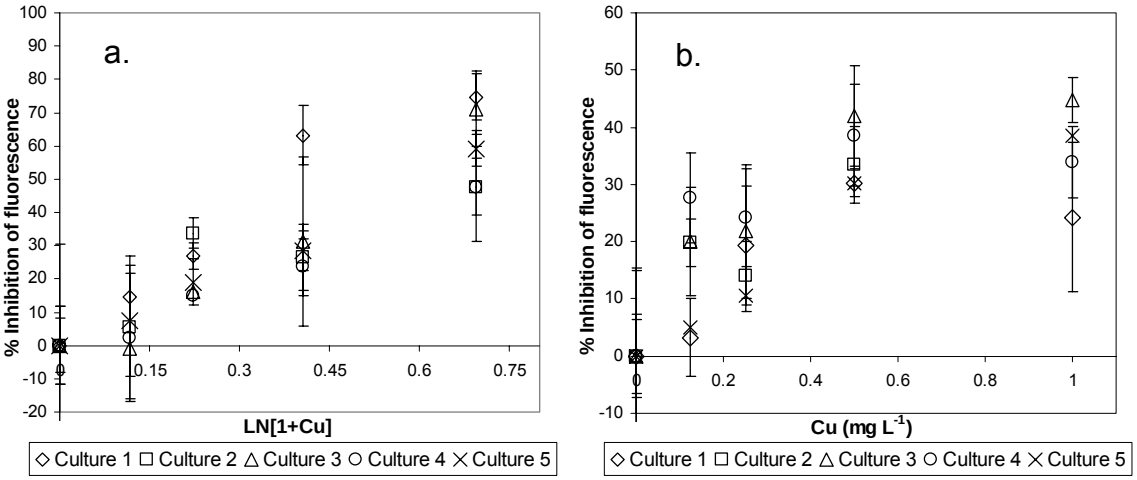
The results of an ANCOVA showed a significant dose-response to 24h copper exposure (Ln(1+Cu), $F_{(1,15)}$=220.32, p<0.001) and a significant effect of culture ($F_{(1,15)}$= 28.56, p<0.001) on esterase activity in *Tetraselmis* sp. using the microtitre plate bioassay. As Cu concentrations increased, esterase activity decreased (Figure 2.5a). The IC_{50} (Cu concentration causing 50% inhibition) was approximately 0.500mg Cu L^{-1}. Within each independent experiment (culture), %CV was low to moderate for all treatment Cu concentrations (Table 2.5), however, %CV was very large between independent cultures at each Cu

treatment concentration (0=0%, 0.125=87.29%, 0.250=36.18%, 0.500=46.81%, 1.00=21.28%).

Similar to the Cu dose-response results for *Tetraselmis* sp., the results of an ANCOVA suggested a significant dose-dependent effect of 24h Cu exposure ($F_{(1,15)}$=23.89, p<0.001) and a significant effect of culture ($F_{(4,15)}$=13.61, p<0.001) on esterase activity for *Chaetoceros gracilis* (Figure 2.5b). The IC_{50} for Cu on esterase activity for *C. gracilis* was determined to lie between 0.125 and 0.250mg Cu L^{-1}. Maximal effect of Cu on esterase activity appeared to occur at 0.500mg Cu L^{-1}, with no additional inhibitory effect of Cu observed at higher Cu concentrations. Within each independent culture, %CV is low to moderate for all treatment Cu concentrations (Table 2.5), and greater to quite large between independent cultures in the esterase activity bioassay at each Cu treatment concentration (0=0%, 0.125=69.66%, 0.250=31.47%, 0.500=14.97%, 1.00=24.42%).

Table 2.5. Percent coefficient of variation (%CV) within each independent experiment (culture) of the microtitre plate bioassay for Cu toxicity to esterase activity at Cu treatment concentrations in *Tetraselmis* sp. and *Chaetoceros gracilis*.

	Trace (in f/2 medium)	**0.125 mg Cu L^{-1}**	**0.250 mg Cu L^{-1}**	**0.500 mg Cu L^{-1}**	**1.00 mg Cu L^{-1}**
***Tetraselmis* sp.**	*%CV*	*%CV*	*%CV*	*%CV*	*%CV*
Culture 1	5.99%	19.80%	2.81%	11.56%	12.60%
Culture 2	33.63%	20.21%	5.77%	11.76%	13.67%
Culture 3	18.93%	29.52%	28.74%	22.18%	20.71%
Culture 4	4.72%	11.40%	24.39%	8.41%	17.09%
Culture 5	11.05%	17.00%	8.05%	7.60%	11.40%
C. gracilis					
Culture 1	7.71%	8.28%	14.93%	3.93%	19.80%
Culture 2	12.88%	8.67%	12.32%	10.36%	N/A
Culture 3	14.24%	11.10%	3.92%	13.60%	6.36%
Culture 4	19.01%	12.76%	13.33%	10.38%	10.73%
Culture 5	14.35%	17.75%	20.50%	10.42%	8.72%



Figures 2.5a and 2.5b. 24h dose-response to Cu on esterase activity (percent inhibition of fluorescence) in (2.5a) *Tetraselmis* sp. (2.5b) *Chaetoceros gracilis* using the microtitre plate bioassay.

Determination of Cu concentrations in the flask protocol by ICP-OES analysis.

Nominal Cu concentrations (trace, 0.125, 0.250, 0.500, 1.00mg Cu L^{-1}), were comparable to actual Cu concentrations as measured by ICP-OES (Table 2.6), eliminating the possibility that lack of dose-response (or tolerance) to Cu in the flask bioassay was due to Cu binding to the Erlenmeyer flasks over time (0, 3, 12, and 24h). Measured Cu concentrations appeared to be slightly higher than the nominal Cu. Standard deviations for all measurements were less than 0.009 mg L^{-1}, indicating sound instrumentation and analytical technique.

Table 2.6. Medium-associated Cu in Erlenmeyer flasks as measured by ICP-OES.

Nominal Cu (mg L^{-1})	Cu (mg L^{-1}) **0h**	Cu (mg L^{-1}) **3h**	Cu (mg L^{-1}) **12h**	Cu (mg L^{-1}) **24h**
Trace	0.005	0.006	0.006	0.006
0.250	0.257	0.259	0.258	0.259
0.500	0.524	0.526	0.529	0.526
1.000	1.060	1.066	1.064	1.066

Discussion

The effect of Cu on microalgal growth.

Decline in cell growth was one of the earliest methods to quantify the effects of pollutants on unicellular microalgae (Blaise et al. 1998a), and toxic levels of copper have been shown to negatively impact microalgal growth (Franklin et al. 2001a, 2001b, Lage et al. 2001, Ismail et al. 2002). Lage et al. (2001) assessed the effect of Cu on the growth of *Amphidinium carterae* (Dinophyceae) using a Coulter counter, and observed that the highest Cu concentrations (3.13μM) significantly inhibited culture growth after 10 days of exposure. Upon microscopic evaluation, the authors thought these cells were dead due to a loss of flagella (loss of motility structures being a proxy for cell death as suggested by Anderson and Morel (1978), cited in Lage et al. (2001)). However, these "dead" *A. carterae* cells exhibited high levels of esterase activity (as measured by fluorescein fluorescence), which is indicative of cellular viability, and upon inoculation into control medium, culture growth was reestablished and there was no further observable structural damage.

Encystment is a survival strategy employed by many dinoflagellates when faced with unfavourable conditions (Lee 1989), and cell count methods (such as a Coulter counter) cannot differentiate between vegetative and encysted cells. Culture growth is a useful endpoint in toxicity studies, though it is more desirable to combine growth with defined physiological endpoints (such as esterase activity) to assess the overall impact of toxic metals on microalgal species, which is examined in the presented chapter. In this research, there was no statistically significant effect of Cu on the growth of *Tetraselmis* sp., though percent growth appeared to decline as Cu concentrations increased.

In many published studies, Cu negatively affected the growth of *Tetraselmis*. However, *Tetraselmis* was usually less affected by high levels of copper than other microalgae. In an evaluation of the 72h growth EC_{50} among six different microalgae, *Tetraselmis* sp. demonstrated the least sensitivity to Cu (Table 2.7) (Franklin et al. 2001a). Similarly, Ismail et al. (2002) also found *Tetraselmis* sp. to be relatively insensitive to Cu exposures of 96 hours (Table 2.7). It is possible that the observed insignificance of Cu on growth

Table 2.7. A summary of the IC_{50} and EC_{50} values for copper toxicity to the growth and esterase activity of microalgae.

Microalga	Phylum	Endpoint	Exposure time (h)	EC_{50}/IC_{50} (mg Cu L^{-1})	Reference
Chaetoceros calcitrans	He	G	96	0.060-0.080	Ismail et al. (2002)
Chlorella sp.	C	G	72	0.0073	Franklin et al. (2001a)
Chlorella sp.	C	G	48	0.006	Franklin et al. (2001b)
Chlorella sp.	C	E	24	0.0450	Franklin et al. (2001b)
Dunaliella tertiolecta	C	E	24	>0.470	Franklin et al. (2001b)
Dunaliella tertiolecta	C	G	48	1.461	Franklin et al. (2001b)
Entomoneis cf. punctulata	He	G	72	0.018	Franklin et al. (2001a)
Isochrysis galbana	Ha	G	96	0.030-0.040	Ismail et al. (2002)
Nitzchia cf. paleacea	He	G	72	0.024	Franklin et al. (2001a)
Phaeodactylum tricornutum	He	G	72	0.009	Franklin et al. (2001a)
Selensatrum capricornutum	C	G	72	0.0075	Franklin et al. (2001a)
Selenastrum capricornutum	C	G	48	0.006	Franklin et al. (2001b)
Selensatrum capricornutum	C	E	24	0.0390	Franklin et al. (2001b)
Tetraselmis sp.	C	G	72	0.146	Franklin et al. (2001a)
Tetraselmis sp.	C	G	96	0.340-0.410	Ismail et al. (2002)

Phylum: He=Heterokontophyta, C= Chlorophyta, Ha=Haptophyta.
Endpoint: G= growth, E= esterase activity.

of *Tetraselmis* sp. is due to different exposure times (24h Cu in this study) compared to 72 and 96h in other published studies. Franklin et al. (2001a) and Ismail et al. (2002) examined the effect of chronic Cu exposure (72 and 96h, respectively), whereas this study examined acute (24h) Cu exposure. In relation to exposure time, the observed variability between experiments would likely be more pronounced during shorter exposure times, whereas during chronic Cu exposures these contributing factors may be less visible (Heimann, pers. comm.). Thus a significant effect of Cu on growth of *Tetraselmis* sp. could be hidden in the large standard errors of the experiments. In addition to exposure time, irradiance, initial inoculation density, culture age, temperature, culture medium, and species strain differed between the above studies (Table 2.8), which may have contributed to the different results. Regardless of these potential methodological differences, it appears that *Tetraselmis* sp. may have the capacity to persist in Cu levels that are toxic to other microalgae.

Table 2.8. Protocol differences between three independent studies examining the impact of Cu exposure on the culture growth of *Tetraselmis*.

Parameter	Blanchette and Heimann (unpub.)	Franklin et al. (2001a)	Ismail et al. (2002)
Irradiance (µmol photons x m^{-2} x s^{-1})	37.68	70.0	50.4
Initial inoculation density (cells mL^{-1})	$1.5x10^5$	$2\text{-}4x10^4$	$1x10^6$
Culture age	2-3weeks#	*	*
Culture and experimental temp. (°C)	24	21	28
Culture medium	Guillard's f/2	Guillard's f/2	Walne's**
Culture strain	NQAIF0012##	CS87†	UMACC 144 and 146‡
Cu exposure time (h)	24	72	96

* Harvested in logarithmic/ exponential growth phase (no age given).
** Synthetic seawater (Marine Environment) enriched with Walne's Solution.
# 2-3 week old cultures used to inoculate fresh medium to specified cell density.
North Queensland Algal Identification/ Culturing Facility, isolated from the Great Barrier Reef, Australia.
† CSIRO (Hobart, Tasmania, Australia) Microalgal Culture Collection.
‡ National Prawn Fry Production and Research Centre, Palau Sayak, Kedah.

It is currently unclear why *Tetraselmis* is less affected by Cu than many other microalgal species, though the use of electron microscopy and X-ray spectroscopy to observe the visible morphological effects of Cu may elucidate Cu speciation within the cell and at the cellular armour-environment interface, which may influence Cu toxicity. An ultrastructural study of control (no pollutant present) *Tetraselmis suecica* cells showed the highest number of lipid-bearing osmiophilic[1] vesicles in the most actively swimming cells (Ballan-Dufrançais et al. 1991). After five days of exposure to 0.050mg Cu L^{-1}, electron probe microanalysis revealed copper accumulate within the vesicles, which suggested that in addition to acting as lipid storage areas (their primary purpose) they may also assist in Cu detoxification due to the capability of the lipids and fatty acids to bind to the Cu (Ballan-Dufrançais et al. 1991). The number of osmiophilic vesicles per cell differed within a population of *T. suecica*, and the cells with the highest number of vesicles showed the most resistance to Cu as measured by visual inspection of the level of disruption to cellular organelles (Ballan-Dufrançais et al. 1991). Forty percent of Cu-exposed *T. suecica* cells showed "massive disorganisation" of cellular contents, with "cell organelles indistinguishable" (Ballan-Dufrançais et al. 1991). In these disrupted cells, the Cu-containing osmiophilic vesicles remained unchanged in comparison to

[1] Having an affinity for osmium tetroxide- a fixative and contrasting agent used in electron microscopy (Ballan-Dufrançais et al. 1991).

control cells, though were generally fewer in number. Sixty percent of Cu-exposed cells, while smaller than control cells, generally exhibited well-developed mitochondria and plastids, but the osmiophilic vesicles were more numerous and significantly larger than observed in control (no Cu) cells, with dilated membranes. The authors suggested that sequestration of Cu to osmiophilic vesicles could assist in heavy metal detoxification, which varies within a population.

Despite the potential of this research to elucidate Cu tolerance mechanisms in the relatively Cu-resistant *Tetraselmis*, there are some aspects of the Ballan-Dufrançais et al. (1991) study which must be more closely considered. The cells were pre-fixed with 3% gluteraldehyde and cooled to 4°C. However, these cells were not preserved by fast freeze fixation (-196°C) followed by freeze-substitution (-90°C), which may have differentially affected the morphological appearance of the cells depending upon how the fixative was added and mixed with the sample (K. Heimann, pers. comm.). An additional consideration is the nature of cell sectioning, which may impact the number of visually obvious vesicles and resolution of membrane structures (K. Heimann, pers. comm.). Ballan-Dufrançais et al. (1991) sectioned their “ultrathin median” cell sections longitudinally from the distal part of the cell, preparing and examining 200-300 cells per treatment. In their discussion, the authors did not acknowledge the potential impact of section position relative to the three-dimensionality of a cell on the results. Despite the potentially negative effect of fixation on organelle structure, the number of cells examined per treatment increases confidence in the results, which must incidentally be taken within the context of where and how the cells were sectioned for analysis.

Nassiri et al. (1996) also investigated the ultrastructural effect of Cu on *T. suecica* (loss of flagella, depletion of starch and all organelles being unrecognisable apart from the nucleus and osmiophilic vesicles) using X-ray spectroscopic studies, and similarly to Ballan-Dufrançais et al. (1991), documented the sequestration of copper in osmiophilic vesicles. Nassiri et al. (1996) also characterised additional detoxification methods: the timing of the ultrastructural changes was a function of Cu exposure, with initial sequestration of Cu within the osmiophilic vesicles, followed by formation of additional thecal layers which adsorbed the Cu, and finally extracellular release of Cu-enriched

organic material. However, the authors did not look for a simultaneous decline in the number and size of osmiophilic vesicles post-organic excretion, which is important to assess the potential role of these vesicles in detoxification via exocytosis. Like the Ballan-Dufrançais et al. (1991) study, these results must also be taken within the context of the methodology: the cells were not cryo-fixed and freeze-substituted prior to embedding, and 50nm longitudinal ultrathin sections were examined. However, the authors did not state how many cells they sampled to arrive at their conclusions. Despite this, both studies may indicate that *Tetraselmis* could employ a suite of mechanisms to deal with the presence of toxic levels of copper.

Bioassays with diatoms as test organisms are well represented in the literature, although *Chaetoceros* is recognised mainly for its value as an aquaculture feed organism (Duerr et al. 1998), and has not been employed in many toxicity bioassays. The research presented in this chapter showed a statistically significant dose-dependent response of *C. gracilis* percent growth to 24h Cu (trace-1.0mg Cu L^{-1}) incubation under normal (approximately 37.68µmol photons x m^{-2} x s^{-1}) irradiances. In comparison to *Tetraselmis* sp., *C. gracilis* is statistically more sensitive to Cu, though both organisms display inhibition of percent growth at higher Cu concentrations. In the few studies published using *Chaetoceros* as a bioassay test organism, this microalga is usually one of the most sensitive (of a suite of microalgae) to a particular contaminant. In addition to the Ismail et al. (2002) study (discussed in previous paragraphs), Romero et al. (2002) found that the growth of *Chaetoceros* sp. (IC_{50}=0.560mg Cd L^{-1}) was consistently more sensitive to a 96h exposure to cadmium (0-100mg Cd L^{-1}) than *Tetraselmis* sp. (IC_{50}=0.630mg Cd L^{-1}), though both organisms displayed similar IC_{50} values, in line with the growth data presented in this chapter. *Chaetoceros* sp. was more affected by a 14 day sublethal Cd (0.050mg L^{-1}), exposure than *Tetraselmis* sp., displaying significant decreases in growth, pigmentation, and cell volume in comparison to control (no Cu) cultures (Romero et al. 2002). *Chaetoceros* has been shown to be highly sensitive to both Cu and Cd, which may be a function of cell wall chemistry. The Bacillariophyceae are characterised by negatively charged siliceous cell walls ([Darley 1974], in Van den Hoeck et al. 1995), which may strongly attract free copper ions and increase toxicity. Microalgal cell wall arrangement and

chemistry may influence metal uptake (Crist et al. 1981), though further comparative studies must be performed.

The effect of toxic levels of Cu and low irradiance on the growth of *Chaetoceros gracilis*.

An important bioassay parameter is ambient light. This research showed a dramatic difference in 24h Cu toxicity between *C. gracilis* grown in normal (approximately 37.68µmol photons x m^{-2} x s^{-1}) and low (approximately 11.50µmol photons x m^{-2} x s^{-1}) irradiances. In normal irradiance conditions, there was a significant dose-response effect of percent growth to Cu, though in low irradiance conditions, only the control (no Cu) organisms showed high levels of growth, which suggests a co-limiting effect of both low irradiance and Cu. In reduced irradiance situations, this photosynthetic diatom would likely be compromised, as insufficient irradiance leads to slower growth (Timmermans et al. 2001) and production of fewer intracellular storage materials (Lawler 1993), which may increase Cu toxicity. Cu is an essential micronutrient, and is a constituent of the primary electron donor in photosystem I; the Cu-protein plastocyanin (Baron et al. 1995). However, at high concentrations, Cu can become toxic. There has been little published research investigating the impact of toxic levels of metals and inadequate irradiance on microalgae, despite the possibility that the presence of metal pollution could affect phytoplankton species composition in natural waters where light may be limiting and competition for enough light to satisfy photosynthetic demands for pollution-stressed cells could be fierce (Reinfelder et al. 2000). Reinfelder et al. (2000) acknowledged the possible impacts associated with co-limitation by toxic levels of metals and low irradiance, but did not incorporate light regime experiments into their study investigating the metabolic responses (heat production and oxygen consumption) to trace metal toxicity in *Thalassiosira weissflogii* (Heterokontophyta).

The great majority of metal/irradiance co-limitation studies focus upon Fe starvation in conjunction with low light, which strive to mimic natural unpolluted conditions in Southern ocean environments (Timmermans et al. 2001). Timmermans et al. (2001) documented a co-limitation by low levels of iron and irradiance on the growth rates of *Chaetoceros brevis* and *Chaetoceros*

calcitrans, a useful study to compare with the *Chaetoceros gracilis* growth data presented in this chapter. Both organisms had reduced growth rates under low irradiance regimes (*C. calcitrans* low light=20µmol photons x m^{-2} x s^{-1}, *C. brevis* low light=15µmol photons x m^{-2} x s^{-1}) in comparison to high and control light levels (*C. calcitrans* high light=200µmol photons x m^{-2} x s^{-1}, *C. brevis* normal light=60µmol photons x m^{-2} x s^{-1}), which increased sigmoidally as Fe ($0.5x10^{-12}$-14 $x10^{-12}$ M) increased. The Timmermans et al. (2001) study corroborates the findings in this chapter that low levels of light negatively affect the growth of *Chaetoceros*. However, the iron levels in the presented research were not limiting ($1x10^{-5}$M, in Guillard's f/2 medium); instead the organisms were exposed to toxic levels of Cu. The decline in percent growth with the addition of Cu (0.250-1.00mg Cu L^{-1}) in low irradiance was more dramatic than the results in the Timmerman et al. (2001) iron-starvation study, which suggests that toxic amounts of Cu in conjunction with low irradiance levels may have a more severe impact on *Chaetoceros* growth than limiting Fe and low irradiance. Future research should focus upon the impact of high levels of metals and limited irradiance.

<u>The effect of experimental design on esterase activity.</u>

In this chapter, experimental design impacted Cu toxicity on esterase activity in both *Tetraselmis* sp., and *C. gracilis*. The results of the flask bioassay showed no consistent dose-response over 6, 12, and 24h of Cu incubation for both organisms. In contrast, the microtitre plate bioassay displayed a significant effect of both Cu and culture on esterase activity in *Tetraselmis* sp. and *C. gracilis*. One difference between the two protocols was the presence of the glass Erlenmeyer flasks in the flask bioassay: it was possible that significant amounts of Cu ions bound to the glassware, thereby reducing the actual Cu concentrations in the medium below nominal values (Blaise et al. 1998, Eisentraeger et al. 2003), resulting in the inconsistent and statistically insignificant Cu dose-response of esterase activity. To test this, the Cu concentrations in the medium in Erlenmeyer flasks were quantified over 24h, using inductively coupled plasma optical emission spectrometry (ICP-OES). According to the ICP-OES data, there was no significant binding of Cu to the glassware during 24h incubation. Measured Cu was actually higher than

nominal copper concentrations, which can be attributed to the stock Cu (154.8mg Cu L^{-1}) having slightly higher copper concentrations than intended (150.0mg Cu L^{-1}), which was outside the combined accuracies of the measuring equipment (scale ± $4.5x10^{-4}$%, measuring cylinder ±1.0%, total=1.0005% error).

Despite the significant dose-dependent effect of Cu on esterase activity in the microtitre plate bioassay, the potential for Cu ions to bind to the microtitre plate wells must be addressed. Blaise et al. (1998) acknowledged binding of Cu to microtitre plate wells as a potential concern in the earliest published bioassays due the polystyrene make-up of the plates and the increase of area to volume ratio created by a 96-well experimental design. However, Blaise et al. (1998) cited the work of Blanck et al. (1984), who addressed this concern by comparing the EC_{50} values of 13 different microalgae for 19 toxins, including Cu, during successive dilutions within the plate wells. These authors saw no effect of chemical adsorption to the plate well, and repeated testing with *Monoraphidium pusillum* (Chlorophyceae) demonstrated that EC_{100}'s did not deviate significantly between dilutions in relation to Cu concentration. Additionally, the plates used in the presented research were new, came with a "low binding" assurance from the Dynex microlite® manufacturers, and were composed of polypropylene, not polystyrene as seen in many earlier studies (Blanck et al. 1984, Blaise et al. 1998). The effect of Cu on *Tetraselmis* sp. and *C. gracilis* showed that there was sufficient bioavailable Cu to elicit a dose-response on esterase activity, but further research should assess the amount of Cu (if any) bound to plate walls using ICP-OES, as in the flask bioassay.

Given the time course of the ICP-OES data on Cu availability in the medium for the flask bioassay, it is unlikely that the difference in Cu toxicity to esterase activity between the two bioassay protocols is due to decrease in Cu concentrations present in the medium. Therefore, these differences are more likely due to a combination of factors such as: culture age (Gilbert et al. 1992) (flask bioassay microalgae were 2-3 weeks old at the time of analysis, microtitre plate bioassay organisms were 7 days old), the impact of sample transfer on esterase activity in the flask bioassay (Minier et al. 1993), and differential adhesion and resuspension of cells in the flask upon sample transfer to the microtitre plates for fluorometric analysis.

Culture age may have affected Cu toxicity to esterase activity between bioassay protocols because more mature cultures (2-3 weeks old in the flask bioassay) are likely to be nutrient-deplete in comparison to younger cultures (7 days old in the microtitre plate bioassay), which may affect esterase activity in cultures that are already stressed (K. Heimann, pers. comm.). Additionally, esterase activity varies according to microalgal culture growth stage (Gilbert et al. 1992). In *Tetraselmis suecica*, esterase activity (per cell) peaked during the exponential growth stage, and declined rapidly during growth stasis (Gilbert et al. 1992). Future research should focus upon using microalgae of identical ages to compare between bioassay protocols.

Sample transfer from the flasks to the microtitre plates for fluorometric analysis in the flask protocol presented difficulties. Despite the fact that inoculation density in the culture flasks was standardised, both *Tetraselmis* sp. and *C. gracilis* tended to adhere in a variable manner to the glassware (pers. obs.), and there was no clear correlation between level of adhesion and Cu concentration. Therefore, the samples in the plates may have not been fully standardised despite the efforts of the author. Standardisation of algal density is critical because toxicity increases if inoculation density decreases due to the increased Cu load per cell (Moreno-Garrido et al. 2000, Franklin et al. 2002). An additional physiological factor is that the transfer of microalgae from the flasks to the plates may have negatively affected esterase activity. Minier et al. (1993) noted that transfer of *Calothrix* PCC (Cyanophyta), *Haslea ostrearia* (Heterokontophyta) and *Prorocentrum micans* (Dinophyta) from batch culture to microtitre plate wells significantly affected esterase activity. Esterase-induced fluorescein fluorescence stagnated (or decreased) for 24 hours after transfer, with a return to control levels after 48 hours of acclimation in the plate wells. However, Minier et al. (1993) did not formally present these measurements in their publication (just stated “data not shown”), which makes comparison to these results difficult.

In contrast to the findings of the research presented in this chapter, many publications report good agreement (as measured by IC_{50} values) between flask and microtitre plate bioassays, though all of them use growth as an endpoint (St. Laurent et al. 1992, Rojickova et al. 1998, Eisentraeger et al. 2003). This presents difficulties when comparing the esterase activity results presented in

this chapter because growth and esterase activity in microalgae may exhibit different sensitivities to contaminants. Additionally, these studies use very different protocols, most notably the continuous agitation of culture flasks (St. Laurent et al. 1992, Rojickova et al. 1998) and microtitre plates (Eisenstraeger et al. 2003) during pollutant exposure, which may have a profound impact upon the differential settling of microalgae within the culture vessels (a physiological parameter that may have contributed to the differences seen between the flask and microplate protocol results in this chapter). Nevertheless, the results of these studies (St. Laurent et al. 1992, Rojickova et al. 1998, Eisentraeger et al. 2003) have led the authors to conclude that microtitre plate methods may be a substitute for traditional flask assays within the parameters of their methodologies.

Using *Selenastrum capricornutum* (Chlorophyceae) to test for the effects of Cu on growth, St. Laurent et al. (1992) noted no significant difference between the 96h growth EC_{50} values of flask (EC_{50}=0.070mg L Cu^{-1}) and microplate (EC_{50}=0.066mg L Cu^{-1}) bioassay designs. Similarly, Rojickova et al. (1998) employed *Raphidocelis subcapita* (Chlorophyceae) to assess the impact of 72h Cu incubation on culture growth in flask, tube, and microtitre plate bioassays. R^2 values in bioassay comparisons based on linear regression of the log_{10} values of EC_{50} for 11 chemicals were sufficiently high (flask and tube R^2=0.980, flask and plate R^2=0.980) for the authors to confidently state that tube and microtitre plate bioassays can substitute flask bioassays in most cases, despite the lack of agreement between EC50 values among the protocols (flask EC_{50}=0.360mg Cu L^{-1}, tube=0.550mg Cu L^{-1}, plate=0.310mg Cu L^{-1}). Eisenstraeger et al. (2003) also performed a comparative study on *Desmodesmus subspicatus* (Charophyceae) using microtitre plate and Erlenmeyer flask growth inhibition assays for 14 different organic and metal contaminants. These authors noted no significant difference between the 24h growth EC_{50} values for all tested contaminants between protocols. In summary, using growth as an endpoint for assessing the toxicity of pollutants in various green algae yielded good agreement between flask and microtitre plate bioassays (St. Laurent et al. 1992, Rojickova et al. 1998, Eisentraeger et al. 2003).

The effect of culture in the microtitre plate bioassay for Cu toxicity to esterase activity.

The results of the ANCOVA for the microtitre plate bioassay protocol with *Tetraselmis* sp. and *C. gracilis* not only demonstrated a significant effect of copper on percent inhibition of fluorescence, but also a significant effect of culture. Each bioassay experiment was independent for culture, microtitre plate, and FDA probe. It is possible that a combination of these independent variables contributed to a significant effect on the quantification of esterase activity. For example, different independent cultures of *Tetraselmis* sp. and *C. gracilis* may differ naturally in esterase activity, despite all cultures being exposed to the same environmental and culture conditions. Craig et al. (2003) also documented a statistically significant effect of independent culture on the inhibition of bioluminescence re-establishment in the dinoflagellate *Pyrocystis lunula* (Dinophyceae) in the presence of Cu. However, these authors acknowledged that the "difference was relatively small and probably not biologically significant," because the differences were within the percent coefficient of variation and did not affect the nature and pattern of Cu toxicity (K. Heimann, pers. comm.). Whether culture-specific differences are or are not biologically significant in microalgae it is an interesting topic for future research and must be considered in experimental designs.

Each bioassay experiment was also independent for FDA working stock preparation; all FDA was from the same stock, though fresh working dilutions were prepared prior to each experiment. Variation in acetone volume and/or stock weight of the enzyme substrate FDA (despite due care) can contribute to higher than usual variability in the quantification of esterase activity (Breeuwer et al. 1995, Franklin et al. 2001a). It is likely that a combination of the above factors contributed to the variability between independent repeats of the microtitre plate bioassay.

Comparison of the Cu sensitivities of the endpoints growth and esterase activity.

The exact toxic mechanism of copper on esterase activity is unknown, but metals generally inhibit enzyme activity by binding to sulphydryl groups (essential for structure and catalytic action), or competing with essential metals

for extracellular binding sites on the cell's surface (Van Assche and Clijsters 1990). Copper may indirectly affect growth by compromising membrane integrity (Franklin et al. 2001a) or inhibiting cell division and/or cytokinesis (Blanchette and Heimann unpublished, see chapter 4 of this thesis). Potential differences between the toxicity endpoints growth and esterase activity additionally contribute to difficulties in comparing the presented data to previously published studies.

I found no significant effect of Cu on growth in *Tetraselmis* sp., though esterase activity was affected in a dose-dependent manner in the microtitre plate bioassay (IC_{50} esterase activity=0.500mg Cu L^{-1}). In contrast, both growth (IC_{50}=0.500mg Cu L^{-1}) and esterase activity (microtitre plate bioassay IC_{50}=0.125-0.250mg Cu L^{-1}) were negatively impacted (statistically) by Cu in *C. gracilis*, suggesting that esterase activity is a more sensitive endpoint than growth in these two microalgae using these protocols. Franklin et al. (2001b) also compared the sensitivity of cell division rate and esterase-induced fluorescein fluorescence (esterase activity) to copper in the chlorophyceaen microalgae *Chlorella* sp., *Selensatrum capricornutum* and *Dunaliella tertiolecta*. In contrast to the findings presented in this chapter and with the exception of *D. tertiolecta* (Table 2.7), the growth of *Chlorella* sp. and *S. capricornutum* was substantially more sensitive to Cu than esterase activity (Table 2.7). Franklin et al. (2001b) conclude that the toxic mechanism and effects of Cu in microalgae could be species-specific, and speculated that the high tolerance of *D. tertiolecta* may be attributed to extracellular exclusion of copper or internal detoxification methods. However, it is difficult to truly compare the sensitivities (as determined by EC_{50}/IC_{50} values) of these endpoints to Cu within the Franklin et al. (2001b) study because the bioassays use different time points, which may impact upon toxicity measurements if survival strategies are time-dependent. Franklin et al. (2001a) also described the relative insensitivity of esterase activity to copper in comparison to growth for the heterokontophytes *Phaeodactylum tricornutum,* and *Nitzchia* cf. *paleacea,* and the chlorophyte *Tetraselmis* sp., observing that esterase activity persists at control levels even in copper concentrations that "severely inhibit cell division rate." Franklin et al. (2001a) did, however, conclude that esterase activity may be an appropriate substitute for cell division rate in the diatom *Entomoneis punctulata* based upon

the similarity of their EC_{50} values (esterase activity 24h EC_{50}=0.0091mg Cu L^{-1}, cell division rate 48h EC_{50}=0.0170mg Cu L^{-1}), despite the inhibition values being sourced from different time points.

Conclusions and outlook.

It is clear from this and other studies that the effect of copper on growth and esterase activity in microalgae may be species- and method-specific. In contrast to other studies, esterase activity appeared to be a more sensitive endpoint for copper toxicity than growth in *Tetraselmis* sp. and *C. gracilis* (microtitre plate bioassay). Despite these conclusions, further questions remain regarding the effectiveness of the microtitre plate bioassay as a tool to quantify Cu toxicity to esterase activity in long-term cultures of *Symbiodinium* (notorious for their strong adhesive properties, making standardisation of initial inoculation density, a critical component of this bioassay, highly challenging), and the effect of pH, salinity, and temperature on esterase activity and Cu toxicity, which are addressed in chapters 3 and 4 of this thesis.

Chapter 3

The use of the microtitre plate bioassay for esterase activity and copper toxicity to evaluate the suitability of initial inoculation density standardisation protocols for *Symbiodinium microadriaticum* (DINOPHYTA).

Introduction

Most ecotoxicological research to date has focused upon temperate ecosystems, despite the fact that tropical systems contain approximately 75% of the total global biodiversity (Lacher and Goldstein 1997). Australia's Great Barrier Reef is a tropical ecosystem that has been subject to increasing anthropogenic pressure, and there is a great need to establish aquatic toxicology monitoring programs in the region (Hutchings and Haynes 2000, Haynes and Michalek-Wagner 2000, Haynes and Johnson 2000, and Esslemont 2000). Haynes and Michalek-Wagner (2000) and Hutchings and Haynes (2000) have called for more research investigating the sublethal effects of heavy metals in the Great Barrier Reef Marine Park (GBRMP) to provide early-warning indicators of metal stress on marine life.

The study of metal toxicity (specifically copper) is particularly relevant to Townsville, North Queensland. Large amounts of copper is mined, refined, and shipped by sea in the region (Esslemont 2000). Copper, in toxic concentrations, can be introduced into the marine environment via antifouling paints at ship grounding (Haynes et al. 2002, Haynes and Loong 2002) and/or maintenance sites (Hanyes and Loong 2002). Haynes and Loong (2002) found high levels of Cu in the Townsville and Cairns harbours "consistent with contamination arising from hull cleaning and painting." Copper can also pollute the North Queensland marine environment via land-based runoff in pesticides, fertilisers, and antifungal compounds (Rayment and Neil 1996, Hutchings and Haynes 2000, Haynes and Michalek-Wagner 2000). Elevated levels of Cu have been measured in cane-cultivated soil (Rayment and Neil 1996) and several Wet Tropics streams (Moss and Bennett 1991). Cu is present at above-background[2] levels in some North Queensland near-shore environments (0.028-0.230mg Cu g^{-1} sediment dry weight, Haynes and Loong 2002), and its effect on marine systems must be investigated.

Zooxanthellae are symbiotic microalgae that contribute to the energy requirements of marine hosts (such as giant clams and corals) through photosynthesis. To date, most of the research quantifying the effect of

[2] Background Cu concentrations in uncontaminated sediments suggested as <0.020mg Cu g^{-1} sediment dry weight (Doherty et al. 2000), in uncontaminated seawater $3.5x10^{-5}$-$1x10^{-3}$mg Cu kg^{-1} seawater (Quinby-Hunt and Wilde 1986-1987) or 0.1µg Cu L^{-1} seawater (Sadiq 1992).

pollutants on zooxanthellae has been based on coral bleaching, which occurs when the coral expels the microalgae as a stress response (Jones 1997). In the past decade, especially in Australia, there has been an increasing amount of research describing the effect of toxins on zooxanthellae *in hospite* using PAM-fluorometry, which quantifies fluorescence yield as a measure of photosystem II electron transport efficiency (Haynes and Michalek-Wagner 2000, Elfwing et al. 2002, Jones and Heyward 2003). However, very little bioassay work has been done on freshly isolated zooxanthellae in culture, and even less is known about more established laboratory cultures.

Given the importance of zooxanthellae in tropical marine ecosystems, and the above background levels of metals in some regions of the GBRMP (Haynes et al. 2002, Haynes and Loong 2002), the lack of ecotoxicological research in the literature on established cultures of these microalgae should be noted. According to Goh and Chou (1997), there had only been one prior study using established zooxanthellae cultures in a heavy metal bioassay. Goh and Chou in 1993 and 1997 used growth as an endpoint in *Montipora verrucosa*-isolated *Symbiodinium microadriaticum*. Goh and Chou (1997) exposed *S. microadriaticum* to levels of Cu (42µg L^{-1}) and Zn (509µg L^{-1}) that significantly depressed growth rates in time-course bioassays lasting up to 28 days. Despite the potential ecological relevance of this study, it may be even more valuable to develop bioassays for sublethal effects, thus providing early physiological indications of metal stress on zooxanthellae.

In 2003, Jones and Heyward developed a bioassay for produced formation water ("PFW," an oil and gas exploration effluent) toxicity on the photochemical efficiency of freshly isolated microalgae from the coral *Heliofungia actiniformis*. Jones and Heyward (2003) analysed PFW for its chemical components, and found a range of benzenes, polycyclic aromatic hydrocarbons, and 13 different metals (including <10µg Cu L^{-1}), with the most prevalent being barium (1.25 mg L^{-1}). *H. actiniformis* was sourced from a sub-tidal reef flat at Magnetic Island, Townsville, and a WATER-PAM was used to quantify photochemical efficiency (variable fluorescence (F_v) to maximal fluorescence (F_m)) as a measure of PFW toxicity exposure. After a 48h exposure to 6.25% PFW, there was a significant decrease in the ratio of F_v to F_m of freshly-isolated zooxanthellae. An additional consideration is that the

viability of zooxanthellae quickly declines after isolation from the host coral due to osmotic and nutritional stress (K. Heimann, pers. comm.); there may have been several factors contributing to the drop in F_v/F_m in the Jones and Heyward (2003) study. Also, given the history of dredging at Magnetic Island, which exposes fringing reefs to high metal concentrations in resuspended fine sediment (Esslemont et al. 2004), their results should be examined within the context of coral exposure history, which may impact the outcome of a bioassay. The presence of research quantifying the effect of metal toxicity on cultured zooxanthellae in the literature is scarce, though studies exploring the effect of oxygen tension (exposure to varying O_2, N_2, and CO_2 gas mixtures, Matta and Trench 1991), nutrient concentrations (Domotor and D'Elia 1984, Sakami 2000, Annis and Cook 2002), temperature, irradiance, and salinity (Sakami 2000) have been published.

It is critical to standardise initial inoculation density (IID) when using microalgae as bioassay test organisms to ensure the results of the bioassays are comparable statistically and biologically. This is especially important when testing for metal toxicity: fewer algal cells exposed to a set level of metal concentration in media generally results in more bioavailable metal per cell, which may increase overall toxicity (Moreno-Garrido et al. 2000, Franklin et al. 2002). Additionally, most microalgae have the ability to chemically modify their habitats by producing negatively charged carboxylic acid- and phosphate-rich metal-complexing compounds (resulting from carbon metabolism of carbohydrates) that may postpone the toxic effects of copper (Lombardi et al. 2002). Therefore, cultures of varying cell densities may produce different concentrations of metal-complexing agents, which can affect overall toxicity. Keeping the effect of IID in mind, the highly difficult quantification of zooxanthellae (specifically *Symbiodinium*) culture density by conventional means (i.e. cell counts using a haemocytometer, pers. obs.) may be one of the reasons why few research papers have been published using zooxanthellae. Many *Symbiodinium* species in established cultures are very tightly clumped and adhere strongly to culture vessels (pers. obs.), and visual quantification is therefore problematic.

Accurate quantification of *Symbiodinium* using a cell counter is nearly impossible at high cell densities, given the three-dimensionality of their group

association. This has not been directly documented in the literature, but Jones and Heyward (2003) had to use “approximations” of cell density (quantified on a haemocytometer) after eight days of zooxanthellae growth in culture. Similarly, Goh and Chou (1997) could not accurately define IID with a haemocytometer, choosing instead to begin their bioassay with “>$2.0x10^4$ cells mL^{-1}.” Aliquots of this mother culture were then counted on a haemocytometer, but it is unclear whether cell density between samples was ever standardised.

The first aim of this chapter is to explore the viability of three different methods (chlorophyll *a* autofluorescence, protein content, and cell count by haemocytometer) to quantify established cultures of *Tridacna maxima*-isolated *Symbiodinium microadriaticum*. The second aim is to use the different cell density quantification methods to standardise initial inoculation density in a microtitre plate bioassay for copper toxicity with esterase activity as an endpoint. Esterase activity has been examined in *Symbiodinium microadriaticum* in only one previous study. Selvin et al. (1988) used the dye fluorescein diacetate (FDA) to explore, via epifluorescence microscopy, optimal culture growth conditions. This chapter presents the only known data quantifying esterase activity in a bioassay for metal toxicity with a host-isolated zooxanthella in culture.

Materials and Methods

Organism culturing.

Symbiodinium microadriaticum (NQAIF ID 0023; CS73) was cultured under sterile conditions, with all procedures performed in a laminar flow (AES Environmental Pty. Ltd). *S. microadriaticum* was isolated from a giant clam (*Tridacna maxima*) by E.M. Deane in 1978, and donated to CSIRO in 1979. *T. maxima* was sourced from Heron Island in the Great Barrier Reef, Australia. At NQAIF (North Queensland Algal Identification/ Culturing Facility), organisms were cultured in sterile-filtered Guillard's f/2 medium (see chapter 2 for medium preparation), and all glassware was acid-washed in 10% HCl and autoclaved. All *S. microadriaticum* cultures used in the bioassays were inoculated with 1mL of 2-3 month old mother culture and 49mL of Guillard's f/2 growth medium. Cultures grew undisturbed for one month before bioassay use in a culture chamber (Contherm Digital Series Cooled Incubator) at 24°C at an approximate irradiance of 11.50µmol photons $m^{-2}s^{-1}$ (measured on a light meter: LI-COR LI-250, LI-COR Inc., Lincoln, Nebraska, USA) in a 12:12 light:dark cycle.

Standardisation of *Symbiodinium* initial inoculation density using the Pierce Protein Assay.

A one-month old culture of *S. microadriaticum* was gently resuspended using a 1mL sterile plastic Pasteur pipette to homogenise the cell suspension, and viewed under a microscope to assess the efficiency of the homogenisation process. No obvious cell damage was observed. A 10mL aliquot of homogenised culture was concentrated by centrifugation (20min, 4°C, 1500rpm, Eppendorf Centrifuge 5810R, rotor type A-4-62). The cell pellet (with residual supernatant) was lysed by the addition of 500µL 2x RIPA buffer (Table 3.1) (1h, on ice, in the dark). Cell lysis was assisted by "tissue" homogenisation (Eppendorf microfuge tissue grinding pestle) for 10 min (2X) with an incubation period between the homogenisations of 20h, 4°C, in the dark. Homogenised pellets were centrifuged to remove cell debris (Eppendorf Centrifuge 5810R, rotor type F45-30-11, 10min, 4°C, 1500rpm) and the supernatant was used for protein content estimation.

Table 3.1: Chemical components of RIPA buffer.

RIPA Buffer	1x	2x
1% Triton X 100	2mL	4mL
1% (Deoxycholate) Tween 20	2mL	4mL
0.1% SDS (10% stock)	2mL	4mL
0.15M NaCl (=150mM)	1.7532g	3.5064g
25mM Tris-HCl	0.605g	1.21g

The remainder of the protocol followed the Pierce Protein Assay Instructions (Smith et al. 1985, Kessler and Fanestil 1986, Wiechelman et al. 1988, and Brown et al. 1989, cited in Pierce Instruction Kit 23225 Rockford, IL 61105, USA). Bovine Serum Albumin (BSA) standards (0, 5, 10, 20, 40µg mL^{-1} albumin standard final concentration) (Table 3.2) and *S. microadriaticum* samples (2.5, 5, 20µL) were prepared in 1.5mL Eppendorf tubes (Table 3.3). BSA standards were made up to 100µL with dI H_2O, while *S. microadriaticum* samples were made up to100µL with 1X RIPA.

Table 3.2: Pipetting scheme for albumin standard curve.

Albumin standard final concentration (µg mL^{-1})	BSA stock (µL)	dIH_2O (µL)
0	0.0	100.0
5	2.5	97.5
10	5.0	95.0
20	10.0	90.0
40	20.0	80.0

Table 3.3: Pipetting scheme for *Symbiodinium microadriaticum* samples.

Symbiodinium sample (µL)	1X RIPA buffer (µL)
2.5	97.5
5.0	95.0
20.0	80.0

S. microadriaticum samples were corrected for background absorbance using a blank (50µL 2X RIPA buffer, 50µL Guillard's f/2 medium). 1mL of Pierce BCA working solution was added to standard, sample, and blanks, followed by incubation for 2h at room temperature in the dark. A spectrophotometer (Varian DMS 90 UV-Vis.) was zeroed at 562nm using the standard curve sample without albumin prior to measuring sample absorbance (cuvettes: pathway length 10mm, standard optical glass, Starma Pty. Ltd., Thornleigh, NSW 2120,

cuvettes rinsed with dIH_2O between each reading). The BSA standard curve linear equation (Figure 3.1) was used to determine amount of protein in *S. microadriaticum* samples. A standard level of protein for use in the esterase bioassays was set based upon the first protein assay; 275µL of *S. microadriaticum*, corrected for the original 10x concentration factor, had 0.7076 µg protein mL^{-1}. Algal concentration for subsequent bioassay inoculation was adjusted based upon this standard (after independent analysis of protein content) using sterile-filtered seawater.

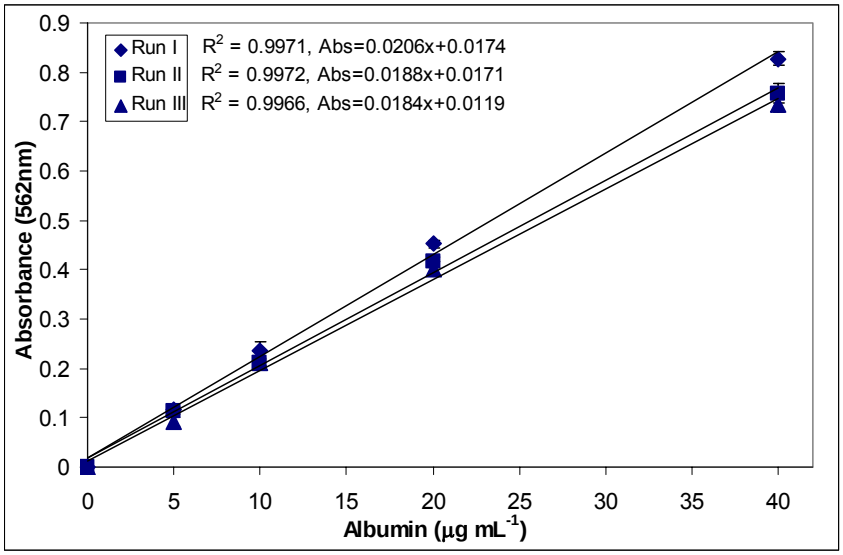


Figure 3.1. Pierce protein assay bovine serum albumin standard curve. R^2 values and equations are presented. n=3.

Standardisation of *Symbiodinium* initial inoculation density using chlorophyll *a* autofluorescence.

Symbiodinium were harvested from the mother culture as per the previous methods section. In a pre-prepared (see chapter 2) microtitre plate, varying concentrations of one-month old *Symbiodinium microadriaticum* culture [0% (275µL seawater only), 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5% (made to 275µL with sterile-filtered seawater), and 100% (275µL culture only)] were added to individual wells in triplicate in a randomised design. All preparation occurred in a sterile laminar flow with acid-washed and autoclaved materials. The inoculated plate was then covered in Parafilm® and dark-adapted (2h, room temperature). After dark adaptation, the plate was light saturated with a camera flash (ASA 100, DIN21, 5m, Comp. Pos. II, ALFOTron

VTC40, Greve & Brummel, Münster, Germany) within the spectrofluorometer (Varian Cary Eclipse) and chlorophyll *a* autofluorescence was analysed (Table 3.4). The *Symbiodinium* seawater dilution used for each esterase bioassay for Cu toxicity had approximately the same chlorophyll *a* autofluorescence (as indicated by overlapping standard error bars) using the standard curve (percent *Symbiodinium* vs. chlorophyll *a* autofluorescence). These particular autofluorescence measurements were used because they corresponded to points on the graph in linear phase; chlorophyll *a* autofluorescence measurements between 75 and 100% organism did not appear significantly different, which may be due to self-shading of organisms in the microtitre plate.

Table 3.4: Varian Cary Eclipse settings for chlorophyll *a* autofluorescence analysis.

Parameter	Setting
Software	Cary Eclipse
Operating program	Kinetics
Excitation wavelength	430nm
Emission wavelength	685nm
Time	252s
PMT detection	High

Standardisation of *Symbiodinium* initial inoculation density using a haemocytometer.

Culture flasks containing *S. microadriaticum* were gently resuspended using a 1mL sterile plastic Pasteur pipette and examined under a microscope to determine homogeneity. A 1mL aliquot of resuspended algae was added to an Eppendorf tube with 1 drop of Lugol's Iodine as a fixative, and then gently vortexed (Combi-spin PCV-2400, Grant-Bio) at 2400rpm for approximately 10s to facilitate cell suspension. Cells were quantified on a Neubauer Improved Haemocytometer (Hirschman EM Techcolor) and adjusted to $1.5x10^5$ cells mL^{-1} using sterile-filtered seawater.

The microtitre plate bioassay for copper toxicity of esterase activity.

After standardisation of *S. microadriaticum* inoculate either by protein content, chlorophyll *a* autofluorescence, or direct cell count, the microtitre plate bioassay was conducted as described in the Materials and Methods section of chapter 2.

Statistics

Statistical advice was provided by Dr. Mike Steele of the James Cook University Mathematics and Physics Department. Due to the exploratory nature of this research and the inconsistency of the relationship between copper and esterase activity (percent inhibition of esterase-induced fluorescein fluorescence), the bioassay data will be described without the use of statistical tests. According to Steele (pers. comm., 2005), the use of statistical tests on such variable data is impractical due to the small sample sizes involved.

Results

Standardisation of *Symbiodinium microadriaticum*.

Pierce protein assay

Determination of protein content in *S. microadriaticum* was not reproducible between independent cultures, and the homogenisation procedure as well as the assay itself failed to accurately quantify protein content in small (2.5-5.0μL) samples (Table 3.5). Theoretically, using the same homongenate, the protein concentration of a 20μL sample should be eight times that of a 2.5μL sample, which was nearly achieved with culture 2 (2.5μL sample=0.420μg mL^{-1} protein, 20.0μL sample=3.807μg mL^{-1} protein, Table 3.5). This could not be confirmed with culture 1 due to conducting the assay at the detection limit of the spectrophotometer for this culture (2.5 and 5.0μL sample=n.d.). Similarly, culture 3 (5.0μL sample=0.096μg mL^{-1} protein, 20.0μL sample=2.234μg mL^{-1} protein, Table 3.5) also failed to accurately report the dilution relationship between 2.5, 5.0 and 20μL of the same homogenate.

Nevertheless, the possibility remained that the assay would be more accurate well above the detection limit, and the bioassay initial inoculation density was standardised according to the protein content in the 20.0μL sample. Protein content of each culture used in bioassays 1-3 was independently calculated using the Pierce protein assay. The protein content of culture 2 (5.234μg protein $275\mu L^{-1}$ undiluted sample) had 7.4 times more protein than culture 1 (0.7076μg protein $275\mu L^{-1}$ undiluted sample). Therefore culture 2 was diluted with sterile-filtered seawater to reflect the protein content in culture 1 for use in the Cu bioassay. Likewise, culture 3 (3.08μg protein $275\mu L^{-1}$ undiluted sample) had 4.4 times more protein than culture 1, so bioassay experiment 3 initial inoculation density was also diluted to the protein concentration of culture 1. Using cultures 2 and 3 diluted with seawater and culture 1 undiluted, the IID for each independent bioassay experiment theoretically had the same protein content. However, the success of standardising IID by protein content can only be ascertained from the bioassay dose-response curves.

Chlorophyll a autofluorescence

Determination of *S. microadriaticum* cell density by chlorophyll *a* autofluorescence was performed independently in triplicate (Figure 3.2). Each data point represents the average of five independent measurements of chlorophyll *a* autofluorescence (chl. *a* autofluorescence) with standard errors. All three curves are quadratic in nature, and all R^2 values exceed 0.95 (culture 1 R^2=0.9554, culture 2 R^2=0.9870, culture 3 R^2=0.9908), indicating a strong relationship between percent *S. microadriaticum* and chl. *a* autofluorescence.

10x concentrated *Symbiodinium* (µL)	Mean absorbency (562nm)	Protein content (µg mL^{-1})	Protein content undiluted (µg 275µL^{-1})
Culture 1			
Blank	0.32	-	
2.5	0.318	n.d.	
5	0.329	n.d.	
20	0.348	0.515	0.7076
Culture 2			
Blank	0.298	-	
2.5	0.323	0.42	
5	0.34	1.342	
20	0.387	3.807	5.234
Culture 3			
Blank	0.341	-	
2.5	0.348	n.d.	
5	0.355	0.096	
20	0.394	2.234	3.08

Table 3.5. Protein content of four-week old *Symbiodinium microadriaticum* cultures based on the Pierce protein assay bovine serum albumin curve (Figure 1). n=3.

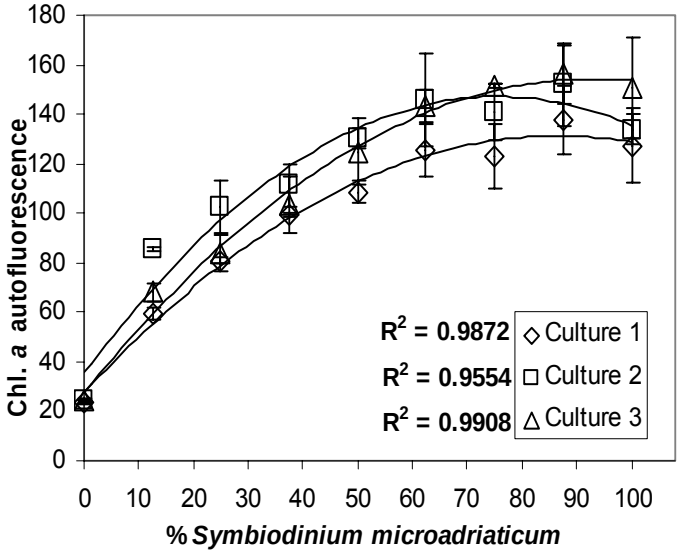


Figure 3.2. Determination of *S. microadriaticum* cell density via chl. *a* autofluorescence. Data points show standard error. n=3.

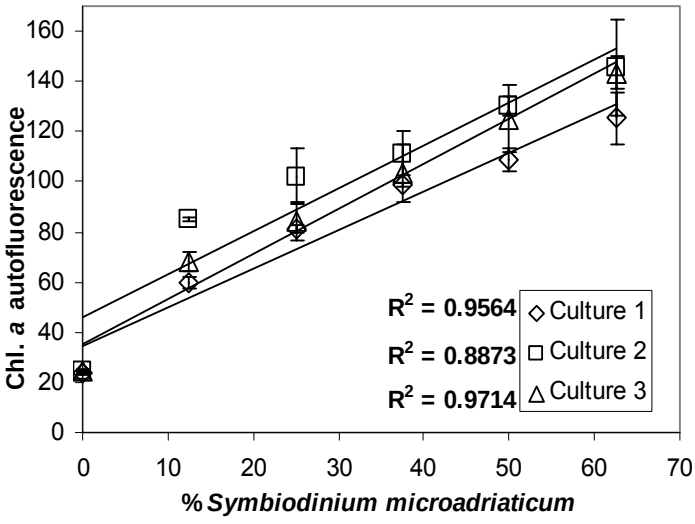


Figure 3.3. Determination of *S. microadriaticum* cell density via chl. *a* autofluorescence. Linear portion of Figure 3.2. Data points show standard error. n=3.

The results of quadratic regression analyses (culture 1 $F_{(1,7)}$=64.27, culture 2 $F_{(1,7)}$=231.21, culture 3 $F_{(1,7)}$=321.99) indicate a significant effect (culture 1 p=0.0015, cultures 2 and 3 p<0.001) of percent *S. microadriaticum* on chl. *a* autofluorescence.

Between experiments, standard error bars overlap, suggesting no significant difference in chl. *a* autofluorescence between 50% *S. microadriaticum* for experiment 1 (culture 1) and 37.5% *S. microadriaticum* in experiments 2 and 3 (Figure 3.2). These three dilutions were therefore chosen as the initial inoculation densities for the esterase bioassays (culture 1=50% sample, culture 2=37.5%, culture 3=37.5%). There appears to be no significant difference in chl. *a* autofluorescence between 62.5 and 100% *S. microadriaticum* within each experiment, thus the three dilutions chosen for the esterase bioassays were below 62.5%, on the linear portion of the curve (culture 1 R^2=0.9564, culture 2 R^2=0.8873, culture 3 R^2=0.9714, Figure 3.3), to avoid the impact of self-shading in the microtitre plate wells.

Cell count via haemocytometer.

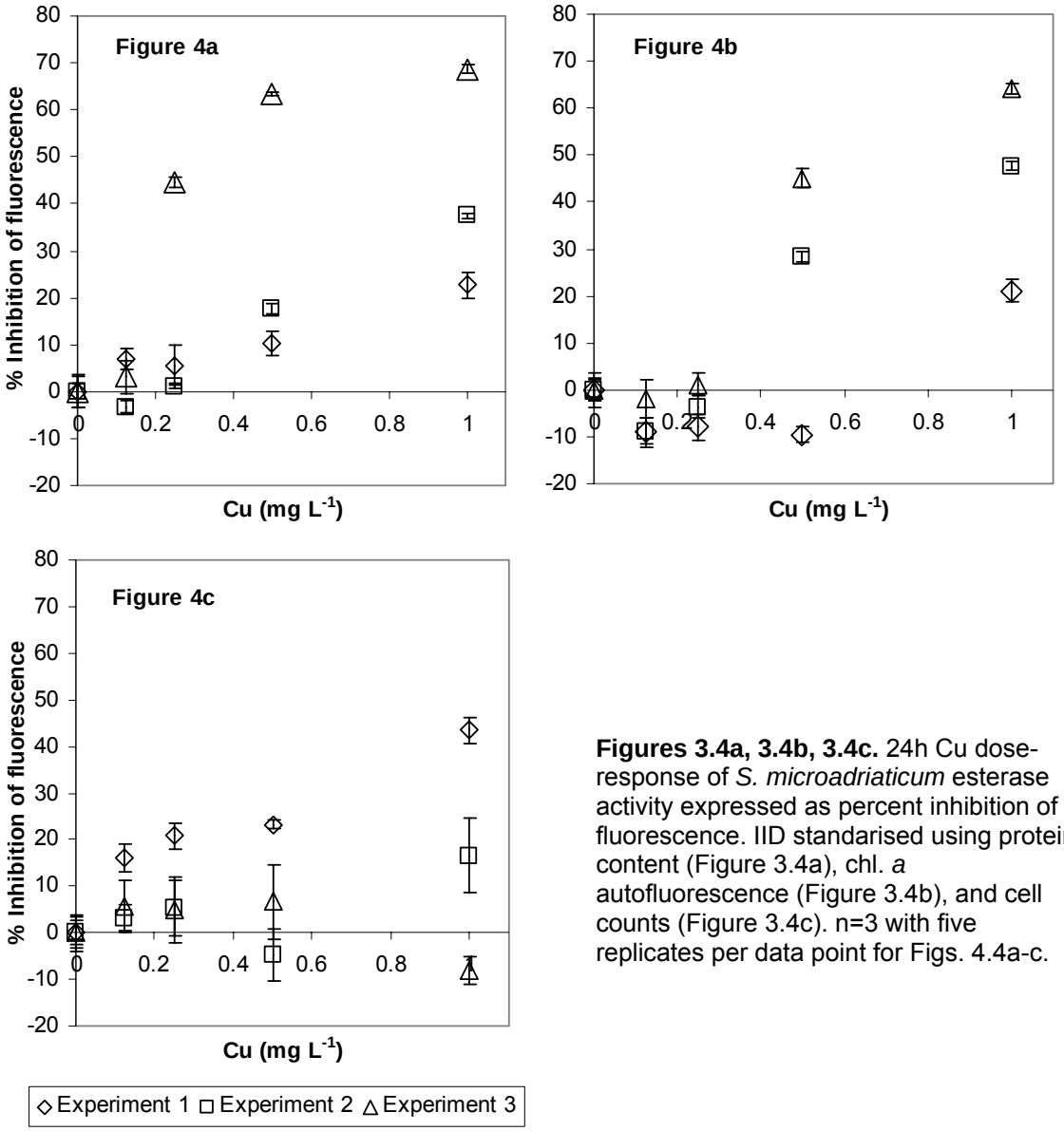
Quantifying *S. microadriaticum* cell density on a Neubauer Improved haemocytometer was difficult due to the strongly self-adhesive nature of the organisms. For this reason, reported cell densities are approximations only. It is also likely that the organisms adhered to the glassware irregularly, because one relatively high cell count came from a very pale culture (experiment 1 mother culture ~$4.93x10^5$ cells mL^{-1}) and two relatively low cell counts were sourced from darkly pigmented cultures (experiment 2 mother culture ~$1.03x10^5$ cells mL^{-1}, experiment 3 mother culture ~$1.10x10^5$ cells mL^{-1}). The within-culture variability was high (standard deviation culture 1 ±$1.53x10^5$, culture 2 ±$2.52x10^4$, culture 3 ±$2.65x10^4$).

<u>Bioassays for Cu toxicity using protein content, chlorophyll *a* autofluorescence, and cell counts to standardise initial inoculation density (IID).</u>

Standardisation of IID using protein content.

The effect of Cu (mg L^{-1}) on esterase activity (percent inhibition of fluorescence (%IF)) in *S. microadriaticum* standardised for IID using protein

content showed a high level of variability between experiments (Figure 3.4a), and all experiments exhibit increased %IF with increased Cu. At 0.250 mg Cu L^{-1}, experiments 1 and 2 exhibited similar %IF (experiment 1=5.56%±4.29, experiment 2=1.41%±0.54), while experiment 3 had 44.6 %IF±1.11. The trend was maintained at 0.500 and 1.0 mg Cu L^{-1} (experiment 1=22.6 %IF±2.83, experiment 2=37.5 %IF ±0.47, experiment 3=68.7 %IF ±0.95). There was little variability within data points, as indicated by the low within-experiment percent coefficient of variations (%CV experiment 1= 11.8, experiment 2= 7.93%, experiment 3= 11.0%).



Figures 3.4a, 3.4b, 3.4c. 24h Cu dose-response of *S. microadriaticum* esterase activity expressed as percent inhibition of fluorescence. IID standarised using protein content (Figure 3.4a), chl. *a* autofluorescence (Figure 3.4b), and cell counts (Figure 3.4c). n=3 with five replicates per data point for Figs. 4.4a-c.

Standardisation of IID using chlorophyll a autofluorescence.

The effect of Cu (mg L^{-1}) on esterase activity (percent inhibition of fluorescence) in *S. microadriaticum* (standardised for IID using chlorophyll *a* autofluorescence) had high variability between experiments, with two dose-response curves displaying similar trends (experiments 2 and 3) and the third (experiment 1) as a relative outlier in the data set (Figure 3.4b). All experiments showed weak (>-10.0% inhibition) increases in esterase-induced fluorescein fluorescence at 0.125 and 0.250 mg Cu L^{-1}, though at 0.500 mg Cu L^{-1} experiments 2 and 3 revealed similar %IF (28.3%±1.05 and 45.0%±2.02, respectively) in contrast to experiment 1 (-9.53% ±1.53). All experiments displayed the highest %IF at 1.0 mg Cu L^{-1} (experiment 1= 21.0%±2.45, experiment 2= 47.6%±0.96, experiment 3=64.1%±1.07). There was little variability within data points, as indicated by the low within-experiment percent coefficient of variations (%CV experiment1= 6.91, experiment 2= 8.05%, experiment 3= 7.52%).

Standardisation of IID using cell count.

The effect of Cu (mg L^{-1}) on esterase activity (percent inhibition of fluorescence) in *S. microadriaticum* (IID standardised using cell counts) was conducted independently in triplicate, with each experiment displaying a different relationship between Cu and %IF (Figure 3.4c). There is little difference in %IF values between 0.125 and 0.500 mg Cu L^{-1} in experiment 1 (16.1%±2.87- 23.3%±1.03), though percent inhibition increased (43.4%±2.85) at 1.0 mg Cu L^{-1}. Experiments 2 and 3 had similar percent inhibition values at 0.125 mg Cu L^{-1} (experiment 2= 3.22%±2.85, experiment 3=5.67%±5.65) and 0.250 mg Cu L^{-1} (experiment 2=5.17%±5.91, experiment 3=4.92%±7.22), diverging at 0.500 mg Cu L^{-1} (experiment 2= -4.74%±5.55, experiment 3=6.72%±7.95). At 1.0 mg Cu L^{-1}, experiment 2 displayed 16.6%±7.93 inhibition of fluorescence, and experiment 3 showed activation of esterase activity (-8.00% ±2.99). In comparison to figures 3.4a and 3.4b, figure 3.4c had larger standard error values in experiments 2 and 3, indicating higher within-culture variability for error, though within-experiment %CV remained low (experiment 1=9.54%, experiment 2=11.0%, experiment 3=8.17%).

Discussion

There is a need to determine the effects of anthropogenic pollution on coral reef systems. In North Queensland, Australia, copper is refined and shipped by sea (Esslemont 2000), and can be introduced to marine ecosystems through antifouling paints at ship grounding sites (Haynes et al. 2002, Haynes and Loong 2002) or as land-based runoff from agricultural areas (Rayment and Neil 1996, Haynes and Michalek-Wagner 2000). Above-background levels of Cu (0.028-0.230mg Cu g^{-1} sediment dry weight) have been measured in some North Queensland near-shore environments (Haynes and Loong 2002). Jones and Heyward (2003) have also identified the need for toxicological studies on tropical reef ecosystems because produced formation water (an effluent from the oil and gas industry) is often discharged near coral communities in the Northwest Shelf of Australia. The challenge of this research lies in the methodology as field-based studies are expensive and often cumbersome. However, the close relationship between corals and symbiotic algae means that if pollution affects the host organism, it will likely affect the microalgae (Jones and Heyward 2003). Therefore, bioassays with cultured algae offer a less expensive alternative to holobiont studies and present the opportunity for cost-effective replication (Blaise et al. 1998).

Despite the practical benefits of working with microalgae, there are few publications on bioassays for metal toxicity in cultured zooxanthellae. In addition to the scarcity of published literature is the tendency of authors to present "pooled" dose-response curves, thereby combining the results of independent experiments and burying the unique relationships that may be revealed between pollutant and endpoint within large standard error bars. This pooled relationship is often accompanied by high R^2 values in the dose-response curves, thus "justifying" the methodology. An example of this method can be observed in Goh and Chou (1997). These authors found a significant inhibitory effect of 40µg Cu L^{-1} on the specific growth rate of *S. microadriaticum* isolated from the coral *Montipora verrucosa* after 23 days of Cu exposure. Goh and Chou (1997) used four independent replicate culture tubes for each of the treatments (20µg Cu L^{-1} and 40µg Cu L^{-1}) and the controls, and calculated the mean specific growth rates with standard deviations. However, the standard

deviations were approximately 50% of the mean values, indicating large within-treatment variability. From their figures and calculations, it cannot be discerned if the relationships between Cu and specific growth rate were consistent between experiments. This presents difficulty in placing my research, which did not combine independent bioassay experiments, within the context of this one potentially comparable study.

Chlorophyll *a* autofluorescence was a relatively successful method to determine *S. microadriaticum* cell density (see below), but this did not translate into independently replicable relationships between percent inhibition of esterase activity and Cu in the microtitre plate bioassay. However, if the bioassay data had been pooled (all three independent experiments combined in the manner of Goh and Chou (1997)), the bioassay would be considered more successful, with percent inhibition of fluorescence positively increasing (R^2=0.9073) with increasing copper concentrations (Figure 3.5), thus revealing the expected dose-response of esterase activity to copper.

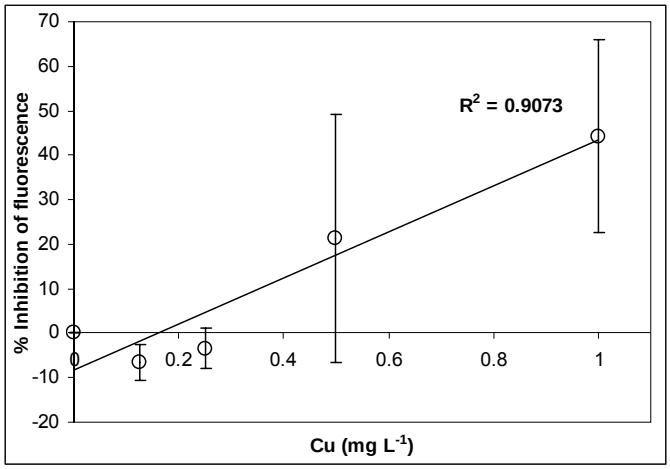


Figure 3.5. 24h Cu dose-response curve of *S. microadriaticum,* IID standardised using chl. *a* autofluorescence*.* Data are presented as three pooled independent replicates per data point with standard deviations.

One of the challenges inherent in working with established cultures of *Symbiodinium* is overcoming its strong adhesive properties (Domotor and D'Elia 1984, Sakami 2000, [McLaughlin and Zahl 1959, Freudenthal 1962, Loeblich and Sherley 1979, *in* Domotor and D'Elia 1984]), which present difficulties when standardising initial inoculation density (IID). Despite the inconsistent relationships between Cu and percent inhibition of esterase activity in this

chapter, all IID approaches showed a 24h dose-response to copper and little within-treatment variability. This indicated that the variability in the relationship between Cu and percent inhibition of esterase activity was likely due to the differences in IID between independent experiments of the bioassay (i.e. increased number of cells decreases overall copper toxicity as each cell is exposed to fewer copper ions). The results obtained indicate that no method used to achieve consistent IIDs (discussed below) overcame the cell enumeration problem associated with the organism's adhesive properties in culture.

Using protein content as a proxy for cell density in independent cultures of *S. microadriaticum,* sample volume and protein content were not well correlated. For culture 1, this relationship could not be examined due to undetectable protein levels in the smaller volumes (2.5, 5.0µL), suggesting that larger sample volumes should be used in this assay to avoid working at the detection limit of the spectrophotometer. Only culture 2 appeared to have good agreement between sample volume and protein content. Another compounding difficulty (besides adhesion) associated with using protein content as a measure of *Symbiodinium* cell density was the challenge of extracting protein from the organisms. It was necessary to expose the cells to multiple homogenisations and strong detergents for 21 hours in order to extract enough protein for analysis. The resistance to the detergents may be due to the thick extracellular armour of *S. microadriaticum*, which has been documented by Schoenberg and Trench (1980). These researchers found that *Symbiodinium microadriaticum* experiences dramatic morphological changes 24h after isolation from the host tissue, with the algal cell wall significantly increasing in thickness and homogeneity. This may also explain the strong adherent properties of cells in long-term cultures.

Other factors influencing between-experiment variability using protein content to standardise IID may have been culture health and/or nutritional status (Valenzuela-Espinoza et al. 2005) leading to variability of protein content. For replicates to be used in bioassays, it is essential that organisms are cultured under identical conditions. Lighting regime, in particular UV-B levels (Yu et al. 2004), can affect the protein content of microalgal cells. Therefore protein content may be a measure of cell health, and not cell density. *Symbiodinium*

also have the ability to change their protein profile based upon age, state of isolation (fresh vs. established cultures), and culture condition (Stochaj and Grossman 1997). The above scenarios, however, were highly unlikely to have jeopardised the use of protein content as a proxy of culture cell density, as culture replicates were of identical age and maintained under the same environmental and nutrient conditions. In conclusion, the protein assay was most likely unsuccessful for evaluating culture cell density due to the assay operating at its detection limit and the detergent-resistant cellular armour of *S. microadriaticum*, necessitating complex homogenisation procedures, which added to the difficulty in standardising IID between different cultures.

Chlorophyll *a* autofluorescence (chl. *a* autofluorescence) appeared to be a more accurate representation of *S. microadriaticum* culture densities. Percent culture (organism) up to 62.5% showed a linear relationship for chl. *a* autofluorescence, while higher culture content revealed a stabilisation in the intensity of chl. *a* autofluorescence due to fluorescence quenching. Increased cell number in microtitre plate wells may interfere with detection of fluorescent light emission by the spectrofluorometer light probe, resulting in cells closer to the surface quenching the fluorescence of those deeper in the well (Blaise et al. 1998). For this reason, initial inoculation densities for esterase bioassays were chosen from the linear portion of the chl. *a* autofluorescence curve. In general, chl. *a* autofluorescence appeared to be a suitable method to quantify *S. microadriaticum* culture cell densities, but this did not translate into replicable experiments in the microtitre plate bioassay for Cu toxicity to esterase activity.

Standardisation of IID using cell counts showed the greatest inconsistency between independent experiments and was the most difficult way to determine initial inoculation density due to the adhesion of cells to each other and the glassware. The unreliability of cell counts for standardizing IID was supported by the results of experiment 1, where a pale culture yielded the highest number of cells per millilitre and copper inhibited esterase activity more than in the other two cultures. This suggests that cell count in this experiment overestimated culture cell density, resulting in fewer cells being exposed to more copper. Given the pale colour of the culture, however, it may also be possible that culture health in general was compromised, resulting in copper being more toxic to esterase activity. In contrast to the use of protein content

and chl. *a* autofluorescence as proxies of culture cell density, IID standardisation using cell counts yielded a higher level of within-culture variability, indicating that cell count via haemocytometer may not be a viable option for established cultures of *S. microadriaticum.*

Cell counts of zooxanthellae using a haemocytometer have been described in the literature, though in these studies (Annis and Cook 2002, Jones and Heyward 2003) the zooxanthellae were freshly isolated from their host organism, and were not in established cultures. Freshly isolated zooxanthellae are physiologically (Schoenberg and Trench 1980, Stochaj and Grossman 1997) different from established cultures of zooxanthellae, which may impact upon their effectiveness in a bioassay (i.e. adherent properties). Additionally, freshly isolated zooxanthellae are more likely to be stressed than established cultures, which may affect the outcome of a bioassay. Past studies have demonstrated that *in hospite Symbiodinium* are also morphologically distinct to their free-living form, with symbiotic cells being exclusively coccoid and surrounded by several thick-layered walls, which cultured cells show both coccoid and gymnidoid stages with simpler cell wall arrangements (Steele 1975, Trench 1993).

Traditional methods of determining cell density, such as direct cell count, may be inappropriate for quantifying microalgae that have strong adhesive properties (such as fouling diatoms and colonial microalgae like *Chrysocystis fragilis*), but are a suitable technique when quantifying culture cell densities for *Tetraselmis* sp. and *C. gracilis* (Chapter 2) to standardise IID in bioassays. The results obtained indicate that chl. *a* autofluorescence might show promise as a method to quantify culture densities of zooxanthellae for standardisation of IID in bioassays. However, the unique properties of the cultured organism do not lend themselves for use in this bioassay. Even if the methodology could be developed to successfully replicate initial inoculation density leading to reproducible bioassay outcomes, the applicability of sensitivity measurements to the holobiont (and pollution monitoring) would still be questionable because the algae that survive extraction from the holobiont and subsequent culturing may not represent the true *in situ* population (Santos et al. 2001). Given the difficulties encountered when working with cultured *S. microadriaticum*, perhaps future research should focus upon measuring metabolic activity *in situ*, despite

the high costs associated with fieldwork and large-scale laboratory experiments. Measurement of produced formation water (Jones and Heyward 2003) and herbicide (Raberg et al. 2003) toxicity to *in hospite* coral-associated zooxanthellae F_v/F_m and effective quantum yield (respectively) have been successfully achieved with PAM-fluorometry, which may be a more viable option for future research.

Chapter 4

The effect of pH, salinity, and temperature on esterase activity and copper toxicity in *Tetraselmis* sp. (CHLOROPHYTA) using the microtitre plate bioassay.

Introduction

The results of a bioassay must be taken within the context of the experiment's controlled variables, and there is a critical need to establish the effect of environmental conditions on the outcome of a bioassay. The addition of effluents to a bioassay protocol can change previously set parameters such as pH and salinity, which can impact upon copper speciation (Stumm and Morgan 1981, Sadiq 1992). Metal species is an important parameter when determining copper bioavailability because free ion concentration is often a predictor of toxicity (Lage et al. 1996, Eriksen et al. 2001, and Paquin et al. 2002). Seawater is a strong buffer against metal toxicity, and copper has been suggested to form stronger complexes with organic ligands in seawater than any other trace metal (Sadiq 1992). The relationship between metal species, toxicity, and temperature is also particularly important to microalgae because membrane permeability can change with fluctuating temperatures (Hassler et al. 2004). The ability of a bioassay to accurately report the effect of a pollutant with the interaction of changing variables will determine its usefulness to environmental monitoring.

Esterase activity is a bioassay endpoint that has been examined using microalgae (Dorsey et al. 1989, Gilbert et al. 1992), and has been employed previously in microtitre plate bioassays (Galgani and Bocquene 1991, Galgani et al. 1992). However, few studies have actually examined the effects of changing variables such as pH, temperature, and salinity to assess the applicability of this endpoint to field monitoring. Minier et al. (1993) were the first to explore the effects of changing environmental parameters (temperature, irradiance, salinity) on esterase activity kinetics using a microtitre plate bioassay with the algae *Calothrix* PCC 7601 (Cyanoprokaryota), *Haslea ostrearia* (Heterokontophyta), and *Prorocentrum micans* (Dinophyta). They described esterase activity as controlled by a photoperiod-dependent circadian rhythm, and measured an enhanced level of fluorescence in *Calothrix* and *H. ostrearia* with increasing irradiance. A temperature increase of 10°C significantly enhanced esterase activity for all species of microalgae (*P. micans*-67.5%, *H. ostrearia*-131.0%, *Calothrix*-50.5%), and esterase activity was generally higher at moderate (25-35%) salinity levels. Minier et al. (1993) highlighted the importance of performing esterase activity bioassays within the context of

environmental parameters. However, Minier et al. (1993) did not explore the effects of changing environmental parameters in the presence of a pollutant.

In contrast, Adams and Stauber (2004) developed a whole-sediment toxicity test using esterase activity as an endpoint in the marine diatom *Entomoneis* cf *punctulata*. They examined the effects of changing salinity and pH on esterase activity after 24h of Cu (25µg L^{-1}) exposure. Using flow cytometry, it was established that esterase-induced fluorescein fluorescence intensity increased with decreasing salinity (35%-5%). Additionally, as pH decreased (8.5-6.0) esterase-induced fluorescein fluorescence intensity also decreased, but only pH 6.0 was significantly different. Adams and Stauber's (2004) salinity study was reflective of marine (33-37% salinity (Libes 1992), and estuarine (5-32% salinity (Libes 1992)) environments, but did not include hyper-saline (>35% (Kinne 1964)) environments. Likewise, the pH levels (8.5-6.0) used may not account for extreme changes in pH associated with the introduction of some acid-based pollutants (Regel et al. 2002). A wider range of salinities and pH levels are needed to fully understand the effects of changing environmental parameters on esterase activity, and its suitability to report the effect of pollutants under such conditions.

Regel et al. (2002) used esterase activity as an endpoint in their field study to examine the effects of acid mine drainage (AMD) and dry-land salinity run-off in a South Australian creek. This study is significant because they used a wide range of pH and salinity levels in a whole-effluent esterase bioassay, but the results must be taken within the context of freshwater chemistry, which differs from seawater chemistry (Sadiq 1992). Regel et al. (2002) applied their esterase bioassay using flow cytometry to a field study with AMD-affected changes in pH (3.0-8.8) and salinity (represented by conductivity in mS cm^{-1}), as a function of distance from an Adelaide mine and source of saline run-off. Upon exposure of *Microcystis aeruginosa* (Cyanoprokaryota) and *Selenastrum capricornutum* (Chlorophyta) to AMD-contaminated river water for one hour, there was a reproducible and predictable relationship between proximity to pollution source and esterase activity (as source proximity increased, esterase activity decreased) that was maintained over 24h. Exposure of both algae to high conductivity (20mS cm^{-1} is ~12ppt salinity) samples showed a significant depression in esterase activity after one hour, but both algae recovered after a

24-hour acclimation period. Despite the environmental relevance of this study, most of the conclusions drawn regarding the individual effects of naturally occurring environmental parameters (pH, conductivity, nutrient load) and metals (Al, Fe, Mn) on esterase activity were sourced from a correlation matrix. Regel et al. (2002) mainly based their conclusions upon statistically significant relationships, and not the direct effects of these parameters on esterase activity in these microalgae.

The purpose of this chapter was to quantify the relationship between esterase activity and copper toxicity in *Tetraselmis* sp. in the presence of the changing environmental parameters salinity, pH, and temperature. Shock exposure experiments using *Tetraselmis* sp. were conducted to mimic the effects of anthropogenic or naturally occurring sudden environmental shifts on esterase activity and Cu toxicity. Two experimental protocols were employed: the first protocol examined the impact of Cu and environmental parameters within independent cultures, and the second protocol examined these impacts between independent cultures. The purpose of these different experimental designs was to ascertain any effects of experimental design on the variability of esterase activity in *Tetraselmis*. Like Minier et al. (1993), it was a kinetics study that explored the effect of environmental variables on esterase activity alone. However, unlike study by Regel et al. (2002), this research quantified the direct effect of these parameters on esterase activity and Cu toxicity in a wide range of pH, salinity (and temperature) levels, which is lacking in the Adams and Stauber (2004) study. The wide range of pH values (2.0-10.0) chosen as experimental treatments reflect industry-produced pH extremes in nature; the leather industry produces waste effluent at pH 2.8-13.0 (Saravanabhavan et al. 2005), and the pulp and paper industries generate waste of pH 2-14 during product treatment (Singh 2002).

Tetraselmis sp. was chosen for this study because it is a hardy, easily cultured organism, and initial inoculation densities can be accurately determined via cell counts. *Tetraselmis* sp. is also an important aquaculture feed organism for commercially lucrative organisms such as oysters and mussels (Ronquillo et al. 1997), and is also used as food for rotifers, with the latter in turn being feed for larval fishes, prawns, and crabs (Luna-Andrade et al. 2002). Additionally, the presence of *Tetraselmis* in Cu-contaminated environments has been shown to

shield rotifers from the toxic effects of the metals (Luna-Andrade et al. 2002). The ease of manipulation and importance to industry makes *Tetraselmis* sp. a logical choice for this bioassay.

Methods and Materials

Tetraselmis sp. culturing

Tetraselmis sp. (NQAIF ID 0003, collected on the Great Barrier Reef, Australia) was cultured in Guillard's f/2 (Sigma-Aldrich) growth medium (see chapter 2 for medium preparation) under sterile conditions in a laminar flow (AES Environmental Pty. Ltd.). All glassware was acid-washed in 10% HCl, rinsed with de-ionized water (Elix 5, Millipore filtration system equipped with a pre-filter and carbon trap), and autoclaved prior to use. The *Tetraselmis* sp. cultures used in the bioassay contained 1mL of a two to three week old mother culture, and 99mL Guillard's f/2 medium. Cultures grew undisturbed for seven days in an environmental growth chamber (Contherm Digital Series Cooled Incubator) at 24°C at an approximate irradiance of 37.68µmol x m^{-2} x s^{-1} (measured on a light meter: LI-COR LI-250, LI-COR Inc., Lincoln, Nebraska, USA) on a 12:12 light:dark cycle. The LI-COR light meter was not fitted with a suitable sensor to measure variability of irradiance in relation to cell depth. For use in the bioassay, *Tetraselmis* sp. cell density was quantified on a Neubauer Improved haemocytometer (Hirschman EM Techcolor) and cell density was adjusted to $1.5x10^5$ cells mL^{-1} with Guillard's f/2 medium.

Preparation of pH shock to esterase activity and Cu toxicity experiments.

The preparation of pH-adjusted media followed the general protocol by Craig et al. (2003) with the following modifications. The pH stock was composed of 980mL (+/- amount HCl or NaOH) pre-filtered (0.45µm Whatman GFC filters) natural seawater, with the addition of either HCl (32%, Analytical grade Ajax Finechem Seven Hills, NSW 2147) or 0.4N NaOH (Analytical grade, Ajax Finechem Seven Hills, NSW 2147) to adjust the pH. pH was measured on a Eutech Instruments/Oakton Instruments pH 510 Bench Meter (Vernon Hills, IL 60061 USA).

On the day of the bioassay, the pH-adjusted seawater was sterile-filtered with Guillard's f/2 growth medium, which was used to standardise *Tetraselmis* sp. cell density to $1.5x10^5$ cells mL^{-1}. Two protocols were employed: the first examined the impact of pH and Cu within three independent cultures (WIC), and the second examined the impact of pH and Cu between fifteen independent

cultures (BIC). After the addition of *Tetraselmis* sp. to the pH-adjusted medium, pH was re-checked and adjusted if needed. The pH levels used in the WIC protocol were: 2.07 (±0.017), 4.13 (±0.006), 6.05 (±0.090), 8.70 (±0.091) and 9.92 (±0.041). The pH levels used in the BIC protocol were: 2.09 (±0.021), 4.25 (±0.065), 6.26 (±0.061), 8.25 (±0.038) and 9.97 (±0.044). A pH of 10.0 was the maximum limit due to significant precipitation occurring above this level. *Tetraselmis* sp. in the pH-adjusted Guillard's f/2 media was used to inoculate the bioassay microplates. Microtitre plate bioassay results are stated in terms of the measured pH at the time of inoculation (before transfer to microtitre plate) because it was impossible to directly measure pH in the microtitre plate wells at the time of the 24h bioassay. It is likely that pH did change over the course of 24 hours (see below), but this was not directly quantifiable.

Due to the ability of microalgae to modify the pH of growth media during the course of a bioassay (Craig et al. 2003), Erlenmeyer flasks were employed to monitor the change in pH during the 24h microtitre plate bioassay. Abiotic factors such as depletion of CO_2 or assimilation of NO_3^- or NH_4^+ may influence pH (Beardall, pers. comm.), though this was not quantified. All flasks were acid-washed and autoclaved, and inoculated in a sterile laminar flow. The flasks were exposed to exactly the same temperature and light conditions as the microtitre plate bioassay. Three flasks contained 150mL of pH-adjusted Guillard's f/2 medium, and three additional flasks contained *Tetraselmis* sp. at a cell density of $1.5x10^5$ cells mL^{-1}, also in the pH-adjusted medium. The experiment was repeated twice with independent cultures and medium. pH was measured at inoculation and after 24h.

The effect of pH on fluorescence without Cu for the WIC protocol was analysed via two-way ANOVA (proc. glm III, Statistica version 7, StatSoft Inc., Tulsa, OK, USA) to determine the effects of both culture and pH on fluorescence (adapted from Craig et al. 2003). The p-value is deemed significant at ≤0.05, and the F-value is written as F(df effect, df error). The influence of pH on percent inhibition of fluorescence by 1.0 mg Cu L^{-1} for the WIC protocol was also measured by two-way ANOVA (proc. glm III), with F- and p-values as above. The effect of pH on fluorescence without Cu in the BIC protocol was analysed via quadratic regression analysis, and the effect of pH on percent inhibition of fluorescence by Cu was determined by one-way ANOVA

(proc. glm III). The p-value is deemed significant at ≤0.05, and the F-value is written as F(df effect, df error). pH was not a precisely replicated variable between independent treatments, but it is unlikely that a difference of 0.006-0.091 in pH values would have a significant impact on the bioassay outcome, especially in shock experiments. Percent coefficients of variation (%CV) within data points and between treatments are reported for both protocols.

Preparation of salinity shock to esterase activity and Cu toxicity experiments.

Two days prior to the bioassay, pre-filtered (0.45µm Whatman GFC filters) natural seawater was adjusted using dIH_2O (Elix 5, Millipore filtration system equipped with a pre-filter and carbon trap) or NaCl (Ajax Finechem, analytical grade, Auburn, NSW 2144) to 25, 30, 35, 40, or 45ppt salinity levels as described in Craig et al. (2003). Salinity was measured on an AO Special Scale salinometer (American Optical Corp., Keene, NH USA). This adjusted seawater was made anticipating the addition of approximately 20mL of a 35ppt *Tetraselmis* sp. inoculate for use in the bioassay. Inoculate volume varied from 14-20mL, and did not have a significant impact on overall salinity. On the day of the bioassay, 980mL of the salinity-adjusted seawater was used to make Guillard's f/2 medium, which was then employed to standardise cell density to $1.5x10^5$ cells mL^{-1}. The effects of salinity and Cu were examined via two different protocols: one varying salinity within three independent cultures (WIC), the second varying salinity between fifteen independent cultures (BIC). *Tetraselmis* sp. in the salinity-adjusted Guillard's f/2 media was used to inoculate the bioassay microtitre plates.

The effect of salinity on fluorescence without Cu and percent inhibition of fluorescence by 1.0mg Cu L^{-1} in the WIC protocol was analysed via two-way ANOVA as per the previous section. The effect of salinity on fluorescence (without Cu), and percent inhibition of fluorescence by Cu in the BIC protocol was determined by one-way ANOVA (proc. glm III, Statistica version 7, StatSoft Inc., Tulsa, OK, USA). F- and p-values are deemed significant as per the previous section. Percent CV within independent data points and between treatments are reported.

Preparation of temperature shock to esterase activity and Cu toxicity.

For temperature shock experiments, temperature of the environmental growth chambers (Contherm Digital Series Cooled Incubator) was adjusted to either 32, 28, 24, 20, or 16°C at least 12h before the bioassay to allow the empty cabinet to achieve temperature stability. Irradiance (approximately 37.68µmol), pH (8.40), salinity (35ppt), and light:dark cycle (12:12) were held as constants. The effect of Cu and temperature within three independent cultures (WIC) and between fifteen independent cultures (BIC) was examined. Temperature was monitored during the 24h shock bioassay via a digital cabinet display and an independent mercury thermometer within the culture chamber.

The impact of temperature on control and percent inhibition of fluorescence by 0.500 and 1.00 mg Cu L^{-1} for the WIC protocol was analysed via two-way ANOVA, with F- and p-values deemed significant as per the pH section of the materials and methods. The effect of temperature on fluorescence (without Cu) and percent inhibition of fluorescence by Cu in the BIC protocol was determined via one-way ANOVA (proc. glm III). F- and p-values are significant as per the pH section of the materials and methods. Additionally, percent inhibition data between (and including) 16 and 28°C was analysed by quadratic regression analysis (with F- and p-values as above) due to the 32°C data being relative outliers (Everingham, pers. comm. 2005). Percent CV within independent data points and cultures, and between treatments are reported.

Prompted by the strong quadratic relationship between percent inhibition of fluorescence and temperature between 16 and 28°C, the effect of 32°C on the morphology of *Tetraselmis* sp. was inspected visually via microscopy (Olympus BX51 with a DP70 Digital camera and 100x oil immersion using Nomarski differential interference contrast, following horizontal transects within the viewing field until 20 cells per treatment were photographed). Additionally, cell length as a proxy for cell size (De Miranda et al. 2005) was analysed via one-way ANOVA, to determine if Cu affects cell size.

Microtitre plate bioassay for copper toxicity to esterase activity in *Tetraselmis* sp.

After variation of pH, salinity, or temperature as described above, the microtitre plate bioassay followed the protocol in chapter 2, the exception being that only 0.500 and 1.00mg Cu L^{-1} were used as pollutant concentrations in the temperature experiments, and 1.00mg Cu L^{-1} in the pH and salinity experiments. The effect of pH, salinity, and temperature on control (no pollutant present) data was also measured.

Results

pH shock experiments

Within three independent cultures (WIC protocol).

The overall effect of pH on esterase activity (fluorescence reported as arbitrary units (a.u.)) without Cu is a weakly (R^2=0.758) quadratic relationship (Figure 4.1a), with the most variability within treatments at pH 8.70 (±0.091) and pH 9.92 (±0.041) (Table 4.1a). There was little variability at all other pH treatments (Table 4.1a). The results of a two-way ANOVA indicated a significant ($p<0.001$, $F_{(4,8)}=14.89$) effect of pH on esterase activity and no significant effect ($p=0.176$, $F_{(2,8)}=2.18$) of culture on fluorescence (a.u.). There was little variability within cultures across all treatments (Table 4.1a).

In the presence of 1.0mg Cu L^{-1} (Figure 4.1b), there was no inhibition of esterase activity at pH 2.07 (±0.017) and 4.13 (±0.006), 78.8% mean inhibition at pH 6.05 (±0.090), 22.3% mean inhibition at pH 8.70 (±0.091), and 36.4% mean inhibition at pH 9.92 (±0.041). The highest variability within a pH treatment occurred at pH 8.70 (±0.091) (Table 4.1a). There was little variability at all other pH treatments (Table 4.1a). Like the effect of pH on esterase activity (Figure 4.1a), the results of a two-way ANOVA indicated a significant ($p<0.001$, $F_{(4,8)}=123.67$) effect of pH on esterase activity, and no significant effect ($p=0.367$, $F_{(2,8)}=1.138$) of culture. There was slightly higher variability within the three cultures across all pH's (Table 4.1a) than seen in the control (no Cu).

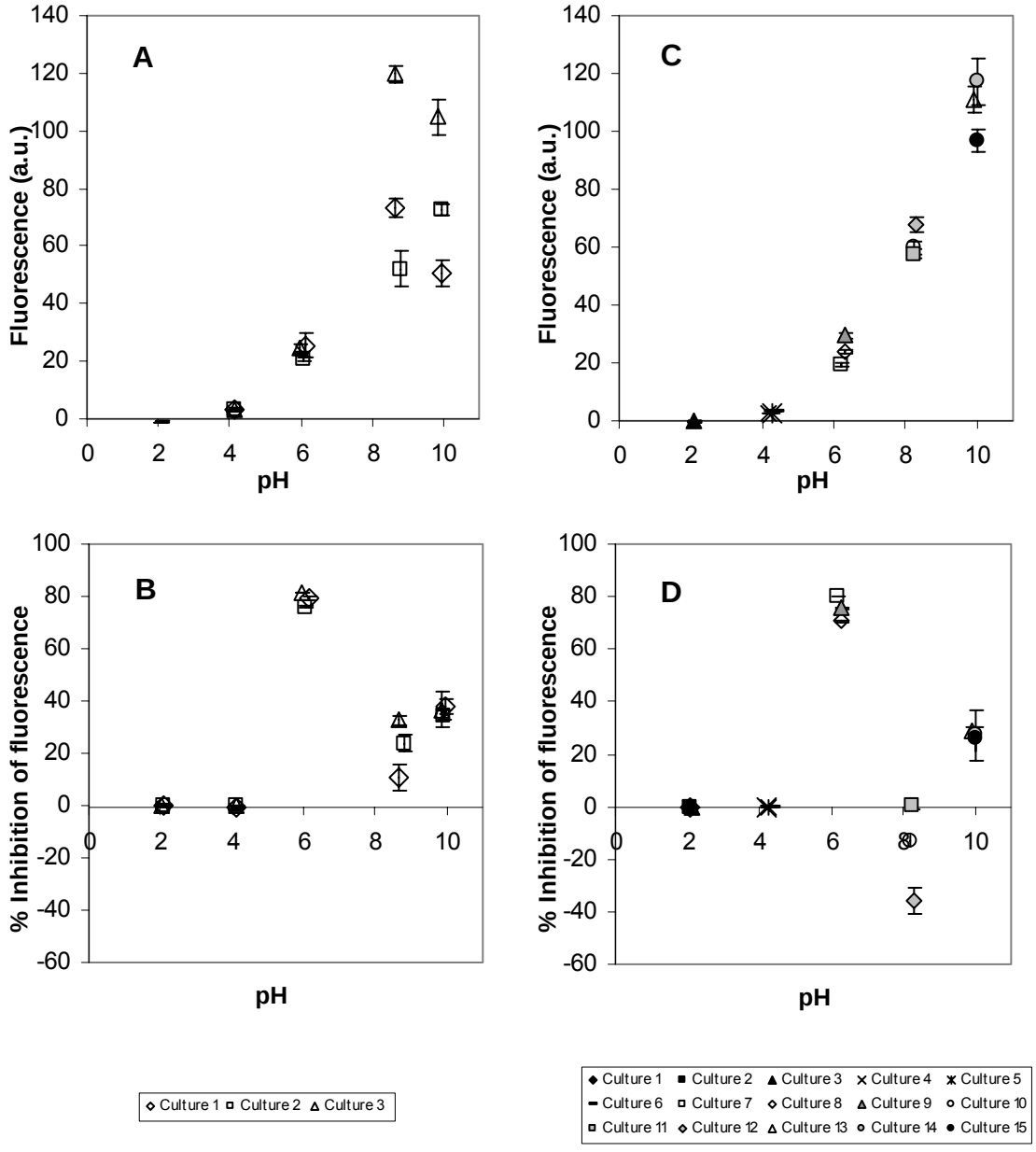
Tables 4.1a and 4.1b: Percent coefficient of variation in pH shock experiments to esterase activity and Cu toxicity within (WIC protocol, Table 4.1a), and between (BIC protocol, Table 4.1b) independent *Tetraselmis* sp. cultures.

a.

WIC Protocol		
Within treatments	**No Cu**	**1.0mg Cu L^{-1}**
pH	*%CV*	*%CV*
2.07(±0.017)	0	0
4.13(±0.006)	6.97	0
6.05(±0.090)	10.46	3.54
8.70(±0.091)	42.51	49.60
9.92(±0.041)	35.82	4.21
Within cultures		
Culture		
1	14.02	18.47
2	12.90	14.00
3	11.58	10.76

b.

BIC Protocol		
Within treatments	**No Cu**	**1.0mg Cu L^{-1}**
pH	*%CV*	*%CV*
2.09(±0.021)	0	0
4.24(±0.065)	17.00	0
6.29(±0.061)	20.63	6.41
8.25(±0.038)	8.47	110.55
9.97(±0.044)	9.73	5.11
Within cultures		
Cultures		
1-3	0.316-0.733	0.319-0.680
4-6	3.32-3.58	1.55-2.09
7-9	0.930-2.67	1.63-3.96
10-12	4.65-6.24	4.77-9.47
13-15	7.41-12.96	3.23-20.62



Figures 4.1a-d. The influence of pH on esterase activity and copper toxicity in *Tetraselmis* sp.

Figures 4.1a and 4.1b show the within-culture experimental design (WIC protocol).
Figures 4.1c and 4.1d show the independent culture experimental design (BIC protocol).
Figures 4.1a and 4.1c display the effect of pH on esterase activity (reported as fluorescence (a.u.)).
Figures 4.1b and 4.1d display the effect of pH on esterase activity (reported as % inhibition of fluorescence) by 1.0mg Cu L^{-1}.

Between 15 independent cultures (BIC protocol).

The effect of a 24h pH shock on esterase activity without Cu between independent cultures of *Tetraselmis* sp. followed a strongly quadratic function (R^2=0.989), with fluorescence readings at pH 2.09(±0.021) and 4.25(±0.065) nearly zero (Figure 4.1c). The results of a quadratic regression ($F_{(2, 12)}$= 532.49)

indicated a significant ($p<0.001$) effect of pH on esterase activity. Within each treatment pH, fluorescence readings showed little variability (Table 4.1b). There was little variability within each independent culture, as indicated by the small error bars and low %CV (Table 4.1b).

The effect of pH on esterase activity in the presence of 1.00mg Cu L^{-1} (Figure 4.1d) displayed zero percent inhibition at pH 2.09(±0.021) and 4.25(±0.065), increasing to an average of 75.4% at pH 6.26(±0.061), and decreasing to -16.3% (average) at pH 8.25(±0.038). In Figure 4.1d, percent inhibition of fluorescence at pH 9.97(±0.044) was 27.3%; not nearly as high as the data for pH 6.26(±0.061). The results of a one-way ANOVA ($F_{(4, 10)}=54.59$) indicated a significant ($p<0.0001$) effect of pH on esterase activity in the presence of Cu. The within-treatment variability for the data set presented was relatively low excepting pH 8.25(±0.038) (Table 4.1b), and variability within independent cultures (data points) was low to moderate (Table 4.1b).

A comparison of the WIC (Figure 4.1a) and BIC protocol (Figure 4.1c) control data showed similar quadratic trends, with esterase activity increasing with higher pH. However, Figure 4.1a displayed more variability in the data at pH's 8 and 10 than Figure 4.1c. In the presence of 1.00mg Cu L^{-1}, the two protocols (Figures 4.1b and 4.1d) showed a remarkably similar toxicity pattern with the exception at pH 8.

Change in pH over 24h.

Over the course of 24h, Erlenmeyer flasks containing Guillard's f/2 growth media in seawater (Figure 4.2a) at pH 9.93 changed to pH 9.45, and pH 8.09 increased to pH 8.17. Standard deviation bars were extremely small, and superimposition of data points occurred due to identical pH levels. Erlenmeyer flasks containing Guillard's f/2 growth media in seawater and the organism *Tetraselmis* sp. experienced more dramatic pH changes (Figure 4.2b). Cultures at the beginning of the experiment with pH 9.95 declined to pH 9.72 (culture 1) and pH 9.82 (culture 2). Cultures at pH 8.30 increased to pH 8.56 (culture 1) and pH 8.84 (culture 2), and cultures starting at pH 6.19 increased to pH 7.25 (culture 1) and pH 6.80 (culture 2). In general, cultures tended to change towards pH 8.

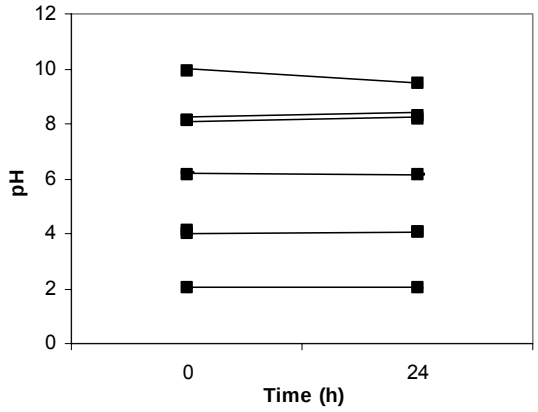


Figure 4.2a. The change in pH over 24h in Erlenmeyer flasks containing only Guillard's f/2 growth media in seawater.

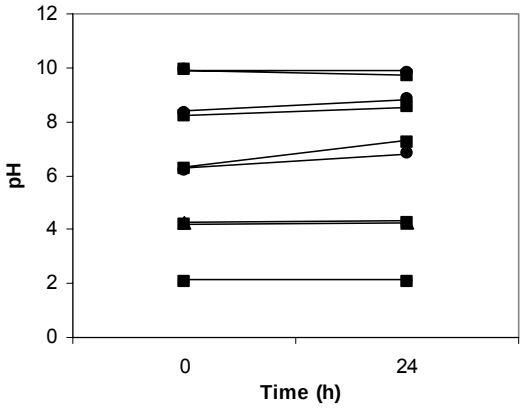


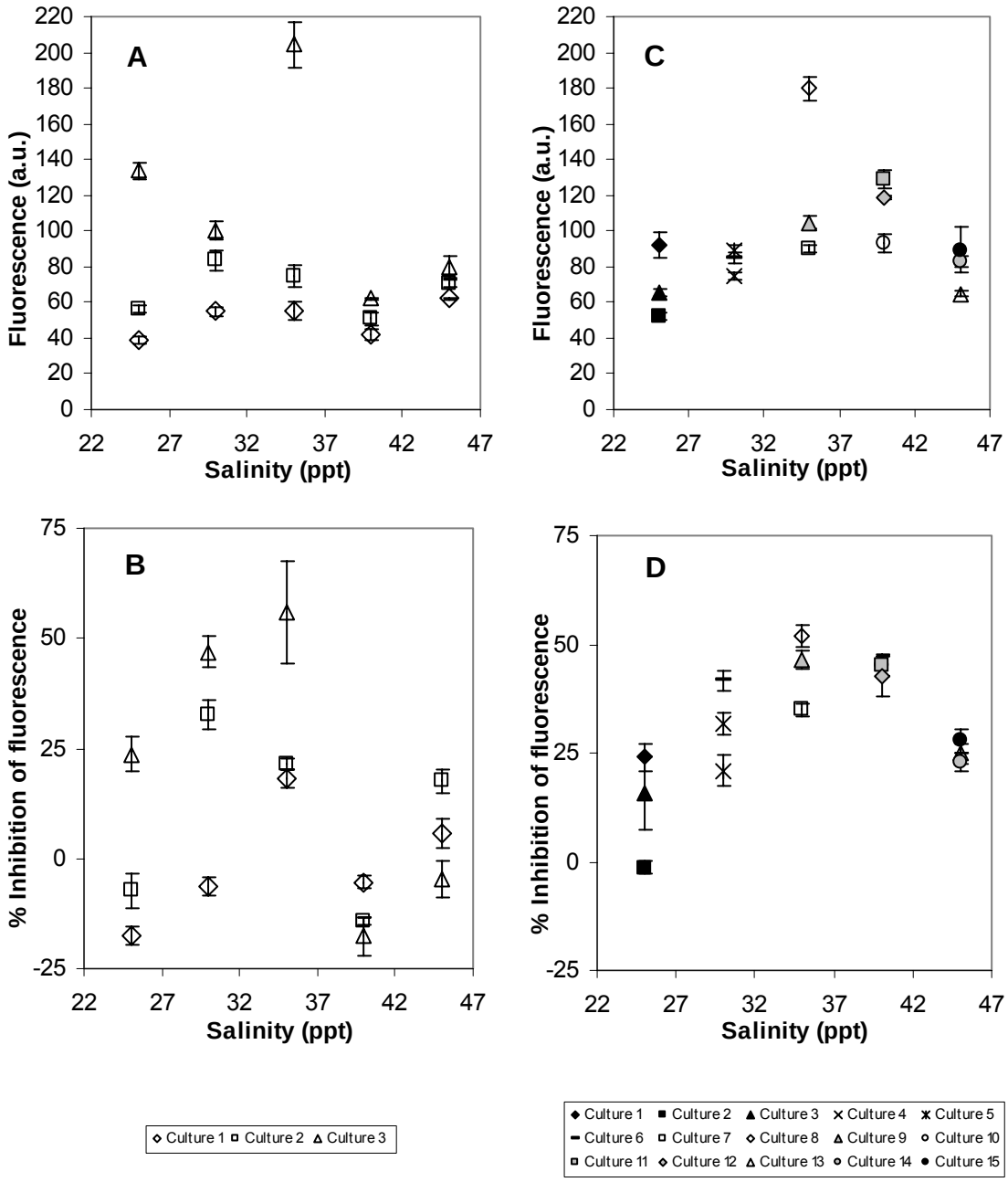
Figure 4.2b. The change in pH over 24h in Erlenmeyer flasks containing Guillard's f/2 media in seawater with two independent *Tetraselmis* sp. cultures 1(■) and 2 (●).

Salinity shock experiments

Within three independent cultures (WIC protocol).

The effect of salinity (ppt) shock on esterase activity within three independent cultures without copper displayed no obvious pattern (Figure 4.3a). Data points (with the exception of two outliers) ranged between 40 and 100 a.u. across all salinities examined. The results of a two-way ANOVA ($F_{(4, 8)}$=1.39) indicated no significant effect (p=0.320) of salinity on fluorescence. In contrast, there was a significant effect of culture on fluorescence ($F_{(2, 8)}$=5.86, p=0.027), though this was likely due to the two outliers being from culture three. %CV within salinity treatments was generally high (Table 4.2a), whereas average %CV within cultures (across all salinity treatments) was low (Table 4.2a).

The impact of salinity on esterase activity in the presence of 1.00mg Cu L^{-1} also displayed no obvious pattern, though percent inhibition appeared to steadily increase from 25 to 35ppt (Figure 4.3b). Results of a two-way ANOVA indicated no significant effect of either salinity ($F_{(4, 8)}$=3.22, p=0.074) or culture ($F_{(2, 8)}$=1.97, p=0.202) on esterase activity in the presence of Cu. %CV within treatments was generally high (Table 4.2a), whereas average %CV within cultures across all salinity treatments was low (Table 4.2a).



Figures 4.3a-d. The influence of salinity on esterase activity and copper toxicity in *Tetraselmis* sp.

Figures 4.3a and 4.3b show the within-culture experimental design (WIC protocol).
Figures 4.3c and 4.3d show the independent culture experimental design (BIC protocol).
Figures 4.3a and 4.3c display the effect of salinity on esterase activity (reported as fluorescence (a.u.)).
Figures 4.3b and 4.3d display the effect of salinity on esterase activity (reported as % inhibition of fluorescence) by 1.0mg Cu L^{-1}.

Tables 4.2a and 4.2b: Percent coefficient of variation in salinity shock experiments to esterase activity and Cu toxicity within (WIC protocol, Table 4.2a), and between (BIC protocol, Table 4.2b) independent *Tetraselmis* sp. cultures.

a.

WIC Protocol		
Within treatments	**No Cu**	**1.0mg Cu L^{-1}**
Salinity (ppt)	*%CV*	*%CV*
25	66.3	51.0
30	28.9	72.0
35	72.8	65.4
40	19.1	51.2
45	12.4	77.8
Within cultures		
Culture		
1	12.7	9.91
2	13.2	9.13
3	10.7	15.4

b.

BIC Protocol		
Within treatments	**No Cu**	**1.0mg Cu L^{-1}**
Salinity (ppt)	*%CV*	*%CV*
25	29.21	100.40
30	9.04	33.11
35	38.45	19.27
40	16.14	3.18
45	15.99	9.73
Within cultures		
Cultures		
1-3	5.84-14.10	3.98-25.20
4-6	5.11-7.26	7.15-8.81
7-9	3.73-7.51	4.07-5.88
10-12	1.89-10.39	5.83-11.80
13-15	4.37-26.33	5.76-7.72

Between 15 independent cultures (BIC protocol).

The impact of a 24h salinity (ppt) shock on esterase activity without Cu in *Tetraselmis* sp. appeared to follow a weak (R^2=0.412) parabolic trend (Figure 4.3c). Esterase activity increased with increasing salinities from 25-35ppt, but declined at higher salinity levels (40-45ppt). Results of a one-way ANOVA ($F_{(4, 10)}$=2.64) indicated no significant effect (p=0.097) of salinity on esterase activity. The variability within salinity treatments was moderate (Table 4.2b), indicating reasonably acceptable accuracy between experiments. The variability within independent cultures (data points) also appears to be relatively low (Table 4.2b).

Like the effect of salinity on esterase activity without Cu (Figure 4.3c), the effect of salinity on esterase activity in the presence of Cu (Figure 4.3d) revealed a parabolic trend, but the relationship was stronger (R^2=0.732), exhibiting an increase in inhibition of esterase activity at salinities from 25-35ppt and less inhibition at 40-45ppt. The results of a one-way ANOVA ($F_{(4, 10)}$=7.54) indicated a significant effect (p=0.005) of salinity on esterase activity in the presence of copper. Variability within salinity treatments in general seemed to be lower compared to the control data, with the exception of data at 25ppt (Table 4.2b). Variability within independent cultures (data points) is also low to moderate (Table 4.2b).

A comparison of the WIC (Figure 4.3a) and BIC protocol (Figure 4.3c) control data showed similar trends, though Figure 4.3c appeared to have less variability (Tables 4.2a and 4.2b). In the presence of 1.00mg Cu L^{-1}, the two protocols (Figures 4.3b and 4.3d) displayed very different toxicity patterns, with Figure 4.3b showing no obvious data trend, and Figure 4.3d displaying a parabolic pattern with the highest toxicity at moderate salinity levels.

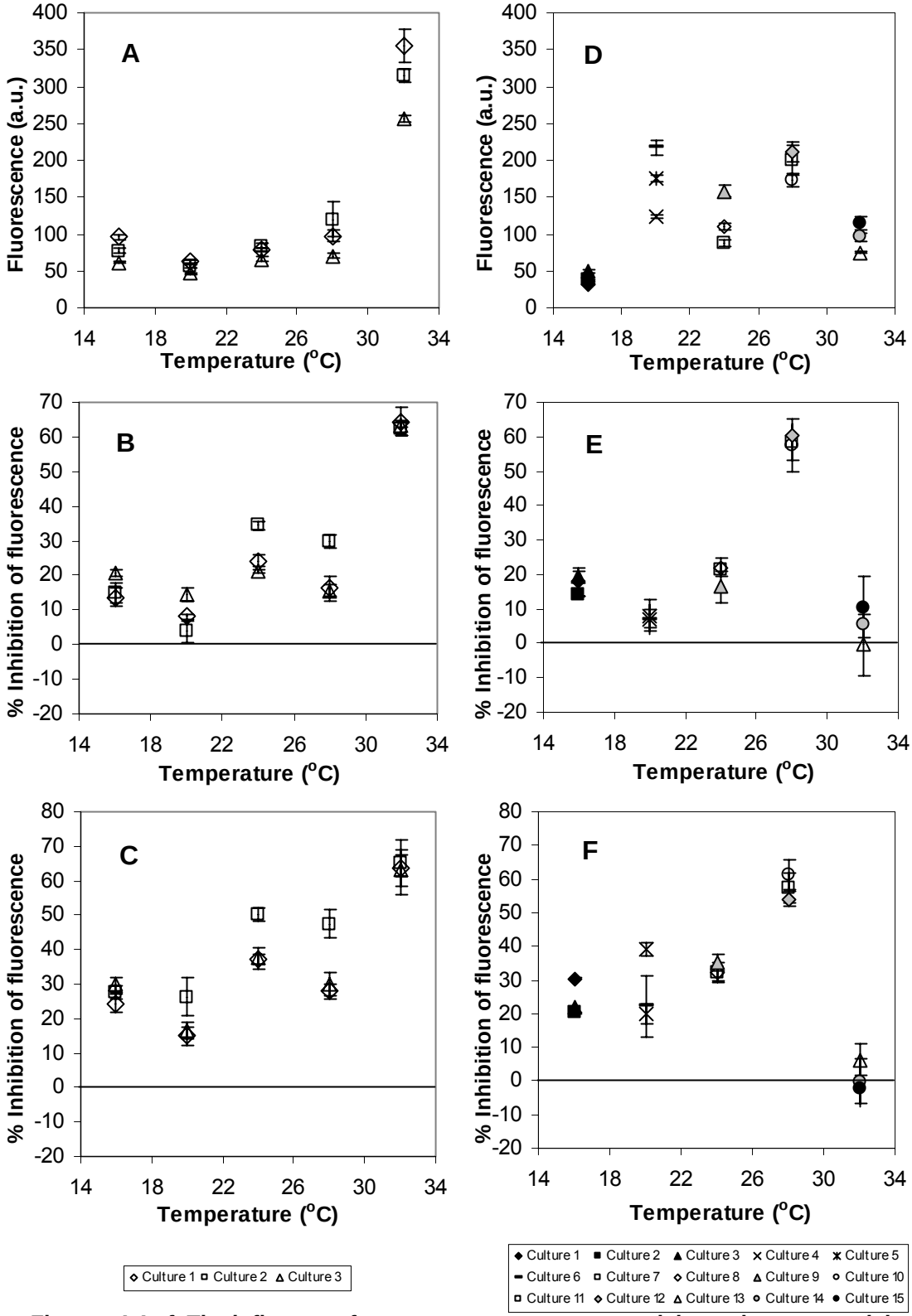
Temperature shock experiments

Within three independent cultures (WIC protocol).

The impact of a 24h temperature shock (16, 20, 24, 28, and 32°C) on esterase activity within three independent *Tetraselmis* sp. cultures in the absence of Cu (Figure 4.4a) displayed a relatively strong quadratic regression (R^2=0.883). Fluorescence measurements between 16 and 24°C were within the 50-100 a.u. range, with a slight increase at 28°C, and a large increase in fluorescence (250-350 a.u.) at 32°C. Results of a two-way ANOVA suggested a significant effect of both culture ($F_{(2,8)}$=5.25, p=0.035) and temperature ($F_{(4,8)}$=85.45, p<0.0001) on esterase activity. The variability within temperature treatments is low to moderate, as indicated by the %CV values (Table 4.3a), with the variability within cultures across all temperature treatments being substantially lower (Table 4.3a).

In the presence of 0.500mg Cu L^{-1} (Figure 4.4b), the data also displayed a quadratic pattern, though weaker (R^2=0.805) than that of Figure 4.4a. Esterase activity appeared to be negatively affected (higher % inhibition of fluorescence) with increasing temperatures; 32°C had an average of 63.0% inhibition. Results of a two-way ANOVA did not find a significant effect of culture on percent inhibition ($F_{(2,8)}$=0.502, p=0.623). However, there was a significant effect of temperature ($F_{(4,8)}$=38.70, p<0.0001). The variability within temperature treatments was moderate to high, as indicated by the %CV values (Table 4.3a), but variability within cultures across all temperatures was markedly lower (Table 4.3a).

The effect of temperature on esterase activity in the presence of 1.0mg Cu L^{-1} (Figure 4.4c) displayed an almost identical pattern to that seen in Figure 4.4b, though 4.4c has a slightly weaker (R^2=0.720) quadratic regression. The results of a two-way ANOVA indicated a significant effect of both culture



Figures 4.4a-f. The influence of temperature on esterase activity and copper toxicity in *Tetraselmis* sp.

Figures 4.4a-c show the within-culture experimental design (WIC protocol).
Figures 4.4d-f show the independent culture experimental design (BIC protocol).
Figures 4.4a and 4.4d display the effect of temperature on esterase activity (fluorescence (a.u.))
Figures 4b and 4e display the effect of temperature on esterase activity (%IF) by 0.5mg Cu L^{-1}.
Figures 4c and 4f display the effect of temperature on esterase activity (%IF) by 1.0mg Cu L^{-1}.

Tables 4.3a and 4.3b: Percent coefficient of variation in temperature shock experiments to esterase activity and Cu toxicity within (WIC protocol, Table 4.3a), and between (BIC protocol, Table 4.3b) independent *Tetraselmis* sp. cultures.

a.

WIC Protocol			
Within treatments	**No Cu**	**0.500mg Cu L^{-1}**	**1.0mg Cu L^{-1}**
Temperature (°C)	*%CV*	*%CV*	*%CV*
16	22.13	22.36	9.87
20	14.15	62.67	31.76
24	12.54	26.38	17.94
28	25.79	38.89	30.61
32	16.23	1.5	1.61
Within cultures			
Culture			
1	10.63	6.47	9.33
2	14.65	7.63	14.08
3	7.25	6.18	13.49

b.

BIC Protocol			
Within treatments	**No Cu**	**0.500mg Cu L^{-1}**	**1.0mg Cu L^{-1}**
Temperature (°C)	*%CV*	*%CV*	*%CV*
16	21.94	15.81	21.98
20	27.37	8.87	38.91
24	30.32	15.64	5.10
28	10.20	2.22	6.21
32	20.97	92.49	112.04
Within cultures			
Cultures			
1-3	2.57-5.09	1.27-19.92	1.09-4.21
4-6	4.24-9.16	4.28-5.86	3.82-10.79
7-9	6.97-9.13	4.46-6.76	4.85-7.84
10-12	10.08-21.04	7.09-17.64	3.96-10.56
13-15	2.79-14.23	6.37-19.39	8.60-12.12

($F_{(2,8)}$=6.35, p=0.022) and temperature ($F_{(4,8)}$=41.54, p<0.0001) on esterase activity. The variability within temperature treatments is low to moderate, as indicated by the %CV values (Table 4.3a), with even lower variability within cultures across temperature treatments (Table 4.3a).

Between 15 independent cultures (BIC protocol).

The impact of temperature (°C) shock on fluorescence (a.u.) (Figure 4.4d) in *Tetraselmis* sp. was significant as indicated by the results of a one-way ANOVA ($F_{(4, 10)}$=13.77, p=0.0005). Dunnett's post-hoc test revealed that the 16°C data group was significantly different from the rest of the data set (20°C p=0.0003, 24°C p=0.0075, 28°C p<0.0001, 32°C p=0.020). Therefore, the ANOVA was re-run excluding the 16°C data. Results of the second ANOVA

($F_{(3,8)}$=5.99) still indicated a significant effect (p=0.019) of temperature on fluorescence (a.u.), which was likely due to the significant difference of the 32°C data set from the 28°C data set (p=0.016) as detailed from Dunnett's post-hoc test. There was no significant difference between data at 20, 24, and 28°C. Variability within treatment groups was moderate (Table 4.3b), as was the variability within independent cultures (Table 4.3b).

The effect of temperature on esterase activity in the presence of 0.500mg Cu L^{-1} (Figure 4.4e) displayed a strongly quadratic relationship between (and including) 16 to 28°C, with data at 32°C being outliers from this relationship. The results of a one-way ANOVA ($F_{(4, 10)}$=145.58) analysing all data points revealed a significant (p<0.0001) effect of temperature on esterase activity in the presence of Cu. The results of a quadratic regression analysis of data points between (and including) 16 to 28°C ($F_{(2, 9)}$= 539.7, R^2= 0.991) also showed a significant (p<0.0001) effect of temperature on esterase activity. There appeared to be relatively little variability within temperature treatments with the exception of 32°C data (Table 4.3b), and relatively low variability within independent cultures (data points) (Table 4.3b).

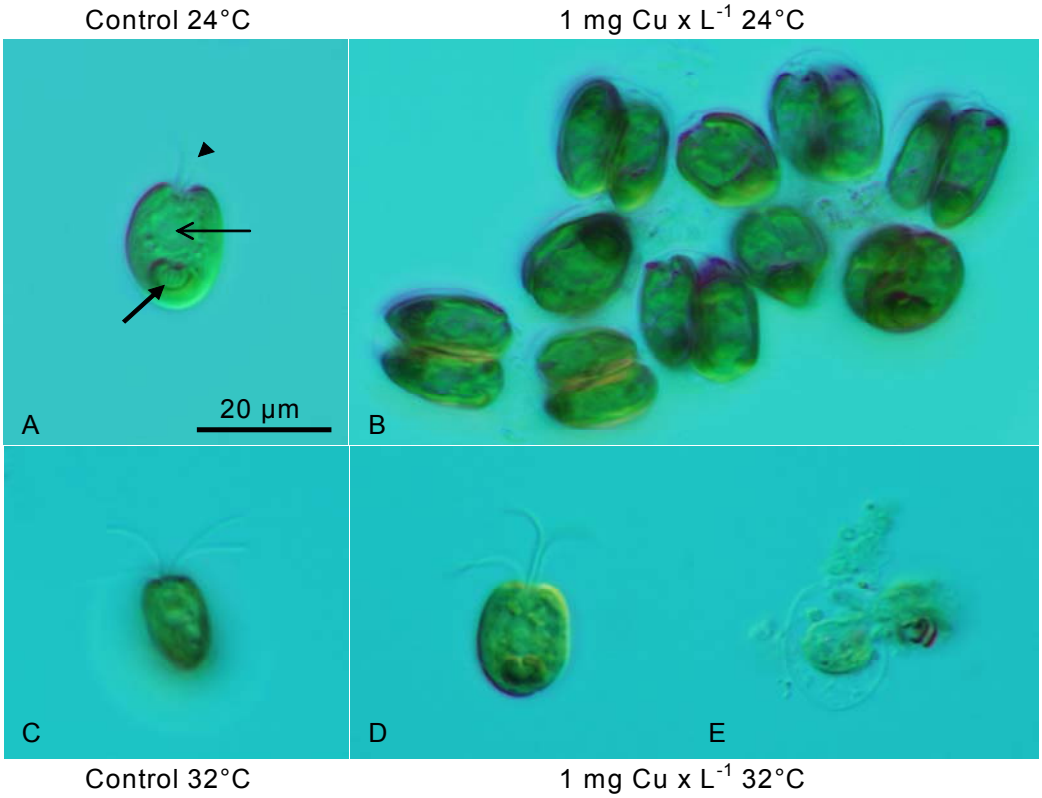
The influence of temperature (°C) on esterase activity in the presence of 1.00mg Cu L^{-1} (Figure 4.4f) displayed a similar, though weaker, (R^2= 0.856) quadratic relationship as in Figure 4.4e, again with data as 32°C being outliers. A one-way ANOVA with all data points ($F_{(4, 10)}$=34.96) showed a significant (p<0.0001) effect of temperature on esterase activity in the presence of 1.00mg Cu L^{-1}. Quadratic regression analysis of points between (and including) 16 to 28°C ($F_{(2, 9)}$=26.85) also indicated a significant (p=0.0146) relationship between temperature and inhibition of esterase activity. There appeared to be relatively low variability within treatments with the exception of the 32°C data (Table 4.3b), and little variability within independent cultures (Table 4.3b).

A comparison of the WIC (Figure 4.4a) and BIC protocol (Figure 4.4d) control data revealed very different data trends, with Figure 4.4a displaying a tight quadratic relationship with increasing temperature, and Figure 4.4d displaying no obvious trend. In the presence of Cu, Figures 4.4b and 4.4c (WIC protocol) show that as temperature increased, toxicity increased, which is similar to the data obtained in the BIC protocol (Figures 4.4e and 4.4f), though

this relationship only held for measurements of esterase activity between 16 and 28°C.

The effect of temperature in the presence of Cu on the morphology of *Tetraselmis* sp.

Microscopic analysis (Figures 4.5a-4.5e) was used to evaluate the effect of temperature (24°C- Figures 4.5a and 4.5b and 32°C- Figures 4.5c-4.5e) on the morphology of *Tetraselmis* sp. in control (Figures 4.5a and 4.5c) and Cu treatment (1.00mg Cu L^{-1}, Figures 4.5b, 4.5d, 4.5e). At 24°C, Cu inhibited (or slowed) cell division and cytokinesis for more than 75% of the analysed cells (Figure 4.5b). At 32°C, most cells exposed to Cu appeared to be healthy (Figure 4.5d), with only one out of 20 cells lysed (Figure 4.5e). Control cells at both temperatures (Figures 4.5a and 4.5c) appeared to have no morphological abnormalities.



Figures 4.5a-e: The effect of temperature and copper on the morphology of *Tetraselmis* sp. Figs. 4.5a & c show micrographs of control cells exposed to 24 and 32°C, respectively. Fig. 4.5b shows that copper at the nominated concentration inhibits or slows cell division and cytokinesis at 24°C for more than 75% of the analysed cells of the treatment. Figs. 4.5d & 4.5e show the effect of the nominated concentration of copper at 32°C. The arrows in Fig. 4.5a point to typical morphologically characteristic features of *Tetraselmis* sp.: the flagella (arrow head), the pyrenoid embedded in the cup-shaped chloroplast (thick arrow), and the nucleolus embedded in the nucleus (round translucent space) (open ended thin arrow). Scale bar is 20 µm and applies to all micrographs.

Table 4.4: Measurement of cell length for *Tetraselmis* sp.

	Control (24°C)	Cu (24°C)	Control (32°C)	Cu (32°C)
Mean cell length (µm)	19.92	19.96	20.08	19.96
Standard deviation	0.8954	0.3152	0.9322	0.6073
Standard error	0.2054	0.0723	0.2139	0.1393

Analysis of cell length as a proxy for cell size revealed no significant difference in mean cell length for any of the Cu treatments (Table 4.4). Average cell length was close to 20µm with little variation from the mean. Analysis of cell lengths via a one-way ANOVA statistically confirmed that there was no significant difference between treatments ($F_{(3,76)}$=0.18, p=0.910). A post-hoc analysis revealed that neither cell length of controls (p=0.900) nor Cu treatments (p<1.0) were significantly different from one another at either temperature regime, and cell lengths were not significantly different between controls and copper treatments (24°C p=0.998, 32°C p=0.955).

Discussion

The addition of toxic effluents may change bioassay parameters such as pH and salinity, which can impact upon metal speciation (Stumm and Morgan 1981, Sadiq 1992). Metal speciation is an important factor in bioassays, because the free ion concentration is believed to be a predictor of metal toxicity (Lage et al. 1996, Eriksen et al. 2001, and Paquin et al. 2002). The ability of a bioassay to effectively quantify the effect of a pollutant despite changes in environmental parameters will determine its usefulness to water quality monitoring. To simulate such environmental shifts that may affect the outcome of a bioassay, shock experiments regarding pH (2-10), salinity (25-45ppt) and temperature (16-32°C) were conducted on *Tetraselmis* sp. (Chlorophyta) in the absence (effect on esterase activity) and presence of copper (effect on Cu toxicity). Additionally, these experiments were conducted using two different protocols: one that tested the above effects within three independent cultures (WIC; following a standard bioassay design, as per Craig et al. 2003), and another which quantified these effects between 15 independent cultures (BIC; a situation likely experienced in the field or during site monitoring situations).

Evaluation of the effect of pH on esterase activity and Cu toxicity.

Increasing pH resulted in a rise of esterase-induced fluorescence (a.u.) in *Tetraselmis* sp. without the addition of copper, exhibiting similar trends in both the WIC and BIC protocols, with zero fluorescence recorded at pH 2 and 4. Increases in esterase activity (fluorescence) with increasing pH have been previously documented. Franklin et al. (2001) described rises in pH from 5.8-8.3 in freshwater, which increased the conversion of FDA to fluorescein by *Selenastrum capricornutum* (Chlorophyta); maximum fluorescence being observed at pH 7.8 and 8.0. In contrast, a rise of pH from 8.0 (approximate pH of seawater) to 9.5 was necessary to increase esterase activity from undetectable to easily measurable in the marine diatom *Phaeodactylum tricornutum* (Franklin et al. 2001). The authors suggested that increases in pH may increase membrane permeability to FDA, the substrate for esterase, in both seawater and freshwater.

Esterase activity as a function of pH was also evaluated in the marine diatom *Entomoneis* cf. *punctulata* (Adams and Stauber 2004). Using flow cytometry to measure FDA conversion to fluorescein, the number of "healthy" cells in a population was quantified. After a 24h exposure to varying pH levels on a relatively fine pH scale (6.0-8.5 at 0.5 pH intervals), no significant difference in the overall fluorescein fluorescence or number of healthy cells was observed at pHs ranging from 6.5-8.5. However, at pH 6.0, the *E.* cf. *punctulata* population exhibited significantly more "unhealthy" cells, and an overall lower fluorescein fluorescence intensity of healthy cells. The described sensitivities of esterase activity in two marine microalgae (Adams and Stauber 2004, marine diatom; and this study, marine chlorophyte) fits well with the pH ranges in normal (~8.5) and moderately polluted (6.0-8.0) marine environments (Regel et al. 2002).

The increase in esterase-induced fluorescein fluorescence concurrent with a rise in pH has been explained in two ways. Franklin et al. (2001) proposed that an increase in fluorescence due to rising pH may be the result of improved membrane permeability for the substrate FDA, resulting in the delivery of more substrate for the esterases and hence increased conversion of FDA to fluorescein, the fluorescent product of esterase activity. In contrast, Adams and Stauber (2004) suggested that marine algae may suffer at suboptimal pH levels

and become “unhealthy.” While the presented research cannot immediately differentiate between the two mechanisms, it is conceivable that marine microalgae could have adapted to the range of pH they typically encounter in their natural environments. Further research into the effects of pH on esterase activity in *Tetraselmis* sp. may include membrane permeability studies or ascertaining the pH optimum of the esterases, as they may be inhibited at acidic pH in marine organisms.

During a 24h exposure to 1.0mg Cu L^{-1}, *Tetraselmis* sp. displayed a similar pattern for both the WIC and BIC protocols. As esterase activity was not observed at pH 2 and 4, it is not surprising that Cu treatment yielded no effect at these pH levels. However, at pH 6, there is a consistently high (80%) percent inhibition of fluorescence, a measure of esterase activity, and less inhibition at pH 10. At pH 8, however, there is a divergence in this pattern, with the WIC protocol displaying 25% inhibition and the BIC protocol showing activation of esterase activity (20% negative inhibition). The results may reflect the inherent variability of seawater collected from the Townsville region, because different batches of seawater were used for the WIC and BIC protocols. It is possible that an unforseen component in the seawater influenced the results. Accounting for the difference at pH 8, the pattern of inhibition at pH 10 is remarkably similar between the two protocols. Excluding copper toxicity data at pH 8 between the two protocols, it can be concluded that esterase activity in *Tetraselmis* sp. appeared to be restricted to pH levels above 6.0, and may not be useful for marine bioassay that test the effects of extremely acidic effluents.

pH has been called the “master variable” in controlling metal toxicity due to its influence on metal speciation and biological surface adsorption (Nalewajko and Olaveson 1998). In this chapter, Cu was consistently more toxic to esterase activity in *Tetraselmis* sp. at pH 6, and less toxic at pH 8 and 10, which is in line with current paradigms regarding metal toxicity and speciation. An increase in toxicity with decreases in pH has been attributed to an increase in free metal ions (Baker et al. 1983, Starodub et al. 1987, DePhilippis et al. 2003), the most biotoxic form of metal (Lage et al. 1996, Eriksen et al. 2001, Paquin et al. 2002). Villaescusa et al. (1997) described increasing Cr(VI) toxicity to luminescent bacteria (using the Microtox® bioassay) with a decrease in pH (9.3-4.6), and attributed this relationship to availability of Cr species. Similarly, Ho et al. (1999)

saw an increase in Cu toxicity to the bacterium *Vibrio fischerii* (also using the Microtox® bioassay) as pH decreased. In contrast, the same authors described an opposite effect of pH on Pb, Ni, Cd, and Zn toxicity to *V. fischerii*, which cannot be explained by current metal bioavailability models (such as the free ion availability model, Campbell (1995)). They resolved this conflict by describing the relationship between metal toxicity and pH as "metal and organism specific," a sentiment shared by Nalewajko and Olaveson (1998). Franklin et al. (2000) attributed a decrease in toxicity at lower pH to competition for biologically significant binding sites between H^+ and metal ions. Franklin et al. (2000) saw that a decrease in pH from 6.5 to 5.7 resulted in decreased Cu toxicity to growth rate (cell divisions) in *Chlorella* sp. (Chlorophyta) (72h EC_{50} pH 6.5=1.5µg Cu L^{-1}, pH 5.7=35 µg Cu L^{-1}). They also measured intracellular and cell-bound Cu, and found lower concentrations of cell-associated Cu at pH 5.7, providing evidence for their hypothesis that Cu and H^+ ions may compete for cellular binding sites.

Microalgal cell walls (or thecae) may be an equally important factor in determining metal toxicity. The theca of *Tetraselmis* sp. is mainly composed of acidic polysaccharides (Becker et al. 1998). DePhilippis et al. (2003) assessed the metal removal capacity of two acidic polysaccharide-encapsulated cyanobacteria *Cyanospora capsulata* and *Nostoc* PCC7936 in media of varying pH, and observed a strong complexing capacity of these acidic polysaccharides for free Cu ions at pH 6.1-6.2. Above pH 7, the number of free Cu ions decreased in media with and without organisms, attributing the decline in Cu concentration without algae to media complexation (DePhilippis et al. 2003). It is possible that at pH 6, *Tetraselmis* sp. was also able to attract and bind more free Cu ions from the media, similar to *C. capsulata* and *Nostoc*. However, at present this statement cannot be substantiated and should be investigated in future research by characterising the amount and location of extracellularly bound and intracellular Cu.

In conclusion, fluorescein fluorescence (a.u.) increased significantly with increasing pH in *Tetraselmis* sp., and 1.00mg Cu L^{-1} is most toxic at pH 6 in the WIC and BIC protocols. The observed increase in toxicity of Cu with decreasing pH may be due to the increased bioavailability of free Cu ions at those pH conditions. However, exposure to the additional stress of surviving outside

optimal pH may also contribute to a higher percent inhibition of esterase activity in *Tetraselmis* sp. Future research may focus upon discerning the amount of copper bound to hydroxide (OH^-) ions through computer-generated modelling systems (Gustafsson et al. 1995). pH modelling is advantageous because it mathematically predicts the relationships of media components and can quantify precipitation and complexation in the system (Gustafsson et al. 1995), thereby providing evidence for hypotheses about metal toxicity to microalgae. EDTA (ethylene dinitrilotetraacetic acid) is essential for delivering iron (a critical microalgal nutrient) to marine microalgae, and is present in Guillard's f/2 medium ($1x10^{-5}$M $Na_2EDTA●2H_2O$, Guillard and Ryther 1962), which was used in the presented research. However, EDTA may also decrease bioavailable Cu in aquatic systems (James et al. 1998), which may compromise metal toxicity studies. It was unadvisable, in this research, to remove EDTA from the growth medium because it is critical for microalgal iron nutrition and culture health, and toxicity results may actually reflect iron limitation resulting in unhealthy/stressed cells rather than the effects of copper (K. Heimann, pers. comm.).

The differences between the WIC and BIC protocols (at pH 8 and 10 for the effect of pH on esterase-induced fluorescein fluorescence, and at pH 8 for copper toxicity) might be attributed to small sample size, in which case further larger-scale studies could prove useful. It could also be possible that the WIC protocol data, a finer spatial scale, might be elucidating the natural variability within a microalgal population of the same species. Pouneva (1997) stained cultures of *Anabaena variabilis* and *Synechocystis miniscula* (Cyanophyceae), and *Chlorella pyrenoidosa, Scenedesmus acuminatus, Coelastrum cambricum*, and *Nephrochloris salina* (Chlorophyceae) with fluorescein diacetate (FDA) under standard conditions, observing, via epifluorescent microscopy, a difference in fluorescence between cells within the same population and species. Using flow cytometry, it was also possible to examine the variability of FDA fluorescence between cells in a population of the same microalgal species, which has been demonstrated to change according to environmental conditions (Adams and Stauber 2004). An additional consideration when designing pH variability experiments with microalgae is the possibility that abiotic and biotic factors (such as depletion of CO_2 or assimilation of NO_3^-) may influence pH level due to the degree of air equilibriation in the medium (Beardall, pers.

comm.). The latter may have been demonstrated in Figure 4.2a, where a slight drop at pH 10 occurred in medium without organisms. However, these experiments were conducted using autoclaved Erlenmeyer flasks with Steri-stoppers to allow for sterile air exchange, thus negating most of the effects of abiotic equilibrium factors. In the study presented, it could not be conclusively determined whether the variability between protocols was due to the inherent variability within a population of algae, sample size, or seawater batch, though future studies designed to address these questions may resolve the issue.

Evaluation of the effect of salinity on esterase activity and Cu toxicity.

A 24-hour salinity shock experiment revealed no significant effect of this environmental parameter on esterase activity (fluorescein fluorescence) in *Tetraselmis* sp. for both the BIC and WIC protocols. This is consistent with results obtained by Adams and Stauber (2004) who measured fluorescein fluorescence intensity in the diatom *Entomoneis* cf. *punctulata* after a 24h salinity (5-35ppt) exposure. In contrast, Minier et al. (1993) observed an effect of salinity (15-45ppt) on esterase activity (fluorescence units min^{-1}) in *Calothrix* PCC 7601 (Cyanophyceae), *Haslea ostrearia* (Bacillariophyceae) and *Prorocentrum micans* (Dinophyceae). However, their data were not formally statistically analysed. Examination of the graphs presented in their publication revealed a slight quadratic relationship between salinity and esterase activity, similar to the relationship between salinity and esterase activity presented in this chapter. Minier et al. (1993) described the highest esterase activity at salinity levels that the organisms were originally cultured in, which may possibly hint to species-specific salinity optima for esterase activity.

There is no conclusive reason why esterase activity was relatively unaffected by fluctuations in salinity, though some *Tetraselmis* species are known to be halotolerant (Erga et al. 2003, Strizh et al. 2004). Sodium ion transport system characteristics are considered to be the most important determinations of salt resistance (Balnokin et al. 1997). Strizh et al. (2004) identified an increase in *Tetraselmis viridis* Na^+-ATPase, responsible for Na^+ export from the cell, when the alga was exposed to increasing levels (0.58-70ppt) of salinity. High salinity levels also resulted in expression of previously undescribed Na^+-ATPase isoforms (Strizh et al. 2004). A study on esterase

isozyme expression in the peanut, *Arachis hypogaea* L, known for its halo-tolerance, revealed the expression of nine different esterase isozymes at high salinity (6.09ppt), whereas at control levels, only five esterase isozymes were detectable (Hassanein 1999). However, from the Hassanein et al. (1999) study, in cannot be concluded if the high-salinity esterase isozymes directly assist with *A. hypogaea* L. survival in saline conditions. Similar studies have not been conducted in *Tetraselmis* sp., but increased numbers of esterase isoforms, if detected and experimentally analysed in reference to an assay endpoint, may provide an adaptive strategy to high-salinity environments.

Copper exists in many forms in seawater, and the complexity of the interactions between the ionic form of the metal and organic and inorganic ligands is evidenced by the lack of agreement on these relationships between studies (Sadiq 1992). Traditionally, it is hypothesised that ionic copper is less toxic at higher salinities because it readily complexes with chloride, thereby becoming less bioavailable (McLusky et al. 1986, Craig et al. 2003) and because it must compete with cations in the seawater for binding sites on the surface of the organism (McLusky et al. 1986). A review by Hall and Anderson (1995) of 173 publications investigating the toxicity of various chemicals in relation to salinity resulted in negative correlations (toxicity increased with decreased salinity) for 55%, no correlation between salinity and toxicity (27%), and positive correlations in 18% of the studies. Most of studies involving metals reported increasing toxicity with decreasing salinity, further supporting the hypothesis of McLusky et al. (1986). Like the effects of pH on toxicity, the effect of salinity is thought to be related to the physiology, ecology, and life history of the species, which confounds efforts to produce one predictive model of toxicity (Hall and Anderson 1995).

The results presented in this study on *Tetraselmis* sp*.* conflict with the McLusky et al. (1986) hypothesis. The highest percent inhibition appeared to occur at control (culturing-35ppt) salinity levels in both the WIC and BIC protocols. The data obtained in this study are, however, in line with other studies also describing an insignificant effect of salinity on metal toxicity in microalgae. Latala and Surosz (1998) documented an inhibition of growth in populations of the freshwater Chlorophyceae *Chlorella vulgaris, Oocystis submarina, Scenedesmus armatus,* and *Stichococcus bacillaris* in response to

Cd, Co, Cu, and Mn at control salinity levels. Increasing salinity (0.15-9ppm) did not correlate with metal toxicity. Similarly, Craig et al. (2003) observed no significant correlation between percent inhibition of bioluminescence by Cu (0.113 mg L^{-1}) and salinity (17-48ppt) in a marine 4-hour *Pyrocystis lunula* (Dinophyceae) bioassay.

It is unclear whether the differences between the WIC and BIC protocols were due to sample size or population heterogeneity, though the results suggested that the *Tetraselmis* sp. used in this study can survive in a broad range of environmental parameters and high copper concentrations.

Evaluation of the effect of temperature on esterase activity and Cu toxicity.

It is well known that esterase activity and copper toxicity (within limits) exhibit positive relationships with temperature due to increased metabolic activity (Mehta et al. 2002). The effect of temperature on esterase activity in *Tetraselmis* sp. was significant, and followed a positive correlation between increases in temperature and esterase activity-induced fluorescein fluorescence, which was also described by Minier et al. (1993) for *Calothrix* PCC 7601 (Cyanophyceae) *Haslea ostrearia* (Bacillariophyceae), and *Prorocentrum micans* (Dinophyceae). A significant increase in esterase activity was observed for all species from 10-16°C, and at 20°C, the cyanobacterium and the diatom showed no (or a small decline) in esterase activity, while the dinoflagellate exhibited a dramatic increase. Thus esterase activity in response to temperature appears to be species-specific.

Traditionally, higher temperatures are thought to increase the biotoxicity of metals (Rao and Khan 2000, Balu et al. 2001, Mehta et al. 2002) because: (1) as temperature increases, metabolic activity increases and transport of metals across the cell membrane is more rapid (Mehta et al. 2002), (2) metal adsorption is thought to be an exothermic reaction and higher temperatures might increase the desorption of metals from particles and ligands increasing bioavailability (Rao and Khan 2000), (3) changes in temperature have been shown to affect the function of biological surface ligands (Gonzalez-Davila et al. 1995) and (4) it has been shown that organisms are physically stressed at temperatures outside their physical optima, which may compound the effects of the pollutant (Rao and Khan 2000, Balu et al. 2001, Craig et al. 2003).

Consistent with the above expectations, the percent inhibition of fluorescence by 0.500 and 1.00mg Cu L^{-1} in *Tetraselmis* sp. increased as temperature increased. With the exception of results obtained at 32°C in the BIC protocol, results presented in this chapter additionally support the hypothesis that a rise in temperature increases metal toxicity, probably due to a combination of the above reasons (1-4).

Much of the work investigating toxicity of metals in relation to temperature has focused on marine invertebrates and fish, with results obtained in accordance to the above expectations (Rao and Khan 2000, Balu et al. 2001). However, there are some studies on microalgae which appear to be in contradiction. Craig et al. (2003) examined the effect of temperature (10-30°C) on the percent inhibition of bioluminescence by Cu (0.229 and 0.457mg L^{-1}) in *Pyrocystis lunula*. They observed a significant effect only at the lowest temperature, attributing the increase in toxicity to a compounding effect of stress. Similarly, Issa et al. (1998) described a significant effect of Cd on the growth of *Scenedesmus obliquus* (Chlorophyta) at 10°C, but not 40°C. Adsorption kinetic studies with *Dunaliella tertiolecta* (Chlorophyta) (Gonzalez-Davila et al. 1995) and *Chlorella vulgaris* (Chlorophyceae) (Mehta et al. 2002) revealed an increase in Cu surface adsorption with increasing temperature, which may or may not translate to increased toxicity. Regardless of the mechanism of toxicity, the finding that increased temperature enhanced Cu toxicity in the microalga *Tetraselmis* sp. suggests that metal toxicity in microalgae in relation to temperature could be species-specific and aligned with particular temperature optima, which needs to be accounted for in bioassay designs.

As with pH and salinity, the variability between the WIC and BIC protocols could be due to population heterogeneity or sample size. However, an additional variable was introduced into the WIC protocol. Due to infrastructure constraints, the 24h effects of only one temperature at a time could be recorded, resulting in progressively older cultures (7-18 days) to be used in the temperature experiments. Although cultures were diluted to the same initial inoculation density for each temperature treatment, it cannot be ruled out that culture age affected the outcome of the bioassay, which may also explain the high variability at 32°C (using 18-day old cultures).

Pane et al. (2001) observed a significant difference in the biomolecular profile of *Tetraselmis suecica* in cultures of different ages. Protein content was significantly different between the exponential (9-14 days old), static (16-23 days old), and senescent (26-28 days old) culture phases, and additionally, the timing of culture phases were different between cultures grown in 14 or 18°C. In 14°C, *T. suecica* exhibited a lag phase of 5 days, with maximum culture density ($2.43x10^6$ cells mL^{-1}) occurring at 21 days. In 18°C, the cultures had a shorter lag phase (2 days) before entering exponential growth, and attained a maximum density of $1.94x10^6$ cells mL^{-1} at 9 days. It is possible that *T. suecica* exploited nutrients faster at the higher temperature, and the culture became nutrient limited more rapidly, which in turn impacted on the biomolecular profile. Pane et al. (2001) concluded that thermostability of *T. suecica* varies with age and incubation temperature due to changes in the protein profile. Potential differences in culture thermostability and/or protein content may thus partially explain the difference between the WIC and BIC protocols.

Culture age also may have an effect on the toxicity of Cu. Mehta et al. (2002) studied the effect of *Chlorella vulgaris* culture age (8, 16, 32 days) on adsorption of Cu at concentrations between 0.64-6.40mg Cu L^{-1}. The older (16 and 32 days) cultures had significantly higher adsorption levels (1.0-2.0 fmol $cell^{-1}$) across Cu concentrations than the 8-day old cultures. Mehta et al. (2002) suggested that the increased adsorption capacity of older cultures of *C. vulgaris* may be due to new and/or additional binding sites on mature cells. There has been no similar study conducted with *Tetraselmis* sp., though increased binding sites in older cultures may explain the differences in Cu toxicity at 32°C between the WIC (18 day old cultures) and BIC (7 day old cultures) protocols. An increase in Cu adsorption with culture age may explain the increase in Cu toxicity at 32°C and hence increased Cu uptake, though this should be confirmed in future studies.

Visual inspection of *Tetraselmis* sp. did not reveal any detectable morphological differences between organisms grown at 24 or 32°C without copper. However, there was an obvious difference between control (no Cu) and treatment (1.0mg Cu L^{-1}) organisms after incubation at 24°C; 75% of observed cells exposed to Cu displayed an arrest after cell division before exiting the mother theca. Nassiri et al. (1996) also documented arrested culture growth in

Tetraselmis suecica exposed to increasing (0.05-1.0 mg L^{-1}) Cu concentrations. As Cu in the media increased, the number of days per cell cycle also increased exponentially, with growth cessation at 1.0mg Cu L^{-1}. Similarly, Franklin et al. (2001) found the 48h EC_{50} for *Tetraselmis* sp. growth to be 83μg Cu L^{-1}, and the 72h EC_{50} was 146μg Cu L^{-1}. It is also worth noting that, in the above study, *Tetraselmis* sp. was significantly more tolerant to Cu at 48 and 72h than the other five organisms examined (*Chlorella* sp. and *Selenastrum capricornutum* (Chlorophyceae), *Phaeodactylum tricornutum, Entomoneis* cf *punctulata, and Nitzschia* cf *paleacea* (Bacillariophyceae)) in the Franklin et al. (2001) study. It is likely that high Cu concentrations slow cellular division or "hatching" (exit from the mother theca) in *Tetraselmis* sp. at 24°C, though in comparison to other organisms (Franklin et al. 2001) is copper-tolerant. It is, however, interesting that high (32°C) temperature in the presence of Cu did not seem to impact on the morphology, cell division and/or post-cytokinesis "hatching" of *Tetraselmis* sp. This suggests that in varying temperature regimes Cu may influence alternative metabolic processes in microalgae, which leads to fascinating future research.

In conclusion, temperature significantly affected esterase activity and Cu toxicity in *Tetraselmis* sp., possibly due to a combination of factors. There was a difference in the results obtained from the WIC and BIC protocols, which may be explainable by culture age. Despite this, esterase activity was observed over a wide range of temperatures, with and without Cu in *Tetraselmis* sp. The hardiness of this alga in the face of high levels of Cu and varying environmental conditions (pH, salinity, temperature) may render it an ideal candidate for metal bioremediation (Malik 2004). Future research could focus upon uptake kinetics and intracellular storage of Cu to elucidate detoxification mechanisms of this organism for copper, which will assist in making an informed decision about the organism's potential in copper bioremediation.

Chapter 5

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

Increased anthropogenic pressure on marine ecosystems necessitates methods to quantify the toxic effects of pollutants on marine organisms. As vital primary producers and coral reef-associated organisms in the tropical marine environment, microalgae communities may be directly impacted by pollution. In North Queensland, Australia, copper is mined and shipped in vast quantities and used as a component of antifouling paints on vessels that regularly trade in the Great Barrier Reef World Heritage Area (GBRWHA). Unacceptably high levels of copper have been measured in some near-shore environments and at ship-grounding sites within the GBRWHA. Therefore, the presence of copper at toxic levels necessitates the development of early-warning systems of copper pollution in marine microalgae.

This thesis directly addressed the need for the detection of copper pollution via bioassays by investigating the effects of copper on the sublethal endpoint esterase activity in three marine microalgae: *Tetraselmis* sp. (Chlorophyceae), *Chaetoceros gracilis* (Heterokontophyceae) and *Symbiodinium microadriaticum* (Dinophyceae). Using *Tetraselmis* sp. and *C. gracilis* as test organisms, the effect of experimental design on esterase activity (using two different protocols) was determined. This research is important because it highlights the impact of experimental design and protocol standardisation on the results and therefore interpretation of pollution studies, which is critical for developing science-based legislation. The results of these experiments demonstrated that experimental design could have a profound effect upon esterase activity: the flask bioassay showed no consistent effect of Cu on esterase activity, whereas the microtitre plate bioassay (using the same species of microalgae) displayed a significant and reproducible dose-dependent effect of Cu on esterase activity. Future research should focus upon standardisation of bioassay protocols such as culture age, bioassay volume, and the potential of free copper ions to bind to untreated culture ware and reduce overall bioavailability. Inductively coupled plasma optical emission spectrometry, however, revealed no binding of Cu to glassware over time in the flask bioassay, though ICP-OES does not shed light upon potential copper complexation by components of the culture medium, i.e. the effect of EDTA on Cu availability. This, in conjunction with the effects of omission of EDTA from

the culture medium on culture health and toxicity test outcome, is an important point of consideration for future metal toxicity studies.

In addition to the sublethal endpoint esterase activity, the effect of copper on culture growth was examined in *Tetraselmis* sp. and *C. gracilis*. Both microalgae displayed a dose-dependent response of culture growth to Cu, though only the *C. gracilis* data was statistically significant. This experiment highlighted the various potential tolerance mechanisms to Cu between different species of microalgae. Therefore, employing a battery of microalgae from diverse phyla is essential to assess the effect of toxic levels of metals on these primary producers. Additionally, the impact of low levels of light and toxic Cu concentrations on the culture growth of *C. gracilis* was examined. There was a dramatically different response of culture growth to Cu between *C. gracilis* in low and high light levels, which further underpins the importance of standardising bioassay methodology. It is also advantageous to employ multiple endpoints when assessing the effects of a pollutant on microalgae, because different endpoints are likely to vary in sensitivity. The microtitre plate bioassay successfully provided a dose-dependent response of esterase activity to Cu toxicity, which appeared to be a more sensitive endpoint than culture growth in both *Tetraselmis* sp. and *C. gracilis*.

The use of sublethal endpoints in toxicity testing is important to provide an early-warning assessment of metal toxicity to microalgae, and the microtitre plate bioassay for Cu toxicity to esterase activity fulfills this need for *Tetraselmis* sp. and *C. gracilis*. However, not all microalgae are suitable for use in the microtitre plate bioassay by virtue of their unique physiologies. *Symbiodinium microadriaticum* is a symbiotic dinoflagellate isolated from a Heron Island (GBRWHA) giant clam *Tridacna maximum*, and as such is essential to reef health. Some *S. microadriaticum* strains established in long-term cultures are highly adhesive, and thus quantification of cell density is extremely challenging. Little research has been published using *Symbiodinium* species as bioassay test organisms, likely because of their adhesive properties, which do not lend themselves to accurate quantification of initial inoculation density; a necessary component of toxicity bioassays. In this thesis, *S. microadriaticum* cell mass was quantified using protein content, chlorophyll *a* autofluorescence, and cell counts on a haemocytometer. Despite the relative success of chlorophyll *a*

autofluorescence in quantifying cell mass, this did not translate into a consistently repeatable bioassay for Cu toxicity to esterase activity using the microtitre plate protocol. Most likely, this was due to the differential adhesion of cells to each other and culture materials, which resulted in inconsistent cell numbers between and within experiments. Despite the results presented in this chapter, the importance of *Symbiodinium* in tropical reef ecosystems demands their use in bioassays for metal toxicity. Future work should focus upon measurement of the effects of toxic levels of metals *in situ*, or upon the development of bioassays that specifically account for the unique adhesive properties of these microalgae.

The addition of toxic effluents to a marine environment can dramatically alter salinity, pH, and/or temperature. It is vital to account for these changing environmental parameters when designing bioassays for metal toxicity because pH and salinity can affect free ion content, assumed to be the most toxic form of metal. Temperature can significantly alter membrane permeability and physiological processes within a cell, which can also impact upon overall metal toxicity to an organism. This thesis addressed these issues by exposing *Tetraselmis* sp. to toxic levels of Cu in addition to changing salinity, pH, and temperature, and fluorometrically quantifying the resulting effects on baseline esterase activity and copper toxicity using the microtitre plate bioassay. This research also explored the impact of experimental design on esterase activity and copper toxicity to esterase activity (within independent cultures; WIC vs. between independent cultures; BIC). In general, pH and temperature had a significant effect on both esterase activity and Cu toxicity in both experimental designs, and salinity generally did not affect Cu toxicity to esterase activity. The effect of culture did not have a consistently significant effect on either control esterase activity 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, an important conclusion of this thesis. It also revealed that *Tetraselmis* sp. may be a suitable candidate for bioremediation of copper in marine and estuarine waters due to its resistance to high levels of Cu (1.0mg Cu L^{-1}) as expressed by its stable esterase activity during Cu exposure even under changing pH, salinity, and temperature regimes.

The use of microalgae in bioassays for metal toxicity is advantageous because these organisms facilitate a high level of experimental replication and provide opportunities for miniaturisation and therefore cost-effectiveness. However, more emphasis must be placed upon standardisation of bioassay protocols in order to effectively implement science-based legislation. The increased levels of anthropogenic pollution in marine environments and the importance of microalgae as primary producers necessitate carefully planned future research.

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