The effects of treated sewage effluent on three species of marine macroalgae

A thesis submitted for the degree of Doctor of Philosophy

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June 2004

Declaration

This thesis is submitted in accordance with the regulations of Victoria University in fulfillment of the requirements for the degree of Doctor of Philosophy. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and no material previously published or written by another person except where duly acknowledged or referenced.

Mary-Anne Shir

June 2004

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Acknowledgements

This thesis would not have been possible without the encouragement and assistance of many people. I am sincerely grateful to the following:

Dr Trevor Burridge for instigating my interest in marine ecotoxicology and motivating me throughout my candidature.

My fellow research students who included Stuart Campbell, Juanita Bite and Sue Pascoe for their support and encouragement. Thanks also to all the laboratory technicians over the years who offered constant assistance.

Jenny Stauber and fellow staff of CSIRO who provided funding for part of my research and gave invaluable feedback on manuscripts.

Victoria University for financial support through an Australian Postgraduate Award Scholarship.

Timothy Lavery for all his patience, love and support.

My family for all their understanding.

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List of Abbreviations and Definitions

ANOVA	Analysis of variance.
Antheridia	Male reproductive organs containing sperm.
Dioecious	Having male and female gametes produced on separate plants.
Diploid	Nucleus containing two sets of chromosomes (2N).
EC_{50}	Effective concentration of toxicant which causes an adverse effect in 50% of a test population.
Embryo	Young multicellular organism, derived from a zygote or spore.
Gamete	A haploid reproductive cell capable of uniting with another such cell to form a diploid zygote.
Gametophyte	Multicellular sexual, gamete-producing phase of the life history of a plant.
Germination	First outward sign of morphogenesis, when a protuberance emerges from the zygote or spore and elongates to form the primary rhizoid.
Haploid	Life history phase of a plant where the nuclei contain a single set of chromosomes.
LC_{50}	Lethal concentration which causes the onset of mortality in 50% of a test population.

LOEC	Lowest observable effect concentration, which causes a statistically significant adverse effect to the test population on the observed parameters.
NOEC	No observable effect concentration, which causes no statistically significant adverse effect to the test population on the observed parameters.
Oogamous	Reproduction involving large non-motile eggs and small motile sperm.
Oogonia	Female reproductive organs, each containing one or more eggs.
Spore	Reproductive cell derived by mitosis or meiosis.
Sporophyte	Diploid (2N) spore-producing multicellular phase of a life history.
Zoospore	Motile reproductive cell not involved in sexual reproduction.
Zygote	Diploid cell resulting from fusion of gametes.

List of Publications and Conference Presentations

The Comparative Effects of Oil and Oil/Dispersant Conjugates on Germination of the Marine Macroalga *Phyllospora comosa* (Fucales: Phaeophyta). *Marine Pollution Bulletin,* **31**, 446-452, 1995.

The Effects of Sewage Effluent on The Early Life Stages of Three Species of Marine Macroalgae. Technical Report. *CSIRO*, August, 1998.

Annual Conference of the Australasian Society for Ecotoxicology. Sydney, June, 1995. *The Effects of Oil and Dispersed Oil on Mortality and Growth of the Marine Macroalga Phyllospora comosa.*

Annual Conference of the Australasian Society for Ecotoxicology. Sydney, June,1996. *The Effects of Oil and Dispersants on Germination of Phyllospora comosa.*

13th Annual Conference of the Australasian Society for Phycology and Aquatic Botany. Hobart, January, 1997. *The Effects of Sewage Effluent on Germination and Growth of Brown Marine Macrophytes.*

2nd International Conference on Marine Pollution and Ecotoxicology. Hong Kong, June, 1998. *The Effects of Sewage Effluent on the Early Life Stages of Three Species of Marine Macroalgae.*

Abstract

The early life stages of the macroalgal species *Phyllospora comosa, Hormosira banksii* and *Macrocystis angustifolia* were exposed to treatments of sewage effluent, copper, chromium and reduced salinity. Germination and mortality of *P. comosa* and *H. banksii* zygotes and embryos, and germination and germination tube growth of *M. angustifolia* spores were utilised as acute toxicity test endpoints. Germination bioassays were of 48 hours duration whilst mortality bioassays were conducted over 96 hours. Growth of all three species and sporophyte production of *M. angustifolia* were utilised as chronic bioassay endpoints. These tests were conducted over 2-3 weeks. It was found that sensitivity of test end points varied depending on exposure time. Generally longer exposure times resulted in significantly lower threshold concentrations. Growth and sporophyte production or reproduction bioassays showed greater sensitivity in the test species, whilst the shorter tests tended to produce higher threshold concentrations. Primary treated effluent exerted greatest effects on the early life stages of the algae followed by chlorinated secondary and secondary treated effluent.

Treatment of sewage effluent significantly reduced toxicity with primary treated effluent consistently exerting greater toxicity. It was found that concentrations of primary treated effluent ranging between 5-15% significantly inhibited zygote and zoospore germination by 50% in all three species. Embryo mortality of 50% of the test populations of *H. banksii* and *P. comosa* were recorded at concentrations ranging between 5-25% primary treated effluent. Threshold concentrations for these assays tended to be lower than those recorded for germination assays and could be attributed to longer exposure times.

Growth rates of *P. comosa* and *H. banksii* embryos exposed to treated effluent were recorded at one and two weeks and results suggested that prolonged exposure significantly affected the development of embryos. Sporophyte production of *M. angustifolia* was assessed after 19-20 days of exposure to the effluent treatments (primary, secondary and chlorinated secondary) and it was found that low concentrations

(2.5-5% effluent) of all three effluent samples significantly inhibited reproduction (sporophyte development and production).

Copper chloride and hexavalent chromium (nominal concentrations diluted in seawater) were employed as the reference toxicants for this study and salinity reduction assays were employed to determine the effects of seawater dilution on the early life stages of the three species. The most sensitive assay endpoints were the two week growth tests for *H. banksii* and *P. comosa*, and sporophyte production for *M. angustifolia*. Further work also concentrated on investigating the effects of effluent treatments at the ultrastructural level. To compliment toxicity test protocol adopted for this study, bioluminescence inhibition assays were also employed to provide a biological reference.

Electron microscopy studies revealed that exposure to toxicants and effluents may cause disruptions in chloroplast development in young plants. There was also evidence of reduced numbers of physodes and Golgi-derived vesicles in young *P. comosa* and interrupted physode development in *H. banksii* embryos cultured in treated effluent. Of particular concern were the effects treatment solutions exerted on cellular membrane health and development. Scanning electron microscopy revealed gross deformities and stunted development in all three species of algae after exposure to varying treatments of sewage effluent.

Sewage effluent has the ability to limit viability of macroalgal populations in coastal environments subject to discharge. This is an outcome of significant environmental relevance and of particular concern. This research study has emphasised that reduced salinity and the presence of toxicants in discharge effluent exert significant negative effects on the early life stages of macroalgal development. The data collected in this thesis may be used to determine potential effects of sewage discharge in local marine environments. The toxicity test endpoints employed may also be used to compliment existing monitoring programmes established in areas receiving large volumes of effluent.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

An erroneous assumption that aquatic environments can absorb and dilute high volumes of wastes due simply to the copious amounts of water held within has long existed and has led to large-scale pollutant contamination of many coastal environments. Wastewater or sewage effluent, discharged into these environments consists of water, dissolved or suspended solids and chemical and biological matter, and has been linked to causing toxic effects in many plant and animal species (Borowitzka 1972, Vidaković 1983, May 1985, Tewari & Joshi 1988, Brown *et al.* 1990). The fate of sewage effluent upon entering the aquatic environment and the toxicological effects on benthic organisms is an area of research that has seen some significant findings in recent times, but an area that has been neglected in Australia (Burridge *et al.* 1995a, 1995b, Burridge *et al.* 1996, Shir & Burridge 1998, Bidwell *et al.* 1998, Burridge *et al.* 1999, Doblin & Clayton 1995).

1.1.1 Effluent discharge

Organisms inhabiting coastal marine environments are subject to the effects of a variety of pollutants such as sewage and industrial effluents, crude oil and accidental chemical discharges. Domestic sewage and industrial effluents have been regarded as the most common sources of aquatic pollution (Tripathi & Shukla 1991), and concern has been expressed over the effects of these types of discharge on marine ecosystems (Plusquellec *et al.* 1991, Anderlini & Wear 1992, Costello & Gamble 1992, Bothner *et al.* 1994). Of major concern is the significant impact that sewage discharge may have on the structure and diversity of aquatic communities in the receiving waters (Fletcher 1974, Littler & Murray 1975, Tewari & Joshi 1988, Brown *et al.* 1990, Seager & Abrahams 1990).

Monitoring studies have shown that negative impacts of sewage outfalls on receiving water quality are significant and can have detrimental effects on surrounding plant and

animal communities (Scanes & Philip 1995, Otway *et al.* 1996). There is also evidence indicating that the effects of sewage can be drastically altered once the effluent enters the marine environment, and that modifications of compounds in complex effluents and conditions of reduced salinity can alter the effects of the discharge (McLean *et al.* 1991, Bridges *et al.* 1994). Effects of sewage pollution on marine biota occur at the cellular, individual, population and community levels of organisation however, the vast majority of effects have been documented at the community level only (Underwood & Peterson 1988, Otway *et al.* 1996). The need for ecotoxicological research in conjunction with monitoring programmes has become essential to ensure that sewage effluent effects can be anticipated before discharge occurs, but also to attempt to minimise any irreversible effects to coastal marine environments. Monitoring programmes can provide information on changes in macroalgal, macroinvertebrate and infaunal communities within the vicinity of an effluent plume and surrounding areas (Fagan *et al.* 1992).

1.1.2 Toxicity testing

A paucity of information exists on the specific effects of sewage effluent on plant and animal species endemic to Australian waters (Richardson & Martin 1994, Martin & Richardson 1995, Burridge *et al.* 1996, Burridge *et al.* 1999). Sewage effluent is a complex mixture of components consisting of industrial, agricultural and domestic discharge (Miller 1990). The constituents of effluent discharges have been linked to causing poor water quality, habitat disruption and significant ecological impacts in aquatic environments (Bascom 1982, Swartz *et al.* 1986, Pols 1989, Seager & Abrahams 1990, Bothner *et al.* 1994). Investigations have stressed the importance of monitoring and effective effluent management to minimise significant environmental impacts (Bascom 1982, Swartz *et al.* 1986, Whitelaw & Solbé 1989, Fagan *et al.* 1992), but greater focus needs to be directed towards ecotoxicological testing. There is little doubt about the usefulness of toxicity testing or toxicity data as complementary tools to routine environmental monitoring and assessment programmes (Chapman & Long 1983, Botterweg & Risselada 1993). The information gained from toxicity testing can be used to determine acceptable levels of discharge leading to effective management (Alloway &

Ayres 1993) via the a-priori prediction of effects of the effluent on test species. Monitoring programmes can then be implemented to ensure that prolonged periods of effluent discharge do not cause deleterious effects to marine organisms and their surrounding environments.

Toxicity tests are routinely employed to evaluate the concentration of a chemical and the duration of exposure required to produce a specific effect (Chapman & Long 1983, Rand & Petrocelli 1985). Acute toxicity tests are performed to determine concentrations of test chemicals that result in deleterious effects in organisms during a short-term exposure period under controlled conditions (Lewis 1991). For these types of tests mortality is often considered as the deleterious effect. Chronic toxicity tests indicate the concentrations of chemicals that produce effects over significant portions of the test organisms' life spans (Rand & Petrocelli 1985). For these types of tests abnormal growth and development are considered as common test endpoints. Generally, concentrations that produce chronic effects are lower than those that produce more readily observable acute effects (Buikema Jr *et al.* 1982, Lewis 1991, 1992). A chronic toxicity test is designed to expose test organisms to a range of chemical concentrations estimated to bracket the threshold for significant effects (Steele & Thursby 1983, Anderson & Hunt 1988, Cairns & Pratt 1993, Forbes & Forbes 1993). Both types of tests can provide informative data, depending on the objectives of the testing.

Increasing volumes of sewage effluent enter marine ecosystems each year (Fagan *et al.* 1992, Otway *et al.* 1994, Pun *et al.* 1995), and consequently the need for routine toxicity testing and monitoring has increased. Bascom (1982) noted that several species of bird and kelp disappeared from areas of southern California (USA) located near deep marine outfalls. Swartz *et al.* (1986) also recorded ecological changes in macrobenthic communities in the same area, and Maurer *et al.* (1991) expressed concern over the increase in contamination of coastal waters along the southern Californian shelf which has caused decreases in plant and animal diversity. Monitoring studies conducted in Australia have also recorded changes in community structures along the southern

coastline of Victoria with the recorded disappearances of intertidal macroalgal species (Manning 1979, Brown *et al.* 1990, Bellgrove *et al.* 1997). Whilst monitoring programmes have provided information on the effects of sewage effluent on several species ranging over different trophic levels, they cannot provide data on the specific toxic effects of the effluent on species affected. To gain this information, controlled ecotoxicological experiments need to be conducted. Most toxicity tests that have been undertaken to date have utilised only a limited number of animal species as the test organisms. Costello and Read (1994) noted that many toxicity tests with sewage effluent have been conducted on North Atlantic fish species such as the herring *Clupea harengus* and cod *Gadus morhua* and crustacean species such as the shrimp *Crangon crangon*. Other researchers have focussed on testing the effects of primary and secondary treated effluent on shell development of larvae of the east Pacific red abalone *Haliotis rufescens* (Hunt & Anderson 1989), and effects of sewage effluent on mortality of juveniles of the fish species *Menidia menidia* (Santoro & Fikslin 1987). Miller *et al.* (1987) tested the lethality of New York and New Jersey (USA) sewage effluent on the copepods *Eurytemora herdmani* and *Psuedoclanus minitus* and larvae of the mysid *Mysidopsis bahia*. Many tests conducted to determine the effects of sewage effluent on marine species have been short-term, single species lethality tests (Costello & Read 1994). Few reports have been conducted on the long-term effects of sewage effluent with the exception of tests conducted on phytoplankton of several months duration (Frithsen *et al.* 1989).

1.1.3 Algal toxicity testing

Whilst some research has been conducted in Australia on impacts of sewage effluent on algal species (Manning 1979, Brown *et al.* 1990, Doblin & Clayton 1995), little work has been documented on the toxic effects of effluent discharge on these species (Burridge *et al.* 1999). Large macroalgal communities can be found inhabiting eulittoral and sublittoral zones of rocky, high energy coastlines (Borowitzka 1972, Cheshire & Hallam 1988, Brown *et al.* 1994, Burridge *et al.* 1999). Their particular sensitivity to sewage effluent has been well documented through monitoring studies noting the disappearance

of these species from once extensively populated areas (Manning 1979, Brown *et al.* 1990, Doblin & Clayton 1995). There is a need to examine why these populations are declining. Costello and Gamble (1992) noted that many reports have examined the toxic effects of sewage effluent on luminescence in bacteria and phytoplankton blooms in mesocosms, but no extensive research on macroalgae has been conducted. The need to investigate the effects of sewage effluent on macroalgae exists, as this type of discharge can either be a source of nutrient supply or an inhibitor of macroalgal growth (Ogawa 1984). The little ecotoxicological research conducted determining the effects of sewage effluent on macroalgal species has been conducted abroad namely USA, Japan and India. Anderson and Hunt (1988) utilised the microscopic life stages of the giant kelp *Macrocystis pyrifera*. They found that germination was inhibited at concentrations above 0.56% primary treated effluent after 48 hours exposure. Ogawa (1984) investigated the effects of secondary treated effluent on the early development of sargassaceous plants and found that germination of zygotes was completely inhibited in secondary treated effluent containing 25-50 mgL^{-1} NH^{+}_{4}-N. Studies such as these suggest that several species of macroalgae are amenable to evaluating the toxicity of a variety of toxicants including sewage effluents.

The advantages of utilising marine macrophytes in routine ecotoxicological testing are that they are sessile in nature and can be used to characterise a specific location. They are easily collected and have been known to readily accumulate toxic compounds present in their surrounding environment, which suggests that they can also be used as bioindicators (Levine 1984). This study investigated the effects of sewage effluents on three macroalgal species endemic to Australian coastal waters. The macrophytes chosen were *Phyllospora comosa* (Labillardiere) C. Agardh (Fucales), *Hormosira banksii* (Turner) Decaisne (Fucales) and *Macrocystis angustifolia* Bory (Laminariales). Tests were also conducted with the marine bacterium *Vibrio fischeri* using the commercially available Microtox® bioluminescence inhibition assay (Microbics Corporation).

Phyllospora comosa is found in sub-littoral zones of high energy coastal waters from Encounter Bay, South Australia to Port Macquarie, New South Wales. It is a large macroalga which forms dense forests and is anchored by a holdfast radiating out from the base of the main axis of the plant. The liguate axis and other branched axes are densely crowded with flat leafy laterals (receptacles) which produce male or female gametes (Burridge & Hallam 1993). *Phyllospora comosa* is dioecious, having separate male and female plants, and is fertile year round. Male and female plants can be differentiated by the appearance of their receptacles (Burridge *et al.* 1993). Gametes are formed in the oogonia and antheridia in conceptacles within receptacles (Burridge & Hallam 1993). Male and female gametes are released simultaneously into the water column, the eggs are retained in stalks at the receptacle surface and the sperm fertilise the eggs in a random fashion. Maximum fertilisation usually takes place in the first 30 minutes, although eggs are retained on receptacles for up to 15 hours to optimise fertilisation (Burridge *et al.* 1993).

Hormosira banksii is a moniliform brown alga endemic to lower eulittoral communities of southern Australia and New Zealand (Clayton *et al.* 1985), it is dioecious and fertile year round (Müller *et al.* 1984). Plants are attached to the substratum by a discoid holdfast and the thallus consists of branched chains of vesicular segments that are joined by slender restrictions (Clayton *et al.* 1985). Reproduction in *H. banksii* is oogamous and eggs and sperm are exuded on the surface of the thalli. Upon contact with seawater, the oogonial membranes dissolve to liberate four eggs per oogonium. Each antheridia releases 64 spermatozoids that swarm around the eggs until fertilisation has been achieved (Müller *et al.* 1984).

Macrocystis angustifolia is found in the cold temperate oceans of the Southern Hemisphere, between 40° and 60°S (Perissinotto & McQuaid 1992). It is anchored by a holdfast and produces a floating canopy of dense vegetative fronds at the surface (Lewis & Neushal 1994). The only type of reproductive structures present on the adult plant (sporophyte) are the unilocular sporangia. *Macrocystis angustifolia* has a life cycle with a heteromorphic alternation of generations, consisting of a microscopic haploid

gametophyte stage and macroscopic diploid sporophyte stage. The sporangia produce 16 to 64 motile zoospores (haploid cells) which are released into the water column where they begin germination by undergoing meiosis followed by mitotic divisions (Tugwell & Branch 1989, Anderson & Hunt 1988).

For this study, the early life stages of *P. comosa, H. banksii* and *M. angustifolia* were utilised in determining the toxic effects of treated sewage effluent.

1.2 Aims of this study

The aims of this study were to:

1. determine the effects of primary, secondary and chlorinated secondary treated effluents on the early life stages and endpoints of the marine macroalgal species; *Phyllospora comosa, Hormosira banksii* and *Macrocystis angustifolia*. Zygote and zoospore germination, embryo and spore germination tube growth, embryo mortality, and sporophyte production (reproduction) and growth were investigated.

2. validate all tests conducted by conducting separate toxicity tests with the reference toxicants copper chloride and hexavalent chromium.

3. compare results obtained from differently treated effluents and to determine the extent to which treatment alters toxicity.

4. establish standard ecotoxicological tests which can be routinely employed for the determination of sewage effluent toxicity.

5. to develop toxicity tests with marine macrophytes which can be quickly and easily conducted and provide reproducible results.

6. to investigate the specific toxic effects of treated effluent at the ultrastructural level of development of *P. comosa, H. banksii* and *M. angustifolia.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Collection

Fertile samples of *Phyllospora comosa* , *Hormosira banksii* and *Macrocystis angustifolia* were collected at low tide from Sorrento ocean beach (38°22'S, 144° 44'E), Sorrento, Victoria (Fig. 2.1).

2.2 Test species and toxicity test endpoints

2.2.1 *Phyllospora comosa*

Healthy and mature male and female plants were gathered, sexed and receptacles from each plant were placed into 25 ml polyethylene vials containing fresh seawater. Male and female plants were separately differentiated on the basis of the appearance of conceptacles within receptacles (Burridge & Hallam 1993). Female conceptacles appeared dark when held towards the light whilst male conceptacles appeared milky white when held just below the surface of the water. Forty vials, each containing four same-sex receptacles, were placed on a bed of crushed ice and transported to the laboratory in darkness to reduce the risk of premature gamete release.

Gamete release for *P. comosa* was initiated according to the procedure of Burridge and Hallam (1993). Vials containing *P. comosa* receptacles were placed on ice under a direct light source of 190 $\mu E\ m^{-2}\ s^{-1}$; gamete release then occurred within 3-6 hours. Female gamete release was observed when eggs and stalks became visible on the receptacle surface. Male gamete release was observed when a spermatozoid discharge was visible close to the receptacle surface. Prior to attempting fertilisation, samples of sperm were viewed under a compound microscope to verify viability based on motility, and sperm densities were recorded with the use of an ocular micrometer (1 ml of sperm solution was placed onto the micrometer and sperm numbers averaged).

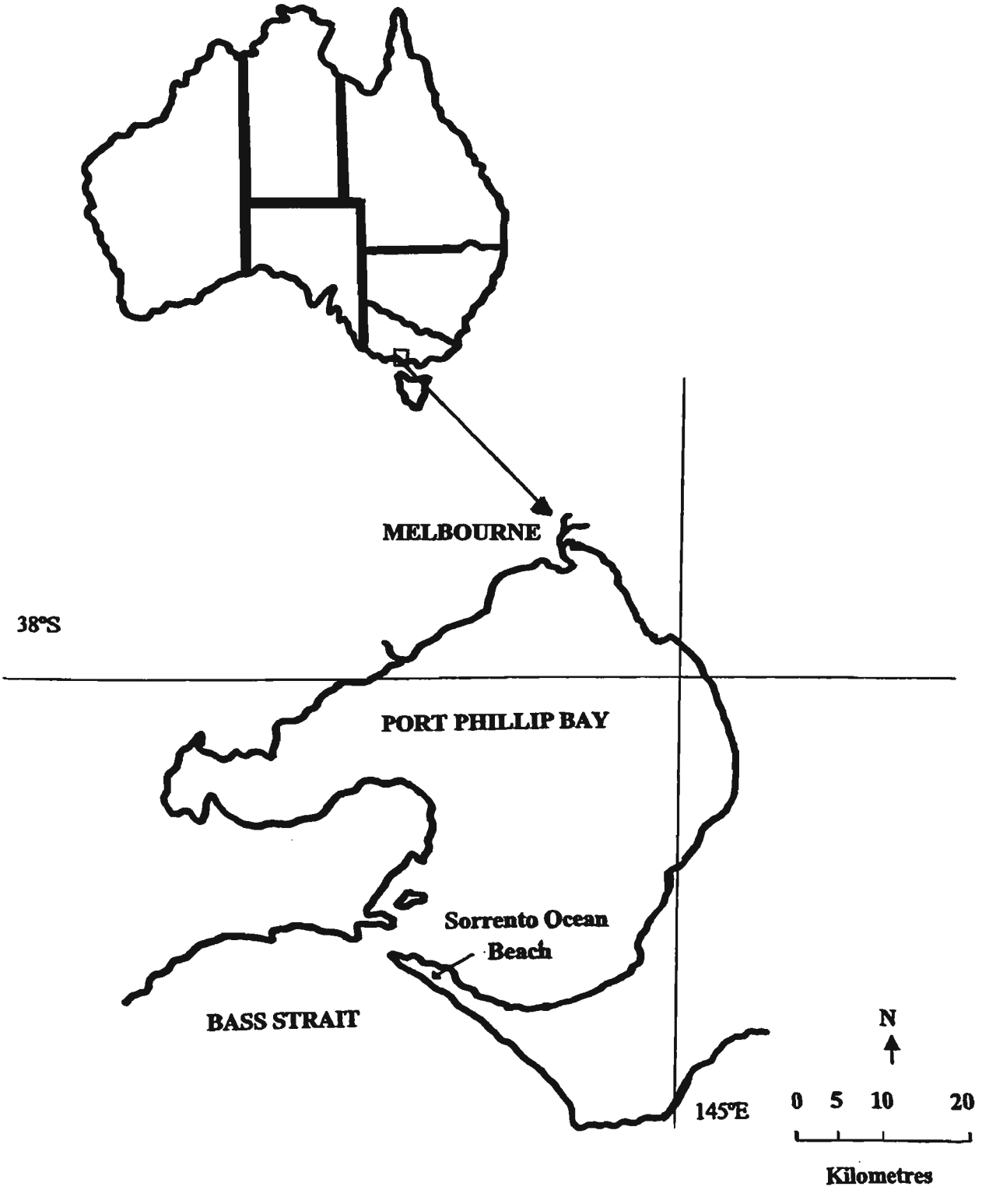


Fig. 2.1. Collection site of macroalgal species

Fertilisation for toxicity tests was achieved by following the procedure of Burridge and Shir (1995). Male and female gametes were directly added to 25 ml filter paper-lined beakers (the filter paper acted as the substratum). Beakers were filled with filtered oceanic seawater. One 2 cm segment of a female receptacle was added to each beaker. A volume of 1 ml of the seawater/spermatozoid mixture was added to each beaker. One ml contained approximately 10^6 sperm per aliquot (sperm densities less than 10^6 cells ml^{-1} were discarded). All beakers were covered with Parafilm® and placed in a refrigerated incubation cabinet and maintained at 15°C under a cool-white fluorescent light source of 120 $\mu E\ m^{-2}\ s^{-1}$ in a light:dark regime of 12:12 hours. The cultures were agitated periodically for the next 12 hours at which time receptacle tissue was removed and unattached zygotes could be observed scattered over the substratum surface.

Following fertilisation and receptacle tissue removal, cultures were left for 6 hours to allow zygotes to adhere to the substratum. Beakers were gently agitated and seawater and non-viable (unattached and therefore ungerminated) zygotes decanted and treatment solutions added. Four replicates were established for each treatment. Zygote germination was recorded at 48 hours, zygote mortality at 96 hours and embryo growth was measured at one and two weeks with a compound microscope. Germination rates were assessed on the basis of the presence or absence of a germination tube and mortality rates on the basis of a clearly necrotic appearance. The first 80 zygotes encountered during scoring were assessed in each replicate. Growth of embryos was measured with the aid of an objective graticule and the first 20 embryos encountered were measured (the entire length of each embryo was measured, that is, lengths of rhizoidal axes recorded).

2.2.2 *Hormosira banksii*

Healthy and mature *H. banksii* specimens were collected from the rock platform at the collection site. Samples were placed directly into plastic bags filled with seawater. These bags were placed on a bed of crushed ice and transported to the laboratory in darkness.

Gamete release of *H. banksii* followed a modification of the procedure of Gunthorpe *et al.* (1995). Bags containing samples were stored at 4°C overnight. The chilled algae were rinsed briefly with tepid (20-23°) tap water and placed on trays under a direct light source as described in Section 2.2.1. Male and female gametes exuded onto the surface of *H. banksii* segments within 1 hour. Sexed fronds were placed separately into two 1 L beakers filled with seawater. Egg and sperm suspensions were viewed under a compound microscope to verify their presence. Viability of sperm was also assessed by their motility. Densities of eggs and sperm utilised were approximately 150 eggs/cm^2 and 10^7 sperm/ml respectively, providing an egg:sperm ratio of 1:1000. The gamete suspensions were then mixed and allowed to stand for 10-15 minutes, after which time fronds were removed. The gamete suspensions were added to 25 ml beakers containing coverslips. Beakers were covered with Parafilm® and placed in a refrigerated incubation cabinet at 15°C and 12:12 hours light:dark regime overnight as described in Section 2.2.1. Following adhesion, the gamete suspension was decanted and replaced with treatment solutions. Four replicates of each treatment were established. Germination, embryo mortality and one and two week growth rates were assessed as described in Section 2.2.1. Treatment solutions were changed every three days for all *P. comosa* and *H. banksii* growth tests.

2.2.3 *Macrocystis angustifolia*

Healthy and mature *M. angustifolia* plants were collected from the edge of the rock platform. Several plants were collected at any one time. Reproductive sporophylls were removed from the base of the plants and layered between sheets of paper towelling, according to the method of Anderson and Hunt (1990) used for bioassays with the macroalga *Macrocystis pyrifera.* Great care was taken to ensure that sporophylls did not come in contact with each other as this could lead to premature zoospore release. When layered, the bundles of sporophylls and paper towelling were quickly immersed in seawater, sealed in plastic bags and placed on a bed of crushed ice for transportation to the laboratory.

For zoospore release of *M. angustifolia,* sporophylls were washed in filtered oceanic seawater and blotted dry with paper towel to remove any epiphytes (Anderson & Hunt 1990). Sporophylls were then laid on paper towelling at room temperature for 1 hour and then placed into a 1 L beaker filled with seawater to allow zoospore release. Release occurred at times ranging between 10 and 60 minutes and the zoospore suspension was checked every 10 minutes after desiccated sporophylls had been added to the seawater. Zoospore densities utilised for the tests were a minimum of 10^5 cells/ml.

Four replicates containing each treatment were established. A volume of 2 ml of the zoospore suspension was added to each 25 ml beaker containing the treatment solution and a coverslip which acted as a substratum. Forty-eight hours after zoospore addition, spore germination and growth were recorded for the first 80 gametophytes encountered, utilising a compound microscope and objective graticule. At this time analytical reagent grade nutrients (Ajax Chemicals, Australia) were added (minus EDTA-chelated iron) as well as germanium dioxide (0.10 mg/L) to control diatom growth. The nutrient medium used was prepared following the method of Steele and Thursby (1988) and contained sodium nitrate (12.7 g/L), sodium phosphate (1.28 g/L), sodium citrate (103 mg/L) and ferrous sulphate (97 mg/L) dissolved in seawater. The stock was then autoclaved at standard pressure and temperature for 15 minutes. For each litre of algal culture medium used, 5-10 ml of nutrient medium was added. Test solutions were changed every four days. Sporophyte production (reproduction) was scored and sporophyte growth of the first 20 individuals encountered, measured on day 20. For all germination and mortality assays, >80% germination/survival in control treatments was consistently recorded and used as the benchmark for test acceptability.

2.2.4 *Vibrio fischeri*

The effects of all treatments were also tested on the marine bacterium *Vibrio fischeri* utilising the Microtox® test. These tests were performed as specified by Microbics Corporation (Anon. 1992). Dilutions of test chemicals were added to suspensions of

bacteria and incubated for 5 and 15 minutes at 15°C prior to the measurement of luminous activity in the model 2205 Microtox® instrument. Tests were repeated 3-5 times for each treatment and the phenol standard test was performed each time a treatment was tested. All supplies and accessories, including the bacterium used for these tests were provided by Microbics Corporation. Microtox® reconstitution solution (specially prepared distilled water) was used to reconstitute the bacterium (reagent). Microtox® diluent (specially prepared 2% NaCl) was used to dilute the sample and reagent and Microtox® osmotic adjustment solution (specially prepared 22% NaCl) was used to adjust the osmotic pressure of the sample to 2% NaCl.

2.3 Dilution water

Oceanic seawater was used as the culture medium and dilution solution in all tests conducted. Seawater was supplied by Queenscliff Marine Station, Victoria. The seawater (temperature: 15 ± 1°C; salinity: 34 ± 2°/oo; pH: 8 ± 1; DO: 7 ± 1 mg/L) was filtered through a 0.2 μm membrane filter and refiltered upon arrival at Victoria University where it was stored in a 5000 L tank until required.

2.4 Test solutions

The effects of two reference toxicants, three sewage effluents and salinity reduction were tested on the early life stages of *P. comosa, H. banksii* and *M. angustifolia.* The reference toxicants were analytical reagent grade copper chloride ($CuCl_2.2H_2O$) and hexavalent chromium ($K_2Cr_2O_7$) (Ajax Chemicals, Australia). Stock solutions of these compounds were prepared with seawater immediately prior to the commencement of each test. From these stock solutions, serial dilutions were made to cover the predetermined concentration ranges for the tests conducted. The stock solution concentrations remained the same for the range of tests performed. The concentration of copper stock was 100 mg/L $CuCl_2.2H_2O$ and chromium stock was 1000 mg/L $K_2Cr_2O_7$ in seawater. From this point all nominal concentrations of these compounds will be

expressed as the individual copper and chromium ions. It should be noted that only nominal concentrations were required to assess background variability of algal responses over the test period. All toxicity tests performed with reference toxicants were conducted using polyethylene beakers to minimise absorptive losses of the metals (Anderson & Hunt 1988).

The sewage effluent samples were provided by the South Eastern Purification Plant (SEPP), Carrum, Victoria, 24 hours prior to the commencement of each test. At each collection, data were made available for chemical analyses performed on the effluent by the SEPP (discussed further in future chapters). Primary, secondary and chlorinated secondary treated effluent samples were collected. Two types of tests were conducted with the effluent samples: modified and unmodified. Modified effluent tests refer to the salinity adjustment of the samples with commercially available sea salts ('Marine-mix', Marine Enterprises, Baltimore, USA). For these tests the effluent samples were adjusted to the salinity of seawater (34 ± 2°/oo). Salinity was measured using a CO150 Conductivity Meter Model 50250 (Hach Company). Following adjustment, serial dilutions with seawater of the 100% effluent stock were performed. For unmodified tests, the salinity of the effluent was not adjusted, dilutions with seawater of this 100% stock were performed for toxicity tests. For all effluent samples, the pH was adjusted to that of seawater with HCl and NaCl buffers and dissolved oxygen to saturation. To determine whether the sea salts had any effect on the algae, distilled water controls were used. These involved dissolving the sea salts in distilled water until the salinity of seawater was obtained. It was found that the commercial sea salts exerted no significant effects on any of the algal species and so results for both types of controls have been combined to form one control result in future chapters. To determine whether salinity reduction had any effects on the algae, seawater was mixed with distilled water to obtain salinities corresponding to the unmodified effluent test concentrations. All sewage effluent and salinity reduction tests were conducted using glass beakers (Anderson & Hunt 1988).

During the course of this study, cultures exposed to effluent treatments were showing signs of bacterial contamination after 8-12 days. To overcome this problem and to determine whether the presence of bacteria had a significant effect on algal growth, measures were taken to inhibit bacterial contamination. A suite of tests was conducted at different times with the same effluent samples at the same concentrations on the three algal species. Two ways of controlling bacterial growth were attempted: effluent samples were autoclaved for 30 minutes followed by 24 hours of aeration, and a second batch of the original effluent samples were treated with antibiotics. Antibiotics tested were streptomycin sulphate (50 mg/L), penicillin G (100 mg/L) and chloramphenicol (50 mg/L). To determine whether these treatments altered the toxic effects of the effluent samples, a third batch of the original effluent was left untreated. All three batches (autoclaved, treated with antibiotics, untreated) of each of the effluent samples (primary, secondary and chlorinated secondary treated effluent) were tested at different times (due to logistical reasons) during the course of this study. No significant differences in growth of any of the algae were recorded. The advantage to 'treating' the effluent samples was that bacterial contamination no longer obstructed the view of the algal cultures, particularly with *M. angustifolia* gametophytes due to their small size. Autoclaving effluent samples was the easier option and was adopted for controlling bacterial growth. No 'treatment' of the effluent samples was required for the shorter germination and mortality assays as bacterial contamination was not evident at 48 and 96 hour counts.

2.5 Data analysis

Data were checked for normality and homogeneity of variances using box plots and residual plots. When these criteria were not met, arcsine or $\log_{10}$ transformations were performed to improve linearity in regression. Analyses of variance (ANOVA) were performed on arcsine transformed germination and mortality data and tested at the 0.05% significance level. The statistical power of each test was determined and found to be >0.99, unless otherwise stated. Dunnett's test was used to determine NOEC (no observable effect concentrations) and LOEC (lowest observable effect concentrations) and Spearman-Karber or Probit to determine median effect concentrations (EC_{50}) for

germination and median lethal concentrations (LC_{50}) for mortality data (TOXCALC software 1992). Growth data were $\log_{10}$ transformed, analysed and EC_{50} concentrations determined. The Microtox® Analyser System software (1992) was used to determine EC_{50} concentrations for the bacteria tests.

2.6 Specimen preparation for light and electron microscopy

2.6.1 Preparation of cultures

Cultures employed for light and electron microscopy were prepared in the same manner as those for toxicity testing described in Sections 2.2.1-2.2.3. Algal samples for transmission electron microscopy were grown on filter paper and those for scanning electron microscopy were cultured on glass coverslips. Beakers housing algal cultures were transported on ice to the Department of Ecology and Evolutionary Biology, Faculty of Science, Monash University, Clayton Campus, Victoria, where electron microscopy procedures were conducted.

2.6.2 Fixative

Fixation of algal samples for electron microscopy was carried out in modified Karnovsky's Fixative (Karnovsky 1965). The fixative consisted of 4% paraformaldehyde, 4% glutaraldehyde and 1% caffeine in 0.05M sodium cacodylate buffered distilled water with an adjusted pH of 7.2-7.3. Samples were fixed for 2-4 hours in screw-top glass vials, at which time they were washed 3 times at 5 minute intervals with a 1% sodium cacodylate and 1% caffeine seawater buffer adjusted to pH of 7.2-7.3.

2.6.3 Tissue preparation for light microscopy

Live algal tissue samples for light microscopy were fixed whilst still attached to the filter paper substratum. Filter paper pieces containing algal tissue were no larger than 5x5 mm prior to fixing and were cut using a sharp razor. Great care was taken to ensure that algal

tissue was not damaged, and samples were prepared under a shallow bed of seawater to reduce the risk of samples drying. Samples were fixed as described in Section 2.6.2, and dehydrated through a graded, 10% stepped ethanol series. Samples were dehydrated in each concentration for 15 minutes at which time the ethanol was removed by pipette and replaced with the next concentration. Samples were maintained in screw-top glass vials throughout this procedure. At the 100% ethanol step, samples were dehydrated for 30 minutes with 3 changes of absolute dry ethanol.

After dehydration, samples were infiltrated in a stepped series of LR White resin dilutions (London Resin Company) diluted with 100% ethanol. Two changes of resin were conducted each day with concentrations of LR White increased by 20% daily up to 100%. Samples were stored at 4°C and inverted several times a day. At the final 100% resin step, samples were infiltrated for 3 days, with resin being changed twice daily. Samples were then polymerised at 60-70°C overnight.

2.6.4 Sample preparation for scanning electron microscopy

Fixing of samples for scanning electron microscopy (SEM) followed the same procedure as described in Section 2.6.2. Algal samples for SEM research were cultured on glass slides. Prior to fixing, these slides were cut by scoring with a diamond tipped knife and snapping the slides. Again great care was taken during this procedure to ensure that samples did not dry out. After fixing, samples were dehydrated through a graded ethanol series starting at 10% ethanol and working through to 100% ethanol. Samples were held at each concentration for 15 minutes and three 30 minute changes in 100% ethanol were made. After the final 100% ethanol change, samples were critical point dried with a CO_2 exchange for ethanol for 30-60 minutes at which time the temperature was raised from ambient to 40°C and pressure from 800-1200 psi. Samples were then mounted on aluminium stubs with double sided tape. Stubs and attached samples were then sputter

coated with gold using a Balzers SCD-005 Sputter Coater. Once sputter coated, samples were stored in a dessicator until required.

2.6.5 Sample preparation for transmission electron microscopy

For transmission electron microscopy (TEM), live algal tissue growing on filter paper was cut into 1x1 mm pieces followed by fixing and rinsing in the same manner described in section 2.6.2. Samples were held in 1% osmium tetroxide in seawater for 1 hour followed by dehydration through a graded acetone series in 5% steps. They were held in each concentration for 15 minutes and three 30 minute changes with 100% acetone were made. Samples were infiltrated with graded steps of Spurr's resin with 5% increases in resin per step. The low-viscosity epoxy embedding resin was made following the procedure of Spurr (1969). Firm grade or standard medium was prepared by mixing 10 g vinyl cyclohexene dioxide, 6 g Dow Epoxy Resin 736, 26 g nonenyl succinic anhydride and 0.4 g dimethylamonoethanol. Spurr's resin was diluted with 100% acetone for sample infiltration. Two 5% resin changes were made daily after which time resin concentrations were increased by 5% until a concentration of 100% resin was reached. Samples were held in 100% Spurr's resin for 7 days, with at least 3 changes and were stored below -4°C. During this procedure, samples were inverted several times a day. Samples were then polymerised at 60-70°C overnight.

2.7 Sectioning

2.7.1 Light microscopy

For light microscopy, sections of 0.2-0.25 μm were cut with glass knives on Ultracut microtomes. Sections from LR White resin blocks were placed on a drop of distilled water on a glass slide and heat-adhered to the slide using a hot plate. Sections were stained with toluidine blue pH 4.5, for 30 seconds (Dawes 1971), rinsed with distilled water and viewed under a compound microscope.

2.7.2 Transmission electron microscopy

For TEM, sections were cut with glass knives on an Ultracut microtome. The sections were floated on water and those showing gold or silver interference colours were mounted on uncoated 200 mesh grids. Sections were stained for ten minutes with uranyl acetate followed by ten minutes with lead citrate (Venable & Coggeshall 1965).

2.8 Microscopy

2.8.1 Light microscopy

Light micrographs were taken using a 35 mm SLR Nikon camera mounted on a Nikon compound light microscope. Micrographs were taken on Agfa black and white film and developed on Ilford photographic paper (Horder 1958). Films were developed in Agfa Rodinal for 8 minutes, washed under running water with 3 changes over 1 minute and then placed into fixer (Ilford Hypam 1:4 and Ilford Hypam Hardner 1:75) for 4 minutes at which time they were rinsed under running water for 30 minutes. For photographic printing, paper was placed into developer (Ilford Multigrade 1:9) for 1 minute, washed for 15 seconds with water and acetic acid and placed into fixer (Ilford Ilfospeed 1:3) for 30 seconds before being washed under running water for 2 minutes.

2.8.2 Scanning electron microscopy

Tissue sections were examined using a Hitachi S570 SEM at an accelerating voltage of 10 kV. Micrographs were taken using secondary electron detection and developed using Kodak 120 or 220 black and white film following the method described in Section 2.8.1.

2.8.3 Transmission electron microscopy

Grids were examined in a JEOL 200CX TEM operating on an accelerating voltage of 80-160 kV. Electron micrographs were taken and developed using Kodak 4489 electron microscope film following the method described in Section 2.8.1.

CHAPTER 3
THE EFFECTS OF SEWAGE EFFLUENT ON GERMINATION OF *PHYLLOSPORA COMOSA, HORMOSIRA BANKSII* AND *MACROCYSTIS ANGUSTIFOLIA*

3.1 Introduction

Monitoring of species population dynamics is a widely used tool in determining biological effects of pollutants on marine environments (Blackstock *et al.* 1986). Biotic responses to contaminant influences can be detected at a number of different levels of biological organisation. These test species can prove to be extremely important screening tools, providing information on the bioavailability and effects of discharged effluents through standardised toxicity tests. The most relevant evidence however, can be gained by using indigenous species in bioassays along with biomonitoring surveys of areas where the species are found (Chapman & Long 1983).

Fucalean and laminarian algal species are often considered as important primary producers and habitat formers in shallow marine communities of temperate waters (Clendenning 1971, North 1979, Mann 1973, James *et al.* 1987). Their presence in these communities is vital for the survival and well being of countless animal assemblages and communities. Studies have suggested that these algal species can be utilised successfully in routine toxicity testing (North & James 1987, Burridge & Shir 1995, Burridge *et al.* 1995a, b, Bidwell *et al.* 1998). They have also been extensively used in studies of growth rates, nutritional requirements and responses to light and temperature (Hsiao & Druehl 1973, Lünning & Neushul 1978, James *et al.* 1987). In recent decades, the increased discharge of sewage effluent and other pollutants into marine environments has been indirectly linked to contributing to declines in these and other algal communities worldwide (Chapman 1995, Santoro & Fikslin 1987, Anderson & Hunt 1988, Costello & Read 1994). Consequently this study has focussed on developing short-term bioassays which can be quickly and easily conducted under laboratory conditions utilising endemic algal species, with germination as an important test endpoint.

Recent studies utilising *Phyllospora comosa* as the test species have focussed on the effects of crude oil and diesel fuel on germination of zygotes (Shir 1994, Burridge & Shir 1995). It is from these short, simple tests that longer and more involved tests can be developed. Exposing test organisms for longer periods of time provide researchers with data that would not normally be made available. Since the composition of sewage effluent can change readily, short-term bioassays are more useful as the data generated can be used to record changes in effluent toxicity over time. These short-term bioassays can provide a mechanism for monitoring sewage effluent effects (Burridge *et al.* 1999). Such tests were conducted by Burridge *et al.* (1996) who investigated the effects of treated sewage effluent on the species investigated for this study. They found that algal responses varied with time and that this was most probably due to changes in effluent toxicity. Copper and chromium were chosen as such reference toxicants due to the abundance of literature available on the two with reference to ecotoxicicity testing (Stauber & Florence 1985, 1986, 1987, 1989). They were also considered as they are commonly found in discharged sewage effluent and have shown to be particularly toxic to aqautic organisms (Smith & Simpson 1993, Pun *et al.* 1995).

Studies have also been conducted utilising *Hormosira banksii.* These studies investigated the effects of treated sewage effluent on germination and growth of the species (Doblin & Clayton 1995, Burridge *et al.* 1996). Doblin and Clayton (1995) investigated the effects of secondary treated effluent on germination and growth of *H. banksii,* but neglected to include a reference toxicant as a treatment. Their study was however, informative if not from a strictly toxicological viewpoint then certainly from a descriptive biological one.

Ecotoxicological studies conducted utilising *Macrocystis angustifolia* as the test species have been scarce in Australia. Most studies have been conducted in USA utilising *M. pyrifera* (Anderson & Hunt 1988, Anderson *et al.* 1990). Burridge *et al.* (1996) did include *M. angustifolia* in their study and found the species to be most suitable for use in routine ecotoxicological research.

Germination in macroalgal species is an important stage in early development of zygotes and spores. The use of germination as a test endpoint has been widely accepted in ecotoxicological research worldwide (James *et al.* 1987, Anderson & Hunt 1988, Anderson *et al.* 1990, Costello & Read 1994, Burridge *et al.* 1996, Bidwell *et al.* 1998). The test is quick, simple and offers substantial ecological significance.

3.1.1 Aims of this chapter

The aims of this chapter were to:

1. determine the effects of treated sewage effluent on germination of *Phyllospora comosa* and *Hormosira banksii* zygotes, and germination and germination tube growth of *Macrocystis angustifolia* spores.

2. investigate possible seasonal variation in algal germination responses by conducting bioassays with two reference toxicants.

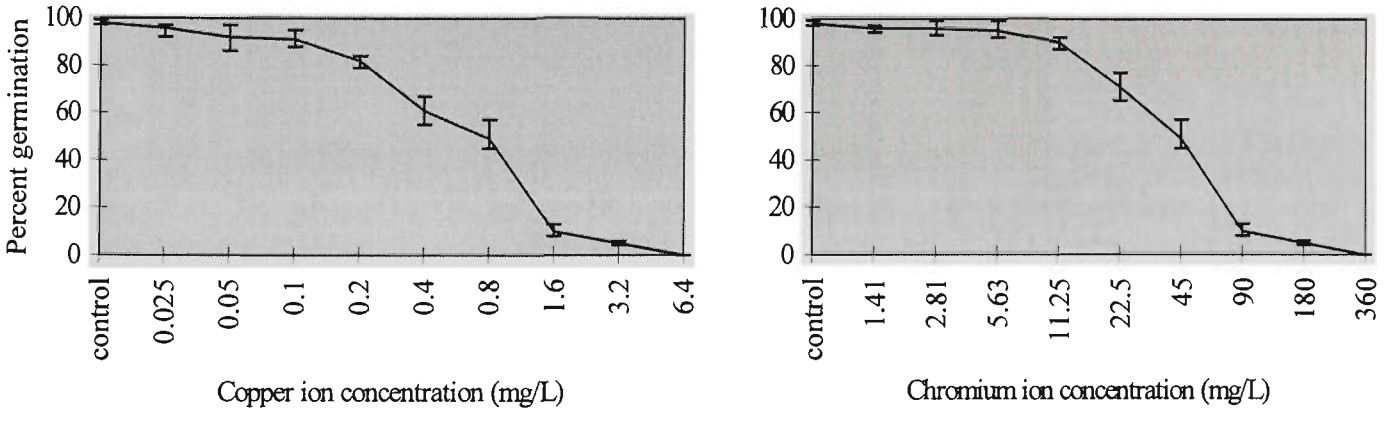
3.2 Results

Germination bioassays conducted with the reference toxicants, copper and chromium were performed over a 15 month period beginning 2/9/96 and concluding 31/11/97, these test dates are denoted as a-e and 1-20 in the following sections. All other germination bioassay dates are denoted as 1-20 with corresponding dates ranging between 15/11/96-31/11/97. Exact dates of each test commencement have been listed as Appendix 1.

3.2.1 *Phyllospora comosa*

Reference toxicants

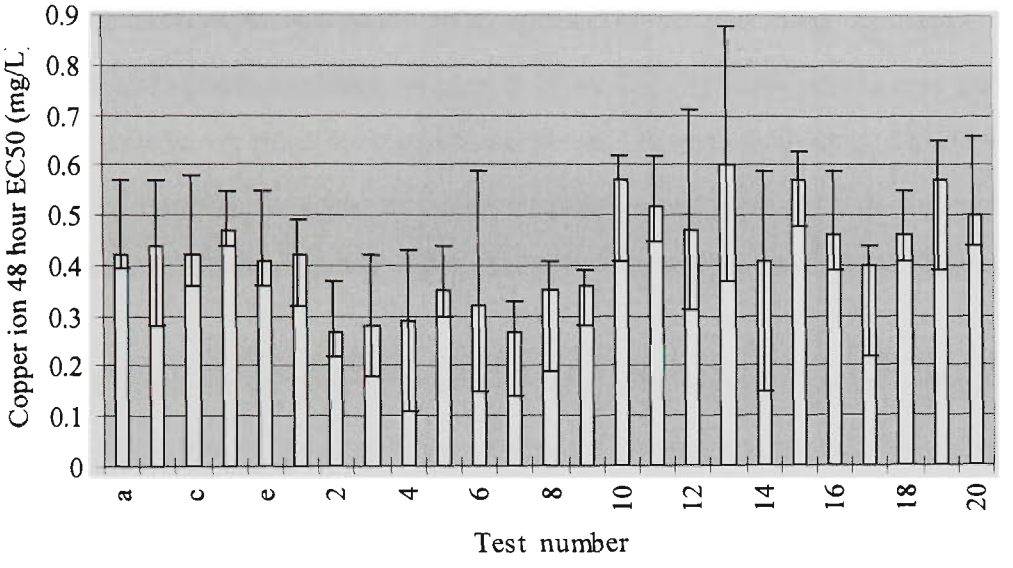
The reference toxicants copper and chromium exerted significant effects on germinating *P. comosa* zygotes (Fig. 3.1). The NOEC values for zygotes exposed to copper ranged from 0.05-0.20 mg/L and the LOEC values ranged from 0.10-0.40 mg/L (Appendix 2.1). The NOEC values for zygotes exposed to chromium ranged from 5.63-22.50 mg/L and LOEC values ranged from 11.25-45.00 mg/L (Appendix 2.1). The 48 hour EC_{50} values for zygotes exposed to copper ranged from 0.27-0.60 mg/L (Fig. 3.2) with a mean EC_{50} value of 0.42 mg/L and a C.V. of 23%. For zygotes exposed to chromium, the 48 hour EC_{50} values ranged from 28.4-68.0 mg/L (Fig. 3.2) with a mean EC_{50} of 50.3 mg/L and a C.V. of 28%.



(a) (b)

Fig 3.1 Germination (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* zygotes exposed to copper (a) and chromium (b) for 48 hours.

(a)



(b)

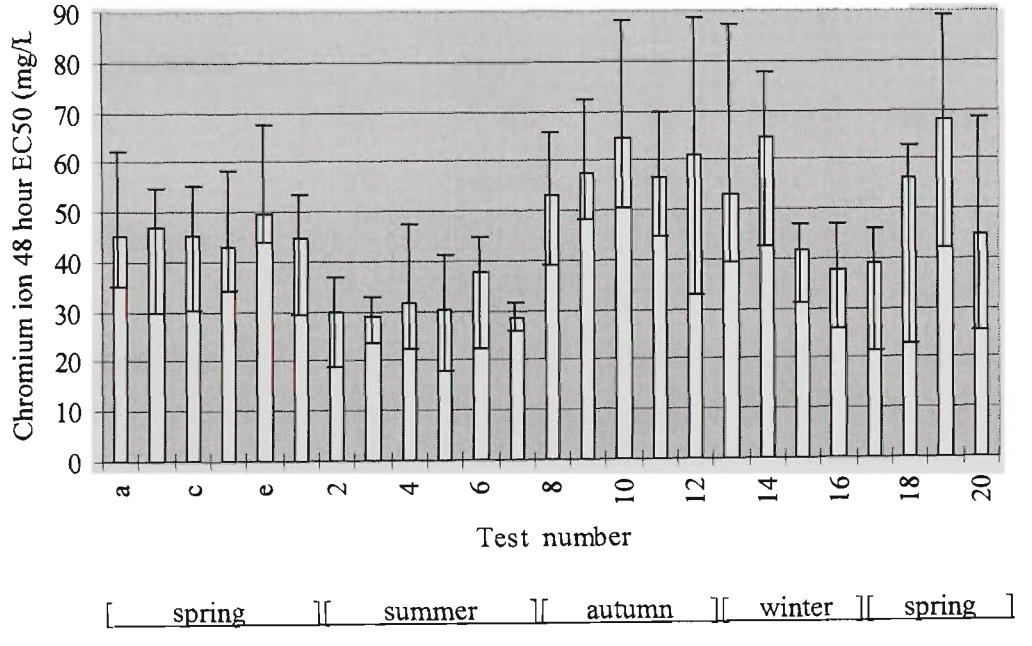


Fig. 3.2 Response over time of *Phyllospora comosa* zygotes exposed to copper (a) and chromium (b) for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Repeated measures ANOVA suggested highly significant variation in *P. comosa* response over time for tests conducted with copper and chromium (Table 3.1). Further two factor ANOVA indicated that variations in the data set were due to seasonal responses of zygotes exposed to copper ($P<0.05$, $F=14.28$, $df=3$) and chromium ($P<0.05$, $F=16.23$, $df=3$). A post hoc significance test (Tukey) indicated significant differences in response of zygotes between summer (highest sensitivity) and the remaining seasons, but no significant differences in zygote response between winter, autumn and spring.

Table 3.1 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 25) data of *Phyllospora comosa* zygotes exposed to copper (Cu) and chromium (Cr) for 48 hours.

Factor	df	MS		F ratio		P	
		Cu	Cr	Cu	Cr	Cu	Cr
Between Subjects							
Treatment	9	1.400	2.180	166.87	102.114	0.000	0.000
Error	30	0.008	0.021				
Within Subjects							
Test	24	0.013	0.011	4.321	5.275	0.000	0.000
Test x Treatment	216	0.009	0.005	3.068	2.840	0.000	0.000
Error	720	0.003	0.002				

Primary treated effluent

Tests conducted with primary treated effluent varied significantly over time for both modified (n=20) and unmodified (n=20) effluent treatments. There were no significant differences in germination response however, between zygotes exposed to modified and unmodified treatments (Fig 3.3) of the same effluent sample for each test conducted except for test 6 ($P=0.000$, $F=89.38$, $df=1$). For this reason, all data were pooled together for statistical analyses as there were also no significant differences in variance between

data sets. The NOEC values for germinating zygotes ranged from 1-4% primary treated effluent (modified and unmodified) and LOEC values ranged from 2-8% primary treated effluent (modified and unmodified) (Appendix 2.2).

The 48 hour EC_{50} values determined for these assays ranged from 4-16% primary treated effluent (Fig. 3.4) with a mean EC_{50} of 11% and C.V. of 43%. This high C.V. was expected since the response of zygotes varied so significantly over time (Table 3.2). The corresponding salinities have been listed in Appendix 3 for the unmodified effluent EC_{50} values. Since there were no significant differences between zygote responses for the modified and unmodified effluent tests, it was concluded that salinity did not exert a significant effect at these concentrations. The significant interaction between test date and treatment would be attributed to changes in effluent toxicity over time. Post hoc significance test (Tukey) indicated no seasonally influenced trends in algal response although such trends were evident for bioassays conducted with the reference toxicants. It should not be dismissed though, that seasonal influences may have contributed to the effects recorded.

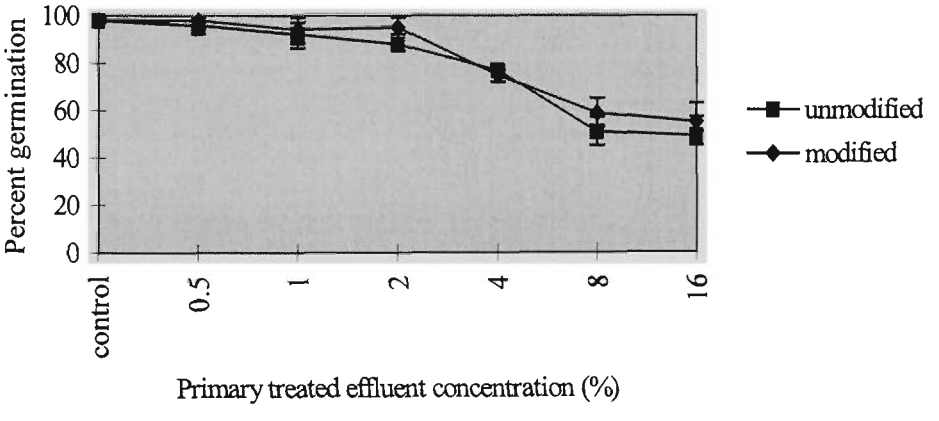
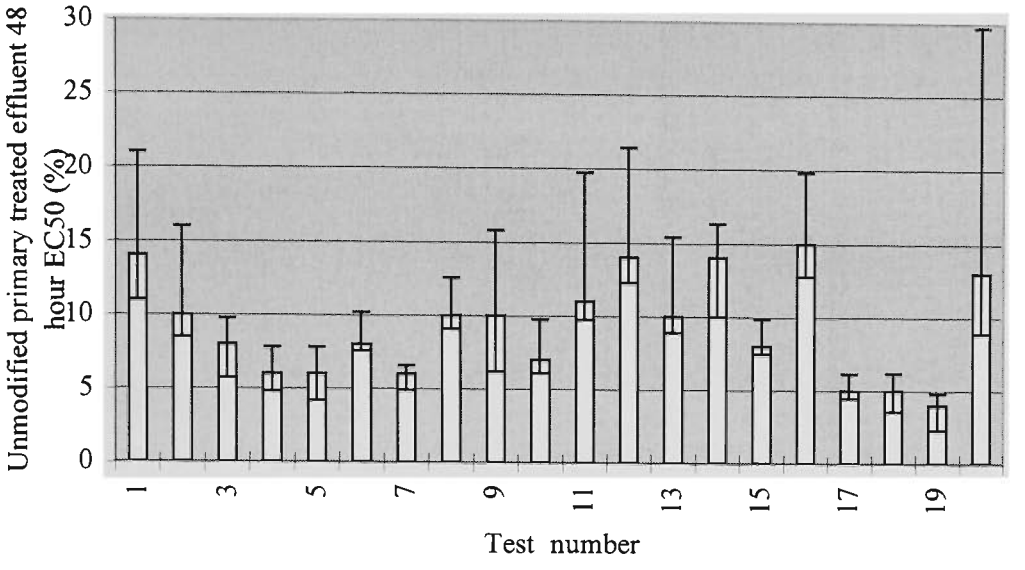


Fig. 3.3 Germination (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* zygotes exposed to modified and unmodified primary treated effluent.

(a)



(b)

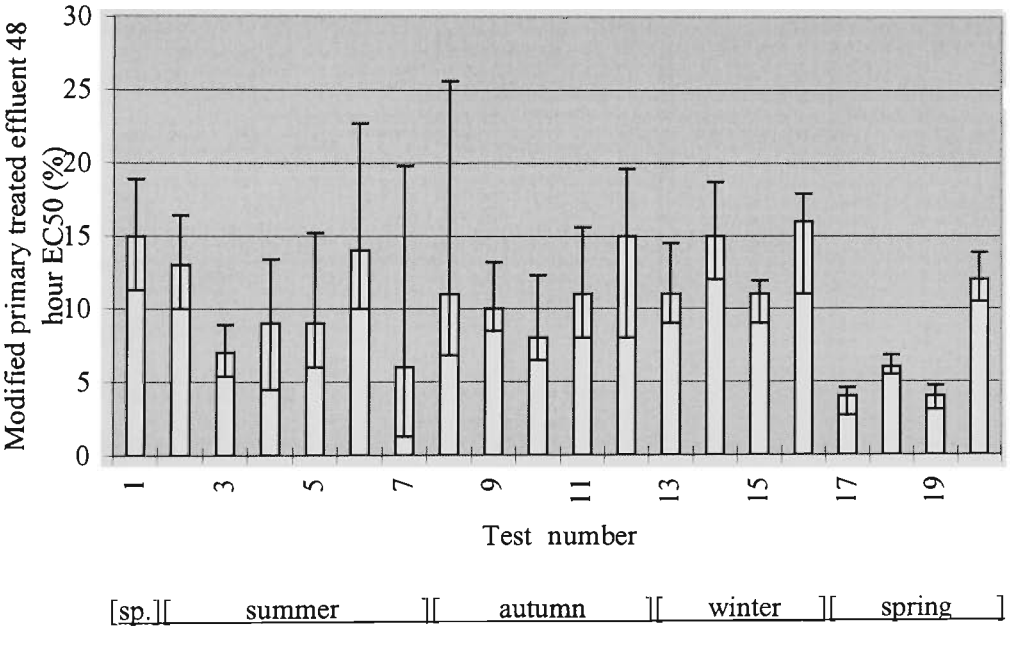


Fig. 3.4 Response over time of *Phyllospora comosa* zygotes exposed to unmodified (a) and modified (b) primary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 3.2 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 40) *hyllospora comosa* zygotes exposed to primary treated effluent for 48 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	3.501	284.82	0.000
Error	18	0.012		
Within Subjects				
Test	39	0.261	33.147	0.000
Treatment × Test	195	0.040	7.310	0.000
Error	702	0.006		

Secondary treated effluent

The results for germination tests conducted with secondary treated effluent (Fig. 3.5) were significantly different to the primary treated effluent as was expected. For modified secondary effluent, NOEC values ranged from 5-40% effluent and LOEC values from 10-80% effluent (Appendix 2.3). The 48 hour EC_{50} values ranged from 28->100% effluent (Fig. 3.6) with a mean EC_{50} of 70% effluent and a C.V. of 29% (EC_{50} values >100% were taken to equal 100% for these calculations). For tests conducted with unmodified secondary treated effluent, the NOEC values ranged from 1-4% effluent and LOEC values ranged from 2-8% effluent (Appendix 2.3). The 48 hour EC_{50} values ranged from 15-42% (Fig. 3.3) with corresponding salinities of 26.88-20.80 ppt (Appendix 2.3). A mean EC_{50} of 30% effluent was recorded and a C.V. of 24%. Germination response of *P. comosa* zygotes varied with time for all secondary treated effluent tests conducted (Table 3.3) and there were significant differences between responses of *P. comosa* zygotes exposed to modified and unmodified secondary treated effluent for each individual sample tested ($P<0.05$, $n=20$).

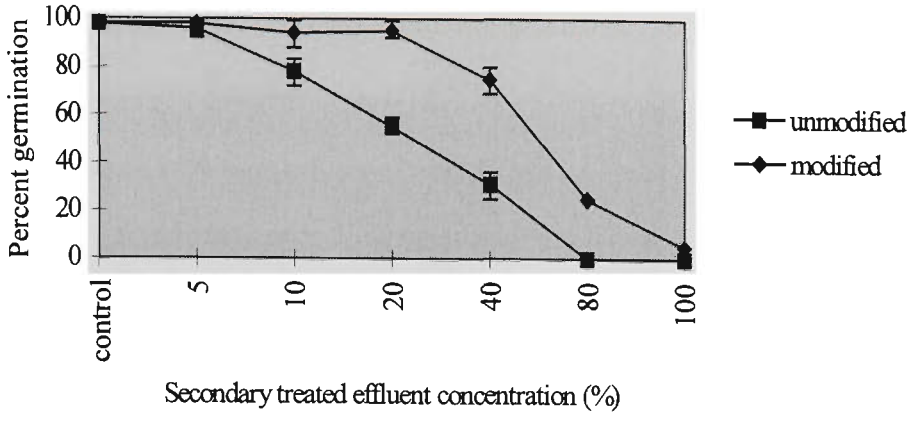
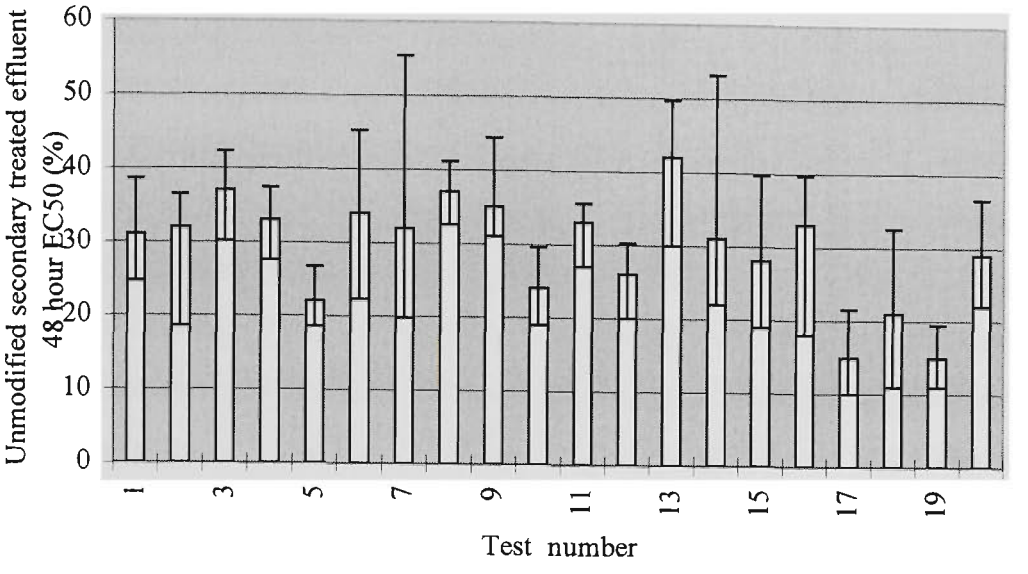


Fig. 3.5 Germination (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* zygotes exposed to modified and unmodified secondary treated effluent.

Table 3.3 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Phyllospora comosa* zygotes exposed to modified (mod) and unmodified (un) secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	1.425	2.356	247.89	159.36	0.000	0.000
Error	18	0.029	0.040				
Within Subjects							
Test	19	1.121	0.135	13.44	14.17	0.000	0.000
Treatment × Test	95	0.304	0.037	3.667	4.17	0.000	0.000
Error	342	0.083	0.009				

(a)



(b)

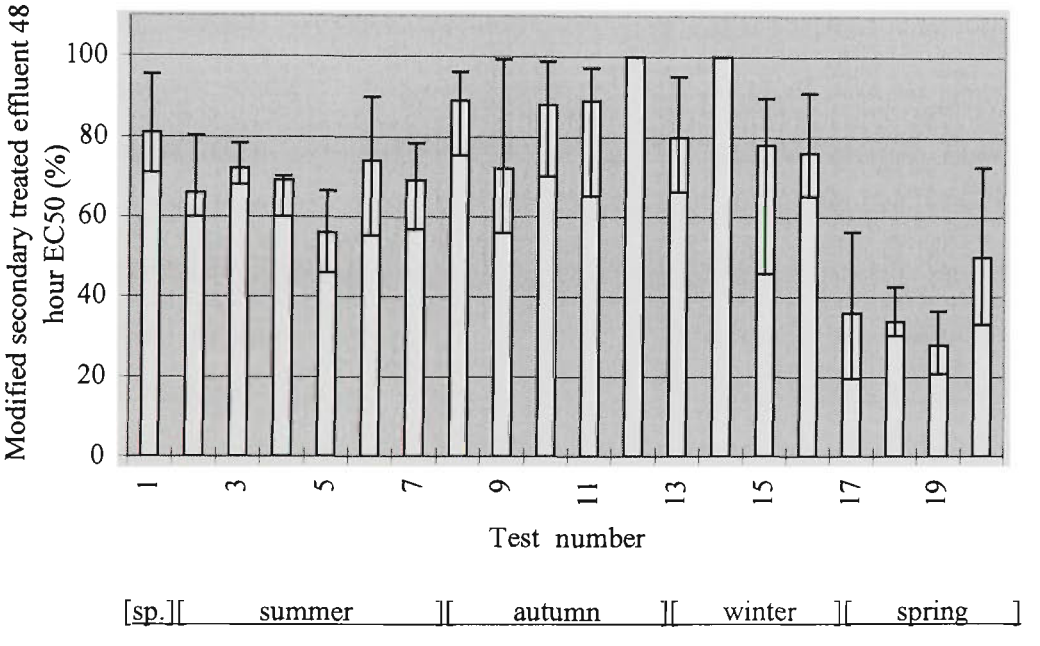


Fig. 3.6 Response over time of *Phyllospora comosa* zygotes exposed to unmodified (a) and modified (b) secondary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Chlorinated secondary treated effluent

Chlorinated secondary treated effluent exerted varied effects on germinating *P. comosa* zygotes (Fig. 3.7) and these effects were significantly different to those recorded for primary and secondary treated effluent samples. Both modified and unmodified effluent samples induced significant effects on zygotes for each of the tests conducted. Percentage germination of zygotes after exposure to modified effluent was significantly higher than for those exposed to unmodified effluent. ANOVA were also performed between all secondary treated effluent samples (modified and unmodified) and corresponding chlorinated secondary treated effluent samples (modified and unmodified) for each batch of effluent tested. Significant differences in zygote response were identified within each batch (i.e. test date) ($P<0.05$) except for test numbers 9 and 17 ($P>0.05$).

Zygote response to chlorinated secondary treated effluent also varied significantly over time (Table 3.4). The NOEC values for zygotes exposed to modified effluent ranged from 1-10% effluent and LOEC values ranged from 2-20% effluent (Appendix 2.4). The 48 hour EC_{50} values ranged from 6-82% modified effluent (Fig. 3.8) with a mean EC_{50} of 41% and a C.V. of 48%. Zygotes exposed to unmodified effluent also produced NOEC values ranging from 1-10% effluent and LOEC values ranging from 2-20% effluent (Appendix 2.4). The 48 hour EC_{50} values however, ranged from 5-31% effluent (Fig. 3.8) with corresponding salinities of 29.63-23.28 ppt (Appendix 2.4). A mean EC_{50} of 22% effluent was recorded and a C.V of 34%. There were no indications of seasonally influenced responses for any tests conducted with chlorinated secondary treated effluent, but it should be emphasised that such influences were recorded for test conducted with the reference toxicants.

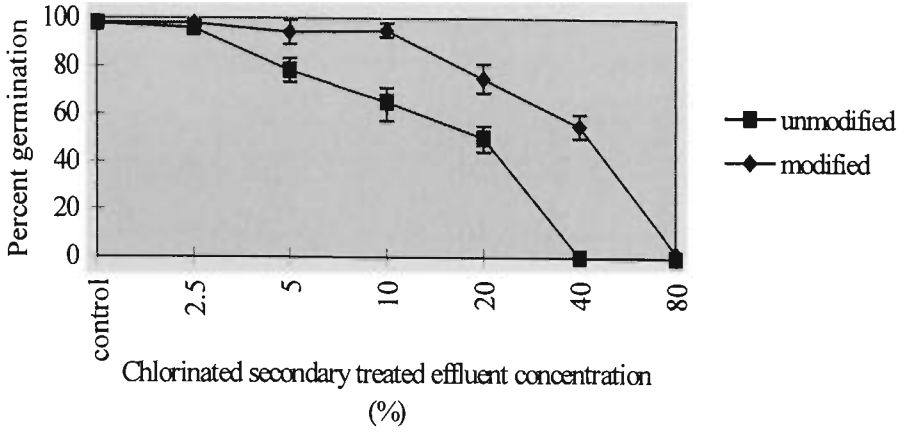
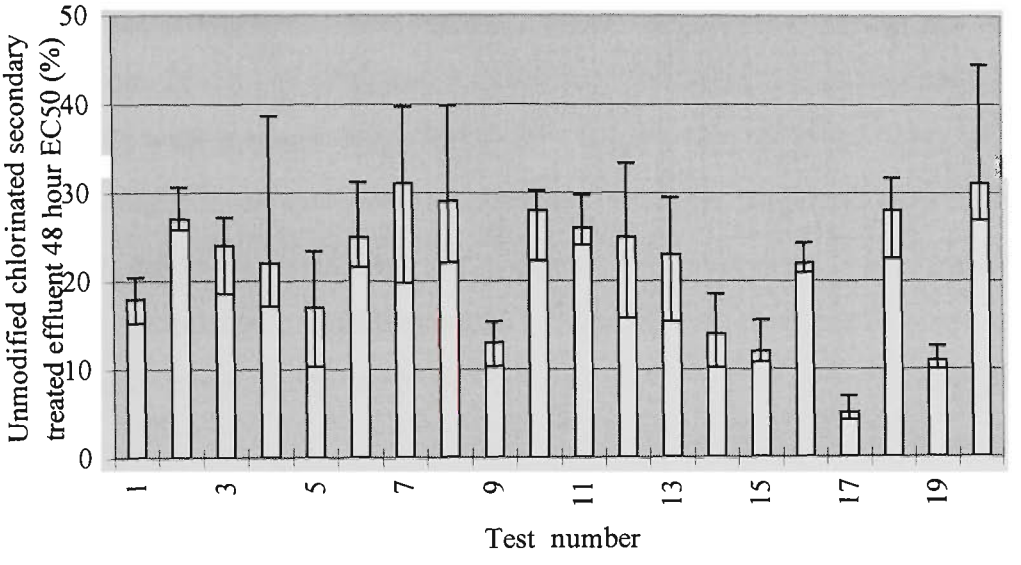


Fig. 3.7 Germination (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* zygotes exposed to modified and unmodified chlorinated secondary treated effluent.

Table 3.4 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Phyllospora comosa* zygotes exposed to modified (mod) and unmodified (un) chlorinated secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	4.975	7.752	158.62	183.56	0.000	0.000
Error	18	0.102	0.093				
Within Subjects							
Test	19	0.356	0.109	19.77	12.11	0.000	0.000
Treatment × Test	95	0.148	0.081	8.20	9.01	0.000	0.000
Error	342	0.018	0.009				

(a)



(b)

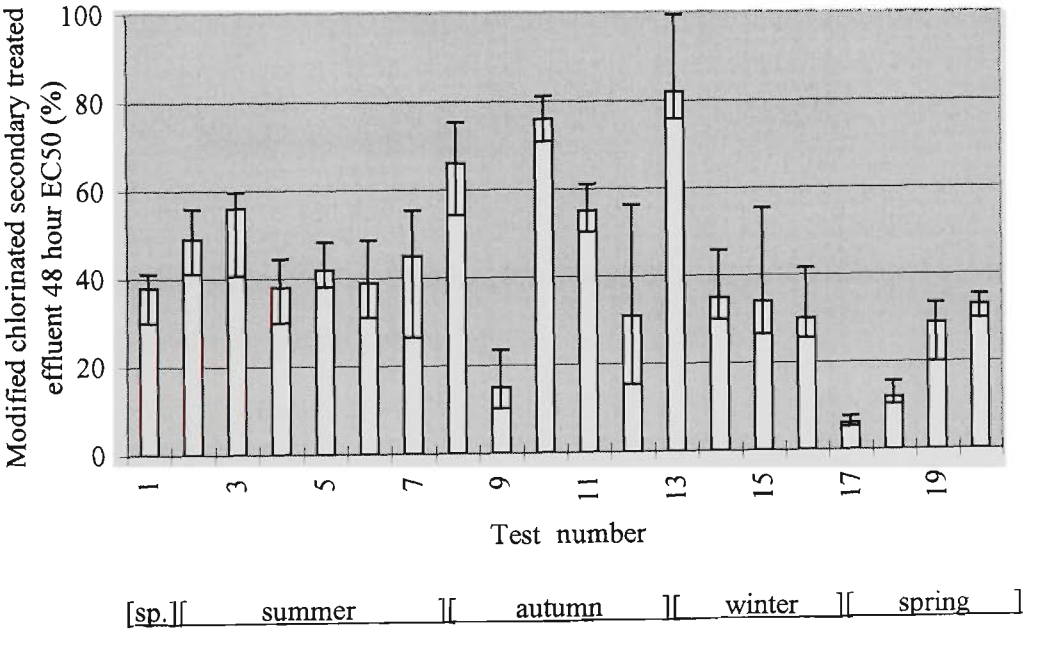


Fig. 3.8 Response over time of *Phyllospora comosa* zygotes exposed to unmodified (a) and modified (b) chlorinated secondary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

Salinity

Salinity reduction exerted significant effects on germinating *P. comosa* zygotes (Fig. 3.9) for all tests conducted. The NOEC values ranged from 28-20 ppt and LOEC values ranged from 26-18 ppt (Appendix 2.5). The 48 hour EC_{50} values ranged from 24-15 ppt (Fig. 3.10) with a mean EC_{50} value of 19 ppt and a C.V. of 13%. *P. comosa* zygotes exhibited significant variation in response to salinity reduction over time (Table 3.5) and a further ANOVA indicated germination response may have been due to seasonal variation. A post hoc significance test (Tukey) indicated that zygotes were significantly more sensitive to salinity reduction in summer ($P<0.05$) than any other season.

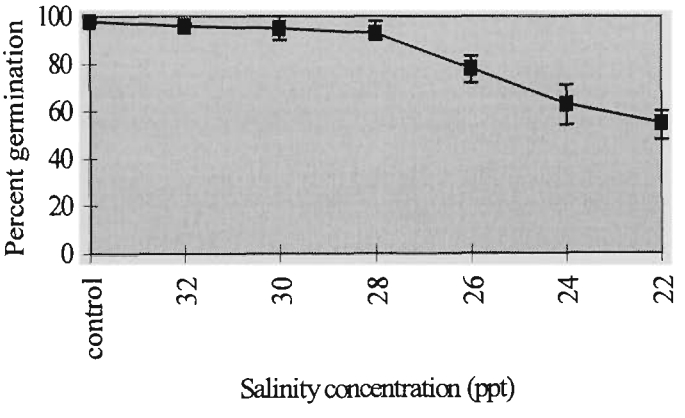


Fig. 3.9 Germination (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* zygotes exposed to varying salinity.

Table 3.5 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Phyllospora comosa* zygotes exposed to treatments of reduced salinity for 48 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	1.112	37.56	0.000
Error	18	0.030		
Within Subjects				
Test	19	0.010	8.33	0.000
Treatment × Test	95	0.003	4.99	0.000
Error	342	0.000		

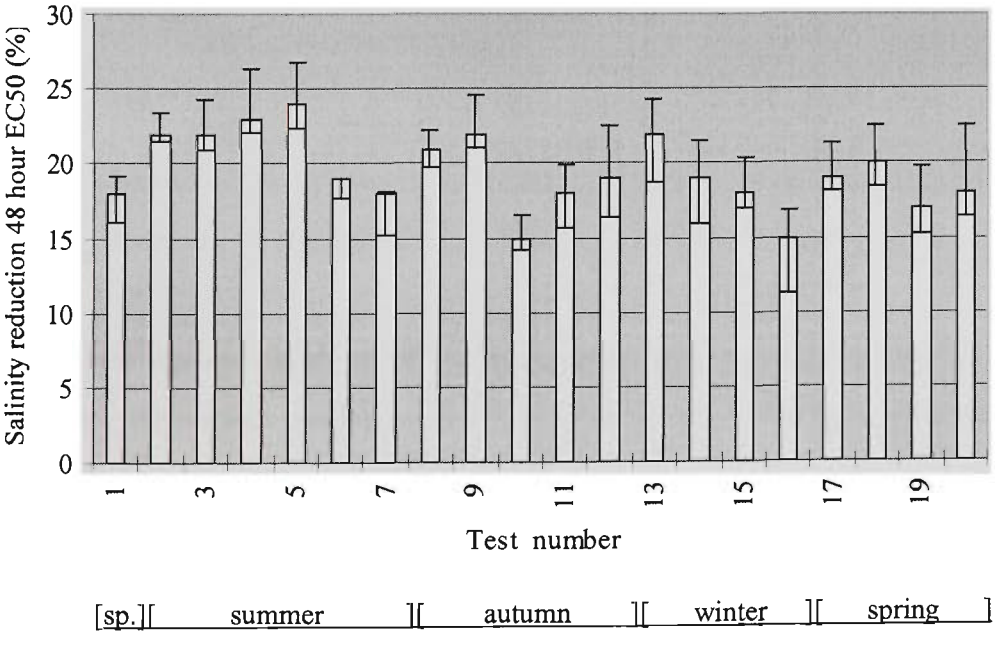
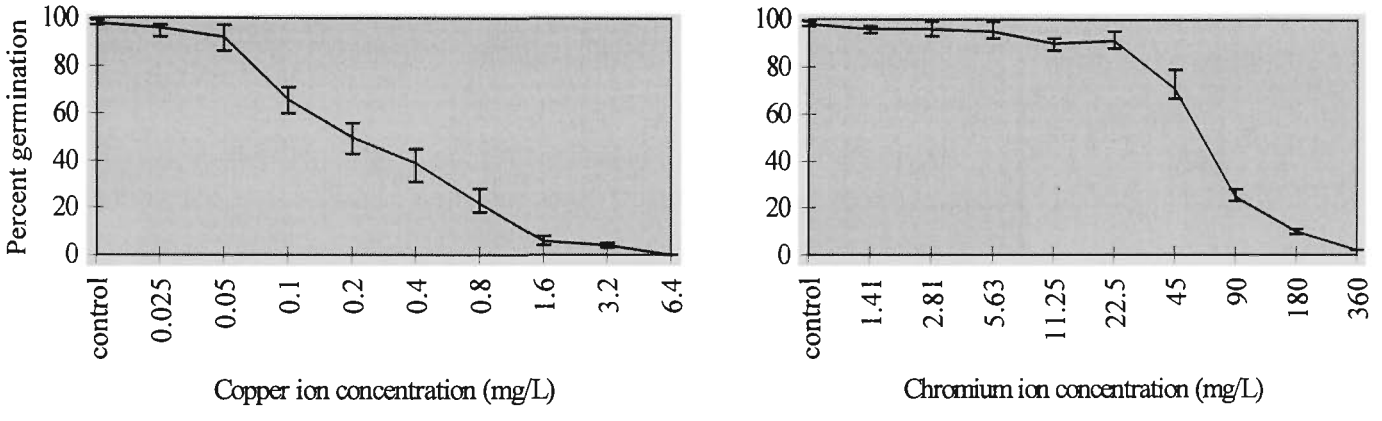


Fig. 3.10 Response over time of *Phyllospora comosa* zygotes exposed to treatments of reduced salinity for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

3.2.2 *Hormosira banksii*

Reference toxicants

Both reference toxicants, copper and chromium, exerted significant effects on germinating *H. banksii* zygotes (Fig 3.11). The recorded NOEC values for zygotes cultured in copper treatments ranged from 0.02-0.09 mg/L and LOEC values ranged from 0.04-0.18 mg/L copper for the tests conducted (Appendix 2.6). The NOEC values for zygotes cultured in chromium treatments ranged from 5.6-22.5 mg/L and LOEC values ranged from 11.3-45.0 mg/L chromium (Appendix 2.6). The 48 hour EC_{50} values for copper tests ranged from 0.09-0.27 mg/L copper (Fig. 3.12) with a mean EC_{50} value of 0.18 mg/L and C.V. of 31%. For chromium tests, EC_{50} values ranged from 41.2-98.7 mg/L chromium (Fig. 3.12) with a mean EC_{50} of 69.8 mg/L and a C.V. of 21%.

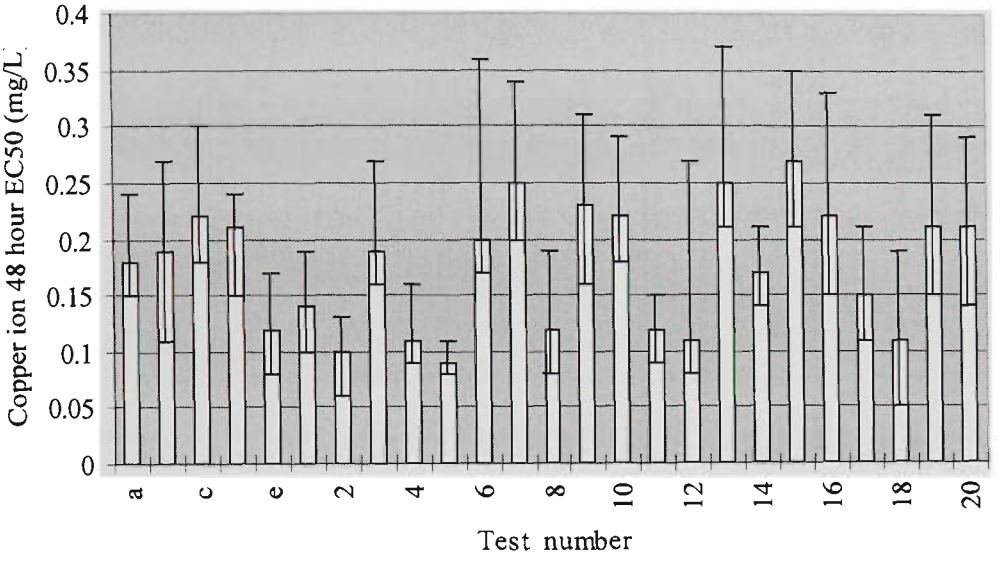


(a) (b)

Fig 3.11 Germination (Mean ± Standard Error) (n = 4) of *Hormosira banksii* zygotes exposed to copper (a) and chromium (b) for 48 hours.

Analyses of variance suggested that responses of *H. banksii* zygotes to both copper and chromium treatments varied over time (Table 3.6). Further analyses suggested that variations in response may have been due to seasonal influences as a significant interaction between treatments and test dates existed for zygotes cultured in both copper ($P<0.05$, $F=23.41$, $df=3$) and chromium ($P<0.05$, $F=21.58$, $df=3$). A post hoc significance test (Tukey) indicated significant differences between zygote responses to copper in summer (where sensitivity was amplified), winter and spring/autumn, but not

(a)



(b)

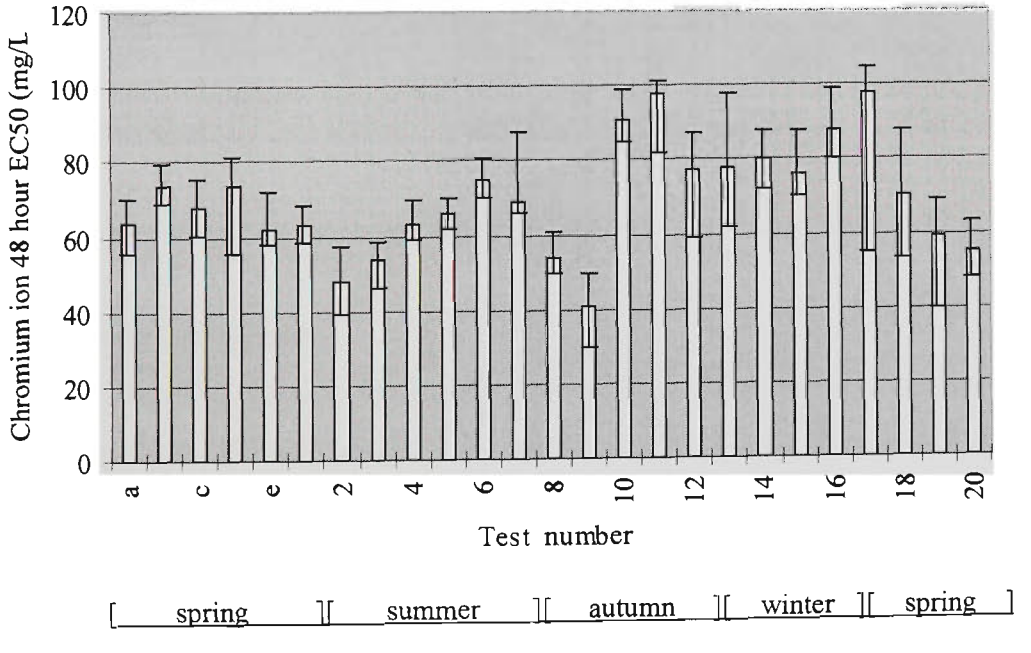


Fig. 3.12 Response over time of *Hormosira banksii* zygotes exposed to copper (a) and chromium (b) for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

between spring and autumn. For zygotes cultured in chromium, Tukey's test indicated significant differences in response in spring/summer and winter/autumn, but not between winter and autumn, and spring and summer where sensitivity was greatest.

Table 3.6 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 25) data of *Hormosira banksii* exposed to copper zygotes (Cu) and chromium (Cr) for 48 hours.

Factor	df	MS		F ratio		P	
		Cu	Cr	Cu	Cr	Cu	Cr
Between Subjects							
Treatment	9	1.906	2.818	233.33	120.88	0.002	0.000
Error	30	0.008	0.023				
Within Subjects							
Test	24	0.112	0.446	7.48	6.32	0.000	0.001
Treatment × Test	216	0.055	0.299	3.66	4.21	0.003	0.000
Error	720	0.015	0.071				

Primary treated effluent

Hormosira banksii zygotes illustrated significant sensitivity to treatments of modified and unmodified primary treated effluent. For simultaneous tests of modified and unmodified effluent, there were no significant differences in response of *H. banksii* zygotes within tests (Fig. 3.13), except for test numbers 8 and 15. For this reason, the results for modified and unmodified primary treated effluent were pooled together for statistical analyses. The NOEC values for primary treated effluent ranged from 1-4% effluent and LOEC values ranged from 2-8% effluent (Appendix 2.7). The EC_{50} values for germinating zygotes exposed to primary treated effluent ranged from 3-17% effluent (Fig. 3.14) with a mean EC_{50} value of 10% effluent and C.V. of 40%. A repeated measures ANOVA indicated that a significant interaction between treatment and test date

existed (Table 3.14), and this was due to fluctuating toxicities of the effluent tested and possibly also to seasonal influences. Salinities of corresponding unmodified effluent treatment EC_{50} values have been included in Appendix 2.7.

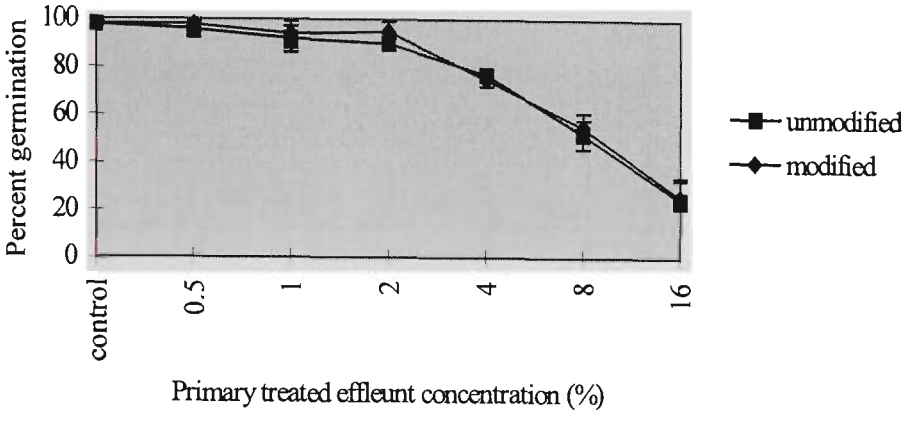
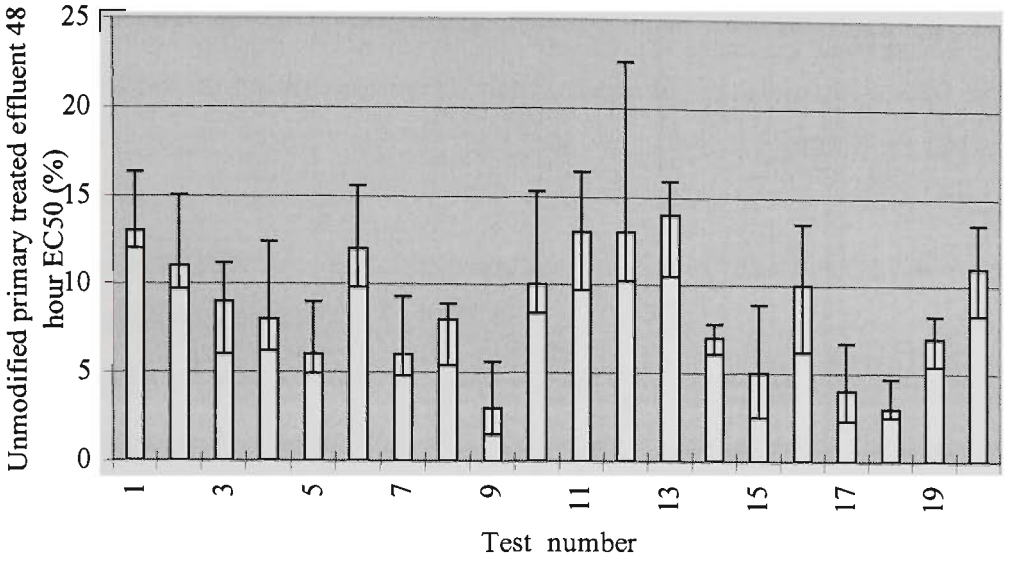


Fig. 3.13 Germination (Mean ± Standard Error) (n = 4) of *Hormosira banksii* zygotes exposed to modified and unmodified primary treated effluent.

Table 3.7 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 40) data of *Hormosira banksii* zygotes exposed to primary treated effluent for 48 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	0.201	84.82	0.000
Error	18	0.002		
Within Subjects				
Test	39	0.016	13.147	0.000
Treatment × Test	195	0.004	3.310	0.000
Error	702	0.001		

(a)



(b)

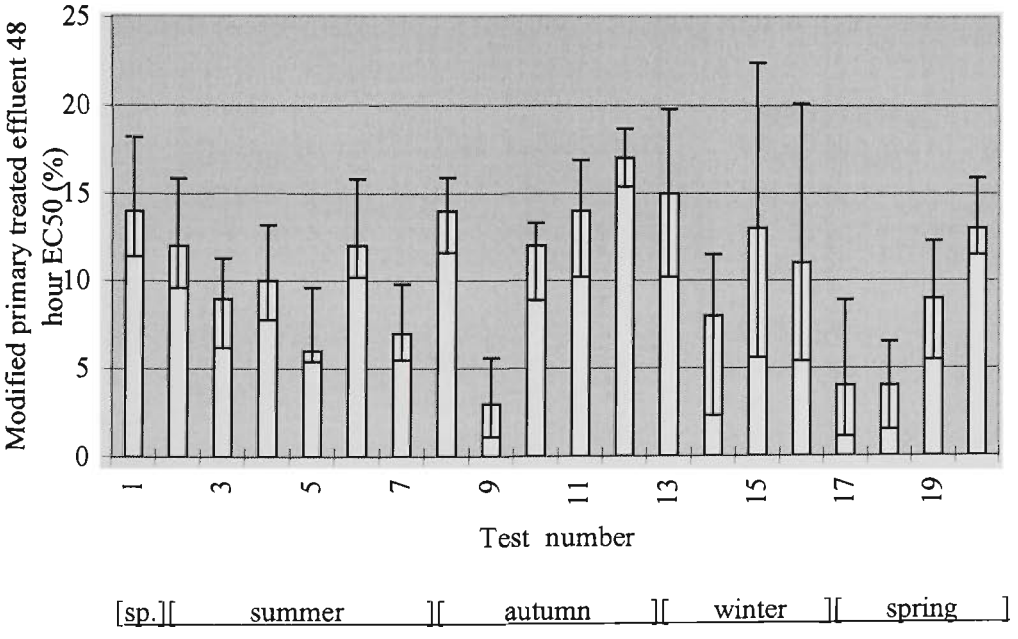
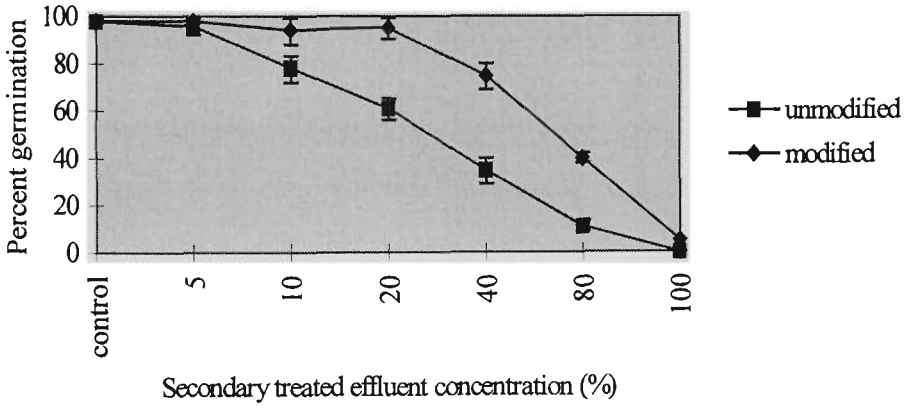


Fig. 3.14 Response over time of *Hormosira banksii* zygotes exposed to unmodified (a) and modified (b) primary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Secondary treated effluent

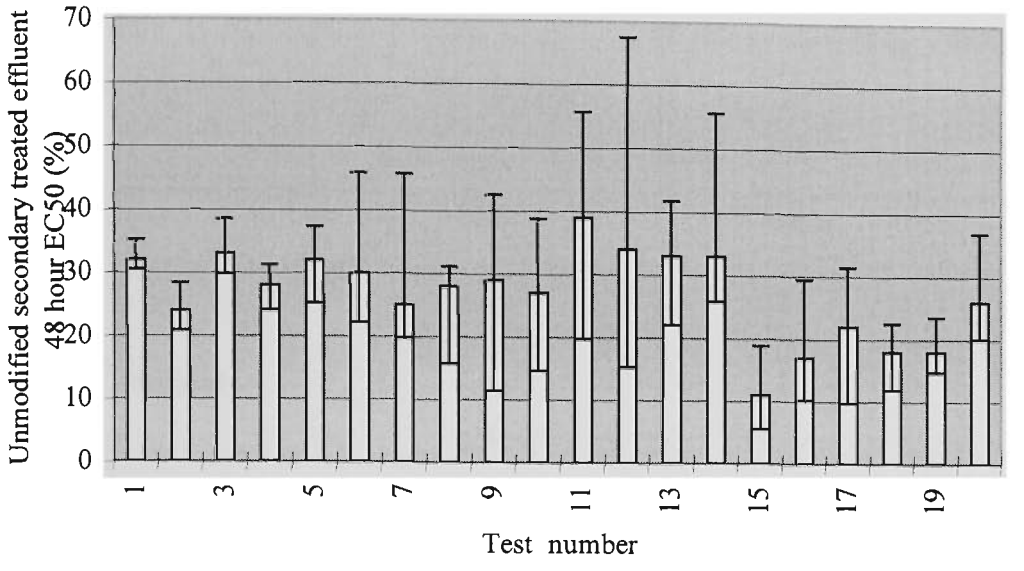
Treatments of secondary treated effluent exerted significant effects on germinating *H. banksii* zygotes for tests conducted with modified and unmodified effluent (Fig. 3.15). These responses were also significantly different to those recorded for primary treated effluent. Overall, percentage germination of zygotes exposed to unmodified secondary treated effluent was lower than for those exposed to modified effluent. The NOEC values for modified effluent ranged from 10-40% effluent and LOEC values ranged from 20-80% effluent (Appendix 2.8). The EC_{50} values for these tests ranged from 48->100% effluent (Fig. 3.16) with a mean 48 hour EC_{50} of 70% and a C.V. of 20% (EC_{50} values of >100% were taken to equal 100% for these calculations). For unmodified secondary treated effluent, the NOEC values ranged from 2-6% and LOEC values ranged from 4-12% effluent (Appendix 2.8). The EC_{50} values ranged from 11-39% effluent (Fig 3.16) with corresponding salinities of 28.88-21.48 ppt (Appendix 2.8). A mean EC_{50} of 27% effluent was recorded and a C.V. of 26%. A repeated measures ANOVA indicated that



zygote response varied over time (Table 3.8) and this was probably due to the fluctuating toxicity of the effluent and also seasonal influences. over the test period.

Fig. 3.15 Germination (Mean ± Standard Error) (n = 4) of *Hormosira banksii* zygotes exposed to modified and unmodified secondary treated effluent.

(a)



(b)

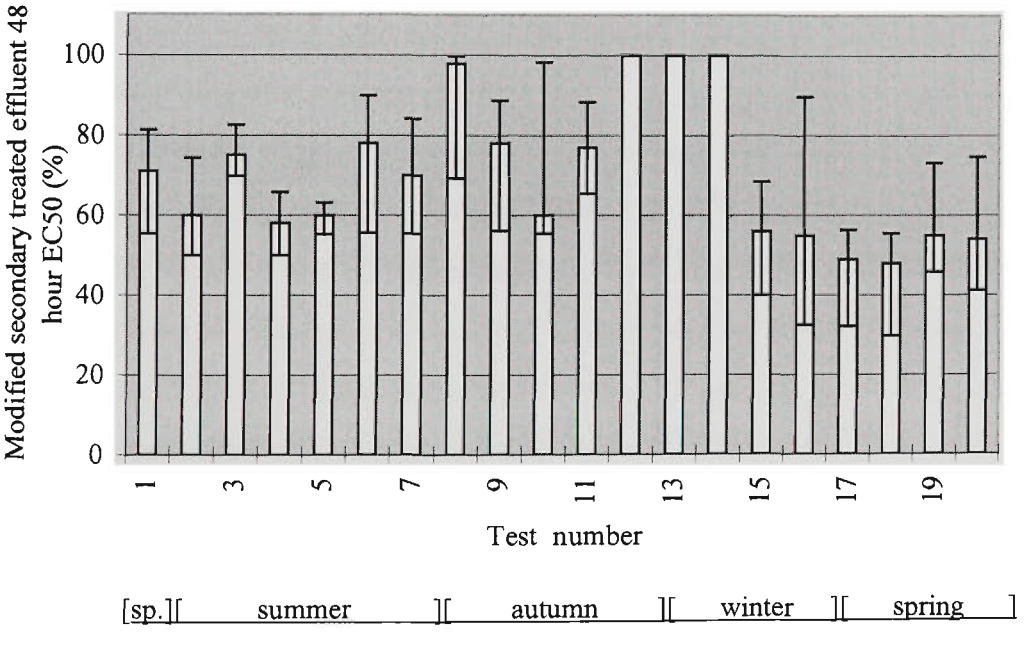


Fig. 3.16 Response over time of *Hormosira banksii* zygotes exposed to unmodified (a) and modified (b) secondary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 3.8 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Hormosira banksii* zygotes exposed to modified (mod) and unmodified (un) secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	0.501	0.269	72.38	81.25	0.000	0.000
Error	18	0.007	0.003				
Within Subjects							
Test	19	0.069	0.178	23.12	22.25	0.000	0.000
Treatment × Test	95	0.015	0.022	5.85	4.43	0.000	0.000
Error	342	0.003	0.005				

Chlorinated secondary treated effluent

Hormosira banksii zygotes exposed to chlorinated secondary treated effluent exhibited significant sensitivity to both modified and unmodified effluent (Fig. 3.17). The effects recorded for these tests were also significantly different to those recorded for primary and secondary treated effluent. For tests conducted with modified effluent, the NOEC values ranged from 5-20% effluent and LOEC values ranged from 10-40% effluent (Appendix 2.9). The recorded EC_{50} values ranged from 28-78% (Fig. 3.18) with a mean EC_{50} of 52% and C.V. of 34%. The NOEC values for tests conducted with unmodified effluent ranged from 2-5% effluent and LOEC values ranged from 4-10% effluent (Appendix 2.9). The EC_{50} values for unmodified effluent ranged from 12-37% (Fig.3.18) with corresponding salinities of 28.75-21.93 ppt. A mean EC_{50} of 23% and C.V. of 34% were also recorded. For all tests conducted over the test period there were consistently significant differences between zygotes exposed to both modified and unmodified treatments of any one batch of effluent tested. Zygote response also varied significantly

over time (Table 3.9). An interaction between test date and treatment was identified and caused by fluctuating toxicities of the effluent samples and possibly seasonal variations.

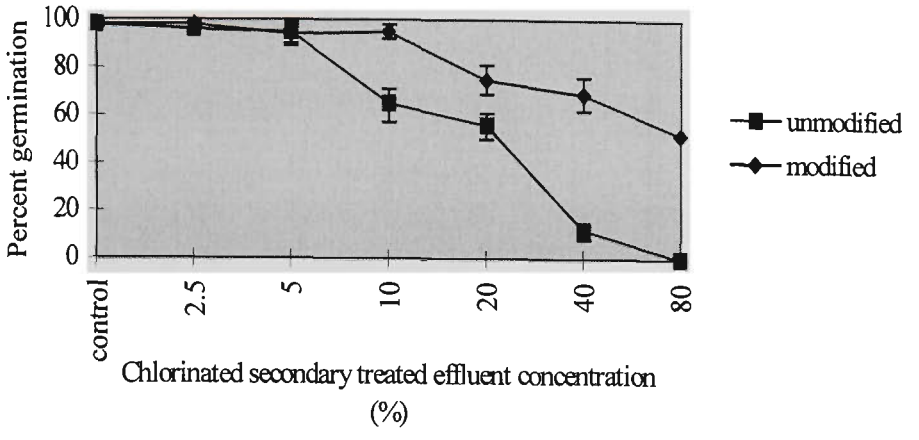
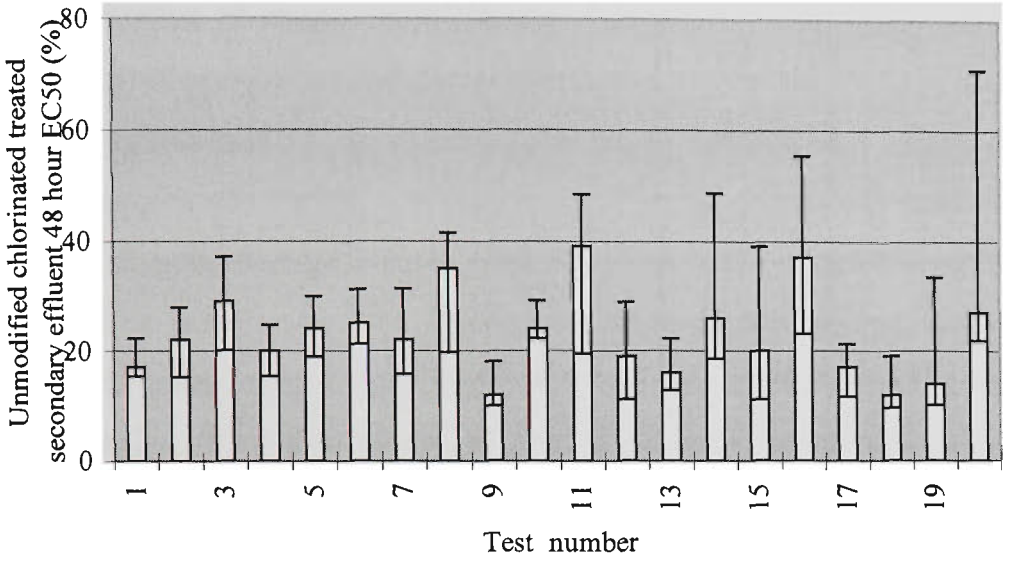


Fig. 3.17 Germination (Mean ± Standard Error) (n = 4) of *Hormosira banksii* zygotes exposed to modified and unmodified chlorinated secondary treated effluent.

Table 3.9 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Hormosira banksii* zygotes exposed to modified (mod) and unmodified (un) chlorinated secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	0.854	0.564	62.77	52.31	0.000	0.000
Error	18	0.014	0.019				
Within Subjects							
Test	19	0.049	0.089	24.52	19.25	0.000	0.000
Treatment × Test	95	0.009	0.025	4.85	8.46	0.000	0.000
Error	342	0.001	0.004				

(a)



(b)

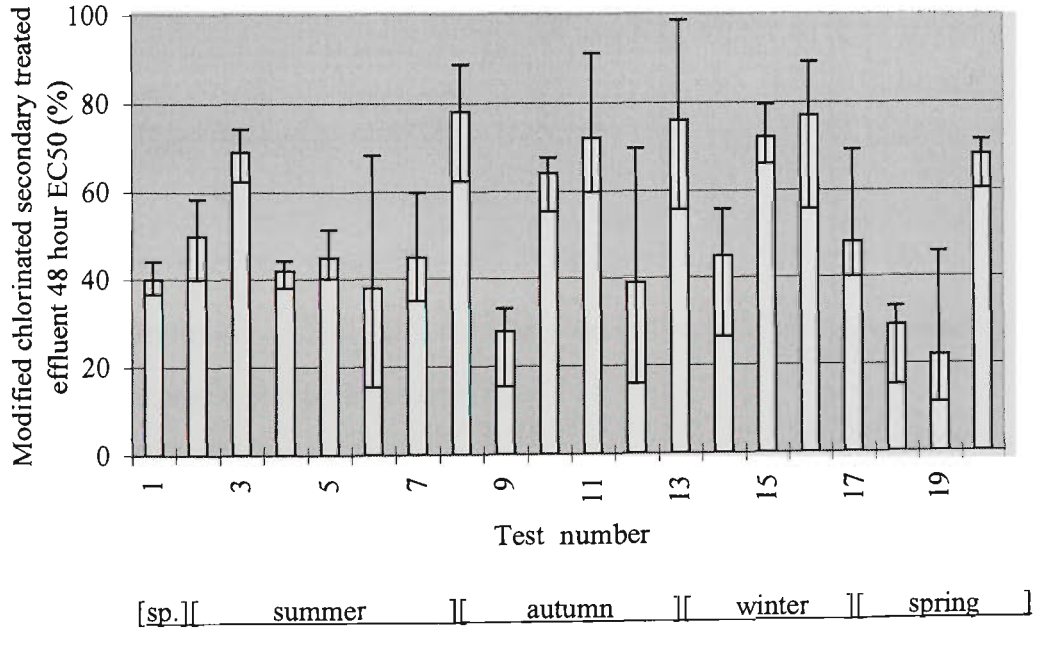


Fig. 3.18 Response over time of *Hormosira banksii* zygotes exposed to unmodified (a) and modified (b) chlorinated secondary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

Salinity

Reduced salinity exerted significant effects on germinating *H. banksii* zygotes (Fig. 3.19). Variation in zygote response was also evident over time (Table 3.10) with the suggestion of seasonal influences on germination ($P<0.05$, $F=22.5$, $df=3$). A post hoc (Tukey) significance test indicated no significant differences in zygote response between winter, autumn and spring, but significant differences between these three seasons and summer. Zygotes appeared most sensitive to treatments of reduced salinity in the warmer months. The NOEC values for zygotes exposed to reduced salinity ranged from 28-20 ppt and LOEC values ranged from 26-18 ppt (Appendix 2.10). The EC_{50} values ranged from 24-13 ppt (Fig. 3.20) with a mean EC_{50} of 19 ppt and C.V. of 16%.

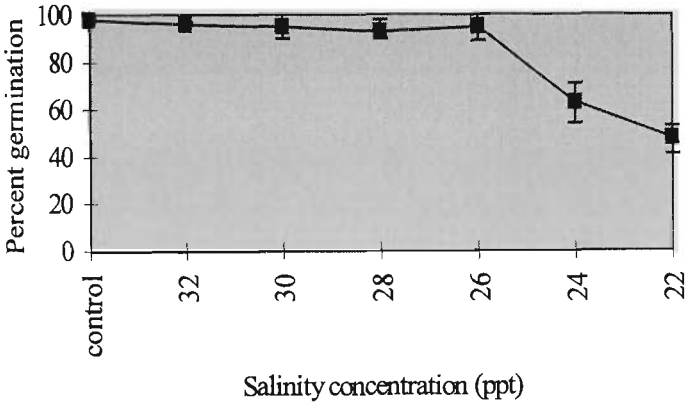


Fig. 3.19 Germination (Mean ± Standard Error) (n = 4) of *Hormosira banksii* zygotes exposed to varying salinity.

Table 3.10 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (n = 20) data of *Hormosira banksii* zygotes exposed to treatments of reduced salinity for 48 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	1.316	30.51	0.000
Error	18	0.043		
Within Subjects				
Test	19	0.022	8.774	0.000
Treatment × Test	95	0.004	2.003	0.000
Error	342	0.002		

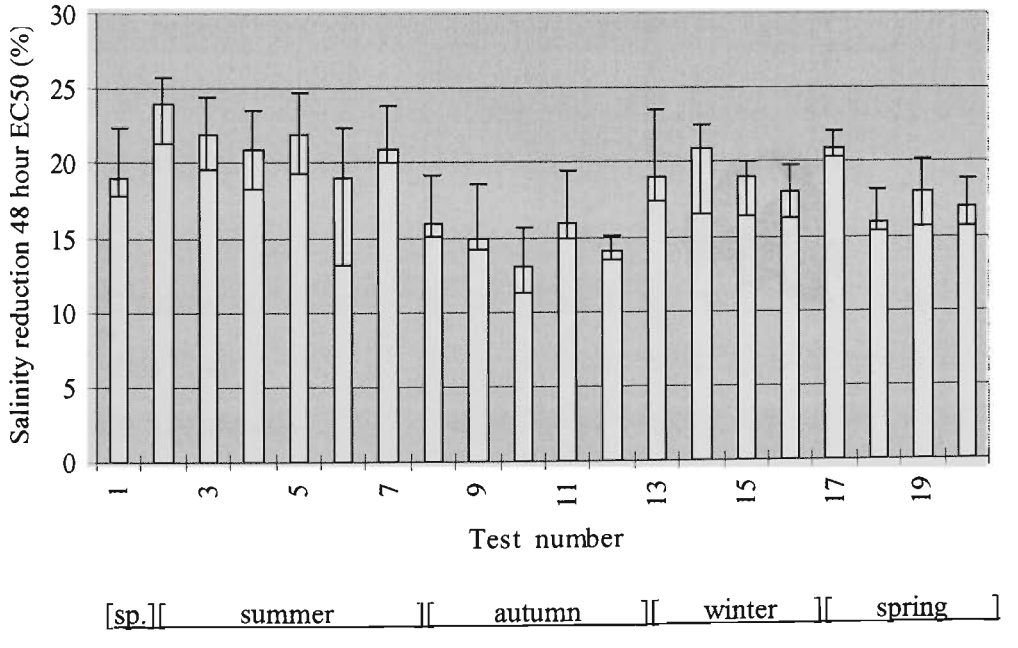


Fig. 3.20 Response over time of *Hormosira banksii* zygotes exposed to treatments of reduced salinity for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

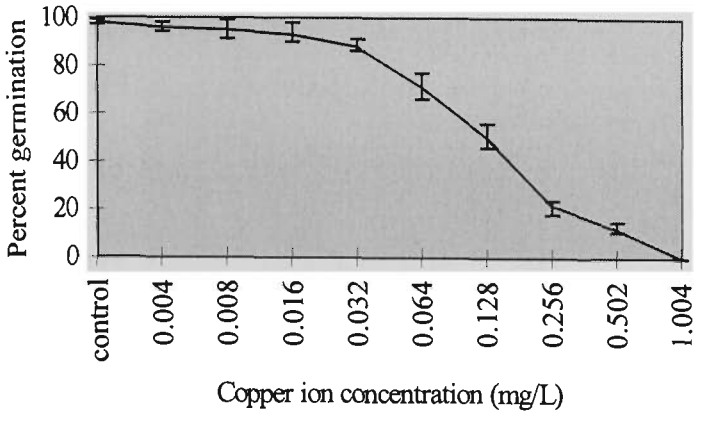
3.2.3 *Macrocystis angustifolia*

Two ecotoxicological test endpoints were investigated utilising *M. angustifolia* as the test species; germination and germination tube growth.

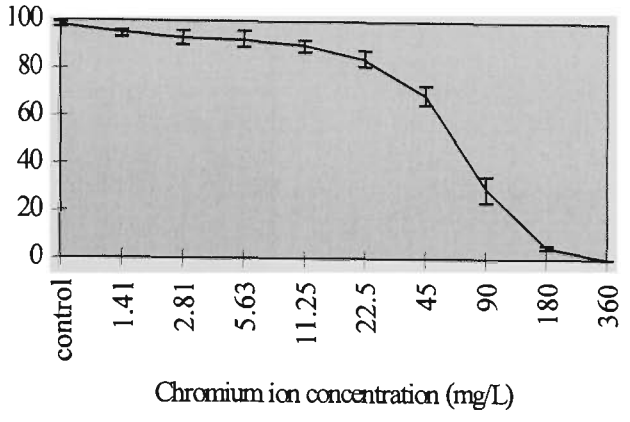
Reference toxicants

Both copper and chromium exerted significant effects on germination (Fig. 3.21) and growth of *M. angustifolia* spores. The NOEC values for spores exposed to copper ranged from 0.02-0.06 mg/L for the 48 hour germination bioassays and <0.0005-0.002 mg/L copper for the growth assays (Appendix 2.11). The LOEC values ranged from 0.04-0.12 mg/L for germination and from 0.0005-0.004 mg/L for growth (Appendix 2.11). The EC_{50} values ranged from 0.09-0.18 mg/L with a mean of 0.15 mg/L and C.V. of 19%, and 0.01-0.07 mg/L with a mean of 0.05 mg/L and C.V. of 38%, for germination and germination tube growth bioassays respectively (Fig. 3.22). ANOVA indicated significant differences in response of spores to copper concentrations over time (Table 3.11). A post hoc significance test (Tukey) indicated that response sensitivity of spores in summer was significantly greater compared to any other season (there were no significant differences in response between winter, autumn and spring). Analysis of variance was also conducted to determine any significant differences between germination and growth responses of spores for each test conducted, and it was found that the growth endpoint was significantly more sensitive than the germination endpoint.

The NOEC values recorded for *M. angustifolia* spores exposed to chromium ranged from 5.65-22.5 mg/L and 2.82-11.3 mg/L chromium for germination and growth bioassays respectively (Appendix 2.12). The LOEC values ranged from 11.3-45.0 mg/L for germination assays and from 5.65-22.5 mg/L for growth assays (Appendix 2.12). The EC_{50} values ranged from 49.7-90.4 mg/L with a mean of 66.1 mg/L and C.V. of 14%, and 22.3-62.7 mg/L with a mean of 41.2 mg/L and C.V. of 26% for germination and growth respectively (Fig 3.23). There was significant variation in response of spores to the treatments over time (Table 3.12) and as for treatments of copper, spores exhibited greatest sensitivity in summer. It was also found that the germination endpoint was the least sensitive of the two tested.



(a)



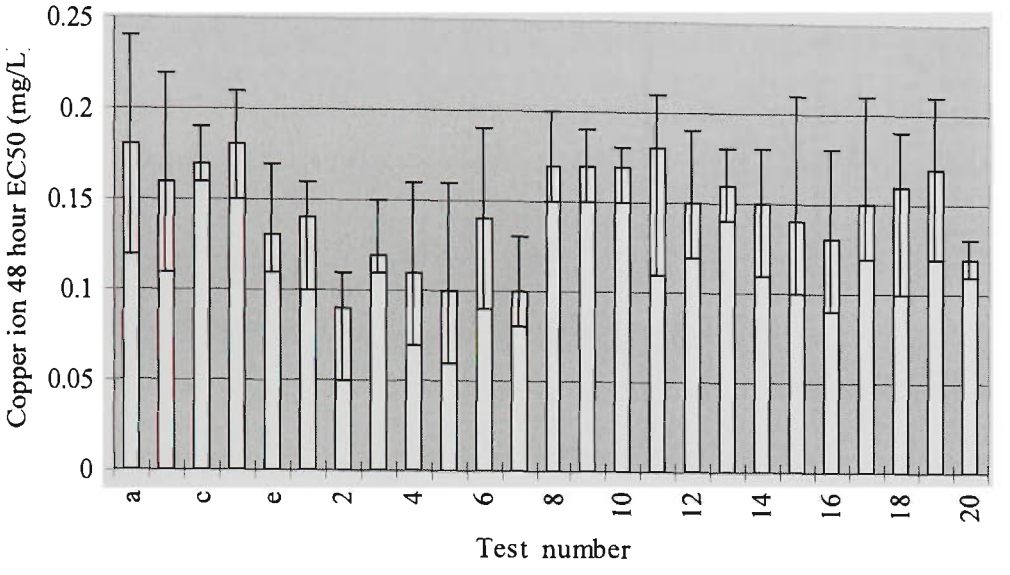
(b)

Fig. 3.21 Germination (Mean ± Standard Error) (n = 4) of *Macrocystis angustifolia* spores exposed to copper (a) and chromium (b) for 48 hours.

Table 3.11 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 25) and $\log_{10}$ transformed germination tube growth (growth) (n = 25) data of *Macrocystis angustifolia* spores exposed to copper for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	9	0.960	0.331	241.63	353.62	0.000	0.000
Error	30	0.004	0.000				
Within Subjects							
Test	24	0.022	0.009	11.07	8.87	0.000	0.000
Treatment × Test	216	0.008	0.004	4.23	5.14	0.000	0.000
Error	720	0.002	0.001				

(a)



(b)

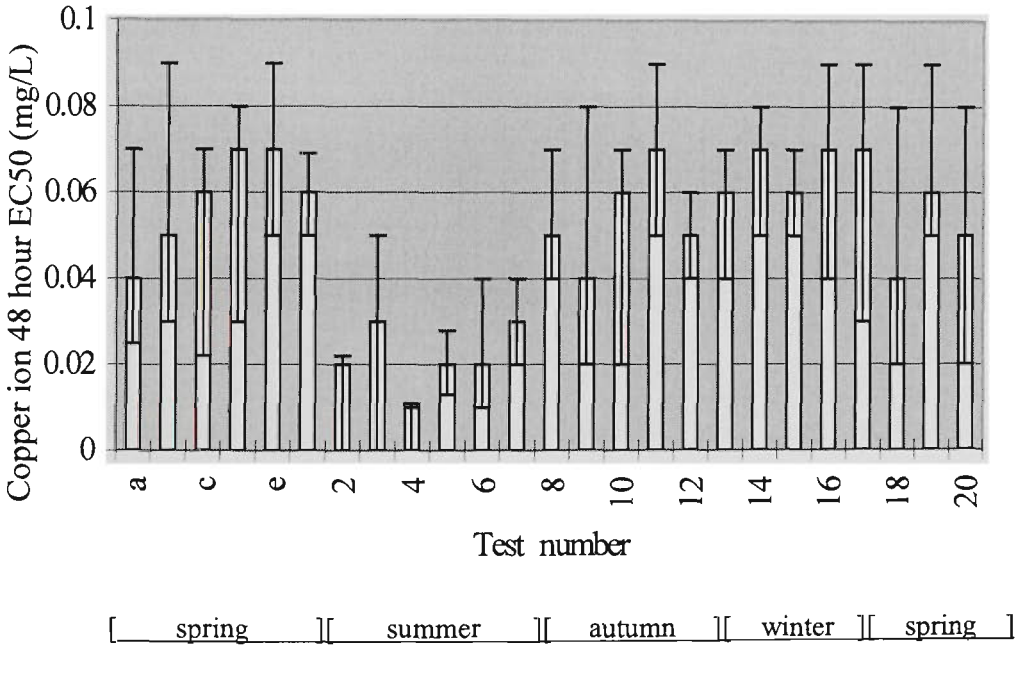
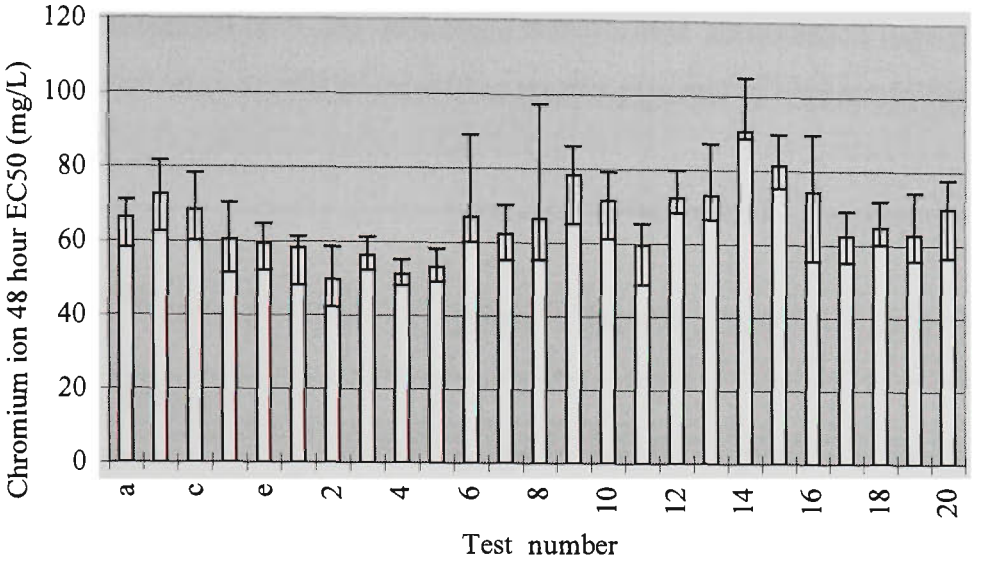


Fig. 3.22 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to copper for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

(a)



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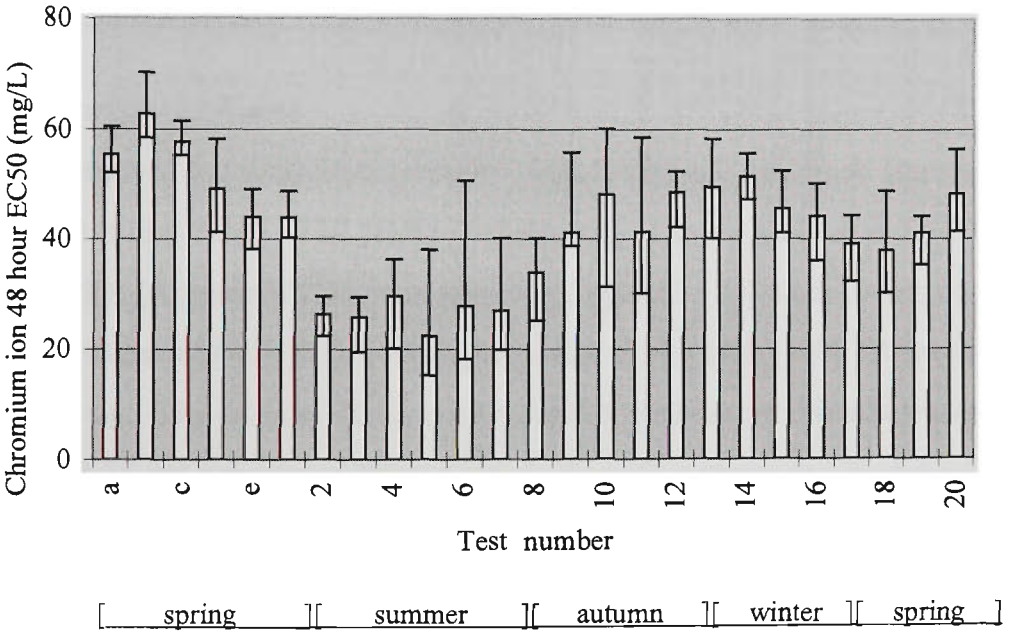


Fig. 3.23 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to chromium for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 3.12 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 25) and log_{10} transformed germination tube growth (growth) (n = 25) data of *Macrocystis angustifolia* spores exposed to chromium for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	9	0.624	0.429	223.1	143.5	0.000	0.000
Error	30	0.045	0.057				
Within Subjects							
Test	24	0.009	0.011	8.514	3.966	0.000	0.000
Treatment × Test	216	0.007	0.007	4.997	2.989	0.000	0.000
Error	720	0.000	0.002				

Primary treated effluent

Spores exposed to unmodified primary treated effluent exhibited significant sensitivity to treatments (Fig. 3.24). The NOEC values ranged from 1-2% and 0.5-2% effluent for the germination (Appendix 2.13) and growth (Appendix 2.14) endpoints respectively and the LOEC values ranged from 2-4% (Appendix 2.13) and 1-4% (Appendix 2.14) effluent respectively. The 48 hour EC_{50} values for the germination bioassays ranged from 3-10% (30.00-28.75 ppt salinity) effluent (Fig. 3.24) with a mean of 6% and a C.V. of 42%. For the growth bioassays, EC_{50} values ranged from 2-10% (31.00-29.00 ppt salinity) effluent, with a mean of 4% and C.V. of 40% (Fig. 3.25). Corresponding salinities at each of these EC_{50} values have been listed in Appendices 2.13 and 2.14. ANOVA between germination and growth endpoints indicated no significant difference in response at any one time (except for tests 3,12,13 and 14, $P<0.05$). Repeated measures ANOVA indicated significant differences in response of spores over time (Table 3.13).

Table 3.13 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and log_{10} transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to unmodified primary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	5	0.555	0.898	284.2	224.5	0.002	0.000
Error	18	0.001	0.000				
Within Subjects							
Test	19	0.012	0.033	5.995	8.250	0.001	0.000
Treatment × Test	95	0.007	0.015	3.510	3.750	0.000	0.001
Error	342	0.002	0.004				

The NOEC values for modified primary treated effluent ranged from 1-4% effluent germination and 0.5-4% effluent for growth assays (Appendices 2.13 & 2.14). The LOEC values for germination and growth bioassays were 2-8% and 1-8% effluent respectively (Appendices 2.13 & 2.14). ANOVA indicated no significant differences in response between the germination and growth endpoints for these tests and repeated measures ANOVA indicated significant interactions between test date and treatment. These however, were not due to seasonal influences (Table 3.14). The 48 hour EC_{50} values for the germination assays ranged from 3-11% with a mean EC_{50} of 6% and C.V. of 45% and 2-9% with a mean of 5% and C.V. of 41% for the growth assays (Fig. 3.26). Comparing differences between modified and unmodified effluent samples at any one time indicated minor differences for each toxicity test endpoint. There were no significant differences in germination response between the unmodified and modified effluent treatments except for test 14 ($P<0.05$, $F=56.7$, $df=1$). For the growth assays there

were also no significant variations in response between the two treatments at any one time, with the exception of test 12 ($P<0.05$, $F=417.2$, $df=1$).

Table 3.14 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and $\log_{10}$ transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to modified primary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	5	0.421	0.745	233.49	245.81	0.000	0.000
Error	18	0.025	0.048				
Within Subjects							
Test	19	0.009	0.029	6.147	8.995	0.000	0.000
Treatment × Test	95	0.005	0.012	4.857	4.001	0.000	0.000
Error	342	0.000	0.003				

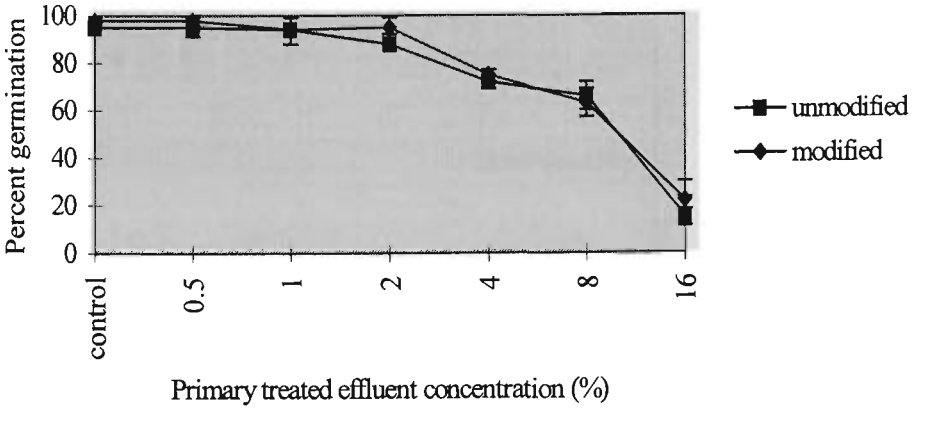
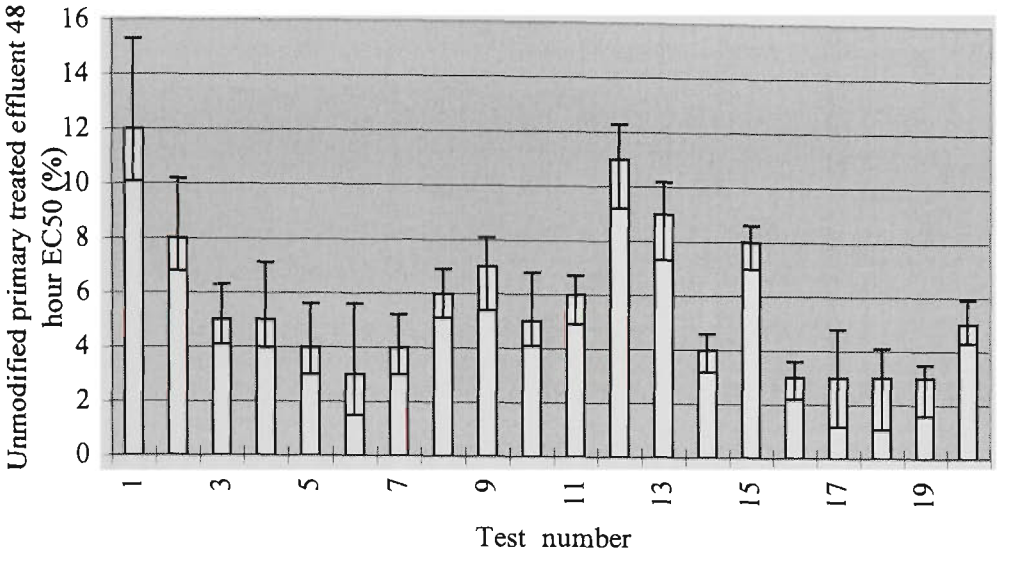


Fig. 3.24 Germination (Mean ± Standard Error) (n = 4) of *Macrocystis angustifolia* spores exposed to modified and unmodified primary treated effluent.

(a)



(b)

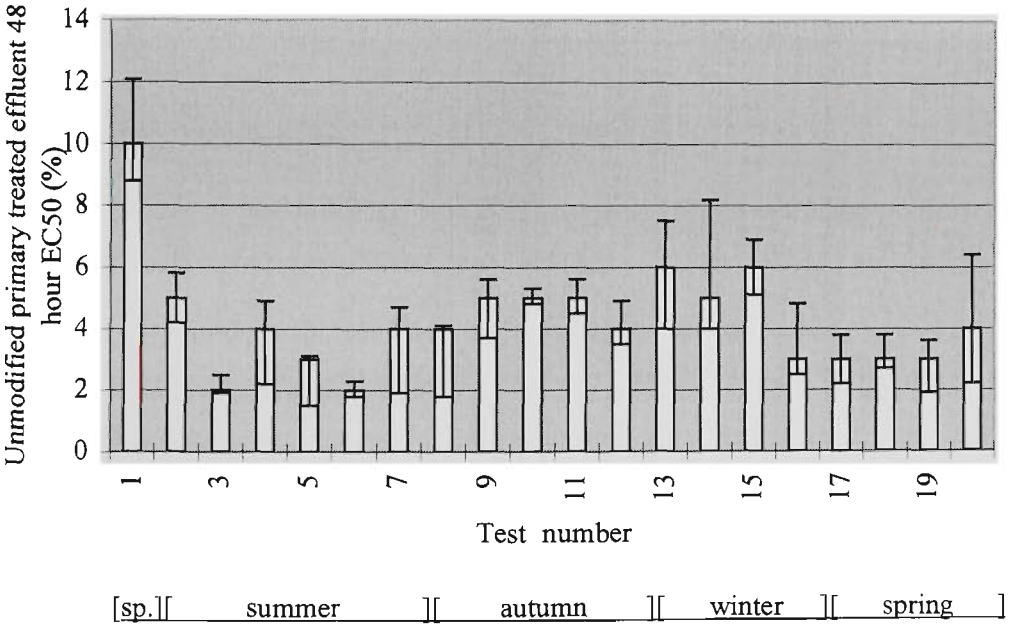
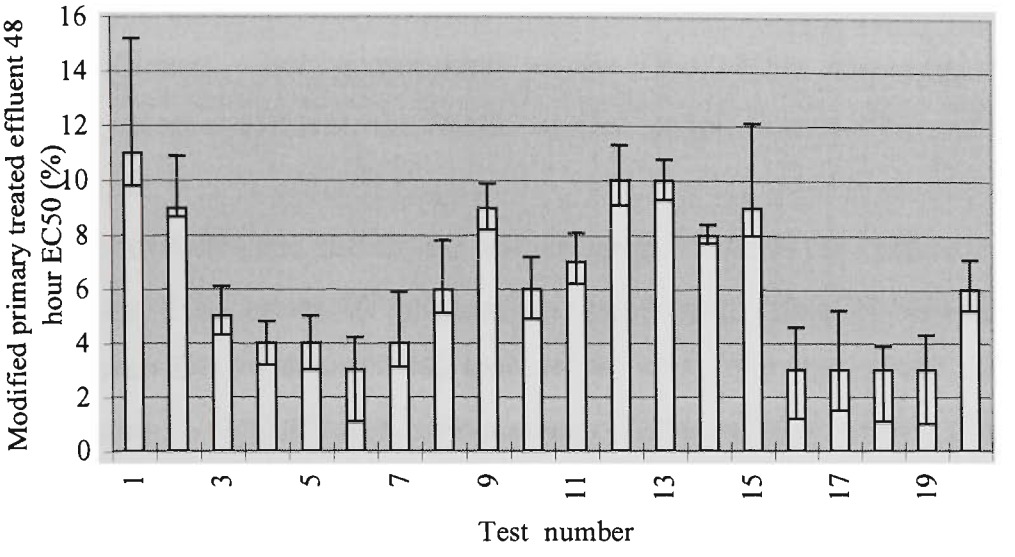


Fig. 3.25 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to unmodified primary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

(a)



(b)

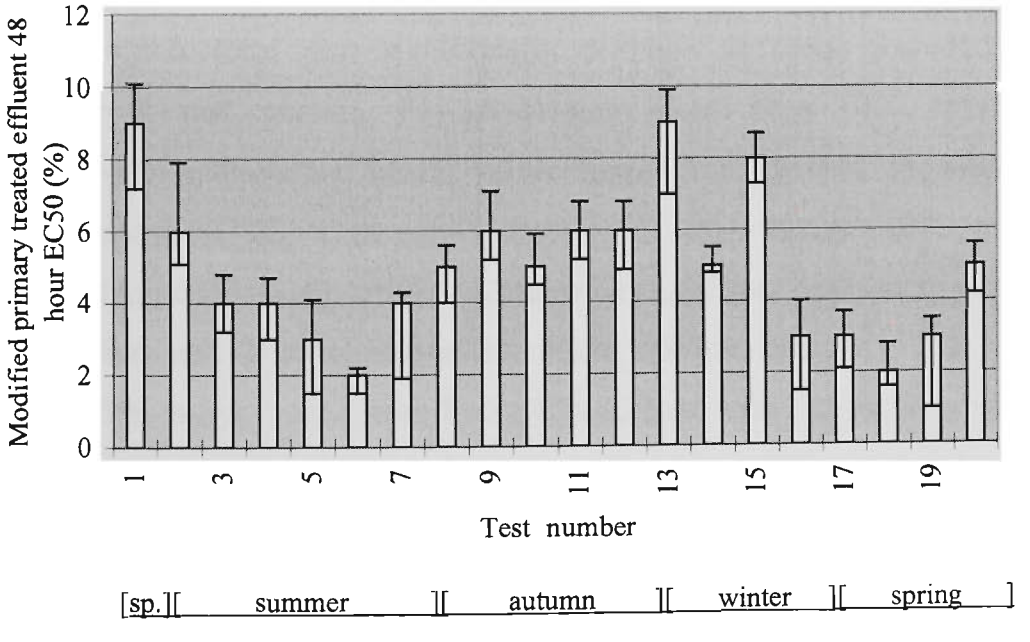


Fig. 3.26 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to modified primary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

Secondary treated effluent

Secondary treated effluent exerted significant effects on *M. angustifolia* spores and these effects were significantly different to those recorded for assays conducted with primary treated effluent. For germination assays (Fig. 3.27) conducted with unmodified secondary treated effluent, the NOEC values ranged from 4-10% and the LOEC values ranged from 8-20% effluent (Appendix 2.15). For the growth assays, the NOEC values ranged from 4-8% and the LOEC values ranged from 8-16% effluent (Appendix 2.16). The 48 hour EC_{50} values for germination assays ranged from 27-51% effluent (Fig. 3.28), with a mean EC_{50} value of 35% and a C.V. 19%. For the growth assays, EC_{50} values ranged from 15-31% effluent, with a mean of 22% and C.V. of 24% (Fig. 3.28). ANOVA indicated significant differences in both germination and growth response of spores exposed to the same unmodified treatments. Repeated measures ANOVA indicated significant interaction between test date and treatment which may have been caused by seasonal influences together with fluctuating effluent toxicity (Table 3.15).

Modified secondary treated effluent also exerted significant effects on *M. angustifolia* spores, which were also significantly different to those recorded for unmodified secondary treated effluent. For germination assays (Fig. 3.27), NOEC values ranged from 10-40% effluent and LOEC values ranged from 20-80% (Appendix 2.15), and for growth assays NOEC values ranged from 5-40% effluent and LOEC values ranged from 10-80% (Appendix 2.16). ANOVA indicated significant differences in response between germination and growth assays for each effluent sample. The EC_{50} values for germination assays ranged from 75->100% effluent with a mean EC_{50} value of 92% and a C.V. of 10% (for these calculations concentrations >100% were taken as 100%). For growth assays, EC_{50} values ranged from 58-95% effluent with a mean of 76% and a C.V. of 20%. Repeated measures ANOVA indicated significant interactions between test date and treatment and like with unmodified effluent these may have been caused by temporal variability together with fluctuating effluent toxicity (Table 3.16).

Table 3.15 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and $\log_{10}$ transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to unmodified secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	5	1.225	745.25	117.45	143.67	0.000	0.000
Error	18	0.012	0.088				
Within Subjects							
Test	19	0.485	66.25	44.09	12.67	0.000	0.000
Treatment × Test	95	0.087	14.51	7.95	2.99	0.000	0.000
Error	342	0.011	5.25				

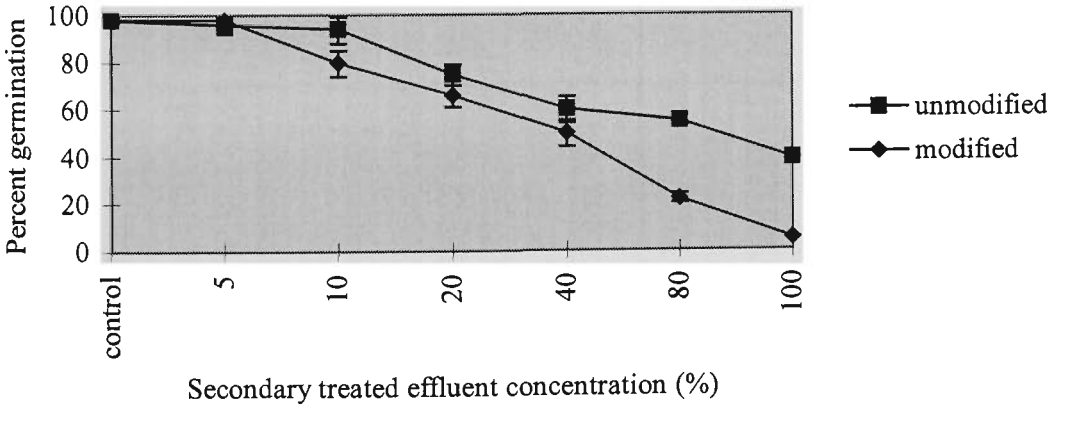
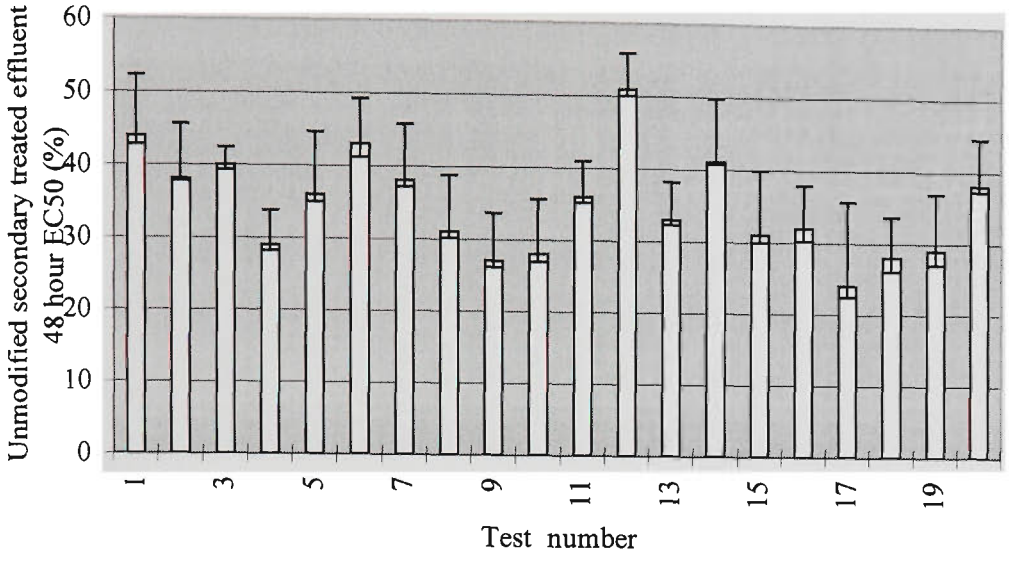


Fig. 3.27 Germination (Mean ± Standard Error) (n = 4) of *Macrocystis angustifolia* spores exposed to modified and unmodified secondary treated effluent.

(a)



(b)

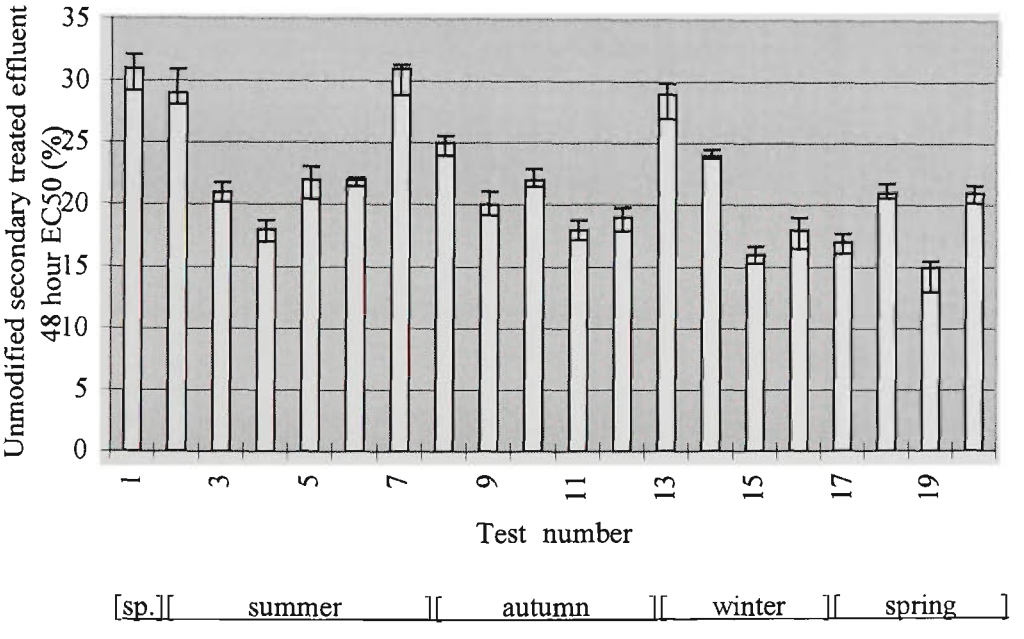
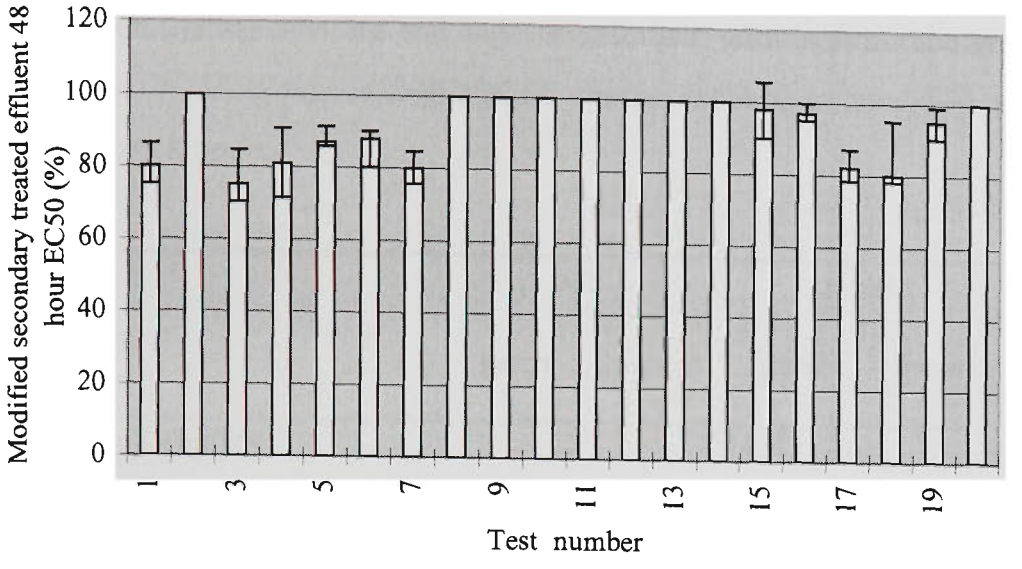


Fig. 3.28 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to unmodified secondary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

(a)



(b)

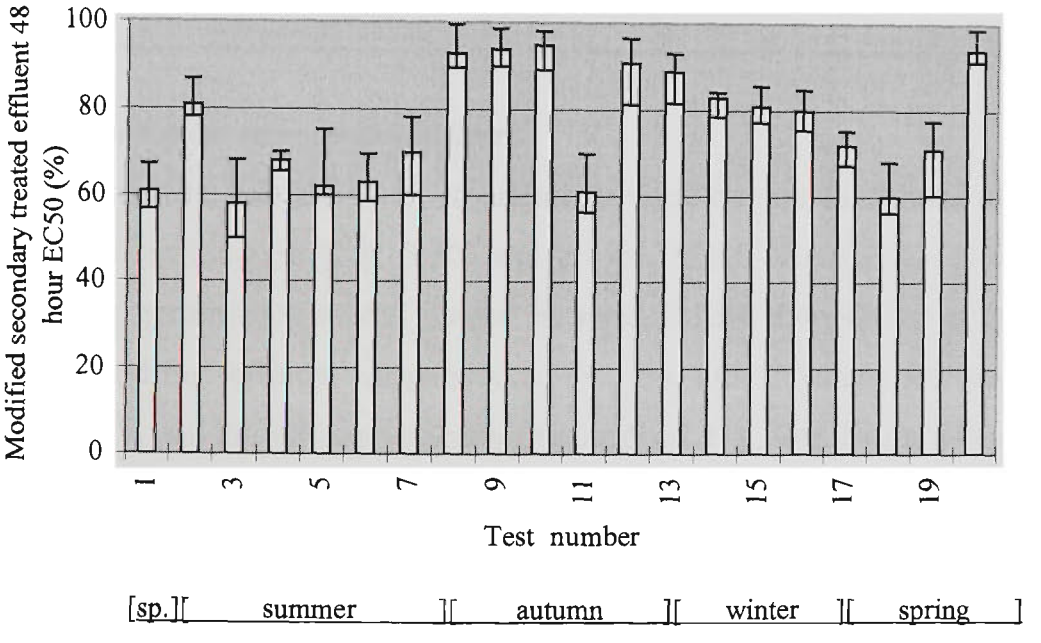


Fig. 3.29 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to modified secondary treated effluent for 48 hours. EC_{50} ± 95% confidence intervals (n = 4).

Table 3.16 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and log_{10} transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to modified secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	growth	germ	growth
Between Subjects							
Treatment	5	2.459	558.23	273.22	132.60	0.000	0.000
Error	18	0.054	0.999				
Within Subjects							
Test	19	0.681	48.59	75.67	11.54	0.000	0.000
Treatment × Test	95	0.066	10.27	7.34	2.44	0.000	0.000
Error	342	0.009	4.21				

Chlorinated secondary treated effluent

Both unmodified and modified, chlorinated secondary treated effluent exerted significant effects on germination (Fig. 3.30) and growth of *M. angustifolia* spores. The NOEC values for germination assays ranged between 3-12% unmodified effluent and 10-40% for the modified effluent (Appendix 2.17). The LOEC values for these assays ranged from 6-24% and 20-80% respectively (Appendix 2.17). For the growth assays the NOEC values for unmodified effluent ranged between 2-8% and for modified effluent 3-12% (Appendix 2.18). The subsequent LOEC values ranged from 4-16% and 6-24% respectively (Appendix 2.18). The EC_{50} values for spores exposed to unmodified effluent ranged between 22-49% effluent (Fig. 3.15) with corresponding salinities of 25.30-19.18 ppt (Appendix 2.17) for the germination assays, with a mean EC_{50} of 36% and a C.V. of 23%. For the growth assays, EC_{50} values ranged from 15-39% effluent corresponding salinities of 28.73-21.48 ppt (Appendix 2.18) with a mean EC_{50} value of 26% and a C.V. of 28% (Fig. 3.15). For germination tests conducted with modified effluent, the EC_{50}

values ranged from 50-98% effluent with a mean EC_{50} of 79% and a C.V. of 20%. The EC_{50} values for growth tests ranged from 22-57% effluent with a mean EC_{50} of 38% and a C.V. of 38%. Zoospore response varied significantly over the test period for both germination and growth bioassays as is evident by significant interactions between test date and treatment (Table. 3.16). Interactions were due to fluctuating toxicities of effluent samples tested and possible temporal variability as was evident with tests conducted with the reference toxicants.

Comparisons of zoospore response after exposure to chlorinated secondary treated effluent were made with responses to primary and secondary treated effluent samples. It was found that zoospore germination and growth responses to primary and secondary treated effluent samples were significantly different to those recorded for chlorinated secondary effluent. Overall, the effects of primary treated effluent wee greatest, followed by chlorinated secondary treated effluent and finally secondary treated effluent.

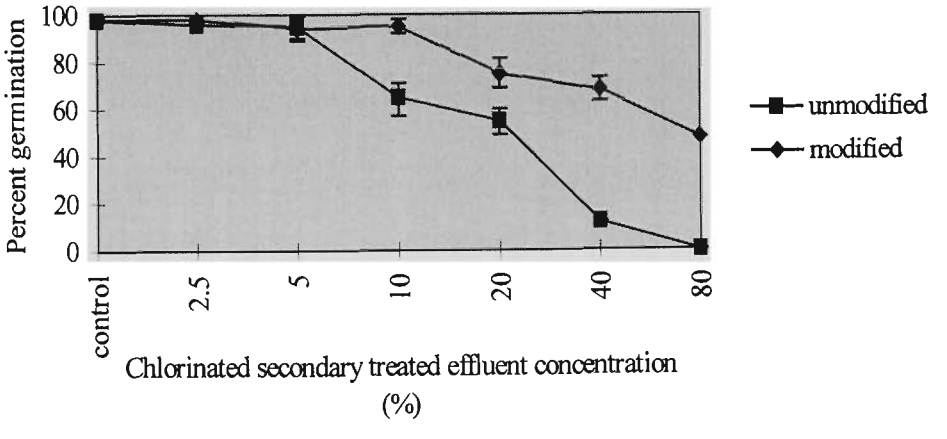
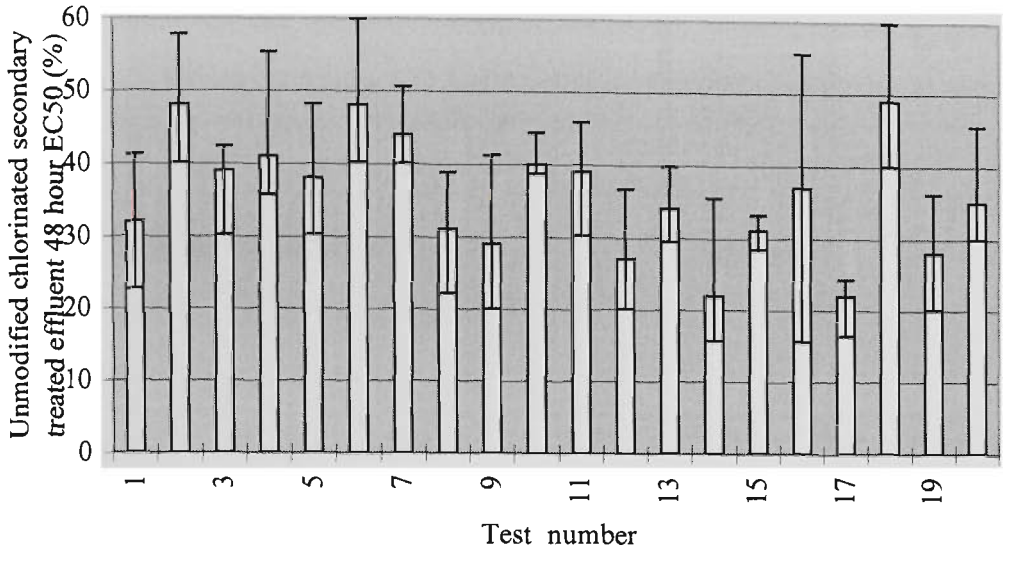


Fig. 3.30 Germination (Mean ± Standard Error) (n = 4) of *Macrocystis angustifolia* spores exposed to modified and unmodified chlorinated secondary treated effluent.

(a)



(b)

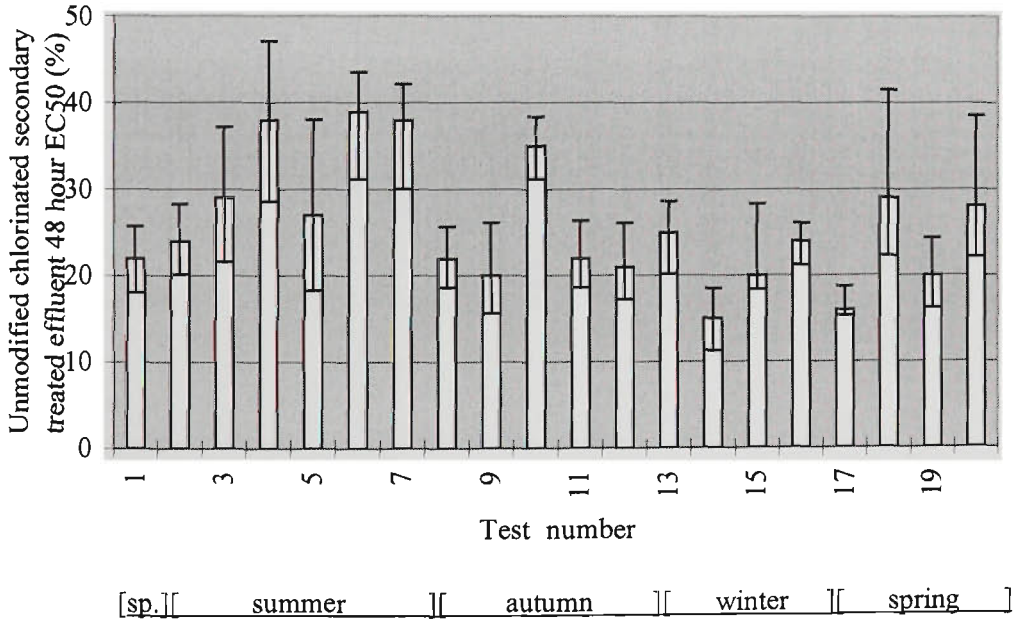
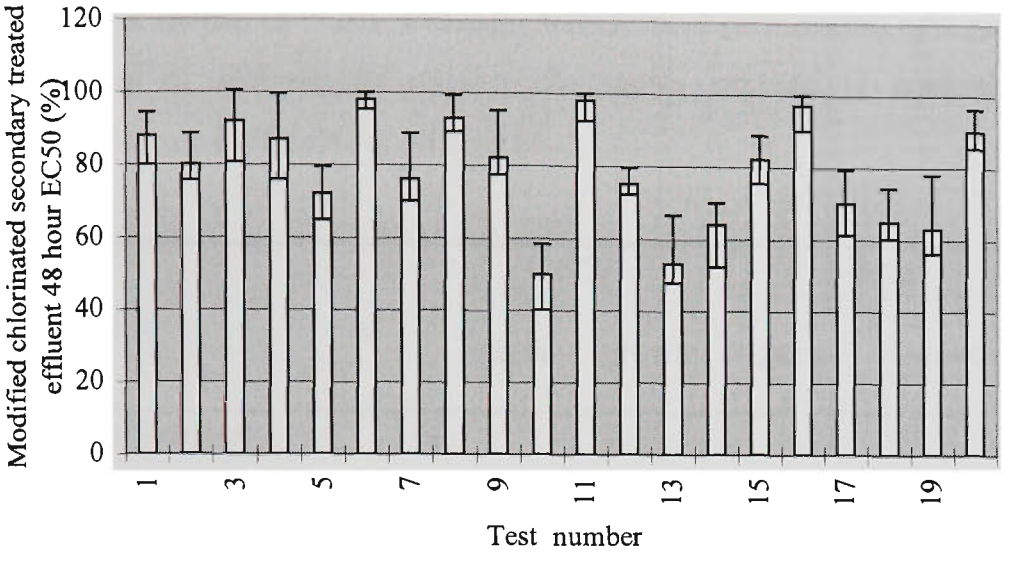


Fig. 3.31 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to unmodified chlorinated secondary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

(a)



(b)

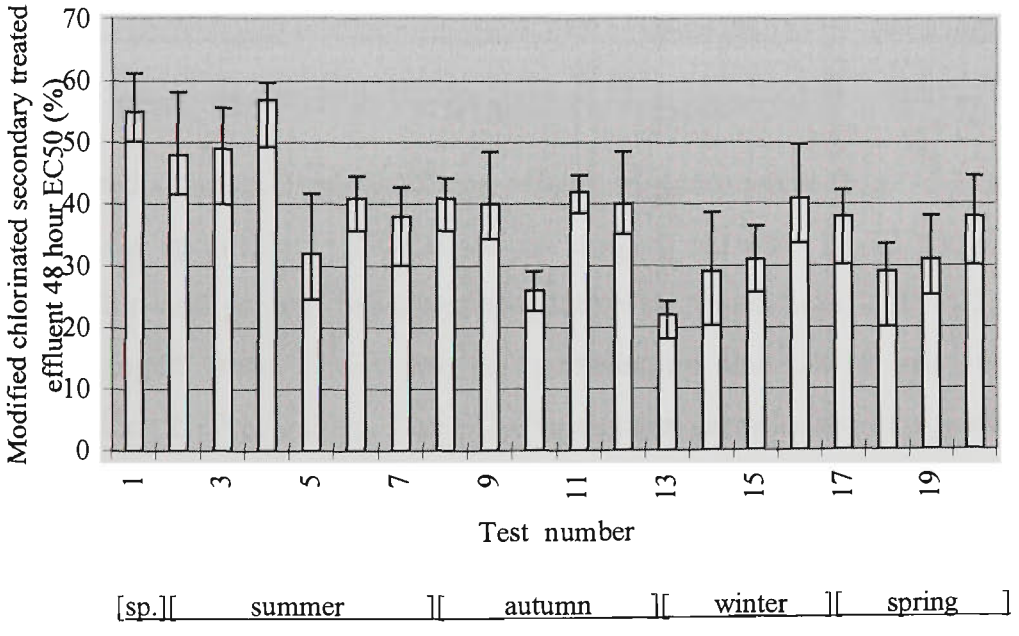


Fig. 3.32 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to modified chlorinated secondary treated effluent for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 3.17 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and log_{10} transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to unmodified chlorinated, secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	Growth	germ	growth
Between Subjects							
Treatment	5	0.982	647.22	122.75	66.594	0.000	0.000
Error	18	0.547	0.671				
Within Subjects							
Test	19	0.661	68.37	82.63	9.428	0.000	0.000
Treatment × Test	95	0.019	15.121	2.382	2.085	0.000	0.000
Error	342	0.008	7.252				

Salinity

Salinity reduction exerted significant effects on germination (Fig. 3.33) and growth of *M. angustifolia* spores, with NOEC values ranging between 24-18 ppt and 28-20 ppt for germination and growth bioassays respectively (Appendices 2.19 & 2.20). The LOEC values ranged from 22-16 ppt for germination and 26-18 ppt for growth assays (Appendices 2.19 & 2.20). The EC_{50} values for germination assays ranged from 22-16 ppt (Fig. 3.19) with a mean EC_{50} value of 18 ppt and a C.V. of 12%. The EC_{50} values for growth assays ranged from 24-16 ppt (Fig. 3.34) with a mean EC_{50} value of 20 ppt and a C.V. of 12%. Whilst analysis of variance indicated a significant interaction between test date and treatments (Table 3.19), post hoc significance testing (Tukey) did not detect significance differences in spore germination or growth responses due to seasonal variation.

Table 3.18 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and $\log_{10}$ transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to modified chlorinated secondary treated effluent for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	Growth	germ	growth
Between Subjects							
Treatment	5	0.982	647.22	122.75	66.594	0.000	0.000
Error	18	0.547	0.671				
Within Subjects							
Test	19	0.661	68.37	82.63	9.428	0.000	0.000
Treatment × Test	95	0.019	15.121	2.382	2.085	0.000	0.000
Error	342	0.008	7.252				

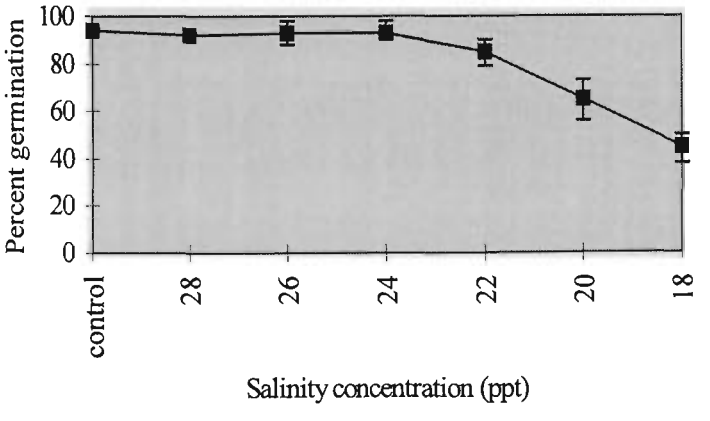
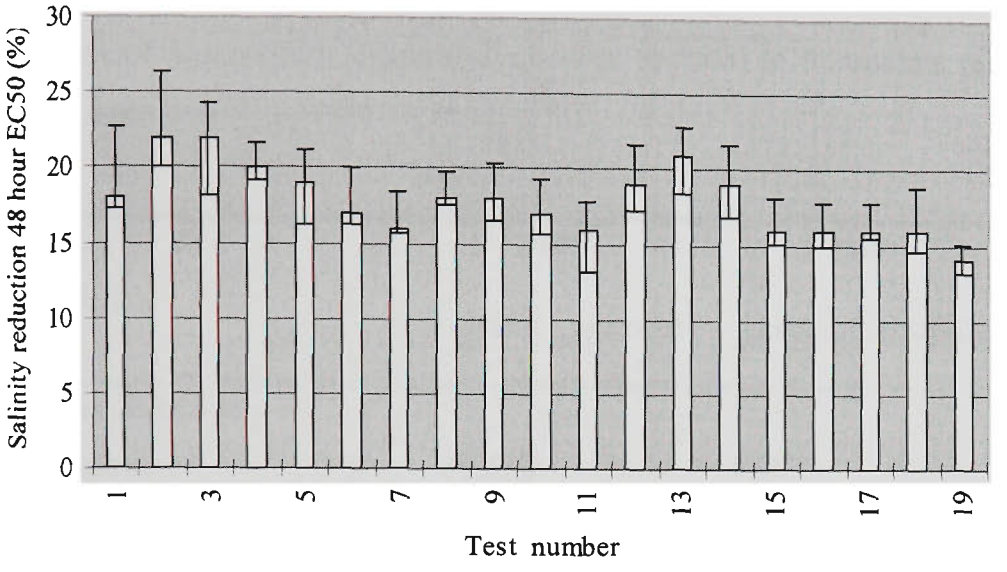


Fig. 3.33 Germination (Mean ± Standard Error) (n = 4) of *Macrocystis angustifolia* spores exposed to varying salinity.

(a)



(b)

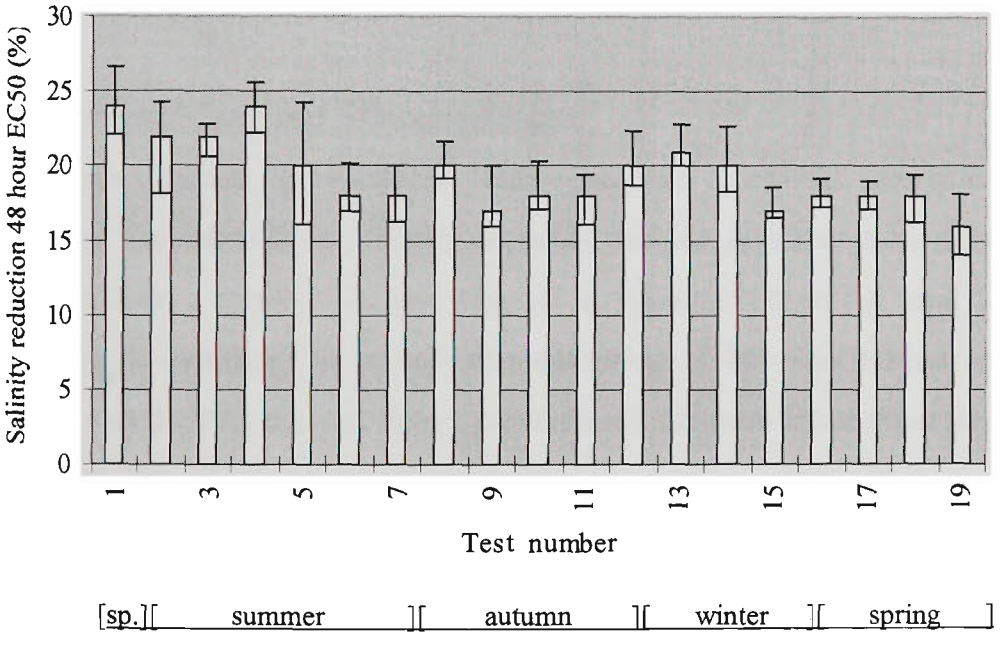


Fig. 3.34 Germination (a) and growth (b) response over time of *Macrocystis angustifolia* spores exposed to treatments of reduced salinity for 48 hours. $EC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 3.19 Table of repeated measures analysis of variance on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage germination (germ) (n = 20) and log_{10} transformed germination tube growth (growth) (n = 20) data of *Macrocystis angustifolia* spores exposed to treatments of reduced salinity for 48 hours.

Factor	df	MS		F ratio		P	
		germ	growth	germ	Growth	germ	growth
Between Subjects							
Treatment	5	0.582	21.33	122.78	41.79	0.000	0.000
Error	18	0.005	0.071				
Within Subjects							
Test	19	0.092	6.59	11.57	6.66	0.000	0.000
Treatment × Test	95	0.017	2.34	2.13	2.37	0.000	0.000
Error	342	0.008	0.508				

3.2.4 Sewage effluent and EC_{50} correlations

The South Eastern Purification Plant conducts chemical analyses of chlorinated secondary treated effluent. These analyses include concentration determinations of a variety of heavy metals, oils and organic compounds including total chlorine (ranging 0.1-0.4 mg/L over the test period) concentrations of the final effluent before discharge. Pearson correlations did not detect correlations between these chlorine levels and algal responses. The EC_{50} values obtained from *H. banksii* germination assays significantly correlated with concentrations of copper (analysis range = 0.001-0.05 mg/L) (r=0.512, s_r=0.087 r0.05(2) 18=0.444) (r = the sample correlation coefficient, s_r = the standard error of r) and lead (r=0.412, s_r=0.055, r0.05(2)18=0.444). The EC_{50} values obtained from *P. comosa* germination assays significantly correlated with mercury (analysis range = 0.00001 - 0.0008 mg/L) concentrations only (r=0.678, s_r=0.053 r0.05 (2)18=0.444). All germination and growth assays conducted with the three species of algae produced EC_{50}

values that significantly correlated with reduced salinity (analysis range = 34-12 ppt) ($r>0.378$, $n=18$, $r_{0.05}(2)18=0.444$).

3.2.5 Test species sensitivity

Relative sensitivities of *P. comosa*, *M. angustifolia* and *H. banksii* embryos have been tabulated below. These comparisons refer to sensitivities between not within species.

Table 3.20 Table of relative sensitivities of *Phyllospora comosa, Macrocystis angustifolia* and *Hormosira banksii* zygotes/spores after exposure to various treatments for 48 hours (germination data only) (scale: *** refers to highest sensitivity, ** refers to moderate sensitivity, * refers to lowest sensitivity).

Species	Treatment	Sensitivity
P. comosa	Copper	**
H. banksii	Copper	***
M. angustifilia	Copper	***
P. comosa	Chromium	***
H. banksii	Chromium	**
M. angustifilia	Chromium	**
P. comosa	Primary treated effluent (modified/unmodified)	***
H. banksii	Primary treated effluent (modified/unmodified)	***
M. angustifilia	Primary treated effluent (modified/unmodified)	***
P. comosa	Secondary treated effluent (modified/unmodified)	***
H. banksii	Secondary treated effluent (modified/unmodified)	***
M. angustifilia	Secondary treated effluent (modified/unmodified)	**
P. comosa	Chlorinated secondary effluent (modified/unmodified)	**
H. banksii	Chlorinated secondary effluent (modified/unmodified)	*
M. angustifilia	Chlorinated secondary effluent (modified/unmodified)	***
P. comosa	Salinity	***
H. banksii	Salinity	***
M. angustifilia	Salinity	**

3.3 Discussion

3.3.1 Relative sensitivity of test species

This study has shown germination of *P. comosa, H. banksii* and *M. angustifolia* to be an excellent toxicity test endpoint. Variation in germination response between species whilst significant was not surprising and illustrates differences in response between species. Variation within species however, was minimal. Recorded coefficients of variation about the mean EC_{50} for bioassays conducted ranged 10-48%. According to Cher (1994) the acceptability of the algal assays can be considered 'good' (C.V. of 35-60%) to excellent (C.V. of <35%). Germination tube growth bioassays conducted with *M. angustifolia* produced greatest variation, but this was expected as ratio scale growth measurements were recorded rather than simple positive or negative binomial germination counts.

With respect to germination sensitivity, *M. angustifolia* spores and *H. banksii* zygotes were more susceptible to the effects of copper than *P. comosa* zygotes. However, the most sensitive endpoint found in this study was germination tube growth. Anderson *et al.* (1990) exposed spores of *M. pyrifera* to copper ions. They recorded NOEC values ranging between 0.019->0.100 mg/L copper for germination assays and <0.018-<0.040 mg/L copper for germination tube growth assays. Since the study of Anderson *et al.* (1990) was merely an investigation of interpopulation comparisons and temporal variability, they only chose to compare NOEC values between tests conducted. The NOEC values recorded for germination assays in this study were similar (0.02-0.04 mg/L) to those recorded by Anderson *et al.* (1990). This may suggest similarities in germination response between *M. angustifolia* and *M. pyrifera* spores exposed to copper. The NOEC values recorded by Anderson *et al.* (1990) for growth assays were inconclusive as they recorded threshold concentrations below the lowest concentrations tested. An important point to arise from the two studies is that germination tube growth threshold concentrations are consistently lower than germination threshold concentrations. This suggests that growth test endpoints utilising *Macrocystis* species are more sensitive than germination in routine toxicity testing.

Results obtained from tests conducted with chromium suggest that *P. comosa* was the most sensitive of the three species even though least sensitive to copper. A number of studies investigating the effects of chromium have been conducted with a variety of aquatic species. Comparisons with such studies suggest that macroalgal germination and germination tube growth bioassays, are less sensitive to the effects of chromium. A bioassay based on inhibition of cell division rates in the microalga, *Nitzschia closterium* (Stauber *et al.* 1994) produced a mean 72 hour EC_{50} value of 2.43 ± 0.18 mg/L, a much more sensitive test species and endpoint when compared to the macroalgae used in this study. Similarly, Dorn *et al.* (1987) found that the mysid *Mysidopsis bahia* and daphnid *Daphnia pulex* were sensitive to chromium, with 48 hour EC_{50} values ranging from 4.21-7.23 mg/L for *M. bahia* and 0.024-0.17 mg/L for *D. pulex.* A number of explanations may be offered as to the differences between and within algal responses recorded ranging from; mode of toxicant entry, metabolism, and physical features of the algae such as size of the early life stages. Other factors which may influence variations between species response are life history characteristics such as the frequency of reproduction, number of modes of reproduction and life span of individuals (Doblin & Clayton 1995). Also recovery from disturbances can be seasonally influenced as found during this study. This may in turn lead to significant reductions in germination and growth of certain species at particular times of the year after or during toxicant exposure.

The effects of sewage effluent treatments varied depending on both species and ecotoxicological endpoints used. For the germination bioassays, primary treated effluent (both modified and unmodified) exerted similar toxicity on all three species, with 48 hour EC_{50} values ranging from 5-10%. Secondary treated effluent was less toxic than primary treated effluent, with 48 hour EC_{50} values ranging from 26->100%. In general, chlorination of the secondary treated effluent increased effluent toxicity, but was still less toxic than the primary treated effluent. Unmodified secondary treated effluent was generally more toxic than modified effluent, with similar toxicity to *P. comosa* and *H. banksii. M. angustifolia* appeared to be slightly less sensitive, but this may not be statistically significant due to the large coefficients of variation over the twenty effluent sampling periods. Much of the bioassay variability was most probably due to effluent

variability over the year long test period. These observed effects of reduced salinity and sewage effluent exposure on the early life stages of the algae assayed have the potential to alter recruitment in the field and possibly affect adult distributions. A number of studies have addressed these issues (Hopkin & Kain 1978, Doblin 1992, Doblin & Calyton 1995) with focus on effluent dilution in the field which can range on average from 0.5-5%. It is likely that the overall responses of the macroalgal species bioassayed for this study to sewage effluent were dependant not only on their physiological tolerances, but also the composition, salinity and concentration of the effluent. It is thought that reduced salinity in particular can lead to stunted growth and growth abnormalities through the inadequate regulation of turgor pressure (Wright & Reed 1990). The inhibition of germination and retardation of normal development of zygotes and zoospores may also lead to reduced competitive ability in the field through reducing the strength of germination rhizoids leading to the dislodgement of zygotes and zoospores in the field. It should also be noted though, that both effluent composition together with reduced salinity contributed to the toxic effects observed during this study as the magnitude of these effects was not reciprocated in reduced salinity controls.

The introduction of effluent into the marine environment can impact local algal communities. Brown *et al.* (1990) recorded reductions in algal species diversity and biomass in areas located near the Eastern Treatment Plant's effluent outfall at Boags Rocks. *M. angustifolia, H. banksii* and *P. comosa* have been shown to be particularly sensitive to the effects of treated effluent and long term exposure could lead to mortality of early life stages at low concentrations. In addition, lower concentrations may impact the population structure leading to diminished recruitment by the disruption of growth and reproduction. Treated sewage effluent is a highly complex mixture and so the actual expression of toxicity of components cannot be determined.

The addition of chlorine to treated sewage effluent may pose a greater threat to algal communities than previously thought. Studies have shown that residual chlorine inhibits germination in other algal species such as *Sargassum horneri, S. hemiphyllum* and *S. thunbergii* (Ogawa 1984) and has shown to interfere in growth of *Ulva californica* and

Phyllospadix torreyi (Kindig & Littler 1980). The exact modes of toxicity of chlorine are not fully understood.

The salinity reduction assays, provided useful information on the effects of reduced salinity on the three species. Salinity reduction of seawater is a direct result of sewage effluent contamination near and around the point of discharge. The combined effects of effluent and reduced salinity increased the overall effects of the unmodified effluent samples suggesting synergistic effects. Salinity reduction did not exert a significant effect in the primary treated effluent tests conducted, as the species exhibited significant sensitivity to the effluent at low concentrations. From these assays it can be concluded that toxic effects were caused by the effluent mixture alone without salinity as a confounding factor. Studies have shown that previously dominant species such as *H. banksii* and *Durvillaea potatorum* have disappeared from areas immediately surrounding an outfall at Boags Rocks, Victoria (Brown *et al.* 1990). The copious volumes of freshwater effluent being discharged into these areas significantly reduce the salinity of surrounding waters. This along with effluent constituents can be attributed to the disappearance of these algal species.

3.3.2 Temporal variability

The results of reference toxicant and reduced salinity bioassays indicate that there was temporal variability in the sensitivity of zygotes of *P. comosa, H. banksii* and spores of *M. angustifolia*. Bioassay results indicated greatest sensitivity of the algal species in the summer months of the year. This temporal variability influenced the statistical analyses which suggested significant interactions between test dates and copper, chromium and salinity treatments.

Few studies have addressed the issue of temporal variability in algal responses (Kennelly 1987, Anderson *et al.* 1990). One source of variability may be the seasonal fluctuations of nutrients in seawater (Anderson *et al.* 1990). Some essential nutrients may be limiting during certain times of the year. This in turn may influence the health of adult plants. Although surrounding water temperature may have a greater effect on increasing algal

sensitivity (Clayton *et al.* 1987, Cheshire & Hallam 1988). Biological processes such as competition and succession may influence reproductive strategies of particular algal species at certain times of the year. One or more of the factors mentioned may have contributed to response variations. These along with the added stresses of toxic compounds may have influenced germination and growth.

Gunthorpe *et al.* (1995) observed reduced fertilisation rates of *H. banksii* gametes from January to April, and suggested that fertilisation of *H. banksii* gametes varied with season, becoming increasingly more variable as air temperature increased. As an intertidal species, *H. banksii* remains exposed to environmental conditions during low tide and exposure during the summer months may have influenced the sensitivity of the zygotes in this study. Also, the warmer months are not a favoured time for reproduction of other kelp species such as *Durvillaea potatorum* (Clayton *et al.* 1987, Cheshire & Hallam 1988) and *Ecklonia radiata* (Kennelly & Larkum 1983). Munda (1982) suggested that the susceptibility of algae to sewage effluent may increase in accordance with the vertical distribution of a species. The results of this study do no invalidate these findings, however the vertical distribution of a species is only one of many possible factors which may contribute to varying responses recorded for this study. Certain species may favour particular seasonal influences such as temperature and light intensity during reproduction and recruitment.

For routine toxicity testing it is essential to know when sensitivity of a test species may vary and to a certain extent the possible reasons why. During times of increased algal sensitivity it is important to note whether algal responses are due to a combination of toxic effects or seasonal variability of the species or both.

3.3.3 Toxicity test endpoint comparisons

The results suggest that the germination test endpoint for *P. comosa* and *H. banksii* and the growth and germination test endpoints for *M. angustifolia* can be used successfully in routine toxicity testing. These early life stages are ecotoxicological endpoints which offer substantial ecological significance.

Other work has previously focussed on the effects of chromium on fertilisation of *H. banksii* gametes (Gunthorpe *et al.* 1995). This assay relied on zygotes developing a cell wall, however, up to 24 hours after gamete release a high proportion of unfertilised eggs (~70%) can secrete a cell wall (Ashburner *et al.* 1997). This suggests that a high proportion of the 'fertilised' zygotes, may in fact be unfertilised and developing via parthenogenic development. Further, non-production of a primary cell wall does not necessarily mean that cells have not been fertilised, the toxicant may simply be blocking development of the primary cell wall. Such bioassays may be more correctly called cell viability assays, which use inhibition of cell wall development as the endpoint. The EC_{50} concentrations obtained by Gunthorpe *et al.* (1995) ranged from 297-434 mg/L hexavalent chromium in seawater. These concentrations are substantially higher than those obtained in this study, suggesting that the endpoints utilised for this study are more sensitive to chromium than the fertilisation inhibition endpoint.

3.3.4 Treatment toxicity

Hexavalent chromium is a highly soluble compound that can readily penetrate biological membranes (Reidel 1985) and bind and/or oxidise polypeptides and proteins, leading to cellular disruption (Patel & Saxena 1983). The effects of copper have been well documented (Bartlett *et al.* 1974, Christensen *et al.* 1979, Stauber & Florence 1985, Stauber & Florence 1986, Stauber & Florence 1987, Guilizzoni 1991, Wong & Chang 1991) with the metal being linked to inhibiting growth and photosynthesis in numerous algal species (Chung & Brinkhuis 1986, Wong & Chang 1991). Copper may also affect permeability of plasma membranes causing loss of potassium from algal cells (Chung & Brinkhuis 1986). Moreover, higher concentrations of copper may affect mitochondrial respiration and other general metabolic pathways (Strömgren 1979, Chung & Brinkhuis 1986) and disrupt the use of stored photosynthate in turn inhibiting germination (Anderson *et al.* 1990). Picton and Steer (1982) investigated germination and germination tube growth in *Fucus* zygotes exposed to copper treatments. They suggested that copper may disrupt calcium ion transport across the cell membrane which plays an important role in germination and growth. A clear finding has been that the chemical state of copper greatly influences toxic effects in algal species (Jackson & Morgan 1978,

Chung & Brinkhuis 1986, Wong & Chang 1991). The more free copper ions available in solution, the more toxicity is exerted.

Pearson correlations calculated between recorded EC_{50} values and compound concentrations within secondary treated effluent, indicated significant positive correlations for assays conducted with *P. comosa* and *H. banksii.* It was found that *P. comosa* zygotes were significantly sensitive to increased concentrations of both copper and lead and *H. banksii* zygotes to mercury. The complexity of sewage effluent makes it difficult for researchers to predict the exact compounds causing significant effects within given species. Wong and Chang (1991) showed that bimetallic combinations of heavy metals found in sewage effluent even at low concentrations can greatly increase the toxicity of the other and vise versa. These can interact to cause synergistic or antagonistic effects. Although levels of heavy metals from sewage effluent are greatly reduced once they are discharged into aquatic environments, they can be accumulated and become toxic to present organisms. The other restriction in determining exact toxic effects of effluent is that not all compounds are tested for in a given sample of effluent. Assumptions can only be made to suggest which compounds are of concern.

3.3.5 Conclusion

The data presented for this study suggest that *P. comosa, H. banksii* and *M. angustifolia* can be used successfully in routine ecotoxicological research. The 48 hour germination endpoint is easily recognised and the tests can be carried out quickly with minimal expense. Germination in macroalgae is an important early life stage with significant ecological relevance. Marine macroalgae are important primary producers in nearshore habitats. If zygotes and spores do not germinate then the continuation of the life cycle is disrupted which can ultimately lead to decreased recruitment. Restricted recruitment may have long term effects on algal population numbers and invertebrate and fish taxa which depend on these communities for food and shelter. Macroalgal communities located at or nearby effluent outfalls are continually susceptible to the effects of sewage discharge. Routine toxicity testing in the form of germination bioassays can help predict the effects of discharge on these local communities.

CHAPTER 4
THE EFFECTS OF SEWAGE EFFLUENT ON MORTALITY OF *PHYLLOSPORA COMOSA* AND *HORMOSIRA BANKSII*

4.1 Introduction

Short-term bioassays, such as 48 hour germination and 96 hour mortality tests, can be used to complement wastewater evaluation procedures based on chemical data (Garric *et al.* 1993). In the past, these bioassays have almost exclusively been conducted on fish (Franklin 1983, Fava *et al.* 1985, Santoro & Fikslin 1987) and crustacean (Costello & Read 1994) species with some utilising algal species. The use of macroalgae as test species has become widely accepted especially in tests where germination, growth and mortality are the assessed endpoints. The information gained from short-term bioassays can be of use in the management of effluent discharges for the purposes of predicting environmental effects, comparing toxicant effects and regulation (Buikema Jr *et al.* 1982).

Many monitoring studies have been conducted on the impacts of sewage effluent on species of marine algae (Borowitzka 1972, Ogawa, 1984, Tewari & Joshi 1988, Brown *et al.* 1990, Tegner *et al.* 1995). The main criterion used for assessing water quality for these types of studies has been in determining impacts on species diversity (Borowitzka 1972). The quality of the surrounding waters in question has gained much attention, as this type of wastewater is either a source of nutrients or an inhibitor of algal growth (Ogawa 1984). For monitoring programmes to be successful, two important criteria need to be met. Firstly, the indicator species used for the study must by causally linked to the input being assessed and secondly the entire area being monitored needs to be logistically accessible at all times (Ogawa 1984, Tewari & Joshi 1988, Brown *et al.* 1990, Tegner *et al.* 1995).

Mortality bioassays can prove useful in; establishing the initial benchmark toxicity of a substance; comparing relative toxicities of substances; and as tools for monitoring changes in toxicity of substances over time (Boedeker *et al.* 1993). The latter point is an important advantage when testing for the effects of effluent. The utilisation of species such as macralgae in mortality bioassays also offers the advantage of conducting routine static tests, which only require the toxicant to be added to the algae under controlled conditions (Shir 1994). The system is then left to stand for the required period at which time results are scored.

It is accepted that sub-lethal toxicity tests, such as germination and growth bioassays, are of greater environmental significance than acute toxicity tests (Chapman & Long 1983). For the purpose of this research, germination bioassays were conducted for 48 hours, at which time results were recorded. Mortality bioassays were then conducted to determine any effects of additional exposure to the toxicants. The point to be made here, is that although a sub-lethal concentration can be determined sooner, it can prove irrelevant if the onset of significant mortality rates are recorded a short time later and at lower concentrations.

The principle objective of an ecotoxicological approach to testing effluent toxicity is to maintain or improve the quality of receiving waters (Garric *et al.* 1993). Cause and effect relationships between effluent discharge and the surrounding ecosystem can then be defined. A major criticism of laboratory based studies however, is the lack of connection to real-world conditions due to the many modifying factors that exist in the environment. Laboratory based studies can provide benchmark results, these can then be applied as an aid in predicting possible outcomes of effluent discharge particularly when any other evidence is lacking. Tests, such as the 96 hour mortality bioassay, allow the determination of toxicity parameters such as no observable effect concentration values, which are a helpful basis for the limitation of effluent discharges (Cairns & Pratt 1993).

4.1.1 Aims of this chapter

The aims of this chapter were to:

1. determine the effects of sewage effluent on mortality of *Phyllospora comosa* and *Hormosira banksii* embryos.

2. investigate possible temporal variation in algal mortality responses by conducting bioassays with two reference toxicants.

4.2 Results

Mortality bioassays conducted with the reference toxicant copper were performed over a 15 month period beginning 4/9/96 and concluding 2/12/97, these test dates are denoted as a-e and 1-20 in the following sections. All other mortality bioassay dates, denoted as 1-20, correspond to dates ranging between 17/11/96-2/12/97. Exact dates of each test commencement have been listed as Appendix 1.

4.2.1 *Phyllospora comosa*

Reference toxicants

For mortality assays, copper (Fig. 4.1) only was utilised as the reference toxicant, as mortality assays conducted with chromium produced extremely variable results with an unacceptably high C.V. (>50%). In addition, *P. comosa* embryos exhibited low sensitivity when exposed to chromium. Embryos were exposed to concentrations of chromium in excess of 10,000 mg/L with no signs of mortality even though they had not germinated. In the initial stages of this study, cultures for these germination and mortality bioassays were maintained and kept for a further 96 hours after mortality had been scored to investigate possible delayed germination, and if no germination occurred, then the expected onset of mortality of ungerminated embryos. These embryos did begin to die between days 5 and 6.

Copper worked well as a reference toxicant with an overall C.V. of 22% for the 96 hour LC_{50} values recorded. These 96 hour LC_{50} values ranged from 0.33-0.92 mg/L (Fig. 4.2). The NOEC values ranged from 0.05-0.40 mg/L and LOEC values ranged from 0.10-0.80 mg/L copper (Appendix 22). Copper treatments exerted significant effects on mortality of *P. comosa* for all tests conducted.

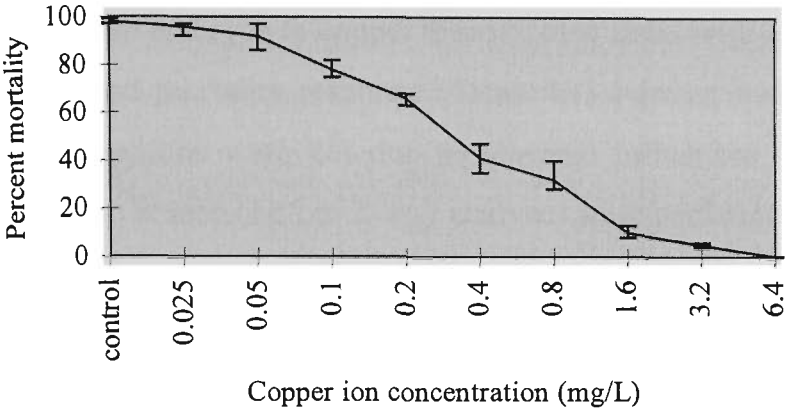


Fig. 4.1 Mortality (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* embryos exposed to copper for 96 hours.

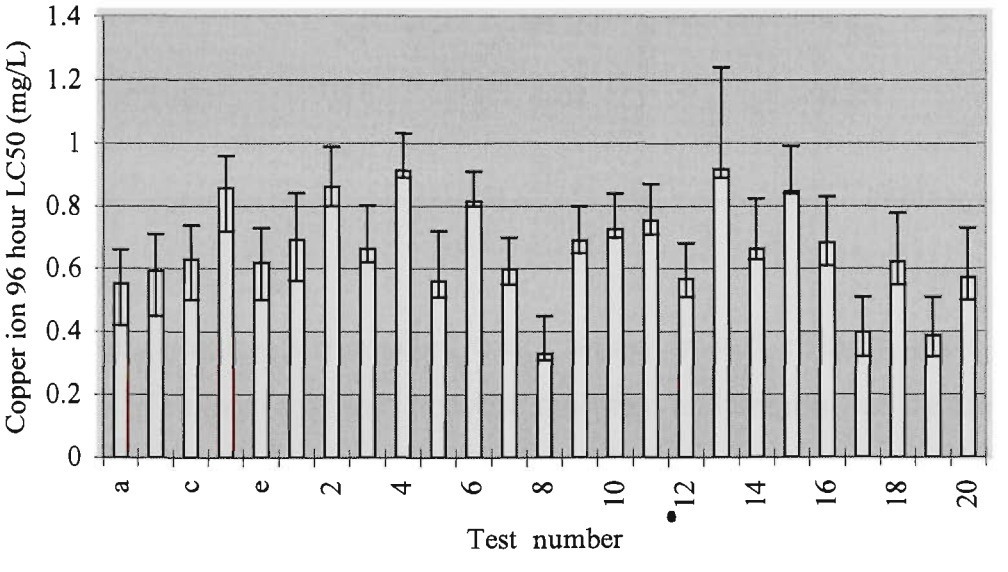


Fig. 4.2 Mortality response over time of *Phyllospora comosa* embryos exposed to copper for 96 hours. LC_{50} ± 95% confidence intervals (n = 4).

Repeated measures ANOVA suggested that there were significant variances in response of *P. comosa* embryos to copper toxicity over time, with a significant interaction between test date and mortality response (Table 4.1) Further ANOVA confirmed however, that these interactions were not due to seasonal influences (for these ANOVA, data were divided into seasons before 2-way analyses were performed).

Table 4.1 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Phyllospora comosa* embryos exposed to copper for 96 hours.

Factor	Df	MS	F ratio	P
Between Subjects				
Treatment	9	500.10	43.88	0.000
Error	30	11.39		
Within Subjects				
Test	24	35.52	7.81	0.000
Treatment × Test	216	4.01	0.89	0.040
Error	750	4.51		

Primary treated effluent

For mortality bioassays conducted with primary treated effluent, there were significant differences in embryo response between modified and unmodified treatments (Fig. 4.3) at any one time based on recorded NOEC and LOEC values (Appendix 23). Unmodified primary treated effluent exerted significant effects over time on mortality of *P. comosa* embryos (Table 4.2). The NOEC values for embryos exposed to unmodified primary treated effluent ranged from 0.5-4.0% effluent and the LOEC values ranged from 1-8% effluent (Appendix 23). The 96 hour LC_{50} values ranged from 4-19% effluent (Fig. 4.4) with a mean LC_{50} value of 12%, C.V. of 40% and corresponding salinities ranging from 29.75-25.98 ppt (Appendix 23).

Modified primary treated effluent also exerted significant effects on survival of *P. comosa* embryos at 96 hours over time (Table 4.2). The LC_{50} values ranged from 5-57% (Fig. 4.3.2) with a mean LC_{50} value of 16% effluent and C.V. of 46%. The NOEC values ranged from 1-4% effluent and LOEC values ranged from 2-8% effluent (Appendix 23).

For the suite of assays conducted with both modified and unmodified primary treated effluent, repeated measures ANOVA indicated a significant interaction between test date and algal response. As there was no such interaction evident in the reference toxicant test, the algal response could be due solely to the fluctuating toxicity of the effluent tested over time

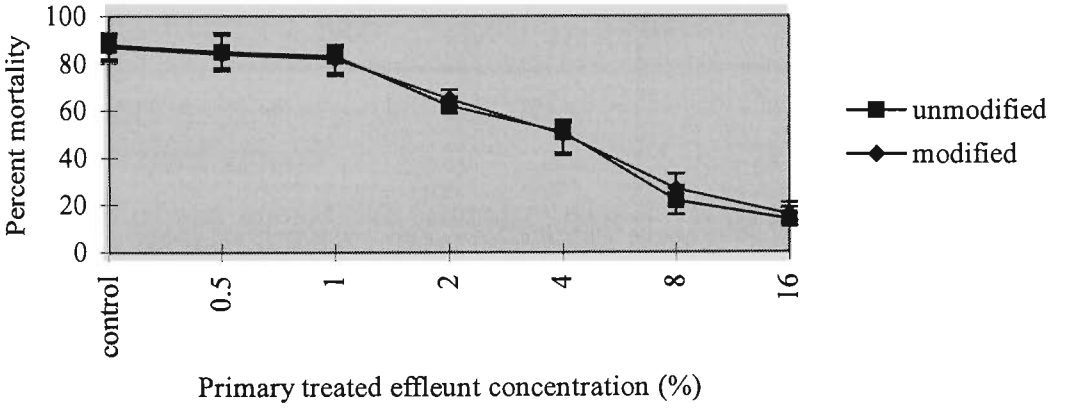


Fig. 4.3 Mortality (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* embryos exposed to primary treated effluent for 96 hours.

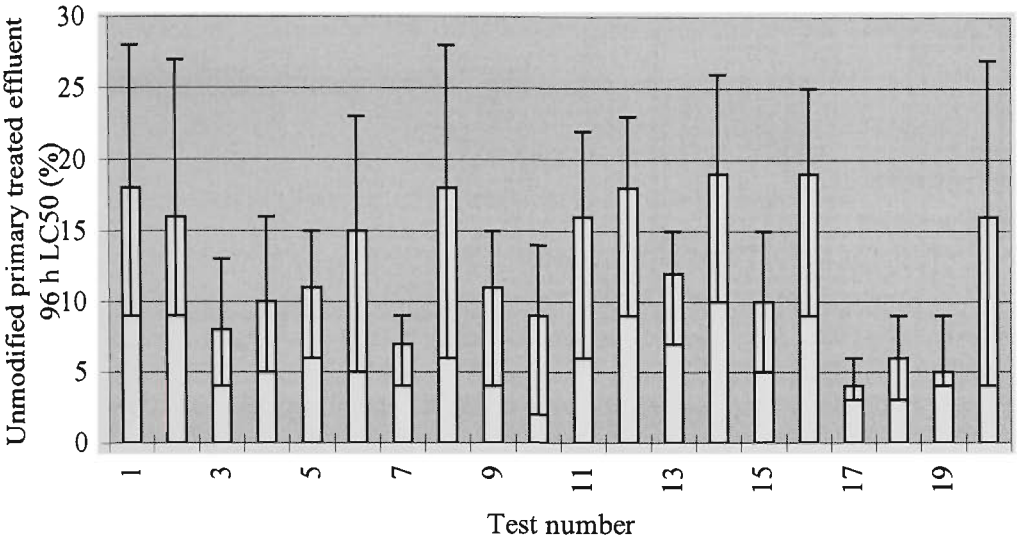
Table 4.2 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Phyllospora comosa* embryos exposed to modified (mod) and unmodified (un) primary treated effluent for 96 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	50.98	71.25	25.28	15.47	0.000	0.000
Error	18	2.02	4.61				
Within Subjects							
Test	19	5.25	3.15	3.09	1.85	0.019	0.002
Treatment × Test	95	2.39	2.44	1.43	1.39	0.008	0.031
Error	342	1.67	1.70				

Secondary treated effluent

Both modified and unmodified secondary treated effluent exerted significantly different effects on mortality of *P. comosa* embryos (Fig 4.5). Overall, higher mortality rates were recorded for embryos exposed to unmodified effluent as opposed to modified effluent. The NOEC values for unmodified treated effluent tests ranged from 2.5-10% effluent and LOEC values ranged from 5-20% (Appendix 24). The 96 hour LC_{50} values ranged from 11-37% effluent (Fig. 4.6), with corresponding salinities ranging from 28.88-21.93 ppt (Appendix 24). A mean LC_{50} of 23% was recorded and a C.V. of 33%. The NOEC values for tests conducted with modified treated effluent these tests ranged from 5-20% effluent and LOEC values ranged from 10-40% (Appendix 24). The 96 hour LC_{50} values ranged from 19-70% effluent (Fig. 4.6) with a mean of 48% and a C.V. of 39%.

(a)



(b)

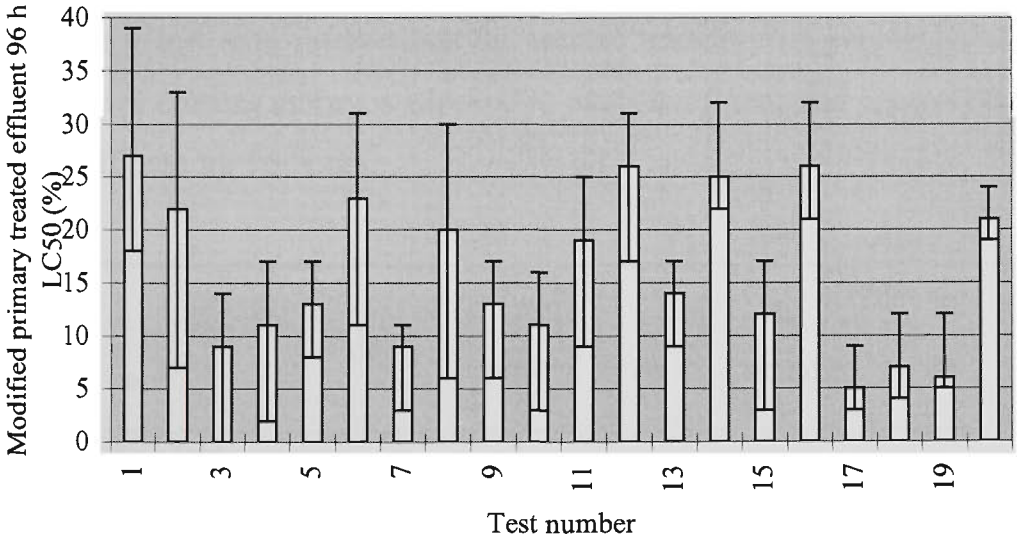


Fig. 4.4 Response over time of *Phyllospora comosa* embryos exposed to unmodified (a) and modified (b) primary treated effluent for 96 hours. $LC_{50} \pm 95\%$ confidence intervals (n = 4).

Repeated measures ANOVA indicated that *P. comosa* embryo response varied over time (Table 4.3). A significant interaction between test date and response was also indicated and this was most probably due to changes in effluent toxicity over time.

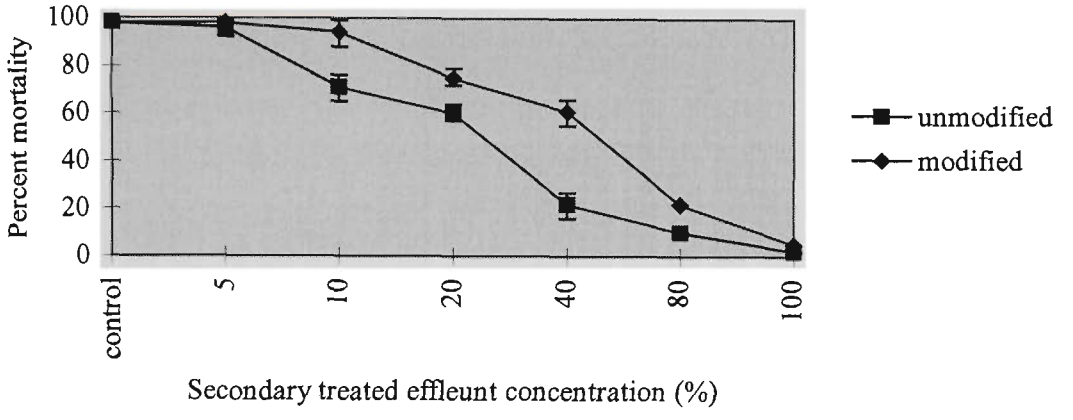
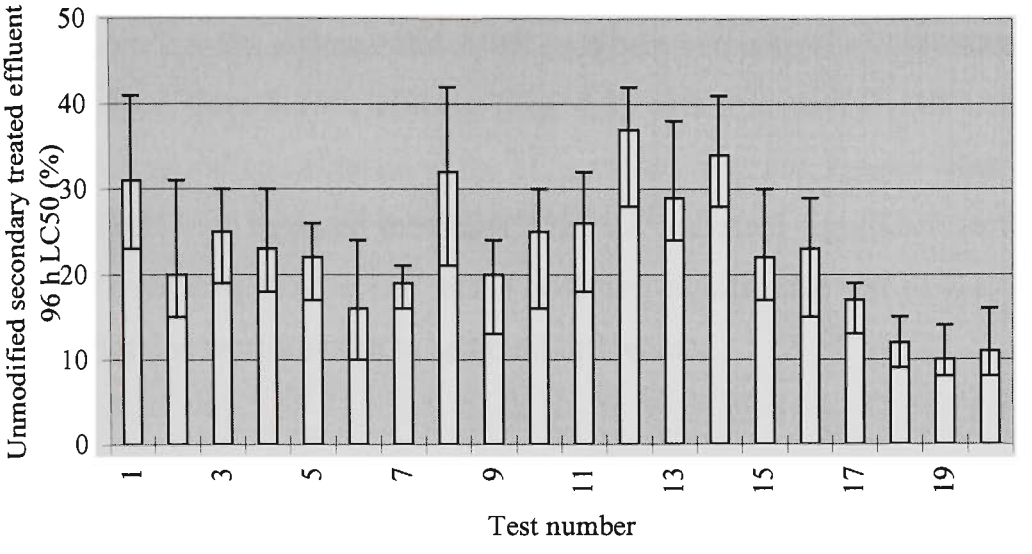


Fig. 4.5 Mortality (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* embryos exposed to secondary treated effluent for 96 hours.

Table 4.3 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Phyllospora comosa* embryos exposed to modified (mod) and unmodified (un) secondary treated effluent for 96 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	1.255	2.669	20.26	83.63	0.000	0.000
Error	18	0.062	0.0335				
Within Subjects							
Test	20	0.452	0.214	23.78	27.05	0.000	0.000
Treatment × Test	100	0.066	0.028	3.47	3.27	0.000	0.000
Error	274	0.019	0.008				

(a)



(b)

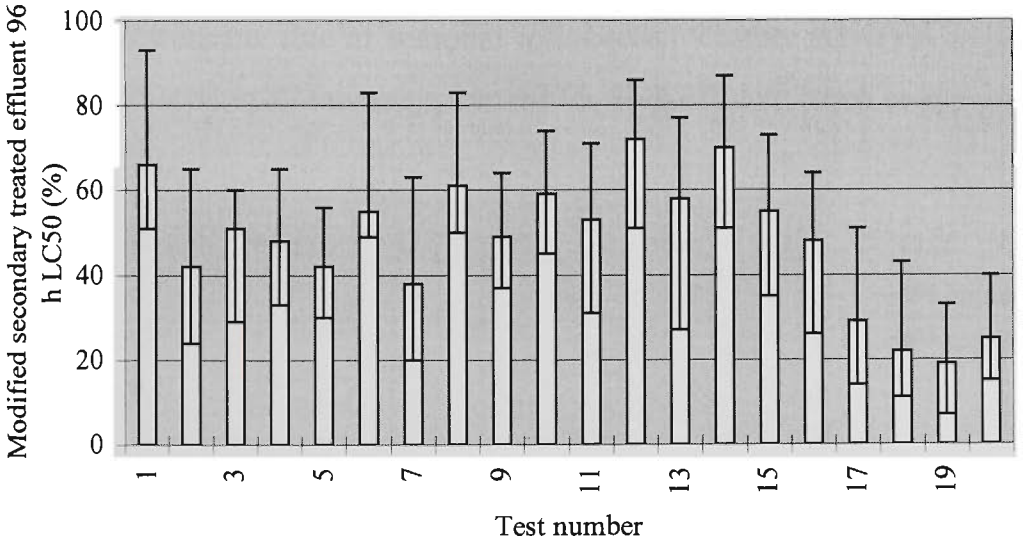


Fig. 4.6 Response over time of *Phyllospora comosa* embryos exposed to unmodified (a) and modified (b) secondary treated effluent for 96 hours. $LC_{50} \pm 95\%$ confidence intervals (n = 4).

Chlorinated secondary treated effluent

Unmodified chlorinated secondary treated effluent exerted significant effects on mortality of *P. comosa* embryos (Fig. 4.7). The NOEC and LOEC values recorded for these assays ranged from 2.5-5% effluent and 5-10% effluent respectively (Appendix 25). The LC_{50} values ranged from 8-15% effluent (Fig. 4.8) with a mean LC_{50} of 11% and a C.V. of 28%. Corresponding salinities to the LC_{50} values recorded, ranged from 29.75-28.38 ppt (Appendix 25). A repeated measures ANOVA indicated significant variation in embryo response over time (Table 4.4), but a post hoc significance test (Tukey) confirmed that these variations were not due to seasonal influences.

Modified chlorinated secondary treated effluent also exerted significant effects on *P. comosa* embryos which were also found to be statistically different to those recorded for unmodified effluent (Fig. 4.7). The NOEC and LOEC values recorded ranged from 2.5-10% effluent and 5-20% effluent respectively (Appendix 25). The LC_{50} values ranged from 10-40% effluent (Fig 4.8) with a mean LC_{50} of 21% and a C.V. of 34%. A repeated measures ANOVA indicated variations in embryo response over time (Table 4.4), but again these were not due to seasonal influences. Further ANOVA indicated significant differences between all bioassays carried for each effluent batch at any one time.

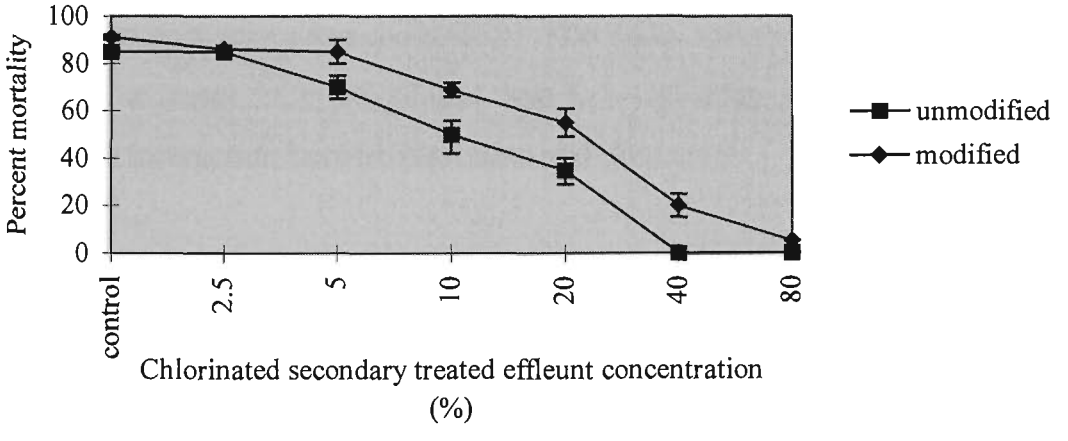


Fig. 4.7 Mortality (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* embryos exposed to chlorinated secondary treated effluent for 96 hours.

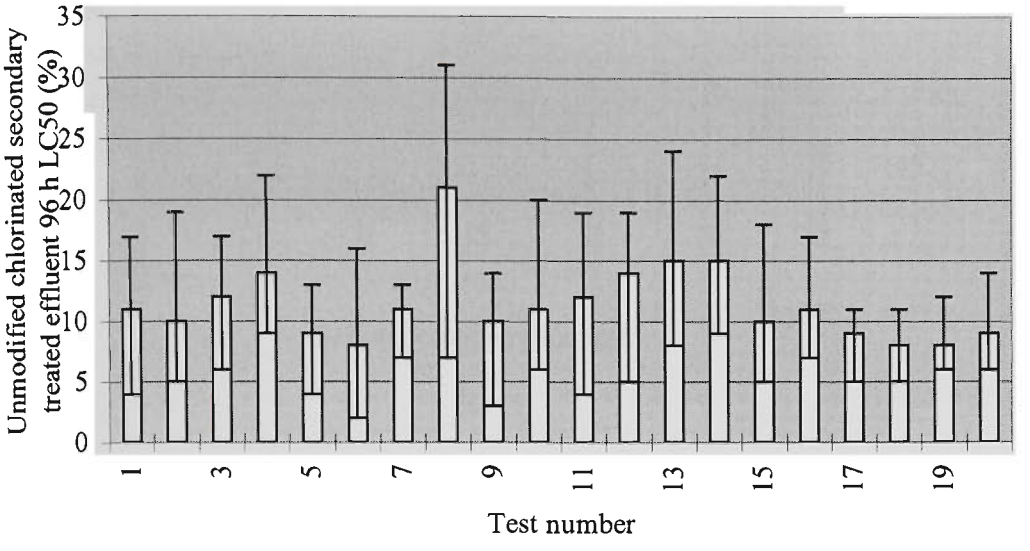
Table 4.4 Table repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Phyllospora comosa* embryos exposed to modified (mod) and unmodified (un) chlorinated secondary treated effluent for 96 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Treatment	5	0.419	0.954	85.62	102.54	0.000	0.000
Error	18	0.004	0.009				
Within Subjects							
Test	19	0.015	0.018	15.84	22.39	0.000	0.000
Treatment × Test	95	0.002	0.011	5.55	3.48	0.000	0.000
Error	342	0.000	0.000				

Salinity reduction

Treatments of reduced salinity exerted significant effects on mortality of *P. comosa* embryos (Fig. 4.9) (Table 4.5). The NOEC and LOEC values ranged from 30-24 ppt and 28-22 ppt respectively (Appendix 26). The LC_{50} values ranged from 25-18 ppt (Fig. 4.10), with a mean LC_{50} of 22 ppt and C.V. of 12%. Repeated measures ANOVA indicated no interaction between test date and treatment.

(a)



(b)

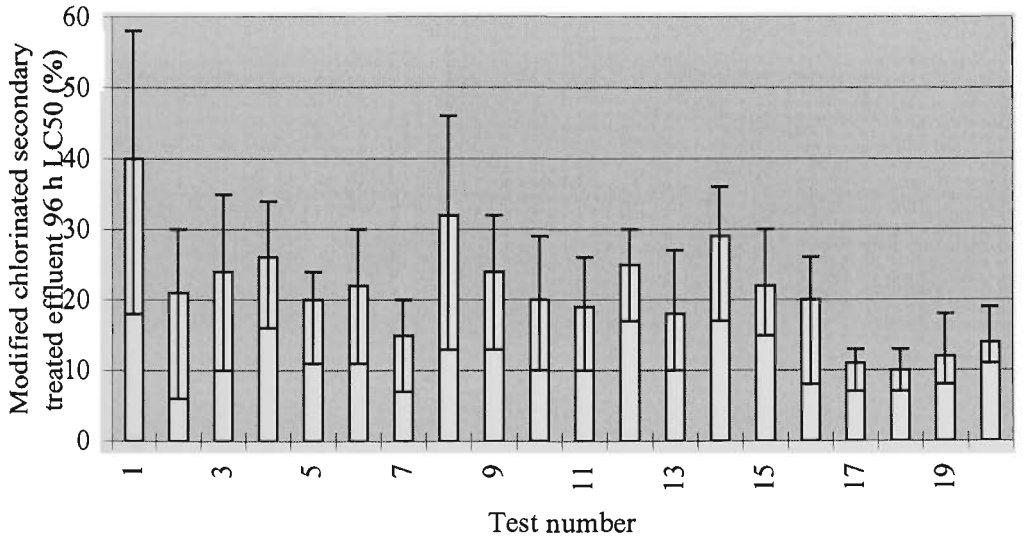


Fig. 4.8 Response over time of *Phyllospora comosa* embryos exposed to unmodified (a) and modified (b) chlorinated secondary treated effluent for 96 hours. LC_{50} ± 95% confidence intervals (n = 4).

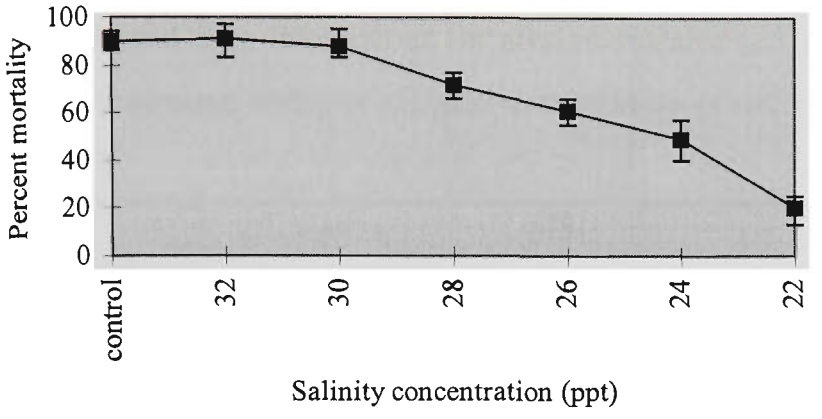


Fig. 4.9 Mortality (Mean ± Standard Error) (n = 4) of *Phyllospora comosa* embryos exposed to reduced salinity for 96 hours.

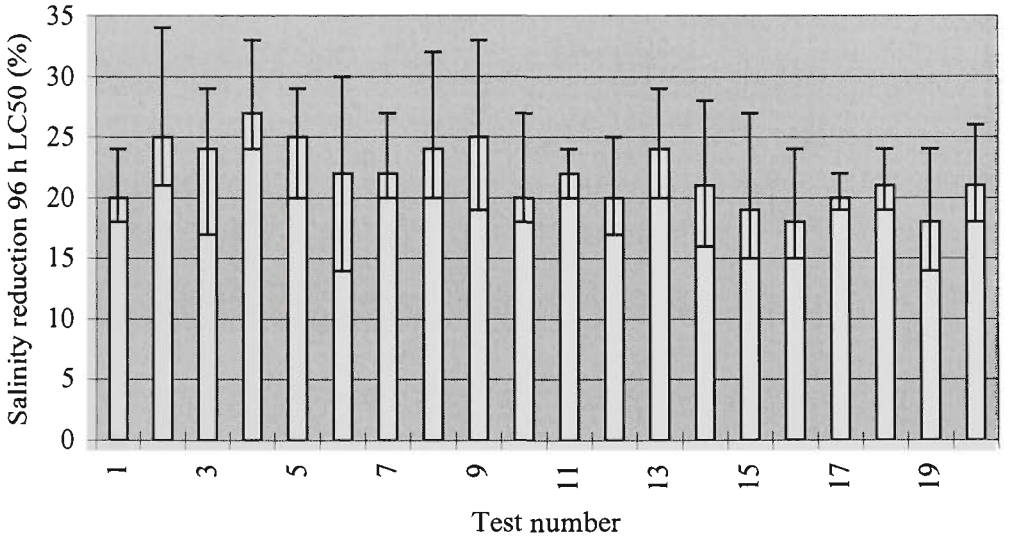


Fig. 4.10 Response over time of *Phyllospora comosa* embryos exposed to treatments of reduced salinity for 96 hours. EC_{50} ± 95% confidence intervals (n = 4).

Table 4.5 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Phyllospora comosa* embryos exposed to treatments of reduced salinity for 96 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	0.718	42.59	0.000
Error	18	0.016		
Within Subjects				
Test	19	0.009	6.37	0.000
Treatment × Test	95	0.001	2.98	0.051
Error	342	0.000		

4.2.2 *Hormosira banksii*

Reference toxicants

Treatments of copper exerted significant effects on mortality of *H. banksii* embryos (Fig 4.11), with chromium once again exerting highly variable effects (for this reason data collected for bioassays with chromium have been excluded). The NOEC and LOEC values for these bioassays ranged from 0.25-1 and 0.5-2 mg/L copper respectively (Appendix 27). The LC_{50} values ranged from 0.82-1.93 mg/L copper (Fig. 4.12) with a mean LC_{50} of 1.26 mg/L and a C.V. of 30%. ANOVA indicated significant variation in embryo response to copper over time (Table 4.6). However, further analyses confirmed that variations in embryo response were not due to seasonal influences (for these ANOVA, data were divided into seasons before 2-way analyses were performed).

.

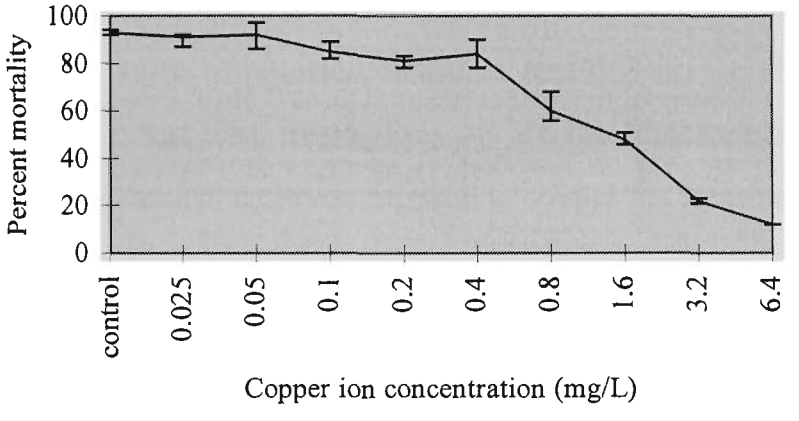


Fig. 4.11 Mortality (Mean ± Standard Error) (n = 4) of *Hormosira banksii* embryos exposed to copper for 96 hours.

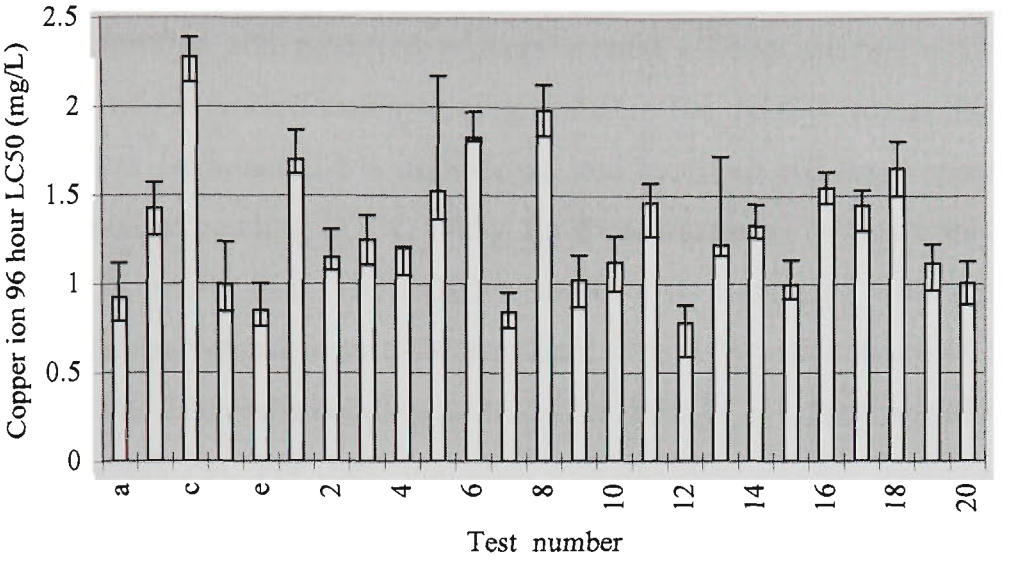


Fig. 4.12 Mortality response over time of *Hormosira banksii* embryos exposed to copper for 96 hours. $LC_{50} \pm 95\%$ confidence intervals (n = 4).

Table 4.6 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Hormosira banksii* embryos exposed to copper for 96 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	9	1.055	41.25	0.000
Error	30	0.026		
Within Subjects				
Test	24	0.019	22.61	0.000
Treatment × Test	216	0.007	5.99	0.030
Error	720	0.001		

Primary treated effluent

Both unmodified and modified primary treated effluent exerted significant effects on mortality of *H. banksii* embryos (Fig. 4.12). The NOEC values for these bioassays ranged from 1-4% and 2-8% unmodified and modified effluent respectively (Appendix 28). The corresponding LOEC values for these bioassays ranged from 2-8% and 4-16% effluent (Appendix 28). The LC_{50} values for tests conducted with unmodified primary treated effluent ranged from 6-24% effluent (Fig. 4.7) with a mean of 14% effluent and C.V. of 37%. The corresponding salinities for these LC_{50} values ranged from 29.50-24.85 ppt (Appendix 28). For tests conducted with modified primary treated effluent, the LC_{50} values ranged from 7-26% effluent (Fig. 4.13), with a mean LC_{50} of 19% and a C.V. of 32%.

ANOVA indicated that embryo response to both unmodified and modified treatments of effluent varied significantly over time (Table 4.7). Analyses were also performed between embryo responses for tests conducted with the two treatments of primary effluent at any one time. It was concluded that there were significant differences in responses of embryos exposed to modified and unmodified treatments of the same effluent sample.

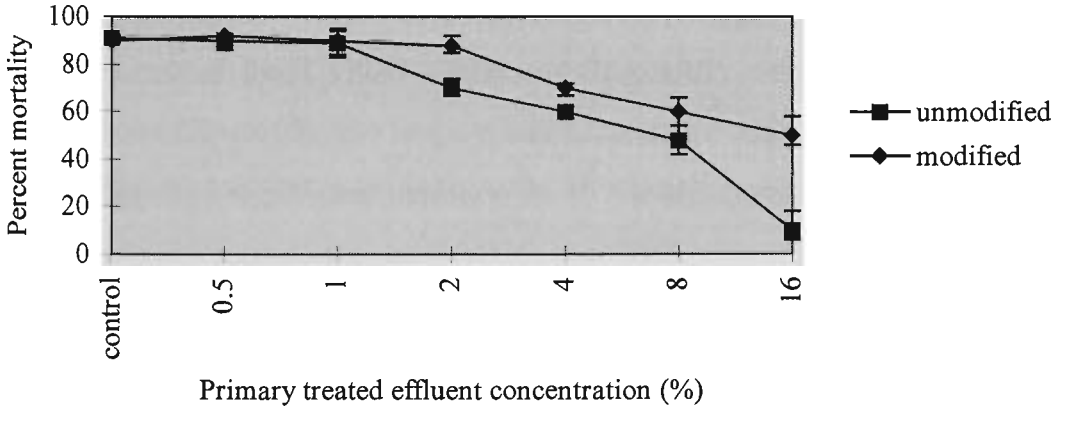


Fig. 4.13 Mortality (Mean ± Standard Error) (n = 4) of *Hormosira banksii* embryos exposed to primary treated effluent for 96 hours.

Table 4.7 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Hormosira banksii* embryos exposed to modified (mod) and unmodified (un) primary treated effluent for 96 hours.

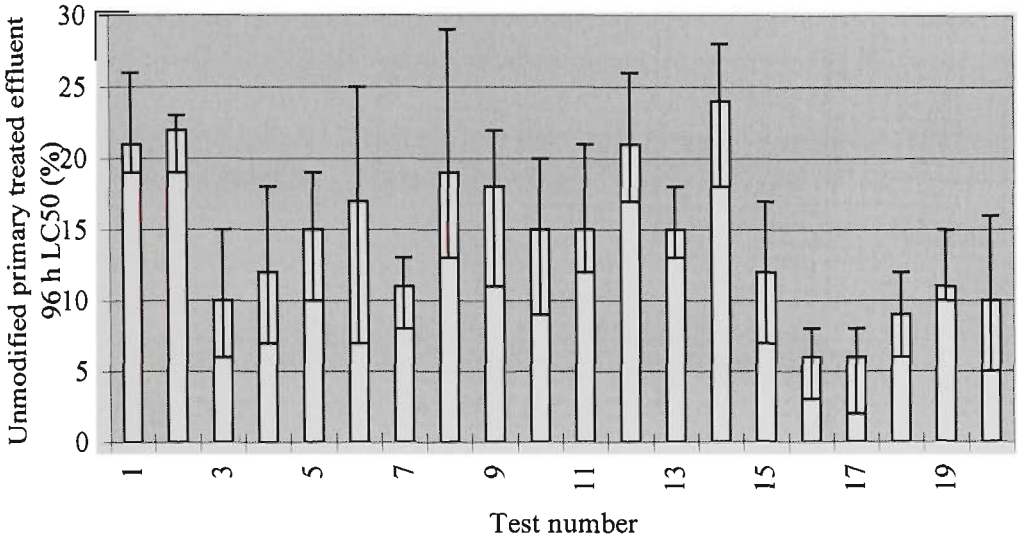
Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	1.282	3.457	85.22	95.36	0.000	0.000
Error	18	0.015	0.036				
Within Subjects							
Test	19	0.255	0.111	31.87	56.27	0.000	0.000
Treatment × Test	95	0.013	0.009	1.63	6.24	0.000	0.000
Error	342	0.008	0.000				

Secondary treated effluent

Unmodified secondary treated effluent exerted significant effects on mortality of *H. banksii* embryos (Fig. 4.14). The NOEC values for these bioassays ranged from 2.5-10% effluent and the LOEC values ranged from 5-20% effluent (Appendix 29). The 96 hour LC_{50} values ranged from 11-39% effluent (Fig 4.15) with corresponding salinities of 28.88-21.48 ppt (Appendix 29) and a mean LC_{50} value of 24% effluent and C.V. of 30%. ANOVA suggested significant variation in *H. banksii* embryo response over time (Table 4.8).

Modified secondary treated effluent also exerted significant effects on *H. banksii* embryos (Fig. 4.14). The NOEC values ranged from 5-10% effluent and LOEC values ranged from 10-20% effluent (Appendix 29). The 96 hour LC_{50} values ranged from 15-39% effluent (Fig. 4.15) with a mean of 26% and a C.V. of 26%. ANOVA suggested significant variation in embryo response over time (Table 4.8).

(a)



(b)

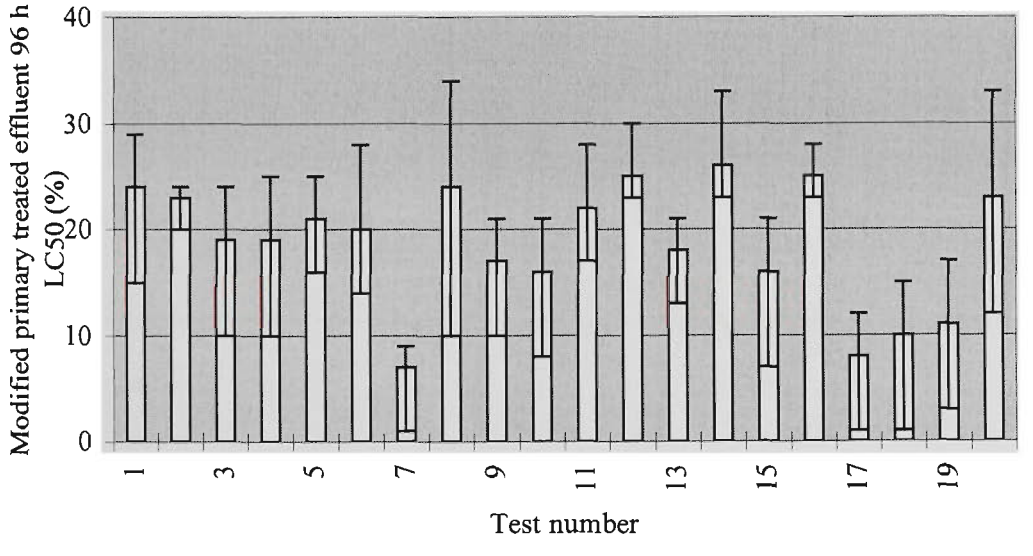


Fig. 4.14 Response over time of *Hormosira banksii* embryos exposed to unmodified (a) and modified (b) primary treated effluent for 96 hours. $LC_{50} \pm 95\%$ confidence intervals (n = 4).

A final ANOVA was also conducted between tests with modified and unmodified secondary treated effluent of the same sample to determine any similarities between results. It was found that significant differences lay between any tests conducted at any one time.

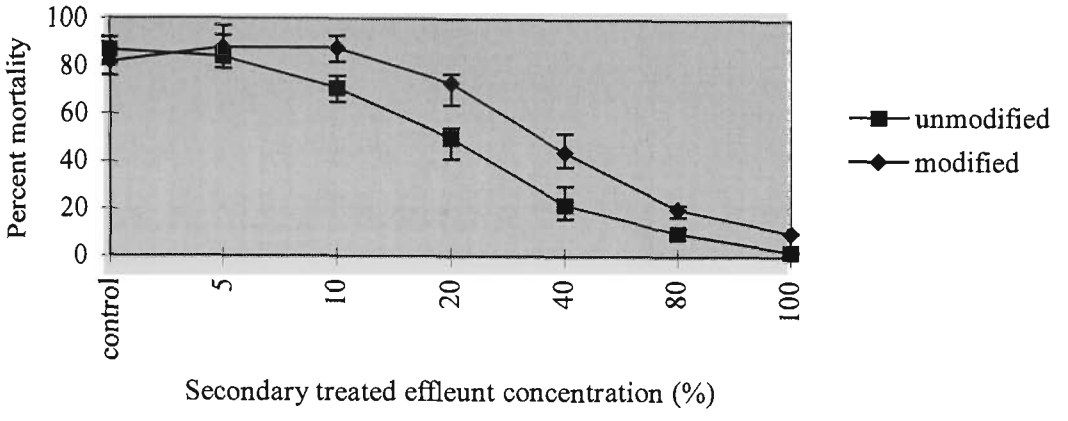
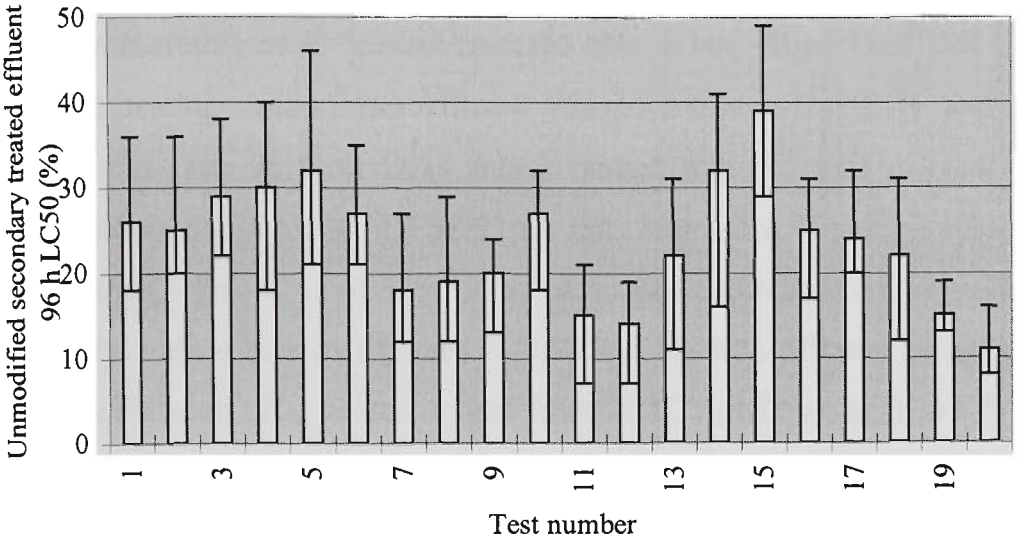


Fig. 4.15 Mortality (Mean ± Standard Error) (n = 4) of *Hormosira banksii* embryos exposed to secondary treated effluent for 96 hours.

Table 4.8 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Hormosira banksii* embryos exposed to modified (mod) and unmodified (un) secondary treated effluent for 96 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	1.578	3.453	88.56	87.29	0.000	0.000
Error	18	0.018	0.039				
Within Subjects							
Test	19	0.245	0.044	35.27	19.52	0.000	0.000
Treatment × Test	95	0.018	0.009	2.57	2.09	0.000	0.000
Error	274	0.006	0.004				

(a)



(b)

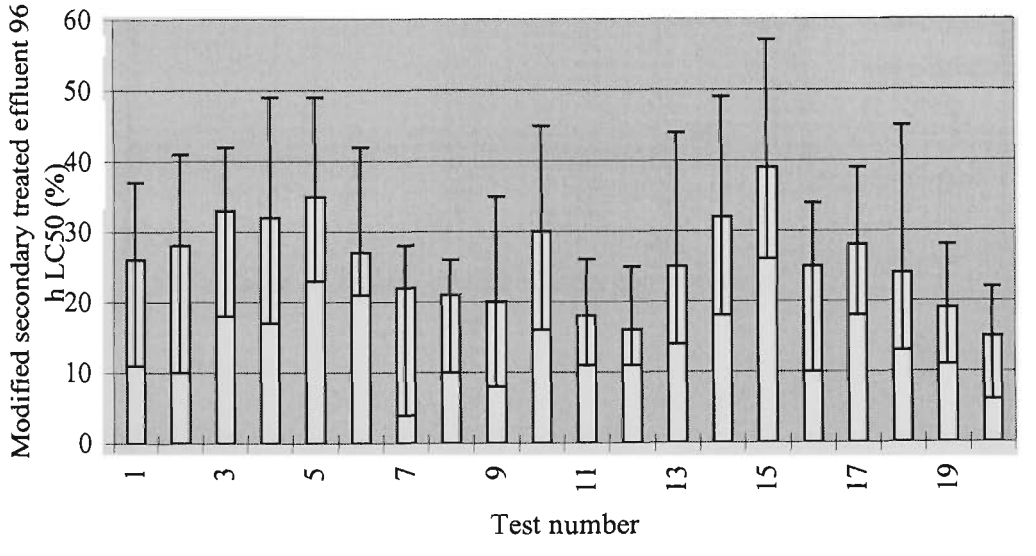


Fig. 4.16 Response over time of *Hormosira banksii* embryos exposed to unmodified (a) and modified (b) secondary treated effluent for 96 hours. $LC_{50} \pm 95\%$ confidence intervals (n = 4).

Chlorinated secondary treated effluent

Both modified and unmodified chlorinated secondary treated effluent exerted significant effects on mortality of *H. banksii* embryos (Fig. 4.16). The NOEC and LOEC values for bioassays testing unmodified effluent ranged between 2.5-10% and 5-20% effluent respectively. The 96 hour LC_{50} values ranged from 15-38% effluent (Fig 4.17) with corresponding salinities of 23.38-21.69 ppt (Appendix 31) and a mean LC_{50} value of 27% effluent with a C.V. of 32%. For tests conducted with modified effluent, the NOEC and LOEC values ranged between 5-20% and 10-40% effluent respectively (Appendix 30). The 96 hour LC_{50} values ranged from 20-67% effluent (Fig. 4.17), with a mean of 46% and C.V. of 30%. ANOVA suggested significant variation in *H. banksii* response over time (Table 4.9). Further ANOVA indicated no similarities between any tests conducted at any one time.

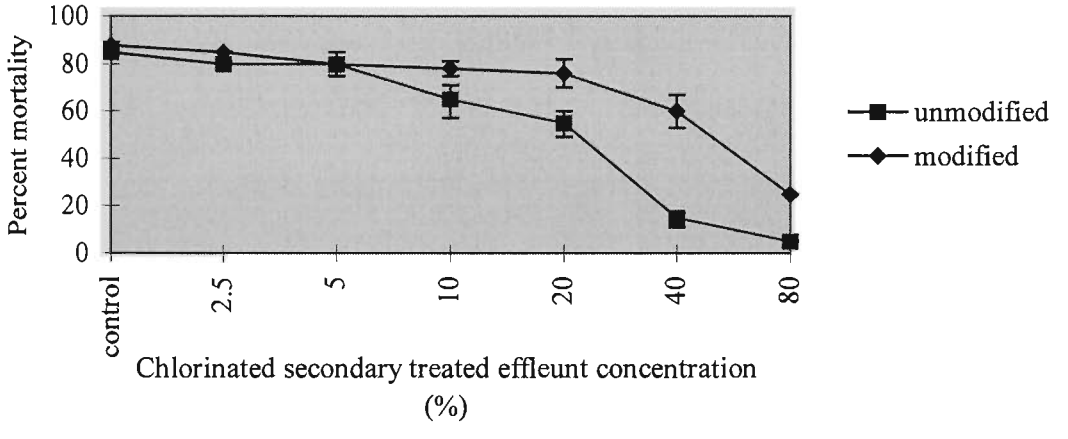
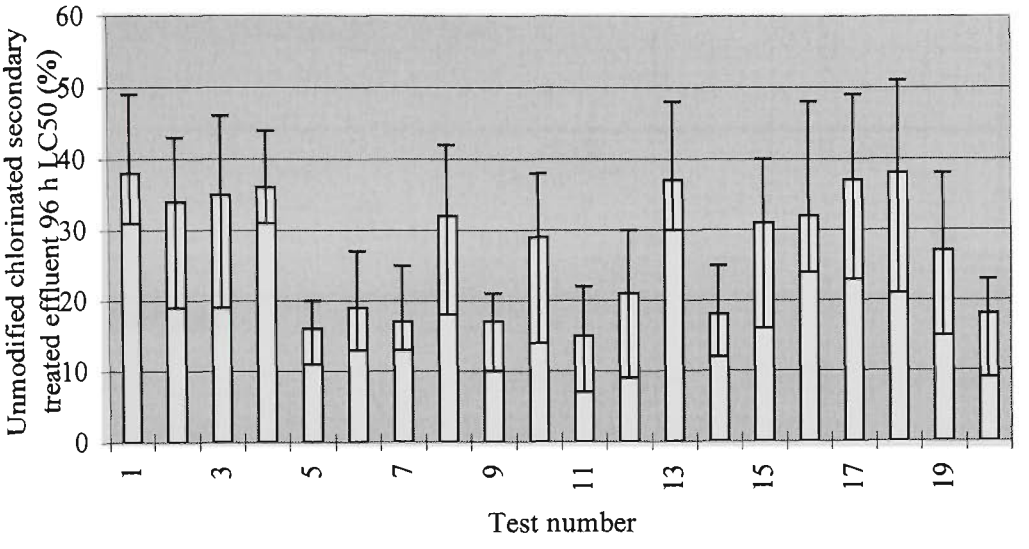


Fig. 4.17 Mortality (Mean ± Standard Error) (n = 4) of *Hormosira banksii* embryos exposed to chlorinated secondary treated effluent for 96 hours.

(a)



(b)

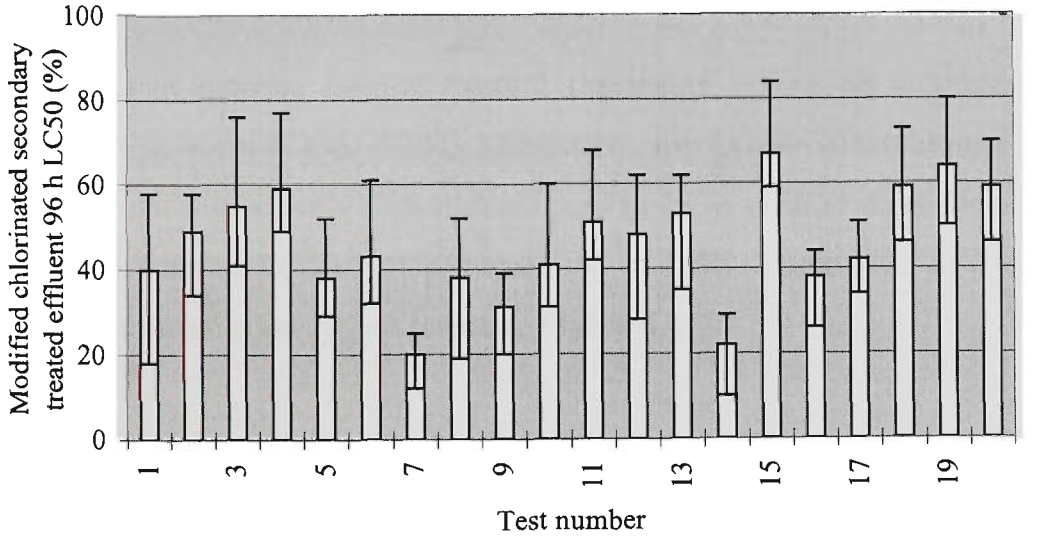


Fig. 4.18 Response over time of *Hormosira banksii* embryos exposed to unmodified (a) and modified (b) chlorinated secondary treated effluent for 96 hours. LC_{50} ± 95% confidence intervals (n = 4).

Table 4.9 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Hormosira banksii* embryos exposed to modified (mod) and unmodified (un) chlorinated secondary treated effluent for 96 hours.

Factor	df	MS		F ratio		P	
		mod	un	mod	un	mod	un
Between Subjects							
Treatment	5	0.898	1.254	154.23	148.56	0.001	0.000
Error	18	0.005	0.008				
Within Subjects							
Test	19	0.594	0.987	85.36	101.36	0.000	0.000
Treatment × Test	95	0.089	0.054	5.89	10.25	0.000	0.000
Error	342	0.007	0.010				

Salinity

Treatments of reduced salinity exerted significant effects on mortality of *H. banksii* embryos (Fig. 4.18) (Table 4.10). The NOEC and LOEC values ranged from 26-20 ppt and 24-18 ppt respectively (Appendix 31). The LC_{50} values ranged from 20-12 ppt (Fig. 4.19), with a mean LC_{50} of 17 ppt and C.V. of 13%. There were also no indications of temporal influences on zygote response (Table 4.10).

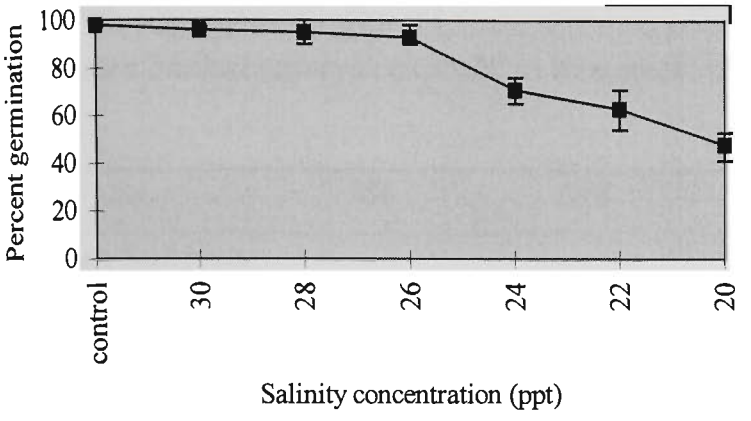


Fig. 4.19 Mortality (Mean ± Standard Error) (n = 4) of *H. banksii* embryos exposed to reduced salinity for 96 hours.

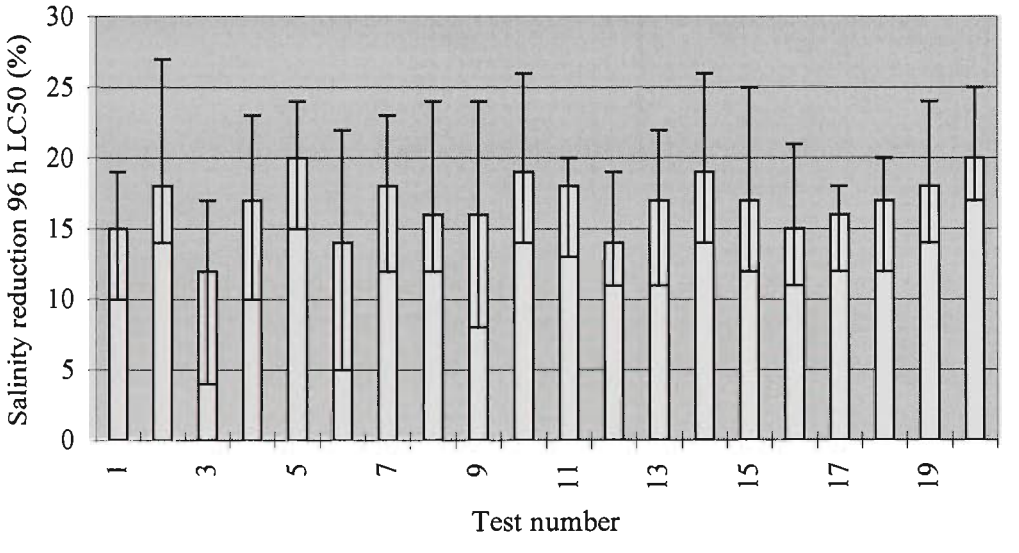


Fig. 4.20 Response over time of *Hormosira banksii* embryos exposed to treatments of reduced salinity for 96 hours. LC_{50} ± 95% confidence intervals (n = 4).

Table 4.10 Table of repeated measures ANOVA on the effect of treatment, test date and treatment × test date interactions on arcsine transformed percentage mortality data of *Hormosira banksii* embryos exposed to treatments of reduced salinity for 96 hours.

Factor	df	MS	F ratio	P
Between Subjects				
Treatment	5	0.522	64.89	0.000
Error	18	0.011		
Within Subjects				
Test	19	0.009	5.32	0.000
Treatment × Test	95	0.001	2.99	0.060
Error	342	0.000		

4.2.3 Sewage effluent and EC_{50} correlations

As for Chapter 3, possible correlations between recorded LC_{50} values and chemical levels of chlorinated secondary treated effluent tested were analysed. Pearson correlations with adjusted probabilities indicated that positive correlations between LC_{50} values and certain compounds existed. The LC_{50} values obtained for *H. banksii* mortality assays significantly correlated with copper concentrations (r=0.518, s_r=0.066, r0.05(2) 18=0.378) (analysis range = 0.001-0.05 mg/L) whilst LC_{50} values obtained from *P. comosa* assays significantly correlated with mercury concentrations (r=0.712, s_r=0.096, r0.05(2)18=0.378) (analysis range = 0.00001-0.0008 mg/L). Mortality bioassays conducted with both species produced LC_{50} values that significantly correlated with reduced salinity (r>0.378, n=25, r0.05(2)18=0.378) (analysis range = 34-12 ppt).

4.2.4 Test species sensitivity

Relative sensitivities of *P. comosa* and *H. banksii* embryos have been tabulated below. These comparisons refer to sensitivities between species not within species.

Table 4.11 Table of relative sensitivities of *Phyllospora comosa* and *Hormosira banksii* embryos after exposure to various treatments for 96 hours (scale: *** refers to highest sensivity, ** refers to moderate sensitivity, * refers to lowest sensitivity).

Species	Treatment	Relative Sensitivity
P. comosa	Copper	***
H. banksii	Copper	**
P. comosa	Primary treated effluent (modified/unmodified)	***/**
H. banksii	Primary treated effluent (modified/unmodified)	**/**
P. comosa	Secondary treated effluent (modified/unmodified)	**/**
H. banksii	Secondary treated effluent (modified/unmodified)	***/**
P. comosa	Chlorinated secondary effluent (modified/unmodified)	***/***
H. banksii	Chlorinated secondary effluent (modified/unmodified)	*/*
P. comosa	Salinity	***
H. banksii	Salinity	**

4.3 Discussion

4.3.1 Relative sensitivity of test species

The results suggest that *P. comosa* embryos were more sensitive to the effects of copper than those of *H. banksii*. A similar trend was evident with assays conducted with modified and unmodified primary treated effluent samples. For unmodified secondary treated effluent, both species demonstrated similar sensitivities to the treatments tested. *P. comosa* embryos however, were significantly less sensitive to the effects of modified secondary treated effluent than embryos of *H. banksii*. For tests conducted with chlorinated secondary treated effluent and reduced salinity, *P. comosa* was the more sensitive of the two species.

Most research into toxicological effects of heavy metals and effluents utilising mortality as the test endpoint have focussed on animal species. The majority of work has been conducted on fish (Franklin 1983, Fava *et al.* 1985, Costello & Gamble 1992), crustacean (Franklin 1983, Fava *et al.* 1985, Santoro & Fikslin 1987) and mollusc (Costello & Read 1994) species. The use of macroalgae in routine toxicity testing has in the past been criticised due to the perceived insensitivity of test species to many toxicants (Thursby *et al.* 1993). Research has shown that the early life stages of these species are in fact highly sensitive to a wide range of toxicants (Steele & Hanisak 1978, Anderson *et al.* 1990, Burridge *et al.* 1996). A great deal of research on embryo mortality after toxicant exposure in large brown macroalgal species has not yet been conducted. Most studies have concentrated on germination (Anderson & Hunt 1990, Costello & Read 1994, Burridge & Shir 1995, Burridge *et al.* 1996, Bidwell *et al.* 1998), growth (Anderson *et al.* 1990, Doblin & Clayton 1995), fertilisation (Gunthorpe *et al.* 1995) and reproduction (Anderson *et al.* 1990) of these species. There has been some limited work conducted on mortality of *P. comosa* embryos (Lavery 1994, Shir 1994). Research into the effects of crude oil and dispersants on mortality of embryos has also been successfully conducted on the species. It was found in this study that embryos demonstrated varied sensitivity to the treatments tested. In several instances the mortality endpoint was more sensitive than germination. This was also highlighted in a study exposing *P. comosa* embryos to a mix of the chemical dispersant Corexit 7664 (Exxon Corporation) in seawater. After

exposure to this mix, the 48 hour EC_{50} value was recorded at 10,500 mg/L whereas the 96 hour LC_{50} value was recorded at 1600 mg/L (Shir 1994). In these instances it would be premature to dismiss data available from the 96 hour mortality bioassays. Basing, for example, safe discharge levels of toxicants solely on 48 hour EC_{50} values may prove inappropriate if at significantly lower concentrations the onset of mortality rates are recorded shortly afterwards. The observation of differences in sensitivity between mortality and sublethal endpoints tends to contradict the norm. This however, can be explained by the longer exposure times for mortality bioassays as opposed to those of germination. The variation between species sensitivity can be attributed to a number of factors ranging from speed of recruitment between *P. comosa* and *H. banksii* embryos to subtle differences in modes of reproduction. The life spans of these species may also explain the greater sensitivity of one species over the other (Doblin 1992).

The sensitivity of *H. banksii* and *P. comosa* embryos to the effects of copper, treated sewage effluent and reduced salinity suggest that these species could be used in routine toxicity testing. Mortality bioassays are quick and simple to perform as they can be incorporated with germination assays and hence be used as an added tool in assessing coastal water quality. It would be recommended however, that these tests be utilised in conjunction with the 48 hour germination bioassay.

The results obtained from this study suggest that acute toxicity tests such as the mortality bioassay are useful in establishing initial benchmarks of treatment toxicity, comparing relative treatment toxicities and as a tool for monitoring changes in treatment toxicity over time. Although sub-lethal concentrations are often considered of greater environmental interest than acute lethal concentrations, there are still good reasons for determining LC_{50} values, especially for determining effluent toxicity. Lethal concentrations can be more convenient for routine monitoring. It can also be possible to establish fairly constant relationships between LC_{50} values and the lowest concentrations where disturbances can be registered (Boedeker *et al.* 1993).

4.3.2 Treatment toxicity

This study demonstrated that although chromium exerted no detectable toxic effects on embryos of *H. banksii* and *P. comosa*, copper exerted significant effects on mortality of the species. The effects of copper have been linked to inhibiting growth, photosynthesis, and respiration in algal species (Stauber & Florence 1987). Research into the effects of copper on marine unicellular algae have shown that copper reacts at the cell membrane affecting cell division and permeability (Stauber & Florence 1985). A positive correlation existed between copper concentrations in effluent treatments and mortality response in *H. banksii* embryos tested in this study. For *P. comosa* however, a positive correlation existed between mortality response and mercury concentrations in the effluent treatments. Mercury has been shown to inhibit growth in the planktonic alga *Dunaliella minuta* (Gotsis 1982) and may cause synergistic effects when present with other heavy metals.

Treatments of sewage effluent exerted varied effects on mortality of *H. banksii* and *P. comosa* embryos. The cause of toxicity cannot be attributed to any one single component. Reduced salinity also played a major part in increasing the toxicity of effluents tested. As previously mentioned, a lack of data exists on the lethal effects of treated effluent on macroalgal species with most mortality tests being conducted on fish larvae (Costello & Gamble 1992).

4.3.3 Conclusion

The mortality bioassays conducted in this study were not aimed to attempt to identify possible long-term or cumulative effects of treated sewage effluent. These short-term and partial life-cycle tests can however, be used to help better understand and predict the effects of discharged effluent. Mortality tests can be regularly used to evaluate concentrations of treatments required to produce the criterion effect and to determine and detect the ecotoxicological effects of these treatments. These bioassays are important tools in understanding fully the impacts of potential toxicants such as discharged sewage effluents.

CHAPTER 5
THE EFFECTS OF SEWAGE EFFLUENT ON GROWTH OF *PHYLLOSPORA COMOSA* AND *HORMOSIRA BANKSII,* AND GROWTH AND REPRODUCTION OF *MACROCYSTIS ANGUSTIFOLIA.*

5.1 Introduction

Growth and reproduction (Thursby *et al.* 1985, Thursby & Steele 1986, Anderson & Hunt 1988, 1990, Doblin 1992, Lavery 1994, Shir 1994, Burridge *et al.* 1995b, Doblin & Clayton 1995, Stauber 1995) of marine algal species have been extensively utilised as test endpoints in ecotoxicological testing. These bioassays can provide information on the effects of toxicants after longer exposure times and normally produce lower threshold concentrations. These lower concentrations may reflect real toxicant concentrations present in the environment giving a better indication of actual effects in exposed areas. Sub-lethal toxicity tests, such as growth and reproduction inhibition, can also be used to complement wastewater evaluation procedures based on chemical data and monitoring programmes (Garric *et al.* 1993).

Monitoring programmes have long been relied upon to provide information on impacts of sewage effluent on marine ecosystems. These programmes have been extensively conducted along Australian coastlines (Borowitzka 1972, Manning 1979, Brown *et al.* 1990, Bellgrove *et al.* 1997). However, it has become apparent that monitoring programmes alone cannot predict outcomes of continuous effluent discharge. Laboratory testing is required to complement monitoring to determine detrimental effects of compounds on sensitive species (Levine 1984). Monitoring studies have shown that differences in population dynamics lie between polluted and unpolluted sites (Austen *et al.* 1989, Smith & Simpson 1993) and it is now essential to apply laboratory based findings in the form of sub-lethal ecotoxicological testing to predict these outcomes before they occur.

Reductions in survival and reproductive success of macroalgae are of ecological importance as they can lead to diminished populations and less diverse communities (Buikema Jr *et al.* 1982). Through chronic toxicity testing, the lowest observable effect concentration is experimentally determined and can be applied to the determination of 'safe' discharge levels. Thus, chronic toxicity tests can be used to monitor sub-lethal responses of species that are of convincing ecological significance (Buikema Jr *et al.* 1982).

5.1.1 Aims of this chapter

The aims of this chapter were to:

1. determine the effects of sewage effluent on growth of *Phyllospora comosa* and *Hormosira banksii* embryos and growth and reproduction of *Macrocystis angustifolia* sporophytes.

2. investigate possible temporal variation in algal growth and reproduction responses by conducting bioassays with two reference toxicants.

5.2 Results

5.2.1 *Phyllospora comosa*

Reference toxicants

Copper (Fig. 5.1) and chromium (Fig. 5.2) exerted significant effects on growth *of P. comosa* embryos after exposure for one and two weeks. Repeated measures ANOVA indicated significant differences in embryo response over the 12 month test period where for both treatments, significant differences in embryo response existed after exposure to treatments of copper and chromium. Repeated measures ANOVA with a grouping factor of treatment concentrations were applied to detect time (month) and treatment interactions for the tests conducted over the 12 months. It was found that significant interactions between time (month) and treatment existed for tests conducted with copper (P=0.000) and chromium (P=0.000) for both one and two week exposure times. ANOVA were applied to detect differences in embryo growth rates between one and two week exposure times. Growth rates of embryos significantly decreased by the second week, with a reduction amplified with increasing concentrations. Single Factor ANOVA were conducted for all tests individually and it was found that increasing treatment concentrations exerted significant effects on growth of *P. comosa.* Significant effects were noted for all tests with NOEC and LOEC values ranging from 0.001-0.002 mg/L and 0.002-0.004 mg/L respectively after one week exposure to copper treatments and <0.001-0.001 mg/L and 0.001-0.002mg/L respectively after two weeks exposure. The corresponding mean EC_{50} values for these tests were found to be 0.007 mg/L copper with a C.V. of 46% for week one and 0.003 mg/L copper with a C.V. of 47% for week two. The recorded EC_{50} values reflect a 50% reduction in growth rates of the embryos. For growth tests conducted with chromium, NOEC and LOEC values were found to range from 5-10 mg/L and 10-20 mg/L for week one respectively and 2.5-5 mg/L and 5-10 mg/L respectively for week two. The mean calculated EC_{50} values were 18 mg/L chromium with a C.V. of 55% for week one and 10 mg/L chromium with a C.V. of 49% for week two.

To determine where differences existed, data from specific months were analysed. This was repeated until significant differences for specific months were indicated. For these

data sets, significant differences in embryo response fell into seasons. It was found that significant increases in embryo growth rates were found in the winter months compared to the remaining seasons and no significant differences in response between summer, autumn and spring months.

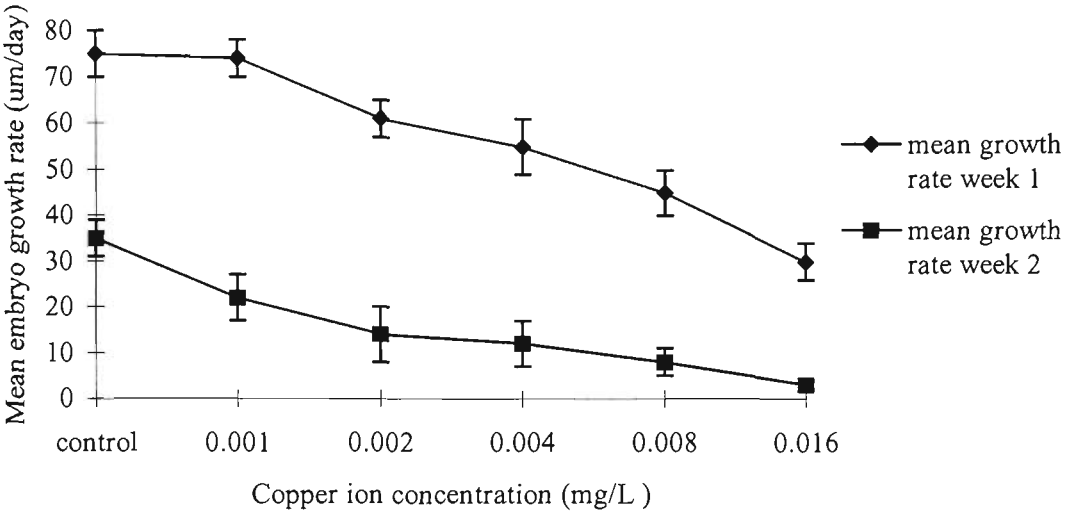


Fig. 5.1 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to copper.

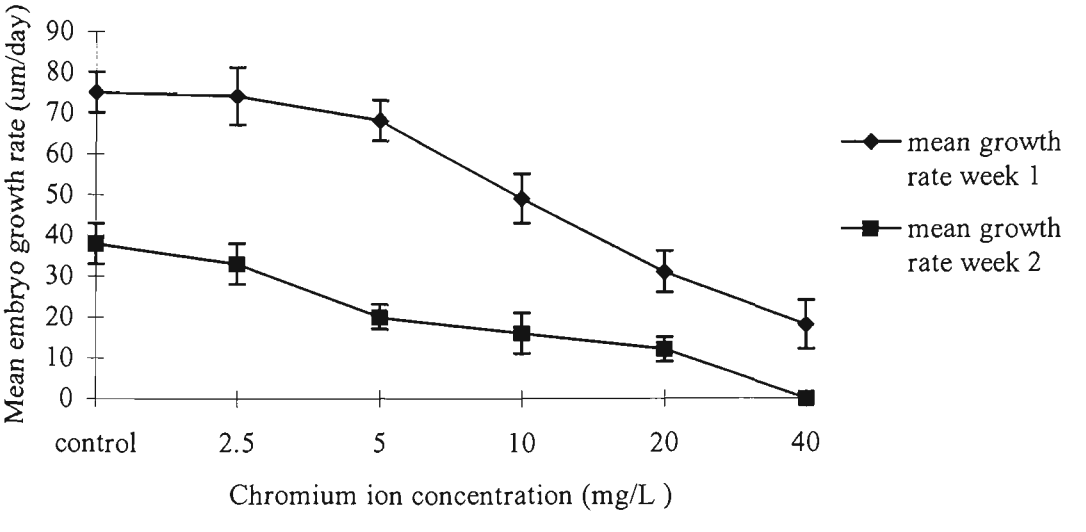


Fig. 5.2 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to chromium.

Primary treated effluent

Growth rates of *P. comosa* embryos were significantly reduced by primary treated effluent after exposures of one and two weeks. Severe deformities and stunted growth of embryos were observed at these stages in concentrations greater than 0.5% for both modified and unmodified primary treated effluent. Both unmodified (Fig. 5.3) and modified (Fig. 5.4) primary treated effluents significantly affected growth rates of the embryos. ANOVA performed on all tests showed that increasing treatment concentrations significantly inhibited growth of *P. comosa.* Repeated measures ANOVA detected significant differences in response of embryos over the 12 month test period and interactions between time (month) and treatments ($P=0.039$ for unmodified effluent tests and $P=0.042$ for modified effluent tests). It was found however, that these interactions were due to fluctuating toxicities of effluents tested rather than seasonal influences. Further analyses indicated no differences between algal responses to modified and unmodified primary treated effluent at any one time. The NOEC values recorded for embryos exposed to both modified and unmodified primary treated effluent for week one, ranged from 2-4% effluent with LOEC values ranging from 4-8%. At two weeks, the recorded NOEC values were found to be 0.5-1% effluent and LOEC values ranged from 1-2% effluent. The mean EC_{50} value for embryos exposed to primary treated effluent for one week was found to be 8% effluent with a C.V. of 59%. For week two, the mean EC_{50} was found to be 4% with a C.V. of 58%. Again ANOVA identified significant differences in embryo growth rates between one and two weeks, where by the second week, growth rates declined substantially.

Secondary treated effluent

Secondary treated effluent exerted significant effects on growth rates of *P. comosa* embryos. During the two week exposure period, deformities in development and stunted growth of embryos were generally observed in treatments of greater than 2.5% for both modified and unmodified secondary treated effluent. Repeated measures ANOVA detected differences in response over the 12 month test period for both modified ($P=0.041$) and unmodified ($P=0.038$) effluent samples. Interactions between time (month) and treatment for both modified and unmodified effluent samples were also

detected, but there was no evidence of seasonal influences. ANOVA were also used to determine differences in algal response between modified and unmodified secondary treated effluent and also between one and two week growth rates.

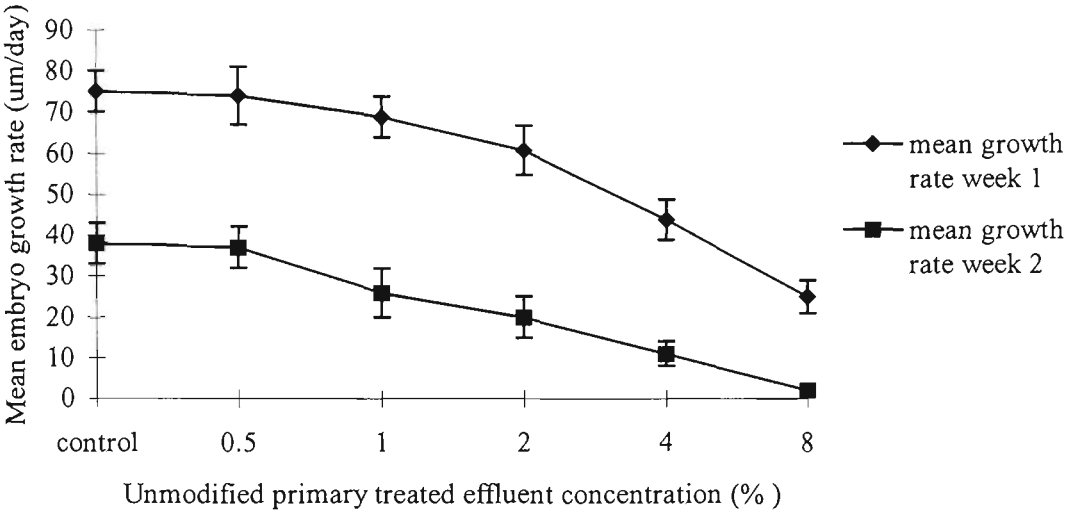


Fig. 5.3 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to unmodified primary treated effluent.

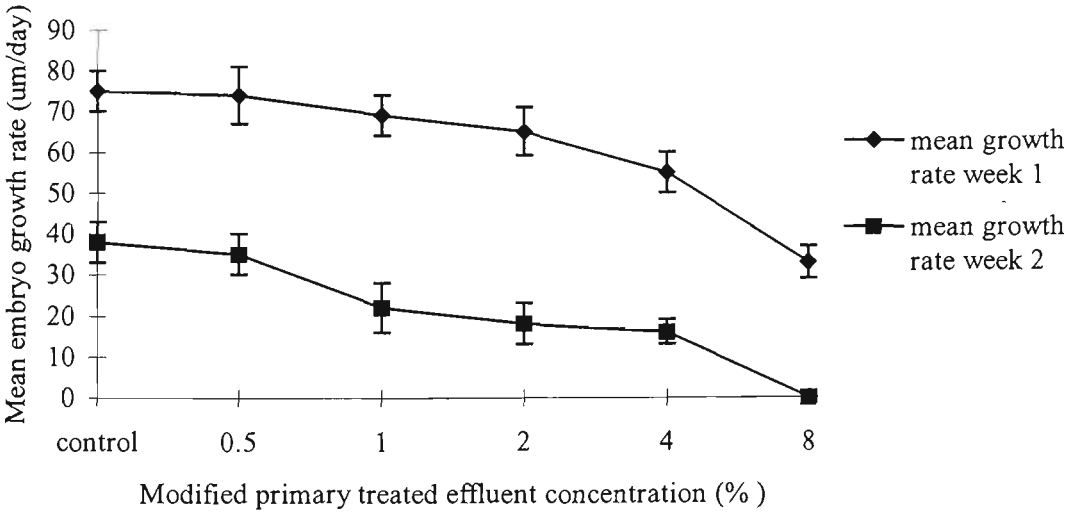


Fig. 5.4 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to modified primary treated effluent.

Single factor ANOVA showed that increasing concentrations of both unmodified (Fig. 5.5) and modified (Fig. 5.6) effluent significantly inhibited growth rates of *P. comosa* embryos. It was also found that reduced salinity in the unmodified effluent samples together with longer exposure significantly increased toxicity of the effluent. The NOEC values for embryos exposed to unmodified effluent ranged from 5-10% effluent in week one and 2.5-5% effluent in week two. The LOEC values for these tests ranged from 10-20% effluent and 5-10% effluent respectively. The mean EC_{50} value for week one was found to be 15% effluent with a C.V. of 49% and corresponding salinity of 28.38 ppt, and for week two, the mean EC_{50} value was found to be 9% with a C.V. of 48% and corresponding salinity of 29.13 ppt. The NOEC values for embryos exposed to modified effluent ranged from 10-20% effluent in week one and 5-10% effluent in week two. The corresponding LOEC values ranged from 20-40% effluent and 5-10% effluent respectively. The mean EC_{50} value for week one was found to be 26% effluent with a C.V. of 48% and for week two the mean EC_{50} value was found to be 19% with a C.V. of 49%.

Chlorinated secondary treated effluent

Chlorinated secondary treated effluent significantly effected growth rates of *P. comosa* embryos. Repeated measures ANOVA showed differences in response over the test period for both modified (P=0.045) and unmodified (P=0.039) effluent samples. Interactions between time (month) and treatment for both modified (P=0.000) and unmodified (P=0.000) effluent were also detected, but no seasonal trends were evident. Single factor ANOVA showed that increasing concentrations of both unmodified (Fig. 5.7) and modified (Fig. 5.8) effluent and longer exposure significantly inhibited growth rates of embryos. It was also found that salinity reduction was a contributing factor in increasing toxicity of the effluent. The NOEC values for embryos exposed to unmodified effluent ranged from 0.5-1% effluent in both weeks. The corresponding LOEC values for these tests ranged from 1-2% effluent. The mean EC_{50} value for week one was found to be 10% effluent with a C.V. of 50% and corresponding salinity of 29.00 ppt, and for week two, the mean EC_{50} value was found to be 9% with a C.V. of 58% and corresponding salinity of 29.13 ppt. The NOEC values for embryos exposed to modified

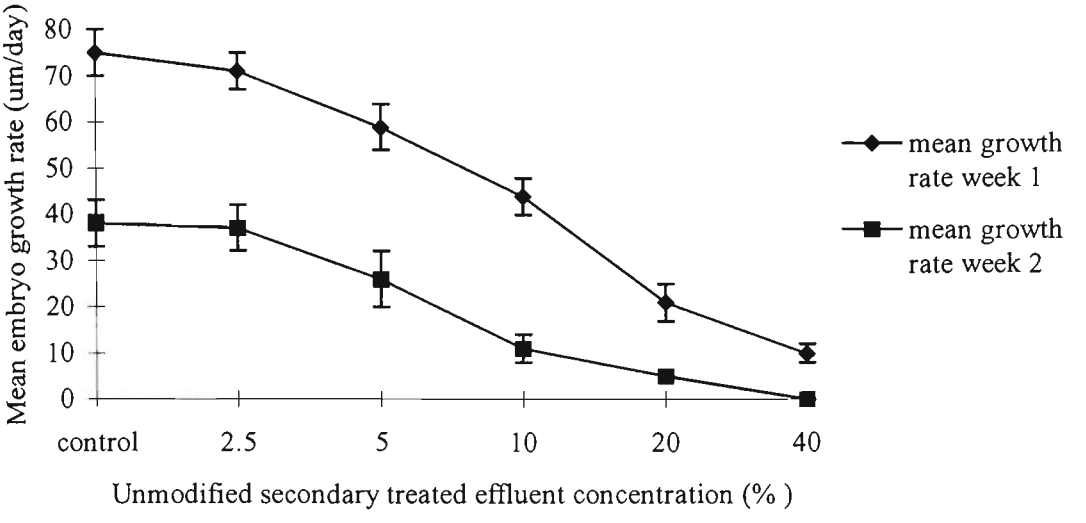


Fig. 5.5 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to unmodified secondary treated effluent.

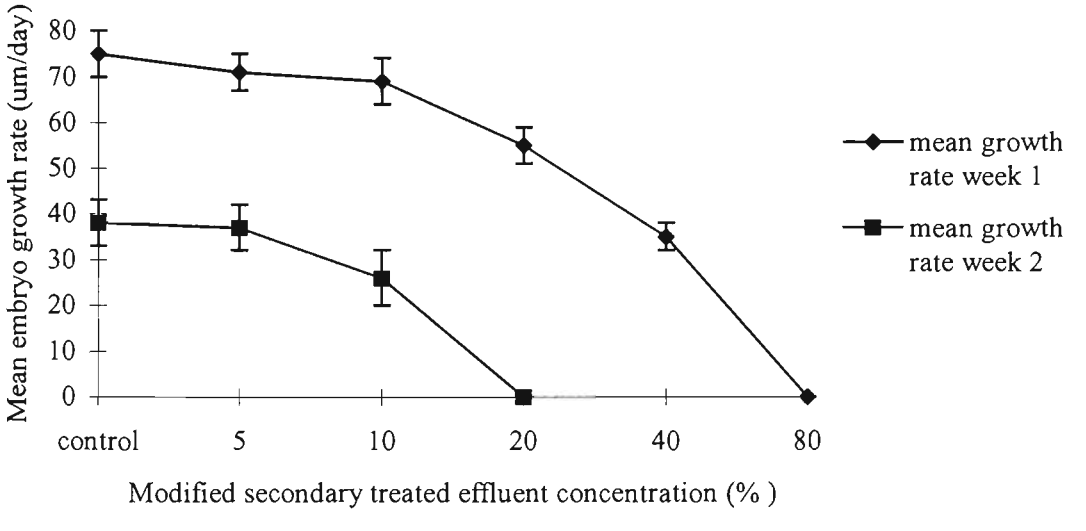


Fig. 5.6 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to modified secondary treated effluent.

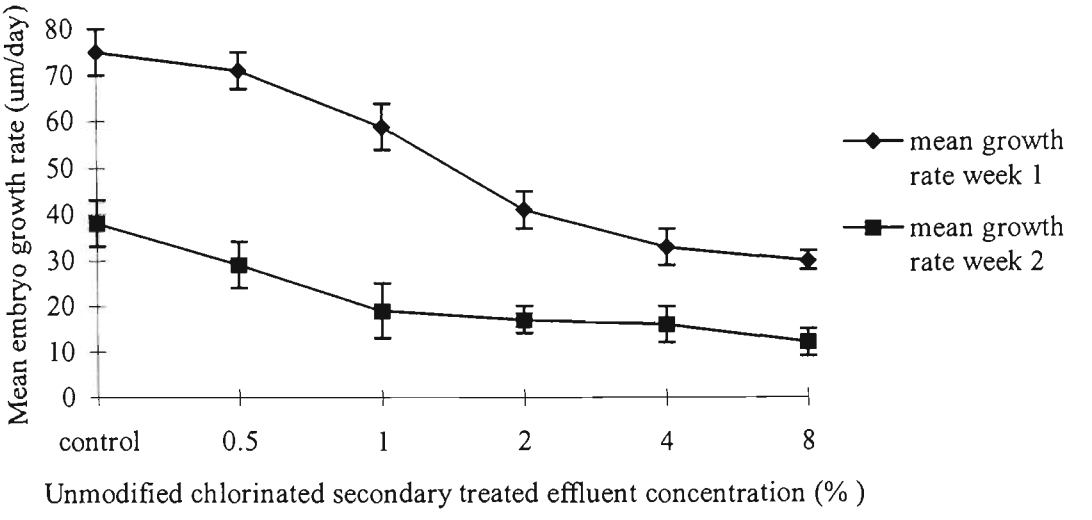


Fig. 5.7 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to unmodified chlorinated secondary treated effluent.

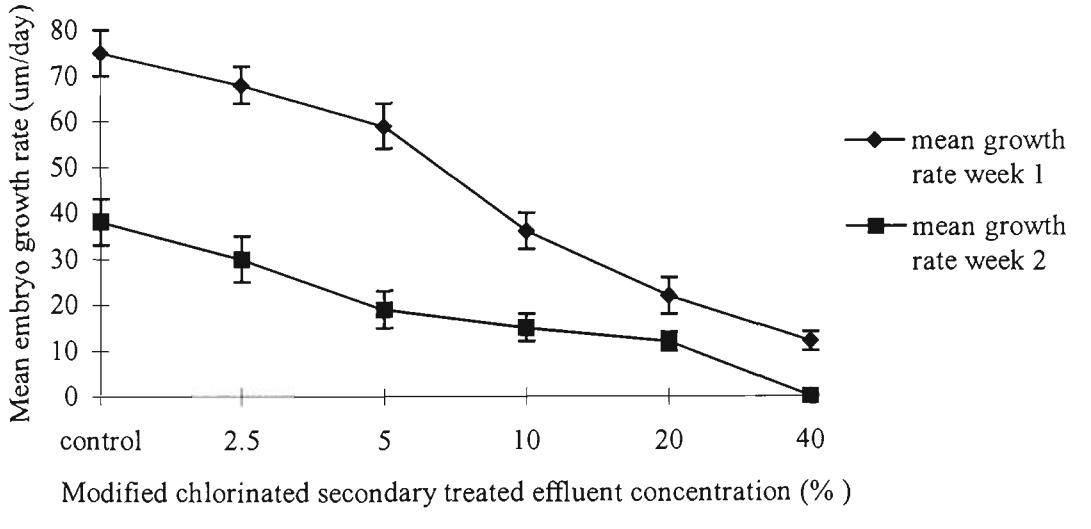


Fig. 5.8 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to modified chlorinated secondary treated effluent.

effluent ranged from 5-10% effluent in week one and 2.5-5% effluent in week two. The corresponding LOEC values ranged from 10-20% effluent and 5-10% effluent respectively. The mean EC_{50} value for week one was found to be 18% effluent with a C.V. of 48% and for week two the mean EC_{50} value was found to be 13% with a C.V. of 51%.

Salinity

Salinity reduction significantly inhibited growth rates of *P. comosa* embryos (Fig 5.9). Repeated measures ANOVA showed differences in response over the test period of 12 months. Interactions between time (month) and treatment were detected (P=0.001). It was found that differences in embryo response fell into seasons. Pronounced embryo growth rates were evident in winter months, compared to the remaining seasons and no significant differences in responses between summer, autumn and spring months. Single factor ANOVA indicated that decreasing salinity concentrations significantly inhibited growth rates of embryos. The NOEC values for embryos exposed to reduced salinity ranged from 30-28 ppt in week one and 32-30 ppt in week two. The corresponding LOEC values for these tests ranged from 28-26 ppt and 30-28 ppt. The mean EC_{50} value for week one was found to be 23 ppt with a C.V. of 40%, and for week two, the mean EC_{50} value was found to be 28 ppt with a C.V. of 33%.

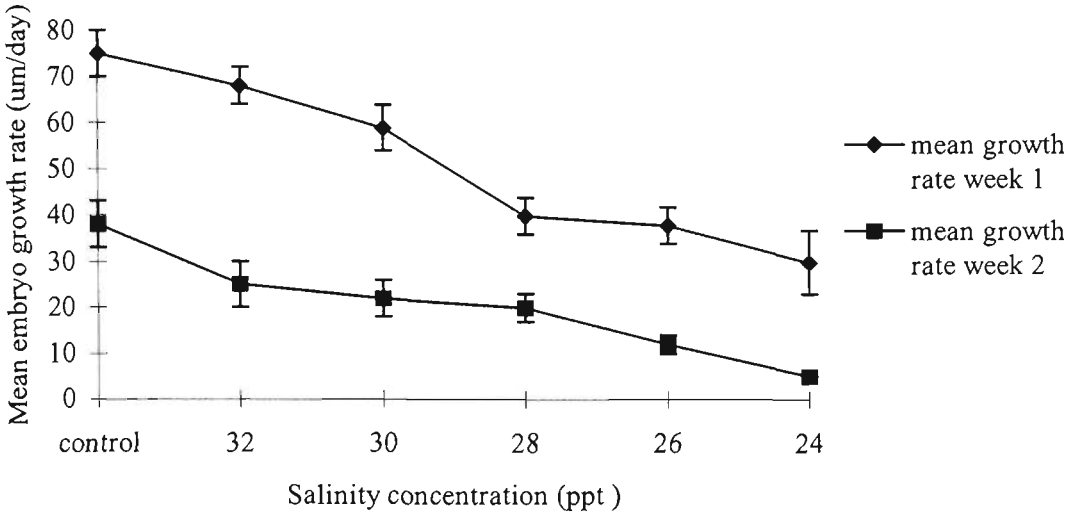


Fig. 5.9 Growth rates (Mean ± Standard Error) (n=4) of *Phyllospora comosa* embryos exposed to reduced salinity.

5.2.2 Hormosira banksii

Reference toxicants

The reference toxicants, copper (Fig. 5.10) and chromium (Fig. 5.11) inhibited growth and caused severe deformities of *H. banksii* embryos at one and two weeks. Repeated measures ANOVA detected significant differences in embryo response over the 12 month test period. Repeated measures ANOVA with a grouping factor of treatment concentrations were then applied to detect time (month) and treatment interactions. It was found that significant interactions between time (month) and treatment existed for tests conducted with copper (P=0.000) and chromium (P=0.001). Data were analysed in the same manner as for *P. comosa* and it was found that significant differences in embryo growth rates were found in the winter months compared to the remaining seasons and no significant differences in response between summer, autumn and spring. Growth rates of embryos were significantly greater during the winter months. Single Factor ANOVA determined that increasing treatment concentrations exerted significant effects on growth of *H. banksii.* Significant effects were noted for all tests with NOEC values ranging from 0.016-0.032 mg/L copper and LOEC values ranging 0.032-0.064 mg/L copper respectively after one week of exposure. NOEC and LOEC values ranged from 0.004-0.008 mg/L and 0.008-0.016 mg/L copper respectively after two weeks of exposure. Corresponding mean EC_{50} values for these tests were found to be 0.1 mg/L copper with a C.V. of 49% for week one and 0.03 mg/L copper with a C.V. of 43% for week two. For growth tests conducted with chromium, recorded NOEC and LOEC values were found to range from 1-2 mg/L chromium and 2-4 mg/L chromium for week one respectively and 0.5-1 mg/L chromium and 1-2 mg/L chromium respectively for week two. The mean calculated EC_{50} values were 11 mg/L chromium with a C.V. of 41% for week one and 4 mg/L chromium with a C.V. of 48% for week two.

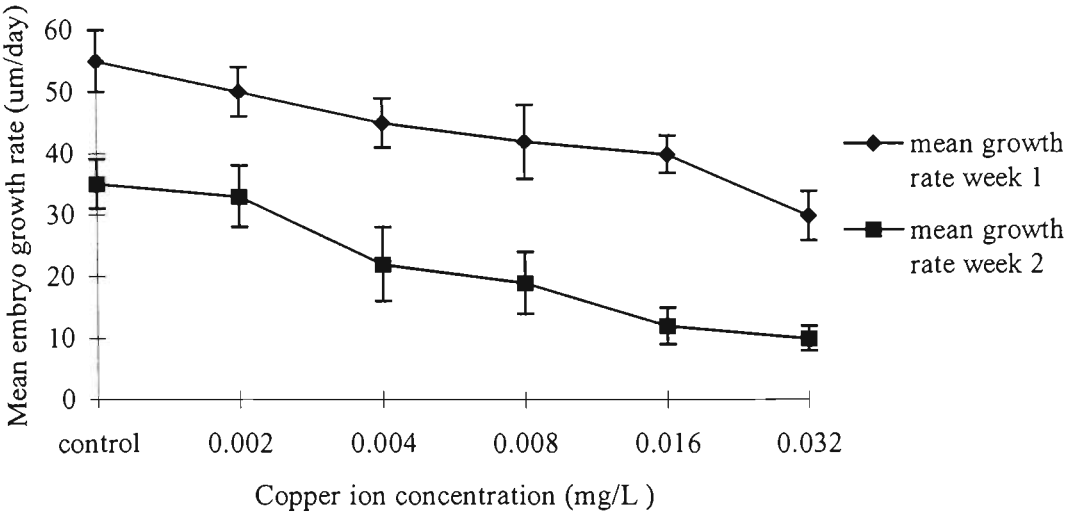


Fig. 5.10 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to copper.

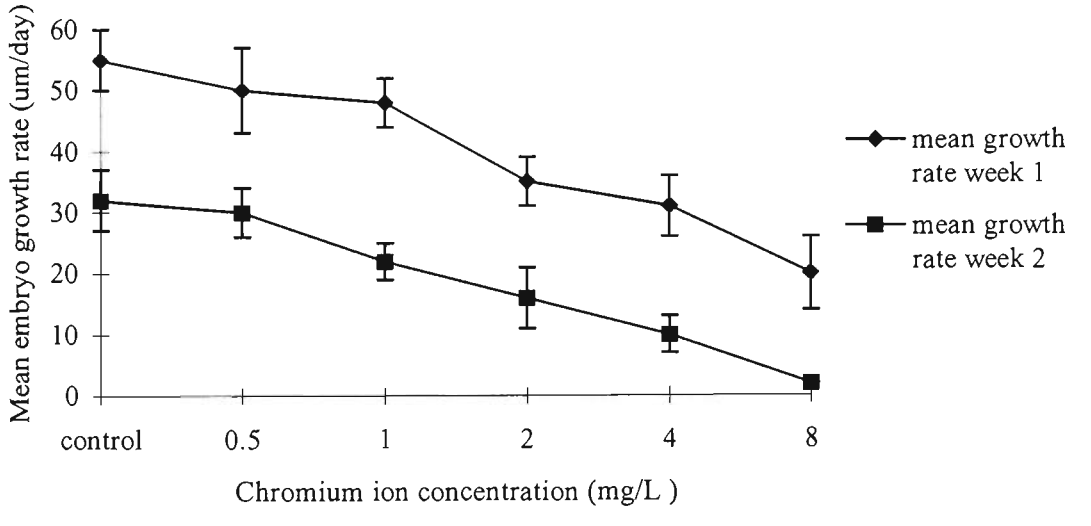


Fig. 5.11 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to chromium.

Primary treated effluent

Primary treated effluent exerted significant negative effects on growth rates of *H. banksii* embryos. There was also evidence of stunted and deformed growth of embryos in cultures of greater than 0.5% effluent. Both unmodified (Fig. 5.12) and modified (Fig. 5.13) primary treated effluents significantly affected growth rates of the embryos. Single factor ANOVA showed that increasing treatment concentrations significantly inhibited growth of *H. banksii.* Repeated measures ANOVA indicated interactions between time (month) and treatments (P=0.041 for unmodified effluent tests and P=0.044 for modified effluent tests), but there were no seasonal trends evident in embryo responses. No significant differences were detected between tests conducted with the same batch of effluent. The NOEC values recorded for embryos exposed to both modified and unmodified primary treated effluent for week one, ranged from 1-2% effluent with LOEC values ranging from 2-4% effluent. At two weeks, the recorded NOEC values were found to be 0.5-1% effluent and LOEC values ranged from 1-2% effluent. The mean EC_{50} value for embryos exposed to primary treated effluent for one week was found to be 10% effluent with a C.V. of 58%. For week two, the mean EC_{50} was found to be 5% with a C.V. of 49%. It was evident that growth rates were significantly inhibited with increasing exposure times.

Secondary treated effluent

Secondary treated effluent exerted significant effects on growth rates of *H. banksii* embryos. Repeated measures ANOVA detected differences in response over the test period for both modified (P=0.039) and unmodified (P=0.035) effluent samples. Interactions between time (month) and treatment for both modified (P=0.000) and unmodified (P=0.000) effluent were also detected, but were not due to seasonal influences, but rather the fluctuating toxicities of effluent samples. Single factor ANOVA suggested that increasing concentrations of both unmodified (Fig. 5.14) and modified (Fig. 5.15) effluent significantly inhibited growth rates of *H. banksii* embryos. It was also found that reduced salinity in the unmodified effluent samples significantly increased toxicity of the effluent. The NOEC values for embryos exposed to unmodified

effluent ranged from 5-10% effluent in week one and 2.5-5% effluent in week two. The LOEC values for these tests ranged from 10-20% effluent and 5-10% effluent

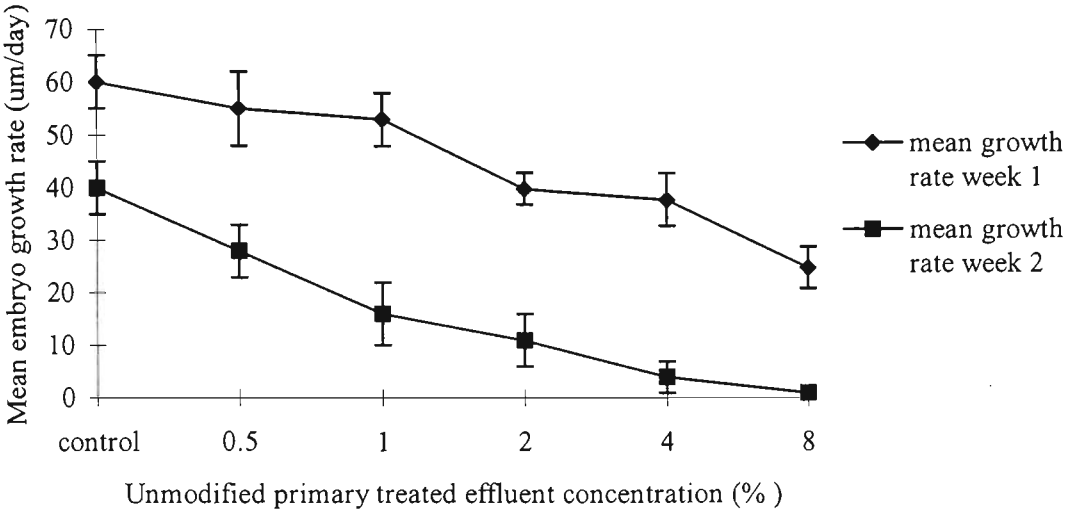


Fig. 5.12 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to unmodified primary treated effluent.

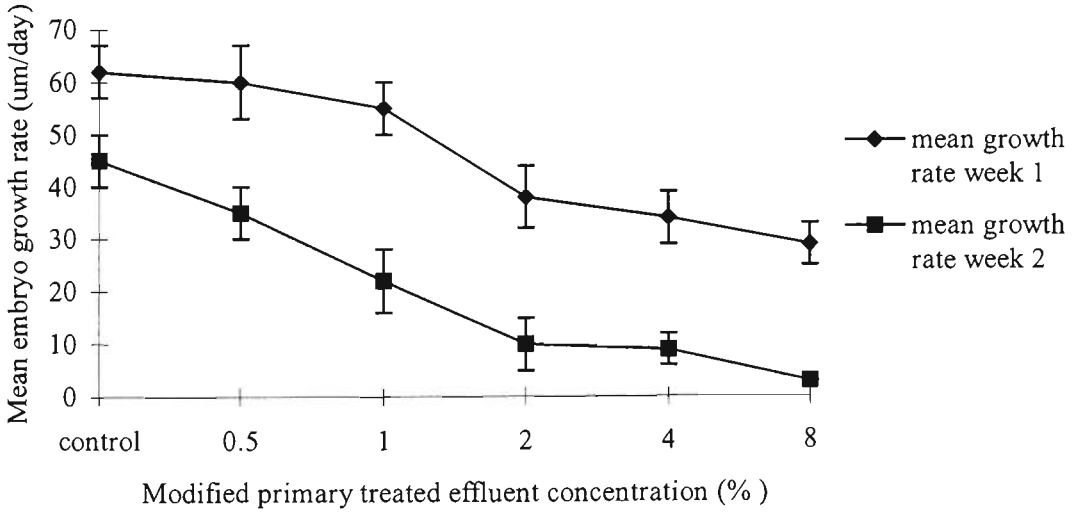


Fig. 5.13 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to modified primary treated effluent.

respectively. The mean EC_{50} value for week one was found to be 15% effluent with a C.V. of 45% and corresponding salinity of 28.38 ppt, and for week two, the mean EC_{50} value was 11 % with a C.V of 45% and corresponding salinity of 28.88 ppt.

The NOEC values for embryos exposed to modified effluent ranged from 10-20% effluent in week one and 5-10% effluent in week two. The corresponding LOEC values ranged from 20-40% effluent and 10-20% effluent respectively. The mean EC_{50} value for week one was found to be 39% effluent with a C.V. of 48% and for week two the mean EC_{50} value was found to be 21% with a C.V. of 49%.

Chlorinated secondary treated effluent

Chlorinated secondary treated effluent exerted significant effects on growth rates of *H. banksii* embryos. Repeated measures ANOVA showed differences in response over the test period for both modified (P=0.044) and unmodified (P=0.040) effluent samples. Interactions between time (month) and treatment for both modified (P=0.021) and unmodified (P=0.009) effluent were also detected, but not due to seasonal influences. Single factor ANOVA showed that increasing concentrations of unmodified (Fig. 5.16) and modified (Fig. 5.17) effluent significantly inhibited growth of embryos. It was also found that salinity reduction was a contributing factor in increasing toxicity of the effluent. The NOEC values for embryos exposed to unmodified effluent ranged from 1-2% effluent in both weeks. The corresponding LOEC values for these tests ranged from 2-4% effluent. The mean EC_{50} value after one week of exposure was found to be 13% unmodified effluent with a C.V. of 63% and corresponding salinity of 28.63 ppt. For week two, the mean EC_{50} value was 9% with a C.V. of 61% and corresponding salinity of 29.13 ppt. The NOEC values for embryos exposed to modified effluent ranged from 5-10% effluent in week one and 2.5-5% effluent in week two. The corresponding LOEC values ranged from 10-20% effluent and 5-10% effluent respectively. The mean EC_{50} value for week one was found to be 30% effluent with a C.V. of 60% and for week two the mean EC_{50} value was found to be 13% with a C.V. of 56%.

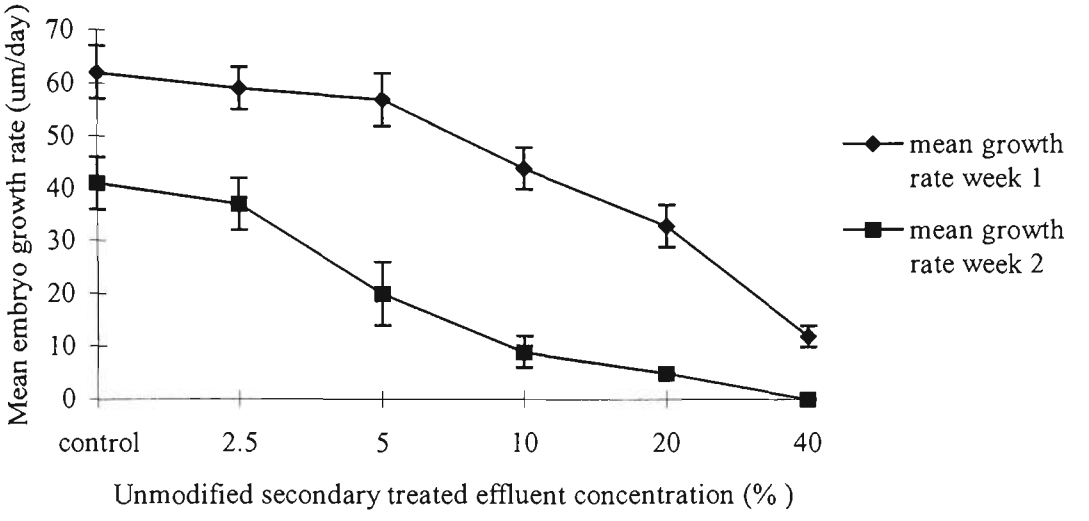


Fig. 5.14 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to unmodified secondary treated effluent.

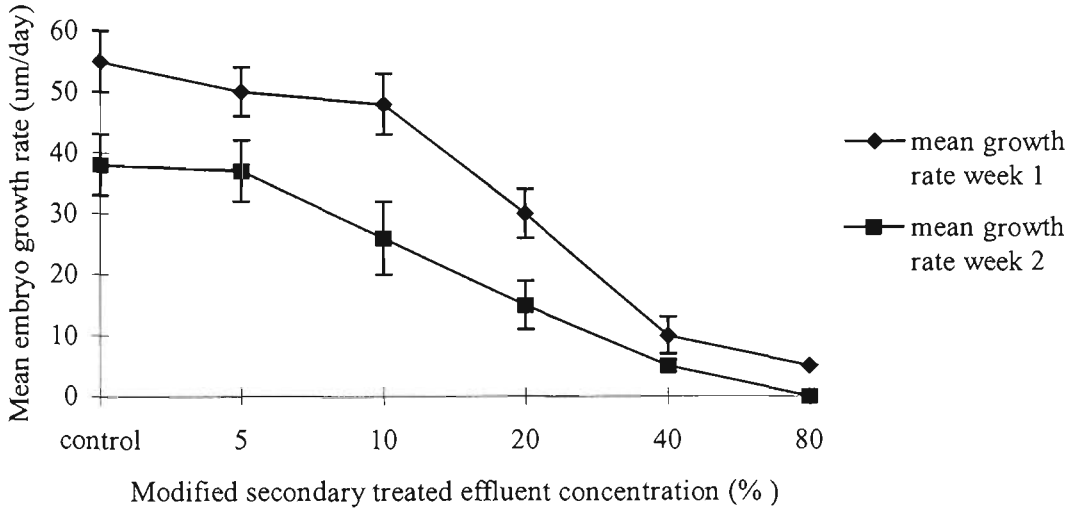


Fig. 5.15 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to modified secondary treated effluent.

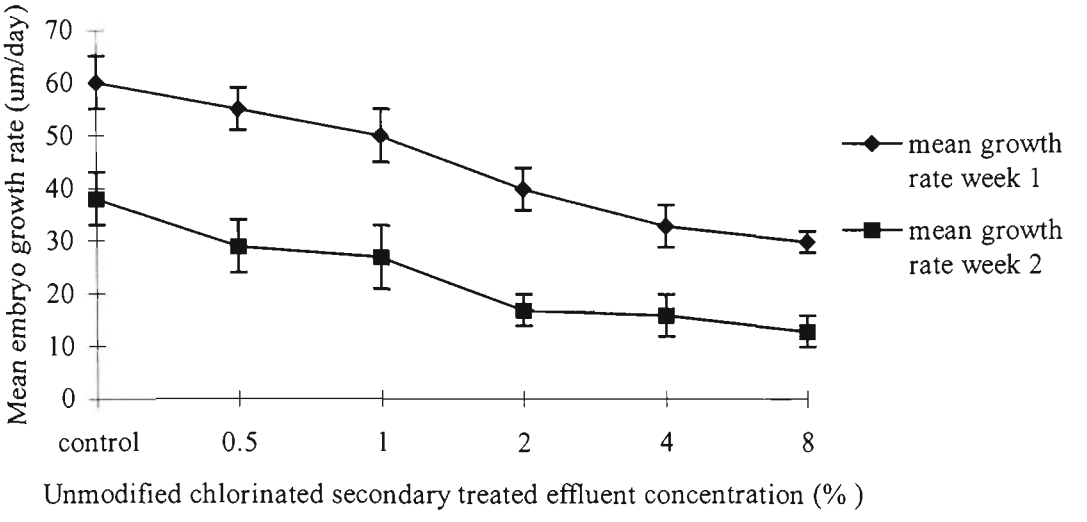


Fig. 5.16 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to unmodified chlorinated secondary treated effluent.

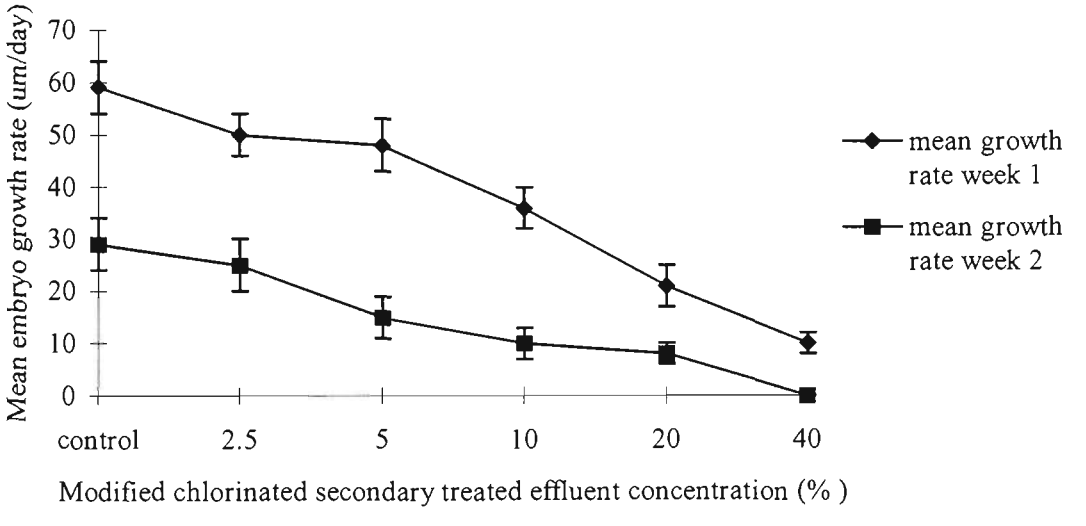


Fig. 5.17 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to modified chlorinated secondary treated effluent.

Salinity

Salinity reduction exerted significant effects on growth rates of *H. banksii* embryos (Fig 5.18). Repeated measures ANOVA showed differences in response over the test period and interactions between time (month) and treatment were also detected (P=0.008). Differences in embryo response fell into seasons, where embryo growth rates in the winter months were found to be significantly greater than remaining seasons, and no significant differences in response between summer, autumn and spring months were detected. Single factor ANOVA showed that decreasing salinity concentrations significantly inhibited growth rates of embryos. The NOEC values for embryos exposed to reduced salinity ranged from 20-18 ppt in week one and 22-20 ppt in week two. The corresponding LOEC values for these tests ranged from 18-16 ppt and 20-18 ppt salinity. The mean EC_{50} value for week one was found to be 14 ppt salinity with a C.V. of 41%, and for week two, the mean EC_{50} value was found to be 18 ppt with a C.V. of 38%. Again longer exposure resulted in greater inhibitory effects.

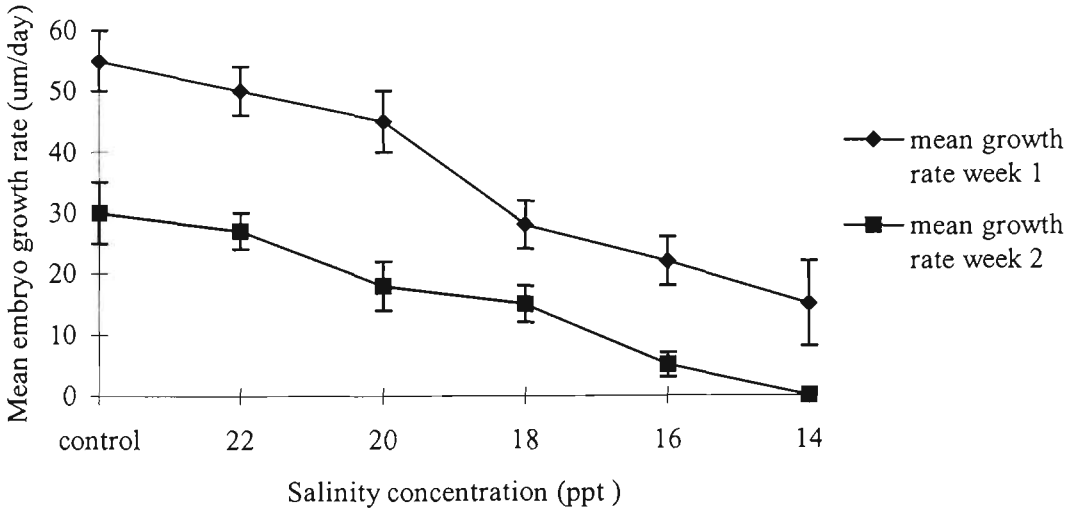


Fig. 5.18 Growth rates (Mean ± Standard Error) (n=4) of *Hormosira banksii* embryos exposed to reduced salinity

5.2.3 Macrocystis angustifolia

Reference toxicants

Copper exerted significant effects on growth (Fig. 5.19) and production (Fig. 5.20) of *M. angustifolia* sporophytes. Repeated measures ANOVA were used to detect any significant interactions in sporophyte response over the 12 month test period. No significant interactions between time (month) and treatment were recorded for either sporophyte growth (P=0.071) or production (P=0.099) bioassays. Single Factor ANOVA were conducted for all tests and it was found that increasing treatment concentrations exerted significant effects on sporophyte growth and reproduction. Significant effects were noted for all tests with NOEC and LOEC values ranging from 0.001-0.002 mg/L copper and 0.002-0.004 mg/L copper respectively for growth tests and <0.001-0.001 mg/L copper and 0.001-0.002mg/L copper respectively for sporophyte production tests. The corresponding mean EC_{50} values for these tests were found to be 0.03 mg/L copper with a C.V. of 51% and 0.01 mg/L copper with a C.V. of 48%.

For tests conducted with chromium, recorded NOEC and LOEC values were found to range from 1-4 mg/L chromium and 2-8 mg/L chromium for sporophyte growth tests and 0-2 mg/L chromium and 1-4 mg/L chromium respectively for sporophyte production tests. The mean calculated EC_{50} values were 13 mg/L chromium with a C.V. of 53% for growth assays and 5 mg/L chromium with a C.V. of 45% for sporophyte production assay. No interactions between time (month) and treatment were evident for any sporophyte growth (P=0.066) or sporophyte production (P=0.059) tests conducted with chromium. Single factor ANOVA did detect that increasing concentrations of chromium significantly affected growth (Fig. 5.21) and production of sporophytes (Fig 5.22).

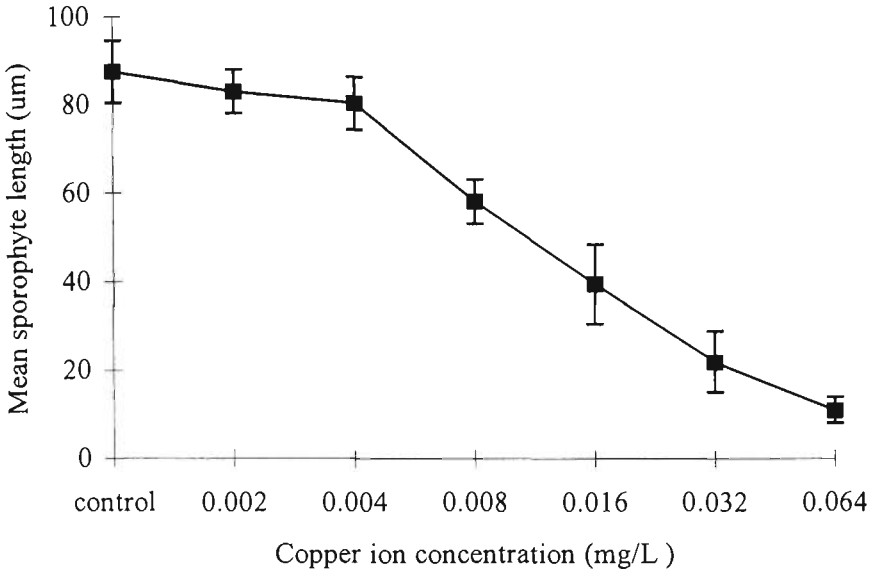


Fig. 5.19 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to copper.

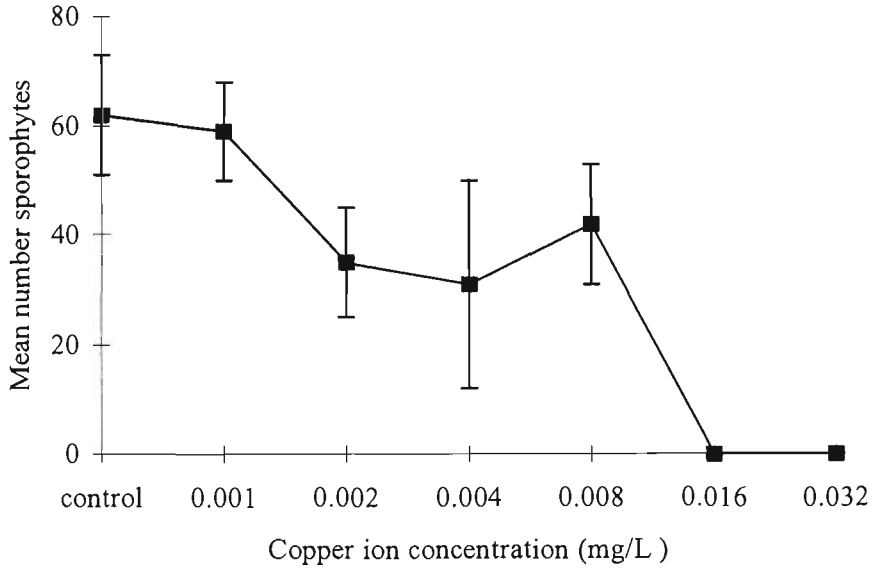


Fig. 5.20 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to copper.

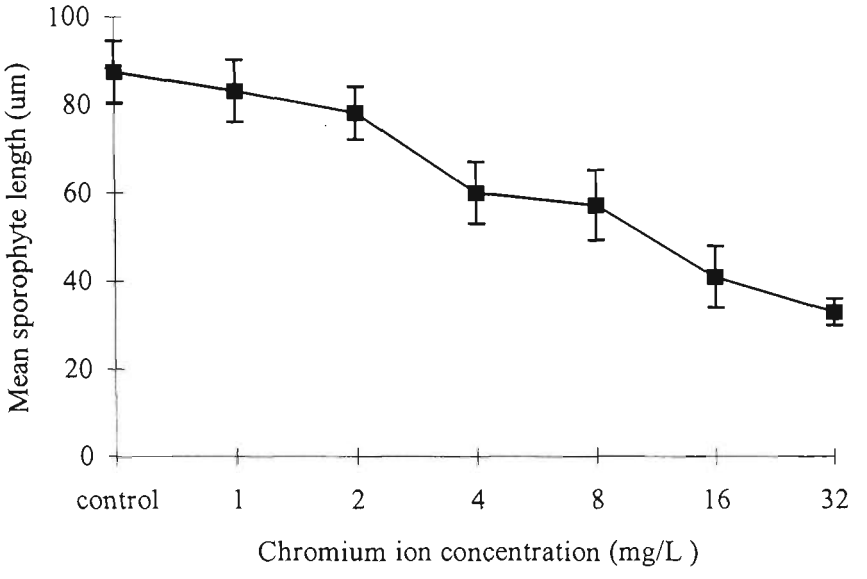


Fig. 5.21 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to chromium.

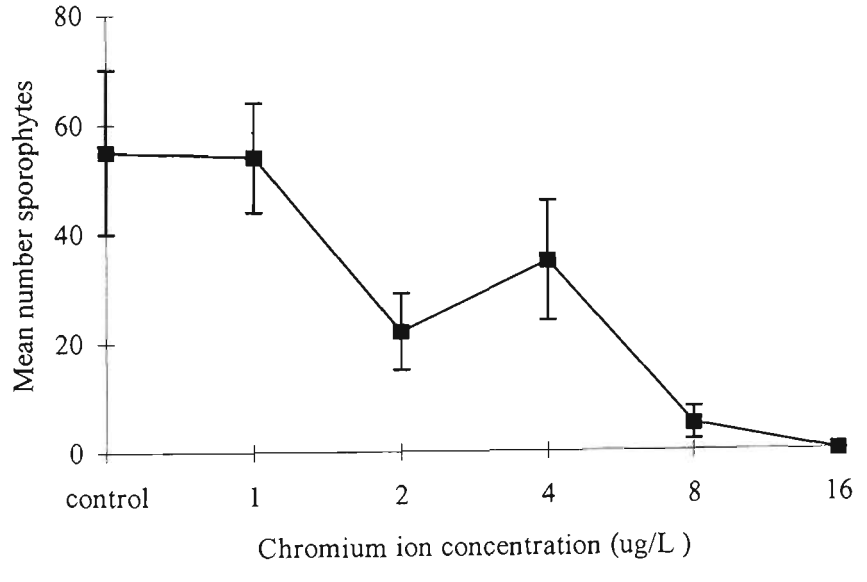


Fig. 5.22 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to chromium.

Primary treated effluent

Primary treated effluent exerted significant effects on sporophyte growth (Fig. 5.23) and reproduction (Fig. 5.24) of *M. angustifolia.* Both unmodified and modified primary treated effluents affected sporophyte growth and production. Single factor ANOVA performed on all tests showed that increasing treatment concentrations significantly inhibited growth and production of sporophytes. Repeated measures ANOVA detected differences in sporophyte growth (P=0.002 and P=0.006 for tests conducted with modified and unmodified effluent respectively) and production (P=0.009 and P=0.004 for tests conducted with modified and unmodified effluent respectively) over the 12 month test period and interactions between time (month) and treatments. Further analyses were conducted to determine differences between algal responses to modified and unmodified primary treated effluent at any one time. No significant differences were detected between tests conducted with the same batches of effluent samples. The NOEC values recorded for sporophyte growth assays exposed to both modified and unmodified primary treated effluent ranged from <0.25-0.25% effluent with LOEC values ranging from 0.25-0.50% effluent. The recorded NOEC values for sporophyte production assays were also found to range from <0.25-0.25% effluent and LOEC values ranged from 0.25-0.50% effluent. The mean EC_{50} value for growth assays was found to be 4% primary treated effluent with a C.V. of 69%. For sporophyte production assays, the mean EC_{50} was found to be 1% with a C.V. of 68%. Salinity reduction played no part in exerted toxic effects recorded for these assays

Secondary treated effluent

Secondary treated effluent exerted significant effects on growth (Fig. 5.25) and production (Fig 5.26) of *M. angustifolia* sporophytes. Repeated measures ANOVA detected differences in response over the 12 month test period for both modified and unmodified effluent samples. Interactions between time (month) and treatment for both modified and unmodified effluent were also detected for growth (P=0.000 and P= 0.006 for modified and unmodified effluent respectively) and reproduction (P=0.002 and P= 0.008 for modified and unmodified effluent respectively) assays, but were not attributed to seasonal influences. ANOVA were also used to determine significant differences in

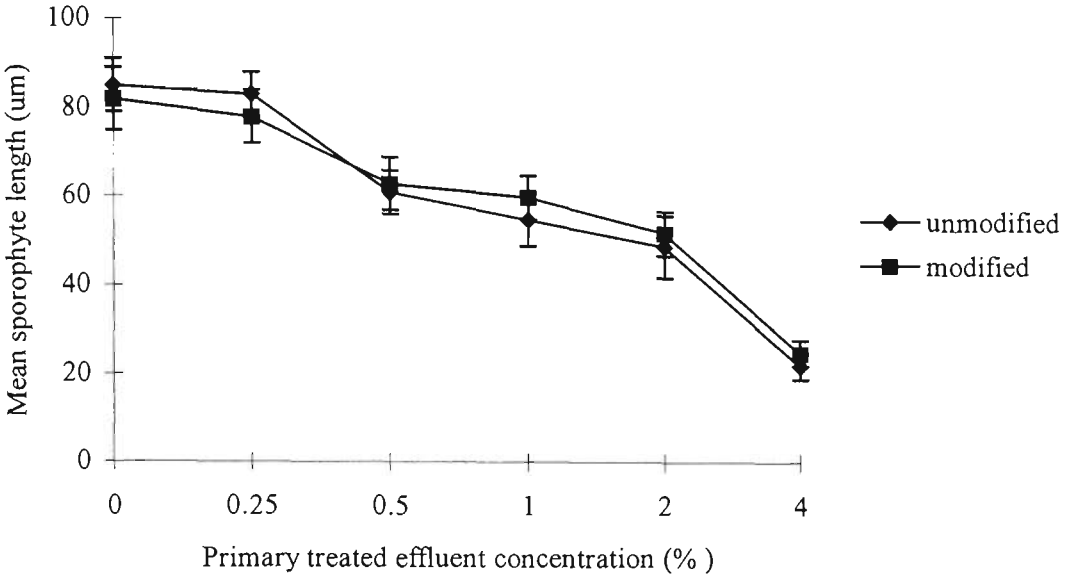


Fig. 5.23 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to primary treated effluent.

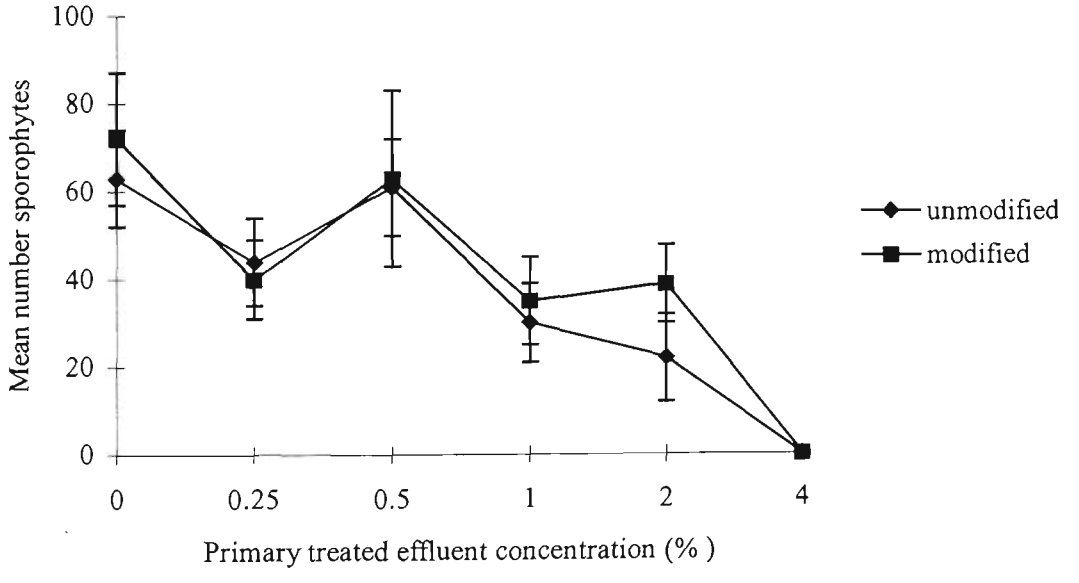


Fig. 5.24 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to primary treated effluent.

algal response between modified and unmodified secondary treated effluent. Single factor ANOVA showed that increasing concentrations of both unmodified and modified effluent significantly inhibited growth and production of *M. angustifolia* sporophytes. It was also found that reduced salinity of the unmodified effluent samples significantly increased toxicity of the effluent. The NOEC values for growth of sporophytes exposed to unmodified effluent ranged from 1.5-3% with LOEC values ranging 3-6% effluent with a mean EC_{50} value of 15% and C.V. of 52% and corresponding salinity of 28.38 ppt. For sporophyte production assays the NOEC and LOEC values ranged from <0.75-1.5% and 0.75-3% unmodified secondary effluent respectively. The mean EC_{50} value was found to be 5% with a C.V. of 42% and corresponding salinity of 29.63 ppt. For growth assays conducted with modified secondary treated effluent, the NOEC and LOEC values ranged from 3-6% and 6-12% effluent respectively. The mean EC_{50} value was found to be 21% effluent with a C.V. of 42%. For sporophyte production assays, the NOEC and LOEC values ranged from <0.75-0.75% and 0.75-1.5% effluent respectively. The mean EC_{50} value was found to be 9% effluent with a C.V. of 41%.

Chlorinated secondary treated effluent

Chlorinated secondary treated effluent exerted significant effects on sporophyte growth (Fig 5.27) and production (Fig. 5.28). Repeated measures ANOVA showed differences in response over the test period for both modified and unmodified effluent samples. Interactions between time (month) and treatment for both modified and unmodified effluent were also detected for all tests and this could be attributed to the fluctuating toxicity of effluent samples. Single factor ANOVA showed that increasing concentrations of both unmodified and modified effluent significantly inhibited sporophyte growth and production. It was also found that salinity reduction was a contributing factor in increasing toxicity of the effluent. The NOEC values for sporophyte growth assays ranged from 1.5-3% effluent for both modified and unmodified samples. The corresponding LOEC values ranged from 3-6% effluent. The mean EC_{50} value for assays conducted with unmodified effluent was found to be 12% effluent with a C.V. of 60%

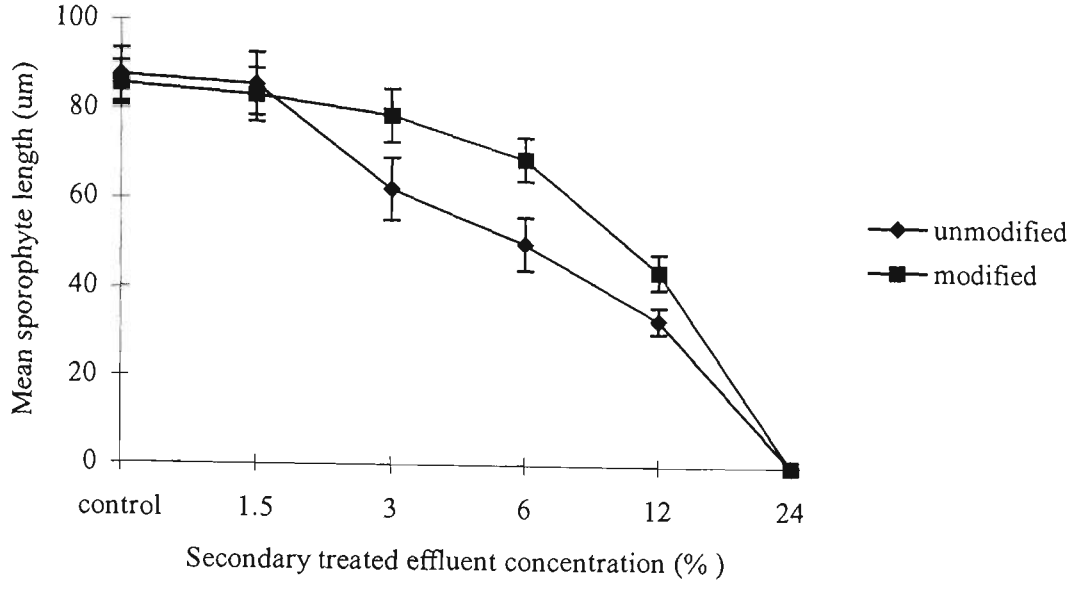


Fig. 5.25 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to secondary treated effluent.

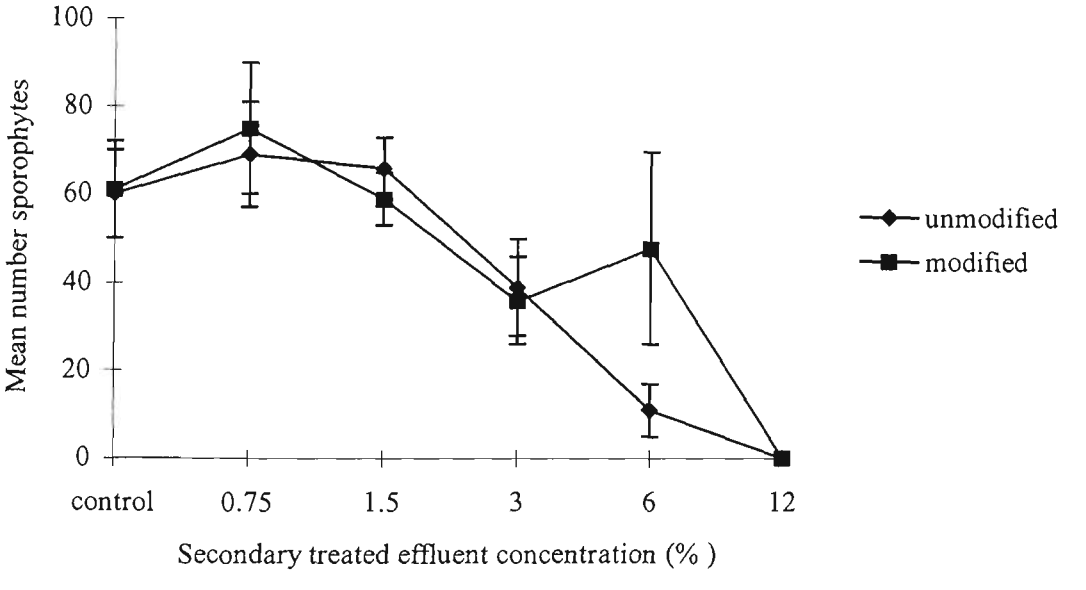


Fig. 5.26 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to secondary treated effluent.

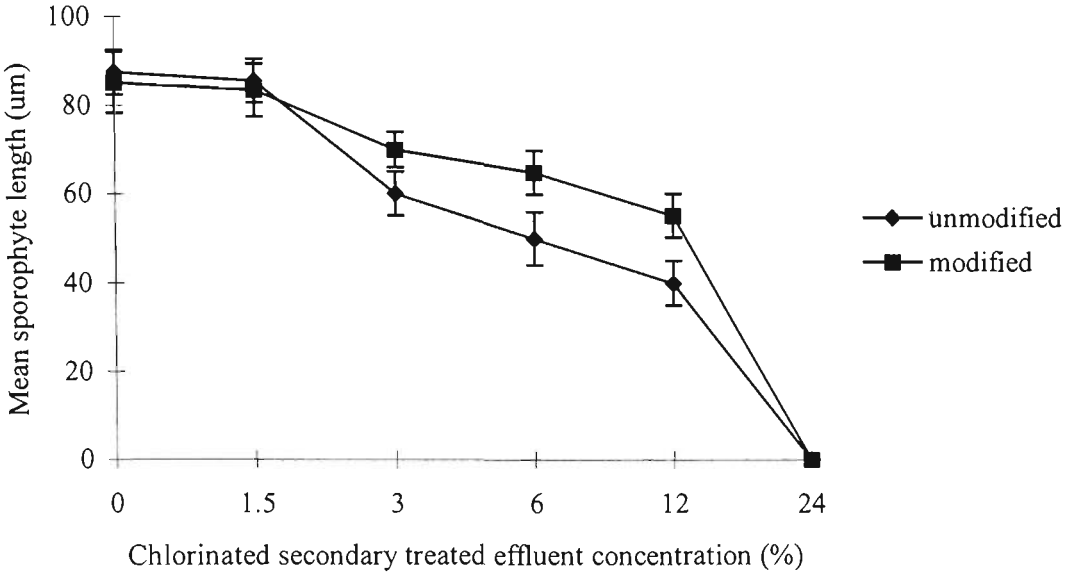


Fig. 5.27 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to chlorinated secondary treated effluent.

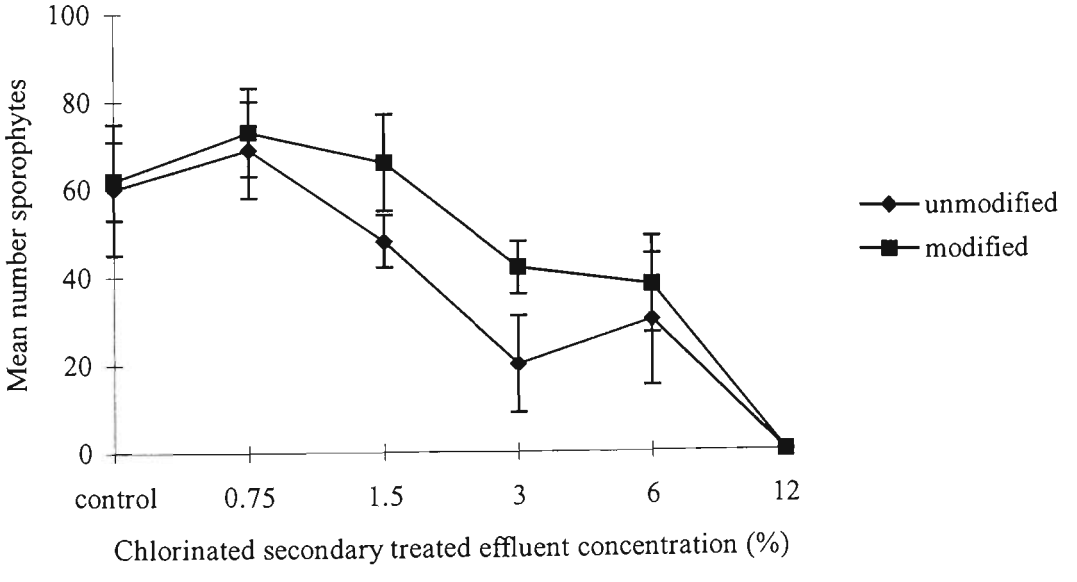


Fig. 5.28 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to chlorinated secondary treated effluent.

and corresponding salinity of 28.75 ppt. For assays conducted with modified effluent, the mean EC_{50} value was found to be 19% with a C.V. of 62%. The NOEC values for sporophyte production assays were found to range from <0.75-0.75% for both modified and unmodified effluent. The corresponding LOEC values ranged from 0.75-1.5% effluent. The mean EC_{50} value for assays conducted with modified effluent was found to be 7% , with a C.V. of 47%. For unmodified effluent, the mean EC_{50} value was 5% with a C.V. of 40% and corresponding salinity of 29.63 ppt.

Salinity

Salinity reduction exerted significant effects on sporophyte growth (Fig. 5.29) and production (Fig 5.30) of *M. angustifolia*. Repeated measures ANOVA showed differences in response over the 12 month test period. Interactions between time (month) and treatment were also detected, but no seasonal trends in response were evident (P=0.062 for growth assays and P=0.077 for reproduction assays). Single factor ANOVA showed that decreasing salinity concentrations significantly inhibited growth and production of sporophytes. The NOEC values for sporophytes exposed to reduced salinity ranged from 30-28 ppt for growth assays and 32-30 ppt for reproduction assays. The corresponding LOEC values for these tests ranged from 28-26 ppt and 30-28 ppt salinity. The mean EC_{50} value for growth assays was found to be 23 ppt salinity with a C.V. of 38%, and for reproduction assays, the mean EC_{50} value was found to be 26 ppt with a C.V. of 30%.

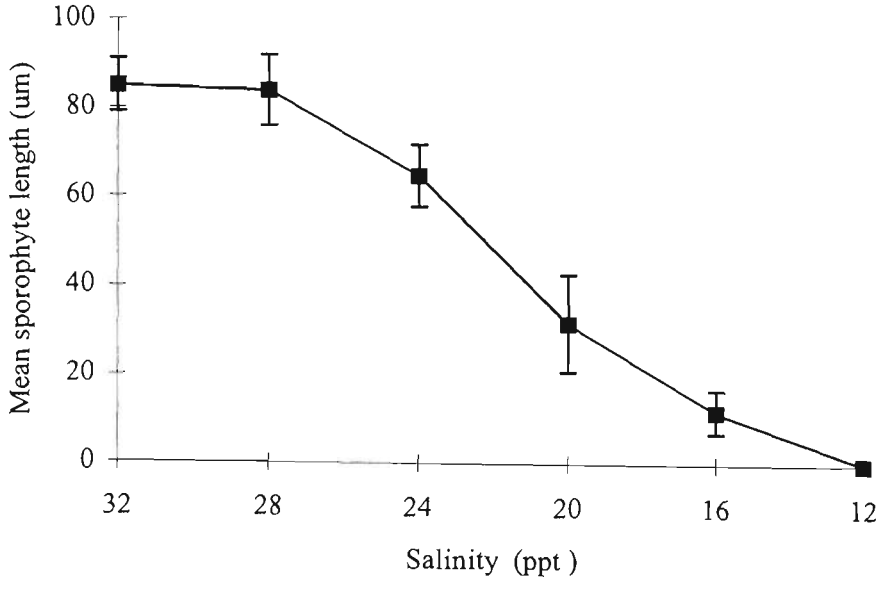


Fig. 5.29 Growth (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to reduced salinity.

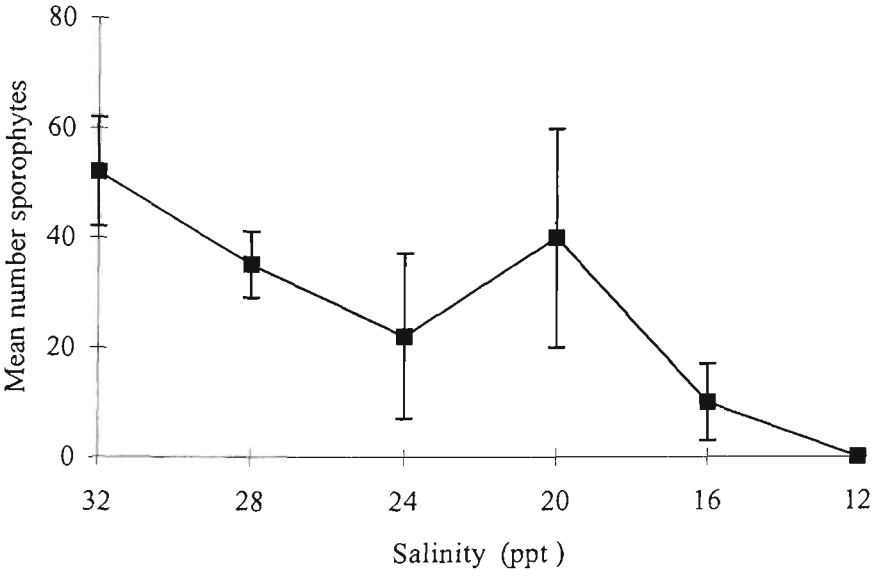


Fig. 5.30 Production (Mean ± Standard Error) (n=4) of *Macrocystis angustifolia* sporophytes exposed to reduced salinity.

5.2.4 Sewage effluent and EC_{50} correlations

As for previous chapters, chemical analyses were conducted by the South Eastern Purification Plant of chlorinated secondary treated effluent. Analyses included the determination of several heavy metal, oil, grease and organic compound levels at fortnightly intervals. Pearson correlations with adjusted probabilities indicated that positive correlations between EC_{50} values and certain compounds existed for results obtained with *P. comosa* and *H. banksii* growth bioassays. For *H. banksii* growth assays, significant correlations existed between EC_{50} values and concentrations of copper (analysis range 0.001-0.05 mg/L) detected in chlorinated secondary treated effluent ($r=0.555$, $s_r=0.086$, $r0.05(2)18=0.378$) after two weeks of exposure. The EC_{50} values obtained for *P. comosa* growth assays also significantly correlated with copper ($r=0.512$, $s_r=0.095$, $r0.05(2)18=0.378$) and mercury concentrations (analysis range 0.00001-0.0008 mg/L) ($r=0.481$, $s_r=0.063$, $r0.05(2)18=0.378$) after two weeks exposure. No such correlations were detected for either *M. angustifolia* growth or reproduction assays. All bioassays conducted with *H. banksii, P. comosa* and *M. angustifolia* produced EC_{50} values which significantly correlated with reduced salinity (analysis range 34-12 ppt) ($r>0.378$, $n=20$, $r0.05(2)18=0.378$).

5.2.5 Test species sensitivity

Analyses of variance indicated that growth responses of the early life stages of *H. banksii*, *P. comosa* and *M. angustifolia* to the reference toxicants were significantly different. It was determined that *P. comosa* embryos were most sensitive to copper followed by *M. angustifolia* sporophytes and *H. banksii* embryos. Tests conducted with chromium revealed that *H. banksii* and *M. angustifolia* were most sensitive followed by *P. comosa*. Growth and reproduction responses to treated sewage effluent varied between the three species. *M. angustifolia* sporophytes were found to be more sensitive to the effects of primary treated effluent. The *M. angustifolia* reproduction assay was found to be the most sensitive assay for testing the effects of secondary treated effluent. For chlorinated secondary treated effluent bioassays, *M. angustifolia* was the most sensitive of the test species. For salinity reduction assays, *P. comosa* embryos and *M. angustifolia* sporophytes demonstrated greater sensitivity than *H. banksii* embryos.

5.3 Discussion

5.3.1 Relative sensitivity of test species

This study has shown that sporophyte and embryo growth and sporophyte production are valuable endpoints for use in routine toxicity testing as negative effects on algal reproduction may result in recruitment declines. Although coefficients of variation were unacceptably high (Cher *et al.* 1994) in the series of tests, the data gathered was essential in determining effects of sewage effluent on early development of the test species. The high coefficients of variation could be attributed to the type of data collected rather than their credibility. Growth data are measurements of individuals. These results can be more complex and variable than simple positive or negative binomial results.

The test species utilised in this study exhibited responses to treatment exposure comparable to those of other studies. The sensitivities of these macroalgal species may have been attributed to specific effects of certain constituents of the effluent samples. Comparisons of algal sensitivities are confounded by unknown constituents and the concentration of these present in discharged effluent. The possibility of synergistic and antagonistic responses also exists. These responses may have been further influenced by algal physiology, life history or reproductive phases. The combined effect of these constituents with reduced salinity may have exerted differing effects on the algae leading to the varied biological responses recorded.

The test species utilised for this study were highly sensitive to the effects of all treatments tested. Strömgren (1979, 1980) noted significant reductions in growth of the macroalgae *Pelvetia canaliculata* and *Fucus spiralis* cultured in copper concentrations as low as 0.012 mg/L. These results are comparable to those recorded for tests conducted with *H. banksii* in this study yet much higher than those gathered for *P. comosa* and *M. angustifolia*. Anderson *et al.* (1990) exposed *M. pyrifera* gametophytes to concentrations of copper and recorded NOEC values of <0.01 mg/L. The NOEC values recorded for this study ranged from <0.001-0.001 mg/L with LOEC values ranging 0.001-0.002 mg/L copper. The study of Anderson *et al.* (1990) neglected to conduct tests at lower concentrations rendering these test results somewhat inconclusive when considering

threshold responses. The results obtained in this study support their finding to a degree as all threshold concentrations recorded for tests with *M. angustifolia* fall below those found for *M. pyrifera*.

Other studies utilising laminarian species have also concentrated on the effects of copper on sporophyte growth. Thompson and Burrows (1984) exposed *Lamanaria saccarina* to copper and found that growth was completely inhibited at 0.03 mg/L and reproduction at 0.01 mg/L copper. Florence and Stauber (1986) exposed the marine diatom *Nitzschia closterium* to copper concentrations and noted a 50% reduction in growth of the diatom at 0.02 mg/L. Another study by Burridge *et al.* (1996) exposed the three species utilised in this study to various treatments of effluent and the reference toxicant 2,4-dichlorophenoxyacetic acid. They recorded differences in species sensitivity not dissimilar to trends found in this study.

Growth bioassays conducted with effluent samples generally produced threshold concentrations between 1-10% effluent. Not only were reductions in growth rates recorded during the test period, but deformities in development were observed also. These observations were most profound in cultures of *P. comosa* and *H. banksii* embryos. Doblin and Clayton (1995) noted that *H. banksii* embryos growing in 28% unmodified chlorinated secondary treated effluent showed signs of abnormal and stunted development. The threshold concentrations recorded in this study were significantly lower than those found by Doblin and Clayton (1995). This may be attributed to the fluctuating toxicity of effluent tested and differences in test protocol.

It was found in this study that all three species of macroalgae utilised were more sensitive to the effects of unmodified sewage effluent compared to modified effluent. Reduced salinity and the added stress of treated effluent increased the sensitivity of all three species. Andersson *et al.* (1992) found that lowering salinity can lead to reduced growth rates and decreased photosynthesis in macroalgal species. It was noted from the data gathered in this study that salinity reduction bioassays produced lower EC_{50} values than corresponding salinities of EC_{50} values recorded for unmodified effluent bioassays. It is

clear that reduced salinity combined with effluent constituents exerted at least an additive if not synergistic effect on the early life stages of the algae tested.

5.3.2 Temporal variability

Growth tests conducted with *P. comosa* and *H. banksii* indicated differences in embryo response after exposure to copper, chromium and reduced salinity over the 12 month test period. The results suggested greatest embryo sensitivity during the warmer seasons of summer, spring and autumn. Higher growth rates and reduced sensitivity were evident in embryos during the cooler winter months. Temporal variability in algal growth is an area which has seen little research (Kennelly 1987). There are many reasons as to why growth rates in algae fluctuate during different times of the year. These range from influences of surrounding water temperatures (Clayton *et al.* 1987) to limitations of essential nutrients (Anderson *et al.* 1990). To validate test results it is more necessary to determine when these influences may affect growth rates of test species rather than the reasons why.

Although temporal variability in response was evident in *P. comosa* and *H. banksii* embryos, this was not the case with sporophytes of *M. angustifolia*. There was no evidence to indicate seasonal influences on sporophyte growth or production. This finding contradicts that of Anderson and Hunt (1990) who found differences in *M. pyrifera* sporophyte growth and production assays conducted between seasons.

5.3.3 Toxicity test endpoint comparisons

It was apparent from the data collected for this study that prolonged exposure of all three macroalgal species to treatments increased sensitivity. For growth assays conducted with *P. comosa* and *H. banksii*, treatment threshold concentrations were lower in week two as opposed to week one. These trends are evident with other studies conducted with growth of *P. comosa* after exposure to various treatments (Lavery 1994, Shir 1994, Burridge *et al.* 1995b). Shir (1994) found that growth rates of *P. comosa* embryos exposed to 1000 mg/L of the chemical dispersant Corexit 7664 halved by week two compared to those cultured in control treatments. Similar trends were recorded by Burridge *et al.* (1995b) who exposed *P. comosa* embryos to formaldehyde. Passive and/or active uptake of

toxicants exert varying effects on algal species. At certain concentrations potential toxicants may increase growth rates (Chung & Brinkhuis 1986), but at higher concentrations, growth, photosynthesis, mitochondrial respiration and other essential metabolic activities can be significantly reduced.

Tests conducted with *M. angustifolia* showed that sporophyte production was a more sensitive test endpoint compared with sporophyte growth. This is consistent with Anderson *et al* (1990) who found that reproduction of sporophytes was a more sensitive endpoint than growth in *M. pyrifera*. Other studies conducted with various laminarian species have also shown this trend (Smith & Harrison 1978, Thompson & Burrows 1984).

Generally, sub-lethal testing will produce significantly lower threshold concentrations than acute toxicity testing. In this study lower threshold concentrations were evident with prolonged exposure which suggests that neither species showed signs of recovery during the treatment periods. This information may be used in relating trends within the laboratory to those of algal species colonising waters receiving constant volumes of sewage effluent.

The data generated from growth tests conducted for this study indicate a high degree of reproducibility for individual species. Comparisons of effluent toxicities could be drawn since median effect concentrations were determined and common end-points investigated. These results were also consistent with threshold responses for other Australasian species (Hopkins & Cain 1978, Gledhill *et al.* 1997).

5.3.4 Treatment toxicity

Concentrations of copper exerted significant effects on all three macroalgal species. Studies investigating the effects of copper on algal species have shown that the heavy metal can inhibit growth, reduce fecundity and cause the onset of mortality (Romeril 1977, Bryan & Langston 1992, Gledhill *et al.* 1997). Copper is an essential element required for metabolic processes and photosynthesis (Gledhill *et al.* 1977), but at high

enough concentrations it can inhibit photosynthesis (Stauber & Florence 1987), disrupt gametophyte development (Garman *et al.* 1994) and restrict growth (Florence & Stauber 1986, Anderson & Hunt 1990). Significant reductions in gametophytic development were evident in this study at concentrations as low as 0.001 mg/L copper. Growth inhibition in all three species was evident in concentrations ranging 0.001-0.016 mg/L copper. Strömgren (1979, 1980) investigated the effects of copper on several fucalean species and found copper concentrations ranging between 0.012-0.050 mg/L caused reductions in growth. Studies conducted with both copper and chromium have failed to determine the exact modes of toxicity of these metals. Chromium has been linked to causing cellular disruptions (Patel & Saxena 1983, Stauber & Florence 1985) which in turn can also lead to decreased growth rates.

5.3.5 Conclusion

The toxicity of sewage effluent is difficult to quantify due its variability and presence of unknown constituents (Kallqvist *et al.* 1989, Sanchez *et al.* 1988, Pun *et al.* 1995). Unmodified effluent samples tended to exert greater toxicity to the three algal species and this can be attributed to the added effect of reduced salinity. Treated effluent can comprise of nitrogenous compounds, orthophosphates, surfactants, trace metals, chlorine and hydrocarbons all of which can have deleterious effects on early life stages of algal species (Doblin & Clayton 1995). Surfactants affect cell membrane permeability (Hotchkiss 1946) and can depress chlorophyll *a* content in algal species (Fabregas *et al.* 1984). Heavy metals such as copper and chromium can reach high levels through accumulation (Edwards 1972) and chlorine is toxic to a wide variety of organisms (Saito 1972). This study aimed to investigate the effects of prolonged exposure of treated sewage effluent on growth and reproduction of three macroalgal species. A greater insight into the chronic effects of effluent on algal early life stages was achieved. Results of this study can be used to better understand the potential effects of sewage discharge on algal communities colonising receiving waters.

CHAPTER 6
THE EFFECTS OF SEWAGE EFFLUENT ON BIOLUMINISCENCE OF *VIBRIO FISCHERI*

6.1 Introduction

Microorganisms have been used extensively to evaluate the toxicities of various compounds present in aquatic systems (McFeters *et al.* 1983). Toxicity assays utilising microorganisms have ranged from the Ames Test, which detects mutagenicity (Chang *et al.* 1981), to the monitoring of bacterial populations after the introduction of a potential toxicant (Mayfield *et al.* 1980). An addition to the suite of tests available utilising microorganisms, or more specifically bacteria, is the bioluminescence inhibition assay (Microtox® Test). This system has been successfully used in the determination of effects of various toxicants (McFeters *et al.* 1983), including water-soluble fractions of hydrocarbons (Poremba 1992) and industrial effluents (Sanchez *et al.* 1988). An advantage to the bioluminescence inhibition assay is its sensitivity and reproducibility (Poremba 1992).

The bioluminescence inhibition assay employs lyophilised bacteria (*Vibrio fischeri*) as the test organism (Anon. 1992). The Microtox® Test System, which is a specially designed photometric instrument, then measures the light output of the bacteria after they have been challenged by a toxicant (Chang *et al.* 1981). Although the test is quick (5-15 minutes) with claimed high precision (Anon. 1992), it should not be solely relied upon when absolute toxicity values are required (Din & Abu 1993). The test has been proven to be useful in determining preliminary estimates of relative toxicities of toxicants including effluents (Chang *et al.* 1981, Din & Abu 1993), but should be used in conjunction with other forms of toxicity test protocols (McFeters *et al.* 1983, Eisman *et al.* 1991). For the purpose of this study, the bioluminescence inhibition assay was used as a screening tool to provide immediate information on effluent toxicity. These data were then used to support and compliment the strongest evidence gained by the utilisation of endemic species as presented in previous chapters.

6.1.1 Aims of this chapter

The aim of this chapter was to:

determine the effects of sewage effluent on the marine bacterium *Vibrio Fischeri* utilising the commercially available Microtox® Test System.

6.2 Results

Bioluminescence inhibition assays were conducted over a 12 month period beginning 17/11/96-2/12/97 and have been denoted as 1-20. Exact dates of each test commencement have been listed as Appendix 1. Please note: due to the nature of the bioluminescence assay, only modified effluent samples were tested, that is, the salinities of samples were adjusted to that of seawater.

Reference toxicants

The reference toxicants, copper (Fig 6.1) and chromium (Fig 6.2), exerted significant effects on bioluminescence of *Vibrio fischeri*. The mean EC_{50} values for tests conducted with copper were found to be 0.17 mg/L after 5 minutes of exposure and 0.15 mg/L after 15 minutes of exposure with corresponding C.V. of 11% and 10% respectively. For these tests, it did not appear that greater exposure time influenced toxicity. The mean EC_{50} values for tests conducted with chromium were 970 mg/L and 186 mg/L after 5 and 15 minutes exposure respectively. The corresponding C.Vs. were 6% and 19%, respectively. For tests conducted with chromium, it was evident that toxicity increased with increasing exposure time.

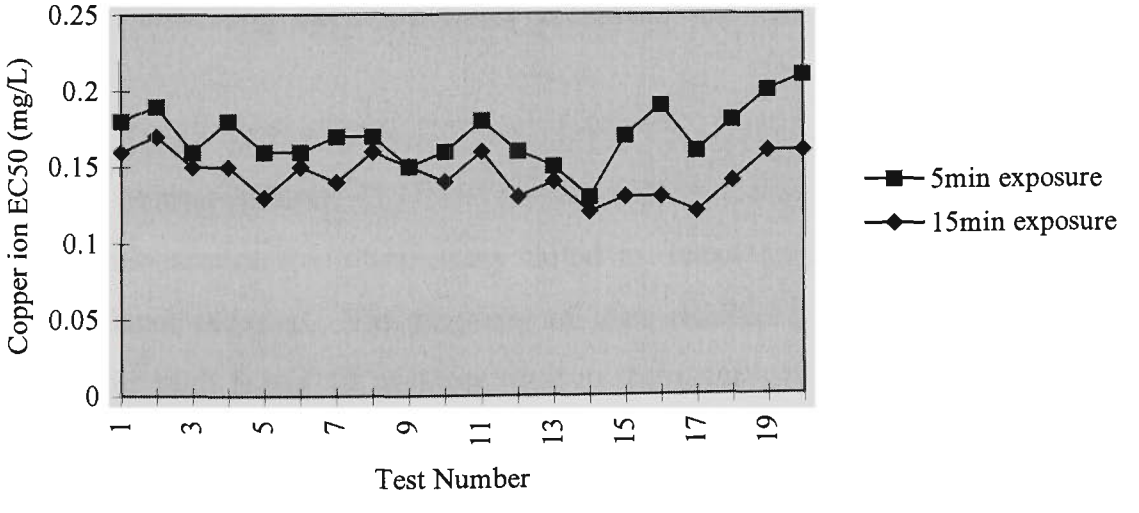


Fig. 6.1 Bioluminescence response over time of *Vibrio Fischeri* bacteria exposed to copper ions for 5 and 15 minutes.

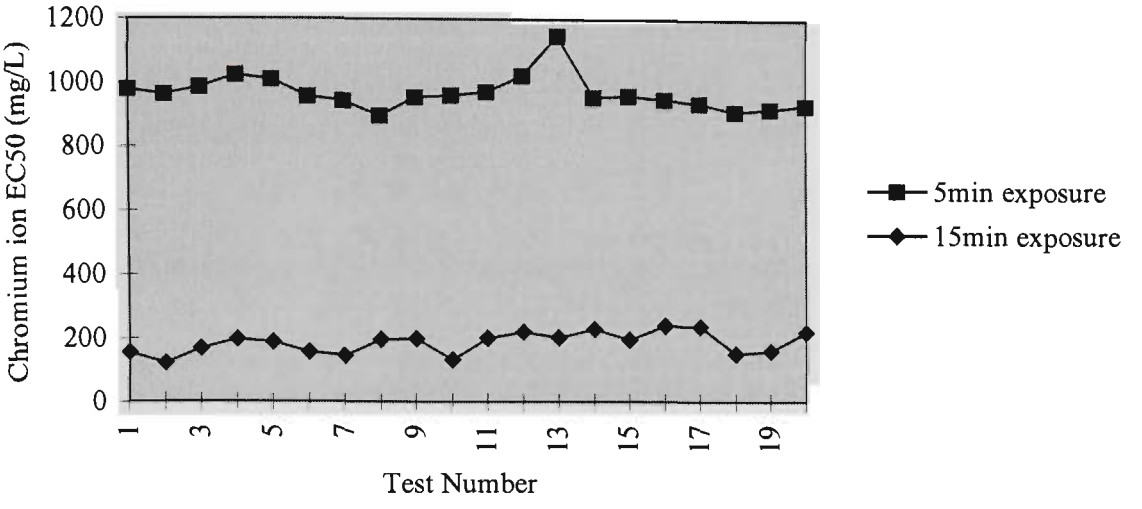


Fig. 6.2 Bioluminescence response over time of *Vibrio Fischeri* bacteria exposed to chromium ions for 5 and 15 minutes.

Primary treated effluent

Primary treated effluent exerted significant effects on bioluminescence of *Vibrio fischeri* (Fig. 6.3). The mean EC_{50} values for tests conducted with primary treated effluent were 30% after 5 minutes of exposure and 20% after 15 minutes of exposure with corresponding C.V. of 43% and 49% respectively. As found with tests conducted with chromium, increasing exposure times increased the toxic effects of primary treated effluent.

Secondary treated effluent

The bioluminescence inhibition assay failed to detect significant toxicity of secondary treated effluent samples. The majority of tests resulted in EC_{50} values of >90% after exposure for both 5 and 15 minutes (due to the nature of this bioassay, 100% effluent samples could not be tested). Of the remainder of tests (test numbers 4, 5, 7, 17, 18, 19) recorded EC_{50} values ranged from 75-88% after 5 minutes of exposure and 68-81% after 15 minutes of exposure.

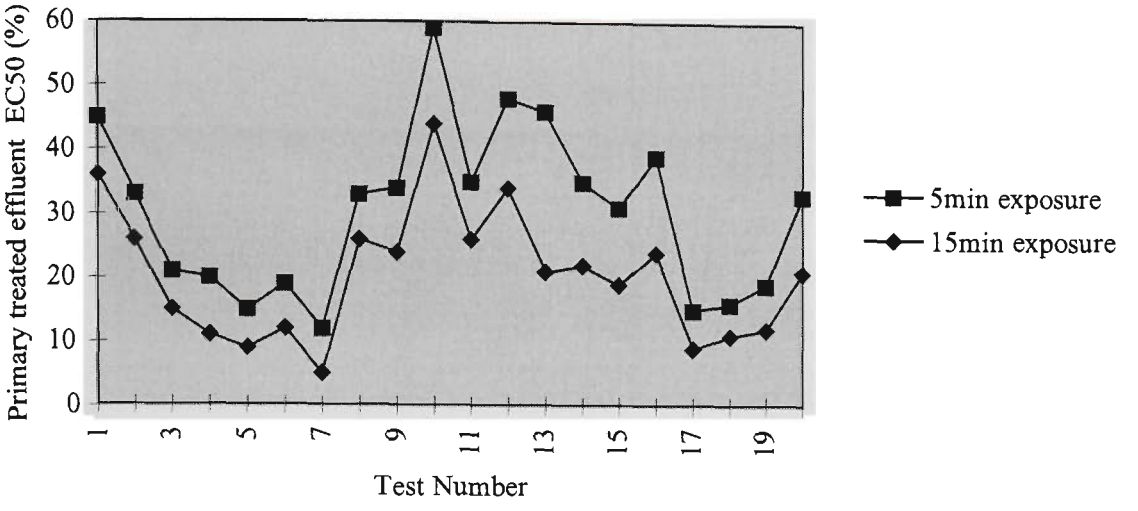


Fig. 6.3 Bioluminescence response over time of *Vibrio Fischeri* bacteria exposed to primary treated effluent for 5 and 15 minutes.

Chlorinated secondary treated effluent

Chlorinated secondary treated effluent exerted significant effects on bioluminescence of *Vibrio fischeri* (Fig. 6.4). The mean EC_{50} values for tests conducted with the effluent were 60% after 5 minutes of exposure and 49% after 15 minutes of exposure with corresponding C.V. of 39% and 41% respectively. As found with previous test results, increasing exposure times increased the toxic effects of chlorinated secondary treated effluent.

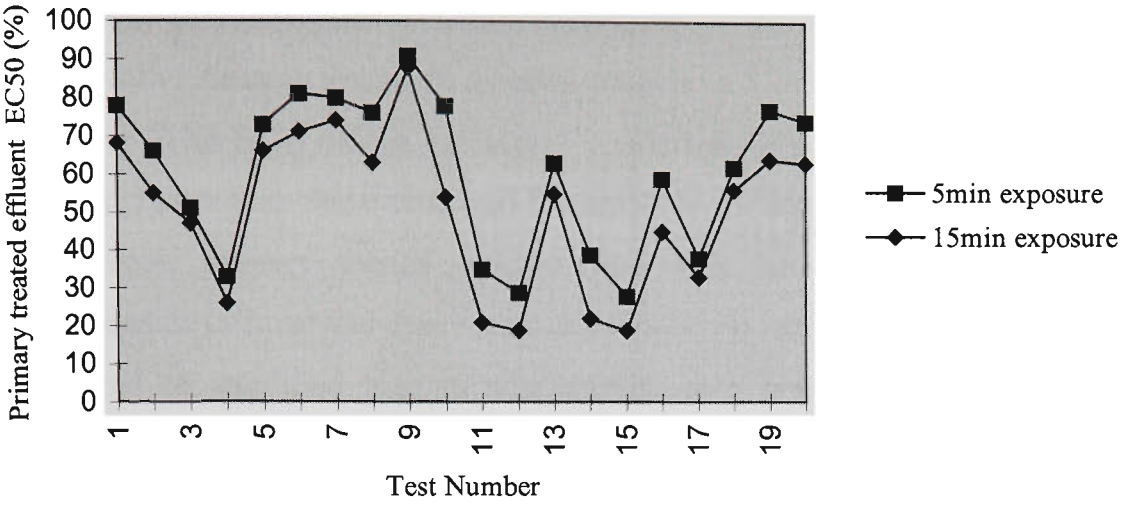


Fig. 6.4 Bioluminescence response over time of *Vibrio Fischeri* bacteria exposed to chlorinated secondary treated effluent for 5 and 15 minutes.

6.3 Discussion

The sensitivity of *Vibrio fischeri* varied depending on the treatment tested. For assays conducted with reference toxicants, greatest sensitivity was recorded for tests conducted with copper. The EC_{50} values for assays conducted with chromium were up to five magnitudes greater than those produced for tests conducted with copper. Of the effluent samples tested, primary treated effluent was most toxic followed by chlorinated secondary treated effluent and then secondary treated effluent. For all tests conducted the general trend of amplified toxicity was evident with longer exposure times. These instances of amplified toxicities may have been as a result of a greater number of sites available for toxicant transport into the cells of the bacterium (Strömgren 1980). Greater exposure time would result in a higher number of these sites being inundated with toxic ions present in the reference toxicant and effluent solutions.

Studies have shown that the bioluminescence inhibition assay can be used as a tool in routine toxicity testing (Bulich & Isenberg 1980, McFeters *et al.* 1983, Poremba 1992, Din & Abu 1993). McFeters *et al.* (1983) compared the effectiveness of the bioluminescence inhibition assay with other microbial bioassays available. They found that the bioluminescence inhibition assay was a useful test for the assessment of suspected toxic compounds present in aquatic systems. They tested a range of toxicants including copper and chromium. The recorded EC_{50} values for these two toxicants fell at 24.9 mg/L and 2412.5 mg/L, respectively. By determining the copper and chromium ion concentrations through back calculations, the results obtained for chromium assays in this study are comparable to those found by McFeters *et al.* (1983). The effects of copper on *V. fischeri* in this study however, were more pronounced. The difference in copper toxicity may be explained by differences in toxicant preparation which were not clearly explained by McFeters *et al.* (1983). Also the concentration of free metal ions may be influenced in the saline diluent used for this study. It has been established that trace metal toxicity to aquatic organisms is not governed by the total metal concentration, but by free metal activity (Sunda & Guillard 1976, Florence 1986, Stauber & Florence 1987). Complexing agents decrease metal toxicity by decreasing free metal ion activity. The

pronounced effects of copper in this study may have been attributed to the absence of such complexes in the diluent used.

Of the effluent samples tested, primary treated effluent exerted the greatest effect followed by chlorinated secondary treated effluent and finally secondary treated effluent. The degree of treatment explains the reasoning behind the primary treated effluent being most toxic to the bacteria. It could also be assumed that this should be true for secondary treated effluent. However, the further treatment of the secondary treated effluent through chlorination increased its overall toxicity. The toxicity of chlorine to a variety of marine organisms has been well documented (Pratt *et al.* 1988, Kallqvist *et al.* 1989, Cairns *et al.* 1990). Kindig and Littler (1980) investigated the toxic effects associated with chlorinated effluent on productivity in several macrophytic algae and found that during long-term tests, chlorine had little effect, yet during short-term exposure tests the toxicity of chlorine was pronounced. This may have been the case with tests conducted with *V. fischeri*. Although secondary treated effluent had little effects on the bacteria, the addition of chlorine significantly increased toxicity of the effluent.

The bioluminescence inhibition assay is an effective test and a quick method for assessing toxicities of sewage effluents. The test species however, was not nearly as sensitive to the effects of the treatments tested as the algal species utilised in previous chapter. Although not as sensitive, the bacteria did provide a measured response, which may be correlated with higher species (Lankford & Smith 1994). The results obtained from these bioassays however, cannot always be translated into realistic measures of effects in the environment. Advantages to the use of bioluminescence inhibition assay are reproducibility of results and highly controlled conditions. The test can be used effectively in monitoring effluent toxicity from one day to the next. Sewage effluent tends to biodegrade over time (Lankford & Smith 1994) so a quick toxicity screening test is also useful in determining changes in toxicity with time. Changes in toxicity can then be used as a warning of variations in effluent composition.

CHAPTER 7
THE EFFECTS OF SEWAGE EFFLUENT ON THE MORPHOLOGY OF *PHYLLOSPORA COMOSA, HORMOSIRA BANKSII* AND *MACROCYSTIS ANGUSTIFOLIA.*

7.1 Introduction

A significant number of research studies have investigated the effects of potential toxicants, such as sewage effluent, on the early life stages of marine macroalgae (Ogawa 1984, Anderson & Hunt 1988, Doblin & Clayton 1995, Burridge *et al.* 1996, Bidwell *et al.* 1998, Burridge *et al.* 1999). Few however, have included research into specific effects of these potential toxicants at the ultrastructural level of early algal development. Although research studies have investigated the biology of gametophyte and zygote development and growth of the early life stages of algae to the embryo and sporophyte stage (Garman 1994), few have concentrated on specific pathways of toxicants at these levels.

Researchers have speculated in explaining the possible effects of toxicants based on recorded findings. Studies have focussed on growth and primary productivity of macroalgae using photosynthetic rates of plants as the test endpoint (Littler & Murray 1975, Kindig & Littler 1980). Tewari *et al.* (1990) investigated the effects of industrial effluent on the biochemical composition of two species of macroalgae and suggested that algal metabolism of carbon and nitrogen was inhibited during exposure to the discharge. Gledhill *et al.* (1997) investigated the effects of several heavy metals and associated these toxicants with photosynthesis inhibition, electron transport disruptions and negative effects to permeability of plasma membranes. They also noted that a lack of information exists on toxic effects of potential toxicants to specific species. Stauber & Florence (1986) have also indicated a paucity in information available, more specifically to the effects of copper on species of algae. These and other studies investigating the effects of discharged waters on algal species have concentrated more on inhibitory effects of toxicants readily visible in laboratory cultures (Ogawa 1984). There is a need to

investigate specific toxic effects of sewage to fully understand the implications associated with discharge into highly populated areas.

Investigating exposed early life stages of macroalgae at the ultrastructural level may help to determine and establish specific effects of potential toxicants.

7.1.1 Aim of this chapter

The aim of this chapter was to:

> investigate effects of sewage effluent on the early life stages of *Phyllospora comosa, Hormosira banksii* and *Macrocystis angustifolia.*

7.2 Results

7.2.1 *Phyllospora comosa*

Scanning and transmission electron and light microscopy revealed significant effects on the morphology of *P. comosa* zygotes and embryos after toxicant exposure. At 48 hours, *P. comosa* cultured in control treatments of natural seawater showed the protuberance of a germination rhizoid (Fig. 7.1, 7.2 & 7.8) with evidence of cellular division along the longitudinal axis (Fig. 7.2). Cultures exposed to varying treatments of sewage effluent produced high percentages (>20% of the test population) of ungerminated zygotes as evident by the absence of a germination rhizoid (Fig. 7.3). There was also evidence of stunted growth and underdevelopment of attachment rhizoids (Fig. 7.4 & 7.5) in embryos cultured in treatments of primary, secondary and chlorinated secondary treated effluent. The onset of mortality was evident with clearly necrotic appearances of both zygotes and embryos (Fig. 7.6, 7.7 & 7.11) made obvious with cells having leaked their contents (Fig 7.11).

Transmission electron micrographs revealed reduced densities of chloroplasts, physodes and Golgi-derived vesicles in embryos and unhealthy appearing cellular membranes and walls (that is, clear evidence of damage and lacking definite structure) cultured in treatment solutions (Fig. 7.9 & 7.12) compared with those cultured in natural seawater (Fig. 7.10); those cultured in natural seawater appeared healthy (Fig. 7.10). Chloroplast and physode densities were substantially decreased in zygotes and embryos cultured in copper and chromium concentrations of greater than 0.4 mg/L and 22.5 mg/L respectively. Primary treated effluent concentrations of greater than 4% significantly reduced numbers of chloroplasts, physodes and Golgi-derived vesicles in embryos as did concentrations greater than 10% secondary and chlorinated secondary treated effluent. Reduced salinity played an important role in producing toxic responses in developing zygotes and embryos. The reductions in numbers of the above-mentioned organelles were evident in treatments below 26 ppt salinity. It was clear however, that both modified and unmodified effluent treatments exerted similar effects although these effects were amplified with reduced salinity.

Chronic growth tests indicated that prolonged treatment exposure of maturing embryos increased sensitivity of the young life stages. Healthy plants were evident by an elongated thallus and a dense collection of holdfast material at the base of the plant (Fig. 7.14 & 7.15). Plants cultured in treatment solutions however, showed signs of severe deformation and stunted development (Fig. 7.13). These observations were made in cultures of greater than 0.001 mg/L copper, 5 mg/L chromium and salinities below 30 ppt for both one and two week exposure times. Longer exposure tended to increase the sensitivity of plants with pronounced stunted growth, unhealthy membranes and the onset of mortality by the second week. Treated effluent concentrations of greater that 0.5% primary, 2.5% secondary and 1% chlorinated secondary treated effluent significantly affected growth and development of young plants. The added stress of reduced salinity of unmodified effluent tests enhanced the effects of the treatment solutions. Treatment solutions of copper and effluent caused significant decreases in chloroplast and physode densities of one week old plants and an incidence of void and empty spaces within cells (Fig 7.17) compared to those of control treatments (Fig. 7.16). By the second week, control embryos appeared healthy with pronounced and elongated thalli. In some copper (concentrations of less than 0.001 mg/L) and modified secondary treated effluent (concentrations of less than 5%) treatment cultures, many plants seemed to recover at the ultrastructural level, although this was not visible in observed cultures. There was evidence however, of more void spaces in young plants at the ultrastructural level although the density of cellular organelles appeared to gradually increase.

Figure 7.1 *Phyllospora comosa*. A scanning electron micrograph of a 48 hour old germinated embryo cultured in natural seawater.

Figure 7.2 *Phyllospora comosa*. A 48 hour old germinated embryo showing signs of longitudinal division (arrow) cultured in natural seawater.

Figure 7.3 *Phyllospora comosa*. Germinated and ungerminated (asterisk) 48 hour old embryos cultured in 1% unmodified primary treated effluent.

Figure 7.4 *Phyllospora comosa*. A scanning electron micrograph of a 48 hour old embryo cultured in 2% modified primary treated effluent.

Figure 7.5 *Phyllospora comosa*. A scanning electron micrograph of a 48 hour old embryo cultured in 15% unmodified secondary treated effluent.

Figure 7.6 *Phyllospora comosa*. Germinated and ungerminated 48 hour old embryos cultured in 20% modified secondary treated effluent.

Figure 7.7 *Phyllospora comosa*. Necrotic (arrow) and ungerminated 48 hour old embryos cultured in 50% unmodified secondary treated effluent.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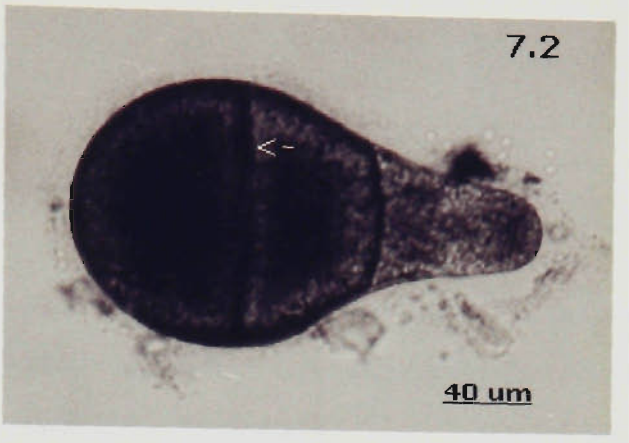
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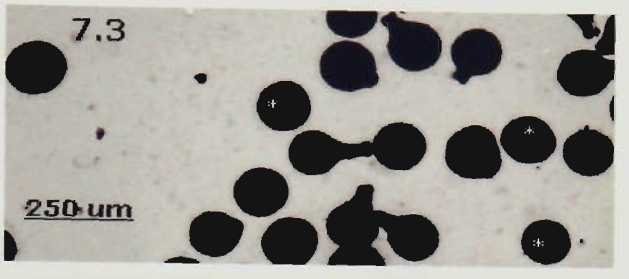
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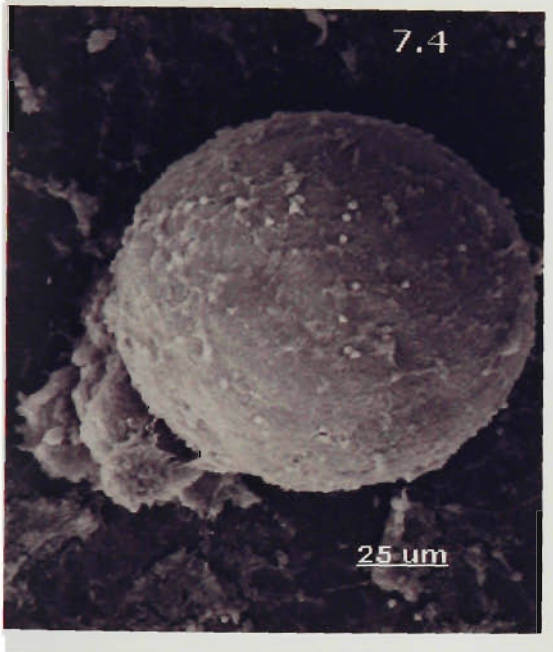
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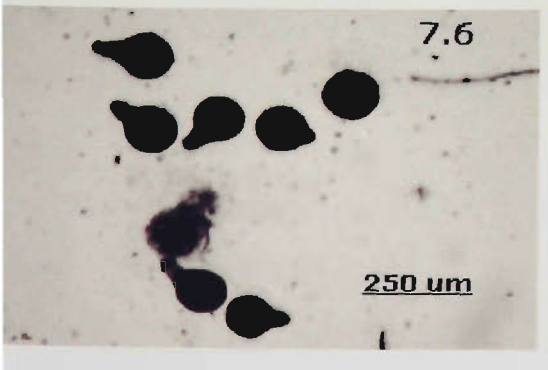
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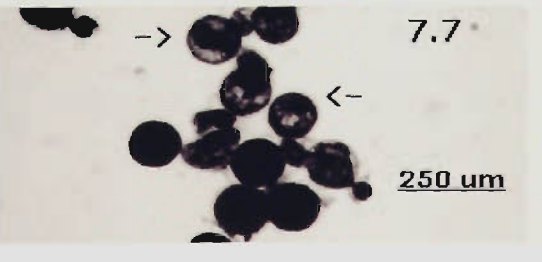
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Figure 7.8 *Phyllospora comosa*. 48 hour old embryos cultured in natural seawater.

Figure 7.9 *Phyllospora comosa*. A transmission electron micrograph of a 48 hour old embryo cultured in 0.2 mg/L copper showing chloroplasts (c), physodes (p), cell wall (arrows) and unhealthy appearing cellular membranes (asterisks).

Figure 7.10 *Phyllospora comosa*. A transmission electron micrograph of a 48 hour old embryo cultured in natural seawater showing chloroplasts (c), physodes (p), Golgi-derived vesicles (v) and cell wall (arrow).

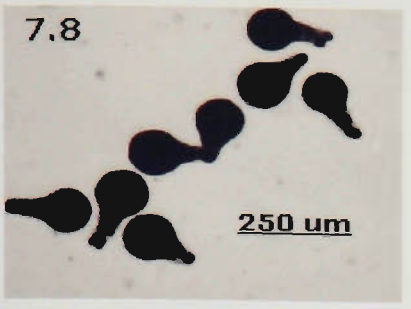
Figure 7.11 *Phyllospora comosa*. Ungerminated and necrotic (arrow) 48 hour old embryos cultured in 50% modified chlorinated secondary treated effluent.

Figure 7.12 *Phyllospora comosa*. A transmission electron micrograph of a 48 hour old embryo cultured in 5% unmodified, chlorinated secondary treated effluent, showing chloroplasts (c), unhealthy appearing cell wall (arrow) and membrane (asterisk) and disrupted vesicles.

Figure 7.13 *Phyllospora comosa*. A scanning electron micrograph of a two week old embryo cultured in 2% modified primary treated effluent.

Figure 7.14 *Phyllospora comosa*. A one week old embryo cultured in seawater.

Figure 7.15 *Phyllospora comosa*. A scanning electron micrograph of two week old embryos cultured in seawater.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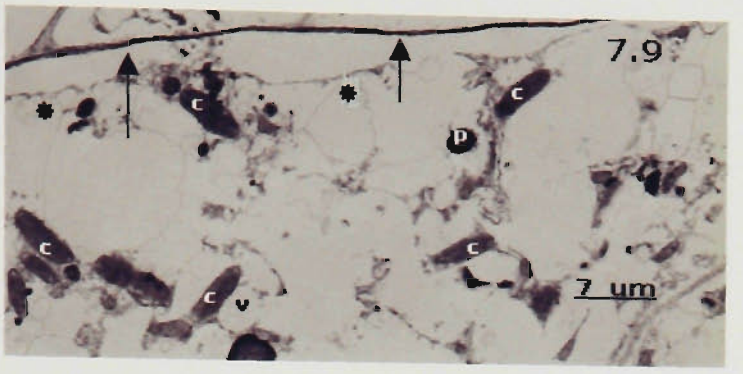
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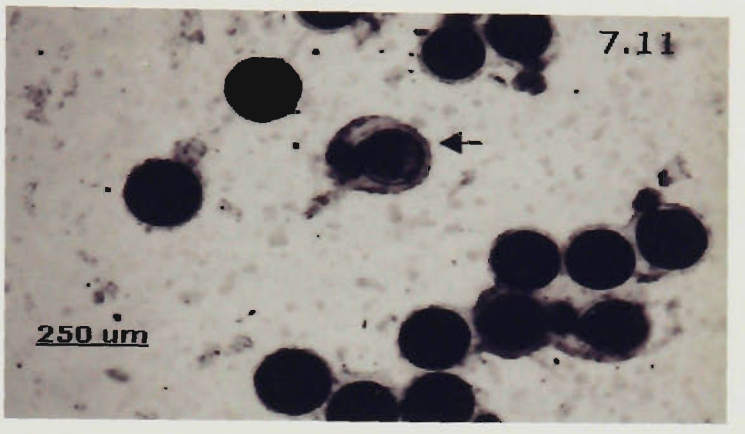
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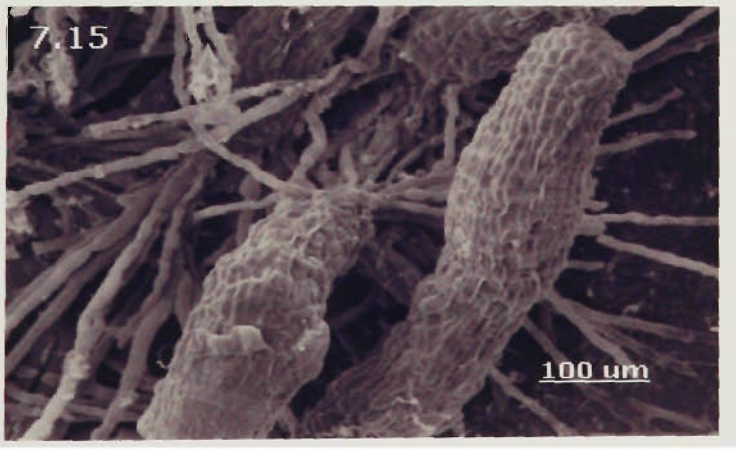
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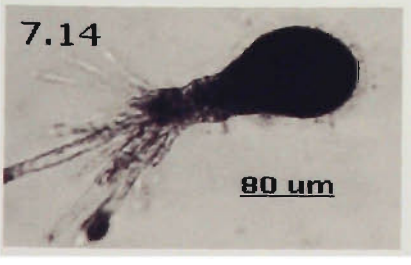
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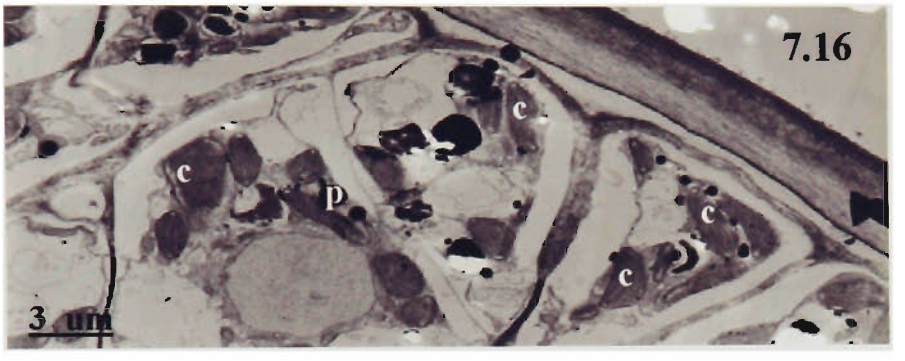
Figure 7.16 *Phyllospora comosa*. A transmission electron micrograph of a one week old embryo cultured in seawater showing chloroplasts (c) and physodes (p).

Figure 7.17 *Phyllospora comosa*. A transmission electron micrograph of a one week old embryo cultured in 1% unmodified primary treated effluent showing reduced numbers of chloroplasts (c) and physodes (p) and an increased incidence of void empty spaces within cells.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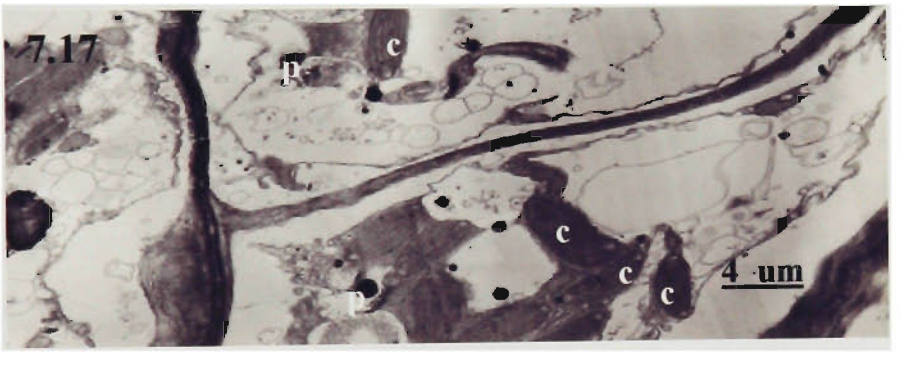
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7.2.2 *Hormosira banksii*

Scanning and transmission electron and light micrographs revealed significant effects on the morphology and development of *H. banksii* zygotes and embryos during and after toxicant exposure. After 48 hours of exposure, *H. banksii* zygotes cultured in control treatments of natural seawater showed signs of germination by the means of a developing rhizoid (Fig. 7.18, 7.19, 7.22 & 7.25). There was also clear evidence of cellular division along the longitudinal axis (Fig. 7.18). Zygotes and embryos (>30% of the test population) cultured in treatments of sewage effluent showed signs of stunted development through significantly shorter (Fig 7.23) and deformed (7.24) germination rhizoids compared to healthy embryos cultured in seawater control treatments. Copper and chromium treatments of greater than 0.1 mg/L and 22.5 mg/L respectively, substantially inhibited germination in zygotes. The same was evident in treatments of greater than 2% primary, 20% secondary and 10% chlorinated secondary treated effluents. Reducing salinity in unmodified effluent treatments enhanced the effects of the treatments tested. The onset of mortality was evident with clearly necrotic appearances of both zygotes and embryos with many having leaked their contents.

Transmission electron micrographs revealed reduced densities of chloroplasts and physodes in embryos cultured in treatment solutions with the two being almost nonexistent in many cells (Fig. 7.21) compared with those cultured in natural seawater. Chloroplast and physode densities were substantively decreased in zygotes and embryos cultured in copper and chromium concentrations of greater than 0.2 mg/L and 45 mg/L respectively. Primary treated effluent concentrations of greater than 4% reduced numbers of chloroplasts and physodes in embryos, as did concentrations of greater than 40% secondary treated effluent and 20% chlorinated secondary treated effluent. The appearance of chloroplasts in these cultures also differed from those in control treatments. Chloroplasts appeared pale and swollen in treatments of reduced salinity. Cellular membranes also seemed affected and appeared unhealthy. Although reduced salinity amplified the effects of effluent samples tested, it was clear that both modified and unmodified effluent treatments exerted similar effects on developing zygotes and embryos.

Long-term growth tests indicated that the longer exposure time increased sensitivity of the young life stages. Healthy plants were evident by an elongated rhizoid (Fig. 7.26), development of a secondary rhizoid (Fig. 7.27 & 7.32) and advanced cellular divisions (Fig. 7.20). Embryos cultured in treatment solutions showed signs of severe deformation and stunted development (Fig. 7.28, 7.29, 7.33 & 7.34). These observations were made in cultures of greater than 0.004 mg/L copper, 2 mg/L chromium and salinities below 20 ppt for both one and two week exposure times. Longer exposure tended to increase the sensitivity of plants with pronounced stunted growth and the onset of mortality by the second week and the incidence of damaged and unhealthy appearing membranes also increased. Treated effluent concentrations of greater that 1% primary, 5% secondary and 5% chlorinated secondary treated effluent significantly affected growth and development of young plants. Reduced salinity of unmodified effluent tests again enhanced the effects of the treatment solutions. Treatment solutions of copper, chromium and effluent caused significant decreases in chloroplast densities after two weeks of exposure (Fig 7.31) compared to those of control treatments (Fig. 7.30). Unlike embryos of *P. comosa,* those of *H. banksii* showed no signs of recovery at any stage during the test period.

Figure 7.18 *Hormosira banksii*. A 48 hour old embryo cultured in seawater showing signs of cellular division (arrow).

Figure 7.19 *Hormosira banksii*. A 48 hour old embryo cultured in seawater.

Figure 7.20 *Hormosira banksii*. A one week old embryo cultured in seawater.

Figure 7.21 *Hormosira banksii*. A transmission electron micrograph of a 48 hour old embryo cultured in 2% primary treated effluent showing unhealthy appearing chloroplasts (c) and a high incidence of void spaces within cells.

Figure 7.22 *Hormosira banksii*. A scanning electron micrograph of a 48 hour old germinated embryo cultured in seawater.

Figure 7.23 *Hormosira banksii*. A scanning electron micrograph of a 48 hour old embryo cultured in 5% unmodified primary treated effluent.

Figure 7.24 *Hormosira banksii*. A scanning electron micrograph of a 48 hour old embryo cultured in 20% unmodified secondary treated effluent.

Figure 7.25 *Hormosira banksii*. A scanning electron micrograph of a 48 hour old embryo cultured in seawater.

Figure 7.26 *Hormosira banksii*. A one week old embryo cultured in seawater.

Figure 7.27 *Hormosira banksii*. One week old embryos cultured in seawater showing developed secondary rhizoids.

Figure 7.28 *Hormosira banksii*. A scanning electron micrograph of one week old embryos cultured in 50% modified chlorinated secondary treated effluent.

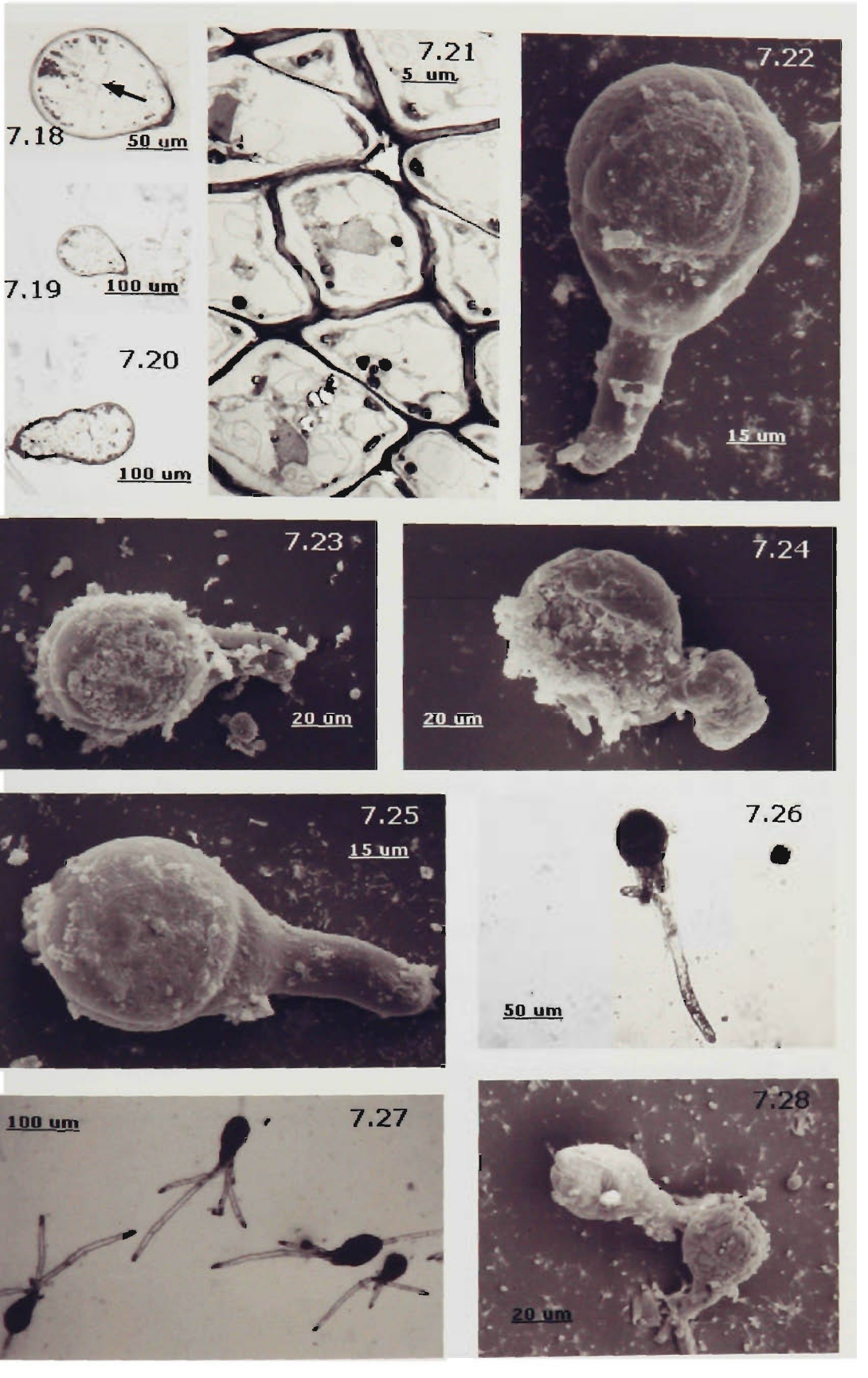
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Figure 7.29 *Hormosira banksii*. A scanning electron micrograph of one week old embryos cultured in 5% unmodified secondary treated effluent.

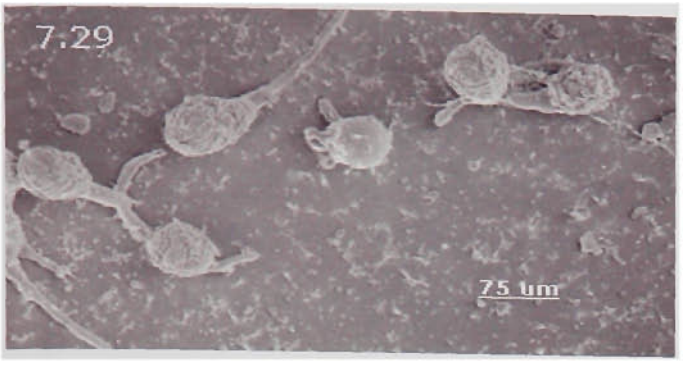
Figure 7.30 *Hormosira banksii*. A transmission electron micrograph of a one week old embryo cultured in seawater showing healthy chloroplasts (c), physodes (p) and cellular membranes (arrows).

Figure 7.31 *Hormosira banksii*. A transmission electron micrograph of a one week old embryo cultured in 2% unmodified primary treated effluent showing unhealthy appearing chloroplasts (c) and membranes (arrows) and large empty spaces.

Figure 7.32 *Hormosira banksii*. A scanning electron micrograph of one week old embryos cultured in seawater.

Figure 7.33 *Hormosira banksii*. A scanning electron micrograph of one week old embryos cultured in 10% modified secondary treated effluent.

Figure 7.34 *Hormosira banksii*. A scanning electron micrograph of one week old embryos cultured in 20% modified, chlorinated secondary treated effluent.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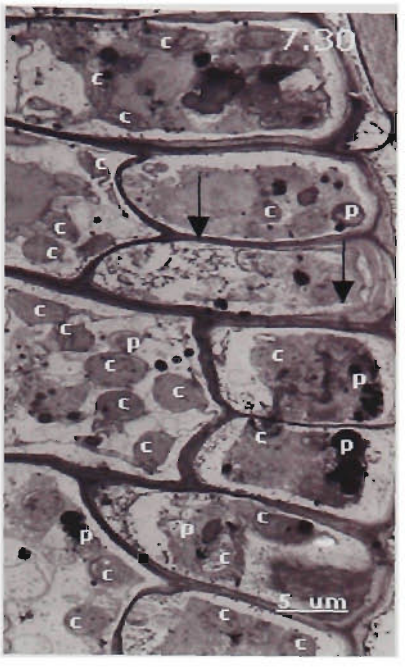
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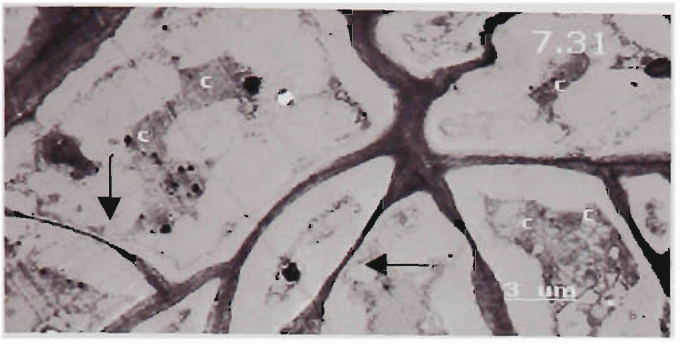
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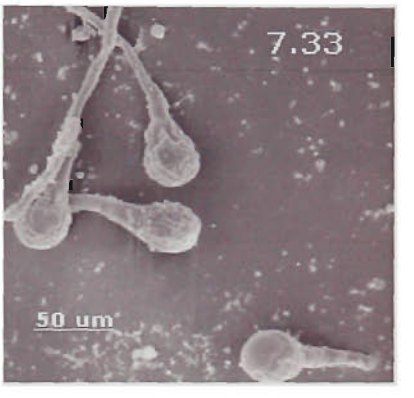
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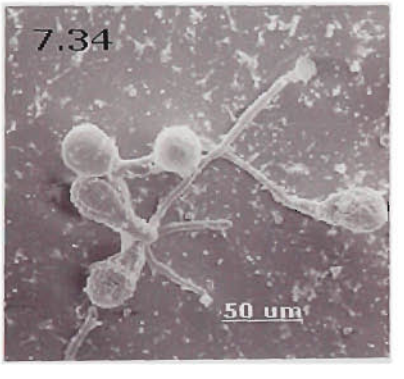
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7.2.3 *Macrocystis angustifolia*

Scanning and light micrographs revealed that treatment solutions exerted substantive effects on the morphology of the early life stages of *Macrocystis angustifolia* spores and sporophytes. After 48 hours, spores could be seen germinated in control cultures (Fig. 7.35). This unique dumbbell appearance of spores is a characteristic of laminarian species, the germination tube then elongates as the young spores develop (Fig. 7.36). In cultures housing spores exposed to treatment solutions, germination was stunted and growth inhibited (Fig. 7.37) (>30% of the test population) which may suggest disruptions in cellular division and imply reduced rates of photosynthesis and increased rates of respiration. This was most evident in treatments of copper and chromium at concentrations greater than 0.032 mg/L and 22.5 mg/L respectively. For spores cultured in treated effluent, inhibition of germination and growth occurred at concentrations of greater than 2% primary, 10% secondary and 15% chlorinated secondary treated effluent.

For sporophyte growth and reproduction assays, concentrations exerting effects were lower than those of the short-term bioassays. Before reproduction of *M. angustifolia,* male and female gametophytes could be seen in abundance in control treatments (Fig. 7.38) and after reproduction, healthy sporophytes were produced (Fig. 7.39 & 7.42). In some treatment cultures however, reproduction did not occur and in others, stunted development and inhibited growth of sporophytes were recorded (Fig. 7.40 & 7.41). These gametophytes seemed to lack healthy definition suggesting disruptions in cellular division. Gametophytes exposed to copper and chromium concentrations of greater than 0.008 mg/L and 4 mg/L respectively did not reproduce. This trend was also evident in treatments of effluent concentrations of greater than 2% primary and 6% secondary and 5% chlorinated secondary treated effluents. Concentrations lower than those mentioned tended to produce significant numbers of deformed sporophytes.

Figure 7.35 *Macrocystis angustifolia.* Germinated and ungerminated 48 hour old spores cultured in natural seawater.

Figure 7.36 *Macrocystis angustifolia.* A scanning electron micrograph of a 48 hour old spore cultured in natural seawater.

Figure 7.37 *Macrocystis angustifolia.* A scanning electron micrograph of a 48 hour old spore cultured in 10% unmodified secondary treated effluent.

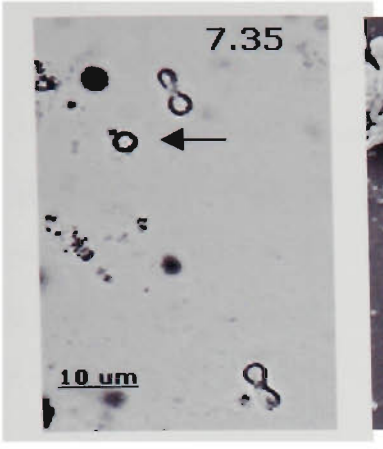
Figure 7.38 *Macrocystis angustifolia.* Two week old male and female gametophytes cultured in natural seawater.

Figure 7.39 *Macrocystis angustifolia.* A scanning electron micrograph of a sporophyte cultured in natural seawater.

Figure 7.40 *Macrocystis angustifolia.* A scanning electron micrograph of a sporophyte cultured in 1% unmodified primary treated effluent.

Figure 7.41 *Macrocystis angustifolia.* A scanning electron micrograph of a sporophyte cultured in 12% modified, chlorinated secondary treated effluent showing a lack of definition in development.

Figure 7.42 *Macrocystis angustifolia.* A sporophyte cultured in natural seawater.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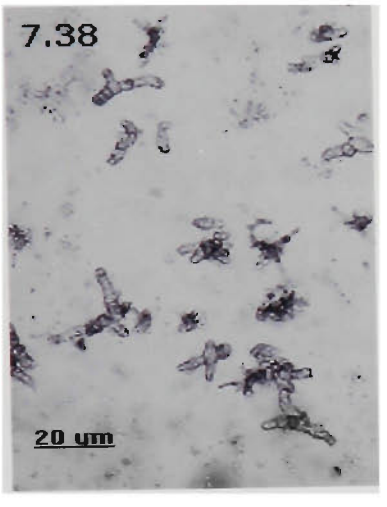
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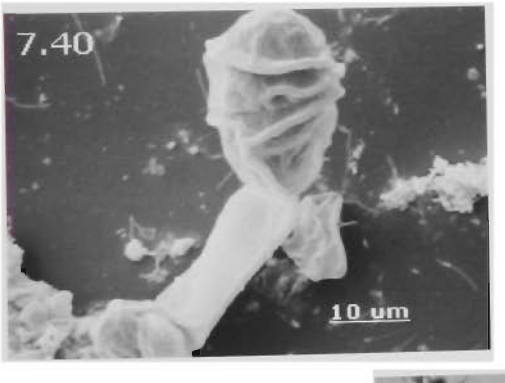
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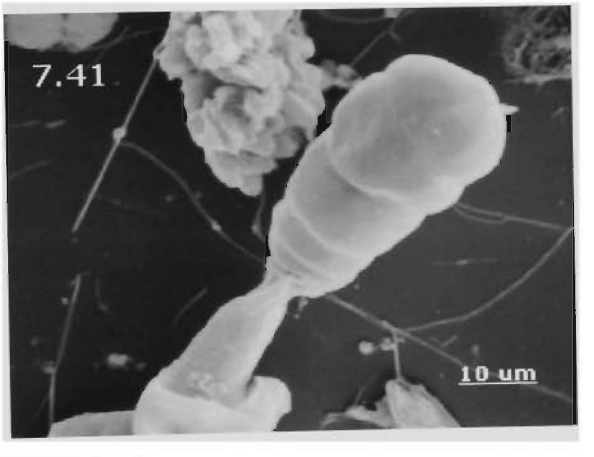
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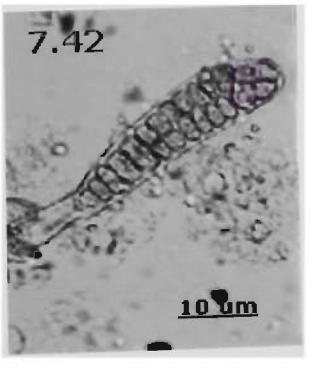
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7.3 Discussion

Treatment solutions exerted significant effects on the development of the early life stages of *P. comosa, H. banksii* and *M. angustifolia.* Scanning electron and light micrographs clearly showed morphological effects of treatments whilst transmission electron micrographs indicated possible effects at the ultrastructural level. Along with effects recorded at the cellular level, severe deformities and stunted growth were common outcomes as a result of toxicant exposure.

Findings of this study are consistent with other investigations addressing effects of treated sewage effluent on several algal species (Burridge *et al.* 1995a, 1995b, Gunthorpe *et al.* 1995, Doblin & Clayton 1995). Doblin and Clayton (1995) investigated the effects of chlorinated secondary treated effluent on germination and growth of *H. banksii* embryos. They recorded stunted growth and signs of plasmolysis in one week old embryos cultured in 28% effluent. They also noted degeneration of two week old plants cultured in effluent concentrations of 40% and higher. Similar observations were made in this study for all three species. Significant morphological differences between control and treatment cultures were observed. Kavekordes and Clayton (pers. comm.) investigated the effects of secondary treated effluent on embryo development of *H. banksii.* Their findings are consistent with those found for this study. They noted discolouration of chloroplasts, the onset of plasmolysis and general degeneration of cells by the end of the first week of exposure. They also emphasised that decreased salinity significantly affected the health of cells. The chlorination of secondary treated effluent suggested increased toxicity compared with unchlorinated effluent however, since this study is merely investigative and based on descriptive observations, statistical inferences cannot be drawn.

The ultrastructural effects of effluent were evident in *H. banksii* and *P. comosa* zygotes and embryos. Effects ranged from changes in chloroplast densities and appearances to the reduction of physode numbers within cells. The void spaces observed in some cells may have been due to those cells having partially leaked their contents. These may also suggest cellular breakdown and membrane disruption.

Copper and chromium exerted negative effects on developing *P. comosa* and *H. banksii* embryos at the ultrastructural level. Reduced numbers of chloroplasts, physodes and Golgi-derived vesicles were common outcomes in such treatments with copper exerting greater toxicity. Morphological effects included deformed development and stunted growth of zygotes and embryos. Sunda and Guillard (1976) researched the toxicity of copper to an estuarine diatom *Thalassiosira pseudonana* and green alga *Nannachloris atomus*. They recorded the inhibition of growth in both species after copper exposure. Hopkin and Kain (1978) investigated the effects of copper on the survival of the alga *Laminaria hyperborea*. They recorded decreased growth rates, respiration and survival at concentrations ranging from 0.025-0.075 mg/dm^3. Ragan *et al.* (1979) researched the chelation of divalent metal ions by brown algal species. They focussed on the ability of these species to concentrate heavy metal ions and to localise these in physodes. This may lead to young plants having the ability to regulate the intake of certain heavy metals and in turn limiting toxic effects during short exposure times. These physodes being reactive with ionic forms of several heavy metals including copper and chromium. It was found in this study that *P. comosa* embryos appeared to recover at the ultrastructural level after two weeks of exposure to copper and effluent treatments suggesting that the alga may possess a delayed ability to regulate heavy metal intake.

Although utilised as reference toxicants for this study, heavy metals such as copper and chromium are commonly detected in discharged waters. Copper has been linked to causing reduced cell division rates in the marine diatom *Nitzschia closterium* (Stauber & Florence 1989). Andersson and Kautsky (1996) investigated the effects of copper on reproduction in the fucalean species *Fucus vesiculosus*. They noted a lack of research into the physiological effects of copper on macroalgal species. They did speculate that copper may cause the degradation of chloroplast membranes causing inhibition of photosynthesis and consequently growth. Chloroplast densities were definitely reduced in both *P. comosa* and *H. banksii* embryos, which may explain reductions in growth rates of the young plants. Other work has suggested that the presence of other compounds found in discharged waters may alleviate the toxic effects of certain heavy metals (Sunda *et al.* 1981, Habermann 1969, Stauber & Florence 1985). Stauber and Florence (1985)

found that manganese and iron effectively uptake copper at surface membranes of algal species and prevent penetration into the cell. Neither however, was able to reverse copper toxicity nor inhibit the toxicity of copper complexes.

Modification of cation uptake and membrane permeability is also likely to have profound effects on growth and survival of embryos. A considerable body of evidence (Sunda 1976, Ragan *et al.* 1979, Stauber& Florence 1985, 1986, 1989) implicates cation uptake and regulation as critical for germination and growth and the effects of toxicants that modify such ionic regulation is likely to inhibit both processes. Further, stress resulting from such inhibition may result in less resources being allocated to growth and production of intracellular resources in the form of physodes, chloroplasts and other vesicular/membrane bound material. Added issues associated with reduced salinity and osmotic stress would compound such problems and presumably account for the synergism observed for particular toxicants under conditions of reduced salinity. There is also significant potential for the disruption of intracellular channeling within cells (Burridge pers. comm., Burridge *et al.* 1999) and membrane damage may account for the reduced incidence of vesicle bound material within treatment cells. These membrane chanelling systems requiring close association between membranes of Golgi-derived bodies and other critical organelles (such as vesicles and endo reticulum) within cells (Burridge 1990).

This study clearly illustrated the negative effects of sewage effluent on the development of three species of macroalgae. Discharged waters have the potential to cause severe deformities within cells, which can then lead to the hindrance or prevention of normal cellular processes. Electron micrographs of *P. comosa, H. Banksii* and *M. angustifolia* clearly showed the effects of effluent discharge on normal growth and development of the three species. Information gained form these micrographs add a further dimension to understanding the effects of discharged waters, as the utilisation of this tool to date has been lacking.

CHAPTER 8

GENERAL DISCUSSION

8.1 Discussion

During the course of this study, it was found that treated sewage effluent, in particular primary and chlorinated secondary treated effluent, exerted toxic effects on the early life stages of *Phyllospora comosa, Hormosira banksii* and *Macrocystis angustifolia*. Both reference toxicants also exerted similar effects, but of greatest concern were the effects of reduced salinity on the young plants. Observed effects included germination inhibition, mortality, deformed development and reduced growth rates. These observations concur with other studies investigating similar treatments on aquatic plants where disruption in growth, respiration and reproduction have been recorded (Stauber & Florence 1989, Anderson & Hunt 1990, Andersson *et al.* 1992, Andersson & Kautsky 1996, Bidwell *et al.* 1998, Burridge *et al.* 1999). It was also found that these toxic effects may have been caused by reductions in organelle densities, specifically chloroplasts and physodes and perhaps most importantly disruptions in membrane development and functionality.

Negative effects of the sewage treatments and especially reduced salinity at the cellular level of the algae utilised were profound. Chloroplasts, physodes and membranes appeared stressed and severely affected. Chloroplasts are essential to cells, as they are the sites for photosynthesis and a reduction of these organelles equates to decreased levels of photosynthate and chlorophyll which are essential for growth. Physodes are thought to be responsible for maintenance of healthy cells and waste metabolites and possibly have antibiotic and antiviral functions (Clayton & King 1981). Poor membrane health may lead to reduced essential resources permeating into cells, resulting again in reduced photosynthesis, cell division and germination (Stauber & Florence 1985). It has been proposed that cation movement across cell membranes in the basal pole of *Fucus* zygotes is a precursor to germination (Robinson & Jaffe 1975, Jaffe *et al.* 1976) and modification

of osmotic potential and cation uptake is likely to severely inhibit embryo physiology. Such inhibition may therefore inhibit the mechanisms responsible for cellular polarisation, consequently inhibiting or delaying germination (Burridge *et al.* 1996). This may then lead to limited growth, reduced Darwinian fitness and mortality.

Effects at the ultrastructural level would presumably also have detrimental effects at the population and community level. Chung and Brinkhuis (1986) noted that heavy metals present in wastewater may decrease the total primary production of algal systems due to failures in recruitment and decreased growth. Abnormalities in the critical early life stages of algal species such as reductions in chlorophyll content and disruptions in cellular membrane health and function may cause potential deleterious impacts on primary production of an entire macroalgal population. Secondly some species of brown algae have shown the ability to concentrate ions of metals from surrounding discharged water. These bioaccumulated metals may then be transferred to the detrital foodweb (Chung & Brinkhuis 1986). Microbial activity may then be decreased which in turn may affect the total production of the system as it is this activity which provides the regeneration of nutrients back into the water column (Babich & Stotzky 1985).

Treated sewage effluent is comprised characteristically of nitrogenous compounds, orthophosphates, detergents, trace metals and residual chlorine (Andrews 1976). Being so complex, the potential for synergistic and antagonistic effects between constituents exists. The effects of effluent treatments tested in this study varied depending on both species and ecotoxicological endpoints used. For the germination bioassays, primary treated effluent (both modified and unmodified) exerted similar toxicity to all three species with 48 hour EC_{50} values ranging from 5-10% effluent. As there were no significant differences in toxicity of modified and unmodified primary treated effluent samples tested, it can be proposed that toxic effects were a direct result of actual effluent toxicity. Secondary treated effluent was less toxic than primary treated effluent with 48 hour EC_{50} values ranging from 26->100% effluent. In general, chlorination of the secondary treated effluent increased effluent toxicity. Unmodified secondary treated effluent was generally more toxic than modified effluent, with similar toxicity to *P.*

comosa and *H. banksii*. *M. angustifolia* appeared to be slightly less sensitive, but this may not be statistically significant due to the high coefficients of variation. Much of the bioassay variability was probably due to effluent variability over the sampling period as the coefficients of variation were higher for tests conducted with treated effluent compared with the reference toxicants. Salinity effects were similar to all three species, but of considerable concern as salinity reduction of surrounding seawater is a direct result of sewage effluent discharge. Reduced salinity is known to affect growth (Burrows 1964), production of fertile tissue (Mathieson 1982) and the release of propagules in other species of marine macroalgae (Doblin & Clayton 1995). It will almost certainly cause osmotic shock and plasmolysis of cell membranes (Burridge *et al.* 1999).

Treated sewage effluent is a highly complex mixture so the actual expression of toxicity of components cannot be assumed. It is difficult to compare the sensitivity of these macroalgal bioassays to sewage effluent with other studies due to variations in effluent quality. Anderson and Hunt (1988) found that germination in *M. pyrifera* was significantly inhibited at concentrations of primary treated sewage effluent of greater than 1% effluent. Burridge *et al.* (1996) exposed *H. banksii, P. comosa* zygotes and *M. angustifolia* zoospores to primary and secondary treated effluents. They found that the responses recorded varied significantly between and within species. The salinity reduction assays performed for this study provided useful information on the effects of reduced salinity levels on the three species. The combined effects of effluent and reduced salinity increased the overall effects of the unmodified effluent samples. Salinity reduction did not exert significant effects in the primary treated effluent tests conducted, as the algae were sensitive to the effluent at low concentrations.

The effects of sewage effluent on coastal ecosystems has been investigated in many locations around the world (Littler & Murray 1975, Tewari & Joshi 1988, Doblin & Clayton 1995). The most noticeable changes caused by this type of disturbance are reductions in species diversity and significant shifts in species dominance. Outfall

regions are susceptible to constant fluctuations in salinity, temperature and water quality. These changes have lead to the disappearance of species, particularly large brown algae which are sensitive to sewage pollution, from areas located near such outfalls. The South Eastern Purification Plant discharges chlorinated, secondary treated effluent at the Boags Rocks outfall. Since the commencement of discharge at the site in 1975, notable changes in species composition and dominance have occurred. This study revealed the significant effects of reduced salinity on the species once dominant at the outfall. It is estimated that up to 440 ML of effluent is discharged each day, this together with chlorine, heavy metals and other compounds present in the effluent contribute to its overall toxicity (Doblin & Clayton 1995). The results obtained from the sewage effluent bioassays suggest that the introduction of treated effluents into the marine environment can impact algal communities. Brown *et al.* (1990) recorded reductions in algal species diversity and biomass in areas located near the Boags Rocks outfall. *M. angustifolia, H. banksii* and *P. comosa* have been shown to be particularly sensitive to the effects of treated effluent and long-term exposure could lead to mortality of the early life stages at low concentrations. Furthermore, concentrations which would not normally cause mortality may impact on the population structure leading to diminished recruitment by the inhibition of germination, growth (*P. comosa* and *H. banksii)* and reproduction (*M. angustifolia*).

Toxicity tests conducted with the reference toxicants and effluent treatments concurrently provided a baseline for temporal variation. Some seasonal variations in algal responses were noted, especially during the warmer months of the year, where young plants exhibited greater sensitivity. Species which exhibit year round reproduction such as those utilised in for this study are best suited for routine toxicity testing, although the seasonal variation recorded for these species must be considered. It is not fully understood why temporal variation occurs, but provided test reproducibility remains high and consistent, the scope for continued development of macroalgal ecotoxicologial assays exists. Overall however, results confirmed the changing toxicity of treated sewage effluent over time. Without reference toxicant data, inaccurate conclusions may be drawn regarding effluent toxicity.

Findings of tests conducted with the reference toxicants were consistent with those of other researchers. Bidwell *et al.* (1998) investigated the effects of copper on germination and growth of the macroalga *Ecklonia radiata*. They found that germination of *E. radiata* was significantly inhibited at 48 hour with EC_{50} concentrations ranging from 0.28-0.39 mg/L copper, similar to results found for this study. Anderson *et al.* (1990) also recorded similar sensitivity of germinating *M. pyrifera* with a mean 48 hour EC_{50} concentration of 0.3 mg/L. They investigated the effects of copper on spore growth and reproduction of the macroalga and found that sporophyte production was the most sensitive (NOEC < 10.2 µg/L), followed by sporophyte growth and germ tube growth (NOEC = 10.2 µg/L) and finally germination (NOEC = 50.1 µg/L). These threshold concentrations were similar to the NOEC values of 0.03-0.07 mg/L copper for *M. angustifolia* germination assays recorded in this study. Copper has been shown to be toxic to a number of marine algae (Stauber & Florence 1985). It is thought that the metal reacts at cell membranes affecting mitosis and inactivating the Hill Reaction in photosynthesis or inactivating essential enzymes (Huntsman & Sunda 1980). Anderson *et al.* (1990) proposed that copper may disrupt membrane integrity and alter capacity for cation uptake during germination. Electron microscopy research conducted for this study confirmed that copper can cause deleterious effects by targeting cellular membranes.

Although not highly toxic compared to copper, chromium exerted some toxic effects on developing *H. banksii, P. comosa* and *M. angustifolia*, the most sensitive endpoints being embryo growth and sporophyte production. Greatest sensitivity to chromium was observed in growth tests where the mean EC_{50} concentrations ranged from 13 mg/L chromium (*M. angustifolia* sporophyte production assay) to 5 mg/L chromium (*M. angustifolia* sporophyte growth assay). The macroalgal bioassays however, were less sensitive to the effects of chromium compared to a bioassay based on inhibition of cell division rates in the microalga, *Nitzschia closterium* (Stauber *et al.* 1994). The mean 72 hour EC_{50} concentration for the *Nitzschia* bioassay was 2.43 mg/L chromium, a much more sensitive test species and endpoint when compared to the macroalgae used in this study. Similarly, Dorn *et al.* (1987) found that the mysid *Mysidopsis bahia* and daphnid

Daphnia pulex were sensitive to chromium, with 48 hour EC_{50} values ranging from 4.21-7.23 mg/L chromium for *M. bahia* and 0.024-0.17 mg/L chromium for *D. pulex.* The exact mode of toxicity of chromium is unknown, it is however, a highly soluble compound that can readily penetrate biological membranes (Reidel 1985) and bind and/or oxidise polypeptides and proteins, leading to cellular disruptions (Patel & Saxena 1983).

The sensitivities of the three algal species varied for the different treatments tested, particularly the sensitivities to the reference toxicants copper and chromium. The mean 48 hour EC_{50} concentration for *P. comosa* zygotes exposed to copper was 0.42 mg/L and for *H. banksii* and *M. angustifolia,* 0.18 and 0.15 mg/L respectively. For chromium, the mean 48 hour EC_{50} concentrations for *P. comosa* and *M. angustifolia* were 50.3 and 49.7 mg/L respectively yet for *H. banksii* the mean 48 hour EC_{50} concentration was 69.8 mg/L chromium. Although *P. comosa* was less sensitive to copper compared to the other two algae, it was more sensitive to chromium together with *M. angustifolia.* Mode of toxicant entry, metabolism, and physical features of the algae could influence differences between and within algal responses recorded. As too can growth conditions, media composition, culture conditions and micronutrient content (Stauber & Florence 1985).

Bioassays based on germination and mortality endpoints were less sensitive to all the treatments tested, compared with the longer term bioassays. However, variability of these growth and sporophyte production tests was high, with coefficients of variation ranging from 37-63%, compared with 16-34% for the 48 hour germination tests with copper, chromium and reduced salinity. The shorter germination and mortality bioassays exhibited greater reproducibility and required less expertise and time. Longer bioassays cannot be used as effectively as the shorter germination assay to reflect the possible effects of sewage effluent at any one particular time. This is due to the fluctuating toxicity of sewage effluent over time as was most evident during the course of this study.

The Microtox® bioluminescence inhibition assay was not as sensitive as the algal test endpoints, but results were made available significantly more quickly. This is advantageous when the monitoring of effluent toxicity from one day to the next is required. The bioluminescence inhibition assay conducted with *Vibrio fischeri* revealed that although the bacterium was sensitive to copper (mean 5 minute EC_{50} of 0.17 mg/L) the opposite was true for chromium (mean 5 minute EC_{50} of 970 mg/L). These results however, were of no great concern as greater emphasis was placed on the test protocol's reproducibility. The low coefficients of variance recorded for both copper (10%) and chromium (6%) indicated the test's suitability for routine toxicant screening.

This study successfully utilised three macroalgal species endemic to Australian waters in routine toxicity testing. The suitability of these species could not be surpassed as all three species are true representatives of typical high energy coastal environments of southern Australia. The bioassays used, particularly germination and mortality, produced data of high reproducibility with some seasonal variation which must be recognised and monitored. The longer, chronic toxicity tests gave an insight into prolonged effects of sewage effluent. Although the ecological relevance of the bacteria *V. fischeri* is questionable, the Microtox® test system was useful in enabling quick screening of treated effluent samples over time.

Toxicity testing is an important tool in understanding the effects of potential toxicants on receiving ecosystems. The data collected for this study suggest that the early life stages of *P. comosa, H. banksii* and *M. angustifolia* can be successfully used in routine toxicity testing. Germination and growth inhibition, mortality and sporophyte production and growth, are ecotoxicological endpoints which offer substantial ecological significance (Burridge *et al.* 1996). The species utilised for this study colonise areas, which are susceptible to the effects of sewage effluent. This being the case, these species are true representatives of ecosystems either currently or potentially exposed to discharged waste water.

8.2 Conclusions

The following conclusions were drawn from this study.

1. Early life stages of macroalgae were suitable for assessing the toxicity of sewage effluents. Longer-term tests such as growth and sporophyte production were more sensitive, but less reproducible than shorter 48 hour germination and 96 hour mortality bioassays.

2. Treatment of sewage effluent significantly reduced its toxic effects, although chlorinated secondary treated effluent was more toxic than secondary treated effluent before chlorination.

3. Copper chloride and hexavalent chromium were suitable reference toxicants.

4. Toxic effects of the treated sewage effluent were greatly exacerbated by reduced salinity of the effluent which was most evident with bioassays conducted with secondary and chlorinated secondary treated effluent.

5. Electron microscopy revealed significant negative effects of treated sewage effluent of the macroalgal species tested ranging from severe physical deformities in development to disrupted and unhealthy appearing cellular membranes and reduced numbers of critical organelles at the ultrastructural level.

6. The Microtox® bioassay was less sensitive to effluent than macroalgal bioassays, but was a useful substitute when rapid results were required.

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Appendix 1

Toxicity test labels as quoted in Chapters 3-5 and their corresponding dates of commencement.

Test number	Test date
a	2/9/96
b	15/9/96
c	2/10/96
d	18/10/96
e	1/11/96
1	15/11/96
2	4/12/96
3	22/12/96
4	2/1/97
5	17/1/97
6	5/2/97
7	26/2/97
8	5/3/97
9	28/3/97
10	15/4/97
11	7/5/97
12	28/5/97
13	16/6/97
14	10/7/97
15	28/7/97
16	28/8/97
17	18/9/97
18	9/10/97
19	1/11/97
20	24/11/97

Appendix 2

Tables of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa, Hormosira banksii* and *Macrocystis angustifolia* germination and germination tube growth bioassays

Appendix 2.1

Table of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa* copper and chromium germination bioassays based on data presented in Section 3.2.1.

Test number	copper				chromium			
	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.
a	0.10	0.20	0.42	0.39-0.57	11.25	22.50	45.2	35.3-62.3
b	0.20	0.40	0.44	0.28-0.58	11.25	22.50	46.9	30.7-55.8
c	0.10	0.20	0.42	0.36-0.57	11.25	22.50	45.4	30.3-56.1
d	0.10	0.20	0.47	0.44-0.54	11.25	22.50	43.2	33.4-58.4
e	0.10	0.20	0.41	0.37-0.54	11.25	22.50	49.8	43.4-68.2
1	0.10	0.20	0.42	0.32-0.49	11.25	22.50	44.6	29.2-55.8
2	0.05	0.10	0.27	0.22-0.37	5.63	11.25	29.8	18.4-36.9
3	0.05	0.10	0.28	0.18-0.42	11.25	22.50	28.9	23.2-33.5
4	0.05	0.10	0.29	0.11-0.43	5.63	11.25	31.5	22.3-47.1
5	0.05	0.10	0.35	0.30-0.44	5.63	11.25	30.2	18.1-41.3
6	0.05	0.10	0.32	0.15-0.59	5.63	11.25	37.6	22.2-45.5
7	0.05	0.10	0.27	0.14-0.33	5.63	11.25	28.4	25.6-31.5
8	0.10	0.20	0.35	0.19-0.41	11.25	22.50	53.0	39.2-66.8
9	0.05	0.10	0.36	0.28-0.39	11.25	22.50	57.3	48.6-72.1
10	0.10	0.20	0.57	0.41-0.63	22.50	45.00	64.4	49.8-88.2
11	0.10	0.20	0.52	0.45-0.62	22.50	45.00	56.6	44.2-69.3
12	0.10	0.20	0.47	0.31-0.71	22.50	45.00	60.9	32.9-88.5
13	0.20	0.40	0.60	0.37-0.88	11.25	22.50	53.3	39.2-87.5
14	0.10	0.20	0.41	0.15-0.59	22.50	45.00	64.5	42.5-78.7
15	0.10	0.20	0.57	0.48-0.63	11.25	22.50	41.6	31.3-46.8
16	0.20	0.40	0.46	0.39-0.59	11.25	22.50	37.7	25.8-46.9
17	0.10	0.20	0.40	0.22-0.44	11.25	22.50	38.9	21.5-46.2
18	0.10	0.20	0.46	0.41-0.55	11.25	22.50	56.4	22.9-63.8
19	0.10	0.20	0.57	0.39-0.65	11.25	22.50	68.0	42.8-89.7
20	0.20	0.40	0.50	0.44-0.66	11.25	22.50	44.6	26.6-69.0

Appendix 2.2

Table of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa* primary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.1.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	4	8	15	11.3-18.9	4	8	14	11.2-21.3	28.50
2	2	4	13	10.2-16.4	2	4	10	8.63-15.8	29.00
3	1	2	7	5.60-8.92	2	4	8	5.40-9.87	29.25
4	2	4	9	4.50-13.4	2	4	6	4.86-7.80	29.50
5	1	2	9	6.30-15.2	1	2	6	5.20-7.80	29.50
6	4	8	14	10.8-22.7	2	4	8	7.60-10.2	29.25
7	1	2	6	2.30-19.8	1	2	6	4.90-6.60	29.50
8	1	2	11	5.75-25.6	1	2	10	9.10-12.6	29.00
9	4	8	10	8.50-13.2	2	4	10	6.32-15.8	29.00
10	2	4	8	6.50-12.3	2	4	7	6.10-9.80	29.38
11	4	8	11	8.54-15.6	4	8	11	9.80-18.7	28.88
12	4	8	15	8.75-19.6	4	8	14	12.3-21.4	28.50
13	2	4	11	9.60-14.5	2	4	10	8.90-15.4	29.00
14	4	8	15	12.3-18.7	4	8	14	10.2-16.3	28.50
15	1	2	11	8.90-11.9	1	2	8	7.50-9.86	29.25
16	4	8	16	11.7-17.9	4	8	15	12.8-19.8	28.38
17	1	2	4	2.90-4.60	1	2	5	4.50-6.15	29.63
18	1	2	6	5.50-6.80	1	2	5	3.60-6.20	29.63
19	1	2	4	3.10-4.70	1	2	4	2.23-4.80	29.75
20	4	8	12	10.5-13.9	2	4	13	8.90-29.7	28.63

Appendix 2.3

Table of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa* secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.1.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	20	40	81	70.1-95.4	4	8	31	24.7-38.5	23.28
2	10	20	66	60.4-80.2	4	8	32	18.6-36.4	23.05
3	20	40	72	68.4-78.4	4	8	37	30.1-42.3	21.93
4	10	20	69	58.3-70.2	4	8	33	27.6-37.4	22.86
5	10	20	56	46.1-66.4	2	4	22	18.6-26.7	25.30
6	20	40	74	55.2-89.9	2	4	34	22.3-45.2	22.60
7	20	40	69	56.8-78.3	4	8	32	19.8-55.3	23.05
8	20	40	89	75.4-96.2	2	4	37	32.5-41.2	21.93
9	20	40	72	55.9-99.3	4	8	35	30.6-44.4	22.50
10	20	40	88	70.2-98.6	2	4	24	19.8-29.6	24.85
11	20	40	89	65.3-97.2	4	8	33	28.7-35.6	22.86
12	40	80	>100	na*	2	4	26	20.3-30.2	24.40
13	20	40	80	66.3-95.1	4	8	42	30.1-49.7	20.80
14	40	80	>100	na*	4	8	31	22.5-53.1	23.28
15	20	40	78	45.9-89.7	4	8	28	19.5-39.7	23.85
16	20	40	76	65.2-89.2	4	8	33	18.7-39.7	22.86
17	10	20	36	19.5-56.3	1	2	15	10.3-21.6	23.38
18	10	20	34	30.5-42.6	2	4	21	11.3-32.5	25.53
19	5	10	28	21.8-36.5	4	8	15	11.3-19.5	23.38
20	20	40	50	33.3-72.5	2	4	29	22.1-36.5	23.70

(na* refers to not applicable)

Appendix 2.4

Table of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.1.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	5	10	38	30.1-41.2	1	2	18	15.3-20.4	26.20
2	5	10	49	41.2-55.8	5	10	27	23.6-30.2	24.18
3	5	10	56	40.7-59.6	5	10	24	22.8-27.6	24.85
4	5	10	38	30.1-44.5	5	10	22	15.3-28.4	25.30
5	5	10	42	38.2-48.4	5	10	17	12.2-33.6	26.43
6	5	10	39	31.2-48.7	5	10	25	21.6-31.2	24.63
7	5	10	45	26.5-55.4	10	20	31	19.8-39.7	23.28
8	10	20	66	54.2-75.4	5	10	29	22.1-39.8	23.73
9	2	4	15	10.2-23.6	2	4	13	10.3-15.4	27.30
10	10	20	76	70.9-81.2	5	10	28	22.3-30.2	23.85
11	10	20	55	50.2-61.2	5	10	26	24.1-29.7	24.40
12	5	10	31	15.4-56.3	5	10	25	15.8-33.3	24.63
13	10	20	82	75.8-99.6	5	10	23	15.4-29.4	25.08
14	5	10	35	30.1-45.8	5	10	14	10.2-18.5	28.50
15	5	10	34	26.5-55.4	2	4	12	10.8-15.6	28.75
16	5	10	30	25.6-41.6	5	10	22	21.0-24.3	25.30
17	1	2	6	5.20-7.60	1	2	5	4.20-6.91	29.13
18	2	4	12	10.3-15.6	5	10	28	22.6-31.6	23.85
19	5	10	29	20.1-33.6	2	4	11	10.0-12.6	28.88
20	10	20	33	29.9-35.4	5	10	31	26.9-44.4	23.28

Appendix 2.5

Table of test NOEC, LOEC and EC_{50} values for *Phyllospora comosa* salinity reduction germination bioassays based on data presented in Section 3.2.1.

Test number	NOEC (ppt)	LOEC (ppt)	EC_{50} (ppt)	95% C.I.
1	22	20	18	19.2-16.1
2	26	24	22	23.4-21.5
3	26	24	22	24.3-20.9
4	26	24	23	26.4-22.1
5	28	26	24	26.8-22.4
6	26	24	19	19.8-17.7
7	24	22	18	18.9-15.2
8	24	22	21	22.3-19.8
9	26	24	22	24.6-21.1
10	20	18	15	16.5-14.2
11	22	20	18	19.9-15.6
12	22	20	19	22.6-16.4
13	26	24	22	20.3-18.7
14	22	20	19	21.5-15.9
15	22	20	18	20.3-17.0
16	20	18	15	16.9-11.2
17	22	20	19	21.3-18.1
18	24	22	20	22.5-18.4
19	20	18	17	19.8-15.2
20	22	20	18	22.5-16.4

Appendix 2.6

Table of test NOEC, LOEC and EC_{50} values for *Hormosira banksii* copper and chromium germination bioassays based on data presented in Section 3.2.2.

Test number	copper				chromium			
	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.
a	0.02	0.04	0.18	0.15-0.24	11.25	22.50	64.2	55.4-70.2
b	0.05	0.10	0.19	0.11-0.27	11.25	22.50	74.4	68.9-79.8
c	0.05	0.10	0.22	0.18-0.30	11.25	22.50	68.9	60.3-75.4
d	0.05	0.10	0.21	0.15-0.24	22.50	45.00	74.4	55.7-81.2
e	0.02	0.04	0.12	0.08-0.17	11.25	22.50	62.5	57.3-71.9
1	0.02	0.04	0.14	0.10-0.19	5.60	11.25	63.2	54.2-68.7
2	0.02	0.04	0.10	0.07-0.13	5.60	11.25	48.2	40.3-57.3
3	0.05	0.10	0.19	0.16-0.27	5.60	11.25	54.1	50.1-58.7
4	0.02	0.04	0.11	0.09-0.16	11.25	22.50	63.7	59.2-69.9
5	0.02	0.04	0.09	0.08-0.11	11.25	22.50	66.9	61.3-70.2
6	0.09	0.18	0.20	0.17-0.36	11.25	22.50	75.8	55.2-81.2
7	0.09	0.18	0.25	0.20-0.34	22.50	45.00	69.4	72.5-88.7
8	0.02	0.04	0.12	0.09-0.19	5.63	11.25	54.8	50.2-61.8
9	0.09	0.18	0.23	0.16-0.31	5.63	11.25	41.2	30.1-49.8
10	0.02	0.04	0.22	0.18-0.29	22.50	45.00	91.4	85.1-99.3
11	0.05	0.10	0.12	0.09-0.15	22.50	45.00	98.5	82.5-101.5
12	0.05	0.10	0.11	0.08-0.27	11.25	22.50	77.6	55.9-87.2
13	0.09	0.18	0.25	0.21-0.37	11.25	22.50	78.5	52.1-98.7
14	0.05	0.10	0.17	0.14-0.21	22.50	45.00	80.4	72.8-88.1
15	0.05	0.10	0.27	0.21-0.35	22.50	45.00	76.4	70.2-88.9
16	0.09	0.18	0.22	0.15-0.33	22.50	45.00	88.5	80.1-99.6
17	0.05	0.10	0.15	0.11-0.21	22.50	45.00	98.7	55.8-105.6
18	0.05	0.10	0.11	0.05-0.19	22.50	45.00	70	53.2-88.7
19	0.09	0.18	0.21	0.15-0.31	11.25	22.50	59	40.1-69.3
20	0.09	0.18	0.21	0.14-0.29	11.25	22.50	55	48.7-63.2

Appendix 2.7

Table of test NOEC, LOEC and EC_{50} values for *Hormosira banksii* primary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.2.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	2	4	14	11.5-18.2	2	4	13	11.9-16.3	28.63
2	2	4	12	9.63-15.8	2	4	11	9.71-15.0	28.88
3	2	4	9	6.21-11.3	2	4	9	6.00-11.2	29.13
4	2	4	10	7.83-13.2	1	2	8	6.21-12.4	29.25
5	1	2	6	5.14-9.57	1	2	6	4.91-8.99	29.50
6	3	3	12	10.2-15.8	3	6	12	9.80-15.6	28.75
7	2	4	7	5.50-9.80	2	4	6	1.10-9.80	29.50
8	1	2	14	11.6-15.9	3	6	8	5.40-8.90	29.25
9	1	2	3	1.10-5.61	1	2	3	1.50-5.60	30.00
10	3	6	12	8.90-12.3	3	6	10	9.70-15.6	29.00
11	3	6	14	10.2-16.9	3	6	13	9.70-16.4	28.63
12	3	6	17	15.4-18.7	3	6	13	10.2-22.6	28.63
13	4	8	15	10.2-19.8	4	8	14	10.5-15.9	28.50
14	1	2	8	2.30-11.5	1	2	7	5.80-15.4	29.38
15	3	6	13	5.60-22.4	1	2	5	2.50-8.90	29.63
16	2	4	11	5.40-20.1	2	4	10	5.60-15.7	29.00
17	1	2	4	1.10-8.90	1	2	4	2.30-6.70	29.75
18	1	2	4	1.50-6.50	1	2	3	2.60-9.71	30.00
19	2	4	9	5.50-12.3	2	4	7	1.10-6.70	29.38
20	4	8	13	11.5-15.9	4	8	11	3.50-15.3	28.88

Appendix 2.8

Table of test NOEC, LOEC and EC_{50} values for *Hormosira banksii* secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.2.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	20	40	71	55.5-81.4	3	6	32	30.5-35.2	23.05
2	20	40	60	50.1-74.3	3	6	28	25.9-32.4	23.85
3	20	40	75	69.8-82.5	4	8	33	29.8-38.5	22.86
4	10	20	58	50.1-65.8	3	6	24	20.2-27.4	24.85
5	10	20	60	55.3-63.2	3	6	29	22.3-34.3	23.70
6	25	50	78	55.6-89.9	3	6	30	22.3-45.8	23.50
7	25	50	70	55.4-94.2	4	8	25	19.8-45.6	24.63
8	25	50	98	69.3-99.9	4	8	28	15.8-31.2	23.85
9	20	40	78	56.2-88.7	6	12	29	11.5-42.5	23.70
10	20	40	60	55.4-98.2	6	12	27	14.7-38.7	24.18
11	20	40	77	65.4-88.4	4	8	39	19.8-55.6	21.48
12	40	80	>100	na*	6	12	34	15.4-67.4	22.60
13	40	80	>100	na*	6	12	33	22.1-41.7	22.86
14	25	50	>100	na*	4	8	33	25.9-55.6	22.86
15	20	40	56	40.1-78.4	2	4	11	5.60-18.9	28.88
16	20	40	55	32.5-89.8	2	4	17	10.2-29.5	26.43
17	10	20	49	32.1-66.3	4	8	22	9.80-31.5	25.30
18	10	20	48	29.8-55.4	2	4	18	11.9-22.6	26.20
19	20	40	55	45.8-72.1	3	6	18	14.8-23.6	26.20
20	20	40	54	41.3-66.7	3	6	26	20.1-36.9	24.40

(na* refers to not applicable)

Appendix 2.9

Table of test NOEC, LOEC andEC_{50} values for *Hormosira banksii* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.2.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	5	10	40	36.8-44.2	2	4	17	15.4-22.3	26.43
2	5	10	50	40.1-58.3	2	4	22	15.3-27.8	25.30
3	10	20	69	62.3-74.2	2	4	29	20.1-37.1	23.73
4	5	10	42	38.2-44.3	2	4	20	15.4-24.7	25.75
5	5	10	45	40.2-51.3	2	4	24	18.9-29.9	24.85
6	5	10	38	15.4-68.2	4	8	25	21.3-31.2	24.63
7	10	20	45	35.2-59.8	5	10	22	15.8-31.3	25.30
8	10	20	78	62.3-88.7	5	10	35	19.7-41.5	22.50
9	5	10	28	15.4-33.3	4	8	12	10.1-18.1	28.75
10	20	40	64	55.4-66.6	4	8	24	22.3-29.1	24.85
11	2	40	72	59.7-91.2	2	4	39	19.4-48.5	23.28
12	5	10	39	15.9-69.7	4	8	19	11.2-27.9	25.98
13	20	40	76	55.7-98.7	4	8	16	12.9-22.2	27.65
14	5	10	45	26.5-85.7	2	4	26	18.6-48.7	24.40
15	20	40	72	65.9-79.5	2	4	20	11.2-39.1	25.75
16	20	40	77	55.6-89.2	4	8	37	23.1-55.4	21.93
17	5	10	48	40.1-69.1	4	8	17	11.7-21.3	26.43
18	5	10	29	15.2-33.4	2	4	12	9.70-19.1	28.75
19	5	10	22	11.2-45.8	3	6	14	10.2-33.5	28.50
20	20	40	68	60.2-71.4	2	4	27	21.9-41.0	24.18

Appendix 2.10

Table of test NOEC, LOEC and EC_{50} values for *Hormosira banksii* salinity reduction germination bioassays based on data presented in Section 3.2.2.

Test number	NOEC (ppt)	LOEC (ppt)	EC_{50} (ppt)	95% C.I.
1	22	20	19	22.4-16.8
2	28	26	24	25.7-21.3
3	26	24	22	24.4-19.6
4	24	22	21	23.6-18.3
5	26	24	22	24.7-16.2
6	22	20	19	22.4-18.1
7	24	22	21	23.8-16.2
8	20	18	16	19.2-15.1
9	20	18	15	18.6-14.2
10	20	18	13	15.6-11.2
11	20	18	16	19.4-15.0
12	20	18	14	15.1-13.5
13	24	22	19	23.6-17.4
14	24	22	21	22.6-15.6
15	22	20	19	20.1-16.4
16	22	20	18	19.8-16.3
17	24	22	21	22.1-20.3
18	22	20	16	18.1-15.3
19	24	22	18	20.2-15.6
20	22	20	17	18.9-15.7

Appendix 2.11

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* copper germination and growth bioassays based on data presented in Section 3.2.3.

Test no.	germination				growth			
	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.
a	0.04	0.08	0.18	0.12-0.24	0.001	0.002	0.04	0.02-0.07
b	0.04	0.08	0.16	0.11-0.22	0.002	0.004	0.05	0.03-0.09
c	0.04	0.08	0.17	0.16-0.19	0.002	0.004	0.06	0.02-0.07
d	0.04	0.08	0.18	0.15-0.21	0.002	0.004	0.07	0.03-0.08
e	0.04	0.08	0.13	0.11-0.17	0.002	0.004	0.07	0.05-0.09
1	0.04	0.02	0.14	0.10-0.16	0.002	0.004	0.06	0.05-0.07
2	0.02	0.04	0.09	0.05-0.11	<0.001	0.001	0.02	0.01-0.03
3	0.02	0.04	0.12	0.10-0.15	0.0005	0.001	0.03	0.01-0.05
4	0.02	0.04	0.11	0.07-0.16	<0.0005	0.0005	0.01	0.09-0.03
5	0.02	0.04	0.10	0.06-0.16	0.0005	0.001	0.02	0.01-0.27
6	0.02	0.04	0.14	0.09-0.19	0.0005	0.001	0.02	0.01-0.04
7	0.02	0.04	0.10	0.08-0.13	0.001	0.002	0.03	0.02-0.05
8	0.04	0.08	0.17	0.15-0.20	0.001	0.002	0.05	0.04-0.07
9	0.03	0.06	0.17	0.15-0.19	0.001	0.002	0.04	0.02-0.08
10	0.03	0.06	0.17	0.15-0.18	0.002	0.004	0.06	0.02-0.07
11	0.06	0.12	0.18	0.11-0.21	0.002	0.004	0.07	0.05-0.09
12	0.03	0.06	0.15	0.12-0.19	0.002	0.004	0.05	0.04-0.06
13	0.03	0.06	0.16	0.14-0.18	0.002	0.004	0.06	0.04-0.07
14	0.04	0.08	0.15	0.11-0.18	0.002	0.004	0.07	0.05-0.08
15	0.04	0.08	0.14	0.10-0.21	0.002	0.004	0.06	0.05-0.07
16	0.04	0.08	0.13	0.09-0.18	0.002	0.004	0.07	0.04-0.09
17	0.04	0.08	0.15	0.12-0.21	0.002	0.004	0.07	0.03-0.09
18	0.03	0.06	0.16	0.10-0.19	0.001	0.002	0.04	0.02-0.08
19	0.04	0.08	0.17	0.12-0.21	0.002	0.004	0.06	0.05-0.09
20	0.04	0.08	0.12	0.11-0.13	0.002	0.004	0.05	0.02-0.08

Appendix 2.12

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* chromium germination and growth bioassays based on data presented in Section 3.2.3.

Test number	germination				growth			
	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)	95% C.I.
a	11.3	22.5	66.3	58.2-70.1	5.65	11.3	55.4	52.1-60.4
b	22.5	45.0	72.1	62.3-81.4	11.3	22.5	62.7	58.4-70.2
c	11.3	22.5	68.4	60.2-78.2	5.65	11.3	57.7	55.3-61.4
d	11.3	22.5	60.3	51.4-70.3	5.65	11.3	49.2	41.1-58.2
e	11.3	22.5	59.4	52.1-64.8	5.65	11.3	44.1	38.2-49.2
1	5.65	11.3	58.3	48.2-61.3	5.65	11.3	44.0	40.2-48.7
2	5.65	11.3	49.7	42.3-58.7	2.82	5.65	26.3	22.3-29.6
3	5.65	11.3	56.3	52.4-61.4	2.82	5.65	25.8	19.4-29.4
4	5.65	11.3	51.2	48.2-55.3	2.82	5.65	29.6	20.1-36.3
5	5.65	11.3	53.4	49.4-58.3	2.82	5.65	22.3	15.3-38.1
6	11.3	22.5	66.8	60.2-88.9	2.82	5.65	27.8	18.2-41.4
7	5.65	11.3	62.3	55.3-70.1	5.65	11.3	26.7	19.8-39.9
8	11.3	22.5	66.5	55.4-97.3	2.82	5.65	33.9	25.2-40.1
9	22.5	45.0	78.2	65.2-86.1	2.82	5.65	41.2	38.7-55.6
10	22.5	45.0	71.4	61.2-79.3	5.65	11.3	48.1	31.2-60.1
11	22.5	45.0	59.6	48.7-65.4	5.65	11.3	41.3	30.1-58.6
12	22.5	45.0	72.4	68.4-79.8	11.3	22.5	48.5	42.1-52.3
13	22.5	45.0	73.2	66.6-87.1	11.3	22.5	49.5	40.1-58.3
14	22.5	45.0	90.4	88.5-105	11.3	22.5	51.4	47.3-55.6
15	22.5	45.0	81.3	75.3-89.8	5.65	11.3	45.6	41.2-52.4
16	11.3	22.5	74.2	55.7-89.8	5.65	11.3	44.2	36.1-50.1
17	11.3	22.5	62.4	55.3-69.1	5.65	11.3	39.1	32.3-44.3
18	15.0	30.0	64.8	60.2-71.9	2.82	5.65	37.8	30.1-48.7
19	22.5	45.0	62.9	55.9-74.3	5.65	11.3	41.1	35.3-44.2
20	22.5	45.0	70.0	56.5-77.7	5.65	11.3	48.3	41.4-56.4

Appendix 2.13

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* primary treated effluent (modified and unmodified corresponding salinity) germination bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	2	4	11	9.80-15.2	2	4	12	10.1-15.3	28.75
2	2	4	9	8.77-10.9	2	4	8	6.81-10.2	29.25
3	2	4	5	4.31-6.12	1	2	5	4.19-6.37	29.63
4	1	2	4	3.22-4.82	1	2	5	4.087.11	29.63
5	1	2	4	3.01-5.12	1	2	4	3.08-5.60	29.75
6	1	2	2	1.11-4.21	1	2	3	1.59-5.66	30.00
7	1	2	4	3.12-4.97	1	2	4	3.02-5.21	29.75
8	2	4	4	5.10-7.81	2	4	6	5.17-6.98	29.50
9	2	4	4	8.21-9.97	2	4	7	5.41-8.18	29.38
10	2	4	6	4.90-7.22	2	4	5	4.10-6.85	29.63
11	2	4	4	6.21-8.12	2	4	6	4.92-6.78	29.50
12	2	4	10	8.09-11.2	2	4	11	9.21-12.3	28.88
13	4	8	10	9.31-10.8	2	4	9	7.31-10.2	29.13
14	2	4	8	7.72-8.41	1	2	4	3.22-4.67	29.75
15	2	4	9	8.01-12.1	2	4	8	7.09-8.67	29.25
16	1	2	3	1.20-4.68	1	2	3	2.21-3.54	30.00
17	1	2	3	1.53-5.24	1	2	3	1.29-4.82	30.00
18	1	2	3	1.11-3.95	1	2	3	1.12-4.19	30.00
19	1	2	3	1.02-4.22	1	2	3	1.65-3.55	30.00
20	2	4	6	5.21-7.10	2	4	5	4.30-5.91	29.63

Appendix 2.14

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* primary treated effluent (modified and unmodified and corresponding salinity) germination tube growth bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	2	4	9	7.20-10.1	2	4	10	8.82-12.3	29.00
2	2	4	6	5.10-6.90	2	4	5	4.22-5.84	29.63
3	1	2	4	3.20-4.80	0.5	1	2	1.92-2.57	31.00
4	1	2	4	3.00-4.70	1	2	4	2.22-4.98	29.75
5	1	2	3	1.50-4.10	1	2	3	1.51-4.12	30.00
6	1	2	2	1.50-2.21	1	2	2	1.88-2.37	31.00
7	1	2	4	1.92-4.35	1	2	4	1.94-4.71	29.75
8	2	4	5	4.02-5.62	2	4	4	1.82-4.11	29.75
9	2	4	6	5.24-7.18	2	4	5	3.72-5.62	29.63
10	2	4	5	4.55-5.96	2	4	5	4.82-5.37	29.63
11	2	4	6	5.22-6.87	2	4	5	4.59-5.60	29.63
12	1	2	6	4.95-6.82	1	2	4	3.59-4.91	29.75
13	4	8	9	6.00-9.97	1	2	6	4.09-7.55	29.50
14	2	4	5	4.81-5.51	0.5	1	5	4.08-8.22	29.63
15	2	4	8	7.31-8.74	2	4	6	5.12-6.98	29.50
16	1	2	3	1.54-4.08	1	2	3	1.58-4.83	30.00
17	1	2	3	2.19-3.78	1	2	3	1.21-4.84	30.00
18	0.5	1	2	1.64-2.81	1	2	3	2.74-3.86	30.00
19	1	2	3	1.08-3.55	1	2	3	1.99-3.61	30.00
20	2	4	5	4.22-5.67	2	4	4	2.28-6.45	29.75

Appendix 2.15

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	20	40	80	75.1-86.3	10	20	44	40.1-52.3	20.34
2	40	80	>100	na*	10	20	38	30.7-45.6	21.69
3	20	40	75	70.2-84.7	10	20	40	35.8-42.4	21.26
4	20	40	80	71.3-90.6	5	10	29	26.3-33.7	23.70
5	20	40	87	85.7-91.3	10	20	36	30.8-44.6	22.16
6	25	50	88	80.2-90.1	10	20	43	33.3-49.2	21.03
7	10	20	80	75.6-84.7	10	20	38	32.3-45.8	21.69
8	25	50	>100	na*	10	20	31	25.6-37.8	23.28
9	25	50	>100	na*	4	8	27	19.8-33.6	24.08
10	40	80	>100	na*	10	20	32	28.7-36.9	23.05
11	40	80	>100	na*	8	16	28	20.0-35.6	23.85
12	20	40	>100	na*	10	20	51	45.2-55.9	18.64
13	20	40	>100	na*	5	10	33	25.6-37.2	22.86
14	20	40	>100	na*	5	10	41	30.2-49.8	21.03
15	20	40	98	90.1-105	6	12	31	28.6-39.9	23.28
16	20	40	97	95.2-99.9	10	20	32	26.3-37.9	23.05
17	20	40	82	78.3-87.1	5	10	27	22.2-38.7	24.08
18	10	20	80	78.2-95.2	8	16	28	20.1-33.6	23.85
19	40	80	95	90.2-98.9	4	8	29	21.5-6.9	23.61
20	20	40	>100	na*	6	12	38	29.7-44.5	21.69

Appendix 2.16

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* secondary treated effluent (modified and unmodified and corresponding salinity) germination tube growth bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	20	40	61	56.8-67.2	8	16	31	28.7-35.6	23.28
2	20	40	81	78.3-87.1	8	16	29	25.6-30.2	23.70
3	5	10	58	50.1-68.3	4	8	21	18.7-27.7	25.53
4	10	2	68	65.7-70.2	4	8	18	16.3-22.4	26.20
5	10	20	62	60.1-75.3	4	8	22	15.3-30.1	25.30
6	10	20	63	58.6-69.7	4	8	22	18.7-25.6	25.30
7	20	40	70	61.2-78.3	8	16	31	25.2-36.1	23.28
8	40	80	93	89.8-99.6	8	16	25	21.7-30.1	24.63
9	40	80	94	90.1-98.7	4	8	20	17.3-24.6	25.75
10	40	80	95	89.3-98.2	4	8	22	20.1-25.7	25.30
11	20	40	61	56.2-69.8	4	8	18	15.6-30.2	26.20
12	40	80	91	81.4-99.6	4	8	19	14.7-21.3	25.98
13	20	40	89	81.7-93.2	8	16	29	27.6-32.3	23.70
14	20	40	83	78.6-84.3	8	16	24	20.2-29.8	24.85
15	20	40	81	77.3-85.7	4	8	16	11.6-27.1	27.65
16	20	40	80	75.6-84.9	4	8	18	12.3-22.4	26.20
17	5	10	72	68.2-75.6	4	8	17	13.6-18.7	26.43
18	10	20	60	56.2-68.1	4	8	21	19.2-23.4	25.53
19	10	20	71	60.3-77.7	4	8	15	14.6-18.7	28.73
20	20	40	94	91.4-98.7	4	8	21	18.4-26.4	25.53

Appendix 2.17

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) germination bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	20	40	88	80.0-94.3	6	12	32	22.8-41.2	2.05
2	20	40	80	75.6-88.4	12	24	48	40.1-37.8	19.41
3	20	40	92	80.7-100	10	20	39	30.1-42.3	21.48
4	20	40	87	78.2-99.6	12	24	41	35.7-55.3	21.03
5	10	20	72	65.3-79.4	6	12	38	30.2-48.1	21.72
6	40	80	98	95.3-99.9	12	24	48	40.2-59.9	19.41
7	10	20	76	90.2-96.3	12	24	44	40.1-50.6	20.33
8	20	40	93	70.1-88.6	4	8	31	22.2-41.2	23.28
9	20	40	82	77.5-95.2	4	8	29	20.0-41.2	23.70
10	10	20	50	40.2-58.3	5	10	40	38.7-44.3	21.26
11	40	80	98	92.3-99.9	5	10	39	30.2-45.8	21.49
12	10	20	75	72.1-79.8	4	8	27	20.1-36.7	24.08
13	10	20	53	48.9-66.6	5	10	34	29.5-39.9	22.60
14	20	40	64	52.3-70.0	4	8	22	15.8-35.6	25.30
15	10	20	82	75.4-88.6	6	12	31	28.4-33.2	23.28
16	40	80	97	90.0-99.9	6	12	37	15.7-55.5	21.95
17	10	20	70	61.3-79.3	5	10	22	16.4-24.3	25.30
18	25	50	65	60.2-74.2	12	24	49	40.0-59.8	19.18
19	25	50	63	56.2-78.0	4	8	28	20.1-36.2	23.85
20	20	40	90	85.4-96.3	6	12	35	30.0-45.5	22.50

Appendix 2.18

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) germination tube growth bioassays based on data presented in Section 3.2.3.

Test no.	modified				unmodified				Salinity (ppt) at EC_{50}
	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	EC_{50} (%)	95% C.I.	
1	12	24	55	50.2-61.3	4	8	22	18.1-25.7	25.30
2	6	12	48	41.4-58.2	4	8	24	20.2-28.3	24.85
3	6	12	49	40.1-55.7	4	8	29	21.6-37.2	23.73
4	12	24	57	49.3-60.2	8	16	38	28.6-47.2	21.72
5	6	12	32	24.6-41.8	4	8	27	18.3-38.1	24.18
6	6	12	41	35.7-44.3	4	8	39	31.2-43.6	21.48
7	6	12	38	34.4-48.6	4	8	38	30.2-42.1	21.72
8	6	12	41	22.7-29.1	4	8	22	18.6-25.7	25.30
9	6	12	40	38.6-44.6	4	8	20	15.6-26.1	25.75
10	3	6	26	22.7-29.1	2	4	35	31.2-38.4	22.50
11	6	12	42	38.6-44.6	4	8	22	18.6-27.3	25.30
12	6	12	40	35.2-48.6	4	8	21	17.3-26.1	25.53
13	3	6	22	18.2-24.3	2	4	25	20.2-28.6	24.63
14	6	12	29	20.3-38.7	2	4	15	11.3-18.5	28.73
15	6	12	31	25.6-36.4	4	8	20	18.4-28.3	25.75
16	3	6	41	34.3-49.8	2	4	24	21.2-26.1	24.85
17	6	12	38	30.2-42.3	2	4	16	15.3-18.7	27.65
18	6	12	29	20.1-33.6	4	8	29	22.3-41.6	23.73
19	6	12	31	25.2-38.1	2	4	20	16.2-24.3	25.75
20	6	12	38	30.1-44.6	4	8	28	22.1-38.6	2.85

Appendix 2.19

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* salinity reduction germination bioassays based on data presented in Section 3.2.3.

Test number	NOEC (ppt)	LOEC (ppt)	EC_{50} (ppt)	95% C.I.
1	24	22	18	22.7-17.3
2	24	22	22	26.4-20.1
3	24	22	22	24.3-18.2
4	24	22	20	21.7-19.1
5	24	22	19	21.2-16.3
6	20	18	17	20.0-16.2
7	20	18	16	18.4-15.1
8	22	20	18	19.8-17.5
9	22	20	18	20.3-16.5
10	22	20	17	19.3-15.7
11	22	20	16	17.9-13.2
12	24	22	19	21.6-17.3
13	24	22	21	22.9-18.4
14	24	22	19	21.6-16.9
15	20	18	16	18.2-15.1
16	22	20	16	17.9-15.5
17	20	18	16	17.8-15.0
18	22	20	16	18.9-14.6
19	18	16	14	15.1-13.2
20	22	20	18	19.5-16.2

Appendix 2.20

Table of test NOEC, LOEC and EC_{50} values for *Macrocystis angustifolia* salinity reduction germination tube growth bioassays based on data presented in Section 3.2.3.

Test number	NOEC (ppt)	LOEC (ppt)	EC_{50} (ppt)	95% C.I.
1	28	26	24	26.7-22.1
2	26	24	22	24.3-18.2
3	26	24	22	22.9-20.7
4	28	26	24	25.6-20.2
5	24	22	20	24.3-16.1
6	22	20	18	20.2-17.0
7	22	20	18	20.0-16.3
8	24	22	20	21.7-19.1
9	20	18	17	20.1-16.0
10	22	20	18	20.3-17.1
11	22	20	18	19.4-16.1
12	24	22	20	22.4-18.7
13	24	22	21	21.9-20.1
14	24	22	20	22.7-18.3
15	22	20	17	18.6-16.5
16	22	20	18	19.2-17.2
17	22	20	18	19.0-17.1
18	22	20	18	19.5-16.3
19	20	18	16	18.2-14.1
20	24	22	20	21.3-16.1

Appendix 3

Tables of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* and *Hormosira banksii* mortality bioassays.

Appendix 3.1

Table of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* copper mortality bioassays based on data presented in section 4.2.1.

Test number	Copper			
	NOEC (mg/L)	LOEC (mg/L)	LC_{50} (mg/L)	95% C.I.
a	0.20	0.40	0.55	0.42-0.66
b	0.10	0.20	0.59	0.45-0.71
c	0.20	0.40	0.63	0.50-0.74
d	0.20	0.40	0.86	0.73-0.96
e	0.10	0.20	0.62	0.50-0.73
1	0.10	0.20	0.69	0.56-0.84
2	0.20	0.40	0.86	0.80-1.01
3	0.05	0.10	0.66	0.62-0.79
4	0.10	0.20	0.91	0.89-1.05
5	0.10	0.20	0.56	0.51-0.68
6	0.10	0.20	0.81	0.80-0.94
7	0.05	0.10	0.60	0.55-0.70
8	0.05	0.10	0.33	0.31-0.45
9	0.10	0.20	0.69	0.65-0.80
10	0.05	0.10	0.73	0.70-0.84
11	0.10	0.20	0.76	0.71-0.87
12	0.10	0.20	0.57	0.51-0.68
13	0.40	0.80	0.92	0.89-1.24
14	0.10	0.20	0.66	0.63-0.79
15	0.10	0.20	0.85	0.84-0.99
16	0.10	0.20	0.68	0.61-0.82
17	0.05	0.10	0.40	0.32-0.51
18	0.10	0.20	0.62	0.55-0.78
19	0.05	0.10	0.39	0.32-0.51
20	0.05	0.10	0.57	0.50-0.73

Appendix 3.2

Table of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* primary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.1.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	4	8	27	16.3-26.1	2	4	18	9.11-28.2	26.20
2	4	8	22	7.55-35.2	4	8	16	9.25-27.1	27.65
3	2	4	9	0.00-13.2	1	2	8	4.55-13.6	29.75
4	2	4	11	2.25-17.8	1	2	10	5.59-16.8	29.00
5	2	4	13	8.25-17.4	1	2	11	6.47-15.2	28.88
6	4	8	23	11.6-31.4	2	4	15	5.63-23.5	28.38
7	2	4	9	3.25-11.4	1	2	7	4.11-9.25	29.38
8	4	8	20	6.36-30.2	2	4	18	6.58-28.5	26.20
9	2	4	13	7.77-18.4	1	2	11	5.25-15.4	28.88
10	2	4	11	4.45-16.5	1	2	9	2.25-14.8	29.13
11	4	8	19	9.98-25.5	2	4	16	6.23-22.9	27.65
12	4	8	26	17.4-31.5	4	8	18	9.59-23.5	26.20
13	2	4	14	9.99-17.2	1	2	12	7.14-15.2	28.75
14	4	8	25	22.5-33.6	2	4	19	10.2-26.3	25.98
15	2	4	12	3.23-17.8	1	2	10	5.45-15.8	29.00
16	4	8	26	21.5-33.1	2	4	19	9.89-25.8	25.98
17	1	2	5	3.37-9.78	0.5	1	4	3.36-6.59	29.75
18	1	2	7	4.45-13.2	0.5	1	6	3.15-9.87	29.50
19	1	2	6	5.59-13.5	0.5	1	5	4.48-9.25	29.63
20	4	8	21	19.9-24.3	2	4	16	4.59-27.2	27.65

Appendix 3.3

Table of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* secondary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.1.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	10	20	66	51.5-93.2	10	20	31	24.1-41.5	23.28
2	10	20	42	24.9-65.8	5	10	20	15.7-31.3	25.75
3	10	20	51	29.8-60.0	5	10	25	19.9-30.2	24.63
4	10	20	48	33.6-65.8	5	10	23	18.8-30.0	25.08
5	10	20	42	30.1-56.7	5	10	22	17.1-26.2	25.30
6	20	40	55	49.0-83.2	5	10	16	10.6-24.4	27.65
7	10	20	38	20.7-63.2	5	10	19	16.5-21.0	25.98
8	20	40	61	50.2-83.1	10	20	32	21.6-42.5	23.05
9	10	20	49	37.9-54.8	5	10	20	13.2-24.5	25.75
10	20	40	59	45.6-64.2	10	20	25	16.3-30.2	24.63
11	20	40	53	41.1-71.5	10	20	26	18.8-32.1	24.40
12	10	20	72	61.5-86.8	10	20	37	28.2-42.2	21.93
13	10	20	58	27.7-77.4	10	20	29	24.4-38.9	23.70
14	20	40	70	51.2-87.2	10	20	34	28.4-41.1	22.60
15	10	20	55	75.1-73.9	5	10	22	17.5-30.6	25.30
16	10	20	48	26.5-64.8	5	10	23	15.5-29.8	25.08
17	5	10	29	14.1-51.2	5	10	17	13.1-19.8	26.43
18	5	10	22	11.3-43.4	2.5	5	12	9.25-15.4	28.75
19	5	10	19	7.28-33.3	2.5	5	10	8.45-14.5	29.00
20	10	20	25	15.7-40.9	2.5	5	11	8.76-16.4	28.88

Appendix 3.4

Table of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.1.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	5	10	11	5.12-17.2	10	20	40	18.9-62.3	21.25
2	2.5	5	10	5.45-19.8	5	10	21	6.56-30.2	25.53
3	5	10	12	6.36-17.4	5	10	24	10.9-35.9	24.85
4	5	10	14	9.99-22.5	5	10	26	16.5-34.2	24.40
5	2.5	5	9	4.14-13.2	5	10	20	11.1-24.9	25.75
6	2.5	5	8	2.25-16.3	5	10	22	11.5-30.2	25.30
7	2.5	5	11	7.45-13.2	5	10	15	7.77-20.0	28.38
8	5	10	21	7.89-31.2	10	20	32	13.1-46.9	23.05
9	2.5	5	10	3.25-14.5	5	10	24	13.2-32.5	24.85
10	2.5	5	11	6.66-20.0	5	10	20	10.1-29.9	25.75
11	2.5	5	12	4.56-19.8	5	10	19	10.2-26.8	25.98
12	5	10	14	5.55-19.8	10	20	25	17.4-30.5	24.63
13	2.5	5	15	8.45-24.6	5	10	18	10.5-27.0	26.20
14	5	10	15	9.56-22.3	5	10	29	17.8-36.9	23.70
15	2.5	5	10	5.55-18.2	5	10	22	15.1-30.5	25.30
16	2.5	5	11	7.17-17.4	5	10	20	8.38-26.2	25.75
17	2.5	5	9	5.66-11.2	2.5	5	11	7.58-13.2	28.88
18	2.5	5	8	5.48-11.2	2.5	5	10	7.14-13.6	29.00
19	2.5	5	8	6.26-12.5	2.5	5	12	8.23-18.7	28.75
20	2.5	5	9	6.12-14.8	5	10	14	11.1-19.4	28.50

Appendix 3.5

Table of test NOEC, LOEC and LC_{50} values for *Phyllospora comosa* salinity reduction mortality bioassays based on data presented in Section 4.2.1.

Test number	NOEC (ppt)	LOEC (ppt)	LC_{50} (ppt)	95% C.I.
1	24	22	20	18.5-24.5
2	28	26	25	21.2-34.5
3	28	26	24	17.8-29.9
4	30	28	27	24.5-34.5
5	28	26	25	20.6-29.8
6	28	26	22	14.1-30.0
7	26	24	22	20.5-26.5
8	28	26	24	20.0-33.2
9	28	26	25	19.8-33.6
10	24	22	20	18.4-26.5
11	26	24	22	20.8-24.6
12	22	20	20	17.8-25.6
13	28	26	24	20.5-29.6
14	24	22	21	16.6-24.5
15	24	22	19	15.6-26.4
16	24	22	18	15.4-24.1
17	24	22	20	19.6-22.8
18	28	26	21	19.7-24.6
19	24	22	18	14.6-24.6
20	24	22	21	18.5-26.9

Appendix 3.6

Table of test NOEC, LOEC and LC_{50} values for *Hormosira banksii* copper mortality bioassays based on data presented in Section 4.2.2.

Test number	Copper			
	NOEC (mg/L)	LOEC (mg/L)	LC_{50} (mg/L)	95% C.I.
a	0.25	0.50	0.92	0.72-1.05
b	0.50	1.00	1.42	1.04-1.57
c	0.50	1.00	2.28	2.16-2.48
d	0.25	0.50	0.99	0.75-1.14
e	0.25	0.50	0.85	0.70-0.94
1	0.25	0.50	1.70	1.53-1.62
2	0.50	1.00	1.15	0.99-1.22
3	0.50	1.00	1.25	1.10-1.39
4	0.50	1.00	1.20	1.19-1.35
5	1.00	2.00	1.52	0.87-1.68
6	0.50	1.00	1.82	1.67-1.95
7	0.50	1.00	0.84	0.73-0.85
8	0.50	1.00	1.98	1.82-2.11
9	0.25	0.50	1.02	0.88-1.17
10	0.25	0.50	1.12	0.96-1.27
11	0.25	0.50	1.46	1.35-1.55
12	0.25	0.50	0.78	0.68-0.97
13	0.50	1.00	1.22	0.72-1.28
14	0.50	1.00	1.33	1.10-1.39
15	0.25	0.50	0.99	0.85-1.07
16	0.50	1.00	1.54	1.45-1.63
17	0.50	1.00	1.44	1.35-1.58
18	0.25	0.50	1.66	1.50-1.81
19	0.25	0.50	1.12	1.00-1.26
20	0.25	0.50	1.01	0.88-1.12

Appendix 3.7

Table of test NOEC, LOEC and LC_{50} values for *Hormosira banksii* primary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.2.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	8	16	24	19.6-26.5	4	8	21	11.1-39.0	25.53
2	8	16	23	19.8-24.5	4	8	22	10.2-41.2	25.30
3	4	8	19	6.66-15.2	2	4	10	19.5-42.3	29.00
4	4	8	19	7.82-17.4	4	8	12	18.8-49.6	28.75
5	8	16	21	10.0-18.5	4	8	15	22.2-49.8	28.38
6	4	8	20	7.98-25.3	2	4	17	20.0-41.1	26.43
7	2	4	7	7.99-13.1	1	2	11	2.22-28.4	28.88
8	8	16	24	14.2-28.1	4	8	19	10.0-25.6	25.98
9	2	4	17	11.3-22.2	4	8	18	8.89-35.6	26.20
10	4	8	16	9.98-20.0	2	4	15	16.1-45.1	28.38
11	8	16	22	12.5-21.3	4	8	15	11.6-27.4	28.38
12	8	16	25	17.1-26.3	4	8	21	11.1-26.3	25.53
13	4	8	18	13.1-17.4	2	4	15	13.3-44.4	28.38
14	8	16	26	18.5-27.9	4	8	24	18.8-49.2	24.85
15	4	8	16	7.78-17.1	2	4	12	26.3-58.9	28.75
16	4	8	25	3.33-7.25	2	4	6	10.0-35.2	29.50
17	2	4	8	2.25-7.36	1	2	6	1.11-13.2	29.50
18	4	8	10	6.12-12.2	2	4	9	1.15-15.8	29.13
19	4	8	11	10.2-15.9	2	4	11	3.29-17.4	28.88
20	8	16	23	5.59-16.3	4	8	10	12.5-33.2	29.00

Appendix 3.8

Table of test NOEC, LOEC and LC_{50} values for *Hormosira banksii* secondary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.2.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	5	10	26	18.1-36.2	2.5	5	26	11.5-38.9	24.40
2	10	20	28	20.0-36.2	5	10	25	10.0-41.2	24.63
3	10	20	33	22.5-38.7	2.5	5	29	18.9-42.1	23.70
4	10	20	32	18.8-40.1	5	10	30	17.8-49.6	23.50
5	10	20	35	21.5-46.6	2.5	5	31	22.2-49.8	23.28
6	5	10	27	21.1-35.6	2.5	5	27	21.5-42.3	24.18
7	5	10	22	12.3-28.7	2.5	5	18	3.25-28.9	26.20
8	5	10	21	12.5-29.8	2.5	5	19	10.2-25.6	25.98
9	5	10	20	13.2-24.3	2.5	5	20	8.88-35.2	25.75
10	10	20	30	18.7-33.5	5	10	27	16.4-46.3	24.18
11	5	10	18	8.15-21.6	2.5	5	15	11.2-26.5	28.38
12	5	10	16	8.59-19.7	5	10	22	11.5-24.9	25.30
13	5	10	25	11.2-31.9	2.5	5	22	13.3-44.4	25.30
14	10	20	32	16.5-41.9	5	10	32	18.5-49.7	23.05
15	10	20	39	29.5-49.7	5	10	39	16.4-35.4	21.48
16	5	10	25	18.5-31.2	2.5	5	25	10.9-35.4	24.63
17	10	20	28	20.3-32.6	5	10	24	18.8-39.6	24.85
18	10	20	24	12.6-31.6	2.5	5	14	12.3-45.8	28.50
19	5	10	19	14.9-18.5	2.5	5	15	11.2-28.5	28.38
20	5	10	15	8.15-16.5	2.5	5	11	5.26-22.8	28.88

Appendix 3.9

Table of test NOEC, LOEC and LC_{50} values for *Homosira banksii* chlorinated secondary treated effluent (modified and unmodified and corresponding salinity) mortality bioassays based on data presented in Section 4.2.2.

Test no.	modified				unmodified				Salinity (ppt) at LC_{50}
	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	NOEC (%)	LOEC (%)	LC_{50} (%)	95% C.I.	
1	10	20	40	31.2-49.8	5	10	38	18.5-58.3	21.70
2	10	20	49	19.9-43.2	5	10	34	36.3-58.7	22.60
3	10	20	55	19.8-46.5	5	10	35	42.1-76.3	22.50
4	20	40	59	31.2-45.0	5	10	36	50.2-77.4	22.27
5	10	20	38	11.1-20.3	2.5	5	16	30.6-51.2	27.65
6	10	20	43	12.8-28.6	2.5	5	19	34.0-62.3	25.98
7	5	10	20	12.5-25.6	2.5	5	17	10.2-14.8	26.43
8	20	40	38	18.4-42.2	10	20	32	19.5-51.2	23.05
9	10	20	31	10.0-22.3	5	10	17	20.1-38.4	26.43
10	10	20	41	13.2-39.6	5	10	29	31.2-60.5	23.70
11	10	20	51	7.48-22.5	5	10	15	42.6-69.7	28.38
12	10	20	48	9.56-30.6	2.5	5	21	28.4-62.5	25.53
13	20	40	53	30.2-48.5	5	10	37	36.2-62.1	21.93
14	5	10	22	12.6-25.4	2.5	5	18	10.2-30.2	26.20
15	20	40	67	17.9-40.2	5	10	31	58.6-84.7	23.28
16	10	20	38	24.6-48.7	5	10	32	28.6-44.4	23.05
17	10	20	42	23.3-49.6	5	10	37	36.2-52.4	21.93
18	10	20	59	21.6-51.2	5	10	38	47.9-73.1	21.70
19	20	40	64	15.2-38.6	5	10	27	50.3-80.7	24.18
20	10	20	59	9.85-23.6	5	10	18	47.3-69.1	26.20

Appendix 3.10

Table of test NOEC, LOEC and LC_{50} values for *Hormosira banksii* salinity reduction mortality bioassays based on data presented in Section 4.2.2.

Test number	NOEC (ppt)	LOEC (ppt)	LC_{50} (ppt)	95% C.I.
1	22	20	15	10.2-19.8
2	22	20	18	14.5-27.8
3	20	18	12	4.45-17.4
4	22	20	17	10.2-23.9
5	26	24	20	15.2-24.9
6	22	20	14	5.23-24.1
7	24	22	18	13.3-23.9
8	24	22	16	13.2-24.7
9	24	22	16	8.79-24.9
10	24	22	19	14.3-26.5
11	22	20	18	13.2-20.9
12	22	20	14	11.1-16.5
13	24	22	17	11.9-22.3
14	24	22	19	14.2-26.5
15	22	20	17	12.3-25.3
16	22	20	15	11.9-21.8
17	22	20	16	12.7-17.4
18	22	20	17	12.6-20.3
19	24	22	18	14.9-24.6
20	26	24	20	17.7-25.4