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THE USE OF BIOCIDAL AGENTS AS POTENTIAL CONTROL MECHANISMS FOR THE EXOTIC KELP *UNDARIA PINNATIFIDA*

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SUMMARY

To assess the potential use of chemical agents as a control mechanism for the marine macroalga *Undaria pinnatifida,* toxicity assays were conducted using five biocide/algicide agents. Three terrestrial herbicides, Diuron, Simazine and Glyphosate, a commercial antifoulant, Sea nine 211™ and a red algal extract, Furanone 281 were assayed for effect on zoospore germination and gametophyte mortality of *U. pinnatifida* and the closely related *Ecklonia radiata*. Germination inhibition and gametophyte mortality were employed as end points in screening bioassays and concentrations determined at which complete inhibition of germination occurred. The biocides which proved most effective were then assayed for effect on gametophyte mortality. The results suggest relative insensitivity for both algae to the three herbicides and maximum sensitivity for the antifoulant. For both species complete inhibition of germination of zoospores and mortality of gametophytes was achieved for concentrations of Sea nine 211™ in excess of 1.6 mgl^{-1}. For Furanone 281 complete inhibition of zoospore germination, and gametophyte mortality occurred between 20 mgl^{-1} and 40 mgl^{-1}.

CONTENTS

1 INTRODUCTION

Undaria pinnatifida (Harvey) Suringer (Phaeophyta: Laminariales, Alariaceae) is a temperate, brown, macroalgae endemic to coastlines of Korea, Japan and China (Hay 1991; Yamanaka and Akiyama 1993; Oh and Koh 1996). Commonly known as "Japanese kelp", this large macrophyte forms dense stands in near shore sublittoral habitats and, depending on habitat, attains a length of 1–3 m (Hay 1991). *U. pinnatifida* also exhibits a seasonality in growth where proliferation of the large sporophyte phase of the plants life cycle is generally associated with colder climatic, and therefore colder water conditions (Saito 1975; Hay and Villouta 1993; Oh and Koh 1996; Campbell and Burridge in press).

U. pinnatifida has also become established in a number of locations outside of Japan and northern Asia and has been identified as an exotic pest species. Since 1971 *U. pinnatifida* has spread to France (Flo'ch *et al.* 1991, 1996; Castric-Fey *et al.* 1993), New Zealand (Hay 1990; Hay and Villouta 1993; Brown and Lamare 1994), Tasmania, (Australia) (Sanderson and Barrett 1989), England (Fletcher and Manfredi 1995), Argentina (Casa and Piriz 1996), and most recently to Port Phillip Bay, Victoria, Australia (Campbell and Burridge in press). Whilst the initial introduction into France is believed to have been inadvertent introduction with oyster spat (Flo'ch *et al.* 1991, 1996; Castric-Fey *et al.* 1993), the primary mechanism for spread between sites of infestation is believed to be via ballast water and/or hull fouling of international shipping and other seaborne traffic (Hay 1990; Sanderson and Barrett 1989; Campbell and Burridge in press).

Information on the impacts of introduction of and infestation by *U. pinnatifida* in Australasia is extremely limited and in most cases anecdotal only. The tendency of the plant sporophyte to form a dense forest during autumn to spring (Sanderson and Barrett 1990; Hay 1991; Campbell and Burridge in press) suggests that competition for space and light may lead to exclusion and displacement of native plants and animals. Displacement of native taxa and alteration in floristic structure may also impact on grazing animals and detrital food chains.

The potential for eradication of *U. pinnatifida* following introduction is also extremely limited. Attempts at local eradication or control of the sporophytes via physical removal has met with very limited success (pers. comm., Victorian Fisheries; CSIRO Hobart), whilst the life cycle of the plant suggests that eradication via removal of the large sporophyte frond only, may be problematic at best. *U. pinnatifida* possesses a heteromorphic alternation of generations where the adult, macroscopic plant produces microscopic (approximately 6 µm) zoospores which adhere to the substratum, germinate and grow into microscopic, haploid gametophytes. The dioecious gametophytes produce eggs and sperm and following fertilisation the eggs develops in-situ into the adult sporophyte. Each adult sporophyte can release up to 106 spores (Saito 1975), suggesting that removal of the sporophytes without simultaneous removal of the very large number of gametophytes growing on the substratum is in effect dealing with only half of the plant population. Further, clearing of the sporophyte canopy may only serve to accommodate increased recruitment of sporophytes from the gametophyte population and allow greater rates of growth of the juvenile, understorey plants (Dean *et al.* 1989; Burgman and Gerard 1990; Campbell and Burridge in press).

A possible mechanism of control and/or eradication of *U. pinnatifida* infestations may be through application of an algicidal agent targeted at the haploid gametophytes. Whilst use of an algicide to remove the adult sporophytes may be problematic, the gametophytes may be both more susceptible to such agents and perhaps more easily treated. While adult macrophytes have been described as relatively insensitive to pollutants and toxicants the smaller reproductive stages have been shown to be substantially more sensitive (Jensen 1984; Thursby and Steele 1986, 1989). The habit of the gametophytes, very small and growing immediately over the substratum, also lends itself to use of an algicidal agent targeted at this component and location of the *U. pinnatifida* life cycle.

The use of algicides in marine habitats and the targeting of sporophytes/macrophytes is difficult owing to the often turbulent and dynamic conditions prevailing in such habitats. The establishment of a dosing regime which might eliminate *U. pinnatifida* sporophytes via direct application of the toxic agent into the water column may require the use of unacceptably high concentrations of the algicide. If the algicidal agent could be delivered immediately to the substratum however, perhaps via a pelletised delivery system (Steinberg pers. comm., UNSW), then mortality of the gametophytes might be achieved at an acceptably low dosing regime and concentration. The seasonal nature of growth of the sporophyte population during colder weather only also suggests that elimination of the gametophytes might be possible when no sporophytes are present and no further recruitment of gametophytes is occurring. In addition, as sub-lethal responses such as (spore and zygote) germination would be expected at concentrations much less than that required to achieve mortality (Burridge *et al.* 1995a, 1995b), dosing in the manner described may also serve to markedly diminish sporophyte recruitment from surviving gametophytes.

The aim of this study is to assess the suitability of a number of biocidal/algicidal agents for use as possible control agents for *U. pinnatifida,* and to determine concentrations required to achieve inhibition of zoospore germination and gametophyte mortality of *U. pinnatifida.* Initial assays were performed using the perennial kelp, *Ecklonia radiata* (C. Ag.) J. Agardh (Phaeophyta: Laminariales, Alariaceae), a laminarian macroalgae closely related to *U. pinnatifida.* The year-round availability of *E. radiata* and a life cycle identical in all respects to *U. pinnatifida* allows development of assay methodology during the period when reproductive *U. pinnatifida* sporophytes are not available (January to June) in Port Phillip Bay.

The biocidal agents assayed were Sea nine 211™, Furanone 281, Diuron, Simazine and Glyphosate. Sea nine 211™, is a recently developed antifoulant paint and contains the active compound, RH-5287 (4,5-Dichloro-2-n-octyl-4-isothiazolin-3-one), (Jacobson *et al.* 1993; Rohm and Haas 1994). RH-5287 is a member of the 3(2H)-isothiazolone class of compounds and has been found to be extremely effective against marine taxa commonly involved in hull fouling, i.e. invertebrate larvae, microalgae and bacteria. It has a relatively low retention rate in the environment with a biological half life of 24–48 h (Callow and Willingham 1996; Rohm and Haas 1992) in the water column and is rapidly partitioned into sediments (Jacobson *et al.* 1993; Burridge unpublished data). Sea nine 211™, is currently being considered by the Environmental Protection Agency (EPA) for release onto the Australian market.

Furanone 281 is a combination of three halogenated furanones isolated from the marine red alga, *Delisea pulchra* (Greville) Montagne (de Nys *et al.* 1992; de Nys *et al.* 1995). These

naturally occurring plant metabolites are utilised by the alga as a natural antifouling agent preventing colonisation by epiphytes and grazing of the alga. Eight halogenated furanones have been isolated from the red alga with varying antifouling capabilities (de Nys *et al.* 1995); these compounds are currently being investigated for possible use as an antifoulant additive for hull paint products (Steinberg pers. comm., UNSW). The combination of halogenated furanones provided for this study are metabolites 1, 2 and 8, referred to here as Furanone 281.

The remaining three biocidal agents tested were the herbicides Diuron [3-(3,4-dichlorophenyl)1-1-dimethylurea, >97%, Sigma, Melbourne)], Glyphosate (N-phosphonomethyl glycine, 99.9%, Monsanto, Melbourne), and Simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine, 90%, Nufarm Ltd., Melbourne). Glyphosate and Diuron are non-selective terrestrial herbicides, acting by absorption through foliage and roots of the targeted plant. Simazine selectively controls weeds and grasses by entering through the roots of terrestrial plants.

2 MATERIALS AND METHODS

2.1 Collection and culture maintenance

Fertile plants of *Ecklonia radiata* were collected by SCUBA diving *at* Queenscliff, Victoria, (144°69'S, 38°25'E) from a depth of 3 to 5 m. *Undaria pinnatifida* was collected from 2–3 m, two kilometres north-east of Point Wilson, Port Phillip Bay, Victoria, (38°04'S, 144°31'E). Samples were sealed in plastic bags and maintained on ice while transported to the laboratory. Plants of both species were used either fresh or after being held in aquaria in which water quality was maintained using an activated carbon filter operating at a flow rate of 7 l min^{-1}. *E. radiata* was maintained for a maximum of 5 days, *U. pinnatifida* was maintained for a maximum of 24 h. *U. pinnatifida* could also be maintained for up to 48 h in sealed plastic bags stored at 4°C with no apparent effect on spore viability or motility.

All cultures and assays were maintained at a temperature of 15 ± 1°C, at a salinity of 30 ± 1‰, and a pH of 8.2. Temperature, salinity and pH were measured using a Hach CO150 Conductivity Meter, Model 50150, and a Eutech Cybernetics pH meter. All glassware was soaked overnight in detergent (Pyroneg Diversy, Melbourne), acid washed for a minimum of 48 h in 10% HCL, triple rinsed in distilled water and oven dried for at least 24 h prior to use.

2.2 Zoospore release and settlement

Zoospore release for both *E. radiata* and *U. pinnatifida* was achieved using a modification of the methodology employed by Anderson and Hunt (1988) for zoospore release in *Macrocystis pyrifera* (see also Burridge *et al.* 1996). Fertile *E. radiata* thallus/sporangial tissue was cut into individual 4 x 4 cm pieces which were rinsed with fresh tap water and rubbed gently to remove epiphytic organisms and initiate osmotic shock. Each piece of tissue was then blot-dried with absorbent paper and allowed to air dry on trays at 20°C for 45 min. Zoospore release was then undertaken by immersing 30 sorus pieces in 3 l of 0.2µ m membrane filtered seawater at 15°C (10 sorus pieces per 1 l of water).

For *U. pinnatifida*, five healthy, mature sporophylls (7 cm in diameter) were rinsed with fresh, 0.2 µm membrane filtered sea water and rubbed vigorously to remove epiphytic organisms and prevent contamination of cultures. Zoospore release was achieved by placing the sporophylls into 5 l of 0.2 µm membrane filtered seawater at 15°C (1 sporophyll per 1 l of water). Five minutes after addition of plant material into the beakers zoospore density for each species was determined by placing 1 ml of zoospore suspension onto a plankton counter (Standard stage micrometer, 1 mm^2 gridation) and observing under a compound microscope. This was then repeated every 5 minute until the zoospore density reached at least 10^4 zoospores ml^{-1}, at which time the sporophytes were removed. Spore suspensions were then diluted to 10^4 spores ml^{-1} and stirred continually until used, to minimise adhesion of zoospores to the beaker surface.

To prepare germination inhibition assays, 25 ml of zoospore suspension was added to 25 ml glass beakers, each of which contained a 1 cm^2 microscope coverslip which acted as an artificial substratum for zoospore adhesion. Beakers were placed on black trays, covered with transparent plastic wrap and placed on shelves under 110 E m^{-2} s^{-1} illumination for 1 h

with a 12:12 h light:dark regime. This facilitated zoospore settlement and adhesion to the coverslips. After 1 h the zoospore suspension was gently decanted off.

2.3 Germination inhibition assays

Forty eight hour germination inhibition assays were initiated by the addition of 20 ml of the test biocide concentration (treatment) into each 25 ml glass beaker immediately after the removal of the zoospore suspension. Four replicates were employed for each treatment, and each assay included a seawater control and five or six treatment concentrations. Diuron was dissolved directly into seawater. Glyphosate, Simazine, Sea nine 211™, and Furanone 281, were first dissolved in dimethylsulphoxide (60 µl DMSO mg^{-1} of biocide agent), then dissolved in seawater.

Range finding tests were performed for each biocide using a log concentration series. For the biocides initially dissolved in DMSO, controls containing DMSO at the same concentration as in the range finding assay series were also employed. As no significant difference was found between the DMSO controls and the filtered seawater controls ($p<0.05$), these were discontinued for the definitive assays. Subject to results of the range finding tests, definitive tests were then conducted using Sea nine 211™ and Furanone 281, employing the same experimental design used in the range finding tests. For *E. radiata*, definitive concentrations of 10, 20, 40, 60 and 80 μgl^{-1} (Sea nine 211), and 100, 250, 500, 750 and 1000 μgl^{-1} (Furanone 281) were employed. For *U. pinnatifida*, definitive concentrations of 1, 5, 10, 20, and 40 μgl^{-1} (Sea nine 211), and 100, 250, 500, 750 and 1000 μgl^{-1} (Furanone 281) were utilised.

Following addition of the biocide treatments, all beakers were covered with transparent plastic wrap and maintained as previously described. After 48 h, the proportion of germinated to non-germinated zoospores was assessed. The coverslip from each beaker was removed and examined under a compound microscope (x10 objective), and the first 30 zoospores sighted were scored for the presence or absence of a germination tube. The presence of a germination tube greater than one spore radius in length was used as an indicator of successful germination (Anderson and Hunt 1988). Sizes of germinated zoospores in controls (spore and germ tube) was also recorded using an ocular graticule and stage micrometer.

2.4 Gametophyte mortality assays

Mortality assays were conducted on 14 day old gametophytes of both species. Following zoospore settlement 20 ml of fresh, filtered seawater was added to each 25 ml beaker. All beakers were incubated under the same culture conditions employed in the germination inhibition assays. After 48 h, 20 ml of a nutrient solution (Steele and Thursby 1988) was added and replenished every 3 days thereafter for the remainder of each assay up until introduction of the biocidal agents.

After 14 days of gametophyte growth, cultures were dosed with the test biocide(s). Stock solutions for the mortality assays were prepared as for the germination inhibition assays and four replicates per biocide treatment were employed. Range finding tests were conducted using a log scale, and definitive concentrations established for *E. radiata* were 50, 100, 150, 200 and 250 μgl^{-1} (Sea nine 211™), and 1, 2, 4, 8 and 16 mgl^{-1} (Furanone 281). For *U.*

pinnatifida, definitive concentrations for Sea nine 211 were, 200, 400, 800, 1200 and 1600 μgl^{-1}, and a single definitive assay for Furanone 281 was conducted with concentrations of 5, 10, 20, 40 and 80 mgl^{-1}.

To assess degree of mortality for each biocide treatment, the number of live gametophytes per unit area was determined by scoring the number per (randomly selected) 1 mm^2 on each coverslip; using a compound microscope, and minigrid (Southern Biological Services, P/L). A density in controls of approximately 200–300 gametophytes per mm^2 was indicative of healthy growth for both *E. radiata* and *U. pinnatifida*. Dead gametophytes were assessed as those having lost all pigmentation and possessing a clearly necrotic appearance. Comparison of treatment densities with controls then provided a measure of proportional mortality of the gametophyte population. The range of gametophyte lengths for both species was also recorded.

For *E. radiata*, the assays were scored at three day intervals, 17, 23 and 30 days after germination, that is 3, 9 and 16 days after dosing. Following scoring at 3 days post dosing, the coverslips and attached gametophytes were placed back into biocide free culture medium and maintained as previous, with culture medium changed every 3 days. This was intended to mimic the likely disappearance of the biocidal agent in the field within 3 days of any (initial) dosing.

For *U. pinnatifida*, eight assays were also dosed at 14 days. Two assays (Test 1) were scored at 17 days (3 days after dosing) and two were dosed again at 17 days (Test 2) and scored at 20 days. Test 3 involved two assays which were dosed at 14 and 20 days then scored at 23 days, or 9 days after initial dosing. In Test 4, two assays were dosed at 14, 17 and 20 days then scored at day 23; 9 days after initial dosing at day 14. Following scoring for gametophyte mortality each assay series was replenished with nutrients every 3 days and scored again at 30 days, 16 days after the initial treatment at day 14.

2.5 Statistical analyses

Percentage germination data were arcsine-transformed and subjected to single-factor analysis of variance (ANOVA). Mortality data were converted into a proportion of control response (gametophyte density) and arcsine transformed prior to single factor and two-factor ANOVA. For all data, no observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) were established via pairwise comparisons using Dunnett's test (Zar 1984). Median and maximum effect (EC_{50} and EC_{99}) concentrations were calculated using Probit or trimmed Spearman Karber analysis (Hamilton *et al.* 1977). Statistical power was determined according to Zar (1984).

3 RESULTS

3.1 *Ecklonia radiata* assays

3.1.1 Forty eight hour germination inhibition assay

The results of each *Ecklonia radiata* germination inhibition assay carried out from March through July are summarised in Tables 3.1 and 3.2. Table 3.1 documents assay results for Sea nine 211™ and Furanone 281, while Table 3.2 summarises assays conducted using Diuron, Simazine and Glyphosate. *E. radiata* zoospores showed a high degree of consistency in germination success for controls in all assays conducted, and the mean control germination for all assays conducted was 87% with a coefficient of variation of 11%. This includes two relatively low values obtained for the first two assays conducted using Sea nine 211™ (refer Table 3.1). The length of germinated zoospores within control assays ranged between 11 and 16 μm.

Sea nine 211™ was the most effective biocide tested with 48 h EC_{50} concentrations substantially lower than Furanone 281, and at least two orders of magnitude lower than Diuron, Simazine or Glyphosate. Dose response curves for germination inhibition using Sea nine 211™ are shown in Figure 3.1. Dose response characteristics for each of the four assays conducted are consistent, even allowing for the relatively low control germination observed for the two assays carried out on 12/03/97 and 24/03/97. With these two assays included, mean control germination was 78% for the four assays with a coefficient of variation of 19%. The mean 48 h EC_{50} for Sea nine 211™ was 17 μgl^{-1} with a coefficient of variation of 28%. The mean 48 h EC_{99} was 60 μgl^{-1} with a coefficient of variation of 25%. Threshold effects were observed at less than 10 μgl^{-1}, i.e. between the NOEC (control) and LOEC of 10 μgl^{-1}.

Dose response curves for four Furanone 281 *E. radiata* germination inhibition assays are shown in Figure 3.2. As for the Sea nine 211™ assays, there is uniformity between the four dose response curves with threshold effects being expressed between 100 and 250 μgl^{-1}. Control germination for these assays produced a mean of 92%, with a coefficient of variation of 4%. The mean 48 h EC_{50} for Furanone 281 was 271 μgl^{-1} with a coefficient of variation of 24%. The mean 48 h EC_{99} was 705 μgl^{-1} excluding assays 1 and 4 where EC_{99} concentrations could not be calculated owing to lack of data convergence required for probit analysis to calculate this value.

Dose response curves for Diuron, Simazine and Glyphosate are presented in Figure 3.3. Of these three agents/toxicants, Diuron was the most effective with Glyphosate appearing to be the least effective. Threshold effects for Diuron were exhibited between 1 and 10 mgl^{-1}, and for Simazine between 10 and 100 mgl^{-1}. The mean control germination for the two diuron assays was 93%, and for the two simazine assays was 87.5%. Mean 48 h EC_{50} concentrations were 3.2 mgl^{-1} and 24 mgl^{-1} for Diuron and Simazine respectively. Zoospores were not as sensitive to Glyphosate when compared to all of the biocidal agents tested, and threshold effects were observed at concentrations above 10 mgl^{-1}. There was also substantial disparity between the two assays conducted, with one of the assays showing 100% inhibition of germination at a (relatively high) concentration between 100 and 1000 mgl^{-1}. The mean control germination for the two glyphosate assays was 89%, whilst median effect

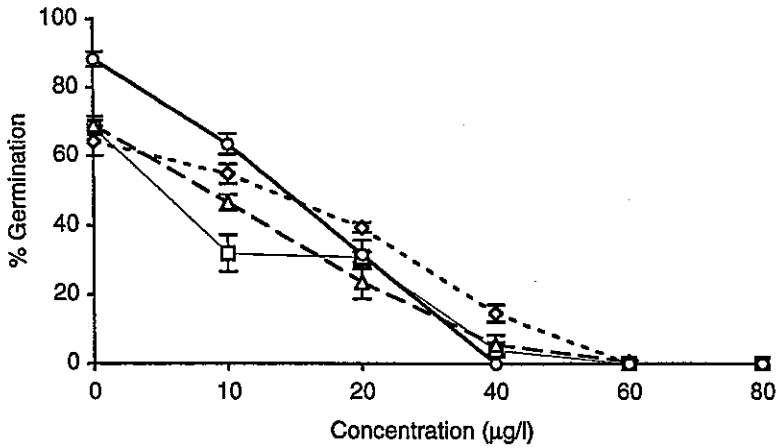

Figure 3.1. Dose response of four *Ecklonia radiata* germination inhibition assays. Zoospores were dosed with Sea nine 211™ for 48 h.

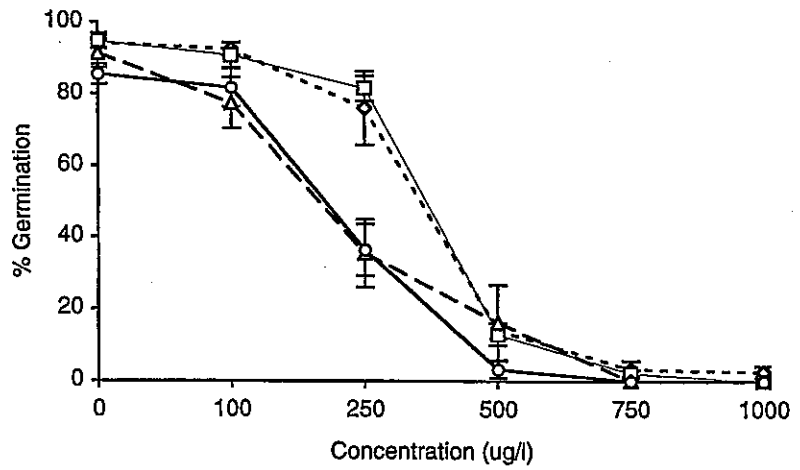

Figure 3.2. Dose response of four *Ecklonia radiata* germination inhibition assays. Zoospores were dosed with Furanone 281 for 48 h.

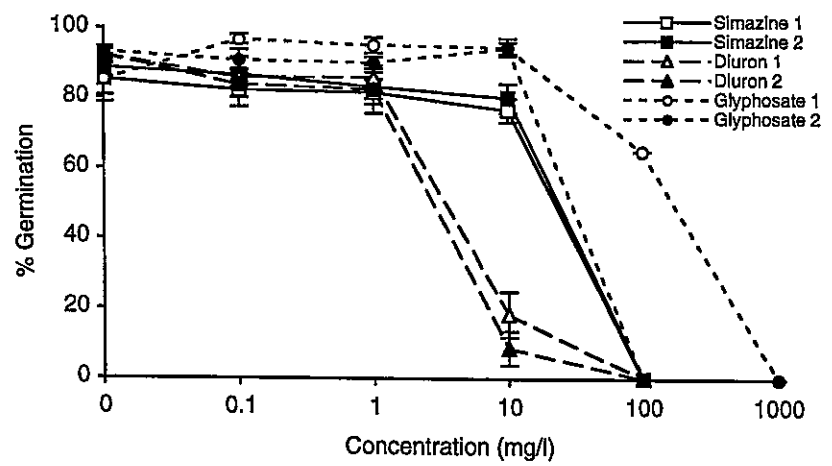

Figure 3.3. Dose response of four *Ecklonia radiata* germination inhibition assays. Zoospores were dosed in two series with simazine, diuron or glyphosate for 48 h.

concentrations (48 h EC_{50}) could not be calculated due to a lack of significant difference between control and treatment concentrations at all but the highest concentrations.

Table 3.1 Summary of *Ecklonia radiata* zoospore germination inhibition assays using the biocides, Sea nine 211™ and Furanone 281. Assays were conducted over 48 h.

Toxicant	Assay	Control germination (%)	48 h EC_{50} (µg/l)	95% Confidence interval	48 h EC_{99} (µg/l)	95% Confidence interval
Sea nine 211™	1	68	15	11.4–17.6	77	58.7–116.4
	2	64	24	19.7–27.4	76	63.2–100.1
	3	90	15	13.1–16.6	47	39.7–60.8
	4	92	14	11.9–15.0	47	39.2–61.1
Furanone 281	1	94	275	241.5–306.9	n/a	953.8–1422.6
	2	95	362	332.7–389.6	788	704.2–916.0
	3	92	214	97.3–313.6	n/a	627.2–4329.7
	4	86	232	198.8–258.5	622	526.4–812.1

Table 3.2 Summary of *Ecklonia radiata* zoospore germination inhibition assays using the biocides/ herbicides diuron, simazine and glyphosate. Assays were conducted over 48 h.

Toxicant	Assay	Control germination (%)	48 h EC_{50} (µg/l)	95% Confidence interval
Diuron	1	89	23	19.5–127.8
	2	86	25	20.8–29.7
Simazine	1	93	4	2.9–4.7
	2	93	3	2.2–3.4
Glyphosate	1	85	n/a	n/a
	2	93	n/a	n/a

3.1.2 Gametophyte mortality assays

Dose response curves for Sea nine 211™ and Furanone 281, 17 day gametophyte mortality assays are shown in Figures 3.4 and 3.5. The results for each assay are also summarised in Table 3.3. Sea nine 211™ was the most effective (toxic) of the two compounds, and the four assays at 17 days produced a mean LC_{50} (3 days after dosing) of 89 μgl^{-1} with a coefficient of variation of 35%. This relatively high coefficient of variation reflects a substantive degree (or at least greater degree) of variation between dose responses for these assays, when compared to the germination inhibition assays. The mean 17 day LC_{99} was 163 μgl^{-1} with a coefficient of variation of 37%, and threshold effects were generally observed at less than 50

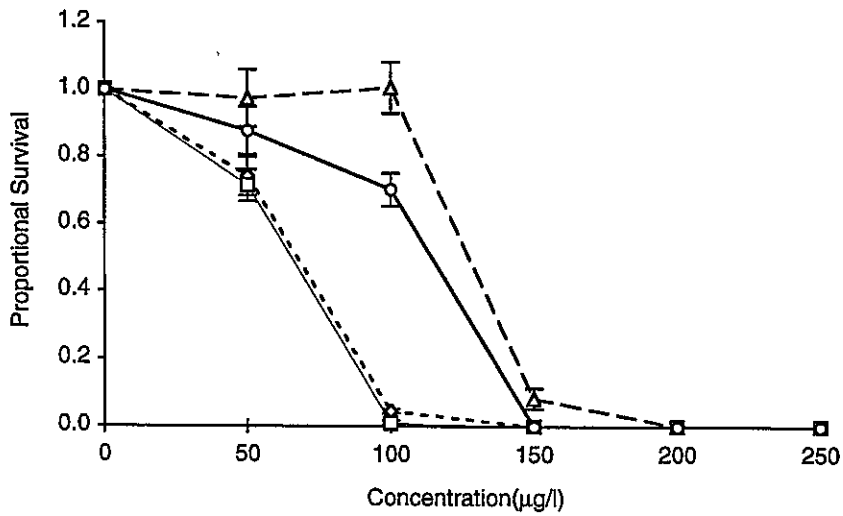

Figure 3.4. Dose reponse of four *Ecklonia radiata* gametophyte mortality assays after dosing with Sea nine™. Mortality was measured at day 17.

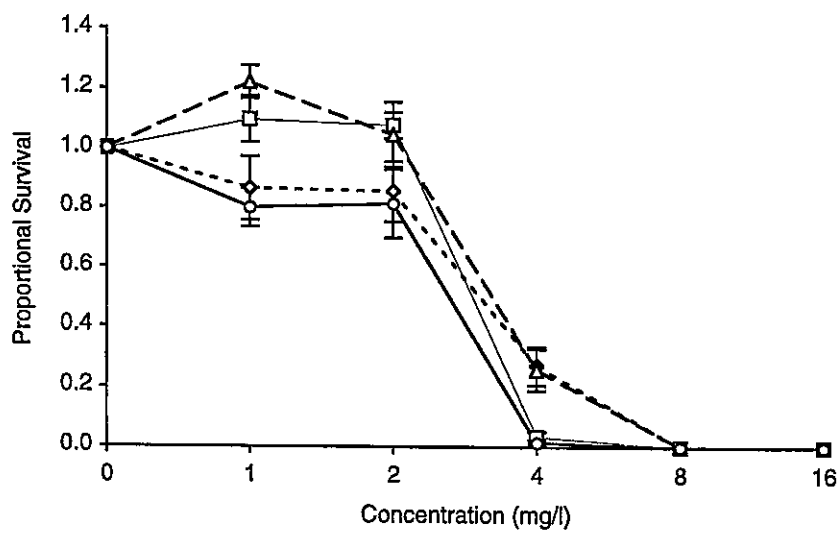

Figure 3.5. Dose reponse of four *Ecklonia radiata* gametophyte mortality assays after dosing with Furanone 281. Mortality was measured at day 17.

Table 3.3 Summary of *Ecklonia radiata* gametophyte assays using the biocides, Sea nine 211™ and Furanone 281. All tests were dosed at day 14 and gametophyte density was measured at day 17.

Toxicant	Assay	17 day LC_{50} (µg/l)	95% Confidence interval	17 day LC_{99} (µg/l)	95% Confidence interval
Sea nine 211™	1	64	60–67	123	107–146
	2	61	57–65	102	89–128
	3	125	123–128	203	n/a
	4	105	98–113	223	n/a
Furanone 281	1	1500	1000–2100	5300	n/a
	2	2900	2600–3200	1100	n/a
	3	2400	2200–2600	6000	n/a
	4	3400	3200–3600	n/a	n/a

gl^{-1}. Analysis of data using a two-factor ANOVA indicated significant interaction ($p<0.05$) between 17 and 30 days for the 50 μgl^{-1} to 150 μgl^{-1} treatments, but these changes were relatively minor and did not substantially alter the LC_{50} concentrations established at three days after dosing. The mean LC_{50} for 30 day mortality was 75.45 μgl^{-1} with a coefficient of variation of 13%.

The 17 day mortality assays using Furanone 281 (Figure 3.5) produced very similar dose response characteristics with a mean 17 day LC_{50} of 2.5 mgl^{-1} and a 32% coefficient of variation. The mean 17 day LC_{99} was 4.1 mgl^{-1} with a 42 % coefficient of variation (n=3). Analysis of data at 3, 9 and 16 days (after dosing) using two-factor ANOVA showed no significant difference between concentrations over time and no significant interaction ($p>0.05$). In these assays male gametophytes also had a much more elongate or filamentous morphology than the thicker or bulbous-appearing females and for increasing concentrations of both Sea nine 211™ and Furanone 281, the number of males declined relative to the females. At 150 μgl^{-1} Sea nine 211™ and at 4 mgl^{-1} Furanone 281 total mortality of males had occurred leaving only female gametophytes. Sizes of male and female gametophytes in control cultures ranged from 25–130 µm, and 25–50 µm respectively.

3.2 *Undaria pinnatifida* assays

3.2.1 Germination inhibition assays

Dose response curves for germination inhibition of *U. pinnatifida* using Sea nine 211™ and Furanone 281 are shown in Figures 3.6 and 3.7 respectively and the results of all tests performed using Sea nine 211™ and Furanone 281 are summarised in Table 3.4. The mean control germination for all assays conducted was 92% with a coefficient of variation of 2%. The length of germinated zoospores within control assays ranged from 20 to 38 µm. Threshold effects for Sea nine 211™ were generally expressed between 5 and 10 μgl^{-1} and total inhibition for all four assays occurred at 40 μgl^{-1}. There was uniformity in calculated 48 h EC_{50} concentrations with a mean of 13.3 μgl^{-1} and a coefficient of variation

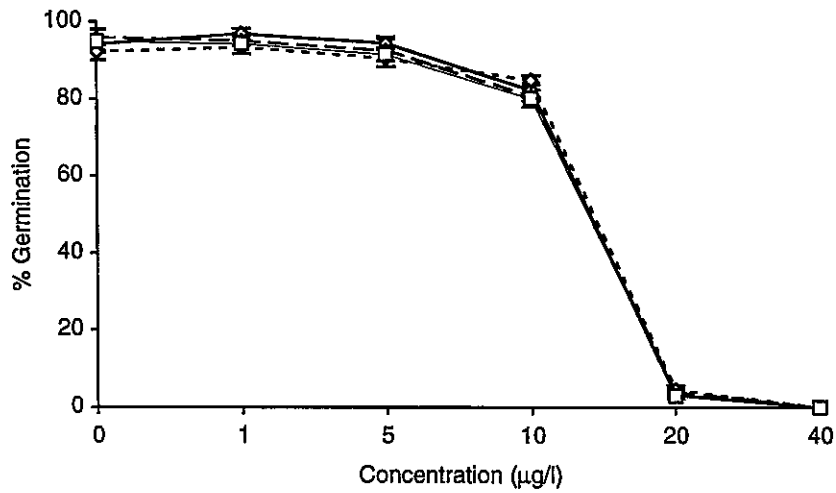

Figure 3.6. Dose response curves for four *Undaria pinnatifida* germination inhibition assays. Zoospores were dosed with Sea nine 211™ and incubated for 24 h.

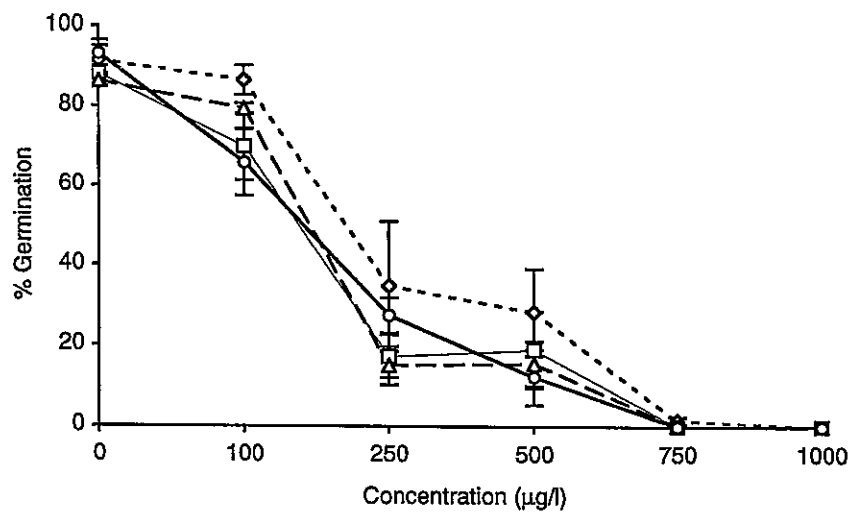

Figure 3.7. Dose response curves for four *Undaria pinnatifida* germination inhibition assays. Zoospores were dosed with Furanone 281 and incubated for 24 h.

Table 3.4 Summary of *Undaria pinnatifida* zoospore germination inhibition assays using the biocides, Sea nine 211™ and Furanone 281. Assays were conducted over 48 h.

Toxicant	Assay	Control germination (%)	48 h EC_{50} (µg/l)	95% Confidence interval	48 h EC_{99} (µg/l)	95% Confidence interval
Sea nine 211™	1	92	14	13.1–14.9	n/a	n/a
	2	96	13	11.8–13.5	21	18.8–24.6
	3	93	14	12.7–14.9	24	21.4–27.0
	4	95	15	12.1–13.8	23	20.4–26.4
Furanone 281	1	92	249	5.4–449.8	n/a	615.4–n/a
	2	88	169	12.1–313.5	989	474.7–n/a
	3	87	204	29.8–342.3	935	510.3–n/a
	4	93	165	52.9–268.3	n/a	596.2–n/a

of only 5%. The mean 48 h EC_{99} concentration was 22.4 μgl^{-1}, with a coefficient of variation of 6% (n=3). Testing with Furanone 281 produced threshold effects at concentrations less than 100 μgl^{-1}. There was substantial uniformity in assay results with a mean 48 h EC_{50} of 196.8 μgl^{-1} and a coefficient of variation of 17%. The mean 48 h EC_{99} was 962.3 μgl^{-1} for two assays only, and total inhibition of germination occurred for all assays at concentrations between 750 μgl^{-1} and 1000 μgl^{-1}.

3.2.2 Gametophyte mortality assays

The results of eight definitive, 30 day gametophyte mortality assays using Sea nine 211™ are summarised in Table 3.5, and dose response curves for each of the assays are presented in Figure 3.8 (two assays per figure in each of Tests 1, 2, 3 & 4). There was substantial uniformity between each of the tests (dosing regimes) with significant but relatively minor differences between LC_{50} concentrations. Test 1 (dosed once only) produced the highest LC_{50}, but based on 95% confidence intervals this was not significantly different from Test 4 (dosed three times). The dose response curves suggest that the increased dosing regime for Tests 2, 3 and 4 has increased the effect of the biocide (toxicant) on rate of mortality resulting in total mortality at (approximately) 800 μgl^{-1}; as opposed to total mortality at 1200 μgl^{-1} and 1600 μgl^{-1} for Test 1. Threshold responses were generally exhibited at (concentrations) less than 200 μgl^{-1} for all assays and there was substantial uniformity in dose response at concentrations less than 400 μgl^{-1}. As observed with *E. radiata*, there was a preferential mortality for male gametophytes compared to females with decreasing numbers of males as concentration of the biocide agents increased and, as for *E. radiata*, at the higher concentrations (at which some gametophytes survived), all males had died. The lengths (or at least overall diameter of the filaments) of *U. pinnatifida* male and female gametophytes in control cultures ranged between 50–150 µm and 20–120 µm respectively.

Two-factor ANOVA indicated significant difference (in mortality) between dosing regimes, as well as significant interaction between dosing regime and mortality ($p<0.05$), indicative of the increased response for Tests 2, 3, and 4 at concentrations greater than 400 μgl^{-1}. Mean

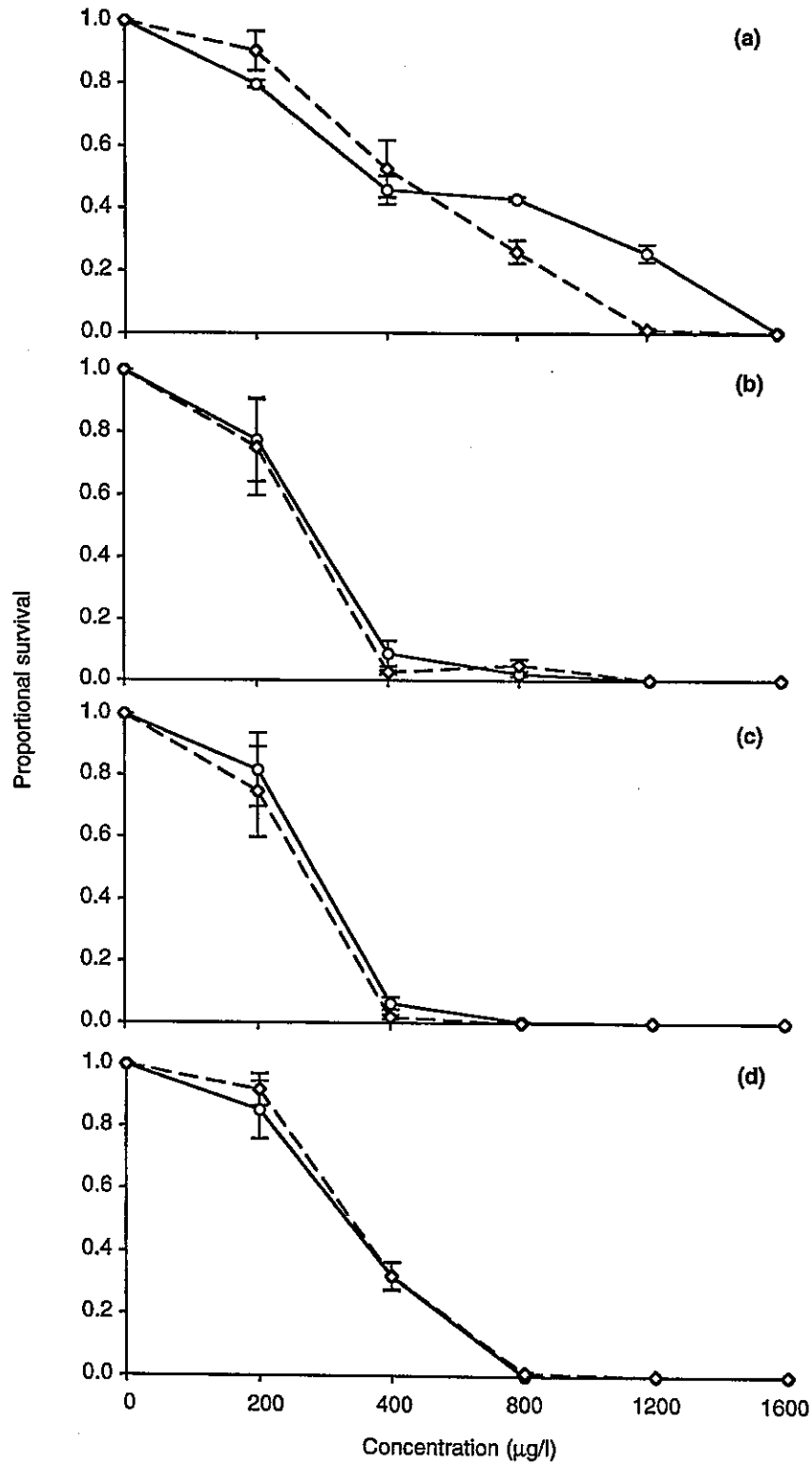

Figure 3.8. Dose reponse curves of eight *Undaria pinnatifida* gametophyte mortality assays after dosing with Sea nine 211™ at (a) day 14, (b) day 14 and 17, (c) day 14 and 20, and (d) day 14, 17 and 20. Mortality was scored after day 30.

Table 3.5 Summary of *Undaria pinnatifida* gametophyte mortality results after dosing with Sea nine 211™. The four dosing regimes and corresponding scoring times are described in the text.

Test	Assay	17 day LC_{50} (µg/l)	95% Confidence interval	20 day LC_{50} (µg/l)	95% Confidence interval	23 day LC_{50} (µg/l)	95% Confidence interval
1. One dose (day 14)	1	756	699.3–816.7	n/a	n/a	n/a	n/a
	2	434	399.0–473.0	n/a	n/a	n/a	n/a
2. Two doses (day 14 &17)	1	n/a	n/a	220	173.8–277.9	n/a	n/a
	2	n/a	n/a	388	330.9–424.9	n/a	n/a
3. Two doses (day 14 & 20)	1	n/a	n/a	n/a	n/a	335	301.9–371.2
	2	n/a	n/a	n/a	n/a	318	288.2–350.7
4. Three doses (day 14,17 & 20)	1	n/a	n/a	n/a	n/a	515	435.5–584.7
	2	n/a	n/a	n/a	n/a	476	398.3–582.8

LC_{50} concentrations for Test 1 (dosed day 14), Test 2 (dosed day 14 and 17), Test 3 (dosed day 14 and 20) and Test 4 (dosed day 14, 17 and 20) were 595 μgl^{-1}, 304 μgl^{-1}, 326 μgl^{-1} and 495 μgl^{-1} respectively. The mean 30 day LC_{50} concentration for all eight assays was 425 μgl^{-1} with a coefficient of variation of 19%. LC_{99} concentrations could not be calculated for these assays owing to lack of data convergence required for probit analysis to calculate this statistic. Concentrations for total mortality can be derived directly from dose response curves and complete mortality was achieved at concentrations greater 1600 μgl^{-1} for a single dose (day 14) and at greater than 1200 μgl^{-1} for more than a single dose.

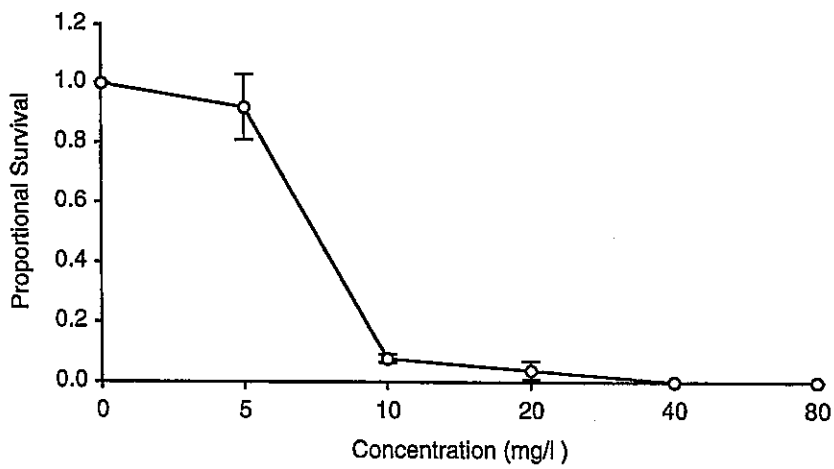

Figure 3.9. Dose response curve of one *Undaria pinnatifida* gametophyte mortality assay after dosing at 14 days with Furanone 281. Mortality was scored at 17 days.

A single dose response curve for a 17 day assay using Furanone 281 is shown in Figure 3.9. Threshold response for this assay occurred between 5 mgl^{-1} and 10 mgl^{-1}, the 17 day LC_{50} for this assay was 7.7 mgl^{-1} and complete gametophyte mortality was observed at concentrations greater than 40 mgl^{-1}.

4 DISCUSSION

The results presented for *Ecklonia radiata* suggest that the assays performed on zoospores and gametophytes offer reproducible mechanisms for assessing toxicity of potential algal control agents. There is substantial consistency in the median effect doses established for both the germination and gametophyte mortality assays using each of the biocidal agents assayed. Where variability is present between assays using the same biocide (i.e. for Furanone 281 and gametophyte mortality, 1100 μgl^{-1} compared to 5300 μgl^{-1} and 6000 μgl^{-1}) the values obtained are still of the same order of magnitude and perhaps reflect normal variability which might be expected in such biological assays. The consistency between threshold responses for both of the assays and the relatively low coefficients of variation also supports the reproducible nature of the two tests. The general consistency in control germination for the germination inhibition assay is also indicative of consistency in assay result; the two low values for the 12/03/97 and 24/03/97 (68% and 64% respectively) perhaps suggesting irregularities in methodology during assay development. Further work incorporating a larger number of tests would better elucidate natural variability in response, whilst coefficients of variability (of the larger number of assays) would accommodate a greater level of quality assurance and confidence in assay result.

Concentrations of Sea nine 211™ and Furanone 281 required to achieve total gametophyte mortality for both biocides were substantially greater than that required for total zoospore germination inhibition. Whilst selective sensitivity may be possible for the different stages of development, greater sensitivity would be expected for the sub-lethal germination endpoint compared to mortality (Burridge *et al.* 1995a; 1995b), the differential response may also be due to the larger size of the gametophytes and a greater surface area to volume ratio associated with the developing gametophyte. This could also account for the apparent greater rate of mortality observed for the smaller male gametophytes (for both *E. radiata* and *U. pinnatifida*). The females with a somewhat larger diameter than the more filamentous males would possess a smaller surface area to volume ratio and may be less susceptible to the toxicant at lower concentrations.

These findings are consistent with other toxicological studies on marine macrophytes where unicellular stages of growth have proved to be substantially more sensitive to exogenous compounds and toxicants than larger embryos and juvenile plants (Steele and Thursby 1986; Burridge *et al.* 1995a). Whilst adults plants (i.e. kelps) have been described as relatively insensitive to toxicants and exogenous pollutants (Thursby and Steele 1988), the smaller phases of these plants life cycle, and especially sub-lethal endpoints such as germination, are generally more sensitive. Burridge *et al.* (1995a) showed much greater sensitivity of *Phyllospora comosa* (Labilladiere) C. Agardh unicellular zygotes when compared to 7 day old embryos, where 7 day old embryos exhibited mortality to 2,4 dichlorophenoxyacetic acid and formaldehyde at concentrations an order of magnitude greater than those causing germination inhibition in the unicellular zygote.

Of the five agents tested, Sea nine 211™ was the most toxic (or effective), whilst Diuron, Simazine and Glyphosate appear to offer the least suitability as potential control agents. Furanone 281 also has potential, but would need to be used at higher concentrations than Sea nine 211™. There is very little information available on the toxicity of Furanone 281 or on mechanisms of toxic expression by the halogenated furanones isolated from *Delisea*

pulchra. De Nys *et al.* (1995) reported on broad spectrum effects of halogenated furanones and reported an EC_{50} of 25 μgl^{-1} for inhibition of settling of cyprid larvae of the barnacle *Balanus amphitrite* Darwin, and strong inhibition of settlement and growth of gametes of *Ulva lactuca* Linnaeus at concentrations as low as 25 $ngcm^{-2}$ (surface treatment).

De Nys et al. (1996) reported on toxic effects of two isolates from the marine red alga *Laurencia rigida* and showed complete inhibition of settling by *Ulva lactuca* gametes at concentrations less than 10 gcm^{-2}; inhibition of settlement of cyprid larvae of the barnacle *Balanus amphitrite* at concentrations between 0.001 gcm^{-2} and 1.0 gcm^{-2}; complete inhibition of settling for larvae of the bryozoan *Bugula neritina* at concentrations less than 1.0 gcm^{-2} and complete mortality of nauplii larvae of *Balanus amphitrite* at concentrations less than 0.1 gcm^{-2}. Whilst direct comparison between these 'surface contact' assays and results obtained here are problematic owing to differences in dosing mechanisms, greater sensitivity might be expected in the former owing to direct contact with biocide leachate from the substratum, and direct contact with cell wall and/or membranes.

De Nys *et al.* (1996) have also reported inhibition of growth of the marine bacterium *Vibrio fischeri* at concentrations less than 0.1 mgl^{-1} while Gram *et al.* (1996) reported inhibitory effects for *Delisea* secondary metabolites on swarming motility of the bacterium *Proteus mirabilis* at concentrations in the 10 mgl^{-1} to 50 mgl^{-1} range. Gram *et al.* (1996) further suggested that the inhibitory effects on swarming may be due to suppression by the halogenated furanone on cell-cell signalling based on *N*-acyl-homoserine lactones (AHLs). Possible implications of this feature of halogenated furanones activity on macroalgal spores or gametophytes is uncertain although potential impacts on cell membranes and membrane permeability are possible.

The mean value for total germination inhibition of *E. radiata* (0.6 mgl^{-1}) using Sea-nine 211™ is consistent with documented results for growth, germination and settlement inhibition for a range of other algal taxa and benthic organisms. Sea-nine 211™ has been documented as having 'minimum inhibitory concentrations' for growth of freshwater algae (*Chlorella, Chlorococcum, Scenedesmus* and *Ulothrix*) at concentrations of 0.06 mgl^{-1} to 1.3 mgl^{-1}. Minimum inhibitory concentrations for growth of blue green algae (*Anabaena, Synechoccus, Nostoc, Scytonema, Microcystis* and *Oscillatoria*) have been established at 0.3 mgl^{-1} to 0.6 mgl^{-1}, while an LC_{50} of 0.34 mgl^{-1} has been documented for the barnacle *Balanus amphitrite* (Rohm and Haas 1992). Inhibition of germination has also been described for two common (marine) fouling Chlorophyta algae, (*Enteromorpha sp.*) at concentrations of 0.1 mgl^{-1} and 0.2 mgl^{-1} (Rohm and Haas 1992). De Nys *et al.* (1996) showed that Sea nine 211™ inhibited settlement of *Ulva lactuca* Linnaeus gametes at concentrations of 0.001 gcm^{-2} to 0.1 gcm^{-2}; inhibited settlement of *Balanus amphitrite* cypris larvae at 0.01 gcm^{-2} to 1.0 gcm^{-2}; inhibited settlement of the bryozoan *Bugula neritina* at 0.01 gcm^{-2} to 1.0 gcm^{-2} and produced complete mortality of *Balanus amphitrite* cypris larvae between 0.0001 gcm^{-2} and 0.1 gcm^{-2}. Inhibition of growth of the marine bacterium *Vibrio fischeri* at concentrations as low as 0.1 mgl^{-1} was also reported.

The mechanism of toxicity for Sea nine 211™ is uncertain. Target organisms for this formulation are generally soft-fouling invertebrate larvae and microscopic algae, and anecdotal evidence suggests that settlement prevention by Sea nine 211™ may be due to direct toxicity and perhaps inhibition of the electron transport chain in oxidative phosphorylation and photophosphorylation (Steinberg pers. comm., UNSW). Development

of benthic organisms involves settlement and germination and presumably involves a high respiratory demand for the developing organism, and any inhibition of respiratory or photosynthetic capacity may restrict settlement and germination. Certainly any physiological restrictions placed on developing embryos or spores may restrict germination via inhibition of development of the polar axis associated with adhesion and germination. Burridge *et al.* (1995a) have suggested that tributyltin (TBT) may inhibit germination of *Phyllospora comosa* embryos through inhibition of the normal polar expression associated with germination (Burridge *et al.* 1993). Burridge *et al.* (1996) have also indicated that exposure to the herbicide 2,4 dichlorophenyl may prevent algal germination through alteration of cation uptake kinetics presumably associated with normal apico-basal polar expression.

The relatively high concentrations of Diuron, Simazine and Glyphosate required to achieve germination inhibition of *E. radiata* (when compared to Sea nine 211™ and Furanone 281) suggests that extremely high concentrations would be required for gametophyte mortality. This is consistent with the findings of Burridge *et al.* (1995a) where differential response between different sizes for the same plant might be expected and also suggests that use of these compounds as an *Undaria* control agent may be limited. Literature on the toxicity of these compounds to marine organisms is limited, with the focus of such work primarily on terrestrial organisms. The most effective (here) of these three compounds, Diuron, has been applied as an antifoulant targeted at marine algae (Callow and Willingham 1996). It is widely accepted that Diuron blocks the electron transfer between the primary electron acceptor of photosystem II (Ashton and Craft 1981), suggesting possible uncoupling of photophosphorylation and oxidative phosphorylation.

Simazine is a member of the triazine class of compounds which are documented as environmental contaminants of coastal waters (Gough *et al.* 1994; Readman *et al.* 1993) and persistent within sediments (Meakins *et al.* 1995). Bester *et al.* (1995) showed that the triazine compound, atrazine, at concentrations above 120 ngl^{-1} uncoupled photo-phosphorylation by inhibition of electron transport in noncyclic photophosphorylation. Triazine compounds also influence plant hormone metabolism and can cause foliar chlorosis (Ashton and Craft 1981). Glyphosate is a commonly used herbicide which causes foliar chlorosis and inhibits pathways responsible for providing precursors for plant lignin, flavonoids and a host of secondary metabolites (Ashton and Craft 1981; Puttwain and Collins 1989). Mammalian toxicity (LD_{50}) of glyphosate is reported in the high 'milligram per kilogram' to 'gram per kilogram' concentration range, varying for test species (Merck 1983), while concentrations of 121.5 mgl^{-1} (48 h LC_{50}) have been described for tadpole mortality (Bidwell and Gorrie 1995).

The results of the germination inhibition assays for *U. pinnatifida* are consistent in all respects with the results obtained for *E. radiata,* using both Sea nine 211™ and Furanone 281. The EC_{50} concentrations for both biocides are (statistically) the same for each algal species, while the concentrations required to achieve complete inhibition of germination show minor but in the context of this exercise inconsequential differences only. This factor and the similarity in dose response between the two species (for both biocides) further suggests that the mechanism of toxicity and uptake kinetics are probably very similar. The consistently high control germination response and low coefficients of variability for both

control and median effect doses also supports consistency in assay outcome and high reproducibility of the assay.

The results of the germination inhibition assays using Sea nine 211™ do however, contrast markedly with the results of the gametophyte mortality assays. For *U. pinnatifida,* the median lethal doses were in general three to four times higher than for *E. radiata,* while the concentration at which total mortality would be achieved at 1.6 mgl^{-1} was eight times the concentration required to achieve the same outcome for *E. radiata* (200 μgl^{-1}). This result appears to be counter-intuitive, given the very similar response for the germination inhibition assays conducted on both taxa. It is presumably due in part to inter-specific differences in response but may also be due to the greater rate of growth exhibited by the *U. pinnatifida* gametophytes; the larger gametophytes having a lower sensitivity than the smaller *E. radiata* gametophytes. This would be consistent with the findings of previous work where reduced sensitivity for larger life stages of the same algal species has been reported (Anderson and Hunt 1988; Burridge *et al.* 1995a).

The lack of substantive difference between the four *U. pinnatifida* mortality assay treatments (dosing regimes) suggests that effects may be expressed at initial dosing and that sensitive individuals are removed at this time. The interaction which is present (over time) in the results is relatively minor, and as Test 4 (three doses) produced LC_{50} concentrations effectively higher than Tests 2 & 3 (two doses) it seems likely that the differences between the 4 tests may be due primarily to inherent variability in response. Another possible confounding factor is that the individuals which have not been removed by the initial dosing represent the more tolerant members of the gametophyte population and that as these are growing (over the duration of the test) they are increasing in size and may be developing a reduced sensitivity to the exposure concentrations employed. As indicated previously, a larger number of assays could be employed to better elucidate variation in assay outcome, and gametophyte size might be employed as a covariate for statistical analyses to assess the effect of size on mortality.

The LC_{50} established for Furanone 281 at 7.7 mgl^{-1} is consistent with the results of the mortality assays using *E. radiata* (mean LC_{50} of 2.5 mgl^{-1}) and contrasts with the differential response of the two algal taxa to Sea nine 211™. This suggests that gametophyte size may not be of major significance to toxic response using Furanone 281 and is perhaps indicative of different mechanisms of toxicity for the two biocidal agents. The consistency in median effect concentration and dose response for *U. pinnatifida* and *E. radiata* when exposed to Furanone 281 does however suggest similarity in toxic expression and uptake kinetics for the two taxa.

5 CONCLUSIONS

The results indicate that of the five biocidal agents tested Sea nine 211™ and Furanone 281 may provide the most acceptable options for chemical control of *Undaria pinnatifida*. The results of the germination inhibition assays using *Ecklonia radiata* indicate relative insensitivity for the herbicides Diuron, Simazine and Glyphosate and also suggest that gametophyte mortality may require unacceptably high application rates of these agents. The results of the *U. pinnatifida* germination assays are very consistent with the *E. radiata* assays using the same endpoint, whilst differences between the two species (reduced sensitivity for *U. pinnatifida*) for the mortality endpoint may be due to larger size of the *U. pinnatifida* gametophyte at the same age (following spore germination). Complete mortality would be expected for *U. pinnatifida* gametophytes for concentrations of Sea nine 211™ greater than 1.6 mgl^{-1} and for *E. radiata* at concentrations in excess of 200 μgl^{-1}. The latter concentration would also produce complete inhibition of germination for both species.

The results of the germination inhibition assays for both algae using Furanone 281 are also very consistent and complete inhibition of germination would be expected at 1.0 mgl^{-1}. *E. radiata* gametophytes appear to be slightly more sensitive to Furanone 281 than *U. pinnatifida* gametophytes, although difference between the two species is relatively minor. For both species complete inhibition of germination and gametophyte mortality would be expected at concentrations in excess of 40 mgl^{-1}.

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