Development of a rapid *Hormosira banksii* bioassay using chlorophyll *a* fluorescence

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ABSTRACT

A wealth of information exists for chlorophyll *a* fluorescence applications in eco-physiology. However, the use of chlorophyll *a* fluorescence in marine ecotoxicology has been limited and this is especially true of macroalgal ecotoxicology.

The primary objective of this thesis was to develop and describe a bioassay protocol that uses improvements on past macroalgal assay techniques to allow the photosynthetic capacity of *Hormosira banksii* gametes to be assessed using chlorophyll *a* fluorescence measurements. Two protocols (using *H. banksii* eggs or sperm) have been developed that allow for rapid assessment of toxic impact (less than 8 h) on macroalgal gametes. This rapid time-to-result allows for timely management decisions to be made. This is significant since past macroalgal bioassays have necessitated up to a 48 h delay before results are available to decision makers.

In addition to this much improved rapidity of result, comparison between a germination based endpoint and the fluorescence endpoint has shown the fluorescence measure to be more sensitive to some classes of toxicant, more precise (in terms of Coefficient of Variation), and can also offer information on mechanistic pathways of toxicants.

In order to validate 'real-world' use of the new fluorescence protocol, the sperm fluorescence bioassay was effectively applied to the assessment of interactive effects displayed by mixtures of anti-foulant compounds. Furthermore, a level of eco-relevance was demonstrated for the chlorophyll *a* fluorescence endpoint %PSII Inhibition. This is significant in that eco-relevance has not previously been experimentally demonstrated for a chlorophyll *a* fluorescence endpoint in ecotoxicology and the demonstrated link to higher level effects may have favourable implications as to future acceptance of fluorescence data into water quality guidelines.

Essentially, this work describes the development of, and also the successful application of, a novel, fluorescence macroalgae bioassay that not only has advantages over currently employed methods, but also offers a powerful tool in both the rapid assessment of toxic impact on near-shore macroalgal communities, and as an effective toxicity screening tool.

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"affranchissant l'esprit et pesant les mondes sans haine, sans peur,

sans pitié, sans amour et sans Dieu."

'releasing the spirit and weighing up worlds without bitterness, without fear,

without pity, without love and without God.'

Description of the scientific mind
Gustav Flaubert (1821-1880)

"I have gotten a lot of results. I know several thousand things that won't work."

Thomas Edison (1847-1931)

"Hey, back off man. I'm a scientist."

Dr. Peter Venkman (Bill Murray)
Ghostbusters, 1984

Chapter 1

Those parts relating to 'fluorescence ecotoxicology' are interpreted or re-printed from the paper: Ralph, P.J., Smith, R.A., Macinnis-Ng, C.M.O., Seery, C.R. 2005. Review: Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems. Toxicological and Environmental Chemistry. *In press*

1.0 General Introduction

1.1 Biological Testing for Marine Contaminants

It has been recognised that the oceans of the planet constitute the ultimate sink for many of the chemicals produced and used by humankind (Pritchard, 1993). With more than 70 000 chemicals in use worldwide and over 30 000 in Australia alone (Chapman, 1995a), a thorough understanding of the impacts caused by contamination, and an effective means by which to assess ecological consequences, is imperative in developing protective legislation for receiving waters (Lam and Gray, 2001). With ever increasing awareness of the harm caused to marine environments by anthropogenic activities, a number of approaches have been devised to assess this impact. These approaches can either aim to identify contaminant situations where remedial action should be commenced or identify areas of potential detriment or harm. The early identification of potential harm is important is it can result in protective measures being implemented before significant impacts are observed.

Traditionally, assessments of environmental harm have been largely comprised of chemical analysis and characterisation. This approach results in data consisting of 'what is present' and 'how much is there' (Munawar, *et al.*, 1989a). From this type of data, regulatory guidelines have been established but they were criticised for lacking the dynamic toxicity information needed to determine the bioavailability of contaminants to the biota within an ecosystem (Munawar, *et al.*, 1989a). Furthermore, while guidelines were often developed in the Northern Hemisphere, they were adopted by governments in Australia and New Zealand for the protection

of vastly different ecosystems to which they were intended (Johnston, *et al.*, 1990; Sunderam, *et al.*, 1992; Davies, *et al.*, 1994; Norris and Norris, 1995; Markich and Camilleri, 1997; Mulhall, 1997; Rose, *et al.*, 1998; Phyu, 2004; Westbury, *et al.*, 2004).

Pollution is a complex problem, where the impact of individual chemicals is influenced by a number of water chemistry variables (pH, dissolved oxygen, organic content, etc.) and where the water sample often comprises an unknown cocktail of chemicals at minute concentrations (Slabbert and Venter, 1999). Given this complexity, chemical analyses alone are often inadequate in providing information on the potential, or indeed actual, effects of pollution to organisms (Munawar, *et al.*, 1989b; Cairns, 1995). In contrast, tests that measure the biological responses of living organisms to toxic challenges (from either single chemicals or complex chemical mixtures) are able to provide vital information that is lacking with chemical analyses (Slabbert and Venter, 1999).

It was only since the late 1980's that the actual effects on biota within an ecosystem have been used as quantitative metrics of contaminant exposure and impact. By the 1990's it was widely recognised that monitoring and testing that focuses on the biota within an ecosystem is what is needed to effectively assess the condition of aquatic systems and to develop legislative guidelines to protect these resources (Maltby and Calow, 1989; Cairns and Pratt, 1993; Cairns, 1995; Slabbert and Venter, 1999).

In order to effectively protect ecosystems, the biological testing undertaken needs to be comprehensive, cost effective and defensible. That is, it needs to encompass as many factors as practicable and give detailed, useful information on how the ecosystem would be affected by pollution. For legislative and regulatory reasons the testing needs to be beyond scientific reproach (as with all good science) with accepted methodology and clear outcomes. For these attributes to be met, a number of factors need to be addressed: specificity, sensitivity, repeatability, precision and relevance. So while the effects of pollution have been considered and investigated from an ecophysiology viewpoint for many decades, these studies have often lacked a thorough assessment of these factors that are required to effectively provide data to be used in water quality guidelines or give adequate information on the threats posed by contamination. Thus, standardised ecotoxicological bioassays were developed to better provide the defensible, thorough data required to support protective legislation (Cairns, 1995; Chapman, 2002).

1.2 Ecotoxicological Bioassays

Ecotoxicology is a field of science concerned with measuring and predicting the effects of pollutants on ecological systems and the components within these systems. Using fundamental chemical and ecological principles, ecotoxicologists aim to investigate the effects of chemicals on individual organisms, through to resultant ecological effects (Chapman, 2002; Forbes and Calow, 2002).

The term ecotoxicology has generally been credited to Professor Truhaut, who in the early 1960's derived the word from 'ecology' and 'toxicology' (Walker, *et al.*,

1997). Truhaut was a toxicologist with the vision to recognise the need for investigations into the fate and effects of chemicals in ecosystems. It reflected a time when there was growing concern over the harmful effects of chemicals within the context of ecology (Walker, *et al.*, 1997). While it was in the late 1960's that ecotoxicology was formally conceived as a coherent field of science, it would be remiss not to acknowledge the numerous studies that would now be recognised as ecotoxicological, but were undertaken much earlier.

The earliest recorded bioassay (though not strictly ecotoxicological) was performed by Aristotle (Chapman, 1995b), where he exposed 'sludge worms', collected from freshwater downstream from Athens, to salt water and observed 100% mortality. It is thought that by the late 1800's, when the effects of anthropogenic impact became evident, a simple 96 hour acute toxicity test with fish had been developed (Adams, 1993). Indeed, the marked rise in observable effects prompted one early environmentalist, G. P. Marsh, to write in 1864: "The ravages committed by Man subvert the relations and destroy the balance that nature had established."

Despite these early beginnings and the subsequent efforts of many to develop a science that incorporated a number of disciplines, it was not until the 1970's that ecotoxicology attempted to 'break free' from being viewed as a sub-discipline of medical toxicology. However, as late as 1983, Moriarty noted that very little had changed and that "the only difference between toxicology and ecotoxicology appears to be in the species selected for toxicological tests: acute toxicity is measured on the water flea instead of on the laboratory rat".

The regulatory nature of ecotoxicology throughout the 1980's may have been responsible for the reluctance to change that Moriarty observed. Governments (in Europe and the U.S.A.) were starting to develop legislation and water quality guidelines using data from biological tests and needed these tests to be beyond scientific reproach and defensible in a court of law. The only way to achieve this is to have tests that are well defined, replicable and accurate. A test with these characteristics resulted in significant compromise, where the tests consisted of prescriptive scenarios with as few variables as possible; such as standardised water chemistry (salinity, nutrient content), standardised abiotic factors (temperature, light, etc.), and regulated feeding rates. It also meant that tests were most often limited to being performed with a few, laboratory-amenable test species; such as the ubiquitous water flea that Moriarty (1983) commented upon. However, while these tests lacked what has since been termed 'eco-relevance' (the degree to which the test can be directly related or linked to actual field events), they provided a significant step forward to the now widespread practice of incorporating biological testing into water quality guidelines (Wells, 1999; ANZECC/ARMCANZ, 2000). These early tests also provided the impetus to expand biological testing to include a range of endpoints of differing sensitivity, a wider range of test species from a number of taxa being used, thus incorporating a greater level of eco-relevance.

Generic, single species ecotoxicological bioassays typically consist of a particular species being exposed to a single chemical over a range of concentrations, with all other factors (abiotic and biotic) being held constant, and measuring a biological response. While this description may be somewhat simplified, a toxicity test is essentially a very simple, reproducible and relatively inexpensive method of

assessing biological impact (though there are exceptions to this, such as, for example, a full fish life-cycle test which can be complicated and expensive). Because of the ability to design and replicate these types of studies, a well designed and implemented toxicity test can be used to demonstrate a cause-effect link between adverse biological effects and the presence of pollution (Cairns, *et al.*, 1994).

The cause-effect relationship that can be demonstrated with a bioassay offers an efficient and effective means of understanding the risk chemicals pose towards aquatic ecosystems. This understanding comes from the bioassay's ability to rank the relative toxicity of chemicals to a particular organism or determine the concentration of a given chemical that elicits a defined biological effect (Cairns, *et al.*, 1994). Furthermore, this approach has proven remarkably effective at quantifying a number of aquatic pollution issues, such as: routine monitoring and surveillance of wastewater toxicity to determine compliance with license requirements (Farré and Barceló, 2003); identification of waste streams or tributaries contributing inputs of toxicants (Yoder and Rankin, 1995); and offering a means by which new chemicals can be tested and predictions of impact made before allowing widespread use (ANZECC/ARMCANZ, 2000). Above all, though there are vast theoretical deficiencies in using single species ecotoxicological bioassays to estimate responses at high levels of biological organisation, they have proven to be particularly effective in doing just that (Cairns, 1983); such as their use in Species Sensitivity Distribution (SSD) curves that aim to protect 95% of species in a system (Hose and Van den Brink, 2004). So while single species bioassays are often criticised for lacking biological complexity and their need for far-reaching extrapolation (Forbes, *et al.*, 2001), they have still formed the backbone of ecotoxicology for more than 20 years.

1.2.1 Macroalgal Bioassays

As explained above, while routine toxicity testing and bioassay protocols have been in use for quite some time, only recently (since the 1990's) have marine macroalgae been widely used in toxicity tests (Anderson, *et al.,* 1990; Burgess, *et al.,* 1993; Eklund, 1993; Doblin and Clayton, 1995; Gunthorpe, *et al.,* 1995; Burridge, *et al.,* 1996). It is even more recently (since approximately 2000) that macroalgae have begun to be investigated in their use as bioindicators (the presence or absence of a species indicates level of pollution impact; Melville, *et al.,* 2005) and biomonitors (organism's tissue concentration of pollutant used to assess relative bioavailability of pollutant; Gosavi, *et al.,* 2004). As such, standardised protocols for macroalgae toxicity testing are limited and this is especially true of Australian macroalgal species (Gunthorpe, *et al.,* 1997).

While the early work of Thursby and Steele (1984; 1985; 1986) pre-dated the wide-spread use of macroalgal bioassays, their development of tests measuring reproductive success laid the groundwork for the eventual USEPA protocols for the estuarine macroalga *Champia parvula* (USEPA, 1994) and the marine macroalga *Macrocystis pyrifera* (USEPA, 1995). Yet while these USEPA protocols exist, as do individually derived protocols for various red and brown algae (Eklund, 1993; Eklund, 1998; Thursby and Steele, 1995), there is no "internationally standardised" protocol that exists for any macroalgae species (Eklund and Kautsky, 2003). The scarcity of macroalgal ecotoxicological data is clearly inconsistent with their ecological importance to many near-shore marine ecosystems.

Microalgal toxicity testing currently enjoys a much more prominent role in marine ecotoxicology, primarily through the use of the internationally standardised test method that utilises *Phaeodactylum tricornutum* (ISO, 1995). However, macroalgae are often the dominant species in hard bottom temperate coastal regions, forming the base of the ecosystem by acting as primary producers (Eklund and Kautsky, 2003), and by providing food, shelter and habitat to a range of other marine organisms (Osborn, 1948; Bidwell, *et al.*, 1998). As such, pollution and toxic impact on macroalgae has far-reaching consequences including bioaccumulation of toxicants (Bryan and Langston, 1992), decreases in species biomass, abundance and diversity (Kevekordes, 2001), or significant shifts in the dominance of a particular species and ecosystem structure (Doblin and Clayton, 1995). Indeed, the ecological importance of macroalgal canopy cover has been investigated by a number of authors (Underwood, 1998; Milazzo, *et al.*, 2004; Schiel, 2004). Decreases in or removal of canopy closure has been found to result in effects on invertebrate and crypto-benthic fish densities (Milazzo, *et al.*, 2004) and shifts in algal community structure (Brown, *et al.*, 1990). To maintain a closed canopy, the brown macroalgae *Hormosira banksii* needs >1 recruit (ca. 2mm in length) per cm^2; however, the number of developing settlers needed to produce these recruits is in the range of 250-750 germlings per cm^2 (Schiel, 2004). So not only is the continued health of mature thalli important, but also the viability of macroalgal gametes is imperative in maintaining canopy cover and preserving ecosystem structure.

Given the ecological importance of macroalgal assemblages, a thorough understanding of the risks single chemicals and wastewater streams pose to macroalgae and the development of methods to assess these risks are vitally

important for the protection of coastal ecosystems. Yet, despite this reasoning, there remains a paucity of toxicological data for Australian macroalgal species and as such, limited data to implement protective measures for these species.

This paucity of macroalgal toxicity data could be a result of early beliefs that invertebrates were more sensitive than aquatic plant species, where it was assumed that any management decision made on the basis of fish and invertebrate testing would automatically protect the associated plant species (eg. Kenaga and Moolenaar, 1979). However, it has since been shown that there was no such universal truth; some plants are more sensitive to toxic impact than invertebrates. For example, Benenati (1990) showed that algae were more sensitive than animal species in 50% of the reports and less sensitive in 30%, while Cairns (1984) and Lewis (1995) both reached the conclusion that sensitivity was dependant on the nature of the chemical as well as the species used in a toxicological study, and that no 'most sensitive' species could hold true over a range of conditions and toxicants. Consequently, there has been wide development of bioassay methods for a range of organisms, and though infrequent, a limited number for Australian macroalgae.

The few methods using macroalgae that do exist in Australia focus predominately on early life stage development (for fucoid macroalgae life cycle (*H. banksii*), refer to Figure 1.1), such as fertilisation success, germination of zygote or zygote mortality (Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Bidwell, *et al.*, 1998; Kevekordes, 2001). Germination has been the most widely used test endpoint, which gauges the effects of contaminants on the developing embryo (also including impacts

on the separate unfertilised gametes, still observed as egg and sperm). Measures of germination inhibition offer biological endpoints of substantial ecological significance as inhibition of these critical life stages will lead to diminished recruitment, population declines and changes in community structure; therefore leading to failure to sustain natural populations (Fletcher, 1992; Burridge *et al.*, 1996; Gunthorpe *et al.*, 1997). Such endpoints have been employed with the Australian macroalgae species *Macrocystis angustifolia* (Burridge *et al.*, 1996), *Ecklonia radiata* (Bidwell, *et al.*, 1998), *Durvillaea potatorum* (Doblin and Clayton, 1995) and *H. banksii* (Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Kevekordes, 2001).

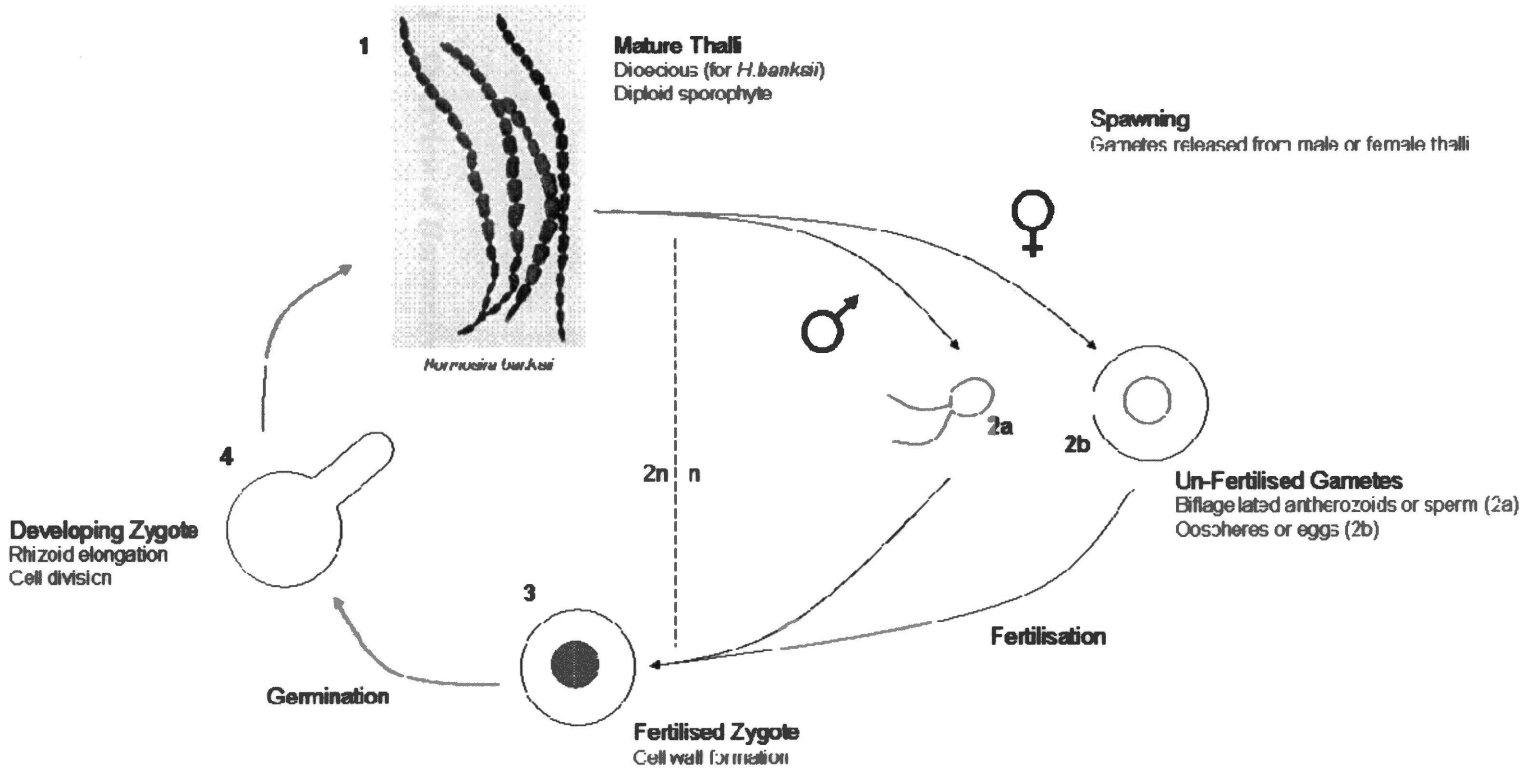

Figure 1.1: Life cycle for the fucoid macroalgae *Hormosira banksii*.

1.3 Role of *Hormosira banksii* in Ecotoxicological Bioassays

Hormosira banksii (Turner) Decaisne (Phaeophyta) is a distinctive, brown macroalga common to the lower intertidal areas of temperate Australia. A review of the literature suggests that it is one of the most studied macroalga in Australia and New Zealand, with more than fifty peer-reviewed articles pertaining to various physiological, ecological and ecotoxicological aspects of *H. banksii* being published from 1948 to 2005, and as such there is considerable information available on it's life history (Osborn, 1948), structure of adult and early life stages (Forbes and Hallam, 1978; Clayton, *et al.*, 1985), different forms and ecotypes (Clarke and Womersley, 1981; Ralph, *et al.*, 1998; Macinnis-Ng, *et al.*, 2005) and use as a test species in toxicity testing (Doblin and Clayton, 1995; Kevekordes and Clayton, 1996; Gunthorpe, *et al.*, 1997; Myers, *et al.*, 2005; Seery, *et al.*, 2006). Following is a brief overview of ecophysiological aspects of, and a summary of the toxicity testing performed to date with, *H. banksii.*

1.3.1 Overview of *Hormosira banksii* Ecophysiological

Hormosira banksii is a brown macroalgae of the order Fucales and is the sole representative of the Family Hormosiraceae. It is endemic to temperate Australasia, being recorded along the southern Australian coastline (Albury, West Australia to Port Macquarie, New South Wales, including north-eastern Tasmania) and the coasts of New Zealand, Lord Howe Island, Norfolk Island, and the Kermadec and Chatham Islands (Osborn, 1948; Womersley, 1987; Miller and Kraft, 1994; Ralph, *et al.*, 1998). The distinctive thallus comprises branched chains of spherical to elongate receptacles (Figure 1.2). While it is generally restricted to exposed rock platforms

and tidal pools of the eulittoral zone, it has also been reported in the sublittoral zone and within the muddy flats of estuaries (King, 1981; Ralph, *et al.,* 1998; Macinnis-Ng, *et al.,* 2005).

Figure 1.2: Photo of *Hormosira banksii* thallus, showing holdfast and receptacles. While foreground of photo is predominately one individual, the nature of canopy formation can be observed with many individuals overlapping to result in coverage. Scale: 2:3cm.

Given the broad geographical range, a considerable degree of morphological variation has been observed. Some six different forms or ecotypes have been described in populations of *H. banksii* (Womersley, 1987). Despite early reports that these ecotypes were in fact different species, which was largely based on receptacle morphology, it is now generally accepted that the various forms are a direct result of habitat acclimation (wave energy, desiccation rates etc.) (Womersley, 1987; Ralph, *et al.,* 1998).

H. banksii is a dioecious alga and is typically reproductive throughout the year. Gametes are released from a diploid sporophyte and gametes can be identified as either 'male' or 'female' by the colour of the extruded packets. Male gametes (antherozoids or sperm) are orange and can easily be distinguished from the green female gametes (oospheres or eggs) (Osborn, 1948). This species has been found to readily spawn under laboratory conditions and can generally be relied upon to provide year-round supply of gametes (with exception to extreme weather events; Gunthope, *et al.*, 1997).

Procedures for obtaining gametes and subsequent fertilization and growth of developing embryos have been well documented (Osborn, 1948; Clarke and Womersley, 1981; Gunthope, *et al.*, 1997), as has gamete structure (Forbes and Hallam, 1978; Clayton, *et al.*, 1985). The considerable amount of information available on *H. banksii* life-cycle and early life stages has proven useful in the development of toxicity testing protocols for this species.

1.3.2 *Hormosira banksii* Ecotoxicology

As has been established above, there has been recognition of the need for macroalgae toxicity data and as such, macroalgae toxicity test protocols have been developed. Of the protocols developed for Australian species, those developed with *H. banksii* are the most prominent (based on citations in peer-reviewed publications: *H. banksii* bioassays have >40 citations, whereas the *Macrocystis angustifolia* and *Ecklonia radiata* bioassays have <10 and <20 citations, respectively). This is most likely because of the comparative wealth of information available on *H. banksii* in

comparison to other macroalgae. Furthermore, it has also been recognised that *H. banksii* "possesses the qualities of a routine bioassay organism" (Gunthorpe, *et al.*, 1995). Though not specific to macroalgae, other authors have compiled lists of attributes an ideal test species would have. Table 1.1 is compiled from Environment Canada (1995) and Calow (1993) and summarises the attributes sought after in an ideal test species.

While these characteristics should be sought after, very rarely are all of them present in one organism. Furthermore, some of these characteristics that make an organism suitable are, paradoxically, the very reason why they can not be used as a test species. For instance, ecological importance may be as a result of a rare or endangered status, thus precluding them from being used as a test species (Calow, 1993). However, *H. banksii* does indeed fit the majority of these criteria (Table 1.1) and subsequently has been incorporated into a number of toxicity studies.

Table 1.1: Desirable characteristics of an ideal test species for ecotoxicological testing. Compiled from Environment Canada (1995) and Calow (1993).

Desired Charateristic	Present in *Hormosira banksii*	Reference
Demonstrated contaminant response	Yes	Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Kevekordes and Clayton, 1996
Commercial, recreational, or ecological relevance	Yes	Underwood, 1998
Amenable to laboratory culture/techniques	Yes	Osborn, 1948
Previous bioassay use	Yes	Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Kevekordes and Clayton, 1996; Kevekordes, 2000; Kevekordes and Clayton, 2000; Kevekordes 2001; Myers, *et al.*, 2005
Readily available	Yes	Gunthorpe, *et al.*, 1995
Short life cycle	No (adult) Yes (gamete viability)	Osborn, 1948
Well known life history	Yes	Osborn, 1948
Distribution within area of interest	Yes	Gunthorpe, *et al.*, 1995; Ralph, *et al.*, 1998
Representative of ecosystem	Yes	Underwood, 1998
Ease of collection and handling	Yes	Osborn, 1948; Gunthorpe, *et al.*, 1995
Known target organisms	Yes	Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Kevekordes and Clayton, 1996

H. banksii bioassays started to appear in the literature by the mid 1990s (Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Kevekordes and Clayton, 1996) and they were all based on assessment of the development of morphological features during early life stages. The methods described in Doblin and Clayton (1995) included assessment of zygote germination, embryo growth and embryo mortality as indicators of the effect of secondarily-treated sewage. The methods in Gunthorpe, *et al.*, (1995) used fertilisation of *H. banksii* gametes as the endpoint for toxic impact (of phenol, 4-chlorophenol and hexavalent chromium), while Kevekordes and Clayton (1996) described a number of different life stages that could be measured over 14 days to form the basis of a bioassay system. All of these publications demonstrate the success of *H. banksii* as a test species used in various bioassay systems. Subsequent publications include: Gunthorpe, *et al.*, 1997; Kevekordes, 2000; Kevekordes and Clayton, 2000; and Kevekordes, 2001, which all use the above methods as published or very close variations thereof. The most recent to be published is Myers, *et al.*, 2005, where a germination endpoint is investigated and standarised for *H. banksii* using four reference toxicants (ammonia, phenol, copper and zinc).

As previously noted, the disadvantage of these endpoints is that protocols assessing early life stages such as germination or embryo growth require 24-72 h incubation before they can be assessed. This period of time is often too long if urgent management decisions are reliant on a result, such as after a contamination event (for example a spill or other unplanned release of potentially toxic chemicals or wastewaters). Though the fertilisation endpoint described in Gunthorpe, *et al.*, (1997) can be assessed after 8 h, it has been noted that this bioassay is labour intensive and

can give variable results (Myers, *et al.*, 2005). This observed variability may be due to the fact that *H. banksii* eggs have the capacity to develop a primary cell-wall parthenogenetically (Clayton, *et al.*, 1998). This confounding effect and subsequent variability is particularly important in the context that there is a tendency to use variability as the single most important determinant on which the value of a bioassay is judged (Myers, *et al.*, 2005).

Another recognised shortcoming of the existing bioassay protocols based on morphological events is the lack of experimentally confirmed eco-relevance (Burridge and Bidwell, 2002). It is a an accepted logical progression that inhibition of early life stages will lead to diminished recruitment, population declines and a failure to sustain natural populations (Fletcher, 1992; Burridge *et al.*, 1996; Gunthorpe *et al.*, 1997). Yet there is no experimentally-derived evidence to support that this progression is actually what occurs in pollution impacted populations of macroalgae. Thus, a major future research direction, and current gap in knowledge, is the investigation into the relationships between contaminant effects on early life-stages of macroalgae as they relate to population and ecosystem structure, which would in turn give valuable confirmation that the logical progression suspected as a result of early life-stage inhibition does indeed hold true (Anderson, *et al.*, 1998).

A rapid bioassay that can yield environmentally relevant results with regard to toxicant concentrations and biologically 'meaningful' effects with regard to endpoint, would be a valuable addition to the armoury of macroalgal ecotoxicology and more economically viable from a commercial aspect.

1.4 Chlorophyll *a* Fluorescence – A Measure of Photosynthesis

Photosynthesis is one of a plant's most fundamental processes and chlorophyll *a* fluorescence is a well-established technique in the field of plant physiology that enables rapid analysis of photosynthetic activity.

Fundamentally, chlorophyll *a* fluorescence is a simple, effective means of measuring the amount of absorbed energy used in photochemical processes (photosynthesis). Each quantum of light absorbed by a chlorophyll *a* molecule raises an electron from the ground state to an excited state. This excitation energy can be used in one of three ways (Figure 1.3); 1) it can be transferred down through the electron transport chain to ultimately fix carbon (photochemical quenching), 2) it can be dissipated as heat (non-photochemical quenching, NPQ), or 3) it can be re-emitted at a slightly longer wavelength in the form of fluorescence (< 5% of absorbed energy). Thus, chlorophyll *a* fluorescence enables a wide range of photochemical processes linked with photosynthesis to be monitored, as fluorescence emission is complementary to the alternative pathways of de-excitation. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation, which in turn provides an insight into the organism's overall "health".

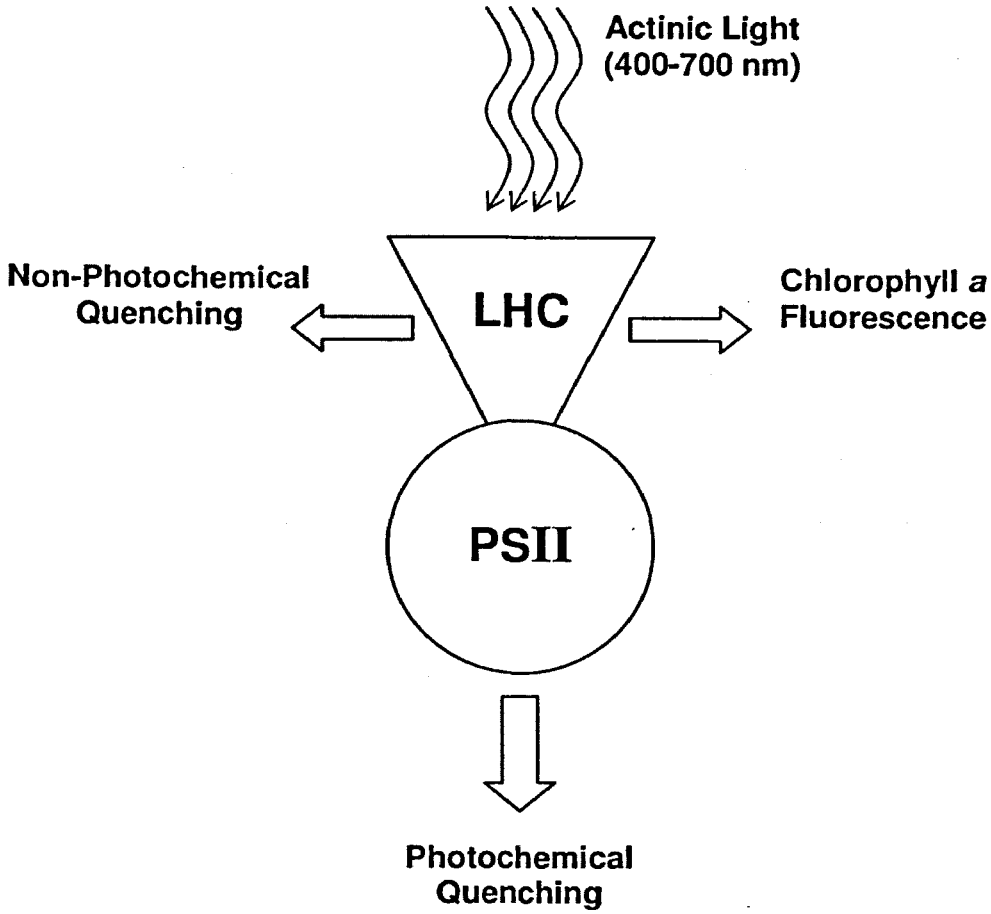

Figure 1.3: De-excitation pathways for Photosystem II (PSII; LHC represents light harvesting complex).

Chlorophyll *a* fluorescence provides biologically significant information: a pollutant that affects a plant's capacity to photosynthesise will inevitably lead to a reduction in production of photosynthates and electrochemical reductants (ATP/NADPH) which are essential to plant metabolism and growth (Krause and Weis, 1991). Therefore, changes to chlorophyll *a* fluorescence can be used as the basis of an ecotoxicological indicator. Indeed, Miles (1991) suggested a simple ecotoxicology test using a variety of measured chlorophyll *a* fluorescence parameters as endpoints to assess the magnitude of toxic impact.

1.4.1 Analysis of Chlorophyll *a* Fluorescence Signal

PAM Fluorometry

Improvements in the technology of optics and photonics have facilitated the development of Pulse Amplitude Modulated (PAM) fluorometers (Schreiber, 1986; Schreiber, *et al.*, 1986) and their increasingly effective use in the analysis of photosynthetic efficiency. PAM fluorometers are commonly used instruments that measure chlorophyll *a* fluorescence via an amplifier selectively synchronised with a modulated light source (Schreiber, 2004). These instruments are capable of providing information on dark-light transitions and also the steady-state performance of photosynthesis in continuous light (Schreiber, 2004).

Fluorescence Parameters

Dark-adaptation of a sample for several minutes (10 – 20 min) before fluorescence measurement allows the photosystem II (PSII) reaction centres to open or become fully oxidised. In turn, the electron transport chain also becomes fully oxidised, photoprotective mechanisms become relaxed, and the pH gradient across the thylakoid membrane is depleted. Upon application of a saturating flash (8000 μmol photons m^{-2} s^{-1} for < 1 s), fluorescence signal rises from the ground state value (given as F_o) to its maximum value, F_m. At this time, Q_A, the plastoquinone molecule acting as the first electron acceptor of PSII, is fully reduced. This allows the determination of maximum quantum yield of PSII ($F_v/F_m = [F_m-F_o]/F_m$). F_v/F_m is a measure of the maximum PSII photochemical efficiency. A decrease in the F_v/F_m ratio can be as a result of an increase in minimum fluorescence (F_o) and/or a decrease in maximum fluorescence (F_m) or a decrease in both parameters. A drop in F_v/F_m is a sign that the

photosystems are under some sort of stress, that is, the lower proportion of reaction centres that are available results in decreased photosynthetic efficiency (Schrieber, 2004).

Effective quantum yield of PSII ($\Delta F/ F_m' = [F_m'-F]/F_m'$) is similar to maximum quantum yield, except that F_m' (maximum fluorescence) and F (minimum fluorescence) are both measured under ambient light conditions. The primary difference between the two quantum yields is that $\Delta F/ F_m'$ requires the photosystems to be operational, which means that the sample is exposed to some level of light rather than being dark-adapted. Interpretation of $\Delta F/ F_m'$ also provides an indication of the amount of energy used in photochemistry and while $\Delta F/F_m'$ values will be lower than F_v/F_m values (due to influence of non-photochemical quenching (NPQ)), for toxicological investigations, $\Delta F/ F_m'$ would usually be the preferred measure of quantum yield (Ralph, *et al.*, 2005).

This is because in controlled laboratory experiments, where the light conditions are consistent, $\Delta F/ F_m'$ is a more sensitive indicator of toxic impacts than F_v/F_m, particularly for PSII herbicides (Juneau and Popovic, 1999; Macinnis-Ng and Ralph, 2003). The dark acclimation period required to measure F_v/F_m removes the light stress from the PSII reaction centres, reducing the influence of non-photochemical quenching. Therefore, F_v/F_m measures the optimal photosynthetic efficiency (by removing light stress) and doesn't reflect the true nature of PSII activity under normal light conditions.

Further details on the principles of chlorophyll *a* fluorescence, and how they relate to issues beyond ecotoxicology, are beyond the scope of this thesis, but can be found in numerous review articles (e.g. Krause and Weis, 1991; Schreiber, *et al.*, 1994; Schreiber, 2004); see also Govindjee (1995) for a historical overview.

1.5 Chlorophyll *a* Fluorescence as Indicator of Toxic Impact

Most of the early 'pollution physiology' work using chlorophyll *a* fluorescence techniques was focussed on herbicides that were designed to specifically target the photosynthetic apparatus. This is based on the fact that when a toxicant interrupts the process of electron transport, the fluorescence emission will change. For example, the phenylurea herbicide, Diuron (DCMU) reversibly inhibits photosynthetic electron flow to the plastoquinone in photosystem II (PSII) by blocking the electron transport chain just after the primary electron acceptor (Q_A) (Miles, 1991; Falkowski and Raven, 1997). This process causes a simultaneous decrease in photochemical (qP) and non-photochemical quenching (NPQ), which allows fluorescence to be an incredibly sensitive and effective means of measuring Diuron impact (Brack and Frank, 1998). Indeed, it has been approximated that 50% of all herbicides are designed to act on or target PSII in a similar fashion to that described above (Koblizek, *et al.*, 1998), so fluorescence is an ideal measure for more than half of the herbicides in use today.

The photosynthetic impact of metals is not as clearly defined as herbicides, although a direct effect on PSII has been well established. Metal impacts on PSII have been thoroughly examined (Mohanty, *et al.*, 1989; Baron, *et al.*, 1995; Kupper, *et al.*,

2002), but a clear understanding of specific impact sites still remains controversial. For example, Baron, *et al.*, (1995) conducted a review of the relationship between copper and photosystem II, summarising the research which has tried to determine the impact sites of copper at toxic concentrations. They found most authors agreed that the target site of Cu impact on PSII was at its oxidizing side. However other authors have suggested Cu impact sites include a target at the Pheo-Q_A-Fe^{2+} domain, the PSII reaction centre, the Hill reaction and water splitting.

Irrespective of the specific impact sites, fluorescence parameters have been shown to be an effective measure of metal phytotoxicity (Samson and Popovic, 1988; Ralph, *et al.*, 2005). Apart from direct impacts on PSII, metals have also been found to inhibit CO_2 fixation (Clijsters and Van Assche, 1985), photophosporylation (Ceniceros-Gomez, *et al.*, 1999) and enzyme activity (Franklin, *et al.*, 2001), as well as the reduction of chlorophyll content (Prasad and Strzalka, 1999; Clijsters and Van Assche, 1985). These indirect processes may also be detected by fluorescence parameters, but with less certainty.

Similarly, the mechanism of action and impact sites of petrochemicals and their associated breakdown products are still poorly understood. However, toxic impacts of oils have still successfully been detected using fluorescence parameters in microalgae (Brank and Frank, 1998), seagrass (Ralph and Burchett, 1998; Macinnis-Ng and Ralph, 2003), freshwater macrophytes (Huang, *et al.*, 1997; Babu, *et al.*, 2002; Marwood, *et al.*, 2001) and phytoplankton (Marwood, *et al.*, 1999). While it has been suggested that oils exposed to light impact on photosystem I, not PSII, the

resulting blockage in electron transport ultimately results in photo-oxidative stress on photosystem II which will be observed by fluorescence parameters (Huang, *et al.*, 1997).

So although the mechanisms of action and specific impact sites remain unconfirmed for some pollutant classes, PSII (which can effectively be monitored using chlorophyll *a* fluorescence) is generally the most sensitive target site in the photosynthetic arrangement (Baron, *et al.*, 1995). As such, fluorescence parameters can effectively demonstrate a dose-response relationship with a variety of toxicants.

1.5.1 Current Applications of Chlorophyll *a* Fluorescence in Ecotoxicology

Chlorophyll *a* fluorescence has only recently been seen as an effective endpoint in ecotoxicology (Cullen, *et al.*, 1986; Samson and Popovic, 1988; Miles, 1991), even though it has been used for approximately twenty years in aquatic and even longer in terrestrial ecophysiological studies of pollution impacts. Some of the main advantages of chlorophyll *a* fluorescence are that it is non-invasive, non-destructive and data are simple to collect. These traits have seen the application of fluorescence as a bioassay endpoint progress rapidly over the past couple of years. This has resulted in a range of methods for conventional bioassays (Miles, 1991), monitoring plant health in polluted ecosystems (Udy and Dennison, 1997), detecting pollutants in water samples using chlorophyll *a* fluorescence as part of a biosensor (Vedrine, *et al.*, 2003; Bengtson-Nash, *et al.*, 2005), and fluorescence methods have been applied *in situ* (Macinnis-Ng and Ralph, 2002). A number of comparisons conducted between chlorophyll *a* fluorescence bioassays and other, well-known, standard

ecotoxicological methods (chiefly growth or morphological endpoints) have confirmed the sensitivity and expediency of chlorophyll *a* fluorescence bioassays (for example, Samson and Popovic, 1988; Radix, *et al.*, 2000; Seery, *et al.*, 2006).

During the initial developmental phase of chlorophyll *a* fluorescence ecotoxicology, research was directed to understanding the impact of the pollutant on the photosynthetic processes of aquatic phototrophs and the subsequent impact on growth and mortality (Miles, 1991; Juneau and Popovic, 1999). Further research saw increased complexity in the analysis of chlorophyll *a* fluorescence endpoints. Effects of light intensity, species selection, exposure period and the choice of the most appropriate fluorescence parameter were the basis of many studies. Conrad, *et al.*, (1993) examined a number of variables that influenced the sensitivity of an aquatic chlorophyll *a* fluorescence bioassay and found microalgal culture age and density, test light conditions, and water chemistry can influence the fluorescence response to a toxicant. El Jay, *et al.*, (1997) also demonstrated the effect that the test light intensity can have on the sensitivity of the fluorescence response. The effect of light intensity has been further investigated by a number of authors (Kupper, *et al.*, 2002; Vavilin, *et al.*, 1995; Peterson and Kusk, 2000; Artetxe, *et al.*, 2002) and is now recognised as a critical aspect of defining standardised protocols for chlorophyll *a* fluorescence bioassays.

The use of different fluorescence parameters in determining the most sensitive endpoint has been a main focus of recent research. Brack and Frank (1998) highlighted the benefits of using different chlorophyll *a* fluorescence parameters as

an endpoint to distinguish between classes of pollutants based on their modes of action. From an investigation of 21 pollutants, including nitro aromatic compounds, aldehydes, volatile organic compounds and herbicides, it was found that different compounds gave different fluorescence responses which could be measured from a number of parameters. They suggested that the detailed analysis of fluorescence patterns may be of great help in identifying phytotoxic pollutants in complex samples. Furthermore, Juneau, *et al.,* (2001) stated that the significance of different parameters for particular pollutants "may confirm again that the sensitivity and usefulness of different fluorescence indicators is highly dependent to specific pollutant inhibitory mechanisms" and the choice of the parameter will be dependent on the pollutant. The use of specific parameters for the determination of toxicity for particular pollutants has been further supported by a number of studies (Dorigo and Leboulanger, 2001; Frankart, *et al.,* 2002; Juneau, *et al.,* 2003).

What is also evident from recent publications and continued development of fluorescence ecotoxicology is the expanding range of test species. Chlorophyll *a* fluorescence has been applied to a range of test species; to date, these include numerous species of microalgae (including phytoplankton and periphyton), macrophytes (seagrass and freshwater species), and coral symbionts (Ralph, *et al.,* 2005). The majority of species used in chlorophyll *a* fluorescence bioassays have been microalgae, most likely due to their ease of use in testing and culturing, their sensitivity to pollutants and their fast growth rate and generation time. While chlorophyll *a* fluorescence has been used to assess the effects of UV light on macroalgal gametes (Coelho, *et al.,* 2001), before this body of work was undertaken,

there were no studies that had used chlorophyll *a* fluorescence to investigate the effects of pollution on macroalgal gametes.

1.6 Research Objectives and Significance

The primary objective of this thesis is to develop and describe a bioassay protocol that uses adaptations on past fertilisation assay techniques (Gunthorpe, *et al.*, 1995) to allow the photosynthetic capacity of *H. banksii* gametes to be assessed by chlorophyll a fluorescence measurements. This new protocol will allow for rapid assessment of toxic impact, which in turn allows for timely management decisions to be made. This is significant in that past macroalgal bioassay systems have necessitated up to a 48 h delay before results are available to decision makers.

In addition to the development of a new bioassay protocol, other aims of this work include demonstrating a level of eco-relevance for chlorophyll *a* fluorescence endpoints and using the newly developed bioassay in applied research. These secondary aims are significant in that eco-relevance has not previously been experimentally demonstrated for any chlorophyll *a* fluorescence endpoint in ecotoxicology, and the use of this bioassay in assessing interactive toxicity of mixtures shows successful application and validates the methodology.

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Chapter 2

2.0 *Development and Optimisation – Including Common Protocols*

2.1 Introduction

Ultimately, the aim of this PhD project was to develop a commercially viable bioassay. To achieve this, a number of elements had to be investigated, such as species selection, selection of macroalgal population, adaptation of existing methods for spawning macroalgae, optimisation of fluorescence technique to measure dose-response relationships, and finally developing a workable protocol that achieved all the requirements necessary of a rapid, commercial bioassay; these requirements being rapidity of result, sensitivity to a range of toxicants, reproducibility, etc. Often, this was an ongoing process where elements were sequentially improved over time, or shifts to an alternative methodology were made to better accommodate these requirements. However, there are many elements that are common to the methods described throughout this thesis and as such they are collated here for brevity (although Chapters that are reproduced from published or submitted papers necessitate some repetition). This chapter will also detail some of the technical details unique to particular methods such as instrument settings and modifications.

2.2 Development and Optimisation

2.2.1 Population Selection

As was established in Chapter 1, *H. banksii* has been proven to be a reliable and effective test species for ecotoxicological investigations (Doblin and Clayton 1995; Gunthorpe, *et al.*, 1995; Kevekordes and Clayton, 1996; Gunthorpe, *et al.*, 1997;

Kevekordes, 2000; Kevekordes and Clayton, 2000; Kevekordes 2001; Myers, *et al.*, 2005). While *H. banksii* fits the requirements of a test species (Table 1.1) and is also readily available to the eventual end-users of the developed bioassays (at PIRVic's ecotoxicology laboratory based at Queenscliff, Victoria; ARC Linkage Industry Partner), the developmental work was based at the UTS laboratory in Sydney, New South Wales. This required the identification of a population of *H. banksii* to be located in NSW that was in close proximity to the UTS laboratories.

The two geographically closest populations of *H. banksii* are situated at Fairlight Beach (151°16'E, 33°47'S) and Dee Why (151°18'E, 33°45'S) (Figure 2.1). However, these populations were too small, with less than 100 mature plants each, and given the frequent collection that would be involved over a 3 year experimental period (PhD candidature) would most likely decimate these populations. Obviously, this was deemed to be too ecologically threatening and so other populations were sought.

Previous studies performed at the UTS laboratory on ecophysiological attributes of *H. banksii* (Macinnis-Ng, *et al.*, 2005) were done with samples collected from Paradise Beach, Pittwater (151°19'E, 33°37'S) (Figure 2.1). However, it was concluded that the Paradise Beach samples were infertile, as gametes could not be extruded from numerous samples collected and as such were unsuitable for use in this project. Literature shows that some populations of *H. banksii*, normally those associated with estuarine or mangrove environments with limited wave action, can be infertile and reproduce via fragmentation (King, 1981). Vegetative reproduction

(i.e. fragmentation) was possible given that Paradise Beach is in fact situated within an estuary with limited wave action, but it was also noted that this beach experiences considerable stormwater run-off from adjacent residential properties which may result in undesirable effects on the variability of the bioassay. These considerations necessitated further searching for a healthy, gamete producing population of a size that would allow for frequent collection.

A *H. banksii* population between Newport and Bilgola beaches (151°19'E, 33°38'S) was found with abundant macroalgae (>1000's) which was sufficiently large enough to supply frequent samples (Figure 2.1, 2.2). There were no immediate pollution sources identified at the headland (such as stormwater or sewage outfalls), and subsequent testing showed reliable, healthy gamete release from adult *H. banksii*.

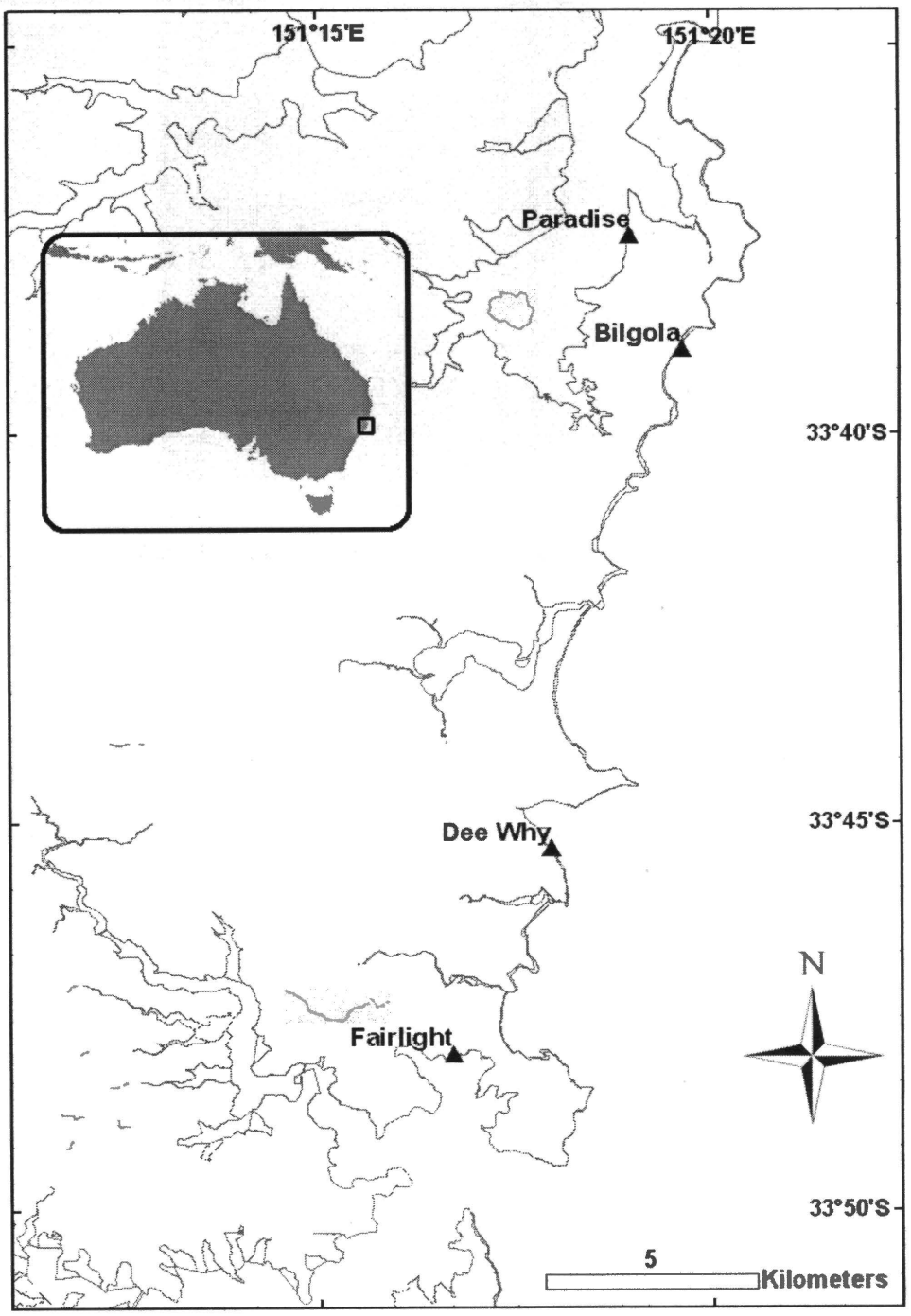

Figure 2.1: New South Wales coastline showing Sydney beaches: Dee Why, Fairlight Beach, and Paradise Beach.

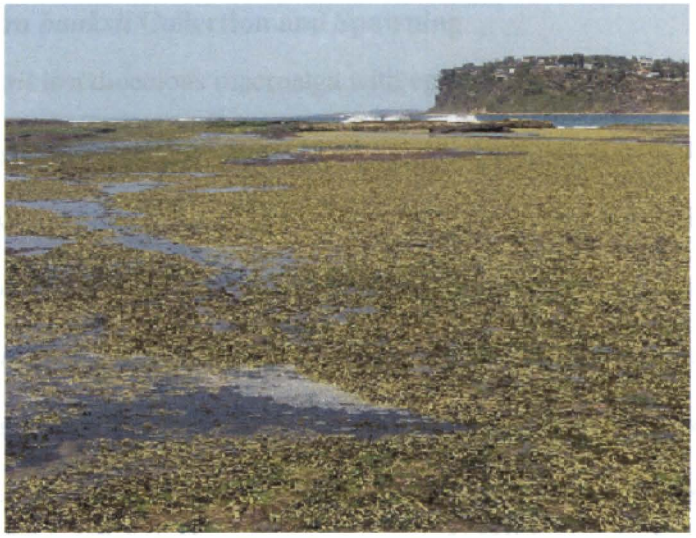

Figure 2.2: Photograph of Bilgola rock platform showing vast population of *Hormosira banksii*.

2.2.2 Toxicants and Solutions

All toxicant solutions were prepared from analytical grade chemicals, as detailed in each chapter. All references to seawater (or filtered seawater) are in reference to the UTS supply, which is collected from Rose Bay, Sydney (151°20'E, 33°31'S), and stored in 5000 L cement tanks. When this seawater was used in experiments described within this thesis, it was filtered (5 µm) and aerated for at least 24 h prior to use. Chemical analysis performed on stock supply showed common contaminant levels to be negligible or below detection limit. Additionally, dissolved oxygen, pH, salinity and temperature were measured at conclusion of all experiments and values are stated within each chapter.

2.2.3 *Hormosira banksii* Collection and Spawning

Hormosira banksii is a dioecious macroalga with eggs or sperm released from a diploid sporophyte after each low tide (Osborn, 1948). Samples of adult thalli were collected approximately three hours before low tide, to avoid natural desiccation-induced release of gametes, from Bilgola Beach, north of Sydney, Australia (location as detailed above). Approximately 50 strands of *H. banksii,* where a strand represents a portion containing 5–20 receptacles of a mature individual, were collected haphazardly from the intertidal sandstone reef and placed in an inert plastic bag for transportation to the laboratory. In the laboratory, the strands were arranged flat on paper towel and allowed to dry for 2 h, after which time they were placed in brown paper bags and stored for 12-18 h at 4°C before use (as per the Gunthorpe, *et al.,* (1995) method detailed below).

Spawning of gametes was performed following the method described by Gunthorpe, *et al.,* (1995). To initiate spawning of gametes, *H. banksii* strands were removed from storage (4°C) and these chilled individuals were soaked in approximately 1000 mL of warm seawater (30 – 40°C) for 20-30 s with gentle agitation. The washed macroalgae were then arranged on a clean, dry surface so as not to be touching each other, and gamete extrusion normally occurred within 3–10 min.

Upon spawning, gametes are identified as either male or female by the colour of the extruded 'packets'. Female gametes (eggs) are green and can be distinguished from the orange male gametes (sperm) (Osborn, 1948). The strands that exhibited spawning were placed into either the sperm or egg designated beaker, each with approximately 150 mL filtered seawater (20°C). Approximately 200 receptacles from

actively spawning strands were put into each beaker to give final solutions of approximately 4 x10^3 eggs mL^{-1}, and approximately 2 x10^6 sperm mL^{-1}. Egg counts were made on a Sedgwick-Rafter cell (Myers, *et al.*, 2005) and sperm counts were performed with a haemocytometer (Seery, *et al.*, 2006). Any further dilutions of gamete stock solutions were performed as required and are detailed below in protocols.

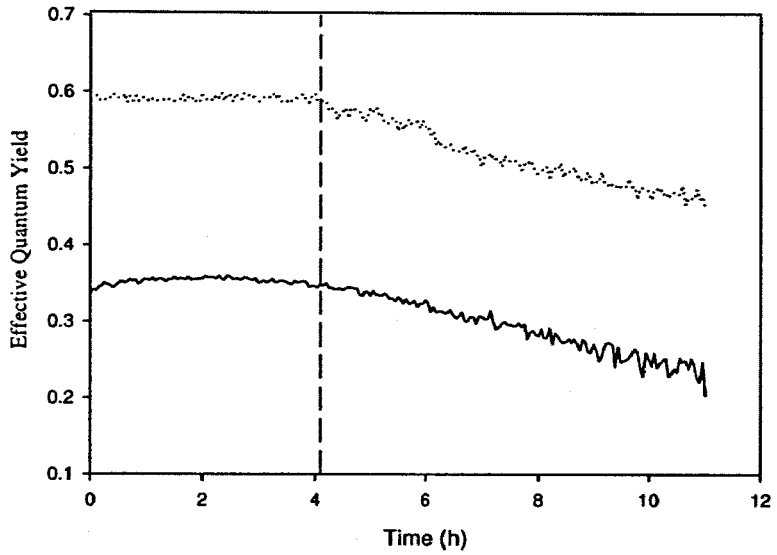

Figure 2.3: Effective Quantum Yield of *Hormosira banksii* gametes over time, as measured with ToxY-PAM at 5 min intervals. Cuvettes contained 1 mL of either sperm (2 x10^6 sperm mL^{-1}) or egg (4 x10^3 eggs mL^{-1}) at 20°C. Solid line represents sperm, dashed line represents eggs.

These stock gamete solutions were filtered through a 200 µm mesh to remove gross plant material. The egg solution (stored under ambient laboratory conditions: 20 ± 1°C, 5 ± 2 µmol photons $m^{-2}s^{-1}$) was used within 40 mins and the sperm solution (stored in dark) within 20 mins. These storage conditions are based on those used in previous studies (Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995) where it was

reasoned that sperm are much more sensitive to light than eggs and need to be kept in the dark. The maximum storage times of 40 and 20 min for eggs and sperm, respectively, are also based on previous studies (Doblin and Clayton, 1995; Gunthorpe, *et al.*, 1995; Myers, *et al.*, 2005), to ensure viability of gametes. However, Figure 2.3 shows that both sperm and eggs remain photosynthetically viable for more than 4 h, which ensures consistent health and viability for duration of the fluorescence bioassays.

2.2.4 The ToxY-PAM Fluorometer

Instrument Overview

ToxY-PAM (Walz GmbH, Effeltrich, Germany) is a dual-channel fluorometer specifically developed for detection of phytotoxic substances in water samples containing phytoplankton or isolated thylakoid membranes (Schreiber, *et al.*, 2002), where channel 1 measures the treatment sample and channel 2 measures the control. Effective quantum yield of PSII ($\Delta F/F_m' = [(F_m'-F)/F_m']$) is calculated for each channel by measuring the minimum fluorescence (F1, F2 where the number denotes channel 1 or 2) and the maximal fluorescence ($F_m'1$, $F_m'2$) taken during a 0.4 s saturating pulse of blue light (2000 µmol photons $m^{-2}s^{-1}$ at 470 nm). $\Delta F/F_m'$ values for each channel are represented as Y1 or Y2 and these values are used to calculate %PSII Inhibition = 100x[(Y2-Y1)/Y2], which corresponds to percent inhibition of the treatment relative to the control sample.

Modifications for use with **Hormosira banksii** *gametes*

The ToxY-PAM was developed for use with microalgal suspensions where the cells are assumed to remain in suspension for the duration of the measurements (Schreiber, *et al.,* 2002). The fast settling rates of *H. banksii* eggs (0.93±0.06 cm min^{-1}) (Forbes and Hallam, 1978) resulted in the eggs collecting at the base of the cuvette. With the fluorescence measurement field of the ToxY-PAM focussed at a point 4 mm above the fluorescence detector, the eggs were consistently falling below the plane of focus which resulted in poor signal strength. With stirrers (commercially available for other PAM instruments) proving ineffective, it was found that elevating the cuvette so that the collected eggs lay within the measurement field resulted in stronger fluorescence signal and improved measurement of effective quantum yield (Figure 2.4 A and B). Thus, the negatively buoyant eggs necessitated the use of custom-built collars to raise the base of the cuvette and position the eggs within the measurement field (Figure 2.5).

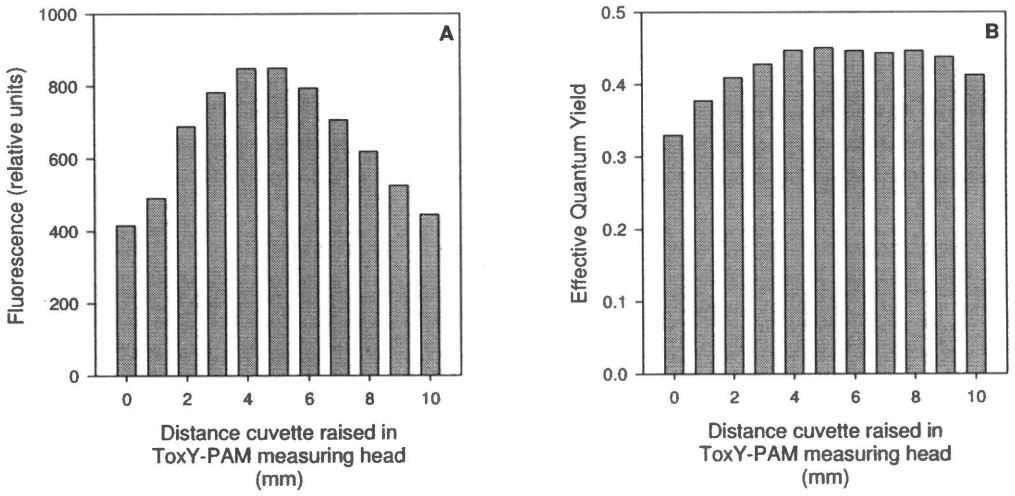

Figure 2.4: Fluorescence signal (A) and effective quantum yield (B) of *Hormosira banksii* eggs with increasing elevation of cuvette (n=1).

Figure 2.5: Left: Diagram of custom-built collars used to elevate cuvettes and raise collected eggs into fluorescence measurement field. Right: Comparison between cuvette with and without collar as used with the ToxY-PAM unit. Apex of each red triangle represents the focussed point of fluorescence measurement from the photomultiplier (PM).

General settings for ToxY-PAM

While ToxY-PAM lacks the flexibility in user-defined settings that some of the other PAMs provide, there are still some important parameters that need to be optimised in order to ensure valid data are collected. The following is a list of the parameters available to ToxY-PAM users (adapted from Schreiber, 2001; Schreiber, *et al.*, 2002) and details the settings used for all measurements of *H. banksii* gametes (eggs and sperm).

Measuring Light (ML) Frequency: this parameter defines the frequency of the 5 μs pulses of the measuring light. It effectively controls the intensity of the measuring light, which increases linearly with the ML Frequency. That is, with ML Frequency set at 4, the intensity equates to 4 μmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). The measuring light intensity employed by ToxY-PAM is relatively higher compared to that of other PAMs in order to induce slight photosynthetic activity, so that inhibition at any step in the electron transport chain will cause a drop in $\Delta F/ F_m'$. ML Frequency was set at 4 for all ToxY-PAM measurements of *H. banksii* gametes.

Saturation Width: This parameter controls the width (or time) of saturation pulses applied in the assessment of F_m values. The standard setting of 0.4 s usually ensures reliable F_m values will be obtained. However, with light sensitive samples, repetitive F_m measurements using a longer saturation pulse may result in a gradual decrease observed in $\Delta F/ F_m'$. The standard setting of 0.4 s was employed for all saturation pulses on *H. banksii* gametes and no decrease in $\Delta F/ F_m'$ was observed with repeated measures.

Sampling Time: Sampling time is the time period over which the fluorescence yields of each channel are averaged to give F1 and F2. At a given ML Frequency, increasing the sampling time will result in increased accuracy of F1 and F2 measurements, although it is accompanied by more time being required for a single measurement. The standard setting of 3 s was used for all *H. banksii* gamete fluorescence measurements.

Clock Time: This feature allows for a set number of measurements to be taken automatically at defined intervals. For example, it can be set to take 3 saturating pulses with a 30 s interval in between each pulse, as was the case for the *H. banksii* bioassays.

2.2.5 Common Protocols

The process of developing a fluorescence bioassay using *H. banksii* gametes involved investigating the suitability of both eggs and sperm to act as the photosynthetic material required for fluorescence assessment. A bioassay using eggs was first investigated as their green colouring immediately suggested sufficient chlorophyll content for fluorescence measurements. Sperm, however, was also investigated, as testing showed that sperm was more sensitive to a greater range of toxicants (such as trace metals); thus broadening the range of toxicants the bioassay would be effective in assessing. This resulted in two protocols that are similar, but given the different requirements of egg versus sperm, there are significant differences in some of the specifics. As such, the two protocols are described separately below.

Fluorescence bioassay protocol* – Hormosira banksii *eggs

For this fluorescence bioassay, each test chamber consisted of a 20 mL glass vial with 5 mL of test solution. The bioassay consisted of 3 replicates of each test concentration (at least 5), a seawater control and a solvent control, where appropriate. The bioassay was initiated by adding 500 µL of 4 $x10^3$ eggs mL^{-1} gamete solution to each test chamber (already containing the test solution). The vials

were collectively covered with clear plastic film and left in an incubator (Labec, Sydney, Australia) for 2 h at 18 ± 0.5°C under 23 ± 2 µmol photon $m^{-2}s^{-1}$ illumination. These exposure conditions are taken from Gunthorpe, *et al.,* 1995, and the exposure period of 2 h is inline with keeping the total time-to-result under 8 h; thus allowing for a result within one working day and fulfilling one of the primary requisites of the developed bioassay.

After the 2 h exposure period, eggs from the seawater control were transferred to a cuvette via glass pipette to give a total liquid volume of 1-1.5 mL and a minimum fluorescence reading (F2) of between 800 and 1500 (relative units) as determined by ToxY-PAM. The fast sinking rates of *H. banksii* eggs resulted in inconsistent egg concentration being drawn from shaken treatment vials, so in order to reduce this variability, the fluorescence signal (F) was used as a proxy for chlorophyll content and thus egg concentration. The range of 800 to 1500 was found to be the range that corresponded to the egg concentration giving the highest and most accurate $\Delta F/ F_m'$ readings (Figure 2.6).

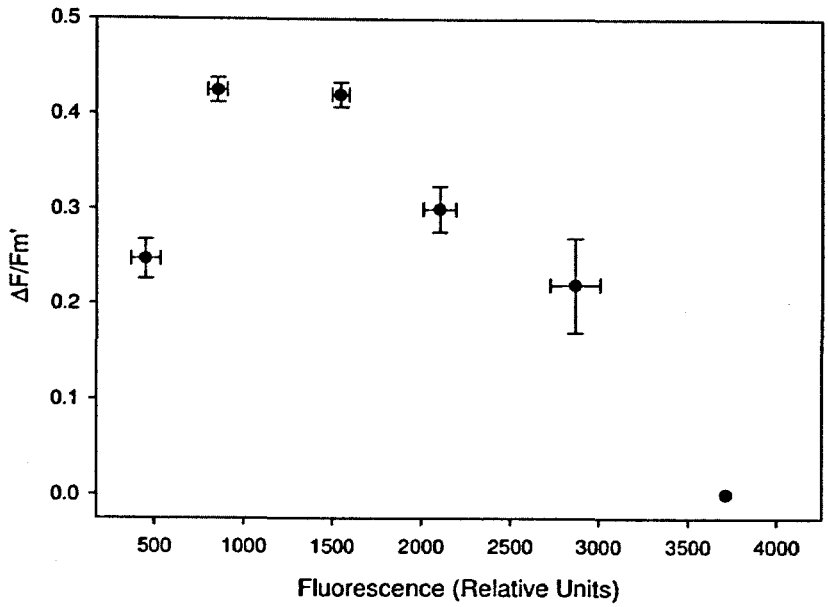

Figure 2.6: Effective quantum yield ($\Delta F/ F_m'$) of *Hormosira banksii* eggs in relation to fluorescence signal (F) being used as proxy for egg concentration. Error bars represent standard deviation (n=3).

It should be noted, however, that the low $\Delta F/ F_m'$ values observed in Figure 2.6 at higher fluorescence levels is not a physiological effect of egg concentration but rather a limitation of the PAM electronics. The maximum capability of fluorescence detection is approximately 4000; therefore, samples with F above 3000 will have falsely lowered F_m values (being recorded as 4000 despite in actual fact being much higher) which in turn gives inaccurately low $\Delta F/ F_m'$ values. In contrast, the lower $\Delta F/ F_m'$ obtained with low starting F values does appear to be a concentration effect, with insufficient photosynthetic material available for accurate fluorescence measurement. The range of 800 – 1500 avoids both of these problems and ensures accurate fluorescence measurements.

When the appropriate amount of eggs had been added to the measurement cuvette to give minimum fluorescence between 800 and 1500, a saturating pulse was performed to obtain effective quantum yield ($\Delta F/F_m'$). If this value was above 0.45, the control eggs were deemed to be healthy and thus used as the reference solution. This health criterion was determined with >300 measurements of control egg solutions, where the mean $\Delta F/F_m'$ was 0.45 ± 0.01. If the seawater control failed to give an $\Delta F/F_m'$ greater than 0.45, test results were discarded, as the eggs or seawater were considered to be of poor quality and unsuitable for use in the bioassay.

Once the confirmation of control $\Delta F/F_m'$ had been performed, eggs from each test chamber were transferred, in turn, to the test cuvette, using the volume determined during the control check, for fluorescence measurement. A % PSII Inhibition measurement, as described above in Chapter 2.2.4 (Instrument Overview), consists of comparative saturating pulses to both the reference and test cuvette. Three pulses were given to each replicate to give a mean % PSII Inhibition for each replicate. These values were then used as the response variable, from which dose-response curves were fitted.

Fluorescence bioassay protocol* – Hormosira banksii *sperm

This fluorescence bioassay comprised at least 5 test concentrations, a zero treatment, a solvent control (if applicable) and a reference control (used in Toxy-PAM's channel 2, explained in detail below). Each test chamber consisted of a 20 mL glass vial containing 5 mL total test solution (sperm and toxicant solutions) and each test chamber was considered a replicate. There were 3 replicates for each test concentration, seawater control and solvent control. The 'chamber 2 reference

replicate' was identical to seawater controls and was used as the reference solution in all ToxY-PAM measurements for that test.

The bioassay was initiated by adding aliquots of the prepared sperm solution and the toxicant solution to each test chamber in various ratios to bring the total test volume to 5 mL, while ensuring the appropriate test concentration was administered as well as maintaining consistent sperm concentrations (1.2×10^6 sperm mL^{-1}) across all treatments. With all additions completed, the test chambers were collectively covered with clear plastic film and incubated for 2 h at 18 ± 0.5°C under 12 ± 1 µmol photons $m^{-2}s^{-1}$ illumination. Sperm concentration of 1.2×10^6 sperm mL^{-1} was chosen based on Figure 2.7, where that concentration resulted in highest control $\Delta F/ F_m'$ values. While 12 ± 1 µmol photons $m^{-2}s^{-1}$ illumination for exposure period is based on Figure 2.8 where sperm $\Delta F/ F_m'$ was found to significantly decrease ($p<0.05$ (1-way ANOVA)) with higher light levels.

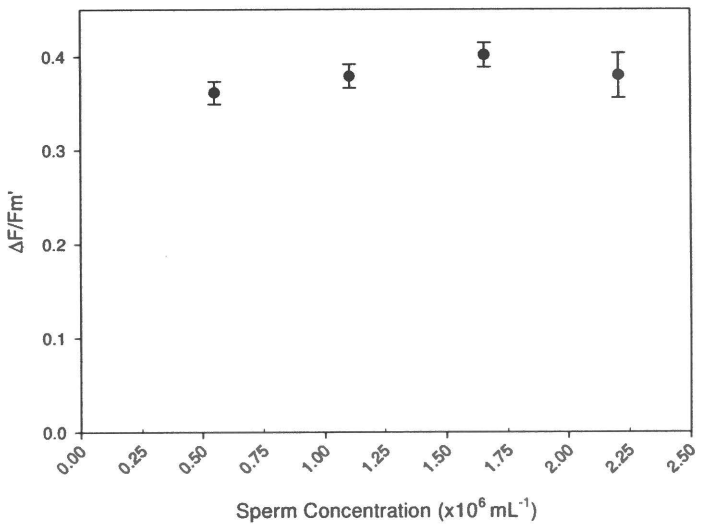

Figure 2.7: Effective quantum yield ($\Delta F/ F_m'$) of *Hormosira banksii* sperm in relation to sperm concentration. Error bars represent standard deviation (n=3).

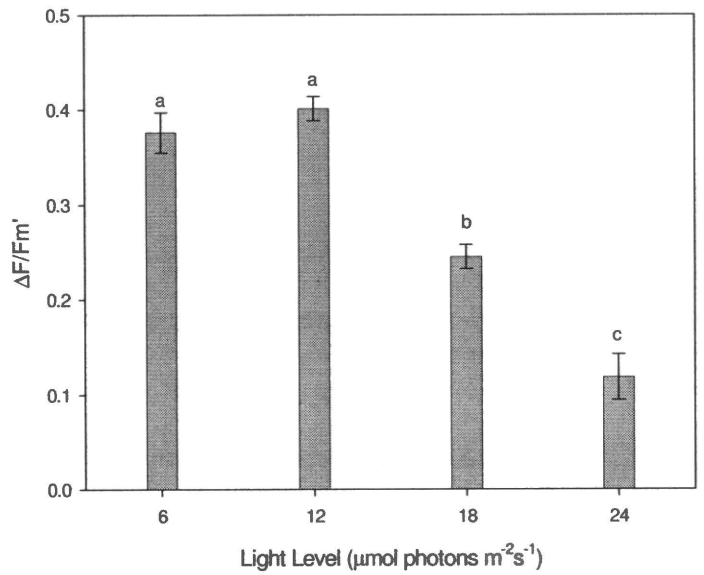

Figure 2.8: Effective quantum yield ($\Delta F/ F_m'$) of *Hormosira banksii* sperm after 2 h exposure to various light levels. Error bars represent standard deviation (n=3).

After the 2 h exposure period, 1 mL of the reference control solution was transferred to channel 2 of ToxY-PAM, and 1 mL of each zero treatment was added, in turn, to channel 1 of ToxY-PAM, where a control %PSII Inhibition value was generated (following the 3 saturation pulses method described above). As opposed to the 'target F' value of 600 – 1600 used in the egg bioassay, a standardised volume of 1 mL can be used for sperm solutions because the cells stay in suspension and thus a consistent sperm concentration can be maintained across treatments.

Also different to the egg bioassay, rather than having just one reference cell that must be above a set control value (0.45 for egg control, as stated above), the sperm bioassay used a zero treatment to confirm the health of the reference control. This method of quality assurance ensures a number of control measurements are taken into account instead of just one measurement being performed on a sole control cuvette. This reduces the chance of a single anomalous result rendering the whole test invalid, and makes the process of quality control more robust (with a greater number of measurements/replicates). A test was considered to be invalid if the level of PSII Inhibition calculated for any of the three zero treatment replicates was greater than 10% when compared to the reference control. This would indicate that the adult thalli, sperm or seawater were of poor quality. However, if the zero treatment varied by no more than 10% from the reference solution (i.e. < 10 %PSII Inhibition) the test would be considered valid. 1 mL from each of the test concentration chambers would then be transferred, in turn, to the test cuvette for fluorescence measurement. The resultant %PSII Inhibition values were plotted as the response variable for each test concentration to provide a dose-response curve.

Germination bioassay protocol – **Hormosira banksii** *gametes*

The bioassay method used for the germination endpoint in this study was similar to that of the published protocol for the *H. banksii* fertilisation endpoint (Gunthorpe, *et al.*, 1995). The principal exception to the original fertilisation method was a longer exposure period of 48 h, as necessitated by the use of a later-stage developmental event such as germination. A single test chamber consisted of an 80 mL beaker containing a glass coverslip on which the *H. banksii* embryos would settle and germinate, 75 mL test solution and measured aliquots of egg and sperm solution. Four replicate chambers were used for each toxicant concentration, seawater control and solvent control (if solvent control was applicable). Bioassays were initiated with the addition of 1 mL of the diluted 2 x10^3 egg mL^{-1} egg solution and 200 µL of the stock sperm solution into each test chamber, which contained the test solution. Once all additions had been performed, the beakers were placed on a tray, covered with clear plastic film and placed in an incubator (Labec, Sydney, Australia) for 48 h at 18 ± 0.5°C. Illumination was maintained at 23 ± 2 µmol photons $m^{-2}s^{-1}$ using cool white fluorescent tubes (Crompton) on a light/dark cycle of 12:12. Both temperature and illumination levels are based on the methods of Gunthorpe, *et al.*, (1997). After the 48 h exposure period, gametes were microscopically scored for germination by placing each coverslip under 30x magnification of a dissecting microscope. A total of 100 gametes were randomly scored for germination on each coverslip, where germination was determined by the initiation of a rhizoid, a dark cell colour and a clear cell wall (Gunthorpe, *et al.*, 1997). This traditional scoring of germination gives percent germination as the response variable. For ease of plotting germination data alongside fluorescence data, we have used the inverse of percent germination, which results in the response variable of percent inhibition of germination. For a test to be

considered valid, the controls must show a mean percent inhibition of germination of less than 20%, otherwise the test was abandoned. A similar germination method has since been published by Myers, *et al.,* (2005), with minor differences in control assurances and liquid volumes.

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Chapter 3

As reprinted from: Seery, C.R., Gunthorpe, L., Ralph, P.J. 2006. Herbicide impact on *Hormosira banksii* gametes as measured by fluorescence and germination bioassays. Environmental Pollution. 140, 43-51.

3.0 Hormosira banksii *Egg Bioassay*

3.1 Abstract

The innovative bioassay described here involves chlorophyll *a* fluorescence measurements of gametes from the macroalgae, *H. banksii*, where gametes (eggs) were exposed to Diuron, Irgarol 1051 and Bromacil. Response was assessed as percent inhibition from control of effective quantum yield ($\Delta F/F_m'$) of photosystem II, herein referred to as % PSII Inhibition. This was measured with the dual-channelled pulse amplitude modulated (PAM) fluorometer, ToxY-PAM. The fluorescence bioassay was run simultaneously with an established *H. banksii* germination bioassay to compare sensitivity, precision, and time-to-result. The fluorescence bioassay gave highly sensitive results, as evidenced by EC_{50}s (% PSII Inhibition) for Diuron, Irgarol 1051 and Bromacil being three, four and three orders of magnitude (respectively) lower than EC_{50}s generated from the germination bioassays. Precision of the fluorescence bioassay was demonstrated with low coefficient of variations (<30%) for all three toxicants. With regard to time, the fluorescence bioassay gave results within 6 h, as opposed to more than 50 h for the germination bioassay.

Chapter 4

As reprinted from: Seery, C.R. and Ralph, P.J. 2005. A Novel Chlorophyll *a* Fluorescence Bioassay for the Rapid Screening and Monitoring of Coastal Pollutants Using *Hormosira Banksii* Antherozoids. Aquatic Toxicology. *In review*

4.0 Hormosira banksii *Sperm Bioassay*

4.1 Introduction

Standardised acute toxicity tests have long played an important role in aquatic risk assessments, especially at the 'screening' level of evaluation (Toussaint, *et al.*, 1995). These tests have for the most part employed fish and macroinvertebrates as the test organism, though over recent years there have been a number of alternative tests that have proven to be effective for screening assays that utilise microorganisms such as bacteria (Microtox; Nacci, *et al.*, 1986), microalgae (Bengtson Nash, *et al.*, 2005) or propagules of macroalgae (Gunthorpe, *et al.*, 1995). These alternative tests are experimentally simple, sensitive, reproducible, usually only require a short exposure time and are generally rapid in terms of time-to-result (Wells, *et al.*, 1998). These characteristics are attributed more to the endpoint being measured rather than the organism employed.

It is not intended that rapid toxicity tests should replace established, standard acute toxicity tests such as the 48 h microalgal growth test, but rather be part of a pollution management strategy that includes a rapid assessment of toxicity, on which management decisions can be made. Rapid toxicity assessment protocols are most useful in situations where their speed and relatively low cost make it practical to screen large numbers of samples for preliminary indications of toxicity. Situations requiring this type of assessment could include identifying and mapping discrete areas of pollution (Thomas, *et al.*, 1986), conducting Toxicity Identification Evaluation (TIE) tests (Farré and Barceló, 2003) or evaluating temporal variations in waste streams or outputs (Toussaint, *et al.*, 1995).

Chlorophyll *a* fluorescence has been shown to be a rapid indicator of phyto-toxicity with herbicides (Haynes, *et al.*, 2000; Ralph, 2000; Macinnis-Ng and Ralph, 2003a), petrochemicals (Macinnis-Ng and Ralph, 2003b) and trace metals (Ouzounidou, 1993; Frankart, *et al.*, 2002). At the commencement of this research, there were no standardised chlorophyll *a* fluorescence-based protocols for the assessment of pollutants in coastal ecosystems. Furthermore, while there are USEPA protocols for the estuarine macroalgae *Champia parvula* (USEPA, 1994) and the marine macroalgae *Macrocystis pyrifera* (USEPA, 1995), there is no "internationally standardised" protocol that exists for any macroalgae species, irrespective of the endpoint or method (Eklund and Kautsky, 2003). Aquatic plant toxicity testing has largely been ignored in preference for invertebrate or fish testing: this is clearly inconsistent with the ecological importance of aquatic plants, which constitute the base of many benthic food webs (Mohan and Hosetti, 1999) and the fact that plants have been found to be more sensitive than invertebrates in some situations (Benenati, 1990).

In this study, we describe a novel bioassay that expands on earlier chlorophyll *a* fluorescence assay techniques to allow a wide range of toxicants to be tested using sperm (antherozoids) from the brown macroalga, *H. banksii* (Seery, *et al.*, 2006). We demonstrate the bioassay's versatility by using three different classes of toxicant: a herbicide, a metal and a petrochemical. In addition to being a rapid indicator of toxicity, the bioassay discussed here can also improve our understanding of the physiological process involved in toxicant impact on macroalgal gametes.

4.2 Materials & Methods

4.2.1 Test Solutions

Stock solutions were prepared with analytical grade chemicals; dichlorophenyldimethyl urea (DCMU, but herein referred to by its common name, Diuron), copper (Cu_2SO_4) and Masa crude oil were supplied by Sigma, Ajax Chemicals and Shell Australia, respectively. Preparation of 10 mg L^{-1} Diuron stock solution required use of acetone as a solvent and was then dissolved in reverse osmosis (RO) water. The highest percentage of solvent in test solutions was less than 1 ppt; as such, solvent controls were performed containing filtered seawater with this concentration of acetone. Copper stock solution was prepared to 1.0 g L^{-1}, and was also dissolved in RO water. The protocol for petrochemical preparation was similar to methods described by Durako, *et al.*, (1993), Ralph and Burchett (1998) and Macinnis-Ng and Ralph (2003). Prior to experiments, crude oil was aged by mixing 9.0 g fresh Masa crude in 900 mL of filtered seawater in a conical flask to give a 1% (w/v) stock crude oil mixture. The conical flask holding the mixture was sealed to prevent loss of the volatile fraction and agitated for 24 h using a vortex magnetic mixer (Jenway 1002, United Kingdom). The mixture was left to settle for 1 h, at which time the water soluble fraction (WSF) was separated. The unmixed oil was pipetted from the surface and aliquots of the WSF were decanted and used as the Masa crude oil stock solution.

These stock solutions were then diluted with filtered seawater to make fresh 'working solutions' each time a bioassay was performed. These fresh solutions were then further diluted to give appropriate test solutions for each bioassay. Seawater,

collected from Rose Bay, Sydney, Australia (151°20'E, 33°31'S), and kept in darkened 5000 L cement tanks, was filtered (5 μm) and aerated for at least 24 h before being used as the diluent in all working solutions.

Dissolved oxygen, pH, salinity and temperature were measured in each test concentration at the conclusion of each test and ranged between 80-90%, 8.0-8.5, 32-34 ppt and 18.0-19.2°C, respectively.

4.2.2 Fluorescence Assessment

ToxY-PAM (Walz GmbH, Effeltrich, Germany) is a dual-channel fluorometer specifically developed for detection of phytotoxic substances in water samples (Schreiber, *et al.*, 2002), where channel 1 measures the treatment sample and channel 2 measures the control. Effective quantum yield of PSII ($\Delta F/F_m' = [(F_m'-F)/F_m']$) is calculated for each channel by measuring the minimum fluorescence (F1, F2 where the number denotes channel 1 or 2) and the maximal fluorescence ($F_m'1$, $F_m'2$) taken during a 0.4 s saturating pulse of blue light (470 nm). $\Delta F/F_m'$ values for each channel are represented as Y1 or Y2 and these values are used to calculate %PSII Inhibition = 100x[(Y2-Y1)/Y2], which corresponds to percent inhibition of the treatment relative to the control sample.

4.2.3 Gamete Collection

H. banksii (Turner) Decaisne (Phaeophyta) is a dioecious macroalga with eggs or sperm released from a diploid sporophyte. Mature *H. banksii* samples were collected approximately three hours before low tide, to avoid natural desiccation-induced release of gametes, from Bilgola Beach, north of Sydney, Australia (151°19'E, 33°38'S). Approximately 50 strands of *H. banksii* (where a strand represents a portion of an individual plant containing 5–25 receptacles) were collected haphazardly from the intertidal sandstone reef and placed in an inert, opaque plastic bag for transportation to the laboratory.

In accordance with the method described by Gunthorpe, *et al.*, (1995) for spawning of gametes, the strands were arranged flat on paper towel in the laboratory and allowed to dry for 2 h. They were then collectively placed in brown paper bags and stored for 12-18 h at 4°C before spawning was induced. To initiate spawning of gametes, the chilled *H. banksii* strands were soaked in warm seawater (30-40 °C) for 20-30 s with gentle agitation. The washed algae were then arranged on a clean, dry surface, so as not to touch each other, and gamete extrusion typically occurred within 3–10 min.

Upon spawning, gametes were identified as either male or female by the colour of the extruded 'packets'. Male gametes (antherozoids or sperm) are orange and can be distinguished from the green female gametes (oospheres or eggs) (Osborn, 1948). The strands that exhibited spawning of male gametes were placed into a beaker with approximately 150 mL filtered seawater (20°C). Approximately 200 receptacles from

actively spawning strands gave a final solution with sperm concentration > 2 x10^6 sperm mL^{-1} (sperm concentration was assessed using a haemocytometer), which was then adjusted to 1.2 x10^6 sperm mL^{-1}. Sperm solutions were filtered through 200 µm mesh filter to remove gross plant material and stored in darkness until needed (not longer than 5 min).

4.2.4 Bioassay Protocol

Table 4.1 summarises the details of the *H. banksii* sperm chlorophyll *a* fluorescence bioassay. The bioassay comprises at least 5 test concentrations, a zero treatment, a solvent control (only applicable for Diuron) and a reference control to be used in Toxy-PAM's channel 2. Each test chamber consisted of a 20 mL glass vial containing 5 mL total test solution (sperm and toxicant solutions) and each test chamber was considered a replicate. There were 3 replicates for each test concentration, seawater control and solvent control. The 'chamber 1 reference replicate' was identical to seawater controls and was used as the reference solution in all ToxY-PAM measurements for that test.

The bioassay was initiated by adding aliquots of the prepared sperm solution and the toxicant solution to each test chamber in various ratios to bring the total test volume to 5 mL, while ensuring the appropriate test concentration was administered (target toxicant concentration) as well as maintaining consistent sperm concentrations across all treatments. With all additions completed, the test chambers were collectively covered with clear plastic film and incubated for 2 h at 18 ± 0.5°C under 12 ± 1 µmol photons $m^{-2}s^{-1}$ illumination.

After the 2 h exposure period, 1 mL of the reference control solution was transferred to channel 2 of ToxY-PAM, and 1 mL of each zero treatment was added, in turn, to channel 1 of ToxY-PAM, where a control %PSII Inhibition value was measured. As described above, a %PSII Inhibition measurement consists of three concurrent saturating pulses to both the reference (channel 2) and test (channel 1) cuvette at 30 sec intervals. The mean %PSII Inhibition taken from the three pulses given to each replicate is then averaged again across the three replicate chambers to give a %PSII Inhibition value for that particular concentration, in this case the zero treatment. If the level of PSII Inhibition was greater than 10% for any seawater control, the test results were considered invalid and the adult thalli, sperm or seawater were considered poor quality and unsuitable for use in the bioassay. If the zero treatment varied by no more than 10% from the reference solution (i.e. < 10 %PSII Inhibition), then 1 mL from each of the test concentration chambers were transferred, in turn, to the test cuvette for fluorescence measurement following the same procedure as the control %PSII Inhibition measurements. The resultant %PSII Inhibition values were plotted as the response variable for each test concentration to provide a dose-response curve.

4.2.5 Statistical Analysis

Dose effects were fitted for each toxicant to a logistic dose-response model:

$$\%\text{PSII Inhibition} = a / (1 + \exp[-((x - EC_{50})/b)])$$

Here a denotes the maximum inhibition at infinite concentrations of toxicant, x denotes the concentration of toxicant, EC_{50} represents the concentration of toxicant that results in a 50% measurable effect in the test population, and b is proportional to

the slope of the curve around EC_{50} (see Streibig, *et al.*, 1993). Each dose-response curve presented below fitted all three parameters well (standard error/parameter < 1) (Cedergreen, *et al.*, 2004). Data were expressed as proportions of the control and EC_{50} values were calculated according to the equation above using least squares regression. To monitor precision of the bioassay, coefficients of variation (CV) were generated for the EC_{50}s from each toxicant set (n=5) as suggested by Environment Canada (1990) and Cherr, *et al.*, (1994), where a CV less than 30 or 35% (respectively) was considered acceptable.

Table 4.1: Summary of test protocol for the *Hormosira banksii* sperm fluorescence bioassay.

Attribute	Protocol Requirement
Test type	Static
Temperature	18 ± 0.5 °C
Light quality	Cool White Fluorescent Lighting
Light intensity	12 ± 1 µmol photon $m^{-2}s^{-1}$
Test chamber size	20 mL
Test solution volume	5 mL
Renewal of test solution	None
Age/stage of test organism	Gamete (antherozoid/sperm)
Sperm concentration	1.2 x10^6 sperm mL^{-1}
Replicates per test concentration	3
Diluent	5 µm filtered seawater, aerated >24 h
Salinity	33 ± 1 ppt
Test concentrations	Minimum 5, plus zero treatment, solvent control (Diuron only) and reference control
Dilution factor	0.3 or 0.5
Exposure period	2 h
Endpoint	%PS II Inhibition
Summary parameter	EC_{50} (2 h %PSII Inhibition)
Test acceptability criteria	>10% Inhibition in zero treatment
Instrument	ToxY-PAM (Heinz Walz GmbH)
Measurements per replicate	3
Time-to-result (complete test duration)	6-7 h (excluding thallus collection)

4.3 Results

Inhibition of photosynthesis was evident in all bioassays conducted with each of the three toxicants. Within the concentration range tested, a significant decline in $\Delta F/F_m'$ was observed after 2 h exposure to all toxicants and therefore a dose-response relationship for %PSII Inhibition was produced (Figure 4.1, A, B and C). For all Diuron bioassays conducted (the only toxicant of the three tested needing a solvent control), the solvent control was never significantly different to the seawater control ($p > 0.05$), and as such, solvent controls are not presented elsewhere.

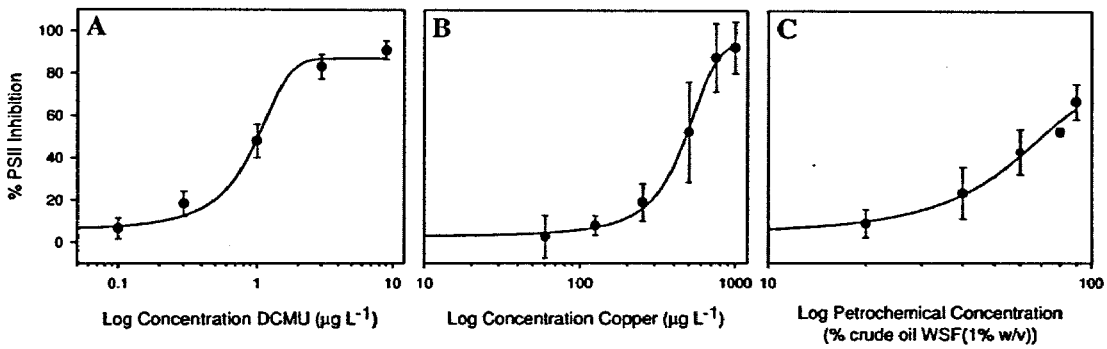

Figure 4.1: Dose-response relationships for %PSII Inhibition of *Hormosira banksii* sperm exposed (for 2 h) to Diuron, copper, or water soluble fraction (WSF) of Masa crude oil (A, B and C, respectively). Data points represent mean ± standard deviation (n=5). Fitted curve used a 3 parameter logistic equation.

Mean EC_{50} values (n=5) for the three toxicants tested are presented in Table 4.2, along with associated 95% confidence intervals. Analysis of variance of these EC_{50}s demonstrated significant differences in sensitivity of *H. banksii* sperm to Diuron and

copper ($p = 0.0001$) and a *post hoc* multiple comparison test ranked Diuron as more toxic than copper with respect to PSII Inhibition. Masa crude oil could not be included in these comparisons due to how the toxicant was quantified; it was measured as %WSF of 1% (w/v), rather than $\mu g\ L^{-1}$.

Coefficients of Variation (CV) for the Diuron and copper bioassays were both under the recommended 30% level (Table 4.2), whereas the petrochemical bioassay generated a CV of 33.6% which falls above this 30% level recommended by Environment Canada (1990), but below that of the 35% suggested by Cherr, *et al.*, (1994).

Table 4.2: Summary parameter table showing EC_{50} value for each toxicant tested and associated Coefficient of Variation (CV) value.

Toxicant	Mean EC_{50} (n=5)	Lower-Upper 95%CI	Coefficient of Variation (n=5)
Diuron	0.92 $\mu g\ L^{-1}$	0.73 – 1.11	14.8
Copper	446 $\mu g\ L^{-1}$	430 – 500	22.6
Masa Crude Oil	55.9 % WSF	38.9 – 73.0	33.6

The dose-response curves all increase monotonically (Figure 4.1) as a result of increasing %PSII Inhibition which, as explained previously, is calculated as a ratio of control and treatment effective quantum yields (or the $\Delta F/F_m'$). Figure 4.2 shows F and F_m' measures for treated samples and when observed in conjunction with dose-response curves, demonstrates how changes in F and F_m' affect effective quantum yield and in turn %PSII Inhibition.

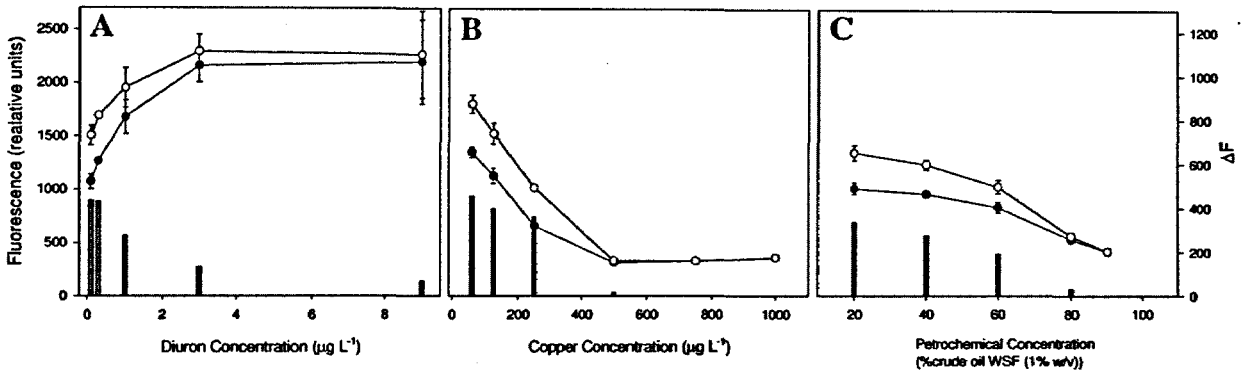

Figure 4.2: Minimal and maximal fluorescence for Diuron, copper and Masa crude oil bioassays. Each panel (A, B and C) shows minimal (F or F1) and maximal (F_m' or $F_m'1$) fluorescence response with increasing toxicant exposure for Diuron, copper and Masa crude oil, respectively. Closed circles (•) represent minimal fluorescence (F); open circles (○) represent maximal fluorescence (F_m') with corresponding axis shown on left of figure (Fluorescence (relative units)). Data points represent mean ± standard deviation (n=3). Vertical bars on each panel correspond to the value ΔF ($\Delta F = F_m' - F$), showing the difference between the plotted minimal and maximal fluorescence. Vertical bar values correspond to the axis shown on right of figure ($\Delta F = F_m' - F$).

All measures of F and F_m' of control treatments (not shown) demonstrated near parallel relationships, where a consistent difference was maintained between F and F_m, thus there is little to no change in $\Delta F/F_m$' over the repeated measures. The F and F_m' measurements for increasing toxicant concentration (Figure 4.2, A, B and C) differ in that the difference between F and F_m' becomes less as more toxicant is present, which is reflected in lower $\Delta F/F_m$' and consequently higher %PSII Inhibition observed at higher toxicant concentrations in the dose-response curves (Figure 4.1, A, B, and C).

In the Diuron treatments (Figure 4.2 A), both F and F_m' increase with toxicant concentration and converged until there is little difference between them. This reduction in difference between F and F_m', or decreasing ($\Delta F = F_m'-F$) as shown by vertical bars, results in lowered $\Delta F/F_m$' and a consequential increase in %PSII Inhibition (Figure 4.1 A). In contrast, the copper treatments show a decrease in F and F_m' with increasing toxicant concentration (Figure 4.2 B). At lower copper concentrations, F and F_m' decrease together (Figure 4.2 B) and so the difference between them, shown as vertical bars ($\Delta F = F_m'-F$), is consistent and there is little change observable in the early data points of $\Delta F/F_m$' (Figure 4.1 B). It is only at higher copper concentrations that the difference between F and F_m' is reduced (Figure 4.2 B) and a marked change in $\Delta F/F_m$' is evident (Figure 4.1 B). Similarly, F and F_m' only converged after equivalent change and eventual decrease at higher concentrations of Masa crude oil WSF (Figure 4.2 C). Subsequently, it is only at higher concentrations than 60% WSF that a significant change in $\Delta F/F_m$' is observable (Figure 4.1 C).

4.4 Discussion

The results presented here demonstrate that chlorophyll *a* fluorescence of *H. banksii* sperm is a rapid indicator of toxicity to macroalgae for three different classes of toxicant. The method is rapid, experimentally straight-forward and displays acceptable sensitivity and precision. The protocol would be suitable for rapid toxicity screening or repetitive, large volume testing over wide spatial areas where a toxic result would warrant further investigation.

H. banksii sperm were photosynthetically inhibited by 50% when exposed to 0.92 μg L^{-1} Diuron. These results agree well with other chlorophyll *a* fluorescence studies. There is only one other published macroalgal EC_{50} for Diuron where Seery, *et al.*, (2006) generated an EC_{50} of 1.65 μg L^{-1} for *H. banksii* eggs. Other EC_{50}s that exist for Diuron exposure have been generated from experiments with coral symbionts or microalgae, and these values are also comparable with the results presented here. EC_{50} values ranging from 4-6 μg L^{-1} have been generated from four species of coral exposed to Diuron after 10 h exposure, using $\Delta F/F_m'$ as the response variable (Jones, *et al.*, 2003) and Bengtson Nash, *et al.*, (2005), also using ToxY-PAM, found 3.7 μg L^{-1} Diuron inhibited photosynthesis of *Phaeodactylum tricornutum* by 50%. While these EC_{50}s are for photosynthetic coral symbionts and a microalga, they are consistent with the EC_{50} of 0.92 μg L^{-1} for Diuron, as generated from the fluorescence-based, macroalgal bioassay presented here. In Australia and New Zealand, the interim trigger value for Diuron in marine waters is currently set at 1.8 μg L^{-1} (ANZECC/ARMCANZ, 2000). As water column concentrations of Diuron have been documented to be as high as 6.7 μg L^{-1} in boating marinas (Thomas, *et al.*,

2001), the testing range of the fluorescence bioassay allows it to be run at environmentally realistic concentrations and provide relevant results in regards to these guideline values.

H. banksii sperm shows a 50% reduction in photosynthesis at 460 μg L^{-1} copper. While this EC_{50} is quite high with regard to the Australian water quality trigger value of 1.3 μg L^{-1} copper for marine waters (ANZECC/ARMCANZ, 2000), it is within the range of results reported within other chlorophyll *a* fluorescence investigations of copper phytotoxicity. Juneau, *et al.*, (2002) generated a 5 h EC_{50} of 30 μg L^{-1} for copper-exposed *Selenastrum capricornutum* using ΔF/Fm'. Yet in contrast, van der Heever and Grobbelaar (1998) were unable to find significant differences between *S. capricorni* exposed for 4 h to 2 mg L^{-1} and control treatments (using an index of toxicity they termed F_t (where $F_t = (F_m - F)/(F_m - F_o)$) and thus were not able to generate an EC_{50}. Germination tests using *H. banksii* by Myers, *et al.*, (2005) generated a mean EC_{50} of 170 μg L^{-1} for copper. While this result indicates greater sensitivity than the fluorescence bioassay, 170 μg L^{-1} is still well above the water quality trigger value of 1.3 μg L^{-1}. Additionally, the germination endpoint requires 48 h exposure and thus would not be suitable for applications of rapid toxicity assessment described previously.

While a number of studies have reported petrochemical toxicity, tests performed with crude oil mixtures are difficult to compare due to the wide variation in test solution preparation methods. For example, Burridge and Shir (1995) have reported a nominal EC_{50} of 130 μg L^{-1} for crude oil with respect to macroalgal germination and Karydis

(1982) demonstrated inhibited growth and photosynthesis in two marine microalgae at 10 – 100 mg L^{-1} crude oil. However, neither of these studies can directly be compared to the results presented here as both use a test solution consisting a small amount of crude oil plus seawater, as opposed to using an aged, water soluble fraction (WSF) as was performed here. As such, the composition of toxic compounds could be vastly different and thus it is inappropriate to directly compare the results. Macinnis-Ng and Ralph (2003) have used the same method of crude oil preparation as is presented here, but due to a limited concentration range they do not calculate an EC_{50}. However, they do report that 0.1 and 0.25 % WSF of a similar crude oil significantly inhibited seagrass (*Zostera capricorni*) photosynthesis as measured with chlorophyll *a* fluorescence.

While the changes in the fluorescence parameters F and F_m' were presented here to demonstrate how these changes affect the summary parameter of %PSII Inhibition, they can also give some insight as to the mode of action for each toxicant. The rise in minimal fluorescence (F) observed in the Diuron treatments is typical of phenylurea derivatives (Schreiber, *et al.*, 2002). Diuron binds to the 32 kD protein of the PSII reaction centre complex which prevents electron transfer from Q_A to Q_B, thus electron transport is blocked giving give rise to an increase in fluorescence emission and thus an increase in F (Schreiber, *et al.*, 2002).

With regard to copper, many authors have shown F and F_m' to decrease with increasing copper concentration or with time of exposure (Ouzounidou, 1993; Kupper, *et al.*, 1998; Boucher and Carpentier, 1999). This has been attributed to the

formation of non-fluorescent copper-chlorophyll complexes (Kupper, *et al.*, 1998) and copper's interference with pigment and lipid biosynthesis (Baron, *et al.*, 1995). Parallel decreases of F and F_m' have also been demonstrated by Kupper, *et al.*, (2002) upon exposure to copper, where $\Delta F/F_m$' remained unchanged until a very late stage of damage, thus a misleadingly low %PSII Inhibition level would be observed at the early stages of impact. This explains why this copper dose-response curve (Figure 4.2) shows no inhibition at lower test concentrations and as a consequence decreases the bioassay's sensitivity to copper. This could be rectified by using a fluorescence parameter that doesn't rely on the ratio between F and F_m, such as monitoring the change in F_m' (Kupper, *et al.*, 2002). However, the use of F_m' presents new problems, as the usual cause of a decrease in F_m' is photoinhibition due to high light (Schreiber, 2004). Thus, using this parameter would require careful consideration of both the toxic impact as well as the physiological response to high light.

The decrease in F and F_m' exhibited in the petrochemical tests indicate increased non-photochemical quenching (NPQ), as has been speculated by Macinnis-Ng and Ralph (2003) and Ralph and Burchett (1998). NPQ can increase via increased heat dissipation (Macinnis-Ng and Ralph, 2003) or by fewer PSII reaction centres being available per unit area (Huang, *et al.*, 1996), both of which have been linked with exposure to petrochemicals or their components. So while it is not the primary focus of the fluorescence bioassay described here, it is possible that careful analysis of fluorescence parameters may offer insight into a toxicant's mode of action.

Chlorophyll *a* fluorescence endpoints have been shown to provide a faster estimation of toxicity than the more traditional developmental endpoints such as germination (Seery, *et al.*, 2006). Furthermore, Radix, *et al.*, (2000) has reported that in 56% of cases, 72 hour growth tests assessing toxicity of sixteen different chemicals to the green microalgae *Pseudokirchneriella subcapitata* could be predicted within 5 hours using measurements of direct fluorescence. These photosynthetic endpoints can therefore be effective in tests where the time-to-result is important or could be used as rapid estimators of toxicity. The more traditional and accepted endpoints such as microalgal growth inhibition or macroalgal germination, may then be used as confirmatory tests in concurrent or subsequent assessments.

In conclusion, this study demonstrates the capacity of a novel chlorophyll *a* fluorescence-based technique for determining dose-response relationships with *H. banksii* sperm for a number of classes of toxicant. However, before this method becomes widely accepted, a number of issues must be addressed. Specifically, the biological relevance of $\Delta F/F_m'$ as an endpoint for toxicity tests must be further demonstrated. Additionally, given that *H. banksii* is fertile all year-round and testing can be performed over vastly different weather conditions, the effect of seasonal variation on the consistency of the bioassay also needs to be addressed. The *H. banksii* fluorescence bioassay has the potential for expansion to include other toxicants such as whole effluents, but the effects of coloured samples and the presence of interfering (auto-fluorescing) compounds would need to be assessed. With these advances, the *H. banksii* fluorescence bioassay can provide a powerful tool in the rapid assessment of toxic impact on near-shore macroalgal communities, and as an effective toxicity screening tool for coastal management.

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Chapter 5

5.0 *Applied use of Method: Interactive Effects of Mixtures*

5.1 Introduction

Anthropogenic chemicals are an omnipotent threat to all coastal ecosystems in close proximity to human habitation. Many of these chemicals have been shown to have deleterious effects to marine species (Bayne, *et al.*, 1985). Of particular interest with regard to marine toxicity are those chemicals used as part of antifouling applications on marine vessels. While the need for antifoulants in the protection of submersed marine structures (eg. buoys, oil rig super-structure and ship's hulls) has been acknowledged (Evans, *et al.*, 2000; Boxall, *et al.*, 2000; Konstantinou and Albanis, 2004), so too has the toxic impact these chemicals have on adjacent marine ecosystems (Fent, 1996).

The severe impacts observed as a result of low levels of Tributyltin (TBT; a common organo-tin antifoulant) has led to it being internationally regulated, culminating with a multinational ban that came into effect January 1st, 2003 as a result of actions on behalf of the International Maritime Organisation (IMO, 1998). However, the need for effective antifoulants remained, so there has been a marked increase in use of antifoulant coatings containing copper combined with organic biocides (Yebra, *et al.*, 2004). Among the biocides in use are the herbicides Diuron and Irgarol 1051 which have been shown to have individual toxic effects on marine organisms such as microalgae (Fernandez-Alba, *et al.*, 2002), seagrasses (Scarlett, *et al.*, 1999a; Ralph, 2000; Macinnis and Ralph, 2003), corals (Owen, *et al.*, 2002; Jones, *et al.*, 2003; Owen, *et al.*, 2003), sea urchins (Kobayashi and Okamura, 2002) and other species (for review see Konstantinou and Albanis, 2004). Given the demonstrated toxicities

of these TBT replacements, non-target toxicity of antifoulant applications continues to be an issue of concern (Boxall, *et al.*, 2000).

Irgarol 1051 and Diuron , two of the most commonly-used biocides have been shown to occur in coastal waters and sediments throughout the world (Thomas, *et al.*, 2001; Konstantinou and Albanis, 2004). Irgarol 1051 has been reported in coastal waters of many European countries including the United Kingdom (Boxall, *et al.*, 2000; Scarlett, *et al.*, 1997; Thomas, *et al.*, 2001), France (Readman, *et al.*, 1993), Germany (Biselli, *et al.*, 2000), as well as in other countries around the world such as USA (Gardinali, *et al.*, 2002), Japan (Okamura, *et al.*, 2000) and Australia (Scarlett, *et al.*, 1999b; Haynes, *et al.*, 2000; Chesworth, *et al.*, 2004). Among the highest concentrations recorded were those in the southern coast of the United Kingdom (up to 1421 ng/l; Thomas, *et al.*, 2001), the Caribbean (up to 1300 ng/l; Carbery, *et al.*, 2005) and Cote d' Azur, France (up to 1700 ng/l; Readman, *et al.*, 1993). Similarly, Diuron has been reported in waters off the United Kingdom (Boxall, *et al.*, 2000; Thomas, *et al.*, 2001), Sweden (Dahl and Blanck, 1996), Japan (Okamura, *et al.*, 2003) and Australia (Haynes, *et al.*, 2000) at levels as high as 6742 ng/l (UK; Thomas, *et al.*, 2001). These levels were of such concern that Diuron has been banned from use as an active component of antifoulant paints, on any size of vessel in the UK, while use of Irgarol 1051 has been limited to use on vessels greater than 25 m in length (Thomas, *et al.*, 2002). Other countries have followed with similar bans, with Denmark and Sweden restricting use of paints comprising Irgarol 1051 and Diuron to vessels greater than 25 m in length (Thomas, *et al.*, 2002; Konstantinou and Albanis, 2004), and the current Australian National Registration Authority for Agricultural and Veterinary Chemicals review of Diuron may result in

similar restrictions. However, the continued use of Diuron in land-based crop applications, in addition to the persistence of Irgarol 1051 and Diuron, means that the herbicides still present a very real threat to marine ecosystems. This prompted Bermuda, a country with significant reef ecosystems, to pass legislation in 2005 banning the use of antifoulant applications containing Irgarol 1051 (Carbery, *et al.*, 2005). Meanwhile, copper remains amongst the most common of trace metal pollutants in marine and estuarine systems due to anthropogenic sources such as industrial outfall, stormwater inputs and agricultural activity (Environment Australia, 2002). Not least of these sources is that attributed to the use of copper in antifoulant applications. Copper has been used as an antifoulant since the mid 1800's (Yebra, *et al.*, 2004) and continues to be used in the TBT-replacement antifoulants in conjunction with organic biocides (Evans, *et al.*, 2000). Thus, copper remains a major concern with regard to the polluting effects of antifoulant use.

Not only do pollutants co-exist in the environment, but in this circumstance, both Irgarol 1051 and Diuron co-exist with copper in antifoulant products. As such, with the chemicals so closely associated, chemical or toxic interactions via combined exposure are not just possible, they are almost unavoidable. So while the individual toxicities of Diuron, Irgarol 1051 and copper have been assessed with a number of marine species (Juneau, *et al.*, 2002; Jones, *et al.*, 2003; Jones and Kerswell, 2003; Bengtson Nash, *et al.*, 2005; Myers, *et al.*, 2005; Seery, *et al.*, 2006) little is known about interactive effects of these chemicals at low concentrations (low being in the order of ppm). Pollutants can interact to give synergistic, additive or antagonistic results, which can in turn render guidelines based on single toxicant testing to be under- or over-protective. An interaction between antifoulant compounds was

demonstrated by Bonnemain and Dive (1990), where ziram (a dithiocarbamate pesticide) and copper were found to act synergistically towards a ciliate. In contrast, Chesworth, *et al.*, (2004) found Diuron and Irgarol 1051 acted additively or antagonistically with respect to the seagrass *Zostera marina*. Similarly, Teisseire, *et al.*, (1999) investigated combined exposure of Diuron and copper to an aquatic macrophyte (*Lemna minor*) and found additivity or non-significant antagonism. Here, we assess the interactive effects of Irgarol 1051, Diuron and copper by use of a chlorophyll *a* fluorescence bioassay developed as a means of rapid assessment of coastal pollutants.

5.2 Materials & Methods

5.2.1 Test Solutions

Stock solutions were prepared with analytical grade chemicals; Irgarol 1051 (2-[*tert*-butylamino]-4-[cyclopropylamino]-6-[methylthio]-1,3,5-triazine), Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) and copper (Cu_2SO_4) supplied by Cigbey Australia, Sigma and Ajax Chemicals, respectively. Preparation of Irgarol 1051 and Diuron stock solutions with reverse osmosis (RO) water required use of acetone as an initial solvent. As such, solvent controls were included in the test; however, the minimal amount of acetone used and the further dilution to final concentrations would result in the highest percentage of solvent in any test solution being less than 10^{-4}. Copper stock solution was prepared to 1.0 g L^{-1}, also dissolved in RO water. Seawater, collected from Rose Bay, Sydney, Australia (151°20'E, 33°31'S), and kept in 5000 L cement tanks, was filtered (5 μm) and aerated for at least 24 h before being used as the diluent in all working solutions.

Dissolved oxygen, pH, salinity and temperature were measured in each test concentration at the conclusion of each test and ranged between 90-95%, 8.0-8.5, 32-34 ppt and 18.0-18.8°C, respectively.

5.2.2 Collection and Preparation of Macroalgae

Collection of *H. banksii* and subsequent spawning of gametes was performed as per methods detailed in Chapter 2.2.3 (*H. banksii* collection and spawning).

5.2.3 Individual Toxicant Bioassays

Individual toxicity tests were performed with Irgarol 1051, Diuron and copper as per method described in Chapter 2.2.5 (Fluorescence bioassay protocol - *H. banksii* sperm).

5.2.4 Toxicant Interaction Bioassays

Based on the individual toxicant bioassays, dose-response curves were fitted for each toxicant and concentrations approximating the EC_{20} (concentration resulting in a 20% reduction in photosynthetic capacity) for each toxicant were then recorded for use in the interaction bioassay. Bioassays were then performed as per the individual toxicant tests with exception that test solutions consisted of three, two-way mixtures, plus controls/references: 0.10 $\mu g\ L^{-1}$ Irgarol 1051 + 0.3 $\mu g\ L^{-1}$ Diuron; 0.10 $\mu g\ L^{-1}$ Irgarol 1051 + 125 $\mu g\ L^{-1}$ copper; 0.3 $\mu g\ L^{-1}$ Diuron + 125 $\mu g\ L^{-1}$ Copper; non-toxicant reference; zero control; solvent control; and Diuron reference toxicant (at EC_{50} concentration).

Toxicant interactions were assessed using Abotts' formula (Gisi, 1996; Teisseire, *et al.*, 1999; Chesworth, *et al.*, 2004). The model compares expected and observed inhibitions, which then allows calculation of a ratio of inhibition. The expected inhibition resulting from the mixture bioassay, termed C_{exp}, is expressed as:

$$C_{exp} = A + B - (AB/100),$$

Where A and B represent the inhibitions resulting from the toxicants acting alone (taken from individual toxicity bioassays). The ratio of inhibition (RI) is thus calculated for each toxicant mixture with:

$$RI = \text{observed inhibition} / C_{exp.}$$

The resultant interactive effect is evaluated by comparing the RI value with 1. RI values significantly greater than 1 are indicative of synergism; RI values not significantly different to 1 indicate additivity; while RI values significantly below 1 are indicative of antagonism. RI values were calculated for each replicate of the three mixture treatments and the mean and standard deviation calculated. To be considered significantly different from 1, the mean RI had to be greater or less than one standard deviation from 1; otherwise the interactive effect of the two chemicals was assumed to be not significantly different from additivity (as per Gisi, 1996; Teisseire, *et al.*, 1999; and Chesworth, *et al.*, 2004).

5.3 Results

5.3.1 Individual Toxicant Bioassays

Photosynthetic stress was evident in all bioassays conducted with each of the three toxicants (Figure 5.1, A, B and C). Within the concentration range tested, a significant decline in $\Delta F/F_m'$ was observed after 2 h exposure to all toxicants and therefore a dose-response relationships for %PSII Inhibition were produced (Figure 5.1). For Irgarol 1051 and Diuron bioassays, the solvent control was never significantly different to the seawater control ($p > 0.05$), and as such, solvent controls are not presented in figures.

Mean EC_{20} values (n=5) for the three toxicants tested are presented in Table 5.1, along with associated 95% confidence intervals. Analysis of variance of these EC_{20}s demonstrated significant differences in sensitivity of *H. banksii* sperm to Irgarol 1051, Diuron and copper ($p < 0.005$) and a *post hoc* multiple comparison test ranked toxicity as Irgarol 1051 > Diuron > copper with respect to PSII Inhibition.

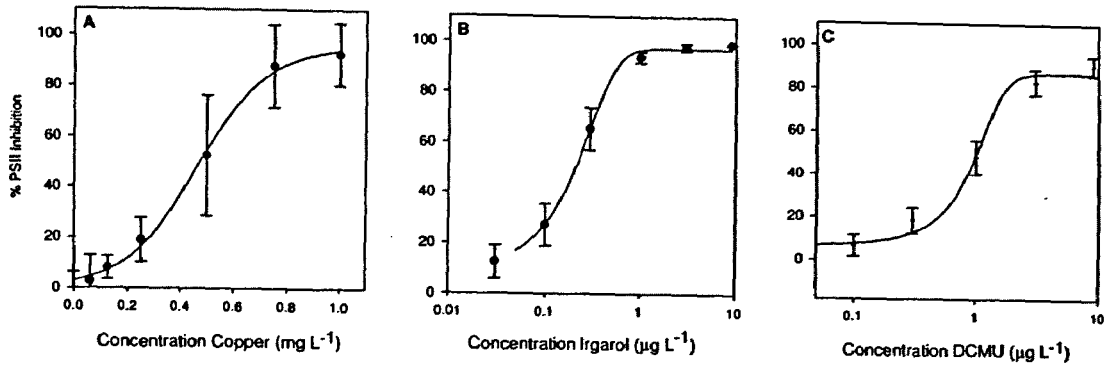

Figure 5.1: Dose-response relationships for %PSII Inhibition of *H. banksii* sperm exposed (for 2 h) to copper, Irgarol 1051, or Diuron (A, B and C, respectively). Data points represent mean ± standard deviation (n=5). Fitted curve used a 3 parameter logistic equation.

Table 5.1: Summary parameter table showing EC_{20} value for each toxicant tested and associated 95% Confidence Intervals (CI).

Toxicant	Mean EC_{20} (n=5)	Lower-Upper 95%CI
Diuron	0.30 µg L^{-1}	0.21 – 0.39
Copper	125 µg L^{-1}	112 – 148
Irgarol 1051	0.10 µg L^{-1}	0.02 – 0.19

5.3.2 Toxicant Interaction Bioassays

As was observed in the individual toxicity bioassays, photosynthetic stress was evident in all mixture treatments. After 2 h exposure period, all toxicant mixtures resulted in a reduced photosynthetic capacity as indicated by elevated %PSII Inhibition values in comparison to control (Figure 5.2). The reference toxicant, Diuron at concentration corresponding to EC_{50} (1.0 μg L^{-1}), gives an expected result of approximately 50% PSII Inhibition, thus bioassay results are validated.

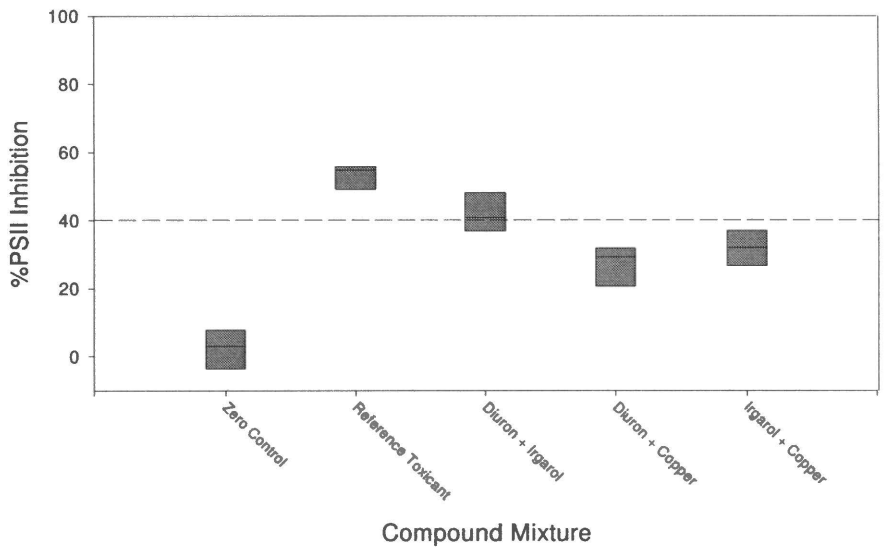

Figure 5.2: %PSII Inhibition of *Hormosira banksii* sperm exposed (for 2 h) to mixtures of Diuron and Irgarol 1051, Diuron and copper, and Irgarol 1051 and copper. Boxes represent median (line within box, n=3) and 95% confidence interval (upper and lower edge of box). Reference line (dashed) represents expected activity of mixtures based on EC_{20} + EC_{20}.

Mixtures containing Diuron and Irgarol 1051 displayed an additive interaction as reflected by RI values given in Table 5.2. In contrast, those mixtures containing copper showed antagonistic effects between copper and both Diuron and Irgarol 1051 (Table 5.2). *Post hoc* comparison ($p<0.05$) shows the order of toxicity from most (and therefore most effective) to least toxic combination as: Diuron + Irgarol 1051 > Irgarol 1051 + copper > Diuron + copper.

Table 5.2: Summary interaction table showing RI values for each toxicant mixture tested and associated interaction term (n=3).

Toxicant	RI ± Standard Deviation	Interaction
Diuron + Irgarol 1051	1.038 ± 0.1426	Additive
Diuron + copper	0.8077 ± 0.1734	Antagonistic
Irgarol 1051 + copper	0.7793 ± 0.1279	Antagonistic

5.4 Discussion

The results presented above show that antagonism occurs for each of the herbicides, Diuron and Irgarol 1051, when mixed with copper. This data is supported by other studies as Teisseire, *et al.*, (1999) also observed antagonism for mixtures of Diuron and copper as assessed with Abott's formula; though their results showed non-significant antagonism (with RI value not significantly different from 1, $p > 0.05$).

While there has been little research directed in to the mechanisms behind toxicant interactions of herbicides and metals, perhaps the antagonism observed with mixtures of Diuron and copper, and Irgarol 1051 and copper, can be explained in terms of impact sites. When a number of toxicants are acting simultaneously and their modes of action or impact sites overlap or compete, interactions of toxicants within that mixture may be influenced by these overlaps, or competition between modes of action.

As was described in Chapter 1, 50% of all herbicides in use today are designed to act on or target photosystem II (PSII; Koblizek, *et al.*, 1998). Both Diuron and Irgarol 1051 reflect this as they both inhibit photosynthetic electron flow to the plastoquinone, Q_B, in PSII by blocking the electron transport chain just after the primary electron acceptor (Q_A) (Miles, 1991; Falkowski and Raven, 1997). In contrast, a review of the relationship between copper and PSII concluded that while the main decree amongst authors is that the primary target sites of copper impact on PSII are at its oxidizing side, other suppositions amongst authors as to copper impact sites include sites at the Pheo-Q_A-Fe^{2+} domain, the PSII reaction centre, the Hill reaction and water splitting (Barón, *et al.*, 1995). With Diuron and Irgarol 1051 blocking the electron chain at Q_A, copper ions acting at the target sites falling downstream of Q_A would result in that copper impact going un-noticed or rather being unobservable (with regard to PSII Inhibition). This masking of copper impact would account for the antagonistic interaction observed in this and other studies.

There has, however, been some research directed into the additive nature of two herbicides acting together. Chesworth, *et al.*, (2004) observed similar results to those presented here, where Diuron and Irgarol 1051 interact additively when present in a mixture. Both herbicides act in a very similar way, as detailed above, so additivity makes sense in that when the two herbicides are mixed it is as though either one of the single herbicide concentrations is doubled (as opposed to competition or blocking). However, there is evidence that suggests that if two herbicides have similar modes of action, they will experience greater competition for binding sites and will in fact interact antagonistically (Jansen, *et al.*, 1993; Chesworth, *et al.*, 2004). Though not observed here, increased competition can result from Irgarol 1051's higher affinity for binding sites, which would result in a reduced Diuron impact and a possible antagonistic interaction (Chesworth, *et al.*, 2004).

A further issue in the assessment of interactive effects arises in light of evidence that the interaction term assigned to a mixture can differ with changes in relative concentrations of components within that mixture (Teisseire, *et al.*, 1999; Chesworth, *et al.*, 2004). While beyond the scope of the work presented here, it would be interesting and useful to assess whether varying the concentrations of mixture components affected the interaction of the mixture components. It would also be interesting to assess if the assigned interaction (additive, synergistic or antagonistic) holds for different endpoints (biochemical, growth, mortality, etc.). If the interaction term assigned to a mixture differs with regard to sensitivity or specificity of the endpoint employed, there may be a 'most sensitive' endpoint by which the interaction can be determined. This 'most sensitive' endpoint could be important as interactions of toxicants can have implications on water quality guidelines.

Given that water quality guidelines are established for single toxicants acting alone, there is no accommodation for the interactive effects of chemicals co-existing in the environment. As Teisseire, *et al.,* (1999) notes, synergism is the most important effect to protect against as the resultant increase in toxicological effect can not be predicted from just ambient water chemistry or single toxicant concentrations measured for a given ecosystem. Additivity is also of similar concern because while single chemical concentrations may be present in an ecosystem at levels below water quality guidelines, if two or more chemicals are present in that ecosystem, each will exert their own toxic effect. The additive result of all individual impacts may be enough to pose serious risk, despite no water quality guidelines having been breached. Thus, the interaction of chemicals is an important factor in determining 'true' risks of co-existing pollutants.

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Chapter 6

6.0 Eco-relevance of Chlorophyll a Fluorescence Endpoints

6.1 Introduction

While the single species bioassay has been and continues to be the backbone of ecotoxicology (Cairns, 1983), there has been fervent and ongoing debate as to the appropriateness of using this data to infer higher level (*supra*-organism) effects of pollution. This debate dates back some twenty years, to the mid-1980's, where many argued over what data and at what level of organisation could, or should, be used as the basis for Water Quality Guidelines (WQG) and whether there was sufficient ecological focus in ecotoxicology (Gray, 1989; Maltby and Calow, 1989; Cairns and Pratt, 1993).

More recent times have seen a greater acceptance of single species tests, particularly of sub-lethal biomarkers (and other tests recently termed microscale toxicity testing, see Wells, *et al.*, 1998 for overview), and recognition of their important role as *part* of an effective environmental protection program (Wells, 1999; Wells, *et al.*, 2001; Galloway, *et al.*, 2002). However, as recently as 2004, there are some that continue to challenge the use of single species toxicity tests as an effective means of assessing potential impacts of pollution at higher levels of biological organisation (Schmidt, 2004). For the purposes of this chapter, higher-levels of biological organisation will be defined as those greater than the individual organism; ie. population- and ecosystem-level.

The primary argument against single species tests is that toxicant-induced changes in individual-level variables are not easily translated into higher-level responses (Forbes and Calow, 2002; Schmidt, 2004), and as such, some go as far to suggest toxicity testing should be exclusively performed at least at the population-level (without actually proposing how this can be done). The weakness in this argument is that incorporating a number of generations, different life stages and inherent variability makes it extremely difficult to perform testing at this complex population-level of biological organisation (Sih, *et al.*, 2004).

While it has been demonstrated that population-level effects of toxicants can be experimentally studied (Forbes and Calow, 2002), it has only been possible to do so with small invertebrates with a generation time of a few weeks. As Sih, *et al.*, (2004) correctly point out, "...comparable studies on [larger, longer lived organisms] would take years, probably decades...and doing it for...tens of thousands of registered chemicals is infeasible."

Indeed, even expert ecologists such as Dr David Schindler (1996) have stated, "It is unrealistic to expect that complex ecosystem-level assessments will ever become a part of routine ecotoxicological procedures. The vast number of environmental stresses that must be monitored, the high cost and long duration of ecosystem-scale tests, and the problem of assessing synergisms, antagonisms and other interactive effects dictate that, for the foreseeable future, the prediction of ecosystem stress must rely heavily on bioassays and monitoring at simpler scales."

With this reliance on "bioassays and monitoring at simpler scales", one way to ensure that using these bioassays does not ignore or overlook higher-level effects is to make sure they have ecological relevance to the receiving environment with respect to the organism selected and the endpoint measured (Anderson, *et al.*, 1990). Thus, a level of 'eco-relevance' is established for the bioassay. For the purposes of this work, eco-relevance is defined as: the degree to which a bioassay can be directly related or linked to actual field events, whereby incorporating higher-level effects (population- and ecosystem-level) that may be inferred beyond that which is immediately measured. Again, for the purposes of this work, the level of eco-relevance for any given bioassay may be based on two primary elements: 1. The relevance the test species/organism has to the greater ecosystem. That is, is the test species an integral part of the ecosystem; will an affect on it result in greater effects to the system as a whole? And, 2. The relevance the endpoint measured has to the organism, the population, and the ecosystem. Does the endpoint represent a significant effect to not only the individual, but also infer effects at higher levels through demonstrated links? Therefore, it is evident that eco-relevance is an important concept if bioassays operating at 'lower' levels of biological organisation are to be used to make decisions that are meant to protect the ecosystem as a whole. As such, it is important for relatively new endpoints to demonstrate a level of eco-relevance if they are to be accepted as routine ecotoxicological tools (Anderson, *et al.*, 1990; Lam and Gray, 2001). This is especially true for those endpoints that measure sub-lethal stresses and which don't immediately have the same obvious consequences as mortality in terms of impact to the individual (eg. fluorescence parameters).

In a recent review of chlorophyll *a* fluorescence ecotoxicology, Ralph, *et al.*, (2005) found that while chlorophyll *a* fluorescence had frequently been used as an indicator of pollution or phyto-toxic stress, at no time has a level of eco-relevance, beyond immediate effect to the individual, been experimentally demonstrated for fluorescence parameters in their use as ecotoxicological endpoints. As such, presented below are a series of experiments that demonstrate how chlorophyll *a* fluorescence measurements of toxicant-impacted macroalgae gametes can be linked to germination success and in turn, how this can have effects at higher levels of biological organisation. Thus, a level of eco-relevance for the *H. banksii* chlorophyll *a* fluorescence bioassays described in Chapters 3 and 4 can be established: macroalgal gamete health can infer effects beyond those experienced by individual gametes and can be linked to population responses, and that chlorophyll *a* fluorescence parameters represent a biologically significant measure of impact. Accordingly, a level of eco-relevance exists for the chlorophyll *a* fluorescence bioassays that is at least equal to that of the germination-based bioassay.

6.2 Materials & Methods

6.2.1 Test Solutions

Toxicant solutions of the herbicides Diuron and Irgarol 1051 were prepared as per the methods described in Chapter 3.3.1 (Test Solutions). All experiments were performed with solvent controls, however the solvent controls were never significantly different to the seawater control ($p > 0.05$) and as such, data for solvent controls are not presented.

6.2.2 Collection and Preparation of Macroalgae

Collection of adult *H. banksii* and subsequent gamete spawning were both performed as described in Chapter 2.2.3 (*H. banksii* collection and spawning).

6.2.3 Photosynthetic Condition of Germinating Gametes Exposed to Toxicant Stress

H. banksii gametes were exposed to a toxicant stress throughout the process of germination. That is, unfertilised eggs and sperm were dosed with a toxicant solution and left to progress through fertilisation and germination, until which time eggs resembled germlings (48 h; steps 2 to 4 of the life cycle shown Figure 1.1). During this exposure period, measures of photosynthetic health were assessed using Microscope-PAM (Walz GmbH, Effeltrich, Germany; a pulse amplitude modulated fluorometer capable of taking fluorescence measurements of single cells).

The period of exposure was performed within petri dishes, with three controls (containing filtered seawater; see Chapter 2.2.2 (Toxicants and solutions)), and three treatment petri dishes (containing 7.5 mg L^{-1} Diuron; based on EC_{50} values from pilot study, data not shown). *H. banksii* eggs and sperm were added to petri dishes at optimal ratio for germination (1:200 egg:sperm; Gunthorpe, *et al.*, 1995). A glass microscope slide was placed at the bottom of each dish so that germlings would settle on them, thus facilitating measurements taken under Microscope-PAM. Solution levels in all petri dishes remained at a level where glass slides and gametes/germlings were immersed at all times, except for short period (approximately 8 min) when slides were removed and measurements were taken.

Petri dishes were kept at 18 ± 0.5°C and illumination at 23 ± 2 µmol photons $m^{-2}s^{-1}$ on light:dark cycle of 16:8 for 48 h. During this time, measures of photosynthetic capacity were made at regular intervals using Microscope-PAM. The parameters measured were $\Delta F/ F_m'$ (effective quantum yield; a measure of PSII efficiency) and ETR_{max} (maximum electron transport rate; a measure of ability to cope with light stress), where the instrument settings mimicked those validated with ToxY-PAM as much as possible (with saturation-width and light intensity being identical). At 48 h, germination was recorded as successful if there was evidence of rhizoid elongation, a dark cell colour, and a clearly visible cell wall (as per Gunthorpe, *et al.,* 1997). All measurements and recording of germination performed for each petri dish were done on the same individual germling for the full 48 h (from initial unfertilised egg through to fertilised germling). This was made possible by simply marking the underside of the slide to circle the designated gamete/germling.

6.2.4 Manipulations of Gamete $\Delta F/ F_m'$ and Corresponding Germination Success

Freshly spawned solutions of *H. banksii* gametes (eggs and sperm) were manipulated with the addition of known amounts of Irgarol 1051 to give samples of eggs or sperm ranging from 'healthy' to 'heavily inhibited'. Irgarol 1051 was used in this experiment as it irreversibly binds to target sites and would not be 'washed out' as would occur with Diuron (Jones and Kerswell, 2003; which would make manipulations less permanent and much harder to control). Eggs were exposed to Irgarol 1051 at the following concentrations of 0, 0.2, 0.5 and 5 µg L^{-1} to give final target $\Delta F/ F_m'$ values of 0.5, 0.3, 0.1 and 0; sperm samples were exposed to Irgarol at

the following concentrations of 0, 0.1, 0.3 and 3 μg L^{-1} to give target $\Delta F/ F_m'$ values of 0.3, 0.2, 0.1 and 0. Healthy eggs and sperm were classified as having a $\Delta F/ F_m'$ of 0.5 and 0.3, respectively (based on mean of >100 control measurements for this experiment). The concentrations of Irgarol 1051 required to give desired inhibitions were determined from dose-response curves presented in Chapters 3 and 5. All actual $\Delta F/ F_m'$ values for the samples were within 10% of target value (actual values are plotted in results). These gametes of varying 'health' were then added to healthy samples of the complimentary gametes (at appropriate ratio of 200:1, sperm:egg (Gunthorpe, *et al.*, 1995)) and the resultant level of germination was recorded after 48 h. A diagram of experimental setup is shown in Figure 6.1.

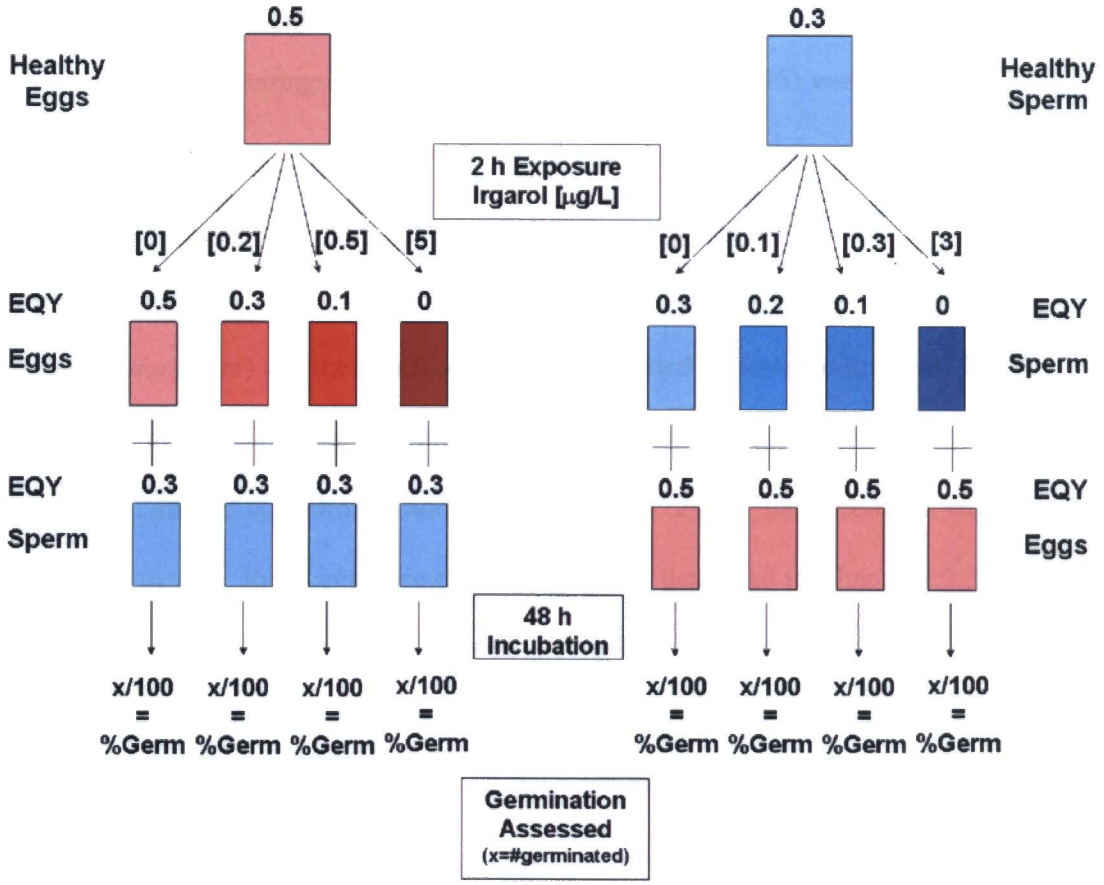

Figure 6.1: Diagram for the method of manipulating gamete solutions to give varying degrees of health, and subsequent assessment of germination success. Irgarol concentrations (nominal) are given in square brackets in µg L^{-1}.

6.2.5 Effect of Parental Toxic Exposure on Gamete Condition

To investigate the link between toxic exposure of adult thallus on gametes, approximately 60 mature thalli of *H. banksii* were collected at mid-high tide as per the method and location described in Chapter 2.2.3 (*Hormosira banksii* collection and spawning). These adults were then divided into 4 groups of 15 plants and initial $\Delta F/ F_m'$ was measured using Diving-PAM (10 randomly selected plants from each

group measured with a single saturating pulse; Diving-PAM measurements were performed with all settings at the default values). Adult thalli (15) were then immersed in each treatment solution: 0, 10, 100 and 1000 μg L^{-1} Irgarol 1051 for a period of 3 h (the remaining period of high tide at time of collection). After this 3 h exposure period, $\Delta F/ F_m'$ was measured on adult thalli for each treatment (as per initial measurement) and then each solution was emptied to simulate low tide. The adult thalli were left without seawater for 3 h to semi-desiccate, as would happen in field before normal collection of adult thalli, and then $\Delta F/ F_m'$ measurements were taken. At this time, the plants were chilled for 18 h as described in Chapter 2.2.3 (*Hormosira banksii* collection and spawning). To follow the procedure for collecting gamete samples, spawning was initiated with warm water rinse after this 18 h chilling and the final adult $\Delta F/ F_m'$ measurements were taken immediately prior to gamete release. Adult thalli that exhibited spawning were grouped as male or female (within treatment) to give three egg solutions and three sperm solutions per treatment. These gamete solutions were then assessed in ToxY-PAM to give $\Delta F/ F_m'$ values for gametes spawned from toxicant-exposed parents (ToxY-PAM settings were as described in Chapter 2.2.4 (General settings for ToxY-PAM)).

6.3 Results

6.3.1 Photosynthetic Condition of Germinating Gametes Exposed to Toxicant Stress

The germlings exposed to Diuron show a marked reduction in $\Delta F/ F_m'$ and maximum electron transport rate when compared to control (Figure 6.2 and 6.3). Figure 6.2 shows the Diuron exposed samples exhibited an immediate decline in $\Delta F/ F_m'$ while the value of $\Delta F/ F_m'$ for control samples did not significantly differ over the 48 h. Figure 6.3 shows a similar photoinhibitory effect where ETR_{max} of the toxicant exposed germlings significantly declined, implying an inability to cope with light stress, whereas the control samples again show a steady 'healthy' response.

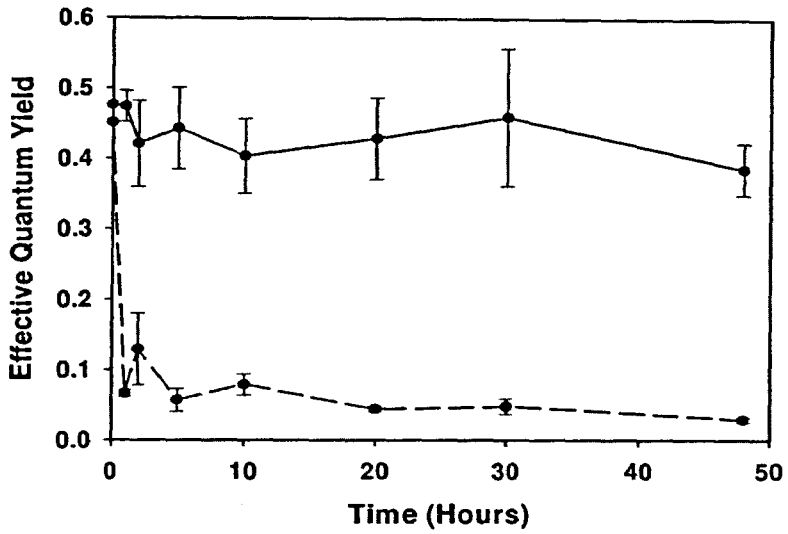

Figure 6.2: Photosynthetic condition of *Hormosira banksii* gametes throughout germination process. Solid line represents Effective Quantum Yield of control (seawater) germination and dashed line represents Effective Quantum Yield of toxicant (7.5 mg L^{-1} Diuron) inhibited germination. Data points represent mean ± standard deviation (n=3).

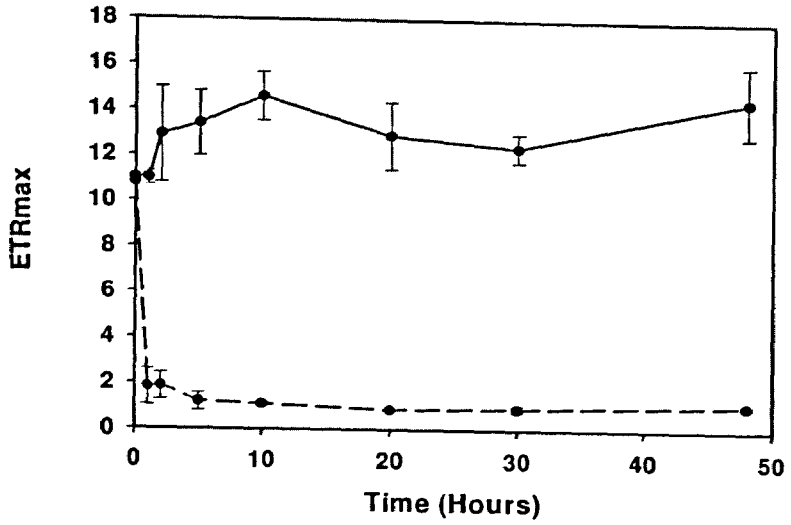

Figure 6.3: Photosynthetic condition of *Hormosira banksii* gametes throughout germination process. Solid line represents Maximum Electron Transport Rate of control (seawater) germination and dashed line represents Maximum Electron Transport Rate of toxicant (7.5 mg L^{-1} Diuron) inhibited germination. Data points represent mean ± standard deviation (n=3).

In terms of germination success, all three control samples exhibited germination success (100%), yet only one of the three Diuron exposed samples resulted in germination (33%).

At time zero, all germlings within control and treatment samples were indistinguishable in terms of photosynthetic health; all started at what would be considered a healthy level as determined by fluorescence parameters (based on control measurements for this experiment). At the first measurement, 2 h into the experiment, a decline in $\Delta F/ F_m'$ and ETR_{max} values occurred in the toxicant exposed treatments. This decline immediately differentiated the treatments from the control,

meaning that after just two hours of toxicant exposure, fluorescence measurements were able to conclude that a deleterious effect had taken place as a result of Diuron exposure. In order to make this same conclusion based on germination success, the experiment had to be conducted for 48 h, at which time rhizoid elongation could be assessed.

6.3.2 Manipulations of Gamete $\Delta F/ F_m'$ and Corresponding Germination Success

Figures 6.4A and 6.4B show a clear relationship between photosynthetic health and germination success ($r^2 = 0.92$ and 0.95 for sperm and egg, A and B, respectively). Photosynthetically healthy gametes, when mixed with healthy counterparts, successfully germinated at a rate of more than 90%, which is above the expected control germination rate as used by and described in *H. banksii* germination bioassay protocols (Gunthorpe, *et al.*, 1995). It can also be seen (Figure 6.4) that germination rates steadily decline with relation to decreasing photosynthetic health, for both eggs ($p < 0.05$) and sperm ($p < 0.05$). That is, if a gamete has a reduced $\Delta F/ F_m'$, there is an association with reduced germination success.

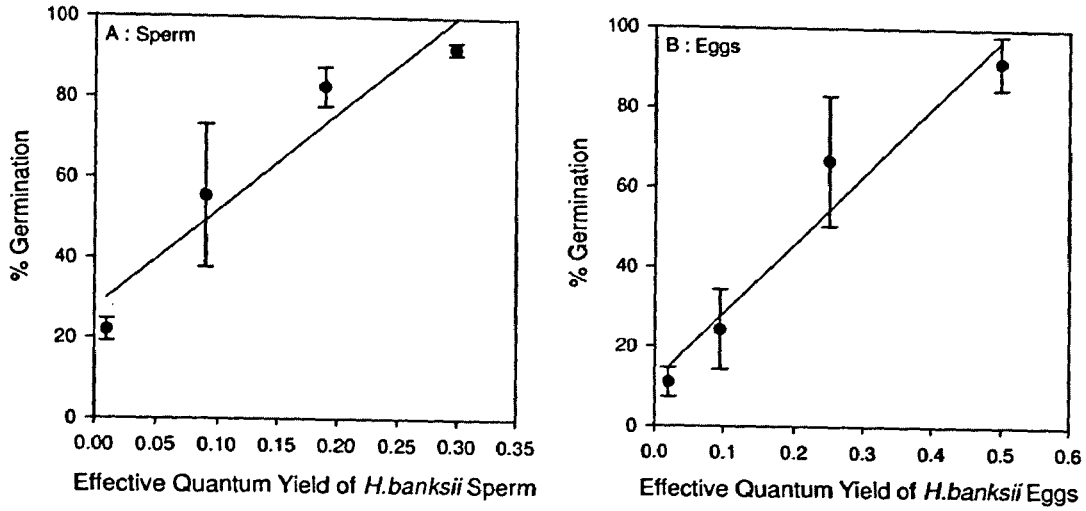

Figure 6.4: Effect of gamete health on ability to germinate. A and B represent sperm and eggs, respectively. Data points represent mean ± standard deviation (n=3). Lines represent 1 order regression for each graph.

6.3.3 Effect of Parental Toxic Exposure on Gamete Condition

It can be seen from Figure 6.5 that this experiment resulted in four distinct levels of inhibition in the toxicant impacted adult *H. banksii* that correspond to the four treatments. All mature thalli started as healthy ($\Delta F/ F_m' > 0.65$) and Irgarol 1051 exposure did indeed photosynthetically inhibit all treatment levels so that the final photosynthetic health of each treatment was decrementally worse than that preceeding it (hence the four distinct levels of inhibition). It can also be seen that the collection process itself did not significantly alter the health of the adults and that final $\Delta F/ F_m'$ values are a true representation of those taken immediately after toxic exposure. That is, there was no washing out or lessening of the toxic effect during the desiccation and chilling steps when Irgarol 1051 was used.

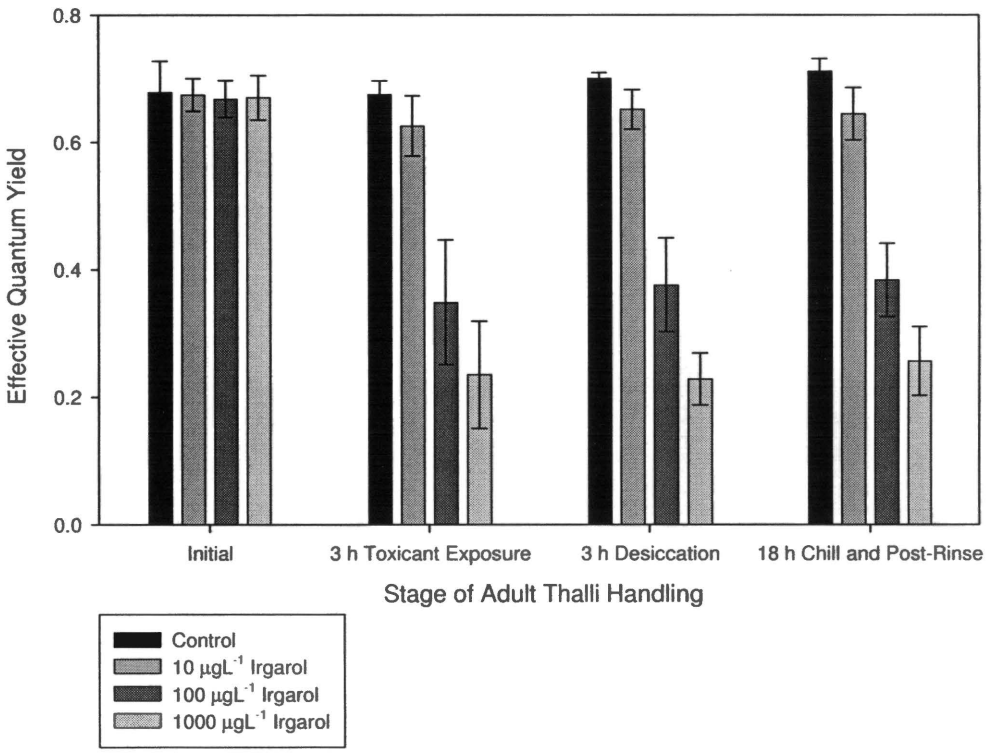

Figure 6.5: Effective quantum yield of adult *Hormosira banksii* throughout Irgarol 1051 exposure and 'collection' process. Bars represent mean ± standard deviation (n=10).

Figure 6.6 shows the $\Delta F/ F_m'$ values for gametes (sperm or eggs, A and B respectively) spawned from toxicant-impacted adults. Kruskal-Wallis analysis of variance shows that the two higher concentrations of toxicant resulted in significantly lower ($p < 0.05$) $\Delta F/ F_m'$ for *H. banksii* eggs and that all toxicant impacted adults spawned sperm with significantly lower $\Delta F/ F_m'$ ($p < 0.05$). As was demonstrated in the previous section (6.3.2), gametes with a reduced $\Delta F/ F_m'$, as seen with the eggs from 1000 µg L^{-1} Irgarol 1051 treatment and the sperm from the 100 and 1000 µg L^{-1}

Irgarol 1051 treatments, are much less likely to be associated with successful germination.

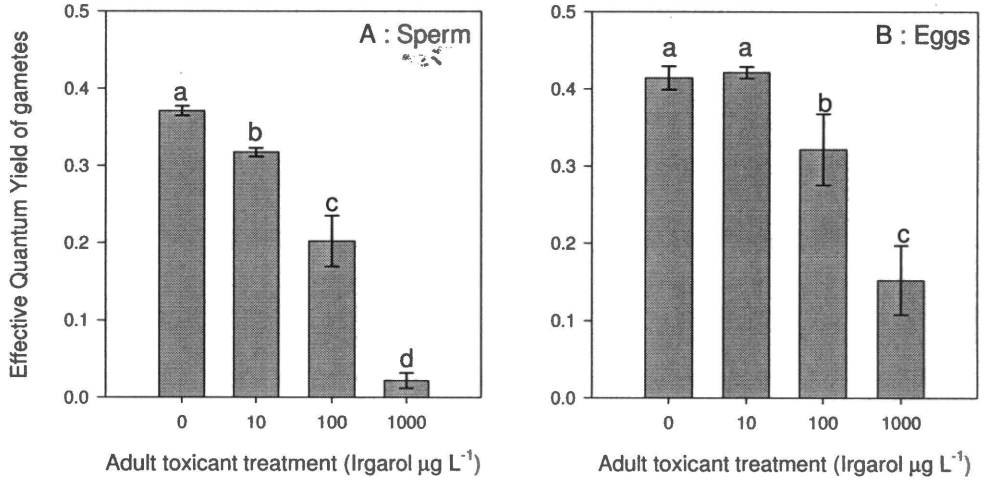

Figure 6.6: Effective quantum yield of gametes spawned from toxicant exposed adult *Hormosira banksii*. A and B represent sperm and eggs, respectively. Bars represent mean ± standard deviation (n=3).

6.4 Discussion

6.4.1 Photosynthetic Condition of Germinating Gametes Exposed to Toxicant Stress

This thesis has previously discussed how the majority of toxicity testing performed with macroalgae is done so with endpoints based on physiological development of early life stages and that these bioassays are well accepted in terms of eco-relevance (in that the endpoints can be linked to population-level effects). So well accepted is

the notion that reduced germination results in population decline (due to a reduction in recruitment) that test protocols using early life stage development of kelp are significant components of state and national pollution monitoring programs in the United States (Anderson, *et al.*, 1998). A fundamental premise of this thesis is that a macroalgal gamete with a reduced photosynthetic health represents the same resultant decline in population numbers.

So while the *H. banksii* fluorescence bioassays described in Chapters 3 and 4 have many advantages over the germination based bioassays, a link between fluorescence parameters and population effects must be made in order to establish a level of eco-relevance that is at least equal to that of the germination bioassays. What is required to make this causal link is a demonstration that fluorescence parameters (specifically $\Delta F/ F_m'$) can effectively predict the likelihood of failed germination, and can do so before the actual event of germination occurs (thus enabling one of the major advantages cited earlier of fluorescence bioassays: the rapidity of the fluorescence endpoint).

In terms of time-to-result, the results described in 6.3.1 show that fluorescence parameters were able to conclude after just 2 h that the Diuron exposed germlings were significantly impacted, whereas the germination endpoint took 48 h to arrive at the same conclusion. While lacking direct evidence that fluorescence parameters are linked to germination success, this result suggests that fluorescence parameters can give an indication of the likelihood of germination success (Figures 6.2 and 6.3), and can do so much earlier than the actual event of germination. The next step in linking

fluorescence and germination is to establish the level of photosynthetic inhibition at which germination is also inhibited and to do so at environmentally realistic pollution levels.

6.4.2 Manipulations of Gamete $\Delta F/ F_m'$ and Corresponding Germination Success

Since inhibition of germination is accepted to have population-level effects with regard to macroalgae (Anderson, *et al.*, 1998), if there is an established link, or correlation, between a fluorescence parameter and germination response, then it follows that fluorescence can also be considered to represent population-level effects. The bioassays described in Chapters 3 and 4 both use $\Delta F/ F_m'$ to measure photosynthetic health of *H. banksii* gametes (eggs and sperm respectively) and ToxY-PAM's capability to make comparisons to real-time controls gives the parameter PSII Inhibition (see Chapters 2, 3 and 4). As such, this current experiment aimed to establish a link between $\Delta F/ F_m'$ of *H. banksii* gametes and the ability of those gametes to successfully germinate. The reasoning being that if it can be determined that once eggs or sperm reach a certain level of photosynthetic damage (lower $\Delta F/ F_m'$) they are unable to germinate, and that a relationship holds for $\Delta F/ F_m'$ and ability to germinate, then the fluorescence parameter can in fact predict the likelihood of germination success.

Indeed, the results presented here show that a gamete with reduced photosynthetic health (PSII inhibition) when crossed with a 'healthy gamete' leads to a reduced germination success and that a relationship between $\Delta F/ F_m'$ and germination success

does exist. Given that a reduction in germination has been linked to population effects, these results, in turn, mean that taking fluorescence measurements allows for rapid assessment to be made as to the toxicity of a sample and that these conclusions have now been shown to infer a higher level effect. That is, the rapid, fluorescence-based endpoint used in the protocols described in Chapters 3 and 4 has thus been demonstrated to have a level of eco-relevance at least equal to that of germination-based bioassays.

6.4.3 Effect of Parental Toxic Exposure on Gamete Condition

The final experiment in this series addressed the question of whether toxicant impacts experienced by adult *H. banksii* macroalgae affect the gametes contained within those adult thalli. Adult thalli are considered to be tolerant to relatively high concentrations of pollution (Thursby and Steele, 1995), which is why macroalgal bioassays predominately use the most sensitive life stage: the gametes (Thursby and Steele, 1995; Gunthorpe, *et al.*, 1997). The assumption is that gamete bioassays simulate the conditions of recently-spawned gametes being released into the water column where they experience toxic impact. However, this overlooks an important factor in that gametes may already be impacted as a result of developing inside a toxicant-exposed adult. While the adult thalli may be able to tolerate this exposure, the developing gametes contained within may be affected, and upon release, without any further toxic assault, display signs of reduced health or viability.

While it is still clear that the gametes of *H. banksii* are the most sensitive lifestage to direct toxic impact, this result demonstrates that toxic impact on the 'tolerant' adult

stage can also adversely affect the un-released gametes. This has implications in the use of field collected macroalgae for bioassay testing as the gametes may already be under toxic stress due to adult exposure. To avoid this, collection sites should be carefully chosen to ensure minimal adult exposure to toxins and good control gamete health should be confirmed and not assumed. The use of real-time controls in ToxY-PAM, as described in the fluorescence bioassay protocols described in Chapters 3 and 4, provides information on the control gametes' health and it can be seen from the beginning of a test whether gametes are already experiencing stress. The germination bioassays, in comparison, have no way of assessing pre-test exposure until control germination is assessed at the end of the 48 h test. Thus, resources and time may be wasted by performing a test that is invalid, but the test needed to be completed to be able to determine this. Whereas, the fluorescence bioassays are able to ascertain whether certain aspects of control gamete health, as relates to photosynthesis, are adequate from the first measurements (and at worst it is only a matter of hours that has been spent), or even better, a sample of the control gamete solution can be tested immediately after spawning to ensure test will be performed with valid control health.

6.5 Conclusions

The three experiments described in this chapter show that fluorescence parameters are capable of predicting germination success when macroalga are exposed to herbicides that inhibit PSII and can do so much sooner than the actual event of germination; that a decline in effective quantum yield is associated with a reduced germination success; and that fluorescence bioassays are capable of detecting

reduced gamete health and viability resulting from adult herbicide exposure. These results are clear indications that fluorescence response and germination are associated in alga exposed to herbicides, and that fluorescence is able to infer biological effects beyond that of just immediate gamete photosynthetic health.

This link showing that a decrease in $\Delta F/ F_m'$ is associated with reduced germination, as has been previously discussed, means that fluorescence and in particular $\Delta F/ F_m'$ of gametes, is associated with higher ecological effects by way of reduced recruitment. Thus, the fluorescence endpoint has a level of eco-relevance at least equal to that of the germination endpoints currently used in pollution monitoring programs in the United States. This is an important step in fluorescence ecotoxicology's recognition as an effective, meaningful measure of pollution effects.

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Chapter 7

7.0 General Conclusions

7.1 Development of Chlorophyll *a* Fluorescence Bioassay with *Hormosira banksii* Gametes

The primary objective of this thesis was to develop and optimise a bioassay protocol that uses improvements on past macroalgae bioassay techniques to allow the photosynthetic capacity of *H. banksii* gametes to be assessed by chlorophyll *a* fluorescence measurements. This primary aim has been met in that two bioassay protocols have been developed using gametes from *H. banksii.*

Both protocols define the use of a PAM (Pulse Amplitude Modulated) fluorometer, ToxY-PAM, to measure photosynthetic inhibition of *H. banksii* gametes exposed to a range of toxicants (herbicides, trace metals and petrochemicals). The protocols differ in the use of either *H. banksii* oospheres (eggs; described in Chapter 3) or antherozoids (sperm; described in Chapter 4 and featured in Chapter 5) as the photosynthetic material. Each of these chapters have illustrated the successful development of a fluorescence based bioassay using *H. banksii* that can be used for rapid assessment of toxic impact on near-shore macroalgal communities, and as a valuable toxicity screening tool for effective coastal management.

ToxY-PAM has been shown to be an effective tool for real-time comparisons of control and treatment photosynthetic measurements, and for the first time has been applied to macroalgal investigations rather than microalgal (such as Bengtson Nash, *et al.*, 2005).

In addition to method development and protocol definition, this work incorporated a series of experiments where the new, fluorescence-based bioassay using *H. banksii* eggs was directly and concurrently compared to the established germination-based macroalgae bioassay (detailed in Chapter 2.2.5 (Germination bioassay protocol – *H. banksii* gametes)). The results of these experiments show the fluorescence bioassay to have substantially greater sensitivity, higher level of precision and a much faster time-to-result than the germination bioassay. The *H. banksii* sperm bioassay gave equally impressive results that also exhibited higher levels of precision and a much faster time-to-result than the germination bioassay for some toxicants, but it had the added advantage over the *H. banksii* egg bioassay in that it could be applied to a wider range of toxicants, and was also able to offer valuable information on the toxic mechanism of the tested toxicants with little variation to standard protocol (described in Chapter 4).

7.2 Experimental Demonstration of Eco-Relevance for a Chlorophyll *a* Fluorescence Endpoint

Expanding the protocol beyond development, one of the secondary objectives of this work was to establish a level of eco-relevance was demonstrated for chlorophyll *a* fluorescence endpoint. This is significant in that this has not previously been published and confirmation of eco-relevance can have favourable implications as to future acceptance of chlorophyll *a* fluorescence bioassay data into water quality guidelines (Anderson, *et al.*, 1990; Lam and Gray, 2001).

The results described in Chapter 6 show that fluorescence parameters are associated with germination success and can occur earlier when compared to the actual event of germination. Moreover, it was experimentally shown that a decline in effective quantum yield of PSII can be directly linked to germination success and that fluorescence bioassays are capable of detecting reduced gamete health and viability resulting from adult herbicide exposure. These results are clear indications that fluorescence response and germination are associated when *H. banksii* is exposed to a PSII inhibiting herbicide, and that fluorescence is, to a certain extent, linked to effects beyond that of immediate photosynthetic health of the gamete.

7.3 Application of Bioassay Method to Applied Research

To further validate the fluorescence based sperm bioassay, the method was applied to a 'real-world' issue, where the interactive effects of anti-foulant compounds (Irgarol 1051, Diuron and copper) were assessed (Chapter 5). The bioassay was successfully used to determine the interactions of these compounds and highlighted the concern, also acknowledged by other authors (Teisseire, *et al.*, 1999; Chesworth, *et al.*, 2004), that interactive effects can have serious implications for the effectiveness of water quality guidelines, such as under- or over-protection.

Given that the bioassay provided useful data and allowed for definitive conclusions, it can be concluded that the *H. banksii* chlorophyll *a* fluorescence bioassay provides a powerful tool in the rapid assessment of toxic impacts and can be effectively used in applied research investigations.

7.4 Future Research Directions

The *H. banksii* fluorescence bioassays described in this thesis have the potential for expansion to include other toxicants such as metals and whole effluents, but the effects of auto-fluorescing humic substances and the interferences caused by coloured or turbid samples need to be assessed.

Another area of potential research involves the comparative sensitivities of eggs and sperm to toxic assault. While it can be concluded that *H. banksii* sperm are generally more sensitive than eggs based on the data presented in this thesis, Eklund (1998) showed that when *Ceramium strictum* gametes are exposed to phenol, the eggs were more sensitive than sperm. Thus there could be differences based on species, toxicant, or a combination or cell physiology and toxicant mode-of-action. Further research into the comparative sensitivities of different life stages of macroalgae could provide valuable information on which aspects of the life-cycle are most vulnerable to toxic impact. So too, given that there are six accepted eco-types of *H. banksii*, a comparison between the relative sensitivities of the eco-types (or indeed different populations of the same eco-type) would also be valuable in validating this bioassay for use nationally.

Seasonal variation has been shown to affect variability in the *H. banksii* germination bioassay due to parental exposure to environmental variations (Gunthorpe, *et al.*, 1995; Myers, *et al.*, 2005). While there is no experimental data for temporal effects on the chlorophyll *a* fluorescence bioassays described here, there is anecdotal evidence that only extreme weather events impact the bioassay variability (such as

when 5 days of >40°C weather effectively 'cooked' adult macroalgae). However, experimental determination of seasonal effects would allow for quality control limits to be established, similar to the advice given by Gunthorpe, *et al.,* (1995) where it is suggested that no testing be performed during the 5 days following an extreme weather event.

7.5 Final Conclusions

Essentially, this work described the development of, and also the successful application of, a novel, fluorescence macroalgae bioassay that not only has advantages over currently employed methods, but also offers important information on macroalgal tolerance to pollution. The *H. banksii* chlorophyll *a* fluorescence bioassay has been shown capable of providing a powerful tool in both the rapid assessment of toxic impact on near-shore macroalgal communities, and as an effective toxicity screening tool.

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Appendix A

Attribute	Sperm Protocol	Egg Protocol	Germination Protocol
Test type	Static	Static	Static
Temperature	18 ± 0.5 °C	18 ± 0.5 °C	18 ± 0.5 °C
Light quality	Cool White Fluorescent Lighting	Cool White Fluorescent Lighting	Cool White Fluorescent Lighting
Light intensity (μmol photon $m^{-2}s^{-1}$)	12 ± 1	23 ± 2	23 ± 2
Test chamber size	20 mL	20 mL	80 mL
Test solution volume	5 mL	5 mL	75 mL
Renewal of test solution	None	None	None
Age/stage of test organism	Gamete (antherozoid/sperm)	Gamete (oosphere/egg)	Fertilised gametes
Test material concentration	1.2 x10^6 sperm mL^{-1}	4 x10^3 eggs mL^{-1}	2 x10^3 eggs mL^{-1} 2 x10^6 sperm mL^{-1}
Replicates per test concentration	3	3	4
Diluent	5 µm filtered seawater, aerated >24 h	5 µm filtered seawater, aerated >24 h	5 µm filtered seawater, aerated >24 h
Salinity	33 ± 1 ppt	33 ± 1 ppt	33 ± 1 ppt
Test concentrations	Minimum 5, plus zero treatment, solvent control (if needed) and reference control	Minimum 5, solvent control (if needed) and reference control	Minimum 5, solvent control (if needed) and reference control
Dilution factor	0.3 or 0.5	0.3 or 0.5	0.3 or 0.5
Exposure period	2 h	2 h	48 h
Endpoint	%PS II Inhibition	%PS II Inhibition	% Germination Inhibition
Summary parameter	EC_{50} (2 h %PSII Inhibition)	EC_{50} (2 h %PSII Inhibition)	EC_{50} (48 h % Germination Inhibition)
Test acceptability criteria	>10% Inhibition in zero treatment	>10% Inhibition in controls	>90% Germination in controls
Instrument	ToxY-PAM (Heinz Walz GmbH)	ToxY-PAM (Heinz Walz GmbH)	Microscope
Measurements per replicate	3	3	1
Time-to-result (complete test duration)	6-7 h (excluding thallus collection)	6-7 h (excluding thallus collection)	> 50 h (excluding thallus collection)