



Institute for Water
& Environmental
Resource Management



University of Technology, Sydney

FINAL REPORT:

RESEARCH INTO EFFECTS OF OIL AND DISPERSED OIL ON TEMPERATE SEAGRASSES

by

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Executive summary

This report summarises a series of experiments designed as proof-of-concept of a scaling experiment to develop a model for assessing the impact of oil and dispersants on seagrasses. This particular report forms the introductory component of a larger research program involving additional granting agencies and post-graduate students. The toxic impact of oil and dispersants on seagrasses was measured *in situ* using chlorophyll a fluorescence and this was later compared to a range of surrogates of varying complexity. These surrogates included seagrass leaf sections, isolated seagrass thylakoid membranes, and microalgal cells. The main finding of this study was that seagrasses were mostly unaffected by oil (Gippsland crude) and dispersant (Slickgone NS). When the *in situ* seagrass data was compared to leaf segment data, leaf segments were found to be more sensitive, while microalgae were the most sensitive tissues to the water soluble fraction (WSF) of oil. This preliminary finding suggests that microalgal bioassays on oil and dispersants are sufficiently sensitive to clearly identify low concentrations in the field that would affect *Zostera capricorni*. Within the larger project (ARC Linkage - LP0561633) other seagrass species will be tested as will other oil dispersant combinations. This pilot project has developed a set of techniques that allow the assessment of oil and dispersant to seagrasses (and potential surrogates).

Introduction

Seagrasses support the majority of the world's near-shore coastal habitats (Wilkinson, 2002). Of the common pollutants entering estuaries and coastal habitats, petrochemicals are ranked amongst the greatest threat (Short and Wyllie Echeverria, 1996; Peters et al., 1997). Petrochemicals or polyaromatic hydrocarbons (PAHs) enter the near shore ecosystem in a number of ways including oil spills, urban street runoff, marina activity, oil drilling mud, oil refinery waste water and leaking storage facilities. Generally, when seagrasses are exposed to petrochemicals, sub-lethal quantities are incorporated into the tissue, causing a reduction in tolerance to other stress factors (Zieman et al. 1984). Subtidal seagrasses are only exposed to the oil in the water column. The primary phytotoxic effect of oil is induced by the absorption of the water-soluble fraction (WSF). The toxic components of petrochemicals are thought to be the poly-aromatic hydrocarbons (PAH), which are lipophilic, so they are able to pass through lipid membranes and tend to accumulate in the thylakoid membranes of the chloroplasts (Ren et al. 1994).

Dispersants consist of a surfactant in a carrier or solvent (Hatcher and Larkum 1982) and can be toxic in their own right, but the solvent can also encourage the breakdown of the waxy cuticle, allowing greater penetration of oils into seagrass leaves thereby increasing phytotoxicity. If an oil spill poses a serious threat to a sensitive coastal area such as a seagrass community, then the most appropriate clean up techniques must be rapidly identified and implemented. The type of oil spilt, the prevailing weather and the local geography will largely influence selection of the most suitable mediation or control technique (containment and recovery, or chemical dispersion). The decision of dispersant usage is generally based on the relative potential damage caused by floating oil slicks or dispersed oil droplets.

In this study field experiments were designed to test the effects of WSF and WSF+dispersant (hereon in referred to as petrochemicals) on the photochemical efficiency of *in situ* seagrass under near natural conditions. By measuring the response of seagrass exposed to a range of concentrations, the relation between treatment concentration and physiological response can be determined, and from this relation predictions can be made of the response to any concentration within the range of concentrations tested.

Field experiments with seagrasses require considerable resources (personnel and time) and are constrained by tides and time of day. Examination of the response of individual seagrass leaves to petrochemicals is more convenient. Results of experiments are likely to provide data comparable with that derived *in situ*. Moreover, by conducting experiments under controlled conditions one can determine the importance of environmental factors such as irradiance and temperature on the effect of oils and dispersants on seagrasses. Therefore, we conducted experiments with leaf segments in this study with a view to obtaining data obtained under well-defined environmental conditions that can be directly compared with the results of *in situ* seagrass exposures.

The response variable (fluorescence yield) that is integral to this study is measured at a subcellular level, that is we are measuring changes in the fluorescence emission of chlorophyll molecules that are residing within the chloroplast, an organelle situated within plant cells. Therefore, experiments examining the fluorescence response of whole plants is necessarily biased initially towards the responses of the outermost cells, and over time deeper subsurface cells may become affected and respond accordingly. This is what happens in the field, hence the importance of assessing seagrass responses to petrochemicals *in situ*. However, for us to better understand the responses of photosynthetic organisms to petrochemicals, examination of single-celled organisms such as microalgae is appropriate. Results of these experiments are uninfluenced by the lag in response characteristic of multicellular organisms such as seagrasses. By conducting similar petrochemical exposure experiments on a microalga we aim to derive a better understanding of how a complete fully functioning cell responds to petrochemicals, and thereby better interpret (and predict) responses of whole seagrass plants.

With a similar objective to experiments with microalgae, further experiments were carried out to determine the sensitivity of *Zostera capricorni* thylakoids to petrochemicals and if possible characterise this sensitivity in terms of EC50. Thylakoids are membranes that reside in the chloroplast of each seagrass (and microalgal) cell. Embedded within the thylakoid membrane are the photosystems PSI and PSII and these photosystems contain chlorophyll. In an intact chloroplast, light energy is transferred from the photosystems to energy-carrying molecules within the chloroplast (ATP and NADPH). A portion of the unused light energy is reemitted as fluorescence, and the extent of this fluorescence rises with increased irradiance. Seagrass thylakoid experiments were devised to examine the effect of petrochemicals in a way that is not influenced by the relatively slow flux of petrochemicals to the inner cells. One might see the thylakoid experiments as analogous to the microalga experiments in that there are no

delayed responses due to complex leaf structure, but the experiments are conducted with seagrass “cells” (i.e. thylakoids) instead of microalgae cells.

In this study we applied WSF at concentrations to 0.5% w/v (or 1.0% in the laboratory). This means that 5 grams (or approximately 6 ml) of oil is mixed in a litre of seawater, and our technique ensured that the oil was well mixed (stirring for 24 hours). Such a concentration is unlikely to represent a real life oil spill scenario unless the spill occurs under conditions where the oil rapidly mixes with the seawater and is relatively contained. These requirements are somewhat mutually exclusive, as open waters can experience considerable turbulence but the oil will disperse rapidly, while contained areas are generally sheltered and will not experience severe turbulence due to wind and wave action. Dispersant applications were used in accordance with recommended dose levels. Therefore, the most concentrated treatments used in the field experiments in this study represent the worst case scenario and would be unlikely to ever be encountered.

This study aimed to determine the effects of the water soluble fraction (WSF) of oil in seawater (and WSF + dispersant) on the photosynthesis of seagrasses at a range of biological scales, from whole plants in the field, to segments of blades, to a microalgae analogue, to the basic components of the photosynthetic apparatus, the thylakoids themselves. By conducting these experiments we wanted to determine whether the logistically simpler experiments on seagrass segments, microalgae or thylakoids could be used to derive similar values as derived from the field experiments. If these experiments provided comparable values, then seawater samples collected from field oil spills could potentially be used in laboratory experiments to rapidly determine the potential toxicity of an oil spill. This work formed the pilot project of a larger project for which an application was submitted to the ARC Linkage Program and was subsequently funded (2005-2007).

Methods

Seagrass species

Both *Zostera capricorni* and *Halophila ovalis* samples were collected from Paradise Beach (Pittwater, NSW) and maintained in recirculating aquaria at the Gore Hill Research Laboratories (University of Technology, Sydney). Field experiments were conducted *in situ* at Paradise Beach at a site separate to the collection site. Growth trials were performed on samples of *Heterozostera tasmanica* shipped from Port Phillip Bay, Victoria; these were successful (however no petrochemical trials were performed on those samples).

Preparation of oil and dispersant water soluble fraction

The protocol for petrochemical preparation followed a modified version used in Ralph & Burchett (1998) and Macinnis-Ng and Ralph (2003a). Briefly, the oil (Gippsland Crude Oil or MFO 380 Bunker oil) was aged by mixing 50 g fresh oil in 5 L filtered seawater (0.45 µm) in a conical flask to give a 1% (w/v) oil mixture. The mixture was rapidly agitated for 24 h using a vortex magnetic mixer and sealed with a plastic (or rubber) stopper during this period to prevent the loss of the volatile fraction of petrochemicals.

The mixture was allowed to settle for one hour, and the oil then removed from the surface with a pipette (light crude oil) or paper towel strips (bunker oil). The remaining solution is referred to here as the water soluble fraction (WSF). When oils and dispersant (Slickgone NS) were used together, the same procedure was adopted except that the dispersant was added to the oil-seawater mixture 10 minutes before the end of stirring, and then the solution allowed to settle for one hour as for the oil-only mixtures.

The Mackay test apparatus is designed to study the effectiveness of dispersants on oil introduced into a seawater medium. The device enables close simulation of environmental conditions such as sea state temperature etc. Simple stirring vessels were budgeted at about \$100 each and we were prepared to purchase these and evaluate their effectiveness; however delivery delays would have severely compromised the timely completion of this project. So, we would have liked to have compared these two devices in this project; however financial and time restraints meant we were unable to evaluate these alternate WSF devices. Ultimately, the protocols listed in Ralph & Macinnis-Ng were adopted as we believe the mixing ratio used (1 part oil to 100 parts seawater) to be equivalent to a worst case scenario. Given the limited impact of 1:100 exposure, future experiments should test the 1:20 ratio, as well as a shorter mixing period (less than 24 h). Both these alternate WSF preparations are expected to yield stronger responses. In this series of experiments we didn't have sufficient time to trial alternative WSF preparations; however this is within the scope of the ARC Linkage project outlined below.

Photosynthetic health assessment

We measured the photosynthetic response of the seagrass to oil and dispersants under light, and used this response as an assessment of its photosynthetic health. Photosynthetic attributes were measured using pulse amplitude modulated (PAM) chlorophyll fluorescence techniques (Schreiber, 2004). Fluorescence measurements were performed using two instruments; a Diving-PAM for seagrasses and a Phyto-PAM for microalgal solutions (Walz GmbH, Effeltrich, Germany). The Diving-PAM employs a 3 μ s pulse of red light from a light emitting diode (LED) with a peak emission at 650 nm as the measuring light. Chlorophyll fluorescence is detected at wavelengths above 710 nm. The Phyto-PAM uses LED's of four different wavelengths (470, 520, 645 and 665 nm). Both fluorometers measured F (light-adapted transient fluorescence), Fm' (light-adapted maximum fluorescence) and derived effective quantum yield ($\Delta F/Fm' = \Phi_{PSII} = [Fm' - F]/Fm'$). Effective quantum yield is a commonly used estimate of photosynthetic health.

Seagrass field experiments

Clear acrylic open-bottomed cylinders were placed in a *Zostera capricorni* meadow at Paradise Beach, Pittwater, and leaf clips and 2 mm acrylic fibre-optics installed so that fluorescence measurements could be made from outside without disturbing the seagrass within the cylinders (see Macinnis-Ng and Ralph (2002) for details). The chambers were pushed 50 mm into the sediment and pegged with stays. Bilge pumps (12 V "Rule" pumps) were attached to custom-built clear plastic lids and powered by a sealed power supply, the pumps ran continuously at one third maximum speed while the lids were in place to ensure water movement. Clear lids allowed light penetration.

The chambers were installed in the field during the afternoon of the day before the experiment. The following morning pre-exposure $\Delta F/F_m'$ measurements were taken of each leaf, and then WSF added. WSF was contained within black polythene bags; bags were placed in the chamber and gently pushed down into the chamber. The largest volume was half the chamber volume (0.5% w/v treatment); lower concentrations required smaller volumes of added WSF. After untying the loosely knotted bag and securing the lid, the bag was removed through a hole in the side of the chamber and the blanking plug reinstalled. This procedure was repeated for all treatments, and the time noted when the bag was removed. One hour after removal of the bag, fluorescence measurements ($\Delta F/F_m'$) were again taken on each leaf, and then subsequent measurements taken at hourly intervals for up to 6 hours. At the end of the day the chambers were removed, the clips were left attached to the samples and leaves were monitored daily for up to five days. Fluorescence readings were corrected for background fluorescence attributable to each plastic fibre.

Each treatment comprised three replicate chambers where a single leaf was measured within each chamber. In addition to chambers with various concentrations of WSF or WSF+dispersant, there were three control chambers (no WSF addition) and three procedural control chambers (no blanking plugs, lids nor WSF addition), and three external controls (no chambers and no additions).

Seagrass leaf segment experiments

Zostera capricorni was collected from Paradise Beach, Pittwater, NSW and maintained in an indoor aquarium at University of Technology, Sydney, Gore Hill Laboratories prior to use (12:12 L:D tungsten floodlight $\sim 100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$, 22 °C). Green blades free of epiphytes were selected, cut to 10 cm lengths and bathed in seawater for at least one hour at $\sim 30 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ before experimentation. This period was incorporated into the protocol to allow wound recovery under non-stressful conditions. Experiments were conducted in 100 ml clear plastic sample vials; 100 ml WSF solution was added (at a range of concentrations) and the vial inverted so that irradiance penetrated through the clear base to the sample within. Samples were fixed in position against the wall of the vial using a plastic clip to facilitate fluorescence measurements ($\Delta F/F_m'$). All treatments were performed in triplicate. Measurements were taken using a Diving-PAM (Heinz Walz GmbH, Germany) set up with the fibre optic bundle positioned with a retort stand.

Microalgae experiments

Dunaliella tertiolecta (CSIRO Marine Laboratories collection) was cultured in $\sim 100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ and used in experiments when growing in logarithmic phase. 250 μl of culture was added to 10 ml seawater/WSF, and added to 25 ml clear plastic vials. The final chlorophyll *a* concentration was approximately $75 \mu\text{g l}^{-1}$. At this concentration a faint green tinge was just discernable; this low concentration was used to ensure there was an excess of WSF relative to algal cells. The transparent vials were placed on an orbital shaker upside down so that the culture received irradiance from above (fluorescent light bank, $\sim 60 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) and incubated for fixed intervals up to 3 hours.

Experiments performed at irradiance below growth irradiance to limit light stress from masking contaminant (WSF) photosynthetic stress. All treatments were performed in triplicate. When conducting each $\Delta F/F_m'$ measurement, a 1 ml aliquot of culture was added to the cuvette supplied with the Phyto-PAM (Heinz Walz GmbH, Germany) and installed in the Phyto-ED (emitter-detector head). The minimal fluorescence was then allowed to stabilise for 30 s before applying the saturating pulse to obtain $\Delta F/F_m'$ estimate.

Both time-course and concentration response experiments were conducted. In the former, samples were introduced into the cuvette and a stirrer used to exclude ambient irradiance and maintain stirring. $\Delta F/F_m'$ measurements were made every 2 min for at least 60 min. Concentration-response experiments included concentrations up to 1% WSF. Exposure intervals varied from three hours to 90 s.

Thylakoid experiments

Seagrass (*Zostera capricorni*) collected from Narrabeen lagoon was cleaned of epiphytes and necrotic material removed, then the remainder frozen at -20 °C. Samples were collected from Narrabeen (equally pristine to Paradise Beach), as this was closer to UTS and we didn't require deployment of chambers. Experiments performed by members of the Aquatic Photosynthesis Group have demonstrated that seagrasses from this region are not exposed to substantial anthropogenic contamination. Buffers for thylakoid preparation were made following the procedures of Patsikka et al. (1998) (see below); experiments used 100 μM ferricyanide as an electron acceptor and water as an electron donor. Initial experiments were conducted with English spinach to test the technique (spinach is widely used as a source of thylakoids), and then preparations made with seagrass (*Z. capricorni*) were then used.

Leaves were homogenised in buffer 1 with acid-washed sand (Buffer 1: 50 mM Hepes-KOH at pH 7.6, 300 mM sorbitol, 5 mM MgCl_2 , 1 mM EDTA, 0.5 Betaine) with the addition of 1% (w/v) BSA just before isolation. The homogenate was then filtered through miracloth, centrifuged at 1000g for 5 min, and the chloroplasts (pellet) resuspended in buffer 2 on ice for 5 minutes to cause osmotic shock (Buffer 2: 20 mM Hepes-KOH (pH 7.4), 5 mM MgCl_2). This was then centrifuged for 5 min at 1000g and resuspended in storage solution (Buffer 3: 100 mM sucrose, 25 mM Tris-HCl pH 8.5, 5 mM NaCl, 10 mM MgCl_2). PSII activity was measured in buffer 4 at a chlorophyll concentration of $\sim 10 \mu\text{g ml}^{-1}$ (Buffer 4: 40 mM Hepes-KOH at pH 7.6, 330 mM sorbitol, 5 mM NaCl, 5 mM MgCl_2 , 0.5 M Betaine, 1 mM KH_2PO_4 , 5 mM NH_4Cl). Ferricyanide (250 μM) was used as the electron acceptor.

Toxicity assessment

Concentration-response experiments provide an estimate of the toxicologically relevant parameter EC_{50} , which represents the Effective Concentration of toxicant that causes a 50% decline in the test parameter value (in this case we used $\Delta F/F_m'$ as the test parameter). In order to derive an EC_{50} value with confidence, one should conduct an experiment with at least five different concentrations, and examine three replicate

treatments at each concentration. Here we derived estimates of EC₅₀ using a variable slope sigmoid equation (defined in Sigmaplot statistical software as the three parameter logistic equation) with appropriate replication and number of treatments (Motulsky and Christipoulos 2003, Runcie and Riddle *in press*).

Data analysis

As described above, concentration-response experiments were analysed using a variable slope sigmoid equation to determine EC₅₀, and the mean value of three estimates was calculated and presented with standard error. All treatments were replicated three times. Differences between values were assessed for significance using t-tests or one-way ANOVA; significance was assumed when the probability of the result being attributable to chance (and not the treatment) was calculated to be less than 5%. Data were tested for homogeneity of variance prior to tests using Minitab software, and transformed using the Boxcox transformation where required.

Results

Seagrass field experiments

Range-finder experiments were not performed in this situation as preliminary experiments showed that a petrochemical impact on *Zostera* would require a large dose (> 1% w/v) to achieve a fluorescence response and that lower concentrations provided no significant effect (0% response). *Zostera capricorni* was exposed to WSF *in situ* at a range of concentrations with a maximum of 0.5%. Two concentration-response experiments were conducted; one at approximately 2 m depth (Figure 1) and another just below the low tide mark (Figure 2). The first experiment ran for 3 days and showed no significant treatment effect during the first 24 hours, although both the procedural and natural control had significantly lower values of $\Delta F/F_m'$ relative to all other treatment values after two days exposure (F=9.39, p=0.000). There were no significant differences between any treatments after three days exposure (F=0.80, p=0.598). The second field experiment ran only for one hour due to equipment failure, and also demonstrated no significant treatment effect (F=1.48, p=0.272). A third field experiment was conducted to determine the effect of WSF+dispersant relative to control and WSF only treated material. This experiment was conducted in shallow water (~0.3m at low tide), the same depth as the second field experiment. This experiment focussed on the highest concentrations of WSF versus controls and ran for 80 minutes; again there were no significant differences between treatments and controls (Figure 3; F=0.63, p=0.562). These results indicate Gippsland crude oil, and Gippsland crude oil + dispersant (Slickgone NS) have little if any effect on the photosynthetic efficiency of *Zostera capricornii* when exposed *in situ* at concentrations up to 0.5% w/v WSF.

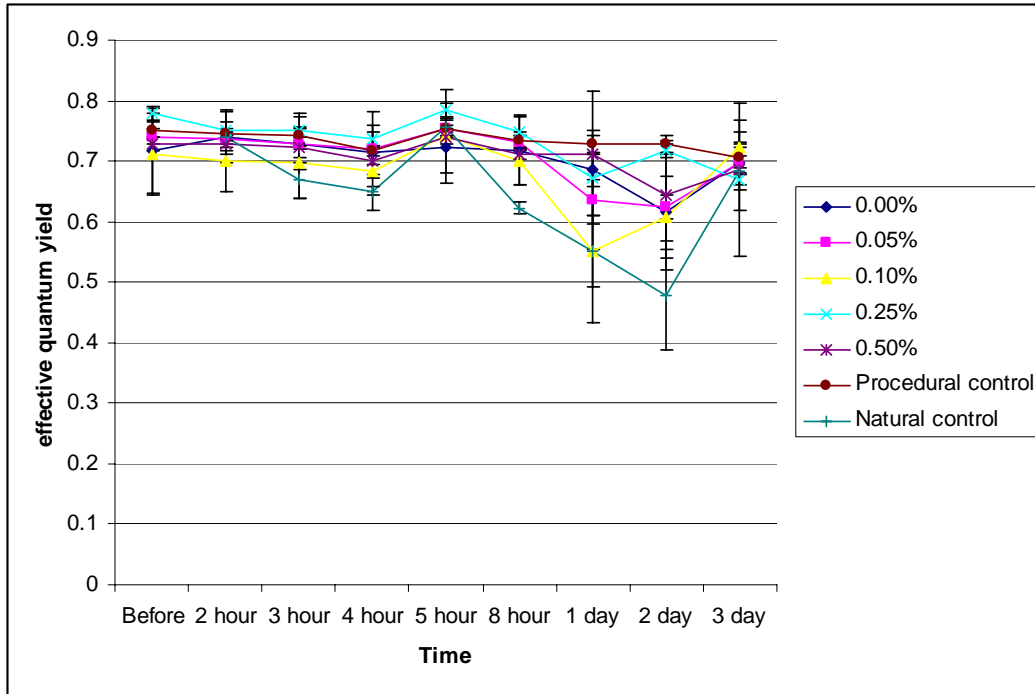


Figure 1. *Zostera capricorni*: changes in effective quantum yield after exposure to a range of WSF concentrations at 2 m depth. Measurements were made over 3 days; note x axis is not to scale; values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

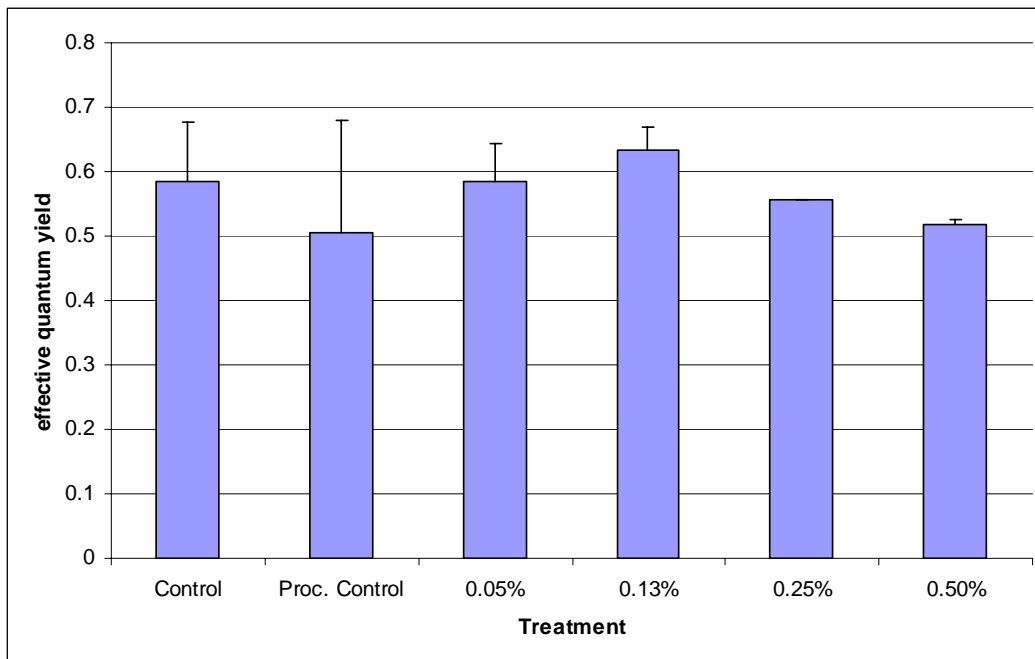


Figure 2. *Zostera capricorni*: changes in effective quantum yield after exposure to a range of WSF concentrations at 0.5 m depth. Measurements were made after one hour exposure. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

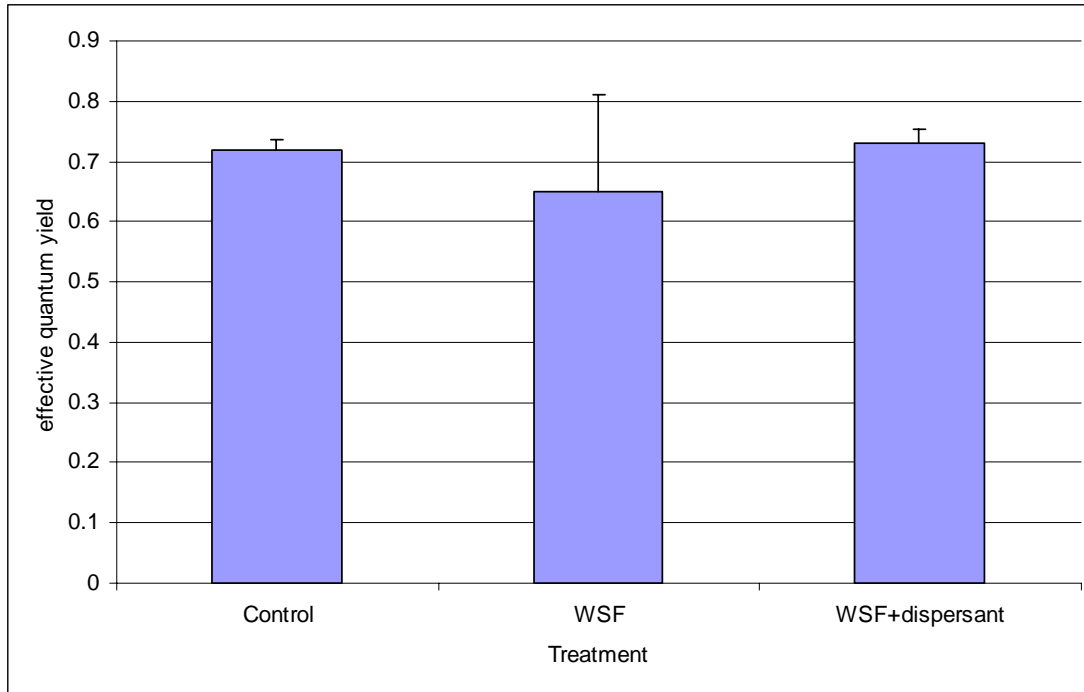
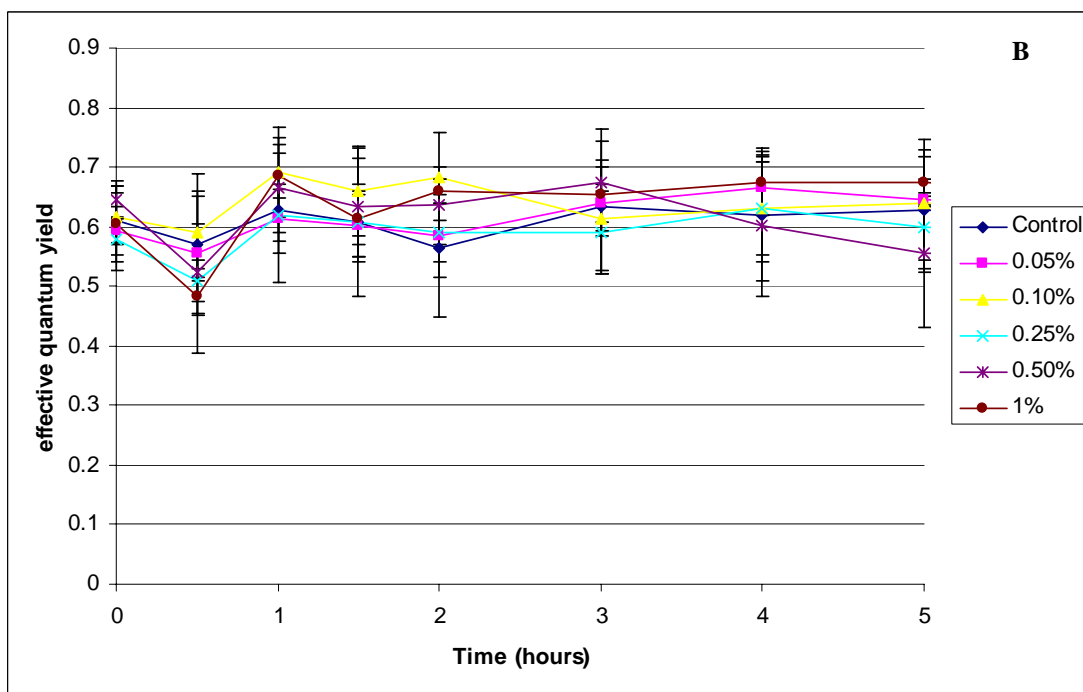
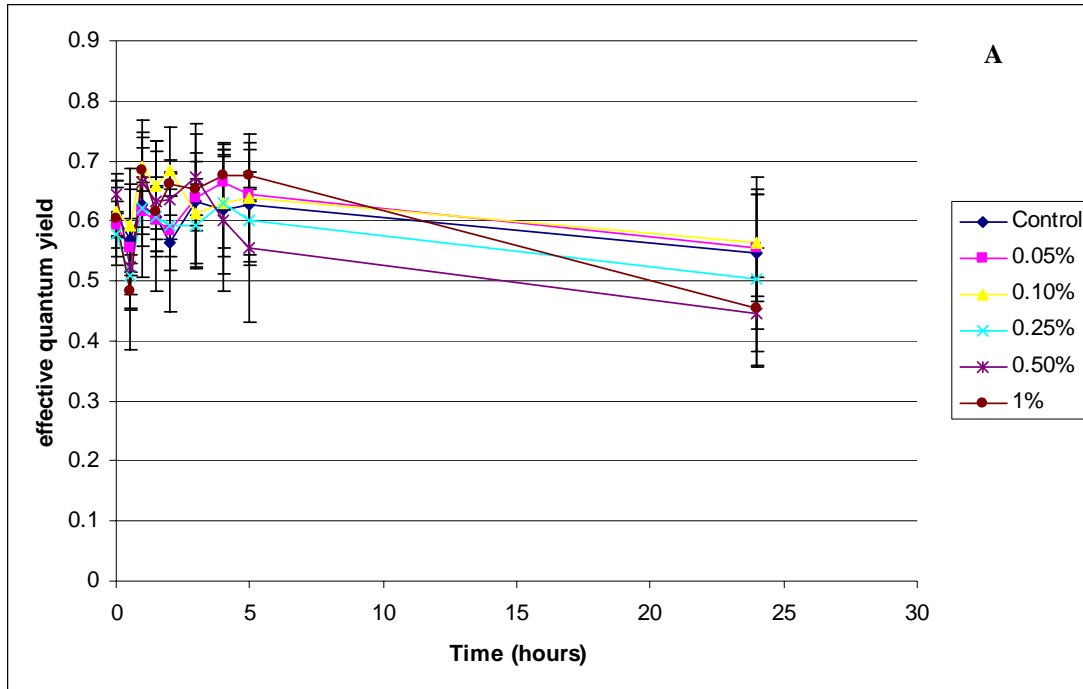


Figure 3. *Zostera capricorni*: changes in effective quantum yield after exposure to WSF (0.5%) and WSF+dispersant at 0.5 m depth. Measurements were made after 80 minutes exposure. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil. Note, no procedural control used in this experiment.

Seagrass leaf segment experiments

A concentration response experiment was conducted on seagrass leaf segments with 30 minute measurements for the first 2 hours, then measurements at hourly intervals for the next 3 hours (Figure 4). While a decline in yield was observed at the greatest concentrations after 30 minutes exposure, differences between controls and treatments were not significant ($F=1.39$, $p=0.276$) then or later in the experiment. A further experiment was conducted with a five minute exposure interval (Figure 5). In an attempt to define the upper toxicity threshold, in the leaf segment experiment we were able to employ a higher concentration of WSF. In this experiment, the 1% WSF treatment demonstrated a significantly lower effective quantum yield relative to the control leaf segments ($F=4.71$, $p=0.02$). However, the 0.5% treatment showed an even stronger response to the WSF, so it is not clear whether a five minute exposure is optimal for leaf segments.



Figures 4A and B. *Zostera capricorni* leaf segments: changes in effective quantum yield after exposure to a range of WSF concentrations in the laboratory; A) all measurements, B) measurements within the first five hours. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

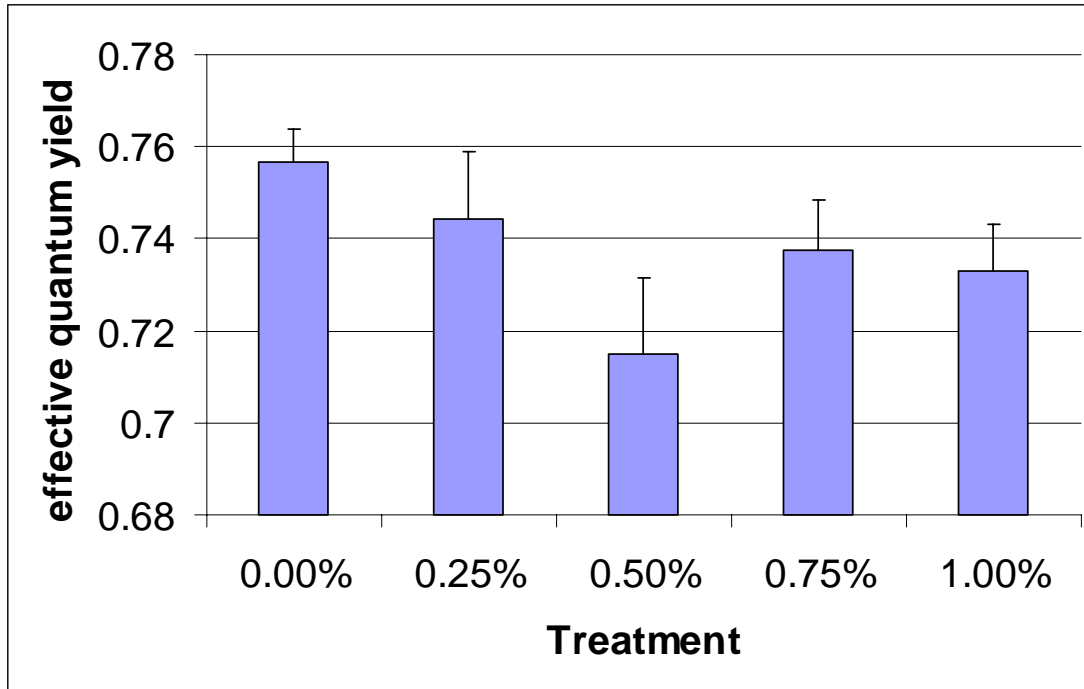


Figure 5. *Zostera capricorni* leaf segment concentration-response experiment: changes in effective quantum yield after exposure to a range of WSF concentrations after five minutes exposure. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

Microalgae experiments

Similarly to the leaf segment experiments, exposures conducted over a three hour interval showed the greatest response after one hour (Figure 6), where $\Delta F/F_m'$ values of algae exposed to 1% WSF after one hour exposure were significantly lower than values of the same algae after two and three hours exposure ($F=148.36$, $p=0.000$). A concentration-response experiment (Figure 7) examining the response of the alga to a range of concentrations after a thirty minute exposure yielded an EC_{50} of 1.66 ± 0.19 % w/v WSF.

Subsequent experiments used shorter exposure intervals in an attempt to capture the greatest effect (Figure 8). Time course experiments were conducted at the highest concentration (1% WSF) to determine a suitable exposure interval. The experiments showed a rapid (within minutes) and significant decline in $\Delta F/F_m'$ when exposed to the WSF. This decline was followed by a gradual recovery of $\Delta F/F_m'$ until pre-exposure values were reached after approximately 60 minutes (Figure 8b). It is unclear whether this is due to a recovery of the algae from petrochemical insult, or whether there is a decline in toxicity of the petrochemicals due to volatilisation.

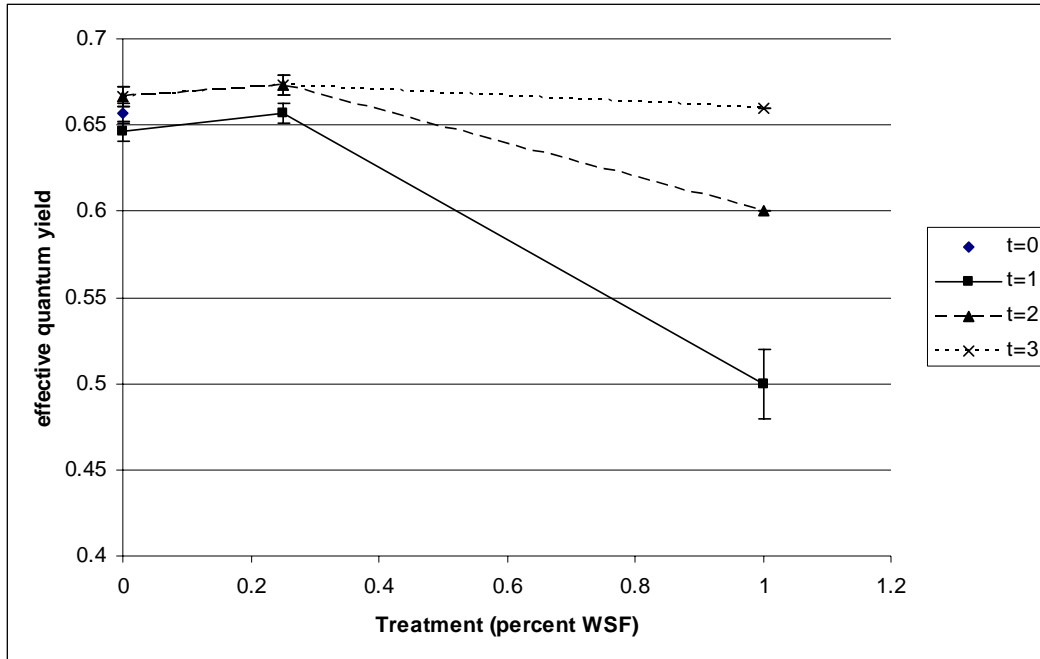


Figure 6. *Dunaliella tertiolecta* time course experiment: changes in effective quantum yield during exposure to 0, 0.25% and 1% WSF over 1, 2 and 3 hours. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

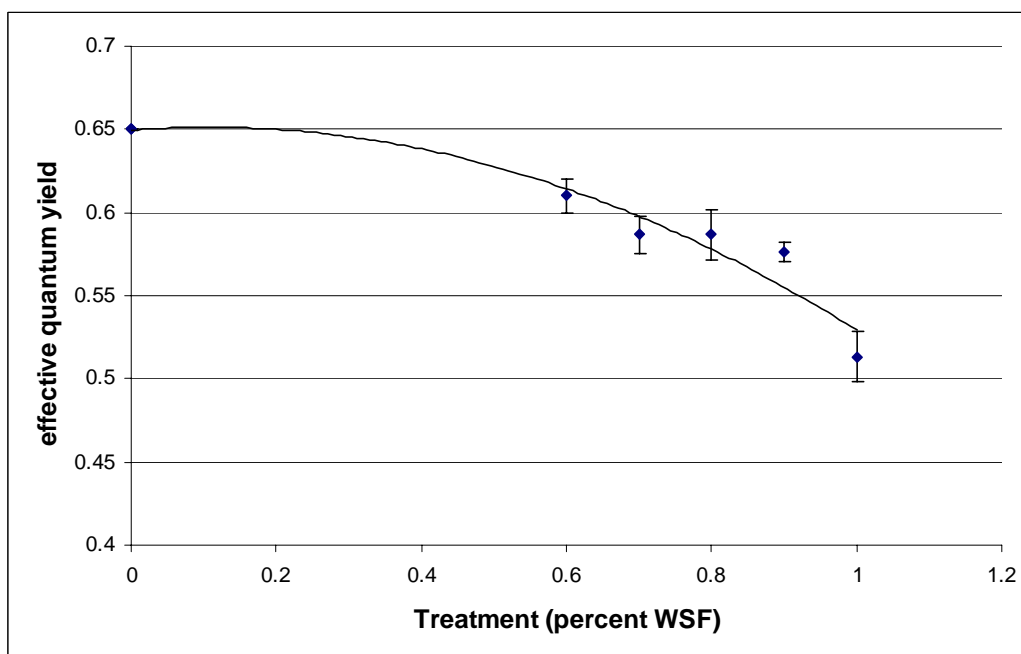


Figure 7. *Dunaliella tertiolecta* concentration-response experiment: changes in effective quantum yield after exposure to a range of WSF concentrations after 30 minutes exposure. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

When algae exposed to 1% WSF were illuminated (actinic irradiance was $164 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) after 5 minutes, $\Delta F/F_m'$ declined rapidly and then recovered to control values, although the rate of recovery was slower than the control and similar to that of darkened WSF-exposed material (Figure 8c). This suggests that the rate of recovery from the WSF insult is slower and masks the potential rate of recovery from illumination. Thus, the effect of WSF on $\Delta F/F_m'$ of *D. tertiolecta* is both short-lived and apparently independent of a decline in $\Delta F/F_m'$ due to irradiance. As *Dunaliella tertiolecta* responded rapidly to the WSF, 90 s exposures were also conducted at concentrations to 1% WSF (Figure 9). A mean EC_{50} value of $1.18 \pm 0.08 \%$ w/v for the water soluble fraction of Gippsland crude oil was derived from three identical experiments. This value is similar to that derived from 30 minutes exposures.

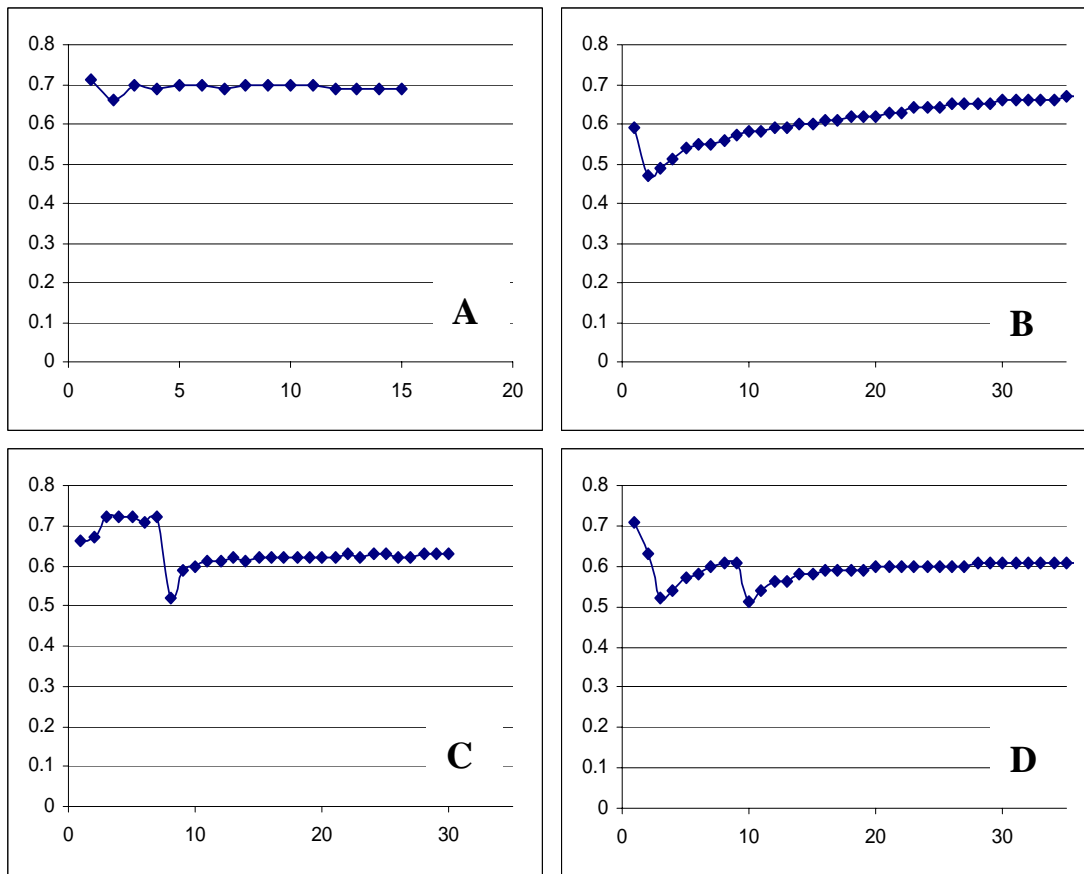


Figure 8. *Dunaliella tertiolecta* time course experiments examining response of effective quantum yield to irradiance and WSF. A) no light and no WSF (control), B) no light and 1% WSF addition, C) light on at 10 minutes, no WSF, D) light on at 10 minutes, 1% WSF added at start. Values are means \pm SD. WSF prepared from Gippsland crude oil.

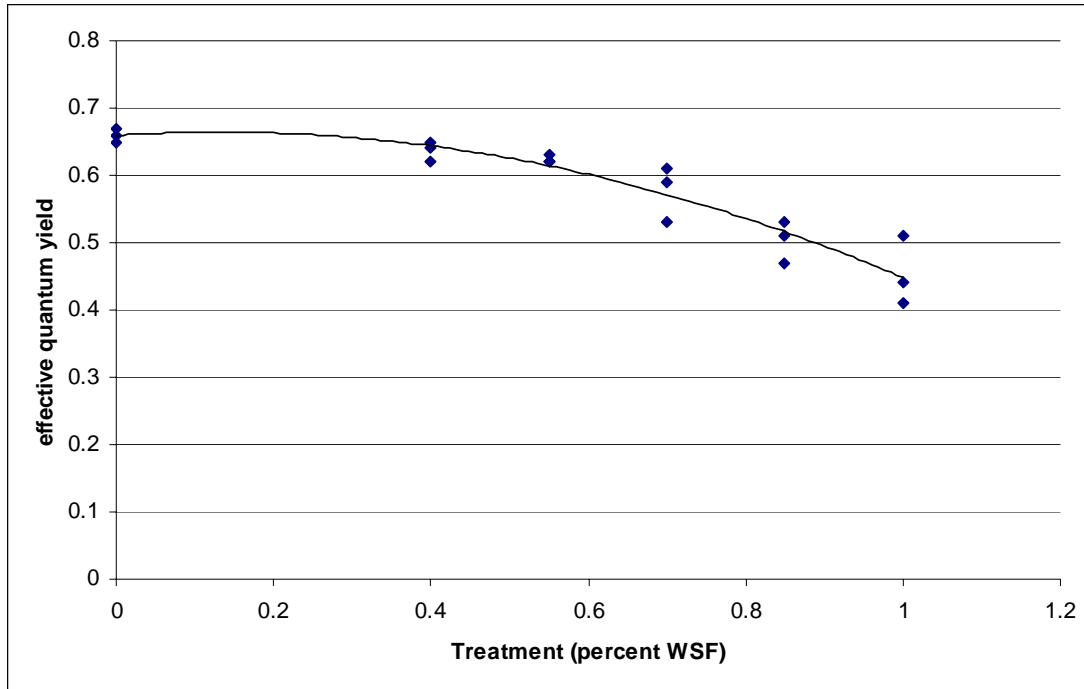


Figure 9. *Dunaliella tertiolecta* concentration-response experiment: changes in effective quantum yield after exposure to a range of WSF concentrations after 90 seconds exposure. Values are means \pm SD, n=3. WSF prepared from Gippsland crude oil.

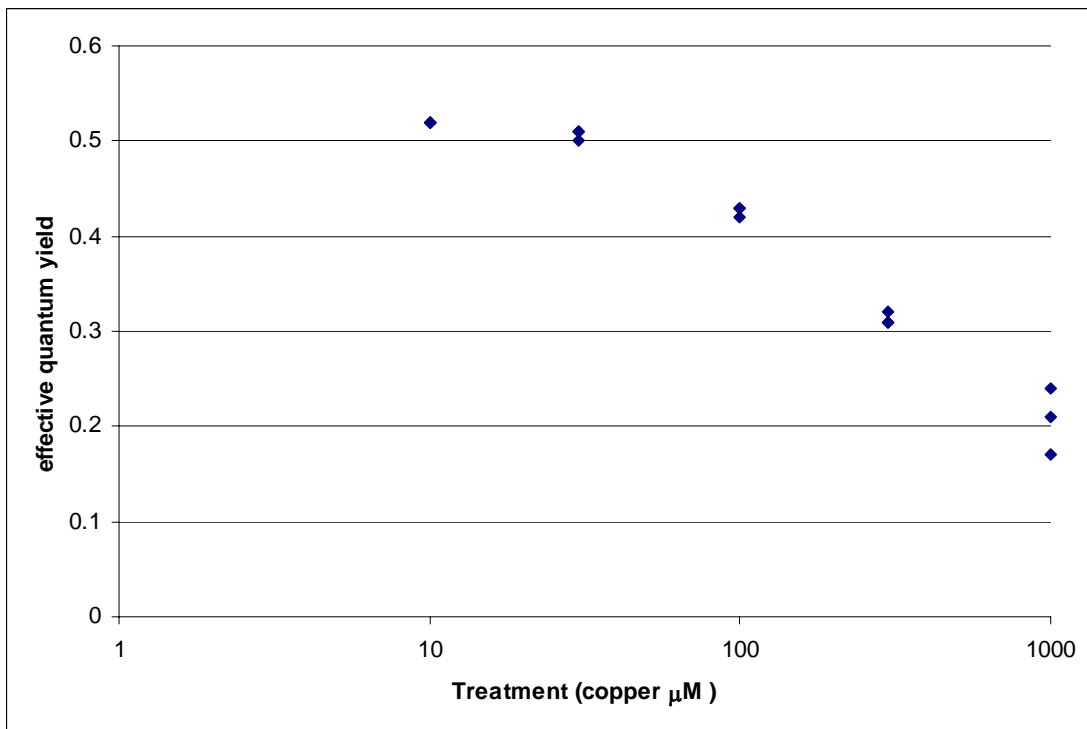


Figure 10. Spinach thylakoid suspension: changes in effective quantum yield after exposure to a range of copper concentrations after 10 minutes exposure. Values are means \pm SD, n=3.

Thylakoid experiments

Initial experiments were conducted with spinach to refine the thylakoid preparation technique. A predictable response of spinach thylakoids to copper sulphate confirmed the technique was optimised (Figure 10; EC_{50} of three experiments was $1655 \pm 1567 \mu\text{M}$). Subsequently, a thylakoid preparation of *Zostera capricorni* was prepared, but there was no detectable effect of WSF. Further tests of the thylakoid preparation suggested the presence of tannins in high concentrations (Figure 11). Tannins are a natural product of this seagrass and interfere strongly with the ferricyanide technique (e.g. Lovley and Coates 1996). Further attempts to use *Zostera* thylakoids for toxicity assessment were abandoned.

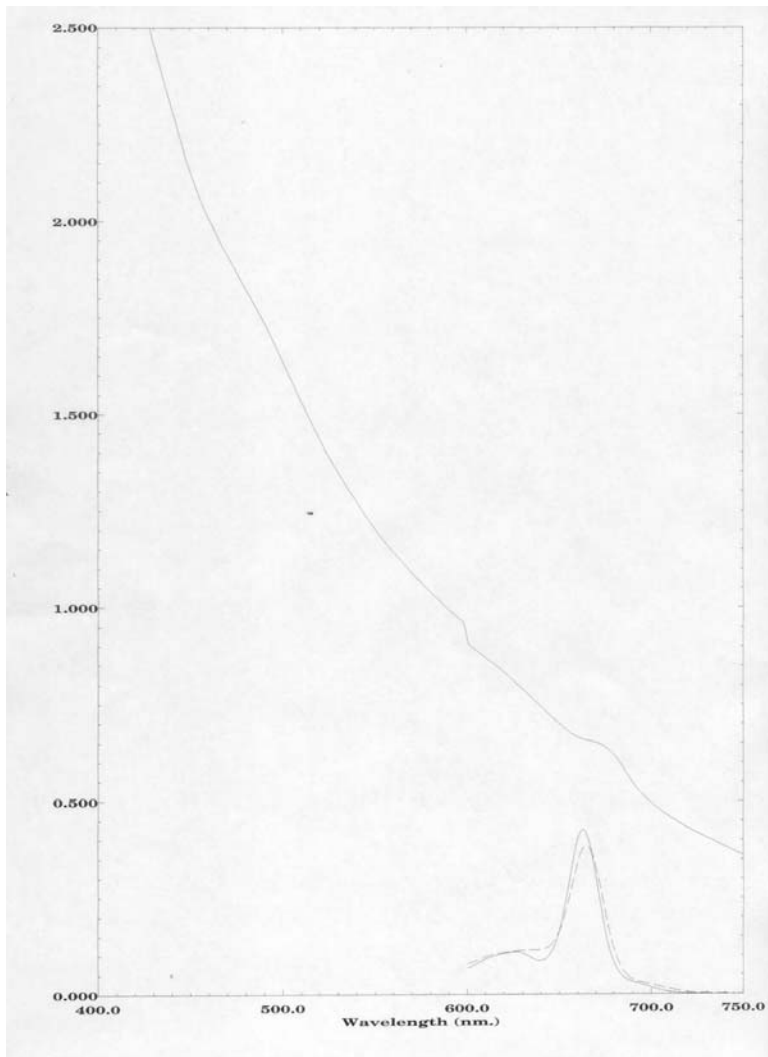


Figure 11. Absorbance spectrum of *Zostera capricorni* thylakoid suspension showing high tannin concentration (upper line) and chlorophyll a absorption maximum (lower line). Y-axis is absorbance.

Discussion

Seagrass field experiments

Experiments conducted in very shallow (<0.5 m, 80 min exposure) and slightly deeper water (~2 m, 5 hr exposure) yielded similar results, where the WSF of Gippsland crude oil had no detectable effect on effective quantum yield of *Z. capricorni*. There is the possibility that experiments conducted in shallower water may demonstrate significant differences over longer time periods than were used in this study (Macinnis-Ng and Ralph 2003). A reduction in irradiance (and hence UV radiance) due to the thicker overlying water column at the deeper site may have caused a reduction in negative effects of the petrochemical treatment in deeper waters. However, the natural daily variations in irradiance during the course of these field experiments can be greater than any depth-related reduction in irradiance; therefore we cannot distinguish depth related effects from variation between experiments due to differences in daily irradiance. Further experiments under controlled conditions using UV filters would enable the significance of depth on the response to petrochemical insult to be determined. It could be speculated that the relatively low values of $\Delta F/F_m'$ on the second day of the first field experiment of treatments with no lid suggest that UV may protect seagrasses from declining $\Delta F/F_m'$ due to sun damage; however this requires further validation.

Laboratory and field experiments by Macinnis-Ng and Ralph (2003) show that petrochemicals and dispersants influence the seagrasses within the first few hours of exposure. The results from this study are in contrast with those of the comparable field study by Macinnis-Ng and Ralph (2003), who demonstrated a significant decline in the effective quantum yield of *Z. capricorni* in response to *in situ* exposure to the WSF of Champion Crude oil. Gippsland Crude oil was used in the present study: both this oil and Champion Crude are light oils with a (presumed) considerable percentage of volatile hydrocarbons. Gippsland crude oil is subject to rapid evaporation. Modelling by ADIOS showed that 12 hours after spillage of 1000 tonnes of Gippsland crude oil into a 2 m sea and 10 knot wind at 20 degrees C, 38% of the oil evaporated (Weathering of Oil at Sea – www.amsa.gov.au). In this study we examined only the water-soluble fraction of oil, so one would expect a far higher proportion of petrochemical to be volatile and therefore evaporate quickly immediately after commencing the exposures. The highly volatile nature of Gippsland Crude oil used in this study, and the lower concentrations used relative to the laboratory experiments are the likely reasons why the field exposures demonstrated no significant effect of WSF on the seagrass.

Although a similar study for Champion Crude oil is not readily accessible at the time of writing, its similar viscosity suggests that it may also be subject to rapid evaporation. However, results from Macinnis-Ng and Ralph (2003) demonstrate significant effects of Champion crude on seagrass photosynthesis, so there may be constitutional differences between these oils that are responsible for the observed differences in seagrass response.

There are also several methodological issues that may help to explain differences in results between the two studies. Firstly, preparation of the water soluble fraction of oil as described by Macinnis-Ng and Ralph (2003) was adopted in this study, as it has clearly demonstrated its success and provides effective mixing of oil and seawater. Discussion with the National Plan Environment Working Group led the authors to investigate alternate means of preparing WSF, however the vortex technique used by Macinnis-Ng and Ralph (2003) was finally adopted as it provided the best mixing environment. The use of stoppers and controlled temperature during vortex mixing meant that the mixing process was unlikely to have resulted in a large loss of volatiles. The process might be improved by the application of more vigorous stirrers that ensure the oil surface layer is brought down to the stir bar on the base of the flask, however the change in colour of water samples suggested effective mixing of oil and seawater using this technique. While storage of treated water for intervals in excess of a few days may have led to the loss of significant portion of toxic volatile compounds, this is unlikely to be the prime reason for no detectable effects, as the second and third field experiments used treated water prepared only a day or two before deployment.

Secondly, the use of chambers in the field in this study required the application of large volumes of WSF and dispersant (up to half the volume of the chamber). The addition of this fluid via a thin polythene bag proved to be effective, and dye tests showed minimal loss of treated water from the chamber during the transfer procedure. Field experiments could only use a maximum WSF mix of 0.5%. This is because the addition of WSF to the field chambers is carried out by the insertion into each field chamber of a bag with half the volume of the chamber. The bag could not be larger or it would disturb the sample holder. As we were reluctant to assume that a 1:50 oil:seawater mixture prepared in the laboratory would contain exactly twice the WSF of oil as a 1:100 solution, we were constrained to the maximum WSF concentration of 1%. When inserted in the field chambers this solution was diluted to 0.5%. The third field experiment using dispersant, employed the chambers used by Macinnis-Ng and Ralph (2003) – these chambers have a superior seal between the top of the chamber and the lid. As there was no significant response in this experiment it is unlikely the modified design of additional chambers constructed for this study led to losses of petrochemicals such that no significant effect was detected.

In short, the same technique as described in Macinnis-Ng and Ralph (2003) was used in this study, yet no significant effects were detected. The only difference was in the oil and dispersant used in these two studies. Therefore, further studies using different oils (and potentially Champion crude to confirm the success of the lab and field techniques) are recommended for the next stage of this study. We would suggest further laboratory studies be conducted to determine the rates of evaporation of Gippsland crude oil and other oils potentially used in this study under conditions of the field experiments conducted in this study. We suggest Gippsland crude oil may be sufficiently volatile that the WSF of this oil presents no serious threat to the seagrass *Z. capricorni*.

Seagrass leaf segments

Exposure of leaf segments to the WSF showed a response in terms of effective quantum yield within 30 minutes. Longer exposures showed no significant effects. Therefore, the WSF of Gippsland crude oil appears to have a rapid but short-lived effect on the photosynthetic apparatus of the seagrass segment (Figure 5). Experiments with bunker oil suggest an initial depression of effective quantum yield greater than that of the controls, followed by a rapid recovery similar to the controls. Interestingly, the decline in effective quantum yield of the bunker oil affected segments is slower than that of controls during the remainder of the time course experiment. This may be due to the properties of the bunker oil WSF inhibiting transport of carbon dioxide into the plants and causing a downturn in photosynthetic rates (the leaf segment experiments were performed in closed chambers). The petrochemicals may also increase the diffusion boundary layer by placing a film of oil over the leaf surfaces thereby slowing down the rate of flux of essential solutes into and out of the leaves.

The rapid recovery after petrochemical insult may be due to a decline in petrochemical concentration due to volatilisation and adsorbance of oil to experimental surfaces, or it may be the result of a biological process within the seagrass leading to a recovery in $\Delta F/F_m'$. An increase in $\Delta F/F_m'$ suggest that non-photochemical quenching processes activated by the insult are no longer activated. This hypothesis could be examined further in the laboratory.

The experiments conducted in this study are not readily comparable with laboratory experiments conducted by Macinnis-Ng and Ralph (2003) as those authors used whole *Z. capricorni* plants including rhizomes, while this study used leaves cut in 100 mm lengths. The difference between leaf segments and the whole seagrasses *in situ* are primarily that the segments are cut and cleaned of epiphytes, while the *in situ* material are accompanied by many alternate sinks for petrochemicals (sediment, epiphytes, bacteria chamber and pump; Macinnis-Ng and Ralph 2003). The leaf segments may be more vulnerable to petrochemicals via their cut surfaces, and this could be tested with scratching of leaves prior to exposure. Regardless of this difference in susceptibility (which could also be tested in a field experiment with cut leaves exposed to WSF relative to uncut control leaves), the rapid recovery of effective quantum yield suggests either a rapid recovery mechanism, or a rapid loss of volatiles and therefore a decline in petrochemical concentration. However, as a rapid recovery is also observed for *D. tertiolecta*, it would seem unlikely that cut surfaces are a significant factor in the rapid response of seagrass segments to petrochemical insult. The alternate explanation is discussed in more detail in the following two sections.

A possible means of obtaining a more significant effect of petrochemicals on the seagrass leaves in the laboratory may simply be exposure of the leaves to greater irradiances. In the field, the midday sun provides irradiances of up to $2000 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ at the sea surface, and as some seagrasses may be not be submerged during the low tide there is potential for severe synergistic effects of high irradiance and petrochemicals. Clearly the combined effects of light quality and intensity with petrochemical exposure require

further examination. The decline in yield for seagrass segments was derived from a decline in F_m' , while F (data not provided) remained relatively constant. This suggests a clear impact of the petrochemicals on PSII function and implies generation of some form of alternate quenching of incoming radiant energy (NPQ).

Microalgae

Experiments where the microalga *D. tertiolecta* was exposed to the WSF of Gippsland crude oil demonstrated a rapid and brief decline in effective quantum yield, followed by a recovery phase lasting approximately three hours. The response of the microalgae was more repeatable than experiments with seagrass segments or seagrass *in situ*, and EC_{50} values could be determined with reasonable confidence. The EC_{50} values varied from 1.2 % WSF after 90 seconds to 1.7% WSF after 30 minutes. These results clearly show that the toxicity of the WSF solution declines significantly over a 30 minute interval, as the concentration required to elicit a 50 % reduction in effective quantum yield increased over this time. Moreover, these values suggest that relatively high concentrations of WSF are required to have an effect on organisms that are intrinsically susceptible to influx of contaminants due to their high surface area to volume ratio (unicellular algae such as *D. tertiolecta*). Alternatively, the waxy cuticle of the seagrass may have prevented the rapid diffusion of petrochemicals into the seagrass tissues. Any effects on more complex organisms such as seagrasses would therefore require higher concentrations or sustained exposure periods at lower concentrations in order for the contaminants (petrochemicals) to reach thylakoid membranes and cause the decline in PSII activity that is evidenced by a decline in effective quantum yield.

The tests with *D. tertiolecta* demonstrate a clear response of the microalga to relatively high concentrations of WSF. While it is unlikely *D. tertiolecta* will experience such concentrations in the field, these high exposures allow us to derive a more accurate estimate of EC_{50} than if we only conducted experiments with low more ecologically relevant concentrations. The EC_{50} values are the final product of these tests and show that at the EC_{50} concentration there is a significant effect of the petrochemicals (i.e. a 50% decline in $\Delta F/F_m'$). These concentrations of WSF (1.2 to 1.7%) equate to a dilution of 12 to 17 grams per litre of seawater (or 15 to 21 ml per litre assuming a specific density of 0.8). This concentration is likely to be far in excess of what would typically be encountered in the field. Future studies with microalgae will examine the responses of *D. tertiolecta* to other oil types, dispersants, and oil-dispersant combinations in accordance with advice from the National Plan Environment Working Group.

The determination of specific uptake mechanisms of seagrasses and microalgae would require considerable resources, but an empirical approach may provide adequate data describing apparent rates of uptake of petrochemicals (both oils and dispersants and combination of the two). Labelled carbon might be a useful tracer in this regard, where the incorporation of ^{14}C -labelled hydrocarbons in the seagrass leaves could be assessed.

The microalgae seemed to suffer more light damage than the seagrasses (greater relative decline in $\Delta F/F_m'$), which could mean the higher irradiance treatments would be more suitable for experiments with seagrasses. When irradiance is very high, photosynthetic organisms generally cope with the excess light energy by diverting this energy to non-photochemical (NPQ) processes while they continue to allocate reductant to photosystem repair. By allocating energy to NPQ the organism restricts the production of damaging reactive oxygen species (see Dummermuth et al. 2003). However with increasing irradiance, repair rates become limited, and the gradually increasing levels of reactive oxygen species that are a consequence of too much irradiance ultimately lead to damage of the photosystem and a decline in $\Delta F/F_m'$. Regardless of these details, experiments with *D. tertiolecta* demonstrate that microalgae are more sensitive to petrochemicals than seagrasses, and we recommend further work be directed to determining a repeatable and comparative relationship between seagrasses and a microalgal species, where an EC_{50} obtained for the microalga is directly translated to an EC_{50} for the seagrass.

Thylakoid experiments

Experiments with thylakoid preparations were difficult to optimise. Initial experiments with spinach thylakoids were necessary in order to demonstrate successful production of viable thylakoids – the chemistry of thylakoid preparation and variable fluorescence measurements is not documented anywhere and this part of the study is a significant innovation. EC_{50} values derived from exposures of the spinach thylakoids to dissolved copper were conducted as a pilot study and yielded significant although high EC_{50} values (Figure 12; EC_{50} was $1655 \pm 1567 \mu\text{M}$). However, any attempt to isolate seagrass thylakoids and conduct similar experiments failed, likely due to high tannin levels (Figure 11). We would suggest that if thylakoid experiments are to be pursued, then experiments with spinach be used instead of attempted seagrass preparations, as these are well-studied preparations and can be compared within an extensive body of literature (Walker 1987).

Conclusion

Field experiments demonstrated no significant effect of Gippsland crude oil on *Z. capricorni*, and this may have been due to the highly volatile nature of this oil. Experiments with Gippsland crude oil and dispersant (Slickgone NS) also demonstrated no significant effect, further studies under a range of environmental conditions may help elucidate whether this combination may have an effect under other conditions likely to be experienced in the field. The use of seagrass segments provided a more dynamic response, but the use of *D. tertiolecta* yielded the most useful values of EC_{50} .

Future work

See attachment 1 for details of the ARC Linkage Project that will continue much of this work in 2005-2008. A major outcome of the current project was that research be directed to establishing clear links between the responses of whole seagrass plants *in situ* and *D. tertiolecta* in the laboratory to WSF of the petrochemicals and dispersants of interest.

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Research Proposal – PhD project by Kim Wilson

Seagrass Tolerance of Oil Spills

Introduction

Limited research exists on the toxic effects of different petroleum hydrocarbons on differing Australian seagrasses (Ralph and Burchett, 1998; Macinnis-Ng and Ralph, 2003). The Australian Maritime Safety Authority (AMSA) has provided funds to establish new procedures to understand the impact of oil spills on seagrass meadows.

Background

Seagrasses are flowering aquatic plants, living fully submerged in seawater and are found throughout the world in coastal and estuarine zones (den Hartog, 1970). Seagrasses are highly productive, remove sediment and nutrients from coastal waters, provide shelter and habitat for fish and invertebrates and act to stabilise the substrate through their anchoring root system (Kuo, 1982, Poiner *et al.* 1993).

Australia has the fifth largest shipping load in the world in terms of cargo shipped and distance travelled (www.amsa.gov.au). Due to the size of our shipping industry the risk of oil spills is high.

Oil affects seagrasses through the absorption of the water soluble fraction (WSF) which leads to a reduction in tolerance to other stress factors (Zieman *et al.*, 1984). Studies have also shown that dispersants can have deleterious impacts on marine vegetation (Thorhaug, 1988; Howard *et al.*, 1989). Previous research suggests that amongst seagrass species there is a wide diversity of responses to petrochemicals (Thorhaug, 1988). The need to define information on the toxicity of petroleum hydrocarbons to different seagrasses is essential if successful mitigation regarding an oil spill is to occur.

Aims

The aims of the project are to provide appropriate data so our industry partner can make informed management decisions when an oil spill occurs by:

- Quantify the relative sensitivities of different seagrass species to various petrochemicals (bunker and crude oil) and petrochemical/dispersant mixtures.
- Define a clear protocol for ecotoxicological toxicity testing of seagrasses to be used Australia-wide.
- Determine whether less time-consuming methodologies such as microalgae testing can be used as a surrogate for seagrass ecotoxicology testing.

Objectives

The objectives of the study are to determine:

- 1) the relative sensitivity of three seagrass species*,
- 2) the toxicity of a common bunker oil (380cSt) and crude oil (Arabian crude) to these seagrasses,
- 3) the toxicity of the following oil + dispersant mixtures to these seagrasses:
 - a. 380cSt + Slickgone NS,
 - b. 380cSt + Corexit 9500,
 - c. Arabian crude + Corexit 9527,
 - d. Arabian crude + Ardrex 6120,
- 4) a toxicity scaling system for the different organismal levels of seagrass.

*The seagrass species to be tested in this study are *Zostera capricorni* Aschers., *Herterozostera tasmanica* (Martens ex Aschers.) den Hartog and *Halophila ovalis* (R.Br.) Hook.

Methodology

Field experiments will be conducted in Port Phillip Bay (Victoria) and Pittwater (New South Wales). Field dosing chambers will be placed in seagrass meadows and oil stock solution will be added. The effective quantum yield of photosystem II will be measured as an indicator of stress. The resulting data will be analysed with ANOVA to detect differences between treatments and EC₅₀ values will be calculated for comparisons between species and oil/ dispersant types.

Chlorophyll *a* fluorescence will be used as an indicator of photosynthetic efficiency in the seagrasses. This technique has been demonstrated in ecotoxicological work on photosynthetic growth by Macinnis-Ng and Ralph (2003, 2004) and Marwood *et al.* (2001).

To establish a seagrass toxicity testing protocol, laboratory experiments will initially determine standard responses on a standard test organism *Phaeodactylum* sp.. *Phaeodactylum* will be exposed to the herbicide DCMU, to oils and to oils and dispersants. The standard responses from *Phaeodactylum* will be compared to the seagrass response for oil and dispersant exposure.

Communication of Results

The project progress will be communicated to the industry partners at regular intervals through progress reports and seminars. Results will also be published in marine science journals, conference presentations and public forums.

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