The Toxicity and sub-lethal Effects of Persistent Pesticides on Juvenile Prawns and a Common Inter-tidal Seagrass Species

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1.0 Non-technical Summary

2000/163	**The Toxicity and sub-lethal Effects of Persistent Pesticides on Juvenile Prawns and a Common Inter-tidal Seagrass Species**

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OBJECTIVES:

1. To determine the identity and concentration of insecticides in water samples taken seasonally from inshore nursery areas in Spencer Gulf
2. To determine the levels of adsorbed residual insecticides and herbicides adsorbed onto top-soil, sampled from selected farms adjacent to recognised marine nursery areas
3. To develop a GIS database identifying the land units adjoining Spencer Gulf, together with point sources such as creeks and other discharge points that have the potential to contribute pollutants into recognised nursery habitats
4. To detemine the toxic and sub-lethal effects on juvenile prawns of the common persistent insecticides used
5. To determine the toxic and sublethal effects of a persistent herbicide used in broad-scale agriculture on *Zostera muelleri*, a common inter-tidal seagrass
6. To prepare and implement an extension program, to communicate results and recommendations of the project to stakeholders.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE:

The entry of chlorpyrifos into inshore marine environments may pose a threat to both Western King Prawn and King George Whiting juvenile populations in South Australia. The prawns are more sensitive than the whiting juveniles to this pesticide. The whiting juveniles accumulated chlorpyrifos at 150 times the background rate, which may pose a threat to their predators. More research is needed to fully ascertain the effects of organophosphorus pesticides on marine organisms in southern Australia.

Juvenile prawns and whiting were shown to be effective indicators of the potential impact of organophosphorus insecticides, but their effectiveness is limited by their availability for testing.

There appear to be significant concentrations of 2,4D-like-herbicides in most of the soil samples surveyed in this study. Conclusions in relation to the impact of these herbicides on the seagrass *Zostera muelleri* are not conclusive. At the concentrations found, oxygen production is impaired over a short period and this is believed to affect the ability of the seagrass to resist the toxicity of the sediments it grows in.

Land-management practices need to take the possibility of herbicide residue build-up into account in weed control activities.

The proposed development of a database of sources of pollutants was not progressed, owing to the sensitivity of property owners over responsibility for persistent pesticides.

KEYWORDS: **Seagrass, juvenile prawns, persistent pesticides, toxicity, sub-lethal effects, inshore marine ecosystems**

Persistent herbicides used in terrestrial activities have been shown worldwide to be impacting on inshore seagrass beds. The impact of pollution from terrestrial pesticides on a seagrass species and juveniles of two commercially important marine species in the South Australian inshore marine environment was examined. The study undertook testing to determine whether the soil dust fraction contained significant amounts of residual pesticides. The toxicity of organo-phosphorus insecticide chlorpyrifos, used in broad-scale agriculture in the region, was tested on juvenile prawns and whiting and a seagrass species.

Juvenile Western King Prawns, *Penaeus latisulcatus*, between 20mm and 40mm long were obtained using a beam trawl at Port Pirie on Spencer Gulf. Juvenile post-larval King George Whiting, *Sillaginodes punctata*, were collected at various locations on the Port River Estuary. Acute toxicity testing was carried out by subjecting 10 prawns and three whiting to a range of chlorpyrifos concentrations with four replications and replicated controls. The 96-hour LC_{50} for chlorpyrifos toxicity to juvenile Western King Prawns was determined to be 0.44 µg/L. The acute toxicity (96-hour LC_{50}) of chlorpyrifos to juvenile King George Whiting was determined to be 9.05 µg/L, compared with values of 1.7 - 136 µg/L for northern hemisphere fish species. Preliminary findings from this study suggest that in the case of King George Whiting, chlorpyrifos can accumulate up to 150-fold from ambient levels.

Some 100 soil samples, together with herbicide histories for the previous 5 years, were obtained from some 60 properties across the Yorke Peninsula. Residual herbicide levels in the potential "dust fraction" were high for a few soils, with trifluralin and a sulfonylurea, metsulfuron-methyl, being the most significant herbicides found. A significant finding was the presence of 2,4-D congeners, particularly in alkaline and calcareous soils, reflecting the extensive use over many years of this cheap "out-of patent" herbicide. Wind-blown dust containing these herbicide residues could contaminate the inter-tidal zone.

The potential of impact of these residues on the inter-tidal seagrass *Zostera muelleri* was examined. *Zostera muelleri* is a significant component of the habitat of juvenile prawns and juvenile whiting which is reported to be in serious decline in many areas of South Australia. The outcome of these bioassays was that 2,4-D and 2,4-D like herbicides have the potential to act as auxins or growth promoters while interfering with oxygen production by the grass. Oxygen production was significantly reduced when seagrass, growing in 100 % sediment, was exposed to low levels of 2,4-D.

None of the seawater sampled contained a known pesticide at or above the limit of detection. The only persistent organic pollutants found were trace amounts of phthalates, which are used industrially as plasticisers, and are ubiquitous in the environment. While the levels of residual herbicides found in this study were not high enough to be immediately toxic, introduction of dust containing residual herbicides is

a factor which can contribute to environmental stress and decline of this important seagrass habitat and its associated species, including prawns and whiting.

2.0 Acknowledgments

The assistance of numerous members Agricultural Bureaus and independent farmers on the York Peninsula with the extensive soil collection is gratefully acknowledged.

The assistance of staff of the South Australian Research and Development Institute at West Beach, in particular Neil Carrick and Tony Olsen, with the collection of juvenile Western King Prawns and juvenile King George Whiting and also the provision of holding facilities is also gratefully acknowledged.

The findings reported here are largely the work of Sam Gaylard and Gareth Lewis, and their enthusiasm and patience is gratefully acknowledged.

3.0 Background

3.1 Seagrasses, their Habitat and the Impact of Urbanization

Seagrass (*Zostera muelleri*) meadows constitute a climax community that grows in the highly productive environment of the intertidal zone. Coastlines are stabilised by this flowering plant that makes an important contribution to productivity in coastal regions (Klumpp, Howard et al. 1989). Kirkman (1992) has estimated that the seagrasses of Western Australia cover the same area as rainforests in the whole of Australia.

The inter-tidal zone provides a nursery area for prawns, fish and crustacea (Bell and Pollard 1989). Fronds of seagrass serve as habitat for the many epiphyte species and algae (Ducker, Food et al. 1977). Prawns graze on epiphytes (Morgan 1980) while larger animals such as dugongs and green turtles also forage and use seagrass meadows as habitat.

The intertidal zone therefore forms part of a coastal ecosystem that is rich in biodiversity. Small fish and crustacea that forage and seek shelter in the seagrasses that pervade this zone in turn provide food for migratory birds that use these mudflats as feeding grounds. Seagrass meadows therefore provide a habitat, foraging ground and nursery area for many fauna of the intertidal zone and make a significant contribution to recycling of organic matter in this region. Any negative impact upon *Zostera*, as a representative seagrass species, is therefore likely to also negatively impact upon co-dependant species within this intertidal environment.

Significant losses of seagrass, amounting to 20% in many areas, have occurred world-wide. Indeed, world losses of seagrass have been likened to a 'wasting disease'. Such losses have been noticed since the late 1930's and perhaps more so since that time with increasing urbanization and industrial activity in coastal regions. It is likely that the loss of seagrass has had an economic impact as well as an ecological impact. Reduced fishing yields and attrition of this habitat have raised concern in the fishing industry and in the scientific community as to a possible cause(s) and impacts of such losses.

While point source contamination has been identified in many coastal areas of the world as possible causes of seagrass loss, there is currently no *single* cause of seagrass loss in areas that are well removed from sites of industrial activity and coastal urbanisation. This research project has therefore focussed on a possible cause of seagrass loss in such remote regions.

The intertidal environment, by definition, is regularly inundated by tidal flows and is therefore subjected to many of the physical and chemical influences associated with both land and marine environments. Biota within the intertidal environment are therefore subjected to the same 'influences'. It is surmised that susceptible biota are negatively impacted by chemicals, such as herbicides and insecticides, derived from land.

Because of its location in the inter-tidal zone, *Zostera muelleri* is subjected to physicochemical environmental forces or 'stress factors' within both marine and terrestrial environments. Stress factors imposed by the marine environment such as light-attenuation, wave action and herbivory are all likely to negatively impact upon seagrass survival. Additionally, stress factors imposed by the terrestrial environment such as desiccation and ultraviolet (UV-B) light (Trocine, Rice et al. 1981) are also likely to limit growth of *Zostera muelleri* within this zone. Other stress factors *within the sediment* such as heavy metal toxins (Faraday 1979; Prange and Dennison 2000), anaerobic condition, nitrite and sulphite toxicity (Jonkers, van et al. 2000), and a limitation in the supply of nutrients such as bioavailable phosphate (Craven and Hayasaka 1982) are also likely to limit seagrass growth through the sediment profile as well as along a hypothetical surface transect within the intertidal zone.

3.1.1 Loss of Seagrass Meadows

It is known that seagrass is being lost from such coastal environments with losses in some cases amounting up to 60% of historical acreage values. However, the cause(s) of such seagrass loss has been the subject of much research. 'Point source' contamination and 'diffuse source' contamination (Bester, 2000) have often been the focus of this research. Point source is used here to describe a known source of contamination of an environment where cause and effect are clearly defined or established.

Diffuse sources of contamination cannot be precisely defined in terms of their cause and effect. Indeed, many point sources of contamination events may result in a diffuse source contamination within a receiving environment.

In an EPA of study of the South Australian Metropolitan Coastline, between Largs Bay and Glenelg, a progression in sand has been noted and correlated to regions of seagrass loss along the metropolitan coast. The correlation is further developed in a chronology of coastal developments such as sewage treatment work discharge outfalls and stormwater drain construction that '*may have contributed* to seagrass loss. While it is not possible to definitively ascribe loss of seagrass to these developments, it is known that losses of seagrass can be attributed to eutrophication (caused by nutrient outflows), storm damage (Preen, Lee Long et al. 1995; Longstaff and Dennison 1999) and possibly herbicides (Bester 2000).

While the chronology correlates with the aerial photography data other *contributing factors* may have also acted in synergy with coastal developments to result in the documented seagrass losses along the metropolitan coastline. While the observations can be correlated with an event(s), it does not necessarily follow that the observation was caused by the event. Indeed, it is likely that seagrass losses are caused by the interaction (possibly synergistic) of many factors within an environment.

Some losses of seagrass can be accounted for when couched in terms such as 'point source' contamination and 'diffuse source' contamination. However losses that occur in regions that are well removed from sites of industrial activity and urbanisation (Bester 2000; Seddon, Connolly et al. 2000) are less easily defined in their 'cause and effect'. Additionally, the preceding description of environmental contamination is

an oversimplification since it is now known that environmental contaminants can be transformed, into secondary 'bioactive chemicals,' during transit or within the receiving environment.

These transformations are often the result of an interaction between the source chemical and the physical, chemical and microbial factors present within the receiving environment. This process is exemplified in studies of mercury poisoning of biota in Minimata Bay, Japan. Non-toxic mercury was released into the bay and subsequently bio-converted to the highly neurotoxic methyl-mercury by micro-organisms within the Bay's sediment. It is therefore worthwhile at this point to make the distinction between 'environmental contamination' and 'environmental pollution'. The terms 'environmental contamination' and 'environmental pollution' are not to be used interchangeably since the former merely refers to the presence of a chemical within an environment whereas the latter refers to a biological impact(s) of a chemical.

3.2 Interactions in the Intertidal Zone

Sea-farming and the fishing industry in general depend on a seasonal supply of a breeding stock and the maintenance of habitats in which juveniles and adults can forage and avoid predation. The intertidal zone is one such habitat. As mentioned earlier, it is a tract of coastal land (at low tide) that is positioned between low tide and the mangrove line (littoral sub-littoral). While seagrass does have a direct commercial value in the manufacture of mats it is considerably more valuable, in commercial terms, as habitat for small fish and crustacea. Indeed, it is known that prawn (*Penaeus*) prefer seagrass habitat to denuded regions of seabed (Subramaniam 1990).

Seagrass is a preferred habitat of fin-fish such as *Sillaginodes punctata* (Cuv. & Val.) (King George whiting) (Connolly 1994) and prawn (Morgan 1980; Subramaniam 1990).
Indeed, it has been demonstrated that a greater percentage of prawns remained in seagrass regions over those in denuded seabeds. Prawns use seagrass as habitat in which to graze and shelter from predators (Mizushima and Takaya 1999). Therefore it is likely that any depletion of seagrass acreage will also negatively impact upon the preferred habitat of prawn and, indirectly, on commercial prawn fishing yields.

Analogous 'interactions' also occur in terrestrial farming. The extensive removal of trees from arable land can lead to weakened soil structure and elevated water tables creating land erosion and rising salt respectively. These factors have then negatively impacted upon the *useful attributes* of land as a region in which to grow commercial crops such as wheat (*Triticum*), barley (*Hordeum vulgare*).

3.2.1 Zostera muelleri – Growth, Photosynthesis and Culture

Studies on the physical adaptations of seagrass have provided an insight into the importance of light. Indeed, Longstaff and Dennison (1999) noted that light attenuation, within the range 1 μE $m^{-2}s^{-1}$ to 972 μE $m^{-2}s^{-1}$, caused by cyclic (annual) pulse-turbidity events are likely implicated in seagrass loss in esturine environments.

The authors note that one species, *Halodule pinifolia*, was better adapted to such cyclic events than *Halophila ovalis*. While morphological responses, such as blade length, were correlated with these events, physiological (biochemical) responses are thought to precede such morphological responses.

It is likely that these changes in biochemistry may provide an 'early warning' indication of stress being imposed on seagrasses. It is particularly interesting that decreases in the chlorophyll a/b ratio, from 2.5 to 2.1, are known to accompany light deprivation caused by these turbidity events. This reduction in chlorophyll a/b ratio was also noted in light attenuation studies conducted by Dennison and Alberte (1982) in which *Zostera marina* exhibited a ratio of 2.2 at the shallow station and 1.8 at the deep stations. The authors think it likely that the increase of chlorophyll 'a' relative to chlorophyll 'b' somehow increases the light capturing efficiency of chloroplasts.

The results of many laboratory (Moore and Wetzel 2000) and field studies (Ralph, Gademann et al. 1998; Pollard 1999; Shafer 1999; Asmus, Sprung et al. 2000) attest to the importance of light in seagrass productivity (Clough and Attiwill 1980; Pollard 1999) and turnover. Additionally, the nutritional requirements of seagrass have been studied under laboratory (Bird, Johnson et al. 1998) and field conditions (Haeder, Kumar et al. 1998; Wear Donna, Sullivan Michael et al. 1999).

The results from such studies indicate that seagrasses, like all green plants, have a primary requirement for a sufficient supply of light and nutrition under the prevailing environmental conditions. The word 'sufficient' is used here, and especially in relation to light, to emphasise an amount that reaches the leaf surface of the plant and is photo-utilised, primarily in photosynthesis and photorespiration, by the plant under normal growing conditions. Additionally, a sufficient supply of nutrient is defined here as meaning that level of nutrient uptake and utilisation under 'normal' growing conditions.

3.2.2 Light Requirements of Seagrass

The requirement for adequate lighting is associated with the amount of photosynthetically active radiation (PAR) reaching the leaf surfaces of seagrass. Photosynthetically active radiation or PAR is defined as the photon flux per unit area between 400 and 700 nm; full sunlight is approximately 2000 $\mu E\ m^{-2}s^{-1}$; one mole of photons being equal to one Einstein (S.Tyerman 2002). It is well known in the literature that above to below sediment biomass ratio is greater in limited light conditions in the seagrasses *Thalassia testudinum* (Dawes and Tomasko 1988; Lee and Dunton 1997) and *Halodule wrightii* (Dunton 1994). It is anticipated that the higher incident PAR and water temperatures make a positive contribution to increased growth and subsequent storage (of carbohydrate) in root tissues during the summer months. Indeed it is likely that under normal environmental conditions, the process of photosynthesis is upregulated under conditions of increased lighting and temperature

It is however also known (Stryer 1980) that carbon loss via photorespiration and an up-regulated oxidase activity of RUBISCO occurs at higher temperatures. This oxidase activity increases at a greater rate than does its carboxylase activity. It is

also believed that enzymes associated with the photosynthetic process become more efficient at their optimal and higher temperatures. In the winter months when light is limited and water temperatures are lower seagrass growth is reduced. Light can also be reduced by means other than a change in season. Epiphyte overgrowth, for example, of leaf surfaces can occur in waters that receive a high nutrient inflow such as sewage outflows.

It is thought that a high nutrient inflow, often from anthropogenic activity, causes eutrophication of seagrass containing waters producing increased epiphyte growth (Wear Donna, Sullivan Michael et al. 1999). Epiphytes attenuate the available light by covering a greater proportion of the leaf's surface area (Day et al. 1989; Kemp et al. 1988; Sand-Jensen and Sondergaard 1981). This attenuation results in reduced productivity (Kemp et al. 1988; Tomasko 1993). Attenuation by epiphytes on *T. testudinum* is documented as being as much as 33% - 56% of PAR (Dixon and Kirkpatrick 1995) at depth in west central Florida. It also noted by Dixon (2000) that approximately 13% to 14% of the surface irradiance is sufficient to maintain *T. testudinum*. Algal overgrowth of seagrass leaves *can reduce* the amount of PAR by as much as 60% reaching the leaf surface.

In effect, the effective photosynthetic surface area of the leaf is reduced thus reducing seagrass growth. Dixon (2000) noted 36% light attenuation at a 'deep station' in *Thalassia testudinum* meadows with the seagrass being provided with lighting of 3730 moles m^{-2} yr^{-1} at a light stressed station before epiphyte attenuation and 2350 moles m^{-2} yr^{-1} after epiphyte attenuation. These results translate to approximately 236 µE $m^{-2}s^{-1}$ at the light stressed station before epiphyte attenuation and 149 µE $m^{-2}s^{-1}$ after epiphyte attenuation for a 12 hour photoperiod per with one mole of photons being equal to one Einstein. Sites that maintained the seagrass were provided with 4910 moles m^{-2} yr^{-1} before epiphyte attenuation and 3120 moles m^{-2} yr^{-1} after epiphyte attenuation.

In this case, the results translate to approximately 311 µE $m^{-2}s^{-1}$ before epiphyte attenuation and 197 µE $m^{-2}s^{-1}$ after epiphyte attenuation for a 12 hour photoperiod per day. Similar studies have been conducted by Czerny and Dunton (1995) on *T. testudinum* in which the authors found that lighting of 6500 moles m^{-2} yr^{-1} (413 µE $m^{-2}s^{-1}$) was sufficient to maintain the plants while 1819 moles m^{-2} yr^{-1} (116 µE $m^{-2}s^{-1}$) caused a decline in plants.

A decrease in biomass in the species *Halodule* and *T. testudinum* in Texas (Dunton 1994; Czerny and Dunton 1995) is also thought to be associated with a long-term reduction in PAR to 5 moles m^{-2} d^{-1} (116 µE $m^{-2}s^{-1}$) during seasonal changes. A decreased photosynthetic efficiency may be responsible for the noted decrease in tissue biomass as the winter approaches. This is particularly important since a temporal change in lighting could possibly act in synergy with a herbicidal insult to impact upon the photosynthetic efficiency of seagrasses. It is further hypothesised that a seasonal use of herbicide may coincide with seasonal fluctuations in PAR to have a negative impact on seagrass survival. Maximal PAR was associated with maximum growth rates (LRGR) for T. testudinum.

However maximum growth rates are not wholly dependent on PAR since the author noted similar shoot growth rates in plants that received maintenance light regimens.

Others have also noted the 'decoupling between growth rates and available light' (Czerny and Dunton 1995; Hall et al. 1991). Therefore growth is not a reliable indicator of low light stress. It is suggested that thermal cues (Tomasko and Hall 1999) and *endogenous seasonal rhythms* may supplant light availability.

It is anticipated that any anthropogenic stress factors, such as a herbicidal insults, will reduce the efficiency of the photosynthetic process. It is expected that a negative impact upon carbon reserves (Neely 2000) and oxygen production will also negatively impact on respiration, phosphorus metabolism (Craven and Hayasaka 1982) and sediment detoxification (Iizumi, Hattori et al. 1980; Ralph and Burchett 1998; Erskine and Koch 2000; Prange and Dennison 2000). It is particularly interesting that the study by Erskine and Koch (2000) into long-term exposures of *Thalassia testudinum* to sulfides was thought to result in a carbon drain from root tissues. These processes will not only affect individual plants but the species as a whole.

An underlying theme that can be identified from these studies is that, if all other factors are constant, a sufficient light regime is essential for seagrass growth and survival. This phenomenon is not restricted to seagrasses. Indeed, it is known that commercially grown terrestrial crops such as grapevines have a minimum light requirement with a net photosynthesis of zero at 30 to 50 $\mu E\ m^{-2}s^{-1}$, and a light saturation point at 700 to 800 $\mu E\ m^{-2}s^{-1}$ (Tyerman 2000). These data suggest that lighting of 250 $\mu E\ m^{-2}s^{-1}$ will be sufficient to maintain *T. testudinum* and possibly other seagrass species such as *Zostera muelleri*.

3.2.3 Nutritional Requirements of Seagrass

Healthy seagrass stands also require an adequate supply of inorganic carbon (Andrews and Abel 1979; Millhouse and Strother 1987; Neely 2000), nitrogen (Touchette and Burkholder 2000) and phosphorus (Craven and Hayasaka 1982; Touchette and Burkholder 2000). Additionally, trace elements may also be required. Axenic cultures of *Halophila decipiens* have been grown under laboratory conditions in modified agar supplemented with nutrients (Bird, Johnson et al. 1998). However, replicating all environmental conditions normally present in *Zostera muelleri's* natural environment would prove to be a difficult task. Furthermore, this would prevent the study of possible synergistic interactions between *Zostera muelleri* and sediment. It was therefore decided to study pollutant impacts on *Zostera* plants as: 'free-floating' (short-term cultures), 'modified sediment' (medium term) and 100% sediment (medium term) cultures. It is likely that any unusual nutritional requirements would be met within these short and medium-term culture techniques.

3.2.4 Carbon Fixation

Carbon dioxide gas has limited diffusion in seawater and is unlikely to supply, directly, sufficient inorganic carbon for the photosynthetic process within seagrass. Carbon can however be partitioned into several coexisting forms. At a seawater pH of 8.1, sodium bicarbonate is the predominant form. Some investigators also believe that the bicarbonate ion is taken up by seagrass and is subsequently lysed by a carbonic anhydrase to produce carbon dioxide and water.

An additional or ancillary 'carbon concentrating mechanism' entitled the C4 pathway permits the shunting of carbon as C4 precursers, such as oxaloacetate, from the mesophyll cells to the bundle sheath as malate. An ($NADP^+$ -linked malate dehydrogenase releases carbon dioxide from malate) enzymic reaction within the bundle sheath cells then converts the oxaloacetate to 'free' carbon dioxide and pyruvate, a C3 compound that is then recycled to the mesophyll cells. This is an energy demanding process noted earlier. However, the process permits the concentration of carbon dioxide in the bundle sheath under high sunlight and arid conditions that are present in the intertidal zone.

The sequestered carbon is then utilised as carbon dioxide in the photosynthetic reactions described by the Calvin Cycle. Products of the Calvin Cycle are simple sugars, starches and the by-product molecular oxygen. Sugar production and its impact upon phosphate metabolism will be discussed in a following section. Losses of carbon from the Calvin Cycle by the process of photorespiration can be within the range 25% and 50% of the captured carbon in terrestrial plants such as grapevines (Tyerman, 2000). It is noteworthy that *Zostera* has been described as both a C3 plant and a C3/C4 plant.

3.2.5 Nitrogen Fixation and Recycling

Inorganic nitrogen is obtained from degraded bacterial detritus that forms part of the sediment. Organic nitrogen in dead leaf matter can also be recycled via bacterial activity within the sediments (Caffrey and Kemp 1990; Rysgaard, Risgaard et al. 1996; Touchette and Burkholder 2000). Storm events will likely increase the outflow of nitrogen by causing excessive leaf loss via weakened abscission zones. While nitrogen is not likely to be limited near sewage outflows these regions are likely to provide eutrophic conditions conducive to algal and epiphyte overgrowth of leaves as discussed previously. Such overgrowth will then indirectly and adversely affect plant growth by light limitation (light attenuation) effects.

It should be noted at this point that increased rates of leaf senescence and the development of abscission zones (Feung, 1976) are caused by the auxin-like activity of herbicides such as 2,4-D (Wernicke, Gorst et al. 1986; Sandmann, De Beer et al. 1991), quinclorac (Grossmann 1998) and picloram (Musiyaka 1995). It is likely that the physical action of storm events will increase the outflow of such nutrients by causing excessive leaf loss from weakened abscission zones. An increased rate of leaf senescence caused by auxin-like activity of 2,4-D or 2,4-D like chemicals will decrease the 'pool' of available nitrogen within the sediment.

Additionally, production and utilisation of ammonium ions as a source of nitrogen is believed to occur within sediment and is made available to seagrass by absorption through its root system. In a study by Blackburn (1983) the author notes that ammonium ions are utilised as part of an 'elemental cycle.'

3.2.6 Phosphate Bio-availability and the Rhizosphere

Microbial activity, within intertidal sediments of seagrass meadows, has been implicated in the sequestering of phosphorus from sediments. Obligate aerobic bacteria that have a capacity to metabolise hydroxyapatite (calcium phosphate) have

been detected in sediments associated with *Z. marina* seagrass beds (Craven and Hayasaka 1982). It is interesting to note that the authors discovered the presence of glucose and acetic acid in the same sediments Craven and Hayasaka (1982) hypothesise that the simple sugar, glucose, is metabolised by aerobic bacteria into acetic acid that may then facilitate solubilisation of phosphate from hydroxyapatite.

3.2.7 Sulphate Reducing Bacteria: Possible Toxic Implications

Blackburn (1983) has noted that most of the sulphate reducing bacterial activity is restricted to the top 12 cm of seabed in *Zostera* seagrass meadows. Most activity occurs in the top 6 cm with 20% of this value occurring in the 6 cm to 12 cm zone and little occurring at greater depths. Such activity may therefore be restricted to the root zone and may be co-associated with nitrite reducing bacteria. Supplied molecular oxygen, from *Zostera's root zone*, may then serve to detoxify sediments and synthesise nitrate and sulphates from nitrites and sulfites respectively.

In summary, growth and survival of *Zostera* are dependent on a *sufficient* supply of light and nutrients. The word sufficient is used to emphasise that captured light must be photosynthetically active radiation (PAR) of an intensity and duration necessary for 'normal' tissue maintenance and growth. Similarly, nutrients must also be *bio-available* to the plant. The nutritional maintenance and growth requirements are co-dependant, in part, on a sufficient supply of molecular oxygen reaching the rhizosphere to abate sediment toxicity and to facilitate a symbiotic or co-habitation relationship between *Zostera* and obligate aerobes in sediment of the root zone.

Under normal conditions, *Zostera* supplies sufficient oxygen to the sediment in the process of photosynthesis. Therefore, light availability and nutritional status should be viewed as *interacting processes*. It is hypothesised that herbicide pre-treatment of *Zostera* will result in an impairment in the synthesis, transport and release of molecular oxygen or glucose to the rhizosphere. An insufficient supply of oxygen into the intertidal sediment will then compromise the bioavailability of phosphate to the growing plant and possibly restrict root growth and render the plant susceptible to the toxic effects within sediment.

3.3 Photosynthesis in Aquatic Plants, the Partial Pressure Of Oxygen Gas within Lacunal Canals, and its Significance for Plant Growth and Survival

Photosynthesis is the synthesis of carbohydrate by using light energy to capture carbon dioxide. During this process oxygen is made as a by-product. Photosystem II and Photosystem I comprise the 'light reactions' of photosynthesis. Carbon dioxide is then condensed with a five carbon sugar (ribulose 1,5-bisphosphate) by the enzyme ribulose 1,5-bisphosphate carboxylase (RUBISCO) in the 'dark reactions' of the Calvin Cycle within the stroma. The initial products of this process are two three-carbon organic compounds (3-PGA) which can either be processed into fructose, glucose and subsequently starch or it can be used to 'regenerate' ribulose 1,5-bisphosphate. Carbon dioxide is supplied to the Calvin Cycle as CO_2 or via a C4 pathway to give rise to the C3 and C4 carbon acquisition-systems, seagrass has been referred to as both a C3 and C3/C4 plant.

Several studies on seagrasses have used *chlorophyll fluorescence* as a measurement of some components of photosynthetic efficiency in plants subjected to 'stress factors' such as photo-inhibition (Ralph and Burchett 1995), heavy metals (Ralph and Burchett 1998), osmosis (Ralph 1998), petrochemicals (Ralph and Burchett 1998) and combinations of factors (Ralph 1999). The impact of herbicides on chlorophyll fluorescence in the seagrasses *Halophila ovalis* (Ralph Peter 2000) and *Zostera capricorni* (Haynes, Ralph et al. 2000) have also been investigated. It should however be noted that effects on photosynthesis are often inferred from measured differences in incident and emitted light wavelength and intensity from a leaf's surface as a result of changes in electron flow through the photosystems rather than a direct measurement of carbohydrate produced.

Hill (1939) demonstrated that isolated chloroplasts are able to lyse water and produce oxygen with ferricyanide being used as the terminal electron acceptor in place of carbon dioxide. Therefore electron-flow, and presumably chlorophyll fluorescence, does not necessarily equate with carbohydrate produced. This point will be considered further when the results of the bioassays are discussed.

The roots of aquatic plants are immersed in water and are therefore limited in their supply of oxygen from the external sediment. Indeed, the sediments in which aquatic plants grow are often anoxic. Aquatic plants have therefore adapted to these waterlogged environments by producing oxygen, in their leaves by photosynthesis, and then transporting the gas via lacunae to the roots to facilitate respiration and to prevent the possible toxic effects of sulphide-containing sediments. Sand-Jensen et al. (1982) quantified the release of oxygen from the roots of several aquatic plants. The authors presented the results as a percentage of total oxygen released by the plant. They noted that *Zostera marina L.* released 1%, of its total released oxygen, into the root zone of the plant with the remainder being released into seawater above the sediment zone.

The authors also point to the limited capacity of the lacunae in this species. It is therefore likely that this species will have a limited ability to store and use oxygen for respiratory processes, since oxygen is used as a co-substrate in mitochondrial respiration. Additionally, the authors hypothesise that the partial pressure of oxygen within lacunae is higher during the photoperiod than in darkness. Indeed, Laing (1940) studied the increase in partial pressure of gasses within aquatic plants when plants were subjected to artificial lighting. The increase in the partial pressure of oxygen within *Zostera*'s lacunae may be the result of a low diffusion coefficient of oxygen within water that causes a poor gas-exchange of oxygen into seawater. This slow exchange of oxygen does not occur in terrestrial plants because the diffusion process between the lacunae and the surrounding atmosphere is facilitated by the stomata and a root system that is not surrounded by water.

3.3.1 Does the partial pressure of oxygen within lacunae affect carbohydrate synthesis?

In the presence of oxygen, RUBISCO, the carbon-capturing enzyme of the Calvin Cycle, can act as both an *oxygenase* as well as a *carboxylase* (its 'normal' function). Indeed, it is known that this enzyme preferentially binds oxygen over carbon dioxide.

It is particularly interesting that the oxygenase activity of RUBISCO increases at a faster rate than does its carboxylase activity as temperature is increased (Stryer, 1983). This may have implications on plant growth in exposed environments such as the intertidal zone. Clough and Attiwill (1980) noted a 30% reduction in net photosynthesis in *Zostera muelleri* in plants that were exposed to sunlight as opposed to plants that were submerged. They concluded that the reduction in photosynthesis may have been caused by self-shading of prostrate leaves (exposed condition) or by a change in the photosynthesis-respiration balance.

Photorespiration under the exposed condition may have contributed to their observed results. Additionally the results of pulse-chase experiments, using C^{14} labelled sodium bicarbonate, show that a significant proportion of the label is partitioned into photo-respiratory intermediates (Andrews and Abel 1979). These data suggest that photorespiration is likely a significant contributing process to the efflux of carbon from seagrasses.

The oxygenase activity of RUBISCO is not such a problem for terrestrial plants since oxygen easily diffuses from the lacunal canals and stomates. However, even in terrestrial plants between 25% and 50% of the 'captured carbon' is lost by the process of photorespiration that relies on a supply of substrate, glyoxylate obtained from ribulose 1,5-bisphosphate, and the oxygenase activity of RUBISCO. The known increase in lacunal gas pressure within aquatic plants that are irradiated with photosynthetic light suggests that it is likely that RUBISCO would be further inhibited under 'normal conditions' in aquatic plants. It is surmised that any increased oxygen production would exacerbate this carbon-loss process and would place the plant in a 'carbon deficit state.'

3.4 Stress factors that can act upon *Zostera*

Plants and animals that inhabit the intertidal environment are constrained by stringent growth conditions imposed on then by their environment. They have therefore adapted to a variety of 'stress factors' imposed upon them by their environment. *Zostera*'s growth and survival is dependent on adaptations within *Zostera* and the interactions between *Zostera* and its surroundings; both biotic and abiotic. It is the efficacy of these adaptations and interactions that determine whether *Zostera* can survive in the intertidal zone. A major change in environmental conditions or a major change in *Zostera's* ability to cope with the prevailing environmental conditions is likely to negatively impact on *Zostera's* survival: as a plant and as a species.

Stress factors such as light availability are known to have a direct effect on *Zostera*'s growth and survival. Light availability, or more correctly photosynthetically active radiation (PAR), can become limited by epiphyte overgrowth of leaves, water clarity and water depth (Dennison). While light attenuation caused by epiphyte overgrowth can be controlled to some extent by grazing arthropods, the remaining light attenuating factors are largely controlled by seasonal wave action, storm events (Longstaff and Dennison 1999) tidal movements and global climate changes (Beardall, Beer et al. 1998; Haeder, Kumar et al. 1998; Short and Neckles 1999). Terrestrial plants and 'aquatic' plants such as *Zostera* can also be directly affected by ultraviolet light (UV-B) (Ralph and Burchett 1995). Plants produce pigments (Dawson and Dennison 1996) that dissipate the high-energy incident light that would

otherwise induce photo-damage by photo-bleaching of organelles such as chloroplasts.

Alternately some seagrasses, such as *Halophila engelmanni Aschers*, can alter the grouping or positioning of chloroplasts so as to reduce the impact of such high-energy radiation on the photosynthetic apparatus (Trocine, Rice *et al.* 1981). However, such adaptations come at an 'energy cost' to the plant. While *Zostera muelleri* is ideally suited to the intertidal environment, experiencing heat exposure during low tide and seasonal freshwater inflows in winter, it has a limited capacity to cope with extremes in temperature and salinity (Kerr and Strother 1985). These 'heat stress factors' and 'osmotic stress factors' will negatively impact on a plant's survival by causing leaf desiccation and perturbations in the osmotic balance that the plant has established within its environment.

Any stress factor that causes a reduction in the synthesis, transport or release of oxygen and carbohydrate into the root zone will subsequently initiate a 'nutritional stress factor' by reducing the bioavailability of phosphorus from hydroxyapatite. This was discussed previously. Bacteria within the root zone have also been implicated in the cycling of nitrogen, as ammonium ions, from the anoxic sediment to *Zostera* and in the detoxification of nitrites and sulphites within the sediment that are known to have an adverse effect on *Zostera* as previously discussed.

Indeed, the monitoring of sulphide levels during growth and senescence phases of benthic macroflora has revealed that seasonal changes (Viaroli, Bartoli et al. 1996) in this potentially toxic metabolite are linked to the production of oxygen by the seagrass *Zostera noltii*. However results of studies conducted under laboratory conditions on the seagrass *Thalassia testudinum* (Erskine and Koch 2000) suggest that a 10 mM pulse of sulfide over a 48 hour period is insufficient to cause mortality in this species. This raises the possibility that while sulfides may contribute to seagrass loss, other factors may act in synergy with sulphides to cause losses in short-term exposure events. It is particularly interesting that the authors point to a significant reduction in leaf elongation rates of 43% at 2 mM sulphide. The authors conclude that sulphides may play a critical role as a *root carbon drain* over long-term exposures.

Other toxicants such as heavy metals (Malea 1994; Malea, Kevrekidis et al. 1995; Warnau, Ledent et al. 1995; Prange and Dennison 2000) are also thought to have an adverse effect on seagrass survival.

Under normal growth conditions a plant (organism) will enter into a homeostatic state with its environment. However, the interaction of endogenous and exogenous stress factors can limit or restrict a plant's (organism's) suitability to its environment if all other factors remain constant. Plants such as seagrasses therefore live within a 'tolerance zone' created by the interaction of stress factors within their environment. A physicochemical treatment or pre-treatment of a plant can modify its susceptibility to such stress factors, which, in turn, will modify its tolerance zone. In effect, treated plants can no longer cope with their 'normal' tolerance zone(s) and are no longer suited to their environment.

It is believed that chemicals exhibiting auxin-like activity act on seagrasses that inhabit the narrow environmental band of the intertidal zone and influence their ability to survive in the intertidal zone. It is further hypothesised that seagrass losses will negatively impact upon the survival of animals that use seagrass meadows as habitat and a foraging ground.

3.4.1 How does Zostera cope with known stress factors?

The known toxic stress of heavy metals (Ralph and Burchett 1998; Prange and Dennison 2000), sulphites (Erskine and Koch 2000) and nitrites (Iizumi, Hattori et al. 1980) imposed on seagrass by sediments of the intertidal zone raised the '*obvious* question': 'How does seagrass survive in such a habitat?' The literature answers this question, in part, by presenting evidence that is consistent with the hypothesis that seagrass detoxifies its own environment (Lee and Dunton 2000) by synthesising, transporting and releasing molecular oxygen into the intertidal mud. This process is believed to occur in a diurnal fashion that prevents toxic sulphide intrusion into the roots of seagrasses. Seagrass therefore modifies its own environment (Jonkers, van *et al.* 2000) and helps create a non-toxic microenvironment adjacent to the root zone of the plant, thereby providing protection from any toxic effects within the sediment.

These literature observations underpinned the formulation of a model for seagrass loss. It is likely that the lack of a sufficient, and a maintained, detoxification process within the root zone of seagrass will subject the plant to toxic factors and result in a 'toxic exposure', and an associated toxic stress, being imposed on the plant. It is also likely that molecular oxygen within the plant is allocated primarily to 'detoxifying the sediment' and to mitochondrial respiration as measured in meadows of *Zostera muelleri* (Clough and Attiwill 1980). It is thought that a 'fixed quotient' of any biosynthesised oxygen is allocated to the detoxification process irrespective of whether it is day or night. The remaining oxygen is likely to be used for energy production in dark respiration in daylight and night-time periods. It can be inferred that any treatment that influences the synthesis, transport and utilisation of oxygen can impact on a plant's survival and the species as a whole.

Growth and survival of seagrass are also affected by 'the carbon budget' (Neely 2000). In periods of high productivity a larger proportion of the plant is dedicated to photosynthesis and energy is re-directed to plant growth with 'excess' being directed to storage. Carbohydrate storage is accompanied by less tissue mass above the sediment (in the winter months). A critical point is thought to exist beyond which a plant is either in 'carbon credit' or in 'carbon deficit'. The maintenance of the carbon budget on presumably 'a controlled growth pattern' raised the question: 'would an uncontrolled growth pattern perturb the carbon budget and impact upon a plant's survival?'

The notion that either sediment kills the plant (oxygen deprivation of roots) or that an imbalance in the carbon budget does are not mutually exclusive. Indeed, these two 'toxic processes' are connected in their production and utilisation of oxygen and carbon in the biochemical pathways: photosynthesis, photorespiration and respiration. Additionally, the interplay of microbial ecology with available oxygen within anoxic sediment of the intertidal zone can determine the magnitude of toxic

stress imposed on these intertidal plants by sulphites and nitrites within the sediments.

The 'stress factors' noted above have lower and upper limits that restrict *Zostera*'s growth within the intertidal zone. It is anticipated that such stress factors interact with one another to create a 'tolerance zone' (Figure 5) within which *Zostera muelleri* can grow. It is further expected that any perturbation affecting *Zostera*'s ability to cope with the prevailing stress factors within this tolerance zone will move *Zostera muelleri* out of its tolerance zone. It is unlikely that *Zostera muelleri* can relocate or initiate large changes in adaptation within a short period of time. Therefore, plants that are subjected to such changes in their ability to cope with the prevailing environmental conditions are unlikely to survive. If such a change adversely affects the fundamental physiology or biochemistry of the plant, such as the carbon budget, this may also affect seed production and dispersal. It is likely that sexual reproduction, gene pool size, and re-colonisation of denuded intertidal zones will become restricted if the carbon budget is adversely affected.

Furthermore, any limitation in gene pool size will then render the species susceptible to further risk by a transient contamination event. The tolerance zone is therefore defined by the prevailing physicochemical factors that are known to negatively impact upon seagrass survival. The tolerance zone for seagrass can be envisaged as a subset of the prevailing environmental conditions.

To maintain its environment *Zostera muelleri*, along with other marine aquatic plants therefore modifies the environment of the sediment adjacent to their root zones by releasing oxygen produced during photosynthesis. The released oxygen is then used to detoxify nitrites and sulphites and likely provides oxygen for growing root tips. Additionally phosphate absorption is facilitated by the action of obligate aerobic bacteria that partially acidify the sediment thus converting hydroxyapatite to bioavailable phosphate.

3.4.2 Lacunal gas pressure, leaf morphology, and induced stress imposed on ribulose 1,5-bisphosphate carboxylase

Lacunae form continuous gas vessels from the leaves to the root zone of *Zostera muelleri* (Kuo, Ridge et al. 1990). They are segmented, at regular intervals, by a series of septa composed of parenchymal cells that are a few cell layers in thickness. The parenchymal cells are metabolically active containing both mitochondria and chloroplasts. These septal cells are arranged in such a way that small gaps between them permit gas flow into the adjacent 'lacunal compartment' within *Zostera muelleri*. Similar structures may also exist in other seagrasses such as *Zostera marina* and *Halodule pinifolia*. Indeed, studies of the freshwater aquatic plant *Nuphar advenum* (Laing 1940), or water lily, have demonstrated similar structures positioned between the petiole and the young root. As the petiole enlarges the abscission zone becomes narrower and permits gas flow between the mature leaf and the root lacunal gas space.

Diurnal changes in oxygen and carbon dioxide gases within the lacunal spaces were noted in this study. Laing (1940) likened these gas spaces to 'leaky reservoirs'. Hartman et al. (1967) also found experimental evidence that gases extracted from

fresh water hydrophytes, *Elodea canadensis* and *Ceratophyllum demersum*, vary in a diurnal fashion with a reservoir of oxygen being stored within the gas space under low light intensities. This is consistent with the known oxygen storage capacity of lacunae in the seagrass *Thalassia testudinum,* as studied by Thursby and Pederson, (1987). It is therefore likely that the septa within *Zostera muelleri* regulate gas flow between the leaves and the roots of this marine intertidal plant as a strategy or adaptation to their aquatic environment. In effect, the septa act as a series of 'two-way biological gas valves' positioned within lacunae between the leaves and the roots. It is proposed that changes in the size of the parenchymal cells, that comprise the septa, will modulate gas flow within the lacunae. It is likely that the parenchymal cells will become more or less turgid as a function of their metabolic activity and the osmotic potential of the surrounding medium (seawater).

If the osmotic potential (seawater concentration) remains constant in daylight (a.m.) PAR will induce photosynthetic cells, such as the parenchymal cells, to produce sugars such as glucose. This will increase the osmotic potential within the cells and cause a steady influx of freshwater into the cells. They will then become more turgid and the intercellular gas spaces between cells will become smaller restricting gas flow. As the photosynthetic period decreases at the end of the day (p.m.) a series of gas (oxygen) filled compartments will be present within the lacunae for use at night in the process of mitochondrial respiration. A decrease in the osmotic potential of the parenchymal cells, as sugars are utilized, will result in them becoming less turgid, causing a concomitant increase in the gas flow through the lacunae prior to the next cycle of photosynthesis.

This hypothesis is consistent with the results discussed above in Hartman's study (1967) of freshwater hydrophytes. If we now consider a second hypothetical scenario in which *Zostera muelleri* is placed in a hyposaline environment in which the osmotic potential is decreased. It is anticipated that a freshwater inflow into the septal parenchymal cells will occur since the solute concentration within the cells will be greater than that of the surrounding environment (diluted seawater). The parenchymal cells will then become more turgid and gas (oxygen) flow through the septa between the leaves and the roots will become restricted. Alternately, a freshwater inflow at night will restrict gas (carbon dioxide) exchange between the roots and the leaves. These events are likely to cause a deficiency in mitochondrial respiration and carbohydrate synthesis respectively within the plant which, in turn, will lead to an energy deficit. These hypotheses are consistent with the known and restricted growth patterns of *Zostera marina L.* in salinity gradients (Pinnerup 1980).

Lacunal gas volumes within *Zostera muelleri* represent between 16 and 20% of the leaf volume (approximately between 1.7 mm^3 and 1.8 mm^3 per leaf) in the 'intertidal form'(Kuo, Ridge et al. 1990) and up to 36% of the leaf volume (approximately between 30 mm^3 and 33 mm^3 per leaf) in the 'sub-tidal form'. It is interesting to note that an incremental increase in oxygen produced during photoperiod will likely produce a greater incremental increase in oxygen partial pressure within the lacunae of the intertidal form. This can be deduced if one considers the lacunal chamber as a sealed gas vessel in which the ideal gas equation ($PV = nRT$) applies (Atkins 1976; Fullick 1994).

It is considered that an increase in gas pressure within the lacunae will cause a competitive inhibition of RUBISCO, a key enzyme involved in the photosynthetic process (Bowes 1972). It is known that a 12% and 30% inhibition of the enzyme (soybean ribulose 1,5-bisphosphate carboxylase) occurs in an atmosphere containing 20% and 100% oxygen respectively, air contains approximately 21% oxygen. While RUBISCO displays its maximum enzyme activity at 55 °C it is known that photosynthesis decreases sharply in *Zostera muelleri* on increasing the ambient temperature to 42 °C from 30 °C (Kerr and Strother 1985). Since *Zostera muelleri* grows on the intertidal mudflat and is exposed to high temperatures at low tide during the summer months it is likely that RUBISCO operates at near its maximum efficiency.

Furthermore, an increase in the partial pressure of oxygen would likely occur within the lacunae under normal growth conditions. However, any treatment that increases oxygen production under the prevailing environmental conditions would have a marked negative effect on photosynthesis in *Zostera muelleri*. The notion that *Zostera muelleri* is inhibited by stored oxygen within the lacunae is supported by the observations of Clough and Attiwill (1980) in which net photosynthesis is reduced by 30% in exposed plants when compared to submerged plants. When RUBISCO acts as an *oxygenase* carbon is diverted to the process of photorespiration. It is known that as much as a 30% loss in carbon-capturing efficiency can occur by photorespiration in commercially important terrestrial plants such as grapevines (Tyerman, 2002). There has been much investment in research in minimising carbon losses from other terrestrial crops such as wheat and barley. Similar processes operating within seagrass physiology and biochemistry will compromise plant survival and possibly species survival. Indeed, Neely (2000) points to the importance of a 'positive carbon budget' in seagrass survival as noted previously.

Some herbicides, such as the triazines, atrazine and simazine, have direct and negative effect upon the photosynthetic process by inhibiting PSII. It is therefore likely that less oxygen is produced in plants subjected to these herbicides. However, other xenobiotics such as the phenoxyalkanoic acids 2,4-D and 2,4,5-T act as herbicides at high concentrations and as plant auxins at lower concentrations. It is likely that photosynthesis and possibly oxygen production will be enhanced in plants subjected to an auxin-induced growth phase.

3.5 Herbicide Usage, Mode(s) of Action and Half-lives

Herbicides are pesticides that negatively impact upon the growth and survival of weeds. Many herbicides are described as being specific in their application and usage. This terminology is often more closely related to the spatial and temporal attributes of the weed in relation to the crop plant of a farm rather than some intrinsic or innate property of the weed and its interaction with the herbicide. Although there are broad-leaf herbicide applications that rely on the large surface areas of target plants to allow maximum uptake in preference to non-targeted plants.

A widely used phenoxyalkanoic acid herbicide, 2,4-D, was first registered as an agricultural herbicide in 1947 (Wilson, Geronimo *et al.* 1997) while other phenoxyalkanoic acid herbicides, such as 2,4,5-T, were developed later. These herbicides are applied to soil surfaces in intensively farmed regions and are used as

a pre-emergent herbicide in the control of broad-leafed weeds during intensive farming practices. "Herbicides comprise nearly half of the 5 billion (10^9) pounds of pesticide *active ingredients* used worldwide annually" (Tominack, 2000). This means that 2.5 billion pounds of herbicide active ingredients are used worldwide annually.

It is known that 5% of surface applied herbicide 2,4-D is 'lost' as dust from farmland (Larney Francis, Cessna Allan et al. 1999). Therefore, 1.5 million (150,0000) pounds of 2,4-D are lost annually in USA from agricultural land, which is equivalent to 1826 kg each day. This is a sufficient amount to cover an intertidal zone of 370 million square metres at 5 mg per square metre or 37000 hectares at 5 mg per square metre *each day* of the year. This calculation is likely an underestimation of herbicide transport and deposition since farming practices, herbicide usage and transport would occur in a shorter time frame.

It is likely that intertidal seagrass species such as *Zostera muelleri* are at risk from the action of 2,4-D if this 'loss' is partly transported to the intertidal zone and if *Zostera muelleri* is susceptible to this xenobiotic. Additionally, it should also be noted that this calculation is an underestimation of phenoxyalkanoic herbicide transport since 2,4,5-T was not considered together with other herbicides, such as quinclorac, that are specifically designed as auxin-type herbicides. Amino acid conjugates (discussed below) formed as 'by products' of phenoxyalkanoic usage are also likely to be present in soil humus made from dead plant material such as weed debris.

Information in the literature detailing the specific mode(s) of action of the phenoxyalkanoic acid herbicides such as 2,4-D and 2,4,5-T is limited. However, it is known that 2,4-D interacts with model membranes (liposomes) and human erythrocytes (Suwalsky, Benites et al. 1996) when applied within the concentration range 10 µM to 1000 µM causing membrane perturbation and the formation of 'echinocytes'. The authors conclude that the amphipathic molecular structure of 2,4-D permits its insertion into the outer monolayer of erythrocytes. This point will become relevant later in a discussion of seagrass bioassay results.

It is known that phenoxyalkanoic acid herbicides can act as herbicides when applied at high (400 µM) concentrations and as a plant auxins (Davidonis, Arjmand et al. 1979; Davidonis, Hamilton et al. 1982) when applied to plant tissue at lower concentrations (1 µM to 10 µM). 2,4-D has been used commercially as a ripening agent by 'setting' fruit over a short time-frame.

One such herbicide, quinclorac, acts as an *auxin herbicide* (Grossmann 1998) by selectively inducing 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity during auxin-induced ethylene biosynthesis (Grossmann and Scheltrup 1997). ACC is subsequently oxidised to cyanide within plant tissues by ACC oxidase (Grossmann 1996).

Plants, such as weeds, subjected to a phenoxyalkanoic acid treatment may also respond to a herbicidal insult by chemically compartmentalising the herbicide into a series of phenoxyalkanoic acid conjugates biosynthesised from amino acids and glycosylated sugars. It is known that the L-amino acid conjugates are often as active or more active than the parent herbicide in their auxin-like activity often exhibiting maximum activity within the range 1 µM to 10 µM (Davidonis, Hamilton et al. 1982).

Indeed, some maximum activities are expressed at 0.1 µM (Davidonis, Arjmand et al. 1979). What is particularly interesting is that pre-treatment of plant tissues with 2,4-D-L-amino acid conjugates *enhances* the uptake of a subsequent C^{14} radiolabelled 2,4-D insult in plant tissue studies (Davidonis, Hamilton et al. 1982). It is likely that if *Zostera* is sensitive to an auxin-like activity of phenoxyalkanoic acid herbicides, and their amino acid conjugates, one would anticipate a change in the growth pattern of plants subjected to such treatments.

Other xenobiotics such as simazine and atrazine are designed to specifically impact upon photosystem II while herbicides such as sulfonylureas are acetolactatesynthetase (ALS) inhibitors and affect the synthesis of branched chained amino acids. It is possible that a deficient ALS system will cause deficient or dysfunctional enzyme systems. It is known that a key enzyme in photosynthesis, rubulose 1,5-bisphosphate carboxylase (RUBISCO), can represent up to 30% of the cellular protein. This specialised protein is involved in photosynthesis and may be rendered dysfunctional in plants that are treated with sulfonylurea herbicide. A dysfunctional, or less than optimal functioning, RUBISCO would then compromise the efficiency of the photosynthetic process.

Persistent xenobiotics, such as simazine and atrazine, have long half-lives typically of the order of years. They are less easily degraded by the physical, chemical and biological factors present within an environment than are the less persistent xenobiotics such as phenoxyalkanoic acids, that have a reported half-lives ranging from weeks (Thompson, Stephenson et al. 1984) to three years. The considerable variation in half-life estimates of 2,4-D is attributable in part to the variability in pH, organic and moisture contents of the soils studied. It is known that 2,4-D is metabolised to 2,4-dichloropheol in soil and possibly in intertidal sediment. It should be noted that no distinction is made in this report between a xenobiotic that has a demonstrated long half-life and one that has a short half-life but is frequently used.

It is surmised that if a chemical is transported and arrives in an environment containing susceptible biota then a biological effect would likely occur in the biota regardless of the absolute value of half-life of the xenobiotic. With the documented increasing herbicide resistance of weeds to conventional herbicides, higher doses are often used. Additionally, with the advent of molecular biology, commercial plants can now be designed to better cope with doses of herbicide (for example roundup ready Canola). However, it seems unlikely that all plants within the biosphere, including aquatic plants, will express the same degree of tolerance. Indeed those plants that have already adapted to stringently controlled environments, such as the intertidal zone, may find it difficult to develop the anthropogenically-constructed adaptations present in engineered plants.

3.6 Spatial and Temporal Attributes of Pesticide Usage and Transport Mechanisms

For herbicides to act on weeds they must first be transported to the weeds and then be absorbed. They can then be translocated within the plant after which they may then be detoxified. Alternately, the herbicide may act on the plant's biochemistry affecting the functional attributes of biochemical pathways or organelle function.

Herbicides are designed to maximise their impact on weeds and minimise their impact on farm produce.

For herbicidal environmental contaminants to have an impact on non-target organisms they must be first transported. There is no shortage of potential transport mechanisms for herbicides from intensively farmed regions. Indeed, it is well known in the literature that transport of herbicide can occur by 'spray drift', runoff, and wind erosion of soil. Off-target transport of 2,4-D is well known. Many regulations are now in place to reduce the incidences of 'spray drift'. However, in a study by Sandmann et al. (1991) the off-site transport of auxin-like herbicides; such as non-volatile (salts) 2,4-D, 2,4,5-T, was monitored in the Tala Valley (Natal) in South Africa. This transport and biological impact on a non-target species (vegetable farming area) resulted in restrictions in the use of auxin-type herbicides within this region.

However, despite a zone (approximating 25km by 25 km) restriction within the valley and a subsequent restriction, approximating 20 km beyond the first for phenoxyalkanoic acid usage, auxin-type herbicides were still detected in air, rain and dew. The maximum concentrations in air and rain were 0.5 $\mu g/m^3$ and 430 µg/L respectively, with as much as 6 g/ha 2,4-D and 7 g/ha 2,4,5-T being deposited in non-target areas. The decrease from the maximum concentrations of auxin-type herbicides in air in successive rain samples is likely the result of a 'wash-out' process of particulate material from air. It is known that such herbicides are extensively used in sugar-cane growing regions beyond the outer (second) boundary set in the Tala valley. The authors conclude that auxin-type herbicides can be transported many kilometres beyond their point of usage to non-target areas where they can have a negative biological impact on non-target species. However, constructing a direct correlation and attributing a delayed (or 'lag period') biological effect in non-target species to such herbicide transport is a "futile" exercise as noted by Farwell et al. (1987).

Farwell's point is relevant here since dust transport processes in South Australia in relation to seagrass loss can also be described as an opportunistic exercise. In a report by De Beer, (1992) on air monitoring studies of the Natal Valley, the author notes that the parent iso-octyl ester of 2,4-D used in the sugar cane industry was not detected. However, polar forms of the herbicide were regularly detected as adsorbed suspended particulate material and a 'chemical transformation from the ester to the polar forms may have occurred. In a study of Canadian soils of the prairies by Larney et al. (1999) the author notes that up to 5% of this surface-applied herbicide is 'lost' as 'wind-eroded sediment' from the application area.

Particulate material can be transported much further distances. In a 'Brown Snow' event, dust and the herbicide trifluralin was transported from Western China to the Canadian Arctic with the constituent particulate-material being identified by its mineral and pollen content (Welch, Muir et al. 1991). The authors concluded that either the herbicide was transported from China or was collected in transit from the northern mid west of the U.S.A. prior to deposition in Canada.

The transport of 'dust' or alluvial erosion is a global phenomenon, indeed dust from Asia contributes to the nutrient input of the Amazon rainforests. Dust transport can often occur over shorter distances and often over agricultural regions. Recently, a

dust storm traveled eastwards from an intensively farmed (sugarcane and wheat) region of Queensland Australia towards the east coast. Dust transport in South Australia occurs regularly with an average of fifteen to seventeen dust storms per annum. In one dust storm event in 1994, ten to fifteen million tonnes of topsoil was transported. Minor dust storms often receive little attention from the news-media and the general population (personal observation) and are regarded as part of the 'normal' weather pattern. Indeed, the transport of dust towards Adelaide contributes to a steady rate of pedogenesis with an annual accession of atmospheric dust in the range of five to ten tonnes per km^2 (Tiller, Smith et al. 1987).

It is clear that dust from intensively farmed regions of the York Peninsula (South Australia), often driven towards Adelaide across Spencer Gulf and the Gulf St. Vincent by westerly winds, can act as transport mechanism for herbicides. Even if dust was not a significant transport vehicle for herbicides there is still the possibility of spray drift and 'particulate' transport processes that have occurred in the Natal Valley as discussed above.
The concept underpinning this study is that 'particulate material' and 'dust' can act as transport mechanisms for herbicides.

3.6.1 Can Some Losses in Seagrass Acreage be Attributed Specifically to Herbicide Usage?

In a study by Bester (2000), the author noted extensive losses of seagrass beds in the Wadden Sea and suggested that such losses were associated with the presence of detected herbicides in the same region. However, the presented results could be interpreted as data referring to environmental contamination rather than environmental pollution since a definitive and causal relationship between the presence of herbicides and the occurrence of seagrass loss was not firmly established. The author pointed to several regions in the study area that contained low levels of herbicide. Water inflows from agricultural and industrial regions are thought to be the source of this environmental contamination.

Whether such trace levels of herbicide had a biological effect on the native seagrass species is speculative. Interestingly, in a laboratory study by Schwarz (19--) much higher concentrations of the herbicide atrazine were found to increase the chlorophyll content of the seagrass *Zostera marina* from 6.67 to 7.0 mg/L when applied at a rate of 2.46 mg/L in a chronic exposure study lasting 40 hrs. While the presented results were not statistically analysed they suggest that the increased chlorophyll content may be the result of a stress-induced response (possibly an up-regulation in synthesis) imposed upon the seagrass by the herbicide exposure.

Synergistic interactions of the herbicide treatment with UV-B, osmotic stress and other physicochemical variables were not investigated. Triazine herbicides, that comprise a component of the ship antifouling paint Irgarol, have also been implicated in seagrass losses in estuarine areas (Scarlett, Donkin *et al.* 1999). In laboratory studies bioconcentration of Irgarol 1051 within *Zostera marina* L. tissue was measured as being in excess of 300 times the water concentration causing a 50% decrease in photosynthetic efficiency in plants subjected to 0.2 and 2.5 µg/L as measured by chlorophyll fluorescence. Estuarine waters in S.W. England contain less than 0.003 µg/L Irgarol, however this herbicide bioconcentrates within leaf tissue

at 25000 times the ambient level when results are expressed on a dry tissue weight basis. The authors speculate on possible risks imposed by siting marinas near seagrass meadows.

Laboratory studies conducted by Haynes et al. (2000) on the seagrasses *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capricorni* have also concluded that electron flow through the photosystems, measured as a depression in effective quantum yield by chlorophyll fluorescence, is perturbed by herbicide (diuron) treatment. This effect was observed when diuron was used within the concentration range 0.1 to 100 µg/L. Interestingly, the effect remained five days after the initial exposure when plants were returned to fresh seawater.
It is important to note at this stage that electron flow and chlorophyll fluorescence measurements do not necessarily equate with photosynthesis when the latter is defined as the synthesis of carbohydrate. Indeed, Hill (cited by Stryer, 1983) demonstrated that isolated and illuminated chloroplasts produce oxygen when ferricyanide is used as the terminal electron acceptor in place of carbon dioxide. In this study oxygen was manufactured (photosynthetic 'light reactions') in the absence of carbohydrate synthesis (photosynthetic 'dark reactions').

This point will become relevant later in a discussion of bioassay results.
Notwithstanding the former, results of laboratory and field studies suggest that seagrasses can be negatively impacted upon by herbicides.

3.7 Experimental Approaches Used in Studying Seagrasses

Two general methods have been used in the study of biological and biophysical phenomena associated with seagrasses. In the first, a part of the environment is *studied in-situ* under the prevailing environmental conditions. These studies have provided valuable data relating to the light requirements (Dixon) of seagrass. Indeed, some investigators have imposed a light attenuation stress (Dennison 1982) on seagrass, *Zostera marina L.* (Eelgrass), in its natural environments to assess the possible impact of such imposed stress. Osmotic stress (Pinnerup, 1980) and nutritional requirements of seagrasses have also been investigated in field experiments. Additionally, geosurvey studies have quantitated seagrass loss by using satellite imagery (Dr Megan Lewis, pers comm). Results obtained from such studies have a direct application in re-establishing seagrass meadows in regions that have suffered acreage losses (.

In the second general study method seagrasses are grown or used under defined laboratory conditions where all 'environmental conditions' can be monitored and varied. The impact of a varying environmental conditions such as light (Ralph and Burchett 1995), temperature and osmotic potential (Kerr and Strother 1985) can then be studied and results assessed. Additionally, chemical treatments such as heavy metal stress (Ralph and Burchett 1998; Prange and Dennison 2000) have also been shown to negatively impact on seagrass survival under laboratory conditions.

Each method of study has its associated advantages and disadvantages. In-situ studies allow results obtained to be applied directly to the studied environment under the prevailing environmental conditions. However, such results may apply only to a specific environment and the specific conditions within that environment. Transient

'contamination events' or transient 'stress factors' may not be observed during the experimental phase of such studies. Additionally, the interaction or synergy of many smaller stress factors may have an observable impact on seagrass that is not obvious in its cause and effect. While geosurveys permit the mapping of large areas it is difficult to interpret individual cause and effect relationships from such data. A diffuse contamination event causing a fundamental change in seagrass biochemistry may manifest itself as seagrass necrosis some time after the contamination event.

Satellite imagery may overlook such cause and effect relationships. Laboratory studies permit the investigator to manipulate, observe and measure the effects of one or more stress factors on seagrass cultures. However, results obtained may apply only to the specific laboratory conditions that may not occur in the natural environment. The results of one study method therefore provide only 'part of the picture'. Results obtained from different study methods augment or supplement one another to provide a perspective view of events that cause seagrass loss.

The specific nutritional requirements of *Zostera muelleri* are not described in detail in the literature. Therefore, agar culture methods used in laboratory cultures of seagrasses (Ducker, Food *et al.* 1977; Bird, Johnson *et al.* 1998) would prove difficult to achieve within the time frame of this study. Additionally, study of any possible synergistic interaction between treatment and environment may be compromised in such a constructed culture system. This is relevant in light of the known toxicity of 'sediment' to seagrass noted earlier. It was therefore decided to use sediment from the same location as the collected seagrass in all culture methods as detailed in the 'Materials and Methods' section.

3.7.1 Study of Seagrass and Impact of Herbicides

Soils were collected from intensively farmed regions of the York Peninsula and analysed for commonly used herbicides. A dry sieving process was used to assess their potential to form fine particulate material or 'dust'. Dust obtained was analysed for commonly used herbicides. Bioassays were then conducted using *Zostera muelleri* to assess the biological impact(s) of identified herbicides. These data were analysed to provide a measure of environmental impact caused by the transport and deposition of surface applied chemicals, used in intensively farmed regions, upon biota of the intertidal zone.

3.7.2 What is a Bioassay, What should a Zostera muelleri Bioassay Measure and How should it be Performed?

A bioassay is a test designed to assess whether a treatment has an effect on a living organism'. There are two broad types of bioassay. In the first type, native species are subjected to a treatment under controlled conditions and results obtained are extrapolated to the same species in its 'natural' environment. In the second type, a readily available species is selected that is known to be sensitive to the treatment under controlled laboratory conditions. Each approach has its merits, the first provides information on environmental pollution at a specific locality for an native species. However extrapolating the results to other localities and other species is problematic.

The second type permits bioassays to be performed at many locations. Additionally, uniform results are assured (all other factors being constant) since only one species is used in the bioassay. However, extrapolating results obtained to a given locality and assessing its relevance to native species at that locality is difficult since environmental conditions and species sensitivity to environmental pollutants are often variable.

The present study will use a native seagrass species, *Zostera muelleri*, in the bioassay. This intertidal species is confined within a restricted coastal environment both locally and globally. Additionally, it is known to grow in anoxic sediments both locally and globally. It is therefore likely that bioassay results obtained will be relevant to the local, regional and global coastal environments. The literature suggests that light, or more specifically photosynthetically active radiation (PAR), is important for seagrass survival. Indeed seagrass growth has been monitored under both field and laboratory conditions.

In a light attenuation field study by Dennison and Alberte (1982) the authors noted an increase in the total chlorophyll content in the controls between the shallow (1.8mg chlorophyll dm^{-2}) and deep (2.2 mg chlorophyll dm^{-2}) stations in the seagrass *Zostera marina* L. Interestingly, the chlorophyll a/b ratio is greater in the shallow (2.0) than the deep station controls (1.8) with the deep station value increasing (to 2.0) on 'reflector treatment'. It is thought that this is a response to an increase in PAR caused by the reflector treatment (Kirk and Tilney-Bassett 1978; Abal 1996; Lee and Dunton 1997). Additionally, leaf turnover time (in days) was greater while leaf production rates (measured as g(dry) m^{-2} day^{-1}, dm^2 m^{-2} day^{-1}, and cm^2 $shoot^{-1}$ day^{-1}) were less in the deep station controls when compared to the shallow station. Leaf turnover time was increased further while leaf production rates were decreased further than the deep station controls by shade treatment. The photosynthetic rate, measured with a Clarke electrode, was greater at the shallow site (0.84 µmole oxygen/dm^2/min) than at the deep site (0.71 µmole oxygen/dm^2/min).

It was proposed by the authors that these physiological and biochemical responses are adaptations to a limitation in PAR. Indeed, survival of seagrasses at depth is directly related to the availability of light and the efficiency of the light-capturing process (Dennison 1987; Kensworthy and Haunert 1991; Zimmerman, Reguzzoni et al. 1991). Ralph et al. (1998) also demonstrated a difference between the photochemical and non-photochemical quenching abilities of shallow (*Posidonia australia*) and deep-water seagrasses (*Thalassodendron pachyrhizum*, *Posidonia angustifolia*) by measuring chlorophyll a fluorescence. It is suggested that seagrasses in shallow water receiving greater amounts of PAR have a higher capacity for non-photochemical quenching and light protection than do the deep-water species. Natural events such as increases in water turbidity caused by freshwater inflows (Longstaff and Dennison 1999) and cyclonic seas (Preen, Lee Long et al. 1995) have also been implicated in seagrass losses.

In a laboratory study by Kerr and Strother (1985) it was found that *Zostera muelleri* increased its 'apparent' photosynthesis as the PAR was increased from 17 to 185 µE m^{-2} s^{-1}. Interestingly, in a separate study by Ralph and Burchett (1995), 'high irradiance stress' was used to impose photo-inhibition on the seagrass *Halophila*

ovalis (R.Br.) and was quantified by chlorophyll-a fluorescence and oxygen measurements.

The 'timing of biological events' can be determined by an interaction of the prevailing environmental conditions with organism's stage of development. This phenomenon has been described by Brouns (1985) as the 'plastochrone interval'. In a study of seagrass productivity the author discusses key biological events, such as time of fruiting that the experimenter can measure under control and test conditions in seagrasses. Indeed, Kerr and Strother (1989) used the same concept to study leaf growth rates in *Zostera muelleri* finding that leaf turnover can occur in as little as 7 days in summer and 29 days in winter months. This senescence rate was mirrored by a higher leaf length growth rate in summer (7-8 mm per day per shoot) than in the winter months (2-3 mm per day per shoot).

It was decided to quantify carbohydrate (acid-soluble) and photosynthetic oxygen produced, as a measure of photosynthetic efficiency, in a comparison between test and control tissue samples. It was also decided to present the results per chlorophyll (mg) content and per unit leaf area (dm^2) as 'independent' methods. A *Z. muelleri* bioassay was designed to monitor directly the products of photosynthesis, carbohydrate and oxygen, and relate these data to the functional components of photosynthesis such as total chlorophyll and leaf area.

It was expected that measurements of leaf area, chlorophyll, oxygen and acid-soluble carbohydrate *on the same leaf sample* would likely reduce the inherent variability associated with results obtained from bioassay designs. The second-youngest leaf within a leaf sheath of *Zostera muelleri* will be selected as biological material to be used in a bioassay this procedure is anticipated to reduce the variability associated with the plastochrone interval discussed in the previous section. It is likely that this will reduce the known variability in chlorophyll contents that occurs between leaves within the same plant and even along the length of a single leaf. Leaf tips will be left intact when leaves are sectioned from the leaf tips and towards the leaf base. Newly synthesised oxygen and carbohydrate will be quantified and results expressed per unit mass of chlorophyll and per unit leaf area. Sand et al. (1982) has studied the release of oxygen from seagrass species, such as *Zostera marina* L., into the surrounding sediment and overlying seawater.

However, the interrelationship between photosynthesis, carbohydrate production and herbicidal impact in *Zostera muelleri* is less well researched. Other submerged aquatic vegetation, such as the freshwater aquatic macrophyte *Elodea* produce visible gas bubbles on its leaves when the plant is provided with a source of 'sunlight'. It is anticipated that *Zostera muelleri* leaf sections will also produce a volume of photosynthetic gas when exposed to adequate lighting. Indeed, it is generally known that oxygen is produced as a by-product of photosynthesis. Therefore, it was thought that measurements of gas volumes produced by illuminated leaf sections would provide an indication of photosynthetic efficiency. It was further reasoned that measurements of carbohydrate content, chlorophyll content and leaf area within the same leaf section would then allow gas volume measurements to be expressed relative to photosynthetic production. It was anticipated that any perturbation in gas volume production rates caused by treatment would be

measurable as a difference, between 'test' and 'control' samples, in carbohydrate produced.

3.7.3 Pulse-Chase Versus Chronic Exposure Events

A fundamental premise of this thesis is the notion that 'dust', from arable land, can act as a transport mechanism for land-use chemicals to the intertidal zone. The presented literature evidence together with the known frequency, ten to seventeen per annum, of dust storms in South Australia suggests that any exposure of the intertidal seagrass *Zostera muelleri* would occur as a transient event rather than a chronic exposure in waters that have a frequent 'turnover'.

However, it should also be noted that the warm summer waters of the upper Spencer Gulf South Australia are poorly 'turned over', and may be subjected to a low but chronic exposure to 'dust-transported' herbicide when these chemicals are used and transported from broad-acre farming regions. Indeed, the known losses and transport of herbicides such as 2,4-D from farmland discussed previously may well produce 'background' levels of contamination and environmental pollution. It is proposed that bioassays will be constructed as a pulse-chase design on the premise that environmental contamination occurs as a transient event.

4.0 Need

Four of the eight fishing industry sectors have identified pollution as one of the priorities for improvement. Although the pollution types and sources are not further defined, the impacts from persistent organic pollutants are becoming more common worldwide. Toxic effects arising from exposure to chemical pollutants are frequently reported. In addition, contamination by these chemicals can lead to discrimination and/or rejection of the product in the marketplace.

The need was identified for a properly funded study that examined the quality of inshore seawater in a defined area and from which links could be established between cause and effect. For the reasons set out below, the study undertaken was seen as Stage 1 of a multi-stage process which would enable the Fishing Industry to understand where it stands currently as far as water pollution by organic chemicals is concerned and the impacts these chemicals might have on specific ecosystem components.

Need for Stage 1 Study

There is increasing evidence from other States in Australia, and worldwide, that persistent herbicides arising from terrestrial activities are impacting on the growth and productivity of inshore seagrass beds. The toxicity of agricultural chemicals, principally insecticides, has been demonstrated on fish species that are indigenous to the Northern Hemisphere but no study has looked at the toxicity of persistent agricultural chemicals to species found in Australia. And, more importantly, the toxicity of widely used persistent agricultural chemicals to species of commercial importance in South Australia has not been studied.

Recruitment of juveniles from the inshore nursery areas where persistent agricultural chemicals are most likely to be found could be significantly compromised. Modern pesticides, intended for terrestrial use, are toxic at extremely low concentrations.

The levels of persistent herbicides found in marine environments elsewhere in the world are significant, and similar levels would be expected to occur in Australian inshore waters given the extensive use of herbicides in Australian agriculture. The toxicity of persistent herbicides to inter-tidal seagrass species has not been studied in Australia.

A study linking the concentration of a key persistent organic insecticide in the soil, its concentration in the adjacent marine environment and its toxicity to a key marine indicator species such as the prawn, represents a good model for the study of the impact of a non-point source pollutant over a relatively small area.

The contribution by wind-blown topsoil from adjacent farm areas, which can act as a carrier of considerable quantities of adsorbed persistent organic pesticides has not been examined in South Australia. The role of dust-storm events in the transport of toxic chemicals elsewhere in the world is well recognised. The concentration in seawater of persistent organic pollutants such as insecticides has not been determined on a seasonal basis. Dose-response data for a major persistent insecticide and a key indicator marine species such as prawns, combined with

knowledge of the concentration of the pesticide in seawater, will provide a scientific basis for proposing modification of land management practices.

The demonstration of significant levels of persistent pesticides in fine farm topsoil and identification of those pesticides in seawater, combined with demonstrated toxicity effects on a key marine species of commercial significance, would provide further support for proposing changes in land-care strategies designed to mitigate these inputs.

Stage 2

The focus of Stage 2 would be to examine the impact of bio-available inorganic chemicals and, separately, increased levels of nutrients on specified key ecosystem components. A subsequent stage could examine the impact of identified industrial chemicals on appropriate indicator species. The aim would be to appreciate the sensitivity of (South) Australian marine ecosystems to pollution from a variety of sources and the impact on market share.

5.0 Objectives

5.1 The identity and concentration of insecticides in water samples taken seasonally from inshore nursery areas in Spencer Gulf, and also downstream from point sources such as creeks, will be determined throughout the duration of the project. This objective was modified to focus on inshore areas above and below Port Broughton following advice from the Extension Specialist as a result of stakeholder meetings. A further modification was to sample principally from in-shore areas that appeared likely to receive dust and/or capture run-off from adjacent agricultural properties.

5.2 The levels of adsorbed residual insecticides and herbicides adsorbed onto the <200 mesh fraction of top-soil, sampled from selected farms adjacent to recognised marine nursery areas, will be determined.

5.3 To develop a GIS database identifying the land units adjoining Spencer Gulf likely to contribute wind borne soil to Gulf waters, together with point sources such as creeks and other discharge points that have the potential to contribute pollutants into recognised nursery habitats. The extreme sensitivity of this objective was quickly recognised and as a result it was decided that collection of soil samples would be done in a manner that did not identify the property owner nor give rise to concerns as to responsibility for any persistent pesticides. Consequently development of a database suitable for use in a GIS was not progressed.

5.4 The toxic and sub-lethal effects on juvenile prawns of the common persistent insecticides used in broad-scale agriculture and in local government pest-control programs will be determined. Other juveniles, such as blue crabs and an indicator scale-fish, may be tested if time and availability of test organisms allows.

5.5 The toxic and sublethal effects of a major persistent herbicide used in broad-scale agriculture on *Zostera muelleri*, a common inter-tidal seagrass species, will be determined.

5.6 Having determined what if any organic pesticide pollutants are in seawater and in conjunction with the toxicology data and residue levels found in soil, to then establish the possible mechanisms for their appearance in seawater and to recommend ways of mitigating the impacts. The objective here is to prepare and implement an extension program, which communicates effectively the results and recommendations of the project to local coast-care groups, local government and appropriate agricultural industry groups and other stakeholders.

6.0 Methods and Materials

6.1 Recovery of Pesticides from Seawater

It did not prove possible to arrange for the collection and transport of seawater samples following rainstorm events leading to discharge from creeks as was originally envisaged due to unavailability of personnel to carry out the sampling on an *ad hoc* basis. As a consequence, duplicate seawater samples were collected in 1 litre brown bottles at 8 sites located from north of Fisherman's Bay to south of Port Broughton, adjacent to Kanyaka Plain, at the mid-point of each season for one year. The location of the sampling points, largely centered on the Port Broughton area, is shown in Figure 7.1. The seawater was transported to the laboratory in portable freezers and stored at 1 ^{0}C until analyzed.

Seawater samples with a pH 8.1 (no pH adjustment was made) were filtered through a Gelman Funnel with a 0.45 µm cellulose nitrate filter. A 500 mL sample of the filtered seawater was then processed through a C_{18} Sep-Pak which had been previously been activated using 5.0 mL of pesticide grade acetonitrile followed by 5.0 mL of Milli-Q water as specified by the manufacturer at a rate of *ca.* 3 mL/minute. After processing the seawater through the activated Sep-Pak, it was then 'washed' (Milli-Q water, 2.5 mL) and the absorbed analytes eluted using acetonitrile (7.5 mL). The eluent was then concentrated and the analytes re-suspended in acetonitrile (100 µL).

The efficiency of pesticide recovery was established by using clean seawater spiked with a standard reference herbicide mixture. Seawater (500 mL from SARDI laboratories at West Beach) was spiked with a pesticide mixture containing trifluralin, simazine, atrazine, diuron, diclofop methyl, chlorsulfuron (technical), metsulfuron (technical) and triasulfuron at ca. 500 ng of each component in acetonitrile (100 µL). This loading is equivalent to 1000 ng per litre (1 µg/L, 1 ppb). The spiked seawater was sonicated (21 ^{0}C, room temperature for 5 minutes) to ensure complete dissolution of the components. Anthracene, at 8 ng/µL, was used as an external standard by injecting a constant volume of 1 µL in triplicate before and after each seawater analysis.

This procedure provides a concentration factor of 5000 fold (50 mL/100 µL). Trifluralin, simazine and atrazine (and possibly diclofop methyl) were recovered at greater than 95% by this procedure as indicated in the chromatograms A1 (SIM mode) and A2 (SCAN mode). All analytes were identified by using the ChemStation software and the NBS75K.L library or authentic materials where available.

6.2 Soil Sample Collection, Preparation and Storage

The collection of some 100 soil samples was coordinated and facilitated with the Agricultural Bureaus on Yorke Peninsula. Appropriate sampling instructions were distributed with the sample containers. Land-holders collected soil samples from land that they considered as "good cropping" and "poor cropping" in accordance with these directions. Surface soil samples (30 cm x 30 cm x 30 cm) were taken with a spade and placed in self-sealing polyethylene bags within labeled metal cans (4L

capacity) that were in turn sealed. Samples were stored in a cool darkened area until collection, transportation and analysis.

All soil samples were dried to a constant weight at 55^{O}C in an oven and then placed into a clean self-sealing polyethylene bag within the original metal can.

Whole soils (50 g) were 'doped', at two loading levels, with a mixture of herbicides that are used in broad acre farming. Two spiking levels, a low QC (quality control, of 10 ppb (µg/kg)) level and high QC (quality control, of 70 ppb (µg/kg)) standard loading was used to assess the efficiency of the solvent extraction procedure from whole soil matrices. Anthracene was used as an internal standard at a loading of 640 ng/100mL of extracting solvent as described in the next section

Instrumental and bioassay techniques for soil analyses
Non-biological (whole soil, dust, intertidal sediment, and seawater) and biological samples (*Zostera muelleri*) were examined by using instrumental and bioassay techniques respectively. Instrumental analytical techniques are divided into physical-soil-separating techniques (dry-sieving), chromatography and spectrophotometric methods while bioassays focussed on the biological impact of herbicides on photosynthesis in the seagrass *Zostera muelleri.*

6.2.1 Soil pH Measurements

Soil pH was measured according to the method published by the Soil Science Department of the University of Adelaide. Briefly, soil (50g or 100g) was placed in a glass beaker and stirred by using a magnetic stirrer (50g sample) or a glass stirring rod (100g sample) for 15 minutes at room temperature (21 °C to 25 °C). The mixture was allowed to settle for fifteen minutes and a pH reading was taken using a PHM62 Standard pH meter, Radiometer, Copenhagen. The pH meter and electrode were calibrated (pH 4.0 and pH 7.0) before and after each sample reading.

6.2.2 Size-Fractionation and the Physical Stability of Dried Soils

Dried whole soils were subjected to a sieving procedure that fractionated soil particles into five size ranges: fraction 1 (> 2 mm), 2 (1 to 2 mm), 3 (0.5 to 1 mm), 4 (0.25 to 0.5 mm) and 5 (< 0.25 mm). This was carried out as triplicate replicates, by placing one hundred grams of dried whole soil into the uppermost sieve (for particles> 2 mm) of a 'sieve stack'. The sieve stack was comprised of four working sieves (2 mm to 0.25 mm size apertures). Sandwiching a double layer of aluminium foil between the lowermost sieve and a capping sieve sealed the lower surface of the lower sieve. This permitted the capturing of particles less than 0.25 mm in size. Additionally, a capping sieve also sealed the upper surface of the uppermost sieve in the same manner. This custom-built apparatus permitted two soil samples to be sieved in triplicate. An elastic strap was used to hold the six stacks together while they were agitated at a maximum setting for fifteen minutes. Sieve fractions were weighed and then placed into self-sealing plastic bags. Fraction 5 (< 0.25 mm) was later tested for its pesticide content.

6.2.3 *Gas Chromatography with Mass Spectral Detection (GC-MS)*

'Persistent' herbicides, such as atrazine, simazine and dichlofop-methyl, were obtained in-house while less persistent herbicides such as sulfonylurea herbicides were supplied by courtesy of the appropriate manufacturers. Authentic standards of the methyl esters of the phenoxy-alkanoic acids were purchased from SIGMA. Additionally, simple esters (from C1 to C18) of 2,4-D were also prepared by the method of Sanchez et al. (1991).

While the method of Sanchez proved successful for the synthesis of shorter chain ester derivatives (up to C16) of 2,4-D it was less effective in preparing the longer chained aliphatic analogues. Therefore, longer-chained simple esters were prepared by heating 2,4-D free acid with excess aliphatic alcohol to drive water from the reaction mixture as an azeotrope, resulting in the formation of the simple ester.

$$ROH + 2,4\text{-}D \rightarrow 2,4\text{-}D\text{-ester} + H_2O\uparrow$$

All pesticide ester standards were positively identified using the ChemStation software provided with the Hewlett-Packard 6890 GC-MS analytical system.

All pesticide residue analyses were performed on a Hewlett Packard HP 6890 Series GC System fitted with a HP-5MS silicone column (30 m x 0.25 mm x 0.25µm) and a Hewlett Packard HP 6890 Series Mass Selective Detector. The mass spectrometer was either operated in *scan mode* (from 35 to 350 amu) or *selective ion monitoring* mode (SIM) at a data collection rate of 0.5 amu/sec. The mass spectrometer was routinely tuned to *maximum sensitivity* according to the manufacturers specifications prior to each analysis.

Ions used in SIM mode were either selected from those obtained by chromatography of authentic standards that were run under scan mode or from those listed in the ChemStation software libraries. High and low concentration quality control sample mixtures were co-analysed before, during and after sample analyses. Any drift in instrument sensitivity was therefore monitored throughout this analytical procedure.

Solvent and reactant controls (blanks) were included in all analytical procedures. Electron impact, at 70 eV and 220°C, was used to ionize analytes eluting from the column. Helium was used as the carrier gas at a flow rate of 1mL/min.

The oven temperature was programmed as a three-ramp system. Following sample injection the initial oven temperature was maintained at 50 °C for three minutes and then programmed to 210 °C at a rate of 20 °C per minute. The rate of temperature increase was then immediately adjusted to 5 °C per minute to attain a temperature of 260 °C. A final ramp of 30 °C per minute increased the oven temperature to 300 °C which was held for thirteen minutes before cooling and commencement of the next cycle.

Samples were either injected manually or by auto injector (Hewlett Packard HP 6890 Series Injector) into an injection port held at 250 °C and operated in splitless mode.

A low (0.01 ppm) and a high concentration (1.0 ppm) quality control (QC) standard mixture of pesticides were used to monitor the efficiency of the gas chromatography system. This allows monitoring for any 'drift' in retention time or change in the response factors of each pesticide under study.

6.2.4 Recovery of Herbicide Standards from 'Whole' Soils

Whole soils were "spiked" at two levels with a mixture of herbicides that were identified in the paddock history reports by the respective farmers as being used in their broad acre farming practices. A low QC (quality control, ca. 10 µg/kg) level and high QC (quality control, ca. 70 µg/kg) standard loading was used to assess the efficiency of the solvent extraction procedure from whole soil matrices.

6.2.5 Gas Chromatographic Analysis of Whole Soils

Whole soil was dried at X^0C for Y days to a moisture content of Z% (table). A whole soil sample (50 g) was then placed in a glass volumetric flask (250 mL capacity) and an internal standard was then added (anthracene, 640 ng as an 80 µL aliquot, made up in acetonitrile at 8ng/µL). Acetonitrile (100 mL) was then added and the flask was capped, placed in an oscillating extraction device for X hrs at room temperature, following which the contents were allowed to settle for two hours. Two x 24 mL aliquots of the supernatant were then transferred to glass scintillation vials (24 mL capacity) and centrifuged (2000 rpm) at room temperature for ten minutes). A total volume of 40 mL acetonitrile extract was then transferred to a pear-shaped flask and the solvent was removed by evaporation and the flask allowed to cool to room temperature. The residue was re-suspended in acetonitrile (0.5 mL) and a sample (1.0 µL) was injected on-column.

6.2.6 Gas Chromatographic Analysis of Dust

Dust (50 g) was placed in a glass volumetric flask (250 mL capacity) and anthracene internal standard was then added. Residues were then extracted by the method previously described for whole soils.

6.2.7 Confirmatory Analyses Using Gas Chromatography with Electron Capture Detection (GC-ECD)

Gas chromatography analysis, with electron capture detection, was also performed on a Perkin-Elmer GC fitted with a HP-5MS silicone column (30 m x 0.25 mm x 0.25 µm) and an electron capture detector (ECD). Temperature programme conditions were identical to those listed in the GCMS analytical procedure. However in this case, the sample was dried (UHP nitrogen at room temperature) and re-suspended to the same concentration in hexane (500 µL). This method was used to detect electrophilic atoms, such as fluorine, chlorine and bromine that might be present in solvent extracts of whole soils.

6.2.8 *Analysis of Acid and Base Hydrolysates by Gas Chromatography*

Analytes, in a random selection of gas chromatography samples, are subjected to a sequential acid then alkaline hydrolytic treatment prior to gas chromatography analysis of concentrated (10 X) samples. This hydrolytic procedure aids in characterising chemicals as being labile or refractory.

The GC sample was first dried (UHP nitrogen at room temperature), resuspended in acetonitrile (200 µL) and transferred to a GC vial glass insert (250 µL capacity). The sample within the GC glass insert was then re-dried and 50% sulphuric acid and hexane (50 µL) was added and the contents mixed by using a syringe (250 µL capacity fitted with a teflon plunger, SGE). An emulsion formed which separated into an acid layer and a hexane layer when the insert was gently tapped onto the bench top over a fifteen-minute period. An aliquot (2 µL) of the hexane layer was then injected onto the GC for analysis.

The hexane extract of the acid hydrolysis procedure was transferred to a second glass insert by using successive washes (3 x 100 µL) of n-hexane. The extract was then dried (UHP nitrogen, room temperature) and aqueous sodium hydroxide (0.5 M, 50 µL) and n-hexane (50 µL) were then added. The contents were then mixed by using a syringe (250 µL capacity fitted with a teflon plunger, SGE) and allowed to stand (20 minutes at room temperature) with occasional tapping of the insert onto the bench top to facilitate phase separation. An aliquot (2 µL) of the hexane layer was then injected onto the GC for analysis.

6.2.9 *Isolation of 2,4-D from Amicide® 500*

Amicide 500, the dimethylamine salt of 2,4-D and is one formulation of this phenoxy-alkanoic acid herbicide used in farming for the control of broad-leaf weeds and is used as a pre-emergent application. It is supplied at 500 g of active ingredient (2,4-D) per litre in a mixture of surfactants and spreading agents. The active ingredient was extracted from acidified Amicide 500 into an organic solvent.

Briefly, Amicide 500 (40 mL) was added to a separating funnel containing Milli-Q water (400 mL) and acidified (to pH 1.0) with concentrated hydrochloric acid (16 mL). This mixture was then extracted into diethyl ether (5 X 200 mL) and the combined ethereal extracts taken to dryness. The white crystalline solid was dried over silica gel in a desiccator for 24 hrs. This solid was positively identified as 2,4-D by its melting point (141 °C +/- 0.5 °C) and mass-spectral pattern. Isolated 2,4-D acid was subsequently used in the synthesis of 2,4-D derivatives.

6.2.10 *Synthesis of a Homologous Series of 2,4-D Esters*

Simple esters of 2,4-D were synthesized by the method of Sanchez et al. (2000). Briefly, the free acid of 2,4-D (25 mg) was added to a GC vial and an aliphatic alcohol (800 µL (or 800 mg for solids) from C1 (methanol) to C_{18} (oleoyl)) was then added. Acetyl chloride (200 µL, analytical grade) was then *carefully* added and the vial was capped with a teflon-coated rubber seal.

The mixture was then heated to 100 °C (sand bath), cooled to room temperature and acetate buffer (0.1 mM, pH 4.6, 1.0 mL) was then added. The contents of the vial were then transferred to a separating funnel containing Milli-Q water (400 mL) and the mixture extracted with diethyl ether (3 x 200 mL).

The combined ethereal extracts were taken to dryness at 35 °C and the residues transferred to a volumetric flask (25 mL capacity) with several 'washes' of toluene (7 x 2 mL). The contents of the flask were then adjusted to 25 mL with toluene. Synthesized esters were identified by their mass spectral patterns and were also compared to the reactant materials (2,4-D free acid and the aliphatic alcohols).

6.2.11 Synthesis of L-Amino Acid Conjugates of 2,4-D

Synthesis of 2,4-D-aspartate and 2,4-D-glutamate was achieved by using the method of Wood and Fontaine (1952). The 2,4-dichlorophenoxy-acetyl chloride (2,4-D-Cl) required for this synthesis was prepared by the method of Freed (1946).

a) Preparation of the acid chloride of 2,4-D

A modification of the method of Freed (1946) was used to prepare the acid chloride of 2,4-D. Freed notes an unusual feature of 2,4D-Cl is its reluctance to react with water, alcohol or amines at lower temperatures, in sharp contrast to other acid chlorides. In the preparation the dried free acid of 2,4-D 15 g was placed in a 100 mL round-bottomed flask fitted with a reflux condenser and positioned in a fume hood. A dried cotton-wool plug was fitted into the top of the reflux assembly.

Thionyl chloride ($SOCl_2$, 75 mL redistilled) and anti-bumping granules were then added to the flask and the mixture was gently refluxed for one hour. A previous attempt at removing excess thionyl chloride by the method of Freed resulted in some pyrolysis of material within the flask. A Buchi rotary-evaporator was therefore *modified* and positioned in a fume hood.

The modification consisted of placing a water trap between the venturi system and the Buchi. The water trap was manufactured from a two-necked round-bottomed flask fitted with glass stoppers through which were placed glass tubing with the tube nearest (outlet) the Buchi being of a shorter length than the inlet glass tube. The water trap was mounted on a retort stand and positioned upright. The apparatus excess thionyl chloride was carefully removed on the *modified* Buchi system at a low temperature (50 °C) over a ten-minute period.

This procedure prevented pyrolysis of the 2,4-D acid-chloride product. In Freed's preparation the acid chloride was then distilled at 180 mm from a Claisen flask to produce a white crystalline product in the receiver. In this work, the un-recrystallised liquid 2,4-D acid chloride was used as a 'crude' product in the subsequent syntheses of 2,4-D-amino acid conjugates as detailed below. Partial synthesis of such analogues would allow their subsequent purification and use as standards in a chromatographic analysis of unidentified analytes within soil, dust and sediment samples.

b) *Preparation of L-amino acid conjugates of 2,4-D*

First, the appropriate amino acid (0.181 mole) was dissolved in a mixture of sodium hydroxide (1 M, 556 mL) and Milli-Q water (970 mL) and cooled to 5 °C on ice. The acid chloride of 2,4-D (0.181 mole) was then added drop wise over a three-hour period to the stirred (magnetic stirring apparatus) alkaline amino acid solution. The preparation was then wrapped in aluminium foil and left for two days in a cold room at 5 °C. A solid cream-coloured mass was present in the glutamate preparation while a white-coloured mass was present in the aspartate preparation.

In a preparation by Feung *et al.* (1971) the authors found that the *ether soluble* (Feung, Hamilton et al. 1973) amino acid conjugate of 2,4-D could be recrystallized from water to produce white crystals with a melting point of 178 °C to 179.5 °C. However, the prepared cream and white solids provided melting points within the range 133 °C to 136 °C while the melting point of the 2,4-D standard was 140 °C and consistent with the literature value.

A solvent mixture (mix A) was therefore designed to preferentially dissolve 2,4-D (free acid) and amino acids at acidic pH (diethyl ether (100 mL): petroleum spirit (300 mL): milli-Q water (100 mL): concentrated hydrochloric acid (2.5 mL); pH 1.0. The solids from the 2,4-D-glutamate and 2,4-D-aspartate preparations were washed (5 x with mix A) in separating funnels and the white solid interfacial material was retained. Milli-Q water (250 mL) was then added to each separating funnel and the contents extracted into diethyl ether (3 x 500 mL).

The combined ethereal extracts were taken to dryness at 60 °C on a Buchi. The dried (oven 55 °C three days then at room-temperature over silica in a desiccator overnight) white solids from the 2,4-D-glutamate and 2,4-D-aspartate preparations provided yields of 1.6 g and 0.8 g respectively. The solids from the 2,4-D-glutamate and 2,4-D-aspartate preparations have melting points of 179 +/- 0.5°C and 202 +/-1.0 °C respectively and are consistent with the literature. High performance thin layer chromatography and infrared analyses verified the identity of the amino acid conjugates of 2,4-D.

6.2.12 HPTLC Analyses of L-amino Acid Conjugates of 2,4-D

The solvent mixture used was butanol, acetic acid and water 30:20:10 v/v.

The 2,4-D-amino acid conjugates (2,4-D-glutamate and 2,4-D-aspartate) were prepared by the method of Wood and Fontaine (1952) and analysed by the method of Fueng (1973). Briefly, the free acid of 2,4-D was first converted into the acid chloride by the method of Freed (1946) and then reacted with an amino acid at pH 4.6.

6.3 Database for Land Units and Point Pollution Sources

Achieving this objective in the manner originally envisaged was not possible. It became clear that no land-owner would be willing to have their land-unit(s) identified in a database that could potentially be linked to point or non-point sources of pollution. Especially where any potential impact was likely to occur off-farm. Comments and advice from several land-care advisers made that position very strongly. They also indicated that for economic reasons, many land-holders were reverting to previous land-management practices that had been shown to adversely effect soil structure. The advisers also commented that many farmers were, also for economic reasons reverting to cheaper out-of-patent herbicides rather than using the more expensive modern pesticide chemicals.

In light of the concerns raised by a number of people, the locations of those soil samples for which reasonably accurate location data were provided were plotted using G.I.S. A resolution was used which showed the distribution of soil samples across the region, but did not allow specific identification of the farm or soil's source. For this reason, a database is not tabulated in the report.

Figure 7.3.1 shows the distribution of sample sites across Yorke Peninsula.

6.4. Toxicity of the insecticide chlorpyrifos to juvenile Western King Prawns *Penaeus latisulcatus* and juvenile King George Whiting *Sillaginodes punctata*

6.4.1 Collection of Organisms

6.4.1.1 Western King Prawns

Juvenile prawns of the species *Penaeus latisulcatus* were collected using a beam trawl towed behind a 20ft run-about boat at Port Pirie, about 250km north of Adelaide on Spencer Gulf. The 8th of May was chosen for trawling as it presented suitable tidal forecasts where high tides leave about one metre of water over the intertidal regions. Five-minute 'shots' were run parallel to the mangrove line and the resulting prawns were held in 80L drums with constant aeration and transported immediately to Adelaide.

This site and method were recommended as suitable for catching maximum numbers of juvenile prawns (N. Carrick pers. comm.). Upon arrival at the laboratory the prawns were sorted for size (20mm < x > 40mm) and placed in a 90 L holding tank, which was continuously aerated. One third of the water was renewed every two days to reduce the build up of excreta and toxic NH_3 and NH_4^+ species. The prawns were fed opened cockles (*Donax spp.*) daily until 24 hours before their use in experiments.

6.4.1.2 King George Whiting

Sillaginodes punctata were collected from the Port River Estuary near Adelaide throughout August. Samples were taken from various locations around the northern end of Torrens Island, with each site supporting extensive beds of seagrass adjacent to small sandy beaches. The post-larval fish were caught using a small beach seine net, (mouth diameter 5m, semicircular perimeter of 7m, a drop of 2m and mesh size of 1 mm with float and lead lines keeping the net upright) (Fowler & Short, 1997). The net was hauled by hand over 40m transects, closed and samples sorted and transferred to aerated holding tanks and transported to the Laboratory as soon as possible (A. Fowler, pers comm.). The transect length was later changed to 20m due to high mortality over longer trawl distances.

When required for experiments, additional numbers of post-larval KGW were obtained from an aquaculture facility at South Australian Research and Development Institute (SARDI) - Aquatic Sciences facility at West Beach.

6.4.2 Exposure Experiments

6.4.2.1 Physicochemical Parameters

All experiments were carried out in a temperature-controlled laboratory set at 18 ^{0}C, which varied no more than 1 ^{0}C. Water was collected from SARDI (West Beach) and the salinity was made constant for all test chambers at 33ppt (1.022 specific gravity). At all times the dissolved oxygen was above 60% saturation and the pH was always in the range of 7.0-8.0. These parameters are in accordance with guidelines for

enzyme bioassay protocols and toxicity testing (Cripe, 1994; Kumar & Chapman, 1998).

6.4.2.2 Acute Toxicity Tests

All solutions of chlorpyrifos were prepared by accurately weighing the solid pesticide (Dow AgroSciences, Dursban XP Technical (98% chlorpyrifos) which was then dissolved in pesticide grade acetone and capped. All solutions were kept in a refrigerator (at 40 °C) for no longer than one week to prevent loss due to degradation and volatilization. The acute toxicity tests were performed with either five or six different concentrations and a zero concentration control. All glassware was rinsed with acetone, and hexane before use in all biological and analytical procedures.

The acute lethality of chlorpyrifos to *P. latisulcatus* and *S. punctata* was determined by semi-static acute toxicity tests, by exposing individuals to a concentration of chlorpyrifos for 96 hours based on the procedure outlined by Adams (1995). The test solutions were renewed at 24-hour intervals to keep the pesticide concentration relatively constant and to reduce the potentially harmful affects of ammonia/ ammonium toxicity building up through excrement. The test chambers were not aerated due to the potential for additional loss of chlorpyrifos through volatilization.

6.4.2.2a Range Finding Test - *P. latisulcatus*

A range finding test was carried out to gauge the relative sensitivity of the organisms, which would allow the concentrations for the actual testing to be made with more accuracy and without killing excessive numbers of organisms (Walker *et al*, 1996).

The test organisms, all of similar size, were taken from the holding tank and placed randomly into the test chambers. Five animals were placed into each chamber and tests were carried out over a 96-hour period.

6.4.2.2b Acute Toxicity Tests- *P. latisulcatus*

The acute toxicity tests consisted of a control and five concentrations, each test consisted four replicates. Ten juvenile prawns were randomly placed in each chamber providing 40 animals per concentration and 240 animals (average wet weight = 0. 1665g ± 0. 0 191 SD) per test. Animals were exposed to 5 test concentrations of chlorpyrifos ranging from 0.05-3.0 µg/L and a control for a period of 96 hours. Each solution was renewed every 24 hours for the duration of the test. Dead organisms were removed as soon as they were detected.

6.4.2.3a Range Finding Test- *S. Punctata*

The test organisms, all of similar size, were taken from the holding tank and placed randomly into the test chambers. Three animals were placed into each chamber and tests were carried out over a 96-hour period using different known concentrations of chlorpyrifos. Each chamber was renewed every 24 hours to ensure the test concentration remained constant.

6.4.2.3b Acute Toxicity Tests- *S. punctata*

To determine the LC_{50} for King George Whiting, a control and six test concentrations of chlorpyrifos were used, with concentrations ranging from 1.56-50 µg/L as determined from the range finding tests. Three fish (average w/w 0.2349 g SD 0.1506 g) were randomly placed in each chamber providing 28 chambers (4 replicates per concentration + 4 control replicates). The solutions were renewed every 24 hours for a 96-hour period. Dead organisms were removed as soon as detected.

6.4.3 Acety1cholinesterase Inhibition

The activity of the enzyme acety1cholinesterase was measured by the method of Ellman *et al*, (1961). In this assay, AChE hydrolyses an artificial substrate acetylthiocholine to acetate and thiocholine. Activity of AChE is measured by an increase in 5-thio-2-nitrobenzoate, a yellow anion produced when thiocholine reacts with dinitrobenzoic acid (DTNB). This colour change, and hence enzyme activity, can then be measured spectrophotometrically.

Acetylthiocholine	(enzyme) →	Thiocholine + Acetate
Thiocholine + dithio-bisnitrobenzoate	→	yellow colour.

Figure 6.4.3. Reaction of Acetylcholinesterase with acetylthiocholine to assess Enzyme Activity. [Ellman et aL, 1961]

It has been noted that with this procedure it is possible that Butyry1cholinesterase (a known pseudocholinesterase) may contribute to enzyme activity measurements (Van der Wel & Welling, 1989). However it has also been recorded that this contribution is generally negligible (Van der Wel & Welling, 1989). The AChE activity was standardized to the amount of protein in the sample using the method of Lowry et al., (1951), using bovine serum albumin as a reference standard. This procedure gives acetylcholinesterase activity expressed as micromoles per minute per gram protein (µmol/min/g).

An AChE inhibition of greater than 10% has been proposed as being indicative of organophosphorus or carbamate exposure (Gibson, 1969).

6.4.3.1 Exposure

Sixty fish weighing an average 0.3868g ± 0.0253g were randomly allocated to 4 experimental chambers and 2 control chambers, with each chamber containing 27 litres of artificial seawater (33ppt), giving 15 fish per 27-litre aquarium. Fish were acclimated to the test chambers for three days before the addition of chlorpyrifos, which was at a concentration of 12.5 µg/L. The fish were fed *Artemia* during both acclimation and experimentation constituting 1% of the weight of the fish (Zinkl et al., 1987; Kumar & Chapman, 1998). The test solutions were renewed every 24 hours, as were the control aquariums.

6.4.3.1a Determination of AChE Inhibition

Samples of 8 fish were collected by taking 2 animals, at random, per test chamber, on each sampling occasion. Each organism was euthanased by immersion in liquid nitrogen. Each sample was stored at –20°C for no longer than 48 hours before analysis for AChE activity. For analysis, the brain of each fish was removed and combined so that 2 fish constituted one replicate. This was further prepared by homogenizing in a few drops of phosphate buffer (pH 8.0) using a manual glass homogenizer. Each sample was kept on ice until analysis for enzyme activity and protein analysis. The activity of acety1cholinesterase was measured using a Varian Superscan 3 spectrophotometer at 410 nm by combining acetylthiocholine iodide (20 µl), DTNB (100 µl), phosphate buffer (pH 8) (3mL) and 20 µl of homogenized tissue samples in a test tube as *per* Ellman *et al,* (1961). All samples were run in duplicate and results averaged.

6.4.3.1b Protein Measurement

To allow for differences in fish weights, size and protein concentrations the enzyme procedure was standardized to the amount of protein in each of the treatments. The analysis of metabolic products was carried out using a HP6890 GC-MS system.

Analyses of the chlorpyrifos solutions used, and concentrations in the aqueous systems, was carried out using a HP 5890 GC with electron capture detection. The HP5890 GC-ECD was fitted with a DB1 100% Dimethyl Polysiloxane 'General Purpose' column with nitrogen as the carrier gas at a flow rate of 60ml/min. The oven was programmed as follows: the initial temperature was set at 120 ^{0}C which was held for 1 minute, then increased at 35^{0}C/Min to 240^{0}C which then was slowed to a rate of 4.5^{0}C/min to 255^{0}C. This was then increased again at the rate of 45^{0}C/min until 300 ^{0}C where it was held for two minutes. Data was recorded and integrated using ChemStation software. Using these parameters the limit of reporting was 20 parts per billion.

The HP6890 GC-MS was set up with a 30m x 0.25jam BPX 5.5% Phenol (equiv.) Polysilphenylene siloxane column. Helium was used as a carrier gas at 10psi. The detector was a 5972 Mass Selective Detector where ionization was by electron impact at approximately 70 eV Volts. The detector was programmed to scan from 35 to 350 m/z.

6.4.3.2 Statistical Analysis

All mortality data were analyzed by the Trimmed Spearman-Karber method of (Hamilton et al, 1977) using a trim of 10% where possible. All ANOVA calculations were carried out using the TOXSTAT 3.3 computer package (Western Ecosystems, 1994). Tests for normality and homogeneity were performed through a Chi-squared test and Bartlett's test. Helena Oakey and Michelle Lorimer from the Biometrics Unit of the University of Adelaide carried out additional statistical analyses.

6.5 Seagrass Collection, Preparation and Culture Methods

6.5.1 Collection of Zostera muelleri

Seagrass was collected from the Barker Inlet, near St. Kilda South Australia, at latitude 34° 46' and longitude 138° 32' during the summer months. It was collected along a transect paralleling the shoreline and positioned half-way between the low tide water level and the upper reaches of the meadow adjacent to the mangrove line. Sampling sites were allocated every five metres along the transect. "Turf blocks" (0.1m by 0.1m by 0.03m) were taken with care so as not to disturb the roots of the seagrass.

The turf blocks were then washed free of sediment at the collection site by moving the turf back and forth through seawater. Sediment-free plants were then placed in aerated seawater collected at the site (20.0 L, aeration being supplied by a portable battery-operated aeration pump) and transported to the laboratory. Plants were stored overnight in an aerated seawater holding tank (500.0 L capacity polypropylene tank (1.8m x 1.4m x 1.6m) filled to 500.0 L) positioned 1.0 m above the laboratory floor on an angle-iron frame. Plants were subsequently freed of any remaining sediment, in the holding tank by using the same washing procedure, and allocated to a working tank for experimentation.

6.5.2 Establishing Laboratory Culture Methods for Zostera muelleri

The laboratory study of *Z. muelleri* culture was designed to investigate the possible effects of 'land-use herbicides' on biota of the intertidal zone. It was decided to observe and quantify any differences between test and control *Zostera* under defined, laboratory-controlled conditions approaching those that can occur in the natural environment. Changes to the sediment content by addition of clean coarse sand and/or herbicides would reflect the changes in toxic stress 'normally' imposed on plants in the intertidal zone. Since *Zostera* is known to propagate by vegetative means, free-floating plants (Buoyant Aquatic *Zostera* (BAZ) were proposed as a test system to remove any possible synergistic interaction between treatment and sediment in this growth system.

6.5.2.1 Equipment and seawater

Since large volumes of seawater were required for seagrass culture methods and for bioassay experimental designs, Filtered Seawater was collected from S.A.R.D.I. (West Beach, South Australia) in polypropylene 50.0 L bins. It was considered that this seawater had a similar chemical profile to that present in the collection area for seagrass samples. All seawater collected was premixed in a holding tank prior to use in any experiments to minimise any effects of batch-to-batch variations that may occur.

Four polypropylene tanks 1.8m x 1.4m x 1.6m were used to store and grow seagrass. Three 'working tanks' were filled to a depth of 1.5 m with seawater while the fourth tank, which served as a holding tank, was filled with 500 L of seawater. Each test tank was provided with artificial lighting and an aeration system.

6.5.2.2 *pH measurements*

pH was routinely measured and compared with standards by using a PHM62 Standard pH meter Radiometer, Copenhagen. Standards (pH 4.0 +/- 0.05 at 25 °C and pH 7.0 +/- 0.05 at 25 °C) were purchased from Labchem, Ajax Chemicals. The pH measurements provided baseline data that assisted in the development of seagrass culture methods. The measurement of pH helped define 'normal' conditions for seagrass growth under laboratory condition relative to seawater. Seawater typically has a pH of 8.1.

6.5.2.3 *Lighting*

Lighting was provided over all test-tanks at 77 cm above the water's surface but not over the holding tank. Initially lighting was provided by "Grolux" fluorescent tubes. However photometer (Li-COR, Li-170 Quantum Radiometer Photometer) readings indicated that approximately one hundred fold greater light intensity (photometer measurements) was available in full sunlight 2852 $\mu E\ m^{-2}\ s^{-1}$. Additionally, a preliminary culture of a seagrass turf in an aquarium positioned adjacent to a laboratory window performed better than the "Grolux" lamp.

It was therefore decided to increase the light intensity by mounting a sodium lamp (Sylvania 17, 400 W, Belgium SHP-TS-400W) equipped with a transformer over the centre of each working tank at a height of 90 cm above the water's surface. The PAR light flux was 250 $\mu E\ m^{-2}\ s^{-1}$ at the water's surface. This represents approximately one quarter of the PAR normally available to *Zostera* under summer conditions. A mechanical timer was used to control the lighting on a 12 hr on - 12 hr off regime (6 am to 6 pm summertime, South Australia).

6.5.2.4 *Temperature*

Laboratory and seawater temperature were maintained at 21 ^{0}C throughout the laboratory culture and experimental period.

6.5.2.5 *Aeration*

A mechanical timer was used to control the aeration on a 12 hr on – 12 hr off cycle (6 pm to 6 am summertime, South Australia). The rationale for the design of this aeration regime was that oxygenation of the water during daylight hours is likely provided by any photosynthetic activity within green leaf material and by physico-chemical entrapment of oxygen from air by wave action. However during night-time oxygenation is likely provided by entrapment of oxygen from air by wave action alone. The aeration system therefore acted as a substitute for wave action.

Aeration was provided by a pump equipped with 'feed pipe' (2 cm diameter) and many smaller silicone tributary pipes fitted with flow regulators, an aeration stone and connected to the feed pipe by using irrigation screw fittings commonly available from hardware stores. On-off taps were positioned on the tributary pipes to allow ancillary

experimentation. Air-flow was measured at each tank, as displaced seawater from an inverted measuring cylinder (1.0 L capacity) as 3 L min^{-1} in all tanks.

6.5.3 Preliminary Experimentation with Z. muelleri *and the Effects of Sediment Content*

Below sediment (root) and above sediment (shoot) tissues were sectioned and dried to provide an indication of tissue biomass and water contents. Plant material was washed briefly under Milli-Q water then tissue-dried. Sectioned plant material was wrapped in aluminium foil and dried (85°C) for four weeks.

Tests were carried out to determine whether *Zostera* could be grown, as 100% sediment-bound plants, in jars under laboratory conditions. Plants were placed in a 10 cm depth of 100% intertidal sediment in large jars. The lighting, temperature and aeration regime previously described was employed to maintain the plants. The results of preliminary experiments suggested that *Zostera* could not be cultured under these laboratory conditions.

6.5.4 Modified Laboratory Growth Conditions

Three sediment regimens, with increasing sediment loading, were developed.

6.5.4.1 Buoyant Aquatic Zostera *(BAZ) using no sediment*

Up to one hundred plants were 'grown', or incubated over a two to three week period, in test tanks as sediment-free plants under the lighting, temperature and aeration regimens described above. Plants were carefully 'turned over' by hand, at least twice per (photoperiod) day, to prevent self-shading. Seawater was replaced twice weekly and plant leaf litter was removed daily to minimise bacterial activity and any subsequent eutrophication. Plants remained at the bottom of the tank on commencement of the experiment. However, plants became 'free-floating', or buoyant, during the incubation period.

6.5.4.2 Preparations of Zostera muelleri *using 30% sediment*

Preliminary attempts to grow *Zostera* in 100% (undiluted) sediment in glass jars had been unsuccessful, and results obtained from studies of 'free-floating plants' (BAZ) were likely to be limited in any analysis of cause and effect in plants that normally grow in the 'solid' substrate of the intertidal zone. Plants growing in 30% sediment would be affected by toxic factor(s), such as sulphites (Erskine and Koch, 2000), nitrites (Iizumi et al., 1980), and heavy metals (Ralph and Burchett, 1998, Prange and Dennison, 2000) within sediment which impose restrictions on plant growth. Partial replacement of pore water in the sediment by laboratory-controlled 'tidal movement' was designed to allow the plants to grow successfully, yet still be subjected to the toxic stresses of the sediment.

Coarse sand, 40 kg, was placed in a 50 litre plastic drum fitted with a lid. Seawater, 40 litres, was then added and the lid fitted. The drum was then placed on its side and rolled several times and particulate material was allowed to settle for ten minutes. Seawater was then decanted from the drum by carefully removing the lid

whilst the open mouth of the drum that was held at an angle of approximately five degrees to the horizontal. This 'washing procedure' was repeated a total of seven times prior to use of the coarse sand.
Sediment (obtained from the same location as the seagrass) and washed coarse sand were then blended in a ratio of 30% to 70% by weight as a 57 kg batch within a horizontally placed 50 litre plastic drum by using a narrow-bladed spade. Washed jars were then filled to a 10 cm depth (the root zone would be 20 cm) with this mixture. Four zostera plants were then carefully planted in the sediment of each jar.

Seawater was then carefully added to the jars by siphoning, and the jars were then provided with artificial lighting (250 $\mu E\ m^{-2}\ s^{-1}$) during the 'daylight hours' and aeration during the 'night-time hours.' Brackish water and any leaf debris was then removed from each jar by a careful siphoning and replacement regime each morning for a period of five days. The water was clear and free of suspended material after five days treatment. Jars were then left in the artificial lighting and aeration system previously described. Plants that did not survive this transplanting procedure were replaced with new plants and then left to acclimate for a period of no less than six weeks.

6.5.4.3 Preparations of Zostera muelleri *in baskets using 100% sediment*

Zostera grows in 100% sediment in the 'natural' environment of the intertidal zone, despite the toxicity of the sediment. Drainage of the sediment and percolation of fresh water through the sediment during tidal flows may be essential for survival of *Zostera*. Attempts to grow *Zostera* in undiluted sediment in glass jars failed, possibly due to lack of adequate drainage of pore water from sediment. It was therefore decided to construct *porous vessels* in which seagrass could be grown in native (100%) sediment that could be periodically purged of any 'toxic factors' that may be present within interstitial water within the sediment.

Baskets were modified by removing the metal handles and trimming the longer and upper edges of the basket with a coping saw. Baskets were lined with two rectangular pieces of shade cloth of a sufficient size to fully cover the inside surfaces of the baskets in a 'north-south' then 'east-west' direction. Shade-cloth on the inside corners of each basket was sewn together with fishing line. Safety glasses were worn during sediment handling procedures to reduce the possibility of eye injury. An 'intact' turf of seagrass (sediment plus seagrass) was transferred, in one motion into the basket by using one hand positioned on the upper surface (seagrass of the turf and then quickly inverting the container. This procedure reduced sediment fragmenting during the handling and transfer process.

Four baskets were randomly allocated to each working tank and seawater was filled to10 cm above the upper edge of the baskets and this served as laboratory 'high-tide.' Laboratory 'low tide', lasting 45 minutes, was achieved initially four times each week, by *slowly* lifting the baskets and then positioning each on stands (inverted empty baskets) within the working tanks. This procedure permitted vertical movement of seawater relative to sediment within seagrass turfs, in an attempt to mimic the tidal flow in the natural environment of the intertidal zone and would replace a portion of interstitial pore water with fresh seawater.

The seawater was replaced with fresh seawater three times per week for a period of three weeks at first, but subsequently tank seawater was replaced once per week with 'low-tides' remaining at four per week. Plant debris was removed daily by using a hand-held net and sediment debris was removed from the bottom of the tank by using a wide-bore siphon. This cleanup procedure reduced the likelihood of onset of eutrophic processes within the aquaculture system. The 'basket preparations' were subjected to the lighting (250 $\mu Em^{-2} s^{-1}$) and aeration (12hrs; 6:00 pm to 6:00 am) regimes previously described.

This turf growth system, while labour-intensive, provided a microcosm resembling the 'natural growth conditions' of *Zostera muelleri*. Macrofauna such as crabs, starfish and snails were removed from this growth system within the first two weeks of culture to reduce the biological oxygen demand within the tank. Tube-worms remained as an integral part of the sediment mass, which also reduced the likelihood of subjecting the seagrass to an anoxic environment. The occasional crab was detected weeks after the initial set-up. These qualitative observations suggest that conditions within the tank approached those within the natural environment. After an initial phase of leaf loss, resembling that which occurred in the jar preparations of *Zostera* above, seagrass plants had similar morphology to 'wild-type' seagrass. Extended periods of laboratory culture resulted in some narrowing of the leaves (Dennison, 1987).

6.5 Bioassays

Measurements of gas volumes produced by illuminated leaf sections can provide an indication of photosynthetic efficiency. Analysis of carbohydrate and chlorophyll content, and measurement of leaf area *within the same leaf section,* would then allow gas volume measurements to be expressed relative to photosynthetic production. The newly synthesised photosynthetic gas and carbohydrate can be quantified and results expressed per unit mass of chlorophyll and per unit leaf area.

It is anticipated that any changes in gas volume production rates caused by treatment will be measurable as differences between 'test' and 'control' samples, in relation to the amount of carbohydrate produced.

The second-youngest leaves within leaf-sheaths of *Z. muelleri* were selected as biological material to be used in bioassays. This procedure is likely to reduce variability by allowing the selection of plant material within a plastochrone interval (Brouns, 1985), and reduce the known variability in chlorophyll content that can occur between leaves within the same plant and even along the length of a single leaf. Leaf tips were left intact when leaves were sectioned from the stem of the plant.

6.5.1 Pulse-Chase Versus Chronic Exposure Events

Arising from the background material presented in Section 3, it is proposed that dust storms can produce transient exposure of *Z. muelleri* in South Australia to herbicides transported from broad-acre farming, in addition to chronic exposure from contaminated sediments. Therefore bioassays were constructed in a pulse-chase design.

Bioassay experiments were therefore designed to test the 'maximum' photosynthetic response of leaf sections under artificial lighting and defined laboratory conditions. Toxic factors that are known to affect seagrass survival and growth in its natural environment, as discussed in previously, were either removed or were maintained under laboratory control. It was anticipated that bioassay measurements of gas volumes (mm^3), leaf area (dm^2), chlorophyll ('a' and 'b' as μg), acid-soluble carbohydrate (measured as 'glucose equivalents' in μg), and gas identities (mass spectrometry) would provide indices with which to monitor the photosynthetic processes.

Measurements of individual leaf sections (measuring between 5 and 7 cm in length), obtained as the second-youngest leaves within a leaf sheath, would reduce any potential errors that may occur when comparing 'control' and 'treatment' groups. The variables measured in the bioassays were therefore gas volume, leaf area and chlorophyll in buoyant aquatic *Zostera* analyses (BAZA) and gas volume, leaf area, chlorophyll and acid-soluble carbohydrate in sediment-bound *Zostera* analyses (30% sediment and 100% sediment).

6.5.2 *Bioassay 1 - Photosynthesis by Zostera leaves in free-floating culture*

6.5.2.1 *Gas volume measurements, manometer design and usage in free-floating Zostera cultures*

The photosynthetic by-product oxygen is poorly soluble in seawater. It is therefore likely that any gases produced by leaf sections of *Zostera muelleri* while placed under artificial lighting will contain oxygen, and measured gas volumes would provide an indication of the photosynthetic capacity of *Z. muelleri* leaf sections. A manometer was designed from long-nosed glass Pasteur pipettes by sealing the tip of the pipette using a propane flame. A piece (ca. 4 cm) of transparent adhesive tape (cellotape) was then folded along the length of the 'thin end' of the manometer to provide a surface on which to etch gas volumes produced by using a on-sided razor blade.
Measured gas volumes were taken from the etch marks on completion of the experiment. The manometer was initially filled with seawater by holding it at its largest end and by topping it up by using a Pasteur pipette. It was possible to move seawater to the tip of the manometer by flicking the liquid to its tip in one motion, as one would do to move mercury to the bulb of a thermometer.

Leaf sections were positioned with their oblique cut ends closest to the tip of the manometer. Preliminary experiments indicated that it was possible to place a leaf section within an inverted manometer and then place excess seawater, as a convex meniscus, within the manometer at its largest end. The manometer could be inverted *without the entrapment of ambient air* by placing a wet index finger over the open end, inverting, and transferring the apparatus with its enclosed leaf section into a reservoir of seawater. Manometers were held in place by using a clamping system. The surface tension of seawater prevented water drainage from the manometer's tip. Leaf sections were buoyed in seawater and travelled towards the manometer's thin tip but were restricted by the narrowing of diameter at the inflexion surface of the manometer.

Each vertically positioned manometer was rotated one-third of a turn around its long vertical axis every 30 minutes to provide 'equal lighting' to both surfaces of the leaf section. Gas bubbles produced during experiments could be coalesced and 'coaxed' to the tip of the manometer by inserting a piece of nylon fishing line positioned past the leaf section and to the manometer's tip; care was taken not to introduce gas bubbles into the manometer during this procedure. Gas volumes were etched at the meniscus onto the cellotaped surface by using a one-sided razor blade.

On completing the experiments leaf sections were removed and temporarily stored, at 5 ^{o}C, prior to leaf area measurements. Manometers were sequentially washed with Milli-Q water (7x), methanol (7x) and acetone (7x), dried at room temperature and weighed. Manometers were then filled to the etch mark with Milli-Q water (at 25 ^{0}C) and then re-weighed. The difference in mass between the two measurements allowed a calculation of gas volume (in μL) to be made.

6.5.2.2 Identification of photosynthetic gases during the photo-period

Gas volumes produced during the photoperiod were first quantified, by etching the manometer, and then diluted with 500 μL of ultra high purity nitrogen (UHP N_2) by using a modified gas-tight syringe (1000 μL capacity fitted with a teflon plunger, SGE). The modified syringe was made by constructing a 1.0 m flexible extension to the needle (25 G) from a piece (15 cm) of large gauge DB-5 column (0.53 mm I.D.) and a piece (90 cm) of small gauge DB-5 column (0.22 mm I.D.). Parts of the extension were glued together with epoxy resin. An aliquot (250 μL) of the diluted gas was then transferred to a second inverted vessel (manometer minus cellotape) filled with seawater for subsequent gas analyses. Diluted gases were stored temporarily in darkness in seawater containing sodium cyanide (1 mg/L) to prevent microbial growth.

Instrument grade air was used to calibrate the GC-MS instrument that was operated in SIM mode. The selected ions were m/z = 32 (oxygen) and m/z = 44 (carbon dioxide). A gas-tight syringe (250 μL capacity fitted with a teflon plunger, SGE) was used to manually inject 250 μL of calibration gas onto the GC-MS system and integrated peak areas were then used to calculate response factors and quantitate oxygen and carbon dioxide in standard gas samples.

A modified syringe (250 μL capacity fitted with a teflon plunger, SGE) was also constructed from a 250 μL capacity gas tight syringe (SGE). An aliquot (250 μL) of the diluted gas was then transferred from the gas storage vessel onto the GC-MS system by inserting the small gauge DB-5 column into the GC septum, withdrawing some carrier (helium) gas and then fully depressing the plunger. Care was taken not to break the DB-5 'injection column'. In all gas transfer procedures syringes were purged of air by repeatedly flushing the syringes (7x) with UHP N_2.

6.5.2.3 Leaf area measurements

Primary production occurs in leaf tissue. The amount of sunlight energy captured by leaf tissue can determine the amount of carbohydrate manufactured in the

photosynthetic process. Seagrasses can adapt their leaf morphology to accommodate the prevailing light conditions(Dennison, 1987, Kensworthy and Haunert, 1991, Zimmerman et al., 1991). Therefore leaf area measurements are relevant to any differences in carbohydrate synthesis between 'treatment' and 'test' groups. Seagrass leaf areas have been measured using scanning devices and simple measurement of leaf area calculated by multiplying leaf length by leaf width at half-length.

Though *Zostera* has narrow leaves, they have planar morphology and a photocopy method was developed to calculate leaf area measurements. Leaves from the manometer study were 'dried' briefly with tissue paper, placed onto a scanning device (Hewlett Packard ScanJet 5200C) and photocopied. Leaves were then immediately stored in acetone (10 mL) at – 20 °C for chlorophyll analysis. Scanned images were magnified (four times) together with 'standard rectangular blackened areas' onto a second sheet of paper. Areas were then cut from the sheets and weighed. Leaf areas were calculated from a standard graph of area vs. weight of paper.

6.5.2.4 Chlorophyll Measurements

The chlorophyll content of chloroplasts within leaf tissue contributes to the efficiency of primary production in leaf tissue. All other factors being constant, the chlorophyll content of leaves will determine, at least in part in part, the amount of carbohydrate manufactured in the photosynthetic process.

The bioassay described above detects the volume of gases produced by leaf sections under standard conditions in a comparison between test and controls. The effect of treatments such as with 2,4-D Amicide on gas volume production is then assessed between test and controls.

6.5.2.5 Measurement of Chlorophyll in Zostera *leaves*

Chlorophyll was measured spectrophotometrically by the method of Dennison (1990). Seagrass leaves were first homogenized in 80% acetone and 20 % Milli-Q water containing magnesium carbonate (10 µM) by using a glass homogenizer. The homogenate was immediately centrifuged (2000 g, room temperature for 15 minutes) and absorbance readings taken at 663 nm, 645 nm and 725 nm. Chlorophyll 'a', 'b' and total chlorophyll concentrations were calculated using equations derived by Arnon (1949).

Chl-a (µg/mL) = $12.7 \times A_{663} - 2.69 \times A_{645}$
Chl-b (µg/mL) = $22.9 \times A_{645} - 4.68 \times A_{663}$
Chl-a + b (µg/mL) = $8.02 \times A_{663} + 20.2 \times A_{645}$
Dennison (1990).

Absorbance readings at 663 nm for chlorophyll a were compared to standard absorbances for chlorophyll a in the extraction solvent. Samples were stored at 5 °C in a refrigerator after chlorophyll analyses until they were analyzed for their acid-soluble carbohydrate contents.

6.5.2.6 *Acid-soluble carbohydrate measurements*

The carbohydrates D-Glucose and starch are manufactured by green leaf tissue in the photosynthetic process. These energy storage molecules are later used in cell maintenance, repair and somatic tissue growth processes. Additionally, microcosms of obligate aerobic bacteria in the anoxic sediment of the intertidal zone also use manufactured glucose. *Zostera* may supply glucose and oxygen to the obligate aerobes to facilitate the localised acidic solubilisation of phosphorus from hydroxyapatite within sediment. Therefore measurements of acid-soluble carbohydrate may provide an indication of the efficiency of the photosynthetic process and the ability of *Zostera muelleri* to metabolize phosphate from hydroxyapatite within 'control' and 'treatment' groups.

6.5.2.7 *Measurement of acid-soluble carbohydrate*

Measurement of acid-soluble carbohydrates, as 'glucose equivalents', was achieved by using a modification of the method of Dawes and Kensworthy (1990). Briefly, a washed (1x with Pyroneg detergent then 10 x with Milli-Q water) anti-bumping granule was added to each of the stored samples from chlorophyll analyses. Samples were then placed in a sand bath and the solvent driven off, until approximately 0.2 mL of the original liquid remained, in a spark-proof fume hood at 100 °C and then cooled to room temperature. Acid-soluble carbohydrate was then dissolved from the dried residue by using warm trichloroacetic acid (5%, 10 mL). Phenol (10 mL, 5 %) and subsequently concentrated sulphuric acid (1 mL,) was *carefully* added to an aliquot (0.2 mL) of the acid-soluble carbohydrate. The exothermic reaction was then cooled to room temperature and the absorbance read at 490 nm. A standard curve of glucose concentration versus absorbance allowed the calculation of acid-soluble carbohydrate in leaf sections to be expressed as 'glucose equivalents'.

6.6.1. Root Hair Growth Evaluation

6.6.1.1 *A sulfonylurea bioassay*

Preliminary experiments were also conducted on BAZ by using the sulfonylurea herbicide chlorsulfuron. Briefly, chlorsulfuron was dissolved in seawater at 15 mg/L and plants were chronically exposed for two weeks with aeration and adequate lighting. It was noted, in a qualitative assessment, that new root hair growth was markedly inhibited in the test group. A second preliminary experiment was therefore conducted in which plants were randomly selected and all rhizomes were removed except one positioned at the distal end of the woody root growth. These 'newly synthesised' rhizomes had no root hair growth. Plants were placed in transparent plastic containers (440 mL capacity, Sarstedt®) and subjected to either a chlorsulfuron or an atrazine treatment, as a negative control, insult under chronic exposure conditions (15 mg/L).

However, the high loading used of this new class of 'sulfonyl urea' herbicide could not explain the known losses of seagrass that have occurred since the 1930's in the natural environment. Additionally the difficulty in quantitating root hair growth suggested that an alternate bioassay should be sought. It was therefore decided to

focus the research on measurements of photosynthesis and the widely used 'safe' herbicide 2,4-D.

6.6.2 *Bioassay 1: Pulse-chase Design: 5ppm 2,4-D (as Amicide), Effects of Herbicides on* Zostera *in Free-floating Culture*

Zostera muelleri were first laboratory acclimated as free-floating plants for two weeks and then subjected to an overnight 2,4-D exposure. Gas volumes, leaf areas and chlorophyll contents of leaf sections were then compared between the test group and the control group.

2,4-D is supplied and used in many formulations. Amine salts of 2,4-D are relatively inexpensive and are still widely used in intensive farming practices. It is known that 2,4-D is lost from land at the rate of 5% of the applied rate of 1-2 kg active ingredient per hectare (Larney Francis et al., 1999). It was therefore decided to test a commercially available 2,4-D preparation rather than 2,4-D free acid in the following bioassays, since results obtained are likely to be relevant to possible ecological impacts in the field.

6.6.2.1 Loading of seawater with 2,4-D

Amicide 500 (50 μL) was added to a volumetric flask (5.0 L capacity) and made to the mark with fresh seawater to provide a solution of 5 mg/L (5ppm) 2,4-D as active ingredient. The flask was capped, inverted several times and the modified seawater used in bioassay experiments.
Free-floating plants were randomly allocated to test and control groups with a minimum of 12 plants in each group. Each plant was allocated to a clean jar and either fresh seawater (control group) or seawater doped with 2,4-D at 5 ppm acid equivalents (test group) was then added. Both sets of plants were placed in the same (lighting) environment as test tanks and were left overnight in darkness. This treatment is equivalent to a nine-hour exposure period. Plants were removed from their jars and placed in clean jars containing clean seawater. Each plant was then rinsed seven times with fresh seawater. It is assumed that this procedure removed any residual 2,4-D following the overnight soak, since it is known that 75% of the dimethylamine salt of 2,4-D can be lost from test plots in a 'first run-off event' in surface transport field studies (Ma et al., 1999).

Using the concept of the 'plastochrone' interval (Brouns, 1985; Kerr and Strother, 1989), leaf material sectioned from the second-youngest leaf within leaf sheaths of *Zostera muelleri* was selected as the bioassay test specimens to help reduce biological variability. Any epiphyte loading on leaf sections was then removed by using a moistened cotton wool bud on samples placed on a seawater-dampened paper towel to help reduce any osmotic stress.

Cleaned leaf sections were then randomly allocated to manometer positions in air-saturated seawater under the same laboratory lighting used in the aquaculture of *Zostera muelleri*. This bioassay was conducted over a nine-hour period. Measurements of gas volumes, chlorophyll and acid-soluble carbohydrates were made for each *leaf section* to determine any differences between 'test' and 'control' treatments.

6.6.3 *Bioassay 2: Effect of Herbicides on Photosynthesis by Sediment-Bound* Zostera

6.6.3.1 *Pulse-chase experimental design: 5ppm 2,4-D (as Amicide) on* Zostera *in 30% sediment (jars)*

Zostera muelleri was laboratory acclimated for two weeks and then treated with an overnight exposure to 2,4-D doped seawater (5ppm acid equivalents) in a similar manner to BAZA above. Twelve jars were selected randomly, and distributed evenly between control and test groups. The steeped plants were then carefully washed free of any residual solution after the overnight treatment with seven rinses of fresh seawater. Interstitial water, within the sediment, was also removed during this washing procedure by using a venturi system as described previously. Fresh seawater (2.0 L) was carefully added to each jar to minimize sediment disturbance. Jars were allocated randomly to a position under the same laboratory lighting and aeration regime used in the aquaculture of *Zostera muelleri.* Leaf sections were periodically removed from plants, cleaned of epiphytes and randomly allocated to a manometer and lighting position prior to gas production analyses. Each bioassay was conducted over a nine hour period for the 14 day duration of the experiment. Analysis of leaf area, chlorophyll and acid-soluble carbohydrates was as described earlier.

6.6.3.1 *Pulse-chase experimental design: 5ppm 2,4-D (as Amicide) on* Zostera *in 100% sediment (baskets).*

Eight basket preparations of *Zostera muelleri* were selected at random and evenly distributed between control and test groups. Interstitial water was removed from the sediment of each basket preparation by placing them on inverted baskets as previously described in 'Modified laboratory growth conditions'. Each basket was then placed in an aquarium containing either fresh seawater (control) or 2,4-D loaded (5ppm acid equivalents) seawater (test) for a period of nine hours. Following the overnight soak, basket preparations were washed free of any residual solution with seven rinses with fresh seawater. Interstitial water, within the sediment, was also removed during this washing procedure by placing aquaculture baskets on empty and inverted baskets as previously described in 'Modified laboratory growth conditions'.

Two test and two control basket preparations were then positioned randomly in a working tank containing fresh seawater and provided with the same laboratory lighting and aeration regime used in the aquaculture of *Zostera muelleri.* This procedure was repeated in a second working tank and six leaf sections were periodically removed from test and control plants at predetermined time intervals. Leaf sections were cleaned of epiphytes, as described previously, and randomly allocated to a manometer position for gas production analyses. Each bioassay was conducted over a nine hour period for the duration (14 day) of the experiment. Again gas volume measurements were correlated with leaf area, chlorophyll and acid-soluble carbohydrate measurements for *each* leaf section.

6.6.3.2 *Bioassay 3: a chronic exposure study using 1ppm 2,4-D (as Amicide) of Zostera in 30% sediment (jars)*

Zostera muelleri which had been laboratory acclimated for two weeks was treated with a chronic exposure to 2,4-D doped seawater (1ppm acid equivalents) for a period of four weeks. Six jars were selected at random and distributed evenly between control and test groups. Jars were then allocated randomly to a position under the same laboratory lighting and aeration regime used in the aquaculture of *Zostera muelleri*. Leaf sections were periodically removed from plants, cleaned of epiphytes and randomly allocated to a manometer and lighting position prior to gas production analyses. Each bioassay was conducted over a nine-hour period for the duration of the experiment. Gas volume measurements were again correlated with leaf area, chlorophyll and acid-soluble carbohydrate measurements for *each* leaf section.

7.0 Results and Discussion

7.1 Seawater Analysis Using Sep-Pak C18 Cartridges

The seasonal duplicate seawater samples were collected, at the mid-point of each season, for one year, from some 8 sampling points centered roughly upon the Port Broughton area. Figure 7.1.1 shows the locations at which the samples were collected.

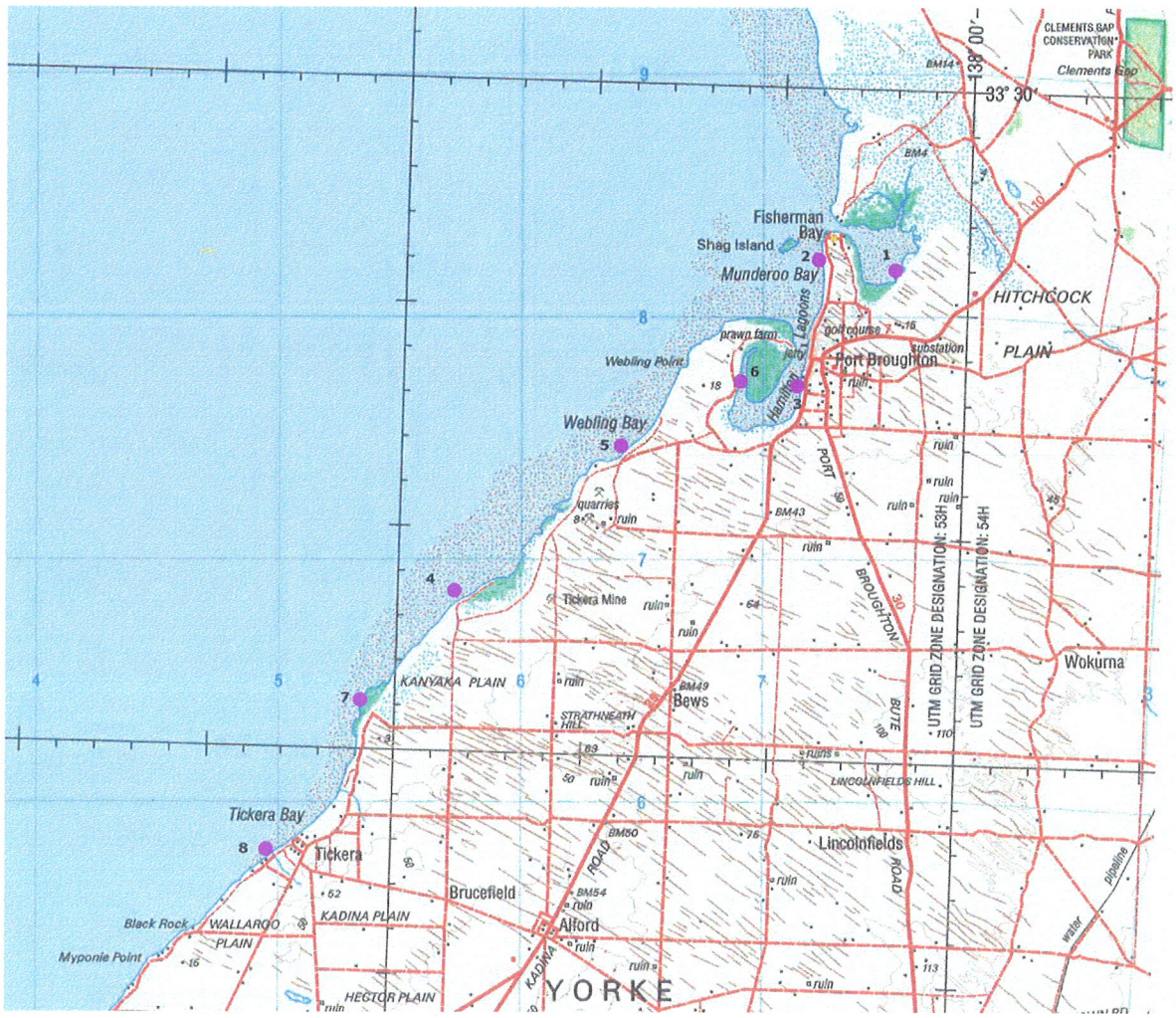

Figure 7.1.1. Location of seawater samples taken for pesticide residue monitoring.

Following GC-MS analysis, extremely low levels (less than 1 ppb) of pesticide residues were detected in all seawater samples as indicated in representative chromatograms 'B1' (SIM) and 'B2' (SCAN). Levels were therefore below detection limits for all samples taken. However, dibutyl-phthalate was detected and identified (96% confidence level) in several seawater samples.

A typical seawater GC-MS trace is shown in Figure 7.1.2 below for illustrative purposes (no axes shown).

Figure 7.1.2. A representative seawater GC-MS trace following SepPak® C18 extraction.

7.1.1 Scan versus SIM GC-MS analysis

It was found that SIM mode analyses provided 'cleaner' chromatograms where the signal-to-noise ratio was lower. A concurrent SCAN mode was performed immediately following all SIM analysis. This technique allows the positive identification (using ChemStation software and appropriate standards) of analytes that may not have appeared in the selective SIM analysis. The figures below show typical results obtained in both "SIM" mode, Figure 7.1.3, and scan mode Figure 7.1.4.

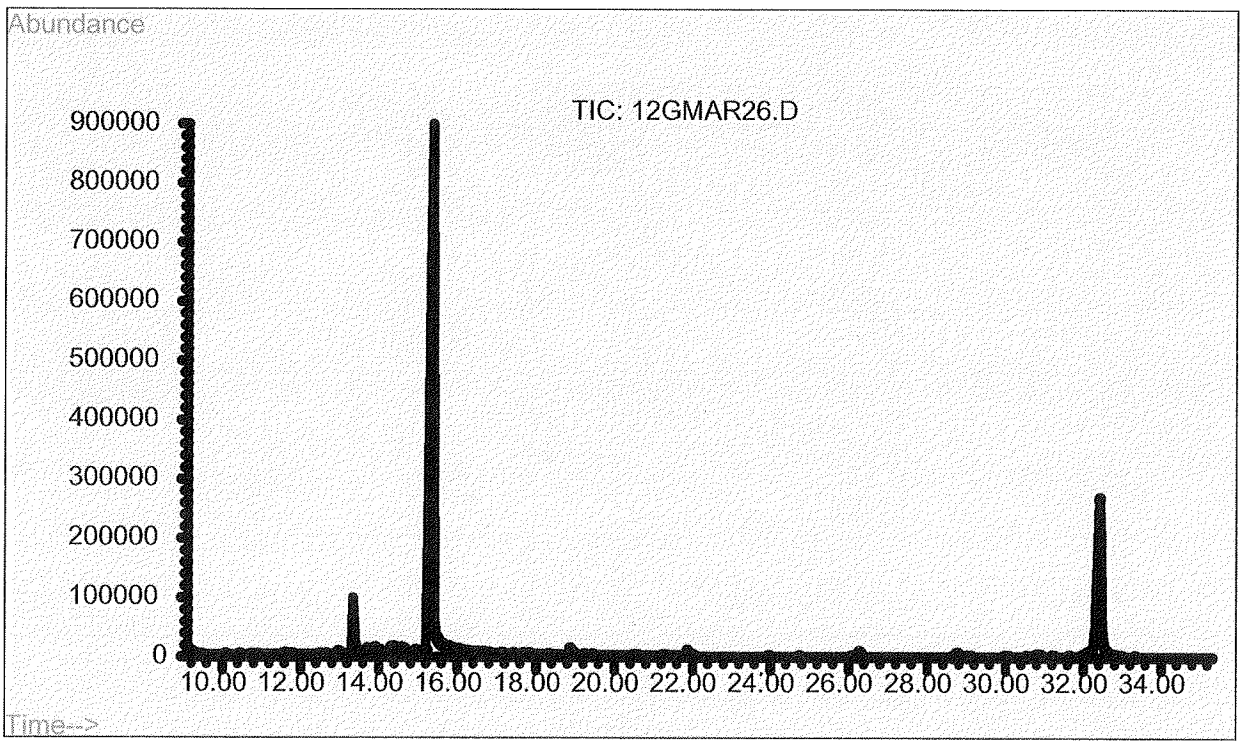

Figure 7.1.3. Seawater sample scanned in Sim mode: SIM B1

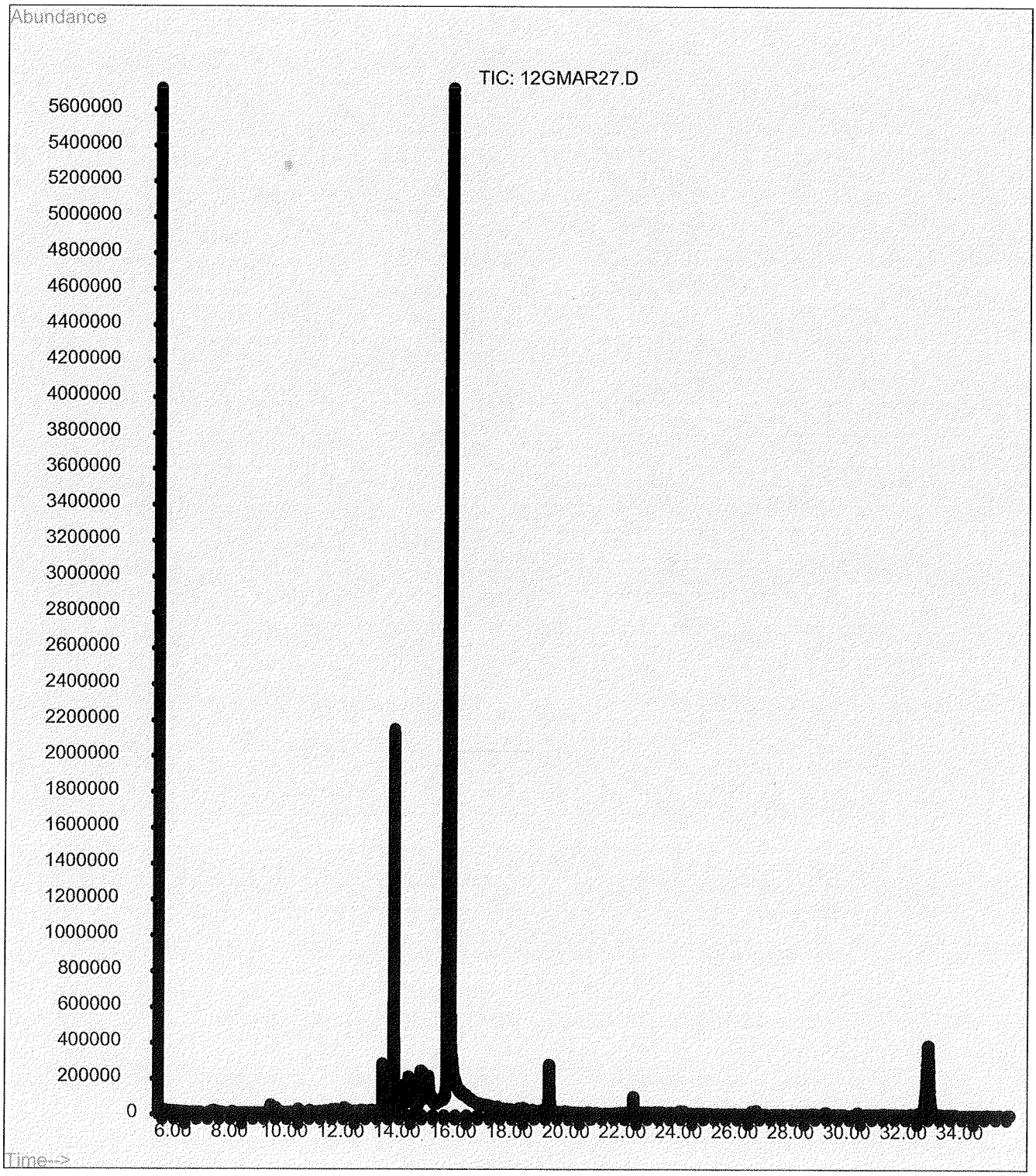

Figure 7.1.4. Seawater sample in scan mode: SCAN B2

No pesticide residues above detection limit were identified in any of the seawater samples over the course of one year. As a result, further sampling was discontinued. This finding does not exclude the possibility of *ad hoc* contamination occurring between sampling events, nor the possibility that more localized pollution effects could be occurring.

7.2 Sources and Levels of Residual Pesticides in Soil and Dust Fractions

Direct loss of pesticides from farm-land to seawater was not evident from the sampling and analysis of seawater during a one year period. Anecdotal evidence presented to us suggests that the loss of dust from farms and land-units is common and widespread. Dust has been clearly recognized as a vehicle by which residual and/or persistent organic chemicals, among other types, can be transported. Often this dust fraction travels large distances and delivers persistent organic pollutants to ecosystems far removed from the source.

Tillage practices in broad-acre farming under dry conditions may contribute to the formation of dust. This dust-forming process may be enhanced or impeded by the soil type present since larger particles of soil may reduce soil erosion. It is likely that farms that have a high potential to form dust will also have a high potential for 'off-site' transport of surface-applied herbicides such as 2,4-D, and soil-incorporated herbicides such as sulfonylureas and trifluralin (Larney Francis et al., 1999).

The "dust fraction" is generally defined as that material passing through a 200 mesh sieve. In order to assess the potential or actual dust fraction in the soil samples, all samples were sieved and the results shown in Table 7.2.1. The pH of the soils sampled ranged from 5.2 through to 8.7.

Farmed soils of the York Peninsula fall into three broad categories when they are sieved under a dry-sieving process. Some have a high potential to form 'dust' (<250 µm) while others have a medium and low potential. It is assumed that the tilling of dried soils will mimic, to some extent, the dry-sieving process that was conducted under laboratory conditions.

Some indication of the variation in the amount of potential "dust fraction' between soil types is shown in the figures below. Loamy soils represented by soil # 38 have relatively small amounts of fine dust (Figure 7.2.1). The grey loams had relatively more fine material comparison, although retaining a considerable proportion of larger aggregates (Figure 7.2.2), while the sandy loams in contrast gave a higher proportion of "dust-like" material (Figure 7.2.3).

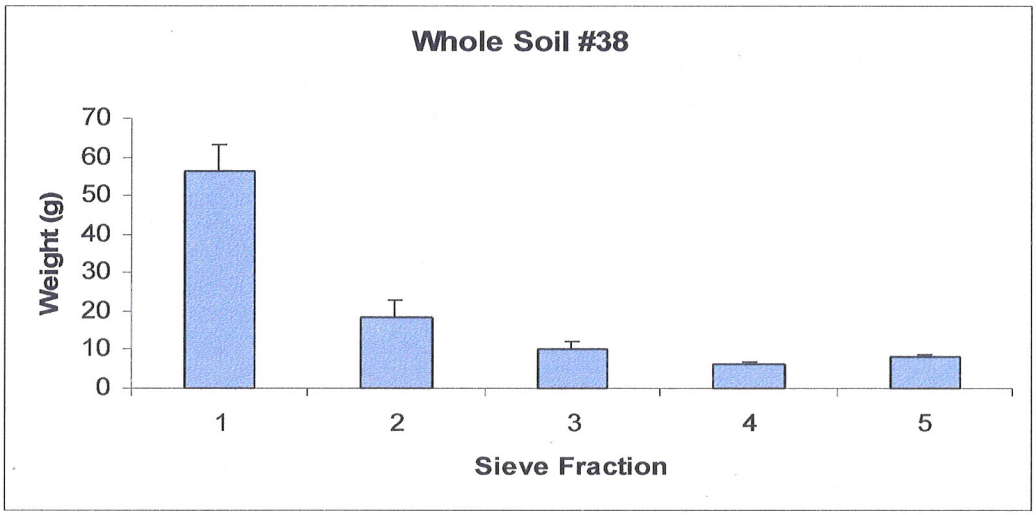

Figure 7.2.1. Sieving results for a red loam soil type

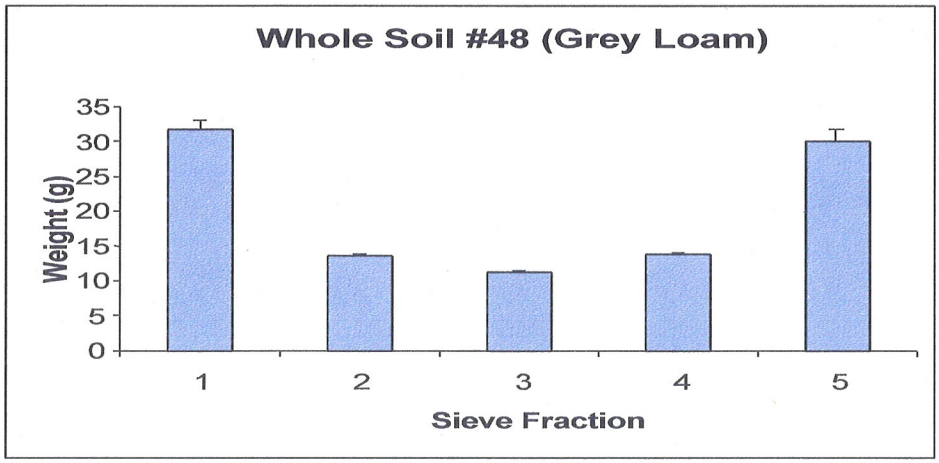

Figure 7.2.2. Sieving results for a grey loam soil type

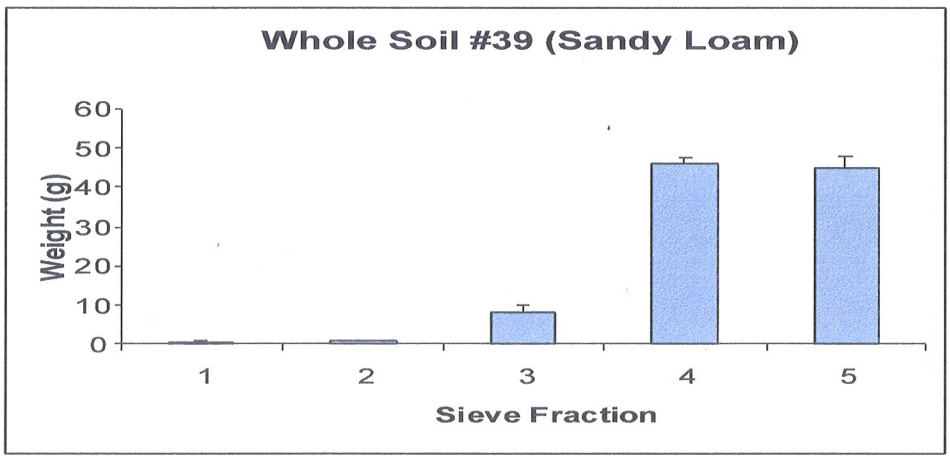

Figure 7.2.3. Sieving results for a sandy loam soil type

Little pesticide, less than 0.5 ppm, was detected in 'whole soil'. Trace amounts of trifluralin the active ingredient in Treflan™ were found in a small number of samples and occasionally a sulfonylurea (metsulfuron-methyl) was found. However, 2,4-D-like compounds were detected in whole-soil samples and dust *i.e.* the less than 250

micron particle size sieve fraction, in all samples by using SIM techniques. Support for these findings was obtained by analysing the same samples on a second gas chromatograph equipped with an electron capture detector. The 2,4-D like materials appeared at longer retention times. A typical trace obtained from a whole soil extract is shown in Figure 7.2.4.

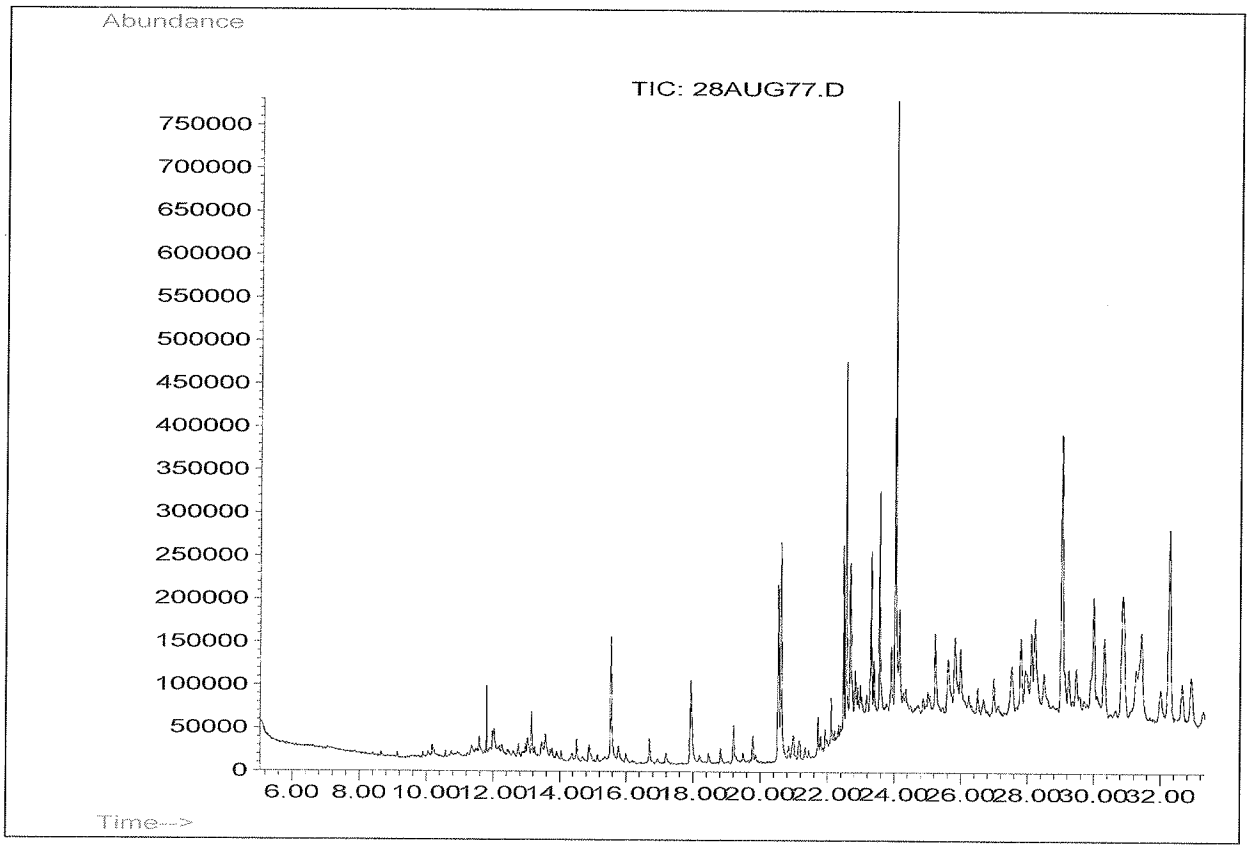

Figure 7.2.4. A typical trace obtained for a whole soil extract

While little parent herbicide was detected in soil samples, this result does not preclude the possibility of herbicidal chemicals being present within the samples in a "crypto" form. Indeed, the ester derivatives of quinclorac, an auxin herbicide (Grossmann, 1998), prove ineffective against leafy spurge plants when applied to the foliage (Rusness et al., 1998). However, the esters are converted to the parent material by a series of omega- and beta-oxidations carried out by soil micro-organisms. It is thought that this conversion is not merely a simple ester hydrolysis. Additionally, it is known (Feung C.-S., Hamilton R.H., Mumma R.O., 1973) that when plants metabolise 2,4-D, the herbicide is chemically compartmentalised into amino acid conjugates and oils (Chkanikov et al., 1984).

This is particularly interesting inasmuch as 2,4-D moieties can potentially be transported from land as 'stable' conjugates within organic debris. It is suggested that such material can then be converted to the parent material (the phenoxy herbicide or merely 2,4-dichlorophenol) in the intertidal zone where it will elicit a biological effect on intertidal seagrasses, such as *Zostera muelleri*, causing plant death in the short-term of weeks.

Confirmation of the presence of materials derived from 2,4-D precursors was obtained by SIM scanning of the peaks occurring at the longer retention times and recording those peaks giving rise to the ions derived from pure 2,4-D. The principal ions originating from 2,4-D had been identified using authentic material. These ions at m/Z 133, 145, 161 and 178 are associated with the presence of the 2,4 dichlorobenzene moiety.

Figure 7.2.5 shows that these ions are present in all peaks that had retention times greater than 22.0 minutes as shown in the trace in Figure 7.2.4 above.

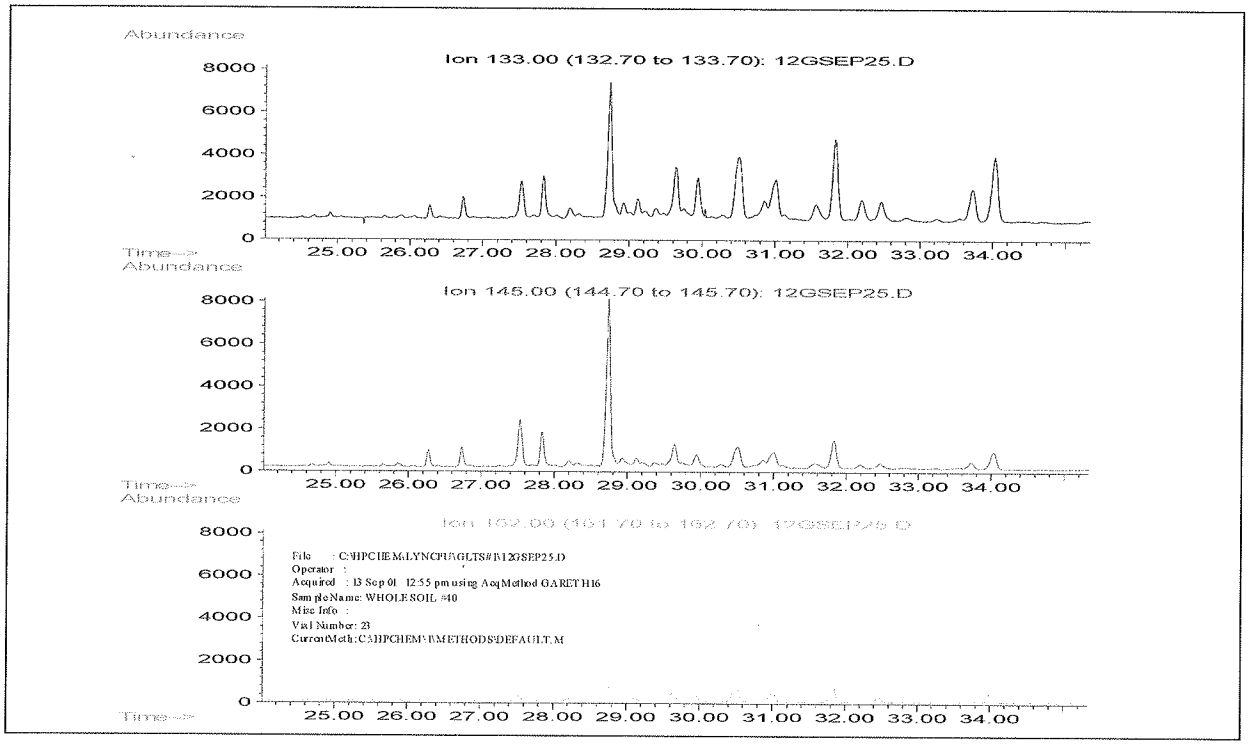

Figure 7.2.5. Simultaneous SIM scans for ions: 133, 145 and 161 of peaks appearing after 22.0 minutes.

Further analysis of the GC traces for these longer retained peaks showed that the expected chlorine 35:37 ratios were present indicating that chlorine was present in these ions.

Different soils displayed a variable range of peaks at 22.0 minutes or later as Figure 7.2.6 shows. This suggests that whatever the mechanism is that is giving rise to these peaks containing the 2,4-dichlorophenol moiety, different soils give a greater range of 2,4-D like homologues than in other soils. There was some evidence that the 2,4-dichlorophenol moiety is attached to a relatively long hydrocarbon fragment and that this would account for the increasing retention times for these materials. It was suggested that humic acid could be associated with these peaks however a separate GC-MS analysis of humic acid showed that it was not soluble in the extraction solvent used and also did not give rise to any of the ions found in the soil extracts.

It is possible that the extensive use of 2,4-D and related herbicides in previous years has led to a build-up of the degradation/breakdown products which may have been coupled to fatty alcohols (waxes) in the soil. Indeed, a Kovats-type plot of log retention time vs. carbon number was linear, as expected, giving strong support for the notion that the 2,4-dichlorophenol moiety derived from the 2,4-D and 2,4,5-T classes of herbicides was associated with a hydrocarbon component in the soil.

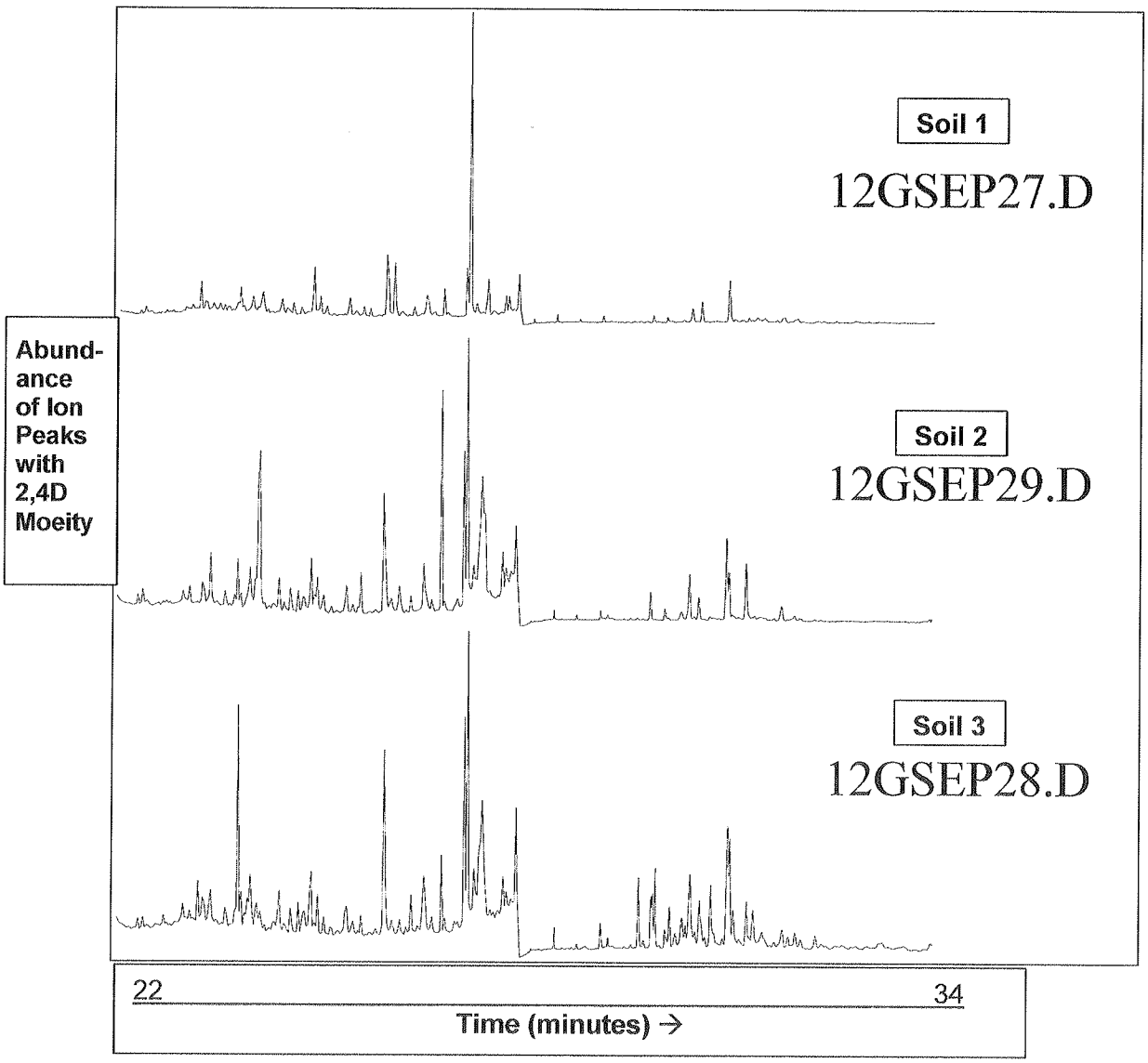

Figure 7.2.6. Three different soils, showing the variation in the number of peaks containing the 133, 145 and 161 ions in the "2,4-D homologues region".

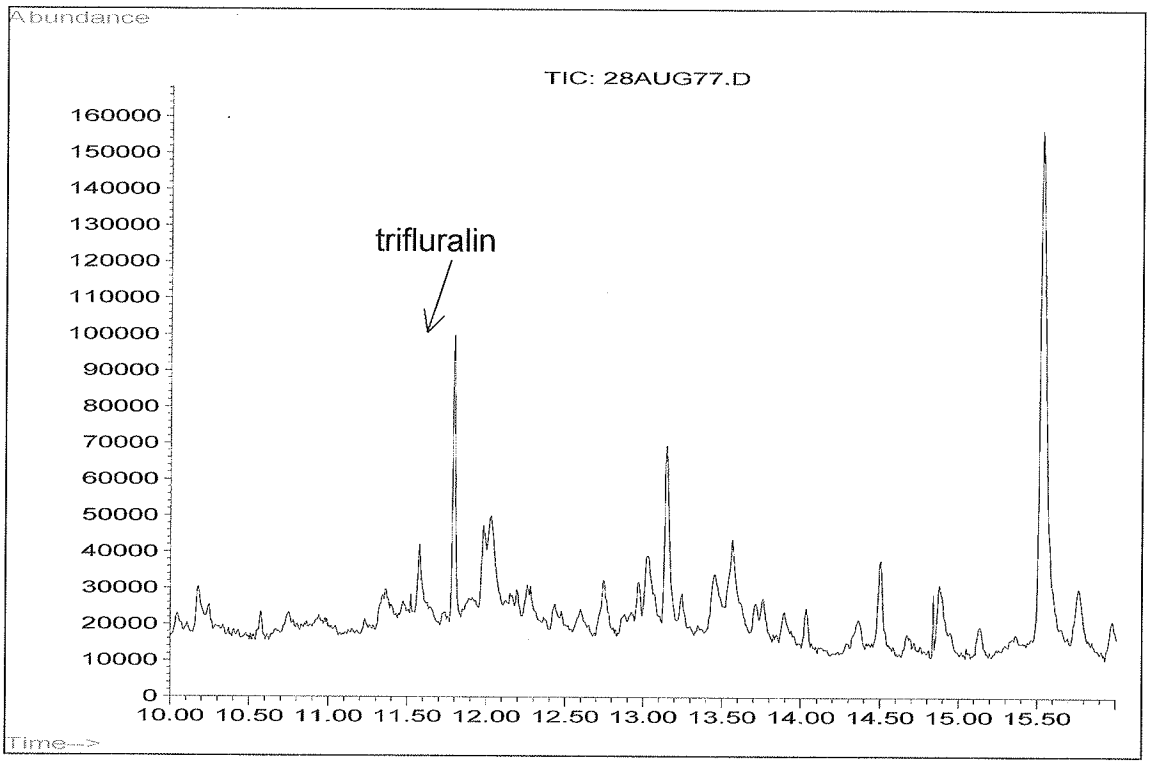

Figure 7.2.7. Chromatogram of a soil containing residual trifluralin.

The presence of trifluralin residues in some soil samples was confirmed by mass-spectral matching of the peak occurring at 11.78 minutes with library data for the pure material. The mass spectrum of trifluralin is shown below in Figure 7.2.8.

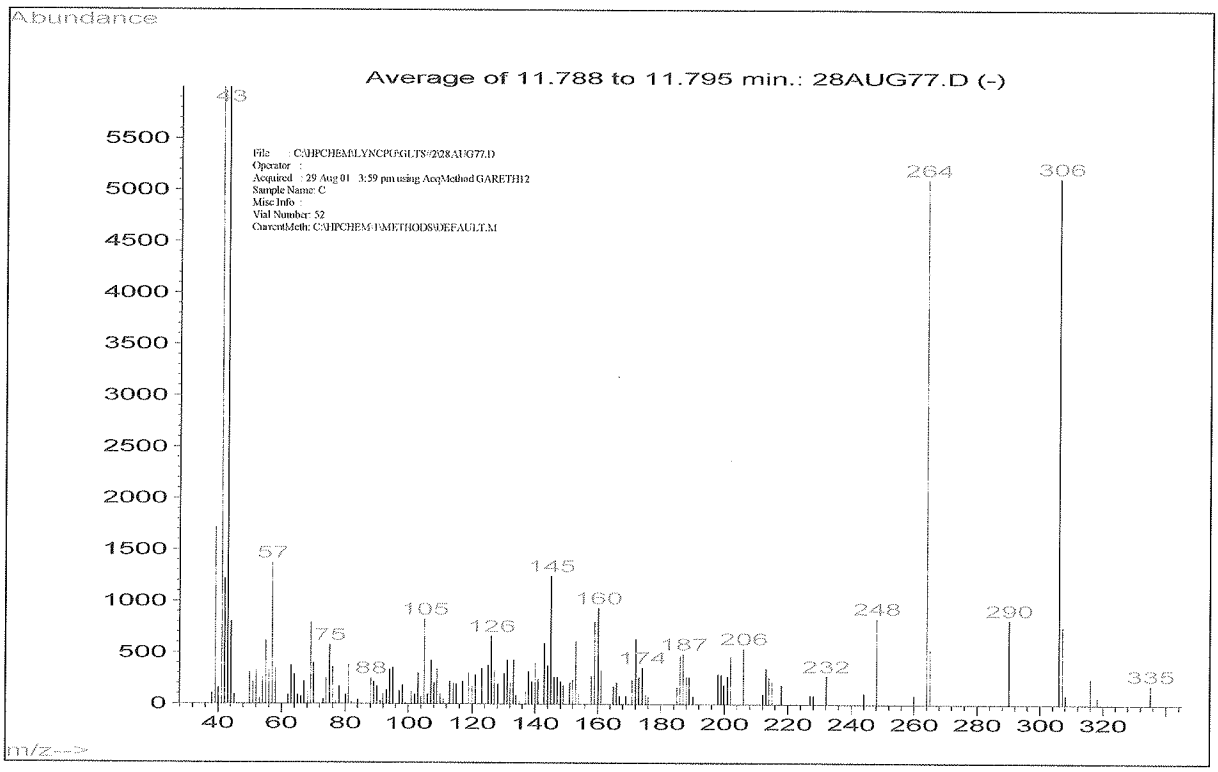

Figure 7.2.8. Mass spectrum of trifluralin.

7.3 Location of Soil Sample Sites (Possible Data Base)

The soil samples used in the "dust study" and to investigate the presence of herbicide residues, were obtained from a range of soil types across the Yorke Peninsula and with a reasonably wide spatial distribution.

Figure 7.3.1 shows the distribution of the sampling locations across the Yorke Peninsula. Initially it was seen as desirable to try and sample as many soil types as possible in order to assess both the potential amount of the dust fraction in each case, and also to determine the presence and identity of any residual herbicides associated with the dust fraction.

It was not possible in this study to select soil samples according to soil type, although ultimately the majority of soil types found across the Yorke Peninsula were included in the 100 or so samples available.

Because land-management practices, in particular tillage practices, ultimately determine how extensively the soil structure is reduced is size, land-care officers were consulted to ascertain what strategies were recommended for the region of interest in this study. It was clear that much effort had been employed in ensuring that land-holders were aware of the need to avoid extensive comminution of soil. The earlier plots in Section 7.2 show how the different soil types give rise to greater or smaller amounts of dust.

Given the intentions of this study were, *inter alia*, was to inform and educate stakeholders in the event that adverse findings, especially in relation to residual herbicides were found, it was decided that any maps and or databases would not allow identification of individual land-holders. With that caveat in mind the map in figure 7.3.1 has been sized to prevent the identification of individual land-holdings. Databases, if subsequently seen as useful, are probably best done as a cooperative activity involving all stakeholders. Also given that no residual herbicides were found in seawater, accurate mapping of creeks and rivers was not proceeded with.

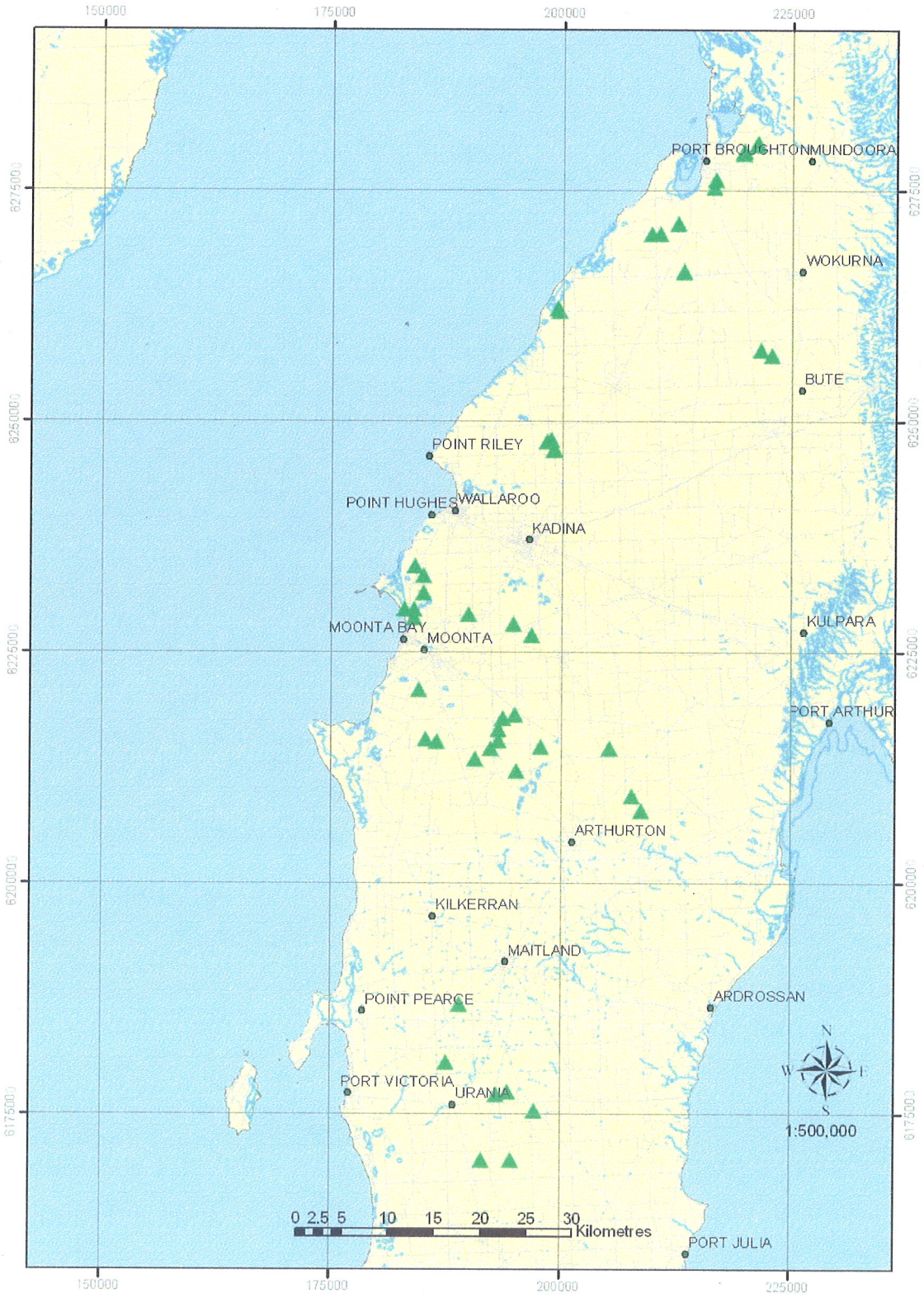

Figure 7.3.1. Yorke Peninsula showing location of soil sample sites used in this study. Sample sites are not numbered or further identified.

7.4 Results of Aquatic Species Toxicity studies

7.4.1 Acute Toxicity

7.4.1.1a Penaeus latisulcatus

At all time intervals all physico-chemical parameters were within acceptable limits in all test chambers as set out by Cripe (1994) and Kumar & Chapman (1998).

The 96-hour LC_{50} for these juvenile Western King Prawns was calculated to be 0.44 µg/L. Chlorpyrifos toxicity was proportional to time of exposure through a logarithmic relationship (slope = 2.079 r^2 = 0.98), as seen in figure 7.4.1. This showed a No Observed Effect Concentration (NOEC) of approximately 0.275 µg/L. At all time intervals there were insignificant mortalities in the control chambers.

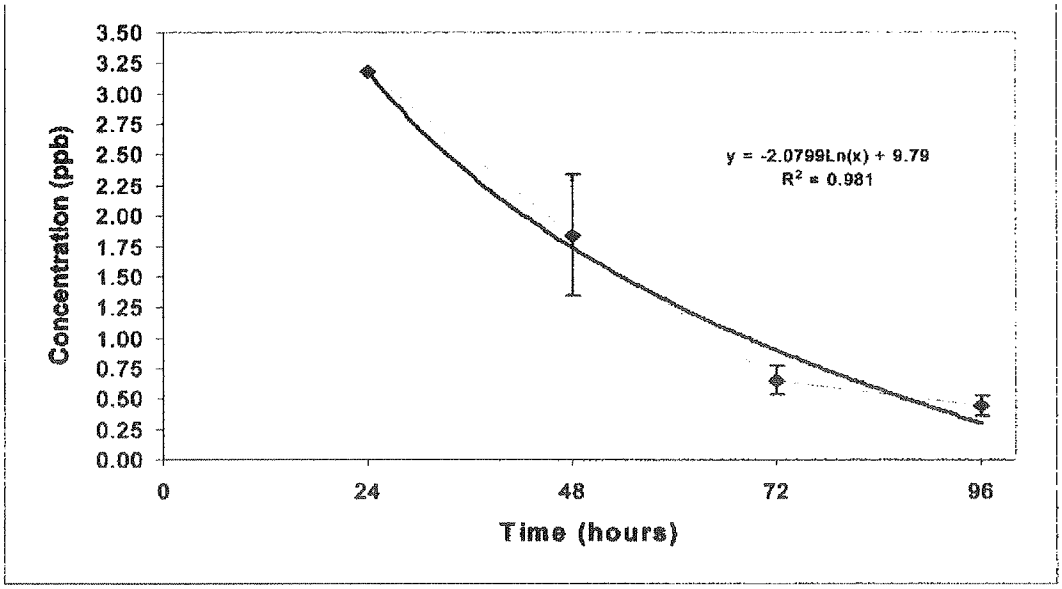

Figure 7.4.1a. Acute Toxicity LC_{50} values for *P. Latisulcatus* on exposure to chlorpyrifos over time. Error bars represent 95% confidence intervals.

7.4.1.1b Sillaginodes punctata

At all time intervals all physico-chemical parameters were within acceptable limits in all test chambers. The 96-hour LC_{50} for these juvenile King George Whiting was found to be 9.05µg/L which was also directly proportional to time of exposure by a logarithmic relationship (slope = 9.775 r^2 = 0.96). This can be seen graphically in Figure 7.4.1b.. Additionally, the NOEC was calculated to be approximately 6.25 µg/L. At all time intervals there was zero mortality in all control chambers.

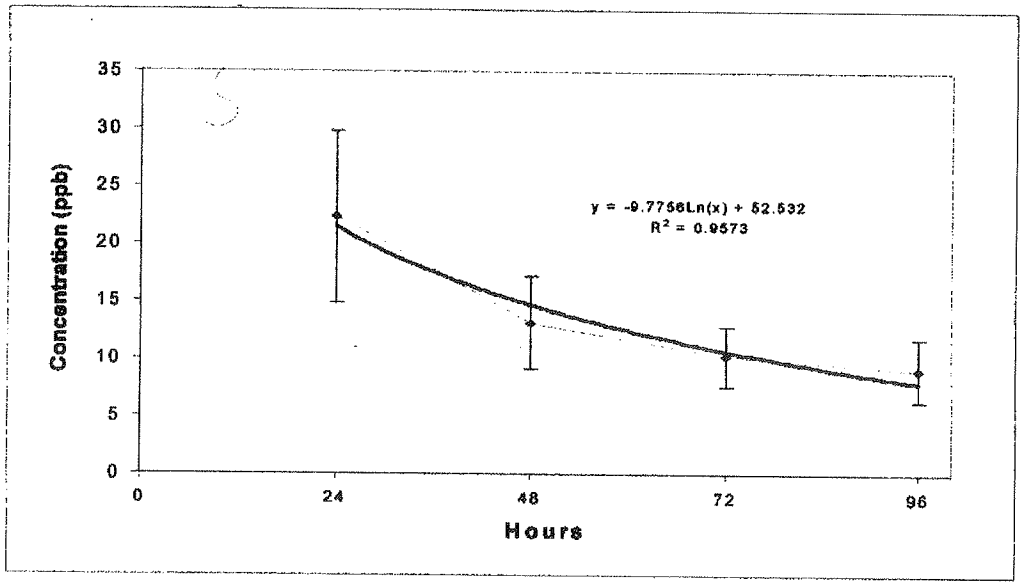

Figure 7.4.1b. Acute toxicity LC_{50} for *S. punctata* on exposure to chlorpyrifos over time. Error bars represent 95% confidence intervals.

7.4.2 Inhibition of Acetylcholinesterase

Throughout all time intervals the control fish brain AChE activity did not significantly differ and the solvent control was also not significantly different to the fish controls. At all time intervals the physico-chemical parameters were also within acceptable limits.

The juvenile King George Whiting used in these experiments showed that when brain AChE activity dropped below 21600 µmol/min/g ± 11100 SD or it was inhibited by more than 66.3% ± 1.19 SD, death resulted. This is shown in Figure 7.4.2 by the "Max" line throughout all time intervals. Secondly, brain AChE reached maximum inhibition after 48 hours, then approached an asymptote at approximately 40% of control activity, shown by the equation to the trend-line in Figure 7.4.2 (slope = 89.379, $r^2 = 0.85$).

All experimental fish examined for AChE inhibition showed significant difference from the controls, while at 24 hours there was a significant difference apparent ($p<0.05$). During these experiments mortality was between 40 and 60%, which was anticipated from the LC_{50}.

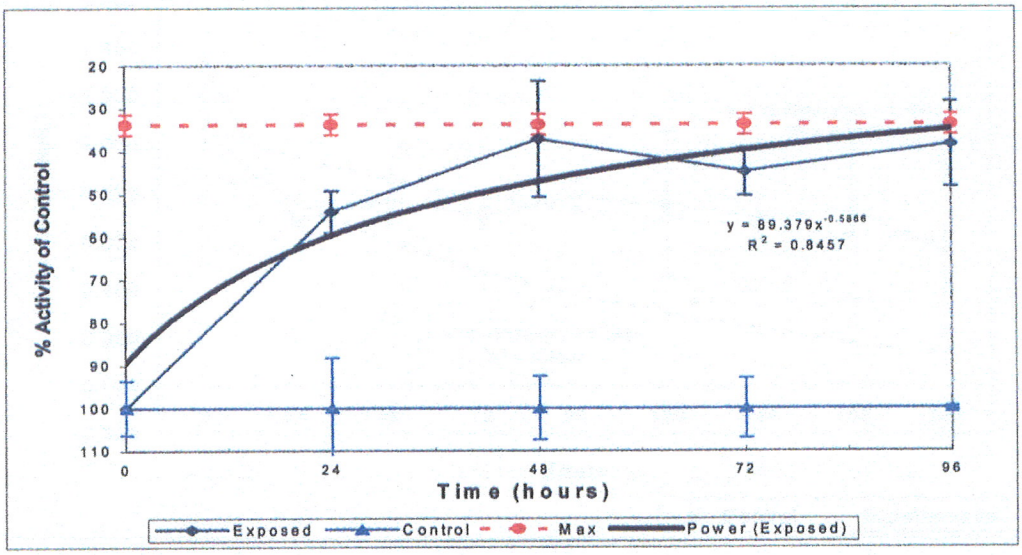

Figure 7.4.2. Inhibition of *S. punctata* brain acetyl-cholinesterase on exposure to 12.5 µg/L chlorpyrifos over time. (± 2SE).

7.4.3 Bioaccumulation

All physico-chemical parameters were within acceptable limits during all time intervals during these tests.
The body burden of chlorpyrifos in muscle, after 96 hours exposure, for the juvenile *S. punctata* was 1.785mg/kg. Figure 7.4.3 shows the rate of uptake and the concentration in muscle following exposure to 12.5 µg/L chlorpyrifos as a function of time. The rate of uptake was directly proportional to the time of exposure (slope = 0.017 r^2 = 0.99). At all time intervals, the residue concentration was significantly different from the control ($p<0.05$). Spiked extract recoveries were approximately 61%.

Simultaneous injection of standard (4ppm) chlorpyrifos solution together with the solvent extract from the 48-hour fish bioaccumulation showed an identical GLC trace with no shoulders, confirming the identity of the peak found as chlorpyrifos. No metabolic products could be identified using GC-ECD or GC-MS.

Due to lack of fish, this experiment could not be replicated. This reduces the statistical certainty of the results, but the trend shown is still applicable. All significant differences are approximate only.

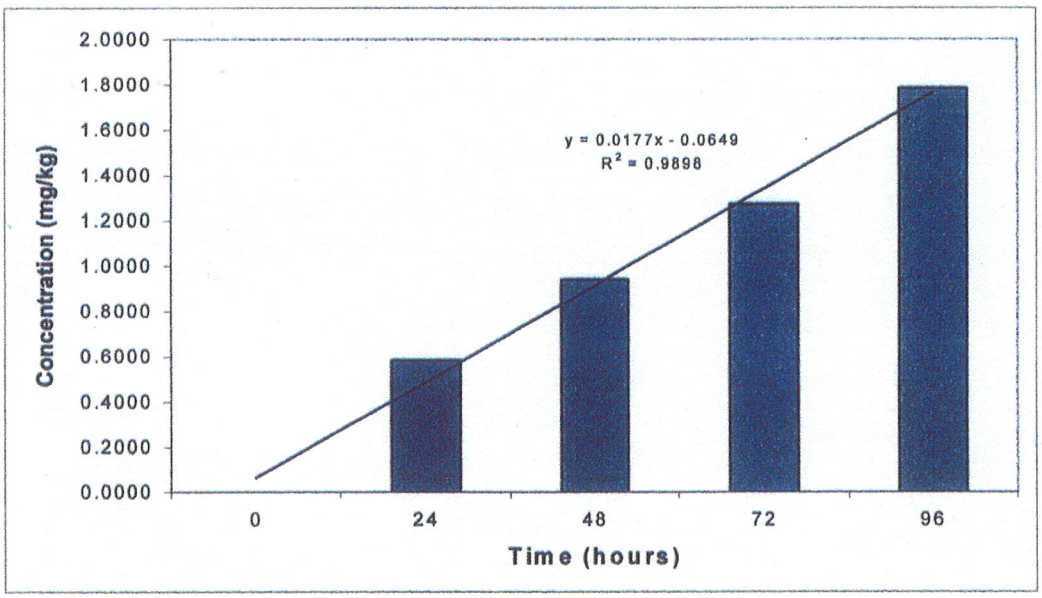

Figure 7.4.3. Rate of uptake (line) and residue concentration (bars) in *S. Punctata* muscle on exposure to 12.5 µg/L chlorpyrifos over time.

7.4.4 Discussion

This study compared two toxicological endpoints for acute lethality of chlorpyrifos to the Western King Prawn and King George Whiting. These endpoints are the 96-hr LC_{50} and sub-lethal AChE inhibition. In addition to these endpoints, short-term acute AChE inhibition over time and the resultant uptake of Chlorpyrifos in tissue was also measured.

The use of indigenous species in toxicity testing is becoming of paramount concern to scientists worldwide. Many recent researchers such as Chapman (1991) and Cairns (1993) firmly believe that the use of foreign species as a surrogate for analysis of local impacts is not appropriate due to the uncertainty of interspecies variation. The use of resident species should be applied wherever possible to eliminate the extrapolation for variation between species (Chapman, 1995; Kumar & Chapman, 1998).

However, a major challenge in ecotoxicology has been to find a test species that is acceptable not only in the laboratory but also through the whole ecosystem as an indicator or 'benchmark' of toxicity. Calow (1993) has compiled a list of characteristics that are ideal for such a quintessential test species; these are given as being:

- Representative of the ecosystem
- Widely distributed
- Economically and/or ecologically significantly sensitive
- Amenable to laboratory culture.

These characteristics are sought after but very rarely will they all be present in the one species, therefore compromises need to be made (Calow, 1993; Ross, 1997].

One commonly used strategy to ensure that as many of these characteristics are present as possible, is the use of juvenile organisms in toxicological testing. This has many advantages over adult organisms. Juveniles are commonly the most sensitive life-stage of an organism's life cycle (Connell, 1972; Cripe, 1994). Consequently, management must be focused on the most sensitive life stage, as it will be affected by contamination first (Connell, 1972; Heath et al, 1997). Secondly, juveniles are usually smaller and therefore require smaller test chambers and holding tanks thus using less space, less chemicals, and are as a result more cost effective.

7.4.4.1 Penaeus latisulcatus *as an indicator of Toxicity*

To investigate the use of Penaeus latisulcatus as an indicator species for marine ecosystem health it was compared to the criteria outlined by Calow (1993) above. It was found that *P. latisulcatus* corresponds to four of the five of Calow's criteria for indicator species. The correspondence is outlined below.

The effects of toxic materials on crustaceans in marine environments have traditionally been evaluated with the mysid shrimp, *Mysidopsis bahia*, or the prawn species *Palaemon serratus* or *Penaeus duorarum*, predominantly in the Northern Hemisphere (Schimmel, 1983; Cripe, 1994; Bocquene & Galgani, 1991). The freshwater shrimp *Paratya australiensis* has also been used in the Southern Hemisphere (Abdullah et al, 1994; Olima et al, 1997). A review of acute toxicities for estuarine organisms has shown that *Penaeidae* are most frequently the family of organisms displaying the greatest sensitivity to toxic materials, especially OP's (Cripe, 1994). In addition juvenile or larval stages of the test organisms have been shown to be up to 1,000 times more sensitive to toxicants than adult forms of the same species (Cripe, 1994; Conner, 1972; Heath et al, 1997).

The WKP can be found throughout tropical Australia, south to the two Gulfs of South Australia and also throughout the Indo Pacific region (Edgar, 1997). Thus the WKP could be regarded as widely distributed. Marine crustaceans, including Penaeid prawns have been used for marine bioassays for the last 15 years. They also been found to be relevant for determining the effects of toxic compounds on marine food chains (Saroglia & Scarano, 1979). The WKP can also be seen as being economically significant from the viewpoint of the $35 million dollar fishing industry it supports in South Australia alone.

Throughout this study, there was found to be very little mortality among the juvenile Western King Prawns arising from transport and laboratory handling, contrary to commonly proffered advice. They were found to be a robust and easily handled organism. This may be due to the carapace providing protection from abrasion. The prawns also seem to be comfortable feeding on commercial food pellets or broken cockles.

However, the juvenile WKP can only be caught during the summer months of the year, due to the fact that they burrow into the sediment to escape the colder water during winter. This reduces the time that the prawn can be captured to

approximately three months in a year (N. Carrick (SARDI) pers. comm.). Collection of organisms throughout this study involved night trawling of a net in the shallow mangrove fringed tidal regions, a process which was both time consuming and laborious, requiring at least four people.

Juvenile *P. latisulcatus* may be suitable for use as an indicator species for a whole ecosystem if a suitable method of aquaculture is developed, as this would eliminate the restrictions on availability.

Secondly, due to a sensitive life-stage living in shallow intertidal areas that are regularly inundated by runoff, they are likely to be exposed to any pollutants present in the runoff at this susceptible stage in their life cycle. As a result, the use of juvenile *P. latisulcatus* as indicators of the health of the larger WKP population could also be warranted. Using the juvenile prawn to monitor and assess toxicities also may be useful in the ongoing management of the WKP Fishery.

7.4.4.2 Sillaginodes punctata *as an Indicator of Toxicity.*

Comparing *S. punctata*, (KGW) with Calow's criteria for a suitable bio-indicator species, it was found that the juvenile KGW fulfills three of Calow's five test species criteria.

In the past, the only comprehensive tests utilizing a marine fish as an indicator of toxicity have been limited to *Cyprinodon variegatus*. Extensive work has been done on freshwater species such as, *Gambusia spp.*, *Ictalurus spp.*, *Pimephales spp.* and *Poecilia spp.*, which are all primarily northern-hemisphere species (Coppage, 1972; Straus & Chambers, 1995; Boone & Chambers, 1997; Heath et al, 1997). While the freshwater fish, *Melanotaenia sp.* has been used in Australia (Kumar & Chapman, 1998). However, very few studies have looked at marine fish indigenous to the Southern Hemisphere as indicators of pollution, or their relative sensitivity to pollutants.

Sillaginodes punctata can be found throughout southern Western Australia, through South Australia and to northern NSW and also northern Tasmania (Edgar, 1997). This could be said to be representative and also widely distributed through southern Australia. The KGW is the most valuable scale-fish commercially and recreationally caught in southern Australia (Edgar, 1997), hence it could be said that this constitutes economic significance.

As there are no published toxicity data for native Australian marine fish, the sensitivity of *S. punctata* could only be determined by comparing with northern hemisphere species, which showed 96hr LC_{50}'s ranging from 1.7-136µg/L (Schimmel et al., 1983) compared to 9.05µg/L in the present study. This demonstrates that the KGW are in fact relatively sensitive to chlorpyrifos compared with other marine species.

However, throughout these experiments there were numerous mortalities during both collection and transport. This is a serious impediment for their use in toxicity testing as it means that excessive numbers must be taken in order to ensure statistically

valid testing procedures, whilst also allowing for 10-20% mortality through collection and transport. The collection of the animals again limits the applicability of *S. punctata* for use in toxicity testing.

In South Australia, post-larval settlement occurs in two phases through June-July to September-November (Fowler & Short, 1996). This, as was the case with the WKP, limits the time during which suitable fish can be caught, to approximately three to four months of the year.

Therefore, the use of juvenile *S. punctata* may only be suitable for use as an indicator species for whole ecosystem health if the necessary aquaculture processes are perfected, as this would remove the restrictions on availability.

Secondly, its use can also be justified in the context that it gives an indication of the population health for the King George Whiting.

7.4.5 Acute Toxicity Experiments

7.4.5.1 Penaeus latisulcatus

Chlorpyrifos was shown to be acutely toxic to juvenile *P. latisulcatus* at levels of 0.44 µg/L. While there are currently no published toxicity data utilizing *P. latisulcatus* and chlorpyrifos, the 96hr LC_{50} seen can be cautiously related to 96hr LC_{50} values found by Cripe (1994), for diazinon to post-larval *Penaeus duorarum* of 21 µg/L. However diazinon, while belonging to the O.P family of insecticides as does chlorpyrifos, has a different chemical structure and may be metabolized differently through the additional nitrogen atom in the pyrimidine ring in that case.

The secondary metabolite is less reactive towards electrophilic substitution and this may increase its persistence in an organism due to slower elimination. The values seen in the freshwater shrimp, *Paratya australiensis*, to chlorpyrifos range from 0.08-0.28µg/L (Olima et al., 1997). This shows that juvenile *P. latisulcatus* is less sensitive to chlorpyrifos than *P. australiensis*, but this must be compared cautiously as there are vast differences in the fresh and marine environments.

It can be seen that there is a definite relationship between the LC_{50} and time of exposure (Fig. 7.4.1a). This is a logarithmic relationship that has a relatively shallow slope (2.079), indicating that the prawns are sensitive over a small range of concentrations.

Although replication of this experiment could not be achieved due to lack of availability of organisms, the LC_{50} value found of 0.44ppb is of concern as this concentration could possibly occur in the environment, especially when concomitant AChE inhibition may occur at significantly lower concentrations as demonstrated by Jarvinen et al., (1983), Heath et aL, (1997) and Kumar & Chapman (1998).

Kumar & Chapman (1998) reported that the main biological factor for determining the acute lethal effect of an organic contaminant may be the cholinergic system, particularly acety1cholinesterase inhibition. Their study also noted that this finding, together with residue analysis could allow the determination of relative sensitivities of

various species avoiding the extrapolation arising from full acute 96hr toxicity tests. This gives an indication of the bioavailability and biological relevance of a contaminant instead of the hit and miss of mortality testing.

The use of *P. latisulcatus* in this study was originally designed to be used for acety1cholinesterase inhibition experiments as well as bioaccumulation. These tests however could not be carried out, due to the unavailability of the organisms throughout the colder months and uncertainty in the availability of the trawl boat necessary.

7.4.5.2 Sillaginodes punctata

The 96-hour acute toxicity of chlorpyrifos to juvenile KGW was observed to be 9.05µg/L at 18°C. When looking at the logarithmic relationship between LC_{50} and time (Fig. 7.4.1b), the slope was observed to be 9.775. This indicates that *S. punctata* could tolerate a larger range of chlorpyrifos concentrations than *P. latisulcatus*. There are currently no published toxicity data on *S. punctata*, but cautious comparisons can be made to similar estuarine fish, taking into account interspecies variation. Schimmel (1983) observed that the 96hr LC_{50} for *Cyprinodon variegatus* to chlorpyrifos is 136µg/L (at 31°C), also *Fundulus simifis* (longnose killifish) 4.1µg/L (30 °C), *Medinia medinia* (Atlantic silverside) 1.7µg/L (27.5°C) and *Mugil cephalus* (striped mullet) 5.4µg/L (24.8°C). This shows that *S. punctata* (LC_{50} 9.05µg/L at 18°C) has relatively similar sensitivities to toxicants compared to most northern-hemisphere fish.

The values seen in the literature show great variability in sensitivities, which may be due in part to temperature influences. In this study, the effect of chlorpyrifos to *S. punctata* was tested at 18°C while the studies reported by Schimmel (1983) varied in temperature. The toxicity of chlorpyrifos, increases significantly with increasing temperature [Macek et al., 1972]. Therefore the organisms tested by Schimmel may have shown higher LC_{50}'s due to the higher test temperatures used. However the species with the greatest tolerance (*C. variegatus*) also showed the highest LC_{50} concentration. This may be attributed to a biological tolerance to chlorpyrifos by *C. variegatus* similar to that found by Boone & Chambers (1997) in the freshwater fish *Gambusia affinis*.

The toxicity of chlorpyrifos to freshwater fish has been investigated in some detail, with the 96hr LC_{50} for the mosquito fish (*G. affinis*) for example, found to be 0.15mg/L (ppm) [Boone & Chambers, 1997]. However, as mentioned above, the mosquito fish has proved to be relatively tolerant to chlorpyrifos compared with other freshwater species such as the bluegill sunfish (*Lepomis macrochirus*). This latter fish is the most sensitive, with a 96hr LC_{50} of approximately 2µg/L, whereas Rainbow trout (*Oncorhynchus mykiss*) had a 96hr LC_{50} of 10.5µg/L (NRA, 1999).

These values vary greatly depending on test temperature, system used (static or flow through), contamination background and size of the organism. They also show that while there may not be a significant difference between marine and freshwater species there is a significant difference through interspecies variation. Nevertheless, it is clear that *S. punctata* is susceptible to chlorpyrifos in the environment in relatively low concentrations.

This variability highlights the need for standardized toxicity testing protocols for marine and freshwater species endemic to regions such as temperate Australia.

7.4.6 Acety1cholinesterase Inhibition Experiments

For these experiments, a concentration of 12.5µg/L was chosen for two reasons:

(i) Chlorpyrifos is sprayed onto crops at certain predetermined occasions throughout the year, depending on the pest species to be controlled. This spraying dose will be acutely lethal to the pest (between 0.5-1.5kg/ha) and application is usually made by air. It has been shown that up to 4% can run off from aerial spraying and this value could be exceeded after heavy rainfall or high winds (NRA, 1999). Therefore, if contaminated runoff enters a marine ecosystem it will be in relatively high concentration over a relatively short time period following the rainfall event.

Chlorpyrifos is relatively short-lived in the environment, half-lives ranging from 2.5 hours to 13 days depending on environmental factors. This suggests that any single contaminated runoff event would not sustain a significant concentration over the longer, traditionally tested chronic time span such as 30-60 days.

However, a higher concentration occurring for a shorter period of time would be more environmentally relevant for estuarine environments, a view supported in studies such as Gluth *et al*, (1985) Escartin & Porte (1996a), Sancho *et al*, (1998), Kumar & Chapman (1998), and Serrano *et al*, (1997).

(ii) Currently there are no reported toxicological data on the KGW, or even a marine fin-fish native to temperate Australia, involving organophosphorus pesticides. Hence the use of a higher concentration ensures that the full spectrum of the dose-response relationship will be observed. This allows comparisons to be made to Northern-Hemisphere species that have been rigorously tested, such as the sheepshead minnow (*Cyprinodon variegatus*).

The lethal brain inhibition in *S. punctata* was found to be 66.3%, i.e.: 33.7% of normal brain AChE activity (Fig. 7.4.2). This area of research has proved to be a source of debate. For example, Coppage (1972), has stated that 17.7% of normal brain activity (83% inhibited) caused death in *Cyprinodon variegatus*, regardless of concentration and exposure time. On the other hand, Jarvinen et al, (1983) demonstrated that the fish *Pimephales promelas* will survive in excess of 200 days with brain AChE inhibition of above 80%, while Weiss (1958) has reported fish death due to AChE inhibition of merely 5.4%.

However, Kumar & Chapman, (1998) have shown that in the freshwater fish *Melanotaenia duboulayi*, a short term (24hr) acute exposure to profenofos caused mortality due to the inhibition of AChE by 72%. In the same study, a sub-lethal exposure caused inhibition of up to 90% over 14 days. The short-term exposure figure of 72% is comparable to the results seen in this study of 66.3%. This shows that the time of exposure is more crucial to inhibition than exposure concentration. During longer exposure times with lower concentrations the fish may have the ability to metabolize and adapt to the pesticide.

The inhibition of brain AChE in *S. punctata* shows a rapid inhibition phase during the first 24 to 48 hours (slope 89.37), indicating there is rapid uptake and distribution to the brain. The inhibition then reaches a plateau after 48 hours and then remains relatively stable just below the maximum inhibition rate of 66%. This may indicate that the fish AChE inhibition is at equilibrium with the system (i.e. at steady state).

Throughout this study it was observed that the exposed fish had several physiological and behavioral differences from controls. These differences were not tested for, but were consistent throughout the replicates and different experiments.

Exposed fish appeared to be sluggish, showed a decreased food intake, an occasional loss of equilibrium, an increase in gill movement, erratic swimming or would swim in spirals. Fish close to death often displayed convulsions. This behavior is supported by the literature, where, for example, Kumar & Chapman (1998) state that all of these symptoms are associated with cholinesterase poisoning. This raises the question of skeletal muscle AChE inhibition. The symptoms seen here are partly due to over-stimulation of skeletal muscles causing erratic swimming and possibly an increase in gill movement/ respiration. It is not known whether skeletal muscle inhibition is similar to the residue uptake that is: linear or logarithmic as seen in brain AChE (shown in Fig. 9).

7.4.7 Bioaccumulation of chlorpyrifos

Bioaccumulation of chlorpyrifos was observed in the muscle tissue of these juvenile *S. punctata* at a concentration of 142.8 times that of the ambient environment after 96 hours.

The uptake of an organic chemical into a fish occurs by passive diffusion across gill membranes (McKim et al, 1985). The rate that this uptake occurs is dependent on the chemical's log P value. Generally, the higher the log P the more lipophilic, and therefore the more readily it will pass across the lipid layers of the gill membrane. Eventually, as log P increases there is a transition point where the rate of uptake control is passed from the membrane to the diffusion layer. However, once the log P values reach a point where the compound is very fat-soluble, the molecule will not move out of the lipid membrane. Therefore it may not become available to affect the target tissue (McKim et al, 1985). Additionally, chemicals with log P values greater than 3 are so lipophilic that excretion back across the gill membrane is greatly reduced (McKim et al, 1985).

Chlorpyrifos has a log P value of 4.7. This puts it into the range where it is fat soluble enough for its passage not to be hindered by the lipid membrane but not so lipophilic that it will not move out of the membrane. Therefore this may present a dangerous combination where the chemical's uptake will be rapid and its progress to the target tissue unhindered.

The results found in this study show that after 96 hours chlorpyrifos can be accumulated in concentrations approximately 142.8 times (1.785mg/kg) that of the ambient environment, where it was at a concentration of 12.5µg/L. It has been shown that the primary route of uptake is through the water, while food is only a

minor influence (McKim et al, 1985). Excretion has been shown to be insignificant with respect to loss of chemical (Welling & DeVries, 1992). Figure 7.4.2 shows that after 96 hours, the uptake of chlorpyrifos had not reached equilibrium. Authors such as Serrano et al, (1997) have noted equilibrium was reached after 15-17 days at a concentration of 1ppm.

At concentrations of approximately 0.3 µg/L rainbow trout reached steady state after 10 days (NRA, 1999). Chlorpyrifos accumulation would continue until steady state is reached. Therefore, while the results of this study show a bioaccumulation of approximately 150 times the ambient environment before steady-state, it is reasonable to suggest that chlorpyrifos could potentially bioaccumulate in excess of 150 times.

A bioconcentration factor can be calculated for a chemical to give an indication of the tendency for that chemical to concentrate in the tissues of an organism that is exposed to it. Chlorpyrifos was shown to have a bioconcentration factor in guppies of around 1600-1700 (Welling & DeVries, 1992), but values from 58 to 6000 have been published (NRA, 1999).

A bioconcentration factor however, relies on the organism being at steady state with the contaminant in the system. From the results observed in this study, it cannot be concluded that the system was at equilibrium as the residue concentrations were still rising after 96 hours. There are many factors that influence when a system reaches a steady state. These are rate of uptake, concentration in the ambient environment and rate of excretion (Walker et al, 1996).

While the system was not at steady state, this model can be used to predict the relative accumulation potentials of each species. Chlorpyrifos, has a log P of 4.7 and the BCF for *S. punctata* after 96 hours was 142.8 (not at steady state).

The literature suggests that steady state occurs approximately at 16 days for a 1 ppm solution and 10 days for a 0.3 ppb solution (NRA, 1997; Serrano et al, 1997). Assuming all things are equal, this would give an approximate steady state time of around 12 days for 12.5 ppb. Using the equation generated in Fig. 7.4.3 of $y = 0.0177x + 0.0649$, the hypothetical concentration at steady state (12 days) would be 5.1625mg/kg assuming the relationship is linear. This gives a log BCF of 2.616. This shows an accumulation potential greater than that found in this experiment due to the theoretical steady state giving a faster rate of bioaccumulation (slope = 0.5553). This may be closer to the actual rate when the system is at steady state.

While the slope of this line is still lower than the other species seen in the literature, it shows that the potential for KGW to accumulate chlorpyrifos or other lipophilic compounds is as much of a threat these fish as it is for other species.

The rate of uptake for S. punctata muscle tissue was shown to fit a linear relationship (slope = 0.0177, $r^2 = 0.99$). This does not correspond with results seen by Welling and De Vries (1992) who observed a semi-logarithmic relationship between uptake of Chlorpyrifos in guppies (*Poeciffia reticulata*), whilst Serrano and co-workers (1997), and Kumar and Chapman (1998) observed logarithmic relationships between the uptake of chlorpyrifos in mussels (*Myfflus galloprovincialis*) and profenofos in rainbow

fish (*Melanotaenia duboulayi*). However all of the reported experiments were carried out using whole organisms, with the exception of Welling and De Vries who measured uptake indirectly as a result of loss from water.

The results from this study with *S. punctata* were done using fish with the brain and a small section of the skull extracted. It is possible that this may account for the linear relationship seen. This experiment shows that the amount of chlorpyrifos in tissues that have lower lipid concentrations such as muscle, is less than that of tissues with high lipid concentration such as the brain. Therefore the lipid soluble chlorpyrifos may have been concentrated in the phospholipids of the brain. It is possible that chlorpyrifos may then be distributed through to the muscle as a secondary process accounting for the lower concentration at earlier time intervals.

A second possibility is that the brain of a fish needs a high supply and flow rate of blood. This would then skew the chlorpyrifos concentration into the brain (due to higher exposure), instead of being evenly distributed throughout the body that would be anticipated to produce a logarithmic relationship, possibly as a result of even blood flow throughout the organism.

These arguments gain further support when compared to the AChE inhibition values that were seen in *S. punctata*. This relationship is logarithmic and appears to plateau after approximately 48 hours. However this equilibrium was not seen in the bioaccumulation experiment. This may indicate that chlorpyrifos affects the brain first and then is distributed through the muscle, which could account for the delay.

The metabolism of chlorpyrifos occurs through a pathway identical to the degradation pathway (Feng et al, 1998). Enzymatic metabolism occurs through the mixed function oxidase enzymes (MFO). These enzymes are responsible for the oxidation of chlorpyrifos to chlorpyrifos oxo form, activating its toxicity. Secondly hydrolysis occurs (also through phase I MFO) producing the primary metabolite 3.5,6-trichloropyridinol TCP and diethyl phosphate (Walker et al., 1996). The bulk of this metabolism occurs in the liver and plasma and is activated by cytochrome P450 enzymes (Richardson, 1995; Straus & Chambers, 1995). TCP is then further metabolised by phase H MFO enzymes which bio-transform the molecule into more water-soluble fractions that will allow its transport out through excretion via the kidneys.

The TCP metabolite is less toxic than the parent compound showing LC_{50} (96hr) values of ranging from 12.5mg/L to 58.4mg/L in Bluegill Sunfish and Atlantic Silverside respectively (NRA, 1999).

In this study, the primary metabolite was unable to be identified by GC-MS in any of the samples. This was also seen by Serranjo *et al*, (1997), who could not identify any metabolites after a short-term exposure (96 hours). This may be accounted for by the method of analysis or sample workings.

Due to the high temperature required to volatilise the sample, compounds may pyrolyse in the injector before being passed onto the column. When chlorpyrifos, is pyrolysed, it breaks into two compounds, 3,5,6 trichloro-pyridinol (TCP) and diethyl-

phosphate. These are also the products of metabolism. Due to the chromatographic similarity of TCP to Chlorpyrifos, it is extremely difficult to separate the two peaks. This means the chlorpyrifos peak will 'mask' any TCP peak seen through products of metabolism.

The bioaccumulation seen in KGW has the potential to be hazardous to other marine organisms that may predate on contaminated fish. While the results suggest that the persistence of chlorpyrifos is not long enough to biomagnify through a food chain, highly contaminated fish could pose a threat to larger fish and marine mammals that use *S. punctata* as a food source, especially in organisms with inadequate MFO systems.

7.5 Impact of Herbicides on the Seagrass *Zostera Muelleri*

Seagrasses live under *stringently controlled growth conditions* within their environments. These stringent growth conditions in the intertidal environment are defined, in part, by toxic sediment (Viaroli *et al.*, 1996), heavy metals (Faraday, 1979), photo-bleaching caused by ultraviolet light (Trocine *et al.*, 1981, Dawson and Dennison, 1996) and other factors that negatively impact upon the photosynthetically available radiation (PAR) such as eutrophication (Wear Donna *et al.*, 1999) and water turbidity (Longstaff and Dennison, 1999).

Maintenance of a 'positive carbon budget' (Neely, 2000) in seagrasses such as *Halodule wrightii* is determined by such physicochemical factors. Indeed, it is the interaction of these stringent growth factors that define a *tolerance zone* in which *Zostera muelleri* can grow within the intertidal zone. Any physicochemical factor(s) that is able to modify the intertidal environment of *Zostera* or negatively impact upon *Zostera's ability to cope with the prevailing intertidal environment* will displace *Zostera* out of its tolerance zone. Indeed, it is known that the seagrasses, *Zostera capricorni*, *Halophila ovalis* and *Cymodocea serrulata*, are negatively impacted upon by herbicides under laboratory controlled conditions (Haynes et al., 2000). Haynes noted changes in the fluorescence parameters associated with the photosynthetic process and inferred a negative impact upon the seagrass. Haynes also points out that it is likely that similar effects occur in the natural environment in the coastal waters of Queensland in which herbicides residues have been detected.

Interestingly, dust as a possible transport mechanism for herbicides to the marine environment was not addressed in the study by Haynes even though dust storms are known to travel from farmland to the coastal environment. In the present study it is known that dust storms occur in South Australia with a frequency of fifteen to seventeen per annum often traveling from the Eyre Peninsula and the York Peninsula towards Adelaide. In one dust storm event in 1994, ten to fifteen million tonnes of topsoil was transported. Dust as a transport mechanism is not a recent phenomenon; indeed there is a measurable accession of atmospheric dust of five to ten tonnes per square kilometre per annum in Adelaide (Tiller *et al.*, 1987). It is likely that this 'transport mechanism' has occurred for some time before commencement of intensive farming practices, and its associated herbicide usage, on the peninsula.

Dust transport of surface applied herbicides, such as 2,4-D and trifluralin, have been studied in soils of the Canadian prairies with up to 5% of the surface-applied

herbicide being 'lost' as dust (Larney Francis *et al.*, 1999). This equates to a 5 mg loss of 2,4-D (as acid equivalents) from each square metre of treated farmland at an application rate of one kilogram per hectare. Short-range (Sandmann *et al.*, 1991) and long range (Welch *et al.*, 1991) transport, 25 and 6000 km respectively, of herbicides have also been studied by other research groups. It is noted that all farms in the present study are within 25 km of the coastline.

Additionally, results obtained from laboratory studies indicate that *Zostera* is negatively impacted upon by 2,4-D at 5 ppm (acid equivalents). This negative impact is likely caused by a carbohydrate deficit induced in *Zostera* by the 2,4-D treatment. A mechanism by which this happens can be constructed by considering the auxin-like activity of 2,4-D. Briefly, *Zostera* is first induced into an up-regulated growth pattern by the auxin-like activity (Davidonis *et al.*, 1979, Davidonis *et al.*, 1982) at low concentrations (1-10 µM) of 2,4-D. A subsequent decreased synthesis (photosynthesis) and an increased usage (mitochondrial respiration) of carbohydrate in an up-regulated growth pattern will likely cause a carbohydrate deficit in affected plants. A decreased oxygen production and storage process within the lacunae of *Zostera* will then subject the plant to the toxic factors within sediment that are normally prevented by a slow leakage of oxygen from the roots into the adjacent sediment. This is 'detoxification' of sediment was noted by Lee and Dunton (2000) in their study of *Thalassia testudinum* seagrass beds.

Furthermore, a nutrient deficit is also likely to occur as symbiotic aerophilic bacteria that are associated with the root zone of some species of *Zostera*, such as *Zostera marina*, will be less able to produce soluble phosphate from hydroxyapatite that is held within sediment (Craven and Hayasaka, 1982). These induced 2,4-D effects will shift *Zostera* out of its tolerance zone and compromise somatic cell growth, cellular repair processes and seed production.

Other possible herbicidal effects may be imposed on *Zostera muelleri* by Treflan® and triasulfuron which were detected in some whole soil extracts by GCMS (Figures 4,5 and 6) and by HPLC (UV-vis) analyses. Treflan® was detected in seven of thirty-five soil extracts analysed and was present at 5–20 ppb (µg/kg) while triasulfuron was detected in three soils at 10 ppb. Additionally, a series of 2,4-D like compounds was detected in most, over 90%, soil extracts. These chemicals may act as a source of 2,4-D or they may display a similar biologically activity as displayed by 2,4-D.

A bioassay was constructed by using *Zostera muelleri* and chlorsulfuron as a representative sulfonylurea herbicide at the relatively high concentration of 10 ppm. Results indicate that photosynthesis was adversely affected some time after the chronic exposure to chlorsulfuron. It is likely that the effect on photosynthesis is the result of ALS inhibition by chlorsulfuron preventing the formation of branched chain amino acids. A key enzyme (protein), RUBISCO, active in photosynthesis can represent as much as 30% of the cellular protein and a branched chain amino acid deficit may adversely affect its function.

It is particularly interesting to note that losses of intertidal seagrasses in the upper Spencer Gulf in 1994 were attributed to the extreme weather conditions present at that time (Seddon *et al.*, 2000). Dust transport of herbicides (2,4-D, Treflan® and sulfonylureas) may have contributed to seagrass losses in this region.

The current and increasing use of 'herbicides' (Sunohara and Matsumoto, 1997, Grossmann, 1998) are likely to have a continuing direct negative impact upon intertidal seagrass species such as *Zostera muelleri.* Additionally, losses of this intertidal seagrass will have an indirect negative impact on fishing yields since juvenile fish use seagrass meadows as habitat (Bell and Pollard, 1989).

7.5.1 Results of Bioassay Experiments

Seagrass was exposed overnight to the herbicide (at 5 ppm, and 1 ppm) for a 12-hour period. Residual herbicide was then 'washed' from the system by using fresh seawater (seven washes). Leaves were sampled and their maximum photosynthetic capacity, under artificial lighting (250 $\mu E/m^2/s$), was measured during an eight-hour photoperiod by using a manometer technique. Gas volumes (predominantly oxygen) were expressed per unit leaf area and per chlorophyll content.

Sulfonylurea treatment of sediment-bound seagrass with Chlorsulfuron inhibited photosynthetic gas production by Zostera muelleri in the medium term (weeks). In the case of 2,4-D, an up-regulation in photosynthesis was detected in 2,4-D treated *Zostera* leaves in all experimental designs. This up-regulation was deduced from the measured increase (50 to 100%) in gas production of test plants beyond that of the control plants. It is however noted that this increased metabolic rate is not sustained but subsides to zero during time-course studies using sediment-bound plants as Figure 7.5.1.

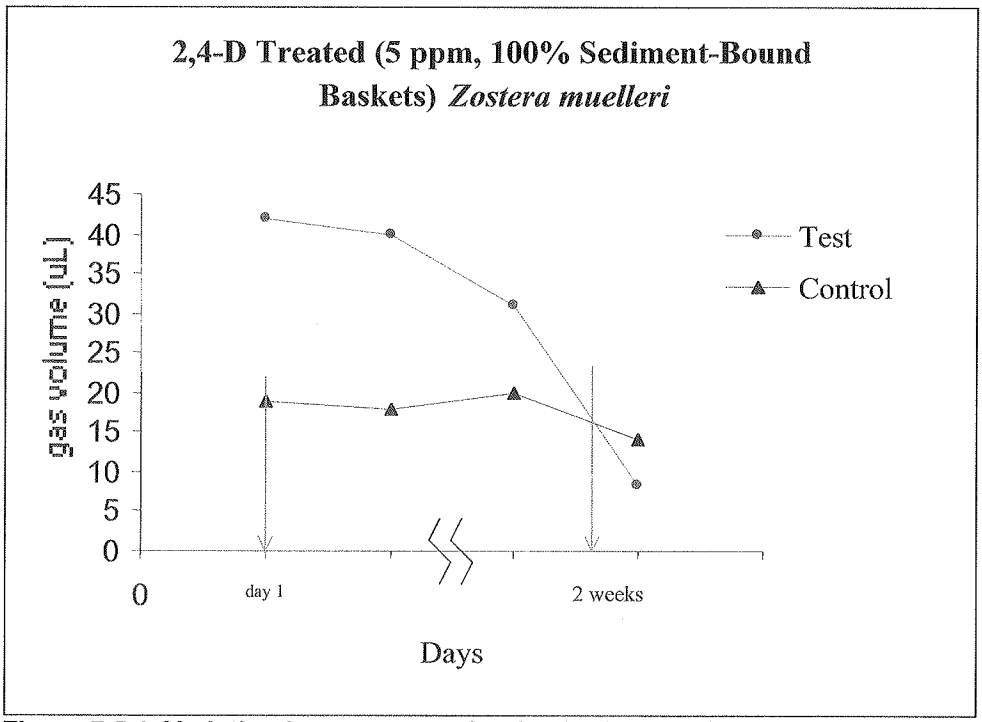

Figure 7.5.1. Variation in oxygen production by Zostera following exposure to 2,4-D.

A structural defect developed within the leaf sheath of *Zostera* subsequent to, and in addition to, the induced effect on photosynthesis. Green leaves are easily removed from the growing plant and close inspection of the base of the removed leaf sheath

revealed a possible abscission zone. It is particularly noteworthy that some algal overgrowth was present on the leaves of *Zostera* in experimental design "(b)" above. Shedding of green leaves results in a smaller photosynthetic area in *Zostera*. A smaller photosynthetic leaf area then renders the plant susceptible to the known toxic effects of sediment in the intertidal zone. The partial overgrowth of seagrass leaves by algae in experimental design "(b)" may also have contributed to a decreased photosynthetic efficiency (and oxygen production) of *Zostera* in this experiment.

The main outcome in this series of experiments was that 2,4-D has an effect on oxygen production and possibly utilisation (sediments) in *Z. muelleri*. Such 'pulse-chase' type treatments cause seagrass death in the short-term of weeks

7.5.2 Carbon Reserves and 2,4-D Treatment

The notion of carbon loss by photorespiration is consistent with results obtained in this study. Acid-soluble carbohydrate levels for 2,4-D treated leaf sections of *Zostera* are approximately two-fold less than a calculated value of acid-soluble carbohydrate for a leaf section containing an equivalent total chlorophyll content in the control group (Figure 7.5.2). The calculated carbon deficit in the test group, and the subsequent death of 2,4-D treated plants, provides a possible explanation for an 'induced ill health' in the treatment group.

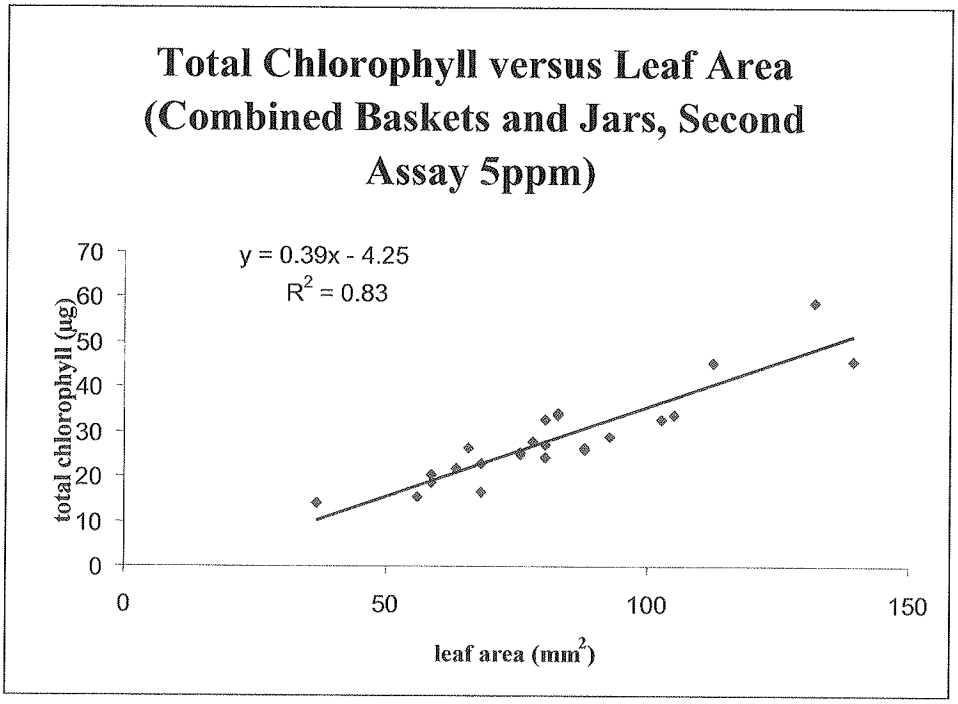

Figure 7.5.2. Variation in chlorophyll levels and leaf area on exposure to 5 ppm 2,4-D.

7.5.3 Auxin-like activity on Photosynthesis: 2,4-D Treatment

It is likely that 2,4-D, at 10 ppm (45 µM), is acting as an auxin in promoting plant growth. It is known that 2,4-D analogues, at 30-50 ppm (ca. 100 µM), (such as 2,4-D-glutamic acid and 2,4-D-aspartic acid) are able to induce 'fruit setting' in tomato plants (Wood and Fontaine, 1952). These results were later supported by the

studies of Feung et al. (1973) in which they found that 2,4-D-glu and 2,4-D, at 100 µM, had similar auxin-like effects on *Avena coleoptile* in cell elongation. An increase in oxygen production is a reflection of an up-regulated photosynthetic process as a prelude to plant growth. This 'growth phenomenon' is not sustainable and *Zostera* outgrows its environment within 2-3 weeks post pulse-chase exposure.

Figure 7.5.3 shows how oxygen production is affected by a range of herbicides and known photo-system inhibitors such as cyanide. Each herbicide treatment was at a concentration of 5 ppm. It is interesting to note how 2,4-D acts as an auxin to stimulate oxygen production whereas its degradation product, 2,4-dichlorophenol, which is an un-coupler of photosynthesis, reduces oxygen production. The synergistic effect of both 2,4-D and 2,4-dichlorophenol was not examined in this study.

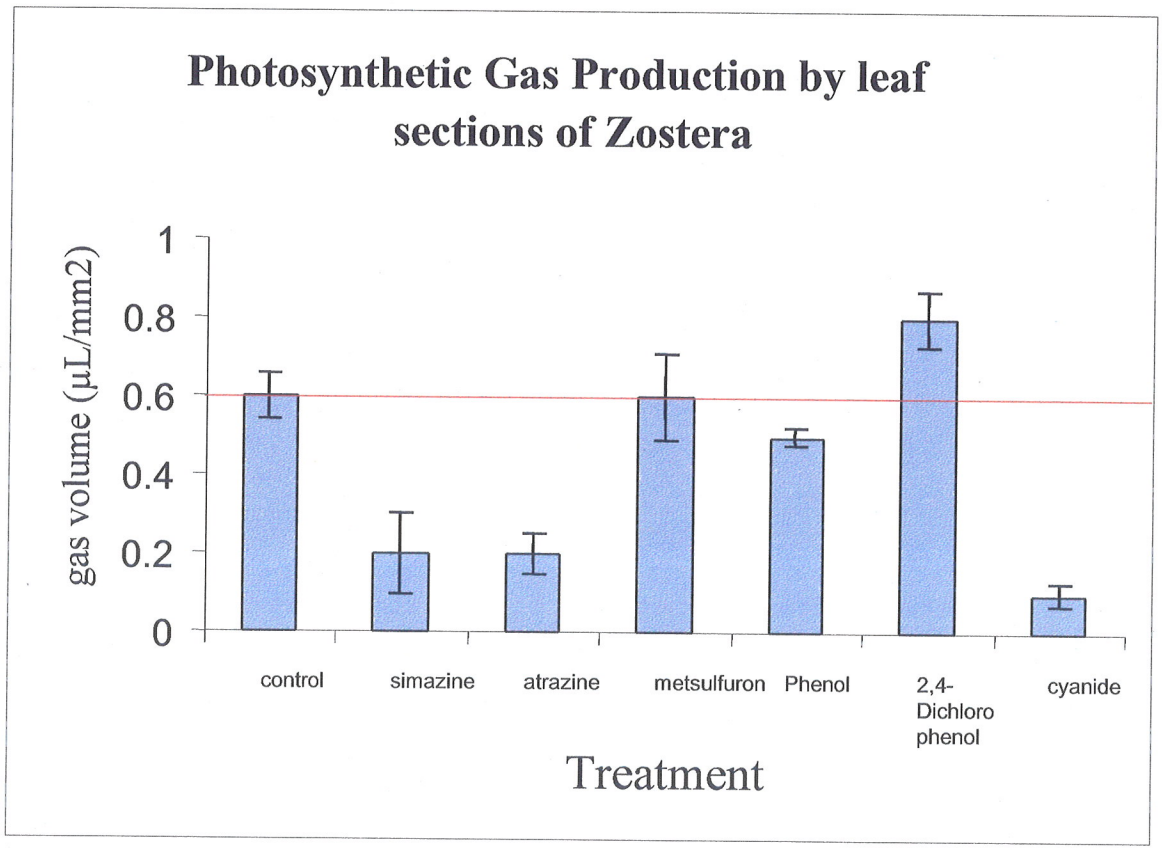

Figure 7.5.3. Variation in oxygen production on exposure to various herbicides at 5 ppm.

7.5.4 Impairment of Phosphate Metabolism caused by impairment of Photosynthesis

Photosynthesised glucose is metabolised by obligate aerobic bacteria in the anoxic root zone of *Zostera muelleri* in the intertidal sediment. The increased, and localised, acetic acid content adjacent to the root zone permits the solubilisation of phosphorus from hydroxyapatite (Craven and Hayasaka, 1982). An inhibition of RUBISCO would limit this process. Accumulated oxygen within the lacunae would compete with carbon dioxide in binding to RUBISCO. RUBISCO would then function as an oxygenase rather than a carboxylase thus promoting photorespiration and wastage

of carbon from the plant. Photo-respiratory processes in terrestrial plants are detrimental to productivity. Additionally, any respiratory processes associated with growing root tips would require an adequate supply of oxygen and carbohydrate. A 2,4-D treated plant would, after an initial photosynthetic 'burst', be limited in its ability to supply such substrates.

Zostera propagates asexually by rhizomes and sexually by seed production. It is known that seagrasses such as Zostera marina are subject to storm damage in shallow waters of one to three metres depth (AIOI KEIKO et al. Seagrass Biology Workshop '96', p. 233). Indeed the eelgrass responded with a larger seedling set subsequent to such damage. If eelgrass is structurally weakened at the base of the leaf sheath by auxin treatment (2,4-D and its analogues) and wave action causes loss of mature green leaves, it is unlikely that mature leaves will develop to produce seedpods. Sexual reproduction will be repressed and recovery of the species from storm damage will be reduced.

Arising from this study, it is suggested that 2,4-D prevents the development of 'mature leaves' and seed production. Such impairment will reduce the production of new plants and will likely reduce the size of the gene pool thereby 'weakening' the species.

For the two herbicides tested, the bioassay results indicate that oxygen metabolism is modified in seagrass that has received a prior treatment with such herbicides. A decreased oxygen production rate was noted in leaf sections from sulfonylurea-treated seagrass (*Z. muelleri*). The decreased oxygen production in this study suggested that photosynthesis is affected by treatment.

An increased oxygen production rate and a decreased potential acid-soluble carbohydrate content in 2,4-D treated seagrass suggest an effect on plant growth. It is known that 2,4-D can affect growth and fruit setting in terrestrial plants. However, the effect of the phenoxy herbicide on *Zostera* is acute in the short-term *viz:* weeks, when the plant dies, after a short-term (12 hours) exposure to 2,4-D. It is thought that the phenoxy-herbicide acts as an auxin inducing an uncontrolled growth in seagrass. Photosynthesis is likely to be up-regulated as a prelude to plant growth. It is then believed that the initial two-fold increase in the oxygen production rate increases the pressure within the lacunae of Z. muelleri. This increased oxygen pressure then inhibits a key enzyme, Rubilose 1,5 bisphosphate carboxylase (RUBISCO), in the carbon sequestering process in photosynthesis. This inhibition then reduces the amount of carbohydrate manufactured.

It is therefore proposed that any short-term gain in the synthesis of carbohydrate (such as glucose) will be quickly (within a week) offset by a decline in carbohydrate synthesis as oxygen, a by-product of photosynthesis, accumulates within the lacunae competitively inhibiting the enzyme RUBISCO. The auxin-like activity of 2,4-D is also known to modify the carbohydrate content of terrestrial plants. In a study on cowpea shoot (Singh et al., 1998) the authors note a reduction of 12 to 28% in carbohydrate (starch) after treating the plants with 2,4-D.

The effects of herbicide, whether acting as a herbicide or where possible as an auxin, compromises *Zostera's* survival in the intertidal zone. Given the widespread and

frequent usage of the herbicides 2,4-D and sulfonylureas in broad acre farming, it is possible to present an assessment of risk to *Zostera muelleri* meadows. Furthermore, and by deduction an assessment of risk to prawn fishing yields can be calculated. If the spatial and temporal usage patterns of the tested herbicides in terrestrial farming are coincident with the spatial and temporal distribution of *Zostera muelleri* an impact on seagrass survival will occur providing a transport mechanism operates between these two farming regions.

It is known that dust storms occur in South Australia with a frequency of ten to seventeen per annum. Indeed, dust storms can transport fine particles many thousands of kilometres. It is also known that herbicides can be transported in dust. It is therefore likely that such herbicide usage will impact upon seagrass survival in South Australia. These results have implications on intertidal seagrass survival regionally, nationally and possibly internationally. Furthermore, since prawns prefer seagrass as habitat, (Alberto J. Sanchez, Seagrass Biology Workshop '96', p. 195) Pink Shrimp (*Penaeus duorarum*), it is also likely that any reduction in seagrass acreage will also adversely affect prawn fishing yields in South Australia.

Light availability is particularly important for seagrass survival and growth (Longstaff and Dennison, 1999, Dennison, 1982). It is interesting that morphological adaptations of seagrass to light attenuation in winter may result in a reduction in the mitochondrial respiratory demand of leaf tissue. Seagrass leaves can become shorter and narrower under such light attenuating growth conditions. These adaptations may therefore be designed to reduce carbohydrate usage as the winter months approach and light availability becomes more limiting. By inducing a 'growth spurt' in seagrass, with 2,4-D, the plant may be pushed into a carbon deficit growth cycle.

It is proposed that the extensive use of 2,4-D on broad acre farms has contributed to the formation of a hidden pool of phenoxy herbicides that can be transported to the intertidal zone where they are converted into the parent herbicide or a xenobiotic with herbicidal activity. Laboratory results have established that the parent herbicide, 2,4-D, will induce a negative and fatal biochemical and biological effect in the seagrass Zostera muelleri. Loss of seagrass caused by this (indirect) auxin-like activity will likely reduce habitat for small fish and crustacea such as prawns. It is well known that prawns prefer seagrass meadows as foraging ground and shelter over bare seabed regions. It has been said that this region provides a nursery area for prawns, fish and custacea (Bell and Pollard 1989BD).

Additionally, when plants metabolise this herbicide the products can be chemically compartmentalised into amino acid conjugates (Feung C.-S., Hamilton R.H., Mumma R.O.) and oils (Chkanikov et al., 1984). This is particularly interesting because 2,4-D moieties can be potentially transported from land as 'stable' conjugates. It is hypothesised that such material can then be converted to the parent material (phenoxy herbicide) in the intertidal zone where it will induce a biological effect on intertidal seagrasses, such as *Zostera muelleri*, causing plant death in the short-term of weeks. Re-activation of parental herbicidal activity is not unusual. Esters of quinclorac are converted to the parent material by a series of omega- and beta-oxidations carried out by soil micro-organisms (Rusness et al., 1998). This conversion is not merely a simple ester hydrolysis.

This notion of bioconversion of 2,4-D metabolites into the parent 2,4-D is particularly important when we consider a potential impact on intertidal seagrasses, since a transport mechanism is available, via wind-blown dust, to this environment and micro-organisms are present within sediment of the intertidal zone. It is therefore hypothesised that the extensive use of 2,4-D (at one to two kilograms per hectare acid equivalents) in broad acre farming has contributed to the formation of a 'hidden' pool of 'potential' herbicidal activity as 2,4-D conjugates. These conjugates can be transported to the environment of the intertidal zone where they will likely act as substrates for bacteria to produce an 'active' form of 2,4-D that will act upon seagrass biochemistry at the level of auxin-like activity. This will result in an induced and uncontrolled growth pattern in *Zostera muelleri* that is not sustainable with the plant being forced into a 'carbon deficit' and an increased susceptibility to known toxic factors; such as sediment anoxia (Terrados et al., 1999), sulphides (Erskine and Koch, 2000), nitrites and heavy metal, within intertidal sediment. This then causes plant death within a period of weeks.

8.0 Benefits and Adoption

While this study has not been able to identify any persistent pesticides in seawater, it has however identified the presence of homologues of the auxin herbicide 2,4-D in many soils over a wide area of the Yorke Peninsula. In some soils, there was also evidence for persistence of other herbicides including trifluralin and the sulfonyl-urea, metsulfuron-methyl.

It was not expected that any industry sector would derive immediate benefit from the research. However, greater community and industry awareness of the impact of persistent organic pollutants generally and, in particular, lipophilic pesticides, on juvenile marine species would be a positive outcome. The toxicity of the organophosphorus insecticide chlorpyrifos to juvenile prawns and juvenile whiting underlines the need for caution in their use in situations adjacent to or with the potential to impact on juvenile prawn and whiting nursery areas.

Changes in land-management practices to protect the sustainability and clean-green image of fishing in the gulfs will be outcomes that will be driven at least by coast-care and land-care groups.

A more proactive role for coast-care and land-care groups in implementing programs monitoring discharges in streams and other waterways

Further research work to better understand the significance of the persistent "auxin-like" herbicide residues found in the soil on seagrass species is required. The research points to *Zostera* being affected by these herbicide residues, although the extent to which other seagrass species would be affected is not known.

9.0 Further Development

Support for additional studies designed to assess the tolerance or otherwise of the other major inter-tidal seagrass species to some of the commonly used herbicides would be necessary. Results from such studies would broaden our understanding of which chemicals have the potential to impact on seagrass beds.

Coordination between groups or organisations wishing, for example, to control mosquitos by spraying, and fisheries agencies would ensure that no chemicals which have the potential to affect juvenile marine species are used. This would require that the impact of agrichemicals on local marine species is known or understood. This in turn requires an understanding of nursery locations for marine species coupled with knowledge of adjacent terrestrial activities. Local Council staff could have a role in coordinating this.

It is recognised that any activity in this broad field requires a sensitive approach. Farmers have enough "on their plate" without being further linked to in-shore marine decline or pollution. Equally, prawn-fishers and other industry sector operators expect that their "harvest" is free of contaminants and that the industry is sustainable.

More talks and/or workshops, at the level of the layperson, stressing the interaction between terrestrial activities and unintended outcomes in adjacent marine ecosystem would be desirable. During this research, it became clear that many operators/farmers/land-holders had not thought about links of this kind nor in detail about the role of land-derived persistent organic pollution on marine ecosystems.

10.0 Planned Outcomes

One of the planned outcomes of this project was to enhance the management of those fisheries that are most directly affected by pollution from persistent organic chemicals, especially pesticides, originating from land. In order to do this it was necessary to examine seawater to determine what if any pesticides were present and then to link these with possible sources on land.

While this study did not find any pesticides in seawater over the course of the research, several pesticides with the potential to impact on marine species were identified in soil samples from land units adjacent to important marine habitats.

A further outcome was to suggest possible processes by which these pollutants could enter the marine environment. Research elsewhere had identified dust as a possible vehicle for the transport of persistent organic pollutants. Examination of the soils sampled in this study showed the presence of residual "auxin-like" herbicide residues in many of them, together with infrequent occurrences of other persistent herbicides such as trifluralin and sulfonylureas. Detailed examination of the dust fraction of these soils, points to dust as a potential source of persistent herbicides in the in-shore marine ecosystem. Persistence of herbicides in soil is due to many factors, however an awareness of the possibility would at least inform soil management practices.

This finding is consistent with previous findings from overseas studies. However common-sense will be required to make management changes because of the sensitivies involved in proposing changes in land management practices especially where the impact is located in another ecosystem. Examples in the literature report on how easily dust has transported pesticides over considerable distances leading to contamination of environments far removed from the source.

The impact of any persistent pesticides on marine species, including juvenile prawns and a widespread in-shore seagrass, was also seen as a relevant outcome. Arising from the toxicity testing undertaken during this study, it was clear that juvenile South Australian marine species such as Western King Prawns and King George Whiting would be adversely affected by the presence of pesticides in their in-shore habitat. These species, particularly prawns, are at the upper end of sensitivity to OP derived insecticides compared with other species recorded in the literature. Whiting were impacted at levels similar to other fin-fish.

The extension work was directed to ensuring that the findings were shown to all stakeholders, and that the management practices which would need to be modified are carefully considered.

11.0 Conclusion

The conclusions that can be drawn from the toxicology section of this study are as follows:

- The 96-hour LC50 for *P. latisulcatus* to chlorpyrifos is 0.44 µg/L. The 96-hour LC_{50} for *S. punctata* to chlorpyrifos is 9.05 µg/L.
- The brain enzyme acetylcholinesterase must be inhibited by more than 66.3% i.e.: 33.7% of normal activity to cause death in *S. punctata.*
- Chlorpyrifos will bioaccumulate in the muscle tissue of *S. punctata* at a level 142.8 times (1.785 mg/kg) that of the ambient environment when exposed to 12:5 µg/L for 96 hours, which may present a hazard for predator organisms.
- The use of juvenile *P. latisulcatus* as an indicator species for toxicity testing of temperate marine environments is limited to its availability.
- The use of *S. punctata* as an indicator species for the health of temperate marine ecosystems is again limited to its availability.
- *P. latisulcatus* may be a good indicator of the potential impact of organophosphorus pesticides on the larger Western King Prawn population.
- *S. punctata* may also be a good indicator of the potential impact of Organophosphorus insecticides on the larger King George Whiting population.

The entry of Chlorpyrifos may pose a threat to both the WKP and KGW juvenile populations that reside in estuary regions. However more research is needed to fully ascertain the effects of organophosphorus pesticides on marine organisms in southern Australia.

Conclusions in relation to the impact of 2,4-D like herbicides on the seagrass *Zostera muelleri* are not as clear cut. Herbicides of this class appear to function as auxins at low levels and more directly as photosynthesis inhibitors to kill the plant at higher levels. Oxygen production is impaired over a short period and this is believed to affect the ability of the seagrass to resist the toxicity of the sediments it grows in.

There appear to be significant concentrations of these crypto-herbicides in most of the soil samples surveyed in this study.

Land-management practices need to take the possibility of herbicide residue build-up into account in weed control activities.

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Appendix 2: Staff

Mr Gareth Lewis was employed on an Australian Postgraduate Industry Award for the duration of this project.

Appendix 3: Sampling Results

The results of the soil sieving procedure for the 100 soil samples are shown in the following tables.

100 g weight within 1 percent		x > 2 mm 1	mm < x < 2 mm 2	um < x < 1 mm 3	um < x < 500 um 4	x < 250 um 5	Total weight (g)	Numbers Alone Total weight (g)
1.00	39 a	0.1	0.4	7	44.5	48.1	100.1	100.1
1.00	39 b	0.5	0.6	8.3	46.3	44.7	100.4	100.4
1.00	39 c	0.6	1	9.8	47.3	41.6	100.3	100.3
1.00	40 a	11.8	12.3	12.1	20.9	42.8	99.9	99.9
1.00	40 b	10.3	10.5	11.7	21.5	46.4	100.4	100.4
1.00	40 c	7	9.5	11.6	21.8	50.4	100.3	100.3
1.00	55 a	50.7	13.5	10.1	9.7	16	100.0	100
0.99	55 b	46.3	13.2	10.7	9.9	18.8	98.9	98.9
1.00	55 c	35.3	17	12.7	12.4	23	100.4	100.4
1.00	56 a	26	14.8	13.6	16	29.9	100.3	100.3
1.01	56 b	24.5	14.8	13.4	15.8	32	100.5	100.5
1.00	56 c	24.9	14.2	13.3	15.6	31.8	99.8	99.8
1.00	9 a	0.2	0.2	1.6	40.5	57.8	100.3	100.3
1.00	9 b	0.3	0.2	1.9	42.3	55.2	99.9	99.9
1.00	9 c	0.1	0.1	2.2	42.2	55	99.6	99.6
1.00	10 a	15.6	10.9	11.7	21.7	40	99.9	99.9
1.00	10 b	17.1	11.7	13	20.2	37.7	99.7	99.7
1.00	10 c	21.5	11.3	12.2	19.2	36	100.2	100.2
1.00	37 a	18.4	16.9	14.4	15.2	35.1	100.0	100
1.00	37 b	19.4	13.4	14.6	17.1	35.1	99.6	99.6
1.00	37 c	18.2	15.7	14.3	17.3	34.3	99.8	99.8
1.00	38 a	49.8	22.7	12.6	7	8.3	100.4	100.4
1.00	38 b	62.9	14.6	8.8	5.9	8	100.2	100.2
1.00	38 c	57.1	18.5	9.6	6.3	8.7	100.2	100.2
1.00	75 a	7	9.9	15.2	28.5	39.3	99.9	99.9
1.00	75 b	11.4	9.2	14.3	28.6	36.3	99.8	99.8
1.00	75 c	8.2	9.7	14.3	28.8	39.2	100.2	100.2
0.99	76 a	17.1	16.1	13.7	17.1	35.4	99.4	99.4
0.99	76 b	13.7	16.2	14.7	17.6	37.2	99.4	99.4
0.99	76 c	14.4	15.2	14.8	18.2	36.4	99.0	99
0.99	47 a	28.1	13.5	12.4	14.4	30.5	98.9	98.9
1.00	47 b	32.7	12.9	11.6	13.8	29.3	100.3	100.3
0.99	47 c	28.4	14.1	10.8	13.9	32.2	99.4	99.4
1.00	48 a	33.2	13.7	11.1	13.8	27.9	99.7	99.7
1.01	48 b	31.4	13.2	11.2	14.2	30.7	100.7	100.7
1.00	48 c	30.5	13.5	11.4	13.7	31.2	100.3	100.3
0.99	79 a	14.3	14.8	15.5	18	36	98.6	98.6
1.00	79 b	12.6	14.9	16.5	18.1	38.2	100.3	100.3
1.01	79 c	12.2	15.5	15.5	18.8	38.5	100.5	100.5
1.00	80 a	0.1	0.3	0.6	19.1	80.3	100.4	100.4
1.00	80 b	0.1	0.2	0.6	17.9	81.4	100.2	100.2
1.00	80 c	0.1	0.2	0.6	17.2	82	100.1	100.1
1.00	71 a	25.6	13.6	13.1	16.4	31.3	100.0	100
1.01	71 b	33.1	12.9	11.7	14.8	28.1	100.6	100.6
1.00	71 c	32.2	12.5	11.8	15.6	28.1	100.2	100.2
1.00	72 a	33.5	19.2	13	14.7	19.1	99.5	99.5
1.00	72 b	35.1	19.3	14.1	14.2	17	99.7	99.7
1.00	72 c	31	17.6	14.7	15.8	20.5	99.6	99.6
1.00	89 a	0	0.3	9.5	38.7	51	99.5	99.5
1.00	89 b	0.1	0.3	9.9	41.7	48.3	100.3	100.3
1.00	89 c	0.1	0.3	10.6	41.2	47.6	99.8	99.8
1.00	90 a	30.4	19.6	18.3	16	16	100.3	100.3
1.00	90 b	27.2	20.7	19.4	15.6	17.3	100.2	100.2
1.00	90 c	27.6	22.2	19.4	14.2	16.3	99.7	99.7
0.96	63 a	36.7	17.1	12.9	11.1	18.4	96.2	96.2
0.99	63 b	30.7	17.2	13.8	12.2	25.4	99.3	99.3
1.00	63 c	28.4	17.7	14.2	13.1	26.9	100.3	100.3
1.00	64 a	31	17.4	13.6	12.4	25.2	99.6	99.6
1.00	64 b	27	17.3	14.6	13.2	27.7	99.8	99.8
1.00	64 c	28.9	17.6	15.1	12.5	25.7	99.8	99.8
1.00	97 a	30.5	17.1	14.5	12.7	25.2	100.0	100
1.00	97 b	32.1	16.3	13.8	12.3	25.3	99.8	99.8
1.00	97 c	29.2	18.1	15	13	25.1	100.4	100.4
1.00	98 a	19.1	12.1	11.9	17.2	39.6	99.9	99.9
1.00	98 b	24.6	10.6	10.6	15.3	38.8	99.9	99.9
1.00	98 c	30.3	12	10.9	14	32.6	99.8	99.8
1.00	85 a	18.2	15.3	17.4	23	25.7	99.6	99.6
0.99	85 b	11.8	12.8	16.9	25.3	31.9	98.7	98.7
1.00	85 c	15.7	14.5	17.5	24.5	27.4	99.6	99.6
1.00	86 a	0.2	0.4	7.4	51.3	40.5	99.8	99.8
0.99	86 b	0.1	0.3	7.4	51.5	40	99.3	99.3
1.00	86 c	0.3	0.4	8.1	49.1	42.2	100.1	100.1
0.95	43 a	30	27.1	18.5	11.7	7.7	95.0	95
1.00	43 b	24	25.7	20.3	14.7	15.7	100.4	100.4
0.99	43 c	33.1	20.9	16.8	13.4	15	99.2	99.2
1.00	44 a	10	9.7	12.7	26	41.6	100.0	100
0.99	44 b	7.9	9.8	13.8	25.1	42.2	98.8	98.8
0.99	44 c	8.1	10.6	14.7	25.2	40.7	99.3	99.3
0.99	61 a	13.5	12.5	11.6	18.7	43	99.3	99.3
0.99	61 b	13.8	10.9	11.6	19	43.9	99.2	99.2
1.00	61 c	12.8	12.1	11.5	19.1	44.1	99.6	99.6
0.99	62 a	2.9	7.2	11	26.6	51.4	99.1	99.1
1.00	62 b	3.6	8.2	11.9	26	49.9	99.6	99.6
1.00	62 c	5	8.7	12.9	25.7	47.8	100.1	100.1
0.94	99 a	43.1	22.3	16.1	9.1	3.2	93.8	93.8
1.01	99 b	35.3	20.7	18.6	12.9	13.1	100.6	100.6
1.00	99 c	40	21.9	17.3	10.7	10.4	100.3	100.3
1.00	100 a	24.4	22.7	18.6	18	16.3	100.0	100
0.99	100 b	27.1	21	17.8	17.5	16	99.4	99.4
1.00	100 c	27.3	20.7	17.8	17.3	16.7	99.8	99.8

	Percentage Weights							
1	2	3	4	5	"5/4"	"5/1"		
0.1	0.4	7.0	44.5	48.1		1	481	481
0.5	0.6	8.3	46.1	44.5		1	89	89
0.6	1.0	9.8	47.2	41.5		1	69	69
11.8	12.3	12.1	20.9	42.8		2	4	4
10.3	10.5	11.7	21.4	46.2		2	5	5
7.0	9.5	11.6	21.7	50.2		2	7	7
50.7	13.5	10.1	9.7	16.0		2	0	0
46.8	13.3	10.8	10.0	19.0		2	0	0
35.2	16.9	12.6	12.4	22.9		2	1	1
25.9	14.8	13.6	16.0	29.8		2	1	1
24.4	14.7	13.3	15.7	31.8		2	1	1
24.9	14.2	13.3	15.6	31.9		2	1	1
0.2	0.2	1.6	40.4	57.6		1	289	289
0.3	0.2	1.9	42.3	55.3		1	184	184
0.1	0.1	2.2	42.4	55.2		1	550	550
15.6	10.9	11.7	21.7	40.0		2	3	3
17.2	11.7	13.0	20.3	37.8		2	2	2
21.5	11.3	12.2	19.2	35.9		2	2	2
18.4	16.9	14.4	15.2	35.1		2	2	2
19.5	13.5	14.7	17.2	35.2		2	2	2
18.2	15.7	14.3	17.3	34.4		2	2	2
49.6	22.6	12.5	7.0	8.3		1	0	0
62.8	14.6	8.8	5.9	8.0		1	0	0
57.0	18.5	9.6	6.3	8.7		1	0	0
7.0	9.9	15.2	28.5	39.3		1	6	6
11.4	9.2	14.3	28.7	36.4		1	3	3
8.2	9.7	14.3	28.7	39.1		1	5	5
17.2	16.2	13.8	17.2	35.6		2	2	2
13.8	16.3	14.8	17.7	37.4		2	3	3
14.5	15.4	14.9	18.4	36.8		2	3	3
28.4	13.7	12.5	14.6	30.8		2	1	1
32.6	12.9	11.6	13.8	29.2		2	1	1
28.6	14.2	10.9	14.0	32.4		2	1	1
33.3	13.7	11.1	13.8	28.0		2	1	1
31.2	13.1	11.1	14.1	30.5		2	1	1
30.4	13.5	11.4	13.7	31.1		2	1	1
14.5	15.0	15.7	18.3	36.5		2	3	
12.6	14.9	16.5	18.0	38.1		2	3	
12.1	15.4	15.4	18.7	38.3		2	3	
0.1	0.3	0.6	19.0	80.0		4	803	
0.1	0.2	0.6	17.9	81.2		5	814	
0.1	0.2	0.6	17.2	81.9		5	820	
25.6	13.6	13.1	16.4	31.3		2	1	
32.9	12.8	11.6	14.7	27.9		2	1	
32.1	12.5	11.8	15.6	28.0		2	1	
33.7	19.3	13.1	14.8	19.2		1	1	
35.2	19.4	14.1	14.2	17.1		1	0	
31.1	17.7	14.8	15.9	20.6		1	1	
0.0	0.3	9.5	38.9	51.3		1	#DIV/0!	
0.1	0.3	9.9	41.6	48.2		1	483	
0.1	0.3	10.6	41.3	47.7		1	476	
30.3	19.5	18.2	16.0	16.0		1	1	
27.1	20.7	19.4	15.6	17.3		1	1	
27.7	22.3	19.5	14.2	16.3		1	1	
38.1	17.8	13.4	11.5	19.1		2	1	
30.9	17.3	13.9	12.3	25.6		2	1	
28.3	17.6	14.2	13.1	26.8		2	1	
31.1	17.5	13.7	12.4	25.3		2	1	
27.1	17.3	14.6	13.2	27.8		2	1	
29.0	17.6	15.1	12.5	25.8		2	1	
30.5	17.1	14.5	12.7	25.2		2	1	
32.2	16.3	13.8	12.3	25.4		2	1	
29.1	18.0	14.9	12.9	25.0		2	1	
19.1	12.1	11.9	17.2	39.6		2	2	
24.6	10.6	10.6	15.3	38.8		3	2	
30.4	12.0	10.9	14.0	32.7		2	1	
18.3	15.4	17.5	23.1	25.8		1	1	
12.0	13.0	17.1	25.6	32.3		1	3	
15.8	14.6	17.6	24.6	27.5		1	2	
0.2	0.4	7.4	51.4	40.6		1	203	
0.1	0.3	7.5	51.9	40.3		1	400	
0.3	0.4	8.1	49.1	42.2		1	141	
31.6	28.5	19.5	12.3	8.1		1	0	
23.9	25.6	20.2	14.6	15.6		1	1	
33.4	21.1	16.9	13.5	15.1		1	0	
10.0	9.7	12.7	26.0	41.6		2	4	
8.0	9.9	14.0	25.4	42.7		2	5	
8.2	10.7	14.8	25.4	41.0		2	5	
13.6	12.6	11.7	18.8	43.3		2	3	
13.9	11.0	11.7	19.2	44.3		2	3	
12.9	12.1	11.5	19.2	44.3		2	3	
2.9	7.3	11.1	26.8	51.9		2	18	
3.6	8.2	11.9	26.1	50.1		2	14	
5.0	8.7	12.9	25.7	47.8		2	10	
45.9	23.8	17.2	9.7	3.4		0	0	
35.1	20.6	18.5	12.8	13.0		1	0	
39.9	21.8	17.2	10.7	10.4		1	0	
24.4	22.7	18.6	18.0	16.3		1	1	
27.3	21.1	17.9	17.6	16.1		1	1	
27.4	20.7	17.8	17.3	16.7		1	1	

Proportions from the sieving as a percentage of the soil