CHAPTER 1: INTRODUCTION

1.1 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 in the Barker Inlet, South Australia (Figure 1.1). Coastlines are stabilised by this flowering plant that also makes an important contribution to productivity in coastal regions (Klumpp *et al*., 1989).

Figure 1.1: View, looking eastwards of the sample collection site for *Z. muelleri*.

It has been estimated that the seagrasses of Western Australia cover the same area as rainforests in the whole of Australia (Kirkman, 1992). This region provides a nursery area for prawns, fish and crustacea (Bell & Pollard, 1989). Fronds of seagrass serve as habitat for the many epiphyte species and algae (Ducker *et al*., 1977). Prawns also graze on epiphytes (Morgan, 1980). Indeed, it is known that prawns (*Penaeus*) prefer seagrass habitat to denuded regions of seabed (Young, 1978; Subramaniam, 1990). Larger

animals such as dugongs and green turtles also forage in seagrass meadows and use it 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 provide food for migratory birds that use these mudflats as feeding grounds. Seagrass meadows therefore provide a habitat and foraging ground for many fauna of the intertidal zone and make a significant contribution to the recycling of organic matter in this region. Any negative impact upon *Z. muelleri*, 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 worldwide. Indeed, world losses of seagrass have been likened to a ‘wasting disease’ (Giesen *et al*., 1990). Such losses have been noticed since the late 1930’s and perhaps more so since that time with increasing urbanisation and industrial activity in coastal regions. It is likely that the loss of seagrass has had an economic as well as an ecological impact. The gross value of production derived from the commercial fishing in Australia was $ 2.2 billion in 2002 – 03 (abare inovation in economics). 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) 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. The present 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’. This study has demonstrated that it is likely that susceptible biota of the intertidal environment are negatively impacted upon by land-use chemicals, such as herbicides, derived from land.

The seagrass *Z. muelleri* grows in a region of the intertidal zone that is bounded by a low water level (low tide) and a high water level (high tide). The beginning of a mangrove habitat circumscribes the high water level of the intertidal zone of the study region. A transect can be drawn at right angles to the shoreline, through the mudflat and towards the mangrove line (Figure 1.2).

Figure 1.2: View, looking eastwards, of the sample collection site for *Z. muelleri*. The yellow line represents a transect extending from the marine to the terrestrial environment.

This transect is also represented schematically in an idealised model for *Z. muelleri* growth in the intertidal zone in Figure 1.3. The model is based on a (hypothetical) normal distribution (Clarke, 1980) derived from the equation: -

$f(x) = (1/\sigma\sqrt{2\pi})e^{-(x-\mu)^2/2\sigma^2}$

Where $\sigma = 8$, $\mu = 51$, with curves 'A' and 'C' being offset by two standard deviations from curve 'B'. The abscissa is omitted to permit the use of the model in a generalised discussion.

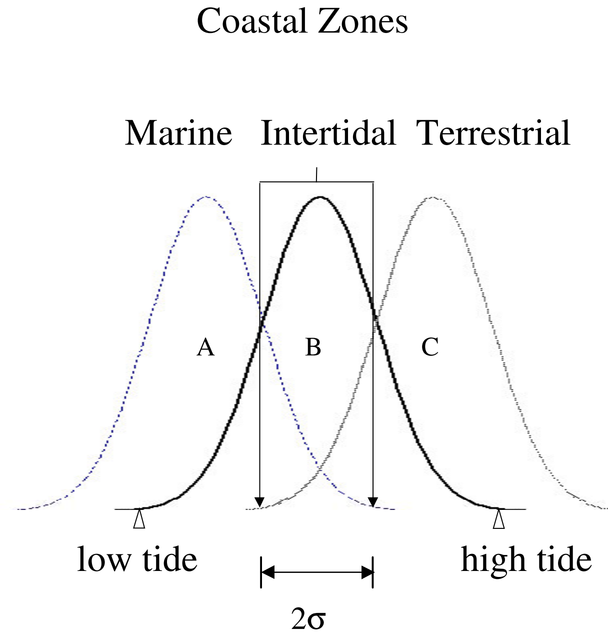


Figure 1.3: Coastal zones at the sample collection site for *Z. muelleri*.

It can be seen that approximately 63 % of the plants grow within one standard deviation of a mean value in a hypothetical normal distribution 'B'. This model has some merit in that light attenuation at the 'low tide line' (caused by the action of wave turbulence and an upwelling of sediment (Longstaff & Dennison, 1999)), epiphyte overgrowth of leaves and water depth (Dalla *et al.*, 1998) are known to reduce the photosynthetic capacity of seagrass leaves and compromise seagrass survival. Additionally, desiccating forces acting at the 'high tide line' are likely to impose osmotic stresses on *Z. muelleri*.

In this model *Z. 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 (Perez *et al*., 1994) and ultraviolet (UV-B) light (Trocine *et al*., 1981) are also likely to limit growth of *Z. muelleri* within this zone. Other stress factors *within the sediment* such as heavy metals (Faraday, 1979; Prange & Dennison, 2000), anaerobic conditions, nitrite and sulfide toxicity (Jonkers *et al*., 2000), and a limitation in the supply of nutrients, such as bioavailable phosphate (Craven & 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.

1.2 Losses of seagrass meadows

Seagrass is being lost from such coastal environments, with losses amounting to 60 % of historical acreage values. The cause(s) of such seagrass loss have been the subject of much research. 'Point source' contamination (Scarlett et al., 1999; Sargent et al., 2000) and 'diffuse source' contamination (Bester, 2000) by herbicide formulations have often been the focus of this research. Point source is used here to describe a known source of contamination, such as herbicide (triazine)-based antifouling agents used on ship hulls, of an environment where cause and effect are clearly established. Diffuse sources of contamination cannot be precisely defined in terms of their cause and effect. Indeed, many point sources of contamination result in a diffuse source contamination for the receiving environment.

In an Environment Protection Agency (EPA South Australia, 1998) study of the South Australian metropolitan coastline between Largs Bay and Glenelg, it was noted that regions of seagrass have been lost causing an increase in exposed seabed. The study was supported by aerial photographic data. The report of the study states that sewage effluent and stormwater drain construction '*may have impacted'* on seagrass loss (EPA South Australia, 1998, page 13). The authors' caution in terminology is justified since it is known that losses of seagrass can also be attributed to eutrophication (caused by nutrient outflows), storm damage (Preen *et al*., 1995; Longstaff & Dennison, 1999) and possibly

herbicides (Scarlett *et al*., 1999; Bester, 2000). While these observations can be correlated with an event(s), it does not necessarily follow that the observation was caused by the event. 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 and urban activity (Bester, 2000; Seddon *et al*., 2000) are less easily defined in their 'cause and effect relationships'. Environmental contaminants can be transformed by chemical, physical and microbial processes into secondary "bioactive chemicals", as seen for example in Minamata Bay where non-toxic mercury was transformed into the highly toxic methyl mercury by micro-organisms in the sediment (Harada, 1972; Takizawa, 1979; Mitra, 1986).

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 in this thesis, since the former is defined as merely the presence of a chemical within an environment whereas the latter is defined as a biological impact(s) of a chemical.

1.3 Is the intertidal zone a farm?

A 'farm' is defined for the purpose of this thesis as a region that has *spatial* and *temporal* attributes suitable for the growth of products for the marketplace. Geographic location and altitude can affect the spatial attributes of farms while temporal attributes can be affected by seasonal changes, time of planting, timing of pesticide usage and crop harvest (Figure 1.4). The intertidal zone is restricted spatially between the upper mangrove line and the low tide mark. It therefore fulfils the first requirement in the definition of a farm. Furthermore, seagrass can be cropped and sold at market for such purposes as making mats, thus fulfilling the second requirement in the above definition of a 'farm'. Interestingly, the aquaculture of oysters is also described as a *farming* practice in the English dictionaries.

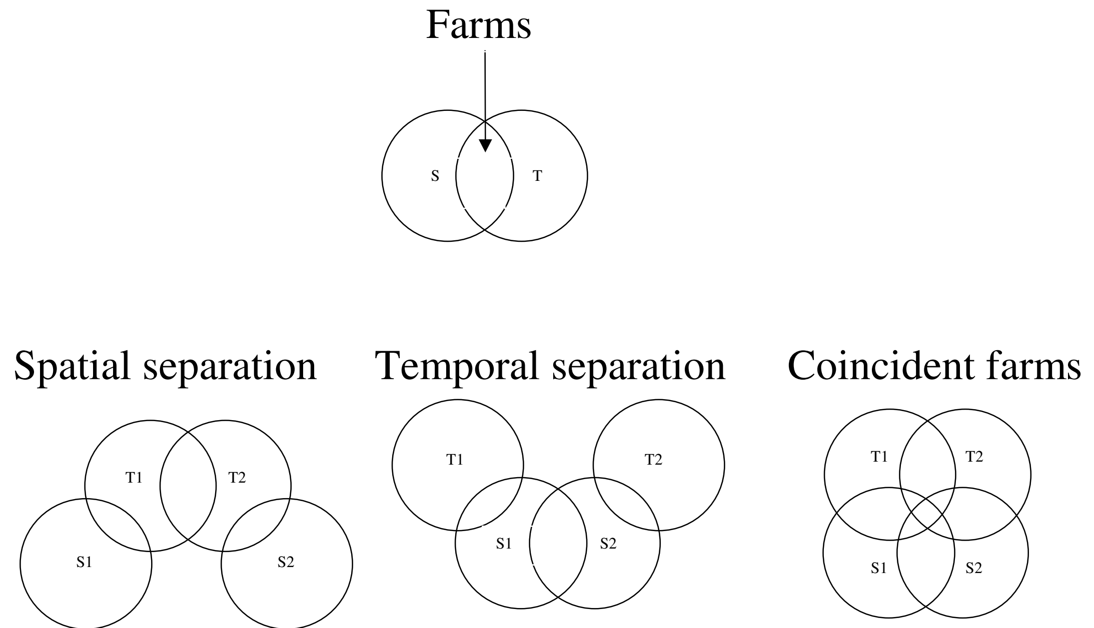


Figure 1.4: Spatial and temporal attributes of farms. Farms have spatial (S) and temporal (T) attributes. Two adjacent farms can interact with one another in their spatial and temporal attributes.

In this thesis the interaction and interrelationship between seagrass and other biota of the intertidal zone are considered by assessing the possible biological impact(s) of a physicochemical insult. For instance, seagrass is a preferred habitat of gilled fish such as *Sillaginodes punctata* (Cuv. & Val.) or King George whiting (Connolly, 1994) and prawn (Morgan, 1980; Subramaniam, 1990). Indeed, it has been demonstrated that a greater percentage of grass shrimp (*Palaemonetes*) remained in 'seagrass' regions over those in denuded seabeds (Eggleston *et al*., 1998). Prawns use seagrass as habitat in which to graze and shelter from predators (Mizushima & 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 has weakened soil structure and has raised the water table creating land erosion and rising salt respectively. These factors have then negatively impacted upon the *useful attributes* of land as a region on which commercial crops, such as wheat and barley, can be grown.

1.4 *Zostera muelleri* – growth, photosynthesis and culture

1.4.1 Evidence of light adaptation and physiological responses

Studies of the physical adaptations of seagrass have provided an insight into the importance of light by noting that light attenuation, within the range 1 to 972 $\mu E\ m^{-2}s^{-1}$, caused by cyclic (annual) pulse-turbidity events are probably implicated in seagrass loss in estuarine environments (Longstaff & Dennison, 1999). The authors note that one species in particular, *Halodule pinifolia*, was better adapted than other species, e.g. *Halophila ovalis*, to such cyclic events. 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. Interestingly, decreases in the chlorophyll a/b ratio, from 2.5 to 2.1, are known to accompany light deprivation caused by these turbidity events (Longstaff & Dennison, 1999). A reduction in the chlorophyll a/b ratio has also been noted in light attenuation studies of *Zostera marina* in which ratios of 2.2 and 1.8 were measured at shallow and deep stations respectively (Dennison & Alberte, 1982). The authors think it probable that the increase of chlorophyll 'b' relative to chlorophyll 'a' somehow increases the light capturing efficiency of chloroplasts.

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

The results of 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 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 used here as meaning the level of nutrient uptake and utilisation under 'normal' growing conditions.

1.4.2 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 is defined as the photon flux per unit area between 400 and 700 nm; full sunlight is approximately 2000 μE $m^{-2}s^{-1}$; one mole of photons being equal to one Einstein (Tyerman, 2002). It is well documented that the biomass above the sediment when expressed as a ratio to the biomass below the sediment surface is greater in limited light conditions in the seagrasses *Thalassia testudinum* (Dawes & Tomasko, 1988; Lee & Dunton, 1997) and *Halodule wrightii* (Dunton, 1994). It is anticipated that both the higher incident PAR and water temperature 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 up-regulated under conditions of increased light and temperature.

It is, however, also known that carbon loss via photorespiration and an up-regulated oxidase activity of ribulose 1,5-bisphosphate carboxylase (RUBISCO) occurs at higher temperatures (Stryer, 1981; Bowes *et al*., 2002). It is known that RUBISCO's 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. A high nutrient inflow, often from anthropogenic activity such as sewage outfall, causes eutrophication of seagrass-containing waters producing increased epiphyte growth (Wear *et al*., 1999). Epiphytes attenuate the available light by covering a greater proportion of the leaf's surface area (Sand-Jensen & Sondergaard, 1981; Kemp *et al*., 1988; Day *et al*., 1989) thereby reducing seagrass productivity (Kemp *et al*., 1988; Tomasko, 1993). Attenuation by epiphytes on *Thalassia testudinum* has been documented to be as much as 33 % - 56 % of PAR (Dixon & Kirkpatrick, 1995) at depth in west central Florida (USA). It has also been noted that approximately 13 % of the surface irradiance is sufficient to maintain *T. testudinum* (Dixon, 2000).

Algal overgrowth of seagrass leaves *can reduce* the amount of PAR by as much as 60 % reaching the leaf surface. Such light attenuation effects and maintenance lighting conditions in *T. testudinum* have been corroborated by others (Czerny & Dunton, 1995). Additionally decreases in biomass in the species *H. wrightii* and *T. testudinum* in Texas (Dunton, 1994; Czerny & Dunton, 1995) are also thought to be associated with a long-term reduction in PAR to 5 moles m^{-2} d^{-1} (116 $\mu E\ m^{-2}s^{-1}$) during seasonal changes. Decreased photosynthetic efficiency may have been responsible for the noted decrease in tissue biomass with the onset of winter. Maximal PAR was associated with maximum growth rates (LRGR) for *T. testudinum*. However maximum growth rates are not wholly dependent on PAR. Others have also noted the 'decoupling between growth rates and available light' (Hall *et al.*, 1991; Czerny & Dunton 1995). Therefore growth is not a reliable indicator of low light stress. It is suggested that thermal cues (Tomasko & Hall, 1999) and *endogenous seasonal rhythms* may supersede light availability. Nevertheless, an underlying theme that can be identified from these studies is that, all other factors being constant, a sufficient light regime is essential for seagrass growth and survival. This phenomenon is not restricted to seagrasses and is known to apply to commercially grown terrestrial crops as well, for example grapevines, that 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, 2002).

These data suggested that lighting of 250 $\mu E\ m^{-2}s^{-1}$ was sufficient to maintain *T. testudinum* and possibly other seagrass species, such as *Z. muelleri*, under a light-stressed condition or a minimum maintenance condition.

1.4.3 Nutritional requirements of seagrass

Healthy seagrass stands require an adequate supply of inorganic carbon (Andrews & Abel, 1979; Millhouse & Strother, 1987; Neely, 2000), nitrogen (Touchette & Burkholder, 2000) and phosphorus (Craven & Hayasaka, 1982; Touchette & Burkholder, 2000). Trace elements, as enzyme cofactors, are also likely to be required for normal seagrass growth. Axenic cultures of *Halophila decipiens* have been grown under laboratory conditions in modified agar supplemented with nutrients (Bird *et al.*, 1998). However replicating all environmental conditions normally present in

Z. muelleri's natural environment is a difficult task. Additionally, this would prevent the study of possible synergistic interactions between *Z. muelleri* and its sediment environment.

1.4.4 Carbon fixation

Carbon dioxide (CO_2) gas has limited diffusion in seawater (Hellblom, 2000) and is unlikely to *directly* supply sufficient inorganic carbon for the photosynthetic process within seagrass. Carbon can, however, be partitioned into bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) anions as coexisting forms. At the pH of seawater (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 CO_2 and water (Badger & Price, 1994; Falkowski & Raven, 1997). An additional or ancillary 'carbon concentrating mechanism', entitled the C4 pathway (Raven, 1997; Bowes *et al*., 2002), permits the shunting of carbon as C4 precursors (such as oxaloacetate) from the mesophyll cells to the bundle sheath as malate. An NADP+ -linked malate dehydrogenase releases CO_2 from malate, and enzymic reaction within the bundle sheath cells then converts the oxaloacetate to 'free' CO_2 and pyruvate, a C3 compound that is then recycled to the mesophyll cells. This is an energy demanding process but permits the concentration of CO_2 in the bundle sheath under high sunlight and arid conditions that are present in the intertidal zone.
The sequestered carbon is then utilised as CO_2 in the photosynthetic reactions described by the Calvin Cycle. Products of the Calvin Cycle are simple sugars, starches and the by-product molecular oxygen (O_2). Sugar production, by seagrass, and its possible release into sediment may impact upon phosphate metabolism (see Section 1.4.6). Losses of carbon from the Calvin Cycle by the process of photorespiration range between 25 % and 50 % of the captured carbon in terrestrial plants such as grapevines (Tyerman, 2002). It is noteworthy that seagrasses have been described as both a C3 plant and a C3/C4 plant (Touchette & Burkholder, 2000).

1.4.5 Nitrogen fixation and recycling

Inorganic nitrogen is obtained from degraded bacterial detritus that forms part of the sediment (Blackburn, 1990). Organic nitrogen in dead leaf matter can also be recycled via bacterial activity within the sediments (Caffrey & Kemp, 1990; Rysgaard *et al*., 1996; Touchette & Burkholder, 2000). Storms are likely to increase the outflow of nitrogen by causing excessive leaf loss via weakened abscission zones. Increased rates of leaf senescence and the development of abscission zones in terrestrial plants are caused by the auxin-like activity of herbicides such as 2,4-D (Wernicke *et al*., 1986; Sandmann *et al*., 1991), quinclorac (Grossmann, 1998) and picloram (Musiyaka, 1995). 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 (Blackburn, 1990).

1.4.6 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 ($Ca_5(PO_4)_3OH$) have been detected in sediments associated with *Zostera marina* seagrass beds (Craven & Hayasaka, 1982). It is interesting to note that the authors discovered the presence of glucose and acetic acid in the same sediments. They hypothesised that the simple sugar, glucose, is metabolised by aerobic bacteria into acetic acid that may then facilitate the dissolution of phosphate (PO_4) from hydroxyapatite.

1.4.7 Sulfate-reducing bacteria: possible toxic implications

It has been noted that most sulfate-reducing bacterial activity is restricted to the top 12 cm of seabed in *Zostera* seagrass meadows (Blackburn, 1990). 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. Molecular oxygen, supplied from

Zostera's root zone, may then serve to detoxify sediments and synthesize nitrate and sulfates from nitrites and sulfides 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 an amount of 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 both dependent, in part, on a sufficient supply of O_2 reaching the rhizosphere to negate sediment toxicity and to facilitate a symbiotic or co-habitation relationship between *Zostera* and obligate aerobes in the sediment of the root zone. Under normal conditions, *Zostera* supplies sufficient O_2 to the sediment through 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 O_2 or glucose to the rhizosphere. An insufficient supply of O_2 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 toxic effects within the sediment.

1.5 Photosynthesis in aquatic plants, the partial pressure of O_2 gas within lacunal canals, and its significance for plant growth and survival

Photosynthesis is the *synthesis of carbohydrate* by using light energy to capture CO_2. During this process O_2 is made as a by-product (Figure 1.5). 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 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 used to 'regenerate' ribulose 1,5-bisphosphate. Carbon dioxide is supplied to the Calvin Cycle as CO_2 (g) 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 (Touchette & Burkholder 2000).

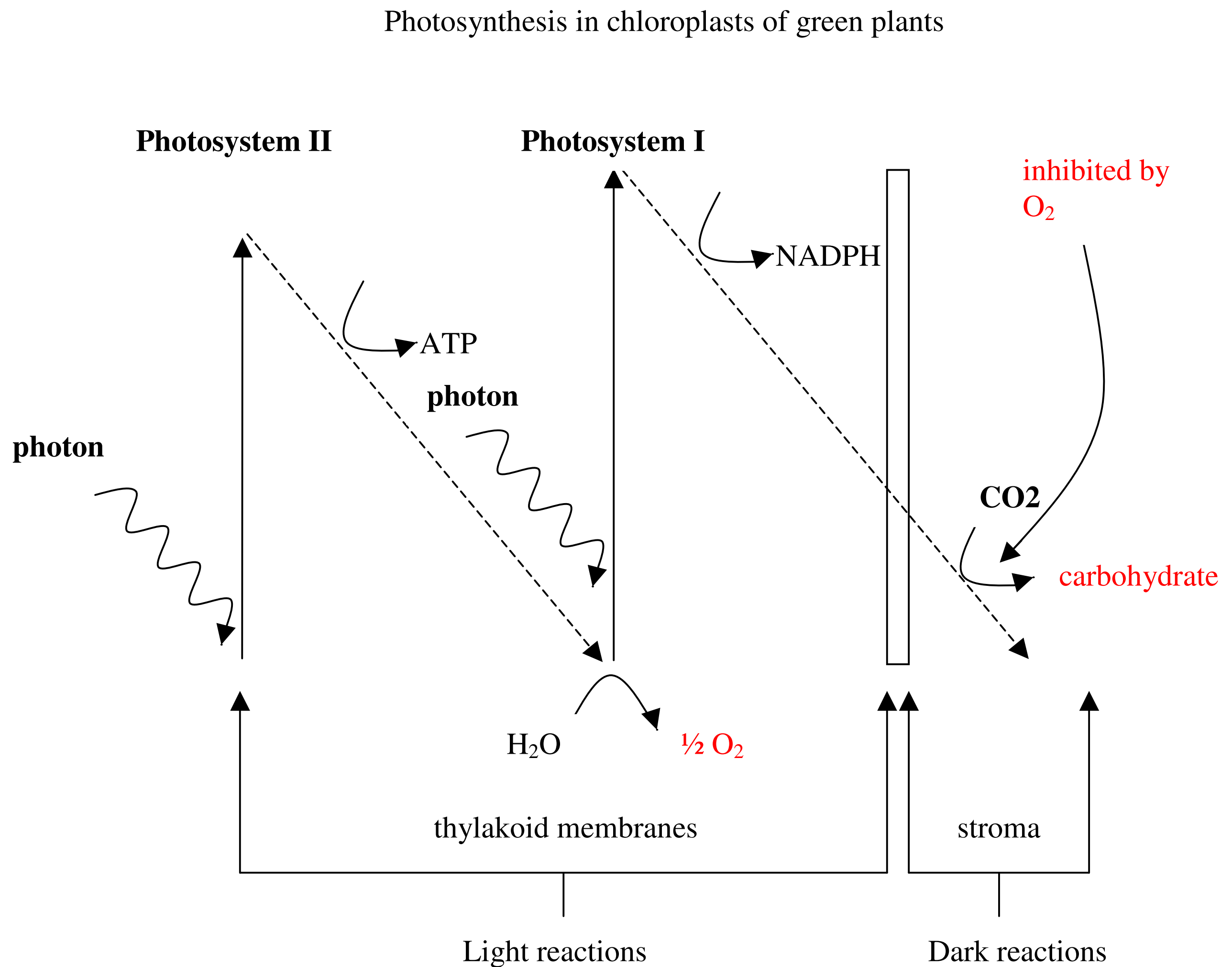


Figure 1.5: Photosynthesis in aquatic plants (adapted from Stryer, 1981).

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 photoinhibition (Ralph & Burchett, 1995), heavy metals (Ralph & Burchett, 1998b), osmosis (Ralph, 1998), petrochemicals (Ralph & Burchett, 1998a) and combinations of factors (Ralph, 1999). The impact of herbicides on chlorophyll fluorescence in the seagrasses *Halophila ovalis* (Ralph, 2000) and *Zostera capricorni* (Haynes *et al*., 2000)

has also been investigated. However, 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. For instance, Hill (from Stryer, 1981) demonstrated in 1939 that isolated chloroplasts are able to lyse water and produce O_2 (Figure 1.5) with ferricyanide being used as the terminal electron acceptor in place of CO_2. Therefore electron-flow, and presumably chlorophyll fluorescence, does not necessarily equate with carbohydrate production. Other investigators (Maxwell & Johnson, 2000) have addressed such issues associated with the appropriate use of chlorophyll fluorescence in the measurement of photosynthesis. The authors point to conditions under which higher levels of O_2 (> 2 %) may contribute to a photorespiratory loss of carbon. It is therefore crucial to measure the concentrations of CO_2 and O_2 together with produced carbohydrate in order to relate fluorescence measurements (electron transport) to photoproductivity (production of carbohydrate).

The roots of sediment-bound benthic aquatic plants are immersed in water and are therefore limited in their supply of O_2 from the external sediment. Indeed, the sediments in which aquatic plants grow are often anoxic (Pregnall *et al*., 1984; Lee & Dunton, 2000). Aquatic plants have therefore adapted to these waterlogged environments by producing O_2, in their leaves by photosynthesis, and then transporting the gas via lacunae to the roots to facilitate respiration and to prevent possible toxic effects from sulfide-containing sediments (Lee & Dunton, 2000). Release of O_2 from the roots of several aquatic plants has been quantified and the results presented as a percentage of total O_2 released by plants (Sand-Jensen *et al*., 1982). The authors noted that Z. *marina* released 1 % of its total released O_2, into the root zone of the plant with the remainder being released into seawater above the sediment zone. The authors also pointed out the limited capacity of the lacunae in this species. It is therefore likely that this species has a limited ability to store and use O_2 for respiratory processes, since O_2 is used as a co-substrate in mitochondrial respiration. Additionally, the authors hypothesise that the partial pressure of O_2 within lacunae is higher during the photoperiod than in darkness. An increase in the partial pressure of gases within freshwater aquatic plants has also been recorded when plants were subjected to artificial lighting (Laing, 1940). The

increase in the partial pressure of O_2 within *Zostera*'s lacunae may be the result of a low diffusion coefficient and a poor gas-exchange of O_2 into seawater. This slow exchange of O_2 does not occur in terrestrial plants since 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.

1.5.1 Can the partial pressure of oxygen within lacunae affect carbohydrate synthesis?

In the presence of O_2, RUBISCO can act as both an *oxygenase* as well as a *carboxylase* (its 'normal' function, Figure 1.6). In fact this enzyme preferentially binds O_2 over CO_2.

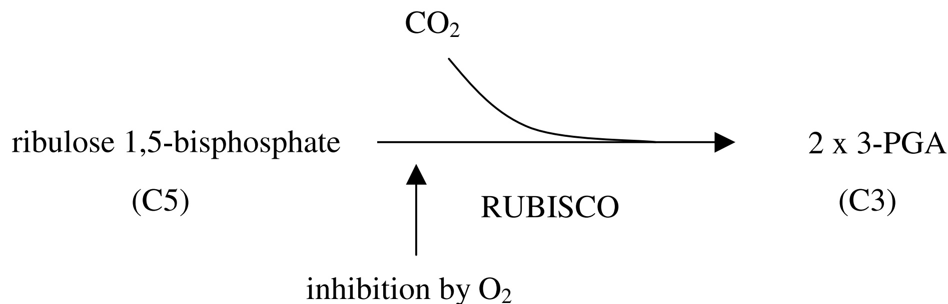


Figure 1.6: Inhibition of the Calvin Cycle by oxygen.

As the temperature is increased, the oxygenase activity of RUBISCO increases at a faster rate than does its carboxylase activity (Stryer, 1981). This may have implications for plant growth in exposed environments such as the intertidal zone.

A 30 % reduction in net photosynthesis in *Z. muelleri* has been noted in plants that were exposed to sunlight as opposed to plants that were submerged (Clough & Attiwill, 1980). The authors 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 ^{14}C labelled sodium bicarbonate, show that a significant proportion of the label is partitioned into photorespiratory intermediates (Andrews & Abel, 1979).

Such data suggests that photorespiration is likely to make a significant contribution to the efflux of carbon from seagrasses.

The oxygenase activity of RUBISCO is less problematic for terrestrial plants since O_2 easily diffuses from the lacunal canals and stomata. 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 hypothesised that any increased O_2 production would exacerbate this carbon-loss process and would place the plant in a 'carbon deficit state'.

1.6 Stress factors that can act upon *Z. muelleri*

Plants and animals that inhabit the intertidal environment are constrained by the stringent growth conditions imposed on them by their environment and have therefore adapted to a variety of 'stress factors'. *Zostera*'s growth and survival is dependent on innate adaptations within *Zostera* and the interactions between *Zostera* and its biotic and abiotic surroundings. It is the efficacy of these adaptations and interactions that determine whether it can survive in the intertidal zone.

Stress factors such as light availability are known to have a direct effect on *Zostera*'s growth and survival (Dennison, 1979; Dennison & Alberte, 1982). Photosynthetically active radiation (PAR), can become limited by epiphyte overgrowth on leaves (Dixon & Kirkpatrick 1995), reduced water clarity (Longstaff & Dennison, 1999) and increased water depth (Dennison & Alberte, 1986). Light attenuation can be caused by epiphyte overgrowth and is controlled, in part, by grazing gastropods (Neckles *et al*., 1993). Additional light attenuating factors are also caused by seasonal wave action, storms (Longstaff & Dennison, 1999), tidal movements and global climate changes (Beardall *et al*., 1998; Haeder *et al*., 1998; Short & Neckles 1999). Terrestrial plants (Li *et al*., 1993) and 'aquatic' plants such as *H. ovalis* can also be directly affected by ultraviolet (UV-B) light (Ralph & Burchett 1995). Plants produce pigments that dissipate the high-energy incident light that would otherwise induce photodamage by photobleaching of organelles

such as chloroplasts (Dawson & Dennison, 1996). Alternatively, 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 *et al*., 1981). However, such adaptations come at an 'energy cost' to the plant. While *Z. 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 & 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 O_2 and carbohydrate into the root zone will subsequently initiate a 'nutritional stress factor' by reducing the bioavailability of phosphorus from hydroxyapatite. Furthermore, monitoring of sulfide levels during growth and senescence phases of benthic macroflora has revealed that seasonal changes (Viaroli *et al*., 1996) in this potentially toxic metabolite are linked to the production of O_2 by the seagrass *Zostera noltii*. However results of studies conducted under laboratory conditions on the seagrass *Thalassia testudinum* (Erskine & Koch, 2000) suggest that a 10 mM pulse of sulfide over a 48 hour period was 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 sulfides 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 sulfide. They concluded that sulfides may play a critical role as a *root carbon drain* over long-term exposures.
Other toxicants such as heavy metals (Malea, 1994; Malea *et al*., 1995; Warnau *et al*., 1995; Prange & Dennison 2000) are also thought to have an adverse effect on seagrass survival.

1.6.1 How does *Z. muelleri* cope with known stress factors?

In order to survive in a habitat that contains heavy metals (Ralph & Burchett, 1998b; Prange & Dennison, 2000), sulfides (Erskine & Koch, 2000) and nitrites (Iizumi *et al*., 1980) seagrasses detoxify their own environment (Lee & Dunton, 2000). This is

achieved by synthesizing, transporting and releasing O_2 into the intertidal mud. This process is believed to occur in a diurnal fashion that prevents toxic sulfide intrusion into the roots of seagrasses. Seagrass creates a non-toxic microenvironment adjacent to its root zone, thereby providing protection from any toxic effects within the sediment. It is also likely that O_2 within the plant is used primarily for 'detoxifying of the sediment' and for mitochondrial respiration (as measured in meadows of *Z. muelleri* by Clough & Attiwill, 1980). 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 stress. 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. In the winter months carbohydrate storage is accompanied by less tissue mass above the sediment. A critical point is thought to exist beyond which a plant is either in 'carbon credit' or in 'carbon deficit'. The interplay of microbial ecology with available O_2 within anoxic sediment of the intertidal zone can determine the magnitude of toxic stress imposed on these intertidal plants by sulfides and nitrites within the sediments.
The 'stress factors' noted previously 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 1.7) within which *Z. muelleri* can grow.

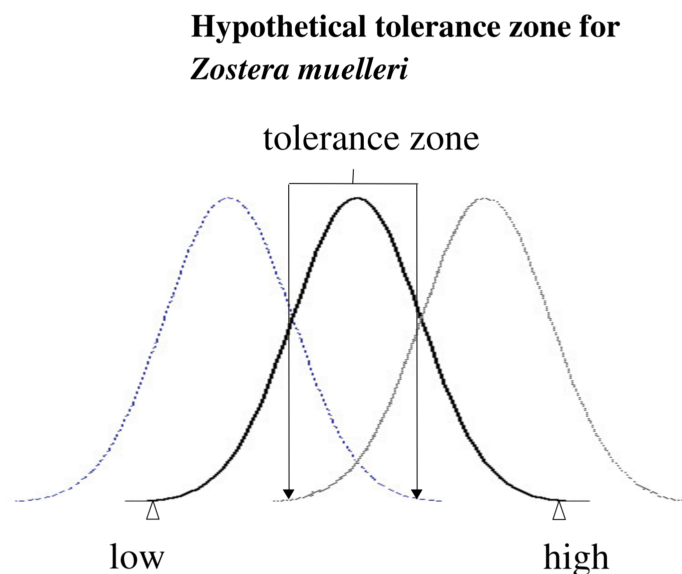


Figure 1.7: A tolerance zone for *Z. muelleri*.

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. To maintain its environment, *Z. muelleri*, along with other marine aquatic plants, therefore modifies the environment of the sediment adjacent to the root zone by releasing O_2 produced during photosynthesis. The released O_2 is then used to detoxify nitrites and sulfides and probably also provides O_2 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 (Craven & Hayasaka, 1982).

1.6.2 Lacunal gas pressure and leaf morphology; an *induced stress* imposed on ribulose 1,5-bisphosphate carboxylase (RUBISCO)?

Lacunae are continuous gas vessels from the leaves to the root zone of *Z. muelleri* (Kuo *et al*., 1990). They are segmented, at regular intervals, by a series of septa composed of parenchymal cells that are a few cell layers thick. The parenchymal cells are metabolically active containing both mitochondria and chloroplasts. The septal cells within *Z. muelleri* are arranged in such a way that small gaps between them permit gas flow into the adjacent 'lacunal compartment' within *Z. muelleri*. Similar structures may also exist in other seagrasses such as *Z. marina* and *Halodule pinifolia*. Indeed, studies of the freshwater aquatic water lily (*Nuphar advenum*; Laing 1940)), have demonstrated that similar structures are 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 O_2 and CO_2 gases within the lacunal spaces were also noted in this study. The author likened the gas spaces to 'leaky reservoirs'. Other investigators (Hartman *et al*., 1967) have 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 O_2 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 (from Lee & Dunton 2000). It is therefore likely that

septa within *Z. muelleri* regulate gas flow between the leaves and the roots of the plant as a survival strategy or adaptation to their aquatic environment. Since seagrasses lack stomata, it is also likely that free gas exchange between the leaves of *Z. muelleri* and their surrounding environment will be restricted (Kuo *et al*., 1990).

1.7 Herbicide usage, mode(s) of action and half-lives

Herbicides are defined, for the purpose of this thesis, as being a subset of the group 'pesticides'. Many herbicides are described as being *specific* in their application and usage. Broad-leaf weedicides, such as Amicide 500^R, rely on the large surface areas of target plants to allow maximum uptake. The active component in Amicide 500^R is 2,4-D (Figure 1.8) and it is one of the widely used phenoxyalkanoic acid herbicides on thirty-one of the fifty studied farms of the Yorke Peninsula. It was first registered as an agricultural herbicide in 1947 (Wilson *et al*., 1997) while other phenoxyalkanoic acid herbicides, such as 2,4,5-T (Figure 1.8), were developed later.

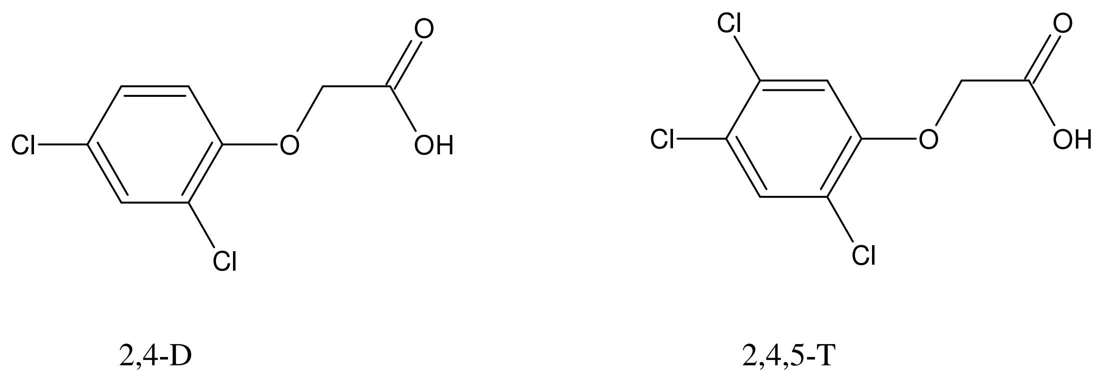


Figure 1.8: Structures of 2,4-D and 2,4,5-T.

These herbicides are applied to soil surfaces in intensively farmed regions and are used as pre-emergent treatments in the control of broad-leafed weeds during intensive farming practices. 'Herbicides comprise nearly half of the 5 billion (5×10^9) pounds of pesticide *active ingredients* used worldwide annually' (Tominack, 2000). It is estimated that 500 million pounds of *herbicide* was used on two principal agricultural crops, soybean and corn, in the U.S.A. with most being applied to corn. Fifty eight million pounds of 2,4-D was used annually in USA (Aspelin, 1997), with 36 million pounds being utilised in agricultural farming, 9 million pounds in suburban use and 13 million

pounds in industrial and commercial use. It is known that of the order of 5 % of surface applied herbicide 2,4-D is 'lost' as dust from farmland (Larney *et al*., 1999). Therefore, 1.8 million pounds of 2,4-D are lost annually in USA from agricultural land, which is equivalent to 2192 kg each day. It is possible that intertidal seagrass species, such as *Z. muelleri* (in South Australia), are at risk from the action of 2,4-D if any of this 'loss' is transported to the intertidal zone and if *Z. muelleri* is susceptible to it. The above calculation does not include 2,4,5-T and other herbicides, such as quinclorac, that are specifically designed as auxin-type herbicides. Amino acid conjugates (discussed below) are formed as 'by-products' of phenoxyalkanoic usage and may be present in soil humus made from dead plant material such as weed debris.

The literature provides details of the specific modes of action of the triazine herbicides (Grossmann *et al*., 2001) that are principally directed at discussions on the inhibition of photosystem II. However, details of the mode of action of the phenoxyalkanoic acid herbicides are anomalous. That is, 2,4-D expresses auxin-like activity on plant tissues when it is applied at low (1 µM to 10 µM) concentrations (Davidonis *et al*., 1979; Davidonis *et al*., 1982) and herbicidal activity when applied at higher (400 µM) concentrations. Indeed, low concentrations have been used commercially to initiate 'fruit setting' in tomato plants (Wood & Fontaine, 1952). These data are supported by studies of lettuce (Meinhardt *et al*., 1991) in which seedlings showed symptoms of leaf epinasty and hyponasty after a single exposure to 2,4-D dimethylamine (10 pg dm^{-3}) in artificial rain simulation experiments. The auxin-induced ripening produces ethylene and cyanide, which is normally detoxified by the ACC (1-aminocyclopropane-1-carboxylic acid synthase) synthase pathway (Yip & Yang, 1998). Auxin-like herbicides are now designed that rely on an induced stress caused by the tissue accumulation of cyanide in an up-regulated ACC pathway (Grossmann & Scheltrup, 1997; Grossmann & Kwiatkowski, 2000). Authors hypothesize that this is the result of a reduced bioconversion of cyanide to L-3-cyanoalanine derivatives by the enzyme L-3-cyanoalanine synthase (Yip & Yang, 1998). Since 2,4-D also interacts with model membranes and cell membranes, such as erythrocytes (Suwalsky *et al*., 1996), it is likely that it interacts, at the molecular level, at multiple sites within target cells. Plants can chemically compartmentalise phenoxyalkanoic acids into conjugates of amino acids and

sugars (Feung *et al*., 1971; Feung *et al*., 1972; Feung *et al*., 1973(a); Feung *et al*. 1973(b)). It is known that the L-amino acid conjugates of 2,4-D are at least as active as the parent herbicide in their auxin-like activity and exhibit maximum activity within the range 0.1 μM to 10 μM (Davidonis *et al*., 1979; Davidonis *et al*., 1982). 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 ^{14}C radiolabelled 2,4-D insult in plant tissue studies (Davidonis *et al*., 1982). If *Z. muelleri* is sensitive to an auxin-like activity of phenoxyalkanoic acid herbicides, and their amino acid conjugates, then it is likely that a change in the growth pattern of plants will occur as a result of such exposure.

While xenobiotics such as simazine and atrazine are designed to specifically impact upon photosystem II, other herbicides such as sulfonylureas are acetolactatesynthetase (ALS) inhibitors and affect the synthesis of branched chained aminoacids (Whitcomb, 1999). It is possible that a deficient ALS system will cause deficient or dysfunctional enzyme systems. It is known that RUBISCO in photosynthesis can represent up to 30 % of the cellular protein. This specialised protein 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.

It is well known that persistent xenobiotics, such as simazine and atrazine, have long half-lives that are typically in 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 reported half-lives ranging from weeks (Thompson *et al*., 1984) to months (Que Hee & Sutherland 1981). The shorter half-lives are probably the result of the parent herbicide interacting with proteins and subsequently forming conjugates (Forgacs *et al*., 2000). The considerable variation in the half-life estimates of 2,4-D in soils is attributable, in part, to the variability in soil types, their moisture and organic carbon contents. It is known that 2,4-D is metabolised to 2,4-dichlorophenol in soil and possibly in intertidal sediment. It should be noted that *no distinction is made in this thesis between a xenobiotic that has a demonstrated long half-life and one that has a short half-life but is frequently used*. The rationale used here is 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.

1.7.1 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 be detoxified. Alternately, the herbicide may act on the plant's biochemistry, affecting the functional attributes of biochemical pathways (Dahroug & Mueller 1990; Nakata, 1991; Gallaher *et al*., 1999; Whitcomb, 1999) or organelle function (Droppa *et al.*, 1981; Duke *et al*., 1984). Herbicides are designed to maximise their impact on weeds and minimise their impact on farm crops. Many herbicides have half-lives that extend well beyond four weeks. Indeed the half-life of atrazine has been reported as being between 50 days (Arthur *et al*., 2000) and 385 days (Jones *et al*., 1982). Additionally, as previously noted for 2,4-D, herbicides are often converted into metabolites that are just as active and sometimes more active than the original herbicide. Therefore, the temporal separation feature of herbicide usage on farms, that are spatially related, becomes less relevant and is less likely to assure minimum impact between one farm and another as the half-life of the herbicide and its active metabolites increases. The availability of a transport mechanism for matter between farms also reduces any protection provided by spatial separation.

Transport mechanisms such as 'spray drift', 'runoff', and 'wind erosion' permit herbicides and their metabolites to impact upon non-target organisms. Transport of pesticides is well studied in the literature (Bidleman, 1999) with herbicide transport being reported in many countries. A 15 % loss, as spray drift, in 2,4-D usage has been recorded (Yates & Akesson, 1966) in which the herbicide travelled beyond 2640 ft (800 m) to a non-target region. Many regulations are now in place to reduce the incidences of 2,4-D 'spray drift'. However, the off-site transport of auxin-like herbicides, such as non-volatile forms (salts) of 2,4-D and 2,4,5-T, were monitored in the Tala Valley (Natal) in South Africa (Sandmann *et al*., 1991), resulting in restrictions in the use of auxin-type herbicides within this region. Despite a zone restriction for phenoxyalkanoic acid usage

within the valley, firstly of 25 km square and later of 45 km square, auxin-type herbicides were still detected in air, rain and dew. The maximum concentrations in air and rain were 0.5 μg m^{-3} and 430 μg L^{-1} respectively with as much as 6 g ha^{-1} 2,4-D and 7 g ha^{-1} 2,4,5-T being deposited in non-target areas. The authors concluded that auxin-type herbicides could be transported many kilometres beyond their point of usage to non-target areas where they could have a negative biological impact on non-target species. In an air monitoring study of the Tala Vallley investigators noted that a parent iso-octyl ester of 2,4-D, used in the sugar cane industry, was not detected (De Beer *et al*., 1992). However, polar forms of the herbicide were regularly detected as adsorbed, suspended particulate material and it was concluded that a 'chemical transformation' from the ester to the polar forms might have occurred. Furthermore, results obtained from a study of the soils of the Canadian prairies indicated that up to 5 % of surface-applied herbicide (2,4-D) and 1.5 % of soil-incorporated herbicide (trifluralin) was 'lost' from the application area as 'wind-eroded sediment' (Larney *et al*., 1999). Interestingly, the same author studied the transport of endosulfan in Northern New South Wales (Australia) where cotton was grown as a commercial crop (Larney, *et al*., 1999). In a 'brown snow' event, dust was transported from Western China to the Canadian Arctic, with the constituent particulate-material being identified by its mineral and pollen content (Welch *et al*., 1991). The authors concluded that the herbicide treflan (trifluralin) was either transported from China or was collected in transit from the north mid west U.S.A. prior to deposition in Canada. Particulate material can therefore be transported over long distances. Dust transport can also occur over shorter distances and often over agricultural regions. Recently, a dust storm travelled eastward from an intensively farmed sugarcane and wheat region of Queensland, Australia, and progressed towards the east coast (Figure 1.9 taken from Visible Earth). Dust transport in South Australia occurs regularly with an average of seventeen to nineteen dust storms per annum (News in science health & medical). Minor dust storms (Figure 1.10) 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 *et al*., 1987; CSIRO).

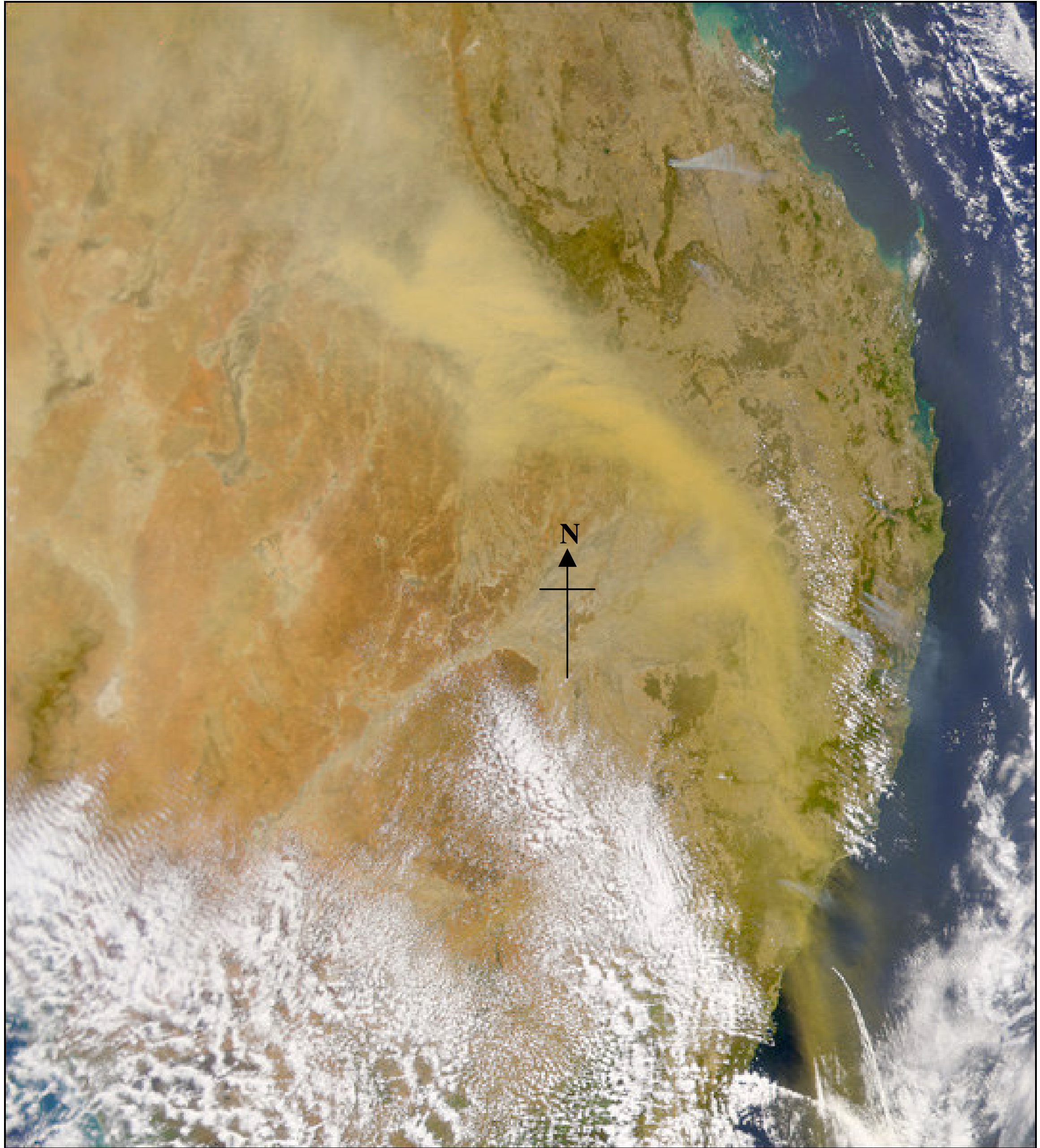


Figure 1.9: Dust storm in Queensland, Australia, heading towards the east coast (Visible Earth).

Figure 1.10: Dust over the Adelaide Plains (25/4/01) 1:05 p.m., View from Target Hill Rd looking west; South Australia. Onshore breeze (dust travelling from the west).

Photography and satellite imagery have therefore provided visual evidence for dust transport that occurs locally (News in science health & medical, CSIRO and Climate Education), regionally (Visible Earth) and globally (Science@NASA and Geology News). It was concluded that dust from intensively farmed regions of the Yorke Peninsula (South Australia), is driven towards Adelaide and across the Gulf of St. Vincent by easterly winds and can act as a transport mechanism for herbicides. It is estimated (from calculations made by Larney *et al*. 1999) that 2,4-D can potentially be transported to the intertidal zone at 5 mg per square metre.

1.8 Experimental approaches used in studying seagrasses

The study of seagrasses can be undertaken by two general methods: *in situ analysis* and *laboratory growth conditions*. Each method has its merits.

In situ analysis has allowed light attenuation stress (Dennison & Alberte, 1982) and osmotic stress (Pinnerup, 1980) to be studied in *Z.marina L.* under field conditions. Data collected has provided valuable insight relating to the light and salinity requirements of *Z marina L.* Additionally, geosurvey studies have quantified seagrass losses in the Spencer Gulf (South Australia) by using satellite imagery (Dunk & Lewis 2000). 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' are monitored and varied. The impact of varying environmental conditions such as light (Ralph & Burchett 1995), temperature and osmotic potential (Kerr & Strother 1985) can then be studied and results assessed. Additionally, chemical treatments such as heavy metal stress (Ralph & Burchett, 1998b; Prange & Dennison, 2000) have also been shown to negatively impact on seagrass survival under laboratory conditions.

It is generally agreed that *in situ* studies provide site and environment specific data relevant to the studied species within a limited time frame. This method may not be suitable for the study of suspected transient 'contamination events' or transient 'stresses' that may be imposed on biota. Additionally while geosurveys permit the mapping of large areas it is difficult to interpret individual *cause and effect relationships* from such data. A diffuse contamination causing a fundamental change in seagrass biochemistry may manifest itself as seagrass necrosis some time after the actual event.

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 translate directly to the natural environment.

The results of one study method therefore provide only 'part of the picture'. Results obtained from the different study methods complement one another to provide a composite view of events that cause seagrass loss. Laboratory cultures of seagrasses in

agar (Ducker *et al*., 1977; Bird, *et al*., 1998) would prove difficult to achieve within the time frame of this study since the specific nutritional requirements of *Z. muelleri* have not been established. Additionally, the 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 (Iizumi *et al*., 1980; Ralph & Burchett, 1998b; Erskine & Koch, 2000; Prange & Dennison, 2000). It was therefore decided to use sediment from the same location as the collected seagrass in all culture methods described in this thesis.

1.9 What is a bioassay, what should a *Z. muelleri* bioassay measure and how should it be performed?

Laboratory-based bioassays are designed to test whether a treatment has an effect on a living organism. Bioassays therefore help distinguish environmental pollution from environmental contamination.

There are two broad types of bioassay. In the first, *native species* are subjected to a treatment under controlled conditions and the results obtained are extrapolated to the *same species* in its 'natural' environment. In the second, *a readily available species* is selected that is known to be sensitive to the treatment under controlled laboratory conditions.

Each construct has its merits; the first provides information on environmental pollution at a specific locality for a native species. However extrapolating the results to other localities and other species is problematic. The second construct 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 may be variable.

Data obtained from the literature suggest that light, or more specifically PAR, is important for seagrass survival. Indeed seagrass growth has been monitored under both field and laboratory conditions. In a light attenuation *field study* the authors noted an increase in the total chlorophyll content in the controls between the shallow (1.8 mg

chlorophyll dm^{-2}) and deep (2.2 mg chlorophyll dm^{-2}) stations in the seagrass *Z. marina L.* (Dennison & Alberte 1982). Interestingly, the chlorophyll a/b ratio was greater in control plants obtained from the shallow station (2.0) than those of the deep station (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 & Tilney-Bassett, 1978; Abal, 1996; Lee & Dunton 1997). Additionally, leaf turnover time (in days) was longer 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 slower in the deep station controls in comparison to the shallow station. Shade treatment, of the (shallow) control group, made leaf turnover time longer and leaf production rates slower than the deep station control group. The photosynthetic rate, measured with a Clark electrode, was greater at the shallow site (0.84 µmole oxygen dm^{-2} min^{-1}) than at the deep site (0.71 µmole oxygen dm^{-2} min^{-1}). The authors hypothesized that these physiological and biochemical responses are adaptations to a limitation in PAR. Indeed, survival of seagrasses at greater depth is directly related to the availability of light and the efficiency of the light-capturing process (Dennison, 1987; Kensworthy & Haunert, 1991; Zimmerman *et al*., 1991). Ralph *et al.* (1998a) also demonstrated, by measuring chlorophyll a fluorescence, that there was a difference between the photochemical and non-photochemical quenching abilities of shallow (*Posidonia australia*) and deep-water (*Thalassodendron pachyrhizum*, *Posidonia angustifolia*) seagrasses . It was 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 & Dennison, 1999) and cyclonic seas (Preen *et al*., 1995) have also been implicated in seagrass losses. In a *laboratory study* it was found that *Z. muelleri* increased its 'apparent' photosynthesis as the PAR increased from 17 to 185 µE m^{-2} s^{-1} (Kerr & Strother 1985). However 'high irradiance stress' *photoinhibition* was imposed on the seagrass *H. ovalis* (R.Br.) and was quantified by chlorophyll a fluorescence and O_2 measurements (Ralph & Burchett, 1995), demonstrating that there is a limit to the increase in photosynthesis with increasing PAR.

The 'timing of biological events' can be determined by an interaction of the prevailing environmental conditions with the organism's stage of development. This phenomenon has been described as the 'plastochrone interval' (Brouns, 1985). In a study of seagrass productivity, the author discussed key biological events such as time of fruiting, that the experimenter can measure under control and test conditions in seagrasses. Other investigators have used the same concept to study leaf growth rates in *Z. muelleri,* finding that leaf turnover can occur in as little as 7 days in summer and 29 days in winter months (Kerr & Strother, 1985). The 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).

Additionally, studies of the release of O_2 from seagrass species, such as *Z. marina L.*, into the surrounding sediment and overlying seawater has provided valuable insight into photosynthesis in seagrasses (Sand-Jensen *et al*., 1982). However the interrelationship between photosynthesis, carbohydrate production and herbicidal impact in *Z. muelleri* is less well researched.

1.10 Experiments

Soils were collected from intensively farmed regions of the Yorke Peninsula (Figures 1.11 and 1.12) and subjected to a dry sieving process to assess the potential of soil to form fine particulate material or 'dust'. Whole soil pH was measured by using a pH electrode. Pesticides and pesticide-like chemicals were extracted and analysed from whole soil, fractionated soil (dust), seawater and intertidal sediment by chromatography techniques. Fourier transform infrared spectrometry (FTIR) was used to provide ancillary data relevant to the structural classification of chemicals.

An homologous series of 2,4-D-like chemicals (simple esters) was synthesized from 2,4-D free acid and a series of aliphatic alcohols. Amino acid conjugates of 2,4-D (2,4-D-aspartate and 2,4-D-glutamate) were also synthesized permitting the testing of the limitations of the supplied ChemStation software and the identification of 2,4-D moieties within chemicals extracted from the studied matrices.

Bioassays were constructed by using *Z. muelleri*, as a representative species of the intertidal zone, to assess the biological impact(s) of 2,4-D (a known transported

herbicide) and any other detected herbicides, upon the intertidal zone. It was reasoned that these data would provide a measure of environmental pollution or potential environmental pollution within the intertidal zone caused by the transport and deposition of surface applied herbicides used in intensively farmed regions.

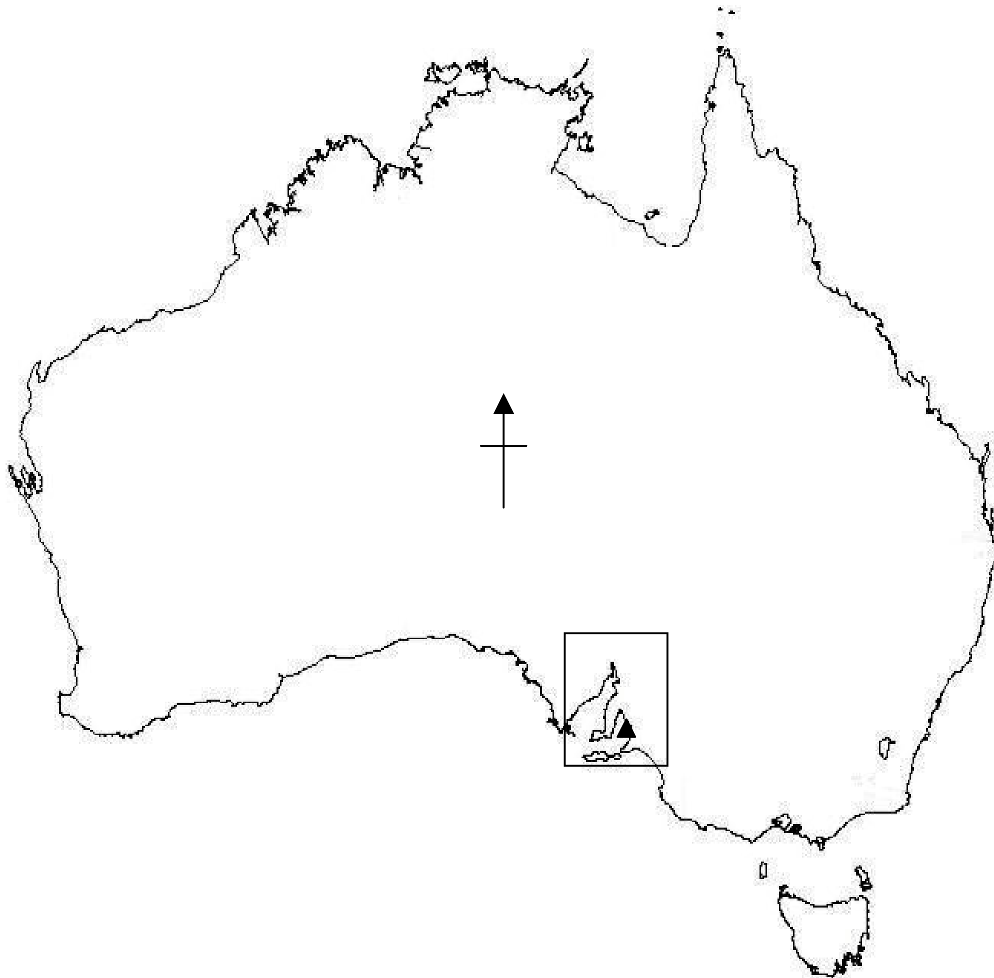


Figure 1.11: Soil sampling sites and seagrass collection sites. The Yorke Peninsula (in South Australia) and the soil-sampling sites are positioned in the middle of the inset (see **Figure:** 1.12) while the seagrass collection site is represented by a filled triangle.

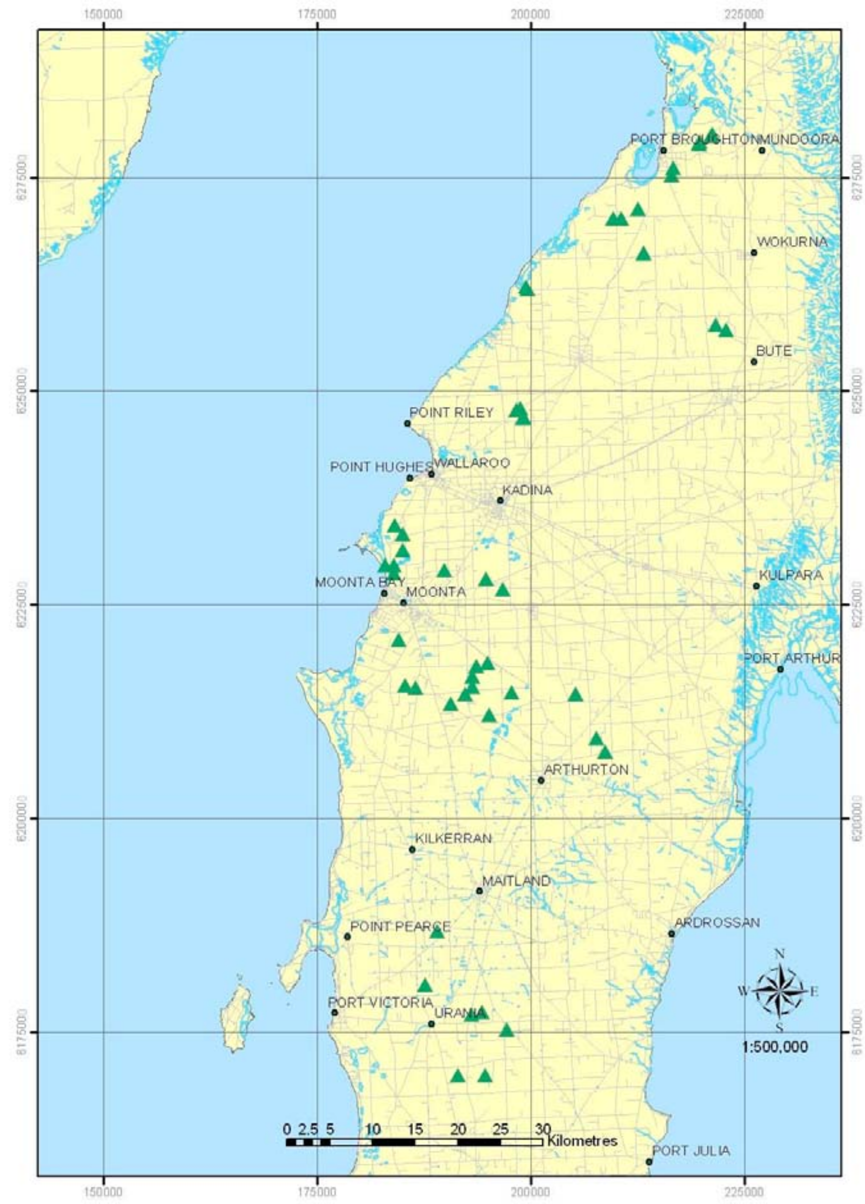


Figure 1.12: Soil collection sites on the Yorke Peninsula (indicated by the green triangles).

CHAPTER 2: MATERIALS & METHODS, PART A: PHYSICOCHEMICAL ANALYSES OF SOIL AND SEAWATER

2.1 Soil sample collection, preparation and storage

Farmers collected soil samples from land that they considered as good cropping and poor cropping in accordance with directions. Surface soil samples (ca. 20 cm x 20 cm x 20 cm) were taken with a spade and placed in self-sealing polyethylene bags within metal cans (4L capacity) that were then sealed. This effort was coordinated by the Agricultural Bureaux and personnel of the Field Laboratory (Adelaide University, Waite Campus). Samples were stored in a cool darkened area until collection, transportation and analysis.

At the laboratory each soil sample was oven-dried to a constant weight at 55 ^{o}C and then placed into a clean self-sealing polyethylene bag within its metal can.

2.2 Instrumental and bioassay techniques

Samples, other than plant material, (whole soil, dust, intertidal sediment, and seawater) were analysed using instrumental techniques while plant material (*Z.muelleri*) was analysed using bioassay techniques. Instrumental analytical techniques were divided into whole soil pH measurements, physical-soil-separating techniques (dry sieving), chromatography and spectrophotometric methods. Bioassays focussed on the biological impact of herbicides on products of the photosynthetic apparatus in the seagrass *Z. muelleri*.

2.2.1 Soil pH measurements

Soil pH was measured by placing soil (50 g and 100 g) in a glass beaker with Milli-Q water (250 mL, adjusted to pH 7 with 0.01M NaOH). The mixture was stirred with a magnetic stirrer (50g sample) or a glass stirring rod (100 g sample) for 15 minutes at room temperature (21 ^{o}C). The mixture was then allowed to settle for fifteen minutes and a pH reading was taken by using a pH meter (PHM62 Standard pH meter, Radiometer Copenhagen). The pH meter and electrode were calibrated with

pH standards (AJAX buffer solutions pH 4.0 ± 0.05 at 25 ^{o}C, Labchem 2490; and Laboratory chemical 2491, pH 7.0 ± 0.05 at 25 ^{o}C) before and after each sample reading.

2.2.2 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). The rationale used here was to ascertain whether soils of the Yorke Peninsula behaved differently from one another when they were subjected to a dry-sieving process. This was achieved, as triplicate replicates, by placing 100 g of dried whole soil into the uppermost sieve (for particles> 2 mm) of a 'sieve stack' shown in Figure 2.1. The sieve stack comprised four working sieves (2.0 mm to 0.25 mm size apertures) and two capping sieves. 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 rope was used to hold the six stacks together while they were agitated on a modified 'Endecotts Test Sieve Shaker' at 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.

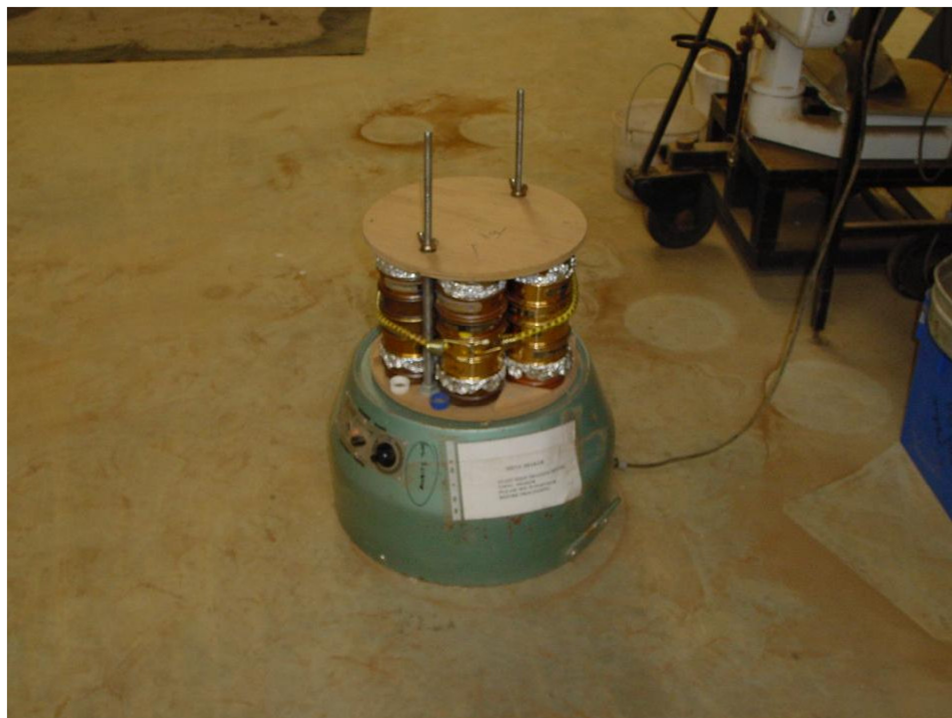


Figure 2.1: Sieve stack used to sieve 'dust' from dried whole soil samples.

2.2.3 Gas chromatography with mass spectral detection (GCMS)

Mass spectral gas chromatographic 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 0.25 μm) and a Hewlett Packard HP 6890 Series Mass Selective Detector. The mass spectrometer was either operated in a *scan mode* (from 35 to 350 a.m.u.) or *selective ion monitoring* mode (sim) at a data collection rate of 0.5 seconds per scan (2 μscans, radio frequency voltage 1.1 MHz and 7.5 kV). When discussing mass spectral data, the term 'ion' refers to 'radical cation' (generated within the mass spectrometer). Automatic gain control from 78 μs to 25 ms with a solvent delay of three minutes was also used. The mass spectrometer was routinely tuned to *maximum sensitivity* according to the manufacturer's specifications prior to each analysis. Ions monitored in sim mode were either selected from those obtained by chromatographing 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, equivalent to 25 and 175 ppb, were co-analysed before, during and after sample analyses. This allowed any drift in instrument sensitivity to be monitored throughout the 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^{-1}.

The oven temperature was programmed as a three-ramp system. On sample injection the initial oven temperature was maintained at 50 ^{o}C for three minutes and then programmed to 210 ^{o}C at a rate of 20 ^{o}C per minute. The rate of temperature increase was then immediately reduced to 5 ^{o}C per minute to attain a temperature of 260 ^{o}C. A final ramp of 30 ^{o}C per minute increased the oven temperature to 300 ^{o}C, which was held for thirteen minutes before cooling and commencement of the next 35.33-minute GC cycle. Samples were either injected manually or by autoinjector (Hewlett Packard HP 6890 Series Injector) into an injection port held at 250 ^{o}C operated in splitless mode. ChemStation software was used on the GCMS analytical system to positively identify all pesticide standards.

2.2.4 Isotope ratio analyses of standards and 'unknowns'

Mass spectrometry permits the detection of chlorine isotopes within analyte molecules. Indeed, *di-chlorinated* analytes produce a characteristic mass-spectral pattern, with the isotopic abundance of M : M+2 : M+4 ions being in the ratio of 9:6:1 respectively. This ratio is directly related to the isotopic abundance of ^{37}Cl being 32.4 % that of ^{35}Cl (Davis & Frearson, 1990). It was therefore anticipated that this technique would provide additional structural information on chlorine-containing ion fragments within 2,4-dichlorophenoxy standards and other 2,4-D- like compounds.

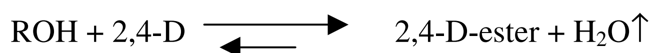
2.2.5 Kovats analyses of retention time data in a homologous series

The retention times of chemicals in a GC trace often follow a log-linear relationship with the increasing molecular weights of analytes within a series (Kovats, 1958). The log-retention times of a series of fatty acid methyl ester standards was plotted against the

number of carbon atoms in the standards. The same technique was used to analyze the series of synthesized simple esters of 2,4-D and other possible chemical series within samples.

2.3 Chromatography standards

Herbicides were either purchased or synthesized in the laboratory. Standard herbicides used in GCMS analyses were atrazine (Chem Service, purity 99 %, Lot 1858A), simazine (Chem Service, purity 98 %, Lot 2-87), diclofop-methyl (Chem Service, technical grade 94 %), and treflan (trifluralin) (Chem Service, technical grade 40 % (as 400 g L^{-1})). Authentic standards of the methyl esters of the phenoxyalkanoic acids (Herbicide Ester Mixture 8270, catalogue No. 48474 containing the methyl esters of 2,4-D, Silvex and 2,4,5-T) were purchased from the Sigma Chemical Company (U.S.A.). Additionally, simple aliphatic esters (from C1 to C18) of 2,4-D were prepared from isolated 2,4-D free acid and analytical grade aliphatic alcohols (Sanchez *et al.,* 1991). While the method of Sanchez proved successful for the synthesis of shorter chained ester derivatives of 2,4-D, up to C16, it was less effective in preparing the longer chained aliphatic analogues. It was noted that longer-chained simple esters could also be prepared by heating the free acid of 2,4-D with excess aliphatic alcohol (18:1) to drive water from the reaction mixture, as an azeotrope, and push the equilibrium to 'the right' resulting in the formation of the simple ester.



Humic acid was purchased from Fluka (AG. CH-9470 Buchs, 35069 887 53680, 50 g, Asche 10 – 15 %, Mr 600 - 1000).

The standard herbicides used in HPLC analyses were chlorsulfuron (technical grade, 98.0 %, Dupont), metsulfuron-methyl (technical grade, 98.9 %, Dupont), and triasulfuron (analytical grade, 99.5 %, Dupont) dissolved in acetonitrile (HiperSolv, BDH for HPLC) or methanol. The sulfonylureas were prepared in both methanol and acetonitrile because of the uncertainty of their stability in protic solvents such as methanol.

2.3.1 Isolation of 2,4-D 'free acid' from Amicide 500^R

Pure 2,4-D was prepared from Amicide 500^R, the dimethyl amine salt of 2,4-D used as one formulation of this phenoxyalkanoic acid herbicide in the control of broad-leaf weeds in intensive farming practices. This formulation 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^R into an organic solvent. Briefly, Amicide 500^R (40 mL) was added to a separating funnel (2 L capacity) containing Milli-Q water (400 mL) that was acidified (to pH 1.0) by adding 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 at 60 ^oC on a rotary evaporator. The white crystalline solid was dried over silica gel in a desiccator for 24 hrs and was identified as 2,4-D by its melting point, infra-red and mass spectral analyses (section 3.6). The isolated 2,4-D free acid was subsequently used in the synthesis of 2,4-D derivatives that were then used to identify putative 2,4-D-like analytes.

2.3.2 Synthesis of an homologous series of 2,4-D esters

Simple esters (Figure 2.2) of 2,4-D were synthesized from the free acid and aliphatic alcohol by using acetyl chloride as the catalyst (Sanchez *et al*., 991). 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 C18 (oleoyl alcohol)) was then added. Acetyl chloride (200 µL, analytical grade, B.D.H.) was then *carefully* added and the vial was capped with a Teflon-coated rubber seal. The mixture was then heated to 100 ^oC (sand bath for one hour), cooled to 21 ^oC and 1.0 mL of acetate buffer (pH 4.6) containing 51 mM acetic acid and 49 mM sodium acetate 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 ^oC on a rotary evaporator and transferred to a volumetric flask (25 mL capacity) with several 'washes' of n-hexane (7 x 2 mL). The contents of the flasks were then adjusted to 25 mL with n-hexane. The synthesized esters were identified by

their mass spectral patterns, which were also compared to those of the reactant materials: 2,4-D free acid and the constituent aliphatic alcohols.

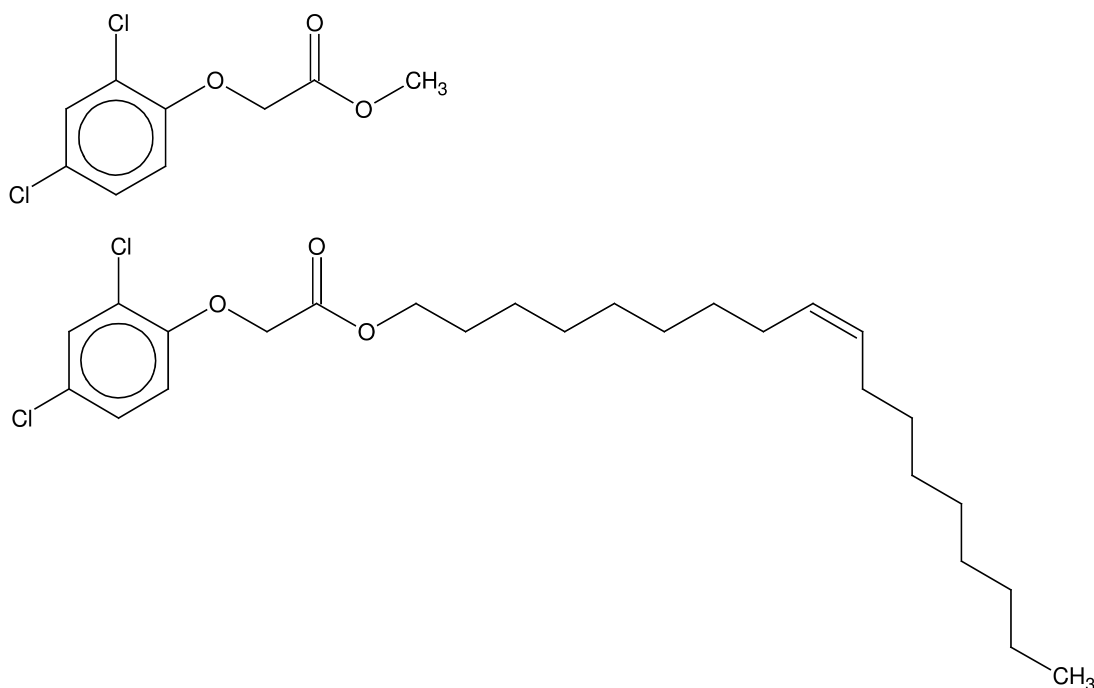
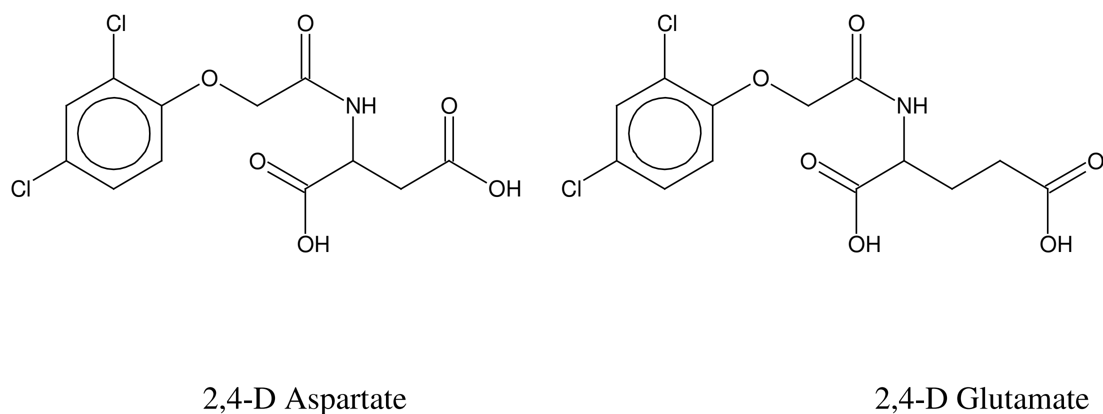


Figure 2.2: Structures of the first (methyl, C1) and last (oleoyl, C18) members of a synthesized homologous series of 2,4-D derivatives.

2.3.3 Synthesis of L-amino acid conjugates of 2,4-D

Synthesis of 2,4-D-aspartate and 2,4-D-glutamate (Figure 2.3) was achieved by condensing the amino acid with the acid-chloride of 2,4-D (Wood & Fontaine, 1952). The 2,4-dichlorophenoxyacetyl chloride (2,4-D-Cl) required for this synthesis was prepared from thionyl chloride and the free acid of 2,4-D (Freed, 1946).



2,4-D Aspartate

2,4-D Glutamate

Figure 2.3: Structures of synthesized amino acid conjugates of 2,4-D.

2.3.4 Preparation of the acid chloride of 2,4-D

The dried free acid of 2,4-D (40 g) was placed in a round bottomed flask (500 mL capacity) 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 ($SOCl_2$, 200 mL, B.D.H.) 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 (Freed, 1946) resulted in some pyrolysis of material within the flask. To overcome this problem, a water trap consisting of a 2-necked round-bottom flask was placed between the vacuum source and a rotary evaporator, and the excess thionyl chloride was removed at 50 ^{o}C under reduced pressure over a ten minute period, during which the apparatus was under constant observation. 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 the current work, the 'crude' uncrystallized liquid 2,4-D acid chloride was used for subsequent synthesis of 2,4-D-amino acid conjugates as detailed below. It was reasoned that even 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.

2.3.5 Preparation of L-amino acid conjugates of 2,4-D

Firstly, the appropriate amino acid (aspartic or glutamic 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 ^{o}C on ice. The acid chloride of 2,4-D (0.181 mole), containing 5 % benzene, 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 ^{o}C. A solid cream-coloured mass was present in the glutamate preparation while a white-coloured mass was present in the aspartate preparation. Previous studies had shown that the *ether soluble* amino acid conjugate of 2,4-D could be recrystallized from water to produce white crystals with a melting point of 178 ^{o}C to 179.5 ^{o}C (Feung *et al*., 1971; Feung *et al*., 1973). However, during the present study, the prepared cream and white solids provided melting points within the range 133 ^{o}C to 136 ^{o}C while the melting point of the pure 2,4-D standard was 141 ^{o}C and consistent with the literature value (Que Hee & Sutherland, 1981). These results suggested that a mixture of materials was present within the cream and white coloured solid products causing the phenomenon of 'melting point depression'. A solvent mixture (mix A) was therefore designed to preferentially dissolve 2,4-D (free acid) and amino acids at acidic pH. The mixture comprised diethyl ether (100 mL), petroleum spirit (300 mL), Milli-Q water (100 mL) and concentrated hydrochloric acid (2.5 mL) at pH 1.0. The solids from the 2,4-D-glutamate and 2,4-D-aspartate preparations were washed 5 times with mix A in separating funnels (1 L capacity) and the white insoluble material remaining at the interface was retained. Milli-Q water (250 mL) was then added to each separating funnel and the contents of the interface were extracted into diethyl ether (3 x 500 mL). The combined ethereal extracts were taken to dryness at 60 ^{o}C on a rotary evaporator, then dried in an oven at 55 ^{o}C for three days followed by drying over silica in a desiccator overnight. The dried 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 had melting points of 179 ± 1 ^{o}C and 202 ± 1 ^{o}C respectively and were consistent with literature values (Que Hee & Sutherland, 1981). The findings from high performance thin layer chromatography (HPTLC) and infrared analyses (FTIR) support

the identification of L-amino acid conjugates of 2,4-D and are described in the following sections.

2.4 HPTLC analyses of the L-amino acid conjugates of 2,4-D; the octyl and the oleoyl ester of 2,4-D and 'unknowns' in soil extracts

Two sets of aluminium-backed silica gel HPTLC plates (HPLC-Alufolien, Kieselgel 60 F 254, Merck) were prepared using the solvent system 1-butanol, acetic acid and water (90:20:10; vol:vol:vol; Feung *et al*., 1973). In the first set the glutamate and aspartate L-amino acid conjugates of 2,4-D were run individually with their respective amino acid and the free acid of 2,4-D. The second set consisted of acetonitrile extracts of whole soils that were run adjacent to the 2,4-D octyl ester standard. Blank tracks were positioned between standards and unknowns in all HPTLC analyses to minimize cross-contamination. Detection of amino acid standards was achieved by excising the tracks and spraying them with 0.3 % ninhydrin in solvent (isopropanol : acetic acid, 90 : 10 vol. %). Analytes were removed from the HPTLC plates by using scissors to cut a 1.0 cm wide strip from the solvent front of the air-dried plates. Analytes were then eluted from the strips by placing them into screw topped culture test tubes containing diethyl ether (10 mL). The test tubes were then sonicated (20 minutes) and centrifuged (500 *g*, 21 ^{o}C). Residues obtained from the second set were dried (rotary evaporator, 35 ^{o}C), resuspended in acetonitrile (50 µL) and analysed by GCMS run in scan mode.

2.5 Detection of standard non-phenoxyalkanoic acid herbicides

Purchased and synthesized standards were analysed by the GCMS methods noted in Section 2.2.3.

2.6 Recovery of herbicide standards from whole soils and seawater

Whole soils (50 g) were 'spiked', at two loading levels, with a mixture of herbicides that are used in broad acre farming. Low QC (quality control), 10 µg kg^{-1}, level and high QC (quality control), 70 µg kg^{-1}, standard loadings were 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 $100mL^{-1}$ of extracting solvent as described in the following sections.

Seawater was similarly doped with a pesticide mixture and pesticides were recovered using a Sep-Pak C18 solid-phase extraction technique. Seawater (500 mL, from the Aquatic Sciences Centre, West Beach, South Australia) was spiked with a pesticide mixture containing 500 ng of each component; treflan, simazine, atrazine, diuron, diclofop methyl, chlorsulfuron (technical), metsulfuron-methyl (technical) and triasulfuron, in acetonitrile (100 µL). This loading is equivalent to 1000 ng per litre (1 µg L^{-1}, 1 ppb). The spiked seawater was then sonicated (21 ^{o}C, for 5 minutes), to dissolve the components, and filtered through a cellulose nitrate filter (0.45µm, Gelman). No pH adjustment was made from pH 8.1 (seawater). The pesticides were extracted by passing the seawater through an activated (5.0 mL acetonitrile followed by 5.0 mL of Milli-Q water as indicated by the manufacturer) Sep-Pak (C18) at 3 mL min^{-1}. The Sep-Pak was then 'washed' (Milli-Q water, 2.5 mL) and the bound analytes were eluted with 7.5 mL acetonitrile. The eluent was then concentrated (rotary evaporator, 35 ^{o}C, 10 mins.) and the analytes resuspended in acetonitrile (100 µL). Analytes were therefore concentrated by a factor of 5000 (500 mL divided by 0.1 mL). Anthracene, at 8 ng $µL^{-1}$, was used as an external standard by injecting a constant volume of 1 µL in triplicate before and after each seawater analysis. This technique provided an indication of the reproducibility of injection volume and on-column loading.

2.7 Preliminary GCMS analyses: detection of treflan and 2,4-D- like chemicals in whole soils?

The results of preliminary soil analyses suggested the presence of treflan and 2,4-D- like chemicals in solvent extracts of whole soils. Therefore the range of 2,4-D analogues, previously purchased or synthesized, were also analysed by GCMS. Data obtained, comprising *retention times*, *mass spectral patterns* (hydrocarbon profiles and ions specific to 2,4-D), *chlorine isotope ratios*, and Kovats analyses were then used to compare and partially characterize analytes in solvent extracts of whole soils and dust. Xenobiotics were not detected in intertidal sediment and seawater, analyses were therefore restricted to whole soil and dust matrices.

2.8 Extraction, detection and characterization of xenobiotics in whole soils and dust obtained from whole soils

2.8.1 GCMS analysis of whole soils

Soil samples were extracted into acetonitrile and analysed by GCMS (Liao *et al*., 1991). The dried whole soil samples (50 g) were placed in glass volumetric flasks (250 mL capacity) together with an internal standard (anthracene, 640 ng as an 80 μL aliquot, made up in acetonitrile at 8ng μL^{-1}). Acetonitrile (100 mL) was then added, the flask was stoppered and placed in an oscillating extraction device for 4 hrs at 21 ^{o}C and the contents left to stand for a further two hours. Two 24 mL aliquots of the supernatant were then transferred to glass scintillation vials (24 mL capacity) and centrifuged (500 *g*) at 21 ^{o}C 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 rotary evaporator treatment (35 ^{o}C, full speed rotation, five minutes) and then allowed to cool to 21 ^{o}C. The residue was re-suspended in acetonitrile (0.5 mL) and a sample (1.0 μL) was injected on-column. This provided an on-column loading of 0.5 ng of internal standard per analysis.

Total 2,4-D was also extracted from whole soils (Renberg, 1974) and the isolated residues were then derivatized into the 2-butoxyethyl ester (Sanchez *et al.,* 1991). The ion fragments with m/z values of 133, 145, 161 and 162 (Figure 2.4) were used to identify 2,4-D- like analytes. The characteristic molecular ion of 2,4-D butoxyethyl ester, with an m/z of 320, was then used to quantify the amount of available 2,4-D in the soil by comparing its peak area with a 2,4-D Amicide 500^{R} (2,4-D dimethyl amine) standard.

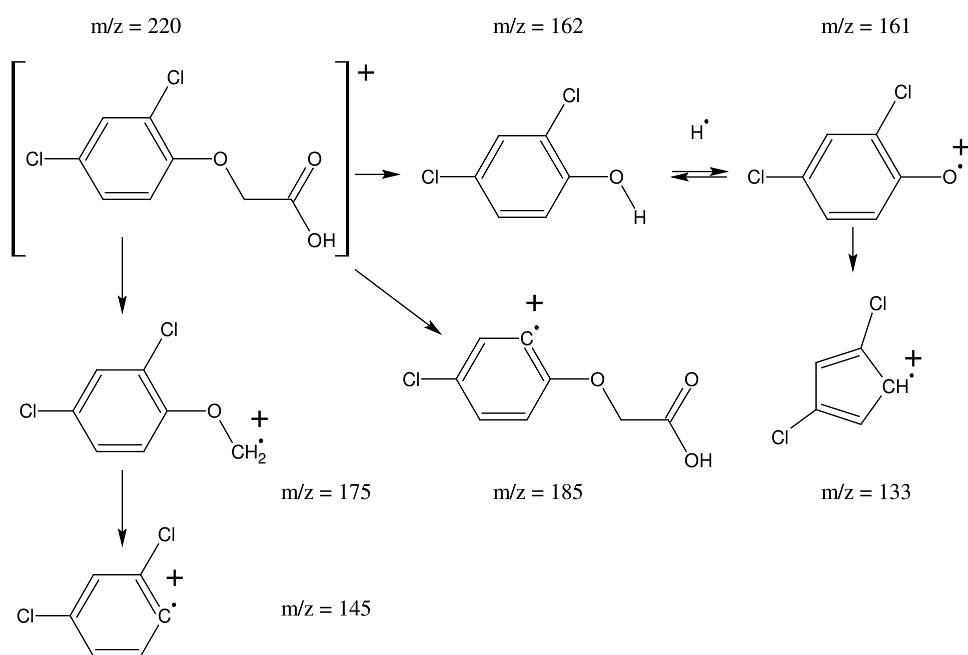


Figure 2.4: Some of the mass spectral fragments of 2,4-D (adapted from Que Hee & Sutherland, 1981).

2.8.2 GCMS analysis of dust

Dust (50 g) was placed in a glass volumetric flask (250 mL capacity) and anthracene internal standard was then added. Subsequently, herbicides and 2,4-D- like chemicals were extracted by the method previously described for whole soils.

2.9 Identifying and quantifying 2,4-D- like chemicals in whole soil and dust by GCMS

2,4-D- like compounds were identified on the basis of detected mass fragments 133,145 and 161 (Figure 2.4) together with the detection of their isotope ‘daughter’ fragments with m/z values of 135, 137, 147, 149 and 163, 165 respectively. Additionally, the detection of specific ion fragments, such as those produced in McLafferty rearrangements (McLafferty & Turecek, 1993) provided additional insight into the molecular structure of standard and unidentified compounds. Scan mode analyses also provided some detail on the presence of hydrocarbon moieties within molecular structures. The cumulative area of mass fragments with m/z values of 133, 145 was used to estimate the amount of 2,4-D- like chemicals that were present in soils of the Yorke

Peninsula. This was achieved by assuming a response factor of one hundred-fold less than that of the purchased 2,4-D methyl ester (Supelco) standard for the selected mass fragments. The cumulative areas for these mass fragments then provided an estimate of analyte concentration from a 160 ng μL^{-1} standard injection of the 2,4-D methyl ester standard (Suplelco).

2.10 Confirmatory analyses using gas chromatography with electron capture detection (GCECD)

Gas chromatography analysis (GCECD) 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) with electron capture detection (ECD). Temperature programme conditions were identical to those listed in the GCMS analytical procedure. However, the sample was dried (UHP nitrogen at 21 ^{o}C) and resuspended to the same concentration in n-hexane (500 μL). This method was used to detect electrophilic atoms, such as fluorine, chlorine, bromine and iodine that may be present in solvent extracts of whole soils.

2.11 Hydrophobic interaction chromatography (HIC) analyses of 2,4-D-like chemicals in whole soils

Organic chemicals such as herbicides sorb to soil particles and to water-soluble organic material (Madhun *et al*., 1986a; Madhun *et al*., 1986b) such as fulvic acid within soil. Indeed, studies have shown that pesticides can be transported on particulate matter in water (Bowmer, 1982) and in air (De Beer *et al*., 1992; Van Dijk & Guicherit, 1999). It is likely that pesticides sorb to soil particles by physicochemical interactions with soil particles. The forces that bind pesticides to particulate matter can often be described as both ionic and van der Waals interactions.

Hydrophobic interactions between particles can be measured by using commercially available Sep-Pak cartridges (Sigma). Indeed, the same technique is often used as a solid phase extraction technique and concentration step in the analysis of trace components in water matrices. The results of preliminary GCMS and HPTLC data suggested that non-polar 2,4-D- like chemicals contained hydrocarbon (hydrophobic) moieties. Therefore, a C18 Sep-Pak technique was used to isolate hydrophobic chemicals from an acetonitrile

extract of whole soil and to place a qualitative estimate on their hydrophobicity. Briefly, the contents of randomly selected GC sample vials were placed onto activated Sep-Pak cartridges that were then sequentially eluted with 5 ml of aqueous phase containing an increasing content of acetonitrile (0 %, 25 %, 50 % and 100 %). The cartridge was then given a final wash with n-hexane (5 mL). Eluents were then dried (rotary evaporator at 35 ^{o}C), resuspended in acetonitrile and analysed by GCMS. Additionally in a large-scale (duplicate) preparation, organic chemicals were extracted from soil by using acetonitrile. Briefly, soil (1 kg) was placed in a volumetric flask (5 L capacity) and acetonitrile (2.5 L) was added. The vessel was capped and repeatedly inverted over a three-hour period and left to stand overnight. The combined supernatants were then centrifuged (500 *g*, 21 ^{o}C) and an aliquot (1L) was evaporated to ca. 10 mL on a rotary evaporator (35 ^{o}C) transferred to a volumetric flask (20 mL capacity) and brought to volume with acetonitrile. A preparative HIC (hydrophobic interaction chromatography) column was made by adding the contents of six C18 Sep-Paks to a Hirsch funnel. The C18 packing was activated by sequentially washing it with acetonitrile and Milli-Q water in accordance with the manufacturer's directions. The acetonitrile extract of whole soil was loaded onto the preparative HIC column that was then sequentially washed with acetonitrile (500 mL) and n-hexane respectively (500 mL). The 'washings' were taken to dryness on a rotary evaporator (1hr, 35 ^{o}C), resuspended in acetonitrile (500 µL) and analysed by GCMS in both sim and scan modes and by FTIR. The simple (aliphatic) esters of 2,4-D were then compared to the n-hexane eluent obtained from the HIC column by using FTIR.

2.12 Analyses of acid and base hydrolysates of chemicals within gas chromatography vials

Six gas chromatography samples, consisting of 500 µL acetonotrile soil extracts, were selected randomly and subjected to a sequential acid then alkaline hydrolytic treatment prior to gas chromatography analysis. It was anticipated that this hydrolytic procedure would help characterise the constituent chemicals as being either labile or refractory to these caustic chemical treatments.

The GC sample was first dried (UHP nitrogen at 21 ^{o}C), 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 (1:1 vol. / vol. with Milli-Q water, 50 μL) and n-hexane (50 μL) were 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. This procedure provided a twenty-fold increase in sample loading on to the GC since a 1 μL injection (from 500 μL acetonotrile) was used in previous analyses. The n-hexane extract of the acid hydrolysis procedure was then transferred to a second glass insert by using successive washes (3 x 100 μL) of n-hexane. The extract was then dried (UHP nitrogen, 21 ^{o}C) and aqueous sodium hydroxide (0.5 M NaOH, 50 μL) and n-hexane (50 μL) were then added. The contents were mixed by using a syringe (250 μL capacity fitted with a Teflon plunger, SGE) and allowed to stand (20 minutes at 21 ^{o}C) with occasional tapping of the insert onto the bench top to facilitate phase separation. A purchased mixture of methyl esters of the chlorophenoxyalkanoic acids (Herbicide Ester Mixture 8270, the Sigma Chemical Company (U.S.A.)) was also subjected to the alkaline hydrolysis procedure for comparative purposes. An aliquot (2 μL) of the hexane layer was then injected onto the GC for analysis.

2.13 Are putative 2,4-D- like chemicals structurally related to a commercially available humic acid?

The 2,4-D- like series detected in the present study was similar to previously identified compounds (Bollag, 1992) in which 2,4-dichlorophenoxy moieties were covalently bound to humic substances such as syringic acid. In Bollag's *in vitro* study, incorporation of 2,4-dichlorophenoxy moieties into humic substances was achieved by co-incubating 2,4-dichlorophenol, and syringic acid with a laccase from *Rhizoctonia praticola*. In the present study, a commercially available humic acid (20 mg, Fluka, AG. CH-9470 Buchs, 35069 887 53680, Asche 10 – 15 %, Mr 600 - 1000) was partly soluble (ca. less than 1 %) in 20 mL of acetonitrile with the aid of sonication. However, it was

noted that the same material was completely soluble in Milli-Q water (pH 6.5). The acetonitrile-soluble material was then taken to dryness (rotary evaporator at 35 ^{o}C) and resuspended in acetonitrile (200 μL) prior to GCMS analysis under the same run conditions that were used in the analysis of whole soil, dust and seawater.

2.14 Fourier transform infra-red spectroscopy (FTIR)

Isolated 2,4-D 'free acid', synthesized L-amino acid conjugates of 2,4-D, the oleoyl ester of 2,4-D and 2,4-D- like compounds isolated from the HPTLC preparations, were prepared for FTIR analyses. Potassium bromide disks containing the samples were prepared by grinding a mixture of the solid analyte (ca. 1-10 mg) and dry spectroscopic grade potassium bromide (ca. 100 mg) into a fine powder with an agate mortar and pestle. The powder was placed into a disk press and pressure was then applied to it, by turning the screws of the press within a bench-top clamping device, to convert it into a transparent to translucent disk. The barrel of the disk press, containing the sample disk, was then placed into the FTIR spectrometer for sample analysis. FTIR spectroscopy was performed on a Perkin Elmer instrument (Spectrum One B, FT-IR Spectrometer) and 'sample disks' were scanned between 4000 and 400 cm^{-1}.

2.15 High performance liquid chromatography (HPLC): sulfonylurea analyses

Preliminary analyses indicated that the sufonylureas were thermally unstable and decomposed when they were analysed by gas chromatography (Figure A5, Appendix A). These observations are supported by the results of previous studies (Long *et al*., 1990). Therefore, sulfonylurea herbicides were extracted from twelve randomly selected whole soils and were analysed by a modified HPLC technique (Sarmah & Kookana, 1999). Briefly, soils (100 g) were extracted with deionized water (100 mL) by inversion mixing. The extracts were then centrifuged (25000 *g*), filtered (glass fibre filters), pH adjusted to 2.2 with hydrochloric acid (1 M) and then eluted through preconditioned Sep-Pak cartridges at a flow rate of 10 mL per minute. Bound analytes were then 'washed' and eluted from the Sep-Pak columns by using Milli-Q water (2.5 mL) and acidified methanol (2.5 mL, pH 2) respectively. Eluted analytes were then adjusted to

pH 7 with aqueous sodium hydroxide (0.5 M), concentrated to 50 μL (21 ^{o}C, under a stream of U.H.P. nitrogen) and then analysed by HPLC.

Reverse phase HPLC (Waters Nova – Pak C18, 3.9 x 15 cm column) was performed on a Waters instrument operated under isocratic conditions with a flow rate of 1.4 mL per minute (4000 psi, 21 ^{o}C). The mobile phase was composed of Milli-Q water, methanol and glacial acetic acid in the ratio 54.5 % : 45 % : 0.5 %, vol. / vol. respectively. The injector was equipped with a 25 μL sample loop and a programmable multiwavelength detector (Waters 490, 0.1 a.u.f.s.) set at 230 nm was used to monitor the eluent. An integrator (Waters 745/745B, data module) was set to an attenuation of 512 to record the results. The HPLC technique system was calibrated by using standard concentrations of chlorsulfuron (30 ng μL^{-1}, 15 ng μL^{-1} and 10 ng μL^{-1}, 3.3 ng μL^{-1}), metsulfuron-methyl (19 ng μL^{-1}, 9 ng μL^{-1} and 6 ng μL^{-1}, 2 ng μL^{-1}) and triasulfuron (34 ng μL^{-1}, 17 ng μL^{-1} and 11 ng μL^{-1}, 3.7 ng μL^{-1}). Quality controls containing 250 ng and 83 ng of sulfonylurea were analysed prior to and subsequent to each sample analysis. Sample concentrations were calculated by comparing peak areas with those of the standards.

2.16 Statistical analyses

Statistical analyses were performed by using 'Excel^{R}' software (Donnelly, 2004) or by adapting t-tests from Clarke (1980). The level of significance for each test was defined by the value of 'α', which should be taken as 0.05 unless otherwise indicated.

CHAPTER 3: RESULTS OF PHYSICOCHEMICAL ANALYSES OF SOIL AND SEAWATER

3.1 Soil pH measurements

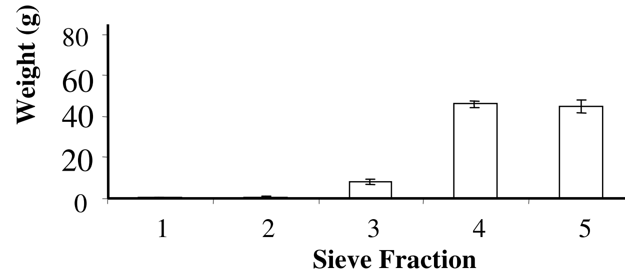
Soils of the Yorke Peninsula are mostly alkaline (mean pH = 7.8 ± 1.0, n = 12) with ten of the twelve analysed soils having a pH ≥ 7.0. This is probably caused by the calcareous parent materials that pervade the region (Talbot & Nesbitt, 1968).

3.2 Size-fractionation and the physical stability of dried soils

Soils can be placed into three broad groups: those that contain a large percentage (typically 40 %) of material smaller than 250 µm, those that contain an intermediate percentage (e.g. 20 %) and those that contain little fine material (Figure 3.1, and Appendix B Figure B1 and Table 1). The distribution of dry weights of particles (grams per 100 grams of whole soil) sieved in fractions 4 and 5 can be described by a polynomial equation ($y = 0.0006x^3 - 0.0994x^2 + 4.8339x - 22.804$, $R^2 = 0.5$, Figure 3.2). This equation can be solved to provide an apex at (37,50). Results of a student's 't-test' indicate that the distribution of particles in fraction 4 ($0.25 < x < 0.5$ mm) do not differ significantly ($t_{29} = 1.24$, $P < 0.15$) from a normal distribution that has a sample mean of 21 (Clarke, 1980). Furthermore, a weak positive correlation is evident when fraction 5 ($x < 0.25$ mm) is expressed as an inverse function of fraction 4 ($y = -461.59x + 58.747$, $R^2 = 0.6$, Figure 3.3) for twenty-eight of the thirty data points analysed. Additionally, an F-test indicated that significant differences were present in the weights of dust (< 250 µm, Appendix B Table 1) obtained from the dry-sieving process.

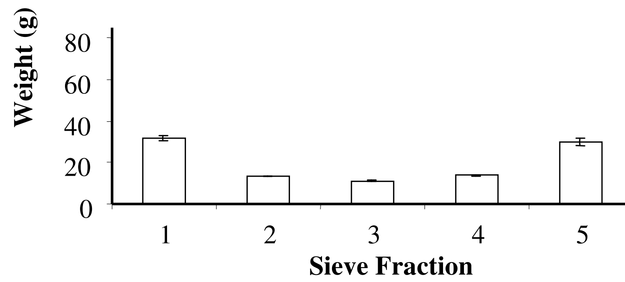
High ‘dust content’ soil

Whole Soil #39 (Sandy Loam)



Intermediate ‘dust content’ soil

Whole Soil #48 (Grey Loam)



Low ‘dust content’ soil

Whole Soil #99 (Loam)

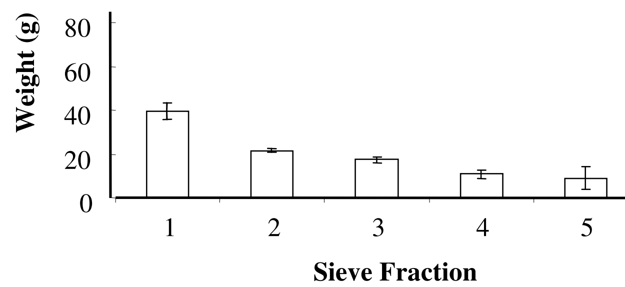


Figure 3.1: Dust content of dried soils of the Yorke Peninsula.

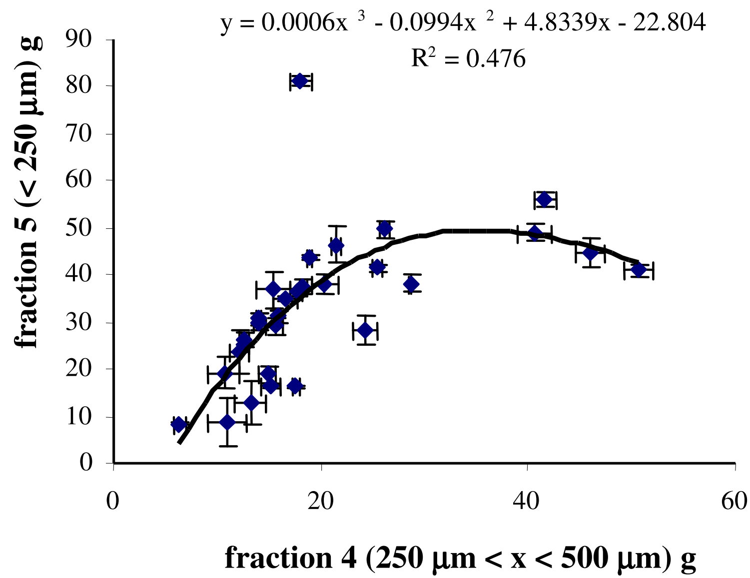


Figure 3.2: Particle size distribution (grams per 100 g soil) in dried soils of the Yorke Peninsula.

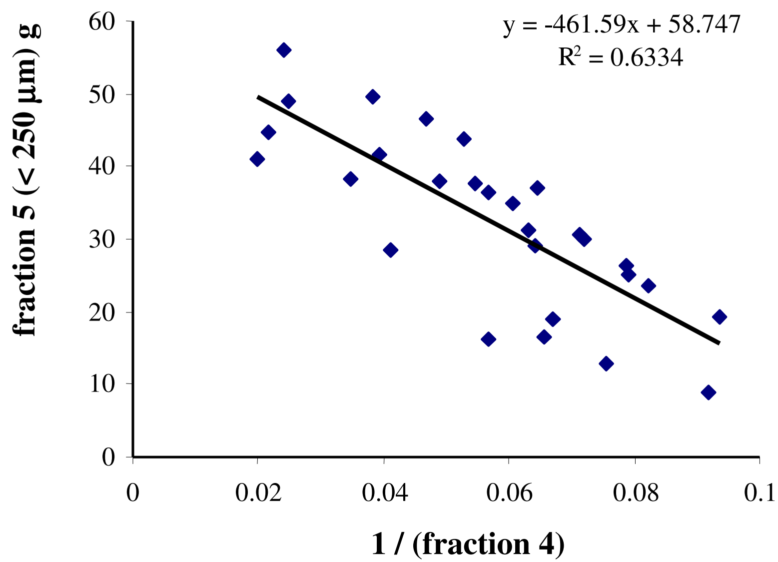


Figure 3.3: Sieve 'fraction 5' as a function of 'fraction 4' (grams per 100 g soil).

3.3 High performance thin layer chromatography (HPTLC) analyses of the L-amino acid conjugates of 2,4-D; the octyl and the oleoyl ester of 2,4-D and 'unknowns' in soil extracts

The L-amino acid conjugates of 2,4-D had R_f values greater than the parent amino acids. The R_f values of L-amino acid conjugates of 2,4-D were 0.9 for both 2,4-D Glu and 2,4-D Asp while the parent amino acids had R_f values of 0.51 and 0.42 for Glu and Asp respectively (Figures A1 and A2, Appendix A). The R_f value of 2,4-D free acid was 1.0. These data are consistent with the literature (Feung *et al.,* 1973). Additionally, the octyl ester of 2,4-D and unidentified chemicals in soil extracts migrated near the solvent front with an $R_f = 1$. The R_f value of the 2,4-D octyl ester standard was verified by GCMS. Interestingly, the unidentified chemicals from soil extracts had similar mass spectral profiles and (chlorine) isotope ratios (Figure A4 Appendix A) to the synthesized simple esters (Figure A7, Appendix A) of 2,4-D. These data are consistent with the unidentified 2,4-D- like series having hydrocarbon and non-polar characteristics.

3.4 GCMS analyses of herbicide standards

3.4.1 Detection of standard non-phenoxyalkanoic acid herbicides and diclofop-methyl

Treflan , atrazine, simazine and diclofop methyl were detected and positively identified as individual chemicals by the ChemStation software when the mass spectrometer was operated in scan mode. Additionally, a mixture of the same compounds was analysed under sim mode using the selected ions listed in Table 1 at 1 ng μL^{-1} for each component on-column as a low quality control. This column loading is equivalent to 25 ppb ($\mu g\ kg^{-1}$) analyte in dry soil. Analyses performed in sim are typified by a 'step' in the chromatogram as the software switches from the detection of 'group 1 ions' to the detection of 'group 2 ions'.

Group	Selected ions used in GCMS analyses
1	43, 69, 72, 76, 77, 89, 103, 110, 111, 120, 123, 125, 140, 141, 145, 155, 156, 173, 174, 175, 178, 184, 186, 187, 188, 191, 200, 201, 215, 219, 232, 235, 253, 264, 306, 335, 340
2	133, 145, 161, 162, 181, 197, 208, and 220

Table 1: Selected ions used for the detection of herbicides.

Selected ions indicated the presence of each analyte at a specific retention time (Figure 3.4) within a sequence of scanned ions. All analytes provided a linear response over the tested concentration range.

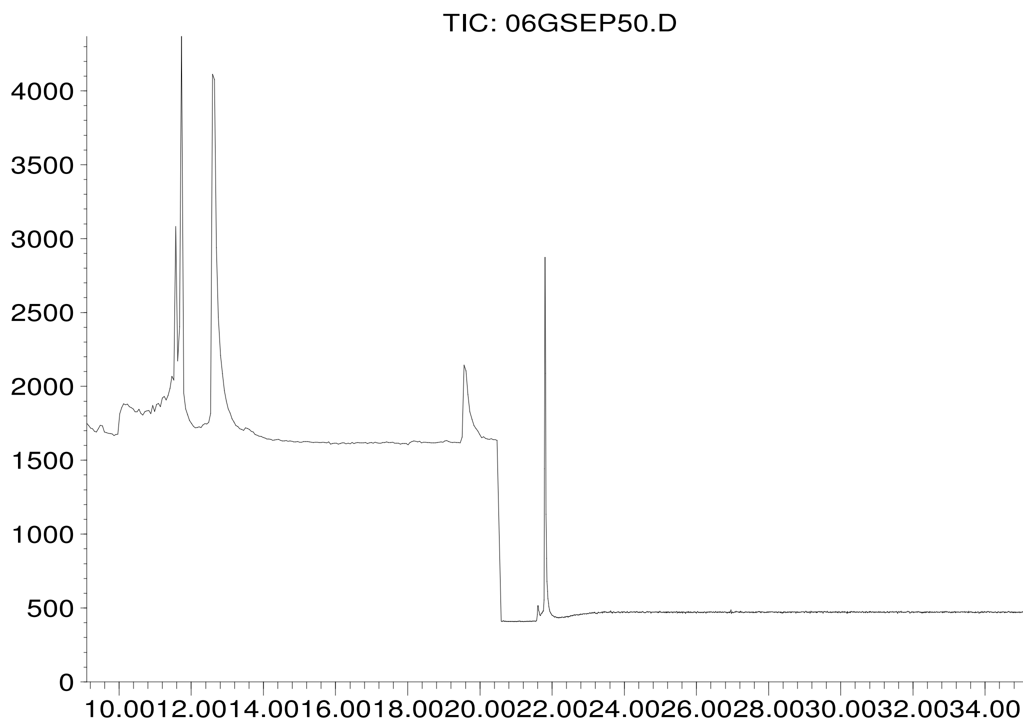


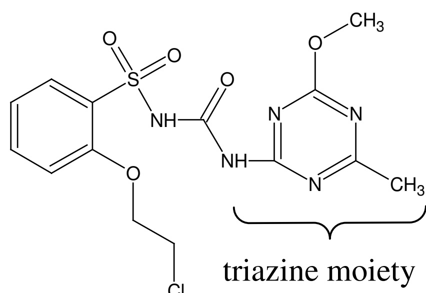
Figure 3.4: Detection of standards in sim mode.

3.4.2 Recovery of herbicide standards from whole soils and seawater

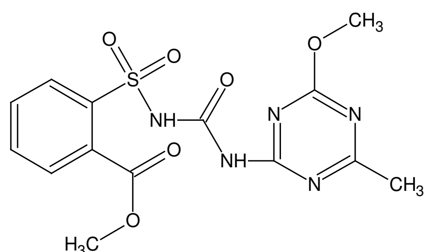
An efficient recovery (105 %) of the internal standard showed that a rotary evaporator treatment concentration step could be used without compromising the internal standard content. This procedure permitted a final loading of 0.512 ng anthracene on-column with each one microlitre injection. Reproducibility (with a coefficient of variation of 3 %, n = 20) of the injection technique was established by repeatedly injecting an anthracene standard (8 ng μL^{-1}) before and after each sample analysis. Treflan, simazine and atrazine were also recovered at 86 %, 92 % and 89 % respectively of the soil ‘spike’ by this procedure. A typical chromatogram is indicated in Figure 3.4 (sim mode). Additionally, standards were analysed in scan mode by using the ChemStation software.

Sulfonylureas are thermally unstable (Long *et al.* 1990). Indeed, they pyrolyse in the heated (250 ^{o}C) injection port of a gas chromatograph to produce two fragments. One fragment contains a ‘triazine moiety’ while the other fragment contains the remainder of the molecule (Figure 3.5).

triasulfuron



metsulfuron-methyl



chlorsulfuron

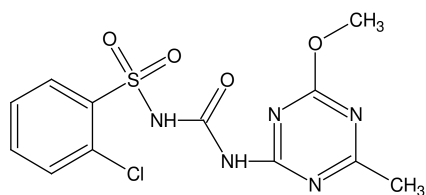


Figure 3.5: Sulfonylurea herbicide structures.

Investigators have therefore quantified sulfonylureas by detecting the triazine moiety on a gas chromatographic system equipped with a nitrogen-phosphorus detector. In the present study the pyrrolysis products of all sulfonylureas could be detected by GCMS

(Figure A5, Appendix A). However, the relatively high column loading (ca. 80 ng on-column) required for efficient detection and the high 'background noise' within soil matrices suggested that an alternate method of analysis (HPLC UV/Vis) should be used (Sarmah & Kookana, 1999).

3.5 Preliminary GCMS analyses: detection of treflan and 2,4-D- like chemicals in whole soils

Treflan (trifluralin) was detected and identified by the ChemStation software in scan mode of whole soil extracts (Figure 3.6). Furthermore, the same analyte was also detected in several soils by using sim mode analyses (Figure 3.7). It is calculated that soils of the Yorke Peninsula contain between 9 and 94 ppb (µg kg^{-1}) treflan.

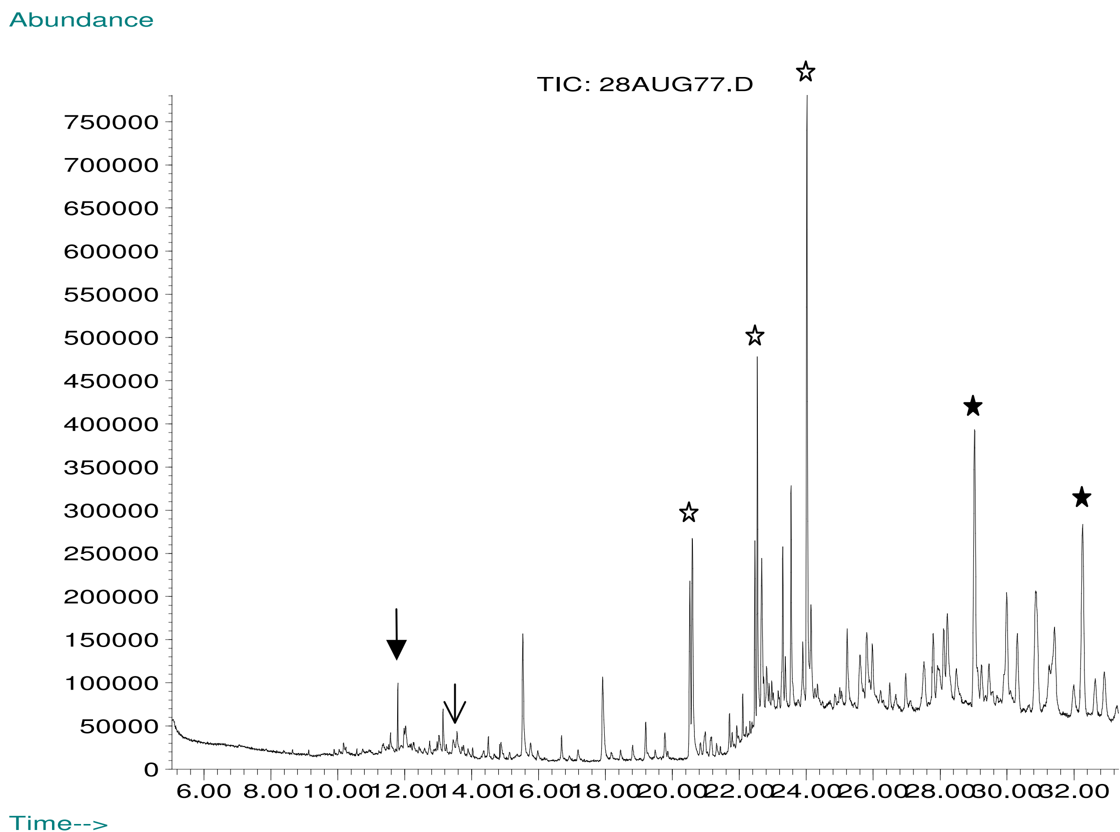


Figure 3.6: Detection of treflan in soil in scan mode. Treflan (bold arrow); internal standard (open arrow); possible sterols (open stars) and 2,4-D- like compounds (closed stars).

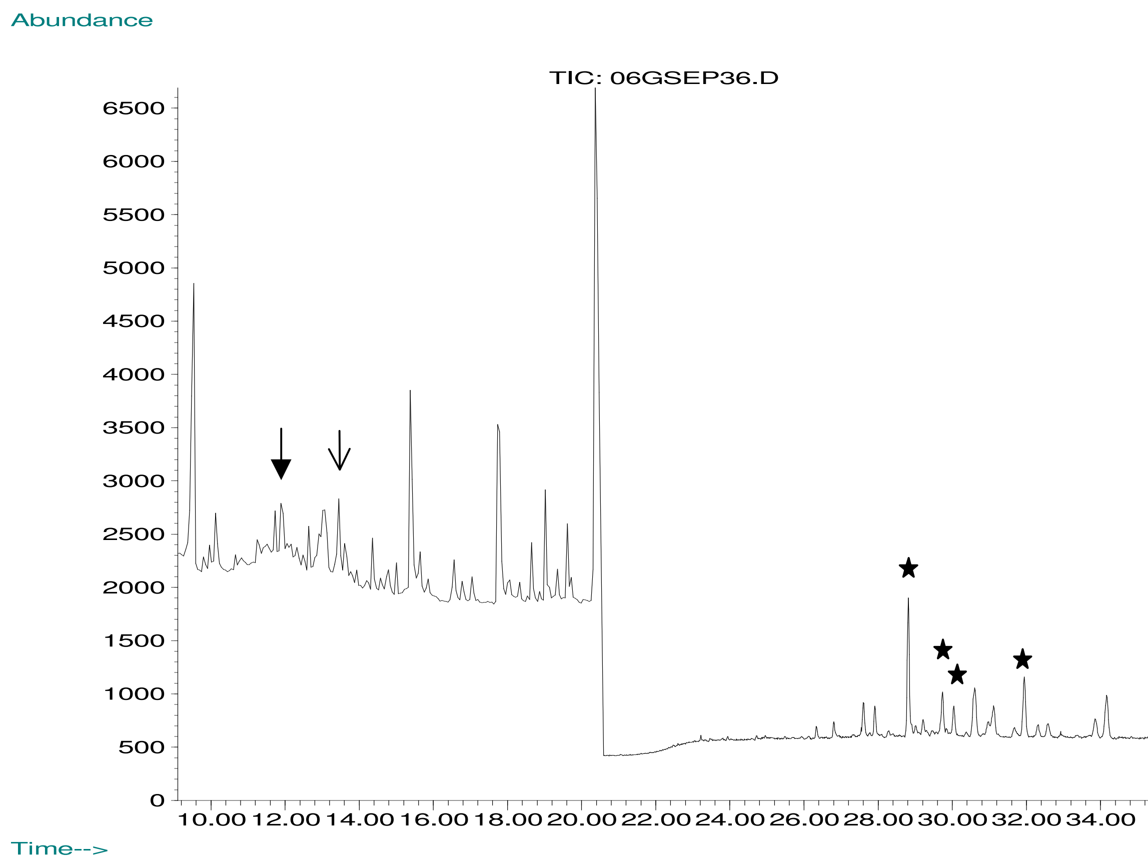


Figure 3.7: Detection of treflan in sim mode. Treflan (bold arrow); internal standard (open arrow) and 2,4-D- like compounds (closed stars).

3.6 Detection of phenoxyalkanoic acid herbicide standards and their derivatives

The results of melting point (141 ^{o}C ± 0.5 ^{o}C), mass-spectral (Figure 3.8) and infra-red analyses (Figure A13, Appendix A) indicated that the white crystalline solid isolated in Section 2.3.1 was the free-acid of 2,4-D. A high yield (97 %) of 2,4-D free acid was obtained during the extraction procedure. This result suggested that the phenoxy bond is stable to acidic (1.4 M HCl) treatment.

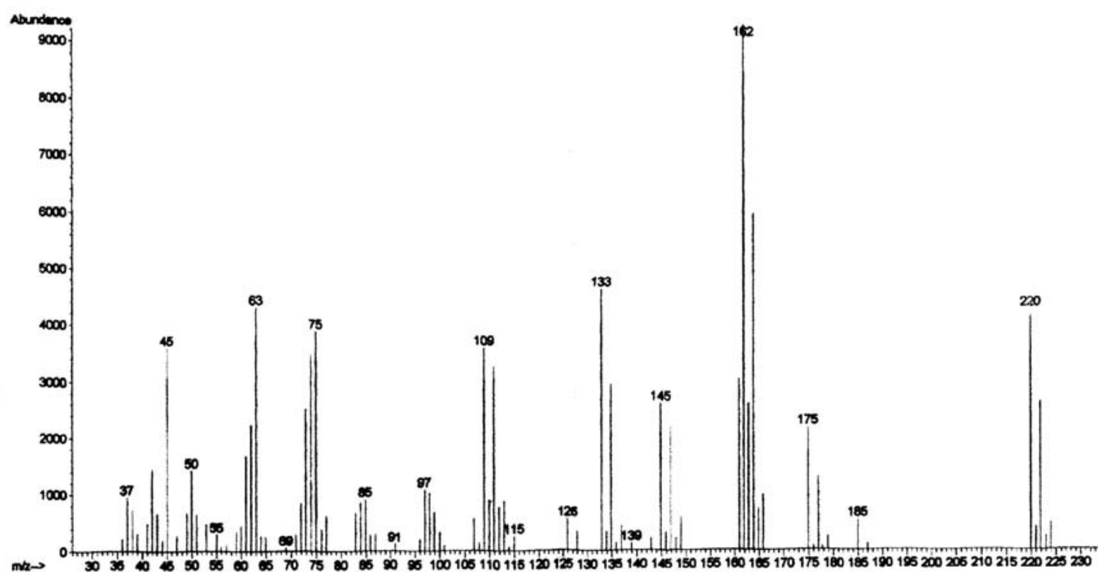
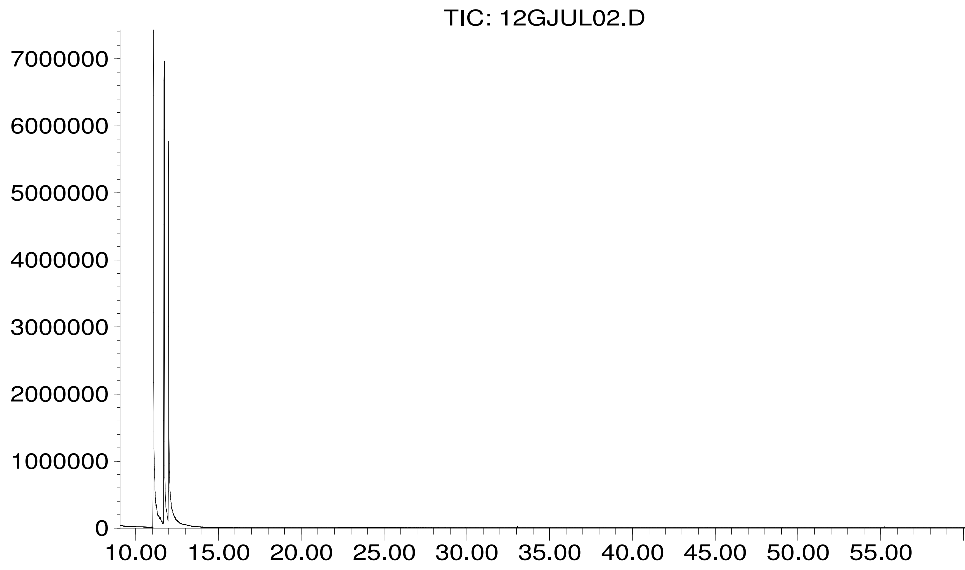
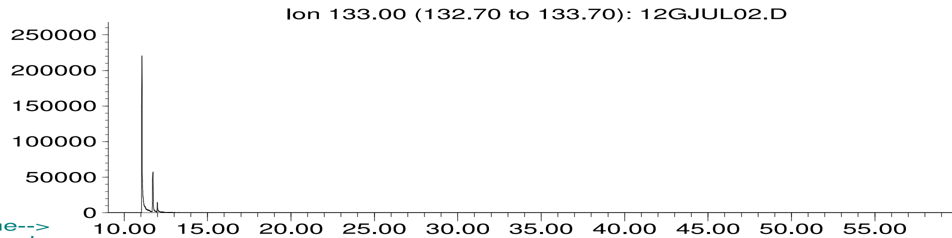
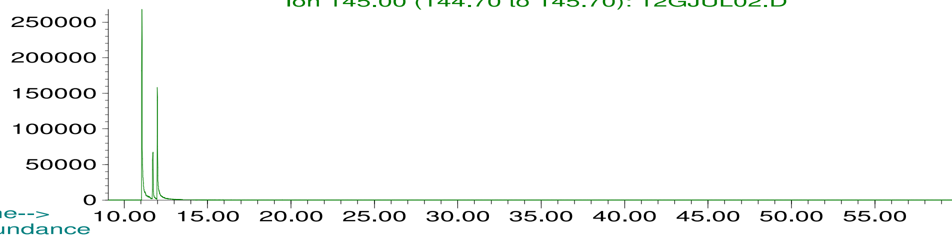


Figure 3.8: Mass Spectrum of isolated 2,4-D free acid.

The GCMS software (ChemStation) identified the methyl esters of phenoxyherbicide standards purchased from SIGMA by using a library-matching algorithm. Indeed the ratios of ion abundance m/z 133:m/z 145:m/z162 remained the same for each analyte regardless of whether detection was performed in 'scan' or ''sim' modes (Figures 3.9 and 3.10).







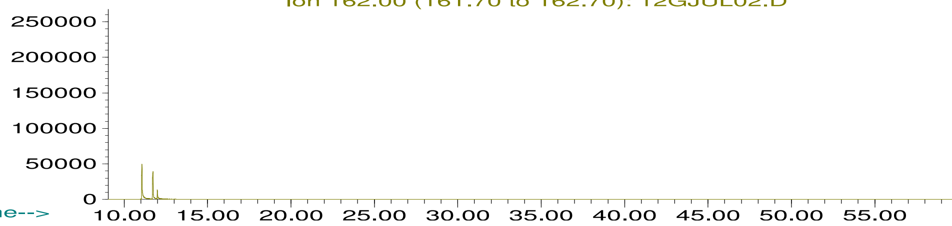
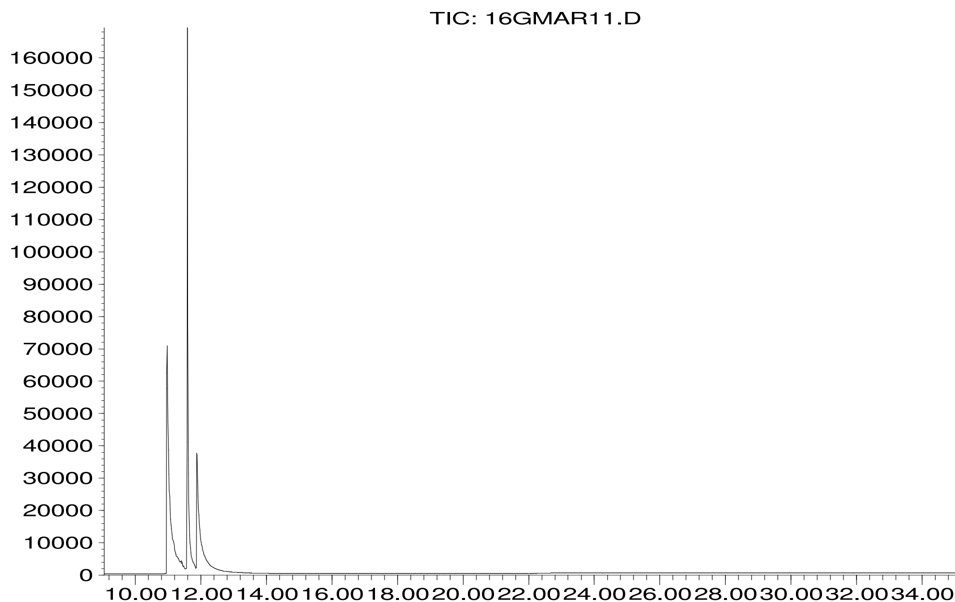
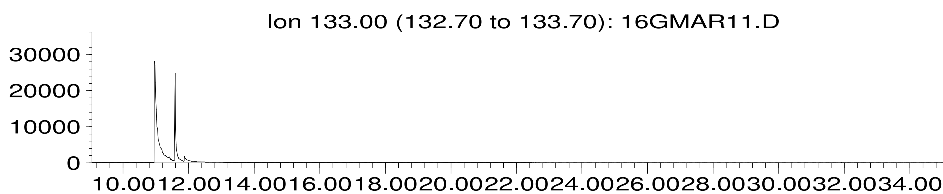
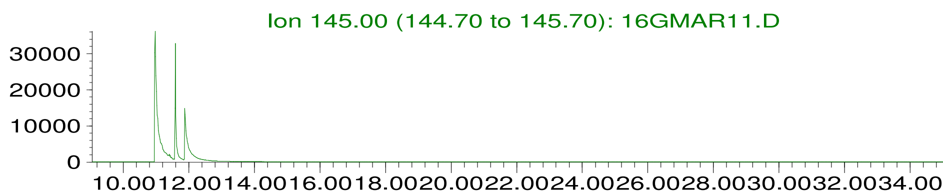


Figure 3.9: Detection of purchased chlorophenoxy herbicide methyl ester standards in scan mode.







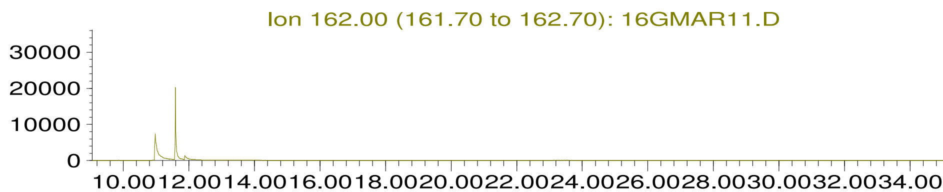


Figure 3.10: Detection of purchased chlorophenoxy herbicide methyl ester standards in sim mode.

Synthesized (Sanchez *et al*., 1991) short-chain (C1) to long chain (C18:1 oleoyl ester) aliphatic monoester standards of 2,4-D (free acid) were also analysed under scan and sim detection. Again the ratios of ion abundance m/z 133 : m/z 145 : m/z 161 : m/z 162 : m/z 220 remained unchanged for each analyte irrespective of whether detection was performed in scan or sim modes (Figure 3.11). It was also noted that characteristic ions present in the 2,4-D methyl ester were absent in the internal standard regardless of whether ions were 'extracted' from a total ion chromatogram or detected *during* a sim analysis using the specific ions m/z 133, m/z 145 and m/z162 (Figure 3.12). The synthesized methyl ester was positively identified by the GCMS 'ChemStation' software and compared satisfactorily with the purchased standard (Supelco) in its retention time and mass spectral pattern (Figure 3.12). The yield of methyl ester obtained by using the method of Sanchez *et al.* was 46 %. It is thought that some losses of this volatile ester may have occurred in preparation during the rotary evaporator treatment.

These data suggested that no matter whether the synthesized aliphatic simple esters were analysed in scan or sim modes the ratio of *isotopic abundances* of selected ions remained constant. This point will be developed further in an analysis of 2,4-D-like chemicals. The scan mode provided molecular detail of eluted analytes if the response was greater than for background and interfering peaks. However, such interference often occurs when analytes are present at low concentrations in complicated matrices. The sim mode focused on selected ions and the mass spectral software (ChemStation) spent a greater proportion of its time 'looking for' the selected ions and provided a mass spectral pattern with little interference. However, sim analyses do not provide molecular detail on the eluted analytes. Therefore, in the present study both modes were necessary in an analysis of low concentrations of pesticides and 2,4-D- like chemicals.

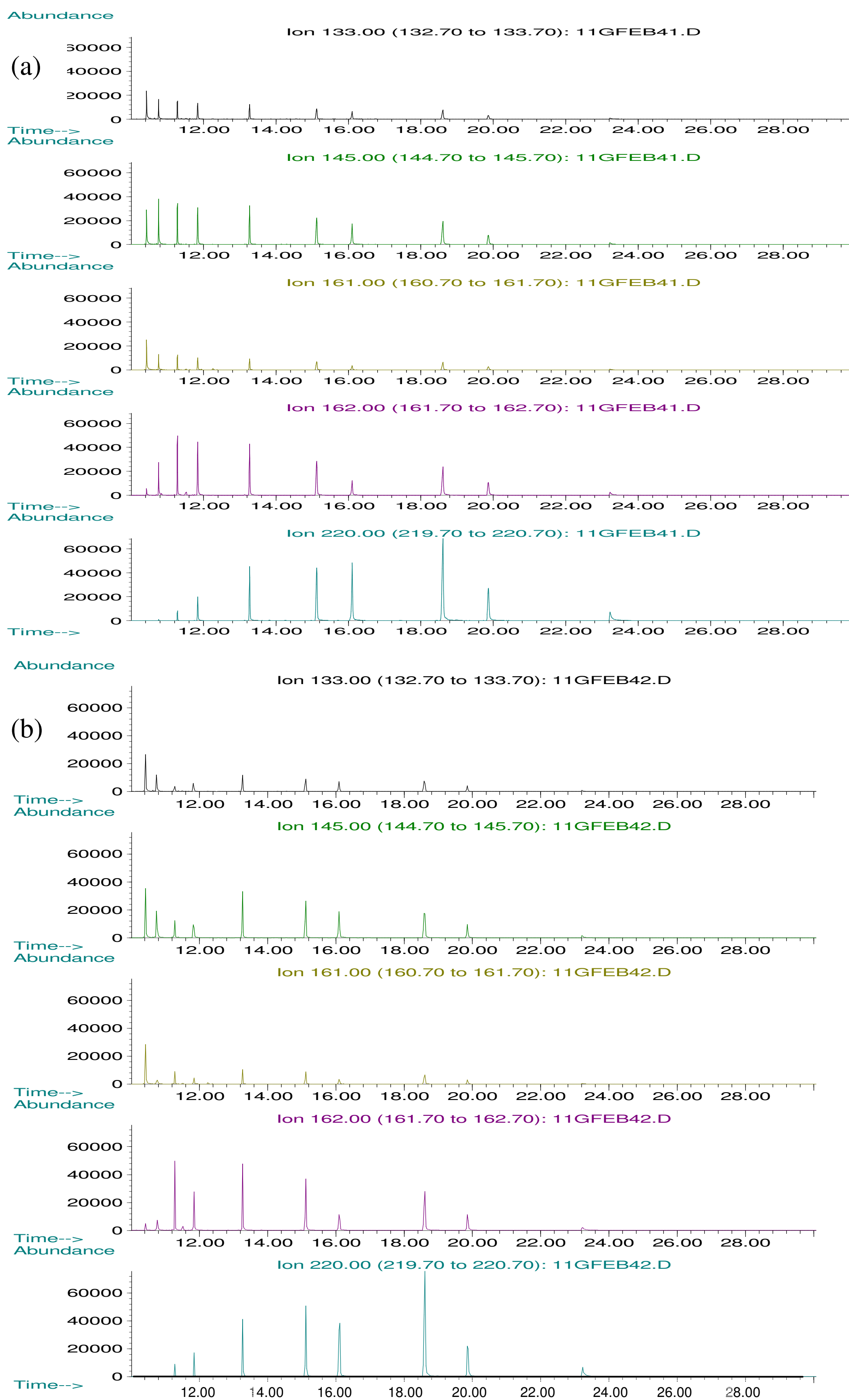
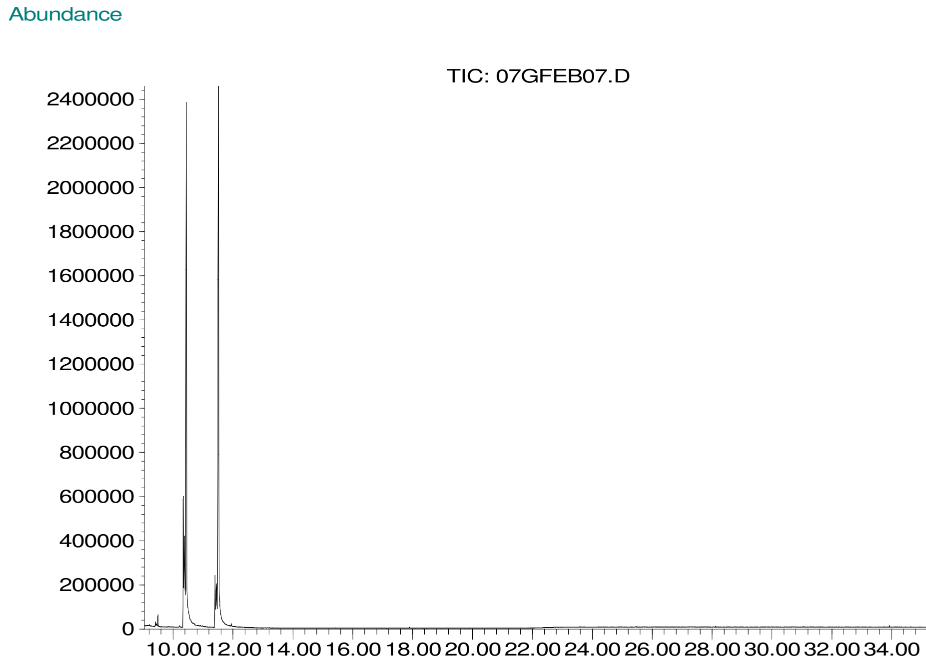
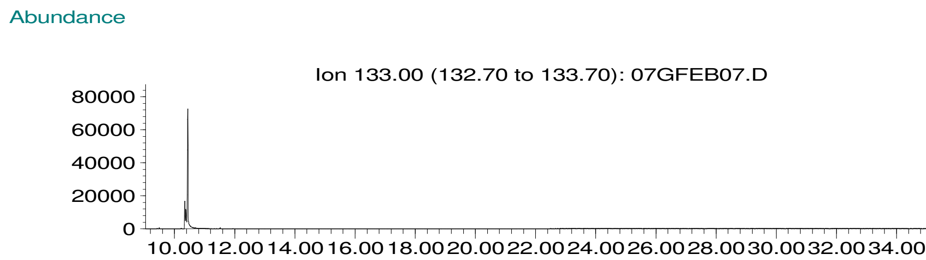
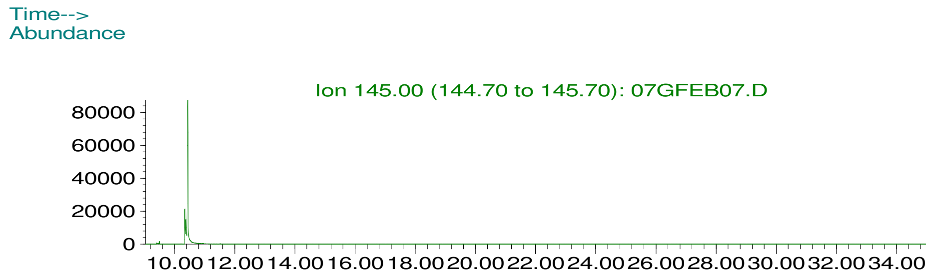


Figure 3.11: scan (a) and sim (b) analysis of an aliphatic ester series of 2,4-D.







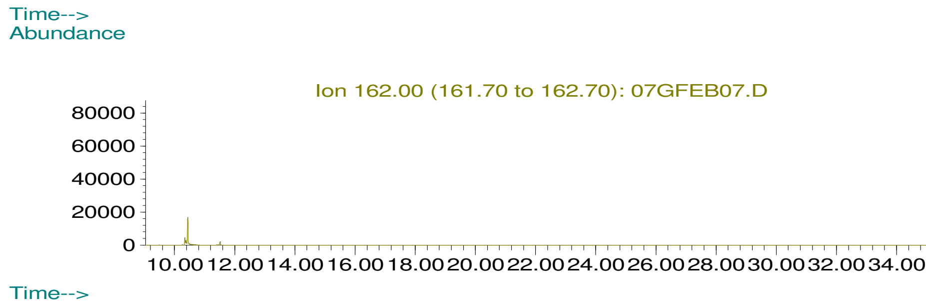


Figure 3.12: Scan mode of the synthesized methyl ester of 2,4-D plus internal standard.

3.7 Hydrocarbons within synthesized long-chain simple esters of 2,4-D

The hydrocarbon 'tails' of later-eluting long-chained standards make an increasing contribution to the mass spectral fragmentation pattern (Figure 3.13). This was demonstrated in both an homologous series of methyl esters of fatty acids and an homologous series of aliphatic alcohols (Figure A6, Appendix A). It should be noted that the same aliphatic alcohols were used in the synthesis (Sanchez *et al.*, 1991) of the aliphatic ester series of 2,4-D (Figure A7, Appendix A). However, the ions m/z 133, m/z 145 and m/z162, that are characteristic of 2,4-D analogues, were absent in the aliphatic alcohol series and were present in low abundance in the fatty acid methyl ester series. Chlorine *isotope ratio* analyses later showed that the fatty acid methyl ester series did not contain multiple ion fragments (M, M+2 and M+4) that are characteristic of compounds that contain two chlorine atoms.

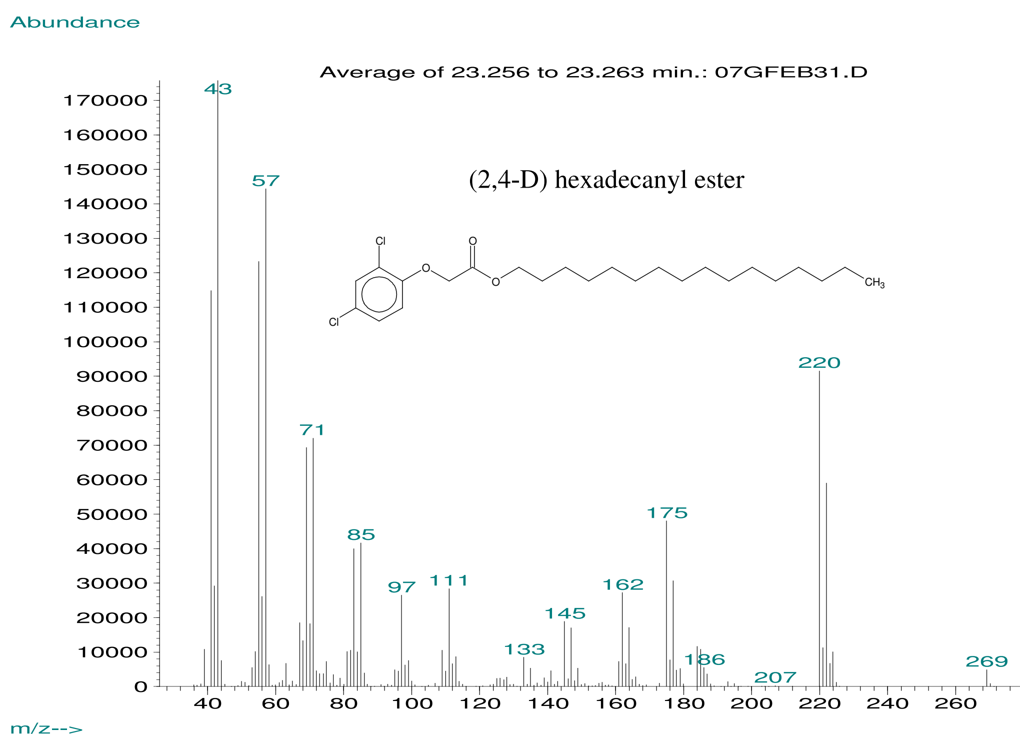


Figure 3.13: Scan of the synthesized hexadecanyl ester of 2,4-D.

It is apparent that hydrocarbon moieties produce characteristic mass spectral patterns when analysed by GCMS. This pattern is similar irrespective of whether the hydrocarbon is contributed by a fatty acid (also known as carboxylic acids) or by a 'long-chained' aliphatic alcohol at an ester linkage. Hydrocarbon moieties effectively reduce the *relative response* of the 'fingerprint' 2,4-D ions m/z 133, m/z 145 and m/z 162 in the mass spectral profile. Additionally, in the mass spectral profiles of aliphatic esters of 2,4-D, a characteristic ion fragment with an m/z of 220 is also produced as a result of a McLafferty (McLafferty & Turecek, 1993) rearrangement. Six atoms are involved in the reaction centre of a McLafferty rearrangement. In carbonyl compounds such as the hexadecanyl ester of 2,4-D, the rearrangement results in the transfer of a single hydrogen atom from the γ position to the oxygen of the carbonyl group, forming the parent 2,4-D acid (Figure 3.14). Such reactions are characteristic of carbonyl compounds.

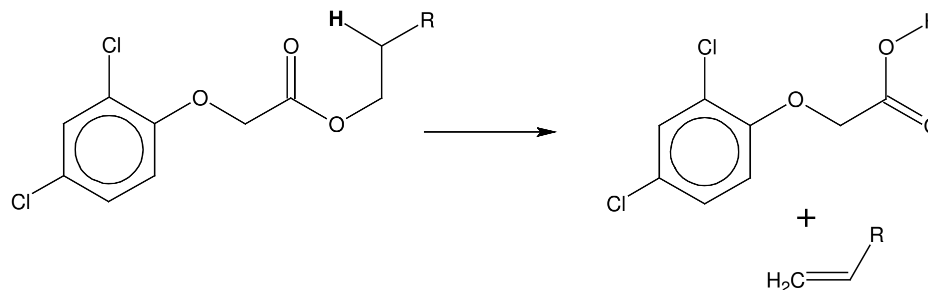


Figure 3.14: McLafferty rearrangement producing 2,4-D acid within the mass spectrometer.

While the ChemStation software positively identified synthesized simple esters of 2,4-D up to C4 (butyl), the longer chained esters, such as C16, were not identified. Indeed, the software often identified these long-chained simple esters as alkanes. It is therefore important to note that the synthesized homologous series of 2,4-D esters in the present

study test the limitations, with respect to analyte identification, of the ChemStation software when the mass spectral library databases are restricted to the supplied 'WILEY275.L' and 'NBS75K.L' libraries. To summarize, as the hydrocarbon chain length increases, a smaller *relative contribution* is made by the 2,4-D fingerprint mass fragments noted above. This is relevant to the analysis of 'unknown' 2,4-D- like compounds detected in soil matrices.

3.8 Isotope ratio analyses of standards and 'unknowns'

The natural *isotopic abundance* of ^{35}Cl : ^{37}Cl is 100 : 32.4 (Davis & Frearson, 1990). A detailed analysis of chlorine isotopes in 2,4-dichlorophenoxy standards, both purchased and synthesized (Figures A7 and A8, Appendix A), and the putative 2,4-D- like series in whole soil (Figures A4 and A9, Appendix A) supports the notion that such compounds contain *two* chlorine atoms. Furthermore, it is likely that the chlorine atoms are in the ortho (2) and para (4) positions since the mass spectral profiles, with m/z values of 133, 145, and 161, most closely resemble the 2,4 rather than the 2,3 or the 3,4 stereoisomers (Que Hee & Sutherland, 1981). The same unidentified series of chemicals were also detected in dust obtained from whole soil (Figure A10, Appendix A). Additionally, the isotopic abundance in the characteristic ion patterns with m/z values of **133**, 135, 137; **145**, 147,149 and **161**, 163, 165 were in the ratio of 9:6:1 for M : M+2 : M+4 and are in agreement with the calculations of Davis and Frearson (1990). The authors provide a formula for calculating the isotopic ratio as follows:

$(a + b)^n = a^2 + 2ab + b^2$

where 'a' is the relative abundance of the light isotope (^{35}Cl)

'b' is the relative abundance of the heavy isotope (^{37}Cl) and

'n' is the number of halogen atoms present.

3.9 Kovats analyses of retention time data in a homologous series

The log-retention times of the methyl ester derivatives of fatty acids and the aliphatic ester derivatives of 2,4-D were linearly correlated with the number of carbon atoms in the analytes of each series (Figure A11, Appendix A). A similar relationship was also

found between the log-retention times of a 2,4-D- like series of chemicals and an arithmetic carbon series.

3.10 Identifying and quantifying 2,4-D and 2,4-D- like chemicals in whole soil and dust by GCMS

The co-eluting characteristic molecular fragments (m/z 133, 145, 161 and 162) of the 2-butoxyethyl ester of 2,4-D (Figure 3.15) allowed the detection and quantification of 2,4-D within soil matrices. It is noted that the isotopic ratio of ^{35}Cl : ^{37}Cl permits the formation of a characteristic mass spectral pattern when this derivative is fragmented. Additionally, the derivatisation procedure produced an identifier ion with an m/z value of 320.

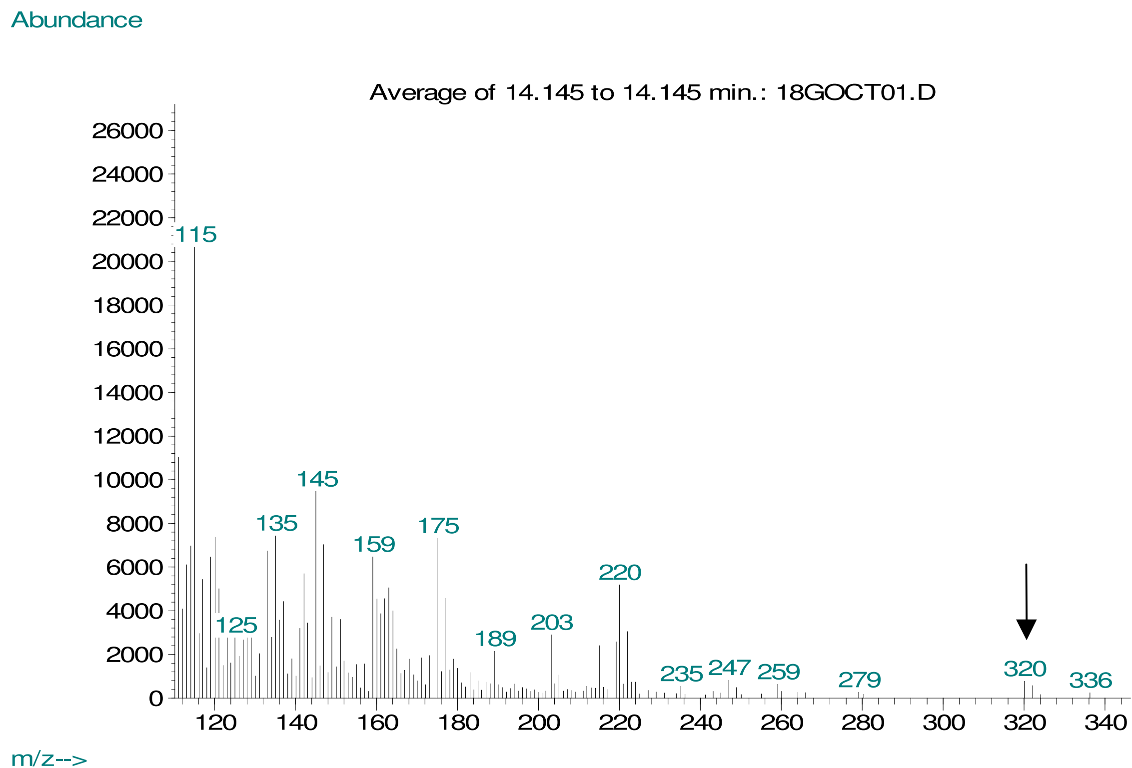


Figure 3.15: Detection of 2,4-D in a soil extract (note the presence of the 2-butoxyethyl ester of 2,4-D at m/z = 320).

Mass spectral patterns similar to those produced by 2,4-D simple esters (Figure A7, Appendix A) were also observed in the 'unknown' 2,4-D chemical series that was extracted from whole soils (Figure A9, Appendix A), and 'dusts' derived from whole soils (Figure A10, Appendix A) of the Yorke Peninsula. Co-eluting ion fragments were present with m/z values of 133, 145 and 161. It is particularly interesting to note that many analytes produced ion fragments with an m/z value of 189 (to be compared with m/z 175) possibly indicating the presence of an additional methylene group attached to the 2,4-dichlorophenoxy moiety (Figure 3.16).

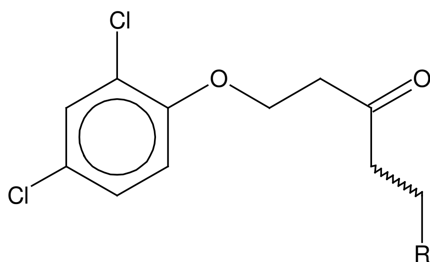


Figure 3.16: A possible 'generic' metabolite of 2,4-D (a 2,4-D- like compound).

It is known that 2,4-D is metabolised in plants into (long chained) derivatives as part of a defence mechanism (Linscott & Hagin, 1970). The detection of an ion with an m/z value of 218 also suggested the presence of a *methylene bridge* separating a 2,4-dichlorophenoxyacetyl moiety (m/z 203) from the remainder of the molecule. A γ-hydrogen is probably made available for the formation of a hexagonal reaction centre in a McLafferty rearrangement to produce a 2,4-dichlorophenoxyacetyl enol molecular fragment (Figure 3.17). However, in this case the γ hydrogen transfer produces an enol which rearranges into the methyl ketone of 2,4-D (m/z = 218). It is currently unclear

whether the ion with an m/z value of 218 was created directly from the original 2,4-D-like molecules or whether it was the result of a rearrangement reaction within a metabolite of 2,4-D.

Isotope ratio analyses of standards and whole soil extracts also suggest the presence of 2,4-D- like chemicals in these matrices. Additionally, a 'peak purity' (ChemStation software) analysis of this unknown series indicated that a chlorophenoxy moieties are covalently bound to hydrocarbon(s) and is not merely co-eluting with hydrocarbon analytes.

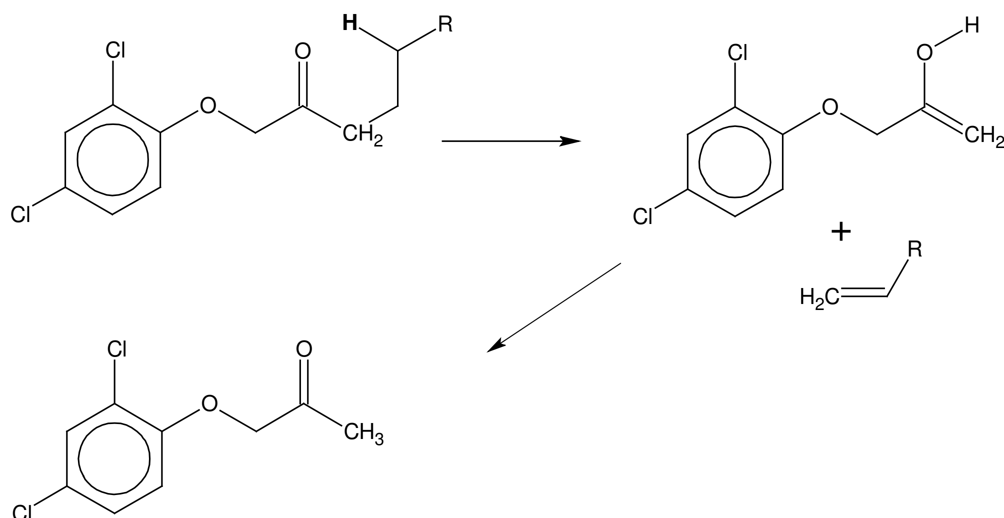


Figure 3.17: A McLafferty rearrangement producing a methyl ketone.

It is calculated from the cumulative areas of analytes, containing co-eluting fragment ions with m/z values of 133 and 145, that 2,4-D- like chemicals are present at concentrations of between 10 and 1022 ppb (185 ± 177 ppb, n=100) in whole soils of the Yorke Peninsula.

3.11 Confirmatory analyses using gas chromatography with electron capture detection (GCECD)

Chemicals detected eluting late in the chromatogram contained halogen atoms. It is important to note that the retention times and their peak profiles, as a set, are similar to those in the GCMS analyses (Figure 3.18). These data support the notion that 2,4-D analogues are present in whole soil extracts and are also probably present on dust (< 250 μm) obtained from whole soils.

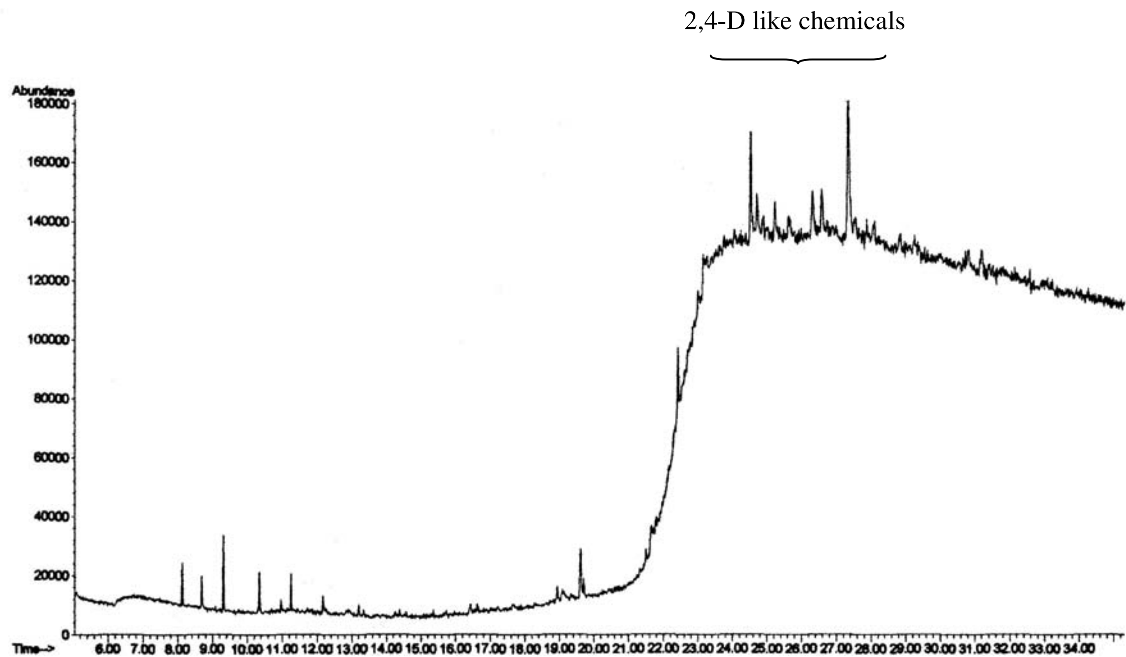
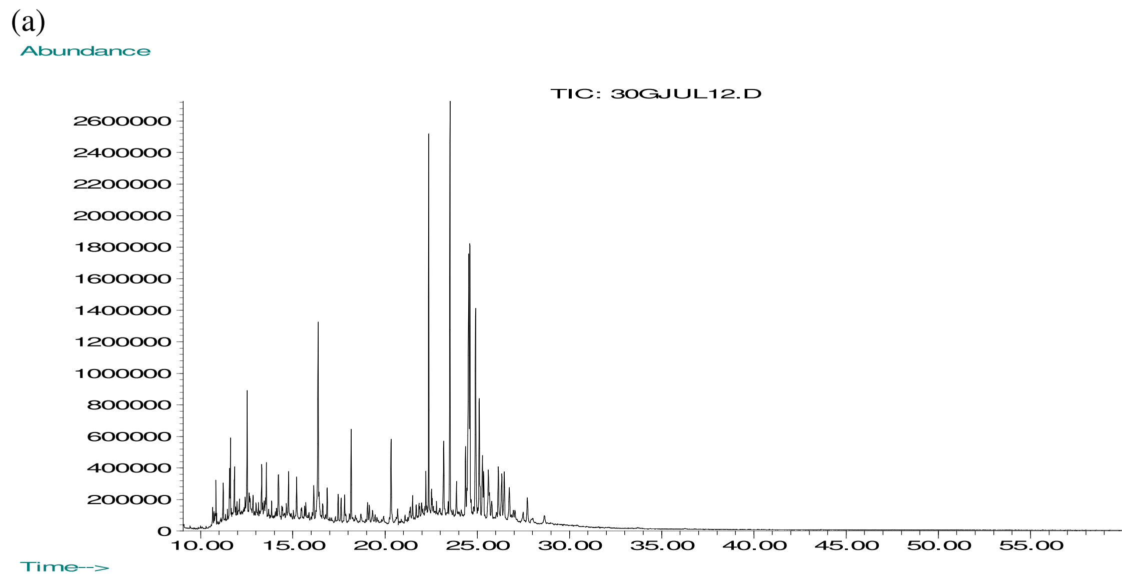


Figure 3.18: GCECD analysis of a whole soil extract.

3.12 Hydrophobic interaction chromatography (HIC) analyses of 2,4-D- like chemicals in whole soils

Poor recovery in the stepped solvent gradient profile while using increasing concentrations of acetonitrile indicated that the members of the 2,4-D- like series were highly hydrophobic. Indeed, only the n-hexane eluent contained the 2,4-D- like series (Figure 3.19). The mass spectral profiles of eluents, analysed under both scan and sim modes, were similar to those of the previously detected 2,4-D- like series (Figure 3.20). FTIR results also suggested that these hydrophobic 2,4-D- like chemicals had hydrocarbon (aliphatic) moieties associated with them (Section 3.15).

(a)



(b)

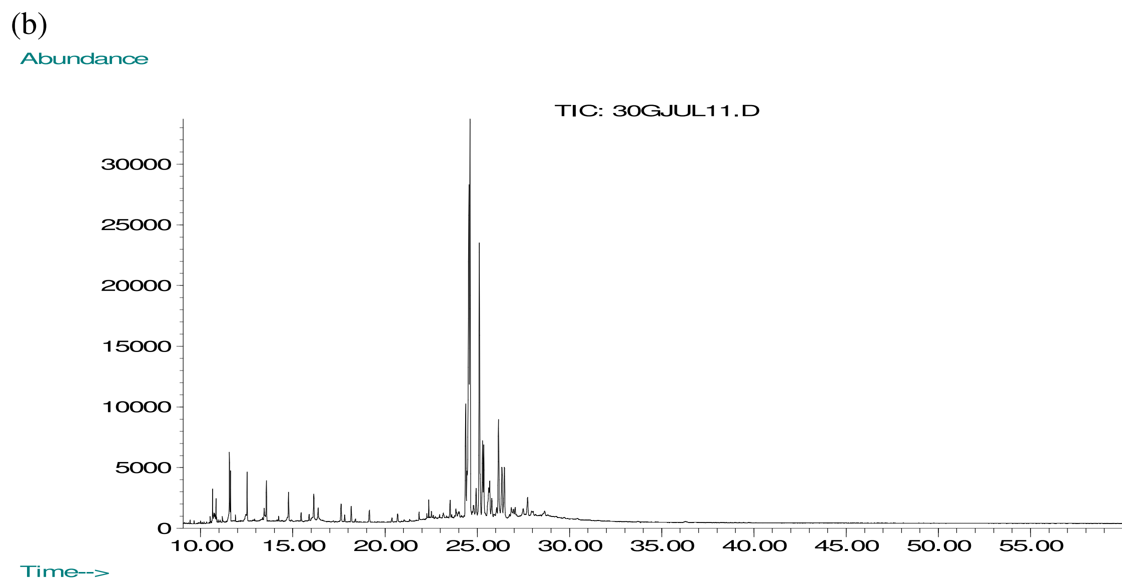


Figure 3.19: n-hexane eluent of a HIC column in scan (a) and sim (b) mode.

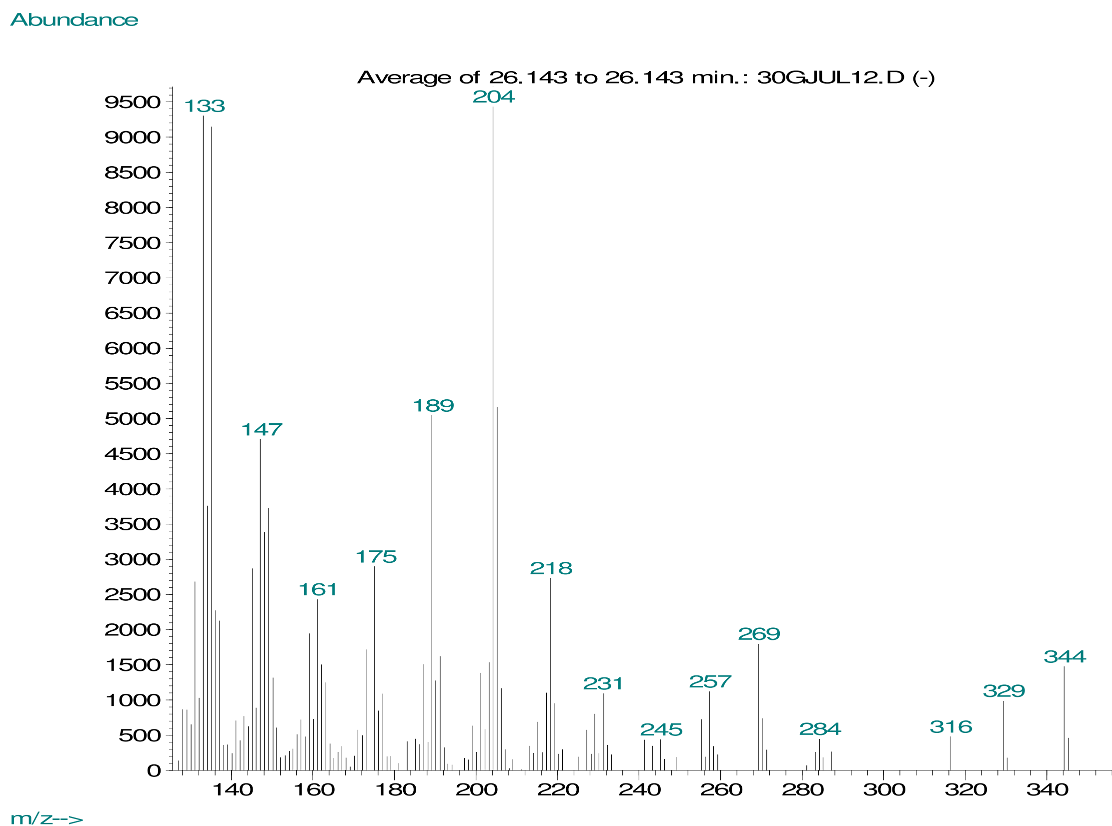
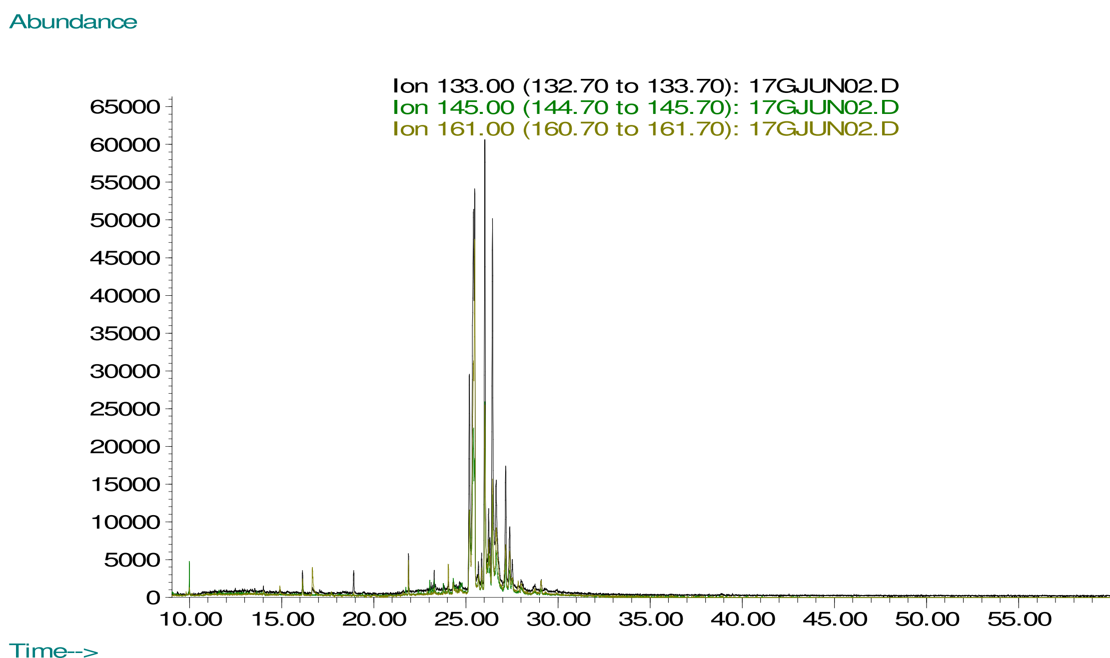


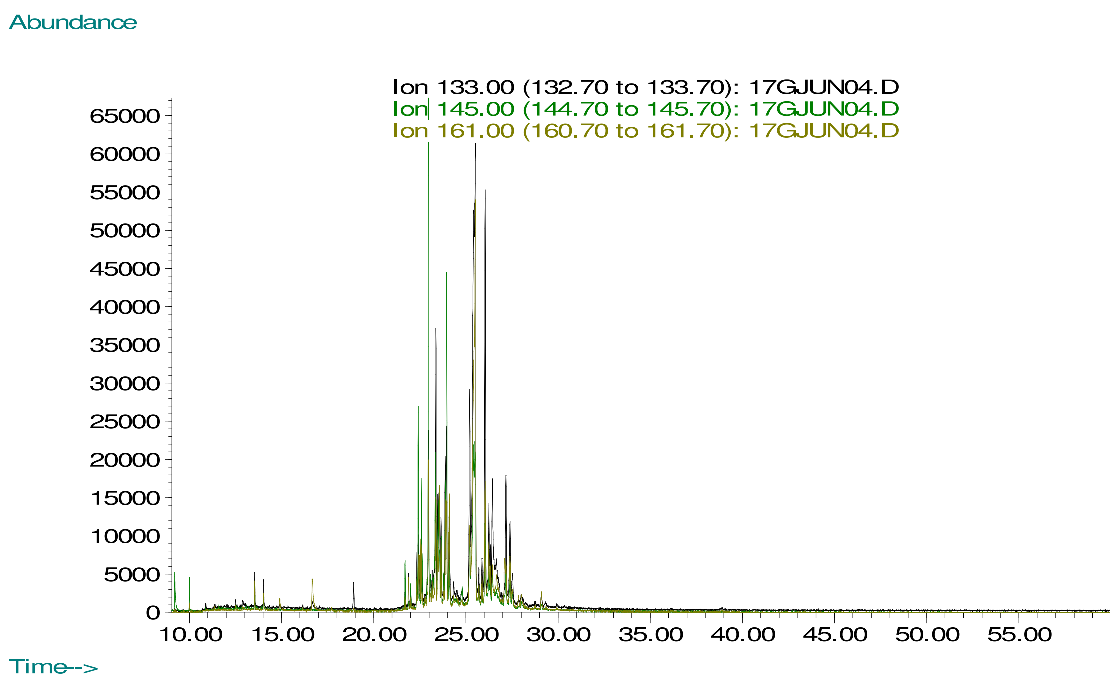
Figure 3.20: The mass spectral profile of an n-hexane eluent from a HIC analysis.

3.13 Analysis of acid and base hydrolysates of detected 2,4-D- like chemicals extracted from whole soils

The acid (50 % H_2SO_4) and subsequent base (0.5 M NaOH) treatment of soil extracts provided additional information in relation to the chemical characterization of the 2,4-D-like series detected at the end of each GCMS profile. The acid treatment resulted in the production of a second series of 2,4-D- like compounds that eluted earlier than the parent group (Figure 3.21 and Figure A12, Appendix A). Subsequent base treatment of the hexane extract obtained from the acid hydrolysate removed this earlier eluting group.

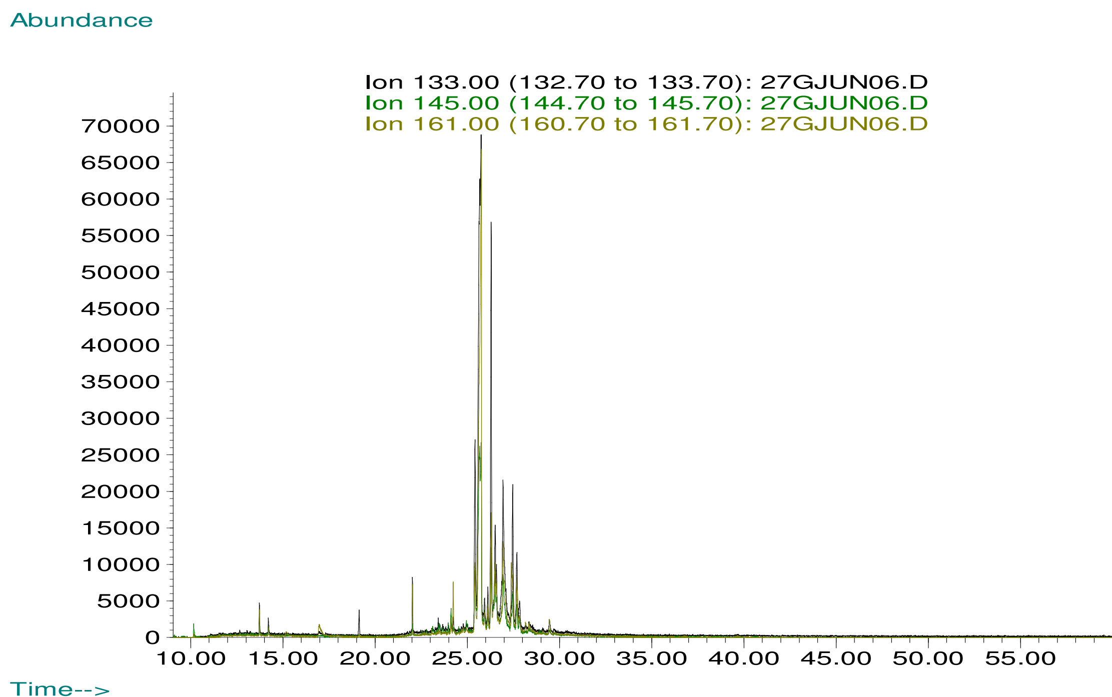


(a) Scan of an extract of whole soil #58, no treatment.



(b) Scan of an extract of whole soil #58 (extracted ion m/z =145) after an acid hydrolysis treatment.

Figure 3.21: Scans of acid and alkali treatments of soil extracts.



(c) Scan of an extract of whole soil #58 (extracted ion m/z =145) after sequential treatments with acid then alkali.

Figure 3.21 (continued): Scans of acid and alkali treatments of soil extracts.

A similar susceptibility to base treatment was also shown by a purchased mixture of chlorophenoxy methyl ester standards (Herbicide Ester Mixture 8270) with 95 % of the parent material being degraded by the treatment.

3.14 Are putative 2,4-D- like chemicals structurally related to a commercially available humic acid?

A GCMS analysis of a standard humic acid (Fluka, AG. CH-9470 Buchs, 35069 887 53680, Asche 10 – 15 %, Mr 600 - 1000) extracted into acetonitrile (Section 2.13) did not detect the complete 2,4-D- like series noted in the present study. However, a single

compound was detected with a *similar* retention time (26.84 mins.) to the 2,4-D series (Figure 3.22). It is likely that it contained a dichlorophenoxy moiety since the *isotopic ratios* that characterize 2,4-D ions (m/z **133**, 135, 137, **145**, 147, 149; and **161**, 163, 165) were present in the mass spectral profile. However, the ion abundances were one-hundredfold less than those observed in the present study (Figure 9, Appendix A) and an alkaline hydrolysis had little effect on the mass spectral profile of the detected analyte.

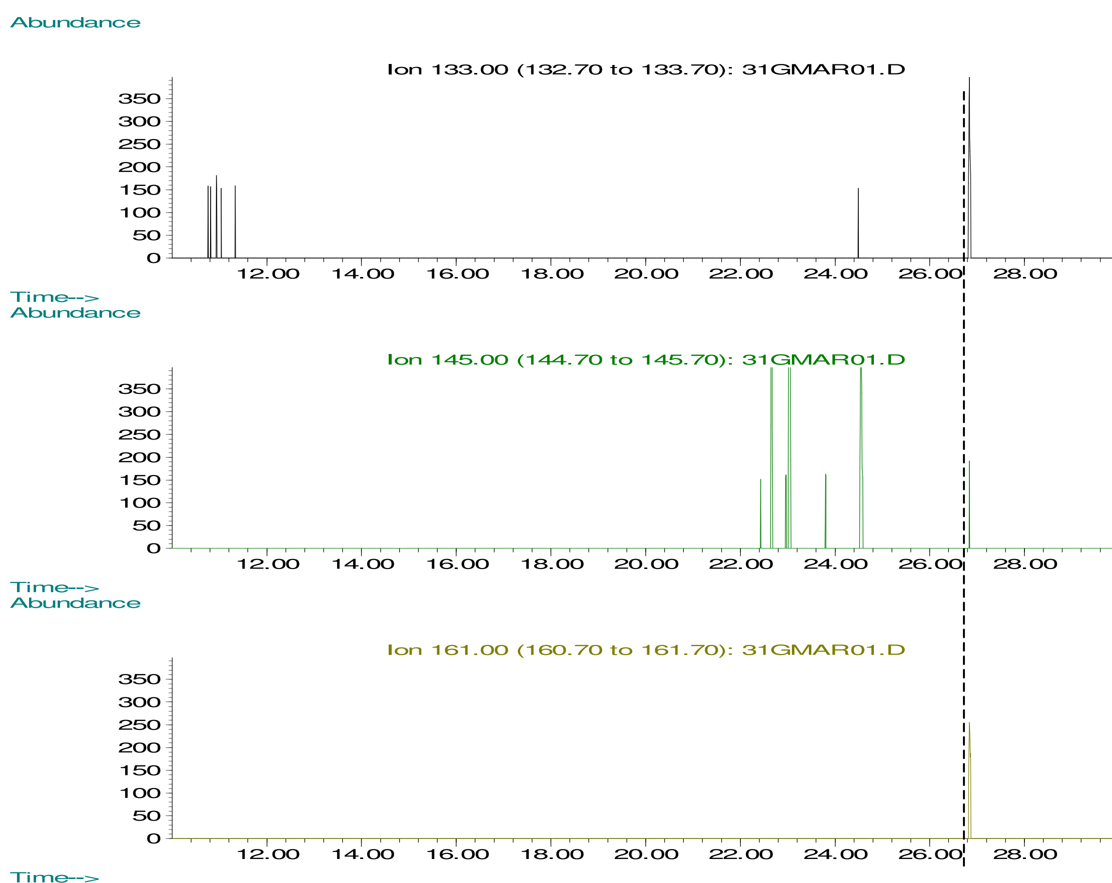


Figure 3.22: Humic acid analysed by GCMS. Note the low abundance of ions that are specific for 2,4-D- like chemicals.

3.15 Fourier transform infra-red spectroscopy (FTIR)

2,4-D free acid absorbed at 3000 cm^{-1} (OH), 1736 cm^{-1} (carbonyl stretching), 1479 cm^{-1} (C-O) and 721 cm^{-1} (C-Cl) (Figure A13, Appendix A). These results are comparable to those presented by Que Hee and Sutherland (1981).

2,4-D-aspartate and 2,4-D-glutamate showed strong absorbance bands at 3375 –3398 cm^{-1} (N-H stretching), 1741 cm^{-1} (carbonyl stretching), 1630 - 1640 cm^{-1} (C-N), and 759 - 760 cm^{-1} (C-Cl). These data are consistent with the compound being a secondary amide (Figure A14, Appendix A).

The oleoyl ester of 2,4-D absorbed at 2950 cm^{-1} (saturated C-H stretching), 2855 cm^{-1} (C-H aryl stretching), 1744 cm^{-1} (carbonyl stretching), 1466 cm^{-1} (possibly a C-O stretching) and 723 cm^{-1} (C-Cl); (Figure A15 (a), Appendix A). These data are similar to those published by Que Hee and Sutherland (1981) for the methyl ester of 2,4-D in which there was a reduction in the 2950 cm^{-1} (saturated C-H stretching) band.

The soil extract had an infra red spectrogram that was similar to that of the oleoyl ester of 2,4-D. Strong absorbance was shown at 2954 cm^{-1} (saturated C-H stretching), 2853 cm^{-1} (C-H aryl stretching), 1745 cm^{-1} (carbonyl stretching), 1465 cm^{-1} (possibly a C-O stretching) and 721 cm^{-1} (C-Cl) (Figure 3.23 and Figure A15 (b), Appendix A).

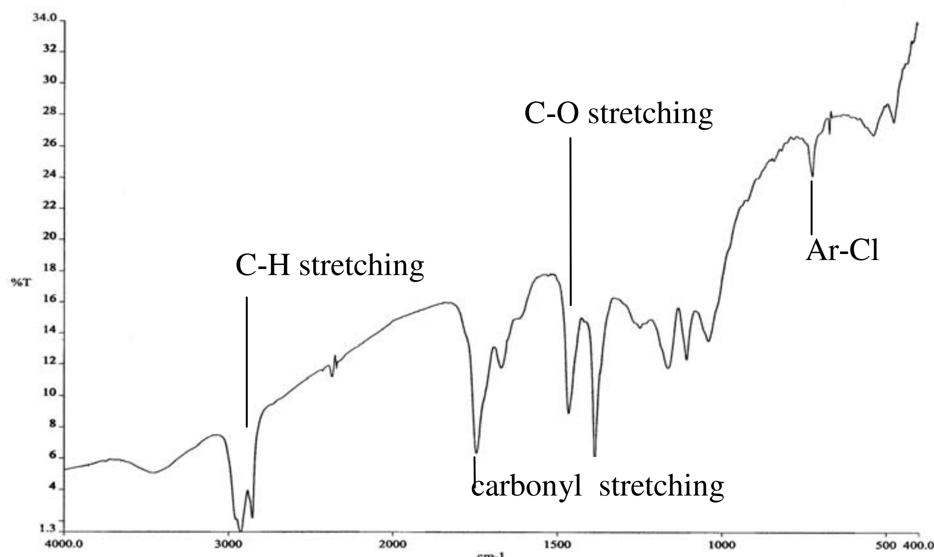


Figure 3.23: FTIR analysis of a soil extract.

3.16 High performance liquid chromatography (HPLC)

Metsulfuron-methyl was detected in three of the twelve soils analysed (Figure A16, Appendix A). These data are consistent with the known usage and 'persistence' of sulfonylurea herbicides on farmland containing alkaline soils (Sarmah *et al*., 1998). The 'residual' sulfonylurea content of whole soil was calculated as being between 2.5 g ha^{-1} and 7.5 g ha^{-1} (assuming a soil density of 1500 kg m^{-3} and depth of 10 cm containing uniformly dispersed metsulfuron-methyl).

3.17 Summary of herbicide and herbicide-like chemicals detected in whole soil and dust by chromatographic analyses

Little or less than the quantifiable limit (4 ppb) of persistent herbicides, such as simazine and atrazine, was detected on soils of the Yorke Peninsula. However, treflan was positively identified, in scan mode, in soil samples and was subsequently detected in seven of the 90 analysed soils by using sim analysis. Levels of treflan were between 9 and 94 ppb in soil.

While simple esters of the phenoxyalkanoic acid esters were not detected, chemicals that resemble 2,4-D were detected in over 90 % of analysed soils. Moreover the same chemicals were also detected in dust (fraction 5, < 250 µm) obtained from a representative sample of six soils. Results from hydrophobic interaction chromatography indicate that the 2,4-D- like chemicals are hydrophobic and the results of isotope ratio analyses and mass spectral patterns suggest that these chemicals contain 2,4-dichlorophenoxy moieties bound to hydrocarbon moieties. Furthermore, the acid and base hydrolysis treatments of the 2,4-D- like chemicals suggest the presence of acid-labile and base-labile bonds within molecular structures that contain 2,4-dichlorophenoxy moieties. It is estimated that soils of the Yorke Peninsula contain between 10 and 1022 ppb 2,4-D- like chemicals.

Sulfonylurea herbicides were also detected by HPLC (UV-VIS detection) in tested soils and were present at between 2.5 g ha^{-1} and 7.5 g ha^{-1}.

CHAPTER 4: MATERIALS & METHODS, PART B: SEAGRASS COLLECTION, PREPARATION, CULTURE METHODS AND THE DEVELOPMENT OF BIOASSAYS

4.1 Collection of *Z. muelleri*

Seagrass was collected from the Barker Inlet, St. Kilda South Australia, at latitude 34° 46′ S and longitude 138° 32′ E on 15/3/2001 in summer. It was collected at low tide (11:30 a.m.) along a ten metre wide 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 (Figure 1.1). Sampling sites were allocated every five metres along the transect line.

The root zone of *Z. muelleri* extended to 20 cm below the sediment surface so turfs (30 cm x 30 cm x 30 cm) were taken with care so as not to disturb the roots of the seagrass. The turfs were then washed free of sediment at the collection site by loosely holding the seagrass and bound sediment then drawing water flow over the sediment surface by moving the turf back and forth through seawater. Sediment-free plants were then placed in aerated seawater collected at the site (20 L), aeration being supplied by a portable battery-operated aeration pump (Otto), and then transported to the laboratory. Free-floating plants were stored overnight in an aerated seawater holding tank (500 L capacity polypropylene tank (0.8 m x 0.8 m x 0.8 m) filled to 500 L) positioned 1 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 washing procedure described above. Additionally, intact turf samples were stored overnight in aerated seawater in 20 L plastic drums. Plants were then allocated to working tanks for experimentation.

4.2 Laboratory culture of *Z. muelleri*

4.2.1 An overview of seagrass culture methods and sediment loading

Three sediment loading regimes were used. In Buoyant Aquatic *Zostera* (BAZ) analyses no sediment was used in a short-term, two-week culture, of ‘free floating’ plants. Sediment-bound *Zostera* comprised plants either grown in 30 % sediment (jars) or in

100 % sediment (baskets). The 'solid' substrate of *Zostera* grown in jars was composed of 30 % (intertidal) sediment and 70 % seawater-washed coarse sand. In 'jar' experiments plants were laboratory-acclimated for 12 months prior to treatment with herbicide. *Zostera* grown in baskets in 100 % sediment were laboratory-acclimated for at least one month prior to use. It was anticipated that the nutritional requirements of *Z. muelleri* were satisfied in both these short and medium-term culture techniques.

4.2.2 Purpose of the seagrass culture study

The laboratory study of *Z. muelleri* culture was designed to investigate the possible effects of 'land-use herbicides' on biota of the intertidal zone. The seagrass, *Z. muelleri*, was selected as a representative species of the intertidal zone. It was decided to observe and quantify any differences between test and control *Zostera* under defined, laboratory-controlled conditions. Seagrass was grown under laboratory conditions that approached those of the natural environment. The rationale used here was that the known toxicity of intertidal sediment to *Zostera* (Faraday, 1979; Jonkers *et al*., 2000; Prange & Dennison, 2000) may be diminished by 'diluting' the sediment or by mimicking the natural water flow that occurs in seagrass meadows. It seemed likely that by modifying the sediment content the experimenter would be able to modify any toxic stress 'normally' imposed on plants in the intertidal zone. *Zostera marina* is known to propagate both sexually and by vegetative means (Phillips *et al*., 1983). It was therefore likely that free-floating *Z. muelleri* (Buoyant Aquatic *Zostera*, (BAZ)) could be used as a test system. Furthermore, it was reasoned that results obtained would be removed from any possible synergistic interaction between treatment and sediment in this growth system. Next, plants were grown in jars by using a mixture of 30 % sediment and 70 % coarse sand. This allowed the study of possible synergistic interactions between a 'low-sediment loading' and treatment on seagrass growth and survival. Seagrass was also grown in 'turfs' (100 % sediment) to allow the study of a possible synergistic interaction between a 'high-sediment loading' and treatment on seagrass growth and survival.

4.3 Establishing laboratory culture methods for *Z. muelleri*

4.3.1 Equipment and seawater

Four polypropylene tanks (0.8 m x 0.8 m x 0.8 m) were used to store and grow seagrass. All tanks were mounted on angle-iron frames to allow ease of access; the total height of each assembly was 1.4 m. Three 'working tanks' were filled to 0.4 m depth with seawater (250 L) while the fourth tank, which served as a holding tank, was filled to 500 L with seawater. Each working tank was provided with artificial lighting and an aeration system. Glass jars (2.5 L) were supplied by Corning.

It was calculated that large volumes of seawater would be required for seagrass culture methods and for bioassay experimental designs. Filtered seawater was therefore collected from S.A.R.D.I. (West Beach, South Australia) by using food-grade polypropylene 50 L bins. It was reasoned that the collected seawater would have a similar chemical profile to that which was present at the collection site for seagrass samples since seagrass species grow along the metropolitan coastline. 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 have occurred.

4.4 Lighting, temperature and aeration regime

4.4.1 Lighting

Initially, lighting was provided by using 'Grolux' fluorescent tubes (36 W) over all working tanks but not over the holding tank. However, the light intensity was then increased to 250 $\mu E\ m^{-2}\ s^{-1}$ by using a sodium lamp (Sylvania 17, 400W, Belgium SHP-TS-400W) equipped with a transformer and positioned over the centre of each working tank at a height of 90 cm above the water's surface. This represented approximately one quarter of the PAR normally available to *Z. muelleri* under summer growth conditions. It is also consistent with measured PAR in *Z. muelleri* meadows (Clough & Attiwill, 1980) and PAR used in seagrass culture techniques (Mc Millan, 1978). A mechanical timer was used to control the lighting on a 12 hr – 12 hr lighting regime (6 a.m. to 6 p.m. summer time, South Australia).

4.4.2 Temperature

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

4.4.3 Aeration

Aeration was used as a substitute for wave action to ensure adequate oxygenation of the seawater. A mechanical timer was used to control aeration on a 12 hr – 12 hr regime (6 p.m. to 6 a.m. summer time, South Australia). Aeration was provided by a pump (Dynavac Pty Ltd) equipped with a main 'feed pipe' (2 cm diameter) to which were connected many smaller silicone tributary tubes fitted with flow regulators and aeration stones. Air flow was maintained at 3 L min^{-1} in all tanks and 0.5 L min^{-1} in jar preparations.

4.5 Preliminary experimentation with Z. *muelleri* and the effects of sediment loading (large jars containing 100 % sediment)

Tests were carried out to determine whether *Z. muelleri* 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 *Z. muelleri* could not be cultured under these laboratory conditions. It is likely that turbulence, caused by the aeration system, may have disturbed sediment particles and prevented sufficient light from reaching the leaf surfaces. This preliminary experiment was therefore repeated, without aerating individual jars, and the jars were placed in a large volume of aerated seawater (250 L) sufficient to cover the upper surface of the jars. Again, *Zostera* did not grow under these conditions.

4.6 Modified laboratory growth conditions using different sediment loadings.

Three sediment-loading regimes were used in the study of *Z. muelleri* in Sections 4.7, 4.8 and 4.9.

4.7 Buoyant Aquatic *Zostera* (BAZ): Using 0 % sediment

Up to one hundred plants were 'grown', or incubated, in test tanks as sediment-free plants, under the lighting, temperature and aeration regimens described previously over a two to three week period. Plants were carefully 'turned over' by hand, at least twice per (photoperiod) day, to prevent self-shading (Clough & Attiwill, 1980). 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 initially, but became 'free-floating', or buoyant, during the incubation period.

4.8 Jar Preparations of *Z. muelleri*: Using 30 % sediment

4.8.1 Rationale and aim

Previous preliminary attempts at growing *Z. muelleri* in 100 % (undiluted) sediment in glass jars had proved unsuccessful (Section 4.5). Since tidal changes of pore water in sediment do not occur in isolated jars, plants were grown in 30 % sediment in order to improve oxygenation, to evaluate the effects of toxic factor(s) in sediment and to have plants survive for reasonable periods. It was reasoned that toxic factor(s), such as sulfides (Erskine & Koch, 2000), nitrites (Iizumi *et al*., 1980), and heavy metals (Ralph & Burchett, 1998b; Prange & Dennison, 2000) within sediment may have imposed restrictions on plant growth. Similar restrictions were likely to operate in *Zostera's* natural environment. It was thought that diluting the sediment with sand would reduce toxicity.

4.8.2 Materials

Sediment was obtained from the same collection site as *Z. muelleri* at the Barker Inlet. Golden Grove coarse sand and plastic siphon tubing (1 cm (i.d.) x 3 m) were purchased from a garden centre. Glass Jars (2.5 L) were obtained from Corning Pty Ltd. A Venturi system was constructed from an Erlenmyer Flask (250 mL), a rubber stopper fitted with inlet and outlet tubing and a 30 cm length of (rigid) polypropylene tube (garden irrigation tubing) was fitted to the water 'inlet tubing' when a vacuum was applied.

4.8.3 Method

The water contents of wet sediment (27.8 % ± 0.4, n=4) and supplied coarse sand (3.9 % ± 0.1, n=4) were first calculated by drying samples (100 g) in an oven (122 ^{o}C) for 39 hrs. 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. Particulate material was allowed to settle for ten minutes. Seawater was then decanted from the drum and this 'washing procedure' was repeated a total of seven times prior to use of the coarse sand. Sediment (23 kg) and washed coarse sand were then blended by using a narrow-bladed spade to a consistency of mortar to produce a ratio of 30 % sediment and 70 % coarse sand when expressed on a dry weight basis. This mixture contained 20.0 ± 0.2 % (n = 6) interstitial water.

All glass culture vessels were washed with detergent (Extran 300), soaked in 5M NaOH for 24 hrs and then soaked in 10 % nitric acid for 24 hrs; an extensive rinsing regime, with tap water and Milli-Q water accompanied each stage of this sequential washing procedure. Glass jars were then dried and filled to a 10 cm depth with 30 % sediment mixture.

A large spatula was then used to place four *Z. muelleri* plants carefully into the sediment in a 'north-south' and 'east-west' pattern. Plant roots were tightly surrounded by modified sediment by agitating the jar along its long axis on a level surface. Seawater (2 litres) was carefully added to the jars by siphoning; the jars were then provided with artificial lighting (250 μE m^{-2} s^{-1}) during the 'daylight hours' and aeration during the 'night-time hours' to minimise light attenuation caused by suspended particulate matter during the photoperiod. Brackish water and any leaf debris were then removed from each jar by a careful siphoning and replacement regime each morning over a period of five days. Jar water became transparent after the five-day washing regime. The jars were left in the artificial lighting and aeration system described previously. Plants that did not survive this transplanting procedure were replaced with new plants that were then left to acclimatise for a period of no less than six weeks.

4.9 Basket Preparations of *Z. muelleri*: using 100 % sediment

4.9.1 Rationale and aim

Zostera muelleri grows in 100 % sediment in its 'natural' environment of the intertidal zone. There is presumably some exchange of pore water with fresh seawater during these cyclic tidal flows that may dilute toxic factors held within sediment. Possible toxic factors are sediment anoxia (Terrados *et al*., 1999) and the possible toxicity associated with sulfides (Erskine & Koch, 2000), that are thought to be present in intertidal mud. Attempts to grow *Z. muelleri* in such undiluted sediment in glass jars had failed so it was decided to construct *porous vessels* in which seagrass could be grown in native (100 %) sediment. The vessels could then be periodically purged of any such 'toxic factors' that may have been present within interstitial water within the sediment.

4.9.2 Materials

Shopping (supermarket) baskets (polypropylene 29 cm x 22 cm x 39 cm) were purchased from a wholesale supplier. Shade cloth (80 % shading capacity, polypropylene) and siphon tubing (3 cm i.d.) were purchased from a garden centre, while an aquarium net was purchased from a pet store.

4.9.3 Method

Baskets were modified by removing their metal handles and by 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. An 'intact' turf of seagrass, having a wet weight of 18 ± 1 kg (adjusted with 100 % sediment for each preparation), was transferred, in one motion, from the storage bins into a basket by rapid inversion (Figure 4.1). Four baskets were allocated randomly to each working tank, and seawater was filled to 10 cm above the upper edge of the baskets and served as laboratory 'high-tide'. Laboratory 'low tide', lasting 45 minutes, was achieved initially four times a week, by *slowly* lifting the baskets and then positioning each on stands (inverted empty baskets)

within the working tanks This allowed replacement of a portion of the interstitial pore water with fresh seawater.

Figure 4.1: *Z. muelleri* grown in baskets.

Brackish tank seawater was replaced by fresh seawater three times per week, for a period of three weeks, at low tide on commencement of this aquaculture system. Subsequently tank seawater was replaced once per week with low ‘tides’ remaining at four times per week. Plant debris was removed daily with a hand-held net and sediment debris was removed from the bottom of the tank by using a wide-bore siphon. The ‘basket preparations’ were then subjected to the lighting (250 $\mu Em^{-2}s^{-1}$) and aeration (12hrs; 6:00 p.m. to 6:00 a.m.) regimes previously discussed.

The ‘turf’ growth system provided a microcosm resembling the ‘natural growth conditions’ of *Z. muelleri*. Macrofauna, such as crabs, starfish and snails, were removed from this growth system within the first two weeks of culture, while tube worms remained an integral part of the sediment mass. The occasional crab was still detected weeks after the initial set-up. This qualitative observation suggested that conditions within the tank were similar those within the natural environment.

4.10 Baseline measurements

4.10.1 pH measurements

pH was routinely measured 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. 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 conditions relative to seawater.

4.10.2 Dissolved oxygen

Dissolved O_2 was measured with a ‘Dissolved oxygen / Temp. Meter (model MC-82, TPS fitted with an L-331 probe)’. The machine was calibrated at its ‘zero point’ with 2.1 % (w/v prepared in Milli-Q water) sodium sulfite solution in accordance with instructions supplied by the manufacturer.

4.10.3 Salinity (sodium chloride) measurements

A chloride-specific probe and a reference electrode (ICI instruments) were connected to a pH meter (PHM62 Standard pH meter, radiometer Copenhagen) that was operated in mV mode. A standard curve was constructed from a dilution series by using analytical grade sodium chloride (Sigma Chemical Company, U.S.A.) in Milli-Q water. A typical regression equation was $y = -51.088x + 239.3$, $R^2 = 0.99$, where ‘x’ is log concentration (ppm) and ‘y’ is the response (mV) of the chloride-specific electrode. Seawater samples were selected at random throughout the experimental period and were diluted (350 times) with Milli-Q water prior to analysis.

4.10.4 The dry matter content of *Z. muelleri*

Plants (n = 12) were washed free of sediment, quickly rinsed with Milli-Q water and dried with tissue paper and wet weights were recorded. Plants were then sectioned into green leaf material and root tissues (below the base of the leaf sheath) and the weighed tissues were individually wrapped in aluminium foil and dried (85 ^{o}C for one week) and 'dry' tissue weights were then recorded. Dry weights were then expressed as a percentage of wet tissue weights.

4.11 Bioassays

4.11.1 Root hair growth – a preliminary study of a sulfonylurea bioassay

Preliminary experiments were conducted on BAZ using the sulfonylurea herbicide chlorsulfuron. Briefly, chlorsulfuron was dissolved in seawater at 15 mg L^{-1} and free-floating 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 selected randomly and all rhyzomes were removed except one positioned at the distal end of the woody root growth. These 'newly synthesized' rhyzomes had no root hair growth. Plants were placed in transparent plastic containers (440 mL capacity, Sarstedt) and subjected to either a chlorsulfuron or a negative control (atrazine) treatment under chronic exposure conditions (15 mg L^{-1}). Results were then compared to those for control plants.

It was found that a high loading of this class of 'sulfonylurea' herbicide was required to have an effect on root hair growth and it was therefore decided to focus the research on measurements of photosynthesis and the widely used 'safe' herbicide 2,4-D.

4.11.2 An overview of a photosynthesis bioassay technique for *Z. muelleri*

The second youngest leaf within leaf-sheaths of *Z. muelleri* was selected as biological material to be used in bioassays. This procedure reduced variability by allowing the selection of plant material within a plastochrone interval (Brouns, 1985). The procedure therefore reduced the known variability in chlorophyll contents that can occur between

leaves within the same plant and even along the length of a single leaf (Dalla & Sturmbauer, 1998). Leaf tips were left intact when leaves were sectioned from the leaf tips and towards the leaf base.

Gas volumes produced by illuminated leaf sections provided an indication of photosynthetic efficiency. Additionally, measurements of acid-soluble carbohydrate content, chlorophyll content and leaf area *within the same leaf section* then permitted gas volume measurements to be expressed as photosynthetic production. Newly synthesized photosynthetic gas and acid-soluble carbohydrate were quantified and results expressed per unit mass of chlorophyll and per unit leaf area. Differences in gas volume production rates and acid-soluble carbohydrate contents were measured between 'test' and 'control' samples and were attributed to treatment.

4.11.3 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 and any exposure of the intertidal seagrass *Z. muelleri* would occur as a *transient event*. It was therefore decided to construct bioassays in a pulse-chase design on the premise that environmental contamination occurs as a transient event.

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 Section 1.1, were either removed or were maintained under laboratory control.

Measurements of gas volumes (mm^3), leaf area (dm^2), chlorophyll ('a' and 'b' as μg), acid-soluble carbohydrate (measured as 'glucose equivalents' as μg), and gas identities (mass spectrometry) provided indices with which to monitor the photosynthetic processes. Potential errors were reduced by performing such measurements on *individual leaf sections* (measuring between 5 and 7 cm in length), obtained as the second-youngest leaves within a leaf sheath, and then comparing results obtained from 'control' and 'treatment' groups.

The variables measured under bioassay designs were gas volume, leaf area, leaf width and chlorophyll in buoyant aquatic *Zostera* analyses (BAZ) and gas volume, leaf width, leaf area, chlorophyll and acid-soluble carbohydrate in sediment-bound *Zostera* analyses (30 % sediment and 100 % sediment).

4.12 Gas volume measurements, manometer design and usage

Since O_2 is poorly soluble in water, any gases produced by submerged leaf sections of *Z. muelleri* while placed under artificial lighting in air-saturated seawater should contain O_2. Hence measured gas volumes should 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 to be etched using a one-sided razor blade. Measured gas volumes were taken from the etch marks on completion of the experiment. Gas volume (µL or mm^3) measurements were equated with the weight (mg) of an equivalent volume of Milli-Q water at 21 ^oC. Manometer calibration was achieved by using standard additions of Milli-Q water and air. The volume (x, µL) of a 'standard addition' of Milli-Q water added to an empty manometer was correlated with the weight (y, mg) of added water ($y = 1.0051x - 0.3222$, $R^2 = 0.99$, n=3). There was no significant difference between calculated gas volumes, from weights of Milli-Q water, and standard additions of air at 25 µL and 50 µL to seawater filled manometers ($P < 0.05$). These data indicated that the weights (mg) of Milli-Q water and gas volumes (µL or mm^3) were equivalent measurements within a manometer under the experimental conditions.
During experimentation the manometer was initially filled with seawater by holding it at its largest end and filling it by using a Pasteur pipette. Any air bubbles were removed from the tip of the manometer by 'flicking' it in one motion. Leaf sections were positioned with their oblique cut ends closest to the tip of the manometer. It was possible to place a leaf section within an inverted manometer and then place excess seawater, as a convex meniscus, at the manometer's largest end. By placing a finger over the end of the manometer it was possible to invert the assembly and transfer the apparatus with its enclosed leaf section into a reservoir of seawater *without the entrapment of ambient air*.

Manometers were then clamped vertically in place. The air pressure of reservoir seawater prevented water drainage from the manometer's tip. Each 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, on completion of the experiment, were 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 of displaced seawater onto the cellotaped surface by using a one-sided razor blade. Leaf sections were then removed and temporarily stored, at 5 degrees centigrade, prior to leaf area measurements. Manometers were sequentially washed with Milli-Q water (7x), methanol (7x) and acetone (7x), dried at 21 ^{o}C and weighed. Manometers were then filled to the etch mark with Milli-Q water (at 21 ^{o}C) and then re-weighed. The difference in mass between the two measurements allowed a calculation of gas volume (in µL (mm^3)) to be made.

4.13 Identification of captured photosynthetic gases produced by *Z. muelleri* during the photo-period: A GCMS method

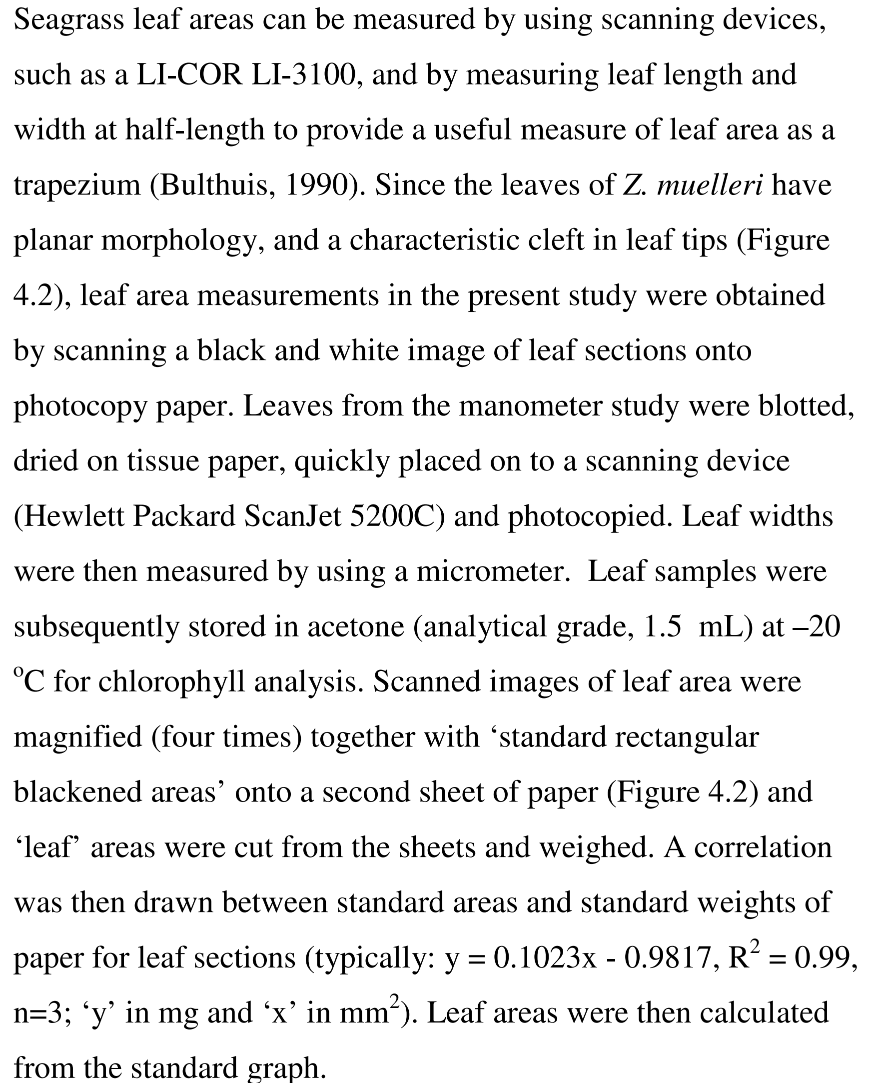
Captured gases were diluted with ultra high purity (UHP) nitrogen (500 µL), transferred to a surrogate manometer filled with metabolically inactive seawater (containing sodium cyanide, 1 mg L^{-1}) and stored in darkness. Transfer was achieved by using a piece of capillary tubing connected to a syringe (1000 µL capacity fitted with a Teflon plunger, SGE).

Instrument grade air was used to calibrate the GCMS instrument that was operated in sim mode. The selected ions were m/z = 32 (oxygen) and m/z = 44 (CO_2). 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 GCMS system and integrated peak areas were then used to calculate response factors and quantify O_2 in standard gas samples.

A modified syringe (250 µL capacity fitted with a Teflon plunger, capillary tubing and a needle) was then used to inject an aliquot (250 µL) of the diluted sample gas into the GCMS system. Care was taken not to break the capillary tubing in the injection

procedure. In all gas transfer procedures, syringes were purged of air by repeatedly flushing them (7x) with UHP nitrogen.

4.14 Leaf area and leaf width measurements



Seagrass leaf areas can be measured by using scanning devices, such as a LI-COR LI-3100, and by measuring leaf length and width at half-length to provide a useful measure of leaf area as a trapezium (Bulthuis, 1990). Since the leaves of *Z. muelleri* have planar morphology, and a characteristic cleft in leaf tips (Figure 4.2), leaf area measurements in the present study were obtained by scanning a black and white image of leaf sections onto photocopy paper. Leaves from the manometer study were blotted, dried on tissue paper, quickly placed on to a scanning device (Hewlett Packard ScanJet 5200C) and photocopied. Leaf widths were then measured by using a micrometer. Leaf samples were subsequently stored in acetone (analytical grade, 1.5 mL) at –20 ^{o}C for chlorophyll analysis. Scanned images of leaf area were magnified (four times) together with 'standard rectangular blackened areas' onto a second sheet of paper (Figure 4.2) and 'leaf' areas were cut from the sheets and weighed. A correlation was then drawn between standard areas and standard weights of paper for leaf sections (typically: $y = 0.1023x - 0.9817$, $R^2 = 0.99$, n=3; 'y' in mg and 'x' in mm^2). Leaf areas were then calculated from the standard graph.

Figure 4.2: A photocopied leaf section of *Z. muelleri* (2x actual size).

4.15 Chlorophyll measurements

It was expected that the chlorophyll contents of chloroplasts within leaf tissues would contribute to the efficiency of primary production in leaf tissue. Therefore, the chlorophyll content of *Z. muelleri* leaves was measured spectrophotometrically (Dennison, 1990). Seagrass leaves were first homogenized in 80 % acetone and 20 % Milli-Q water containing magnesium carbonate (10 μM) by using a glass dounce homogenizer. The homogenate was immediately centrifuged (2000 *g*, 21 °C for 15 minutes) and absorbance readings taken at 663 nm, 645 nm and 725 nm on a UV-VIS spectrophotometer (Shimadzu UV-1201).

A_{725} (a measure of turbidity) was subtracted from A_{663} and A_{645} and the corrected values were used to calculate the chlorophyll concentrations (Dennison, 1990):

$$\text{chl a } (\mu g\ mL^{-1}) = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{chl b } (\mu g\ mL^{-1}) = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{chl a + b } (\mu g\ mL^{-1}) = 8.02 \times A_{663} + 20.2 \times A_{645}$$

Calculated chlorophyll a concentrations were correlated ($R^2 = 0.9$, $y = 1.23x - 0.03$) with the actual concentrations of chlorophyll a in standards (obtained from *Anacystis nidulans* algae, SIGMA, EC No. 207-536-6, F.W. 893.5) made up in the extraction solvent. Samples were then stored (in a 20 mL glass vessel fitted with a Teflon-lined cap) at 5 °C in a refrigerator after chlorophyll analyses until they were analysed for their acid-soluble carbohydrate contents.

4.16 Acid-soluble carbohydrate measurements

Measurement of acid-soluble carbohydrates, as 'glucose equivalents', was achieved spectrophotometrically by using a modification of a phenol-sulphuric acid technique (Dawes & 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 (at 100 °C) and the solvent was dried until 0.2 mL of the original liquid remained. The remaining solvent evaporated on cooling the samples to 21 °C. Acid-soluble carbohydrate was then dissolved from the dried residue by using warm (80-90 °C) trichloroacetic acid (5 %, 10 mL for 3 hrs). Vessels were then returned to their original volume with Milli-Q water

and centrifuged (500 *g* for ten minutes). Phenol (1 mL, 5 %) followed by concentrated sulphuric acid (5 mL,) was *carefully* added to an aliquot (0.2 mL) of the acid-soluble carbohydrate. The exothermic reaction was then cooled to 21 ^{o}C 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'.

4.17 Measurement of chlorosis in leaf sections of *Z. muelleri*

Since it was intended that photosynthetic gas production would be expressed per milligram of chlorophyll, a change in this ratio could be caused by either variable. Therefore an analysis of variance was performed on the total chlorophyll content, expressed as mg dm^{-2}, of the leaf sections for the control and treatment groups to provide a measure of chlorosis.

4.18 Bioassays: buoyant (free-floating) aquatic *Zostera muelleri* (BAZ) and sediment-bound *Zostera* experiments

4.18.1 Xenobiotic treatments

It was 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. Amicide 500^{R} (500 grams per Litre 2,4-D as the dimethylamine (salt)) was supplied by Nufarm Ltd (Adelaide). Atrazine (99 % purity) and simazine (98 %) were supplied by Chem Service while metsulfuron-methyl (technical grade, 98.9 %) was supplied by Dupont. 2,4-dichlorophenol (99 %), a metabolite of 2,4-D, and sodium cyanide (analytical grade) were purchased from the Sigma Chemical Company (U.S.A.).

4.18.2 Loading of seawater with xenobiotics

Amicide 500^{R} (50 µL) was added to a volumetric flask (5 L capacity) and made up to the mark with fresh seawater containing 0.01 % vol. / vol. Tween 80 detergent to provide a solution of 5 mg L^{-1} (5 ppm) 2,4-D as active ingredient. The flask was capped, inverted several times and the modified seawater used in bioassay experiments. Similar

solutions containing simazine, atrazine, metsulfuron-methyl, 2,4-dichlorophenol or sodium cyanide were also prepared.

4.19 A bioassay using buoyant (free-floating) aquatic *Z. muelleri* (BAZ) in a pulse-chase experimental design: 5ppm 2,4-D (as Amicide 500^{R})

Zostera muelleri can propagate by vegetative means. It was therefore decided to conduct a simple bioassay by using free-floating plants in a short-term bioassay. Plants were first laboratory-acclimated for two weeks and then subjected to an overnight exposure to xenobiotic at 5 ppm active ingredient.

Free-floating plants were allocated randomly to test and control groups with three plants being placed in each group. Each plant was allocated to a clean jar and either fresh seawater containing 0.01 % vol. Tween 80 (control group) or seawater doped with xenobiotic at 5 ppm acid equivalents (test group) was then added. Plants were placed in the dark in the same environment as test tanks and were left overnight. This treatment was equivalent to a nine-hour exposure period. Plants were removed from their jars and placed into clean jars containing fresh seawater. Each plant was then rinsed seven times with fresh seawater. Since 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), it was anticipated that little residual xenobiotic would be present following the extensive washing procedure.

Leaf material was then sectioned from the second-youngest leaf within leaf sheaths of *Z. muelleri*. Any epiphyte loading on leaf sections was then removed by using a moistened cotton wool bud on leaf sections placed on a seawater-dampened paper towel to help reduce any osmotic stress. Cleaned leaf sections were then allocated at random to manometer positions under the same laboratory lighting used in the aquaculture of *Z. muelleri*. This bioassay was conducted over an eight-hour period. It was anticipated that gas volumes produced by illuminated leaf sections could be correlated with leaf areas and chlorophyll measurements for the *same leaf section*. A one-way ANOVA and Scheffe's statistical test were then used to compare 'test' and 'control' leaf-section results in an assessment of treatment effects.

4.20 Pulse-chase experimental designs using sediment-bound *Z. muelleri* in jars and basket preparations

4.20.1 5ppm 2,4-D (as Amicide 500^R) on *Z. muelleri* in 30 % sediment (jars)

Zostera muelleri was laboratory-acclimated for two years and was treated with an overnight exposure to 2,4-D doped seawater (5 ppm acid equivalents) in a similar manner to BAZ above. Twelve jars were allocated randomly and evenly distributed between control and test groups. The steeped plants were washed free of any residual solution after the overnight steep with seven rinses of fresh seawater. Interstitial water, within the sediment, was also removed during this washing procedure by using a Venturi system on jars placed at a ten-degree angle to the horizontal. Fresh seawater (2 L) was then 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 *Z. muelleri*. Leaf sections were removed periodically from plants, cleaned of epiphytes and allocated randomly to a manometer and lighting position prior to gas production analyses. Four bioassays were conducted, each lasting eight hours, over the duration of the 14-day experiment.

It was anticipated that the gas volume produced by *each* illuminated leaf section would be correlated with leaf area, chlorophyll and acid-soluble carbohydrate measurements for the *same leaf section*. A comparison was made between 'test' and 'control' leaf-section results in an assessment of treatment.

4.20.2 5ppm 2,4-D (as Amicide 500^R) on *Z. muelleri* in 100 % sediment (baskets)

Eight basket preparations of *Z. muelleri* were allocated randomly and distributed evenly 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 Section 4.9.3. Each basket was placed in an aerated aquarium containing either fresh seawater (control) containing 0.01 % Tween 80 or 2,4-D loaded (5 ppm acid equivalents) seawater (test) containing 0.01 % Tween 80 for a period of nine hours. Following the overnight steep, basket preparations were washed free of any residual solution with seven rinses of fresh seawater. Interstitial water, within the sediment, was removed during this washing procedure by placing the aquaculture baskets on empty and

inverted baskets as described in Section 4.9.3. Two test and two control basket preparations were then positioned at random in a working tank containing fresh seawater and provided with the same laboratory lighting and aeration regime used in the aquaculture of *Z. muelleri*. This procedure was repeated in a second working tank and six leaf sections were removed from test and control plant groups at predetermined time intervals. Leaf sections were cleaned of epiphytes, as before, and allocated randomly to a manometer position for gas production analyses. Each bioassay was conducted over an eight-hour period for the 14 days of the experiment. Gas volume measurements were correlated with leaf area, chlorophyll and acid-soluble carbohydrate measurements for *each* leaf section. A qualitative assessment of the number of leaves 'lost' from the control and treatment groups was also made. Statistical analyses were performed according to the methods outlined in Section 2.16.

CHAPTER 5: RESULTS OF BIOASSAY STUDIES

5.1 Collection and culture of *Z. muelleri*

It was found that the leaves of *Z. muelleri* collected from the Barker Inlet often had algal growth covering a substantial proportion (ca. 5 %) of their leaf areas. This growth could easily be removed either by running a one-sided razor blade across the leaf's surface or by using a wetted cotton-wool bud while samples were kept damp on a seawater-wetted paper towel surface. Such growth was largely absent from the laboratory cultures described below.

5.2 Preliminary experimentation with Z. *muelleri* and the effects of sediment loading (large jars containing 100 % sediment)

The results of preliminary experiments, collected during several earlier attempts at growing seagrass, suggested that replacing the seawater twice weekly for the first three weeks increased the survival rates of seagrass cultures grown in 100 % sediment in jars. It was reasoned, furthermore, that increasing the water-body volume was insufficient to prevent the action of toxic 'elements' within sediments from causing seagrass death. It is particularly noteworthy that 'inclusion bodies' (gas pockets) were detected near the root zone of the sediment-bound plants. An annular pink-purple layer appeared at the edges of these inclusion bodies during this experiment.

It was concluded that a larger scale growth of seagrass turfs under laboratory conditions would be problematical unless a process could be developed to 'detoxify' the intertidal sediment.

5.3 Modified laboratory growth conditions

5.3.1 Buoyant Aquatic *Zostera* (BAZ): Using 0 % sediment

Zostera muelleri can be grown, at least in the short-term, as free-floating plants (BAZ) under laboratory conditions. It is believed that photosynthesis, induced by the artificial lighting, caused *Zostera* to produce O_2 within the plant's tissue rendering plants less dense than seawater. Indeed, the observed (visual and binocular light microscopy) gas

production by leaf sections was subsequently used as a basis for the development of a bioassay for this seagrass.

5.3.2 Jar Preparations of *Z. muelleri*: Using 30 % sediment

Larger plants, exhibiting pronounced root and shoot growth, survived (observation) the transplanting process better than did the smaller plants which exhibited less pronounced growth attributes. Leaf death, caused by the transplanting process and leaf senescence, in *Zostera* was best assessed by the appearance of blackened leaf tips (Philips, 1990). Additionally, it was noted that compromised plants eventually lost most of their 'above-sediment' biomass within weeks of being transplanted. Leaves became narrower and shorter during extended growth periods in both the jar system and basket preparations (Section 5.6.4). Green leaves were buoyant in seawater. When leaves were cut, by using a pair of scissors under water, a gas bubbled from both cut edges and the removed leaf section rose to the water's surface. This result indicated that gas pressure within the leaf was *greater than one atmosphere*. This point is important in later discussions. 'Inclusion bodies' (Figure 5.1) were produced in modified sediment during the first two weeks of this aquaculture growth system. These 'gas pockets' were in close proximity to the roots of *Zostera* that were adjacent to the glass wall of the culture vessel and are thought to be associated with the root zone of *Zostera* under normal growing conditions. An annular pink-purple layer was visible, in white light, around each inclusion body in each jar preparation.

Figure 5.1: The presence of inclusion bodies (arrows), in the modified sediment of seagrass cultures. These are 'pockets of gas' probably created by the release of oxygen from the roots of *Z. muelleri*.

5.3.3 Basket Preparations of *Z. muelleri*: Using 100 % sediment

Leaf morphology of the outermost (older) leaves within the leaf sheath appeared to have changed little during the first two to three weeks of the culture procedure. However, some narrowing of leaves occurred in cultures that were older than three weeks (Section 5.6.4). The chlorophyll content of leaf sections was 3.9 ± 0.4 (n=6) mg dm^{-2} and was similar to previously reported literature values (Dennison, 1990). After an initial phase (occurring within 2 weeks) of leaf loss, resembling that which occurred in the jar preparations of *Zostera* (using 30 % sediment), seagrass plants had similar morphology

to 'wild-type' seagrass. However, extended periods of laboratory culture resulted in some narrowing of the leaves (Figure 5.9).

5.4 Baseline measurements

5.4.1 Seawater pH: A diurnal pH cycling in the aquaculture of *Z. muelleri*

The pH of seawater at the collection site was 8.1 ± 0.4 (n=6). The mean seawater pH was 7.36 ± 0.4 (n=6) in the white bins (20 L capacity) containing seagrass turfs that were left overnight while the mean pH in the 'large volume' yellow bins was 7.83 ± 0.3 (n=6). After an acclimatisation period of two weeks the pH of seawater (at 21 ^{o}C) in the working tanks, that contained *Z. muelleri* in baskets, cycled in a diurnal fashion between pH 8.05 in the morning and pH 8.15 at night (Figure 5.2). It is known that seawater has a pH of 8.2 (Hellblom, 2000). The measured lower pH, in the present study, during the daylight hours could possibly be explained by microbial activity within sediment. Craven & Hayasaka (1982) suggest that obligate aerobic bacteria in sediment adjacent to the roots of *Zostera* are able to utilise glucose and O_2 to create a microacidic environment in which insoluble hydroxyapatite is converted into soluble phosphate that is then utilised by *Zostera marina*. This diurnal variation was not observed in a holding tank that contained seawater alone. While there was a marginal diurnal fluctuation in pH, the mean pH was 8.1 in this seagrass culture system.

5.4.2 Dissolved oxygen

Dissolved O_2 was significantly lower (5.8 ± 0.1 mg L^{-1}, n = 3, $P < 0.05$) in the large storage growing systems that contained four control baskets than in those that contained two control baskets (6.7 ± 0.1 mg L^{-1}, n = 4)

5.4.3 Salinity (sodium chloride) measurements

The sodium chloride content of seawater was 34.9 ppt ± 1.7 (n =6)

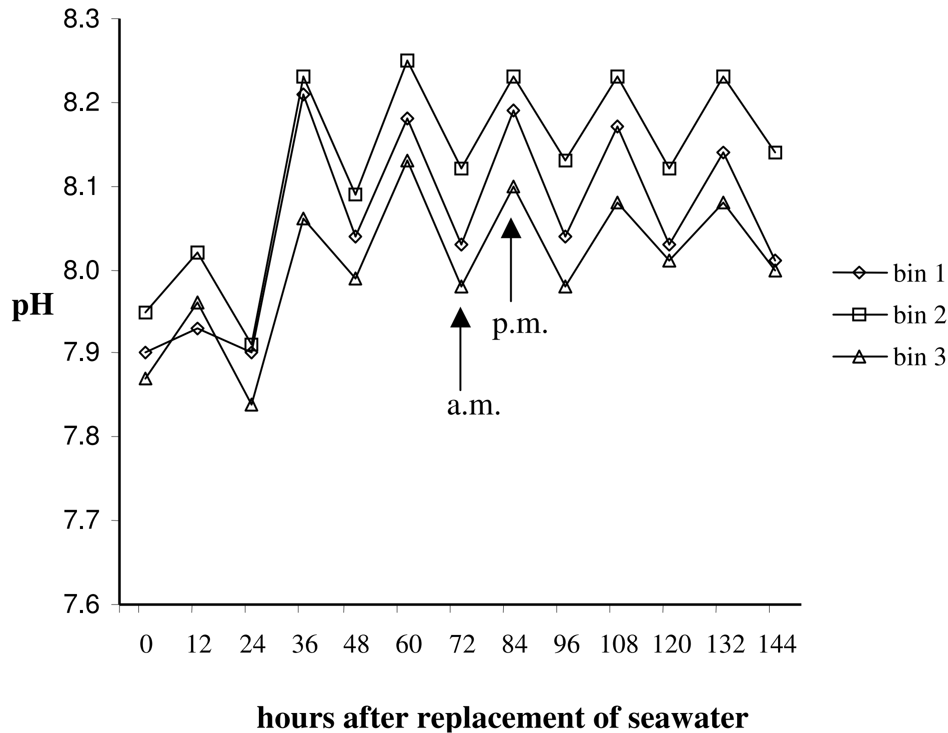


Figure 5.2: Diurnal pH cycling in the aquaculture of *Z. muelleri*.

5.4.4 The dry matter content of *Z. muelleri*

Dry tissue weights of shoots, roots and whole plants were 17.1 ± 1.1 %, 15.6 ± 1.7 % and 16.0 ± 1.2 % respectively of the wet tissue weights (n = 12 in each group, Figure 5.3).

An analysis of variance (Sheffe's test) indicated that there was no significant difference between the dry matter contents of the analysed tissues.

5.5 Bioassays

5.5.1 Root hair growth – a preliminary study of a sulfonylurea bioassay

Photosynthetic gas production in chlorsulfuron-treated seagrass was inhibited after an 11-day exposure as measured using a manometer technique that was described in Chapter 4.

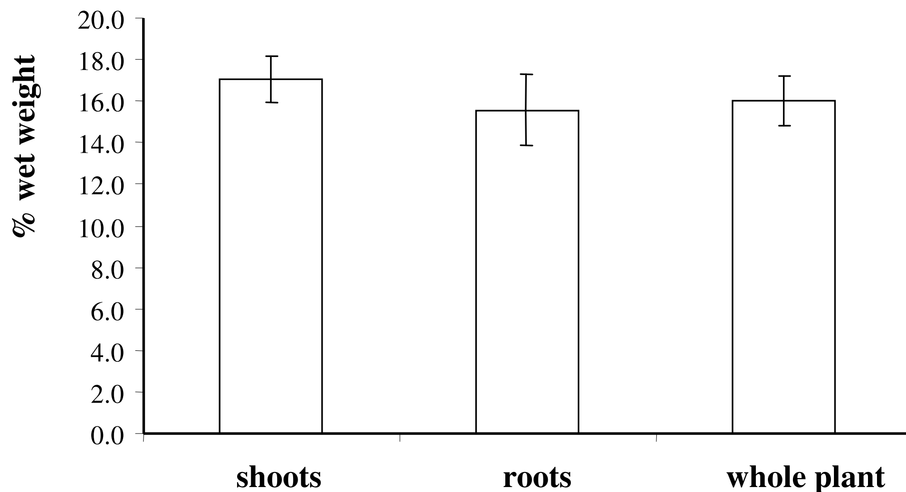


Figure 5.3: The dry matter content of *Z. muelleri*.

It was noted, in a qualitative assessment, that new root hair growth was markedly inhibited in the test groups and the negative control. Additionally, cut green-leaf sections from the test groups (sulfonylurea and atrazine) did not release O_2 when the submerged sections were viewed under a binocular microscope whilst O_2 release was clearly visible in the control group. It seemed likely that metabolic activity associated with the photosynthetic process in *Z. muelleri* is affected by a prolonged exposure of the plant to the sulfonylurea herbicide chlorsulfuron.

5.6 Photosynthesis bioassay techniques for *Z. muelleri*

5.6.1 Lacunal gas composition

The lacunae in the control and test *Z. muelleri* groups contained 100 % O_2. These results contrast with those of previous studies (Roberts & Moriarty, 1987) in which the gas compositions in the lacunae of other seagrass species were 33 % for O_2 and 67 % for nitrogen. The three seagrass species studied by Roberts and Moriarty were *Zoster capricornia Aschers., Cymodocea serrulata (R. Br.) Aschers. & Magnus and Syringodium isoetifolium (Aschers.).*

5.6.2 A bioassay using buoyant (free-floating) aquatic *Z. muelleri* (BAZ)

Baseline data were collected in a BAZ study of 'control plants' measuring normal rates of photosynthetic gas production (n=21). These data showed that the rate of photosynthetic gas production was 0.10 ± 0.03 µmol O_2 chl mg^{-1} min^{-1}, which is equivalent to 0.46 ± 0.13 µmol O_2 dm^{-2} min^{-1}. Total chlorophyll (mg) was poorly correlated with leaf area (dm^2), (y = 4x, R^2 = 0.4, n=21), which is consistent with the known and variable chlorophyll contents of seagrass leaves between plants and even the chlorophyll content along a single leaf (Dalla & Sturmbauer, 1998). However, it was also noted that the gas production rate, expressed in µmol O_2 chl mg^{-1} min^{-1} was linearly correlated (y = 4x + 877, R^2 = 0.8, n=21) with gas production expressed in µmol O_2 dm^{-2} min^{-1} (Figure 5.4). These results were consistent with previously published work (Ralph & Burchett, 1995). Additionally, chlorophyll a and chlorophyll b were also linearly correlated (Figure 5.5) and these results were similar to those observed in *Z. marina* (Dennison & Alberte, 1982).

A student's t-test showed that the controls used in a BAZ experiment (Section 4.19) did not differ significantly (P < 0.05) from the baseline control data in the photosynthetic production of gas. Therefore the observed differences between treatment means (Figures 5.6 A and 5.6 B) in the present experiment may have been caused by the xenobiotic treatments.

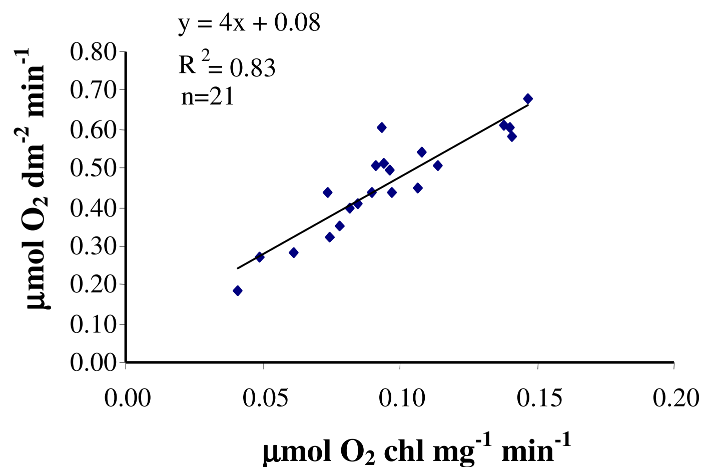


Figure 5.4: Photosynthetic gas production in control *Z. muelleri*.

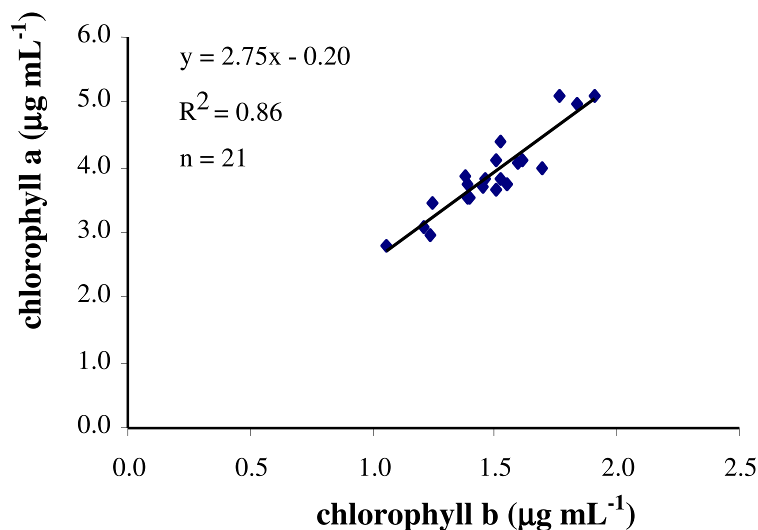


Figure 5.5: Chlorophyll a versus chlorophyll b in Z. muelleri.

A one-way analysis of variance (ANOVA) was also used to test the data obtained from this experiment (Figure 5.6). Scheffe's statistical test (α= 0.05) showed that the amount of photosynthetic gas produced over an eight-hour period was influenced by the treatments. Simazine, atrazine and sodium cyanide treatments used as positive controls, reduced gas production by 62, 60 and 83 % respectively while metsulfuron-methyl had little effect. An unexpected finding, that was not significantly different from the control value, was the production of 31 % less gas by the 2,4-dichlorophenol treatment group and a 16 % increase in the 2,4-D treatment group.

5.6 A

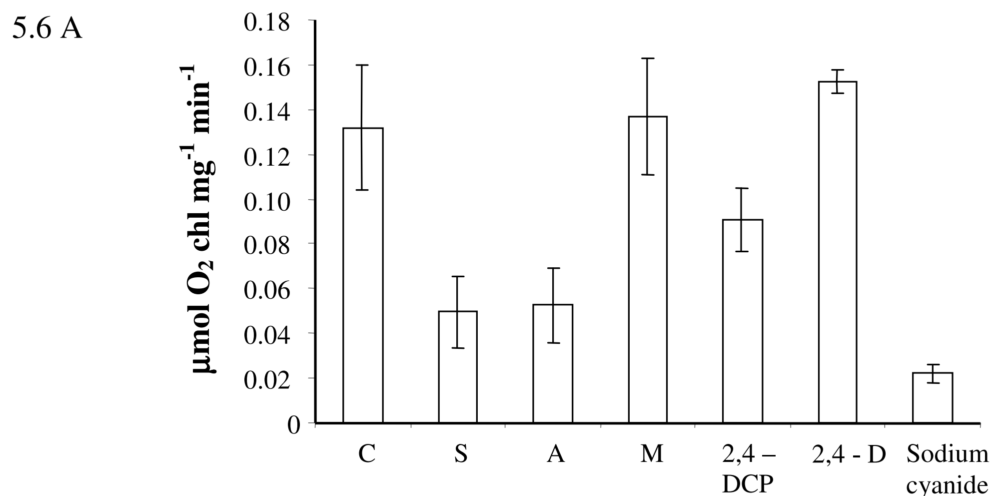


Figure 5.6: Photosynthetic gas production by *Z. muelleri* (n=3). Control (C), simazine (S), atrazine (A), metsulfuron-methyl (M), 2,4-dichlorophenol (2,4-DCP), 2,4-D and sodium cyanide.

5.6 B

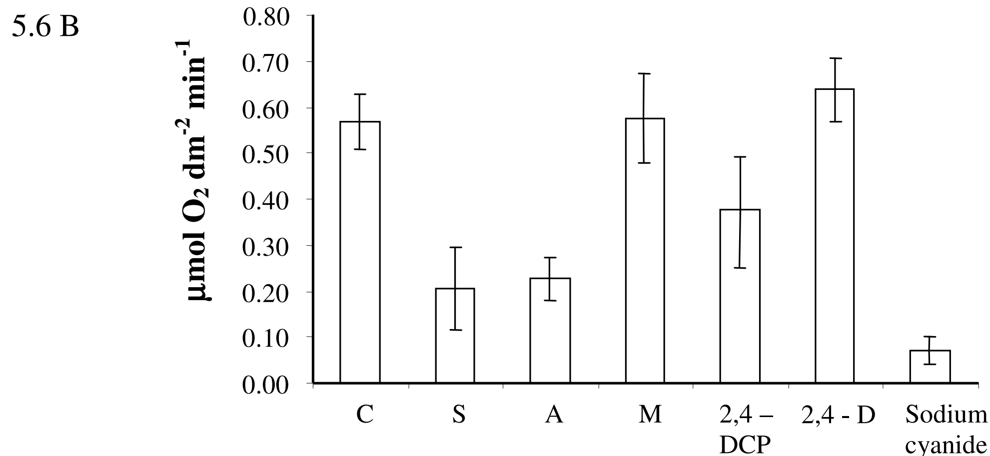


Figure 5.6: (continued)

5.6.3 Bioassays using sediment-bound *Z. muelleri* in pulse-chase experimental designs

Results of a one-way analysis of variance showed that treatment means were not significantly different from their respective controls in their photosynthetic production of gas in the jar (30 % sediment) and basket (100 % sediment) preparations after 5 days post-exposure to 2,4-D. However, the treatment mean (0.113 ± 0.046 µmol O_2 chl mg^{-1} min^{-1}, n = 6) in the basket preparation was significantly greater than the control value (0.059 ± 0.062 µmol O_2 chl mg^{-1} min^{-1}, n = 6) one day post-exposure of the 2,4-D insult ($\alpha = 0.10$, $p = 0.06$) (Figure 5.7).

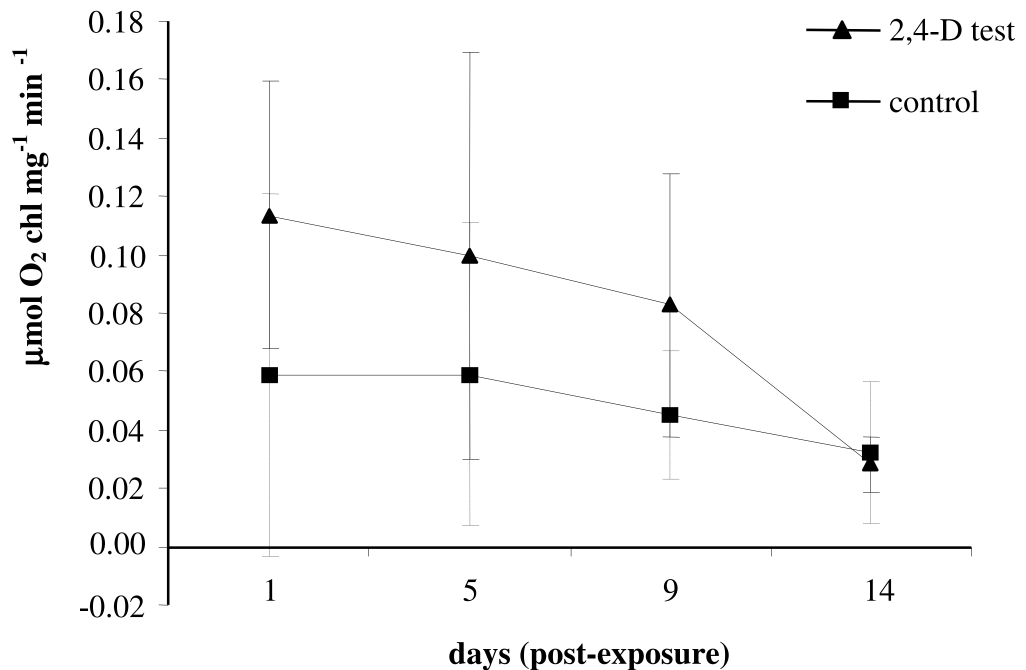


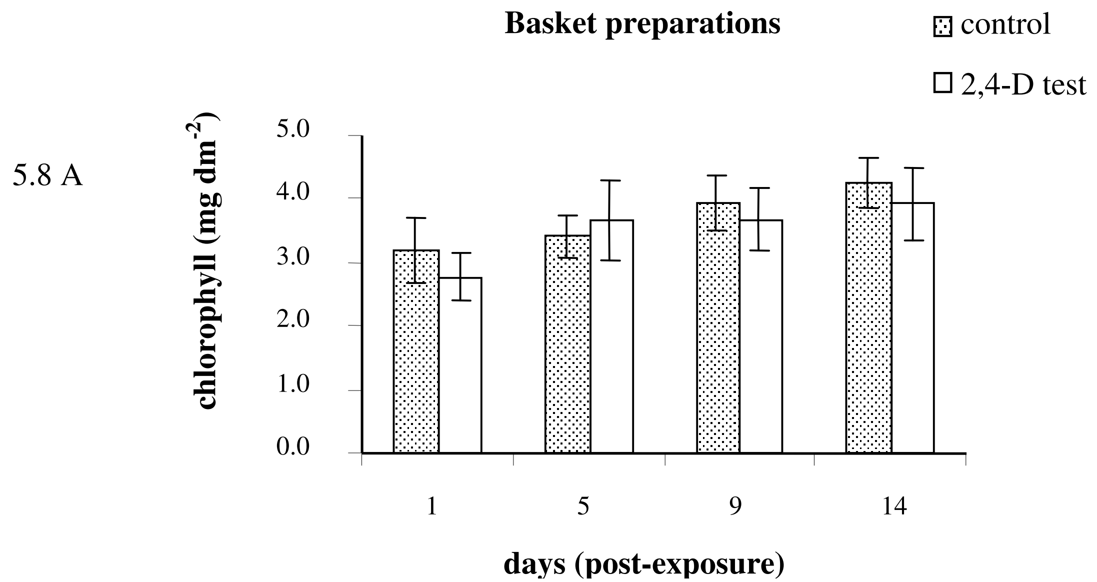
Figure 5.7: Photosynthetic gas production by 2,4-D- treated *Z. muelleri* in sediment-bound plants.

5.6.4 Are chlorosis and changes in leaf width evident in *Z. muelleri* leaves that have been treated with 2,4-D?

An analysis of variance indicated that little or no chlorosis had occurred in leaf sections taken from the treatment groups in the jar and basket preparations when values were compared with their respective control groups (Figure 5.8). Chlorosis was also absent in BAZ experiments.

An analysis of variance also indicated that leaf widths in the control groups of 'wild type' *Z. muelleri* (2.2 ± 0.3 mm, n=12) were significantly greater ($P < 0.05$) than those obtained from *Zostera* cultured in baskets (1.7 ± 0.4 mm, n=12) and jars (1.5 ± 0.3 mm, n=12) (Figure 5.9). However, there was no significant difference between the leaf widths of the control groups of *Z. muelleri* cultured in baskets and jars.

5.8 A



5.8 B

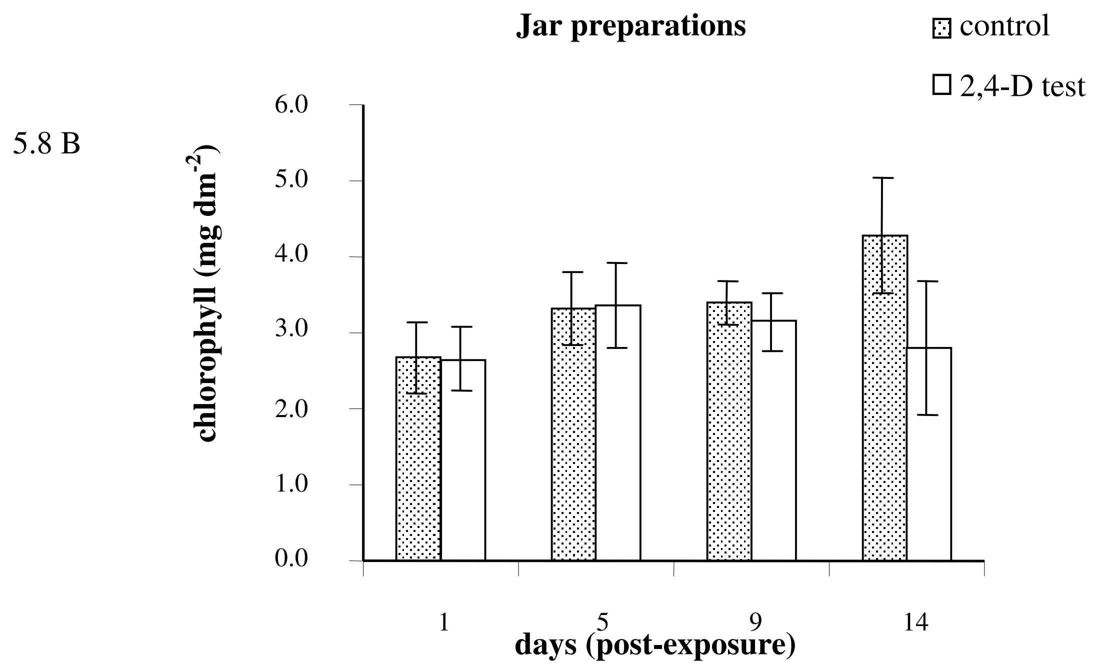


Figure 5.8: The chlorophyll contents of *Z. muelleri* leaves. Hatched and non-hatched areas are controls and 2,4-D test groups, respectively.

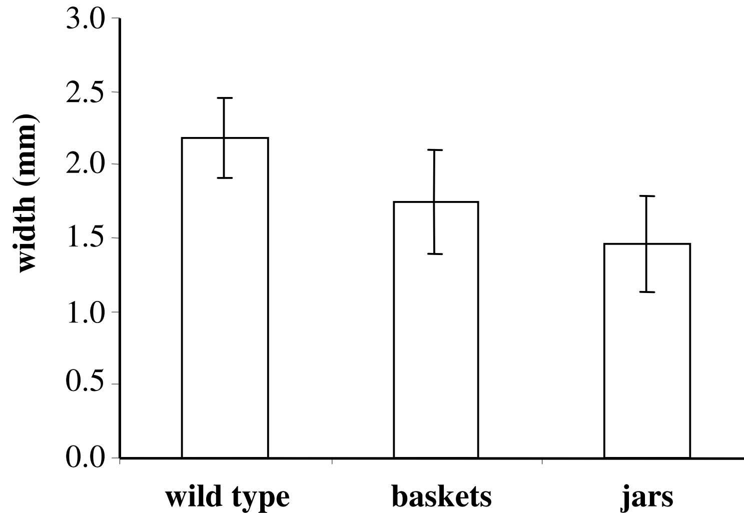


Figure 5.9: Leaf widths, at half-length, of *Z. muelleri*.

5.6.5 Acid-soluble carbohydrate measurements

Plots of total chlorophyll versus acid-soluble carbohydrate are presented in Figure 5.10. The plots provide the following linear regression equations:

$y = 0.0281x - 1.9874$, $R^2 = 0.92$ (test jar) [A]

$y = -0.0116x + 18.59$, $R^2 = 0.26$ (control jar) [B]

$y = 0.0363x + 4.0373$, $R^2 = 0.92$ (test basket) [C]

$y = -0.0165x + 56.9$, $R^2 = 0.54$ (control basket) [D]

Where ‘y’ and ‘x’ represent total measured chlorophyll (µg) and acid-soluble carbohydrate (µg), as ‘glucose equivalents’, respectively.

It was calculated, from each regression equation, that as the total chlorophyll contents approached zero the acid-soluble carbohydrate contents *of the test groups* also approached zero with 71 µg and – 111 µg being the calculated values for the jar and basket preparations respectively. Conversely, the *control group* regression equations provide large and positive calculated acid-soluble carbohydrate values of 1602 and 3448 µg for the jar and basket preparations respectively.

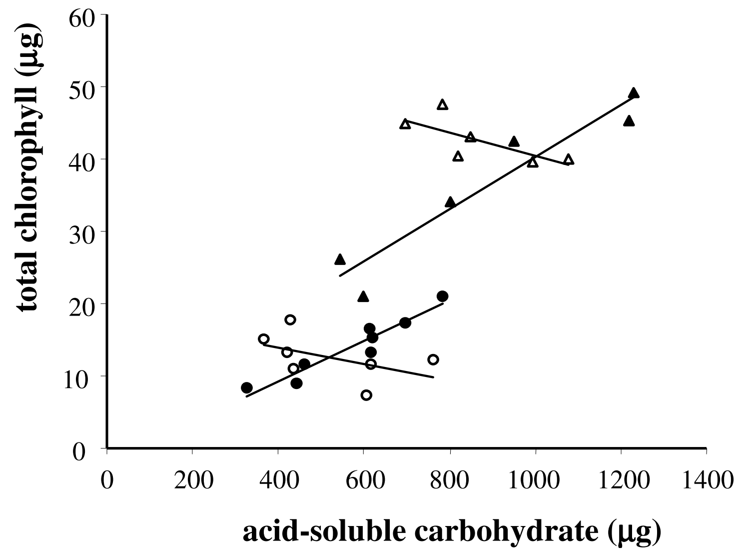


Figure 5.10: Total chlorophyll versus acid-soluble carbohydrate (measured as 'glucose equivalents') contents in basket (triangles) and jar (circles) preparations. 2,4-D- treated baskets (▲), control baskets (△); 2,4-D- treated jars (•) and control jars (o).

The near zero values in the test groups may indicate a *carbohydrate deficient state* (Neely, 2000) in the treated leaf sections while the positive values in the control groups are consistent with there being a *carbohydrate credit* state within leaf sections. The larger control value in the basket preparations of *Z. muelleri* may indicate a larger energy requirement of plants grown in 100 % sediment.

Furthermore, paired linear equations '[A]' with '[B]'and '[C]' with '[D]' were solved in order to calculate the intercept (*equivalence point*) chlorophyll and acid-soluble carbohydrate values in the jar and basket preparations respectively. The acid-soluble carbohydrate contents (expressed as µg glucose equivalents chlorophyll μg^{-1}), at the equivalence points, were 41 for the jar preparation and 25 for the basket preparation. The same results can be expressed on a molar basis, assuming that all carbohydrate is in the form of glucose. At the equivalence point, the acid-soluble carbohydrate contents were 201 µmoles chlorophyll $\mu mole^{-1}$ for the jar preparation and 124 µmoles chlorophyll $\mu mole^{-1}$ for the basket preparation. There was no evidence of chlorosis (Section 5.6.3 and Figure 5.8 at 9 days post-exposure) between the control and test

preparations. It is probable that the measured differences in carbohydrate content were caused by a fundamental change(s) in carbohydrate chemistry within leaf sections from the test groups rather than an artefact of a change in chlorophyll content of leaf sections. Furthermore, at the equivalence point, the acid-soluble carbohydrate results within the basket preparations are consistent with a greater usage (or less synthesis) of carbohydrate when expressed per chlorophyll content and when compared to the jar preparations. Additionally, a one-tailed student's t-test showed that the ratio of acid-soluble carbohydrate (μg) to total chlorophyll (μg) was significantly greater ($\alpha = 0.10$, $p = 0.06$) in the test group (24.5 ± 2.8, n = 6) than the control group (20.7 ± 4.6, n = 6) in 100 % sediment-bound plants (baskets). Therefore, it is probable that a *greater synthesis and greater usage* of acid-soluble carbohydrate occurred in the test group of the basket preparation when compared to the jar preparations.

5.7 Summary of bioassay results

5.7.1 Buoyant (free-floating) aquatic *Z. muelleri* (BAZ)

An overnight immersion of 'whole plants' in seawater loaded with herbicides or a herbicide metabolite affected photosynthesis in *Z. muelleri*. This effect was evident as either a decrease (simazine, atrazine or 2,4-dichlorophenol) or an increase (2,4-D treatment) in net photosynthetic gas production.

5.7.2 Sediment-bound plants

An overnight immersion of plants in a sub-lethal dose of 2,4 D affected photosynthesis in *Z. muelleri*. The effect was evident as an initial increase in the amount of O_2 produced by *Z. muelleri*. There was then a post-treatment decrease in net O_2 production. The carbohydrate (as glucose equivalents) contents of leaf sections from treated plants were correlated with their total chlorophyll contents while those of the control group were poorly correlated. It was noted that the carbohydrate contents of the treated leaves approached zero or was negative as the total chlorophyll contents of the leaves approached zero.

The net photosynthetic O_2 production in leaf sections removed from *control plants* grown in 100 % sediment was 48 % less than that of controls, as a non-significant

difference (at P = 0.05), obtained from freshly isolated sediment-free plants. Gas production in the 2,4-D treatment group was greater by 115 % than that of controls at 1 day post-exposure (Figure 5.7). The difference between test and control photosynthetic gas production rates became less marked as time progressed from 1 to 14 days. Therefore, 2,4-D had a marginal, but not a statistically significant, effect on free-floating *Zostera* (BAZ) by increasing the volume of photosynthetic gas produced. Additionally, 2,4-dichlorophenol, a metabolite of 2,4-D, marginally decreased photosynthetic gas production. The increased gas production was also evident in sediment-bound plants, in 100 % sediment (basket preparations) but not in 30 % sediment (large jars), in the 2,4-D treatment group. Furthermore, an acid-soluble carbohydrate analysis of leaf sections showed that total chlorophyll was correlated with acid-soluble carbohydrate in the 2,4-D treatment group. Indeed, carbohydrate in the basket preparations (100 % sediment) was significantly greater than in the control group when the results were expressed relative to their total chlorophyll contents. While total chlorophyll, when expressed per unit area, was the same in the control and treatment groups, for each bioassay; the *leaf widths* in the jar preparations were 33 % smaller than those of the wild type *Zostera*. Indeed, a rank order is evident between leaves obtained from the wild type (2.2 ± 0.3 mm), basket (1.7 ± 0.3 mm) and jar preparations (1.5 ± 0.3 mm).

5.8 A qualitative assessment of leaf attachment in sediment-bound *Z. muelleri*

It was noted that more leaves were sloughed from the treatment groups of the sediment-bound plants than from the control groups (Figure 5.11).

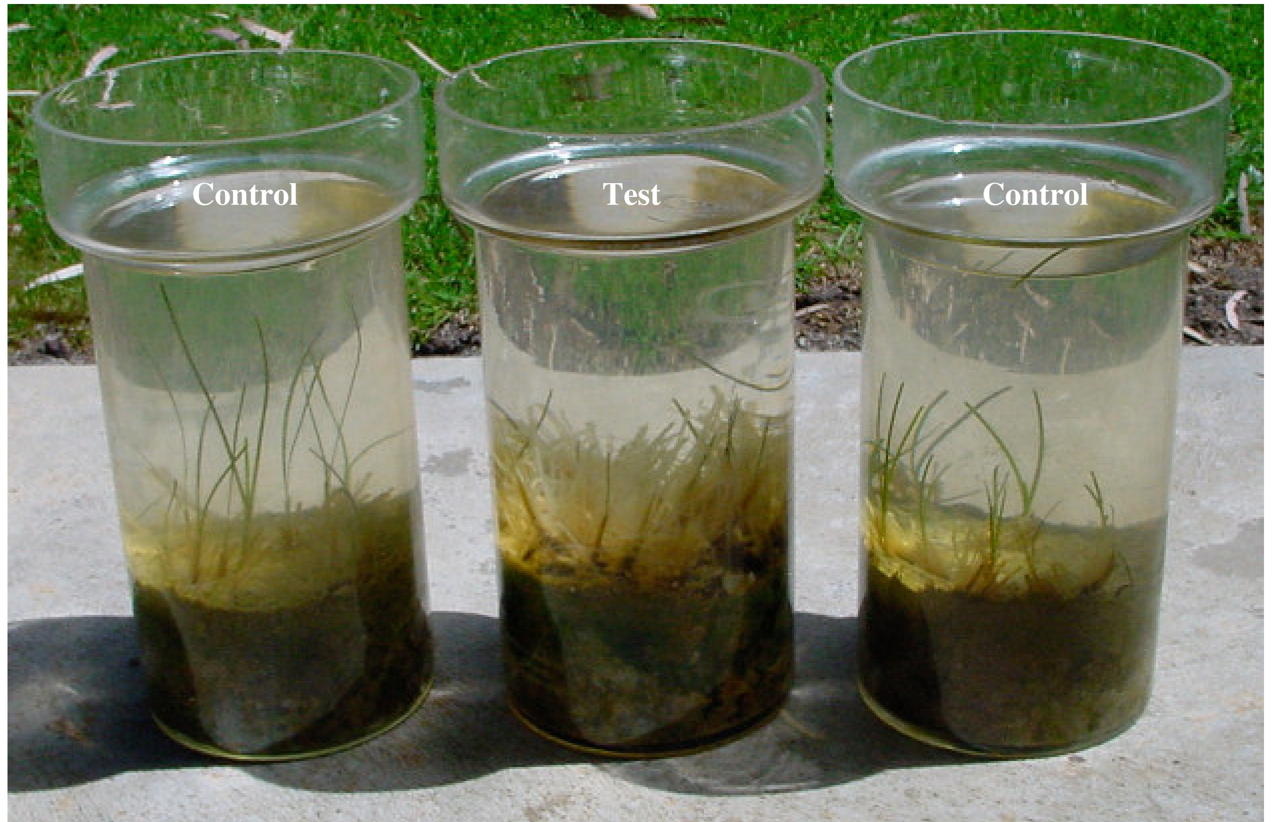


Figure 5.11: *Z. muelleri* grown in jars (acclimatized to laboratory conditions for sixteen months prior to treatment with 2,4-D). There are less green leaves within the sheaths of the 2,4-D treated plants.

Additionally, the development of a possible abscission zone at the base of treated leaves within the leaf sheath was detected (Figure 5.12). Black blemishes were also visible on some treated leaves but were absent from *all* control leaves. This is an interesting observation since fungal hyphae are known to be associated *only* with the sub-tidal form of *Z. muelleri* (Kuo *et al*., 1990).

Figure 5.12: A possible 2,4-D- induced leaf abscission zone in *Z. muelleri.*

CHAPTER 6: DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Discussion

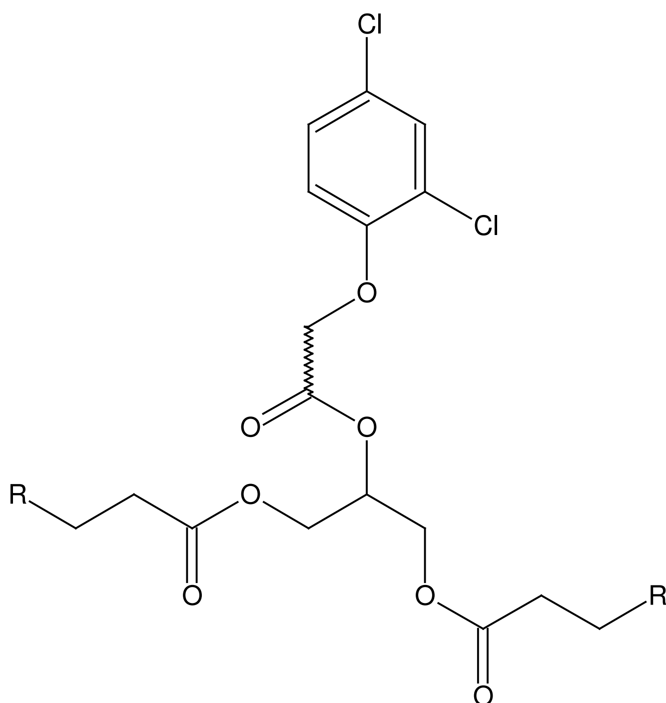
Herbicides are used extensively in land-based agricultural farming with over one million tonnes of active ingredient being used annually world-wide (Tominack, 2000). Their usage is defined by the season, weed infestation and the intended crop. On the Yorke Peninsula 2,4-D is primarily applied to farmland between the months of June and August in preparation for the summer canola and wheat crops.

Pesticide research is largely driven by the commercial need of farms to be profitable in competitive markets. Therefore it is not surprising that early pesticide research had focussed on the development of long lasting pesticide formulations. The half-life of many herbicides used on farmland ranges from years, in the case of *persistent* chemicals such as simazine and atrazine (Jones *et al*., 1982), to months with current-use herbicides. The half-life of 2,4-D is estimated as being between weeks (Thompson *et al*., 1984) and months (Que Hee & Sutherland, 1981) and is dependent on the soil pH, the organic carbon content and the moisture content of soils. It is likely that 2,4-D persists, at least for a short period, in the dry soils that can occur on the Yorke Peninsula. Likewise, the sulfonylureas persist in the alkaline soils that pervade the Yorke Peninsula (Sarmah *et al*., 2000) because of their slower hydrolysis under alkaline conditions. In this thesis no distinction was made between a herbicide that is persistent and one that is frequently used since both will have a negative effect on susceptible non-target biota if a transport mechanism is made available from the point of usage to the area of the non-target biota (Bidleman, 1999). In the present study the herbicides treflan (between 9 and 94 ppb), 2,4-D (250 ppb) and metsulfuron-methyl (2.5 g ha^{-1} and 7.5 g ha^{-1}) were detected in whole soil samples obtained from the Yorke Peninsula. Additionally, chemicals that resemble 2,4-D were also detected in whole soil at 10 to 1022 ppb and in dust obtained from whole soil at 200 ppb. The 2,4-D- like chemicals may act as precursors of 2,4-D, 2,4-dichlorophenol or as bioactive molecules on susceptible biota. Indeed it has been demonstrated that quinclorac (Rusness *et al*., 1998), also an auxin-like herbicide (Grossmann, 1998), can be 're-synthesized' from its less active long-chain esters by microbial action within soil.

In the present study it is currently uncertain whether the detected 2,4-D was present in whole soil as the original applied formulation (for example Amicide 500^{R}) or whether it was present as a conjugate. This uncertainty arises from the *caustic treatments* employed in the extraction procedure of Renberg (1974). Notwithstanding the former, 2,4-D or its progenitor(s) is present on or in soils of the Yorke Peninsula. Indeed, the mass spectral signatures of 2,4-D (Figure 1.8) and 2,4-D- like compounds (Figure 3.16), such as the synthesized simple aliphatic ester series (Figure A7, Appendix A), are unique. They are defined, in part, by the presence of *two* chlorine atoms within a phenoxy moiety and the natural abundance of chlorine being 75 % and 25 % for ^{35}Cl and ^{37}Cl respectively. Therefore, it is expected that a di-chlorinated molecule would contain two ^{35}Cl, one ^{35}Cl and one ^{37}Cl or two ^{37}Cl atoms in the molecular ratio 9:6:1 (as calculated by Davis & Frearson, 1990). This pattern was observed in 2,4-D- like chemicals detected in soil extracts (Figures A4 and A9, Appendix A) and in dust (Figure A10, Appendix A) obtained from soil. Additionally, the abundance of the principal ions, with m/z values of 133, 145 and 161, also suggested that the chlorine atoms occupied the *ortho* and *para* positions in the phenoxy ring since the mass spectral results are consistent with the observed pattern in 2,4-D (Que Hee & Sutherland, 1981). The presence of electrophilic atoms, such as chlorine, within extracted chemicals was also independently verified by using a GCECD capillary analytical system and the results showed a similar chromatographic profile to the mass spectral data (Figure 3.18).

It is known that aliphatic long-chained esters of 2,4-D can be formed on land by an ultraviolet light-catalyzed condensation of the chlorophenoxy acid and aliphatic alcohols (Que Hee & Sutherland, 1981). However, it is unlikely that the detected 2,4-D-like chemical series of the present study are simple aliphatic esters of 2,4-D since m/z 220, created by a McLafferty rearrangement (Figure 3.14), was only detected at near background levels in the mass spectral profiles. The formation of this characteristic ion was shown in the synthesized series of long-chained esters of 2,4-D (Figure A7, Appendix A) that had an aliphatic carbon chain length equal to or greater than three carbon atoms (propyl ester). GCMS analysis did detect the presence of an ion with an m/z value of 218 that was also probably created by a McLafferty rearrangement (McLafferty & Turecek, 1993) within 2,4-D- like compounds (Figure 3.17).

The presented mass spectral results differ from those previously published (Bollag, 1992) in a study of the metabolic products of 2,4-D. Bollag detected 2,4-dichlorophenoxy moieties that *abutted* syringic acid, a humic acid monomer. The results of the present study indicate that a 2,4-dichlorophenoxy carbonyl moiety is possibly bound to a hydrocarbon moiety via a *methylene bridge*. Alternately, metabolites of 2,4-D (Linscott & Hagin, 1970) generally produce an ion with an m/z value of 218 within the mass spectrometer as a result of a rearrangement process (Figures 3.16 and 3.17). Results from hydrophobic interaction chromatography and FTIR analyses also suggest that the 2,4-dichlorophenoxy moiety is connected to a highly hydrophobic hydrocarbon moiety with FTIR absorbencies at 2954 cm^{-1} (saturated C-H stretching), 2853 cm^{-1} (C-H aryl stretching), 1745 cm^{-1} (carbonyl stretching) and 721 cm^{-1} (C-Cl) (Figure 3.23 and Figure A15 (b), Appendix A). It is unlikely that the 2,4-D- like compounds contained an amide bond since the characteristic 'N-H' stretching and bending frequencies, 3375 - 3398 cm^{-1} and 1630 - 1640 cm^{-1} respectively, (Figure A14, Appendix A) were absent in FTIR spectra of extracts of whole soil (Figure 3.23 and Figure A15 (b), Appendix A). While some similarities exist between the previously detected *low molecular weight* humic acid adducts of 2,4-D (Bollag, 1992) it should be noted that such compounds were *synthesized in vitro* by fungal enzymes whereas analytes detected in the present study were extracted from whole soil and dust matrices. Additionally, 2,4-dichlorophenoxy moieties were *released* on acidification of soil extracts while a subsequent alkali treatment removed the released 2,4-D- like series (Figure 3.21 and Figure A12, Appendix A). It was demonstrated that the *purchased* methyl ester of 2,4-D is unstable in alkaline media (Section 3.13). It was also demonstrated that the phenoxy (ether) bond in 2,4-D is stable to acid treatment (Section 3.6). It is known that ester bonds are labile to both acidic and base treatments (Morrison & Boyd, 1974). It was thought that the identified 2,4-D- like chemicals are possibly large molecules that can be cleaved into smaller molecules by acidic treatment and then subsequently cleaved by an alkaline treatment.



It is likely that the detected 2,4-D series contains a 2,4-dichlorophenoxy moiety, a carbonyl moiety and a hydrocarbon moiety. This is evident in the mass spectral data of the unknowns (Figures A4 and A9, Appendix A) and standards (Figure A7, Appendix A) and in the FTIR data of the unknowns and standards (Section 3.15). Furthermore, the presence of acid labile and base labile bonds is also consistent with the 'unknowns' being ester molecules.

Figure 6.1: A hypothetical structure for the detected 2,4-D- like series. 'R' represents the hydrocarbon portion of conjugated fatty acids. It is currently unclear whether the 2,4-dichlorophenoxy moiety is connected to position 1, 2 or 3 on the glycerol backbone.

The detected compounds are not simple esters since m/z 220 (formed during a McLafferty rearrangement) is not displayed in the mass spectral profiles. It is possible that the detected 2,4-D series are beta - keto ester compounds that are known to be unstable in acidic or basic media (Vogel, 1996). Notwithstanding the former, the data are also consistent with the structure drawn in Figure 6.1 which should be compared to the 'preliminary' structure drawn in Figure 3.16, derived from a mass spectral analysis alone. It is interesting to note that other investigators (Chkanikov *et al.*, 1984) have detected 2,4-D that was conjugated to *alkali-labile oils* in what is thought to be part of a plant's detoxification system in response to a 2,4-D insult.

It is known that soil-surface applied herbicides, such as 2,4-D, are 'lost' from land at 5 % while soil-incorporated herbicides such as treflan, are lost at 1.5 % of the applied rate by wind erosion processes (Larney *et al.*, 1999). Also, herbicides can be transported,

as particulate matter, over short (25 km, Sandmann *et al.*, 1991) and longer (6000 km, Welch *et al.*, 1991) distances . Dust storms occur in South Australia with a frequency of seventeen to nineteen dust storms per annum (News in science health & medical). These data, together with the studied annual rate of pedogenesis of 5 - 10 tonnes per square kilometre, in Adelaide (Tiller, Smith *et al.* 1987) make it likely that herbicides are transported to species of the intertidal zone in the Barker Inlet. The alkaline and friable nature of the dried soils of the Yorke Peninsula, and the current tilling practices used on some farms, are likely to contribute to dust problems in this region. Since most farms of the Yorke Peninsula are within 25 km of the coastline, wind-driven dust is a likely transport mechanism for not only pesticides but also nutrients to the intertidal environment.

Z. muelleri grows in a 'tolerance zone' within the intertidal environment. The tolerance zone is defined by the interaction of *physical stress* factors, such as desiccation (Perez *et al*., 1994) and ultraviolet (UV-B) light (Trocine *et al*., 1981) with *chemical stress* factors such as heavy metals (Faraday, 1979; Prange & Dennison, 2000), anaerobic conditions and nitrite and sulfide toxicity (Jonkers *et al*., 2000). The maintenance of a sufficient amount of O_2 within the lacunae of *Z. muelleri* is required, not only for respiratory purposes but also to abate the toxic effect of sulfides within sediment that may impact upon the 'carbon drain over long-term exposures' (Erskine & Koch, 2000). The maintenance of photosynthesis within *Z. muelleri* is therefore necessary for its survival within its ecological niche.

Photosynthesis was defined, for the purpose of this thesis, as being not merely the production of O_2 in green plants but rather the production of carbohydrate, with O_2 being synthesized as a 'by-product'. Indeed, Hill demonstrated in 1939 that isolated chloroplasts synthesized O_2 in the *absence* of any synthesized carbohydrate (Hill, 1939; from Stryer, 1981). The present study has shown that a transient exposure of *Z. muelleri* to 2,4-D negatively impacted upon carbohydrate synthesis and O_2 production. This may have caused a carbohydrate deficit (Neely, 2000) and a subsequent energy deficit within affected plants. The deficit is evident in Figure 5.10 where the acid-soluble carbohydrate contents of leaf sections are negative or approach zero as the total chlorophyll contents approach zero in the 2,4-D-treated test groups. Furthermore, the crossover between the

test regressions and their respective controls possibly represents that point at which the treated plant enters a carbohydrate-deficit state. Whether this was caused by a decrease in the synthesis of carbohydrate or a greater usage of carbohydrate is currently unknown. One interpretation of the results is that *Z. muelleri* simply *outgrows its environment*. It is important to note that either a carbohydrate deficit *or* an O_2 deficit can cause an energy deficit in *Z. muelleri* since *both* are required for the process of respiration. An increase in the chlorophyll ‘a’ to chlorophyll ‘b’ ratio, and presumably the efficiency of photosynthesis in seagrasses (Dennison & Alberte, 1982), did not occur on 2,4-D treatment of *Z. muelleri* and is therefore unlikely to account for measured differences in acid-soluble carbohydrate. It has been noted that *Z marina* released only 1 % of its photosynthesized O_2 into the sediment (Sand-Jensen *et al*, 1982). If *Z. muelleri* behaved similarly then it is likely that inhibition of RUBISCO, a key carbon-sequestering enzyme in the Calvin Cycle of photosynthesis, would occur since the internal gas pressure would increase with increasing synthesis and storage of O_2 within the lacunae. Such changes in internal gas pressures have been noted in freshwater aquatic plants that were subjected to increased illumination (Laing, 1940; Hartman & Brown, 1967).

The morphology of seagrass leaves can vary between species and even within a species. This is an important point when discussing possible herbicidal or auxin-like effects on *Z. muelleri* since it has been demonstrated that the lacunae represent 16.12 % and 36.34 % of the leaf tissue volume in the intertidal and subtidal forms respectively (Kuo *et al.*, 1990). Any changes in O_2 production and storage within the lacunae are therefore likely to have a direct effect on lacunal gas pressures since stomata are absent in seagrasses (Kuo *et al.*, 1990). In the present study, the initial increase in O_2 pressure (greater than one atmosphere, since bubbles were released on collection of leaf sections) within the lacunae probably caused a greater than 25 % inhibition in the enzyme activity of RUBISCO (Bowes & Ogren, 1972) and an increased carbon loss by the process of photorespiration. This may have then been followed by a decreased O_2 availability to the roots caused by the auxin-like activity of 2,4-D acting upon the metabolically active parenchymal cells (Kuo *et al.*, 1990) of the septa and resulting in a decreased gas flow between the lacunal compartments. Indeed, it is known that 2,4-D can act by causing abnormal cell growth to such an extent that transport within the xylem and phloem is

impeded (Hess, 1993; Zimdahl, 1993). The differences in the relationship between acid-soluble carbohydrate and total chlorophyll between the 'jar' and 'basket' preparations may have been the result of morphological changes in leaf structure that had occurred on extended culture under light (PAR)-deficient (Dennison & Alberte, 1982) or nutrient-deficient conditions.

Results of the present study highlight the differences between quantum yield fluorescence measurements of photosynthesis in seagrasses (Ralph, 2000), that provide one measure of photosynthesis as electron flow (Snel *et al*., 1998), and the manufacture or usage of carbohydrate. Therefore, the relationship:

photosynthesis α f(chlorophyll fluorescence) α f(carbohydrate produced) α f(oxygen produced)

should be considered as sufficient only when *all variables* are quantified.

While it was not quantified, the strength of leaf attachment to the base of the sheath was weakened in *Z. muelleri* by the 2,4-D pulse treatment. This may have been caused by the development of an abscission zone initiated by 2,4-D's auxin-like activity (Figure 5.12). Additionally, the appearance of 'black spots' on the leaves of 2,4-D treated plants may have indicated the presence of an opportunistic fungus that is not normally present within the intertidal forms of *Z. muelleri* (Kuo *et al*., 1990).

It is likely that an increased susceptibility to toxic components within the sediment also occurred with impaired photosynthesis. In effect, *Z. muelleri* was 'frame-shifted' out of its tolerance zone by a transient exposure to 2,4-D. *Zostera's* ability to cope with the prevailing environmental conditions had changed as a result of such exposure.

6.2 Conclusions

The frequent dust storms that occur in South Australia can provide a *transport mechanism* for herbicides such as treflan, sulfonylureas and phenoxyalkanoic acid herbicides to the intertidal zone. Soils from the Yorke Peninsula, South Australia, have the potential to form fine particulate matter ('dust') and this may be exacerbated by farming practices. In the present study, treflan, sulfonylureas, 2,4-D and 2,4-D- like

xenobiotics have been detected in whole soils of the Yorke Peninsula. It is therefore likely that herbicides are transported ‘off-site’ from farmland. The results of laboratory studies indicated that the intertidal seagrass *Z. muellerei* is negatively impacted upon by a transient exposure to the herbicide 2,4-D and possibly its metabolite 2,4-dichlorophenol. While 2,4-D is regarded as a herbicide at high (mM) concentrations, it exhibits auxin-like activity at the lower (μM) concentrations (Davidonis *et al*., 1979; Davidonis *et al*., 1982; Wernicke *et al*., 1986; Sandmann *et al*., 1991) that were used in the present study. This auxin-like effect was evident as an initial increase, followed by a decrease, in the production of O_2 at two-weeks post-exposure to 2,4-D. Additionally, the treatment affected *carbohydrate metabolism* and likely caused a *deficiency* in carbohydrate in leaf sections. A *transient* exposure of *Z. muelleri* to 2,4-D therefore affected O_2 synthesis and transport together with carbohydrate metabolism.

Survival of *Z. muelleri* in the intertidal zone is dependent on a *sufficient* synthesis and translocation of O_2 into the ‘toxic’ sediment. It is also likely that any limitation in carbohydrate and O_2 would negatively impact upon the *energy balance* within *Z. muelleri*. This may have a ‘flow-on’ effect in reducing seed production and plant vigour. The Western King Prawn (*Penaeus latisulcatus*) inhabits seagrass meadows and uses them as foraging grounds and as a nursery region. It is probable that a negative impact of transported herbicide on *Z. muelleri* would also negatively impact upon prawns. Furthermore, the present study probably underestimates the potential negative impact of herbicides on intertidal species since synergistic effects between xenobiotics and physicochemical factors present within this region were not studied.

While current farming practices and herbicide usage are not the only cause of seagrass losses, they contribute to such losses and subsequently impact upon fishing yields. Furthermore, intertidal seagrasses will continue to be negatively impacted upon by a possible increased usage of auxin-like herbicides on herbicide-resistant weeds (Weedscience.com) on farmland.

Close collaboration between the farming industries, government regulators and farm-chemical suppliers is now needed to help reduce ‘off-site transport’ and minimize environmental pollution in neighboring environments, such as the intertidal zone.

6.3 Future Directions and Possible Experimental Designs

6.3.1 Stable isotope analysis

To investigate the photosynthetic process at the level of carbohydrate synthesis, the stable isotope ^{14}C within sodium bicarbonate (Na HCO_3) could be used. It would be possible to monitor 'newly synthesized' acid-soluble carbohydrate. Isolated carbohydrate could then be monitored by nuclear magnetic resonance or converted to $^{14}CO_2$ which could be analysed by GCMS with selective ion monitoring at an m/z value of 46.

It is likely that data obtained would allow the experimenter to investigate induced stress caused by physico-chemical treatment(s). Results obtained could then be correlated with current methods of measuring photosynthetic efficiency such as fluorimetry.

6.3.2 Collection and measurements of pesticides on dust

Micro-filtration devices positioned along the east coast regions of the Yorke Peninsula and the Adelaide coastline could provide a measure of dust and herbicide transport to the intertidal zone. These data would help quantify the spatial and temporal transport of xenobiotics used on farmland.

6.3.3 Further characterization of the 2,4-D- like chemicals

It is likely that tandem mass spectrometry and ^{13}C nuclear magnetic resonance will help characterise the molecular structures within the 2,4-D- like series detected in the present study.

6.3.4 *In vivo* measurement of RUBISCO inhibition by pressure-transduction

In vitro studies, on enzyme extracts, have demonstrated that RUBISCO, a key enzyme in the photosynthetic process, is inhibited by 25 % at 1 atmosphere O_2 (Bowes & Ogren, 1972). It is likely that greater O_2 pressures will cause further inhibition of RUBISCO. The following equation may relate changes in lacunal O_2 (partial) pressure to an *in vivo* inhibition of RUBISCO:

$$f(\Delta P/\Delta t) = k\ (\%\ \text{inhibition of RUBISCO}) + C$$

Diurnal changes in lacunal gas pressures (inferred from the research of Lee & Dunton, 2000) can probably be measured by using a pressure transducer connected to a cut end of a *Z. muelleri* leaf. Additionally, this methodology would allow the experimenter to monitor the effects of photosynthesis on lacunal gas pressure and conversely the effects of lacunal gas pressure on photosynthesis, i.e. detect any synergistic effects of physico-chemical treatments on the photosynthetic process.

6.3.5 Use of Fluorimetry

A pulse amplitude fluorimeter (PAM) would allow measurement of quantum efficiencies associated with photosystem 2 and the assimilation of CO_2 under laboratory conditions. Results obtained would permit an assessment of the impact(s) of physicochemical treatment(s), such as herbicide and ultraviolet light, on the carbon budget (measured as acid-soluble carbohydrate) under laboratory conditions. It would then be possible to compare the results obtained from *in situ* fluorimetry measurements on *Z. muelleri* 'conducted in the field' with laboratory results. This would allow measurement of the carbon budget in Z. *muelleri* and provide an assessment of its importance for seagrass survival when *Zostera* is impacted upon by physico-chemical stress factors.

6.3.6 Measurements of the strength of leaf attachment

Development of a 'tensile strength device would allow quantification of the strength of attachment of leaves to the leaf base in control and xenobiotic treated plants. It is likely that data, collected from the second youngest leaf within a leaf sheath, will be correlated with any weakening in leaf attachment caused by an auxin-like activity of xenobiotics acting upon *Z. muelleri*. It is anticipated that such a device may be more broadly useful in plant research.

6.3.7 A direct biological effect of 2,4-D- like compounds on prawns (*Penaeus*)?

Organophosphorus pesticides, such as profenofos, interfere with the catabolism of acetylcholine by inhibiting the enzyme acetylcholine esterase (Kumar & Chapman, 1998). The enzyme inhibition then causes neural dysfunction and death in affected animals.

It is interesting to note that 2,4-D can be metabolised to 2,4-D acetylcholine (2,4-D-ACh), a false cholinergic messenger (Sastry *et al.*, 1997). Furthermore, 2,4-D also causes an impairment of operant learning in affected rats (Lakshmana & Raju, 1996). The authors suggest that the measured differences between test and control groups can be explained by differences in acetylcholinesterase (AChE) activity. Such enzyme activity may also be adversely affected in prawns subjected to a transient exposure to 2,4-D or 2,4-D- like chemicals.

6.3.8 Redox studies of jar preparations in modified and unmodified sediment

It is possible to continually monitor the redox potential of modified (diluted with sand) sediments by placing a redox probe through a glass jar and within the sediment zone. This would permit the continual monitoring of a treatment, such as a ‘pulse’ of herbicide, on the redox potential of modified sediment. Changes in the redox potential, especially adjacent to the root zone, may provide data that relates to changes in the bioavailability of phosphorus (Craven & Hayasaka, 1982) and possibly other nutrients. However, use of unmodified sediment would require water flow (inlet and an outlet), on a 12 hr –12 hr cycle, through the sediment to remove accumulated ‘toxins’ within the interstitial water. ‘Tidal flows’ could be mimicked in this proposed laboratory system.

6.3.9 Redox studies of basket preparations in unmodified sediment

The previously mentioned redox studies could also be performed on *Z. muelleri* cultures grown in basket preparations. Again, results obtained are likely to provide information that could be related to environmental contamination events that may occur in the ‘natural environment’.