Microalgae and mangroves as carbon sources for estuarine invertebrates:
evidence from stable isotope enrichment experiments

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Abstract

The importance of different sources of carbon (energy) supporting secondary productivity in estuaries and coastal waters is still rather uncertain, despite decades of research on this topic. One of the major uncertainties is the relative importance of carbon from macrophyte detritus and microalgae, particularly diatoms and cyanobacteria within sediments (microphytobenthos, MPB). Existing methods have been unable to determine their relative contributions to consumer nutrition. This thesis addresses some of the limitations of existing methods, and then uses an innovative combination of techniques to determine the relative contributions of detritus and MPB to the nutrition of consumers within a mangrove forest in southeast Queensland, Australia. To ensure wide applicability, techniques were developed for both mud and sand, encompassing the range of sediments encountered in estuaries and protected coastal waters.

Stable isotope analysis is commonly used to resolve food web issues. Difficulty extracting MPB from sediment to obtain a pure carbon isotopic signature ($\delta^{13}$C), however, has hampered efforts to determine the importance of MPB as a carbon source for consumers. I showed that compound-specific isotope analysis of phytol, part of the chlorophyll molecule, can be used to estimate MPB $\delta^{13}$C with enough precision for most food web studies: within 1-1.8‰ of actual values (95% CI) at normal levels of replication (n = 5 or 10). Although the majority of phytol in sediments is produced in situ by MPB, part of the phytol pool is derived from detritus (e.g. 33% for mud, 17% for sand in the current study). In some situations, this could lead to errors in estimating MPB $\delta^{13}$C, realistically by about 1‰ (in sand) or 2‰ (in mud), but an adjustment can be made where necessary. Compound-specific isotope analysis of phytol (the phytol method) can therefore be confidently used to estimate MPB $\delta^{13}$C values in estuarine environments.

Where $\delta^{13}$C values of detritus and MPB are similar, natural abundance stable isotopes are unable to resolve their contribution to consumer nutrition. Enrichment of producers with the heavy $^{13}$C isotope generates distinct $\delta^{13}$C values that can be traced into consumers. Studies aiming to enrich MPB using $^{13}$C-enriched sodium bicarbonate have, however, observed simultaneous enrichment of bacteria. This may
simply be due to use of MPB-derived carbon by bacteria, but if bacteria acquire the label directly, enrichment of consumers would indicate use of bacteria, rather than MPB, as a primary carbon source. Application of $^{13}\text{C}$-enriched bicarbonate resulted in enrichment of MPB in both mud and sand and subsequent secondary enrichment of bacteria. Direct bacterial uptake was trivial in comparison to MPB uptake. Any labelling of animals in subsequent enrichment experiments could therefore be attributed to use of MPB as a primary carbon source.

I used a combination of the phytol method, stable isotope enrichment, and compartment modelling within a mangrove forest to examine use of detritus and MPB as carbon sources for common consumers; crabs (*Australoplax tridentata* and *Parasesarma erythrodactyla*) and foraminifera (*Ammonia beccarii* and *Trochammina inflata*). Compartment modelling quantified the extent of use through comparison of producer and consumer enrichment over time. The main carbon source for *T. inflata* was unable to be established, but all other species acquired virtually all of their carbon locally (within a 1 m radius), from MPB and/or mangrove detritus. The majority of carbon for *A. tridentata* was derived from MPB (99%), whereas *P. erythrodactyla* and *A. beccarii* predominantly utilised mangrove detritus (88 and 84%, respectively). For the latter two species, the remainder of carbon was derived from MPB. The different strategies in carbon utilisation by the crab species indicate feeding selectivity that may be a strategy to avoid interspecific competition for food resources. The high abundance of benthic invertebrates and their rapid assimilation of local carbon sources highlight their potential importance in influencing carbon cycling and productivity in estuaries. Consumption of benthic invertebrates by more mobile, higher consumers may also allow for transport of locally-produced carbon to adjacent waters via trophic relay.

The methods demonstrated in this thesis have potential application in resolving food web issues in a variety of habitats. Further application of these techniques at a variety of temporal and spatial scales would help to develop a broader understanding of the importance of macrophyte detritus and MPB, and establish general patterns in utilisation of carbon sources in estuarine systems.
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Declaration

The material presented in this thesis has not been previously submitted for a degree or diploma in any university, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

Joanne Oakes
Chapter 1. Introduction

1.0 General introduction
Increasing pressure from encroaching coastal urban development has resulted in increased emphasis on the conservation of estuarine habitats (Valiela et al. 2004). Habitats such as seagrass beds, saltmarsh and mangrove forests have long been valued for the services they provide. Coastal estuarine habitats assist with removal of land-derived nutrients and contaminants from runoff after rainfall (Valiela et al. 2004) and also help to stabilise the shoreline (Newell et al. 1995). The importance of these habitats for supporting juveniles of fisheries species, including prawns and fish, has also been recognised (Young 1978, Herzka et al. 2001, de la Moriniere et al. 2003). One of the main attributes used to assess the importance of these habitats is their contribution to the nutrition of recreationally and commercially important fisheries species.

For decades, researchers have tried to determine the sources of nutrition (especially carbon) supporting consumers in coastal ecosystems. In habitats where macrophytes are dominant, macrophyte material is considered to be a poor quality food resource (Skov & Hartnoll 2002, Thongtham & Kristensen 2005). Microbial degradation, however, enhances the quality of food (Werry & Lee 2005). For this reason the prevailing paradigm in estuarine ecology has been that consumers derive their nutrition via a microbially-mediated, detritus-based pathway (Odum & Heald 1975).

Although macrophytes are the most conspicuous source of production available to estuarine consumers, diatoms and cyanobacteria in the upper layer of sediments, collectively referred to as microphytobenthos (MPB), provide a further potential carbon source in the sediments of macrophyte-dominated habitats and on intertidal mudflats and sandflats devoid of conspicuous vegetation. In southeast Queensland, which is the focus of this thesis, Webb (1999) reported productivity of MPB to vary from 18.3-691.8 mg C m$^{-2}$ h$^{-1}$. This data is unpublished but demonstrates that MPB within subtropical southeast Queensland may be highly productive. MPB may also be very productive in temperate regions (12.7 mg C m$^{-2}$ y$^{-1}$, Cahoon 1999). It is this generally high productivity and the abundance of MPB that has led to recognition of
their potentially important role in providing nutrition to estuarine consumers (e.g. Currin et al. 1995, Herman et al. 2000, Leguerrier et al. 2003).

Despite efforts in seagrass (Fry 1984, Kitting et al. 1984, Moncreiff & Sullivan 2001), saltmarsh (Currin et al. 1995, Wainright et al. 2000) and mangroves (Lee 2000, Bouillon et al. 2004a), the relative importance of detritus and microalgae as carbon sources for estuarine consumers remains unresolved. This is in large part due to problems encountered with existing techniques, giving inconclusive results. The purpose of this thesis is to address some of the problems of existing techniques for exploring the trophic importance of detritus and MPB. To ensure wide applicability, new techniques were developed and tested in both sandy and muddy sediments, encompassing the range of sediments encountered in estuarine environments. The suitability of an innovative combination of methods to resolve food web issues involving detritus and MPB was then demonstrated in a case study using consumers in a mangrove forest in southeast Queensland, Australia.

1.1 Sandy sediments
Sandy sediments are characterised by large grain sizes and low organic matter content (Cartaxana et al. 2006). Because sandy sediments tend to be associated with high hydrodynamic energy (MacIntyre et al. 1996), resuspension of MPB and burial due to sediment mixing leads to characteristically low biomass of MPB relative to muddy sediments (Herman et al. 2001). A lack of macrophytes also means that there is no autochthonous production of macrophyte detritus. Detritus transported to sandy sediments from adjacent habitats and autochthonous MPB production are therefore likely to be the main sources of carbon. Because of their low productivity, sandy shores, particularly beaches, have traditionally attracted less research attention than muddy sediments and habitats dominated by macrophytes. Although some work has been done on sandy beaches, including in Australia (James & Fairweather 1996), the focus has historically been on establishing distributions and abundances of animals (McLachlan & Jaramillo 1995). It is only recently that considerable effort has been put into researching factors affecting MPB productivity in sandy sediments (Cook & Roy 2006) and the trophic relationships between consumers and carbon sources on extensive sandy shores. Much of this research effort has been in Europe, where MPB on sandy shores has been shown to be utilised by nematodes (Middelburg et al. 2000),
and macroalgal detritus deposited in sandy sediments has been shown to be a carbon source for a variety of meiofauna (Rossi 2007). In Hong Kong, manipulative experiments to alter the availability of mangrove detritus have investigated impacts on abundance and biomass of fauna within sandy sediments (Lee 1999). In Australia, however, despite vast expanses of sandy habitat available, there has been little research looking at trophodynamics of sandy shores. Methods facilitating the conclusive determination of the carbon sources of consumers in sandy sediments would assist in the development of an understanding of these habitats.

1.2 Muddy sediments
In contrast to sandy sediments, muddy sediments typically occur where low hydrodynamic energy allows settlement of fine material. Often, muddy sediments are found around and adjacent to saltmarshes, seagrass beds and mangrove forests, where vegetation slows water movement and helps to trap and stabilise sediments (Newell et al. 1995). Compared to sandy sediments, muddy sediments typically have a greater content of organic matter (Cartaxana et al. 2006), derived locally or transported from adjacent environments (Bouillon et al. 2004b). Low resuspension rates due to low hydrodynamic energy, and the typically greater water content of muddy sediments, also encourage the development of high MPB biomass (MacIntyre et al. 1996). MPB therefore has the potential to be a major source of carbon for consumers within muddy sediment habitats. The contribution of locally-derived detritus can also be significant in these environments, as described below in more detail for mangroves in particular.

1.3 Mangrove forests
Mangrove forests are widely distributed along the coastlines of tropical and subtropical regions and have long been considered as highly productive habitats that maintain fisheries productivity through provision of organic matter (carbon) to animals (consumers). The dominant paradigm in early mangrove ecology was that material was outwelled from mangrove forests to support consumers offshore (Odum & Heald 1975). However, a lack of evidence to support this theory, and evidence that consumers in some systems utilise carbon sources in their immediate vicinity (Guest et al. 2004), has led to the more recent idea that mangroves supply carbon locally to the benthic invertebrates that are abundant within them. Mangroves themselves are not the sole source of primary production available to these consumers, however. The
main purpose of this thesis was therefore to investigate potential carbon sources within mangrove forests and to determine their contribution to the nutrition of common invertebrate consumers.

1.3.1 Potential carbon sources in mangrove forests
The most conspicuous producers within mangrove forests are mangrove trees. However, despite high productivity (e.g. 831-922 g leaf litter m\(^{-2}\) yr\(^{-1}\) in southeast Queensland, Australia; Mackey & Smail 1995), mangrove detritus is considered to be a poor quality food resource. The high tannin content of mangrove leaves acts as a feeding deterrent and the high C:N ratio is considered insufficient to meet the nutritional requirements of consumers (Skov & Hartnoll 2002, Thongtham & Kristensen 2005). A less refractory, more palatable carbon source is offered by benthic diatoms and cyanobacteria (MPB), within the upper layer of mangrove forest sediments. Though microscopic, MPB are very abundant and typically have a rapid turnover rate, potentially resulting in very high productivity. Further carbon can be imported into mangrove forests as phytoplankton or macrophyte detritus from adjacent habitats (Bouillon et al. 2004b). In southeast Queensland, where the current study is focussed, however, Guest & Connolly (2006) found little evidence of transportation of material from adjacent saltmarsh habitats more than 5 m into mangrove forests. The overall contribution of allochthonous sources to consumers within mangrove forests in southeast Queensland is therefore likely to be minor in comparison to local (autochthonous) production. MPB and mangrove detritus are likely to be the major forms of production available to consumers in mangrove forests in this region.

Despite numerous attempts to determine the ultimate source of carbon for consumers in and around mangrove forests in Australia (Loneragan et al. 1997) and elsewhere (Rodelli et al. 1984, Primavera 1996, Chong et al. 2001, Bouillon et al. 2002a, Bouillon et al. 2002b, Hsieh et al. 2002, Bouillon et al. 2004b), studies to date have found it difficult to unequivocally resolve the contributions of mangroves and MPB.

1.3.2 Potential nutritional importance of mangroves
Although mangrove leaf litter is considered to be a poor quality food resource, laboratory feeding experiments (Kwok & Lee 1995, Ashton 2002) and field studies
(Robertson 1986, Camilleri 1992, Micheli 1993, Ashton 2002) have established that some consumers ingest large quantities of leaf litter in mangrove forests. Crabs of the family Grapsidae (grapsid crabs), including those of the subfamily Sesarminae, are found with large quantities of mangrove leaf material in their guts (Dahdouh-Guebas et al. 1999). They have also been shown to rapidly remove much of the litter that falls to the forest floor (e.g. 79% reported by both Robertson & Daniel 1989 and Ashton 2002). It is thought that fragmentation of leaves through crab feeding encourages microbial colonisation and decay, thereby improving food quality by decreasing the C:N ratio (Skov & Hartnoll 2002, Werry & Lee 2005). Mangrove material that has been improved in this way can then be utilised, in the form of crab faecal pellets, by other consumers (Lee 1997). However, although we know that consumers can use mangrove leaf litter, the assimilation efficiency for mangrove leaves tends to be low (9-50%, Kwok & Lee 1995, Lee 1997, Thongtham & Kristensen 2005), and feeding experiments have shown that crabs do not thrive on a diet of pure mangrove leaves (e.g. Kwok & Lee 1995). In sandy sediments, addition of mangrove detritus to sediments as a potential food source has been found not to affect the abundance and biomass of macrobenthos (Lee 1999). Kwok & Lee (1995) suggested that crabs within mangrove forests may utilise mangrove detritus, but must also rely on a further source of nutrition.

### 1.3.3 Potential nutritional importance of microphytobenthos

In the past, little emphasis has been placed on the importance of MPB, despite the fact that areas devoid of macrophyte vegetation, where MPB could be the only carbon source, often support a greater abundance of certain key fish and prawn species than adjacent seagrass beds and mangrove forests (e.g. Halliday 1995, Kenyon et al. 1995, Gray et al. 1996). MPB may be consumed as part of the sediment (e.g. by invertebrates, Montagna et al. 1995, Ford & Honeywill 2002), and may also become available to pelagic consumers and filter feeders as a major component of water column algae following resuspension by wind and tides (de Jonge & van Beusekom 1995, Lucas 2003). Within mangrove forests, crabs are often observed grazing surface sediment (Micheli 1993) and, for ocypodid crabs (family Ocypodidae), MPB is generally considered to be a major source of nutrition (Rodelli et al. 1984, France 1998, Hsieh et al. 2002, Reinsel 2004). Studies have also shown that MPB is likely to be a major source of carbon for other benthic invertebrates (e.g. Newell et al. 1995,
Dittel et al. 1997, Lee 2000, Hsieh et al. 2002, Bouillon et al. 2004a, Guest et al. 2004, Kieckbusch et al. 2004). In these studies, however, the utilisation of MPB is usually not able to be demonstrated unequivocally and it is feasible that consumers utilise alternative sources of nutrition. Even where utilisation of MPB is clearly shown, it has not been possible to demonstrate the extent of the contribution of MPB to consumer nutrition. The value of MPB as a carbon source to consumers in mangrove forests is therefore yet to be determined conclusively.

1.4 Consumers within mangrove forests

At low tide, the exposed mud of mangrove forests can be seen to harbour an abundance of crabs and other invertebrates. A variety of shorebirds feed in mangrove forests (Martinez 2004) and fish take advantage of tides that inundate the forest to feed on benthic invertebrates (Sheaves & Molony 2000). These benthic invertebrates can include snails (Bouillon et al. 2004a, Guest et al. 2004, Alfaro 2006), bivalves (Bouillon et al. 2004a), and meiofauna (e.g. nematodes, amphipods, copepods, foraminifera; Couch 1989, Coull 1999), typically dominated by a small number of taxa (Hodda & Nicholas 1985). In addition to providing a potential food resource for higher consumers, benthic invertebrates provide a number of other ecosystem services, including enhancing remineralisation of organic matter and cycling of nutrients (Coull 1999). The abundance, biomass and distribution of benthic invertebrate taxa, and hence their influence on ecosystem processes and availability as a food source, can be influenced by sediment type, salinity and temperature (Coull 1999). The current study investigates consumers which reside in the mangrove forests of southeast Queensland where the most abundant benthic invertebrates for macro- and meiofauna are, respectively, species of crab and foraminifera (pers. obs.)

1.4.1 Crabs

The most widespread and abundant consumer taxa found in mangrove forests are brachyuran crabs, primarily grapsids and ocypodids (Snelling 1959, Tan & Ng 1994, Hartnoll et al. 2002, Skov et al. 2002). Through their feeding and burrowing activities, crabs perform a number of important services in the functioning of mangrove forests and have therefore been described as ecosystem engineers (Jones et al. 1994). The abundance of crabs means that the impact of crabs on mangrove ecosystems can be significant. Feeding by crabs leads to changes in soil topography
and particle size (Warren & Underwood 1986), and changes to soil chemistry through burrowing activities can impact mangrove growth and production (Smith et al. 1991, Kristensen & Alongi 2006). Even mangrove zonation and colonisation can be affected by crab feeding through selective predation on mangrove propagules (Smith 1987). Grazing by crabs on the surface of estuarine sediments may also influence productivity by removal of MPB (Taylor & Allanson 1993). As stated previously, crabs can consume a large proportion of the litter which falls to the forest floor (Ashton 2002), enhancing litter decomposition (Werry & Lee 2005) and producing faecal pellets which make mangrove material available to other consumers within the system (Lee 1997). This potentially prevents significant export of mangrove material to adjacent environments (Lee 1995). It was the realisation of the extent of removal of mangrove litter by crabs that first led researchers to question the older outwelling paradigm. Note, however, that consumption of large numbers of crabs by mobile consumers might nevertheless result in carbon being transported to coastal waters via trophic transfer (Sheaves & Molony 2000).

The crabs *Parasesarma erythrodactyla* (family Grapsidae) and *Australoplax tridentata* (family Ocypodidae) are dominant in the southeast Queensland region of Australia (Snelling 1959) and were the most abundant macrofauna in the mangrove forest that was the focus of Chapters 5 and 6 of this thesis (pers. obs.). Camilleri (1992) observed *P. erythrodactyla* feeding on mangrove leaves, and both *P. erythrodactyla* and *A. tridentata* feed on superficial sediments (pers. obs.). Whereas ocypodids are thought to rely upon MPB (Rodelli et al. 1984, France 1998), grapsid crabs are believed to rely primarily upon leaf litter for their carbon (Robertson et al. 1992, Lee 1997, Lee 1998, Thongtham & Kristensen 2005). A study by Guest et al. (2004) looking at *P. erythrodactyla* and *A. tridentata* within a southeast Queensland saltmarsh found δ13C values of the two species to be similar. Whereas in a saltmarsh both species appeared to utilise MPB as a primary source of carbon, the same crab species within adjacent mangrove forests were considered to be reliant upon MPB or a combination of MPB, mangrove material and detritus from the saltmarsh (Guest et al. 2004). The ultimate carbon source of these species within mangrove forests therefore remains unresolved.
Due to the great abundance of crabs and their importance in mangrove forests, numerous studies have aimed to establish their nutrition sources. However, work to date only demonstrates simple ingestion, not assimilation, and/or encounters other problems (as discussed in section 1.5.3 below) that prevent resolution of the issue. The sources of carbon for crabs, and their relative contributions, therefore remain poorly defined.

1.4.2 Foraminifera

In addition to MPB, mangrove forest sediments are host to a variety of abundant meiofauna. The high biomass and replacement rates of meiofauna means that predation by higher consumers does not significantly impact meiofaunal populations (Coull 1999). Meiofauna are therefore a potentially valuable source of nutrition within mangrove forests and estuarine habitats in general. Foraminifera are often an abundant component of the meiofauna of estuarine sediments (e.g. 72-400 individuals per gram of wet sediment; Ellison 1984). *Trochammina inflata* and *Ammonia beccarii* are foraminifera with widespread global distributions and in sediments of the mangrove forests in southeast Queensland, where this study was done, they are among the most abundant meiofauna species (pers. obs.). Although no studies have specifically addressed the role of foraminifera in mangrove ecosystems, it has been demonstrated that foraminifera in deep-sea sediments can be rapid processors of phytodetritus (Moodley et al. 2000, Gooday et al. 2002, Moodley et al. 2002). Foraminifera can also provide nutrition for higher consumers (Chong & Sasekumar 1984, Branco et al. 2002, Stergiou & Karpouzi 2002, Abed-Navandi et al. 2005). The great abundance of foraminifera within sediments means that they may significantly affect ecosystem processes. For this reason it is important to determine the sources of nutrition supporting foraminifera in mangrove forests.

Fatty acid analysis has indicated that foraminifera can assimilate material from detritus, MPB and bacteria (Topping et al. 2006), and studies to date have indicated that diet differs among species (Topping et al. 2006), but the relative contributions of sources to the diet of foraminifera species have not been quantified.
1.5 Stable isotope analysis for resolving food web issues

Previous studies aiming to resolve food web issues pertaining to consumers in mangrove forests have often relied upon observations of feeding in the field or laboratory (Micheli 1993, Ashton 2002) or gut content analysis (Malley 1978, Dahdouh-Guebas et al. 1999). However, these methods are only able to indicate the food that has been ingested. Studies using natural abundance stable isotopes are able to indicate the food that has been assimilated by an organism and which is, therefore, of nutritional importance (Thomas & Cahoon 1993).

1.5.1 General theory

The analysis of stable isotope ratios is one of the most commonly applied techniques in trophodynamic studies (e.g. Hesslein et al. 1991, Machás & Santos 1999, Kharlamenko et al. 2001, Moncreiff & Sullivan 2001), due to the advantages over other methods.

Stable isotope analysis uses the ratio of a heavy, rare isotope (e.g. $^{13}$C, $^{15}$N or $^{34}$S), to a light, common isotope of an element (e.g. $^{12}$C, $^{14}$N or $^{32}$S), compared to that of an international standard. This ratio is referred to as the isotopic signature and is expressed in units of per mil ($\delta$). The isotopes most commonly analysed in ecological studies are those of carbon, nitrogen and sulfur. The isotopic signatures of these elements are shown as $\delta^{13}$C, $\delta^{15}$N and $\delta^{34}$S, respectively. To clarify interactions at lower trophic levels using stable isotope analysis, isotopic signatures of available autotrophic producers must be distinct from one another. Differences in producer signatures result from differences in the source of nutrients and/or discrimination against heavy or light isotopes of an element (fractionation) during uptake and assimilation. For example, the carbon isotopic signatures of producers within estuarine environments may vary due to different sources of carbon dioxide, obtained from either air or water, and different photosynthetic pathways (C$_3$ or C$_4$) which favour the light isotope ($^{12}$C) to varying extents (Peterson & Howarth 1987). The signature of a heterotrophic consumer reflects that of its food (Peterson et al. 1985), and a comparison of the isotopic signatures of consumers and producers can therefore reveal the ultimate source of organic matter even after multiple trophic transfers (Peterson et al. 1986). Where a consumer has assimilated organic matter originating
from two distinct producers, its signature lies between those of the producers, depending on the relative contribution of each source to the diet.

Fractionation occurring in the transfer of elements from the source to consumer tissues may confuse interpretation of data. Although fractionation of carbon is relatively minor, with consumers on average only $0.5\%$ (± S.E. $0.13\%$) enriched relative to their food source (McCutchan et al. 2003), nitrogen is progressively enriched by an average of $2.3 ± 0.18\%$ with each trophic transfer (McCutchan et al. 2003). This nitrogen fractionation can be used to advantage, where the source of nutrition is known, to assist in resolving trophic levels of consumers (Peterson 1999). Where the aim is to determine the contribution of available producers to the nutrition of consumers, however, stable isotope analysis of carbon is preferable.

1.5.2 Stable isotope studies in mangrove habitats

Numerous studies have applied natural abundance stable isotope analysis to explore food web issues in mangrove ecosystems. Although contribution of mangrove material to the nutrition of some consumers, such as juvenile prawns (Newell et al. 1995, Loneragan et al. 1997, Chong et al. 2001), has been reported, most studies suggest that MPB is utilised by a wider variety of species and to a greater extent (e.g. Bouillon et al. 2002a, Hsieh et al. 2002). Bouillon et al. (2002a), for example, found that of 22 species of benthic invertebrate, most relied predominantly upon carbon from MPB. Of the species studied, very few, including a grapsid crab (*Parasesarma asperum*) and gastropods (*Melampus fasciatus, Cassidula mistelina* and *Pythia plicata*), derived significant carbon from mangrove detritus (Bouillon et al. 2002a).

With the exception of Chong et al. (2001), who estimated an 84% contribution of mangroves to the diet of juvenile prawns (*Penaeus merguiensis*) using mixing models, studies to date have been unable to quantify the contribution of mangroves to consumer diets and none have quantified the contribution of MPB. All natural abundance isotope studies are also compromised by a number of problems that prevent food web issues being resolved, particularly in and around mangrove forests.
1.5.3 Limitations of natural abundance stable isotope analysis

Accurate use of stable isotope analysis relies upon clear definition of the isotopic signature of the producers of interest (Peterson 1999). Although stable isotope analysis has great potential for tracing the movement of MPB-derived carbon through foodwebs, isotopic signatures assumed for MPB have been questionable due to problems with methods used to isolate MPB from the sediment matrix for stable isotope analysis (discussed in detail in Chapter 2). Contamination of MPB samples with non-MPB material such as meiofauna and detritus result in incorrect δ¹³C values. In studies of mangrove forest food webs, error in MPB δ¹³C may result in MPB being estimated as a more or less significant nutrition source, relative to mangrove material, than it actually is. There is therefore a need for a method to accurately determine carbon signatures of MPB, in mangrove forests and more generally.

In addition to being well-defined, producer δ¹³C values for natural abundance isotope studies also need to be distinct from one another (Peterson 1999). Mixing models are then used to determine the contribution of different producers to the diet of the consumer of interest (Phillips & Gregg 2001, Phillips & Gregg 2003). Because MPB δ¹³C is thought to be influenced by the presence of depleted DIC in mangrove forests (Bouillon et al. 2004b), this may present a problem for mangrove forest ecosystems. Depleted δ¹³C of MPB may then be more similar to δ¹³C of phytoplankton and/or mangroves, decreasing resolution of sources. Another problem is that where there are more than two producer sources, as is the case in most mangrove forests where detritus from adjacent environments and phytoplankton can also provide carbon, mixing models usually cannot provide a unique solution. In the case of Guest et al. (2004), for example, the ultimate source of carbon for the crabs Parasesarma erythrodactyla and Australoplax tridentata were unable to be conclusively determined because either MPB or a combination of saltmarsh and mangrove material could have explained the observed δ¹³C values for consumers. Natural abundance stable isotope studies using carbon have therefore been unable to conclusively determine the relative contributions of MPB and mangrove detritus to the nutrition of consumers in mangrove forests.
1.6 Stable isotope enrichment experiments

In situations where natural abundance stable isotopes are unable to resolve food web issues, manipulation of producer $\delta^{13}C$ values, through enrichment with the heavy $^{13}C$ isotope, can help to overcome these limitations.

Stable isotope enrichment experiments were first used in the early 1990s in freshwater systems (e.g. Kling 1994, Hall 1995). The first use of stable isotope enrichment for an estuarine or marine application was by Winning et al. (1999), who demonstrated the ability to use $^{15}N$-enrichment to create isotopically distinct $\delta^{15}N$ values for the seagrass, *Zostera capricorni*, and its epiphytes. Since this initial foray into isotope enrichment in estuarine and marine environments, many studies have demonstrated the ability of stable isotope enrichment to resolve food web ambiguities. Additions of $^{13}C$-enriched phytodetritus (labelled, killed diatoms) to deep-sea (Moodley et al. 2002, Aberle & Witte 2003, Witte et al. 2003) and intertidal sediments (Moodley et al. 2002) assisted in identification of the groups of fauna that utilise organic material settling in these environments. Labelling of living microalgae (MPB) with $^{13}C$ in the laboratory (Herman et al. 2000) and the field environment (Middelburg et al. 2000, Moens et al. 2002) demonstrated that MPB is utilised by macrofauna and meiofauna in both muddy and sandy intertidal sediments. On a larger scale, understanding of the cycling of nitrogen and its use as a source of nutrition for consumers has been improved through $^{15}N$-enrichment of an estuary (Hughes et al. 2000) and a tidal freshwater marsh (Gribsholt et al. 2005, Gribsholt et al. 2007). Although stable isotope enrichment techniques have been useful in solving food web issues, no studies to date have utilised stable isotope enrichment in mangrove forests. Further, recent developments in methodology and interpretation of stable isotope experiments (Hamilton et al. 2004) provide scope for future studies to gain even greater insight into trophodynamic relationships in estuarine systems.

1.6.1 General theory

Stable isotope enrichment alters the isotopic signature of a producer through addition of a substrate enriched in the heavy isotope (e.g. $^{13}C$ for carbon) that is capable of being utilised by the producer of interest, thereby creating a unique isotopic signature (e.g. Hall 1995). This distinct isotopic signature then acts as a label that can be traced into consumers to demonstrate use of the producer of interest as a nutrition source.
Even where labelled isotopes have been successfully used as tracers, quantification of the extent of consumer reliance on a source has proven difficult. It has been assumed that to quantify the extent of use of an enriched producer by a consumer, enrichment experiments need to maintain constant enrichment of the producer of interest for sufficient time for the consumer to attain equilibrium with its food source. However, this is rarely possible due to spatial and temporal variations in producer enrichment (e.g. Raikow & Hamilton 2001), and the expense associated with running experiments over a long period. It can also be difficult to determine when a consumer has reached equilibrium with its carbon source. Recently, however, Hamilton et al. (2004) demonstrated the application of compartment modelling, using the program WinSAAM, to model the contribution of a labelled producer to consumers, without the need to maintain enrichment or for the consumer to reach equilibrium. This was done for a freshwater stream enriched with $^{15}$N. Whereas enrichment experiments are able to unequivocally demonstrate use of a carbon source by a consumer, compartment modelling of experimental results allows quantification of the contribution of the producer of interest. However, to date, only one study (van Oevelen et al. 2006b) has used compartment modelling in a marine environment, and this was to determine the extent of use of bacterial carbon by consumers. Compartment modelling, in combination with stable isotope enrichment, has great potential to resolve food web issues in other marine or estuarine environments.

1.6.2 Studies using enrichment

Enrichment studies have been used for a number of years in estuarine environments. Winning et al. (1999) enriched seagrass and epiphytes with $^{15}$N, and a number of studies have since enriched MPB with carbon (e.g. Herman et al. 2000, Middelburg et al. 2000, Moens et al. 2002) or phytoplankton with carbon and/or nitrogen (e.g. Moodley et al. 2002). Enrichment experiments have demonstrated that invertebrates on mudflats use MPB as a carbon source (Middelburg et al. 2000) and deep-sea foraminifera have been shown to utilise phytoplankton detritus (Aberle & Witte 2000). Few studies have examined use of refractory vascular plant detritus, although Abed-Navandi et al. (2005) used $^{15}$N and $^{13}$C-enrichment in the laboratory to demonstrate that thalassinidean shrimp could assimilate seagrass detritus.
I am not aware of any published studies using isotope enrichment experiments specifically aiming to resolve food web issues in mangrove forests. Manipulation of mangrove detritus and MPB $\delta^{13}C$ values in combination with compartment modelling does, however, have clear potential to resolve the source of carbon supporting consumers within mangrove forests and is the strategy used in this thesis. I also address some of the methodological aspects of enrichment experiments. For example, Middelburg et al. (2000) aimed to enrich MPB, but also reported enrichment of bacteria. Although it was assumed that the bacteria had acquired label via MPB, there has not been a concerted effort to determine if label uptake by bacteria may be direct, in which case bacteria may provide an additional primary source of carbon for consumers in mangrove forests.

1.7 Overall rationale of the thesis
Estuarine habitats can be highly productive habitats supporting an abundance of benthic invertebrates that provide a food resource of potential importance in maintaining biodiversity and fisheries production. Even on sandy beaches, which are relatively poorly understood, allochthonous poduction by MPB may support meio- and macrofauna. It is important to establish the source of carbon for invertebrates within estuarine habitats, but studies to date have been hampered by limitations of the methodologies applied, and have therefore been unable to unequivocally determine the sources of carbon supporting these consumers. The main purpose of this thesis was to develop and apply novel techniques which can be applied in both muddy and sandy habitats to resolve the long-standing confusion over the role of detritus and MPB in consumer nutrition. Using a case study in southeast Queensland, Australia, I intended to demonstrate the application of these techniques to determine the ultimate source of carbon used by consumers in a mangrove forest.

1.8 Structure of the thesis
To enable stable isotope investigation of the role of MPB in mangrove forest food webs, a compound-specific method was developed in Chapter 2 to accurately determine MPB carbon signatures, circumventing issues in previous studies where MPB isotope determinations were problematic. Due to concern that detritus within sediments may interfere with estimates of MPB $\delta^{13}C$ made using this new method, the potential for such interference was examined in Chapter 3. Chapter 4 compares the
uptake of $^{13}$C label by MPB and non-photosynthetic bacteria to determine if bacteria are capable of direct uptake of label and might thus act as an additional primary source of carbon for consumers. This was necessary to ensure that in Chapter 5, which details an experiment to label MPB with $^{13}$C, MPB was the only primary source of labelled carbon available to consumers. The MPB enrichment experiment was combined with compartment modelling to quantify the importance of MPB as a carbon source for consumers in mangrove forests. Chapter 6 was the complement of the previous chapter, examining the importance of mangrove leaf litter by adding $^{13}$C-enriched mangrove detritus to sediments and modelling its uptake by consumers. Chapter 7 provides a summary of the main findings of the thesis and discusses their implications.

These chapters are intended to provide a logical progression of experiments towards an overall conclusion, but there is also an intention to subsequently publish some chapters as separate components. At the time of thesis submission, Chapter 2 had been published and is presented here essentially as published, with only minor alterations for consistency of style in the thesis. Note, however, that to avoid substantial changes to the text, section headings remain as published and therefore vary from those used in other chapters of this thesis. Co-authors on this publication contributed scientific advice and editorial guidance.

Chapter accepted for publication in peer-reviewed journal:


2.0 Abstract
Carbon stable isotope analysis is the best method for validating theories about the role microphytobenthos (MPB) plays in estuarine food webs. However, difficulties in extracting MPB from sediments to determine a pure isotope signature have hampered such studies. We have developed compound-specific isotope analysis (CSIA) of phytol $\delta^{13}C$ ($\delta^{13}C_{\text{phytol}}$) as an accurate proxy for $\delta^{13}C$ of bulk MPB ($\delta^{13}C_{\text{bulk}}$). Since in most circumstances MPB is the main source of phytol in estuarine sediments, our method circumvents the need to extract MPB. We have demonstrated in the laboratory the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$, and could predict $\delta^{13}C_{\text{bulk}}$ within 1.8‰ (95% CI) of observed values at moderate replication ($n = 5$). If water temperature is included, $\delta^{13}C_{\text{phytol}}$ predicts $\delta^{13}C_{\text{bulk}}$ within 1.3‰ (95% CI). With greater replication ($n=10$), predictions are extremely good (within 1.0‰). A two-source mixing model assessed the usefulness of each method for estimating MPB contribution to consumer nutrition. At moderate replication ($n = 5$), for a gap between MPB and another producer of 6.0‰, estimates were more precise (i.e. 95% CI were smaller) when both $\delta^{13}C_{\text{phytol}}$ and temperature were used (95% CI = 0.40) rather than only $\delta^{13}C_{\text{phytol}}$ (0.46). The greatest difference in precision was for a gap of 6.0‰, close to the average gap in the literature (5.5‰). Given the difficulties in extracting MPB from sediment, carbon isotope studies of estuarine food webs could benefit by using CSIA of phytol, especially in conjunction with simple measurements of water temperature at the time of collection. The advance in methodology will allow a re-evaluation of the trophic importance of MPB.
2.1 Introduction

Increasing anthropogenic pressure on coastal areas has led to an emphasis on the conservation of estuarine habitats, especially those of nutritional importance for fisheries species. Although macrophytes such as seagrasses and mangroves are relatively well-protected, little consideration is given to the abundant cyanobacteria and diatoms (known collectively as microphytobenthos (MPB)) in superficial sediments. Although inconspicuous, MPB can be highly productive (up to 10 Kcal m\(^{-2}\) yr\(^{-1}\); Kennish 1995). Consequently, it has been proposed that MPB may be an important source of nutrition for many estuarine consumers (e.g. Riera & Richard 1996).

To determine the contribution of MPB to consumer diets, recent studies have applied stable isotope analysis (SIA). Unlike more traditional methods (e.g. gut content analysis), SIA is able to distinguish between food which is merely ingested by an organism and that which is assimilated, and is able to trace the ultimate source of nutrition for a consumer (Thomas & Cahoon 1993). This is particularly useful in the case of MPB, which may be utilised via microbe intermediaries.

Carbon stable isotope analysis utilises the ratio of the rare, heavy isotope \(^{13}\text{C}\) to the common, lighter isotope \(^{12}\text{C}\) of producers and consumers. Producer signatures differ depending on the source of nutrients and fractionation (discrimination between heavy and light isotopes) during uptake and assimilation. Since the signature of a heterotrophic consumer reflects that of its food (Peterson et al. 1985), comparison of the consumer signature with those of potential autotrophic sources can indicate the ultimate source(s) of organic matter even after multiple trophic transfers (Peterson et al. 1986).

Studies using stable isotope analysis have supported the assimilation of MPB by both lower level consumers (e.g. nematodes, Montagna et al. 1995, Riera et al. 1999, Riera & Hubas 2003) and higher order species (e.g. oysters such as *Crassostrea gigas*, Riera & Richard 1996, and the commercially harvested sand whiting, *Sillago ciliata*, Melville & Connolly 2003). However, accurate use of stable isotope analysis relies upon clear definition of the isotopic signatures of potential sources and the signatures assumed for MPB in previous studies are questionable (Table 2.1).
In studies to date, estimation of $\delta^{13}C$ of bulk MPB (i.e. whole algal cells) has been either through analysis of material scraped from the sediment surface (sediment or MPB mats) or attempts to physically separate MPB from sediments prior to analysis. Where isotopic signatures of bulk sediment are used as a proxy for MPB $\delta^{13}C$ (e.g. Dittel 2000), signatures are contaminated by detritus and meiofauna within the sediment. Although contamination may be minimised by allowing diatoms to migrate into nylon screens (e.g. Couch 1989) or silica powder (e.g. Riera & Richard 1996), discrimination against non-motile MPB or other components of the community may give biased isotopic signatures (e.g. Cook et al. 2004). The ‘sieve-and-spin’ method that separates MPB by centrifuging the sediment-MPB mixture in colloidal silica (S.Y. Lee unpubl. data) does not discriminate against non-motile species, but is time-consuming and the sieving process removes larger MPB from the sample (pers. obs.). Contamination is reduced, but detritus and meiofauna may still be significant in the sample obtained (S.Y. Lee unpubl. data). Such uncertainty regarding MPB $\delta^{13}C$ may have resulted in misinterpretation of trophic linkages. There remains, therefore, a need for a reliable method for determining MPB $\delta^{13}C$ in studies of marine food webs.

The isotopic signature which is required for comparison to that of consumers is $\delta^{13}C$ of the whole algal cell (MPB $\delta^{13}C_{bulk}$). Microalgae are comprised of many different compounds, however, and MPB $\delta^{13}C_{bulk}$ is simply the weighted average of the signatures of each of these compounds. The determination of the isotopic abundances of individual compounds such as chlorophyll-$a$ (chl-$a$; Sachs et al. 1999), phytol (Riebesell et al. 2000) and fatty acids (Uhle et al. 1997) has been made possible by the recent development of compound-specific isotope analysis (CSIA). If the isotopic signature of a compound uniquely associated with MPB (i.e. a biomarker) is predictably related to that of the whole algal cell, then extraction and analysis of this compound may be used to estimate MPB $\delta^{13}C_{bulk}$.

Compound-specific isotope analysis has been used in only a few studies of trophodynamics to date (e.g. Middelburg et al. (2000) used CSIA of fatty acids to trace $^{13}C$ uptake by bacteria). However, no trophic studies have utilised CSIA of MPB. The current study describes the development and validation of a method to predict MPB $\delta^{13}C_{bulk}$ using CSIA of phytol to effectively isolate the living autotrophic
component of sediment (i.e. MPB). This negates the need for physical isolation of algal cells from the sediment matrix.

Studies indicate that chl-\(a\) \(\delta^{13}C\) is an effective proxy for \(\delta^{13}C_{\text{bulk}}\) of phytoplankton (e.g. Laws et al. 1995), however, extraction of chl-\(a\) from sediments is relatively complex (see Sachs & Repeta 2000). In comparison, phytol can be extracted using fewer steps (see current chapter), reducing the possibility of fractionation. Phytol is a side chain of chlorophyll, and its \(\delta^{13}C\) is therefore expected to be predictably related to MPB \(\delta^{13}C_{\text{bulk}}\). Although phytol is found within all green plants, rapid degradation of phytol in the water column and senescent cells of detritus (Rontani & Volkman 2003) suggests that phytol within sediments is relatively recent and most likely produced predominately \textit{in situ} by MPB.

A relationship between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) exists for terrestrial plants (van Dongen et al. 2002) and marine phytoplankton (Sakata et al. 1997, Riebesell et al. 2000) and macroalgae (van Dongen et al. 2002; Fig. 2.1), but has not been shown for MPB. Sources, uptake and assimilation of carbon may differ for benthic autotrophs (i.e. MPB), therefore the relationship between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) may also differ to that described for other producers. Temperature (Hinga et al. 1994), taxon-specific biosynthetic pathways and the size, membrane permeability (Laws et al. 1995) and geometry (Popp et al. 1998) of cells may affect the uptake and assimilation of carbon in MPB. The composition of MPB communities and the temperatures to which they are exposed are likely to be both temporally and spatially variable (Currin et al. 1995). Therefore, the relationship between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) may be both species- and temperature-dependent.

This study aimed to:

a) determine if a predictive relationship exists between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) for MPB then compare this to the literature regression for terrestrial plants, and marine phytoplankton and macroalgae,

b) investigate potential influences of growth temperature and species on the phytol-bulk relationship, and

c) demonstrate the precision and effectiveness of measuring \(\delta^{13}C_{\text{bulk}}\) using
\[ \delta^{13}C_{\text{phytol}} \] through determining the expected difference between predicted and observed \[ \delta^{13}C_{\text{bulk}} \] and, using a two-source mixing model, assess the change in 95% confidence intervals for estimations of MPB contribution to consumer nutrition.

Figure 2.1: Relationship between \[ \delta^{13}C \] of bulk plant material and \[ \delta^{13}C \] of phytol for literature data (terrestrial plants and marine macroalgae, van Dongen et al. 2002; marine phytoplankton, Sakata et al. 1997; Richesell et al. 2000; regression: \( R^2=0.96 \), \( F_{1,15}=392 \), \( p<0.001 \)) and for MPB grown in the laboratory (regression: \( R^2=0.68 \), \( F_{1,56}=117 \), \( p<0.001 \)).
Table 2.1: Methods for obtaining MPB $\delta^{13}$C used in various studies, estimates of levels of contamination with bacteria, detritus and meiofauna (0=insignificant, 1=low, 2=medium, 3=high) and expected discrimination against components of the MPB community.

<table>
<thead>
<tr>
<th>General method</th>
<th>Specifics</th>
<th>Contamination</th>
<th>MPB discriminated against</th>
<th>Reference</th>
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<td>Bulk sediment</td>
<td>3</td>
<td>3</td>
<td>None</td>
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<td></td>
<td>MPB colonies/mats</td>
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<td>3</td>
<td>Non-colonial</td>
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<td>Gut contents of mudskipper known to</td>
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<td>Physical separation of MPB from</td>
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<td>1</td>
<td>Non-motile</td>
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<td>Sieve-and-spin</td>
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<td>Use of biomarker</td>
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<td>1</td>
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2.2 Materials and procedures

2.2.1 Algal culture

To maximise chances of identifying species differences, different taxa and various sizes and shapes of MPB were selected, namely: the cyanobacterium, Oscillatoria sp., and the diatoms Nitzschia closterium, Nitzschia frustulum, two naviculoid species (sp.1 and sp.2), Pleurosigma sp., Gyrosigma sp., and Bacillaria paxillifer. All species were isolated from sandy sediment from Moreton Bay, southeast Queensland, with the exception of N. frustulum (CSIRO Collection of Living Microalgae; Strain CS-258/1 http://www.marine.csiro.au/microalgae/collection.html), which was isolated from Beaver Cay, north Queensland, Australia in 1987.

MPB species were grown in flasks containing 300mL of sterile F2 culture medium (Guillard & Ryther 1962) on a light:dark cycle of 12:12 h. As MPB $\delta^{13}C_{\text{bulk}}$ typically varies with growth rate, which increases with temperature (Fielding et al. 1998), diatoms were cultured at a range of temperatures to deliberately extend the applicable range of the relationship. Each species was cultured at average temperatures for each flask of 10, 15, 20, 25, 30 or 35°C (one flask for each species per temperature). To mimic field conditions, temperatures for each of these treatments varied within 2°C, increasing slightly during the light period and decreasing during the dark period. These conditions were essentially stable throughout the culture period and are termed “constant” from hereon. To determine if temperature fluctuations between days would affect the $\delta^{13}C_{\text{phytol}} - \delta^{13}C_{\text{bulk}}$ relationship, the temperatures for another 10 flasks of each of Nitzschia closterium, Gyrosigma sp. and Oscillatoria sp. were varied by as much as 10°C from day to day so that the growth temperature was not constant for the culture period. Cells were harvested when most cultures appeared to have attained sufficient biomass for analysis; after 16 days for Nitzschia frustulum, 24 days for other MPB species grown at constant temperature, and 14 days for MPB grown with variable temperature.

Algae were collected by filtration onto pre-combusted (450°C, 12 h) 25 mm GF/F filters. Degradation of phytol is minimal in frozen samples (Sun et al. 1998), so filters were stored frozen in aluminium foil until processed (up to 7 days). Approximately
25% of the filtered material was retained on filter papers for bulk analysis. The remainder was extracted for phytol.

2.2.2 Extraction of phytol

The sterol fraction, within which phytol is found, was extracted using an adaptation of the method of Bligh & Dyer (1959). Shredded filters were sonicated for 10 min and then centrifuged at 2000 rpm for 3 min (x3) in 30mL, 40mL and 30mL of 3:6:1 dichloromethane:methanol:H₂O. The extracts were combined in a separating funnel. Dichloromethane (DCM; 30mL) and milli-Q H₂O (30mL) were added and the phases allowed to separate. The bottom layer was drawn off into a round-bottom flask and the process was repeated using 15mL of DCM. Total extracts were evaporated to near dryness at 40°C on a rotary evaporator, then transferred to test tubes. The extract was blown to dryness with N₂ and saponified with 5% KOH in methanol:H₂O (80:20; 3mL) to give a pH of 12-14. Samples were flushed with ultrapure N₂ and heated (80°C, 2 h). Following cooling, 3mL of milli-Q H₂O and 3mL of 4:1 hexane:DCM were added and samples were centrifuged at 2000rpm for 2 min. The top solvent layer was transferred to a test tube. This was repeated twice more with 2mL of hexane:DCM. The volume was reduced with N₂ and the extract transferred to a vial. In preparation for gas chromatography, samples were blown to dryness with N₂ and derivatised (60°C, 2 h) with 100µL of pure bis(trimethylsilyl)trifluoroacetamide (BSTFA).

2.2.3 Gas chromatography and gas chromatography-mass spectrometry

Each extract was analysed by gas chromatography to ensure there was sufficient phytol for analysis and that there were no co-eluting compounds which could interfere with analysis. Gas chromatography (GC) was initially performed using a Varian CP 3800, interfaced with Galaxie chromatography software. For analysis of the neutral fraction, the gas chromatograph was equipped with a 50 m x 0.32 mm i.d. cross-linked 5% phenyl-methyl silicone (HP5, Hewlett Packard) fused-silica capillary column; hydrogen was the carrier gas. Samples were injected through a hot injector. The initial oven temperature was 45°C with a 30°C min⁻¹ ramp rate to 140°C and then a 3°C min⁻¹ ramp rate to 310°C which was held for 5 min. Sterol fractions were analysed using a flame ionisation detector, with 5β(H)-cholan-24-ol as the internal
standard. Initial peak identifications were based on retention times relative to authentic and laboratory standards and subsequent GC-MS analysis. Verification of the identity of individual sterols by GC-MS analysis was performed on a Thermoquest / Finnigan GCQ-Plus benchtop mass spectrometer fitted with a direct capillary inlet and an automated on-column injector. Data were acquired in scan acquisition or selective ion monitoring and processed using Xcalibur software supplied with the instrument. The nonpolar column (HP5) and operating conditions were the same as that described above for GC-FID analyses, except that helium was used as the carrier gas.

2.2.4 Stable isotope analysis

2.2.4.1 Bulk analysis
Algal samples on glass-fibre filters were dried at 60°C for several hours, before being punched and packed into tin cups (Elemental Microanalysis Ltd., Okehampton, UK) for analysis. Prior to the cups being closed, a few drops of sulphurous acid were added to remove any carbonates present and the samples re-dried. Samples were then analysed for δ13C using a Carlo Erba NA1500 CNS analyser interfaced via a Conflo II to a Finnigan Mat Delta S isotope ratio mass spectrometer operating in the continuous flow mode. Combustion and oxidation were achieved at 1090°C and reduction at 650°C. Samples were analysed at least in duplicate. Results are presented in standard δ notation:

\[
\delta^{13}C(\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]

where R = \(^{13}\text{C}/^{12}\text{C}\). The standard for carbon is Vienna Pee Dee Belemnite (VPDB). The reproducibility of the stable isotope measurements was ~0.2‰ for C.

2.2.4.2 Compound-specific isotope analysis
Not all species of MPB grew to sufficient biomass for compound-specific isotope analysis of phytol at all temperatures. Only those samples where a sufficiently strong, pure phytol peak was obtained were analysed. Compound-specific isotope ratio mass spectrometry was performed on sterol extracts using a Hewlett Packard 5890 series II gas chromatograph, which was coupled via a Finnigan MAT GC combustion interface.
to the isotope ratio mass spectrometer described above. The gas chromatograph was
equipped with a 60 m J&W DB-1, 0.32 mm i.d. column with He as the carrier gas.
Samples were injected on-column via a cold on-column injector (“Duck Bill”,
Hewlett Packard). The initial oven temperature of 40°C was maintained for 1 min
followed by a 30°C min⁻¹ ramp rate up to 120°C followed by a 4°C min⁻¹ ramp rate to
315°C, which was held for 15 min. Samples were co-injected twice with C₁₆ and C₂₄
deuterated n-alkanes of known isotopic composition, the average of the two injections
are reported here. To obtain the ratio for phytol, the carbon isotope ratios of the
derivatised samples were corrected for the number of carbon atoms that had been
added during derivatisation. Results are presented in standard δ notation as described
above.
2.3 Assessment

2.3.1 Establishing a predictive $\delta^{13}C_{\text{phytol}} - \delta^{13}C_{\text{bulk}}$ relationship

Both phytol and bulk $\delta^{13}C$ values were obtained for a total of 58 samples. For all species pooled, and regardless of whether algae were grown at constant or fluctuating temperature, $\delta^{13}C_{\text{phytol}}$ was linearly correlated with $\delta^{13}C_{\text{bulk}}$ (Fig. 2.1):

$$\delta^{13}C_{\text{bulk}} = 0.638 \times \delta^{13}C_{\text{phytol}} - 6.135$$  
(Equation I)

This regression (Equation I) was not significantly different to that for literature data (ANCOVA: test for heterogeneity of slopes, $F_{1,71} = 3.72$, $p = 0.057$; test for differences in elevations, $F_{1,72} = 0.03$, $p = 0.853$; Fig. 2.1). The similarity of the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ for such diverse producers as MPB, terrestrial plants, marine phytoplankton and marine macroalgae suggests that the observed relationship is widely applicable. There was more variation in the relationship for MPB than for literature data for these other plant groups. This may be because the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ for MPB is inherently more variable or, alternatively, the source of this variation may be fractionation arising from the additional procedures required to extract phytol from the complex sediment matrix, compared to simple plant samples.

2.3.2 Influence of species effect on the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$

Of the MPB species studied, three (Oscillatoria sp., Pleurosigma sp. and Bacillaria paxillifer) had insufficient data for regression analysis ($n < 5$). Regression analyses showed the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ for Nitzschia closterium (Fig. 2.2) and close to significant for Nitzschia frustulum (Fig. 2.2). Regressions for the remaining species were not significant. The relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ did not significantly differ among species (ANCOVA: slopes, $F_{4,4} = 5.80$, $p = 0.058$; elevations, $F_{1,8} = 3.14$, $p = 0.115$; Fig. 2.2).

The similarity of the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ among MPB species indicates that the biosynthetic pathways responsible for partitioning carbon into phytol within cells is similar for all species studied.
2.3.3 Influence of temperature on the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$

For the 37 cultures grown at constant temperature, $\delta^{13}C_{\text{phytol}}$ was generally depleted relative to $\delta^{13}C_{\text{bulk}}$. The offset between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ was smaller at greater culture temperatures (Fig. 2.3). The relationship between offset and culture temperature was similar regardless of whether the measure of temperature used was the average temperature, or the temperature extremes, either during the light or the dark periods. Sachs et al. (1999) reported that the offset between $\delta^{13}C_{\text{bulk}}$ and chl-$\alpha$ $\delta^{13}C$ of marine phytoplankton was influenced by growth rate, with use of more enriched carbon sources by chl-$\alpha$ at faster growth rates. However, growth rate is often positively correlated with temperature (Fielding et al. 1998), which was not considered separately in the study by Sachs et al. (1999). Hinga et al. (1994) found growth rate to have no effect on carbon isotope fractionation during uptake by marine phytoplankton, but fractionation increased with temperature. The relationship observed by Sachs et al. (1999) may therefore be attributable to differences in
temperature rather than growth rate. Although Sachs et al. (1999) studied marine phytoplankton, chl-\(a\) is also found in MPB. Assuming that the enrichment of chl-\(a\) they reported is in fact due to increased temperature, this may account for the change in offset between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) observed in the current study at increased temperatures. As phytol is a side chain of chl-\(a\), enrichment of chl-\(a\) at greater temperatures would also result in phytol becoming less depleted relative to bulk MPB. Nevertheless, the current paper demonstrates that growth conditions can affect cell components independent of the whole cell.

![Graph showing relationship between growth temperature and the offset between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\).](image)

**Figure 2.3: Relationship between growth temperature and the offset between \(\delta^{13}C_{\text{phytol}}\) and \(\delta^{13}C_{\text{bulk}}\) (regression: \(R^2=0.17, F_{1,35}=7.12, p=0.011\)).**

Using both \(\delta^{13}C_{\text{phytol}}\) and temperature as predictors (Equation II), explained more of the variation in \(\delta^{13}C_{\text{bulk}}\) values (80\%; multiple regression: \(R^2 = 0.80, F_{2,34} = 69.81, p < 0.001\)) than when \(\delta^{13}C_{\text{phytol}}\) alone was used (Equation I; 68\%). The multiple regression equation (Equation II) was:

\[
\delta^{13}C_{\text{bulk}} = 0.671 \times \delta^{13}C_{\text{phytol}} + 0.136 \times \text{temperature} - 8.799 \quad \text{(Equation II)}
\]

There was no indication of collinearity between \(\delta^{13}C_{\text{phytol}}\) and temperature.
2.3.4 Predictive ability of the $\delta^{13}C_{\text{phytol}} - \delta^{13}C_{\text{bulk}}$ relationship

Although there is a significant regression between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ (Equation I), and between temperature, $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ (Equation II), the ability to predict $\delta^{13}C_{\text{bulk}}$ using these equations needs to be further assessed to ensure usefulness in food web applications.

2.3.4.1 Difference between observed and predicted $\delta^{13}C_{\text{bulk}}$

To determine the average difference expected between observed and predicted $\delta^{13}C_{\text{bulk}}$ with different numbers of samples, a small (three), average (five) or large (ten) number of replicate $\delta^{13}C_{\text{phytol}}$ values were randomly selected from those measured for the algae cultured in the current study. For each level of replication, this was repeated 100 times to form 100 groups. Within these groups, each $\delta^{13}C_{\text{phytol}}$ value was entered into either Equation I or II to predict $\delta^{13}C_{\text{bulk}}$. The difference between the $\delta^{13}C_{\text{bulk}}$ value predicted and that measured for the sample (observed $\delta^{13}C_{\text{bulk}}$) was calculated. The mean difference for each group was determined and these values were used to calculate the overall mean difference between observed and predicted $\delta^{13}C_{\text{bulk}}$ for the 100 groups for each level of replication (Table 2.2). The difference between observed and predicted $\delta^{13}C_{\text{bulk}}$ expected in 95% of cases was also determined (Table 2.2).

In every scenario, when temperature and $\delta^{13}C_{\text{phytol}}$ are both used as predictors for $\delta^{13}C_{\text{bulk}}$ (Equation II), the difference between observed and predicted $\delta^{13}C_{\text{bulk}}$ is smaller than when $\delta^{13}C_{\text{phytol}}$ is the sole predictor (Equation I). For example, when 5 samples are used, $\delta^{13}C_{\text{bulk}}$ predicted from Equation I will typically lie within 0.8‰ of observed values, and will be within 1.8‰ 95% of the time. Predicted $\delta^{13}C_{\text{bulk}}$ from Equation II will typically be within 0.6‰ of observed values and within 1.3‰ 95% of the time. To resolve food webs where the gap between $\delta^{13}C$ of MPB and a second producer is small, greater precision of MPB signatures will be required and Equation II will therefore be applied.
Table 2.2: Precision of $\delta^{13}$C<sub>bulk</sub> estimates relative to observed $\delta^{13}$C<sub>bulk</sub> values when replication is small (n=3), average (n=5) or large (n=10) using either Equation I or II. The mean difference between observed and predicted $\delta^{13}$C<sub>bulk</sub> and the 95% confidence intervals of predictions are shown for each scenario.

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<th>n</th>
<th>Mean</th>
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<tr>
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<td>Equation I</td>
<td>Equation II</td>
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Resolution of MPB signatures can also be improved by increasing replication. In all cases, the expected mean difference between observed and predicted $\delta^{13}$C<sub>bulk</sub> was greater when replication was decreased.

2.3.4.2 Influence of gap distance

A two source mixing model (Phillips & Gregg 2001) was used to assess the usefulness of each of the two equations in estimating source contribution to consumer nutrition. This model incorporates variation in consumer and producer $\delta^{13}$C, sample size and gap distance (the differences between mean $\delta^{13}$C of sources) and calculates 95% confidence intervals for estimates of source contribution. Where the precision of these estimates is greater, 95% confidence intervals are smaller.

Modelling was simulated assuming three, five or ten replicates, where the variation was calculated for $\delta^{13}$C<sub>bulk</sub> predictions using either Equation I or II and gap distances between MPB and a second producer varied from 2 to 20‰. The sample sizes (ten for both) and standard deviations (1.00 and 1.09, respectively) specified for the consumer and the second producer were typical values for Moreton Bay, from which MPB samples were taken.

For all gap distances greater than 2‰, the smallest (i.e. best) 95% confidence intervals were obtained for a sample size of ten where Equation II was used for
predictions (Fig. 2.4). The greatest benefit gained from the addition of temperature as a predictor for $\delta^{13}C_{bulk}$ was at a sample size of three. When the sample size was ten, there was only a slight improvement in confidence intervals at any gap distance when Equation II was applied in preference to Equation I. The greatest difference between confidence intervals among the methods was when gap distance was 6.0‰. As the average offset between producers is 5.5‰ (calculated from available literature), great benefit is expected from taking extra samples and/or measuring temperature for inclusion in Equation II. However, the method selected will depend on the needs of the researcher, availability of resources, and the gap distances between $\delta^{13}C$ of MPB and other potential producers.

Figure 2.4: Improvement in 95% confidence intervals for predictions of MPB contribution to consumer nutrition with increased sample size and gap size for Equation I and Equation II.
2.4 Discussion

We have demonstrated that MPB $\delta^{13}C_{\text{bulk}}$ can be determined from measurements of $\delta^{13}C_{\text{phytol}}$ in sediments (Equation I) or, with greater confidence, through measurement of both $\delta^{13}C_{\text{phytol}}$ and temperature (Equation II). When ten $\delta^{13}C_{\text{phytol}}$ measurements are made and temperature is included as a further predictor, $\delta^{13}C_{\text{bulk}}$ can be estimated within 1.0‰ of observed values 95% of the time (Table 2.2). Estimation of MPB $\delta^{13}C$ from $\delta^{13}C_{\text{phytol}}$ therefore potentially allows adequate distinction from other producers with a signature within 1.0‰ of the true MPB signature. Regardless of the equation used, the confidence of $\delta^{13}C_{\text{bulk}}$ predictions is greater when replication is increased.

Values of MPB $\delta^{13}C_{\text{bulk}}$ determined using the $\delta^{13}C_{\text{phytol}}$ method are more reliable than those measured using alternative methods, where the influence of contamination on MPB $\delta^{13}C$ is unknown. Use of values from the relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ in trophic studies may thus lead to a re-evaluation of the importance of MPB as a source of nutrition for consumers in estuarine systems.
2.5 Comments and recommendations

The advantage of the $\delta^{13}C_{phytol}$ method for MPB $\delta^{13}C_{bulk}$ determination is the reliability of the MPB $\delta^{13}C_{bulk}$ values obtained. Previous methods have been compromised by contamination with detritus and meiofauna (e.g. Dittel et al. 2000) and/or discrimination against some components of the MPB community (e.g. Cook et al. 2004; Table 2.2). The $\delta^{13}C_{phytol}$ method for MPB $\delta^{13}C_{bulk}$ determination incorporates all components of the MPB community, and use of phytol as a biomarker removes the possibility of contamination by meiofauna. However, although most phytol in detritus degrades before it enters sediments (Rontani 2001), residual phytol has the potential to interfere with predictions, especially where detrital fragments are large (Cuny et al. 1999). Where necessary, sieving sediment through appropriate mesh sizes can be used to remove large detritus particles (e.g. < 1 mm) and possibly minimise the potential for such interference.

The temperatures to which diatoms are subjected, and the composition of MPB communities in the field, vary considerably both spatially and temporally (Currin et al. 1995). In the current study, variation in bulk signatures in the range of -15‰ to -32‰ was achieved by manipulation of temperature. Therefore, variation in temperature has great potential to cause considerable variation in MPB $\delta^{13}C_{bulk}$ spatially and temporally in estuarine environments. For example, at lower temperatures in shaded areas of intertidal zones, or during winter, MPB signatures may be relatively depleted in $^{13}C$. Overall MPB $\delta^{13}C$ may also become more depleted if a community becomes dominated by a species with more depleted $\delta^{13}C$ values. This emphasises the need to estimate MPB signatures on a case-by-case basis using the equations described in the current paper. In applying Equation II to food web studies, it is advised that the measure of temperature used should be that of the superficial sediment layer (i.e. upper 5 mm) at the time of collection. Fluctuations in temperature do not affect the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ (and therefore the predictive ability of Equation I), however the $\delta^{13}C$ of cell components is influenced by the temperature at which they are synthesised (Hinga et al. 1994). It should be noted that if the temperature recorded at the time of sample collection differs markedly to when biomass was created (i.e. when temporal temperature fluctuations are large) this may cause errors in estimations made using Equation II.
Further investigation is required to determine the extent to which this occurs and, if necessary, methods for incorporating this effect into predictions made using Equation II.

The precision of estimates of MPB $\delta^{13}C$ made using the equations described in the current paper is sufficient to allow discrimination of MPB from other potential sources of nutrition in food web studies. The relationship between $\delta^{13}C_{\text{phytol}}$ and $\delta^{13}C_{\text{bulk}}$ is similar for different species of MPB across a range of growth temperatures and for such diverse producers as terrestrial plants and marine macroalgae and phytoplankton (Fig. 2.1) and should therefore have wide applicability in predicting MPB $\delta^{13}C$ in food web studies in estuarine and marine environments. The method may also be useful in predicting $\delta^{13}C_{\text{bulk}}$ of other producers.
Chapter 3. The contribution of detritus to error in MPB $\delta^{13}C$ estimates using compound-specific isotope analysis of phytol in estuarine sediments

3.0 Abstract

Studies assessing trophic interactions in coastal waters using stable isotopes have struggled to accurately determine carbon signatures ($\delta^{13}C$) for microphytobenthos (MPB). Compound-specific isotope analysis of phytol may be useful in estimating MPB $\delta^{13}C$, but this method relies upon phytol in sediments being primarily derived from MPB. Contamination of sediment with phytol from non-MPB sources would result in error in estimates of MPB $\delta^{13}C$. Phytol is found in macroalgae and higher plants, as well as MPB, but, following senescence, is rapidly degraded by exposure to light in the water column, and microbial activity and invertebrate grazing in sediments. Phytol in sediments is therefore expected to primarily be produced in situ by MPB. The aim of the current study was to quantify the contribution of MPB and different size fractions of detritus (< 53 $\mu$m, 53-125 $\mu$m, 125-500 $\mu$m, 500-1000 $\mu$m, 1000-2000 $\mu$m and > 2000 $\mu$m) to the phytol pool of estuarine sediments, and to determine if sieving to remove size fractions of the sediment improves estimates of MPB $\delta^{13}C$. This was done for sandy and muddy sediments, because these have photic zones of different depths which could influence photodegradation of phytol. Total phytol content was greater for mud (1.24 ± S.E. 0.33 $\mu$g.g$^{-1}$) than for sand (0.05 ± 0.02 $\mu$g.g$^{-1}$) and the proportion of phytol derived from detritus was also greater for mud (33.0 ± 4.2%) than for sand (16.8 ± 5.2%). On average, for both mud and sand, most of the phytol from detritus was from the 53-125 $\mu$m fraction (8.9 ± 1.8% and 5.4 ± 1.9%, respectively). Two-source mixing models showed that, when MPB was separated from another producer by 6.5‰, MPB $\delta^{13}C$ estimates would be within 2.1‰ of true values for mud and 1.1‰ for sand. Sieving to remove detritus >1000 $\mu$m improved estimates only for sand (1.0‰). Error was improved for both sediment types by removal of detritus > 500 $\mu$m (1.4‰ error for mud, 0.8‰ for sand) and detritus > 125 $\mu$m (1.3‰, 0.6‰). However, concurrent removal of some MPB phytol may bias the MPB analysed. Even without sieving, detritus will in many situations not be a major source of error for MPB $\delta^{13}C$ estimates. Compound-specific isotope analysis of phytol can therefore be confidently used to estimate MPB $\delta^{13}C$ in both muddy and sandy estuarine sediments.
3.1 Introduction

In Chapter 2, I showed that the carbon signature ($\delta^{13}C$) of phytol, which is a side chain of chlorophyll-$a$, can be used to predict the carbon signature of bulk microphytobenthos (MPB) material in cultures where MPB is the only potential phytol source. However, phytol is also synthesised by macroalgae and higher plants (Hörtensteiner 1999) and sediments may contain not only phytol produced in situ by MPB, but also phytol from non-MPB sources, viz. phytoplankton, macroalgae, seagrass, mangroves, saltmarsh and terrestrial plants. To predict $\delta^{13}C$ of bulk MPB in the field using phytol $\delta^{13}C$ (the phytol method; refer Chapter 2), the phytol extracted from sediments should be predominantly from MPB. Depending on the difference between $\delta^{13}C$ of phytol from MPB and the contaminant phytol, contribution of non-MPB phytol would shift the predicted MPB $\delta^{13}C$ away from true values. Although phytol degrades rapidly in senescent plant cells before they enter the sediment (Rontani 2001), quantification of the contribution of non-MPB phytol to the sediment pool is required to determine its potential impact on estimates of MPB $\delta^{13}C$ made using the phytol method.

The processes by which phytol is degraded are similar for different autotrophs, including algae and higher plants (Hörtensteiner 1999). In senescent plant cells, the close association of phytol with chlorophyll-$a$ makes it susceptible to rapid degradation by photosynthetically-active radiation (PAR) in sunlight (Rontani & Volkman 2003). In living cells, PAR excites chlorophyll but this energy is then dissipated by the thylakoid carotenoids in cell membranes as it enters the reactions of photosynthesis (Rontani 2001). When these membranes are destroyed following cell senescence, however, photoprotective mechanisms cease to function (Rontani 2001). As the energy from PAR can no longer be dissipated effectively, excess singlet and triplet oxygen molecules accumulate and may attack cell components (Cuny et al. 1999). Without the containment of cell membranes, chlorophyll becomes associated with other hydrophobic cell components. In this hydrophobic environment, the lifetime of destructive excited chlorophyll and singlet oxygen is increased, enhancing their photooxidative capacity and leading to greater phytol degradation (Rontani 2001). Rapid degradation of phytol with exposure to PAR has been observed in laboratory experiments with, for example, 99% of phytol in senescent cells of the Chlorophyceae
*Dunaliella tertiolecta* expected to degrade within 1 day, assuming a surface daily irradiance of 60 mol m⁻² (PAR) and a residence time of 1 day in the euphotic zone (Cuny et al. 1999).

Significant photodegradation of phytol occurs throughout the euphotic zone of the water column, where exposure to sunlight is greatest (Cuny et al. 1999). Residence time within the euphotic zone is increased for smaller particles, due to their slow settling velocity (McCave 1975, Cuny et al. 1999) and, in contrast to larger particles, internal shading is minimal (Cuny et al. 1999). This increases the effective exposure of phytol in small particles to PAR, thereby enhancing phytol degradation (Cuny et al. 1999). As particulate organic matter in the world’s oceans is typically smaller than 20 µm with a settling velocity of approximately 1.3 m per day (McCave 1975), the majority of phytol in phytodetritus is expected to degrade before entering the sediment (Rontani 2001). This is especially the case at higher temperatures, where the diffusive velocity of destructive singlet oxygen is increased (Cuny et al. 1999), enhancing its ability to degrade phytol.

Photodegradation ceases when all photosensitisers (e.g. chlorophyll) have been destroyed, or when the particles are no longer exposed to light (Rontani & Volkman 2003). In shallow waters, the entire water column may be photic. In sandy sediments with larger grain sizes, light may also penetrate the upper few centimetres (MacIntyre et al. 1996), allowing photodegradation to continue after organic matter is incorporated into sediment. In muddy sediments, light penetration is limited to the upper few millimetres (MacIntyre et al. 1996). Below this level, and deep in sandy sediment, no photodegradation is possible, but other degradation processes occur. Although some phytol makes it into these layers of the sediment intact, various processes, including both aerobic (Rontani et al. 1999) and anaerobic (Grossi et al. 1998) microbial degradation, and feeding by invertebrates (Sun et al. 1993, Sun et al. 1998, Sun et al. 1999) may ultimately degrade residual phytol.

Due to rapid degradation of phytol in the water column and continued degradation in the sediment, the majority of phytol extracted from sediment is expected to be produced *in situ* by MPB. However, for the purposes of applying compound-specific isotope analysis of phytol to estimate δ¹³C of bulk MPB, the potential contamination
of sedimentary phytol with phytol from other producers requires quantification
(Chapter 2). The contribution of detritus to the sediment phytol pool may differ
between mud and sand because photodegradation of detritus may extend deeper in
sand where light penetrates deeper into the sediment (MacIntyre et al. 1996). Greater
re-suspension of particles in sand (MacIntyre et al. 1996) may also influence the
quantities of detritus and associated phytol within these sediments. For this reason,
for both mud and sand, the aim of this experiment was to assess:

a) the contribution of detritus to the phytol pool in estuarine sediments
b) the error in MPB $\delta^{13}C$ estimates made using the phytol method, resulting from
   any detritus-derived phytol, and
c) if sieving to remove larger size fractions of the sediment could remove detritus
   and therefore reduce this error.
3.2 Methods

3.2.1 Sample collection and separation
Three sites with sandy sediments and three sites with muddy sediments were haphazardly selected kilometres apart in subtropical Moreton Bay, southeast Queensland, Australia (Fig. 3.1). The tidal range for Moreton Bay is approximately 2 m. Muddy sites were in clearings amongst stands of mangroves, predominantly *Avicennia marina*. Sandy sites occur on more exposed shores with more terrestrial vegetation alongside (e.g. *Casuarina equisitifolia*), rather than mangroves. Nevertheless, sandy sites were selected so that, as much as possible, sites representing the two habitat types were interpersed to avoid confounding due to potential spatial patterns in detritus and MPB characteristics. From each muddy site and each sandy site, three replicate samples were collected 10s of metres apart. MPB for stable isotope analysis is typically collected from the upper few millimetres of sediment, as this is where most MPB is concentrated (deJonge & Colijn 1994). This upper layer is also best used to extract phytol for determining MPB $\delta^{13}$C using the phytol method (Chapter 2). In the current experiment, therefore, surface (upper 5 mm) sediment was scraped to collect sufficient sediment (approx 50mL) for extraction of phytol and fatty acids. Sediment was stored frozen before processing, which results in minimal phytol degradation (Sun et al. 1998).

Sediment was sieved to retain size fractions < 53 $\mu$m, 53-125 $\mu$m, 125-500 $\mu$m, 500-1000 $\mu$m, 1000-2000 $\mu$m and > 2000 $\mu$m. To allow determination of the weights of plant material in each size fraction, non-plant material was removed manually under a dissecting microscope for samples > 500 $\mu$m. For samples of smaller size fractions, manual removal of non-plant material, including sediment, was not feasible, so separation was achieved via centrifugation (4000 rpm, 5 min) using density separation in silica gel (LUDOX). Further separation of the remaining material into detrital and MPB fractions was unable to be achieved; densities were not sufficiently distinct to allow separation in silica gel, and for larger size fractions, colonisation of detritus by MPB (Wannigama et al. 1981, Mfilinge et al. 2005) prevented separation. Size fractions therefore consisted of both detritus and MPB. Following homogenisation, a subsample of each detritus/MPB sample and a subsample of total sediment was dried.
(60°C to constant weight) and weighed to allow quantification of phytol and fatty acids per mass of sediment (refer section 3.3.3).

Figure 3.1: Map of southern Moreton Bay indicating sandy (squares) and muddy (triangles) sampling sites.
3.2.2 Extraction of phytol and fatty acids

The neutral sterol fraction, within which phytol is found, was extracted using an adaptation of the method of Bligh & Dyer (1959), as described in Chapter 2. Phytol within each sample could have been derived from either MPB or detritus. Fatty acid biomarkers for MPB (16:1(n-7); Cook et al. 2004) and detritus from terrestrial plants (26:0; Cook et al. 2004) were therefore examined for quantification of the amount of MPB or detritus in each sample which contributed to the measured phytol. Although 16:1(n-7) may not be present in all components of MPB, it is the accepted biomarker for diatoms (Cook et al. 2004), which comprise the vast majority of MPB at the sites studied. The fatty acid 16:1(n-7) was therefore considered to be representative of the MPB of interest.

Due to the degradation of phytol in senescent plant material, it is the most recently deposited detritus within sediments that is most likely to contain significant phytol. This was therefore the fraction of detritus that was of most interest in the current study. The 26:0 fatty acid selected as a marker for terrestrial plant detritus also degrades following senescence (Wannigama et al. 1981) and was therefore considered to be a suitable marker for the recently deposited plant detritus that is most likely to contribute phytol to sediments.

Total fatty acids for the purpose of identifying sources of phytol (MPB or detritus) were separated from the sediment using a modified version of the method of Bligh & Dyer (1959). Following saponification and removal of neutrals (see chapter 2), the remaining aqueous phase was acidified with 500 µL of concentrated HCl to give a pH < 5. For each sample, 3mL of 4:1 hexane:dichloromethane (DCM) was added, and samples were then shaken and centrifuged at 2000 rpm for 2 min. The top solvent layer was transferred to a test tube. This process was repeated twice more with 2mL of 4:1 hexane:DCM. The extract was then methylated (80°C, 2 h) with 3mL of 10:1:1 methanol:HCl:DCM. Once cool, 3mL of milli-Q H₂O was added. Methylated total fatty acids were extracted by adding 3mL of 4:1 hexane:DCM, shaking and centrifuging at 2000 rpm for 2 min. This was repeated twice with 2mL of 4:1 hexane:DCM. The volume was reduced with N₂ and the total fatty acid extract was transferred to a vial. In preparation for gas chromatography, samples of neutral sterols for phytol analysis were blown to dryness with N₂ and derivatised (60°C, 2 h).
with 100 µL of pure bis(trimethylsilyl)trifluoroacetamide (BSTFA). Total fatty acids were converted to their methyl esters by treatment with acidified methanol.

3.2.3 Gas chromatography
Gas chromatography for sterol and total fatty acid fractions was done using a Varian CP 3800 interfaced with Galaxie chromatography software, as described in Chapter 2. The internal standard for the sterol fraction was 5β(H)-cholan-24-ol (Chiron AS, Norway) and the injection standard for fatty acids was the C23 fatty acid methyl ester. Peak identifications were based on retention times relative to authentic and laboratory standards and subsequent GC-MS analysis, as outlined in Chapter 2. Peak areas were determined from the chromatograms using the computer program Galaxie and were then related to measurements of the mass of the total sediment and each size fraction to determine the mass of each compound per mass of dry sediment (µg.g⁻¹) and how this was distributed among size fractions of detritus/MPB.

3.2.4 Phytol content of MPB and detritus
Because detritus and MPB were unable to be separated and were therefore both present in all samples, I needed to be able to attribute the measured phytol to either of these components. To do this, I determined which sample had the least MPB relative to detritus based on the smallest ratio of 16:1(n-7) to 26:0. Assuming that this sample contained 100% detritus, I used it to determine the mass of phytol contained in detritus per mass of 26:0. This ratio was then used to calculate how much phytol in all other samples was from detritus, based on the 26:0 content of each sample. The remaining phytol was assumed to be from MPB. Although this method is unlikely to be 100% accurate, it is impossible to get an entirely clean sample of detritus. The described method was therefore selected as being least likely to give false conclusions as it was believed that, if anything, the contribution of detritus to sediments would be overestimated using this method.

3.2.5 Impacts of detritus phytol on MPB δ¹³C estimations using the phytol method
The total contribution of detritus to phytol in mud and sand was compared using t-tests, and the relative proportion of phytol derived from each size fraction was also compared using a series of t-tests comparing mud and sand. For mud and sand
separately, the total contribution of MPB and detritus to phytol in sediments was calculated. Two-source mixing models can be used to determine the proportional contributions of two sources to a mixture (e.g. the contributions of two food sources to the diet of a consumer; Phillips & Gregg 2001). Equal contribution by two sources would produce a mixture with a δ¹³C value midway between the δ¹³C values of the two sources, whereas greater contribution by one source would produce a mixture with δ¹³C more similar to that source. Similarly, a two-source mixing model was used to determine the change in MPB δ¹³C predictions made using the phytol method resulting from the presence of detritus-derived phytol in sediments.

The change in MPB δ¹³C predictions (error) was determined for the average difference between δ¹³C of MPB and another producer, based on literature values (6.5‰; Connolly et al. 2004). This very nearly coincided, incidentally, with the difference between MPB and mangrove δ¹³C in the present study (6.7‰). Two-source mixing models were then also used to simulate the effects of sieving to remove large size fractions of sediment, as suggested in Chapter 2, to determine if MPB δ¹³C estimations could be improved by reducing the contribution of detritus-derived phytol.
3.3 Results

3.3.1 Phytol content of MPB and detritus
The detritus/MPB sample with the lowest content of MPB relative to detritus was a > 2000 µm sample from sandy sediments with a 16:1(n-7) to 26:0 ratio of 0.13. Each 1 µg of 26:0 equated to 2.9 µg of phytol.

The total phytol content of mud (1.24 ± S.E. 0.33 µg g⁻¹ dry sediment) was significantly greater than for sand (0.05 ± 0.02 µg g⁻¹ dry sediment; t-test: t₁₆, 0.05 = 2.12, p = 0.002). The proportion of the total phytol pool contributed by detritus was also greater for mud (33.0 ± 4.2%) than for sand (16.8 ± 5.2%; t-test: t₁₆, 0.05 = 2.12, p = 0.03; Fig. 3.2). The majority of phytol, however, was derived from MPB for both sediment types.

Phytol from detritus was distributed similarly among size fractions within each sediment type, with the 53-125 µm fraction contributing the greatest proportion of detritus-derived phytol in both mud and sand (8.9 ± 1.8% and 5.4 ± 2.0%, respectively; Table 3.1). Detritus-derived phytol was distributed relatively evenly across the remaining size fractions for both sediment types, although the > 2000 µm size fraction from muddy sediments also contributed a considerable proportion of phytol to sediments (6.8 ± 3.0%).
Figure 3.2: Relative proportions (mean ± S.E.) for mud and sand of sediment phytol contributed by detritus and MPB in different size fractions, and total overall contribution from MPB and detritus.
Table 3.1: Relative % contribution (mean ± S.E.) of detritus and MPB in different size fractions of muddy and sandy sediments to the total phytol pool in sediments.

<table>
<thead>
<tr>
<th>Fraction (µm)</th>
<th>Mud Detritus</th>
<th>Mud MPB</th>
<th>Sand Detritus</th>
<th>Sand MPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 2000</td>
<td>6.8 ± 3.0</td>
<td>15.2 ± 4.0</td>
<td>2.4 ± 0.8</td>
<td>6.1 ± 3.0</td>
</tr>
<tr>
<td>1000-2000</td>
<td>3.7 ± 1.2</td>
<td>1.4 ± 1.1</td>
<td>1.4 ± 0.4</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>500-1000</td>
<td>4.3 ± 0.6</td>
<td>5.2 ± 2.3</td>
<td>2.1 ± 0.8</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>125-500</td>
<td>3.4 ± 1.0</td>
<td>7.7 ± 1.1</td>
<td>3.0 ± 1.0</td>
<td>4.2 ± 2.7</td>
</tr>
<tr>
<td>53-125</td>
<td>8.9 ± 1.8</td>
<td>19.4 ± 5.6</td>
<td>5.4 ± 2.0</td>
<td>20.0 ± 7.2</td>
</tr>
<tr>
<td>&lt; 53</td>
<td>5.7 ± 3.4</td>
<td>18.1 ± 5.5</td>
<td>2.7 ± 0.6</td>
<td>58.5 ± 11.2</td>
</tr>
</tbody>
</table>

3.3.2 Impact of detritus phytol on MPB δ¹³C estimations using the phytol method

Since the proportion of phytol in sandy sediments derived from detritus was relatively small, detritus contamination of the phytol pool had a minor impact on theoretical estimates of MPB δ¹³C determined using the phytol method in sand. Although there was a greater contribution of detritus-derived phytol in muddy sediments, error estimates of MPB δ¹³C for mud were also not excessive. For the average difference between δ¹³C of two producers (6.5‰), detritus-derived phytol contamination would result in MPB δ¹³C estimates made using the phytol method being within 2.1‰ of true values for mud and within 1.1‰ for sand (Table 3.2). For mud, removing the > 1000 µm fraction from sediment made no difference to estimates. Removal of detritus > 2000 µm, however, caused greater error in MPB δ¹³C estimates (estimates within 2.3‰ of true values). This reflects the removal of, not only detritus, but also a significant proportion of the MPB-derived phytol associated with it (15.2 ± 4.0% of phytol within muddy sediments was derived from MPB in this size fraction; Table 3.1). The error associated with MPB δ¹³C estimates would be improved, however, with removal of the > 2000 µm size fraction for sand (1.0‰) or, for mud and sand, removal of the > 500 µm (1.4‰ and 0.8‰, respectively) or > 125 µm size fraction (1.3‰ and 0.6‰, respectively; Table 3.2). In these cases, improved estimates result from a smaller proportion of the remaining phytol being derived from detritus. Removal of the fraction > 125 µm, for example, resulted in 20% of the remaining
phytol for mud and 10% for sand being derived from detritus, with the remainder derived from MPB.

Table 3.2: The amount of phytol within the sediment pool estimated to be contributed by detritus when different size fractions (and therefore phytol from both detritus and MPB) are removed. The error in estimations of MPB δ¹³C, determined using a two-source mixing model, is shown for a difference between MPB and another source of 6.5‰.

<table>
<thead>
<tr>
<th>Sediment type</th>
<th>Fraction</th>
<th>Phytol contributed by detritus (%)</th>
<th>MPB δ¹³C estimation error (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mud</td>
<td>Total</td>
<td>33</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>&lt; 2000 µm</td>
<td>35</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>&lt; 1000 µm</td>
<td>32</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>&lt; 500 µm</td>
<td>22</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>&lt; 125 µm</td>
<td>20</td>
<td>1.3</td>
</tr>
<tr>
<td>Sand</td>
<td>Total</td>
<td>17</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>&lt; 2000 µm</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 1000 µm</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 500 µm</td>
<td>12</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>&lt; 125 µm</td>
<td>10</td>
<td>0.6</td>
</tr>
</tbody>
</table>
3.4 Discussion
The current study confirms that the majority (~70%) of phytol found within muddy and sandy sediments is derived from MPB. Rapid photodegradation of phytol in senescent plant material in the water column (Cuny et al. 1999) and further degradation within sediments (Rontani & Volkman 2003) limit the input of detritus-derived phytol to mud and sand. Some phytol from detritus does, however, enter both muddy and sandy sediments intact, possibly compromising estimates of MPB δ¹³C made using the phytol method.

Accurate estimation of MPB δ¹³C values using compound-specific isotope analysis of phytol requires that all, or nearly all, phytol within sediments is from MPB. In the current study, only a small proportion of the phytol pool within the sediment was derived from detritus, and the error in MPB δ¹³C estimates made using the phytol method was therefore minor, with estimated MPB δ¹³C values within 2.1‰ of true values for mud, and within 1.1‰ of true values for sandy sediments. Given that MPB δ¹³C values estimated to date have typically been compromised by contamination with meiofauna and detritus and/or exclusion of part of the MPB assemblage (refer Table 2.1, Chapter 2), it is difficult to establish how the error in estimates made using the phytol method may affect the ability to isotopically distinguish between MPB and another producer. However, where the expected difference in δ¹³C between MPB and another source is relatively large (e.g. 6.7‰ between MPB and mangroves for the site used in the current study), and variation is small, the expected error for MPB δ¹³C estimates of up to 2.1‰ is unlikely to impact on the ability to separate two producers for food web analysis. In a study by Bouillon et al. (2002a), the difference between δ¹³C of MPB (-17.3 ± 0.8‰) and mangroves (-28.6 ± 0.3‰) was 11.3‰. A similar difference between MPB (-14.4 ± 0.3‰) and surrounding macrophytes (-26.0 ± 0.2‰) was also reported by Creach et al. (1997). In circumstances such as these, particularly given the small variation in δ¹³C for both producers, the two sources would be easily separated despite error arising from contamination with detritus-derived phytol. However, this error would have resulted in an inability to distinguish between δ¹³C of MPB (-14.8‰) and Spartina spp. (-12.6‰) in a study by Stribling & Cornwell (1997). In such cases, sieving to remove larger fractions, thereby improving MPB δ¹³C estimates to within 1.3‰ for muddy sediments and 0.6‰ sandy sediments,
may be required. However, this should be applied with caution, as the introduction of new sources of error through sieving may counteract the perceived improvement.

Although sieving removes larger fragments of detritus, thereby reducing the contribution of detritus-derived phytol to the sample, MPB phytol may also be removed. Close association between detritus and colonising MPB (Wannigama et al. 1981) would result in some MPB also being removed by sieving. Taxa of MPB which are large or colonial may be retained on sieves and would therefore also be excluded from the phytol sample for MPB $\delta^{13}$C estimation, particularly for smaller sieve sizes. Because the size of MPB (Popp et al. 1998) and their ability to form colonies or to colonise detritus (Wannigama et al. 1981) may be taxon-specific, sieving would preferentially remove some components of the MPB assemblage. Where $\delta^{13}$C values of MPB are taxon-specific, exclusion of components of the assemblage which are relatively enriched or depleted in $^{13}$C would introduce error to MPB $\delta^{13}$C estimates made for the entire MPB community.

Taxon-specific variation in $\delta^{13}$C values for MPB may result from a number of factors. Carbon in MPB is obtained by transport of CO$_2$ or HCO$_3^-$ into cells (Burkhardt et al. 1999). Ordinarily, MPB discriminate against the heavy $^{13}$C isotope, which diffuses more slowly than the lighter $^{12}$C. However, variations in discrimination during carbon uptake, transport and assimilation influence $\delta^{13}$C of MPB cells, with greater discrimination against $^{13}$C giving more depleted MPB $\delta^{13}$C, and less discrimination giving more enriched $\delta^{13}$C values. Such variations in discrimination may be due to carbon limitation, in which case fixation of almost all carbon results in more $^{13}$C being assimilated, or may be due to taxon-specific characteristics including cell size and shape (Popp et al. 1998), growth rate (Laws et al. 1995), cell membrane permeability (Laws et al. 1995), and species-specific carbon assimilation mechanisms (Burkhardt et al. 1999, Vuorio et al. 2006).

Under a given set of growth conditions, taxon-specific growth rates may result in variation in $\delta^{13}$C values among components of the MPB assemblage. Smaller taxa tend to grow more rapidly (Admiraal 1977, Korb et al. 1996) and, more generally, the conditions (e.g. light regime and temperature) which result in optimum growth may
vary among taxa (Admiraal 1977). At faster growth rates, demand for carbon is increased, and almost all available carbon is used, resulting in a greater proportion of $^{13}$C being assimilated than at slower growth rates (Burkhardt et al. 1999). This would be reflected in greater enrichment of faster growing cells and species (Laws et al. 1995). For larger cells, depending on shape (Popp et al. 1998), greater carbon content within cells can also increase carbon demand, but supply is limited by the smaller relative surface area and thicker boundary layer through which carbon must pass (Laws et al. 1995). Discrimination against $^{13}$C would be limited, leading to more enriched $\delta^{13}$C values (Laws et al. 1995, Popp et al. 1998, Burkhardt et al. 1999).

Taxon-specific differences in cell membrane permeability and biosynthetic pathways (Laws et al. 1995) would also affect carbon transport into the cell and its assimilation into cell compounds.

Due to the potential for taxon-specific $\delta^{13}$C values, it is important to include all components of the MPB for analysis when estimating MPB $\delta^{13}$C values using the phytol method. Exclusion of a particular component of the MPB, which may be enriched or depleted compared to the remainder of the assemblage, would increase the discrepancy between true and estimated MPB $\delta^{13}$C values, giving predictions of $\delta^{13}$C which are not representative of the entire MPB community. For example, Cook et al. (2004) found that their method for obtaining an MPB sample for $\delta^{13}$C analysis (migration of MPB into lens tissue) resulted in a sample where relatively $^{13}$C-enriched cyanobacteria (Oscillatoria sp.) were over-represented. This would result in a $\delta^{13}$C value for MPB which is more enriched than the true $\delta^{13}$C of the MPB community within the sediment. The potential bias in MPB $\delta^{13}$C estimates caused by incidental removal of certain MPB taxa by sieving requires that the advantages of sieving (i.e. less contamination by detritus-derived phytol) must be weighed against the disadvantages (i.e. biasing the MPB community measured). Given the large range in $\delta^{13}$C values among MPB taxa grown under similar conditions (demonstrated in Chapter 2), in most cases it will be preferable to adjust for the expected error in MPB $\delta^{13}$C estimates, rather than risk biasing against some components of the MPB assemblage by sieving to remove detritus-derived phytol. Sieving to improve MPB $\delta^{13}$C estimates is best reserved for situations where it is expected that sieving will not selectively remove components of the MPB assemblage (although this is probably
rare) or that the contribution of detritus-derived phytol to the sediment will be unusually large.

The contribution of detritus-derived phytol to sediments may be increased: a) where sediment has greater input of detritus containing phytol, and/or b) where the detritus entering sediment contains unusually high quantities of phytol. High detrital input is expected where supply is greater (e.g. within a mangrove forest or seagrass meadow; Holmer et al. 2004), but settlement of detritus can also be influenced by wind and hydrodynamic movement. Sediments protected from such movement, typically muddier environments, tend to have greater detrital input, reflected in a higher organic matter content (Cartaxana et al. 2006, also refer Chapter 4). Greater re-suspension and removal of detritus on more exposed, sandy shores (MacIntyre et al. 1996), limits the input of detritus to sediments. This may be the reason behind there being a greater contribution of detritus-derived phytol relative to MPB-derived phytol observed in muddy sediments than in sandy.

Regardless of the quantity of detritus entering the sediment, degradation of phytol should prevent significant contamination of the sediment phytol pool. However, much of this degradation is due to photodegradation through exposure of senescent cells to sunlight (Rontani 2001). Where photodegradation is prevented or suppressed, the contribution of detritus-derived phytol to sediments may be increased. In highly turbid waters, the photic zone of the water column is very shallow and may prevent exposure of detritus to sunlight. This may be counteracted, however, by the high energy typical of turbid waters. Hydrodynamic movement may extend the time for which detritus is suspended in the water column thereby increasing the susceptibility of phytol to degradation within this photic zone. Where detritus is not exposed to light, degradation of phytol is significantly decreased (Cuny et al. 1999). However, because of the small size of most detritus particles (< 20 μm; McCave 1975), their residence time in the photic zone should be sufficient for phytol to be substantially photodegraded. For larger particles of detritus, slower settling velocity and increased internal shading may result in more phytol entering sediment intact (Cuny et al. 1999). This would be of even greater concern at higher latitudes, where cooler water would further slow the reactions that lead to photodegradation (Cuny et al. 1999). Phytol is expected to have largely degraded before entering sediments even where these
conditions exist, however, and would be further degraded within the sediment (Rontani & Volkman 2003). It is therefore more likely that only a combination of the aforementioned conditions would result in a significant increase in the contribution of detritus-derived phytol to sediments, so sieving to improve MPB $\delta^{13}C$ estimates should not be necessary in the majority of cases.

The error in MPB $\delta^{13}C$ values predicted to result from contamination of the sediment phytol pool by detritus in the current study is relatively minor, even for samples of bulk sediment. Nevertheless, it is likely that the anticipated error is overestimated, and MPB $\delta^{13}C$ estimates made using the phytol method are, in reality, more precise than indicated. The contribution of phytol from MPB and detritus in different size fractions of muddy and sandy sediment was determined using the detritus sample with the smallest algal content. However, MPB is known to be capable of colonising mangrove detritus (Wannigama et al. 1981, Mfilinge et al. 2005), and was observed on and within larger detrital fragments in the current study (pers. obs.). The presence of a small amount of the 16:1(n-7) algal biomarker in the cleanest detritus sample further indicates the presence of MPB, probably as cells unable to be removed through sieving due to their close association with detritus. A small and unknown fraction of the phytol attributed to detritus on the basis of this sample being ‘clean’ can therefore be attributed to MPB, in which case the phytol content of detritus, calculated from the 26:0 to phytol in the clean sample, would be overestimated.

The ratio of 26:0 to phytol reported by Wannigama et al. (1981) for dead leaves of *Avicennia marina*, the dominant macrophyte at the muddy sites in the current study, was similar to that in the current study (3.7 µg (current study) compared to 2.9 µg of phytol per 1µg of 26:0). However, Wannigama et al. (1981) also found fatty acid evidence that MPB had colonised the dead leaves, thereby increasing their apparent phytol content, as in the current study. Wannigama et al. (1981) did not describe the age or condition of the leaves studied. If the leaves studied by Wannigama et al. (1981) were whole, a lower phytol content would be expected for the detritus in the current study due to the smaller size and, particularly if it is assumed that these smaller fragments isolated from sediment are typically older. Detritus of greater age would have had more time for phytol to degrade, and the smaller size of fragments
would have made phytol more susceptible to degradation, in part due to decreased internal shading (Cuny et al. 1999). It is therefore likely that detritus in the current study would contain less phytol per µg 26:0 than the dead leaves studied by Wannigama et al. (1981). My estimates of phytol content were based on larger detritus fragments (> 2000 µm), and the greater degradation potential for smaller detritus sizes was not incorporated into estimates of detritus phytol content. Therefore, there is even greater potential for phytol content, particularly in smaller size fractions, to have been overestimated, and the relative contribution of MPB to the phytol pool in sediments to have been underestimated.

In conclusion, the contribution of detritus to the phytol pool in sediment is inconsequential for both muddy and sandy sediments. The resulting error in estimates of MPB δ¹³C made using the phytol method is minimal. Furthermore, my calculations of detrital contributions to the phytol pool are, if anything, overestimates. Removal of larger size fractions can improve the accuracy of estimates, but this is probably unnecessary for most food web applications. For the food web studies in southern Moreton Bay described in the following chapters, compound-specific isotope analysis of phytol can be used confidently in both muddy and sandy sediments.
Chapter 4. Relative uptake of $^{13}$C-enriched sodium bicarbonate by microphytobenthos and non-photosynthetic bacteria: does direct bacterial uptake lead to false conclusions in $^{13}$C-enrichment experiments?

4.0 Abstract

Microphytobenthos (MPB) is an inconspicuous, but highly productive, potential source of food for animals in estuarine sediments. Recent studies to establish the nutritional importance of MPB have applied $^{13}$C-enriched sodium bicarbonate to surface sediments to label MPB with $^{13}$C. Subsequent labelling of consumers is assumed to indicate use of MPB as a primary nutrition source. In such studies, bacteria also acquire the $^{13}$C label, but it is uncertain if this uptake is direct or indirect. Indirect uptake by bacteria may occur through use of extracellular polymeric substances (EPS) exuded by MPB, in which case bacteria are simply another trophic link. However, if the uptake is direct, for example by chemoautotrophic bacteria, labelling of consumers may indicate the use of bacteria and the role of MPB may be overestimated. The current study investigated the potential for bacteria to directly assimilate carbon from sodium bicarbonate applied to sandy and muddy sediments. Uptake by MPB, indicated by enriched phytol $\delta^{13}$C values, occurred when labelled sediments were exposed to light, but not when sediments were kept in the dark. In all cases, enrichment of phytol was lower and less variable in muddy sediments (mean 34.4‰, coefficient of variation (CV; calculated using atom %) = 6.5%) than in sand (1087.4‰, CV = 24.0%). Variation in labelling indicated differences in photosynthetic rates of, and therefore photoassimilation of carbon by, MPB. This may be due to sediment and site characteristics, such as shading and grain size, affecting light availability and temperature, and thereby $^{13}$C uptake. Bacterial biomarkers (i+a15:0) were enriched only in sandy sediments, where MPB enrichment was greatest. Greater enrichment of bacteria in labelled sediments exposed to light (mean 75.9, S.E. ± 15.6‰) shows that bacteria rely upon MPB and/or EPS as a carbon source. Slight enrichment of bacteria in labelled dark plots (-17.3 ± 1.6‰ compared to -20.6 ± 0.8‰ for unlabelled dark plots), where MPB remained unlabelled, shows that bacteria can directly assimilate carbon from sodium bicarbonate. However, direct uptake by bacteria is trivial relative to that by MPB. Therefore, at usual levels of enrichment, any labelling of consumers can confidently be attributed to the use of MPB as a primary source of nutrition either directly or indirectly via trophic links which may include bacteria.
4.1 Introduction

Much of the carbon content of estuarine sediments can be attributed to highly productive microphytobenthos (MacIntyre et al. 1996) and non-photosynthetic bacteria (Findlay et al. 1992). Whereas MPB is responsible for primary production, non-photosynthetic bacteria are consumers, and therefore a source of secondary production (e.g. Boschker et al. 2005). Consequently, studies to establish the primary carbon source for estuarine consumers have focussed on the importance of MPB, more recently by labelling MPB with the rare, heavy carbon isotope, $^{13}$C, and then tracing the transfer of this label into consumers (e.g. Middelburg et al. 2000). In these studies, dissolved $^{13}$C-enriched sodium bicarbonate (NaH$^{13}$CO$_3$) is sprayed onto the sediment surface, where MPB is concentrated during the day at low tide (MacIntyre & Cullen 1995, Easley et al. 2005) and the $^{13}$C label is assimilated by MPB during photosynthesis. Approximately 42-73% of this carbon is then excreted by MPB as $^{13}$C-enriched extracellular polymeric substances (EPS) in mucous (Goto et al. 1999). Consumers take on the label by directly consuming enriched MPB or EPS (Decho & Moriarty 1990, Decho & Lopez 1993, Kawamura et al. 1998), or by ingesting other consumers that have done so.

Several studies have demonstrated that bacteria utilise carbon derived from MPB (e.g. Boschker et al. 1999, Bouillon et al. 2004a, Cook et al. 2004), and that EPS is a major contributor to the carbon supporting bacteria in sediments (Goto et al. 2001). Bacteria themselves are a potential source of carbon for higher consumers (van Oevelen et al. 2006a). In intertidal sediments, bacteria have been shown to contribute, on average, 8% of the carbon required by meiobenthos and 11% of the carbon for macrobenthos (van Oevelen et al. 2006b). Kharlamenko et al. (2001) used fatty acid analysis as evidence of bacterial assimilation by a bivalve (Ruditapes philippinarum) and gastropod (Homalopoma sangarense), and Epstein and Shiaris (1992) used fluorescence-labelled bacteria to show that the majority of benthic organisms (e.g. nematodes, ciliates, harpacticoid copepods) in muddy sediment derive nutrition from bacteria. Bacterial carbon may be transferred to micro-, meio- and macrofauna via trophic transfer, or bacteria may be directly consumed along with the particulate organic matter with which they are often associated (Hall & Meyer 1998). Although bacteria are very small, high turnover rates and productivity suggest that they may play an important role in carbon transfer within benthic food webs. If use of MPB-
derived carbon by bacteria is significant, then bacteria may provide an important link between MPB and higher consumers.

Although most enrichment studies to date have looked at the importance of MPB as a source of nutrition for consumers in muddy sediments, there is increasing interest in the trophodynamics of sandy sediments (e.g. Middelburg et al. 2000). To trace movement of MPB-derived carbon into consumers, Middelburg et al. (2000) enriched MPB in both muddy and sandy sediments. However, not only MPB but also non-photosynthetic bacterial polar-lipid derived fatty acids (PLFAs: i14:0, a15:0, i15:0, i16:0, 18:1(n-1)7), also rapidly became $^{13}$C-enriched. The enrichment of bacteria, which was evident within four hours of application of $^{13}$C-sodium bicarbonate, was proposed to be due to consumption by bacteria of enriched EPS from MPB.

It is typically assumed that direct uptake of $^{13}$C-sodium bicarbonate is only by MPB, and that any labelling of consumers therefore indicates use of MPB as the primary nutrition source. Where bacterial enrichment is indirect (i.e. through assimilation of EPS secreted by MPB) bacteria are simply another link in the trophic transfer of carbon. Any subsequent labelling of consumers then represents utilisation of MPB as a primary source of carbon. However, bacteria and MPB are able to utilise the same carbon sources in some instances. For example, Veuger et al. (2006) reported that PLFAs of both bacteria and, to a lesser extent, algae became enriched following addition of $^{13}$C-enriched glucose. Similarly, although MPB may dominate initial uptake, some bacteria, particularly chemoautotrophs, may be capable of directly assimilating $^{13}$C from sodium bicarbonate (Engel et al. 2001, Boschker & Middelburg 2002, Wuchter et al. 2003). If direct bacterial uptake is substantial, labelling of consumers may, in fact, be indicative of use of bacteria as a primary source of nutrition. In this case, interpretation of consumer enrichment as evidence of use of MPB-derived carbon would be flawed.

As evidence to support their claim that enrichment of bacteria resulted from assimilation of MPB-derived carbon, and not direct assimilation of $^{13}$C from sodium bicarbonate, Middelburg et al. (2000) stated that there was no uptake of $^{13}$C-enriched sodium bicarbonate by bacteria in sandy sediments in the dark (i.e. when MPB are unable to photoassimilate the label and exude it as EPS), implying that bacteria do not
directly assimilate the $^{13}$C label from sodium bicarbonate. However, no details were provided as to how this was established so it is difficult to ascertain if this is likely to be the case at all levels of enrichment and in other habitat and sediment types.

In the sandy sediments studied by Middelburg et al. (2000) it is likely that the interface between the anoxic and oxic zones of the sediment, where chemoautotrophic bacteria are typically found (Howarth 1984), would be deep within the sediment. However, in muddy sediments, this interface may be within a few millimetres of the surface (MacIntyre et al. 1996), increasing the potential for substantial direct uptake of $^{13}$C label by bacteria.

There appear to be no published records of replicated, controlled studies assessing whether direct uptake of $^{13}$C-sodium bicarbonate by bacteria is significant relative to that by MPB, and no studies comparing uptake for sandy and muddy sediments. The aim of the current study was therefore to compare uptake of $^{13}$C from sodium bicarbonate by MPB and bacteria:

a) where MPB was able to directly photoassimilate $^{13}$C (sediment was exposed to light) and release EPS for consumption by bacteria,

b) where MPB was unable to assimilate $^{13}$C (sediment was in the dark), enriched EPS was unavailable, and the only possible bacterial uptake was direct, and

c) as a comparison between muddy and sandy sediments.
4.2 Methods

The sites used for the current study were those described in Chapter 3 (Fig. 3.1). Spatial interpersion of sites representing each habitat type prevented confounding due to patterns in water quality and MPB characteristics. Three samples of surface sediment (upper 5 mm) per site were used to characterise organic matter content (loss on ignition, 500°C for 3 hours) and grain size distribution of sediment at each site.

Within each site, three replicates of the experiment were set up in the intertidal zone, approximately 30 cm above mean low tide. Each replicate consisted of four circular plots (40 cm diameter), one for each of four treatments: 1) dark spiked (DS), 2) dark unspiked (DU), 3) light spiked (LS), and 4) light unspiked (LU). Within each replicate, these treatments were clustered in a block design to counteract potential spatial variability within sites so that it would not mask small variations in $\delta^{13}C$ due to treatment.

For the dark treatments (DS and DU), photoassimilation of the $^{13}C$ label by MPB was prevented by placing black plastic covers over plots for one hour prior to labelling and throughout the experiment. A portable dark room allowed access to dark plots for labelling and sample collection. Temperatures were similar (within 1°C) in covered and uncovered plots during the experiment, an important consideration given that temperature differences might have affected carbon assimilation by algae and bacteria.

Labelled plots (DS and LS) were enriched with $^{13}C$-enriched sodium bicarbonate dissolved in filtered seawater, sprayed onto the sediment surface (0.8 g NaH$^{13}$CO$_3$ m$^{-2}$). Unspiked plots did not have label added and acted as a control for spiking. Although bacterial uptake in LS plots could be indirect via bacterial consumption of MPB exudates (EPS) as proposed by Middelburg et al. (2000), bacterial uptake in DS plots could only occur via direct uptake, given that MPB are unable to photoassimilate $^{13}C$ in the dark.

Four hours after spiking, the time after which Middelburg et al. (2000) observed label uptake by MPB and bacteria, the upper 5 mm of sediment within 15 cm of the centre of each plot was collected. Samples collected from dark plots were stored within dark
bags to prevent exposure to light. All samples were immediately placed into ice to slow metabolism of MPB and bacteria, ensuring that carbon uptake following collection would be negligible compared to that before collection. Samples were stored frozen prior to processing.

Uptake of $^{13}$C by MPB and bacteria was assessed through compound-specific isotope analysis of biomarkers extracted from sediment using the modified method of Bligh & Dyer (1959) described in Chapter 2. Although phytol is present in all green plants, it was selected as the biomarker for MPB because rapid degradation (as discussed in Chapter 3) means that phytol within sediments should primarily be derived from MPB. *Iso-* and *anteiso-* branched 15:0 polar-lipid derived fatty acids are well established as being of bacterial origin (e.g. Boschker et al. 1999), and were chromatographically distinct in the samples collected, so these fatty acids, in combination (i+a15:0), were selected as the biomarker for bacteria.

For each of phytol $\delta^{13}$C and bacterial $\delta^{13}$C, data were log$_{10}$x transformed to make variances more homogeneous, and means were compared using a split-plot ANOVA (4 factors: habitat, site within habitat, treatment, block). Where significant differences were detected, pairwise comparisons among treatment means were done using Tukey tests. Coefficients of variation, calculated as SD/mean x 100, provide a measure of variation standardised by the mean (Quinn & Keough 2002) and were used to describe the variability in phytol $\delta^{13}$C. The delta notation typically used for reporting stable isotope values is a ratio of enrichment of samples relative to a standard. Atom % values which are an absolute measure of the % of carbon atoms within a sample that are present as the $^{13}$C isotope, were therefore used to calculate these coefficients of variation.
4.3 Results

Sediment characteristics differed between muddy and sandy habitats (Table 4.1). Organic matter content was significantly greater and more variable in muddy than sandy sites (t-test: t₁₆ = 3.1, p = 0.006) and sediment grain size distributions were generally more variable for mud than for sand. Grain size was smaller for mud (74.9% < 250 μm) than for sand (65.8% 250 μm - 500 μm).

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<tr>
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<th>Mud</th>
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<tr>
<td></td>
<td>Mean (%)</td>
<td>CV (%)</td>
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<tr>
<td>Organic matter</td>
<td>16.7</td>
<td>92.5</td>
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<td>Sediment size fraction:</td>
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<td>&gt; 1000 μm</td>
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<td>500–1000 μm</td>
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<td>250–500 μm</td>
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<td>98.7</td>
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<td>&lt; 63 μm</td>
<td>26.8</td>
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Pairwise comparisons showed that phytol δ¹³C in LS plots was clearly enriched relative to all other treatments in both habitat types (p < 0.001 for all comparisons; Fig. 4.1). Phytol δ¹³C values in LS plots appeared more enriched and more variable for sandy sediments (mean 1087.4‰, coefficient of variation, CV = 24.0%) than for muddy sediments (mean 34.4‰, CV = 6.5%; Fig. 4.1), however for both phytol δ¹³C values (split-plot ANOVA: F₃,₁₅₈ = 29.5, p < 0.001) and bacterial biomarker (i+a₁₅:₀) δ¹³C values (F₁,₁₂₂ = 80.2, p < 0.001; Fig. 4.2) there was a significant interaction between habitat and treatment. Sandy and muddy habitats were therefore considered separately in further analysis of phytol and i+a₁₅:₀ δ¹³C. Phytol δ¹³C differed among blocks within site, regardless of treatment (Block factor: F₃,₁₅₈ = 3.9, p = 0.021).
Fortunately, this small-scale variability was able to be partitioned statistically so as not to obscure treatment effects. Such small-scale variability was not evident for bacteria, for which the block effect was not significant ($F_{3,58} = 0.6$, $p = 0.542$).

For the bacterial biomarker (i+a15:0), pairwise comparisons revealed no difference among treatments in mud. In sandy sediments, however, LS plots appeared more enriched than other treatments. Pairwise comparisons revealed that i+a15:0 in LS plots was more enriched than in non-enriched plots and DS plots ($p < 0.001$ in all cases), but DS plots were more enriched than LU plots ($p = 0.010$). There was no significant difference, however, between DS and DU plots (Fig. 4.2).
Figure 4.1: Phytol $\delta^{13}$C (mean ± S.E.) for treatments within mud and sand. Lettering represents Tukey results, where the same letter indicates no significant difference.
Figure 4.2: Bacterial biomarker (i+a15:0) $\delta^{13}$C (mean ± S.E.) for treatments within mud and sand. Lettering represents Tukey results, where the same letter indicates no significant difference. N.B. y-axis for sandy sediment is split to allow differences in light and dark unspiked plots and spiked dark plots to be seen. The scale is different on either side of this split.
4.4 Discussion

This study provides evidence that bacteria are capable of directly assimilating carbon from $^{13}$C-enriched sodium bicarbonate, but that this uptake is relatively minor. In DS plots, no enrichment of phytol was observed, indicating that MPB was unable to assimilate $^{13}$C from sodium bicarbonate in the dark, presumably due to inhibition of photosynthesis. Enriched EPS would therefore not have been available for use by bacteria as a carbon source. However, in sandy DS plots, bacterial biomarkers became enriched, demonstrating the ability of bacteria to directly take up $^{13}$C. The low enrichment of bacterial biomarkers in DS plots compared to LS plots, where MPB was highly enriched, indicates that of the carbon bacteria derive from sodium bicarbonate, the majority is obtained through use of MPB and/or the EPS they exude. Relatively little carbon is obtained via direct assimilation of $^{13}$C from sodium bicarbonate. Similar direct assimilation of carbon by bacteria may also occur in muddy sediments, but in the current study may have been below detectable limits due to the overall lower enrichment of muddy sediments relative to sand. It is possible that only the massive overall enrichment of sandy sediments allowed direct uptake to be detected in the current study. Similar direct uptake by bacteria may also occur in mud, but in the current study these sediments may not have been sufficiently enriched to detect this. Nevertheless, direct uptake of carbon plays a relatively minor role for bacteria, so at usual levels of enrichment, labelling of consumers following application of $^{13}$C-enriched sodium bicarbonate can confidently be attributed to the use of MPB as a primary source of nutrition.

In all cases where phytol $\delta^{13}$C was enriched, bacterial biomarkers (i+a15:0) did not attain the same level of enrichment. There was no evidence of bacterial enrichment in muddy sediments, and even in sandy sediments where phytol was enriched to over 1000‰ in LS plots, bacterial biomarkers were far less enriched than phytol. This difference in enrichment may be due to a number of reasons. Faster turnover and consequently greater assimilation of $^{13}$C by MPB compared to bacteria could explain differences in direct uptake. However, I have demonstrated that this is unlikely given that even in the absence of assimilation by MPB, direct uptake by bacteria is minimal. The greater enrichment of MPB compared to bacteria is more likely a result of mechanisms involved in the transfer of carbon between these. Smith and Underwood (1998) found that the time from uptake of carbon by MPB during photosynthesis to its
excretion in EPS could be short (< 1 h), but EPS could be produced for up to 4 h after carbon uptake. Any lag time between photosynthesis and EPS production would be evident in a lower enrichment of bacteria relative to MPB at any given point in time. Differences in enrichment may also be influenced by the retention of the heavy $^{13}$C isotope by MPB, and discrimination against $^{13}$C by bacteria during carbon assimilation. The variability in discrepancies between $\delta^{13}$C values of phytol and bacteria biomarkers, particularly between the two habitat types, however, hints at variations in the extent to which MPB-derived carbon contributes to bacterial biomass. Variation in enrichment of phytol between sandy and muddy sediments also points to differences in carbon uptake by MPB.

The lack of enrichment of bacterial biomarkers in all muddy plots and the low enrichment of MPB in muddy sediments compared to sand reflects the very different characteristics of these sites. Phytol was naturally more depleted in DU and LU plots (where no label was applied) in mud than in sand. This might well be due to the use of depleted DIC from surrounding mangroves at muddy sites (Bouillon et al. 2002a). Proximity to mangrove trees may also account for the lower enrichment observed in LS plots in mud compared to sand. Shading inhibits production by MPB (Stutes et al. 2006) and, by reducing light availability, mangrove trees may also influence sediment temperatures, thereby affecting carbon turnover rates of MPB (e.g. Barranguet et al. 1998, Montani et al. 2003). The impact of shading would be further exacerbated by the smaller grain size of mud. Light penetration is greatly decreased in smaller-grained muddy sediments compared to larger-grained sand (MacIntyre et al. 1996), leading to a shallow euphotic zone that limits the region within which MPB can photosynthesise. This might then lead to decreased photosynthetic rates (Barranguet et al. 1998) and greater uptake of $^{13}$C. Slower turnover due to the shallow euphotic zone, shading and perhaps lower sediment temperature, would limit the ability of MPB to take up $^{13}$C, leading to the observed lower enrichment observed for phytol in muddy sediments. Other characteristics of the sediment that affect photosynthetic rates, such as water content (MacIntyre et al. 1996), may also be responsible for the differences in enrichment observed for phytol in muddy and sandy sediments. However, further studies would be required to determine which sediment characteristic, or combination of characteristics, are responsible for the differences in phytol enrichment observed.
As aforementioned, the low enrichment of MPB may have resulted in the inability to detect subsequent enrichment of bacterial biomarkers, particularly if the differences in MPB and bacterial enrichment are similar to those observed for sand. However, differences in the use of MPB-derived carbon in mud and sand may offer a further explanation. Whereas MPB is often the major primary source of carbon available in sandy sediments, muddy sediments typically have much higher organic matter content, reflecting the availability of alternative carbon sources, primarily in the form of macrophyte detritus (Cartaxana et al. 2006). Bouillon and Boschker (2006) found that the carbon sources of bacteria were variable, with $\delta^{13}C$ values of bacterial biomarkers generally corresponding to $\delta^{13}C$ values of the available organic matter. This was demonstrated by Köster et al. (2005) who showed that, in sediments with low organic matter content, there was a greater dependency of bacteria on microalgae within the sediments. The lack of enrichment of bacterial biomarkers in muddy sediments in the current study, even where MPB was enriched, may therefore reflect the lack of reliance of bacteria upon MPB in sediments with high organic matter content.

Further insight may be gained through the calculation of values of absolute carbon uptake based on biomass measurements for MPB and bacteria, and knowledge of the abundance of biomarkers (i.e. phytol and i+a15:0) within this biomass (see, for example, Middelburg et al. 2000). These measurements were not made in the current study, however absolute uptake calculations would be a valuable addition to future studies with a specific focus on investigating the processes and interactions within assemblages of MPB and bacteria in estuarine sediments.

In general, assimilation of carbon from sodium bicarbonate by bacteria appears to be primarily through consumption of EPS from MPB, as indicated by the greatest bacterial enrichment being when MPB were also enriched (sandy LS plots). Bacteria appear to be simply another trophic link from MPB to higher consumers. In sandy sediments, where enrichment was huge, some enrichment of bacteria was observed in the absence of enriched MPB, indicating that bacterial uptake may occur in the absence of enriched MPB, and is therefore direct. However, in comparison to uptake by MPB, this enrichment is negligible, and, at the usual levels of enrichment attained, would be unable to be detected (as observed for muddy sediments) in bacteria or
animals utilising bacteria as a primary food source. Whether uptake is direct or indirect, carbon applied as sodium bicarbonate is rapidly taken up by MPB, giving MPB a far more enriched signature than bacteria. Even in the dark, when there was no competition with MPB for carbon, bacteria do not rapidly assimilate label. Consequently, enrichment of consumer $\delta^{13}C$ can confidently be assumed to indicate use of MPB as the primary food source. Although bacteria is a food source for some consumers (e.g. van Oevelen et al. 2006a & b), negligible enrichment at usual spiking levels would prevent any trace of label in consumers utilising this carbon source.

Overall, it appears that in studies assessing the nutritional importance of MPB through $^{13}C$-enrichment using sodium bicarbonate, there is little concern that bacteria will directly assimilate $^{13}C$. At usual levels of enrichment, labelling of consumers can confidently be attributed to the use of MPB as a primary source of nutrition either directly or indirectly via trophic links that may include bacteria.
Chapter 5. The contribution of microphytobenthos to the nutrition of estuarine consumers in mangroves: $^{13}$C-enrichment and compartment modelling

5.0 Abstract
Despite the conspicuous biomass of mangroves, few studies have demonstrated a major contribution of mangrove carbon to animals in mangrove forests. Carbon stable isotope analysis suggests that microphytobenthos (MPB), which is also abundant in mangrove forests, may be the main source of carbon. However, natural abundance stable isotope studies have been unable to determine the relative importance of the two sources because MPB and mangrove carbon isotope signatures ($\delta^{13}$C) are too variable and/or similar, or because of confusion resulting from additional producers within the system. To circumvent this, I labelled MPB in a mangrove forest with $^{13}$C and monitored $^{13}$C uptake by consumers: crabs (*Parasesarma erythrodactyla* and *Australoplax tridentata*) and foraminifera (*Ammonia beccarii* and *Trochammina inflata*) over 35 days. Crab muscle, gill and hepatopancreas tissues were analysed separately. Treatment (spiked with $^{13}$C-enriched bicarbonate at days 0, 7, and 14), procedural control (non-enriched bicarbonate) and control plots were destructively sampled every 7 days. All species (and all tissues) were measurably enriched within 7 days, with maximum enrichment at 28 days, or 35 days for *P. erythrodactyla*. For crabs, enrichment was generally greatest in the hepatopancreas, then gills, then muscle. All tissues of *A. tridentata* were more enriched (e.g. $521\%$ for hepatopancreas at day 28) than those of *P. erythrodactyla* ($92\%$). *Ammonia beccarii* ($245\%$) was more enriched than *T. inflata* ($12\%$). Compartment modelling using $\delta^{13}$C of hepatopancreas tissue indicated that 99% of the nutrition of *A. tridentata* was derived from MPB, compared to 35% for *P. erythrodactyla*. MPB also provided more nutrition to *A. beccarii* ($34\%$) than to *T. inflata* (minimal). Application of compartment modelling to trophic ecology is relatively new and in this study confirms that MPB are an important source of primary nutrition to consumers in mangrove forests.
5.1 Introduction

Mangrove forests are highly productive systems widely thought to be important for maintaining fisheries productivity. Mangrove trees, the most conspicuous primary producers within these forests, are very productive (e.g. 831-922 g leaf litter m\(^{-2}\) yr\(^{-1}\) in southeast Queensland, Australia; Mackey & Smail 1995), and are commonly assumed to be the main producers supporting consumers in these systems (e.g. Camilleri 1992). However, diatoms and cyanobacteria within sediments (MPB) can also be highly productive, but offer a more labile potential carbon source. This has led to debate over whether mangrove trees or MPB are the main source of primary nutrition for consumers in mangrove forests.

Despite their potential importance in ecological processes and studies using various techniques including stable isotopes (e.g. Newell et al. 1995, Bouillon et al. 2002a, Bouillon et al. 2004a), the carbon source for invertebrates within mangrove forests remains subject to debate. Crabs, which tend to dominate mangrove fauna, are among the more frequently studied consumers and are thought to be important in processing leaf material so that it can more easily be utilised by other consumers (Werry & Lee 2005), and may also affect development of MPB communities (Webb & Eyre 2004). Broadly, grapsid crabs (family Grapsidae) are considered to feed on leaf litter (e.g. Twilley et al. 1997, Lee 1998) whereas ocypodid crabs (family Ocypodidae) are thought to rely on bacteria (Dye & Lasiak 1986, 1987), MPB (Rodelli et al. 1984, France 1998) or plant matter (Hsieh et al. 2002). Meiofauna are less frequently studied, most likely due to their small size, but may also serve an important role in mangrove forests. Foraminifera, for example, are abundant throughout shallow-water sediments (e.g. 72-410 per g of wet sediment; Ellison 1984) and their rapid response to additions of phytodetritus (e.g. Moodley et al. 2000, Moodley et al. 2002, Suhr & Pond 2006) suggests that they may be important in carbon cycling within sediments (Gooday et al. 2002, Moodley et al. 2002). Both meiofauna (Gee 1989) and crabs (Sheaves & Molony 2000, Hollingsworth & Connolly 2006) may provide a food source for more mobile consumers, such as fish (Stergiou & Karpouzi 2001) and birds (Martinez 2004), thereby providing a pathway for movement of carbon from mangrove forests to adjacent estuarine habitats (Kneib 1997).
Studies aiming to establish the primary sources of nutrition (i.e. the primary producers supporting the base of the food web) for consumers in mangrove forests have most commonly used natural abundance stable isotopes. Stable isotope analysis has an advantage over stomach content analysis because it can indicate what food has been assimilated by the consumer, rather than what was merely ingested (Petersen 1999). Natural abundance stable isotopes apparently indicate that mangroves provide the majority of carbon for some species (e.g. prawns, Chong et al. 2001), but it is more common for studies to find only minor contributions of mangroves. For example, Bouillon et al. (2002a) showed that few consumers (including gastropods and the sesarmid crab, *Parasesarma asperum*) within an Indian mangrove forest assimilated significant amounts of mangrove carbon, and Kieckbusch et al. (2004) used mixing models to show that mangroves were not the major carbon source for consumers in fringe mangroves of subtropical lagoons. Both Bouillon et al. (2002a) and Kieckbusch et al. (2004) suggested that algal carbon may be a more important source. However, natural abundance stable isotope studies, such as those described above, rely upon similarities between carbon signatures of consumers and their food sources. Where there are more than two alternative food sources, a consumer signature similar to that of either mangroves or MPB may be produced by a diet derived from a combination of other producers within the system, or transported into the forest. For example, Guest et al. (2004) found that carbon signatures of crabs within a mangrove forest in southeast Queensland, Australia, were more similar to those of MPB than mangroves, but cautioned that these signatures may also result from assimilation of carbon derived from a combination of mangroves and saltmarsh plants, which may be transported into the habitat, with or without a MPB contribution.

Natural abundance stable isotopes have been used successfully in environments where there are few producer sources and where stable isotope signatures of producers are distinct (e.g. Thresher et al. 1992). Where isotopic signatures of producers are similar, however, the addition of a substrate enriched in heavy, rare isotopes (e.g. $^{15}$N, $^{13}$C) can be used to alter producer signatures. For example, Winning et al. (1999) demonstrated that addition of $^{15}$N as $^{15}$N-enriched potassium nitrate (> 98% $^{15}$N) differentially labelled, and therefore gave distinct $\delta^{15}$N values, for seagrass and their epiphytes, for which natural abundance $\delta^{15}$N values were similar. Where carbon
sources are of interest, $\delta^{13}C$ values can be manipulated through addition of substrates (usually NaHCO$_3$) enriched in $^{13}C$. Labelling using $^{13}C$ can be used to give MPB distinct $\delta^{13}C$ values (e.g. Herman et al. 2000, Middelburg et al. 2000, Moens et al. 2002). This $^{13}C$ label can then be traced into consumers which utilise MPB as a carbon source. The relative abundance of the light carbon isotope ($^{12}C$) in the environment is much greater than for the heavier $^{13}C$ isotope. Depending on uptake and assimilation pathways, this is reflected in primary producers which photoassimilate carbon. Stable isotope enrichment experiments add compounds where almost all (99.9%) of the carbon is $^{13}C$, altering the ratio of $^{12}C$: $^{13}C$ available for uptake by primary producers. This alters the $\delta^{13}C$ of producers able to use the compound, resulting in a unique signature which is then transferred to consumers that are directly or indirectly (via trophic transfer) reliant upon MPB as a source of nutrition.

Although enrichment experiments solve some of the problems associated with natural abundance stable isotope studies, they have some of their own problems (detailed by Hamilton et al. 2004). Enrichment experiments generally show which consumers utilise a particular source, but are unable to quantify the extent of utilisation. Quantifying utilisation would rely on the consumer of interest attaining equilibrium with its food source, but enrichment experiments are rarely run for sufficient time for this to occur (e.g. Herman et al. 2000, Moens et al. 2002). In other cases, the degree of producer enrichment fluctuates temporally or spatially (e.g. Raikow & Hamilton 2001), preventing equilibrium from being reached. For less mobile meiofauna, collection using large cores can mitigate against this by encompassing variability in labelling of meiofauna and thereby giving an average for the labelled area. Where consumers of interest are more mobile, feeding throughout the enriched area would result in less variation in $\delta^{13}C$ among individuals of the one species, as they would feed across patches of variable producer enrichment. Where mobile consumers are able to forage outside of the enriched area, however, their label would be diluted and the contribution of carbon by the enriched producer species would be underestimated. Although some studies have circumvented this problem by confining animals in the laboratory (e.g. Herman et al. 2000) or using physical barriers in the field (e.g.
Middelburg et al. 2000, Moens et al. 2002), these procedures may alter the feeding behaviour of the consumers studied.

Hamilton et al. (2004) solved many of these problems in a freshwater system using a compartment modelling approach that avoids the need to wait for animals to reach equilibrium, thus allowing for variable enrichment over time. The model describes compartments relating to 1) the consumer of interest, 2) the producer of interest, and 3) all other potential food sources, as well as 4) the rates of exchange of carbon among the compartments. Having first defined a compartment model to describe the system, an equation-solving program (WinSAAM) compares consumer signatures to those of producers over the time of the experiment and iteratively seeks the parameters which give the best fit of the model to the observed consumer values (according to fractional standard deviations, FSDs). Hamilton et al. (2004) successfully described the contribution of microbial and algal food sources to consumers within a stream system, but also suggested further improvements to their approach, viz.: 1) looking at different body tissues and using the one with the fastest turnover, and 2) separating algae and microbes as food sources. In the current study, however, we were interested in the primary sources of carbon supporting consumers in mangrove forests. The latter of these two recommendations is therefore irrelevant, as I demonstrated in Chapter 4 that bacteria in mangrove systems of southeast Queensland do not directly assimilate carbon from the NaHCO$_3$ which is used to apply the $^{13}$C label to sediments. Transfer of $^{13}$C to bacteria and then to higher consumers would indicate that MPB are the primary carbon source and bacteria are simply a trophic link from MPB to higher consumers.

Although stable isotope enrichment techniques have been applied to sandy and muddy tidal flats (e.g. Middelburg et al. 2000, Moens et al. 2002), coastal marshes (Carman & Fry 2002) and freshwater systems (e.g. Hall & Meyer 1998, Mulholland et al. 2000, Raikow & Hamilton 2001, Hamilton et al. 2004), only one study (van Oevelen et al. 2006b) has utilised compartment modelling in a marine environment and I have found no published studies of $^{13}$C-enrichment of MPB within mangrove forests. In the current study, I aimed to use the compartment modelling approach previously applied by Hamilton et al. (2004) to freshwater systems, in combination with $^{13}$C-enrichment
of MPB, to quantify the importance of MPB as a carbon source for consumers (crabs and meiofauna) within mangrove forests.

5.2 Methods

5.2.1 13C-labelling

Within a clearing in a mangrove forest in Coombabah Lake in southeast Queensland, Australia (near the most westerly mud site shown on Fig 4.1), 18 circular, 1 m diameter plots were haphazardly selected, and randomly assigned to one of three treatments: 1) enriched, 2) procedural control or 3) control (6 plots per treatment). For enriched plots, 13C-enriched sodium bicarbonate (NaH13CO3) was dissolved in 1 L of filtered seawater. This was then sprayed evenly over the sediment surface, with 0.8 g of sodium bicarbonate applied per m2 of sediment at the beginning of the experiment and then at days 7 and 14 to maintain enrichment of MPB. The same method was applied for procedural controls, except that non-enriched sodium bicarbonate was used. Procedural controls were used to ensure that plots were sufficiently far apart to prevent movement of 13C label among plots and to test for changes in use of MPB by consumers through monitoring of consumer δ13C. Addition of carbon may have enhanced MPB growth, leading to greater use of MPB as a carbon source for consumers, and thus changes in δ13C of animals. Control plots were not treated and were used to obtain background values against which to compare MPB and consumer δ13C values.

5.2.2 Sample collection

Plots for each treatment were further allocated to a time for destructive sampling, which was done at time 0, 7, 14, 21, 28 or 35 days. At each time, sediment was scraped from the surface of one of each type of treatment plot (to 5 mm depth) for determination of MPB δ13C using the phytol method (described in Chapter 2). Carmen & Fry (2002) suggested using a core greater than 10 cm² to minimise the effects of patchy distributions of isotope label and meiofauna, and so meiofauna were collected from the centre of each plot using a 5 cm deep, 15 cm diameter core. The dominant meiofauna (the foraminifera Ammonia beccarii and Trochammina inflata) were handpicked from sediment, dried at 60°C to constant weight, and acid-washed (5% HCl, as per Moodley et al. 2002) prior to stable isotope analysis. Enough
individuals were used to obtain sufficient material for isotope analysis (typically 10 - 30 individuals). At each sampling time, crabs (*Parasesarma erythroductyla*, family Grapsidae and *Australoplax tridentata*, family Ocypodidae) with carapace widths ranging from 8 to 13 mm were collected within 20 cm of the plot centre. For crabs with burrows near the centre of the plot, the plot diameter was sufficient to incorporate their entire home range over the 5 week period (Guest et al. 2006), so no confinement of crabs was required. Crab hepatopancreas, gill and muscle tissues were dissected, dried at 60°C to constant weight, ground, and placed in tin cups for stable isotope analysis on an isotope-ratio mass-spectrometer (IRMS). Sediment samples and cores for meiofauna (but not crabs) were also collected 4 hours after initial treatment, using only a small area of a randomly selected plot of each treatment type. Sediment and animals were stored frozen until processed.

5.2.3 Estimation of MPB $\delta^{13}C$

Due to rapid uptake and depuration, $\delta^{13}C$ of MPB would ideally be monitored constantly throughout the experimental period. As this was unable to be done, data from a pilot study run over 18 days at the same site, using the same application of enriched sodium bicarbonate, was used to determine rates of uptake (20.325‰ h$^{-1}$, described by a linear equation) and depuration (0.009‰ h$^{-1}$, described by an exponential equation) of label by MPB. These rates were then used to estimate the response of MPB to enrichment over time. To verify these predictions, samples of MPB collected during the experiment were compared to those predicted (Fig. 5.1). With the exception of day 7, all estimates were within 100‰ of observed MPB $\delta^{13}C$ values. A similar method was used by Hamilton et al. (2004) to predict $\delta^{15}N$ of algae and microbes in an isotope addition experiment in a freshwater stream.

5.2.4 Modelling

Modelling was done for each species using the Windows version of Simulation Analysis and Modelling (WinSAAM 3.0.7), an equation-solving program developed by the US National Institute of Health (see [www.winsaam.com](http://www.winsaam.com)). Compartments within the model were: 1) the MPB carbon source, 2) the consumer of interest, and 3) all alternate, unknown carbon sources (Fig. 5.2). The predicted MPB $\delta^{13}C$ values were used as a forcing function, and parameters defining the exchange rates among
the compartments were varied between 0 and 1. The transfer rates of interest were from producer (MPB) to consumer \(k(2,1)\), alternate carbon sources to the consumer \(k(2,3)\) and out of the consumer to the system in general \(k(0,2)\); Fig. 5.2). The contribution of MPB to the nutrition of each consumer of interest was determined from the ratio of \(k(2,1)\) to \(k(0,3)\). These transfer rates were determined through modelling with WINSAAM. WinSAAM sought a generalised least squares fit of the compartment model to the consumer \(\delta^{13}C\) values, using equal weightings for the observations. The best fit of the model to the data was determined to be when feasible parameters were obtained that gave the smallest fractional standard deviations (FSDs). Fractional standard deviations provide an estimate of statistical precision, similar to the standard error used in inferential statistics, and equate to the standard deviation divided by the mean (Pawlosky et al. 2001). Low FSD values indicate that model parameters are well defined and estimates made using the model are robust and give realistic values. FSDs < 0.5 are considered to indicate a good fit of the model to the data (Stefanovski et al. 2003, Faichney et al. 2004).

Figure 5.1: Predicted (solid line) and observed (triangles) \(\delta^{13}C\) values for MPB showing variation in enrichment throughout the experimental period. Additions of \(^{13}C\) label are indicated by arrows.
Figure 5.2: Schematic diagram depicting the relationship between compartments in the model. Numbers within circles indicate compartment numbers as referred to in the text. 

$k$ = rate constant for transfer of $^{13}$C into and out of compartments, in the direction indicated by arrows. Numbers in brackets indicate the direction of transfer that rate constants refer to, e.g. $k(2,3) =$ rate of transfer of $^{13}$C to compartment 2 from compartment 3.
5.3 Results
Within 4 h of initial label addition, MPB in treatment plots was enriched (-18.2‰) relative to the average δ13C of MPB in control plots (-22.2‰). The maximum MPB enrichment during the experimental period was calculated to be approximately 720‰, shortly after the final addition of label on day 14 (Fig. 5.1). Throughout the experimental period there was no evidence of MPB or consumer enrichment in either control or procedural control plots, for which δ13C values were similar, confirming that there was no movement of the 13C label among plots and that additional carbon did not alter the extent of use of MPB by consumers (i.e. no shift in natural abundance δ13C values of consumers could be detected).

In treatment plots, consumers responded rapidly to enrichment. The foraminifera Ammonia beccarii showed evidence of enrichment (38.7‰ compared to -24.2‰ for control plots) within 4 h of initial label addition and, for both crab species, all tissues were enriched within 7 d, with the hepatopancreas most enriched, followed by gill, then muscle (Fig. 5.3). Hamilton et al. (2004) recommended that the tissue type with most rapid turnover be used, and so modelling was done using the hepatopancreas values for both crab species.

The hepatopancreas of Australoplax tridentata was enriched to 231.5‰ by day 7 and attained a maximum enrichment of 521.6‰ at day 28. Modelling using WinSAAM (Fig. 5.4) showed that the transfer rates from MPB to A. tridentata (k(2,1)) and from A. tridentata to the system (k(0,2)) were 0.0102 and 0.0103, respectively (Table 5.1). The ratio of these transfer rates revealed that A. tridentata derives most of its nutrition (99%) from MPB. These parameter values gave a good fit of the model to the observed data, as indicated by FSDs of < 0.5.

The response of Parasesarma erythrodactyla to MPB enrichment was less pronounced. By day 7, the hepatopancreas was enriched to 1.7‰ and the maximum enrichment was 110.5‰ at day 35. Compartment modelling (Fig. 5.4) gave transfer rates of 0.0008 and 0.0022 for k(2,1) and k(0,2). From these rates it was calculated that P. erythrodactyla derives approximately 35% of its carbon from MPB. FSD values > 0.5, however, demonstrate that the model parameters are not as well-defined.
as for *A. tridentata*, and there is less confidence in the estimate of MPB contribution (Stefanovski et al. 2003; Table 5.1).

Both foraminifera species showed evidence of enrichment (Fig. 5.5), but *Ammonia beccarii* became much more enriched than *Trochammina inflata*. For *A. beccarii*, the $\delta^{13}C$ value after 7 days was 38.7‰, and the maximum enrichment was 245.3‰ at day 28. The best fit of the compartment model to the data (Fig. 5.6) was achieved using transfer rates of 0.0007 for $k(2,1)$ and $k(0,2)$ (Table 5.1). The model showed that 34% of the carbon for *A. beccarii* is derived from MPB. However, FSDs were again > 0.5, indicating that model parameters are not well defined and that there is less confidence in this estimate.

*Trochammina inflata* showed very little evidence of enrichment until day 28 when an enriched sample (11.7‰) was obtained. Such a value could not occur naturally, indicating some degree of uptake of MPB carbon by *T. inflata*. However, no feasible fit of the model to the observed data could be established, so transfer rates were unable to be determined. The presence of enriched values at day 28 does indicate that *T. inflata* utilises MPB-derived carbon to some extent, however the contribution of MPB to the carbon of this foraminifera is likely to be minimal, perhaps resulting from accidental ingestion of MPB along with a major alternative carbon source.
Table 5.1: Natural abundance $\delta^{13}$C and maximum enrichment of MPB and consumers, and calculated parameters and results of WinSAAM modelling. FSDs in brackets. n.a. = not applicable, - = no model generated.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Natural abundance ($\delta^{13}$C, ‰)</th>
<th>Maximum enrichment ($\delta^{13}$C, ‰)</th>
<th>Transfer producer to consumer $k(2,1)$</th>
<th>Transfer consumer to system $k(0,2)$</th>
<th>% contribution of MPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microphytobenthos</td>
<td>-25.5</td>
<td>720.0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>A. tridentata</em> Hepatopancreas</td>
<td>-28.0</td>
<td>521.6</td>
<td>0.0102 (0.33)</td>
<td>0.0103 (0.39)</td>
<td>99</td>
</tr>
<tr>
<td><em>P. erythroductyla</em> Hepatopancreas</td>
<td>-26.8</td>
<td>110.5</td>
<td>0.0008 (0.85)</td>
<td>0.0022 (1.45)</td>
<td>35</td>
</tr>
<tr>
<td><em>A. beccarii</em></td>
<td>-24.2</td>
<td>245.3</td>
<td>0.0007 (1.51)</td>
<td>0.0020 (2.94)</td>
<td>34</td>
</tr>
<tr>
<td><em>T. inflata</em></td>
<td>-23.0</td>
<td>11.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.3: δ¹³C values for hepatopancreas, muscle and gill tissues of the crab species, *Australoplax tridentata* and *Parasesarma erythodactyla* throughout the experimental period. Grey bars represent δ¹³C values for hepatopancreas in control plots (mean ± S.E.).
Figure 5.4: Line depicting output from the model which best fit the observed $\delta^{13}C$ values for the hepatopancreas of the two crab species, *Australoplax tridentata* and *Parasesarma erythrodactyla*. 
Figure 5.5: $\delta^{13}C$ values of *Ammonia beccarii* and *Trochammina inflata* throughout the experimental period. Grey bar represents $\delta^{13}C$ of *A. beccarii* in control plots (mean ± S.E.). Values for *T. inflata* in control plots were similar to those for *A. beccarii*.

Figure 5.6: Line depicting output of model which best fit the observed $\delta^{13}C$ for *Ammonia beccarii*. 
5.4 Discussion
Labelling of MPB with $^{13}$C effectively demonstrated the transfer of MPB carbon to macroinvertebrates and meiofauna, within mangrove forests. Although the extent of $^{13}$C uptake varied among taxa, all consumers (Ammonia beccarii, Trochammina inflata, Australoplax tridentata and Parasesarma erythrodactyla) became labelled during the experimental period. MPB and consumers in procedural control plots did not become enriched, indicating that there was no contamination among plots and that addition of carbon did not impact upon the use of MPB by consumers enough to alter natural abundance isotope values. The enrichment of consumers in treatment plots therefore shows that MPB contributes carbon to the diets of all consumers studied. The enrichment of the foraminiferan, A. beccarii, within 4 h of initial label addition demonstrates that the transfer of carbon from MPB to consumers can be rapid. Despite the abundance of an alternate carbon source, in the form of mangrove litter, compartment modelling indicated that the majority of carbon for A. tridentata, and a significant proportion of carbon for P. erythrodactyla and A. beccarii was derived from MPB. T. inflata derived only a very minor amount of carbon from MPB.

Previous studies have suggested that litterfall is the dominant food source for consumers within mangrove forests (e.g. Camilleri 1992). Feeding experiments have shown that large quantities of mangrove leaf litter are consumed and processed by crabs, particularly grapsids (Lee 1998, e.g. Micheli 1993, Ashton 2002). However, such feeding experiments demonstrate consumption, rather than assimilation, of mangrove material and are often laboratory-based without alternative food sources available. Although crabs can survive and grow on a diet of mangrove leaves, Kwok & Lee (1995) found that the grapsid crab species Chiromanthes bidens and Parasesarma plicata did not produce any reproductive output on a pure mangrove leaf diet. This suggests that mangrove leaves alone do not provide sufficient energy and nutrients for these crabs, and that an alternative food source must be utilised in the environment. In addition to shredding leaves, crabs are also often seen to graze on the surface of mud. Sediments within mangrove forests contain detritus, which may be colonised by microbes, as well as MPB and invertebrates, all of which have been observed in the guts of crabs (Dahdouh-Guebas et al. 1999). The current study suggests that MPB contributes a significant portion of the carbon required by the ocypodid crab (Australoplax tridentata) and grapsid crab (Parasesarma
erythrodactyla). This could be either through direct ingestion of MPB or via ingestion of invertebrates which have themselves consumed MPB. The greater use of MPB by A. tridentata than by P. erythrodactyla concurs with previous findings that the nutrition source of grapsid crabs, such as P. erythrodactyla (Meziane et al. 2006), is different to that of ocypodid crabs. Whereas ocypodid crabs have previously been described as being reliant upon MPB carbon (e.g. Rodelli et al. 1994, France 1998), grapsids are thought to typically consume leaf litter (e.g. Neopisesarma versicolor; Thongtham & Kristensen 2005). The current study provides the first unequivocal evidence that ocypodids derive the majority of their nutrition from MPB. I have also shown, however, that grapsid crabs are not entirely reliant upon leaf litter, with P. erythrodactyla deriving approximately a third of its carbon from MPB. Bouillon et al. (2002a) similarly found that, although the grapsid Parasesarma asperum is typically considered to depend upon leaf litter, discrepancies with mangrove carbon signatures indicated that another source contributed to the diet of P. asperum. The current study demonstrates that MPB is a feasible alternative source of carbon for grapsids, such as P. asperum, that do not entirely rely upon mangrove carbon.

Investigation of sources of carbon supporting foraminifera is complicated by their relatively small size. However, recent fatty acid analyses have determined that intertidal benthic foraminifera rely upon phytodetrital material, bacteria and MPB (Gooday et al. 2002, Topping et al. 2006). Topping et al. (2006) further identified differences in the carbon sources of two species of foraminifera, with both Haynesina germanica and Ammonia beccarii relying upon bacteria and diatoms, but A. beccarii assimilating carbon from a wider range of MPB. Although potential food sources have been identified, fatty acid studies to date have not quantified the relative contributions of these sources. The current study confirms that A. beccarii utilise MPB and that this is a significant source of carbon for the species. However, the source of the majority of carbon for both A. beccarii and Trochammina inflata remains unclear.

For all taxa except Australoplax tridentata, a source other than MPB supplies a large part of the carbon requirements. The most obvious potential alternative source of carbon is leaf litter from Avicennia marina, the dominant mangrove species at the study site. Although mangrove leaves are typically considered a poor quality food
source, leaves of *A. marina* have greater nitrogen content and lower tannin levels than other mangrove species, making them a better quality food source (Kwok & Lee 1995). Observations of detritus consumption by benthic foraminifera (Schmiedl et al. 2000) and *Parasesarma erythrodactyla* (Camilleri 1992), further suggest that mangrove leaves are a feasible alternative carbon source. Alternatively, transport of detritus from adjacent habitat (i.e. seagrass or saltmarsh) may provide the additional carbon. Connolly et al. (2005) for example, found that fish (yellowfin whiting, *Sillago schomburgkii*) over mudflats are reliant upon material transported from adjacent seagrass beds. A study by Guest et al. (2004) of southeast Queensland mangrove forests, was also unable to discount the use of seagrass and saltmarsh material transported into mangrove forests as a potential food source for consumers in a southeast Queensland mangrove forest. Further investigation, through enrichment of alternative food sources, is required to determine where the remainder of the carbon is obtained (refer Chapter 6).

Regardless of potential contribution of carbon by other sources, the current study provides evidence that all consumers examined utilise MPB-derived carbon to varying extents. The use of MPB by both meiofauna and crabs offers a potential pathway for movement of carbon to habitats adjacent to mangroves. Both meiofauna (Gee 1989) and crabs (Sheaves & Molony 2000) are a potentially important source of food for mobile consumers which utilise inundated intertidal habitats. Foraminifera, for example, are consumed by fish (Stergiou & Karpouzi 2002), thalassinidean shrimp (*Pestarella tyrhrhena*; Abed-Navandi et al. 2005) and crabs (*Callinectes ornatus*; Branco et al. 2002). Hollingsworth and Connolly (2006) found that glassfish, *Ambassis jacksoniensis*, moving onto a southeast Queensland saltmarsh on the spring high tide were heavily reliant upon crab zoea released by crabs on the marsh. Fish such as the banded toadfish (*Torquigener pleurogramma*), which were observed in the inundated mangrove forest in the current study (pers. obs.) also consume meiofauna and crabs (Hughes 1984). Grapsid crabs dominate the diet of groupers (*Epinephelus coioides* and *Epinephelus malabaricus*) and snapper (*Lutjanus argentimaculatus*; Sheaves & Molony 2000) in northeastern Australian mangrove forests, and crabs comprise a large part of the diet of birds in Brazilian mangrove swamps (Martinez 2004). Considerable use of MPB by crabs and the foraminifera,
Ammonia beccarii, therefore offers scope for significant transportation of carbon, via trophic relay, to adjacent estuarine habitats.

In the current study, although I was able to quantify the contribution of MPB to the nutrition of consumers, for all consumers except Australoplax tridentata the FSD values for compartment model parameters were > 0.5, indicating that observed values did not fit the developed model particularly well. Although the uptake of $^{13}$C label by all species clearly demonstrates that there is some reliance on MPB-derived carbon for all species examined, the confidence in my determinations of the extent of MPB contribution is high only for A. tridentata. The inability to accurately fit a model to the observations for some species implies that factors other than those assessed may have contributed to the variations in consumer $\delta^{13}$C which were observed. This may be due to variations in the contribution of MPB to individual animals, particularly because animals (especially crabs) varied in size. Alternatively, spatial variation in MPB enrichment and/or use of MPB by consumers may have resulted from the necessary destructive sampling of plots, which resulted in enriched plots for each sampling time necessarily being set slightly apart. For A. tridentata, which appear to rely almost solely on MPB, spatial variation would be less of an issue, provided that MPB was available. For all other taxa, which appear to utilise a further carbon source in addition to MPB, spatial variation in the relative abundance of the alternate food source may affect the proportion of carbon which is derived from MPB. Further sources of error in my estimates of MPB contribution to consumer diets may result from variation between estimated and true MPB $\delta^{13}$C.

Where spatial variation in labelling of MPB and consumers is an issue, sufficient replication of plots to encompass some of this variability and/or using ecosystem-scale labelling (e.g. Pace et al. 2004, Gribsholt et al. 2005, Kritzberg et al. 2006, Gribsholt et al. 2007) would diminish this problem. Although both the current study and Hamilton et al. (2004) relied upon simulation of producer labelling throughout the experimental period, this potentially introduces further error to estimates of producer contribution. Enrichment experiments using compartment modelling would be improved by direct determination of producer labelling either by 1) increasing the frequency of sampling events to capture variation in producer $\delta^{13}$C throughout the
experimental period, or, to minimise extra effort, 2) strategically aiming to capture the 
peaks and troughs in producer labelling based on prior simulation of producer 
signatures. Future studies should also aim to establish contribution of producers to 
consumers at multiple times and locations. Ontogenetic diet shift (Lukoschek & 
McCormick 2001) and/or seasonal or spatial variation in mangrove or MPB 
production may influence the carbon sources supporting consumers. Given that 
_Australoplax tridentata_ appears almost wholly reliant upon MPB carbon, it would be 
interesting to observe if the distribution of this species is correlated with MPB 
abundance, or if the composition of the diet of _A. tridentata_ is different where MPB 
are less abundant. Guest et al. (2004) found that δ¹³C of _A. tridentata_ varied across 
the boundary between mangrove forests and saltmarshes. This may reflect changes in 
MPB δ¹³C with proximity to mangrove forests and therefore depleted dissolved 
inorganic carbon (Bouillon et al. 2002a) if MPB remains the sole carbon source across 
this interface. However, variation in δ¹³C may also be due to a shift in the diet of 
_A. tridentata_ in saltmarsh. This could be resolved through further experiments 
labelling MPB with ¹³C.

Although there is scope for further improvement, the current study demonstrates the 
application of a relatively novel combination of techniques (¹³C-enrichment and 
compartment modelling) to quantify the importance of MPB as a carbon source. 
Another feature of the experiment was that I was able to enrich the entire home range 
of consumers. I thus avoided the potential for interference with natural foraging 
behaviours that has been encountered in previous enrichment studies in other habitats 
that examined consumers within a laboratory (e.g. Abed-Navandi et al. 2005) or 
confined by barriers in the field (e.g. Herman et al. 2000). Although the source of 
significant carbon for some consumer species could not be determined, this may be 
derived from mangrove leaf litter. Further pulse-chase studies that enrich these 
alternate carbon sources should help to determine the alternative sources of carbon. 
Nevertheless, the current study has shown, unequivocally, that MPB can be an 
important carbon source for consumers within mangrove forests.
Chapter 6. $^{13}$C-enrichment of mangrove detritus to trace its contribution to the nutrition of estuarine consumers in mangrove forests

6.0 Abstract
The experiment using $^{13}$C-enrichment of algae demonstrated that some invertebrate species within a mangrove forest derive much of their nutrition from microphytobenthos (MPB). However, the source of a substantial amount of the carbon of some invertebrate species was unable to be determined, and mangrove detritus was hypothesised to be the alternative source. In this study, I added $^{13}$C-labelled mangrove detritus to sediment within a mangrove forest and monitored, over 35 days, the transfer of $^{13}$C to consumers: crabs (Parasesarma erythrodactyla and Australoploax tridentata) and foraminifera (Ammonia beccarii and Trochammina inflata). Mangrove detritus was initially enriched (-11.5‰) relative to control plots (-29.4‰), but 7 days after detritus addition there was no trace of enrichment, possibly due to rapid processing of detritus by some consumers. Within 7 days of detritus addition, A. beccarii (-21.0‰ compared to -25.8 for controls) and all tissues of P. erythrodactyla (e.g. hepatopancreas, -21.2‰ compared to -27.6‰ for controls) were noticeably enriched. Compartment modelling indicated that 88% of the nutrition of P. erythrodactyla and 84% for A. beccarii was derived from mangrove detritus. For these species, the contribution of carbon from mangrove detritus appears to be the complement of that derived from MPB. For A. tridentata a lack of enrichment indicated only a very minor contribution of mangrove detritus to the diet, and this species derives its carbon substantially from MPB. T. inflata also showed no enrichment, but for this species MPB is not a major carbon source either. This species either relies on a further carbon source (e.g. mangrove root exudates) or lives so deep within sediments that material at the surface is not utilised. The $^{13}$C-labelling of mangrove detritus was successful in demonstrating the extent of utilisation of detritus by invertebrates, and showed that for some species mangrove detritus is the major carbon source.
6.1 Introduction

The high productivity of mangroves (e.g. 832-922 g leaf litter m\(^{-2}\) yr\(^{-1}\) in southeast Queensland, Australia; Mackey & Smail 1995) potentially offers a significant source of carbon for consumers, but there remains contention regarding how this production might enter the food web. An early paradigm of mangrove ecology was that the majority of mangrove material was transported offshore via outwelling to support a detritus-based food web (see review, Lee 1995). Stable isotope studies to date, however, have provided little evidence that consumers offshore from mangrove forests derive nutrition from mangroves (Rodelli et al. 1984, Newell et al. 1995, Loneragan et al. 1997).

More recently, it has been accepted that much of the production in some mangrove systems is retained within the forest and utilised on a more local scale. It has been suggested that retention of mangrove material within forests may occur through rapid processing of large quantities of mangrove material by crabs (Lee 1998), and subsequent use of crab faecal pellets as a food source by other invertebrates (Lee 1997). Ingestion of mangrove material by crabs is supported by field observations (Camilleri 1992), leaf litter in crab stomachs (Dahdouh-Guebas et al. 1999), and field experiments showing that crabs remove 28% of the mangrove litter in *Rhizophora* sp. forests (Robertson 1986). Bouillon et al. (2004b) provided stable isotope evidence that much of the carbon required by some crab species within mangrove forests is assimilated from mangrove material, although the extent to which this occurs varies among species, with some more reliant upon MPB. Other invertebrates, including juvenile prawns which are found within mangrove-lined creeks (Rodelli et al. 1984, Loneragan et al. 1997), also appear to have some contribution of mangrove carbon (Newell et al. 1995). However, limitations with natural abundance carbon stable isotope analysis, upon which these studies relied, mean that the extent of contribution of mangrove carbon to consumers remains uncertain. Natural abundance isotope analysis relies upon there being few potential carbon sources (i.e. primary producers) in the system of interest. These producers must have distinct carbon signatures (\(\delta^{13}C\)) which are used in mixing models to determine a feasible combination of sources that could produce the observed consumer \(\delta^{13}C\) value. In some studies however, not all end members are considered (e.g. Chong 2001). Phytoplankton (Bouillon et al. 2004b) and material transported inshore (Connolly et al. 2005) can provide additional
sources of primary production but are often not incorporated into mixing models. Inclusion of these producers may give alternative solutions to mixing models and thus different estimates of the contribution of mangroves to consumers.

To circumvent the requirement to identify and characterise distinct $\delta^{13}C$ for all available producers, enrichment experiments have been used in the past to assess the use of detritus as a carbon source for consumers. Addition to sediments of detritus from $^{13}C$-enriched phytoplankton (Moens et al. 2002, Moodley et al. 2002, Aberle & Witte 2003, Witte et al. 2003) and $^{13}C$- and $^{15}N$-enriched macroalgae (Rossi 2007) has demonstrated rapid and considerable uptake by various meiofauna and macrofauna species. However, because algal material is more labile than vascular plant material, it is likely to be easier for consumers to assimilate (Alongi et al. 1992, Lee 1999, Moens et al. 2002). In a laboratory-based experiment which is the only study thus far to assess the use of vascular plant detritus by consumers through stable isotope enrichment, $^{13}C$- and $^{15}N$-enriched seagrass detritus was assimilated by one of two thalassinidean shrimp examined (Abed-Navandi et al. 2005). Although it is possible to alter the carbon isotope composition of mangrove leaves (e.g. with $^{14}C$; Newell et al. 1995), no studies have added enriched mangrove detritus to sediments to monitor and quantify its assimilation by consumers.

The pulse-chase stable isotope enrichment experiment in Chapter 5 provided unequivocal evidence of use of a producer as a carbon source by a consumer. Compartment modelling allowed the extent of this use to be quantified. Although in Chapter 5 I was able to quantify the contribution of MPB to three of the consumers of interest, the main carbon source for the foraminifera *Trochammina inflata* was unable to be established. The source of a large part of the carbon in the foraminifera *Ammonia beccarii* and the grapsid crab *Parasesarma erythrodactyla* also remained unaccounted for. Even for *Australoplax tridentata*, there was the possibility of some very minor contribution from alternative sources. I hypothesised that mangrove leaf litter provided the remainder of carbon for these consumers. In the current study, I used a pulse-chase enrichment experiment with $^{13}C$-enriched mangrove detritus, in combination with compartment modelling, to quantify the extent to which mangrove detritus is used as a carbon source for consumers within a mangrove forest.
6.2 Methods

6.2.1 Preparation of mangrove detritus

For $^{13}$C-enrichment of mangrove leaves, groups of juvenile trees (< 1 m tall) of the dominant species at the experimental site (*Avicennia marina*) were completely enclosed in bottomless plastic chambers (80 cm (l) x 60 cm (w) x 40 cm (h)) which were pushed 5 cm into the sediment to form a seal at the base. The transparent top of the chambers allowed adequate exposure to light, although to avoid death of plants due to heat stress, mangroves were selected so that tubs would be at least partially shaded by surrounding trees. Chambers were secured to the sediment with ropes and pegs to prevent movement with tides. A knife was used to cut around the perimeter of the plot to a depth of 20 cm to ensure that mangrove plants could not obtain carbon via aerial roots from outside of the chambers. Sealed containers were placed within the chambers to enclose a volume of air and thereby minimise the volume of air remaining that would require CO$_2$ enrichment.

Every two days for 14 days, 30 – 40 mL of $^{13}$C-enriched CO$_2$ was injected into the chamber via septa. At the end of this period, both brown and green leaves were collected. These were combined and dried at 60°C to constant weight. A subsample was analysed on an EA-IRMS and found to have a $\delta^{13}$C value of 220‰.

Non-enriched detritus, for use as a procedural control, was prepared using leaves collected from other mangrove trees adjacent to the enrichment chambers.

6.2.2 Experimental set-up

At the same location as described in Chapter 5, 18 circular, 1 m diameter plots were haphazardly selected. These were randomly assigned to one of three treatments: 1) enriched, 2) procedural control or 3) control (6 plots per treatment). Enriched detritus was evenly spread over the surface of enriched plots and pushed into the surface of the sediment. To control for this treatment, and to test for additional detritus causing a shift in consumer diet and therefore $\delta^{13}$C, procedural controls were similarly treated, with non-enriched detritus added to the sediment. Control plots were left untouched.
The quantity and size distribution of detritus to add to sediment was determined from an initial assessment of the quantity and size composition of detritus naturally occurring in sediments at the study site. This was done for three replicate samples of sediment by sieving sediments (53-125 µm, 125-250 µm, 250-500 µm, 500-1000 µm, 1000-2000 µm) and separating detritus from other material through manual removal or, for smaller size fractions, via density separation through centrifugation with silica gel (as described in Chapter 3). Mangrove leaf litter was then dried (60°C to constant weight) and weighed. Although this mangrove litter may not exactly mimic the detritus naturally available to consumers, this method is routinely used to degrade leaf litter before offering it to consumers (e.g. see review by Tenore et al. 1982). Regardless, natural decay processes would have impacted litter degradation throughout the current study following its initial addition to sediment.

The mass of detritus already present in sediment at the study site averaged 9.9 g.m⁻², with the bulk of detritus belonging to the smaller size classes (Table 6.1). Enriched and non-enriched material was ground to give different sizes of detritus which were added to the sediment in proportions equal to those observed to occur naturally. Approximately 3.2 g.m⁻² of detritus was added to each enriched and procedural control plot, resulting in approximately 25% of the detritus available to consumers having been added. For enriched plots, this resulted in an overall expected δ¹³C for available detritus of 33‰.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>Mass (g)</th>
<th>% of total detritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000-2000 µm</td>
<td>0.9 ± 0.3</td>
<td>8.9</td>
</tr>
<tr>
<td>500-1000 µm</td>
<td>0.5 ± 0.2</td>
<td>4.6</td>
</tr>
<tr>
<td>250-500 µm</td>
<td>1.3 ± 0.3</td>
<td>13.3</td>
</tr>
<tr>
<td>125-250 µm</td>
<td>2.0 ± 0.4</td>
<td>19.9</td>
</tr>
<tr>
<td>53-125 µm</td>
<td>5.3 ± 0.9</td>
<td>53.4</td>
</tr>
<tr>
<td>Total</td>
<td>9.9 ± 0.2</td>
<td>100</td>
</tr>
</tbody>
</table>
6.2.3 Sample collection and analysis

Plots of each treatment were further allocated to a time for destructive sampling, which occurred prior to detritus addition then 7, 14, 21, 28, and 35 days after addition. Just after addition of detritus to sediments, a sample of surface sediment for determination of detritus $\delta^{13}C$ was obtained from a randomly selected treatment plot. At all other sampling times, for one plot of each treatment type, a sample of surface sediment (to 5 mm depth) was obtained for separation of detritus for $\delta^{13}C$ analysis. All sediment samples were derived from a number of smaller subsamples from throughout the plot that were pooled and homogenised to offset any impact of non-homogeneous application of labelled detritus. Samples of foraminifera (*Ammonia beccarii* and *Trochammina inflata*) and crabs (*Australoplax tridentata*, family Ocypodidae and *Parasesarma erythrodactyla*, family Grapsidae) were collected, prepared and analysed for $\delta^{13}C$ as described in Chapter 5.

Different size fractions of mangrove detritus (53-125 $\mu$m, 125-250 $\mu$m, 250-500 $\mu$m, 500-1000 $\mu$m, 1000-2000 $\mu$m) were separated from sediment using the methods described above. The $\delta^{13}C$ values for each size fraction of detritus, at each time, and for each treatment type, were determined separately by analysis on an EA-IRMS.

6.2.4 Modelling

WinSAAM modelling was done for each $^{13}C$-enriched consumer, as described in Chapter 5. Compartments within the model were: 1) the mangrove detritus carbon source, 2) the consumer of interest, and 3) all alternate, unknown carbon sources (as shown in Fig. 5.2 (Chapter 5) except that for the current study compartment 1 denotes enriched mangrove detritus, not MPB). The transfer rates of interest were from producer (detritus) to consumer ($k(2,1)$), alternate carbon sources to the consumer ($k(2,3)$) and out of the consumer to the system in general ($k(0,2)$). The contribution of detritus to the nutrition of each consumer was determined from the ratio of $k(2,1)$ to $k(0,3)$. To determine the values of these parameters (transfer rates) for crabs, modelling was done, as described in Chapter 5, using hepatopancreas $\delta^{13}C$ values. This tissue was identified in Chapter 5 as having the fastest turnover rate, and therefore being the most suitable tissue for monitoring uptake of $^{13}C$ (Hamilton et al. 2004). As described in Chapter 5, a good fit of the model to the data was when fractional standard deviations (FSDs) associated with each parameter in the model...
were < 0.5. Discrepancies between observed (refer Table 2) and predicted (33%) δ\(^{13}\)C values for detritus just after addition hinted at similar contamination with non-detrital material to that observed in Chapter 3. Particularly for smaller size fractions, for which removal of non-enriched, non-detrital material is more difficult, inclusion of contaminants with mangrove detritus would dampen the observed δ\(^{13}\)C, as observed (Table 6.2). The δ\(^{13}\)C values for the 1000-2000 µm size fraction of detritus were therefore used in the model, as these were considered to be least affected by contamination with non-detrital material.

Table 6.2: Values of δ\(^{13}\)C observed for size fractions of detritus just after addition of enriched detritus demonstrating greater divergence from the expected δ\(^{13}\)C value (33%) for smaller size fractions.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>δ(^{13})C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000-2000 µm</td>
<td>-11.5</td>
</tr>
<tr>
<td>500-1000 µm</td>
<td>-22.9</td>
</tr>
<tr>
<td>250-500 µm</td>
<td>-24.3</td>
</tr>
<tr>
<td>125-250 µm</td>
<td>-24.9</td>
</tr>
<tr>
<td>53-125 µm</td>
<td>-26.9</td>
</tr>
</tbody>
</table>
6.3 Results
The most enriched $\delta^{13}C$ value (-11.5‰) obtained for the 2 mm size fraction of detritus was removed from sediment collected immediately after addition of detritus. One week following addition of detritus, however, 2 mm detritus in the enriched plots (-28.8‰) was within 1‰ of that in control plots. Thereafter, the $\delta^{13}C$ values of the three treatment types were indistinguishable (Fig. 6.1). Throughout the experiment $\delta^{13}C$ values of detritus and consumers within control plots did not vary. Similar $\delta^{13}C$ values for consumers in control and procedural control plots also show that addition of detritus, and the processes involved, did not alter the diet of consumers in a way that altered their isotope signatures. The consistent values for controls and procedural controls also indicate that there was no significant transfer of label among treatment types.

Figure 6.1: Observed $\delta^{13}C$ values throughout the experimental period for detritus in the 1000-2000 µm size fraction in enriched plots (triangles). Grey bar represents $\delta^{13}C$ values of detritus of the same size in control plots, centred around the mean and with width of bars showing ± S.E.
The tissues of *Australoplax tridentata* became only marginally enriched relative to control plots during the experiment (Fig. 6.2). However, all tissues of *Parasesarma erythrodactyla* were enriched within 7 days of the addition of enriched detritus, when the peak in enrichment was observed (Fig. 6.2). Muscle tissue became only marginally enriched (-21.3‰ at 7 days, compared to –23.2‰ in controls). Although gill and hepatopancreas tissues responded similarly to enrichment (-19.0‰ and -23.9‰ at 7 days, respectively), the difference between δ13C in control and treatment plots at this time was smaller for gill (4.9‰) than for hepatopancreas (5.2‰), confirming the greater turnover rate of the hepatopancreas, as observed in Chapter 5. For both tissue types, a second, lower peak in 13C-enrichment was evident (-22.6‰ and -24.6‰, respectively) at day 21.

Of the foraminifera, only *Ammonia beccarii* became enriched. There was no evidence of enrichment for *Trochammina inflata* throughout the experimental period with δ13C values for individuals in enriched plots (average = -25.1‰) remaining similar to values for *T. inflata* in control plots (-24.7‰) (Fig. 6.3). *A. beccarii* attained a maximum δ13C of -21.0‰, noticeably enriched compared to control plots (-26.1‰) at day 7, and had a second, lower peak in 13C-enrichment (-22.7‰) at day 21.

Compartment modelling was applied to the two consumers that became enriched during the experimental period (*Parasesarma erythrodactyla* and *Ammonia beccarii*). For *P. erythrodactyla*, WinSAAM modelling using δ13C of the hepatopancreas (Fig. 6.4) determined the transfer rates from detritus to *P. erythrodactyla* (k(2,1)) and from *P. erythrodactyla* to the system (k(0,2)) to be 0.3464 and 0.3916, respectively (Table 6.3). The ratio of these showed that 88% of the nutrition of *P. erythrodactyla* was derived from mangrove detritus. However, FSDs > 0.5 (2.63 and 2.79, respectively) indicate a rather poor fit for the model, and therefore a lower confidence in the estimate of detritus contribution to consumer nutrition (Stefanovski et al. 2003).

Modelling with WinSAAM (Fig. 6.5) showed transfer rates from detritus to *Ammonia beccarii* (k(2,1)) and from *A. beccarii* to the system (k(0,2)) to be 0.8175 and 0.9727 (Table 6.3). Based on these parameters, *A. beccarii* was found to derive 84% of its nutrition from mangrove detritus. However, FSD values > 0.5 (6.95 and 7.14, respectively) again demonstrate the rather poor fit of the model. There is a
greater error associated with the estimate of detritus contribution to the nutrition of A. beccarii than for P. erythrodactyla.

Figure 6.2: $\delta^{13}$C values for hepatopancreas, muscle and gill tissues of the crab species, Australoplax tridentata and Parasesarma erythrodactyla in enriched plots throughout the experimental period. Grey bars represent $\delta^{13}$C values for hepatopancreas in control plots (mean ± S.E.).
Figure 6.3: δ¹³C values for *Ammonia beccarii* and *Trochammina inflata* in enriched plots throughout the experimental period. Grey bar represents δ¹³C of *A. beccarii* in control plots (mean ± S.E.).

Figure 6.4: Line depicting output from the model that best fit the observed δ¹³C values for *Parasesarma erythrodactyla*. 
Table 6.3: Natural abundance $\delta^{13}C$ and maximum enrichment of detritus and consumers, and calculated parameters and results of WinSAAM modelling. FSDs in brackets. n.a. = not applicable, - = no model generated.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Natural abundance ($\delta^{13}C$, ‰)</th>
<th>Maximum enrichment ($\delta^{13}C$, ‰)</th>
<th>Transfer producer to consumer $k(2,1)$</th>
<th>Transfer consumer to system $k(0,2)$</th>
<th>% contribution of detritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. marina detritus</td>
<td>-29.4</td>
<td>-11.5</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>A. tridentata hepatopancreas</td>
<td>-26.2</td>
<td>-24.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. erythrodactyla hepatopancreas</td>
<td>-26.4</td>
<td>-21.2</td>
<td>0.3464 (2.63)</td>
<td>0.3916 (2.79)</td>
<td>88</td>
</tr>
<tr>
<td>A. beccarii</td>
<td>-26.1</td>
<td>-21.0</td>
<td>0.8175 (6.95)</td>
<td>0.9727 (7.14)</td>
<td>84</td>
</tr>
<tr>
<td>T. inflata</td>
<td>-24.9</td>
<td>-24.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6.5: Line depicting output of model that best fit the observed $\delta^{13}$C values for *Ammonia beccarii*.
6.4 Discussion

This study confirmed that not all consumers utilise mangrove detritus as a carbon source, but mangrove detritus is the major source of carbon utilised by the grapsid crab, *Parasesarma erythrodactyla*, and the foraminifera, *Ammonia beccarii*.

Previous studies have suggested that mangrove detritus is the main source of nutrition for grapsid crabs, with observations of crabs, including *Parasesarma erythrodactyla* in southeast Queensland (Camilleri 1992), grazing on large quantities of mangrove litter (Robertson 1986). However, such studies have primarily relied upon laboratory-based feeding experiments (Abed-Navandi et al. 2005), gut content analysis (Dahdouh-Guebas et al. 1999) and observations in the field (Camilleri 1992). None of these methods are able to indicate assimilation of material, and laboratory-based experiments, in particular, may interfere with feeding behaviour (Micheli 1993). As discussed in Chapter 5, even natural abundance stable isotope studies, though capable of showing assimilation, may give false conclusions if not all available carbon sources are recognised. The manipulative field-based enriched experiment used in the current study, however, avoids these pitfalls.

The current study provides solid evidence that a grapsid crab, in this case *Parasesarma erythrodactyla*, is predominantly reliant upon mangrove leaf litter for its carbon requirements. As expected, modelling also showed that this is not the sole source of nutrition, with an alternative source estimated to provide 12% of the carbon requirements of this species. In Chapter 5, I estimated that 35% of the diet of *P. erythrodactyla* was derived from MPB. Although this exceeds the proportion estimated to be obtained from alternative sources in the current chapter, FSDs > 0.5 associated with compartment model parameters for *P. erythrodactyla* in both experiments indicate that there is some error in estimates of producer contribution. Given that FSDs associated with parameters in Chapter 5 are smaller (i.e. better) than those in the current study, it is more likely that the contribution of detritus to the diet of *P. erythrodactyla* is somewhat lower than predicted. Nevertheless, it appears that the bulk of the carbon required by *P. erythrodactyla* is derived from mangrove detritus, with remaining carbon derived from MPB. It is possible that *P. erythrodactyla* may be actively feeding on both MPB and detritus. Alternatively, crabs may be selecting for detritus and incidentally ingesting MPB which has colonised detritus (Wannigama et al. 1998). They may also be selectively or
incidentally ingesting other consumers, including meiofauna, which have themselves consumed detritus and/or MPB.

Due to the relatively small size of foraminifera, direct determination of the composition of their diet is complicated. Fatty acid analysis has shown that foraminifera can assimilate phytodetrital material, bacteria and MPB to varying extents (Gooday et al. 2002, Topping et al. 2006) and stable isotope enrichment experiments in deep-sea sediments have demonstrated that foraminifera rapidly process microalgal detritus (Gooday et al. 2002, Moodley et al. 2002). However, the relative contribution of these sources to the diet of foraminifera has not previously been determined. The current study shows that the foraminiferan *Ammonia beccarii*, within a mangrove forest, has a similar dietary composition to that of *P. erythrodactyla*, deriving the majority of its carbon from mangrove detritus. It must be noted, however, that this is not the sole source of carbon; 16% of the carbon required by *A. beccarii* is contributed by a further carbon source. This carbon, as determined in Chapter 5, is provided by MPB. In the current study, FSDs > 0.05 indicate that model parameters are not very well defined, but this was less of a problem for the model describing uptake of $^{13}$C from MPB. In any case, carbon in *A. beccarii* is primarily derived from mangrove detritus and is supplemented by MPB. As for *P. erythrodactyla*, the δ$^{13}$C values observed do not necessarily indicate direct consumption of MPB and/or mangrove material by *A. beccarii*. For example, *A. beccarii* may obtain mangrove and MPB carbon via trophic transfer through consumption of faecal pellets from crabs with a diet of mangroves and MPB, and/or through consumption of meiofauna or bacteria with such a diet. *A. beccarii* has previously been reported as being reliant upon bacteria and MPB (Topping et al. 2006), but there is no record of reliance upon mangrove carbon. The wide geographic distribution of *A. beccarii* (e.g. United Kingdom, Castignetti 1996; Scandinavia, Alve & Murray 1999; Australia, Cann et al. 2002), and its tolerance of a wide variety of sediment characteristics (Alve & Murray 1999) means that it also occurs in habitats other than mangrove forests. In such cases, where carbon from mangrove detritus is unavailable, *A. beccarii* may acquire its carbon from detritus of other macrophytes. *A. beccarii* may be a generalist and therefore able to switch its diet to rely upon algae and/or detritus, depending on the dominance of the carbon sources available.
Any use of mangrove detritus by *Australoplax tridentata* or *Trochammina inflata* was minimal. The lack of enrichment in *A. tridentata* was not unexpected, given that I showed, in Chapter 5, that this species relies almost entirely upon MPB as a source of nutrition. The considerable difference in dietary composition of the two crab species (*P. erythrodactyla* and *A. tridentata*), despite them having access to the same carbon sources, provides evidence of feeding selectivity. Both crab species have been seen feeding on surface sediments containing MPB and mangrove detritus (pers. obs.). Whereas the diet of *P. erythrodactyla* reflects this, being a combination of MPB and detritus, *A. tridentata* appears to be more specialised, selectively feeding primarily on MPB. Such feeding selectivity has been observed in other ocypodid crabs which utilise specialised mouthparts to separate MPB from the sediment (Doi et al. 2005) and may be a way of partitioning resources to avoid competition, allowing species to co-exist (Putman 1994).

Although the foraminifera *Trochammina inflata* was found in Chapter 5 to derive only a small proportion of its carbon from MPB, there was no discernible contribution of mangrove detritus in the current study. The lack of enrichment could suggest that *T. inflata* utilises a further carbon source other than MPB or mangrove detritus (e.g. phytoplankton or root exudates), but more likely indicates that *T. inflata* did not have access to the enriched material. Previous studies have reported that *T. inflata* is a shallow (0-3 cm, Goldstein et al. 1995) or deep (to 60 cm, Hippensteel et al. 2002) infaunal species. In the current study, *T. inflata* individuals were collected from cores of the upper 5 cm of sediment. Although foraminifera are capable of migrating within sediments (Heinz et al. 2001), it is possible that *T. inflata* in the current study remained in deeper layers (i.e. closer to 5 cm depth) and so did not have access to the surface layers where enriched MPB and detritus was located. This highlights the need, in studies such as this, to have a good understanding of the movement of consumers of interest. The small transfer of $^{13}$C label from MPB in Chapter 5 may indicate an overlap in range with MPB migrating downwards into the sediment. Although detritus can also be transported downwards within sediments (Heinz et al. 2001), the $^{13}$C label in the current study disappeared so rapidly that this is unlikely to have occurred to any great extent. Determination of the contribution of detritus to the nutrition of *T. inflata* was further complicated by the initial low enrichment of mangrove detritus.
The initial $^{13}$C value of mangrove detritus within sediments in enriched plots (-11.5‰) was well below that expected (33‰). This may be due to there being more mangrove detritus naturally occurring in the sediment than anticipated, but this is unlikely given that sediment was well-characterised for the study site before addition of detritus. Non-homogeneous application of labelled detritus may also have resulted in sampling of an area with a lower content of enriched detritus, however this is also unlikely as efforts were made during sample collection to minimise the chance of this occurring. It therefore appears more likely that the relatively depleted signature immediately after addition of enriched detritus arose from contamination of detritus samples with non-enriched, non-detrital material unable to be separated from the detritus size fractions. Although the effects of this were minimised by modelling using only the largest size class of detritus (2 mm), for which removal of contaminants was easier, the presence of even small quantities of non-enriched non-detrital contaminants mean that the $\delta^{13}$C value of mangrove detritus within sediments may have been overestimated in the current study. Modelling may then have overestimated the contribution of mangrove detritus to consumers, as consumers would need to assimilate a greater quantity of less-enriched detritus to attain the same $\delta^{13}$C value. This may account for the high FSD values associated with model parameters in the current study, and the estimated combined contribution of MPB (Chapter 5) and detritus of over 100%. Taking into account the probable overestimation of mangrove detritus contribution, and the better FSDs for parameters in the models of MPB utilisation (Chapter 5), it is likely that both A. beccarii and P. erythrodactyla derive approximately 30% of their nutrition from MPB and 70% from mangrove detritus. Future studies may be able to limit overestimation of producer contribution by 1) obtaining a cleaner detritus fraction, 2) using fatty acids to quantify the level of contamination, and adjust $\delta^{13}$C accordingly, or 3) using compound-specific isotope analysis of a mangrove detritus biomarker to estimate $\delta^{13}$C of detritus in sediments for use in modelling. Further applications of $^{13}$C-enriched detritus should also aim for a more enriched initial detritus $\delta^{13}$C value to assist in detecting even minor use of detrital material by consumers. This should be achieved via addition of detritus which is more enriched, rather than through addition of a greater quantity of detritus, which may alter food availability to consumers. Bouillon et al. (2004b) found that $\delta^{13}$C values of consumers varied depending on the composition of organic matter in the sediment, reflecting differences in utilisation of
food sources brought about by altered availability. Significantly increasing the availability of mangrove detritus relative to other sources within the sediment would similarly affect utilisation of detritus, potentially giving misleading results in experiments such as that described in the current study.

Although there is scope for improvement of the methods applied in the current study, I found unequivocal evidence for the reliance of some consumers in southeast Queensland mangrove forests (e.g. *Parasesarma erythrodactyla* and *Ammonia beccarii*) on mangroves as a major carbon source. The considerable use of mangrove-derived material hints at a possible mechanism for transfer of carbon from mangroves to offshore environments. Near the site for the current study in Moreton Bay, southeast Queensland, Melville and Connolly (2003) found evidence that carbon from mangrove detritus contributes a minor, but measurable, amount to the nutrition of offshore fish. Regardless of the trophic pathways leading to assimilation of mangrove-derived carbon by benthic invertebrates, consumption of these invertebrates as a major component of the diet of more mobile consumers such as birds (Martinez 2004) and fish (Gee 1989, Sheaves & Molony 2000) offers potential for carbon from mangrove forests to be transported offshore via trophic relay (Kneib 1997). Similarly, consumption of MPB by benthic invertebrates may also result in MPB carbon being transported offshore via trophic relay to support offshore foodwebs. Therefore, although it may be true that material from mangrove forests is transported offshore, as has been the dominant paradigm (Odum & Heald 1975), the mechanism for this may not be direct transport of detritus, but rather trophic transfer.

The current study is the first to add $^{13}$C-labelled vascular detritus to coastal sediments to trace transfer of carbon to consumers. It was demonstrated that addition of $^{13}$C-enriched detritus to sediments, in combination with compartment modelling, can quantify the contribution of mangrove detritus to consumer nutrition. The methods used in the current study would be useful in resolving similar debates regarding the contribution of detritus to foodwebs in other systems, such as seagrass beds and saltmarsh. Within mangrove forests, however, wider application of the described method at different times of year, and for different consumers, would assist in the development of a broader understanding of the carbon sources of consumers in mangrove forests.
Chapter 7. General conclusions

In this chapter I first review the conclusions of the thesis and discuss their relevance to, and implications for, current issues in estuarine food web research. I then elaborate on the limitations of the current study, and highlight areas for further research.

7.1 Summary of findings

This thesis used a case study of a mangrove forest in southeast Queensland to demonstrate the use of an innovative combination of techniques to determine the relative extent of use of macrophyte detritus and microphytobenthos (MPB) as carbon sources for estuarine consumers.

The methods applied in the current study are expected to be useful for foodweb work in habitats with sandy or muddy sediments. Compound-specific isotope analysis of phytol was successfully applied to both muddy (Chapters 4 and 5) and sandy sediments (Chapter 4), although, for natural abundance stable isotope studies, some adjustment to δ\(^{13}\)C estimations may be needed to account for contamination with detritus-derived phytol. Application of \(^{13}\)C-enriched sodium bicarbonate was also shown to be effective in tracing transfer of carbon from MPB in sandy and muddy sediments. Direct assimilation of \(^{13}\)C by bacteria from bicarbonate applied to sediments was relatively minor. Label detected in consumers would therefore ultimately be derived from MPB, with bacteria as a potential intermediate trophic link.

Following verification of methods, experiments to enrich MPB and mangrove detritus with \(^{13}\)C and to trace subsequent uptake by consumers showed that either source can be important in the nutrition of consumers. The relative extent of MPB and mangrove detritus as a carbon source varied among consumer species. Whereas the ocypodid crab *Australoplax tridentata*, for example, was found to derive almost all of its carbon from MPB, the majority of carbon for the grapsid crab *Parasesarma erythrodactyla* was derived from mangroves. The carbon from MPB or mangroves was not necessarily ingested directly by consumers, and may have been assimilated via consumption of other food sources (e.g. meiofauna).
This has therefore been an important study for developing techniques to better understand trophic relationships of consumers and producers in estuarine habitats. For mangrove forests in southeast Queensland, it has provided solid evidence for the relative importance of macrophyte detritus and MPB as carbon sources for selected benthic invertebrates.

7.2 Implications for understanding estuarine food webs

In addition to resolving the carbon sources of consumers within a mangrove forest, the current study has greater implications for foodweb studies in general, in terms of the way in which carbon is utilised within estuarine systems.

I showed in Chapters 5 and 6 that for all consumer species studied except *Trochammina inflata*, MPB and mangrove material within a 1 m radius of where individuals were found accounted for virtually all of their carbon requirements. Pelagic sources or local sources that were not studied presumably provide carbon for *T. inflata*. For the most part, however, consumers apparently derive their carbon locally. This corresponds with the findings of Guest et al. (2004), who showed that *Australoplax tridentata* and *Parasesarma erythrodactyla*, the same crab species I studied here, relied upon carbon from within metres of where they were found in southeast Queensland mangrove forests. However, the natural abundance stable isotopes used by Guest et al. (2004) were unable to distinguish between two potential solutions which might explain the consumer δ¹³C values observed. Carbon was determined to be derived either solely from local sources (MPB within the mangrove forest) or from a mixture of local material (MPB and mangroves) and material (saltmarsh grass) imported from adjacent saltmarshes (Guest et al. 2004). Enrichment of potential carbon sources within a mangrove forest, described in Chapters 5 and 6 of this thesis, demonstrated that carbon for *A. tridentata* and *P. erythrodactyla* is obtained from within a 1 m radius and is therefore assimilated at the fine scale defined by Guest et al. (2004). This pattern of reliance on sources in the surrounding few metres does not occur in all estuarine environments, or even in all mangrove systems. In an Indian mangrove forest, for example, Bouillon et al. (2004b) observed that consumers derived carbon from organic matter imported from surrounding environments where it was available. This is indicative of the potential for assimilation of carbon within estuarine environments on a larger scale than that
observed in this thesis, and also hints that the scale of carbon acquisition and assimilation may depend on the availability of alternative carbon sources.

The utilisation of locally-derived organic matter demonstrated in this thesis may account for the rapid removal of labelled detritus observed in Chapter 6. Mangrove detritus can be removed from sediment either by tidal transport (i.e. outwelling, Lee 1995) or ingestion by consumers (Robertson 1986, Ashton 2002). Although there is little evidence to support tidal transport from macrophyte-dominated estuarine habitats (reviewed by Lee 1995), the rapid appearance of label in the consumers *Parasesarma erythrodactyla* and *Ammonia beccarii* reported in this thesis, provides evidence that detritus was removed via processing by benthic invertebrates. Individual consumers may assimilate only small quantities of carbon from mangrove detritus, however the great abundance of some benthic invertebrates (e.g. 30 crabs m$^{-2}$ in southeast Queensland, Snelling et al. 1959; 72-410 foraminifera per gram of wet sediment, Ellison 1984) means that the overall impact of consumers feeding on detritus can be considerable. Crabs within mangrove forests have been found to remove 79% of litterfall per day (Ashton 2002). Processing by foraminifera in mangroves appears not to have been measured, but benthic foraminifera have been found to be rapid processors of organic material in studies on the deep sea (e.g. removing 8.8 mg C m$^{-2}$ d$^{-1}$, Nomaki et al. 2006). Similar rates of processing in the current study would account for the observed rapid disappearance of labelled detritus, particularly given that less abundant consumers which were not studied may also collectively consume large quantities of mangrove detritus. The rapid assimilation of macrophyte-derived carbon by consumers indicated in this thesis provides a mechanism for carbon retention within a forest, as opposed to direct export of particulate organic matter to adjacent waters. The conversion of macrophyte-derived material to invertebrate biomass would then make this carbon available to higher consumers that predate upon benthic invertebrates during tidal inundation (Sheaves & Molony 2000). This emphasises the potentially important role of meiofauna and macrofauna in the functioning of estuarine ecosystems dominated by macrophytes and may explain why, despite high productivity, many systems show little evidence of outwelling of detrital material (Rodelli et al. 1984, Newell et al. 1995, Loneragan et al. 1997, and see review by Lee 1995).
In addition to influencing detritus processing, benthic invertebrates may also influence MPB assemblages. Although utilisation of MPB carbon by consumers such as Australoplax tridentata may be indirect (i.e. via trophic transfer), the presence of microalgae in the guts of some invertebrates (Dahdouh-Guebas et al. 1999, Fleeger et al. 1999, Azovsky et al. 2005) means that it is possible that MPB carbon is obtained directly, via consumption, thereby potentially impacting upon MPB assemblages. There are numerous instances of studies finding that consumers make far greater use of carbon from microalgae than from surrounding vascular plants, possibly due to the less refractory, more palatable nature of algae (Lee 1999, Moens et al. 2002).

Evidence comes from habitats dominated by mangroves (Lee 2000, Bouillon et al. 2004a), seagrass (Fry et al. 1984, Kitting et al. 1984, Moncreiff & Sullivan 2001) and saltmarsh (Sullivan & Moncreiff 1990). Benthic invertebrates may be highly abundant, potentially creating intense grazing pressure on MPB (Rzeznik-Orignac et al. 2003, Webb & Eyre 2004), such that exclusion of grazers increases algal biomass in estuarine habitats (Gacia et al. 1999, Mak & Williams 1999, Blanchard et al. 2001). Of the consumers studied in this thesis, individuals of A. tridentata were found to be most reliant on MPB, and might therefore be considered to exert the major part of grazing pressure in this system, given that the species is also very abundant. However, Parasesarma erythrodactyla, Ammonia beccarii and Trochammina inflata also derived some carbon from MPB. In situations where these species are far more abundant than A. tridentata, grazing by these species may have a greater overall impact on algal productivity. This is not believed to be the case in the main study site for this thesis, but emphasises the importance of considering not only the dietary composition, but also the abundance of a consumer species when assessing the overall impact of a consumer species on carbon cycling in estuarine environments.

From this study it can be seen that consumers with the same available carbon sources can selectively utilise either MPB or mangrove detritus as their primary source of carbon. This feeding selectivity is clearly evident in the two crabs studied, with Australoplax tridentata almost solely reliant upon MPB and Parasesarma erythrodactyla obtaining most of its carbon from mangrove detritus. The wide recognition that many ocypodids rely upon MPB (Rodelli et al. 1984, France 1998, Doi et al. 2005) and grapsids upon mangrove leaf litter (Lee 1998, Thongtham &
Kristensen 2005) highlights that feeding selectivity may be a common phenomenon among crabs and suggests that there may be an advantage for these crabs to rely upon different diets rather than competing for the more palatable, less refractory MPB. A fundamental theory of community ecology is that similar co-existing species must have minimal niche overlap in order to co-exist (Pianka 1974, Putman 1994). The term ‘niche’ refers to the resources utilised by an organism (Putman 1994). By differing in their requirements, species are able to share available resources, thereby avoiding competitive exclusion (Pianka 1974). This phenomenon has been demonstrated through gut-content analysis of sesarmine crabs that feed in the mangrove canopy (Cannicci et al. 1999). The selective feeding of the two crab species studied in this thesis may be a strategy allowing the species to co-exist.

Although the diet of consumers may be influenced by competition with other species (Pianka 1974), carbon sources may also be determined by availability of food (Wainright et al. 2000). This may result in spatial and temporal differences in the relative use of potential carbon sources even within a consumer species or ecosystem type. Production of detritus by mangroves (Micheli 1993, Twilley et al. 1997) and saltmarshes (Currin et al. 1995) is known to vary seasonally. MPB can also be influenced by seasonal factors, as variation in shading by macrophytes impacts light availability, thereby affecting MPB biomass and productivity (Wainright et al. 2000, Doi et al. 2005). Tidal regime may also influence availability of carbon sources by allowing material from adjacent habitats to be imported to the system, but can also result in increased export of material from estuarine environments (Twillie 1985, Slim et al. 1997). Export of material may be further enhanced by heavy rainfall (Twillie 1985, Slim et al. 1997). Where this rainfall flows to an estuary from catchments with urban or agricultural areas, runoff can also introduce nutrients to the system (van Dover et al. 1992, Waldron et al. 2001, Nixon & Buckley 2002), potentially enhancing MPB growth at a time when detritus may be less available due to increased export. Differences in availability of carbon sources resulting from such factors may be reflected in consumer diets, provided that consumers are capable of switching diets. Species with less flexibility in diets will be affected, in terms of abundance and distribution, by changes in food availability. For example, if *Australoplax tridentata* which was found to rely heavily upon MPB for its carbon, was unable to vary its diet, shading of the area, thereby reducing MPB productivity,
may also lead to decreased abundance of *A. tridentata*. This effect might also manifest as small-scale variation in abundances of *A. tridentata* at any one time.

When available food sources change, many consumers are capable of switching their diets. In the natural estuaries of southeast Queensland, for example, snub-nosed garfish (*Arrhamphus sclerolepis*) consume seagrass and aquatic crustaceans (Waltham & Connolly 2006). In artificial canals adjoining these estuaries, however, seagrass is largely unavailable and this species was found by Waltham & Connolly (2006) to replace their natural diet with algae and terrestrial ants. As mentioned above, Bouillon et al. (2004b) also provided evidence of diet-switching, with epifauna, including crabs, making use of material imported to the mangrove forest when it was available. For crabs, this was reflected in the correlation between $\delta^{13}C$ values of sediment organic matter and those of the sesarmid crabs (Bouillon et al. 2004b). Similarly, Doi et al. (2005) found that $\delta^{13}C$ values of gastropods and an ocypodid crab reflected increased availability of suspended organic matter in the upper reaches of an estuary. Micheli (1993) also found that carbon sources utilised by crabs depended on the availability of different sources. Crabs maintained in the laboratory fed selectively on more decayed leaves whereas, in the field, feeding was less selective due to a shortage of food (Micheli 1993). The foraminifera in the current study are also expected to be capable of varying their diet. Both *Ammonia beccarii* and *Trochammina* spp. are widespread, and are found in diverse habitats, including those having no association with mangroves (e.g. intertidal mudflats in Cornwall, England; Ellison 1984). With the exception of MPB, available carbon sources are likely to be very different to those in the current study. For *A. beccarii*, which was found in this study to derive the majority of its carbon from mangrove forests, individuals found elsewhere must utilise an alternative food source, perhaps other forms of macrophyte detritus. The potential for spatial and temporal variation in the utilisation of carbon sources by various consumers highlights the importance of replicating studies, such as that described in this thesis, at different times and locations. Evidently, the results of a single study in one location cannot be extrapolated to all estuarine systems, or even all representatives of the one type of system.
Regardless of the ultimate source of carbon for consumers in estuarine environments, use of locally-derived carbon by benthic invertebrates ultimately makes this carbon available to mobile higher consumers. Many studies have provided evidence that benthic invertebrates, including foraminifera (Chong & Sasekumar 1984) and crabs (Davis 1985, Salini et al. 1990), are important prey items for fish and crustaceans. Some of these nekton species are of commercial importance (Robertson & Blaber 1992), utilising estuarine habitats when they are inundated by high tides. Larvae of crabs are highly abundant at certain times of year (Mazumder et al. 2006) and in Australia it has been shown that these are a major source of food for fish moving onto inundated saltmarshes (Hollingsworth & Connolly 2006). Any locally-derived carbon utilised by crabs in saltmarsh would be passed to the larvae, and then transferred to fish that consume them. This provides scope for considerable export of locally-derived carbon to offshore systems where it may support higher order consumers. The extent to which benthic invertebrates utilise MPB and macrophytes may thereby determine the contribution of these sources to the nutrition of higher consumers. This type of scenario should be imbedded, eventually, within the concept of trophic relay (Kneib 1997). Trophic relay describes the possibility that a series of predator-prey interactions results in a net transfer of organic matter from intertidal to deeper waters in the bodies of animals.

Evidence for the use of mangrove carbon by offshore fish was found by Melville and Connolly (2003) in southeast Queensland, near the study site for this thesis, and, in Malaysia, Chong et al. (2001) reported use of mangrove carbon by penaeid prawns offshore from mangroves. Many more studies, however, have been unable to find evidence of mangrove carbon supporting fisheries in adjacent and offshore environments (Rodelli et al. 1984, Loneragan et al. 1997, Bouillon et al. 2004a, and review by Lee 1995). Mangrove δ¹³C values in tissues of offshore species may also reflect a combination of any number of other sources. Mobile consumers are more likely to rely on a mixture of multiple sources, and the contributions of these sources are unlikely to be able to be resolved through direct comparison. In situations such as estuaries with numerous potential sources, newer modelling routines for analysis of isotope data, such as IsoSource (Phillips & Gregg 2003), are proving useful (Benstead et al. 2006). Isotope analysis, perhaps in combination with other markers such as fatty acids (Meziane et al. 1997), may help to resolve ultimate food sources.
supporting fisheries. Nevertheless, in the current study I managed to demonstrate that mangrove material and MPB within the mangrove forest are an important source of carbon for benthic invertebrates known to be ingested by mobile consumers, including species harvested commercially. Efforts to determine the importance of mangrove forests in maintaining fisheries must also, therefore, consider the potential role of this MPB. Given previous difficulties with obtaining $\delta^{13}C$ values for MPB, and the potential for MPB $\delta^{13}C$ values to vary with species and growth conditions (e.g. MPB $\delta^{13}C$ values from -15 to -31‰ resulted from differences in species and growth temperature in Chapter 2), it is unlikely that studies would have been able to demonstrate assimilation of MPB by higher consumers, even if this source had been considered. The phytol method used in conjunction with large-scale isotope enrichment experiments (e.g. Cole et al. 2002, Pace et al. 2004, Gribsholt et al. 2004, Kritzberg et al. 2006), may help to characterise local MPB sources and their importance in supporting higher consumers.

Although for some consumers (e.g. Australoplax tridentata) mangroves themselves are not an important source of carbon, mangroves are important in maintaining an environment for the alternative carbon source, MPB, that they do utilise. Mangroves trap fine particles and stabilise sediments, allowing the development of mudflats that favour MPB production (Tomlinson 1986). Where sediment is less silty, MPB still occurs, but biomass is generally lower. In Chapter 3, for example, I showed that sandy sediments contained less phytol, reflecting a lower biomass of MPB. Even for consumers which rely only on MPB, this lower autochthonous production may limit animal abundances. The potentially greater relative availability of autochthonous material may also result in changes to the diet of existing species and/or the development of a different suite of species to what would otherwise exist. Given the potential importance of invertebrates in the nutrition of higher consumers, maintenance of biodiversity and fisheries productivity may rely on sustaining not only highly productive MPB communities, but also mangroves to physically support MPB and provide an additional carbon source for some invertebrates.

7.3 Recommendations for future studies
The combination of methods I used in this thesis to quantify the extent of use of MPB and macrophyte detritus within a mangrove forest have wide applicability, and could
be applied to resolve other food web issues in various estuarine environments. This includes habitats with sandy sediments, which have been largely overlooked until very recently.

The phytol method could be instrumental in developing a greater understanding of the role in MPB in a variety of estuarine environments, and how this role might vary among times and locations. In the past, studies examining the importance of MPB have estimated $\delta^{13}C$ values for MPB using a variety of methods. However, as explained previously, these methods may be affected by contamination with non-MPB material (e.g. Dittel et al. 2000) and/or may bias against some components of the MPB assemblage (e.g. Cook et al. 2004), potentially leading to inaccurate estimates of $\delta^{13}C$ values and thus to incorrect estimates of the contribution of MPB to consumer nutrition. The subjective nature of past methods probably also means that some of the temporal and spatial variability reported for MPB is an artefact of biases inherent in the procedures. An advantage of the phytol method is that any error due to contamination with detritus can be accounted for and adjustments made to give accurate estimates of MPB $\delta^{13}C$ values. The greater objectiveness of the phytol method also allows more meaningful comparison of MPB $\delta^{13}C$ values across studies and laboratories.

Given that MPB $\delta^{13}C$ values can vary spatially and temporally, with proximity to enriched or depleted DIC sources (Bouillon et al. 2002a) and among species (Chapter 2), the availability of a widely applicable method is an advantage. Previous studies have sometimes been unable to obtain a sample of MPB for isotopic analysis and have resorted to using MPB $\delta^{13}C$ values from earlier studies for interpretation of their results (e.g. Hsieh et al. 2002 used MPB values from Currin et al. 1995 even though they worked on different continents). The potentially large variation in MPB $\delta^{13}C$ values over different temporal and spatial scales, however, means that these values may not be representative of the site being studied. The phytol method would provide $\delta^{13}C$ values for MPB where other methods are unsuccessful, thereby eliminating the error likely to be associated with using substitute MPB $\delta^{13}C$ values.

Because phytol does not contain nitrogen, it is limited to stable isotope studies of carbon. For studies examining nitrogen isotopes, compound-specific isotope analysis
of alternative biomarkers containing nitrogen would allow for the determination of δ¹⁵N values of MPB. A biomarker containing both carbon and nitrogen, such as chlorophyll, would have the potential to allow simultaneous determination of both δ¹³C and δ¹⁵N values. Chlorophyll degrades more rapidly than phytol (Rontani 2001), so contamination of samples with detritus-derived material would be less of an issue than for the phytol method. Previous use of chlorophyll as a proxy for phytoplankton (Sachs et al. 1999) also implies that a predictable relationship between chlorophyll δ¹³C and δ¹⁵N signatures and those of bulk algal material is likely. Existing methods for extracting chlorophyll from sediment for isotopic analysis (Sachs & Repeta 2000), however, are time-consuming and require equipment more specialised than for phytol extraction and analysis. If extraction and isolation of chlorophyll from sediments can be simplified and made more routine, compoundspecific isotope analysis of chlorophyll to determine δ¹³C and δ¹⁵N of MPB may be a viable option. The phytol method, or similar methods using alternative biomarkers such as chlorophyll, may also assist in determining the food web role of pelagic microalgae, particularly in offshore environments, however this would only be possible following validation of a predictive relationship between δ¹³C of bulk algal material and the selected biomarker.

The use of enrichment experiments and compartment modelling in this thesis proved to be an effective way to determine the contribution of carbon sources to consumer diets. Typically, food web studies using natural abundance isotopes are unable to specify food sources with certainty and are unable to quantify the extent of use of resources other than whether they are dominant or minor contributors, particularly where there are many available sources. Enrichment of available producers allows certainty in identification of carbon sources. Compartment modelling then allows for the use of these sources to be quantified, whilst removing the need for maintaining a constant enrichment of the producer and waiting for the consumer to attain equilibrium (Hamilton et al. 2004). As previously discussed, there is a need for replication on a small scale to characterise study sites and develop models with a greater degree of certainty, but larger scale variability among locations and seasons also needs to be addressed. Nevertheless, the current study demonstrates the first application of this combination of techniques to a macrophyte-dominated estuarine environment. These techniques have previously been applied to a freshwater system.
(Hamilton et al. 2004), and for labelling bacteria on an intertidal flat (van Oevelen et al. 2006b), but have potential to be used in other situations where natural abundance stable isotopes are unable to confidently resolve the source of nutrition for consumers. In all but the simplest systems, isotope enrichment and compartment modelling seem to be the best way to quantify the use of a producer by a consumer.

Previous studies aiming to establish the importance of macrophyte detritus to consumers using natural abundance stable isotopes have often been unable to distinguish between detritus and living plant material (e.g. saltmarsh, Currin et al. 1995; seagrass, Loneragan et al. 1997) because, for a variety of macrophytes (e.g. seagrass, Zieman et al. 1984; Currin et al. 1995; mangroves, Sheaves & Molony 2000, Werry & Lee 2005), carbon $\delta^{13}C$ values do not change significantly with decomposition. Determination of the relative contributions of microalgae and detritus can also be problematic (Fry et al. 1982). Enrichment, as described in the current study, is a relatively simple option for determining the role of detritus in consumer nutrition and has potential to be used in further situations, such as seagrass beds and saltmarshes, where the role of detritus is debated.

In seagrass beds, seagrass or epiphytes are the main sources of production available to consumers. However, similar to the situation in mangroves, living seagrass material is considered to be a refractory food source. Decreased C:N ratios of seagrass material generated during decomposition (Holmer & Olsen 2002) may improve its quality as a nutrition source, but epiphytic algae is often found to be the major source of carbon for consumers (Fry et al. 1984, Kitting et al. 1984, Moncreiff & Sullivan 2001). Natural abundance stable isotopes, however, are often unable to distinguish between seagrass and their epiphytes (Fry et al. 1982, Loneragan et al. 1997). Winning et al. (1999) demonstrated that $^{15}N$-labelling of seagrass and epiphytes resulted in distinct $\delta^{15}N$ values for these sources. This method, or a similar method to label seagrass with $^{13}C$, could also be used to generate labelled detritus to ascertain its importance in providing nutrition to consumers. Abed-Navandi et al. (2005) recently demonstrated utilisation of seagrass detritus by thalassinidean shrimp using such a method. Their laboratory study is weakened somewhat by the possibility that laboratory conditions may have affected feeding behaviour, particularly if availability of alternative sources was altered compared to the natural environment (Micheli
Labelling of seagrass detritus in situ would be preferable, and concomitant use of compartment modelling would help ascertain the extent to which such detritus provides a carbon source for consumers.

Enrichment of detritus in combination with compartment modelling might also be of use in studies of north-American saltmarshes and in sandy sediments. The high productivity of MPB in North American saltmarshes is widely acknowledged (Sullivan 1975, Sullivan & Moncreiff 1988, Newell et al. 1995), and stable isotopes indicate that MPB may be a dominant source of nutrition for estuarine and coastal invertebrates in and adjacent to saltmarshes (e.g. Sullivan & Moncreiff 1990, Currin et al. 1995). However, measurements of MPB $\delta^{13}$C values to date have been uncertain, as previously discussed, and though MPB may indeed be the major carbon source for consumers, there has been no definitive quantification of the role of saltmarsh detritus in providing nutrition. Use of the compound-specific isotope analysis of phytol may help to confirm the role of MPB, but enrichment of detritus, in combination with compartment modelling, is likely to be the best way to quantify any role of saltmarsh material in supporting consumers.

In sandy sediments, Rossi (2007) used enriched detritus to demonstrate the use of macroalgae as a source of nutrition by invertebrates. Despite the potential for macrophyte detritus to enter sandy sediments, however, there have been no attempts to look at the assimilation of added vascular plant detritus by benthic invertebrates on sandy sediments to date. Lee (1999) added mangrove detritus to sandy sediments and found that it did not affect the abundance or biomass of macrobenthos in the sediment. Stable isotope enrichment experiments may, however, demonstrate that the consumers present utilise vascular plant detritus when it is available. Such a study would help determine if allochthonous or autochthonous sources contribute to nutrition of meiofauna and macrofauna found in sandy sites.

In the enrichment experiments described in this thesis, I selected the crab tissue (hepatopancreas) which had the fastest turnover rate to enhance detection of label uptake by crabs. Previous studies have found similar differences in the stable isotope turnover rates of different tissue types to those observed among crab tissues in the current study (Tieszen et al. 1983, Hobson & Clark 1992). The potential difference in turnover rates of tissues within a consumer of interest demonstrates the importance of
selecting a tissue type with a turnover rate appropriate to the temporal scale of the research question of interest. Although, in this study, the hepatopancreas was useful in showing carbon that was recently assimilated, use of tissues with slower turnover rate (e.g. muscle or gill tissue in the case of crabs), might be more appropriate for studies to determine the source of food for consumers over previous weeks or months (Hesslein et al. 1993).

Knowledge of the range of movements of a consumer of interest is of importance in enrichment studies in the field to prevent the need to confine consumers, which might otherwise alter their feeding behaviour and/or the food sources available (Micheli 1993). In the current study, enrichment covered the entire range of the consumers (crabs) believed to be most mobile. Although the general lack of enrichment of the foraminiferan Trochammina inflata might indicate use of a further non-enriched food source within the enriched area, it may also indicate that the range of vertical movement of T. inflata did not bring it into contact with the enriched material. This emphasises the need to understand movement, both horizontally and vertically, for consumers of interest. For more mobile consumers only larger-scale enrichment or containment of food sources and/or animals will allow enrichment experiments to be successful.

As discussed previously, changes in food availability may alter the diet or distribution of consumer species in estuarine environments. Manipulative studies would help examine this. Using mangrove forests as an example, it may be possible to decrease shading in an area of mangroves to determine the impact on the availability of MPB and mangrove carbon and monitor if changes in availability are reflected in the diet and/or species composition of the consumers present. If shading was increased in the site used in the current study, for example, decreased MPB productivity may lead to a loss of Australoplax tridentata, for which MPB is normally thought to be the primary carbon source. Alternatively, we may see a shift in the diet of A. tridentata resulting in a greater reliance on mangrove detritus. Competition with Parasesarma erythrodactyla for mangrove detritus may then lead to exclusion of one of these species. This demonstrates the difficulty of predicting changes in animal distributions with small-scale changes in mangrove forest attributes using our current, limited foodweb understanding.
7.4 Final conclusion and recommendation

Mangroves provide services such as shoreline protection and can act as a sink for nutrients from catchments (Ewel et al. 1998, Dahdouh-Guebas et al. 2005). To date, however, despite calls for conservation of mangroves based on their role in providing nutrition to fisheries, there has been little irrefutable evidence that consumers utilise mangrove detritus as a source of nutrition (Fry & Ewel 2003). I have demonstrated the use of a combination of techniques which can be applied to show the extent to which consumers utilise carbon from producers in mangrove forests.

In this thesis, I showed that both mangroves and the MPB within the mangrove system can be major forms of nutrition for benthic invertebrates, though the relative importance of these varies among consumers. Benthic invertebrates such as those studied constitute a major source of nutrition for higher consumers, including species of economic significance in deeper water adjacent to mangroves (Robertson & Blaber 1992). This points to a pathway for carbon to be transported out of mangrove forests via trophic transfer. Destruction of mangrove habitats and subsequent loss of benthic invertebrates may therefore impact negatively upon fisheries.

The methods demonstrated in this thesis have potential application in resolving food web issues in other estuarine habitats, particularly to explore patterns in use of MPB and macrophyte detritus. Further application of these techniques at other locations and at different times would help to develop a broader understanding of trophic relationships between consumers and producers and to establish general patterns in utilisation of available carbon sources in estuarine systems.
8.0 References


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