Biomarkers (lipids, fatty acids and stable isotopes) to examine diet of the sea urchin *Evechinus chloroticus*.

Erica Vanesa Zarate

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Abstract

This thesis describes the use of biomarkers as tools to better understand the diet preferences and trophic position of *Evechinus chloroticus*, the dominant grazer of New Zealand rocky reefs. Biomarkers, such as lipids, fatty acids and stable isotopes, were combined to improve the knowledge of feeding habits of wild sea urchins from north-eastern New Zealand, revealing a high contribution from *Ecklonia radiata* to *E. chloroticus* diets, but also a contribution from *Carpophyllum plumosum* in the Hauraki Gulf.

The use of lipid and fatty acid composition of *E. chloroticus* gonads collected from three locations within the Hauraki Gulf revealed a digestive pathway from the brown seaweed, especially *E. radiata*, to gut contents, gut and gonads, confirming the storage role for nutrients played by gonads in sea urchins. Biosynthesis and selective retention of essential fatty acids seen in the gonads confirm their dual function, as a reproductive and storage organ. This pattern was consistent at the three locations. However, lipid and fatty composition showed a variation between different populations of *E. chloroticus* within the Hauraki Gulf, outer (Great Barrier Island), intermediate (Matheson's Bay) and inner gulf (Rakino and Rangitoto Islands), suggesting that environmental conditions, such as water temperature, light, turbidity, salinity and nutrients affect the lipid and fatty acid composition of seaweed. Additionally, artificial diets also had an effect on the fatty acid composition of *E. chloroticus*, indicating that this sea urchin species was able to biosynthesise and selectively retain essential fatty acids when they are present in high amounts in the diets.

Seasonal lipid variation was observed in three of the dominant seaweed species *E. radiata, C. plumosum and Cystophora torulosa*, from the Hauraki Gulf, with higher levels of lipids during winter and spring, when *E. chloroticus* is accumulating nutrients to prepare for gametogenesis. Application of a mixing model to the stable isotope data allowed quantification of the contribution of these potential food items to *E. chloroticus* diet. Almost 40% of the diet is derived from *E. radiata* and 40% from *C. plumosum*, making them the principal contributors to urchins feeding in Matheson's Bay. A very low contribution to its diet was made by *C. maschalocarpum* and *C. torulosa*. Additionally, a trophic level of 1.5 indicated that *E.*

chloroticus is also feeding on microorganisms living on the seaweeds or possibly has nitrogen-fixing bacteria resident in the gut.

A comparison of the fatty acid composition of two different species of sea urchins, *E. chloroticus* and *A. dufresnii*, from different geographical locations revealed that not only the diet, but also the environmental conditions and the reproductive stage of the animals affect their fatty acid composition. Both sea urchin species showed similar fatty acid composition to the dominant brown seaweeds of those environments, indicating that *E. chloroticus* feeds on *E. radiata* and *A. dufresnii* on *Undaria pinnatifida*.

The information presented in this thesis is valuable not only for ecological purposes but for future application in aquaculture as *E. chloroticus* is the only sea urchin commercially harvested in New Zealand.

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Chapter 1

General Introduction

One of the principal grazers of the subtidal rocky reefs of north-eastern New Zealand, the sea urchin *Evechinus chloroticus*, was the topic for this PhD thesis. Different biochemical approaches were used to better understand its diet and feeding habits to expand the ecological knowledge. The knowledge achieved in this thesis is described in the chapters that follow. Firstly, basic information about sea urchins in general and more particularly the biochemical analyses used are presented here to facilitate a clearer comprehension of the thesis as a whole.

1.1 Sea urchins

Sea urchins are part of the Phylum Echinodermata, Class Echinoidea. The name Echinoidea, which means "like a hedgehog", is derived from the fact that their spherical armless body is covered with spines (Barnes et al., 1968). Sea urchins are brown, black, purple, green, white and red, and some are multi-coloured. The sea urchin body can be divided into an aboral and an oral hemisphere, with the parts arranged radially around the polar axis. The oral pole bears the mouth and is directed against the substratum. The aboral pole contains the anal region.

Sea urchins are ecologically important as they play a central role in determining the structure and functioning of macroalgal forest communities (Lawrence, 1975; Schiel et al., 1986). Grazing activity by sea urchins can turn a kelp forest area into a region which lacks large brown seaweed, often called an "urchin barren" (Lawrence, 1975).

1.1.1 Distribution and habitat

Echinoids are exclusively marine and form a conspicuous element of the benthic megafauna as significant habitat formers of temperate rocky reefs (reviewed in Lawrence (2007)). Most sea urchins are adapted for an existence on rocks and other types of hard bottoms, and spines are used when moving. Some of them tend to seek rocky depressions, and some species are actually capable of excavating burrows in rocks. Sea urchins are typically found from the intertidal to hadal depths, and are common in most marine environments, excluding estuaries or on very exposed coasts (Lawrence, 2007).

1.1.2 Diet

Sea urchins feed with a highly developed scraping apparatus called Aristotle's lantern. The majority of sea urchins are grazers, scraping the substrate surface on which they live in with their lantern. They are described as generalist feeders (Lawrence, 2007) and are opportunistic in their mode of feeding (De Ridder et al., 1982). In fact, of all echinoderm taxa, sea urchins feed on the widest range of organisms, from soft-bodied organisms (e.g. plants) to hard-bodied animals (e.g. bivalves) (De Ridder et al., 1982). Their grazing activity often controls the distribution of kelps and other encrusting species so that, in the most extreme cases, sea urchin populations can, through over-grazing, turn productive kelp dominated marine communities into sea urchin barrens, devoid of kelp and associated species (Lawrence, 1975).

The food taken in by sea urchins is initially digested in the intestine but then is stored inside nutritive phagocyte cells in the gonads, primarily as lipid (Castell et al., 2004; Cook et al., 2000; Iverson, 2009; Liyana-Pathirana et al., 2002). In general, lipids are stored in animal bodies in reservoirs and these often substantial stores can later be mobilized to provide fuel for short or long-term energy demands (Pond, 1998). Lipids consist of a large group of chemically heterogeneous compounds, the majority of which include esters of fatty acids as part of their structure; these fatty acids are considered the "building blocks" of lipids (Iverson, 2009). Lipids and their component fatty acids are incorporated from the diet and accumulate over time representing an integration of dietary intake over days, weeks, or months, depending on the organism and its intake and storage rates (Iverson, 2009). The storage organs in sea urchins are the gonads, which have a multifunctional role and are known to reflect both the nutritive and reproductive status of individuals.

1.1.3 Economic importance

In addition to their ecological and physiological importance, sea urchin gonads are highly valued and considered as delicacies in many parts of the world. Sea urchins are prized for their yellow or orange gonads which have a caviar-like appearance and a bitter-sweet flavour (Fig. 1.1). In Asia they are eaten either as sushi (uni) or used in the preparation of soups, sauces, pastes and omelettes (Lawrence, 2007; Liyana-Pathirana et al., 2002), and in South American and the Mediterranean countries they are eaten raw and macerated in lemon, although several other local preparations are known. They have a high nutritional value,

which is attributed to their elevated contents of carotenoids, lipids, proteins and carbohydrates (Lawrence, 2007). In general, the edible species are found in shallow waters but not all of them have the same palatability; thus some abundant species like *Tetrapygus niger* from Chile, are not consumed as they are not palatable (Lawrence et al., 1998). In recent years, the increasing demand for sea urchin gonads has, in many cases, resulted in the overexploitation of natural stocks. This decline has concentrated worldwide interest in both the development of methods for sea urchin aquaculture to increase gonad yield and quality (Liyana-Pathirana et al., 2002) and the utilisation of previously unfished wild stocks of sea urchins. The only sea urchin species commercially harvested in New Zealand is the echinoid *Evechinus chloroticus*.

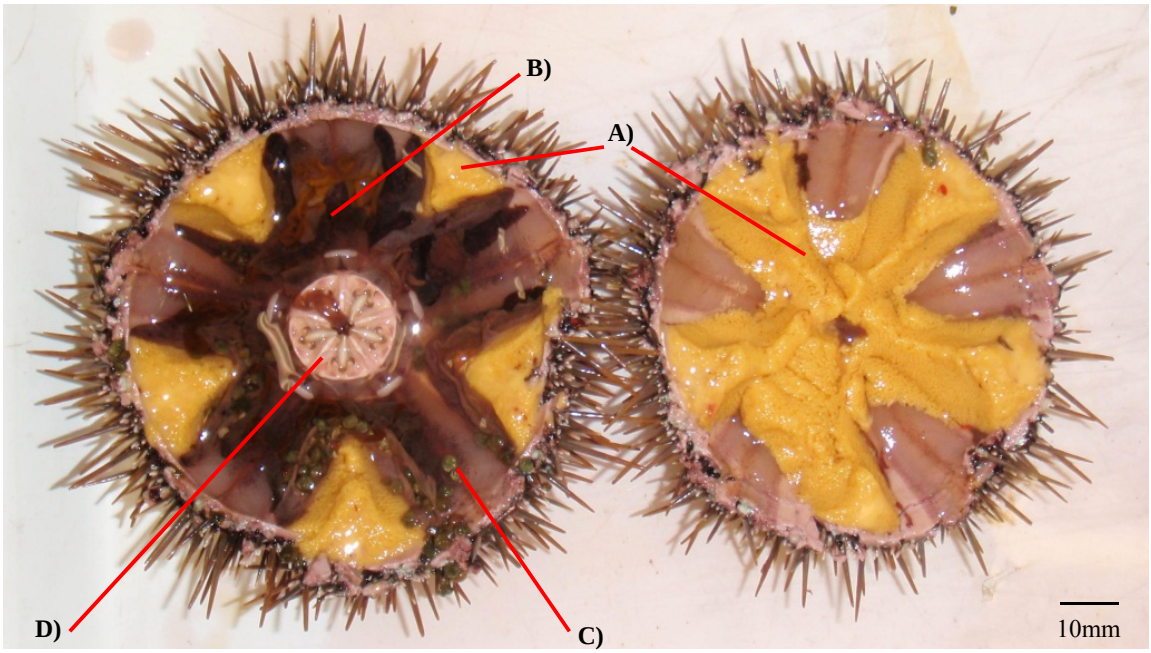


Figure 1.1. Wild *E. chloroticus* collected from the Hauraki Gulf showing: A) yellow gonad sacs, B) gut, C) gut contents and D) Aristotle's lantern.

1.2 Biomarkers

Biomarkers are compounds or groups of compounds, that can be used as an indicator of a biological state or condition in individual organisms or groups of individual organisms (Parrish et al., 2000). Molecular and isotopic biomarkers have being recently used as trophic biomarkers in ecological studies in marine ecosystems (Dethier et al., 2013; Parrish, 2013). These biomarkers are easily identified and metabolically stable, so they are transferred from

one trophic level to the other relatively unchanged, giving crucial information (Dalsgaard et al., 2003).

1.2.1 Lipids

Lipids are compounds that can store more energy per unit volume than other compounds as they are very rich in carbon, making them one of the most important source of energy in marine ecosystems (Parrish et al., 2000). It is hard to find a simple definition for lipids in literature, and most authors have used the chemical definition related to their solubility, defining lipids as a group of compounds that have in common that they are soluble in organic solvents such as hydrocarbons, chloroform, benzene, ethers and alcohols (Budge et al., 2006). Christie et al. (2010) define lipids as: fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds. Although, it is hard to define lipids without mentioning their constituent fatty acids, a description of fatty acids will be given in Section 1.2.2.

Lipids play essential functions in cells, where they serve as a major form of energy storage in animal and plant tissues and are responsible for maintaining the structural integrity of cells as a major component of cell membranes (Christie et al., 2010). The most common energy storage lipids are Triacylglycerols or Wax esters, whereas the most common structural lipids are Phospholipids and Sterols as part of the cell membrane (Fig. 1.2) (Budge et al., 2006; Christie et al., 2010).

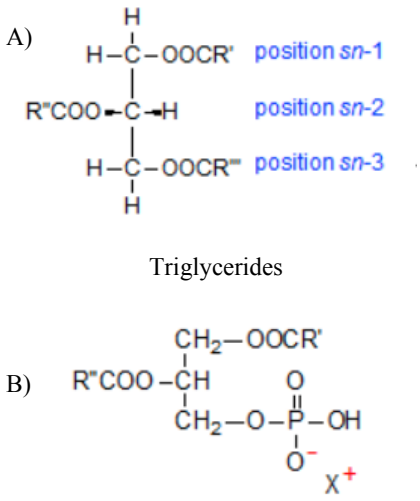


Figure 1.2. Structure of some lipids: A) Example of energy lipids and B) Example of structural lipid. Modified from Christie (2012).

The analysis of lipids has become more important in ecological studies as they are involved in many vital biological processes in animals, plants and microorganisms. Lipid provides the densest form of energy and their synthesis and accumulation by algae is the principal source of energy in marine ecosystems (Lee et al., 1998). Several studies have analysed lipids in marine environments, studying the total lipids or separating lipid classes, indicating the presence of certain types of organisms in their diets or the physiological state of the organism (Lee et al., 1972; Parrish, 2013; Sargent et al., 1977). It has been reported that wax esters are the energy reserves in various marine invertebrates like copepods (Sargent et al., 1988), whereas triglycerides serve as the main energy reserve in sea urchins (Liyana-Pathirana et al., 2002; Sewell, 2005).

1.2.2 Fatty acids

Fatty acid (FA) analysis is a powerful ecological tool for discerning trophic relationships in terrestrial and aquatic ecosystems (Budge et al., 2006; Dalsgaard et al., 2003; Iverson, 2009; Kelly et al., 2008). It has advantages over traditional stomach content analysis in that it can be used to examine either long-term dietary sources or those from recent feeding activity depending on the type of tissue selected (Fukuda et al., 2001; Iverson, 2009; Sargent et al., 1988). Consumers break down their dietary lipids into their constituent fatty acids and these are incorporated relatively unchanged into the tissues of the consumer (Howell et al.,

2003; Iverson, 2009; Lee et al., 1972). FAs are the largest constituent of neutral lipids, such as triacylglycerols and wax esters (the energy storage lipids) as well as of the polar phospholipids (primarily membrane structural components) (Iverson, 2009). They are compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex (Christie et al., 2010). In general, they contain an even number of carbon atoms in straight chains, although the synthesis can also produce to some extent odd- and branched-chain of fatty acids when supplied with appropriate precursors (Christie et al., 2010). Fatty acids can be grouped depending on the grade of unsaturation of their carbon chain into: 1) saturated fatty acids (SFA), those with no double bonds in the carbon chain, 2) monounsaturated FA (MUFA), with one double bond present and 3) polyunsaturated FA (PUFA) with two or more double bonds present (Budge et al., 2006) (Fig. 1.3).

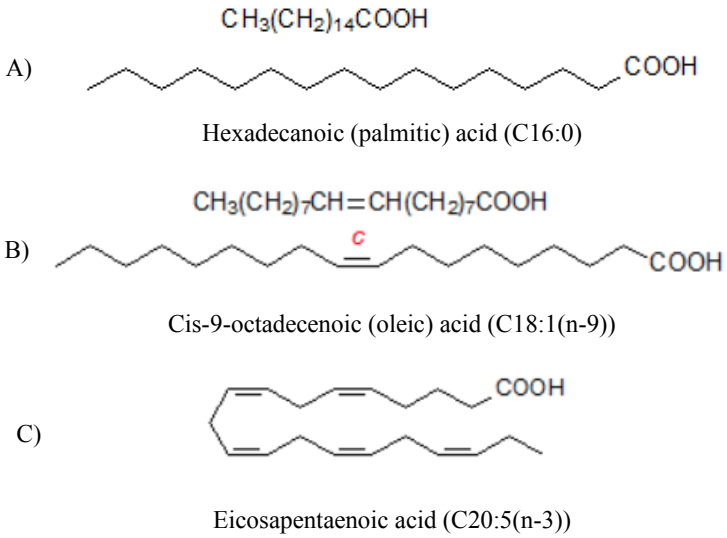


Figure 1.3. Structure of some fatty acids present in sea urchins: A) saturated fatty acids (SFA), B) monounsaturated fatty acids (MUFA) and C) polyunsaturated fatty acids (PUFA) (Christie, 2012).

Certain FAs are derived from specific sources, so the presence of unique classes of FAs or characteristic ratios of selected FAs can act as 'biomarkers', making it possible to identify the dietary source such as diatoms, dinoflagellates, bacteria, land plants and macroalgae (Iverson, 2009). Primary producers such as unicellular phytoplankton and seaweeds (macroalgae) typically produce in the chloroplasts FAs ranging from 14 to 24 carbon atoms with various degrees of unsaturation, and are essentially the only organisms that possess the enzyme needed for synthesizing long-chain PUFA (Iverson, 2009). These FAs flow through the marine food web to higher trophic levels, since animals are not capable of inserting a double bond between the terminal methyl end and the n-9 carbon which is needed to form PUFA (Iverson, 2009). In combination with additional data from stomach

contents, fatty acid analysis can provide information on the ecology of species and help in determining species specific importance in ecosystems as well as determining ecosystem structure and functioning (Iverson, 2009).

Several researchers have used FA analysis to understand animal diets and trophic relationships, especially in marine benthic habitats, where it is unpractical to discern the feeding behaviour of animals from direct observation (Iverson, 2009; Kelly et al., 2012; Parrish, 2013). In general, most of these studies have used information on the FA composition of possible food items and related this to the FA composition of the consumers (Budge et al., 2002; Wessels et al., 2012). Many authors have reported the FA composition and determined biomarkers for the primary producers, macroalgae (Khotimchenko et al., 2002; Li et al., 2002). For example, a recent review by Kelly et al. (2012) revealed that brown seaweeds are characterised by having high levels of C18:1(n-9), C18:4(n-3) and C20:4(n-6); green seaweeds have relatively high levels of C18:3(n-3) and red algae contain high levels of C16:0 and C20:5(n-3). The authors also found that higher levels of C18:1(n-9) distinguishes brown algae from other types of algae. Thus, using this information it is possible to discern the feeding habits of herbivorous like amphipods (Crawley et al., 2009; McLeod et al., 2013; Wessels et al., 2012), polychaetes (Drazen et al., 2008) and crustaceans (Budge et al., 2002). In rocky subtidal food webs most of the studies have focused in the identification of diets in different species of sea urchin, like *Psammechinus miliaris* (Hughes et al., 2005), *Strongylocentrotus droebachiensis* (Wessels et al., 2012), *Paracentrotus lividus* and *Arbacia lixula* (Hughes et al., 2005; Martínez-Pita et al., 2010a).

1.2.3 Stable isotopes

Fatty acid analysis is complemented by stable isotope analysis to clarify the trophic structure of marine systems (Guest et al., 2010; Kharlamenko et al., 1995). Stable isotope analysis, particularly carbon and nitrogen, is based on the fact that as carbon changes very little between successive trophic levels, the carbon isotope can often indicate the ultimate source of primary production at the base of a consumer's diet. The nitrogen isotope experiences greater fractionation per trophic level and is thus used to infer the trophic status of a consumer (Guest et al., 2010; McCutchan et al., 2003). Both isotopes can be used in different ways. Fractionation of $\delta^{13}C$ is limited to about 1‰ per trophic level, and it can therefore be used to identify ultimate carbon sources as it changes a little through the food web (Fry et al., 1984; Peterson et al., 1985). Conversely, the $\delta^{15}N$ content of consumers is

typically enriched by 3 to 4‰ relative to their prey, so results can be used to infer trophic connections in systems where feeding relationships are unknown (Hobson et al., 1992; Vander Zanden et al., 1999). Thus, combined measurements of carbon and nitrogen isotopes can provide information on both source materials and food web structure (Fredriksen, 2003). Therefore, when combined with biomarkers, such as lipids and FAs, stable isotopes can lead to a better understanding of a particular food web and the complex trophic relationships that occur in marine environments (Dethier et al., 2013).

1.3 The species to be studied

1.3.1 *Evechinus chloroticus*

Evechinus chloroticus (Echinoidea: Echinometridae), also known as kina, is endemic to the New Zealand region, ranging along the whole coast of the New Zealand mainland and nearby islands (Dix, 1970; Mortesnsen, 1943; Pawson, 1961). Kina typically dwells on rocky bottoms, but it may be found on other hard stable substrates, shelly sand, fine sand or mud (Dix, 1970; Fell, 1952; Morton et al., 1968). In the North Island, kina is most commonly found on shallow rocky reefs, strongly associated with kelp forests, being the most dominant grazer in these habitats (Barker, 2013; Dix, 1970; Schiel, 1982). These reefs are dominated mainly by brown algae of the order Fucales, which includes the genera *Carpophyllum* and *Cystophora*, and Laminariales with *Ecklonia radiata* as the most common species (Barker, 2013; Dix, 1970; Schiel, 1982). In general terms, kelp forests are extensively distributed along the north eastern coast of New Zealand, including offshore islands (Schiel, 1988).

Most ecological research on *E. chloroticus* has concentrated on the effects of its herbivory on algal communities, demonstrating a strong inverse correlation between sea urchins and the abundance of large brown algae (laminarians and fucoids) (Andrew et al., 1982; Ayling, 1978; Choat et al., 1982; Dix, 1970; Kerrigan, 1987; Walker, 1977). Herbivory by *E. chloroticus* has important consequences, not only for algal assemblages, but also for the local abundance of molluscan herbivores and several fish species (Andrew, 1988; Andrew et al., 1982; Choat et al., 1986; Don, 1975; Jones, 1984; Schiel, 1982). Feeding experiments have indicated that this sea urchin possesses a selective mode of feeding, being able to distinguish between algal species but with a preference for *E. radiata* (Cole et al., 2000; Don, 1975; Schiel, 1982). However, it can also feed on encrusting organisms, such as sponges, when algal food is scarce (Ayling, 1978).

Even though there is an abundant natural supply of kina in New Zealand (Booth et al., 2003) it has only been exploited for the domestic market (Barker, 2013). Attempts to develop an export market have been unsuccessful, as the gonads are variable in size and colour, and have been reported to have an intermittent bitter taste (McShane et al., 1994). Several studies have focused on methods for the improvement in the quality of *E. chloroticus* gonads; conducting laboratory feeding experiments as well as in the wild, but their gonads have not yet reached the requirements of the export market (James, 2006; James et al., 2008; Phillips et al., 2010; Woods et al., 2008). Consequently, both the sensory quality and consistency of *E. chloroticus* gonads need to be enhanced in order to develop a successful export industry in New Zealand (Goebel et al., 1998; Phillips et al., 2010).

1.3.2 *Arbacia dufresnii*

Arbacia dufresnii is a common sea urchin in the south-western Atlantic Ocean (Brogger et al., 2010). It distributes from Río de la Plata (~35°S) to Patagonia and the Malvinas Islands and around Tierra del Fuego to Puerto Montt in Chile, at a depth range between 0 and 315 m (Bernasconi, 1953; Brogger et al., 2010). In shallow water reefs communities in southern Chile, *A. dufresnii* plays an important ecological role through herbivory by altering benthic assemblages (Newcombe et al., 2012). On Argentinean coasts, *A. dufresnii* is usually found in areas of coarse sediments and hard substrates (Brogger et al., 2013), being the most abundant sea urchin in the north Patagonian gulfs (Zaixso et al., 2000), with its abundance diminishing towards the south. It has been described as a carnivorous in circalittoral mussel beds (Penchaszadeh et al., 1998); however, its ecological role at shallow coastal waters has not been studied. This sea urchin species can be considered herbivorous when algae constitute the only feeding source, grazing mainly on *Macrocystis pyrifera* (Vasquez et al., 1984). Furthermore, laboratory experiments showed that *A. dufresnii* from Nuevo Gulf, Patagonia, Argentina can also feed on the invasive algae, *Undaria pinnatifida* (Teso et al., 2009).

Thesis structure and research objectives

Overall objective

The principal aim of this thesis is to improve understanding of the feeding habits of sea urchins exposed to different environmental and artificial conditions by the use of different biomarker tools. Lipids, fatty acids (FA) and stable isotopes (SI) were investigated as biomarker tools to better understand the ecology, diet preferences, and trophic level of *E. chloroticus* and provide new data to increase the knowledge of seaweed-herbivore interactions in northern New Zealand, especially within the Hauraki Gulf. This information is valuable knowledge for future application in aquaculture, as *E. chloroticus* is the only edible sea urchin species that is commercially harvested in New Zealand. Thus, knowledge of the diet of wild *E. chloroticus* from different populations around northern-eastern New Zealand, the way that they assimilate lipids, FAs and stable isotopes from their potential food source in the wild and in controlled feeding experiments, and the comparison with other species is valuable information for future aquaculture in New Zealand.

Specific aims

Chapter Two

Lipid and fatty acid composition of *Evechinus chloroticus* gonad, gut, gut contents and possible food items (two brown seaweed species: *Ecklonia radiata* and *Carpophyllum maschalocarpum*) were examined to understand the energy flow from the diet to the sea urchin's storage organ, the gonad. The main aims of this chapter were: 1) study the assimilation and the accumulation of lipids and FAs from the brown seaweeds through gut contents, gut and gonad tissues of *E. chloroticus* as differences in the FA composition of tissues provide information to the tissue-specific FA requirements of animals and 2) determine if this pattern was consistent at three different locations from the Hauraki Gulf.

Chapter Three

The lipid (energy and structural lipid) and FA (SFA, MUFA and PUFA) composition of gonads of wild *Evechinus chloroticus,* as well as *Ecklonia radiata* plants, were used to explore trophic interactions of different populations around the Hauraki Gulf. To address this

aim, wild sea urchins and kelp plants were collected from two locations in the inner and the outer part of the Hauraki Gulf.

Chapter Four

The FA composition of *Evechinus chloroticus* gonads was used to examine how manufactured diets, differing in protein composition, influence the fatty acid composition of their gonads. To address this aim, sea urchins fed either one of two manufactured diets (high protein) or wild-collected seaweed (low protein) as a natural diet for a period of 13 weeks in sea cages were analysed and compared with wild sea urchins as control. The results of this study are valuable information for future aquaculture of *E. chloroticus* in New Zealand.

Chapter Five

Lipid composition of the dominant brown seaweeds from north-eastern New Zealand was used to determine seasonal variation in these potential food items of *E. chloroticus*. To address this aim, plants of *Ecklonia radiata*, *Cystophora torulosa*, *Carpophyllum plumosum* and *Carpophyllum maschalocarpum* were collected from the Hauraki Gulf, New Zealand during the four seasons of 2010. This is valuable information to better understand how the lipid composition of the possible food items of *E. chloroticus* changes throughout the year and how does this relate to the biochemical composition and reproductive stage of the sea urchin.

Chapter Six

Stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$) of *Evechinus chloroticus* tissues (gonad and gut) and its potential diet items, the brown seaweed species *E. radiata*, *C. torulosa*, *C. plumosum* and *C. maschalocarpum*, was analysed to clarify the feeding habits of *E. chloroticus*. Understanding diet preferences as well as the trophic level of *E. chloroticus* is important to better understand its ecology and provide new data on seaweed-herbivore interactions in northern New Zealand, complementing the results from previous chapters.

Chapter Seven

The aim of this chapter was to determine how the natural diet of sea urchins influences the FA composition of their gonads by sampling two sea urchin species from different habitats with different diets. Both sea urchin species differ in their diets: the Argentinean *Arbacia dufresnii* being omnivorous or herbivorous depending on the food availability, while *E. chloroticus*, endemic to New Zealand, is mainly herbivorous. By studying two species with different diet compositions the general applicability of this approach for diet analysis in sea urchins was examined and tested for *de novo* biosynthesis of fatty acids as recently described in other sea urchin species.

Chapter Eight

In the General Discussion, the use of biomarkers, lipid and FA composition and also stable isotopes of different tissues (gonad, gut and gut contents) of *E. chloroticus* from the wild as well as feeding experiments are brought together with information about their potential food items, the dominant brown seaweeds. This chapter summarizes the information and conclusions of the previous chapters and provides direction for future research.

Chapter 2

Lipid and fatty acid profile of *E. chloroticus* gonad, gut, gut contents and potential food items from the Hauraki Gulf, New Zealand.

2.1 Introduction

Sea urchins form a conspicuous element of the benthic megafauna and are significant habitat formers of temperate rocky reefs, reviewed by Lawrence (2007). They are typically found from the intertidal to 60-80m depth, and in most marine environments, excluding estuaries, or on very exposed coasts (Lawrence, 2007). Sea urchins are described as generalist feeders (Lawrence, 2007) and are opportunistic in their mode of feeding (De Ridder et al., 1982). In fact, of all echinoderm taxa, sea urchins feed on the widest range of organisms, from soft-bodied organisms (e.g. plants) to hard-bodied animals (e.g. bivalves) (De Ridder et al., 1982). Additionally, their grazing activity often controls the distribution of kelps and other encrusting species, so that in the most extreme cases, sea urchin populations can, through over-grazing, turn productive kelp dominated marine communities into low productivity sea urchin barrens, devoid of kelp and associated species (Lawrence, 1975).

The sea urchin *Evechinus chloroticus* (Echinoidea: Echinometridae), also known as kina, is endemic to the New Zealand region, ranging along the coast of the New Zealand mainland and nearby islands (Dix, 1970; Mortesnsen, 1943; Pawson, 1961). Kina is normally found in water less than 12-14m deep and is typically a rocky bottom dweller, but it may also be found on other hard stable substrates or on shelly sand, fine sand and mud, and in the rocky intertidal zone (Dix, 1970; Fell, 1952; Morton et al., 1968). In addition, its abundances generally increase with increasing exposure to wave action, except in the most exposed locations, where densities are reduced (Barker, 2013; Choat et al., 1987; Choat et al., 1982).

Most ecological research on *E. chloroticus* has concentrated on the effects of its herbivory on algal communities, demonstrating a strong inverse correlation between the abundance of large brown algae (laminarians and fucoids) and *Evechinus* (Andrew et al., 1982; Ayling, 1978; Choat et al., 1982; Dix, 1970). Herbivory by *E. chloroticus* has important consequences, not only for algal assemblages (Andrew et al., 1982; Don, 1975; Schiel, 1982), but also for the local abundance of molluscan herbivores (Andrew et al., 1982;

Choat et al., 1986) and several fish species (Andrew, 1988; Choat et al., 1987; Jones, 1984, 1988). Laboratory feeding experiments suggest that *E. chloroticus* consumes a large range of brown seaweeds, but has reference for *Ecklonia radiat*a (Barker, 2013; Don, 1975; Schiel, 1982).

In addition to their ecological and physiological importance, sea urchins are highly valued and considered as delicacies in many parts of the world (Lawrence, 2007). Sea urchins are prized for their yellow gonad sacs which have a caviar-like appearance and a bitter-sweet flavour. In Asia they are eaten either as sushi (uni) or used in the preparation of soups, sauces, pastes and omelettes (Lawrence, 2007; Liyana-Pathirana et al., 2002), whereas in South American and the Mediterranean countries they are generally eaten raw or macerated with lemon (Lawrence, 2001). The gonads, as the storage organ in sea urchins, have a high nutritional value, which is attributed to their elevated contents of carotenoids, carbohydrates, proteins and lipids (Lawrence, 2007). The food taken in by sea urchins is initially digested in the intestine, but then is stored inside nutritive phagocyte cells in the gonads, primarily as lipid (Castell et al., 2004; Cook et al., 2000; Iverson, 2009; Liyana-Pathirana et al., 2002). Thus, gonads have a multifunctional role and their biochemical composition reflects both the nutritive and reproductive status of individuals (Hughes et al., 2006; Russell, 1998; Walker et al., 2007; Walker et al., 2001). Furthermore, their dual function appears to result in greater biochemical and fatty acid modification than in the lipid storage areas of other invertebrate herbivores (Hughes et al., 2006; Iverson, 2009; Kelly et al., 2008).

Fatty acid (FA) analysis is a powerful ecological tool for discerning trophic relationships in terrestrial and aquatic ecosystems (Budge et al., 2006; Dalsgaard et al., 2003; Iverson, 2009; Kelly et al., 2008). It has advantages over traditional stomach content analysis in that it can be used to examine either long-term dietary sources or those of recent feeding activity depending on the type of tissue selected (Fukuda et al., 2001; Sargent et al., 1988). Consumers break down their dietary lipids into their constituent fatty acids and these are incorporated relatively unchanged into the tissues of the consumer (Howell et al., 2003; Lee et al., 1971). Fatty acids are released from lipid molecules during digestion, but are generally not degraded, and are taken by tissues in their basic form (Iverson, 2009). They represent the "building blocks" of lipids and are the largest constituent of neutral lipids, such as triacylglycerol (TAG) and wax esters (WE), which are common energy storage lipids, as well as of the polar phospholipids (PL), which primarily form structural components such as cell

membranes (Iverson, 2009). Therefore, certain fatty acids come from specific sources, so the presence of unique classes of fatty acids or characteristic ratios of selected fatty acids act as 'biomarkers', making it possible to identify a dietary source such as diatoms, dinoflagellates, bacteria, land plants and macroalgae (Iverson, 2009; Iverson et al., 2004; Kelly et al., 2008; Parrish et al., 2000).

Primary producers such as unicellular phytoplankton and seaweeds (macroalgae) typically produce *de novo* fatty acids, ranging from 14 to 24 carbons with various degrees of unsaturation, and are the only organisms that possess the enzyme acetyl-CoA necessary for producing long-chain polyunsaturated fatty acids (PUFA) (Iverson, 2009). For example, high concentrations of some PUFA, such as C20:5(n-3) or EPA, C20:4(n-6) or ARA, C18:1(n-9) and C18:4(n-3), characterise brown seaweeds (Kelly et al., 2012; Khotimchenko, 1998). These fatty acids flow through the marine food web to higher trophic levels, as animals are not capable of inserting a double bond between the terminal methyl end and the n-9 carbon to form PUFA (Iverson et al., 2004). However, some invertebrate taxa, such as sea urchins, are capable of modifying dietary FA and elongating long chain FA from lower FA precursors (Kelly et al., 2012; Kelly et al., 2008; Liyana-Pathirana et al., 2002). Feeding experiments have shown that *Strongylocentrotus droebachiensis* is capable of biosynthesizing FA that were not present in the diets and suggested that the dual function of the gonads increases this effect (Kelly et al., 2008; Liyana-Pathirana et al., 2002). Sea urchins are also capable of selective retention of some FAs that are present in low or trace concentrations in the diets (Castell et al., 2004; Kelly et al., 2012; Kelly et al., 2008). For example, *Psammechinus miliaris* selectively retained C20:4(n-6) and C20:5(n-3) when these PUFA were present in very low concentrations in the diets (Cook et al., 2000; Kelly et al., 2012).

As it is well known that the lipid energy flows from lower to higher trophic levels through the marine food web, the aim of this study was to examine how the lipid and FA composition changed from the potential food of *E. chloroticus* (brown seaweed), through the gut contents, gut and finally the storage organ, the gonads. Dethier et al. (2013) showed that the FA profile in marine macrophytes differs among sites and dates and it can be used as a biomarker to distinguish macrophyte taxa. For this reason, in the present study two of the most common brown seaweed species (*Ecklonia radiata* and *Carpophyllum maschalocarpum*) and *E. chloroticus* tissues (gonad, gut and gut contents) were collected from three different locations around the Hauraki Gulf. This investigation was focused,

firstly, in the possible capability of *E. chloroticus* tissues (gut contents, gut and gonad) to biosynthesise and/or selectively retain particular lipids and FAs, as differences in the FA composition of tissues provide information to the tissue-specific FA requirements of animals (Castell et al., 2004); and secondly, if this pattern was consistent at the three locations.

2.2 Methodology

2.2.1 Sample collection and storage

Wild *E. chloroticus* and two of the most dominant species of brown seaweed (*Ecklonia radiata* and *Carpophyllum maschalocarpum*), as potential diet items, were collected by snorkelling from 3 different locations in the Hauraki Gulf, New Zealand during the austral summer of 2010. The locations were: Matheson's Bay (36^0 29'S, 174^0 53'E) collection date 11th of February 2010; Rakino Island (36^0 72'S, 174^0 95'E), collection date 24th of January 2010 and Rangitoto Island (36^0 80'S, 174^0 86'E), collection date 17th of February 2010. In total, 20 sea urchins were collected at each location along with five individuals of each of the two seaweed species, from which two blades were sampled per individual.

After collection, the sea urchins, together with the seaweed species, were transported alive to the laboratory in plastic buckets filled with seawater and sea ice. Wet weight (blotted with a paper towel before weighing to the nearest mg) and test diameter (using a Toledo Vita calliper to nearest mm) of the whole sea urchin was recorded prior to dissection. The test was cut around the equator to separate the oral and aboral surfaces, and the Aristotle's lantern was removed and weighed together with the wet test. The coelomic fluid was drained to waste and the gut contents were transferred to a 50ml polypropylene tube. The 5 gonads, together with the gut, were removed using tweezers and they were weighed and stored separately in 50ml polypropylene tubes in the -80 freezer, along with the gut contents and seaweed, for further analysis. Gonad and gut indices were determined as in Phillips et al. (2009).

From the 20 sea urchins collected at each location, 10 were randomly selected for lipid and fatty acid (FA) analyses. Gonads, gut tissue, gut contents and seaweed material were lyophilized for 72 hours to a constant mass in a freeze-dryer (VirTis Bench Top 2k). The samples were cryogenically ground using a MM301 Mixer Mill (Retsch), to a ground

particle size of <5µm. Lyophilized, ground samples were stored in sealed polypropylene 10ml tubes under desiccant at -20°C until the lipid or FA analysis.

2.2.2 Lipid analysis

Lipid determination was carried out using an Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods following the protocols defined by Parrish (1997, 1999) with minor modifications. Lyophilized tissue was weighed (~8 mg for gonad, ~12 mg for gut, ~15 mg for gut contents and ~17 mg for seaweed) into 1ml V-vials (Wheaton). Lipid extraction was performed as described in Sewell (2005) with the following modification: 250µl of ultrapure water, 25µl of ketone in chloroform (used as internal standard, as natural concentrations are low in marine tissues), 100µl chloroform and 250µl methanol (final ratio of water: chloroform: methanol 2:1:2) were added to the V-vial before 20 minutes of sonication on ice in an ultrasonication bath (Sanyo Soniprep 150). After vigorous shaking the V-vials were centrifuged at 2000 RPM for 5 minutes at room temperature. Both the aqueous and chloroform fractions were transferred with a drawn Pasteur pipette to a clean glass V-vial, leaving the solid non-lipid material behind. An additional 250µl water and 250µl chloroform were added and the vial was shaken for a further 1 to 2 minutes. Subsequently, the sample was re-centrifuged at 2000 RPM for 5 minutes at room temperature. Most (90%) of the upper water and methanol fraction was removed with a Pasteur pipette and discarded, with little disturbance to the interface between the aqueous and chloroform layers. The lower chloroform layer (ca. 375µl = [100 + 250 added in extraction] + 25µl from internal standard) was transferred to a third V-vial using a 200µl Eppendorf pipette with a chloroform-rinsed tip. This V-vial containing the lipids suspended in chloroform layer was again stored in a -20^{o}C freezer until used for the Iatroscan analysis. All V-vials used in the extraction process were cleaned with 3x methanol and 3x chloroform washes as recommended by Parrish (1999), and all solvents used in lipid extraction were HPLC-grade. Immediately before spotting onto the Chromarods, the lipid extract was dried down in a stream of instrument grade nitrogen gas and an exact concentration of chloroform (75µl for gonad, 50µl for gut and 25µl for gut contents and seaweed) was added using a Gilson positive displacement pipette. Four separate samples were processed on each run (2 replicate Chromarods of each sample; total 8 rods). The remaining 2 Chromarods were unspotted blanks to test for contamination of the development solvents.

Chromarods were developed as described in Parrish (1999), except that instead of a triple development process, we used a double development system that resulted in two chromatograms. After spotting, the rack of Chromarods was placed inside a constant humidity chamber (CHC) for 5 minutes and then transferred to Development tank 1 (69.3ml of Hexane, 0.7ml of Diethyl-ether and 0.035ml of Formic Acid; 98.95:1:0.05) for 24 minutes. After this, the rack was placed again for 5 minutes in the CHC before returning to Development tank 1 for a further 19 minutes. The Chromarods were dried for 5 minutes in a Rod Dryer TK-8 (Iatron Laboratories) at 60°C and were then run in the Iatroscan set to Partial Scan Mode (PPS 27). After recording the first chromatogram, the rack was placed in the CHC for 5 minutes followed by 33 minutes in Development tank 2 (55.3ml of Hexane, 14ml of Diethyl-ether and 0.7ml of Formic Acid; 79:20:1). The Chromarods were dried for 5 minutes at 60°C and then they were run in the Iatroscan using a full 30 second scan and settings of 2000ml min–1 O_2 and 160 ml min–1 H_2settings as in Sewell (2005).

Quantification of the lipid in the sample was based on multilevel calibration curves generated for each lipid class found in the samples, plus the internal standard, on the rack of 10 Chromarods. Rods were calibrated with a 10-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform to cover the range of lipid concentrations present in the samples. Lipid classes were: Aliphatic Hydrocarbon (AH: Nonadecane), Wax Ester (WE: Miristyl dodecanoate), Methyl Ester (ME: Methyl palmitate), Ketone (KET: 3- hexadecanone), Triacylglycerol (TAG: tripalmitin), Free Fatty Acid (FFA: Palmitic acid), Sterols (ST: cholesterol), Diacylglycerol (DAG: 1,2 Dipalmitoyl-rac-glycerol), acetone-mobile polar lipids (AMPL: 1-monopalmitoyl-rac-glycerol) and Phospholipid (PL: L-α-phosphoditylcholine). AMPL includes glycolipids, pigments and any remaining neutral lipids from the PL and is normally separated in the acetone third development which was not used here (Parrish, 1987). However, the second development used here, separated a peak from PL that it was confirmed contained monoacylglycerols when spiked with the AMPL standard 1-monopalmitoyl-rac-glycerol, and is referred to as AMPL here. The stock solution of purified lipids was prepared using 5 mg of each lipid class dissolved in 1000 µl of chloroform using a Gilson Microman positive displacement pipette. Five serial dilutions were prepared from the initial stock solution, giving a total of 6 different dilutions of the lipid standards. The dilutions were run in the Iatroscan using the same methodology as was used for the samples. Quadratic regressions were used for lipid quantification as the FID response (Y-axis in a chromatogram) is curved at low

concentrations (< 1 to 5 µg) (Delmas et al., 1984; Parrish, 1987; Parrish et al., 1985). Peak areas from the calibration curves were calculated based on the mean of 3 separate Chromarods (r^2> 0.9968 for all lipid classes).

Lipids were quantified for each tissue sample (gonad, gut and gut contents) from each of the ten sea urchins and 2-3 individuals of seaweed species (2 extractions per sample and 2 rods per extraction) and analysed with the Iatroscan. The concentration of each lipid class per sample was determined using the percentage recovery of the internal standard in each sample and the calibration curve appropriate for each lipid class. Total lipid was calculated by the sum of the concentrations of each lipid class (AH, WE, ME, TAG, FFA, DAG, ST, AMPL, and PL) for each sample. Lipid classes can be grouped into energy storage and structural lipids. The concentration of energy storage was calculated by summing the concentration of TAG, DAG, FFA, AH, ME and WE; whereas the concentration of structural lipids was calculated by summing the concentration of PL, ST and AMPL. Values are presented as the mean (± SE) of the different tissue samples (n=10 for gonad, n=9-10 for gut and gut contents and n=2-3 for seaweed) in units of µg lipid per mg of lyophilized sample.

2.2.3 Fatty acid analysis

Fatty acid analysis was conducted using the one-step reaction from Lepage and Roy (1986), also known as the direct transesterification method. The same 10 sea urchins used for the lipid analysis were used for the FA analysis. Lyophilized tissue, sea urchin gonad (~50 mg), gut (~100 mg), gut contents (~150 mg), and seaweed (~150 mg) were weighed using a Sartorius balance (LE244S; max 240g; d=0.1mg) in pre-weighed glass tubes (KIMAX, culture tubes; 16x125mm; 20ml with phenolic caps and cemented rubber liners). An internal standard consisting of 50 µg to 300 µg of tridecanoic acid (C13:0) and tricosanoic acid (C23:0) dissolved in 2 ml of methanol-toluene 4:1 (v/v) was precisely weighed and added to the biological samples. These FAs were used as internal standards as used in previous studies of sea urchins (Chen, 2005; Cook et al., 2007) and seaweeds (Crawley et al., 2009). A small magnetic stirring bar was added to each tube and, while stirring, 200 µl of acetyl chloride were slowly added over a period of 1 min. The tubes were tightly closed and subjected to methanolysis at 100°C for 1 hour; the tubes were re-weighed after heating to check for leakage, if this was the case, the samples were restarted from lyophilized tissue.

After the tubes had been cooled in tap water to room temperature, 5 ml of 6% K_2C0_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged for five minutes at 2000 RPM, and an aliquot of the toluene upper phase was transferred to an autosampler vial and then injected into an Agilent GC 7890 gas chromatograph equipped with a mass spectrometry detector (MSD 5975c). Separation was performed with a 35-m fused silica column (internal diameter of 0.32 mm), wall-coated with 0.20 mm SP-2330, and with helium as the carrier gas. After injection at 60°C, the oven temperature was raised to 150°C at a rate of 40°C min^{-1}, then to 230°C at 3°C min^{-1}, and finally held constant for 30 minutes. Fatty acid methyl ester (FAME) peaks were identified by comparing their retention times with those of authentic 37 fatty acid methyl ester (FAME) standards (Supelco Inc.). The mass spectra of FAMEs not present in the standard mix were compared with those from the National Institute of Standards and Technology mass spectra library (NIST MS Search 2.0), as well as the lipid library of Christie (2012). Total FA was calculated by the sum of the concentrations of each individual FA for each sample. FAs can be grouped into Saturated FA (SFA: FA with no double bond in the carbon chain), Monounsaturated FA (MUFA: FA with one double bond present) and Polyunsaturated FA (PUFA: FA with two or more double bonds present). Each value is presented as the mean (± SE) of the different tissue samples (n=10 for gonad, n=9-10 for gut and gut contents and n=2-3 for seaweed) in units of µg FA per mg of lyophilized sample and also as the percentage of the total fatty acids identified.

2.2.4 Statistical analysis

For the statistical analysis, the two seaweed species (*E. radiata* and *C. maschalocarpum*) as well as gonad, gut and gut contents of *E. chloroticus* were classified as five different tissues. Lipid and FA profiles were compared among tissues as well as among locations (Rakino Island, Matheson's Bay, Rangitoto Island) using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). Gut and gonad samples were regarded as independent at the time of sampling for the analysis of the PERMANOVA. The data was left untransformed and converted into similarity matrices using Euclidean distances. Similarity patterns in the data were visualised using multidimensional scaling (MDS). Two-way PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the permutation method, was performed to examine differences in the lipid and FA profile among tissues and locations; pairwise comparisons were conducted when there

was a significant effect. The similarity percentages procedure (SIMPER) was used to explore the differences among tissues by determining which lipid classes and FAs contributed most to differences in the multivariate signature. Those lipids and FAs identified by the SIMPER were further compared by Univariate two-way PERMANOVA analysis. We used this approach as this test avoids the assumptions of the traditional one-way analysis of variance (Underwood, 1997) and assumes that the samples are exchangeable, meaning independent and identically distributed, under a true hypothesis (Anderson, 2003).

2.3 Results

2.3.1 Lipid Analysis

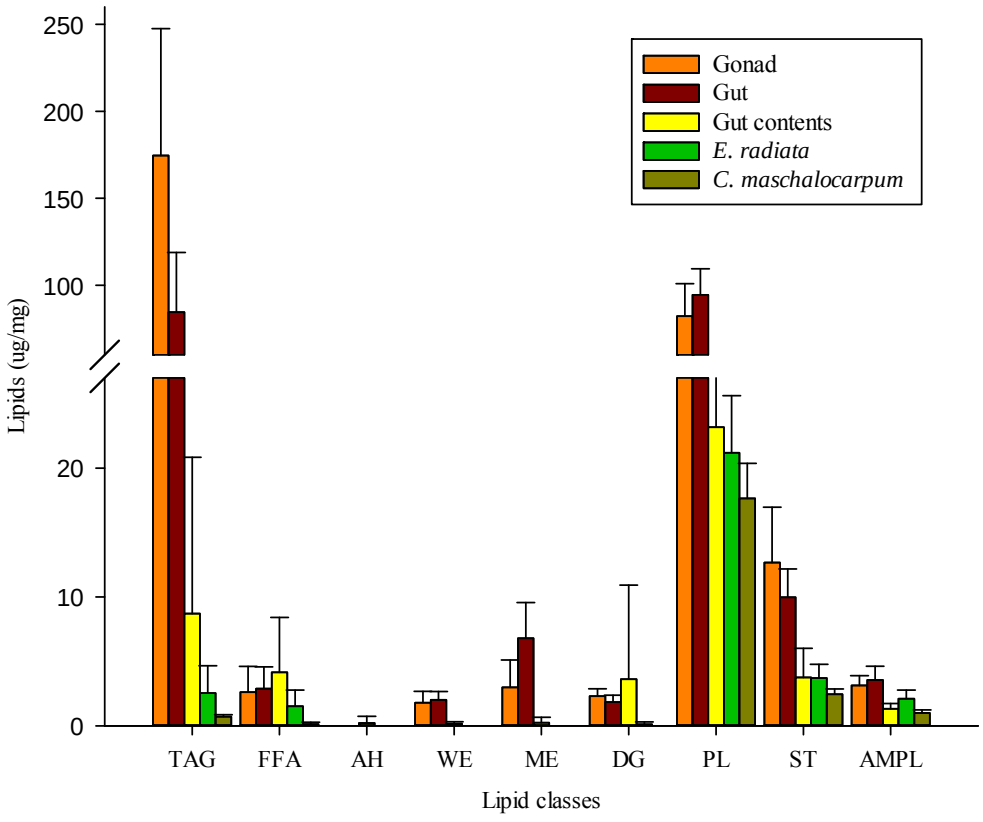
A total of nine lipid classes were detected in the different tissues (two different species of seaweed: *E. radiata* and *C. maschalocarpum* and *E. chloroticus*: gut contents, gut and gonad). Six different classes of energy lipids were identified: TAG, FFA, DAG, AH, WE and ME. However, these six classes were not present in all of the tissues and their concentrations also varied among tissues (Table 2.1; Fig. 2.1). TAG was the energy lipid present in the highest concentration and with the greatest variation amongst tissues (~172-0.8 µg/mg of dry mass; Table 2.1; Fig 2.1). The remaining energy lipids (FFA, DAG, ME and WE) were present in small or trace concentrations (<10 µg/mg of dry mass) and in some tissues were not detected. Three classes of structural lipids (PL, ST, and AMPL) were detected in all tissues; however, their concentrations also varied among tissues (Table 2.1; Fig. 2.1). PL was the structural lipid always present in the highest concentration, followed by ST and then AMPL (Table 2.1; Fig. 2.1).

The concentration of total lipid and also the concentration of energy and structural lipid varied significantly among tissues but not among locations (Table 2.2, Fig. 2.2). For this reason, the mean of the total, energy and structural lipid of the three locations was calculated for the following analyses. The seaweed *C. maschalocarpum* showed the lowest concentration of total lipid followed by the concentration in *E. radiata* and sea urchin gut contents which were not significantly different (Table 2.1; Table 2.2.D). Sea urchin gut had a relatively higher concentration of total lipid and *E. chloroticus* gonad showed the highest concentration of total lipid among all tissue types (Table 2.1; Table 2.2.D).

Table 2.1. Lipid classes (µg/mg of lyophilized weight) present in *E. chloroticus* gonad, gut, gut contents and two species of seaweed (*E. radiata* and *C. maschalocarpum*) from three locations (Matheson's Bay, Rakino Island and Rangitoto Island). Triacylglycerol (TAG), Free Fatty Acid (FFA), Aliphatic Hydrocarbon (AH), Wax Ester (WE), Methyl Ester (ME) and Diacylglycerol (DAG) as Energy Lipids and Phospholipids (PL), Sterols (ST) and Acetone-mobile Polar Lipids (AMPL) as Structural Lipids. ND: not detected. Data are combined for the 3 locations and represent the mean (±SE) of ten (n=30) samples of gonad, 29 for gut and gut contents and 6 for seaweed.

Lipid Class	***C. maschalocarpum***	***E. radiata***	**Gut content**	**Gut**	**Gonad**
TAG	0.71±0.15	2.54±2.12	8.71±12.13	84.53±34.28	174.64±72.86
FFA	0.13±0.15	1.52±1.25	4.15±4.26	2.88±1.69	2.61±2.00
AH	ND	ND	0.22±0.51	ND	ND
WE	ND	ND	0.16±0.15	2.02±0.65	1.79±0.88
ME	ND	ND	0.23±0.42	6.80±2.77	2.98±2.12
DAG	ND	0.12±0.19	3.62±7.29	1.85±0.53	2.30±0.57
Energy Lipid %	3.72	13.55	32.25	46.49	62.71
PL	17.64±2.73	21.18±4.44	23.16±5.62	94.48±14.65	82.23±18.67
ST	2.45±0.41	3.71±1.06	3.75±2.27	9.98±2.19	12.67±4.29
AMPL	1.00±0.23	2.10±0.67	1.32±0.42	3.55±1.06	3.13±0.76
Structural Lipid %	96.16	86.45	67.75	53.51	37.29
Total Lipid	21.93±3.30	31.17±5.34	45.33±23.89	206.08±42.10	282.36±76.76

Figure 2.1. Lipid classes present in *E. radiata, C. maschalocarpum* and *E. chloroticus* gut contents, gut and gonad from the 3 locations and represent the mean (±SE) of ten (n=30) samples of gonad, 29 for gut and gut contents and 6 for seaweed. TAG (Triacylglycerol), FFA (Free Fatty Acid), AH (Aliphatic Hydrocarbon), WE (Wax Ester), ME (Methyl Ester), DAG (Diacylglycerol), PL (Phospholipid), ST (Sterols) and AMPL (acetone-mobile polaripids)



Seaweed species and gut contents had a greater concentration of structural lipid than energy lipid (Table 2.1; Fig. 2.2.A-C). The seaweed *C. maschalocarpum* contained only trace concentrations of energy lipids (Table 2.1; Fig. 2.2.A). In the case of sea urchin gut, the concentration of energy lipid was similar to the concentration of structural lipid (Table 2.1; Fig. 2.2.D). In contrast, sea urchin gonad had a greater concentration of energy lipid than structural lipid (Table 2.1; Fig. 2.2.E).

Table 2.2. Results of Univariate two-way PERMANOVA comparing the concentrations of: A) total lipid, B) energy lipid, C) structural lipid among two brown seaweed species, sea urchin gut contents, gut and gonad and among locations and D) Pairwise comparisons between tissues. Significant results ($p<0.05$) are showed in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	1104100	276040	110.79	**0.0001**	9948
Location	2	5349.3	2674.7	1.08	0.3450	9948
Tissue x Location	8	20042	2525.2	1.01	0.4447	9935
Residual	85	211310	2486			
Total	99	134000				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	514720	128680	60.33	**0.0001**	9943
Location	2	3639	1819.5	0.86	0.4272	9947
Tissue x Location	8	12500	1562.5	0.73	0.6685	9938
Residual	85	181310	2133.1			
Total	99	712100				

C)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	140530	35133	189.66	**0.0001**	9956
Location	2	454.91	227.46	1.23	0.2996	9958
Tissue x Location	8	7474.5	934.31	5.04	**0.0002**	9926
Residual	85	15746	185.24			
Total	99	164210				

D)

	Total Lipid			Energy Lipid			Structural Lipid		
Tissues	t	P(perm)	Unique perms	t	P(perm)	Unique perms	t	P(perm)	Unique perms
Gonad, Gut	4.76	**0.0001**	9857	5.76	**0.0001**	9855	2.30	**0.0281**	9831
Gonad, Gut contents	15.95	**0.0001**	9840	12.05	**0.0001**	9847	18.36	**0.0001**	9839
Gonad, *E. radiata*	7.65	**0.0001**	9831	5.80	**0.0001**	9855	8.54	**0.0001**	9749
Gonad, *C. maschalocarpum*	7.90	**0.0001**	9831	5.91	**0.0001**	9856	9.14	**0.0001**	9847
Gut, Gut contents	19.82	**0.0001**	9811	11.61	**0.0001**	9817	29.93	**0.0001**	9833
Gut, *E. radiata*	10.67	**0.0001**	9816	6.43	**0.0001**	9836	14.61	**0.0001**	9816
Gut, *C. maschalocarpum*	11.18	**0.0001**	9823	6.66	**0.0001**	9822	15.55	**0.0001**	9822
Gut contents, *E. radiata*	1.62	0.0947	9849	2.02	0.0618	9848	0.46	0.6588	9851
Gut contents, *C. maschalocarpum*	2.56	**0.0257**	9842	2.54	**0.0325**	9848	2.31	**0.0318**	9855
E. radiata, C. maschalocarpum	3.60	**0.0162**	8923	8.37	**0.0002**	8969	2.36	0.0622	8956

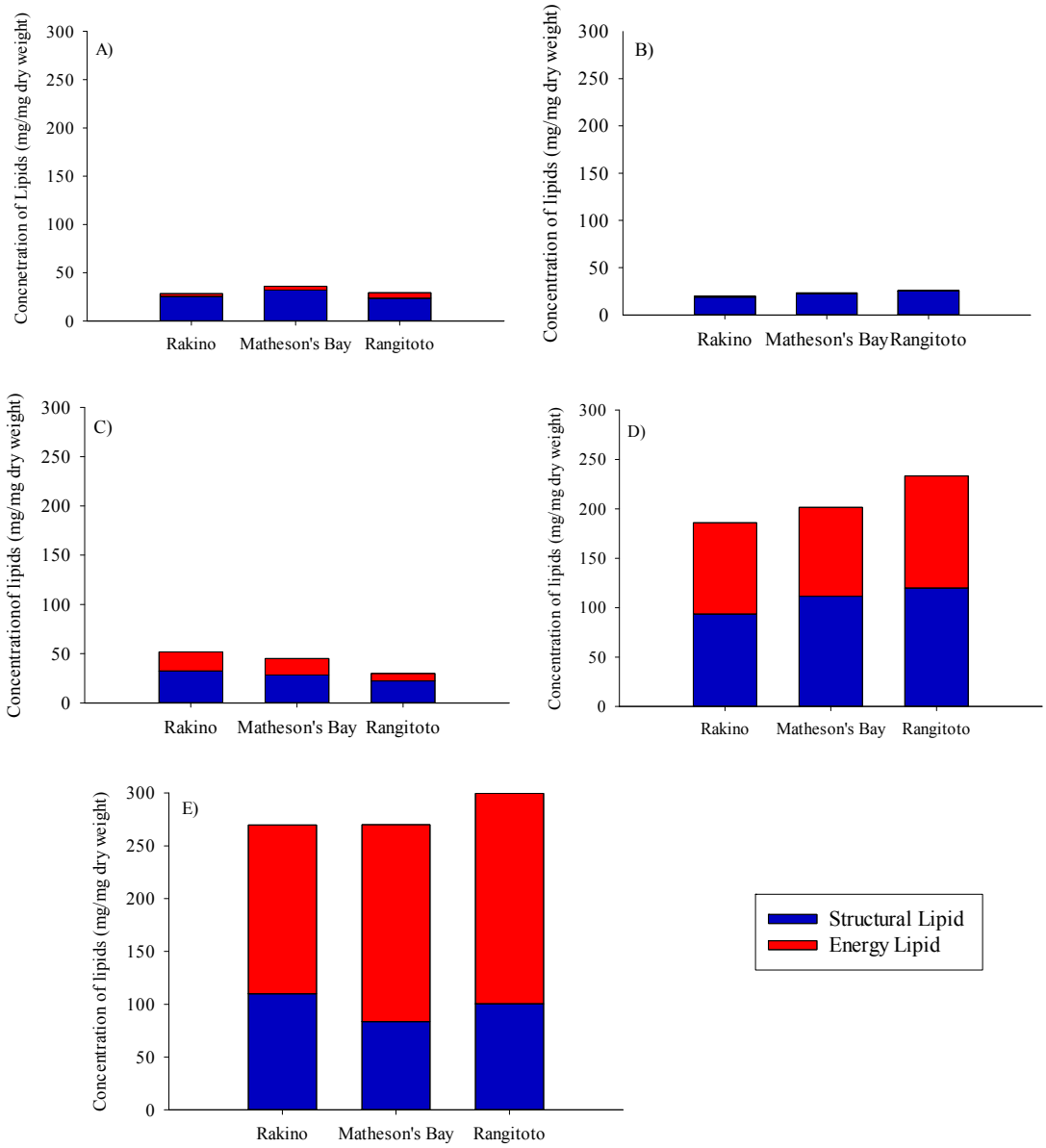


Figure 2.2. Comparison across all locations (x axes: Rakino, Matheson's Bay and Rangitoto) between energy lipids including: AH, TAG, FFA, WE, DAG and ME and structural lipids including: ST, AMPL, PL of seaweed species: A) *E. radiata* and B) *C. maschalocarpum* and tissues of *E. chloroticus:* C) gut contents, D) gut and E) gonad.

Two-way PERMANOVA analysis revealed that the lipid profile was also significantly different among tissues (Table 2.3), with the separation among tissues clearly shown in the MDS plot (Figure 2.3). No significant differences were, however, found among the three different locations, and the interaction term was also not significant (Table 2.3). For this reason, the three locations were pooled and the average was calculated to show the differences among the tissues in later figures and statistical analyses. Pair-wise comparisons revealed no significant differences in the lipid profile between the seaweed species and between *E. radiata* and gut contents; however, the lipid profile was significantly different among the remaining tissues ($p<0.05$; Table 2.3.B).

Table 2.3. Results of A) Multivariate two-way PERMANOVA and B) Pair-wise comparisons comparing lipid profile among seaweed species, *E. radiata, C. maschalocarpum* and *E. chloroticus* gut contents, gut and gonad from different sites in the Hauraki Gulf. A) Two-way PERMANOVA results including tissues and locations. B) Pair-wise comparisons among tissues. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	601000	150200	66.88	**0.0001**	9952
Location	2	5498.7	2749.3	1.22	0.2865	9957
Location x Tissue	8	15403	1925.4	0.86	0.5666	9937
Residual	85	190970	2246.7			
Total	99	812870				

B)

Tissues	t	P(perm)	Unique perms
Gonad, Gut	5.90	**0.0001**	9927
Gonad, Gut contents	12.47	**0.0001**	9922
Gonad, *C. maschalocarpum*	5.75	**0.0001**	9926
Gonad, *E. radiata*	5.83	**0.0001**	9934
Gut, Gut contents	14.53	**0.0001**	9939
Gut, *C. maschalocarpum*	7.14	**0.0001**	9929
Gut, *E. radiata*	7.35	**0.0001**	9962
Gut contents, *C. maschalocarpum*	1.38	**0.0445**	9932
Gut contents, *E. radiata*	1.90	0.1221	9924
C. maschalocarpum, *E. radiata*	2.06	0.0506	9072

SIMPER analysis showed that three lipid classes contributed to the differences among tissues, two energy lipid classes (TAG and DAG) and one structural lipid (PL) (Table 2.4). In all tissues, TAG made the greatest contribution to the energy lipid, its concentration was statistically different among tissues (Pseudo-$F_{4,99}$=58.98, P(perm)=0.0001) but not among locations (Pseudo-$F_{2,99}$=1.19, P(perm)=0.3063) and the interaction term was not statistically significant (Pseudo-$F_{8,99}$=0.56, P(perm)=0.815). Pairwise comparisons showed that the concentration of TAG, the major storage lipid, increased from the seaweeds (*E. radiata* and *C. maschalocarpum*) and gut content to the gut and the gonad presenting the greatest concentration of TAG of all the tissues (Table 2.1; Table 2.4).

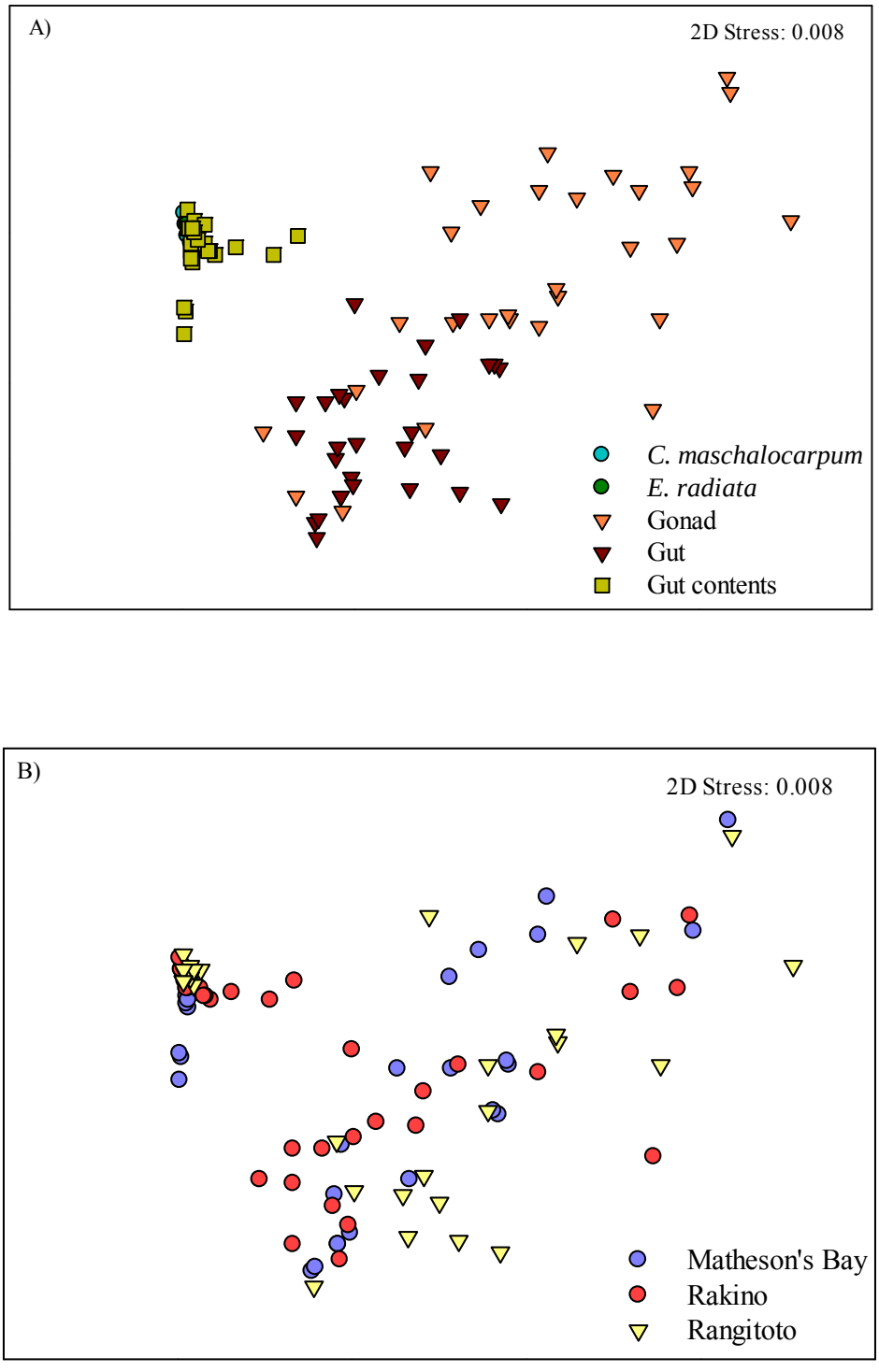


Figure 2.3. Multidimensional scaling (MDS) plot of Euclidean distance of lipid profile of: A) Tissues: seaweed species (*E. radiata* and *C. maschalocarpum*) and *E. chloroticus* (gut contents, gut and gonad) and B) Locations: Matheson's Bay, Rakino and Rangitoto. Seaweed and gut contents data are overlaying each other in the left part of the graph.

No significant differences were found in the concentrations of DAG between tissues (Pseudo-$F_{4,99}$=2.50, P(perm)=0.0541), although there was a significant difference among locations (Pseudo-$F_{2,99}$=6.65, P(perm)=0.0012) and the interaction term was also significant (Pseudo-$F_{8,99}$=3.82, P(perm)=0.0097). Pairwise comparisons showed significant differences between Matheson's Bay and Rakino (t=2.60, P(perm)=0.0022) and also between Matheson's Bay and Rangitoto (t=2.60, P(perm)=0.0028), but no differences were observed between Rakino and Rangitoto (t=0.023, P(perm)=0.9842); however, the pattern is unclear, being higher in some locations in one tissue and different in the other location. DAG was present in trace concentrations in *E. radiata* from Rakino, 0.35±0.04µg/mg of dry weight, but not detected in the other locations. DAG was not detected in *C. maschalocarpum* at all three locations (Table 2.1). The gut contents of sea urchins from Matheson's Bay showed relatively higher concentrations of this lipid class (9.39±10.41µg/mg of dry weight) compared to the concentrations in the other locations (0.26±0.08µg/mg of dry weight in Rangitoto and 0.95±0.57µg/mg of dry weight in Rakino). In the gut, this lipid class had values of 1.57±0.19µg/mg dry weight in Rakino and 2.14±0.84µg/mg of dry weight in Rangitoto. In the gonad, the concentrations of DAG ranged between 2.06±0.66µg/mg of dry weight in Rakino to 2.45±0.56µg/mg of dry weight in Matheson's Bay.

As part of the structural lipids, PL was the lipid class present in the highest concentration in all tissues, and significant differences were found in the concentration of this lipid class between tissues (Pseudo-$F_{4\text{-}99}$=192.56, P(perm)=0.0001) but not between locations (Pseudo-$F5_{\text{-}99}$=0.998, P(perm)=0.3688). Even though this lipid class did not contribute to the differences between gonad and gut (Table 2.4), it was higher in the gonad than in the gut (t=2.78; P(perm)=0.0078) and these concentrations were higher than the concentration of PL present in the gut contents, *E. radiata* and *C. maschalocarpum* as shown in the pairwise comparisons (Table 2.1; Table 2.4). The remaining lipid classes did not contribute greatly to the differences among tissues as they were present in very small concentrations (< 13 µg/mg of dry weight) (Table 2.1).

Table 2.4. Contribution of individual lipid class to multivariate differences in lipid profile between *E. chloroticus* gonad, gut, gut contents and possible food items (brown seaweed species) as determined by SIMPER. t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor for each lipid class and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Tissues: Groups 1 & 2	Average squared distance	Lipid	Average Value Group 1	Average Value Group 2	Av.Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Gonad & Gut	14842.40	TAG	175	84.50	14000	0.97	94.12	94.12	5.99	**0.0001**	9827
Gonad & Gut contents	37126.09	TAG	175	8.71	33100	1.41	89.26	89.26	12.15	**0.0001**	9847
		PL	82.2	23.20	3790	1.64	10.22	99.48	16.33	**0.0001**	9825
Gut & Gut contents	12737.87	TAG	84.5	8.71	7100	1.15	55.75	55.75	11.41	**0.0001**	9818
		PL	94.5	23.20	5450	2.15	42.75	98.50	24.05	**0.0001**	9827
Gonad & *C. maschalocarpum*	39958.43	TAG	175	0.71	35400	1.45	88.54	88.54	5.63	**0.0001**	9827
		PL	82.2	18.05	4420	1.73	11.07	99.60	8.25	**0.0001**	9802
Gut & *C. maschalocarpum*	14263.24	TAG	84.52	0.71	8160	1.29	57.21	57.21	5.77	**0.0001**	9827
		PL	94.5	18.5	5960	2.89	41.82	99.03	12.26	**0.0001**	9784
Gut content & *C. maschalocarpum*	387.29	TAG	8.71	0.71	207	0.35	53.56	53.56	19286	0.0530	9926
		PL	23.2	18.5	74.4	0.54	19.22	72.68	1.96	0.0589	9809
		DAG	3.62	ND	64.4	0.36	16.64	89.42	1.20	0.1037	9643
Gonad & *E. radiata*	38977.14	TAG	175	2.54	34700	1.44	89.07	89.07	5.57	**0.0001**	9835
		PL	82.2	21.20	4130	1.59	10.60	99.67	7.89	**0.0001**	9839
Gut & *E. radiata*	13550.34	TAG	84.5	2.54	7850	1.27	57.94	57.94	5.65	**0.0001**	9810
		PL	94.5	21.20	5580	2.40	41.20	99.14	11.78	**0.0001**	9765
Gut contents & *E. radiata*	333.01	TAG	8.71	2.54	118	0.33	56.54	56.54	1.5	0.0965	9907
		DAG	3.62	0.12	64.3	0.35	19.30	75.84	1.16	0.1061	9796
		PL	23.2	21.2	46.3	0.45	13.90	89.75	0.80	0.4212	9783
C. maschalocarpum & *E. radiata*	46.28	PL	21.2	18.5	32.2	1.03	69.61	69.61	1.24	0.2330	336
		TAG	2.54	0.71	7.03	0.51	15.20	84.81	1.97	**0.0094**	9081

2.3.2 Fatty Acid Analysis

Fifty seven FAs were identified in the different tissues: seaweed species, *E. radiata* and *C. maschalocarpum,* and *E. chloroticus* gut contents, gut and gonad from the three locations in the Hauraki Gulf (Table 2.5; Table 2.6). The FAs that contributed to more than 2% of the total FA are shown in Figure 2.4, with the addition of C22:6(n-3) or DHA, which is considered an essential FA.

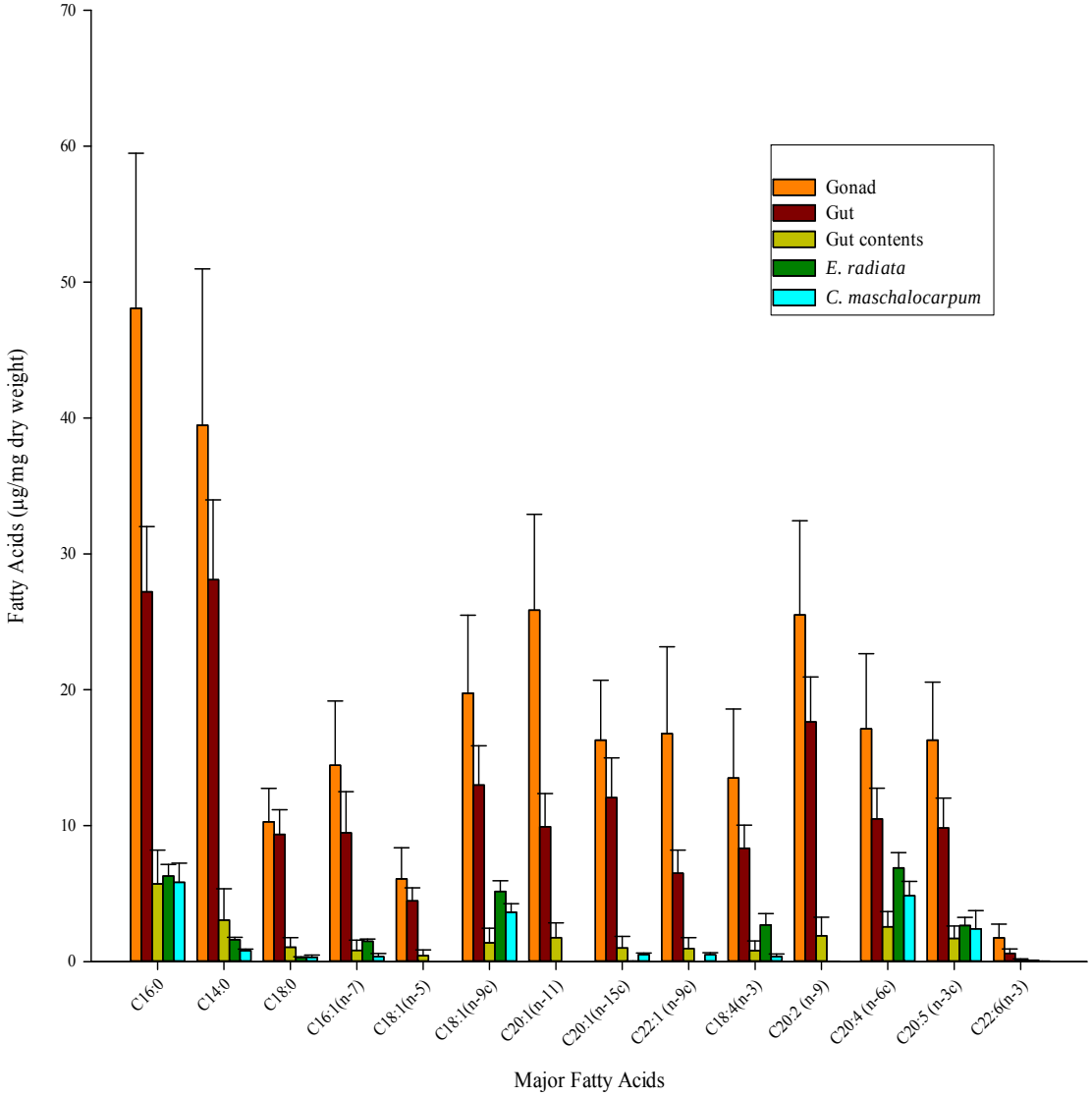


Figure 2.4. Fatty acids contributing more than 2% of the total FA in *E. chloroticus* gonad, gut, gut contents and seaweed species, *E. radiata* and *C. maschalocarpum* from three locations in the Hauraki Gulf combined (as there were not significant differences between locations; see Table 2.7). Data represent the mean (±SE) of n=30 samples of gonad, n=29 of gut and n=22 of gut contents and n=7 of seaweed.

Table 2.5. Fatty acid composition (% of the total FA) of seaweed species, *E. radiata* and *C. maschalocarpum and E. chloroticus* gut contents, gut and gonad from the three locations in the Hauraki Gulf combined (as there were not significant differences between locations; see Table 2.7). Data represent the mean (±SE) of n=30 samples of gonad, n=29 of gut and n=22 samples of gut contents and n=7 of seaweed. ND = not detected.

Fatty acids	*C. maschalocarpum*	*E. radiata*	Gut contents	Gut	Gonad
C12:0	0.03±0.02	0.02±0.02	0.14±0.05	0.12±0.04	0.05±0.02
C14:0	3.30±0.62	5.10±0.68	9.34±3.09	14.35±2.31	11.86±1.13
C14:1	ND	ND	0.20±0.09	0.13±0.06	0.06±0.02
C14:1(n-5)	ND	ND	0.84±0.35	1.24±0.65	0.55±0.11
C15:0	0.21±0.03	0.24±0.07	0.57±0.19	0.17±0.03	0.43±0.06
C16:0	23.53±1.38	19.75±0.72	18.88±2.49	13.87±1.33	14.59±1.12
C16:1	ND	ND	ND	0.25±0.06	0.15±0.02
C16:1	ND	ND	0.50±0.86	0.16±0.05	0.17±0.02
C16:1	0.86±0.77	ND	2.35±1.26	0.97±0.46	0.57±0.09
C16:1(n-7)	1.60±1.05	4.79±0.85	3.38±1.29	4.80±1.20	4.33±0.39
C17:0	ND	ND	1.22±2.40	0.10±0.03	0.12±0.01
C16:2(n-6)	ND	ND	ND	ND	0.05±0.01
C16:3(n-6)	ND	ND	ND	ND	0.02±0.00
C16:3(n-3)	ND	ND	ND	ND	0.08±0.02
C18:0	1.18±0.36	0.93±0.14	3.25±0.86	4.79±0.69	3.22±0.49
C18:1(n-5)	ND	ND	1.21±0.57	2.28±0.36	1.82±0.14
C18:1(n-9t)	ND	ND	5.52±3.10	0.48±0.12	0.51±0.05
C18:1(n-9c)	14.96±2.03	16.23±1.67	4.15±1.54	6.58±0.84	5.92±0.30
C18:1(n-7)	0.47±0.18	0.34±0.16	0.32±0.13	0.56±0.10	0.68±0.04
C18:2	ND	ND	ND	ND	0.07±0.00
C18:2(n-6t)	ND	ND	0.53±0.28	1.03±0.29	1.05±0.17
C18:2(n-6c)	3.73±0.42	4.27±0.88	2.07±0.96	0.24±0.06	0.20±0.06
C19:1	ND	ND	0.16±0.05	0.22±0.06	0.22±0.03
C18:3	ND	ND	0.22±0.06	0.29±0.08	0.30±0.04
C19:1	ND	ND	0.88±1.16	0.29±0.07	0.16±0.01
C18:3(n−6)	0.33±0.05	0.60±0.17	0.29±0.36	0.04±0.01	0.05±0.00
C18:3(n-3)	11.54±1.60	5.78±1.07	2.08±1.04	0.16±0.05	0.26±0.03
C20:0	0.76±0.07	2.07±0.70	1.25±0.40	1.21±0.37	0.58±0.06
C20:1(n-15)	2.05±0.11	0.13±0.04	2.89±1.09	6.14±1.08	4.96±0.23
C18:4(n-3)	1.32±0.56	8.26±1.62	2.34±0.91	4.25±0.57	4.04±0.32
C20:1(n-11)	ND	ND	5.41±1.26	5.02±0.75	7.85±0.71
C20:1(n-9)	ND	ND	0.70±0.36	1.17±0.30	1.80±0.06
C20:2(n-9)	ND	0.04±0.01	5.79±1.70	9.01±1.16	7.69±0.51
C20:2	ND	ND	0.83±0.35	0.98±0.34	1.14±0.17
C20:2	ND	ND	0.31±0.11	0.48±0.08	0.66±0.03
C20:2	ND	ND	ND	0.19±0.07	0.25±0.02
C20:2(n-6)	0.30±0.11	0.20±0.02	0.54±0.12	0.57±0.15	0.88±0.12
C20:3(n-9)	ND	ND	0.93±0.41	1.69±0.60	1.63±0.29
C21:1	ND	ND	1.38±0.85	0.64±0.11	1.10±0.05
C20:3(n-6)	0.41±0.04	0.43±0.09	0.26±0.11	0.10±0.03	0.26±0.04
C20:4(n-6)	19.89±2.34	21.51±0.85	8.76±2.44	5.39±1.01	5.41±1.34
C20:3(n-3)	ND	ND	0.42±0.17	0.54±0.12	0.99±0.13
C22:0	1.29±0.18	ND	0.36±0.28	0.11±0.05	0.04±0.00
C20:4(n-3)	0.47±0.14	0.66±0.11	ND	0.12±0.06	0.40±0.22
C22:1	ND	ND	0.39±0.24	ND	ND
C22:1(n-9)	2.02±0.17	ND	2.88±0.94	3.28±0.49	6.01±0.12
C20:5(n-3)	9.00±3.29	8.20±0.96	5.60±1.33	5.04±0.97	5.08±0.91
C23:1	ND	ND	ND	0.20±0.05	0.20±0.00
C20:5	ND	ND	ND	ND	0.32±0.19
C22:4	ND	ND	ND	0.11±0.08	0.19±0.05
C24:0	0.53±0.13	0.24±0.08	0.35±0.34	0.05±0.02	0.04±0.00
C22:3	ND	ND	ND	0.06±0.07	0.08±0.03
C24:1(n-9)	0.25±0.08	ND	0.19±0.17	0.15±0.03	0.16±0.01
Unknown PUFA	ND	ND	ND	0.09±0.06	0.19±0.05
Unknown PUFA	ND	ND	ND	ND	0.03±0.00
C22:6(n-3)	0.08±0.04	0.19±0.13	0.34±0.19	0.31±0.18	0.54±0.18
SFA	**30.80±1.72**	**28.36±1.60**	**35.36±2.48**	**34.75±2.89**	**30.93±1.86**
MUFA	**22.18±2.18**	**21.49±2.24**	**33.32±2.79**	**34.55±2.14**	**37.22±1.54**
PUFA	**46.96±3.00**	**50.15±3.74**	**31.34±3.59**	**30.69±3.87**	**31.85±2.94**

Table 2.6. Fatty acid composition (μg/mg of dry weight) of seaweed species, *E. radiata* and *C. maschalocarpum* and *E. chloroticus* gut contents, gut and gonad from the three locations in the Hauraki Gulf combined (as there were not significant differences between locations; see Table 2.7). Data represent the mean (±SE) of n=30 samples of gonad, n=29 of gut and n=22 samples of gut contents and n=7 of seaweed. ND = not detected.

Fatty acids	*C. maschalocarpum*	*E. radiata*	Gut contents	Gut	Gonad
C12:0	0.01±0.00	0.01±0.01	0.04±0.02	0.23±0.09	0.17±0.03
C14:0	0.79±0.13	1.61±0.17	3.06±2.29	28.10±5.88	38.63±1.49
C14:1	ND	ND	0.05±0.03	0.26±0.14	0.21±0.06
C14:1(n-5)	ND	ND	0.26±0.17	2.45±1.50	1.82±0.40
C15:0	0.05±0.01	0.07±0.01	0.17±0.07	0.33±0.06	1.35±0.19
C16:0	5.84±1.41	6.30±0.85	5.71±2.49	27.21±4.80	46.96±1.65
C16:1	ND	ND	ND	0.49±0.13	0.51±0.06
C16:1	ND	ND	0.15±0.25	0.31±0.12	0.53±0.06
C16:1	0.25±0.26	ND	0.94±0.42	1.91±1.08	1.89±0.37
C16:1(n-7)	0.36±0.23	1.50±0.15	0.83±0.75	9.48±3.02	14.15±1.79
C17:0	ND	ND	0.36±0.71	0.20±0.05	0.38±0.02
C16:2(n-6)	ND	ND	ND	ND	0.16±0.06
C16:3 (n-6)	ND	ND	ND	ND	0.05±0.02
C16:3(n-3)	ND	ND	ND	ND	0.22±0.07
C18:0	0.31±0.17	0.30±0.05	1.06±0.70	9.36±1.81	10.07±1.83
C18:1(n-5)	ND	ND	0.43±0.43	4.46±0.96	5.99±0.47
C18:1(n-9t)	ND	ND	1.61±1.07	0.94±0.29	1.67±0.28
C18:1(n-9c)	3.63±0.63	5.16±0.79	1.38±1.08	12.99±2.89	19.37±2.07
C18:1(n-7)	0.12±0.07	0.11±0.06	0.11±0.09	1.11±0.27	2.17±0.31
C18:2	ND	ND	ND	ND	0.23±0.03
C18:2(n-6t)	ND	ND	0.18±0.17	2.01±0.65	3.50±0.83
C18:2(n-6c)	0.93±0.24	1.36±0.32	0.59±0.35	0.48±0.15	0.66±0.24
C19:1	ND	ND	0.05±0.03	0.43±0.12	0.71±0.03
C18:3	ND	ND	0.07±0.05	0.56±0.14	0.96±0.22
C19:1	ND	ND	0.28±0.38	0.57±0.21	0.53±0.03
C18:3(n−6)	0.09±0.02	0.19±0.06	0.08±0.11	0.08±0.03	0.15±0.01
C18:3(n-3)	2.93±1.03	1.87±0.50	0.56±0.31	0.32±0.11	0.85±0.18
C20:0	0.19±0.06	0.66±0.23	0.40±0.24	2.39±0.88	1.90±0.34
C20:1(n-15)	0.51±0.12	0.04±0.01	1.00±0.86	12.06±2.93	15.97±0.70
C18:4(n-3)	0.36±0.21	2.69±0.84	0.80±0.72	8.34±1.70	13.25±1.35
C20:1(n-11)	ND	ND	1.76±1.09	9.92±2.44	25.32±2.42
C20:1(n-9)	ND	ND	0.25±0.25	2.34±0.84	5.85±0.52
C20:2(n-9)	ND	0.01±0.00	1.91±1.36	17.64±3.30	25.06±0.69
C20:2	ND	ND	0.28±0.25	1.83±0.74	3.66±0.49
C20:2	ND	ND	0.10±0.09	0.90±0.24	2.20±0.30
C20:2	ND	ND	ND	0.41±0.25	0.84±0.13
C20:2(n-6)	0.07±0.01	0.06±0.01	0.18±0.12	1.13±0.43	2.83±0.56
C20:3(n-9)	ND	ND	0.31±0.28	3.26±1.15	5.18±1.13
C21:1	ND	ND	0.40±0.22	1.27±0.34	3.59±0.28
C20:3(n-6)	0.10±0.03	0.14±0.04	0.08±0.05	0.19±0.07	0.84±0.17
C20:4(n-6)	4.86±1.04	6.89±1.13	2.56±1.11	10.50±2.25	16.80±4.77
C20:3(n-3)	ND	ND	0.14±0.13	1.07±0.32	3.24±0.69
C22:0	0.31±0.06	ND	0.09±0.06	0.20±0.07	0.13±0.00
C20:4(n-3)	0.12±0.07	0.21±0.06	ND	0.23±0.10	1.25±0.71
C22:1	ND	ND	0.11±0.06	ND	ND
C22:1(n-9)	0.50±0.15	ND	0.96±0.80	6.50±1.70	19.36±1.86
C20:5(n-3)	2.41±1.35	2.64±0.60	1.70±0.91	9.84±2.20	15.91±3.62
C23:1	ND	ND	ND	0.40±0.12	0.66±0.06
C20:5	ND	ND	ND	ND	0.98±0.55
C22:4	ND	ND	ND	0.22±0.17	0.61±0.19
C24:0	0.13±0.02	0.08±0.02	0.09±0.07	0.10±0.04	0.12±0.02
C22:3	ND	ND	ND	0.10±0.12	0.24±0.10
C24:1(n-9)	0.06±0.01	ND	0.05±0.03	0.29±0.08	0.51±0.08
Unknown PUFA	ND	ND	ND	0.19±0.13	0.58±0.16
Unknown PUFA	ND	ND	ND	ND	0.10±0.01
C22:6(n-3)	0.02±0.01	0.06±0.04	0.11±0.09	0.60±0.33	1.71±0.72
SFA	7.63±1.80	8.98±1.08	10.98±5.38	68.11±11.04	99.70±4.25
MUFA	5.42±1.08	6.84±0.86	10.62±5.75	68.18±13.31	120.81±10.33
PUFA	11.86±3.89	16.14±3.19	9.66±4.85	59.92±10.58	102.06±15.85
Total FAMEs	26.10±10.10	31.97±0.53	31.26±15.62	196.20±29.43	322.57±35.42

The concentration of total FA (µg/mg dry weight) differed significantly between tissues but not between locations (Table 2.7.A). Both seaweed species and gut contents had the lowest concentration of total FA (~30µgmg dry weight), with no significant differences in pair-wise tests (Table 2.7B). Sea urchin gut tissues had relatively higher concentrations (~196µgmg dry weight), whereas the gonad was the tissue with the highest concentration of total FA (~328µg/mg of dry weight), and these tissues were different from each other and all other tissues (Table 2.6; Table 2.7.B).

Table 2.7. Results of A) Univariate two-way PERMANOVA and B) Pair-wise comparisons comparing total concentration of FA (µg/mg dry weight) between seaweed species (*E. radiata* and *C. maschalocarpum*) and *E. chloroticus* gut contents, gut and gonad from three locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	14513	3628.20	165.8	**0.0001**	9956
Location	2	51.14	25.57	1.168	0.3155	9959
Tissue x Location	8	37.04	4.63	0.21	0.9873	9927
Residual	79	1728.70	21.88			
Total	93	16330				

B)

Tissue	t	P(perm)	Unique perms
Gonad, Gut	8.96	**0.0001**	9829
Gonad, Gut contents	18.59	**0.0001**	9836
Gonad, *E. radiata*	10.27	**0.0001**	9848
Gonad, *C. maschalocarpum*	9.71	**0.0001**	9839
Gut, Gut contents	24.14	**0.0001**	9837
Gut, *E. radiata*	13.88	**0.0001**	9880
Gut, *C. maschalocarpum*	13.38	**0.0001**	9799
Gut contents, *E. radiata*	0.15	0.8868	9829
Gut contents, *C. maschalocarpum*	1.22	0.2359	9835
E.radiata, C. maschalocarpum	2.30	0.0567	9828

The total concentration of SFA and MUFA was also significantly different between tissues, but not between locations; the interaction term was also not significant (Table 2.8.A.B). The concentration of SFA was not significantly different between seaweed species and gut contents; however the concentration of MUFA was smaller in *C. maschalocarpum,* followed by the concentration in *E. radiata* and then the concentration in the gut contents (Table 2.6; Table 2.8.D; Fig. 2.5). In both cases (SFA and MUFA), these concentrations were smaller in these tissues than sea urchin gut and the gonad was the tissue with the highest concentration of SFA and MUFA (Table 2.6; Table 2.8.D; Fig. 2.5).

Significant differences were also found in the total concentration of PUFA between tissues as well as between locations; the interaction term was not significant (Table 2.8.C). Gut contents was the tissue with the lowest concentration of PUFA, followed by the

concentration in *C. maschalocarpum, E. radiata*, then the concentration in the gut and, as with SFA and MUFA, the highest concentration of PUFA was in the gonad (Table 2.6; Table 2.8.D; Fig. 2.5). Pairwise results also showed that the concentration of total PUFA was lower in the gonads from Matheson's Bay (290.12±65.44 µg/mg dry weight) than the gonads from Rakino (>330µg/mg dry weight; t=2.62, P(perm)=0.0153) and also the concentration of PUFA was higher in the gut contents from Rakino (46.42µg/mg dry weight) than Rangitoto (<25µg/mg dry weight; t=2.60, P(perm)=0.0147). The concentration of PUFA did not show significant differences in the remaining tissues between locations (p>0.05).

Table 2.8. Results of Univariate two-way PERMANOVA comparing the concentrations of: A) SFA, B) MUFA and C) PUFA among two brown seaweed species, sea urchin gut contents, gut and gonad and between locations. D) Pairwise comparisons among tissues. Significant results (p<0.05) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	141580	35396	139.51	**0.0001**	9949
Location	2	111.48	55.74	0.22	0.8005	9948
Tissue x Location	8	302.94	37.87	0.15	0.1493	9940
Residual	79	20044	253.72			
Total	93	120400				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	211960	52989	135.4	**0.0001**	9951
Location	2	784.3	392.15	1	0.3699	9949
Tissue x Location	8	735.93	91.99	0.24	0.9826	9930
Residual	79	30917	391.35			
Total	93	244390				

C)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	139640	34909	183.96	**0.0001**	9962
Location	2	1279.2	639.59	3.37	**0.0379**	9962
Tissue x Location	8	2329.1	291.13	1.53	0.1553	9934
Residual	79	14991	189.77			
Total	93	158240				

D)

	SFA			MUFA			PUFA		
Tissues	t	P(perm)	Unique perms	t	P(perm)	Unique perms	t	P(perm)	Unique perms
Gonad, Gut	6.74	**0.0001**	9829	8.80	**0.0001**	9852	10.21	**0.0001**	9831
Gonad, Gut contents	17.01	**0.0001**	9808	16.78	**0.0001**	9795	20.99	**0.0001**	9846
Gonad, *E. radiata*	9.63	**0.0001**	9835	9.58	**0.0001**	9861	10.86	**0.0001**	9858
Gonad, *C. maschalocarpum*	9.02	**0.0001**	9839	8.96	**0.0001**	9821	10.53	**0.0001**	9842
Gut, Gut contents	22.49	**0.0001**	9840	19.41	**0.0001**	9808	20.30	**0.0001**	9828
Gut, *E. radiata*	13.34	**0.0001**	9828	11.74	**0.0001**	9854	10.10	**0.0001**	9826
Gut, *C. maschalocarpum*	12.60	**0.0001**	9813	11.08	**0.0001**	9789	10.30	**0.0001**	9841
Gut contents, *E. radiata*	1.20	0.2449	9849	2.31	**0.0262**	9829	3.64	**0.0021**	9836
Gut contents, *C. maschalocarpum*	1.90	0.0644	9856	2.91	**0.0132**	9856	1.19	0.237	9842
E. radiata, C. maschalocarpum	1.88	0.0905	9840	8.72	**0.0288**	9801	2.29	0.0578	8966

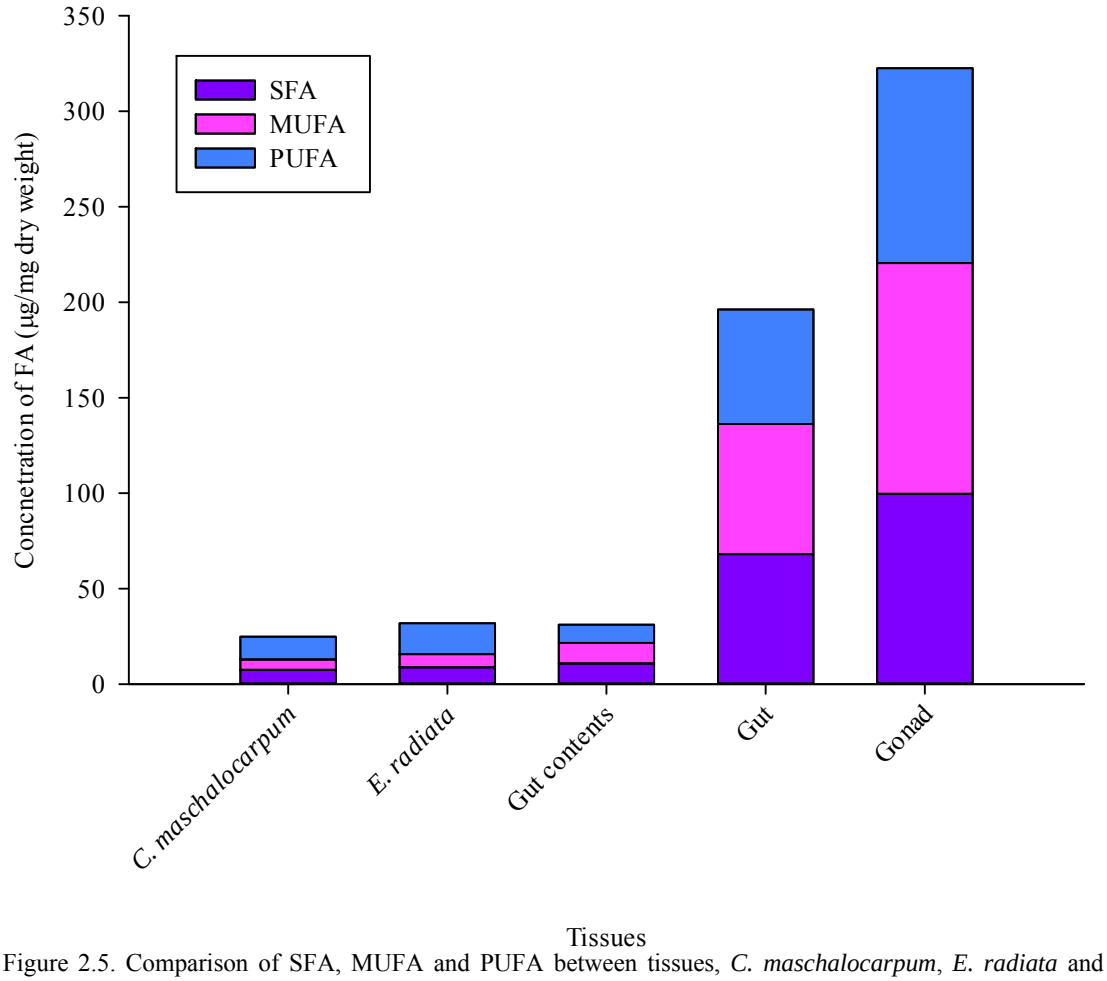


Figure 2.5. Comparison of SFA, MUFA and PUFA between tissues, *C. maschalocarpum*, *E. radiata* and *E. chloroticus* gut contents, gut and gonad from three locations in the Hauraki Gulf combined. Data represent the mean (±SE) of n=30 samples of gonad, n=29 of gut and n=22 of gut contents and n=7 of seaweed.

Similarly, significant differences were found in the FA profile between tissues as shown in the MDS plot (Fig. 2.6); but no significant differences were found between locations and the interaction term was also not significant (Table 2.9.A). Pairwise comparisons revealed a significant difference in the FA profile between all the tissues (Table 2.9.B). As there was no significant difference between the three locations, the locations are pooled together in the summary tables and figures.

Table 2.9. Results of A) Multivariate two-way PERMANOVA and B) Pair-wise comparisons comparing FA profile among seaweed species (*E. radiata* and *C. maschalocarpum*) and *E. chloroticus* gut contents, gut and gonad from three locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

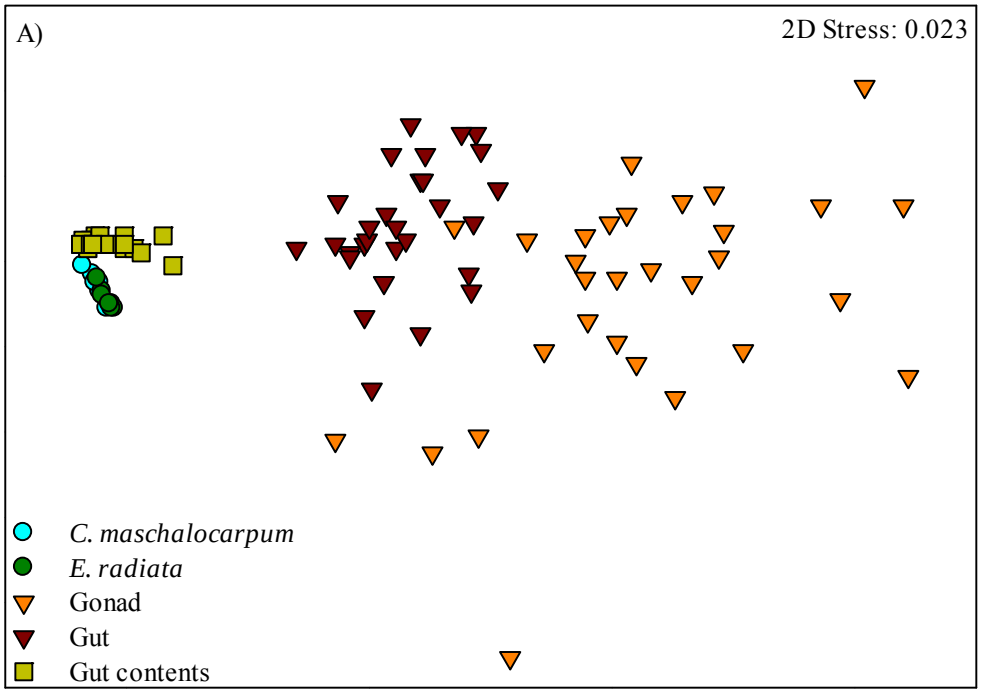
A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	105040	26261	109.50	**0.0001**	9943
Location	2	527.99	263.99	1.01	0.3345	9958
Tissue x Location	8	1130.9	141.36	0.59	0.8155	9934
Residual	79	18946	239.82			
Total	93	125650				

B)

Tissues	t	P(perm)	Unique perms
Gonad, Gut	74145	**0.0001**	9929
Gonad, Gut contents	15.18	**0.0001**	9923
Gonad, *E. radiata*	8.42	**0.0001**	9927
Gonad, *C. maschalocarpum*	7.94	**0.0001**	9933
Gut, Gut contents	18.34	**0.0001**	9923
Gut, *E. radiata*	10.54	**0.0001**	9936
Gut, *C. maschalocarpum*	10.09	**0.0001**	9951
Gut contents, *E. radiata*	4.78	**0.0001**	9937
Gut contents, *C. maschalocarpum*	3.49	**0.0002**	9944
E. radiata, *C. maschalocarpum*	3.41	**0.0013**	9926

The FA profile of *E. radiata* consisted of 22 identified FAs (Table 2.5; Table 2.6). Seven SFAs were present in this species of seaweed, dominated by C16:0, followed by C14:0, and with the other SFAs present in trace concentrations, <2% of the total FA. Four MUFAs were identified in this brown seaweed with C18:1(n-9c) as the dominant followed by C16:1(n-7); all other MUFAs identified were present in small percentages, < 1% of total FA. In *E. radiata* a total of 11 PUFAs were identified: C20:4(n-6) was the PUFA present in the highest percentage, followed by C20:5(n-3) and C18:4(n-3). C18:3(n-3) and C18:2(n-6c) had percentages of ~4-6% of total FA and the remaining identified PUFAs were less than 1% of the total FA (Table 2.5; Table 2.6).



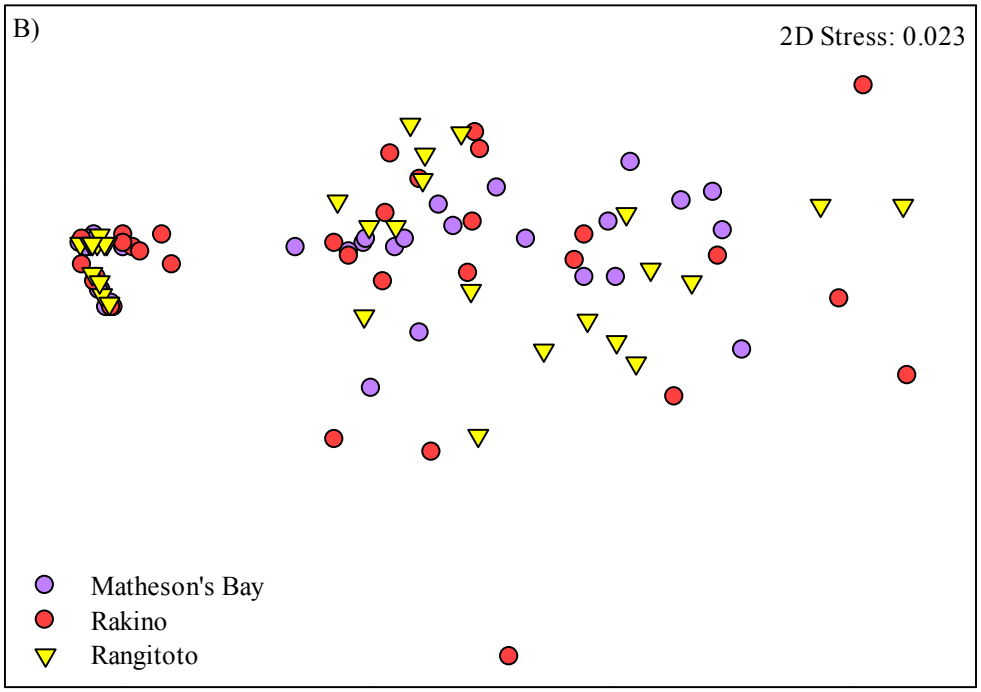


Figure 2.6. Multidimensional scaling (MDS) plot of Euclidean similarities of FA profile of A) Tissues: *E. chloroticus* gonad, gut, gut contents and seaweed species: *E. radiata* and *C. maschalocarpum* and B) Locations: Rakino, Matheson's Bay and Rangitoto. Seaweed and gut contents data are overlaying each other in the left part of the graph.

In total 24 FAs were present in the FA profile of *C. maschalocarpum*: 8 SFAs, 7 MUFAs and 9 PUFAs (Table 2.5; Table 2.6). The dominant SFA was C16:0 followed by C14:0; the rest of the SFAs were present in trace concentrations, < 2% of total FA (Table 2.5; Table 2.6). Among the MUFAs, C18:1(n-9c) was present in the highest percentage followed by C20:1(n-15) and C22:1(n-9) with percentage of ~2% of total FA; the other 4 MUFAs were present with less than 2% of total FA (Table 2.5). Among the PUFAs, C20:4(n-6) was the dominant (~19% of total FA), followed by C18:3(n-3) and C20:5(n-3) with concentrations >9% of total FA. C18:2(n-6c) was present with relatively smaller percentages, ~3% of total FA. The remaining PUFAs were present in concentrations less than 2% of total FA (Table 2.5; Table 2.6).

The FA profile of *E. chloroticus* gut contents consisted of 43 identified FAs. C16:0 was the major SFA present, with C14:0 and C18:0 present in relatively high concentrations. The rest of the SFAs were present in trace concentrations, less than 2% of total FA. A total of 18 MUFAs were identified in gut contents: C18:1(n-9t) and C20:1(n-11) were the MUFAs present in the highest percentages (~5% of total FA). C16:1(n-7), C18:1(n-9c), C20:1(n-15) and C22:1(n-9c) were also present in relatively high percentages (~3-4% of total FA). The rest of the MUFAs identified were present in small percentages or trace concentrations. Sixteen different PUFAs were identified: C20:4(n-6) and C20:5(n-3) were the PUFAs present in the highest percentages (~8% of total FA), followed by and C20:2(n-9), with relatively high percentage (~5% of total FA). C18:3(n-3), C18:2(n-6) and C18:4(n-3) were PUFAs also present in high percentages (~2% of total FA). The rest of the PUFAs identified were present in trace concentrations, less than 1% of total FA (Table 2.5; Table 2.6).

In total, 49 different FAs were identified in the *E. chloroticus* gut tissue. Nine SFAs were identified, with C16:0 and C14:0 presenting the highest percentages (~14% of total FA) and C18:0 showing also relatively high percentage (~5% of total FA). The rest of the SFAs were present at less than 1.5% of total FA. Among the 19 MUFAs identified in this tissue, six were the major MUFAs present: C16:1(n-7), C18:1(n-5), C18:1(n-9c), C20:1(n-15), C20:1(n-11) and C22:1(n-9), with percentages ranging from ~3-6% of total FA. The rest of the MUFAs were present in trace concentrations, less than 1.5% of total FA. In total, 21 PUFAs were identified with C18:4(n-3), C20:2(n-9), C20:4(n-6) and C20:5(n-3) present in the highest percentages, varying between ~4-8% of total FA. The rest of the PUFAs were present in trace concentrations, less than 2% of total FA (Table 2.5; Table 2.6).

The FA profile of *E. chloroticus* gonad contained almost all the identified FAs (57) and with the highest concentrations of the majority of FAs when compared to the other tissues (Tables 2.5; Table 2.6). Among the nine identified SFAs, C16:0 (~15% of total FA) and C14:0 (~12% of total FA) were dominant (Tables 2.5; Table 2.6), with smaller percentages of C18:0 (~3% of total FA) and the remaining SFAs were present in trace concentrations (<1% of total FA). Nineteen MUFAs were identified in this tissue, with C20:1(n-11), C22:1(n-9), C18:1(n-9c), C20:1(n-15) and C16:1(n-7) in the highest percentages (~4-8% of total FA). Fourteen other MUFAs were present in trace concentrations, less than 2% of total FA (Table 2.5; Table 2.6). In total, twenty seven PUFAs were identified in the sea urchin gonad, with C20:2(n-9), C20:4(n-6), C20:5(n-3) and C18:4(n-3) being the dominant (~4-8% of total FA). The remaining PUFAs, ranging from C16:2(n-6) to C22:6(n-3), were present in less than 2% of the total FA (Table 2.5; Table 2.6).

The SFA, MUFA and PUFA profile significantly differed between tissues (Table 2.10), as clearly shown in the MDS plot (Fig 2.7), but there was no difference between locations and the interaction term was also not significant. Pairwise comparisons showed that SFA profile was not significantly different between *C. maschalocarpum* and *E. radiata,* nor between *E. radiata* and gut contents (Table 2.10.D); however, there was a significant different SFA profile between the remaining tissues.

SIMPER analysis revealed that the dominant SFAs (C14:0 and C16:0) contributed the most to the differences between tissues (Table 2.11); but were not significantly different between locations (Table 2.12). The concentrations of these two SFAs followed the same pattern as the total concentration of SFA: increasing from the seaweed species to the sea urchin tissues, with sea urchin gonad showing the highest concentrations of all tissues (Table 2.6; Table 2.12). Even though C18:0 was not considered as one of the contributors of the differences by SIMPER, its concentration was also significant between tissues (Pseudo-$F_{4,79}$=174.63, P(perm)=0.0001) but not between locations (Pseudo-$F_{2,79}$=.010, P(perm)=0.0541), being present in very low concentration in the seaweeds (~1% of total FA) and in relatively higher percentages in *E. chloroticus* tissues (>3% of total FA).

Table 2.10. Results of Multivariate two-way PERMANOVA comparing A) SFA profile, B) MUFA profile, C) PUFA profile and D) Pair-wise comparisons among *E. radiata* and *C. maschalocarpum* and *E. chloroticus* gut contents, gut and gonad from three different locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	54529	13632	114.36	**0.0001**	9956
Location	2	79.68	39.84	0.33	0.7262	9956
Tissue x Location	8	316.94	39.62	0.33	0.9599	9927
Residual	79	9417	119.2			
Total	93	64342				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	30090	7522.40	107.71	**0.0001**	9952
Location	2	187.21	93.61	1.34	0.2626	9952
Tissue x Location	8	254.6	31.83	0.46	0.9168	9933
Residual	79	5517.1	69.84			
Total	93	36049				

C)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissue	4	20308	5077	100.93	**0.0001**	9948
Location	2	245.36	122.68	2.44	0.0796	9951
Tissue x Location	8	526.6	65.83	1.31	0.2320	9923
Residual	79	3974	50.30			
Total	93	25054				

D)

	SFA			**MUFA**			**PUFA**		
Tissues	t	P(perm)	Unique perms	t	P(perm)	Unique perms	t	P(perm)	Unique perms
Gonad, Gut	6.92	**0.0001**	9939	8.82	**0.0001**	9944	6.29	**0.0001**	9944
Gonad, Gut contents	15.45	**0.0001**	9920	15.07	**0.0001**	9918	14.74	**0.0001**	9925
Gonad, *E. radiata*	8.65	**0.0001**	9924	8.40	**0.0001**	9930	7.90	**0.0001**	9930
Gonad, *C. maschalocarpum*	8.11	**0.0001**	9937	7.85	**0.0001**	9939	7.65	**0.0001**	9952
Gut, Gut contents	19.63	**0.0001**	9910	15.9	**0.0001**	9931	18.42	**0.0001**	9909
Gut, *E. radiata*	11.52	**0.0001**	9928	8.96	**0.0001**	9948	10.24	**0.0001**	9931
Gut, *C. maschalocarpum*	10.92	**0.0001**	9920	8.53	**0.0001**	9945	10.14	**0.0001**	9906
Gut contents, *E. radiata*	1.64	0.8940	9960	6.28	**0.0001**	9958	6.71	**0.0001**	9951
Gut contents, *C. maschalocarpum*	2.05	**0.0282**	9952	4.24	**0.0002**	9944	4.77	**0.0001**	9948
E. radiata, C. maschalocarpum	1.81	0.0790	9942	5.00	**0.0008**	9921	3.54	**0.0020**	9931

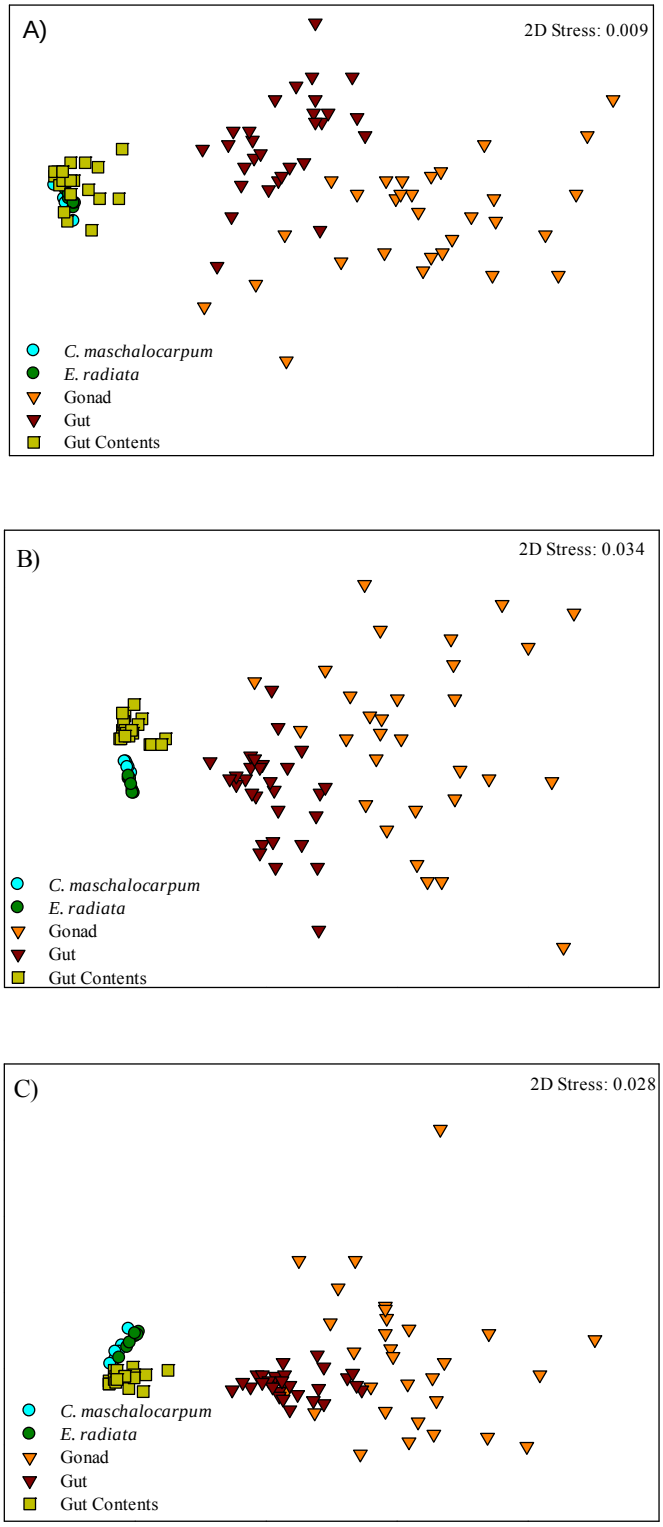


Figure 2.7. Multidimensional scaling (MDS) plot of Euclidean similarities of A) SFA profile, B) MUFA profile and C) PUFA profile of seaweed species: *E. radiata* and *C. maschalocarpum* and *E. chloroticus* gut contents, gut and gonad. All three locations (Matheson's Bay, Rakino and Rangitoto) were included in the analysis. Seaweed and gut contents data are overlaying each other in the left part of the graph

Table 2.11. Contribution of individual SFA to multivariate differences in fatty acid profile between *E. chloroticus* gonad, gut, gut contents, *E. radiata* and *C. maschalocarpum* as determined by SIMPER (>10% of contribution). t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor for each SFA and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Tissues: Groups 1 and 2	Average squared distance	FA	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Gonad & Gut	887.08	C16:0	48.1	27.20	584	1.12	65.80	65.80	8.83	**0.0001**	9839
		C14:0	39.5	28.10	291	0.81	32.77	98.57	4.66	**0.0001**	9817
Gonad & Gut contents	3481.66	C16:0	48.1	5.71	1930	2.00	55.33	55.33	16.52	**0.0001**	9826
		C14:0	39.5	3.06	1460	1.74	41.90	97.23	14.16	**0.0001**	9836
Gut & Gut contents	1233.33	C14:0	28.1	3.06	665	2.15	53.95	53.95	19.86	**0.0001**	9820
		C16:0	27.2	5.71	490	2.10	39.73	93.68	19.27	**0.0001**	9810
Gonad & *E. radiata*	3543.53	C16:0	48.1	6.30	1870	2.01	52.83	52.83	9.05	**0.0001**	9838
		C14:0	39.5	1.61	1560	1.82	44.07	96.90	8.13	**0.0001**	9822
Gut & *E. radiata*	1284.52	C14:0	28.1	1.61	735	2.40	57.23	57.23	11.83	**0.0001**	9841
		C16:0	27.2	6.30	460	2.22	35.81	93.01	10.92	**0.0001**	9845
Gut contents & *E. radiata*	15.87	C14:0	3.06	1.61	7.14	0.44	45.00	45.00	2.34	**0.0321**	9857
		C16:0	5.71	6.30	6.87	0.91	43.30	88.31	0.68	0.5103	9820
Gonad & *C. maschalocarpum*	3646.75	C16:0	48.1	5.84	1910	2.02	55.42	52.42	8.44	**0.0001**	9841
		C14:0	39.5	0.80	1620	1.85	44.53	96.95	7.67	**0.0001**	9820
Gut & *C. maschalocarpum*	1350.35	C14:0	28.1	0.80	779	2.46	57.68	57.68	11.26	**0.0001**	9842
		C16:0	27.2	5.84	481	2.22	35.58	93.27	10.70	**0.0001**	9823
E. radiata & *C. maschalocarpum*	3.58	C16:0	6.3	5.84	2.48	0.68	69.35	69.35	0.77	0.4779	9857
		C14:0	1.61	0.80	0.70	2.16	19.60	88.95	13.33	**0.0001**	9807
Gut contents & *C. maschalocarpum*	19.58	C14:0	3.06	0.80	10.2	0.52	51.89	51.89	3.37	**0.0086**	9833
		C16:0	5.71	5.84	7.58	0.76	38.70	90.59	0.13	0.9006	9846

Table 2.12. Results of Univariate two-way PERMANOVA analysis of individual MUFA that contributed to the differences between *E. radiata, C. maschalocarpum*, sea urchin gut contents, gut and gonad from three locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

SFA		Tissue	Location	Tissue x Location
C14:0	Pseudo-F	99.60	0.48	0.32
	P(perm)	**0.0001**	0.6200	0.9595
C16:0	Pseudo-F	127.81	0.03	0.22
	P(perm)	**0.0001**	0.9721	0.9877

Six MUFAs contributed most to the differences among tissues (Table 2.13). The concentrations of C18:1(n-9c), C18:1(n-9t), C20:1(n-11), C20:1(n-15) and C22:1(n-9) varied significantly between tissues but not between locations; the interaction term was also not significant (Table 2.14). C20:1(n-11) was not detected in *E. radiata* or *C. maschalocarpum*, was present in low concentrations in the gut contents, followed by the concentration in the gut and the highest concentration was detected in the gonad (Table 2.6; Table 2.13). The concentration of C18:1(n-9c) was present in trace amounts in the gut contents, followed by the concentration in *C. maschalocarpum* and *E. radiata,* then by the concentration in the gut, and was highest in the gonad (Table 2.6; Table 2.13). C18:1(n-9t) was not detected in the seaweed species; but was present in low concentrations in the gut, followed by the concentration in the gut contents and in the gonad with similar concentrations in both tissues (Table 2.6; Table 2.13). C22:1(n-9) and C20:1(n-15) were present in trace concentrations, or not detected, in both seaweed species *E.radiata* and *C. maschalocarpum* and gut contents; however their concentrations were higher in the gut tissue, with the highest concentrations detected in the gonad (Table 2.6; Table 2.13). The sixth MUFAs that contributed to the differences was C16:1(n-7), showing significant differences between tissues and also between locations (Table 2.14), again showing the same pattern, increasing from the brown seaweeds to the gonads. However, the significance between locations showed that the tissues from Matheson Bay presented relatively smaller concentrations than the tissues from Rakino ($t=2.19$; P(perm)=0.0305) and from Rangitoto ($t=2.88$, P(perm)=0.0068).

Table 2.13. Contribution of individual MUFA to multivariate differences in fatty acid profile among *E. chloroticus* gonad, gut, gut contents and the seaweed species (*E. radiata* and *C. maschalocarpum*) as determined by SIMPER (>10% of contribution). t, P(perm) and Unique perms were obtain from PERMANOVA on a single factor for each SFA and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Tissues: Groups 1 and 2	Average squared distance	MUFA	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Gonad & Gut	737.62	C20:1(n-11)	25.8	9.92	308	1.17	41.70	41.70	11.41	**0.0001**	9850
		C22:1(n-9)	19.8	6.5	205	1.29	27.74	69.44	5.40	**0.0001**	9841
		C18:1(n-9c)	19.7	13	85.6	0.84	11.60	81.04	5.70	**0.0001**	9830
Gonad & Gut contents	1938.34	C20:1(n-11)	25.8	1.76	630	1.73	32.49	32.49	15.66	**0.0001**	9818
		C22:1(n-9)	19.8	0.96	380	1.82	19.61	52.10	15.22	**0.0001**	9835
		C18:1(n-9c)	19.7	1.38	370	1.69	19.10	71.20	14.62	**0.0001**	9833
		C20:1(n-15)	16.3	0.10	253	1.67	13.07	84.27	15.51	**0.0001**	9834
		C16:1(n-7)	14.4	0.83	207	1.51	10.70	94.98	13.28	**0.0001**	9824
Gut & Gut contents	503.38	C18:1(n-9c)	13	1.38	144	1.95	28.58	28.58	19.59	**0.0001**	9835
		C20:1(n-15)	12.1	0.10	131	1.88	26.11	54.69	17.52	**0.0001**	9817
		C16:1(n-7)	9.48	0.83	84.1	1.44	16.70	71.36	15.06	**0.0001**	9808
		C20:1(n-11)	9.92	1.76	73.6	1.61	14.61	86.01	15.11	**0.0001**	9831
Gonad & *E. radiata*	1963.39	C20:1(n-11)	25.8	ND	716	1.86	36.49	36.49	9.26	**0.0001**	9838
		C22:1(n-9)	19.8	ND	417	1.93	21.22	57.71	9.08	**0.0001**	9863
		C20:1(n-15)	16.3	0.042	283	1.79	14.40	72.11	9.10	**0.0001**	9846
		C18:1(n-9c)	19.7	5.16	245	1.40	12.48	81.59	6.40	**0.0001**	9831
Gut & *E. radiata*	489.25	C20:1(n-15)	12.1	0.042	153	2.09	31.23	41.23	10.60	**0.0001**	9826
		C20:1(n-11)	9.92	ND	104	2.05	21.30	52.53	10.45	**0.0001**	9838
		C16:1(n-7)	9.48	1.5	72.4	1.36	14.80	67.33	7.70	**0.0001**	9816
		C18:1(n-9c)	13	5.16	69.9	1.39	14.28	81.61	7.36	**0.0001**	9826
Gut contents & *E. radiata*	30.17	C18:1(n-9c)	1.38	5.16	15.9	1.80	52.79	52.79	12.44	**0.0001**	9828
		C20:1(n-11)	1.76	ND	4.22	0.84	13.99	66.78	5.62	**0.0001**	9845
		C18:1(n-9t)	1.61	ND	3.68	0.82	12.20	78.98	3.79	**0.0026**	9853
Gonad & *C. maschalocarpum*	2005.68	C20:1(n-11)	25.8	ND	716	1.86	37.72	35.72	8.55	**0.0001**	9822
		C22:1(n-9)	19.8	0.50	397	1.88	19.79	55.51	8.39	**0.0001**	9804
		C8:1(n-9c)	19.7	3.63	292	1.52	14.55	70.07	6.53	**0.0001**	9850
		C20:1(n-15)	16.3	0.51	268	1.74	13.36	83.42	8.16	**0.0001**	9808
		C16:1(n-7)	14.4	0.36	220	1.57	10.97	94.40	6.99	**0.0001**	9819
Gut & *C. maschalocarpum*	516.83	C20:1(n-15)	12.1	0.51	142	2.01	27.44	27.44	9.41	**0.0001**	9835
		C20:1(n-11)	9.92	ND	104	2.05	20.17	47.61	9.65	**0.0001**	9844
		C18:1(n-9c)	13	3.63	96	1.64	18.57	66.18	8.16	**0.0001**	9847
		C16:1(n-7)	9.48	0.36	92	1.53	17.80	83.97	8.13	**0.0001**	9846
Gut contents & *C. maschalocarpum*	18.84	C18:1(n-9c)	1.38	3.63	6.49	1.40	34.46	34.46	7.33	**0.0001**	9837
		C20:1(n-11)	1.76	ND	4.22	0.84	22.41	56.87	5.18	**0.0003**	9814
		C18:1(n-9t)	1.61	ND	3.68	0.82	19.53	76.40	3.49	**0.0042**	9846
E. radiata & *C. maschalocarpum*	5.21	C18:1(n-9c)	5.16	3.63	3.21	1.07	61.60	61.60	3.88	**0.0065**	9844
		C16:1(n-7)	1.5	0.36	1.37	2.31	26.37	87.96	12.47	**0.0003**	1840

Table 2.14. Results of Univariate two-way PERMANOVA analysis of individual MUFA that contributed to the differences between *E. radiata, C. maschalocarpum and E. chloroticus* gut contents, gut and gonad from three locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

MUFA		Tissue	Location	Tissue x Location
C16:1(n-7)	Pseudo-F	76.64	3.43	0.62
	P(perm)	**0.0001**	**0.0364**	0.7606
C18:1(n-9c)	Pseudo-F	88.80	2.28	0.36
	P(perm)	**0.0001**	0.1055	0.9414
C18:1(n-9t)	Pseudo-F	19.53	0.11	0.98
	P(perm)	**0.0001**	0.898	0.4438
C20:1(n-11)	Pseudo-F	129.35	0.2	0.61
	P(perm)	**0.0001**	0.813	0.7775
C20:1(n-15)	Pseudo-F	107.29	0.18	0.38
	P(perm)	**0.0001**	0.8327	0.9364
C22:1(n-9)	Pseudo-F	119.24	0.33	0.27
	P(perm)	**0.0001**	0.7165	0.9759

Five PUFAs contributed to the differences between tissues, which were C18:3(n-3), C18:4(n-3), C20:2(n-9), C20:4(n-6) or ARA and C20:5(n-3) or EPA (Table 2.15). Even though the concentration of C18:3(n-3) was relatively low in all tissues, significant differences were found between tissues but not between locations; however, the interaction term was also significant making the analysis hard to describe as there was not a clear pattern. *C. maschalocarpum* presented the higher concentrations (~3µg/mg dry weight), followed by the concentrations in *E. radiata* (~2µg/mg dry weight) in every location (Table 2.6; Table 2.16). Following were C18:3(n-3) concentrations of gonads from Rakino and Rangitoto (~1µg/mg dry weight) and the concentration of gut contents from Matheson Bay (0.74µg/mg dry weight). Then, the gut contents concentrations from Rakino and Rangitoto (~0.4-0.6µg/mg dry weight) and gonads from Matheson Bay (0.65µg/mg dry weight). The gut tissue presented the smallest concentration in all locations (~0.2-0.4µg/mg dry weight) (Table 2.6; Table 2.16).

The concentrations of C18:4(n-3) and C20:2(n-9) were significantly different between tissues but not between locations; the interaction term was also not significant (Table 2.16). C18:4(n-3) was present in very low concentrations in *C. maschalocarpum*, followed by the concentration in the gut contents, then in *E. radiata*, gut and the highest concentration was found in the gonad (Table 2.6; Table 2.15). C20:2(n-9) was not detected in *C. maschalocarpum*, was present in very low concentrations in *E. radiata* (~0.01 µg/mg dry weight), followed by the concentration in the gut contents (~2µg/mg dry weight). Much higher concentrations were found in the gut (~17µg/mg dry weight), and with the highest concentrations in the gonad (~25µg/mg dry weight) (Table 2.6; Table 2.15).

The concentrations of ARA and EPA were significantly different between tissues, between locations and the interaction term was also significant (Table 2.16). The interaction effect was a result of the variation in the concentration of these PUFAs in the gonad between locations, as the remaining tissues showed no significant differences between locations ($p>0.05$). ARA was present in relatively small levels in the gut contents, increasing in *C. maschalocarpum*, *E. radiata*, gut and again the highest concentration was found in the gonad, regardless of location (Table 2.6; Table 2.15). However, the concentration of this PUFA in the gonad from Matheson's Bay was slightly smaller than the concentration in the gonad from Rakino ($t=3.7213$, $P(perm)=0.0008$) and Rangitoto ($t=3.898$, $P(perm)=0.0009$). The lowest concentration of EPA was found in the gut contents and *C. maschalocarpum*, followed by the concentration in *E. radiata*, gut and gonad, showing this pattern in every location. Nevertheless, gonads from Rakino contained higher concentrations than gonads from Matheson's Bay ($t=3.2387$, $P(perm)=0.0029$) and from Rangitoto ($t=2.7498$, $P(perm)=0.0067$).

Table 2.15. Contribution of individual PUFA to multivariate differences in fatty acid profile among *E. chloroticus* gonad, gut, gut contents and brown seaweed species (*E. radiata* and *C. maschalocarpum*) as determined by SIMPER (>10% of contribution). t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor analysis for each SFA and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Tissues: Groups 1 and 2	Av. squared distance	PUFA	Av. Value Group 1	Av. Value Group 2	Av. Sq.Dist	Sq.Dist/SD	Contribution%	Cumulative%	t	P(perm)	Unique perms
Gonad & Gut	351.60	C20:2(n-9)	25.5	17.60	119	0.81	33.77	33.77	5.40	**0.0001**	9851
		C20:4(n-6)	17.1	10.50	78.2	0.60	22.25	56.03	7.19	**0.0001**	9827
		C20:5(n-3)	16.3	9.84	63.9	0.75	18.17	74.20	8.40	**0.0001**	9836
		C18:4(n-3)	13.5	8.34	54.3	0.68	15.46	89.66	5.12	**0.0001**	9829
Gonad & Gut contents	1351.54	C20:2(n-9)	25.5	1.91	605	1.77	44.76	44.76	15.22	**0.0001**	9821
		C20:4(n-6)	17.1	2.56	243	1.17	17.96	62.72	15.03	**0.0001**	9827
		C20:5(n-3)	16.3	1.70	231	1.57	17.11	78.83	18.91	**0.0001**	9836
		C18:4(n-3)	13.5	0.80	187	1.25	13.83	93.66	11.41	**0.0001**	9841
Gut & Gut contents	481.42	C20:2(n-9)	17.6	1.91	260	2.26	53.97	53.97	21.98	**0.0001**	9834
		C20:5(n-3)	9.84	1.70	71.7	1.74	14.89	68.85	16.31	**0.0001**	9825
		C20:4(n-6)	10.5	2.56	69.1	1.61	14.36	83.22	14.79	**0.0001**	9829
		C18:4(n-3)	8.34	0.80	60.2	1.95	12.50	95.72	19.63	**0.0001**	9822
Gonad & *E. radiata*	1273.08	C20:2(n-9)	25.5	0.01	696	1.92	54.69	54.69	9.08	**0.0001**	9827
		C20:5(n-3)	16.3	2.64	204	1.47	16.03	70.72	9.88	**0.0001**	9819
		C18:4(n-3)	13.5	2.69	142	1.08	11.19	81.90	5.34	**0.0001**	9820
		C20:4(n-6)	17.1	6.89	135	0.83	10.63	92.54	5.89	**0.0001**	9805
Gut & *E. radiata*	460.19	C20:2(n-9)	17.6	0.01	321	2.68	69.80	69.80	13.90	**0.0001**	9815
		C20:5(n-3)	9.84	2.64	56.7	1.58	12.33	82.13	8.25	**0.0001**	9830
Gut contents & *E. radiata*	36.37	C20:4(n-6)	2.56	6.89	21	1.75	57.68	57.68	8.58	**0.0001**	9843
		C20:2(n-9)	1.91	0.01	5.36	0.72	14.72	72.41	5.17	**0.0001**	9850
		C18:4(n-3)	0.80	2.69	4.68	1.36	12.87	85.27	6.74	**0.0001**	9836
Gonad & *C. maschalocarpum*	1385.97	C20:2(n-9)	25.5	ND	697	1.92	50.28	50.28	8.39	**0.0001**	9818
		C20:5(n-3)	16.3	2.41	212	1.47	15.28	65.57	9.29	**0.0001**	9826
		C18:4(n-3)	13.5	0.36	198	1.29	14.27	79.84	6.01	**0.0001**	9835
		C20:4(n-6)	17.1	4.86	181	0.98	13.04	92.88	6.55	**0.0001**	9819
Gut & *C. maschalocarpum*	519.64	C20:2(n-9)	17.6	ND	322	2.68	61.91	61.91	12.85	**0.0001**	9826
		C18:4(n-3)	8.34	0.36	66.6	2.15	12.82	74.73	10.92	**0.0001**	9853
		C20:5(n-3)	9.84	2.41	61.4	1.52	11.81	86.54	7.90	**0.0001**	9864
Gut contents & *C. maschalocarpum*	23.67	C20:4(n-6)	2.56	4.86	7.37	1.04	31.12	31.12	4.65	**0.0002**	9842
		C18:3(n-3)	0.56	2.93	6.58	1.36	27.81	58.93	12.33	**0.0001**	9830
		C20:2(n-9)	1.91	0	5.41	0.72	22.85	81.78	4.80	**0.0012**	9837
		C20:5(n-3)	1.7	2.41	2.81	0.79	11.88	93.66	1.87	0.0693	9842
E. radiata & *C. maschalocarpum*	16.67	C20:4(n-6)	6.89	4.86	6.09	1.20	36.55	36.55	3.15	**0.0213**	9828
		C18:4(n-3)	2.69	0.36	6.09	1.69	36.52	76.09	5.85	**0.0016**	9821
		C18:3(n-3)	1.87	2.93	2.23	0.83	13.39	86.48	2.80	**0.0256**	9843
		C20:5(n-3)	2.64	2.41	1.88	0.88	11.30	97.78	0.63	0.5540	9822

Table 2.16. Results of Univariate two-way PERMANOVA analysis of individual PUFA that contributed to the differences among *E. radiata, C. maschalocarpum and E. chloroticus* gut contents, gut and gonad from three locations in the Hauraki Gulf. Significant results ($p<0.05$) are shown in bold.

MUFA		Tissue	Location	Tissue x Location
C18:3(n-3)	Pseudo-F	88.09	0.39	4.66
	P(perm)	**0.0001**	0.6916	**0.0019**
C18:4(n-3)	Pseudo-F	62.70	0.74	0.28
	P(perm)	**0.0001**	0.4913	0.9718
C20:2(n-9)	Pseudo-F	119.24	0.33	0.27
	P(perm)	**0.0001**	0.7152	0.9765
C20:4(n-6)	Pseudo-F	84.34	6.24	3.71
	P(perm)	**0.0001**	**0.0030**	**0.0020**
C20:5(n-3)	Pseudo-F	137.83	6.17	2.94
	P(perm)	**0.0001**	**0.0032**	**0.0065**

2.4 Discussion

Lipid and FA analysis in *Evechinus chloroticus* revealed a clear pathway of assimilation from the seaweed to the sea urchin tissues. For instance, when feeding on both potential food items, *Ecklonia radiata* and *Carpophyllum maschalocarpum*, the lipid (total and energy lipids) and FA (SFA, MUFA and PUFA) concentrations in the *E. chloroticus* tissues increase from the gut content to the gut and gonad, with the gonads having the highest concentrations. . This was expected, as the gonad is considered a storage organ in sea urchins as well as having a reproductive function. This dual function can be explained by the capability of this organ to modify lipids and FAs according to their role (Hughes et al., 2006; Walker et al., 2001). This pattern of concentration and modification was consistent across the three locations sampled in the Hauraki Gulf. Although histological information is not available in the present study, the literature indicates that at the time of collection, i.e. from the end of January to the beginning of February, partial spawning has occurred, suggesting that the reproductive stage of the animals were similar in the three locations analysed here (Walker, 1982).

The seaweed species showed higher concentrations of structural lipid (>86% of total lipids) than energy lipid (<15% of total lipids). Although both seaweed species showed the same structural lipid classes with PL being dominant, followed by ST and AMPL; *E. radiata* (Laminariales) contained slightly higher concentrations of them than *C. maschalocarpum* (Fucales). Furthermore, the energy lipids were also different between these two brown seaweed species, three energy lipids were detected in *E. radiata* (TAG, FFA and DAG) in relatively small concentrations, while *C. maschalocarpum* had a single energy class (TAG in trace amounts). It is not surprising the variation seen in the lipid and fatty acid composition,

between these two brown seaweeds species as they belong to different orders, *E. radiata* being part of the Laminariales and *Carpophyllum* of Fucales (Schiel, 1988). This agreed with the stable isotope results presented in Chapter 6. A previous study showed that the polar lipid composition divided the brown algae in two groups, the Laminariales containing more phospholipids, whereas the Fucales presenting more glycolipids and the authors suggested a connection with taxonomic position of brown seaweeds (Vaskovsky et al., 1996). In the present study, the polar lipids were not analysed in this level of detail, but the taxonomic difference could explain such a variation found here.

The number of lipid classes as well as total and energy lipid increased from seaweed to the gut contents, which showed nine lipid classes and, also, the concentration of total and energy lipid was higher in this tissue than in the algae. Gut of *E. chloroticus* showed a total of eight lipid classes: TAG, FFA, WE, ME and DAG as energy lipids and ST, PL and AMPL as structural lipids and their levels were higher than the gut contents. Previously, Lawrence et al. (1989) found that the lipid content in the sea urchin gut decreased during starvation, suggesting that the gut acts as a storage organ responsive to immediate needs as well as a region for immediate deposit of excess nutrients. Starved sea urchins, *Lytechinus variegatus*, showed that the lipid content in their gut (stomach and intestine) increased after feeding treatment and the authors suggested that the gut has a specific functional organization prior to the transfer of nutrients to the gonads (Bishop et al., 1992). Sea urchin gonad presented the highest concentration of all lipid classes as well as the highest concentration of total and energy lipids. As the storage organ, the gonad is expected to contain a great concentration of energy lipids, with TAG as the main energy reserve (Liyana-Pathirana et al., 2002) as in most other animals (Iverson, 2009). Here, the differences in the lipid profile among tissues were principally driven by the presence and concentration of this lipid class, with levels showing the same pattern, being present in very low concentration in *C. maschalocarpum* and *E. radiata* followed consecutively by the gut contents, gut and gonad of *E. chloroticus.* Thus, lipid energy, in terms of TAG, is transferred from seaweeds to sea urchin gut contents, digested by the gut and stored in the gonad (Castell et al., 2004; Cook et al., 2000). However, it is also possible that the high concentration of dietary phospholipids are being metabolized and stored as neutral lipid in the gut and gonad, as previously suggested for *Lytechinus variegatus* (Gibbs et al., 2009).

All those findings are supported by the FA results. Phospholipids usually contain high amounts of PUFA, being mainly the structural components of cell membranes (Budge et al., 2006). In contrast, SFA and MUFA are mostly an important fraction of the neutral lipids, such as TAG, which normally act as energy reserves (Budge et al., 2006). The total FA, SFA, MUFA and PUFA and individual FAs followed the same pattern as lipids: small levels were found in the seaweed, increasing in the gut contents, gut and gonads. However, the percentage of PUFA was higher in the seaweed (~50%) than in the remaining tissues (~30%), which presented higher percentages of SFA and MUFA. These results indicate an increase of SFA and MUFA and decrease of PUFA in the digestion process from the seaweed to the sea urchin tissue, probably due to biosynthesis occurring in these tissues or special storage of some FAs present in low concentrations in the algae material. This is supported by the variation in the percentage or concentration of some particular FAs from the seaweed to the sea urchin tissues, as it is well known that the FA composition of sea urchin gonads is affected by the diet, both artificial and natural (Castell et al., 2004; Cook et al., 2000; Kelly et al., 2008; Liyana-Pathirana et al., 2002).

The levels of some SFAs revealed the selective retention and possible biosynthesis of FAs occurring in the gut and gonads. C14:0 was presented in relatively high percentages in the seaweed, but even higher in the tissues (gut and gonad), where the concentration was one of the highest, suggesting an especial accumulation of this SFA. The percentages of C16:0 decreased from the seaweed to the gut and gonad tissue suggesting that this SFA is probably been used as a precursor of the elongated C18:0, which was present in very low levels in the seaweed but higher level in the sea urchin tissues. It is well known that some SFAs, especially C16:0, are elongated and desaturated to produce longer and unsaturated FAs (Kelly et al., 2012). High levels of these SFAs were also found in the gonads of several sea urchin species like *S. droebachiensis* (Liyana-Pathirana et al., 2002), *Psammechinus miliaris* (Cook et al., 2000; Cook et al., 2007) and *Paracentrotus lividus* (Cook et al., 2007).

Furthermore, *E. chloroticus* seems to be able to synthesize a variety of MUFAs or bioaccumulate them when they are present in very low concentration in their diets, as revealed by the moenes C16:1(n-7), C20:1(n-15), C22:1(n-9) being present in very small concentrations in the seaweeds and gut contents but in higher levels in the gut and gonad. Particularly, the high dietary levels of C16:0 could explain the high levels of C16:1(n-7) in the sea urchin tissues, as it usually desaturates from C16:0 and tends to accumulate at

relatively high concentrations in animal tissues (Kelly et al., 2012). The biosynthesis of long-chain MUFA could be related to the high dietary levels of the precursor C18:1(n-9) as previously described in *S. droebachiensis* (Castell et al., 2004). Similar results were reported for *S. droebachiensis* where the high levels of C20:1(n-15) in their gonads suggested that this MUFA was biosynthesised as it was absent in the diet used in the feeding experiment (Liyana-Pathirana et al., 2002). A different study on *S. droebachiensis* showed high levels of C20:1(n-11), C20:1(n-9) and C22:1(n-9) in their gonads but very low levels in the diets, suggesting that they are biosynthesised or specifically retained by these sea urchins (Castell et al., 2004).

Additionally, the high levels of C18:1(n-9) found in the brown seaweeds (~4-5μg/mg dry weight) and high levels in the sea urchin tissues (~13-19μg/mg dry weight), and the decrease in its percentages from the seaweed to the sea urchin gut and gonad, indicate a possible selective assimilation of this MUFA by sea urchin tissues and elongation to synthesise longer MUFAs. These results are supported by previous findings in *S. droebachiensis*, where high levels of C18:1(n-9) in the diets seams to increase the levels of C20:1(n-9) in the sea urchin gut and gonad (Castell et al., 2004). Furthermore, the conversion of C18:1(n-9) to C20:1(n-9) appears to be accelerated by high levels of n-3 PUFA in the diets (Castell et al., 2004).

E. chloroticus seems to be able to assimilate and store long-chain PUFA when they are available in high concentrations in the diet, as they have important structural functions in cell membranes (Floreto et al., 1996; Hughes et al., 2005). In particular, the high levels of C18:4(n-3), C20:4(n-6) (Arachidonic acid or ARA) and C20:5(n-3) (Eicosapentaenoic acid or EPA) in the seaweed, as well as in sea urchin tissues, indicate that *E. chloroticus* is possibly feeding on brown seaweeds and selectively retaining these FAs. However, *E. chloroticus* appears to be grazing on *E. radiata* more than *C. maschalocarpum* as proposed by Don (1975), based on the relatively higher levels of these PUFAs in *E. radiata*. The FA profile of *E. radiata* agreed with previous reports of *E. radiata* collected in the south of New Zealand (McLeod et al., 2013) as well as *E. radiata* from Australia (Guest et al., 2010); although, the fatty acid profile of *C. maschalocarpum* has not been described in previous literature.

It is well known that some FAs can be used as biomarkers and tracked from the sources to the consumers (Iverson, 2009). Thus, all these findings are supported by the common knowledge that brown seaweeds are characterised by high levels of C18:4(n-3),

C20:4(n-6) and C20:5(n-3) (Kelly et al., 2012; Khotimchenko et al., 2002). Moreover, Hanson et al. (2010) suggested that ARA can be a useful biomarker to trace *E. radiata* through the food web. Consequently, high levels of these PUFAs in the sea urchin tissues indicate a diet based on brown seaweed as reported previously for other sea urchin species like *P. miliaris* (Cook et al., 2000), *S. droebachiensis* (Liyana-Pathirana et al., 2002) and *P. lividus* (Cook et al., 2007). Furthermore, dietary lipids had a great influence on the FA composition of gut and individual PUFAs, such as C20:4(n-6), C20:5(n-3) and C22:6(n-3) which were preferentially deposited in the gonads of *S. droebachiensis* (González-Durán et al., 2008).

The higher levels of some FAs in the sea urchin tissues than in the seaweed could also be explained by the possibility of bacteria living in the gut and synthesising FAs as previously reported for *S. nudus* (Iwanami et al., 1995). Bacteria play an important role in providing essential nutrients and particularly synthesising long-chain PUFA in marine environments (Nichols, 2003). An analysis of the isolated bacteria *Vibrio* sp. from *S. nudus* suggested an aerobic desaturation and elongation of C18:1(n-9) to form C20:5(n-3) through C18:3 (Iwanami et al., 1995). Unfortunately, no literature was found investigating the bacteria from *E. chloroticus*, but this particular *Vibrio* sp. has been isolated from other sea urchin species like *Echinus esculentus* (Unkles, 1977), *S. intermedius* (Sawabe et al., 1995) and *S. nudus* (Iwanami et al., 1995; Sawabe et al., 1995). Further studies are needed to better understand if this bacteria is present in the sea urchin flora and also if it plays a role in the synthesis of these essential FAs in *E. chloroticus*.

Summarizing, this study is highlighting the clear ability of *E. chloroticus* to convert food sources into nutrient reserves, as shown by the lipid and FA analysis. There was a clear increment in the concentration of energy lipid, in terms of TAG, as it passed through the digestive process from the brown seaweeds (possible food items) where the concentrations of energy lipids were relatively low, to *E. chloroticus* gut and finally to the gonad, where the energy lipids were present in relatively high concentrations. Consequently, it seems that *E. chloroticus* is able to convert dietary structural lipids (PL) into energy lipids (TAG). In addition, due to the increase in the proportion of triglycerides from seaweed to gut to gonad, the energy content from these FA followed the same pattern, increasing from the potential food to the gut contents, then to the gut until it finally reaches the storage organ, the gonad. The digestive pathway was revealed by the variation in the percentages and concentrations of

particular beneficial or required FAs. The high levels of PUFA in the diet are assimilated by the sea urchin and possibly favour the biosynthesis of SFA and MUFA. *E. chloroticus* tissues seems to assimilate and accumulate and probably synthesize some FA, as there were some FAs present in the gut and gonad and absent in the diets or present in relatively higher concentration than in the diets. Nevertheless, further studies using different diets are needed to better understand these processes. Thus, using lipid and FA analysis it was possibly to identify *E. chloroticus* diet, suggesting that *E. radiata* is probably more suitable for consumption providing more nutrients, indicated by the higher level of some PUFA than *Carpophyllum* and so accumulate more of those PUFA observed in their gonads.

Chapter 3

Lipid and fatty acid composition of *Evechinus chloroticus* gonads from different locations in the Hauraki Gulf, New Zealand

3.1 Introduction

Gonads of sea urchins, also known as roe or uni, are highly valued seafood, consumed principally as sushi in many regions around the world, particularly Asia, North and South America and the Mediterranean (Lawrence, 2001; Liyana-Pathirana et al., 2002). They are considered delicacies because of the caviar-like appearance of the yellow gonad sacs as well as their particular bitter-sweet flavour and aroma (Chen, 2005; Lawrence, 2007; Liyana-Pathirana et al., 2002; Phillips et al., 2010; Verachia et al., 2012). Sea urchin gonads are also of a relatively high nutritional value, attributed to their elevated contents of carotenoids, carbohydrates, proteins and especially lipids (Hughes et al., 2006; Lawrence, 2007). This is because sea urchin gonads have a multifunctional role: they are the nutritive and, also, reproductive organs, and therefore their biochemical composition reflects both functions (Hughes et al., 2006; Russell, 1998; Walker et al., 2007; Walker et al., 2001). Furthermore, because of this dual role gonads probably undergo a greater biochemical and fatty acid (FA) modification than in the lipid storage organs of other herbivorous invertebrates (Hughes et al., 2006; Iverson, 2009).

The sea urchin species *Evechinus chloroticus*, also known as kina, is endemic to the New Zealand region (Barker, 2013) and is the only sea urchin species commercially harvested for the domestic market (Woods et al., 2008). Kina is widely distributed along the entire coast of the New Zealand mainland and nearby islands (Dix, 1970; Mortesnsen, 1943; Pawson, 1961), generally in waters <12-14 metres deep (Barker, 2013). Kina typically dwells at the rocky bottom but it may be found on hard stable substrates, shelly sand, fine sand and mud (Dix, 1970; Fell, 1952; Morton et al., 1968). Generally, its abundance increases with increasing exposure to wave action except in the most exposed locations, where densities are reduced (Choat et al., 1987; Choat et al., 1982). However, it is difficult to make broad generalisations about the habitat of *E. chloroticus* through New Zealand because it has different patterns of distribution in relation to kelp and other invertebrates. In the North Island, kina is most commonly found on shallow rocky reefs, strongly associated with kelp

forests, often dominated by the laminarian *Ecklonia radiata* and in very shallow waters fucaleans such as *Carpophyllum maschalocarpum* and *C. angustifolium* (Barker, 2013; Dix, 1970; Schiel, 1982). *E. chloroticus* is mostly herbivorous, being one of the dominant grazers in northern New Zealand feeding on a great variety of brown seaweed species (Barker, 2013). Early studies revealed that kina has a preference for *E. radiata* (Don, 1975; Schiel, 1982). However, when algae is limited, they can graze on a wide range of encrusting organisms, like sponges (Ayling, 1978).

Identifying the diet of animals is important to understand their basic ecology and to characterise their trophic interactions (Kelly et al., 2012). There are several methods of studying the diet of animals, such as analysis of the gut contents or fecal pellets. However, these approaches can underestimate previously digested food items and also overestimate the most recent feeding activity (Iverson et al., 2004; Kelly et al., 2012). In addition, in marine ecosystems it is difficult to characterise diets due to the inaccessibility of free-ranging organisms and the inability to directly observe the interactions of species (Iverson, 2009). Thus, a recently developed approach is the use of lipid and FA signatures to analyse animal diets. Lipids are important energy reserves since they can store more energy per unit volume than either proteins and carbohydrates (Parrish, 2013). Additionally, they consist of a large group of nonpolar compounds, of which FAs are the "building blocks" (Iverson, 2009). FAs are the largest constituent of neutral lipids, such as the energy storage lipids triacylglycerols and wax esters, and the primary membrane structural components, the polar phospholipids (Iverson, 2009). Generally, FAs can be grouped into: 1) saturated FA (SFA), those with no double bonds in the carbon chain, 2) monounsaturated FA (MUFA), with one double bond present and 3) polyunsaturated FA (PUFA), with two or more double bonds present (Budge et al., 2006).

In general, animals cannot synthesise FAs, especially long-chain PUFA, so these are assimilated from their diets (Iverson, 2009). Primary producers, like seaweed, are the only organisms that possess the enzymes to produce long-chain PUFA; hence, they are considered as a rich source of PUFA in marine ecosystems (Iverson, 2009; Kelly et al., 2012). For example, brown seaweeds are characterised by high levels of PUFAs like 18:3(n-3), 18:4(n-3), 20:4(n-6) and 20:5(n-3) (Floreto et al., 1996), so that these PUFAs can be used as biomarkers to identify algal diets (Kelly et al., 2012). Thus, several studies have used FA signatures to identify the diet of different marine invertebrates, like copepods (Sargent et al.,

1988), bivalves (Guest et al., 2008), polychaetes and crustaceans (Drazen et al., 2008) and sea urchin species (Hughes et al., 2005). The food taken in by sea urchins is initially digested in the intestine (= gut) and then is stored inside nutritive phagocyte cells in the gonads, primarily as lipid which is the major nutrient component in sea urchin gonads (Castell et al., 2004; Cook et al., 2000; Iverson, 2009; Liyana-Pathirana et al., 2002). Feeding experiments have shown that the FA composition of sea urchin gonads is affected by the sea urchin's diet (Castell et al., 2004; Cook et al., 2000; Kelly et al., 2008; Liyana-Pathirana et al., 2002). Consequently, previous studies have used the gonadal FA composition to study the diet of sea urchins from different sites and regions (Guest et al., 2010; Hughes et al., 2005). For example, *Psammechinus miliaris* showed a significant variation in the FA signature between sea urchins collected at two different depths as well as between two different locations (Hughes et al., 2005). A different study revealed that wild *Strongylocentrotus droebachiensis* collected from kelp beds presented a different FA signature than those collected from the sea urchin barrens (Kelly et al., 2008).

Algal communities are strongly influenced by environmental conditions such as water temperature, light, turbidity, salinity, and nutrients (Grace, 1983). These factors also affect the lipid and fatty acid composition in brown seaweeds (Gerasimenko et al., 2010; Nelson et al., 2002). There is a clear differential distribution of brown seaweeds around New Zealand coast, as well as within Hauraki Gulf, due to a variation in the environmental conditions (Schiel, 1988). In the North Island of New Zealand, the East Auckland Current flows southeast down the eastern side of the island (Heath, 1985). Grace (1983) described that the outer Hauraki Gulf presents "ocean" conditions, whereas the inner Hauraki Gulf shows more "harbour" conditions, where there is a reduction in salinity and the waters are more turbid. Due to the restricted light penetration and more sheltered zones in the inner gulf, sea urchin barrens are absent and replaced by *Carpophyllum flexuosum* forests; while these barrens are more extended at more exposed offshore islands (Grace, 1983). Thus, a great variation in the algal community structure is found in north-eastern New Zealand, where the reefs are dominated by the large brown algae *E. radiata* and *C. maschalocarpum* as well as *E. chloroticus* as the most abundant sea urchin (Shears & Babcock, 2004).

The present study addressed the hypothesis that the lipid (energy and structural lipid) and FA (SFA, MUFA and PUFA) composition of *E. chloroticus* varies among different populations within the Hauraki Gulf due to a variation in the composition of its preferred diet

(*E. radiata*). To test this hypothesis, we collected wild sea urchins as well as *E. radiata* plants from four locations, two of them from the inner part (Rakino and Rangitoto) and the remaining from the outer part of the Hauraki Gulf (Great Barrier Island and Matheson Bay).

3.2 Methodology

3.2.1 Sample collection and storage

Wild *E. chloroticus* were collected by snorkelling (<20m depths) from four different locations from the Hauraki Gulf, New Zealand: Great Barrier Island (36^0 10'S, 175^0 25'E), collection date 4th February 2010; Matheson's Bay (36^0 29'S, 174^0 53'E), collection date 11th of February 2010;as the outer part of the Hauraki Gulf and Rakino Island (36^0 72'S, 174^0 95'E), collection date 24th of January 2010; and Rangitoto Island (36^0 80'S, 174^0 86'E), collection date 17th of February 2010 as part of the inner part of the Hauraki Gulf. All the collection sites were shallow rocky reef dominated by brown seaweeds. In total, 20 sea urchins were collected at each location where *E. radiata* was highly abundant; therefore it was most likely the main food of the sea urchins. For this reason, two plants of *E. radiata* were collected in the same locations as possible food items.

After collection, the sea urchins were transported alive to the laboratory in plastic buckets filled with seawater and sea ice, together with the seaweed. The wet weight (to the nearest mg after patting dry with a paper towel) and test diameter (using a Toledo Vita calliper to nearest mm) of the whole animal were recorded prior to dissection. The test was cut around the equator into the oral and the aboral halves, the coelomic fluid was drained, and the Aristotle's lantern was removed and then weighed together with the wet test. The five gonads were removed using tweezers, weighed and stored in 50ml polypropylene tubes in the -80°C freezer until analysis. Seaweed plants were washed with seawater and small pieces (10x5cm) of the blades were placed in the same polypropylene tubes and also stored in -80^{o}C freezer.

From the 20 sea urchins collected at each location, 10 were randomly selected for lipid and fatty acid (FA) analysis. Gonads (five gonads from each individual were pooled) and seaweed material were lyophilized for 72 hours to a constant mass in a freeze-dryer (VirTis Bench Top 2k) and then cryogenically grounded using an MM301 Mixer Mill (Retsch), which resulted in a ground particle size of <5µm. Lyophilized, ground samples

were stored in sealed polypropylene 10ml tubes under desiccant at -20°C until lipid or FA analysis.

3.2.2 Lipid analysis

Lipid determination was carried out using an Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods following the protocols defined by Parrish (1997, 1999) with minor modifications. Lyophilized gonads were weighed (~8 mg for gonads and ~17mg for seaweed material) into 1ml V-vials (Wheaton). Lipid extraction was performed as described in Sewell (2005) with the following modification: 250 µl of ultrapure water, 25 µl of ketone in chloroform (used as an internal standard as natural concentrations are low in marine tissues), 100 µl chloroform and 250 µl methanol (final ratio of water: chloroform: methanol 2:1:2) were added to the V-vial before 20 minutes of sonication on ice in an ultrasonication bath (Sanyo Soniprep 150). After vigorous shaking, the V-vials were centrifuged at 2000 RPM for 5 minutes at room temperature. Both the aqueous and chloroform fractions were transferred with a drawn Pasteur pipette to a clean glass V-vial, leaving the solid non-lipid material behind. An additional 250 µl water and 250 µl chloroform were added and the vial was shaken for a further 1 to 2 minutes. Subsequently, the sample was re-centrifuged at 2000 RPM for 5 minutes at room temperature. Most (90%) of the upper water and methanol fraction was removed with a Pasteur pipette and discarded, with little disturbance to the interface between the aqueous and chloroform layers. The lower chloroform layer (ca. 375µl = [100 + 250 added in extraction] + 25µl from internal standard) was transferred to a third V-vial using a 200µl Eppendorf pipette with a chloroform-rinsed tip. This V-vial containing the lipids suspended in a chloroform layer was stored in a -20^{o}C freezer until used for the Iatroscan analysis. All V-vials used in the extraction process were cleaned with 3x methanol and 3x chloroform washes as recommended by Parrish (1999), and all solvents used in lipid extraction were HPLC-grade. Immediately before spotting onto the Chromarods, the lipid extract was dried down in a stream of instrument grade nitrogen gas and an exact amount of chloroform (75 µl) was added using a Gilson positive displacement pipette. Four separate samples were processed on each run (two replicate Chromarods of each sample; total eight rods). The remaining two Chromarods were unspotted blanks to test for contamination of the development solvents.

Chromarods were developed as described in Parrish (1999), except that, instead of a triple development process, we used a double development system that resulted in two

chromatograms. After spotting, the rack of Chromarods was placed inside a constant humidity chamber (CHC) for five minutes and then transferred to Development tank 1, (69.3 ml of Hexane, 0.7 ml of Diethyl-ether and 0.035ml of Formic Acid; 98.95:1:0.05) for 24 minutes. After this, the rack was placed again for 5 minutes in the CHC before returning to Development tank 1 for a further 19 minutes. The Chromarods were dried for 5 min in a Rod Dryer TK-8 (Iatron Laboratories) at 60°C and were then run in the Iatroscan set to Partial Scan Mode (PPS 27). After recording the first chromatogram, the rack was placed in the CHC for 5 minutes followed by 33 minutes in Development tank 2 (55.3 ml of Hexane, 14 ml of Diethyl-ether and 0.7 ml of Formic Acid; 79:20:1). The Chromarods were dried for 5 minutes at 60°C and then they were run in the Iatroscan using a full 30 second scan and settings of 2000ml min^{-1} O_2 and 160 ml min^{-1} H_2 settings as in Sewell (2005).

Quantification of the lipid in the sample was based on multilevel calibration curves generated for each lipid class found in the samples, plus the internal standard, on the rack of 10 Chromarods. Rods were calibrated with a 10-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform to cover the range of lipid concentrations present in the samples. Lipid classes were: Aliphatic Hydrocarbon (AH: Nonadecane), Wax Ester (WE: Miristyl dodecanoate), Methyl Ester (ME: Methyl palmitate), Ketone (KET: 3- hexadecanone), Triacylglycerol (TAG: tripalmitin), Free Fatty Acid (FFA: Palmitic acid), Sterols (ST: cholesterol), Diacylglycerol (DAG: 1,2 Dipalmitoyl-rac-glycerol), Acetone-mobile polar lipids (AMPL: 1-monopalmitoyl-rac-glycerol) and Phospholipid (PL: L-α-phosphoditylcholine). We did not use the acetone third development (Parrish, 1987). However, the second development separated a peak from PL that was confirmed to contain glycolipids and monoacylglycerols when spiked with an AMPL standard (1-monopalmitoyl-rac-glycerol). A stock solution of purified lipids was prepared using 5 mg of each lipid class dissolved in 1000 µl of chloroform using a Gilson Microman positive displacement pipette. Five serial dilutions were prepared from the initial stock solution, giving a total of six different dilutions of the lipid standards. The dilutions were run in the Iatroscan using the same methodology as was used for the samples. Quadratic regressions were used for lipid quantification as the FID response (Y-axis in the chromatogram) is curved at low concentrations (< 1 to 5 µg) (Sewell 2005). Peak areas from the calibration curves were calculated based on the mean of three separate Chromarods (r^2> 0.9968 for all lipid classes).

Lipids were quantified for each of the 10 sea urchins and 2-3 individuals of seaweed species (two extractions per sample and two rods per extraction) and analysed with the Iatroscan. The concentration of each lipid class per sample was determined using the percentage recovery of the internal standard in each sample and the calibration curve appropriate for each lipid class on each rack of Chromarods. Total lipid was calculated by the sum of the concentrations of each lipid class (AH, WE, ME, TAG, FFA, DAG, ST, AMPL, and PL) for each sample. Lipid classes can be grouped into energy storage and structural lipids. The concentration of energy storage lipid was calculated by summing the concentration of TAG, DAG, FFA, AH, ME and WE; whereas the concentration of structural lipids was calculated by summing the concentration of PL, ST and AMPL. Values are presented as the mean (± SE) of 10 gonads and 2 plants of *E. radiata* per location in units of µg lipid per mg of freeze-dried sample.

3.2.3 Fatty acid analysis

Fatty acid analysis was conducted using the one-step reaction from Lepage and Roy (1986), also known as the direct transesterification method, on the same ten sea urchins gonad and two *E. radiata* plants used in the lipid analysis. Lyophilized gonads (~50 mg) and seaweed (~150mg) were weighed using a Sartorius balance (LE244S; max 240g; d=0.1mg) in pre-weighed glass tubes (KIMAX, culture tubes; 16x125 mm; 20 ml with phenolic caps and cemented rubber liners). An internal standard consisting of 50 µg to 300 µg of tridecanoic acid (C13:0) and tricosanoic acid (C23:0), dissolved in 2 ml of methanol-toluene 4:1 (v/v) was precisely weighed and added to the biological samples. These FAs were used as internal standards in previous studies on sea urchins (Chen, 2005; Cook et al., 2007) and seaweeds (Crawley et al., 2009). A small magnetic stirring bar was added to each tube and, while stirring, 200 µl of acetyl chloride was slowly added over a period of one minute. The tubes were tightly closed and subjected to methanolysis at 100°C for one hour. The tubes were re-weighed after heating to check for leakage. If leaks occurred, the process was repeated with a new lyophilized tissue.

After the tubes had been cooled in tap water to room temperature, 5 ml of 6% K_2C0_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged for five minutes at 2000 RPM, and an aliquot of the toluene upper phase was transferred to an autosampler vial and then injected into an Agilent GC 7890 gas chromatograph equipped with a mass spectrometry detector (MSD 5975c). Separation

was performed with a 35-m fused silica column (internal diameter of 0.32 mm), wall-coated with 0.20 mm SP-2330, and with helium as the carrier gas. After injection at 60°C, the oven temperature was raised to 150°C at a rate of 40°C min^{-1}, then to 230°C at 3°C min^{-1}, and finally held constant for 30 minutes. Fatty acid methyl ester (FAME) peaks were identified by comparing their retention times with those of authentic 37 fatty acid methyl ester (FAME) standards (Supelco Inc.). The mass spectra of FAMEs not present in the standard mix were compared with those from the National Institute of Standards and Technology mass spectra library (NIST MS Search 2.0), together with the lipid library of Christie (2012). Total FA was calculated by the sum of the concentrations of each individual for each sample. FAs were grouped into Saturated FA (SFA: FA with no double bond in the carbon chain), Monounsaturated FA (MUFA: FA with one double bond present) and Polyunsaturated FA (PUFA: FA with two or more double bonds present). Each value is presented as the mean (± SE) of 10 urchin gonads and two *E. radiata* blades per location in units of µg FA per mg of freeze-dried sample and also, as the percentage of the total fatty acids identified.

3.2.4 Statistical analysis

Lipid and FA profiles were compared among sea urchin gonads as well as *E. radiata* samples from different locations (Great Barrier Island, Matheson's Bay, Rakino Island and Rangitoto Island) using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). The data was left untransformed and converted into similarity matrices using Euclidean distances. Similarity patterns in the data were visualised using multidimensional scaling (MDS). Multivariate one-way PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the permutation method, was performed to examine differences in the lipid and FA profiles among locations. Univariate one-way PERMANOVA analysis was used to compare the concentration of total, energy and structural lipid, as well as the concentration of total FA, SFA, MUFA and PUFA among locations. Pair-wise comparisons were then conducted to compare significant differences in lipid and FA among locations. In the case of less than 100 permutations, Monte Carlo test was used. The similarity percentages procedure (SIMPER) was used to explore the differences in the lipid and FA profile among locations by determining which lipid classes and FAs contributed most to differences in the multivariate signature. Univariate PERMANOVA analysis was used to test the differences of each of these important lipids and FAs. We used this approach as this tests avoids the assumptions of the traditional one-way

analysis of variance or ANOVA (Underwood, 1997) and assumes only that the samples are exchangeable, i.e. independent and identically distributed, under a true hypothesis (Anderson, 2003).

3.3 Results

3.3.1 Lipid Profile

The lipid profile of the seaweed *E. radiata* consisted of three energy lipids (TAG, FFA and DAG) and three structural lipids (PL, ST and AMPL) with the energy lipids being present in very small concentrations compared to the structural lipids (Table 3.1; Fig. 3.1).

Table 3.1. Lipid classes (µg/mg of dry weight) present in *E. radiata blades* from four locations in north-eastern New Zealand (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Triacylglycerol (TAG), Free Fatty Acid (FFA), and Diacylglycerol (DAG) as Energy Lipids and Phospholipids (PL), Sterols (ST) and Acetone-mobile Polar Lipids (AMPL) as Structural Lipids. Data represent the mean (±SE) of two plants per location. ND=not detected.

	Location			
	Outer Hauraki Gulf		**Inner Hauraki Gulf**	
Lipid Classes	**Great Barrier**	**Matheson's Bay**	**Rakino**	**Rangitoto**
TAG	0.98±0.15	1.75±0.70	1.80±0.27	2.87±2.16
FFA	2.27±0.69	2.29±0.40	0.68±0.29	3.0±0.08
DAG	0.08±0.12	ND	0.35±0.09	ND
Energy Lipid	3.33±0.73	4.04±0.30	2.83±0.06	5.98±2.09
PL	14.51±1.04	26.04±0.14	19.60±4.72	17.91±1.55
ST	2.49±0.23	4.17±1.04	3.97±1.34	2.98±1.09
AMPL	0.67±0.11	1.69±0.24	1.93±0.65	2.69±0.85
Structural Lipid	17.67±1.38	31.90±1.42	25.49±6.71	23.58±3.49
Total Lipid	20.99±2.11	35.94±1.12	28.33±6.77	29.56±5.58

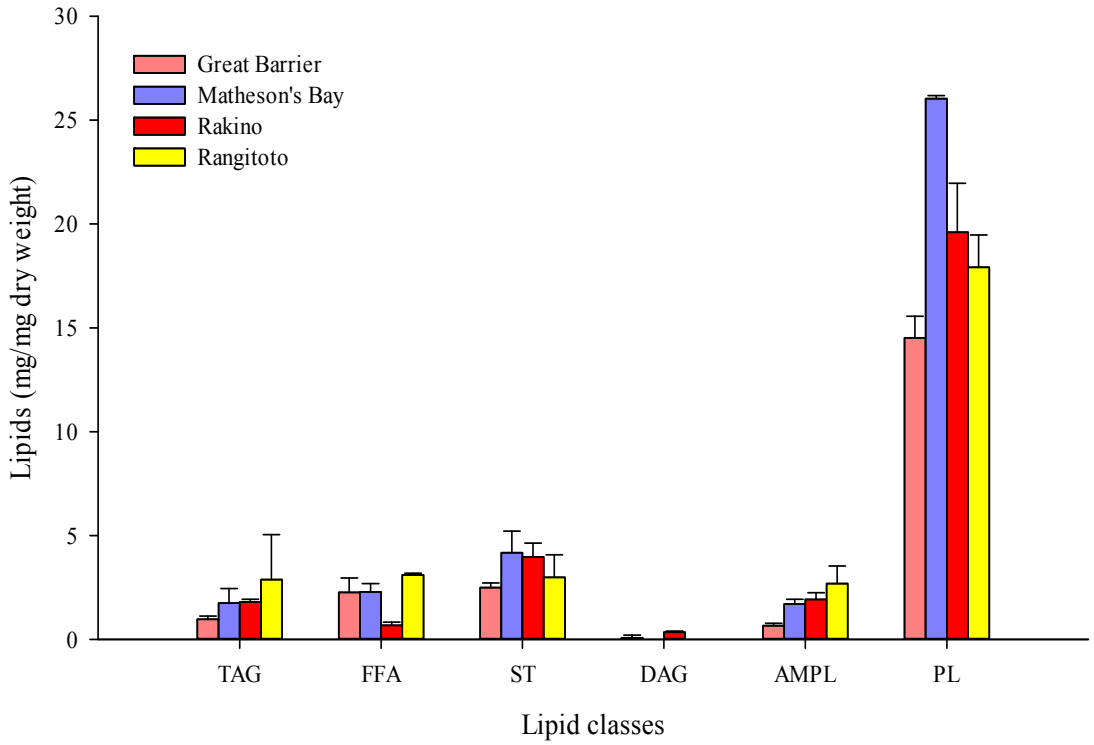


Figure 3.1. Lipid classes present in *E. radiata* from different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-east New Zealand. Data represent the mean (±SE) of 2 plants per location. TAG (Triacylglycerol), FFA (Free Fatty Acid), DAG (Diacylglycerol), PL (Phospholipid), ST (Sterols) and AMPL (acetone-mobile polar lipids).

Even though significant differences were not found in the concentrations of total lipid (Table 3.2.A) and structural lipid (Table 3.2.B) of *E. radiata* collected from the four different locations, plants from Great Barrier showed a trend for slightly smaller concentrations of total and structural lipid than the remaining locations (Table 3.1). The concentrations of energy lipid were, however, significantly different among locations overall (Table 3.2.C), but pairwise comparisons did not revealed any significant differences (Table 3.2.D). The significant differences found in the energy lipids could be related to the concentration of TAG of *E. radiata* from Rangitoto that was slightly higher compared to plants from Great Barrier, showing very small concentration (Table 3.1; Fig. 3.1), despite the fact significant differences were not found in the concentration of TAG between locations (Pseudo-$F_{3,4}$=0.92, P(perm)=0.4865).

The lipid profile of *E. radiata* was also not significantly different between locations (Table 3.2.E; Fig. 3.1). PL was the lipid class present in the highest concentration in *E. radiata* plants regardless of location (Table 3.1; Fig. 3.1), which varied significantly among locations (Pseudo-$F_{3,4}$=7.24, P(perm)=0.0482), where the smallest concentrations were found in the plants collected from Great Barrier and the highest in Matheson's Bay (Table 3.1; Fig. 3.1).

Table 3.2. Results of Univariate one-way PERMANOVA comparing: A) total lipid, B) structural lipid, C) energy lipid, D) Pair-wise comparisons of energy lipid (with Monte Carlo simulation) and E) Multivariate one-way PERMANOVA analysis of the lipid profile and among *E. radiata* plants collected in four different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	224.49	74.83	3.85	0.1057	105
Residual	4	77.69	19.42			
Total	7	302.18				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	206.48	68.83	4.50	0.1020	105
Residual	4	64.14	15.29			
Total	7	267.62				

C)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	9.03	3.01	3.70	**0.0403**	105
Residual	4	3.26	0.81			
Total	7	12.29				

D)

Location	t	P(perm)	Unique perms	P(MC)
Great Barrier, Rakino	0.96	0.6624	3	0.4448
Great Barrier, Matheson's Bay	1.28	0.6609	3	0.3283
Great Barrier, Rangitoto	1.84	0.3359	3	0.2067
Rakino, Matheson's Bay	5.66	0.3275	3	0.257
Rakino, Rangitoto	2.44	0.3396	3	0.1356
Matheson's Bay, Rangitoto	1.38	0.3345	3	0.3066

E)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	162.61	54.20	4.14	0.0583	105
Residual	4	52.39	13.10			
Total	7	215				

The lipid profile of *E. chloroticus* gonads consisted of five energy lipids (TAG, FFA, WE, ME and DAG) and three structural lipids (PL, ST and AMPL) with TAG and PL contributing to the highest proportions to the total lipid, regardless of location (Table 3.3; Fig.3.2).

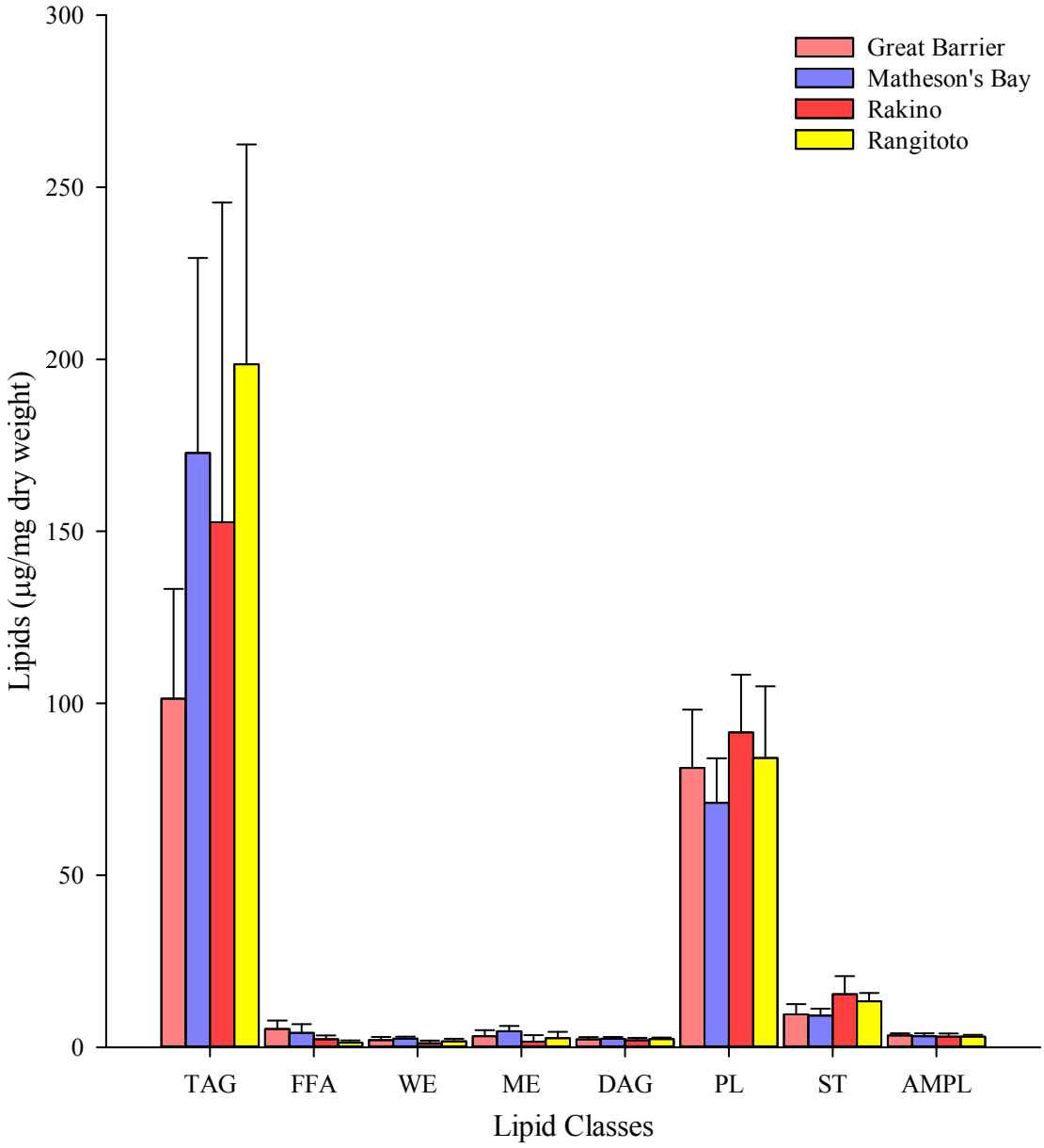


Figure 3.2. Lipid classes present in *E. chloroticus* gonads from different locations (Outer Hauraki Gulf: Great Barrier, Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern, New Zealand. Data represent the mean (±SE) of 10 sea urchin gonads per location. TAG (Triacylglycerol), FFA (Free Fatty Acid), WE (Wax Ester), ME (Methyl Ester), DAG (Diacylglycerol), PL (Phospholipid), ST (Sterols) and AMPL (acetone-mobile polar lipids).

Table 3.3. Lipid classes (µg/mg of dry weight) present in *E. chloroticus* gonads from four locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Triacylglycerol (TAG), Free Fatty Acid (FFA), Aliphatic Hydrocarbon (AH), Wax Ester (WE), Methyl Ester (ME) and Diacylglycerol (DAG) as Energy Lipids and Phospholipids (PL), Sterols (ST) and Acetone-mobile Polar Lipids (AMPL) as Structural Lipids. Data represent the mean (±SE) of ten sea urchin gonads per location.

	Location			
	Outer Hauraki Gulf		**Inner Hauraki Gulf**	
	Great Barrier	**Matheson's Bay**	**Rakino**	**Rangitoto**
Lipid Classes				
TAG	101.36±31.88	172.76±56.57	152.62±92.92	198.54±63.89
FFA	5.31±2.48	4.15±2.59	2.33±1.09	1.35±0.64
WE	2.07±0.96	2.49±0.58	1.16±0.78	1.72±0.75
ME	3.19±1.77	4.68±1.5	1.63±1.87	2.65±1.82
DAG	2.29±0.67	2.45±0.56	2.06±0.66	2.39±0.44
Energy Lipid	114.22±33.77	186.53±56.39	159.79±93.83	206.65±63.92
PL	81.22±16.93	71.06±12.95	91.52±16.82	84.11±20.84
ST	9.54±3.01	9.22±1.98	15.43±5.23	13.35±2.41
AMPL	3.43±0.62	3.23±0.81	3.07±0.96	3.10±0.51
Structural Lipid	94.19±20.12	83.52±14.20	110.01±21.17	100.57±22.28
Total	208.41±54.35	270.05±59.18	269.80±95.99	307.21±72.40

The concentration of total lipid was significantly different among gonads from different locations (Table 3.4.A), with gonads from Great Barrier containing a lower concentration of total lipid than gonads collected at Matheson's Bay and Rangitoto (Table 3.3; Table 3.4.B). Similarly, the concentration of gonadal energy lipids was also significantly different between locations (Pseudo-$F_{3,36}$=3.68, P(perm)=0.026). Yet again, pairwise comparisons revealed that sea urchin gonads collected at Great Barrier showed smaller concentrations of energy lipids than gonads from Matheson's Bay and Rangitoto (t=3.43, P(perm)=0.019 and t=3.99, P(perm)=0.0011, respectively; Table 3.3). Additionally, the concentration of structural lipids was significantly different among locations (Pseudo-$F_{3,36}$=3.11, P(perm)=0.04); however, this time pairwise comparisons showed that gonads from Rakino had higher concentrations of structural lipids than gonads from Matheson's Bay (t=3.29, P(perm)=0.0032;).

Table 3.4. Results of A) Univariate one-way PERMANOVA and B) Pair-wise comparisons of Total lipid (µg/mg dry weight) of *E. chloroticus* gonads from four different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	50276	16759	3.21	**0.0357**	9953
Residual	36	188200	5227.9			
Total	39	238480				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Rakino	1.76	0.0955	9337
Great Barrier, Matheson Bay	2.43	**0.0236**	9317
Great Barrier, Rangitoto	3.45	**0.0028**	9309
Rakino, Matheson Bay	0.10	0.9942	9341
Rakino, Rangitoto	0.98	0.3342	9344
Matheson Bay, Rangitoto	1.26	0.2237	9365

Significant differences were also found in the multivariate lipid profile of *E. chloroticus* gonads from the four locations (Table 3.5.A) as revealed in the MDS plot (Fig. 3.3). Pairwise comparisons revealed that the lipid profile of the gonads from Great Barrier was significantly different to the lipid profile of the gonads from Matheson's Bay and Rangitoto (Table 3.5.B).

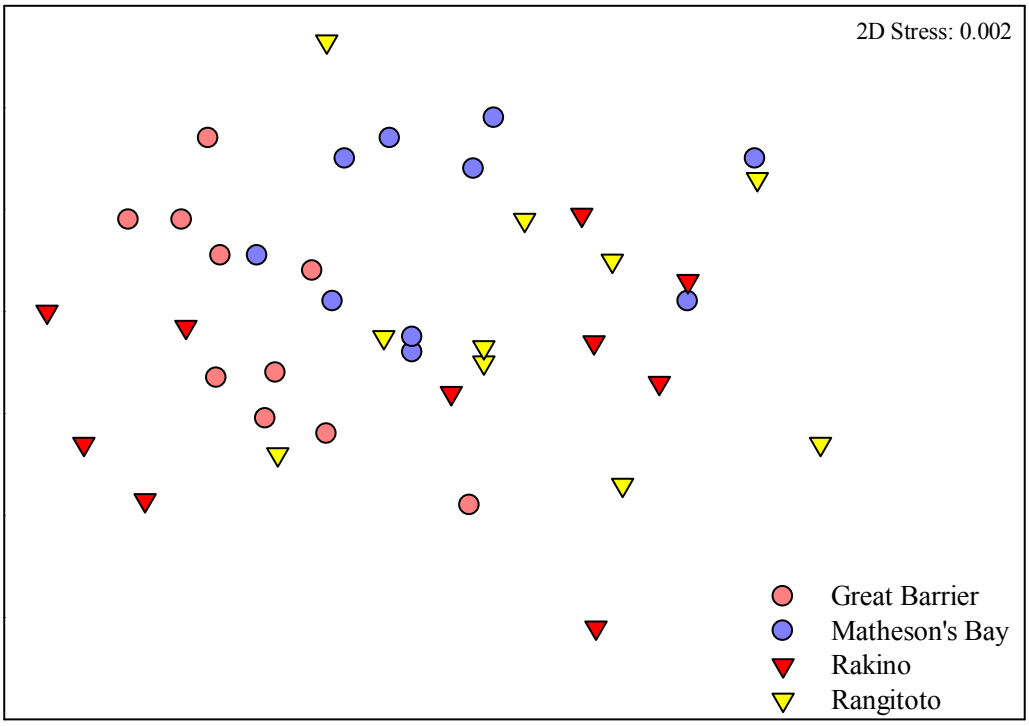


Figure 3.3. Multidimensional scaling (MDS) plot of lipid profile of *E. chloroticus* gonads from the four locations: Outer Hauraki Gulf (Great Barrier and Matheson's Bay) and Inner Hauraki Gulf (Rakino and Rangitoto).

Table 3.5. Results of A) Multivariate one-way PERMANOVA and B) Pair-wise comparisons of lipid profile of *E. chloroticus* gonads from four different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	53451	17817	3.89	**0.0141**	9959
Residual	36	265080	4585.5			
Total	39	218530				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Rakino	1.63	0.1092	9432
Great Barrier, Matheson's Bay	3.27	**0.0015**	9414
Great Barrier, Rangitoto	3.98	**0.0008**	9435
Rakino, Matheson's Bay	0.84	0.4093	9418
Rakino, Rangitoto	1.27	0.2087	9427
Matheson's Bay, Rangitoto	1.04	0.3047	9394

SIMPER analysis showed that the energy lipid TAG was the lipid class that contributed to the differences among locations (Table 3.6). The concentration of TAG was significantly different among gonads collected in the different locations (Pseudo-$F_{3.36}$=3.98, P(perm)=0.0152), with a higher concentration of TAG in gonads from Matheson's Bay and Rangitoto than gonads from Great Barrier (Table3.3; Table 6.6).

Table 3.6. Contribution of individual lipid classes to multivariate differences in lipid profiles among *E. chloroticus* gonads from four different locations (Outer Hauraki Gulf: Great Barrier and Matheson Bay; Inner Hauraki Gulf: Rakino and Rangitoto) as determined by SIMPER. t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor for each lipid and pairwise comparison. Significant results ($p<0.05$) are shown in bold.

Locations: Groups 1 & 2	Average squared distance	Lipid class	Average Value Group 1	Average Value Group 2	Av.Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Great Barrier & Rakino	12159.45	TAG	101	153	11400	1.15	93.87	93.87	1.64	0.1204	9301
Great Barrier & Matheson's Bay	9581.42	TAG	101	173	9000	0.76	93.97	93.97	3.43	**0.0013**	9359
Rakino & Matheson's Bay	11987.38	TAG	153	173	11100	0.85	92.32	92.32	0.59	0.5752	9331
Great Barrier & Rangitoto	14880.56	TAG	101	199	14100	0.96	94.99	94.99	4.26	**0.0007**	9322
Rakino & Rangitoto	14301.24	TAG	153	199	13600	0.79	94.78	94.78	1.29	0.2121	9309
Matheson's Bay & Rangitoto	7993.21	TAG	173	199	7230	0.81	90.44	90.44	0.96	0.3557	9384

3.3.2 Fatty Acid profile

The FA profile of plants of the seaweed *E. radiata* contained 22 identified FAs at all locations (Table 3.7). The FA profile consisted of: 7 SFAs, where C14:0 and C16:0 were the dominant SFAs; 4 MUFAs, dominated by C16:1(n-7) and C18:1(n-9c), and 11 PUFAs with C18:4(n-3), C20:4(n-6) or ARA (Arachidonic Acid) and C20:5(n-3) or EPA (Eicosapentaenoic Acid) present in the highest concentrations (Table 3.7).

Table 3.7. Fatty acid composition of *E. radiata* from 4 different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). Fatty acid values are presented as μg/mg of dry weight. Mean values and standard error for 2 plants per location.

FAs	Great Barrier	Matheson's Bay	Rakino	Rangitoto
C12:0	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00
C14:0	1.12±0.01	1.42±0.12	1.77±0.03	1.67±0.14
C15:0	0.05±0.01	0.07±0.01	0.07±0.01	0.07±0.01
C16:0	3.58±0.39	6.45±0.90	6.89±0.22	6.28±0.78
C16:1(n-7)	1.03±0.14	1.47±0.22	1.42±0.05	1.59±0.23
C18:0	0.21±0.03	0.30±0.06	0.35±0.01	0.25±0.03
C18:1(n-9c)	2.64±0.42	5.11±0.82	6.08±0.12	4.57±0.60
C18:1	0.21±0.02	0.07±0.01	0.19±0.00	0.06±0.00
C18:2(n-6)	0.67±0.13	1.34±0.25	1.19±0.06	1.74±0.24
C18:3(n−6)	0.05±0.01	0.15±0.04	0.19±0.02	0.27±0.04
C18:3(n-3)	0.73±0.14	1.65±0.31	2.08±0.10	2.30±0.31
C20:0	0.31±0.06	0.82±0.14	0.81±0.01	0.35±0.05
C20:1(n-15)	0.04±0.01	0.03±0.01	0.04±0.00	0.06±0.01
C18:4(n-3)	1.04±0.18	2.22±0.40	3.57±0.14	2.95±0.40
C20:2(n-9)	0.02±0.01	0.01±0.00	0.02±0.00	0.01±0.01
C20:2(n-6)	0.05±0.00	0.06±0.01	0.07±0.00	0.06±0.00
C20:3(n-6)	0.05±0.01	0.13±0.03	0.15±0.01	0.18±0.02
C20:4(n-6)	4.02±0.60	6.86±1.01	7.75±0.15	7.11±0.72
C20:4(n-3)	0.19±0.03	0.22±0.05	0.29±0.01	0.17±0.02
C20:5(n-3)	1.96±0.32	2.83±0.46	3.23±0.11	2.37±0.27
C24:0	0.07±0.01	0.08±0.01	0.09±0.01	0.05±0.00
C22:6(n-3)	0.02±0.00	0.01±0.00	0.09±0.01	0.06±0.01
SFA	**5.27±0.49**	**9.05±1.23**	**9.96±1.02**	**8.67±1.02**
MUFA	**3.92±0.58**	**6.58±1.04**	**6.28±0.84**	**6.28±0.84**
PUFA	**8.80±1.43**	**15.41±2.55**	**17.23±2.03**	**17.23±2.03**
TOTAL	**18.07±2.54**	**31.31±4.87**	**36.35±1.08**	**32.18±3.91**

The FA profile of *E. radiata* was statistically different among locations, (Pseudo-$F_{3,4}$=12.45, P(perm)=0.039), with the MDS plot showing a clear separation between the FA profiles of *E. radiata* collected at Great Barrier compared with seaweed collected in the rest of the Hauraki Gulf (Fig. 3.4). SIMPER analysis revealed that the FAs that contributed to the difference among locations (>10%) were C16:0, C18:1(n-9), C18:4(n-3) and C20:4(n-6) (Table 3.8). *E. radiata* from Great Barrier had smaller concentrations of C20:4(n-6), C18:1(n-9) and C18:4(n-3) (Pseudo-$F_{3,4}$=11.3, P(perm)=0.0397; Pseudo-$F_{3,4}$=13.85, P(perm)=0.0382, and Pseudo-$F_{3,4}$=25.42, P(perm)=0.0092, respectively) than the brown seaweed collected in

other parts of the Hauraki Gulf (Table 3.7). In contrast, concentrations of C16:0 were not statistically different among *E. radiata* from different locations (Pseudo-$F_{3,4}$=11.16, P(perm)=0.1152) (Table 3.7).

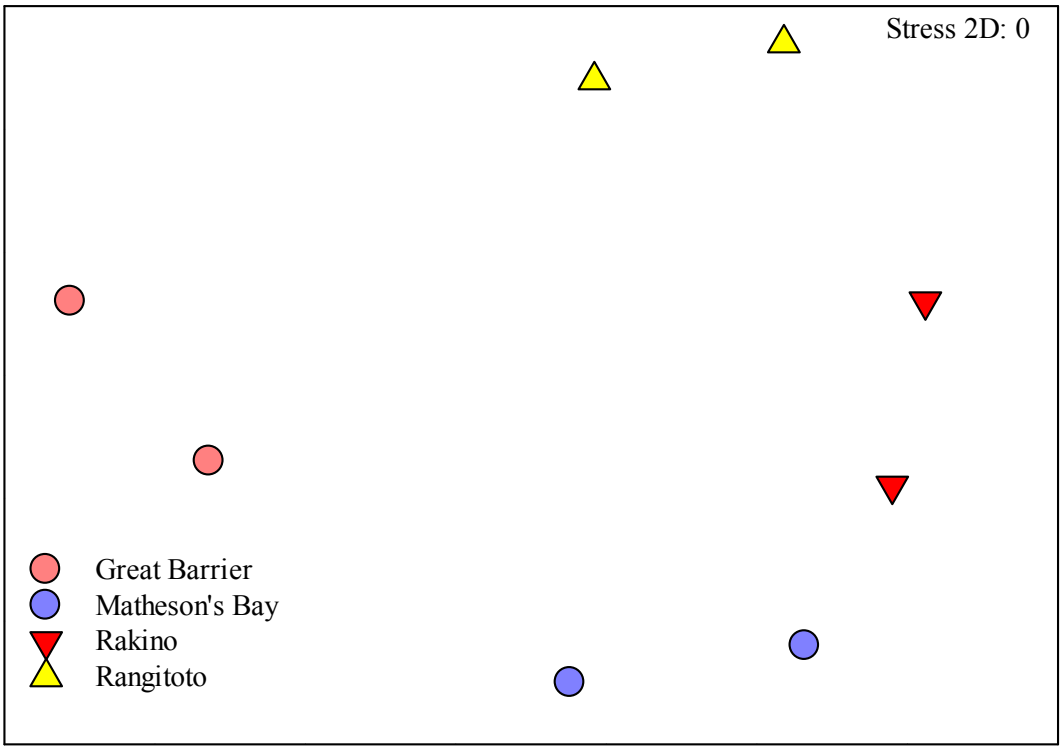


Figure 3.4. Multidimensional scaling (MDS) plot of FA profile of *E. radiata* from the four locations: Outer Hauraki Gulf (Great Barrier and Matheson's Bay) and Inner Hauraki Gulf (Rakino and Rangitoto).

Table 3.8. Contribution of individual FAs to multivariate differences in FA profile between *E. radiata* from different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) as determined by SIMPER (>10% of contribution).

Groups 1 & 2	Average squared distance	FAs	Av.Value Group 1	Av.Value Group 2	Av.Sq. Dist	Sq.Dist/ SD	Contribution %	Cumulative %
Great Barrier & Matheson Bay	28.52	C20:4(n-6)	4.02	6.86	8.77	1.59	30.74	30.74
		C16:0	3.58	6.45	8.74	1.89	30.63	61.37
		C18:1(n-9c)	2.64	5.11	6.55	1.75	22.97	84.34
Great Barrier & Rakino	48.35	C20:4(n-6)	4.02	7.75	14.2	3.73	29.27	29.27
		C18:1(n-9c)	2.64	6.08	12	4.83	24.75	54.02
		C16:0	3.58	6.89	11.1	4.59	22.97	76.99
		C18:4(n-3)	1.04	3.57	6.43	6.69	13.30	90.29
Great Barrier & Rangitoto	30.17	C20:4(n-6)	4.02	7.11	10	2.09	33.13	33.13
		C16:0	3.58	6.28	7.69	1.98	25.48	58.61
		C18:1(n-9c)	2.64	4.57	4.02	1.72	13.33	71.95
		C18:4(n-3)	1.04	2.95	3.75	2.74	12.42	84.37

Fifty five different FAs were identified in *E. chloroticus* gonads at all locations; however, the concentration of some of the FAs varied among locations (Table 3.9). The FAs that contributed to more than 2% of the total FA are shown in Figure3.5.

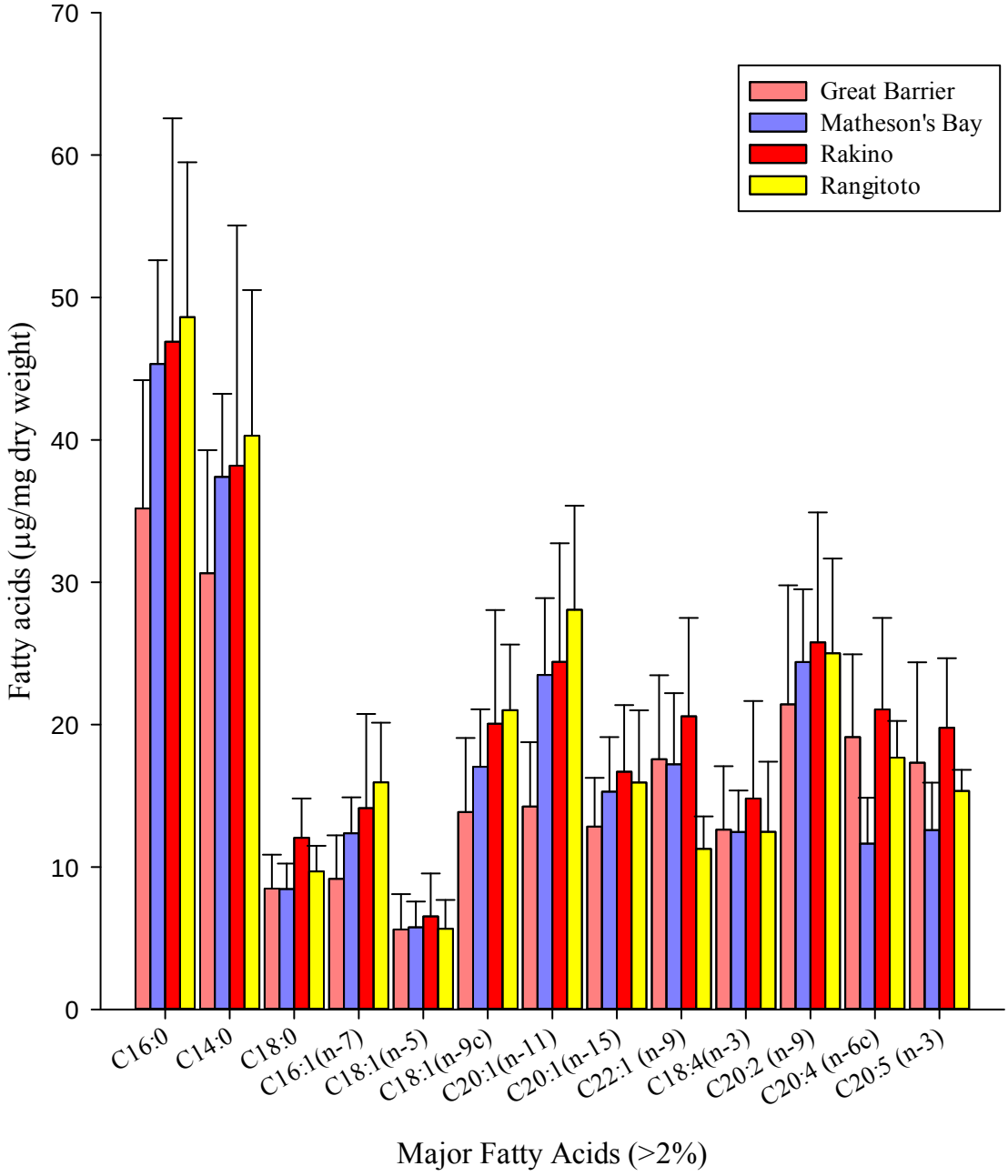


Figure 3.5. Fatty acids contributing more than 2% of the total FA in *E. chloroticus* gonads from four locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern, New Zealand. Data represent the mean (±SE) of 10 sea urchins per location.

Table 3.9. Fatty acid composition of *Evechinus chloroticus* gonads from 4 different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern New Zealand. Fatty acid values are presented as μg/mg of dry weight. Mean values and standard error for 10 sea urchins per location.

FAs	Great Barrier	Matheson's Bay	Rakino	Rangitoto
C12:0	0.17±0.06	0.16±0.03	0.16±0.10	0.20±0.11
C14:0	30.41±8.87	37.41±5.83	38.19±16.88	40.29±10.23
C14:1	0.16±0.07	0.18±0.06	0.17±0.09	0.28±0.13
C14:1(n-5)	1.34±0.46	1.49±0.39	1.69±0.93	2.27±0.67
C15:0	1.07±0.21	1.33±0.44	1.18±0.35	1.55±0.36
C16:0	35.19±9.01	45.33±7.28	46.90±15.68	48.63±10.87
C16:1	0.42±0.18	0.47±0.12	0.48±0.22	0.57±0.20
C16:1	0.47±0.16	0.51±0.12	0.49±0.20	0.61±0.23
C16:1	1.59±0.69	1.50±0.47	1.94±1.06	2.24±0.43
C16:1(n-7)	9.18±3.05	12.37±2.53	14.13±6.61	15.94±4.19
C17:0	0.32±0.13	0.38±0.10	0.36±0.06	0.40±0.07
C16:2(n-6)	0.12±0.08	0.10±0.04	0.18±0.10	0.21±0.10
C16:3 (n-6)	0.20±0.13	0.03±0.01	0.05±0.03	0.07±0.02
C16:3(n-3)	0.34±0.21	0.19±0.05	0.30±0.14	0.19±0.06
C18:0	8.48±2.39	8.46±1.79	12.05±2.77	9.69±1.80
C18:1(n-5)	5.62±2.48	5.77±1.81	6.53±3.02	5.66±2.03
C18:1(n-9t)	2.20±0.85	1.35±0.40	1.88±0.77	1.78±0.34
C18:1(n-9c)	13.87±5.20	17.04±4.04	20.06±7.99	21.01±4.61
C18:1(n-7)	1.29±0.53	1.83±0.38	2.42±0.63	2.26±0.50
C18:2	0.23±0.18	0.19±0.05	0.25±0.11	0.25±0.06
C18:2(n-6t)	2.32±1.28	2.58±0.69	3.72±1.91	4.19±1.28
C18:2(n-6c)	1.23±0.49	0.42±0.17	0.90±0.29	0.65±0.20
C19:1	0.76±0.21	0.73±0.12	0.72±0.23	0.68±0.09
C18:3	0.81±0.39	0.71±0.17	1.08±0.41	1.09±0.30
C19:1	0.51±0.26	0.50±0.15	0.56±0.24	0.53±0.16
C18:3(n−6)	0.49±0.31	0.14±0.03	0.17±0.08	0.16±0.06
C18:3(n-3)	1.78±0.86	0.65±0.34	0.99±0.54	0.92±0.33
C20:0	1.60±0.58	1.57±0.53	2.25±0.89	1.88±0.67
C20:1(n-15)	12.85±3.41	15.29±3.83	16.69±4.68	15.94±5.07
C18:4(n-3)	12.62±4.46	12.46±2.92	14.81±6.85	12.47±4.93
C20:1(n-11)	14.24±4.52	23.49±5.40	24.41±8.34	28.07±7.32
C20:1(n-9)	3.45±1.26	5.25±0.91	6.09±2.34	6.21±1.64
C20:2(n-9)	21.42±8.37	24.40±5.12	25.78±9.14	25.01±6.67
C20:2	3.19±1.30	3.71±0.94	4.12±1.25	3.14±0.97
C20:2	1.46±0.61	1.85±0.61	2.40±1.31	2.35±0.79
C20:2	0.51±0.29	0.70±0.19	0.86±0.40	0.96±0.34
C20:2(n-6)	3.09±1.15	2.19±0.69	3.08±0.92	3.23±0.49
C20:3(n-9)	4.09±1.48	4.07±1.26	6.33±2.05	5.14±0.96
C21:1	3.31±0.94	3.29±0.84	3.64±1.33	3.84±0.87
C20:3(n-6)	1.03±0.41	0.67±0.16	0.84±0.24	1.01±0.18
C20:4(n-6)	19.12±5.82	11.65±3.21	21.07±6.42	17.69±2.57
C20:3(n-3)	2.95±1.33	2.44±0.86	3.61±1.34	3.68±0.98
C22:0	0.13±0.06	0.13±0.08	0.14±0.05	0.13±0.06
C20:4(n-3)	1.09±0.39	0.54±0.27	1.96±0.83	1.24±0.38
C22:1(n-9)	17.58±5.90	17.22±4.99	20.59±6.90	20.26±3.07
C20:5(n-3)	17.33±7.05	12.60±3.33	19.78±4.88	15.35±1.48
C23:1	0.81±0.21	0.60±0.28	0.72±0.29	0.66±0.16
C20:5	0.75±0.33	1.52±0.50	0.99±0.74	0.42±0.10
C22:4	0.91±0.51	0.39±0.19	0.73±0.27	0.71±0.15
C24:0	0.13±0.05	0.10±0.07	0.14±0.09	0.11±0.05
C22:3	0.22±0.07	0.17±0.06	0.35±0.07	0.21±0.04
C24:1(n-9)	0.52±0.17	0.43±0.14	0.60±0.28	0.51±0.14
Unknown PUFA	0.54±0.18	0.43±0.09	0.74±0.46	0.56±0.12
Unknown PUFA	0.29±0.51	0.11±0.07	0.09±0.04	0.10±0.04
C22:6(n-3)	2.06±0.98	1.02±0.29	2.46±1.36	1.66±0.56
SFA	**77.49±20.66**	**94.87±14.89**	**101.36±33.18**	**102.88±22.35**
MUFA	**90.16±27.75**	**109.31±22.50**	**123.80±41.84**	**129.31±25.06**
PUFA	**100.32±33.29**	**85.94±15.99**	**117.62±26.76**	**102.62±17.38**
TOTAL	**267.85±80.11**	**290.12±50.20**	**342.78±97.46**	**334.82±62.94**

The concentration of the total FA was not significantly different among locations (Pseudo-$F_{3,36}$=1.78, P(perm)=0.1629, Table 3.9). However, the FA profile of *E. chloroticus* gonads was significantly different among the four locations (Table 3.10.A) as clearly shown in the MDS plot (Fig. 3.6). Pairwise comparisons showed that gonads from Great Barrier presented a different FA profile compared to gonads from Matheson's Bay and Rangitoto (Table 3.10.B).

Table 3.10. Results of A) Multivariate one-way PERMANOVA and B) Pair-wise comparisons of FA profile of *E. chloroticus* gonads from different locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto). 10 sea urchins were used for the analysis from each location. Significant results ($p<0.05$) are shown in bold

A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	4729	1576.3	2.89	**0.0229**	9954
Residual	36	19627	545.2			
Total	39	24356				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Matheson's Bay	2.71	**0.0048**	9417
Great Barrier, Rakino	1.75	0.0744	9425
Great Barrier, Rangitoto	2.61	**0.0067**	9418
Matheson's Bay, Rakino	1.08	0.2875	9433
Matheson's Bay, Rangitoto	0.98	0.3588	9415
Rakino, Rangitoto	0.71	0.5638	9385

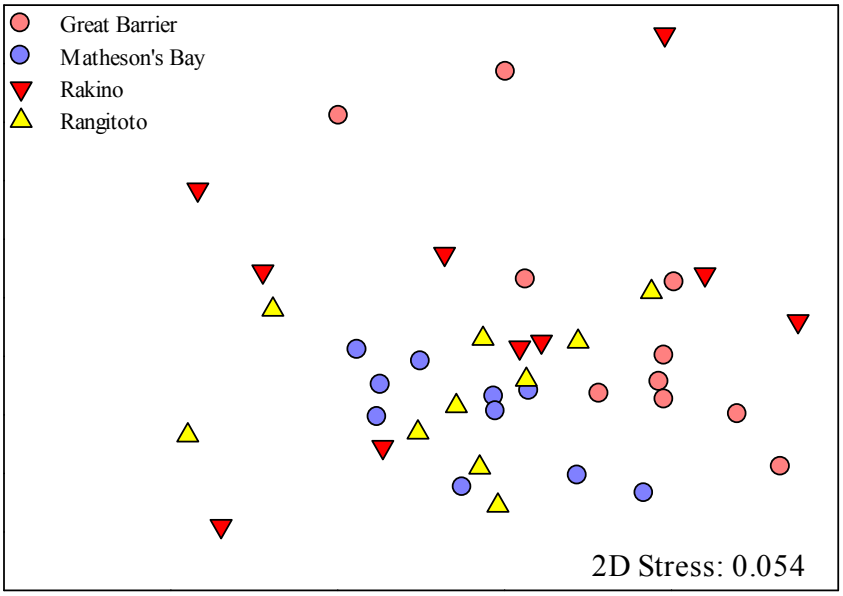


Figure 3.6. *Evechinus chloroticus*. Multidimensional scaling (MDS) plot of Euclidean similarities of FA profile of sea urchins gonads from the four locations: Outer (Great Barrier and Matheson's Bay) and Inner (Rakino and Rangitoto).

The total concentration of SFA (Table 3.11.A) and the SFA profile (Table 3.9; Table 3.11.B; Fig. 3.7) were not significantly different between locations. In total nine SFAs were identified in the sea urchin gonads at all locations (Table 3.9) with C14:0 and C16:0 being the dominant SFAs.

Table 3.11. Results of A) Univariate one-way PERMANOVA of the concentration (µg/mg dry weight)of SFA and B) Multivariate one-way PERMANOVA of SFA profile comparing sea urchin gonads from four different locations in the Hauraki Gulf, New Zealand.

A)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	4505.5	1501.80	2.67	0.0607	9948
Residual	36	20246	562.39			
Total	39	24751				

B)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	1985.1	661.81	2.59	0.0626	9946
Residual	36	9205.3	255.7			
Total	39	11190				

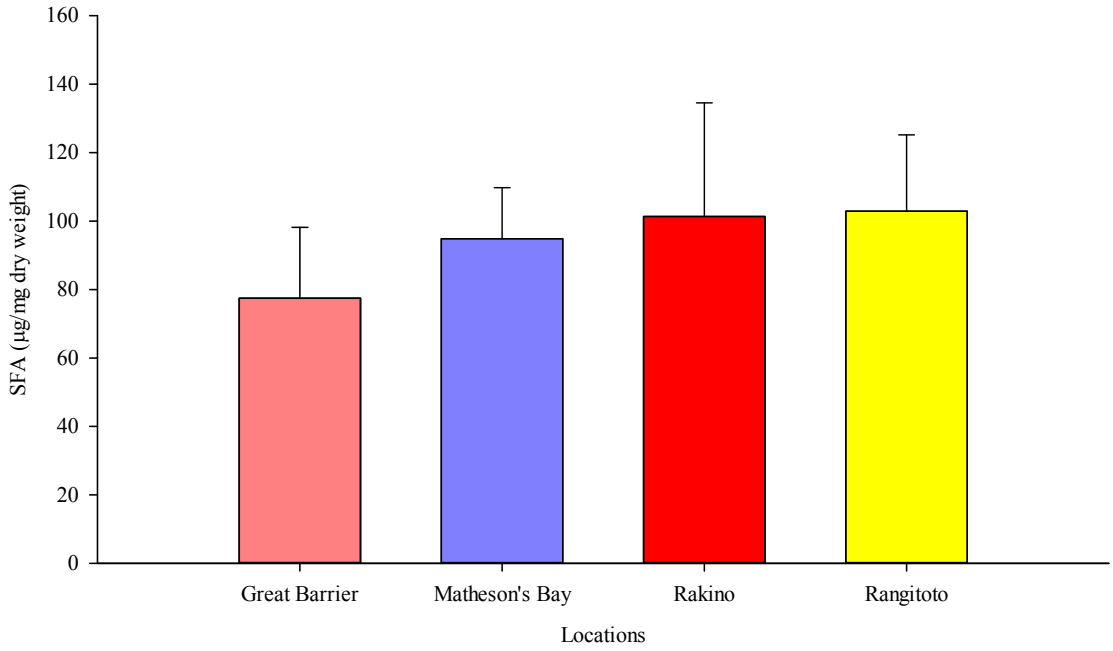


Figure 3.7. Concentration (µg/mg dry weight) of the total saturated FA (SFA) of *E. chloroticus* gonads from four locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern New Zealand. Data represent the mean (±SE) of 10 sea urchins per location.

The total concentration of MUFA was significantly different among gonads from different locations (Table 3.12.A; Fig. 3.8); gonads from Great Barrier showed the smallest concentration of MUFA of all locations, as revealed by pairwise comparisons (Table 3.9; Table 3.12.B).

Table 3.12. Results of A) Univariate one-way PERMANOVA and B) Pair-wise comparisons comparing the total concentration of MUFA (μg/mg dry weight) among sea urchin gonads from four different locations in the Hauraki Gulf, New Zealand. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	9017.4	3005.80	3.29	**0.0363**	9954
Residual	36	32892	913.67			
Total	39	41909				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Matheson's Bay	2.34	**0.0337**	9331
Great Barrier, Rakino	2.12	**0.0461**	9317
Great Barrier, Rangitoto	3.31	**0.0046**	9315
Matheson's Bay, Rakino	0.48	0.6385	9332
Matheson's Bay, Rangitoto	1.19	0.2533	9326
Rakino, Rangitoto	0.36	0.7197	9310

In total19 MUFAs were identified in *E. chloroticus* gonads at all locations (Table 3.9). Significant differences were found in the MUFA profile in gonads from different locations (Table 3.13.A), with gonads from Great Barrier having a different MUFA profile than the gonads from the rest of the locations, as shown by pairwise analysis (Table 3.13.B).

Table 3.13. Results of A) Multivariate one-way PERMANOVA and B) Pair-wise comparisons comparing MUFA profile among sea urchin gonads from four different locations in the Hauraki Gulf, New Zealand. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	1862.4	620.80	4.05	**0.0037**	9957
Residual	36	5521.4	153.37			
Total	39	7383.8				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Matheson's Bay	2.85	**0.0046**	9441
Great Barrier, Rakino	2.30	**0.0216**	9419
Great Barrier, Rangitoto	3.55	**0.0005**	9398
Matheson's Bay, Rakino	0.54	0.7361	9415
Matheson's Bay, Rangitoto	1.12	0.2807	9423
Rakino, Rangitoto	0.70	0.5929	9421

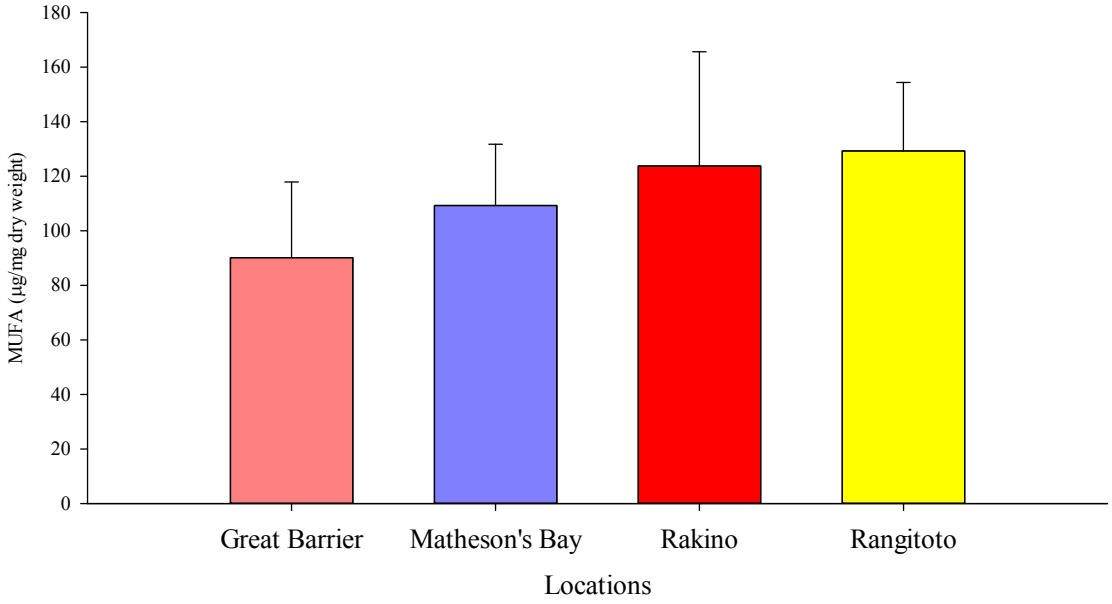


Figure 3.8. Concentration (µg/mg dry weight) of monounsaturated FA (MUFA) of *E. chloroticus* gonads from four locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern New Zealand. Data represent the mean (±SE) of 10 sea urchins per location.

Of the six MUFAs present in the highest concentrations (>2% of total FA; Fig. 3.5; Table 3.9) SIMPER analysis showed that four MUFAs contributed to the differences among the four locations in the Hauraki Gulf (Table 3.14). There was a significant variation in the concentrations of C20:1(n-11) between locations (Pseudo-$F_{3.36}$=3.29, P(perm)=0.0321) where gonads from Great Barrier contained smaller concentrations of this MUFA than the gonads from Matheson's Bay, Rangitoto and Rakino (Table 3.9; Table 3.14). Significant differences were also found in the concentrations of C18:1(n-9c) in gonads from different locations (Pseudo-$F_{3.36}$=3.13, P(perm)=0.0355), being present in higher concentrations in the gonads from Rangitoto than sea urchin gonads collected from Great Barrier (Table 3.9; Table 3.14). The concentrations of C16:1(n-7) were also different among gonads from different locations (Pseudo-$F_{3.36}$=4.26, P(perm)=0.0121), with the gonads from Great Barrier showing the lowest concentration of this MUFA (Table 3.9; Table 3.14). The last MUFA that contributed to the differences was C22:1(n-9) but its concentrations were not significantly different among locations (Pseudo-$F_{3.36}$=0.71, P(perm)=0.5513).

Table 3.14. Contribution of individual MUFAs to multivariate differences in fatty acid profile among *E. chloroticus* gonads from different locations (Outer Hauraki Gulf: Great Barrier and Matheson Bay; Inner Hauraki Gulf: Rakino and Rangitoto) as determined by SIMPER (>10% of contribution). t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor analysis for each FA and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Groups 1 & 2	Average squared distance	MUFA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist/SD	Contribution%	Cumulative%	t	P(perm)	Unique perms
Great Barrier & Matheson's Bay	360.92	C20:1(n-11)	14.2	25.1	162	0.95	44.89	44.89	4.86	**0.0004**	9359
		C18:1(n-9c)	13.9	18.2	57.5	0.90	15.94	60.83	2.06	0.0554	9350
		C22:1(n-9)	17.6	18.5	54.6	0.70	15.12	75.95	0.37	0.7197	9314
Great Barrier & Rakino	541.61	C20:1(n-11)	14.2	24.4	184	0.88	34.01	34.01	3.39	**0.0048**	9350
		C18:1(n-9c)	13.9	20.1	120	0.93	22.19	56.20	2.06	0.602	9267
		C22:1(n-9)	17.6	20.6	83.2	0.72	15.37	71.57	1.05	0.3114	9320
		C16:1(n-7)	9.18	14.1	72.3	0.83	13.35	84.92	2.15	**0.0453**	9354
Great Barrier & Rangitoto	540.14	C20:1(n-11)	14.2	28.1	258	0.93	45.29	45.29	5.08	**0.0002**	9357
		C18:1(n-9c)	13.9	21	94.6	0.83	16.63	61.92	3.25	**0.0058**	9306
		C16:1(n-7)	9.18	15.9	70	0.92	12.96	78.18	4.13	**0.011**	9315

Table 3.15. Contribution of individual PUFAs to multivariate differences in fatty acid profile among *E. chloroticus* gonads from different locations (Outer Hauraki Gulf: Great Barrier and Matheson Bay; Inner Hauraki Gulf: Rakino and Rangitoto) as determined by SIMPER (>10% of contribution). t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor analysis for each FA and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Groups	Average squared distance	PUFA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist/SD	Contribution%	Cumulative%	t	P(perm)	Unique perms
Great Barrier & Matheson's Bay	302.69	C20:2(n-9)	21.4	25.7	105	0.91	34.76	34.76	1.39	0.1889	9307
		C20:4(n-6)	19.1	12.6	82.2	0.83	27.14	61.9	3.10	**0.0073**	9327
		C20:5(n-3)	17.3	13.7	67.6	0.48	22.34	84.25	1.46	0.1561	9385
Matheson's Bay & Rakino	369.34	C20:4(n-6)	12.6	21.1	118	0.66	31.93	31.93	3.72	**0.0007**	9342
		C20:2(n-9)	25.7	25.8	98.7	0.86	26.73	58.65	0.01	0.9905	9374
		C20:5(n-3)	13.7	19.8	68	0.75	18.4	77.06	3.24	**0.0032**	9284
		C18:4(n-3)	13.2	14.8	52.4	0.77	14.18	91.24	0.67	0.5119	9307

The total concentration of PUFA was not significantly different in gonads from different locations (Table 3.9; Table 3.16; Fig. 3.9).

Table 3.16. Results of A) Univariate one-way PERMANOVA comparing concentration of PUFA (µg/mg dry weight) among sea urchin gonads from four different locations in the Hauraki Gulf, New Zealand.

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	3482.7	1160.90	1.95	0.1323	9940
Residual	36	21417	594.91			
Total	39	24899				

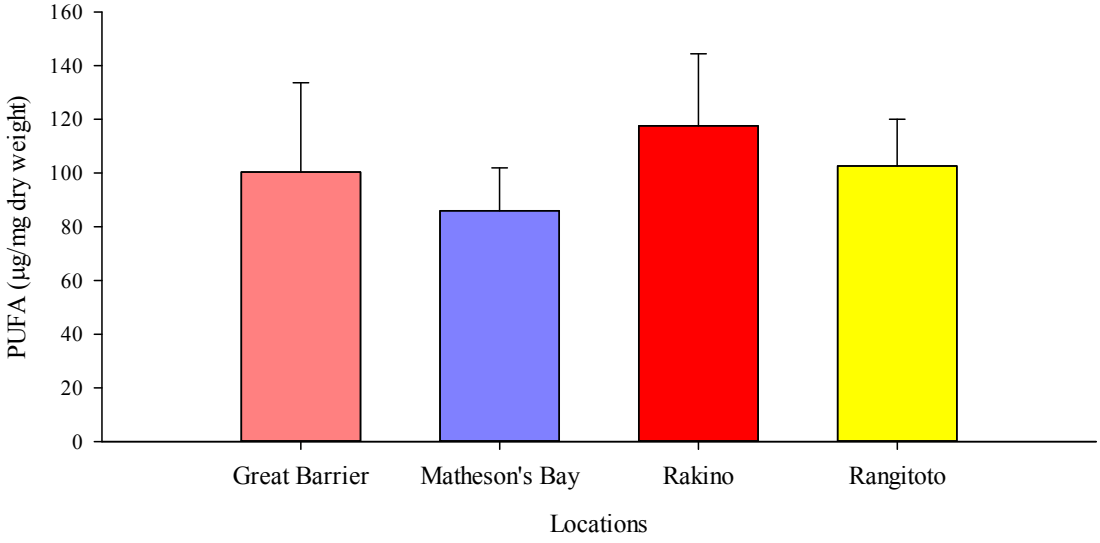


Figure 3.9. Concentration (µg/mg dry weight) of polyunsaturated FA (PUFA) of *E. chloroticus* gonads from four locations (Outer Hauraki Gulf: Great Barrier and Matheson's Bay; Inner Hauraki Gulf: Rakino and Rangitoto) in north-eastern New Zealand. Data represent the mean (±SE) of 10 sea urchins per location.

Twenty seven PUFAs were identified in the gonads of *E. chloroticus* at all locations (Table 3.9); however, there was a significant difference in the PUFA profile among locations (Table 3.17.A) with gonads from Matheson's Bay showing a different PUFA profile compared to the gonads from Great Barrier and Rakino (Table 3.17.B).

Table 3.17. Results of A) Multivariate one-way PERMANOVA and B) Pair-wise comparisons comparing PUFA profile among sea urchin gonads from four different locations in the Hauraki Gulf, New Zealand. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Location	3	881.48	293.83	2.16	**0.0454**	9933
Residual	36	4900.7	136.13			
Total	39	5782.1				

B)

Location	t	P(perm)	Unique perms
Great Barrier, Matheson's Bay	1.80	**0.0425**	9436
Great Barrier, Rakino	1.06	0.3127	9460
Great Barrier, Rangitoto	0.10	0.3473	9409
Matheson's Bay, Rakino	2.11	**0.0061**	9419
Matheson's Bay, Rangitoto	1.61	0.0746	9438
Rakino, Rangitoto	1.18	0.2363	9416

Of the 27 PUFAs identified, four contributed to the differences among gonads collected from different locations: C18:4(n-3), C20:2(n-9), C20:4(n-6) and C20:5(n-3) which were also the dominant PUFAs (Table 3.154). The concentrations of ARA and EPA were significantly different in gonads from different locations (Pseudo-$F_{3,36}$=5.68, P(perm)=0.002 and Pseudo-$F_{3.36}$=3.14, P(perm)=0.0292, respectively). Pairwise comparisons revealed that that the concentration of ARA was lowest in the gonads from Matheson's Bay and the concentration of EPA was higher in the gonads from Rakino compared with the gonads from Rangitoto and Matheson's Bay (Table 3.9; Table 3.15). In contrast, the concentrations of C18:4(n-3) and C20:2(n-9) were not statistically different among locations (Pseudo-$F_{3,36}$=0.46, P(perm)=0.71 and Pseudo-$F_{3,36}$=0.77, P(perm)=0.5212, respectively) (Table 3.9; Table 3.15).

3.4 Discussion

There is a clear distinction in the lipid and FA profiles of *E. chloroticus* gonads from different locations in the Hauraki Gulf, New Zealand, probably due to changes in the FA composition of their main diet item (*E. radiata*) related to variation in the environmental conditions. Other authors have also showed a spatial variation in the lipid and FA profiles of different sea urchin species, attributing these differences to the food available in each location, which affected the lipid composition in the gonads of sea urchins (Chen, 2005; Hughes et al., 2005). Furthermore, it has been suggested that differences in the diets from diverse habitats seem to explain the FA variation of wild populations of sea urchins (Hughes et al., 2006).

Gonads of sea urchins feeding in areas with lower algal lipid contents, such as Great Barrier, had comparatively less total lipid, especially energy storage lipid (i.e. triacylglycerols, TAG) than areas with higher algal lipid content such as Matheson's Bay and Rangitoto. Under normal conditions of gonadal growth, relatively high concentrations levels of TAG are expected to be found in the gonads of sea urchins (Hughes et al., 2006; Iverson, 2009; Liyana-Pathirana et al., 2002). Previous diet experiments on *E. chloroticus* found that sea urchins collected from the wild and fed seaweed contained higher concentrations of TAG and total lipid than sea urchins fed manufactured diets, suggesting that this lipid class was assimilated from the seaweed diets (Phillips et al., 2010). According to the results of this previous study, one of the contributing factors to the differences in the gonadal lipid compositions among locations could be the nutrient composition of the food being consumed by the sea urchins, mainly *E. radiata* (Don, 1975; Schiel, 1982). *E. radiata* plants coming from Great Barrier showed slightly lower concentrations of total lipids, including TAG and PL than the rest of the locations. Even though, to our knowledge, there are few studies comparing biochemical composition of seaweeds between different sites in north-eastern New Zealand, there is a great variation in the algal community structure (Shears, Babcock, et al., 2004). In general terms, kelp forests are extensively distributed along the north-eastern coast of New Zealand, including offshore islands (Schiel, 1988). However, there is a considerable variation in the seaweed species composition and abundances among and within different localities around New Zealand as well as within the Hauraki Gulf (Choat et al., 1982; Schiel, 1988). The different conditions such as salinity and light penetration due to the turbidity in the Hauraki Gulf (Grace, 1983) could explain the variation in the lipid composition of *E. radiata* collected in the different locations. However, the findings of the current study suggest that Matheson Bay may be an intermediate location between Great Barrier Island considered as outer and Rangitoto and Rakino Islands as inner. Further studies are needed to better understand the effect of these factors in the lipid composition of brown seaweeds in the Hauraki Gulf.

In addition to the differences in the total lipid and storage lipids, the FA profile was also different between *E. chloroticus* gonads collected at Great Barrier compared to gonads from Matheson's Bay and Rangitoto. Although gonads of *E. chloroticus* collected from the four locations contained the same FAs, the concentration of MUFA and PUFA were different between locations. Gonads of sea urchins collected at Great Barrier showed smaller concentrations of MUFAs and gonads of sea urchins collected in Matheson's Bay showed

smaller concentrations of PUFAs than the remaining locations. Our results are in accordance with a report of *P. miliaris* where the differences in the FA profiles of their gonads between two locations in the west coast of Scotland as well as between intertidal and subtidal populations were attributed to the food composition (Hughes et al., 2005). As mentioned before, the variability in the algal distribution, composition and abundance due to differences in the environmental conditions within the Hauraki Gulf (Grace, 1983; Schiel, 1988) could be the explanation in the differences found here.

In particular, gonads of *E. chloroticus* contained C14:0 and C16:0 as the dominant SFAs regardless of location and significant differences were not found in the SFA profile or total concentration of SFA between locations. These results are consistent with a previous study on the gonads of *E. chloroticus* where these two SFAs were also dominant and their concentrations did not differ between kina collected from the north tip of the New Zealand's South Island and kina collected in the southern tip of the North Island (Chen, 2005). Similar results were also found in S. *droebachiensis* gonads, where diet experiments showed C16:0 and C14:0 as the major SFAs and the authors suggested that diet did not have a significant effect on these FAs (Liyana-Pathirana et al., 2002).

The MUFA profiles were different among locations, possibly reflecting differences in diets. The total concentration of MUFA as well as the concentrations of the dominant MUFAs C16:1(n-7), C18:1(n-9c) and C20:1(n-11) showed the same pattern as for lipids, being lower in the gonads from Great Barrier compared to the remaining locations. *E. radiata* contained relatively high concentrations of C18:1(n-9), but the rest of the MUFAs were present in very low levels in the kelp compared to the sea urchins gonads. Furthermore, *E. radiata* plants collected in Great Barrier showed relatively lower levels of this MUFA than plants collected from the remaining locations. Feeding experiments on *S. droebachiensis* showed that their gonads presented higher levels of the monoenes C20:1(n-11), C20:1(n-9) and C22:1(n-9) than their diets (Castell et al., 2004). The authors suggested that these long-chain monoenes may either be produced by the sea urchins or specifically retained by these animals. It is known that C18:1(n-9) is the precursor of longer chain monoenes and it seems that diets with high levels of long-chain PUFA facilitates the elongation of C18:1(n-9) to C20:1(n-9) (Castell et al., 2004). Therefore, C18:1(n-9) ingested through *E. radiata* could be especially retained and used to synthesize longer chain monoenes by *E. chloroticus*. Thus, the lower concentration of these two MUFAs in the gonads of *E. chloroticus* collected from

Great Barrier is probably explained by lower concentrations of C18:1(n-9) in *E. radiata* collected at this location.

In contrast, the PUFA profiles showed a different pattern between locations. Although the total concentration of PUFA was not significantly different among locations, gonads from Matheson's Bay and Great Barrier had lower levels than the locations in the inner part of the Hauraki Gulf. The dominant PUFAs C20:4(n-6) or ARA and C20:5(n-3) or EPA were present at highest levels in the gonads of sea urchins collected in Rakino and the lowest levels were present in the gonads collected from Matheson's Bay. In general, brown seaweeds as the dominant diet item of *E. chloroticus* (Barker, 2013; Don, 1975), are characterised by high levels of PUFAs, particularly C18:3(n-3), C18:4(n-3), C20:4(n-6) and C20:5(n-3) (Floreto et al., 1996; Kelly et al., 2012). Additionally, ARA and EPA have been considered as an indicator of macroalgal material in the diet of marine organisms as they are present in relatively high levels in seaweeds (Cook et al., 2000; Sargent et al., 1987). A previous study of *S. droebachiensis* revealed relatively high levels of ARA and EPA in their gonadal lipids and the authors suggested that it was an indication of their diet, particularly *Laminaria* which presents high levels of these two PUFAs (Liyana-Pathirana et al., 2002). *E. radiata* collected for the current study showed C18:4(n-3), ARA and EPA as the dominant PUFAs, being present in relatively smaller concentrations in the plants collected in Great Barrier than the remaining locations. It is well known that FA biosynthesis in macrophytes occurs in the chloroplasts and environmental conditions like light, salinity and water temperature affect their FA composition (Floreto et al., 1998), explaining the variation in the FA profile of the plants collected from locations with different environmental conditions (Grace, 1983).

However, it is not yet clear why PUFA profiles followed a different pattern in the gonads of sea urchins from the different locations. One possible explanation could be that *E. chloroticus* from the inner part of the Hauraki Gulf is feeding on algae with more microalgal film growing on them compared with the remaining locations, as these PUFAs are also indicators of diatoms (Sargent et al., 1987). Another possible explanation is that *E. chloroticus* from the inner part of the Hauraki Gulf could be synthesizing more of these PUFAs as they have important structural functions in cell membranes. Floreto et al. (1996) reported that the white sea urchin *Tripneustes gratilla* was able to purposely synthesize long-chain PUFAs. Later, Hughes et al. (2005) suggested that the use of PUFAs as dietary indicators can lead to a misinterpretation, as not only primary producers are a source of

PUFAs but bacteria also play an important role in synthesizing these compounds, as was shown for *Strongylocentrotus nudus* (Iwanami et al., 1995).

Thus, several factors might explain the variation in the lipid and FA composition of *E. chloroticus* gonads between the inner and outer part of the Hauraki Gulf. As mentioned before environmental conditions have an effect in the FA composition of seaweeds (Floreto et al., 1998) and indirectly in the sea urchin gonads due to the effect of the reproductive stage of the animals (Hughes et al., 2006). Seawater temperature influences the fluidity of the cell membrane and animals can modify this by altering the lipid concentrations (Parrish, 2013). Higher seawater temperatures lead to a decrease in the levels of C20:5(n-3) possibly to maintain the membrane fluidity as reported for *P. lividus* and *A. lixula* (Martínez-Pita et al., 2010b). The water temperature is typically 1-2ºC warmer at the offshore islands in the north-eastern New Zealand (Shears & Babcock, 2004), thus the possible variances in the water temperature as well as nutrient conditions between the inner and outer part of the Hauraki Gulf can lead to differences in the reproductive stages of *E. chloroticus* and influence the variation in the lipid and FA profile. Although histological work was not conducted in the present study, Walker (1982) reported a variation in the period between appearance of mature gametes in the gonads and spawning between a population from Rangitoto compared with populations from nearby islands within the Hauraki Gulf. Little is known about the seasonal impact on the FA composition of *E. chloroticus* and their possible relation to reproduction and environmental factors. It has been shown that FA signatures change over time in different sea urchin species. For example, *Psammechinus miliaris* (Hughes et al., 2006), *Paracentrotus lividus* (Carboni et al., 2013) and *Arbacia lixula* (Martínez-Pita et al., 2010b) revealed a temporal variation in the FA profile related to the gonadal maturity. Kina has an annual reproductive cycle (Brewin et al., 2000) and it has been shown that the biochemical composition is highly affected by the seasons, especially lipids (Verachia et al., 2012). Yet, no seasonal analysis on the FA profile has been investigated for *E. chloroticus* gonads. For this reason, the sampling was only focused on the four locations during the austral summer, before spawning, minimizing the seasonal factor.

In conclusion, the lipid and FA profile of *E. chloroticus* gonads showed a variation among sites within the Hauraki Gulf, in New Zealand possibly due to the variability in the lipid and FA composition of *E. radiata* possibly related to differences in the environmental conditions among locations. However, no clear pattern was found between the inner and the

outer part of the Gulf; instead the difference was between Great Barrier and Matheson's Bay considered as outer and the inner Gulf locations. However, Matheson's Bay might be considered as intermediate location between Great Barrier Island and the remaining locations. This study highlights the importance of understanding the small scale variation in the lipid and FA composition of diets as well as sea urchin gonads and how these changes may affect the reproductive ecology of sea urchins. Thus, further studies are needed on the reproductive stage as well as the gender of the gonads of *E. chloroticus* to better understand the variation in the lipid and FA composition described here. The findings of the current study report valuable lipid and FA information of the only sea urchin species commercially harvested in New Zealand.

Chapter 4

The effect of manufactured diets on the fatty acid composition of the gonads of the sea urchin *Evechinus chloroticus*.

4.1 Introduction

The gonads of sea urchins, also known as roe or uni, are highly valued seafood, consumed principally as sushi in many regions around the world, particularly Asia, North and South America and the Mediterranean (Lawrence, 2007). They are considered delicacies because of the caviar-like appearance of the yellow gonads sacs, their particular bitter-sweet flavour and aroma and their high nutritional value (Chen, 2005; Lawrence, 2007; Liyana-Pathirana et al., 2002; Phillips et al., 2010; Verachia et al., 2012). The overexploitation of wild populations of sea urchins has increased due to the high demand for their gonads in Japan and elsewhere in the world. In order to satisfy the requirements of the international market there is a need to develop sustainable fisheries and aquaculture for both traditionally exploited, as well as new species of sea urchins (Chen, 2005; Cook et al., 2007; Liyana-Pathirana et al., 2002; Phillips et al., 2010).

In New Zealand, there is an abundant supply of the endemic *Evechinus chloroticus* (Booth et al., 2003). This species of sea urchin, known by the Maori as Kina, is widely distributed along the whole coast of the New Zealand mainland and nearby islands (Dix, 1970; Mortesnsen, 1943; Pawson, 1961). Kina is typically a rocky bottom dweller but it may be found on other places such as hard stable substrates or shelly sand, fine sand and mud (Dix, 1970; Fell, 1952; Morton et al., 1968). Generally its abundance increases with increasing exposure to wave action except in the most exposed locations, where densities are reduced (Choat et al., 1987; Choat et al., 1982).

Kina was harvested by the Maori prior to the arrival of Europeans in New Zealand and more recently has been exploited in commercial fisheries for a domestic market (Barker, 2013). Although natural stocks of kina are abundant around New Zealand, the development of an export market has been unsuccessful as their gonads are variable in size and colour and have an intermittent bitter taste (McShane et al., 1994; Phillips et al., 2010). In order to develop a successful and sustainable commercial cultured export market in New Zealand, the

standards of quality (colour, taste, texture and freshness) and consistency of *E. chloroticus* gonads need to be enhanced (Goebel et al., 1998; Phillips et al., 2010).

As the gonad size, colour, taste and biochemical composition of sea urchins depend on diet (Cook et al., 2007; Cook et al., 1998; Hughes et al., 2006; Phillips et al., 2010), experiments have focused on determining the effects on the gonads of sea urchins fed different natural algal species and manufactured diets (Chen, 2005; Cook et al., 2000; Cook et al., 2007; Kelly et al., 2008; Liyana-Pathirana et al., 2002; Phillips et al., 2010; Verachia et al., 2012). Manufactured diets with high levels of protein have been associated with improved growth and taste in sea urchin gonads (Cook et al., 2000; Cook et al., 2007; Phillips et al., 2010). Thus, to meet the requirements of the New Zealand export market, laboratory experiments have focused on comparing manufactured diets with a high protein content with the natural diet of sea urchins as well sea urchins collected from the wild (Chen, 2005; James, 2006; James et al., 2008; Phillips et al., 2009; Phillips et al., 2010; Woods et al., 2008). For example, an artificial diet with fish skins as the biggest component, improved the urchin roe quantity (gonad index, GI) and colour of *E. chloroticus* in land-based tanks or sea-cages (James, 2006). A related study of the same species comparing two prepared diets differing in the protein source (fish-skin and seaweed material), found that while the animal-based protein diets increased the GI, the macroalgal-based protein diets gave a more desirable taste (Woods et al., 2008).

Recent experiments on *E. chloroticus* compared the gonad yield, biochemical composition and sensory quality of gonads from wild sea urchins and urchins fed two manufactured diets (high protein content) and seaweed (low protein content) and wild sea urchins (Phillips et al., 2010). Phillips et al. (2010) found that the manufactured diets increased the gonad yield, glycogen and protein concentration, but a higher total lipid and triglyceride concentration was found in sea urchins fed seaweed or collected from the wild. A sensory panel found the ovaries from sea urchins fed manufactured diets had a particularly bitter taste when compared to wild sea urchins or those fed seaweed. The authors concluded that the manufactured diets were not ideal for gonad quality enhancement, particularly in terms of the sweet and unami taste preferred by the export market (Phillips et al., 2010).

It is well known that the fatty acid (FA) composition in sea urchin gonads is also affected by the diet (Castell et al., 2004; Kelly et al., 2008; Liyana-Pathirana et al., 2002). FAs are the major components of lipids and can be grouped into: 1) saturated FA (SFA),

those with no double bonds in the carbon chain, 2) monounsaturated FA (MUFA), with one double bond present and 3) polyunsaturated FA (PUFA), with two or more double bonds present (Budge et al., 2006). Different studies have suggested that sea urchins are able to elongate and desaturate shorter fatty acids to longer chain FA (Castell et al., 2004; Cook et al., 2000; Cook et al., 2007). Dietary experiments on *Psammechinus miliaris* showed that proportions of individual FA were significantly affected by diet type. For example, sea urchins fed salmon pellets as well as those collected from mussel lines had high proportions of C22:6(n-3) or DHA and sea urchins fed the kelp *Laminaria saccharina* showed high proportions of C20:4(n-6) (Cook et al., 2000). A different study on *Strongylocentrotus droebachiensis* revealed that the levels of 20:5(n-3), 18:2(n-6) and 20:1(n-15) changed during a 9-week feeding experiment using a grain-based manufactured diet (Liyana-Pathirana et al., 2002). Furthermore, Kelly et al. (2008) suggested that FA signatures of sea urchin gonads are affected by diet and can be used to differentiate sea urchins fed in the laboratory from those in the field.

Thus, the present study extends the research of Phillips et al. (2010), and analyses the same *E. chloroticus* gonads from the South Island of New Zealand used in this previous study to investigate the effect of two different manufactured diets and seaweed diet on the fatty acid composition in terms of SFA, MUFA and PUFA of *E. chloroticus* gonads by comparing them with wild sea urchin gonads.

4.2 Methods

4.2.1 Sample collection and store

The sample collection and feeding experiments are described in Phillips et al. (2010). Briefly, sea urchins were collected during June 2006 from Dieffenbach Point at the Queen Charlotte Sound entrance to the Tory Channel (14^0 14’ 10” S, 174^0 08’ 65” E), off the northeast coast of the South Island of New Zealand. Sea urchins were transported to D’Urville Island (40^0 49’ 60” S, 173^0 52’ 0” E) in 20 litre plastic buckets filled with seawater. The diet experiments were conducted as described in detail by Phillips et al. (2010). Briefly, thirty sea urchins were placed in each of the nine metal-framed cages covered in plastic mesh (600 mm x 600 mm x 250 mm) and the cages were suspended along a mussel line for a period of 13 weeks in Catherine Cove (40° 51' 0" S, and 173° 52' 59" E). Sea urchins within the cages were fed either two different manufactured diets or wild-collected

seaweed as a natural diet. Manufactured diets were prepared at the Product Development Research Centre, University of Otago and consisted of a semi-moist product cooked in sealed plastic sausage casings for 25 minutes at an internal temperature of 75^0C (Table 4.1). Wild-collected seaweed included *Macrocystis pyrifera*, *Carpophyllum maschalocarpum*, *Ulva sp*., *Sargassum sp., Marginariella sp*., and *Xiphophora sp.* At weekly intervals, sea urchins were fed either unwashed seaweed harvested locally or manufactured diets. At the end of the 13-week feeding trial, sea urchins were packed in polystyrene bins and maintained at a temperature of 6^0C while transported to the Department of Food Science at the University of Otago. Wild sea urchins were also collected from the Tory Channel in September 2006 as a post-experiment comparative sample.

For physical and biochemical analysis, groups of 10-18 sea urchins (one gonad per urchin) were analysed per treatment. Before dissection, test weight and the diameter of the whole animal were measured and the Aristotle's lantern removed. The test was then cut in half. Gonads were removed intact, rinsed in seawater, drained and weighed. One gonad from each sea urchin was used for the biochemical analysis. For the analysis each gonad was individually homogenized using an Ultra-Turrax T25 with an 18G blending head at 8000 rpm for one minute. The homogenate was divided into aliquots of approximately 1g, and they were placed into microcentrifuge tubes and stored at -80^0C until further analysis. Five of these gonads were randomly selected to be used for the fatty acid analysis. For this purpose the gonads were lyophilized to a constant mass on a freeze-dryer (VirTis Bench Top 2k). Using a MM301 Mixer Mill (Retsch), they were then cryogenically grounded to a particle size of <5μm (Retsch). Lyophilized, ground gonads were stored in new microcentrifuge tubes under desiccant at -20°C until the analysis.

Table 4.1. Composition of the two manufactured diets (Percent in diet) (Phillips et al., 2010).

Ingredients	Diet 1	Diet 2
Arcon SM (concentrate)	19	11
Wheaten Cornflower	27.9	35
Flaxseed oil	5.4	5.9
Water	22.3	20.2
Kelp (wet)	19.5	22.1
Vitamin premix	0.3	0.3
Mineral premix	0.3	0.3
Algro	0.3	0.3
Glycine	0	1.2
MSG	0	1.2
$CaCO_3$	0	0.4
Sodium alginate	0	2
GLA acid	0	0.1
Glycerol	5	0
Proximate composition		
Total protein (dry basis)	22.8	17.8
Total carbohydrate (dry basis)	51.7	60.3
Total fat (dry basis)	10.8	11.4
Total water	42.5	42.8

4.2.2 Fatty acid analysis

Fatty acid analysis was conducted using the one-step reaction from Lepage and Roy (1986), also known as the direct transesterification method. Sea urchin freeze-dried gonads (~50 mg) were precisely weighed using a Sartorius balance (LE244S; max 240 g; d=0.1 mg) in pre-weighed glass tubes (KIMAX, culture tubes; 16x125 mm; 20 ml with phenolic caps and cemented rubber liners). An internal standard consisting of 50 µg to 300 µg of Tridecanoic acid (C13:0) and Tricosanoic acid (C23:0) were dissolved in 2 ml of methanol-toluene 4:1 (v/v). The standard was precisely weighed and added to the biological samples. These FAs were used as internal standards in previous studies (Chen, 2005; Cook et al., 2007; Crawley et al., 2009). A small magnetic stirring bar was added to each tube and, while stirring, 200 µl of acetyl chloride was slowly added over a period of one minute. The tubes were tightly closed and subjected to methanolysis at 100°C for 1 hour. The tubes were re-weighed after heating to check for leakage.

After the tubes had been cooled in tap water until the reach room temperature, 5 ml of a 6% K_2C0_3 solution was slowly added to stop the reaction and neutralize the mixture. The

tubes were then shaken and centrifuged for 5 minutes at 2000 RPM. An aliquot of the toluene upper phase was transferred to an autosampler vial and then injected into an Agilent GC 7890 gas chromatograph equipped with a mass spectrometry detector (MSD 5975c). Separation was performed with a 35 m fused silica column (internal diameter of 0.32 mm), wall-coated with 0.20 mm SP-2330, and with helium as the carrier gas. After injection at 60°C, the oven temperature was raised to 150°C at a rate of 40°C min^{-1}, then to 230°C at 3°C min^{-1}, and finally held constant for 30 minutes. Fatty acid methyl ester (FAME) peaks were identified by comparing their retention times with those of the authentic 37 FAME standards (Supelco Inc.). The mass spectra of FAMEs not present in the standard mix were compared with those from the National Institute of Standards and Technology mass spectra library (NIST MS Search 2.0), together with the lipid library of Christie (2012). Each fatty acid is expressed as the percentage of the total fatty acids identified.

4.2.3 Statistical analysis

The fatty acid profiles of wild-collected sea urchins and sea urchins fed seaweed and the two manufactured diets were compared using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). The data (the percentage of the total fatty acids characterised) was left untransformed and converted into similarity matrices using Euclidean distances. Similarity patterns in the data were visualised using multidimensional scaling (MDS). PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the permutation method, was performed to examine differences in FA composition between the gonads of sea urchins fed different diets and from the wild. Pair-wise comparisons were then performed to compare significant differences in FA between diets. The similarity percentages procedure (SIMPER) was used to determinate which FA contributed most to the differences in the multivariate signature. Those FA identified by the SIMPER were further compared between diets by one-way PERMANOVA on a single variable to test the differences between diets. One-way PERMANOVA on a single variable analysis was used to test the differences between each of these important lipids and FA. We used this approach as it avoids the assumptions of the traditional one-way analysis of variance or ANOVA (Underwood, 1997) and assumes only that the samples are exchangeable, i.e. independent and identically distributed, under a true hypothesis (Anderson, 2003).

4.3 Results

Forty different types of FAs could be identified from the gonads of *Evechinus chloroticus* harvested from the wild and those fed a seaweed diet and two manufactured diets (Table 4.2). The FAs that contributed to more than 2% of the total FA are shown in Figure 4.1, with the addition of the common marine PUFA C22:6(n-3), which is a long polyunsaturated chain produced by the elongation and desaturation of the essential fatty acids C18:1(n-9) and C18:2(n-6).

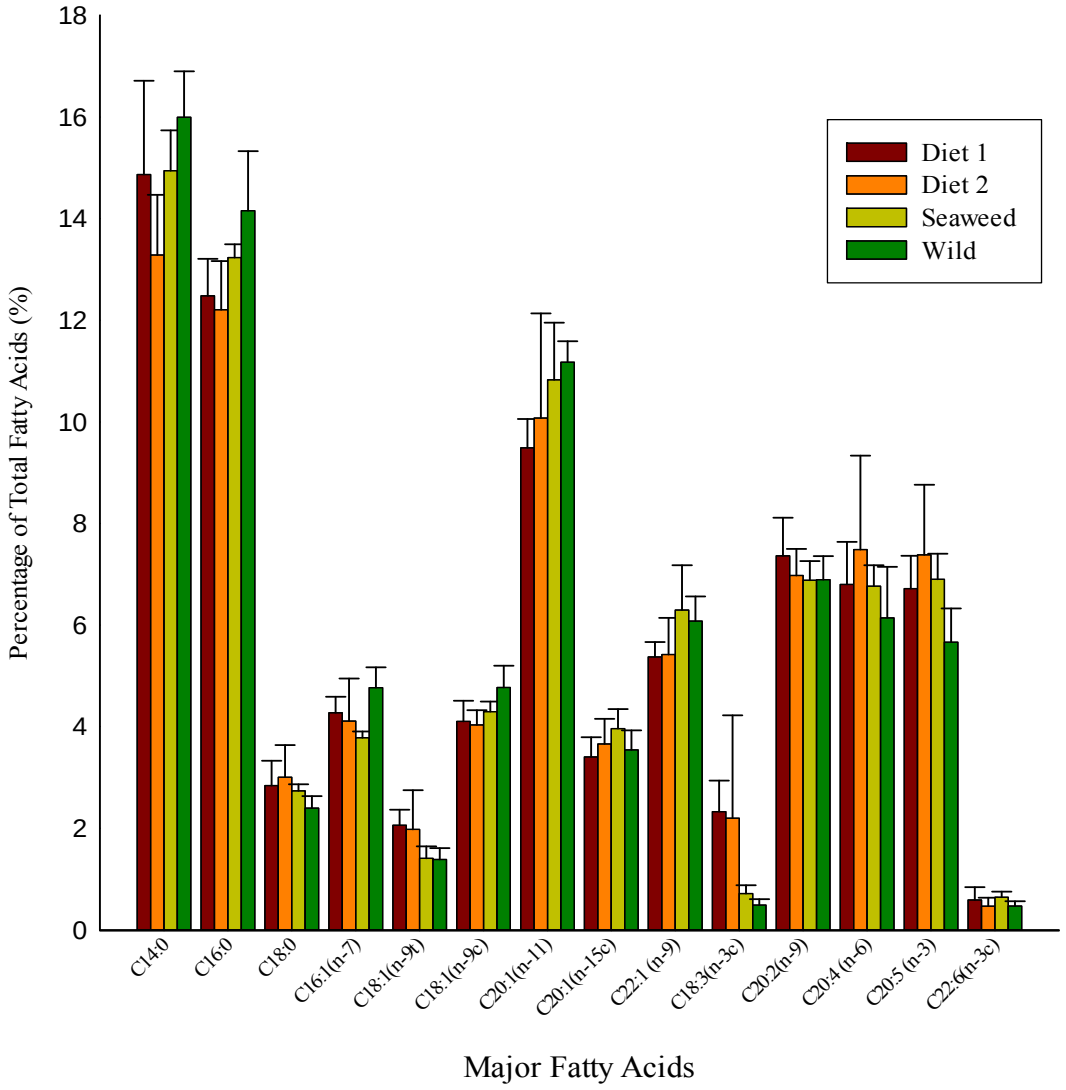


Figure 4.1. Fatty acids contributing more than 2% of the total FAs in the gonads of sea urchins collected from the wild and sea urchins fed manufactured Diet 1, Diet 2, and Seaweed. Results are mean values ± standard deviation (n=5 gonads per treatment, except for seaweed (n=4)

Table 4.2. Fatty acid composition of the gonads of *E. chloroticus* harvested from the wild and sea urchins fed seaweed and two different manufactured diets. Fatty acid values are presented as the percentage of the total fatty acids. Mean values and standard error for 5 sea urchins per treatment, except sea urchins fed seaweed where 4 sea urchins were used.

FA	Diet 1	Diet 2	Seaweed	Wild
C12:0	0.10±0.02	0.09±0.02	0.12±0.01	0.12±0.02
C14:0	14.87±1.84	13.29±1.18	14.95±0.79	16.00±0.90
C14:1(n-5)	0.81±0.09	0.70±0.18	0.70±0.11	0.82±0.09
C15:0	0.32±0.10	0.32±0.11	0.34±0.07	0.38±0.07
C16:0	12.48±0.73	12.21±0.96	13.24±0.26	14.16±1.17
C16:1	0.15±0.03	0.12±0.03	0.14±0.03	0.16±0.02
C16:1	0.14±0.06	0.08±0.03	0.18±0.03	0.13±0.02
C16:1	0.14±0.02	0.16±0.05	0.16±0.02	0.17±0.01
C16:1	0.58±0.05	0.55±0.06	0.64±0.05	0.67±0.07
C16:1	0.12±0.03	0.12±0.05	0.14±0.01	0.10±0.02
C16:1(n-7)	4.28±0.31	4.11±0.84	3.78±0.12	4.77±0.40
C17:0	0.08±0.01	0.08±0.02	0.09±0.00	0.09±0.02
C18:0	2.84±0.48	3.00±0.63	2.74±0.13	2.40±0.24
C18:1(n-5)	1.75±0.33	1.58±0.37	1.87±0.23	1.56±0.27
C18:1(n-9t)	2.06±0.31	1.98±0.77	1.41±0.23	1.39±0.22
C18:1(n-9c)	4.10±0.41	4.03±0.29	4.30±0.20	4.77±0.43
C18:1(n-7)	0.53±0.08	0.56±0.09	0.47±0.02	0.59±0.07
C18:2(n-6t)	0.98±0.10	0.80±0.20	0.98±0.09	0.96±0.13
C18:2(n-6c)	1.18±0.26	1.18±0.50	0.67±0.10	0.54±0.05
C18:3	0.37±0.10	0.32±0.11	0.36±0.11	0.32±0.04
C18:3(n−6)	0.12±0.05	0.10±0.05	0.14±0.03	0.09±0.02
C18:3(n-3)	2.33±0.61	2.20±2.03	0.71±0.17	0.49±0.12
C20:0	0.56±0.06	0.50±0.07	0.63±0.08	0.54±0.06
C20:1(n-15)	3.40±0.39	3.66±0.49	3.96±0.38	3.54±0.38
C20:1(n-11)	9.49±0.56	10.08±2.06	10.83±1.12	11.18±0.41
C20:1(n-9)	0.99±0.16	1.12±0.19	1.26±0.19	1.38±0.18
C20:2(n-9)	7.37±0.75	6.97±0.53	6.88±0.38	6.89±0.47
C20:2	1.03±0.06	0.93±0.14	0.95±0.18	0.98±0.10
C20:2	0.56±0.06	0.53±0.07	0.59±0.04	0.64±0.11
C20:2(n-6)	1.54±0.14	1.85±0.33	1.32±0.10	1.35±0.26
C20:3(n-9)	1.76±0.32	1.59±0.22	1.76±0.29	1.57±0.33
C21:1	0.87±0.21	1.03±0.05	1.01±0.23	1.10±0.18
C20:3(n-6)	0.41±0.08	0.47±0.13	0.39±0.05	0.38±0.06
C20:4(n-6)	6.80±0.84	7.49±1.85	6.76±0.42	6.14±1.00
C20:3(n-3)	1.30±0.10	1.54±0.59	1.06±0.12	0.87±0.08
C20:4(n-3)	0.69±0.14	0.67±0.33	0.43±0.11	0.43±0.10
C22:1(n-9)	5.37±0.29	5.42±0.72	6.29±0.88	6.08±0.48
C20:5(n-3)	6.71±0.65	7.39±1.38	6.90±0.51	5.66±0.66
C24:1(n-9)	0.22±0.14	0.13±0.05	0.19±0.03	0.14±0.03
C22:6(n-3)	0.59±0.25	0.46±0.17	0.64±0.11	0.47±0.09
Total SFA	**31.25±2.42**	**29.50±0.83**	**32.10±0.72**	**33.68±1.56**
Total MUFA	**35.01±1.45**	**35.42±2.79**	**37.33±1.57**	**38.54±1.30**
Total PUFA	**33.74±2.86**	**34.50±3.59**	**30.57±0.90**	**27.78±1.96**
Total FAMEs	**30.67±7.39**	**26.93±7.60**	**35.95±11.52**	**39.75±6.55**

The FA profile of *E. chloroticus* gonads was significantly different for the two manufactured diets, seaweed diet and wild, sea urchins as revealed by one-way PERMANOVA analysis (Table 4.3.A), although there was no significant difference in the total amount of FA between diets (Table 4.4). All 40 identified FAs were present in the sea urchin gonads regardless of diet, but differed in their percent contribution as shown in the MDS plot (Fig. 4.2.A; Table 4.2).

Pair-wise comparisons revealed significant differences in the FA profile between sea urchins fed Diet 1 and urchins fed seaweed or from the wild as well as sea urchins fed Diet 2 and sea urchins from the wild (Table 4.3.B). In contrast, no significant differences were observed in the FA profile between sea urchins fed Diet 1 and Diet 2, between sea urchins fed Diet 2 and the seaweed diet and between sea urchins fed the seaweed diet and those harvested from the wild (Table 4.3.B).

Table 4.3. Results of A) one-way PERMANOVA and B) Pair-wise test comparing FA profile of *E. chloroticus* gonads of wild sea urchins and sea urchins fed two manufactured diets and seaweed. All 40 FA identified were included in the analysis. 5 sea urchins were used for the diet treatments except for Seaweed treatment, where 4 sea urchins were used. Significant results are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	81.29	27.10	3.06	**0.0017**	9936
Residual	15	132.68	8.85			
Total	18	213.96				

B)

Groups	t	P(perm)	Unique perms
Diet 1, Diet 2	0.95	0.528	126
Diet 1, Seaweed	1.68	**0.026**	126
Diet 1, Wild	2.40	**0.010**	126
Diet 2, Seaweed	1.38	0.072	126
Diet 2, Wild	2.29	**0.009**	126
Seaweed, Wild	1.66	0.059	126

Table 4.4. Results of one-way PERMANOVA comparing total fatty acids (μg/mg dry-weight) between *E. chloroticus* gonads of sea urchins from the wild and sea urchins fed seaweed and two manufactured diets.

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	47381	15794	2.37	0.1116	9942
Residual	15	99985	6665.7			
Total	18	147000				

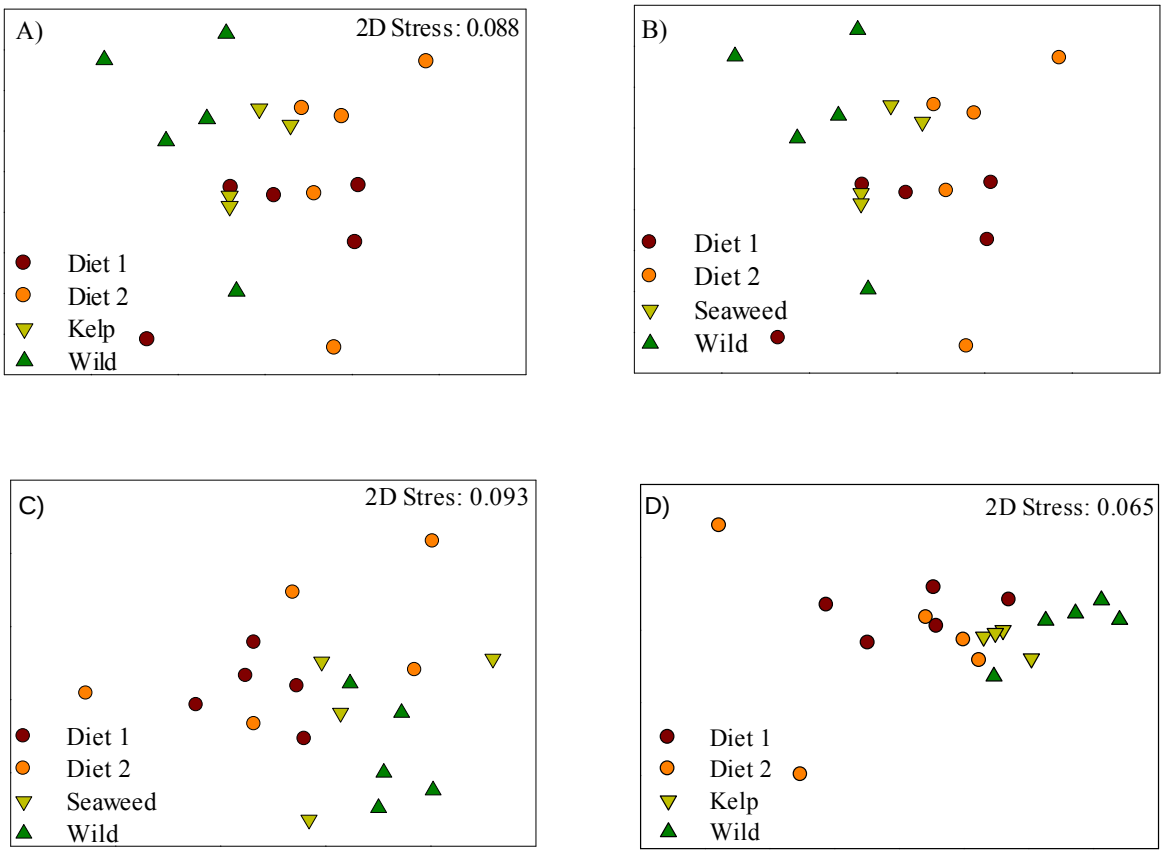


Figure 4.2.. Multidimensional scaling (MDS) plot of Euclidean similarities of FA profile of *E. chloroticus* fed different diets based on all 40 identified FA: Diet 1, Diet 2, Seaweed, Wild sea urchins. A) All identified fatty acids (N= 40); B) Saturated FA (N=7); C) Monounsaturated FA (N=17) and D) Polyunsaturated FA (N=16).

Seven identified saturated fatty acids (SFA) were present in the FA profile of all sea urchin gonads, regardless of diet (Table 4.2). The major SFAs were C14:0 (~13-16% of total FA) and C16:0 (~12-14% of total FA). Smaller amounts of C18:0 (~2-3% of total FA) and C20:0 were present (<1% of total FA) and trace amounts (<0.5% of total FA) of C12:0, C15:0 and C17:0 (Table 4.2). One-way PERMANOVA analysis showed significant differences in the SFA profile between the gonads of sea urchins fed different diets and sea urchins from the wild (Table 4.5.A). The MDS plot revealed the separation in the SFA profile among diets (Fig. 4.2.B). Pairwise results showed that the lipid profile of the gonads of sea urchins fed Diet 2 was different to the lipid profile of sea urchin gonads fed seaweed and sea urchin gonads from the wild (Table 4.5.B).

Table 4.5. Results of one-way PERMANOVA comparing SFA profile of *E. chloroticus* gonads fed the two manufactured diets, seaweed and sea urchins from the wild. All the saturated fatty acids identified were included in the analysis (N=7). 5 sea urchins were used for the diets treatments except for Seaweed, where 4 sea urchins were used.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	31.17	10.39	4.24	**0.0084**	9953
Residual	15	36.75	2.45			
Total	18	67.92				

B)

Diets	t	P(perm)	Unique perms
Diet 1, Diet 2	1.43	0.1823	126
Diet 1, Seaweed	0.70	0.6236	126
Diet 1, Wild	1.83	0.0765	125
Diet 2, Seaweed	2.34	**0.0162**	126
Diet 2, Wild	3.59	**0.0096**	126
Seaweed, Wild	1.71	0.1020	126

SIMPER analysis showed that the two major SFAs, C14:0 and C16:0 were the greatest contributors to this difference (Table 4.6) and their amounts showed significant differences between sea urchins fed different diets (Table 4.7). The gonads of sea urchins from the wild and those fed seaweed had a higher percentage of C14:0 than the gonads of sea urchins fed Diet 2 (Table 4.2; Table 4.6.A). Similarly sea urchins from the wild had a higher percentage of C16:0 than sea urchins fed the two manufactured diets and also sea urchins fed seaweed had a higher percentage than sea urchins fed Diet 2 (Table 4.2; Table 4.6.B).

Table 4.6. Contribution of individual SFA to multivariate differences in SFA profile between gonads of *E. chloroticus* fed different diets, Diet 1, Diet 2, seaweed and sea urchins from the wild as determined by SIMPER. t, P(perm) and Unique perm were obtained from a pairwise comparison analysis on a single variable. Significant results ($p<0.05$) are shown in bold.

Diets: Groups 1 and 2	Average squared distance	SFA	Average Value Group 1	Average Value Group 2	Average Sq. Dist	Sq. Dist /SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Diet 1 & Diet 2	7.73	C14:0	14.9	13.3	6.14	0.67	79.47	79.47	1.66	0.1401	126
		C16:0	12.5	12.2	1.05	0.71	13.64	93.11	0.55	0.6084	126
Diet 1 & Seaweed	4.47	C14:0	14.9	14.9	3.19	0.87	71.39	71.39	0.09	0.9281	126
		C16:0	12.5	13.2	1.04	0.84	23.34	94.73	1.96	0.1009	126
Diet 2 & Seaweed	6.23	C14:0	13.3	14.9	4.15	0.84	66.60	66.6	2.57	**0.0297**	126
		C16:0	12.2	13.2	1.66	0.76	26.73	93.33	2.35	**0.0296**	126
Diet 1 & Wild	9.42	C14:0	14.9	16	4.64	1.03	49.29	49.29	1.23	0.2652	126
		C16:0	12.5	14.2	4.33	1.03	45.90	95.19	2.73	**0.0326**	126
Diet 2 & Wild	15.11	C14:0	13.3	16	8.93	1.13	59.09	59.09	4.32	**0.0071**	126
		C16:0	12.2	14.2	5.45	1.02	36.07	95.16	3.04	**0.0239**	126
Seaweed & Wild	4.42	C14:0	14.9	16	2.22	0.81	50.37	50.37	1.83	0.0993	126
		C16:0	13.2	14.2	1.99	1.03	45.09	95.46	1.53	0.1722	126

Table 4.7. Results of one-way PERMANOVA on a single variable comparing the percentage of the SFA A) C14:0 and B) C16:0 between gonads of *E. chloroticus* fed two manufactured diets, seaweed diet and sea urchins from the wild.

A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	18.73	6.242	4.01	**0.0256**	9957
Residual	15	23.33	1.56			
Total	18	42.05				

B)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	11.41	3.80	5.40	**0.0073**	9953
Residual	15	10.56	0.70			
Total	18	21.97				

Monounsaturated fatty acids (MUFA) contributed at least 35% of the total FAs in the sea urchin gonads (Table 4.2), with 17 MUFAs identified. C20:1(n-11) and C22:1(n-9) were the major MUFAs present (>5% of total FA) followed by relatively high percentages of C16:1(n-7), C18:1(n-9c) and C20:1(n-15) (Table 4.2). The remaining 12 MUFAs were present in very low percentages, less than 2 % of total FA (Table 4.2). The One-way PERMANOVA analysis revealed a statistically significant separation in the identified MUFAs in gonads of sea urchin fed different diets (Table 4.8.A). The separation is also clearly shown in the MDS plot (Fig 4.2.C). Pairwise comparisons showed significant differences between the gonads of sea urchins fed Diet 1, sea urchin fed seaweed and sea urchins from the wild (Table 4.10.B). SIMPER analysis showed that the MUFAs that contributed more than 10% to the differences in the MUFA profile were C16:1(n-7), C18:1(n-9c), C20:1(n-11) and C22:1(n-9) (Table 4.10). However, PERMANOVA on a single variable showed that the only MUFA that had significant differences in the percentage between the gonads of sea urchins fed different diets and from the wild was C18:1(n-9c) (Table 4.12). Pairwise comparisons revealed that the percentage of this MUFA was higher in the gonads of sea urchins from the wild than sea urchins fed Diet 2 (Table 4.2; Table 4.12). This analysis also showed that C16:1(n-7) had smaller percentages in gonads of sea urchins fed seaweed than sea urchins fed Diet 1 or sea urchins from the wild. Additionally, C20:1(n-11) showed smaller percentages in the gonads of sea urchins fed Diet 1 than sea urchins fed seaweed or sea urchins from the wild and C22:1(n-9) had higher percentage in the gonads of sea urchins from the wild than those fed Diet 1 (Table 4.2; Table 4.11).

Table 4.8. Results of one-way PERMANOVA comparing MUFA profile (N=17) of gonads of *E. chloroticus* fed the two manufactured diets, seaweed diet and those from the wild.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	18.98	6.33	2.29	**0.0371**	9933
Residual	15	41.49	2.77			
Total	18	60.46				

B)

Diets	t	P(perm)	Unique perms
Diet 1, Diet 2	0.61	0.7885	126
Diet 1, Seaweed	2.23	**0.0075**	125
Diet 1, Wild	3.27	**0.0086**	126
Diet 2, Seaweed	0.99	0.4291	126
Diet 2, Wild	1.44	0.1261	126
Seaweed, Wild	1.50	**0.0414**	126

Table 4.9. Results of PERMANOVA on a single variable comparing the percentage of the MUFA that contributed to the differences between *E. chloroticus* gonads fed two manufactured diets, seaweed and sea urchins from the wild. A) C16:1(n-7), B) C18:1(n-9c), C) C20:1(n-11) and D) C22:1(n-9). Significant differences are shown in bold ($p<0.05$)

A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	2.32	0.772	3.33	0.0560	9972
Residual	15	3.49	0.23			
Total	18	5.81				

B)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	1.67	0.56	4.19	**0.0248**	9962
Residual	15	1.99	0.13			
Total	18	3.66				

C)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	8.39	2.80	1.83	0.1835	9964
Residual	15	22.97	1.53			
Total	18	31.36				

D)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	2.97	0.99	2.85	0.0747	9953
Residual	15	5.22	0.35			
Total	18	8.19				

Table 4.10. Contribution of individual MUFA to multivariate differences in fatty acid profile between *E. chloroticus* gonads fed different diets, Diet 1, Diet 2, Seaweed and sea urchins from the wild as determined by SIMPER. t, P(perm) and Unique perm were obtained in pairwise comparison analysis on a single variable. Significant results ($p<0.05$) are shown in bold.

Diets: Groups 1 and 2	Average squared distance	Fatty acid	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/ SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Diet 1 & Diet 2	6.5	C20:1(n-11)	9.49	10.1	4.05	1.09	62.32	62.32	0.61	0.5588	126
		C16:1(n-7)	4.28	4.11	0.59	1.03	9.07	71.39	0.45	0.6665	126
		C18:1(n-9t)	2.06	1.98	0.46	0.83	7.01	78.40	0.26	0.8431	126
		C22:1(n-9)	5.37	5.42	0.39	0.88	6.03	84.43	0.15	0.8718	126
		C20:1(n-15c)	3.4	3.66	0.37	0.74	5.68	90.11	0.93	0.3907	126
Diet 1 & Seaweed	6.53	C20:1(n-11)	9.49	10.8	3	0.74	45.86	45.86	2.35	**0.0333**	126
		C22:1(n-9)	5.37	6.29	1.50	1.12	22.98	68.84	2.21	0.0698	126
		C20:1(n-15c)	3.40	3.96	0.54	0.90	8.29	77.13	2.16	0.0681	126
		C18:1(n-9t)	2.06	1.41	0.54	1.19	8.28	85.41	3.50	**0.0242**	126
		C16:1(n-7)	4.28	3.78	0.33	1.10	5.08	90.49	2.97	**0.0311**	126
Diet 2 & Seaweed	8.94	C20:1(n-11)	10.1	10.8	4.96	0.76	55.48	55.48	0.65	0.5779	126
		C22:1(n-9)	5.42	6.29	1.67	0.87	18.64	74.13	1.73	0.1458	126
		C18:1(n-9t)	1.98	1.41	0.74	0.61	8.22	82.34	1.57	0.1038	126
		C16:1(n-7)	4.11	3.78	0.60	0.89	6.72	89.06	0.82	0.4485	126
		C20:1(n-15c)	3.66	3.96	0.39	0.82	4.3	93.36	1.02	0.3175	126
Diet 1 & Wild	6.53	C20:1(n-11)	9.49	11.2	3.23	1.37	49.43	49.43	5.39	**0.0084**	126
		C22:1 (n-9)	5.37	6.08	0.76	1.32	11.56	60.99	2.79	**0.0330**	126
		C18:1(n-9c)	4.10	4.77	0.73	0.93	11.13	72.13	2.54	0.0512	126
		C18:1(n-9t)	2.06	1.39	0.57	1.20	8.69	80.82	3.99	**0.0189**	126
		C16:1(n-7)	4.28	4.77	0.45	0.84	6.85	87.67	2.17	0.0777	126
		C20:1(n-15c)	3.4	3.54	0.26	0.92	3.92	91.59	0.57	0.5624	126
Diet 2 & Wild	9.01	C20:1(n-11)	10.1	11.2	4.79	0.79	53.14	53.14	1.16	0.2913	126
		C16:1(n-7)	4.11	4.77	1.05	0.89	11.63	64.78	1.69	0.1299	126
		C22:1(n-9)	5.42	6.08	0.94	0.79	10.45	75.22	0.43	0.6782	126
		C18:1(n-9c)	4.03	4.77	0.79	0.94	8.76	83.98	3.00	**0.0227**	126
		C18:1(n-9t)	1.98	1.39	0.76	0.62	8.41	92.39	1.83	0.0643	126
Seaweed & Wild	4.41	C20:1(n-11)	10.8	11.2	1.20	1.18	27.12	27.12	0.62	0.5245	126
		C16:1(n-7)	3.78	4.77	1.11	1.43	25.18	52.30	4.70	**0.0162**	126
		C22:1(n-9)	6.29	6.08	0.82	0.89	18.53	70.84	0.47	0.6368	126
		C20:1(n-15c)	3.96	3.54	0.40	0.64	9.14	79.98	1.64	0.1422	126
		C18:1(n-9c)	4.30	4.77	0.40	0.81	9.1	89.09	2.05	0.0955	126
		C18:1(n-5)	1.87	1.56	0.20	0.77	4.45	93.54	1.85	0.1127	126

There were 16 identified polyunsaturated fatty acids (PUFA) in *E. chloroticus* gonads (Table 4.2). The PUFAs that contributed >5% of total FA of *E. chloroticus* gonads were C20:4(n-6) or ARA (Arachidonic Acid) and C20:5(n-3) or EPA (Eicosapentaenoic Acid) (Table 4.2) and C20:2(n-9). Minor amounts of C18:3(n-3) or ALA (Alpha-linoleic Acid) and C22:6(n-3) or DHA (Docosahexaenoic Acid) were detected and the rest of the 11 PUFAs were present in small or trace amounts (<2.5% of total FA; Table 4.2). In the one-way PERMANOVA analysis significant differences in the PUFA profile were seen between gonads of sea urchins fed different diet types (Table 4.13.A). The differences are also shown in the MDS plot (Fig. 4.2.D). Pairwise comparisons showed statistical differences between the gonads of sea urchins fed Diet 1, sea urchins fed seaweed and those from the wild (Table 4.13.B). SIMPER analysis showed that C18:3(n-3), C20:2(n-9), ARA and EPA were the PUFAs that contributed more than 10% to the difference (Table 4.15). The percentage of C18:3(n-3) was significantly different between sea urchins fed different diets and sea urchins from the wild (Table 4.14.A), with a lower percentage in the gonads of sea urchins from the wild than sea urchins fed the two manufactured diets. The percentage was also higher in the gonads of sea urchins fed Diet 1 than sea urchins fed seaweed (Table 4.2; Table 4.15). The percentage of EPA also varied significantly between diet treatments (Table 4.14.D), with higher percentages in the gonads of sea urchins fed Diet 2 and seaweed compared to sea urchins from the wild (Table 4.2; Table 4.15). However, the concentration of this PUFA was slightly higher in the gonads of sea urchin fed seaweed (25.22±9.09µg/mg dry weight) and from the wild (22.20±1.68µg/mg dry weight) compared to sea urchins fed the manufactured diets (~20µg/mg dry weight). Even though there was no significant difference in the percent of ARA (Table 4.14.B) or C20:2(n-9) (Table 4.14.C) between diets, their concentrations were slightly higher in the sea urchins fed seaweed and those from the wild (>24µg/mg dry weight) compared to sea urchins fed manufactured Diet 1 and Diet 2 (<20µg/mg dry weight).

Table 4.11. Results of PERMANOVA comparing PUFA profile (N= 16) of the gonads of *E. chloroticus* fed the two manufactured diets, seaweed diet and *E. chloroticus* from the wild. 5 sea urchins were used for the diets treatments except for Seaweed, where 4 sea urchins were used.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	31.14	10.38	2.86	**0.0045**	9939
Residual	15	54.44	3.63			
Total	18	85.58				

B)

Diets	t	P(perm)	Unique perms
Diet 1, Diet 2	0.79	0.7622	126
Diet 1, Seaweed	2.08	**0.0165**	126
Diet 1, Wild	2.62	**0.0152**	126
Diet 2, Seaweed	1.28	0.0712	126
Diet 2, Wild	2.05	**0.0161**	126
Seaweed, Wild	1.79	0.0634	126

Table 4.12. Results of PERMANOVA comparing the percentage of the PUFA that contributed to the difference between the gonads of *E. chloroticus* fed two manufactured diets, seaweed diet and *E. chloroticus* from the wild. A) 18:3(n-3), B) ARA, C) C20:2 (n-9) and D) EPA. Significant results are shown in bold ($p<0.05$)

A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	13.43	4.48	4.42	**0.0112**	9941
Residual	15	15.18	1.01			
Total	18	28.61				

B)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	4.55	1.52	1.29	0.3187	9956
Residual	15	17.71	1.18			
Total	18	22.26				

C)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	0.77	0.26	0.85	0.5114	9951
Residual	15	4.54	0.30			
Total	18	5.31				

D)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Diets	3	7.85	2.62	3.67	**0.0295**	9960
Residual	15	10.69	0.71			
Total	18	18.54				

Table 4.13. Contribution of individual PUFA to multivariate differences in PUFA profile between the gonads of *E. chloroticus* fed the two manufactured diets, Seaweed diet and *E. chloroticus* from the wild as determined by SIMPER. t, P(perm) and Unique perm were obtained in pairwise comparison analysis on a single variable. Significant results ($p<0.05$) are shown in bold

Diets: Groups 1 & 2	Average squared distance	Fatty acid	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/ SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Diet 1 & Diet 2	10.22	C20:4(n-6)	6.80	7.49	3.11	0.59	30.45	30.45	0.85	0.4718	126
		C18:3(n-3)	2.33	2.02	3.03	0.77	29.61	60.06	0.15	0.9340	126
		C20:5(n-3)	6.71	7.39	2.08	0.72	20.37	80.43	1.05	0.3464	126
		C20:2(n-9)	7.37	6.97	0.80	0.56	7.84	88.27	0.98	0.4670	126
		C20:3(n-3)	1.30	1.54	0.29	0.51	2.87	91.14	1.01	0.4431	126
Diet 1 & Seaweed	5.83	C18:3(n-3)	2.33	0.715	2.93	1.66	50.21	50.21	5.08	**0.0171**	126
		C20:2(n-9)	7.37	6.88	0.79	0.55	13.55	63.76	1.18	0.3712	126
		C20:4(n-6)	6.80	6.76	0.70	0.82	12.01	75.77	0.08	0.9439	126
		C20:5(n-3)	6.71	6.90	0.57	0.93	9.72	85.49	0.46	0.6519	126
		C18:2(n-6c)	1.18	0.67	0.32	1.30	5.48	90.97	3.70	**0.0207**	126
Diet 2 & Seaweed	11.47	C18:3(n-3c)	2.20	0.715	4.93	0.56	42.96	42.96	1.58	0.0782	126
		C20:4(n-6)	7.49	6.76	2.72	0.55	23.74	66.70	0.87	0.5433	126
		C20:5(n-3)	7.39	6.90	1.72	0.72	15.02	81.72	0.72	0.5089	126
		C20:3(n-3)	1.54	1.06	0.47	0.58	4.09	85.81	1.76	**0.0498**	126
		C18:2(n-6c)	1.18	0.67	0.45	0.65	3.93	89.74	2.08	**0.0315**	126
		C20:2(n-6)	1.85	1.32	0.36	1.17	3.17	92.92	3.24	**0.0253**	126
Diet 1 & Wild	9.36	C18:3(n-3c)	2.33	0.49	3.70	1.88	39.49	39.49	6.63	**0.0091**	126
		C20:4(n-6)	6.80	6.14	1.80	0.9	19.26	58.76	1.12	0.2871	126
		C20:5(n-3)	6.71	5.66	1.80	0.98	19.2	77.96	2.53	0.0585	126
		C20:2(n-9)	7.37	6.89	0.85	0.56	9.08	87.04	1.21	0.3097	126
		C18:2(n-6c)	1.18	0.536	0.47	1.61	5	92.04	5.49	**0.0089**	126
Diet 2 & Wild	17.44	C18:3(n-3c)	2.20	0.49	5.64	0.59	32.33	32.33	2.07	**0.0071**	126
		C20:4(n-6)	7.49	6.14	4.69	0.65	26.86	59.20	1.59	0.1336	126
		C20:5(n-3)	7.39	5.66	4.61	0.88	26.46	85.65	2.69	**0.0388**	126
		C20:3(n-3)	1.54	0.87	0.66	0.7	3.93	89.58	2.81	**0.0069**	126
		C18:2(n-6c)	1.18	0.54	0.60	0.75	3.44	93.02	3.01	**0.0074**	126
Seaweed & Wild	4.23	C20:5(n-3)	6.90	5.66	2.07	1.24	48.93	48.93	3.06	**0.0327**	126
		C20:4(n-6)	6.76	6.14	1.32	1.27	31.17	80.10	1.15	0.2964	126
		C20:2(n-9)	6.88	6.89	0.28	0.75	6.68	86.78	0.03	0.9750	125
		C20:3(n-9)	1.76	1.57	0.18	0.80	4.33	91.11	0.90	0.3930	126

4.4 Discussion

In the previous study by Phillips et al. (2010), i.e. that used the same *E. chloroticus* gonad samples as used here, they described how the two manufactured high protein diets affected the biochemical composition of *E. chloroticus* gonads, increased the gonad yield and resulted in gonads with a bitter taste. Additionally, they found that differences in the total lipid and triacylglycerol (TAG) concentration in gonads between sea urchins fed different manufactured diets and wild sea urchins, with higher TAG in the gonads of both sea urchins fed seaweed and wild sea urchins than in sea urchins fed manufactured Diet 1. Phillips et al. (2010) further suggested that sea urchins use precursors from seaweeds to make triacylglycerol.

The principal goal in the present study was to determine if the FA profile in the gonads of *E. chloroticus* was affected by these manufactured diets and to compare the profile with a seaweed diet or from wild populations. Our multivariate analyses showed a significant difference in FA profile with diet, even though the total amount of fatty acids was similar between treatments. In particular, two SFAs (C14:0 and C16:0), four MUFAs (C16:1(n-7); C18:1(n-9c), C20:1(n-11) and C22:1(n-9)) and three PUFAs (C18:3(n-3), C20:4(n-6) or ARA and C20:5(n-3) or EPA) were important in the FA profile differences for sea urchins fed different diets and from the wild.

In *E. chloroticus* gonads, the dominant SFAs (C14:0 and C16:0) contributed to the differences between diet treatments. This has been found in previous studies on the same species (Chen, 2005) as well as in the gonads of other sea urchin species (Cook et al., 2007; Kelly et al., 2008; Liyana-Pathirana et al., 2002). The percentage of C14:0 and C16:0 was higher in the gonads of sea urchins collected from the wild than sea urchins fed manufactured diets. Kelly et al. (2008) found evidence that sea urchins in barrens obtain much of their nutrition from non-macroalgal sources such as diatoms, and that these sources may be more nutritious than seaweed. For example, cyanobacteria is generally dominated by C16:0 and C14:0 (Martínez-Pita et al., 2010a). This could explain the difference in the higher percentage of these SFAs in the gonads of sea urchins from the wild, suggesting that they graze on the kelp, but also incorporate FAs from associated microorganisms.

Among the MUFAs, C16:1(n-7), C8:1(n-9c), C20:1(n-11) and C22:1(n-9) were the FAs present in relatively high percentages in sea urchin gonads and they contributed to the differences in the FA profile of sea urchins fed different diets. C20:1 isomers are present in

high concentrations in sea urchin gonads even though they are either absent or at present in very low levels in seaweed, the natural diet of sea urchins; it has been suggested that the formation of these MUFAs in sea urchin gonads may be of biosynthetic origin (Chen, 2005; Kelly et al., 2008; Liyana-Pathirana et al., 2002; Wessels et al., 2012). *S. droebachiensis* gonads contained higher amounts of C20:1(n-11), C20:1(n-9) and C22:1(n-9) than the dietary lipids (<1%), suggesting that this sea urchin species was able to synthesize these monoens or can specifically retain them (Castell et al., 2004). On the other hand, copepods have the ability to synthesize large amounts of long chain MUFA, such as C20:1(n-11), C20:1(n-9), C22:1(n-11) and C22:1(n-9), and it has been suggested that large amounts of this MUFA indicate the direct consumption of copepods (Iverson, 2009; Sargent et al., 1988). However, it is highly unlikely that sea urchins consume mobile prey such as copepods. Here, the percentages of C20:1(n-11) and C22:1(n-9) were higher in the gonads of sea urchin from the wild and sea urchins fed kelp than sea urchins fed Diet 1. The brown seaweeds commonly consumed by *E. chloroticus* had very low concentrations of C20:1(n-15), C20:1(n-9) and C20:1(n-11), with some of these FAs not detected in previous studies on brown seaweeds (Guest et al., 2010; Hanson et al., 2010; McLeod et al., 2013). These results suggest that this sea urchin species is also able to either biosynthesise these long-chain MUFA or specifically retain them when they are present in very low percentages in the diet as suggested for *S. droebachiensis* (Castell et al., 2004). However, it is also possible that they are consuming indirectly amphipods or isopods that are very abundant in these brown seaweeds (Taylor et al., 1994) when they are feeding on the kelp, thus incorporating and storing these MUFAs (Drazen et al., 2008).

C16:1(n-7) was also present in relatively high percentages and contributed to the differences between treatments being present in smaller percentages in the gonads of sea urchins fed seaweed than those fed Diet 1 and sea urchins from the wild. Diet experiments on *S. droebachiensis* showed that this MUFA was also one of the contributors among the differences between treatments with higher levels in the sea urchin gonads than in the seaweed (Kelly et al., 2008). Previous studies on brown seaweeds reported relatively high levels of C16:1(n-7) (Guest et al., 2010; Hanson et al., 2010; McLeod et al., 2013). Additionally, this MUFA is present in high concentration in diatoms and is considered indicative of animals feeding on diatoms possibly being part of the microalgal films growing on seaweeds (Dalsgaard et al., 2003; Kharlamenko et al., 1995). However, it can also be synthesized from the SFA by the animal (Iverson, 2009; Iverson et al., 2004). The results here indicate that *E. chloroticus* is able to selectively retain C16:1(n-7) when it is present in

the diet as this MUFA is an important precursor for longer chain FA as suggested before (Iverson et al., 2004; Kattner et al., 2012).

PUFA are considered to be important structural and physiological components of cell membranes (Cook et al., 2007; Liyana-Pathirana et al., 2002) and are essential FAs in all animals, because they lack desaturase enzymes for converting short chain into long chain PUFA (Castell et al., 2004). It has been suggested in a previous study on *S. droebachiensis* that sea urchins are able to synthesize highly unsaturated FAs using both elongase and desaturase enzymes (Castell et al., 2004; Cook et al., 2007). Feeding experiments on *Paracentrotus lividus* showed that the amounts of EPA were higher in the gonads of animals fed kelp than those fed the pellet diet even though this PUFA was absent from the kelp diet. Furthermore, the amount of ARA was higher in the gonads than in the diet itself, indicating that this sea urchin has the ability to synthesize these long-chain PUFA (Carboni et al., 2013). However, some PUFAs can also be assimilated from the diet (Cook et al., 2000; Cook et al., 2007). Liyana-Pathirana et al. (2002) suggested that the high content of ARA and EPA in *S. droebachiensis* gonads was possibly derived from the diet, particularly *Laminaria* sp. which has a high proportion of these two PUFAs. Here we found that ARA, EPA and C18:3(n-3) were the dominant PUFAs in *E. chloroticus* gonads and were the FAs involved in the observed differences between diets. With the exception of C18:3(n-3), the rest of these PUFAs were present at higher concentrations in the gonads of sea urchins fed seaweed and those from the wild compared to those fed the manufactured diets; even though they showed no significant differences in the percentages among treatments. It is known that brown seaweeds are characterised by relatively high levels of the PUFAs ARA and EPA (Johns et al., 1979; Kelly et al., 2012; Khotimchenko et al., 2002). Therefore, we can suggest that this sea urchin species is assimilating these essential FAs from the brown seaweeds present in Tory Channel, and incorporating and storing them in the gonads when they are present in high levels in the diet. This suggestion has also been made for *S. droebachiensis* (Liyana-Pathirana et al., 2002).

Additionally, the PUFA C20:2(n-9) also contributed to the differences between the treatments, being present in higher concentration in the gonads of sea urchins from the wild and those fed seaweed compared to those fed the manufactured diets. This PUFA is a pathway between C18:1(n-9) and C20:3(n-9); Δ6 desaturase activity on C18:1(n-9) produces C18:2(n-9), which is elongated to C20:2(n-9) and then Δ5 desaturase converts this in C20:3(n-9) (González-Durán et al., 2008). All of these FAs were present in higher concentrations in the gonads of sea urchins from the wild or fed seaweed compared to those

fed manufactured diets. The precursor of all these FAs is the MUFA C18:1(n-9) which is generally present in relatively high concentrations in brown seaweeds (Kelly et al., 2012). This precursor in present in high concentration in the potential food of *E. chloroticus*, indicating that this sea urchin species is able to particularly accumulate it and then elongate it to produce longer FAs.

Castell et al. (2004) found a correlation between the dietary and tissue lipid content of C18:3(n-3) showing that the higher the content of this PUFA in the diet the higher content in the tissue lipids. This PUFA is considered essential FA, because animals lack of the enzymes to synthesize it and also because it is the precursor of C20:5(n-3) (Castell et al., 2004). Here we found that 18:3(n-3) was present in higher concentrations in the gonads of sea urchins fed manufactured diets than sea urchins from the wild, suggesting that the manufactured diets had a higher concentration of this precursor. We were not able to analyse the FA profile of the manufactured diets, however flaxseed oil, which is a major component (Table 4.1), contains large amounts of ALA. Thus, we hypothesise that *E. chloroticus* is capable of storing this essential PUFA when it is present in high concentration in the diet.

C22:6(n-3) is considered another essential FA because animals in general are not able to synthesize it and need to incorporate it from their diets (Kelly et al., 2012). However, in this study it contributed <0.6% to the total fatty acid content. This fatty acid is present in certain classes of flagellated microalgae (Sargent et al., 1997); however, it has also been associated with carnivorous feeding in sea urchins (Cook et al., 2000; Kharlamenko et al., 1995; Takagi et al., 1986). In diet experiments on *Psammechinus miliaris,* 22:6(n-3) was present in higher concentrations in the gonads of sea urchins fed salmon and mussels than in sea urchins fed seaweed (Cook et al., 2000). Our results are more similar to the findings in feeding experiments on *S. droebachiensis* (Castell et al., 2004; Liyana-Pathirana et al., 2002) where the authors suggested that sea urchins do not have a requirement for high levels of this n-3 PUFA. The low presence of this PUFA in the present study could be explained by these sea urchins being fed manufactured diets high in protein based on soy (Phillips et al., 2010), compared to animal protein; seaweed with a low protein content (Smith et al., 2010) or sea urchins from the wild fed a primarily macroalgal diet (Barker, 2013; Schiel, 1982).

In conclusion, the current study clearly showed the effect of the manufactured diets on the fatty acid profile of *E. chloroticus* gonads and the ability of this species of sea urchin to either store essential fatty acids when they are present in high concentration in the diets or synthesize *de novo* essential fatty acids when they are either absent in the natural diet or

present in very small quantities. Although we were unable to provide the fatty profile of the manufactured diets, the results showed that sea urchins fed seaweed diets and sea urchins from the wild accumulated higher amounts of essential PUFAs. Therefore, using manufactured diets, this study has shown evidence for biosynthesis of several FAs and that some of the FA in the wild sea urchins may be coming from non-seaweed sources. These results are valuable as a variation in the lipid and fatty acid composition of *E. chloroticus* gonads due to different diets may also be a factor in gonad quality/taste. Knowing the importance of the PUFA in the food chain and also in human health, further feeding experiments on *E. chloroticus* using different manufactured diets higher in PUFA content need to be done in order to improve the intake and accumulation of the essential FA in the gonads. The information presented in the current study is valuable and would enable better management of sea urchins in aquaculture.

Chapter 5

Seasonal changes in the lipid composition of some dominant brown seaweed from the Hauraki Gulf, New Zealand.

5.1 Introduction

Large brown macroalgae or Phaeophytes, particularly in the Order Laminariales (true kelps or laminarians) and Fucales (fucoids) are commonly the dominant intertidal and subtidal seaweeds on many temperate and boreal shores, providing biomass, habitat and food for a suite of herbivores, including gastropods and sea urchins (Choat et al., 1982). Marine macroalgae are also one of the living renewable food sources from the oceans, providing beneficial nutrients such as vitamins, trace minerals, amino acids and dietary fibre and lipids; all of which form part of a healthy diet for humans (Dawczynski et al., 2007; Norziah et al., 2000; Tabarsa et al., 2012). Moreover, within the macroalgae, Phaeophyte are the most nutrient rich, with high energy, protein and lipid contents (Kaehler et al., 1996). Brown seaweeds are also used in practical applications as a source of structurally and functionally unique polysaccharides, alginic acids and their salts (alginates), which are widely used in the food-processing industry, and for biotechnology and medicine (Shevchenko et al., 2007). As algae are considered important primary producers in a marine food web (Guschina et al., 2006), knowledge of their chemical composition is important to evaluate potential sources of protein, carbohydrate and lipid to marine invertebrate and vertebrate herbivores (Renaud et al., 2007). Furthermore, seaweeds are the principal source of energy in marine ecosystems because they are the only organisms, together with phytoplankton, that are able to synthesize and accumulate essential long chain fatty acids, being the fatty acids the principal components of lipids (Iverson, 2009; Lee et al., 1998). Consequently, many marine animals, especially herbivores, select their food items in order to maximise the consumption of nutritive components that suit their specific needs and that they are not themselves able to synthesize (Kaehler et al., 1996).

It is well known that the chemical composition (i.e. protein, carbohydrate and especially lipid) of brown seaweeds is influenced by environmental factors such as water temperature, salinity, light and nutrients, and therefore this composition changes seasonally, bathymetrically and latitudinally in different type of habitats in which they grow. (Adams et al., 2011; Gerasimenko et al., 2010; Honya et al., 1994; Marinho-Soriano et al., 2006; Nelson et al., 2002; Renaud et al., 2007; Rodríguez-Montesinos et al., 1991). Additionally, different

seaweed species present dissimilar biochemical compositions, and differences between stipe and the blade have been also found within the same plant (Black, 1950; Renaud et al., 2007). Several researchers around the world have focused on the variation in the lipid composition of different brown algae species between the seasons; nevertheless, not all the species show the same pattern (Gerasimenko et al., 2010; Honya et al., 1994; Nelson et al., 2002). In some algae, like *Macrocystis pyrifera,* the lipid levels increase during winter and decrease in summer (Rodríguez-Montesinos et al., 1991); while, some species show no seasonal variation at all (Renaud et al., 2007). The chemical composition of macroalgae available at different times of the year, may play an important role affecting the fitness of the herbivores that live in those areas (Kaehler et al., 1996). Thus, study of the variation in the chemical composition of ecologically important brown seaweed species is essential to better understand the implications on the herbivores that feed on those species. To our knowledge, no information is available on the chemical composition, particularly lipid composition, of the ecologically important macroalgae from New Zealand, especially their seasonal variability and their potential quality as dietary components through the year.

Large brown macroalgae are generally the dominant organism in the temperate rocky reef around the New Zealand coast, providing food and shelter for many invertebrate and vertebrate herbivores (Andrew, 1986; Babcock et al., 1993; Choat et al., 1986; Novaczek, 1984). Species of the genera *Carpophyllum* and *Cystophora* are the most conspicuous fucaleans, whereas *Ecklonia radiata* is the only common laminarian in the north-eastern New Zealand (Schiel, 1988). Laminariales generally occur subtidally whereas most fucoids are found in the intertidal and very shallow subtidal zones, due to their high light requirements (Schiel et al., 2006). Algal community structure varies greatly around north-eastern New Zealand, with *E. radiata* and *C. maschalocarpum* the most dominant species found at all locations, although some other macroalgae species were also abundant in some locations but not in others (Shears & Babcock, 2004). The variation in the algal community structure was related to different environmental variables like wave exposure and turbidity and also to the presence and density of *Evechinus chloroticus*, which was the most ubiquitous sea urchin in these locations (Shears & Babcock, 2004).

Previous chapters have focused on the analysis of *Evechinus chloroticus* gonads using lipid and fatty acids techniques to better understand the diet of sea urchins in the north-eastern New Zealand, suggesting that this sea urchin species is principally obtaining the lipid nutrients from *E. radiata*. However, other brown species are also present in *E. chloroticus* diet. As mentioned before, the seasonal variation in the chemical composition of the

macroalgae play an important role in the herbivorous community, thus, the current study is focused in the total lipid and also the lipid composition of the most common intertidal macroalgae from north-eastern New Zealand analysed throughout the year. The implications of temporal variation in the lipid composition of these brown seaweed species as food source to *E. chloroticus* are studied.

The particular aim of the present study was to compare the total lipid and lipid composition of the common intertidal marine macroalgae, *E. radiata*, *Carpophyllum plumosum*, *C. maschalocarpum* and *Cystophora torulosa* from north-eastern New Zealand and analyse their seasonal variation. As cited before, the chemical composition as well as the lipid composition is affected by environmental factors like light, temperature and nutrients in the seawater; thus, in this study these possible influences were minimized by sampling in the same location during different seasons of the year. The knowledge of the variation in the lipid composition of the dominant brown seaweed species is of importance for the understanding of the algal physiological adaptation to a varying environment as well as the possible implications on the principal grazer, *E. chloroticus*.

5.2 Methodology

5.2.1 Sample collection and storage

Ecklonia radiata (blade), *Ecklonia radiata* (stipe), *Cystophora torulosa*, *Carpophyllum maschalocarpum* and *Carpophyllum plumosum* were collected from Matheson's Bay (36° 18.1' S, 174° 48.65' E) in the Hauraki Gulf, New Zealand. Seaweeds were collected at the end of March, June, September and December, corresponding to austral autumn, winter, spring and summer, respectively. Five individual seaweeds of each species were manually harvested at low tide from depths of one metre or less by cutting the fronds at the base. *E. radiata* consists of a solid cylindrical stipe and a thick blade lamina and it attaches to the substratum by multiple haptera (Babcock et al., 1993; Novaczek, 1984). Because the two parts are very distinguishable, *E. radiata* was divided in the field into stipe and blade and they were considered as different "species" for the lipid analysis. Algal samples (~20 g wet weight) were placed in individually labelled polypropylene 50 ml tubes. The tubes were frozen in liquid nitrogen in the field and transported in liquid nitrogen to the laboratory to prevent biochemical deterioration. Frozen algal samples were stored in the -80 freezer before they were lyophilized to a constant mass in a freeze-dryer (VirTis Bench Top 2k). The samples were cryogenically grounded using an MM301 Mixer Mill (Retsch), which

resulted in a ground particle size of <5µm (Retsch). Lyophilized, ground samples were stored in sealed 10ml tubes under desiccant at -20°C until lipid extraction.

5.2.2 Lipid analysis

Lipid determination was carried out using an Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods following the protocols defined by Parrish (1997, 1999) with minor modifications. Freeze-dried tissue from five different individuals of each seaweed species was weighed (~17 mg) into 1ml V-vials (Wheaton). Lipid extraction was performed as described in Sewell (2005) with the following modification: 250 µl of ultrapure water, 25 µl of ketone in chloroform (used as internal standard as natural concentrations are low in marine tissues), 100 µl chloroform and 250 µl methanol (final ratio of water: chloroform: methanol 2:1:2) were added to the V-vial before 20 minutes of sonication on ice in an ultrasonication bath (Sanyo Soniprep 150). After vigorous shaking, the V-vials were centrifuged at 2000 RPM for 5 minutes at room temperature. Both the aqueous and chloroform fractions were transferred with a drawn Pasteur pipette to a clean glass V-vial, leaving the solid non-lipid material behind. An additional 250 µl water and 250 µl chloroform were added and the vial was shaken for a further 1 to 2 minutes. Subsequently, the sample was re-centrifuged at 2000 RPM for 5 minutes at room temperature. Most (90%) of the upper water and methanol fraction was removed with a Pasteur pipette and discarded, with little disturbance to the interface between the aqueous and chloroform layers. The lower chloroform layer (ca. 375 µl = [100 + 250 added in extraction] + 25 µl from internal standard) was transferred to a third V-vial using a 200 µl Eppendorf pipette with a chloroform-rinsed tip. This V-vial, containing the lipids suspended in chloroform, was again stored in a -20°C freezer until used for the Iatroscan analysis. All V-vials used in the extraction process were cleaned with 3x methanol and 3x chloroform washes as recommended by Parrish (1999), and all solvents used in lipid extraction were HPLC-grade. Immediately before spotting onto the Chromarods, the lipid extract was dried down in a stream of instrument grade nitrogen gas and 25 µl of chloroform was added using a Gilson positive displacement pipette. Four separate seaweed samples were processed on each run (2 replicate Chromarods of each sample; total 8 rods). The remaining two Chromarods were unspotted blanks to test for contamination of the development solvents.

Chromarods were developed as described in Parrish (1999), except that instead of a triple development process, we used a double development system that resulted in two chromatograms (for an example see Fig. 5.1). After spotting, the rack of Chromarods was

placed inside a constant humidity chamber (CHC) for 5 minutes and then transferred to the first development tank, (69.3 ml of Hexane, 0.7 ml of Diethyl-ether and 0.035 ml of Formic Acid; 98.95:1:0.05) for 24 minutes. After this, the rack was placed again for 5 minutes in the CHC before returning to development tank 1 for a further 19 minutes. The Chromarods were dried for 5 minutes in a Rod Dryer TK-8 (Iatron Laboratories) at 60°C and were then run in the Iatroscan set to Partial Scan Mode (PPS 27). After recording the first chromatogram, the rack was placed in the CHC for 5 minutes followed by 33 minutes in development tank 2 (55.3 ml of Hexane, 14 ml of Diethyl-ether and 0.7 ml of Formic Acid; 79:20:1). The Chromarods were dried for 5 minutes at 60°C and then they were run in the Iatroscan using a full 30 second scan and settings of 2000ml min^{-1} O_2 and 160 ml min^{-1} H_2 settings as in Sewell (2005).

Quantification of the lipid in the sample was based on multilevel calibration curves generated for each lipid class found in the samples, plus the internal standard, on the rack of 10 Chromarods. Rods were calibrated with a 10-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform. Lipid classes were: Aliphatic Hydrocarbon (AH: Nonadecane), Wax Ester (WE: Miristyl dodecanoate), Free aliphatic alcohol (FALC: Cetyl alcohol), Ketone (KET: 3- hexadecanone), Triacylglycerol (TAG: tripalmitin), Free Fatty Acid (FFA: Palmitic acid), Sterols (ST: cholesterol), Diacylglycerol (DAG: 1,2 Dipalmitoyl-rac- glycerol), acetone-mobile polar lipids (AMPL: 1-monopalmitoyl-rac-glycerol) and Phospholipid (PL: L-α-phosphoditylcholine). AMPL includes glycolipids, pigments and any remaining neutral lipids from the PL and is normally separated in the acetone third development (Parrish, 1987) which was not used here. However, the second development separated a peak from PL that confirmed the presence of possible glycolipids and monoacylglycerols when spiked with the AMPL standard 1-monopalmitoyl-rac-glycerol (Fig. 5.1).

The stock solution of purified lipids was prepared using 5 mg of each lipid class dissolved in 1000 µl of chloroform using a Gilson Microman positive displacement pipette. Five serial dilutions were prepared from the initial stock solution, giving a total of six different dilutions of the lipid standard, that covered the range of lipid concentrations found in the seaweed. The dilutions were run in the Iatroscan using the same methodology as was used for the samples. Quadratic regressions were used for lipid quantification as the FID response (Y-axis in a chromatogram) is curved at low concentrations (< 1 to 5 µg) (Sewell 2005). Peak areas from the calibration curves were estimated from the mean of 3 separate Chromarods (r^2> 0.9968 for all lipid classes).

Lipid quantification on each sample was performed including four technical replicates, consisting of two extractions of the same sample which were spotted in two separate rods in the Iatroscan. Sample concentration of each lipid class was calculated as the arithmetic mean of the four technical replicates. The concentration of each lipid class technical replicate was determined using the percentage recovery of the internal standard in each sample and the calibration curve appropriate for each lipid class on each rack of Chromarods. Total lipid was calculated by the sum of the concentration of each lipid class (AH, WE, FALC, TAG, FFA, DAG, ST, AMPL, and PL) for each sample. Lipid classes can be grouped into energy storage and structural lipids. The concentration of energy storage was calculated by summing the concentration of TAG, DAG, FFA, AH, FALC and WE; whereas the concentration of structural lipids was calculated by summing the concentration of PL, ST and AMPL. Values are presented as the mean (± SE) of five samples (n=5) per seaweed species in units of μg lipid per mg of freeze-dried sample.

5.2.4 Statistical analysis

Lipid profiles were compared between brown seaweed species and seasons using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). The data (concentration of each lipid class, μg/mg dry weight) was left untransformed and converted into resemblance matrices using Bray-Curtis dissimilarity coefficients, as used by Kelly et al. (2009) and Crawley et al. (2009). Statistical analysis using Euclidean distance was also performed, and produced the same results. Dissimilarity patterns in the data were visualised using multidimensional scaling (MDS). Two-way PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the Permutation Method, was performed to examine differences in the lipid profile between brown seaweed species and seasons. Pair-wise comparisons were then conducted to compare significant differences in the lipid profile between seaweed species and seasons. The similarity percentages procedure (SIMPER) was used to determine which lipid classes contributed most to the differences between species in the multivariate signature. Univariate one-way PERMANOVA was used to test the differences of the total lipid, structural lipid and energy lipid and the concentration of each of the lipid classes that contributed to the differences between species and seasons. Pair-wise multiple comparisons were performed when significant differences ($p<0.05$) were found. This approach was used as this test avoids the assumptions of the traditional one-way analysis of the variance or ANOVA (Underwood, 1997) and only assumes that the samples are exchangeable, i.e. independent and identically distributed, under a true hypothesis (Anderson, 2003).

5.3 Results

5.3.1 General lipid profile

A total of nine lipid classes were detected in the brown seaweed species collected from northeast New Zealand. The structural lipids were dominant and present in all brown seaweeds (~83-97% of total lipid) and included sterols (ST), acetone mobile polar lipids (AMPL) and phospholipids (PL) (Table 5.1; Fig. 5.2). In contrast, energy lipids were present in only small or trace concentrations (~2-16% of total lipid) and included aliphatic hydrocarbon (AH), wax ester (WE), free alcohol (FALC), triacylglycerol (TAG), free fatty acid (FFA) and diacylglycerol (DAG) (Table 5.1; Fig. 5.2). However, not all the energy lipid classes were detected in all the brown seaweed species (Table 5.1).

Table 5.1. Concentration (µg/mg of dry weight) of lipid classes in brown algae species during spring. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as energy lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

	Cystophora torulosa	***Ecklonia radiata* (blade)**	***Ecklonia radiata* (stipe)**	***Carpophyllum plumosum***	***Carpophyllum maschalocarpum***
ST	86.24 ± 6.38	3.43 ± 0.58	3.01 ± 0.32	4.07 ± 0.36	2.73 ± 0.37
AMPL	4.56 ± 0.85	2.62 ± 0.60	1.09 ± 0.19	1.31 ± 0.34	1.32 ± 0.43
PL	22.19 ± 1.89	29.88 ± 6.94	24.42 ± 2.24	24.59 ± 2.2	21.52 ± 1.48
Structural Lipids %	83.37	93.67	96.13	96.66	97.92
AH	7.14 ± 1.22	ND	-	ND	ND
TAG	1.65 ± 0.37	2.16 ± 0.96	0.83 ± 0.24	0.83 ± 0.14	0.54 ± 0.12
FFA	ND	0.27 ± 0.17	0.32 ± 0.43	ND	ND
DAG	1.60 ± 0.40	ND	-	0.21 ± 0.12	ND
WE	0.11 ± 0.01	ND	-	ND	ND
FALC	11.76 ± 4.2	ND	-	ND	ND
Energy Lipids %	16.42	6.33	3.87	3.34	2.08
Total lipid	135.52 ± 13.42	38.36 ± 6.9	29.67± 2.29	31.01 ± 2.26	26.11 ± 1.83

Total lipid differed among species, when all the seasons were combined (Table 5.2.A) and using only a single season (spring) (Table 5.2.B). The same result was obtained when the concentration of energy lipid and structural lipid were analysed separately, including all the seasons (Table 5.2.C.E) and just spring (Table 5.2.D.F).

Table 5.2. Results of Univariate one-way PERMANOVA test of brown seaweed species comparing: A) total lipid across all the seasons, B) total lipid during spring, C) energy lipid across all the seasons, D) energy lipid during spring, E) structural lipid across all the seasons and F) structural lipid during spring. Significant results ($p>0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	60013	15003	493.60	**0.0001**	99955
Residual	95	2887.5	30.40			
Total	99	62900				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	16271	4067.70	135.95	**0.0001**	9941
Residual	20	602.86	30.14			
Total	24	16874				

C)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	156000	38995	126.72	**0.0001**	9936
Residual	95	29234	307.73			
Total	99	185000				

D)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	36542	9135.50	21.9	**0.0001**	9937
Residual	20	8356.20	417.81			
Total	24	44898				

E)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	52072	13018	360.71	**0.0001**	9955
Residual	95	3428.50	36.09			
Total	99	55501				

F)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	14030	3507.40	101.73	**0.0001**	9945
Residual	20	689.58	34.48			
Total	24	14719				

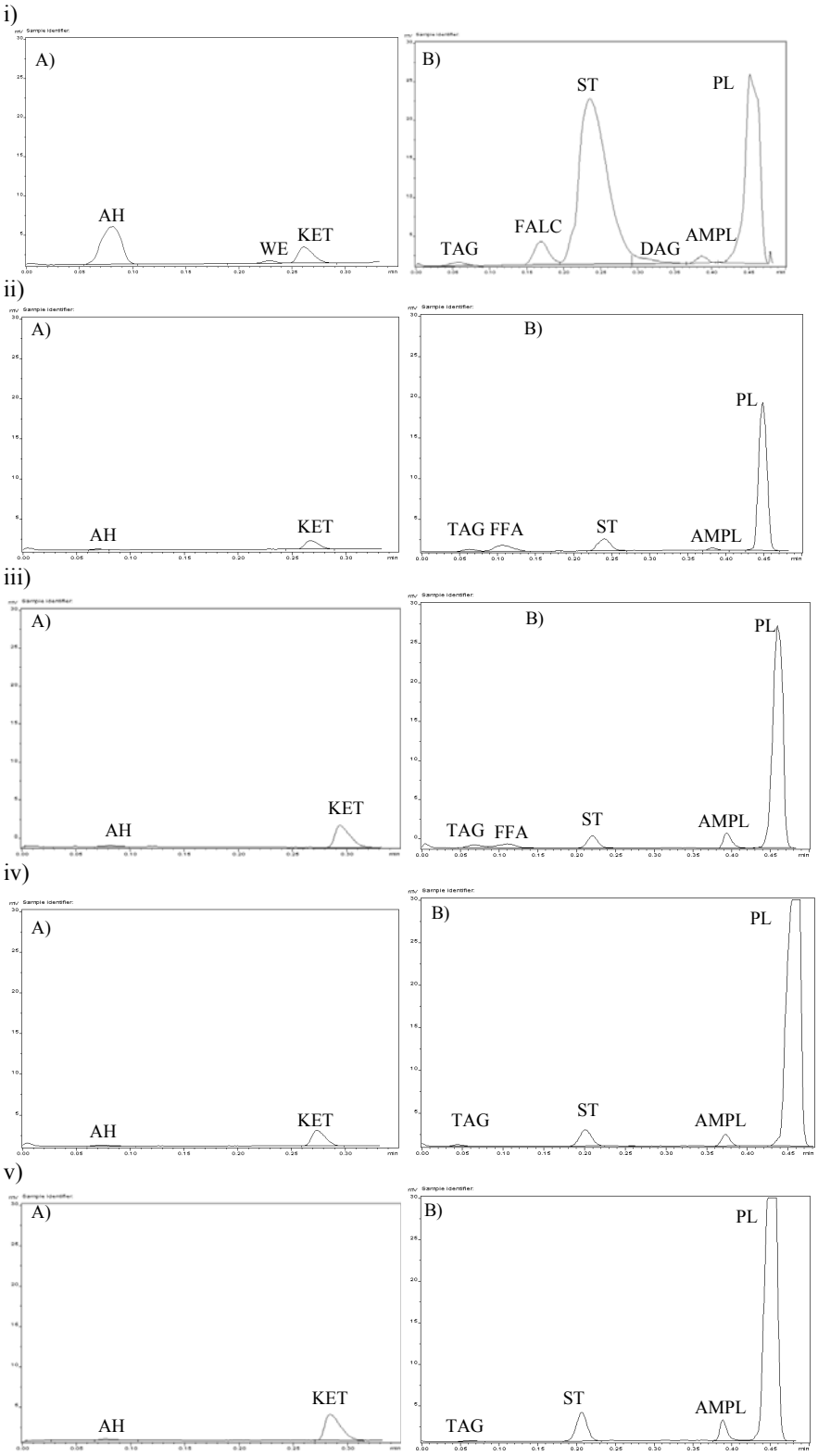


Figure 5.1. Representative TLC/FID chromatograms of lipid extracted from the brown seaweed collected in Matheson's Bay i) *Cystophora torulosa*, ii) *Ecklonia radiata* (blade), iii) *Ecklonia radiata* (stipe), iv) *Carpophyllum plumosum*, v) *Carpophyllum maschalocarpum*. The 2 panels (A-B) are the chromatograms resulting from the 2 scans of the Chromarods: (A) Hexane: diethyl ether: formic acid (98.95:1:0.05), 24 min, 5 min dry, and redeveloped for 19 min. Partial scan on Iatroscan (PPS27). (B) Hexane: diethyl ether: formic acid (79:20:1) for 33 min. Full scan. X-axis is time (s); y-axis shows FID response on the same relative scale. AH: aliphatic hydrocarbon; WE: Wax Ester; FALC: free aliphatic alcohol; KET: ketone (added as internal standard); TAG: triacylglycerol; FFA: Free Fatty acid; ST: cholesterol; DAG: diacylglycerol; AMPL: acetone-mobile polar lipids; PL: phospholipid.

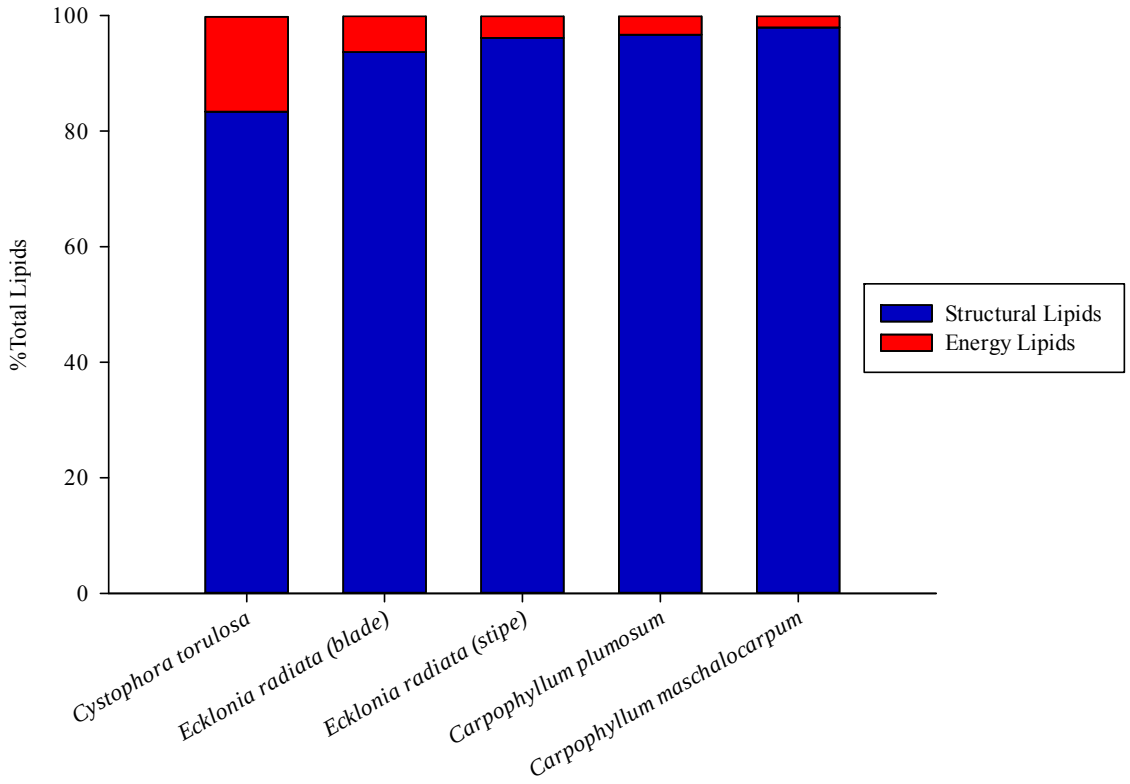


Figure 5.2. Comparison between Energy lipids (AH, TAG, FFA, WE, FALC) and Structural lipids (ST, AMPL, PL) of 4 different brown seaweed species: *Cystophora torulosa*; *Ecklonia radiata* (blade)*; Ecklonia radiata* (stipe)*; Carpophyllum plumosum*; *Carpophyllum maschalocarpum.*

Two-way PERMANOVA analysis showed a significant interaction term indicating a different seasonal variation in the different species (Table 5.2.A). Additionally, lipid profiles of the brown seaweeds were significantly different between species, but also among seasons (Table 5.2.A.B). The strong interaction term suggested analysing these two factors (species and season) independently, studying the variation of one factor (species) at one level of the other factor (season). Variation between species was analysed at spring as this was the season with the most lipid classes present, and is the period of reproductive activity for most of these species (Dunmore, 2006; Novaczek, 1984; Schiel, 1988).

An MDS plot of all seaweed species across the four seasons showed only two clusters, separated by a large distance (Fig. 5.3.A). The left hand point included *C. torulosa*, with the other species overlapping on the right hand side of the plot (Fig. 5.3.A). To reveal differences in lipid profile between *E. radiata* (blade), *E. radiata* (stipe), *C. plumosum* and *C. maschalocarpum,* a second MDS analysis was conducted excluding *C. torulosa* (Fig. 5.3.B) also showing differences among the rest of the seaweed species. The pair-wise comparisons revealed significant differences in the lipid profile between all the seaweed species (Table 5.2.C).

Table 5.3. Results of two-way PERMANOVA and Pair-wise comparisons comparing lipid profile between brown seaweed species and season in Matheson's Bay during 2011. A) Five seaweed species with *Cystophora torulosa* included in the analysis. B) Four seaweed species, *Cystophora torulosa* was excluded from the analysis. C) Pair-wise comparisons, all the species included in the analysis. Significant ($p<0.05$) are showed in bold

A)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	4	68860	17215	411.35	**0.0001**	9951
Season	3	475.33	158.44	3.79	**0.0103**	9951
Species x Season	12	1242.70	103.56	2.47	**0.0063**	9908
Residual	80	3348	41.85			
Total	99	73926				

B)

Source of Variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	3	3283.90	1094.60	26.11	**0.0001**	9942
Season	3	372.24	124.08	2.96	**0.0157**	9954
Species x Season	9	996.85	110.76	2.64	**0.0017**	9912
Residual	64	2683.10	41.92			
Total	79	7336.10				

C)

Seaweed species	t	P(perm)	Unique perms
C. plumosum, C. torulosa	32.42	**0.0001**	9919
C. plumosum, E. radiata (blade)	3.09	**0.0001**	9954
C. plumosum, C. maschalocarpum	5.19	**0.0001**	9950
C. plumosum, E. radiata (stipe)	6.52	**0.0001**	9954
C. torulosa, E. radiata (blade)	27.41	**0.0001**	9923
C. torulosa, C. maschalocarpum	33.40	**0.0001**	9928
C. torulosa, E. radiata (stipe)	33.70	**0.0001**	9926
E. radiata (blade), *C. maschalocarpum*	5.98	**0.0001**	9962
E. radiata (blade), *E. radiata* (stipe)	5.89	**0.0001**	9936
C. maschalocarpum, E. radiata (stipe)	2.64	**0.0050**	9953

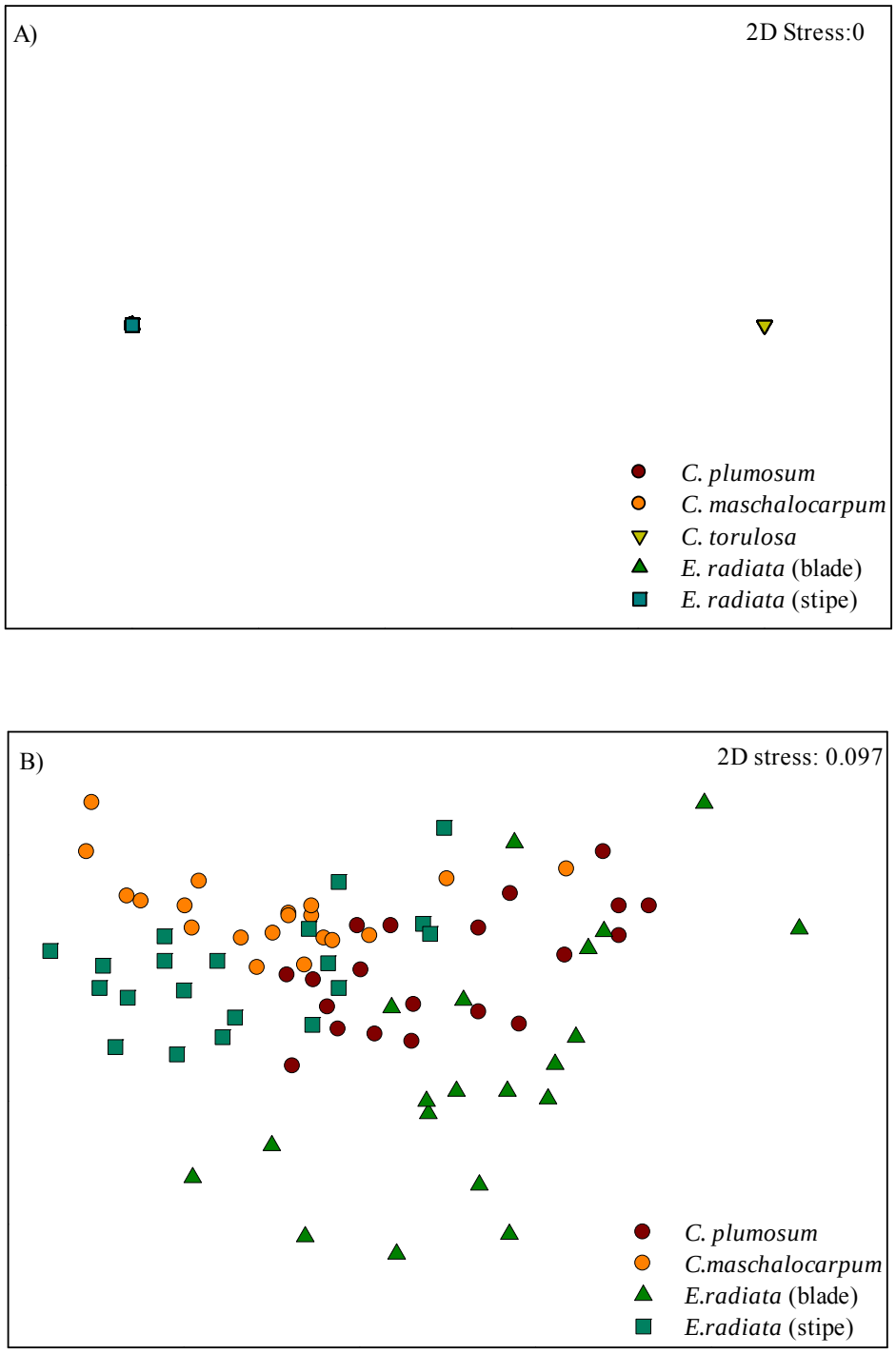


Figure 5.3. Multidimensional scaling (MDS) plot of Bray-Curtis similarities of lipid profile of brown seaweed species: *Cystophora torulosa*; *Ecklonia radiata* (blade); *Ecklonia radiata* (stipe); *Carpophyllum plumosum* and *Carpophyllum maschalocarpum.* A) *Cystophora torulosa* was included in the analysis and B) *Cystophora torulosa* was excluded in the analysis to show differences among remaining algae. All seasons were included in this analysis.

5.3.2-Lipid profile between species

C. torulosa had the highest concentration of total lipid during all the seasons (>110 µg/mg of dry mass) and the highest percentage of energy lipids (~12-16% of total lipid) of the seaweed species (Table 5.1). *E. radiata* (blade) had a smaller concentration of total lipid (~34-38 µg/mg of dry mass) and the energy lipids were also present in smaller percentage that *C. torulosa* (~4-11% of total lipid, Table 5.1). In the case of *E. radiata* (stipe), the total lipid was smaller than *E. radiata* (blade) (~26-29 µg/mg of dry mass). Following in the concentration of total lipid was *C. plumosum*, with ~31-36 µg/mg of dry mass and the percentage of the energy lipids was also small, just ~1-3% of total lipid. *C. maschalocarpum* had the smallest concentration of total lipid (~24-29 µg/mg of dry mass) and the lowest percentage of energy lipids, ~1-2% of the total lipid of the brown seaweed species analysed in the present study (Table 5.1).

Differences in the lipid profiles between species were driven by the presence and concentrations of the energy lipids. *C. torulosa* showed a completely different lipid profile, with 5 classes of energy lipids (AH, WE, TAG, FALC and DAG) and ST, AMPL and PL as structural lipids (Fig. 5.1.i; Fig. 5.4.A). In contrast, the lipid profile of *E. radiata* (blade and stipe) consisted of 6 different lipid classes in total: AH, TAG and FFA as energy lipids and ST, AMPL and PL as structural lipids (Fig. 5.1.ii-iii; Fig. 5.4.B-C). *C. plumosum* and *C. maschalocarpum* contained the same 5 lipid classes (AH and TAG as energy lipids and ST, AMPL and PL as structural lipids, Fig. 5.1.iv-v; Fig. 5.4.D-E), but varied in the concentrations of each class (Table 5.1).

SIMPER analysis showed that two lipid classes contributed to the differences between *C. torulosa* and the rest of the seaweed species (Table 5.4): one structural lipid, ST, and the energy lipid FALC. The concentration of ST was significantly different between species when all the seasons were combined (Pseudo-$F_{4,95}$=1166.3, P(perm)=0.0001), and also when just spring was analysed (Pseudo-$F_{4,20}$=829.74, P(perm)=0.0001). *C. torulosa* had the highest concentration of ST (~80µg/mg of dry mass) and it was the only species where this concentration was higher than PL (~20µg/mg of dry mass). The concentration of ST in the rest of the seaweed species was much smaller (<5µg/mg of dry mass) being present in relatively in higher concentration (~4µg/mg of dry mass) in *C. plumosum*, compared with the concentration in *E. radiata* (stipe) and *C. maschalocarpum* (~3µg/mg of dry mass in both species) (Table 5.1; Table 5.4).

FALC also contributed to the differences between species as this lipid class was only detected in *C. torulosa*, being the most abundant among the energy lipids in this species (~11µg/mg of dry mass). The rest of the energy lipids in *C. torulosa* were present in smaller concentrations, less than 8µg/mg of dry mass (Table 5.1).

In contrast, the lipid classes that contributed to the difference in the lipid profile between *E. radiata* (blade), *E. radiata* (stipe), *C. plumosum* and *C. maschalocarpum* were the structural lipids PL and AMPL, and the energy lipids TAG and FFA (Table 5.4). PL was the lipid class present in the highest concentration in these species, and differed between species when all the seasons were analysed (Pseudo-$F_{3,76}$=10.54, P(perm)=0,0001), and during the spring (Pseudo-$F_{3,16}$=4.02, P(perm)=0.02). Pairwise comparisons showed that the concentrations of PL were statistically higher in *E. radiata* (blade) (>24g/mg of dry mass) than *E. radiata* (stipe) (~20µg/mg of dry mass) and *C. maschalocarpum* (~22µg/mg of dry mass). Additionally, PL was higher in *C. plumosum* (~25µg/mg of dry mass) than *C. maschalocarpum* (Table 5.1; Table 5.4). However, the pairwise comparison showed no significant difference in the concentration of this structural lipid between *E. radiata* (blade) and *C. plumosum,* or between *E. radiata* (stipe) and *C. maschalocarpum* (Table 5.1; Table 5.4).

The other structural lipid that contributed to the difference in the lipid profile of *E. radiata* (blade), *E. radiata* (stipe), *C. plumosum* and *C. maschalocarpum* was AMPL. This class of lipid was found to be significantly different between species when all the seasons were analysed (Pseudo-$F_{3,76}$=41.73, P(perm)=0.0001) and also during spring (Pseudo-$F_{3,16}$=13.75, P(perm)=0.001). Pairwise comparisons showed that this structural lipid was higher in *E. radiata* (blade) (~3µg/mg of dry mass) than *C. plumosum* (~2µg/mg of dry mass), as well as *C. maschalocarpum* (~1.5±0.43µg/mg of dry mass) and *E. radiata* (stipe) (~1µg/mg of dry mass). *C. plumosum* had a higher concentration of AMPL than *C. maschalocarpum* and *E. radiata* (stipe) (Table 5.1; Table 5.4).

The last lipid class that contributed to the differences between *E. radiata* (blade), *E. radiata* (stipe), *C. plumosum*, and *C. maschalocarpum* was TAG. This energy lipid was found to be statistically different among species using all the seasons together (Pseudo-$F_{3,76}$=21.77, p=0.0001) and also during spring (Pseudo-$F_{3,16}$=10.49, p=0.0022). Pairwise results showed that *E. radiata* (blade) had a higher concentration of TAG (>2µg/mg of dry mass) than *E. radiata* (stipe), *C. plumosum* and *C. maschalocarpum* (<1.5µg/mg of dry mass). *C. plumosum*

had a smaller concentration (~0.8µg/mg of dry weight) of this energy lipid than *E. radiata* (stipe) (~1µg/mg of dry weight) but a bit higher than *C. maschalocarpum* (~0.54µg/mg of dry weight) (Table 5.1; Table 5.4).

The last energy lipid contributing to the differences between species was FFA and its presence distinguished *E. radiata* (stipe) with an small concentration of ~0.5µmg of dry mass and *C. maschalocarpum*, where this lipid class was not detected (Pseudo-$F_{3,76}$=32, P(perm)=0.001) (Table 5.1; Table 5.4).

Table 5.4. Contribution of individual lipid class to multivariate differences in lipid profile between brown seaweed species (*E. radiata* (blade), *E. radiata* (stipe), *C. plumosum*, *C. maschalocarpum* and *C. torulosa*) as determined by SIMPER. t, P(perm) and Unique perms were obtained from PERMANOVA on a single factor for each lipid class and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

Species: Groups 1 & 2	Average dissimilarity	Lipid class	Average Value Group 1	Average Value group 2	Average Dissimilarity	Dissimilarity/ SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
C. torulosa & E. radiata (blade)	63.55	ST	77.99	3.79	46.68	14.29	73.45	73.45	34.14	**0.0001**	9788
		FALC	10.88	ND	6.85	4.21	10.78	84.24	17.30	**0.0001**	9745
C. torulosa & E. radiata (stipe)	66.65	ST	77.99	2.96	50	16.11	75.02	75.02	34.58	**0.0001**	9777
		FALC	10.88	ND	7.26	4.19	10.89	85.91	17.30	**0.0001**	9749
C. torulosa &C. plumosum	63.55	ST	77.99	4.24	47.32	13.96	74.46	74.46	34.54	**0.0001**	9774
		FALC	10.88	ND	6.99	4.14	11	85.46	17.30	**0.0001**	9757
C. torulosa & C. maschalocarpum	66.13	ST	77.99	3.05	50.06	15.27	75.7	75.7	34.54	**0.0001**	9780
		FALC	10.88	ND	7.28	4.11	11.01	86.71	17.30	**0.0001**	9733
E. radiata (blade) & E. radiata (stipe)	15.6	PL	26.46	21.74	8.31	1.74	53.25	53.25	4.23	**0.0005**	9854
		AMPL	3.1	1.05	3.25	2.25	20.83	74.08	10.17	**0.0001**	9846
		TAG	2.02	1.03	1.89	1.06	12.13	86.2	3.38	**0.001**	9857
E. radiata (blade) & C. plumosum	12.33	PL	25.67	26.46	6.46	1.44	52.41	52.41	0.65	0.5283	9840
		TG	0.66	2.02	2.01	1.15	16.3	68.71	4.84	**0.0001**	9857
		AMPL	2.41	3.1	1.41	1.31	11.46	80.18	2.61	**0.0121**	9823
E. radiata (blade) & C. maschalocarpum	16.26	PL	26.46	22.04	8.5	1.36	52.32	52.32	3.82	**0.0004**	9829
		AMPL	3.1	1.43	2.67	1.96	16.44	68.76	7.74	**0.0001**	9848
		TAG	2.02	0.46	2.47	1.27	15.22	83.97	5.54	**0.0001**	9868
E. radiata (stipe) & C. plumosum	13.67	PL	21.74	25.67	7.47	1.32	54.67	54.67	4.11	**0.0003**	9826
		AMPL	1.05	2.41	2.29	1.67	16.74	71.41	7.10	**0.0001**	9835
		ST	2.96	4.24	2.13	1.95	15.57	86.98	8.16	**0.0001**	9824
E. radiata (stipe) & C. maschalocarpum	9.6	PL	21.74	22.04	5.69	1.37	59.29	59.29	0.34	0.7436	9855
		TAG	1.03	0.46	1.06	1.39	11.08	70.36	5.99	**0.0001**	9821
		FFA	0.55	ND	1.01	1.49	10.57	80.93	6.69	**0.0001**	9460
C. plumosum & C. maschalocarpum	11.35	PL	25.67	22.04	7.04	1.4	62.03	62.03	3.62	**0.0014**	9827
		ST	4.24	3.05	2	2.15	17.66	79.69	7.59	**0.0001**	9827
		AMPL	2.41	1.43	1.79	1.62	15.79	95.48	4.75	**0.0002**	9830

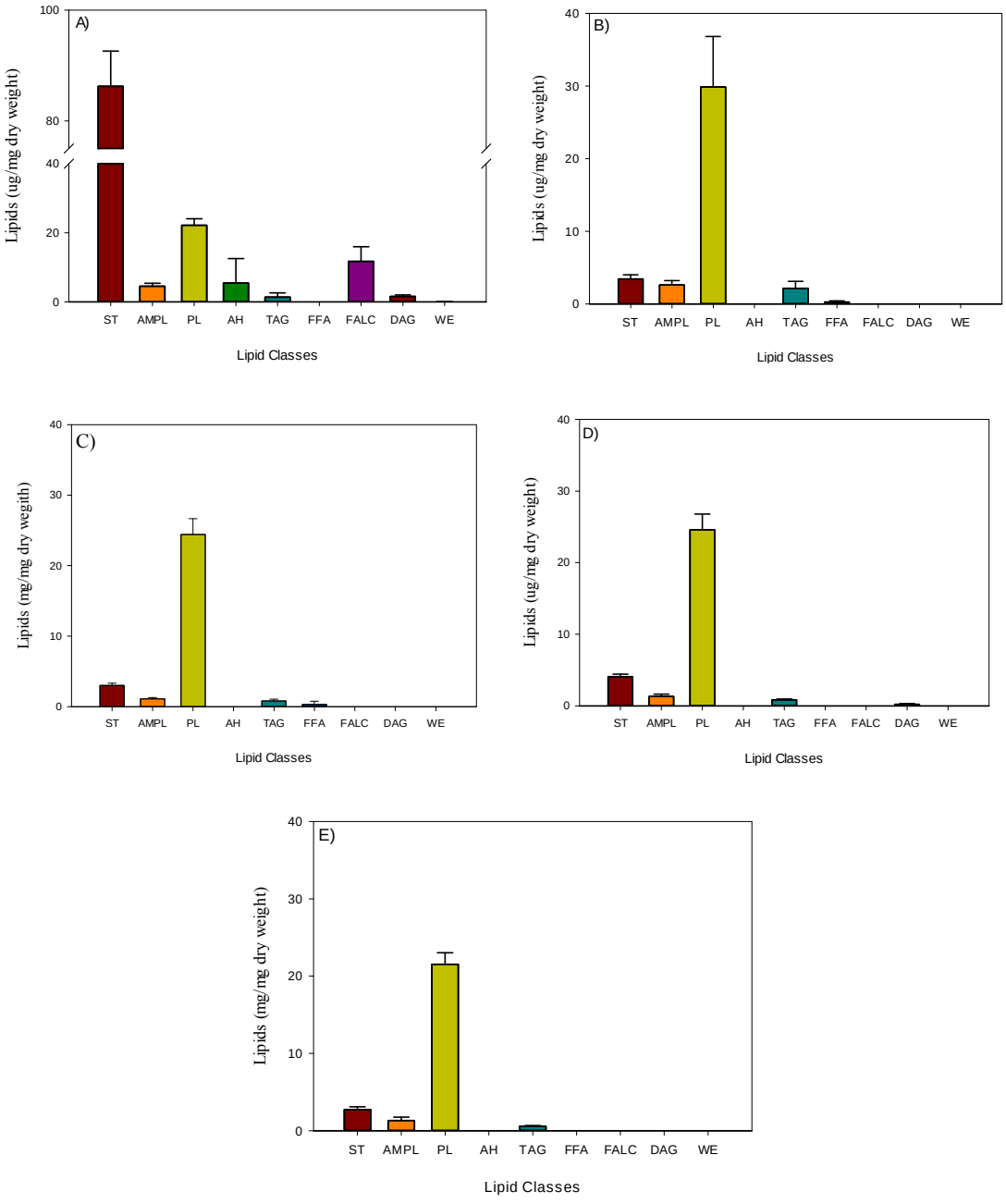


Figure 5.4. Lipid Profile of five seaweeds during spring: A) *Cystophora torulosa*, B) *Ecklonia radiata* (blade), C) *Ecklonia radiata* (stipe), D) *Carpophyllum plumosum* and E) *Carpophyllum maschalocarpum*. Lipids classes are shown on the x axes. Free Sterol (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Free alcohol (FALC), Diacylglycerol (DAG) and Wax Ester (WE) as energy lipids. Mean values of five different individuals (±SE) are shown.

5.3.3-Lipid profile among seasons per brown seaweed species

The significant interaction term in the two-way PERMANOVA (Table 5.2) led this study to conduct the seasonal analysis separately for each seaweed species.

Total lipid in *C. torulosa* was found to be statistically different among seasons (Table 5.6.A; Fig. 5.6.A). Pairwise multiple comparison analysis showed that spring and autumn had a higher concentration of total lipid than summer and winter (Table 5.5; Table 5.6.B; Fig. 5.6.A). When a comparison was made on the concentration of the energy lipids, significant differences were not found among the seasons (Pseudo-$F_{3,16}$=1.55, p=0.228). However, when the concentration of structural lipids was compared among seasons, significant differences were found (Pseudo-$F_{3,16}$=8.69, p=0.001). Multiple pairwise comparisons showed that during summer the concentration of structural lipids was smaller than the concentration in winter (t=2.21, p=0.027), spring (t=4.18, p=0.016) and autumn (t=3.52, p=0.02) and also the concentration in winter was significantly smaller than the concentration during spring (t=3.57, p=0.018) (Table 5.5).

Table 5.5. Concentration (µg/mg of dry weight) of lipid classes of *C. torulosa* among seasons. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as Structural Lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as Energy Lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

Lipid Class	Autumn	Winter	Spring	Summer
ST	79.93±14.46	74.63±1.78	86.24±6.38	71.18±5.60
AMPL	10.26±16.45	3.86±0.42	4.56±0.85	3.91±0.96
PL	23.82±5.77	21.77±0.61	22.19±1.89	18.01±1.13
Structural Lipids	114.01±11.53	100.27±1.93	112.99±7.98	93.10±6.71
AH	4.51±2.01	5.28±0.62	7.14±1.23	4.82±0.30
TAG	1.77±0.44	1.04±0.24	1.65±0.37	1.12±0.13
FFA	ND	ND	ND	ND
DG	1.17±0.34	1.22±0.13	1.60±0.40	1.11±0.63
WE	0.12±0.05	0.11±0.01	0.11±0.01	0.10±0.01
FALC	9.06±1.79	10.93±1.54	11.76±4.20	11.75±2.88
Energy Lipids	16.63±3.91	18.59±1.92	22.26±5.47	18.89±2.75
Total Lipid	130.64±9.03	118.85±3.69	135.52±13.42	111.99±6.24

Table 5.6. Results of total lipid of *C. torulosa* A) Univariate one-way PERMANOVA test and B) Pairwise comparisons among seasons. Significant results (p>0.05) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Season	3	274.02	91.34	7.70	**0.0016**	9950
Residual	16	189.90	11.87			
Total	19	463.92				

B)

Seasons	t	P(perm)	Unique perms
autumn, winter	2.67	**0.0403**	125
autumn, spring	0.63	0.5477	126
autumn, summer	3.75	**0.0217**	126
winter, spring	2.75	**0.0230**	126
winter, summer	2.10	0.0951	126
spring, summer	3.67	**0.0091**	126

The lipid profile of *C. torulosa* also showed significant differences among seasons (Table 5.7.A), as evident in the separation in multidimensional space in the MDS plot (Fig. 5.5.A). Pair-wise comparisons revealed that the lipid profile in winter was significantly different to the lipid profile during spring and summer, and also spring and summer had a statistically different lipid profile (Table 5.7.B).

Table 5.7. Results of lipid profile of *C. torulosa* A) multivariate one-way PERMANOVA analysis and B) Pairwise comparisons among seasons. Significant results (p>0.05) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Season	3	790.72	263.57	2.90	**0.0341**	9945
Residual	16	1455.70	90.98			
Total	19	2246.40				

B)

Seasons	t	P(perm)	Unique perms
autumn, winter	0.85	0.4883	126
autumn, spring	0.97	0.4422	126
autumn, summer	1.42	0.1639	126
winter, spring	3.14	**0.0163**	126
winter, summer	1.66	**0.0274**	126
spring, summer	3.42	**0.0102**	126

SIMPER analysis revealed that the lipid classes that contributed the most to these differences were two structural lipids (ST and PL) and one energy lipid (FALC) (Table 5.8.A). In the case of PL, significant differences were found in its concentration among seasons (Pseudo-$F_{3,16}$=3.12, p=0.0333). Pairwise multiple comparison revealed that summer had significant smaller concentration of PL (~18µg/mg of dry mass) than winter, spring and autumn (>21µg/mg of dry mass) (Table 5.5; Table 5.8.A). However, the concentration of ST was not significantly different among seasons (Pseudo-$F_{3,16}$=3.03; p=0.0611) and no significant differences were found in the concentration of FALC either among seasons (Pseudo-$F_{3,16}$=1.03; p=0.4079) (Table 5.5).

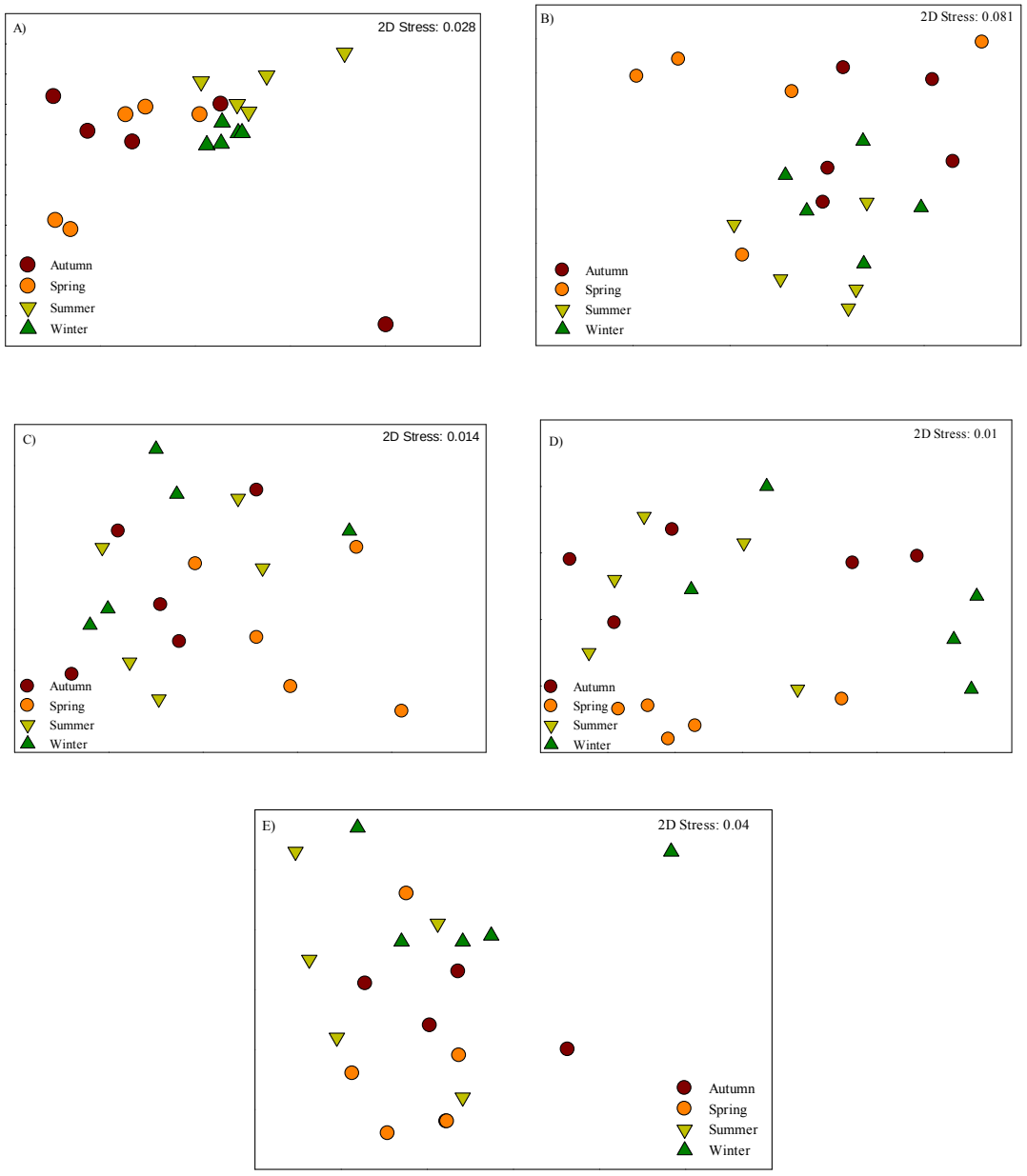


Figure 5.5. Multidimensional scaling (MDS) plot of Bray Curtis similarity for the lipid profile among seasons of brown seaweed species: A) *C. torulosa,* B) *E. radiata* (blade), C) *E. radiata* (stipe), D) *C. plumosum* and E) *C. maschalocarpum*.

Table 5.8. Contribution of individual lipid class to multivariate differences in lipid profile of seaweed species among seasons as determined by SIMPER: A) *C. torulosa*, B) *E.radiata* (blade) and C) *C. plumosum*. P(perm) and Unique perms were obtained from PERMANOVA on a single factor for each lipid class and pairwise comparisons. Significant results ($p<0.05$) are shown in bold.

A)

Seasons: Group 1 & 2	Average squared distance	Lipids	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Autumn & Summer	352.97	ST	79.9	71.2	269	1.15	76.2	76.2			
		PL	23.8	18	61.4	1.70	17.4	93.6	2.20	**0.0410**	126
Winter & Summer	66.1	ST	74.6	71.2	39.6	0.7	59.85	59.85			
		PL	21.8	18	15.5	1.8	23.39	83.25	6.54	**0.0091**	126
		FALC	10.9	11.7	9.2	0.9	13.91	97.16			
Spring & Summer	336.03	ST	86.2	71.2	284	1.1	84.65	84.55			
		PL	22.2	18	21.3	1.2	6.4	91	4.23	**0.0076**	126

B)

Seasons: Groups 1 & 2	Average squared distance	Lipids	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Summer & Autumn	23.67	TAG	2.8	0.9	6	0.8	25.12	81.06	2.45	**0.0142**	126
Summer & Winter	14.79	TAG	2.3	0.9	2.9	1	18.17	82.35	2.71	**0.0234**	126

C)

Seasons: Group 1 & 2	Average squared distance	Lipids	Average Value Group 1	Average Value Group 2	Average Sq.Dist	Sq.Dist/SD	Contribution %	Cumulative %	t	P(perm)	Unique perms
Winter & Spring	35.71	PL	28.9	24.6	31.9	1.1	89.45	89.45			
Winter & Summer	39.01	PL	28.9	24	37.4	1.1	95.77	95.77			

Total lipid of *E. radiata* (blade) showed no significant differences among seasons (Pseudo-$F_{3,16}$=0.72, p=0.56, Fig. 5.6.B), presenting all the seasons relatively similar concentrations of total lipid (~34-38μg/mg of dry mass; Table 5.9; Fig. 5.6.B). Similar results were obtained when the concentration of structural lipids was compared among seasons (Pseudo-$F_{3,16}$=1.23, p=0.35; Table 5.9). In contrast, the concentration of energy lipids was found to be significantly different among seasons (Table 5.10.A). Pairwise results showed that the concentration of energy lipids during summer had a lower concentration (~1μg/mg of dry mass) than in autumn (~4μg/mg of dry mass) and also in winter (~3μg/mg of dry mass) (Table 5.9; Table 5.10.B). SIMPER analysis revealed that TAG was the energy lipid that contributed to the differences, being present in smaller concentrations during summer (<1μg/mg of dry mass) than winter and autumn (>2.3μg/mg of dry mass; Table 5.9; Table 5.8.B). The lipid profile of *E. radiata* (blade) showed no significant seasonal variation (Pseudo-$F_{3,16}$=1.90, P(perm)=0.141) as also shown in the dispersal pattern of the MDS plot (Fig. 5.6.B).

Table 5.9. Concentration (μg/mg of dry weight) of lipid classes of *E. radiata* (blade) by season. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as energy lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

Lipid Class	Autumn	Winter	Spring	Summer
ST	3.42±0.69	4.19±0.52	3.43±0.58	4.14±0.83
AMPL	2.58±0.46	3.55±0.49	2.62±0.60	3.66±1.20
PL	24.45±2.27	25.31±2.03	29.88±6.94	26.21±2.74
Structural Lipids	30.45±3.06	33.05±1.86	35.93±7.23	34±3.25
TAG	2.78±1.70	2.25±0.48	2.16±0.96	0.89±0.29
FFA	1.16±0.27	1.16±0.47	0.27±0.17	0.48±0.19
Energy Lipids	3.94±1.62	3.41±1.18	2.42±1.10	1.37±0.40
Total Lipid	34.39±3.10	36.46±3.26	38.36±6.90	35.37±2.89

Table 5.10. Results of concentration of energy lipids of *E. radiata* (blade) A) Univariate one-way PERMANOVA test and B) Pairwise comparisons among seasons. Significant results (p>0.05) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Season	3	5931.10	1977	4.49	**0.0090**	999
Residual	16	7045.10	440.32			
Total	19	12976				

B)

Seasons	t	P(perm)	Unique perms
autumn, winter	0.49	0.626	126
autumn, spring	1.24	0.161	126
autumn, summer	4.10	**0.011**	126
winter, spring	1.03	0.321	126
winter, summer	4.08	**0.008**	126
spring, summer	1.68	0.113	126

Total lipid of *E. radiata* (stipe) revealed no significant differences among seasons (Pseudo-$F_{3,16}$=1.8206, p=0.1879, Table 5.11). Similarly, no significant differences were found in the concentration of structural lipid (Pseudo-$F_{3,16}$=2.3901, p=0.111) nor in the concentration of energy lipid (Pseudo-$F_{3,16}$=2.9993, p=0.612). Likewise, there were not significant differences in the lipid profile of *E. radiata* (stipe) among the seasons (Pseudo-$F_{3,16}$=3.1089, P(perm)=0.0504) also showed in the MDS plot (Fig. 5.6.C).

Table 5.11. Concentration (µg/mg of dry weight) of lipid classes of *E. radiata* (stipe) by season. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as energy lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

Lipid Class	Autumn	Winter	Spring	Summer
ST	2.83±0.46	3.16±0.23	3.01±0.32	2.83±0.61
AMPL	1.05±0.32	0.92±0.16	1.09±0.19	1.14±0.36
PL	20.52±1.62	20.99±2.81	24.42±2.24	21.03±1.87
Structural Lipids	24.41±2.32	25.07±3.17	28.52±2.38	24±2.62
TAG	0.89±0.33	1.40±0.49	0.83±0.25	0.98±0.31
FFA	0.80±0.41	0.66±0.18	0.32±0.43	0.41±0.17
Energy Lipids	1.69±0.56	2.06±0.31	1.14±0.60	1.4±0.42
Total Lipid	26.1±2.04	27.13±3.26	29.67±2.29	26.40±2.82

Seasonal variation was found in the concentration of total lipid of *C. plumosum* (Table 5.12.A; Fig. 5.6.D). Pairwise comparisons showed that the concentration of total lipid during winter (~37µg/mg of dry mass) was greater than the concentration during spring and summer, presenting a concentration of ~31µg/mg of dry mass in both seasons (Table 5.13; Table 5.12.B). Similarly, the concentration of structural lipids was significantly different among seasons (and Pseudo-$F_{3,16}$=3.6478, p=0.0392, respectively) where winter presented a greater

concentration (~36µg/mg of dry mass) than spring (~30µg/mg of dry mass; t=3.4107, p=0.0163) and summer (~30µg/mg of dry mass; t=3.0588, p=0.0311) (Table 5.13). Although SIMPER results showed that PL was the lipid class that contributed most to these differences (Table 5.8.D), the concentration of this lipid class showed no statistical variation among seasons (Pseudo-$F_{3,16}$= 2.6598, p=0.0853). The concentration of energy lipids was also significantly different among seasons (Pseudo-$F_{3,16}$=8.3378, p=0.0031) and pairwise multiple comparisons revealed that this concentration was greater during spring (~1µg/mg of dry mass) than the concentration during winter (~0.6µg/mg of dry mass; t=3.4798, p=0.0336) and summer (~0.7µg/mg of dry mass; t=3.2218, p=0.039; Table 5.13). However, no statistical differences were found in the lipid profile of *C. plumosum* among seasons (Pseudo-$F_{3,16}$=2.87, P(perm)=0.0616), with significant overlap among seasons as shown in the MDS plot (Fig. 5.6.D).

Table 5.12. Results of concentration (µg/mg of dry weight) of total lipid of *C. plumosum* A) Univariate one-way PERMANOVA test and B) Pairwise comparisons among seasons. Significant results (p>0.05) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Season	3	243.28	81.09	3.54	**0.0393**	9961
Residual	16	366.57	22.91			
Total	19	609.85				

B)

Seasons	t	P(perm)	Unique perms
autumn, winter	0.51	0.186	126
autumn, spring	0.99	0.310	126
autumn, summer	0.83	0.431	126
winter, spring	3.36	**0.017**	126
winter, summer	3.04	**0.032**	126
spring, summer	0.29	0.821	126

Table 5.13. Concentration (µg/mg of dry weight) of lipid classes of *C. plumosum* among seasons. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon (AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as energy lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

Lipid Class	Autumn	Winter	Spring	Summer
ST	4.51±0.52	4.52±0.69	4.07±0.36	3.85±0.37
AMPL	2.76±0.49	2.95±0.44	1.31±0.33	2.61±0.71
PL	25.17±3.85	28.91±3.43	24.59±2.20	24.03±2.27
Structural Lipids	32.44±4.64	36.38±3.38	29.98±2.49	30.48±2.67
TAG	0.57±0.05	0.57±0.12	0.82±0.14	0.65±0.08
DAG	0.16±0.1	ND	0.21±0.12	ND
Energy Lipids	0.73±0.08	0.57±0.12	1.05±0.25	0.65±0.07
Total Lipid	33.18±4.64	36.96±3.34	31.01±2.26	31.13±2.71

C. maschalocarpum showed no significant differences when the concentration of total lipid was analysed among seasons (Pseudo-$F_{3,16}$=2, p=0.143) presenting a range of total lipid of ~25-29µg/mg of dry mass (Table 6.14; Fig. 5.6.E). Additionally, no significant differences were found in the concentration of energy lipids (Pseudo-$F_{3,16}$=1.67, p=0.2217) neither in the concentration of structural lipids (Pseudo-$F_{3,16}$=2.16, p=0.1269) (Table 5.14). Similarly, no significant differences were found in lipid profile among seasons (Pseudo-$F_{3,16}$=1.68, p=0.2032) also shown in the MDS plot (Fig. 5.6.D).

Table 5.14. Concentration (µg/mg of dry weight) of lipid classes of *C. maschalocarpum* among seasons. Sterols (ST), Acetone-mobile Polar Lipids (AMPL) and Phospholipid (PL) as structural lipids. Aliphatic Hydrocarbon AH), Triacylglycerol (TAG), Free Fatty Acid (FFA), Diacylglycerol (DAG), Wax Ester (WE) and Free alcohol (FALC) as energy lipids. ND: not detected. Data represent the mean (±SE) of 5 samples per seaweed species.

Lipid Class	Autumn	Winter	Spring	Summer
ST	3.23±0.28	3.45±0.23	2.73±0.37	2.78±0.41
AMPL	1.19±0.32	1.84±0.45	1.32±0.43	1.42±0.31
PL	22.94±2.47	23.64±4.05	21.52±1.48	20.05±2.60
Structural Lipids	27.35±2.71	28.93±4.42	25.57±1.87	24.25±2.90
TAG	0.43±0.11	0.40±0.10	0.54±0.12	0.47±0.11
Energy Lipids	0.43±0.11	0.40±0.10	0.54±0.12	0.47±0.11
Total Lipid	27.79±2.74	29.33±4.50	29.11±1.83	24.72±2.99

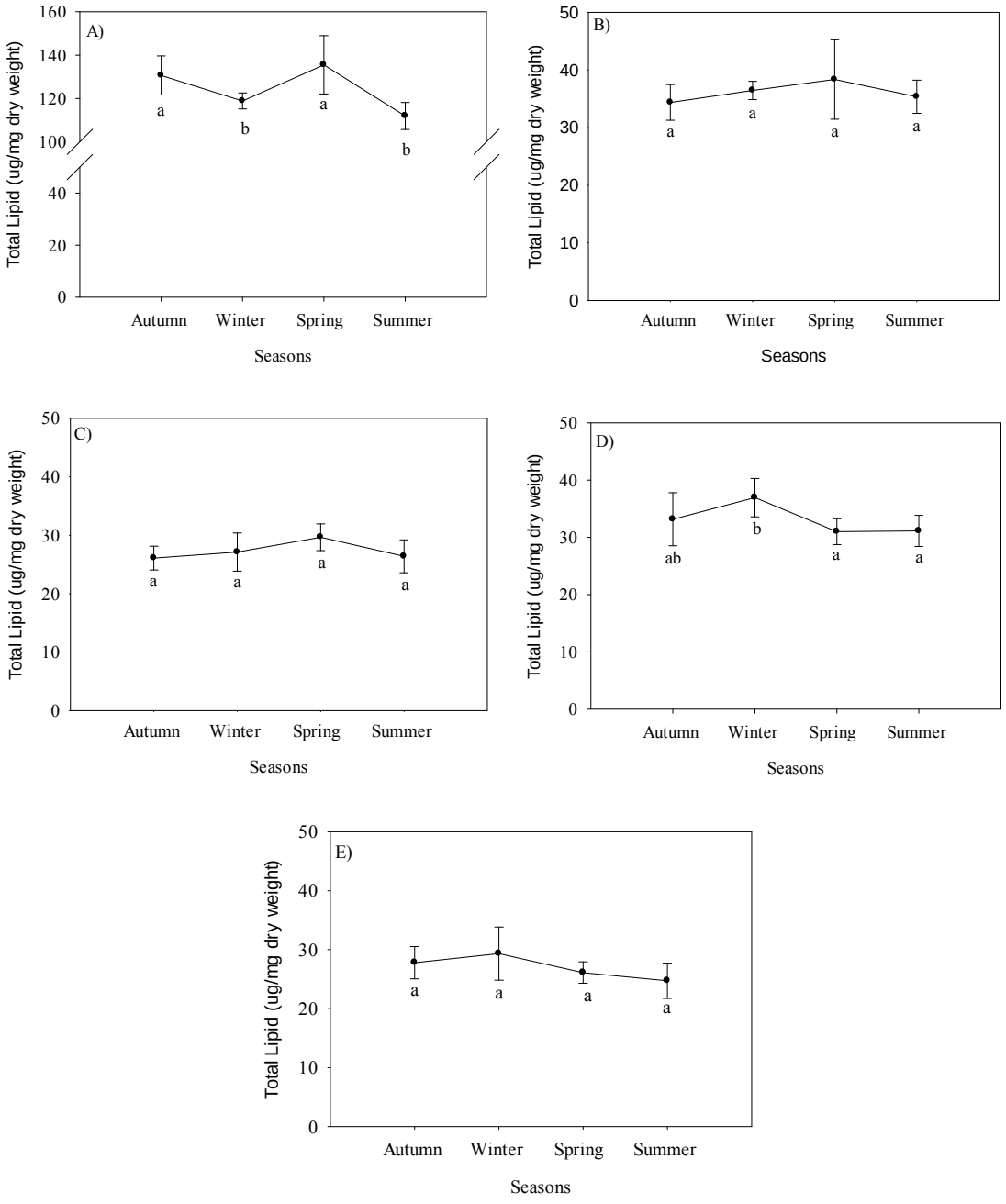


Figure 5.6. Seasonal total lipid of brown seaweeds: A) *C. torulosa*, B) *E. radiata* (blade), C) *E. radiata* (stipe), D) *C. plumosum* and E) *C. maschalocarpum*. Mean values of five different individuals (±SE) per season are shown. Letters bellow error bars that are not in common indicate contents that are significantly different ($p<0.05$)

5.4 Discussion

Measurable differences were observed in the lipid composition of the four dominant brown seaweeds from north-eastern New Zealand analysed here and also between *Ecklonia radiata* blade and stipe. Additionally, seasonal variation was found on the lipid composition of the fucalean species, *Cystophora torulosa* and *Carpophyllum plumosum,* but no variation was observed in the rest of the species. These findings are consistent with previous studies where seasonal variation was found in some seaweeds (Rodríguez-Montesinos et al., 1991) but not in others (Renaud et al., 2007).

Cystophora torulosa presented a completely different lipid profile to the other species analysed here, and also was the only species that demonstrated seasonal variation. This species had a higher concentration of lipid (i.e., total lipid and structural lipids, particularly PL and ST) during spring than in winter and summer. The highest concentration of lipids during early spring (the sampling was made at the beginning of the season, late September) suggests that this fucalean is preparing itself for the reproductive season by storing more lipids when is reproductive from late spring through late summer (Hawes, 2008). These results are in agreement with previous studies on *Costaria costata*, which showed higher lipid proportions during spring, when this algae is preparing itself for the reproductive season (Gerasimenko et al., 2010).

Carpophyllum plumosum also showed seasonal variation in the lipid profile, revealing the highest concentration of total lipids and structural lipids during winter. The fertile period for this brown seaweed occurs from September to January, with most individuals showing no reproductive structures by early December (Schiel, 1988), hence the slightly earlier peak of lipids compared to *C. torulosa*. It seems that *C. plumosum* is able to adjust physiologically earlier in than *C. torulosa* to prepare for the reproductive season. Macroalgae from the Northern Pacific Ocean also showed the highest lipid content during winter and spring and the authors related this to the fact that spring samples contained young recruiting individuals (Nelson et al., 2002). In contrast, *C. maschalocarpum* was the only seaweed species that did not show a seasonal trend in total lipid or any lipid classes. The differences found in the present study with respect to the two *Carpophyllum* species are very intriguing. They showed different lipid composition and *C. plumosum* showed seasonal variation, whereas *C. maschalocarpum* did not. Further studies are needed to better understand this group of fucaleans.

E. radiata blade showed statistical differences in the concentration of energy lipids among seasons, although not significant differences were found in the lipid composition or total lipids. During summer *E. radiata* blade presented the lowest amounts of energy lipids, reflected in the low amount of TAG. *E. radiata* is reproductive for the longest period of the year of the seaweeds studied here, with some members of shallow populations possessing fertile sori from May to late November (Novaczek, 1984). This might explain the higher values of TAG during the reproductive season, from autumn to spring and very low amounts from spring to austral summer. However, further studies on the chemical composition of different parts of this kelp are needed for a better understanding on the accumulation and movements of nutrients within different parts of the plant, as *E. radiata* stipe did not show significant differences between seasons.

C. torulosa presented the greatest concentration of total and energy lipids and a very different lipid profile compared with the rest of the species analysed here with. Total lipids varied from 2.6% to 3.8% of dried mass in the three species (*E. radiata* blade and stipe, *C. plumosum* and *C. maschalocarpum*), which agreed with previous studies of phaeophytes from different parts of the world that reported for lipid content less than 8% of dry weight (Herbreteau et al., 1997; Renaud et al., 2007; Sánchez-Machado et al., 2004; Shevchenko et al., 2007). However, *C. torulosa* contained higher values of total lipid, >13% of dry mass. Furthermore, energy lipids only contributed with ~2-6% to the total lipids of the three species (*E. radiata* blade and stipe, *C. plumosum* and *C. maschalocarpum*), but in *C. torulosa* its contribution was of ~16% of the total lipids. TAG was the principal component of the energy lipids in the rest of the three brown algae, whereas in *C. torulosa* FALC was the principal contributor. Previous studies described TAG as the principal energy storage lipids, as reported for the brown seaweed *Egreria menziesii* (Nelson et al., 2002). It is surprising to find the presence of FALC as a storage lipid, as to our knowledge it was not found in other seaweed species, making this seaweed species very different and interesting for future studies.

Furthermore, *C. torulosa* revealed a very different pattern of structural lipids, showing lower values of PL (16% of total lipids) but higher values of ST as structural lipids (64% of total lipids). The higher amount of sterol in this species was unexpected, and preliminary results (unpublished data) have revealed a high amount of sterols that are more polar with lower molecular weight than the usual sterols found in brown seaweeds, such as fucosterols

(Kamenarska et al., 2003). Differences in the polar lipids between Laminariales and Fucales have previously been found, suggesting a connection with the taxonomic position of brown algae (Vaskovsky et al., 1996). Further studies are needed to better understand the great differences found between *C. torulosa* compared with the rest and thus understand more about how the lipid composition is determined by taxonomy in the New Zealand brown seaweed.

The high levels of lipids present in *C. torulosa* may suggest that this seaweed species would be a great source of nutrients for herbivorous grazers. Previous chapters (Chapter 2 and Chapter 3) have shown the ability of *E. chloroticus* to convert lipids, so they may be able of convert the high energy lipid FALC present in *C. torulosa* to TAG, as the normal energy lipid. However, the principal grazer in New Zealand rocky reef, *E. chloroticus*, seems to have more preference for *E. radiata* (Barker, 2013; Don, 1975), even though it contains fewer amounts of lipids. Feeding experiments on *E. chloroticus* showed that this sea urchin is able to distinguish between seaweed species, preferably choosing *E. radiata* (Cole et al., 2000; Schiel, 1982). Furthermore, other chapters of this thesis (Chapter 2, Chapter 3, Chapter 7), show that the fatty acid profile of *E. chloroticus* is similar to *E. radiata*, indicating that this sea urchin species is feeding on this kelp. Additionally, stable isotope analysis (Chapter 6) revealed that *E. chloroticus* is probably feeding on *E. radiata* but also on *C. plumosum*. So it seems that, despite of the high level of nutrients present in *C. torulosa*, this seaweed species it is not part of *E. chloroticus* diet. Nevertheless, the fatty acid composition of this seaweed species has not been analysed. Therefore, further analyses need to be performed to have a more comprehensive idea of what is in *C. torulosa*, which even though it contains so many nutrients is not chosen by *E. chloroticus*. Probably this is related to the high amount of sterols present in *C. torulosa* compared to the preferred *E. radiata* and *C. plumosum*.

The high levels of lipids present in *E. radiata* and *C. plumosum* during winter/spring indicate that these seaweed species would be a great source of nutrients on this time for *E. chloroticus*, as it corresponds with the season that this sea urchin is preparing for the gametogenesis (Brewin et al., 2000; Walker, 1982). Histology analysis revealed that during this time nutritive phagocytes occupied most of the gonads, providing nutrient material to the future gametes that are formed during spring (Walker, 1982). However, seasonal analysis of the biochemical composition of *E. chloroticus* showed the higher levels of lipids during summer (Verachia et al., 2012), indicating that the high levels of lipids incorporated by the

nutritive phagocytes are being used to form the gametes and these are accumulated during spring until they reach the maximum level in summer.

In conclusion, seasonal variation in the lipid contents depends on the dominant brown seaweed species analysed here. However, in general terms winter and spring were the seasons with higher lipid contents in the brown seaweeds chosen as food by *E. chloroticus*, making them very nutritious at the precise time when this sea urchin species is preparing itself for the gametogenesis (Walker, 1982). On the contrary, it is surprising that *E. chloroticus* is not choosing *C. torulosa* as a food item; regardless the great amount of lipids present. The results also revealed unexpected differences between the *Carpophyllum* species. However, the following chapter of this thesis (Chapter 6) will show that stable isotope contents are also very different between this two *Carpophyllum* species. More studies are needed including other species like *C. angustifolium*, *C. flexuosum* and the hybrids to better understand the lipid differences in this interesting seaweed group. Further studies on the seasonal variation of the chemical composition of these and extra brown seaweed species from different locations around New Zealand are required for a superior understanding of the nutritional value among the seasons. Furthermore, it will be very interesting to determine if the seasonal variation in the chemical composition of brown seaweeds affects the feeding selection of their grazers, like *E. chloroticus*.

Chapter 6

Stable isotopes, a complementary tool to examine the diet of *Evechinus chloroticus.*

6.1 Introduction

Stable isotope (SI) analysis has been widely used for a better understanding of food webs as well as animal feeding patterns and diets in many environments and habitats, and has become a very important tool in trophic ecology (Fredriksen, 2003; Phillips et al., 2006; Wolf et al., 2009). This type of analysis is based on the general assumption that there is a predictable relationship between the isotopic composition of a consumer and its food source (Vanderklift et al., 2006). As carbon changes very little between successive trophic levels (limited to about 1% per trophic level), the carbon isotope signature can often indicate the ultimate carbon source of a consumer if the signature of the possible food items are different from each other (Fry et al., 1984; Hanson et al., 2010; Peterson et al., 1985). In contrast, the signature of nitrogen isotopes experiences greater fractionation per trophic level (enriched by 3 to 4‰ in theory, but in practice it ranges between 2 and 5‰, relative to their prey) and it is thus used to infer the trophic levels of a consumer where feeding relationships in a particular trophic web are unknown (DeNiro et al., 1981; Guest et al., 2010; McCutchan et al., 2003; Wolf et al., 2009). Therefore, combined measurements of carbon and nitrogen isotopes can provide information on both source materials and food web structure (Fredriksen, 2003).

While this tool has been used in trophic studies in many marine environments (reviewed by Wolf et al. 2009), few studies on kelp forest communities have used this approach (Dethier et al., 2013; Fredriksen, 2003; Guest et al., 2010; Kharlamenko et al., 2001). According to those studies, macroalgae provide the habitat for diverse number of marine invertebrates and is the main carbon source of animals with different trophic levels in these communities. Furthermore, primary production by macrophytes, in terms of nutrient supply, plays an important role in determining the diversity density and biomass of marine herbivorous (Iverson, 2009; Renaud et al., 2007).

Large brown macroalgae are generally the dominant organisms on temperate New Zealand coastal reefs where they provide habitat for a diversity of fish and invertebrates (Choat et al., 1982; Guest et al., 2010). In north-eastern New Zealand these reefs are

dominated mainly by brown algae of the order Fucales, which includes the genera *Carpophyllum* and *Cystophora*, and Laminariales with *Ecklonia radiata* as the most common species (Schiel, 1988). There are few grazers in these types of habitats, where the sea urchin *Evechinus chloroticus* is one of the most frequent residents (Barker, 2013; Schiel, 1982, 1988). *E. chloroticus* is principally herbivorous (Barker, 2013; Don, 1975); however, it has been suggested that this sea urchin species will eat a variety of food if algae is scarce such as encrusting sponges (Ayling, 1978). Feeding experiments have indicated that this sea urchin species is able to distinguish between algae species and it has been suggested that it has preference for *E. radiata* (Cole et al., 2000; Schiel, 1982). However, little is known about the contribution of these macrophytes to the dominant grazer, *E. chloroticus*, in these particular rocky reef environments.

Stable isotopes analysis has been proven to be a useful tool for discerning trophic relationships in marine ecosystems (Guest et al., 2010; Johnson et al., 2012). Particularly, some studies have focused in the trophic relationship between macroalgae and sea urchins, such as *Anthocidaris crassispina*, *Echinus esculentus*, *Tetrapygus niger*, *Heliocidaris erythrogramma*, *Centrostephanus tenuispinus*, *Phyllacanthus irregularis*, *Paracentrotus lividus* and *Arbacia lixula* (Agnetta et al., 2013; Fredriksen, 2003; Rodríguez, 2003; Vanderklift et al., 2006; Yatsuya et al., 2004) . Those studies helped to understand the trophic levels and diet preferences of several sea urchin species and have a more comprehensive knowledge of the trophic relationships in those marine environments.

In the current study, the signatures of the most common stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of the dominant primary producers (*Ecklonia radiata, Carpophyllum plumosum, C. maschalocarpum and Cystophora torulosa*) and one of the principal grazers (*Evechinus chloroticus*) of the food webs in a temperate rocky reef in north-eastern New Zealand were used to address the following questions: 1) Do different brown seaweed species have different SI signatures in these temperate environments? 2) Do the SI signatures vary between different organs (gonad and gut) of the dominant grazer, *E. chloroticus*? 3) Can the SI signature of the principal primary producers be used to clarify *E. chloroticus* feeding habits and which of the potential food sources is actually being assimilated by these sea urchin species? To answer this last question the Bayesian mixing model (SIAR: Stable Isotope Analysis in R) was performed, as it was previously used to study trophic ecology and infer about the diet of predators in different environments (Parnell et al., 2008; Phillips,

2012). Consequently, the aim of this study was to gain understanding of diet preferences as well as the trophic level of this sea urchin species to better comprehend its ecology and provide new data to the knowledge of seaweed-herbivore interactions in northern New Zealand.

6.2 Methodology

6.2.1 Sample collection and store

Wild *E. chloroticus* and the most dominant potential diet species of brown seaweed (*Ecklonia radiata*, *Carpophyllum maschalocarpum*, *Carpophyllum plumosum* and *Cystophora torulosa*) were collected by snorkelling from Matheson's Bay (36^0 29' S, 174^0 53' E) in the Hauraki Gulf, north-eastern New Zealand during the austral summer of 2010. In total 20 sea urchins were collected along with five individuals of each brown seaweed species (~10cm of plant). Because *E. radiata* has two parts that are very distinguishable (Babcock et al., 1993) it was divided in the field into stipe and blade (~10cm) and they were analysed separately.

After collection, the sea urchins, together with the seaweed species, were transported live in plastic buckets filled with chilled seawater to the laboratory at the University of Auckland. Wet weight (blotted dry with a paper towel before weighing to the nearest mg) and diameter (using a VITA caliper to nearest mm) of the whole sea urchin was recorded prior to dissection. The test was cut around the equator to separate the oral and aboral surfaces and the Aristotle's lantern was removed and weighed together with the wet test. The coelomic fluid was drained to waste and the gut contents, including solids that were floating in the coelomic fluid, were transferred to a 50 ml polypropylene tube. The five gonads, together with the gut, were removed using tweezers and were weighed and stored separately in the same type of polypropylene 50 ml tube in the -80°C freezer, along with the seaweed species, for further analysis.

From the 20 sea urchins collected five were randomly selected for stable isotope analysis. Gonads, guts, and algae were lyophilized for 72 hours to a constant mass in a freeze-dryer (VirTis Bench Top 2k). The samples were cryogenically grounded using an MM301 Mixer Mill (Retsch), to a ground particle size of <5 μm. Lyophilized, ground

samples were stored in sealed polypropylene 10ml tubes under desiccant at -20°C until the analysis.

Lyophilized tissue was weighed (~3.5 mg of gonad, ~3 mg of gut and ~2.7 mg of each of the seaweed species; dry material) into pressed tin microcapsules (Standard Weight 8x5 mm from Elemental Microanalysis). The samples were then sent for ^{13}C and ^{15}N isotopes analysis at the UC Davis Stable Isotope Facility (Department of Plant Sciences), USA for analysis using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 Isotope Ratio Mass Spectrometer (IRMS; Sercon Ltd., Cheshire, UK). Lyophilized samples were combusted at 1000°C in a reactor packed with chromium oxide and silvered copper oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). A helium carrier then flowed through a water trap (magnesium perchlorate) and an optional CO_2 trap (for N-only analyses). N_2 and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL/min) before entering the IRMS.

During SI analysis, samples were interspersed with several replicates of at least two different laboratory standards. These laboratory standards, which were selected to be compositionally similar to the samples being analysed, have previously been calibrated against NIST Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, USGS-40, and USGS-41). A sample's preliminary isotope ratio was measured relative to reference gases analysed with each sample. These preliminary values were finalised by correcting the values for the entire batch based on the known values of the included laboratory standards. The long term standard deviation was 0.2 permil for ^{13}C and 0.3 permil for ^{15}N. The final delta values were expressed relative to international standards V-PDB (Vienna PeeDee Belemnite) and air for carbon and nitrogen, respectively.

6.2.2 Trophic position

Trophic position was calculated according to a model developed by Hobson and Welch (1992):

$$TL=1 + (\text{sea urchin } \delta^{15}N‰ - \text{brown seaweed } \delta^{15}N‰)/3.8$$

Where TL is the trophic level of the consumer and 3.8 is the $\delta^{15}N‰$ enrichment value per trophic level. According to this model, a TL close to 1 indicates herbivorous feeding, while TL>2 indicates a carnivorous diet.

6.2.3 Statistical analysis

The values of $\delta^{13}C$ and $\delta^{15}N$ for *E. chloroticus* tissues (gonad and gut) and five species of seaweed (*E. radiata* (blade), *E. radiata* (stipe), *C. plumosum*, *C. maschalocarpum* and *C. torulosa*) from Matheson's Bay were analysed using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). The data was left untransformed and converted into similarity matrices using Euclidean distances. Univariate one-way PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the permutation method, was performed to examine differences in stable isotope values (carbon and nitrogen separately) between tissues and seaweed species, followed by pair-wise comparisons. We used this approach as this tests avoids the assumptions of the traditional one-way analysis of variance or ANOVA (Underwood, 1997) and assumes only that the samples are exchangeable , i.e. independent and identically distributed, under a true hypothesis (Anderson, 2003).

6.2.3 Mixing Models

Mixing models were used to explore the contribution of the possible food items to *E. chloroticus* diet. Nitrogen and carbon isotope values for the sources, different species of brown seaweed (*E. radiata*, *C. plumosum*, *C. maschalocarpum* and *C. torulosa*) and the consumer (gonad and gut of *E. chloroticus*) were used in the Stable Isotope Analysis in R (SIAR) package (Parnell et al., 2008). As significant differences in the stable isotope values were not found between the two different parts of *E. radiata* (blade and stipe), the mean of these values was used for this model. The model input parameters adjusts the isotopic values of the consumer (because isotopes can be lost during the conversion of source proteins into consumer tissues) by the use of the trophic enrichment factor (TEF). To estimate the TEF, average values for marine invertebrates were taken from Caut et al. (2009) as reported by Vanderklift et al. (2010). Two different analyses were conducted: (1), using the raw values of $\delta^{13}C$ and $\delta^{15}N$ of the sources and the consumer and (2) using the values corrected for the possible trophic enrichment effect using the TEF as suggested by Fry (2013).

6.3 Results

6.3.1 Stable isotope analysis

The brown seaweed species: *C. torulosa*, *C. maschalocarpum*, *C. plumosum,* and the blade and stipe of *E. radiata* (which were considered as two different species, see methods) as well as *E. chloroticus* tissues exhibited a wide range of carbon isotope values. The $\delta^{13}C$ values in the seaweed ranged from approximately -11‰ in *C. maschalocarpum* to -16.5 ‰ in *E. radiata* (Table 6.1.). The $\delta^{13}C$ mean signature of *E. chloroticus* tissues ranged around -16‰ (Table 6.1).

One-way PERMANOVA on a single variable analysis showed significant differences in the $\delta^{13}C$ between *E. chloroticus* tissues and seaweed species (Table 6.2, Fig. 6.1). Pairwise analysis revealed that *C. maschalocarpum* had the lowest amount of $\delta^{13}C$ (-11.2‰) followed by *C. torulosa* (-13.6‰) and the highest amounts were present in *C. plumosum*, *E. radiata* (blade) and *E. radiata* (stipe) which were not statistically different (~-16‰) (Table 6.1; Table 6.2.B). Therefore, the distinct parts of *E. radiata*, blade and stipe, showed no significant differences ($p>0.05$) in the carbon content (Table 6.1; Table 6.2). In contrast, there were significant differences ($p<0.05$) between the two *Carpophyllum* species with *C. plumosum* showing a higher carbon content (Table 6.1; Table 6.2).

The values of $\delta^{13}C$ were not significantly different between gonad and gut tissues of *E. chloroticus* (~-16‰) showed by Univariate one-way PERMANOVA results. Pairwise comparisons revealed that these values were also similar to the values in *E. radiata* as well as the values in *C. plumosum* and statistically different to the $\delta^{13}C$ values of the rest of the brown seaweed species (Table 6.1; Table 6.2.B). Thus, the carbon assimilated by *E. chloroticus* was derived mainly from the kelp *E. radiata* and *C. plumosum* (Fig 6.1).

Table 6.1. Stable isotope ratios of *E. chloroticus* tissues (gonad and gut) and brown seaweed species *E. radiata* (blade), *E. radiata* (stipe), *C. torulosa*, *C. maschalocarpum* and *C. plumosum*. Data represent mean (±SE) of 5 samples per tissue or seaweed species.

Tissues and Seaweed Species	$\delta^{13}C$	$\delta^{15}N$
E. chloroticus gonad	-15.78±0.68	8.38±0.31
E. chloroticus gut	-16.29±0.54	8.20±0.20
E. radiata (blade)	-15.97±0.64	7.09±0.30
E. radiata (stipe)	-16.51±0.22	6.65±0.32
C. plumosum	-16.54±0.29	7.06±0.40
C. torulosa	-13.65±0.94	6.37±0.60
C. maschalocarpum	-11.20±0.45	4.88±0.61

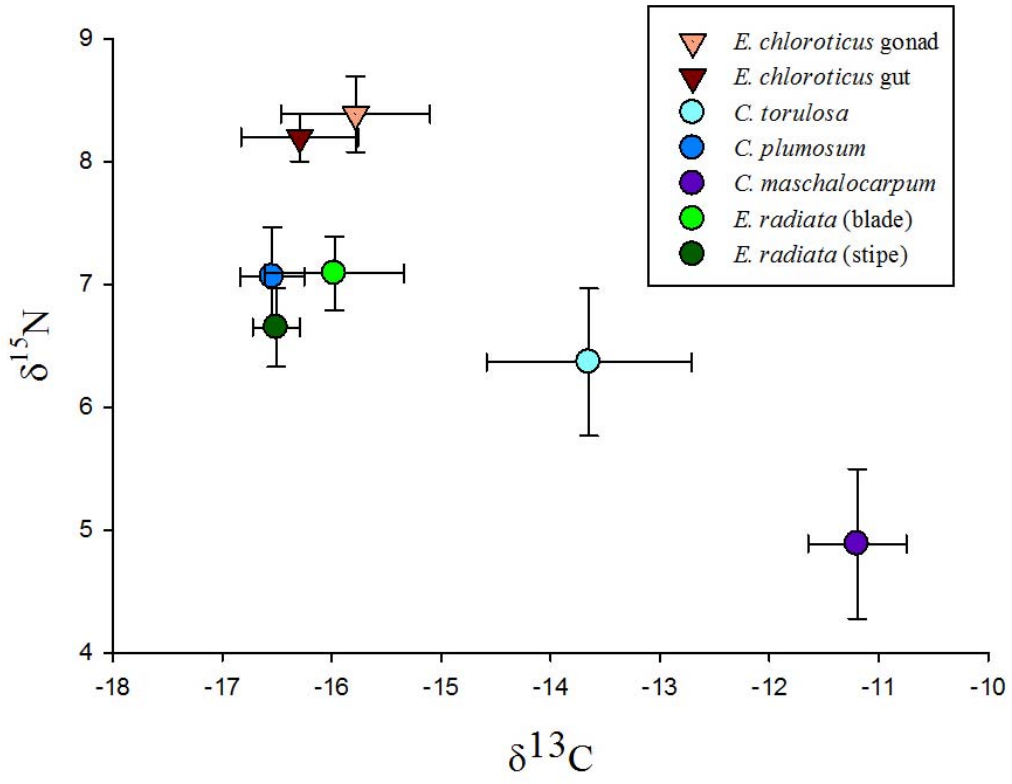


Figure 6.1. Stable Isotope ratio signature (mean ±SE; n=5) of $\delta^{13}C$ and $\delta^{15}N$ for gonad and gut tissues of *E. chloroticus* and 5 brown seaweed species from Matheson's Bay, New Zealand.

Table 6.2. Results of one-way PERMANOVA on a single variable of A) $\delta^{13}C$ values and B) Pairwise comparisons *between E. chloroticus* tissues (gonad and gut) and the different brown seaweed species *E. radiata* (blade), *E. radiata* (stipe), *C. torulosa*, *C. maschalocarpum* and *C. plumosum* (n=5). Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissues/Seaweed Species	6	120.13	20.04	59.17	**0.0001**	9951
Residual	28	9.48	0.34			
Total	34	129.73				

B)

E. chloroticus tissues /Seaweed Species	t	P(perm)	Unique perms
Gonads, Gut	1.31	0.2114	126
Gonads, *C. torulosa*	4.12	**0.0233**	126
Gonads, *C. plumosum*	2.29	0.0572	126
Gonads, *C. maschalocarpum*	12.56	**0.0082**	126
Gonads, *E. radiata* (blade)	0.46	0.6412	126
Gonads, *E. radiata* (stipe)	2.25	0.0689	126
Gut, *C. torulosa*	5.47	**0.0074**	126
Gut, *C. plumosum*	0.92	0.3807	126
Gut, *C. maschalocarpum*	16.31	**0.0070**	126
Gut, *E. radiata* (blade)	0.86	0.4363	126
Gut, *E. radiata* (stipe)	0.82	0.4013	126
C. torulosa, *C. plumosum*	6.58	**0.0078**	126
C. torulosa, *C. maschalocarpum*	5.27	**0.0083**	126
C. torulosa, *E. radiata* (blade)	4.59	**0.0152**	126
C. torulosa, *E. radiata* (stipe)	6.63	**0.0077**	126
C. plumosum, *C. maschalocarpum*	22.27	**0.0088**	126
C. plumosum, *E. radiata* (blade)	1.81	0.0969	126
C. plumosum, E. radiata (stipe)	0.22	0.8324	126
C. maschalocarpum, *E. radiata* (blade)	13.72	**0.0073**	126
C. maschalocarpum, *E. radiata* (stipe)	23.76	**0.0062**	126
E. radiata (blade), *E. radiata* (stipe)	1.76	0.1026	126

The mean $\delta^{15}N$ signatures of the 5 brown seaweed species ranged from ~5‰ to ~7‰ (Table 6.1). Additionally, *E. chloroticus* tissues presented values of ~8‰ for $\delta^{15}N$ (Table 6.1).

One-way PERMNAOVA on a single variable analysis revealed significant differences in the values of $\delta^{15}N$ between seaweed species as well as between *E. chloroticus* tissues (Fig. 6.1; Table 6.3.A). Pairwise results showed that *C. maschalocarpum* contained the lowest amount of $\delta^{15}N$ (4.88‰) followed by the amount in *C. torulosa* (~6‰) which was similar to the amount in *E. radiata* (stipe); however this value was similar to the valued observed for *E. radiata* (blade) (Table 6.1; Table 6.3.B). *C. plumosum* and *E. radiata* were the species with the highest amounts of $\delta^{15}N$ (~7‰) (Table 6.1; Table 6.3.B). All these results were similar to the results obtained for $\delta^{13}C$. There were no significant differences in the nitrogen content

between *E. radiata* blade and stipe and the two different species of *Carpophyllum* varied significantly, with *C. plumosum* showing a higher $\delta^{15}N$ value than *C. maschalocarpum* (Table 6.1; Table 6.3.B).

The $\delta^{15}N$ value of *E. chloroticus* gonad was not significantly different to the value of $\delta^{15}N$ obtained in *E. chloroticus* gut, showed by univariate one-way PERMANOVA analysis (Table 6.3.B). These values (~8‰) were significantly higher than the values observed in the seaweed species (<7‰) revealed by pairwise comparisons (Table 6.1; Table 6.3B).

Using $\delta^{15}N$ values in *E. chloroticus* gonad and primary producers the trophic level calculated for this sea urchin was 1.52, indicating that it feeds mainly on algae, but also on animal material to some extent.

Table 6.3. Results of one-way PERMANOVA on a single variable of A) $\delta^{15}N$ values and B) Pairwise comparisons between the different *E. chloroticus* tissues (gonad and gut) and brown seaweed species (*E. radiata* (blade), *E. radiata* (stipe), *C. torulosa*, *C. maschalocarpum* and *C. plumosum*) (n=5). Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Tissues & Seaweed Species	6	41.72	6.95	39.96	**0.0001**	9940
Residual	28	4.87	0.17			
Total	34	46.59				

B)

E. chloroticus tissues & Seaweed Species	t	P(perm)	Unique perms
Gonads, Gut	1.15	0.3867	126
Gonads, *C. torulosa*	6.71	**0.0074**	126
Gonads, *C. plumosum*	5.88	**0.0077**	126
Gonads, *C. maschalocarpum*	11.47	**0.0082**	126
Gonads, *E. radiata* (blade)	6.75	**0.0089**	126
Gonads, *E. radiata* (stipe)	8.75	**0.0081**	126
Gut, *C. torulosa*	6.50	**0.0090**	126
Gut, *C. plumosum*	5.71	**0.0086**	126
Gut, *C. maschalocarpum*	11.56	**0.0087**	126
Gut, *E. radiata* (blade)	6.89	**0.0088**	126
Gut, *E. radiata* (stipe)	9.20	**0.0096**	126
C. torulosa, *C. plumosum*	2.16	**0.0748**	126
C. torulosa, *C. maschalocarpum*	3.87	**0.0085**	126
C. torulosa, *E. radiata* (blade)	2.41	**0.0488**	126
C. torulosa, *E. radiata* (stipe)	0.93	0.3628	126
C. plumosum, *C. maschalocarpum*	6.67	**0.0082**	126
C. plumosum, *E. radiata* (blade)	0.12	0.9159	126
C. plumosum, E. radiata (stipe)	1.80	0.1198	126
C. maschalocarpum, *E. radiata* (blade)	7.24	**0.0064**	126
C. maschalocarpum, *E. radiata* (stipe)	5.72	**0.0104**	126
E. radiata (blade), *E. radiata* (stipe)	2.23	0.0655	126

6.3.2 Estimates of contribution to diet using mixing models

The relative contributions of each possible food item are presented in Figure 6.2 and Figure 3. The SIAR mixing model indicated that two seaweed species contributed more substantially to *E. chloroticus* diet. There were no differences in the model considering and not considering the trophic enrichment factor and also both tissues (gonad and gut) of *E. chloroticus* showed similar results (Table 6.4). The most important brown seaweed species to the diet appeared to be *E. radiata* (blade and stipe combined) and *C. plumosum*, with contributions of >37% for both tissues (Fig. 6.2; Table 6.4). *C torulosa* had a diet contribution 12-18% and 10-15% for gonad and gut, respectively (Fig. 6.2; Fig. 3; Table 6.4). SIAR output revealed the minimum contribution from *C. maschalocarpum* of <7% for both tissues (Fig. 6.2; Fig. 6.3; Table 6.4).

Similar results were obtained when the data was analysed by SIAR model using the boxplot outputs, indicating that the diet of *E. chloroticus* is represented mainly by *C. plumosum* and *E. radiata* with very little contribution from *C. torulosa* and almost no contribution from *C. maschalocarpum*; again when including or excluding the trophic enrichment value (Fig. 6.3).

Table 6.4. Summary information for the output files from SIAR (Stable Isotope Analysis in R) with the proportional contribution of the possible diet items for *E. chloroticus* gonad and gut including and excluding the Trophic Enrichment Value (TEF) and the deviation standard from each stable isotope.

		Excluding TEF				Including TEF			
Tissues	Seaweed Species	Low 95%	High 95%	Mode	Mean	Low 95%	High 95%	Mode	Mean
Gonad	*Carpophyllum plumosum*	0.04	0.70	0.36	0.38	0.09	0.82	0.41	0.45
	Ecklonia radiata	0.02	0.68	0.37	0.37	0.02	0.71	0.39	0.39
	Cystophora torulosa	0.00	0.38	0.06	0.18	0.00	0.33	0.02	0.12
	Carpophyllum maschalocarpum	0.00	0.19	0.02	0.07	0.00	0.14	0.01	0.05
	SD ^{15}N	0.06	2.01	0.59	0.90	0.00	1.79	0.17	0.68
	SD ^{13}C	0.81	4.59	1.71	2.37	0.31	3.87	1.20	1.79
Gut	*Carpophyllum plumosum*	0.08	0.83	0.44	0.45	0.08	0.75	0.41	0.42
	Ecklonia radiata	0.02	0.77	0.42	0.42	0.03	0.66	0.37	0.37
	Cystophora torulosa	0.00	0.28	0.02	0.10	0.00	0.39	0.03	0.15
	Carpophyllum maschalocarpum	0.00	0.12	0.01	0.04	0.00	0.17	0.01	0.06
	SD ^{15}N	0.00	1.78	0.47	0.76	0.00	1.47	0.14	0.54
	SD ^{13}C	0.68	3.62	1.32	1.89	0.70	5.27	1.88	2.61

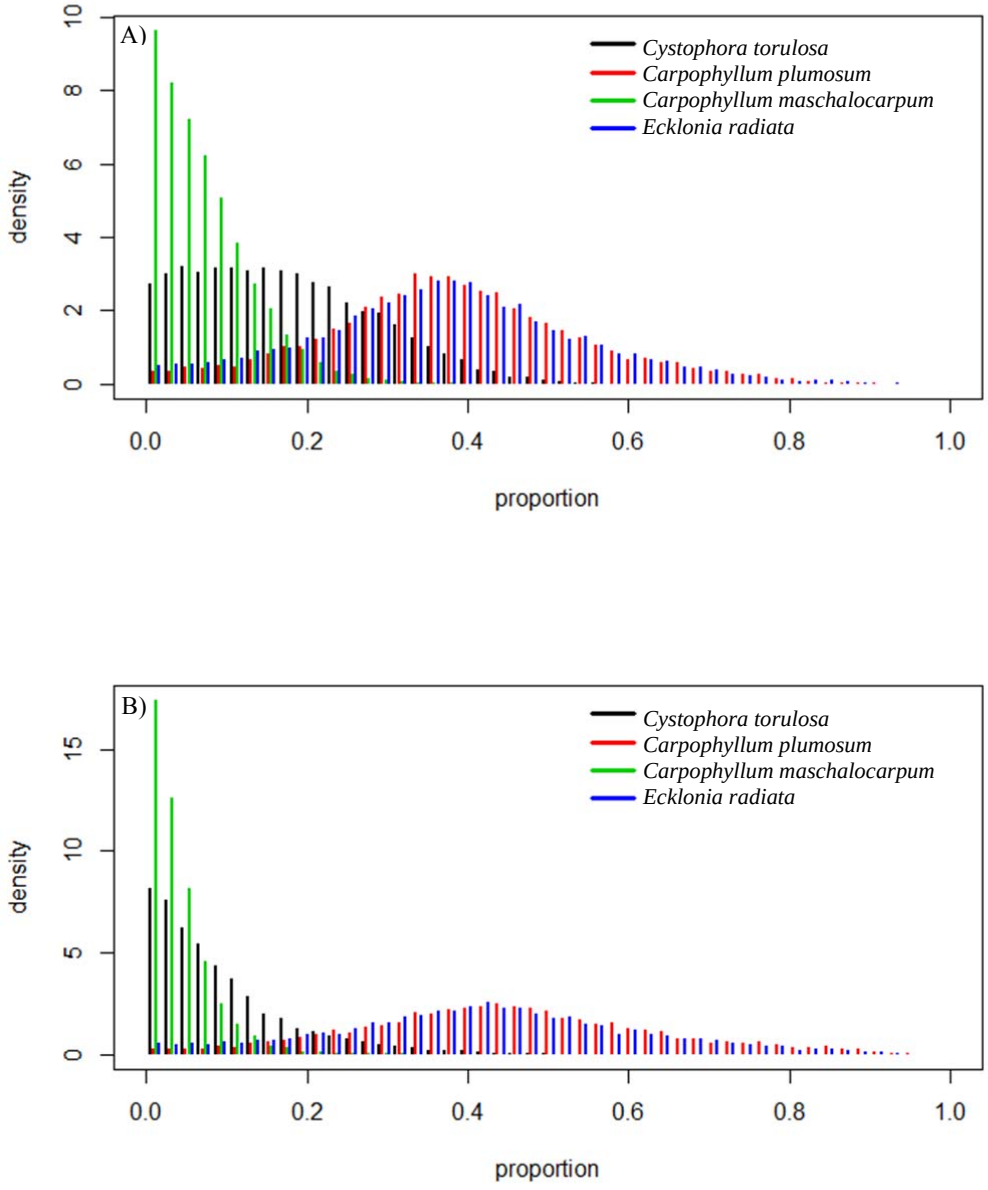


Figure 6.2. Proportional contributions to the diet of *Evechinus chloroticus* for possible food items (*Cystophora torulosa*, *Carpophyllum plumosum*, *C. maschalocarpum* and *Ecklonia radiata*) for 10000 iterations of an isotope mixing model (Stable Isotope Analysis in R, SIAR). A) *E. chloroticus* gonad and B) *E. chloroticus* gut excluding the trophic enrichment factor from the analysis.

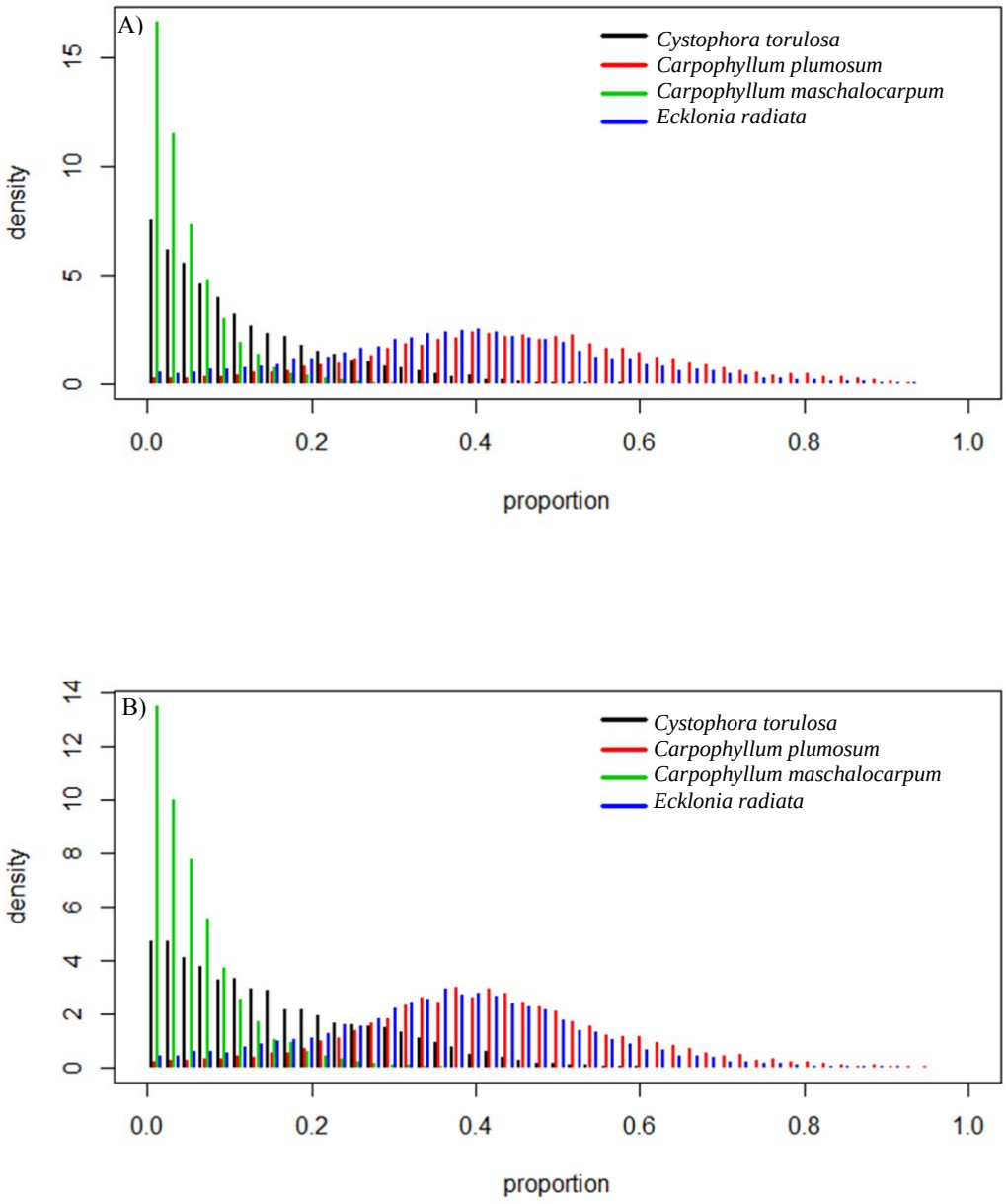


Figure 6.3. Proportional contributions to the diet of *Evechinus chloroticus* for possible food items (*Cystophora torulosa*, *Carpophyllum plumosum*, *C. maschalocarpum* and *Ecklonia radiata*) for 10000 iterations of an isotope mixing model (Stable Isotope Analysis in R, SIAR). A) *E. chloroticus* gonad and B) *E. chloroticus* gut including the trophic enrichment factor in the analysis.

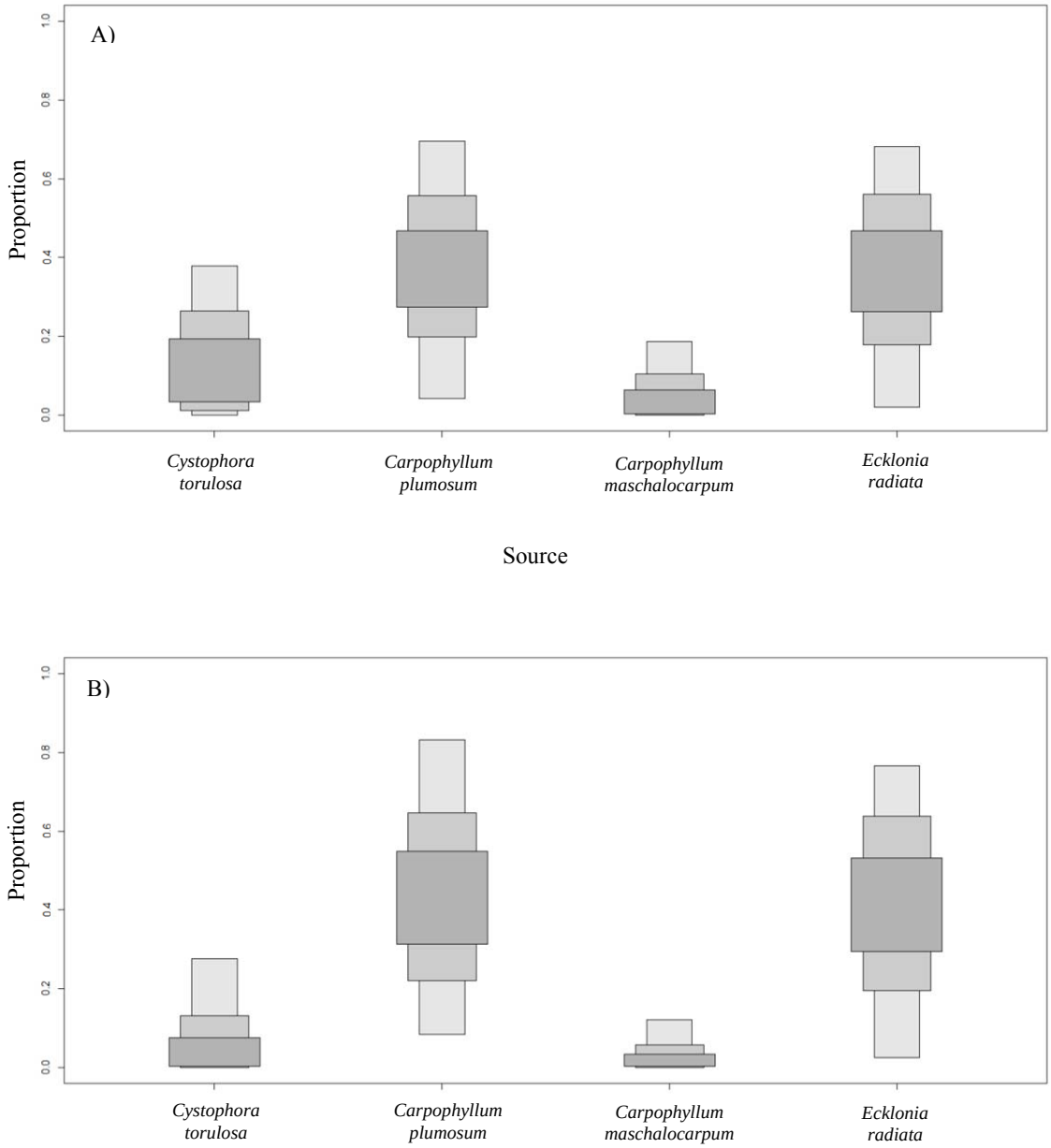


Figure 6.4. Results of SIAR (95, 75 and 50% credibility intervals) illustrating contribution ranges of brown seaweed species (*C. torulosa*, *C. plumosum*, *C. maschalocarpum* and *E. radiata*) to *E. chloroticus* diet. A) *E. chloroticus* gonad and B) *E. chloroticus* gut excluding the trophic enrichment factor from the analysis.

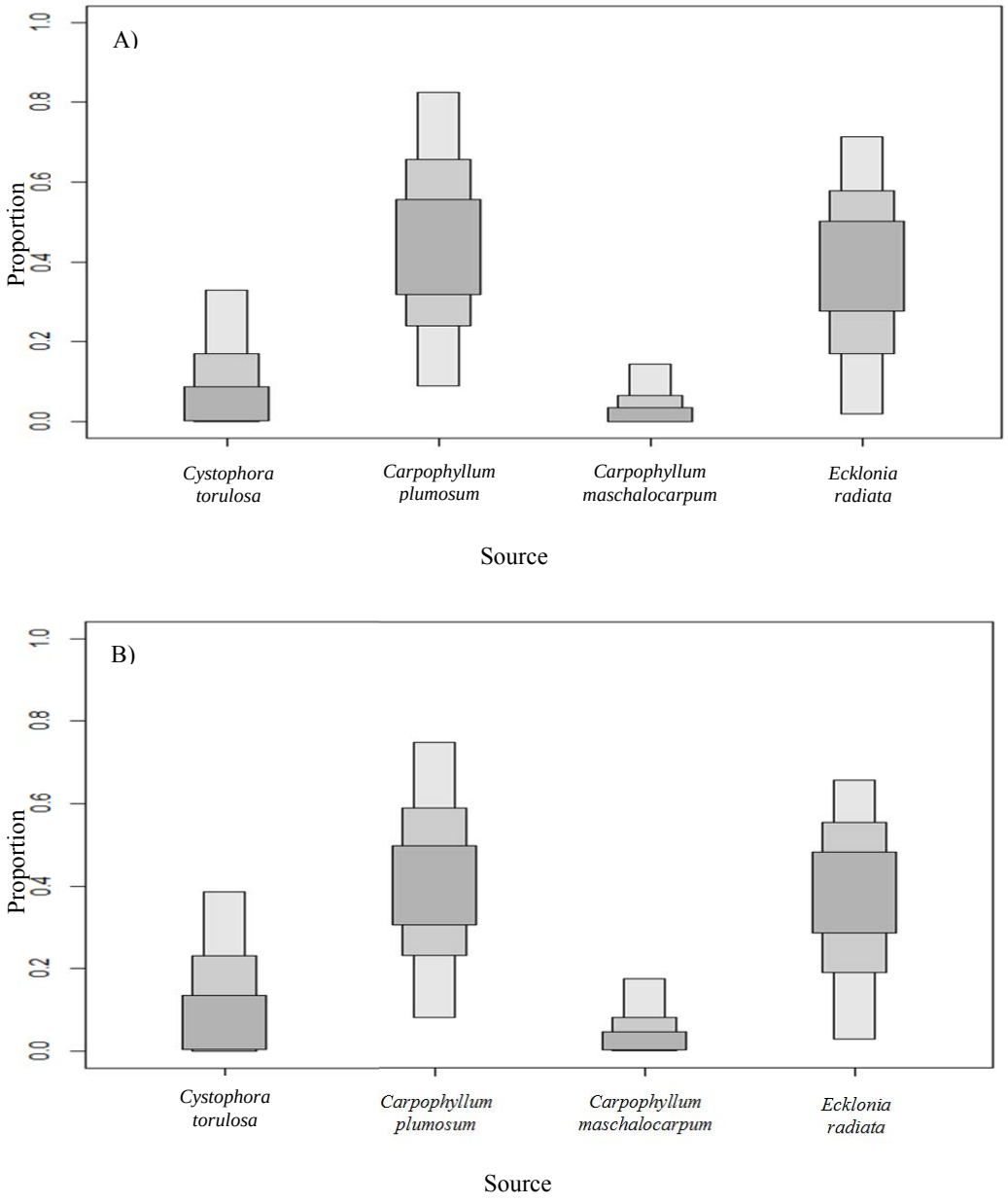


Figure 6.5. Results of SIAR (95, 75 and 50% credibility intervals) illustrating contribution ranges of brown seaweed species (*C. torulosa*, *C. plumosum*, *C. maschalocarpum* and *E. radiata*) to *E. chloroticus* diet. A) *E. chloroticus* gonad and B) *E. chloroticus* gut including the trophic enrichment factor in the analysis.

6.4 Discussion

The current study clearly showed that stable isotopes can be used to identify different seaweed species as food sources for the sea urchin *E. chloroticus* in north-eastern New Zealand. The findings suggest that the carbon assimilated by *E. chloroticus* was derived from *E. radiata* and *C. plumosum*, as similar $\delta^{13}C$ signatures were found between *E. chloroticus* gut and gonad and these brown seaweed species. Besides, the mixing model revealed a contribution of 80% from *E. radiata* and *C. plumosum* (40% each) to *E. chloroticus* diet and very poor contribution from the rest of the brown seaweeds analysed here. Furthermore, there was a clear variation in the stable isotope signatures between the four brown seaweed species, where the signatures of *E.radiata* (blade and stipe) and *C. plumosum* were very different to *C. torulosa* and *C. maschalocarpum* signatures.

The use of SIAR was advantageous to quantify the proportional contribution of the brown seaweed species to *E. chloroticus* diet. It was not surprising the high contribution of *E. radiata*, as it has been reported that *E. chloroticus* being primarily herbivorous, has preference for this kelp (Don, 1975; Schiel, 1982). However, the high contribution of *C. plumosum* to its diet was unexpected , as fucoid seaweeds are known to be more predominant in shallow waters, whereas *E. radiata* is more abundant below 10m together with *E. chloroticus* (Schiel, 1988; Shears & Babcock, 2004). Thus, it is possible that this fucoid is being consumed as drift algae by *E. chloroticus* in Matheson's Bay, probably arriving in the sea urchin areas after easterly storms.

Furthermore, it is striking the significant differences in the stable isotope signature found between the two *Carpophyllum* species, with *C. plumosum* presenting higher values of $\delta^{15}N$ and more negative values of $\delta^{13}C$ than *C. maschalocarpum* and also the low contribution of this last one to *E. chloroticus* diet. Differences in the lipid profile were also found between these two seaweed species, where *C. maschalocarpum* revealed lower amount of total and energy lipids than *C. plumosum* (Chapter 5). The results of the current study revealed more similar isotope signatures between *C. plumosum* and *E. radiata*, also being the main contributors to *E. chloroticus* diet. The findings of this study are supported by the differences found in the fatty acid (FA) composition between *E. radiata* and *C. maschalocarpum*, even though the FA profile between the two *Carpophyllum* species was not analysed (Chapter 2). A previous study on benthic marine primary producers indicated that the $\delta^{13}C$ can discern between algae and seagrass but it was not sufficient to distinguish

between red and brown algae (Hanson et al., 2010). The authors suggested that the use of an extra biomarker, like FA analysis, was required for a better discrimination between these primary producers. Therefore, stable isotope analysis needs to be complemented by other studies, such as FA analysis, in order to have a better understanding of the trophic interactions of marine systems (Guest et al., 2010; Kharlamenko et al., 1995). Thus, many studies have used these two different analyses as complementary tools to examine food webs in different ecosystems. For example, a research using multiple stable isotopes ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) and FA profiles established which primer producer (brown seaweed, red seaweed or seagrass) provide the major source of energy and nutrition in a temperate coastal environment (Crawley et al., 2009). Guest et al. (2010) analysed the spatial variation of SI and FA profiles of primary producers (brown seaweed) and two different consumers (invertebrates and fish). Consequently, this study highlights the importance of the use of FA analysis as a complement of the SI analysis to distinguish between these two *Carpophyllum* species.

Further studies are needed to better understand the dissimilarities found in this endemic genus of New Zealand, in terms of the stable isotope signature as well as biochemical composition of the different species of the genera *Carpophyllum*. A recent research in the Hauraki Gulf, New Zealand reported relatively higher values of $\delta^{15}N$ (~7.9‰) and lower values of $\delta^{13}C$ (~-13‰) for *C. maschalocarpum* collected in different locations within the Hauraki Gulf (Johnson et al., 2012), compared with the values found for *C. maschalocarpum* ($\delta^{15}N$=~5‰; $\delta^{13}C$=~11‰) in the current study. Even though both collections were made in the Hauraki Gulf, the collection sites for each study were different as well as the time of the year for the collection. The samples collected by Johnson et al. (2012) were from Fairchild Reef and Elephant Rock, in the outer part the Hauraki Gulf, sampled during November. Instead, the seaweed species in the current study were collected from the inner part of the Hauraki Gulf during February-March, possibly explaining the different results. This phenomenon has been also described for brown seaweed species collected in different locations relatively proximate to each other, where a variation in the isotope signatures was observed depending of the time of the year and sampling location (Dethier et al., 2013; Guest et al., 2010). Consequently, different collection times and sites and their respective environmental characteristics could explain the different results in the current study with previous findings. Furthermore, variation in the currents, tides as well as

salinity and contributions of fresh water can alter the carbon and nitrogen signatures in macrophytes (Dethier et al., 2013).

The isotopic composition of the two distinguishable parts of *E. radiata*, blade and stipe, showed no variation according with the results from a previous report of *E. radiata* collected in Australia (Guest et al., 2010). Thus, a small section of any part of *E. radiata* may be considered to represent the entire plant, providing a useful guide of how to process this brown seaweed species for trophic studies. Additionally, *E. chloroticus* tissues gut and gonad also showed no significant differences in the stable isotope signatures. A previous study on *Lytechinus variegatus* comparing the stable isotope signatures between these two organs, revealed differences in $\delta^{13}C$ values, where the gut showed relatively higher values (-16.18±0.48‰) than gonad (-17.40±0.47‰), but similar $\delta^{15}N$ values were found (Prado et al., 2012). However, this study used lab feeding experiments possibly explaining the differences with the current study where the sea urchins were collected from the wild. Thus, the no variation in the stable isotope signatures between these two organs found here, suggests a similar assimilation of carbon and nitrogen by *E. chloroticus* gut and gonads.

Differences in the $\delta^{15}N$ signatures between sea urchins and seaweeds were found, with an enrichment of ~2‰, agreeing with previous studies that suggested that even though in theory the enrichment is considered to be around 3-4‰, in practice it ranges between 2 and 5‰ (DeNiro et al., 1981; Prado et al., 2012). Moreover, *E. chloroticus* showed a trophic level (TL) of 1.5, a value slightly higher than expected for an entirely herbivorous (1992), suggesting that *E. chloroticus* is consuming non-algal organisms when they are grazing on kelp, feeding probably on the mobile epifauna, such as amphipods, isopods and gastropods (Taylor et al., 1994). Similar results were found for *Echinus esculentus*, presenting a TL of 1.7 indicating that this sea urchin eats other organisms in addition to algae (Fredriksen, 2003). However, this high trophic level could also be related to the bacteria living in their gut, fixing and incorporating nitrogen to the tissues, as it has been shown that sea urchins have bacteria able of fixing N_2 from the seawater and then incorporated into their tissues (Fong et al., 1980; Guerinot et al., 1977; Pinnegar et al., 2000). Further studies, such as lab feeding experiments and bacteria isolation, are needed to better understand this unpredicted high trophic level.

In conclusion, stable isotope analysis helped to comprehend the diet habits of *E. chloroticus* in Matheson's Bay, indicating a main contribution from *E. radiata* and *C.*

plumosum, probably drift algae, to the diet. These findings highlight the importance of the use of the mixing models as well as a complement the results with FA analysis to a major understanding of trophic interaction in marine environments. The results of the current study also suggested that *E. chloroticus* is possibly eating microorganisms that grow on the seaweeds not behaving as a complete herbivorous grazer or containing bacteria living in the gut, fixing nitrogen. However, further studies are needed to better understand the isotopic composition of more seaweed species around the Hauraki Gulf as well as bacteria possibly fixing nitrogen and incorporate these values to the mixing model. Furthermore, researches on the seasonal variation of stable isotope signature are also necessary both on seaweeds and sea urchins considering also different environmental factors and several locations around the Hauraki Gulf.

Chapter 7

Fatty acid profile of two sea urchin species *Evechinus chloroticus* and *Arbacia dufresnii*.

7.1 Introduction

Fatty acid (FA) analysis is a powerful ecological tool for discerning trophic relationships in terrestrial and aquatic ecosystems (Budge et al., 2006; Dalsgaard et al., 2003; Iverson, 2009; Kelly et al., 2008). It has advantages over traditional stomach content analysis in that it can be used to examine either long-term dietary sources or those of recent feeding activity depending on the type of tissue selected (Fukuda et al., 2001; Sargent et al., 1988). During the digestion process, consumers break down their dietary lipids into their constituent FAs, considered as the "building blocks" of lipids, and these are incorporated relatively unchanged into the tissues of the consumer (Howell et al., 2003; Iverson, 2009; Lee et al., 1971). As different dietary sources have different FA composition, such as the presence of unique FAs or ratios, FAs can be used as biomarkers making it possible to identify dietary sources such as diatoms, dinoflagellates, bacteria, land plants and macroalgae (Iverson, 2009; Iverson et al., 2004; Kelly et al., 2008; Parrish et al., 2000).

Many studies have used the tools of FA analysis to understand animal diets and trophic relationships in marine benthic habitats (Iverson, 2009; Kelly et al., 2012). In these environments, primary producers such as unicellular phytoplankton and seaweeds (macroalgae) typically produce *de novo* fatty acids, ranging from 14 to 24 carbons with various degrees of unsaturation (Iverson, 2009). Photosynthetic organisms are able to produce long-chain polyunsaturated fatty acids (PUFA) such as C20:5(n-3), C20:4(n-6), C18:1(n-9) and C18:4(n-3), which characterise brown seaweeds (Iverson, 2009; Kelly et al., 2012; Khotimchenko, 1998). These fatty acids flow through the marine food web to higher trophic levels as, in general, animals are not capable of inserting a double bond between the terminal methyl and the n-9 carbon to form PUFA (Iverson et al., 2004).

Sea urchins, however, may be capable of modifying dietary FA and synthesizing long chain FA from lower FA precursors due to the dual function of their gonads (Kelly et al., 2012; Kelly et al., 2008; Liyana-Pathirana et al., 2002), as both a nutritive and reproductive organ (Hughes et al., 2006; Walker et al., 2001). Feeding experiments have shown that

Strongylocentrotus droebachiensis is capable of biosynthesising FAs that were not present in the diets, highlighting the ability of sea urchin gonads in biosynthesising and acting as a specific site for lipid storage (Kelly et al., 2008; Liyana-Pathirana et al., 2002). Sea urchins are also capable of selective retention of some FAs that are present in low or trace concentrations in the diets (Castell et al., 2004; Kelly et al., 2012; Kelly et al., 2008). For example, *Psammechinus miliaris* selectively retained the essential FA C20:4(n-6) (Arachidonic Acid or ARA) and C20:5(n-3) (Eicosapentaenoic Acid or EPA) when these PUFAs were present in very low concentrations in the diets (Cook et al., 2000; Kelly et al., 2012).

FA analysis is also a useful tool for discerning feeding habits in different marine environments. *P. miliaris*, for example, showed a clear variation in the gonadal FA composition of two contrasting populations from the Scottish west coast (Hughes et al., 2005), with higher levels of ARA and EPA in the subtidal population associated with a brown algal diet, whereas the intertidal population contained higher levels of C22:6(n-3), associated with filter feeding invertebrates and C18:4(n-3) related to green algae. Hence, the subtidal population was feeding on brown algae suggesting an herbivorous diet, while the intertidal population was more omnivorous feeding on invertebrates and green algae. Thus, the FA composition helped to understand the feeding habits, showing that some species of sea urchins, depending on their distribution and the availability of food, show a more omnivorous and even carnivorous feeding behaviour than herbivorous habits.

Evechinus chloroticus (Valenciennes, 1846), also known as kina, is endemic to the New Zealand region, ranging along the entire coast of the New Zealand mainland and nearby islands (Dix, 1970; Mortesnsen, 1943; Pawson, 1961) and is the most abundant regular echinoid on reefs in northern New Zealand (Andrew, 1988). Kina is normally found in water less than 12-14 metres deep and is typically a rocky bottom dweller, but it may also be found on other hard stable substrates or on shelly sand, fine sand and mud as well as in the rocky intertidal zone (Dix, 1970; Fell, 1952; Morton et al., 1968). Generally, its abundance increases with increasing exposure to wave action, except in the most exposed locations, where densities are reduced (Barker, 2013; Choat et al., 1987; Choat et al., 1982). Kina is considered one of the largest sea urchins with a maximum test diameter of 16-17 cm (Barker, 2013), and has an annual reproductive cycle. Gametogenesis starts in the late austral winter, with gonad size increasing during the austral spring, reaching peaks in mid-summer when

spawning occurs (Brewin et al., 2000; Walker, 1982). Most ecological research on *E. chloroticus* has concentrated on the effects of its herbivory on algal communities, demonstrating a strong inverse correlation with the abundance of large brown algae (laminarians and fucoids) (Andrew et al., 1982; Ayling, 1978; Choat et al., 1982; Dix, 1970; Kerrigan, 1987; Walker, 1977). Laboratory feeding experiments suggest that *E. chloroticus* consumes a large range of brown seaweeds, but having a preference for *E. radiat*a (Barker, 2013; Schiel, 1982). However, when seaweed is scarce it can feed on a wide range of encrusting organisms such as sponges (Ayling, 1978).

In contrast, the green sea urchin *Arbacia dufresnii* (Blainville, 1825), is one of the most abundant echinoids along the southern tip of South America, distributed from the mouth of the Rio de la Plata (35º S) to Patagonia and the Falkland Islands and around Tierra del Fuego, to Puerto Montt in Chile (41° S) at depths of 0-315 meters (Bernasconi, 1953). In the study area in Nuevo Gulf, Argentina,, it is commonly found in the presence of *Macrocystis* algae and shares habitat with another sea urchin species *Pseudechinus magellanicus* in Argentina (Bigatti et al., 2006; Teso et al., 2009). *A. dufresnii* is relatively small, presenting a range of adult size from 1.6 to 4.4 cm (Brogger et al., 2010). This sea urchin species develops an annual reproductive cycle with two spawning events, one partial spawning in spring and another one during summer (Brogger et al., 2010, Epherra et al., unpublished data). *A. dufresnii* was previously considered primarily carnivorous in Argentinean waters, when algae are absent (Penchaszadeh et al., 1998). Analysis of the gut contents indicated that it consumes mainly invertebrates such as serpulid polychaetes, barnacles, sponges and bivalves (Vasquez et al., 1984). However, this sea urchin species can be considered herbivorous when algae constitute the only feeding source, grazing mainly on *Macrocystis pyrifera* (Vasquez et al., 1984), and laboratory experiments indicate that *A. dufresnii* from Nuevo Gulf feeds particularly on *Undaria pinnatifida* (Teso et al., 2009). A study using stable isotope analysis to determine the trophic position of *A. dufresnii*, revealed this sea urchin species to be herbivorous in three different gulfs in Patagonia (Galván et al., 2009). Thus, due to all this evidence, *A. dufresnii* can be considered as omnivorous with a varied diet depending on the food availability in the environment where it is found. Therefore, particularly in Nuevo Gulf, Patagonia Argentina (the study site), *A. dufresnii* is principally herbivorous during all year revealed by analysis of the gut contents (Cadierno et al., 2011).

The aim of the current study was to apply FA analysis to compare mature gonads of these two herbivorous species of sea urchins, *E. chloroticus* and *A. dufresnii* that live in different environments and have distinct species of brown seaweed as diet choices. Thus, further understand the use of this tool in the identification of sea urchin species with variation in their diets and environments. The goal of this study was determine if the FA composition of their gonads was different and therefore link that composition to the different diet choices present in those habitats.

7.2 Methodology

7.2.1 Sample collection and store

In total 20 sea urchins of *E. chloroticus* were collected by snorkelling from Rakino Island (36^0 72'S, 174^0 95'E), Hauraki Gulf, New Zealand, during January 2010 when the gonad size reaches the highest peak (Brewin et al., 2000; Walker, 1982). After collection, the sea urchins were transported live to the University of Auckland in plastic buckets filled with chilled seawater. Wet weight (blotted with a paper towel to nearest mg) and test diameter (using a Toledo Vita calliper to nearest mm) of the whole animal were recorded prior to dissection. The test was cut around the equator to separate the oral and the aboral surfaces and the Aristotle's lantern was removed and weighed together with the wet test. The 5 gonads of each sea urchin were removed using tweezers and after being weighed they were stored separately in 50 ml polypropylene tubes in the -80 freezer until analysis. During the collection of *E. chloroticus* the animals were in the mature/spawning season (Walker, 1982) making possible to distinguish males and females by gonad colour (female orange-like, male white). From the 20 *E. chloroticus* collected and stored, 10 of them were gender identified (7 females and 3 males) and they were used for the fatty acid (FA) analysis; the remaining 10 gonads were in the post-spawning stage making it impossible to identify gender not being used for this analysis. Gonads were lyophilized for 72 hours to a constant mass in a freeze-dryer (VirTis Bench Top 2k) then cryogenically grounded using an MM301 Mixer Mill (Retsch), which resulted in a ground particle size of <5 μm (Retsch). Lyophilized, ground samples were stored in sealed polypropylene 10ml tubes under desiccant at -20°C until FA analysis.

Thirty *A. dufresnii* were collected by SCUBA diving at depths of 5-10 metres (42°46'44" S, 64°59'52"W) during September 2009-2010 from Punta Cuevas, Nuevo Gulf,

Patagonia, Argentina when their gonads showed the mature stage of the reproductive cycle (Brogger et al., 2010 Epherra et al., unpublished data). After collection the sea urchins were immediately transported live to the laboratory at the University of Patagonia, San Juan Bosco, in plastic buckets filled with seawater. Wet weight (blotted with a paper towel to nearest mg) and test diameter (using an Essex calliper to nearest mm) of the whole animal were recorded prior to dissection. The test was cut around the equator to separate the oral and the aboral surfaces. The gender discrimination was done by gonad colour, where ovaries range from light to dark purple and testes show a creamy-orange colour (Brogger et al., 2010). The gonads of each sea urchin were dissected out using tweezers. Of the 30 collected sea urchins 20 of them (ten per year) were weighed and then the individual gonads were stored separately in Eppendorf tubes in the -80 freezer for later FA analysis. Frozen gonads contained in Eppendorf tubes were then sent to INTI (National Institute of Industrial Technology, Mar del Plata, Argentina) where they were lyophilized for 48 hours to constant mass using a Labcono Freeze dry System (Freezone 6 Lts) and they were sent back to the Laboratory at the University of Patagonia San, Juan Bosco, and kept in a -20°C Freezer until the FA extraction.

7.2.2- Fatty acid analysis

Fatty acid analysis was conducted using the one-step reaction from Lepage and Roy (1986), also known as the direct transesterification method. Both species were analysed using the same protocol in the laboratories of both universities. Lyophilized gonads (~50 mg for *E. chloroticus* and ~30 mg for *A. dufresnii*) were weighed using a Sartorius balance (LE244S; max 240g; d=0.1mg) in pre-weighed glass tubes (KIMAX, culture tubes; 16x125mm; 20ml with phenolic caps and cemented rubber liners). An internal standard consisting of ~70 µg of tridecanoic acid (C13:0) and tricosanoic acid (C23:0), dissolved in 2 ml of methanol-toluene 4:1 (v/v) was precisely weighed and added to the *E. chloroticus* samples. These FAs have been used as internal standards in previous studies of *E. chloroticus* (Chen, 2005) and other sea urchin species (Cook et al., 2007). No internal standards were used for *A. dufresnii* samples due to delivery problems in getting the standards in Patagonia. A small magnetic stirring bar was added to each tube and, while stirring, 200 µl of acetyl chloride was slowly added over a period of 1 min. The tubes were tightly closed and subjected to methanolysis at 100 °C for 1 hour; the tubes were re-weighed after heating to check for leakage.

After the tubes had been cooled in tap water to room temperature, 5 ml of 6% K_2CO_3 solution was slowly added to stop the reaction and neutralise the mixture. The tubes were then shaken and centrifuged for five minutes at 2000 RPM, and an aliquot of the toluene upper phase was transferred to an autosampler vial. The toluene aliquots obtained from the *A. dufresnii* samples were dried under N_2 and were then mailed in sealed vials to the University of Auckland for the GC-MS analysis. These toluene aliquots were then injected into an Agilent GC 7890 gas chromatograph equipped with a mass spectrometry detector (MSD 5975c). Separation was performed with a 35-m fused silica column (internal diameter of 0.32 mm), wall-coated with 0.20 mm SP-2330, and with helium as the carrier gas. After injection at 60 °C, the oven temperature was raised to 150 °C at a rate of 40 °C min^{-1}, then to 230 °C at 3 °C min^{-1}, and finally held constant for 30 min. Fatty acid methyl ester (FAME) peaks were identified by comparing their retention times with those of authentic 37 fatty acid methyl ester (FAME) standards (Supelco Inc.). The mass spectra of FAMEs not present in the standard mix were compared with those from the National Institute of Standards and Technology mass spectra library (NIST MS Search 2.0), together with the Lipid Library (Christie, 2012). Due to the absence of the internal standard in the *A. dufresnii* samples, the relative content (as percentage) of the total FA in the samples was used for the analysis and was determined using the peak areas of fatty acids. The identified FAs were then grouped into Saturated FA (SFA: FA with no double bond in the carbon chain), Monounsaturated FA (MUFA: FA with one double bond present) and Polyunsaturated FA (PUFA: FA with two or more double bonds present) for the analysis.

7.2.3- Statistical analysis

FA profiles were compared between sea urchin gonads of the two different species (*E. chloroticus* and *A. dufresnii*) and between genders using Primer v6.1.12 (Clarke et al., 2006) with the PERMANOVA+ v1.0.1 add-on (Anderson, 2003). The data (relative content as proportion of the total FA) was left untransformed and converted into similarity matrices using Euclidean distances. Similarity patterns in the data were visualised using multidimensional scaling (MDS) with the addition of superimposed clusters at different similarity levels. Multivariate two-way PERMANOVA, using 9999 permutations and the unrestricted permutation of raw data as the permutation method, was performed to examine differences in the FA profile between species and genders. Differences in *A. dufresnii* FA data from 2009 and 2010 were also tested using two-way multivariate PERMANOVA

analysis revealing significant differences between genders (Pseudo-$F_{1,16}$=20.83, P(perm)=0.0001) but no significant differences between years (Pseudo-$F_{1,16}$=1.86, P(perm)=0.1523) and the interaction term was also non-significant (Pseudo-$F_{1,16}$=1.33, P(perm)=0.2547). For this reason, both years (2009-2010) were combined for *A. dufresnii* in the species comparison. Univariate PERMANOVA analysis was performed to test differences in the total percentage of SFA, MUFA and PUFA and multivariate PERMANOVA analysis was used to test differences in the profiles of SFA, MUFA and PUFA between species and genders. The similarity percentages procedure (SIMPER) was used to explore the differences between species and gender by determining which individual SFA, MUFA and PUFA contributed most to the differences in the multivariate signature. Univariate one-way PERMANOVA analysis was used to test the differences of each of these important FAs. This approach was used as this test avoids the assumptions of the traditional one-way ANOVA or analysis of variance (Underwood, 1997) and assumes only that the samples are exchangeable, i.e. independent and identically distributed, under a true hypothesis (Anderson, 2003).

7.3 Results

A combined total of 60 different FAs were identified in the gonads of *E. chloroticus* and *A. dufresnii*; however, not all of the FAs were present in both species (Table 7.1). The FAs that were present in more than 2% of the total FAs are shown in the Figure 7.1.

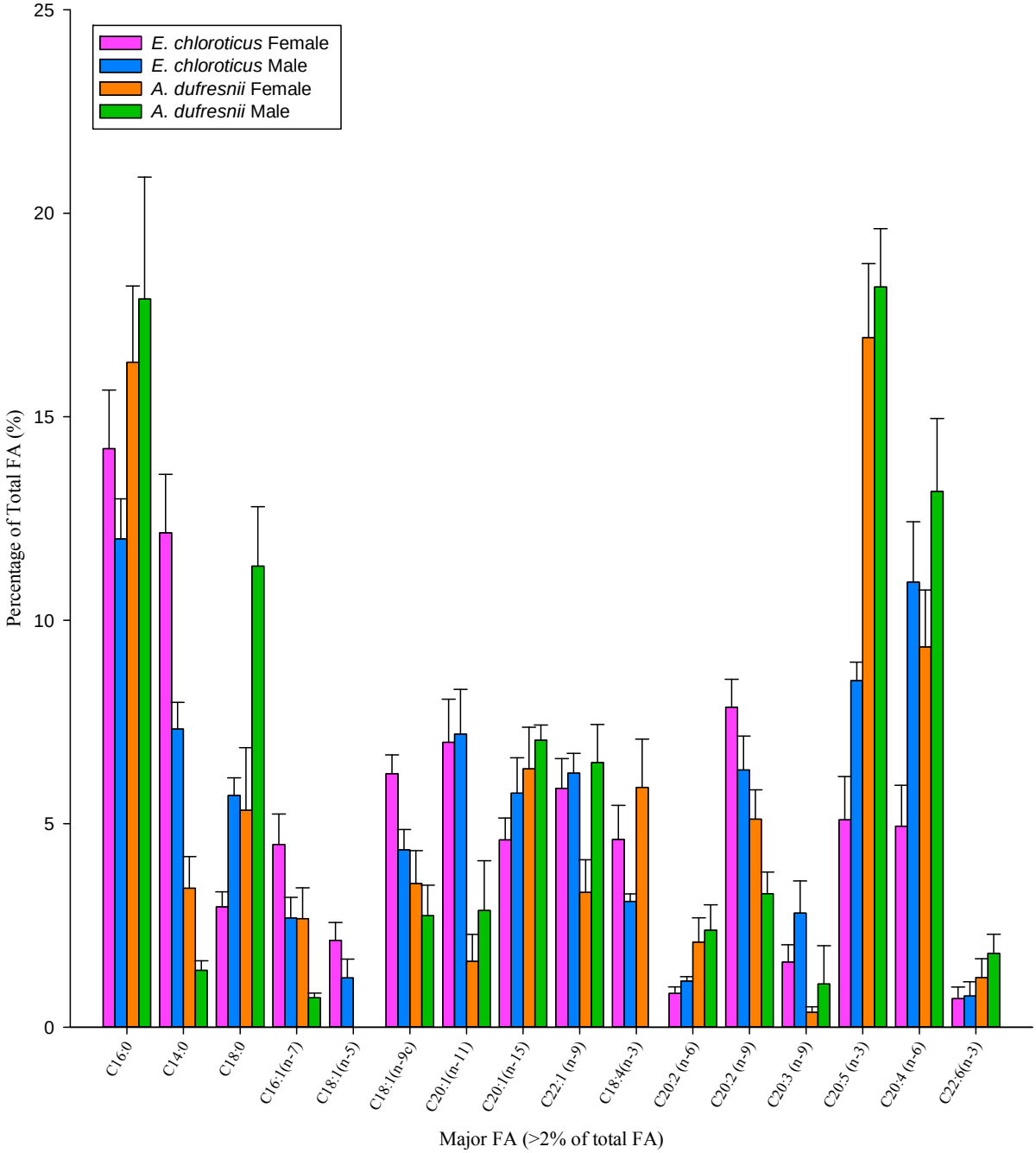


Figure 7.1. Fatty acids contributing more than 2% of the total FA in the gonads of male and female *E. chloroticus* and *A. dufresnii*. Results are mean values ± standard deviation (n=7 females and n=3 males of *E. chloroticus*; n=12 females and n=8 males of *A. dufresnii*).

Table 7.1. Fatty acid composition (% of the total FA) of sea urchin gonads of female and male of two different species: *E. chloroticus* and *Arbacia dufresnii* from New Zealand and Argentina, respectively. Data represent the mean (±SE) of 7 females and 3 males of *E. chloroticus* and 12 females and 8 males of *A. dufresnii*.

	Evechinus chloroticus		***Arbacia dufresnii***	
FA	**Female**	**Male**	**Female**	**Male**
C12:0	0.05±0.01	0.02±0.00	ND	ND
C14:0	12.15±1.44	7.33±0.66	3.42±0.78	1.40±0.23
12-MeC14:0	ND	ND	0.17±0.06	ND
C14:1	0.06±0.01	0.03±0.01	ND	ND
C14:1(n-5)	0.56±0.12	0.24±0.05	ND	ND
C15:0	0.32±0.08	0.43±0.07	0.97±0.22	0.45±0.06
C16:0	14.22±1.44	12.00±0.99	16.34±1.87	17.89±2.99
C16:1	0.16±0.02	0.08±0.02	0.14±0.04	ND
C16:1	0.15±0.01	0.13±0.12	0.16±0.02	ND
C16:1	0.64±0.07	0.25±0.09	0.26±0.03	ND
C16:1(n-7)	4.49±0.75	2.68±0.51	2.67±0.76	0.72±0.11
C17:0	0.09±0.02	0.16±0.03	0.50±0.07	0.55±0.04
C16:2(n-6)	0.06±0.03	0.04±0.02	0.29±0.13	ND
C16:3(n-6)	0.02±0.00	0.01±0.00	ND	ND
C16:3(n-3)	0.06±0.02	0.19±0.01	0.52±0.14	ND
C18:0	2.96±0.37	5.69±0.43	5.33±1.54	11.33±1.46
C18:1(n-5)	2.14±0.44	1.21±0.46	ND	ND
C18:1(n-9t)	0.58±0.08	0.42±0.02	1.65±0.98	0.35±0.30
C18:1(n-9c)	6.23±0.46	4.36±0.50	3.53±0.58	2.85±0.44
C18:1	0.67±0.09	0.85±0.12	ND	ND
C18:2	0.08±0.01	0.05±0.01	ND	ND
C18:2(n-6t)	1.20±0.32	0.67±0.08	ND	ND
C18:2(n-6c)	0.26±0.05	0.28±0.05	1.52±0.40	1.48±0.18
C19:1	0.20±0.04	0.25±0.03	0.48±0.11	0.29±0.25
C18:3	0.34±0.10	0.25±0.02	ND	ND
C19:1	0.17±0.04	0.13±0.01	ND	ND
C18:3(n−6)	0.05±0.01	0.03±0.01	0.34±0.16	0.36±0.30
C18:3(n-3)	0.33±0.09	0.15±0.04	1.41±0.60	0.34±0.22
C20:0	0.65±0.16	0.64±0.17	1.28±0.24	1.96±0.23
C20:1(n-15c)	4.60±0.54	5.75±0.87	6.35±1.02	7.05±0.37
C18:4(n-3)	4.61±0.84	3.09±0.19	5.89±1.18	ND
C20:1(n-11)	7.00±1.06	7.20±1.10	ND	1.98±0.33
C20:1(n-9)	1.80±0.29	1.58±0.22	1.20±0.32	1.27±0.28
C20:2(n-9)	7.86±0.69	6.32±0.83	5.11±0.72	3.28±0.53
C20:2	1.20±0.18	1.20±0.09	0.52±0.11	0.46±0.10
C20:2	0.74±0.27	0.53±0.05	ND	ND
C20:2	0.28±0.05	0.14±0.03	ND	ND
C20:2(n-6)	0.83±0.16	1.14±0.11	2.09±0.60	2.38±0.62
Unknown PUFA	ND	ND	ND	0.27±0.10
C20:3(n-9)	1.60±0.42	2.80±0.79	0.37±0.14	1.06±0.94
C21:1	1.08±0.15	0.95±0.09	1.17±0.37	ND
C20:3(n-6)	0.21±0.02	0.36±0.05	0.54±0.14	0.48±0.07
C20:3	ND	ND	0.47±0.20	ND
C20:4(n-6)	4.94±1.00	10.93±1.49	9.34±1.40	13.17±1.79
C20:3(n-3)	1.04±0.20	1.06±0.11	1.72±0.35	1.12±0.25
C22:0	0.04±0.01	0.05±0.01	0.18±0.07	0.26±0.14
C20:4(n-3)	0.45±0.16	1.05±0.27	0.35±0.13	ND
C22:1	ND	ND	0.15±0.19	ND
C22:1(n-9)	5.87±0.73	6.25±0.48	3.31±0.80	6.51±0.93
C20:5(n-3)	5.10±1.06	8.51±0.46	16.95±1.81	18.19±1.43
C23:1	0.21±0.05	0.19±0.03	ND	ND
C20:5	0.28±0.24	0.38±0.16	0.48±0.30	ND
C22:4(n-6)	0.19±0.05	0.27±0.06	0.54±0.20	ND
C24:0	0.04±0.03	0.03±0.01	ND	0.25±0.25
Unknown PUFA	ND	ND	0.25±0.33	ND
C22:3 or C22:4	0.09±0.01	0.16±0.03	ND	ND
C24:1(n-9c)	0.18±0.06	0.14±0.04	0.81±0.37	0.46±0.53
Unknown PUFA	0.13±0.02	0.54±0.09	ND	ND
Unknown PUFA	0.03±0.00	0.03±0.01	ND	ND
C22:6(n-3c)	0.70±0.28	0.77±0.35	1.22±0.46	1.82±0.47
SFA	**30.53±2.35**	**26.34±0.98**	**28.18±3.17**	**34.10±4.51**
MUFA	**36.79±1.74**	**32.70±1.22**	**21.88±1.61**	**21.48±1.87**
PUFA	**32.68±2.91**	**40.96±1.43**	**49.93±3.29**	**44.42±3.04**

The FA profile of sea urchin gonads was found to be statistically different between the two species (*E. chloroticus* and *A. dufresnii*) as well as between genders in both species; the interaction term was also statistically significant (Table 7.2). The MDS plot clearly showed a separation between species and genders, where the samples were grouped in two different clusters, the first cluster grouped the 2 species and the second cluster grouped females and males within each species (Fig. 7.2). Due to the large effect of species x gender interaction found, the effect of gender was examined for each species separately.

Table 7.2. Results of multivariate two-way PERMANOVA comparing FA profile between female and male sea urchin gonads from two different species: *Evechinus chloroticus* and *Arbacia dufresnii* from New Zealand and Argentina, respectively. Significant results ($p<0.05$) are shown in bold.

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	2114.80	2114.80	124.58	**0.0001**	9941
Gender	1	680.78	680.78	40.10	**0.0001**	9931
Species x Gender	1	122.02	122.02	7.19	**0.0023**	9948
Residual	26	441.38	16.98			
Total	29	3359				

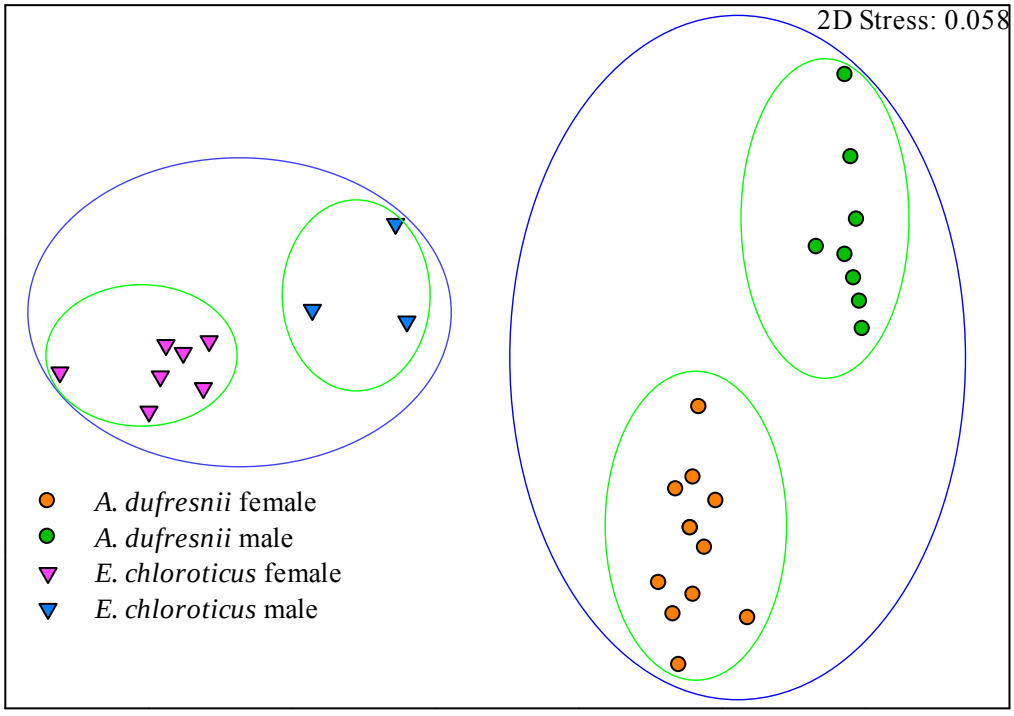


Figure 7.2. Multidimensional scaling (MDS) plot of Euclidean similarities of the gonadal FA profile of *E. chloroticus* female and male and *A. dufresnii* female and male with superimposed clusters at similarity levels of 15% indicating homogenous cluster 1 (species in blue) and 10% indicating homogenous cluster 2 (genders in green).

The FA profile of *E. chloroticus* gonads consisted of 55 different FAs (Table 7.1) regardless of gender. In total 9 SFAs, were identified corresponding to ~26-30% of the total FA (Table 7.1; Fig 7.3), with C14:0 and C16:0 as the dominant SFAs (>7% of total FA) in both male and female gonads (Table 7.1; Fig. 7.1). Nineteen identified MUFAs, contributed ~32-36% of the total FA (Table 7.1; Fig, 7.3), 6 were found to be present in more than 2% of the total FA, and they were: C16:1(n-7), C18:1(n-5), C18:1(n-9c), C20:1(n-11), C20:1(n-15) and C22:1(n-9) regardless of gender (Table 7.1; Fig. 7.1). PUFAs were the dominant type of FA identified (27/55 FA) in *E. chloroticus* female (~32% of the total FA) and male (~41% of the total FA) (Table 7.1; Fig. 7.3). The dominant PUFAs in both genders were C18:4(n-3), C20:2(n-9), C20:3(n-9), C20:4(n-6) and C20:5(n-3) (Table 7.1; Fig. 7.1).

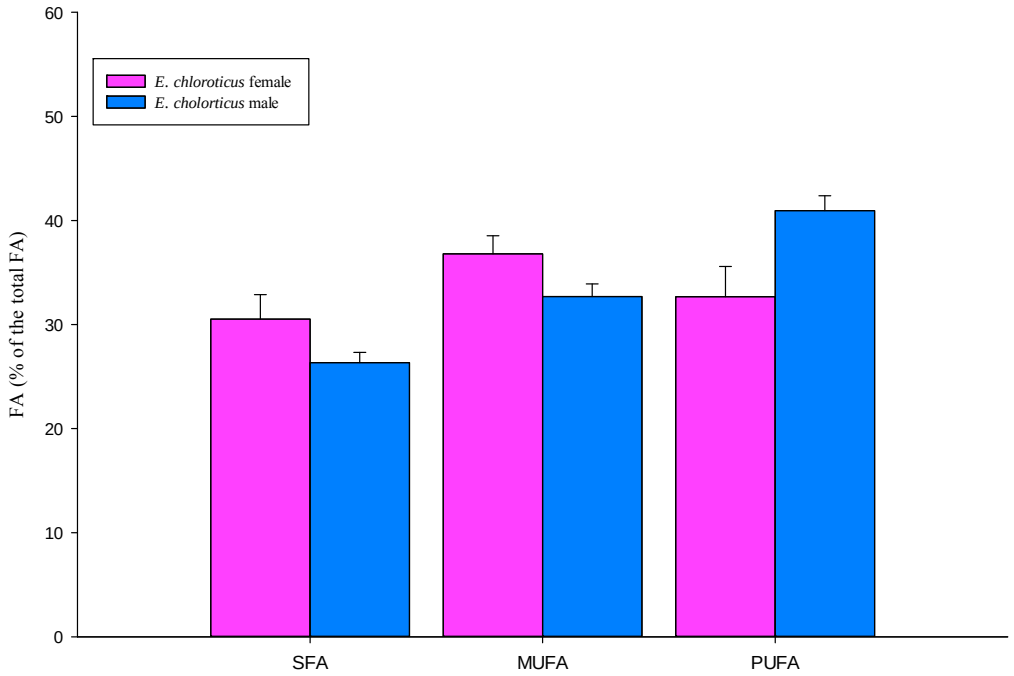


Figure 7.3. SFA, MUFA and PUFA percentages (% of total FA) of *E. chloroticus* female and male gonads, from Rakino Island, New Zealand. Data represent the mean (±SE) of 7 females and 3 males.

A. dufresnii gonads showed a FA profile of 41 identified FAs for females and 30 identified FAs for males (Table 7.1). Females and males contained 8 identified SFAs, contributing ~28-34% to the total FA (Table 7.1; Fig 7.4), with the dominant SFAs C14:0, C16:0 and C18:0 (~1-18% of total FA), regardless of gender (Table 7.1; Fig 7.1). Thirteen different MUFAs were identified in female *A. dufresnii* and 9 in males, corresponding to ~21% of the total FA (Table 7.1; Fig. 7.4), with the highest percentages of C16:1(n-7), C18:1(n-9c), C20:1(n-15), C20:1(n-11) and C22:1(n-9) in both genders (Table 7.1; Fig 7.1). PUFA comprised ~44-50% of the total FA; Table 7.1; Fig. 7.4), with 20 different PUFAs in females and 13 in males (Table 7.1). C20:2(n-9), C20:2(n-6), C20:4(n-6) and C20:5(n-6) were present in the highest percentages regardless of gender, with the addition of C18:4(n-3) in female gonads and C22:6(n-3) in male gonads (Table 7.1; Fig. 7.1).

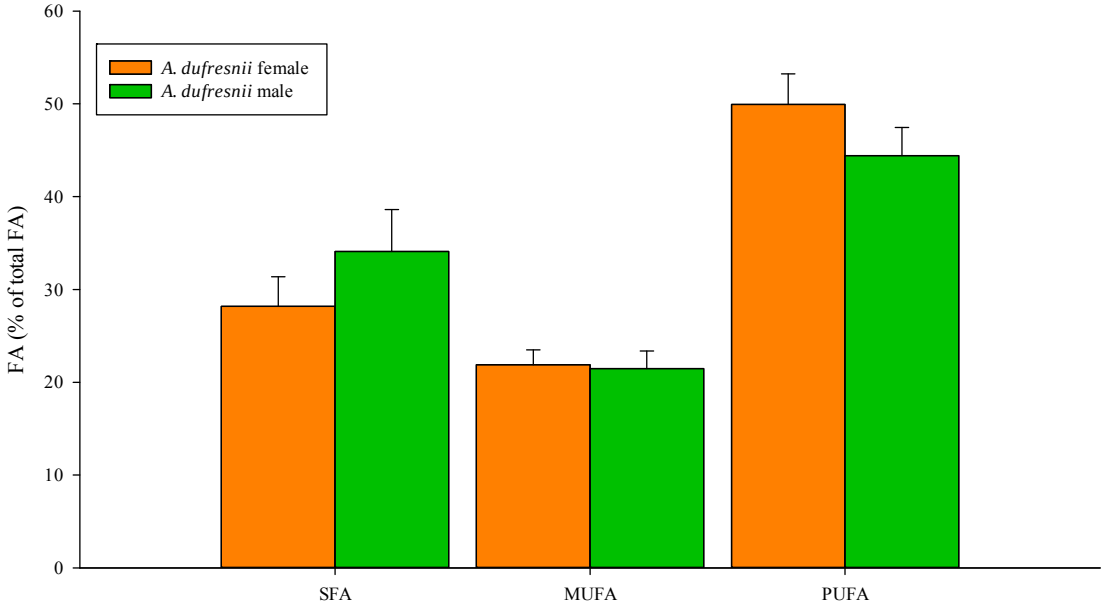


Figure 7.4. SFA, MUFA and PUFA percentages (% of total FA) of *A. dufresnii* female and male gonads, from Nuevo Gulf, Argentina. Data represent the mean (±SE) of 12 females and 8 males.

Significant differences were found in the total percentage of SFA between genders but not between species and the interaction term was also significant showing a different pattern between males and females in the two species (Table 7.3.A). *E. chloroticus* females showed higher total percentage of SFA (~30% of total FA) than males (~26% of total FA) and the opposite occurred in *A. dufresnii*, where the total percentage of SFA was smaller in females (~28% of total FA) than in males (~34% of total FA) (Table 7.1; Table 7.3.A). The SFA profile was also significantly different between species as well as between genders, as revealed by two-way PERMANOVA analysis and shown in the MDS plot (Table 7.3.B, Fig. 7.5). SIMPER analysis revealed that the dominant SFAs, C14:0, C16:0 and C18:0 contributed to the differences between sea urchin species (Table 7.4.A). Univariate one-way analysis showed that the percentages of C14:0 were higher in *E. chloroticus* gonads (>7% of total FA) than *A. dufresnii* gonads (<4% of total FA) (Table 7.1, Fig. 7.1, Table 7.4.A). The percentages of C16:0 were also significantly different between sea urchin species, being higher in *A. dufresnii* (>16% of total FA) than *E. chloroticus* (<14 % of total FA) (Table 7.1; Table 7.4.A). C18:0 was also present in higher percentages in *A. dufresnii* (5-11% of total FA) than *E. chloroticus* (3-5% of total FA) (Table 7.1). The differences between genders were attributed to the same dominant SFA, C14:0 which was 2x higher in females than males in both species (Table 7.1; Table 7.4.A.B). C16:0 was present in higher percentages in *E. chloroticus* females (14% of total FA) than males (12% of total FA) but it did not contribute to the differences between *A. dufresnii* genders (Table 7.1; Table 7.4.A.C). C18:0 was present in higher percentages in males (>5.5% of total FA) than females (<4% of total FA), regardless of species (Table 7.1; Table 7.4.B.C).

Table 7.3. Results of A) Univariate two-way PERMANOVA of SFA percentage and B) Multivariate two-way PERMANOVA of SFA profile comparing the gonads of two sea urchin species *E. chloroticus* and *A. dufresnii* and genders. All the saturated fatty acids identified were included in the analysis. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	10.88	10.88	0.99	0.3439	9835
Gender	1	55.56	55.56	5.01	**0.0362**	9824
Species x Gender	1	149.16	149.16	13.46	**0.0018**	9822
Residual	26	288.15	11.08			
Total	29	503.75				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	626.11	626.11	90.03	**0.0001**	9941
Gender	1	233.43	233.43	33.56	**0.0001**	9951
Species x Gender	1	49.28	49.26	7.09	**0.0042**	9944
Residual	26	180.82	6.96			
Total	29	1089.60				

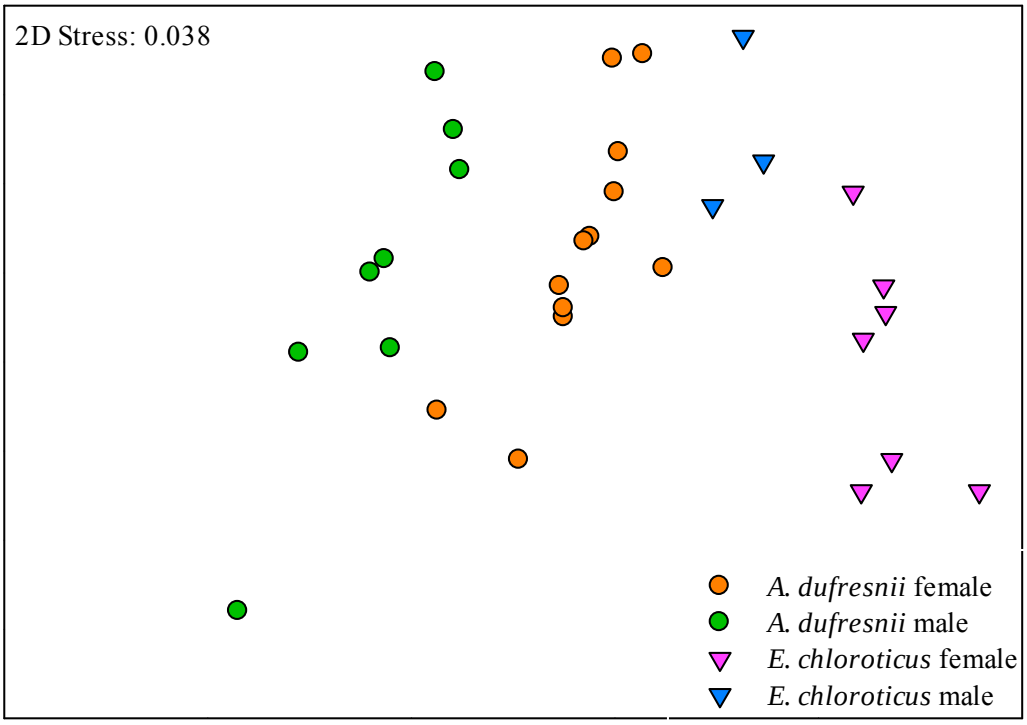


Figure 7.5. Multidimensional scaling (MDS) plot of Euclidean similarities of the gonadal SFA profile of *E. chloroticus* female and male and *A. dufresnii* female and male.

Table 7.4. Contribution of individual SFA to multivariate differences A) between species: *E. chloroticus* and *A. dufresnii* and between genders B) *E. chloroticus* males and females and C) *A. dufresnii* females and males as determined by SIMPER. Pseudo-F, P(perm) and Unique perm were obtained in two-way PERMANOVA on a single variable. Significant results ($p<0.05$) are shown in bold.

A)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
E. chloroticus & *A. dufresnii*	73.06	C14:0	10.7	2.61	69	2.26	68.29	68.29	197.93	**0.0001**	9856
		C16:0	13.6	17	17	0.63	16.83	85.12	15.75	**0.0001**	9834
		C18:0	3.78	7.73	13.7	0.82	13.51	98.64	12.64	**0.0022**	9840

B)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	47.47	C14:0	12.1	7.33	25.3	1.61	62.52	62.52	29.50	**0.008**	120
		C18:0	2.96	5.69	7.73	2.69	19.11	81.63	105.84	**0.009**	120
		C16:0	14.2	12	7.37	0.96	18.20	99.83	5.75	**0.043**	120

C)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	30.73	C18:0	5.33	11.3	40	1.75	67.60	67.60	75.83	**0.0001**	9447
		C16:0	16.3	17.9	13.5	0.62	22.79	90.39	2.06	0.1694	9452

The total percentage of MUFA was significantly different between species as well as between genders and the interaction term was also significant (Table 7.5A). *E. chloroticus* had higher percentages of MUFA than *A. dufresnii* (Table 7.1). Within each species females of *E. chloroticus* showed a slightly higher percentage than males, whereas similar percentages were found in male and female *A. dufresnii* (Table 7.1; Table 7.5.A). Multivariate two-way PERMANOVA analysis showed that the MUFA profile was also significantly different between species and genders, as clearly displayed in the MDS plot (Table 7.5.B; Fig. 7.6). C20:1(n-11) was the MUFA that contributed to the differences between species revealed by SIMPER analysis, being present in higher percentages in gonads of *E. chloroticus* (~7% of total FA) than *A. dufresnii* (<2% of total FA or not detected) (Table 7.1; Table 7.6.A). The differences between *E. chloroticus* genders were attributed to the MUFA C16:1(n-7) and C18:1(n-9c) being present in higher percentages in females than males as well as C20:1(n-15) with higher percentages in males than females (Table 7.1; Table 7.6.B). The gender difference in *A. dufresnii* was attributed to the percentages of C22:1(n-9), C16:1(n-7) and C20:1(n-11) (Table 7.6.C). The percentages of C22:1(n-9) were higher in males than in females as well as the percentage of C20:1(n-11) which was not detected in the females (Table 7.1; Table 7.6.C). C16:1(n-7) revealed higher percentages in *A. dufresnii* females than males (Table 7.1; Table 7.6.C).

Table 7.5. Results of A) Univariate two-way PERMANOVA of MUFA percentage and B) Multivariate two-way PERMANOVA of MUFA profile comparing the gonads of two sea urchin species *E. chloroticus* and *A. dufresnii* and genders. All the monounsaturated fatty acids identified were included in the analysis. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	1277.30	1277.30	447.02	**0.0001**	9842
Gender	1	16.04	16.04	5.61	**0.0258**	9862
Species x Gender	1	19.87	19.87	6.95	**0.0153**	9836
Residual	26	74.29	2.86			
Total	29	1387.50				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	397.09	397.09	127.6	**0.0001**	9953
Gender	1	102.64	102.64	32.98	**0.0001**	9941
Species x Gender	1	23.91	23.91	7.68	**0.0019**	9954
Residual	26	80.91	3.11			
Total	29	604.55				

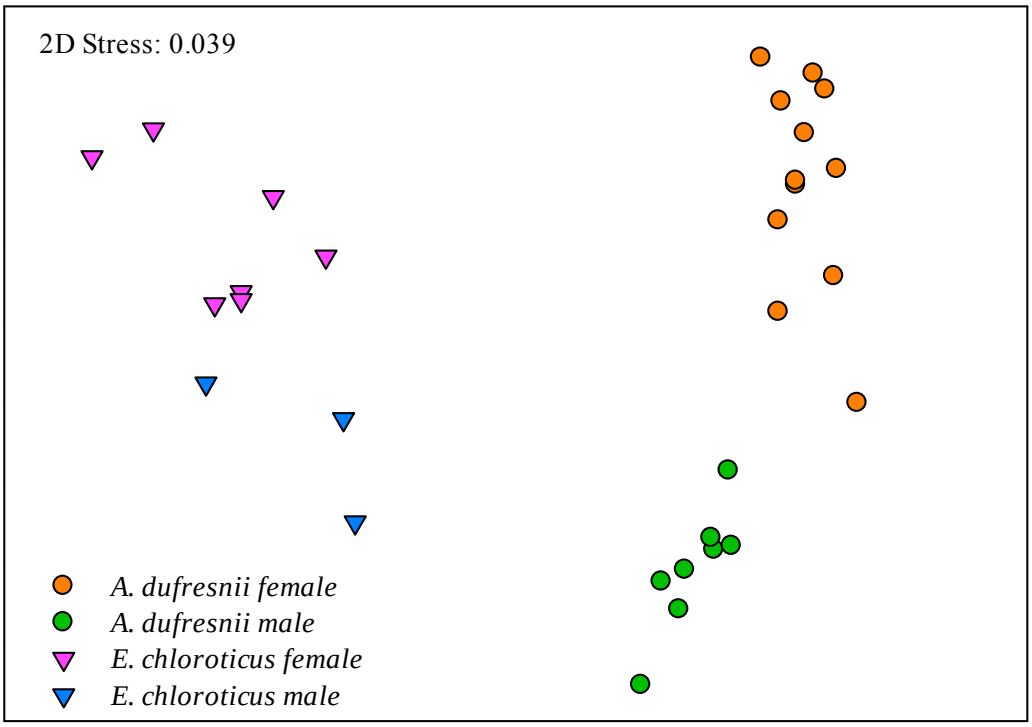


Figure 7.6. Multidimensional scaling (MDS) plot of Euclidean similarities of the gonadal MUFA profile of *E. chloroticus* female and male and *A. dufresnii* female and male.

Table 7.6. Contribution of individual MUFA to multivariate differences A) between species: *E. chloroticus* and *A. dufresnii* and between genders B) *E. chloroticus* males and females and C) *A. dufresnii* females and males as determined by SIMPER. Pseudo-F, P(perm) and Unique perm were obtained in two-way PERMANOVA a single variable. Significant results ($p<0.05$) are shown in bold.

A)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
E. chloroticus & *A. dufresnii*	73.97	C20:1(n-11)	7.06	0.79	45.1	2.80	61.02	61.02	254.76	**0.0001**	9082

B)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	12.98	C16:1(n-7)	4.49	2.68	3.92	1.15	30.21	30.21	14.24	**0.008**	120
		C18:1(n-9c)	6.23	4.36	3.85	1.66	29.65	59.86	33.10	**0.009**	120
		C20:1(n-15)	4.6	5.75	2.07	0.89	15.97	75.83	6.84	**0.026**	120
		C20:1(n-11)	7	7.2	1.81	0.72	13.93	89.75	0.08	0.7822	120

C)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	27.45	C22:1(n-9)	3.31	6.51	11.5	1.54	42.01	42.01	67.23	**0.0001**	9452
		C16:1(n-7)	2.67	0.72	4.32	1.45	15.74	57.75	51.09	**0.0001**	9478
		C20:1(n-11)	ND	1.98	4.02	3.28	14.63	72.38	434.88	**0.0002**	253

Significant differences were found in the total percentage of PUFA between species but not between genders, although there was a significant interaction term (Table 7.7.A). *A. dufresnii* presented higher percentages of PUFA than *E. chloroticus* regardless of gender; however, within each species genders showed different patterns, where *E. chloroticus* males had slightly higher percentages of total PUFA than females and the opposite was observed for *A. dufresnii* gonads (Table 7.1). The PUFA profile was also significantly different between species and genders as shown in the MDS plot (Table 7.7.B; Fig. 7.7). The contribution to the differences between species was made by C20:5(n-3) and C20:4(n-6), revealed by SIMPER analysis. *A. dufresnii* gonads presented higher percentages of these PUFAs (9-18% of total FA) than gonads of *E. chloroticus* (4-10% of total FA) (Table 7.1; Table 7.8.A). The same PUFA attributed to the differences between *E. chloroticus* genders, being higher in males than females (Table 7.1; Table 7.8.B). C18:4(n-3) contributed to the difference between *A. dufresnii* genders, which was present in relatively high percentages in females but not detected in the males and C20:4(n-6) showed higher percentages in males than females (Table 7.1; Table 7.8.C).

Table 7.7. Results of A) Univariate two-way PERMANOVA of PUFA percentage and B) Multivariate two-way PERMANOVA of PUFA profile comparing the gonads of two sea urchin species *E. chloroticus* and *A. dufresnii* and genders. All the polyunsaturated fatty acids identified were included in the analysis. Significant results ($p<0.05$) are shown in bold.

A)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	1052.40	1052.4	114.81	**0.0001**	9858
Gender	1	11.90	11.90	1.30	0.2609	9841
Species x Gender	1	277.89	277.89	30.32	**0.0001**	9823
Residual	26	238.31	9.17			
Total	29	1580.5				

B)

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Species	1	1091.60	1091.60	157.99	**0.0001**	9945
Gender	1	344.71	344.71	49.89	**0.0001**	9936
Species x Gender	1	48.84	48.84	7.07	**0.0042**	9951
Residual	26	179.65	6.91			
Total	29	1664.80				

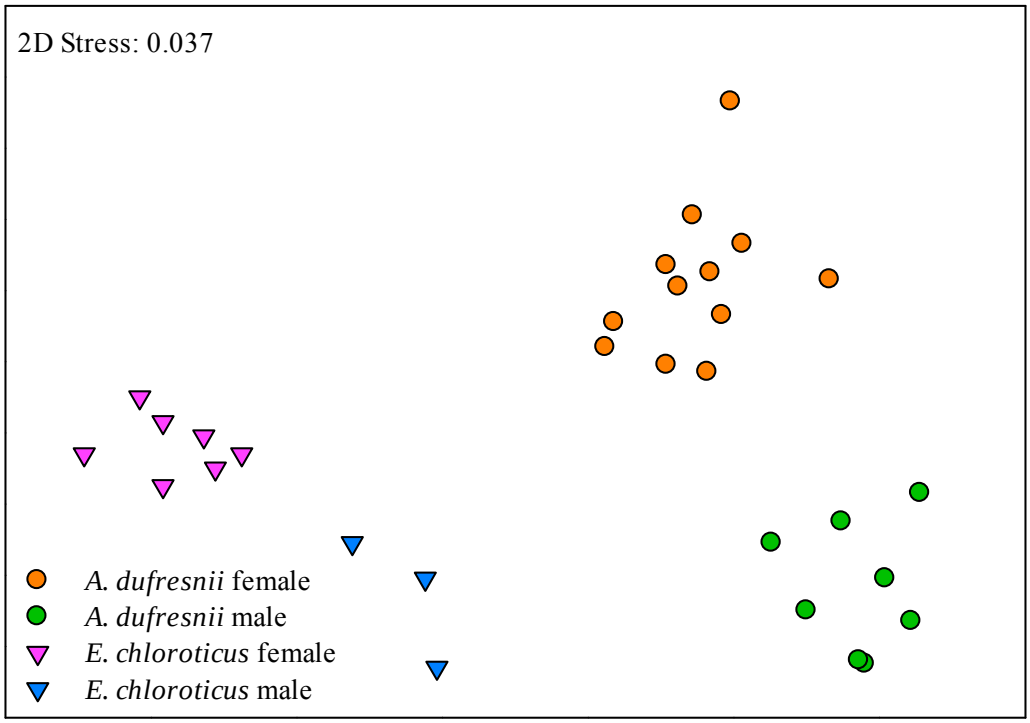


Figure 7.7. Multidimensional scaling (MDS) plot of Euclidean similarities of the gonadal PUFA profile of *E. chloroticus* female and male and *A. dufresnii* female and male.

Table 7.8. Contribution of individual PUFA to multivariate differences A) between species: *E. chloroticus* and *A. dufresnii* and between genders B) *E. chloroticus* males and females and C) *A. dufresnii* females and males as determined by SIMPER. Pseudo-F, P(perm) and Unique perm were obtained in two-way PERMANOVA a single variable. Significant results ($p<0.05$) are shown in bold.

A)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
E. chloroticus & *A. dufresnii*	343.59	C20:5(n-3)	6.12	17.4	134	2.68	74.76	74.76	267.17	**0.0001**	9834
		C20:4(n-6)	6.74	10.9	19.2	1.24	10.74	85.50	15.95	**0.0006**	9820

B)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	60.88	C20:4(n-6)	4.94	10.9	38.2	2.05	62.83	62.83	57.70	**0.0082**	120
		C20:5(n-3)	5.1	8.51	12.8	1.62	21.00	83.82	27.25	**0.0095**	120

C)

Groups 1 & 2	Average squared distance	FA	Av.Value Group 1	Av.Value Group 2	Av.Sq.Dist	Sq.Dist /SD	Contribution %	Cumulative %	Pseudo-F	P(perm)	Unique perm
Female & Male	72.46	C18:4(n-3)	5.89	ND	36	2.39	49.72	49.72	194.39	**0.0001**	2772
		C20:4(n-6)	9.34	13.2	19.2	1.18	26.51	76.23	28.653	**0.0004**	9430

7.4 Discussion

The results of this study demonstrate that fatty acid composition can be used to characterise different species from different environments. There was a clear distinction in the fatty acid (FA) composition of the gonads between the New Zealand (*E. chloroticus)* and Argentinian (*A. dufresnii)* sea urchin species probably due to dissimilar diet choices and environmental conditions in the different marine systems. *E. chloroticus* revealed a higher total percentage of monounsaturated FA (MUFA) and a lower total percentage of polyunsaturated FA (PUFA) than *A. dufresnii,* although no significant differences were found in the total proportion of saturated FA (SFA), indicating that probably particular FAs are contributing to the differences due to the availability of the diet. Additionally, significant differences were also found in the FA signatures between genders in both species. Hughes et al. (2006) suggested that changes in the FA signature in sea urchin gonads are correlated with habitat related diets combined with gender differences due to the dual function of the sea urchin gonads, the reproductive and the storage role.

FA profiles (SFA, MUFA and PUFA) were also significantly different between species with particular FA contributing to the differences. Particularly, C14:0 and C20:1(n-11) were present in higher percentages in *E. chloroticus* while C16:0, C18:0, C20:4(n-6) and C20:5(n-3) were present in lower percentages than *A. dufresnii*. The differences found here between species are probably related to the differences in the diet of each species. Even though both species are herbivorous, with *E. chloroticus* being completely herbivorous, (Barker, 2013) and *A. dufresnii* being herbivorous in the collection site (Teso et al., 2009), they are harvesting different brown seaweed species. *E. chloroticus* principally feeds on *E. radiata* (Don, 1975; Schiel, 1982) and *A. dufresnii* mostly on the invasive *Undaria pinnatifida* (Teso et al., 2009, Epherra personal communication). The FA composition of *E. radiata* from the same location analysed in previous chapters (Chapter 2 and Chapter 3) as well as from other sites (Guest et al., 2010; McLeod et al., 2013) showed a similar FA composition to *U. pinnatifida*, data collected from the literature from different geographical regions (Sánchez-Machado et al., 2004; Vaskovsky et al., 1996) and they were also comparable with the FA profiles reported for several brown seaweed species (Khotimchenko et al., 2002). These profiles were characterised by high levels of C14:0, C16:0,

C18:1(n-9), C18:2(n-6), C18:3(n-3), C18:4(n-3), C20:4(n-6) and C20:5(n-3). Most of these FAs were also present in high percentages in both sea urchin species except for the PUFA C18:2(n-6) and C18:3(n-3) which have been considered as precursors for the synthesis of the long-chain essential FAs C20:4(n-6) and C20:5(n-3) (Bell et al., 2001; Kelly et al., 2012), indicating that both sea urchin species are feeding on those brown seaweeds. This is supported by previous reports where particular FAs were determined as biomarkers of macroalgae (Khotimchenko et al., 2002; Li et al., 2002). A recent review by Kelly et al. (2012) revealed that brown seaweeds are characterised for having high levels of C18:1(n-9), C18:4(n-3) and C20:4(n-6) or ARA (Arachidonic Acid), similar profiles as found in the present study for the brown seaweeds *E. radiata* and *U. pinnatifida*.

The relatively low levels of C22:6(n-3) or DHA (Docosahexaenoic Acid) in both species indicates that they do not have a carnivorous diet in the locations sampled as this PUFA has been previously associated with carnivorous feeding (Kharlamenko et al., 1995). For example, high levels of C22:6(n-3) were found in *P miliaris* collected from scallop lines in Scotland and it was suggested that the high content of this PUFA was probably due to feeding upon the mussel *Mytilus edulis*, which is known to contain high levels of C22:6(n-3) (Cook et al., 2000).

Therefore, the significant differences in the FA composition between species need to be explained by other factors apart from the diet, such as internal or external factors. The internal factors, such as the biosynthesis of macromolecules such as protein and complex lipid in the gonads for either reproductive and storage purposes, are controlled genetically (Hughes et al., 2006; Kelly et al., 2008; Liyana-Pathirana et al., 2002; Walker et al., 2001) . Additionally, external factors may be related with environmental conditions, like seasons, water temperature, light, turbidity and nutrients as they affect the fatty acid composition of the brown seaweeds (Gerasimenko et al., 2010; Honya et al., 1994) and may affect the FA composition of marine invertebrates (Sanina et al., 2002).

One possible explanation of the variation found between the sea urchin species could be due to the ability of sea urchins to biosynthesise long-chain FA from shorter FA (Kelly et al., 2008; Liyana-Pathirana et al., 2002). However, species having different requirements may be able to synthesise distinct FAs. The higher levels of C18:0 in *A. dufresnii* with respect to *E.*

chloroticus suggests that *A. dufresnii* has an improved ability to elongate C16:0 as previously proposed for *A. lixula* when compared with *P. lividus* (Martínez-Pita et al., 2010a). In contrast, *E. chloroticus* appears to be capable of synthesising longer MUFAs probably from C18:1(n-9) (Castell et al., 2004), as this species showed higher percentages of C20:1(n-11) compared low levels in *A. dufresnii*. Artificial diet experiments on *S. droebachiensis* revealed that high dietary levels of C18:1(n-9) increased the levels of C20:1(n-9) and this conversion was accelerated when the diets were also rich in PUFA (Castell et al., 2004).

Water temperature is an environmental factor that may affect the FA composition in marine invertebrates as it has an influence on the membrane fluidity. *A. dufresnii* presented higher percentages of PUFA compared to *E. chloroticus*, probably due to the variation in the water temperature of both locations, as the sampling was done in different seasons of the year (spring vs. summer). Thus, the lower temperature during the austral spring (mean of ~10°C) when *A. dufresnii* was sampled, could explain the higher percentages of C20:5(n-3) related to the lower percentages in *E. chloroticus* sampled during the austral summer (~20°C). Biological membranes possess a lipid bilayer that need to be fluid enough to permit lateral movements of their constituents (Parrish, 2013). There is a clear relationship between unsaturated FAs and membrane fluidity, where low temperatures increase the levels of PUFA, especially C20:5(n-3) to maintain its fluidity (Parrish, 2013; Sanina et al., 2002). Similar results were found for *A. lixula* and *P. lividus* which showed elevated levels of EPA during winter time, probably using this PUFA to balance the effect of the temperatures on membrane lipids (Martínez-Pita et al., 2010a). Furthermore, a different study on *P. lividus* revealed the highest levels of the PUFAs C20:5(n-3) and C20:4(n-3), during winter and spring and the lowest during summer (Arafa et al., 2012).

Likewise, seasons affect the FA composition of sea urchin gonads due to the different reproductive stages of the animals around the year (Hughes et al., 2006). Here, this factor was minimised by the fact that both samplings were accomplished during maturity stage in both species (see Methods). However, little is known about the influence that the reproductive stages have on the FA composition of these two sea urchin species. Wild populations of *P. miliaris*, for example, revealed a temporal variation in the FA signature related to the reproductive maturity of the animals, due to the multifunction of the gonads, being the reproductive and also the

storage organ (Hughes et al., 2006). Furthermore, *P. lividus* seemed to accumulate high levels of long-chain PUFA during gametogenesis when they are available in the diets (Carboni et al., 2013).

Unfortunately, due to problems with the delivery of the standards (see Methods) it was not possible to perform a comparison of the concentrations of the total amount or amounts of particular FAs between the species. An analysis related to the animal/gonadal size is needed as there is a great variation in the size of both sea urchin species. *E. chloroticus* being relatively large, 16-17cm (Barker, 2013) compared with the relatively small size of *A. dufresnii*, 1.6-4.4cm (Brogger et al., 2010). Therefore, a comparative analysis relating the size and also the gonadal index of the animals with the total weight of the FAs of dry gonad is needed to have a more comprehensive understanding of the variation between species.

The results of this research also showed a clear variation in the FA profile between genders, where males showed higher percentages of C18:0, C20:1(n-15), C22:1(n-9), C20:4(n-6) and C20:5(n-3) whereas females showed higher percentages of C14:0, C16:0, C16:1(n-7) and C18:4(n-3), regardless of species. It has been suggested that a variation in the FA profile between genders may reflect metabolic peculiarities of gonad tissue related to specific requirements of spermatogenesis and oogenesis (Martínez-Pita et al., 2010b). Similar findings were reported for *Psammechinus miliaris* where the levels of the essential PUFAs, ARA, EPA and DHA, were higher in males than females (Hughes et al., 2006). This is expected as it is generally recognised that male gametes are composed mostly of structural lipids, especially phospholipids, which are essential parts of the cell membrane (Kozhina et al., 1978; Mita et al., 1989). Female gametes, on the contrary, besides having phospholipids in their cell membranes, have more energetic reserves, which results in the presence of more SFA and MUFA, which are part of the neutral lipids (Morais et al., 2003). Previous studies reported that in both species females contained relatively higher amounts of energy lipids, principally triglycerides whereas males presented higher amounts of structural lipids, especially phospholipids (Diaz de Vivar et al., 2011; Verachia et al., 2012).

In conclusion, the gonadal FA composition showed a great variation between the sea urchins *A. dufresnii* and *E. chloroticus*. These species inhabit completely different geographical

locations, having varied diet choices and different environmental conditions. Despite the fact the variation in the FA composition is normally affected by the diet, in this case both sea urchin species seemed to be feeding on brown seaweeds. The FA composition of both sea urchin species was similar to the FA composition of the most common brown seaweeds from both locations: *U. pinnatifida* in Argentina and *E. radiata* in New Zealand, indicating that they are probably harvesting on them. Therefore, the differences found here might be explained by the capacity of these animals on biosynthesise particular FAs, in concordance with their requirements, particularly *A. dufresnii* elongating C16:0 to C18:0 and *E. chloroticus* converting C18:1(-9) in C20:1(n-11). However, further analyses are desirable to compare the amounts of these FAs between species relating to the size differences between species. Seawater temperature was another factor that appeared to have an impact in the levels of PUFAs in these sea urchin species, incrementing the proportions of EPA with low temperature. The present study highlights the importance of taking into account not only diet choices but also different factors that could affect FA composition when comparing different sea urchin species. Studies on the fatty acid signature during the reproductive cycle of *A. dufresnii* have recently been completed (Zarate et al., article in preparation). However, further studies on the seasonal variation of gonadal FA composition of *E. chloroticus* are needed to better understand the effect on the reproductive stage and also the temperature impact in the FA profiles.

Chapter 8

General Discussion

Using different biomarkers, lipids, fatty acids and stable isotopes, this PhD thesis has provided comprehensive information on the nutritional ecology of *Evechinus chloroticus*, the dominant grazer of the New Zealand subtidal rocky reefs. This new information is presented in six data chapters, whose major findings were:

- The identification of the allocation of nutrients (lipids and fatty acids) from the food source to the storage organ of *E. chloroticus*. The nutrients (in terms of lipids and fatty acids) increase from the diet, preferably *Ecklonia radiata*, to gut contents, gut and gonad, indicating the great ability of the gonads as an organ able to biosynthesise and bio-accumulate lipids and essential fatty acids.

- The lipid and fatty acid composition of *E. chloroticus*, as well as its potential food, *Ecklonia radiata*, showed a variation between different locations around the Hauraki Gulf, where the sea urchins have similar food items to choose from, but probably different environmental conditions, affecting the biochemical composition.

- Feeding experiments showed different lipid and fatty acid composition of *E. chloroticus* gonads fed manufactured and kelp diets compared with sea urchins from the wild, revealing the ability of this sea urchin to biosynthesise or retain particular fatty acids.

- The four brown seaweed species chosen as potential food of *E. chloroticus* showed a different lipid composition and three of them revealed seasonal variation, *Cystophora torulosa* showed a clearly different lipid profile and together with *Carpophyllum plumosum* and *Ecklonia radiata* presented higher levels of lipids during the austral winter/spring.

- Stable isotope analysis confirmed the preference of *E. chloroticus* for *Ecklonia radiata*, and suggested the addition of *Carpophyllum plumosum* as one of the main contributors to its diet in Matheson's Bay.

- The comparison of fatty acid composition of two sea urchin species revealed that even though (or despite) the two species were feeding on brown seaweeds, *E. chloroticus* on *E. radiata* and *Arbacia dufresnii* on *Undaria pinnatifida*, they presented different fatty acid composition, possibly due to the distinct environmental conditions.

8.1. Factors affecting the lipid and fatty acid composition of echinoids

8.1.1 External factors

8.1.1.1 Diet

Different chapters of this thesis have helped to understand how the diet affects the lipid and fatty acid composition of *E. chloroticus*, as seen previously for *S droebachiensis*, *P. miliaris* and *P. lividus* (Castell et al., 2004; Cook et al., 2007; Hughes et al., 2006; Kelly et al., 2008; Liyana-Pathirana et al., 2002). Wild *E. chloroticus* showed a similar fatty acid composition to *E. radiata* (Chapters 2, 3, 7) indicating that this sea urchin species is feeding on the most dominant Laminariales (Schiel, 1988) in the Hauraki Gulf. Feeding experiments on *E. chloroticus* have shown the ability of this sea urchin to distinguish different seaweed species (Cole et al., 2000; Schiel, 1982). A comparison of the fatty acid composition of the two most dominant brown seaweed species, *E. radiata* and *C. maschalocarpum*, revealed a more similar fatty acid profile between *E. radiata* and *E. chloroticus* than *C. maschalocarpum* (Chapter 2). High levels of particular FAs, such as C18:1(n-9), C20:4(n-6) and C20:5(n-3) were present in *E. radiata* and were also present in relatively high levels in *E. chloroticus* gonads (Chapters 2, 3), indicating that this sea urchin was probably harvesting on it. Stable isotope results reinforce the findings of the great contribution of *E. radiata* (40%) to *E. chloroticus* diet (Chapter 6), whereas the contribution of *C. maschalocarpum* was very low (7%). However, these results also indicated that *C. plumosum* was a major contributor (40%) in Matheson's Bay. Unfortunately, the fatty acid composition of this last brown seaweed was not analysed. These findings are supported by

previous studies, where these specific FAs have been considered as biomarkers for brown seaweed diets (Kelly et al., 2012; Khotimchenko et al., 2002; Li et al., 2002). In addition, Hanson et al. (2010) suggested C20:4(n-6) as a biomarker to trace *E. radiata* in this kind of environments.

Likewise, the fatty acid composition of *Arbacia dufresnii* gonads indicated that this sea urchin species was also feeding on brown seaweed but in this case, on the invasive *Undaria pinnatifida* in Nuevo Gulf, Argentina (Chapter 7). It has been previously reported that *A. dufresnii* has different diet habits, depending on the food availability (Penchaszadeh et al., 1998; Vasquez et al., 1984). However, laboratory experiments indicated that *A. dufresnii* from Nuevo Gulf feeds particularly on *Undaria pinnatifida* in the Nuevo Gulf, Argentina suggesting complete herbivorous behaviour (Teso et al., 2009), supporting the findings of this study.

Furthermore, manufactured diets also affected the lipid and fatty acid composition of *E. chloroticus* (Chapter 4). However, it seems that *E. chloroticus* was getting more nutrients, principally long-chain PUFA when it fed on kelp or when sea urchins were feeding in the wild. These results suggested that the manufactured diets chosen were not the ideal for incrementing the levels of essential FAs that are desirable for human consumption (Arts et al., 2001); having implications for future aquaculture purposes.

8.1.1.2 Environmental conditions

Sea urchins collected from four different locations around the Hauraki Gulf showed a variation in the lipid and fatty acid composition, indicating that not only the food but also the location has an effect on the lipid and fatty acid composition of *E. chloroticus* gonads, as the dietary choices were relatively the same in these sampled areas (Chapter 3). Thus, it seems that not only the food affect the lipid and fatty acid composition of *E. chloroticus* gonads, in this case different environmental conditions appear to also have an impact, as shown for different sea urchins species (Martínez-Pita et al., 2010a). The diverse selective retention of some fatty acids, especially long-chain MUFA seen in different populations of *E. chloroticus* gonads are possibly related to variation in the local environmental conditions, such as temperature, light, turbidity, salinity and nutrients affecting the FA composition of the potential food items (Gerasimenko et al., 2010; Nelson et al., 2002). It is commonly known that temperature changes the membrane

fluidity (i.e. low temperatures induce the loss of fluidity of cell membranes) and that it could be reverted by the accumulation of long-chain PUFA like 20:5(n-3) (Parrish, 2013). Thus, the lower levels of C20:5(n-3) in sea urchins collected from places with warmer waters (Great Barrier Island) compared with the higher levels of this PUFA in locations with slightly colder waters (Rakino and Rangitoto) (Shears, Babcock, et al., 2004) support these findings.

The effect of seawater temperature on the fatty acid composition of sea urchin gonads was also supported by the results from Chapter 7. The higher levels of C20:5(n-3) present in *A. dufresnii* could be explained by the lower seawater temperature (~10°C) in Nuevo Gulf, Argentina when these sea urchins were collected during spring. In contrast, the lower levels in *E. chloroticus* were probably related to the summer collection when the seawater temperature (~20°C) was higher.

The lipid and fatty acid composition of sea urchin gonads is also affected by the seasons (Martínez-Pita et al., 2010a). Seasonality was not analysed in the lipid or fatty acid composition of *E. chloroticus* gonads. However, the lipid composition of its potential food, *E. radiata* and *C. plumosum* varied between seasons, showing higher amounts of lipids (TAG and PL) during winter/spring, indicating that they are possibly preparing themselves for the reproductive season. On the other hand, previous seasonal analysis of the biochemical composition of *E. chloroticus* gonads showed the energy lipid TAG did not change significantly with seasons; however, the amount of total lipids, in terms of ST and PL, were higher during summer, when the spawning occurs (Verachia et al., 2012). These results suggest the seasonality found in the diet affects the lipid composition of *E. chloroticus*, suggesting that when this sea urchin feeds on these brown seaweeds during winter/spring it is the time when there is a rapid gametogenesis and when major lipid accumulation occurs in *E. chloroticus* gonads (Lamare et al., 2002; Walker, 1982).

8.1.2 Internal factors

8.1.2.1 Biosynthesis and bioaccumulation

E. chloroticus demonstrated a great ability to biosynthesise lipids and fatty acids and also to retain particular lipids and fatty acids in the gonads (Chapters 2, 3, 4, 7). It is well known, that sea urchin gonads have a multifunctional role, acting as reproductive organ, but also as storage organ and having a great facility for biochemical and fatty acid modification (Hughes et al.,

2006; Kelly et al., 2008; Walker et al., 2001). This gonadal biosynthesis capacity was shown in Chapter 2, where the results revealed a pronounced increase in the lipid and fatty acid content and also a more diverse lipid and fatty profile in the gonads compared with the potential food, *Ecklonia radiata*. *E. chloroticus* seems to be able to store and convert particularly the dietary structural lipid (PL) into the energy lipid (TAG), as seen previously in *Lytechinus variegatus* (Gibbs et al., 2009). Furthermore, it appeared that the high amount of dietary PUFAs stimulated the biosynthesis of long-chain MUFAs, like C16:1(n-7), C20:1(n-15) and C22:1(n-9), as also reported for *Strongylocentrotus droebachiensis* (Liyana-Pathirana et al., 2002). High levels of C18:1(n-9), the precursor of long-chain MUFA (Castell et al., 2004), in the diet but not in *E. chloroticus* tissues indicated selective assimilation and possible elongation to synthesise longer MUFA like C20:1(n-9), present in very small concentrations in the potential diet but found to be high in the sea urchin gonad.

The biosynthesis ability of *E. chloroticus* gonad was also seen in Chapter 3, as a lower dietary levels of C18:1(n-9) seemed to lead to lower levels of C20:1(n-9) in the gonads, as seen for the sea urchins collected from Great Barrier Island. Likewise, feeding experiments showed the capacity for selective retention and biosynthesis of special FAs in *E. chloroticus* gonads (Chapter 4). As part of the already mentioned selective retention and elongation of C18:1(n-9) to produce C20:1(n-9), it seems that *E. chloroticus* is especially storing long-chain PUFAs C20:4(n-6) and C20:5(n-3) when they are present in high levels in the diets, in sea urchins fed kelp and from the wild compared with sea urchins fed manufactured diets. These long-chain PUFAs characterise brown seaweed FA composition (Floreto et al., 1998; Kelly et al., 2012), so when they are available in high concentration in the diet, it appears that *E. chloroticus* selectively retains them as they are considered essential fatty acids and participate in many animal functions (Parrish, 2013).

8.1.2.2 Reproductive stage and gender

The differences found in the lipid and fatty acid profile of *E. chloroticus* collected from four locations around the Hauraki Gulf, were attributed to a minor variation in seawater temperature but possibly also to the distinct reproductive stages of the animals (Chapter 3). Walker (1982) found differences in reproductive stages of populations of *E. chloroticus* from the Hauraki Gulf separated by only few km. Furthermore, he found that the appearance of mature

gametes and spawning was different for *E. chloroticus* from Rangitoto compared with a population from nearby islands. Histological analysis may help to find possible differences in the reproductive stages of these populations sampled in the present study, explaining the variation in the fatty acid composition found here. *Paracentrotus lividus* showed accumulation of long-chain FA during gametogenesis (Carboni et al., 2013). Furthermore, *P. lividus* and *A. lixula* also showed variation in the fatty acid profile related to the reproductive stages, accumulating particular FAs before the gametogenesis (Martínez-Pita et al., 2010a). Nutrients are accumulated and stored in the gonads and then they are used to form the gametes, due to the dual role of this organ (Hughes et al., 2006). Histological analysis has shown that nutritive phagocytes, being the nutritive storage in the gonads, accumulate in amount and size before gametogenesis and then transfer the nutrients to the gametes (Walker et al., 2001). As lipids are the compounds that can store more energy per unit volume, being the most important source of energy (Parrish, 2013), it is expected that they change during the reproductive cycle.

Furthermore, the energy accumulated, especially in terms of lipids and fatty acids, before gametogenesis is extremely important for the development of eggs and sperm. It has been shown how essential is the parental energy source from energy lipid reserve, such as TAG, to the development of *E. chloroticus* larvae (Sewell, 2005). The levels of parental TAG reserve decline from gastrula and during arm formation, as shown for *E. chloroticus* (Sewell, 2005) Additionally, feeding experiments revealed that the fatty acid composition of parental diet was reflected in the fatty acid composition of eggs and larvae of *P. lividus* (Gago et al., 2009). Thus, the knowledge of lipid and fatty acid composition during the reproductive stage is critical to a better understanding of how the nutrients are transferred and used for the larvae.

The analysis of the fatty acid composition revealed differences between genders in *E. chloroticus* and *A. dufresnii* gonads. As expected, males contain a higher amount of phospholipids and therefore PUFAs, as the sperm is composed mainly of structural lipids, the principal components of the cell membrane (Mita et al., 1989). In contrast, females having also cell membranes around the eggs, have more volume inside due to the high levels of energy lipids and fatty acids, particularly SFA and MUFA (Hughes et al., 2005). Similar results were found for other sea urchin species like *P. lividus*, *A. lixula* and *P. miliaris* (Hughes et al., 2005; Martínez-Pita et al., 2010b).

8.1.2.3 Bacteria

It is also possible that *E. chloroticus* possess bacteria living in the gut that synthesise FAs, as seen in *S. nudus* (Iwanami et al., 1995). In Chapter 2, it was shown that the lipid and fatty acid composition of the gut was different to the potential food items, presenting a more diverse FA profile than the seaweeds, particularly in terms of higher levels of PUFA like C20:5(n-3). Bacteria play an important role in providing essential nutrients to marine environments, synthesising for example long-chain PUFAs (Nichols, 2003). Even though to our knowledge there is no study on *E. chloroticus* gut bacteria, previous studies have reported the presence of these microorganisms in sea urchins (Iwanami et al., 1995; Sawabe et al., 1995; Unkles, 1977). A previous study on *S. nudus* suggested that C18:1(n-9) was desaturated and elongated to form C20:5(n-3) by the bacteria *Vibrio* sp. (Iwanami et al., 1995). This bacteria was isolated in sea urchins such as *Echinus esculentus* (Unkles, 1977), *S. intermedius* and *S. nudus* (Sawabe et al., 1995). Furthermore, the stable isotope results (Chapter 6) suggest that levels of nitrogen present in *E. chloroticus* gut and gonads may be related to nitrogen fixation by bacteria living in the gut. This was supported by a previous report on *S. droebachiensis*, where nitrogenase activity was demonstrated in sea urchins feeding on seaweeds and seagrasses (Guerinot et al., 1977; Guerinot et al., 1981).

8.2 Applications of biomarker approach.

8.2.1 Ecological application

The findings of this thesis confirm the value of lipid and FA analysis together with stable isotope composition to, determine the trophic status of sea urchins, especially *E. chloroticus*, the principal grazer on the rocky reefs of north-eastern New Zealand and the only commercially harvested.

The use of variety of biomarkers (lipids, fatty acids and stable isotopes) has provided a better understanding of *E. chloroticus* diet and preferences in the Hauraki Gulf. With a combination of these biomarkers, this study indicated that *E. chloroticus* is feeding principally on *E. radiata* (Chapters 2, 3, 6, 7); however, stable isotope results (Chapter 6) suggested that in addition to *E. radiata* they are also consuming *Carpophyllum plumosum* but not *C.*

maschalocarpum in Matheson's Bay. However, it is surprising that *E. chloroticus* does not appear to feed on *C. torulosa* as this seaweed species showed the highest lipid content and therefore the greatest energy resource of four of the most dominant brown seaweed species in the Hauraki Gulf (Chapter 5). *E. chloroticus* would benefit from lipid high energy contribution provided by preferential ingestion of *C. torulosa*, may be converting the high levels of FALC, another energy lipid, into TAG, the normal energy storage lipid in sea urchins or sterols into energy lipids (Liyana-Pathirana et al., 2002). However, the stable isotopes analysis discussed in Chapter 6 revealed that *E. chloroticus* assimilates carbon from *E. radiata* and *C. plumosum* showing 80% of contribution to its diet and not from *C. torulosa* in Matheson's Bay (<20%).

Furthermore, this thesis resolved the allocation of nutrients such as lipids and fatty acids within *E. chloroticus*. There was a clear increase in concentration of lipids and fatty acids from the seaweed through gut content, gut and gonad, and this pattern was consistent in three different locations from the Hauraki Gulf (Chapter 2). The levels of TAG and particular FAs increased from *E. radiata* to the gut and gonads, highlighting the ability of this sea urchin to accumulate dietary nutrients in the storage organ.

However, these results exposed the difficulty of choosing a representative location to analyse these biomarkers in *E. chloroticus* as they may show different patterns around the New Zealand coast (Chapter 3). Even if the food choices are similar, a variation in the environmental factors as water temperature, light, may affect its lipid and fatty acid composition. Moreover, the variation in the lipid and fatty acid profile related to the environmental changes, like water temperature, indicates the importance of possible use of these biomarkers in the study of climate change. The high ability of sea urchins to biosynthesise and bio-accumulate specific FAs, like C20:5(n-3) to respond to the disordering effect of temperatures on membrane fluidity (Chapter 3) suggests a possible thermal acclimation of sea urchins during global warming. Thus, in the context of climate change, the variation in the membrane fluidity due to alteration of fatty acid composition of the lipid bilayer in marine ecosystems will become more and more significant (Parrish, 2013).

8.2.2 Economic application

Although *E. chloroticus* is the only sea urchin commercially harvested in New Zealand, there is no commercial aquaculture developed for this invertebrate (Phillips et al., 2010; Woods et al., 2008). Valuable information for aquaculture purposes is presented in this thesis. Manufactured diets affected the lipid and fatty acid composition (Chapter 4); however, it seems that *E. chloroticus* is getting more nutrients, principally long-chain PUFA when it feeds on kelp. Thus, manufactured diets must be improved to substitute wild collected macroalgae to develop a commercially and sustainable aquaculture for *E. chloroticus* in New Zealand. Furthermore, brown seaweeds, especially *E. radiata* and *C. plumosum*, should be taken into account when manufactured diets are prepared for aquaculture. Furthermore, it seems that *E. chloroticus* is accumulating and biosynthesising more PUFAs when they are present in high levels in the diets (Chapters 2, 3).

There is a growing interest in the effect of PUFAs in human health, as they are associated with cardiovascular diseases. Some of these PUFAs are considered essential FAs, C20:4(n-6) and C20:5(n-3), as they cannot be synthesised by humans and therefore they need to be consumed from the diet (Arts et al., 2001). Thus, there is a pharmaceutical interest in obtaining PUFA from different sources. Consequently, the findings from this study are very important for commercial purposes as high amounts of PUFA were found in *E. chloroticus* gonads and also they can be increased with different diets. Furthermore, brown seaweeds also contained high amounts of these essential fatty acids, indicating that they could be a commercially viable source of these FAs as nutritional supplements.

8.3 Future directions

Further studies are needed to achieve a better understanding of *E. chloroticus* nutrient assimilation, especially FAs because of their importance for reproduction and nutrition. Furthermore, due to the overexploitation of wild populations of sea urchins there is a world interest in aquaculture. Thus, future studies on the variation in lipid and FA profile during the reproductive cycle are needed on this endemic species for a better management of *E. chloroticus* aquaculture. Additional investigation on seasonal changes in the biochemical composition of the

dominant brown seaweeds is also required, especially in terms of FAs and SI. These biomarkers have been shown to change with seasons in different brown seaweeds around the world (Gerasimenko et al., 2010; Nelson et al., 2002; Renaud et al., 2007). Dethier et al. (2013) found that these biomarkers vary significantly among species, sites and dates in the marine primary producers in Washington state, USA. To our understanding, it is unknown how these biomarkers change seasonally in dominant brown seaweeds from New Zealand and it will be very interesting to determine how they may affect their grazers, especially *E. chloroticus* and its reproductive cycle.

Moreover, further analysis need to be completed using stable isotope and FA analysis on *E. chloroticus* and different brown algae species from different sites around New Zealand, especially the South Island, to better understand the real diet of this sea urchin in the wild and how this affects the FA composition, thus use them to prepare richer artificial diets for aquaculture purposes.

Analyses on the bacteria biota possibly living *E. chloroticus* are also required to have a more comprehensive knowledge of the biosynthesis of FAs that occur in sea urchins. As nitrogen fixation has been reported for *S. droebachiensis* (Guerinot et al., 1981), future studies are needed on *E. chloroticus* to better understand this process.

Chapter 9

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