Evidence for Polyunsaturated Fatty Acid Biosynthesis in the Ragworm (*Nereis virens*) and the Lugworm (*Arenicola marina*)

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Abstract

There is increasing evidence suggesting that heterotrophic marine organisms could also be potential sources of polyunsaturated fatty acids (PUFAs), in addition to marine bacteria and microalgae, including two polychaete species such as the lugworm (*Arenicola marina*) and the ragworm (*Nereis virens*). The capacity for PUFAs production by these species is, therefore, essential to understand the roles of polychaetes in relation to PUFA fluxes in an estuarine food web. Two methods were adopted to study fatty acid biosynthesis by the worms: i) quantifying of PUFA fluxes in closed system (*A. marina* and *N. virens*) and ii) stable isotope labelling and mass spectrometry to investigate possible pathway (*N. virens* only). In the first method, the controlled amount of fatty acid inputs and outputs in the culture worms was determined together with GC/GC-MS analysis to calculate the amount of fatty acid gained per system box after subtracting food given fatty acids. Palmitic acid (C16:0) and monoenoic acids e.g. palmitoleic acid (C16:1n7), oleic acid (C18:1n9) and vaccenic acid (C18:1n7) were prominent fatty acids in the system. EPA was found to be accumulated over the period of experiment in both species. In the stable isotope experiment, ^{13}C-labelled palmitic acid (C16:0) was incorporated into the feed pellets and used to trace the changes in the $^{13}C/^{12}C$ ratio in characteristic peaks of mass spectra. Significant changes in the $^{13}C/^{12}C$ ratio of key peaks in the spectra compared with the controlled start samples: palmitic acid (C16:0), stearic acid (C18:0), arachidonic acid (ARA: C20:4n6) and EPA (C20:5n3) within the 7 days period of experiment revealed a pathway for EPA production in *N. virens*. These findings would therefore corroborate of the concept that some marine animals are able to generate long-chain PUFAs such as ARA, EPA and DHA. *N. virens* and *A. marina* could be one of the very important PUFA sources within estuarine marine ecosystem.

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Abbreviations

ACP	Acyl carrier protein
AGR	Average growth rate
CI	Chemical ionisation
DW	Dry weight
EI	Electron-impact ionisation
FA	Fatty acid
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
PKA	Polyketide synthase pathway
PUFA	Polyunsaturated fatty acid
PWG	Percentage weight gain
SCD	Stearoyl CoA desaturase
SGR	Specific growth rate
WW	Wet weight

List of Fatty Acid Nomenclatures and Abbreviations

Shorthand formula	Common name	Systemic name	Abbreviations
C14:0	Myristic acid	Tetradecanoic acid	-
C16:0	Palmitic acid	Hexadecanoic acid	PA
C16:1n7	Palmitoleic acid	*cis*-9-hexadecenoic acid	-
C18:0	Stearic acid	Octadecenoic acid	SA
C18:1n7	Vaccenic acid	*cis*-11-octadecenoic acid	VA
C18:1n9	Oleic acid	*cis*-9-octadecenoic acid	OA
C18:2n6	Linoleic acid	*cis*,*cis*-9,12-octadecadienoic acid	LA
C18:3n3	α-linolenic acid	*cis*,*cis*,*cis*-9,12,15-octadecatrienoic acid	ALA
C18:3n6	γ-linolenic acid	*Cis*,*cis*,*cis*-6,9,12-octadecatrieonic acid	GLA
C19:0	-	Nonadecanoic acid	-
C20:0	Arachadic acid	Eicosanoic acid	-
C20:1n9	eicosenoic acid	*cis*-11-eicosenoic acid	-
C20:2n6	Eicosadienoic acid	*cis*,*cis*-11,14-eicosadienoic acid	EDA
C20:3n6	Dihomo-gamma-linolenic acid	*cis*,*cis*,*cis*-8,11,14-eicosatrienoic acid	DGLA
C20:3n3	-	*cis*,*cis*,*cis*-11,14,17-eicosatrienoic acid	ETE
C20:4n3	-	*cis*,*cis*,*cis*,*cis*-8,11,14,17-eicosatetraenoic acid	ETA
C20:4n6	Arachidonic acid	*cis*,*cis*,*cis*,*cis*-5,8,11,14-eicosatetraenoic acid	ARA
C20:5n3	-	*cis*,*cis*,*cis*,*cis*,*cis*-5,8,11,14,17-eicosapentaenoic acid	EPA
C22:4n6	Adrenic acid	*cis*,*cis*,*cis*,*cis*-7,10,13,16-docosatetraenoic acid	-
C22:5n6	Osbond acid	*cis*,*cis*,*cis*,*cis*,*cis*-4,7,10,13,16-docosapentaenoic acid	-
C22:5n3	-	*cis*,*cis*,*cis*,*cis*,*cis*-7,10,13,16,19-docosapentaenoic acid	
C22:6n3	-	*cis*,*cis*,*cis*,*cis*,*cis*,*cis*-4,7,10,13,16,19-docosahexaenoic acid	DHA

'n' is used as a symbol to indicate the first double bond position counting from the methyl end of fatty acid.

Chapter 1

Introduction

1.1 *Introduction to Lipid Biochemistry*

Lipids are one of the most important classes of biological molecules in living organisms. Their molecules have special properties which include low solubility (or insolubility) in water and inability to form large polymers (Nelson and Cox, 2008). The principal storage lipids are fats and oils while phospholipids and sterols are the main components in biological membranes (Nelson and Cox, 2008). This section will provide a brief summary of the basic structure, chemical properties, nomenclature and biosynthesis of fatty acids which are the constituents and derivatives of fats and oils in organisms.

1.1.1 Polyunsaturated Fatty Acid (PUFA) Structure and Nomenclature

Fatty acids are carboxylic acids with a long-chain hydrocarbon which contain from 4 to 36 carbon atoms (C_4-C_{36}). There are two major categories: saturated fatty acids and unsaturated fatty acids. Saturated fatty acids contain only a single bond between adjacent carbon atoms in their molecules. These fatty acids have a low reactivity in chemical reactions. Palmitic acid (C16:0) and stearic acid (C18:0) are two relatively abundant examples of saturated fatty acids and their structure is shown in Figure 1.1.

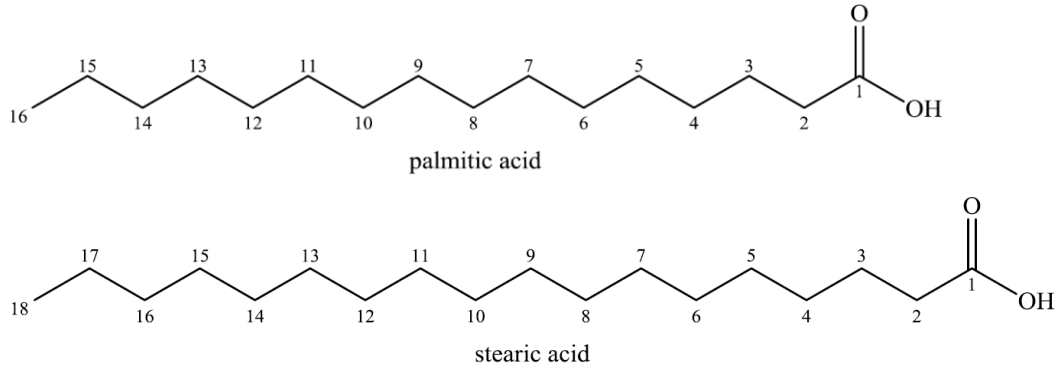
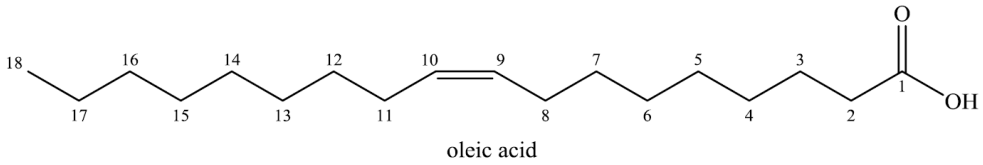


Figure 1.1: Structure of the saturated fatty acids: palmitic acid (C16:0) and stearic acid (C18:0).

In contrast to saturated fatty acids, unsaturated fatty acids contain at least one double bond between adjacent carbons, and they have a higher chemical reactivity. Unsaturated fatty acids which contain only a single carbon/carbon double bond in the

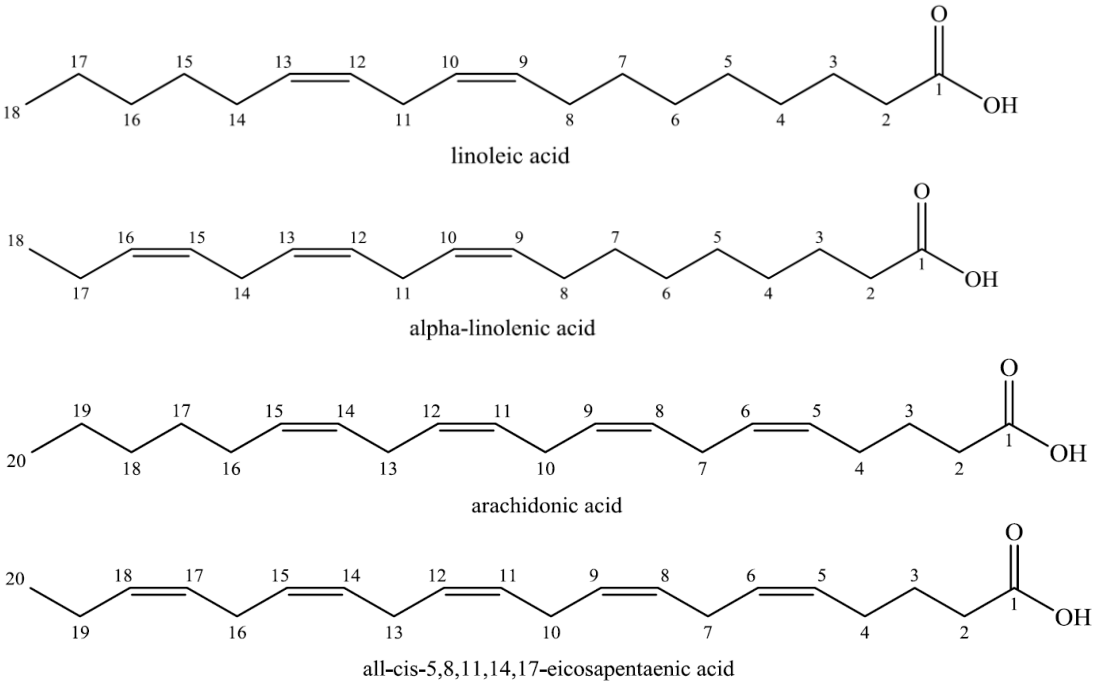
molecule are called monounsaturated fatty acids (MUFAs). An example is an 18 carbon fatty acid, oleic acid (C18:1n9). In this example, the first double bond position is situated between the C_9 and C_{10} position from the carboxyl end (in the middle of the carbon chain for this fatty acid). MUFAs with one double bond between C_9 and C_{10} carbons, as illustrated in Figure 1.2, are commonly found in nature, due to their enzyme specificity properties.



oleic acid

Figure 1.2: The chemical structure of oleic acid, a monounsaturated fatty acid.

The unsaturated fatty acids with more than one double bond are considered as polyunsaturated fatty acids (PUFAs). There are several PUFAs in living organisms, such as linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) which are two major precursors for the synthesis of further long-chain PUFA such as arachidonic acid (ARA) (C20:4n6), eicosapentaenoic acid (EPA) (C20:5n3) and docosahexaenoic acid (DHA) (C22:6n3). The double bonds in PUFAs are normally *cis* double bond (*Z*) and are methylene-interrupted between double bonds. The PUFAs that occur in nature mostly have an even-number of carbon atoms. The chemical structures of some PUFAs are exemplified in Figure 1.3.



linoleic acid

alpha-linolenic acid

arachidonic acid

all-cis-5,8,11,14,17-eicosapentaenic acid

Figure 1.3: The chemical structures of selected PUFAs: linoleic acid (C18:2n6), α-linolenic acid (C18:3n3), ARA (C20:4n6) and EPA (C20:5n3).

The nomenclature of fatty acids is based on their chain length, number of double bonds and the first position of the double bond. For instance, palmitic acid contains 16 carbon atoms and does not have any double bonds in its molecule; the concise formula is, therefore, C16:0 which indicates the number of carbons after 'C' (e.g. C_{16}) and the absence of double bonds is represented by the zero written as '0'. For further example, oleic acid is C18:1 which indicates that this molecule contains 18 carbon atoms together with one double bond.

There are two main ways to specify the position of double bonds in MUFAs and PUFAs. The most precise is the systemic naming system (IUPAC) in which the position of each double bond is indicated counting from carbon 1, which is the carbon position at the carboxyl end of the molecule, as in the nomenclature of other organic compounds. The delta symbol (Δ) is used as an indication for this naming system and is followed by superscript number for the position of each double bond. In the case that there are two or more double bonds, the superscript numbers designating each double bond position are conventionally separated by a comma. Using this system of nomenclature, it is possible to summarise the structure of the entire molecule. For example, linoleic acid has its first and only double bond at the C_9 position from carboxyl end, therefore, it can be written in this shorthand formula as C18:1(Δ^9).

A second, less precise system of nomenclature is also commonly used (particularly in animal nutrition, aquaculture and clinical studies). It will be useful to adopt this system in this thesis to facilitate comparison with studies in these fields of investigation, although the full systemic naming system will be adopted when additional clarity is needed. Instead of counting from the carboxyl end, the first double bond position is counted from the methyl end. Two conventions are used to then identify at which position the first double bond occurs. Either 'n' or 'ω' are used as a symbol to indicate the double bond position. For oleic acid, the written chemical formula is C18:1n9 or C18:1ω9. Note that oleic acid is unusual in the sense that the position of the double bond is represented by the same number in both nomenclature systems. For other fatty acids that is not the case. As shown in Figure 1.3, linoleic acid has two double bonds and the first double bond position is situated at the C_6 position from the methyl end; hence, the designated formula is C18:2n6 or C18:2ω6. Using the systemic naming system, this molecule would be more completely described as: C18:2$\Delta^{9, 12}$. In contrast, α-linolenic acid has three double bonds but the first double bond is positioned at the C_3 from the methyl end, thus the shorthand formula is C18:3n3 or C18:3ω3. This

fatty acid can also be more completely described as C18:3$\Delta^{9, 12, 15}$. In nature, (e.g. animals, plants and some bacteria) it is found that many of the common PUFAs fall into two families of fatty acid according to the position of the first double bond from the methyl end. It is therefore generalised that there are two main classes of PUFA: omega-6 (ω6) and omega-3 (ω3) fatty acids due to the position of their first double bond from the methyl end. In order to understand the naming system for fatty acids, arachidonic acid (C20:4n6) will be used as an example as shown in Figure 1.4.

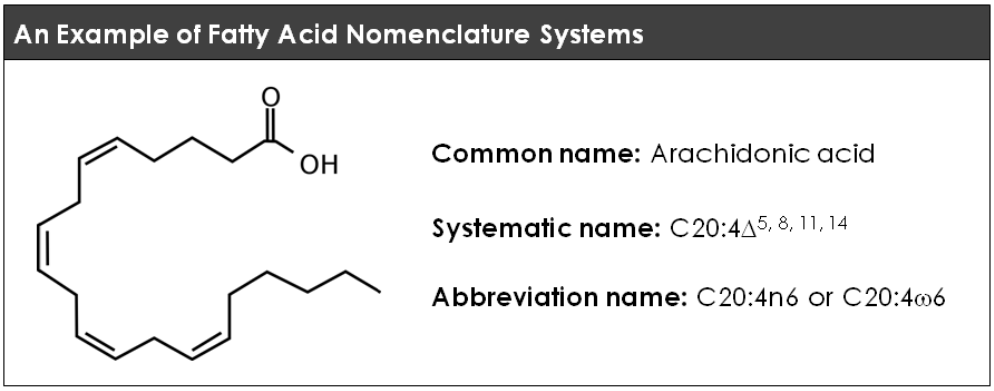


Figure 1.4: Fatty acid nomenclature systems using C20:4n6 as an example

In addition to these formal systems of nomenclature, some fatty acids have their own trivial names such as stearic acid (Gr. *stear*) or palmitic acid (Fr. *palmitique*) and in animal nutrition these trivial names sometimes continue to be used. For convenience, the trivial names will be used from time to time in this thesis, the systemic names being given where necessary for clarity. However, the systemic and the omega system are more widely used to describe PUFA in scientific investigations.

1.1.2 Mechanisms of de novo Fatty Acid Biosynthesis from Two-Carbon Precursors

It is universally accepted that all organisms have the ability to synthesise palmitate (C16:0) or stearate (C18:0) *de novo* from two-carbon organic compounds such as acetate or acetyl coenzyme A using a specific enzyme, fatty acid synthase (FAS). The fatty acid synthase in animals and fungi is a multi-subunit enzyme with seven active sites to catalyse each enzymatic reaction in fatty acid biosynthesis (Nelson and Cox, 2008). The fatty acid biosynthesis requires a specific three-carbon compound called malonyl-CoA, which is formed from acetyl CoA, as a precursor to elongate the carbon chain. Furthermore, this synthetic pathway requires a specific protein, acyl carrier protein (ACP), as a shuttle to make enzymatic reactions occur continuously. These steps in fatty acid biosynthesis are illustrated in Figure 1.5.

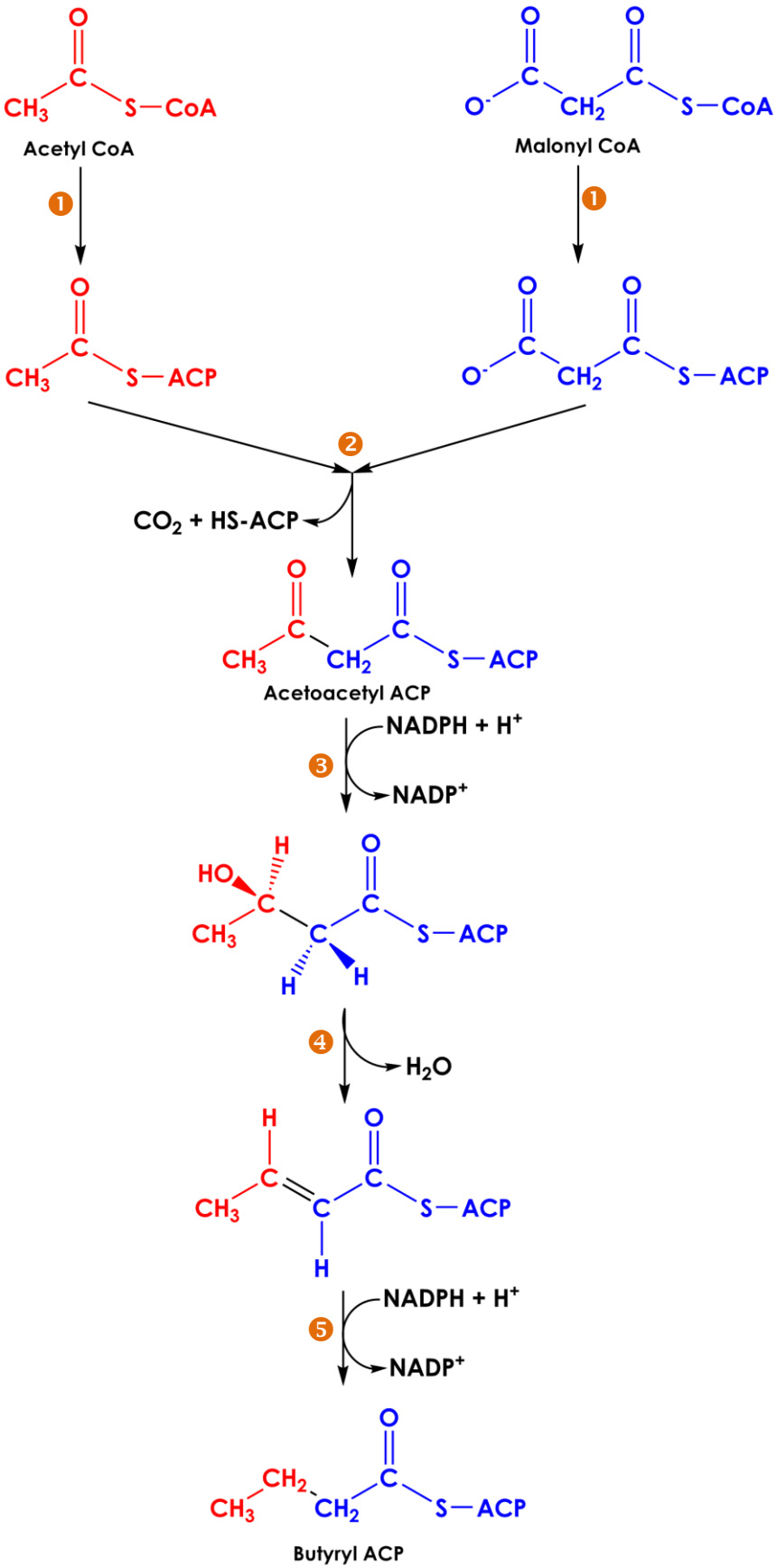


Figure 1.5: Fatty acid biosynthetic enzymatic reactions to synthesise butyryl ACP from acetyl CoA and malonyl CoA (adapted from Garrett and Grisham, 2005 and McMurray and Begley, 2005).

Referring to Figure 1.5, the first step of the anabolic pathway is the conversion of acetyl CoA to acetyl ACP and malonyl CoA to malonyl ACP by ACP transacylase ❶. The products from the first step are more reactive and suitable for further reaction. The condensation of acetyl ACP and malonyl ACP is the second step to generate acetoacetyl ACP as in ❷. Carbon dioxide (CO_2) and ACP are produced at this step. Acetoacetyl ACP is then reduced by NADPH ❸ and followed by dehydration to crotonyl ACP ❹. As the final step, crotonyl ACP is reduced again to produce saturated acyl butyryl ACP ❺. All reactions are repeated for several cycles by adding malonyl ACP in each cycle to increment the carbon chain length resulting in the incorporation of two more carbon atoms in the molecules per cycle. For example, palmitic acid (C16:0) is produced by the pathway being repeated for seven cycles. Accordingly fatty acids found in nature as a result of this pathway have an even-number of carbon atoms. It may be noted that the pathway of fatty acid biosynthesis in bacteria is different which will be explained later on Topic 1.1.3 and can result in the production of odd numbered and branched chain fatty acids (Emmanuel, 1978; Kaneda, 1991; Metz *et al*. 2001).

Palmitic acid is an end-product of catalysis by FAS. However, some animals can further elongate the carbon chain into stearic acid (C18:0) or even longer using a different enzyme system, which is called the fatty acid elongation system (Nelson and Cox, 2008). The various elongase enzymes represent a family of enzymes involved in the pathway that occurs on the endoplasmic reticulum (ER) membrane. The fatty acid elongation system resulting in elongation of the fatty acid chain is quite similar to the palmitate synthesis pathway except that the acetyl-CoA group is used as a carrier instead of ACP. Therefore, there are four main steps: donation of two carbons from malonyl CoA, reduction, dehydration and followed by second reduction as a final step to generate a fatty acid with a chain length two-carbons longer as shown in Figure 1.6. It should be noted that the addition of two-carbon atoms from malonyl CoA occurs at the carboxyl end for the fatty acid and converts the existing carboxylic group carbon into a methylene in the molecule.

In addition to elongation, palmitic acid and stearic acid can be desaturated to generate MUFAs of the same carbon chain length. In this reaction resulting in the formation of a double bond, the double bond is situated between C_9 and C_{10} from the carboxyl end due to the carbon specificity of the enzyme active site. Fatty acyl-CoA desaturase is the enzyme involved directly inserting the double bond into the carbon chain. A series of electron transfers are the main components for this activity, as shown

in Figure 1.7. Oxygen (O_2) is also required for the removal of hydrogens and release as water (H_2O) at the end of process. There are a number of desaturase enzymes and the position of the target carbon adjacent to which the double bond is inserted is used to name and identify the various enzymes. For instance, Δ9 desaturase is an enzyme that catalyses the insertion of a double bond on the 9^{th} position from the carboxyl end as described above. This enzyme is commonly found in most species is able to produce palmitoleate (C16:1n9) and oleate (C18:1n9) from palmitate and stearate, respectively.

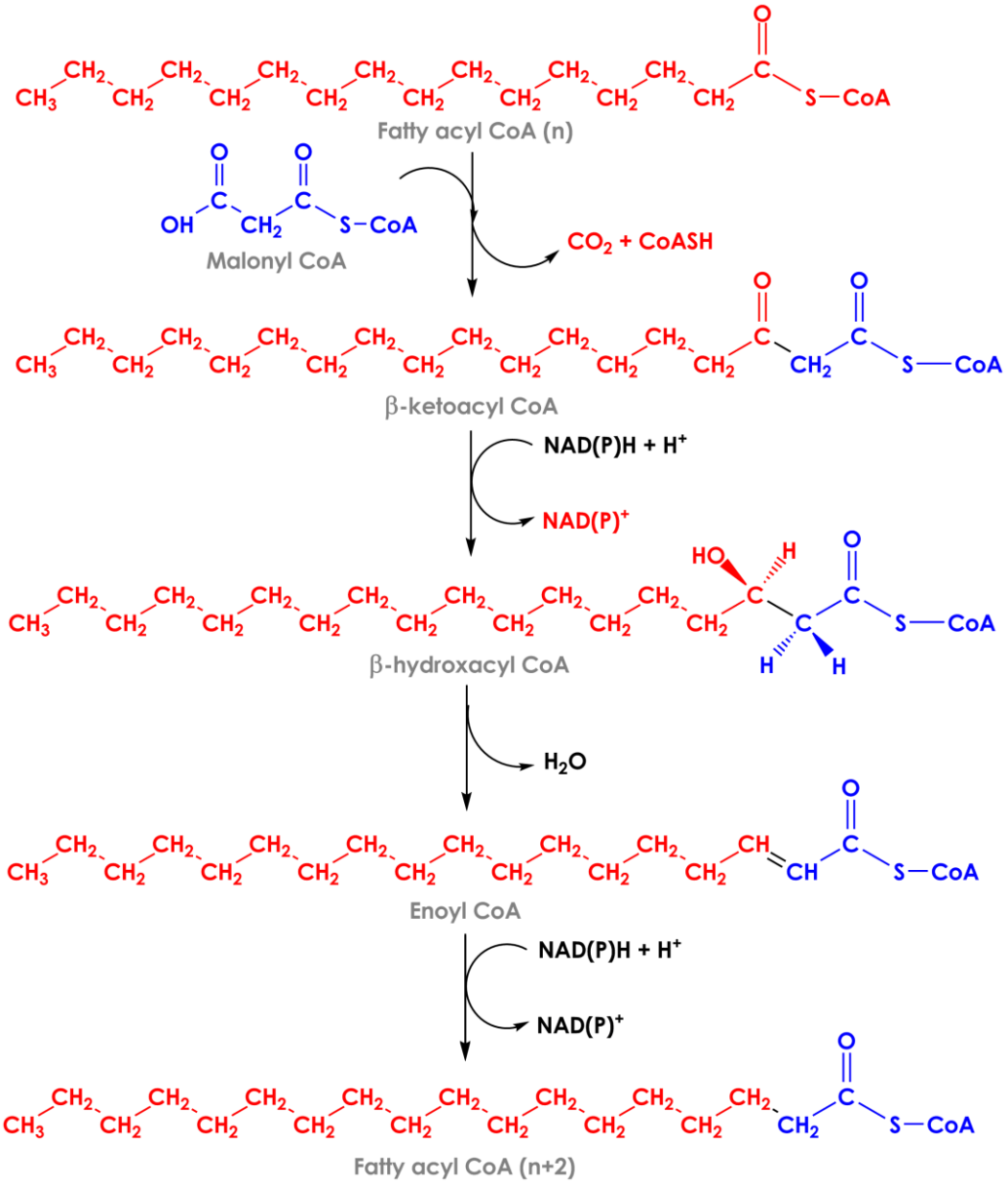


Figure 1.6: The mechanism of fatty acid elongation using fatty acid elongation system.

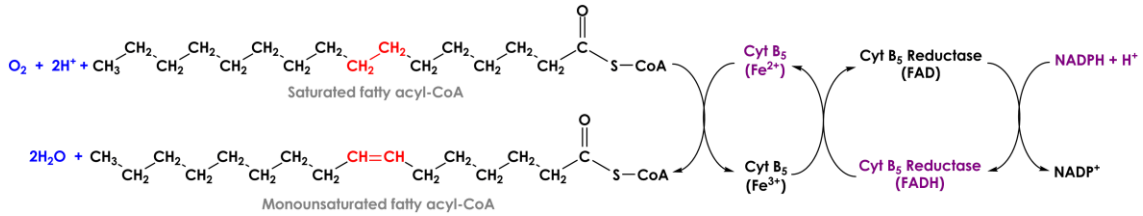


Figure 1.7: Electron transfer in the desaturation process by fatty acyl-CoA desaturase.

1.1.3 A Big Picture of the General PUFA Biosynthetic Pathways

As mentioned earlier in topic 1.1.2, it is generally accepted that all eukaryotes have an ability to synthesise palmitate (C16:0) and/or stearate (C18:0) *de novo* from two-carbon precursors. In contrast, the ability to generate longer chain fatty acids and to desaturate them varies widely between different eukaryotes groups. In this section, an overview of current knowledge of this diversity in PUFA biosynthesis pathways will be reviewed and explained.

Among all organisms two major pathways to synthesise PUFAs are known. In bacteria and some protists, the polyketide synthase pathway (PKS) is used whereas eukaryotes and the aerobic pathways as described briefly above are used. The end products of either of these pathways might be arachidonic acid (ARA; C20:4n6), EPA (C20:5n3) and DHA (C22:6n3). It is therefore necessary to establish which pathway is being used in any organism under investigation. Most of the discussion that follows will refer to the elongation and desaturation pathways of eukaryotes, but some reference will be made to the PKS pathway to avoid confusion.

The PKS pathway is found in both prokaryotes and some lower eukaryotes (Metz *et al.*, 2001). However, this is thought not to be an important pathway in higher organisms such as animals. One of the most interesting points about the PKS pathway is that the desaturation steps occur during the elongation of short-chain fatty acids. In contrast to the PKS pathway, the desaturation in the aerobic pathways occur only to intact fatty acyl chains such as palmitate or longer-chain fatty acids. The PKS pathway is shown in Figure 1.8.

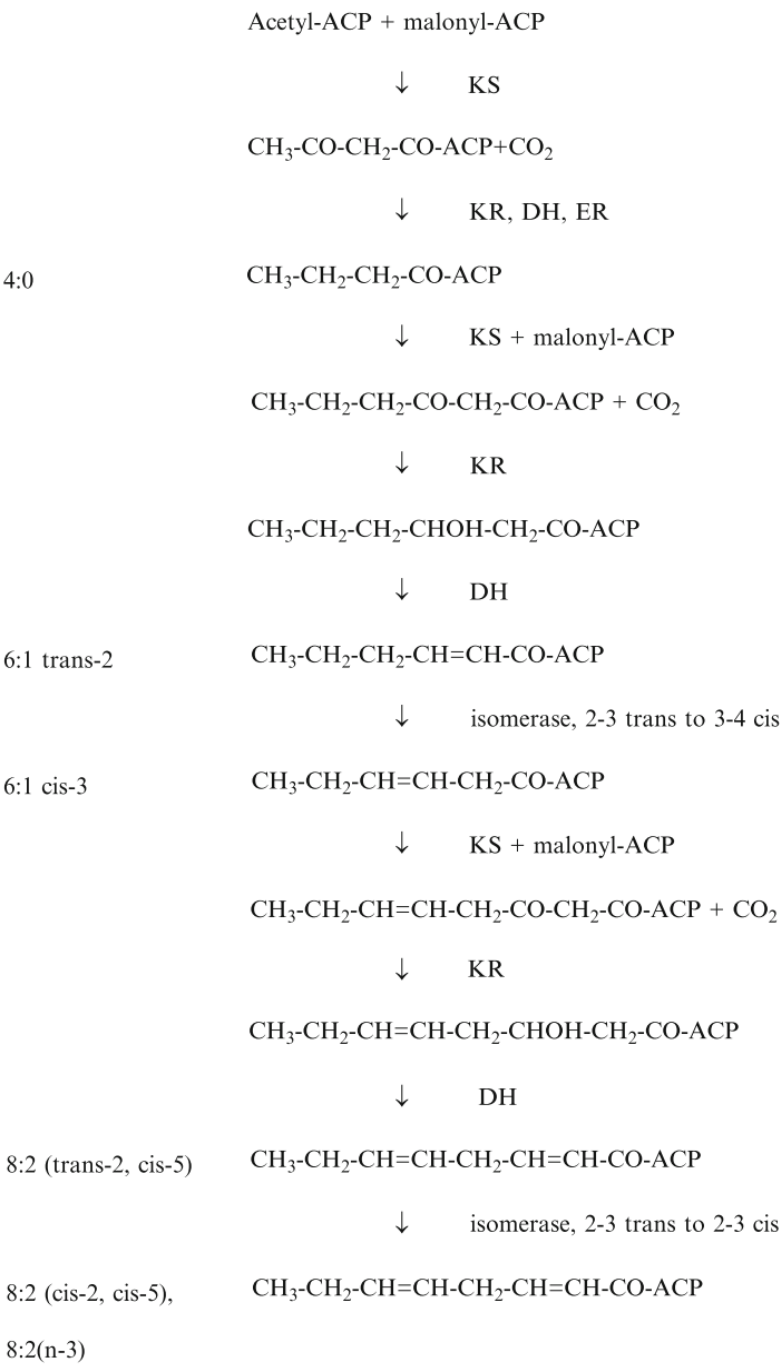


Figure 1.8: Fatty acid synthesis using the polyketide system (PKS). DH = dehydrogenase; ER = enoyl reductase; KR = 3-ketoacyl-ACP-reductase and KS = 3-ketoacyl synthase (Bell and Tocher, 2009)

Marine bacteria include several groups which synthesise EPA and/or DHA but as noted above the biochemical pathways differ from those found in eukaryotes. Gammaproteobacteria such as the marine bacteria, *Shewanella* sp., and Cytophaga-Flavobacterium-Bacteroides (CFB) are the two main lineages of marine prokaryotes that are known, using molecular and genomic evidence, to be able to synthesise ARA, EPA and DHA (David, 2003). Most are psychrophiles, piezophiles, and halophiles and these

conditions are typically found in deep-sea environments. These bacteria produce PUFA, unlike eukaryotes, via the PKS pathway (Metz *et al*., 2001). Interestingly, molecular evidence (gene homology comparison with the *Shewanella* gene) has shown that a thraustochytrid-like marine protist, *Schizochytrium* sp., can also generate DHA (C22:6n3) and C22:5n6 in this way of synthesis (Metz *et al*., 2001; Hauvermale *et al*., 2006).

Most eukaryotic organisms have the ability to synthesise at least some long-chain PUFA via the aerobic pathways using specific desaturase and elongase systems from C_{18} precursors. Several marine organisms have been shown to have the ability to synthesise these long-chain PUFAs (Bell and Tocher, 2009). The details of the various pathways are shown in Figure 1.9.

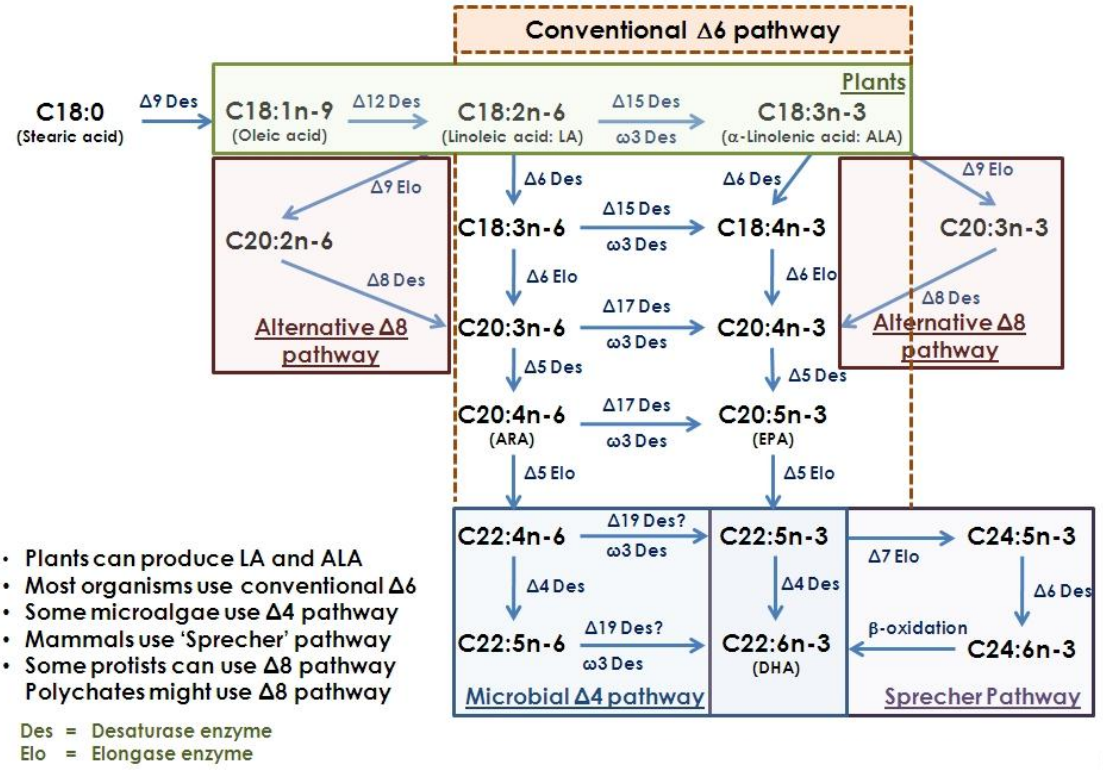


Figure 1.9: An overview of aerobic long-chain PUFA biosynthetic pathways. The various routes for synthesis of ARA, EPA and DHA are shown. The conventional Δ6 pathway and the alternative Δ8 pathway are also illustrated in this diagram. (Adapted from Olive *et al*, 2009 and Venegas-Calaron *et al*, 2010)

In order to understand a general view of the aerobic PUFA biosynthetic pathways, each step will be explained sequentially and by referring back to Figure 1.9. It is interesting to note that desaturases and elongases are the two key families of enzymes that play a critical role in these biosynthetic pathways. In the first step, stearic

acid (C18:0) can be desaturated to oleic acid (C18:1n9) by means of a Δ9 desaturase (stearoyl CoA desaturase). All animals are able to generate the MUFA, oleic acid, from the C_{18} saturated fatty acid precursor. Oleic acid (C18:1n9) can be desaturated further to linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) using the activity of Δ12 and Δ15 (ω3) desaturases, respectively. All plants have the ability to generate these two fatty acids, linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3), however, animals − with some exceptions − are not capable of producing these fatty acids. Linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) are therefore classified as essential fatty acids (EFA) due to the fact that some animals must take these fatty acids from their diets. These two fatty acids are therefore essential as precursors to produce further 'downstream' long-chain PUFA as illustrated in Figure 1.9. In the n-6 series, linoleic acid (C18:2n6) is desaturated and elongated by a series of enzymes; Δ6 desaturase, Δ6 elongase and then Δ5 desaturase, resulting in the synthesis of ARA (C20:4n6). The same sequence can also lead to the production of EPA (C20:5n3) from the α-linolenic acid precursor. These n-6 and n-3 series are also called the 'conventional' Δ6 and Δ3 pathways and several organisms may utilise these pathways to generate ARA and EPA. The pathway to synthesise DHA (C22:6n3) from a C_{20} precursor such as ARA and EPA can occur by way of two different metabolic routes: the 'Δ4 microbial' pathway and the 'Sprecher' pathway. The Δ4 microbial pathway can be found in some microalgae. This pathway uses Δ4 desaturase to generate ω6-docosapentaenoic acid (C22:5n6) from adrenic acid (ADA: C22:4n6) in the n-6 series and DHA (C22:6n3) from ω3-docosapentaenoic acid (C22:5n3) precursor in the n-3 series. In mammals, including humans, the 'Sprecher' pathway or the 'retroconversion' route is used to synthesise DHA (C22:6n3) from the EPA (C20:5n3) precursor. In this route, EPA is elongated to a C_{24} intermediate followed by desatutation and β-oxidation for chain shortening to make DHA as an end product. Some organisms can bypass the conventional Δ6 pathway using the alternative Δ8 pathway. In this pathway, linoleic acid (C18:2n6) is elongated to eicosadienoic acid (C20:2n6) and then desaturated by Δ8 desaturase to eicosatrienoic acid (C20:3n6). In the same way, α-linolenic acid (C18:3n3) is elongated and Δ8 desaturated to C20:4n3.

Different organisms therefore have differing capability for the synthesis of long-chain PUFAs and the ecological importance of this may not at present be fully understood. An exploration of different groups of living organisms and the biosynthetic

pathways they utilise is therefore essential to more fully understand possible fatty acid production cycles in ecosystems including marine and estuarine systems. Due to the fact that all known elongase enzymes are rather similar in their activities and structure, for instance there is a single PUFA elongase gene in all fishes (Bell and Tocher, 2009), the diversity of pathways is likely to be due to differences among the activity and expression control of the desaturase enzymes involved. Therefore, the desaturases are discussed in more detail in this section. Also, the desaturation steps have been considered as the rate-limiting step in PUFA biosynthesis (Pereira *et al*., 2003).

Desaturase structure and function has been reviewed previously by many authors such as Pereira *et al*. (2003), Arts *et al*. (2009) and Meesapyodsuk and Qiu, 2012. The mechanism of desaturase is region-selective to each double bond position and it is an aerobic process (Behrouzian and Buist, 2003). The Δ system is used to designate the introduced double bond position counted from the carboxyl end. For example, Δ9 desaturase involves an incorporation of a double bond in the ninth carbon atom counted from the carboxyl end.

Δ9 desaturase or stearoyl CoA desaturase (SCD) is the first key enzyme to produce oleic acid (C18:1n9) from stearic acid (C18:0). This is one of the best studied desaturase enzymes. SCD has been identified in different species such as insects, fish, mouse and human (Pereira *et al*., 2003). All animals are thought to have the ability to generate oleic acid via SCD because of the universal occurrence of SCD; oleic acid is therefore not considered as an 'essential' fatty acid (Pereira *et al*., 2003 and Castro *et al*., 2011).

In terms of Δ12 desaturase and Δ15 (ω3) desaturase, these enzymes do not occur universally throughout the animal kingdom as in the case for SCD. Higher animals such as the vertebrates fish and many mammals, including humans, lack the ability to generate linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) from oleic acid (C18:1n9) substrate (Pereira *et al*., 2003 and Meesapyodsuk and Qiu, 2012). However, some terrestrial invertebrates such as the land snail (*Bulimulis alternates mariae*), the garden slug (*Arion circumscriptus*), the American cockroach (*Periplanata americana*) and the house cricket (*Acheta domesticus*) have shown the activity of these two desaturases (Blomquist *et al*., 1991; Borgeson *et al*., 1991 and Weinert *et al*., 1993). The invertebrate nematode model, *Caenorhabditis elegans*, has been particularly well investigated and the presence of the Δ12 and Δ15 desaturase activity has been

demonstrated using molecular and genetic evidence (*fat-1* and *fat-2* mutant studies) (Beaudoin *et al*., 2000; Watts and Browse, 2002; Watts, 2009). The presence or absence of these two key enzyme activities is therefore critical information in order to determine what are 'essential fatty acids' in each type of animal.

Activity of the Δ6 desaturase enzyme has been found to be more widespread in the animal kingdom. This enzyme plays a key role in desaturation of linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) to γ-linolenic acid (C18:3n6) and stearidonic acid (C18:4n3), respectively. In mammals, including humans, this enzyme can further desaturate C24:5n3 to C24:6n3 in the special 'Sprecher' pathway prior to β-oxidation to produce DHA (C22:6n3) as an end product. In mammals, the expression of this Δ6 desaturase is controlled by several factors such as nutritional control and/or hormonal and signalling control (Bell and Tocher, 2009). Most animals have the Δ6 desaturase activity to synthesise some fatty acid metabolites. In a similar mechanism, Δ5 can catalyse further reactions on both n-6 and n-3 series pathways. This enzyme can be considered to be one of the most important in the PUFA biosynthetic pathways due to the fact that Δ5 desaturase catalyses the final step in ARA (C20:4n6) and EPA (C20:5n3) production from C20:3n6 and C20:4n3, respectively. In aquatic ecosystems, several fish desaturases have been studied extensively using molecular techniques. Several studies of marine fish indicate that Δ5 desaturase activity is limited in many marine fish species (Tocher and Ghioni, 1999; Tocher, 2003) and differences in the expression/activity of this enzyme among fish species are ecologically important. Some teleost fish species, mainly freshwater and diadromous species such as zebrafish, salmon, trout and tilapia, demonstrate a fully functional ability to synthesise ARA (C20:4n6) and EPA (C20:5n3) (Hastings *et al.*, 2001; Tocher, 2003; Hastings *et al.*, 2004). In contrast, this function appears to have been lost among many marine species, as reviewed by Bell and Tocher (2009), who also note that freshwater teleosts are considered primitive to marine teleost species, suggesting that this may be a secondary 'loss-of-function' in the marine environment.

1.2 *Consideration of the Possible Role of Polychaetes in Marine Ecosystems and Aquaculture in Relation to PUFA Biosynthesis*

1.2.1 PUFAs in Marine Ecology and their Transfer between Trophic Levels

The nature of lipid classes is rather different from other essential nutrients such as proteins or carbohydrates in terms of digestion, assimilation and biosynthesis. Ingested proteins and carbohydrates are generally digested and hydrolysed into smaller monomers: amino acids and monosaccharides, respectively. These smaller subunits are utilised as a precursor to produce a new functional macromolecules. In contrast, lipids are normally digested only into fatty acids and other derivatives and these fatty acids tend to either accumulate in animal tissues or are incorporated into phospholipids in plasma membrane in their basic form. As described earlier in topic 1.1.2 and 1.1.3, organism may have different abilities to modify chain-length and degree of unsaturation. According to the above characteristics, lipids, and more specifically their fatty acid constituents, are therefore potentially very useful tracers in trophic transfer and marine ecology dynamic studies (Iverson, 2009). In order to evaluate the potential for fatty acids to be used to trace trophic steps the degree to which they may be converted by way of elongation and desaturation is of crucial importance. An exploration of each fatty acid in the fatty acid profiles in organisms in each trophic level could therefore be very helpful to understand the changes of fatty acids along the food web and during nutrient transfer. Each synthesised fatty acid can, however, potentially be modified as they transfer up the trophic level in a food chain and this process is generally termed as ‘trophic upgrading’ or ‘trophic repackaging’ by ecologists. For instance, two species of copepods, *Temora longicornis* and *Pseudocalanus elongatus*, have shown rapid growth when fed on the heterotrophic dinoflagellate *Oxyrrhis marina* which was in turn predating the green chlorophycean *Dunaliella*; whereas the copepods failed to grow when fed a monospecific (*Dunaliella* sp.) diet. This was interpreted as being due to the presence and/or absence of some essential fatty acids derived from *Oxyrrhis* sp. (Klein Breteler *et al.*, 1999) not present in the *Dunaliella* diet.

Trophic upgrading may play a key role in fatty acid transfer in marine food webs but the extent to which this occurs is not yet fully understood. In many summaries of marine ecosystems, it is frequently stated that only marine bacteria and marine algae can act as the primary source of unsaturated fatty acids in the ecosystem and that they are subsequently transferred to higher trophic level organisms. For example, Bell and

Tocher (2009) mentioned that virtually all PUFA in marine food webs can be produced by these primary producers thus discounting the possible importance of trophic upgrading. This conclusion should be interpreted with caution however, because several heterotrophic marine organisms have been shown to have the ability to produce some important fatty acids independently of their diet either by desaturation and/or elongation of precursors or by *de novo* synthesis from two-carbon compound precursors. Examples include the marine protist *Schizochytrium* sp. and the parasitic protozoan *Perkinsus marinus*. Some animals have also been shown to have the ability to generate long-chain PUFA such as EPA; in one important example biosynthesis has been shown to occur during the commercial production of a sea urchin, *Psammechinus miliaris* (Bell *et al*., 2001). Several species of terrestrial insects and the genetic model nematode, *C. elegans* have also been extensively studied and the studies have also indicated that these terrestrial animals may be capable of *de novo* PUFA synthesis as described in topic 1.1.3. So far, however, there has been little discussion about PUFA biosynthesis among marine invertebrates.

In estuarine ecosystems, macrofaunal invertebrates are often among the most important constituents in the food web and marine polychaetes in boreal regions often constitute the single most abundant macrofaunal elements. This is true for instance for the two species of benthic polychaete worms, each with different living modes, that are the subject of this thesis. The lugworm (*Arenicola marina* Linnaeus, 1758) and king ragworm (*Nereis virens* Sars 1835), were selected and used as representative marine invertebrates in estuaries because each may be the single most dominant macrofaunal species by biomass. It should be noted that the king ragworm species name will be named and cited as *Nereis virens* (M. Sars, 1835) rather than the taxonomic corrected name, *Alitta virens* (M. Sars, 1835) as revised by Bakken and Wilson (2005), throughout this thesis for recognition and clarification purposes. *A. marina* is a marine polychaete which occupies U- or J- shaped burrows in organic-rich muddy sand (Jacobsen, 1967), whereas *N. virens* lives in a permanent burrow below the sediment surface. The two worm species are also different in terms of feeding mode; *A. marina* is a well-known deposit feeder whereas *N. virens* is a generalist that can be a predator on small animals, omnivorous or even herbivorous depending on the richness of the diets around their burrows (Duangchinda, 2007). In the case of *A. marina*, the worms normally feed on decaying organic materials such as seaweed, microalgae, dead animals and some bacteria (Alyakrinskaya, 2003). *A. marina* is a prey item of several predators including flatfish such as plaice (*Pleuroplectes platessa*) and flounder (*Platichthyes*

flexus) and other marine fishes and birds (de Vlas, 1979). Where *A. marina* and *N. virens* share a habitat the former is also an occasional prey item of the latter.

It should be noted that these two species have different ecological states regarding the seasonal variations. A king ragworm *N. virens* is a semelparous species which they are able to reproduce once in a life span and they will die after reproductive spawning. Normally, the ragworm has the life span for 3 years prior to spawning on spring which the water temperature is about 10-12 °C (Kristensen, 1984). The spawning reproduction process in this species occurs synchronously around April or May. It is rather different in the case of *N. diversicolor* which has the extended spawning period from early spring to summer at the age of 12-18 months. The spawning and breeding season of the lugworm (*A. marina*) is around October during the low water spring tide and they are iteroparous polychaetes which are able to reproduce multiple times in their life cycle (Hardege and Bentley, 1997). These different reproductive cycle is important to the changes in their physiological states including the lipid profile pattern. According to the principle of allocation, when the worm are in mature and reproductive state, their fatty acid profiles could adapt for reproduction. To avoid the effect of reproduction and spawning on the fatty acid profiles, the polychaetes that were used in the experiment were collected as an immature worms (around 1 g weight) and there were no sign of any reproductive maturation changes.

In summary, these two polychaete species, *A. marina* and *N. virens*, occupy the intermediate trophic levels in estuaries. Therefore, an understanding of their lipid profiles and their ability to synthesise PUFA is essential to determine PUFA fluxes and transfer in marine ecosystems. If it were proven that benthic organisms such as these two polychaetes have the ability to carry out chain elongation and desaturation, a re-interpretation of the flux of PUFA in marine food chains would be required.

1.2.2 Problems in Aquaculture and their Alternative Solutions

According to The State of World Fisheries and Aquaculture (SOFIA) report published in 2012 by the FAO, aquaculture is still increasing whereas the contribution of capture fisheries to total world fish supply has been constant (or declining) since at least the end of the 1980s. Whereas total world fish production reached 154 million tonnes in 2011 the continued growth of supply since then has been entirely due to expansion of the aquaculture sector since in contrast to aquaculture, the capture fisheries

component has been rather constant at about 90 million tonnes over the period between in the last six years (2006-2011) as shown in Figure 1.10.

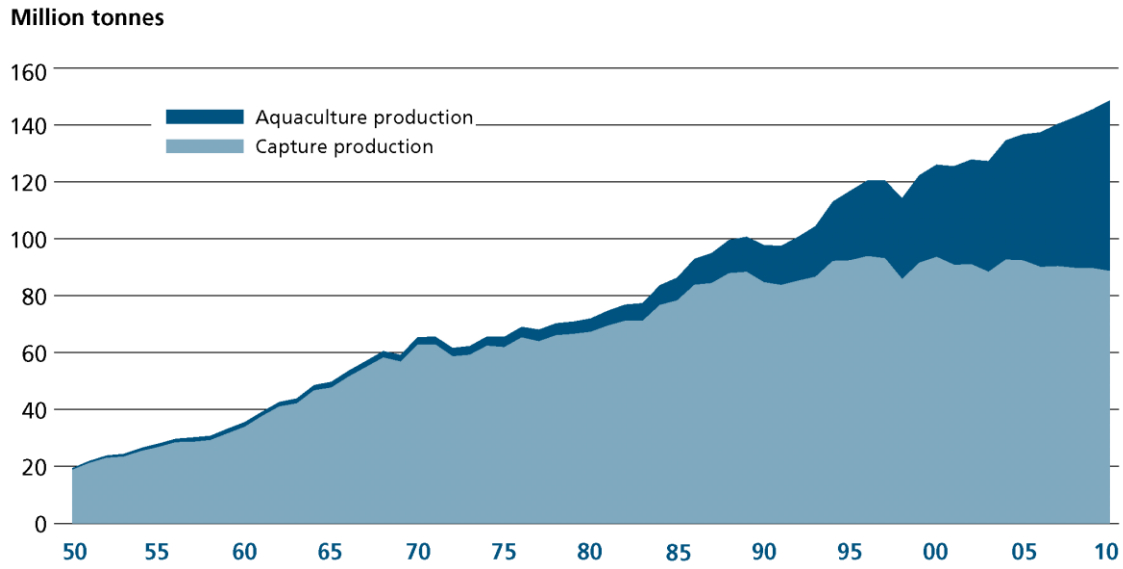


Figure 1.10: Trends of world capture fisheries and aquaculture (FAO report, 2012).

The data summarised by the FAO show that whereas the capture fisheries sector is dominated by marine fisheries, the dominant sector for aquaculture includes mostly inland and freshwater fish production. There are a number of reasons for this difference in balance, which perhaps lie outside the immediate concerns of this thesis; nevertheless concerns have been expressed that the growth of the aquaculture industry may itself contribute to the decline of fishery stocks due to the extensive use of marine raw materials, fish meal and fish oil in formulated fish feeds (Arts *et al.*, 2001). About 25% of fish meal and fish oil production comes from by-products from fisheries according to the International Fish Meal and Fish Oil Organisation (FAO report, 2012). Crustacean and carnivorous finfish farming, for instance, still requires a high-protein and lipid fish meal and fish oil derived from capture fisheries (Tacon *et al.*, 2006). Due to the limited quantity of marine fish meal and fish oil, alternative sources such as plant-derived oil, genetic-engineered plant oil and single-cell oil (SCO) from microalgae are rapidly rising for the aquafeeds (Warude *et al.*, 2006; Naylor *et al.*, 2009). However, these microorganism oils may not be ideal as a replacement for 'marine lipid and fatty acids' as discussed and reported by Miller *et al*. (2008), hence, the development of further alternatives to marine fish oil should be investigated.

The aquaculture of polychaetes was first established in the 1980's in Europe and is now being adopted in a number of countries in SE Asia, including China. Whereas previously, worm aquaculture was developed as a means of providing bait for sea angling and fishing sport, production is now (2012) mostly aimed at the fish and shrimp aquaculture feed sector − cultured worms being used as a component of hatchery diets for fish and shrimp. Polychaete worms are now routinely included in the diets of most hatcheries for the shrimp *Litopeneaus vannamei* either from locally resourced wild collected sources, importation of wild collected sources from China or by way of incorporation of more biosecure cultured sources (UK, Netherlands, Thailand and China). Several lines of evidence indicate that polychaete worms contain moderately high levels of PUFA in their lipid content, and typically are found to have high levels of ARA and EPA along with a broad range of other fatty acids, and to a lesser extent in DHA (Duangchinda, 2007 and García-Alonso *et al*., 2008). Polychaetes are therefore highly suitable as 'natural' fish feed especially for finish and crustaceans (Luis and Passos, 1995; Meunpol *et al*., 2005). Furthermore, Olive *et al*. (2009) have suggested that *A. marina* might have the ability to produce long-chain fatty acids when fed on a diet which lacks them. The implication is that either the worms take up PUFA from associated microorganisms or they have the ability to generate PUFA by chain elongation and desaturation of precursor fatty acids in given feeds (see section 1.1.3 above). However, the study by Ashforth (2008) implied that endosymbiont bacteria in the *A. marina* gut may not play a key role in EPA biosynthesis and the worm could possibly be able to synthesise EPA itself. The evidence obtained in the earlier studies was based on the characteristic fatty acid profiles of the worms grown on a variety of diets and on an analysis of the net gain or loss of key fatty acids in a closed experimental system. This interpretation is based on an experimental analysis of the individual fatty acid content of worms grown in an experimental system in which the inputs of specific fatty acids was measured and on an analysis of bacterial molecular genetics. Hence, an application of biochemical and stable isotope labeling approach is adopted in this thesis to test this concept. In addition, in a study of a closely related nereidid species, *Nereis diversicolor*, by García-Alonso *et al*. (2008), the authors suggested that the fatty acid profiles derived from this species were independent from the diets and seasons − they concluded the worm itself might play a key role in generating EPA and DHA within the tissue. Furthermore, Olive *et al*. (2009) suggested that *A. marina* may utilise the alternative Δ8 pathway (see Figure 1.9) to synthesise EPA basing their interpretation on the gain and presence of the eicosadienoate (C20:2n6) and

the virtual absence of γ-linolenic acid (C18:3n6) in worms grown in the closed experimental systems. Further investigation using ^{13}C-stable isotope labelling together with the working of GC-MS spectra analysis in this thesis were therefore conducted to test this hypothesis. The basic principles of GC/GC-MS and rationale of the mass spectra analysis in order to trace the labelling isotope will be explained in the next section.

1.3 *The Application of GC-MS and ^{13}C Stable Isotope in Fatty Acid Metabolic Profiling Studies*

This section will introduce some relevant basics and principles of stable isotopes and gas chromatography-mass spectrometry (GC-MS). Then, the interpretation of mass spectra of each fatty acid methyl ester (FAME) family will be discussed. The metabolite mass spectra of FAMEs will be explained and clarified. This underlies the interpretation of mass spectra of the FAMEs found in the worm tissues, including those obtained after elongation and desaturation from a given precursor according to the hypothesised PUFA biosynthetic diagram (Figure 1.9). Understanding these principles will be helpful to comprehend the methodology adopted in Chapter 3.

1.3.1 Introduction to Stable Isotopes

Taking a relatively simple view for the purpose of explanation it can be said that the atom is the basic unit of matter. It consists of two main parts, according to the Bohr model, nucleus and surrounding electrons. The nucleus is composed of protons (positive charge) and neutrons (neutral charge) clustered together. Protons and neutrons have an atomic mass of one, whereas electrons (negative charge and with virtually no mass) can be represented as moving around outside the nucleus. Different elements have a different atomic number according to the number of protons found in the nucleus. However, some elements may have a variety of chemical forms due to differences in the number of neutrons in the nucleus. These variant forms of elements are called isotopes. For example, the most common form of the carbon atom in nature is ^{12}C which contains 6 protons and 6 neutrons in the nucleus. Nevertheless, ^{13}C and ^{14}C also occur in nature but in a relatively small amount compared to ^{12}C. For example, ^{13}C has a nucleus constituting 6 protons and 7 neutrons instead of 6 neutrons while ^{14}C contains 8 neutrons as shown in Figure 1.11.

The small changes in the atomic structure of the nucleus can lead to dramatic changes in chemical properties of the element. For instance, ^{13}C is a nonradioactive carbon isotope; in contrast, ^{14}C is radioactive. Because of the stability of the nonradioactive isotope, its relative abundance, which may be expressed as the $^{13}C/^{12}C$ ratio, is constant in organic materials. For example, the ratio will be the same in each animal collected from the natural environment, with the proviso that very small differences might occur due to differences in diet and the effects of isotopic enrichment (McCutchan *et al*., 2003). However, if an external source artificially enriched in ^{13}C is taken into an organism, the $^{13}C/^{12}C$ ratio will change. The changes in the $^{13}C/^{12}C$ ratio in specific molecules can then be used to trace the incorporated isotope through a metabolic pathway.

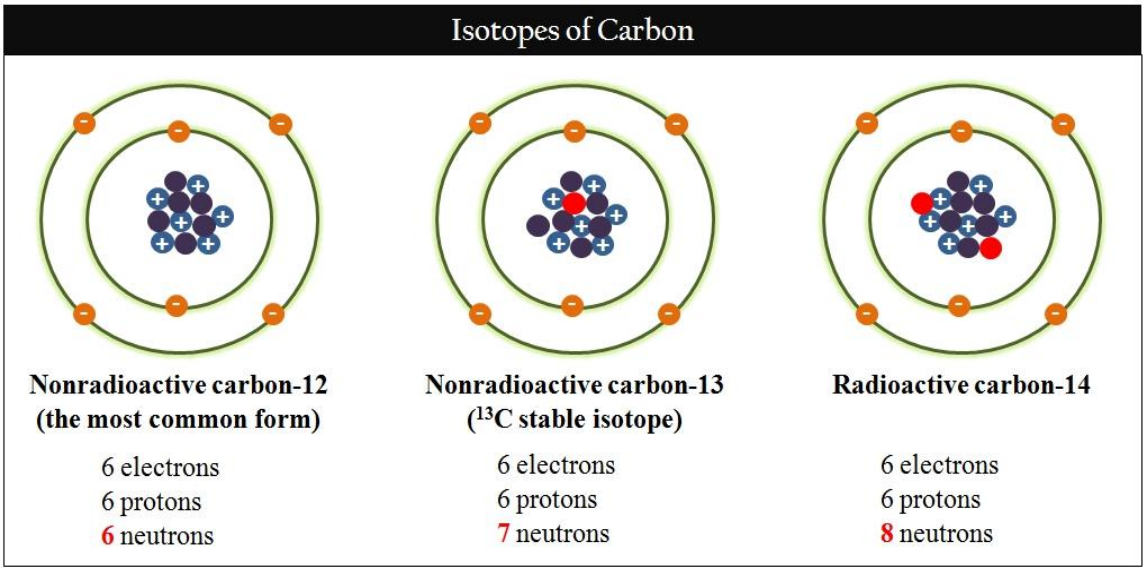


Figure 1.11: Diagram showing the atomic composition in different carbon isotopic forms: nonradioactive ^{12}C, stable isotope ^{13}C and radioactive ^{14}C, respectively.

In the past, ^{14}C radioactive had been adopted widely to trace metabolic pathways in living organisms. Due to the possible risks involved in the use of radioisotopes, the use of the ^{13}C stable isotope is becoming more common, notably in paediatric and clinical research to study changes in fatty acid metabolism (Hellerstein *et al*., 1991; Demmelmair *et al*., 1998; Guo and Jensen, 1998; Brenna, 2002; Larqué *et al*., 2003; Rodriguez *et al*., 2003).

The addition of neutrons in an isotope may affect the kinetics of chemical reactions, especially the enzyme-catalysed reactions, which is also called the isotopic effect. The heavier nucleus requires more activation energy to complete the reaction.

However, the heavier carbon atom is less affected by the addition of one neutron than is the case with relatively much lighter hydrogen atom (Koletzko *et al*., 1998). If only a small quantity of heavier carbon atom is incorporated as part of a larger organic compound such as long-chain fatty acid, the isotopic effect will have a smaller difference compared with their normal forms (Koletzko *et al*., 1997).

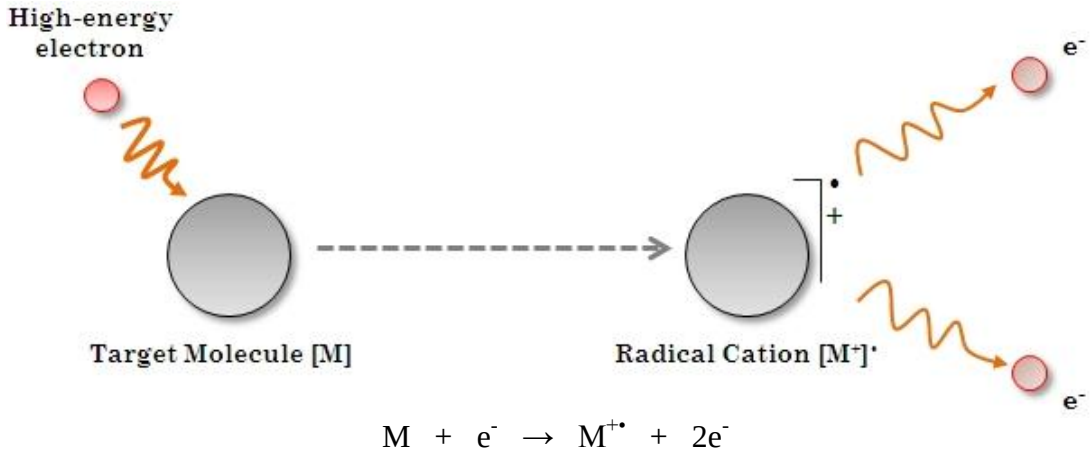
1.3.2 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a combination of two powerful instruments: gas chromatography (GC) and mass spectrometry (MS). The first part is gas-chromatography which is used to separate the volatile molecules, such as fatty acids. Next, the separated molecules will be broken down and identified using the mass spectrometry component. An explanation of the principles of the instruments is necessary to understand the logic of the metabolic tracing experiment that has been adopted.

In GC, the molecules are first heated in the column inlet and evaporated into the gaseous state. The gases are then separated according to mass and size. Like most chromatography techniques, there are two main phases which are involved in the separation process: mobile phase and stationary phase. A mobile phase is a solution or a mixture of the molecules of interest in liquid or gas. In GC procedures, a carrier gas such as helium (He) or hydrogen (H) is adopted as the mobile phase. In contrast to the mobile phase, a stationary phase or immobilized phase is a suite of one or more chemical compounds that are fixed in a place e.g. silica in the GC column. The interaction between the mobile phase and the stationary phase of different molecules leads them to elute at different times which is called the retention time for each molecule of interest. The smaller molecules will separate prior to the molecules having a larger mass and similarly, molecules that are less reactive with the stationary phase will elute prior to more reactive ones.

After the GC procedure, the vaporised and mass-separated gases, which are also called GC eluent, are broken down into radical cations, which have a charge with an odd number (unpaired) of electrons on the ions, in the ionisation source of the mass spectrometer as shown in Figure 1.12. There are several ways to ionise the molecules such as electron impact ionisation (EI) or chemical ionisation (CI); however, EI was adopted in the investigations in this thesis. This technique was selected and applied in the analysis in the experiments (see Chapter 3) because the technique is suitable for

volatile organic molecules which have a molecular weight less than 1000 as is the case for free fatty acid molecules. Furthermore, most of the mass spectra libraries for comparisons and identification of the ions are set using the EI technique. In EI, the electron beam, which has higher energy than the first ionisation energy of the molecule (70-eV), bombards the molecule and turns molecules into cation fragments as shown in Figure 1.12. Some ions could be further ionised into smaller ions. The simplified diagram to summarise the working of GC-MS is illustrated in Figure 1.13.



$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

Figure 1.12: A simplified diagram shows an ionisation process using electron impact ionisation (EI) to create a radical cation. A generalised equation for the EI process is also demonstrated below.

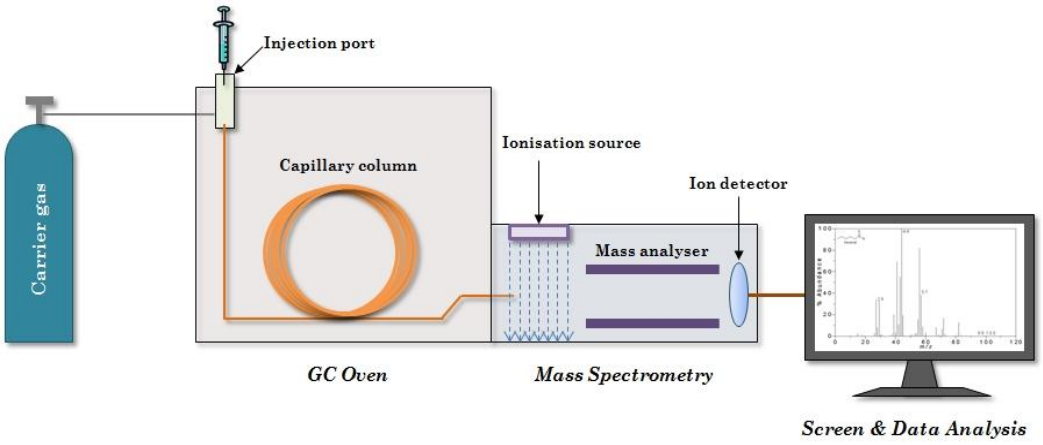


Figure 1.13: A simplified image of gas chromatography-mass spectrometry (GC-MS).

The radical cations are then separated using the mass-to-charge ratio (m/z) in the mass analyser unit which contains electromagnetic field. A mass-to-charge ratio (m/z) is a proportion of an ion mass in terms of atomic mass unit (amu) divided by its charge number. Most of the products derived from EI have only one positive charge in the molecule; therefore, the m/z ratio of the molecule can approximately be its molecular

mass. The smaller m/z fragment will bend more than that of a heavier fragment in a mass analyser electromagnetic field. According to this principle, the fragments with the same m/z value will out at the ion exit slit through the tube. The peak strength in a mass spectrum is therefore depended on the relative abundance of the fragments. The largest m/z value on the scale tends to be the molecular ion $[M]^+$ which has the molecular mass of a whole molecule.

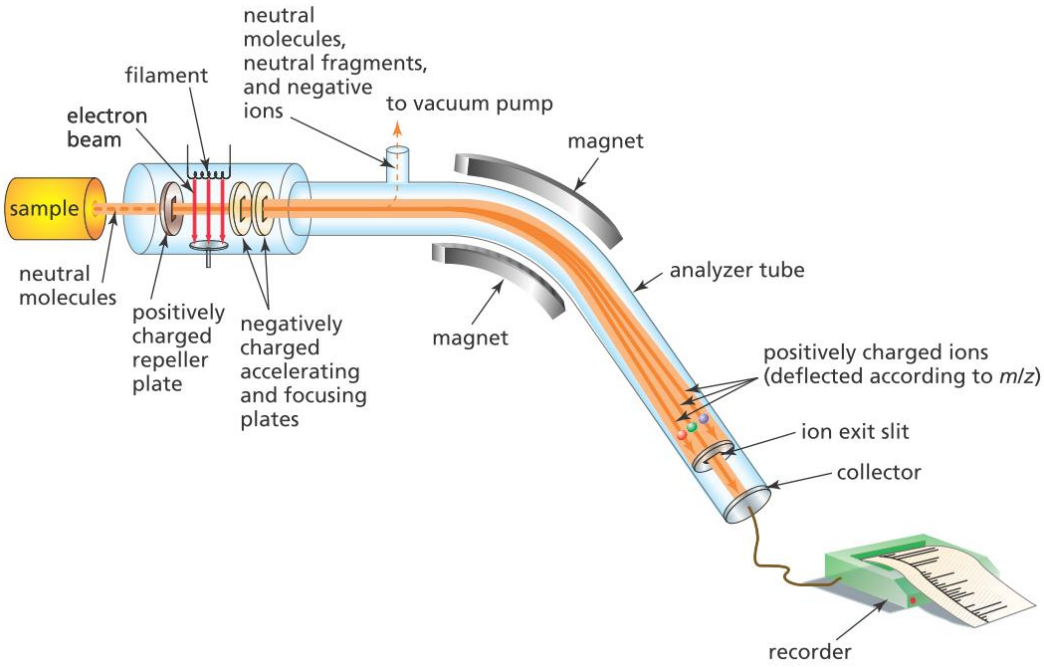


Figure 1.14: Figure a schematic diagram of mass spectrometer. Different m/z values will be detected on the different peak in the mass spectra (Bruice, 2011)

The mass spectrum is a pattern that shows the abundance and m/z of the fragment ions. The X-axis of the spectra is the m/z value of ion peaks while the Y axis is the abundance of the ions. The absolute abundances of the ion peaks were set as a default for analysis; the actual number of the abundance gives greater accuracy in determining the $^{13}C/^{12}C$ ratio. The most abundant ion peak is called the base peak while the peak for the whole molecule is called the molecular peak. The smaller fragments usually cause the stronger peaks and are therefore more abundant in the signal. This scenario is illustrated for idealised ions in Figure 1.15, where C^+ is the smallest and the most abundant ion in the ionisation source; therefore, the C^+ ion peak is the base peak for this molecule. In contrast to C^+, M^+ is the biggest size or the molecular peak, hence, the peak is detected at the farthermost point on m/z ratio scale (X-axis).

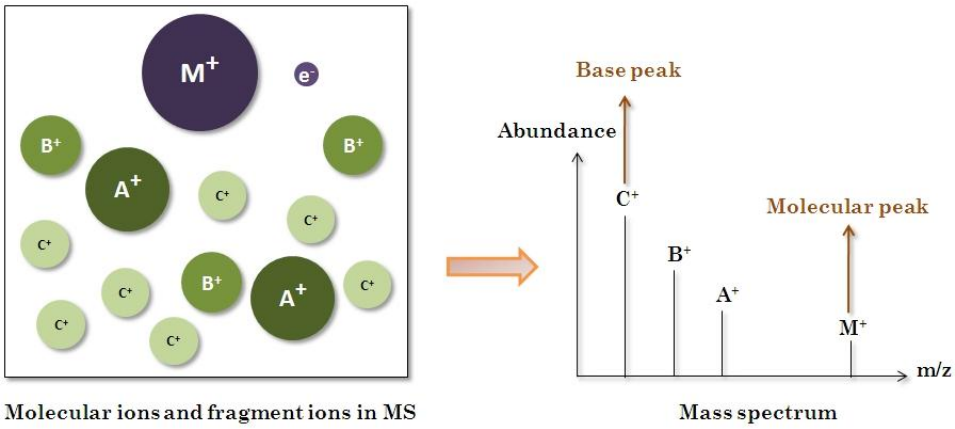


Figure 1.15: An idealised diagram showing the relative abundances of the ions in the mass spectra. The whole molecular peak is designated as M^+ while the rest of the fragment ions are displayed as A^+ B^+ and C^+ on the spectra according to their abundance.

An increase of one in the number of neutrons in an isotope will cause an increase of one unit in the m/z ratio of the fragment ions if all the fragments contain this isotope. The addition of the neutron can be determined using the changes in the $^{13}C/^{12}C$ ratio from the mass spectra. The signal strength of one more atomic mass for each fragment ion will be increased; consequently, an enhancement of the $^{13}C/^{12}C$ ratio could be indicated the presence of ^{13}C in the compounds as demonstrated in Figure 1.16.

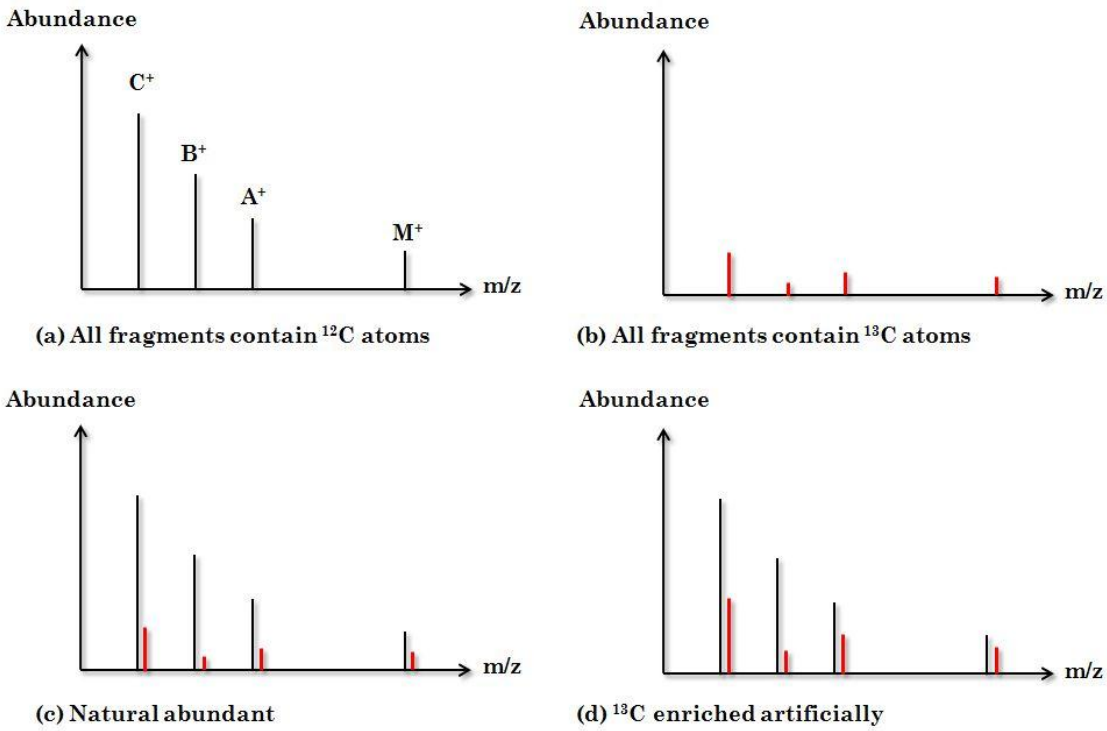


Figure 1.16: The idealised diagrams show the mass spectral patterns on different suppositions when: all fragment products contain only ^{12}C atoms (a), all fragments contain only stable ^{13}C atoms (b), all fragments is in natural condition which both ^{12}C and ^{13}C can be detected in different proportion (c) and all fragments after artificially ^{13}C enriched environment (d). Any significant changes in $^{13}C/^{12}C$ of each fragment will indicate an incorporation of ^{13}C labelled precursors into fatty acid metabolites.

1.3.3 Mass Spectroscopic Interpretation of Fatty Acid Methyl Esters (FAMEs)

According to Christie (1993), all fatty acids derived from the sample is needed to transesterify into fatty acid methyl esters (FAMEs). These FAMEs are more volatile in order to analyse by GC. An understanding of mass spectra derived from FAMEs is therefore essential for an understanding of the logic behind the experimental design and the interpretation of the data. Only some specific ions were used as characteristic peaks for determination of the changes in $^{13}C/^{12}C$ ratio from fatty acids; however, as some peaks are not useful in an interpretation of the changes of the isotope ratio. This section will explain and discuss FAMEs categorised into 4 main groups: saturated, monoenoic, dienoic and polyenoic fatty acid methyl esters. The principles of the interpretation were adopted mainly from the lipid library AOCS (http://lipidlibrary.aocs.org) and many textbooks for mass spectroscopy and mass spectroscopic identification guides such as Silverstein *et al*. (2005) and Dass (2007). Throughout this thesis, it should be noted that the shorthand formula for FAMEs will be followed in which the number of carbons in

the free fatty acid form will be used to describe the corresponding FAME in order to make a clear recognisable name of the fatty acid from which the FAME is derived.

1.3.3.1 An Overview of FAMEs Mass Spectra

As mentioned earlier, some FAME molecules are cleaved and ionised into fragment ions but some molecules can remain whole i.e. as shown by the presence of the molecular ion peak. These peaks are displayed on the mass spectra; therefore, the identification of each dominant fragment ion is a critical step to trace the fatty acid metabolic pathway from a given labelled-isotope precursor.

In long-chain saturated fatty acids (C_{16}-C_{20}), the whole molecular peak ion [M^+] is easily seen on the spectrum at the farthermost point of the m/z value (x axis). This ion peak could be identified from the entire molecular weight of the FAMEs. For example, the molecular weight of methyl palmitate (C16:0) is 270; hence, the molecular peak ion will appear at the m/z = 270 on the spectra. If the same carbon-numbered FAME with only one double bond in its molecule is analysed, the molecular peak ion will have two mass units less than methyl palmitate (C16:0). For instance, methyl vaccenate (C16:1n9) has one double bond between ninth and tenth carbons; hence, the molecular peak should be detected at m/z = 268 compared to the m/z = 270 in methyl palmitate. The number of the double bonds therefore causes a characteristic reduction of m/z value for a whole molecular peak.

Further from the molecular peak, some fragment ions are detected on the spectrum. One of the most common fragment peaks on the mass spectra, especially in saturated FAMEs, is a product derived from a special McLafferty rearrangement, of which the ion is also called McLafferty ions (m/z = 74). The McLafferty rearrangement is one of the most common intramolecular rearrangements via β-cleavage derived from the mass spectrometry. This rearrangement can be found in any organic molecules which contain a carbonyl group (-C=O-) (in this experiment, the ester group in the FAMEs.) The electron shifting mechanism of the McLafferty rearrangement is shown in Figure 1.17. The presence of the McLafferty ion can therefore confirm the ester derivatives of the molecule, in this case, the FAMEs are found in the sample. The other products that are formed by the loss of the McLafferty ion ([M-74$^+$]) are also useful for detecting the isotope. However, the McLaffery ion peak is not a dominant peak in unsaturated fatty acids, particularly in the PUFAs.

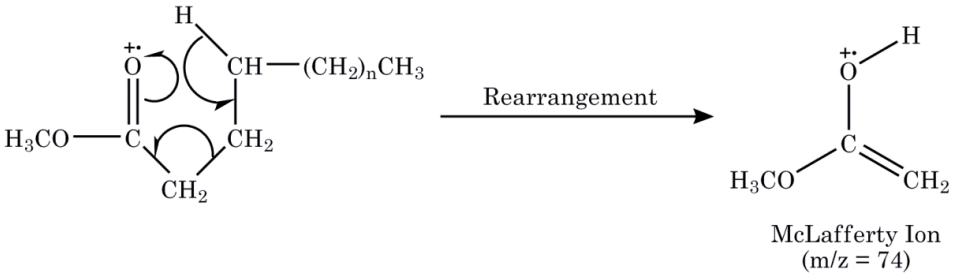


Figure 1.17: A schematic reaction mechanism for production of the McLafferty ion from a long-chain hydrocarbon fatty acid methyl ester (FAME) precursor via the McLafferty rearrangement.

In addition to the products from the McLafferty rearrangement, the loss of the methoxy group (-OCH_3) or methanol (CH_3OH) from the molecular peak ($[M-31/32]^+$) is often detected from the FAMEs spectra. For saturated and dienoic FAMES, the loss of the methoxy ion ($[M-31]^+$) is a major product, whereas the loss of methanol ($[M-32]^+$) is dominant on the monoenoic FAMEs spectra. The rest of the peaks on the mass spectra series are derived from the hydrocarbon series. These series are different for each type of FAMEs and will be discussed in detail later.

1.3.3.2 Mass Spectra of Saturated FAMEs

Straight-chain saturated FAMEs have several characteristic peaks and are easy to identify from the molecular peak ($[M^+]$. The dominant (base) peak from the saturated FAMEs is the McLafferty ions (m/z = 74). The presence of the methoxy ion (m/z = 31) is also found on the spectrum together with the loss of the methoxy peak ion $[M-31]^+$. The long homologous series of methyl ester molecules can be determined in several spectra. The general formula for this ion series is $[CH_3\text{-}OCO\text{-}(CH_2)_n]^+$ where n starts from 2. Each ion will be different by 14 atomic mass unit (amu).

Figure 1.18 shows as an example the methyl palmitate (C16:0) spectrum from the GLC-538 external standard sample. The most abundant peak (base peak) in the spectrum is the McLafferty ion (m/z = 74). As mentioned earlier, the molecular peak is clearly seen in the spectrum which is the m/z = 270 peak ion. Also, the loss of the methoxy ion $[M-31]^+$ (m/z = 239) is also shown on the spectrum. The homologous series according to the general formula $[CH_3\text{-}OCO\text{-}(CH_2)_n]^+$ is started from 87, 101, 115, 129 and so on. The chemical structure of the series is shown in Figure 1.19.

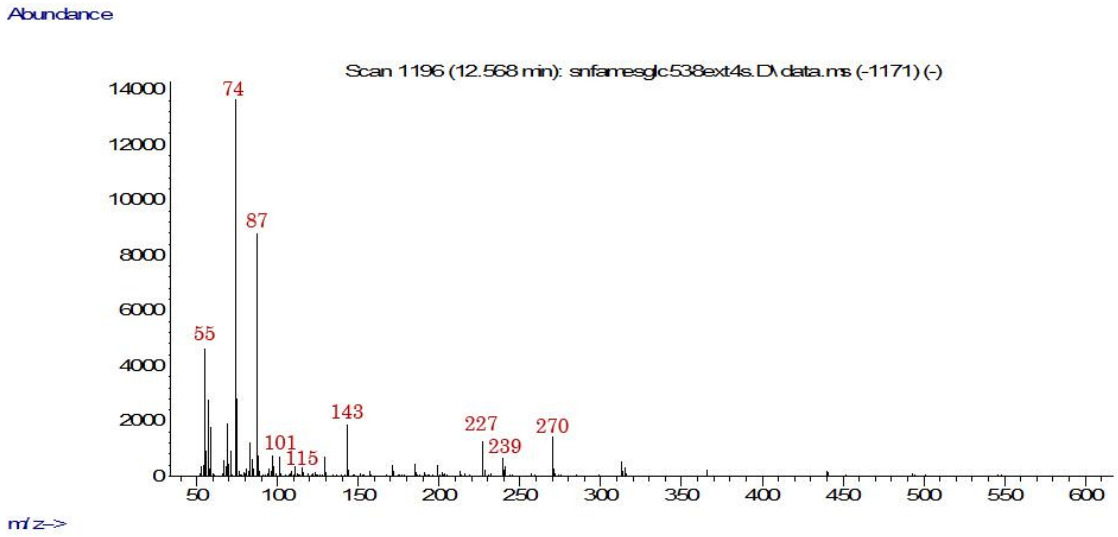


Figure 1.18: The mass spectrum of methyl palmitate (C16:0) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

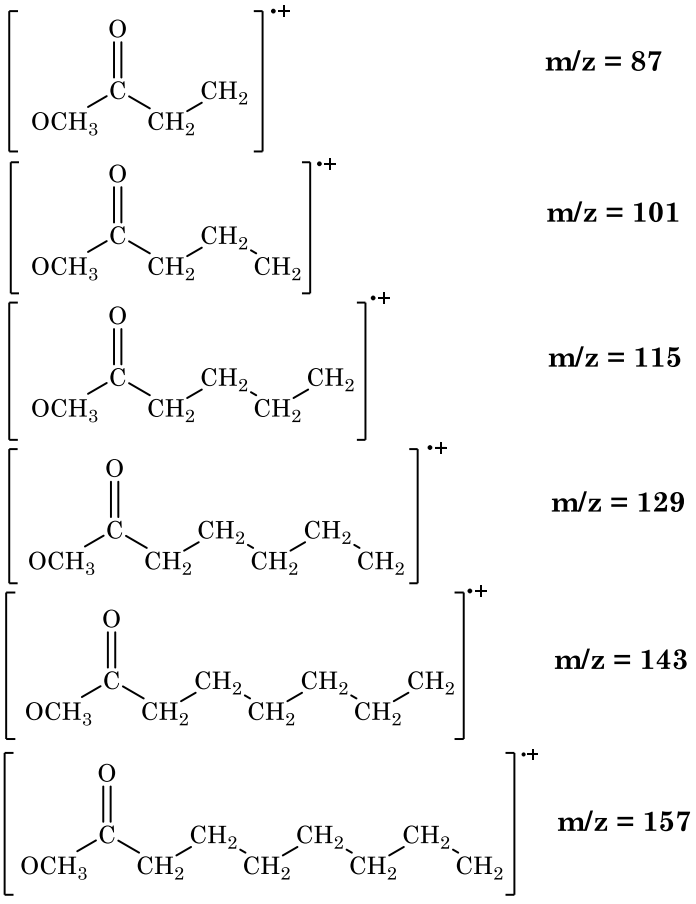


Figure 1.19: The homologous series of fragment ion peaks chemical structures derived from saturated FAMEs.

Another example of a saturated FAME is methyl stearate (C18:0) from the same standard as demonstrated in Figure 1.20. The dominant peak is still the McLafferty ion (m/z = 74). The whole molecular peak is m/z = 298; therefore, the loss of the methoxy ion peak $[M-31]^+$ is m/z = 267 as a result of an increase of the whole molecular weight. However, the series is still the same as all saturated FAMEs because all the fragments are ionised from the carboxyl end of the molecule.

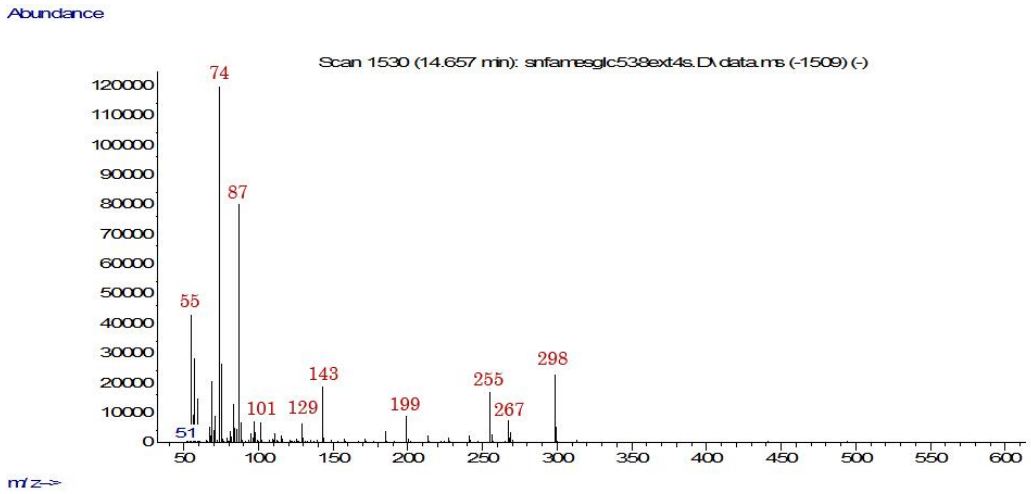


Figure 1.20: The mass spectrum of methyl stearate (C18:0) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

1.3.3.3 Mass Spectra of Monoenoic and Dienoic FAMEs

From this section forward, the discussion and characterisation of mass spectra will be focused mainly on methylene-interrupted double bonds in unsaturated FAMEs.

For monounsaturated and dienoic FAMEs, it is difficult to determine and identify the position of the double bond due to the ability of electron movement over the molecule when they are ionised, which is also called electron delocalisation. For example, methyl vaccenate (C16:1n9) and methyl palmitoleate (C16:1n7) have the same molecular weight which will be shown on the same molecular peak at m/z = 268 but have a different position of the double bonds in their molecules. However, the integration between mass spectra and GC retention time is very helpful to identify the fatty acid methyl esters including some mass spectrum peak ions. Methyl oleate (C18:1n9) will be given as an example of monoenoic fatty acid while methyl linoleate (C18:2n6) and methyl eicosadienoate (C20:2n6) spectra will be discussed as examples of dienoic FAMEs.

For monoenoic FAMEs such as methyl oleate (C18:1n9), the molecular peak (m/z = 296) is still recognisable on the spectra as in Figure 1.21. In contrast to normal saturated FAMEs, monoenoic acids tend to lose methanol (CH_3OH) instead of the methoxy ion ($-OCH_3$); hence, the peak of $[M-32]^+$ (m/z = 264) is more dominant compared to the $[M-31]^+$ (m/z = 265). The series on the spectra is derived from hydrocarbon fragment ions in the general formula $[C_nH_{2n-1}]^+$. The most abundant ion in the spectra is m/z = 55 from the hydrocarbon fragment series instead of the McLafferty ion (m/z = 74). The hydrocarbon fragments contain a double bond in its molecules, thus it is difficult to determine the chemical structure of ions because of the variation of isomeric chemical forms. The methyl oleate mass spectrum is shown in Figure 1.21.

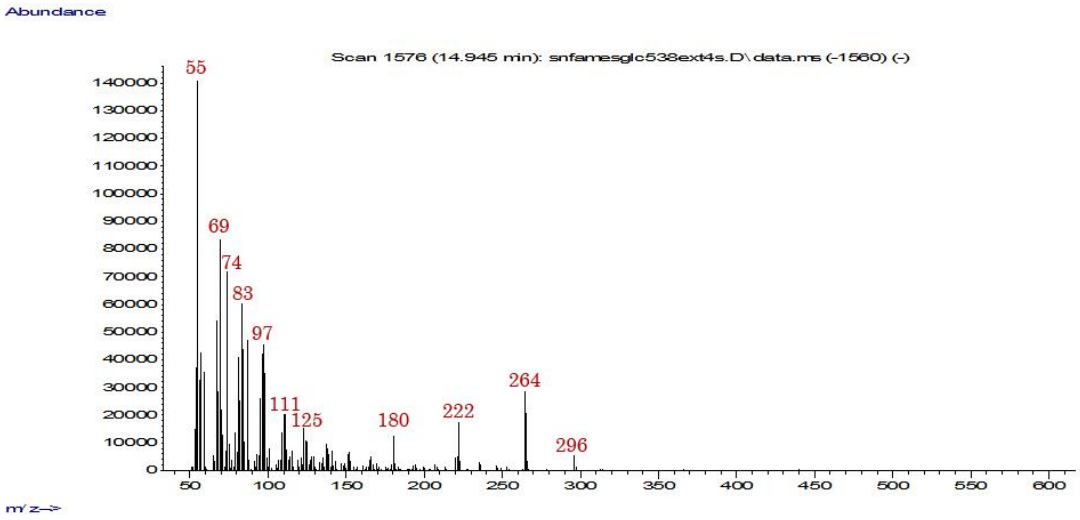


Figure 1.21: The mass spectrum of methyl oleate (C18:1n9) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

The molecular ion peak of methyl linoleate (m/z = 294) is rather prominent on the spectrum as shown in Figure 1.22. The loss of the methoxy ion $[M-31]^+$ (m/z = 220) is also detected, although the McLafferty ion is less likely to be found on the spectrum. The hydrocarbon series formula is similar to the monounsaturated FAMEs except that the number of hydrogen atoms is reduced by two units further, the general formula therefore is $[C_nH_{2n-3}]^+$. The ions from the hydrocarbon series are dominated in the lower m/z range (m/z = 67, 81, 95, etc.).

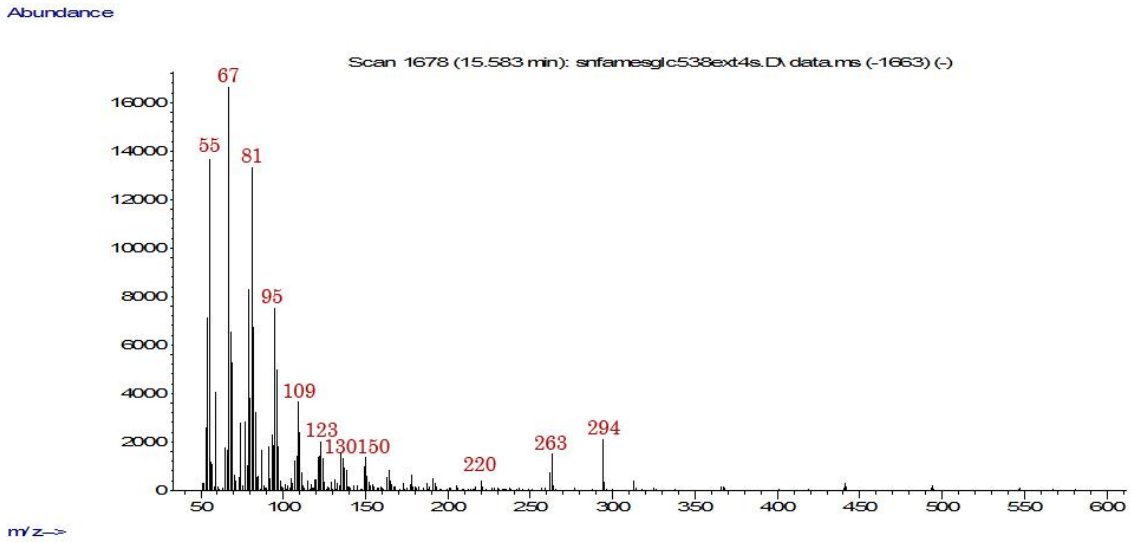


Figure 1.22: The mass spectrum of methyl linoleate (C18:2n6) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

Methyl eicosadienoate (C20:2n6), which is a dienoic FAME with the same number and position of double bonds, shows a similar pattern to the spectrum as in the case of methyl linoleate (C18:2n6). The dominant peak ions are derived from the hydrocarbon series ($[C_nH_{2n-3}]^+$). However, the molecular peak ion (m/z = 322) is also detected on the spectrum together with the loss of the methoxy ion $[M-31]^+$ (m/z = 291). The mass spectrum of methyl eicosadienoate (C20:2n6) is shown in Figure 1.23.

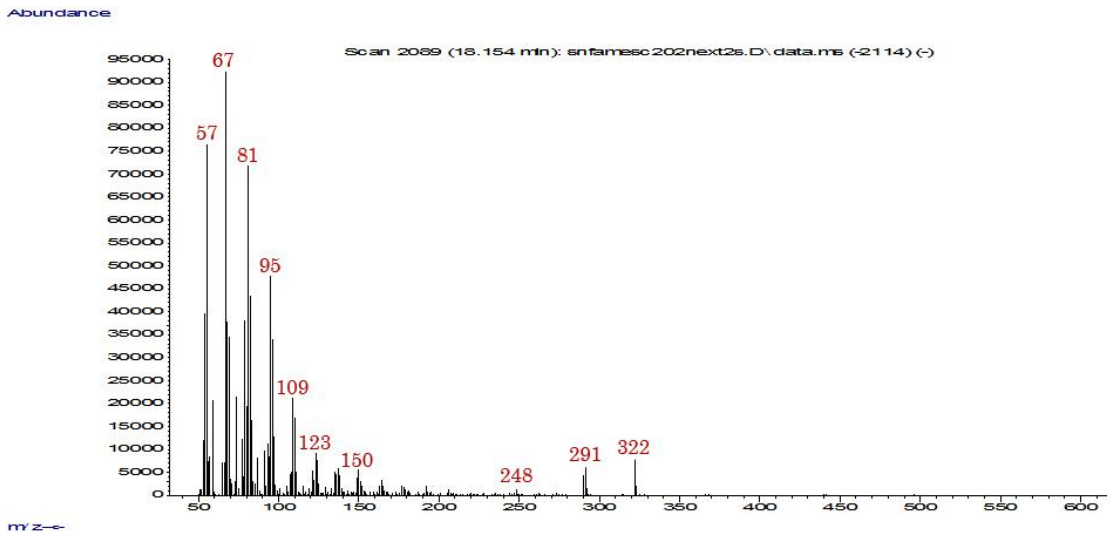


Figure 1.23: The mass spectrum of methyl eicosadienoate (C20:2n6) taken from the eicosadienoate external standard with some m/z value of interesting peaks labelled (author's own).

1.3.3.4 Mass Spectra of Trienoic and Polyenoic FAMEs

In contrast to monoenoic and dienoic FAMEs, the trienoic and polyenoic FAMEs have their own unique 'fingerprint' ions for each family of FAMEs. An understanding of two ion type products from the ionisation process is necessary to know the trienoic and polyenoic FAMEs mass spectra pattern. There are two main ions derived from the cleavage process: ω-ion and α-ion, which are named according to the position of the cleavage site. If the cleavage product occurs from the methyl end, the product will be termed as ω-ion. In contrast, the α-ion is the cleavage product from the carboxyl end of the cleaved molecule. Further details will be considered later in the given examples.

Methyl γ-linolenate (C18:3n6) and methyl α-linolenate (C18:3n3) will be compared and discussed together to show the similarities and differences for each type of trienoic FAMEs. The molecular peak of these two molecules are detectable on the same mass-to-charge (m/z = 292). However, the loss of the methoxy/methanol $[M-31/32]^+$ is rarely seen on the spectrum including the McLafferty ion (m/z = 74). Trienoic FAMEs have one more double bond compared to dienoic FAMEs, therefore, the general formula for the hydrocarbon series becomes $[C_nH_{2n-3}]^+$. The dominant base peak in trienoic spectrum is m/z = 79 which is characterised from hydrocarbon series. The mass spectra of these two FAMEs are displayed in Figure 1.24 and Figure 1.25.

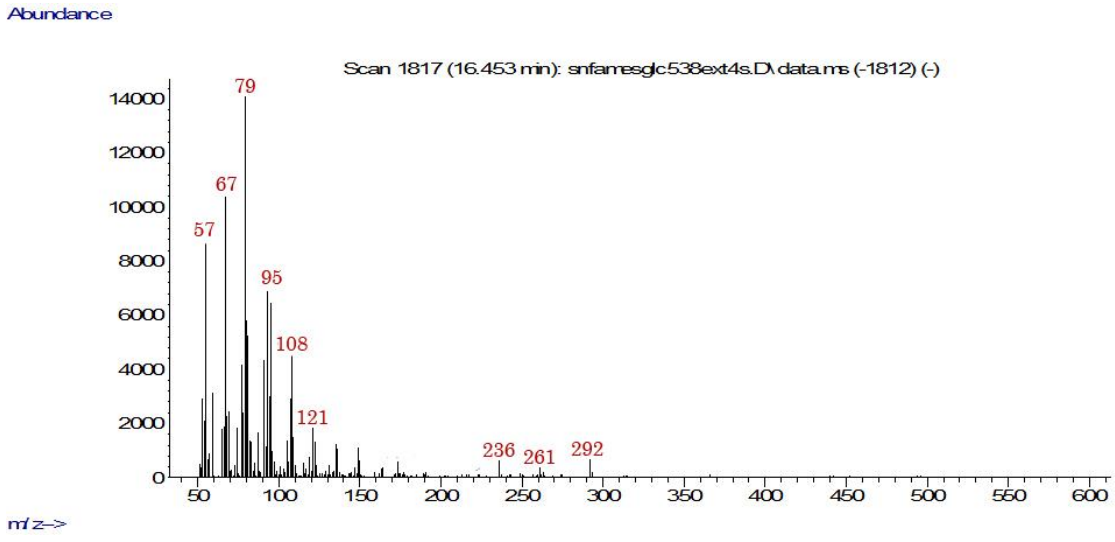


Figure 1.24: The mass spectrum of methyl α-linolenate (C18:3n3) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

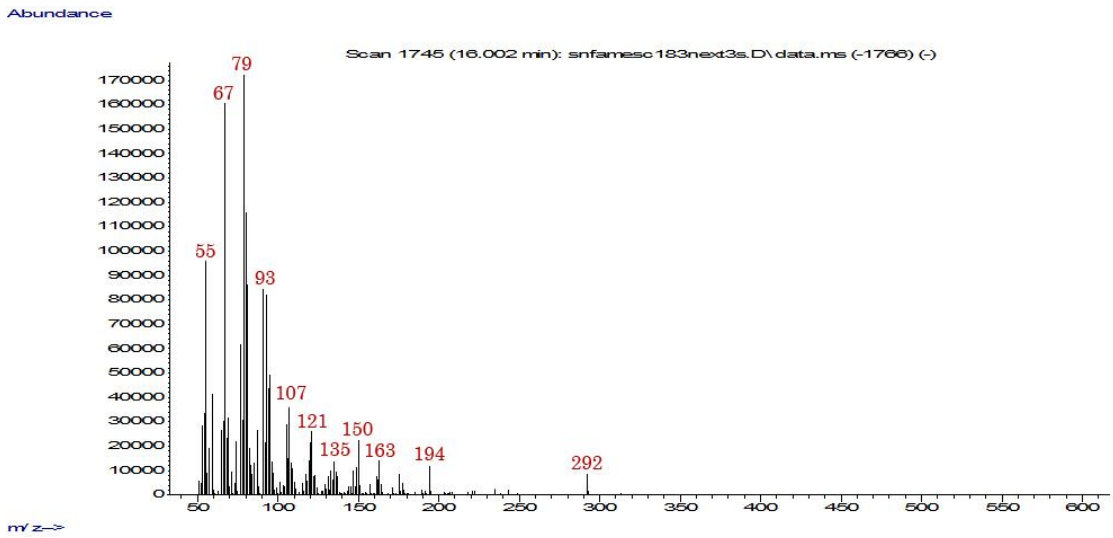


Figure 1.25: The mass spectrum of methyl γ-linolenate (C18:3n6) taken from the γ-linolenate external standard with some m/z value of interesting peaks labelled (author's own).

The interesting point for trienoic and polyenoic FAMEs is the presence of the ω- and α-ion. These two ions are very useful to determine the position of double bonds in methylene-interrupted FAMEs. For ω-ions, it is cleaved at the position as shown in Figure 1.26. The fragment ω-ion for n-6 fatty acids methyl esters is therefore at m/z = 150 while the m/z = 108 ion is a 'fingerprint' in any n-3 fatty acids. These 'fingerprint' ω-ions are constant in each fatty acid family. In contrast to ω-ions, α-ions are variable from each fatty acid family.

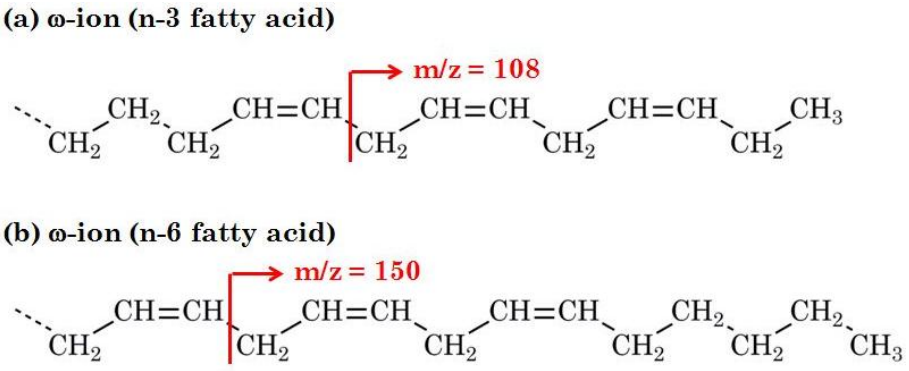


Figure 1.26: A diagram shows how the ω-ions can be produced from different fatty acid families: n-3 or ω-3 fatty acid (a) and n-6 or ω-6 fatty acid (b).

The production of these α-ions is rather similar with the ω-ions except the cleavage has occurred from the carboxyl end as illustrated in Figure 1.27. In methyl γ-linolenate (C18:3n6), the α-ion is shown at m/z = 194 whereas the peak at m/z = 236 is the α-ion of methyl α-linolenate (C18:3n3).

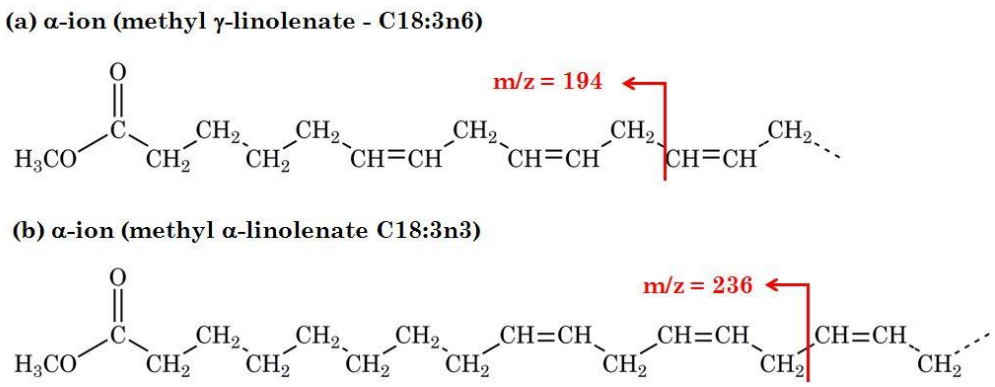


Figure 1.27: Diagram showing how the α-ions can be produced from different fatty acid families: n-3 or ω-3 fatty acid (a) and n-6 or ω-6 fatty acid (b).

Analogous α-ions in different methylene-interrupted FAMEs have a different m/z ratio on the spectra. According to the lipid library AOCS, the m/z ratio of the α-ions is listed in Table 1.1.

Table 1.1: The α-ions in the mass spectra of different families of FAMEs

First two double bond positions	Ion (m/z)
Δ4, 7	166
Δ5, 8	180
Δ6, 9	194
Δ7, 10	208
Δ8, 11	222
Δ9, 12	236
Δ10, 13	250
Δ11, 14	284

The mass spectra of polyenoic FAMEs show a rather similar pattern to that of trienoic FAMEs; however, the molecular peak is very difficult to detect on the spectrum. The ions from cleavage processes are still important to determine the fragment. Three examples of the important FAMEs mass spectra will be explained to show the characteristic ions of each FAMEs: methyl arachidonate (ARA - C20:4n6), methyl eicosapentaenoate (EPA - C20:5n3) and methyl docosahexaenoate (DHA - C22:6n3).

As can be seen from the methyl ARA (C20:4n6) mass spectrum in Figure 1.28, the molecular peak of methyl arachidonate (m/z = 318) is present only in a very small amount that may be insufficient to allow it to be detected on the spectrum. The ω-ion of this FAMEs is shown on the m/z = 150 as a result of cleavage of the ω6 fatty acid methyl esters. According to Table 1.1, α-ion is also detected at the m/z = 180. The other peaks are derived mainly from the hydrocarbon series $[C_nH_{2n-3}]^+$.

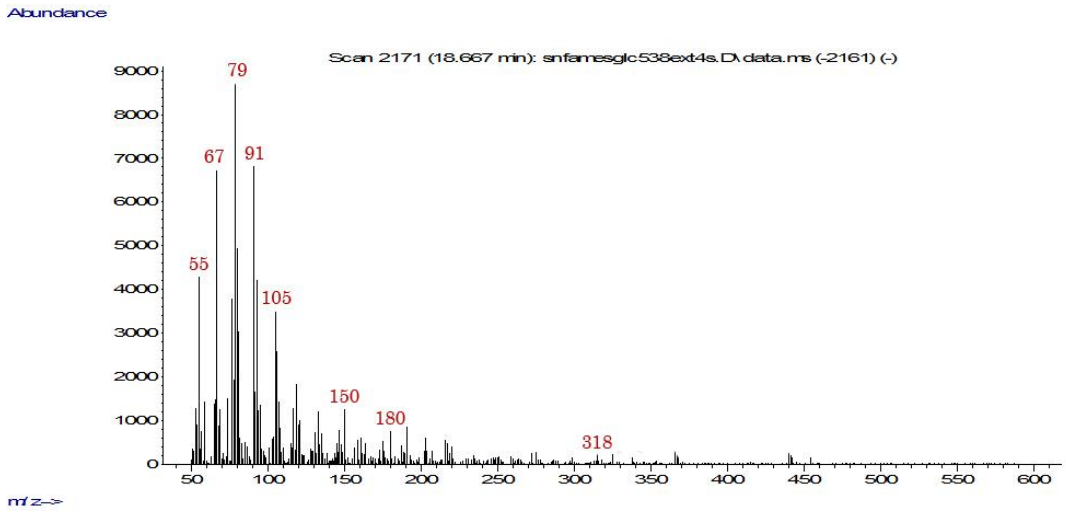


Figure 1.28: The mass spectrum of methyl arachidonate (C20:4n6) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

For polyenoic FAMEs mass spectra such as methyl eicospentaenoate (C20:5n3) (Figure 1.29) and methyl docosahexaenoate (C22:6n3) (Figure 1.30), the higher mass range peaks including the molecular peak (m/z = 316 and 342, respectively) are hardly seen on the spectrum. The hydrocarbon series can also be detected as usual on the spectrum. The prominent peak, which is unique to the polyenoic FAMEs, is a tropylium rearrangement ion (m/z = 91) which is an aromatic compound with an empirical formula of C_7H_7 and its chemical structure is shown in Figure 1.31. Both methyl eicosapentaenoate (C20:5n3) and methyl docosahexaenoate (C22:6n3) have the same ω-ion (m/z = 108) due to the same position of the first double bond from methyl end (ω3 fatty acids). In contrast to the ω-ion, the α-ion of methyl eicosapentaenoate (C20:5n3) is shown at m/z = 180, while the α-ion of methyl docosahexaenoate (C22:6n3) appears at m/z = 166. These two α-ions are small but it stands clear from the background noise on the spectrum.

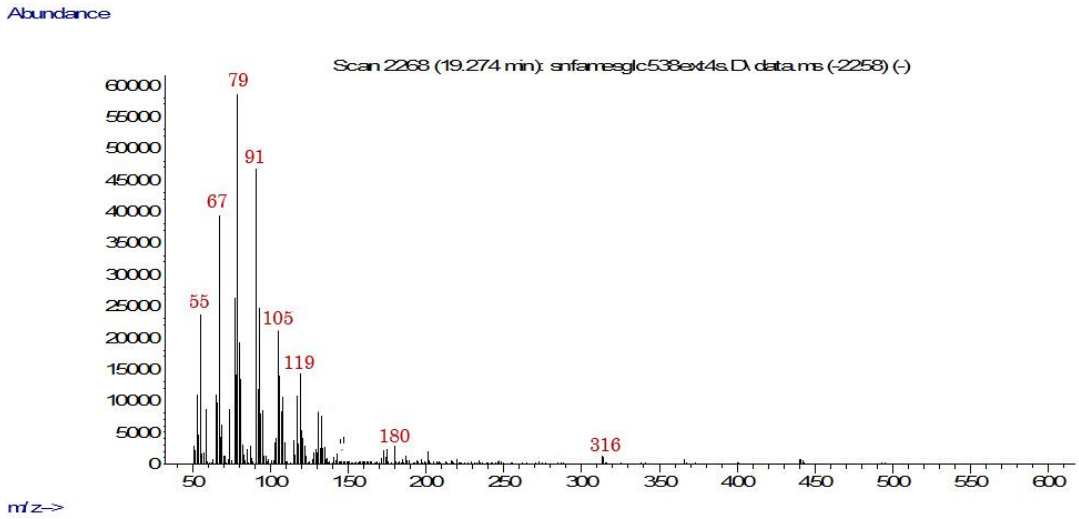


Figure 1.29: The mass spectrum of methyl eicosapentaenote: EPA (C20:5n3) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own).

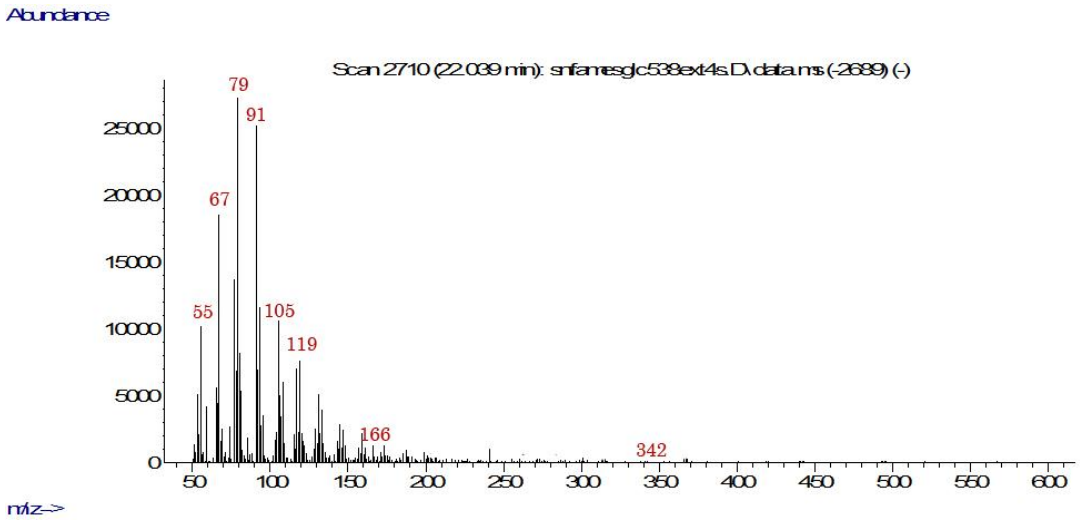


Figure 1.30: The mass spectrum of methyl docosahazaenoate: DHA (C22:6n3) taken from the GLC538 external standard with some m/z value of interesting peaks labelled (author's own) methyl DHA mass spectrum (C22:6n3) from GLC538 standard.

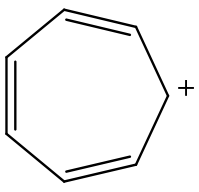


Figure 1.31: A chemical structure of the tropylium ion (m/z = 91).

1.3.3.5 The Application of Stable Isotopes in the Tracing Experiment

In the experiment described in section 3.2 , Chapter 3, 1-^{13}C palmitic acid was introduced and incorporated into fish feed to trace the changes of the putative PUFA biosynthetic pathways in *N. virens*. The position of the ^{13}C atom in palmitic acid will be changed if the fatty acid chain is elongated; therefore, only some peaks are classified as characteristic peaks for interpretation. This section will discuss the characteristic peaks of each important fatty acid in the pathways being investigated. Any significant changes in the $^{13}C/^{12}C$ ratio in these peaks relative to the control group may be taken to indicate the possible ability to generate PUFAs in the worms. The discussion will start with saturated FAMEs followed by unsaturated FAMEs. The fatty acids were transesterified during lipid extraction; hence, the esters of fatty acid will be discussed mainly throughout this chapter.

The comparison between ^{13}C-unlabelled and ^{13}C-labelled methyl palmitate (C16:0) mass spectra is essential to affirm the incorporation of ^{13}C in fish feed and in the worms. The ^{13}C atom is positioned at the first carbon (at the carboxylic group: -COOH); therefore, any fragments that contain ^{13}C from the carboxylic group will have one more atomic mass unit shifted from the normal abundance mass unit. For instance, the McLafferty ion (m/z = 74) has the carboxylic part of the molecules as in Figure 1.17, thus the ^{13}C-label will be present in the McLaffery ion derived from ^{13}C-labelled methyl palmitate as m/z = 75. According to this, the loss of the McLafferty ion ($[M-74]^+$) should not be detected by any change in the $^{13}C/^{12}C$ ratio. The molecular peak of ^{13}C-labelled methyl palmitate could certainly have one more atomic mass unit added to m/z = 271 instead of m/z = 270. Also, the loss of the methoxy group ($-OCH_3$) ion ($[M-31]^+$) will be changed with the addition of one more mass unit because of the presence of ^{13}C atom in the fragment. The $^{13}C/^{12}C$ ratio of methyl ester homologous fragment series will also be increased as a result of the incorporation of ^{13}C in the ions. Figure 1.32 shows the comparison between unlabelled and labelled methyl palmitate fragment ions.

As discussed above, it is widely accepted that fatty acids are elongated by addition of two-carbon atoms at a time on the first carbon (carboxylic carbon) of their molecules. As a result of this addition mechanism, the ^{13}C position from the ^{13}C palmitate will be shifted towards the end of the molecule of methyl stearate as in Figure 1.33. Consequently, some fragment ions will contain the ^{13}C atom.

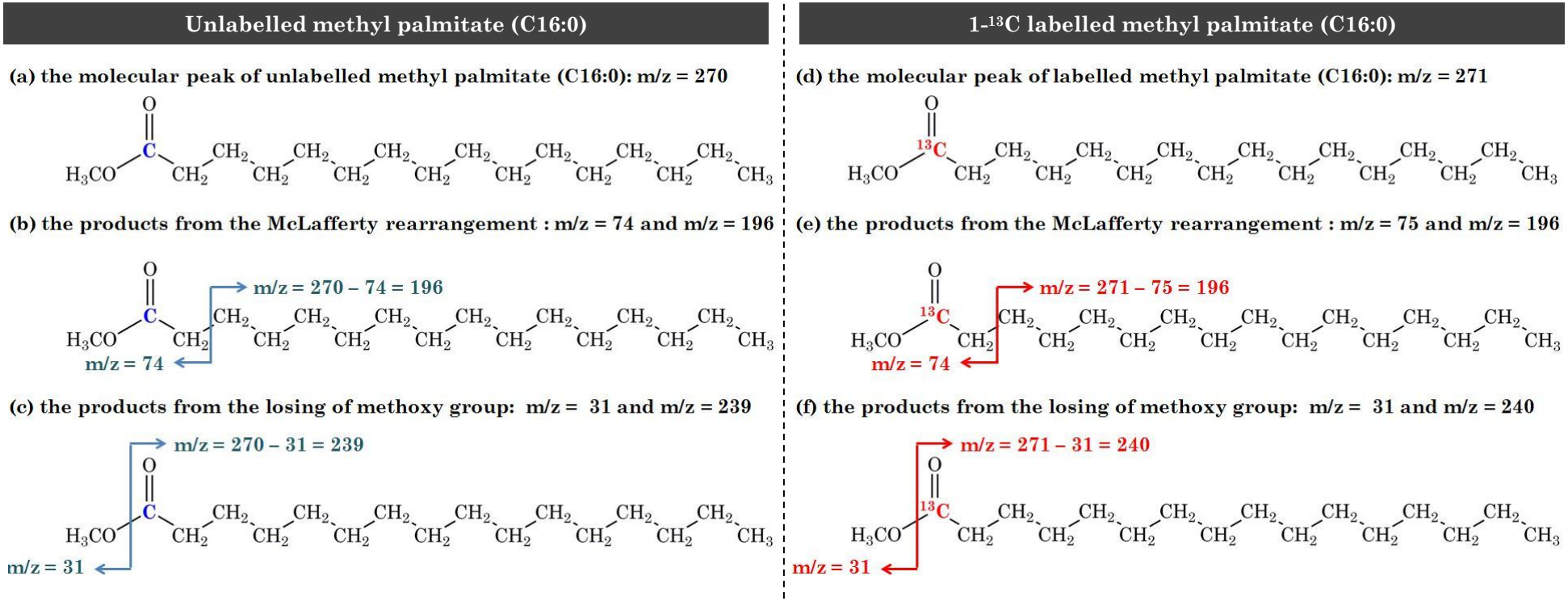


Figure 1.32: The schematic diagrams compare the mass spectrum products derived from ^{13}C-labelled and unlabelled methyl palmitate. The whole molecular peak ions are compared as in (a) and (d) while the McLafferty fragments from ^{13}C-labelled and unlabelled are compared in (b) and (e). The loss of the methoxy ion is also shown the difference between the labelled and unlabelled compound in (c) and (f).

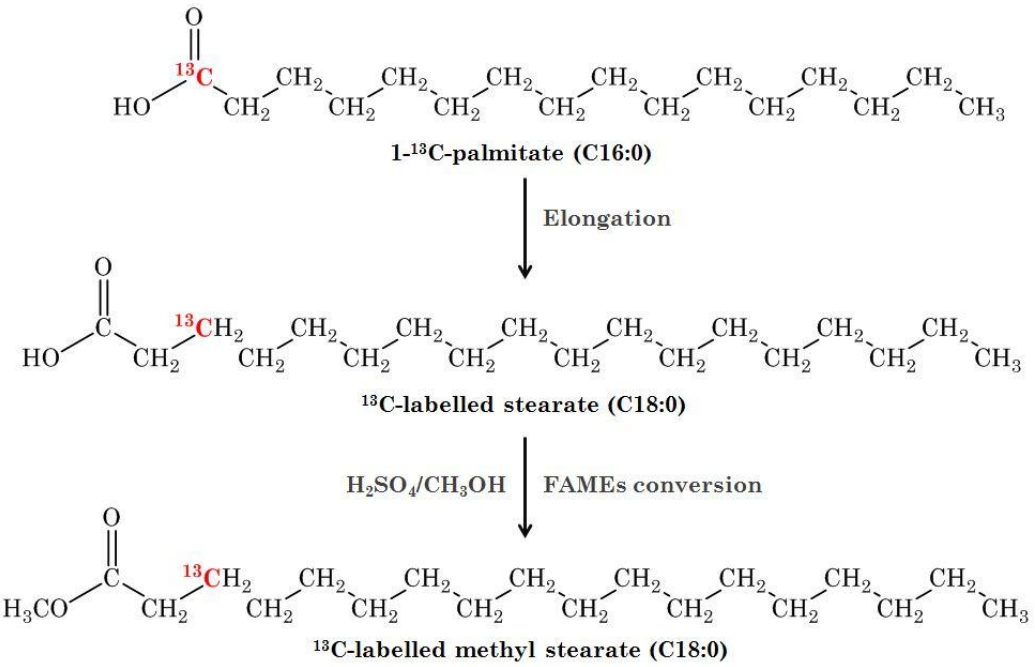


Figure 1.33: The shift of ^{13}C atom after an elongation process of palmitate to stearate and followed by FAMEs conversion to become methyl stearate (C18:0)

For methyl stearate (C18:0), the whole molecular peak will have one more mass unit and will be detected on m/z = 299 instead of m/z = 298, the relative abundance on $^{13}C/^{12}C$ ratio will subsequently be increased compared to the abundance in nature. Furthermore, the McLafferty ion will, in contrast to methyl palmitate (C16:0), have normal molecular mass (m/z = 74) as a result of the elongation and the consequent shifting of the ^{13}C position. The product resulting from the cleavage of the McLafferty ion peak $[M-74]^+$ will have one more atomic mass unit due to the incorporation of the ^{13}C atom in this fragment. The product resulting from the loss of the methoxy group $[M-31]^+$ (m/z = 267) would also have one more m/z value as the methoxy group does not contain the ^{13}C, hence the $^{13}C/^{12}C$ ratio of 268:267 should be changed. Similarly to methyl palmitate (C16:0), the analogous methyl series from m/z = 87 will be altered to m/z = 88 including any further fragment ions. The cleavage and products from elongated methyl stearate (C18:0) is shown in Figure 1.34.

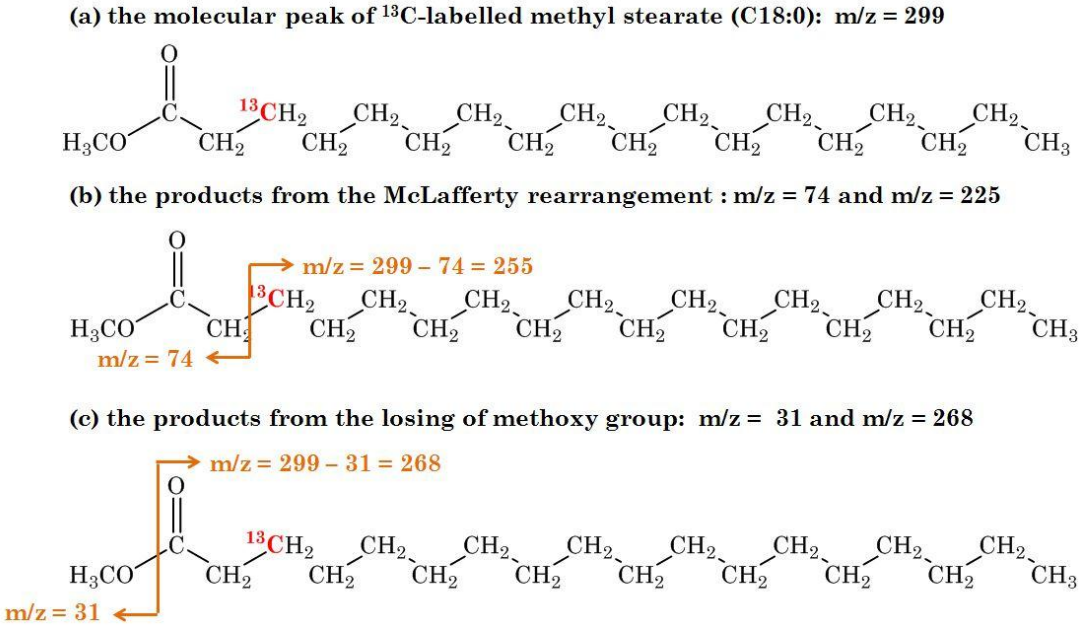


Figure 1.34: A schematic diagram shows molecular peak (a) and some characteristic peaks: the McLafferty products (b) and the loss of the methoxy group products (c) from methyl stearate (C18:0) which is derived from the precursor ^{13}C-labelled palmitate (C16:0)

In summary, an elongation of ^{13}C-labelled palmitate (C16:0) precursor will cause the shift in the ^{13}C atom towards the methyl end of the molecules by two carbon atom units. For example, the position of ^{13}C in the C_{18} fatty acid will be moved by a further two carbon atoms. In the C_{20} fatty acid, the ^{13}C will be situated four carbons further toward the methyl end compared with the ^{13}C-labelled palmitate. The double bond position in the molecule will not have any effects on the ^{13}C position. The diagram summarising the elongation to C_{18} and C_{20} from ^{13}C-labelled palmitate is illustrated in Figure 1.35.

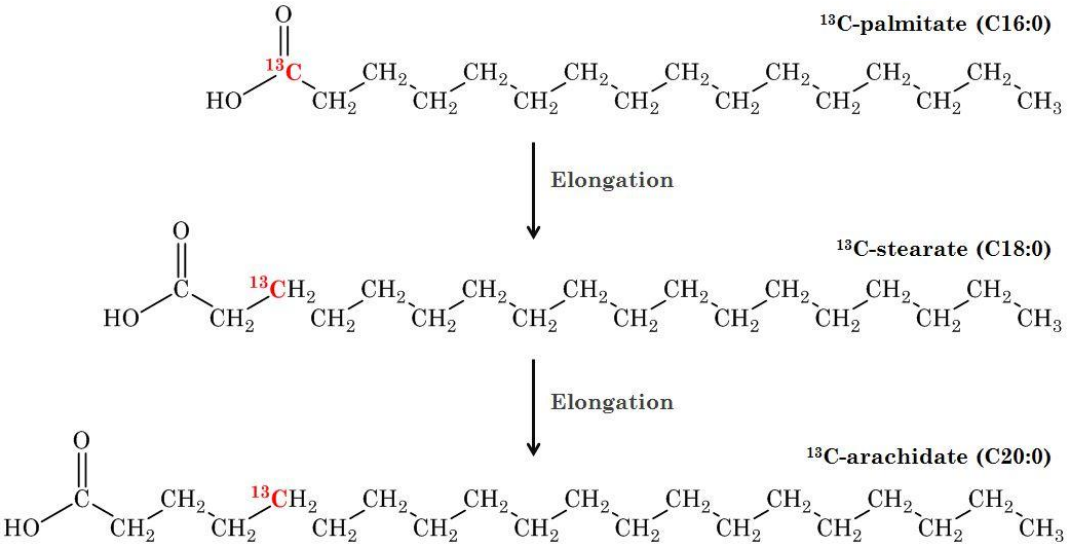


Figure 1.35: The summarised diagram of elongation of ^{13}C-labelled palmitate to further long-chain saturated fatty acids (C_{18} and C_{20} fatty acid)

One of the most important monoenoic fatty acid intermediates in fatty acid biosynthesis is oleic acid (C18:1n9). As mentioned earlier, the elongation products from ^{13}C-labelled palmitate will consist of the ^{13}C atom which positions towards the methyl end. The chemical structure of labelled methyl oleate and their products from ionisation is displayed on Figure 1.36. The abundance of the $^{13}C/^{12}C$ ratio of the molecular peak (m/z = 296 and m/z = 297) will be increased. The McLafferty ion (m/z = 74) therefore does not contain ^{13}C in the fragment and will be unaffected while the product resulting from the loss of the McLafferty ion $[M-74]^+$ (m/z = 222) will show a significant shift of one more atomic mass unit. Also, the loss of methanol $[M-32]^+$ (m/z = 264) ion peak will show as an addition to the atomic mass unit. The $^{13}C/^{12}C$ ratio between m/z 264 and 265 will consequently be changed in the labelled-experimental group. The hydrocarbon series might not be useful to trace the isotopic changes due to the fact that the fragment could occur on either the carboxyl end or the methyl end.

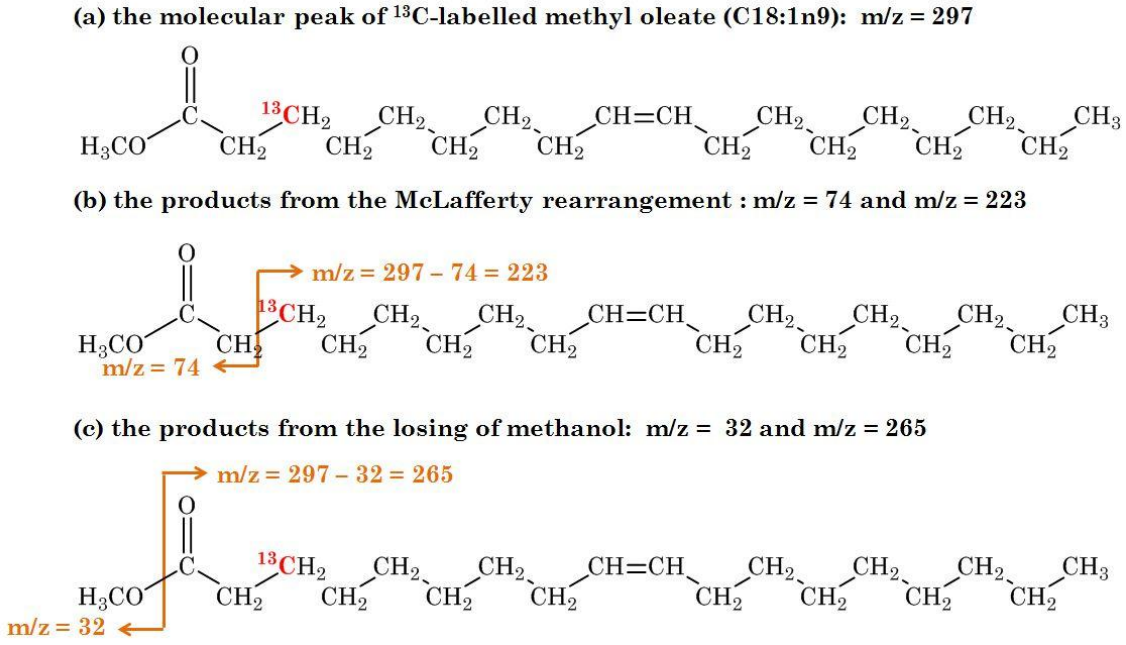


Figure 1.36: A schematic diagram shows molecular peak (a) and some characteristic peaks: the McLafferty products (b) and the loss of the methoxy group products (c) from the methyl oleate (C18:1n9) which is derived from the precursor ^{13}C-labelled palmitate (C16:0)

In a similar manner, linoleic acid (C18:2n6) will have ^{13}C on the two carbon shifted from the carboxyl end as in oleic acid. Consequently, the McLafferty (m/z = 74) and the methoxy (m/z = 31) ions will not contain any ^{13}C atom in their molecules while the loss of these two ion peaks, $[M-74]^+$ (m/z = 220) and $[M-31]^+$ (m/z = 263), will be detected in the changes of the $^{13}C/^{12}C$ ratio as a result of the incorporation of ^{13}C atom into their fragments. The molecular peak (m/z = 294) will certainly be changed in the atomic mass unit. The fragment derivatives from methyl linoleate are demonstrated in Figure 1.37.

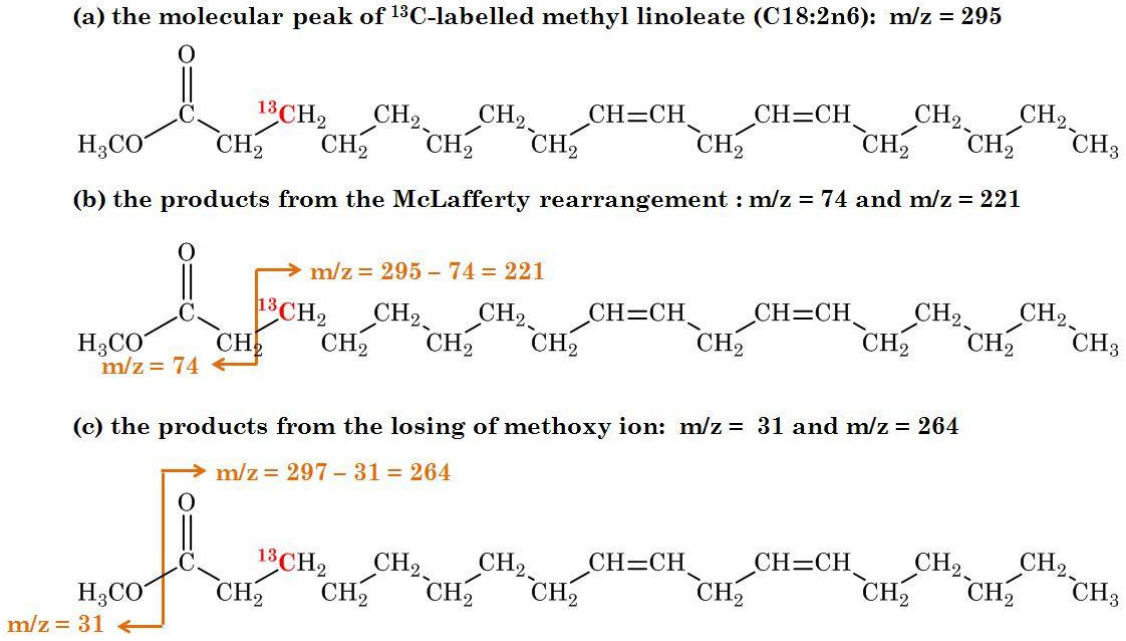


Figure 1.37: A schematic diagram shows molecular peak (a) and some characteristic peaks: the McLafferty products (b) and the loss of the methoxy group products (c) from the methyl linoleate (C18:2n6) which is derived from the precursor ^{13}C-labelled palmitate (C16:0).

As explained in an earlier section, trienoic FAMEs have their own unique fragment ions to identify the molecules. Both γ-linolenic acid (C18:3n6) and α-linolenic acid (C18:3n3) have the same molecular mass and will add one more atomic mass unit, if they were elongated from ^{13}C-labelled palmitic acid (m/z = 293). In the two characteristic peaks (α and ω ions) for trienoic and polyenoic acids, only α-ions are useful to trace the changes in $^{13}C/^{12}C$ isotopic ratio as a result of containing the ^{13}C in their molecules as demonstrated in Figure 1.38 and Figure 1.39, while ω-ions are not helpful in this aspect of the experiment. For the hydrocarbon series, it is also difficult to identify exact isomeric forms of its fragments; therefore, the homologous series are not of interest in the interpretation of changes of the $^{13}C/^{12}C$ ratio.

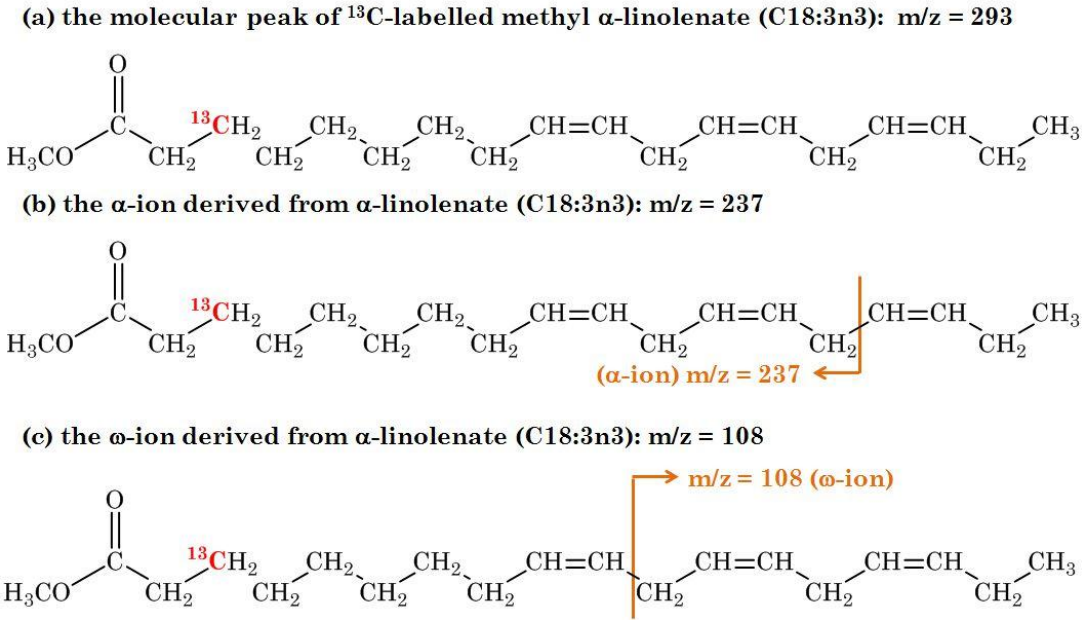


Figure 1.38: A schematic diagram showing the generation of the α-ion and ω-ion fragment ions from ^{13}C-labelled methyl α-linolenate (see Figure 1.39 for comparison). The whole molecular peak is demonstrated in (a) while the productions of α- and ω-ions are shown in (b) and (c), respectively.

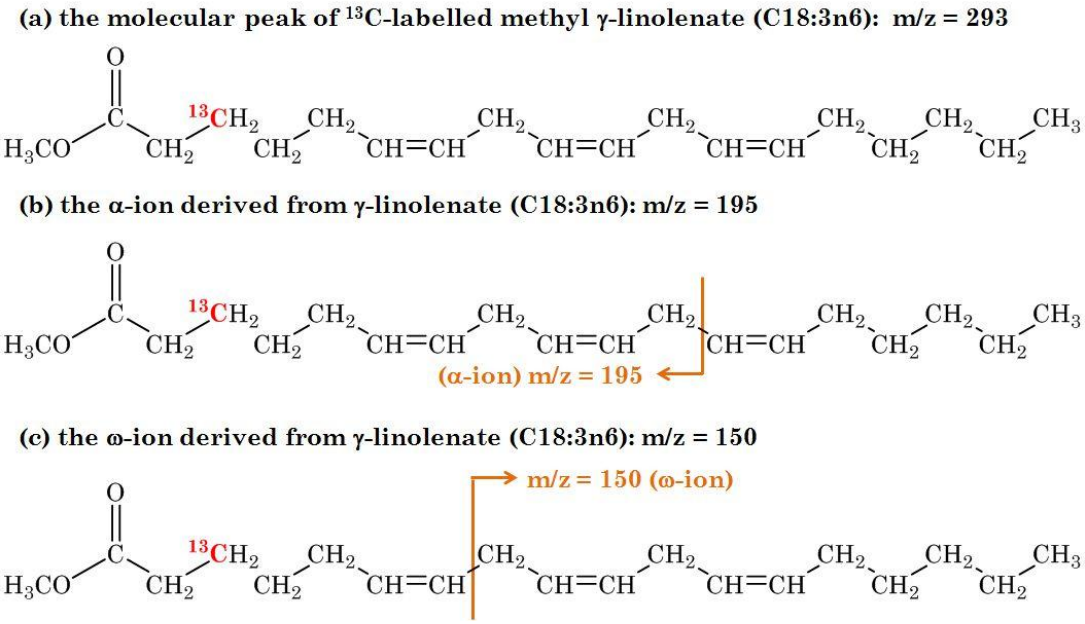


Figure 1.39: Schematic diagram showing the generation of the α-ion and ω-ion fragment ions from ^{13}C-labelled methyl γ-linolenate. The whole molecular peak is demonstrated in (a) while the productions of α- and ω-ions are shown in (b) and (c), respectively.

Polyenoic fatty acids such as ARA (C20:4n6), EPA (C20:5n3) and DHA (C22:6n4) can produce α-ions which have m/z = 180, 180 and 166, respectively. The elongated products from ^{13}C-labelled palmitate will generate α-ions with the increasing mass-to-charge ratio (m/z = 181 in ARA and EPA and m/z = 167 in DHA). These α-ions can therefore be used to trace any changes in $^{13}C/^{12}C$ ratio. The molecular peak can also be useful to imply the changes; however, for these very long chain fatty acid molecules it is difficult to detect the whole molecular ions after ionisation. Figure 1.40 shows the derivative α-ions from ARA, EPA and DHA.

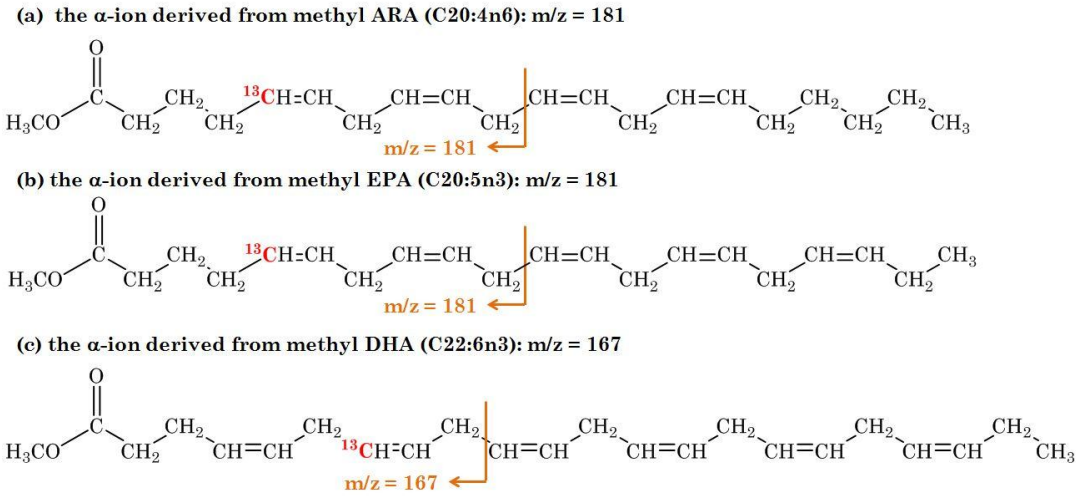


Figure 1.40: Schematic diagram displaying the fragmentation that generates the α-ions from methyl ARA (a), EPA (b) and DHA (c)

1.3.3.6 Conclusion

In summary, GC-MS is an instrument to separate and identify volatile organic molecules. The application of GC-MS can also be used to trace the changes in metabolic pathways using stable isotope labelled precursors. In the key experiment conducted in this thesis (Chapter 3), the ^{13}C-labelled palmitic acid is adopted as a precursor for PUFA biosynthetic pathways. As discussed previously, only some fragment peak ions are useful to investigate the changes in $^{13}C/^{12}C$ ratio after the intake of ^{13}C by *N. virens* due to the incorporation of the ^{13}C atom in the fragment ions. While some other peaks may not be of such interest because they lack the ^{13}C atom, hence no change would be expected to occur in their m/z ratio. Nevertheless, such ions are of interest in providing a negative control and will reveal if the molecules are behaving as expected from the theory. All in all, the characteristic peaks will therefore be carefully considered to affirm the capacity got *N. virens* to produce long-chain fatty acids such as EPA and DHA

1.4 *Research Aims*

The aim of this thesis is to study the ability to synthesise PUFA and the exact biosynthetic pathways in two different polychaetes species, *A. marina* and *N. virens*, using a net gain or loss experiment and a ^{13}C-stable isotope-labelling approach. Also, this research will aim to determine, using the stable isotope-labelling experiment, if, as has been suggested, the worms are able to generate long-chain PUFA (in particular EPA) by chain elongation and desaturation via the 'alternative' $\Delta 8$ pathway.

1.5 *Research Objectives and Hypotheses*

1. To determine the net changes of fatty acid i.e. a gain or loss within the closed system for cultured specimens of both species; A net gain of any specific fatty acid, would indicate the production of that fatty acids within the closed system.

2. To develop a method to trace the pathway of lipid biosynthesis in polychaetes following administration of a specific ^{13}C labelled substance in the labelled-fed and unlabelled-fed worms. Any different in terms of mass spectra and the m/z ratio between the labelled-fed worm and unlabelled-fed worm could support that the new method can distinguish the labelled and the unlabelled pathway.

3. To examine the ability to synthesise long-chain PUFA such as EPA (C20:5n3) from a simple labelled precursor such as ^{13}C-labelled palmitic acid (C16:0) in polychaete species. The significant difference of the $^{13}C/^{12}C$ ratio in the α-ion of EPA would indicate the worm ability to produce EPA.

4. To investigate the PUFA biosynthetic routes that the worms may utilise, if it is proven that they are able to produce long-chain PUFA, by examination of changes in the $^{13}C/^{12}C$ ratio in key intermediate fatty acids. Any statistically significant difference of the $^{13}C/^{12}C$ ratio in characteristic peaks of some key intermediates from the labelled food-fed worms could imply about the pathways and the ability to synthesise long-chain PUFA.

Chapter 2

The Net Gain or Loss of Fatty Acids in the Lugworm (*Arenicola marina*) and the Ragworm (*Nereis virens*)

2.1 *Introduction*

Some polyunsaturated fatty acids (PUFA) such as arachidonic acid (C20:4n6) and eicosapentaenoic acid (C20:5n3) are nutritionally important to several marine organisms (see review by Parrish, 2009). In polychaetes, the fatty acid profiles in several families have been investigated such as *Nereis diversicolor* by Bischoff *et al*. (2009), *N. virens* by Brown *et al*. (2011) and *Arenicola marina* by Duangchinda (2007) and Olive *et al*. (2009). Interestingly, a number of deep sea polychaetes (Drazen *et al*., 2008) have also been characterised in this way. Most of these studies reveal relatively high levels of PUFA, but especially EPA. A study of *N. virens* has shown that a moderately high content of the characteristic PUFA such as EPA (C20:5n3) is maintained when fed on low fatty acid content diets (Olive *et al*., 2009). Ashforth (2008) has also reported that the symbiotic bacteria found associated with the lugworm's burrow may not synthesise EPA nor contribute EPA to *A. marina*. These findings suggest that these two worms may each be able to produce EPA from a simple substrate obtained within their diets. This chapter therefore addresses the question of whether or not the two polychaetes *A. marina* and *N. virens* are able to accumulate certain PUFA in excess of that provided in their diets.

The polychaetes are positioned at intermediate positions in the food chains within marine ecosystems, particularly in estuaries. Therefore, an understanding of any changes in lipid profiles of these two polychaetes species will fulfil a requirement for exploration of PUFA flux transfer between trophic levels. The ability to generate a net gain of some important and intermediate PUFAs under nutritionally controlled experimental conditions, will, in turn, facilitate a re-examination of the role of polychaetes in intertidal and estuarine ecosystems. The findings could also be applied to an evaluation of the role of marine worms in a more sustainable aquaculture industry. Therefore, a study of the growth of *A. marina* and *N. virens* under controlled conditions was carried out to permit quantification of their fatty acid profiles. This was a necessary preliminary experiment, leading to the subsequent investigation of how the worms may synthesise PUFAs as described in Chapter 3. The net gain or loss experiments on both species were carried out at different times but in a similar manner. The results will be

discussed together in order to make a clear comparison between these worm species that exhibit two different modes of living.

2.2 *Materials and Methods*

2.2.1 Animal Collection

a) *A. marina*: The lugworms, together with samples of the organic-rich sand in which they live, were collected from Alnmouth, Northumberland, UK near the mouth of the River Aln (grid reference: NU 24453 10828) in June 2010. These worms were then used in a growth experiment in which sterilised and dried brewer's yeast (supplied by Shoreline Polychaete Farms LLP) was used as a food source creating an organic-rich sand in which bacteria were also able to grow, and which, according to Ashforth (2008), may form the food resource ingested and digested by the lugworms.

b) *N. virens*: Due to their different habitats and diets, the worms were collected at the estuary of the River Blyth, Northumberland, UK (grid reference: NZ 30985 81139) in April 2011. The sterilised and frozen worm feed pellets supplied by the Shoreline company were utilised as a food source in the experiment. The diets were made mainly from fish meal, soya bean meal and cereals and contain high nutrition content sufficient for normal development of the worms.

2.2.2 Experimental Designs

The *A. marina* experiment was conducted in five plastic boxes with the dimensions 30 cm x 20 cm x 15 cm placed in a recirculating research aquarium, housed within Ridley Building 2, Newcastle University. Three boxes were given yeast and organic-rich sand as a food source while no food was added to the two remaining boxes. The three boxes receiving food were supplied by mixing 40 g of dried yeast and 20 g of organic-rich sand together. A layer of sand from Cullercoats Bay, Northumberland (adjacent to the Dove Marine Laboratory; grid reference: NZ 36340 71268) which was oven-dried at 200 $^{\circ}$C for 4 hours and sieved with the 0.10 mm sand sieve prior to the wet set up, was then added until the sand reached 9 cm in depth. This clean, sieved sand was also added to the unfed boxes to the same total depth. A supply of running UV-sterilised artificial sea water (12 ± 0.1°C) was run to each box and the boxes were allowed to stand for a week prior to the experiment after which time the experimental animals were introduced.

The *A. marina* specimens placed in each experimental box were previously depurated, to empty their guts, for a period of 24 hours and weighed before the experiment. Ten specimens (average weight 1 g) were put into each box. Ten similar size depurated worms were weighed and flushed under oxygen-free nitrogen and were kept frozen at -80 °C to prevent further fatty acid oxidation. These ten worms were then used as the time-zero control to make it possible to calculate the net gain or loss of each fatty acid that occurred during the experiment. Samples of dried brewer's yeast as added to the boxes, the organic-rich sand from Alnmouth and clean sand taken from the beach at Cullercoats were also stored under nitrogen at -80 °C for subsequent lipid extraction and analysis of FAMEs.

All boxes were maintained and operated under running artificial sea water for 60 days and were monitored daily. The other conditions of the experiments were 10-12°C in temperature, salinity at 30-32, pH at 8.0-8.3 and dissolved oxygen (DO) at 7.5-8.5 mg/L. The closed-recirculation system in this experiment was a 'System 5000 Marine components' with TMC Commercial UV steriliser P8-440w from Tropical Marine Centre Ltd, Bristol, UK). A black plastic bag was used to cover the boxes and make them as a 'dark condition' for the system to remove some microalgae. After the experiment, each worm was depurated again for 24 hours before being weighed. The weighed worms were then kept in the same manner as the initial worms under nitrogen and frozen at -80°C. After completion of the experiment, the remaining sand was thoroughly mixed by gloved hands for approximately 30 minutes and then stored in the same way as the worms.

The *N. virens* net gain or loss of fatty acids experiment was carried out in six plastic boxes of the same size and placed in the same aquarium system. Three boxes were given feed diets whereas the other three boxes were unfed. The unfed experimental groups were used as the controlled against the fed group. These groups would also be able to exclude the influence from the worm's gut bacteria. The worms were depurated to empty their guts for 24 hours prior to the experiment. Twelve worms were put into each box (0.96 ± 0.17 g). Another ten worms were collected and then frozen in liquid nitrogen and kept frozen at -80°C as a start generation group. The boxes were set in the dark using the black cover and run with UV-sterilised artificial seawater for 60 days. After that, the *N. virens* were depurated for 24 hours and then weighed again after the experiment to compare weight changes during the experimental period. All the worm

samples were kept frozen at -80°C for subsequent lipid extraction and FAMEs analysis, including feed pellet samples.

According to the previous study by Islam (2001), the amount of food given in the *N. virens* experiment was determined at 0.2% of worm body wet weight was a suitable proportion for growing worms without waste of the food. An assumption was made according to the preliminary study conducted by the author to determine the appropriate amount of food to grow the worm as data shown in Appendix A, for an average animal at the start of the experiment for which the initial worm weight was around 1 g, it would have grown to approximately 3 g at the end of experiment after 60 days. Worm weight was also assumed to change every week; therefore, the amount of food for the worms was adjusted every week. The dry weight of feed pellets was started from 0.20 g and reached up to 0.45 g within the experimental period.

2.2.3 Lipid Extraction and FAMEs Conversion

The two most popular methods to extract the lipid and fatty acid contents are Bligh and Dyer (1959) and Folch *et al*. (1957). According to the comparison study between these two methods by Iverson *et al*. (2001), lipid extraction in high lipid content marine samples using Folch *et al*. (1957) were significantly more effective than the Bligh and Dyer (1959) method. Thus, all the lipid extraction in this thesis were followed and adapted from Folch *et al*. (1957) and Duangchinda (2007). The total lipids from the extraction were transesterified to fatty acid methyl esters (FAMEs) for GC analysis as described later.

All worm and feed diet samples were lyophilised in a freeze drier for 48 hours prior to the extraction. Twenty worm samples were individually weighed before and after lyophilisation to determine the regression analysis equation for the gain and loss of fatty acid calculation. The frozen worm tissue and feed samples were extracted and analysed.

All glassware was cleaned using acetone for rinsing and heated in the oven at 200°C overnight to remove any solvents. The samples were homogenised by a small glass homogenizer with methanol and then chloroform (1:2 v/v). In this case, 1 ml of methanol and 2 ml of chloroform were applied to the extraction. The tissue sample was homogenised with methanol for a minute prior to the addition of the chloroform and then allowed to mix using vortex for a further minute. The sample and the solvent were

then kept in the Pyrex tube with PTFE liner caps under an oxygen-free, nitrogen flushed atmosphere, sealed tightly and then stored in the refrigerator overnight (approximately 16 hours).

After overnight extraction, the samples and the solvents were removed by vacuum-filtering through Whatman 2E filter paper. A volume of 0.5 ml of C19:0 stock solution (0.5 mg/ml of hexane) was introduced into the tube using an automatic pipette. Weak salt solution, 0.88% KCl, was added around 25% of the starting volume after the filtration to remove water and some solvent. The mixture was shaken gently and left until two layers clearly separated. The upper layer was drained into the waste bottle by new glass pipette while the lower layer was evaporated under a nitrogen atmosphere at 37°C until dry.

The extracted lipids were then subject to transesterification to fatty acid methyl esters (FAMEs) following the method of acid-catalysis (Christie, 1993). The dried lipids from the extraction were re-suspended in 1 ml toluene and 2 ml of 1% sulphuric acid in methanol, flushed with nitrogen gas to prevent oxidation, mixed thoroughly by vortex and incubated at 80°C for an hour. After cooling, 2 ml of 2% w/v $KHCO_3$ and 5 ml of hexane : diethylether (1:1, %v/v) were added into the vial and allowed to separate into two layers. The upper layer was transferred to a new clean tube and then 5 ml of hexane : diethylether was added again to the lipid, transferred the new upper layer to the same tube. The remaining lower layer was drained into the waste bottle. After that, the upper layer was evaporated under nitrogen gas at 37°C until dry. A quantity (1.5 ml) of hexane was put into the tube to re-suspend the dried FAMEs. FAMEs were then transferred to GC vials, kept frozen under nitrogen at -20°C before GC analysis. GC-MS was also applied to determine some unidentified peaks compared with the mass spectra library using GC/MSD ChemStation program (Agilent Technologies).

2.2.4 GC and GC-MS Analysis

Samples were analysed using an Agilent 7890A gas chromatograph (GC) fitted with an Agilent DB23 fused silica capillary column (30m x 0.25mm I.D. x 0.25μm film thickness) and interfaced to an Agilent 5975C MSD. Samples were injected on to the GC column in pulsed splitless mode (1 min. splitless at inlet pressure 150kPa, then 30ml/min split) using an Agilent 7683B automatic injector.

The GC inlet temperature was 280°C and the GCMS interface temperature was 220°C. Helium was used as carrier gas at a constant flow rate of 1ml/min. The GC oven was heated from 60°C (initial hold time 1 min.) to 140°C (hold time 3 min.) at 20°C/min., then at 10°C/min. to 190°C (hold time 3 min.), then at 10°C/min. to 220°C (final hold time 18 min.).

The MSD was operated in electron impact mode (electron voltage 70eV, source temperature 230°C, quad temperature 150°C, multiplier voltage ca. 1800V). Data was acquired using Agilent Chemstation software, operating in full scan mode (range 50-600 amu/sec) or selected ion monitoring mode (30 ions, 0.8 cps, 35 msec dwell time) after a solvent delay of 5 min. Data processing was performed using ChemStation software and peaks were identified, where possible, by comparison of mass spectra with those in the NIST05 mass spectra library or in the published literature.

Four external standards were used for analysis of the retention time of each fatty acid: (1) Sigma cod liver oil, (2) GLC-538 Nu-Chek prep standard, (3) a single FAMEs of C18:3n6 (γ-linolenic acid) and (4) a single FAMEs of C20:2n6 (eicosadienoic acid). All external standards were run in the same condition as the samples.

The chromatograms from the GC analysis were examined, the characteristic retention time determined and the FAMEs identified by comparison with the external standards. Next, the peak area of each fatty acid was automatically calculated using the Thermo Fisher Scientific's Atlas Chromatography Data System (CID) program.

Quantification analysis of each fatty acid was determined and calculated by comparing each individual peak with the known amount of the internal standard (C19:0). For example, the exact amount of the C19:0 is 0.25 mg and the peak area of the C19:0 is 100. The peak area of a fatty acid of interest of the same sample is X, then the amount of this C16:0 is 0.25X/10 mg as shown in Figure 2.1.

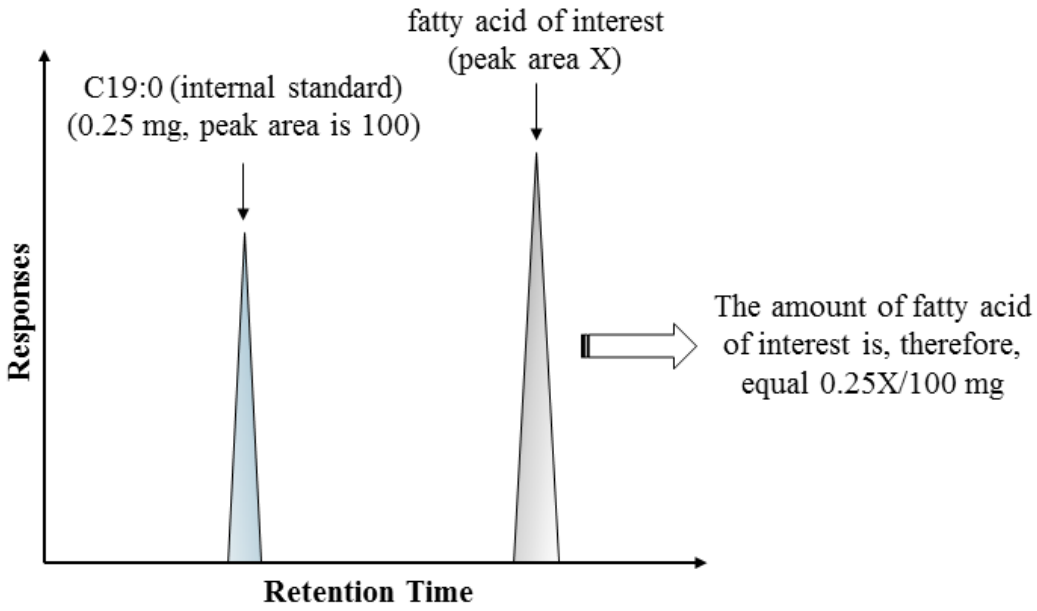


Figure 2.1: A simplified diagram illustrating how to determine the amount of a fatty acid of interest from the internal standard (C19:0).

2.2.5 Growth Calculations

At the end of experiment, the survival rate and a number of different growth rates were calculated and comparisons between the results for fed and unfed boxes carried out. The mortality rate, specific growth rate (SGR), percentage weight gain (PWG) and absolute growth rate (AGR) were applied to the experiment using the following equations (Lazo and Davis, 2000):

The mortality rate (% mortality)

$$\%\ Mortality = \frac{(Initial\ number\ worms - Final\ number\ worms)}{Initial\ number\ worms} \times 100$$

The specific growth rate (SGR)

$$SGR = \frac{(lnW_f - lnW_i)}{\Delta t} \times 100$$

Where; $\ln W_f$ = the natural logarithm of the final worm weight;

$\ln W_i$ = the natural logarithm of the initial worm weight;

Δt = time period between W_f and W_i days (60 days in these cases).

The percentage weight gain (PWG)

$$PWG = \frac{(W_f - W_i)}{W_i} \times 100$$

Where; W_f = the final worm weight;

W_i = the initial worm weight.

The absolute growth rate (AGR)

$$AGR = \frac{(W_f - W_i)}{\Delta t}$$

Where; W_f = the final worm weight ;

W_i = the initial worm weight;

Δt = time period between W_f and W_i days (60 days in these cases).

2.2.6 Bioaccumulation of Fatty Acids and Statistical Analysis

All the fatty acids, which are calculated based on the chromatograms, are given in terms of dry weight as mg g^{-1}. In order to compare the gain or loss of any fatty acid in relation to worm biomass, it is necessary to convert the amount of fatty acid mg g^{-1} dry weight to an estimated amount relative to the wet weight. This is because it is not possible to determine initial values in the worms added to the boxes as they cannot be dried. Accordingly, the regression equation between wet weight and dry weight of 13 worms was determined. Each fatty acid was then converted from mg g^{-1} dry weight into the estimated equivalent mg g^{-1} wet weight using the resulting regression equation.

The bioaccumulation of each fatty acid in *A .marina* was then calculated in terms of total worm wet weight biomass using the following formula:

Bioaccumulation of FA per system box = fatty acid of lugworm (END – START – YEAST)

Where:

START = (FA in g wet weight × number of surviving worms × average worm weight before the experiment);

END = (FA in g wet weight × number of surviving worms × average worm weight after the experiment);

YEAST = (FA in g dry weight × the amount of added yeast at start of the experiment (40 g))

The START term was calculated using the number of surviving worms after the experiment, multiplied by the average worm weight at the start of the experiment rather than the average weight at the start multiplied by the number of animals added at the start to allow for the fact that in a few boxes a small number of animals died during the course of the experiment. Failure to allow for the mortality would tend to underestimate any net gain. In adopting this procedure, the assumption is made and the calculations carried out as if all of the mortalities occurred during the first day of the experiment. The quantity of fatty acids in the sand prior to the addition of yeast and in the homogenised sand after the completion of the experiment was not sufficient to be detected using the GC procedures adopted and is taken to be zero. The YEAST term therefore was the only value used for calculating the exact net gain or loss of each fatty acid during the experiment.

For the growth experiment, means and standard deviations of the *A. marina* wet weights were determined. Independent *t* test of significance using SPSS software was used for the statistical analysis of the worm wet weight data, relating to worm weight before and after the experiment.

Due to a different type of diet in the *N. virens* experiment, the formula is slightly changed from the *A. marina* experiment calculation. The feed pellets were given to the experimental boxes every day instead of mixing yeast and organic-rich sand; therefore, the YEAST term in the equation would be altered to 'FEED'. The 'FEED' term will be determined from the multiplication of the amount of food added and fatty acid content in terms of g dry weight. The adjusted formula for the *N. virens* experiment is shown in the box below.

Bioaccumulation of FA per system box = fatty acid of ragworm (END – START – FEED)

Where:

START = (FA in g wet weight × number of surviving worms × average worm weight before the experiment);

END = (FA in g wet weight × number of surviving worms × average worm weight after the experiment);

FEED = (FA in g dry weight × the amount of added feed pellet over the period of the experiment).

2.3 *Results*

2.3.1 Growth Rates

In the *A. marina* experiment, the average worm wet weight (mean ± SD) at the beginning and at the end of the experiment after 60 days of culture was determined and is shown in Table 2.1, which also shows the calculated growth rates and results of the statistical analysis.

At the end of experiment, the average *A. marina* wet weights in each of the fed food boxes (box 1-3) were 3.01 g, 3.31 g and 3.28 g, respectively. There was a significant increase in wet weight in comparison with the initial weight of the worms (independent *t* test: $p < 0.05$). In contrast, there was no statistically significant difference between the mean worm wet weights from the unfed food boxes (box 4 and 5) before and after the experiment.

The average *N. virens* wet weight at the start of the experiment is shown in Table 2.2. The average final weights at the end of experiment were 3.68, 3.49, and 3.90 g in the fed groups, respectively while the final average wet weight of the unfed groups were 0.63, 0.62 and 0.61 g, respectively. There was a significant difference between the average wet weight before and after the experiment (independent *t* test; $p < 0.05$) in all fed boxes; however, there was no significant difference in the unfed groups. It is interesting to note the high mortality rate was calculated as 41.67% in box 5 but the reasons for this are not known.

The average growth rate and the specific growth rate of the experiments were adjusted to take account of the observed mortality (see methods on topic 2.2.5) and calculated per surviving worm. In *A. marina*, the AGR (mg day^{-1} survived $worm^{-1}$) of the fed groups were the positive value, 2.77, 3.10 and 3.05, respectively while the AGR per survived worm of the unfed groups were -6.25 and -5.70 mg day^{-1} survived $worm^{-1}$. In a similar manner to the *A. marina* experiment, the AGR (mg day^{-1} survived $worm^{-1}$) of the *N. virens* in the fed groups were 4.363, 4.005 and 4.521, respectively whereas the AGR in the unfed groups were -0.575, -0.496 and -0.431.

The specific growth rates and percentage weight gain were also calculated and determined both in *A. marina* and *N. virens* experiments as shown in Table 2.1 and Table 2.2. The positive values were determined on all fed groups while the negative values were determined on all unfed boxes.

Table 2.1**:** The average *A. marina* wet weight at the beginning and at the end of experiment, the growth rates and % mortality in each experimental set (fed = boxes 1-3 and unfed = boxes 4-5).

	Fed Boxes			**Unfed Boxes**	
	Box 1	**Box 2**	**Box 3**	**Box 4**	**Box 5**
Initial wet weight (g)	1.680 ± 0.425^{a}	1.633 ± 0.156^{a}	1.637 ± 0.119^{a}	1.523 ± 0.108^{a}	1.370 ± 0.130^{a}
Final wet weight (g)	3.011 ± 0.292^{b}	3.309 ± 0.264^{b}	3.281 ± 0.299^{b}	1.150 ± 0.823^{a}	1.029 ± 0.952^{a}
% Mortality	20	10	10	20	20
SGR (% day^{-1})	0.973	1.177	1.159	-0.468	-0.478
SGR (% day^{-1} survived worm^{-1})	0.108	0.131	0.129	-0.059	-0.060
PWG (%)	79.237	102.576	100.459	-24.486	-24.936
AGR (mg day^{-1})	22.185	27.923	27.405	-6.215	-5.696
AGR (mg day^{-1} survived worm^{-1})	2.773	3.103	3.045	-0.777	-0.615

Note: Initial and final weight data are in terms of mean ± 95% CI; letters following the numbers indicate statistical differences between groups ($p<0.05$).

Table 2.2: The average *N. virens* wet weight at the beginning and at the end of experiment, the growth rates and % mortality in each experimental set (fed = boxes 1-3 and unfed = boxes 4-5).

	Fed Boxes			**Unfed Boxes**		
	Box 1	**Box 2**	**Box 3**	**Box 4**	**Box 5**	**Box 6**
Initial wet weight (g)	1.059 ± 0.640^{a}	1.090 ± 0.788^{a}	0.921 ± 0.425^{a}	1.014 ± 0.731^{a}	0.829 ± 0.418^{a}	0.842 ± 0.554^{a}
Final wet weight (g)	3.678 ± 1.315^{b}	3.493 ± 1.407^{b}	3.907 ± 1.280^{b}	0.633 ± 0.566^{a}	0.623 ± 0.550^{a}	0.608 ± 0.358^{a}
% Mortality	16.67	16.67	8.33	8.33	41.67	25
SGR (% day^{-1})	2.074	1.942	2.408	-0.783	-0.482	-0.542
SGR (% day^{-1} survived worm^{-1})	0.207	0.194	0.219	-0.071	-0.002	-0.060
PWG (%)	247.186	220.578	324.077	-37.475	-25.127	-27.753
AGR (mg day^{-1})	43.628	40.053	49.735	-6.328	-3.473	-3.885
AGR (mg day^{-1} survived worm^{-1})	4.363	4.005	4.521	-0.575	-0.496	-0.431

Note: Initial and final weight data are in terms of mean ± 95% CI; letters following the numbers indicate statistical differences between groups ($p<0.05$)

2.3.2 Fatty Acid Contents in the A. marina Experiment

The fatty acid content expressed in terms of mg g^{-1} DW ± 95% CI of the initial *A. marina* samples are shown in Figure 2.2. The three most abundant fatty acids from the worm samples before the experiment started are *cis*-vaccenic acid (C18:1n7), palmitic acid (C16:0) and eicosapentaenoic acid (C20:5n3). Some long chain fatty acids such as stearidonic acid (C18:4n3), eicosatetraenoic acid (C20:4n3) and DHA (docosahexaenoic acid: C22:6n3) were not detected in the initial worm tissue. It is of interest that the two critical fatty acids that might be involved in PUFA biosynthesis were also found in the worm tissues, γ-linolenic acid (C18:3n6) and eicosadienoic acid (C20:2n6) as shown in Figure 2.2.

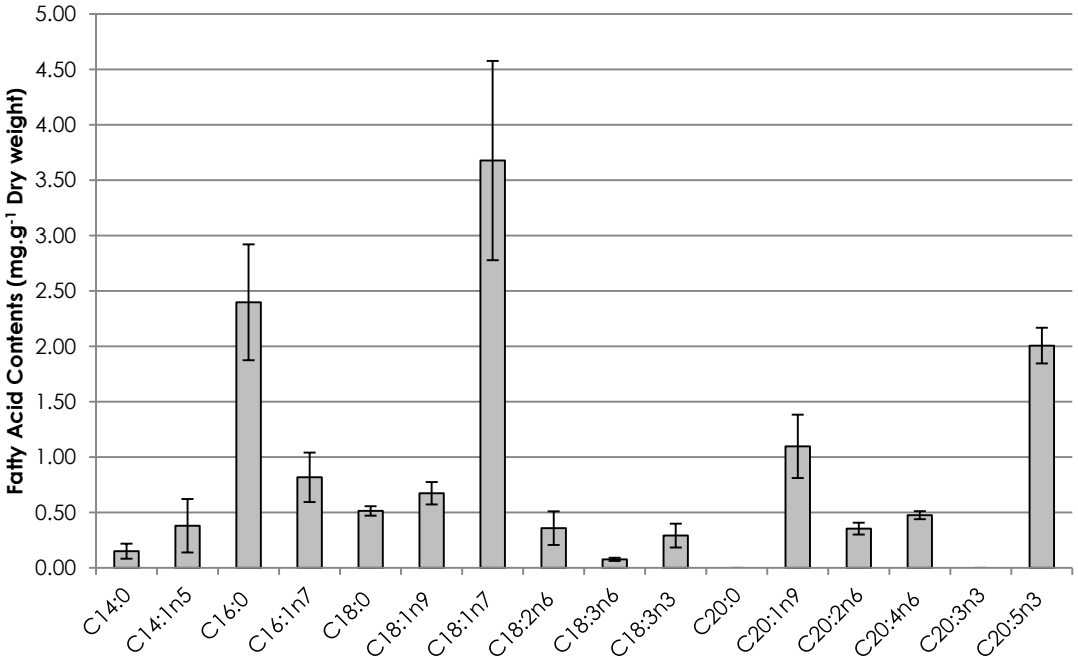


Figure 2.2: Fatty acid contents in the initial *A. marina* tissue samples (mg g^{-1} DW) are shown and 95% confident interval are used as the error bar (n = 10).

In the case of dried brewer's yeast, only C_{16} and C_{18} fatty acids were present in the samples. It was not possible to detect long-chain PUFA such as EPA and DHA on the chromatograms. The most abundant fatty acid in the yeast samples was palmitic acid (C16:0). It should be noted that the critical fatty acid intermediates both γ-linolenic acid (C18:3n6) and eicosadienoic acid (C20:2n6) were not found in the yeast samples. The fatty acid content of the yeast is illustrated in Figure 2.3.

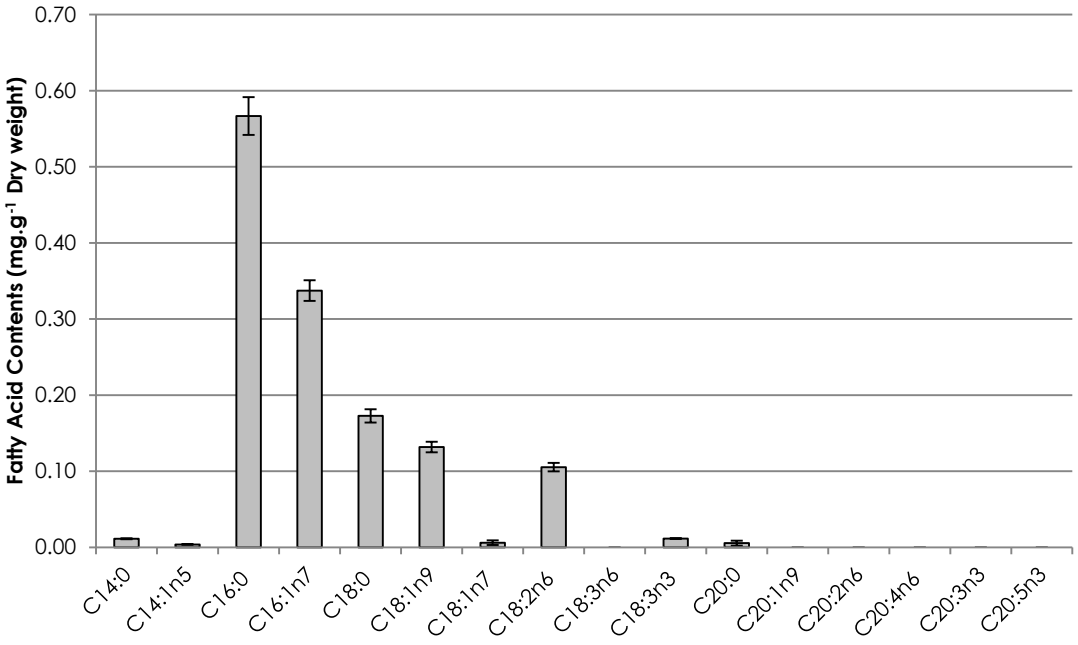


Figure 2.3: Fatty acid content of the dried brewer's yeast (mg g^{-1} DW) are shown and 95% confident interval is used as the error bar (n = 10).

The fatty acid concentration after the experiment was similar to studies by Duangchinda (2007) and Olive *et al.* (2009). In order to discuss the ability of the worm to synthesise long-chain PUFA and the PUFA biosynthetic routes in *A. marina* from the given feed, the net bioaccumulation of each fatty acid at the end of the experiment needed to be determined as explained in topic 2.2.6.

2.3.3 Fatty Acid Contents in the N. virens Experiment

The lipid profile of *N. virens* was rather different compared with *A. marina*. The fatty acid contents in the worm tissue samples (n = 3) is presented in Figure 2.4 with 95% CI used as the error bar. The most abundant fatty acid in the initial worm samples was EPA. Palmitic acid (C16:0), oleic acid (C18:1n9), stearic acid (C18:0) and *cis*-vaccenic acid (C18:1n7) were also detected in the worm samples. In the case of critical intermediate metabolites between γ-linolenic acid (C18:3n6) and eicosadienoic acid (C20:2n6), only C20:2n6 was found in the initial worm samples. Docosahexaenoic acid (C22:6n3) was also detected on the chromatogram.

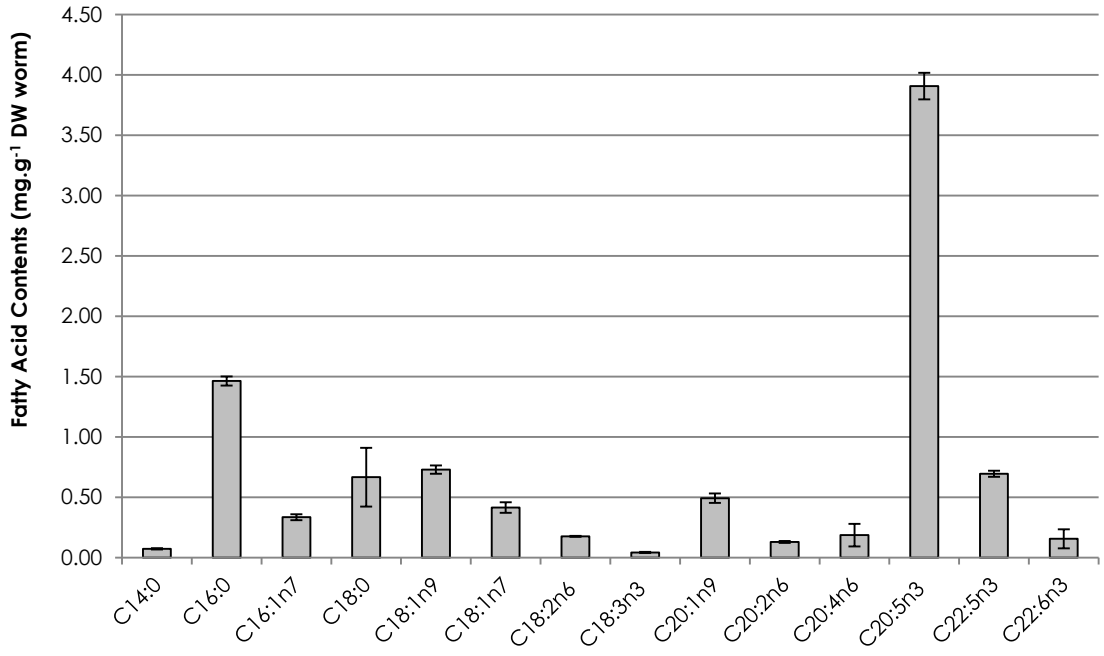


Figure 2.4: Fatty acid content of the initial *N. virens* tissue samples (mg g^{-1} DW) are shown and 95% confident interval are used as the error bar (n = 10).

In the case of fish feed pellets, all carbon-length ranges from C_{14}-C_{22} were detected from the GC chromatograms. The fatty acid contents in the feed pellets are shown in Figure 2.5. The most prominent fatty acid constituent in the feed pellets was palmitic acid (C16:0). Lower concentrations of oleic acid (C18:1n9), EPA (C20:5n3) and DHA (C22:6n3) were also found. Neither γ-linolenic acid (C18:3n6) and eicosadienoic acid (C20:2n6) were determined from the fish feed samples.

The net accumulation of specific fatty acid was calculated and determined in a similar manner to the *A. marina* experiment as explained in topic 2.2.6. The results after calculation are presented in the section 2.3.5.

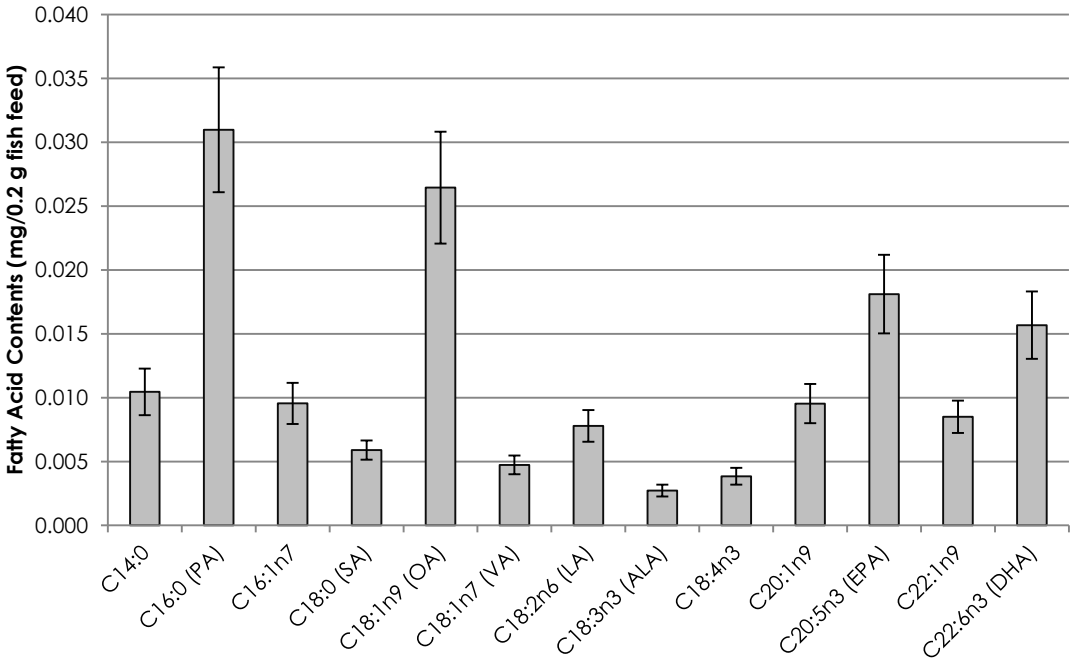


Figure 2.5: Fatty acid contents in the feed pellets (mg g^{-1} DW) are shown and 95% confident interval is used as the error bar (n = 10).

2.3.4 Net Gain or Loss of Fatty Acids in A. marina in Culture Systems.

The thirteen whole worm samples were used for analysing a correlation and regression between the worm wet weight and the worm dry weight. The whole worm samples were weighed before and after freeze-drying. The results were calculated using the linear regression analysis by Microsoft Excel. The regression graph and equation are shown in Figure 2.6 below including R^2 value. The regression equation for the association between the worm wet weight and the worm dry weight was $Y = 0.139X + 0.024$ and R^2 value for the goodness fit of scatter plot was 0.918. Each fatty acid in terms of mg g^{-1} dry weight was then converted to the mg g^{-1} wet weight using the regression equation.

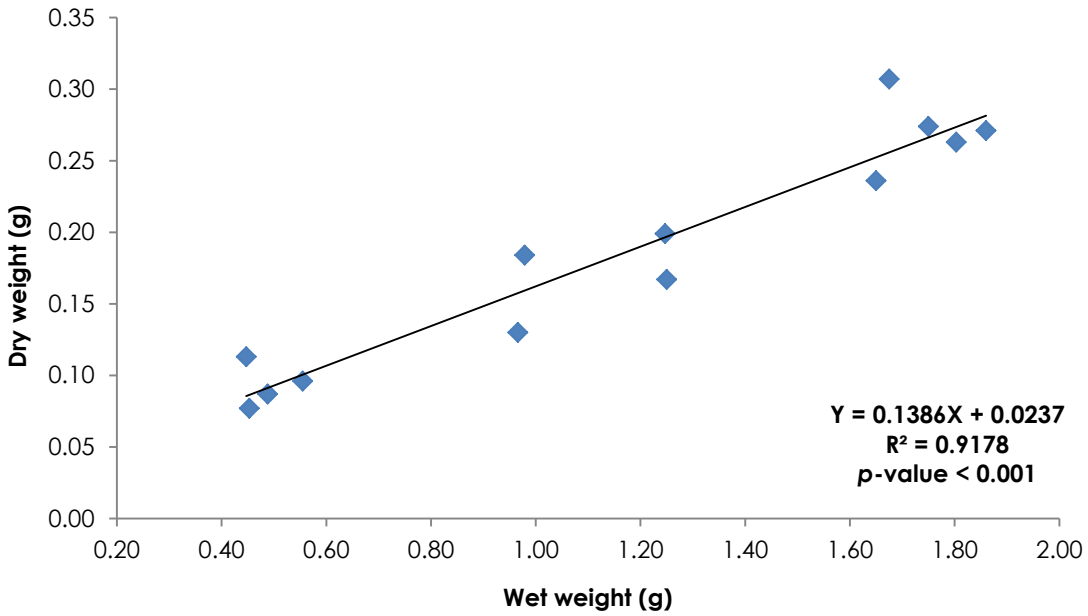


Figure 2.6: The regression graph and equation between whole *A. marina* wet weights and dry weight after freeze drying (n = 13).

After the conversion, the observed change in the quantity of any specified fatty acid was calculated. The change can be referred to as the 'net gain' or 'net loss' of each fatty acid. It should be noted that from this point forward throughout this chapter, any gain in a fatty acid means the net gain of that fatty acid in the system after subtracting the quantity present in the given food and the quantity of that fatty acid present in the worm tissues at the start of the experiment. The calculation steps are shown in section 2.2.6. A net loss may be observed if a specified fatty acid was present in the food and was not assimilated with 100% efficiency by the worms. A net gain can only be observed if there is a capability for the production of the fatty acid in the system on the assumption that the only external sources were the introduced worms and the given food. Negative controls without given food were included in the experimental design to test this assumption.

The result of net gain and loss of fatty acid is illustrated in in Figure 2.8 in terms of g per system unit. The general trend for the net gain or loss of each fatty acid, with a few exceptions, was a net gain in the system boxes provided with food and a loss of fatty acids in the unfed boxes. According to Figure 2.8, the fatty acids that gained the most were palmitic acid (C16:0), palmitoleic acid (C16:1n7), *cis*-vaccenic acid (C18:1n7) and oleic acid (C18:1n9), respectively. In the case of long chain PUFA, ARA (C20:4n6) and EPA (C20:5n3), were also shown to accumulate over the experimental

period. It is also interesting that there were gains in both eicosadienoic acid (C20:2n6) and γ-linolenic acid (C18:3n6). As mentioned earlier, the overall pattern of the unfed boxes was a net decrease in the amount of each fatty acid present in the worm tissues; however, there was a very slight increasing of oleic acid (C18:1n9) in both boxes, 0.04 and 0.01 g. There was no net gain or loss of eicosatrienoic acid (C20:3n3) within the system box.

2.3.5 Net Gain or Loss of Fatty Acids in N. virens

The regression analysis between worm wet weights and worm dry weights was also conducted and determined for *N. virens* in a similar way to *A. marina*. Twenty worms were weighed and freeze-dried to make a data set for regression analysis. The regression line and equation is shown in Figure 2.7. The regression equation between *N. virens* wet weights and dry weight is $Y = 0.220X - 0.027$ and R^2 value for the goodness fit is 0.995. This equation was then used to convert the measured term mg g^{-1} DW to mg g^{-1} wet weight and then to calculate the net gain or loss of fatty acids in *N. virens* in terms of mg in the systems as discussed earlier in topic 2.3.4.

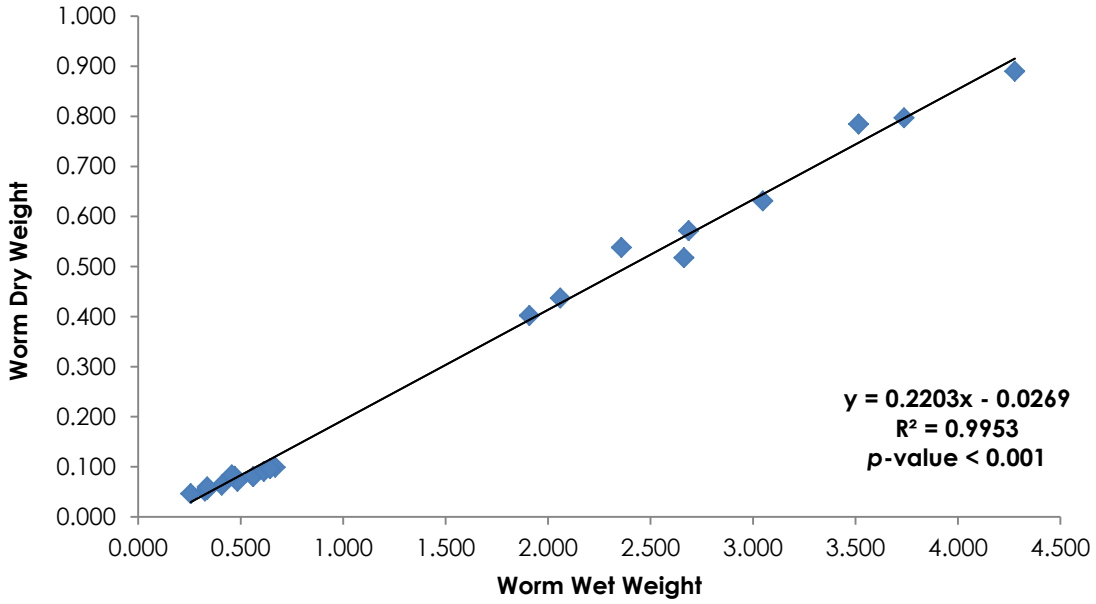


Figure 2.7: The regression graph and equation between whole *N. virens* wet weights and dry weight after the freeze drying (n=20).

An analysis of the bioaccumulation of fatty acids over the course of experiment in *N. virens* was calculated in a similar way to the *A. marina* experiment. The total amount of feed pellets given to the worm was multiplied with the average fatty acid content in the food pellets to determine the absolute amount of each fatty acid added to the system with the food. It was then possible to calculate the net change in the absolute amount of fatty acid within the system (net gain or loss) as being the absolute amount in terms of g present in the worms at the end of the experiment less the amount in the worms at the beginning of the experiment less the amount given in the food. If this value is positive then there must have been some production of the fatty acid within the system during the course of the experiment. In the fed groups, nearly all fatty acids were net accumulated over the experimental period with an exception in the case of myristic acid (C14:0) and C20:1n9. Of the fatty acids showing a net gain, *cis*-vaccenic acid (C16:1n7) increased by the greatest amount followed by oleic acid (C18:1n9) and EPA (C20:5n3). The amount of DHA (C22:6n3) also increased during the experiment but to a lesser degree. Some fatty acids in the fed groups were lost (net loss) during the experiment, for example, myristic acid (C14:0), α-linolenic acid (C18:3n3) and 11-eicosenoic acid (C20:1n9). It is of interest that eicosadienoic acid (C20:2n6) was gained in this experiment but not γ-linolenic acid (C18:3n6). In the unfed groups, all fatty acids, as expected, were lost during the course of the experiment. It is also noticeable that the amount of EPA (C20:5n3) that was lost was the highest, among all the fatty acids that were observed in the unfed groups; it was followed by palmitic acid (C16:0) suggesting that these fatty acids are subject to active metabolic breakdown in starving animals.

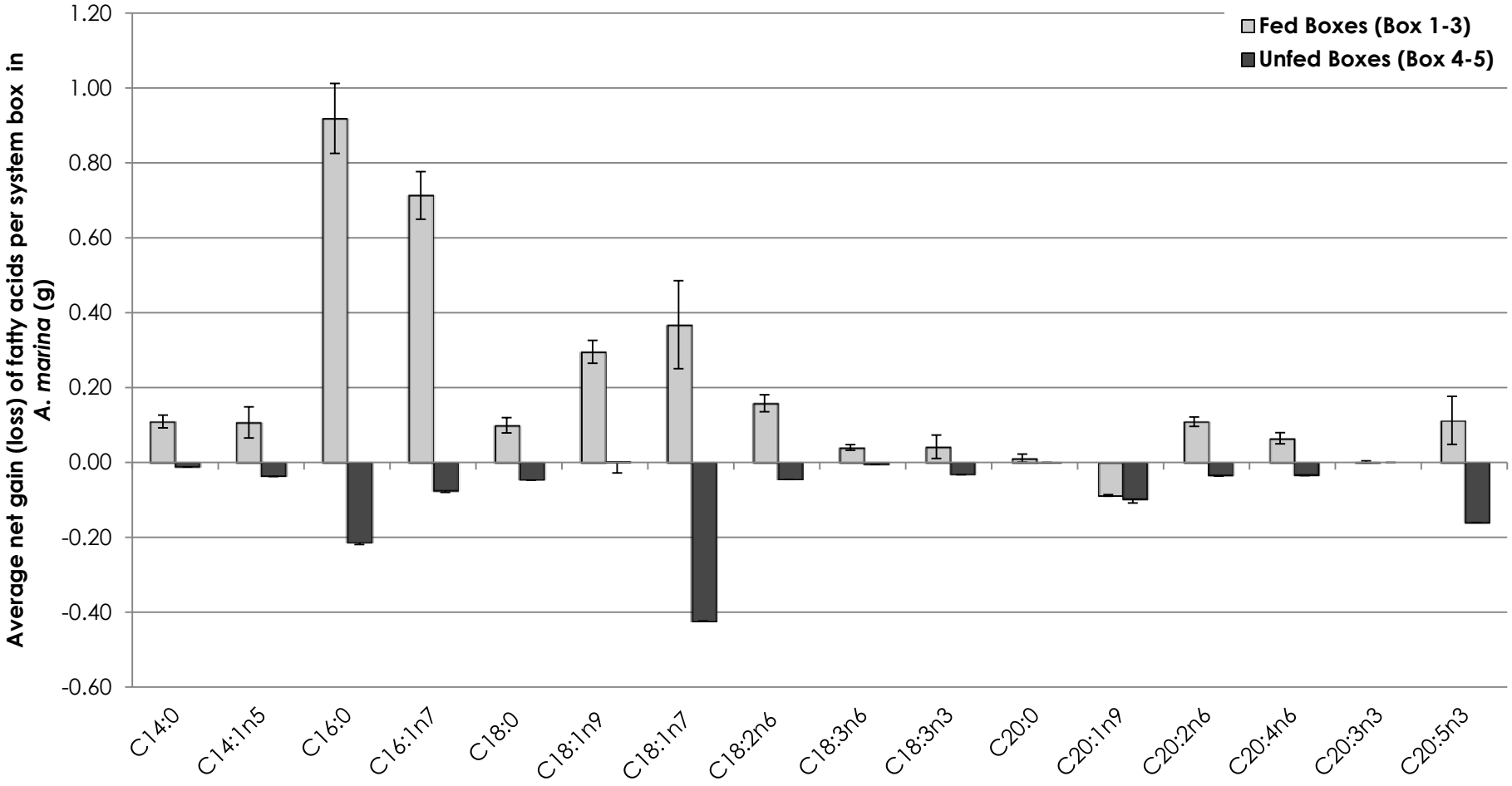


Figure 2.8: The average net bioaccumulation (net gain or loss) of each selected fatty acid in *A. marina* in terms of g using the initial generation as a comparison baseline and subtracted the fatty acid contents from the amount given dried yeast.

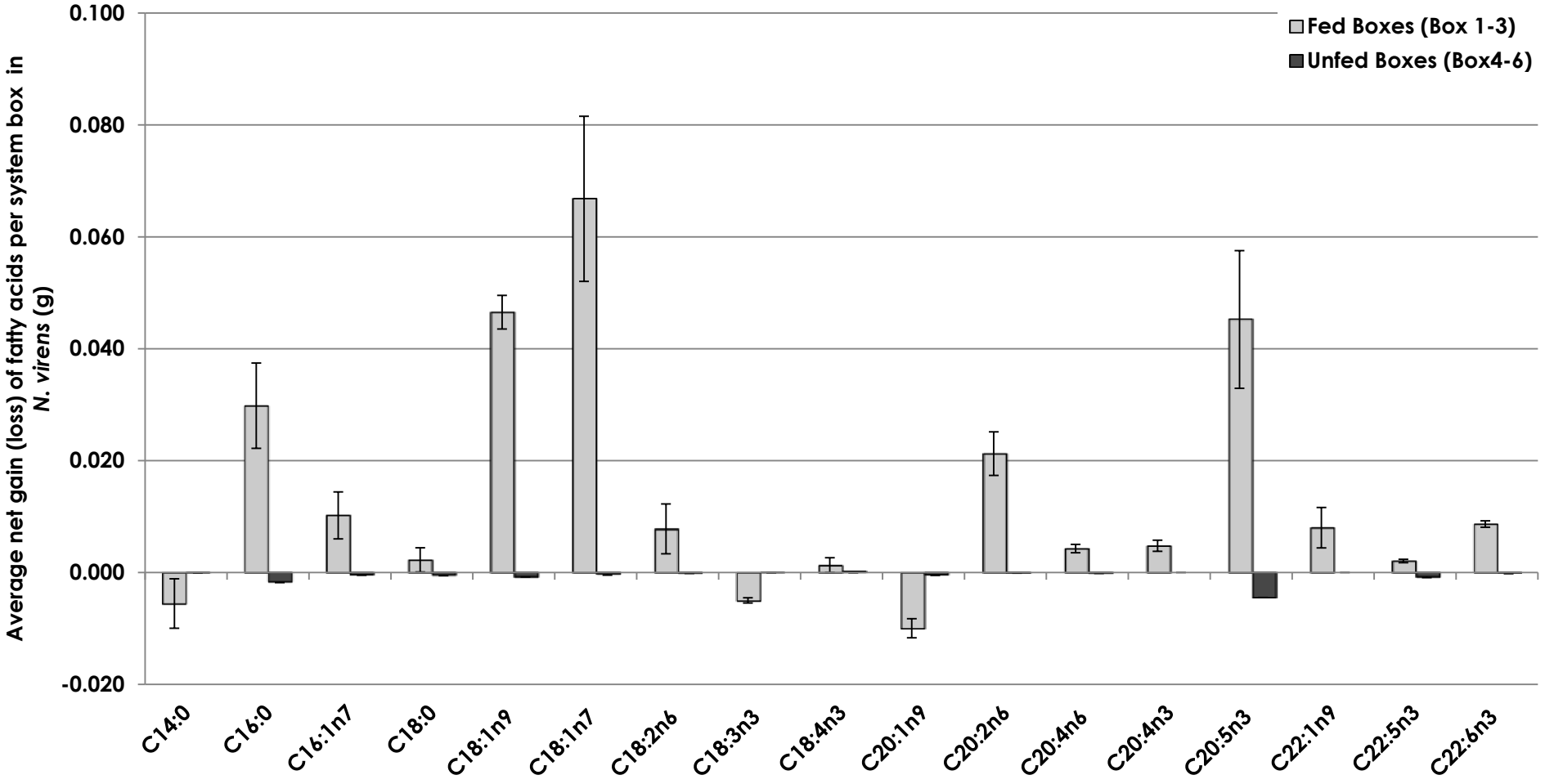


Figure 2.9: The average bioaccumulation (net gain or loss) of each selected fatty acid in *N. virens* in terms of g using the initial generation as a comparison baseline and subtracted the fatty acid contents from the feed pellets.

2.4 *Discussion*

For both the experiments with *A. marina* and with *N. virens*, a significant increase in wet weight in the fed group ($p < 0.05$) occurred confirming that the worms were feeding effectively and that the given diet was sufficient for growth. Furthermore, the evidence from the specific growth rate (SGR), percentage weight gain (PWG) and average growth rate (AGR) in these two worm species were positive numbers which also support the idea of food influences on their growth. The specific growth rate (SGR) per day per survived worm was calculated to determine the exact change of the growth rate over the period of experiment, and the individual growth rate was reflected in the calculated change in biomass for the whole box i.e. the whole-box SGR. In the unfed group, there were no significant differences ($p > 0.05$) between the worm wet weight before and after the experiment in either *A. marina* or *N. virens*. This result indicates that they were able to survive and maintain their living activities even though growth rates expressed as SGR, PWG and AGR were negative. The unfed worms in both species may have utilised the lipid and other nutrients in their tissues for survival.

The fatty acid contents in *A. marina* after the experimental period demonstrated the effects of diets, which were yeast and organic-rich sand, on the lipid profile of the lugworms. Lipid extraction from dried, sterilised yeast diet showed that the range of fatty acids in the food was restricted to a small number of saturated and monounsaturated C_{16} and C_{18} fatty acids and none of the longer chain PUFA were found in the yeast samples. This lipid profile is similar to the previous study in a yeast *Saccharomyces cerevisiae* by Henry (1982) in which they reported that yeast comprised mainly of monoenoic fatty acids such as palmitoleic acid (C16:1n7) and oleic acid (C18:1n9). The study in the same species of yeast suggested that yeast has the ability to generate only monoenoic acids and cannot further desaturated to linoleic acid (C18:2n6), α-linolenic acid (C18:3n6) and even longer chain PUFA (Weete, 1974; Bossie and Martin, 1989). Similarly, neither organic-rich sand nor clean sand were found to have contained any measurable quantity of fatty acids in the samples examined. Therefore, the net gain of fatty acids at the end of experiment could not have been be derived only from that in the yeast and organic-rich sand added to the system. This result can therefore be taken as evidence to suggest that the fatty acid gains within the system box may result from the physiological and biochemical processes of the worm itself or associated-microorganisms.

A wide range of fatty acids in *A. marina* samples showed a net gain over the experimental period when the animals were fed on the 'low fatty acid content' feed (given yeast and sand) and some of these were completely absent in the given food; these include the long-chain PUFA: ARA (C20:4n6) and EPA (C20:5n3). This result suggests that the fatty acids that accumulated within the system were derived and generated independently from the given food; this conclusion was also strongly supported in the study using a similar procedure for *A. marina* by Olive *et al.* (2009). This finding is also similar to that observed in a study in *N. diversicolor* by García-Alonso *et al*. (2008) who showed that the fatty acid profile of the worm did not change regardless of the seasonal changes in the food and where the fatty acid content of the worms was found to be independent of the fatty acid contents of the food. Despite the absence of photosynthetic sources in their diet, these net gains of specific fatty acid results imply that *A. marina* may produce a diverse spectrum of fatty acids within their own system. The bioaccumulation of fatty acids within the system might be produced from the worm itself, or could be synthesised by the bacteria in the system. However, the bacterial study by Ashforth (2008) showed that bacteria associated with *A. marina* burrows are mostly members of the chemolithoautotroph *Epsilonproteobacteria*. For this class of bacteria, it has been reported that they cannot synthesise PUFA and therefore they are unlikely to act as a potential PUFA source for the worms. It might be difficult to control about the gut's bacteria; however, the unfed groups were conducted to compare any changes of the fatty acid profile in the system. There were net loss of most fatty acid in the unfed group while most of fatty acids were gained in the fed groups. This result indicates that the worm gut bacteria may not contribute to a net gain of fatty acid. A net gain of ARA and EPA within the *A. marina* system box therefore suggests that these two fatty acids are essential to the worm and can be synthesised by the worm when not present in the diet. In the case of DHA (C22:6n3), only trace amounts of all C_{22} fatty acids, including DHA (C22:6n3), have been found in the worm samples by previous studies (Lytle *et al.*, 1990; Olive *et al.*, 2009); however, these C_{22} fatty acids were not detected on the chromatograms in this present study. This observation, together with the previous findings, indicates that DHA and other C_{22} fatty acids are not important to *A. marina* and that the lugworm itself might not be able to generate these fatty acids. In the unfed controls, virtually all fatty acids showed a net loss from the system. The loss of these fatty acids from the worm samples indicates that fatty acids in the worm samples might be utilised as energy sources when the worms are in the fatty acid-deficient system. Further implications and contributions of the net gain

of fatty acid results within the system to the PUFA biosynthetic pathways in *A. marina* will be discussed later in Chapter 4.

The results were rather different in the case of *N. virens* in which the given diets contained a wide range of fatty acid species (as shown in Figure 2.5). As explained earlier, the net accumulation of fatty acid calculation procedure was adopted in this experiment to resolve the problem with a various spectra of fatty acid contents within the diets. After the calculation of the net gain in specific fatty acids, the calculations show that there was a net gain of virtually all of the fatty acids present within the fed group. This result also suggests that *N. virens* may have the ability to accumulate and produce some PUFAs within the system despite the fact that some of them were introduced to the system with the food. A relatively high net gain of EPA at about 40-50 mg is suggested, indicating that EPA is one of the most important fatty acids among a range of fatty acids present in the tissues and that the amount of EPA given with the food may be amplified within the system leading to a net gain. The observation of a high EPA content and net gain during the experiment is in agreement with the study in the same species by Brown *et al*. (2011) which showed that EPA is one of the most abundant fatty acids when fed on several types of diet including: commercial fish food, halibut faecal waste, uneaten halibut feed pellets and mixture of faecal waste and feed pellet waste. EPA is also dominant in similar related nereidid species, *N. diversicolor* (Bischoff *et al*., 2009). Fidalgo e costa *et al*. (2000) has suggested that *N. diversicolor* can biosynthesise EPA de novo when fed on low-EPA diet.

Also, Brown *et al*. (2011) found that palmitic acid (C16:0), oleic acid (C18:1n9) and C18:2n6 are among the highest fatty acid concentrations. The results from the net gain calculation in this present study are also rather similar in the case for palmitic acid (C16:0) and oleic acid (C18:1n9). However, linoleic acid (C18:2n6) were gained in a relatively small amount. This rather contradictory result may be due to enough content of linoleic acid in the pellets for the worm's growth.

Surprisingly, there were small but noticeable net gains of DHA (C22:6n3) and other C_{22} intermediate such as C22:5n3 from the system while these species were present in the initial samples only in trace amount. García-Alonso *et al*. (2008) also reported that DHA concentration in *N. diversicolor* was observed and was independent from the different food treatments. It seems possible that these results are due to the ability of the worm to generate DHA. This result is different from that observed in the study of the net gain in *A. marina*, where C_{22} fatty acids including DHA were not found

to be generated within the system. The contributions of this experiment to the PUFA biosynthetic routes in *N. virens* will be further discussed in Chapter 4.

In conclusion, the net gain of certain fatty acids within the closed-recirculating controlled system in the two polychaete species provides further evidence to support the hypothesis that these polychaete worms have the potential to synthesise PUFAs. However, to provide further evidence that a net gain of fatty acids including PUFA: ARA, EPA and/or DHA is a consequence of biochemical processes occurring within the worm a further experiment on worm biochemistry using ^{13}C stable labelling approach was adopted as will be described in the next chapter.

Chapter 3

^{13}C Stable Isotope Labelling Experiments to Trace the PUFA Biosynthetic Pathway

Chapter 2 presented data confirming that a net gain of certain PUFA does occur in *Nereis virens* when grown in the laboratory on a diet of known fatty acid content. These data are consistent with the hypothesis that *N. virens* can synthesise these fatty acids; however, the evidence remains circumstantial. It is possible that the fatty acids may have been synthesised by other organisms co-present in the growth system (e.g. bacteria, diatoms or protists) that are in turn consumed by the worm. A similar criticism can be levelled as the study of PUFA gain in *Arenicola marina* by Duangchinda (2007); although, Ashforth (2008). Although Ashforth *et al*. (2011) did not find evidence for extensive growth of bacterial species capable of PUFA biosynthesis in similar culture systems.

In order to gain clearer insight into the biosynthetic capabilities of the *N. virens* and, importantly, to distinguish between PUFA production in the *N. virens* tissues and uptake from associated organisms, a ^{13}C stable isotope tracer approach was adopted. Further, this technique would potentially aide in characterising the PUFA biosynthetic pathways, if present, in *N. virens*.

In order to explain the experimental approach adopted and to facilitate with the interpretation of the results, an explanation of some theoretical and practical aspects of fatty acids mass spectrometry is presented.

3.1 *Introduction*

A general perception has emerged in some of the animal nutrition literature that the majority of animals are incapable of producing long-chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (C20:5n3) and docosahexaenoic acid (C22:6n3) from more saturated precursors with a shorter chain length (Bell and Tocher, 2009). More recently, studies have emerged that offer contradictory findings about the ability of some animals to synthesise long-chain PUFA. Several techniques have been used to investigate this topic; for example, the molecular studies employed by Watts and Browse (2002) and Watts (2009) using *Caenorhabditis elegans* showed that the nematode may be capable of synthesising EPA from C_{16} substrates. Controlled feeding regimes followed by nutritional analysis have been adopted in several studies including

studies concerning the lugworm, *A. marina* (Olive *et al*., 2009) and also the net gain or loss of fatty acids in *N. virens* in chapter 2. Furthermore, many biochemistry and analytical chemistry techniques have also been widely used to study the patterns of fatty acid biosynthesis. One of the interesting techniques was the application of radioactive carbon ^{14}C as a labelling compound. For instance, the cockroach, *Periplaneta americana*; the termite, *Zootermopsis angusticollis*; and the cricket, *Acheta domesticus*, were studied using this technique and were shown to have the ability to synthesis linoleic acid from ^{14}C-labelled acetate as a precursor (Blomquist *et al*., 1982). Also, ^{14}C-labelled 18:3n3 was also utilised in the wax moth larvae, *Galleria mellonella*, to trace C_{20} fatty acid biosynthesis (Stanley-Samuelson *et al*., 1987).

Latterly, the ^{14}C radioisotope approach has been progressively replaced by stable isotopes on health and safety grounds. Stable isotopes, for example ^{13}C, have proven to be an effective and safe alternative approach to utilise compound-specific labelling pathways, and has been adopted to investigate the lipid biosynthesis pathways on many occasions.

Several series of lipid biochemistry research using ^{13}C-compound specific labelling approaches have been mainly used in mammalian and human studies (Koletzko *et al.*, 1997 and Koletzko *et al.*, 1998). In contrast, few studies have used the ^{13}C-labelling approach to trace any metabolic pathways in invertebrates e.g. the parasitic protozoa (*Perkinsus marinus*) in an oyster using 1,2-^{13}C-acetate and palmitate-d_{31} acid (Chu *et al*., 2002).

As there have been no prior direct studies using ^{13}C labelling technique to trace the metabolic pathway in polychaetes, a preliminary investigation was necessary to determine the amount of ^{13}C to be used, to establish an appropriate diet and to optimise the duration and feeding frequency in the experiment.

3.2 *Materials and Methods*

3.2.1 General Methodology

The worm tissues were homogenised and lipid content extracted as described in Chapter 2. The extracted fatty acids were transesterified to FAMEs using an acid catalysis (H_2SO_4/CH_3OH). The GC was used for initial analysis and then followed by GC-MS to trace the changes in the $^{13}C/^{12}C$ ratio. The instrument was configured to the same conditions as described in Chapter 2. In order to trace the change in the $^{13}C/^{12}C$ ratio, the ion peaks of interest in the mass spectra were extracted using the command 'extract ion chromatography' using an m/z range from 0.30 to 0.70 in the 'ChemStation' program. After that, the extracted peaks were then integrated to determine their own peak area. The integration threshold was set at 7.5 mV to detect the small changes in all longer fragment peak area. The peak area of the characteristic ion peak with normal ^{12}C and ion peak with ^{13}C labelled of the same fragment was calculated to determine the isotope ratio of the peak or the $^{13}C/^{12}C$ ratio.

SPSS version 17.0 was employed for all statistical analyses. A one-way analysis of variance (one-way ANOVA) was used to compare the changes between control and experimental samples on different time periods after intake ^{13}C-labelled palmitic acid. A Dunnett's *t*-test was used to determine the difference of each experimental group against the control group. The samples obtained from the beginning of the experiment were set as a control group in this Dunnett's multiple comparison. A one-tailed test was also used to confirm any increase of fatty acid metabolites after ^{13}C-labelled precursor uptake by the worms as any increase will indicate the production of fatty acid within the worms.

3.2.2 Preliminary Experiment (Single Initial Pulse Feeding)

The preliminary investigations were conducted both in *A. marina* and *N. virens*. The animals were collected from the same site as in the net gain or loss of fatty acid chapter (see topic 2.2.1 page 48). *A. marina* were collected from Alnmouth, Northumberland, UK while *N. virens* were collected from the estuary of River Blyth, Northumberland, UK on October 2011. Each worm was put individually into a seperate experimental jar. The temperature was kept approximately constant (12 ± 0.5^oC) using a supply of running seawater surrounding the experimental jar. Sieved and sterilised sand

to a depth of 2 cm was also added to each experimental jar to reduce any stress that might impact on the animals.

Different methods were applied to introduce the ^{13}C isotope (^{13}C-palmitate) into the body of the two species. In the case of *A. marina*, it was possible to inject the isotope using hypodermic needle into the large and spacious coelomic cavity due to the round body and lack of septa in the trunk region of the lugworm. In contrast, *N. virens* has complete septa throughout the body and the coelom is less spacious than that of *A. marina*; the injection technique therefore proved to be impracticable. However, unlike *A. marina*, *N. virens* will feed on large pellets of food. Therefore, a method of incorporation based on feeding labelled food pellets was successfully adopted.

The ^{13}C-1-palmitic acid (C16:0) was supplied from the Sigma-Aldrich Co. LLC. In *A. marina*, a stock solution of ^{13}C-1-palmitic acid in methanol was prepared to make a final concentration of 500 mg ml^{-1} in methanol. The concentration was calculated and determined from the average palmitate content in the 1 g wet weight of *A. marina* from the net gain or loss experiment in Chapter 2. To reduce any toxic damage to the worms from the methanol, a small volume (0.05 ml) from the stock solution was injected at the dorsolateral side of the body. The injection site was chosen to decrease the risk of damage to the gills and blood vessels. After the injection, the worms were returned individually to the experimental jars. The worm samples were collected 3 hours, 1, 2, 3, 5 and 7 days after injection. In addition, a control worm that had not been injected was sampled and analysed as a baseline to trace any changes in the $^{13}C/^{12}C$ ratio. Only one sample for treatment was utilised in this preliminary investigation.

In contrast to *A. marina*, ^{13}C-1-palmitic acid was incorporated into pelleted fish feed as a precursor for *N. virens* PUFA biosynthesis. The worms were put into an experimental jar with a 2 cm depth of clean sand. The amount of the ^{13}C-1-palmitic acid was also determined from the palmitic acid content in grams per wet weight of *N. virens* from the net gain or loss of fatty acids in Chapter 2. Five grams of feed was ground using a mortar and pestle. Then, 0.125 g of ^{13}C-1-palmitic acid was added and mixed thoroughly, thereby giving a dosage of 25 mg g^{-1}. After that, the incorporated feed diets were re-pelletised and dried in the oven at 200 °C for 3 hours to make a stock of ^{13}C palmitate enriched feed pellets. An average 0.01 g of ^{13}C-labelled palmitate feed pellet was given to each worm at day 0. All worm samples were collected after 3 hours (day 0), 1, 2, 3, 5 and 7 days. Also, an unfed worm was sampled as a baseline to compare the changes in $^{13}C/^{12}C$ ratio.

3.2.3 Repeated Labelling Experiment

N. virens were collected on 12th April 2012 from the same site (River Blyth, Northumberland, UK) as for the single pulse labelling experiment. The worms were reared in the same conditions, as described in chapter 2 and earlier in this chapter (see topic 2.2.1). The lipid extraction and FAMEs conversion were also carried out using the same techniques and analysed by GC and GC-MS as before.

Worms with an average weight of 1 g were put individually into separate experimental jars and placed under controlled experimental conditions for 7 days prior to the start of an experiment. This step was adopted to acclimatise the worms to a new environment allowing them time to burrow and construct their habitat in the sand.

Experimental diets incorporating ^{13}C-palmitate were produced as before using the same proportions of ingredients as described in the single initial pulse feeding experiment. However, the experimental design was modified in response to the results from the single initial pulse feeding experiment by repeated feeding over several days.

Each worm was fed daily with 0.02 g of feed pellets. However, the worms were divided into two groups, one was given ^{13}C-labelled palmitate food pellets (labelled food group) and a second group was given normal unlabelled food pellets (unlabelled food group). Four worms from each group were sampled every other day from 1 day after first feeding to reach 7 days after first feeding (i.e. day 1, day 3, day 5 and day 7). Four individuals (replications) for each treatment (day from first feeding) were used to estimate the between worm error terms in subsequent statistical comparisons. Similarly, four worms were sampled at the start of experiment. This time point was designated as day 0 and the worms were used as the control baseline samples. The fatty acid composition of the fish feed pellets was also analysed to permit a comparison between the normal feed diets and the labelled feed following incorporation of ^{13}C-labelled palmitate. All the worm and feed pellet samples after collection were kept at -80 °C to prevent further oxidation and loss of the double bonds. The experimental design is shown in Figure 3.1.

A one-way analysis of variance (ANOVA) with Dunnett's multiple comparison *post hoc* test was used to compare labelled and unlabelled groups over the experimental period against the control group. The ^{13}C/^{12}C ratio data, like all percentage and proportion data, is limited from 0 to 1; therefore, all the ratio data were arcsine transformed prior to statistical analysis.

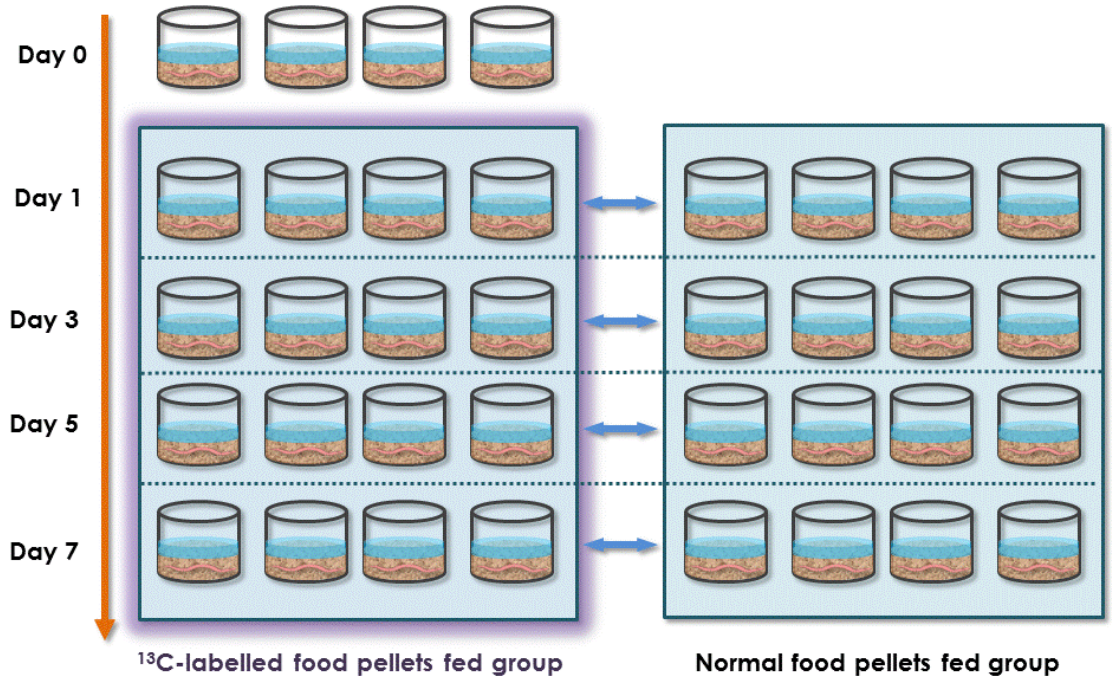


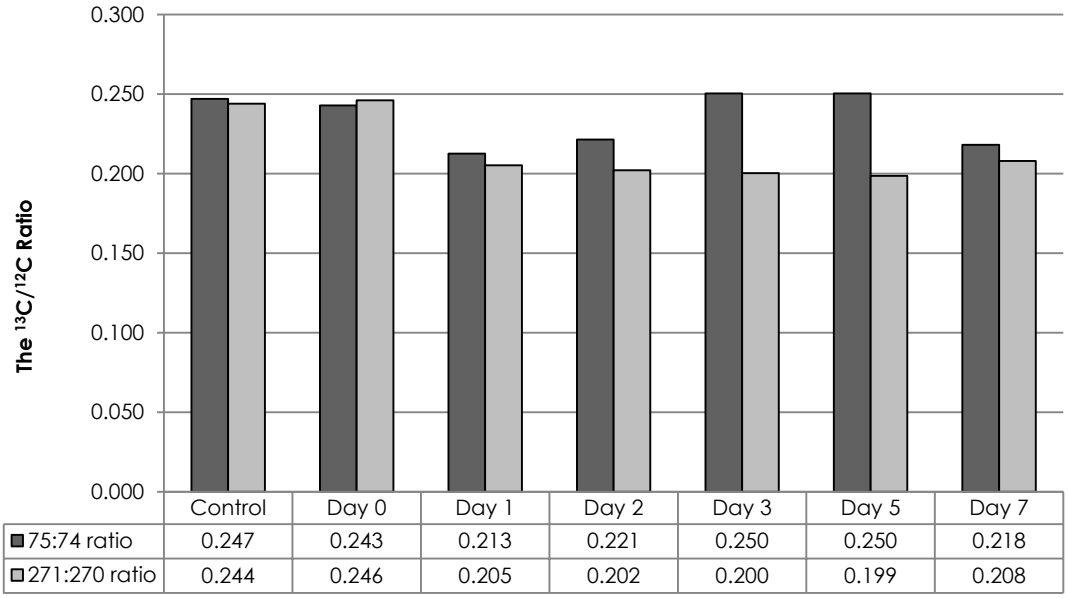
Figure 3.1: Summary of the experimental design. The values for the $^{13}C/^{12}C$ ratio in the fatty acid fragment ions within the labelled feed group and the unlabelled feed group (control group) over the experimental period were compared with the control set at time zero to reveal if any changes took place over time.

3.3 *Results*

3.3.1 Single Initial Pulse Feeding

The $^{13}C/^{12}C$ ratio of the methyl palmitate (C16:0) peak was calculated and compared in *A. marina* and *N. virens* to confirm ^{13}C-labelled palmitate incorporation into their bodies. The results for the *A. marina* are shown in Figure 3.2 while Figure 3.3 displays the data and results for *N. virens*. The McLafferty (m/z = 74), the molecular peak (m/z = 270) and their fragments incorporated with the ^{13}C label (m/z = 75 and m/z = 271) of methyl palmitate were selected to confirm any changes of the $^{13}C/^{12}C$ ratio over the course of the experiment.

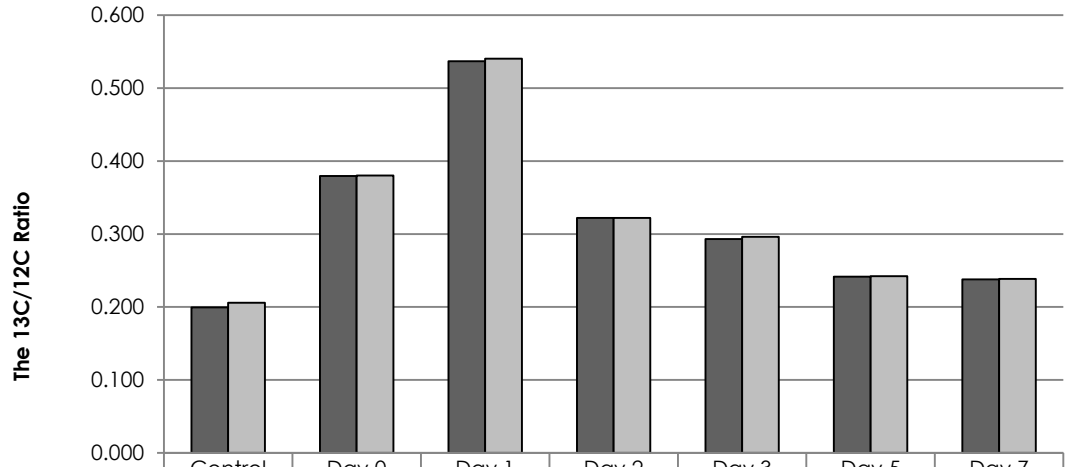
For *A. marina*, the m/z = 75/74 methyl palmitate ratio was relatively constant over the experiment. For the molecular peak, the ratio decreased slightly compared with the control worm as shown in Figure 3.2.



	Control	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
75:74 ratio	0.247	0.243	0.213	0.221	0.250	0.250	0.218
271:270 ratio	0.244	0.246	0.205	0.202	0.200	0.199	0.208

Figure 3.2: The changes of $^{13}C/^{12}C$ ratio of the methyl palmitate (C16:0) in *A. marina*.

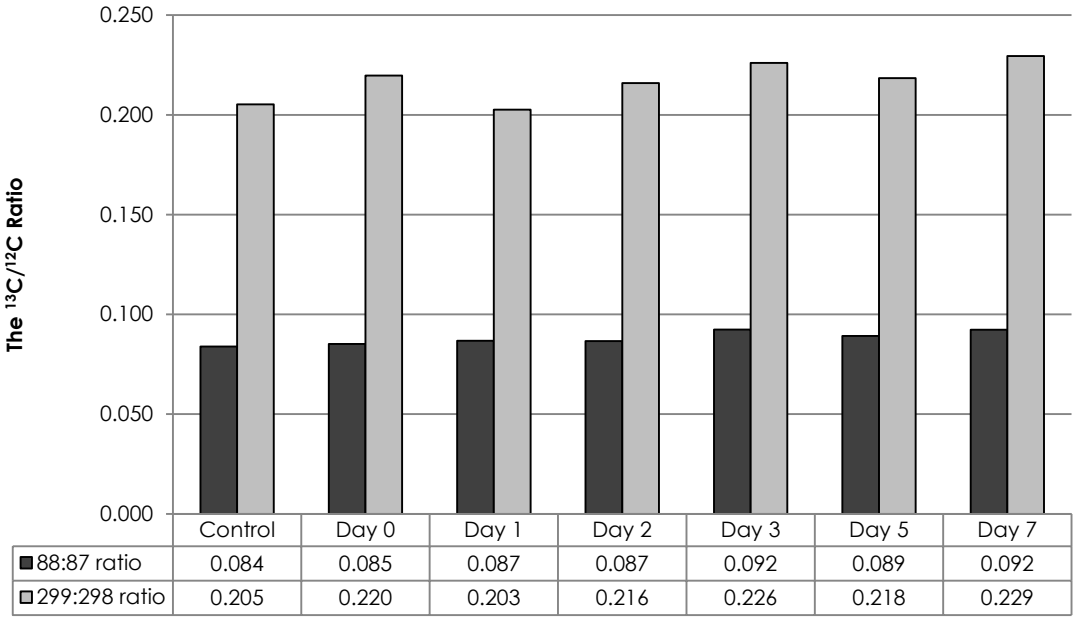
In the case of *N. virens*, the results were notably different from *A. marina*. There were obvious differences making it possible to detect changes both in the McLafferty and the molecular ion peaks. It should be noted however, that as there was no replication within the sampled animal in this preliminary experiment, statistical comparisons between worms were not possible. The McLaffery ion (m/z = 75/74) changed from a ratio of 0.247 at the baseline to 0.380 after 3 hours of injection (day 0) and reached the highest ratio of 0.537 on day 1 as shown in Figure 3.3. Also, note that the changes in the signature ratio in the molecular peak show a similar pattern to that observed for the McLafferty ion.



	Control	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
75:74 ratio	0.200	0.380	0.537	0.322	0.293	0.242	0.238
271:270 ratio	0.206	0.380	0.540	0.322	0.296	0.242	0.238

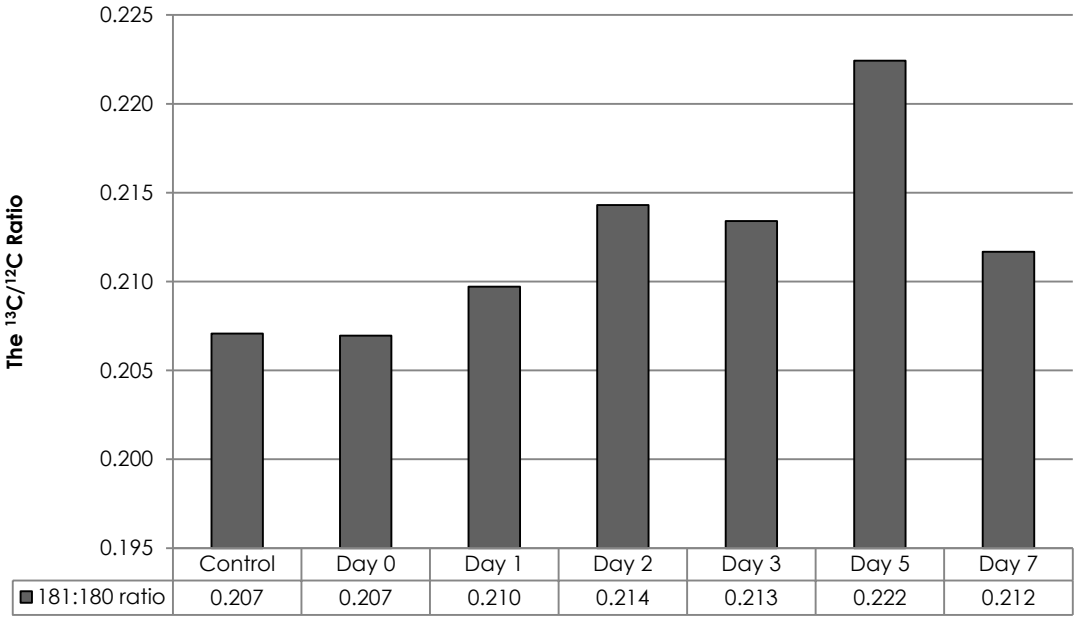
Figure 3.3: The changes of $^{13}C/^{12}C$ ratio of the methyl palmitate (C16:0) in *N. virens*.

Relatively small but noticeable changes in $^{13}C/^{12}C$ ratio peaks of interest for methyl stearate (C18:0) was also detected in *N. virens*. The first hydrocarbon fragment (m/z = 88/87) and the molecular peak (m/z = 299/298) was analysed as the characteristic peak as shown in Figure 3.4. Similar changes were also noticeable for the methyl EPA (C20:5n3) characteristic peak (m/z = 181/180) as shown in Figure 3.5. The most noticeable change observed after injection was an increase in the ratio from that at the beginning of the experiment to the maximum value observed on day 5.



	Control	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
88:87 ratio	0.084	0.085	0.087	0.087	0.092	0.089	0.092
299:298 ratio	0.205	0.220	0.203	0.216	0.226	0.218	0.229

Figure 3.4: The changes of $^{13}C/^{12}C$ ratio of methyl stearate (C18:0) in *N. virens*.



	Control	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
181:180 ratio	0.207	0.207	0.210	0.214	0.213	0.222	0.212

Figure 3.5: The changes of $^{13}C/^{12}C$ ratio of methyl EPA (C20:5n3) in *N. virens*.

Although it is not possible to draw firm conclusions due to the lack of replication, the results obtained were sufficient to indicate that labelled fatty acids were incorporated into the tissues. Discernible changes were detected in the isotopic ratios of the mass spectrum fractions that were predicted to be labelled (see topic 1.3.3, Chapter 1). Equally, changes in isotopic ratios were not observed for the mass spectrum fractions that were not expected to be labelled (data not shown). However the data also suggest that any changes induced in the spectra by incorporation of the labelled carbon were transient and it was decided that an approach using repeated feeding of labelled food, as well as the introduction of multiple worm samples at each time point, was advisable. This is reflected in the design of the second experiment the results of which are presented below.

3.3.2 Repeated Labelling Experiment

In this result section, each fatty acid is presented separately to clarify the observed changes in the $^{13}C/^{12}C$ ratio. The characteristic peaks were selected according to the rationale previously described in topic 1.3.3. It should be noted that the mean obtained from the experiment for each entity is designated in terms of mean ± 95% confident limit of the mean (95% CI). The *p*-value were presented as the tables in Appendix B.

In the case of methyl palmitate (C16:0), there was a statistically significant difference among means for the $^{13}C/^{12}C$ ratio for each of the three characteristic fragment peak ions for samples given repeated labelling but not for the unlabelled group (Table 1B). A one-way ANOVA test showed a significant difference in the $^{13}C/^{12}C$ ratio in the molecular peak (m/z = 271:270), $F(2, 27) = 12.751$, $p < 0.001$. Dunnett's *t* multiple comparison of the molecular peaks in all samples groups indicates that all the samples in the labelled groups had a significantly greater ratio compared with the control ($p < 0.01$). In contrast to the labelled group, none of the isotope ratios in the unlabelled group differed significantly from the control group.

The pattern of results observed for the molecular peak was also observed for the McLaffery ion. One way ANOVA demonstrated a significant difference among means for the McLafferty (m/z = 75:74), $F(2, 27) = 12.426$, $p < 0.001$, and the loss of the methoxy ion (m/z = 240:239), $F(2, 27) = 11.881$, $p < 0.001$, ratio. A Dunnett's *t* post hoc multiple comparisons again indicated a significant difference for all the samples in the labelled groups against the day zero control samples at the start but not for the samples in the unlabelled group ($p < 0.01$).

In the case of methyl stearate (C18:0), the $^{13}C/^{12}C$ ratio of the three characteristic peaks: i) the molecular peak (m/z = 299:298), ii) the McLafferty (75:74) and iii) the first peak in the hydrocarbon series (m/z = 88:87) were analysed. The $^{13}C/^{12}C$ ratio and *p*-value results obtained from the mass spectrum data for methyl stearate are shown in (Table 2B). A one-way ANOVA was performed on the molecular peak that revealed a significant difference among means as $F(2, 27) = 7.812$, $p < 0.001$. A Dunnett's *t* comparison shows that a significant difference in comparison with the day zero control was observed for samples taken on days 3, 5 and 7 among samples in the labelled fed group ($p < 0.05$). However, for the day 1 samples in the labelled group and all samples for the unlabelled feed group, there was no evidence to support a significant difference compared with the control. Also, the isotope ratio for the first hydrocarbon series (m/z = 88:87) changed in a similar way to that observed for the molecular peak and significant differences to the day zero control were observed for the samples at days 3, 5 and 7 as also shown in Table 2B. However, no significant differences between each of the sample means for the McLafferty ion ratio (m/z = 75:74) were observed in comparison with the control using a Dunnett's *t* post hoc test ($p > 0.05$).

For methyl oleate (C18:1n9), the loss of methanol peak ($[M-32]^+$, m/z = 265:264) and the loss of the McLafferty ion peak ($[M-74]^+$, m/z = 223:222) were adopted and analysed as the representative fragment ions. In contrast to the situation found for methyl palmitate (C16:0) and methyl stearate (C18:0), a one-way ANOVA analysis of the methyl oleate (C18:1n9) indicated that there were no significant differences between means both in the case of the loss of the methoxy ion, $F(2, 27) = 0.843$, $p = 0.574$, and the loss of the McLafferty ion, $F(2, 27) = 1.455$, $p = 0.222$. The molecular peak ratio (m/z = 297:297) could not be analysed however, as it could not be detected on the spectra due to the interference of the background noise. The $^{13}C/^{12}C$ ratio of the loss of the McLafferty ion (m/z = 223:222) was quite constant over the period of the experiment as also was the ratio for the loss of the methoxy group ion peak (m/z = 265:264). The statistical data of the methyl oleate are shown in Table 3B.

In the case of methyl linoleate (C18:2n6), the molecular peak (m/z = 295:294) was the only peak ion used in the analysis to trace possible changes in the isotope ratio. A number of ions due to loss would be of interest for this fatty acid but the interesting ions were found only in relatively small amounts and could scarcely be detected. The results from one-way ANOVA analysis pointed out that there were no significant

differences between each groups both in the labelled and unlabelled group, $F(2, 27) = 2.335$, $p = 0.057$. The results of the methyl oleate are shown in Table 4B.

The loss of the methoxy ion' $[M-31]^+$, m/z = 292:291) and the molecular peak ($[M]^+$, m/z = 323:322) were adopted to analyse for methyl eicosadienate (C20:2n6). Both the 'loss of the methoxy ion' and the molecular peak were shown to have very small changes over the experimental period. However, the *F* statistic from a one-way ANOVA was 0.363 and *p*-value was 0.931. These statistic values denoted that there are no significant differences between each sample group. The methyl eicosadienoate (C20:2n6) data is displayed on Table 5B.

The α-ion (m/z = 181:180) of the methyl arachidonate (C20:4n6) was the only ion peak used for tracing the changes of the $^{13}C/^{12}C$ ratio. The molecular peak (m/z = 319:318) and the other long fragment ions were not clear due to background noise on the spectrum. The $^{13}C/^{12}C$ ratio for the α-ion was significantly increased on day 3 and day 5. An analysis from a one-way ANOVA strongly support significant differences $F(2, 27) = 3.145$ and $p = 0.013$. A Dunnett's t multiple comparison test was also supported an increase of the $^{13}C/^{12}C$ ratio on day 3 ($p = 0.001$) and day 5 ($p = 0.047$). The statistical results obtained from the methyl arachidonate are presented in Table 6B.

In a similar way to methyl arachidonate, only the α-ion (m/z = 181:180) was adopted for analysis in methyl eicosapentaenoate EPA (C20:5n3). Strong evidence of an increase was supported by one-way ANOVA, $F(2, 27) = 2.743$ and $p = 0.024$. The $^{13}C/^{12}C$ ratio of the methyl EPA was clearly increased to 0.252 ± 0.015 within only one day compared with the beginning at 0.225 ± 0.004. However, the rest of each treatment did not show any statistically significant differences in comparison with the control group. Further post hoc multiple comparison using Dunnett's t test also confirm a highly significant increase of the day 1 samples than the start samples as shown in Table 7B.

The α-ion (m/z = 167:166) of methyl docosahexaenoate, DHA (C22:6n3) was traced as a characteristic peak. In contrast to methyl ARA and methyl EPA, there was no evidence to support an increase of the $^{13}C/^{12}C$ ratio in the labelled group over the experimental period, $F(2, 27) = 1.078$ and $p = 0.407$. The statistical results for methyl DHA are shown in Table 8B.

A bar graph was used in order to summarise the overall pattern of observed changes in isotope ratio “the big picture”, for some of the fatty acids in the tissues over the experimental period. Figure 3.6 therefore presents the $^{13}C/^{12}C$ ratio data changes in some characteristic fragment peaks of each fatty acid between the labelled-fed groups. It should be noted that the bar graph comparing between the start and the unlabelled-fed group was not present as there were no significant difference between the groups. Some interesting fatty acid methyl ester (FAME) intermediates such as methyl α-linolenate (C18:3n3), γ–linolenate (C18:3n6) and methyl dihomo-γ-linolenate (C20:3n6) could not be identified in this analysis due to only trace amounts of the fatty acids could be found in the tissues. The presence of background noise of the extracted ion spectra was also be found as exemplified from the α-ion (m/z = 237:236) for methyl α-linolenate (C18:3n3) in Figure 3.7.

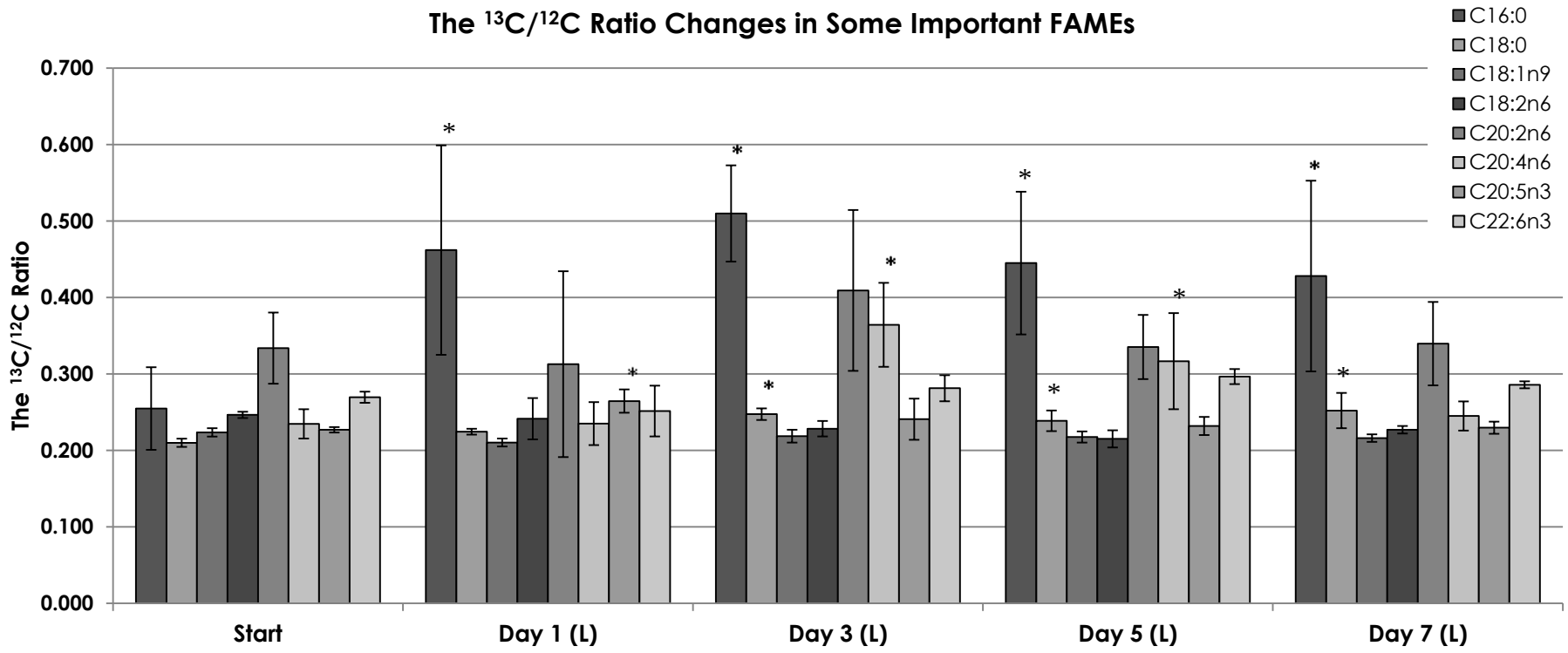


Figure 3.6: The bar graph shows the $^{13}C/^{12}C$ ratio of some selected fragment ions for some important FAMEs. The molecular peaks were adopted in methyl palmitate (C16:0), methyl stearate (C18:0) and methyl linoleate (C18:2n6). The α-ions were selected for methyl arachidonate (C20:4n6), methyl eicosapentaenoate (C20:5n3) and methyl docosahexaenoate (C22:6n3). The loss of the McLafferty ion was used for methyl oleate (C18:1n9) while the loss of the methoxy ion was shown in the case of methyl eicosadienoate (C20:2n6). SD was used as an error bar. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) obtained from the Dunnett's *t* post hoc test. L is abbreviated for labelled pellet fed group.

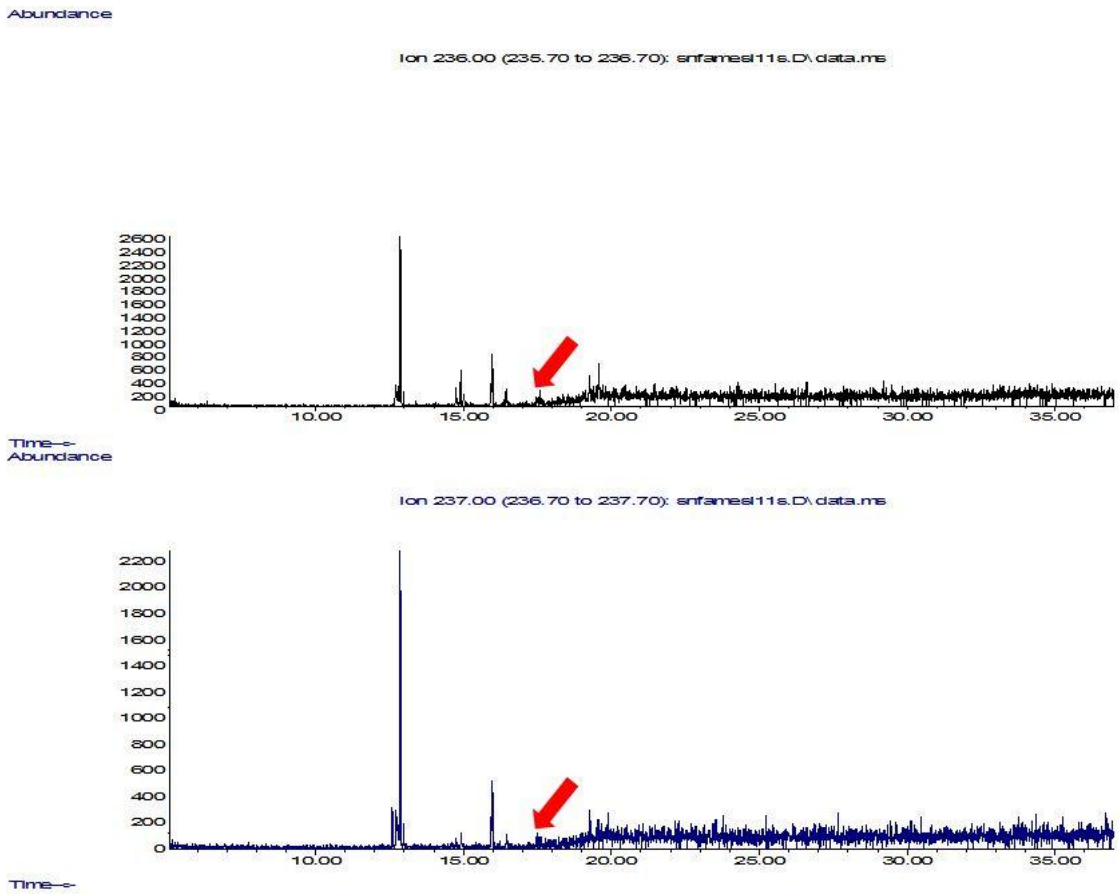


Figure 3.7: An example of the extracted ion mass spectra of the α-ion (m/z = 236/237) for the methyl α-linolenate (C18:3n3). As can be seen, the ion was present only in relatively small amounts and there was a level of noise background that could alter the interpretation. Red arrows indicate the retention time for the methyl α-linolenate.

3.4 *Discussion*

The single pulse feeding experiments for *A. marina* and *N. virens* were preliminary investigations carried out to determine the proper methodology; therefore, only one replicate for each treatment was used. Hence, the results are not sufficient in themselves to support or refute the hypothesis that the animals can synthesise PUFA. They did however provide essential information to modify and adjust the experimental approach in further experiments with sufficient replication to evaluate the hypothesis under test and to allow a more complete analysis of the roles of polychaetes in marine ecosystem in relation to the production and cycling of PUFA.

In the case of *A. marina*, there was insufficient evidence to support any change in the $^{13}C/^{12}C$ ratio of lipids from the worm samples after injection of the ^{13}C-labelled palmitate precursor. It seems possible that these results may be due to an unsuccessful incorporation of ^{13}C-labelled palmitate into the body. Also, after injection the worm

body showed signs of stress caused by the procedure resulting in a squeezing out of some of their body fluid into the external surrounding medium. This would cause the loss of some of the injected ^{13}C-labelled palmitate. In addition to the loss of the precursor due to stress, the inability to inject the precursor to the gut may be another reason for the undetectable changes in the $^{13}C/^{12}C$ ratio as the lipids may not have been taken into the blood stream and distributed to the tissues where lipid metabolism may occur.

Further studies on *A. marina* PUFA metabolic pathways will need to be conducted using a more appropriate labelling technique. The method here may not be strong enough to trace the pathways; for instance, the development of a methodology to allow for a feeding experiment with the labelled isotope precursor in the food should be able to resolve the problems arising from the delivery of the labelled fatty acids to the coelomic fluid rather than inside their guts. For example, an incubation of ^{13}C-labelled acetate could lead to the production of yeast with labelled palmitate and other labelled metabolites. The yeast could then be used as a food source to trace the fatty acid metabolic pathway. Quantifying labelled fatty acid contents would then be necessary to understand any changes in the isotope ratio.

In the case of *N. virens*, the presence of ^{13}C-labelled palmitate after food intake in the labelled fed group confirms the incorporation of ^{13}C-labelled fatty acids into the animal tissues and its retention (at least in the gut). It is also interesting that the highest ratio of the characteristic peaks in methyl palmitate was detected 1 day after the intake. This might result from partial ingestion of some of the feed diets by 3 hours (complete ingestion being likely to be after one day of intake). The detected change in the isotopic ratio of methyl stearate (C18:0) could only result from a conversion of the given labelled C16:0 to C18:0 and therefore provides strong evidence that this chain elongation occurs within the worm tissues and implies an ability of the worm to synthesise stearic acid (C18:0) as has been observed in all organisms. A change in the isotopic ratio of the $^{13}C/^{12}C$ of methyl EPA (C20:5n3) is also of interest as the presence is correlated to the high net gain of EPA in the net gain or loss experiment.

However, this was only a preliminary investigation with one replicate per treatment and further replicated experimentation was required. Some technical limitations and difficulties were also made apparent by this preliminary experiment. For example, other intermediate metabolites in the pathway had shown a marked fluctuation in the isotopic ratio. This result may be caused by a low number of samples and the

variability of the sample. An increase in the number of the samples at each time point would resolve this limitation.

Furthermore, it was not possible to detect any changes in some fatty acid metabolites on the mass spectra. A possible explanation for this might be that the worm may not get enough of certain fatty acids to maintain their steady states of equilibrium; therefore, a repeated labelling feeding was introduced to the experiment for developing the methodology in a more robust investigation. An application of the repeated-feeding experiment should resolve the problem by providing enough food to the worms and the worm can then be in the normal 'unstressed' steady state.

In the case of the actual repeated feeding experiment, any changes in the $^{13}C/^{12}C$ ratio of the interested characteristic peaks is the key parameter to trace and imply the ability of *N. virens* to synthesis some PUFAs. The underlying assumption is that any change in the isotope ratio in any fatty acid species other than the given C16:0 species is due to a metabolic conversion that may involve chain elongation and desaturation of the labelled precursor molecule. The simplest interpretation of such changes is that they occur within the worm tissues due to the action of enzymes derived from the worm genome, but it is also possible that the metabolic changes could be driven by commensal organisms. Most of the possible intermediate fatty acid metabolites (see Figure 1.9) were considered and analysed; however, it was not possible to trace changes in the signature of some of the possible fatty acid metabolites due to their presence only in relatively small amounts or, in some cases, due to their apparent absence. Furthermore, the background noise also masked some peak areas of the chromatographs that would be associated with FAME fragment ion peaks of interest.

The statistically significant accumulation of the methyl palmitate (C16:0) in the labelled fed group over the period of the experiment is strongly indicative of the success of an incorporation of the ^{13}C-palmitate into the *N. virens* body as in Appendix B.

In addition, the absence of an accumulation of the methyl palmitate in the normal fed group is in agreement with this finding. This result is very important as it confirms that the methodology adopted was appropriate and sufficiently reliable for further analysis and discussion.

For the methyl stearate (C18:0), the one-way ANOVA and the Dunnett's *t* multiple comparison showed that the $^{13}C/^{12}C$ ratio in the two characteristic peaks of the methyl stearate on day 3 and day 5 after an incorporation of ^{13}C-palmitate increased significantly. This finding can thus be evidence to support the general concept of the fatty acid elongation from palmitic acid (C16:0) to stearic acid (C18:0) in all organisms (Nelson and Cox, 2008).

It is particularly interesting that there were no significant differences in the $^{13}C/^{12}C$ ratio of the McLafferty ion (m/z = 75:74) derived from methyl stearate (C18:0) yet there were significant differences of the $^{13}C/^{12}C$ ratio of the first hydrocarbon series (m/z = 88:87) and the molecular peak (m/z = 299:298). This is particularly telling due to the fact that chain elongation involves the addition of two carbons in the chain at the carboxyl end, and as a consequence the presence of ^{13}C atoms derived from the ^{13}C-palmitate should not be found in the McLafferty ions ratio (m/z = 75:74); the isotopic signature of this entity is not expected to change as was in fact observed. In contrast to the McLafferty ions, the first hydrocarbon series (m/z = 88:87) and the molecular peak (m/z = 299:298) should contain some ^{13}C atoms if chain elongation has occurred. These findings can therefore be taken as positive evidence and corroborate the concept of how to select certain key ions in order to trace the metabolic pathway of chain elongation and desaturation if evidence is found that this occurs in the worm tissues. Also, these results are in agreement with the general concept that an elongation mechanism normally involves insertion of two carbons at the carboxyl end of the fatty acids as generally accepted (Nelson and Cox, 2008; Berg *et al*., 2012).

It is interesting that there were no changes observed in the isotope ratios of oleic acid (C18:1n9) and linoleic acid (C18:2n6) in this experiment even though changes in some of the supposedly 'downstream' entities was observed as described below. It is difficult to explain this result, but it might be related to the presence of some of these two fatty acids in the feed pellet (see Figure 2.5) which may have diluted the signal in the worm tissues due to uptake of unlabelled fatty acid. These fatty acids levels may have reached the equilibrium state required to supply sufficient titres for their needs. Another possible explanation is that *N. virens* may synthesis these two fatty acids briefly and may not deposit them in their body tissues. However, it was not possible to distinguish between these two alternative explanations.

It is also interesting to note that it was not possible to detect two FAMEs; methyl α-linolenate (C18:3n3) and the methyl γ-linolenate (C18:3n6). This finding could be explained either because these two fatty acids not being present in the samples as the worms were immature or they were present but quantities below the detection threshold. In order to clarify this, the threshold level in the ChemStation program was modified to increase the ability to detect peak areas; however, it was still not possible to detect these fatty acid species. Also, the noise background could be interrupted to get precise peak areas of these two fatty acids as seen in Figure 3.7.

Another important and interesting finding was that there were significant changes in the $^{13}C/^{12}C$ of the α-ion for methyl arachidonate; ARA (C20:4n6) on day 3 and day 5 in the labelled fed group. This result can be said to certainly confirm the capability of *N. virens* to synthesise arachidonic acid (C20:4n6). Although, there is some arachidonic acid content in the pellets, the synthesis of this fatty acid could still be detected. One conclusion that may be drawn from this finding is that arachidonic acid is essential for the worms as a ready source in the conditions in which they were maintained in this experiment.

Besides its functions in relation to phospholipid fluidity, arachidonic acid (ARA) is one of a number of very important metabolites in several marine invertebrates as a precursor of hormones and chemical signaling molecules such as eicosanoids. According to the review by Stanley (2000), eicosanoids, such as prostaglandins, play important roles in the immune system, reproductive physiology and ion transport in several invertebrates. It is generally accepted that arachidonic acid (C20:4n6) is utilised as a major precursor to synthesise further eicosanoids and their derivatives as reviewed by Stanley-Samuelson (1987), Stanley-Samuelson (1994), Stanley and Howard (1998) and Stanley (2000). The eicosanoid biosynthesis involves three different pathways: i) the cyclooxygenase pathway which produces prostaglandins; ii) the lipoxygenase pathway which generates leukotriene and lipoxin; and iii) the epoxygenase pathway which produces some active epoxy compounds as illustrated in Figure 3.8. Prostaglandin derived from the cyclooxygenase pathway could be an important eicosanoid in the polychaetes. For example, Meunpol *et al*. (2005) and Meunpol *et al*. (2010) reported that *Perinereis* sp. contains a high level of prostaglandin E_2 (PGE_2) which could affect oocyte maturation in the shrimp *Penaeus monodon* when they are given food containing nereid worms. These studies also proposed that an increase of PGE_2 as the worm matures could function as a reproductive hormone. Similar to these

findings in *Perinereis* sp., investigations of *A. marina* by Bentley *et al*. (1990) and Bentley and Hardege (1996) have also suggested a reproductive function of arachidonic acid (ARA) together with dihomo-γ-linoleate (C20:3n6) which can act as an oocyte maturation hormone. It is therefore likely that an increase of the ARA during the experimental period could be involved as a synthesis need for a precursor source in eicosanoid biosynthesis to produce prostaglandin E_2.

In the case of methyl eicosapentaenoate; EPA (C20:5n3), there was a significant increase of this fatty acid only in samples obtained on day 1 after provided with the labeled food compared with the control start samples. This result may be explained by the fact that the worms may be in a starvation state during the acclimatisation period prior to the experiment. An inadequate EPA titre could lead to urgent EPA biosynthesis to provide enough long-chain polyunsaturated EPA for maintenance of their equilibrium states. EPA may be incorporated directly to phospholipids to control the fluidity or could also act as a precursor but may be less important than ARA due to its role in the eicosanoid biosynthetic pathways (Stanley, 2000). Further indication is that *N. virens* may have the ability to synthesise EPA from the given precursor ^{13}C-palmitate. The biochemical approach would clarify the idea that some of the polychaetes may be able to generate some of the important fatty acids like EPA under certain conditions.

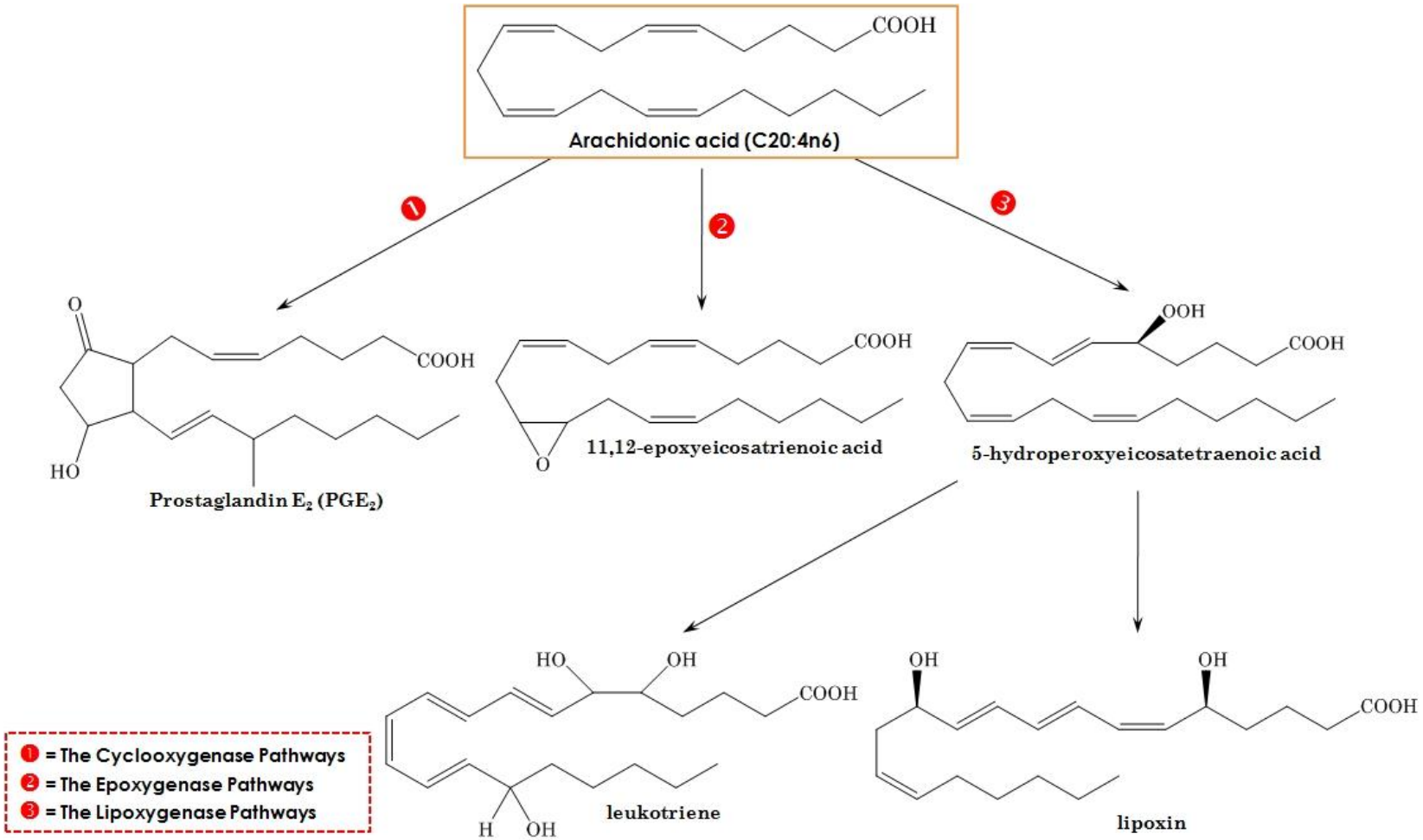


Figure 3.8: An overview diagram of the eicosanoid biosynthesis pathways based on a mammalian background. Arachidonic acid (C20:4n6) is a major precursor in these representative pathways. Three different sub-pathways to produce different products have also been specified (adopted and redrawn by author from Stanley, 2000).

In contrast to EPA, there was no significant increase of the α-ion of methyl docosahexaenoic acid (DHA, C22:6n3) in any groups compared with the control start group. This finding of low DHA levels in the body contents seemed consistent with several studies on polychaetes such as *A. marina* (Olive *et al*., 2009), *N. diversicolor* (Luis and Passos, 1995) and *N. virens* (Duangchinda, 2009). DHA does not appear to be an important metabolite for polychaetes. However, the failure to detect any evidence for biosynthesis of this fatty acid may also be due to the presence of some DHA content in the feed pellet, which may have had an effect on the result; hence caution must be applied, as the findings might not be transferrable to the idea that DHA is not important for their needs. The worms might get sufficient DHA from their food or their diets in nature.

Furthermore, temperature effects on phospholipid composition and membrane fluidity are widely accepted (Becker *et al*., 2012). For example, the deep-sea hydrothermal vent polychaete species *Paralvinella palmiformis* has a greater degree of unsaturation at 17 °C than at 30-40 °C (Taghon, 1988). A lower temperature can increase the content of the unsaturated fatty acids to maintain the fluidity of the membrane. The temperature in this experiment was set at 12 ± 0.1 °C which was similar to the wild condition. Therefore, the worm may produce EPA only during the first day to maintain equilibrium of the membrane fluidity after starvation during the acclimatisation process. After reaching the equilibrium state, the generation of EPA might not be necessary and *N. virens* may switch their energy to other metabolic processes.

The following conclusions can be drawn from this study:

1. *N. virens* can elongate palmitate (C16:0) to stearate (C18:0) from the carboxyl end as in most multicellular eukaryotes;
2. *N. virens*, in the conditions of this experiment, have also shown the ability to synthesise certain long chain unsaturated fatty acids including ARA (C20:4n6) and EPA (C20:5n3).

Chapter 4

General Discussion

4.1 *Potential PUFA Biosynthetic Pathways in the Lugworm (Arenicola marina) and the Ragworm (Nereis virens)*

In order to understand the wider significance of the results presented in this thesis, the general view of aerobic PUFA biosynthesis in eukaryotes is illustrated again in Figure 4.1. The bioaccumulation experiments in Chapter 2 provide circumstantial evidence of PUFA biosynthetic ability in the two worm species *A. marina* and *N. virens*. Further investigations using a ^{13}C stable isotope labelling approach presented in Chapter 3 provided more direct evidence and also corroborated the idea that pathways for PUFA biosynthesis through chain elongation and desaturation exist in *N. virens*. The combination of the results presented in the two chapters enhance understanding of PUFA biosynthetic pathways in polychaetes, provides additional information about invertebrate PUFA biochemistry, and provides further evidence for PUFA biosynthesis in marine invertebrates. In this section, the results will be discussed in more detail, treating each species separately.

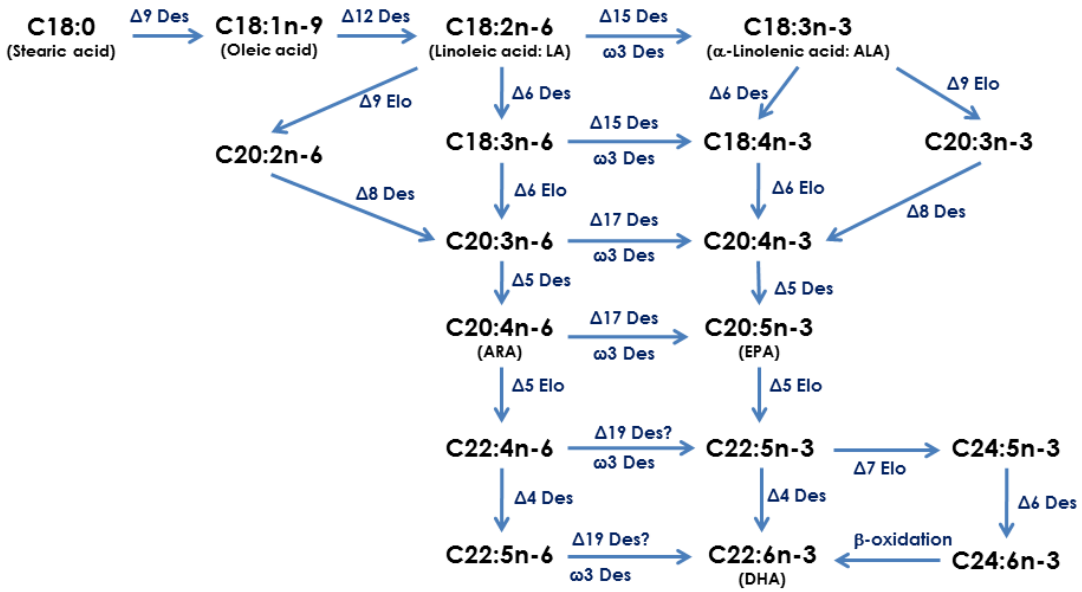


Figure 4.1: An overview of general aerobic PUFA biosynthetic routes in most eukaryotes. Each enzyme family is labelled in each reaction step. (Des = desaturase; Elo = elongase)

4.1.1 The Potential PUFA Biosynthetic Routes in the Lugworm (Arenicola marina)

The bioaccumulation, or more precisely the observed net increase in the tissue concentration, of certain PUFAs observed in specimens grown in the closed system boxes, as described in Chapter 2 (see Figure 2.8), implies PUFA biosynthesis. However, this observation does not provide conclusive evidence that it is the worms themselves that are responsible for the biosynthesis, nor does it provide direct evidence of the pathways involved. In a similar study, Olive *et al.* (2009) examined the entire spectrum of fatty acids present in lugworm tissues and calculated any changes in concentration. On the basis of this indirect evidence, they concluded that a pathway involving the ω9 elongase-Δ8 desaturase pathway (see Figure 4.1) was likely to be involved. Since one of the end products that accumulate in the worm tissue is EPA and this is produced by some marine bacteria, it is possible that symbiotic bacteria present in the lugworm culture system may have been the source of the fatty acid. However, the presence of intermediates for the ω9 elongase-Δ8 desaturase pathway suggested that there may be direct biosynthesis. Ashforth (2008) and Ashforth *et al*. (2011) carried out a study of the bacterial communities in the sand of the same type of closed box recirculating systems subject to bioturbation by *A. marina*. They found evidence that certain bacteria do form the most likely ingested food source following decomposition and breakdown of the given food (Ashforth, 2008; Ashforth *et al*., 2011) but the main species involved were not members of PUFA synthesising bacterial clades. Moreover, these studies found that possible PUFA synthetic bacteria were not abundant in the system, and the results therefore indicate that such bacteria are not likely to contribute significantly to PUFAs accumulating in the worm tissue. Thus, in order to interpret these results it is necessary to focus on the possibility of a within-tissue biosynthetic pathway in polychaete worms as typified by the lugworm and the ragworm species. Duangchinda (2007) and Olive *et al.* (2009) carried out a detailed analysis of the fatty acid profiles associated with the organic sediments before and after bioturbation and did not find evidence for a substantial population of any other PUFA containing microorganisms, which again tends to caution against the concept that the fatty acids are ingested and lending weight to the concept that the worms do indeed carry out the chain elongation and desaturation. In order to discuss the potential pathway, each reaction step will be explained sequentially from the simple substrate towards long-chain PUFA products. It should also be noted that in the case of *A. marina*, the hypothesised pathway will be based mainly on the evidence in Figure 2.8, Chapter 2.

In the case of the saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0), a net gain of both was observed within the system boxes. These results suggest that the worms are able to synthesise palmitic acid and stearic acid. This interpretation is in accord with the concept that all organisms can synthesise both palmitic acid (C16:0) and stearic acid (C18:0) using fatty acid synthase (FAS) (Nelson and Cox, 2008). The observation that a net bioaccumulation of oleic acid (C18:1n9) also occurred within the boxes provides evidence that desaturation by ∆9 desaturase also occurs in *A. marina*, as reported for other animals (Parrish, 2009). Similarly, a net gain of palmitoleic acid (C16:1n7) was also observed over the experimental period. This result implies that ∆9 desaturase might also be active and is able to add a double bond between the C_9 and C_{10} atoms in palmitic acid (C16:0). The addition of a double bond in the C_9 position of palmitic acid to generate palmitoleic acid is commonly found in nature and has been reported for vertebrates, including humans (Ntambi, 1999; Paton and Ntambi, 2009; Guillou *et al*., 2010).

It is interesting to note that there was a moderate gain of *cis*-vaccenic acid (C18:1n7) within the system. *Cis*-vaccenic acid has been considered to be a biomarker that is derived mainly from bacteria. However, it may be difficult to determine the exact origin and source of *cis*-vaccenic acid. In one study the source of *cis*-vaccenic acid had been traced to endosymbiont bacteria, such as bacteria in the ruminant digestive gut (Or-Rashid *et al*., 2007). Among marine invertebrates, a study of the bivalve mollusc *Solemya velum* has shown that the presence of *cis*-vaccenic acid (C18:1n7) in the tissue is almost certainly derived from symbiotic bacteria. It has therefore been suggested that this fatty acid could be used as a biomarker for fatty acid derivation from symbiotic bacteria (Conway and McDowell Capuzzo, 1991). Also, a comparison study between the bacteria-symbiotic brittle star species *Ophiothrix fragilis* and ophiuroid echinoderm *Amphiura chiajei* and the non-bacteria-symbiotic species *Ophiocomina nigra* has shown a marked difference in terms of *cis*-vaccenic acid content. This was interpreted as being an indication of the contribution of symbiotic bacteria to the *cis*-vaccenic acid production (McKenzie *et al*., 2000). Returning to discuss the situation in *A. marina*, two possibilities exist for the production of the moiety of this fatty acid species; it may have a bacterial origin, either from endosymbiont bacteria in the worm gut itself or from the stimulation of bacterial growth by *A. marina*, i.e. bacterial gardening, as postulated by several authors and confirmed by Ashforth *et al* (2011); or, it may be derived from the isomerisation process from oleic acid (C18:1n9). This has been revealed by studies of several plant species such as kaki fruit (*Diospyros kaki*) and brassica species (Shibahara

et al., 1990; Barthet, 2008). Among animal studies, a deuterium labelling study of a cultured human leukemia cell line (MOLT4) demonstrated the presence of both labelled and non-labelled oleic acid (C18:1n9) and *cis*-vaccenic acid (C18:1n7) derived from [2,2 $^{2}H_{2}$] labelled palmitoleate (C16:1n7); this indicates the possible existence of an isomerisation process (oleic acid precursor) and elongation process (using palmitoleate as a precursor) (Shibahara *et al*., 1997). This pathway has also been indicated as a possibility for generation of *cis*-vaccenic acid from palmitoleic acid by an elongation process in several other studies (Holloway and Wakil, 1964; Shibahara *et al*., 1989). In a recent study, heterologous expression of a rat elongase gene in yeast has also shown the ability of this enzyme to convert palmitoleic acid to *cis*-vaccenic acid (Yazawa *et al*., 2011). Further investigations would therefore be required to resolve and clarify the exact origins of *cis*-vaccenic acid accumulating in *A. marina*. The possible origins of *cis*-vaccenic acid by way of three different routes in *A. marina* is summarised in Figure 4.2.

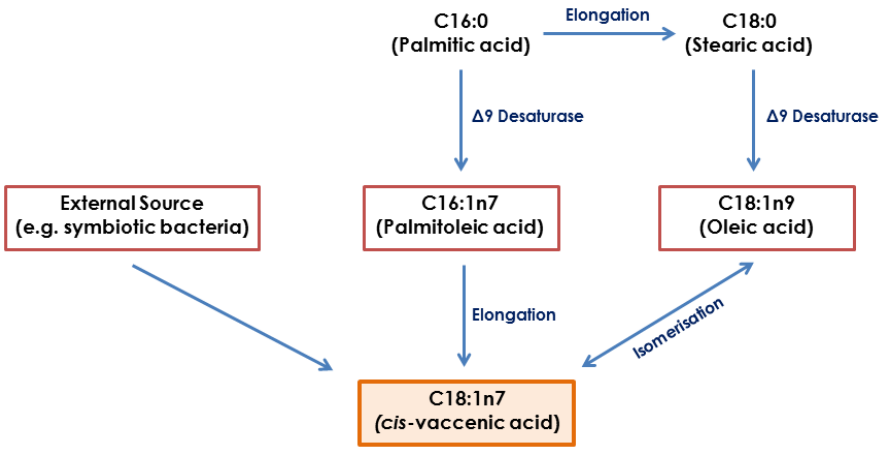


Figure 4.2: The possible origins of *cis*-vaccenic acid in *A. marina* tissues by three different routes: external source contribution, elongation of palmitoleate (C16:1n7) and isomerisation of oleic acid (C18:1n9).

It should be noted that *cis*-vaccenic acid is a major fatty acid in several species of polychaetes as revealed by analysis of their fatty acid content, and clarification of these possible biosynthetic routes is necessary for an evaluation of this finding. On the one hand it may be an indication of bacteriophagy/coprophagy, as this finding is sometimes interpreted e.g. by Olive *et al.*, (2009), but may on the other hand reflect the physiological importance of this fatty acid in polychaetes and the consequent existence of pathways for its biosynthesis. Further investigation is still required to clarify the position in relation to possible PUFA biosynthetic pathways.

In the case of the 'essential' fatty acids, linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3), there was a net gain of these fatty acids over the experimental period. The accumulation of these two fatty acids indicates the ability of *A. marina* to generate these so called 'essential' fatty acids. Although most animals are not able to synthesise these two fatty acids, a few animals have been shown to have the ability to produce them. Terrestrial insects in particular, as described in Chapter 1 and in references cited therein, have been found to have this capability. It is interesting to note that the net gain in *A. marina* was notably greater in the case of linoleic acid in comparison with α-linolenic acid; this result might imply the preferential utilisation of the linoleic acid (C18:2n6) and n6 series rather than α-linolenic acid and n3 series (C18:3n3) to produce PUFA. This finding would seem to be consistent with a recent molecular study on an octopus, *Octopus vulgaris*, which revealed the preferential use of n6 fatty acids over n3 fatty acids (Monroig *et al*., 2012). However, further experiments on molecular biology aspects on differential desaturase activities would still be needed to confirm the preference. According to the results and discussions at this point, the potential PUFA biosynthetic pathway from palmitic acid is shown in Figure 4.3.

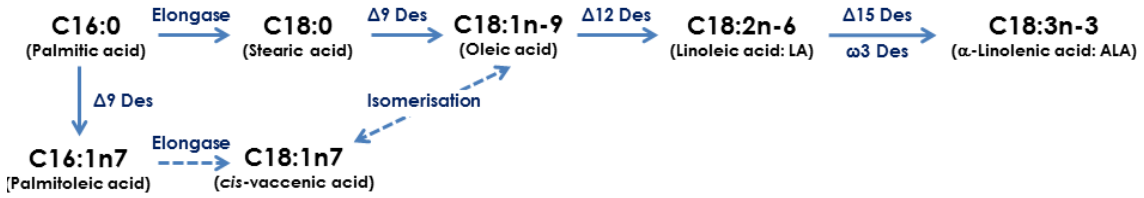


Figure 4.3: The potential biosynthetic pathway in *A. marina* from palmitic acid to 'essential' fatty acid precursors. Dotted lines are used for biosynthesis steps where further investigations are still required for confirmation.

The 'downstream' biosynthesis pathways from linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) to further long-chain fatty acids is also important for an understanding of biosynthetic pathways leading to PUFA. In order to clarify the processes involved the conventional n6 fatty biosynthetic series and the n3 series are discussed separately.

In the case of the n6 series, linoleic acid can either be Δ6 desaturated to γ-linolenic acid (C18:3n6) followed by elongation in the 'conventional' pathway or alternatively it can be first elongated to eicosadienoic acid (C20:2n6) and then Δ8 desaturated to give C20:3n6; this is described as the 'alternative' Δ8 pathway. It is therefore necessary to consider the extent to which there is a net gain of either

eicosadienoic acid (C20:2n6) or γ-linolenic acid (C18:3n6) in *A. marina* tissues to determine the most likely PUFA biosynthetic pathway. In this study, a net gain of both C20:2n6 and C18:3n6 was observed within the system after 60 days of experiment. However, there was a relatively small gain in C18:3n6 (less than 0.05 g per system box, see Figure 2.8) compared with that for C20:2n6 (around 0.10 g per system box). This finding is somewhat different from the results of Lytle *et al.* (1990), Duangchinda (2007) and Olive *et al.* (2009) in that they could not detect the presence of C18:3n6 in *A. marina* samples. This discrepancy may be due to the different diets and experimental conditions, although further investigation is required for confirmation. In all these cases however, it appears that the alternative Δ8 pathway is the more dominant one, but the evidence presented here suggests that *A. marina* may use either the 'conventional' Δ6 or the 'alternative' Δ8 routes to synthesise EPA although the 'alternative' route seems to predominate.

In evaluating which pathways are active in these polychaete worms, the absence as well as the presence of an intermediate fatty acid is important. Therefore, the absence of most of the fatty acids that are intermediates in relation to the conventional n3 fatty acid biosynthesis series: C18:4n3, C20:3n3 and C20:4n3 is important. This finding is consistent with the fatty acid profile study by Duangchinda (2007) who also found that these n3 intermediate fatty acids were not present in either wild or cultured *A. marina*. This result is also in agreement with the fatty acid profile study by Lytle *et al*. (1990) with an exception that the same authors found a trace amount of C20:3n3 in the tissue samples. A possible explanation for this difference may be that the worm samples in the study by Lytle *et al*. (1990) were purchased from a commercial company and would have been subject to different diets and culture conditions from those used in this study. These results all imply that *A. marina* does not utilise the conventional n3 series pathway to generate long chain PUFA including EPA (C20:5n3) and this pathway could therefore be possibly ruled out of the potential biosynthesis pathways. The absence of these n3 fatty acids also rules out the 'cross-links' between the n6 series and n3 series.

Taking into account all of these results, Figure 4.3 is extended in Figure 4.4 to represent the likely pathways for PUFA production in *A. marina*. Although eicosatrienoic acid (C20:3n6) was not detected in this experiment, this fatty acid is known to be essential to *A. marina* in terms of reproductive biology since this fatty acid causes the initiation in spawning and sperm maturation in males (Bentley *et al*., 1990; Pacey and Bentley, 1992). The spawning season of most European populations of *A.*

marina (including the local populations) is in the autumn period (Howie, 1959; Howie, 1961; Frank and Berghuis, 1979) which was rather different from the collection time in June in the net gain experiment. Consequently, the failure to detect eicosatrienoic acid in the experimental samples may be due to the fact that they were not collected during the spawning period.

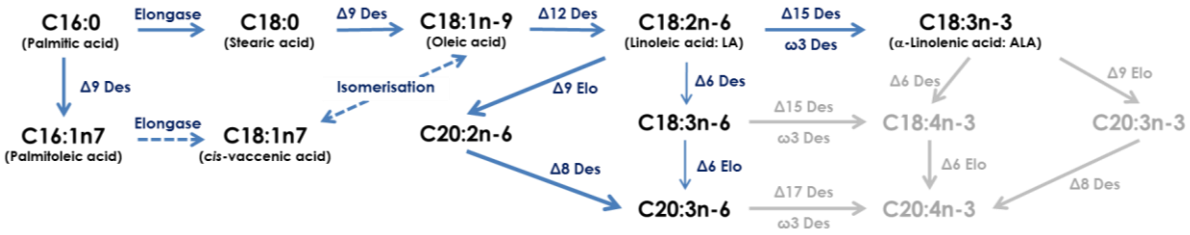


Figure 4.4: The possible routes following either the 'conventional Δ6' or the 'alternative' pathways to produce EPA in *A. marina*. The thicker lines in the 'alternative' pathway indicate the preference of this pathway over the 'conventional' route. The absence of n3 fatty acid intermediates may rule out the possibility of the utilisation of n3 series routes.

The absence of all C_{22} fatty acid intermediates (C22:4n6 and C22:5n6) and DHA (C22:6n3) in samples of wild-caught specimens and those transferred to the experimental system (as described in Chapter 2) implies that the worms may not be capable of, or have only very limited ability to generate DHA. In contrast to this there were clearly net gains of two important fatty acids: ARA (C20:4n6) and EPA (C20:5n3). These results indicate that *A. marina* may use and produce EPA as the end product of their PUFA biosynthetic pathways. In this case, ARA (C20:4n6) is Δ5 desaturated to EPA (C20:5n3). These findings are consistent with the studies by Duangchinda (2007) and Olive *et al*. (2009) as these authors also failed to detect C22:4n6 and C22:6n3 in samples from the closed *A. marina* culture system. Furthermore, the absence of C_{24} fatty acids such as C24:5n3 and C24:6n3 would be an indication that they are not able to synthesise DHA by means of the 'Sprecher' pathway as can be found in mammals, including humans. A modified PUFA biosynthetic diagram is shown in Figure 4.5. It should be noted again that these hypothesised PUFA biosynthetic pathways in *A. marina* are discussed primarily from the net gain results in Chapter 2. Further investigations using biochemistry and molecular approaches would still be required in order to determine the exact PUFA biosynthetic pathways in certain conditions.

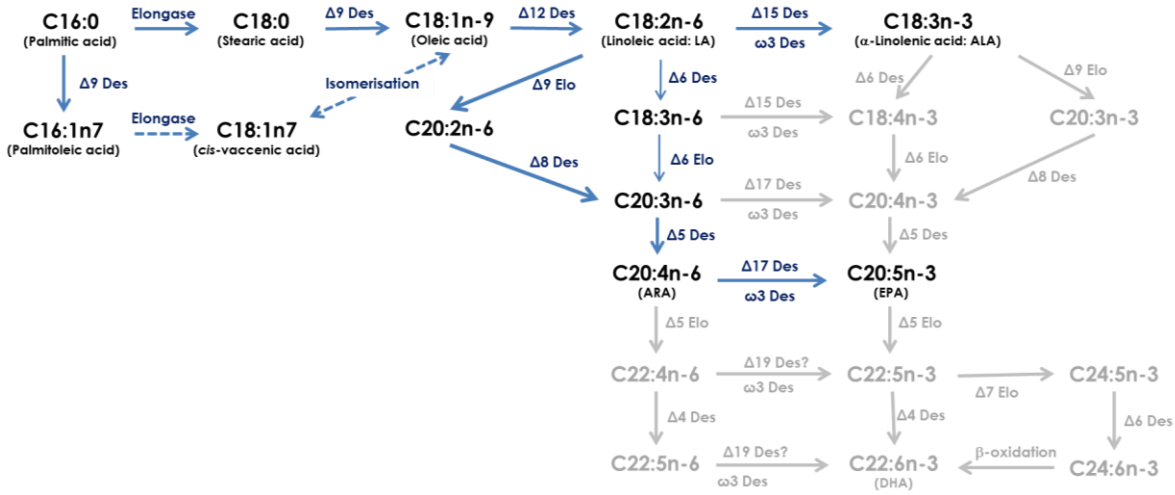


Figure 4.5: The possible EPA biosynthetic routes from palmitic acid (C16:0) precursor in *A. marina*, mainly based on the results in Chapter 2.

4.1.2 The Potential PUFA Biosynthetic Routes in the Ragworm (Nereis virens)

The combination of the results from Chapter 2 and Chapter 3 provides a clearer insight of the PUFA biosynthetic pathways in the case of *N. virens*. The two sets of experiments provide convincing evidence that there was a net gain of the PUFAs, ARA and EPA, within the worm tissues. However, tracing the pathways involved requires a systematic evaluation of the evidence for each fatty acid. There were some differences between the conditions in the stable isotope experiment as described in Chapter 3 and those of the growth experiments described in Chapter 2; these differences might had led to small differences in results. Nevertheless, the two experimental approaches allow further clarification and, importantly, provide more direct evidence of the ability of this species to synthesise certain long-chain PUFA and the pathways involved can be determined more clearly. In order to discuss the PUFA biosynthetic pathways in *N. virens*, a similar structure of the discussion will be adopted as in the previous section for *A. marina.*

In Chapter 2, it was shown that there was a net gain in the system of both of the saturated fatty acids: palmitic acid (C16:0) and stearic acid (C18:0) after subtracting the fatty acid contents in feed pellets and allowing for those present in the worms at the start of the experiment. The accumulation of these fatty acids confirms that *N. virens* is able to bring about *de novo* synthesise of these fatty acids as normally occurs in all eukaryotic organisms. The amount of stearic acid (C18:0) gained within the system boxes in the Chapter 2 experiment was however relatively small. Nevertheless, the stable isotope results also provide support for the concept that *N. virens* has the ability

to generate this fatty acid. The statistically significant changes in the $^{13}C/^{12}C$ ratio of some key peaks in terms of the stearic acid (C18:0) mass spectrum, three days after the addition of labelled food pellets, further corroborates of the general concept that all eukaryotes can elongate palmitic acid (C16:0) to stearic acid (C18:0) (Nelson and Cox, 2008; Voet and Voet, 2011).

The monoenoic acids; palmitoleic acid (C16:1n7), vaccenic acid (C18:1n7) and oleic acid (C18:1n9) were all accumulated within the system boxes as described in Chapter 2. Vaccenic acid (C18:1n7) and oleic acid (C18:1n9) were dominant, with similar quantities, in terms of absolute amount being gained, among the spectrum for the various fatty acids. This result is clearly compatible with the observations of Bischoff *et al*. (2009) who demonstrated that the tissue concentrations of both vaccenic acid (C18:1n7) and oleic acid (C18:1n9) were high in *N. diversicolor* both in wild-caught and in cultured specimens. These fatty acids were also found to be always present in the related polychaete species found in the tropical worm, *Perinereis nuntia* (Limsuwatthanathamrong *et al.,* 2012). These findings can also be taken as evidence that these polychaete worms are able to synthesise oleic acid (C18:1n9) as has generally been found for all eukaryotes (Nelson and Cox, 2008; Voet and Voet, 2011). Although significant changes in the $^{13}C/^{12}C$ ratio of characteristic peaks in oleic acid (C18:1n9) were not observed in the stable isotope experiment, this might be a consequence of the substantial amount of oleic acid present in the feed pellets (as shown in Figure 2.5); this was likely to be sufficient to maintain the steady state of this fatty acid in worm tissues without further biosynthesis. Interestingly, it was not possible to detect vaccenic acid (C18:1n7) in the stable isotope experiment; this result may be due to sample dilution prior to GC-MS analysis. Further investigations, particularly using a molecular biological approach, into the origins of vaccenic acid are still required to confirm whether or not the worm itself or symbiotic bacteria are responsible for the production of this fatty acid that is characteristically so abundant in the worm tissue fatty acids.

Moving on from the monoenoic acids, it should be noted that linoleic acid (C18:2n6) was accumulated over the period of the experiment and this result strongly suggests that *N. virens* is capable of generating this dienoic fatty acid. It is also interesting to note that α-linolenic acid (ALA; C18:3n3), one of the 'essential fatty acids' and precursor of n3 series, was lost both in the fed and unfed groups as described in Chapter 2. This interesting finding is also in agreement with a study of *Perinereis nuntia* in which the authors could not detect ALA in their wild-caught population

samples (Limsuwatthanathamrong *et al.*, 2012). A similar result was also found for *N. diversicolor* in which Bischoff *et al.* (2009) were not able to detect ALA in wild-caught samples but ALA was found in the culture samples. A low level of ALA was also reported by García-Alonso et al. (2008) for *N. diversicolor*. The investigation using the ^{13}C stable isotope approach (Chapter 3) also supports this idea because α-linolenic acid (C18:3n3) was not detected on the chromatogram. Interestingly, although linoleic acid (C18:2n6) was found in the samples, there were no statistically significant differences among the labelled group samples. The explanation for this might be similar to the case for oleic acid since linoleic is also found in the feed pellet (see Figure 2.5); this may have been sufficient for the requirements of the worms during the course of the experiment. In conclusion, the findings in this thesis, along with the other lines of evidence cited, imply that ALA is not an important key fatty acid in nereidid worm species. It is therefore more likely that the worm may utilise the n6 series to generate long chain PUFAs rather than the n3 series, notwithstanding the likelihood that the 'alternative' pathway is of even greater importance as discussed below. To be more certain of this, however, an estimate of the activity of Δ15 (ω3) desaturase is needed to support or refute the ability of the worms to generate ALA from the palmitic acid; this could be achieved using cloning and heterologous expression. In this thesis, the desaturation step from linoleic acid (C18:2n6) to ALA is still questioned as shown in Figure 4.6.

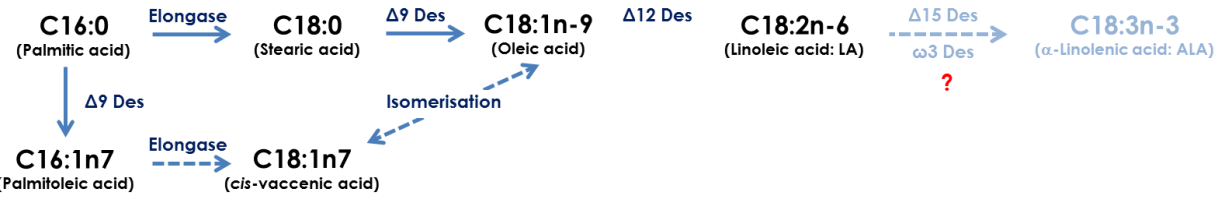


Figure 4.6: The potential biosynthetic pathway in *N. virens* from palmitic acid to 'essential' fatty acid precursors. Dotted lines are used for steps that are considered doubtful and where further investigations are required to confirm or refute the pathway.

Distinguishing between two key intermediates: γ-linolenic acid (C18:3n6) and eicosadienoic acid (C20:2n6), is critical in order to determine the PUFA biosynthetic pathways in *N. virens*. Estimates of the absolute gain of these fatty acids (Chapter 2 Figure 2.9) and the fact that, of the two, only eicosadienoic acid (C20:2n6) can be detected on the chromatograms, both in the wild-caught and the experimental samples, suggests that C20:2n6 is the more important intermediate. This finding is further supported by the stable isotope experiment in Chapter 3 in that eicosadienoic acid

(C20:2n6) was detected but not γ-linolenic acid (C18:3n6). The absence of this γ-linolenic acid has been shown in nearly all studies in polychaete taxa so far, such as, *N. virens* (Duanchinda, 2007; Brown *et al*. 2011), *N. diversicolor* (Garcia-Alonso *et al*. 2008; Bischoff *et al*. 2009) and *P. nuntia* (Limsuwatthanathamrong *et al.* 2012). The absence of this fatty acid is a clear indication that *N. virens* utilises the alternative Δ8 pathway to synthesise long-chain PUFAs. However, the observations presented in Chapter 3 do not provide conclusive evidence of this since no significant differences were observed in the $^{13}C/^{12}C$ ratio of the characteristic mass spectrum peaks of C20:2n6 comparing among the labelled group and between with the unlabelled group within 7 days of the experiment. The failure to detect differences in the isotopic signature of this fatty acid illustrates a difficulty with the experimental approach since the intermediate fatty acids in a pathway may be present only for a very short period and transient changes in the isotope ratio may not have been detected with the sampling times used. Notwithstanding this difficulty, the 'alternative' Δ8 pathway is the most likely PUFA biosynthetic pathway in the case for *N. virens*. The possible PUFA biosynthetic routes in *N. virens* is modified and illustrated in Figure 4.7.

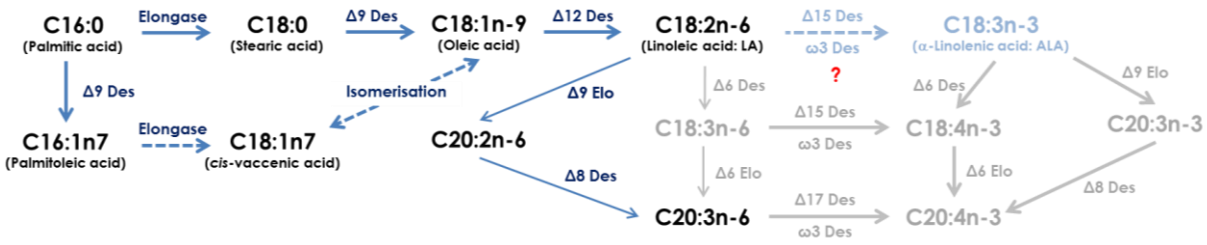


Figure 4.7: The possible PUFA biosynthetic pathways in *N. virens*. Based on the study in this thesis, *N. virens* may use the alternative Δ8 pathway to generate PUFA.

In the case of arachidonic acid (ARA; C20:4n6), there was a relatively small net accumulation of ARA as described in Chapter 2. Interestingly, there was also a significant increase of the isotope ratio in the key peaks of ARA on days 3 and 5 after labelled feed pellets were delivered in the stable isotope experiment. These results strongly indicate that *N. virens* has the ability to synthesise ARA for its own needs and that unlike the intermediate fatty acids in the pathway ARA is accumulated for sufficient time to be detected. ARA could be functioning either in terms of membrane fluidity or as a precursor to synthesise further PUFAs and active eicosanoids as discussed in topic 3.4 .

The relatively large amount of accumulation of EPA at about 0.04 g per system box in the net gain experiment provides strong evidence that *N. virens* is capable of EPA biosynthesis. Furthermore, significant differences of the isotope ratio in characteristic peaks of the EPA mass spectrum on day 1 after delivery of the labelled diets, as described in Chapter 3, provides further evidence that *N. virens* has the ability to produce EPA from a palmitic acid (C16:0) substrate. In all cases where the fatty acid profile has been obtained from studies of polychaete groups, EPA has been found to be one of the most prominent PUFAs within the wide ranges of fatty acids present. For example, EPA is a dominant PUFA in *N. virens* (Duanchinda 2007; Brown *et al*. 2011), *N. diversicolor* (Fidalco e costa *et al*. 2000; Luis and Passos 1995; García-Alonso *et al*. 2008; Bischoff *et al*. 2009) and *P. nuntia* (Limsuwatthanathamrong *et al.* 2012). This could be widened to include deep-sea polychaetes as Drazen *et al.* (2008) reported that two species of polychaetes; *Laetmonius* sp. and *Paradiopatra* sp. have a relatively large amount of EPA compared with other fatty acids present in the worm tissues. EPA is typically found in many groups of marine invertebrates although it is a relative small component compared with DHA (C22:6n3) in some; for example, in a lipid profile comparison study of the lobster (*Homarus americanus*) and the red crab (*Geryon quinquedens*) with marine fish by Budge *et al*. (2002). A study of the lipid profile in a sea star (*Asterius rubens*), a sea urchin (*Echinus esculentus*) and a sea cucumber (*Holothuria forskali*) by Allen (1986) showed that EPA is prominent as well as DHA, both occurring in similar amounts. The relatively high concentration of these two fatty acids (EPA and DHA) is rather different from the pattern found in polychaetes (only EPA in abundance).

In relation to the results presented in Chapter 2, it is interesting to note that there were definite, though relatively small, net accumulations of C_{22} fatty acids such as C22:1n9, C22:5n3 and DHA (C22:6n3) within the system. This result is rather different from those obtained for *A. marina* in which none of these fatty acids were detected on the GC chromatograms. These C_{22} fatty acids derived from *N. virens* samples suggests that these fatty acids may be involved in the DHA biosynthetic route. Furthermore, the absence of C22:4n6 and C22:5n6 in the net gain experiment and the stable isotope experiment suggests that *N. virens* does not utilise the 'microbial' $\Delta 4$ pathway to synthesise DHA. It may be suggested therefore that nereid polychaetes may desaturate C22:5n3 directly to DHA (C22:6n3) albeit in relatively low amounts. Also, the recent study by Brown *et al*. (2011) has shown that DHA levels in *N. virens* fed on fish faecal waste was relatively high; this further supports the concept that *N. virens* has the ability

to produce longer chain to DHA in addition to EPA rather than being restricted to the production of EPA alone as was the case for *A. marina*. The recent study in *N. diversicolor* by Lillebø *et al*. (2012) has shown the presence of both C22:5n3 and DHA (C22:6n3) in all three species. A tropical species, *P. nuntia*, is also similar as DHA can be detected in all farm-raised samples and in wild-caught population, even though only trace amounts of C22:5n3 were found in the samples (Limsuwatthanathamrong *et al.*, 2012). However, direct evidence for this was not found in the present study. The results presented in Chapter 3 did not reveal any changes in the $^{13}C/^{12}C$ ratio in the case of DHA. This may be a consequence of the relatively short duration of the stable isotope experiment or may indicate that in *N. virens*, DHA is not as physiologically important as EPA and is not one of the most needed fatty acids to maintain body functions after the worms have been starved prior to the experiment compared with EPA. For clarification purposes, further research, including a molecular study of Δ4 desaturase activity in *N. virens*, needs to be carried out to establish whether or not it is able to synthesise DHA from EPA. The hypothesised set of possible PUFA biosynthetic pathways by *N. virens* is illustrated in Figure 4.8.

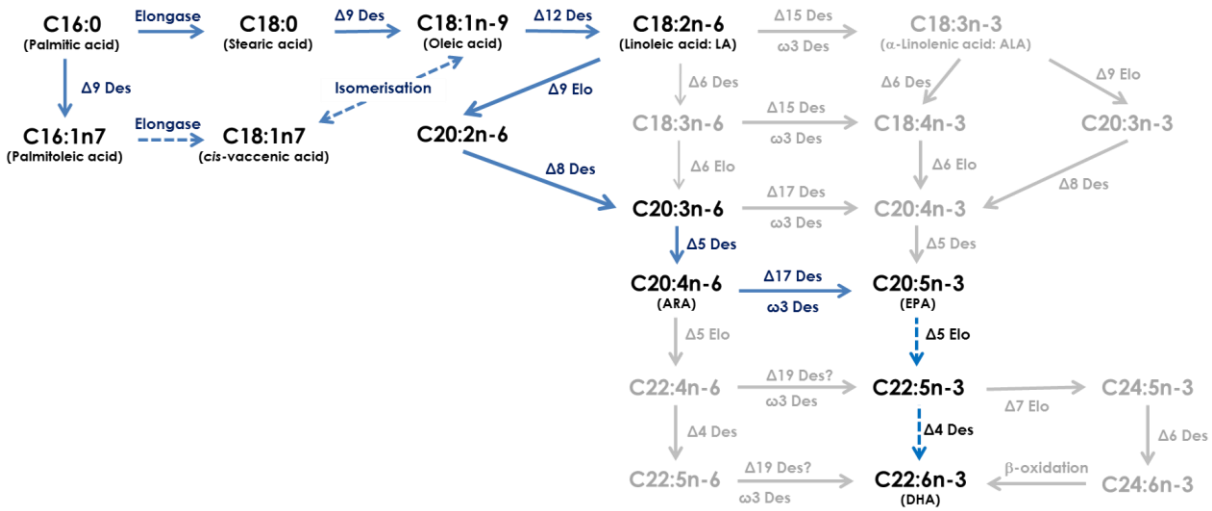


Figure 4.8: The hypothesised possible PUFA biosynthetic diagram in *N. virens* based on the net gain fatty acids in Chapter 2 and the stable isotope experiment in Chapter 3. Dotted line represents where further investigations are required for clarification of the pathway.

4.1.3 Comparison with Selected Invertebrates: Functional and Evolutionary Aspects

The molecular studies conducted by Hashimoto *et al*. (2006) and Hashimoto *et al*. (2008) suggest that despite the desaturases and elongases being highly conserved, substantial functional diversification has nevertheless occurred. The ability to synthesise fatty acids by different group of organisms varies throughout the eukaryotic domain. For example, a recent study of the Δ5 desaturase gene in an octopus (*Octopus vulgaris*), (Monroig *et al*. 2012b) demonstrated that the deduced amino acid sequences are very similar to those of the pacific oyster (*Crassostrea gigas*) at 61% similarity and had 52.2% similarity with the gastropod limpet (*Lottia gigantia*) but there was a low per cent similarity in comparison with other, much less closely related organisms such as thraustochytrids as well as other unrelated invertebrates. A similar study of elongase structure in the same species of octopus also showed a very similar structure in terms of amino acid comparisons among other eukaryotes (Monroig *et al*. 2012). These two studies provide examples that may imply the unity of the desaturase and elongase pattern in each taxonomic clade. Functional/ecological adaptation to a specific environment may be an important driver of the functional diversity of the enzymes within the basic architecture of pathways as shown in Figure 4.1 (Hashimoto *et al*., 2008). Therefore, it may be hypothesised that the observed pattern of fatty acid profiles among different groups of organisms reflects functional/ecological diversification within the core set of pathways. However, further investigations are still required to answer 'why' different groups of eukaryotes employ different mechanisms for PUFA biosynthesis and differ in their fatty acid profiles.

It is currently impossible, and outside the scope of this thesis, to compare the fatty acid biochemistry within all groups of eukaryotes; therefore, a few selected marine invertebrates will be adopted for comparison and discussion. The selected groups of invertebrates are relatively well studied and are selected to represent the major clades of invertebrate phylogeny (Adoutte *et al*., 2000) as shown in Figure 4.9. They are: Parazoa (e.g. sponges), Radiata (e.g octacorals), Lophotrochozoa (e.g. polychaetes), Ecdysozoa (e.g. copepods) and Deuterostomia (e.g. echinoderms).

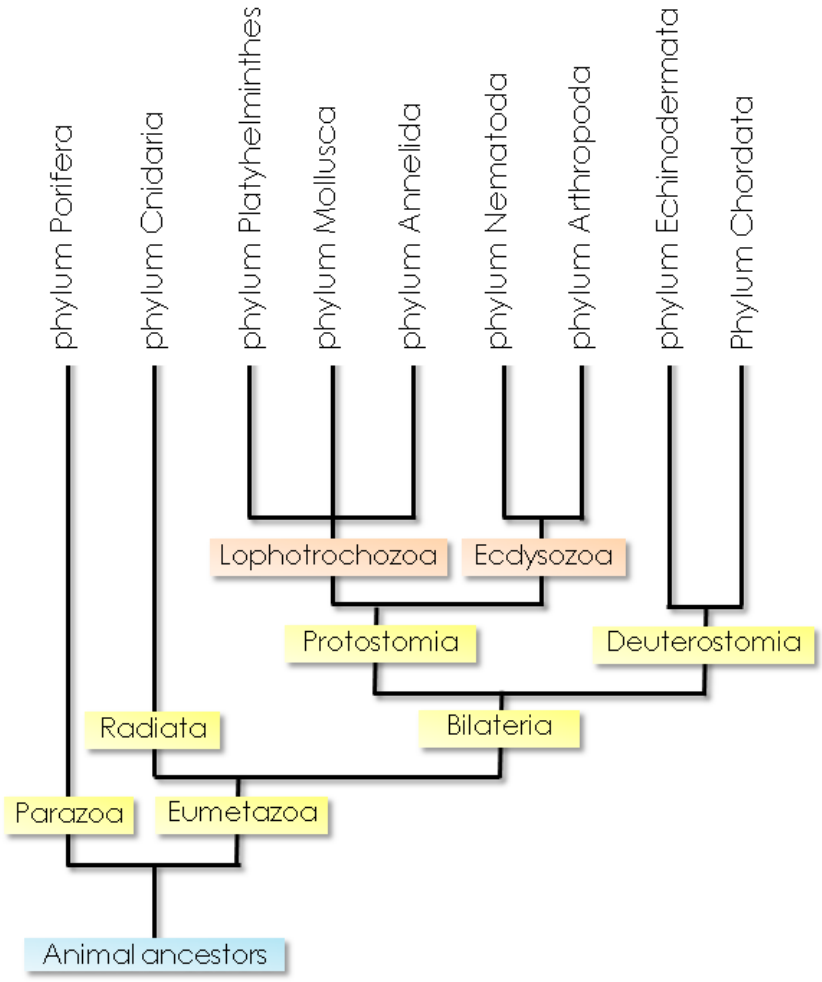


Figure 4.9: The molecular-based metazoan phylogeny. Only some invertebrate phyla are shown in this figure. (Adapted and redrawn from Adoutte *et al*. (2000))

The first group to discuss is that of the marine sponges (Parazoa) which is one of the most primitive groups of multicellular animals. Although most marine sponges do not contain a high concentration of normal methylene-interrupted PUFA (Djerassi and Lam, 1991), they do however, have their own characteristic fatty acids such as demospongic acid (Δ5,9-diunsaturation), other non-methylene-interrupted (NMI) fatty acids and branched-chain fatty acids, all of which occur in this group; as reviewed by Bergé and Barnathan (2005). It was previously thought that demospongic acid was characteristic and found only in the Demospongiae; however, evidence that this fatty acid may not be unique and found only in the Demospongiae has now been obtained; it can be found in some other organisms as reviewed by Bergé and Barnathan (2005) and Komprobst and Barnathan (2010). Notwithstanding the situation as now understood for demospongic acid, marine sponges nevertheless have their own unique NMI fatty acids and branched fatty acids that can be found only in poriferan groups (Bergé and Barnathan, 2005).

In the case for the Radiata, reference will be made to anthozoan cnidarians, particularly octacorals, which are exemplified as being representative of the radiate clades. The octacorals have been extensively studied in terms of comparison between species and analysis of their fatty acid profile; the studied species include *Gersemia rubiformis* (Imbs *et al*., 2006), *Pseudopterogorgia* sp. (Carballeira *et al*. 1996), *Eunicea* sp. (Carballeira *et al*., 1997), and *Heliopora coerulea* (Svetashev and Vysotskii, 1998). In addition, a comparative study between zooxanthellae-free octacoral species, the genera *Acanthogorgia*, *Acabaria*, *Chironephthya*, *Echinogorgia*, *Menella*, *Ellisella*, and *Bebryce*) and zooxanthellae-endosymbiotic octacoral (the genera *Paralemnalia* and *Rumphella*) has been carried out (Imbs *et al*., 2009). It is interesting to note that ARA (C20:4n6) is one of the most dominant fatty acids in these octacorals and that they have a relatively low content of EPA and DHA as observed in all species (both symbiotic and asymbiotic corals). Also, there was the presence of unique tetracosapolyenoic acid fatty acids (C_{24} fatty acids; C24:5n6 and C24:6n3). The authors suggested that octacorals are likely to generate DHA by means of the 'sprecher' pathway via β-oxidation and chain shortening. The high concentration of ARA together with the presence of tetracosapolyenoic fatty acids is characteristic and these lipid profiles can be characterised as representing a chemotaxonomic biomarker in gorgonian corals while C22:5n6 was a marker for Milleporidae corals (Imbs *et al*., 2009; Imbs *et al*., 2010).

In the case of bilateria Lophotrochozoa, marine polychaetes will be used as a representative from this clade. According to this present study, both *A. marina* and *N. virens* have the ability to synthesise and produce EPA as end product of the Δ8 pathway, while *N. virens* and other nereidid species, such as *N. diversicolor*, are able to carry out further chain elongation and desaturation to synthesise DHA but to a more limited extent. Furthermore, even polychaetes found in the deep sea, an ecosystem very different to the euphotic zone, have also been demonstrated to have a relatively high content of EPA, especially in comparison with that in other phyto-detritivorous families (Drazen *et al*., 2008; Würzberg *et al*., 2011). A relatively high content of EPA may be therefore considered as the 'characteristic' and 'marker' fatty acid for marine polychaetes. Further example in a deep-sea gutless polychaete *Ridgeia piscesae*, there were ARA, EPA and lesser extent of DHA presented in the samples (Fullarton *et al*., 1995). The authors suggested that these PUFAs are likely to be derived and transferred from the euphotic zone and the filter feeding larvae then conserve these fatty acids carefully until they reach to adult. A study in the same species by Pond *et al.* (2002) argued that the presence of these PUFAs in the worm tissues may not be derived from

the eutrophic zone; however, it is likely that these PUFAs may originated from chemosynthetic processes from the hydrothermal vent. According to the phylogenetic tree shown in Figure 4.10, *R. piscesae* is considered to be closely related *A. marina* and this present study suggests an alternative possibility that *R. piscesae* may able to *de novo* synthesise and generate long-chain PUFA from simple precursor e.g. palmitic acid (C16:0).

Intense research on zooplankton, particularly in the case of herbivorous calanoid copepods, allows copepod be considered as a representative group of marine ecdysozoa. These copepods are dominant in the polar and high-latitude marine ecosystems (Bergé and Barnathan, 2005). The combination of wax esters, phospholipids and triacylglycerol can be used as the characteristic biomarker for the copepods and these could be used to provide evidence of energetic adaptation between species; however, only fatty acids will be focused on in this discussion. Very high levels of EPA and DHA derived from phospholipids, (accounting for up to 60% of total fatty acids derived from phospholipids), could be one of the most important 'characteristic' fatty acid profiles for the copepods. Also, the presence of C20:1n9 and C22:1n11 monoenoic fatty acid can be considered characteristic for the herbivorous copepods (Sargent *et al*., 1978; Kattner and Hagen, 1995; Bergé and Barnathan, 2005). The strong and clear signal of lipid profile pattern in copepods can be further used as a biomarker between trophic levels (Ederingto *et al*., 1995; Desvilettes *et al*., 1997; Cass *et al*., 2010). A recent study of the copepods, *Calanus finmarchicus*, *C. glacialis*, *Pseudocalanus* sp., *Tisbe furcata* and *Nitokra lacustris* by Parrish *et al*. (2012), lead the authors to suggest that these species of copepods may be able to produce and biosynthesise these fatty acids to maintain their high content of EPA and DHA. This would support the functional diversity of the desaturase and elongase activity.

Echinoderms are taken as a representative group of deuterostome invertebrates. A variety taxa of echinoderms have been investigated by several authors e.g. asterozoans (Kanekiwa *et al*., 1987; Howell *et al*., 2003), sea urchins (Bell *et al*., 2001; Liyana-Pathirana *et al*., 2002) and holothuroideans (Kanekiwa *et al*., 1986; Svetashev *et al*., 1991). These authors all found a similar pattern of lipid profile in all samples and which were very similar to the polychaete lipid profile in terms of having high EPA and low DHA concentrations. A study in a sea urchin, *Psammechinus miliaris*, using deuterium labelling also showed that this species is capable of producing EPA from ALA (C18:3n3) but is not able to generate the longer chain DHA (Bell *et al*., 2001). Another

feature of the echinoderms is their ability to accumulate a high proportion of ARA (C20:4n6) and this high content of ARA together with high EPA and low amounts of DHA can be utilised as a biomarker for echinoderms (Takagi *et al*., 1980).

Taking all of these small examples of invertebrate groups into the interpretation, these findings may lead to the idea that each group of invertebrate has their own 'characteristic' fatty acid profile. Thus, the fatty acid profile may result partially from evolutionary constraints modifying the basic pathway and this in turn may result from selective pressures and adaptive responses in different groups of animals to suit the specific environments. As more data become available, it may become possible to carry out a rigorous analysis using principle component analysis and phylogenetic analysis to determine the relative importance of taxonomic position and ecological position in determining the fatty acid profile of marine invertebrates.

Within the phylum or taxa, different species of invertebrate species may, however, show different fatty acid characteristic pattern showing that divergence and evolutionary changes continue to occur. For instance, the present study of A. marina (this thesis) and the study by Duangchinda (2007) and Olive *et al.* (2009) all suggest that *A. marina* is able to synthesise EPA but not DHA. However, this thesis together with references cited in the earlier section implies that nereidid worms are able to synthesise both EPA and DHA, the latter to a lesser extent.

Although the question 'why' two species of polychaetes show different ability to generate long-chain PUFAs is difficult to answer currently, this thesis will set theories for both evolutionary and ecological views. In terms of the evolutionary aspect, the answer may go back to the Ordovician period (around 400 mya) when polychaetes became diversified and radiated into different clades (Hints, 2000; Hints and Eriksson, 2007). Although polychaete phylogeny has been debated for decades, most studies suggest that *A. marina* (family Arenicolidae) and *N. virens* (family Nereididae) are rather distance in terms of phylogenetic relationship as shown in Figure 4.10 (Almeida *et al*., 2003; Bleidorn *et al.*, 2003; Rousset *et al.*, 2007). This difference possibly hints to their different capability to synthesise PUFA. If the PUFA biosynthesis architecture in Figure 4.1 is primitive, *A. marina* may have retained the ability to generate EPA but lost the ability to further synthesise to DHA during the evolutionary period. A possible explanation may lie within the ecological aspects of their living modes and trophic niche. The ability to synthesise DHA may require more energy expenditure to elongate and desaturate fatty acids regarding to the increased enzyme activity and gene

expression involved. EPA may possibly be essential enough to sustain the survival of *A. marina* while DHA is also required, although with a lesser extent compared with EPA, in the case of *N. virens*. Furthermore, *N. virens* is a well-known omnivorous generalist species; hence, the variety of food ingested may influence their ability to produce DHA. Some of their diets may contain enough DHA; however, the worm may face low-content DHA food within its environment that may change spatially and temporally. The ability to synthesise DHA would, therefore, be retained in *N. virens*. To elucidate this theory, further investigations are very important to be undertaken in order to establish either in evolutionary and ecological viewpoints.

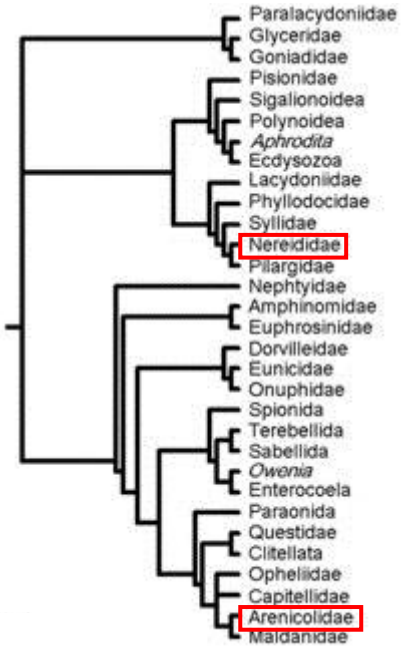


Figure 4.10: An annelid phylogenetic tree from analysis of the positive ordered series (Almeida *et al.*, 2003)

However, functional diversification may be found, even in the same species, in situations where different individuals live in different conditions, as the degree of fatty acid unsaturation in the lipid profile may differ where animals have been living in different temperature regimes. For example, a study of the crab (*Carcinus maenas*) suggests that they can adjust the degree of unsaturation in their fatty acid profile in an acclimation response to temperature (Chapelle, 1978). Such temperature-dependent fatty acid unsaturation has been studied in several marine organisms, for example in the sea scallop (*Placopecten magellanicus*) (Hall *et al*., 2002), Pacific oyster (*Crassostrea gigas*) (Pazos *et al*., 1996), an octopus (*Octopus vulgaris*) (Miliou *et al*., 2006) and even in some deep-sea polychaetes species (Taghon, 1988). These lines of evidence clearly

show that even within the same species, it is possible to demonstrate substantial variation and different activities within the lipid biosynthetic pathways. This would also suggest that these enzymes can be up-regulated or down-regulated in respond to the changes in environmental conditions e.g. temperature, and this may also be possible in response to the PUFA profile of the diet such as a study in *N. diversicolor* (Narciso and da Fonseca, 2000), a brine shrimp *Artemia salina* nauplii (Zhukova *et al*., 1998), a king scallop *Pecten maximus* (Marty *et al*., 1992) and a mussel *Mytilus galloprovincialis* (Pirini *et al*., 2007).

Several biochemistry and molecular studies have indicated that although desaturase and elongase genes named in Figure 4.1 are found throughout the animal kingdom and are conserved throughout the evolutionary history, the different functional diversification and variation in the activities of these enzymes still occurs. Different conditions may lead to diversification of the ability and function of desaturases and elongases as they are important in the control of membrane fluidity. For example, a study of Δ6 desaturase in human liver has revealed that this enzyme can be negatively regulated depending on the presence or absence of C_{18} unsaturated fatty acids (Skrzypski *et al*., 2009). A study in *Lates calcarifer*, a euryhaline marine fish, showed that Δ6 desaturase and elongase gene expression can also be up-regulated (Alhazza *et al*., 2011). This is also similar to a study in the function of Δ5 and Δ6 desaturase in relation to the evolution of jawed vertebrates by Costa *et al*. (2012); these authors suggested that desaturase can be regulated and its expression might be linked to specific habitats and exposure to different environments. The marine nematode and model organism *C. elegans* is also able to regulate Δ9 desaturase activities as can also be found in the studies of humans (Brock and Watts, 2006; Brock *et al*., 2007). These examples all corroborate the concept that desaturase and elongase enzymes can be controlled and regulated and these genes are not constitutively expressed. Further researches required to clarify and determine the unity and the diversity of the PUFA biosynthetic mechanisms - both ecological and evolutionary aspects.

All of this does call into question the assumption that the presence of PUFA necessarily indicates an origin from bacteria, diatom or other protistan sources. The extent to which marine organisms synthesise or recycle fatty acids obtained in their food is complex and should be studied in greater depth as explained in the following section.

4.2 *Implications of Polychaete PUFA Biosynthetic Abilities in Relation to their Roles in Marine Ecology and Aquaculture*

Several physical factors such as salinity, sediment, oxygen availability and temperature could shape diverged habitats within the same estuarine ecosystem; this could also lead to biodiversity of organisms in this complex ecology (Kaiser *et al*., 2005, Dobson and Frid, 2008). In order to discuss to roles of polychaetes according to the findings in this thesis, the relationship between each species will be discussed separately.

Arenicola marina is one of the most well-known marine invertebrates which can be considered to act as an irrigation engineering system within an intertidal zone, especially in relation to the worms cycle of ingestion and defecation (Meysman et al., 2005). This process of irrigation is also an example of the phenomena referred to as 'bioturbation' (Kristensen *et al*., 2012). The animal lives in a J-shaped burrow system in a muddy or sandy substrate ingesting substrate at depth and depositing it later at the surface and periodically driving water into the burrow system. Thus, their burrowing activity and their dwellings lead to the development of special microbial communities by creating oxygen flow within their burrows (Wethey *et al*., 2008; Kristensen *et al*., 2012). Despite the fact that *A. marina* is a non-selective deposit feeder and several food sources might be utilised, recent studies have indicated that bacteria may be particularly important (see Ashforth *et al*., 2011). The microbial communities in the surrounding area might therefore be a potential source of long-chain PUFAs to the worm. However, the analysis of the bacterial communities in a cultured *A. marina* mesocosm (similar in design to that adopted in this study) has suggested that the dominant group of bacteria in the sediment that was the most likely to be used as food, was classified within the Epsilonproteobacteria. This group of bacteria has a similar phylotype, as revealed by a 16S molecular study, to the sulphur-cycling bacteria and they are not known to be able to synthesise long-chain PUFA (Ashforth et al., 2011). This chemoautotroph bacterial group may therefore be a major food source for *A. marina* in the conditions used for the present experiments and as such is not considered to be the likely source of the fatty acids accumulating in the worm's tissues.

Other than bacterial communities, microalgae such as benthic diatoms and other photosynthetic protists are found in the organic rich sands occupied by *A. marina* (Retraubun *et al*., 1996). Though the specific study of PUFA biochemistry of the

lugworm-burrow dwelling microalgae is very limited, the studies by Dunstan *et al*. (1993) and Berge *et al*. (1995) suggest that a diatom, *Skeletonema costatum*, contains high EPA content. In natural ecosystems such organisms could therefore be considered a potential source of EPA. However, both Duangchinda (2007) and Ashforth (2008), in the design of the recirculation systems used, constructed systems in dark conditions in an attempt to exclude the direct influence of photosynthetic organisms. A comparison between fatty acids present in *A. marina* cultured in the 'dark' condition and the 'light' condition did not reveal significant differences in terms of fatty acid content (Olive *et al*., 2009), despite the fact that microalga and other photosynthetic organisms need light as an energy source to drive their energetic metabolic reactions including PUFA biosynthesis. The lack of any differences between the 'dark' and the 'light' system would therefore imply that microalgae are not a necessary source of PUFA for the *A. marina*. In addition, other unusual fatty acids such as n4 series, which are unique to certain algae and diatom (Dunstan *et al*., 1993; Viso and Marty, 1993; and Berge *et al*., 1995), were not present in the worm samples. This present study and Olive *et al.* (2009) also did not find any evidence for the presence of an 'unknown' source of PUFA in the residual sand samples taken from the experimental systems. It is noteworthy that García-Alonso *et al.* (2008) did not find significant differences in the fatty acid constituents of *N. diversicolor* between seasons nor when fed on different diets, which again was taken as evidence that these animals are not directly dependent on photosynthetic cells for their characteristic PUFA. Although most microalgae were not able to grow in the culture, these data must be interpreted with caution because some microalgae are fully capable of mixotrophic metabolism. The stable isotope tracing technique was therefore adopted to the present study.

It is rather difficult to determine specific predators or animals at a higher trophic level compared with *A. marina* as an intermediate level prey, but *A. marina* are consumed by a variety of fish and bird species. However, as explained in topic 1.1.3 Chapter 1, many vertebrates, and in particularly marine fish, do not utilise any ability to desaturate/elongate their fatty acids into longer-chain PUFA such as EPA and DHA when their diets contain enough and/or an excess of 'essential metabolite' fatty acids (Nelson, 1992; Tocher and Ghioni, 1999 and Tocher, 2003). It is widely accepted that marine teleost fish evolved from teleosts adapted to freshwater ecosystems (Pough *et al*., 2011) in which the sources of long-chain PUFAs were less abundant compared with the marine ecosystem (Bell and Tocher, 2009). The ability to convert simple fatty acids into EPA and/or DHA is more necessary for freshwater teleosts rather than marine

teleosts (Bell and Tocher, 2009). The ability of *A. marina* to generate EPA might then be an important PUFA source for many carnivorous fish in marine/estuarine ecosystems where the fish species feeding on the worm populations are primarily marine, not freshwater species. Although the finding in this thesis may not demonstrate the ability of *A. marina* to synthesise DHA, the presence of DHA in higher trophic level predators might result from their generalistic foraging behaviour in which *A. marina* is just one component in their diets. This hypothesis could also apply to the upstream animals in the case of *N. virens* for which the ability to generate EPA and/or DHA from palmitic acid was demonstrated according the results in this thesis.

Nereis virens is considered to be an omnivorous species with a diverse variety of potential foods; therefore, it might be difficult to determine their specific diets in nature. In this specific experiment, the feed pellets were commercially prepared for worm farming and contain a range of different fatty acids (see Figure 2.5). It is known that the animal can be cultured on pellets incorporating fish faecal waste (Brown *et al*., 2011). Thus, the discussion of *N. virens* will be focused on the scavenger aspects of its nutrition. The ability to generate further long-chain PUFA from the used substrate is rather interesting. The faecal waste from animals in higher trophic levels, especially fish, can be modified, upgraded and recycled again when fish predators are fed on *N. virens*.

It should be noted that these data should be interpreted with cautious because the worm samples were collected on specific time and season of the year. They were all in an immature stage, which have only somatic growth. In the mature and reproductive stage worms, the lipid profile in *N. virens* and *A. marina* might have altered because of the changes of their needs for specific fatty acids. For example, the level of C20:3n3 in *A. marina* should be increased regarding its function as the spawning hormone (Bentley and Hardege, 1996). This is rather different from the experiment in Chapter 2, which this fatty acid was not detected on the chromatogram due to the fact that the lugworms were only at an immature stage. According to the results in this thesis, α-linolenic acid (C18:3n3) was only trace amount and cannot be detected in Chapter 3. This may due to the fact that the ragworms were in immature stage which is similar to the results to the reports in García-Alonso *et al.* (2008). Therefore, α-linolenic acid should be found, if the ragworms are in at maturation stage.

The findings in this and the related studies (Duangchinda, 2007; Olive *et al*. 2009; Ashforth *et al*. 2011) raise a question over the general assumption that all PUFAs in marine organisms originate from marine bacteria and/or microalgae (Drazen *et al*., 2008; Drazen *et al*., 2009). In this study, the polychaetes which each play a key role in the middle of food webs might be important in terms of the PUFA origins in the higher vertebrates that feed on them. Hughes *et al*. (2011) have suggested that in addition to macroalgae, a primary consumer e.g. the sea urchins (*Paracentrotus lividus* and *Psammechinus miliaris*) could contribute long chain PUFA to the biogeochemical cycles within marine food webs. Nevertheless, more research on this issue is needed and should be undertaken before this contradiction to general assumptions is more clearly understand.

Because of the finding that both *A. marina* and *N. virens* are now known to be able to produce high levels of EPA (C20:5n3) as demonstrated Chapter 2 and Chapter 3, it is possible to make a preliminary estimate of the amounts that might be contributed to a typical ecosystem containing these worms. The potential amount of EPA production that could be generated may be expressed as an amount per square kilometre per year, by a typical population of worms, and consequently the amount that the worms may contribute to their ecosystem is very interesting and can be estimated to establish their importance as an EPA source to higher trophic organisms. In order to determine the production of EPA in such a way, the annual production of each of the two worm species has been taken from published sources. This information is then adopted to calculate the potential production of EPA by each worm species as two illustrative scenarios. The annual production is estimated using published data on the life histories of the animals. The annual production is preferred for this interpretation rather than the annual standing-crop biomass of the worm because the annual production reflects the population dynamic in relation to biotic interactions e.g. predation and population growth factors (Dolbeth *et al*., 2012). In order to make this estimation, a series of calculations was made as follows. The net gain of EPA within each experimental box in Chapter 2 was converted into g of EPA per g worm wet weight which can be converted by dividing the net amount of EPA gained in the system with the number of surviving worms and worm wet weight gain (weight differences between before and after experiment) for each box. The annual production of each worm in terms of dry weight m^{-2} was converted to wet weight m^{-2} using the same regression equation between the worm wet weight and the worm dried weight described in topic 2.3.4. After that, the amount of EPA gained (g) per g worm wet weight was calculated into g unit per annual

production per m^2 and then into kg per annual production per km^2. Figure 4.11 illustrates the calculation steps.

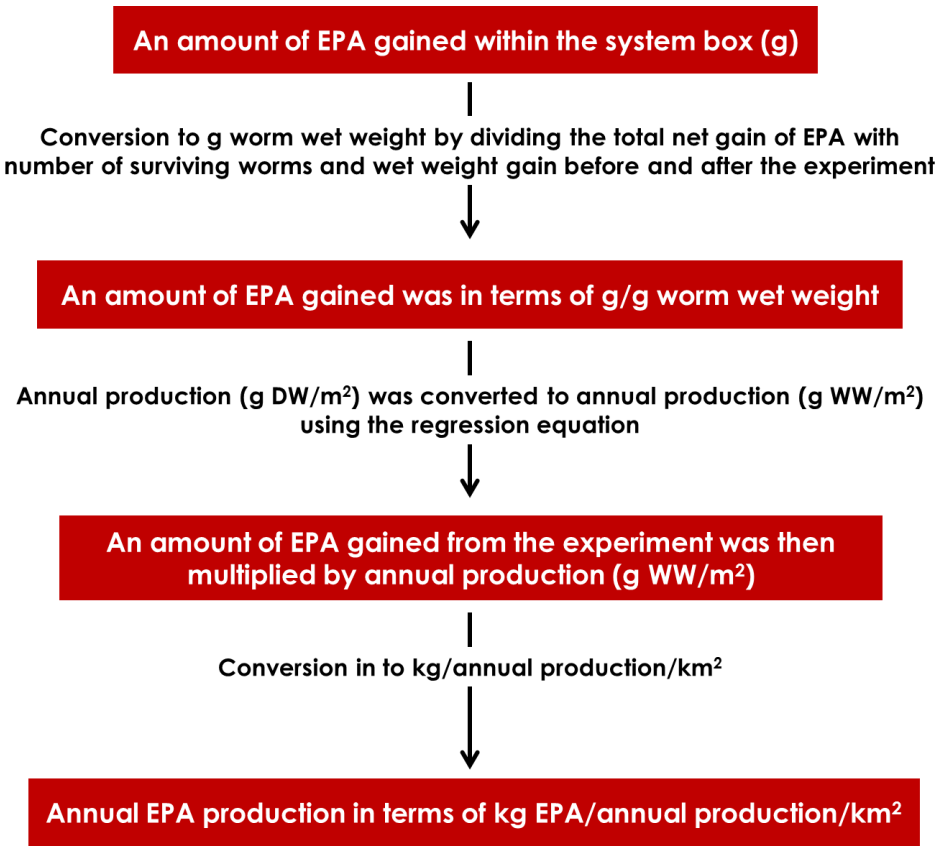


Figure 4.11: The calculation steps of annual EPA production in terms of kg EPA per annual production / km^2 from the amount of EPA gained in Chapter 2.

In the case of *A. marina*, the study by Beukema and De Vlas (1979) provides parameters for a representative natural population of *A. marina* inhabiting in the tidal flats of Dutch Wadden Sea along the coast of Europe and was adopted and exemplified as the scenario of the lugworm. The authors have reported that the production of *A. marina* is at about 5 g/m^2 ash-free DW and this can be converted to 35.904 g WW/m^2 using the regression equation. According to the calculation step and Table 4.1, the EPA production can be estimated at about 244.450 kg km^{-2} y^{-1} or 0.244 g m^{-2} y^{-1} in terms of wet weight.

Table 4.1: Scenario#1 the calculations of EPA gain (kg) / annual production wet weight / km^2 in *A. marina* based on the data from Chapter 2 experiment in this thesis and the study by Beukema and De Vlas (1979) in the Dutch Wadden Sea.

	Net EPA gain (g) / system box	**Net EPA gain (g) / g worm WW**	**EPA gain (g) / annual production (35.904 g WW) / m^2**	**EPA gain (kg) / annual production (35.904 g WW) / km^2**
Box 1	0.072	0.007	0.243	242.777
Box 2	0.105	0.007	0.250	249.928
Box 3	0.180	0.007	0.241	240.643
Mean	0.119	0.007	0.244	**244.450**
SD	0.055	0.000	0.005	4.863

In the second scenario for *N. virens*, the study in estuarine production by the *N. virens* population in Norsminde Fjord, Denmark, the authors report that the annual production of *N. virens* in this specific population can reach up to 23.73 g AFDW/m^2 (Kristensen, 1984). This annual production was then converted into 107.75 g WW/m^2 using the regression equation in Chapter 2. The same calculation steps as in the first scenario were adopted. The amount of EPA produced by the worms in this scenario is calculated as 175.841 kg km^{-2} y^{-1} or 0.176 g m^{-2} y^{-1} in terms of wet weight as shown in Table 4.2.

Table 4.2: Scenario#2 the calculations of EPA gain (kg) / annual production wet weight / km^2 in *N. virens* based on the data from Chapter 2 experiment in this thesis and the study by Kristensen (1984) in Norsminde Fjord, Denmark.

	Net EPA gain (g) / system box	**Net EPA gain (g) / g worm WW**	**EPA gain (g) / annual production (107.75 g WW) / m^2**	**EPA gain (kg) / annual production (107.75 g WW) / km^2**
Box 1	0.059	0.002	0.202	202.357
Box 2	0.037	0.002	0.184	184.341
Box 3	0.039	0.001	0.141	140.826
Mean	0.045	0.002	0.176	**175.841**
SD	0.012	0.000	0.032	31.634

Even though these figures were determined as scenarios, the figures are in agreement with each other and suggest that for any one of the worm species the contribution of EPA to the whole ecosystem could reach more than 100 kg EPA per km^2 per annum (0.1 g m^{-2} y^{-1}). This would mean that the polychaete can be regarded as acting as a potential EPA source within the ecosystem and this would raise the question of how this EPA can be transferred throughout the food web. Although, it is rather difficult and limited but the comparison of annual production with other potential sources would put this figure into ecological context. Therefore, more research on EPA annual production and life histories of polychaetes, microalgae and other potential sources are needed and would help us to establish a greater degree of accuracy concerning the roles of polychaetes within estuarine ecosystems. It would for instance be better to investigate PUFA production in both field and laboratory systems together.

In addition to their role in marine ecosystems, the abilities of the worms to synthesise long-chain PUFAs such as EPA in the case for *A. marina* and/or DHA in the case for *N. virens* are essential for an evaluation of the application of polychaetes in integrated aquaculture production systems. Marine polychaetes have been studied as an aquafeed for several marine organisms including crustaceans and some carnivorous finfish. A commercial sector for polychaete worm production has begun to develop in Europe and more recently in China and SE Asia.

As introduced in Chapter 1, the use of fish meal (FM) and especially fish oil (FO) from wild-caught low trophic level fish are increasingly a matter of concern in relation to the overall sustainability in the aquaculture industry. According to the report by Tacon *et al*. (2011), it was estimated that around 0.78 million tonnes of marine fish oil were consumed in 2008 by the aquaculture industry. If the industry utilises fish oil more than it can net produce, this would not be considered as sustainable (Naylor *et al*., 2000). The search for alternatives to fish oil sources are increasing in order to replace or supplement the FO derived from natural fish caught in pelagic fisheries. In a recent review by Klinger and Naylor (2012), the authors have suggested the use of polychaetes or marine worm as one of the alternative FO replacement choices due to their high long-chain n3 fatty acid content as previously reported by Olive (1999). The authors also added that polychaete aquaculture could be possible using recirculating system (RAS) technologies and integrated multitrophic aquaculture (IMTA). The polychaete genera *Nereis*, *Arenicola*, *Glycera* and *Sabella* are among the polychaete genera that have been suggested to have a high potential for applications in the IMTA industries (Barrington *et*

al., 2009). The findings in this thesis confirms the ability of two of these polychaete genera to generate long-chain PUFA, and shows that they could potentially contribute novel PUFA and draws more attention to the value of polychaete worm aquaculture and utilisation of them as one of the FM and FO sources as discussed below.

According to the study in *A. marina*, the only added food was yeast incorporated into sand, and this contained only a limited range and quantity of fatty acids. However, after the experiment within 60 days, the worms had shown a greater growth rate associated with a high content of fatty acids including EPA (C20:5n3). Though this experiment was conducted at a small scale, this low cost but high productivity is suggestive that *A. marina* could be one of the potential sources for supplementary fish oil in terms of mass production. The study by Olive *et al*. (2009) has also suggested that *A. marina* could contribute to 'fed-aquaculture' as an alternative to fish oil sources of essential fatty acids and EPA.

The findings of the *N. virens* experiment in this thesis also imply that *N. virens* could be considered as one of the supplementary sources for fish oil. The ability to synthesise EPA and even longer-chain DHA in *N. virens* indicates that this worm species could be cultured and utilised as aquafeed given directly to some carnivorous finfish and crustacean farming. These results are in agreement with a recent study in a recirculating aquaculture system for *N. virens* by Brown *et al*. (2011). The author suggests that *N. virens* could grow on faecal waste and/or uneaten halibut feed pellets. The fatty acid contents of the worms produced were of interest containing particularly palmitic acid (C16:0), oleic acid (C18:1n9), linoleic acid (C18:2n6) and EPA (C20:5n3). These fatty acids could be essential for the aquaculture production especially in IMTA (Brown *et al*., 2011). In addition, the study by the same author has shown that a relatively low level of DHA was detected in the worm tissues when fed on these diets. The studies in *N. diversicolor*, a species closely related to *N. virens*, have also shown that this common species contained high contents of fatty acids and could be a potential source for alternative fish oil in terms of aquaculture (Fidalgo e Costa, 1999; García-Alonso *et al.*, 2008). The abilities of these polychaetes to utilise organic wastes such as faecal pellets (Brown *et al*., 2011) or eel sludges (García-Alonso *et al*., 2008), would be beneficial in relation to environmental aspects and can reduce cost in aquaculture industries. Hence, the IMTA system could be one of the most interesting aquaculture systems in order to sustain and create more environmental friendly impacts of aquaculture on the natural ecosystem (Pillay and Kutty, 2005). Taken together, these

results and several lines of evidence on polychaete biochemistry and aquaculture, as discussed above, suggest that polychaete aquaculture would be interesting to be considered as one of the potential 'alternative' fish-oil sources for some economic aspects of marine aquaculture.

4.3 *Future work*

The study in this thesis serves as a base for future studies and it is recommended that further research should be undertaken in order to further the field of fatty acid biosynthesis. According to the difficulty of delivering ^{13}C-labelled stable isotopes directly into the *A .marina* gut as described in Chapter 3, the application of ^{13}C stable isotope labelling approach using known quantities of labeled fatty acid in the food source would be required to clarify the pathway to synthesise PUFA. In this instance a yeast material containing labelled stearic acid might be required. The same technique to analyse and interpret the results as in Chapter 3 for *N. virens* could be then adopted to distinguish the pathway and the ability to generate long-chain PUFA such as EPA (C20:5n3) in *A. marina*.

The increasing use of molecular techniques such as cloning and expression of specific genes in many animals would introduce other techniques to enhance the understanding of PUFA biosynthesis in marine invertebrates. These molecular techniques have been adopted to study desaturase and elongase activities in some animals such as *Caenorhabditis elegans* (Beaudoin *et al*., 2000a; Beaudoin *et al*., 2000b; Watt and Browse, 2002), octopus (*Octopus vulgaris*) (Monroig *et al*., 2009; Monroig *et al*., 2012) and vertebrate and marine fish (Tocher and Ghioni, 1999; Tocher, 2003; Morais *et al*., 2009). These techniques could be adapted to the study of polychaetes to determine the pathways of biosynthesis. Also, the heterologous expression in yeast for instance would be very helpful for two major purposes: effects of differences in either laboratory or environmental conditions on the enzyme activities in the worms and the preferential substrates of these enzymes. For example, the study of elongase gene expression derived from octopus in yeast by Monroig *et al*. (2012) showed that the elongase enzyme efficiently elongated n6 fatty series fatty acids rather than n3 series fatty acids. This technique would help us to establish whether both *A. marina* and *N. virens* desaturase/elongase enzymes can act effectively on the n6 fatty acid precursors rather than in the n3 fatty acid series as has been deduced from the observations reported in this thesis. As discussed earlier in this chapter in discussing the

various possible PUFA biosynthetic routes, each enzyme has its own specific function and contribution to the pathway. For instance, demonstration of the presence of Δ12 and Δ15 (ω3) desaturase activities in the worms would confirm the likelihood of their ability to generate linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) in certain conditions. In order to distinguish the 'downstream' pathway in *A. marina*, the comparison between Δ8 and Δ6 desaturase activities is essentially to establish whether just one of these pathways or both pathways are utilised in specific experimental conditions. The ability to produce DHA in *N. virens* might not be considered to be conclusive from the evidence presented in this study, the molecular cloning and expression of Δ4 desaturase gene would be able to resolve this problem.

In terms of marine ecology, a 'whole-system approach' should be adopted to see the pattern of PUFA flux transfer between the trophic levels both 'upstream' and 'downstream' in the food web. It would be interesting to compare fatty acid profiles and to investigate the fluxes between each trophic level to determine the relative importance of the various sources within the system. For example, the potential sources of fatty acid within the lugworm burrow could be evaluated. The sources include, in addition to the bacteria as investigated by Ashforth *et al*. (2011) associated microalgae around the burrow system, protists and, as shown in this study the biosynthetic capabilities of the worm itself. This exploration might establish a better understanding of PUFA flux transfer within the lugworm habitat. The biotic interaction between each organism related to the polychaetes may provide more information on how the PUFA is transferred throughout the food web.

For future research, it might be possible to study the polychaete worm species with other marine species in relation to integrated multitrophic aquaculture and recirculating aquaculture systems. The ability to utilise wastes as the worm food source such as in this study and previous studies by Bischoff *et al*. (2009) in *N. diversicolor* and Brown *et al*. (2011) in *N. virens* are very important for further application to aquaculture industries. Also, the study of nutrient flow within the system would also be interesting as in the study by Schneider *et al*. (2005). However, the development of polychaete culture will of course be subject to economic and practical considerations.

In summary, while there are many areas of research to conduct and further investigations are still needed to enhance an understanding of PUFA flux transfer within marine ecosystems and the roles polychaete within the system. This thesis has provided evidence to show that both *A. marina* and *N. virens* have the ability to synthesise long-

chain PUFA, especially EPA, using the quantifying input-output fatty acid experiment and the ^{13}C stable isotope labelling experiment. This finding may therefore be used to question the general assumption that only microbial and microalgal communities can contribute and act as PUFA sources to the marine ecosystem. The polychaetes may contribute PUFA to further 'upstream' species in estuarine and marine ecosystems. The high EPA content in both worm bodies would also be interesting to the aquaculture industries in order to make these industries more sustainable.

Appendix A

The effect of food quantities on the growth of the ragworm (*N. virens*) data.

The worms were collected on October, 2009 and were cultured at the Research Aquarium, Ridley Building 2, Newcastle University under the same conditions as described in the methodology section in Chapter 2. Ten worms (1.00 ± 0.23 g) were put into each plastic experimental box. Each experimental box was fed different quantities based on the per cent of food per body weight. There were 6 boxes: unfed, 0.05%, 0.10%, 0.15%, 0.20% and 0.25% of the initial worm body weight. Each box was set for 60 days and the growth rate calculations were determined as the same way in the net gain experiment. The results are shown here in Table A1.

	Worm Groups (b.w. = body weight)					
	0% b.w.	**0.05% b.w.**	**0.10% b.w.**	**0.15% b.w.**	**0.20% b.w.**	**0.25% b.w.**
Initial wet weight (g)	1.505 ± 0.516	1.387 ± 0.552	1.621 ± 0.574	1.263 ± 0.528	1.524 ± 0.520	1.453 ± 0.614
Final wet weight (g)	1.174 ± 0.466	1.504 ± 0.628	1.976 ± 0.764	1.734 ± 0.611	2.018 ± 0.685	2.016 ± 0.878
Weight Difference (g)	-0.331	0.117	0.355	0.472	0.494	0.563
Food Quantities (g)	0.000	5.826	11.493	17.201	22.849	28.600
Survival rate (%)	12.5	12.5	5.0	5.0	12.5	25.0
SGR (% day^{-1})	-0.621	0.203	0.495	0.793	0.702	0.819
PWG (%)	-21.995	8.439	21.904	37.378	32.419	38.750
AGR (g day^{-1})	-0.008	0.003	0.009	0.011	0.012	0.014

According to the data in Table A1, the growth rate of the worms was rather constant from the food quantities of 0.20% body worm wet weight. therefore, the net gain experiment in Chapter 2 was used the amount of food at 0.20% of initial body weight.

Appendix B

Table 1B: Methyl Palmitate (C16:0): The changes in $^{13}C/^{12}C$ ratio of the three characteristic ion peaks; the McLafferty ion (m/z = 75:74), the loss of the methoxy ion ($[M-31]^+$, m/z = 240:239) and the molecular peak ($[M]^+$, m/z = 271:270) over the experimental period. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 75 : 74	**m/z = 240 : 239**	**m/z = 271 : 270**
Start	0.247 ± 0.054	0.247 ± 0.052	0.255 ± 0.054
Day 1 (labelled)	0.450 ± 0.134 (p = 0.003)*	0.467 ± 0.138 (p = 0.002)*	0.462 ± 0.137 (p = 0.002)*
Day 3 (labelled)	0.500 ± 0.070 (p < 0.001)*	0.489 ± 0.065 (p = 0.001)*	0.510 ± 0.063 (p < 0.001)*
Day 5 (labelled)	0.441 ± 0.093 (p = 0.005)*	0.432 ± 0.096 (p = 0.007)*	0.445 ± 0.093 (p = 0.005)*
Day 7 (labelled)	0.420 ± 0.130 (p = 0.012)*	0.421 ± 0.096 (p = 0.012)*	0.428 ± 0.125 (p = 0.010)*
Day 1 (control)	0.198 ± 0.014 (p = 0.989)	0.202 ± 0.013 (p = 0.986)	0.208 ± 0.014 (p = 0.989)
Day 3 (control)	0.185 ± 0.003 (p = 0.995)	0.188 ± 0.007 (p = 0.994)	0.197 ± 0.005 (p = 0.994)
Day 5 (control)	0.185 ± 0.002 (p = 0.995)	0.188 ± 0.006 (p = 0.994)	0.191 ± 0.001 (p = 0.996)
Day 7 (control)	0.184 ± 0.001 (p = 0.995)	0.187 ± 0.005 (p = 0.994)	0.194 ± 0.002 (p = 0.995)

Table 2B: Methyl Stearate (C18:0): The changes in $^{13}C/^{12}C$ ratio of the three characteristic ion peaks; the McLafferty ion (m/z = 75:74), the first hydrocarbon series (m/z = 88:87) and the molecular peak ($[M]^+$, m/z = 299:298) over the experimental period. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 75 : 74	**m/z = 88 : 87**	**m/z = 299 : 298**
Start	0.221 ± 0.006	0.086 ± 0.002	0.210 ± 0.005
Day 1 (labelled)	0.213 ± 0.003 (p = 1.000)	0.088 ± 0.002 (p = 0.712)	0.224 ± 0.004 (p = 0.118)
Day 3 (labelled)	0.212 ± 0.003 (p = 1.000)	0.107 ± 0.007 (p = 0.002)*	0.247 ± 0.008 (p < 0.001)*
Day 5 (labelled)	0.213 ± 0.002 (p = 1.000)	0.108 ± 0.015 (p = 0.001)*	0.239 ± 0.014 (p = 0.001)*
Day 7 (labelled)	0.213 ± 0.003 (p = 1.000)	0.120 ± 0.016 (p < 0.001)*	0.252 ± 0.023 (p < 0.001)*
Day 1 (control)	0.220 ± 0.003 (p = 0.925)	0.084 ± 0.003 (p = 0.935)	0.219 ± 0.008 (p = 0.366)
Day 3 (control)	0.212 ± 0.004 (p = 1.000)	0.084 ± 0.001 (p = 0.931)	0.219 ± 0.003 (p = 0.386)
Day 5 (control)	0.212 ± 0.001 (p = 1.000)	0.086 ± 0.001 (p = 0.876)	0.218 ± 0.005 (p = 0.463)
Day 7 (control)	0.216 ± 0.002 (p = 1.000)	0.086 ± 0.001 (p = 0.890)	0.225 ± 0.009 (p = 0.115)

Table 3B: Methyl Oleate (C18:1n9): The changes in $^{13}C/^{12}C$ ratio of the two characteristic ion peaks; the loss of methanol ($[M-32]^+$, m/z = 265:264) and the loss of the McLafferty ion ($[M-74]^+$, m/z = 223:222) over the experimental period in methyl oleate (C18:1n9). The molecular peak is not presented here. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 223 : 222	**m/z = 265 : 264**
Start	0.224 ± 0.006	0.724 ± 0.017
Day 1 (labelled)	0.210 ± 0.005 (*p* = 1.000)	0.700 ± 0.018 (*p* = 1.000)
Day 3 (labelled)	0.219 ± 0.008 (*p* = 0.995)	0.714 ± 0.010 (*p* = 0.991)
Day 5 (labelled)	0.218 ± 0.007 (*p* = 0.998)	0.712 ± 0.016 (*p* = 0.994)
Day 7 (labelled)	0.216 ± 0.005 (*p* = 0.999)	0.715 ± 0.009 (*p* = 0.983)
Day 1 (control)	0.220 ± 0.004 (*p* = 0.990)	0.708 ± 0.015 (*p* = 0.998)
Day 3 (control)	0.218 ± 0.004 (*p* = 0.998)	0.709 ± 0.011 (*p* = 0.998)
Day 5 (control)	0.220 ± 0.009 (*p* = 0.983)	0.717 ± 0.012 (*p* = 0.976)
Day 7 (control)	0.221 ± 0.005 (*p* = 0.965)	0.719 ± 0.018 (*p* = 0.964)

Table 4B: Methyl Linoleate: The changes in $^{13}C/^{12}C$ ratio of the characteristic ion peaks over the experimental period in methyl linoleate (C18:2n6); the molecular peak ($[M]^+$, m/z = 295:294). The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 295:294
Start	0.246 ± 0.004
Day 1 (labelled)	0.241 ± 0.027 (p = 1.000)
Day 3 (labelled)	0.228 ± 0.010 (p = 0.995)
Day 5 (labelled)	0.215 ± 0.011 (p = 0.998)
Day 7 (labelled)	0.227 ± 0.005 (p = 0.999)
Day 1 (control)	0.224 ± 0.026 (p = 0.990)
Day 3 (control)	0.216 ± 0.008 (p = 0.998)
Day 5 (control)	0.224 ± 0.021 (p = 0.983)
Day 7 (control)	0.223 ± 0.009 (p = 0.965)

Table 5B: Methyl Eicosadienoate (C20:2n6): The changes in $^{13}C/^{12}C$ ratio of the characteristic ion peaks over the period of experiment in methyl eicosadienoate (C20:2n6); the loss of the methoxy ion ($[M-31]^+$, m/z = 292:291) and the molecular peak ($[M]^+$, m/z = 323:322). The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 292 : 291	**m/z = 323 : 322**
Start	0.334 ± 0.047	0.291 ± 0.043
Day 1 (labelled)	0.313 ± 0.121 (p = 0.957)	0.255 ± 0.079 (p = 0.995)
Day 3 (labelled)	0.409 ± 0.105 (p = 0.296)	0.294 ± 0.063 (p = 0.868)
Day 5 (labelled)	0.335 ± 0.042 (p = 0.882)	0.269 ± 0.031 (p = 0.980)
Day 7 (labelled)	0.340 ± 0.055 (p = 0.860)	0.290 ± 0.043 (p = 0.899)
Day 1 (control)	0.312 ± 0.091 (p = 0.958)	0.268 ± 0.018 (p = 0.982)
Day 3 (control)	0.316 ± 0.036 (p = 0.950)	0.287 ± 0.010 (p = 0.919)
Day 5 (control)	0.316 ± 0.042 (p = 0.757)	0.273 ± 0.046 (p = 0.972)
Day 7 (control)	0.312 ± 0.040 (p = 0.958)	0.269 ± 0.025 (p = 0.981)

Table 6B: Methyl Arachidonate (C20:4n6): The changes in $^{13}C/^{12}C$ ratio of the α-ion (m/z = 181:180) characteristic ion peak over the experimental period. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 181 : 180
Start	0.235 ± 0.019
Day 1 (labelled)	0.235 ± 0.028 (*p* = 0.568)
Day 3 (labelled)	0.364 ± 0.055 (*p* = 0.001)*
Day 5 (labelled)	0.317 ± 0.063 (*p* = 0.047)*
Day 7 (labelled)	0.245 ± 0.019 (*p* = 0.678)
Day 1 (control)	0.247 ± 0.062 (*p* = 0.778)
Day 3 (control)	0.280 ± 0.041 (*p* = 0.320)
Day 5 (control)	0.258 ± 0.059 (*p* = 0.639)
Day 7 (control)	0.266 ± 0.008 (*p* = 0.518)

Table 7B: Methyl Eicosapentaenotate (C20:5n3): The changes in $^{13}C/^{12}C$ ratio of the α-ion (m/z = 181:180) characteristic ion peak over the experimental period. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 181 : 180
Start	0.221 ± 0.004
Day 1 (labelled)	0.252 ± 0.015 (*p* = 0.006)*
Day 3 (labelled)	0.240 ± 0.027 (*p* = 0.374)
Day 5 (labelled)	0.231 ± 0.012 (*p* = 0.745)
Day 7 (labelled)	0.225 ± 0.008 (*p* = 0.818)
Day 1 (control)	0.237 ± 0.015 (*p* = 0.125)
Day 3 (control)	0.232 ± 0.006 (*p* = 0.720)
Day 5 (control)	0.223 ± 0.010 (*p* = 0.840)
Day 7 (control)	0.227 ± 0.004 (*p* = 0.890)

Table 8B: Methyl Docosahexaenoate (C22:6n3): The changes in $^{13}C/^{12}C$ ratio of the α-ion (m/z = 167:166) characteristic ion peak over the experimental period. The ratio data are in terms of mean ± SD. The *p*-value for the one-tailed Dunnett's test of each treatment against the start samples is given. An asterisk (*) is attached to *p*-value less than 0.05 ($p < 0.05$) for clarity.

	m/z = 167 : 166
Start	0.269 ± 0.015
Day 1 (labelled)	0.251 ± 0.016 (*p* = 0.703)
Day 3 (labelled)	0.281 ± 0.036 (*p* = 0.249)
Day 5 (labelled)	0.297 ± 0.069 (*p* = 0.105)
Day 7 (labelled)	0.286 ± 0.056 (*p* = 0.197)
Day 1 (control)	0.271 ± 0.049 (*p* = 0.397)
Day 3 (control)	0.258 ± 0.029 (*p* = 0.605)
Day 5 (control)	0.289 ± 0.067 (*p* = 0.170)
Day 7 (control)	0.294 ± 0.037 (*p* = 0.124)

References

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B. and de Rosa, R. (2000) 'The new animal phylogeny: Reliability and implications', *Proceedings of the National Academy of Sciences*, 97(9), pp. 4453-4456.

Alhazzaa, R., Bridle, A.R., Nichols, P.D. and Carter, C.G. (2011) 'Up-regulated desaturase and elongase gene expression promoted accumulation of polyunsaturated fatty acid (PUFA) but not long-chain PUFA in *Lates calcarifer*, a tropical euryhaline fish, fed a stearidonic acid- and γ-linoleic acid-enriched diet', *Journal of Agricultural and Food Chemistry*, 59(15), pp. 8423-8434.

Almeida, W.d.O., Christoffersen, M.L., Amorim, D.d.S., Garraffoni, A.R.S. and Silva, G.S. (2003) 'Polychaeta, Annelida, and Articulata are not monophyletic: articulating the Metameria (Metazoa, Coelomata)', *Revista Brasileira de Zoologia*, 20, pp. 23-57.

Alyakrinskaya, I.O. (2003) 'Some ecological features of the lugworm *Arenicola marina* L. (Annelida, Polychaeta) and its morphological and biochemical adaptations to burrowing', *Biology Bulletin of the Russian Academy of Sciences*, 30(4), pp. 411-418.

Allen, W.V. (1968) 'Fatty-acid synthesis in the echinoderms: *Asterias rubens*, *Echinus esculentus* and *Holothuria forskali*', *Journal of the Marine Biological Association of the United Kingdom*, 48(02), pp. 521-533.

Arts, M.T., Ackman, R.G. and Holub, B.J. (2001) '"Essential fatty acids" in aquatic ecosystems: a crucial link between diet and human health and evolution', *Canadian Journal of Fisheries and Aquatic Sciences*, 58(1), pp. 122-137.

Arts, M.T., Brett, M.T. and Kainz, M.J. (2009) *Lipids in aquatic ecosystems*. USA: Springer Science+Business Media, LLC.

Ashforth, E.J. (2008) *Bacterial diversity in an Arenicola marina L. bioturbated mesocosm*. Newcastle University.

Ashforth, E.J., Olive, P.J.W. and Ward, A.C. (2011) 'Phylogenetic characterisation of bacterial assemblages and the role of sulphur-cycle bacteria in an *Arenicola marina* bioturbated mesocosm', *Marine Ecology Progress Series*, 439, pp. 19-30.

Bakken, T. and Wilson, R.S. (2005) 'Phylogeny of nereidids (Polychaeta, Nereididae) with paragnaths', *Zoologica Scripta*, 34(5), pp. 507-547.

Barrington, K., Chopin, T. and Robinson, S. (2009) 'Integrated multi-trophic aquaculture (IMTA) in marine temperate waters', *FAO Fisheries and Aquaculture Technical Paper*. , (No. 529).

Barthet, V.J. (2008) '(n-7) and (n-9) *cis*-monounsaturated fatty acid contents of 12 Brassica species', *Phytochemistry*, 69(2), pp. 411-417.

Beaudoin, F., Michaelson, L.V., Hey, S.J., Lewis, M.J., Shewry, P.R., Sayanova, O. and Napier, J.A. (2000a) 'Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway', *Proceedings of the National Academy of Sciences of the United States of America*, 97(12), pp. 6421-6426.

Beaudoin, F., Michaelson, L.V., Lewis, M.J., Shewry, P.R., Sayanova, O. and Napier, J.A. (2000b) 'Production of C_{20} polyunsaturated fatty acids (PUFAs) by pathway engineering: identification of a PUFA elongase component from *Caenorhabditis elegans*', *Biochemical Society Transactions*, 28(6), pp. 661-3.

Becker, W.M., Hardin, J., Bertoni, G. and Kleinsmith, L.J. (2012) *Becker's world of the cell*. 8th ed., International ed. Boston, Mass.; London: Benjamin Cummings

Behrouzian, B. and Buist, P.H. (2003) 'Mechanism of fatty acid desaturation: A bioorganic perspective', *Prostaglandins Leukotrienes and Essential Fatty Acids*, 68(2), pp. 107-112.

Bell, M., Dick, J. and Kelly, M. (2001) 'Biosynthesis of eicosapentaenoic acid in the sea urchin *Psammechinus miliaris*, *Lipids*, 36(1), pp. 79-82.

Bell, M. and Tocher, D. (2009) 'Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions', in Kainz, M., Brett, M.T. and Arts, M.T. (eds.) Lipids in Aquatic Ecosystems. Springer New York, pp. 211-236.

Bentley, M.G., Clark, S. and Pacey, A.A. (1990) 'The Role of Arachidonic Acid and Eicosatrienoic Acids in the Activation of Spermatozoa in *Arenicola marina* L. (Annelida: Polychaeta)', *The Biological Bulletin*, 178(1), pp. 1-9.

Bentley, M.G. and Hardege, J.D. (1996) 'The role of a fatty acid hormone in the reproduction of the polychaete *Arenicola marina* (L.)', *Invertebrate Reproduction & Development*, 30(1-3), pp. 159-165.

Berg, J.M., Tymoczko, J.L. and Stryer, L. (2012) *Biochemistry*. 7th edn. W.H. Freeman and Company.

Bergé, J.-P., Gouygou, J.-P., Dubacq, J.-P. and Durand, P. (1995) 'Reassessment of lipid composition of the diatom, *Skeletonema costatum*', *Phytochemistry*, 39(5), pp. 1017-1021.

Bergé, J.-P. and Barnathan, G. (2005) 'Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects', in Ulber, R. and Gal, Y. (eds.) *Marine Biotechnology I*. Springer Berlin Heidelberg, pp. 49-125.

Beukema, J.J. and De Vlas, J. (1979) 'Population parameters of the lugworm, *Arenicola marina*, living on tidal flats in the Dutch Wadden Sea', *Netherlands journal of sea research*, 13(3), pp. 331-353.

Bischoff, A.A., Fink, P. and Waller, U. (2009) 'The fatty acid composition of *Nereis diversicolor* cultured in an integrated recirculated system: Possible implications for aquaculture', *Aquaculture*, 296(3–4), pp. 271-276.

Bleidorn, C., Vogt, L. and Bartolomaeus, T. (2003) 'New insights into polychaete phylogeny (Annelida) inferred from 18S rDNA sequences', *Molecular phylogenetics and evolution*, 29(2), pp. 279-288.

Bligh, E.G. and Dyer, W.J. (1959) 'A rapid method of total lipid extraction and purification', *Canadian Journal of Biochemistry and Physiology*, 37(8), pp. 911-917.

Blomquist, G.J., Borgeson, C.E. and Vundla, M. (1991) 'Polyunsaturated fatty acids and eicosanoids in insects', *Insect Biochemistry*, 21(1), pp. 99-106.

Blomquist, G.J., Dwyer, L.A., Chu, A.J., Ryan, R.O. and de Renobales, M. (1982) 'Biosynthesis of linoleic acid in a termite, cockroach and cricket', *Insect Biochemistry*, 12(3), pp. 349-353.

Borgeson, C.E., Kurtti, T.J., Munderloh, U.G. and Blomquist, G.J. (1991) 'Insect tissues, not microorganisms, produce linoleic acid in the house cricket and the American cockroach', *Experientia*, 47(3), pp. 238-241.

Bossie, M.A. and Martin, C.E. (1989) 'Nutritional regulation of yeast delta-9 fatty acid desaturase activity', *Journal of bacteriology*, 171(12), pp. 6409-6413.

Brenna, J.T. (2002) 'Efficiency of conversion of α-linolenic acid to long chain n-3 fatty acids in man', *Current Opinion in Clinical Nutrition & Metabolic Care*, 5(2), pp. 127-132.

Brock, T.J., Browse, J. and Watts, J.L. (2007) 'Fatty acid desaturation and the regulation of adiposity in *Caenorhabditis elegans*', *Genetics*, 176(2), pp. 865-875.

Brock, T.J. and Watts, J.L. (2006) 'Genetic regulation of unsaturated fatty acid composition in *C. elegans*', *PLoS genetics*, 2(7), p. e108.

Brown, N., Eddy, S. and Plaud, S. (2011) 'Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*', *Aquaculture*, 322–323(0), pp. 177-183.

Bruice, P.Y. (2011) *Organic chemistry*. 6th edn. USA: Prentice Hall.

Budge, S.M., Iverson, S.J., Bowen, W.D. and Ackman, R.G. (2002) 'Among- and within-species variability in fatty acid signatures of marine fish and invertebrates on the Scotian Shelf, Georges Bank, and southern Gulf of St. Lawrence', *Canadian Journal of Fisheries and Aquatic Sciences*, 59(5), pp. 886-898.

Carballeira, N.M., Sostre, A. and Rodríguez, A.D. (1996) 'Phospholipid fatty acid composition of gorgonians of the genus *Pseudopterogorgia*: Identification of tetracosapolyenoic acids', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 113(4), pp. 781-783.

Carballeira, N.M., Sostre, A. and Rodríguez, A.D. (1997) 'Phospholipid fatty acid composition of gorgonians of the genus *Eunicea*: Further identification of tetracosapolyenoic acids', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 118(2), pp. 257-260.

Cass, C., Daly, K.L., Wakeham, S.G. and Van Vleet, E.S. (2010) Proceedings from the 2010 AGU Ocean Sciences Meeting. American Geophysical Union, 2000 Florida Ave., N. W. Washington DC 20009 USA.

Castro, L.F., Wilson, J., Goncalves, O., Galante-Oliveira, S., Rocha, E. and Cunha, I. (2011) 'The evolutionary history of the stearoyl-CoA desaturase gene family in vertebrates', *BMC Evolutionary Biology*, 11(1), p. 132.

Chapelle, S. (1978) 'The influence of acclimation temperature on the fatty acid composition of an aquatic Crustacean (*Carcinus maenas*)', *Journal of Experimental Zoology*, 204(3), pp. 337-346.

Costa, C.L.F., Monroig, O., Leaver, M., Wilson, J., Cunha, I. and Tocher, D.R. (2012) 'Functional desaturase fads1 (δ5) and fads2 (δ6) orthologues evolved before the origin of jawed vertebrate'.

Christie, W.W. (1993) 'Preparation of lipid extracts from tissues', *Advances in lipid methodology*, 2, pp. 195-213.

Chu, F.-L.E., Lund, E., Soudant, P. and Harvey, E. (2002) 'De novo arachidonic acid synthesis in *Perkinsus marinus*, a protozoan parasite of the eastern oyster *Crassostrea virginica*', *Molecular and Biochemical Parasitology*, 119(2), pp. 179-190.

Conway, N. and McDowell Capuzzo, J. (1991) 'Incorporation and utilization of bacterial lipids in the *Solemya velum* symbiosis', *Marine Biology*, 108(2), pp. 277-291.

Dass, C. (2007) *Fundamentals of Contemporary Mass Spectrometry*. US: John Wiley & Sons.

David, S.N. (2003) 'Prokaryotes and the input of polyunsaturated fatty acids to the marine food web', *FEMS Microbiology Letters*, 219(1), pp. 1-7.

de Vlas, J. (1979) 'Secondary production by tail regeneration in a tidal flat population of lugworms (*Arenicola marina*), cropped by flatfish', *Netherlands Journal of Sea Research*, 13(3–4), pp. 362-393.

Demmelmair, H., Baumheuer, M., Koletzko, B., Dokoupil, K. and Kratl, G. (1998) 'Metabolism of $U^{13}C$-labeled linoleic acid in lactating women', *Journal of Lipid Research*, 39(7), pp. 1389-1396.

Desvilettes, C.H., Bourdier, G., Amblard, C.H. and Barth, B. (1997) 'Use of fatty acids for the assessment of zooplankton grazing on bacteria, protozoans and microalgae', *Freshwater Biology*, 38(3), pp. 629-637.

Djerassi, C. and Lam, W.K. (1991) 'Phospholipid studies of marine organisms. Part 25. Sponge phospholipids', *Accounts of Chemical Research*, 24(3), pp. 69-75.

Dobson, M. and Frid, C. (2008) *Ecology of aquatic systems*. Oxford University Press.

Dolbeth, M., Cusson, M., Sousa, R., Pardal, M.A. and Prairie, Y.T. (2012) 'Secondary production as a tool for better understanding of aquatic ecosystems', *Canadian Journal of Fisheries and Aquatic Sciences*, 69(7), pp. 1230-1253.

Drazen, J.C., Phleger, C.F., Guest, M.A. and Nichols, P.D. (2009) 'Lipid composition and diet inferences in abyssal macrourids of the eastern North Pacific', *Marine Ecology Progress Series*, 387, pp. 1-14.

Drazen, J.C., Phleger, C.F., Guest, M.A. and Nichols, P.D. (2008) 'Lipid, sterols and fatty acids of abyssal polychaetes, crustaceans, and a cnidarian from the northeast Pacific Ocean: food web implications', *Marine Ecology Progress Series*, 372, pp. 157-167.

Duangchinda, T. (2007) Aspects of fatty acids of cultured lugworms (*Arenicola marina* L.) and their potential role in aquaculture. Newcastle University.

Dunstan, G.A., Volkman, J.K., Barrett, S.M., Leroi, J.-M. and Jeffrey, S.W. (1993) 'Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae)', *Phytochemistry*, 35(1), pp. 155-161.

Ederington, M.C., McManus, G.B. and Harvey, H.R. (1995) 'Trophic transfer of fatty acids, sterols, and a triterpenoid alcohol between bacteria, a ciliate, and the copepod *Acartia tonsa*', *Limnology and Oceanography*, 40(5), pp. 860-867.

Emmanuel, B. (1978) 'The relative contribution of propionate, and long-chain even-numbered fatty acids to the production of long-chain odd-numbered fatty acids in rumen bacteria', *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 528(2), pp. 239-246.

FAO. (2012), *The state of world fisheries and aquaculture (SOFIA)*. Rome, 2012.

Farke, H. and Berghuis, E.M. (1979) 'Spawning, larval development and migration behaviour of *Arenicola marina* in the laboratory', *Netherlands Journal of Sea Research*, 13(3–4), pp. 512-528.

Fidalgo e Costa, P.F. (1999) 'Reproduction and growth in captivity of the polychaete *Nereis diversicolor* OF Muller, 1776, using two different kinds of sediment: Preliminary assays', *Boletin-instituto Espanol de Oceanografia*, 15(1/4), pp. 351-356.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) 'A simple method for the isolation and purification of total lipides from animal tissues', *The Journal of biological chemistry*, 226(1), pp. 497-509.

Fullarton, J.G., Dando, P.R., Sargent, J.R., Southward, A.J. and Southward, E.C. (1995) 'Fatty acids of hydrothermal vent *Ridgeia piscesae* and inshore bivalves containing symbiotic bacteria', *JMBA-Journal of the Marine Biological Association of the United Kingdom*, 75(2), pp. 455-468.

García-Alonso, J., Müller, C.T. and Hardege, J.D. (2008) 'Influence of food regimes and seasonality on fatty acid composition in the ragworm', *Aquatic Biology*, 4(1), pp. 7-13.

Garrett, R.H. and Grisham, C.M. (2005) *Biochemistry*. 3rd edn. Thomson Learning, Inc.

Gibson, K.J. (1993) 'Palmitoleate formation by soybean stearoyl-acyl carrier protein desaturase', *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 1169(3), pp. 231-235.

Guillou, H., Zadravec, D., Martin, P.G.P. and Jacobsson, A. (2010) 'The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice', *Progress in Lipid Research*, 49(2), pp. 186-199.

Guo, Z. and Jensen, M.D. (1998) 'Intramuscular fatty acid metabolism evaluated with stable isotopic tracers', *Journal of Applied Physiology*, 84(5), pp. 1674-1679.

Hall, J.M., Parrish, C.C. and Thompson, R.J. (2002) 'Eicosapentaenoic acid regulates scallop (*Placopecten magellanicus*) membrane fluidity in response to cold', The *Biological Bulletin*, 202(3), pp. 201-203.

Hashimoto, K., Yoshizawa, A.C., Okuda, S., Kuma, K., Goto, S. and Kanehisa, M. (2008) 'The repertoire of desaturases and elongases reveals fatty acid variations in 56 eukaryotic genomes', *Journal of Lipid Research*, 49(1), pp. 183-191.

Hashimoto, K., Yoshizawa, A.C., Saito, K., Yamada, T. and Kanehisa, M. (2006) 'The repertoire of desaturases for unsaturated fatty acid synthesis in 397 genomes', Genome informatics. *International Conference on Genome Informatics*, 17(1), pp. 173-183.

Hastings, N., Agaba, M., Tocher, D.R., Leaver, M.J., Dick, J.R., Sargent, J.R. and Teale, A.J. (2001) 'A vertebrate fatty acid desaturase with Δ5 and Δ6 activities', *Proceedings of the National Academy of Sciences*, 98(25), pp. 14304-14309.

Hastings, N., Agaba, M.K., Tocher, D.R., Zheng, X., Dickson, C.A., Dick, J.R. and Teale, A.J. (2004) 'Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in atlantic salmon (*Salmo salar*)', *Marine Biotechnology*, 6(5), pp. 463-474.

Hauvermale, A., Kuner, J., Rosenzweig, B., Guerra, D., Diltz, S. and Metz, J.G. (2006) 'Fatty acid production in *Schizochytrium* sp.: Involvement of a polyunsaturated fatty acid synthase and a type I fatty acid synthase', *Lipids*, 41(8), pp. 739-747.

Hellerstein, M.K., Christiansen, M., Kaempfer, S., Kletke, C., Wu, K., Reid, J.S., Mulligan, K., Hellerstein, N.S. and Shackleton, C.H. (1991) 'Measurement of de novo hepatic lipogenesis in humans using stable isotopes', *The Journal of Clinical Investigation*, 87(5), pp. 1841-1852.

Henry, S.A. (1982) 'Membrane lipids of yeast: biochemical and genetic studies', *Cold Spring Harbor Monograph Archive*, 11, pp. 101-158.

Hints, O. (2000) 'Ordovician eunicid polychaetes of Estonia and surrounding areas: review of their distribution and diversification', *Review of Palaeobotany and Palynology*, 113(1–3), pp. 41-55.

Hints, O. and Eriksson, M.E. (2007) 'Diversification and biogeography of scolecodont-bearing polychaetes in the Ordovician', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 245(1–2), pp. 95-114.

Holloway, P.W. and Wakil, S.J. (1964) 'Synthesis of fatty acids in animal tissues II. The occurrence and biosynthesis of *cis*-vaccenic acid', *Journal of Biological Chemistry*, 239(8), pp. 2489-2495.

Howell, K.L., Pond, D.W., Billett, D.S.M. and Tyler, P.A. (2003) 'Feeding ecology of deep-sea seastars (Echinodermata: Asteroidea): a fatty-acid biomarker approach', *Marine Ecology Progress Series*, 255, pp. 193-206.

Howie, D. (1959) 'The spawning of *Arenicola marina* (L). I. The breeding season', *Journal of the Marine Biological Association of the United Kingdom*, 38(02), pp. 395-406.

Howie, D.I.D. (1961) 'The spawning of *Arenicola marina* (L.)', Spawning under experimental conditions. *Journal of the Marine Biological Association of the United Kingdom*, 41, pp. 127-144.

Hughes, A.D., Cook, E.J., Orr, H., Kelly, M.S. and Black, K.D. (2011) 'The transformation of long chain polyunsaturated fatty acids in benthic food webs: The role of sea urchins', *Journal of Experimental Marine Biology and Ecology*, 409(1–2), pp. 229-234.

Imbs, A., Demidkova, D., Dautova, T. and Latyshev, N. (2009) 'Fatty acid biomarkers of symbionts and unusual inhibition of tetracosapolyenoic acid biosynthesis in corals (Octocorallia)', *Lipids*, 44(4), pp. 325-335.

Imbs, A., Demina, O. and Demidkova, D. (2006) 'Lipid class and fatty acid composition of the boreal soft coral *Gersemia rubiformis*', *Lipids*, 41(7), pp. 721-725.

Imbs, A.B., Latyshev, N.A., Dautova, T.N. and Latypov, Y.Y. (2010) 'Distribution of lipids and fatty acids in corals by their taxonomic position and presence of zooxanthellae', *Marine Ecology Progress Series*, 409, pp. 65-75.

Islam, M. (2001) A study of the lipid composition of cultured *Nereis virens* Sars and *Arenicola marina* L. (Annelida: Polychaeta) in relation to their use in aquaculture maturation diets. University of Newcastle upon Tyne.

Iverson, S. (2009) 'Tracing aquatic food webs using fatty acids: from qualitative indicators to quantitative determination', in Kainz, M., Brett, M.T. and Arts, M.T. (eds.) *Lipids in Aquatic Ecosystems*. Springer New York, pp. 281-308.

Iverson, S., Lang, S. and Cooper, M. (2001) 'Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue', *Lipids*, 36(11), pp. 1283-1287.

Jacobsen, V.H. (1967) 'The feeding of the lugworm, *Arenicola Marina* (L.). Quantitative studies', *Ophelia*, 4(1), pp. 91-109.

Kaiser, M.J., Martin, J.A., Jennings, S., Thomas, D.N., Barnes, D.K.A., Brierley, A.S., Polunin, N.V.C., Raffaelli, D.G. and Williams, P.J.l.B. (2005) *Marine Ecology: Processes, Systems, and Impacts*. Great Britain: Oxford University Press.

Kaneda, T. (1991) 'Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance', *Microbiological Reviews*, 55(2), pp. 288-302.

Kanekiwa, M., Okamoto, J. and Takagi, T. (1987) 'Fatty acids in asteroidea. Occurrence of unusual 5-olefinic acids', *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 87(4), pp. 1037-1040.

Kanekiwa, M., Itabashi, Y., Endo, S. and Takagi, T. (1986) 'Fatty acids in holothuroidea: Occurrence of cis-14-tricosenoic acid', *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 84(4), pp. 451-455.

Kattner, G. and Hagen, W. (1995) 'Polar herbivorous copepods – different pathways in lipid biosynthesis', *ICES Journal of Marine Science: Journal du Conseil*, 52(3-4), pp. 329-335.

Klein Breteler, W.C.M., Schogt, N., Baas, M., Schouten, S. and Kraay, G.W. (1999) 'Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids', *Marine Biology*, 135(1), pp. 191-198.

Klinger, D. and Naylor, R.L. (2012) 'Searching for solutions in aquaculture: charting a sustainable course', *Annual Review of Environment and Resources*, 37(1).

Koletzko, B., Demmelmair, H., Hartl, W., Angelika, K., Koletzko, S., Sauerwald, T. and Szitanyi, P. (1998) 'The use of stable isotope techniques for nutritional and

metabolic research in paediatrics', *Early Human Development*, 53(Supplement 1), pp. S77-S97.

Koletzko, B., Sauerwald, T. and Demmelmair, H. (1997) 'Safety of stable isotope use', *European Journal of Pediatrics*, 156(13), pp. S12-S17.

Kornprobst, J.-M. and Barnathan, G. (2010) 'Demospongic acids revisited', *Marine Drugs*, 8(10), pp. 2569-2577.

Kristensen, E. (1984) 'Life cycle, growth and production in estuarine populations of the polychaetes *Nereis virens* and *N. diversicolor*', *Ecography*, 7(3), pp. 249-250.

Kristensen, E., Penha-Lopes, G., Delefosse, M., Valdemarsen, T., Quintana, C.O. and Banta, G.T. (2012) 'What is bioturbation? The need for a precise definition for fauna in aquatic sciences', *Marine Ecology Progress Series*, 446, pp. 285-302.

Larqué, E., Demmelmair, H., Berger, B., Hasbargen, U. and Koletzko, B. (2003) 'In vivo investigation of the placental transfer of ^{13}C-labeled fatty acids in humans', *Journal of Lipid Research*, 44(1), pp. 49-55.

Lillebø, A.I., Cleary, D.F.R., Marques, B., Reis, A., Lopes da Silva, T. and Calado, R. (2012) 'Ragworm fatty acid profiles reveals habitat and trophic interactions with halophytes and with mercury', *Marine Pollution Bulletin*, 64(11), pp. 2528-2534.

Liyana-Pathirana, C., Shahidi, F., Whittick, A. and Hooper, R. (2002) 'Lipid and lipid soluble components of gonads of green sea urchin (*Strongylocentrotus droebachiensis*)', *Journal of Food Lipids*, 9(2), pp. 105-126.

Lazo, J.P. and Davis, D.A. (2000) *Encyclopedia of Aquaculture*. New York: John Wiley & Sons, Inc.

Limsuwatthanathamrong, M., Sooksai, S., Chunhabundit, S., Noitung, S., Ngamrojanavanich, N. and Petsom, A. (2012) 'Fatty acid profile and lipid composition of farm-raised and wild-caught sandworms, *Perinereis nuntia*, the diet for marine shrimp broodstock', *Asian Journal of Animal Sciences*, 6(2), pp. 65-75.

Luis, O.J. and Passos, A.M. (1995a) 'Seasonal changes in lipid content and composition of the polychaete *Nereis (Hediste) diversicolor*', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 111(4), pp. 579-586.

Lytle, J.S., Lytle, T.F. and Ogle, J.T. (1990) 'Polyunsaturated fatty acid profiles as a comparative tool in assessing maturation diets of *Penaeus vannamei*', *Aquaculture*, 89(3-4), pp. 287-299.

Marty, Y., Delaunay, F., Moal, J. and Samain, J.F. (1992) 'Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development', *Journal of Experimental Marine Biology and Ecology*, 163(2), pp. 221-234.

McCutchan, J.H., Lewis, W.M., Kendall, C. and McGrath, C.C. (2003) 'Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur', *Oikos*, 102(2), pp. 378-390.

McKenzie, J.D., Black, K.D., Kelly, M.S., Newton, L.C., Handley, L.L., Scrimgeour, C.M., Raven, J.A. and Henderson, R.J. (2000) 'Comparisons of fatty acid and stable isotope ratios in symbiotic and non-symbiotic brittlestars from Oban Bay, Scotland', *Journal of the Marine Biological Association of the UK*, 80(02), pp. 311-320.

McMurray, J.E. and Begley, T.P. (2005) *The Organic Chemistry of Biological Pathways*. Roberts and Company.

Meesapyodsuk, D. and Qiu, X. (2012) 'The Front-end Desaturase: Structure, Function, Evolution and Biotechnological Use', *Lipids*, 47(3), pp. 227-237.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V. and Browse, J. (2001) 'Production of polyunsaturated fatty acids by potyketide synthases in both prokaryotes and eukaryotes', *Science*, 293(5528), pp. 290-293.

Meunpol, O., Duangjai, E., Yoonpun, R. and Piyatiratitivorakul, S. (2010) 'Detection of prostaglandin E_2 in polychaete *Perinereis* sp. and its effect on *Penaeus monodon* oocyte development in vitro', *Fisheries Science*, 76(2), pp. 281-286.

Meunpol, O., Meejing, P. and Piyatiratitivorakul, S. (2005) 'Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock', *Aquaculture Research*, 36(12), pp. 1216-1225.

Meysman, F., Galaktionov, O.S. and Middelburg, J.J. (2005) 'Irrigation patterns in permeable sediments induced by burrow ventilation: a case study of *Arenicola marina*', *Marine Ecology Progress Series*, 303.

Miller, M.R., Nichols, P.D. and Carter, C.G. (2008) 'n-3 Oil sources for use in aquaculture–alternatives to the unsustainable harvest of wild fish', *Nutrition Research Reviews*, 21(2), pp. 85-96.

Miliou, H., Fintikaki, M., Tzitzinakis, M., Kountouris, T. and Verriopoulos, G. (2006) 'Fatty acid composition of the common octopus, *Octopus vulgaris*, in relation to rearing temperature and body weight', *Aquaculture*, 256(1–4), pp. 311-322.

Monroig, Ó., Guinot, D., Hontoria, F., Tocher, D.R. and Navarro, J.C. (2012a) 'Biosynthesis of essential fatty acids in *Octopus vulgaris* (Cuvier, 1797): Molecular cloning, functional characterisation and tissue distribution of a fatty acyl elongase', *Aquaculture*.

Monroig, Ó., Navarro, J., Dick, J., Alemany, F. and Tocher, D. (2012b) 'Identification of a Δ5-like fatty acyl desaturase from the cephalopod *Octopus vulgaris* (Cuvier 1797) involved in the biosynthesis of essential fatty acids', *Marine Biotechnology*, 14(4), pp. 411-422.

Monroig, Ó., Rotllant, J., Sánchez, E., Cerdá-Reverter, J.M. and Tocher, D.R. (2009) 'Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis', *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1791(11), pp. 1093-1101.

Morais, S., Monroig, O., Zheng, X., Leaver, M.J. and Tocher, D.R. (2009) 'Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5- and ELOVL2-like elongases', *Marine Biotechnology* (NY), 11(5), pp. 627 - 39.

Narciso, L. and da Fonseca, L.C. (2000) 'Growth, survival and fatty acid profile of *Nereis diversicolor* (OF Muller, 1776) fed on six different diets', *Bulletin of marine science*, 67(1), pp. 337-343.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000) 'Effect of aquaculture on world fish supplies', *Nature*, 405(6790), pp. 1017-1024.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K. and Nichols, P.D. (2009) 'Feeding aquaculture in an era of finite resources', *Proceedings of the National Academy of Sciences of the United States of America*, 106(36), pp. 15103-15110.

Nelson, D.L. and Cox, M.M. (2008) *Lehninger's Principles of Biochemistry*. 5th edn. USA: W.H. Freeman.

Nelson, G.J. (1992) *Dietary fatty acids and lipid metabolism*. New York: Marcel Dekker, Inc.

Ntambi, J.M. (1999) 'Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol', *Journal of Lipid Research*, 40(9), pp. 1549-1558.

Olive, P.J.W. (1999) 'Polychaete aquaculture and polychaete science: a mutual synergism', *Hydrobiologia*, 402, pp. 175-183.

Olive, P.J.W., Duangchinda, T., Ashforth, E., Craig, S., Ward, A.C. and Davies, S.J. (2009) 'Net gain of long-chain polyunsaturated fatty acids (PUFA) in a lugworm *Arenicola marina* bioturbated mesocosm', *Marine Ecology Progress Series*, 387, pp. 223-239.

Or-Rashid, M.M., Odongo, N.E. and McBride, B.W. (2007) 'Fatty acid composition of ruminal bacteria and protozoa, with emphasis on conjugated linoleic acid, vaccenic acid, and odd-chain and branched-chain fatty acids', *Journal of Animal Science*, 85(5), pp. 1228-1234.

Pacey, A.A. and Bentley, M.G. (1992) 'The fatty acid 8, 11, 14-eicosatrienoic acid induces spawning in the male lugworm *Arenicola marina*', *Journal of experimental biology*, 173(1), pp. 165-179.

Parrish, C. (2009) 'Essential fatty acids in aquatic food webs', in Kainz, M., Brett, M.T. and Arts, M.T. (eds.) *Lipids in Aquatic Ecosystems*. Springer New York, pp. 309-326.

Parrish, C.C., French, V.M. and Whiticar, M.J. (2012) 'Lipid class and fatty acid composition of copepods (*Calanus finmarchicus*, *C. glacialis*, *Pseudocalanus* sp., *Tisbe furcata* and *Nitokra lacustris*) fed various combinations of autotrophic and heterotrophic protists', *Journal of Plankton Research*, 34(5), pp. 356-375.

Paton, C.M. and Ntambi, J.M. (2009) 'Biochemical and physiological function of stearoyl-CoA desaturase', *American Journal of Physiology - Endocrinology And Metabolism*, 297(1), pp. E28-E37.

Pazos, A.J., Ruíz, C., García-Martin, O., Abad, M. and Sánchez, J. (1996) 'Seasonal variations of the lipid content and fatty acid composition of *Crassostrea gigas* cultured in E1 Grove, Galicia, N.W. Spain', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 114(2), pp. 171-179.

Pereira, S.L., Leonard, A.E. and Mukerji, P. (2003) 'Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 68(2), pp. 97-106.

Pillay, T.V.R. and Kutty, M.N. (2005) *Aquaculture: principles and practices*. Blackwell publishing.

Pirini, M., Manuzzi, M.P., Pagliarani, A., Trombetti, F., Borgatti, A.R. and Ventrella, V. (2007) 'Changes in fatty acid composition of *Mytilus galloprovincialis* (Lmk) fed on microalgal and wheat germ diets', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 147(4), pp. 616-626.

Pond, D.W., Allen, C.E., Bell, M.V., Van Dover, C.L., Fallick, A.E., Dixon, D.R. and Sargent, J.R. (2002) 'Origins of long-chain polyunsaturated fatty acids in the hydrothermal vent worms *Ridgea piscesae* and *Protis hydrothermica'*, *Marine Ecology Progress Series*, 225, pp. 219-226.

Pough, F.H., Janis, C.M.J. and Heiser, J.B. (2011) 'Vertebrate life'. 7th edn. Pearson Education, USA.

Retraubun, A.S.W., Dawson, M. and Evans, S.M. (1996) 'The role of the burrow funnel in feeding processes in the lugworm *Arenicola marina* (L.)', *Journal of experimental marine biology and ecology*, 202(2), pp. 107-118.

Rodriguez, M., Funke, S., Fink, M., Demmelmair, H., Turini, M., Crozier, G. and Koletzko, B. (2003) 'Plasma fatty acids and [^{13}C]-linoleic acid metabolism in preterm infants fed a formula with medium-chain triglycerides', *Journal of Lipid Research*, 44(1), pp. 41-48.

Rousset, V., Pleijel, F., Rouse, G.W., Erséus, C. and Siddall, M.E. (2007) 'A molecular phylogeny of annelids', *Cladistics*, 23(1), pp. 41-63.

Sargent, J.R., Gatten, R.R. and Henderson, R.J. (1978) 'Marine wax esters', *Science Progress Oxford*, 65, pp. 437-458.

Schneider, O., Sereti, V., Eding, E.H. and Verreth, J.A.J. (2005) 'Analysis of nutrient flows in integrated intensive aquaculture systems', *Aquacultural Engineering*, 32(3–4), pp. 379-401.

Shibahara, A., Yamamoto, K., Nakayama, T., Kajimoto, G., Noda, M., Kitano, E. and Kitamura, H. (1997) 'A novel pathway for oleic acid bioformation by an enzymatic double-bond shifting reaction in animal cells (A Rapid Communication)'.

Shibahara, A., Yamamoto, K., Takeoka, M., Kinoshita, A., Kajimoto, G., Nakayama, T. and Noda, M. (1989) 'Application of a GC-MS method using deuterated fatty acids for tracing *cis*-vaccenic acid biosynthesis in kaki pulp', *Lipids*, 24(6), pp. 488-493.

Shibahara, A., Yamamoto, K., Takeoka, M., Kinoshita, A., Kajimoto, G., Nakayama, T. and Noda, M. (1990) 'Novel pathways of oleic and *cis*-vaccenic acid biosynthesis by an enzymatic double-bond shifting reaction in higher plants', *FEBS Letters*, 264(2), pp. 228-230.

Skrzypski, J., Bellenger, S., Bellenger, J., Sinclair, A., Poisson, J.-P., Tessier, C., Rialland, M. and Narce, M. (2009) 'Revisiting delta-6 desaturase regulation by C18 unsaturated fatty acids, depending on the nutritional status', *Biochimie*, 91(11–12), pp. 1443-1449.

Svetashev, V.I., Levin, V.S., Cham Ngok, L. and Do Tuet, N. (1991) 'Lipid and fatty acid composition of holothurians from tropical and temperate waters', *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 98(4), pp. 489-494.

Svetashev, V.I. and Vysotskii, M.V. (1998) 'Fatty acids of *Heliopora coerulea* and chemotaxonomic significance of tetracosapolyenoic acids in Coelenterates', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 119(1), pp. 73-75.

Silverstein, R.M., Webster, F.X. and Kiemle, D.J. (2005) *Spectrometric identification of organic compounds*. 7 edn. Hoboken, NJ: John Wiley & Sons.

Stanley-Samuelson, D.W. (1987) 'Physiological roles of prostaglandins and other eicosanoids in invertebrates', *The Biological Bulletin*, 173(1), pp. 92-109.

Stanley-Samuelson, D.W. (1994) 'The biological significance of prostaglandins and related eicosanoids in invertebrates', *American Zoologist*, 34(6), pp. 589-598.

Stanley-Samuelson, D.W., Jurenka, R.A., Loher, W. and Blomquist, G.J. (1987) 'Metabolism of polyunsaturated fatty acids by larvae of the waxmoth, *Galleria mellonella*', *Archives of Insect Biochemistry and Physiology*, 6(3), pp. 141-149.

Stanley, D.W. (2000) *Eicosanoids in Invertebrates Signal Transduction Systems*. Princeton, New Jersey: Princeton University Press.

Stanley, D.W. and Howard, R.W. (1998) 'The biology of prostaglandins and related eicosanoids in invertebrates: Cellular, Organismal and Ecological Actions', *American Zoologist*, 38(2), pp. 369-381.

Tacon, A.G.J., M.R., H. and R.P., S. (2006) 'Use of fishery resources as feed inputs for aquaculture development: trends and policy implications', *FAO Fisheries Circular no. 1018*.

Tacon, A.G.J., Hasan, M.R. and Metian, M. (2011) 'Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects', *FAO Fisheries and Aquaculture Technical Paper*.

Taghon, G.L. (1988) 'Phospholipid fatty acid composition of the deep-sea hydrothermal vent polychaete *Paralvinella palmiformis* (Polychaeta-ampharetidae): effects of thermal regime and comparison with two shallow-water confamilial species', *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 91(3), pp. 593-596.

Takagi, T., Eaton, C.A. and Ackman, R.G. (1980) 'Distribution of fatty acids in lipids of the common atlantic sea urchin *Strongylocentrotus droebachiensis*', Canadian *Journal of Fisheries and Aquatic Sciences*, 37(2), pp. 195-202.

Tocher, D. and Ghioni, C. (1999) 'Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells', *Lipids*, 34(5), pp. 433-440.

Tocher, D.R. (2003) 'Metabolism and functions of lipids and fatty acids in teleost fish', *Reviews in Fisheries Science*, 11(2), pp. 107-184.

Turpeinen, A.M., Mutanen, M., Aro, A., Salminen, I., Basu, S., Palmquist, D.L. and Griinari, J.M. (2002) 'Bioconversion of vaccenic acid to conjugated linoleic acid in humans', *The American Journal of Clinical Nutrition*, 76(3), pp. 504-510.

Viso, A.C. and Marty, J.C. (1993) 'Fatty acids from 28 marine microalgae', *Phytochemistry*, 34(6), pp. 1521-1533.

Voet, D. and Voet, J.G. (2011) *Biochemistry*. 4th edn. USA: John Wiley & Sons.

Warude, D., Joshi, K. and Harsulkar, A. (2006) 'Polyunsaturated fatty acids: Biotechnology', *Critical Reviews in Biotechnology*, 26(2), pp. 83-93.

Watts, J.L. (2009) 'Fat synthesis and adiposity regulation in *Caenorhabditis elegans*', *Trends in Endocrinology & Metabolism*, 20(2), pp. 58-65.

Watts, J.L. and Browse, J. (2002) 'Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*', *Proceedings of the National Academy of Sciences of the United States of America*, 99(9), pp. 5854-5859.

Weete, J.D. (1974) 'Fungal lipid biochemistry'. Plenum, New York.

Weinert, J., Blomquist, G.J. and Borgeson, C.E. (1993) '*De novo* biosynthesis of linoleic acid in two non-insect invertebrates: The land slug and the garden snail', *Experientia*, 49(10), pp. 919-921.

Wethey, D.S., Woodin, S.A., Volkenborn, N. and Reise, K. (2008) 'Porewater advection by hydraulic activities of lugworms, *Arenicola marina*: A field, laboratory and modeling study', *Journal of Marine Research*, 66(2), pp. 255-273.

Würzberg, L., Peters, J., Schüller, M. and Brandt, A. (2011) 'Diet insights of deep-sea polychaetes derived from fatty acid analyses', *Deep Sea Research Part II: Topical Studies in Oceanography*, 58(1), pp. 153-162.

Yazawa, H., Kamisaka, Y., Kimura, K., Yamaoka, M. and Uemura, H. (2011) 'Efficient accumulation of oleic acid in Saccharomyces cerevisiae caused by expression of rat elongase 2 gene (rELO2) and its contribution to tolerance to alcohols', *Applied Microbiology and Biotechnology*, 91(6), pp. 1593-1600.

Zhukova, N.V., Imbs, A.B. and Yi, L.F. (1998) 'Diet-induced changes in lipid and fatty acid composition of *Artemia salina*', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 120(3), pp. 499-506.