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## **DOCTOR OF PHILOSOPHY**

**The effects of vegetable oil contamination on mussels.**

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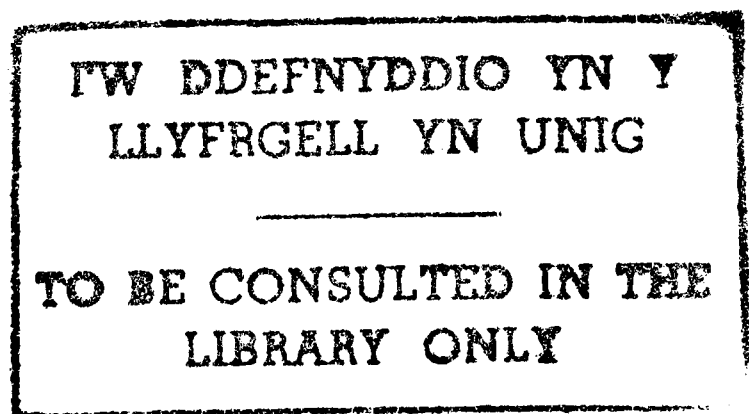
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# The Effects of Vegetable Oil Contamination on Mussels

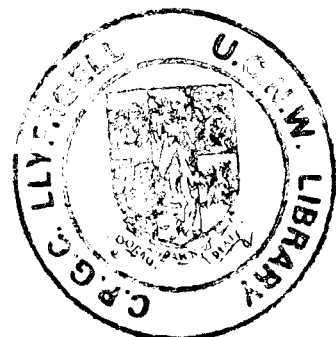
Thesis submitted in accordance with the requirements of the University of Wales for  
the degree of Doctor of Philosophy

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October 1995



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To my beloved husband

Rico

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# Summary

In this study the effects of the vegetable oils rapeseed, linseed, olive and sunflower oil on mussel performance were investigated.

In view of the scarce knowledge of the effects of vegetable oil spills on marine life, unlike petroleum spills which have been extensively studied, this investigation was directed towards an evaluation of the impact of vegetable oil contamination in the marine environment using *Mytilus edulis* as a bioindicator organism.

The growth of mussels, their tolerance to changing salinities and temperatures, their behaviour and vegetable oil metabolism were studied. Fatty acid composition of mussels, microalgae and vegetable oils was also determined.

All the vegetable oils studied had an inhibitory effect on the growth of *Mytilus edulis*, the growth rate of mussels after four weeks of exposure to the oils being 5 times lower than the growth rates of the control mussels. Growth rates were assessed by a photographic method which proved to be practical and provided sufficient precision in detect small increases in growth. Vegetable oils caused mortalities and they changed the fatty acid composition of mussels. Other biological responses of mussels are also affected by sunflower oil exposure: gaping time, tolerance to low salinities and foot extension activity, of which the latter may be of ecological significance. An uptake and accumulation of fatty acids in mussels marked the presence of vegetable oils, however, fatty acid metabolism was only detected after the oils had been removed.

The results of this study indicate that contrary to what is believed, vegetable oils should not be overlooked under the argument of their edibility and biodegradability but instead should be included in oil spill contingency planning because they can cause mortality and disrupt the growth of wild and cultured mussels.

# Contents

<b>1</b>	<b>Introduction</b>	<b>1</b>
1.1	The M. V. <i>Kimya</i> . . . . .	1
1.2	Sensitive areas . . . . .	2
1.3	Mussel economic importance . . . . .	3
1.4	Preliminary studies . . . . .	4
1.5	Aims of this study . . . . .	5
1.6	Thesis organization . . . . .	5
<b>2</b>	<b>Mussel biology, growth and metabolism</b>	<b>7</b>
2.1	Introduction . . . . .	7
2.2	Mussel biology . . . . .	8
2.2.1	Systematics . . . . .	8
2.2.2	Habitat . . . . .	9
2.2.3	Morphology . . . . .	10
2.2.4	Mussel mortality . . . . .	18
2.3	Mussel growth . . . . .	21
2.3.1	Size frequency distributions . . . . .	22
2.3.2	Growth checks on or within the shell . . . . .	23
2.3.3	Physiological measurements . . . . .	24
2.3.4	Direct measurements . . . . .	25
2.3.5	Quantitative expressions of growth . . . . .	26
2.3.6	Allometric growth . . . . .	28
2.3.7	Factors influencing growth . . . . .	30
2.4	Marine lipids and vegetable oils . . . . .	37

2.4.1	Fatty acid: structure and metabolism . . . . .	40
2.4.2	Fatty acid composition in molluscs . . . . .	46
2.4.3	Vegetable oils . . . . .	49
2.5	Summary . . . . .	55
<b>3</b>	<b>Fatty acids in biological samples</b>	<b>56</b>
3.1	Introduction . . . . .	56
3.2	Materials and methods . . . . .	57
3.2.1	Fatty acid general analytical procedure . . . . .	57
3.2.2	Algae . . . . .	62
3.2.3	Mussel collection and preparation . . . . .	63
3.2.4	Vegetable oils . . . . .	64
3.3	Results . . . . .	64
3.3.1	Algae . . . . .	64
3.3.2	Algae fatty acids . . . . .	66
3.3.3	Mussels . . . . .	74
3.3.4	Vegetable oils . . . . .	76
3.4	Discussion . . . . .	79
3.4.1	Algae . . . . .	79
3.4.2	Mussels . . . . .	80
3.4.3	Vegetable oils . . . . .	82
3.5	Summary . . . . .	83
<b>4</b>	<b>The growth of mussels exposed to vegetable oils</b>	<b>84</b>
4.1	Introduction . . . . .	84
4.2	Materials and methods . . . . .	86
4.2.1	Experimental set up . . . . .	86
4.2.2	Mussel area . . . . .	89
4.2.3	Growth rate measurement . . . . .	91
4.2.4	Fatty acid analysis . . . . .	92
4.3	Results . . . . .	94

4.3.1	Temperature and diet . . . . .	94
4.3.2	Mussel growth . . . . .	96
4.3.3	Specific growth rate . . . . .	100
4.3.4	Mortality . . . . .	102
4.3.5	Statistical analysis . . . . .	102
4.3.6	Fatty acids . . . . .	104
4.4	Discussion . . . . .	113
4.4.1	Mussel growth . . . . .	113
4.4.2	Fatty acids . . . . .	114
4.5	Summary . . . . .	116
<b>5</b>	<b>The biological responses of mussels to vegetable oils</b>	<b>118</b>
5.1	Introduction . . . . .	118
5.2	Materials and Methods . . . . .	119
5.2.1	Vegetable oils exposure . . . . .	119
5.2.2	Salinity experiment . . . . .	121
5.2.3	Fatty acid analysis . . . . .	122
5.2.4	Analysis of shell structure . . . . .	122
5.3	Results . . . . .	123
5.3.1	Vegetable oils exposure . . . . .	123
5.3.2	Response to salinities . . . . .	124
5.3.3	Statistical analysis . . . . .	128
5.3.4	Mussel response to temperatures . . . . .	131
5.3.5	Fatty acids . . . . .	133
5.3.6	Shell records . . . . .	143
5.4	Discussion . . . . .	146
5.5	Summary . . . . .	149
<b>6</b>	<b>Vegetable oil metabolism</b>	<b>151</b>
6.1	Introduction . . . . .	151
6.2	Materials and methods . . . . .	152

6.2.1	Mussel sampling . . . . .	152
6.2.2	Mussel extracts preparation . . . . .	153
6.2.3	Incubation and sampling . . . . .	153
6.2.4	Fatty acid analysis . . . . .	154
6.3	Results . . . . .	156
6.3.1	Lipid extracts . . . . .	156
6.3.2	Fatty acids . . . . .	160
6.4	Discussion . . . . .	167
6.5	Summary . . . . .	169
<b>7</b>	<b>The behaviour of mussels exposed to sunflower oil</b>	<b>170</b>
7.1	Introduction . . . . .	170
7.2	Materials and methods . . . . .	171
7.2.1	Experimental setup . . . . .	171
7.2.2	Fatty acid analysis . . . . .	172
7.3	Results . . . . .	174
7.3.1	Laboratory conditions . . . . .	174
7.3.2	Mussel activity . . . . .	175
7.3.3	Fatty acids . . . . .	182
7.3.4	Statistical analysis . . . . .	187
7.4	Discussion . . . . .	189
7.4.1	Mussel activity . . . . .	189
7.4.2	Fatty acids . . . . .	190
7.5	Summary . . . . .	191
<b>8</b>	<b>General conclusions</b>	<b>193</b>
	<b>Appendix A</b>	<b>200</b>
	<b>Appendix B</b>	<b>204</b>
	<b>References</b>	<b>208</b>

# List of Figures

2.1	A-Muscle scars on the inner surface of the left valve of a <i>Mytilus</i> shell, B-Lateral view of the muscles and their distribution (White, 1937) . . .	12
2.2	A- Visceral mass of <i>Mytilus</i> , B-Gonadal canals distributed through the mantle, C-Posterior view of the mantle <i>in vivo</i> , when immersed, showing the two siphons (White, 1937) . . . . .	13
2.3	Walford plot (Cerrato, 1980). . . . .	27
2.4	Metabolic sequences for unsaturated fatty acid synthesis in animals (Gurr and Harwood, 1991). . . . .	43
2.5	Major desaturation pathways for polyunsaturated fatty acid synthesis in plants and algae (Gurr and Harwood, 1991). . . . .	44
2.6	Desaturation pathways for unsaturated fatty acid synthesis in animals (Gurr and Harwood, 1991). . . . .	45
2.7	Structure of a triacylglyceride. R, R' and R'' represent fatty acids. . . .	50
3.1	(a) Average cell density of 5 subsamples of the algae cultures <i>Pavlova lutheri</i> , <i>Tetraselmis chunii</i> and <i>Rhinomonas reticulata</i> and (b) Dry weight of 100 ml subsamples of the same algae. . . . .	65
3.2	Average and standard deviation of the 10 major fatty acids of the microalgae <i>Pavlova lutheri</i> , <i>Tetraselmis chunii</i> and <i>Rhinomonas reticulata</i> . Percentages refer to the total of each group. . . . .	73
3.3	Length (line) and lipid weight (bars) of 10 mussels collected from Beaumaris. . . . .	75
3.4	Water percentage, wet and dry weights of 10 mussels collected from Beaumaris. . . . .	75

3.5	Concentration of the main 10 fatty acids from 9 mussels distributed between three classes of fatty acids. Mean and standard deviations are presented. . . . .	77
3.6	Percentage fatty acid composition of the vegetable oils rapeseed, linseed, olive and sunflower. . . . .	78
4.1	Diagram of the experimental set up . . . . .	87
4.2	Scan of 10 mussels created from a picture using an Apple Macintosh OneScanner and SuperPaint software . . . . .	90
4.3	Chemical structure of the triglyceride and phospholipid standards. . . .	93
4.4	Water temperature in the tanks during mussel acclimation and experiment.	95
4.5	Number of cells in each of the algal cultures used to feed mussels. . . .	95
4.6	Growth of mussel shells under different vegetable oil treatments for eight weeks . . . . .	97
4.7	Growth of mussel shells under different vegetable oil treatments for eight weeks . . . . .	98
4.8	Variation of the average area of 10 mussels from each treatment for 8 weeks. . . . .	99
4.9	Specific growth rates for the control and oil treatments for four weeks. .	100
4.10	Cumulative specific growth rates of mussels exposed to vegetable oils for four weeks with food and no food for another four weeks. . . . .	101
4.11	Mortality among mussels fixed on frames and loose in the tanks, submitted to different treatments during 8 weeks. . . . .	102
4.12	Neutral fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks . . . . .	105
4.13	Neutral fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks . . . . .	106
4.14	Polar fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks . . . . .	110
4.15	Polar fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks . . . . .	111

5.1	Mortality of mussels submitted to different vegetable oil treatments for nine weeks. . . . .	123
5.2	Mean time of opening response of mussels in 32 ‰ salinity, pre-exposed to vegetable oil treatments, before and after being submitted to decreasing salinities. . . . .	124
5.3	Mean time of mussel valve closure in decreasing salinities, pre-exposed to vegetable oil treatments. . . . .	125
5.4	Percentage of mussels closing their valves with decreasing salinities, pre-exposed to different vegetable oil treatments for nine weeks. . . . .	126
5.5	Mean gaping times of mussels in different salinities, pre-exposed to different vegetable oil treatments for nine weeks. . . . .	127
5.6	Means and standard deviations of flowrates and temperatures registered during nine days of experiment. . . . .	132
5.7	Mussel mortality as a function of vegetable oil and temperature exposure.	133
5.8	Fatty acid composition of mussels pre-exposed to different vegetable oils before being exposed to temperature treatments. . . . .	134
5.9	Fatty acid composition of mussels pre-exposed to different vegetable oils before being exposed to temperature treatments. . . . .	135
5.10	Fatty acid composition of mussels pre-exposed to different vegetable oils and submitted to three temperature treatments. . . . .	138
5.11	Fatty acid composition of mussels pre-exposed to different vegetable oils and submitted to three temperature treatments. . . . .	139
5.12	Fatty acid composition of mussels dead during vegetable oils exposure.	142
5.13	Fatty acid composition of mussels pre-exposed to vegetable oils dead during temperature treatments. . . . .	144



5.14	Photomicrographs of acetate peels of mussel <i>Mytilus edulis</i> shells. Scale bars=200 $\mu\text{m}$ (A, C, E, & G) and 100 $\mu\text{m}$ (B, D, F & H). A & B low and higher power appearance, respectively of a shell grown in control (sea-water) conditions. P=periostracum; PL=prismatic layer; Gb=growth bands. C & D low and higher power appearance, respectively of a shell grown in sunflower oil. The marked growth bands (filled arrow in C) represent the point at which the sunflower oil was introduced to the mussel. The open arrow in C indicates the point at which the mussel was possibly no longer exposed to the oil. E & F low and higher power appearance, respectively of a shell grown in linseed oil. G & H low and higher power appearance, respectively of a shell grown in olive oil. . . . .	145
6.1	Length frequency distribution of mussels collected from Beaumaris to be incubated with sunflower oil. . . . .	157
6.2	Length frequency distribution of mussels collected from Beaumaris to be incubated with linseed oil. . . . .	157
6.3	Incubation temperatures of mussel mantles with sunflower and linseed oils.	158
6.4	Mean weight of lipids from three replicates and standard deviations, extracted from mussel mantles incubated with sunflower and linseed oils .	159
6.5	Mean weight of lipids from three control replicates and standard deviations, extracted from mussel mantles incubated with Tris buffer solution	159
6.6	Mean fatty acid composition of mussel mantles incubated with Tris buffer solution and no sunflower oil, at different incubation times. . . . .	161
6.7	Mean and standard deviation of the dominant fatty acids in mussel mantles incubated with sunflower oil, at different incubation times. . . . .	163
6.8	Mean fatty acid composition of mussel mantles incubated with Tris buffer solution and no linseed oil, at different incubation times. . . . .	165
6.9	Mean fatty acid composition of mussel mantles incubated with linseed oil, at different incubation times. . . . .	166
6.10	Mean fatty acid composition of three replicated samples of linseed oil used for incubation with mussel mantles. . . . .	167

7.1	Water temperature registered in both experimental tanks, during the time of experiment. . . . .	174
7.2	Composition of mussel diet during the course of the experiment. . . . .	175
7.3	The number of mussels gaping at 12 hours intervals. Arrows show when food was supplied and sunflower oil added. . . . .	176
7.4	Number of foot extensions displayed by mussels at 12 hour intervals. Arrows show when food was supplied and sunflower oil added. . . . .	176
7.5	Number of mussel movements recorded at 12 hour intervals. Arrows show when food was supplied and sunflower oil added. . . . .	177
7.6	Mussel activity recorded at 24 hour intervals. . . . .	179
7.7	Weight of lipids extracted from the sampled mussels. . . . .	182
7.8	Variation of saturated fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	183
7.9	Variation of oleic and linoleic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	184
7.10	Variation of linolenic and octadecatetraenoic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	185
7.11	Variation of arachidonic and eicosapentaenoic fatty acid methyl esters in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	186
7.12	Variation of docosahexaenoic fatty acid methyl ester (FAME) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	187
7.13	Diagram of the concentration trends of fatty acids in mussels exposed to sunflower oil for 15 days of experiment. . . . .	189

3.9	Percentage composition of selected fatty acids from different authors compared with the ones obtained in this study. . . . .	82
4.1	Percentual errors (cv) on shell area measurements of 2 photographs from each mussel and maximum and minimum standard deviation in mm <sup>2</sup> . . . . .	99
A.1	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) for 8 weeks (T) in the control group. . . . .	200
A.2	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with rapeseed oil for 8 weeks (T). . . . .	201
A.3	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with linseed oil for 8 weeks (T). . . . .	201
A.4	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with olive oil for 8 weeks (T). . . . .	202
A.5	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with sunflower oil for 8 weeks (T). . . . .	202
A.6	Length of reference object as measured in the picture (Pic) and measured with calipers (Real). . . . .	203

7.1	Water temperature registered in both experimental tanks, during the time of experiment. . . . .	174
7.2	Composition of mussel diet during the course of the experiment. . . . .	175
7.3	The number of mussels gaping at 12 hours intervals. Arrows show when food was supplied and sunflower oil added. . . . .	176
7.4	Number of foot extensions displayed by mussels at 12 hour intervals. Arrows show when food was supplied and sunflower oil added. . . . .	176
7.5	Number of mussel movements recorded at 12 hour intervals. Arrows show when food was supplied and sunflower oil added. . . . .	177
7.6	Mussel activity recorded at 24 hour intervals. . . . .	179
7.7	Weight of lipids extracted from the sampled mussels. . . . .	182
7.8	Variation of saturated fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	183
7.9	Variation of oleic and linoleic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	184
7.10	Variation of linolenic and octadecatetraenoic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	185
7.11	Variation of arachidonic and eicosapentaenoic fatty acid methyl esters in control mussels and in mussels exposed to sunflower oil for 15 days . . .	186
7.12	Variation of docosahexaenoic fatty acid methyl ester (FAME) in control mussels and in mussels exposed to sunflower oil for 15 days . . . . .	187
7.13	Diagram of the concentration trends of fatty acids in mussels exposed to sunflower oil for 15 days of experiment. . . . .	189

# List of Tables

1.1	Mussel production (in metric tonnes) from the first ten producers in the world (Hickman, 1992). . . . .	4
2.1	Major lipid classes (Sargent, 1976). . . . .	38
2.2	Percentage of fatty acids in different oils (Menon <i>et al.</i> , 1989; Ackman and Ratnayake, 1989). . . . .	53
2.3	Oilseed production in million tonnes a year and oil content (%) of major oilseeds (Matthews, 1981). . . . .	54
2.4	Production and consumption of some Chinese oils in million tonnes a year (Gunstone, 1994d). . . . .	54
3.1	Operating conditions used in GC-MS and GC analysis. . . . .	61
3.2	Mean and standard deviation of the weight of one single cell and number of cells existing in 1 mg of cell's dry weight. . . . .	65
3.3	Analysis of variance for the number of <i>Pavlova lutheri</i> cells between replicates. . . . .	67
3.4	Analysis of variance for the number of <i>Tetraselmis chuii</i> cells between replicates. . . . .	67
3.5	Analysis of variance for the number of <i>Rhinomonas reticulata</i> cells between replicates. . . . .	67
3.6	Regression parameters and correlation coefficient for the calibration area of FAMES against their concentrations. . . . .	68
3.7	Percentage composition of fatty acids in three microalgae. . . . .	71
3.8	Average percentage fatty acid composition of 10 mussels collected from Beaumaris. . . . .	77

3.9	Percentage composition of selected fatty acids from different authors compared with the ones obtained in this study. . . . .	82
4.1	Percentual errors (cv) on shell area measurements of 2 photographs from each mussel and maximum and minimum standard deviation in mm <sup>2</sup> . . . . .	99
A.1	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) for 8 weeks (T) in the control group. . . . .	200
A.2	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with rapeseed oil for 8 weeks (T). . . . .	201
A.3	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with linseed oil for 8 weeks (T). . . . .	201
A.4	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with olive oil for 8 weeks (T). . . . .	202
A.5	Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with sunflower oil for 8 weeks (T). . . . .	202
A.6	Length of reference object as measured in the picture (Pic) and measured with calipers (Real). . . . .	203

# Chapter 1

## Introduction

### 1.1 The M. V. *Kimya*

The M. V. *Kimya* was a Maltese registered motor vessel en route on the January the 6th of 1991. from Spain to Birkenhead carrying in its tanks 1500 tonnes of crude sunflower oil. Due to stormy weather conditions, described as wind speed of gale force 10 and 25 foot waves, the vessel ran into difficulties and capsized 16 miles southwest of Holyhead, Anglesey with the cost of 10 lives among the 12 members of the crew. The ship drifted for 2 days before grounding off Llanddwyn Island. The cargo started to leak into the sea, and came ashore.

All the unsuccessful trials of salvage, including bringing the vessel to land, righting it and unloaded it safely, resulted in further leaks and the *Kimya*, now a wreck, ended up on a cove at Porth Twyn-Mawr, finally uprighted and leaking sunflower oil.

At the time of the accident local observations made by the coordinator of the Friends of the Earth group, revealed a thin sheen across the surface of the water and later bright yellow streaks of oil drifting north along the coast (Pritchard, 1991). The leaked sunflower oil, water insoluble, formed a sheen on the water surface, visible in the rock pools and an emulsion similar to gum. The smell was intense and spread along a considerable length of the coast.

Some shellfish mortality was registered at Porth Cwyfan and it was suggested that the cause of death was probably the diesel fuel from the wreck. Later that year, almost 11 months after the accident on site observations made possible a reevaluation of the situation. The water was clean, with no signs of oil leaking from the wreck. However

ashore of Porth Gro, remaining oil could be observed in different forms (Salgado, 1992). On the beach and on its more exposed side the oil formed blocks conglomerated with sand appearing like pebbles. In more protected areas, where the wave action probably was not so strong, a layer of sunflower oil penetrated the sand hardening it. In the rock pools the water became black and odorous due to the degradation of the oil. In some other pools closer to the intertidal zone the water was still turbid. In June 92 another survey on the area showed the wreck completely colonized by small mussels, but at Porth Gro beach there was still evidence of the oil contamination on the sands. The oil had penetrated a few centimeters into the sands, hardening it and tainting it of a black and odorous layer.

The presence of the leaked sunflower oil, after more than one year from the accident seemed to be localized but nevertheless persistent. It is its effects as a pollutant of marine biota that precipitated this study.

## 1.2 Sensitive areas

The bivalve culture along the Menai Strait which includes mussels, oysters and clams used both for consumption and research is a resource of economical and scientific significance. Apart from some shellfish mortality registered at Porth Cwyfan which was believed being caused by the release of the diesel from the vessel, it was not known what the impact could be of the sunflower oil to the local marine communities.

The *Kimya* grounded near the National Nature Reserve in the Cefni Estuary, home to migratory and breeding birds. Birds are most affected by oils, in that they cover their feathers, destroying their insulation function and impeding the flight resulting in hypothermia, exhaustion and ultimately death (Smith and Herunter, 1989).

The amenity value of the beaches was diminished, due to the presence of oil on the shore and its unpleasant odour. The visual esthetics of Porth Twyn-Mawr was only affected by the wreck at low tide when it can be seen.

Other vegetable oil spills of rapeseed and soybean oils showed that contrarily to what one may think, the effects of contamination with edible oils on wildlife are overlooked (Smith and Herunter, 1989; Gunstone, 1994a). Considering that the number



of birds dying, can be large and even higher than the number of deaths caused by petroleum spills, vegetable oil spills can not be neglected under the argument that they are biodegradable. Five hundred birds died in Vancouver Harbour in consequence of a rapeseed oil spillage, compared with 50 birds killed by petroleum spills occurred in the same place over a 5 year period. Another discharge of vegetable oil, 2.5 million gallon of soybean oil into the Minnesota River caused 4000 dead and 1300 injured birds. Moreover due to oxidation and polymerization the oils can increase their specific gravity and settle to the bottom where anaerobic conditions can rapidly develop and cause many damage to aquatic life (Gunstone, 1994a). Breeding grounds for fish or other aquatic resources can be seriously affected in these conditions.

### 1.3 Mussel economic importance

Mussels are used in most countries both for bait for fishing and for human consumption. As far as human consumption is concerned, France traditionally has the highest *per capita* of any country, and mytiliculture is a flourishing industry (White, 1937). Mussels now occupy the premier position in the world statistics for aquaculture production (Hickman, 1992). The total world production of mussels exceeded 1.1 million tonnes in 1988 (FAO, 1990). The vast majority of this comes from aquaculture. Over 20 countries now report regular harvests of farmed mussels but world production is dominated by two countries, China, with almost 40% of the total, and Spain with a further 20% (Table 1.1). The Netherlands, which traditionally rivalled Spain in mussels, has seen a dramatic decline in its production since the early 1980s. By far the majority of the farmed crop is the “blue” type of mussel, the various *Mytilus* species. Green mussels of the various *Perna* species are farmed in warmer waters, particularly in Thailand and Philippines, but also in China and New Zealand.

Other exploitable uses of mussels are the shells of the tropical members of the Mytilidae in that they give the pearls employed in jewellery and various ornamental articles. The byssus, too, may be so large in these forms that its fibres are used for the manufacture of cloths where clothing materials are scarce (White, 1937).

Country	Production	Species
China	429,675	<i>Mytilus edulis</i> <i>Perna viridis</i>
Spain	209,687	<i>Mytilus edulis</i> <i>M. galloprovincialis</i>
Italy	85,400	<i>M. galloprovincialis</i>
Netherlands	77,596	<i>Mytilus edulis</i>
Denmark	72,524	<i>Mytilus edulis</i>
France	54,873	<i>Mytilus edulis</i> <i>M. galloprovincialis</i>
U.S.A.	35,724	<i>Mytilus edulis</i> <i>Mytilus californianus</i>
Thailand	35,270	<i>Perna viridis</i> <i>Musculus senhouseni</i>
Germany	30,865	<i>Mytilus edulis</i>
Korea	27,356	<i>Mytilus crassitesta</i>

Table 1.1: Mussel production (in metric tonnes) from the foremost ten producers in the world (Hickman, 1992).

## 1.4 Preliminary studies

The fatty acid composition of mussels from the area of the accident were compared with the fatty acid composition of mussels from around the coast of Anglesey, including areas far away from the wreck to see if there was any difference of fatty acid composition in mussels exposed and non-exposed to sunflower oil (Salgado, 1992).

The results from this study showed clearly marked differences between the fatty acid composition of mussels exposed and non-exposed to the slick (Mudge *et al.*, 1993). The mussels in the vicinity of the wreck had increased amounts of oleic and linoleic fatty acids which are the major components of sunflower oil: 66% of linoleic and 21% of oleic acid, (Merck & Co., 1989), when compared with mussels from other areas. The increase of linoleic acid reached 12 times the normal level. It was demonstrated that linoleic acid can be used as a tracer of sunflower oil contamination because mussels take it up into their tissues reflecting the oil composition.

## 1.5 Aims of this study

Mussels were chosen as the target organism due to their known suitability as bioindicators of pollution (Phillips, 1980) and because they also represent an important local economic resource justifying the investigation of the effects of any pollution threats posed upon their culture. The primary objective of this work was to study the effects of sunflower and other vegetable oils on growth of mussels and survival; since the success of the species depends on adaptations to changing environmental conditions. The second objective was to evaluate vegetable oil interference with mussel tolerance to changes in temperature and salinity. A third objective consisted in determining the fate of the excess of fatty acids resulting from vegetable oil exposure, or in other terms whether mussels are able to metabolize them benefiting from their presence. The effect of sunflower oil on mussel behaviour was the last objective of this work.

## 1.6 Thesis organization

Following this introductory chapter, where the reasons for this work have been explained, Chapter 2 reviews some aspects of the biology and ecology of mussels including their common habitats and distribution. It also provides a description of the methods used to measure mussel growth discussing their advantages and disadvantages. The last part of this chapter is concerned with the biochemical composition of mussels and vegetable oils, describing and comparing fatty acid metabolism in animals with the system in plants. Some uses of the vegetable oils employed in this study are also supplied.

In Chapter 3 fatty acid analysis of the three biological materials used in the experiments is presented as a reference for the following chapters highlighting the characteristic fatty acid composition of each matrix analysed.

The effect of vegetable oils on survival and growth of mussels is studied in Chapter 4 explaining how shell areas rather than shell lengths can be used to measure growth in a reliable and practical way. The influence of vegetable oil exposure on mussel fatty acid composition is also given confirming the ability of mussels to accumulate the oils.

Further investigations on the effect of vegetable oils on the tolerance of mussels to changing salinities measuring the delay to open valves and gaping times is discussed in

Chapter 5. Fatty acid composition and mortality at different changes in temperature is also considered.

Chapter 6 is concerned with sunflower and linseed oil *in vitro* metabolism. Fatty acid composition resultant from the incubation of mantle extracts with the two oils is shown and its variations with time tentatively explained.

Mussel behaviour under sunflower oil exposure is analysed in Chapter 7. Biological activity responses namely gaping, movements and foot extensions activities are used to assess the effect of the oil and the importance of this kind of measurements in biological monitoring stressed.

Finally Chapter 8 concludes the thesis by summarising the main conclusions reached, identifying areas of further research and giving recommendations on the consequences of future vegetable oil spills.

# Chapter 2

## Mussel biology, growth and metabolism

### 2.1 Introduction

Marine mussels are among the commonest molluscs in the coastal zone. They are widely distributed since they can tolerate extreme fluctuations in salinity (Davenport, 1979; Davenport, 1981) and temperature (Almada-Villela, 1984). They are an important economical resource not only in aquaculture, due to their ease of handling and low cost of cultivation (Utting, 1987), but also in the pharmaceutical industry for their biochemical value in polyunsaturated fatty acids (Joseph, 1982). In addition, because they are sessile filter feeders and have been shown to be effective concentrators of trace toxic substances (Lee, 1972; Widdows *et al.*, 1990) they are now widely used as biomonitoring organisms for coastal water quality (Goldberg, 1975; Farrington *et al.*, 1987). Coastal and estuarine areas have received an increase in inputs of chemicals resulting from industrial activity (Widdows and Donkin, 1992), organic waste discharges (Widdows, 1985) and accidental spills at sea and mussels readily accumulate hydrophobic organic contaminants in their tissues with minimal metabolic transformation (Moore *et al.*, 1989). Therefore tissue residues reflect changes in the quality and quantity of contaminants in the environment (Burns and Smith, 1981). Through evolution they have acquired biological and physiological features which have enabled them to adapt to new environments and hence successfully occupy habitats unsustainable for other organisms (Seed, 1980). But do they possess adaptive mechanisms to cope with anthropogenic inputs like vegetable oil spills?

The following sections review the present state of knowledge on aspects of mussel biology, growth and lipid metabolism and will help the reader to understand the purpose and the methods employed in this investigation.

Under the title mussel biology, basic aspects of mussel taxonomy is briefly presented with mention of the main characteristics which distinguish the genus *Mytilus*, the target organism of this study, from other genera. Their geographical distribution is described along with the varied habitats and zonal range where they can be found. The main morphological and anatomical features of mussels are shown and causes of mussel mortality are discussed including vegetable oil spills as potential lethal agents.

The second part is concerned with mussel growth. Growth rate is an overall measure of performance of an organism in a certain environment (Widdows and Johnson, 1988). Changes in biotic or abiotic conditions by natural or accidental causes leading to deterioration in environmental quality can be monitored by measuring changes in growth. Its measurement represents therefore an important index of environmental pollutant effect (Widdows and Johnson, 1988). The methods most used to measure mussel growth are explored and their advantages and disadvantages are discussed. The biological and physical factors which influence growth, are also described.

The third part of this chapter is biochemically oriented since it reviews the lipid composition of mussels, their characteristic fatty acid composition and structure. The major metabolic pathways of fatty acid biosynthesis in animals are compared to the ones in plants emphasizing the enzymatic differences between the two kingdoms and the limited biosynthetic capacity of animals. A summary description of the uses of the four vegetable oils studied and their fatty acid composition is provided. Finally, values for present and future trends in world's vegetable oil production, puts into perspective the increasing necessity for trade and consequently the risks of spill accidents on shipment.

## **2.2 Mussel biology**

### **2.2.1 Systematics**

The sea mussel, *Mytilus edulis* is a Lamellibranch mollusc, belonging to the order Filibranchia, suborder Mytilacea and family Mytilidae. This family is believed to have

its origin as far back as the Devonian era, some 400 million years ago (Gosling, 1992). The genus itself, however, is of relatively recent origin, with records no older than the Pliocene (about 15 million years ago). *Mytilus* is a very old name, probably derived from the Greek word “mitilos”, meaning sea mussel (Gosling, 1992). White (1937) refers to the origin of the word “mussel” as Anglo-Saxonic origin *muscl*, *muscel*, *muscule*, *muscla* meaning that which instantly retires on being touched.

The order Filibranchia is characterized by the structure of the gills and by the presence of a well developed byssus organ (White, 1937). The gills are highly developed structures which enabled lamellibranchs to radiate in other aquatic habitats by freeing themselves from deposit feeding (Seed, 1980). In addition, the development of byssal attachment threads, coupled with the associated evolution of the heteromyarian shaped shell has enabled mytilid mussels to become independent of soft sediments (Seed, 1980).

The family is generally recognised by its shell form and sculpture, hinge structure and muscle scars, nevertheless because shell shape is extremely variable depending on environmental conditions and age of the animal, other internal, physiological and biochemical characteristics sometimes need to be used to correctly identify species, especially in regions of species hybridisation (Seed, 1976).

The genus *Mytilus* differentiates from the other genera in the family by the presence of a pitted resilial ridge, several hinge teeth, the presence of an anterior adductor muscle and a more or less continuous posterior byssus and foot retractor muscle scar (White, 1937).

### 2.2.2 Habitat

*Mytilus edulis* is a common intertidal species. It is widely distributed in the northern hemisphere. Examples occur in European waters extending from the White Sea (Former-Soviet Union) as far south as the Atlantic coast of southern France (Gosling, 1992). On the east coast of North America, *Mytilus edulis* extends from the Canadian Maritimes southwards to Cape Hatteras in North Carolina. Its presence has been confirmed in Iceland and it has been suggested that mussels from South America, Falkland and Kerguelen Islands are the same species (Gosling, 1992). Mussels are distributed worldwide and occupy many different habitats. They can adapt well to live on a broad

variety of microhabitats because mussels are tolerant to a great variation in temperature salinity, desiccation and oxygen tension (Seed and Suchanek, 1992). They extend from mild, subtropical locations to frequently frozen regions (Davenport and Carrion-Cotrina, 1981). Mussels tolerate salinity ranges from oceanic, brackish to nearly fresh waters and their zonal distribution goes from the high intertidal to subtidal regions (Seed and Suchanek, 1992). Adults are always attached by the byssus, to hard or semiconsolidated substrata. Subtidal populations often occur on seamounts piles, piers and platforms (Richardson and Seed, 1990), but *Mytilus edulis* preferentially occupies the intertidal zone. This zonal distribution of mussels is controlled by biological factors of predation and competition, rather than the inability to survive the conditions found in subtidal regions (Seed and Suchanek, 1992). They are commonly found in estuaries, seashores and harbours.

Although coastal areas are nutrient rich, due to the inputs of organic matter from terrigenous and antropogenic origin, they also contain contaminants from the same origin. Mineral and vegetable oil spills are examples of contaminants which entering the marine environment and reaching coastal zones can affect the species inhabiting these zones.

### 2.2.3 Morphology

#### Shell

The Lamellibranchiata are characterized by the presence of a double or bivalve shell which is secreted by the two lobes of the mantle (White, 1937). Primeval bivalves are thought to had single limpetlike shells which evolved into the typical laterally compressed bivalve form and the development of byssal threads for attachment resulted in considerable shell asymmetry (Seed, 1980).

The lamellibranch shell is composed of three layers; the outer, middle and inner layers. The outer layer, also known as periostracum, is composed by conchiolin, an albuminoid which makes the shell horny. This thin layer is frequently worn off in older shells. The middle or prismatic layer, is composed of prisms of calcium carbonate embedded in an organic matrix of conchiolin; the inner layer, a nacreous or pearly



layer, is laminated in structure and calcareous in composition. The calcium deposition in this layer is rhythmic and produces recognizable patterns of deposition (microgrowth bands) related with tidal and daily periodicities (Richardson, 1989). Once the new shell is formed it is possible to measure it as well as the period of time in which its formation took place. This valuable information is used to measure shell growth rates (see Section 2.3.2).

Inside the shell there are muscle scars (figure 2.1A) where the muscles (figure 2.1B) attach to the shell. The size and location of these scars often constitute taxonomical elements for species identification. The posterior adductor muscle scar is large, as is that of the posterior byssal retractor muscle. There is also a large posterior pedal retractor muscle scar located anterior to the byssal retractors but no anterior equivalent. The anterior adductor muscle scar is small and located on the antero-ventral edge of the shell, just anterior to hinge plate with approximately three denticulate hinge teeth. The anterior byssal retractor muscle scar is located on the posterior face of the shell, below the ligament. It is this difference in size of the anterior and posterior adductor muscles which constitutes a taxonomical characteristic between heteromyarian and isomyarian forms: the former is believed to have evolved from the latter (White, 1937).

Often called “blue mussel”, *Mytilus edulis* is especially wellknown for its colour polymorphism, varying from brown to blue-black, sometimes with a radial colour pattern evident (Morton, 1992). The general shape of the shell is that of an elongated triangle (mytiliform) with extreme reduction of the anterior face of the shell, expansion of the posterior face and ventral flattening (Morton, 1992). Shell form of *M. edulis* is, however, highly variable, according to habitat and age (Seed, 1976). Being highly gregarious, the consequent overcrowding prevents free development of any individual. Seen from the ventral side, the shell margin is to some extent sinusoidal. This slight curvature can vary with exposure conditions. Specimens living on exposed situations show a tendency for concavity, whereas animals inhabiting protected areas have a somewhat convex shell margin (White, 1937).

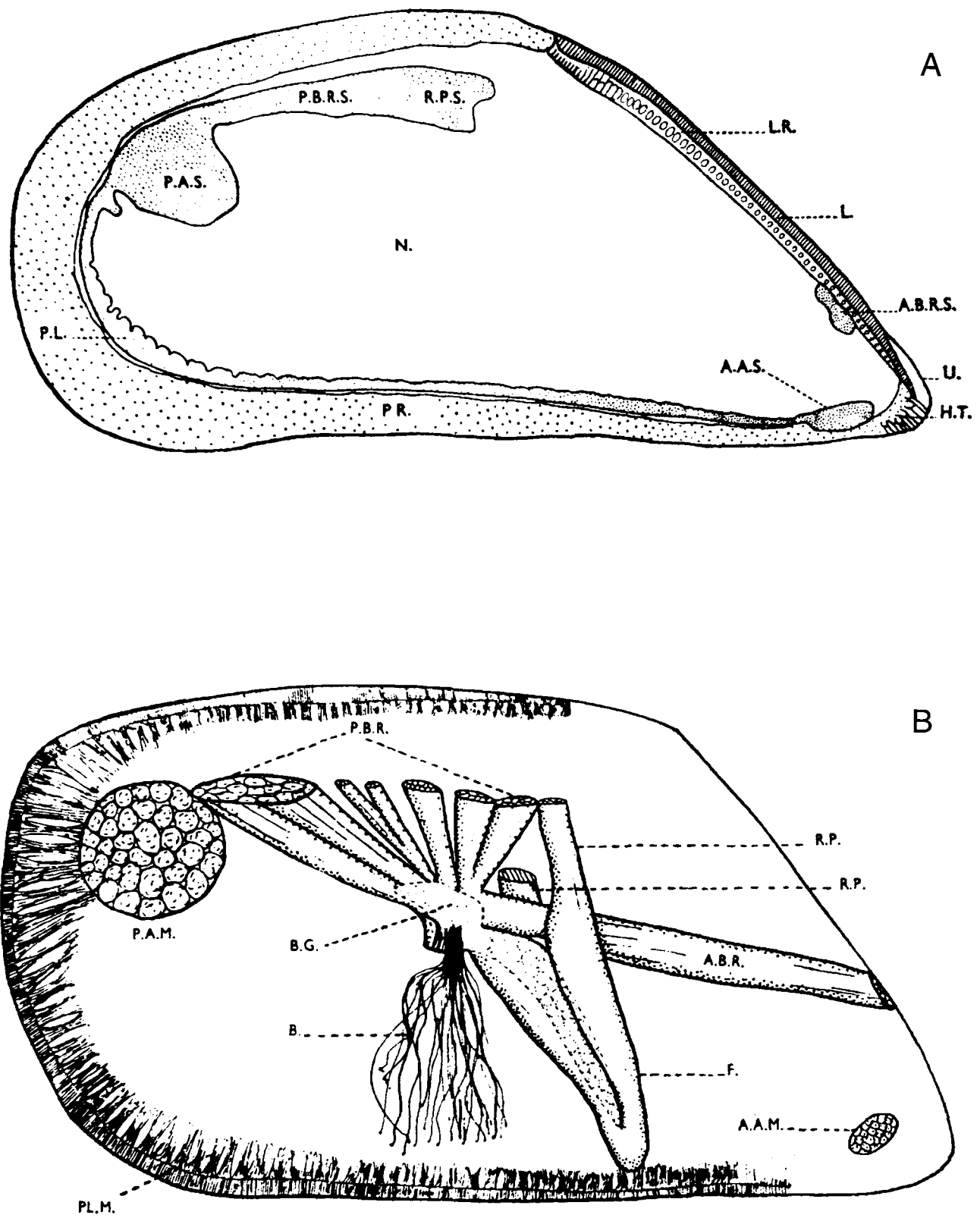


Figure 2.1: A-Muscle scars on the inner surface of the left valve of a *Mytilus* shell, B-Lateral view of the muscles and their distribution (White, 1937). For reference letters see page 14.

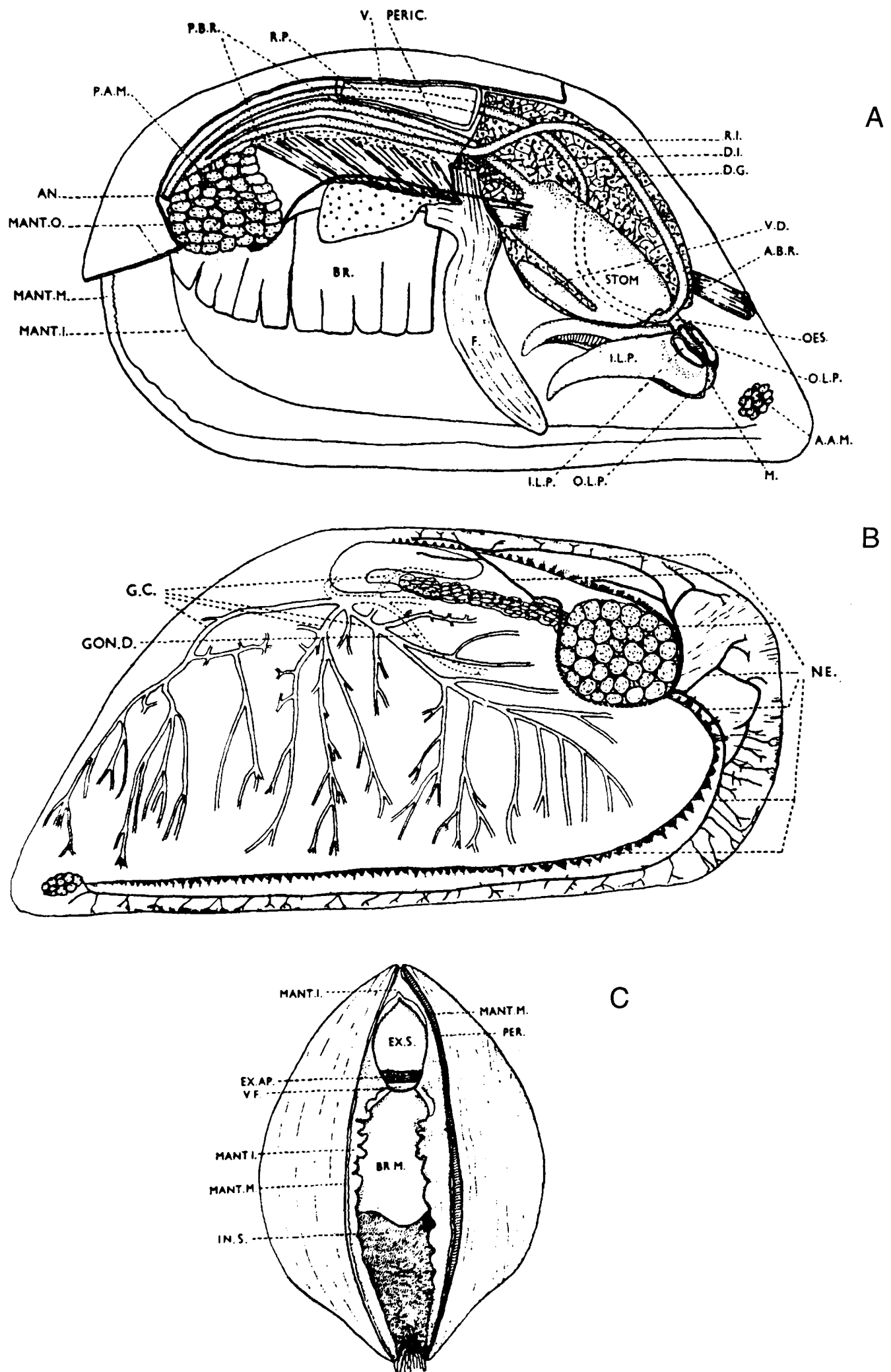


Figure 2.2: A- Visceral mass of *Mytilus*, B-Gonadal canals distributed through the mantle, C-Posterior view of the mantle *in vivo*, when immersed, showing the two siphons (White, 1937). For reference letters see page 14.

## Reference letters for figures 2.1 and 2.2

A.A.M.	Ant. adductor muscle	MANT.O.	Outer border of mantle
A.A.S.	Ant. adductor muscle scar	N.	Nacreous layer of shell
A.B.R.	Ant. byssal retractor muscle	NE.	Nerves
A.B.R.S.	Ant. byssal retractor musc. scar	OES.	Oesophagus
AN.	Anus	O.L.P.	Outer labial palp.
B.	Byssus	P.A.M.	Post. adductor muscle
B.G.	Byssal gland	P.A.S.	Post. adductor muscle scar
BR.	Branchia		
BR.M.	Branchial membrane	P.B.R.	Post. byssal retractor
D.G.	Digestive gland	P.B.R.S.	Post. byssal retractor musc. scar
D.I.	Direct intestine	PER.	Periostracum
EX.AP.	Exhalant aperture	PERIC.	Pericardium
EX.S.	Exhalant siphon		
F.	Foot	P.L.	Pallial line
G.C.	Genital canals	PL.M.	Pallial muscle
GON.D.	Gonoduct	PR.	Prismatic layer of shell
H.T.	Hinge teeth	R.I.	Recurrent intestine
I.L.P.	Inner labial palp	R.P.	Pedal retractor muscle
IN.S.	Inhalant siphon	R.P.S.	Pedal retractor muscle scar
L.	Ligament	STOM	Stomach
L.R.	Ligament ridge	U.	Umbo
M.	Mouth	V.	Ventricle
MANT.I.	Inner border mantle	V.D.	Stomach ventral diverticulum
MANT.M.	Middle border mantle	V.F.	Ventral velum

## Mantle

The mantle of the Lamellibranchs consists of two thin lobes which are attached to one another and to the visceral mass dorsally, and then extend down each side of the body as far as the ventral surface. Through the separated lobes of the mantle there is a deep, densely ciliated, rejectory tract where water, with food and dissolved oxygen enters and leaves with the waste products of respiration, digestion and excretion. The rejected material in the form of a mucus-bound pseudofaecal string, is transported posteriorly towards the inhalant aperture and eventually rejected (White, 1937). The mantle contains much of the gonads extending into both lobes (figure 2.2B), which, during the reproductive season, sometimes increases by a factor of 100 fold in thickness. The reproductive cells, when mature, escape through the rejectory tract in the same way as the waste products. Part of the nervous and vascular systems of the mussel are also found in the mantle (figure 2.2B).

The mantle is normally of a creamy colour except on the posterior edge, but during the period of sexual activity the colour becomes more intense, especially in the female, where it may be a deep reddish orange. The edge of the mantle in *M. edulis* is divided into three typical folds. The outer fold, containing the pallial muscles; the middle fold which secretes the periostracum and a very thick inner fold, which thrusts well out of the shell and can be seen when the mussel is gaping. It forms a dorsal exhalant aperture and a ventral inhalant one (White, 1937). The edges of these apertures are drawn out and form a more or less elongated tube, the exhalant or dorsal siphon and the inhalant or ventral siphon (figure 2.2C). The exhalant siphon is small and conical and releases the waste water; the inhalant siphon is extensive possessing a posterior brown pigmented epithelium with sensory papillae and accepts the water in. Due to the presence of the sensory papillae on the edge of the inhalant siphon, it is very sensitive to stimulation, and retracts at once on being touched or subjected to change of light intensity (White, 1937).

## Ctenidia

The gills, more correctly referred to as ctenidia, are typical of the Mytiloidea in that they are flat and filibranch (Morton, 1992). The ctenidia are involved in both respiration and feeding. Each ctenidium comprises a pair of demibranchs, inner and outer which divide the pallial cavity into inhalant (infrabranchial) and exhalant (suprabranchial) chambers. Each demibranch is constituted by two lamellae, one ascending, the other descending, which are held together by connective tissue junctions. The lamellae are made up of ciliated filaments irrigated by blood vessels which receive the oxygen required for respiration from the water passing through (Morton, 1992).

Water is driven from the inhalant chamber to the exhalant chamber by lateral cilia arranged along the sides of the filaments. Rows of laterofrontal cilia filter the water and transfer particles onto the apex of the filament where they are transported along the food acceptance tracts. It is due to this feeding mechanism that mussels are classified as filter feeders (Morton, 1992). The filtration rate of bivalves, that is the volume per unit time of water drawn into the animal's body is known to be influenced by environmental parameters such as salinity, temperature, dissolved oxygen and concentration of suspended matter (Abel, 1976) and because of that it is often used to assess the effects of pollutants on this particular physiological response (Bayne *et al.*, 1982; Davenport and Woolmington, 1982; Widdows *et al.*, 1987).

## Foot

The foot (figures 2.1B and 2.2A) is of the typical mytilid form, *i.e.* long, highly mobile and plantar (Morton, 1992). Its colour is frequently brown due to the presence of brown pigments in its ciliated epithelium. Its major functions involve adhesion to substrata, excretion and locomotion (White, 1937). On its posterior side the foot has a deep groove which extends the whole of its length. It commences proximally at the aperture of the byssus and terminates distally in a rounded depression. This depression is used as a sucker by means of which the animal can adhere to a solid structure. At rest it projects into the anteriormost reaches of the mantle cavity. Its role in excretion is made possible by the existence of powerful ciliary currents, on the dorsal region of the

foot, which pass unwanted material postero-dorsally and then postero-ventrally to the posterior edge of the visceral mass, where it falls onto the mantle below to be rejected (Morton, 1992). The main bulk of the foot is made up of longitudinal muscle fibres which are direct continuations of the paired pedal retractor muscles. These muscles work as propellers and together with the byssal muscles which slacken or tighten the byssal threads, enable mussels to move (White, 1937).

### **Byssus**

*Mytilus edulis* is the species with maximum development of byssus. They are of great importance to the larval stages in providing attachment and their retention in the adult forms (by neoteny) was determinant in the successful radiation of bivalves (Seed, 1980). They serve two purposes. The most obvious one is to provide attachment of the mussel to the substratum; the other is to enable its locomotion by alternately attaching and releasing the byssal threads. The byssus complex is composed by muscles, a byssogenous cavity where the byssal threads are formed and the byssal threads themselves (figure 2.1B, P.B.R., B.G. and B.). The byssal retractor muscles are paired and inserted anteriorly and posteriorly into the shell (see the scars in figure 2.1A). They work as muscles retractors of the byssus. The byssus cavity contains muscle fibres and glands where the byssal threads originate. The threads are thin transparent fibres moulded together to form the stem or axis. Each thread has an adhesive disc at the most external portion of the byssus with which the mussel attaches itself to the substratum. The foot extends with its tip attached to the substratum and the threads secretion passes through the foot groove until it reaches the substratum. When the threads are in place the foot groove opens and the water becomes in contact with the threads hardening them (White, 1937). A cement-like secretion from the purple foot gland collects on the distal end of the thread forming the adhesive disc. The growth of the stem depends on the strain put upon it by the byssal threads. If it is too strong the stem is cast off and a new one started.

Rate of thread formation can be used as an informative index of mussel activity and as a measure of toxicity of pollutants. The number of threads formed per mussel

per unit time has been applied to examine the effects of environmental factors such as exposure to air, salinity and temperature on byssal thread formation (Winkle, 1970) and in the study of sublethal effects of metals on *M. edulis* byssal thread production (Martin *et al.*, 1975).

### Visceral mass

The visceral mass contains the mouth, the stomach and the gut (figure 2.2A). The mouth is located anteriorly between the inner and outer labial palps and connects to a long oesophagus that opens into the stomach (Morton, 1992). The stomach determines which potential food material is eventually accepted for intracellular digestion in the digestive diverticula using three ciliated food-sorting areas. The midgut leaves the stomach postero-dorsally and returns over the stomach, penetrating postero-dorsally the ventricle of the heart and terminates in an anus over the posterior adductor muscle (Morton, 1992).

### Pericardium

The pericardium of *M. edulis* is a thin membrane continuous with the body wall (White, 1937). It is located just below the dorsal edge of the shell (figure 2.2A, PERIC.). It comprises a three-chambered heart with a single ventricle and two lateral auricles (Morton, 1992). The ventricle occupies the whole length of the pericardium and communicates with the two sided auricles by a wide aperture. This aperture contains a valve which allows the blood flowing from the auricles into the ventricle, but not the reverse flow. Covering the auricles there are two pericardial glands that belong to the excretory system.

#### 2.2.4 Mussel mortality

There are several natural factors responsible for the mortality among mussels. These can be divided into physical and biological factors. Extremes of physical factors such as storms (by dislodgement), temperature and desiccation, and excessive deposition of silt are all known to cause mortality in mussels (Seed and Suchanek, 1992). These factors vary seasonally and their additive effect can occasionally result in mass mortalities



(Seed and Suchanek, 1992). Physiological intolerance to desiccation is probably the single most important factor determining the upper limits of mussel zonation. Storm generated waves can also cause extensive mussel mortality, especially among *Mytilus edulis* which is more vulnerable than *Mytilus californianus* because its shell is less robust and its byssal attachment is weaker (Seed, 1976). High levels of silt, inorganic detritus and biodeposits produced by the mussels themselves can also lead to suffocation (Seed and Suchanek, 1992).

Biological factors causing mussel mortality fall into four main categories: predators, parasites, pathogens and competitors for food and space. Of all the potential mortality factors, predation plays a major role because many species feed on mussels and amongst the most significant are gastropods (Hughes and de Dunkin, 1984), starfish (O'Neill *et al.*, 1983), crabs (Rheinallt, 1986) and birds (Bustnes and Erikstad, 1990). All are known to forage selectively on specific size ranges of *Mytilus*. Consequently, these predators have the potential to influence population size structure as well as abundance and local distribution patterns (Seed and Suchanek, 1992). The way for mussels to escape predation is to grow out of the size range normally taken by the predators. The length of time for which they remain vulnerable will depend on growth rate and this in turn is a function of geographic location and tidal elevation (Seed and Suchanek, 1992). The dogwhelk feeds extensively on mussels from the intertidal zone of exposed rocky shores, whereas the starfish attacks the mussels from the low intertidal. Periodically the numbers of starfish *Asterias rubens* can rise dramatically and lead to a denuding of *Mytilus* from the middle and lower shore (Seed, 1976). These predators influence the distribution of mussels on the lower shore. Among birds oystercatchers, sandpipers, knots, various species of duck and gulls are known to feed on *Mytilus* (Bustnes and Erikstad, 1990). Crabs can also take large numbers of mussels in their diet. Mortality from crab predation is generally most intense in the low shore and sublittoral where crabs are most abundant and where they can feed for longer periods. Mussel mortality is most intense between spring and summer because the littoral crab population migrates into deeper water during winter (Seed and Suchanek, 1992). Prey density seems to influence the feeding habits of crabs and in order to survive in most rocky shores, mussels must settle at densities in excess of 10000 per square metre (Seed,

1976). Some species of fish such as plaice and flounder also feed on mussels, especially in flat sandy areas (Dare and Edwards, 1976). Limpets and sea urchins may account for some mortality on the lower shore and amongst young mussels. In certain localities mammals such as seals, sea otters and walrus have been reported to take limited numbers of mussels (Seed, 1976).

*Mytilus* can harbour numerous parasites that are not generally thought to cause substantial mortality but they usually weaken the host leading to disease and reduced growth (Seed, 1976). Larval trematodes have been found in mussels (Jameson and Nicoll, 1913). Sporocysts of certain forms can damage the gonad and may cause castration (Seed, 1976). Pea crabs commonly encountered in the mantle cavity of *Mytilus* compete with its host for food and can damage the gill tissues (Seed, 1969). The red-worm *Mytilicola intestinalis*, unlike the pea crab, has received the greatest attention because it was thought to be responsible for heavy mortalities on the Dutch beds in 1950 (Seed, 1976). It is generally accepted that the presence of the parasite may cause loss of condition and even death depending on the degree of infestation. Reduced filtration rates and abnormal gonad development have been reported in infested mussels by red-worm (Meyer-Waarden and Mann, 1951; Mann, 1956) but it also destroys the microvillar border of the intestinal epithelium (Giusti, 1967). *Mytilicola* is estuarine, occurring in sandy or muddy bays where water movement is sluggish and salinity lowered. Mussels higher in the littoral zone, and those raised from the bottom, are generally less infected since the infective copepodid stage crawls close to the sea bed (Seed, 1976).

Mussels are the dominant competitors for space amongst the ground-covering organisms on horizontal or gently sloping rocky shores. Only rarely are other species able to displace mussels from mid to lowshore sites (Seed and Suchanek, 1992). On steeper rock faces, mussels tend to be replaced by more effective competitors like barnacles or algae (Lewis, 1964). Interspecific competition can occur between *Mytilus edulis* and *Mytilus californianus* sometimes leading to exclusion of one species (Harger, 1972), but usually they can coexist due to differences in life strategies. Intraspecific competition can also be a major factor of mortality as a result of overcrowding. The underlying mussels are suffocated or deprived of food. This becomes more acute in fast growing populations (Richardson and Seed, 1990). Occasionally, hummocks are formed where

the centrally positioned mussels do not have direct contact with the substratum resulting in instability and easy mussel detachment by strong wave action and tidal scour. However, this is more appropriately called emigration rather than mortality, since some of these mussels will survive to colonise other areas (Seed, 1976).

Fouling organisms are increasingly being recognized as significant sources of mortality in littoral and sublittoral mussel population (Seed and Suchanek, 1992). Mortality usually occurs when mussels are dislodged as a result of the increased weight or shearing stresses in the form of drag or lift imposed on them by the fouling organisms, especially barnacles and seaweeds (Witman and Suchanek, 1984). In general, organisms which bore into the mussel shell may cause mortality indirectly by weakening the shell structure, thus increasing mussel vulnerability to predators (Seed and Suchanek, 1992). Blooms of toxic algae can occasionally result in high mortality although their effects appear to be mainly sublethal (Tracey, 1988). High natural mortality has also been reported in some populations of *M. edulis* at times of metabolic stress (Emmett *et al.*, 1987). This appears to be related to the relatively poor condition of post-spawned mussels when nutrient reserves in the mantle are at their lowest levels (Worrall and Widdows, 1984).

It is believed that pollution only becomes a significant factor of mussel mortality when they are stressed or weakened by disease (Sunila and Lindstrøm, 1985). In addition, mussels are able to flourish near sewage outfalls (Bøhle, 1972) where the organic load is high. Nevertheless the present study indicates that vegetable oils can cause mussel mortality both in laboratory conditions and in the wild (Mudge *et al.*, 1993).

### 2.3 Mussel growth

Growth has been extensively documented in *Mytilus* (Stromgren, 1975; Stromgren, 1976a; Almada-Villela *et al.*, 1982; Almada-Villela, 1984; Gruffydd *et al.*, 1984; Kautsky, 1982; Kiørboe *et al.*, 1981; Loo and Rosenberg, 1983; Manley *et al.*, 1984; Redpath, 1985; Richardson *et al.*, 1990) partly because of its commercial importance and partly because its growth history is permanently recorded in the shell as a series of growth checks, a feature which makes these animals especially amenable to growth studies

(Seed and Suchanek, 1992).

There are two principal methods of assessing growth. Firstly, the size of the whole organism can be related to age determining the absolute growth, or if it represents the percentage increase per unit of time then it is called relative growth. Secondly, allometric growth can be measured, where the rate of growth of one parameter, for instance dry weight, is related with the rate of growth of another (shell growth) (Seed and Richardson, 1990). Since growth is defined as an increase in body size, weight or volume are appropriate parameters for its measurement. However, the shell is such a prominent feature of molluscan anatomy with great variability in shape, thickness and amount of water retained in the mantle cavity, that growth is generally measured in terms of shell length (Seed, 1976). This parameter has the additional advantage of being easily measured without having to detach the animal from the substratum, and can then be related to weight, volume or even energy content by one or more allometric functions (Seed, 1976; Seed and Suchanek, 1992). Several methods have been used to assess absolute growth in bivalves, but some have received particular attention:

- Analysis of size frequency distributions.
- Growth checks on or within the shell.
- Estimates from physiological measurements.
- Other direct measurements of shell growth.

### 2.3.1 Size frequency distributions

When recruitment to the population is seasonal, individual year classes can be identified as distinct modes in plots of size frequency distributions. Changes in the position of these modes over time enable the mean growth rate of each year class to be estimated. This method of growth assessment relies on identification of individual year classes, which is not always feasible. Particularly in *Mytilus* this method has a limited application because recruitment occurs for extended periods and the variable individual growth rates usually result in an inevitable overlapping of age classes (Seed, 1976; Kautsky, 1982). Sometimes this difficulty can be overcome with mathematical techniques, but

the use of this estimate is limited because it only gives information about the average growth of mussels and fails in retrieving information about the growth dynamics of the population (Seed and Richardson, 1990).

### 2.3.2 Growth checks on or within the shell

Many bivalves, including mussels, produce growth rings on the surface of the shell (Seed and Richardson, 1990). They are deposited during periods of suspended shell growth, and maybe related with various environmental factors including seasonal changes in temperature or food availability, prolonged stormy weather, or even with the annual reproductive cycle. Consequently, they can not be assumed to be annual in origin, and even when annual rings are present their use in age determination can be confounded by other non-annual growth checks (Seed and Suchanek, 1992). Also, shell abrasion can restrict the use of this technique for the determination of absolute growth because the early rings may be worn away (Seed and Richardson, 1990). In older mussels the growth is so small that the rings are deposited too close together to be resolved (Seed, 1976).

Microgrowth bands within the shell can be more accurately used to detect variations in individual growth rates. One advantage of this method, relative to more conventional methods such as size frequency analysis and shell surface growth rings, is that with a single population sample is possible to measure growth rates. This is particularly useful in places like offshore platforms where repeated population sampling may be difficult or impracticable (Richardson *et al.*, 1990). The main problem with this method is that in *Mytilus edulis* the growth lines are poorly preserved in the shell. Well defined growth bands are only deposited when mussels are emmersed and therefore clearly seen in intertidal mussel shells. Subtidal mussels show much weaker bands and it seems their deposition is related to an internal rhythm rather than tidal or daily cycles (Richardson, 1989). In these cases, it is necessary to first establish the periodicity of the internal deposition pattern and only then is it possible to determine individual growth rate.

### 2.3.3 Physiological measurements

As an alternative of direct measurement of mussel growth, it is common to use physiological responses as estimates of the growth potential usually termed “scope for growth” (Seed and Richardson, 1990). The energy available for growth is determined from physiological analysis of the energy balance equations 2.1 and 2.2 which include the energy inputs (food intake) and energy expenditures (respiration and excretion) (Widdows and Johnson, 1988)

$$C = P + R + U + F \quad (2.1)$$

$$P = A - (R + U) \quad (2.2)$$

where C is the total consumption of food energy, P is the production of both somatic tissues and gametes, R is the respiratory expenditure, U is the energy lost as excreta, F represents the faecal energy loss and A is the absorbed ration. The result from this balance (P) determines the potential for growth. When integrated over time and applied to individuals of different sizes these data can be used to derive an average growth curve. It provides a good method for quantifying the responses of individual mussels to changing environmental conditions such as food supply, temperature, salinity and contaminants (Seed and Suchanek, 1992). Another advantage is the possibility of growth assessment during short-term experiments. Also it is particularly useful in assessing the biological effects of pollution (Widdows and Johnson, 1988) as it includes several components of the animal physiology (feeding rate, respiration and excretion), which affect changes in growth rate. The disadvantage is that it does not differentiate between energy used for somatic growth and reproductive output (Seed and Suchanek, 1992) and the estimated growth has to be compared with other direct growth determinations. Widdows and Johnson (1988); Widdows *et al.* (1990) used physiological energetics (scope for growth) to quantify the sublethal biological effects along an environmental pollution gradients. In both studies, there was a positive relation between the pollution gradient and the reduced scope for growth mainly due to the lower feeding rate and or enhanced oxygen uptake caused by the presence of pollutants. There is evidence that this method of estimate growth is in good agreement with other more direct methods

(Bayne and Worrall, 1980) and can be used to diagnose the classes of pollutants that cause reduction in growth and their mode of action by determining which component of the energy balance was affected (Widdows *et al.*, 1990).

### 2.3.4 Direct measurements

Experimental cages containing marked mussels have been extensively used to analyse the effects of size, season and different environmental conditions on growth (Seed and Richardson, 1990). Providing that caging itself does not disturb growth rate through its effects on water movement and food supply, this method provides valuable information in the analysis of seasonal growth in mussels of different initial size and in different environmental conditions (Seed, 1976). The caging effect is, according to studies by Harger (Harger, 1970), more critical in intertidal than in subtidal growth studies. Because intertidally wave action is stronger, the amount of open surface of the cage becomes more important in modifying the water currents and food availability for mussels and consequently their growth.

A laser diffraction technique was developed by (Stromgren, 1975) and later adopted by other authors (Manley *et al.*, 1984; Almada-Villela *et al.*, 1982; Gruffydd *et al.*, 1984) to obtain precise measurements of linear growth with a standard error of 3  $\mu\text{m}$ . With this technique the length increase of the shell is measured as a decrease of a slit, 100–600  $\mu\text{m}$  wide, formed between a silver fixed edge and the growth edge of the shell. The physical device used to measure the small decreases in length is a laser of low output power (1 mW) which produces a diffraction pattern when its light passes through the slit. The diffraction pattern consists on a series of regular dark and white bands with an important and useful characteristic. The distance between any two bands in the diffraction pattern is inversely proportional to the width of the slit. In this way the width of the slit can be easily calculated and its decrease with time determined. This reflects the increase of the shell in length. This elegant and precise way of measuring growth has a good aplicability on pollution studies and in the assessment of the effect of various environmental parameters on growth (Almada-Villela *et al.*, 1982; Almada-Villela, 1984; Manley *et al.*, 1984; Gruffydd *et al.*, 1984; Redpath, 1985). There is, however, one major drawback; the width of the slit must be small (100–600  $\mu\text{m}$ ), in

order to obtain a good diffraction pattern, which requires adjustment when the shell grows beyond the silver edge. Other technical constraints exist; the edge must be fixed in a parallel position relative to the edge of the shell and the shell itself needs to be closed at the moment of measurement otherwise gaping shells will affect the optical conditions of the measuring process. Davenport and Glasspool (1987) discuss some of the problems of using this method on a routine basis. The more relevant ones in terms of accuracy and useability seem to be the presence of a fixed pointer in close proximity to the growing shell edge, which may inhibit normal growth in that region of the shell; also the method becomes time consuming when there are tens of shells to be measured. Furthermore, changes in only one dimension (length) will be detected. The same authors proposed a photographic technique for the measurement of short term shell growth in bivalve molluscs. It consists of photographing a number of experimental animals, mounted firmly on a plate, at regular time intervals and digitizing manually the negative images of the shell outlines with the X-Y coordinates of these points stored with the aid of a minicomputer. A reference object of known dimensions needs to be photographed and digitized the same way as the shells to enable the calculation of the shell areas. One main achievement with this technique is that not only information about size is determined but also the changes in shell shape can be monitored over time. Photographs used to determine growth changes are useful, fast, many shells can be photographed at the same time and they are reliable when compared to the laser diffraction method (Davenport and Glasspool, 1987). Nevertheless, the manually operated digitizer employed by Davenport besides being time consuming, it is not satisfactory due to lack of accuracy. The method used in this research, discussed later in Chapter 4, is based on this photographic technique but brings forward an important improvement relying on an electronic scanner rather than a manual process for the acquisition of the image.

### **2.3.5 Quantitative expressions of growth**

Each habitat seems to impose, by its resource limiting environmental conditions, a maximum size beyond which further growth proceeds only slowly, if at all (Seed and Suchanek, 1992). The fast growing individuals approach this limit relatively quickly, whereas in locations of slow growth this limit may be attained only by much older



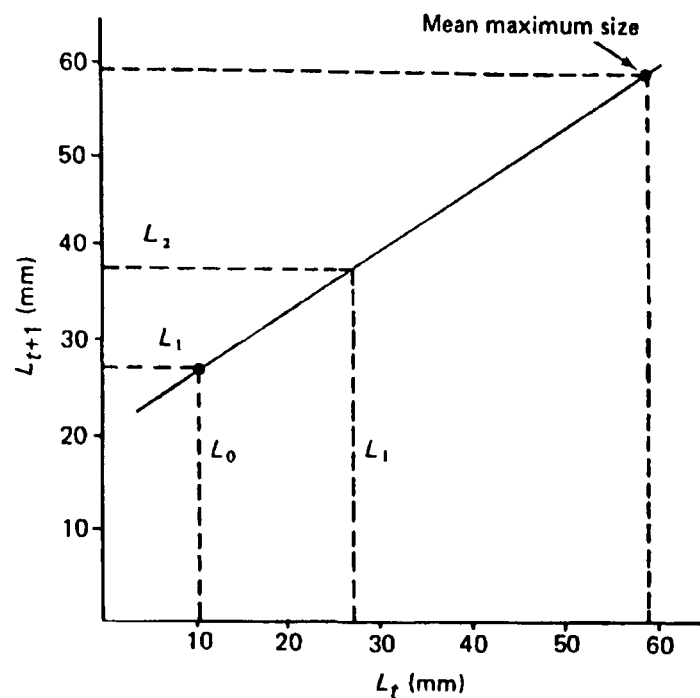


Figure 2.3: Walford plot (Cerrato, 1980).

individuals (Seed, 1976). The maximum potential size ( $L_\infty$ ) of individuals within a population under any set of environmental conditions may be approximated using the Ford-Walford plot, where length at  $t + 1$  years is plotted against length at  $t$  years (Seed and Suchanek, 1992). Maximum size ( $L_\infty$ ) is obtained where the line of best fit intercepts a point of zero growth when  $L_t = L_{t+1}$  (Figure 2.3).

This parameter is basic to many growth equations, two of which, the von Bertalanffy and the Gompertz, presented below, have been widely used in fisheries biology and to describe and compare growth rates in *Mytilus* (Bayne and Worrall, 1980). The von Bertalanffy equation provides a good description of growth when applied to fast growing populations showing no obvious growth inflexion points:

$$L_t = L_\infty [1 - e^{-k(t-t_0)}] \quad (2.3)$$

where  $k$  is the growth rate constant reflecting the rate at which maximum size ( $L_\infty$ ) is approached and  $t_0$  the time when  $L_t = 0$ . The Gompertz equation is similar but uses the logarithms of length rather than length itself:

$$\log L_t = \log L_\infty [1 - e^{-k_1(t-t_1)}] \quad (2.4)$$

where  $k_1$  is the growth rate constant and  $t_1$  is the time when  $L_t = 1$ . Sigmoidal growth curves of the Gompertz type appear to be more characteristic of slower growing popula-

tions (Seed and Richardson, 1990). Both equations assume that growth is determinate and that some maximum attainable size exists for any given population. Yet growth in many bivalves, including mussels, may not always be determinate and may not cease at any fixed adult size, at least over their realized life span (Seed, 1980). It has been suggested (Thiesen, 1973) that von Bertalanffy equation 2.3 may only be valid for *Mytilus* above one-third of their maximum size and that for smaller mussels the Gompertz equation 2.4 should be used instead. Neither of the two equations has into account the seasonal changes of temperature on growth variation although attempts to include a variable relating to temperature and time (day-degrees) have been made. Evidence exists that  $L_{\infty}$  may depend on temperature since results by (Seed, 1976) showed that arctic mussels grow slowly and generally attain a large size (77.5–283.9 mm in Greenland). Despite these difficulties and criticism by some authors, curve fitting by means of these equations is a perfectly acceptable technique providing there is evidence for the existence of a real asymptote and that it is appreciated that some degrees of uncertainty are always associated with such estimates.

### 2.3.6 Allometric growth

So far we discussed methods to determine absolute or relative linear growth rates of mussels in terms of increase in body size. However, mussels, like most organisms, exhibit progressive changes in their relative shell proportions with increase of body size (Seed and Suchanek, 1992). It is clearly evident that body shape does not always change uniformly with an absolute increase in size of the whole animal (Seed, 1980). Of the various environmental factors that are known to influence shell shape in bivalves, (growth rate, population density and degree of exposure to wave action), population density (overcrowding) seems to be particularly important in the case of *Mytilus* (Seed and Suchanek, 1992). The shells of densely packed mussels are proportionately more elongate with greater length to height ratios than those from less crowded conditions. In addition, increases of mussel age and size contribute to the gradual change in body proportions giving rise to disproportionate growth. Older mussels generally have relatively heavy elongate shells where width frequently exceeds shell height. The study of the different ratios of growth between two parameters of the body or between one

parameter and the whole organism is termed allometry and the relationship between any two size variables ( $x$  and  $y$ ) can be expressed by the allometric equation

$$y = ax^b \quad (2.5)$$

where  $a$  and  $b$  are constants. The growth coefficient  $b$  represents the relative growth rate of the two variables and  $a$  is the value of  $y$  when  $x$  is unity. Rewritten in logarithmic form, the allometric equation becomes

$$\log y = \log a + b \log x \quad (2.6)$$

If  $y$  and  $x$  are allometrically related, the plot on logarithmic coordinates gives a straight line being  $b$  the slope and  $\log a$  the intercept. These two parameters of the transformed data are estimated by regression analysis. When the two variables being considered have the same units of measurement, a value of unity for the exponent  $b$  is said to describe an isometric relationship in which the relative growth of the two variables is identical, thereby maintaining geometrical similarity with increasing size. Values of  $b$  greater than unity indicate that  $y$  is increasing relatively faster than  $x$  (positive allometry), whereas values of  $b$  less than unity indicate the reverse (negative allometry). If the dimensions of  $x$  and  $y$  differ, then different criteria for isometry and allometry will apply. As an example if  $y$  is a weight or volume with dimension of ( $L^3$ ) and  $x$  is length with the correspondent dimension ( $L$ ), then the value of  $b$  which makes the relationship isometric is no longer  $b=1$ , but rather  $b=3$  (Seed, 1980). A more or less isometric relationship exists between length and width in *Mytilus edulis*, the rate of increase in shell height decreases in the larger size mussels. Volume, shell and tissue weights on the other hand, exhibit a characteristic exponential relationship. The allometric exponent  $b$  in equation 2.5 remains relatively constant over a wide range of mussel size which means that the rate of increase of the two variables ( $x, y$ ) is proportional. Changes in relative proportions may be associated with the maintenance of physiological favourable surface area to volume ratios as body size increases; or they may reflect adaptive responses to changing environmental conditions.

The allometric equation has found extensive use in physiological investigations and in studies of mussel production for the estimation of flesh weights from shell length

measurements. It provides simple and useful comparisons of the variability in shell proportions among different bivalve populations and valuable insight into relationships between shell shape and environmental change in space and time (Seed, 1980). Nevertheless its use is not exempt from criticism because it only compares two variables in terms of their proportions and does not account for change of shell shape which normally occurs during the growth process (Davenport and Glasspool, 1987).

Although several methods can be used to determine growth, each has its intrinsic problems. Probably the most reliable estimates are those obtained using a combination of methods (Seed, 1976), but that is not always feasible.

### 2.3.7 Factors influencing growth

One of the features of mussel growth is the variation of growth rate. This variation occurs not only between localities but also within similar size and age groups in the same population. Even mussels grown under apparently identical conditions can exhibit widely different growth rates. Variation is such that whilst in ideal conditions *Mytilus edulis* may grow very rapidly, sometimes exceeding 60–70 mm within twelve to eighteen months. In unfavourable conditions growth may be very slow, some individuals measuring only 20–30 mm after perhaps fifteen to twenty years (Seed, 1976). It is known that growth variation is at least determined by genotype, but this source of variation is probably minor compared with those resulting from environmental factors. In the following sections the main biological and environmental factors influencing growth are discussed.

#### Age and size

Increasing age is accompanied by a decline in growth rate. This may occur to such an extent that in very old individuals, growth in length may virtually cease. One possible explanation is the reduced metabolic activity in older mussels or the greater increase in mass relative to shell length. To maintain constant growth in length, larger mussels would require either longer or more efficient feeding periods. Senility itself can not be the primary cause of reduced growth, since transplantation of old, non-growing mussels, to more favourable situations can often result in renewed growth (Seed, 1976).

### Seasonal and annual cycles

In temperate waters, growth is usually influenced by seasons. There is a rapid growth during spring and summer compared with slight or absent growth during the colder winter months. Flesh weight, by contrast, exhibits pronounced seasonal peaks associated with the annual reproductive cycle. Thus, the pattern of growth in temperate water populations consists of alternating increments in shell length during the spring and summer and flesh weight during winter (Seed and Suchanek, 1992). Whether winter cessation in linear growth is related to the gametogenic cycle is uncertain. Reduced availability of food in the winter could be significant, but low temperature is unlikely to be the sole controlling factor since *Mytilus* can live under extreme arctic conditions where the temperature is at or below 0°C for much of the year. In addition to seasonal differences, marked annual variations in growth rates occur according to local conditions.

### Temperature

Temperature has been widely acknowledged as an important factor in controlling growth rate. However, there is a relatively wide range of temperatures for which mussel growth is not impaired. Physiological studies on *M. edulis* (Bayne *et al.*, 1976) have demonstrated that between 10°C and 20°C, water temperature has little effect on scope for growth. These results have been confirmed by examining the effect of constant temperatures on growth of *M. edulis* (Almada-Villela *et al.*, 1982). She found that between 3°C and 20°C linear growth increased logarithmically; above 20°C growth declined sharply, probably due to decrease of other physiological processes e.g. filtration rate, while at lower temperatures (3°C and 5°C) growth proceeded only very slowly. Another observation made by Almada-Villela was that mussels exposed to sinusoidal temperature changes achieved better growth than those subjected to abrupt changes. It was then concluded that the absence of any adverse effects of fluctuating temperature and the ability to acclimate to temperature changes, at least over part of their physiological range, indicates that *Mytilus* is well adapted to life in the constantly changing environmental conditions usually associated with coastal and estuarine waters.

The dependence of growth on temperature is more clearly demonstrated from another point of view and becomes comparable, when shell length is plotted against time in day $\times$ degrees ( $D^\circ\text{C}$ ). In the Danish Wadden Sea, mussels required approximately 6000  $D^\circ\text{C}$ , equivalent to 1.8 years, to reach a length of 50 mm. In the Menai Strait 4000–7000  $D^\circ\text{C}$ , corresponding to a mean of about 1.2 years, were required to reach the same length whilst in Greenland a length of 50 mm was achieved between 6300–9000  $D^\circ\text{C}$ , or the equivalent of 7–10 years (Seed, 1976). For low values of  $D^\circ\text{C}$ , the relationship length vs. day $\times$ degrees becomes linear. However, growth rates expressed in these terms are not always consistent, for Welsh mussels the relationship between length and  $D^\circ\text{C}$  is sigmoidal. Only some portions of the data are linear, suggesting that a doubling of the number of day $\times$ degrees should result in a corresponding increase in size which means that factors other than temperature, possibly food supply, must be involved (Seed, 1976; Seed and Suchanek, 1992). A few other studies add to this last hypothesis: growth patterns of *M. edulis* on a production platform off the Californian coast proved to be determined mainly by variations in phytoplankton, rather than temperature, which could be virtually eliminated as an important growth regulator over the range 10–18  $^\circ\text{C}$  normally experienced by these mussels (Page and Hubbard, 1987). Similarly, in a study of mussels in western Sweden, low temperatures ( $< 5^\circ\text{C}$ ) did not seem to limit growth whenever these coincided with the spring phytoplankton bloom (Loo and Rosenberg, 1983).

A major difficulty in correlating single environmental variables with growth rate in field populations is that the correlation, although obvious, may not be causal and information on other potential variables may be required before any definite conclusion can be withdrawn.

### Salinity

It is well known that mussels can adapt to live in brackish waters of the river estuaries and lagoons. Seed, (1976) suggested that this was the result of favourable feeding conditions, originated by freshwater input of nutrients, rather than a beneficial effect of lower salinity environments. Indeed, low seawater concentrations have been reported

to have detrimental effects on mussel growth or even be lethal in extreme conditions (Almada-Villela, 1984). In the same study by Almada-Villela poor growth rates of *M. edulis* exposed to low steady salinity regimes (6.4 and 16 ‰) were found, but there was growth enhancement at a salinity of 22.4 ‰. Despite the high food levels provided, high mortalities were observed in the lower steady salinity regimes on the range of 1.8–9.6 ‰. These results indicate that low salinities *per se* influence growth. The same author also found a salinity threshold (12.8 ‰) below which shell growth was impaired. Calcification and shell deposition seem to be depressed around the same salinity level as showed by earlier work (Malone and Dodd, 1967). As a general outcome, the study made by Almada-Villela indicates that a gradual acclimation of the mussels to extreme low salinities (5–10 ‰) is possible if salinities are reduced gradually. Gruffydd *et al.*, (1984) showed that mussel shell growth was not effected at all until the salinity minimum reached a level about 50% below seawater.

*M. edulis* can survive considerably reduced salinities and will even grow as dwarfed individuals in the inner Baltic, where salinities can be as low as 4–5 ‰ (Kautsky, 1982). Results from reciprocal transplant experiments suggest that differences in growth rate and maximum size between North Sea and Baltic mussels are mainly due to physiological adaptations to environmental salinity.

Mussels develop a behavioural mechanism to protect themselves from short-term exposure to dangerously low salinities and thus surviving salinity fluctuations typically occurring in estuaries. The shell valves of *Mytilus* are closed tightly all the time the external salinity is low with negligible exchange between the mantle fluid and the external medium. Gaping of the shell valves only takes place when salts at high enough concentration (equivalent to about 20–22 ‰) diffuse slowly through the fine passageway between the closed but incompletely sealed valves (Davenport, 1979; Davenport, 1981). In this way mussels can cope with salinity changes at the cost of some physiological functions. Oxygen depletion and accumulation of metabolites are some of the penalties incurred. Also feeding is stopped while the valves remain shut and growth rate will inevitably be depressed.

### Food supply

Food supply is probably the single most important factor in determining growth rate, since without it sustained growth can not occur (Seed and Suchanek, 1992). If food is scarce, then growth is retarded regardless of all other conditions (Seed, 1976). Mussels are efficient filter feeders removing particles from water down to 2–3  $\mu\text{m}$  with 80–100% efficiency (Mohlenberg and Riisgard, 1977). The particulate material in suspension that *M. edulis* filter, generally called seston, includes bacteria, phytoplankton, fine organic detritus and material of inorganic origin. Dissolved organic matter may also contribute to the energy intake of this bivalve. Growth of *M. edulis* in natural conditions has always exceeded the rates of growth recorded in laboratory studies, irrespective of food supply and temperature. An explanation for this occurrence was presented by Kiørboe *et al.*, (1981) in which they suggest that mussels use resuspended bottom material, natural silt, as an additional food source which increases their clearance potential yielding maximum growth rates. In shallow neritic waters, some resuspended bottom material will always be present, and in fact the concentration of suspended inorganic material exceeds the concentration of particulate organic matter by several orders of magnitude. On the contrary, in laboratory experiments, even when a varied diet was offered, but no silt was present, mussels failed to attain their potential of maximum growth (Kiørboe *et al.*, 1981). The same authors found that when a low concentration of silt and a high algal concentration were added, mussel growth rate was comparable to natural peak rates.

The height on the shore is an important factor determining food availability since mussels only feed when they are submerged. The reduced amount of time available for feeding experienced by mussels in the upper littoral is probably the cause for their slower growth compared with their congeners in the low shore or sublittoral. This factor also determines the upper limit of mussel distribution on the shore because there is a level along the intertidal gradient where the energy made available during the feeding period will not be enough to satisfy the energy requirements for metabolism during exposed periods. The height at which this energetic balance becomes critical depends on the wave splash conditions and water-retaining properties of the shore. A value of 50% aerial exposure is accepted to be the point of zero growth for *M. edulis* (Seed,



1976; Seed, 1980) and seems to coincide with the natural limit of mussel beds on the shore. While growth decreases with tidal exposure, life expectancy often increases, since predation pressure in the upper shore is substantially reduced.

### Other factors

Light has been investigated by some authors in relation to its effects on mussel growth. The results are however apparently contradictory while Seed, (1976) stated that light seems to have a detrimental effect on growth in *Mytilus* by adversely affecting the exposed growing edge of the mantle tissue responsible for shell secretion. Work by Stromgren, (1976a) confirmed an encouragement of linear growth under constant darkness but higher growth rates during the hours of expected daylight than during the night. Mussels exposed to normal conditions of day and night grew more during the day than during the night (Stromgren, 1976a). These rather surprising results indicated that calcification may involve processes inhibited to some extent by light, so that some darkness is necessary for rapid shell growth in *Mytilus*. On the other hand certain light conditions were also capable of enhance growth. Further studies (Stromgren, 1976b) demonstrated that the investigation of light effects upon mussel growth could not be looked at simply in terms of day and night conditions but also the duration and nature of the light i.e. photoperiod, the energy of the light and spectral composition of the light had to be taken into consideration. The author came out with a set of conditions for which mussels attained the best growth rates which can be summarized as follows: photoperiod of 7 hours or less, low light irradiance (energy) up to  $4 \text{ W} \cdot \text{m}^{-2}$  and the favourable wave band of less than 600–700 nm. This range of wavelengths correspond to the colours blue, green and yellow. It was also noted that the red light had an inhibiting effect on growth as well as full light with all the wavelengths present. This indicated that when all wavelengths are present and the irradiance is relatively high, the positive growth effect of the smallest wavelengths is counteracted by red light. When the irradiance is lowered, the negative effect of red light is not observed.

Genotype has ultimately some influence on individual growth rates. Several studies have revealed that more heterozigous *M. edulis* individuals have lower energy re-

quirements for maintenance metabolism, and a higher efficiency for protein synthesis (Diehl *et al.*, 1986; Hawskins *et al.*, 1986). The higher energy status of these individuals may, thus, be reflected in faster somatic growth in juvenile mussels (Koehn and Gaffney, 1984). In addition to growing faster, heterozygous mussels also tend to achieve more uniform average growth rates (Koehn and Gaffney, 1984).

Storms and strong wave action can significantly reduce growth rate of *M. edulis*, presumably by reducing feeding efficiency (Harger, 1970).

Population structure is another factor controlling growth rates. Populations containing mixed ages, where the majority of small mussels are amongst the byssus threads of larger individuals are at a disadvantage in the competition for food and obviously their growth rates will be reduced (Seed, 1976).

Intraspecific competition for space occupation can lead to extreme variations in growth rate. Spat settled in populations containing two year classes grew at less than half the rate of spat settling on bare rock surface (Seed, 1976).

Parasitism also contributes for the growth rate variation in mussels. Pea crabs previously thought to be harmless comensals of *Mytilus* and even sign of good health are now seen as parasites causing reduced growth rates, shell shape distortions and poorer condition on infested mussels compared to non-infested ones (Seed, 1969; Bierbaum and Ferson, 1986). Reduced food intake by the mussel, tissue damage and, or reduced filtration rates are the most likely factors in lowering mussel fitness (Bierbaum and Shumway, 1988).

Pollution caused mainly by the input of anthropogenic contaminants in the marine environment constitutes another cause of significant reductions in mussel growth rate. Tributyltin (Stephenson *et al.*, 1986; Thain, 1986), heavy metals (Stromgren, 1982; Redpath, 1985; Manley *et al.*, 1984) and petroleum hydrocarbons (Stromgren *et al.*, 1986) are a few examples of those contaminants producing effects at very low concentrations. The effect on mussel growth of other compounds like vegetable oils, originated from spills, was the subject of this research work and will be discussed in Chapter 4.

## 2.4 Marine lipids and vegetable oils

The biochemistry of lipid metabolism in bivalves has been the subject of a number of studies (Voogt, 1983; Zandee *et al.*, 1980; Ackman and Ratnayake, 1989; Ackman, 1983; de Zwaan and Mathieu, 1992). Several factors determined its investigation. Bivalves have an important commercial value in human nutrition, their known high content of polyunsaturated fatty acids (PUFA) have made them an attractive food resource for the prevention of cardiovascular diseases (Piretti *et al.*, 1987). Apart from human usefulness, bivalves held an important position in marine food chains and, in addition, lipid biology of bivalve molluscs provides an important link with pollution since lipophilic compounds, such as xenobiotics, are readily taken up into the tissues of bivalves and concentrated to levels greatly above those of the surrounding seawater (Livingstone and Pipe, 1992). Lipids are involved in important functions in metazoans, being fundamental components of membranes, and of membrane transport processes (Ben-Mlih *et al.*, 1992). From a physiological point of view, lipids constitute an important form of nutrient storage. They are the fuel for gametogenesis and are also the main form of energy reserve in the oocytes used by the embryos and by early larval stages (Holland, 1978). The sterol constituents of lipids also provide valid reasons for biological and pharmacological interest, both because they are precursors of hormonally active substances and of vitamins of group D (Goad, 1976). The hypocholesterolemic action of sterols on mammals and birds is also of great importance (Piretti *et al.*, 1987). Other aspects include the role of prostaglandins in the regulation of gamete emission in bivalve molluscs (Osada *et al.*, 1989).

A summary description of the main groups of lipids in marine organisms follows, with special emphasis on their physiological significance. This introduces the fatty acids, the fundamental components of lipids and vegetable oils, which formed the basis of the analytical program of this work.

Lipids are grouped into two major classes with regard to structure and function (Table 2.1), the neutral lipids and polar lipids (Sargent and Whittle, 1981). The former are reserves of fatty acids which can be catabolised to form ATP, thus, having an active function in metabolism. The latter constituted mainly by phospholipids with a major

role in biomembrane structure and function (Sargent and Whittle, 1981). The physical properties of the biomembranes are strongly influenced by their lipid composition, in particular by the nature of their constituents fatty acids (Gurr and Harwood, 1991). Phospholipids can also be catabolised to generate ATP but that only occurs when all the other readily used energy moieties had been exhausted. Polar lipid content is usually a few per cent of the dry weight of the animal and remains fairly constant in fatty acid composition whereas neutral lipid content can range from a few per cent of its dry weight to more than 90% in adipose tissues and vary substantially in fatty acid composition (Sargent and Whittle, 1981).

	Lipid Class	Acyl Acceptor
Neutral Lipids	Sterol esters	Cholesterol
	Triacylglycerols	Glycerol
	Alkyldiacylglycerols	Glycerol
	”	Fatty alcohol
	Wax esters	Fatty alcohol
Polar Lipids	Phosphatidyl serine	Phosphoserine
	Phosphatidyl ethanolamine	Phosphoethanolamine
	Phosphatidyl choline	Phosphocholine
	Plasmalogens	Phosphocholine
	”	Fatty alcohol
	Sphingomyelin	Phosphocholine
	”	Sphingosine
	Cerebroside	Sphingosine
	”	Sugar
	Ganglioside	Sugar
	”	Neuraminic acid
Phosphatidyl inositol	Phosphoinositol	

Table 2.1: Major lipid classes (Sargent, 1976).

The concentration of neutral lipids in an organism is determined by a balance between dietary intake, anabolism and catabolism (Sargent and Whittle, 1981). A large intake of lipids results in substantial deposition of lipid within the organism, so that the

fatty acid composition of tissue neutral lipid reflects that of the diet. Within this group of lipids, triacylglycerols are the most common class in phytoplankton and marine fish; wax esters are typical from marine invertebrates like copepods and alkyldiacylglycerols are found in elasmobranchs.

The concentrations of polar lipids in an organism although subject to turnover, is relatively constant. Its fatty acid composition is also influenced by diet, since the essential fatty acids present can not be synthesised *de novo* by animals (Sargent and Whittle, 1981).

There exists a notable heterogeneity in the lipid composition of different bivalve species. Sedentary species, including *M. edulis*, which are occasionally obliged to survive periods of anaerobiosis, have a lower lipid content than other species (de Zwaan and Mathieu, 1992). Indeed, during anaerobiosis, glycogen would appear to be a better form of energy reserve. Even under these conditions, however, the role of lipids can not be ignored. Taking into account the fact that the lipid content of mussels increases at a much lower rate than carbohydrate and protein content during growth, it was suggested that lipids constitute the principal source of energy for growth (Zandee *et al.*, 1980).

The lipid content of the mussel undergoes only weak seasonal variations. In *Mytilus platensis*, for example, the lipid content varies from 0.9 to 1.8% of the wet weight (DeMoreno *et al.*, 1980). Differences also exist in the lipid content of various organs of the mussel and in the content of polar and neutral lipids. The mantle and the digestive gland generally display the highest neutral lipid content, probably reflecting the storage capacities of these tissues. For populations of mussels from the English Channel, lipids are conserved for gametogenesis between autumn and spring, whereas carbohydrates and proteins are used for both energy production and gametogenesis. The highest values observed in the gonad coincide with gametogenic activity and oocyte maturation. Gamete release is reflected by a sharp fall in lipid concentration (Pieters *et al.*, 1980). In addition, it has been suggested that stored carbohydrates are transformed into lipids stored in the gametes.

The relative proportions of polar and non-polar lipids in whole tissues also undergo seasonal variations. It has been shown (Trider and Castell, 1980) that the lipid content in oysters ranges from 0.5 to 2% of the wet weight and that polar lipids represent

a proportion of the wet flesh weight around 0.5% and did not vary greatly over the seasons. On the other hand, neutral lipids change dramatically according to the sexual cycle (Trider and Castell, 1980).

### 2.4.1 Fatty acid: structure and metabolism

Fatty acids may occur in free form, but they are mostly present in esterified form (Voogt, 1983). They are found in sterol esters, acylglycerols and phospholipids (Table 2.1).

Chemically, fatty acids are compounds of carbon and hydrogen, organized in a chain with a functional carboxyl group ( $-\text{COOH}$ ). They are usually represented by a shorthand notation the  $\omega$  system or the equivalent  $n-$  system. Both notations specify first the number of carbon atoms in the chain and second the number of double bonds. When the fatty acid is unsaturated the number of double bonds is followed by the symbol  $\omega$  or  $n-$  and the number of the carbon atom indicating the position of the first double bond relative to the methyl end of the chain. As an example linoleic acid is represented as 18:2 $\omega$ 6 or 18:2(n-6). The  $\omega$  notation will be adopted here because it makes easier to understand biochemical reactions between fatty acids and it highlights the existence of fatty acid families as explained in section 2.4.1.

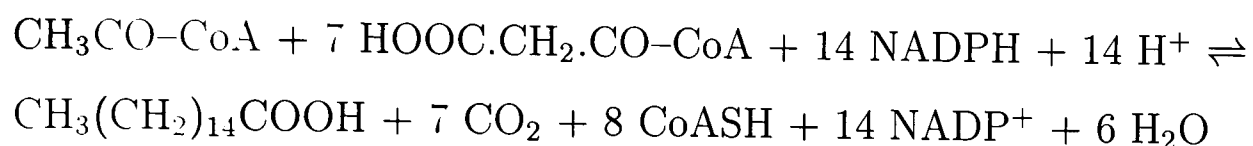
The chemical name of fatty acids ends by a suffix which designates the degree of unsaturation. For saturated fatty acids the suffix is anoic, for monounsaturated the suffix is enoic and for polyunsaturated fatty acids dienoic, trienoic etc. Information about all the positions of double bonds counted from the carboxyl end of the molecule may also be given. The chemical name of linoleic acid is 9,12-octadecadienoic acid.

The geometric structure of double bonds in the unsaturated fatty acids from marine sources are generally accepted as *cis* configuration and in the case of the polyethylenic fatty acids these are considered to have mainly a methylene interrupted arrangement ( $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$ ) as the type structure (Ackman, 1964).

### Fatty acid biosynthesis

Most of the fatty acids occurring naturally in plants and animals have even numbers of carbon atoms and this fact suggests the principle of their biosynthesis by condensation of two carbon units (Gurr and Harwood, 1991). The first step in fatty acid synthesis is the

activation of acetate by *coenzyme* A (CoA) producing acetyl-CoA. The two activated carbon fragment is carboxylated to malonyl-CoA, reaction catalized by the enzyme acetyl-CoA carboxylase. Malonyl-CoA generated this way forms the source of nearly all the carbons of the fatty acyl chain. The fatty acid chain then grows in a series of reactions adding a malonyl unit each time. The overall reaction of fatty acid synthesis can be summarized as:



Six separate enzyme activities are involved. These are known collectively as fatty acid synthetase.

### Elongation

In contrast to the *de novo* formation of fatty acids by fatty acid synthetase enzymes, the chain lengthening of pre-existing fatty acids, either formed in the animal's body or originated from the diet, is catalysed by elongases. Elongation occurs by addition of acetyl-CoA units and NADPH or NADH as the reducing coenzyme. Neither CO<sub>2</sub> nor malonyl-CoA is involved in this pathway (Gurr and Harwood, 1991). One of the most important functions of elongation is the transformation of dietary essential fatty acids (linoleic and  $\alpha$ -linolenic acids) to the higher polyunsaturated fatty acids. The starting point can either be linoleic acid or  $\alpha$ -linolenic acid, the two of which give rise to two series of polyunsaturated fatty acids of distinct and not interconvertible families,  $\omega$ 6 and  $\omega$ 3 respectively. The process involves a sequence of alternate elongations and desaturations represented in Figure 2.4.

### Desaturation

The most widespread pathway of fatty acid desaturation is by an oxidative mechanism, in other words by aerobic desaturation. This pathway is almost universal and is used by bacteria, yeasts, algae, higher plants, protozoa and animals. There are however, fundamental differences, related with the presence of different enzymatic systems, between the desaturation process occurring in animals and in plants or algae.

Plants and algae are able to produce *de novo* all the fatty acids they require, from the monounsaturated fatty acids to the highly polyunsaturated ones without any restrictions, because they possess the enzymatic resources to highly desaturate fatty acids (Figure 2.5).

In this and following sections a  $\Delta x$  notation is employed to designate the desaturase enzyme, where  $x$  is the number of the first carbon atom where the double bond is introduced, counted from the carboxyl end of the chain.

The precursor for polyunsaturated fatty acid formation in plants and algae is oleic acid, 9-18:1 originating from desaturation of stearic acid (18:0) by  $\Delta 9$  desaturase (Figure 2.5). The next double bond is introduced between carbons 12-13 by  $\Delta 12$  desaturase producing linoleic acid (9,12-18:2). One further desaturation at 15-16 position by  $\Delta 15$  desaturase produces  $\alpha$ -linolenic acid (9,12,15-18:3).  $\alpha$ -linolenic acid is the most common fatty acid found in plants and freshwater algae. In marine algae, highly unsaturated 20 carbon acids are predominant, the principal of which are arachidonic and eicosapentaenoic acids.

In animals, unlike in plants and algae, the enzymes present can only introduce new double bonds between an existing double bond and the carboxyl end of the fatty acid (Gurr and Harwood, 1991). Most of the monounsaturated fatty acids produced in animals have a  $\Delta 9$  double bond, resulting from desaturation of readily available saturated fatty acids originating from the diet or biosynthesized *de novo*. The desaturation process of 16:0 and 18:0 in animals, operates on carbons in the  $\Delta 9$ - $\Delta 10$  position by the action of  $\Delta 9$  desaturase. The conversions are: 16:0 to  $\Delta 9$ -16:1 (palmitoleic acid) and 18:0 to  $\Delta 9$ -18:1 (oleic acid) (Ackman, 1983). Vaccenic acid (18:1 $\omega$ 7) can be formed by the elongation of palmitoleic acid (16:1 $\omega$ 7) and similarly the conversion of oleic acid into gondoic acid (20:1 $\omega$ 9), abundant in wax esters of calanoid copepods (Sargent and Whittle, 1981), involves just an elongation reaction with retention of the  $\omega$  structure. Cetoleic acid (22:1 $\omega$ 11) the other monounsaturated fatty acid abundant in zooplankton wax esters is formed by desaturation of arachidic acid (20:0) to 20:1 $\omega$ 11, this reaction being catalyzed by  $\Delta 9$  desaturase followed by elongation to 22:1 $\omega$ 11 (Sargent and Whittle, 1981). The inability of animals to desaturate oleic acid towards the methyl end of the chain gives rise to distinct families of polyunsaturated fatty acids that are not in-



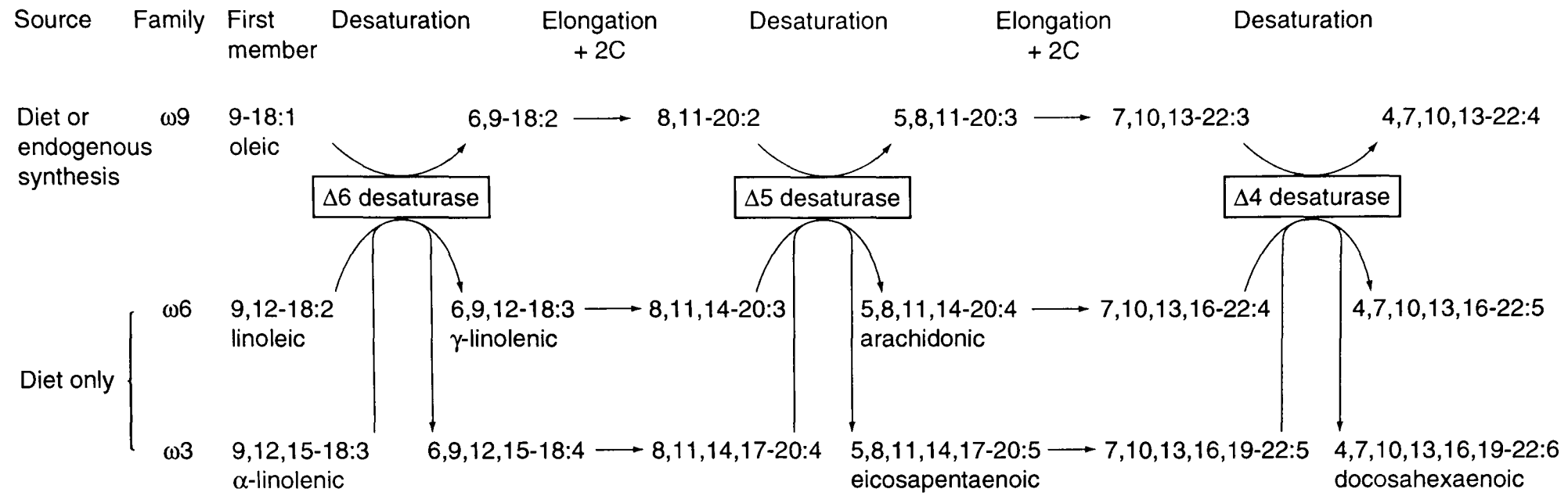


Figure 2.4: Metabolic sequences for unsaturated fatty acid synthesis in animals (Gurr and Harwood, 1991).

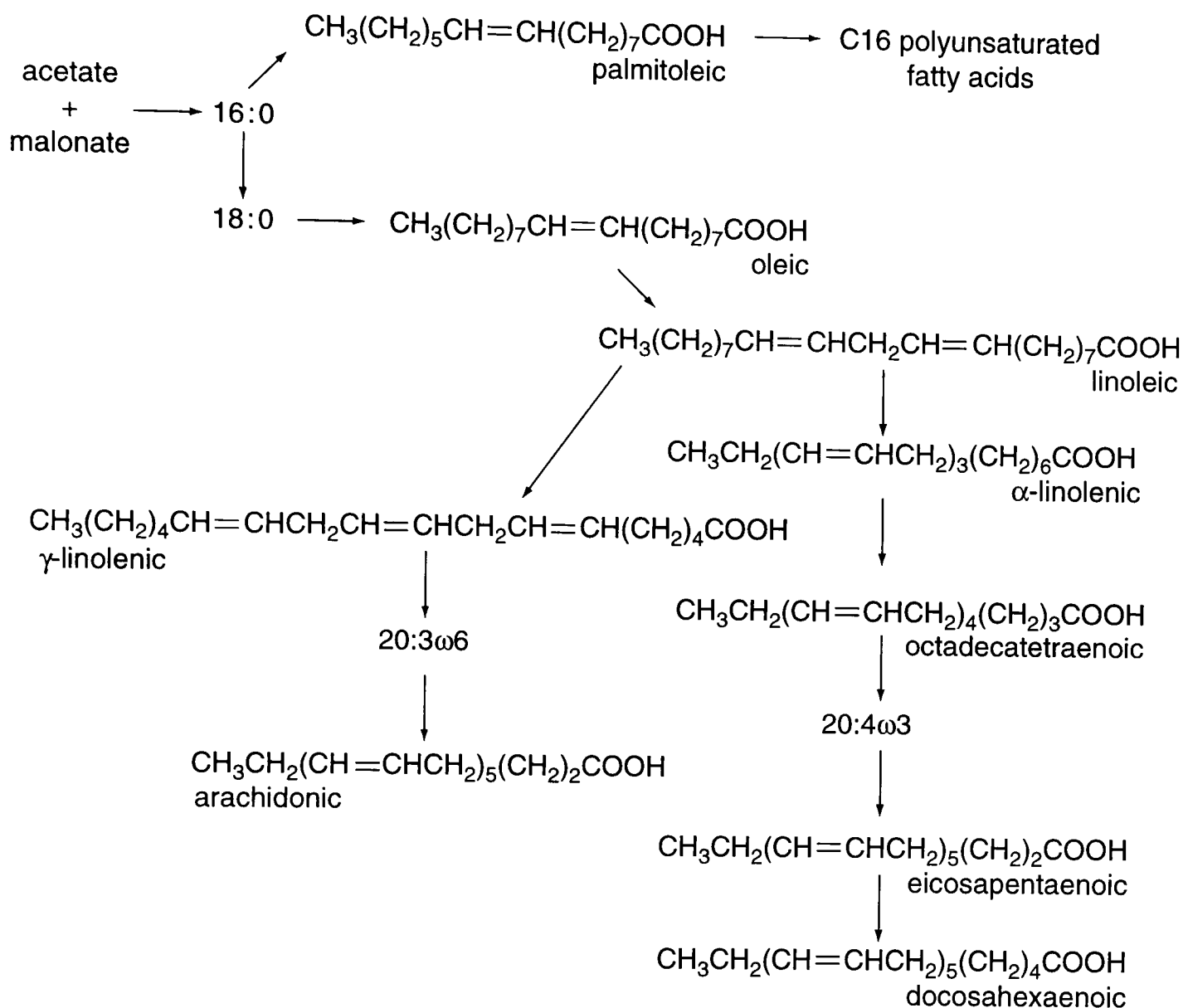


Figure 2.5: Major desaturation pathways for polyunsaturated fatty acid synthesis in plants and algae (Gurr and Harwood, 1991).

terconvertible (Gurr and Harwood, 1991). In general, polyunsaturation in animals is accomplished by three separate desaturases, designated as  $\Delta$ 4,  $\Delta$ 5 and  $\Delta$ 6 because they introduce double bonds between carbon atoms 4-5, 5-6 and 6-7 respectively (Figure 2.6).

The most important substrates for the first polydesaturation in animals are oleic acid originated from diet or endogenously and linoleic and  $\alpha$ -linolenic acids originating from diet only (Gurr and Harwood, 1991). These three fatty acids are the precursors of each of the 3 families of fatty acids. Oleic acid gives rise to a series of  $\omega$ 9 fatty acids, and linoleic and  $\alpha$ -linolenic to a series of  $\omega$ 6 and  $\omega$ 3 fatty acids, respectively (Ackman and Ratnayake, 1989; Gurr and Harwood, 1991). The first polydesaturation is at  $\Delta$ 6 and the sequence is one of alternate elongations and desaturations in the order  $\Delta$ 6,  $\Delta$ 5,  $\Delta$ 4 (Figure 2.4). The limiting step in this pathway is the  $\Delta$ 6 desaturase. Interconversions within a family can occur not only by chain elongation, but by a chain shortening by

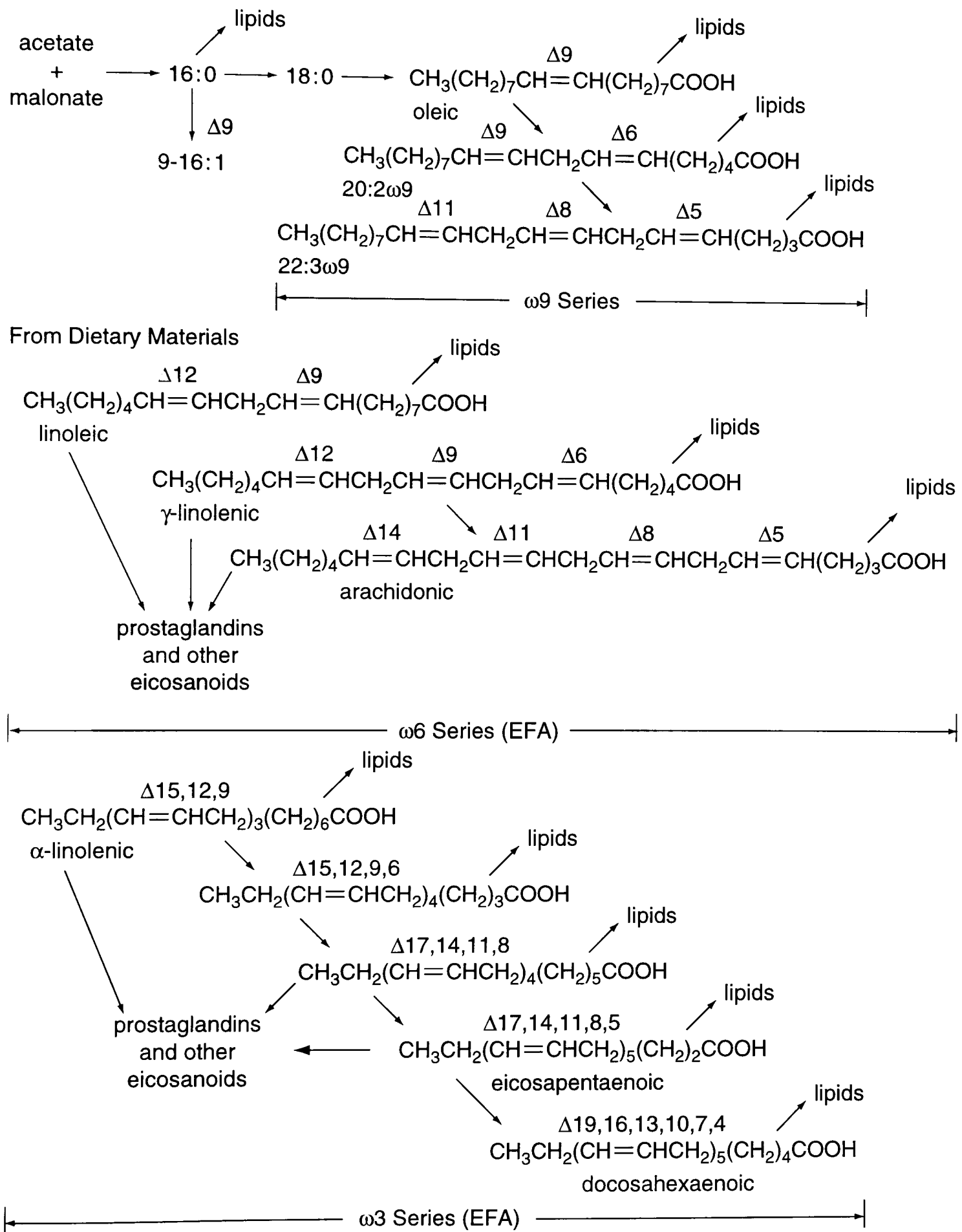


Figure 2.6: Desaturation pathways for unsaturated fatty acid synthesis in animals (Gurr and Harwood, 1991).

two carbon units (Gurr and Harwood, 1991).

### 2.4.2 Fatty acid composition in molluscs

Lipids from marine organisms have a greater fatty acid complexity than most terrestrial organisms. These acids typically range in chain length from 14 to 22 carbon atoms. For each chain length there is usually a mixture of acids of varying degrees of unsaturation which can be up to 6 double bonds in the longer chain fatty acids (Lovern, 1964). The fatty acid composition of molluscs and of marine invertebrates in general, is influenced by a host of environmental and biological factors (Joseph, 1982). Two important environmental factors are temperature and food availability which, together, may contribute to a seasonal variation in fatty acid composition. Among the biological factors are taxonomic relationships, differences in sex and influence of reproductive cycles, diet and distribution of the different esterified lipid classes in specialized body tissues. Therefore, there is an inherent danger in comparing fatty acid compositions of filter-feeders, even within a single species or population (Joseph, 1982). It can be assumed that triacylglycerides have consistent fatty acid patterns throughout the bivalve body, but the distribution of fatty acids in the phospholipids may depend on the function associated with the organ, or on the relative proportion of the two major phospholipids, phosphatidylethanolamine and phosphatidylcholine, respectively (Ackman, 1983). General fatty acid composition of molluscs in marine invertebrates is characterized by a predominance of two polyunsaturated acids: 20:5 $\omega$ 3 and 22:6 $\omega$ 3. This pattern reflects the fact that these organisms utilize organic material elaborated by phytoplankton (Ben-Mlih *et al.*, 1992). The major marine saturated fatty acids of marine molluscs are palmitic acid (16:0) and myristic acid (14:0). Stearic acid (18:0) is only present in trace amounts in marine lipids.

Palmitic acid is freely available in algal diets, and is usually accompanied by approximately half as much 14:0. Stearic acid is a minor component in most algal lipids (Ackman, 1983). It is probable that 14:0 and 16:0 acids are easily assimilated by bivalves and, therefore, it is not surprising that bivalve lipids contain 20-50% saturated acids, of which 16:0 is invariably the major acid. Palmitic acid is extensively biosynthesized *de novo* and its presence in all lipid classes make it ubiquitous in marine life forms

(Sargent and Whittle, 1981). Myristic acid is an abundant fatty acid in neutral lipids of both phytoplankton and marine bivalves but is only present in very small amounts in phospholipids of marine animals. This fact indicates that it is used to obtain metabolic energy and hence it may be a useful marker for lipid metabolic energy transmission through the trophic levels (Sargent and Whittle, 1981).

Marine monounsaturated fatty acids contain a range of isomers. The palmitoleic fatty acid (16:1 $\omega$ 7) is the most abundant isomer in phytoplankton and marine molluscs. Phytoplanktonic 16:1 $\omega$ 7 must contribute significantly to the quantities of this acid present in invertebrates, although the animals themselves are able to biosynthesize it (Sargent and Whittle, 1981). The amount of palmitoleic acid provided by the algae in the wild appears to be of the same order of magnitude as in the mollusc triacylglycerides and it dominates the 16:1 isomers in the triacylglycerides of most bivalves (Ackman, 1983). Phytoplankton contains less but substantial quantities of 18:1 fatty acid when compared with the 16:1, including both the  $\omega$ 9 isomer (oleic acid) and  $\omega$ 7 isomer (*cis*-vaccenic acid). Vaccenic acid is a major fatty acid of benthic algae (Sargent and Whittle, 1981). In mammals, 18:1 fatty acid is generally assumed to be oleic acid and *cis*-vaccenic acid is associated with bacteria such as *E. coli*. Marine fish oils contain both isomers, the latter usually accounting for less than one third of total 18:1. Bivalves have considerable flexibility in their biochemical pathways for lipid synthesis and it may be more economical in terms of energy expenditure to change exogenous 16:1 $\omega$ 7 to 18:1 $\omega$ 7 than to synthesize 18:1 $\omega$ 9 *de novo* (Ackman, 1983). The ratio  $\omega$ 9/ $\omega$ 7 monounsaturates could be useful indices of food chain relationships (Sargent and Whittle, 1981). In phospholipids, the 18:1 isomer ratios are approximately the same as in the triacylglycerides indicating that in most bivalves the 18:1 acids are probably drawn from the same fatty acid pool (Ackman, 1983).

Polyunsaturated fatty acids (PUFA) are major constituents of both neutral and polar lipids in marine organisms, although the animal phospholipids are richer in polyunsaturated fatty acids than animal neutral lipids (Sargent and Whittle, 1981). Phytoplankton contains polyunsaturated fatty acids from C16 to C22. Diatoms and dinoflagellates both contain large concentrations of 20:5 $\omega$ 3 and 22:6 $\omega$ 3; littoral algae, benthic algae and fresh water organisms contain substantial amounts of  $\omega$ 6 polyunsaturated fatty

acids. Littoral algae especially contain 20:4 $\omega$ 6, arachidonic acid. Planktonic algae, on the other hand contain typically marine high concentrations of  $\omega$ 3 polyunsaturated fatty acids. Marine invertebrates are rich in  $\omega$ 3 polyunsaturated fatty acids, especially 20:5 $\omega$ 3 and 22:6 $\omega$ 3. Benthic and inshore invertebrates including molluscs, coelenterates, echinoderms and crustacea contain substantial amounts of  $\omega$ 6 polyunsaturated fatty acids in their polar lipids, particularly 20:4 $\omega$ 6. These  $\omega$ 6 polyunsaturated fatty acids must originate in benthic algae. Pelagic invertebrates including copepods, decapods, mysids and euphausiids contain small concentrations of  $\omega$ 6 polyunsaturated fatty acids reflecting the low concentrations of this fatty acid family in offshore phytoplankton (Sargent and Whittle, 1981). The main polyunsaturated fatty acids present in phytoplankton are C16 and C18. The same fatty acids are, however, present in small concentrations in polar lipids of invertebrates although C18 can reach high concentrations in copepod wax esters. Either the shorter chain polyunsaturated fatty acids in plant material are converted to longer chain polyunsaturated fatty acids in the invertebrates or they are catabolised. Some species of invertebrates possess polyunsaturated fatty acids in their lipids even if they were not present in their diet in significant amounts. This suggests that invertebrates can convert shorter chain dietary polyunsaturated fatty acids to longer chain polyunsaturated fatty acids through chain elongation and desaturation reactions (Sargent and Whittle, 1981) (Figure 2.4).

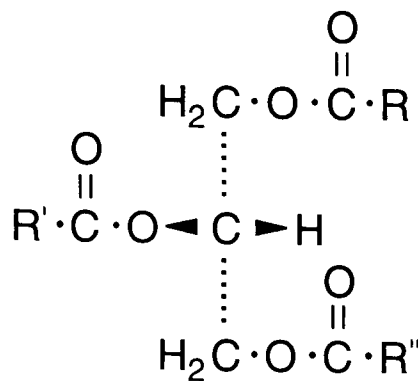
Marine mussels have been extensively studied regarding their fatty acid composition (Pastoriza *et al.*, 1981; DeMoreno *et al.*, 1980; Gardner and Riley, 1972). The studies by DeMoreno showed a small fluctuation in total lipid content of the soft tissues, yet the fatty acid composition changed considerably. There seem to be a reciprocity between the levels of 16:0 and 16:1 $\omega$ 7 and the polyenoic acids 20 and 22. When food is abundant there is an increase of 20 and 22 polyunsaturated fatty acids and a decrease of 16 carbon fatty acids, whereas when food is scarce the reverse occurs. On the other hand the 18:0 and 18:1 $\omega$ 9 remain virtually constant. Diatoms and dinoflagellates provide the polyenoic C20 and 22 fatty acids while the small amount of the saturated and monounsaturated, C14 to 18 fatty acids come from detritus. The abundance of polyunsaturated fatty acids in mussel tissues suggests that phytoplankton are an important food source for these organisms.

### 2.4.3 Vegetable oils

This section is dedicated to describe the general composition of vegetable oils and in particular of the four oils used in this study: linseed, olive, rapeseed and sunflower oils. These oils were the subject of this study because one of them, sunflower oil, was accidentally spilled in the sea contaminating the area of the accident and the near shore including the local fauna. Other vegetable oils are transported by sea for commercial purposes and the possibility of spillages are not remote. Their effects upon mussels were investigated and for reference information, their fatty acid composition, main uses and future marketing prospects are summarized below.

The nutritional value of vegetable oils and fats is determined primarily by their fatty acid composition. The fatty acids in seed oils are esterified mainly in triacylglycerides (TAG) (Figure 2.7), the main form of fat storage in animal tissues. The minor components of oils include sterols, phospholipids, waxes and tocopherols, but these are significantly reduced by refining processes (Ackman and Ratnayake, 1989). The melting point of the fat or oil is determined by the type of fatty acids present in triacylglycerides, the fatty acids chain length, degree of unsaturation and double bond configuration, either *cis* or *trans*. The melting point of the TAG is also an important parameter, that determines the intestinal absorption of the oil by the consumer organism; the rates of absorption decreasing with increasing melting points. Saturated fatty acids with chain length 18 carbons or more and monounsaturated fatty acids with more than 20 carbon are liberated by the lipolytic activity of triacylglyceride lipases from positions 1 and 3 of the triacylglyceride. The resultant free fatty acids are poorly absorbed, whereas the resulting monoacylglycerides, with a fatty acid on position 2 of the triacylglyceride are easily incorporated into micelles and absorbed. The position of the fatty acids in the TAG molecule is thus important in determining their rate of absorption (Menon *et al.*, 1989).

Phospholipids although present in small amounts in processed vegetables oils are hydrolysed by phospholipase A2 on position 2 of the glycerol molecule; the end product is lysophospholipid which is potentially toxic. However, the rate of phospholipid hydrolysis is extremely slow compared to that of TAG and the concentrations of lysophospholipids



1,2,3-Triacylglyceride (TAG)

Figure 2.7: Structure of a triacylglyceride. R, R' and R'' represent fatty acids.

formed in the bowel are extremely low and unlikely to be of any nutritional consequence. Phospholipids are essentially secreted in the bile and play an important role in solubilizing endogenous/exogenous cholesterol and facilitate micellar solubilization of lipolytic products. Thus, lipid constituents other than glycerides do not play a major role in the nutritional properties of oils (Menon *et al.*, 1989).

The common edible oils come usually from cultivated annual crops, perennial plantations (coconut, palm) or from by-products such as rice bran oil and cotton seed oil. Other oils referred to as uncommon edible oils due mainly to inadequate human knowledge about their edible quality, come from forest trees and are used for different purposes other than food consumption (Menon *et al.*, 1989).

### Linseed oil

Linseed oil belongs to the uncommon edible oils category. It comes from the seeds of the plant *Linum usitatissimum*. Its use in human diet is limited, although it is well known as a drying oil and for its extensive applications in paints, varnishes and plastics. Smaller amounts are used in the manufacture of linoleum, oil cloth, printers ink, patent and imitation leather products (Godin and Spensley, 1971). The oil generally contains about 40–56% of 18:3 $\omega$ 3 fatty acid which oxidizes and polymerizes easily. Triacylglycerides that only contains unsaturated fatty acids comprise between 43 and 64% and those that are composed of two unsaturated and one saturated fatty acids form 32–41% of linseed oil. Current Indian Government legislation classifies it as an edible oil (Bajpai *et al.*, 1985). The results from studies made by Menon, (1989) indicated that the use of linseed



oil as an edible oil will not produce any hazardous effects, if sufficient vitamin E is given. Its fatty acid composition is as shown in Table 2.2.

### **Olive oil**

Over 90% of the olives produced in the world are used in extraction of oil. Pure olive oil is used largely for comestible purposes e.g. cooking, salads, etc. and in preservation of foods e.g. sardine canning. It is also used in the textile industry for wool combing, in the manufacture of high class toilet preparations and cosmetics and in the pharmaceutical industry for medical purposes. The fruit when ripe contains 10–60% of oil depending on climatic conditions and care in cultivation (Godin and Spensley, 1971).

The extraction process gives rise to 3 types of oil according to the pressing stage from which the oil is collected. The first pressing yields virgin olive oil, seldom exported in its natural state, but forms the basis of edible export types of the finest quality. Separation of oil from aqueous material is carried out by prolonged settling. The residual pulp is subjected to a second pressing with hot water, yielding an oil of higher free fatty acid content. This oil is submitted to a refining process of decolorization, deacidification and deodorization and is commercially known as refined olive oil and used largely to blend with virgin oils for edible export types. The residue from the third pressing is commonly extracted with a hydrocarbon solvent to produce an oil that is saleable as an edible oil after refining (Godin and Spensley, 1971).

Olive oil is valued for culinary purposes due to a good distinctive flavour, a low cloud point, making it ideal as salad dressing and a low linoleic acid content (slight oxidation of this acid impairs flavour).

The relative importance of olive oil is decreasing due to the rapidly increase production of oilseeds whereas the production of olive oil has not advanced much. Fatty acid composition of olive oil is presented in Table 2.2.

### **Rapeseed oil**

Seeds from *Brassica campestris* or *Brassica napus* yield rapeseed oil which, when refined, is used mainly for edible purposes, on salads or as cooking oil. Hydrogenated

rapeseed oil is used in the preparation of butter substitutes. Inferior qualities are widely used in lubricating and cutting oils, in the manufacture of soap and for lighting. Erucic acid (22:1 $\omega$ 9), present to the extent of 20–45% in rapeseed oil, can serve as a substitute for olein in flotation agents. Ozonizing splitting of erucic acid yields brassylic (*trans* 22:1 $\omega$ 9) and pelargonic acids, which give, by hydrogenation under pressure, a product suitable for synthetic waxes and lubricants (Godin and Spensley, 1971). New applications of rapeseed oil as a renewable raw material for use in the chemical industry have been investigated resulting in high demands of rape breeding and its implementation (Flessner, 1991).

Unlike other oils, rapeseed oil has major amounts of erucic acid (22:1 $\omega$ 9, 23–39%) and gondoic acid (20:1 $\omega$ 9, 9–11%) and minor amounts of long chain saturated fatty acids: arachidic (20:0), behenic (22:0) and lignoceric (24:0) acids (0.4–0.6%) and a low total saturated fatty acid amount of (0.4–3%) (Menon *et al.*, 1989). Investigations had shown that rapeseed oil with a high erucic acid content caused changes in the heart tissue of experimental animals (Godin and Spensley, 1971). A number of countries have therefore limited the rapeseed oil content of their products until the commercial introduction of a new variety of rapeseed oil virtually free of erucic acid, containing only 0.1–0.2% and making it suitable for consumption. This oil was successfully produced through plant breeding, by blocking the extension of oleic acid to C20 and C22 monoethylenic successors. Canola oil is the Canadian registered trade mark for this new variety of rapeseed oil (Ackman and Ratnayake, 1989). Its fatty acid composition is shown in Table 2.2.

### **Sunflower oil**

A great part of the world production of sunflowers is used for the production of edible oil. The sunflower oil of best quality is directly consumed as table or cooking oil, or used in the manufacture of margarine and compound cooking fat. Inferior grades are used for industrial purposes like soap manufacture and employed in blends with linseed and other drying oils in paint and varnishes. The seeds themselves constitute a saleable product. The large seeds are roasted, salted and sold to confectioners and the hulled

kernels are roasted and sold as such or used in sweets or candies. They can be used as food to poultry. The residue left after oil extraction is employed as protein supplement for livestock and as nitrogenous fertilizer (Godin and Spensley, 1971). Its fatty acid composition is shown in Table 2.2.

Fatty acid	linseed	olive	rapeseed (Canola)	sunflower
Palmitic acid	8	10	4	7
Stearic acid	6	3	2	4
Oleic acid	20	80	65	19
Linoleic acid	14	6	18	70
Linolenic acid	51	-	9	-
Arachidic	1	-	-	-
Erucic	-	-	0.1	-
Others	-	1	1.9	-

Table 2.2: Percentage of fatty acids in different oils (Menon *et al.*, 1989; Ackman and Ratnayake, 1989).

## Market

To look at the world oilseed position in more exact terms a list of the major oilseeds is presented (Table 2.3). Rapeseed is the least significant, but in the temperate climate zones where it grows, it does have much greater importance. For the most part, rapeseed is consumed where it is produced, except in Canada, where it is exported to world markets. Rapeseed oil domestic consumption in Canada accounts now for a massive 46% market share. However, Canada produces more oil than required for domestic market and exports it mainly to India (Matthews, 1981). Recently, Canadian producing oil companies are also trying to penetrate European market. Switzerland and UK, but especially Spain have been the target markets for rapeseed oil (Gunstone, 1994c).

From an economic point of view, oil production is likely to increase since world population is expected to rise to 6 billion by the year 2000 (Gunstone, 1994d). In addition, the UN Food & Agriculture Organization recommends the consume of 20 –

Seed	Oilseed production	Oil content
	1980/81	
Soybean	87	16.0
Cotton	25	5.0
Sunflower	15	5.7
Groundnut	12	5.5
Rapeseed	11	4.3

Table 2.3: Oilseed production in million tonnes a year and oil content (%) of major oilseeds (Matthews, 1981).

22 kg of oil per person a year. Assuming everyone consumed about 15 kg of oil a year, the present annual oil production would have to increase by 20 million tonnes. Palm oil will have a major role to play in satisfying future world demand for edible oils due to its high productivity (Gunstone, 1994d). Other oils like rapeseed are expected to be potential competitors. The demand for edible oils in China will also increase at a greater rate than domestic supply, creating export opportunities for other countries, namely Canada. Rapeseed oil is the dominant oil in terms of consumption in China and it is expected to remain dominant, but will probably lose market share because of limited supplies and strong competition from palm and soybean oils (Table 2.4).

Oil	Production		Consumption	
	1988/92	2004/5	1988/92	2004/5
Rapeseed	2.01	3.39	2.32	3.75
Soybean	0.61	1.05	0.94	1.55
Sunflower	0.26	0.39	0.27	0.46
Linseed	0.12	0.15	0.14	0.20
Palm	0.01	0.02	0.82	2.40

Table 2.4: Production and consumption of some Chinese oils in million tonnes a year (Gunstone, 1994d).

With the world's demand for edible oils in perspective, it is fair to say that world trade of oils will increase and with it the danger of edible oil spills. It is now being

recognized that vegetable oil spills are a wildlife threat causing extensive killing and injuries on birds (McKelvey *et al.*, 1980; Smith and Herunter, 1989) and that it can be also detrimental in fish breeding grounds or areas of aqua or mariculture. They cause anoxic conditions in the environment, in the water (Gunstone, 1994a) and sediments (Mudge *et al.*, 1995), with acute consequences for aquatic life specially the sessile organisms as it will be shown in this work.

## 2.5 Summary

In this chapter some concepts of mussel biology, growth and biochemical metabolism were reviewed. regarding mussel position as a resource for economic exploitation and for scientific research. In the first part taxonomic and anatomic aspects of mussel biology were presented. Ecological considerations on mussel habitat and distribution were also described. Factors contributing to mussel mortality, divided into biological and physical factors. were summarized.

The second part was concerned with growth. Different methods available to measure growth were explored, pointing out their advantages and disadvantages. The method used in this research work for measuring mussel growth is also briefly introduced. The main parameters influencing growth were referred with special mention on pollution effects.

In the third and last section of this chapter lipid metabolism in bivalves and fatty acid composition of mussels and vegetable oils were discussed. Variations in lipid content were shown to be related with season and reproductive cycle. Typical fatty acid compositions of bivalves were given showing the interrelationships between food availability and metabolic needs of these species. Metabolic pathways of fatty acid biosynthesis in animals were compared with the ones in plants emphasizing the limited capacity of animals for desaturation reactions which is overcome by dietary sources. Finally a brief reference of the main uses and fatty acid composition of the vegetable oils utilized in this study was presented. Future trends in vegetable oil consumption and trade were put forward together with the perspectives of accidental spills.

# Chapter 3

## Fatty acids in biological samples

### 3.1 Introduction

Fatty acids are the basic components of both mussel lipids and vegetable oils (see Chapter 2), the two primary sources of interest in this investigation. Therefore fatty acids are fundamental to this study. Their analysis is of extreme importance to characterize both vegetable oils and mussel lipids and to monitor their change in mussels exposed to vegetable oils. To assess the degree of oil contamination in mussels the fatty acid content of oils was compared with that of mussel tissues before and after exposure of mussels to oils.

Fatty acid composition of invertebrates, mussels in particular, is very much influenced by their diet and since phytoplankton is their main food source, analysis of microalgae fatty acids were also required. The different species of microalgae *Pavlova lutheri* (Droop) Green, *Tetraselmis chuii* Butcher and (*Rhodomonas baltica*) *Rhodomonas reticulata* and four vegetable oils, linseed, rapeseed, olive and sunflower oil were analysed for their fatty acid composition. These were used as a source of food and as contaminants, respectively, in growth experiments described in Chapter 4. Fatty acid composition of mussels collected from the wild was also established and the natural variation of their composition investigated.

This chapter describes the materials and methods used in the determination of fatty acid methyl esters from their different tissues: mussel soft tissues, oils and algae.

## 3.2 Materials and methods

The materials used in the analysis of fatty acids methyl esters include chemical reagents and instruments. The reagents were purchased from Rathburn (hexane, methanol and pentane HPLC grade, chloroform glass distilled grade) and BDH (boron trifluoride-methanol and magnesium chloride Analar). The methylating agent, trimethylsulfonium hydroxide (TMSH) was acquired from Camlab Ltd. Fatty acid methyl ester standards were acquired from Alltech and Sigma.

Several instruments were utilised namely a Chemlab freeze drier, a MSE Centaur 1 centrifuge, a ZM Coulter Counter, a Buchi RE111 rotary evaporator, heating plate, a Finnigan Mat 4600 gas chromatograph-mass spectrometer (GC-MS) and a Carlo Erba 8160 gas chromatograph (GC). The chromatographic columns were purchased from Alltech and the gases for the chromatographs were provided by BOC, Ltd. Whatman paper filters n.4 and GF/C filters, with 1.2  $\mu\text{m}$  pore size were used.

The biological samples were collected or obtained from different places: *Mytilus edulis* were collected in the south coast of Anglesey-North Wales at low tide; vegetable oils rapeseed and sunflower were kindly offered by Van den Berg and Jurgens; linseed oil was offered by BOCM and olive oil supplied by Leon Frenkell Ltd. Microalgae were reared at the School of Ocean Sciences, Menai Bridge, Anglesey.

### 3.2.1 Fatty acid general analytical procedure

Fatty acid analysis involves two sets of tasks: preparation of the biological material and the choice of the best operating conditions of the analytical instruments. The preparation of the samples is generally carried out in three main steps:

- Solvent Extraction
- Derivatisation
- Analyses

### Solvent extraction

The extraction procedure requires the use of mixtures of polar/non-polar solvents to remove the lipid content from the tissue in analysis where the fatty acids will be found. Throughout this study the extracting solution was chloroform:methanol (2:1 *v/v*). Samples were dried before extraction in order to obtain a water free sample which increased extraction efficiency. Freeze drying was more appropriate than oven drying since the samples were submitted to low temperatures reducing the chances of losing sample material by evaporation.

Whenever quantitative analysis is to be used, it is of good practice to add a fatty acid standard at this stage to check extraction efficiency later. The extraction itself took about 30 minutes in the fridge using the quick method developed by (Folch *et al.*, 1957) and then the sample was cleaned from water soluble salts using a salt aqueous solution (magnesium chloride, 0.017% *w/v*). After this, it was necessary to separate the two phases formed using light centrifugation. The aqueous phase was discarded and another wash with a mixture of polar/non-polar solvents (Folch upper phase solution, chloroform:methanol:water, 3:48:47 *v/v/v*) was required to clean the sample. Any remaining water resulting from the washing solutions was removed with a pasteur pipette under vacuum to ensure that the lipid extract was water free. For quantitative purposes, the lipids were weighed by evaporation of the sample to dryness in a rotary evaporator, dissolved in chloroform and transferred to a preweighed vial, reevaporated under oxygen free nitrogen flow and reweighed.

### Derivatisation

Derivatisation was required for gas-liquid chromatography, in order to permit analysis of compounds with inadequate volatility and stability, in one hand and to improve chromatographic performance and peak shape on the other hand (Knapp, 1979). In the present study the derivatisation used was methylation, which involves a chemical reaction where the hydrogen atom from the carboxyl end of the fatty acid is substituted by a methyl group forming a methyl ester. This reaction is catalysed by boron trifluoride-methanol ( $\text{BF}_3$ ) and is based on the work by Morrison (Morrison and Smith, 1964).



The lipid samples were redissolved in an appropriate volume of chloroform, to make a concentration of lipids of  $1\text{--}2\text{ mg}\cdot\text{ml}^{-1}$  and transferred to Teflon lined screw cap vials with the methylating agent in a nitrogen atmosphere to prevent fatty acid oxidation and heated to  $100\text{ }^{\circ}\text{C}$  for 1 hour. The fatty acids become in this way fatty acid methyl esters (FAMES). After methylation, the methylated fatty acids were recovered and the methylating agent removed. A water-pentane system was used to clean and separate the fatty acids methyl esters. The pentane was evaporated under oxygen free nitrogen flow and the fatty acid methyl esters were finally dissolved in hexane.

Trimethylsulfonium hydroxide (TMSH) in methanol was also used in this study as methylating agent. Unlike the reaction with  $\text{BF}_3$ , the methylation reaction with TMSH takes place in the injector and in consequence does not require long preparation involving heating or removal of the methylating agent, saving time and reagents. Therefore the lipids were just dissolved in the methylating agent, shaken and were ready for injection after a few seconds. A standard fatty acid methyl ester, not present in the unknown samples (tricosanoic fatty acid methyl ester) was added just before injection in the GC or GC-MS as an internal standard, the component relative to which all the other fatty acids were quantitated. It is useful in eliminating injection errors.

### **Injection**

The sample was introduced into the injection chamber where it vaporizes due to high temperature ( $230\text{ }^{\circ}\text{C}$ ) and it is driven by the carrier gas into the chromatographic column. Each component in the sample will distribute itself between the two phases (stationary and moving phase) according to its partition coefficient (ratio of the concentrations in the two phases at equilibrium). The components will begin to separate when the moving phase carries them through the system. At the far end of the column the methyl esters are quantified by the detector and a chromatogram is produced where each component in the initial sample is represented by a peak, the area of which is proportional to its amount in the sample; the retention time is also a characteristic of the compound for the operating conditions applied. This fact has an important application since it becomes possible to identify the compounds through their retention times,

obtained from standards, as long as operational conditions remain unchanged. If the detector is a mass spectrometer (as in a gas chromatograph-mass spectrometer, GC-MS) rather than a flame ionization detector (as in commonly used gas chromatographs, GC), positive identification of the compounds is possible because the mass spectrometer gives information about the masses of the fragments of the molecule, formed by electron bombardment, and among them the mass of the molecular ion identifies the compound. Both types of instrument were used in this study. The working principles of the GC-MS are described in (Salgado, 1992).

### Calibration and operating conditions

When determining the best operating conditions, due consideration must be given to the type of chromatographic column and the oven's temperature programme in order to ensure the optimum running time and peak resolution. In addition, any analytical instrument requires a calibration to be carried out prior to analysis, in order to establish a relationship between the amount of substance in analysis and the response from the instrument. Usually this relationship is a straight line and in this study it represents the relation between concentration of fatty acid methyl esters and the corresponding area of the same compounds obtained from the instrument. The calibration was accomplished by injecting a series of diluted standard solutions of fatty acid methyl esters over a concentration interval where the unknown compounds are expected to fall within. The calibration compounds should be as many and as similar to the ones found in the unknown samples as possible, so that they can be identified and quantitated.

The GC-MS and GC operating conditions used in this work are summarized in Table 3.1.

The injection technique used is explained in detail in (Salgado, 1992) and was based on the work by (Frank *et al.*, 1978).

For the calibration of the GC-MS, equipped with the Carbowax 20M column, a mixture of fatty acid methyl esters was prepared from standards and a stock solution made in chloroform. It contained the FAMES 16:0, 17:0, 18:0, 18:1 $\omega$ 9, 18:2 $\omega$ 6, 18:3 $\omega$ 3, 20:0, 20:1 $\omega$ 9, 20:2 $\omega$ 6, 20:3 $\omega$ 6, 20:4 $\omega$ 6, and 22:6 $\omega$ 3, at the concentration of 500  $\mu\text{g} \cdot \text{ml}^{-1}$

column	GC-MS		GC
	capillary	capillary	capillary
phase	Carbowax 20M	BPX-70	Carbowax 20M
thickness ( $\mu$ m)	0.25	0.25	0.25
length (m)	30	30	30
ID (mm)	0.32	0.32	0.32
carrier	helium	helium	hydrogen
flow ( $\text{ml} \cdot \text{min}^{-1}$ )	1	2.5	1
injector	split/splitless	split/splitless	on-column
temperature ( $^{\circ}\text{C}$ )	230	230	230
detector	MS-EI	MS-EI	FID
temperature ( $^{\circ}\text{C}$ )	230	230	230
energy (eV)	70	70	-
mass scan range ( $m/z$ )	45–400	45–400	-
air pressure (KPa)	-	-	110
hydrogen pressure (KPa)	-	-	60
Sample ( $\mu\text{l}$ )	1	1	1
injection mode	splitless	splitless	splitless
oven temperature (GC-MS)	50 $^{\circ}\text{C}$ $\xrightarrow{40^{\circ}\text{C}/\text{min}}$ 160 $^{\circ}\text{C}$ $\xrightarrow{3^{\circ}\text{C}/\text{min}}$ 190 $^{\circ}\text{C}$ $\xrightarrow{40^{\circ}\text{C}/\text{min}}$ 230 $^{\circ}\text{C}$		
oven temperature (GC)	80 $^{\circ}\text{C}$ $\xrightarrow{50^{\circ}\text{C}/\text{min}}$ 150 $^{\circ}\text{C}$ $\xrightarrow{3.3^{\circ}\text{C}/\text{min}}$ 230 $^{\circ}\text{C}$		

Table 3.1: Operating conditions used in GC-MS and GC analysis.

each compound. From this solution, a second solution was prepared with half the concentration ( $250 \mu\text{g} \cdot \text{ml}^{-1}$ ), and from the latter, 5 dilutions standards were made. The final concentration of the standards were 10, 50, 75, 100 and  $150 \mu\text{g} \cdot \text{ml}^{-1}$ . The internal standard tricosanoic acid methyl ester (23:0) was added before injection in a way that its concentration was the same in all the 5 standards  $49.2 \mu\text{g} \cdot \text{ml}^{-1}$ . They were then injected in the GC-MS to calibrate it.

Another calibration was necessary, when using the column BPX-70 used for the analysis of mussel fatty acids. In this case the FAMES 22:0 and 24:1 $\omega$ 9 were added to the same twelve FAMES used in the previous calibration and a new stock solution was prepared containing  $250 \mu\text{g} \cdot \text{ml}^{-1}$  of each of the fourteen FAMES. A series of dilutions

were prepared to achieve the final concentrations of 12.5, 25, 50, 75 and 100  $\mu\text{g} \cdot \text{ml}^{-1}$ . The internal standard was included in all standards at the same concentration of 49.2  $\mu\text{g} \cdot \text{ml}^{-1}$ . The two new FAMES were added in this calibration because lipids were later separated into their constituent groups, phospholipids and neutral lipids which needed two calibrated yield monitors, the phospholipid, dibehenoyl phosphatidylcholine (22:0) and the triacylglyceride 15-trinervonin (24:1 $\omega$ 9).

### 3.2.2 Algae

Three species of microalgae *Pavlova lutheri* (Droop) Green, *Tetraselmis chuii* Butcher and *Rhinomonas reticulata* (*Rhodomonas baltica*) laboratory reared were chosen as the main food source for mussels throughout the experiments. They were analysed to determine their dry weight and fatty acid composition.

#### Cell counts and Dry weight

Samples of approximately 500 ml of each of the three algae cultures were collected from the cultures in the School of Ocean Sciences, counted and filtered for density and dry weight determinations, respectively. Samples were kept in the fridge for no more than one hour, while subsamples were taken and their cell density determined.

To count the number of cells in the samples, five aliquots of 1 ml each were collected from the three algae cultures, and diluted to 100 ml with filtered seawater. After good homogenization the number of cells from each culture was counted.

Five replicate samples of 100 ml each were filtered through Whatman GF/C filters, 1.2  $\mu\text{m}$  pore size, mounted on a millipore vacuum system. The filters had been previously weighed and washed with methylene chloride in a soxhlet for 24 hours, to remove any organic contamination present, such as fatty acids, that could interfere with fatty acid analysis (Scribe *et al.*, 1991). The filtrates were then freeze dried overnight, and weighed again to calculate the dry weight of the microalgae samples.

#### Fatty Acids

Lipids and fatty acids were extracted from the dried microalgae. The filters were soaked in 20 ml of chloroform-methanol (2:1) solution containing an antioxidant, 2,6-di-*tert*-

butyl-*p*-cresol (BHT), and 0.5 ml of the triacylglyceride triheptanoin (17:0) standard solution ( $0.213 \text{ mg} \cdot \text{ml}^{-1}$ ) added to determine the extraction efficiency. The mixture was stirred and kept in the fridge for 30 minutes. The lipid extract was then washed with 0.2 volumes of magnesium chloride solution 0.017%, to remove hydrosoluble salts and centrifuged at 2000 rpm for 5 minutes to separate clearly the water phase from the organic phase. The water phase was discarded and the organic phase containing the lipids was washed with Folch upper phase, (chloroform:methanol:water, 3:48:47) to clean the lipid extract. Evaporation to dryness in a rotary evaporator at  $40^\circ\text{C}$  enabled lipid weighing before methylation. For the preparation of fatty acid methyl esters, the samples were redissolved in a suitable volume of chloroform; 1 ml of lipid sample was taken and 2 ml boron trifluoride-methanol 14% were added under nitrogen and the solution heated at  $100^\circ\text{C}$  for 1 hour. The resulting fatty acid methyl esters were extracted by adding pentane and water in the proportions 2:1, shaken and the pentane phase recovered after separation of the two phases. Pentane was then evaporated under nitrogen and the methyl esters finally dissolved in hexane to which 0.1 ml of tricosanoic fatty acid methyl ester (23:0) standard solution ( $0.492 \text{ mg} \cdot \text{ml}^{-1}$ ) was added for quantification purposes. The samples were injected in a gas chromatograph mass spectrometer (GC-MS) for analysis.

### 3.2.3 Mussel collection and preparation

Ten specimens of *Mytilus edulis* were collected, at low tide, from the midshore in Beaumaris, with an average length of  $3.4 \pm 0.3$  cm and brought into the laboratory for analysis of fatty acid content. The purpose of this analysis was to characterize the fatty acid composition of mussels from this area and time of year and to investigate their individual fatty acid variability. Thus, the analysis were conducted in each of the 10 mussels separately.

Mussels were measured and shelled to remove the soft tissues. The wet weights of the soft tissues were registered and the whole organisms were freeze dried for 24 hours. Before the lipid extraction was initiated, the dry weight was taken. The whole mussel tissues were ground in pestle and mortar, mixed with 20 ml of chloroform-methanol solution and 0.5 ml of a triacylglyceride (*cis*-15-trinervonin, 24:1) solution ( $0.976 \text{ mg} \cdot$

ml<sup>-1</sup>) to check the extraction efficiency. This triacylglyceride was chosen after being proved its absence in the natural samples. The mixture was then vortexed and left in the fridge for 2 hours. The extracted samples were vortexed again and filtered to separate the solid residues of the mussel flesh from the extract. The test vials were rinsed with 10 ml of extracting solvent to remove all the mussel residues. The lipid extract is a clear yellow solution which from this point onwards, undergoes the same procedure as the one described for the algae. For the methylation, the lipids were redissolved in a volume of chloroform which made the solution no more than 10 mg · ml<sup>-1</sup> in concentration or otherwise diluted to meet this requirement. From this lipid solution, 100 μl were taken and methylated with 25 μl of TMSH 0.2 M in methanol. This methylating agent trimethylsulfonium hydroxide (TMSH) was used, instead of boron trifluoride-methanol, to shorten the methylating process, since this reagent does not require heating or a long wait for the reaction to be completed. It also saves reagents because it uses very small amounts of sample and does not need to be removed from the methylated sample afterwards. The methyl esters were injected in the GC-MS for analysis.

### 3.2.4 Vegetable oils

Because the oils are already in a reasonable pure form of lipids, extraction was unnecessary and did not take place. Samples of the four different vegetable oils, rapeseed, linseed, olive and sunflower were directly methylated to obtain the fatty acids derivatives and were analysed in the GC-MS.

Three drops of oil were weighed in three separate vials and each dissolved in 10 ml chloroform. Only 1 ml from each one of the three solutions was methylated with boron trifluoride-methanol, extracted with pentane and transferred to hexane and analysed.

## 3.3 Results

### 3.3.1 Algae

The average cell density of the 5 algae subsamples was  $(2.82 \pm 0.07) \times 10^6$ ,  $(2.95 \pm 0.05) \times 10^6$  and  $(0.71 \pm 0.03) \times 10^6$  cells · ml<sup>-1</sup> for *Pavlova*, *Tetraselmis* and *Rhinomonas* respectively. Figure 3.1(a) shows the cell density of each of the 5 replicates with standard

deviations.

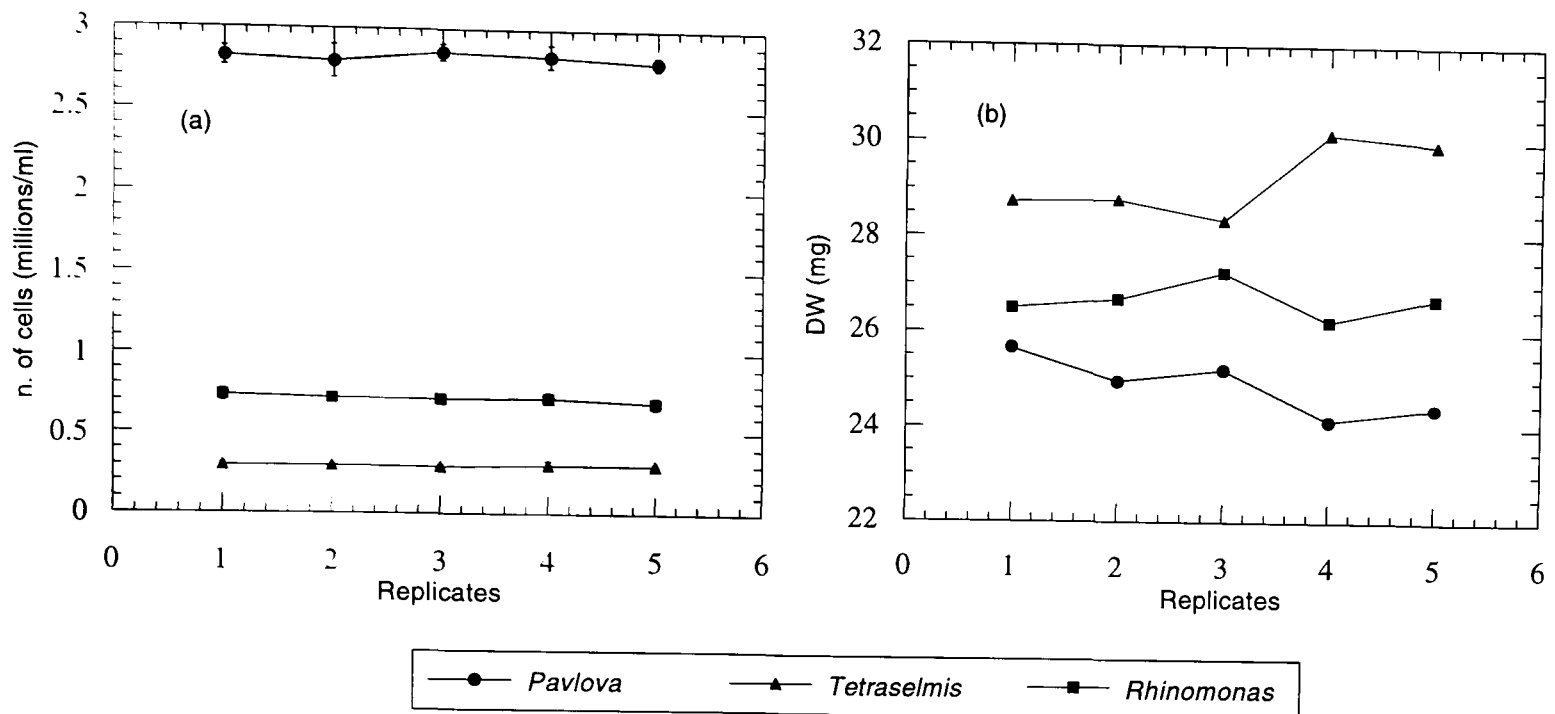


Figure 3.1: (a) Average cell density of 5 subsamples of the algae cultures *Pavlova lutheri*, *Tetraselmis chuii* and *Rhinomonas reticulata* and (b) Dry weight of 100 ml subsamples of the same algae.

The dry weights of the 3 microalgae species are presented on figure 3.1(b). The average dry weight and standard deviation for 100 ml samples was  $24.87 \pm 0.61$ ,  $29.19 \pm 0.81$  and  $26.68 \pm 0.38$  mg for *Pavlova*, *Tetraselmis* and *Rhinomonas*, respectively. Given these values and the results of cell density, the weight of one single cell for each species and the number of cells present in 1 mg of dry weight were calculated. The means and standard deviations of these parameters are shown in Table 3.2. These results indicate that although *Pavlova* culture is far more dense than the other two, the weight of each cell is the lowest and that *Pavlova* cells are the smallest among the three.

Algae	Weight of 1 cell $\pm$ sd (pg)	n. of cells $\pm$ sd in 1 mg DW
<i>Pavlova l.</i>	$88.18 \pm 2.98$	$(11.34 \pm 0.38) \times 10^6$
<i>Tetraselmis c.</i>	$988.80 \pm 198.13$	$(1.01 \pm 0.20) \times 10^6$
<i>Rhinomonas r.</i>	$375.13 \pm 18.60$	$(2.66 \pm 0.13) \times 10^6$

Table 3.2: Mean and standard deviation of the weight of one single cell and number of cells existing in 1 mg of cell's dry weight.

Statistical analysis was performed to test significant differences of cell density be-

tween the replicates of the same algae species. The null hypothesis tested is written as  $H_0 : \mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$ , meaning that the means of the 5 replicates of cell countings, for each species, do not differ significantly. Similarly, differences in the counts within each replicate were tested. Analysis of variance revealed no significant differences in any of the cases, confirming that the method is reliable. The raw data, before any calculations were done, was used in the statistical tests, so the results of the analysis of variance. Tables 3.3 to 3.5, refer to the number of cells counted in  $50\mu\text{l}$  of cell culture. Since no differences were found in the number of cells from the same culture, the pooled mean and standard deviation was calculated and given in  $\text{cells} \cdot \text{ml}^{-1}$ . The calculation of the final standard deviation has been corrected accordingly to take into account the propagation of errors (Miller and Miller, 1989).

### 3.3.2 Algae fatty acids

#### Calibration

In the calibration of the GC-MS for the FAMES straight lines were fitted, using linear regression analysis to the chromatographic area of each FAME versus the known concentration in the standard. The adequacy of this procedure lies on the assumption that the GC-MS detector had a linear response over the range of concentrations used. The standards solutions included the FAMES 16:0, 17:0, 18:0, 18:1 $\omega$ 9, 18:2 $\omega$ 6, 18:3 $\omega$ 3, 20:0, 20:1 $\omega$ 9, 20:2 $\omega$ 6, 20:3 $\omega$ 6, 20:4 $\omega$ 6 and 22:6 $\omega$ 3 each one in five different concentrations: 25, 50, 100, 250 and  $500\mu\text{g} \cdot \text{ml}^{-1}$ . A constant concentration of 23:0 ( $49.2\mu\text{g} \cdot \text{ml}^{-1}$ ) was added as the internal standard in each of the 5 solutions. Table 3.6 shows the parameters obtained from the linear regression which in conjunction with equation 3.1 were used to calculate the concentrations of FAMES from each sample. The meaning of the symbols is as follows: “Conc” is the FAME concentration, “area” is the FAME chromatographic area; “intercept” and “slope” are the regression coefficients given in Table 3.6. and area IS, conc IS are respectively area and concentration of the internal standard.

$$\text{Conc}(\mu\text{g}/\text{ml}) = \frac{\text{area} - \text{intercept}}{\text{slope}(\text{area} \cdot \text{ml}/\mu\text{g})} \quad (3.1)$$



Source of variation	SS	DF	MS	F	P
Replicates	4	3289	822	0.71	0.596
Error	20	23218	1161		
Total	24	26507			

$P > 0.05$  therefore  $H_0$  is accepted

Table 3.3: Analysis of variance for the number of *Pavlova lutheri* cells between replicates.

Source of variation	SS	DF	MS	F	P
Replicates	4	116.2	29.0	0.40	0.809
Error	23	1686.5	73.3		
Total	27	1802.7			

$P > 0.05$  therefore  $H_0$  is accepted

Table 3.4: Analysis of variance for the number of *Tetraselmis chunii* cells between replicates.

Source of variation	SS	DF	MS	F	P
Replicates	4	1525	381	1.43	0.253
Error	24	6376	266		
Total	28	7901			

$P > 0.05$  therefore  $H_0$  is accepted

Table 3.5: Analysis of variance for the number of *Rhinomonas reticulata* cells between replicates.

Fame	slope (area · ml/μg)	intercept (area)	corr
16:0	2036	-3780	0.991
17:0	2247	24	0.982
18:0	2288	7454	0.977
18:1ω9	2189	950	0.976
18:2ω6	2074	-2513	0.978
18:3ω3	1979	-2924	0.978
20:0	2455	29606	0.965
20:1ω9	2324	13669	0.966
20:2ω6	2204	-1005	0.966
20:3ω6	2178	-64	0.966
20:4ω6	2223	-2404	0.967
22:6ω3	2606	-16627	0.954

Table 3.6: Regression parameters and correlation coefficient for the calibration area of FAMES against their concentrations.

The method just described to calculate FAME concentrations is based on the raw areas of the standards regressed against their concentrations. Another method was also used where the raw areas of the compounds were divided by the area of the internal standard and multiplied by its concentration using equation 3.2. This new variable named “resp” is defined as

$$\text{resp}(\mu\text{g/ml}) = \frac{\text{area}}{\text{area IS}} \times \text{conc IS}(\mu\text{g/ml}) \quad (3.2)$$

and was regressed against the standard’s concentrations. Equation 3.1 still applies with “area” substituted for “resp”. This method was used in order to compensate for errors occurring for example in the injections process. However, the error associated with the area of the internal standard must be small otherwise this will be another source of error in the calculation of the concentration of the desired FAME by way of equation 3.1. We observed that for different injections of the same sample, the concentration results obtained using this method indeed showed a large variation. Re-investigation of the data indicated that the areas of the internal standard varied within

a large interval although its concentration and thus, its area should have remained approximately constant for different injections of the sample. The large variations of C23 resulted in large errors in FAME concentrations, when calculated in this way. Comparing the two methods of calibration the following conclusions were withdrawn.

1. The relative amount of FAMES is the same using both calibration methods.
2. The absolute values of FAME concentrations differ with the method. They are higher when calculated with the calibration based on raw areas than with the one based on "resp". However the differences are almost double for *Rhinomonas* whereas for *Pavlova* they are smaller and for *Tetraselmis* are almost insignificant.
3. In terms of percentage error ( $\text{sd}/\text{mean} \times 100$ ), the errors for all the FAMES in *Pavlova* and *Tetraselmis* calculated with raw areas were smaller than when calculated with "resp", but for some FAMES in *Rhinomonas* the opposite occurred. The majority of FAMES presented a smaller error calculated with raw areas, though.

It was decided then to use the raw areas of the standards to built the regression lines since the errors were minimized this way.

Some of the fatty acids detected in the samples were not used in the calibration due to unavailability of the standards and since some of them are quantitatively important, their concentrations were calculated from the closest FAMES present in the calibration. The saturate 14:0 and all the 16's mono and polyunsaturates were calculated from the regression of the 16:0; 18:1 $\omega$ 7 was calculated from 18:1 $\omega$ 9; 18:4 $\omega$ 3 from 18:3 $\omega$ 3 and 20:5 $\omega$ 3 was calculated from the FAME, 20:4 $\omega$ 6. These values should be interpreted with care as the regression coefficients are not the same for each FAME as can be seen in Table 3.6. However, it seemed useful to calculate them even with reservation rather than not having information at all.

### Fatty acid composition

The study of fatty acid composition of the three species of algae is presented in 3 groups, saturated, monounsaturated and polyunsaturated fatty acids. Table 3.7 summarizes

their fatty acid composition in percentage relative to the total. The polyunsaturated fatty acids are the biggest group representing 39 to 64% of the whole fatty acid components, depending on the species. The saturates constitute the next important group with percentages varying from 25 to 37% and the monounsaturated fatty acids group represents the smallest fraction of the fatty acid pool with 7–25%. *Pavlova lutheri* has the most balanced distribution between each group. The saturated and polyunsaturated fatty acids represent approximately the same proportion (37 and 39%) and the monounsaturated fatty acids slightly lower than the previous (25%). In the other two species, *Tetraselmis chuii* and *Rhinomonas reticulata* the differences are markedly larger. The polyunsaturated fatty acids predominate over the saturated and especially over the monounsaturated which represent 13 and 7% of the total fatty acid composition.

The ratio of all polyethylenic acids to all saturated acids (poly/sats) is highest in *Tetraselmis chuii* poly/sats = 2.4, similar to the one in *Rhinomonas reticulata* poly/sats = 2.1 and lowest in *Pavlova lutheri* poly/sats = 1.0. The ratio of polyethylenic acids to monoethylenic (poly/mono) is higher for *Rhinomonas* poly/mono = 9.7 than for *Tetraselmis* poly/mono = 4.7 or *Pavlova* poly/mono = 1.6. These figures show the relative importance of the polyunsaturates over the saturates and especially the monounsaturates. Within the polyunsaturates the fatty acids belonging to the linolenic family ( $\omega 3$ ) dominate the ones from the linoleic family ( $\omega 6$ ), the ratio  $\omega 3/\omega 6$  for the three microalgae were 7, 3.5 and 5.3 respectively for *Pavlova*, *Tetraselmis* and *Rhinomonas*. Fatty acid composition of marine organisms has typically predominance of  $\omega 3$  over  $\omega 6$  fatty acids, which is thought to be originated in plankton and carried along throughout the ocean food chain (Ackman, 1964).

Qualitatively the differences in fatty acid composition between the three microalgae were small, the striking differences being the absence of 22:6 $\omega 3$  and the presence of the polyenoic 16 carbon fatty acids 16:3 $\omega 6$  and 16:4 $\omega 3$  in *Tetraselmis*. Figure 3.2 shows the concentration of the major ten fatty acids present in each species organized in three groups according to their degree of unsaturation and indicating the percentage of the fatty acids shown, from the total of the group. Among the saturates, palmitic and myristic (16:0 and 14:0) were the main fatty acids present, whereas the predominant

Fame	<i>P. lutheri</i>	<i>T. chuii</i>	<i>R. reticulata</i>
saturates			
14:0	12.1	2.5	15.4
15:0	0.5	0.1	0.8
16:0	23.8	22.5	13.1
18:0	0.5	0.2	0.3
sum	36.9	25.3	29.6
monounsaturates			
16:1 $\omega$ 7	21.4	1.1	1.0
16:1 $\omega$ 9	-	0.3	2.9
16:1 $\omega$ 13	-	1.4	-
18:1 $\omega$ 9	1.6	7.8	0.6
18:1 $\omega$ 7	1.6	1.8	2.1
20:1 $\omega$ 9	-	0.8	-
sum	24.6	13.2	6.6
polyunsaturates			
16:2 $\omega$ 6	0.7	0.9	-
16:3 $\omega$ 6	0.5	3.7	-
16:3 $\omega$ 3	-	1.0	-
16:4 $\omega$ 3	-	16.0	-
18:2 $\omega$ 6	1.3	8.4	8.4
18:3 $\omega$ 6	0.8	0.6	1.4
18:3 $\omega$ 3	1.8	13.8	19.9
18:4 $\omega$ 3	10.0	13.1	23.9
20:4 $\omega$ 6	0.4	0.1	0.4
20:5 $\omega$ 3	18.6	4.0	6.1
22:6 $\omega$ 3	4.5	-	3.7
sum	38.6	61.6	63.6

Table 3.7: Percentage composition of fatty acids in three microalgae.

monounsaturates included 16:1 $\omega$ 7 and the 18C, both positional isomers 18:1 $\omega$ 9 and 18:1 $\omega$ 7. The fatty acids found in the polyunsaturates ranged from the 18C to the long chain 20:5 $\omega$ 3 and 22:6 $\omega$ 3, except in *Tetraselmis* which showed no 22:6 $\omega$ 3, but moderate and high amounts of 16:3 $\omega$ 6 and 16:4 $\omega$ 3, respectively. The 18C were mainly 18:2 $\omega$ 6, 18:3 $\omega$ 3 and 18:4 $\omega$ 3. *Pavlova* and *Rhinomonas* were the most similar algae in terms of the 10 major fatty acids and *Tetraselmis* differed from the previous two especially in the polyenoic fatty acids composition.

Quantitatively however, fatty acids are distributed differently amongst the three algae species. In the saturates, palmitic acid (16:0) is the most concentrated fatty acid in both *Pavlova* and *Tetraselmis* while myristic acid (14:0) dominates in *Rhinomonas*.

Within the monounsaturates, palmitoleic acid (16:1 $\omega$ 7) is the most abundant fatty acid in the primnesiophyte *Pavlova* representing 87% of the class. The red microalgae *Rhinomonas*, on the other hand, has the lowest percentage of monounsaturates but palmitoleic acid is also the abundant monounsaturate. The prasinophyte *Tetraselmis*, however, shows oleic acid (18:1 $\omega$ 9) as the dominant monounsaturated. Together with vaccenic acid, these two monoethylenic compounds constitute 72% of the group.

It is in the polyunsaturates that the biggest differences lie, particularly in *Tetraselmis chuii* where the long chain polyunsaturate 22:6 $\omega$ 3 is substituted by the polyenoic 16:3 $\omega$ 6 and 16:4 $\omega$ 3, the latter becoming the highest polyunsaturate of the group. The 18C polyunsaturates come next in concentrations and the highly polyunsaturate 20:5 $\omega$ 3 completes the pool of the polyethylenic acids. For *Pavlova lutheri* the 20:5 $\omega$ 3 is the predominant polyunsaturate followed by 18:4 $\omega$ 3 and 22:6 $\omega$ 3. The other two 18C linoleic (18:2 $\omega$ 6) and linolenic (18:3 $\omega$ 3) acids are the least concentrated in the group contributing with 8% of the polyunsaturates. In *Rhinomonas reticulata* the 18:4 $\omega$ 3 and 18:3 $\omega$ 3 dominate linoleic, 20:5 $\omega$ 3 and 22:6 $\omega$ 3. Another polyunsaturate of 18 carbons is present just in this red algae, among the major 10 fatty acids and that is the 18:3 $\omega$ 6, its concentration being however small compared to the other polyenoics of the class.

Statistical analysis on the major ten fatty acids of the 4 replicates of *Pavlova lutheri* and *Tetraselmis chuii* and the 5 replicates of *Rhinomonas reticulata*, revealed significant differences between the replicates when analysis of variance was applied. These differences were attributed to some loss of the sample during methylation and bad chro-

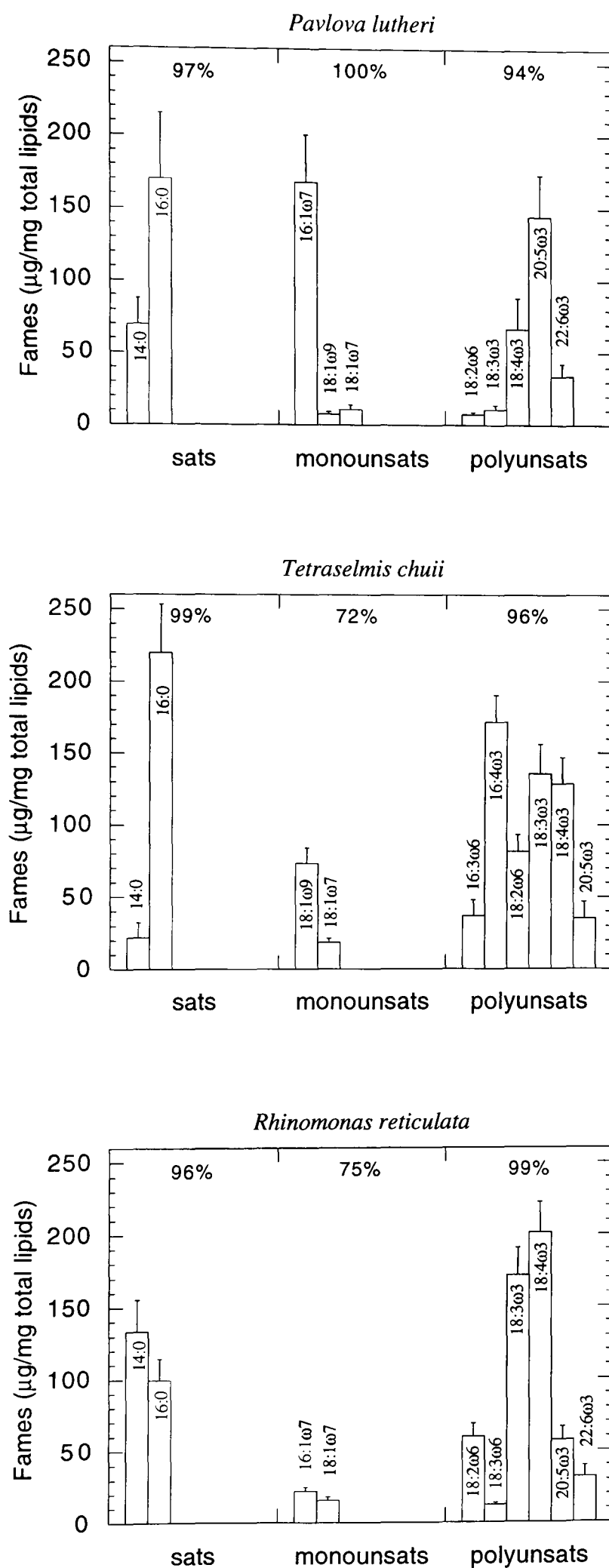


Figure 3.2: Average and standard deviation of the 10 major fatty acids of the microalgae *Pavlova lutheri*, *Tetraselmis chuii* and *Rhinomonas reticulata*. Percentages refer to the total of each group.

matographic response. Since the means of the replicates within one single alga sample should be similar, the replicates whose means were out of the 95% confidence interval were rejected. Sample evaporation during methylation was the probable cause of the large standard deviations from the mean. The pooled mean of the selected values was used in Figure 3.2.

### 3.3.3 Mussels

The size of the 10 mussels analysed ranged from 3.0 to 3.8 cm with an average length of  $3.4 \pm 0.3$  cm, which was large enough to enable individual analysis to be carried out. Figure 3.3 shows the lengths and the weight percentage of total lipids per dry weight of mussels. The percentage in weight of lipids varies from 3 to 9% of dry weight, the mean being  $5.7 \pm 2.0\%$ .

The water content from each mussel was calculated and is shown together with their wet and dry weights in Table 3.8. As it can be seen the water percentage is very high and relatively constant for all mussels ( $85 \pm 3\%$ ). The wet and dry weights vary according to the size of the mussel and its water content.

The percent fatty acid composition of 9 mussels is shown in Table 3.9 distributed among the three groups of increasing degree of unsaturation. One mussel from the initial ten was not considered for quantitative results since the area of the internal standard was abnormally low giving erroneously high fatty acid content. These mussels exhibited high percentage of polyunsaturated fatty acids (50%) and considerable high percentage of saturates (34%), the smallest group being the monounsaturates (16%). The saturated fatty acids of these mussels were characterized by two main components, palmitic acid constituting 23% of the total fatty acids and stearic acid with 7%. The other saturates found, 14:0, 15:0 and 17:0 were minor compounds, the three of them making up 4% of the total. Within the monounsaturated fatty acids, gondoic acid (20:1 $\omega$ 9) was the most concentrated. Not found in appreciable amounts in algae, it represents a considerable 6% of the total fatty acid pool and 38% of the monounsaturates. After gondoic acid, palmitoleic acid (16:1 $\omega$ 7) was the next abundant monounsaturate (4%) and the two 18C, vaccenic (18:1 $\omega$ 7) and oleic (18:1 $\omega$ 9) acids followed with similar percentages 2.7 and 2.5%, respectively. In the polyunsaturates the 20C predominated the highly unsaturates



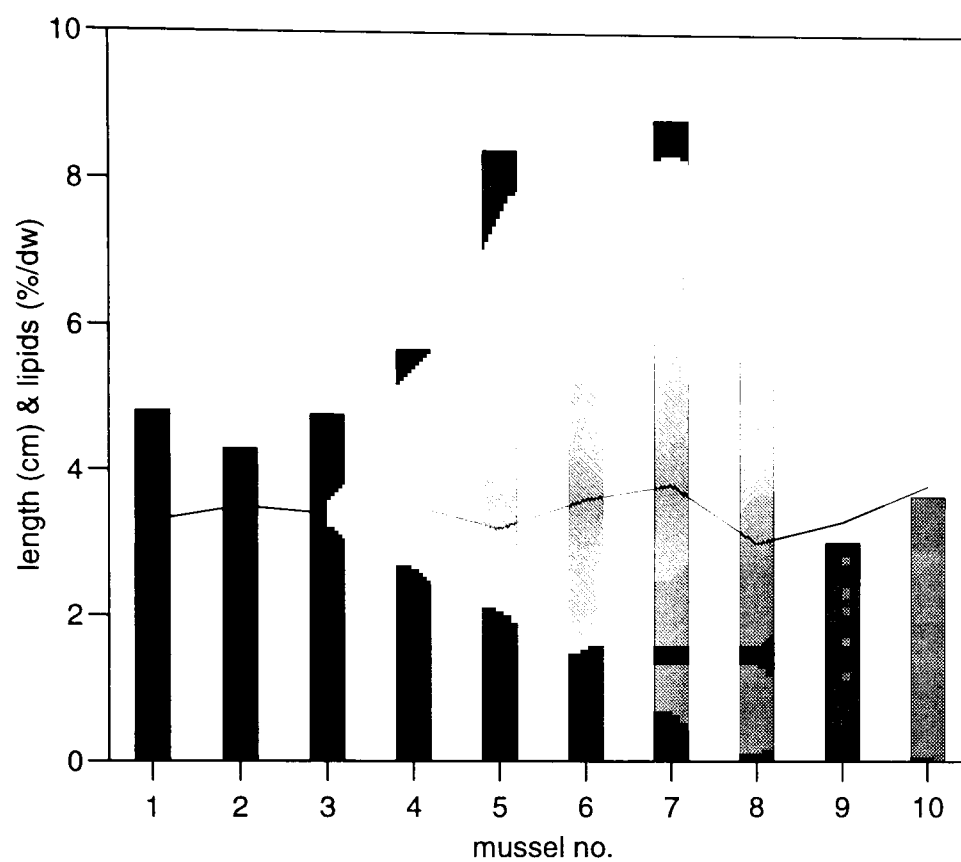


Figure 3.3: Length (line) and lipid weight (bars) of 10 mussels collected from Beaumaris.

Mussel	ww (mg)	dw (mg)	water (%)
1	700	100	86
2	950	150	84
3	1050	150	86
4	900	100	89
5	600	50	92
6	1150	200	83
7	1850	300	84
8	750	100	87
9	600	100	83
10	1350	250	81

Table 3.8: Water percentage, wet and dry weights of 10 mussels collected from Beaumaris.

20:5 $\omega$ 3 and 22:6 $\omega$ 3 were the most abundant with 15 and 13% respectively, followed closely by arachidonic acid (20:4 $\omega$ 6) representing 10% of the total. A dienoic acid with 20 carbons constituted 3% of the polyenoic acids and the rest of the group was composed by 18C and a 16C: linoleic, linolenic, 18:4 $\omega$ 3 and a 16:2 each of which with percentages lower than 2%.

In summary and in quantitative terms the major 10 fatty acids found in the mussels are represented in Figure 3.4 in a similar representation to the fatty acid groups of algae. The two most abundant saturates are palmitic and stearic (18:0) acids with amounts of 47 and 14  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids. Stearic acid is not an abundant fatty acid in microalgae, consequently its presence in second position suggests a different origin other than phytoplankton. The monounsaturates group although with small concentrations, varying from 5 to 12  $\mu\text{g} \cdot \text{mg}^{-1}$ , has a variety of four fatty acids from which gondoic acid, the biggest, is unusual in microalgae. The polyunsaturates are rich in 20C mainly the highly unsaturated ones arachidonic acid and 20:5 $\omega$ 3, the latter dominating the group with 31  $\mu\text{g} \cdot \text{mg}^{-1}$  immediately followed by the 22:6 $\omega$ 3 with 28  $\mu\text{g} \cdot \text{mg}^{-1}$ ; the 20:2 is the smallest of the four with 6  $\mu\text{g} \cdot \text{mg}^{-1}$ . Again in this group such an extent of 20C is not common from phytoplankton, the 18C polyunsaturated or the 16C being more typical. These differences show that mussels do not have a single source of diet, phytoplankton and detritus both contribute to the fatty acid composition of mussels and also that some fatty acids are specific of some species like gondoic and arachidonic acids and they are present even if they are not abundant in the diet.

### 3.3.4 Vegetable oils

The percent fatty acid composition of each of the vegetable oils employed in the experiments is presented in Figure 3.5. Two of the oils, sunflower and linseed have a different predominant fatty acid, respectively linoleic (18:2 $\omega$ 6) and linolenic (18:3 $\omega$ 3) acids; the other two, rapeseed and olive oils, contain the same most abundant fatty acid, oleic acid (18:1 $\omega$ 9), in different proportions. In addition, rapeseed oil has higher amounts of linoleic acid than olive oil, the latter containing almost one third of linoleic acid relative to rapeseed oil. The saturates present are palmitic (16:0) and stearic (18:0) acids, varying from 4 to 11%. Sunflower has the highest level of saturates, 14%, and

saturates	%	monounsaturates	%	polyunsaturates	%
14:0	2.3	16:1 $\omega$ 7	4.4	16:2 $\omega$ 6	1.8
15:0	1.4	18:1 $\omega$ 9	2.5	18:2 $\omega$ 6	1.5
16:0	22.8	18:1 $\omega$ 7	2.7	18:3 $\omega$ 3	1.9
17:0	1.4	20:1 $\omega$ 9	6.0	18:4 $\omega$ 3	1.7
18:0	6.6			20:2 $\omega$ 9	3.1
				20:2 $\omega$ 6	0.9
				20:4 $\omega$ 6	9.8
				20:5 $\omega$ 3	15.0
				22:2	0.9
				22:6 $\omega$ 3	13.4
sum	34.5		15.6		49.9

Table 3.8: Average percentage fatty acid composition of 10 mussels collected from Beaumaris.

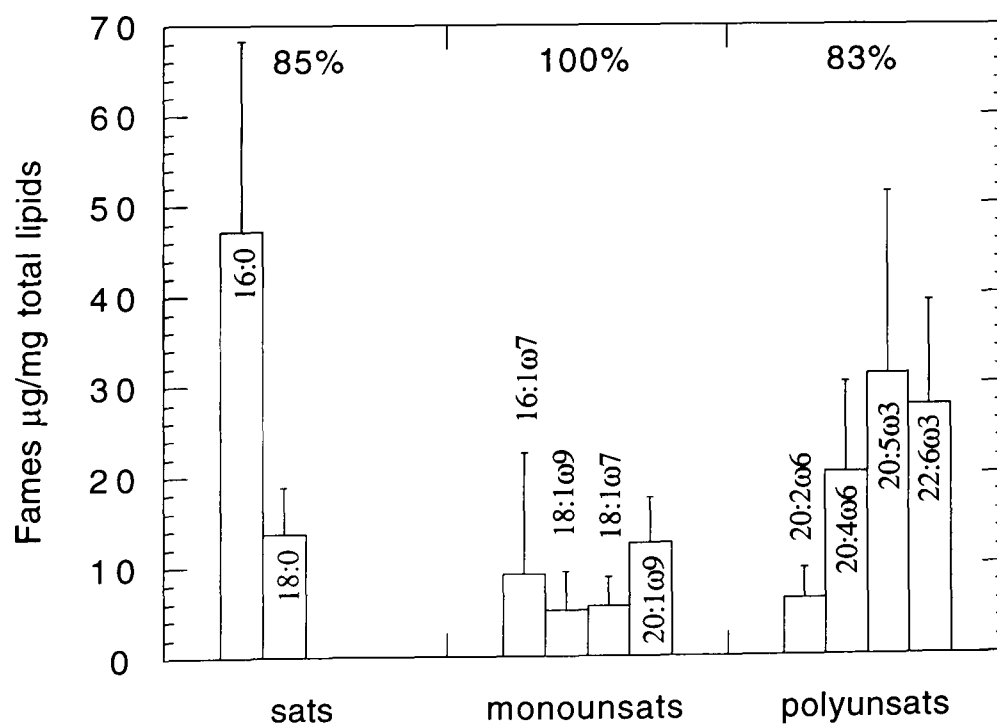


Figure 3.5: Concentration of the main 10 fatty acids from 9 mussels distributed between three classes of fatty acids. Mean and standard deviations are presented.

rapeseed the lowest, 6%, entirely constituted by palmitic acid. Olive and linseed have 12% and 11% respectively. The saturate most abundant in all the oils is palmitic acid whereas stearic acid is only a small or absent component.

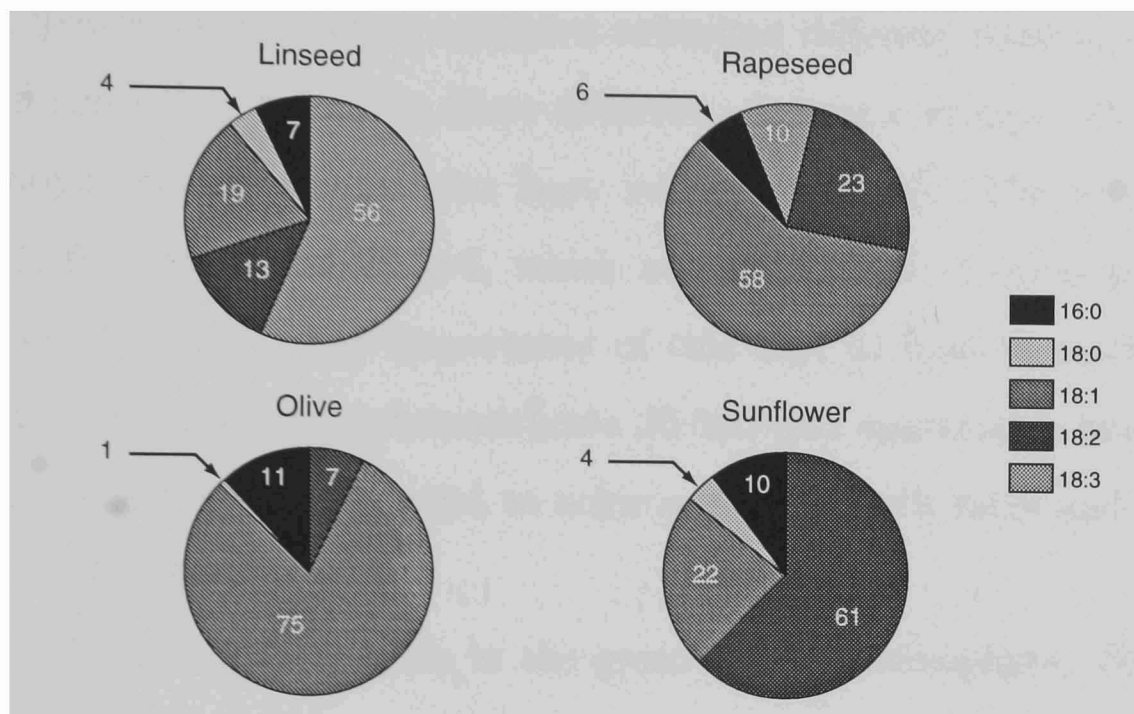


Figure 3.6: Percentage fatty acid composition of the vegetable oils rapeseed, linseed, olive and sunflower.

The only monounsaturate found in these oils was oleic acid with percentages ranging from 20% for the linseed oil to 60% or 70% for rapeseed and olive oils, making the monounsaturates the major group. A brief note should be mentioned here about rapeseed oil as it used to have high amounts of the monounsaturate erucic acid ( $22:1\omega9$ ), which was believed to cause heart problem conditions in experimental animals and because of that new varieties of the oil were produced with erucic content smaller than 0.5%. It is this new variety that was used in this study.

The 18C polyunsaturates were the abundant fatty acids in both linseed and sunflower oils. In linseed oil, 69% of its fatty acid composition is polyunsaturates, linolenic acid representing the largest proportion 56%, and linoleic completing the remaining 13%, whereas in sunflower oil, linoleic acid alone makes the 61% of the polyunsaturates. In both rapeseed and olive oils the polyunsaturates are significantly smaller than the monounsaturates, particularly in olive oil where linolenic acid is absent and linoleic is only 7% of the total fatty acids. Rapeseed has a better balance of polyethylenic acids with 33% distributed between linoleic and linolenic acids.

## 3.4 Discussion

### 3.4.1 Algae

The three species of microalgae analysed exhibited different fatty acid compositions as expected since they belong to three different taxonomic groups. *Pavlova lutheri* is a prymnesiophyte with a particular fatty acid composition. The predominant fatty acids are 16:0, 16:1 $\omega$ 7 and 20:5 $\omega$ 3, which are not typical of other prymnesiophytes (Volkman *et al.*, 1989). The importance of this alga as food in mariculture lies on the high proportions of the polyunsaturate 20:5 $\omega$ 3 and appreciable amount of 22:6 $\omega$ 3 it contains, since they are essential to achieve good growth rates and larval survival (Volkman *et al.*, 1989).

The distribution of fatty acids in the green algae, prasinophyte, *Tetraselmis chuii* is typical of most chlorophytes (Volkman *et al.*, 1989), with the characteristic high proportions of polyunsaturates 16C and 18C, low abundance of C20 polyunsaturated fatty acids (PUFA) and absence of the long chain 22:6 $\omega$ 3. The major fatty acids in this alga are 16:0 and the polyunsaturates 16:4 $\omega$ 3, 18:3 $\omega$ 3, 18:4 $\omega$ 3 and 18:2 $\omega$ 6. Within the monounsaturates oleic acid, 18:1 $\omega$ 9, is the most abundant but there are significant amounts of the vaccenic isomer, 18:1 $\omega$ 7. This fatty acid is well known to be a marker of bacterial presence but it is also a genuine component of the fatty acids of many microalgae as shown by (Volkman *et al.*, 1989) that axenic cultures of green algae exhibit considerable amounts of vaccenic acid. As algal diet, *Tetraselmis* is a good source of carbohydrates but lacks the highly unsaturate 22:6 $\omega$ 3 essential for good growth of cultured organisms.

The major fatty acids of the rhodophyte *Rhinomonas reticulata* are the polyunsaturates 18:3 $\omega$ 3 and 18:4 $\omega$ 3 and the saturates myristic and palmitic acids, (14:0 and 16:0). It also contains important proportions of the highly unsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 which could be appropriate in mariculture diets, but it has low levels of monounsaturates.

The fatty acid composition of *Pavlova lutheri* in this study is close to that reported by Langdon and Waldock, (1981) and Volkman *et al.*, (1989). The main fatty acids are the same with some quantitative dissimilarities, the main divergences occurring for

palmitoleic acid (16:1 $\omega$ 7), 18:4 $\omega$ 3 and 20:5 $\omega$ 3. Langdon and Waldock, (1981) found more palmitoleic acid and less of the other two polyenoic acids, whereas Volkman determined less palmitoleic acid and 18:4 $\omega$ 3 and more 20:5 $\omega$ 3. In the same work (Langdon and Waldock, 1981) the authors analysed cultures of *Tetraselmis suecica* which resemble in qualitative terms the fatty acid composition of *Tetraselmis chuii* analysed in this study. *Tetraselmis chuii* exhibited more 16:4 $\omega$ 3, 18:4 $\omega$ 3 and less 18:1 $\omega$ 9 than either of the *Tetraselmis suecica* cultures, but more 18:2 $\omega$ 6 than *T. suecica* analysed by Langdon and Waldock. (1981) and less than the one analysed by Volkman *et al.*, (1989).

Differences in fatty acid content are not unusual, since rearing laboratory conditions vary. Stage of growth at the time of harvesting and other culture conditions, such as light intensity, nutrient status of the medium and length of the light can change lipid composition (Volkman *et al.*, 1989). Additionally, growing temperatures may cause changes in the fatty acid content and large relative variations have been demonstrated within various algal classes (Ackman *et al.*, 1968).

Nutritional deficiencies in a diet, are usually overcome by using mixed algal diets. Green algae provide a high carbohydrate content and the requirements for 20C and 22C PUFA can be met by other species like *Pavlova lutheri* and *Rhinomonas reticulata* which are rich in these polyunsaturates.

### 3.4.2 Mussels

The analysis of the 3 cm size class mussel population revealed a steady and high water content around 85%, average dry weights of 150 mg and average lipid percentage of 6% of the mussel dry weight or 0.8% of the mussel wet weight. This latter value is comparable with the range of lipids found by (DeMoreno *et al.*, 1980) (0.9–1.8% of the wet weight) in *Mytilus platensis*. It seems that regardless the species and natural habitat, the amount of lipids in mussels have only small variations. The same can not be said about the amount of fatty acids in mussels. The total fatty acid concentration of the 9 mussels collected from Beaumaris was averaged in 13.6  $\mu\text{g} \cdot \text{mg}^{-1}$  of dry weight whereas (Ben-Mlih *et al.*, 1992) found 29.5  $\text{mg} \cdot \text{g}^{-1}$  for Mediterranean mussels. Whether our results, which were half of those obtained by Ben-Mlih were due to differences in the analytical methods employed, in particular the methylation, or because the mussels

from Beaumaris have in fact a lower level of fatty acids, was also investigated during the course of this study.

The analysis of fatty acid composition of *Mytilus edulis* was characterized by the predominance of 16:0 and the polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3. Highly unsaturated C20 and C22 are provided by the phytoplankton diet, diatoms and dinoflagellates respectively, while saturated and monoenoic acids of 14 to 18 carbons are provided by detritus (DeMoreno *et al.*, 1980). Phytoplankton and detritus are the two main sources of food in mussels (Stromgren, 1976a). This same pattern of fatty acid distribution can be found in other mytiloid species from various geographical locations (Joseph, 1982). Besides these three ubiquitous fatty acids in mussels, others present in variable percentages between species are nevertheless common in mussels. These include the monounsaturates palmitoleic, oleic and gondoic acids. Arachidonic (20:4 $\omega$ 6) and gondoic (20:1 $\omega$ 9) acids which we found to be present in substantial amounts are also typical of marine invertebrates but they are probably biosynthesized by mussels from other dietary precursors or as in the case of gondoic acid biosynthesized *de novo* as explained in Chapter 2 under the section of fatty acid biosynthesis.

Our results compared with the results of (Gardner and Riley, 1972; Paradis and Ackman, 1977) for *M. edulis* are listed in Table 3.9. Higher percentages of the 3 major fatty acids 16:0, 20:5 and 22:6 and lower percentages of the monounsaturates 16:1, 18:1 and 20:1 were obtained by us. Arachidonic acid (20:4 $\omega$ 6) in these samples was more than double the percentage found by Paradis and Ackman; Gardner and Riley who only detected a very small percentage (0.6%) in phospholipids.

It seems obvious that there is a natural variation in fatty acid composition even in the same species which has to do most probably with different climatic conditions and food availability. This fact shows clearly the inherent danger in comparing fatty acid composition of filter feeders even within a single species or population (Joseph, 1982). However a major trend of fatty acid distribution can be recognized in mussels as reported here and confirmed by (Gardner and Riley, 1972; Ben-Mlih *et al.*, 1992) and (DeMoreno *et al.*, 1980).

Fame	Gardner & Riley, (1972)	Paradis & Ackman, (1978)	this study
saturates			
16:0	13.6	15.9	22.8
monounsaturates			
16:1 $\omega$ 7	8.3	7.5	4.4
18:1 $\omega$ 9	7.4	11.1	2.5
20:1 $\omega$ 9	8.8	7.5	6.0
polyunsaturates			
20:5 $\omega$ 3	12.9	10.2	15.0
22:6 $\omega$ 3	5.9	13.7	13.4

Table 3.10: Percentage composition of selected fatty acids in *M. edulis* from different authors compared with the ones obtained in this study.

### 3.4.3 Vegetable oils

In all the four vegetable oils analysed, the fatty acid profiles are reduced to a pool of C18 and palmitic acid. This is a characteristic of fatty acids in terrestrial plants. The saturates include, palmitic and stearic acids, being palmitic acid more concentrated than stearic acid. The other 18C are oleic, linoleic and linolenic acids in varied amounts depending on the type of oil. Oleic acid, the single monounsaturate present, constitutes 75% of olive and 58% of rapeseed oils. The polyunsaturates are the predominant group for linseed and sunflower oils both having different major fatty acids which are linolenic and linoleic, respectively. Although rapeseed oil has the same dominant fatty acid as olive oil, their fatty acid composition is different; rapeseed has less oleic acid than olive but about 3 times more linoleic acid and in addition it has linolenic acid which is absent in olive oil. Since all of the oils have a different largest fatty acid or different composition, they were used to investigate the effect on the mussel fatty acid profile (Chapter 4).



### 3.5 Summary

Fatty acid composition of different organisms, relevant to the present study, were presented in this chapter. Three species of microalgae belonging to different taxonomical groups revealed different associations of fatty acids. The prymnesiophyte *Pavlova lutheri* which has as major fatty acids palmitic acid, palmitoleic and the polyunsaturate 20:5 $\omega$ 3, also contains 22:6 $\omega$ 3. The prasinophyte *Tetraselmis chuii* rich in 16C and 18C polyunsaturates, contains 20:5 $\omega$ 3 but no 22:6 $\omega$ 3. The microalgae rodophyte *Rhinomonas reticulata* revealed a good source of polyunsaturates not only 18C but 20C and 22C polyenoic acids too.

Green algae rich in 16C and 18C can provide a high carbohydrate content whereas algae with abundance of the long chain polyunsaturates 20C and 22C, like the prymnesiophyte and the rodophyte provide essential fatty acids for good growth and larval survival. A mixture of the three microalgae should be used as algal diet to meet the nutritional requirements of cultured organisms.

Mussels collected from the midshore in Beaumaris were characterized regarding their fatty acid composition. Predominance of the saturate palmitic acid and the polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 was encountered. Other fatty acids also abundant in marine invertebrates like arachidonic and gondoic acids were found. Although much variation exists a major trend of fatty acid distribution can however be safely recognized characterized by the predominance of palmitic acid and the polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3.

The four vegetable oils analysed revealed palmitic acid and the 18C, saturate and unsaturates as the predominant fatty acids. Linseed and sunflower oils have different major fatty acids: linolenic acid and linoleic acid, respectively. Rapeseed and olive oils have in common oleic acid as the dominant fatty acid, however, the amounts of polyunsaturates are very different. These oils were used in this study because they have three different major fatty acids or different fatty acid composition which allowed us to investigate their affect upon the fatty acid profile of mussels.

# Chapter 4

## The growth of mussels exposed to vegetable oils

### 4.1 Introduction

Mussels have been recognised as good bioindicators in environmental monitoring programmes throughout the world (Phillips, 1980; Widdows and Donkin, 1992). Their particular attributes of being a dominant species in coastal and estuarine areas, sessile and able to concentrate many chemicals in their tissues, are only a few characteristics amongst others which made mussels very attractive for pollution studies including the "Mussel Watch" programme. The mussel watch concept was initiated in North America proposed by (Goldberg, 1975) as a need to collect information about the state of pollution of the water mainly for public health purposes. Although this concept was initially concerned with metal radionuclide and organic contamination, its range of analysis has been extended to include many other chemical compounds and its use is now widespread in many countries of the world (Widdows and Donkin, 1992). In this study mussels have been chosen as an indicator of the polluting effect of vegetable oils spilled in the sea.

Economical development and world population growth require an increase in production and trade of essential goods like food. Vegetable oil production is expected to grow in the future to meet the food requirements of an expanding world population (Gunstone, 1994b; Gunstone, 1994c; Gunstone, 1994d). Increasing trade and shipment leads to the higher risk of spills and consequent environmental contamination and pollution. It becomes important for this reason to be able to predict the impact of veg-

etable oil spills in the marine environment. The effects of petroleum oil spills in marine molluscs are well documented (Lee, 1972; Widdows *et al.*, 1981; Bayne *et al.*, 1982; Widdows *et al.*, 1987; Widdows *et al.*, 1990) and contingency plans exist in coastal areas to become into practice in the case of a petroleum oil spill accident. However, the same is not true for vegetable oil spills. Their potential danger to the marine fauna and wildlife has been neglected (Gunstone, 1994a) because most of them are edible and not considered a threat. Little is known about the effects these compounds might have in the marine environment in particular in bivalves, except for some reports on marine birds (Smith and Herunter, 1989; McKelvey *et al.*, 1980).

Before we proceed it is important to clarify what is pollution and what is contamination since they are distinct concepts. The term "pollution" implies a biological effect, whereas "contamination" is a physical-chemical phenomenon resulting from the discharge to the environment of compounds in excess of normal concentrations (Widdows and Donkin, 1992). The assessment of pollution and environmental quality is therefore dependent on biological measurements, preferably associated with measurements of the chemical contaminants.

Previous studies (Salgado, 1992; Mudge *et al.*, 1993) have shown that mussels exposed to a sunflower oil spill, had increased amounts of linoleic and oleic acids in their fatty acid pool, which are the predominant fatty acids in sunflower oil. This implies that mussels were taking up the oil from the water into their tissues. The consequences of the high concentration of oil in the mussels was not known and led us to investigate its effects and the effects of other vegetable oils upon mussel growth rate. Since the rate of growth of an organism is a fundamental component of physiological fitness it represents an important index of environmental pollutant effect (Widdows, 1985) and a useful biological measurement in this study. Since growth is defined as increase in body size, weight or volume are appropriate parameters for its measurement. However, the shell is such a prominent feature of molluscan anatomy that growth is generally measured in terms of shell length (Seed, 1976). Although shell length measurements are very common in this work we have used shell area measurements which, supported by the technique described in Section 4.2.2, proved to be an easy and convenient method in assessing small increases of mussel growth. In addition, information of shell shape

is also obtained which can be useful to monitor changes as an adaptive response to changing environmental conditions (Seed and Richardson, 1990).

The subject of this chapter is the assessment of the polluting effect of vegetable oils by measuring, in the laboratory, *Mytilus edulis* shell area growth rate as the biological response to vegetable oils exposure and at the same time monitor the levels of fatty acids in mussel tissues, in order to evaluate the extent of the biochemical contamination of the oils.

## 4.2 Materials and methods

The materials employed in these experiments include equipment, both photographic and analytical, phytoplankton cultures, reagents for fatty acid analysis and the four vegetable oils used in growth measurements. For growth measurements it was necessary to set up a system of tanks to maintain, feed and contaminate mussels with oils; photographic equipment, a scanner and a computer were also used to record and process the mussel shell images. Analysis of fatty acid methyl esters (FAMES) were carried out much in the same way as described in Chapter 3 and the instrument used for analysis was a GC-MS.

One laboratory experiment was set up in order to compare mussel growth rate exposed, for 8 weeks, to vegetable oils with growth rate of a control group.

### 4.2.1 Experimental set up

#### Tanks

Five tanks of 11l capacity were set up with an open water system receiving filtered seawater at a flow rate of  $300 \text{ ml} \cdot \text{min}^{-1}$ , kept constant by a header, as shown in Figure 4.1. The water was aerated by introducing compressed air through air stones and the tanks were cleaned weekly to remove the debris and wastes from the mussels. The overflow from each tank was used to deliver seawater to another system of tanks where mussels were placed for tolerances experiments as described in Chapter 5. The water temperature was recorded during the acclimation and experimental periods.

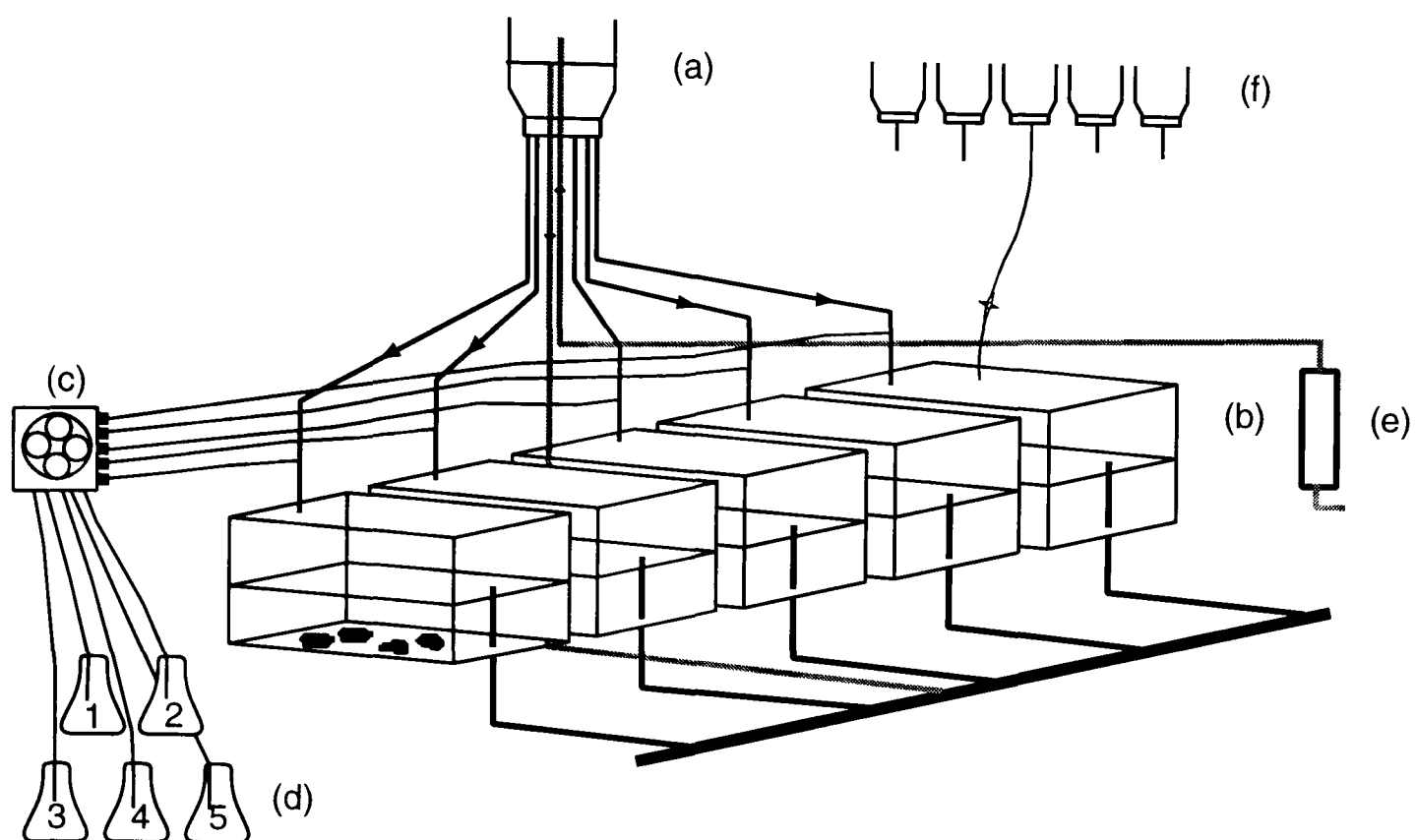


Figure 4.1: Diagram of the experimental set up. a) header, b) tanks, c) peristaltic pump, d) oil containers, e) water filter, f) food delivery.

## Oils

Several methods to introduce the oils in the water were tried in order to make them available for mussels. Since oils are water insoluble and are less dense than water, they floated at the water surface and were rapidly drained before coming in contact with mussels. In the sea wave action and hydrodynamic conditions favour a more prolonged contact with contaminants than could be provided in the laboratory, therefore efforts to improve availability of oil to mussels were envisaged.

Microencapsulation used by some authors (Langdon and Waldock, 1981) was one of the unsuccessful trials. An aqueous solution of gelatin was added to a solution of acacia and oil under a water bath and microcapsules were obtained. However the microcapsules float on the water just like the oils and they were not stable, after a few days in the fridge the microcapsules formed aggregates. In addition, it seemed difficult to pump the solid microparticles through the peristaltic pump without blocking the tubes.

The other method tried was to cover the algae with oil so that the oils would be ingested by the mussels together with the food. The process employed was mechanical stirring and it proved to be inefficient since oil did not adhere to the cells. The droplets

obtained were just the result of oil emulsification which after a few seconds disappeared forming an oil surface layer.

It was then decided to use a surgical needle connected to the tube of a peristaltic pump to deliver small droplets of oil at the bottom of the tanks, close to the mussels. Four different oils, rapeseed, linseed olive and sunflower oil were delivered in this fashion by a peristaltic pump with a flow rate set to  $0.3 \text{ ml} \cdot \text{min}^{-1}$ . On the 20th day of experiment the flow rates were measured again and they had slowed down to  $0.01 \text{ ml} \cdot \text{min}^{-1}$  in rapeseed tank,  $0.02 \text{ ml} \cdot \text{min}^{-1}$  in olive and sunflower tanks and  $0.05 \text{ ml} \cdot \text{min}^{-1}$  in linseed tank. The reason for that was probably the viscosity of the oils offering great resistance to be pumped. The fifth tank received distilled water with a flow rate of  $0.11 \text{ ml} \cdot \text{min}^{-1}$  instead of oil and it was the control group.

### Mussels

Mussels were collected from Bangor pier pillars at low tide and brought into the laboratory where they were left overnight in two tanks of seawater with aeration. The next day, they were cleaned of barnacles and other epifauna and sorted by size. Fifty mussels between 1.5 and 2 cm length were chosen and distributed amongst the 5 tanks. They were permanently fixed on perspex frames with cyanoacrylate glue, 10 per frame per tank. Another set of 25–31 mussels with mean size of  $2.3 \pm 0.6 \text{ cm}$  were placed loose in each tank to be collected throughout the experiment for fatty acid analysis.

### Food

A mixture of microalgae composed by the species *Pavlova lutheri* (Droop) Green, *Tetraselmis chuii* Butcher and (*Rhodomonas baltica*) *Rhinomonas reticulata* was supplied by drip-feeding on a daily basis. The cell density of each algal culture was determined every day with a ZM coulter counter and the different species mixed according to their concentration to have approximately the same number of cells in the tank each day ( $1.6\text{--}2.0 \times 10^9$  cells). This number was calculated according to the tank capacity and based on cell densities used by (Almada-Villela, 1984).

## Procedure

Mussels were kept in the tanks for one week with running seawater to acclimate to the experimental conditions, after which the oils started to flow into the tanks and food supplement was provided daily. The mussels fixed on the frames were taken out of water weekly and the frames carefully cleaned around the mussels before being photographed and they were taken back to the tanks within one hour. At the same time and with the same frequency 3 to 9 loose mussels were removed from each tank to analyse their fatty acid composition.

### 4.2.2 Mussel area

Mussel shell areas were measured by photographing the organisms from each tank weekly, for four weeks and once more after eight weeks. The first photograph was taken before the oils started to flow into the tanks (week 0) and during the four weeks gap, the microalgae supplement was removed and mussels were only exposed to the oils.

Photographing was preceded by careful cleaning of the frames, taking care to not damage or disturb the animals, because the oils stick to the frame surfaces and mussels produce pseudofaeces and byssal threads which are deposited around the shell. This procedure proved to be essential in order to make mussel's edge sharp preventing interferences on the measuring method. The photographs were taken using a Zeiss Tessovar camera held on a stand at a fixed distance from the mussels. A source of light was used underneath the mussel frames to improve the contrast and allow a sharp shell edge. The pictures obtained in this way were scanned using an Apple Macintosh OneScanner at 300 dpi and the images imported into an Apple Macintosh II si computer and processed by appropriate software. In SuperPaint, one of the computer applications used, small particles and fine byssus threads that still persisted on the scans, even after cleaning the frames and did not form part of the shell itself, were deleted not to interfere with the calculation of the shell area. This process was carried out at approximately the resolution at which the photograph was scanned through a fourfold magnification in Superpaint ( $72 \times 4$  dpi) (see Figure 4.2).

The calculation of the area of the mussels was done in FlexiTrace which enables one

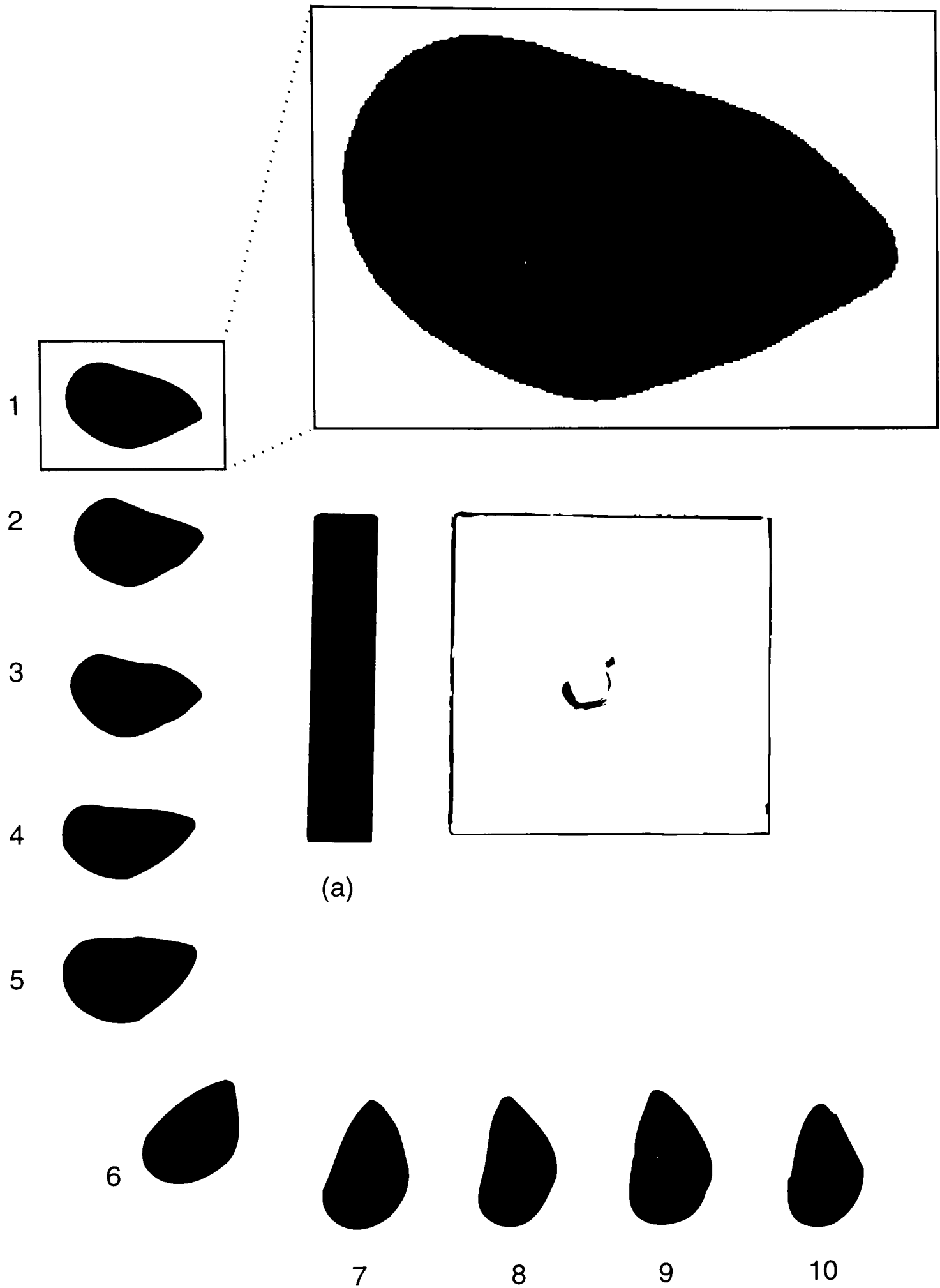


Figure 4.2: Scan of 10 mussels created from a picture using an Apple Macintosh One-Scanner and SuperPaint software to edit and magnify the image on an Apple Macintosh IIsi computer. (a)-reference object.



to count the number of pixels (computer image unity, 72 pixels=1 in) contained within the shell image. The number of pixels can then be converted to area in mm<sup>2</sup>, using a reference object of known dimensions in each photograph. Two pictures from the same plate were processed to define the error in these measurements. The expression to convert pixels in mm<sup>2</sup> is as follows:

$$\text{Area}(\text{mm}^2) = \text{Area}(\text{pixels}) \times F_c^2$$

where Area (pixels) is the mussel area in pixels and  $F_c$  is the conversion factor related to the magnification of the picture given by:

$$F_c = \frac{\text{known length of reference object (mm)}}{\text{length of reference object on the picture (pixels)}}$$

The reference object used was a rectangular piece of wood of known length photographed next to the mussels. The areas in pixels of the mussels, reference object and its real length is given in Table A.6 in Appendix.

### 4.2.3 Growth rate measurement

The rate of growth of an organism is not constant through its life time. It decreases with size or age and depends on many physical and biotic parameters like temperature and salinity, food availability and predation (see Section 2.3). In order to compare the growth of mussels submitted to different vegetable oil treatments, the measurements must be independent of mussel size and referred to the time interval in which the growth was measured. Therefore, they have to be normalized relatively to size and time. Of most interest is the relative growth and differences in growth rate for a short period of time under specific conditions. Growth is thus expressed in terms of specific growth rate (SGR) which is physiologically more appropriate. Others authors (Almada-Villela, 1984; Gruffydd *et al.*, 1984) used this approach to study the increase in length of mussels under different physical and biological conditions. The same definition was used but considering mussel areas rather than lengths. Specific growth rate is defined as the rate of change in area per unit of area and time:

$$SGR = \frac{dArea}{Area \cdot dt}$$

where  $dArea$  is the area increment in  $\text{mm}^2$  after each week ( $t=1,\dots,4$ ),  $Area$  is the mussel area in  $\text{mm}^2$  and  $dt$  is the time interval in weeks. Solving this differential equation, SGR becomes:

$$SGR \int dt = \int \frac{dArea}{Area}$$

Integrating according to the integration limits  $Area_0$  and  $Area_1$  for mussel areas at the beginning and end of the measurement and corresponding times  $t_0$  and  $t_1$ , respectively we obtain:

$$SGR = \frac{\ln(Area_1) - \ln(Area_0)}{t_1 - t_0}$$

This formula was employed throughout the experiment to calculate mussel specific growth rates.

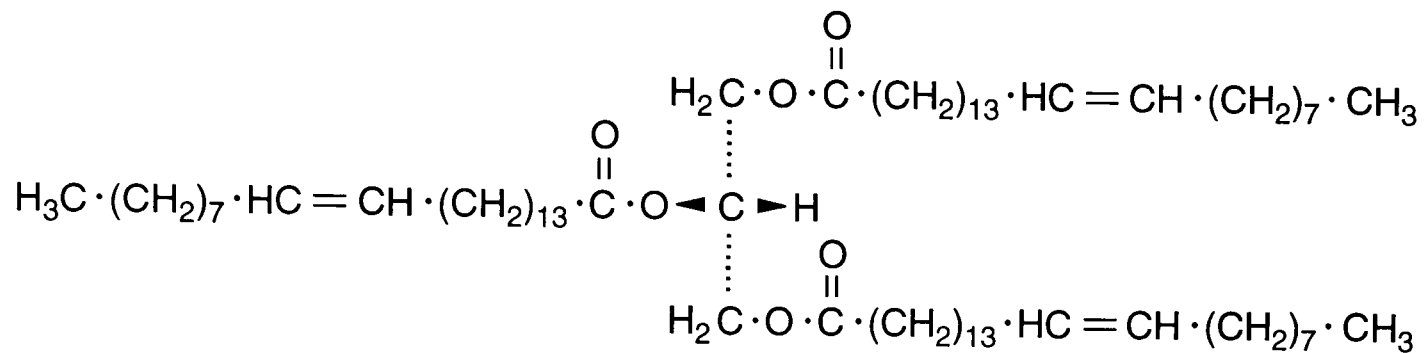
#### 4.2.4 Fatty acid analysis

The fatty acid constituents of mussel tissues were determined in a similar way as described in Section 3.2.3 but in this case the lipids were split into the two classes of lipids: neutral and phospholipids. The purpose of separating the lipids was to characterize the normal distribution of fatty acids between the two classes and to monitor differences on fatty acid distribution in both classes of lipids due to the exposure to vegetable oils.

To accomplish lipid separation a 10 cm long chromatographic glass column was filled with silica gel 40 M and blocked with a glass fiber bud at the end. The lipid sample was introduced and eluted with 30 ml chloroform to remove neutral lipids and 30 ml methanol to remove the polar lipids. After separation, each of the lipid fractions followed the same procedure as the non-separated lipids. They were evaporated until dryness in the rotary evaporator, they were weighed, redissolved in chloroform, methylated with trimethyl sulfonium hydroxide (TMSH) (see Chapter 3) and injected into the GC-MS.

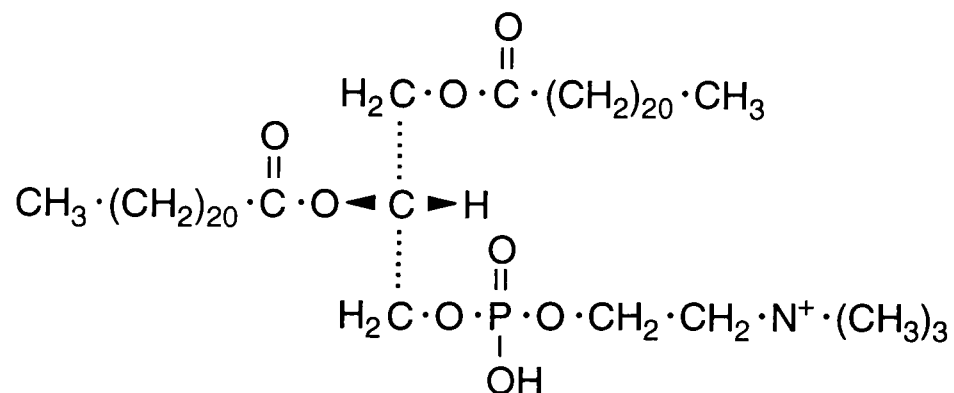
In the calculation of fatty acid methyl esters from neutral and polar lipids, a new calibration of the GC-MS equipped with a BPX-70 column was necessary to include two new standards: a triglyceride representing neutral lipids (*cis*,15-trinervonin, 24:1 $\omega$ 9) and a phospholipid representing the polar lipids (dibehenoyl phosphatidylcholine, 22:0) (Figure 4.3), to enable the extraction efficiency to be calculated. The FAMES in the

standard solutions were: 16:0, 17:0, 18:0, 18:1 $\omega$ 9, 18:2 $\omega$ 6, 18:3 $\omega$ 3, 20:0, 20:1 $\omega$ 9, 20:2 $\omega$ 6, 20:3 $\omega$ 6, 20:4 $\omega$ 6, 22:0, 22:6 $\omega$ 3 and 24:1 $\omega$ 9 prepared in 5 different concentrations: 12.5, 25, 50, 75 and 100  $\mu\text{g} \cdot \text{ml}^{-1}$  of each component and a constant concentration of tricosanoic acid methyl ester (23:0) ( $49.2 \mu\text{g} \cdot \text{ml}^{-1}$ ) was added as the internal standard in each of the 5 solutions.



*cis*, 15-Trinervonin

1,2,3-Tri[*cis*-15-tetracosenoyl]-glycerol



Phosphatidylcholine, dibehenoyl

1,2-Didocosanoyl-*sn*-glycero-3-phosphocholine

Figure 4.3: Chemical structure of the triglyceride and phospholipid standards.

The concentrations of FAMEs in the samples were calculated from

$$\text{Conc} (\mu\text{g} \cdot \text{ml}^{-1}) = \frac{\text{resp}(\mu\text{g} \cdot \text{ml}^{-1}) - \text{intercept}(\mu\text{g} \cdot \text{ml}^{-1})}{\text{slope}} \quad (4.1)$$

where the coefficients “slope” and “intercept” were obtained by linear regression, between areas of FAME standards and their concentrations; “resp” in equation 4.1 gives

the area of each FAME relative to the internal standard (SI) 23:0 and is given by:

$$\text{resp} = \frac{\text{area}}{\text{area IS}} \times \text{conc IS } (\mu\text{g/ml}) \quad (4.2)$$

Conversion to  $\mu\text{g} \cdot (\text{mg lipids})^{-1}$  was achieved by dividing the concentration of FAME by the amount of lipids present in 1 ml of injected sample. For easier comparison of the amount of fatty acids in different samples, FAME concentrations in  $\mu\text{g} \cdot (\text{mg lipids})^{-1}$  were transformed into percentages of the total.

### 4.3 Results

This section reports the experimental conditions of water temperature, mussel diet and oil flow rates employed in the experiments and shows how mussel shell areas varied, under the reported conditions, with time and with each oil treatment. Mussel mortalities for each treatment are also presented. Specific growth rates of mussels exposed to the different oils are compared to the ones from the control group. They were calculated cumulatively after one, two, three, four and eight weeks of treatment and the growth rates after the first 4 weeks of treatment supplied with food were compared with the last 4 weeks of treatment without food provision. The fatty acid composition of mussels exposed and non-exposed to the oils is presented showing their biochemical evolution through the time of the experiment and the differences between the classes of lipids.

#### 4.3.1 Temperature and diet

Water temperature was recorded every two days for the first week of mussel acclimation and less regularly afterwards. The results are plotted in Figure 4.4. The mean temperature was  $9.6 \pm 1.0$  °C.

Mussel diet was composed by the 3 microalgae *Pavlova lutheri* (Droop), *Tetraselmis chuii* (Butcher) and *Rhinomonas reticulata* analysed in Chapter 3. The density employed throughout the experiment is shown in Figure 4.5. The total amount of cells delivered to the tanks every day was approximately constant for the 4 weeks and close to  $2 \times 10^9$  cells with *Pavlova* being the most abundant of the three.

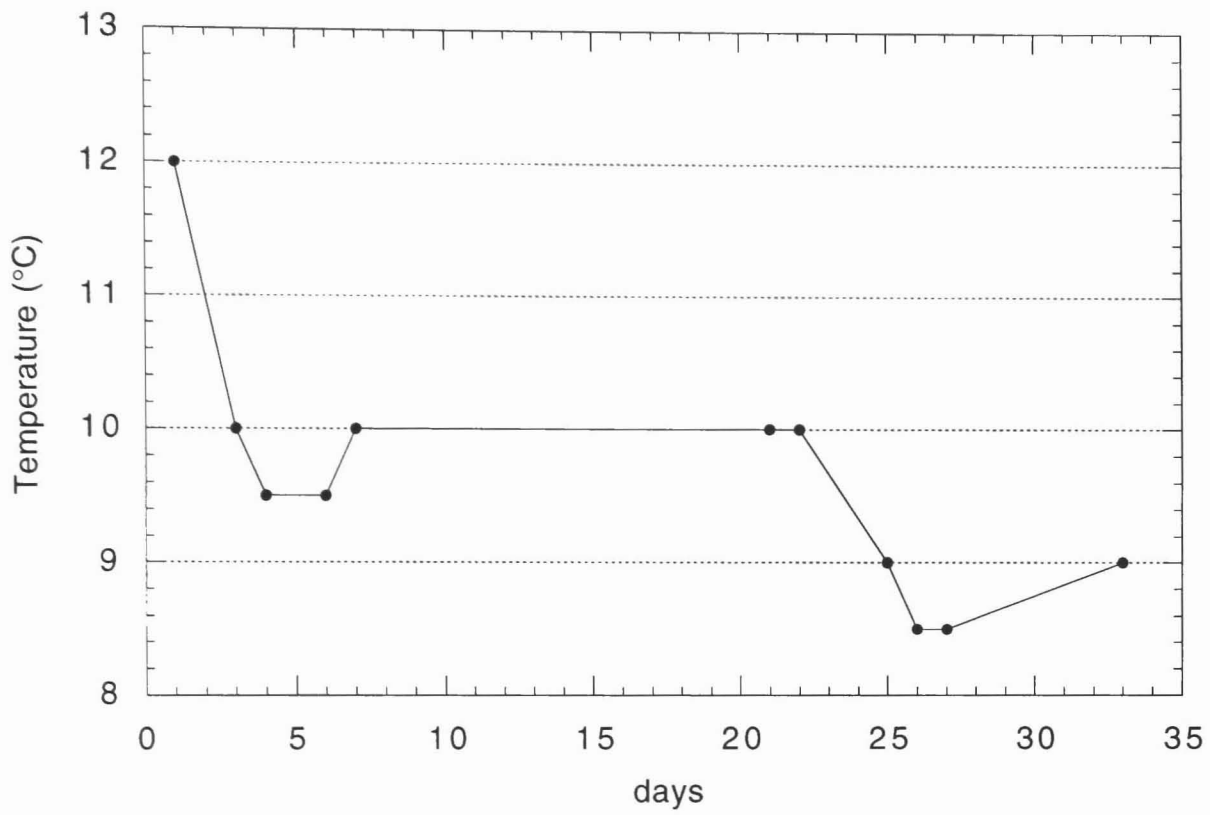


Figure 4.4: Water temperature in the tanks during mussel acclimation and experiment.

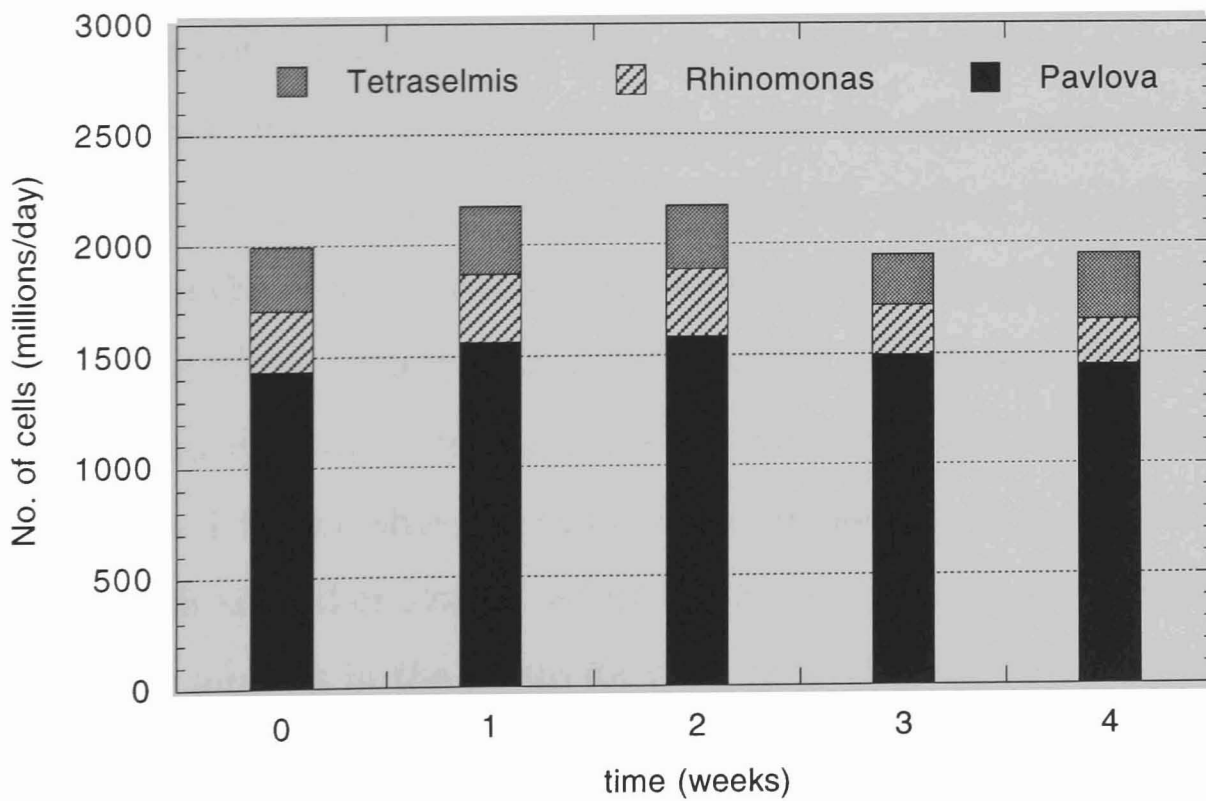


Figure 4.5: Number of cells in each of the algal cultures used to feed mussels.

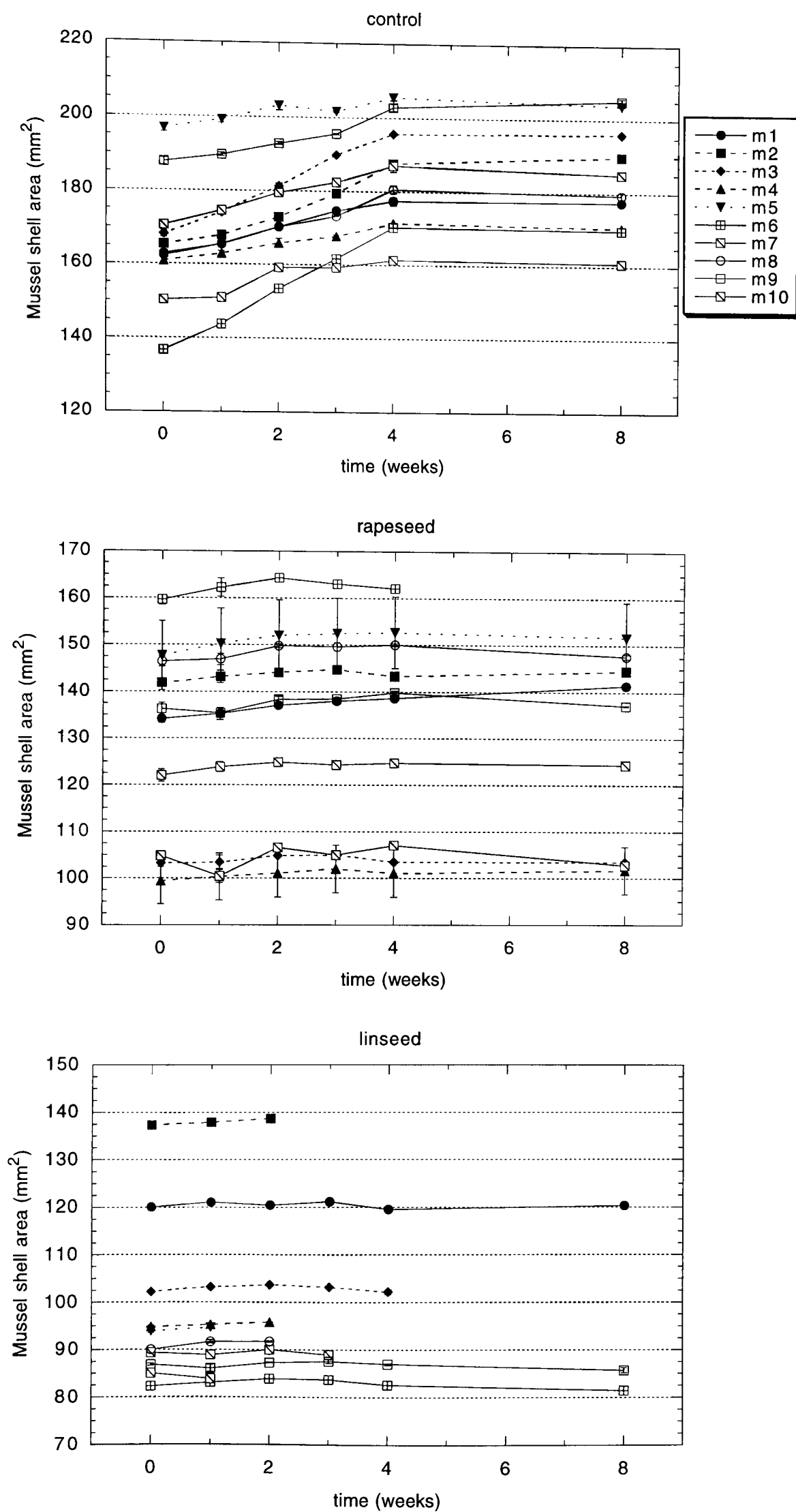


Figure 4.6: Growth of mussel shells under different vegetable oil treatments for eight weeks. Mussels were fed on microalgae for the first 4 weeks and food deprived for the remaining 4 weeks.

### 4.3.2 Mussel growth

Figure 4.2 shows a life size picture of mussel shells as it is obtained from the scanner. The enlarged mussel gives an idea of the magnification applied to all mussels to calculate their areas without any loss of resolution. The raw data for the areas of the shells, in pixels, as well as the dimensions of the reference object in the pictures are provided in Appendix A, from which the areas in  $\text{mm}^2$  presented in the following sections were calculated.

The areas obtained for the mussel shells under the different oil treatments are shown in Figures 4.6 and 4.7. They represent the change in area of 10 shells during 4 consecutive weeks fed on a microalgae diet and exposed to a vegetable oil, or distilled water for the control group and a further 4 more weeks just exposed to the oils or distilled water with no food supplement.

Although these graphs can not serve the purpose of comparing growth rates directly, because the areas are not normalised, it seems clear that the control group exhibited a different pattern of growth when compared to the other groups. During the first 4 weeks of experiment most of the control mussels increased in area notably and on the 4 following weeks, when food supplement was not provided, some of the curves flattened. The increase in area of mussels treated with oils, however exhibited a smaller increase than the control ones. Furthermore, the growth curves for all the mussels submitted to the oil treatment are much flatter when compared to the control. Mussels treated with linseed oil presented the poorest area increase and registered the highest mortality rate.

The graphs also show variation in growth within the 10 mussels of each group. Looking at the control group, whereas mussels number 6, 3 and 2 had a high increase in area on the first 4 weeks, showing large slopes, mussels 1, 4, 7, 8 and 9 revealed a slower increase with smoother slopes. A growth curve was also obtained by averaging the areas of the 10 mussels in the group for each week (Figure 4.8). It shows a clearer picture of the performance of the different groups. The control group grew notably, whereas the mussels treated with oils had smaller increments of area exhibiting smaller slopes between weeks.

The precision associated with the measurement was estimated from the two pho-

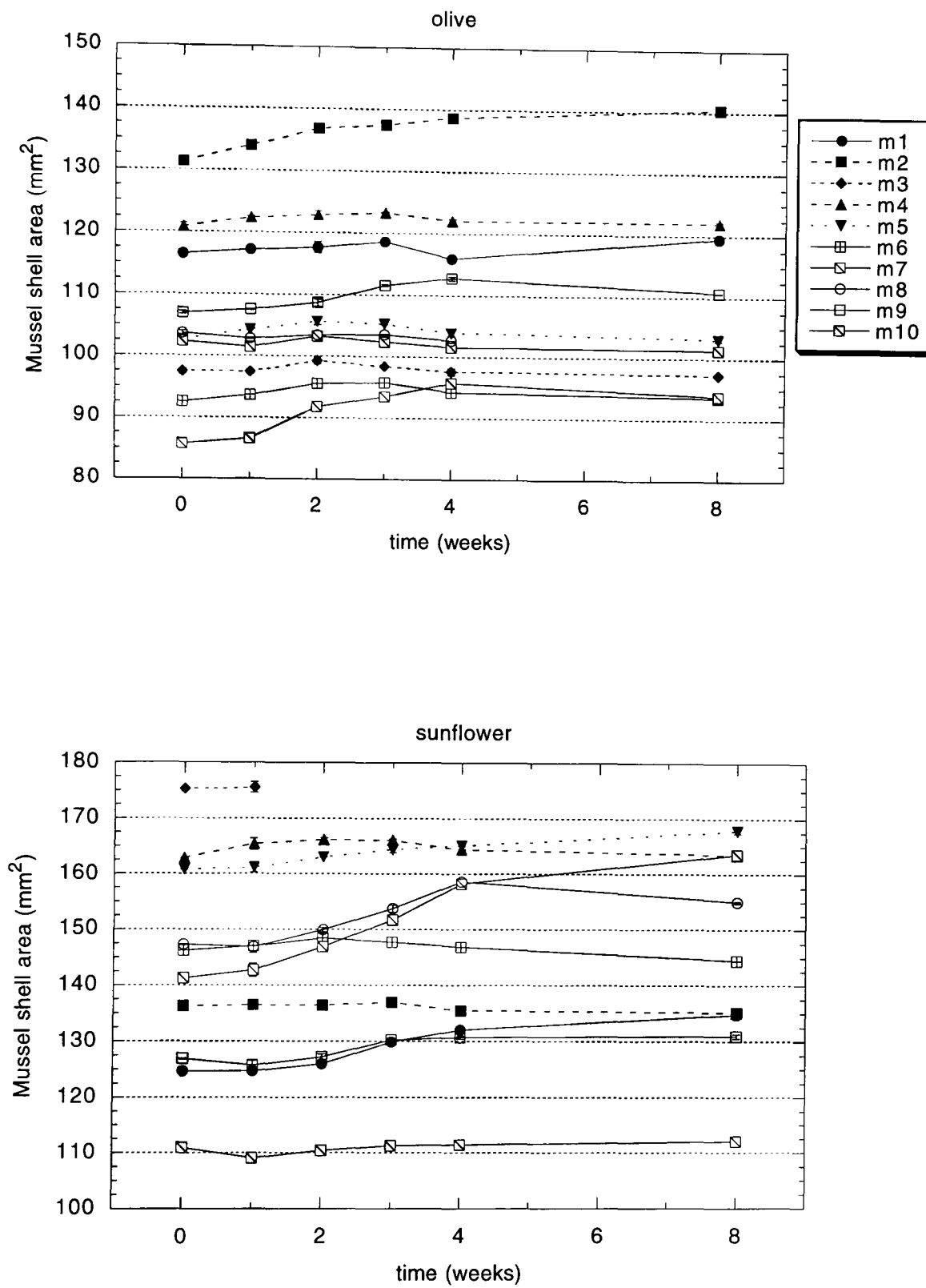


Figure 4.7: Growth of mussel shells under different vegetable oil treatments for eight weeks. Mussels were fed on microalgae for the first 4 weeks and food deprived for the remaining 4 weeks.



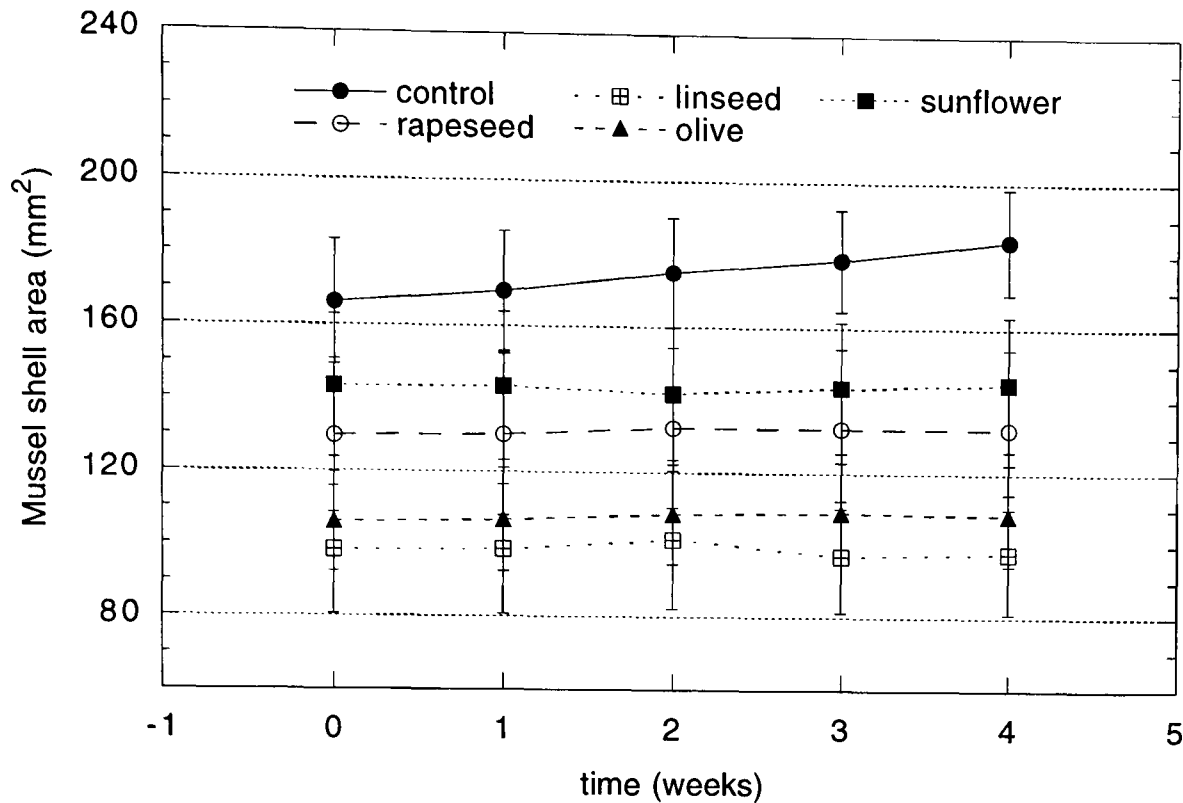


Figure 4.8: Variation of the average area of 10 mussels from each treatment for 8 weeks.

tographs taken from each mussel frame. Since the shells in both photographs were the same, the deviations obtained for mussel area were due to manipulation of the images from the photograph to the computer image. These deviations given by the percentage error ( $cv = sd/mean \times 100$ ) for each pair of photographs determine the error inherent to the measuring process. Table 4.1 gives the percentage errors of all paired measurements grouped according to treatment and week and also shows the largest (sd max) and smallest (sd min) standard deviation in  $mm^2$  obtained from the same paired measurements.

Week	N	cv	sd min	sd max	Treatment	N	cv	sd min	sd max
0	50	0.3906	0.0049	1.3363	Total	274	0.3300	0.0047	1.9496
1	50	0.4951	0.0276	1.9496	Control	60	0.3230	0.0105	1.6640
2	47	0.3334	0.0148	1.2550	Rapeseed	59	0.5176	0.0729	1.9496
3	44	0.2362	0.0049	0.8665	Linseed	40	0.2662	0.0147	0.7282
4	43	0.2942	0.0120	1.6640	Olive	59	0.2714	0.0047	0.9323
8	40	0.1852	0.0097	0.8995	Sunflower	56	0.2470	0.0049	1.1585

Table 4.1: Percentual errors (cv) on shell area measurements of 2 photographs from each mussel and maximum and minimum standard deviation in  $mm^2$ .

The mean percentual error for the 247 measurements was 0.3%, which is similar to that found by Davenport and Glasspool, (1987) using a similar photographic process.

### 4.3.3 Specific growth rate

In order to obtain comparable results, since mussels have different initial areas, individual specific growth rates were calculated for each week, as explained in Section 4.2.3. Figure 4.9 illustrates the average of the individual growth rates of the 10 mussels from each treatment for each of the 8 weeks of experiment cumulatively, between week zero and the next weeks (0-1, 0-2, 0-3, 0-4 and 0-8 weeks). This way of showing the data evens out the weekly variations in shell growth rates of *M. edulis* and allows the general growth trends for that period of time to be revealed.

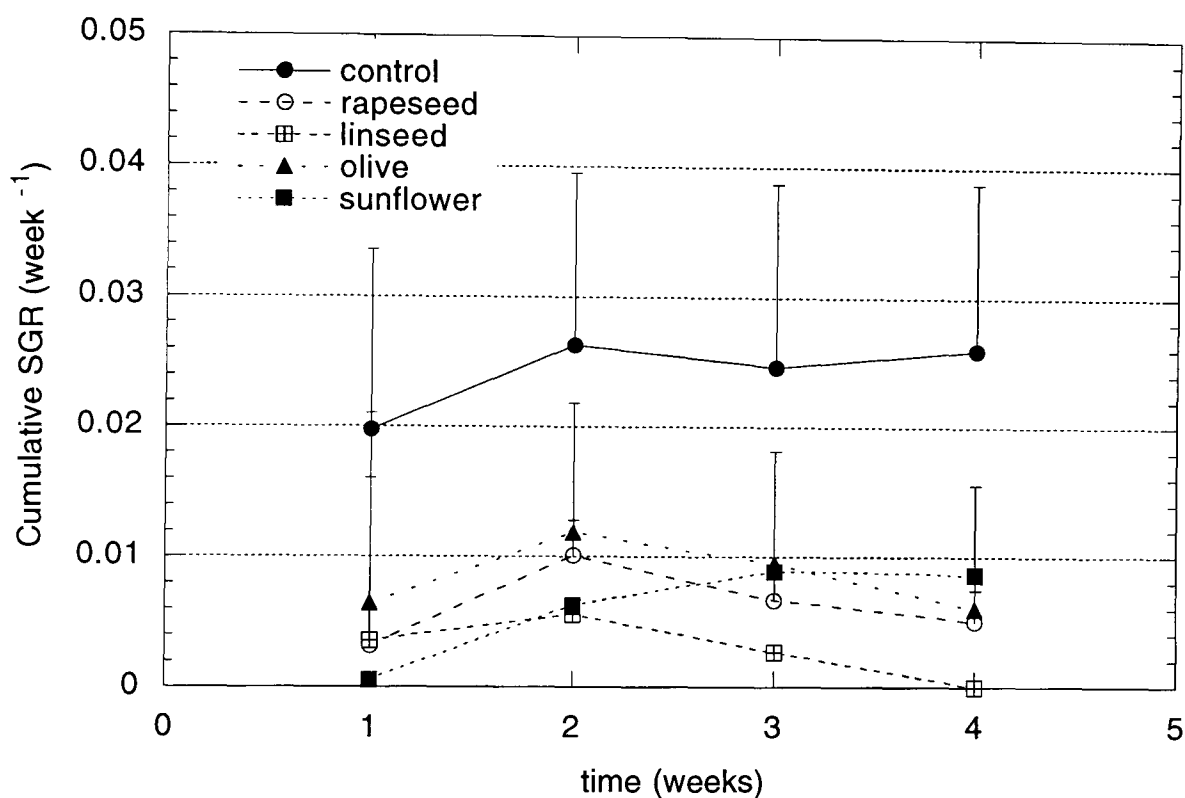


Figure 4.9: Specific growth rates for the control and oil treatments for four weeks.

The higher growth rate of the control group relative to the other groups is clearly seen throughout the experiment and that values of SGR for the mussels subject to the oils are similar. This implies that treatment with oils has impaired mussel growth. The growth rates of mussels in all treatments increased during the second week and after that time, while the control mussels grew at an approximately constant rate of  $\text{SGR} = 0.025 \cdot \text{week}^{-1}$ , mussels treated with oils showed a sharp decrease in growth rate. The only exception to this behaviour was sunflower oil, which exhibited an increase of growth until the third week suggesting some growth recovery maybe due to the decrease of oil flow, or some degree of adaptation. By the fourth week of treatment all the oils

had caused a decline of growth rates.

The overall result of the specific growth rates of mussel shells submitted to different vegetable oil treatments after 4 consecutive weeks is given by the cumulative growth rate calculated between the time interval 0-4 weeks and shown in Figure 4.10. A significant larger growth rate for the control mussels can be seen compared to any of the other oil treatments. Linseed oil treatment caused the lowest specific growth rate on mussels; sunflower, olive and rapeseed oils, in this order, caused decreasing specific growth rates. Even though, growth rates among the oil treatments are not statistically different (see Section 4.3.5), they all have a similar inhibiting effect on mussel growth. The same figure also shows the difference in growth rates of mussels fed on algae and exposed to oils for 4 weeks and further exposed to the oils for another 4 weeks but without any food supplement. It is clear that growth rates decrease drastically in the absence of food supplement. In fact, the growth rate of control mussels, under these starving conditions, is similar to the growth rate of any of the other mussels treated with oils.

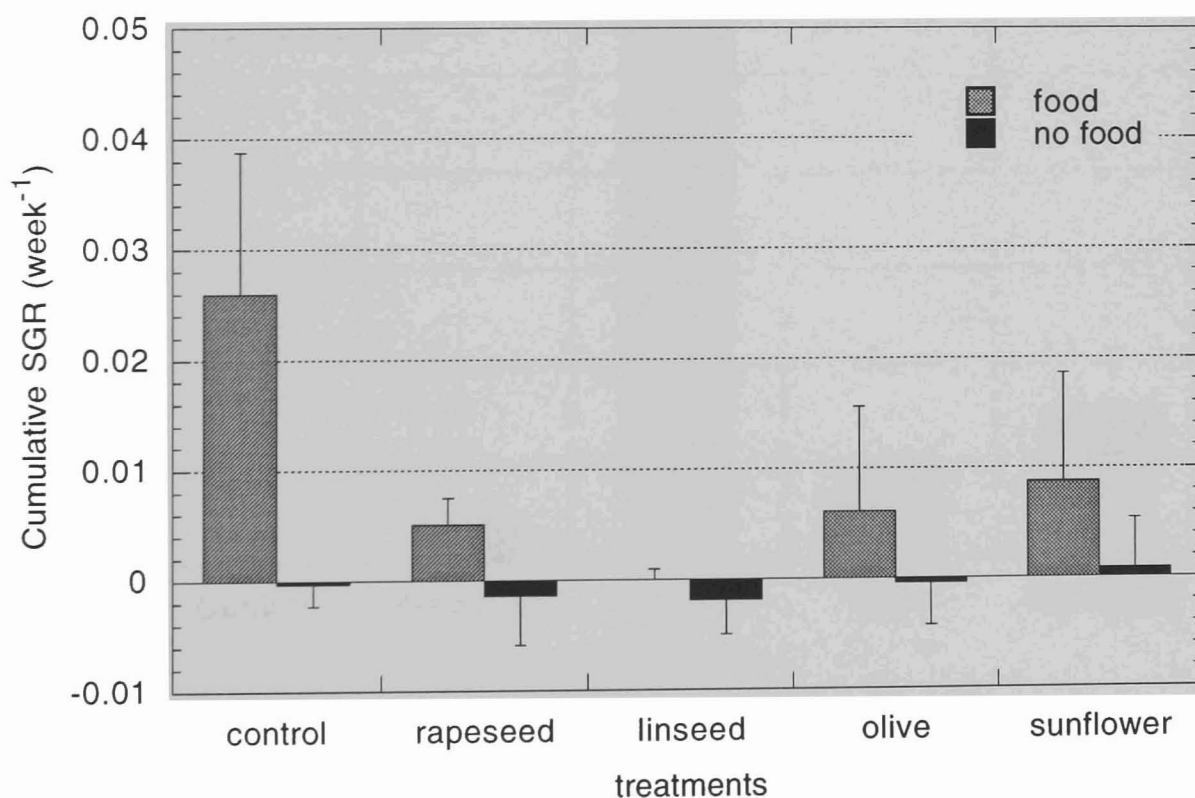


Figure 4.10: Cumulative specific growth rates of mussels exposed to vegetable oils for four weeks with food and no food for another four weeks.

### 4.3.4 Mortality

In terms of mussel mortality, linseed oil was the most detrimental as shown in Figure 4.11. Mussels treated with this oil started to die after the first week of exposure. Two mussels died after the first week, 3 more on the following week, one mussel died after the 3rd week and the last one died after the fourth week. A total of 7 deaths occurred in this group of mussels plus another 5 mussels among the loose ones in the tank. Mussels submitted to the other oils had a higher percentage of survival but still some mortality was registered. From the mussels treated with rapeseed oil only one died after the fourth week of exposure. Two mussels died in olive oil tank, one after the first week and another one after the fourth. Sunflower oil treatment also had one dead mussel after the first week and two more on the third and fourth weeks. In contrast, the control mussels survived the experiment with no mortality indicating that there were good conditions for survival and sustained growth.

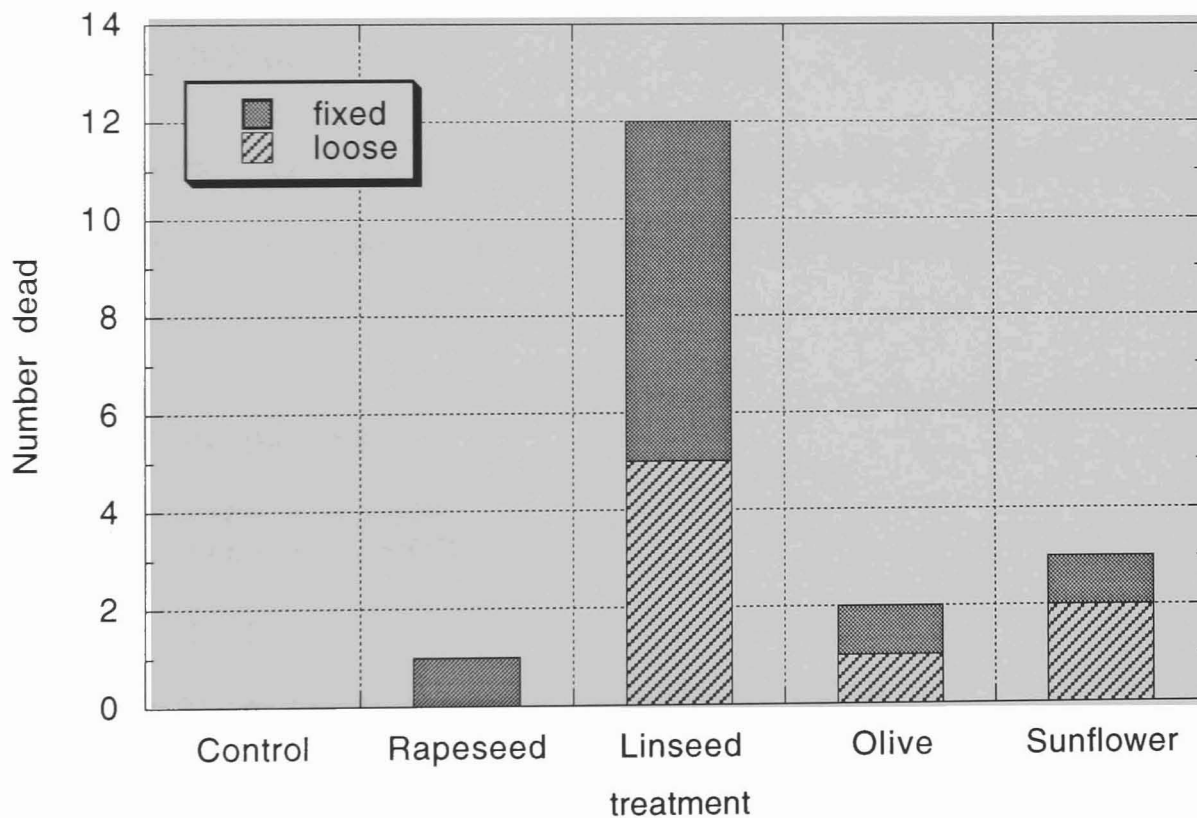


Figure 4.11: Mortality among mussels fixed on frames and loose in the tanks, submitted to different treatments during 8 weeks.

### 4.3.5 Statistical analysis

Differences of specific shell growth rates between treatments were tested with analysis of variance (Anova) using Minitab. The null hypothesis ( $H_0 : \mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$ )

was rejected, that is, the differences between the cumulative growth rate means for each treatment after 4 weeks were significantly different  $P < 0.001$ . A multiple comparison test, Scheffé, was employed after one way analysis of variance in order to know where the differences lie. The test is designed to do all pairwise comparisons of the means. In this way not only the control mean is compared with all the other means but also the means among the oil treatments will be tested. The Minitab output results are shown below.

ANALYSIS OF VARIANCE ON growth					
SOURCE	DF	SS	MS	F	p
treat	4	0.0033608	0.0008402	10.18	0.000
ERROR	38	0.0031373	0.0000826		
TOTAL	42	0.0064981			

				INDIVIDUAL 95 PCT CI'S FOR MEAN	
				BASED ON POOLED STDEV	
LEVEL	N	MEAN	STDEV	-----+-----+-----+-----	
1	10	0.025974	0.012827		(-----*-----)
2	10	0.005059	0.002423	(-----*-----)	
3	4	0.000009	0.000903	(-----*-----)	
4	10	0.006024	0.009533	(-----*-----)	
5	9	0.008674	0.009895	(-----*-----)	
				-----+-----+-----+-----	
POOLED STDEV = 0.009086				0.000	0.012 0.024

From the 95% confidence intervals diagram shown, level 1, the control group is different from all the other levels, the different oils (2-rapeseed; 3-linseed; 4-olive and 5-sunflower).

Scheffe pairwise comparisons

Family error rate = 0.0500  
 Individual error rate = 0.00678

Critical value = 4.05

Intervals for (column level mean) - (row level mean)

	1	2	3	4
2	0.009278			
	0.032551			
3	0.010571	-0.010344		
	0.041359	0.020445		
4	0.008313	-0.012602	-0.021410	
	0.031586	0.010672	0.009379	
5	0.005344	-0.015571	-0.024302	-0.014606
	0.029255	0.008341	0.006971	0.009306

The null hypothesis tested by Sheffé's comparisons was  $H_0 : \mu_B - \mu_A = 0$  where  $B$  and  $A$  are any pairs of means. The result of the test was ten confidence intervals one for each pair of means. The interval which does not contain zero is significant. The intervals comparing the control with the oil treatments are the only ones which do not contain zero, therefore only the control is significantly different from the oil treatments.

The final conclusions can be withdrawn: all the mussels exposed for four weeks to the oil treatments showed a significant lower specific growth rate than the control mussels and among the four oils tested, there were no significant differences. In other words none of the oils produced different growth rates in the mussels over the time of experiment. Therefore they were all similarly inhibiting of mussel growth.

### 4.3.6 Fatty acids

Mussel fatty acid composition was characterized and changes caused by the presence of oils were monitored for four consecutive weeks and a further four weeks during which mussels were food deprived. Fatty acids were analysed from both neutral and polar lipid classes, the results being shown in Figures 4.12 to 4.15.

#### Composition of neutral lipids

Between 3 and 9 mussels were collected from each of the treatment tanks to be analysed. The mean size and standard deviation was  $2.3 \pm 0.6$  cm.

Week zero represents the time of analysis before mussels had been in contact with oils or algal food, hence the fatty acid composition in any of the groups should reflect the fatty acid composition of wild mussels and its natural variation.

The six fatty acids shown in the graphs 4.12 to 4.15 were chosen according to the major fatty acids present in the oils and in the mussels: oleic acid (18:1 $\omega$ 9), the major fatty acid in rapeseed and olive oils, linoleic acid (18:2 $\omega$ 6) the predominant in sunflower oil, linolenic acid (18:3 $\omega$ 3) the highest in linseed oil and the saturate palmitic acid (16:0) and the highly polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 typical of mussels.

In week zero, just before oils and food were supplied, mussel fatty acid composition for any of the groups is similar, with palmitic acid being the predominant fatty acid varying from 19 to 22% of the total fatty acids, followed by the polyunsaturate 20:5 $\omega$ 3

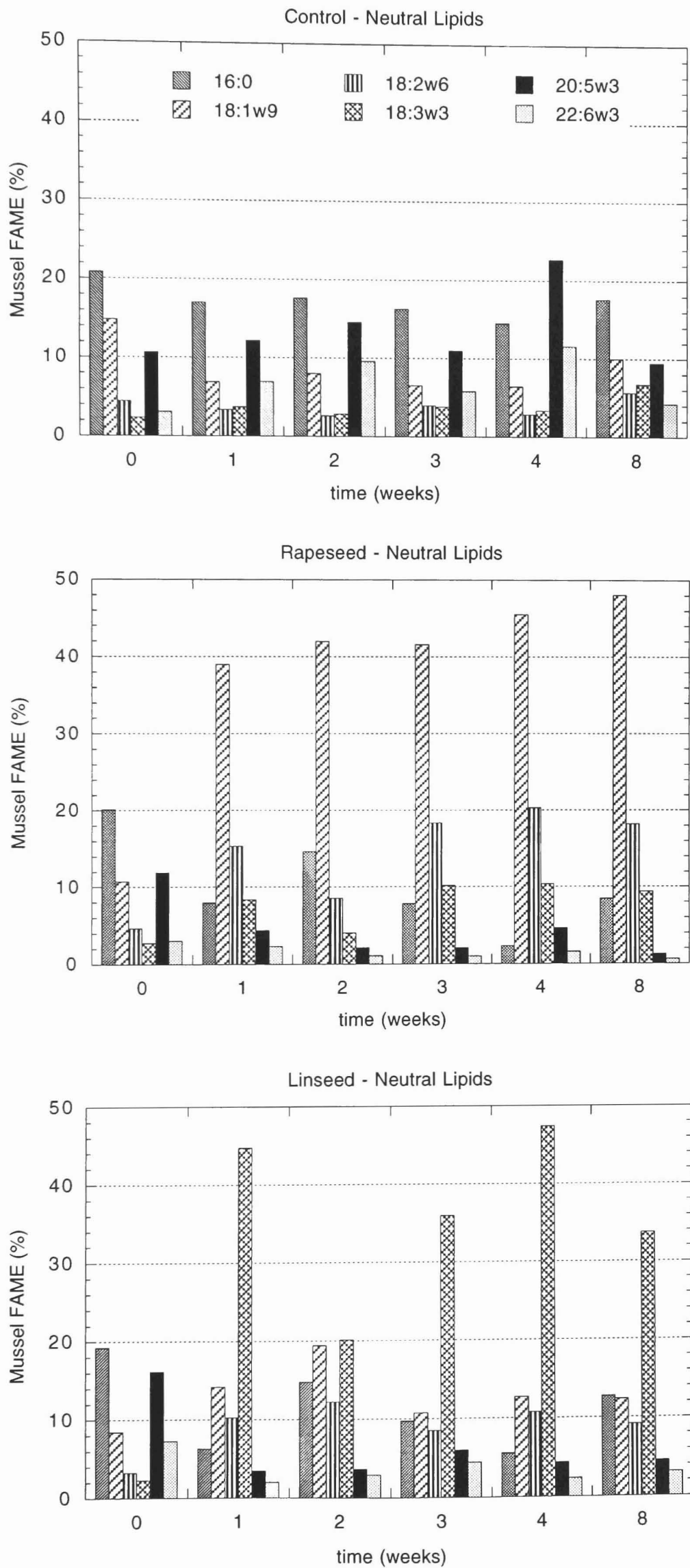


Figure 4.12: Neutral fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks. Food was provided from week one to four and oil was delivered from week one to eight.

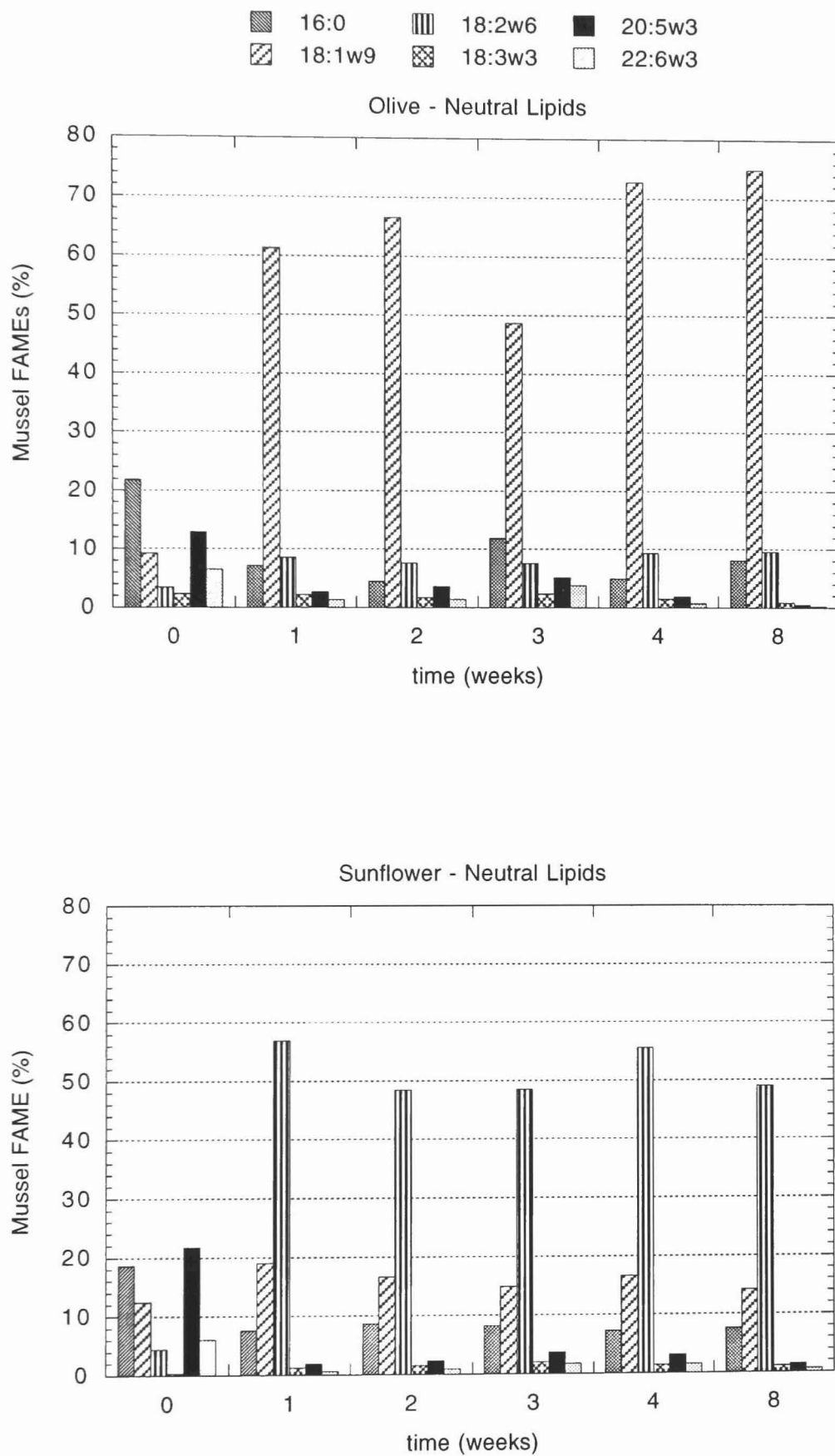


Figure 4.13: Neutral fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks. Food was provided from week one to four and oil was delivered from week one to eight.



which exhibited a higher percentage of variation between 11 and 22%. Third most abundant was oleic acid with percentage between 9 and 15%; the polyenoic 22:6 $\omega$ 3 varies from 3 to 7% occupying the fourth position and the 18C polyenoic, linoleic and linolenic acids complete the 6 fatty acid pool in 5th and last position, respectively. Linoleic acid has the second smallest variation among the 5 treatments, after palmitic acid.

After 1 week of treatment with oils and food supplement, the fatty acid composition changes greatly. In the control group, palmitic acid decreases slightly, oleic acid decreases significantly and both linolenic acid and 20:5 $\omega$ 3 increase slightly, whereas 22:6 $\omega$ 3 increase considerably. During the following 3 weeks the fatty acid profile is similar to the first week with some small fluctuations, palmitic acid being always lower than the value on week zero, oleic acid rising slightly on the second week but decreasing again on the two following weeks after which it stabilizes. The 18C polyunsaturates behave in an opposite way compared to oleic acid they decrease when the former increases in the second week and increase when oleic drops down in the third week. The polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 seem to be the ones benefiting from the decrease of the other fatty acids. They rise slowly from week zero onwards and, except for the third week when there was an unexpected decrease, they culminate on the fourth week with high percentages.

In the last week, week 8, the conditions were different, as food was not provided for 4 weeks and the only source of food for the control mussels was detritus accumulated in the tanks or small particles passing through the glass fiber filter. Fatty acid composition of mussels changed. The 18C increased to percentages close to or slightly higher than initially in week zero at the cost of the highly polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 which decreased to percentages similar to the ones in week zero.

The mussels treated with rapeseed oil revealed a large increase of oleic acid and linoleic acids, the major constituents of the oil. Oleic acid continued to increase from the first to the last week from 39% until it reached 48%, almost half of the total fatty acids. Linoleic acid also increased from 15% in the first week to 18% in the following weeks stabilizing around that percentage. Linolenic acid followed a similar trend as linoleic acid, increasing from 3% to 8% on the first week and then remained steady from weeks

3 to 8 at 10%. To compensate for the increased proportion that these fatty acids make of the total, some other proportions must decrease namely the polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 and palmitic acid. However it is recognized that the absolute concentrations may not change. Palmitic acid proportion decreased to less than half of its initial value and the C20 and C22 polyunsaturates also decreased considerably.

Linseed oil treatment led to a 40% increase of linolenic acid, the predominant fatty acid in the oil, after the first week of exposure. Some fluctuation but still with high percentages varying between 30 and almost 50% of the total fatty acid composition were seen. The fatty acids decreasing in concentration were palmitic and the polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 from the first to the last weeks. However, the last two fatty acids recovered slightly in the third week showing a 2% increase at the cost of all the others. Oleic and linoleic acids also experienced a 2 to 10% increase through the weeks. In the second week palmitic, oleic and linoleic acids increased whereas linolenic acid decreased considerably to half of its value on the previous week.

In the case of olive oil, mussel fatty acid composition was very polarized towards oleic acid. The proportion of oleic acid, again the major component of the oil increased from 9% in week zero with no oil exposure to an enormous 60% after 1 week of treatment and rose until 75% of the total fatty acids. In the third week there was a marked decrease of oleic acid accompanied by a slight increase of the saturate palmitic acid and the polyenoics 20:5 $\omega$ 3 and 22:6 $\omega$ 3. Linoleic acid also increased from 4% of the total fatty acids on week zero to 9% after 1 week and remained fairly constant around 8 and 10% on the following weeks. Palmitic acid and the highly polyunsaturates decreased substantially with the oil treatment.

Sunflower oil exposed mussels exhibited a fatty acid composition different from the control mussels but constant throughout the experiment. The major fatty acid in the oil, linoleic acid rose to high percentages after the first week and it remained like that, high and steady, for the 8 weeks. Its proportion increased from 5% on week zero to an average 52% on the following weeks. Oleic acid behaved very much on the same way, but with a smaller increase of 12% to an average 16%. The decreasing fatty acids were palmitic acid with an average 13% decrease and the polyenoics 20:5 $\omega$ 3 and 22:6 $\omega$ 3 with an average decrease of 12% and 4%, respectively.

### Composition of polar lipids

The same fatty acids studied in the previous section (neutral lipids) were analysed in polar lipids to investigate whether their fatty acid composition was affected differently by the vegetable oils. The results from the analysis of mussel polar lipids under vegetable oil treatments and for the control can be found on Figures 4.14 and 4.15.

Polar lipids showed a much more homogenous composition than neutral lipids. The control group exhibited a fairly constant proportion of palmitic acid throughout the experiment, which was the major fatty acid in this lipid fraction. Its value on week zero was not taken into account because it was marked as a false result due to sample evaporation during the analytical process. Since fatty acids on week zero for all treatments should be similar, because the analysis preceded the treatment, fatty acids from the other treatments can be used as guide values. Polar lipids before treatment had started, were characterized by an average 24% of palmitic acid, 19% of 20:5 $\omega$ 3, 13% of 22:6 $\omega$ 3 and minor percentages of the 18C. Palmitic acid remained constant and close to 20% for the 4 following weeks and dropped slightly after the 4 weeks of starvation. The polyenoic 20:5 $\omega$ 3 increased slowly with time from 11% to 17% and 22:6 $\omega$ 3 also showed a 3% increase. The 18C showed no significant variation.

The influence of rapeseed oil was noticed in polar lipids by the increase of oleic acid after the first week of treatment. It increased by 12%, although it never achieved proportions higher than 22% nor ever became the predominant fatty acid during the time of treatment supplied with food. Palmitic acid was still the dominant fatty acid for the same 4 weeks of treatment although showing large variations. The polyunsaturates C20 and C22 decreased slightly relative to week zero but remained close to an average of 12% of the total fatty acids. The fatty acid 22:6 $\omega$ 3 decreased after the second week and then showed a similar constant behaviour as 20:5 $\omega$ 3 with a lower average proportion of 9%. Linoleic acid and linolenic acids, the second and third fatty acids in rapeseed oil, increased in mussel polar lipids after the first week from 2% to 8% and from 1% to 4%, respectively.

Mussels treated with linseed oil showed an evident increase of linolenic acid from 1% to an average 12%, though not exceeding 16% of the total fatty acid pool. Palmitic

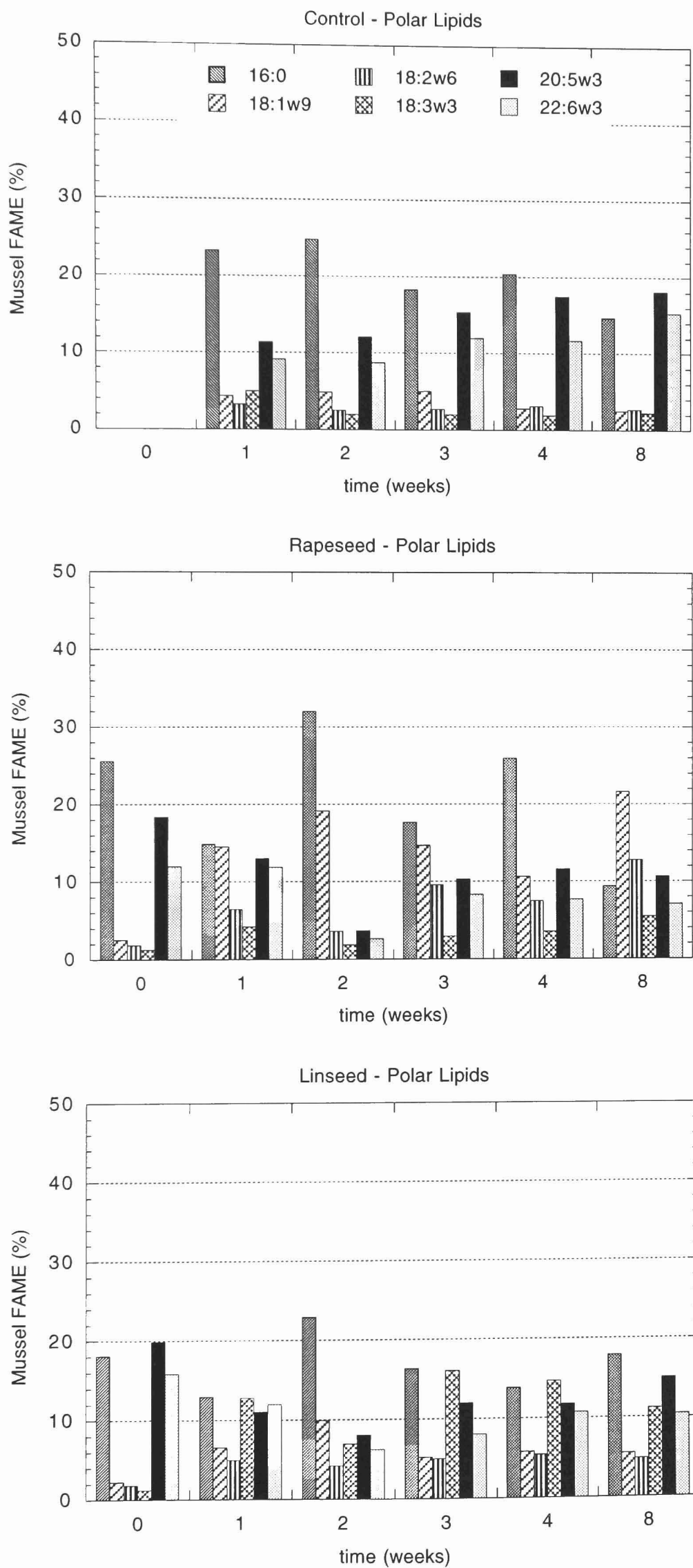


Figure 4.14: Polar fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks. Food was provided from week one to four and oil was delivered from week one to eight.

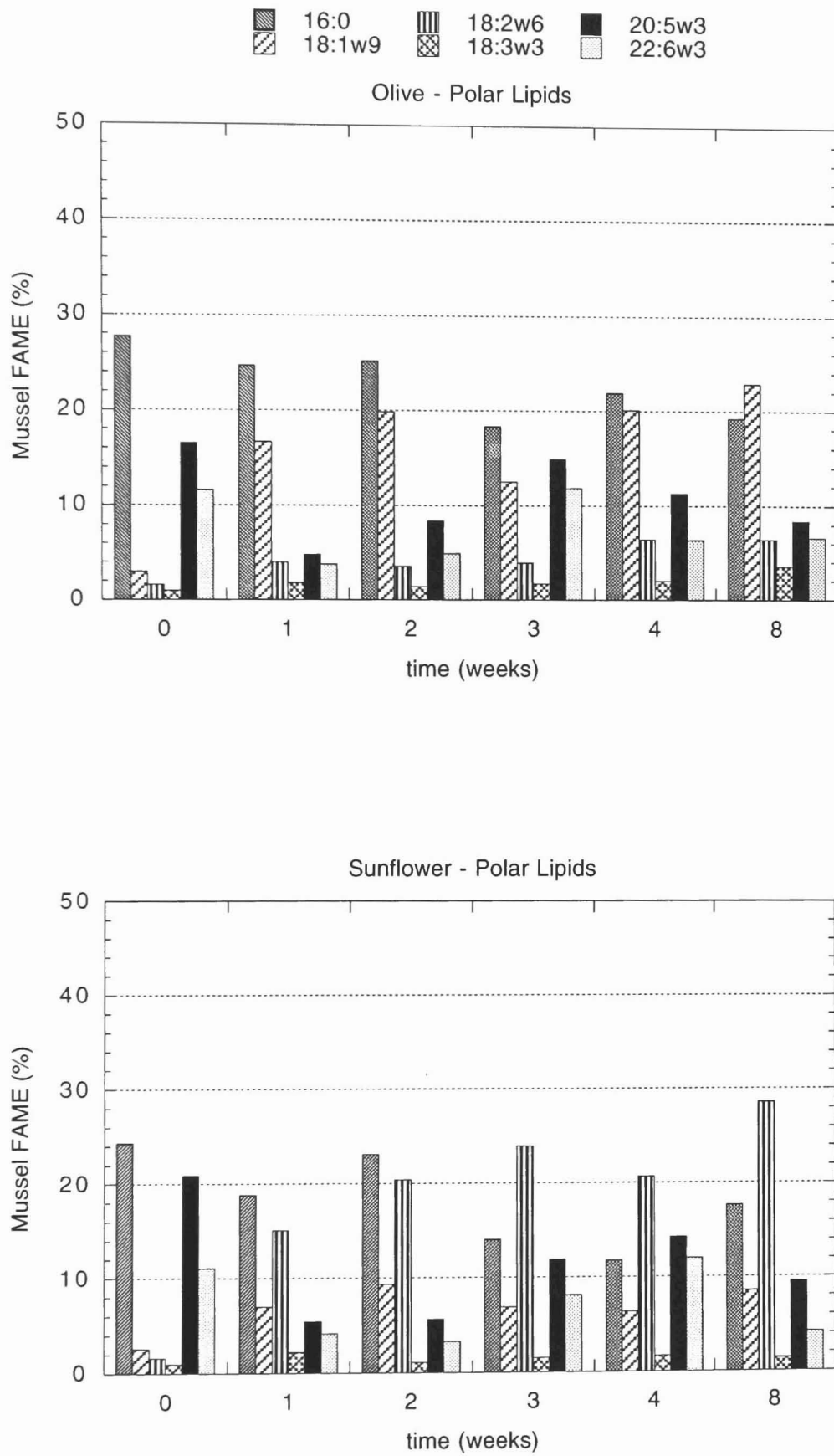


Figure 4.15: Polar fatty acid composition of control mussels and mussels exposed to different vegetable oil treatments during eight weeks. Food was provided from week one to four and oil was delivered from week one to eight.

acid was the dominant fatty acid for almost all the experiment with an average of 17%. The percentage of polyenoic fatty acid methyl esters of 20 and 22 carbons was high during week zero, specially 20:5 $\omega$ 3, and decreased with the introduction of the oil to 12% and 10%, respectively. Oleic and linoleic acids, representing 19 and 13% of linseed oil, respectively caused a 5% and 3% increase of the same fatty acids in mussels.

The treatment with olive oil resulted in an increase in the proportion of oleic acid: from 3% on week zero, its percentage reached 23% as the highest proportion in the eighth week. Palmitic acid was once again the dominant fatty acid with very steady percentages along the weeks with an average of 23%. The highly polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 decreased with the addition of the oil from 17% and 12%, respectively, to less than 10% in both cases.

The polar lipids of mussels exposed to sunflower oil had the highest levels of linoleic acid, almost reaching 30% by the eighth week. The 20% increase was the highest among the oil treatments and the amount of linoleic acid superceeded that of palmitic acid after the third week. Palmitic acid was, however, the first or second fatty acid in the pool. Oleic acid representing 23% of the oil, also increased in the mussels from 3% to 8% in average. The long chain polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 decreased with sunflower oil from 20% to 9% and 11% to 6%, respectively.

In general two main differences can be observed from these results. The change in mussel fatty acid composition caused by the diet and by the exposure to the vegetable oils in one hand and the qualitative and quantitative changes in fatty acids in the two classes of lipids on the other hand. The changes in fatty acids in control mussels were influenced by the diet, while the changes in oil treated mussels were mainly influenced by the presence of oils. Neutral lipids accumulated higher concentrations of fatty acids from external origin than polar lipids and these in turn possessed higher concentrations of palmitic acid and of the two long chain polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 than neutral lipids even after exposure to the oils.

## 4.4 Discussion

### 4.4.1 Mussel growth

Under the experimental conditions of temperature and food supply, control mussels achieved better specific growth rates (Figure 4.9) than any of the other mussels exposed to the vegetable oil treatments demonstrating the inhibiting effect of vegetable oils on growth of *M. edulis*. The marked differences in specific growth rates between the control mussels and the ones treated with oils emphasized the stressing effect of the oils on mussels. The cumulative specific growth rate for four weeks in control mussels was five times larger than that of the mussels treated with rapeseed oil and three times as much as the ones treated with sunflower oil. Although, sunflower oil treatment resulted in an increase of mussel growth rate for the first 3 weeks, suggesting some growth recovery after one more week of exposure the growth rate became constant.

An appropriate food supply was required for mussels to maintain appreciable growth. During the last 4 weeks of experiment when food was not delivered, mussel growth in any treatment was negligible. The effect of one stressor should only be investigated if the organisms present signs of sustained growth.

Mortality data confirms that all the vegetable oils employed in this study can cause mussel mortality. Unlike the control mussels which registered no mortality, all the oils registered mortality to some extent. Linseed oil was the most detrimental in this respect causing the highest mortality among the mussels. It also had the highest flow rate of all the treatments and is known by its drying properties (Godin and Spensley, 1971) which maybe an adverse effect for mussel living tissues such as mussel specialized ctenidia. It is then possible to say that the lethal effect of vegetable oils on mussels is dependent on their properties and also on the levels of exposure.

As discussed in Chapter 2, there are many factors upon which mussel growth rate depends and in this study feeding rate and exposure to oils were the two predominant aspects contributing for the final mussel performance. The pattern of growth for all mussels including the ones of the control was an increase of specific growth rate (SGR) followed by a decrease in the oil treatments and a constancy in the control. Ageing is usually accompanied by a decline in growth rate (Seed, 1976), therefore a decline in the

specific growth rate with time would be expected. In addition to this general decreasing growth trend the vegetable oils depressed mussel growth even further, producing differences of 3 to 5 times less than in control mussels, depending on the vegetable oil. However these differences between oil treatments were not statistically significant and all the oils had a similar inhibiting effect on mussel growth.

#### 4.4.2 Fatty acids

Neutral lipids of mussels were most influenced by the presence of the vegetable oils, in some cases presenting a close resemblance with fatty acid composition of the oils themselves. Olive oil was one example, its uptake by the mussels was large enough to change oleic acid percentage in mussels (see Figure 4.13) to a similar percentage as the one in olive oil (Figure 3.5). The other oils also caused obvious changes in mussel fatty acid composition but the percentages of the major fatty acids in oils did not reach as high percentages in mussels. Invariably the major fatty acid in the oil became the major fatty acid in the mussels producing a clear picture of oil uptake by the mussel neutral lipids. Other fatty acids typical and usually present in appreciable amounts in mussels, like palmitic acid, 20:5 $\omega$ 3 and 22:6 $\omega$ 3 could only be found in comparable percentages in the control group free of the influence of the oils. Polar lipids on the other hand, although showing the influence of the oils by an increase of the major fatty acids of the different oils, they were not visibly predominant at all times, contrarily to what had occurred in neutral lipids. Another difference between neutral and polar fatty acids of mussels treated with oils was that palmitic acid, 20:5 $\omega$ 3 and 22:6 $\omega$ 3 exhibited much higher percentages in polar than in their neutral counterparts, palmitic acid being the leading fatty acid in most treatments and for most of the experiment. Thus, it seems neutral lipids are the major storage of uptake of external substances, upon which metabolic activity takes place, reflecting the changes in the environment and the needs of the organism, and that polar lipids have a much more structural role showing more constant composition resisting to environmental changes.

Within the control mussels the observed variations in fatty acids of neutral lipids with time of experiment were due to the influence of the amount and type of fatty acids present in the diet. The main variations in neutral lipids were detected in palmitic



acid, oleic acid, 20:5 $\omega$ 3 and 22:6 $\omega$ 3. The first two decreased after 1 week of treatment and the last two increased. These variations can be explained if we consider that 72% of the three microalgae species in the diet is composed by *Pavlova lutheri*, as can be seen in Figure 4.5, which is rich in 20:5 $\omega$ 3 and 22:6 $\omega$ 3 and has small amounts of 18C, consequently the polyenoics supplied from the diet increased in mussel neutral lipids and the 18 carbon monounsaturate typically of detritic origin decreased. Palmitic acid proportion decreased slightly due to an increase of the polyunsaturates. At the last week, after being food deprived during 4 weeks, the only source of food being possibly detritus accumulated in the tank and any particles passing through the fibre glass filter, fatty acid composition of control mussels changed again. This time the proportions of the two highly polyunsaturates decreased and the 18C oleic, linoleic and linolenic acids increased, suggesting there was some metabolic activity. Either oxidative reactions of the highly unsaturated fatty acids or desaturations of oleic acid which might have increased by the ingestion of detritus. In polar lipids, the increase of the long chain polyunsaturated and the slight decrease of palmitic acid was probably due to the effect of a diet rich in these first two acids. However, the characteristic stable composition of polar lipids achieved by a balance between palmitic acid and the two highly polyunsaturated was observed at the eighth week when after mussel starvation the two polyenoics still remained high.

The effect of food in changing lipid fatty acid composition was more direct in neutral than in polar lipids but in any case changes were only detected in the control mussels since the influence of oils dominated that of the diet. The continued exposure of mussels to the oils from the first to the eighth weeks produced an increase of fatty acids in mussels corresponding to the major acids in the oils: oleic, linolenic and linoleic acid and the suppression of other neutral fatty acids typical from mussels, namely 20:5 $\omega$ 3 and 22:6 $\omega$ 3 which were provided by the food rich in these last two components. No change was either evidenced in the eighth week after food had been removed because the presence of oils was still the main effect in variation of the neutral fatty acid profiles. However in polar lipids the suppression of palmitic acid, 20:5 $\omega$ 3 and 22:6 $\omega$ 3 did not occur so extensively because of the necessity to keep fairly constant concentrations of polyunsaturates, in particular the long chain ones, in these type of lipids. The

observations made here that polar lipids are less dependent on diet than neutral lipids agrees with work reported previously by Langdon and Waldock (1981) for *Crassostrea gigas* spat.

## 4.5 Summary

The effect of four different vegetable oils on *Mytilus edulis* shell growth was investigated using measurements of shell area with a photographing process over an eight week period. The method employed proved to be precise enough to measure changes in growth during short time periods (7 days) with a maximum error inherent to the method of 1%.

The growth rates of *Mytilus edulis* exposed to the oils were significantly depressed compared to the control mussels demonstrating the growth inhibiting effect of the vegetable oils. Besides growth depression vegetable oils also caused mussel mortalities, high in some cases. Linseed oil was the most detrimental in this respect producing 12 deaths, whereas the other oils, rapeseed, olive and sunflower only registered 1, 2 and 3 dead mussels, respectively. On the contrary control mussels had no mortalities. The concentrations of exposure and type of oil maybe related to the observed mortalities. Diet proved to be essential for mussel sustained growth enabling the assessment of the vegetable oils effect on growth. When food supply was removed the growth rates of mussels in all treatments were negligible (Figure 4.10).

Fatty acid analysis were performed to monitor the effect of the vegetable oils on mussel fatty acid composition. Major differences were noticeable on the levels and quality of fatty acids in the two main classes of lipids: the neutral and polar lipids in mussels treated with oils. In the neutral lipids, the major fatty acid present in the oil predominated in the mussels reducing the levels of the naturally occurring fatty acids like palmitic acid and the long chain polyunsaturates 20:5 $\omega$ 3 and 22:6 $\omega$ 3 and reflecting the influence of the oils. In the polar lipids, the percentage of the fatty acids typical in the oils increased but were not as high as in neutral lipids neither were dominant. Palmitic acid as well as the highly polyunsaturated 20:5 $\omega$ 3 and 22:6 $\omega$ 3 were much higher than in neutral lipids and exhibited a constant pattern throughout the

experiment. The fatty acid composition of the control mussels was quite different from the treated mussels showing the influence of the diet with increased levels of palmitic acid and polyunsaturates and low levels of the 18C predominant in the oils.

# Chapter 5

## The biological responses of mussels to vegetable oils

### 5.1 Introduction

The ultimate objective of ecotoxicological studies is both to predict and diagnose the causes of biological/ecological effects resulting from exposure to chemicals and other stressors in the environment (Widdows and Donkin, 1991). To fulfil this objective, it is necessary to establish a series of stepwise relationships before it can be proved that the concentrations of the chemical in the environment are the cause for a biological effect in the organism. These relationships involve a good knowledge about the bioconcentration factors of the chemical and its mode of action in the organism. Measurements of both the concentration of contaminants in the body tissues and biological effects in the organism are suitable for assessing environmental pollution, since all the information to complete the diagnostic frame is often unavailable.

Mortality and shell valve gape are two physiological responses which have been used to measure the effect of pollutants in bivalves. Valve movement response of mussels has been proposed as a biological monitoring tool (Sloof *et al.*, 1983; Kramer *et al.*, 1989). The rationale is that mussels under optimal conditions remain with their valves open for most of the time in order to pump water for respiration and feeding; whereas in response to environmental stress and many toxicants, mussels close their shells for extended periods of time (Widdows and Donkin, 1992). In addition, it was investigated how prolonged the effect of the oil can be if mussels are exposed to natural changes of salinity and temperature in the environment.

Changes in salinity and temperatures by the presence of freshwater sources and periods of emmersion are most likely to happen amongst intertidal living organisms. The performance of mussels under vegetable oils exposure to these changing conditions was assessed by measuring the biological responses, mortality and shell valve gape. The objective of this chapter was to establish a) whether vegetable oils have a prolonged biological effect on mussel valve gape when they experience a range of change in salinities and b) whether vegetable oils cause mussel mortality when they are submitted to a range of temperatures. The following tolerance experiments were designed to test 1-the effect of different salinities on the time of valve closure of *Mytilus edulis* (L.) pre-exposed to vegetable oils and 2-the effect of different temperatures on mussel mortality after being exposed to the oils.

## 5.2 Materials and Methods

Tolerance experiments consisted on exposing mussels to the four vegetable oils, rape-seed, linseed, olive and sunflower with a control group receiving distilled water instead of oil, for nine weeks after which they were submitted to the tolerance experiments. This consisted of transferring the mussels to containers with clean seawater of a range of decreasing salinities and from there they were transferred to tanks with running seawater at different temperatures for one week.

### 5.2.1 Vegetable oils exposure

The experimental set up for exposure of mussels to the oils was similar to the one used for growth experiments described in Chapter 4, since the period of oil exposure was simultaneous between the two sets of experiments. In the tolerance experiments mussels were distributed among five round bowls of approximately two litres of capacity. They were set up with a flow through seawater system and placed underneath the tanks containing the mussels for the growth experiment, so that the five bowls could receive the seawater outflow from the tanks, saving space, time, money and the amount of oil wastes. In consequence of this system arrangement, the seawater flow, the food supplement and the oils were not directly introduced in the bowls and they were delivered

after passing through the first set of tanks.

### Tanks

The flow rates measured on the outlet of the bowls were 261, 188, 194, 207 and 176 ml · min<sup>-1</sup> for the control, rapeseed, linseed, olive and sunflower bowls, respectively. The flow rate had been set up to 300 ml · min<sup>-1</sup> in the growth tanks and was kept constant by a header. However the oils accumulating in the piping and on the bowl's outlet walls slowed down the waterflow. A layer of oil accumulated at the surface of the water and had to be removed every day to prevent the blocking of the outlet.

### Oils

The oils flowrate set initially in the growth tanks to 0.3 ml · min<sup>-1</sup> slowed down to 0.01 ml · min<sup>-1</sup> in rapeseed tank, 0.02 ml · min<sup>-1</sup> in both olive and sunflower tanks and 0.05 ml · min<sup>-1</sup> in linseed tank into the growth experimental tanks and outflowed into the bowls. The reason for the oil flow decrease was probably the viscosity of the oils offering great resistance to be pumped. The flowrate of distilled water was reduced to 0.1 ml · min<sup>-1</sup> in the control bowl when the oil flowrates were observed to have decreased.

### Mussels

Mussels were collected from Bangor pier pillars during low tide and brought into the laboratory where they were cleaned from epifauna and flora and sorted by size. Sixty six adult mussels between 4 and 7 cm long were placed in each of the 5 tanks. They were kept there with running seawater to acclimate to the experimental conditions for one week, after which the oils started to flow into the tanks as well as food supplement.

### Food

The food, like the water and oils was received from the outflow of the growth tanks and had the same composition. It was composed by the mixture of microalgae *Tetraselmis chuii*, *Pavlova lutheri* and *Rhinomonas reticulata* supplied daily by drip-feeding. The cell density of the algal culture was counted daily with a ZM coulter counter and mixed

according to their concentration to have approximately the same number of cells per day ( $1.6 - 2.0 \times 10^9$  cells).

### 5.2.2 Salinity experiment

Seawater and distilled water solutions were mixed to obtain the different salinities: 32, 25, 20, 15 and 10‰. The final salinity was checked with a refractometer and it varied by  $\pm 1$ ‰. Five containers with approximate capacity of one litre were filled with these different salinity solutions and they were placed in a running seawater tank to keep the water temperature constant and similar to the temperature in the mussel bowls. The water temperatures for each salinity container were 7.7, 7.8, 7.9, 7.7 and 7.8 °C for the decreasing salinities, respectively and the water temperature mussels had been exposed to in the oil tanks were 8.2 and 8.8 for the control and linseed oil groups, respectively. On the second day of experiment the water temperatures in the salinity containers increased to 9.2, 9.7, 9.7, 10.4 and 11.1 °C for the decreasing salinities, respectively and the water temperatures in the oil tanks were 10.4 and 9.7 °C for olive and sunflower oil groups, respectively. Groups of 10 or 11 mussels pre-exposed to the vegetable oils linseed, olive and sunflower oils, for 9 weeks and another group of 10 control mussels with no contact with oil, were transferred to a clean seawater and 32‰ salinity container one at a time and allowed to open their valves. The time mussels took to open their valves under those new conditions was recorded and they were transferred quickly to the decreasing salinities before they could shut their valves by air exposure. Mussels were allowed to stay five minutes maximum time in each salinity before being transferred to the next low salinity. After the 10‰ salinity mussels were put back on 32‰ and allowed to gape, the time they took to gape after being through the salinity series was also registered. The time that mussels took to gape (in 32‰) or close their valves (in the other salinities) was taken as the measurement to assess any difference of behaviour between the control and the oil groups. The following measurements were recorded: the time mussels, took to gape when placed in clean seawater at 32‰, coming from the different oil treatments and coming from the decreasing series of salinity; the time mussels took to shut their valves when transferred to the decreasing salinity series and the salinity at which shell closure occurred; the gaping time between salinities 25 and

10‰ and the percentage of valve closure for the different salinities and treatments.

### **Temperature experiment**

Mussels from each of the tanks contaminated with oils and from the control group were split up into 3 groups to be transferred to 3 tanks of approximate capacity of 9 litres, at 3 different temperatures 5, 10 and 18 °C. The highest temperature was kept constant by controlled heating using a thermostat and the lowest temperature was kept constant by a coiled cooling system with glycol. The tanks had to be insulated with expanded polystyrene foam and covered to maintain the temperatures. A flow through seawater system was set up receiving filtered seawater with a flow rate of 200 ml · min<sup>-1</sup>. The mussels were distributed among the temperature tanks in the total number of 67 from the control group, 8 exposed to linseed oil, 40 exposed to olive oil and 45 exposed to sunflower oil. They were all equally distributed amongst the 3 temperature tanks and placed, within the same temperature tank, in net bags to distinguish the groups formerly contaminated with the different oils. Mortality was the biological response measured to assess the effect of vegetable oils on mussel tolerance to different temperatures.

### **5.2.3 Fatty acid analysis**

To monitor the fatty acid concentrations in the mussel tissues under the new conditions of temperature and oil depuration, fatty acid analysis were performed. Mussel samples were collected just before being transferred to the temperature treatments and 10 days later in the new conditions. Three mussels from each tank/treatment were collected each time, their length measured and the soft tissues weighed and freeze dried. The lipid extraction was done according to the methods described in chapter 3 and the extracted lipids were methylated using trimethyl sulfonium hydroxide (TMSH) and injected in the gas chromatograph mass spectrometer (GC-MS) Finnigan Mat 4600.

### **5.2.4 Analysis of shell structure**

In order to investigate the effect of vegetable oils on shell growth and structure, mussels from both control and oil treatment were selected for study. Mussel shells were air dried and each shell valve was embedded in Metaset SW resin (Buehler UK Ltd) and radial



sections cut manually, using a hacksaw, from the umbo to the growing margin of the shell. The cut surfaces were ground on progressively finer grades of wetted waterproof silicon carbon paper and finally with alumina grit on glass. The sections were washed, dried and polished on a cloth soaked in household metal polish (Brasso). The shells were then etched using 0.01 M HCl for 30 minutes and acetate peels (Agar Scientific Ltd) prepared using the technique described by Richardson *et al.*, (1979). When dry, the peels were removed and mounted on slides, covered with coverslips and examined under the light microscope.

## 5.3 Results

### 5.3.1 Vegetable oils exposure

Between the fifth and sixth weeks of oils exposure, high mussel mortality occurred. Mortality percentages are shown on Figure 5.1 for each of the oils and the control group.

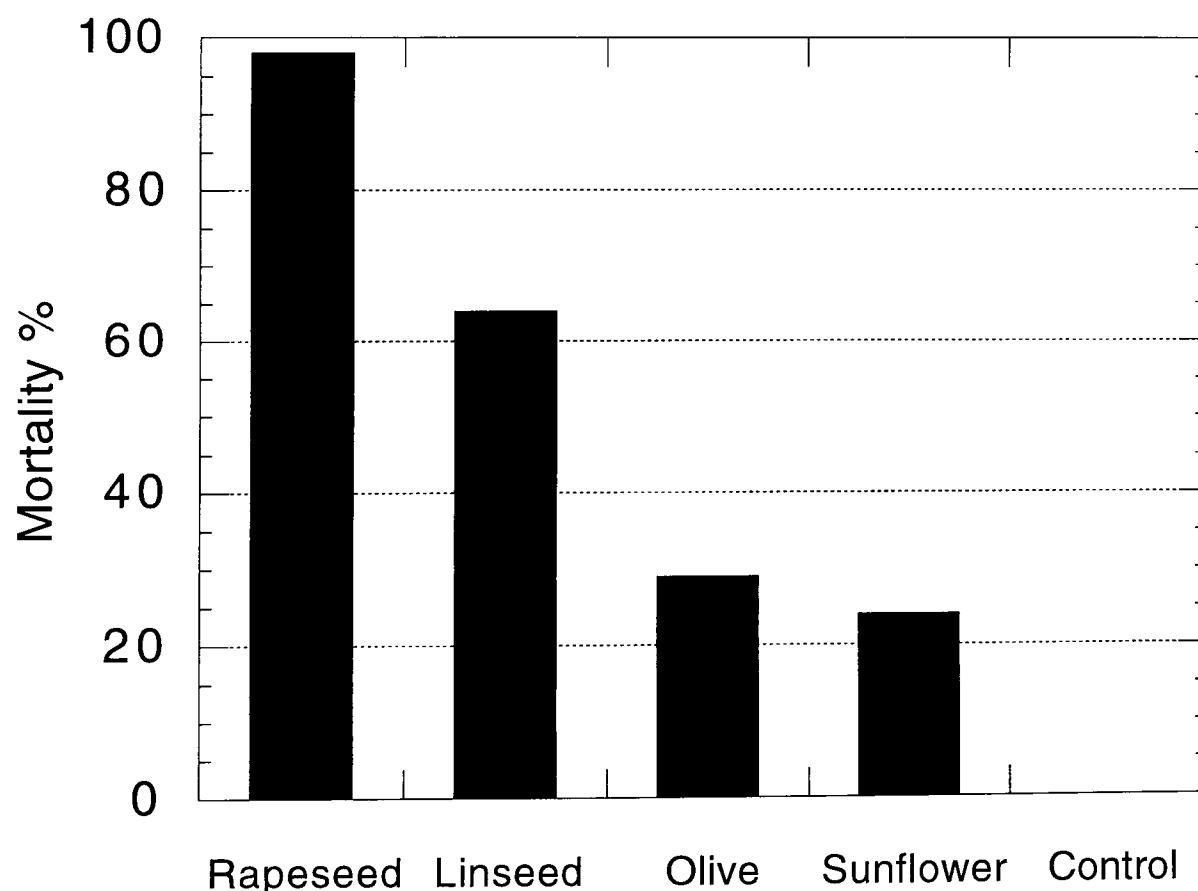


Figure 5.1: Mortality of mussels exposed to different vegetable oil treatments for nine weeks.

All the oil treatments had high mortality varying from 24% to 98%, whereas in the control group all the mussels survived. The stock mussels exposed to rapeseed oil was

severely depleted resulting on the survival of just one specimen. The other oils caused lower but significant mortalities, linseed oil was the next causing 64% of the deaths; olive and sunflower oils a lot less lethal caused 29% and 24% of the mussels to die, respectively. Rapeseed oil was the most detrimental in terms of survival since it caused the highest mortality.

### 5.3.2 Response to salinities

Several measurements were made to assess any differences in mussel response to different salinities between a control group and groups of mussels treated with vegetable oils.

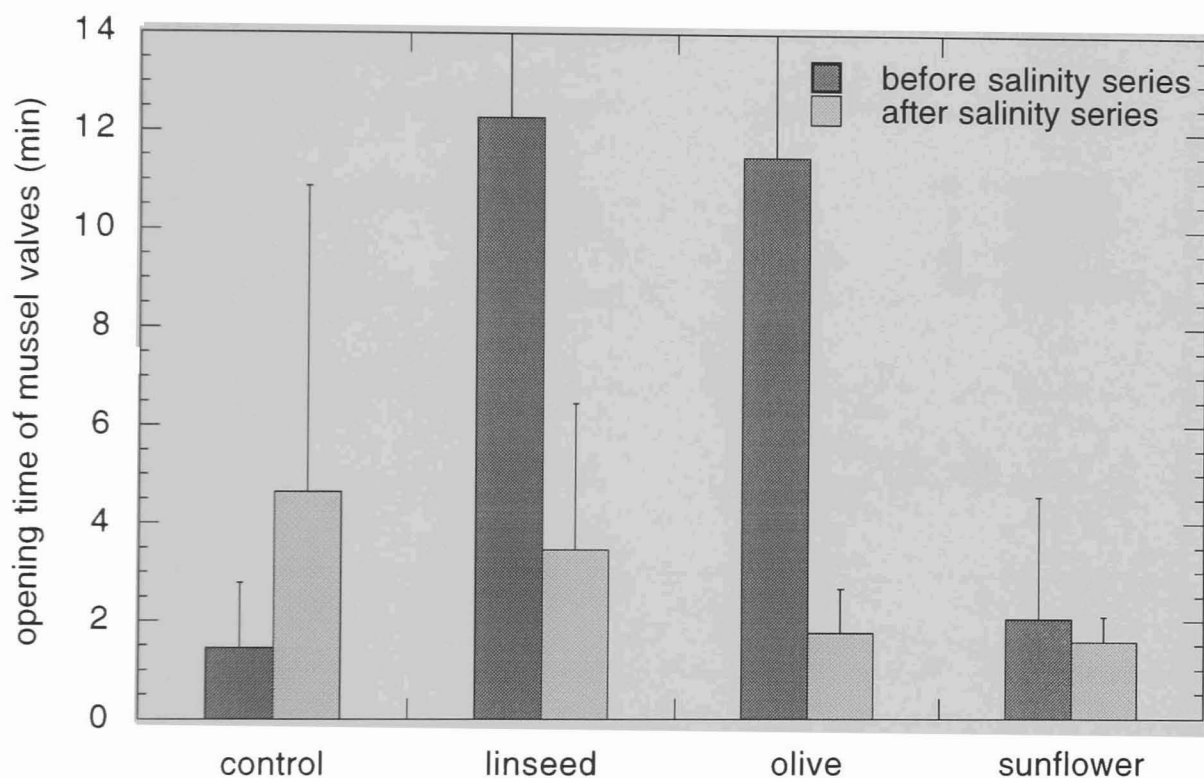


Figure 5.2: Mean time of opening response of mussels in 32‰ salinity, pre-exposed to vegetable oil treatments, before and after being submitted to decreasing salinities.

Figure 5.2 indicates the time mussels took to open their valves when placed in clean seawater at 32‰ salinity, coming from the oil treatments with the same salinity and the time taken by the same mussels to open their valves when transferred back to the salinity 32‰ after being through all the salinity series staying 5 minutes in each. Control mussels were the fastest to open their valves before the salinity series, an average time of 1.5 min was required for the control mussels to start gaping. Mussels exposed to sunflower oil displayed longer time of opening response 2 min and notably smaller than the other two groups, the olive and linseed oil which needed about 12 min until they opened their valves. After the mussels had been exposed to the series of

descendent salinities and put back on salinity 32‰ the groups which had taken longer time to open their valves before salinity series, olive and linseed, decreased drastically the opening time, after the salinity treatment, from 12 min to approximately 2 min and less than 4 min, respectively. The sunflower oil group of mussels also reduced slightly their time of response to less than two minutes. The longest time of opening response was for the control group which increased to 4.5 min. It is worthy to note that high standard deviations were calculated for the time of response at 32‰ for each of the groups of 10 mussels coming from the oil treatments and that they decreased largely after the mussels had been through the salinity series except for the control group which contrarily had an increased standard deviation.

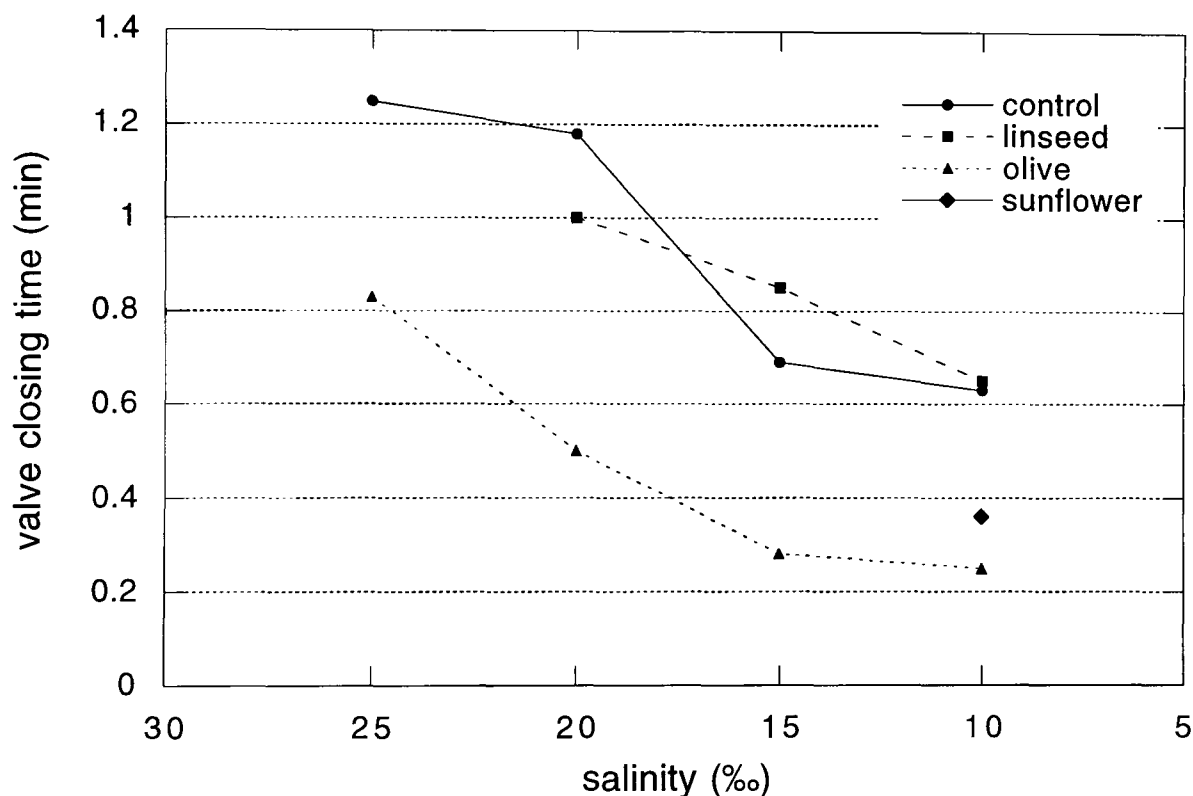


Figure 5.3: Mean time of mussel valve closure in decreasing salinities, pre-exposed to vegetable oil treatments.

Figure 5.3 shows the time mussels from each group needed to close their valves at decreasing salinities. In general, the time of closure decreased with the decrease in salinity. The control mussels closed their valves at approximately 1.2 min for salinities 25‰ and 20‰ and the time of response decreased sharply for 15‰ and decreased again to 0.6 min for 10‰ salinity. None of the mussels treated with linseed oil when moved from salinity 32‰ to salinity 25‰ closed their valves. This only occurred at lower salinity (20‰) where mussels closed their shells after 1 min. The time of response decreased almost linearly for the other two lower salinities being the time of closure for

the 10‰ salinity similar to the one from the control. The response of mussels treated with olive oil was faster than all the other mussels for any of the salinities; they took 0.8 min to close valves at the highest salinity and exhibited a large decrease of closing time for the following lower salinities stabilizing for 10‰ at approximately 0.2 min. This was 3 times faster than control and linseed group. Sunflower treated mussels kept gaping at the three higher salinities closing only for salinity 10‰ in about 0.3 min. It is clear from the graph that salinity 10‰ is an important mark in reducing the time closure of mussels valves.

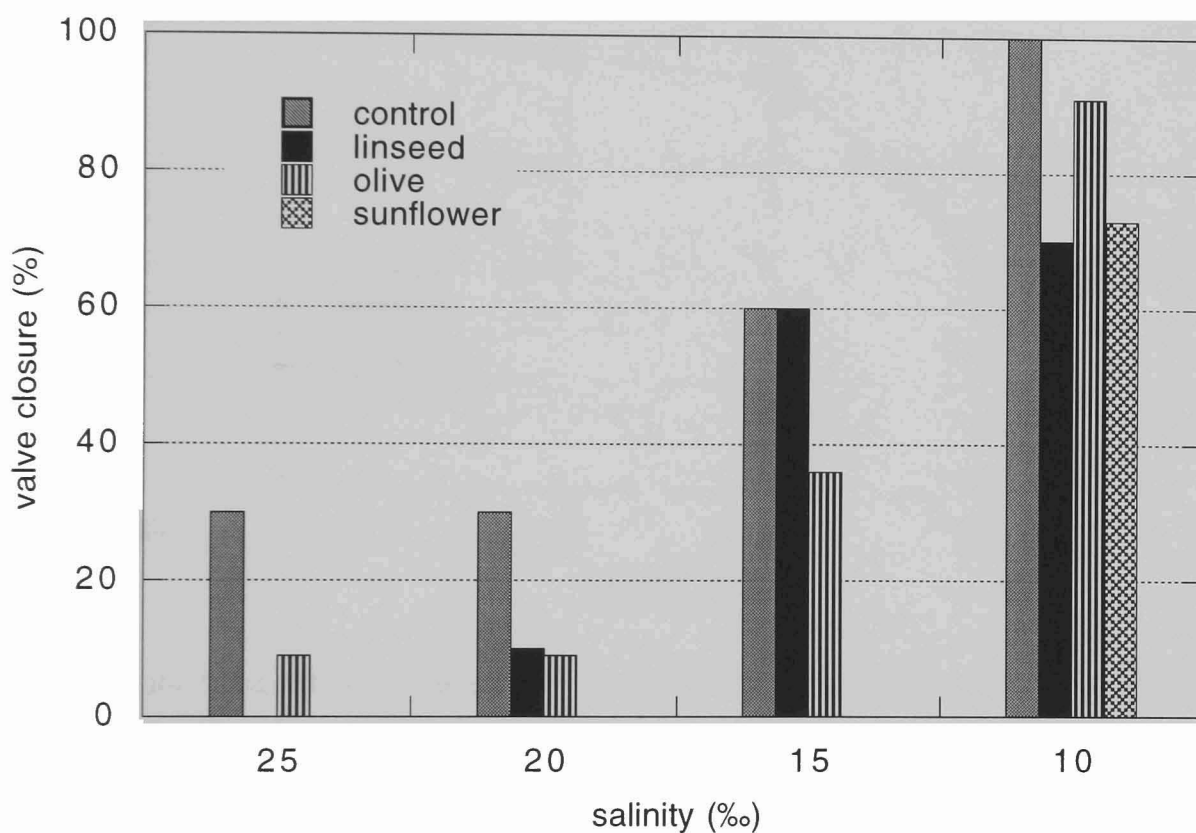


Figure 5.4: Percentage of mussels closing their valves with decreasing salinities, pre-exposed to different vegetable oil treatments for nine weeks.

Figure 5.4 quantifies in percent how many of these mussels closed their shells for each of the salinities they experienced. The percentage of mussels closing their valves increased with the decrease in salinity. Control mussels had the highest percentage of closed mussels for any salinity. It increased greatly at a salinity 15‰ and they all closed at 10‰. Linseed treatment registered lower percentage of closed mussels than the control for any salinity but it increased in similar way as the control with a large percentage of closed mussels for salinities 15‰ and 10‰. Mussels exposed to olive oil started to close their shells from salinity 20‰ showing a large increase of closed mussels for the two lowest salinities. Sunflower oil group of mussels only closed their

shells for the lowest salinity and like all the other groups submitted to the oils the percentage of closure was not 100% but varied between 70% for sunflower and linseed group and 90% for olive group. Out of 42 mussels 43% closed the valves at salinity 10‰ and 26% closed at 15‰, 17% did not close their valves and the remaining 14% were distributed between the 9% of mussels closing at 25‰ and 5% at 20‰.

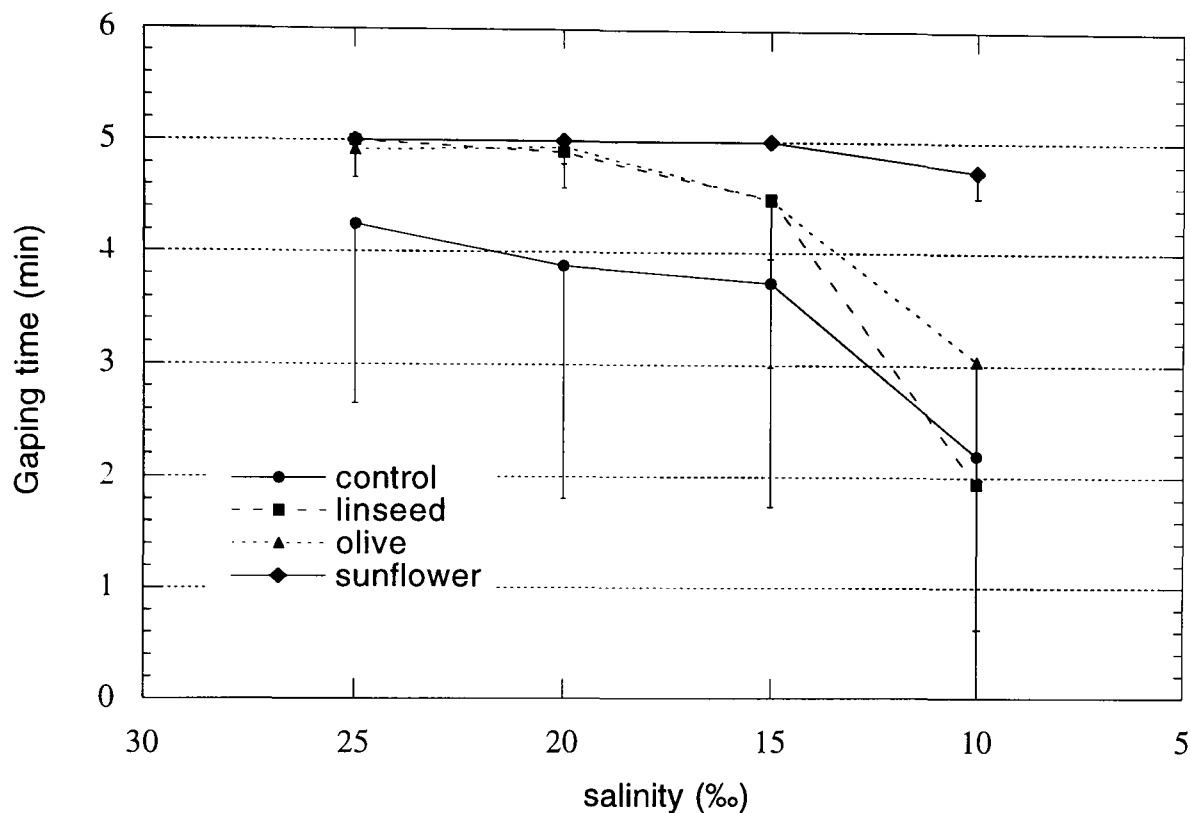


Figure 5.5: Mean gaping times of mussels in different salinities, pre-exposed to different vegetable oil treatments for nine weeks.

Figure 5.5 shows how long the different groups of mussels kept the valves open at the different salinities or in other words the average gaping times of mussels in different salinities. The maximum allowable gaping time at each salinity was five minutes. The groups treated with vegetable oils displayed longer gaping times than the control mussels for any salinity, the only exception was linseed group for the lowest salinity which had a slightly shorter gaping time than the control. Sunflower group, in particular, exhibited the longest gaping periods of 5 min even at salinity 15‰ and at 10‰ it decreased to 4.8 min. All groups showed a clear decrease of gaping time for 10‰ salinity. The standard deviations for the mean gaping times were higher in the control mussels than in the other groups but for the lowest salinity, standard deviations were similarly high in all groups except sunflower group.

### 5.3.3 Statistical analysis

Statistical analyses were used to check the significance of the previous results. Analysis of variance (Anova) was performed to test the three sets of measurements: 1—the differences on the opening time of mussel valves between the control and the oil groups at salinity 32‰ before and after mussels had gone through the salinity series; 2—the differences between the control and oil groups on the closing time of mussel valves in the decreasing salinities and 3—the differences between the control and oil groups on the gaping time of mussels in decreasing salinities. The null hypothesis stated for any of the tests was the following ( $H_0 : \mu_1 = \mu_2 = \mu_3 = \mu_4 = 0$ ) meaning there are no differences on the means of opening time, or the means of closing time or the means of gaping time of mussel valves between the control and the oil groups in any of the different experimental conditions. Whenever the null hypothesis in the analysis of variance test was rejected a Scheffé pairwise comparisons test was performed (Zar, 1974) to decide which mean or group of means were significantly different.

#### Effect of oils and salinities on opening response

In this test the null hypothesis,  $H_0$ : there are no differences on the opening time of mussel valves between the control and the oil groups when placed at salinity 32‰, before and after mussels had gone through the salinity series, was rejected which means the probability that the differences found in the opening times of mussel valves before and after salinities exposure ( $P = 0.018$ ) and between the oil treatments ( $P = 0.034$ ) are due to random error is very small at a level of 95% of confidence. Therefore the differences are due to the salinity treatment and exposure to the different oils. A Scheffé's pairwise comparisons test was called to decide which of the means were different. The following computer output shows the results of the tests.

#### Analysis of Variance for opent

Source	DF	Seq SS	Adj SS	Adj MS	F	P
treat	1	347.89	329.22	329.22	5.87	0.018
oil	3	523.67	510.11	170.04	3.03	0.034
treat*oil	3	619.02	619.02	206.34	3.68	0.016
Error	75	4203.95	4203.95	56.05		
Total	82	5694.53				

Scheffe for opentime(mn) after oils and before and after salinity series.

ROW	CODE	mean	n			
1	1	6.810	42			
2	2	2.818	41			
3	3	3.044	19			
4	4	7.865	20			
5	5	6.540	22			
6	6	1.806	22			
7	7	1.450	10			
8	8	12.267	10			
9	9	11.484	11			
10	10	2.040	11			
11	11	4.639	9			
12	12	3.463	10			
13	13	1.596	11			
14	14	1.572	11			

ROW	CODE	CODE	diffmean	SE	low_ci	up_ci
1	1	2	3.992	1.64365	-4.0751	12.0591
26	3	4	-4.821	2.39844	-16.5926	6.9506
27	3	5	-3.496	2.34472	-15.0039	8.0119
28	3	6	1.238	2.34472	-10.2699	12.7459
37	4	5	1.325	2.31306	-10.0275	12.6775
38	4	6	6.059	2.31306	-5.2935	17.4115
47	5	6	4.734	2.25731	-6.3449	15.8129

The conclusion from the Scheffé's pairwise comparisons test was contradictory relative to the analysis of variance, since it did not reveal any differences on the opening time of mussel valves in any of the oil or salinity treatments. All the 95% confidence intervals for the paired differences contain zero meaning that they are not significant. The likelihood of contradiction increases using this test relative to others (for example the one available from Minitab which test the means against the grand mean) but the protection against *type I errors* also increase. Type one errors occur when the null hypothesis is rejected when it is in fact true (Zar, 1974). It seems, therefore that a *type I error* has been committed and the null hypothesis should be accepted. The response of mussels of opening valves before and after going through the different salinities and pre-exposed to the different oils experiment was similar for mussels treated with oils and for the control mussels and it was not influenced by the salinity treatment. This proves that the capacity of mussels to open valves was not affected on the long term by the oil exposure nor by the short exposure to different salinities. The responses of mussels treated with oils were similar to the ones in the control mussels after the oil source had been removed.

**Effect of oils on closing/gaping response**

The time mussels spent gaping was dependent on salinity. It is reasonable to expect that mussel gaping times would decrease with decreasing salinities since mussels were acclimated to higher salinity. Mussels transferred to the different salinities, responded by closing their valves after a certain time and at a specific salinity. The time they took before closing (gaping time) was recorded and tested for differences between the different oil groups and for the different salinities. The analysis of variance shows the test results. The null hypothesis ( $H_0$ : There are no differences in the closing/gaping time of mussel valves for each oil group and salinity) was rejected ( $P = 0.000$  for both factors), that is the type of oil and salinity both had significant effects on the closing/gaping time response of mussels. However the interaction between the type of oil and salinity was not significant ( $P = 0.406$ ). And after a Scheffé pairwise comparisons test, the sunflower group revealed significant longer time of closure/gaping time than the control group but similar to the other oils. The lowest salinity, 10‰ caused a significant ( $P < 0.001$ ) decrease of closing/gaping time. Higher salinities caused non significant shell closure and the gaping times were statistically similar among them. These results suggest that sunflower oil increased the closing time of mussel valves or the gaping time of mussels pre-exposed to it and that salinity 10‰ determined a significant increase of valve closure or decrease of gaping time. Higher salinities caused non significant shell closure and the gaping times were statistically similar among them.

**Analysis of Variance for closing/gaping time with oil treatment**

Source	DF	Seq SS	Adj SS	Adj MS	F	P
oil	3	58.810	58.810	19.603	7.36	0.000
sali	3	344.792	342.375	114.125	42.84	0.000
oil*sali	9	25.096	25.096	2.788	1.05	0.406
Error	152	404.900	404.900	2.664		
Total	167	833.598				

Scheffe pairwise comparisons between the means of each oil treatment 1-control, 2-linseed, 3-olive, 4-sunflower and salinities 5-25, 6-20, 7-15 and 8-10



ROW	CODE	mean	n
1	1	2.489	40
2	2	3.418	40
3	3	3.266	44
4	4	4.157	44
5	5	1.025	42
6	6	3.261	42
7	7	4.452	42
8	8	4.592	42

ROW	CODE	CODE	diffmean	SE	low_ci	up_ci
1	1	2	-0.929	0.365445	-2.31904	0.46104
2	1	3	-0.777	0.357043	-2.13508	0.58108
3	1	4	-1.668	0.357043	-3.02608	-0.30992
8	2	3	0.152	0.357043	-1.20608	1.51008
9	2	4	-0.739	0.357043	-2.09708	0.61908
14	3	4	-0.891	0.348438	-2.21635	0.43435
23	5	6	-2.236	0.356638	-3.59254	-0.87946
24	5	7	-3.427	0.356638	-4.78354	-2.07046
25	5	8	-3.567	0.356638	-4.92354	-2.21046
26	6	7	-1.191	0.356638	-2.54754	0.16554
27	6	8	-1.331	0.356638	-2.68754	0.02554
28	7	8	-0.140	0.356638	-1.49654	1.21654

In summary, the statistical analysis demonstrated that neither of the vegetable oils nor the salinity treatment affected the time of opening response of mussels in clean seawater at same salinity they were before, showing that vegetable oils did not have a long term effect on valve movements of mussels. Nevertheless, oil treatment and salinity had an effect on the closing/gaping times of mussels. Mussels exposed to sunflower oil showed significantly longer closing/gaping times than the control mussels, indicating that there was an effect of the oil beyond the time of exposure. The lowest salinity employed 10‰ had a significant effect in mussels isolation inside their shells.

### 5.3.4 Mussel response to temperatures

During the time of this experiment the flow rates and temperature in the tanks were checked daily and they were kept constant within  $60 \text{ ml} \cdot \text{min}^{-1}$  and  $0.5^\circ\text{C}$  maximum standard deviation, respectively. Figure 5.6 presents the mean flow rates and water temperatures of the three experimental tanks during nine days of experiment.

The biological response of mussels previously exposed to vegetable oils and transferred to different temperatures was measured in mortality rate. Figure 5.7 shows mussel mortality registered for each temperature and oil treatment.

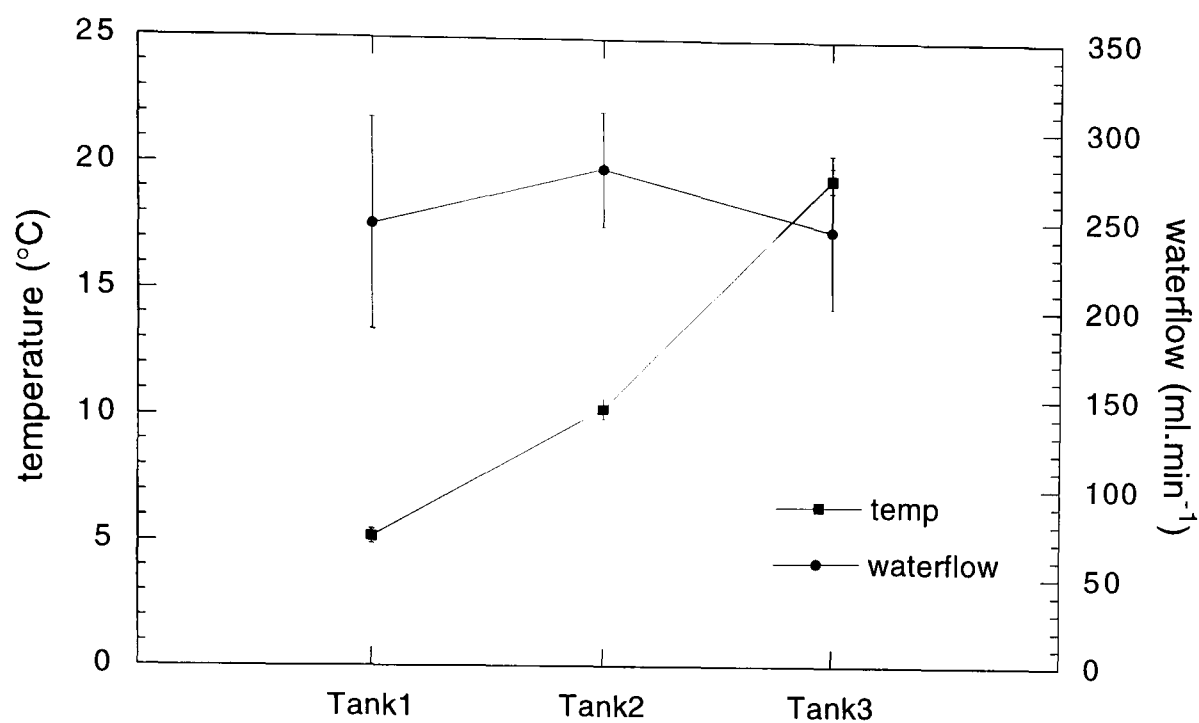


Figure 5.6: Means and standard deviations of flowrates and temperatures registered during nine days of experiment.

It is clear that mortality increased with temperature. All the mussel groups including the control registered mussel mortality for the highest temperature. Sunflower oil group was the only one to have deaths at all the three temperatures. According to the temperatures, one mussel out of fifty two died at 5 °C, two mussels out of fifty one died at 10 °C and six out of fifty three died at 19 °C. According to the oils groups, two mussels out of sixty three died from the control group, one mussel out of eight died from linseed group, 2 mussels out of forty died from olive and 4 out of forty five died from sunflower group. The distribution of deads according to both temperature and oil treatment is shown in Figure 5.7. In terms of oil treatment, sunflower oil caused the highest mortality. From the total nine dead mussels, five died within the first four days of experiment and the other four died 15-38 days after the beginning of experiment. The first mussels to die were three in the tank at 19 °C and one at 10 °C, the latest three deaths occurred one at 19 °C and two at 5 °C. The number of mussels in each oil-temperature treatment was relatively low to conclude anything further. The apparent effect of sunflower oil on mussel mortality can not be related with the effect of temperature, since if there would be any additive effect of oil with temperature, the number of dead mussels treated with sunflower oil exposed to the highest temperature should have been higher.

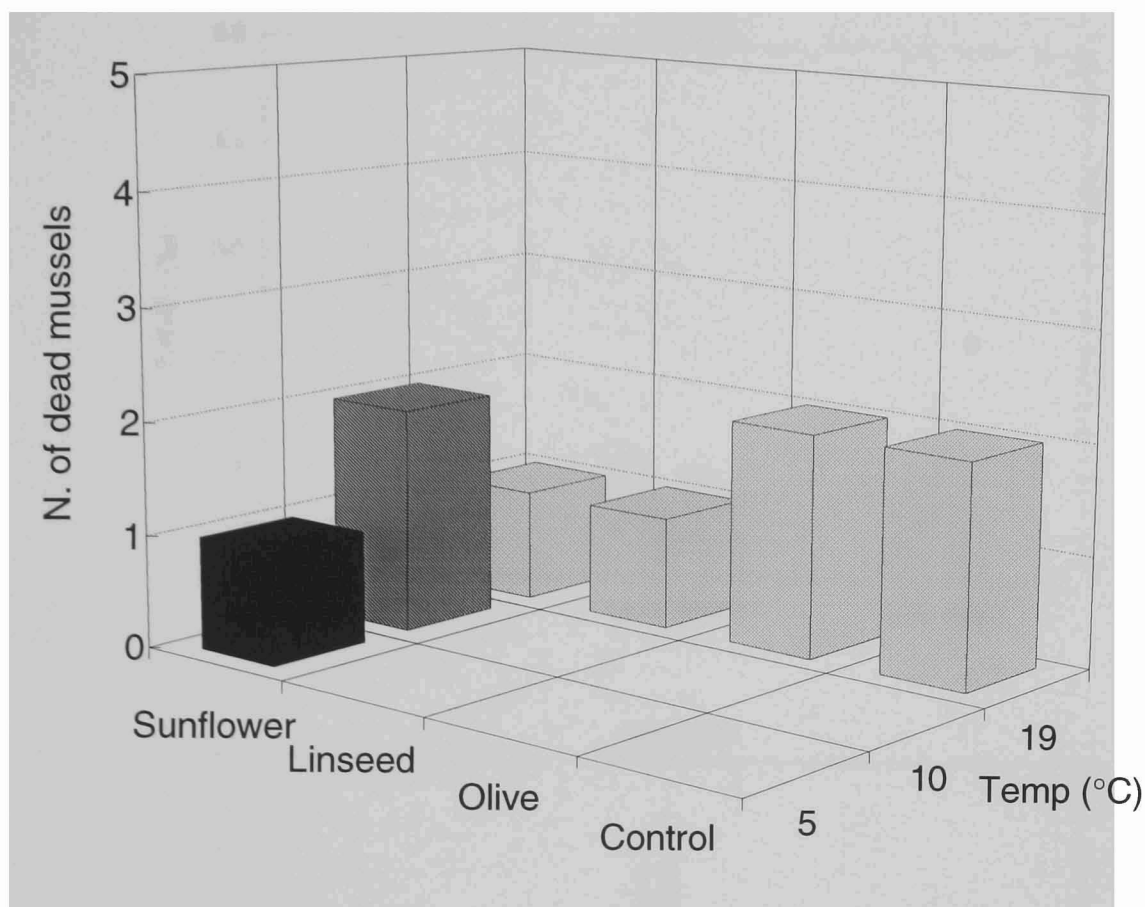


Figure 5.7: Mussel mortality as a function of vegetable oil and temperature exposure.

### 5.3.5 Fatty acids

#### Before temperature treatment

Fatty acid composition of mussels exposed to the vegetable oils treatment for 8 weeks and exposed to clean seawater for three days before the temperature treatment, is shown in Figures 5.8 and 5.9.

The control group had three major fatty acids making about half of the total fatty acids, 32% of palmitic, 11% of palmitoleic and 9% of 20:5 $\omega$ 3. The remaining 7 major fatty acids were distributed among the three groups, stearic and myristic in the saturates; oleic, vaccenic and gondoic in the monounsaturates and linoleic and 22:6 $\omega$ 3 in the polyunsaturates.

The fatty acid composition of the only one live mussel from the rapeseed oil treatment was characterized by a dominance of oleic acid (32%) which together with palmitic acid made 53% of the total fatty acid pool. Palmitoleic and linoleic acids were the next largest fatty acids with 9 and 7%, respectively. Linolenic acid was also present among the ten major fatty acids representing 4% and the polyunsats 20:5 $\omega$ 3 and 22:6 $\omega$ 3 did not exceed 4% of the total.

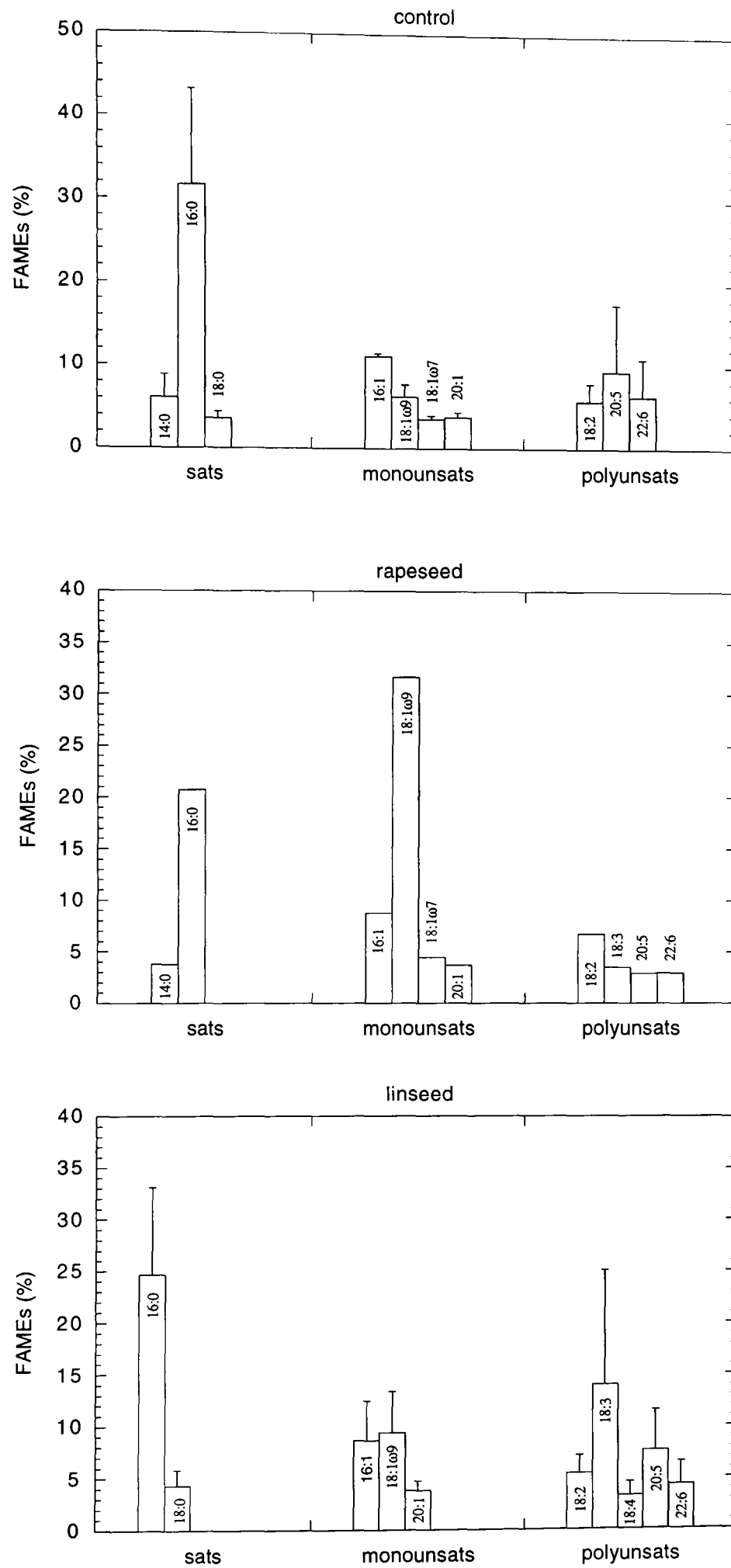


Figure 5.8: Fatty acid composition of mussels pre-exposed to different vegetable oils before being exposed to temperature treatments.

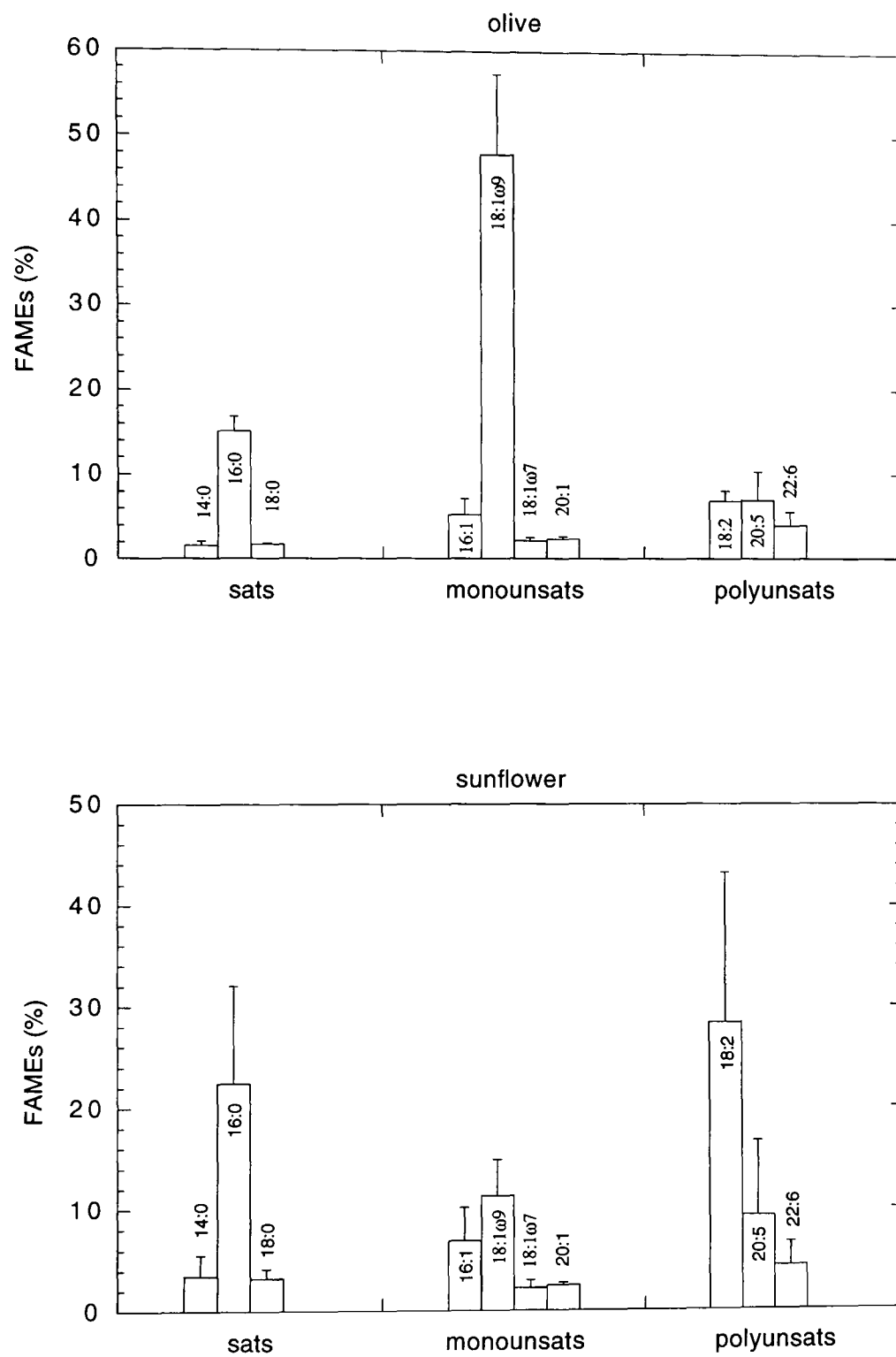


Figure 5.9: Fatty acid composition of mussels pre-exposed to different vegetable oils before being exposed to temperature treatments.

Mussels exposed to linseed oil had palmitic acid as the major fatty acid representing 25%, linolenic was the second with 14% and oleic was the third in the pool with 9%. These three fatty acids constituted 48% of the total fatty acid composition. From the other monounsaturates palmitoleic was the most concentrated with 9% and gondoic acid the least concentrated with less than 5%. The other saturated fatty acid present was stearic acid and the remaining polyunsaturates were rich in 18C and 20C. The long chain 20:5 $\omega$ 3 represented 8% and linoleic 6% the other 18C and 22C, 18:4 $\omega$ 3 and 22:6 $\omega$ 3, respectively, were lower than 5%.

Olive oil treatment led to a fatty acid composition in mussels distinguishable by high amounts of oleic acid (48%), almost half of the total fatty acid composition on its own and a much lower percentage of palmitic acid (15%). Myristic and stearic acids were the other saturates, palmitoleic, vaccenic and gondoic made the other monounsaturates, either of these smaller than 3%. The other polyunsaturates were constituted by 7% of linoleic and the same percentage of 20:5 $\omega$ 3 and 22:6 $\omega$ 3 was slightly smaller.

Mussels exposed to sunflower oil had linoleic and palmitic acids as the major fatty acids constituting 50% of the total pool. Oleic and 20:5 $\omega$ 3 were the next concentrated and the remaining fatty acids in the 3 groups were much smaller and constituted by myristic and stearic in the saturates; palmitoleic, vaccenic and gondoic in the monounsaturates and 22:6 $\omega$ 3 in the polyunsaturated fatty acids.

In summary, the mussels exposed to rapeseed, olive and sunflower oils after 3 days of oil decontamination in clean seawater, still showed the fatty acid markers of the respective vegetable oils. Rapeseed and olive oil groups had oleic acid as the major fatty acid and sunflower oil group had linoleic acid as the major one. The linseed oil group was the only exception being linolenic acid the second largest fatty acid rather than the first. Linolenic acid having a higher degree of unsaturation becomes more susceptible to oxidation than the other 18C. The control group showed a typical fatty acid composition constituted by the 3 main fatty acids, palmitic, palmitoleic and 20:5 $\omega$ 3, although the latter exhibited a smaller percentage than found before (see Chapter 4), which can be explained by the lack of food supplement.

### After temperature treatment

Fatty acid composition of mussels collected after eight weeks of exposure to the vegetable oils and after thirteen days in clean seawater at the same (10 °C) and two other different temperatures (5 and 19 °C) is shown in this section. The major ten fatty acid methyl esters from each oil group and temperature are shown in Figures 5.10 and 5.11.

The control mussels exhibited a similar fatty acid composition in any of the three temperatures, characterized by 22 to 26% of palmitic acid, the major saturated fatty acid; 11–13% of the polyunsaturated acid 20:5 $\omega$ 3 and 8–11% of the monounsaturated palmitoleic acid. These three fatty acids made up 48% of the total amount of fatty acids. Oleic acid came next with 10% and the long chain polyunsaturated 22:6 $\omega$ 3 was in fifth position with 6%. The other constituent fatty acids among the ten were myristic and stearic in the saturates, vaccenic and gondoic in the monounsaturates and a 18C and a 20C in the polyunsaturates. The 18C was either linoleic acid or 18:4 $\omega$ 3 and the 20C was 20:2 $\omega$ 6 or arachidonic acid. This fatty acid profile was similar to the one found previously in mussels (see Chapter 3), with the same high percentage of palmitic acid, followed by 20:5 $\omega$ 3, palmitoleic and oleic acids. Some qualitative departures from the mentioned characteristic composition of control mussels were noticed for the different temperatures: linoleic acid was replaced by 18:4 $\omega$ 3 at 5 °C and 20:2 $\omega$ 6 was replaced by 20:4 $\omega$ 6 at 19 °C.

The fatty acid composition of mussels exposed to linseed oil was different from the one described for control mussels and there are marked differences in composition for the three temperature treatments. Mussels exposed to 5 °C showed the largest amount of 16:0, which together with palmitoleic and oleic acids made 46% of the total fatty acid pool. Myristic and stearic acids completed the group of saturated acids; oleic and gondoic acids completed the monounsaturated group and the polyunsaturates were constituted by 20:5 $\omega$ 3, linolenic acid and 22:6 $\omega$ 3. In the 10 °C temperature oleic acid was the dominant fatty acid rather than palmitic acid in 5 °C, which occupied the second place. Linolenic acid was third with 17%. Stearic acid was the fourth major fatty acid followed by linoleic acid. Within the polyunsaturates linolenic acid was the predominant fatty acid although smaller than oleic acid. The other polyunsaturates

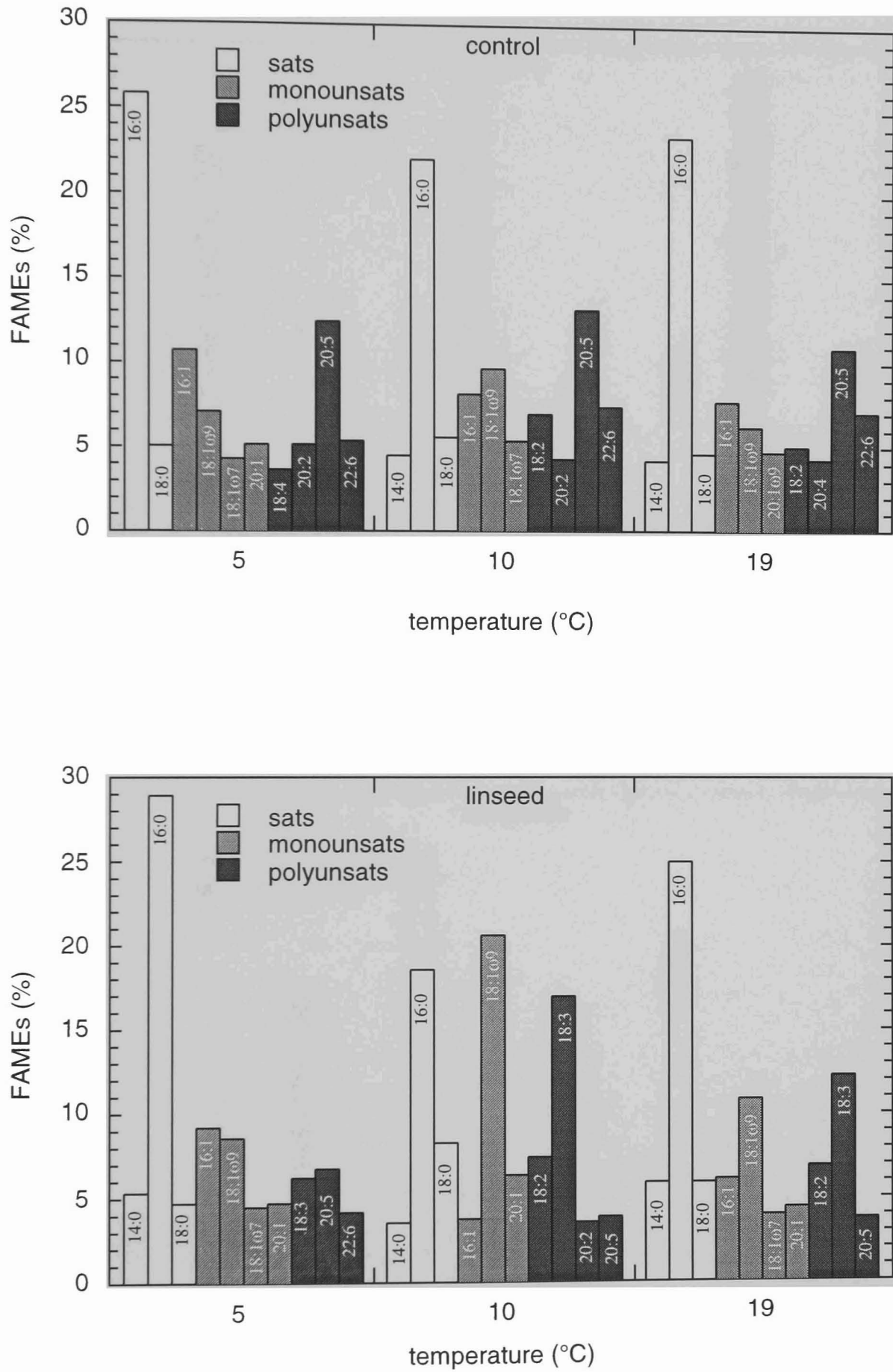


Figure 5.10: Fatty acid composition of mussels pre-exposed to different vegetable oils and submitted to three temperature treatments.



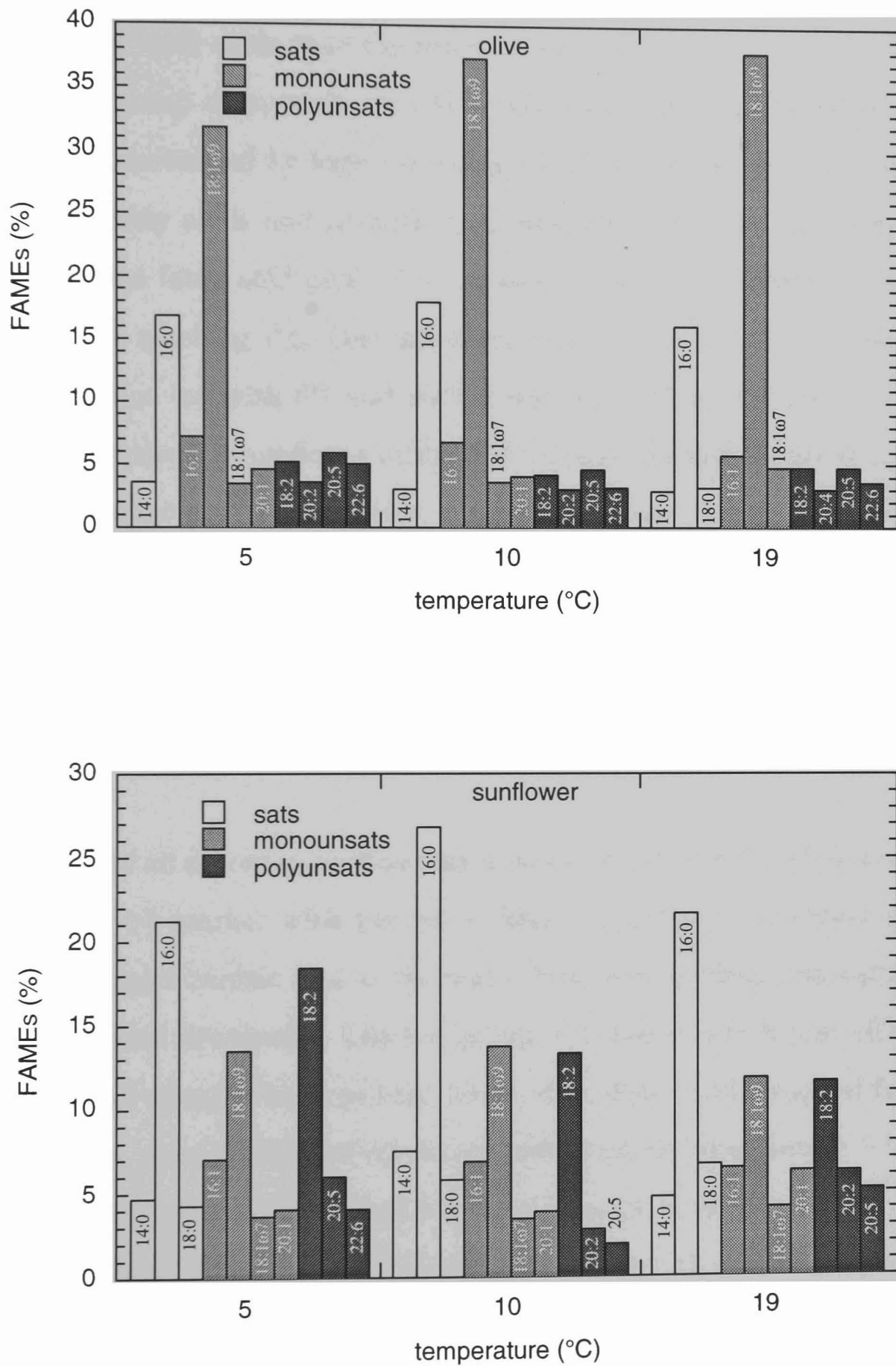


Figure 5.11: Fatty acid composition of mussels pre-exposed to different vegetable oils and submitted to three temperature treatments.

were 20:5 $\omega$ 3 and the dienoic 20C. In the highest temperature, mussels exhibited the same largest three fatty acids as in 10 °C but palmitic acid was the largest between the three, linolenic second and oleic third. The dienoic 20C was replaced by vaccenic acid and the remaining fatty acids were the same as for 10 °C.

The olive oil group of mussels showed a consistent fatty acid composition for all temperatures, characterized by high percentage of oleic acid which predominated with 32–38% of total fatty acids and palmitic acid with 16–18%. The two fatty acids constituted 53% of the fatty acid pool. The remaining fatty acids were much smaller in concentration, not reaching 7%. One difference was noticed for 19 °C: stearic acid was present in the major ten with 6% and 20:2 $\omega$ 6 was replaced by 20:4 $\omega$ 6.

Mussels pre-exposed to sunflower oil showed three major fatty acids at the 3 temperatures. palmitic, oleic and linoleic acids, constituting about 50% of the total. Palmitic acid dominated at all temperatures, linoleic was the second largest at 5 °C but was slightly smaller than oleic in the other two temperatures. The other saturates were myristic and stearic, the remaining monounsaturates were composed by palmitoleic, vaccenic and gondoic acids and the rest of the polyunsats were 20:5 $\omega$ 3 and 22:6 $\omega$ 3 or 20:2 $\omega$ 6.

After 13 days of oil decontamination only mussels pre-exposed to olive oil still showed oleic acid as the oil marker with persistent high percentages, sunflower pre-exposed mussels had no longer linoleic acid as the major fatty acid in their compositions and its percentage decreased drastically. Linseed group of mussels, which just after 3 days in clean seawater had already decreased the levels of linolenic acid, dropped further down its value after the next 10 days of oil decontamination at temperature 5 °C, dropped only slightly at temperature 19 °C and increased slightly at 10 °C.

Comparing mussel fatty acid profiles before and after the temperature treatment, some changes could be noticed. Linseed group of mussels was most sensitive to changes in temperature which were reflected in marked changes in fatty acid composition. The comparison was made for each temperature group and revealed at 5 °C a dramatic decrease of linolenic acid to half of the value obtained before the treatment and an increase of palmitic acid; at 10 °C oleic acid doubled its percentage, linolenic increased slightly and palmitic decreased. For the highest temperature the three major fatty

acids, palmitic linolenic and oleic kept their percentages essentially constant relative to before the treatment.

Mussels treated with olive oil also registered some changes in fatty acid composition after temperature exposure. Oleic acid decreased greatly from 48% to 32%, the lowest percentage of this acid among the temperatures treatment, whereas the other monounsats increased.

Sunflower oil group had increased percentages of palmitic and oleic acids with the temperatures and decreased percentages of linoleic acid.

The control group did not show big changes in fatty acid composition with different temperatures, slight variations occurred with palmitic acid and 20:5 $\omega$ 3. The former decreased with the temperature treatment and the latter increased, the other fatty acids remained basically unchanged.

### **Composition of dead mussels**

The results of fatty acid composition from dead mussels during vegetable oil exposure and temperature treatment are shown in Figures 5.12 to 5.13.

Figure 5.12 shows the fatty acids of mussels which died during the course of the exposure to linseed and sunflower oils. Mussels exposed to linseed oil which died after 2 weeks of exposure exhibited oleic, linoleic and palmitic acids as the main three fatty acids. Linolenic acid the major fatty acid in the oil was only on fourth place. On the third exposing week the dead mussels had a different fatty acid composition characterized by higher percentage of palmitic acid which became the major one and decreased percentages of linoleic and oleic fatty acids. Comparing these two sets of fatty acid composition with the correspondent fatty acids from neutral lipids on weeks 2 and 3 during the oils exposure (see Chapter 4), it is obvious the sharp decrease of linolenic acid in dead mussels in both weeks and the increase of linoleic and oleic acids in their turn.

The dead mussels exposed to sunflower oil, had palmitic acid as the more concentrated fatty acid with similar percentages for both weeks and linoleic acid as the second or third fatty acid depending on the week; oleic acid being the second fatty acid on the

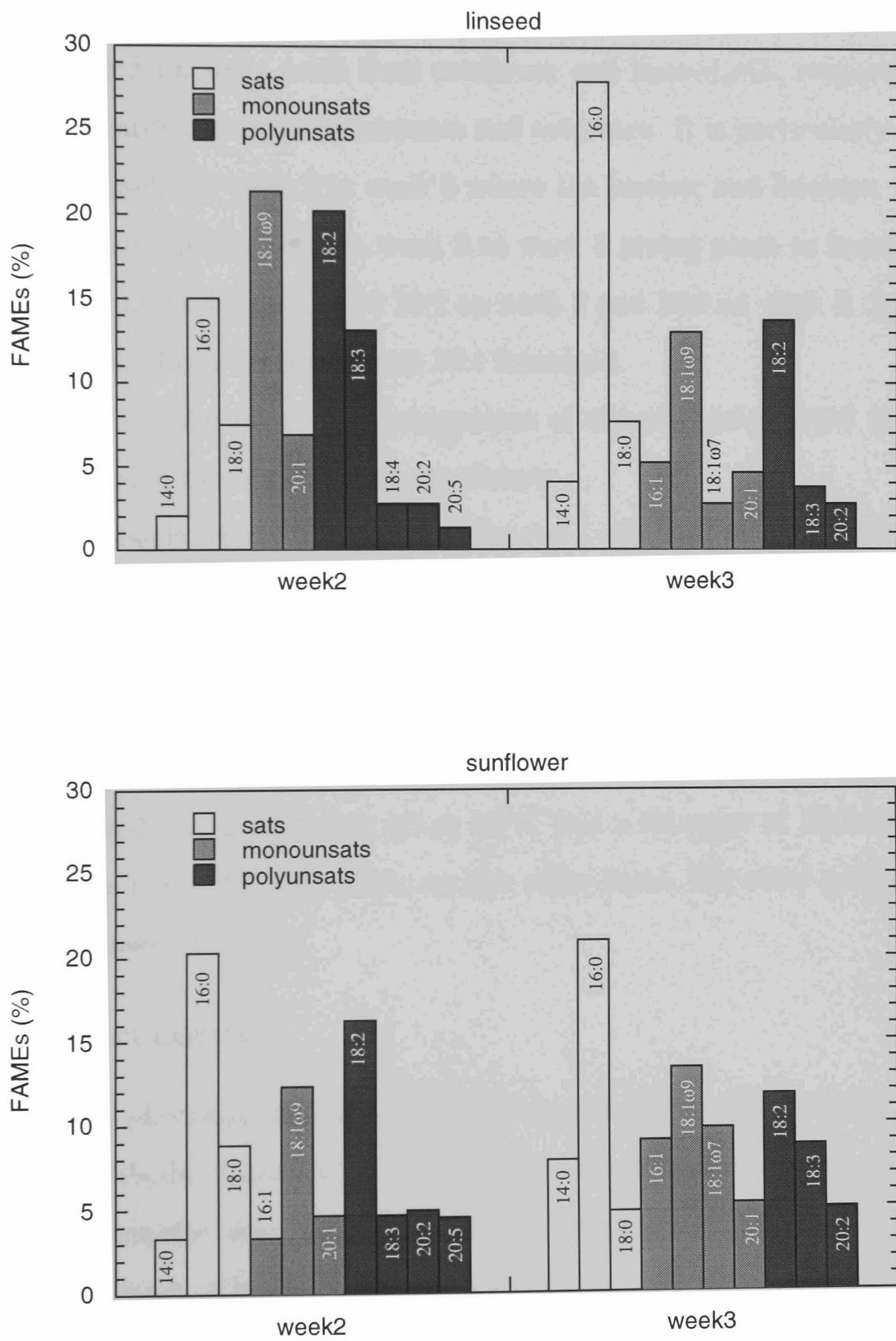


Figure 5.12: Fatty acid composition of dead mussels after vegetable oils exposure.

third week. The neutral lipids of living mussels exposed to sunflower oil for the same weeks were characterized by higher percentages of linoleic and oleic acids and lower percentages of palmitic acid.

These differences suggest that the high concentrations of the polyunsaturates from the oils, linoleic and linolenic acids from sunflower and linseed oils, respectively, were being oxidized towards the monounsaturates and saturates. It is particularly noticeable from the dead mussels on week 2 to week 3 where the linoleic and linolenic acids from each group decreased markedly from week 2 to week 3 giving place to increased levels of the next lower unsaturate, 18:2 and 18:1 on week 2 and 16:0 on week 3. In sunflower group, 18:2 and 18:1 both decreased and 16:1 increased.

Figure 5.13 shows the fatty acid composition of mussels pre-exposed to vegetable oils which died during the temperature treatments.

The control mussels which died at 19°C showed a decrease of palmitic acid and an increase of the 18C. The percentage of oleic acid doubled and the highly polyunsaturates were replaced by the dienoic 20C.

The linseed dead mussels at the same high temperature exhibited no linolenic acid and increased percentages of 18:2 and 20:2.

Mussels perished under sunflower oil at 10°C had a decrease of 18:2 and no signs of 16:0 among the major 10 fatty acids, on the other hand, the other polyunsaturates besides 18:2 increased markedly.

### 5.3.6 Shell records

Figure 5.14 shows photomicrographs of acetate peel of polished and etched radial sections of *M. edulis* shells. Figure 5.14 A and B show the appearance of the microgrowth patterns in the prismatic layer (PL) of the shell of a mussel from the control tank. Note the weak growth bands (Gb) particularly in Figure 5.14 B. Each of Figures 5.14 C-H represents one shell exposed to different vegetable oil treatments i.e. sunflower, linseed and olive oils, respectively. All the shells exposed to the oils show a disturbance to the normal pattern of calcium deposition at the point at which they were exposed to the oils (arrows). The microgrowth bands deposited during treatment with oil are more marked than those deposited prior to and after treatment (e.g. Figure 5.14 C). At least

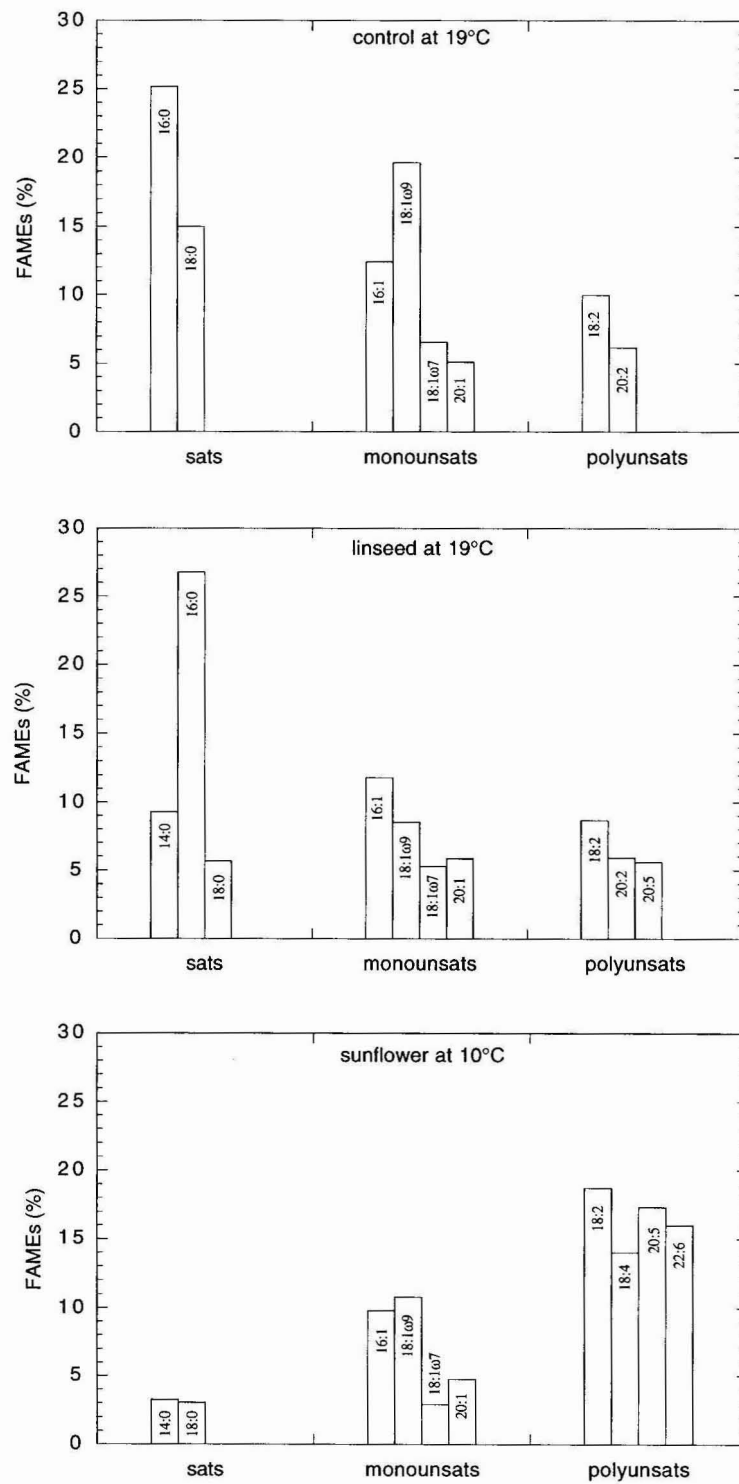
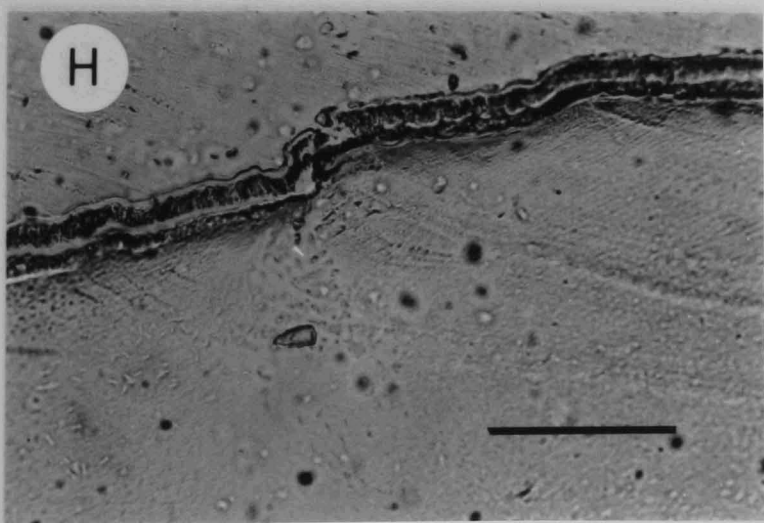
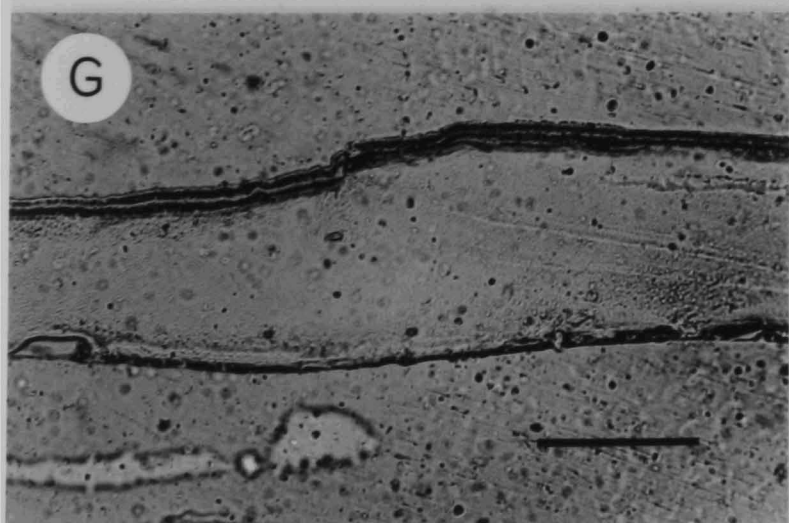
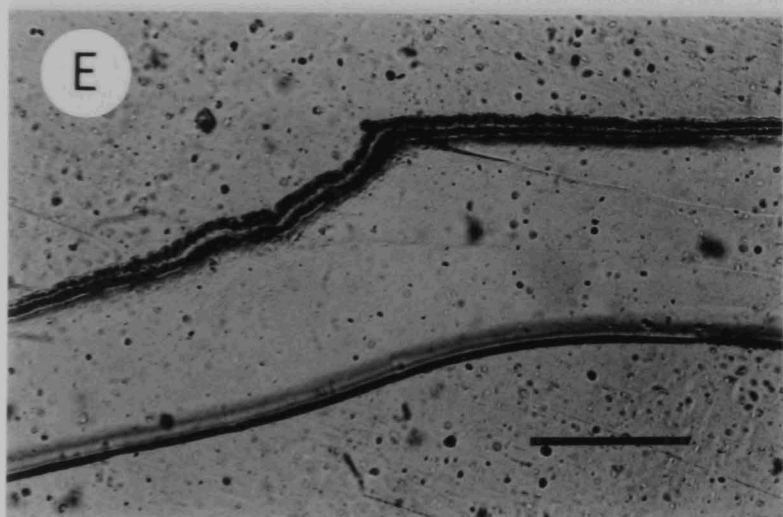
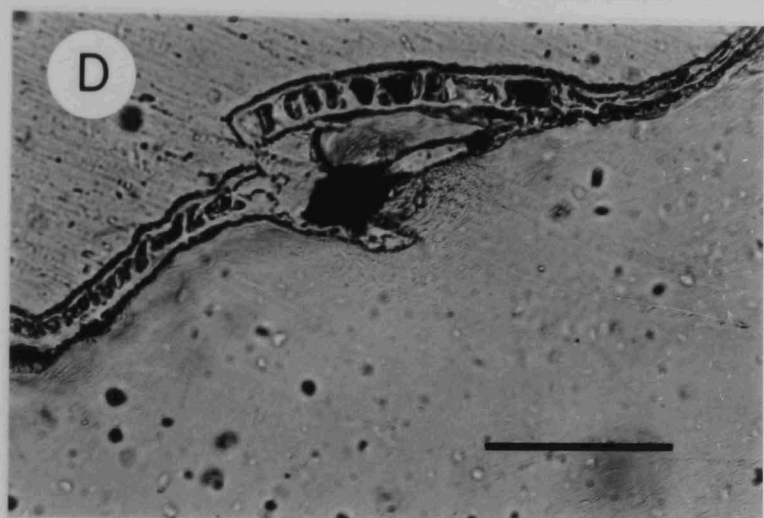
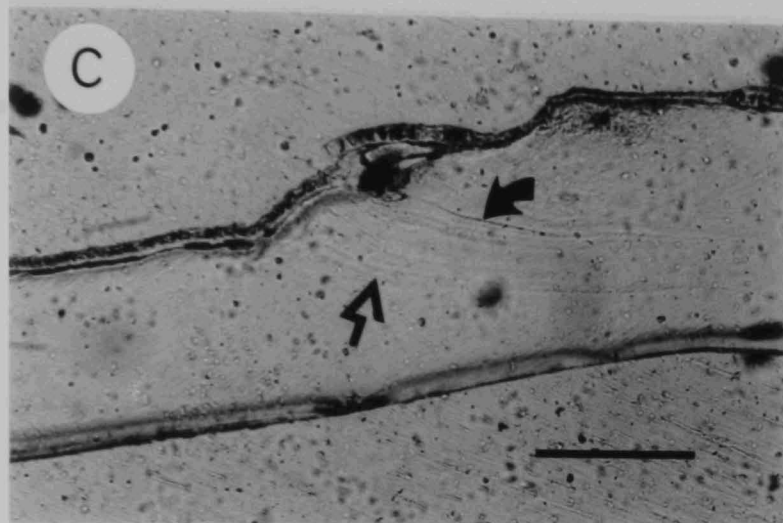
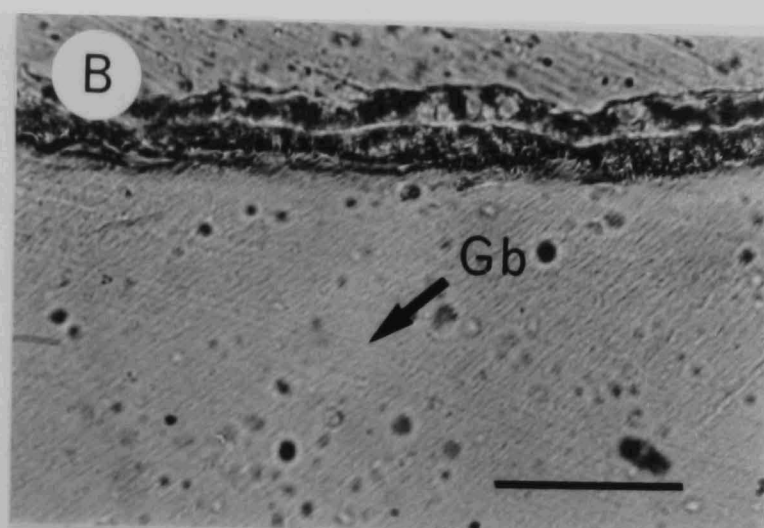
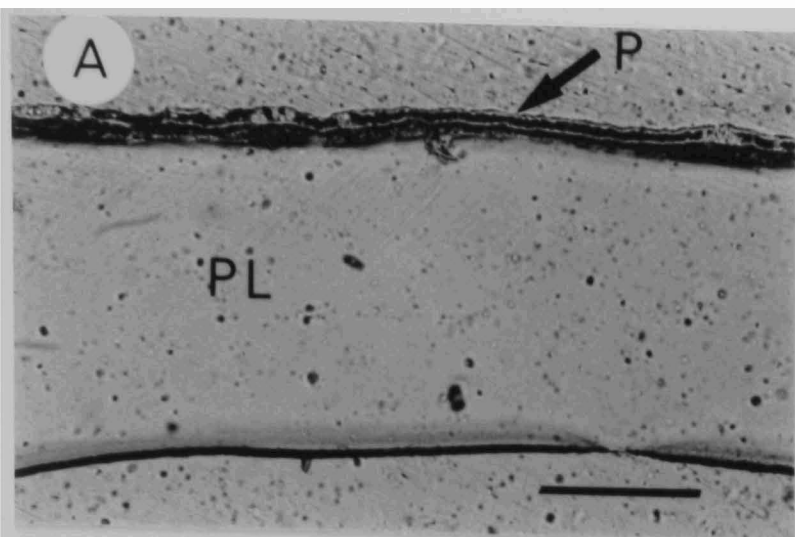


Figure 5.13: Fatty acid composition of mussels pre-exposed to vegetable oils dead during temperature treatments.

Figure 5.14: Photomicrographs of acetate peels of mussel *Mytilus edulis* shells. Scale bars=200  $\mu\text{m}$  (A, C, E, & G) and 100  $\mu\text{m}$  (B, D, F & H). A & B low and higher power appearance, respectively of a shell grown in control (seawater) conditions. P=periostracum; PL=prismatic layer; Gb=growth bands. C & D low and higher power appearance, respectively of a shell grown in sunflower oil. The marked growth bands (filled arrow in C) represent the point at which the sunflower oil was introduced to the mussel. The open arrow in C indicates the point at which the mussel was possibly no longer exposed to the oil. E & F low and higher power appearance, respectively of a shell grown in linseed oil. G & H low and higher power appearance, respectively of a shell grown in olive oil.







two clearly marked bands can be seen in each shell exposed to the oil treatment, possibly indicating the start and finish of exposure to the oil. Although the shells were not intentionally marked before and after oil exposure, it can not be concluded undoubtedly that the cause of this disturbance was vegetable oil exposure. However, the similarity of the pattern of microgrowth bands in the shells exposed to the oils and the interruption and in some cases breaks in the periostracum approximately at the same distance from the growing edge of the shell, which are not seen in the control mussels, indicates that oils were the likely cause for the interrupted calcium deposition.

## 5.4 Discussion

Vegetable oils are hydrophobic and their density is lower than water. Because of these characteristics, they tend to stay on the water surface or to be adsorbed onto solid surfaces with which they come into contact. Low flow rates and low water temperatures enhance these properties resulting in the accumulation of polymerized oil. Once the polymer is formed it prevents the normal water flow and oxygenation of the water between the air-water interface. The recorded high mortality of mussels exposed to all of the oils in the bowls was probably due to these phenomena. The flow rates were lower than in the growth tanks and the capacity of the bowls was much smaller than the tanks concentrating the amount of oils in the bowls. The temperatures were low, between 8 and 10°C. In addition, the task of removing the daily accumulation of oils from the bowl's water surface was interrupted for one week between the fifth and sixth weeks of the experiment, coinciding with high mortality. These results show how detrimental vegetable oils can be in certain conditions of low water renewal and oil concentration, producing high mortality among mussels. A situation like this is likely to occur in the wild for instance in pools left by the low tide, where there is usually a rich and diverse fauna. In the growth tanks also some mortality was observed, not as high because of the more unfavourable conditions for oil to accumulate in the tanks, but in this case linseed oil was the most detrimental in terms of mussel mortality not rapeseed. Although linseed oil flow rate was the highest of the four oils, which may have been the cause of high mortality in mussels in the growth tanks, it did not produce the highest mortality

of mussels in the bowls. Estimation of the amount of oil remaining in the bowls was not possible because it was unknown how much of the oil was actually removed with the flow through system, how much stayed adsorbed to the bowl's walls and how much was uptaken by the mussels. Water and oil flow rates were different for each experimental bowl and this also makes it difficult to predict whether the main cause of mortality by the oils was only their concentration or some other oil feature related with its chemical composition. Furthermore, the internal shell surfaces of mussels treated with oils, were chalky in appearance when compared to the iridescent lustre of the control mussels. This alteration of the pearly appearance is thought to be due to calcium dissolution due to acidic conditions in consequence of long periods of anoxia (Crenshaw, 1980). The vegetable oils had promoted poor oxygenation and eventually death among mussels by anoxia. However the deaths recorded in the growth tanks during exposure to the oils could not have been caused by anoxia since the water was aerated. In contrast to the mussels in the bowls, mussels in the tanks with aeration showed a normal pearly appearance in the internal shells, confirming that oxygen deprivation was not the likely cause of death.

Vegetable oil treatment did not have a long term effect on mussel response to open their valves when transferred to clean seawater. The differences in valve opening time observed were not statistically significant between the oil groups. However, sunflower oil had a long term effect on gaping time of mussels when they were transferred to clean seawater of decreasing salinities. The gaping time was longer for mussels pre-exposed to sunflower oil than for the control mussels suggesting a necessity for oil depuration or to compensate any isolation periods due to the presence of oils when they could not feed. Salinities 25 ‰, 20 ‰ and 15 ‰ did not interfere with either closing times or gaping times, only salinity 10 ‰ was responsible for 70-100% of mussel valve closure according to the oil group and for a shorter isolation time. This result is in agreement with previous data published by Davenport (1979) determining the salinity ( $10.7 \pm 2.9$  ‰) at which complete valve adduction is induced.

Vegetable oils did not have a prolonged effect on mussel mortality when transferred to clean seawater. Mortality in the control group was similar to the mortality registered in the oil groups. However, temperature had a significant effect on mussel mortality.

The temperature 19°C caused the highest mortality, even in the control mussels, indicating that mussels died due to the thermal shock rather than by pre-exposure to the oils. All the mussels were in tanks with water temperatures of 8°C to 10°C before being transferred to the different temperature treatments, a 3 to 5°C difference was not enough to cause high mortalities in tanks at 5°C and 10°C, respectively but a 10°C difference was certainly the cause for increased mortality in any group of mussels in tank 3 at 19°C.

Fatty acid composition of mussels pre-exposed to the vegetable oils and analysed after 3 days of oil decontamination, before being transferred to the temperature treatments, revealed some differences to the fatty acid composition found during oil exposure (see Chapter 4). The control group showed an increase of palmitic acid and a decrease of 20:5 $\omega$ 3. Mussels had been maintained for some weeks without food supplement which had caused a decrease of the long polyunsaturates provided by phytoplankton. In consequence of this decrease palmitic acid increased. In the groups treated with rapeseed, olive and sunflower oils, oleic acid for the first two oils and linoleic acid for the latter, were still the major fatty acids although with lower levels than during oil exposure. These mussels kept the oil's marker fatty acids after 3 days of oil decontamination. Mussels pre-exposed to linseed oil however, did not show the oil marker (linolenic acid) as the dominant fatty acid in their tissues after the same time of oil decontamination. Linolenic acid is a more unstable fatty acid susceptible to oxidation due to its higher degree of unsaturation, therefore it was the first fatty acid to decrease in clean seawater. Linoleic acid and in particular oleic acid, both seemed to be resistant to extensive oxidation because they are lower unsaturated fatty acids. These observations also suggest that there was fatty acid metabolism going on from the largest fatty acids available towards the saturated fatty acid palmitic acid.

After 13 days of oil decontamination in different temperatures, oleic acid in mussels exposed to olive oil was still showing similar percentages as after the 3 days decontamination revealing a great resistance to oxidation and no influence of the temperatures on the fatty acid profiles. Linoleic acid from mussels exposed to sunflower oil, on the other hand decreased in all temperatures but especially on the two highest, increasing oleic and palmitic acids. Linoleic acid possessing 2 double bonds seemed to be the next to

linolenic, more susceptible fatty acid to oxidation among the three fatty acid oil markers (linolenic, linoleic and oleic acids) and higher temperatures seemed to accelerate the process. Fatty acid composition of mussels exposed to linseed oil varied with the temperature and the pattern seem to reflect different rates of metabolic transformation of fatty acids. At 5 °C, palmitic acid rose to high percentages only found in the control group and all the other fatty acids were low, suggesting oxidation of linolenic acid to a great extent but at 10 °C oleic acid dominated the fatty acids and linolenic acid was the third fatty acid with a relatively high percentage. Mussels at this intermediate temperature were probably metabolizing linolenic acid into other fatty acids of lower degree of unsaturation, but still showing high percentages of oleic and linolenic acids, indicating an intermediate metabolic rate. At the highest temperature the metabolic process must have been accelerated because linolenic and oleic acids were much lower than in mussels at 10 °C. A similar pattern of oil decontamination of linseed oil seemed to have occurred at the extreme temperatures and a slower rate of decontamination at 10 °C must be responsible for the higher percentages of oleic and linolenic acids.

Fatty acid composition of dead mussels after vegetable oil and temperature exposure changed notably from either the composition obtained during oil exposure and from the one before the temperature treatment. The results suggest that either 10 °C or 19 °C seem to stimulate fatty acid metabolism, the direction of which is probably dependent on the most concentrated fatty acid available. If it is a saturated fatty acid then the metabolic direction is towards the more unsaturated fatty acids: 16:0 → 18:0 → 18:1 → 18:2 → 20:2, if it is a polyunsaturate fatty acid the direction changes towards the less unsaturated fatty acids increasing linoleic, oleic and palmitoleic: 18:3 → 18:2 → 18:1 → 16:1.

## 5.5 Summary

It was confirmed that oils can cause high mortalities especially if conditions of poor oxygenation occur. The vegetable oils olive and linseed produced an effect on the gaping response of mussels, delaying the time of opening valves, however this effect was reversible once mussels were transferred to clean seawater. A long term effect of the oils

was noticed on the gaping time of mussels, since they spent more time gaping in clean seawater, suggesting a necessity for depuration or feed compensation. The salinity at which most of the mussels adducted their valves was 10 ‰ result not considered affected by oil exposure.

Temperature, rather than oil treatment was determinant on mussel mortality, the highest temperature being the cause of mussel mortality in all the oil treatments.

Fatty acid composition of mussels transferred to clean seawater still showed signs of oil contamination but active oxidative metabolism has occurred because the dominant fatty acids in the oils decreased in all cases. Temperature had some influence in this process accelerating or delaying the decrease of fatty acids oil markers. Oleic acid, nevertheless remained high showing resistance to oxidation. Mussels that died during the oil exposure showed, in general, decreased levels of the fatty acids typical in the oils, but still identifiable fatty acid markers. The decrease was attributed to oxidation by bacterial degradation, rather than catabolic processes prior to death. Unlike these dead mussels, the ones that died after some period of depuration, presented signs of active metabolism prior to death, dependent on temperature, as indicated by the low levels of oil marker fatty acids and in the case of sunflower oil increased percentages of long chain polyunsaturates.

Acetate peels of the the radial sections of *M. edulis* shells revealed an altered pattern of microgrowth band deposition in all the shells exposed to the oils which was not seen in the control shells. The two most distinct growth bands in the shells exposed to the oils possibly indicate the beginning and end of oil exposure. Between these two marks, the periostracum was often interrupted indicating the presence of the oil stressor. The control shells on the other hand showed a constant pattern of calcium deposition with no interruptions.

# Chapter 6

## Vegetable oil metabolism

### 6.1 Introduction

It is known from previous studies (Salgado, 1992; Mudge *et al.*, 1993) and it has been demonstrated in Chapter 4 that when mussels are exposed to rapeseed, linseed, olive and sunflower oils, their fatty acid composition reflects the composition of the oils, indicated by an increase of the major fatty acids present in the oils. During the eight weeks of oil exposure, there were no signs of metabolic reactions leading to the conversion of the increased fatty acids into longer chains or more unsaturated fatty acids or to shorter saturated chains required for their own maintenance, benefiting in some way from oil contact.

Acetyl-CoA carboxylase and fatty acid synthetase are enzyme systems responsible for lipid biosynthesis (Gurr and Harwood, 1991; Kluitmans and Zandee, 1973) and an increase in their activity or their products is an indication of fatty acid metabolism. The balance of the energetic reserves in the blue mussels in different stages of their lives was studied by different authors (Dare and Edwards, 1975; Pieters *et al.*, 1979) and led Kluitmans *et al.* (1985) to conclude that dietary lipids are largely catabolized, while glycogen and proteins are stored. Investigations on lipidic enzymatic activity (Kluitmans and Zandee, 1973) revealed a significant increase of acetyl-CoA carboxylase and fatty acid synthetase during pre-spawning period in female mantles of *Mytilus edulis* but only slight activity in male mantles and virtual absence of activity in other periods of their life. The authors concluded that there must be an adequate balance between supply and breakdown of lipids to justify the inactivity of the biosynthetic enzymes in

the tissues of the blue mussel. Increased enzymatic activity was only detected when intense biosynthesis was required for gametogenesis.

It is important to investigate whether the enhanced concentrations of fatty acids in mussel tissues, caused by the oil exposure can trigger their metabolism. Mussel capability to elongate pre-existing fatty acids has been discussed (see Chapter 2), nevertheless, the route or extent of biochemical transformations that sunflower oil and linseed oil accumulated in mussel tissues can go through is not known. It can either be an anabolic process in which the excess of fatty acids are metabolized into others required for mussel maintenance, or a catabolic process where the metabolic pathway is rather fatty acid breakdown and excretion. To accomplish this objective, mussel mantle extracts were incubated *in vitro* with and without two vegetable oils, sunflower and linseed. The fatty acid composition of the mussel extracts was monitored during the incubation.

## 6.2 Materials and methods

### 6.2.1 Mussel sampling

Thirty mussels with a mean size of  $5.6 \pm 0.3$  cm were collected from the midshore at Beaumaris, Anglesey at low tide and brought into the laboratory where they were washed with seawater and distributed amongst tanks of running seawater at  $13.7^\circ\text{C}$  with a flowrate of  $300 \text{ ml} \cdot \text{min}^{-1}$  and aerated. Mussels were kept for 1 day without food before the beginning of experiment.

Another set of 30 mussels were collected at the same place, later in the year and brought into the laboratory where they received a similar treatment as for metabolism experiments with sunflower oil. They were washed with seawater and distributed amongst tanks with running seawater with a flowrate of  $300 \text{ ml} \cdot \text{min}^{-1}$  and aerated. The water temperature *in situ* was  $3.9^\circ\text{C}$  and in the laboratory was  $7.1^\circ\text{C}$ . The mussels were left in the tanks for 4 hours, without food, before the beginning of experiment.

### 6.2.2 Mussel extracts preparation

Mussel shells were opened by cutting the adductor muscle and mantles were excized from the rest of the mussel tissue on ice. A total amount of 43.72 g of mussel mantles were obtained and kept in the fridge for no more than one night until further processing. The mantles were split into three subsamples reweighed and homogenized separately in a homogenizer with 20 ml of Tris-HCl buffer solution, 25 mM. The homogenates were then centrifuged at 20000 g for 30 minutes, the supernatants recovered and centrifuged again at 100000 g for another 30 minutes. For centrifugation, weights were adjusted with buffer solution when needed. The final supernatant was recovered and kept in the freezer at  $-20^{\circ}\text{C}$  until incubation. The buffer solution was prepared from 50 ml of Tris ([hydroxymethyl]aminomethane) 0.1 M and 29.2 ml of HCl 0.1 M and the volume adjusted to 100 ml. A solution of EDTA, 1 mM, was also prepared to be added at the final Tris solution. It was necessary to adjust the pH with NaOH to pH= 7.43.

The extracts from the second group of mussels were treated in a similar way to the previous group. The shells were measured and the 67.20 g of extract was split into 8 subsamples for easier homogenization. These were homogenized at  $4^{\circ}\text{C}$  in a homogenizer with 20 ml of 25 mM Tris buffer solution adjusted to pH= 7.98 with HCl 0.1 M and 1 mM EDTA (ethylene diaminetetracetic acid) was added to the solution. This buffer solution should ensure all ionic conditions for enzyme activity (Kluytmans *et al.*, 1985; Livingstone, 1981). Following homogenization, the tissues were centrifuged at 50000 g ← for 30 min; the cell fragments were discarded and the supernatants centrifuged again at 100000 g for another 30 minutes. The final supernatant was recovered and distributed equally among 6 test vials.

### 6.2.3 Incubation and sampling

#### Sunflower oil incubation

The mantle extracts were removed from the freezer and allowed to defrost. Ten millilitres of mantle extract were transferred to 5 test vials to which 10 ml of Tris-HCl buffer solution were added. A sixth vial had the remaining 8 ml of mantle extract and the same volume of buffer solution. Half of a millilitre of sunflower oil was delivered into 3



of the 6 test vials. A seventh vial was used as a blank and had 20.5 ml of buffer solution. All the 7 vials were vortexed mixed, covered with aluminium foil and incubated in a running seawater tank at 11.6 °C to simulate natural seawater temperatures. Samples of 3 ml from each vial were collected after 0, 1, 3, 6, 24 and 48 hours of incubation and analysed for fatty acids.

### **Linseed oil incubation**

Twenty seven millilitres of mantle extract were transferred to 6 test vials and 10 ml of Tris-HCl solution added to each vial. A volume of 185  $\mu$ l of linseed oil was delivered to three of the six test vials and a seventh vial was a blank having 37.2 ml of Tris buffer solution. All the 7 vials were vortexed mixed, covered with aluminium foil and incubated in a running seawater tank at 8.0 °C. Samples of 6 ml were taken from each vial at the beginning of incubation, time 0 and after 1, 3, 6, 24 and 48 hours and analysed for fatty acids. The water temperature was recorded at each sampling time.

### **6.2.4 Fatty acid analysis**

The procedure used for analysis of fatty acids was the same for incubation with both sunflower and linseed oils and is detailed below. Each sample from each test vial was mixed with 10 ml of a chloroform-methanol solution (2:1, v/v) with the antioxidant BHT (2,6-di-tert-butyl-p-cresol). A known amount (0.488 mg) of the trygliceride [*cis*]-15-trinervonin (24:1) and 0.4506 mg of the phospholipid phosphatidylcholine, dibehenoyl (22:0) were added as yield monitors. The samples were then vortexed mixed and left in the fridge for 30 min to extract the lipids. The other stages of extraction proceeded in the same way as described in Chapter 3. After extraction the samples were filtered through a Whatman paper filter n.4, 0.2 vol. of  $MgCl_2$  0.017% were added and samples shaken. This step should removes the salts from the lipidic phase. Centrifugation at 2000 rpm for 5 min cleared the two phases. The supernatant was discarded and the lipids washed with Folch upper phase reagent made with 96 ml of Milli-Q water, 94 ml of methanol and 6 ml of chloroform. The lipids were then evaporated to dryness in the rotary evaporator, transferred to a pre-weighed vial and weighed. According to their weight, they were dissolved in an appropriate volume of chloroform to obtain a

concentration of no more than  $1 \text{ mg} \cdot \text{ml}^{-1}$  and methylated using trimethyl sulfonium hydroxide (TMSH). Before analysis  $25 \mu\text{l}$  of tricosanoic fatty acid methyl ester (C23:0)  $0.492 \text{ mg} \cdot \text{ml}^{-1}$  solution was added as an internal standard.

The analyses were completed in a gas chromatograph mass spectrometer, Finnigan Mat 4600, equipped with a BPX-70 capillary column and operation conditions as described in Chapter 3. Gas chromatography was also used for samples incubated with and without linseed oil when the results from sunflower oil incubation proved unsatisfactory in terms of standard deviations from the mean of the replicates. The gas chromatograph used was a Carlo Erba 8160 with a Carbowax 20M capillary column, 30 m long, 0.32 mm of internal diameter and  $0.25 \mu\text{m}$  of film thickness. The operating conditions were as described in Chapter 3.

Identification of the fatty acid methyl esters (FAMES) was done by comparison with standards and quantification was obtained from calibration with the FAME standard solutions.

### Calibration

Standard solutions with the following FAMES 16:0, 17:0, 18:0, 18:1 $\omega$ 9, 18:2 $\omega$ 6, 18:3 $\omega$ 3, 20:0, 20:1 $\omega$ 9, 20:2 $\omega$ 6, 20:3 $\omega$ 6, 20:4 $\omega$ 6, 22:0, 22:6 $\omega$ 3 and 24:1 $\omega$ 9 were used to calibrate both instruments, the GC-MS and the GC. A constant concentration of 23:0 ( $49.2 \mu\text{g} \cdot \text{ml}^{-1}$ ) was added as the internal standard to each of the solutions.

Two calibrations were done, one for each instrument used. In the GC-MS a calibration test was run with 4 different concentrations of the standards: 12.5, 25, 50 and  $75 \mu\text{g} \cdot \text{ml}^{-1}$  each FAME; in the GC the standards covered a wider range of concentrations to make sure the errors between replicated samples were minimized, especially at the lower concentration limits. The standards were 0.5, 2.5, 5, 10, 20, 50, 100, 120, 150, 160 and  $170 \mu\text{g} \cdot \text{ml}^{-1}$ .

The straight lines, for both calibrations, obtained by linear regression analysis between chromatographic area of each FAME and its known concentration in the standard solutions were used to calculate the amount of each FAME in the samples. FAMES in the samples which were not present in the calibration standards were calculated from

the nearest calibrated FAME, as detailed in Chapter 3. The calibration for the GC with 10 standard dilutions resulted in good correlation coefficients, whereas in the calibration for the GC-MS the 4 dilutions used produced lower correlation coefficients. The GC also showed a much more reliable response relative to the GC-MS in terms of reproducibility of chromatographic areas which contributed to the better results with smaller errors in calculating the concentrations of fatty acid methyl esters in the samples.

## 6.3 Results

In this section, the length of mussels, incubation temperatures and chemical analysis are shown. Fatty acid composition of mussel extracts are compared between the two experiments. The size frequency distribution of the two sets of mussels used in the experiments are shown in Figures 6.1 and 6.2. The water temperatures during incubation are also presented in Figure 6.3.

Mussels incubated with sunflower oil had a mean length and standard deviation of  $5.6 \pm 0.3$  cm and the mussels incubated linseed oil had a slightly longer mean length at  $5.9 \pm 0.4$  cm. The water temperatures were very different between the two experiments because they were performed in different times of the year, the incubation of mussel mantle extracts with sunflower oil occurred during the autumn whereas the incubation with linseed oil was during the winter. The variation in temperatures was not big especially if we consider that there was no temperature control in the tanks. The average temperature and standard deviation for the incubation with sunflower oil was  $11.6 \pm 0.2$  °C and for the incubation with linseed oil was  $8.0 \pm 0.2$  °C. There was a bigger water temperature variation when the mussels were brought into the laboratory and when the experiment started 8 days later from  $13.7$  °C to  $11.6$  °C.

### 6.3.1 Lipid extracts

The total weight of lipids extracted from each of the vials at different incubation times is shown in Figures 6.4 and 6.5. Variation in the lipid weights between replicates was noticed and the main cause for that was the difficulty in homogenizing the sample before collection. All the samples were vortexed before an aliquot was removed for analysis

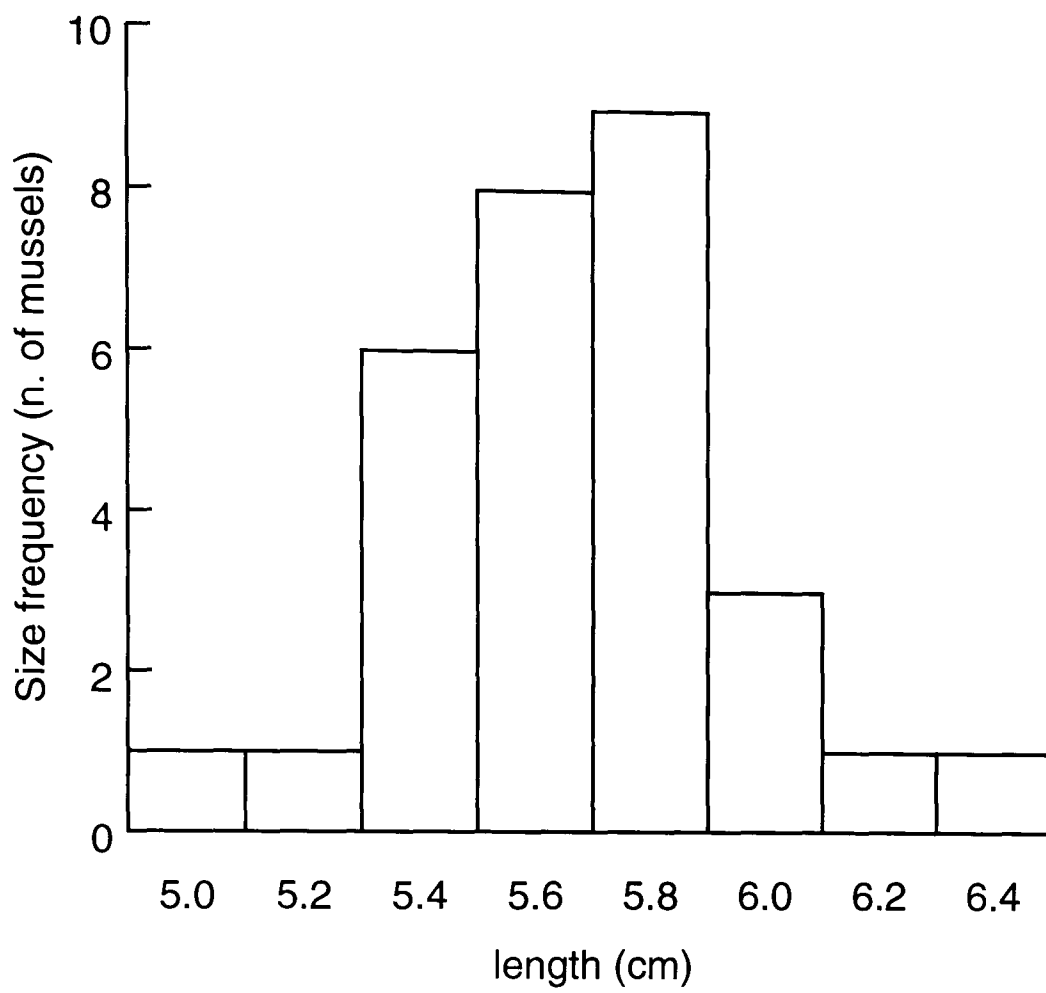


Figure 6.1: Length frequency distribution of mussels collected from Beaumaris to be incubated with sunflower oil.

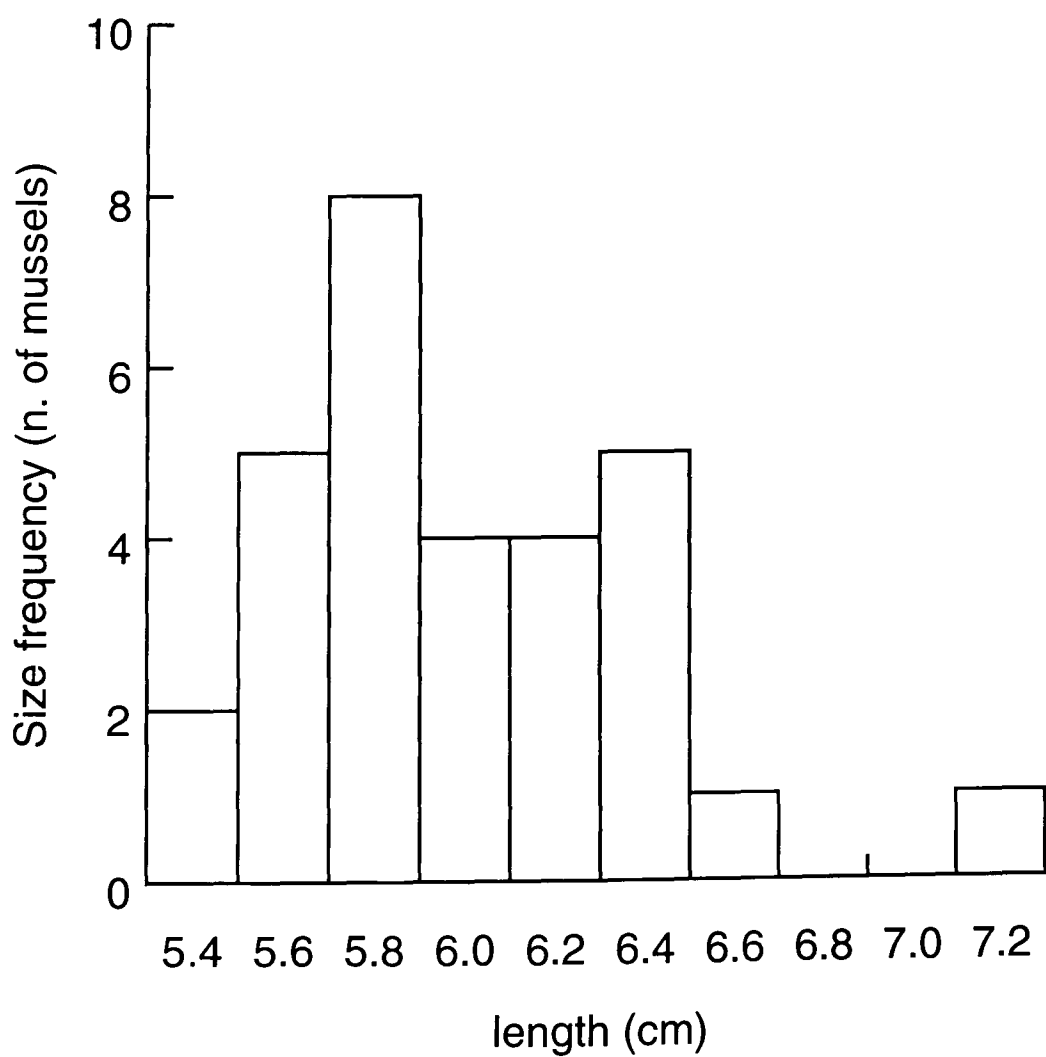


Figure 6.2: Length frequency distribution of mussels collected from Beaumaris to be incubated with linseed oil.

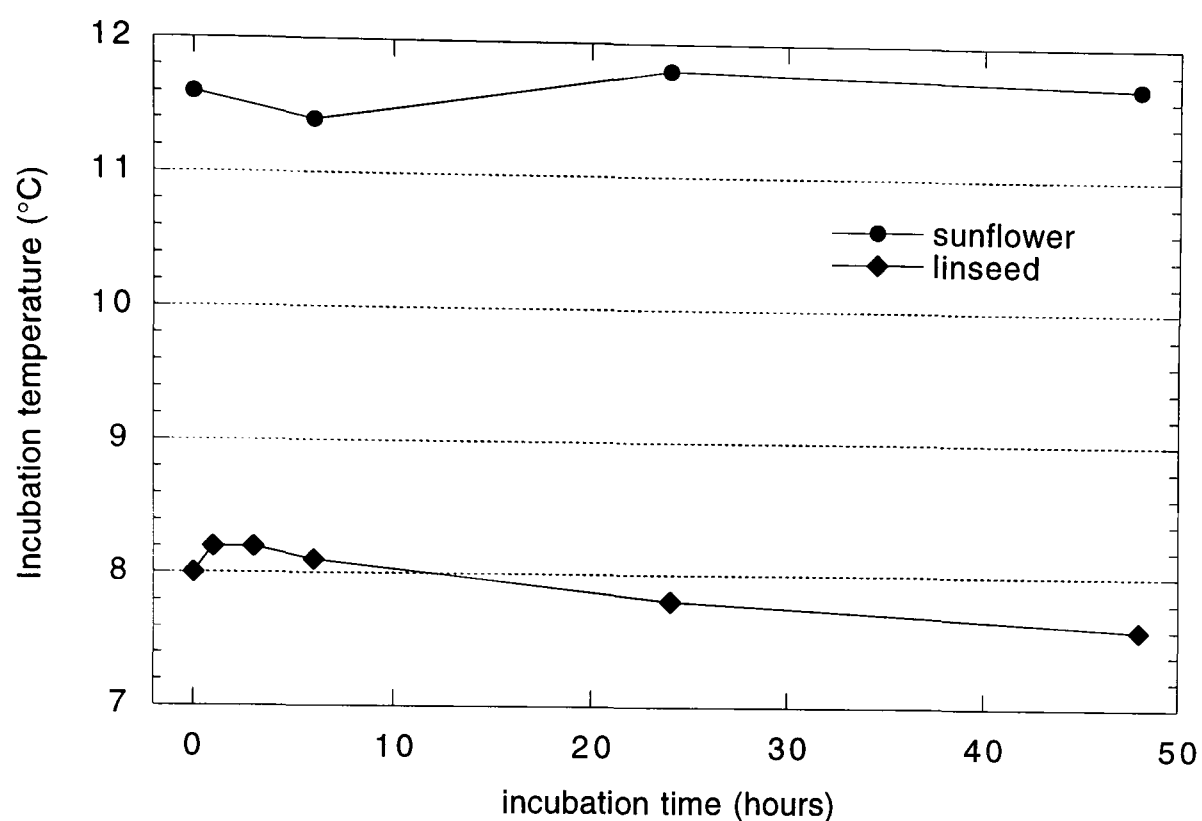


Figure 6.3: Incubation temperatures of mussel mantles with sunflower and linseed oils.

but even so due to the unsolubility of the oil in the aqueous medium a homogeneous sample could not always be obtained.

The amount of lipids extracted from the samples incubated with the vegetable oils (Figure 6.4) was about twenty and ten times larger for sunflower and linseed oils, respectively, than the correspondent extracts incubated just with buffer solution (Figure 6.5). Incubation with sunflower oil produced larger amounts of lipids than the incubation with linseed oil because the initial amount of sunflower oil applied was 5 times larger than the amount of linseed oil. The weight of lipids varied during the incubation time in a similar way in both experiments with the different oils. There was an increase in lipids after 1 hour of incubation time from 13 to 44 mg and from 10 to 35 mg that decreased after 3 hours to 13 and 22 mg and tended to a mean value around 28 mg and 23 mg for sunflower and linseed oils, respectively. The incubation with sunflower oil showed a high amount of lipids after 48 hours which seemed abnormal, investigations on the extraction of these samples revealed that they had been filtered with a Whatman paper filter no.1 instead of the usual no.4. The previous filter retained more the filtrate and the presence of the oil must have been enhanced. In the corresponding samples incubated without sunflower oil that were also filtered with the same filter, the amount of lipids was similar to the concentration at 24 hours but in this case there was no oil

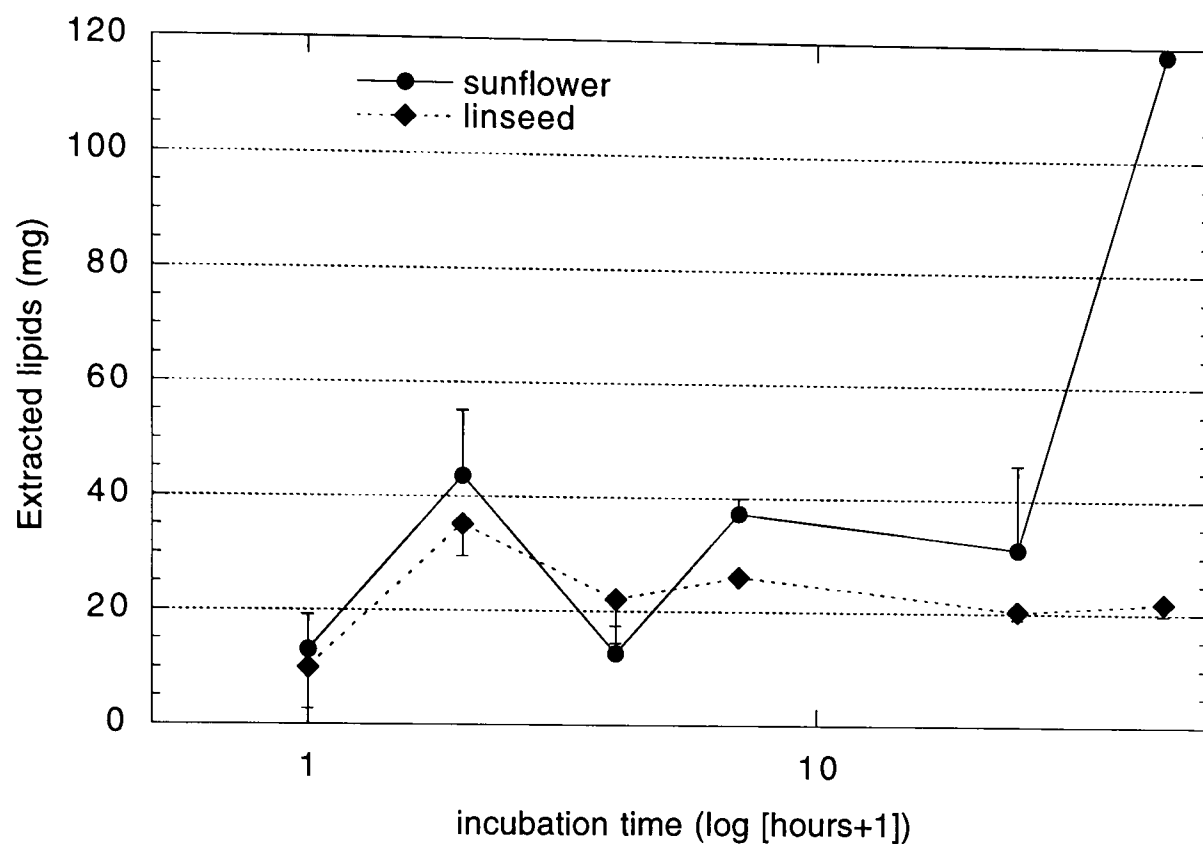


Figure 6.4: Mean weight of lipids from three replicates and standard deviations, extracted from mussel mantles incubated with sunflower and linseed oils, at different incubation times.

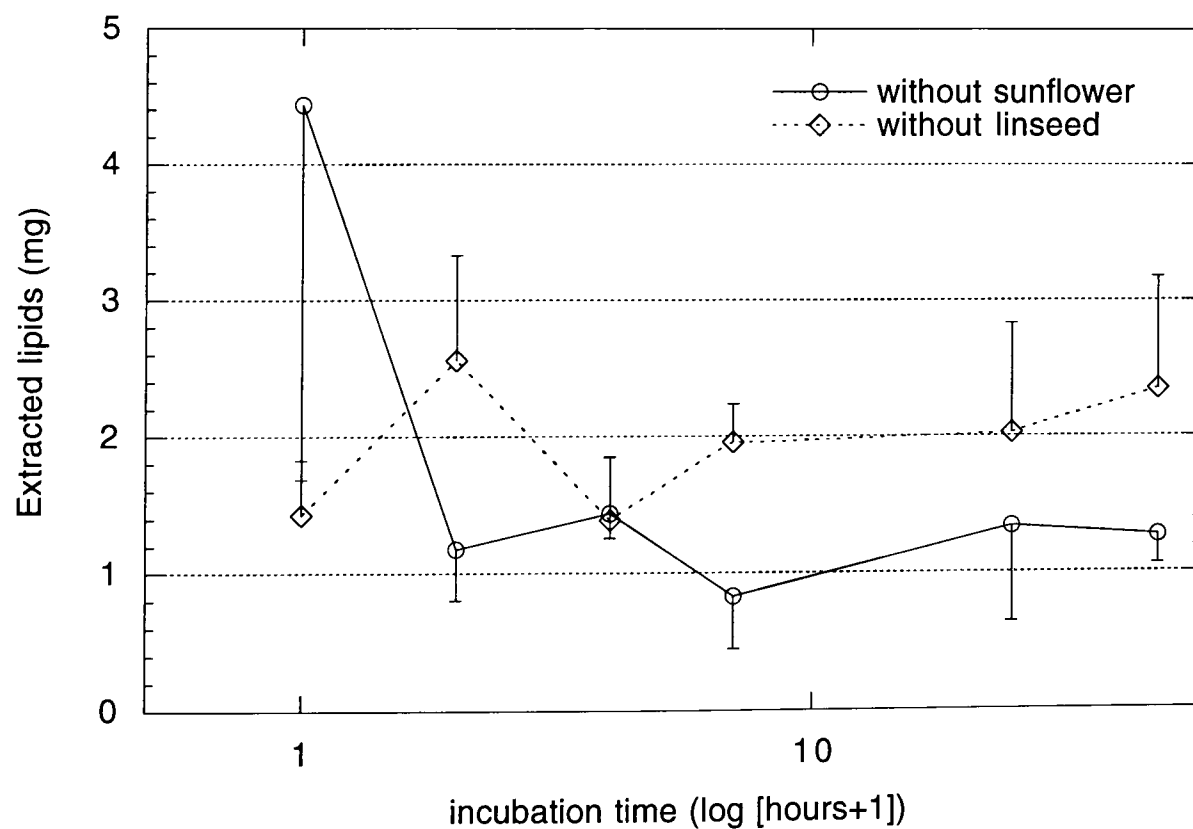


Figure 6.5: Mean weight of lipids from three control replicates and standard deviations, extracted from mussel mantles incubated with Tris buffer solution, at different incubation times.

to interfere. In addition, the 48 h sample was the last one to be collected, emptying the test vial. Oil adsorbed onto the vial could have been removed this way, increasing the amount of oil sampled.

The pattern of lipid variation between samples incubated with and without vegetable oils was similar. During the first 3 hours of incubation there were some fluctuations, a sharp increase after the first hour, followed by a decrease at similar rate after 3 hours leading to a more or less constant weight for the remaining incubation times. Lipids from mantles incubated without sunflower oil showed lower weight of lipids than the incubation without linseed oil and for time zero the weight of lipids was large with large standard deviation reflecting probably poor homogenization of the sample before collection. Apart from this high value, lipids varied between a small interval, 0.8 and 1.4 mg with a tendency to become constant around 1.2 mg for the remaining of the time.

During the first 3 hours of incubation an equilibrium seem to be established between oil-water-mantles after which the weight of lipids became more or less constant.

### 6.3.2 Fatty acids

#### Sunflower oil incubation

Figures 6.6 and 6.7 show the fatty acid composition of mussel mantles incubated without and with sunflower oil, respectively.

The ten most abundant fatty acids found in mussel mantles incubated without sunflower oil are represented in the three graphs (Figure 6.6). The two major ones were palmitic acid and eicosapentaenoic acid (20:5 $\omega$ 3) followed by docosahexaenoic acid (22:6 $\omega$ 3) and palmitoleic acid. Neither of the C18 fatty acids nor 20:4 $\omega$ 6 were detected until three hours of incubation after which there was an increase in all of them. Throughout the incubation period, fluctuations in the concentration of fatty acids were noticed showing a cyclic pattern of increase followed by a decrease. For the first 3 hours there was no notable net increase but from 3 hours onwards all the fatty acids seemed to have increased markedly, although the standard deviations have increased too. After 24 hours, palmitic acid and 20:5 $\omega$ 3 had the largest increase among all the fatty acids from 6 to 40  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids and from 7 to 40  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids,

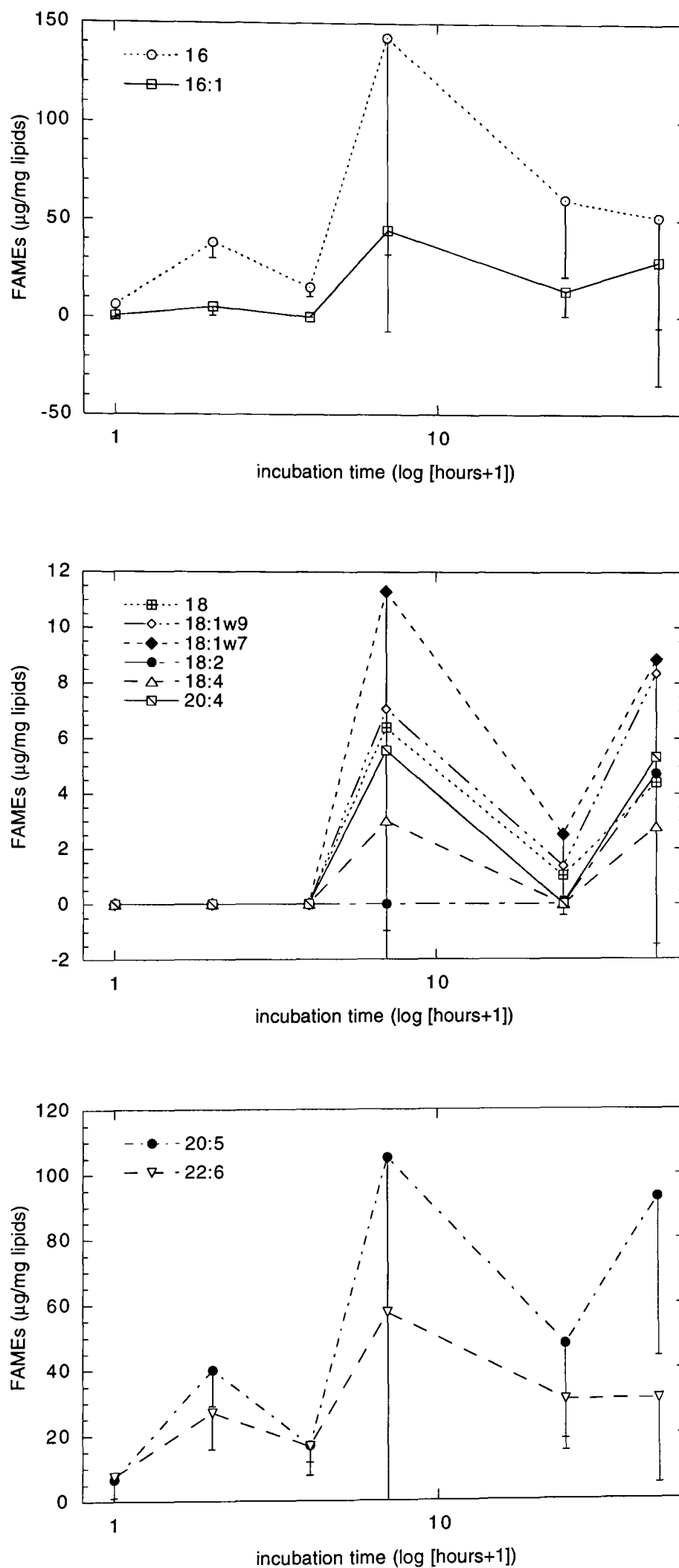


Figure 6.6: Mean fatty acid composition of mussel mantles incubated with Tris buffer solution and no sunflower oil, at different incubation times.



respectively.

The fatty acid composition of the 3 replicates of mantle extracts showed a high standard deviation from the mean. The largest deviations occurred for 6 and 48 hours of incubation time and coincide with the large increase of all fatty acids, which therefore may not be real. Interpretation of the data must, therefore, take this into account.

These results become difficult to interpret considering the large errors obtained, but if the interval 0 to 24 hours of incubation is considered, when the errors were smaller, a net increase of all fatty acids can be seen. Whether this is an indication of metabolic activity occurring in the mantle extracts carried out by the intracellular enzymes using intracellular fatty acids or just the result of random variations is not possible to confirm based on the data presented. Statistical analysis can help to evaluate the magnitude of these variations in order to conclude if they are attributable to random error or if they have a probability of being real. The analysis of variance employed resulted in non significant differences in any of the fatty acids concentrations with time of incubation.

Fatty acids from mussel mantles incubated with sunflower oil can be seen in Figure 6.7. They exhibited a very different profile of fatty acids compared with the mantles incubated without sunflower oil. Sunflower oil dominated the fatty acid composition of the mantle extracts reflecting the predominant fatty acids in the oil *e.g.*, linoleic, oleic, palmitic and stearic acids. Fatty acids typical and abundant in mussels *e.g.*, eicosapentaenoic and docosahexaenoic acids were only present in small amounts at time zero, after which they became undetectable. Linoleic acid was the predominant fatty acid with an average  $669 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids. Likewise, oleic acid showed high concentrations throughout the experiment with an average of  $115 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids. Palmitic and stearic acids were next in concentrations. All fatty acids, except for palmitic acid, had clearly increased concentrations relative to the average value obtained in mantles incubated without oil suggesting that an uptake of sunflower oil into the mantles has occurred. Evidence of metabolism of the surplus amount of these fatty acids, however, was not found. Considering the large standard deviations obtained at times 1 and 24 hours resulting from the large increase of fatty acids, a small weight should be attributed to these values in the interpretation of the data. Statistical differences could not be found between the mean concentrations of any fatty acids along the incubation

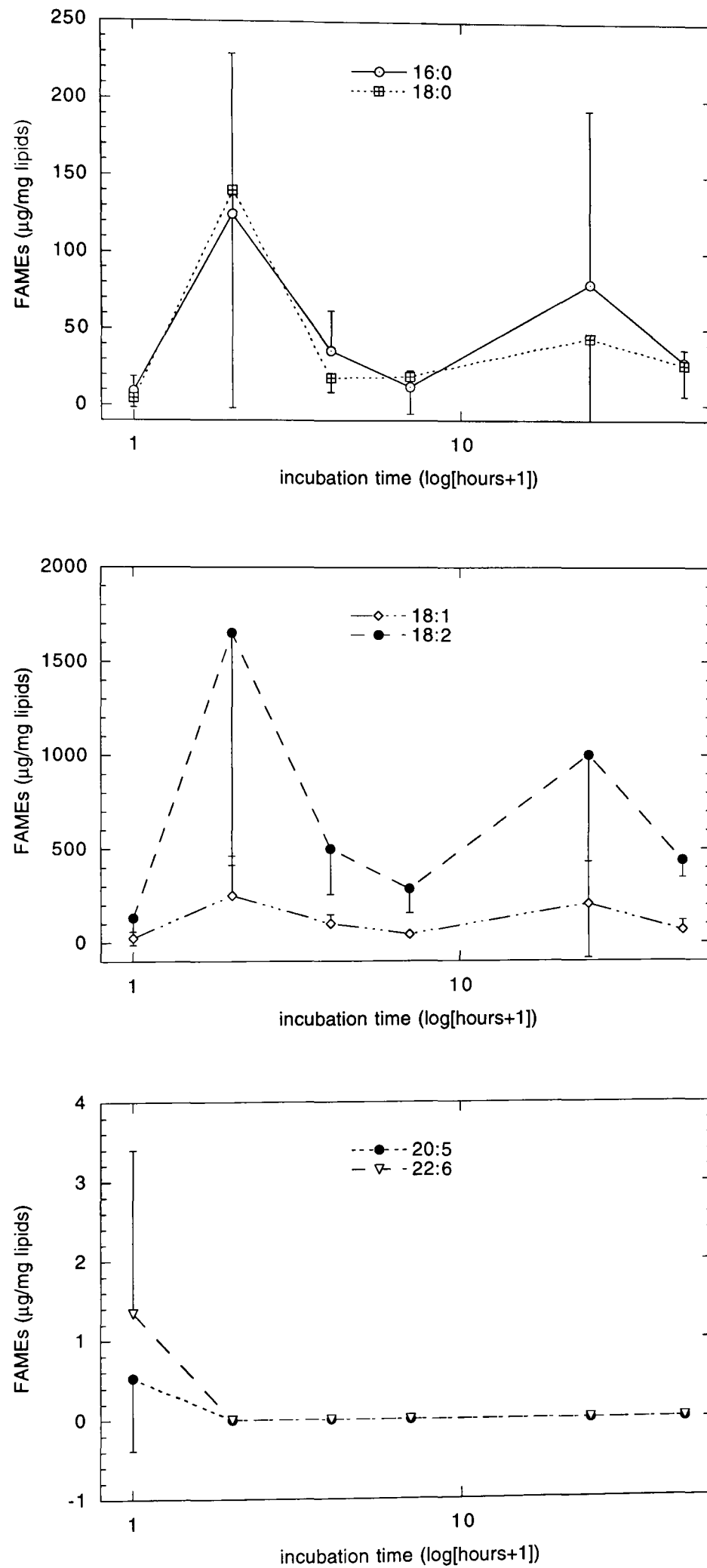


Figure 6.7: Mean and standard deviation of the dominant fatty acids in mussel mantles incubated with sunflower oil, at different incubation times.

time.

### Linseed incubation

The evolution of fatty acid composition in the mantle extracts incubated for 48 hours with Tris-HCl buffer solution and no linseed oil (control) is shown in Figure 6.8. The ten most concentrated fatty acids are represented. Eicosapentaenoic, palmitic, docosahexaenoic and palmitoleic acids were the most abundant starting with concentrations between 112 and 145  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids, at time zero, decreasing sharply after one hour and rising to levels which remained approximately constant for the rest of incubation time between 30 and 55  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids. The same trend was observed in the other fatty acids albeit at lower concentrations. The fatty acids, linolenic and linoleic acid were present at concentrations below 8 and 5  $\mu\text{g} \cdot \text{mg}^{-1}$  of lipids, respectively, after the first hour and the second more concentrated saturated fatty acid was myristic acid rather than stearic.

The incubation of the mussel mantles with linseed oil in Figure 6.9 showed a different fatty acid composition in quantitative terms from the one without oil. Linolenic acid was the predominant fatty acid and together with oleic and linoleic acids constituted from 74% to 86% of the total fatty acids resembling linseed oil fatty acid composition (Figure 6.10). The other fatty acids appeared in small concentrations relative to the abundant C18s. The long chain fatty acids typically abundant in mussels, 20:5 $\omega$ 3, 22:6 $\omega$ 3 and additionally 16:1 were represented in small amounts. The monounsaturate 18:1 $\omega$ 7 and the 18:4 $\omega$ 3 also contributed to the minor fatty acids in mantles incubated with linseed oil and completed the group of ten most concentrated fatty acids.

In terms of evolution with incubation time, all fatty acids undergone a drastic decrease in concentrations after 1 hour and from that time on, a progressive increase was recorded. The incubation with linseed oil seemed to have promoted a gradual uptake of fatty acids from the oil after one hour of incubation, throughout the whole period of incubation, but there were no signs of active metabolic transformations of these increased fatty acids into, for instance, the longer chain ones.

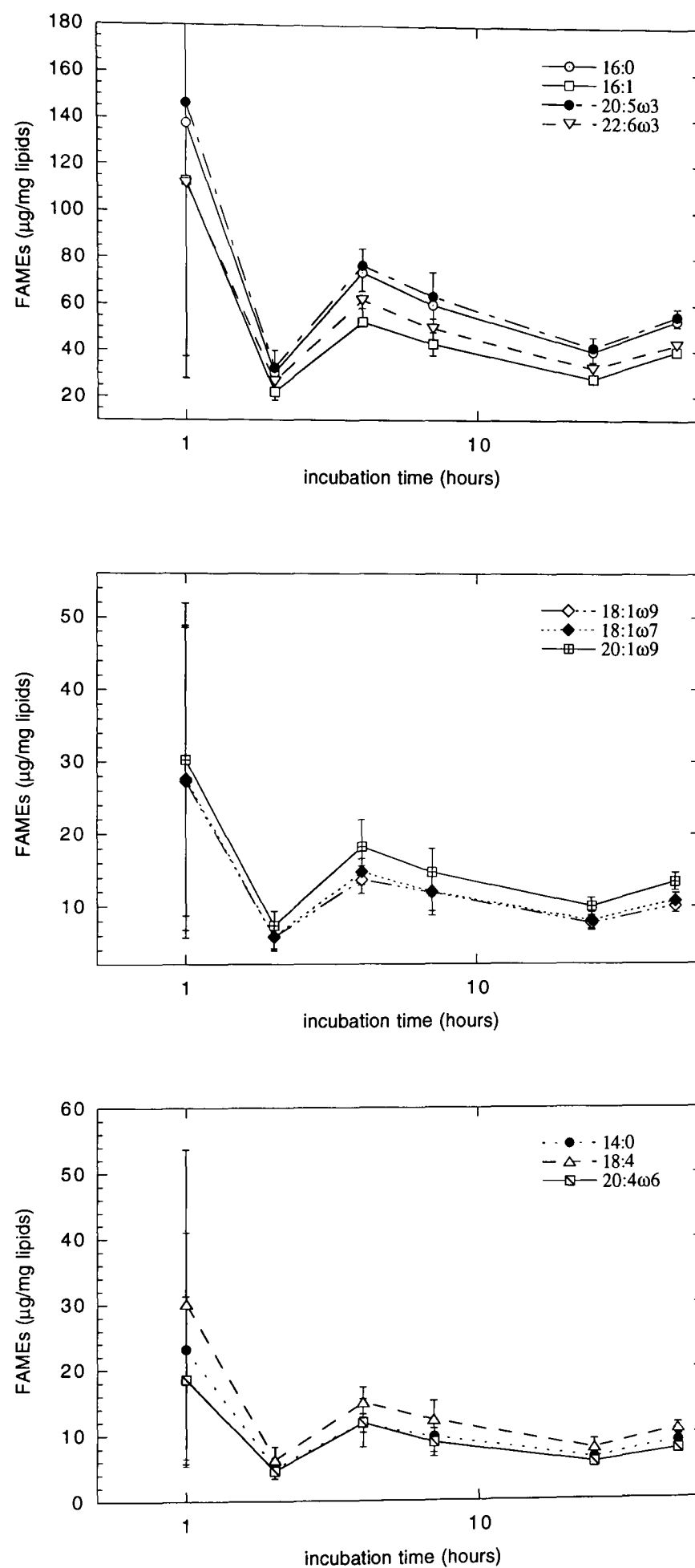


Figure 6.8: Mean fatty acid composition of mussel mantles incubated with Tris buffer solution and no linseed oil, at different incubation times.

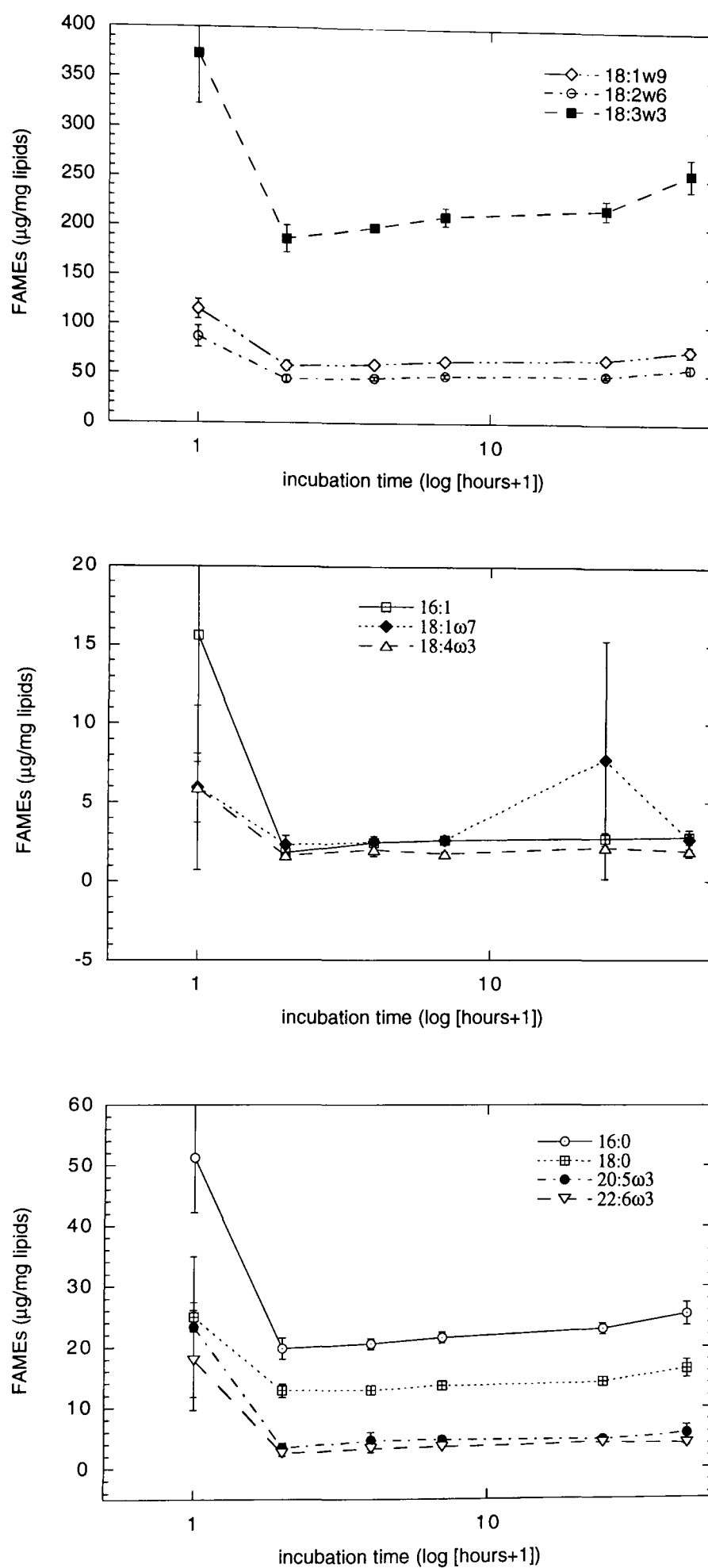


Figure 6.9: Mean fatty acid composition of mussel mantles incubated with linseed oil, at different incubation times.

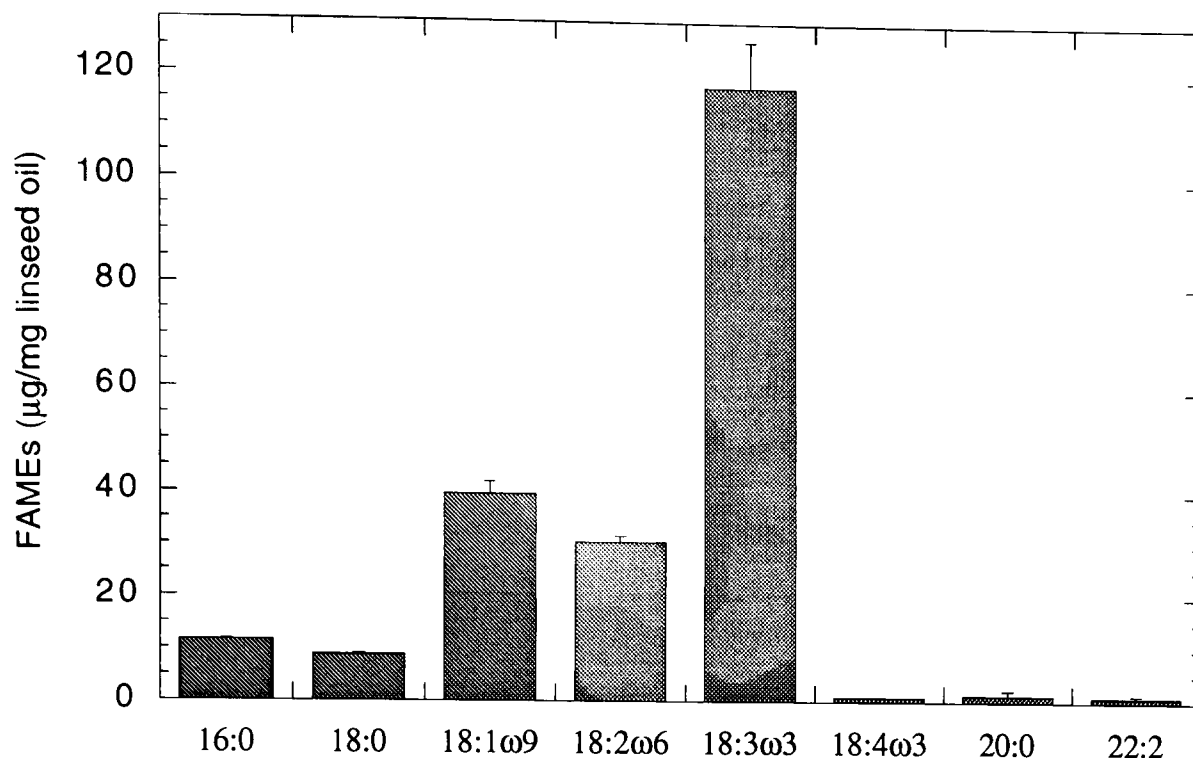


Figure 6.10: Mean fatty acid composition of three replicated samples of linseed oil used for incubation with mussel mantles.

### Statistical analysis

The analysis of variance tests performed with FAME data obtained from the incubation of mantles with and without linseed oil, revealed significant differences with time of incubation. For the control mantles, incubated just with buffer solution, all the FAMES varied significantly with time. The differences were tested with Tukey's all pairwise comparisons test which demonstrated that the different means were registered at time zero. During the remaining time, non-significant differences were found. Likewise, the FAMES in mantles incubated with linseed oil varied significantly. The concentrations of fatty acids at time zero were significantly higher than for the remaining time. Nonetheless, the FAMES linolenic, linoleic, oleic and stearic, showed significant increase at 24 and 48 hours of incubation, confirming the progressive uptake of linseed oil by the mantles. Some of the results from statistical tests are shown in Appendix B.

## 6.4 Discussion

Mussel mantles incubated with the two vegetable oils, sunflower and linseed oils demonstrated an increase of the dominant fatty acids in the oils relative to the fatty acid concentrations in mantles incubated without oils. Although in some cases large variations in fatty acid concentrations were obtained, particularly in sunflower oil incubation, a

trend of fatty acid evolution with time of incubation has emerged. There was an increase of linoleic, oleic and stearic acids in mantles incubated with sunflower oil relative to their without oil counterparts which remained unchanged through the incubation period and a gradual significant increase of linolenic, linoleic, oleic and stearic acids found in mantles incubated with linseed oil. The large variation registered was attributed to the difficulty of obtaining efficient homogenization of the two insoluble phases present, water and oil with the extract suspension and also to the analytical instrument which was substituted in the linseed oil incubation experiment leading to considerable reduction of standard deviations. In the controls, incubated with buffer solution and no oil, the concentrations of fatty acids fluctuated more than in the oil incubations and differently in both experiments. While in the sunflower oil incubation the control exhibited small concentrations of fatty acids for the first 3 hours of incubation, increasing afterwards, the control for the linseed incubation showed the opposite evolution in time, a dramatic decrease followed by non significant changes through the remaining time of incubation. The net result was a decrease of all fatty acids between 0 and 48 hours.

It is not possible to conclude from these results that the increase of fatty acids in both incubations with oils gave rise to active metabolism converting the excess of essential fatty acids into other required fatty acids *e.g.*, long chain polyunsaturated fatty acids.

Similar findings were reported in Chapter 4, relative to the *in vivo* evolution in time of fatty acids in mussels exposed to vegetable oils. During the time of exposure accumulation of the fatty acids present in the oils could be seen but no signs of active metabolic reactions leading to synthesis of other essential fatty acids were observed. It seems that changes in fatty acid composition after oil exposure only start to occur when the oil contamination source is removed, as seen in Chapter 5.

So far it has been difficult to prove the capacity of invertebrates to synthesize *de novo* essential fatty acids but it has been recognized their ability to elongate fatty acids originated from the diet. Field studies on fatty acid composition of mussels exposed to a sunflower oil spill (Mudge *et al.*, 1993) suggested the existence of a correlation between the concentration of 18:2 $\omega$ 6 and other fatty acids, namely 20:2 $\omega$ 6 and 18:3 $\omega$ 3. The results discussed here, however, failed to prove the existence of marked metabolic activity

in consequence of a surplus amount of essential fatty acids and rather an accumulation occurred.

## 6.5 Summary

Mussel mantles incubated with the vegetable oils sunflower and linseed oil showed an increased level of the fatty acids abundant in the oils, reflecting its accumulation.

Control mantles incubated with buffer solution instead of oils revealed some variation in fatty acid concentrations with time which were not significant in sunflower experiment and neither in linseed experiment after the first hour of incubation.

No signs of active metabolism were detected in the mantles incubated with oils in consequence of the increase of essential fatty acids.



# Chapter 7

## The behaviour of mussels exposed to sunflower oil

### 7.1 Introduction

Mussels have been widely adopted in chemical monitoring and surveillance programmes due to their ability to accumulate a variety of chemicals (Goldberg *et al.*, 1978; Widows, 1985) and because they meet the requirements of a suitable indicator organism (Phillips, 1980). The use of mussels as biological sensors to monitor water quality has become important (Kramer *et al.*, 1989) and attempts have been made to include the biological response of organisms in monitoring systems for detection of pollution (Bayne *et al.*, 1985). In contrast to chemical monitoring which requires several weeks until an equilibrium is established before analyses can be performed, physiological and behavioural changes are fast and therefore suitable when a fast response is required in continuous biological monitoring (Kramer *et al.*, 1989). Some studies have contributed to the success of the use of mussel activity as a tool in biological monitoring (Kramer *et al.*, 1989; Sloof *et al.*, 1983).

The shell valve movement of bivalves has recently attracted attention not only to study the rate of physiological processes such as filtration or adaptation to temperature changes but also to detect both natural changes in the environment, *e.g.* the effects of light (Ameyaw-Akumfi and Naylor, 1987), salinity fluctuations (Davenport, 1979; Davenport, 1981) and the effects of pollutants (Sloof *et al.*, 1983; Kramer *et al.*, 1989).

The purpose of this chapter is to investigate whether mussel shell valve gape, foot extensions or movements show a pattern of activity and if the pattern changes with

exposure to sunflower oil and consequently to determine if these features could or could not be used as biological responses to detect the presence of pollutants. In addition, fatty acid analyses were carried out on two individual mussels, collected each day, rather than in a homogenized pool of several mussels sacrificed at the end of the experiment. Using such a methodology I was interested in detecting individual variations in oil uptake and in observing the daily evolution of mussel fatty acid composition in the mussels, in the presence and absence of vegetable oil.

## 7.2 Materials and methods

Much of the equipment employed in these experiments was the same as that used in Chapter 4, namely the experimental apparatus and the fatty acid analysis techniques. A video camera connected to a Panasonic Time Lapse AG-6720 video recorder were used to record the behaviour of control mussels and those exposed to sunflower oil, every second.

### 7.2.1 Experimental setup

Two tanks of 11 l capacity were mounted in a flowthrough system receiving  $300 \text{ ml} \cdot \text{min}^{-1}$  of filtered seawater kept constant by a header. One tank contained a control group of mussels whilst the second contained mussels which were continuously contaminated with sunflower oil delivered by a peristaltic pump at flow rate of  $0.42 \text{ ml} \cdot \text{min}^{-1}$  during the first 7 days of the experiment. After which the sunflower oil was stopped and the mussels exposed for a further 7 days to flowing filtered seawater.

About 100 mussels (length  $60 \pm 5 \text{ mm}$ ) were collected from the midshore at Beaumaris, Anglesey and brought into the laboratory where they were washed with seawater and distributed amongst the two tanks containing running seawater. Mussels were kept in the tanks for 13 days before the beginning of experiment for acclimation and preliminary video-recording trials. The behaviour of ten mussels in each tank were then video-recorded, whilst two others were removed every day, for 14 days, for fatty acid analysis.

Mussels were fed on the day following their arrival at the laboratory and subse-

quently each day for a period of one month with an algal culture composed of a mixture of *Tetraselmis chuii*, *Rhinomonas reticulata* and *Pavlova lutheri*. The total number of cells delivered to each tank, daily, was  $1.7\text{--}2.0 \times 10^9$  cells.

The video camera was fixed on top of the tanks in such a way that the 10 mussels in both, the control and experimental tanks, could be filmed simultaneously. Mussels were filmed in continuous light throughout the recording session. After the 15 day recording session, each video film was observed and hourly observations, were taken of the number of mussels whose valves were gaping, which showed foot extensions and movements in both, the experimental and control tanks. Some difficulty was experienced in deciding whether mussels kept a narrow gape or had their valves closed, due to the filming angle, but changes of activity from closed to gaping valves were easily detected.

### 7.2.2 Fatty acid analysis

Sixty mussels, two per day, with a mean length of  $5.9 \pm 0.5$  cm were removed from the two tanks and individually analysed. Two individuals were removed from both tanks, the control and the experimental tank, every day and analysed individually. The procedure for extraction, methylation and injection of fatty acid methyl esters was similar to the one described in Chapter 3. The methylation was done with boron-trifluoride in methanol and a GC Carlo Erba was the analytical instrument. Each sample was extracted with 10 ml of a chloroform-methanol solution (2:1, v/v) with the antioxidant BHT (2,6-di-tert-butyl-p-cresol). A known amount (0.488 mg) of the triacylglyceride [*cis*]-15-trinervonin (24:1) and 0.4506 mg of the phospholipid phosphatidylcholine dibehenoyl (22:0) were added as yield monitors. The samples were then vortexed and left in a fridge for 30 min to extract the lipids. After extraction the samples were filtered through Whatman paper filters n.4 and a 0.2 vol. of 0.017%  $\text{MgCl}_2$  were added to the filtrate and samples shaken. This step removes the water soluble salts from the lipidic phase. Centrifugation at 2000 rpm for 5 min cleared the two phases. The supernatant was discarded and the lipids washed with Folch upper phase reagent made with 96 ml of Milli-Q water, 94 ml of methanol and 6 ml of chloroform. The lipids were then evaporated to dryness in a rotary evaporator, transferred to a preweighed vial and weighed. According to their weight, they were dissolved in an appropriate volume

of chloroform to obtain a concentration of no more than  $10 \text{ mg} \cdot \text{ml}^{-1}$  and methylated using boron-trifluoride in methanol. Before analysis, tricosanoic fatty acid methyl ester (C23:0) from a solution  $0.492 \text{ mg} \cdot \text{ml}^{-1}$  was added as internal standard.

The analysis were done in a gas chromatograph Carlo Erba 8000, equipped with a on-column injector and a 30 m long Carbowax 20M capillary column. The operating conditions were as follows. The carrier gas was hydrogen flowing at a rate of 20 KPa. Hydrogen was also used at a rate of 60 KPa which together with air at 110 KPa provided the combustion gases for the flame ionization detector. The oven temperature was programmed to start at  $80^\circ\text{C}$  and stay constant for 2 min and increase after that time to  $150^\circ\text{C}$  at a rate of  $49.9^\circ\text{C} \cdot \text{min}^{-1}$  with a second temperature increase to  $230^\circ\text{C}$  at a slower rate of  $3.3^\circ\text{C} \cdot \text{min}^{-1}$  holding at this temperature for 30 minutes. One microlitre of sample was injected 30 seconds after the sweep had been activated.

Identification of the fatty acid methyl esters (FAMES) were done by comparison with standards run in the same conditions as the unknown samples and quantification was obtained from calibration with FAME standard solutions.

### Calibration

Standard solutions containing the FAMES 16:0, 17:0, 18:0, 18:1 $\omega$ 9, 18:2 $\omega$ 6, 18:3 $\omega$ 3, 20:0, 20:1 $\omega$ 9, 20:2 $\omega$ 6, 20:3 $\omega$ 6, 20:4 $\omega$ 6, 22:0, 22:6 $\omega$ 3 and 24:1 $\omega$ 9 were used to calibrate the gas chromatograph. Five different concentrations of the standards were prepared: 10, 50, 100, 150 and  $170 \mu\text{g} \cdot \text{ml}^{-1}$  each FAME. A constant concentration of tricosanoic acid methyl ester 23:0 ( $49.2 \mu\text{g} \cdot \text{ml}^{-1}$ ) was added as the internal standard to each of the 5 solutions and these were injected. The resulting chromatogrammes enabled the building of calibration lines obtained by linear regression analysis between chromatographic area of each FAME and its known concentration in the standard solutions. The amount of each FAME in the unknown samples were calculated this way using the calibration lines and equation 3.1.

## 7.3 Results

### 7.3.1 Laboratory conditions

The water temperature experienced by both mussel groups, the control and the one exposed to sunflower oil, is shown in Figure 7.1.

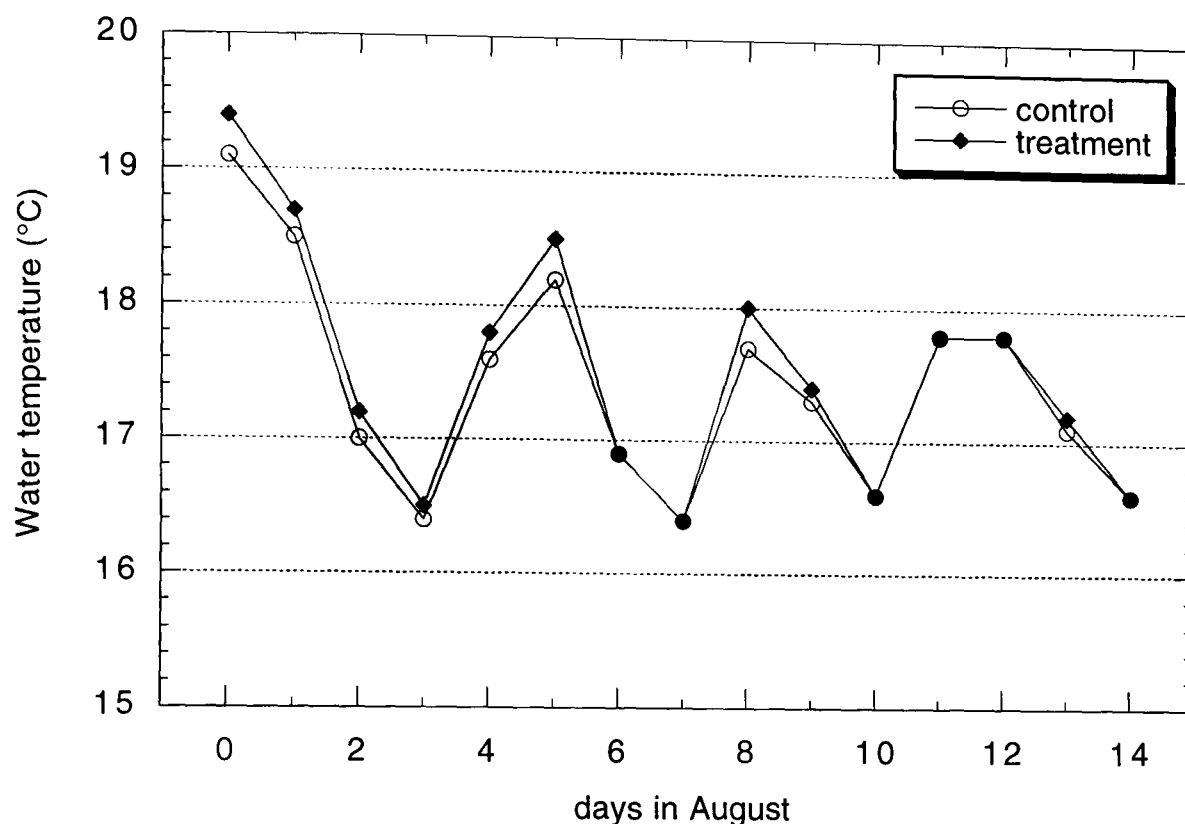


Figure 7.1: Water temperature registered in both experimental tanks, during the time of experiment.

The ambient water temperature showed a cyclic fluctuation between  $16.4^{\circ}\text{C}$  and  $19.4^{\circ}\text{C}$ , the average and standard deviation being  $17.5 \pm 0.8^{\circ}\text{C}$ . The temperature between the two tanks was very similar and the largest deviation registered was  $0.3^{\circ}\text{C}$ . The temperature conditions were thus basically the same for both groups of mussels.

Mussel diet composition in terms of number of cells and species is illustrated in Figure 7.2.

The total number of microalgae cells delivered to the mussels was between  $1.7\text{--}2.0 \times 10^9$  cells except on the fourth day when only a small volume of *Tetraselmis chuii* was available. The cell density was for this reason reduced to less than half of its usual value, but it was reestablished on the following days. In terms of quality of diet, mussels received on average 50% of *Pavlova lutheri*, 30% of *Tetraselmis chuii* and 20% of *Rhinomonas reticulata*. This mixture of microalgae provide mussels with essential fatty acids for normal growth as it was discussed in Chapter 3.

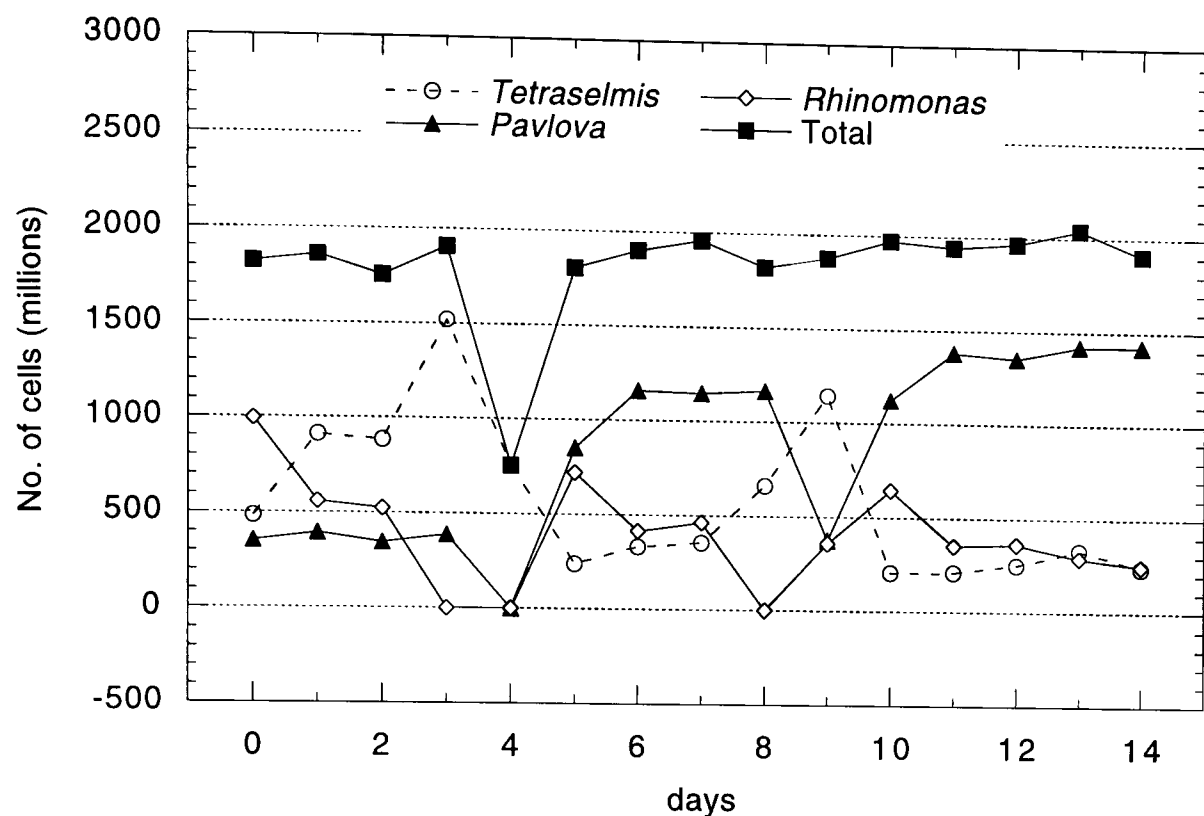


Figure 7.2: Composition of mussel diet during the course of the experiment.

### 7.3.2 Mussel activity

The patterns of mussel behaviour in terms of the gaping shells valves, foot extensions and movements are illustrated in Figures 7.3 to 7.5. Although hourly information was taken from the video recordings, for the sake of clarity 12 hours intervals were chosen to show mussel activity and how it varied with the input of food and under the influence of sunflower oil when compared with the control mussels.

Food influenced mussel activity clearly and in a cyclic way. After food had been distributed (shown by the arrows), more mussels gaped to feed, the number of mussels showing foot extensions increased and to a lesser extent the number of mussel movements also increased. During the course of the experiment, the mussels tended to clump together making it difficult to observe individual mussels. Therefore during the second half of the experiment they were separated from one another and they were relocated, so that they randomly covered the bottom of the tank. The movement of the mussels resulted in a high activity on the 10th day i.e. 228 hours and in the following hours the pattern of activity observed was still disturbed as shown in all graphs from Figure 7.6.

The total number of mussels gaping (Figure 7.3) during the first week of treatment with sunflower oil, did not differ significantly ( $P = 0.567$ ,  $F = 0.37$ ) from the control group of mussels. A range of five to ten control mussels gaped in 12 hours intervals and

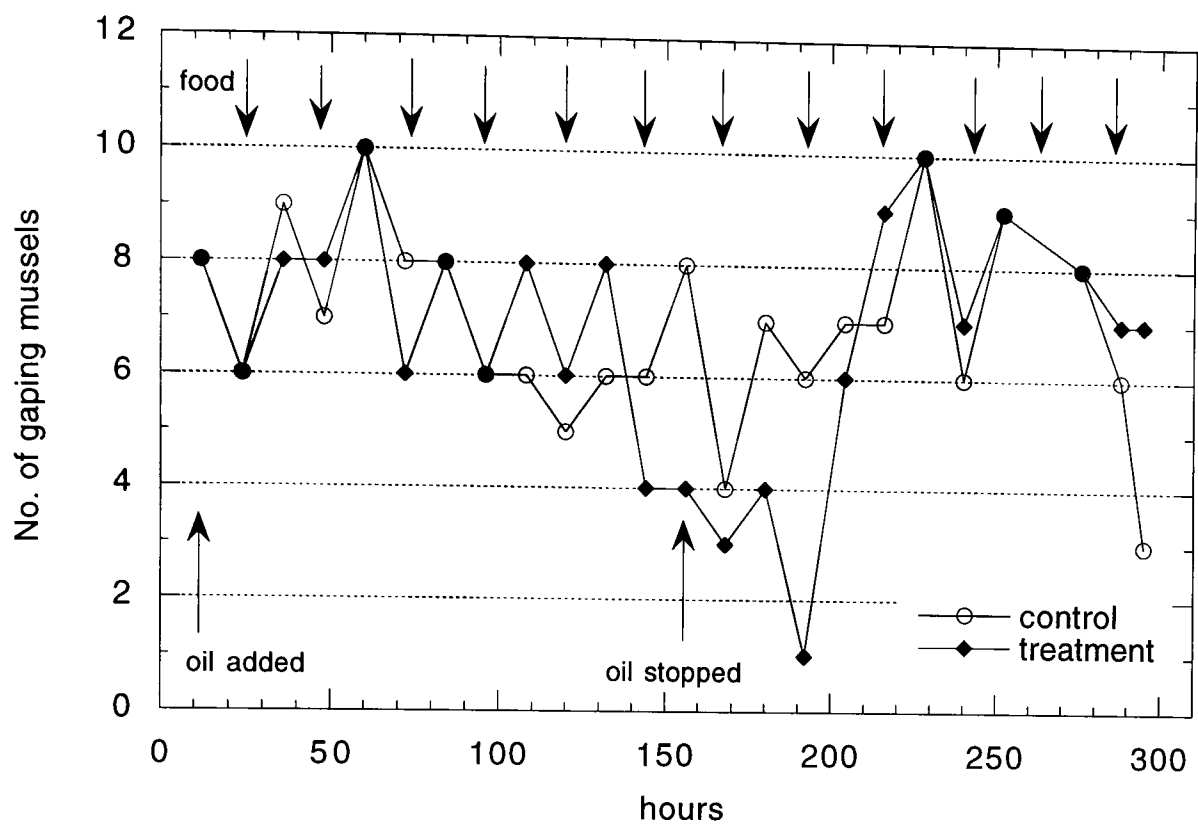


Figure 7.3: The number of mussels gapping at 12 hours intervals. Arrows show when food was supplied and sunflower oil added.

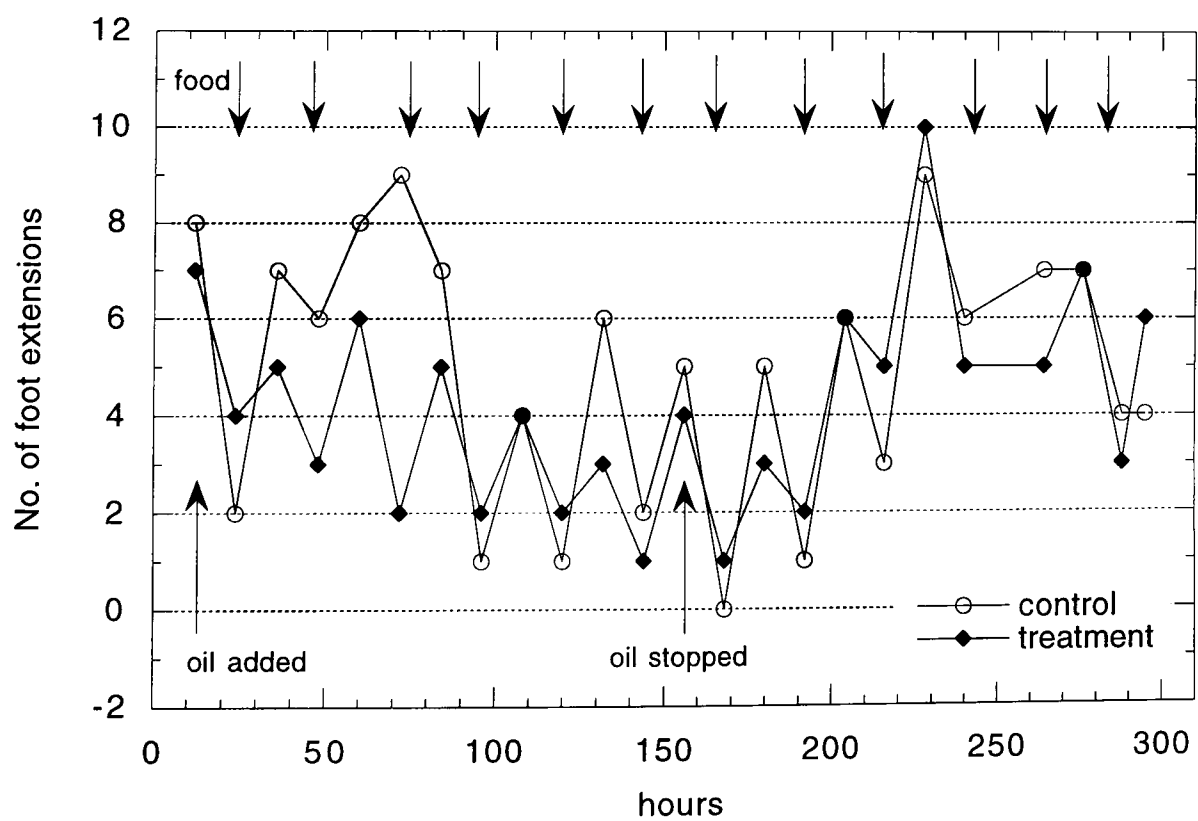


Figure 7.4: Number of foot extensions displayed by mussels at 12 hour intervals. Arrows show when food was supplied and sunflower oil added.

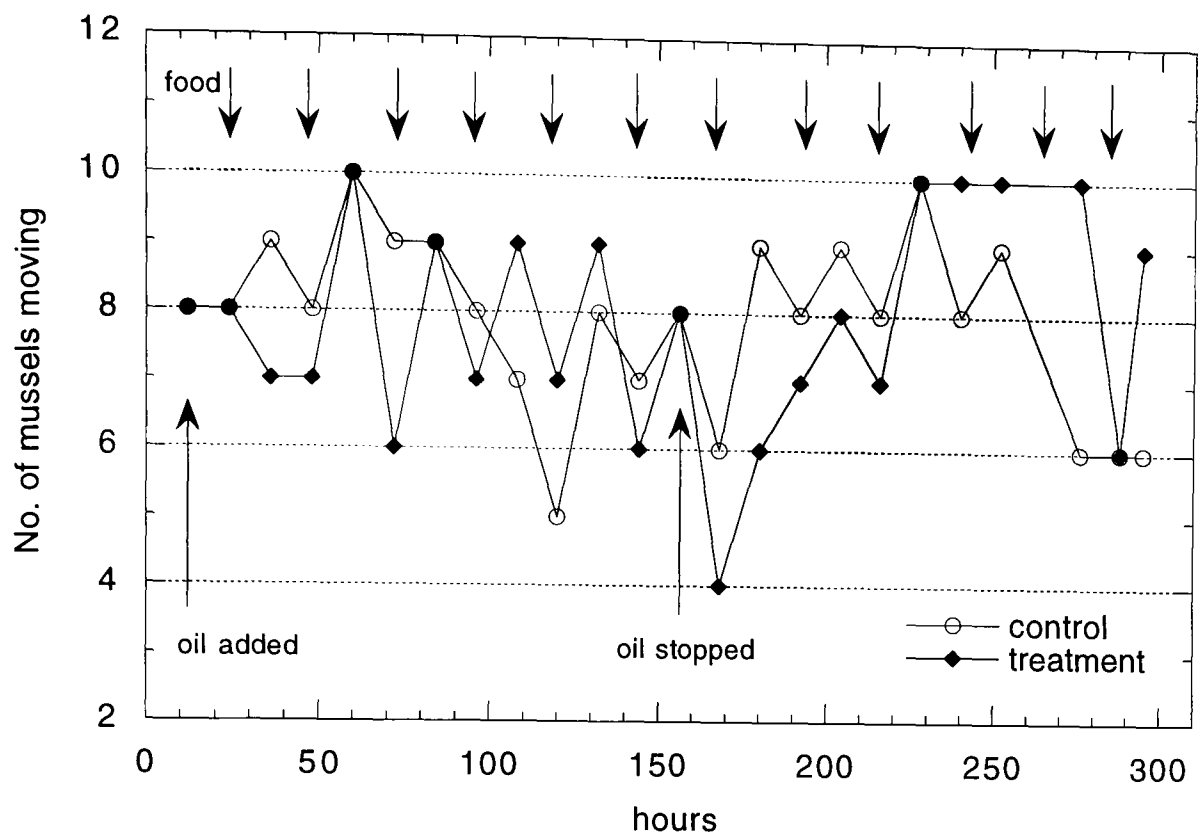


Figure 7.5: Number of mussel movements recorded at 12 hour intervals. Arrows show when food was supplied and sunflower oil added.

a range of four to ten mussels in the experimental tanks gaped in the same time interval. When the oil was stopped after 159 hours the range of control mussels gaping was four to ten. This range was similar to the one for the first half of the treatment, as expected, since the control mussels received the same treatment for the whole experimental time. The group of mussels treated with sunflower oil, however, showed a gradual decrease in the number of mussels with their valves open, so that by 159 hours after first exposure to the oil, only one mussel was observed gaping. After the oil was stopped there was a general increase in the number gaping 33 hours after the oil had been removed. A range of six to ten mussels gaping was exhibited until the end of experiment. Generally, however, mussels treated with oil did not close their valves more than the control group except for a short period just after oil additions had been stopped.

The number of mussels exhibiting foot extensions (Figure 7.4) ranged from zero to nine in the control group and from one to seven in the group of mussels treated with sunflower oil during the time of exposure (159 hours); for the following time when oil additions were stopped, mussels with foot extensions ranged from three to ten. The maximum number of mussels with their foot out in the treatment group decreased steadily from 7 to 3 just before the oil was removed and increased again from 3 to 7



after the oil was stopped. Generally there seems to be a reduction in the number of mussels protruding their foot in the presence of sunflower oil which not only stops in the absence of oil, but also increased to the same or above those of the control values.

The number of mussels moving varied from 5 to 10 mussels every 12 hours in the control group; 6 to 10 mussels under sunflower oil exposure and 4 to 10 mussels after oil had been removed. There was a decrease in the number of mussels moving in the treatment group just after the oil was removed followed by an increase to average values. However during the 60 hours following the absence of oil the treatment group of mussels showed less movements than the control.

In order to examine the data statistically, mussel activity was accumulated in 24 hours intervals, so that differences between mussel behaviour in the control and treatment groups could be tested. Figure 7.6 illustrates the daily variation in gaping, foot extensions and movement for the six days of exposure to sunflower oil and for the following six days in clean seawater.

Similar number of mussels gaped in the control conditions and those exposed to sunflower oil, both decreasing with time. Except for the 7th day when oil was stopped and the following day, the number of mussels gaping in the control or treatment group were similar.

For the foot extensions, control mussels were always more active than the mussels under sunflower oil treatment, although both groups showed a decreasing number of foot extensions. In the absence of oil the number of foot extensions increased to values similar to the control mussels, suggesting that sunflower oil had some inhibiting effect on mussel foot activity.

Mussel movement was the most constant response of the three activities, varying between a small interval of 8 and 10 mussels moving each day and showing no influence in the presence or absence of oil.

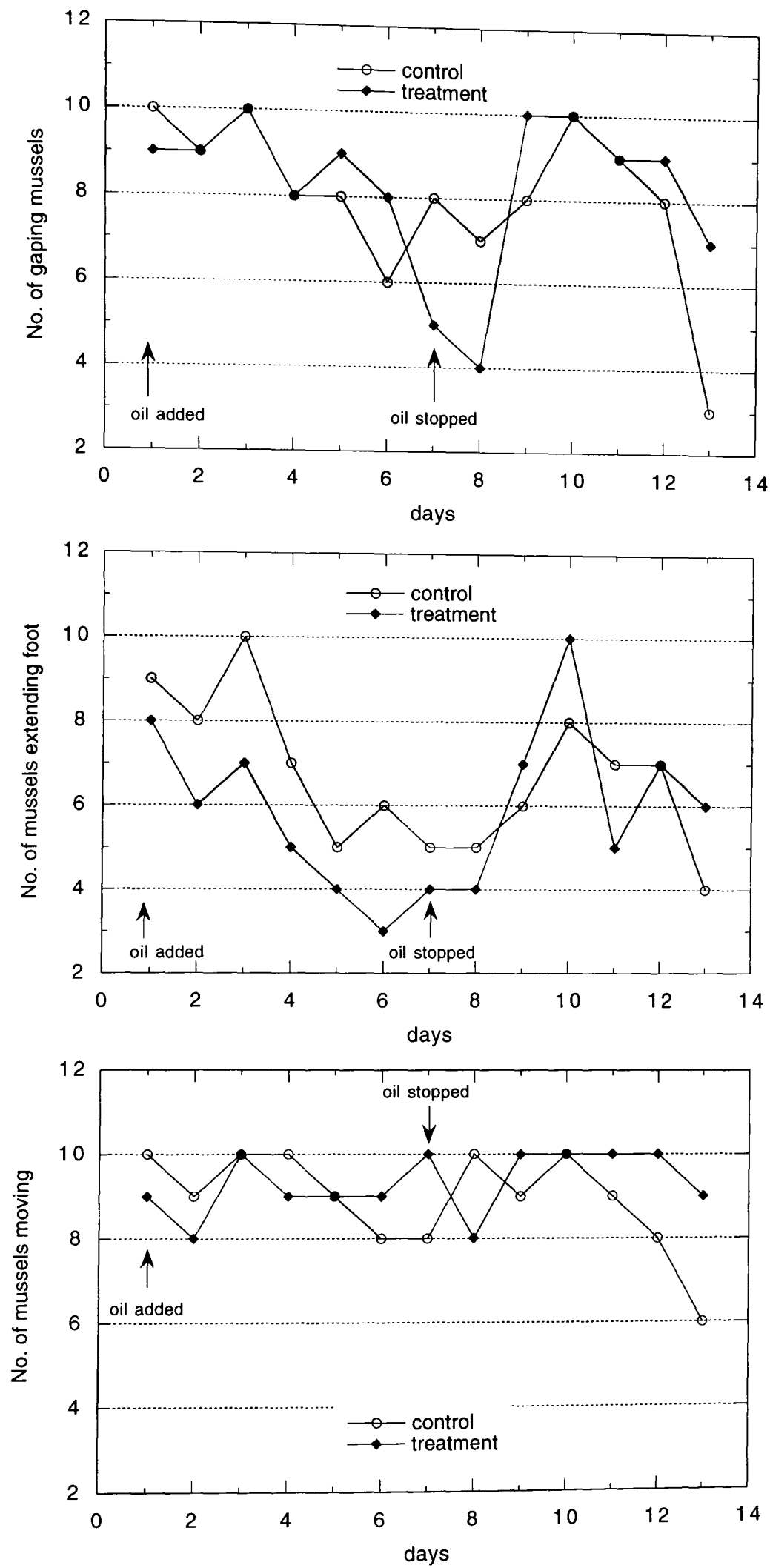


Figure 7.6: Mussel activity recorded at 24 hour intervals.

### Statistical analysis

Statistical analysis was carried out to test if the differences in mussel activity between the control and treatments exposed to sunflower oil were significantly different. Transformation of the data was required since it does not follow a normal distribution (Zar, 1974). The arcsine transformation (angular transformation) is applicable when data represents proportions and it was used in this case.

The arcsine transformed mussel gaping data, in the control and experimental treatments was tested using two way analysis of variance. The two null hypothesis tested were: There is no effect of sunflower oil exposure on the number of mussels gaping and there are no differences on the mean number of mussels gaping during the 6 days of the experiment. The tests were performed using two sets of six day data separately because the group of mussels exposed to the oil received a different treatment on the second week. For the first 6 days of experiment the null hypothesis concerning the effect of the oil was accepted with  $P = 0.567$  and the second hypothesis concerning the effect of time was rejected with  $P = 0.049$ . In order to ascertain in which days gaping activity varied, a Tukey's pairwise comparison test was carried out and day 3 turned out to be different from day 6. The output from statistical tests is shown below.

#### Analysis of Variance for gaping during the first week

Source	DF	SS	MS	F	P
treatment	1	0.003333	0.003333	0.37	0.567
days	5	0.227926	0.045585	5.13	0.049
Error	5	0.044450	0.008890		
Total	11	0.275709			

#### Analysis of Variance for gaping during the second week

Source	DF	SS	MS	F	P
treatment	1	0.00274	0.00274	0.09	0.776
days	5	0.46020	0.09204	3.02	0.125
Error	5	0.15251	0.03050		
Total	11	0.61545			

These results indicate that sunflower oil did not affect the number of mussels gaping and that the number of mussels gaping decreased over the duration of the experiment, the number of mussels gaping on the sixth day being significantly lower than those on the third day. During the last 6 days of the experiment, twoway analysis of variance was performed in the same way as for the first week. The result was non significant

$P = 0.776$  and  $P = 0.125$  for both null hypothesis: *ie.*, there is no effect of sunflower oil, after it has been removed, on the number of mussels gaping and there are no differences in the mean number of mussels gaping with time, therefore the null hypothesis were accepted. The overall conclusion is that sunflower oil did not affect the number of mussels gaping before and after being exposed to it and the number of mussels gaping decreased with time during the first six days of experiment but not during the last 6 days. During the last 6 days, as mentioned before, mussels were moved to their original positions in the tanks which caused an increase in activity in both groups which probably masked any differences with time.

Foot extensions were the only observed mussel activity that was affected by sunflower oil. A twoway analysis of variance for the first 6 days of the experiment revealed that sunflower oil significantly decreased the number of mussels exhibiting foot extensions ( $P = 0.004$ ) and that the same variable also decreased with time of treatment ( $P = 0.007$ ). During the second half of treatment, when the oil was removed, neither of the two factors, treatment ( $P = 0.995$ ) or time ( $P = 0.061$ ) affected mussel foot extensions. It means that the effect of the oil on mussels was reversed when the source was removed.

#### Analysis of Variance for foot extensions during the first week

Source	DF	SS	MS	F	P
treatmen	1	0.162565	0.162565	24.13	0.004
week1	5	0.428962	0.085792	12.73	0.007
Error	5	0.033688	0.006738		
Total	11	0.625215			

#### Analysis of Variance for foot extensions during the second week

Source	DF	SS	MS	F	P
treatm	1	0.00000	0.00000	0.00	0.995
week2	5	0.38002	0.07600	4.56	0.061
Error	5	0.08330	0.01666		
Total	11	0.46332			

The data for mussel movements could not be tested with parametric tests using data transformation because many of the values were 100% (all mussels moved). But examination of the control and treatment data it appears that the number of mussels moving was not affected by either sunflower oil exposure nor the duration of the experiment.

### 7.3.3 Fatty acids

The average and standard deviation of the four groups of mussels, two control and two treated with oil, sampled for fatty acid analysis was  $5.9 \pm 0.5$  cm;  $6.1 \pm 0.6$  cm and  $5.8 \pm 0.3$  cm;  $6.1 \pm 0.4$  cm, respectively. The weight of lipids extracted from the mussel soft tissues is shown in Figure 7.7. The weight of lipid in the treatment groups showed no significant increases ( $P = 0.602$ ,  $F = 0.280$ ) in weight relative to the control group, during the first 7 days of exposure to sunflower oil. The uptake of the oil seemed therefore, to be limited. However, since the analysis were performed in individual mussels with wide variation of lipid weights, it can not be concluded that mussels did not take up the oil.

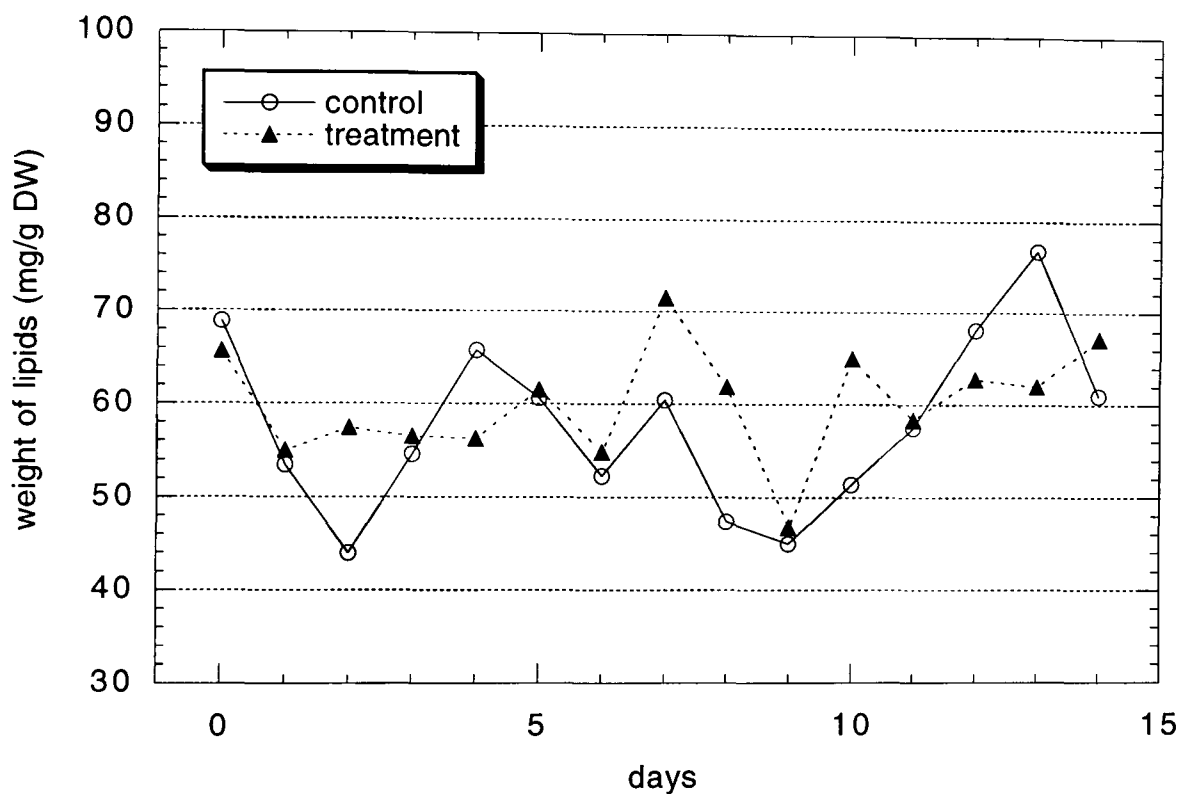


Figure 7.7: Weight of lipids extracted from the sampled mussels.

Variations in the mussel fatty acid composition during the 15 days of the experiment are shown in Figures 7.8 to 7.12. Day zero corresponds to the first day of sampling before sunflower oil had been introduced, therefore concentrations of fatty acids in the control mussels should be similar to the ones in the treated mussels. On the seventh day mussel sampling occurred before sunflower oil had stopped, thus they were still exposed to oil.

The composition of the sunflower oil has been shown previously (Chapter 3) to contain a predominance of linoleic, oleic and palmitic acids. The concentration of fatty

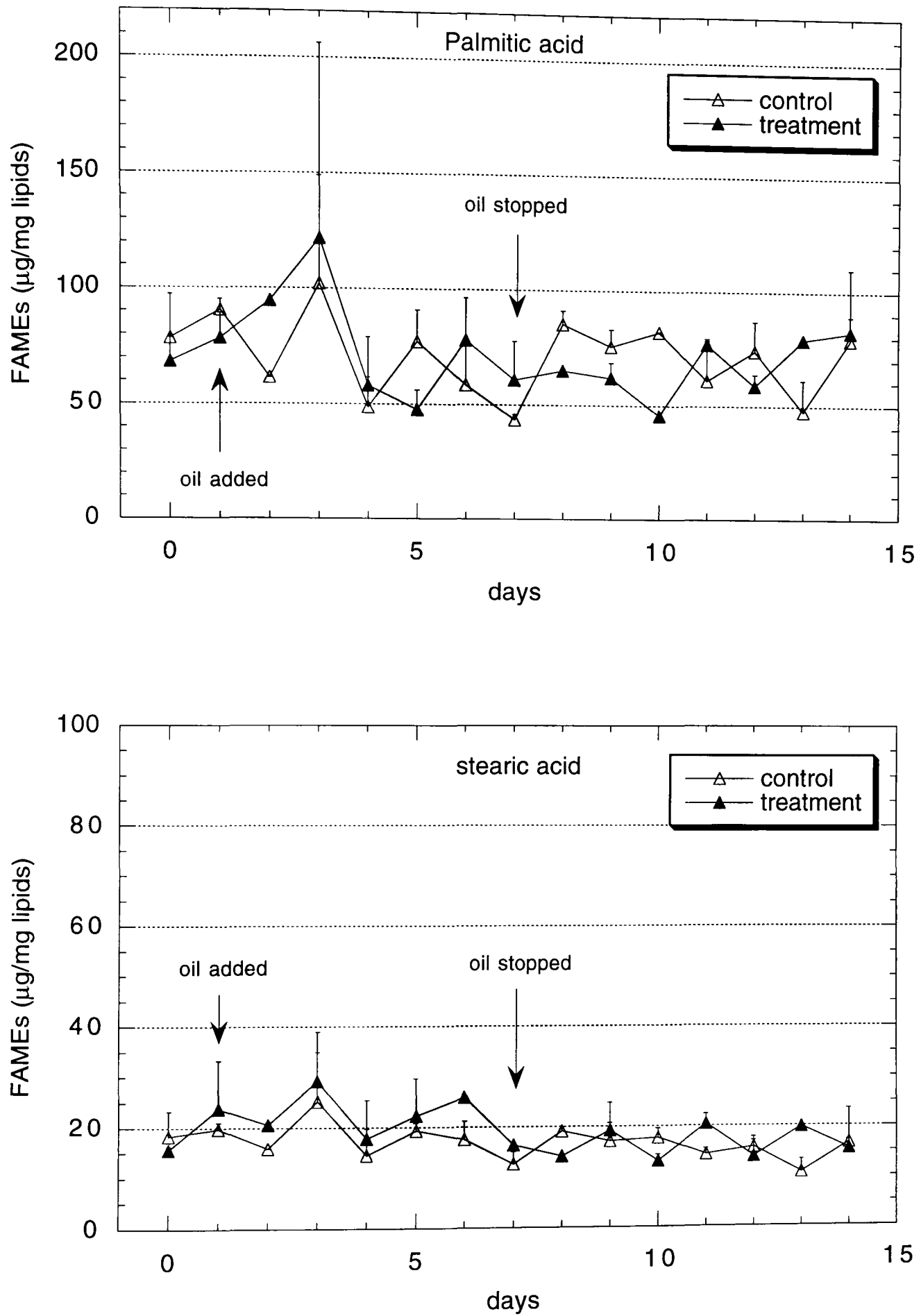


Figure 7.8: Variation of saturated fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days of the experiment.

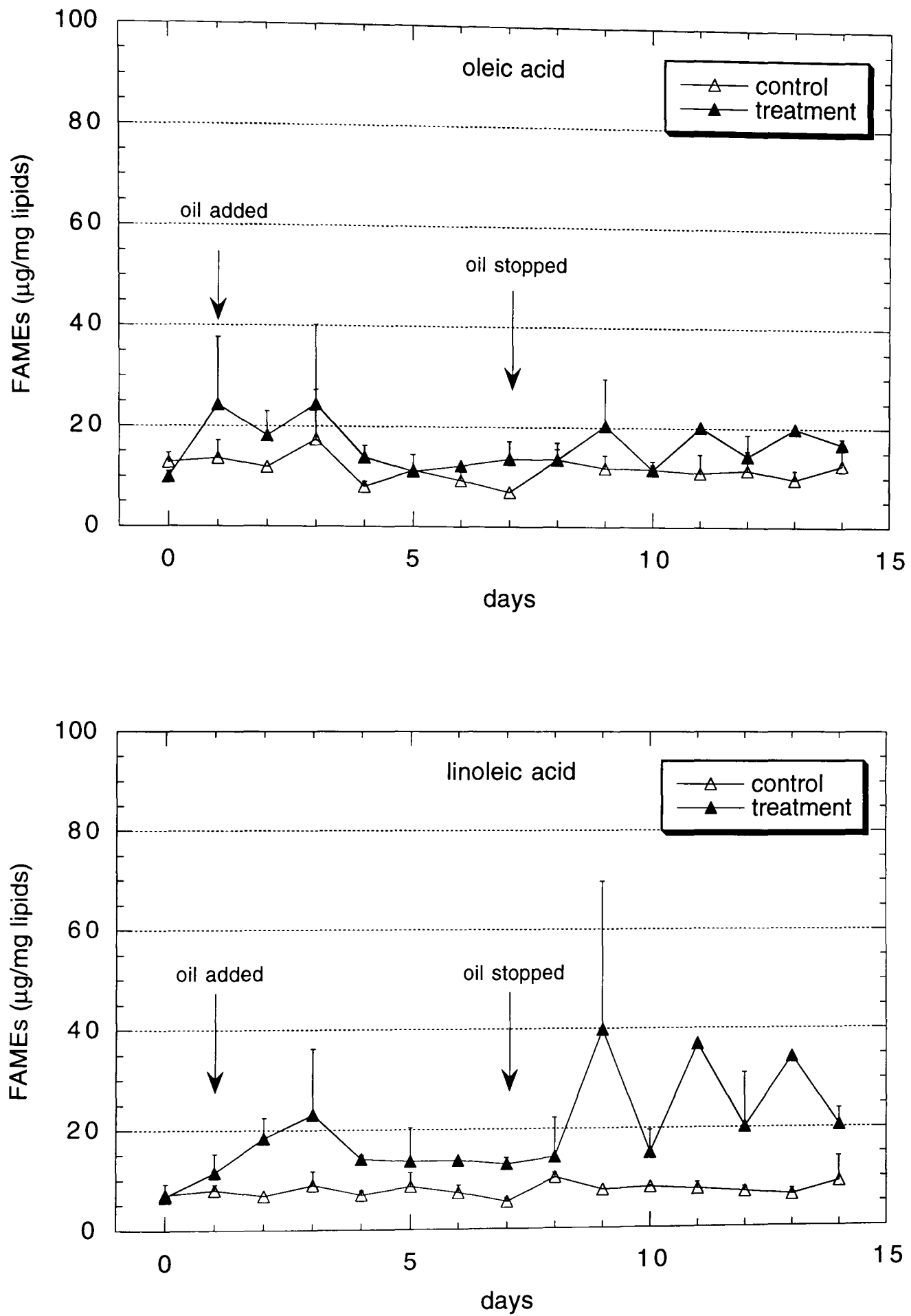


Figure 7.9: Variation of oleic and linoleic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days of the experiment.

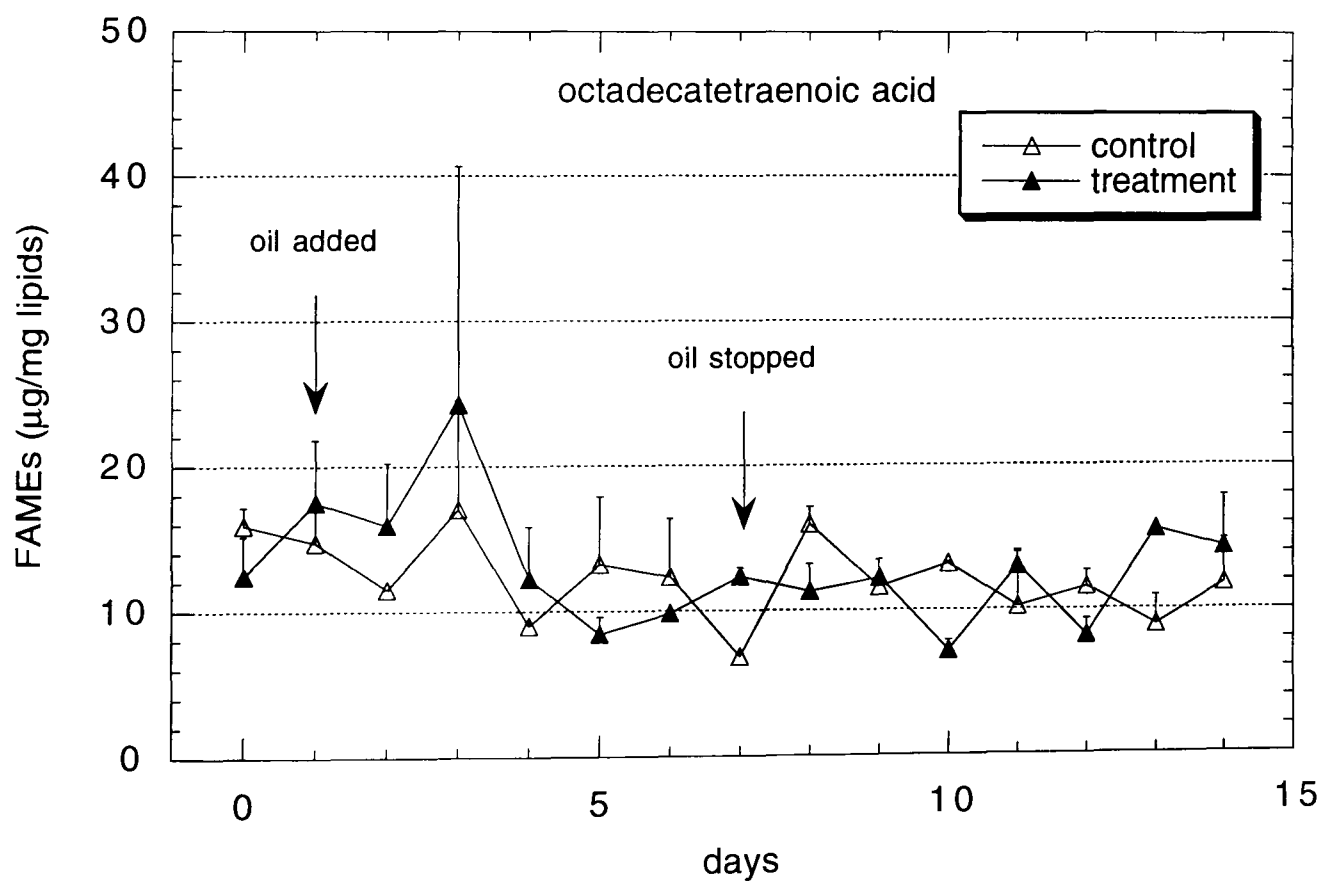
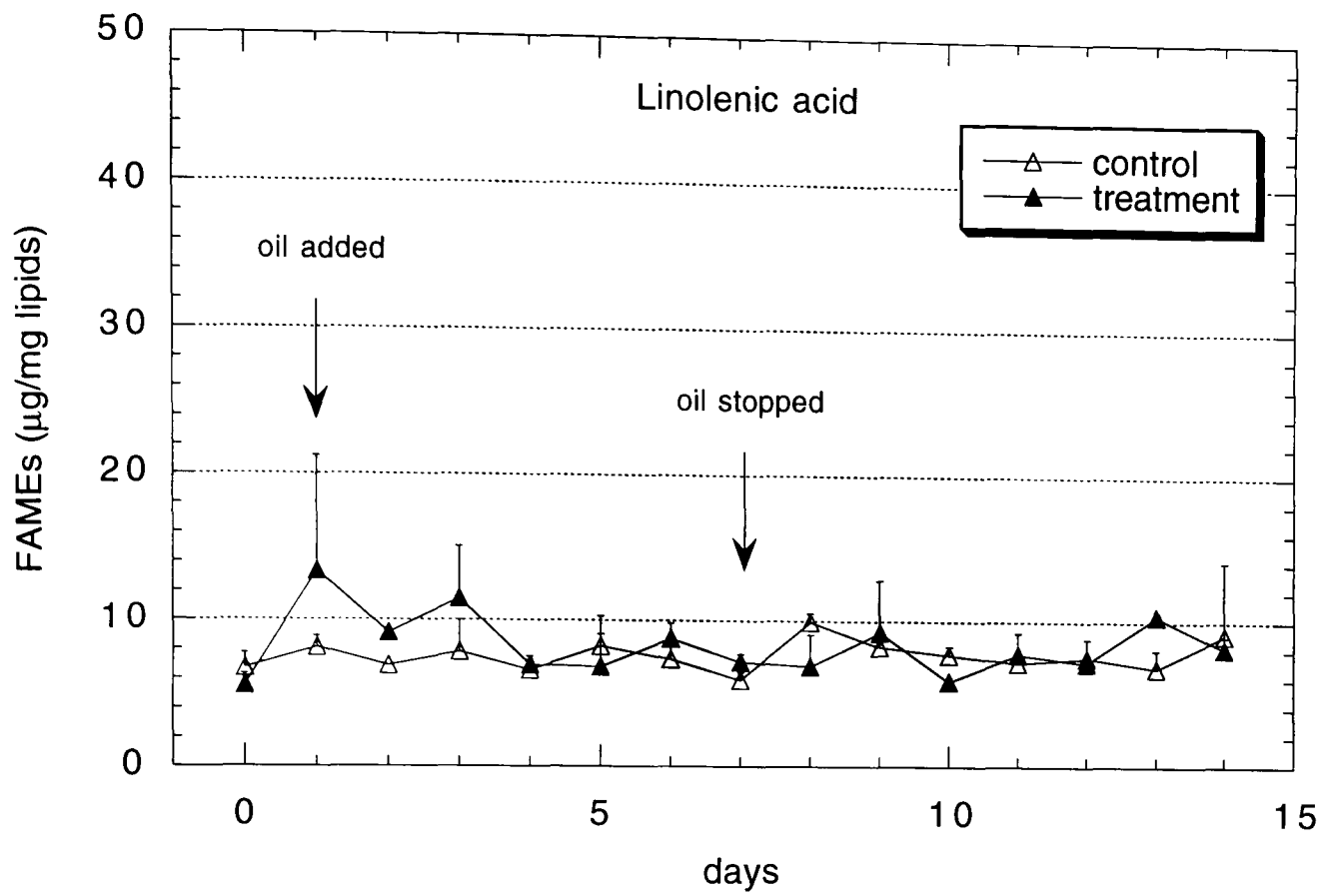


Figure 7.10: Variation of linolenic and octadecatetraenoic fatty acid methyl esters (FAMES) in control mussels and in mussels exposed to sunflower oil for 15 days of the experiment.



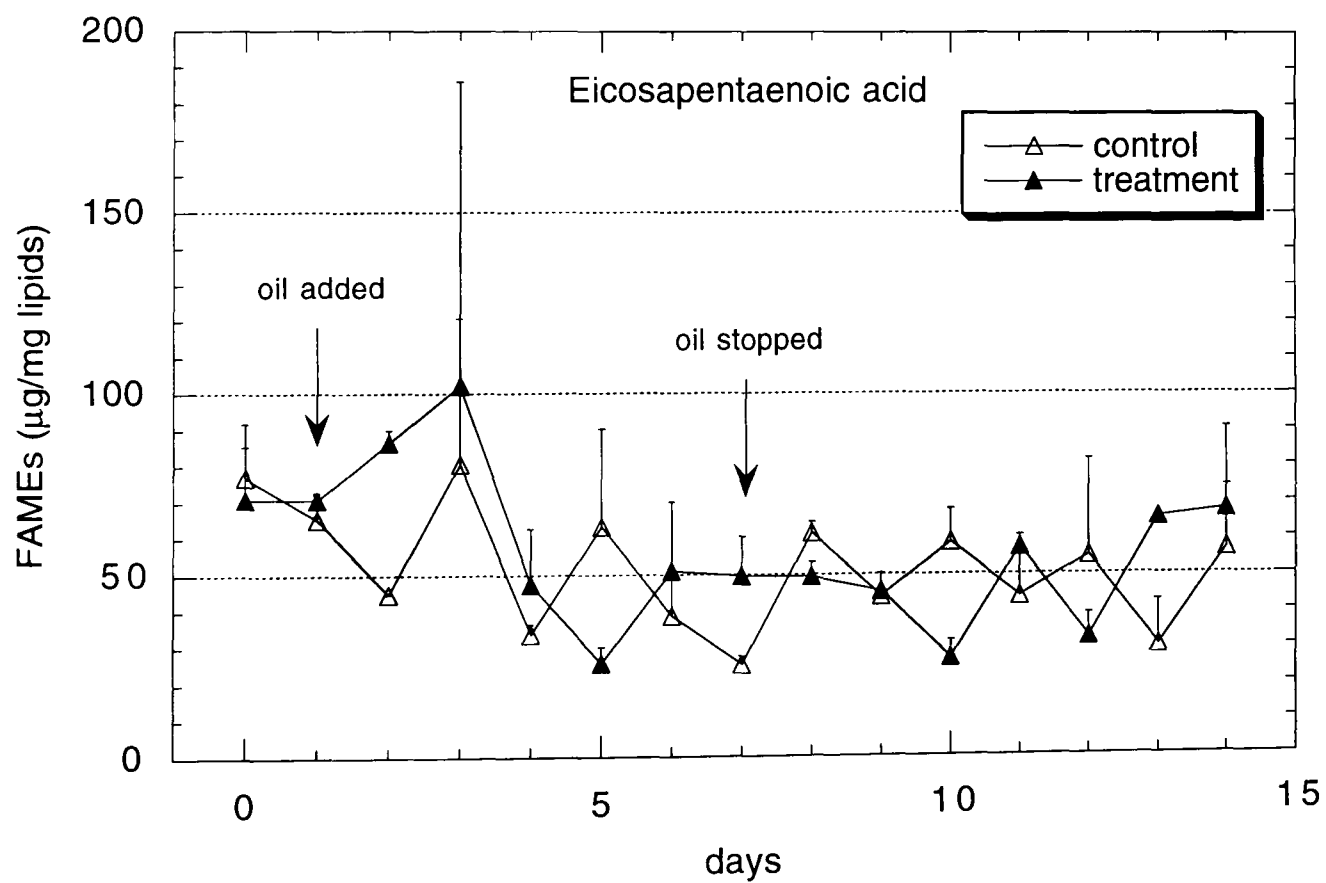
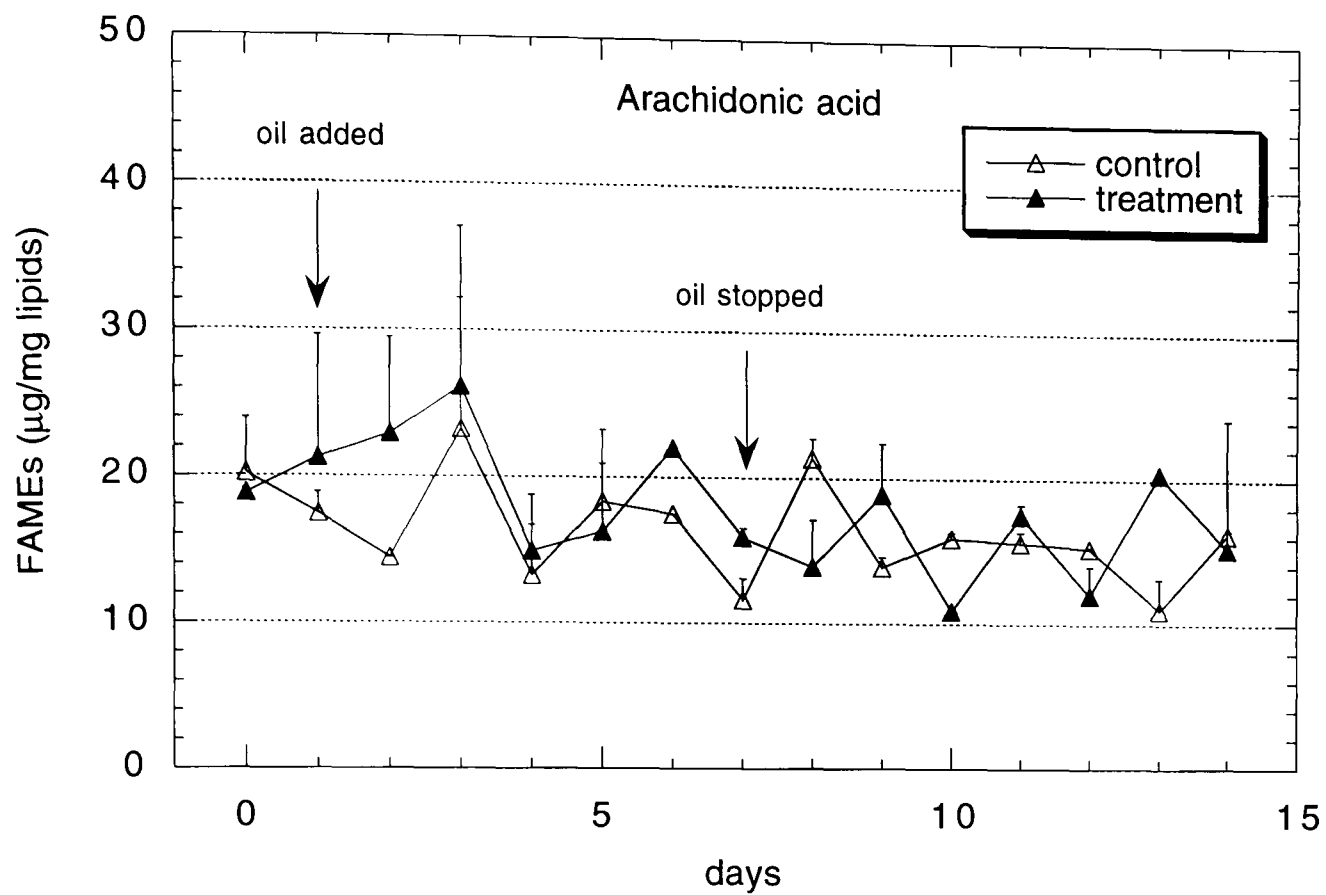


Figure 7.11: Variation of arachidonic and eicosapentaenoic fatty acid methyl esters in control mussels and in mussels exposed to sunflower oil for 15 days of the experiment.

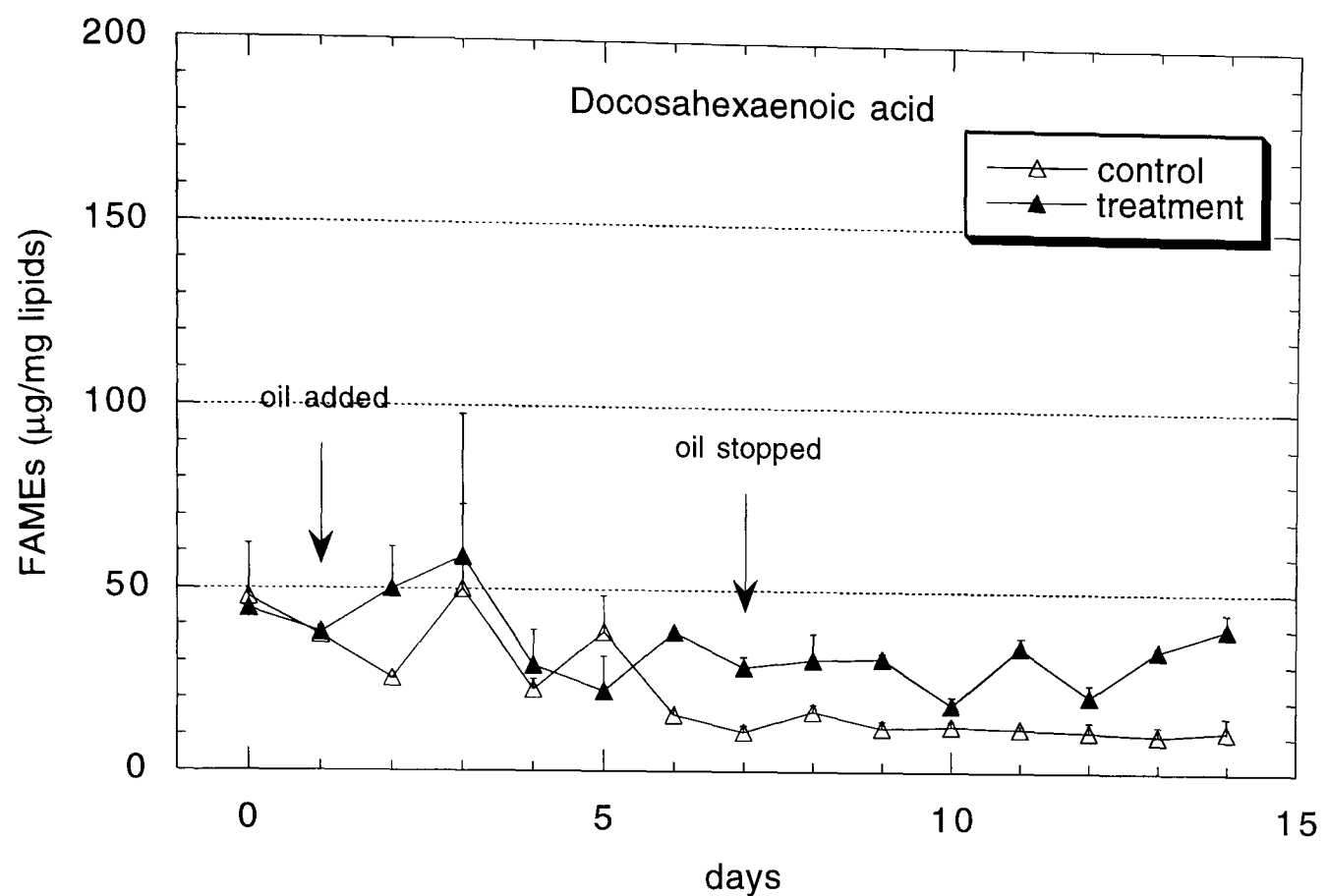


Figure 7.12: Variation of docosahexaenoic fatty acid methyl ester (FAME) in control mussels and in mussels exposed to sunflower oil for 15 days of the experiment.

acids in the control mussels showed no obvious trend with time (Figures 7.8 to 7.12). However, mussels treated with sunflower oil, showed a distinct pattern of fatty acid composition with time. During the first three days all fatty acids increased showing higher concentrations than the control. Linoleic acid in particular exhibited the highest rate of increase ( $4 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids per day); on day 4 there was a decrease which stabilised until day 7 or 8 and then, after the oil was stopped, an oscillation was noted within an interval of concentrations which varied according to the particular fatty acid. For oleic and linoleic acids the interval was wider than for all others.

### 7.3.4 Statistical analysis

Before any conclusions can be drawn from the data, it is imperative that any variations in the fatty acid composition during the experimental period are statistically analysed to verify whether there was an increase in fatty acids in the group of mussels treated with sunflower oil. Two-way analysis of variance was employed. The concentrations of fatty acids in the treatment and control groups were split into two data sets according to the period during which the treatment was received. The first set included fatty acid

concentration during the first 7 days of the experiment when sunflower oil was added to the water in which the mussels were present, whilst the other set included the last 7 days when oil was stopped and the treatment mussels were allowed to return to normal. During the first seven days linoleic acid from mussels treated with sunflower oil was the only fatty acid which significantly differed from the concentration in the control mussels ( $P = 0.002, F = 16.66$ ). For the last 7 days of the experiment, after the oil had been stopped, not only linoleic acid remained significantly higher ( $P < 0.001, F = 23.87$ ) than in the control but also, oleic acid ( $P = 0.008, F = 9.28$ ) and the long chain polyunsaturate docosahexaenoic acid ( $P < 0.001, F = 61.32$ ).

The statistical analysis reveals that linoleic acid indicates the presence of sunflower oil uptake and that mussels continued to accumulate this fatty acid after they were no longer exposed to it. Oleic acid and 22:6 $\omega$ 3 were only significantly higher on the second week when the oil was absent, but both fatty acids showed large variations in concentrations in the control mussels during the first week which decreased considerably in the second week. This variation that did not occur for linoleic acid, contributed to the non significant difference between oleic acid and 22:6 $\omega$ 3 and the control mussels. Since the fatty acid 22:6 $\omega$ 3 is not present in the sunflower oil, an increase during the second week of the experiment relative to the concentration in control mussels suggests that there was metabolism of linoleic acid into this long chain polyunsaturated fatty acid. This must only be apparent because there is no evidence of the presence in marine invertebrates of enzymes which convert  $\omega$ 6 to  $\omega$ 3 fatty acids.

The pattern of fatty acid evolution with time was analysed with a simplified diagram (Figure 7.13) representing a trend of concentrations rather than their calculated values. In this way individual variations are eliminated which make interpretation easier. The first region of the diagram 7.13 corresponds to an uptake of fatty acids from the diet and oil, followed by an active loss on day 4. From day 4 to 7, there was a period when fatty acids were kept constant and after sunflower oil had been removed, on day 7, large variations occurred in linoleic and oleic acids and smaller variations in the other fatty acids.

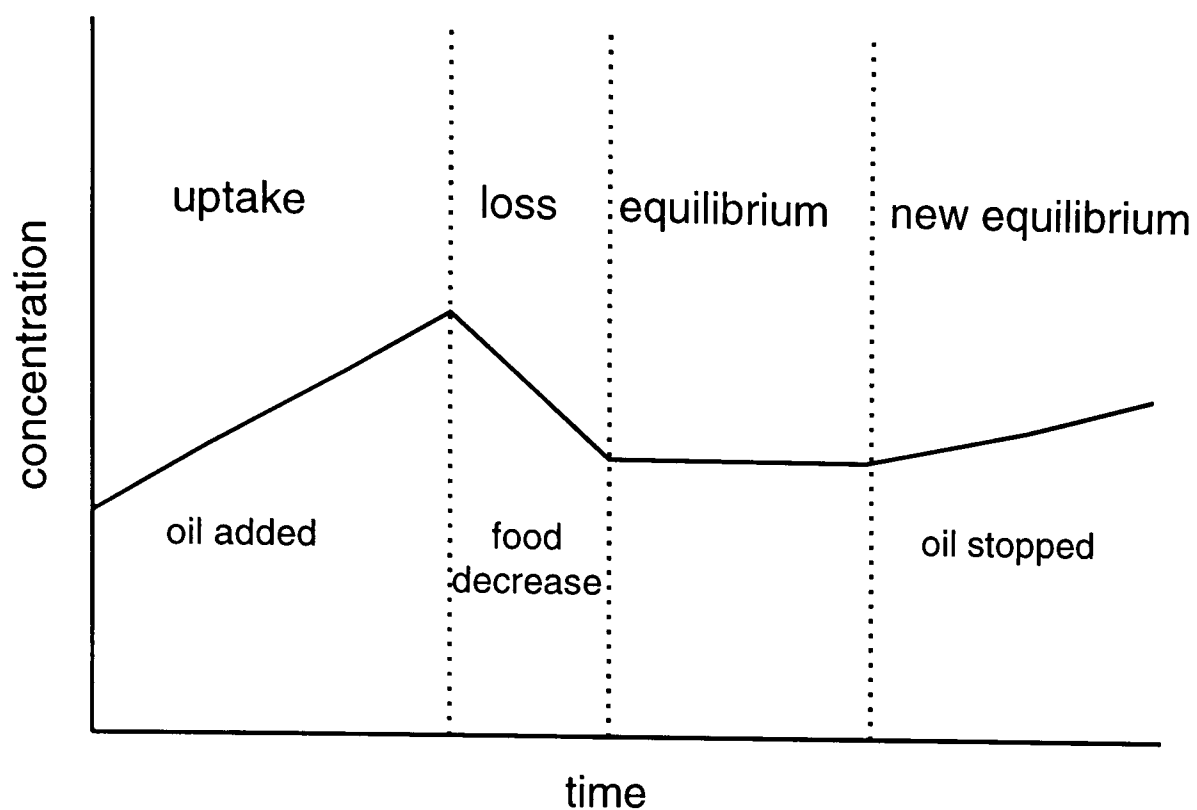


Figure 7.13: Diagram of the concentration trends of fatty acids in mussels exposed to sunflower oil for 15 days of experiment.

## 7.4 Discussion

### 7.4.1 Mussel activity

The three responses of the mussels investigated in this study were designed to show whether sunflower oil inhibited or affected the mussels behaviour. The conclusions from the work suggest that changes in the mussel's behaviour may be indicative of the presence of contaminants. For the present experimental conditions foot extensions but not gaping or shell movement activities were depressed by sunflower oil. Gaping activity was influenced by the addition of microalgae with an increase in the number of mussels gaping on these occasions, however their activity was not affected by the presence of sunflower oil, although there was a tendency for more mussels to close towards the end of the first week of oil exposure, so a longer period of exposure may have a more profound affect. The valve closure response of the freshwater mussel *Dreissena polymorpha* to chemicals, has been demonstrated by Sloof *et al.*, (1983), to be most sensitive to lower concentrations of trace metals. However the detection concentration which triggered valve closure increased when the organisms were pre-exposed to lower concentrations of the chemicals. Similar decrease in susceptibility was observed in *Mytilus edulis* after acclimatization to lower concentrations of copper (Davenport and

Manley, 1978). In addition, the freshwater mussel was more susceptible to metals than to organic compounds (Sloof *et al.*, 1983) indicating a selective sensitivity to specific compounds.

Movement of the mussels was not influenced by exposure to sunflower oil, their activity remaining constant throughout the experiment. Some of the shell valve movements observed were repeated strong movements of gaping and closing of the shell valves which were not related to the presence of the oil since they occurred in both treatment and control groups of mussels. In contrast to the gaping and shell movement activities, foot extension activity decreased in the presence of sunflower oil. Nevertheless the effect was reversed when the presence of the oil was removed. It was noticed during the experiment that only one of the mussels exposed to sunflower oil was not firmly attached by byssus to the bottom of the tank. Foot extensions are an important activity in mussels since it is through this activity that mussels produce byssal threads to attach themselves to the substratum and achieve some limited locomotion (White, 1937; Winkle, 1970). Without byssal threads, mussels become vulnerable to storms and predators and are disadvantaged in areas of strong competition for space. Inhibition of foot extension activity by sunflower oil could therefore, be an undesirable effect which may have consequences at the population level leading in extreme conditions to the loose of purchase on the rock and eventually mortality.

#### **7.4.2 Fatty acids**

Interpretation of the data was difficult because the analyses were performed on individual samples from one mussel introducing the natural variation of concentrations of fatty acids in mussels. However the presence of sunflower oil was reflected by a significant increase in linoleic acid in mussels exposed to the oil compared to the control mussels. The influence of the oil was not limited to the period of time that mussels were in contact with it, but it was prolonged through the following week. The data indicates that the oil was readily taken up by the mussels, as suggested by the rate of increase of linoleic acid of the first few days after exposure to oil. However, this uptake is governed by the amount of food available, since on the fourth day there was a marked decrease of all fatty acids probably due to the lowered number of microalgae cells delivered (Figure

7.2). An active loss of linoleic acid perhaps by metabolism of the oil, suggests an adjustment to reach a concentration of equilibrium which lasted approximately 5 days. After this time the concentration of linoleic acid fluctuated coinciding with the absence of the oil leading to variations in linoleic acid concentrations above those of the control. The overall process resulted in accumulation of the fatty acid relatively to the beginning of exposure and to the control mussels. With oleic acid the same metabolic process could have occurred since its evolution in time was similar to the one observed for linoleic acid. These two fatty acids being the major fatty acids in sunflower oil had exerted a larger influence on the mussels metabolism than other minor fatty acids in the oil like stearic and palmitic acids. The long chain polyunsaturate 22:6 $\omega$ 3 not present in the oil was significantly higher on the second week of the experiment in mussels pretreated with oil than in the control mussels. However, this significance seems to be related with the decrease of the long polyenoic acid in the control mussels rather than a real increase in the treated mussels. Concentrations of 22:6 $\omega$ 3 in the control mussels decreased from about  $50 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids on day zero to  $10 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids on the second week of the experiment, whereas in the treated mussels the decrease of this fatty acid was smaller and concentrations of 22:6 $\omega$ 3 remained close to  $30 \mu\text{g} \cdot \text{mg}^{-1}$  of lipids during the second week of the experiment. The existence of metabolism of the surplus fatty acids i.e. linoleic and oleic acids into longer chain fatty acids was therefore not detected. Unlike the terrestrial mollusc *Cepea nemoralis* (Ackman, 1983) and other aquatic animals (Kanazawa *et al.*, 1979) which are capable of bioconvert linoleic and linolenic acids to highly unsaturated C20 and C22, *Mytilus edulis* and marine bivalves in general, have not been shown to possess this capacity.

## 7.5 Summary

The experiment described in this chapter showed that some biological responses of mussels are selectively influenced by contaminants *i.e.*, sunflower oil exposure did not affect gaping or shell movement activity whereas foot extension activity decreased, as established by video-recording individual mussel behaviour. The fatty acid composition of mussels was monitored at the same time to establish whether the oil could be respon-

sible for any changes in the mussel's activity through confirmation of its presence in the mussel tissues. Linoleic and oleic acids increased in mussels exposed to sunflower oil indicating that they were taking it up. The study of the daily uptake rate of the oil and further metabolism was consequently only possible to put forward as a hypothesis based on the evolution in time of these two fatty acids. The process was described in three phases, the first one of uptake for three days, followed by a decrease of fatty acids in consequence of a decreased number of cells in the diet and loss possibly by metabolism to meet an equilibrium concentration. The third phase consisted in variations of the concentrations in consequence of changing surrounding concentrations due to the removal of sunflower oil. Metabolism of linoleic and oleic acid into the long chain fatty acid 22:6 $\omega$ 3 was not observed despite the significantly higher concentration of the polyenoic fatty acid in treated mussels than in control mussels. The difference was due to a decrease of this fatty acid in control mussels during the second week of experiment.

# Chapter 8

## General conclusions

The effects of the vegetable oils rapeseed, linseed, olive and sunflower oil on mussel performance were investigated during this study. The purpose was the evaluation of the impact of vegetable oil spills in the marine environment using *Mytilus edulis* as bioindicator organism. Growth of mussels exposed to vegetable oils, their tolerance to changing salinities and temperatures after exposure, measured by valve activity and mortality, their behaviour and metabolism during and after the contact with oils were also studied. Fatty acid composition of the different biological samples studied was also determined.

As general conclusions it can be said that all the vegetable oils studied had an inhibitory effect on the growth of *Mytilus*; the growth rates of mussels after four weeks of exposure to the oils was 5 times lower than the growth rate of the control mussels. The mechanism through which vegetable oils affect the growth of mussels was not evaluated in this study. Direct measurements of growth do not provide insight into the physiological components of the energy available for growth such as feeding rate, digestion, respiration and excretion which allow the assessment of the mode of action of the pollutant (Widdows and Johnson, 1988). Vegetable oils have not been the subject of much research regarding toxic effects on marine organisms. However other classes of pollutants have been described as toxic, acting through different mechanisms of toxicity in bivalves e.g. hydrocarbons affecting the ciliary feeding activity by non-specific narcosis (Donkin *et al.*, 1989) and affecting membrane structure and function which reflect on the processes of food digestion and absorption (Widdows *et al.*, 1987); copper affecting the neural control of gill cilia (Howell *et al.*, 1984); TBT (Snoeij *et al.*, 1987) and phe-



nols (Buikema *et al.*, 1979) uncoupling the oxidative phosphorylation with consequent increase of the respiration rate; DBT (Snoeij *et al.*, 1987) and hypoxia (Widdows and Donkin, 1989) inhibiting the oxidative metabolism thus reducing respiration rate. Before any of these toxic effects can be attributed to vegetable oils, more investigation is required.

Bivalve molluscs contain within their shells a complete record of their growth history in the form of microgrowth patterns (Richardson *et al.*, 1979; Richardson, 1989). Changes in environmental conditions e.g. fluctuations in seawater temperature (Richardson *et al.*, 1980b), food availability, salinity, tidal height on the shore (Richardson *et al.*, 1980a), detrimental algal blooms, storm conditions, predator attacks, spawning events and anthropogenic inputs (Thompson and Richardson, 1993). The present work, also confirms that the presence of organic compounds of anthropogenic origin such as vegetable oils have an effect on shell growth and calcium deposition pattern in *Mytilus edulis*, hence growth can be evaluated. Analysis of acetate peels of polished and etched radial sections of *Mytilus edulis* shells exposed to vegetable oils, revealed a disturbance to the normal pattern of calcium deposition. Marked microgrowth bands were deposited during the period of oil treatment and periostracum deformities were observed, whilst microgrowth bands deposited prior to and after treatment were regularly spaced. The control shell by contrast to the treated shells, exhibited through all the experimental period, a regular and weak appearance of banding. These observations confirm the adverse effects of vegetable oils in calcium deposition and shell growth of *Mytilus edulis*.

Mortality was observed in mussels exposed to all the vegetable oils after one week in aerated conditions, whereas all the control mussels survived. There seem to be a dependence of mortality on time of exposure and concentration or type of oil, since mortality was higher in the mussels exposed to the highest flow rate of linseed oil and deaths started to occur after one week. This finding was confirmed by the second exposure to sunflower oil during one week when no mortalities were recorded. Mortality can increase by poor oxygenation of the water, as the oils polymerizing at the surface of the water reducing the oxygen exchange between the air-water interface. The increased mortality recorded in mussels in the bowls exposed to the oils with no aeration, was attributed to smothering when the removal of the polymerized oils accumulated at the

water surface was interrupted. Furthermore, examination of the internal shell of dead and alive mussels subjected to this conditions revealed the loss of the lustre and a chalky appearance which indicate shell dissolution caused by anaerobically production of acid (Crenshaw, 1980). At the temperature of 19 °C the mortality observed in all groups of mussels was probably caused by low oxygen tension due to the increased temperature and absence of aeration, but a thermal shock of 9 °C should also be considered as a possible cause of mortality. Temperatures between 3 and 20 °C promote the growth of *M. edulis* (Almada-Villela *et al.*, 1982) therefore they could not per se be the cause of mussel mortality. At temperatures 5 °C and 10 °C (isothermal) the deaths recorded were in mussels pre-exposed to sunflower oil. Although it may mean that mussels pre-exposed to sunflower oil were less able to adapt to different temperatures than the other groups of mussels, no additive effect of the two factors treatment and temperature was observed *e.g.*, mortality did not increase for the highest temperature.

In terms of mussel tolerances to changing salinities and temperatures, after oil exposure, only mussels pre-exposed to sunflower oil showed longer gaping times than the control mussels suggesting the need for depuration or active filtration to compensate possible periods of valve isolation. This behaviour also suggests a possible mode of action of vegetable oils in the respiration rate of mussels. Mussels exposed to the other oils also showed a tendency to spend longer periods of time gaping but their gaping times were not significantly different from the ones exhibited by the control mussels. Whether this effect was related to the type of oil or not, could not be clear in this investigation.

All the control mussels responded by closing their valves at a salinity of 10‰ whereas only 70 to 90% of the mussels pre-exposed to the oils closed their valves at that salinity. This finding suggests that vegetable oils may increase mussel tolerance to lower salinities either by an inhibition of the salinity receptors or just due to the increased need for filtration to compensate prolonged periods of valve closure. The salinity at which valve adduction was induced is in agreement to that determined by Davenport, (1979).

Mussel behaviour was also affected by sunflower oil exposure. The number of mussels exhibiting foot extensions decreased under the presence of sunflower oil. As the

foot together with the byssal gland have an important function in providing byssal attachment, if foot extension activity is inhibited by vegetable oils, the ability of mussels to attach themselves to the substratum may become impaired and survival endangered by dislodgment (Seed, 1976). Observations made on mussels exposed to sunflower oil indicated that some of them had a small number of byssal threads laid down.

The fatty acid composition of mussels was altered by exposure to the vegetable oils to reflect the fatty acid composition of the oils. The abundant fatty acids in the mussels, palmitic, eicosapentaenoic and oleic acids changed with the microalgae food and especially with the presence of the vegetable oils. After 4 weeks of being fed on a phytoplankton diet, the polyunsaturates eicosapentaenoic acid and docosahexaenoic acid increased in control mussels and the monounsaturate oleic acid decreased. The predominant fatty acids in mussels, after 4 weeks of exposure to the vegetable oils and fed with the same diet as the control mussels, were the abundant fatty acids in the oils: oleic in rapeseed and olive, linoleic in sunflower and linolenic in linseed. The influence of the oils was greater in mussel neutral lipids than in their polar lipids. Mussel polar lipids had an increase in the fatty acids from the oils but their typical composition was less altered than in the neutral lipids. Palmitic acid remained the predominant fatty acid for most of the time of exposure and the polyunsaturated eicoapentaenoic and docosahexaenoic acids were also present in high concentrations.

During the time of exposure to the vegetable oils, fatty acid composition of mussels was influenced by the oils and the increased fatty acids remained high with the time of exposure. Changes in fatty acid composition were only observed when the vegetable oils were removed and mussels were transferred to clean seawater. The oil markers of higher degree of unsaturation, e.g. linolenic acid were the first to decrease in three days of oil decontamination. In the course of the depuration process, linoleic acid followed in decreasing concentration after ten days and only oleic acid remained high in concentration after this time.

Temperature, in addition to the depurating clean seawater, also promoted changes in fatty acid composition of mussels. The lowest temperature of 5 °C stimulated the decrease of linolenic and linoleic acids in mussels pre-treated with linseed and sunflower oils, respectively and the highest temperature of 19 °C apparently promoted

the elongation of linoleic acid into eicosadienoic acid in mussels previously treated with sunflower oil. This reaction only involves the addition of two carbon units within the same fatty acid family and not desaturases which are believed to be absent in invertebrates (Ackman and Ratnayake, 1989). Fatty acid profile of mussels pre-exposed to olive oil was not influenced by temperature treatment, remaining essentially constant under the three different temperatures.

Analysis of fatty acids in dead mussels have identified two routes of metabolism. One route of oxidative degradation following the death of mussels where the unsaturated oil markers were oxidized to saturated and lower unsaturated fatty acids; the other route of active mussel oxidation or elongation depending on the more concentrated fatty acids accumulated from exposure to the vegetable oils.

The metabolic processes carried out by mussels exposed to vegetable oils and transferred to clean seawater seem to be an uptake of fatty acids during oil exposure followed by the loss of the more concentrated fatty acids after oils being removed and further oxidation or elongation with time and temperature.

Uptake of sunflower oil during one week exposure occurred from the first day and the concentration of fatty acids from the oil increased for the first 3 days, after which there was a loss and the establishment of an equilibrium concentration. The second week of experiment consisting of decontamination with clean seawater showed an oscillating concentration of linoleic and oleic acids still increased relative to the concentrations in control mussels.

The *in vitro* metabolic incubations of mussel mantles homogenates with linseed and sunflower oils for 48 hours also confirmed an increased uptake of the oils and no metabolic reactions during the period of contact with the oils.

All the experiments involving fatty acid analysis and their metabolism pointed out for an uptake of fatty acids in the presence of the vegetable oils with no evident metabolism during oil exposure and active metabolic reactions of oxidation and elongation just after removal of the oils. These reactions however do not confirm the existence of the enzyme systems present in higher animals e.g.  $\Delta 6$ ,  $\Delta 5$  and  $\Delta 4$  desaturases (Gurr and Harwood, 1991), required for extensive desaturation of short chain fatty acids to long polyunsaturated fatty acids. Moreover, studies on enzymatic activity of acetyl-CoA

carboxylase and fatty acid synthetase, the two enzymatic systems involved in fatty acid biosynthesis (Kluytmans *et al.*, 1985), demonstrated very low or absent biosynthesis of fatty acids throughout the year. The biochemical mechanisms for fatty acid formation being only activated in the later stages of gametogenesis, especially in the mantle of female animals. Activation of the enzymatic systems were only detected under starvation conditions and exclusively in mantle tissues. The function of lipids in the metabolism of *Mytilus edulis* seem to be important in the maintenance of an adequate supply and breakdown of energy. As an ample supply of fatty acids is provided with the food, their biosynthesis is suppressed. When dietary intake is very low, during late autumn and winter, lipid biosynthesis becomes active (Kluytmans *et al.*, 1985).

This investigation has not exhausted the theme, on the contrary, it opened way for possible further studies to increase the knowledge about the effects of vegetable oils on marine organisms. Some physiological and chemical aspects can be looked at to improve the knowledge on the mechanisms of toxicity of vegetable oils. Filtration rate and byssus production are two responses that maybe affected by these contaminants. The measurement of enzymatic systems activity is another response that may help to identify in which type of contaminants vegetable oils can be included and which are the most likely routes for their elimination from living tissues. The effects of vegetable oils on mussel reproduction and larvae settlement and growth are also aspects of significant importance at the population level.

In the event of another sunflower oil spill similar to that of the *Kimya* the possible consequences can be summarized as:

1. Mussel mortality due to smothering, in areas of low hydrodynamic conditions and/or oil toxicity.
2. Reduced growth of the mussels with economical implications in mussel farms.
3. Possible increased susceptibility to other pollutants and predation due to stress including increase in gaping.
4. Smaller size and loss of stock in mussel farms and expected alteration of the organoleptic properties of the mussels in consequence of changes in fatty acid

composition.

As final recommendations, authorities responsible for the elaboration of spill contingency plans, should be aware of the above physiological effects of vegetable oils on mussels and act accordingly in order to protect mussel cultures.

# Appendix A

This appendix contains the raw data in pixels of the mussel shells and the dimensions of the photographed reference object used to calculate mussel shell areas in  $\text{mm}^2$  and the picture magnification factors (F).

Control group											
T	R	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
0	1	23931	24385	24786	23723	29005	20162	25127	24015	27657	22143
0	2	23936	24401	24801	23713	29023	20140	25178	23983	27690	22190
1	1	23840	24195	25055	23464	28703	20665	25162	23749	27298	21653
1	2	23796	24115	25061	23402	28623	20713	25096	23845	27283	21768
2	1	24509	24875	26048	23773	29140	22045	25784	24455	27763	22937
2	2	24672	25059	26296	24103	29493	22280	26050	24699	27932	23006
3	1	25168	25871	27394	24220	29079	23308	26266	24976	28164	22914
3	2	25292	25942	27436	24222	29130	23362	26412	25061	28299	23070
4	1	25461	26960	28199	24713	29547	24457	26795	25929	29139	23208
4	2	25520	26951	28015	24518	29523	24484	26955	25969	29139	23156
8	1	25711	27519	28432	24726	29600	24637	26876	26116	29828	23367
8	2	25878	27661	28521	24823	29653	24706	26887	26017	29786	23385

Table A.1: Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) for 8 weeks (T) in the control group.

Rapeseed oil treatment											
T	R	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
0	1	19592	20699	15093	14546	21579	23309	17884	21408	19956	15336
0	2	19625	20771	15079	14508	21582	23325	17790	21406	19887	15302
1	1	19361	20505	14760	14322	21514	23184	17746	21053	19404	14325
1	2	19440	20572	14901	14448	21581	23348	17781	21090	19438	14463
2	1	19649	20628	15028	14494	21813	23594	17896	21402	19736	15204
2	2	19653	20715	15034	14482	21767	23517	17908	21556	19932	15358
3	1	19883	20867	15112	14688	21963	23477	17923	21582	19923	15151
3	2	19905	20856	15135	14706	21974	23509	17910	21548	19990	15110
4	1	20016	20702	14938	14581	22058	23366	18021	21653	20193	15460
4	2	20017	20681	14929	14585	22024	23418	17988	21666	20181	15423
8	1	20568	21031	15039	14779	22056	0	18032	21447	19942	14901
8	2	20583	21051	15073	14819	22086	0	18153	21543	19970	15005

Table A.2: Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with rapeseed oil for 8 weeks (T).

Linseed oil treatment											
T	R	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
0	1	17358	19947	14805	13755	13637	11927	12949	13030	12598	12350
0	2	17469	19895	14828	13735	13597	11947	12965	13057	12589	12324
1	1	17293	19687	14686	13552	13487	11830	12653	13037	12205	11927
1	2	17322	19729	14813	13675	13571	11918	12757	13155	12393	12031
2	1	17372	19942	14921	13796	0	12069	12936	13195	12584	0
2	2	17238	19894	14866	13720	0	12027	12915	13137	12483	0
3	1	17432	0	14874	0	0	12047	12815	0	12571	0
3	2	17485	0	14877	0	0	12076	12826	0	12656	0
4	1	17222	0	14723	0	0	11927	0	0	12552	0
4	2	17266	0	14774	0	0	11916	0	0	12551	0
8	1	17459	0	0	0	0	11824	0	0	12477	0
8	2	17487	0	0	0	0	11831	0	0	12416	0

Table A.3: Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with linseed oil for 8 weeks (T).



		Olive oil treatment									
T	R	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
0	1	16787	18908	14016	17381	14757	13315	14730	14916	15408	12354
0	2	16853	18998	14102	17503	14828	13391	14811	14985	15455	12378
1	1	16733	19120	13906	17494	14941	13383	14552	14736	15384	12398
1	2	16766	19170	13930	17476	14860	13375	14448	14657	15348	12338
2	1	16806	19565	14203	17567	15114	13686	14776	14814	15550	13122
2	2	16883	19588	14220	17598	15129	13661	14784	14805	15566	13145
3	1	17097	19790	14176	17783	15199	13799	14752	14936	16096	13470
3	2	17098	19824	14185	17760	15171	13789	14758	14919	16067	13475
4	1	16735	20058	14124	17635	15017	13619	14643	14797	16287	13837
4	2	16743	19981	14058	17623	14982	13584	14678	14845	16276	13802
8	1	17401	20457	14175	17782	15039	13625	14755	0	16123	13639
8	2	17385	20404	14151	17728	14987	13611	14746	0	16112	13667

Table A.4: Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with olive oil for 8 weeks (T).

		Sunflower oil treatment									
T	R	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
0	1	17936	19621	25234	23458	23155	21043	20342	21221	18287	15977
0	2	17955	19658	25260	23436	23154	21069	20346	21210	18268	15983
1	1	17845	19599	25116	23639	23056	21034	20379	20985	17971	15591
1	2	17833	19475	25136	23693	23081	21061	20478	21060	18022	15637
2	1	18091	19602	0	23820	23406	21347	21125	21572	18324	15891
2	2	18088	19597	0	23892	23403	21283	21053	21492	18220	15819
3	1	18791	19838	0	24036	23833	21428	21990	22295	18877	16154
3	2	18824	19841	0	24057	23789	21351	21944	22231	18837	16077
4	1	19065	19566	0	23778	23885	21237	22888	22958	18924	16135
4	2	19110	19612	0	23760	23855	21234	22862	22913	18863	16079
8	1	19651	19722	0	23882	24560	21136	23928	22640	19159	16351
8	2	19697	19724	0	23835	24465	21029	23835	22611	19077	16363

Table A.5: Area in pixels of 10 mussels (m1-m10), from 2 pictures (R) treated with sunflower oil for 8 weeks (T).

Week	Control		Rapeseed		Linseed		Olive		Sunflower	
	Pic (pixels)	Real (mm)	Pic (pixels)	Real (mm)	Pic (pixels)	Real (mm)	Pic (pixels)	Real (mm)	Pic (pixels)	Real (mm)
0	584	48.10	588	48.60	593	49.20	591	49.20	594	49.50
1	605	50.40	604	50.40	603	50.40	603	50.40	603	50.40
2	606	50.40	604	50.40	604	50.40	603	50.40	604	50.40
3	606	50.40	605	50.40	605	50.40	605	50.40	607	50.40
4	604	50.40	606	50.40	606	50.40	606	50.40	606	50.40
8	608	50.40	608	50.40	608	50.40	608	50.40	609	50.40

Table A.6: Length of reference object as measured in the picture (Pic) and measured with calipers (Real).

# Appendix B

Selected statistical tests on fatty acid concentrations of mussel mantles incubated with linseed oil are shown. Analysis of variance and Tukey's pairwise comparisons tests were used.

```
MTB > Oneway '18:0' 'hours';
SUBC> Tukey 5.
```

```
ANALYSIS OF VARIANCE ON 18:0
SOURCE      DF      SS      MS      F      p
hours       5      653.99  130.80  77.56  0.000
ERROR       30      50.59   1.69
TOTAL       35      704.58
```

```
INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV  -----+-----+-----+-----
  0         6      24.979    2.174  (---*---)
  1         6      12.831    0.992  (---*---)
  3         6      12.898    1.384  (---*---)
  6         6      13.623    0.577  (---*---)
 24         6      14.230    0.591  (---*---)
 48         6      16.346    1.345  (---*---)
-----+-----+-----+-----
POOLED STDEV = 1.299      12.0      16.0      20.0      24.0
```

Tukey's pairwise comparisons

Family error rate = 0.0500  
Individual error rate = 0.00487

Critical value = 4.30

Intervals for (column level mean) - (row level mean)

	0	1	3	6	24
1	9.869				
	14.428				
3	9.802	-2.347			
	14.361	2.213			
6	9.077	-3.072	-3.005		
	13.636	1.488	1.555		
24	8.470	-3.679	-3.612	-2.887	
	13.029	0.881	0.948	1.673	

```

48      6.354   -5.794   -5.727   -5.002   -4.395
      10.914   -1.235   -1.168   -0.443    0.164

```

MTB > Oneway '18:1w9' 'hours';  
SUBC> Tukey 5.

ANALYSIS OF VARIANCE ON 18:1w9

SOURCE	DF	SS	MS	F	p
hours	5	14118.0	2823.6	94.89	0.000
ERROR	30	892.7	29.8		
TOTAL	35	15010.7			

INDIVIDUAL 95 PCT CI'S FOR MEAN  
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV	
0	6	115.08	8.87	(---*)
1	6	57.89	4.57	(-*)
3	6	59.44	6.10	(---*)
6	6	62.75	2.74	(-*--)
24	6	65.20	3.09	(---*)
48	6	75.23	4.97	(---*)

POOLED STDEV = 5.45

60                      80                      100                      120

Tukey's pairwise comparisons

Family error rate = 0.0500  
Individual error rate = 0.00487

Critical value = 4.30

Intervals for (column level mean) - (row level mean)

	0	1	3	6	24
1	47.62				
	66.77				
3	46.07	-11.13			
	65.22	8.03			
6	42.76	-14.44	-12.89		
	61.91	4.72	6.27		
24	40.31	-16.89	-15.34	-12.03	
	59.46	2.27	3.82	7.13	
48	30.27	-26.92	-25.37	-22.06	-19.61
	49.42	-7.77	-6.22	-2.91	-0.46

MTB > Oneway '18:2w6' 'hours';  
SUBC> Tukey 5.

ANALYSIS OF VARIANCE ON 18:2w6

SOURCE	DF	SS	MS	F	p
hours	5	7819.5	1563.9	53.87	0.000
ERROR	30	870.9	29.0		
TOTAL	35	8690.4			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
0	6	87.050	10.751	-----+-----+-----+-----+-----+ (--*--)
1	6	44.818	2.764	(--*--)
3	6	45.473	5.097	(--*--)
6	6	48.108	1.997	(--*--)
24	6	49.432	2.391	(--*--)
48	6	56.914	3.909	(--*--)

POOLED STDEV = 5.388

45                  60                  75                  90

Tukey's pairwise comparisons

Family error rate = 0.0500  
Individual error rate = 0.00487

Critical value = 4.30

Intervals for (column level mean) - (row level mean)

	0	1	3	6	24
1	32.774 51.690				
3	32.118 51.035	-10.114 8.803			
6	29.484 48.400	-12.748 6.168	-12.093 6.824		
24	28.159 47.076	-14.073 4.844	-13.418 5.499	-10.783 8.134	
48	20.678 39.595	-21.554 -2.637	-20.899 -1.982	-18.264 0.653	-16.939 1.977

MTB > Oneway '18:3w3' 'hours';  
SUBC> Tukey 5.

ANALYSIS OF VARIANCE ON 18:3w3

SOURCE	DF	SS	MS	F	p
hours	5	143249	28650	57.84	0.000
ERROR	30	14861	495		
TOTAL	35	158110			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
0	6	373.27	44.82	-----+-----+-----+-----+-----+ (--*--)
1	6	186.88	12.41	(--*--)
3	6	198.60	20.34	(--*--)
6	6	210.38	8.95	(--*--)
24	6	218.77	9.33	(--*--)
48	6	255.85	15.11	(--*--)

POOLED STDEV = 22.26

210                  280                  350                  420

Tukey's pairwise comparisons

Family error rate = 0.0500  
 Individual error rate = 0.00487

Critical value = 4.30

Intervals for (column level mean) - (row level mean)

	0	1	3	6	24
1	147.3 225.5				
3	135.6 213.7	-50.8 27.3			
6	123.8 202.0	-62.6 15.6	-50.8 27.3		
24	115.4 193.6	-71.0 7.2	-59.2 18.9	-47.5 30.7	
48	78.4 156.5	-108.0 -29.9	-96.3 -18.2	-84.5 -6.4	-76.2 2.0

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