DOCTOR OF PHILOSOPHY

Bacterial degradation of linseed and sunflower oils in salt marsh sediments.

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Award date:
1999

Awarding institution:
Bangor University

Bacterial Degradation of Linseed and Sunflower Oils in Salt Marsh Sediments

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

by

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January 1999

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Summary

This work investigated the consequences of vegetable oils spills in salt marsh sediments. The role of autochthonous bacteria in the oils degradation and degradative pathways were also studied '*in situ*' and '*in vitro*'.

Simulated spills of sunflower and linseed oils revealed that both oils penetrated the sediments at a rate of 10^{-7} cm^2 s^{-1}. However, whereas 60% of the linseed oil had disappeared from the sediments after 2 months most of the sunflower oil remained after 6 months. Differences were noted in the adsorption of the oils to sediment particles and the depth at which they accumulate and these factors most likely influenced the route of the oil degradation and the sediments properties such as permeability. The contamination of the sediments with vegetable oils lead to a noticeable reduction in the abundance of plant roots and infauna.

The abundance of aerobic, anaerobic and sulphate reducing bacteria in the sediments was increased by the addition of both oils, with linseed oil supporting greater bacterial density than sunflower oil. During the course of the experiment the relative abundance of oil degrading bacteria also increased. As a consequence of the increased bacterial activity, the sediments pH and Eh decreased and anoxic conditions were established, earlier in the case of linseed than that of sunflower oils.

The degradation of the oils appeared to be a sequential process, initiated by the aerobic and/or anaerobic bacteria and continued by the sulphate reducing bacteria which themselves where unable to utilise the raw oils. The original composition of both oils underwent alterations mostly associated with their main fatty acid: the concentration of 18:3ω3 and 18:2ω6 in linseed and sunflower oil, respectively, decreased whereas that of the remaining fatty acids increased.

As a result of the bacterial degradation of the vegetable oils 'new' fatty acids were detected and their identification was attempted using GC-MS analysis of their picolinyl and methyl esters. Various degradative pathways of linseed and sunflower oils involving the formation of the 'new' fatty acids are suggested with isomerisation, hydrogenation and β-oxidation as the primary routes for the degradation.

Contents

Accompanying Material

Pereira, M.G.; Mudge, S.M. & Latchford, J. 1998. Bacterial Degradation Of Vegetable Oils. *Chem. Ecol.* **14**: 291-303.

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Acknowledgments

Firstly I would like to express my gratitude to Drs. Stephen Mudge and John Latchford for their competent supervision, support and encouragement throughout this study.

I am also indebthed to Dr. David J. Harvey (Oxford University, Department of Pharmacology) for is helpful contribution to the identification of the picolinyl esters of the 'new' fatty acids. Dr. Sinclair Buchan assisted with the determination of the sediments permeability for which he is thanked; and Dr. George Floodgate's advice on the sediments microbiology is much appreciated.

Mr. Rhind (Countryside Council for Wales) and Dr. Eifion Jones provided invaluable information concerning Foryd Bay for which they are gratefully acknowledged. S. Wallis is thanked for his help with the diffusion coefficient equation.

I have received the assistance from several members of the technical staff from the School of Ocean Sciences and John Rowlands and Ian Pritchard are specially thanked for their help in the field and laboratory work. I am also grateful to John East for his assistance with the GC-MS and Sylvia East, Vivienne Ellis and Sandra Hague's help at different stages of this work is sincerely appreciated. The construction of equipment by the workshop people for this work is also thanked.

I wish to thank Unitrition International Limited, for their kind donation of the linseed oil used in the field and laboratory experiments.

Finally, I would like to thank specially Rubina Rodrigues for her continued support during this study. Her comments and proof reading of earlier drafts of this thesis were invaluable, for which I am deeply grateful.

This research work was funded by Junta Nacional de Investigação Científica e Tecnológica, Portugal (PRAXIS XXI - CIENCIA - BD/2807/93-IG).

1. INTRODUCTION

1.1. COMMERCIAL USE OF VEGETABLE OILS

Oils and fats are used in the food industry for the manufacture of a wide variety of products ranging from margarines to chocolate or used directly as salad and cooking oils. However, a significant proportion (about 20%) is used in the oleochemicals industry for the manufacture of an equally wide range of products: soap, detergents, toiletries, plastics, paints, ink, *etc*. (Ratledge, 1994). About 75% of the total fats and oils are derived from plants (of which soybean, palm, sunflower and rape oils account for over 70%) and the remainder are from animals which encompass lard, tallow and marine oils (Ratledge, 1994).

Between the mid thirties and the mid eighties, the world exports of edible oils increased five fold from 4 to 20 million tonnes per year (Gunstone, 1989). The increase was due almost entirely to only four vegetable oils: soya, palm, sunflower and rape. The export of all the others vegetable oils rose from 3.1 to only 4.5 million tonnes for the same period, while the growth leaders increased almost 20 fold from 0.8 to 15.2 million tonnes. Soya became significant during 1950's and palm, sunflower and rape oils during the 70's (Gunstone, 1989). World consumption of vegetable oils has increased 4% per year over the

last 15 years, driven mainly by population growth and improved income in large parts of the world and it has been predicted that in the year 2000, world production of vegetable oils and fats will have increased to 85 million tons (van Duyvenbode, 1995). Countries like Malaysia will increase their production of palm oil from 5 to 8 million tonnes per year (Ong, 1989). According to Ong, palm oil will have a major role to play in satisfying the world demand for edible oils because oil palm is the most productive oil producing plant in the world (Gunstone, 1994a). Others oils like rapeseed are expected to be potential competitors. It is also expected that China will have greater demands of edible oils than the domestic supply, creating export opportunities for other countries, namely Canada (Gunstone, 1994b). China is expected to produce a total of 11.7 million tonnes of oils and fats per year from domestic crops by 2004/2005, about 9 million tonnes being seed oils. Consumption of edible oils and fats is projected to reach about some 15.3 million tonnes and 5.1 million tonnes higher than in 1993 (Gunstone, 1994b). Rapeseed is expected to remain the dominant oil in terms of consumption, but will probably lose market share because of limited supplies and strong competition from palm oil and soybean oil (Gunstone, 1994b).

1.2. Regulations Governing Transport and Shipment of Vegetable Oils

The nonpetroleum oil industry is already far from insignificant (see above) and with the world's demand for edible oils likely to increase, it is reasonable to expect that the world trade of oils will increase. Nothing in the way vegetable oils are handled differs significantly from petroleum oils, so the likelihood of spills of vegetable oils is no different from that of petroleum oils (Rigger, 1997). However, the international community regulations of shipboard discharges of petroleum and nonpetroleum oils differ (Hunt, 1997). Nonpetroleum oils are allowed to be discharged from tank cleaning or deballasting operations provided their concentration is no greater than 1 part of the oil in 10 parts of water. These requirements are part of international treaties establishing global standards for regulating discharges from vessels and were established during the International Convention for the Prevention of Pollution from Ships, 1978. Yet, there are no laws in case of losses of entire tanks or entire cargo vessels.

In recent years, this issue has been the subject of much debate in the USA. The Oil Pollution Act of 1990 (OPA 90) is the latest major legislative effort to regulate pollution by oil and hazardous substances (Hunt, 1997; Rigger, 1997). This act applies equally to petroleum and nonpetroleum oils. Lately, the nonpetroleum oil industry sought a change in environmental regulations governing edible oils, requiring that federal agencies differentiate between edible and toxic oils, because the industry considered that the environmental risks associated with the transportations, handling and storage of animal fats and vegetable oils are different from those of toxic oils. However, in 1997 the Environmental Protection Agency (EPA) denied this request based on the belief that a worst case discharge or substantial threat of discharge of animal fats and/or vegetable oils to navigable waters, adjoining shorelines or the exclusive economic zone, could reasonably be expected to cause substantial harm to the environment, including wildlife that may be killed by the discharge of fats or vegetable oils (EPA, 1997).

1.3. Vegetable Oils Spills and Effects

The greatest hazard to the marine environment from vegetable oils occurs during loading and unloading of the cargo, when spillages of small amounts can occur (Hoffman, 1989). Some of the largest fluxes to the marine system include emulsified industrial oils from machinery, wastes from vegetable oil processing and soap manufacture. These influxes constitute continual or chronic pollution (Clarke, 1992). However, larger spills can take place and there are a number of cases reported.

The largest nonpetroleum oil spill documented in the literature was a 2.5 million gallon spill of soybean oil into the Minnesota River and upper Mississippi River, during the winter of 1962-1963 (Public Health Service, 1963). Because the spill occurred during winter when the rivers where frozen, its impact was not felt until the spring thaw. Soybean oil was found as far as 250 miles downstream of the spill after the thaw began. It was estimated that this event caused the loss of 4000 birds and injured 1300 others. Additional problems were caused by the increase in specific gravity of the oil after oxidation and polymerisation. It then settled onto the bottom creating anaerobic conditions, which meant

that the oil was not biodegraded quickly, with harmful consequences for the benthic organisms. As water temperature rose in summer, the settled and floating oil increased the biological oxygen demand.

A spill of nearly 10000 tons of palm and coconut oil and edible raw material such as copra (dried coconut meat) occurred in 1975 on Fanning Island in the Pacific Ocean (Russell & Carlson, 1978). According to these authors, the effects of these oily substances were similar to those occurring after a petroleum oil spill. Fishes, crustaceans and molluscs were killed and an excessive growth of *Enteromorpha* and *Ulva* occurred. However, the exact mechanism leading to the death of these organisms was not identified. The authors suggested that the most likely reason was asphyxiation and clogging of the digestive tract. The algal growth was attributed to the elimination of algal competitors, increased fertilisation from the pollution and reduced grazing pressure.

In the winter 1988/1989, a spill of nonylphenol and vegetable oils (such as palm oil) in the Netherlands was responsible for thousands of seabirds being washed ashore. Many of these birds showed signs of emaciation, aggressive behaviour, bloody stool and a lack of plumage. In spite of intensive attendance, nearly all the birds died. However, in this case the toxicity of nonylphenol markedly contributed to the mortality (Zoun *et al.,* 1991).

Vancouver Harbour in Canada is an area where spills of vegetable oils are well documented. One study made in the 70's (McKelvey *et al.,* 1980) analysed the effects of vegetable oil spills on wintering birds. The authors reported three spills of vegetable oils between 1974 and 1978. The first two of these spills were not officially reported and it was the presence of distressed birds that led to their discovery. The total volume of vegetable oil (one soybean oil spill and two rapeseed oil spills) from the three incidents was estimated at 35 barrels and led to the death of 500 birds, compared with the loss of 50 birds caused by 167 spills of petroleum oil during the same period of time. The authors speculate that vegetable oil spills are more dangerous for birds because: i) they are less visible in the water, ii) vegetable oil lacks strong odour and iii) rapeseed oil might be more damaging than petroleum oils to the waterproofing ability of the bird feathers.

Another Vancouver Harbour study investigated the effects of a small (400 gallon) spill of rapeseed oil during the winter of 1989 (Smith & Herunter, 1989). The bulk of spilled oil

was in the water for several hours before it was removed by mechanical means within 15 hours of discovery. This spill resulted in 88 aquatic birds being oiled, many of which were found dead. Over half of the birds found alive subsequently died during treatment (Smith & Herunter, 1989).

The authors of both reports support the hypothesis that the mechanism by which oiling caused birds to die is fouling plumage, which leads to hypothermia. Hypothermia alone may cause the birds to die or may weaken them and make them more vulnerable to predation, starvation and other causes of death. The researchers have found that vegetable oils generally lack any acute toxicity when ingested by preening birds (acute toxicity is associated with petroleum oils), but this effect is somewhat offset by the chronic effects of ingesting large quantities of nonpetroleum oils and by the fact that cleaning nonpetroleum oils oiled birds is much more difficult than cleaning those fouled with petroleum oils. According to the Tri-State Bird Rescue and Research Organization (Gunstone, 1994c) large quantities of oil are often ingested by birds, when the animals clean themselves, resulting in lipid aspiration pneumonia; furthermore, the oil can act as a laxative and lead to further dehydration and debilitation.

1.4. BACKGROUND

The capsize and wrecking of the M.V. *Kimya* off the coast of Anglesey, in January 1991, led to the release of 1500 tonnes of sunflower oil into the marine environment. It was the loss of the *Kimya* that lead to research studies regarding the fate of vegetable oils in the marine environment, such as the present work in the School of Ocean Sciences. The oil was initially spilled into the water at a slow rate with occasional large releases that covered intertidal regions. The oil continued escaping from the wreck until October 1991 (Mudge, 1996).

Initial assessment suggested that the vegetable oils would be rapidly degraded in the environment (Mudge *et al.,* 1993). In practice, however, the oil formed a polymer in seawater and produced intractable lumps resembling "used chewing gum" (Mudge *et al.,* 1993). On the beach, the oil bound to the sand forming concrete-like aggregations

(Salgado, 1992). These were still on the beach six years after the accident (Mudge, 1997). At the same time, the intertidal pools along the shore, near the wreck site, were found to be anaerobic (Salgado, 1992).

During 1991, Salgado (1992) observed significant mortality of mussels adhering to the intertidal shores, many of whom were found in the anaerobic pools. A survey of the fatty acids in the mussels (*Mytilus edulis*) showed that enhanced concentrations of linoleic acid (principal component of sunflower oil) were present in the mussels tissues up to 3 km from the wreck site. Yet, this high concentration (40% of the total fatty acids) was not responsible for the mussels deaths (Salgado, 1992). Further laboratory studies indicated that vegetable oils had sub-lethal effects in mussels. Organisms contaminated with vegetable oil, even at very low concentrations, showed a very low growth rate. Mussels contaminated with linseed oil showed smaller growth rates than mussels contaminated with olive, rapeseed or sunflower oils (Salgado, 1995). The author found that the mussels that died during these experiments showed no enhancement of the characteristic fatty acids of the oil, whereas, those that survived had a profile similar to the vegetable oil. The mechanisms responsible by the mussels death was not established; however, two reasons were suggested for this: i) some mussels were able to detect the presence of oil and chose not to gape, consequently dying of suffocation; ii) some mussels consumed the oil and metabolised the principal component into a toxic metabolite.

To further comprehend the consequences of vegetable oil spills in marine environments sunflower and linseed oil were applied to salt marsh sediments to reproduce potential spills (Mudge *et al.,* 1995). Sunflower oil polymerised at the surface after 28 days, resulting in the formation of a cap of increased shear strength and reduced permeability to water and oxygen. More than 35 days were necessary for sunflower oil to penetrate down to 30 cm depth. Linseed oil, on the other hand, percolated down to 30 cm in 7 days without a formation of a polymer for the duration of the experiment (35 days). Increased numbers of viable aerobic bacteria were recorded with addition of both oils but were more enhanced with linseed oil than with sunflower oil. Mudge *et al.,* (1995), reported the presence of an unexpected fatty acid (possibly 18:2ω3) resulting from linseed oil degradation, however no confirmation of this was presented at that time.

The fatty acids resultant from the linseed oil degradation and the physical, chemical and microbiological factors that affect this degradation were investigated in laboratory experiments (Pereira, 1993). This work showed that in the set up conditions the oil did not polymerise and aerobic and anaerobic microbial degradation occurred. An increase in saturated and monounsaturated acids and a decrease in polyunsaturated acids which constitute linseed oil was observed, in both aerobic and anaerobic conditions. Pereira (1993), reported the presence of unexpected fatty acids (one 18:2 and two 18:3) resulting from the linseed oil degradation. The 18:2 was suggested to be the 18:2ω3 reported by Mudge *et al.,* (1995). However, the full identification of these fatty acids was not carried out. The mechanism suggested to be responsible for these transformations was isomerisation and biohydrogenation similar to those observed in rumen organisms (Kellens *et al.,* 1986).

1.5. Aim and Objectives

The basic premise of this scientific work is that spills of vegetable oil occurred in the past and they will continue to occur in the future. As it can be perceived from the previous literature search, there is no compelling evidence to challenge this assumption.

Based on the historical record, it is clear that little information is available concerning vegetable oil spills. These reports and the investigation recently developed in the School of Ocean Sciences showed that much more field and laboratory research needs to be carried out to fully establish the consequences of vegetable oil spills in marine environments. The present research project is a continuance of these last works. Therefore, the broad **aim** of this project was to investigate the consequences of linseed and sunflower oils spills in salt marsh sediments and in particular the role of bacteria in their degradation. The specific **objectives** set to accomplish this aim were:

i) to determine the transformations that occurred in linseed and sunflower oils and to the sediment characteristics after spillages to salt marsh sediments. To quantify these alterations with time and depth, various physical, chemical and bacteriological parameters were measured after simulation of small oil spills.

ii) to determine the role of sediment populations of aerobic and anaerobic bacteria in the degradation of these oils.

iii) to identify the fatty acids resultant from the vegetable oils degradation in the studied environment and consequently to establish the pathway(s) of their degradation.

2. Biodegradation of Vegetable Oils and Fatty Acids

2.1. Vegetable Oils

Vegetable oils consist primarily of glycerides, which are esters formed by the reaction of three fatty acid molecules with one molecule of glycerol to yield one triacylglycerol molecule and three molecules of water (Killops & Killops, 1993). Although natural oils contain mainly triacylglycerols, they may include small amounts of sterols, phospholipids and waxes. Fatty acids contribute from 94 to 96% of the total weight of various oils. Because of their preponderant weight in the triacylglycerol molecules and also because they comprise the reactive portion of the molecules, the fatty acids influence greatly both the physical and chemical character of triacylglycerols. In considering the composition of a glyceride it is particularly important to distinguish between the saturated acids, with relatively high melting temperatures, and the unsaturated acids, which melt at low temperatures and are chemically much more reactive (Encyclopaedia Britannica, 1974; Menon *et al.*, 1989).

Without any doubt, the accuracy in determining the fatty acid composition of vegetable oils has improved since the introduction of gas chromatography (GC) and high performance liquid chromatography (HPLC). Even so, variation in reported fatty acid composition occurs because of the differences in measuring techniques (White, 1992). Apart from this, there are also natural variations in the fatty acids produced in any one type of oilseed which may vary with geographic location, soil type, climate, moisture, temperature, maturity of seed and agricultural practice (Chu & Sheldon, 1979).

This chapter presents the fatty acid composition and some other features of the two selected vegetable oils used in this work: linseed and sunflower. These oils are of great importance world-wide. The decision to utilise these oils was based principally in the different degree of unsaturation of the main fatty acid of each one of these oils, with linseed oil being classified as a drying oil and sunflower oil as a semi-drying oil. Furthermore, linseed and sunflower were used previously in work which lead to this research project (Section 1.4).

Linseed oil

In 1988, the world-wide production of linseed oil ranked 12th among major vegetable oils (Haumann *et al.,* 1988). There is only one species of cultivated flaxseed, *Linum usitatissimum* L, containing α-linolenic acid (18:3ω3) at concentration ranging from 40% to 63% or higher (Batta *et al.,* 1985). It is cultivated principally in the USA, Canada, former Soviet Union, India and Argentina (Encyclopaedia Britannica, 1974; Budavari, 1989). Flax is grown primarily for the production of linseed oil, which, because of its high content of 18:3ω3, is an excellent drying oil that is used in paints, varnishes, putty, oilcloths, linoleum, printing inks, artificial rubber, tracing cloth, tanning and enamelling leather (Encyclopaedia Britannica, 1974; Budavari, 1989).

The fatty acid composition of linseed oil is greatly affected by temperature and other climatic conditions (Yermanos *et al.,* 1969). It was found by McGregor & Carson (1961) that in general, flaxseed grown in northern, cooler climates in Canada produced linseed oil with greater degree of unsaturation than that grown in southern, warmer climates (also Canada). A typical fatty acid distribution for linseed oil is shown in Table 2.1.

Current Indian government legislation classifies linseed oil as an edible oil (Bajpai *et al.,* 1985). The results of Menon *et al.,* (1989) indicate that the use of linseed oil as an edible oil will not produce hazardous effects, if sufficient vitamin E is given. However, with its high 18:3ω3 contents, linseed oil has a limited use as edible oil because of its high oxidative unstability (Menon *et al.,* 1989). Due to the nutritional value of the ω3 fatty acids, experiments have been carried out to develop edible linseed oils with a much lower content of 18:3ω3. Research work lead to the development of a low-18:3 linseed oil called Linola containing 3% of 18:3 and 72% of 18:2 (Haumann, 1990).

Table 2.1. Fatty acid composition (weight %) of linseed and sunflower oils (Menon *et al.,* 1989; Budavari, 1989).

Systematic name	Trivial Name	Omega Name	Linseed oil	Sunflower oil
Hexadecanoic acid	Palmitic acid	16:0	8	6.4
Octadecanoic acid	Stearic acid	18:0	6	1.3
cis-9-octadecenoic acid	Oleic acid	18:1ω9	20	21.3
cis-9, *cis*-12-octadecadienoic acid	Linoleic acid	18:2ω6	14	66.2
cis-9, *cis*-12, *cis*-15-octadecatrienoic acid	α-linolenic acid	18:3ω3	51	<0.1
Eicosanoic acid	arachidic	20:0	1	4
Docosanoic acid	behenic acid	22:0	-	0.8

Sunflower oil

Sunflower oil is obtained from the seed of *Helianthus annus* L. which is a native of North America. The plant is grown in large quantities in Russia, Argentina, China, the European Community and the United States (White, 1992). World-wide, the production of sunflower oil is the fourth largest of edible oils, being surpassed by soybean, palm and rapeseed oils (Haumann *et al.,* 1988). Generally, sunflower oil is used for consumption as a cooking and salad oil and in the manufacture of shortenings and margarines. High-oilseed sunflowers (40% oil) are generally grown for oil seed production, whereas low-oilseed sunflowers (about 30% oil) are grown for confectionery, nut and birdseed markets (White, 1992).

A typical fatty acid composition of sunflower oil is shown in Table 2.1. Like linseed oil, the fatty acid composition of sunflower oil is naturally variable, depending upon climate, temperature, genetic factors and position of the seed in the flower head (Campbell, 1983). In fact, few vegetable oils reflect the influence of these factors as significantly as sunflower

oil does. The 18:2 and 18:1 averages of northern grown sunflower oil in the United States compared with the southern varied, with the 18:2 ranging from 44% to 68% and 18:1 from 19% to 47% (Campbell, 1983). The warmer the temperature during maturation of the seed, the lower the 18:2 and the higher the 18:1 levels became. Because of the variability in 18:1 and 18:2 production it is difficult to give an accurate fatty acid content of sunflower oil without actually measuring it (White, 1992).

Treatment of normal varieties of sunflower oil seeds with chemical mutagens and development of their progenies have resulted in hybrids bearing oil with 18:1 contents greater than 80% and 18:2 contents less than 10% (Purdy, 1986). The fatty acid composition of these varieties appear to be unaffected by climatic conditions.

The high polyunsaturated fatty acid content of sunflower oil makes it susceptible to oxidative deterioration and thus reduce flavour stability. The high-oleic sunflower oil was shown to have dramatically improved oxidative unstability compared with normal sunflower oil (Purdy, 1985). This improved stability was attributed entirely to the shift in fatty acids from 18:2 to 18:1.

2.2. Microbial Degradation of Vegetable Oils

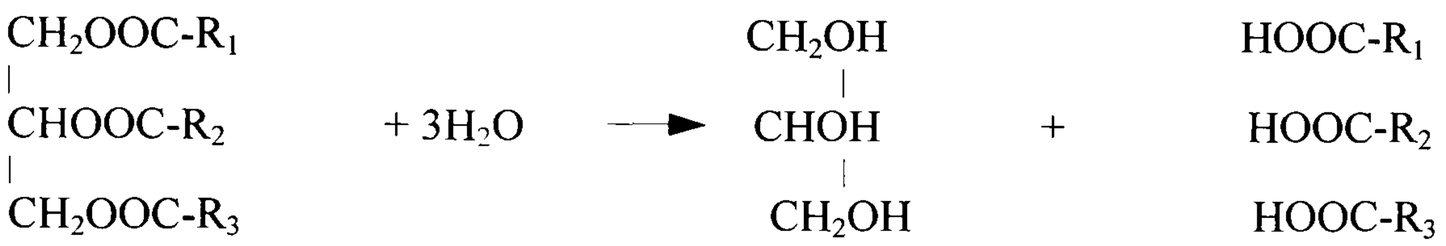
2.2.1. The biochemistry of the oil degradation

Numerous micro-organisms can utilise vegetable oils as the sole source of carbon and energy (Tan & Gill 1985; Koritala *et al.,* 1987; Ratledge, 1994). However, triacylglycerols and their partially hydrolysed products, di- and mono-acylglycerols, are not assimilated as such. Therefore, the first step in the microbial degradation of an oil is its hydrolysis to fatty acids and glycerol (Cornish *et al.,* 1993; Ratledge, 1994). This hydrolysis proceeds *via* the action of extracellular lipases or by lipases that are attached to the cell surface and thus appear to be cell associated, as the triacylglycerol oil is unable to penetrate the cell interior (Ratledge, 1994). Although it is necessary for a micro-organism to be able to produce a lipase for it to be able to grow on an oil, it would appear that most micro-organisms are

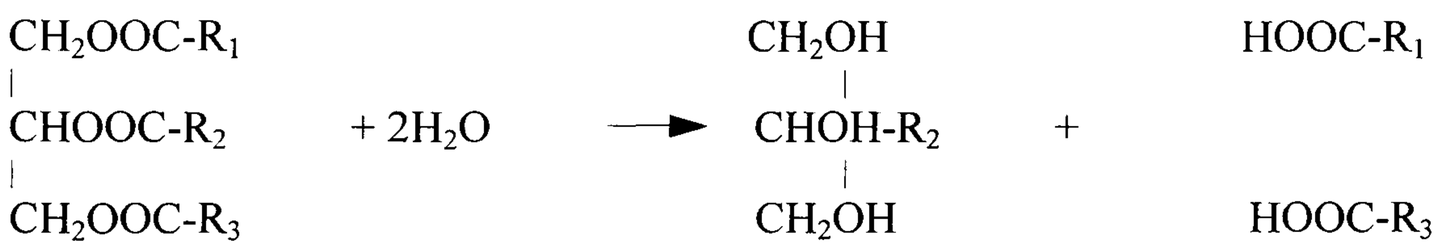
able to do so (Cornish *et al.,* 1993; Rattledge, 1994). The production of lipases is influenced significantly by temperature, ratio of nitrogen to carbon, inorganic salts and oxygen. In general, bacterial lipase synthesis is stimulated by lipids such as lard, olive oil and fatty acids (Ratledge, 1994).

Microbial lipases are classified into three types according to their reaction specificity: non-specific lipases, 1,3-specific lipases and fatty acids specific lipases (Figure 2.1) (Finnerty, 1989).

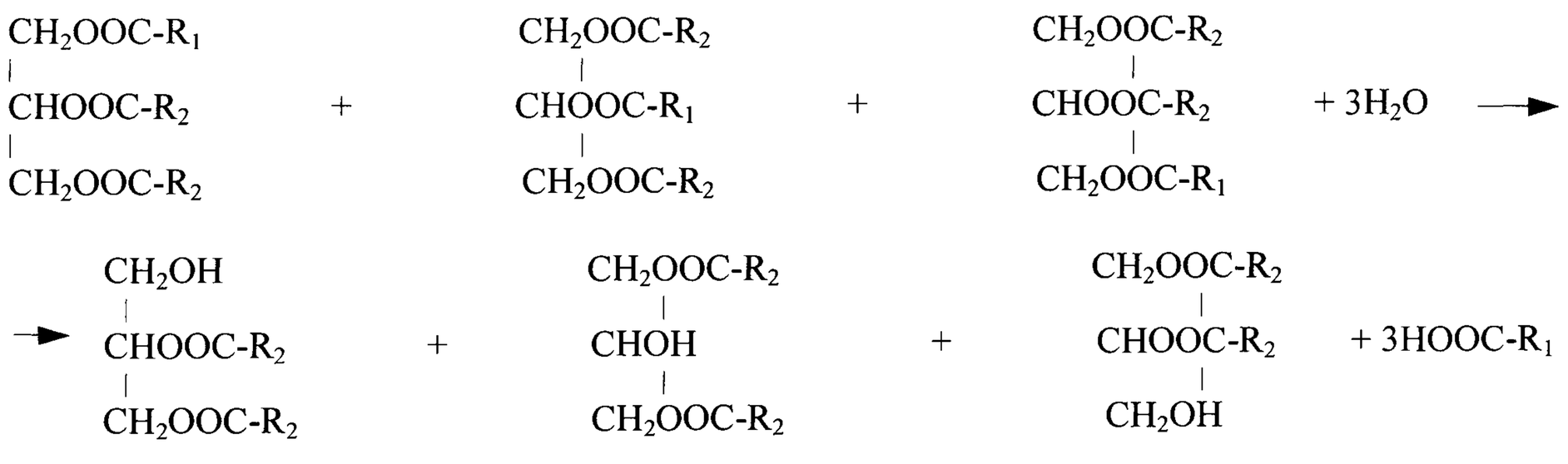
(a) Non-specific lipase reaction



(b) 1,3-specific lipase reaction



(c) Fatty acid specific lipase reaction



In all examples $RCOO^-$ = long chain fatty acyl group

Figure 2.1. Hydrolytic reactions of lipases (Ratledge, 1994).

Non-specific lipases exhibit no specificity to either the fatty acid position on glycerol or to the structure of the fatty acid at any position. These lipases catalyse the total hydrolysis of triacylglycerols to free fatty acids and glycerol. Lipases showing no regio-specificity appear

to be the most commonly found (Macrae & Hammond, 1985) occurring both in bacteria and fungi. This type of lipases will remove fatty acyl groups from all three positions of the glycerol more or less at the same rate (Finnerty, 1989; Ratledge, 1989, 1994).

The 1,3- specific lipases catalyse reactions at the C_1 and C_3 positions of triacylglycerols to yield the free fatty acids 1,2 (2,3)-diacylglycerols and 2- monoacylglycerols. Since 1,2 (2,3)-diacylglycerols and 2- monoacylglycerols are chemically unstable, undergoing acyl migration to yield 1,3-diacylglycerols and 1(3)- monoacylglycerols, 1,3-specific lipases can degrade triacylglycerols to free fatty acids and glycerol (Finnerty, 1989). Only lipases showing 1,3-regio-specificity are known; 2-specific lipases remain to be found (Finnerty, 1989; Ratledge, 1989, 1994). A few lipases are known to show specificity towards the fatty acids themselves. The best known example is the enzyme from *Geotrichum candidum* (Finnerty, 1989; Ratledge, 1989, 1994). This lipase attacks triacylglycerols containing fatty acids with a double bond at the 9 position and then only providing that there is no additional double bond between C_1 and C_9. Fatty acids with either a *cis*-double bond or *cis*-double bonds are hydrolysed more quickly than those with *trans*-double bond. Triacylglycerols containing oleate, linoleate or α-linoleneate groups are more readily hydrolysed whereas those with substituents such as palmitoleate (16:0), stereate (18:0), γ-linoleneate (18:3 *c*6,*c*9,*c*12) or arachidoneate (20:4 *c*5,*c*8,*c*11,*c*14) are not. All the lipases mentioned will hydrolyse tri, di-and monoacyglycerols at approximately equal rates (Ratledge 1989, 1994).

Fatty acids from triacylglycerols can be assimilated as such into the lipids of the cell. Although there are few exceptions, most micro-organisms appear to use the component fatty acyl group with only the minimum amount of modification (Ratledge 1994), that is, there is very little desaturation or elongation of the incoming fatty acids. The incoming fatty acids must repress the synthesis of desaturase enzymes in the same way that they repress *de novo* fatty acids biosynthesis (Gill & Ratledge, 1973). However, if the chain length of the substrate is too short to give fatty acids of sufficient chain length to be satisfactorily incorporated into the microbial lipid, then some modification is essential. Lee *et al.,* (1992) observed the growth of *Apitrichum curvatum* on various substrates: when myristic acid (14:0) was used as such, the yeast oil contained 90% of its acyl groups as this fatty acid. With palmitic acid (16:0) as substrate, elongation to stearic acid (18:0) occurred as well as desaturation of this to both oleic (18:1) and linoleic (18:2) acids. These authors

also examined the growth of this yeast on a different range of fatty acids. Even unusual fatty acids not found in micro-organisms, such as petroselenic acid (16:1 *c*6) or eleostearic acid (18:3 *t*5,*t*8,*c*11) were significantly incorporated into the yeast lipids indicating that there is little problem in accommodating such fatty acids, at least in the storage fraction of the yeast. So, fatty acids that have the necessary chain length and degree of flexibility by virtue of their double bonds can be utilised even though they may not be synthesisable by that particular micro-organism. When a cell grows on a fatty acid or oil, some of the substrate is also metabolised to yield energy for cell synthesis. Thus, there is a partition between assimilation of the substrate directly into the lipids of the cell and its degradation. Glycerol from triacylglycerols is taken up by a process of facilitated diffusion and converted by two steps to the central metabolite, glyceraldehyde-3-phosphate (Cornish *et al.*, 1993).

Fatty acids metabolism

To yield energy, fatty acids can undergo the following oxidation processes.

α-Oxidation

Oxidative decarboxilation of fatty acids has been studied for plants and animals with infrequent reports pertaining to prokaryotic micro-organisms (Harwood & Russell, 1984). The occurrence of bacterial fatty acid α-oxidation is not firmly established and, if present, represents a pathway of minor importance (Finnerty, 1989; Ratledge, 1994). α-Oxidation involves the sequential decrease in carbon number of fatty acid by oxidative decarboxylation. The essential reactions of this cycle are (Ratledge, 1994):

$$R\text{-}CH_2 \cdot COO^- \longrightarrow R\text{-}CHOH \cdot COO^- \longrightarrow R \cdot CHO + CO_2 \longrightarrow R\text{-}COO^-$$

Free fatty acids and not their CoA esters, are involved.

This shortening of chain length results in odd-carbon fatty acids from even-carbon fatty acids and even-carbon fatty acids from odd-carbon fatty acid. Evidence exists in prokaryotic micro-organisms that odd- and even-carbon fatty acids characteristic of cellular lipids arise predominantly, if not exclusively, from *de novo* biosynthesis (Finnerty, 1989).

Studies with *Candida utilis* (Fulco, 1967) and *Arthrobacter simplex* (Yano *et al.,* 1971) indicate that some micro-organisms are capable of α-oxidation. Hydroxy fatty acids, particularly 2-hydroxy fatty acids, represent possible precursors for oxidative decarboxylation of fatty acids. The presence of small amounts of 2-hydroxy-12:0 and 2-hydroxy-14:0 in a number of gram-negative bacteria have been reported as part of the lipopolysaccharides of the cell envelope (Wilkinson, 1988). The occurrence of such acids suggest, but not prove, the presence of an α-oxidation route of fatty acids in bacteria (Ratledge, 1994).

β-Oxidation

The principal route by which fatty acids are oxidised in cells is by the sequence known as the β-oxidation (Figure 2.2). The reactions involved in the cyclic 2-carbon shortening of a fatty acid include an inducible enzyme system consisting of the enzymes listed in Figure 2.2. plus 3-hydroxyacyl-CoA epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase which are involved in the β-oxidation of unsaturated fatty acids (Finnerty, 1989; Ratledge, 1994).

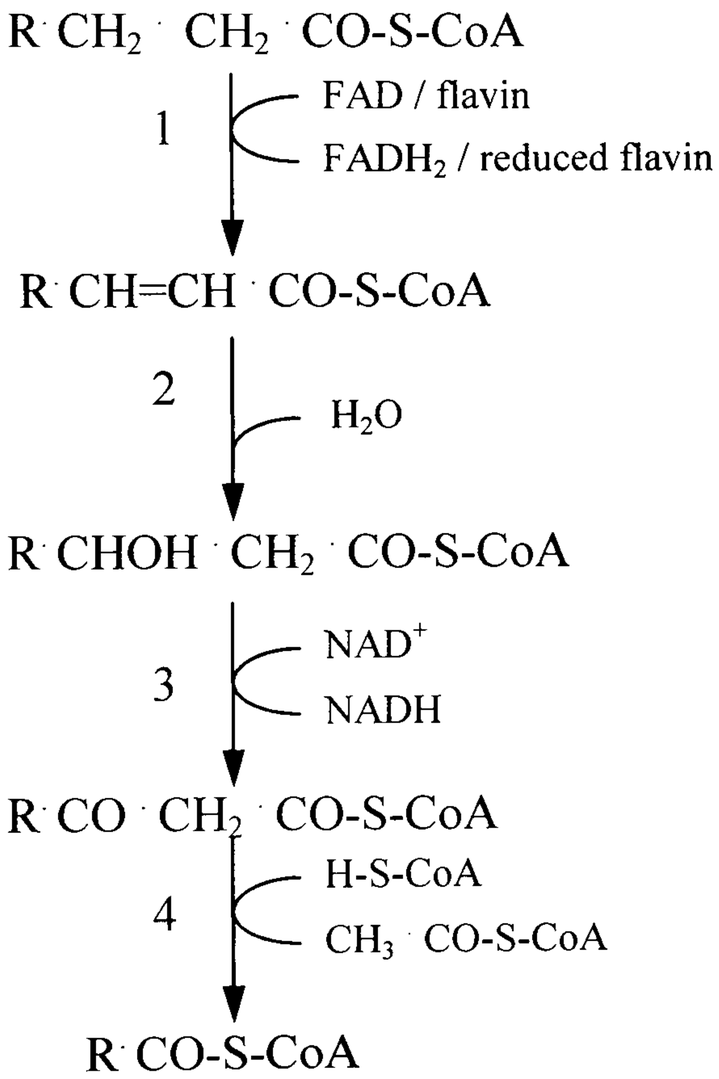


Figure 2.2. β-Oxidation cycle. Individual enzyme reactions:
1. Fatty acyl-CoA dehydrogenase;
2. 2,3-enoyl-CoA hydratase;
3. 3-hydroxyacyl-CoA dehydrogenase (NAD^+ dependent);
4. 3-oxoacyl-CoA thiolase
(Adapted from Ratledge, 1994).

The intermediates formed during a β-oxidation are enzyme bound. However, a detailed explanation of these oxidation reactions of saturated and unsaturated fatty acids is outside the scope of this review. The final reaction of the cycle involves the cleavage of the acyl chain to split off acetyl-CoA leaving an acyl-CoA ester that is now two carbon atoms

shorter that the initial acyl chain (Figure 2.2) (Harwood & Russel, 1984; Mead *et al.,* 1986; Finnerty, 1989; Ratledge, 1994). The shortened fatty acyl-CoA ester then repeats the sequence (see Fig. 2.2) until the acyl chain reaches the C_4 level. The final reaction is, therefore, the cleavage of acetoacetyl-CoA (CH_3COCH_2CO-S-CoA) with the formation of 2 acetyl-CoAs.

ω-Oxidation

The ω-oxidation of fatty acids occurs both in bacteria and yeasts (Finnerty, 1989; Ratledge, 1994). This oxidation is important either when the carboxyl end is unavailable or for the formation of ω-hydroxy fatty acids (Harwood & Russel, 1984). The mechanisms of fatty acids diterminal oxidation to long chain dicarboxylic acid is through an O_2-dependent ω-hydroxylase enzyme system. Long chain dicarboxylation acid results from the ω-hydroxylation of fatty acid to ω-hydroxy fatty acid which is oxidised to aldehydic fatty acid and to α,ω-dicarboxylic acid (Finnerty, 1989). The route of oxidation is (Finnerty, 1989; Ratledge, 1994):

$$CH_3(CH_2)_nCOOH \longrightarrow HOCH_2(CH_2)_nCOOH \longrightarrow OHC(CH_2)_nCOOH \longrightarrow HOOC(CH_2)_nCOOH$$

Yi & Rehm (1988a,b) have demonstrated the conversion of unsaturated fatty acids, oleic (18:1 *c*9) and elaidic (18:1 *t*9) acid to the corresponding unsaturated dicarboxylic acids: *cis*-9- 1,18 octadecanedioic and: *trans*-9- 1,18 octadecanedioic, respectively, by *Candida tropicalis*. It would be reasonable to expect that other mono-, di- or even polyunsaturated fatty acids might not be oxidised similarly.

Although the various oxidations have been discussed as separate processes, it is important to emphasise that in the oxidation of lipids it may be necessary to use a number of different oxidations in order to complete acyl chain catabolism.

In addition, oxidation of carbon atoms within the acyl chain can occur, such as (ω-1)- and (ω-2)- oxidations and mid-chain oxidations, thus forming the various hydroxy, oxo, epoxy, hydropoxy and polyoxygenated derivatives that are found in nature (Harwood & Russel, 1984; Schweizer, 1989). The existence of (ω-1)- hydroxy fatty acids in the lipids of some bacteria (Skerratt *et al.,* 1992) and yeasts suggests that there may be a specific oxidation of

fatty acids at their penultimate terminal carbon atom. An isomeric mixture (ω-1)-, (ω-2)- and (ω-3)- monohydroxy fatty acids was obtained from saturated and monounsaturated C_{12} to C_{18} by *Bacillus megaterium* (Miura & Fulco, 1974, 1975; Nahri & Fulco, 1982, 1986, 1987). Lanser *et al.,* (1992) have investigated the hydroxylation of oleic acid in the whole cells of two strains of *Bacillus pumilus* and, as in the previous case, they could not detect formation of ω-hydroxyoleic acid. They also found that hydroxylation had occurred at the three adjoining carbon atoms, ω-1, ω-2 and ω-3. Mid-chain oxidation of oleic acid to give 12-hydroxyoleic acid (Soda, 1987) and 10-hydroxyoleic acid (Koritala & Bagby, 1992; El-Sharkaway *et al.,* 1992) have been reported, as well as the formation of 9,10 epoxystearic acid from oleic acid (Ruettinger & Fulco, 1981). The fate of (ω-1) and mid-chain hydroxy fatty acids is uncertain. It is possible that (ω-1)-hydroxy fatty acids are not oxidised at this end of the molecule but are degraded by the β-oxidation cycle operating conventionally at the other end. A similar degradation route could be expected in the case of mid-chain hydroxy fatty acids. Their occurrence are, therefore, of most interest for those concerned with biotransformation reactions of fatty acids rather than with their degradation. The utilisation of micro-organisms to produce useful chemical intermediates from fats and oils is increasingly being investigated, because unlike traditional chemical processes, which require extreme temperature and pressure, microbial conversions take place under mild conditions with greater rapidity and specificity.

Another modification of the fatty acids carried out by micro-organisms is the hydrogenation of unsaturated fatty acids.

Biohydrogenation

The hydrogenation of an isolated *cis* double bond in the hydrocarbon chain of a fatty acid appears to be a relatively rare event in nature. Biohydrogenation of polyunsaturated fatty acids is a characteristic biochemical process carried out by micro-organisms of the rumen, which has been known for many years (Wilde & Dawson, 1966), and also by an asacharolytic anaerobe, *Eubacterium lentum*, isolated from rat faeces (Eyssen & Verhulst, 1984; Verhulst *et al.,* 1986). The general pathways are shown in Figure 2.3. Stearic acid is the common end product from all the C_{18} unsaturated fatty acids.

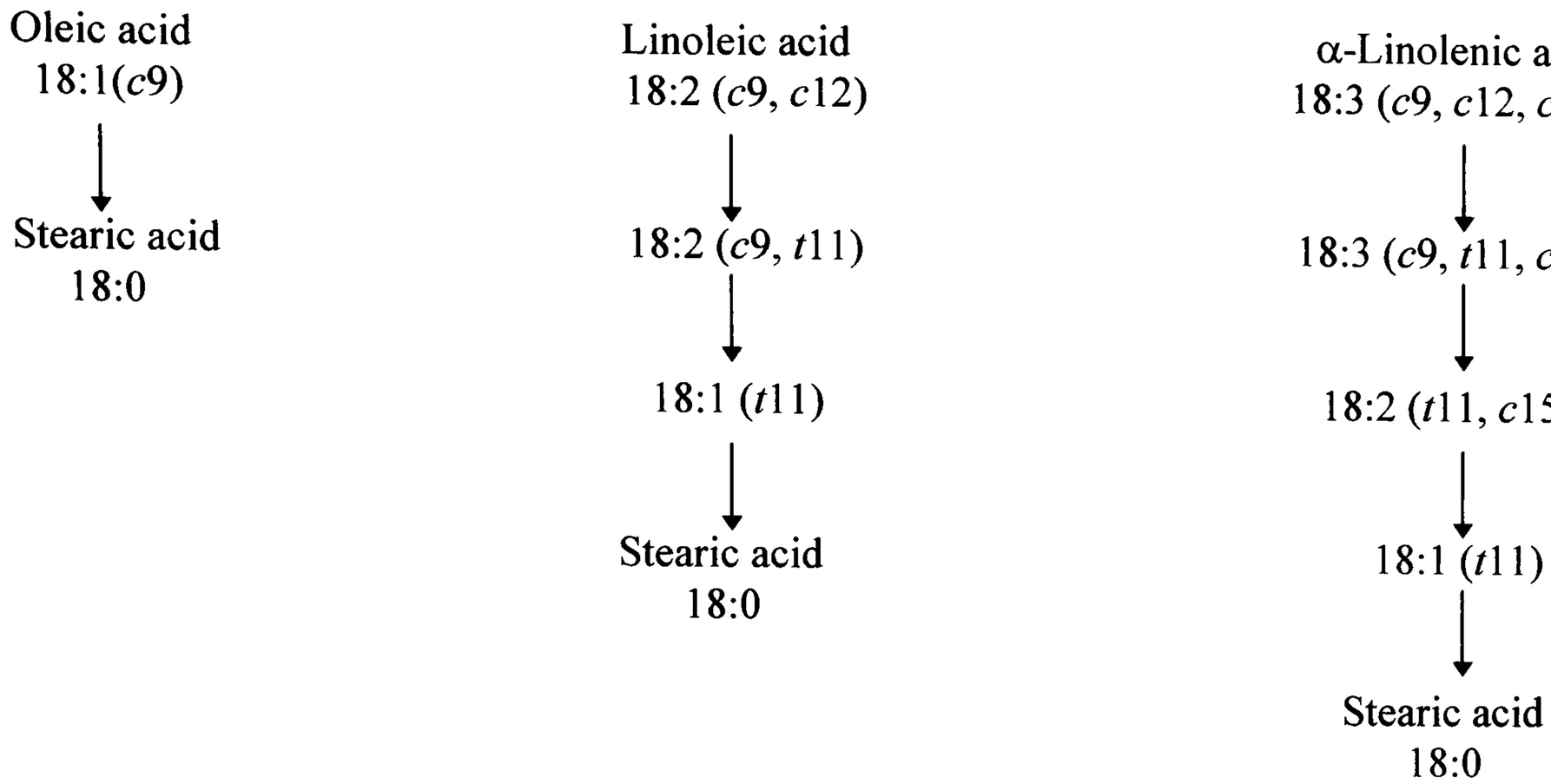


Figure 2.3 General pathway of biohydrogenation of unsaturated C_{18} fatty acids by a mixed culture of rumen micro-organisms (Kellens *et al.*, 1986)

Rumen bacteria, obligate anaerobes, are largely responsible for the hydrogenation - protozoa are of only secondary importance (Dawson & Kemp, 1969; Viviani, 1970). A number of individual rumen micro-organisms have been described that can hydrogenate linolenic and linoleic acids but none of these were capable of completing the hydrogenation beyond the octadecenoic acid stage or converting oleic into stearic acid. These include: *Butyriovibrio fibrosolvens*, the first micro-organism isolated from the rumen (Wilde & Dawson, 1966; Kemp & White, 1968; Kemp & Dawson, 1968); a strain of *Escherichia coli* (Wilde & Dawson, 1966) and a Gram negative micrococcus (Mills *et al.,* 1970). White and collaborators (1970) isolated a Gram-negative capsulated anaerobic bacillus that was able to hydrogenate oleic and linoleic acid to stearic acid. However, linolenic acid was not hydrogenated to stearic acid by this organism, but instead octadecenoic acids were accumulated in the medium where *cis*-15-octadecenoic acid predominated. Kemp *et al.,* (1975) isolated from sheep rumen five (strictly anaerobic) bacteria able to hydrogenate unsaturated fatty acid. One was characterised as *Ruminococcus albus*, two as *Eubacterium* spp. and two as *Fusocillus* spp. The *Fusocillus* organisms were able to hydrogenate oleic and linoleic acid to stearic acid, and linolenic acid to *cis*-15-octadecenoic acid. The *Ruminococcus albus* and the two Eubacteria did not hydrogenate oleic acid but instead converted linoleic and linolenic acids to a mixture of octadecenoic acids where *trans*-11-octadecenoic acid predominated. Therefore, organisms may be used singly or as mixed

cultures (Wilde & Dawson, 1966: Kellens *et al.*, 1986) to effect the complete hydrogenation.

In incubations of linolenic acid with sheep rumen contents, it was observed that the conversion of linoleic acid into 18:1 (*t*11) and subsequently into stearic acid was largely associated with food-particle fractions (Harfoot *et al.*, 1973). Harfoot *et al.*, (1973) rejected the possibility that the hydrogenation could be associated entirely with the bacteria adhering to the food particles and suggested that hydrogenation is effected by extracellular hydrogenases produced by bacteria in suspension. Other authors (Viviani, 1970; Kellens *et al.*, 1986) also observed that complete hydrogenation of polyunsaturated fatty acids present in linseed oil took place *in vitro* only in the presence of complete rumen liquor and the removal of the particulate fraction greatly diminished the biohydrogenation capacity. According to Kellens *et al.*, (1986) the rumen fluid promotes the development of biohydrogenating micro-organisms or the development of the biochemical systems necessary for biohydrogenation in cells that do not necessarily need the rumen fluid for their growth. The presence of co-factors for biohydrogenation in the rumen fluid is another suggestion that has been proposed by several authors (Wilde & Dawson, 1966).

In the case of the biohydrogenation of linoleic acid, the reaction proceeds by isomerization of the *cis-9, cis*-12 to *cis*-9, *trans*-11 octadecadienoic acid. This is followed by reduction of the *cis* bond; the final hydrogenation is that of the *trans* double bond itself. For complete hydrogenation of linoleic to stearic acid in the rumen, two systems must be involved, one specific for the conversion of linoleic acid to a monoenoic acid, and the other for the hydrogenation of the monoene to stereate. Other polyunsaturated fatty acids such as γ-linolenic acid [18:3 (*c*6, *c*9, *c*12)] (Kemp & Landner, 1983) and arachnidonic acid [20:4 (*c*5, *c*8, *c*11, *c*14) (Verhulst *et al.*, 1986) can also be reduced.

Partial purification of the enzyme that catalyses the isomerisation, linoleate Δ^{12} - *cis*, Δ^{11}-*trans* isomerase, has been achieved from the cell envelop of *Butyriovibrio fibrosolvens* (Kepler & Tove, 1967) and some of its properties have been investigated (Kepler *et al.*, 1970). Absolute requirements for the isomeration were shown to be: marked specificity for a free carboxyl group and a *cis*-9, *cis*-12 pentadiene system (Kepler *et al.*, 1970; Verhulst *et al.*, 1986). These studies were facilitated by the finding that this reaction takes place under aerobic conditions. In contrast, the hydrogenation reaction appears to be obligatory

anaerobic (Rosenfelt & Tove, 1971). Rosenfeld & Tove (1971), investigated the reduction of 18:2 (*c*9, *t*11) to 18:1 (*t*11) using punicic acid [18:3 (*c*9, *t*11, *c*13)] and α-eleostearic acid [18:3 (*c*9, *t*11, *t*13)]. In the case of punicic acid both *cis* double bonds were hydrogenated, resulting in the same product as that obtained from linoleic hydrogenation. Yet, in the case of α-eleostearic acid no reaction occurred. This work suggests, therefore, that the configuration of the conjugated *trans* bond imparts a degree of alteration to the molecule so that the organism is incapable of reducing the *cis* bond of the conjugated triene. The hydrogens to reduce the *cis* bond(s) were shown to come from the water (Rosenfeld & Tove, 1971; Hughes *et al.,* 1982). In the mechanism of the reduction, reductants such as NADPH have also been implicated (Hughes *et al.,* 1982). Although this would be an impediment if a cell-free system of biohydrogenation was being contemplated (due to the difficulty of regenerating the reduced co-factor *in situ*), Kellens *et al.,* (1986) have shown that if a growing mixture of rumen bacteria is used under N_2/H_2 atmosphere, anaerobic growth can be self sustaining. Thus the intracellular regeneration of NADPH is achieved by bacteria's own hydrogenase enzyme being coupled with hydrogen.

2.2.2. Degradation in Marine Sediments

a) Vegetable oils

Given that lipases are produced by a wide range of micro-organisms and that the pathways of degradation of glycerol and fatty acids are virtually ubiquitous, high biodegradability of vegetable oils in the marine environment is to be expected (Cornish *et al.,* 1993; Ratledge, 1994). Indeed, some experiments have shown that vegetable oils are quickly degraded (Groenewold *et al.,* 1982; Cornish *et al.,* 1993). However, these tests were usually carried out in the aquatic environment since, according to Cornish and colleages, up to 1993 there was no standard laboratory methods for assessing the fate of oils in sediments. These aquatic screening tests are designed to assess biodegradability of water soluble substances and few methods are suitable for testing oils. Furthermore, these experiments are performed using very small amounts of the substance to test (2-100 mg l^{-1}) (Cornish *et al.,* 1993). In addition, the high biochemical oxygen demand (BOD) observed for vegetable oils also suggest that they are readily degradable (Groenewold *et al.,* 1982). Nevertheless,

the high BOD of vegetable oils poses an increased risk for oxygen depletion and consequently the oil will remain in the environment for a longer period of time.

Biodegradation represents an important route by which these oils can be removed from the environment (Cornish *et al.,* 1993) where transformations by micro-organisms can occur (Mudge *et al.,* 1995). Although microbial degradation rarely occurs under controlled conditions of storage of vegetable oils, micro-organisms can grow on vegetable oil and degrade them as long as environmental conditions are favourable (Ratledge, 1994), with some oils having been found to be not easily degraded in the marine environment (see Section 1.4). Therefore it appears that the biodegradation of an oil is going to depend on the properties of the oil itself and on those of the environment where the oil was spilled.

b) Fatty acids

If hydrolysis of the triacylglycerols present in the vegetable oils occurs, a wide range of pathways for the degradation of fatty acids should be expected. A literature search indicate that most of the studies regarding vegetable oils spills did not focus on the fatty acids degradation, except those mentioned in the background section (section 1.4). However, there is abundant research concerning the degradation of fatty acids in sediments. In sediments, fatty acids are believed to derive from anthropogenic sources and from a wide variety of biological sources, such as plants, animals, algae, microbes and their degradation products. The fatty acids of living systems are, for most part, esterified as waxes, glycerides or phospholipids. Although triacylglycerols represent major components of storage in plant and animals, in view of the existing lypolitic activity in the sediments (Farrington & Quinn, 1973; Norkans & Stettn, 1978), apart from their incidental presence in very recent sediments, triacylglycerols cannot be considered geochemically important as they appear to be easily decomposed (Farrington & Quinn, 1973; De Leeuw & Largeau, 1993).

Even though in a spill of vegetable oils a much higher amount of fatty acids would be present in the sediments, the observed transformations of the fatty acids reported in the literature will provide an insight of what might be the fate of the fatty acids derived from vegetable oil spills.

Degradation rates

Considerable amounts of saturated and unsaturated fatty acids are present in the surface sediments. In general, the individual and total fatty acids decrease in concentration with increasing sediment depth, although the individual fatty acids do not show uniform decrease with depth relative to each other (Parker & Leo, 1965; Parker, 1967; Farrington & Quinn, 1971; Van Vleet & Quinn, 1979 among others). Contrary to the expectations that lipids would be relatively stable, fatty acids are rapidly altered in marine sediments and individual compounds exhibit a range of reactivity. Among the various classes of fatty acids, the following reactivity is generally observed: unsaturated fatty acids > branched fatty acids > saturated fatty acids (Parker & Leo, 1965; Farrington & Quinn, 1971; Johnson & Calder, 1973; Van Vleet & Quinn, 1979; Haddad *et al.,* 1992, Sun & Wakeham, 1994; Canuel & Martens, 1996). Furthermore, within the saturated fatty acids, medium chain fatty acids (C_{14}-C_{19}) have higher rates of degradation than long chain ones (C_{20}-C_{34}) (Farrington *et al.,* 1977; Kawamura & Ishiwatari, 1982; Haddad *et al.,* 1992). Degradation rates are highest in the surface sediments (0-2.5cm), indicating that the reactivity of a given molecule decreases over time, and begins soon after deposition (Parker, 1967; Rhead *et al.,* 1971; Canuel & Martens, 1996). Lipids can undergo rapid alterations during the early stages of deposition, which is referred to as biological diagenesis, brought about largely by microbial activity in the upper layers of recent sediments (Farrington & Quinn, 1973; Johnson & Calder, 1973).

It appears that molecules in the same compound class can degrade at different rates, resulting in preferential preservation of some compounds and the relative degradation of others. Furthermore, based on the observations of differential reactivity of similar compounds (*e.g.* long chain fatty acids are less reactive than medium chain), it seems that organic matter reactivity may be controlled not only by the chemistry of the compound itself, but also by the microbial accessibility to the matrix (either biologically or abiologically produced) in which is incorporated prior to the sedimentary burial. So it appears that the rate and extent of degradation of sedimentary organic compounds are strongly dependent on: molecule structure of the substrate, protective effects by association of organic matter with particle matrices, and sedimentary redox conditions that affect the activity of benthic organisms (Haddad *et al.,* 1992; Sun & Wakeham, 1994; Canuel & Martens, 1996; Sun *et al.,* 1997). The influence of oxygen on the rate at which organic

carbon is degraded by the microbial consortia has been addressed in the literature but there seems to exist some controversy around this issue: several investigations have indicated that significant differences between aerobic and anaerobic decomposition rates exist (Sun *et al.,* 1993; Harvey *et al.,* 1995; Sun *et al.,* 1997), while others found little difference (Westrich & Berner, 1984; Henricks & Reeburgh, 1987; Lee, 1992). Van Vleet & Quinn (1979) showed that even though the fatty acids in bound and unbound form are degraded with depth in the sediments, the unbound fatty acids are preferentially removed leaving a higher percentage of the more protected bound fatty acids at increased depths.

Degradation Pathways

The loss of unsaturated fatty acids in marine sediments have been studied, both *in situ* and in laboratory using labelled fatty acids (Rhead *et al.,* 1971, Gaskell *et al.,* 1976; Harvey *et al.,* 1986; Sun *et al.,* 1997). Sun *et al.,* (1997) showed that 16:0 and 18:1 are degraded through multiple pathways in oxic and anoxic conditions but the dominant fate of fatty acids in recent sediments appears to be degradation via decarboxylation (Gaskell *et al.,* 1976; Harvey *et al.,* 1986; Sun *et al.,* 1997). Gaskell *et al.,* (1976) working in a closed-system incubation experiment using radiolabelled (^{14}C, ^{3}H) oleic acid showed that most (>75%) of this compound in a predominantly anoxic estuarine sediment was remineralised to $^{14}CO_2$ in 10 days. In a similar manner, Sun *et al.*, (1997) found that 80-90% of the labelled 1-^{14}C-palmitic and 1-^{14}C-oleic acids was lost from the sediments, with most of the loss occurring within the first two weeks. Experiments conducted by Harvey *et al.,* (1986) showed that aerobic degradation of uniformly-radiolabelled bacterial membrane lipids produced $^{14}CO_2$ but anaerobic degradation produced both $^{14}CO_2$ and $^{14}CH_4$.

Another pathway in fatty acid degradation is the production of small metabolites. Experiments with labelled (1-^{14}C, 9,10-^{3}H) oleic found low yields (2%) of shorter-chain (C_{12}-C_{18}) saturated fatty acids in only a few days (Rhead *et al.,* 1971, Gaskell *et al.,* 1976). Two biochemical pathways for this alteration were postulated: (1) chain shortening via loss of C_2 units and double bond reduction, and (2) β-oxidation to acetyl-CoA units followed by resynthesis of shorter chain fatty acids. Conversion of labelled oleic acid in recent sediments to a series of saturated fatty acids has shown that hydrogenation and degradation followed by resynthesis is acting in sediments, presumably due to micro-organisms (Rhead *et al.*, 1971). In contrast, Sun *et al.,* (1997) detected no short chain (C_{12}-C_{14}) fatty acids

from 1-^{14}C palmitic acid incubations but found that about 1% of the 1-^{14}C oleic acid may be converted to saturated products. Thus, hydrogenation of unsaturated fatty acids may be more important than chain shortening. Eglinton (1971), Nissenbaum *et al.,* (1972) and Johnson & Calder (1973), observed apparent destruction of the double bonds of unsaturated fatty acid in both oxidising and reducing sediments. In all cases, microbial activity was again suggested as being a major factor in the redistribution of sediment fatty acids. According to Sun *et al.,* (1997), a small amount of the initially added radiolabel was incorporated in non-fatty acid fractions (alkyl esters, diacylglycerols, triacylglycerols, phospholipids, glycolipids, *etc*) and would represent label incorporated into lipids by *de novo* biosynthesis by sedimentary organisms catabolizing ^{14}C containing organic matter.

Another and lesser important fate of the labelled fatty acids in sediments is the transfer of the label from the free to the bound pool (Sun *et al.,* 1997). This transfer could result from either physicochemical association of label-free fatty acids or metabolites with sediment grains and their organic coatings, by chemical esterification, or by incorporation of fatty acid-derived carbon in non-extractable macromolecules or biomass, such as bacterial membranes or biopolymers (Sun *et al.,* 1997).

From these results, it seems that the majority of the C_{12}-C_{18} *n*-alkanoic acids in some aquatic sediments are derived through biological, probably microbiological, degradation and synthesis within the sediment.

3. Salt Marshes

3.1. General Characterisation

Coastal salt marshes may be defined as areas vegetated by herbs, grasses or low shrubs, bordering saline water bodies. Although such areas are exposed to the air for the majority of time, they are subjected to periodic flooding as a result of fluctuations (tidal or non-tidal) in the level of the adjacent water body (Adam, 1993). The definition of coastal salt marshes stresses that the flooding waters are saline - not necessarily seawater but more saline than freshwater (Adam, 1993), in the range of 5-38 psu (Boaden & Seed, 1985). Therefore, coastal salt marshes occupy the interface between land and sea, and develop most readily on low energy coasts in temperate and high latitudes (Allen & Pye, 1992). The coastal salt marsh environment has some features of both land and sea and its biota, both flora and fauna, has both marine and terrestrial elements. Nevertheless, the organisms that are essential for the recognition of salt marshes are the vascular plants which are terrestrial in origin (Adam, 1993). Colonisation by such plants cannot occur unless the sediment is stable enough to permit rooting and growth. Coarse to medium sands are not conducive to

marsh formation, partly because they tend to dry out. There is requirement of some admixture of silt (Boaden & Seed, 1985).

Salt marshes are highly dynamic environments subject to erosion, accretion and progradation (Adam, 1993). The location, character and dynamic behaviour of salt marshes is governed essentially by four physical factors: sediment supply, tidal regime, wind-wave climate, and movement of relative sea level (Allen & Pye, 1992). To these may be added the variable but secondary role played by marsh vegetation in acting both as a source and as a retainer of sediment (Allen & Pye, 1992). Colonisation of sand or mudflats by vegetation can only begin once the level of the surface has been raised to a sufficiently high level in the tidal frame by physical sedimentation processes. Once vegetation is established, the rate of sedimentation frequently increases as more of the incoming sediment is intercepted and trapped by the greater surface roughness (Stumpf, 1983; Stenvenson *et al.,* 1988). Resuspension of deposited material is reduced for the same reason, and organic matter is added to the marsh surface (Allen & Pye, 1992).

Waterlogging

Marshes tend to be quite flat, and their mean elevation is near or slightly below the mean high tide level. Thus, they are frequently waterlogged and anaerobic (Clarke & Hannon, 1967, 1969; Howarth, 1993). The anaerobic conditions are also accompanied by considerable changes in soil chemistry. Nevertheless, the burrows of many species serve to increase the regions of aerobic sediment, which may provide habitats for meiofauna and locally stimulate root growth. However, despite the activities of burrowing animals and radial oxygen loss from roots, much of the subsurface sediment in most salt marshes is anaerobic; even in a largely aerobic sediment there will be numerous anaerobic microniches (Jϕrgensen, 1977a).

Succession, zonation and marsh structure

Various plants can act as primary colonisers of the salt marshes (for example *Salicornia* and *Spartina*) (Boaden & Seed, 1985; Adam, 1993). Following primary colonisation, other species can become established due to environmental changes induced or accentuated by the pioneers. These changes and the subsequent arrival of further species may lead to the habitat becoming unsuitable for some or all the earlier species. Thus a series of fairly predictable changes in community composition, termed a succession, are induced.

The dominant changes in salt marshes are associated with sediment accretion, leading to a raising of the marsh surface and thus to an increase in drainage and to a decrease in submersion by salt water. There are also factors which change with distance inland (and hence height) from the marsh edge. The succession pattern is therefore often paralleled by the marsh zonation (Boaden & Seed, 1985). The actual zonation or inferred succession has been studied in many areas. The usual net result is the description of a three - or four - stage system consisting of pioneers on the upper mudflat which may be bounded by a low salting cliff, a fairly level general salt marsh with creek and pans (salt marsh pools) and often with definable lower and upper zones and, finally, a high marsh zone bordering more terrestrial shrubby vegetation (Figure 3.1) (Boaden & Seed, 1985). Variation in species composition with elevation occur in the microfauna, the fauna, but most obviously, in the vascular plants (Adam, 1993).

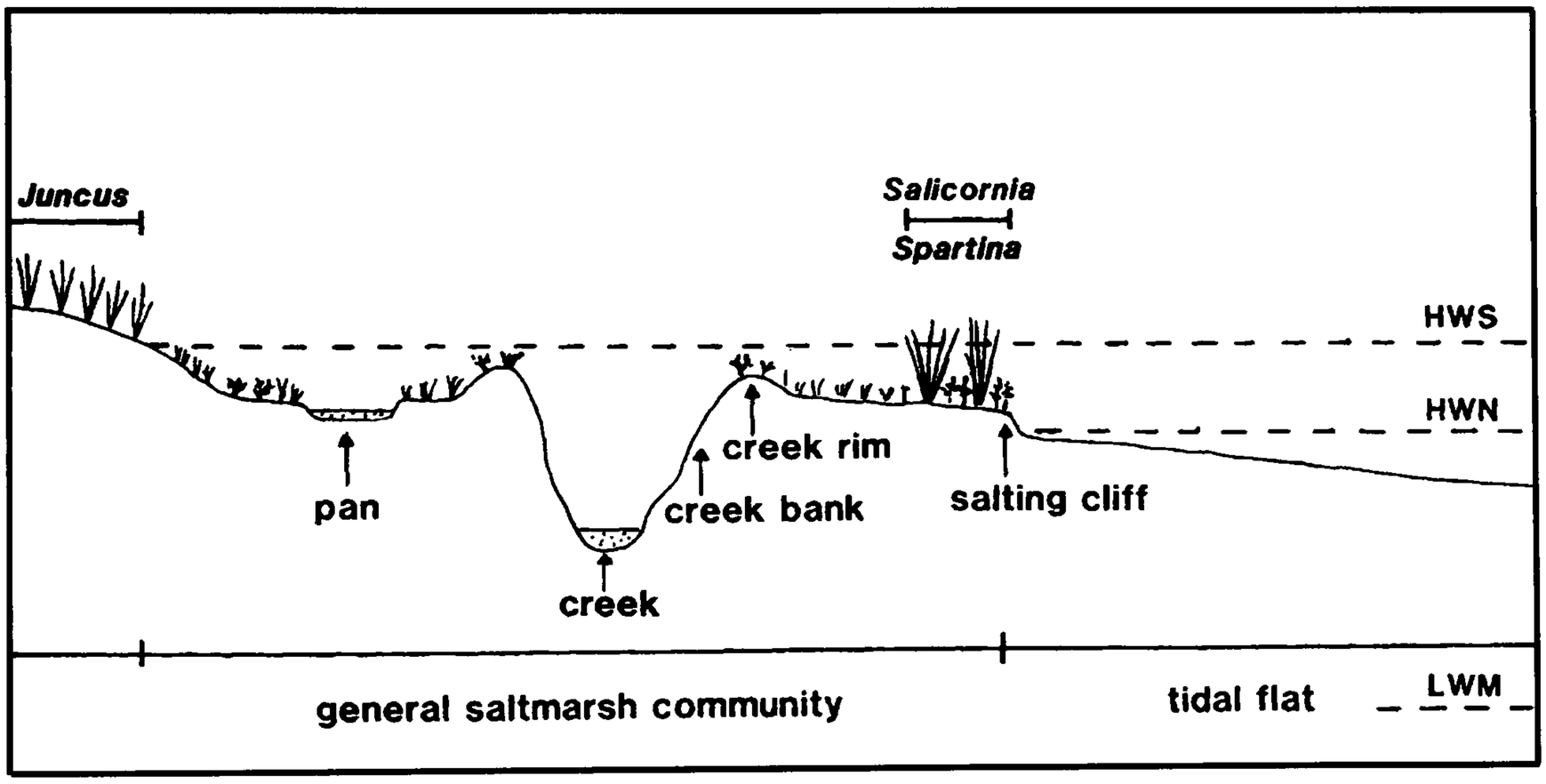


Figure 3.1. Generalised transect of a North European salt marsh (Boaden & Seed, 1985).

As described, the development of a salt marsh follows a fairly well defined successional sequence, starting with a pioneer phase leading to a middle marsh and eventually to a mature marsh (Brereton, 1971). Each of these phases is characterised by a particular assemblage of plants. The pioneer phase is characterised by a variety of halophytic annuals including glasswort (*Salicornia* spp.) and sea-blite (*Suaeda maritima*). The middle marsh is dominated by common salt marsh grass (*Puccinellia maritima*) and red fescue (*Festuca*

rubra), and the upper mature marsh contains a wide diversity of plants including the sea rush (*Juncus maritimus*). Middle and mature salt marshes provide very good feeding and roosting areas for birds (especially migratory species), as well as nesting sites for waders such as dunlin (*Calidris alpina*). Salt marshes are also a key habitat and nursery area for certain marine species.

3.2. Salt Marsh Biota

3.2.1. Bacteria

Bacteria are an important component of the salt marshes microflora. As far as ecosystem functioning is concerned, it could be claimed justifiably that it is bacteria that are responsible for those features of nutrient cycling that are unique to salt marshes (Adam, 1993). Microbial processes in salt marshes have been extensively studied, and both rates and controls of these processes are among the best known for any ecosystem. Nonetheless, much is still unknown, in part because of the great diversity of processes found. Almost every microbial process found in any ecosystem can be found in salt marshes. A more detailed account of the bacteria indigenous from salt marshes and their metabolism will be given later.

3.2.2. Fungi

There are only a few works regarding the fungi in salt marshes and most of the information available relates to North America and British salt marshes (Adam, 1993). The range of soil fungi isolated from various British and European marshes is similar (Pugh, 1960; 1962; Pugh & Beeftink, 1980). In studies of soil fungi from British salt marshes, Pugh (1960, 1962) divided the flora in two categories: 'salt marsh inhabitants' which were isolated with increasing frequency in an upshore direction and 'salt marsh transient' which were regularly carried into the marsh by the tide and showed a downshore increase in frequency of isolation. The fungal flora increase in abundance and species richness upshore (Pugh, 1960; 1962).

One widely recorded specie is *Dendryphiella salina* which is characteristic of high salinity environments. In places where salinity decreases, it is replaced by *Giocladium roseum*. There is also a considerable number of cases of parasitic relationships between fungi and plants, such as mildew and rusts with *Uromyces limonii* being an example of the latter (Pugh, 1979).

3.2.3. Flora

Algae

Although the immediate usual impression of salt marshes is provided by the vascular plants, algae are often an abundant component of the flora. Algae are found attached to vascular plants, on and in the upper layers of the sediment, and as free living forms. The total number of salt marsh algae species is very much larger than the number of vascular plants (Adam, 1993). These communities are rarely coterminous with vascular plant communities and are organised at different spatial scales (Polderman, 1979). In addition, the extent of temporal change in algae communities is considerably greater than within the vascular plant communities (Polderman, 1979). The biomass of algae is much lower than that of vascular plant communities, although the productivity of algae may be a much larger fraction of the total because of more rapid turnover. In addition, as algae lack the lignification of vascular plants they may break down and decay more rapidly than the higher plants and play an important role in nutrient cycling. They are important as food sources, not only for fauna of marine origin but also for many of the invertebrates of terrestrial ancestry (van Wingergen *et al.*, 1981). Algae may also play a crucial role in the establishment of salt marshes. Coles (1979) has shown that stabilisation of mudflats is promoted by the mucilaginous nature of the dominant algae, particularly diatoms. Experimental reduction in grazing pressure on the mudflats, permitting greater algae growth, was followed by increased sedimentation. Algae, can therefore, be claimed to facilitate the subsequent colonisation by vascular plants (Adam, 1993).

The common occurrence are algal mats dominated by cyanophyles in the summer and by the green algae in spring and autumn (Zedler, 1982). Some red and brown algae have adapted to saltings, for example *Bostrychia* attaches to other salt marsh plants, and some

genera including *Fucus* and *Pelvetia* have species with dwarf forms occurring at quite high marsh levels (Boaden & Seed, 1985).

Vascular plants

Although there are considerable differences in species and community composition between different marshes, certain general features of salt marshes vegetation can be recognised and the most frequent is that it is species-poor. The lower marsh is universally species-poor in terms of vascular plants, with the lowest communities generally consisting of a very small number of species. Often the lowest community forms a clear zone dominated by a single species, but there may occasionally be a coarse mosaic of communities with each community forming large patches dominated by a single species. With increasing elevation, species diversity tends to increase as well as the number of communities (Adam, 1993). Much upper marsh vegetation variation can be related to recurrent microscale environmental patterns, such as the change from creek level to inter-creek basins. Similar environmental patterns are also found in the lower marsh, but are frequently less obviously reflected in the vegetation. This may be a consequence of the species paucity of the lower marsh, but also suggest that the frequency of tidal flooding in the lower marsh overrides any point-to-point variation in the environment. The extreme upper marsh and the transition to other habitats, is a region of considerable environmental heterogeneity, strong environmental gradients and considerable temporal variability (Adam, 1993). The two genera which are most prominent as pioneer salt marsh are *Salicornia*, often known as glasswort, samphire or pickle-weed, and *Spartina* or cord-grass. *Puccinellia*, *Scirpus* and *Juncus* species are also often present (Boaden & Seed, 1985).

In relation to the florescent vegetation, the general trend in English marshes is from low-marsh populations of plants which first flower two or more years after germination, in August-September, and produce heavy fruit with no innate dormancy, to the upper-marsh populations with a high proportion of first-year flowering plants which flower in later September-October and produce light fruit- a high proportion of which require a chilling treatment to break dormancy. *Aster tripolium* is an example of a flowering plant, a Compositae, of wide ecological and geographical amplitude in coastal marshes in Europe.

3.2.4. Fauna

The salt marsh fauna can be subdivided in various ways: taxonomically, by ecological affinity (the salt marsh fauna includes groups with marine, freshwater, and terrestrial affinities and evolutionary origins), trophically by subhabitat occupied, or by residence status. Some animals are permanently resident in salt marshes; other are visitors, some seasonally (as many migratory birds), some only at high tide, others at low tide, some at particular stages in their life cycle, many only casually (Adam, 1993).

Invertebrates

In the pioneer zone of the Wadden Sea marshes, Dijkema (1984) found the polychaete *Nereis diversicolor*, the bivalve mollusc *Macoma baltica*, the gastropod *Hydrobia ulvae* and the amphipod *Corohium* sp. The continuously vegetated lower marsh offers much greater habitat diversity than the pioneer zone and supports a rich fauna - for example crabs (Adam, 1993). Crabs are not the only organisms to induce great bioturbation in the salt marsh sediments; a great variety of benthic animals disturb the texture and chemistry of those sediments (Kristensen, 1988) depending on their specific feeding type, mobility and life cycle (Kristensen *et al.,* 1985). The resulting physical and chemical changes in habitats conditions are known to enhance microbial activities and growth rates (Kristensen *et al.*, 1985). Marsh crabs burrow frequently in some areas, perhaps once per day per crab, and cause turnover and local aeration of intertidal sediment to depths of 10 to 100 cm (Basan & Frey, 1977; Bertness, 1985). The burrows may therefore stimulate the growth of *Spartina*, and crab burrows provide a habitat for a variety of symbiotic organisms. Fiddler crabs can aerate the top half centimetre of marsh when feeding at very low tide. Such bioturbation may stimulate primary production by bringing diatoms into close contact with light and nutrients. Detrital decomposition and microbial nutrient recycling may be stimulated into the aerobic zone where fiddler crabs have grazed frequently (Montagna *et al.,* 1981; Bertness, 1985).

The fauna of the midmarsh is considerably richer in species than that of the lower marsh, and, except in creeks and pans, is almost entirely of terrestrial origin. Insects are of relevance in the niche as are arachnids.

The composition of the upper marsh strongly depends on the nature of the terrestrial vegetation; for example, the fauna of brackish upper-marsh reedswamps is very different in character from that in a dry grassland transition between salt marsh and sand dune (Adam, 1993).

Vertebrates

Fish, birds and mammals are the more important and characteristic groups of vertebrates in salt marshes. Reptiles and amphibia are not normally characteristic of salt marshes (Adam, 1993). At low tide, fish are restricted to pans and permanently flowing creeks but at high tide they are able to move over the main marsh surface (Adam, 1993). The fish community of salt marshes contain a number of species which are permanent residents and juveniles of other species which, as they mature, move offshore. Salt marshes provide an important nursery habitat for juveniles of many species which are of economic importance in coastal fisheries (for example see Morton *et al.,* 1987). Creek channels are an important fish habitat, for example gobies (*Pomatoschistus* sp.), menhaden (*Brevoortia tyrannus*), mullet (*Mullus* sp.) and mummichog (*Fundulus heteroclitus*), though not necessarily throughout all of their life. The frequency of tidal flooding limits the value of salt marshes as habitat for nesting birds. Nevertheless, upper and midmarsh may be free from tidal flooding for periods of weeks, or even months, sufficient to allow a number of species to breed (Adam, 1993). Salt marshes are probably best known not for their breeding birds but for the large flocks of other species which may utilise the salt marsh at various times of the year. Wildfowl, both ducks and geese, congregate on salt marshes in large numbers, for both breeding and roosting. The impact of grazing and trampling by wildfowl may be considerable (Smith & Odum, 1981; Smith, 1983). Winter use of North European salt marshes is not restricted to wildfowl but to waders and a large number of passerines (Adam, 1993). Seed-eating finches are prominent, particularly linnets (*Acanthis cannabina*) and greenfinches (*Carduelis chloris*). Salt marshes in eastearn England, particularly around the Wash are the major overwintering habitat for twite (*Acanthis flaverostris*) which feed on seeds of *Salicornia* spp. and *Aster tripolium* (Adam ,1993). The mammals present in salt marshes have considerable impact on salt marsh vegetation. They mainly include red deer (Scotland), rabbits, hares, rates, moles, voles *etc*. depending on the area where the salt marsh is located (Boaden & Seed, 1985; Adam, 1993).

3.3. Sources and Degradation of Organic Matter

Salt marshes are among the most productive ecosystems in the world, with high rates of net production of carbon by the dominant grasses (Schubauer & Hopkinson, 1984), algae (Pomeroy *et al.,* 1981) and in some marshes by chemoautotrophic sulphur-oxidising bacteria (Howarth, 1984). Most of this organic matter is decomposed *in situ*, fuelling other microbial processes (Howarth, 1993). According to various authors (Pomeroy *et al.,* 1981; Schubauer & Hopkinson, 1984) at least half, and often much more, of all the primary production occurs as the below-ground production of roots and rhizomes. In addition, most of the production by chemoautotrophic bacteria in marshes probably also occurs below-ground in the sediments (Howarth, 1984). Thus, marshes have very large inputs of newly produced, relatively labile organic matter directly into their sediments.

Degradation and recycling of organic matter in many habitats is shared by bacteria, fungi, and protozoa together with larger meio- and macrofauna (Atlas & Bartha, 1981; Westermann, 1993). A common feature of all these organisms is their ability to mineralise organic molecules completely to carbon dioxide. Under anaerobic conditions, bacteria are mainly responsible for biological mineralisation, while protozoa and fungi are considered less important.

Micro-organisms are responsible for the recycling of most of the very abundant but difficult to digest biopolymers, such as cellulose and lignin (Atlas & Bartha, 1981). Cellulose degradation occurs under both aerobic and anaerobic conditions. Under aerobic conditions, various fungi as well as aerobic and facultative anaerobic bacterial populations are involved in cellulolytic activities. The main products of cellulose degradation under aerobic conditions are carbon dioxide, water and cell biomass. Anaerobic fermentation of cellulose results in the production of low molecular weight fatty acids as well as carbon dioxide, water and cell biomass. Respiratory metabolism yields more energy to the cell than fermentative metabolism. In aerobic habitats, it tends to be more prevalent than fermentation because this requires a greater consumption of organic matter to support the same biomass than does respiration.

Complete mineralisation does not take place within an organism, but within associations of physiologically different types of micro-organisms. Each cell exploits only a part of the detrital energy and supplies the rest, in the form of excreted metabolic end products, to the next step in the detrital food chain. Thus, the anaerobic detrital energy flow is carried by small extracellular molecules, which to large extent are inorganic (Jørgensen, 1980).

3.4. Oxygen and Aerobic Respiration

When saturated with water, marsh sediments are anoxic below the top few millimetre (King, 1988; Christian *et al.,* 1981). This is a result both of high rate of oxygen consumption by marsh sediments and the very slow rate of oxygen diffusion through waterlogged pores. In fact, no oxygen may actually reach the sediment when it is completely waterlogged (Bedford *et al.,* 1991).

Oxygen penetration into marsh sediments probably varies greatly from marsh to marsh, and even within a marsh, due to differences in frequency of tidal flooding, porosity of the sediment, and distance to creeks (Howarth, 1993). At low tide, air can enter some of the pore spaces of marsh sediments, due both to lateral drainage of pore waters to creeks (Anderson & Howell, 1984; Agosta, 1985) and to loss of sediment pore water during evapotranspiration by the marsh grasses (Dacey & Howes, 1984; Morris & Whiting, 1985). This greatly speeds the rate of oxygen diffusion in the sediment, both from the sediment surface and from the grass rhizosphere. Salt marsh grasses have hollow internal channels which allow oxygen diffusion in a gas phase from the air to the rhizosphere (Armstrong, 1975), and oxygen diffusion from the plant roots to the sediment is probably faster when the sediment containing gas-filled pores (Howarth & Hobbie, 1982).

Aerobic respiration using oxygen as the electron acceptor can occur when oxygen penetrates into partially drained sediments. Aerobic respiration can also occur at the sediment surface and perhaps at the surface of grass roots in the rhizosphere, if oxygen actually diffuses out the roots (Bedford *et al.,* 1991).

Oxygen serves as a terminal electron acceptor not only in the degradation of organic matter but also in the oxidation of reduced inorganic chemicals used as energy sources by chemiolitotrophs (Atlas & Bartha, 1981). The aerobic degradation of organic matter usually proceeds according to the generalised formula:

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 138O_2 \longrightarrow 106CO_2 + 122H_2O + 16HNO_3 + H_3PO_4$$

This type of reaction is responsible for very fast turnover of organic matter in oxygenated environments. Most of the O_2 produced *via* photosynthesis is exhausted by this process in aquatic and terrestrial aerobic environments (Krumbein & Swart, 1983). Besides its importance as the major site of litter degradation, the aerobic environment plays an important role in the cycling of nitrogen and sulphur (Westermann, 1993).

3.5. Anaerobic Metabolism

Anaerobic mineralisation of organic molecules demands the interaction of a complex microbial food web, where the products of one microbial group serve as a substrate for the subsequent microbial group, and when the consumption of a product regulates its type and formation rate. Figure 3.2 shows the main features of anaerobic metabolism, and the microbial groups responsible for the degradation of organic matter if various electron acceptors are present. In the absence of oxygen, bacterial respiration can proceed using other electron acceptors. The energy available to bacteria carrying out respiration depends upon the particular electron acceptor used and generally decreases according to the following list of acceptors: oxygen, nitrate, manganese, iron, sulphate, and carbon dioxide (Jørensen, 1980; Laanbroek, 1990). Generally, competition among bacteria for substrates will tend to lead to a domination by respiration that uses the more energetically favourable electron acceptors if they are available. However, this idealised separation of the use of electron acceptors does not always hold in nature, perhaps because competition among bacteria for substrates is not that severe and other factors are partially controlling bacteria activity. For example, methanogenesis and sulphate reduction often co-occur in marine sediments (Ivanov *et al.,* 1989), which perhaps should not be surprising since the energy yields for the two processes are not that different. Perhaps more surprisingly, sulphate

reduction has been found to occur in the presence of oxygen in microbial mats (Canfield & Des Marais, 1991).

However, if no external electron acceptors are present different groups of organisms are active in the conversion of complex organic molecules to methane, carbon dioxide and water (Westermann, 1993) (Figure 3.2).

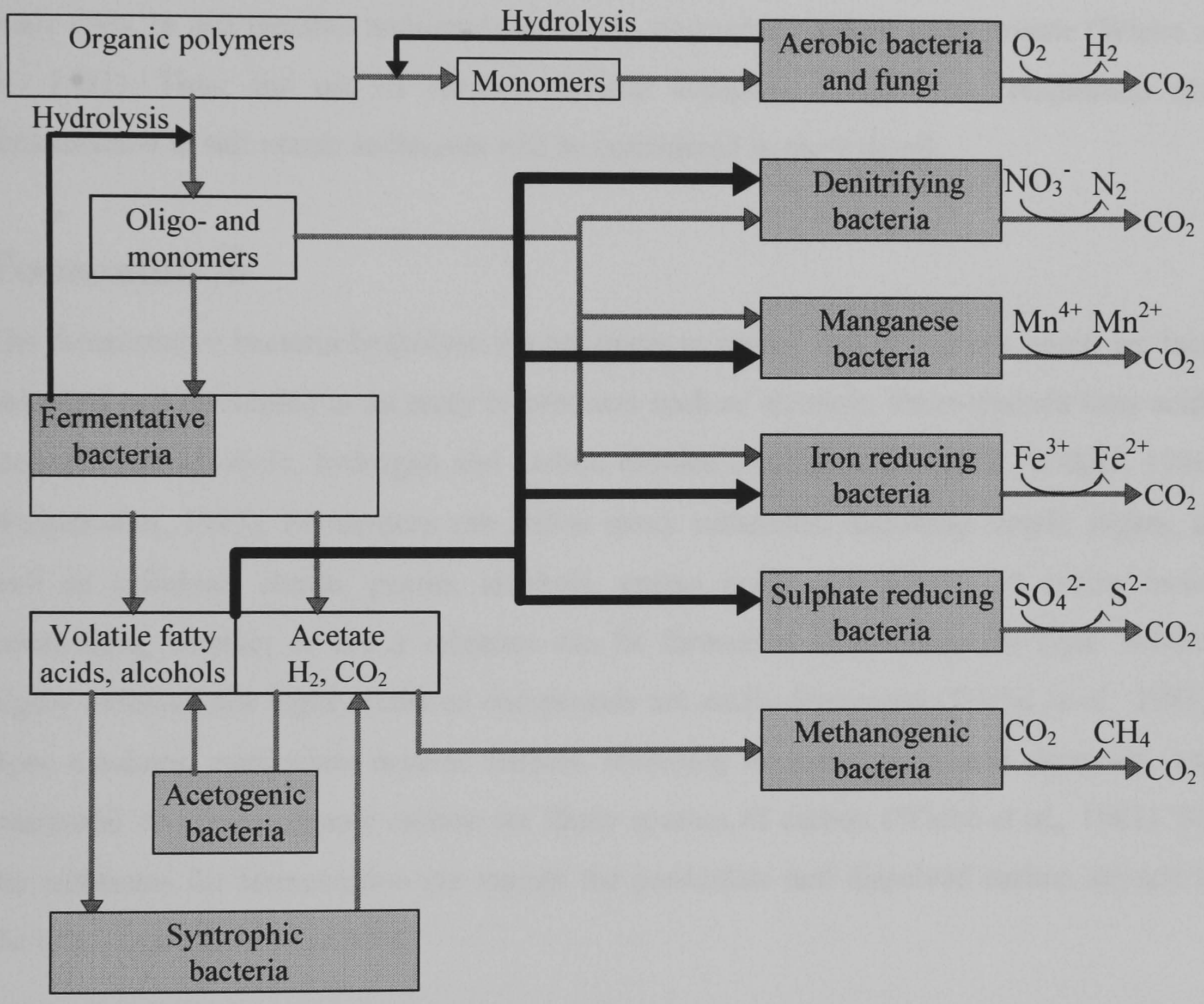


Figure 3.2. Main pathways of the degradation of organic matter in wetlands (Westermann, 1993).

For instance, the end-products of fermentation such as volatile fatty acids and alcohols can be oxidised by hydrogen-producing syntrophic bacteria, leading to the formation of hydrogen, acetate, and carbon dioxide (Figure 3.2). The syntrophic bacteria can only metabolise the fermentative products if the partial pressure of hydrogen is kept low by carbon dioxide-reducing methanogenic bacteria, as this proton reducing oxidation process

is energetically unfavourable under standard conditions. Acetate is also produced in large amounts directly by the fermentative bacteria when the hydrogen partial pressure is low, and is finally split into methane and carbon dioxide by aceticlastic methanogens. Acetogenic bacteria might also produce acetate from hydrogen and carbon dioxide, or even higher fatty acids from acetate and other C_1 and C_2 compounds (Westermann, 1993).

Anoxic decomposition may represent a major pathway for energy flow in salt marshes, particularly in view of the large standing crop of below-ground biomass recorded from many sites. In salt marshes sediments and soils, anaerobic habitats predominate (Wiebe *et al.,* 1981). Thus, the use of various electron acceptors in anaerobic respiration and fermentation in salt marsh sediments will be considered in more detail.

Fermentation

The fermentative bacteria hydrolyse the polymers to mono- and oligomers which are then absorbed and fermented to an array of products such as alcohols, short-chained fatty acids (acetate) and alcohols, hydrogen and carbon dioxide (Wiebe *et al.,* 1981, Widdel, 1986; Westermann, 1993). Fermentors can utilise many substrates, including simple sugars, as well as cellulose, starch, pectin, alcohols, amino acids and purines. A major factor determining whether or not a substrate can be fermented is its oxidation state. Neither highly oxidised nor highly reduced compounds are easily fermented (Wiebe *et al.,* 1981). Root exudates, particulate organic carbon, including decaying roots and rhizomes, and interstitial dissolved organic carbon are likely sources of carbon (Wiebe *et al.,* 1981). So, the substrates for fermentation are mainly the particulate and dissolved carbon already in the soil (Christian *et al.,* 1978).

Although a wide variety of organisms are capable of fermentation reactions, bacteria and yeasts are responsible for most of this activity in salt marshes. They may be facultative or obligatory anaerobic; the former are capable of either aerobic respiration or fermentation while the latter are inhibited or killed by oxygen. The obligate anaerobic fermenters are heterotrophs that dispose of excess electrons by producing reduced fermentation products (Kaspar & Tiedje, 1982).

Dinitrification

Dissimilatory nitrogenous oxide reducers (DNOR) is a term which encompasses three specific pathways (Figure 3.3) (Wiebe *et al.,* 1981; Blackburn, 1983; Koike & Sϕrensen, 1988). The nitrogenous oxides serve as a terminal electron acceptors during cytochrome-catalysed oxidation of organic matter. Nitrate serves as a terminal electron acceptor and it is first reduced to nitrite. Nitrite may further be reduced to N_2O and eventually to N_2 and this process is called **denitrification** (Wiebe *et al.,* 1981; Koike & Sϕrensen, 1988). In some cases, however, the NO_2^- may be further reduced to NH_3, and this process is called dissimilatory ammonia production (Wiebe *et al.,* 1981). This last process can be quantitatively important in nature (Buresh & Patrick, 1978), especially in coastal sediments (Koike & Hattori, 1978; Sϕrensen, 1978).

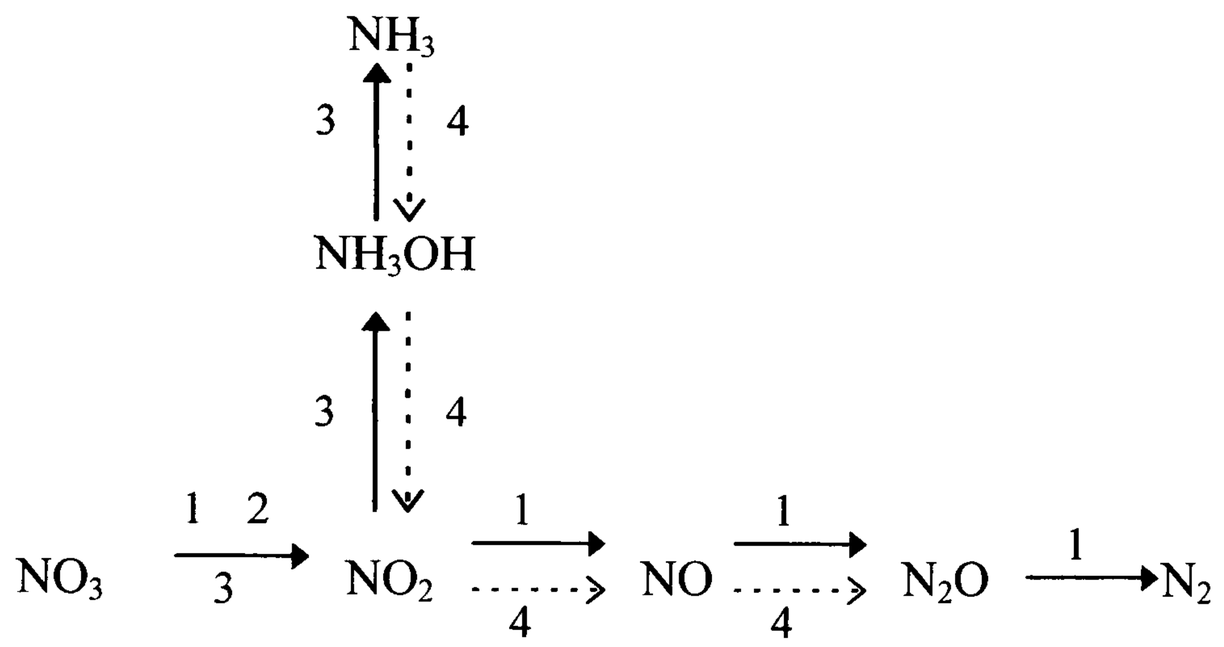


Figure 3.3. Pathways of dissimilatory nitrogenous oxide reduction.
1. Denitrification.
2. Dissimilatory reduction (terminates at NO_2).
3. Dissimilatory ammonia production.
4. " Nitrification" N_2O pathway: ammonia to nitrous oxide.
(Adapted from Wiebe *et al.,* 1981).

An ideal environment for microbial nitrate reduction is where nitrate is in ample supply to substitute oxygen in the processes of organic matter degradation (Koike & Sϕrensen, 1988). The most common denitrifying bacteria in natural ecosystems are aerobes belonging to the genera *Pseudomonas* (Gamble *et al.,* 1977; Westermann, 1993) and *Alcaligenes* (Westermann, 1993). Dissimilatory reduction of nitrate to ammonia is mainly carried out by enterobacteria, *Clostridium* and *Bacillus* species which are typically obligate and facultative anaerobes with fermentative metabolism (Tiedje, 1988).

Dissimilatory nitrogenous oxide reducers can utilise a wide variety of substrates for growth. Theoretically, they can utilise the compounds almost as efficiently as aerobic organisms, because the oxidation of substrates by dissimilatory nitrogenous oxide reducers is cytochrome catalysed (Wiebe *et al.,* 1981). The dissimilatory nitrogenous oxide-reducing

enzymes are induced by the absence of oxygen, whether or not nitrogenous oxides are present.

Denitrification occurs in salt marshes, but tends to be limited by a relatively slow rate of supply of nitrate (Howarth, 1993). Denitrification is a major sink for nitrogen in marshes, which is important because both salt marshes and the coastal marine ecosystems with which they interact are frequently nitrogen limited (Whitney *et al.*, 1981). However, the consumption of organic matter during denitrification appears to be quite small. Since rates are limited by the supply of nitrate, any addition of nitrate will increase rates dramatically (Lindau & DeLaune, 1991). It seems likely that at least some nitrification (the oxidation of ammonium to nitrate by bacteria) occurs in the rhizosphere of marsh grasses since oxygen and high concentration of ammonium may co-exist there. Any nitrate which is thus formed can rapidly diffuse into the adjacent anoxic sediments and be denitrified. In fact, the rhizosphere of marsh grasses seems like an ideal location for coupled rates of nitrification and denitrification (Reddy *et al.*, 1989; Lindau & Delaune, 1991).

Iron and manganese reduction

Fe and Mn are considered together since they have many properties in common. The transformations of Fe and Mn in the environment essentially consist of a simple change between the most reduced forms Fe (II), Mn (II) and the most oxidised forms Fe (III) and Mn (IV) (Grant & Long, 1981). Ferric iron can be chemically reduced by many compounds, some of which are characteristic excretion products of microbes grown anaerobically (*e.g.* formate or sulphide) as well as by lowering the Eh and pH of the environment and establishing conditions that favour reduced over oxidised phases of iron (Nealson, 1983). In addition, many bacteria have the capability to reduce iron under conditions in which it would not be spontaneously reduced. The bacteria that reduce iron are widely distributed. *Bacillus* spp. are particularly widespread, abundant and active in iron reduction, although many other bacteria have this capability (Grant & Long, 1981, Nealson, 1983).

In some pure-culture studies and some sediments, iron-reducing bacteria have been found to outcompete sulphate reducing bacteria for substrates (Lovley, 1987). However, in salt marsh sediments the dominant process appears to be the reduction of Fe (III) by chemical

reaction with hydrogen sulphide. According to Howarth (1993), Jacobson (1990) found that in salt marsh sediments there is little, if any, competition between the process of sulphate reduction and iron reduction. Jacobson (1990) also observed that the addition of molybdate (to inhibit sulphate reduction) to salt marsh sediments slowed the rate of iron reduction, because less sulphide was available for the chemical reduction of iron. If iron reduction in the marsh was predominantly bacterial, the accumulation of fatty acids resulting from inhibiting sulphate reduction would have been expected to increase the rate of iron reduction. Sulphides from sulphate reduction seem to be abundant enough in the marsh sediments to chemically react with iron (III) before it can become available to any iron-reducing bacteria.

Not much attention will be given to the use of oxidised manganese as an electron acceptor for bacterial respiration in salt marsh sediments, since it appears from a literature search that this issue has not been much researched. However, in freshwater wetlands the reduction of Mn is thought to be largely a chemical process (Laanbroek, 1990). Therefore, in salt marsh sediments, it should be expected that Mn would be reduced chemically before becoming available to bacteria, as in the case of iron (Howarth, 1993).

Sulphate Reduction

Sulphate reducing bacteria (SRB) are a diverse group of anaerobic bacteria, unified by their use of sulphate as a terminal electron acceptor in the oxidation of organic matter (Wiebe *et al.,* 1981; Jϕrgensen, 1983; Jones, 1986; Battersby, 1988; Macfarlane & Gibson, 1991). In these reactions, sulphate is stoichiometrically reduced to sulphide according to the equation (Macfarlane & Gibson, 1991):

$$2CH_2O + SO_4^{2-} \longrightarrow 2HCO_3^- + H_2S$$

The bacteria which are able to perform respiratory sulphate reduction have been isolated from a wide range of anoxic environments. They have been grouped in two genera, *Desulfovibrio* and *Desulfotomaculum* (LeGall & Postgate, 1973). The substrates used for sulphate reduction in marine sediments are fermentation end-products (*e.g.* short-chain fatty acids and hydrogen) produced by other sedimentary bacteria. The sulphate reducing bacteria were, until recently, believed to be quite restricted in the organic compounds which they can use as energy sources for growth. The well known strains of *Desulfovibrio*

utilised mainly lactate, pyruvate and C_4 dicarboxylic acids such as malate succinate, but rarely carbohydrates (LeGall & Postgate, 1973). The carbon of the organic substrates is not oxidised completely to CO_2 by *Desulfovibrio* but is excreted partly as acetate (Jørgensen, 1983; Widdel, 1986). The respiratory metabolism of lactate is:

$$2CH_3CHOHCOO^- \text{ (Lactate)} + SO_4^{2-} \longrightarrow 2CH_3COO^- \text{ (Acetate)} + 2HCO_3^- + H_2S$$

Most *Desulfovibrio* strains have hydrogenases and can also utilise H_2 as an energy substrate (Jϕrgensen, 1983; Widdel, 1986):

$$4H_2 + SO_4^{2-} + 2H^+ \longrightarrow 4H_2O + H_2S$$

Hydrogen is an important product in anaerobic fermentation and H_2 formation may be stimulated by its uptake by *Desulfovibrio* (Jϕrgensen, 1983). In most cases, the ability of *Desulfovibrio* spp to grow well with lactate is associated with the capacity to utilise H_2 in the presence of CO_2 and acetate or another organic carbon source (Laanbroek & Pfenning, 1981; Widdel, 1986). In the absence of sulphate, or when iron limits the formation of cytochrome C_3, some strains may ferment pyruvate to acetate, H_2 and CO_2 (Vosjan, 1975).

$$CH_3COCOO^- + H_2O + OH^- \longrightarrow CH_3COO^- + HCO_3^- + H_2$$

Also, some species of *Desulfotomaculum* oxidise lactate and pyruvate to acetate and CO_2. *Desulfobacter* spp and *Desulfotomaculum acetoxidans*, however, may oxidise short-chain fatty acids, such as acetate, completely to CO_2 (Widdel & Pfenning, 1977, Widdel, 1986):

$$CH_3COO^- + SO_4^{2-} \longrightarrow 2HCO_3^- + HS^-$$

Many new strains of sulphate reducing bacteria with a wide spectrum of metabolism and morphology were recently isolated by Widdel (1986). For the classification of these new isolates five new genera were suggested (*Desulfobacter, Desulfobulbus, Desulfococcus, Desulfonema, Desulfosarcina*) as well as a revision of the genus *Desulfovibrio*. The work of Widdel (1986) has revealed the potential versatility in the substrate utilisation of sulphate reducing bacteria in nature. The organic substrates may be oxidised completely to carbon dioxide by individual strains, or incompletely producing acetate and providing a substrate for acetate oxidising sulphate reducers. Thus, all the saturated *N*-fatty acids

ranging from formate, acetate, propionate, butyrate *etc.*, up to stearate, as well benzoate, hydroxybenzoates and urate, can now be added to the list of substrates.

Data by Sϕrensen *et al.,* (1981) and Christensen (1984) indicate that the quantitative contribution of the various substrates to sulphate reduction in marine sediments is: acetate (40-65%), propionate (10-20%), butyrate (5-20%), isobutyrate (8%) and H_2 (5-10%). If the oxidation of butyrate and propionate is incomplete, however, then acetate oxidation can account for almost two thirds of the sulphate reduction occurring in the sediments. Sulphate reducing bacteria therefore have four important roles in anaerobic marine food chains: acetate oxidation, diverse substrate oxidation, hydrogen production (under conditions of sulphate limitation) and hydrogen utilisation.

Sulphate reduction is of particular importance in the degradation of organic matter in salt marshes, degrading perhaps twelve times more organic matter than oxygen respiration and denitrification combined (Howarth & Teal, 1979). In relatively waterlogged areas of two well studied salt marshes (Sapelo Island, Georgia and Great Sippepewisset Marsh, Massachusetts) the sulphate reduction accounts, respectively, for 67% (Howarth & Giblin, 1983) and 80% (Howarth & Teal, 1979) of all the respiration. Sulphate reduction rates often vary between two to three fold within a given marsh, with higher rates in waterlogged sediments (Howarth & Giblin, 1983; King, 1988) but organic carbon availability rather than sulphate regulates rates of sulphate reduction (Wiebe *et al.,* 1981).

Factors controlling sulphate reduction and associated fermentations

Given the importance of sulphate reduction and associated fermentations in the metabolism of salt marshes, it is important to examine the controls of these processes. Temperature is one controlling factor, with rates of sulphate reduction within a marsh increasing with seasonal increases in temperature (sulphate reducing bacteria grow best between 25 and 35°C). However, temperature alone does not explain differences observed within a marsh. The supply of substrates, as already mentioned, is of great importance in salt marshes where there is enough sulphate available. Oxidation-reduction potentials are frequently mentioned as a factor controlling sulphate reduction rates in wetlands and other sediments, with sulphate reduction occurring only at relatively low redox potential (Eh). In the literature, empirical values of Eh (Eh corrected to pH=7) have been established, above which sulphate reduction should not occur: +100mV (Stenvenson 1986) and -120 to -

180mV (Laanbroek, 1990). However, high rates of sulphate reduction have been found in surface layers of some marine sediments having measured Eh values as high as +400mV. It is important to distinguish between theoretical oxidation-reduction potential and the potential measured in marsh sediments. In salt marsh sediments, Eh electrodes respond principally to the electroactivity of sulphide ions, and high values often just reflect the low concentration of sulphide resulting from rapid precipitation of pyrite (Howarth *et al.*, 1983).

Conversely, Jφrgensen (1977b) attributed the sulphate reducing bacteria activity when the Eh measured was +250mV to +350mV to the presence of microniches. According to the author, these microniches with 50-200 μm diameter are not registered by the redox measurements because they are much smaller than the platinum electrode used to perform the measurements.

Methanogenesis

Methanogenesis mediates the last step and often the rate of anaerobic mineralisation of organic matter (Kaspar & Tiedje, 1982). The methanogenic bacteria are a group of organisms of considerable taxonomic diversity which nonetheless form a very distinct group on the basis of their physiology and ecology. Methanogenic bacteria utilise only a limited range of substrates for growth and methanogenesis (Hamilton, 1979; Wiebe *et al.*, 1981; Kaspar & Tiedge, 1982; Archer & Harris, 1986). The latter is a means of synthesising ATP, although the free energy yield associated with methanogenesis (Table 3.1) is low (Thauer *et al.*, 1977) and consequently growth yields are low. In nature, hydrogen, carbon dioxide and acetate are the major substrates for the methanogenic bacteria, although formate (Strayer & Tiedje, 1978), methylamines and methanol (Miller & Woline, 1983) may be produced by non-methanogenic bacteria at levels high enough to serve as significant energy sources for the methanogens.

Methanogenic bacteria use CO_2 or a methyl group as a terminal electron acceptor and the product of the CO_2 reduction is CH_4 (methane) (Kaspar & Tiedge, 1982). These organisms require a low Eh to grow in pure cultures of -350mV and strict anaerobic conditions (Wiebe *et al.*, 1981; Winfley, 1984).

Table 3.1. Free energy yields of some methanogenic reactions.

Reaction	ΔG°' (Kj/ reaction)
$Acetate + H_20 \longrightarrow CH_4 + HCO_3^-$	*-31.0
$4\ Formate + H_2O + H^+ \longrightarrow CH_4 + 3HCO_3^-$	-130.7
$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O$	-135.6
$4CH_3NH_3^+ + 3H_2O \longrightarrow 3CH_4 + HCO_3^- + 4NH_4^+ + H^+$	-225.4
$4CH_3OH \longrightarrow 4CH_4 + HCO_3^- + H^+ + H_2O$	-315.1

* Values are for standard conditions, pH 7 (Archer & Harris, 1986).

Methane fluxes from salt marshes are inversely correlated with the concentration of sulphate in the sediments (Bartlett *et al.,* 1987). At one time it was thought that this was just a result of sulphate reducing bacteria outcompeting methanogenic bacteria for substrates (Abram & Nedwell, 1978; Oremland & Taylor, 1978; Jørgensen, 1980) which may indeed be the case in salt marsh sediments. However, in many subtidal marine sediments, high rates of methanogenesis and sulphate reduction can co-exist; the relatively low fluxes out of sulphate-rich marine sediments are often a result of anaerobic methane oxidation rather than inhibition of methane formation (Ivanov *et al.,* 1989).

Methanogenesis, as denitrification, iron reduction and manganese reduction, also appear to be a minor process in the marsh carbon cycle.

3.6. FORYD BAY

Foryd Bay is a sheltered bay which extends south at the western end of the Menai Strait and was notified as a Special Site of Scientific Interest (SSSI) in 1971 (Countryside Council for Wales, 1971) (Figure 3.4). Covering an area of approximately 283 hectares, the bay forms an extensive area of intertidal mud and salt marsh, with latter occupying an area of 123 hectares (Burd, 1989).

The salt marsh serves as an estuary for three rivers: Afon Carrog, Afon Gwyrfai and Afon Foryd. The largest of these rivers is the Afon Gwyrfai which flows from Llyn Gwyrfai in Snowdonia and runs along the eastern side of the salt marsh.

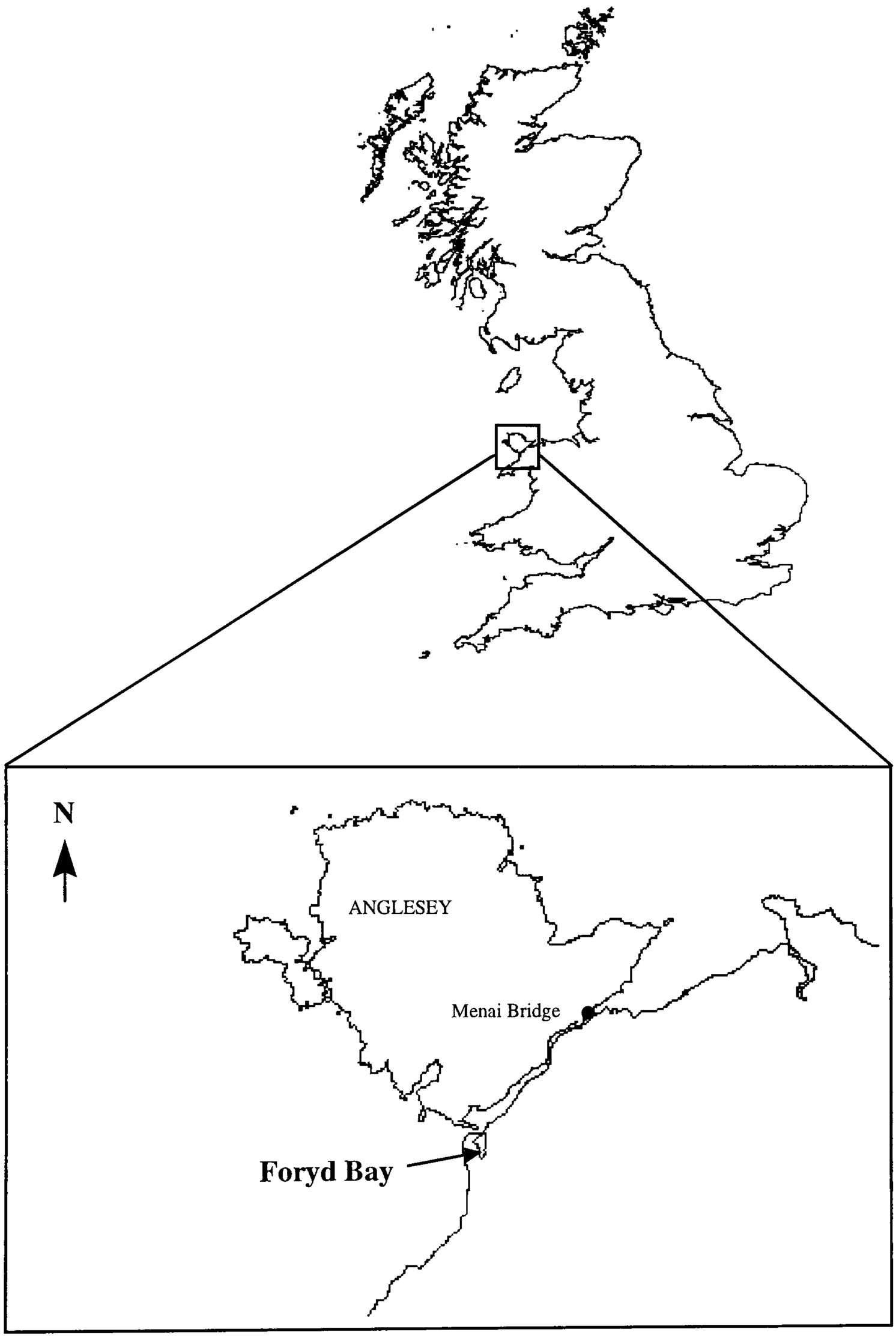


Figure 3.4. Location of Foryd Bay.

In this area there is a continuous gradient between the clean sands near the entrance of the sea and the muds or muddy sands in the sheltered extremes. According to a report by Huckbody *et al.*, (1992) in the Caernarfon and Cardigan Bays, the communities of burrowing invertebrate that colonise Foryd Bay are similar to the other flats of this stretch of coast. Dense populations of polychaete worms (*e.g.* lugworms and ragworms), crustaceans, bivalve molluscs (*e.g.* cockles and file shells), gastropod molluscs (*e.g. Hydrobia ulvae*) and other groups of invertebrates are present over extensive areas. Consequently, these locations are important feeding grounds for birds. Foryd Bay is locally important for wintering wildfowl (winter maxima 700) and waders (winter maxima 1400). Large flocks of wigeon, shelduck, oystercatcher, lapwing, curlew and redshank may occur (Countryside Council for Wales, 1971).

The development of the salt marsh at Foryd Bay was similar to other salt marshes along this coast line. The factors controlling the vegetation development of this salt marsh during the initial stages of Foryd Bay succession were salinity and waterlogging which were associated with different soil texture (Brereton, 1971). This author found that *Salicornia* tended to occur in highly saline non-waterlogged areas whereas *Pucinella* tended to occur in non-saline waterlogged areas.

According to Jones (*pers. commun.*), until 20 years ago it was possible to observe a succession of vascular plants- with *Salicornia*, *Spartina maritima* and *Juncus*, from the lower to the upper marsh. Presently this salt marsh is extensively colonised by cord grass (*Spartina anglica*) as occurs in most salt marshes along this coast line (Huckbody, 1992). The extent of the salt marsh is increasing due to *Spartina* invasion which occupies 70% of the total marsh area (Burd, 1989). The area of intertidal mudflat has, therefore, been reduced as a consequence of the invasion by *Spartina*. Mudflats support a large fauna and the replacement of this community by a monoculture of *Spartina* with few associated animals has aroused concern (Adam, 1993).

The experimental work was carried out in the salt marsh at Foryd Bay. The location of the simulation of the spills of vegetable oils was approximately 53° 06.175'N 004° 19.045'W. This area is located in the upper part of the salt marsh which is a mud zone or muddy sands as opposed to the lower marsh that has sandy sediments. The experiment site is uncovered at low tides and is flooded in the mean high water (Figure 3.5).

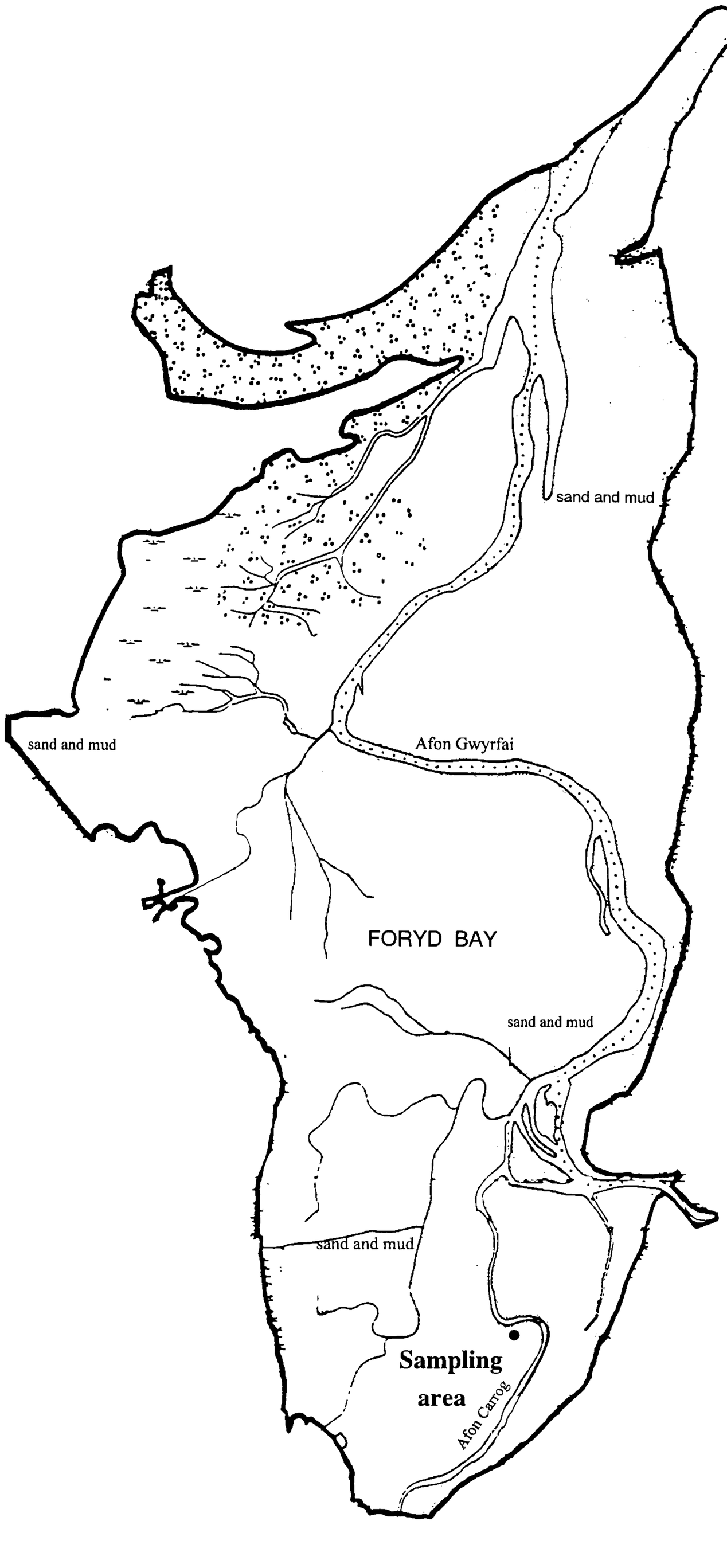


Figure 3.5. Location of the sampling area at Foryd Bay.

4. FIELD EXPERIMENTS SIMULATING VEGETABLE OIL SPILLS

With regard to the first of the three objectives established in chapter 1 (to determine the transformations that occurred to linseed and sunflower oils and to the sediment characteristics after spills in a salt marsh environment), the main hypotheses to test in the practical work presented in this chapter were:

- Spills of linseed and sunflower oils in salt marshes sediments affects both the sediment and the oil characteristics.
- Autochthonous aerobic and/or anaerobic bacteria are able to breakdown and utilise these oils.

4.1. EXPERIMENTAL STRATEGY

Spill simulation

In January 1995, an experiment simulating a spill of linseed oil was carried out in a 4 m^2 area. One year later, a similar spill was simulated in an adjacent area, using sunflower oil.

In the following section, the experimental strategy of these experiments is described. The specific vegetable oil will be mentioned only if there was any differences in the protocol.

Plastic collars of 15 cm length and 30 cm diameter were used to simulate small spills of vegetable oils. Seven collars for linseed oil and eight collars for sunflower oil were placed at random in the selected area. These collars were pushed into the sediments only down to 3 cm depth. The delimitation of this area further down, would prevent any loss of oil by advection. In each collar, one and a half litres of oil was poured simulating a spill of approximately 2 cm height. The collars were covered with a muslin screen to avoid the oil being removed by birds (Mudge *et al.,* 1995).

These reservoirs were left in the field for different lengths of time and sediment samples were collected after 3, 7, 14, 21, 28 and 60 days for the linseed oil experiment and also after 180 days for the sunflower oil assay.

Sampling

On every sampling occasion, before (day 0) and after the oil spill simulation, seven sediment cores of 5 cm diameter and 40 cm length were collected from each collar. Samples to assay anaerobic parameters were sealed in the field, kept and transported in air tight conditions. The concentrations measured at day 0 represent the background levels of each parameter before the sediments were contaminated with oil.

Temperature and pH values were measured in the field. All other parameters were analysed in the laboratory. Samples were transported to the laboratory within two hours of collection and were kept at 5 °C until further analysis. Samples for fatty acids analysis were kept at -20°C. Bacterial numbers were measured as soon as the samples arrived at the laboratory.

Temperature, pH and redox potential measurements were carried out every centimetre from the surface down to 10 cm depth and every 2 cm from 10 cm to 30 cm depth. All other parameters were analysed every 5 cm from the surface down to 30 cm depth. To obtain these samples, the sediment was pushed out of the corers by mechanical action and sections 2 cm thick (0-2, 4-6, 9-11, 14-16, 19-21, 24-26 and 29-31 cm) were sliced. These depths will in future be regard to as depths 1, 5, 10, 15, 20, 25 and 30 cm.

4.2. METHODS

During this work, standard methods and techniques were adopted whenever possible. Although the highest achievable accuracy was sought, higher regard was given to the repeatability of each measurement (precision), since differences between samples were of more interest than the absolute values of the estimates.

4.2.1. Redox Potential (Eh) of the sediments

The redox potential was measured using a platinum electrode (AGB-51), with an operating range between 0 and ± 2000 mV, accuracy of +1 mV and repeatability of 0.5 mV.

The electrode was inserted horizontally into the sediment at the specific depths through predrilled holes in the corer, which were covered during sampling. The probe fitted closely into the holes minimising the entry of air and the mixing of oxygenated and reduced layers. The final reading was recorded 5 minutes after insertion of the electrode, when drift was minimum. The electrode was routinely standardised with a ZoBell solution (0.003M potassium ferrocyanide, 0.003M potassium ferricyanide and 0.1M potassium chloride), with a redox potential of +430 mV at 25°C (ZoBell, 1946).

4.2.2. pH of the sediments

The pH was measured with a glass combination electrode (AGB-51, Portable data-logging pH meter) with an operating range between -19.99 and 19.99, accuracy of +0.01 and repeatability of 0.01.

The pH measurements were taken in a similar fashion to those of the Eh, with the electrode inserted horizontally into the sediments at specific depths, through predrilled holes. The pH value was recorded when the reading in the instrument was stable. Prior to use, the pH meter was calibrated using three standard buffers, with a pH of 4, 7 and 9.

4.2.3. Temperature of the sediments

The temperature was measured also using the AGB-51 meter, which has an operating ranges of -200 to +200 °C, an accuracy of 0.1°C and repeatability of 0.1°C.

The temperature was measured at the same time as the pH, in adjacent predrilled holes on the core.

4.2.4. Enumeration of Bacteria

Aerobic and anaerobic bacteria have a significant role in the carbon recycling in marsh sediments (Howarth, 1993), and possibly play an important part in the degradation of vegetable oils in such environments. Representatives of both bacteria groups were studied using standard techniques.

4.2.4.1. Aerobic Bacteria

The media and dilution solutions used in the enumeration of aerobic and anaerobic bacteria techniques were prepared with a mixture of filtered (0.2 μm) non-aged seawater (from the Menai Strait) and distilled water, giving a final salinity of 26 psu. This mixture of Menai Strait water and distilled water will be referred in future as **dilution fluid**. The salinity of the dilution fluid was close to that of the *in situ* conditions, established after measurements of the sediments pore water salinity at the surface and 30 cm depth, were carried out. The salinity at these depths did not vary by more than 1 psu.

Sample preparation

From each sample representing each depth, 25 g of sediment were weighed and diluted in $25cm^3$ of dilution fluid. In order to remove bacteria from the sediment particles, this mixture was sonicated for 5 minutes in a water bath sonicator (Sonicor, 420 watts). This sonication time was established after a test was performed to investigate the effect of the duration of the sonication on the number of viable cells present in the liquid phase. After sonication, sediments were allowed to settle until the water was virtually colourless and

sample dilutions were prepared down to 10^{-5} using dilution fluid. The number of dilutions prepared varied between samples depending on the depth and sampling occasion.

4.2.4.1.1. Heterotrophic Aerobic Bacteria (HAB)

Experimental Procedure

The enumeration of viable heterotrophs was performed using the pour plate technique with a standard marine media: ZoBell's medium 2216 (ZoBell, 1941) (Appendix). Three replicates of each of three dilutions were prepared and 1 cm^3 of each was plated. The plates were incubated at 20°C for 14 days. These incubation conditions showed, in preliminary tests, to give maximal colonies development. Colony forming units (CFU) were counted in the dilution where 30 to 300 colonies per plate had grown.

4.2.4.1.2. Aerobic Oil Degrading Bacteria (AODB)

The number of oil degrading bacteria was estimated by the most probable number (MPN) technique (Melchiorri-Santolini, 1972; Jones, 1979).

Experimental Procedure

One aliquot (1 cm^3) of each sample was inoculated in triplicate in "universal bottles" containing 9 cm^3 fortified seawater (FSW) (Appendix) and vegetable oil. Each of the replicates was diluted by 10-fold increments until the bacteria were diluted down to 10^{-5} and 10^{-6}. Samples were incubated at 20°C for 14 days and the growth was determined by turbidity. Statistical tables (Rodina, 1972) were used to calculate the MPN of cells.

4.2.4.2. Anaerobic Bacteria

In the present study, an anaerobic cabinet (Don Witley Scientific, G2154, Model MKII) provided an oxygen-free environment in which conventional bacteriological techniques could be applied for the isolation and manipulation of obligate anaerobes in conditions of strict and continuous anaerobiosis.

Anaerobiosis in the cabinet was achieved with a palladium catalyst and a non-explosive oxygen-free gas (10% hydrogen, 10% carbon dioxide and 80% nitrogen) maintained at a slightly positive pressure. The cabinet works on the principle that any oxygen that enters the chamber was removed when the atmosphere was circulated over the palladium catalyst by reaction with hydrogen to form water. The water vapour, created not only from the continuous catalysis but also from the large bulk of media, was removed by the presence of a humidistat. The humidistat controls the operation on an external circulation system through which the atmosphere of the cabinet is pumped. In this external system, silica gel was present which was re-charged whenever it was necessary. Methylene blue, a redox indicator, was used inside the chamber to indicate the current status of the cabinet atmosphere.

To ensure that the first basic requirement for the cultivation of anaerobic bacteria was achieved, oxygen was eliminated from the dilution fluid and media. Solutions were heated (60-70°C), placed inside the anaerobic cabinet and bubbled through with the gas mixture. They were then sterilised and the flasks tightly closed as soon as they were taken out of the autoclave. To ensure that no oxygen remained in the dilution fluid and in the FSW for the determination of oil degraders, the day before sampling the same procedure was followed again and the tubes were kept inside the anaerobic cabinet until their utilisation. A similar procedure was followed for the platting medium whilst still in the fluid state.

Sample preparation

The sediment cores were handled inside the anaerobic cabinet. Sediment samples and dilutions were prepared in the same way as for the aerobic bacteria but in this oxygen-free atmosphere. Sample sonication took place outside the cabinet in tightly closed flasks.

4.2.4.2.1. Heterotrophic Anaerobic Bacteria (HAnB)

The heterotrophic anaerobic bacteria were grown in thioglycollate medium (Merck) (Appendix), which is specific for the cultivation of obligate and facultative anaerobes. The reducing agents thioglycollate and cysteine present in the medium provided conditions suitable even for fastidious anaerobes. This medium also contained resazurin, which is a redox indicator, allowing any increase in the oxygen content of the medium to be detected.

Experimental Procedure

A procedure identical to that used for the heterotrophic aerobic bacteria was followed inside the anaerobic cabinet, with the petri dishes incubated in anaerobic conditions at room temperature (approximately 20°C) for 14 days.

4.2.4.2.2. Anaerobic Oil Degrading Bacteria (AnODB)

To estimate the number of anaerobic bacteria with the capability to breakdown the vegetable oils, the most probable number technique was used.

Experimental Procedure

A procedure identical to that used for the aerobic oil degraders was followed. The composition of the medium is described in the Appendix. The tubes were incubated inside the anaerobic cabinet at room temperature (approximately 20°C) for 14 days.

4.2.4.2.3. Sulphate Reducing Bacteria (SRB)

In the oxygen-free atmosphere of the anaerobic cabinet, SRB can be plated using standard techniques which in the present case involved the use of Postgate medium E (Postgate, 1979) (Appendix). This is a standard solid medium suitable for the growth of these bacteria, mainly *Desulfovibrio* and *Desulfotomaculum* spp. (Herbert & Glibert, 1984; Battersby *et al.,* 1985).

Experimental Procedure

A procedure identical to that used for the heterotrophic anaerobic bacteria was followed and the same anaerobic incubation conditions used.

4.2.5. Particle Size Analysis (PSA)

PSA is the measurement of the size distribution of particles in a sediment sample. Various systems of size classification have been used to define arbitrary limits and ranges of soil particle sizes (Gee & Bauder, 1986). Sediment particles smaller than 2000 μm are generally

divided into three major groups: sands, silts and clays. These groups can be subdivided in smaller size classes. There are various classification systems to relate particle size and size class (Table 4.1).

Table 4.1. Classification of the sizes of sediment particles (After, Grant & Long, 1981).

Texture class	**International Class (μm)**	**American Class (μm)**
Gravel	> 2000	>2000
Sand	2000 - 20	2000 - 50
Silt	20 - 2	50-2
Clay	<2	<2

The objective of the PSA in the present study was to establish the ratio between sand and finer particles. The separation of the two fractions was determined both in untreated and in samples pre-treated with hydrogen peroxide. The pre-treatment of samples to enhance separation or dispersion of aggregates is a key step in PSA and is generally recommended since many soils contain aggregates that are not readily dispersed, such as organic matter and often iron oxides and carbon coatings that bind particles together (Gee & Bauder, 1986). In the present case both methods were adopted to investigate the binding effects of the vegetable oils.

Experimental Procedure

The pre-treatment consisted of the addition of 6 % v/v hydrogen peroxide (20 volumes) (AnalaR®, BDH Ltd) to samples which were then kept at 60°C for 48 hours (Day, 1965).

Untreated and pre-treated samples were analysed in a similar fashion. Sediments were dried overnight at 100°C and ground with a pestle and mortar. A small amount of sample was passed through a 500 μm sieve to remove small fragments of plants, and subsequently through a 63 μm sieve. Samples were shaken manually for 5 minutes. The sand fraction (>63 μm) and the mud fraction (<63 μm) were then weighted separately and the percentage contribution of each fraction calculated. For every sample three replicates were analysed.

4.2.6. Permeability

The permeability of a soil is a measure of its capacity to allow the flow of a fluid through it. The fluid may be either a gas or a liquid, the liquid being usually understood to be water (Head, 1982). The degree of permeability is determined by applying a hydraulic pressure difference across a sample of soil fully saturated, and measuring the consequent rate of flow of water. The coefficient of permeability is expressed in terms of velocity. There are two types of laboratory tests for the direct measurement of soil permeability: i) constant head test - used for soils with high permeability, such as sand; ii) falling head test: for soils of intermediate and low permeability, such as silts and clays.

In the present study the test used to measure the coefficient of permeability of the sediment samples did not fall strictly into either of the above types. Constant head tests are not suitable for sediments with high contents of fine particles and the equipment to carry out falling head tests, suitable for finer sediments, was not available. Consequently, and due to the comparative aspect of this parameter a test suggested by Sinclair Buchan (*pers. comm.*) and based on both types described was used.

Apparatus

The system consisted of a 4 cm diameter sintered glass fibre funnel (porosity 1, 100-160 μm) attached to a 69.5 cm head of distilled water in a glass tube at one end, and to a vacuum pump at the other (Figure 4.1).

Experimental Procedure

Slices of sediment of 4 cm diameter and 2 cm thickness were placed into the sintered glass funnel, distilled water added and a vacuum applied. Experimental tests showed that the vacuum used did not need to be higher than 20 KN m^{-2} since higher pressures did not reduce the time for 1 cm^3 of water to percolate through the sediment sample. Samples were under these conditions for an hour to achieve saturation. The time taken for 1 cm^3 of distilled water to penetrate the sediment under the set up conditions was then measured three times per sample.

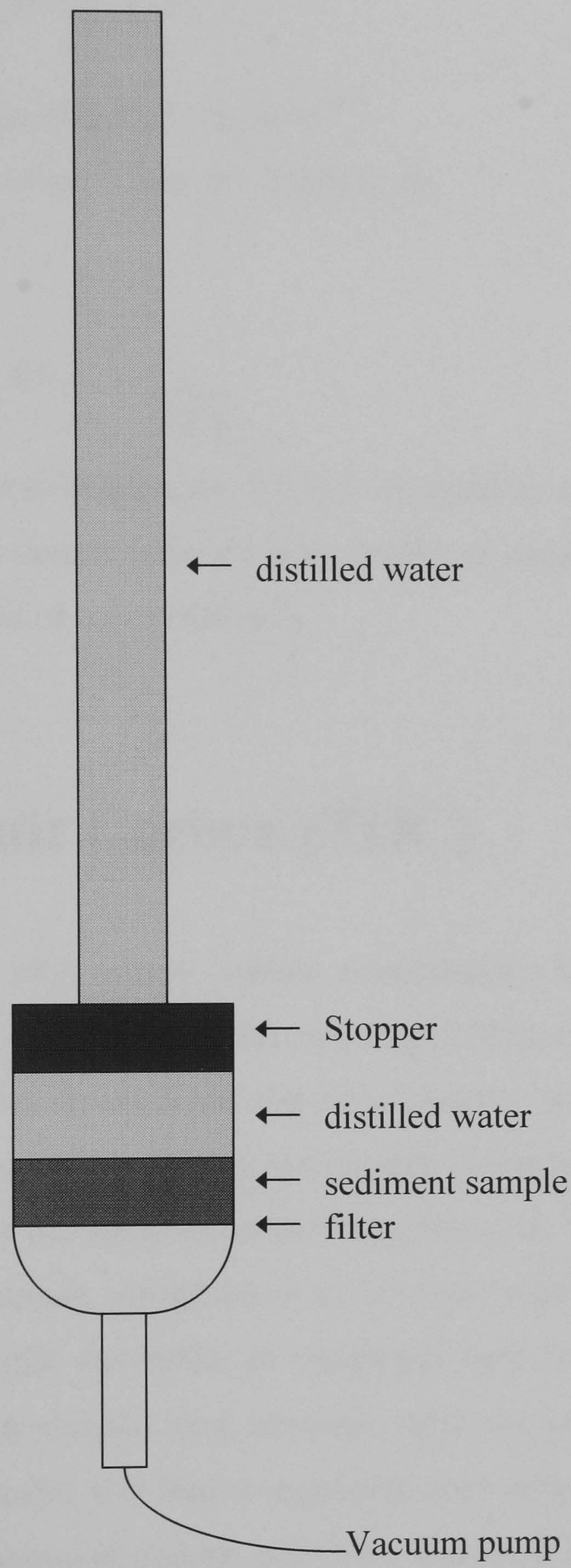


Figure 4.1. Schematic representation of the permeability apparatus.

The coefficient of permeability was determined according to equation (4.2) based in the equation of the constant head test (4.1) because it was legitimate to assume that the head of water in the present case was constant (Sinclair Buchan, *pers. commun.*).

$$K = \frac{q}{tA} \times \frac{l}{h} \qquad (4.1)$$

Since p (KN m^{-2}) = h (m) × ρ_{water} (mg m^{-3}) × g (m sec^{-2})

and ρ_{water} = 1 mg m^{-3} , g = 9.81 m sec^{-2} then p = h (m) x 9.81

so, h (mm) = 102 p

From (4.1)

$$K = \frac{q}{tA} \times \frac{l}{102p} \qquad (4.2)$$

where K is the coefficient of permeability (mm s^{-1}), q is the quantity of flow (mm^3), t is the time (sec), A is the area of the sample (mm^2), l is the length of sample (mm) and p is the pressure of the vacuum and head of water (KN m^{-2}).

4.2.7. Total Organic Carbon (TOC)

For the determination of the total organic carbon concentration in the sediments, the classical method of Schollenberber (1927), as described by el Wakeel & Riley (1956) was followed. This technique, which involves heating of a chromic acid-sediment mixture, gives quantitative results and is widely used by investigators because of its simplicity and rapidity compared to the wet or dry combustion methods. However, the $K_2Cr_2O_7$ methods are subject to interference by certain constitutes of soils, which lead to inaccurate results. For instance, chloride, ferrous iron and oxides of manganese have been shown to undergo oxidation-reduction reactions in chromic acid mixtures. Also, the presence of significant amounts of Fe^{2+} or Cl^- in sediments will lead to a positive error whereas MnO_2 will result in a negative error and low estimates of organic carbon (el Wakeel & Riley, 1956).

In this method, the organic carbon in sediments is oxidised to carbon dioxide by treatment with a mixture of potassium dichromate ($K_2Cr_2O_7$) and sulphuric acid (H_2SO_4) according to the equation:

$$2Cr_2O_7^{2-} + 3C + 16H^+ \longrightarrow 4Cr^{3+} + 3CO_2 + 8H_2O \qquad (4.3)$$

The time and the temperature to which the chromic acid-sediment mixture needs to be heated is critical and has to be standardised (Nelson & Sommers, 1982). el Wakeel & Riley (1956) found that consistent results for organic carbon in mud were obtained if the oxidation was carried out at 100°C for 15 minutes.

After reaction (4.3) has taken place, the excess dichromate ($Cr_2O_7^{2-}$) is titrated with ferrous ammonium sulphate [$Fe(NH_4)_2.6H_2O$]. The dichromate reduced during the reaction with the sediment is assumed to be equivalent to the organic carbon present in the sample, according to the equation:

$$Cr_2O_7^{2-} + 6Fe^{2+} + 14H^+ \longrightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \qquad (4.4)$$

Sample Preparation

Samples were kept at 105°C until dry (given by the constancy of their weight) and then finely ground. el Wakeel & Riley (1956) suggest that salt in sediments should be removed by carefully washing them with distilled water on a sintered glass funnel (Porosity 4). In the present study this step was not performed as washing the salts from the sediments would also remove the vegetable oils. The interferences caused by the salts were not of great concern since what was required was not so much absolute estimates but how TOC levels compared among samples with the same origin.

Experimental Procedure

The solutions used were prepared according to the instructions of el Wakeel & Riley (1956). The reagents were purchased from BDH (AnalaR®).

Chromic acid (10 cm^3) was added to 0.20-0.30 g of dried sediment and samples were heated for 15 minutes in a boiling water bath. After cooling, the mixture was poured into 200 cm^3 of distilled water. Six drops of ferrous-phenanthroline indicator were added and titrations were performed with a 0.2N ferrous ammonium sulphate solution until a brick-red colour just persisted. Three replicates were used for each sample. The same procedure was followed for the same number of "blanks".

Calculation of TOC Concentrations

The organic carbon concentrations were calculated according to the following expression:

$$TOC\ (mg\ C) = 1.15 \times 0.6\ (V_B - V_S) \qquad (4.5)$$

Where 1.15 is the empirical correction factor determined by el Wakeel & Riley (1956) to correlate the carbon content obtained with this method with that by dry combustion, to bring the results into agreement with the actual carbon content. This correlation factor established by el Wakeel & Riley (1956) is similar to that obtained by Allison (1935). In the TOC calculations, the use of the empirical correction factor was more accurate than the use of a calibration curve, since this factor was determined for fine sediments and a calibration curve would have to be established using a chemical (such as glucose) (David Assinder, *pers. commun.*). However, to test the method efficiency, this practical procedure was applied to a standard (glucose) of known concentration. The results obtained varied by no more than 4% of the true concentration.

In the above expression, the factor 0.6 is used since 1 cm^3 of 0.2N ferrous ammonium sulphate is equivalent to 0.6 mg of carbon. V_B is the volume of 0.2N ferrous ammonium sulphate used for titrating the "blanks". V_S is the volume of 0.2N ferrous ammonium sulphate used for titrating the sample.

4.2.8. Fatty Acids

Principles of Gas Chromatography (GC)

Gas chromatography is a form of partition chromatography in which the compounds to be separated are volatilised and passed in a stream of inert gas (the mobile phase) through a column in which active groups are co-valently bonded onto a solid supporting material (the stationary phase). The substances are separated according to their partition coefficients, which are dependent on their volatilities and their relative solubilities in the liquid phase. They emerge from the column as peaks of concentration, ideally exhibiting a Poisson distribution. These peaks are detected by some means, which converts the concentration of the component in the gas phase into an electrical signal, which is amplified and passed through a continuous recorder so that a tracing is obtained with an individual peak for each component as it is eluted. With a suitable detector, the areas under the peaks bear a direct linear relationship to the mass of the components present.

Principles of Mass Spectrometry (MS)

Considered in its simplest form, a MS is a device for producing and measuring mass ions (Evershed, 1992). The principle of the technique is that the compounds in the vapour phase, are bombarded with electrons and form positively charged ions, which can fragment in a number of different ways to give smaller ionised identities. These ions are propelled through a magnetic or electrostatic field and are separated according to their mass to charge (*m/z*) ratio; they are collected in sequence as the ratio increases; the ion current is amplified and then displayed by some means. The largest (or base) peak is given an arbitrary intensity value of 100, and the ion from the parent molecule is termed the molecular ion (M^+). Molecules do not fragment in an arbitrary manner but tend to split at weaker bonds, such as those adjacent to specific functional groups, or according to certain complex rules which have been formulated empirically from studies with model compounds (Christie, 1989a).

Viewed in its simplest form, a MS consists of i) sample inlet system (in the present case a GC), ii) source of ions (in the present case electron ionisation), iii) mass separation system (in these instances a quadrupole analyser), iv) means of ion detection (electron multiplier and photo mutiplier), amplification and recording (v) (Figure 4.2). Mass spectrometers operate at reduced pressure (typically $<10^{-6}$ torr) to enable detection of small amounts (sub-nanogram to microgram) of material that are analysed (Evershed, 1992).

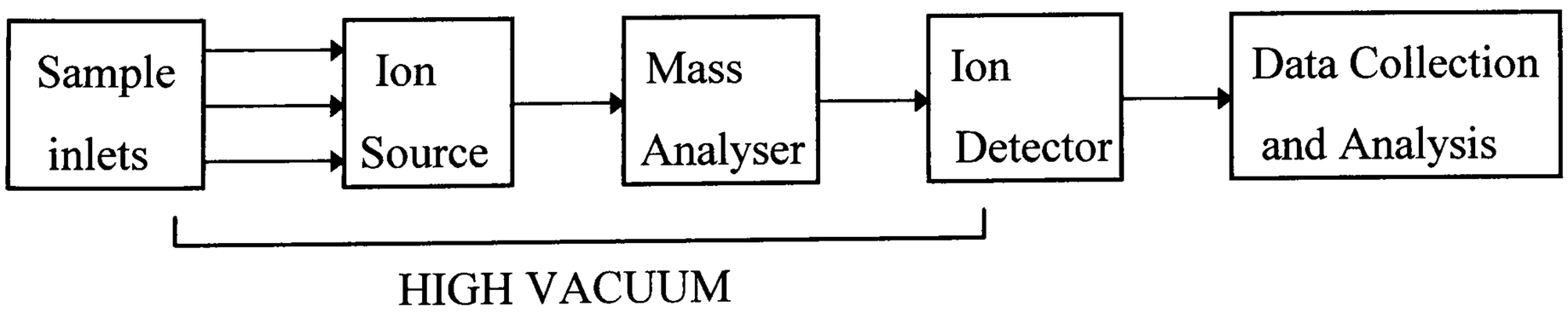


Figure 4.2. Components of a mass spectrometer (After Evershed, 1992).

Mass spectrometry is widely used as a detector in the GC analysis of lipids (Evershed, 1992). The advantage of combined GC and MS is that the mass spectra are recorded continuously during GC analysis, with the result that, in many instances, complete structure identification can be made on eluting compounds. GC and MS are especially

complementary as both are gas phase techniques, displaying optimum performances with the sample sizes at the nanogram range.

Compound identification

Complete structure assignments can frequently be made by consideration of mass spectra and GC retention data alone. The availability of computer library databases of mass spectra of many of the commonly occurring fatty acids provides a very convenient means of mass spectral interpretation. However, the results of computerised library searches are by no means unambiguous and they must be assessed in conjugation with retention times and elution orders. The identification of uncommon fatty acids is by far more difficult than that of common fatty acids and will be addressed in chapter 6.

Experimental Procedure

Sample Preparation

Sediment samples were dried before extraction in order to obtain a water free sample. Samples were freeze dried (for 48 hours), which is more appropriate than oven drying since it prevents evaporation of the compounds of interest. After this step, they were stored at -20°C until extraction. Prior to the extraction, the sediments were homogenised using a glass pestle and mortar and sieved through a 500 μm mesh to remove any sizeable plant debris.

Extraction

Lipids were extracted from 20-30 g of dried sediment by soxhlet extraction with 120 cm^3 of chloroform (Rathburn) which dissolves triacylglycerides and free fatty acids (Ackman, 1992). Whatman cellulose, single thickness (25-100 mm) extraction thimbles were used.

Prior to the field experiments, the efficiency of the extraction was tested using various amounts of oil and various extraction times. The mean and standard deviation obtained for a 10 mg sample of oil are presented in Figure 4.3. In accordance to this test the extraction time selected to be used in this work was 3.5 hours.

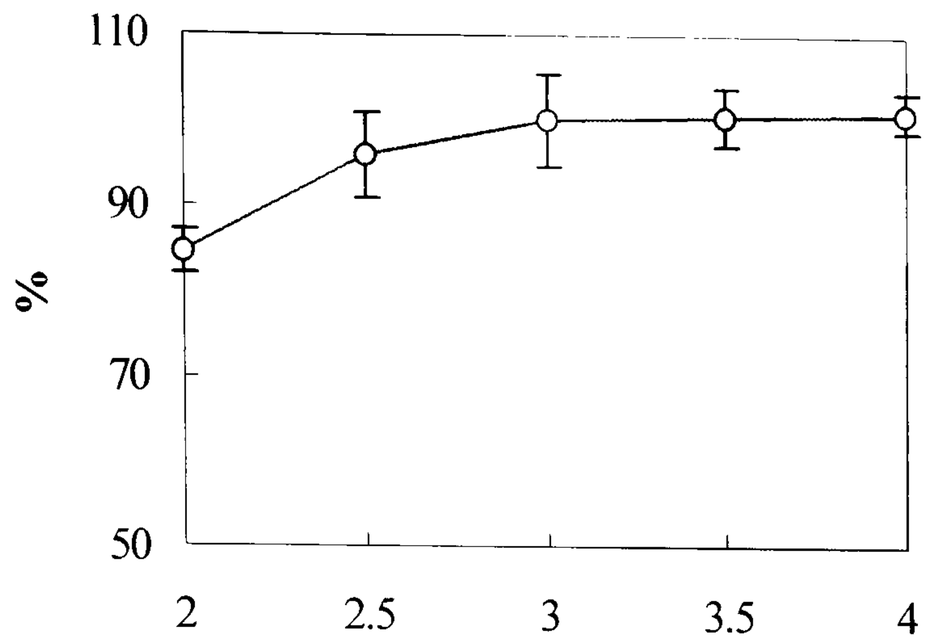


Figure 4.3. Percentage of total lipids recovered for various extraction times.

After extraction, the organic solvent was evaporated in a rotary evaporator. For quantitative purposes, the dried sample was redissolved in chloroform and transferred to a pre-weighted vial. The organic solvent was again evaporated under an oxygen free nitrogen (BOC) flow and reweighted.

Derivatisation

Because of their low volatility, fatty acids are not suitable for GC analysis and they have to be converted to more volatile derivatives, such as methyl esters which are the most widely used compounds for this purpose. In this particular type of derivatisation, the hydrogen atom of the carboxylic group of the fatty acid is substituted by a methyl group. Direct esterification using boron trifluoride in methanol and heating is frequently used. In this method, 50 µl of the methylation agent, TMSH (trimethylsulfonium hydroxide) (Camlab Ltd), was added to 1 mg of lipids in hexane (Rathburn) (Butte, 1983; Schulte & Weber, 1989). The testing of the efficiency of the methylation was not carried out by the author. However, the efficiency had been previously found to be greater than 90% (Mudge, *pers. commun.*).

After derivatisation, samples were analysed without delay so that any deterioration was minimised. Each sample was injected in triplicate in the GC-MS. Prior to the fatty acids methyl esters (FAMEs) analysis, a known amount of a standard FAME (nonadecanoic acid methyl ester, Sigma), not present in the samples, was added to them. This internal standard (IS) was used in the fatty acids quantification. The presence of the internal standard also allowed corrections for injection errors to be made.

GC-MS Operating Conditions

In the GC-MS analysis of fatty acids, due consideration must be given to the operating conditions. In the field studies, the fatty acids were analysed using two different instruments: a Finnigan MAT 4600 and a Fisons MD 800, for the linseed and the sunflower

experiments, respectively. The GC-MS operating conditions used in the field work are summarised in Table 4.2.

Table 4.2. GC-MS operating conditions during the present study.

Operating conditions	**Finnigan MAT 4600**	**Fisons MD 800**
Column	Capillary	Capillary
phase	BPX-70	BPX-70
type	Bonded phase	Bonded phase
material	Fused silica	Fused silica
film thickness (μm)	0.25	0.25
length (m)	25	50
I.D. (mm)	0.22	0.32
Carrier gas	helium	helium
flow rate (ml min^{-1})	2	2
Sample injection		
volume size (μl)	1	1
injector	split/splitless	on-column
temperature (°C)	230	-
injection mode	splitless	cold on-column
Oven temperature	Isothermal at 50°C for 1.7 minutes, 40°C min^{-1} to 170°C, 1°C min^{-1} to 180°C, 10°C min^{-1} to 230°C and isothermal at 230°C for 10 minutes.	Isothermal at 80°C for 2 minutes, 40°C min^{-1} to 160°C, 0.5°C min^{-1} to 170°C, 10°C min^{-1} to 250°C and isothermal at 250°C for 10 minutes.
Detector	EI (electron ionisation)	EI (electron ionisation)
temperature (°C)	230	250
ion source (eV)	70	70
mass scan range (*m/z*)	45 - 400	45 - 400

Fatty acids quantification

The quantification of the fatty acids was facilitated by the presence of the internal standard and also by the establishment of a calibration curve of the main fatty acids present in linseed and sunflower oils.

Calibration

When performing instrumental analysis, a calibration has to be carried out in order to establish a relationship between the concentration of the compound to analyse (in this case FAMEs) and the response from the instrument (area of peak). Usually this relationship is linear until the detector is saturated. The calibration compounds should be as many as

possible and identical to those found in the samples, so that they can be identified and quantified.

In the present case, the calibration was accomplished by injecting a series of standard solutions of FAMEs over a concentration range within which the unknown compounds were expected to fall. The FAMEs used were the principal components of linseed and sunflower oils (16:0; 18:0; 18:1ω9; 18:2ω6; 18:3ω3) (Alltech and Sigma) and the IS (19:0). Standard solutions of each one in the concentrations of 2.5; 5.0; 10.0; 15.0; 20.0; 25.0; 30.0; 40.0; 60.0; 80.0; 100.0; 120.0 and 140.0 μg ml^{-1} were prepared in hexane and injected in triplicate in the GC-MS.

To test if the GC-MS detector had a linear response to the range of concentrations used, the "best" straight line was fitted, using regression analysis of the chromatographic area of each FAME versus the concentration of the standard. The results of the regression analysis showed that there was a linear relationship between these two parameters for all FAMEs (correlation coefficient >0.99 for all). To assess experimental errors associated with the injection step, the repeatability of three standard injections was measured. The results showed that in 52.6% of the cases the coefficient of variation was less than 10% and in the remaining cases was between 10% and 20%.

To compensate for these injection errors another regression analysis was performed using the assistance of the IS values. In this method the areas of the compounds were divided by the area of the internal standard and multiplied by its concentration using the following equation,

$$\text{Fatty acid concentration } (\mu g\ cm^{-3}) = \frac{\text{fatty acid area x IS concentration } (\mu g\ cm^{-3})}{\text{IS area}} \qquad (4.6)$$

and regressed against the actual fatty acids concentration. Regression analysis indicated a significant linear relationship between the two variables for the various fatty acids of linseed and sunflower oil ($F= 1442\text{-}9830$; $p= <0.001$) and residual plots showed a good fit to the model.

The injection of the three replicates showed that the coefficient of variation was always less than 10%.

After this preliminary analysis, the concentration of each fatty acid in the samples was determined using the equation (4.7), which is a modification of expression (4.6), corrected for the response of the instrument to each of the fatty acids analysed, by division by the slope given in the above regression analysis.

$$\text{Fatty acid concentration } (\mu g\ cm^{-3}) = \frac{\text{fatty acid area x IS concentration } (\mu g\ cm^{-3})}{\text{IS area x slope}} \qquad (4.7)$$

To determine the concentration of the fatty acids as mg of fatty acid per gram of dry weight of sediment, the value obtained from the previous equation was multiplied by the dilution factor and divided by the weight of sediment used in the extraction.

4.3. Study of the Spatial Variability of some of the Sediments Properties in an Area of the Studied Salt Marsh

The area (4 m^2) of the salt marsh sediments where the spill of sunflower oil was simulated, was delimited and divided into 4 subzones. From each of this subzones two cores (5 cm diameter and 40 cm length) were taken at random making a total of eight samples. The sediments were sliced every 5 cm down to 25 cm depth (as described in section 4.1) and analysed for total organic carbon concentrations, particle size, pH and Eh, using the techniques described in the methods section (4.2).

The total organic carbon content was determined only at the surface sediments and the results are shown in Table 4.3. One-way ANOVA was used to test for differences in the TOC between the 8 sediment samples, revealing that there were significant differences ($F=267.94$; $p<0.001$) in the TOC contents between the samples.

Table 4.3. Concentration of total organic carbon (mg C g^{-1} dry sediment, mean ± sd, n=3) at surface sediments for samples taken at random from the studied salt marsh.

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
17.8 ± 0.32	19.4 ± 0.12	15.1 ± 0.11	18.4 ± 0.39	23.2 ± 0.28	19.2 ± 0.16	16.2 ± 0.38	16.8 ± 0.16

The results of the percentage of mud at each sediment depth are shown in Table 4.4. One-way ANOVA was used to test for differences in the grain size between the 8 sediment samples, indicating no significant differences for the depths of 1, 5 and 10 cm (1 cm: $F= 0.75$, $p = 0.635$; 5 cm: $F=0.97$, $p=0.485$; 10 cm: $F=0.25$, $p=0.966$) but differences in the grain size of the various samples were significant for the depths of 15, 20 and 25 cm (15cm: $F= 8.09$, $p<0.001$; 20cm: $F= 7.20$, $p=0.001$; 25cm: $F=11.84$, $p<0.001$).

Table 4.4. Percentage of mud in the sediments (mean ± sd, n=3) at the various depths for samples taken at random from the studied salt marsh area.

Depth (cm)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
1	36.7±1.6	36.8±0.6	36.6±0.8	35.1±1.6	36.5±0.8	37.2±1.2	36.2±1.4	36.1±1.4
5	40.7±0.6	41.0±0.6	41.9±1.7	41.1±1.7	40.6±1.0	41.7±1.9	39.5±1.5	40.2±1.4
10	37.0±1.4	37.3±1.4	38.0±1.0	37.7±1.1	38.0±1.6	37.2±1.9	38.0±1.7	38.0±1.3
15	29.4±0.7	32.3±0.9	30.9±0.8	31.9±1.3	33.8±0.9	29.8±0.3	32.8±0.9	30.8±1.3
20	10.0±0.6	12.9±1.1	13.7±0.7	14.5±0.8	13.2±0.9	11.4±0.7	12.9±1.5	12.0±0.4
25	7.9±0.3	8.4±0.3	8.2±0.1	10.7±0.3	7.8±0.6	6.6±0.9	7.3±1.2	8.1±0.2

Values of pH and redox potential (Eh) were also taken for the same depths; however, for these parameters replicate measurements were not performed. The results presented in Table 4.5 are the estimated means (± sd) of the 8 samples. The coefficient of variation of the pH measurements was at most 4% of the sample mean.

In the case of the redox potential, the standard deviation was equally small in relation to the mean, for depths between 10 cm and 25 cm. However, the surface sediments were more heterogeneous, with a coefficient of variation of 30%, and of 14% at the depth of 5 cm.

Table 4.5. pH and Eh (mV) of the sediment (mean ± sd, n=8) for the various depths in samples taken at random from the studied salt marsh area.

	1 cm	5 cm	10 cm	15 cm	20 cm	25 cm
pH	7.34 ± 0.29	6.67 ± 0.16	6.59 ± 0.10	6.63 ± 0.14	6.75 ± 0.10	6.83 ± 0.17
Eh	274.2 ± 80.0	398.6 ± 56.7	424.7 ± 14.9	425.1 ± 14.1	428.4 ± 10.3	424.7 ± 11.6

In summary, these results show that significant differences existed between upper and deeper layers. Also, for the same depth, sediment characteristics may vary from zone to zone within the sampling area. Consequently, this should be taken into consideration when analysing data resulting from the linseed and sunflower experiments.

4.4. LINSEED OIL RESULTS

4.4.1. Redox Potential of the Sediments

Temporal variation

The Eh values varied appreciably with time, ranging from +444 mV (day 0) to -231mV (day 60) (Figure 4.4). Changes in Eh were noticed within 3 days after the addition of oil, when 80% of the values recorded were lower than those measured at day 0. The decrease in Eh was particularly evident in the upper 2 cm, where negatives values were recorded. These negative values were generally maintained throughout the experiment. By day 14, this band of negative Eh had extended down to 6 cm, and after 2 months it could also be observed at approximately 20 cm depth.

Variation with depth

Between the surface and the bottom sediment layers, differences of 400 to 500 mV were observed, prior to and after the addition of linseed oil. For most sampling occasions, the redox potential was lowest at the top 6 cm and highest in the deeper layers (Figure 4.4).

4.4.2. pH of the sediments

Temporal variation

The pH values varied with time from a minimum of 5.9 to a maximum of 7.8 (Figure 4.4). On all sampling occasions, the top 6 cm were the depths most affected by the addition of oil, and the pH values measured after its addition were consistently lower than at day 0, specially at 1 cm depth where a decrease of 1.08 was recorded after 28 days. This decrease

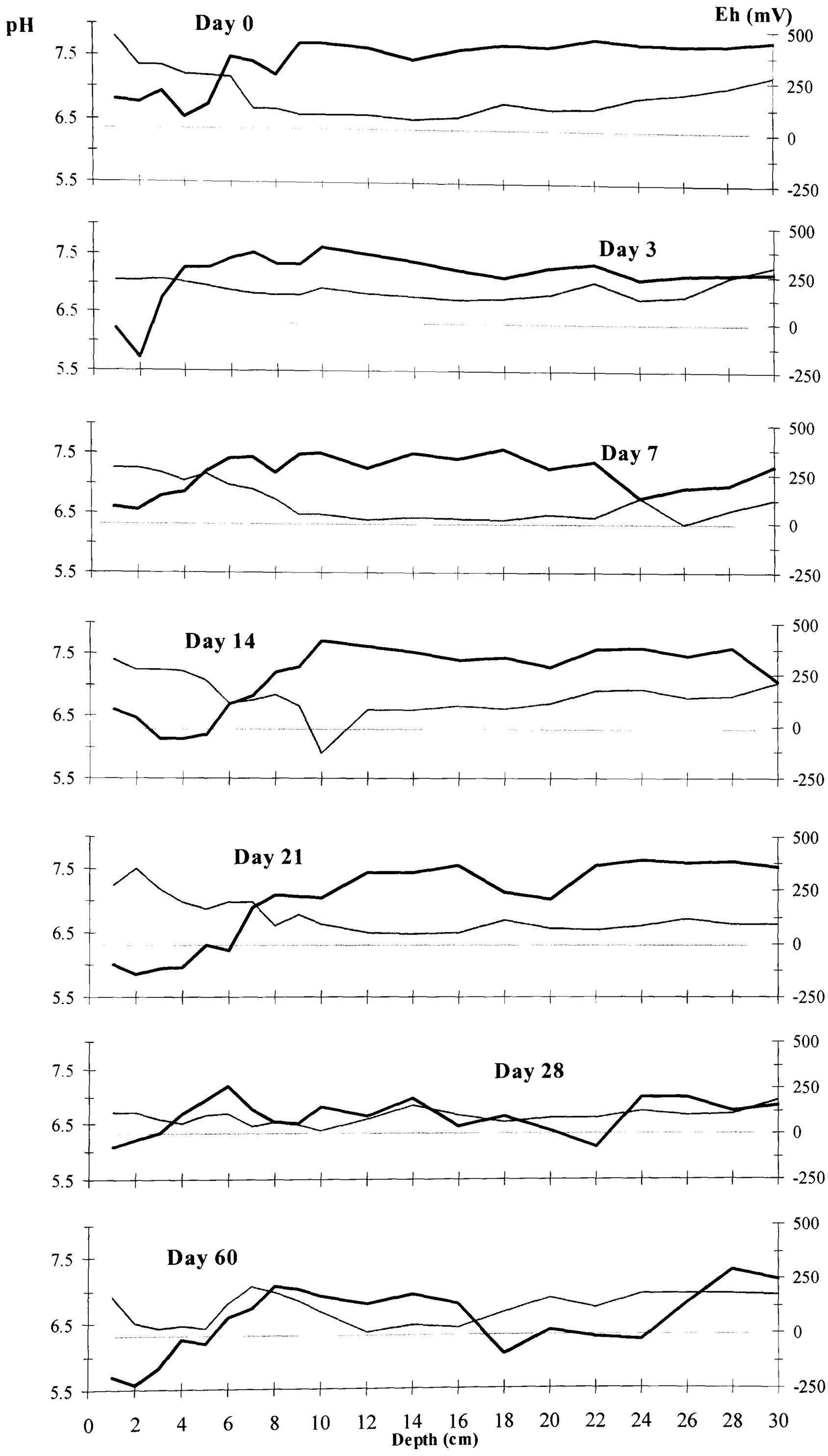


Figure 4.4. Variation of pH (thin line) and Eh (thick line) with time and depth in the sediments of the studied salt marsh previous to (day 0) and after the addition of

at the surface was first perceived after 3 days of the addition of the oil, but showed no linear correlation with time.

In deeper layers (7-30 cm), small decreases in the pH were also observed (maximum decrease 0.65 between day 0 and 7). These alterations were first observed after 7 days and the magnitude of decrease did not change with time.

Variation with depth

The pH values did not vary extensively with depth (Figure 4.4). At the start of the experiment (D0), the pH values were slightly higher (difference of 1 unit) at the top 6 cm than in deeper sediments, but these differences became less marked by day 28, with pH values similar in all depths. This trend was maintained until the end of the experiment.

4.4.3. Temperature of the sediments

Temporal variation

The temperature of the sediments varied between 6.1 and 9.2°C over the course of the experiment (two month period), with the only exception of day 14 when a value of 4.2°C was measured. The difference in the time of the day at which the sampling was carried out could have contributed to the wide scatter of the measured temperatures.

Variation with depth

Temperature did not change greatly with depth for most of the duration of the experiment, varying less than 0.5°C. However, on days 0 and 14, a difference of 2°C was observed between upper and deeper sediments, with the lowest values obtained between the surface and 18 cm depth and the highest towards the deeper layers. This was probably due to a sharp decrease in the air temperature, affecting more markedly the most upper sediments than the lower.

4.4.4. Enumeration of Bacteria

4.4.4.1. Aerobic Bacteria

4.4.4.1.1. Heterotrophic Aerobic Bacteria (HAB)

Temporal variation

An increase of 3 (depths 5, 25 and 30 cm) to 5 fold (for the remaining depths) in the number of aerobic bacteria was observed within 3 days after adding the oil (Figure 4.5). At the surface, this initial growth was sustained and enhanced throughout the experiment with maximum numbers observed on day 60 (15 times higher than those recorded at day 3). For the range of depths between 5 and 20 cm HAB maxima abundances were observed after 3 (5 cm) and 7 (10, 15, 20 cm) days and these values were sustained until the 14^{th} (5 cm) and the 21^{st} days (10, 15, 20 cm) of the experiment respectively, after which they declined to the original background numbers. For deeper sediments, bacteria peaked after 14 (25 cm) and 60 days (30 cm) corresponding, respectively, to an increase of 11 and 24 fold in relation to the numbers observed at day zero.

Variation with depth

During the course of the experiment the surface layers of the sediments contained consistently more bacteria than any of the other layers (Figure 4.5). Prior to the addition of oil, the number of HAB was high at the top 5 cm (3.5×10^4 CFU. g^{-1} wet weight of sediment) but decreased continuously with depth; at 10 cm depth, the numbers were 20%, at 15 cm were 13% and for depths below 15 cm were less than 10% of those counted at the surface.

In the presence of oil, this profile of higher number of bacteria at the top than at the bottom sediments did not change, but the differences were more marked between the 1 cm and the other depths (Figure 4.5).

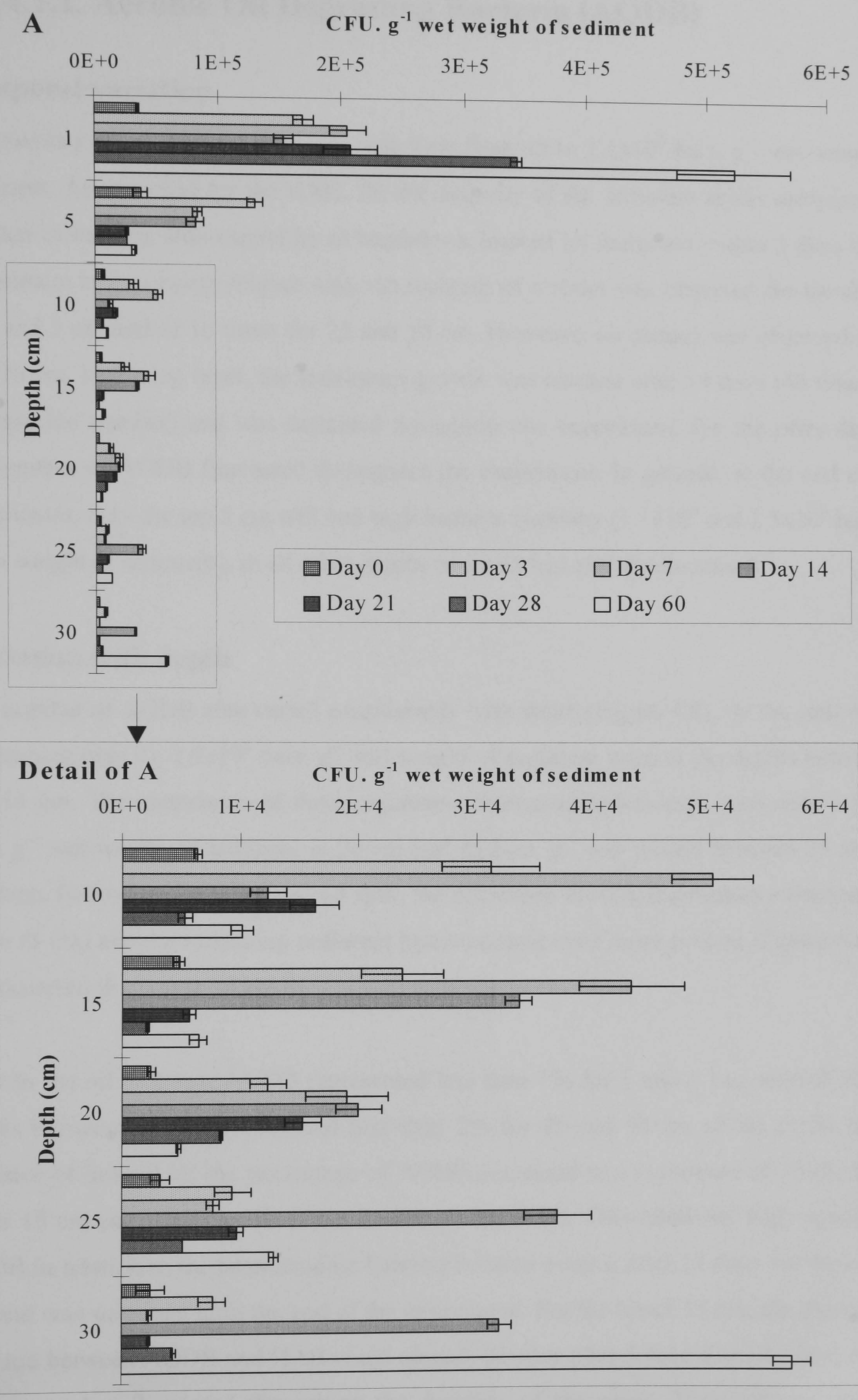


Figure 4.5. Number of heterotrophic aerobic bacteria (± sd) present at the various sediment depths previous to (day 0) and after the addition of linseed oil.

4.4.4.1.2. Aerobic Oil Degrading Bacteria (AODB)

Temporal variation

The number of AODB varied widely with time from 45 to $1.1x10^4$ bact. g^{-1} wet weight of sediment. As observed for the HAB, for the majority of the sediment layers analysed, the number of bacteria with capability to breakdown linseed oil increased within 3 days of the experiment commencing (Figure 4.6). An increase of 6 times was observed for the depths of 1 and 5 cm and of 10 times for 25 and 30 cm. However, no change was observed at 15 and 20 cm. In the top layer, the maximum growth was reached after 14 days (40 times the background number) and was sustained throughout the experiment. For the other depths, the number of AODB fluctuated throughout the experiment. In general, at the end of the experiment, only the top 5 cm still had high bacteria numbers ($1.1x10^4$ and $2.5x10^3$ bact. g^{-1} wet weight of sediment); in all other depths numbers had already decreased.

Variation with depth

The number of AODB also varied considerably with depth (Figure 4.6). At the start of the experiment (day 0), $2.5x10^2$ bact. g^{-1} wet weight of sediment were at the depths between 1 and 15 cm. The abundance of these organisms decreased downwards, with only $1.5x10^2$ bact. g^{-1} wet weight of sediment at 20 cm and 45 bact. g^{-1} wet weight between 25 and 30 cm deep. Following the simulated oil spill, the difference in AODB abundance between the upper (1 cm) and the remaining sediment layers became even more evident (Figure 4.6), as had occurred in the case of HAB.

Prior to the oil addition, AODB represented less than 1% for 1 and 5 cm; around 5% for depths between 10 and 20 cm; and less than 2% for 25 and 30 cm of the HAB. In the presence of linseed oil the percentage of AODB increased to a maximum of <10% in the upper 15 cm and <20% in the lower 15 cm, of the HAB. This relatively high number of AODB in relation to the heterotrophic bacteria became evident after 14 days for the top 15 cm, and was sustained until the end of the experiment. For the lower 15 cm, the increase in the ratio between AODB and HAB could already be seen after 3 days from the start of the experiment but fluctuated throughout the duration of the work. This indicates that an exposure to linseed oil induced an increase in both the proportion and the number of oil degraders.

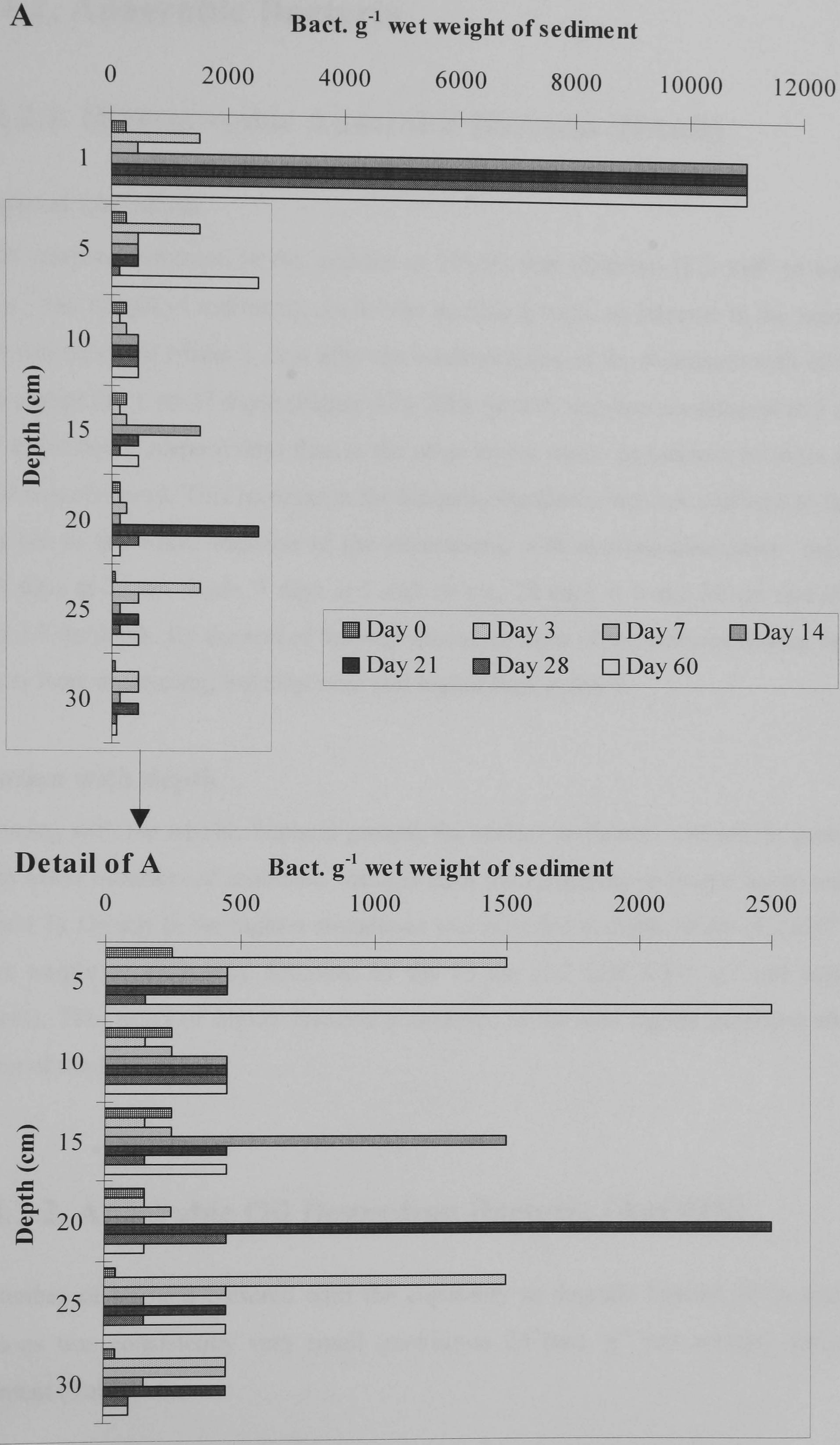


Figure 4.6. Number of aerobic oil degrading bacteria present at the various sediment depths previous to (day 0) and after the addition of linseed oil.

4.4.4.2. Anaerobic Bacteria

4.4.4.2.1. Heterotrophic Anaerobic Bacteria (HAnB)

Temporal variation

A wide temporal variation in the number of HAnB was observed (3.2 $x10^2$ to 6.6 $x10^4$ CFU. g^{-1} wet weight of sediment). As for the aerobic groups, an increase in the number of HAnB was recorded within 3 days after the contamination of the sediments with oil for all depths except the 1 cm (7 days) (Figure 4.7). This growth was less accentuated at 5 and 10 cm (5 and 4 times, respectively) than at the other layers where an increase between 10 and 65 fold was observed. This increase in the bacteria abundance was not confined to the first 3 days but to the whole duration of the experiment, with maxima abundance registered after 3 days at 25 cm depth, 7 days at 1 and 10 cm, 28 days at 5 and 30 cm and after 60 days at 15 cm depth. By the end of the experiment, in most of the sediment layers, bacteria numbers were decreasing, but they were still higher than at day 0.

Variation with depth

Contrasting with the aerobic bacteria groups, the surface sediments seemed, in general, to contain lower numbers of anaerobic bacteria than the remaining sediment layers analysed (Figure 4.7). On day 0, the highest abundance was recorded at depth 10 cm (5.2 $x10^3$ CFU. g^{-1} wet weight of sediment), followed by the 15 cm (1.7 $x10^3$ CFU. g^{-1} wet weight of sediment). This trend of higher bacteria abundance at the mid depths persisted after the addition of the linseed oil.

4.4.4.2.2. Anaerobic Oil Degrading Bacteria (AnODB)

The number of bacteria cultured with the capability to degrade linseed oil in anaerobic conditions was consistently very small (maximum 25 bact. g^{-1} wet weight) during this experiment (Table 4.6).

At day zero, and for all depths analysed, a similar number of bacteria was recorded (2.5 bact. g^{-1} wet weight). Some increase in numbers was observed but this was not uniform for

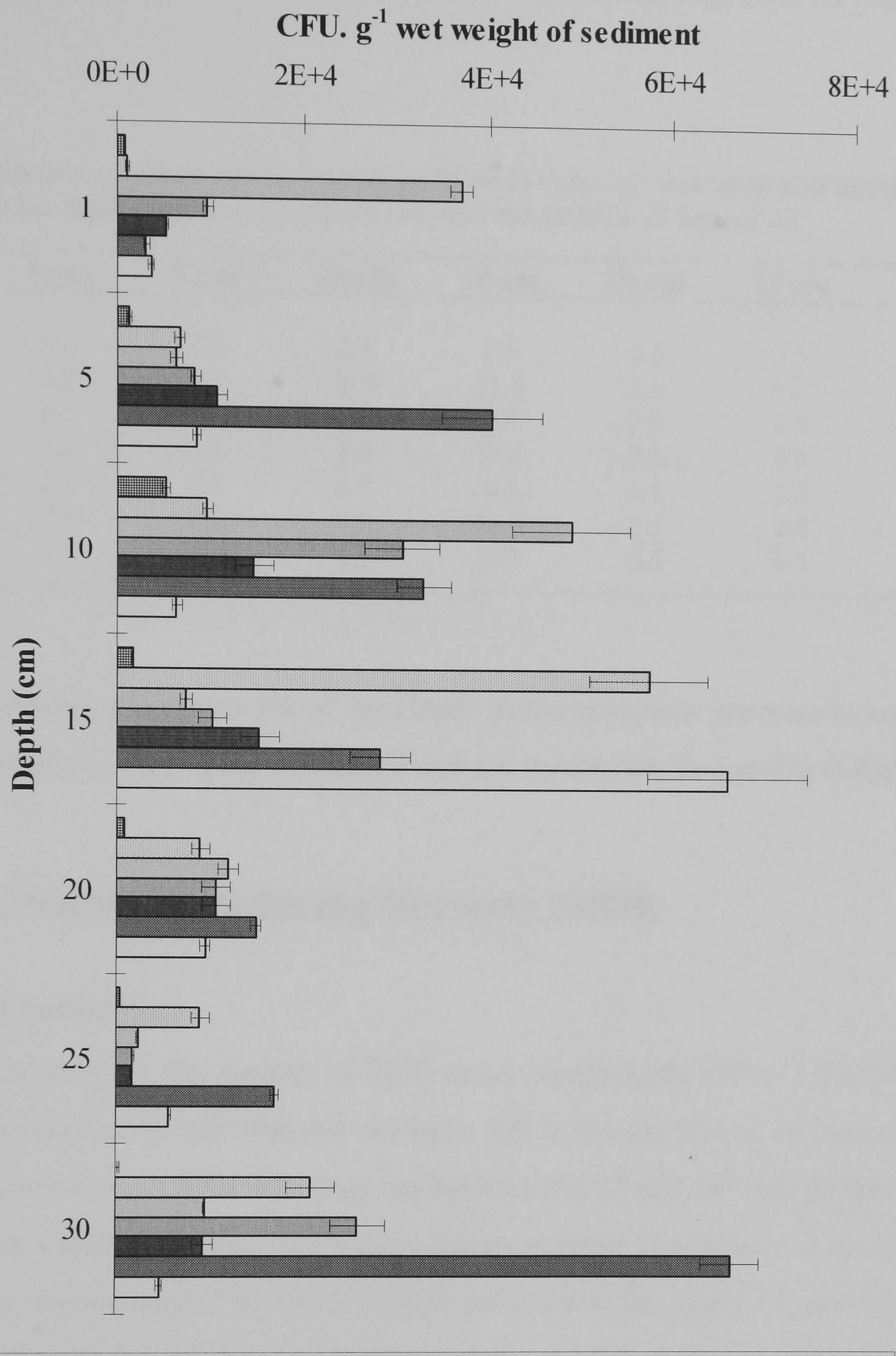


Figure 4.7. Number of heterotrophic anaerobic bacteria (± sd) present at the various sediment depths previous to (day 0) and after the addition of linseed oil.

the duration of the experiment, with 4.5 bact. g^{-1} wet weight recorded for all depths at the end of the experiment. A maximum of a 10 times increase was registered for depths 10 and 15 cm.

Table 4.6. Number of anaerobic oil degrading bacteria (bact. g^{-1} wet weight of sediment) at the various sediment depths previous to (day 0) and after the addition of linseed oil.

Days	1 cm	5 cm	10 cm	15 cm	20 cm	25 cm	30 cm
0	2.5	2.5	2.5	2.5	2.5	2.5	2.5
3	2.5	9.5	25.0	25.0	2.5	2.5	2.5
7	4.5	2.5	2.5	2.5	2.5	2.5	2.5
14	2.5	2.5	2.5	2.5	2.5	2.5	2.5
21	4.5	4.5	4.5	4.5	4.5	2.5	2.5
28	2.5	2.5	2.0	25.0	2.5	2.5	2.5
60	4.5	4.5	4.5	4.5	4.5	4.5	4.5

AnODB represented less than 1% of the HAnB in the sediments previous to and after the linseed oil addition, suggesting that this oil was not particularly favourable to their growth.

4.4.4.2.3. Sulphate Reducing Bacteria (SRB)

Temporal variation

During the experiment, the number of SRB varied considerably (10 to 5.9 $x10^3$ CFU. g^{-1} wet weight of sediment) and is shown in Figure 4.8. In the top 20 cm, an increase (5 to 64 times) in the numbers of SRB was observed between the 3rd and 14th day. However, for the 25 and 30 cm depth more time was needed for an increase in numbers to be detected (60 and 28 days, respectively). The initial growth observed at the upper 10 cm was sustained and enhanced until 21- 28 days, with the numbers of SRB declining afterwards. For the depths of 15 and 20 cm the number of SRB fluctuated throughout the experiment with a maximum number recorded at day 60.

Variation with depth

A small number of SRB were present in the sediments at day 0, with a maximum value of 160 CFU. g^{-1} wet weight of sediment recorded at 1 cm, decreasing progressively with depth (10 CFU. g^{-1} wet weight of sediment at 30 cm depth). After the addition of oil, the depth

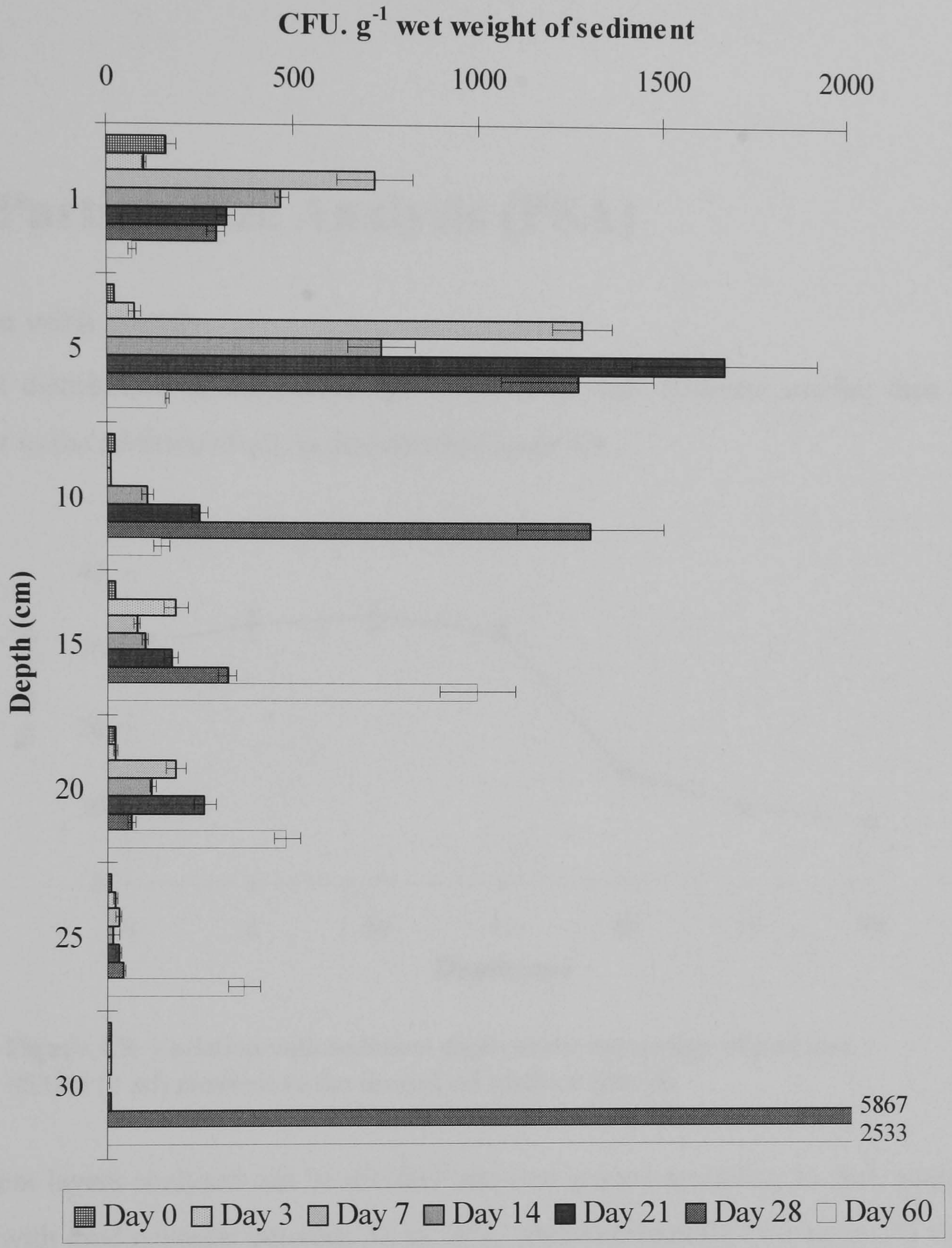


Figure 4.8. Number of sulphate reducing bacteria (± sd) present at the various sediment depths previous to (day 0) and after the addition of linseed oil.

profile varied throughout the experiment. In general, during the first 21 days the maximum bacteria abundance was observed at the 5 cm depth, followed by the 1 cm depth. This profile was modified after 28 days with the maximum concentration measured at the 30 cm depth.

4.4.5. Particle Size Analysis (PSA)

Variation with depth

The spatial distribution of the percentage of particles with diameter smaller than 63μm (mud) prior to the addition of oil, is depicted in Figure 4.9.

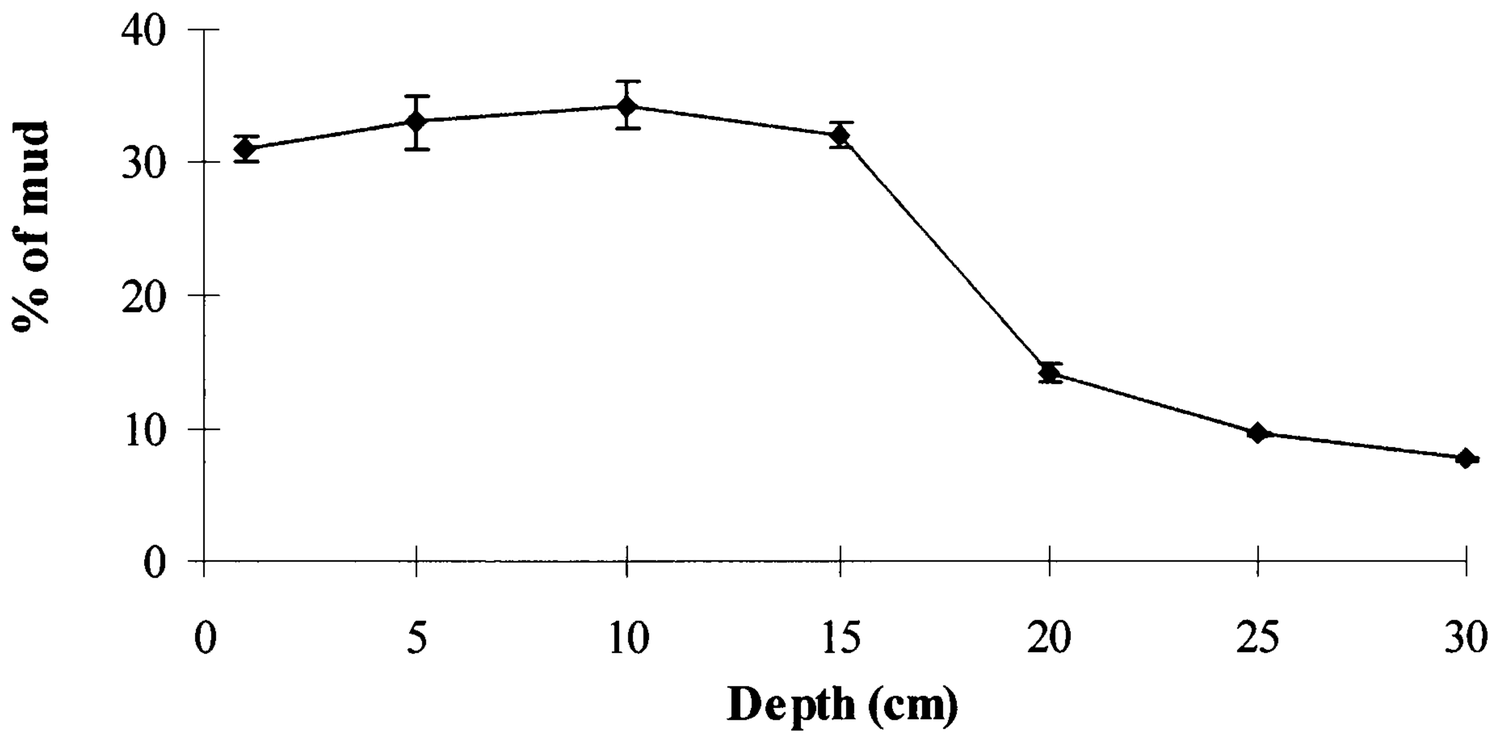


Figure 4.9. Variation with sediment depth of the percentage of particles <63μm (± sd) previous to the linseed oil addition (day 0).

The sediment layers analysed can be divided into two groups according to their grain size: one group with mud contents between 31 to 35%, which consisted of the upper 15 cm, and the other with 8.3 to 14.8% of mud which included the layers from 20 to 30 cm. This latter group showed a decrease in the mud contents with the depth.

This general pattern of variation with depth was not altered by the presence of linseed oil.

Temporal variation

The ratio between mud and sand did not vary markedly with time (Figure 4.10). The temporal variation observed can be attributed to 3 factors: i) errors in the practical

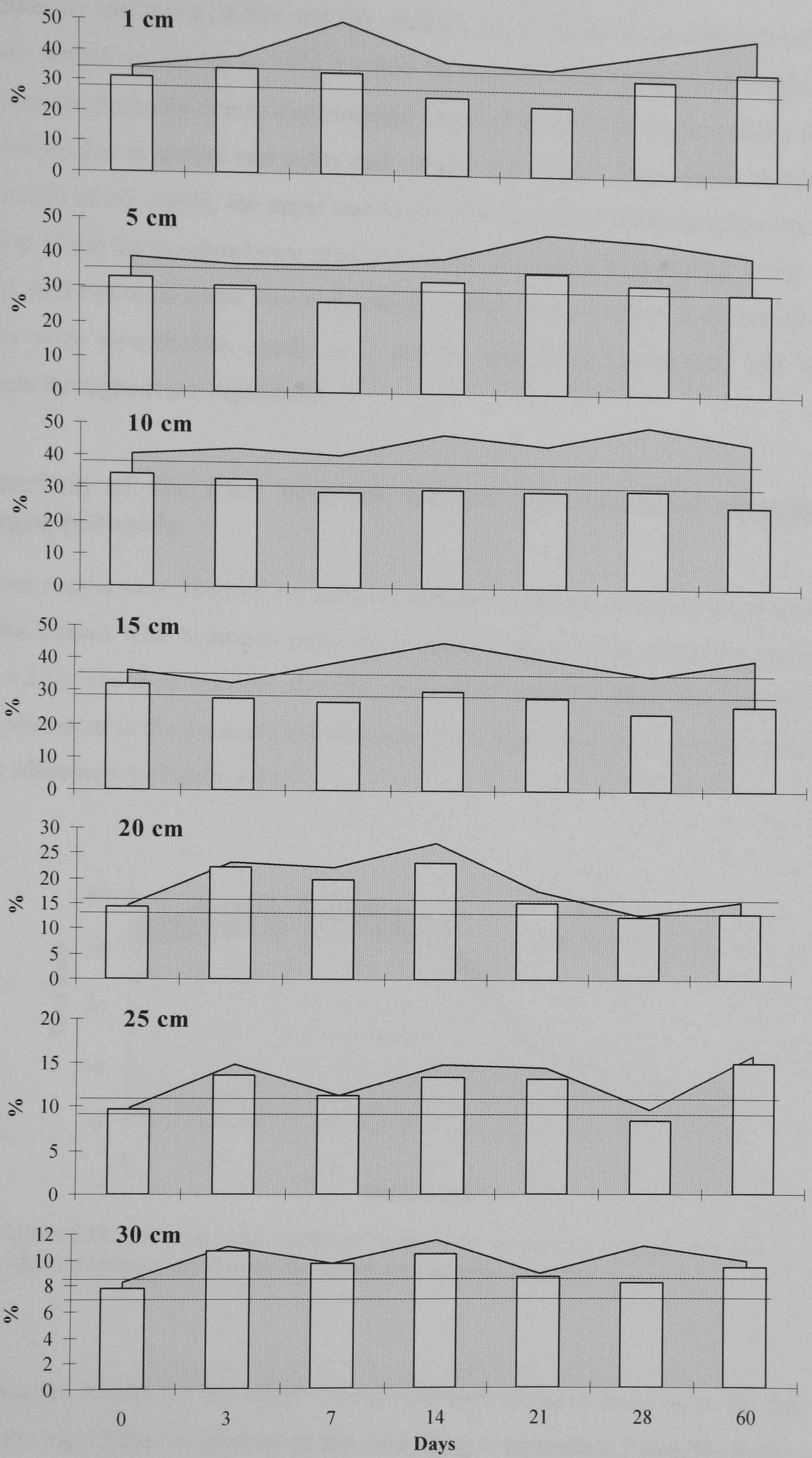


Figure 4.10. Percentage of mud at the various sediment depths before (bars) and after (area) treatment of the sediments with hydrogen peroxide, prior to (day 0) and after the addition of linseed oil. The lines represent 10% above and below the value obtained at day 0.

procedure; ii) spatial variability and iii) presence of oil. Since the coefficient of variation for each sample varied up to 10% it could be assumed that values within this range of variability are probably due to experimental errors (Figure 4.10). Values above this range are probably due to spatial variability and values below result from spatial variability and the presence of oil. Again, the upper and lower 15 cm showed different behaviour. Whilst, at the top 15 cm the measurements recorded outside this range were mostly lower than the original, in the bottom layers they were mostly higher. The accretion or decline in the mud contents were nevertheless small, as it can be seen from Figure 4.10 and were not consistent throughout the experiment.

Comparison of the PSA between treated and untreated samples with hydrogen peroxide

The above results were obtained for samples without treatment; however, the same samples were also treated with hydrogen peroxide to remove the organic matter (as described in section 4.2.5). The mud fraction, after the removal of organic matter, was higher than that prior to treatment in the presence and absence of oil. The results of both set of data for day zero are illustrated in Figure 4.11.

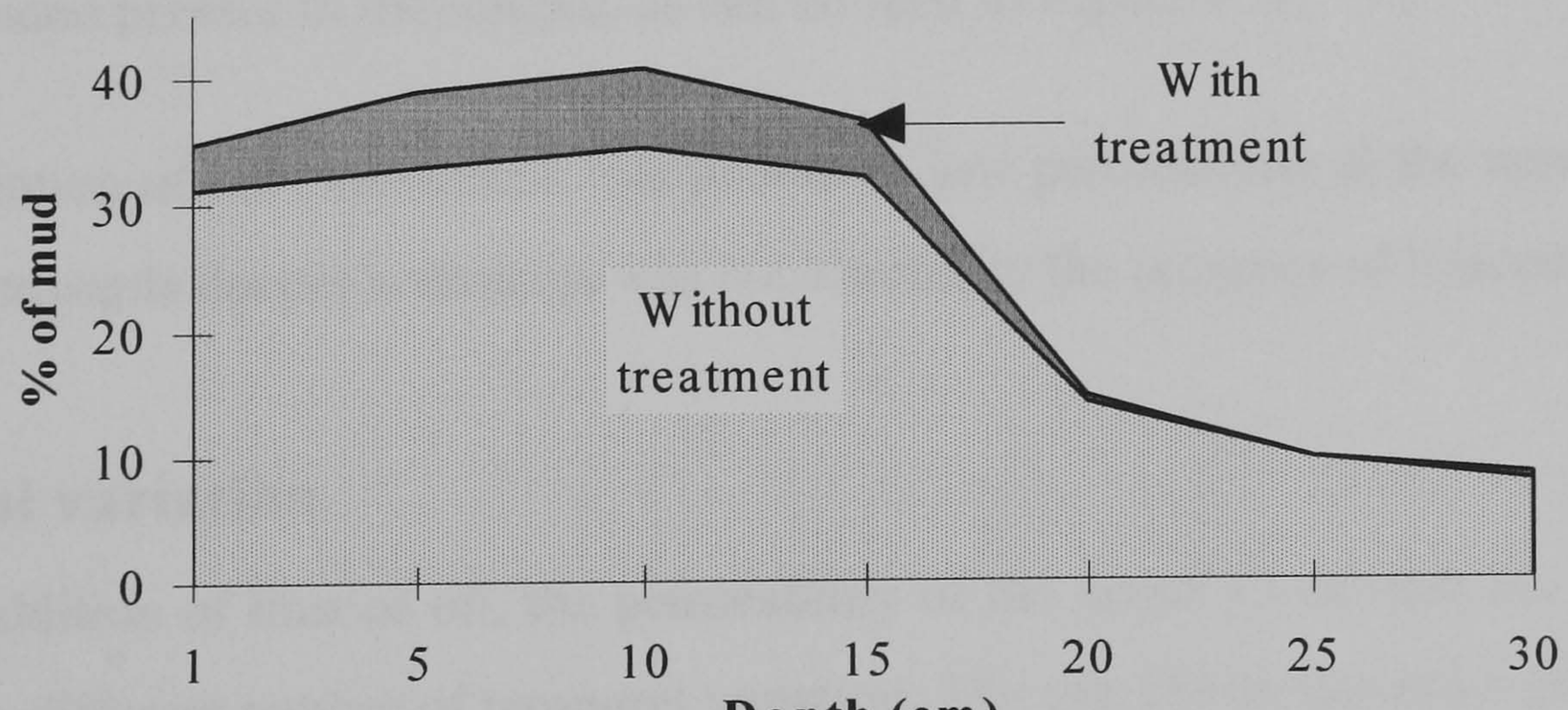


Figure 4.11. Variation with sediment depth of the percentage of mud with and without treatment previous to the linseed oil addition (day 0).

Once again, the results for the upper 15 cm sediments differed from those for the lower layers. In the top 15 cm, an increase in the mud fraction between 3.7 to 6.4% in relation to the results without treatment was observed, whereas in the deeper sediments, only a small increase occurred (up to 0.5%).

The increase in mud contents measured at the top 15 cm at day 0 was enhanced by the addition of linseed oil, constituting an increase of up to 19.8% (Figure 4.10). The first effects of the presence of the oil could be observed from day 7 and lasted until day 60.

At depths between 20 and 30 cm, the increase in the mud fraction after the oil addition was at the most 3.9% and was not consistent throughout the experiment, with a mean accretion of 2.1% at 20 cm depth, 1.0% at 25 cm depth and 0.8% at 30 cm depth. These values suggest a reduction in the increase in organic matter with depth and also that the linseed oil had adsorbed to the sediment particles, mainly to those with grain size <0.63μm.

4.4.6. Permeability

Variation with depth

The results regarding the coefficient of permeability recorded at day zero are illustrated in Figure 4.12. A similar coefficient of permeability of 10^{-7} m s^{-1} was measured for all depths in the upper 15 cm, increasing with depth: 10^{-6} m s^{-1} at the 20 cm layer and 10^{-5} m s^{-1} in the 25 and 30 cm layers. As expected, the permeability results are directly related to the amount of sand present in the sample, as can be seen in Figure 4.12.

For the duration of the experiment, this pattern of low permeability at the upper sediments increasing towards deeper sediments was not altered by the presence of linseed oil.

Temporal variation

With the addition of linseed oil, the permeability of the upper 15 cm and the lower 15 cm exhibited a different pattern of temporal variation. The top 15 cm showed a decrease in the permeability of 30-68% from day 0 to day 3; however, the results fluctuated throughout the experiment (Figure 4.13A). In general, a reduction in the coefficient of permeability from background values was observed, with 50% of the measurements having the permeability reduced to between a quarter and a half of the original values. At the end of the experiment, the permeability appeared to be increasing in the top 5 cm. At depths greater than 15 cm (20 to 30 cm) the permeability was not altered by the presence of the oil (Figure 4.13B).

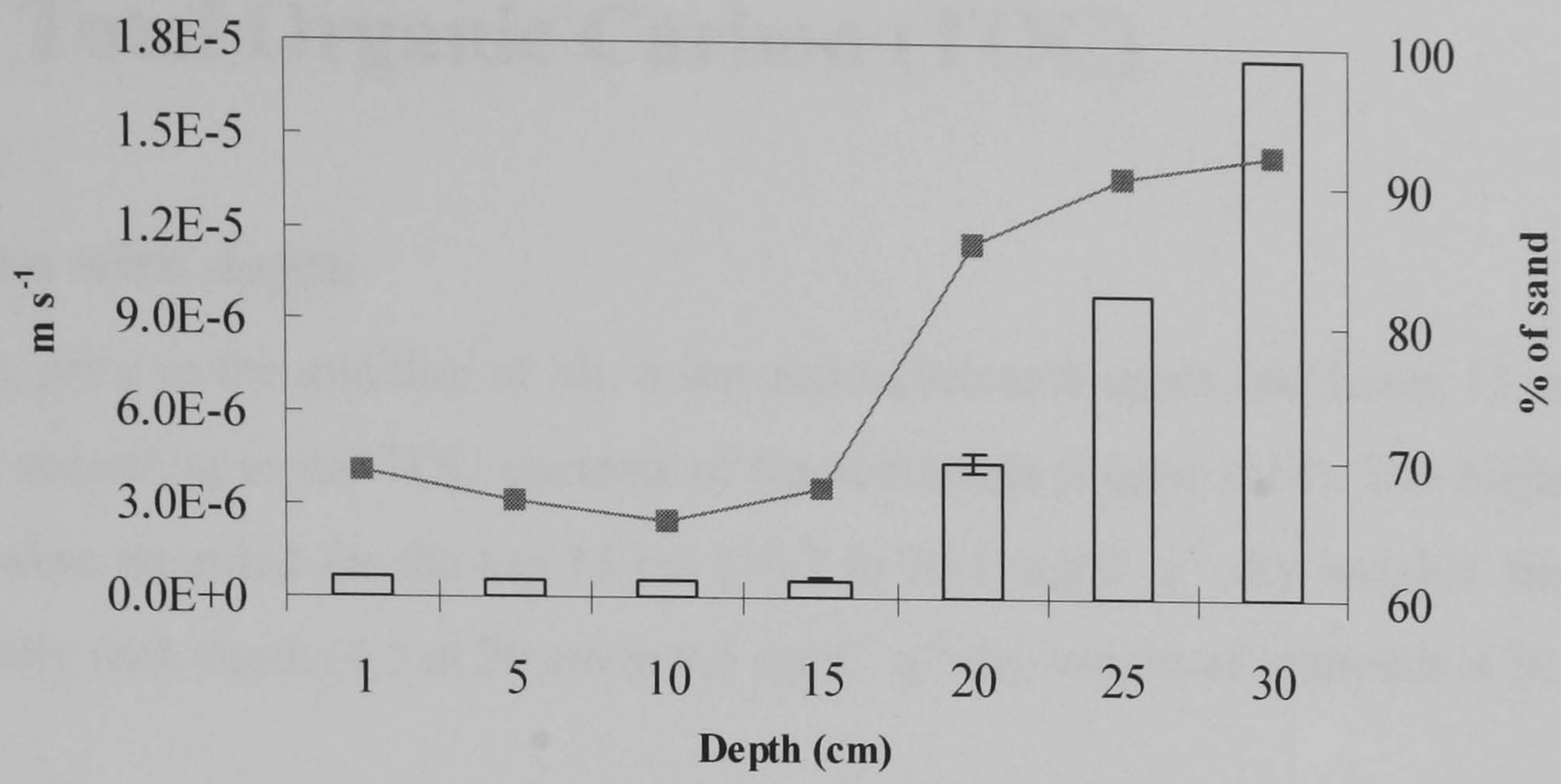


Figure 4.12. Variation with the sediment depths of the coefficient of permeability (bars, ± sd) and sand (line) prior to the linseed oil addition (day zero).

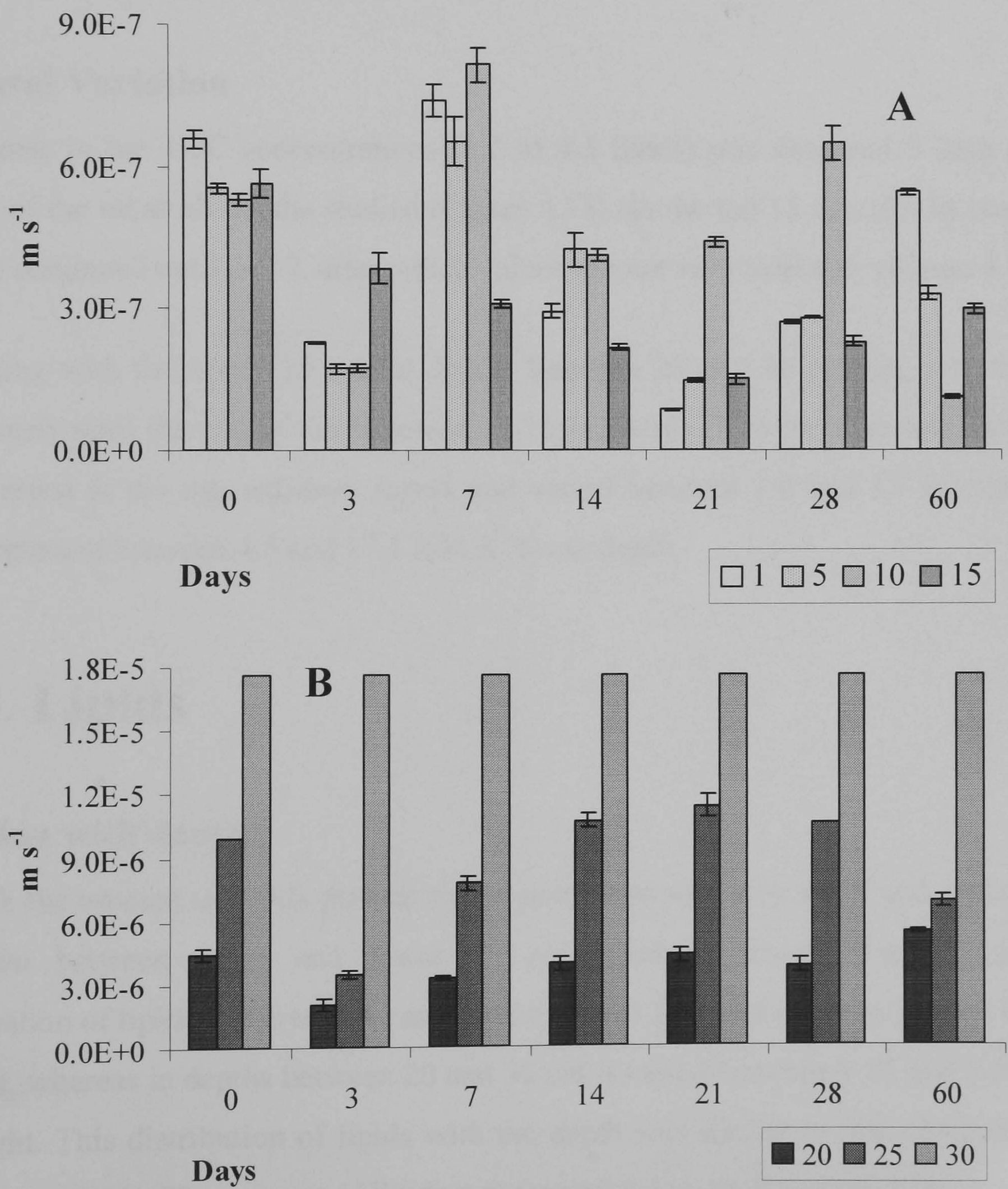


Figure 4.13. Coefficient of permeability (m s^{-1}, ± sd) for sediment depths of 1 to 15 cm (A) and 20 to 30 cm (B) prior to (day 0) and after the additon of linseed oil.

4.4.7. Total Organic Carbon (TOC)

Variation with depth

As before, prior to the addition of oil, a separation between upper and lower 15 cm could be drawn according to the TOC contents of the sediments (Figure 4.14). The highest TOC contents were recorded for the top 15 cm (14.3 to 20.1 mg C. g^{-1} dry weight), decreasing continuously with depth (4.5 at 20 cm to 0.5 mg C. g^{-1} dry weight of sediment at 30 cm).

In the presence of linseed oil, this pattern of higher concentration of TOC in the upper 15 cm in relation to the lower 15 cm continued to be observed. However, this separation was less marked than prior to the addition of oil.

Temporal Variation

An increase in the TOC concentrations (1.2 to 4.5 times) was observed 3 days after the addition of the oil at all depths studied (Figure 4.15). In the top 15 cm, this increase (1.2 - 2.8 fold) continued until day 7, after which values did not vary markedly (Figure 4.15).

Contrasting with the upper 15 cm, at depths between 20 and 30 cm the TOC increased continuously until the end of the experiment (Figure 4.15). This increase was greater than that observed at the top sediment layers and varied between 1.9 and 3.7 fold at 20 and 25cm depth and between 4.5 and 17.1 fold at 30 cm depth.

4.4.8. Lipids

Variation with depth

At day 0, the amount of lipids present in the sediments was very small and, once more, a distinction between upper and lower 15 cm could be drawn (Figure 4.16). The concentration of lipids in the top 15 cm varied between 1.14 and 1.64 mg. g^{-1} dry weight of sediment, whereas in depths between 20 and 30 cm it varied between 0.25 and 0.20 mg. g^{-1} dry weight. This distribution of lipids with the depth was similar to that observed for the TOC (Figure 4.16). The amount of lipids represented 5.4 to 10.5% of the TOC at all depths studied, except at 30 cm where this percentage increased to 38.5%.

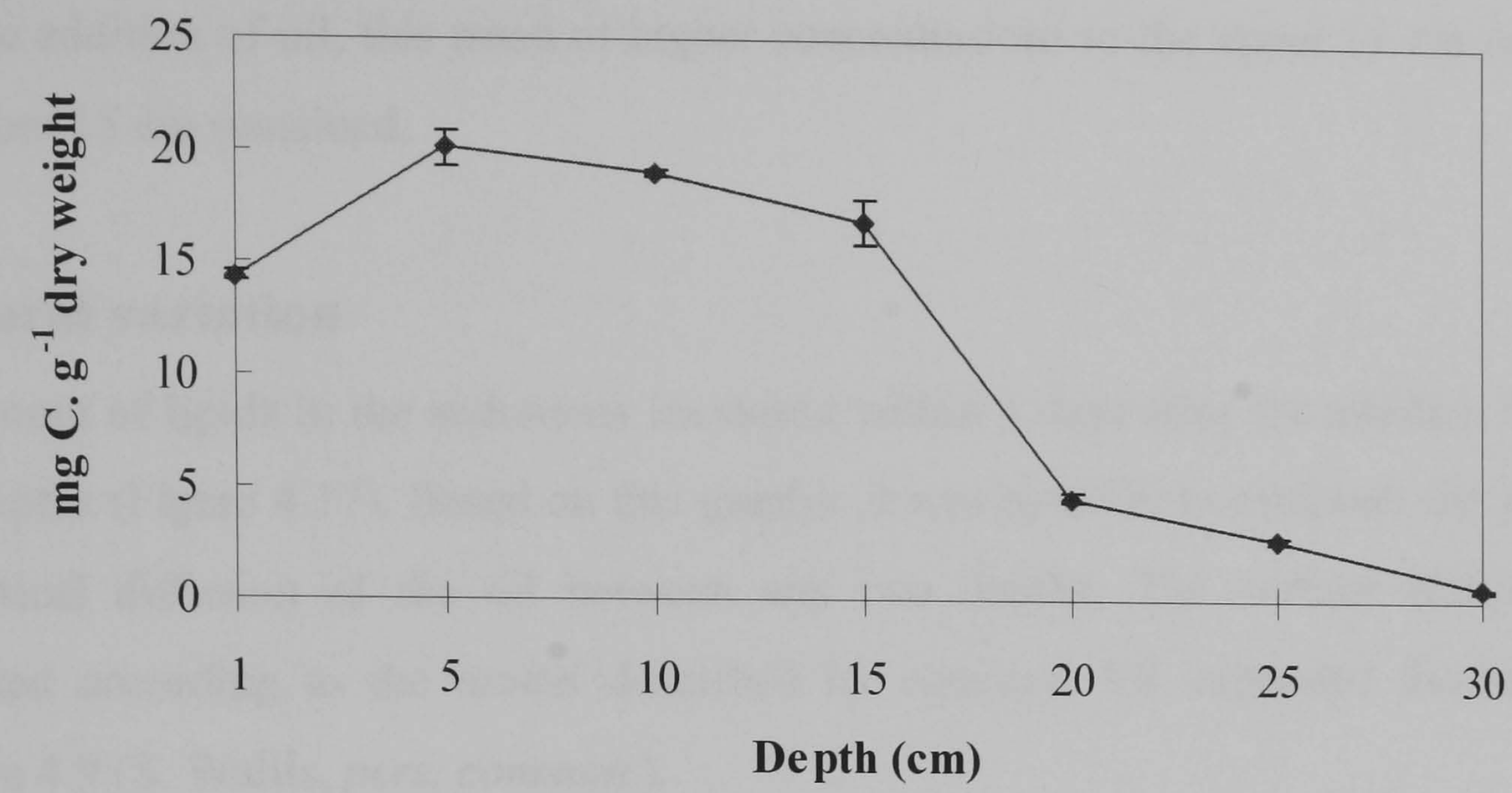


Figure 4.14. Variation with sediment depths of total organic carbon (± sd), prior to the linseed oil addition (day zero).

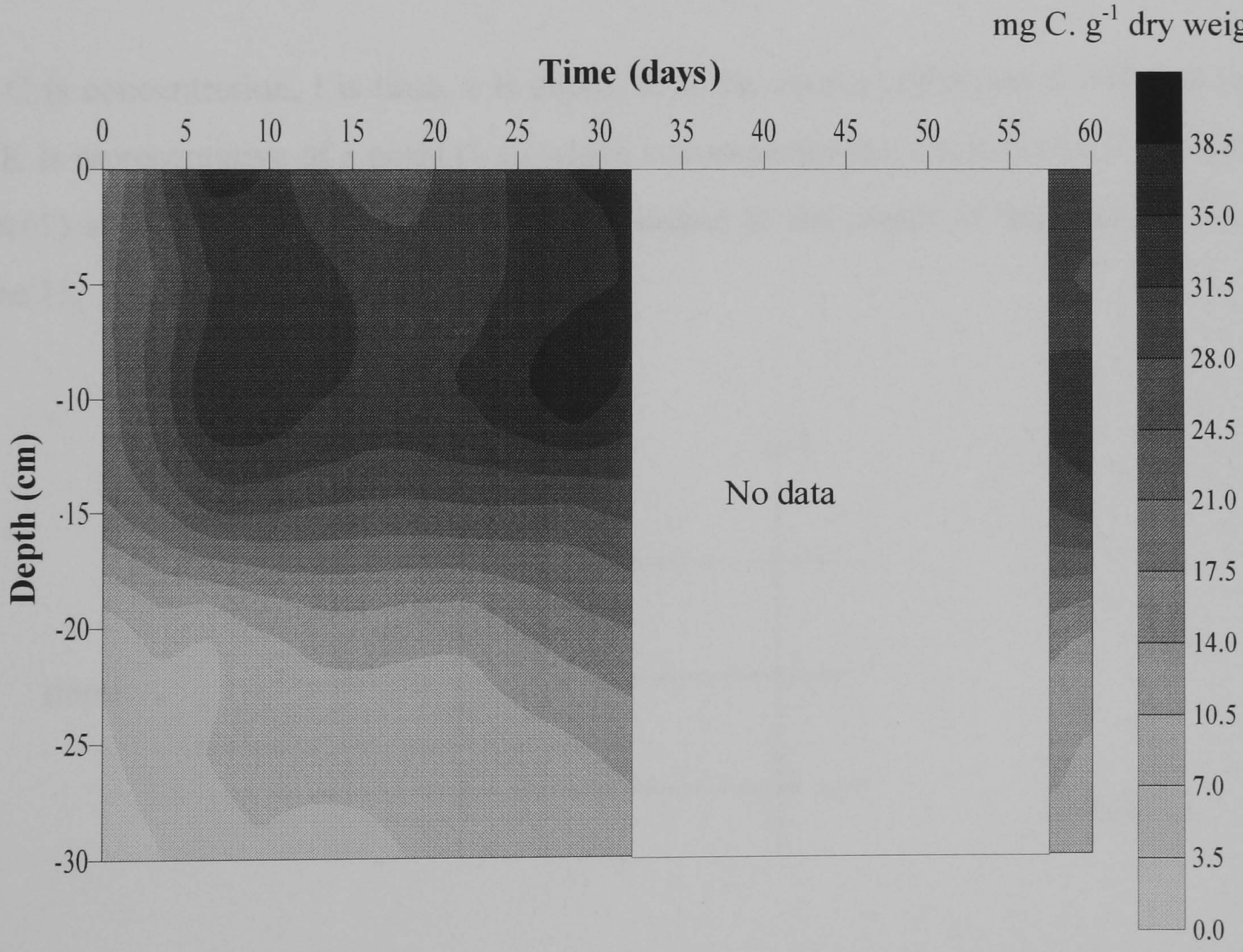


Figure 4.15. Total organic carbon for the sediment depths prior to (day 0) and after the linseed oil addition.

After the addition of oil, this trend of higher concentrations in the upper 15 cm relative to the bottom 15 cm remained.

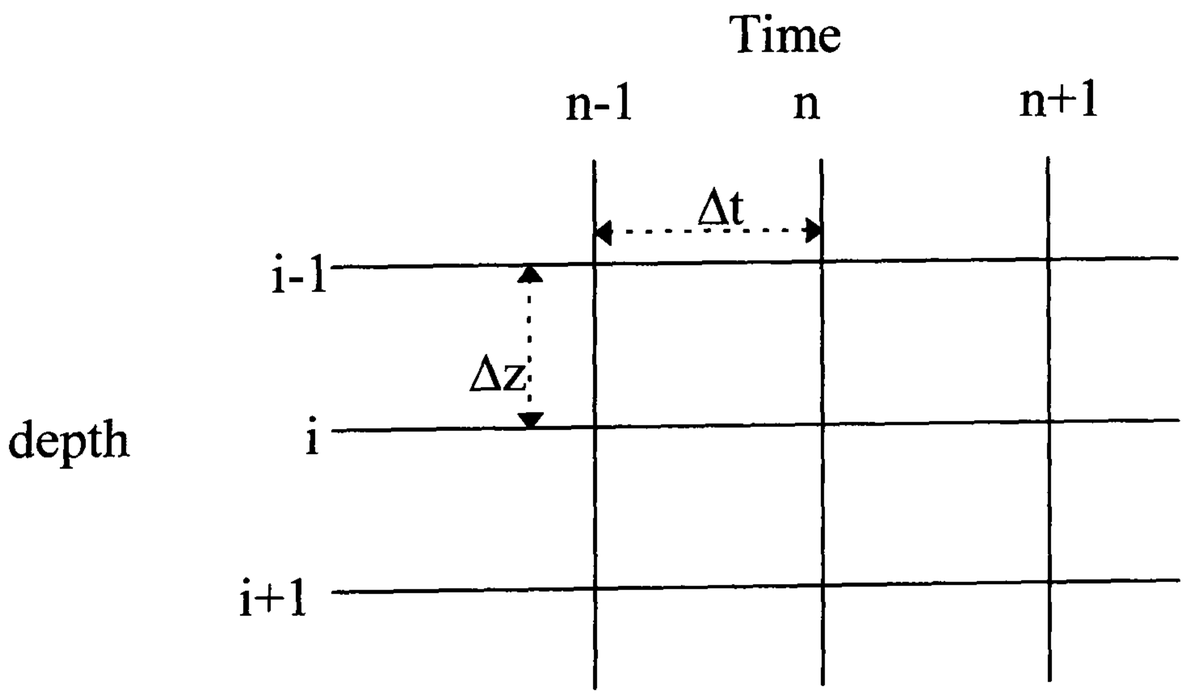
Temporal variation

The amount of lipids in the sediments increased within 3 days after the addition of the oil at all depths (Figure 4.17). Based on this graphic it was possible to establish the pattern of the vertical diffusion of the oil between any two depths. The vertical diffusion was calculated according to the model described by equation 4.8 expanded discretely into equation 4.9 (S. Wallis, *pers. commun.*).

$$\frac{\partial C}{\partial t} = \frac{\partial (K \, \partial C/\partial z)}{\partial z} \qquad (4.8)$$

$$\frac{C(i, n+1) - C(i, n-1)}{2\Delta t} = K \frac{[C(i+1, n) - C(i-1, n) + 2C(i, n)]}{\Delta z^2} \qquad (4.9)$$

Where C is concentration, t is time, z is depth, K is the vertical diffusion coefficient (cm^2 day^{-1}). K is representative of a point (i, n) which encompasses the co-ordinates (i+1,n), (i-1, n), (i, n+1) and (i, n-1) and the value of K is linked to the centre of this area, *i.e.* (i, n) (Scheme 1).



Scheme 1.

The results showed that, generally, the oil penetrated the sediments at a rate of 10^{-7} cm^2 s^{-1}, which indicates a rapid penetration into the sediments.

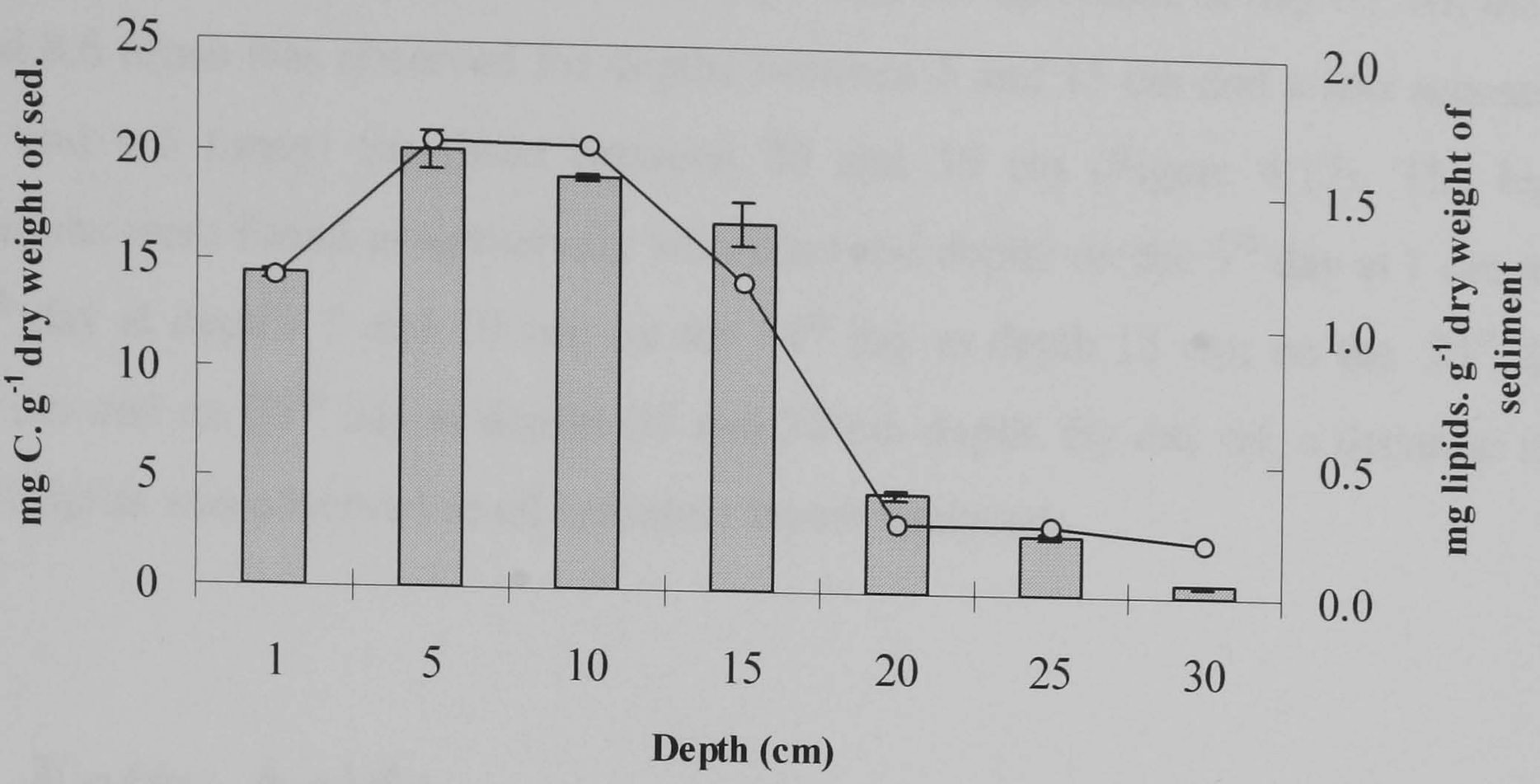


Figure 4.16. Variation with sediment depth of lipids (line) and total organic carbon (bars, ± sd), prior to the addition of linseed oil (day zero).

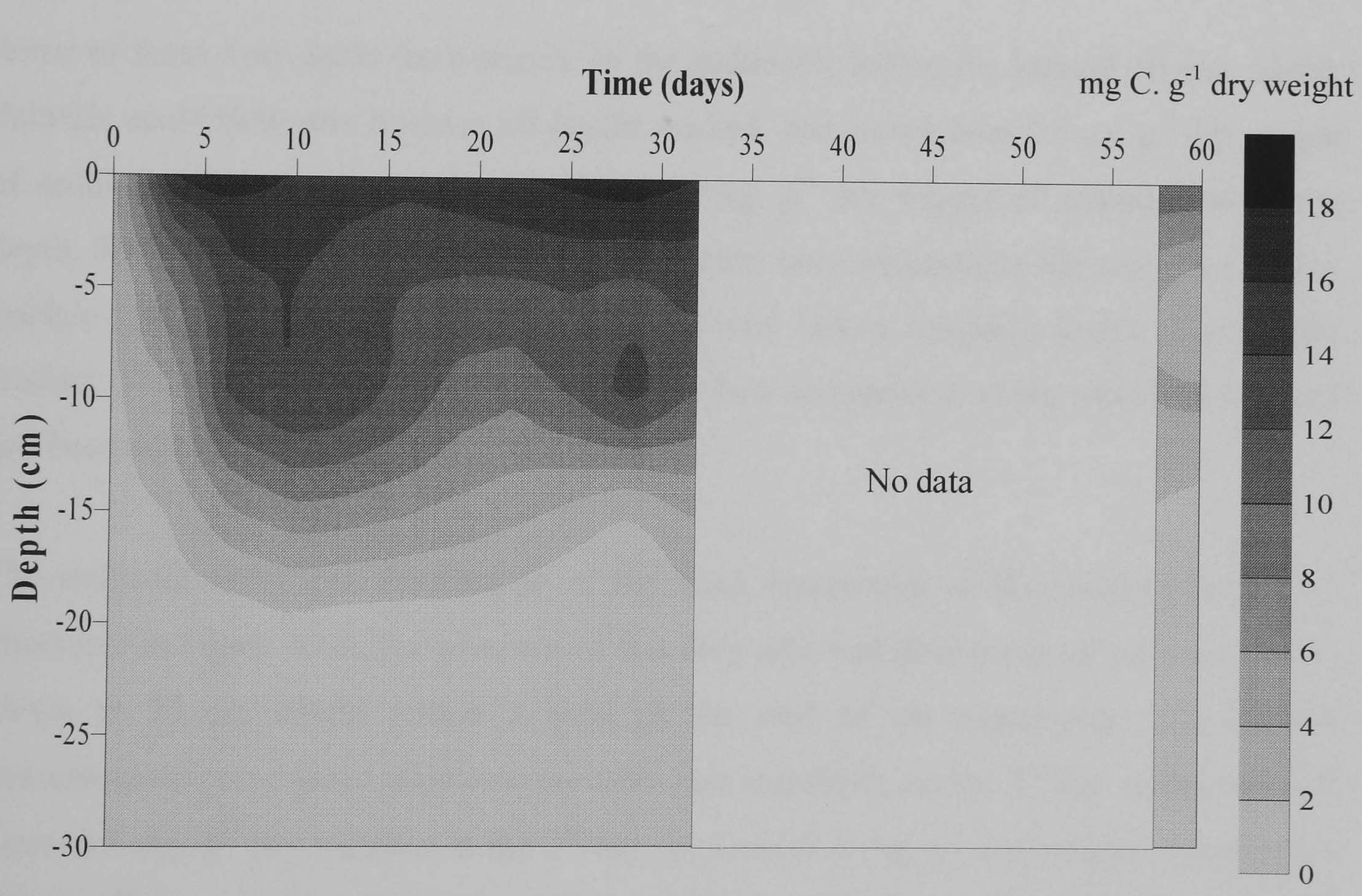


Figure 4.17. Lipids (mg. g^{-1} dry weight of sediment) present at the various sediment depths prior to (day 0) and after the addition of linseed oil.

The greatest increase (10 and 16.5 fold) in the lipids concentration was recorded in the top 1 cm, with the maximum value obtained at day 3 and the minimum at day 60. An increase of 1.2 and 8.6 times was observed for depths between 5 and 15 cm and a less accentuated one (1.1 and 6.6 times) for those between 20 and 30 cm (Figure 4.17). The highest concentrations were found progressively with time and depth: on the 3rd day at 1 cm depth; on the 7th day at depths 5 and 10 cm; on the 14th day at depth 15 cm; on the 21st day at depth 20 cm and on 28th day at depths 25 and 30 cm depth. By day 60, a decrease in the amount of lipids was observed in all sediment layers analysed.

4.4.9. Fatty Acids

In this section particular attention is given to the principal fatty acids of linseed oil: 16:0; 18:0; 18:1ω9; 18:2ω6 and 18:3ω3.

Some of these fatty acids were present in the sediments before the linseed oil was added. Palmitic acid (16:0) was found at all depths studied and varied from 0.3 μg. g^{-1} dry weight of sediment at the surface sediments to 0.019 μg. g^{-1} dry weight of sediment at 30 cm depth. Stearic (18:0) and oleic (18:1ω9) acids were only observed in the top 5 cm, whilst linoleic (18:2ω6) and linolenic (18:3ω3) acids were below detection limits at all depths studied. These concentrations were negligible when compared to those measured after oil had been added.

The temporal and depth distribution of the main component of linseed oil (18:3ω3) is illustrated in Figure 4.18. The presence of this fatty acid was detected in all sediment layers (down to 30 cm depth) within 3 days of the start of the experiment. The highest concentrations were found progressively with time and depth: on the 3rd day at the depth of 1 cm (6.3 mg. g^{-1} dry weight); at the 7th day, at 5 cm (5.3 mg. g^{-1} dry weight of sediment) and at 14th day at 10 cm (4.7 mg. g^{-1} dry weight of sediment) depth, indicating a vertical migration of 18:3ω3 within the top 15 cm with time.

During the first 21 days, the 15 cm layer separated high (0.5 to 6.3 mg. g^{-1} dry weight) from low (0.0006 to 0.5 mg. g^{-1} dry weight of sediment) concentrations. However, after 3 weeks,

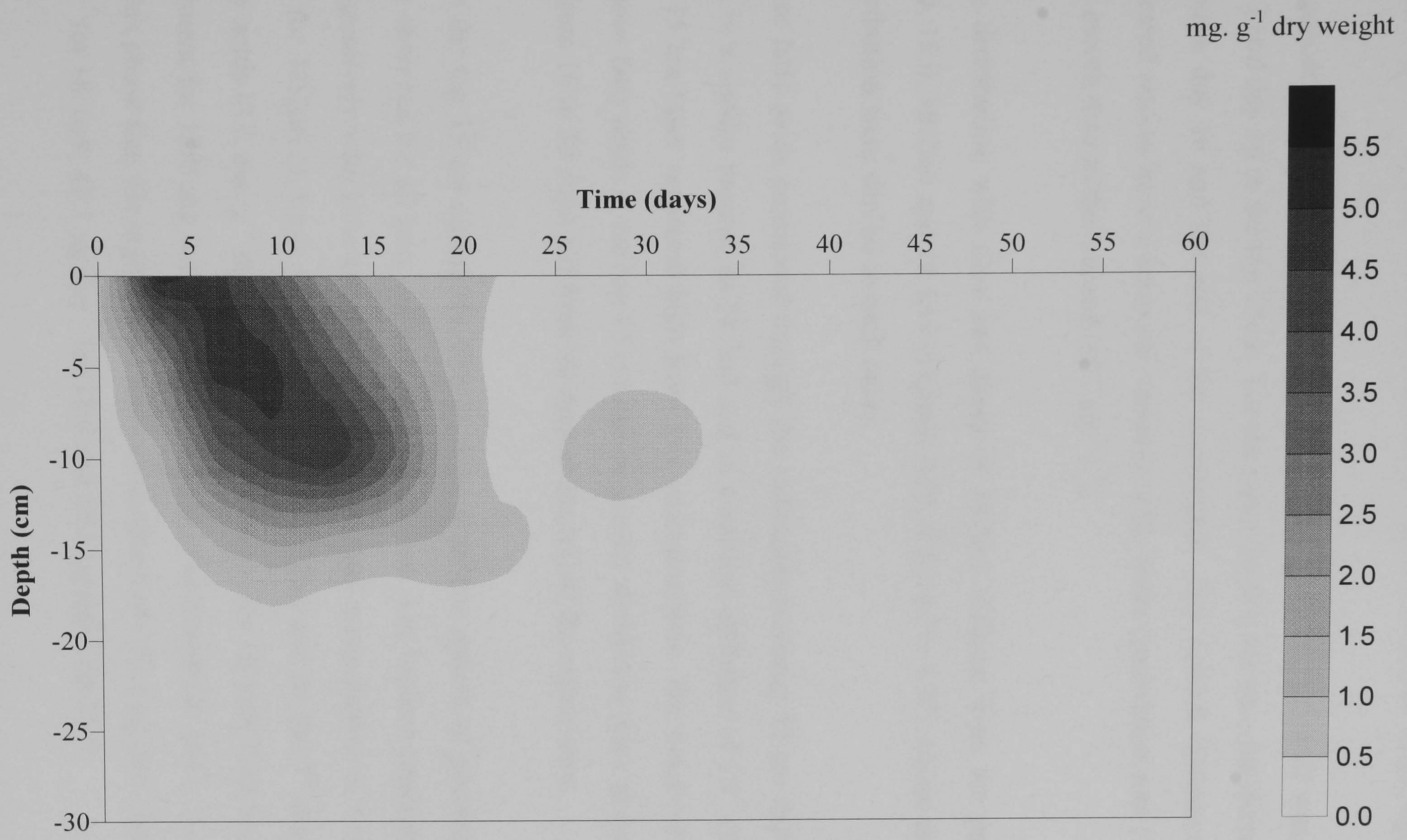


Figure 4.18. Temporal distribution with sediment depth of 18:3ω3 (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

concentrations become equally small at all sediments depths. The only exception was observed at depth 10 cm on day 28 where the concentration varied between 0.5 and 1 mg g^{-1} dry weight of sediment. This accumulation was also mirrored by the lipids contents.

Consequently, the highest loss rate (420 μg day^{-1}) of this fatty acid was observed between day 3 and day 16 in the top 15cm. For the same depths, the loss rate decreased (20 μg day^{-1}) between day 16 and the end of the experiment. The vertical diffusion of this fatty acid appeared also to have a temporal variation with faster penetration rate (10^{-6} cm^2 s^{-1}) in the first month than in the second (10^{-7} cm^2 s^{-1}).

This distribution with time and depth of 18:3ω3 differed from the profile observed for 16:0, 18:0, 18:2ω6 and 18:1ω9 (Figures 4.19, 4.20, 4.21, 4.22) whose temporal and spatial distribution were similar to each other.

These fatty acids permeated through the sediments reaching 30 cm depth in three days or less in a similar manner as 18:3ω3 and at a vertical diffusion of 10^{-7} cm^2 s^{-1}. Once again, the 15 cm layer separated high from low concentrations. The temporal distribution of the various fatty acids at the top 15 cm can be grossly divided in three phases: i) up to day 16; ii) from 16 to 30 days; iii) from 30 days to the end of the experiment.

i) In the top 15 cm during the first 16 days, a similar pattern of penetration to the 18:3ω3 was observed for all fatty acids, except for 18:2ω6. The highest concentrations were found progressively with time and depth, with the highest concentration at 1 cm depth on the 3rd day for 18:2ω6 (1.3 mg. g^{-1} dry weight of sediment) and on the 7th day for the remaining fatty acids (3.2 mg. g^{-1} dry weight of the sediment for 18:1ω9; 0.54 mg. g^{-1} dry weight of sediment for 18:0 and 0.69 mg. g^{-1} dry weight of sediment for 16:0). As for 18:3ω3, it was in this phase that the highest loss rates were observed: 82.3 μg day^{-1} for 18:2ω6; 272.3 μg day^{-1} for 18:1ω9; 45.1 μg day^{-1} for 18:0 and 58.3 μg day^{-1} for 16:0.

ii) From 16 to 30 days, the concentration of the various fatty acids in the upper 15 cm appeared to decrease but at a much lower rate than that observed for 18:3ω3. An exception to this pattern was the high concentrations observed on day 28 for 10 cm depth, which also had high values of 18:3ω3 and lipids concentrations.

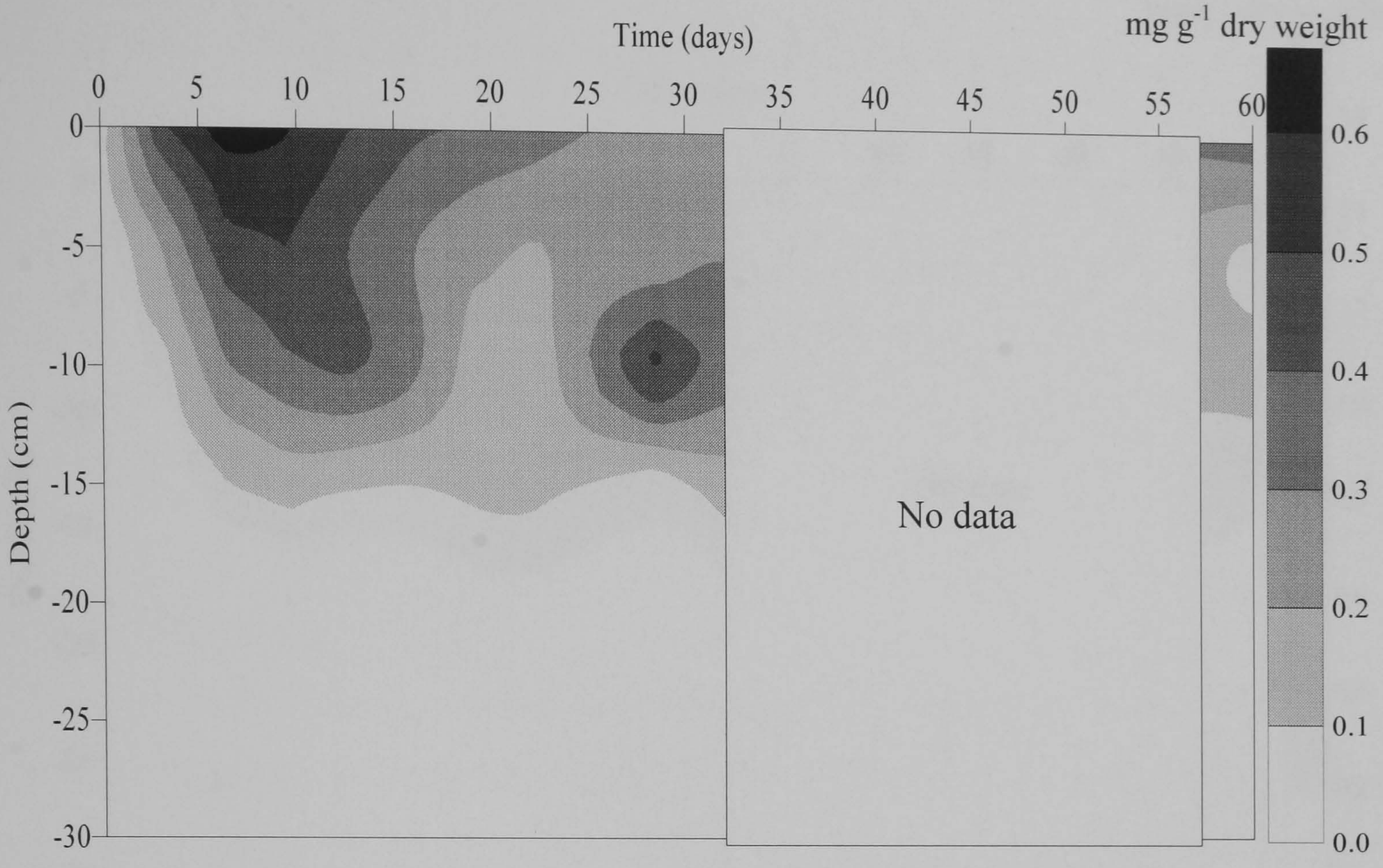


Figure 4.19. Temporal distribution with sediment depth of 16:0 (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

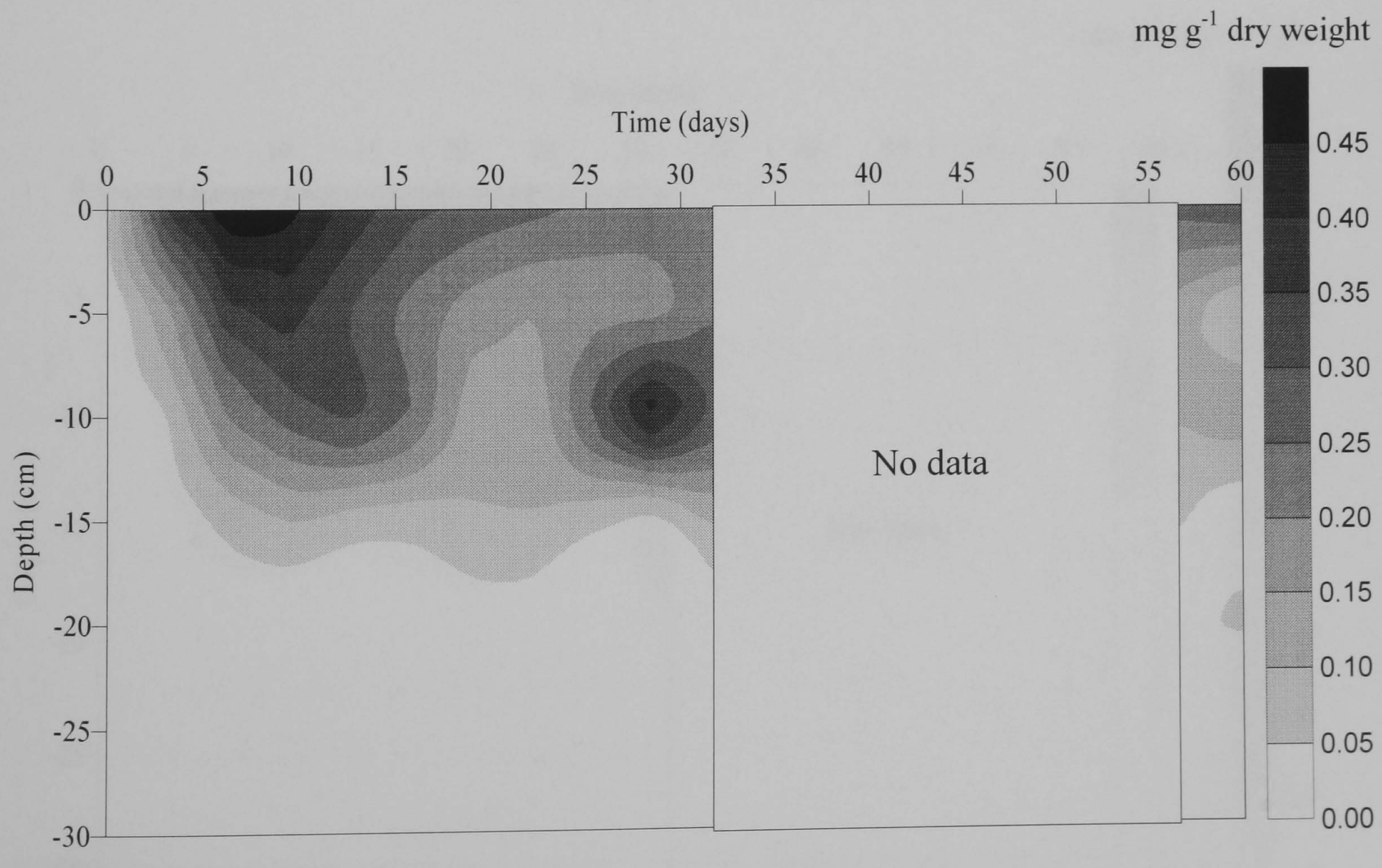


Figure 4.20. Temporal distribution with sediment depth of 18:0 (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

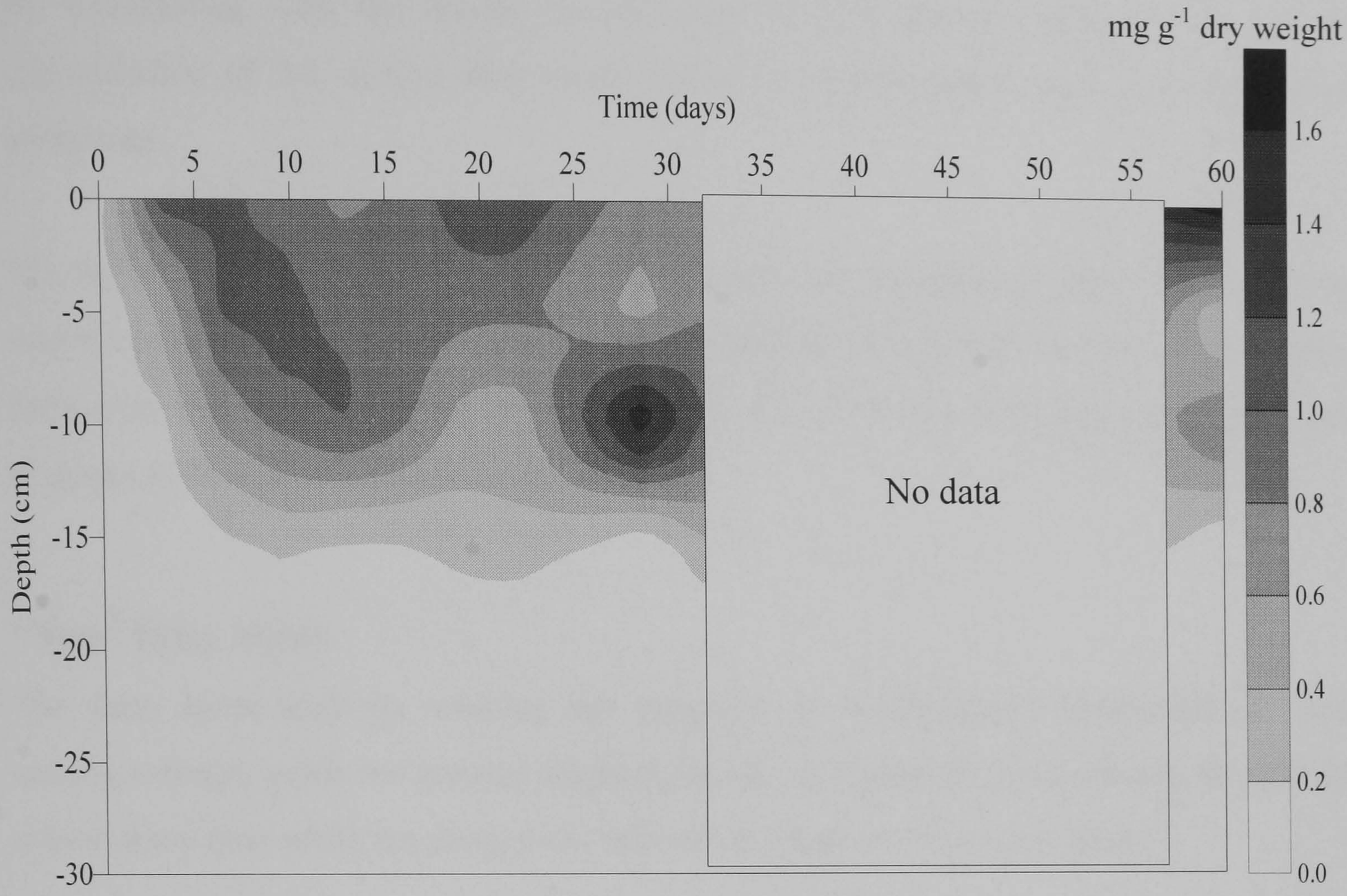


Figure 4.21. Temporal distribution with sediment depth of 18:2ω6 (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

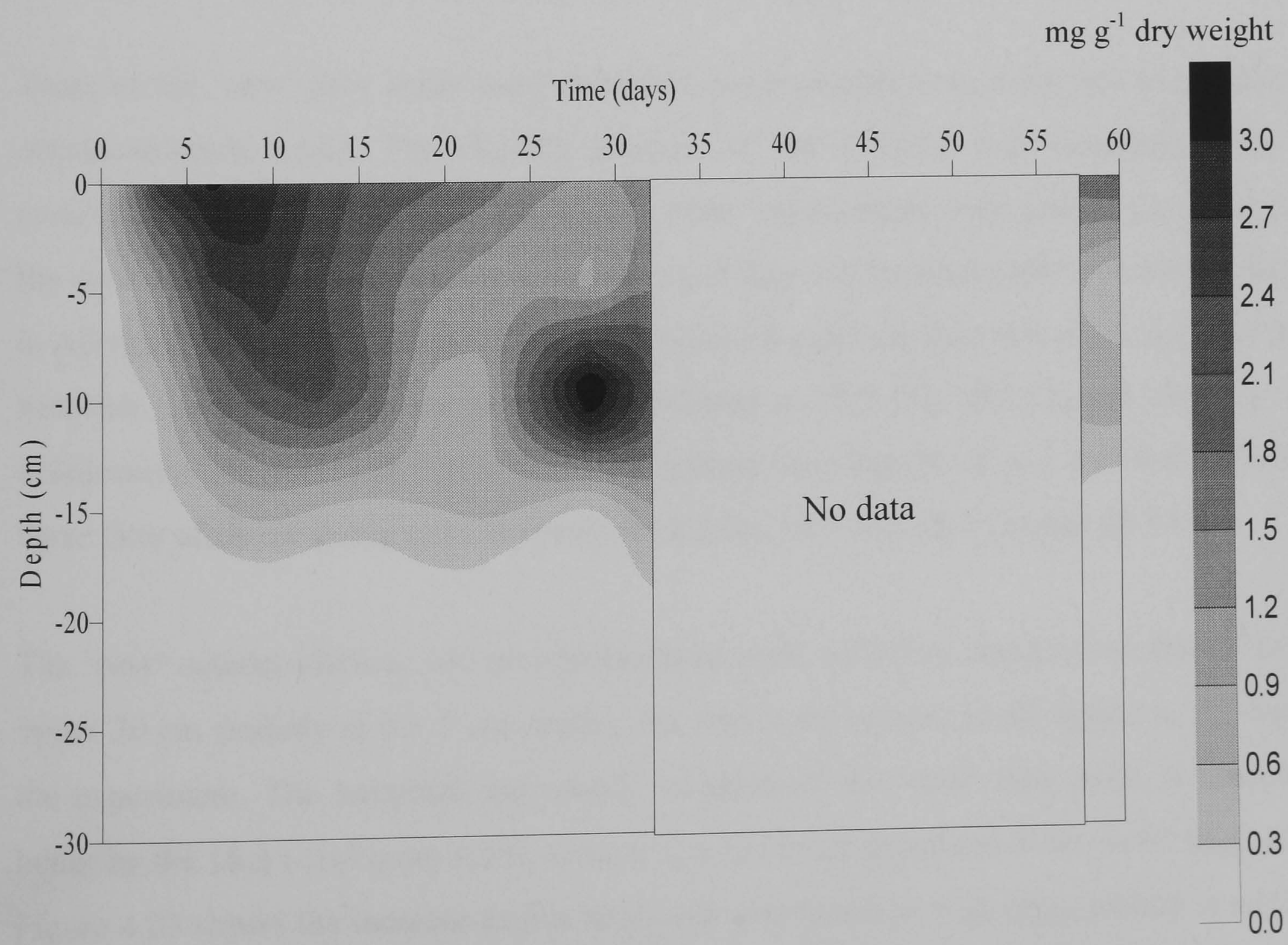


Figure 4.22. Temporal distribution with sediment depth of 18:1ω9 (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

iii) Contrasting with the results obtained for 18:3ω3, between days 30 and 60, the concentration of the various fatty acids started to increase again, mainly in the surface sediments.

The lower 15 cm were, once more, characterised by the presence of small amounts of the various fatty acids. However, between day 30 and day 60 a small increase in the various fatty acids was observed for depths 15 to 20 cm or 15 to 25 cm, depending on the fatty acid (Figures 4.19, 4.20, 4.21 and 4.22).

'New' fatty acids

The fatty acids analysis revealed the presence of octadecenoic, octadecadienoic and octadecatrienoic acids not present originally in the sediments or in the linseed oil. For this reason these fatty acids are going to be referred in future as 'new' fatty acids.

The octadecenoic acid was identified as 18:1ω7 and was present throughout the experiment. Nevertheless, its quantification was not always possible since its elution time was very close to the 18:1ω9, making it difficult to separate both fatty acids.

Three of the 'new' fatty acids were identified as octadecadienoic acids and four others as octadecatrienoic acids. The GC-MS analysis of the FAMEs was insufficient for the complete identification of these fatty acids. More experiments were carried out to identify the double bond position of these fatty acids and they will be discussed in detail in Chapter 6. All three octadecadienoic acids had a retention time greater than that of 18:2ω6 and from hereafter these fatty acids are going to be referred as 18:2 (1), 18:2 (2) and 18:2 (3). The octadecatrienoic acids had a retention time greater than that of 18:3ω3 and from hereafter these fatty acids are going to be referred as 18:3 (1), 18:3 (2), 18:3 (3) and 18:3 (4).

The 'new' octadecadienoic and octadecatrienoic acids were first observed on day 21 in the upper 20 cm (mainly at the 5 cm depth), but they were present in all depths at the end of the experiment. The temporal and spatial variation of the 'new' fatty acids is illustrated better by the 18:3 (4) (Figure 4.23), since it was the most abundant of the 'new' fatty acids. Figure 4.23 shows the increase in this fatty acid concentration with time, mostly at surface, but also in deeper sediments. Nonetheless, a separation between high concentrations at the

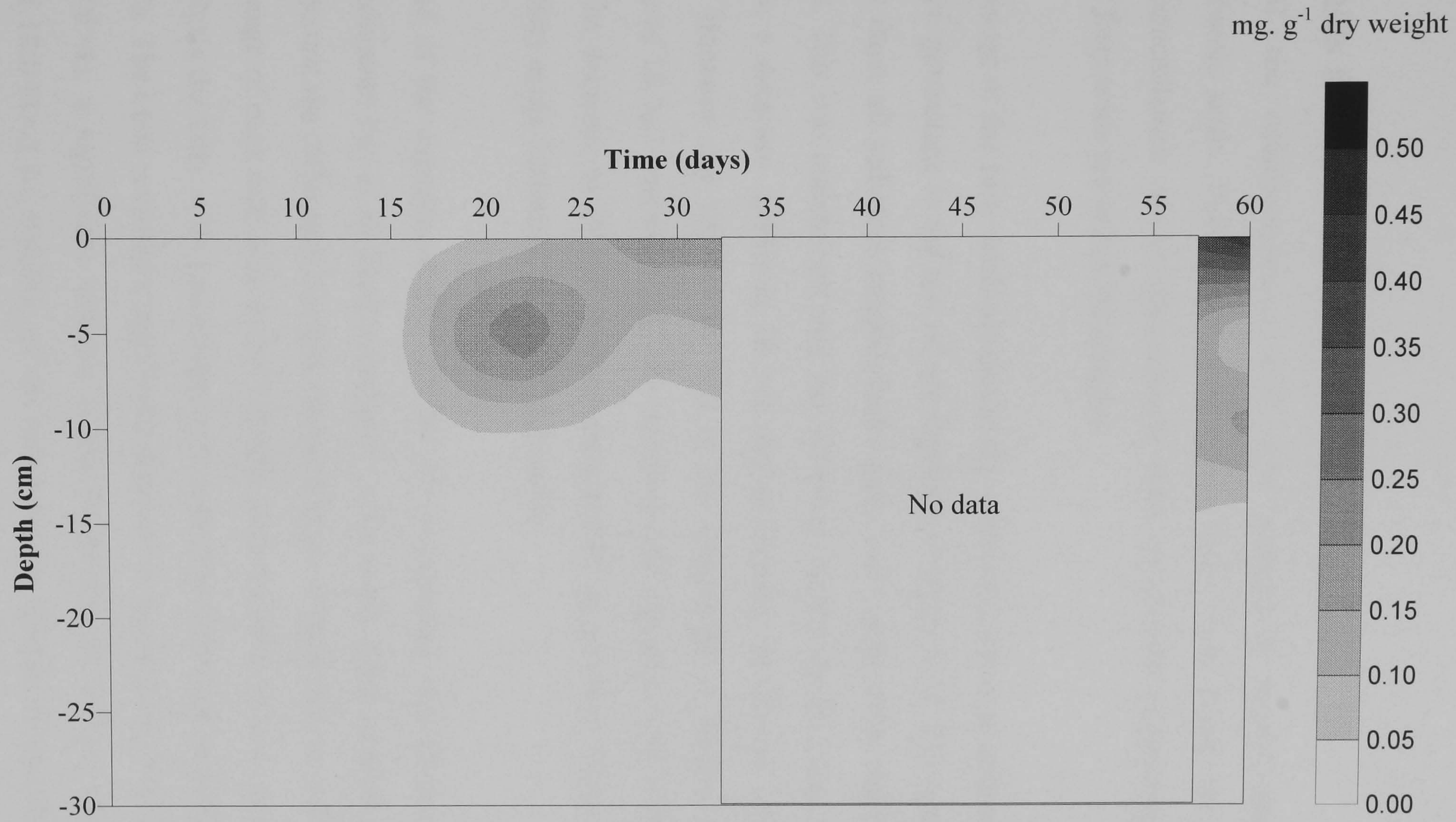


Figure 4.23. Temporal distribution with sediment depth of a 'new' octadecatrienoic acid (mg. g^{-1} dry weight of sediment) after the addition of linseed oil.

top 15 cm and low concentration at the bottom can be drawn, like for all the other acids described before. The observations regarding this fatty acid apply to all other 'new' fatty acids.

Fatty Acids Relative Abundance

In general, the octadecatrienoic acids were present in higher amounts than the octadecadienoic acids. This can be observed in Figure 4.24, where the total amount of 'new' octadecadienoic and octadecatrienoic acids are plotted as percentages of the total amount of fatty acids present in the samples.

The percentage of the fatty acids present in the sediments after contamination with linseed oil and their percentage in the raw oil are depicted in Figure 4.24. The results showed that, after three days, all sediment samples had a fatty acid composition similar to that of the linseed oil. This was maintained until day 14 for all depths except 1 and 30 cm, where, at the former, a decrease (39%) in 18:3ω3 and an increase in 18:1ω9, 18:0 and 16:0 was observed. Between day 14 and the end of the experiment, a further reduction in the percentage of 18:3ω3 was recorded, with this fatty acid less than 13% of the total fatty acid content. The decrease in 18:3ω3 was accompanied by an increase in the percentage of all the other fatty acids, including the 'new' fatty acids.

By the end of the experiment (day 60) the oil composition was altered not only at the surface sediments but at all depths analysed. A 2-sample t-test assuming equal variance was used to test the difference between the percentage of each fatty acid in the pure oil and the percentage of each fatty acid at the 7 depths analysed after 60 days of incubation. Prior to the analysis the fatty acids percentage were normalised through arcsine-transformations (Fry, 1993). The t-test revealed a significant decrease in the proportion of 18:3ω3 ($T=13.44$; $P<0.001$; $DF=8$), a significant increase in the proportion of 18:1ω9 ($T=-6.82$; $P=0.0001$; $DF=8$) and 18:0 ($T=-2.40$; $P=0.043$; $DF=8$) between pure linseed oil and the result obtained for the 7 depths analysed at day 60.

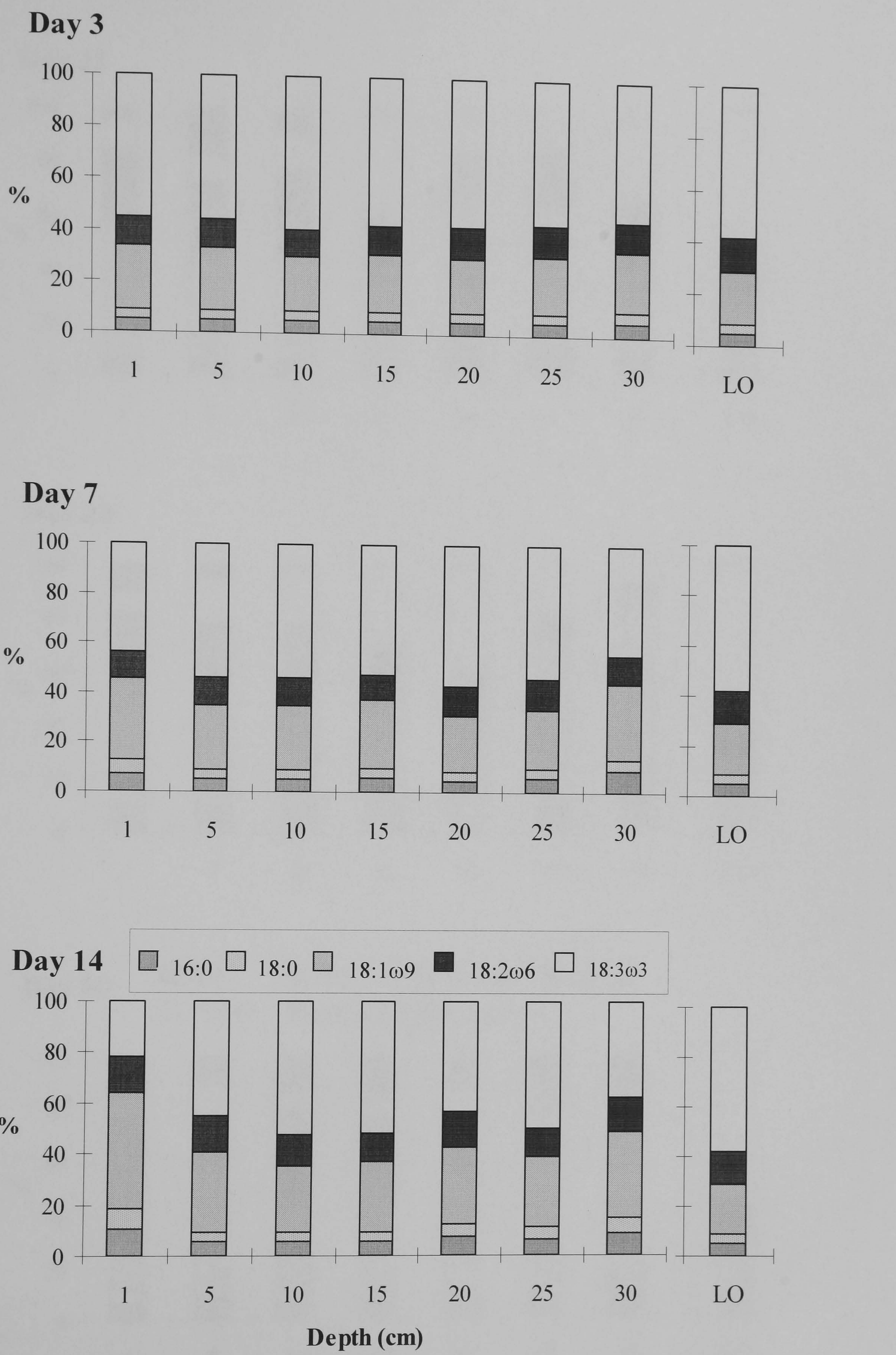


Figure 4.24. Distribution with sediment depth of the relative abundance of the main fatty acids of linseed oil and 'new' fatty acids (weight %) after the addition of linseed oil. LO - Linseed oil; total (18:2) - sum of all 'new' 18:2; total (18:3) - sum of 'new' 18:3.

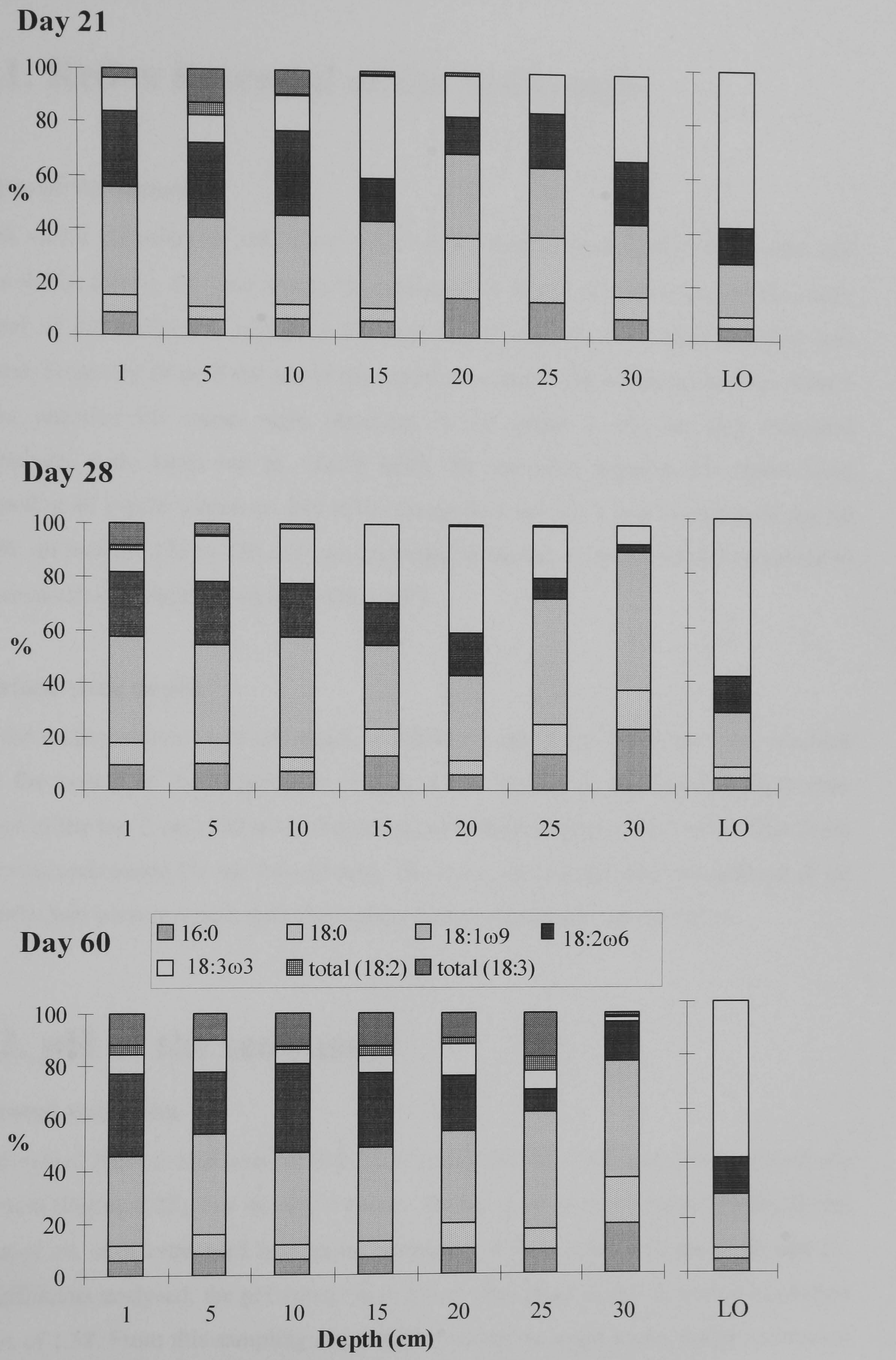


Figure 4.24. Continuation

4.5. SUNFLOWER OIL RESULTS

4.5.1. Redox Potential of the Sediments

Temporal variation

The Eh varied considerably with time, with values ranging from -240 mV to +444 mV (Figure 4.25). During the first month, the presence of oil did not affect greatly the redox potential of the sediments, except at the top 2 to 5 cm, where a small decrease was observed. From day 28 until the end of the experiment the Eh decreased noticeably. After 2 months, negative Eh values were observed in the upper 5 cm but they increased progressively with depth (up to +222.5 mV). By day 180, negative Eh values were registered at all depths (down to -240 mV), except at 1 cm (Eh = 6 mV). Between day 60 and 180, an increase (72 to 136 mV) was recorded at the top 4 cm but values continued to decrease at all other depths (-46.5 to -438.5 mV).

Variation with depth

From the surface down to 30 cm depth, a difference of 133 to 354.5 mV was recorded during the course of the experiment (Figure 4.25). At day 0, the lowest values were obtained in the top 2 cm (230 mV), increasing with depth (up to +422.5 mV). This depth profile was maintained for the first 60 days. However, six months after the addition of oil this profile had been reversed, with the highest values obtained at the top 14 cm.

4.5.2. pH of the sediments

Temporal variation

The pH varied from a minimum of 5.61 to a maximum of 7.97 over the course of the experiment (Figure 4.25). For the first 14 days, the top 8 cm were the most affected by the addition of oil, with a recorded maximum decrease of 0.79 in relation to day 0. By day 21, in all sediments analysed, the pH values were lower than those at day 0, with a maximum decrease of 1.58. From this sampling day until the end of the experiment, the pH decreased only slightly. The final pH was much lower than the initial.

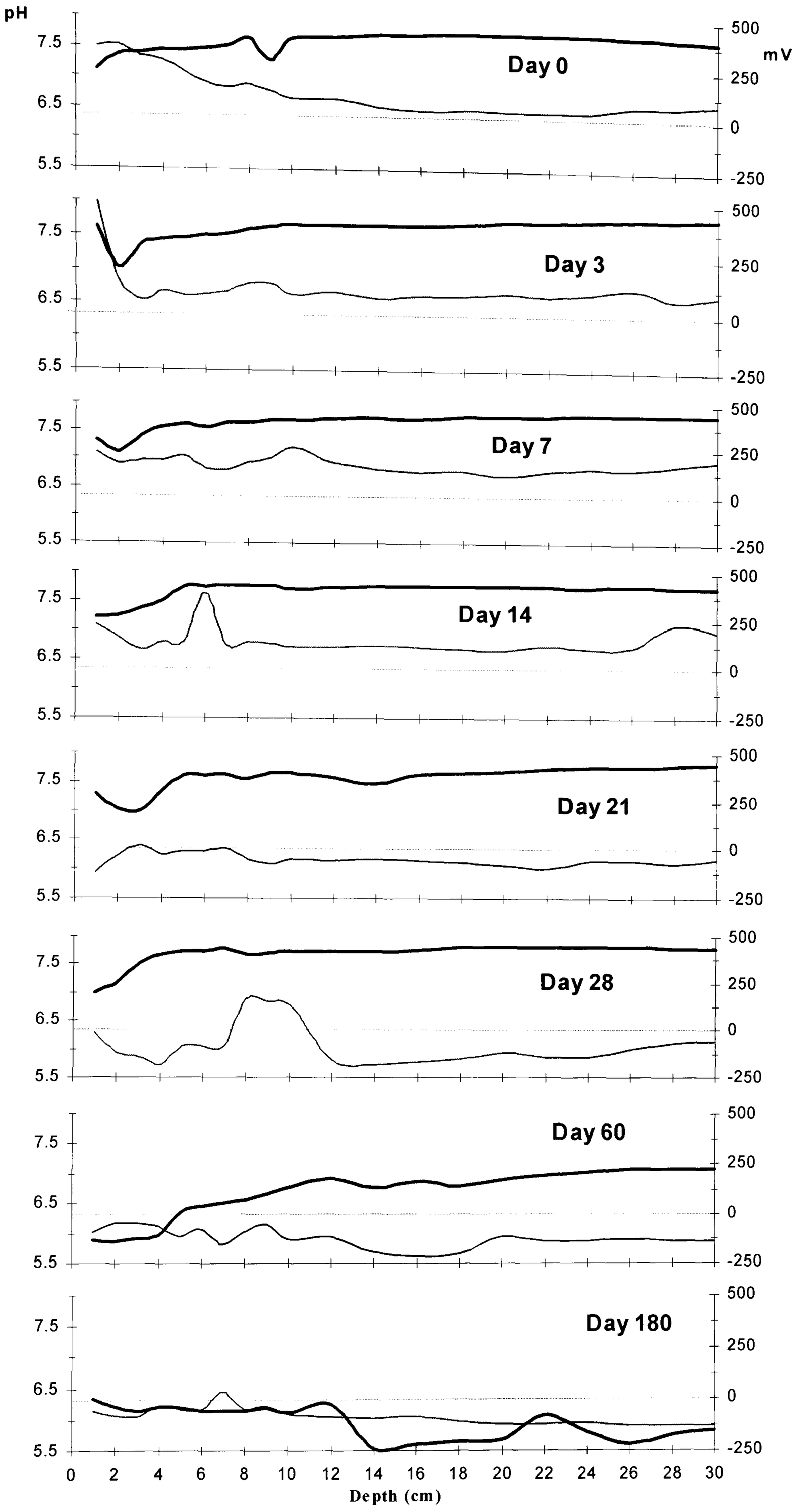


Figure 4.25. Variation of the pH (thin line) and Eh (thick line) with time and depth in the sediments of the studied salt marsh previous to (day 0) and after the addition of sunflower oil.

Variation with depth

Values of pH did not vary markedly with depth for the duration of the experiment (Figure 4.25). At the start of the experiment, higher values were recorded in the upper 8 cm (up to 1.05) than in the sediments layers below, with identical values measured at these other depths (Figure 4.25). Within 3 days after the addition of oil, the band of higher values was restricted to the top 2 cm. By day 180, all depths studied had a pH between 5.9 and 6.45.

4.5.3. Temperature of the sediments

Temporal variation

Temperature varied markedly throughout the experiment, with a lowest value of 0°C (day 21) and a highest of 20.5°C (day 180) (Figure 4.26).

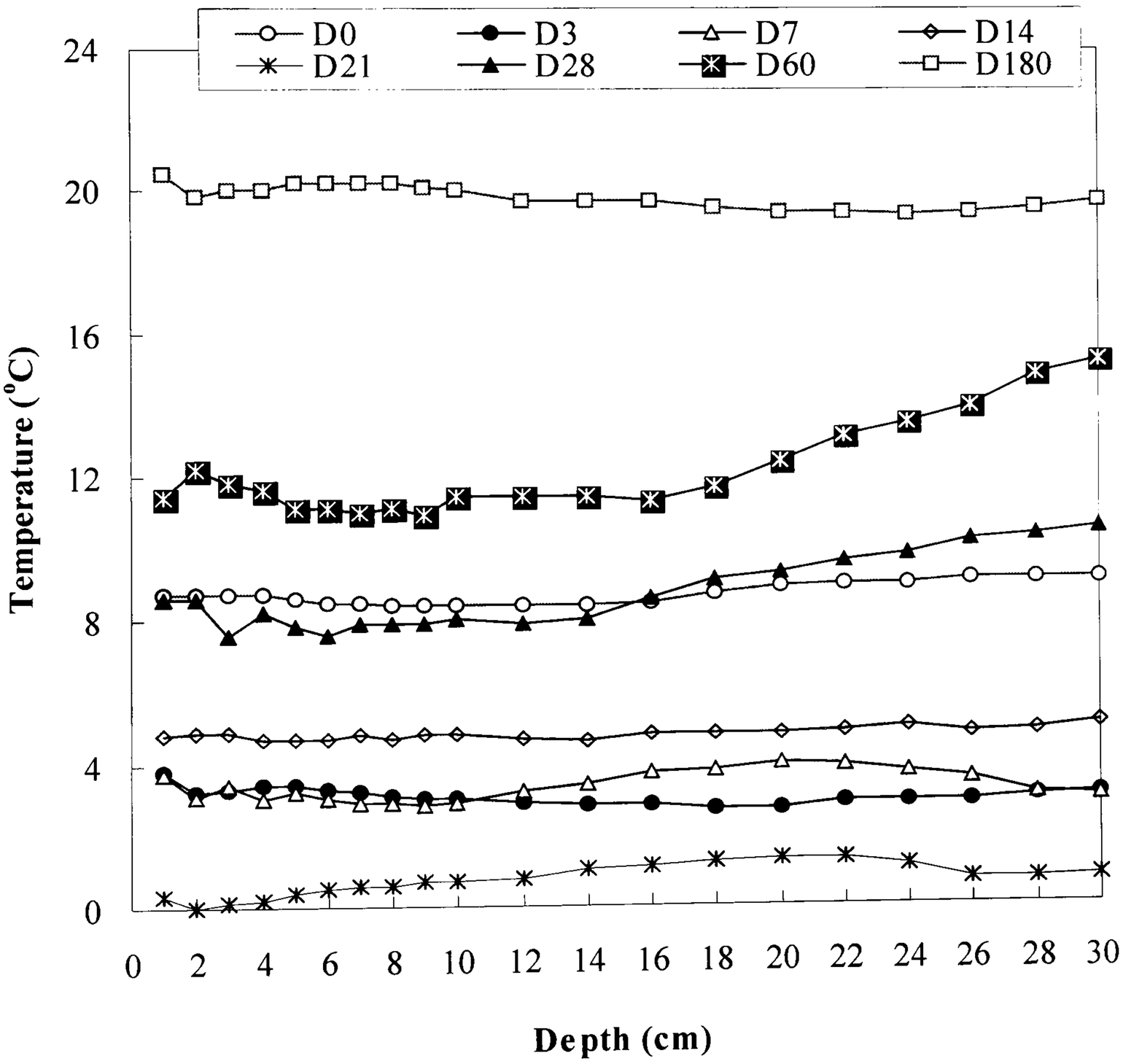


Figure 4.26. Variation of the temperature (°C) with time and depth in the sediments of the studied salt marsh previous to (day 0) and after the addition of sunflower oil.

It was expected that the temperature would increase from day 0 to 180, since sample collection began in winter and finished in summer. Still, changes in temperature were also observed at a much smaller time scale: at day 0, the temperature values fluctuated from 8.4 to 9.1°C with depth. Within 3 days a decrease of approximately 5.5°C was observed, which was maintained until day 7. An increase (4.6 to 5.1°C) was recorded from the 7th to the 14th day and was followed by a decline to values ranging from 0 to 1.3°C at day 21. From then until the end of the experiment, the temperature increased continuously up to 20.5°C. These changes occurred as a result of the alteration in weather conditions and also of the time of sampling.

Variation with depth

For most of the sampling occasions, temperature values varied by 0.5-1.3°C with depth and did not appear to follow a specific pattern (Figure 4.26). The exceptions were days 28 and 60 when there was an increase (2.8 to 4.3°C) in the temperature between the depths of 18 and 30 cm.

4.5.4. Enumeration of Bacteria

4.5.4.1. Aerobic Bacteria

4.5.4.1.1. Heterotrophic Aerobic Bacteria (HAB)

Temporal Variation

The number of HAB varied markedly with time with a minimum of 8.2 x10^{2} CFU. g^{-1} wet weight of sediment and a maximum of 2.1 x10^{6} CFU. g^{-1} wet weight of sediment (Figure 4.27 and 4.28). An increase of 5 and 2.5 fold was registered within 3 days for depths of 1 and 5 cm, respectively. For these sediment layers this initial growth was not sustained and by day 7 a decline to approximately background numbers (1.1x10^{5} and 3.8 x10^{4} CFU. g^{-1} wet weight of sediment) was observed. However, at the 1 cm depth the same bacteria

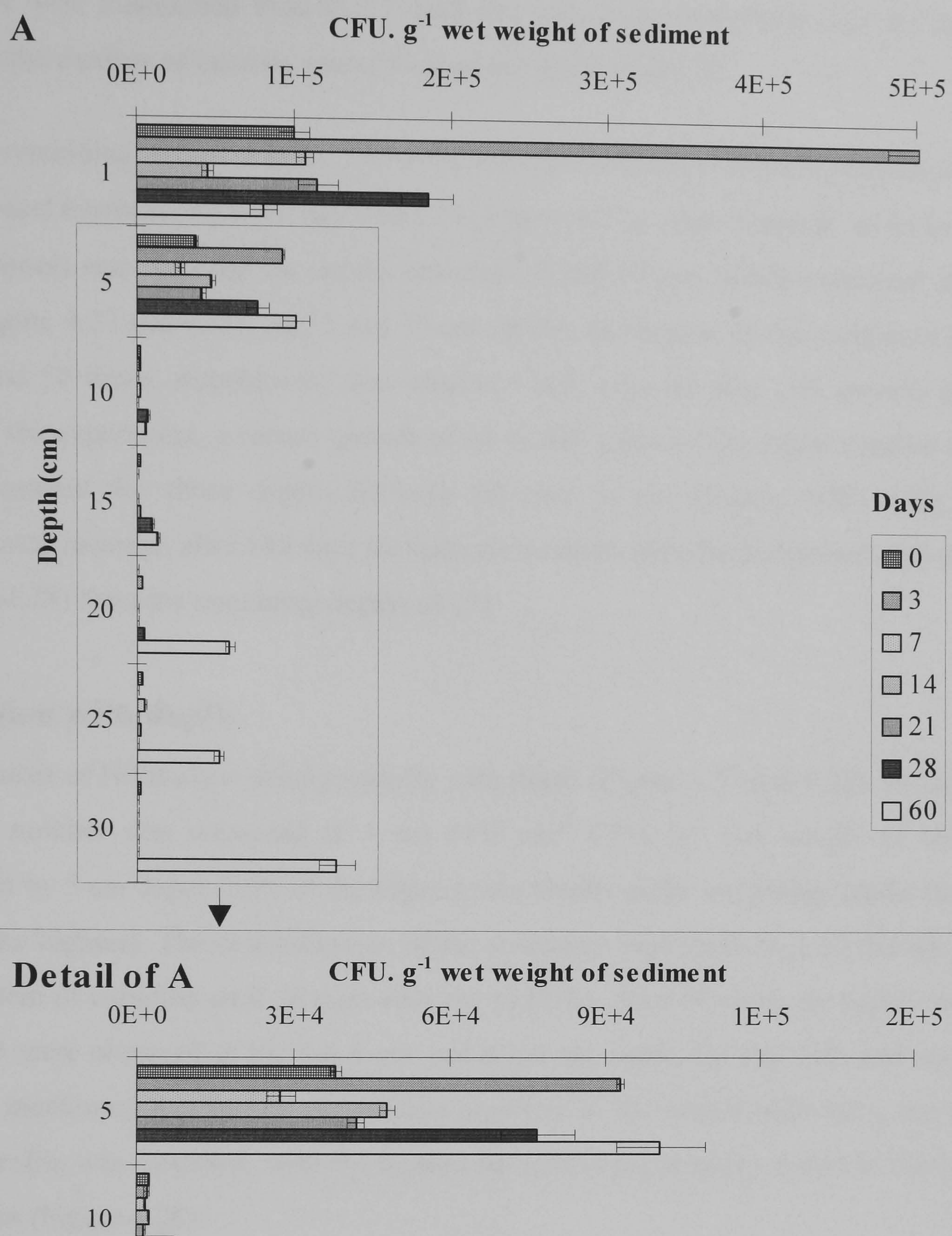


Figure 4.27. Number of heterotrophic aerobic bacteria (± sd) present at the various sediment depths prior to (day 0), and up to 60 days after, the addition of sunflower oil.

numbers were maintained from day 7 until the end of the experiment, but for the 5 cm fraction the number of bacteria started to increase again at day 28.

For the remaining sediment layers (10 to 30 cm) the abundance of HAB fluctuated around background numbers, between day 0 and 28 (Figure 4.27). After 1 month, an increase (2.7 to 4.1 times) was recorded for depths between 10 and 20 cm, which continued until day 180 (Figure 4.27 and 4.28). At 25 and 30 cm depths, an increase in the number of bacteria (15.3 and 50 times, respectively) was observed only after 60 days. Six months after the start of the experiment, a record growth of 60 to 840 times of the initial number of HAB was measured for those depths between 10 and 30 cm (Figure 4.28). Due to this accentuated increase, after 180 days the bacteria numbers have been depicted on a different Figure (4.28) from the remaining depths (4.27).

Variation with depth

The number of HAB also varied markedly with depth (Figure 4.27 and 4.28). At day 0, the highest number was measured at 1 cm (1.0 $x10^5$ CFU. g^{-1} wet weight of sediment) followed by 5 cm depth (38% of the highest) and finally at the remaining depths (less than 5% of the highest). The contamination of the sediments with sunflower oil did not change this pattern of variation until 28 days after the addition. After 60 days, the highest numbers of HAB were observed at the top 5 cm and at 30 cm depth. By day 180, and due to the already mentioned increase in the bacteria numbers in the deeper sediments, the original depth profile was modified, with the highest bacteria concentrations found in the 15 to 30 cm layers (Figure 4.28).

4.5.4.1.2. Aerobic Oil Degrading Bacteria (AODB)

Temporal Variation

As observed for the HAB, the number of bacteria with the capability to degrade sunflower oil varied markedly with time, from 9.5 to 9500 bact. g^{-1} wet weight of sediment (Figure 4.29). An increase in the number of bacteria was observed within 3 days of the addition of oil, particularly at the 1 cm depth where a 10 fold increase was recorded. This initial growth continued, with maximum numbers reached after 7, 14, 21 and 28 days, depending

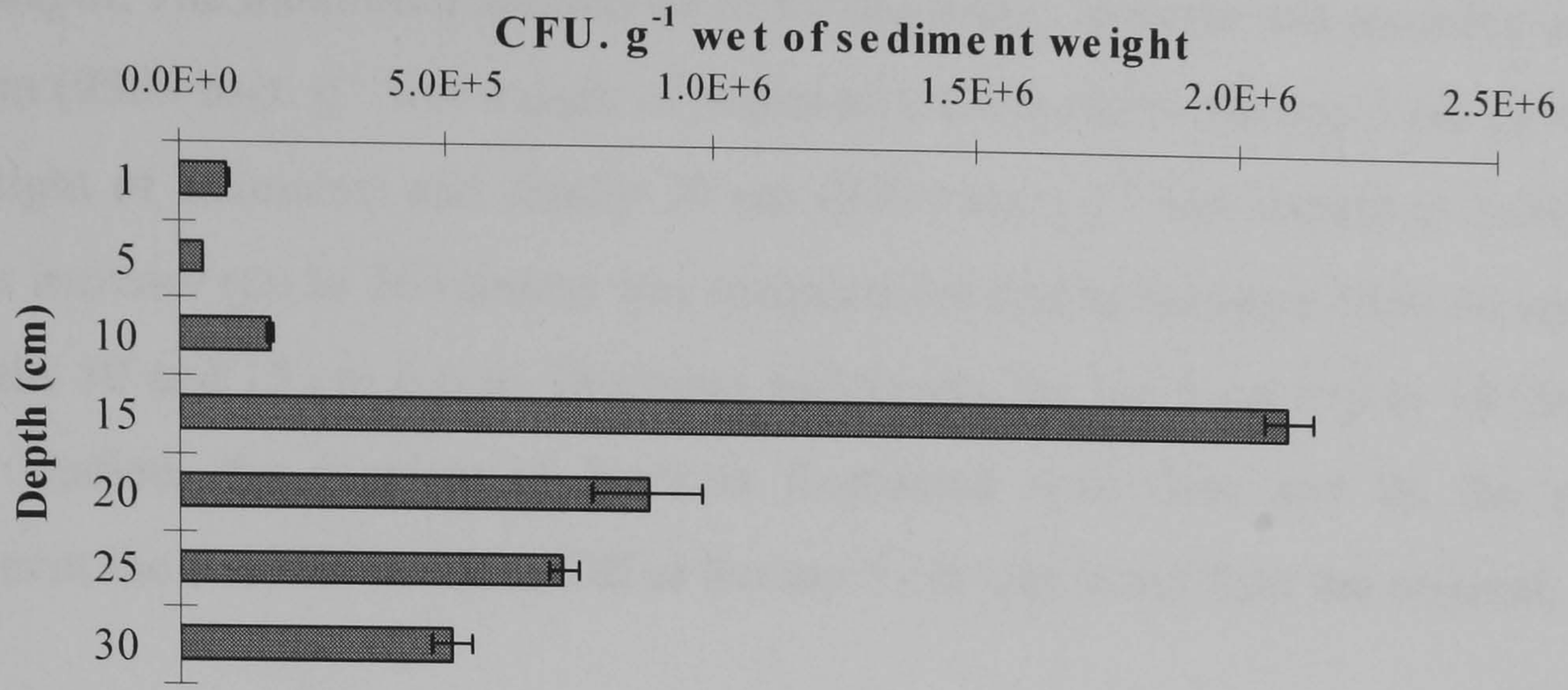


Figure 4.28. Variation with the sediment depth of the number of heterotrophic aerobic bacteria (± sd), 180 days after the addition of sunflower oil.

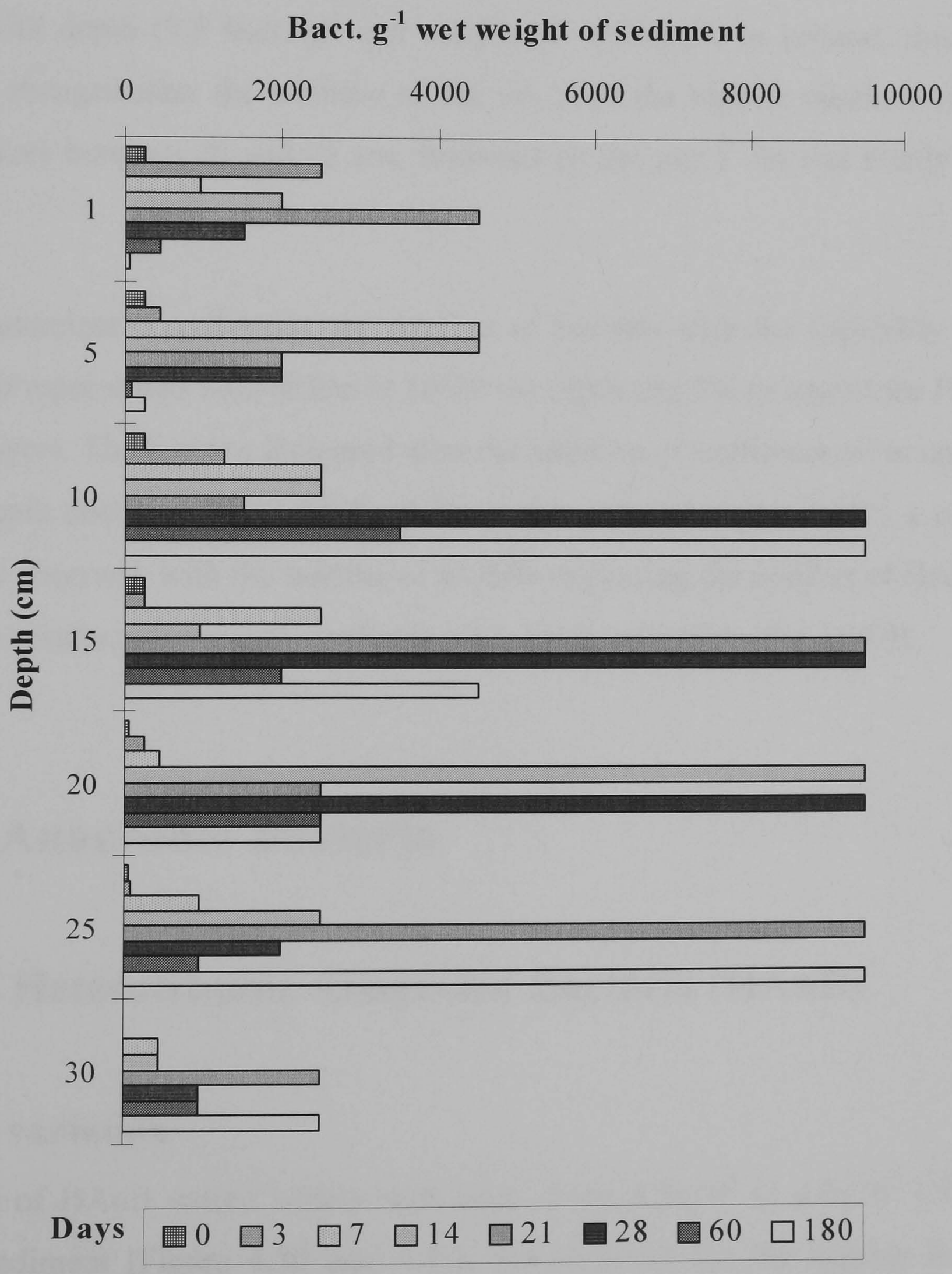


Figure 4.29. Number of aerobic oil degrading bacteria present at the various sediment depths previous to (day 0) and after the addition of sunflower oil.

on the depth. The maximum abundance of oil degrading bacteria was recorded at depths 10 to 25 cm (9500 bact. g^{-1} wet weight of sediment), followed by the top 5 cm (4500 bact. g^{-1} wet weight of sediment) and finally 30 cm (2500 bact. g^{-1} wet weight of sediment). The greatest increase (up to 263 times) was recorded for depths between 20 to 30 cm, followed by depths 10 and 15 cm (up to 38 times) and finally the top 5 cm (up to 18 times). At all depths studied, the number of bacteria fluctuated with time and by the end of the experiment the abundance of AODB at the top 5 cm was lower than the original.

Variation with depth

The number of AODB varied considerably with depth (Figure 4.29). At day 0, the highest abundance was recorded in the upper 15 cm (250 bact. g^{-1} wet weight of sediment) and decreased with depth (9.5 bact. g^{-1} wet weight of sediment). In general, this pattern of distribution changed after the addition of the oil, with the highest numbers recorded for sediment layers between 10 and 25 cm, followed by the top 5 cm and finally the 30 cm depth.

In the uncontaminated sediments, the number of bacteria with the capability to degrade sunflower oil represented 10% or less at 10-20 cm depth and 1% or less of the HAB for the remaining layers. These ratios increased after the addition of sunflower oil to up to 4.4% at the 1 cm depth and to 16% at the 5 cm layer. For the remaining depths a considerable increase was observed, with the number of AODB surpassing the number of HAB up to 6.5 times. These results indicate that sunflower oil is being utilised by the AODB.

4.5.4.2. Anaerobic Bacteria

4.5.4.2.1. Heterotrophic Anaerobic Bacteria (HAnB)

Temporal variation

The number of HAnB varied widely with time, from $3.5x10^2$ to $4.0x10^5$ CFU. g^{-1} wet weight of sediment (Figure 4.30 and 4.31). As observed for the aerobic bacteria, the number of HAnB increased with the addition of oil at all depths analysed. The initial

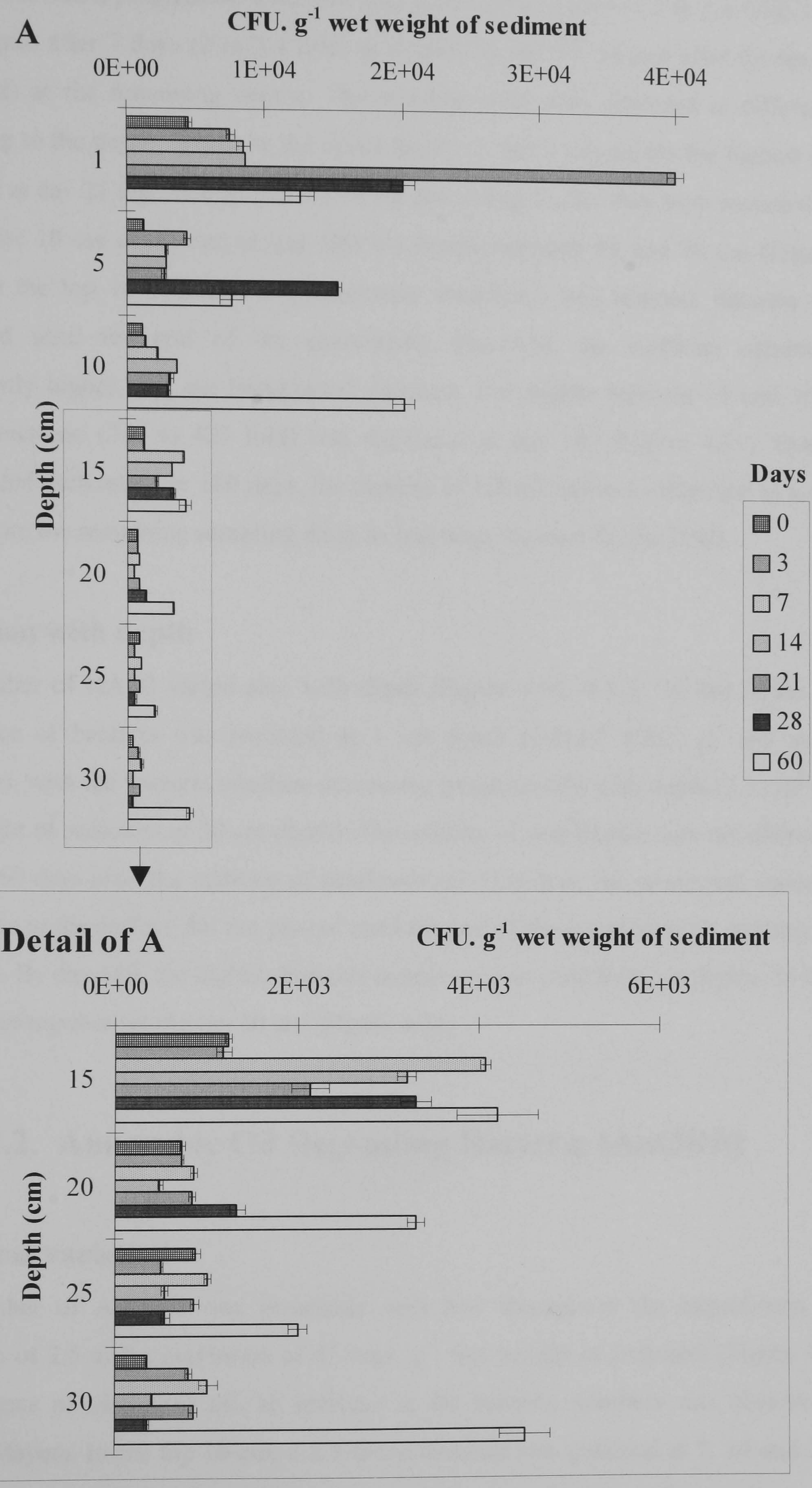


Figure 4.30. Number of heterotrophic anaerobic bacteria (± sd) present at the various sediment depths prior to (day 0), and up to 60 days after, the addition of sunflower oil.

increase showed a progression with time and depth: after 3 days (1.7 to 3.6 fold) in the top 5 cm depth, after 7 days (2 to 3.4 fold) at depths 10 and 15 cm and after 60 days (2.2 to 12.9 fold) at the remaining depths. The maxima were also observed at different times according to the depth. Whilst at the upper layers (1 and 5 cm depth) the highest numbers occurred at day 21 and 28 respectively, at the remaining depths they were recorded later: at day 60 for 10 cm depth and at day 180 for depths between 15 and 30 cm (Figure 4.30, 4.31). In the top 10 cm, after the maximum abundance was reached, bacteria numbers decreased until the end of the experiment. However, the numbers obtained were consistently higher than the background numbers. For depths between 15 and 30 cm the highest increase (216 to 427 fold) was registered at day 180 (Figure 4.31). Due to this accentuated increase after 180 days, the number of HAnB had to be depicted in a different figure from the remaining sampling days, as had been the case for the HAB.

Variation with depth

The number of HAnB varied also with depth (Figure 4.30, 4.31). At day 0, the greatest abundance of bacteria was recorded at 1 cm depth ($4.5x10^3$ CFU. g^{-1} wet weight of sediment), with the bacteria numbers decreasing progressively with depth ($3.5x10^2$ CFU g^{-1} wet weight of sediment at 30 cm depth). This pattern of distribution was not altered during the first 60 days after the addition of sunflower oil. However, the prominent concentration of bacteria at the surface did not prevail until the end of the experiment as was the case for the HAB. By day 180, the highest bacteria concentrations were found at depths 15 to 30 cm with lower numbers at the top 10 cm (Figure 4.31).

4.5.4.2.2. Anaerobic Oil Degrading Bacteria (AnODB)

Temporal variation

The number of AnODB was invariably very low throughout the experiment, with a minimum of 2.5 and a maximum of 45 bact. g^{-1} wet weight of sediment (Figure 4.32). In the presence of sunflower oil, an increase in the bacteria numbers was observed in all sediment layers. In the top 10 cm, a 2.5 times increase was obtained at 7, 14 and 28 days, with the lag time increasing with the depth. This growth led to the maximum values recorded, which were maintained until day 60 for the 1 cm depth, whereas at 5 and 10 cm

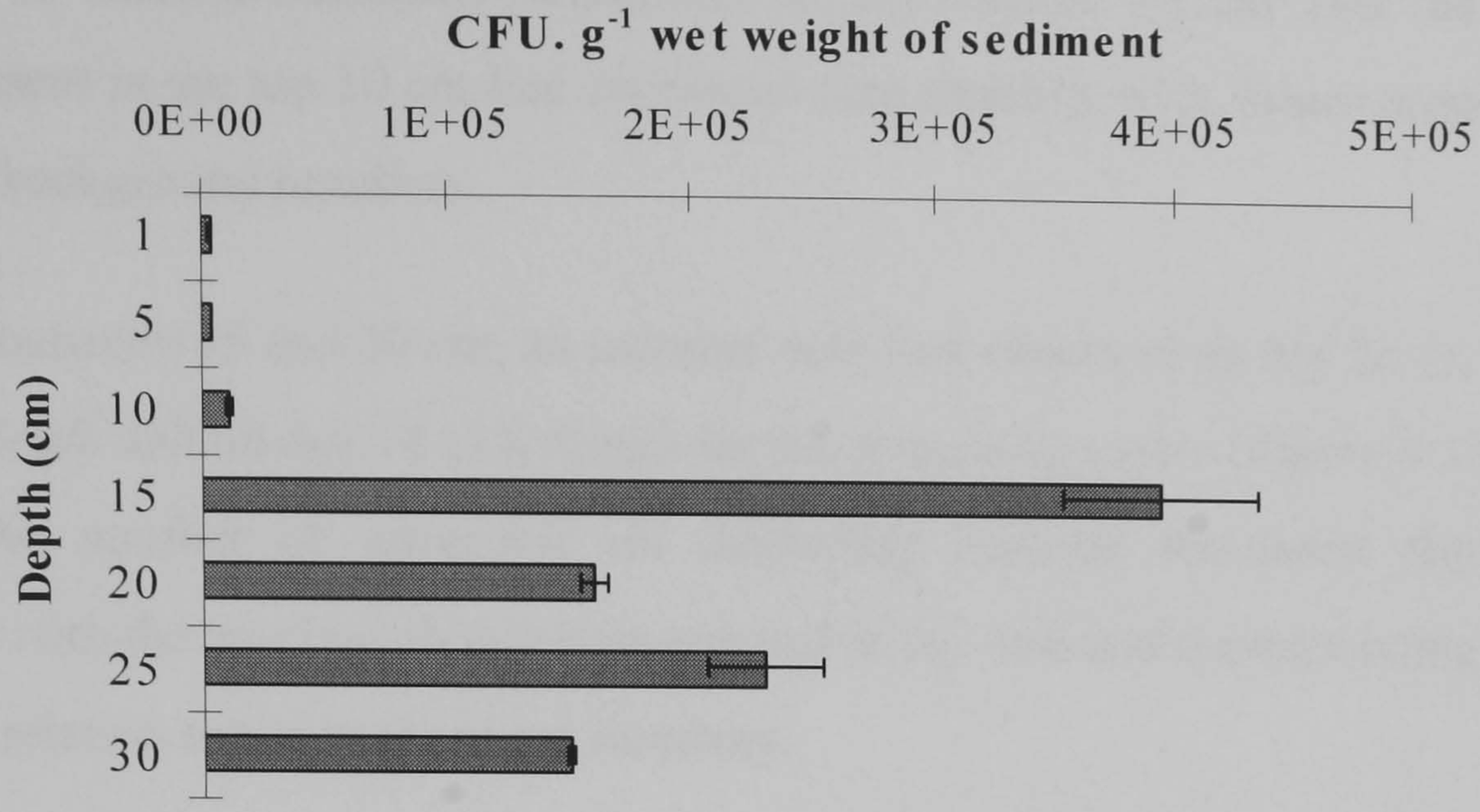


Figure 4.31. Variation with depth of the number of heterotrophic anaerobic bacteria (± sd) 180 days after the addition of sunflower oil.

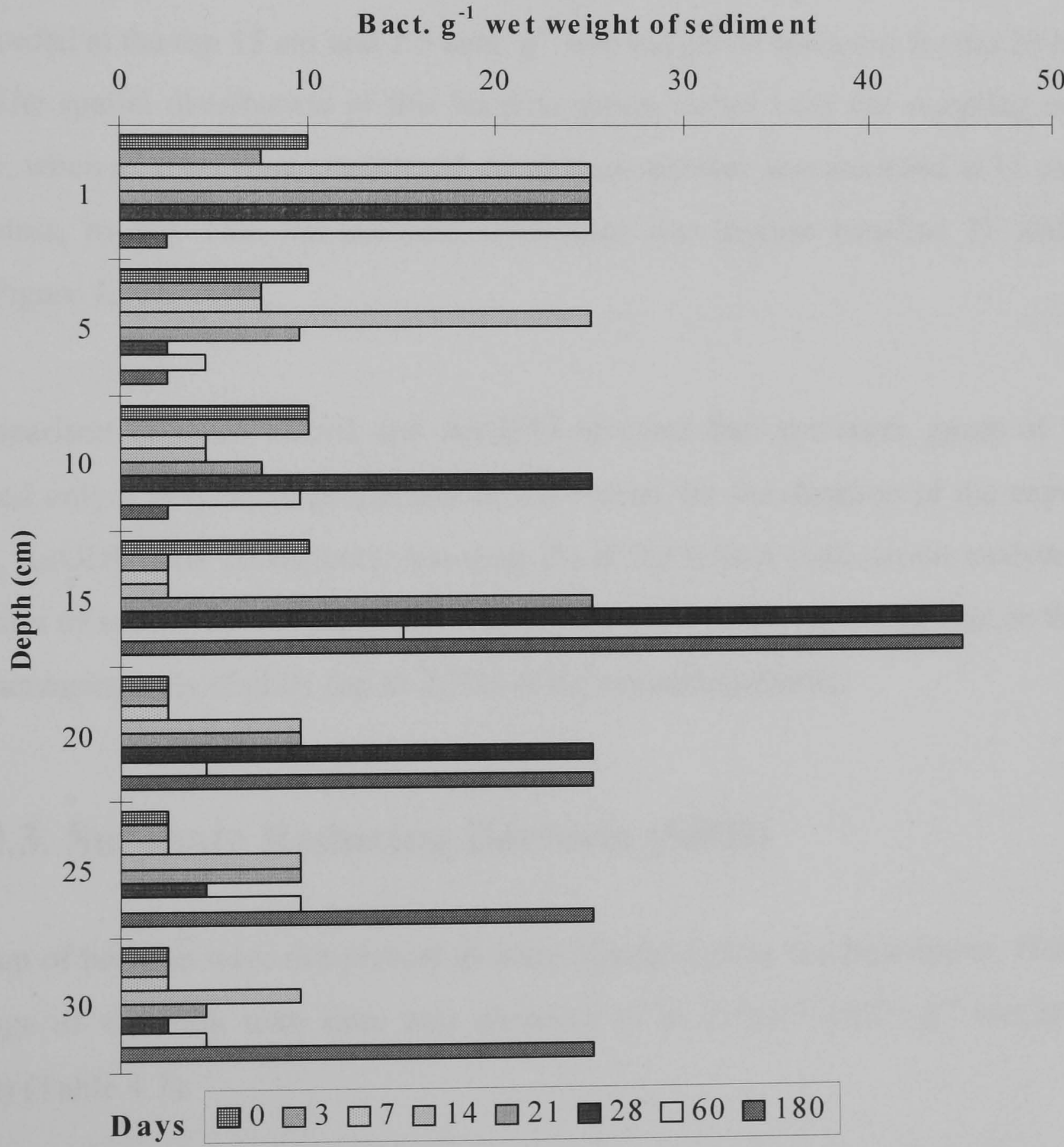


Figure 4.32. Number of anaerobic oil degrading bacteria present at the various sediment depths previous to (day 0) and after the addition of sunflower oil.

the number of bacteria fluctuated throughout the experiment. By day 180, the numbers of bacteria present in the top 10 cm had decreased considerably, with values corresponding to 25% of the background numbers.

For depths between 15 and 30 cm, an increase was first observed on day 21 (2.5 times) for the 15 cm depth and on day 14 (3.8 times) for the remaining layers (Figure 4.32). After this increase, the number of anaerobic oil degrading bacteria fluctuated throughout the experiment with the maxima abundances reached at day 180 and corresponding to a 10 fold increase in relation to the background numbers.

Variation with depth

In the uncontaminated sediments, spatial variability in the number of bacteria with capability to degrade sunflower oil was very limited: 10 bact. g^{-1} wet weight of sediment were recorded at the top 15 cm and 2.5 bact. g^{-1} wet weight of sediment for the 20 to 30 cm depths. The spatial distribution of this bacteria group varied with the sampling occasion. However, when all days were considered, the highest number was recorded at 15 cm depth. Nevertheless, by day 180, the bacterial abundance was highest between 15 and 30 cm depths (Figure 4.32).

The comparison between HAnB and AnODB revealed that the latter group of bacteria constituted only a very small proportion of the former for the duration of the experiment. At day 0, AnODB were consistently less than 1% of the HAnB at all depths analysed. After the addition of sunflower oil, the relative abundance of HAnB did not change in the upper 10 cm, increasing only slightly (up to 2.3%) at the remaining depths.

4.5.4.2.3. Sulphate Reducing Bacteria (SRB)

This group of bacteria were not present at many depths during the experiment. However, a wide range of variation with time was observed (0 to $1.9x10^4$ CFU. g^{-1} wet weight of sediment) (Table 4.7).

At day 0, SRB were only present at the 1 cm depth. With the addition of oil, their numbers increased considerably (64 times), with the highest concentrations recorded after 21 days,

after which numbers declined. By day 180, the numbers were 9.5 times higher than the original. At the depths of 5 and 10 cm a small number of bacteria was first observed after 14 and 28 days, respectively.

Table 4.7. Number of sulphate reducing bacteria (CFU. g^{-1} wet weight of sediment) present at the various sediment depths previous to (day 0) and after the addition of the sunflower oil.

Days	1 cm	5 cm	10 cm	15 cm	20 cm	25 cm	30 cm
0	27	0	0	0	0	0	0
3	85	0	0	0	0	0	0
7	4	0	0	0	0	0	0
14	10	4	0	0	0	0	0
21	1733	2	0	0	0	0	0
28	200	26	1	0	0	0	0
60	233	17	38	0	0	0	0
180	257	417	507	11000	8000	18733	8000

The coefficients of variation never exceeded 10% for 3 replicates.

However, representatives of SRB were only concomitantly present at all sediment depths after 180 days of the addition of oil (Table 4.7). In this instance, the maxima abundances were observed between 15 to 30 cm, as occurred for the heterotrophic aerobic and anaerobic bacteria.

4.5.5. Particle Size Analysis (PSA)

Variation with depth

The mud contents (< 63 μm) of each sediment layer at day 0 is illustrated in Figure 4.33. The spatial distribution pattern in this area was slightly different from that in the location used for simulating the linseed oil spill since, in the present case there was a less clear separation between upper and lower layers, with a continuous decrease in the mud fraction with depth (Figure 4.9).

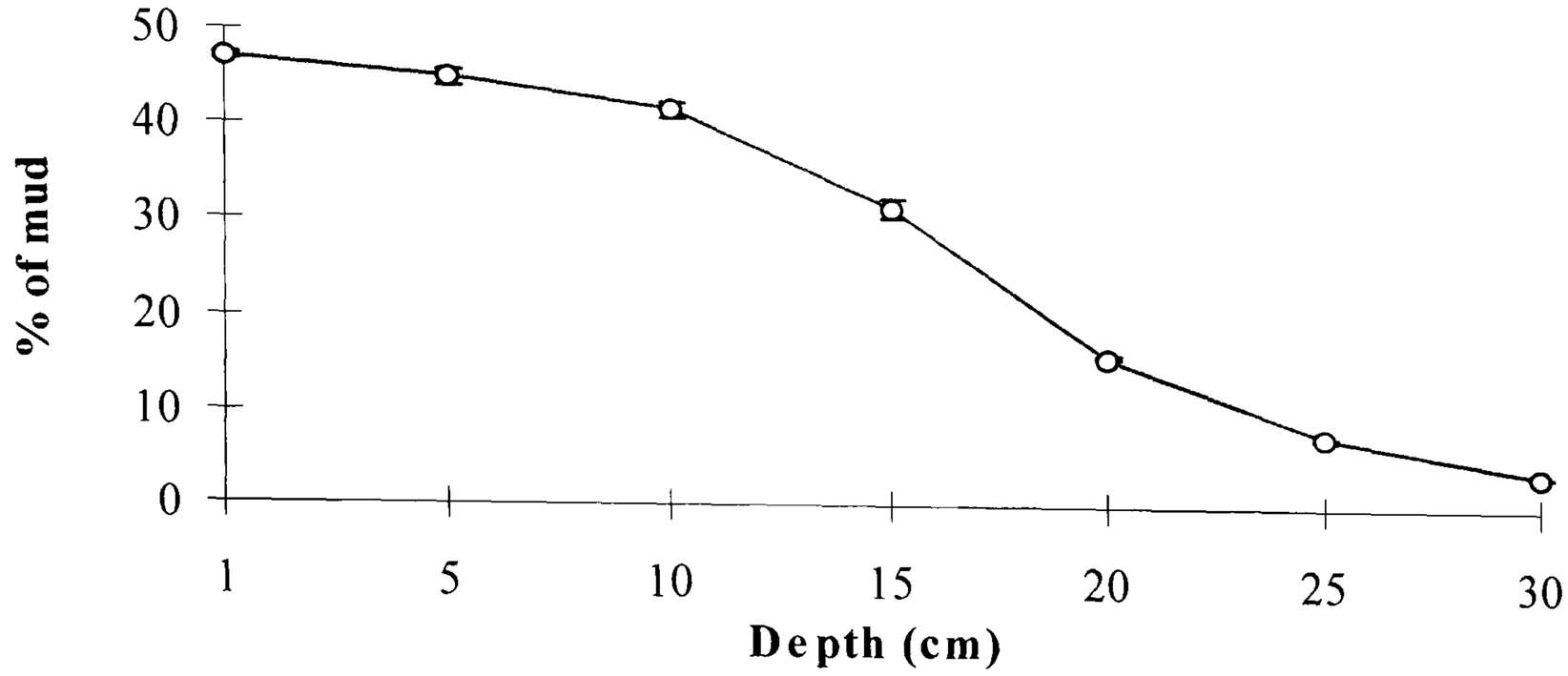


Figure 4.33. Variation with sediment depth of the percentage of particles <63μm, previous to the oil addition (day 0).

The amount of mud varied from 47% down to 31.5% in the top 15 cm and from 16% down to 3.6% between 20 and 30 cm depth.

This pattern of higher mud contents in the top than in the bottom layers was not altered by the addition of the sunflower oil (Figure 4.34).

Temporal variation

The presence of oil appears to have affected differently the various sediment layers analysed (Figure 4.34). Alterations in the mud:sand ratio were registered at 1 cm and between 20 and 30 cm depth, whilst for the depths of 5 and 15 cm no noticeable change was observed. In the upper layer (1 cm depth), the mud contents decreased continuously with the time, from the initial 47% down to 25.9%, recorded at day 180. In the lower layers (20 to 30 cm) the proportion of mud present in the sediments fluctuated throughout the experiment. Nevertheless, the mud fraction was, in most cases, smaller than that observed at day 0. After 60 and 180 days, no particles were retained in a 63μm sieve for depth 25 and 30 cm. The changes observed at these depths seem to happen as a result of the addition of sunflower oil since the background variability is much smaller (Table 4.4).

Comparison of the PSA between treated and untreated samples with hydrogen peroxide

As observed for linseed oil, the removal of organic matter increased the proportion of the mud fraction, in samples with and without oil (Figures 4.34 and 4.35). In the absence of oil,

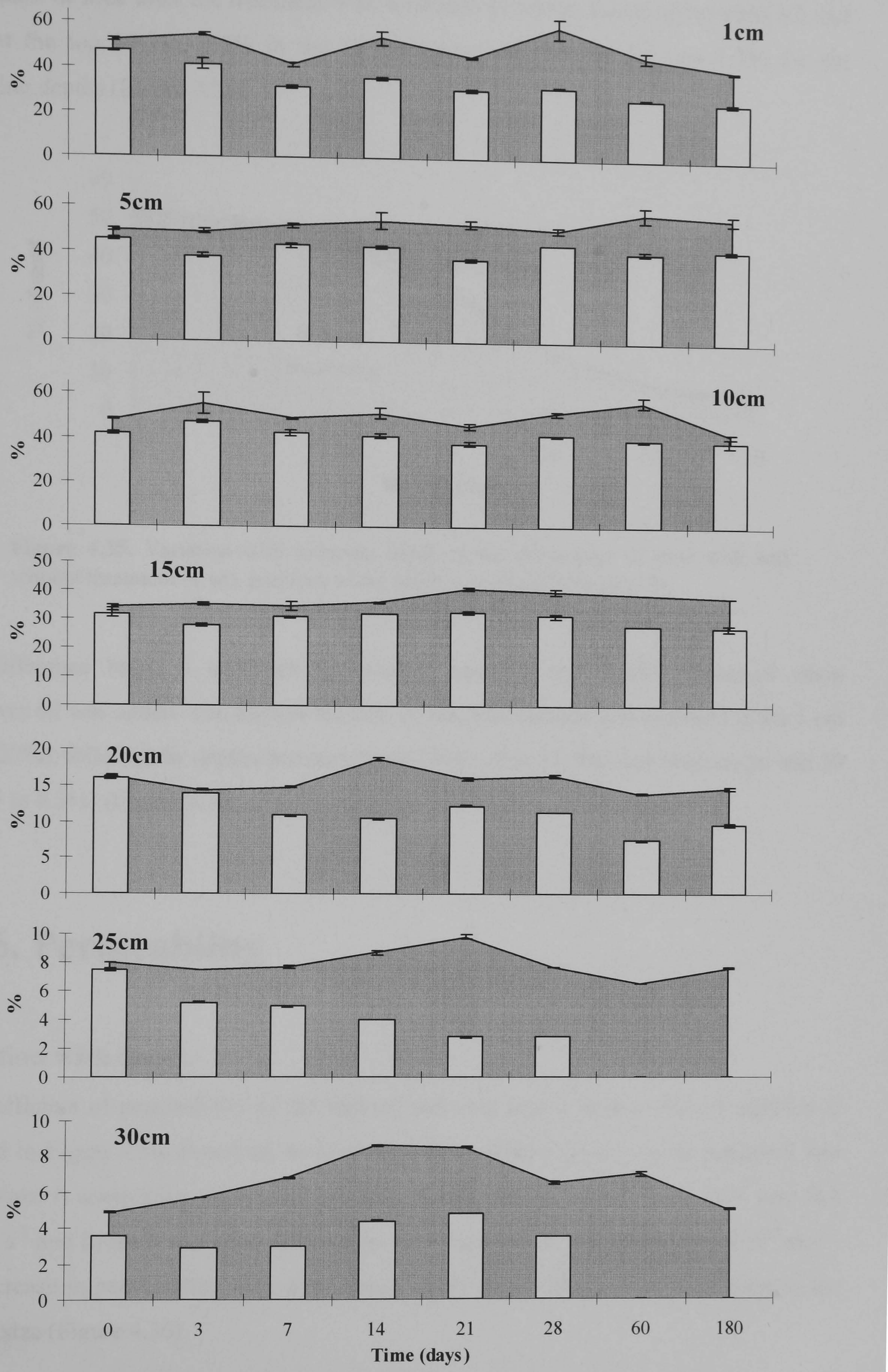


Figure 4.34. Percentage of mud (± sd) at the various sediment depths before (bars) and after (area) treatment of the sediments with hydrogen peroxide, prior to (day 0) and after the addition of sunflower oil.

the amount of mud after the treatment with hydrogen peroxide increased between 4.5 and 6.5% at the top 10 cm, 2.5% in the 15 cm layer and between 0.3 and 1.3% for the remaining depths (Figure 4.35).

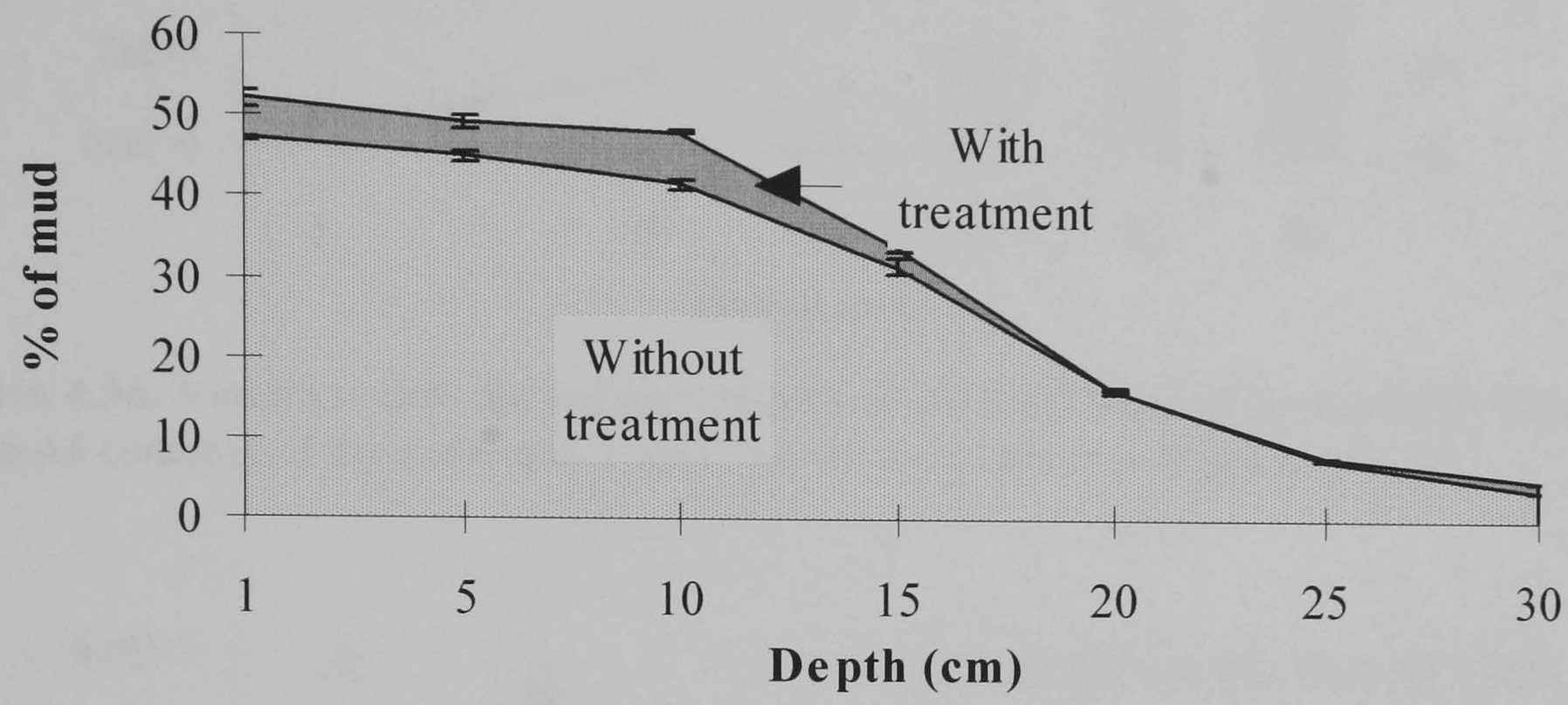


Figure 4.35. Variation with sediment depth of the percentage of mud with and without treatment (± sd), previous to the sunflower oil addition (day 0).

This difference between untreated and treated samples was further enhanced when sunflower oil was added. The highest increase in the mud content was recorded at the 1 cm depth (27%), followed by depths between 5 and 15 cm (3 to 17.7%) and between 20 and 30 cm (0.5 to 8.3%) (Figure 4.34).

4.5.6. Permeability

Variation with depth

The coefficient of permeability of the various sediment layers prior to the oil addition is depicted in Figure 4.36. Based on these results, the sediment layers can be separated into two groups: i) comprising the top 15 cm, with values varying between 2.6 $x10^{-7}$ and 8.1 $x10^{-7}$ m s^{-1} and ii) the lower 15cm with values varying between $2.3x10^{-5}$ and $9.0x10^{-5}$ m s^{-1}. This increase in permeability with depth has a direct relationship with the increase in the particle size (Figure 4.36).

With the addition of sunflower oil, the basic profile of low permeability in the upper layers and medium permeability in the deeper layers was not altered (Figure 4.37).

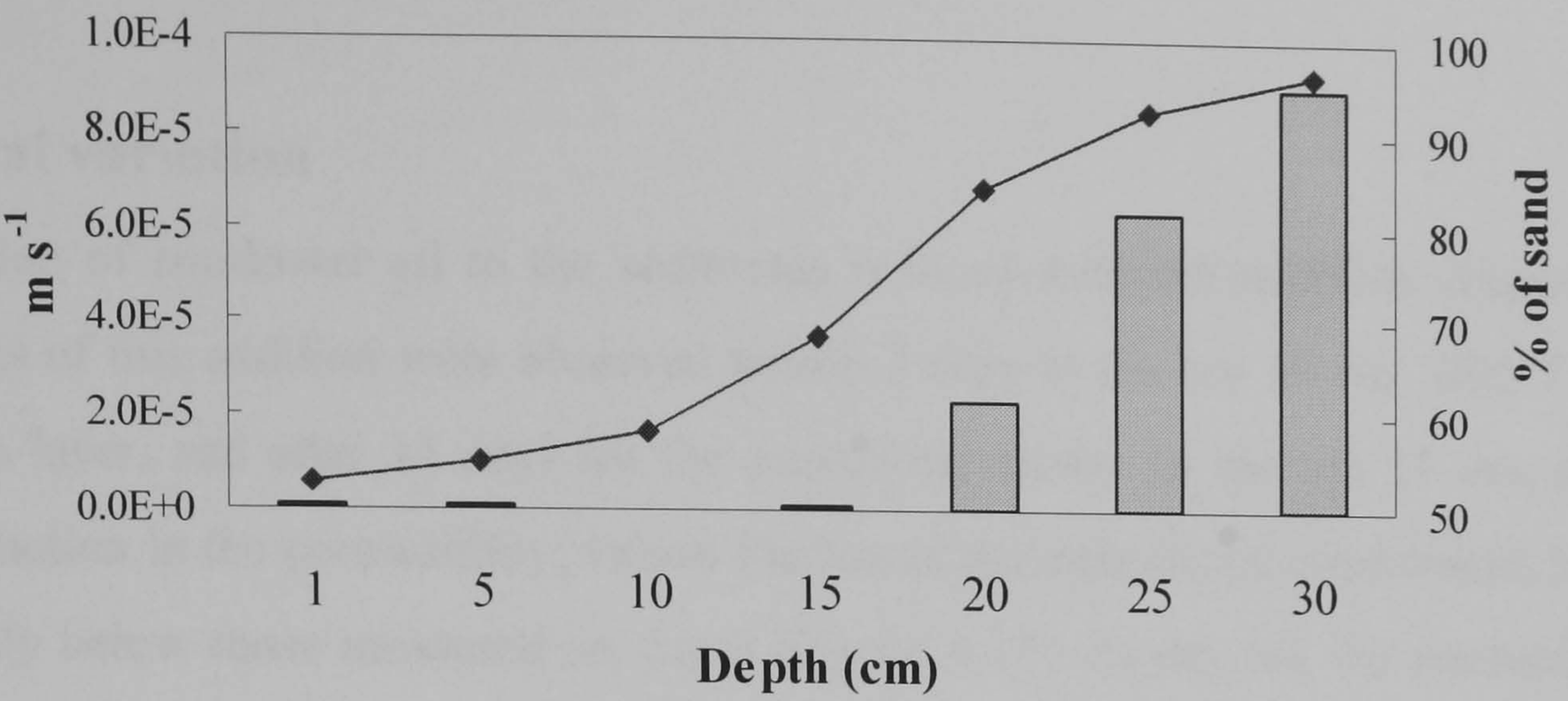


Figure 4.36. Variation with the sediment depth of the coefficient of permeability (bars) and sand contents of the sediments (line) prior to the sunflower addition (day 0).

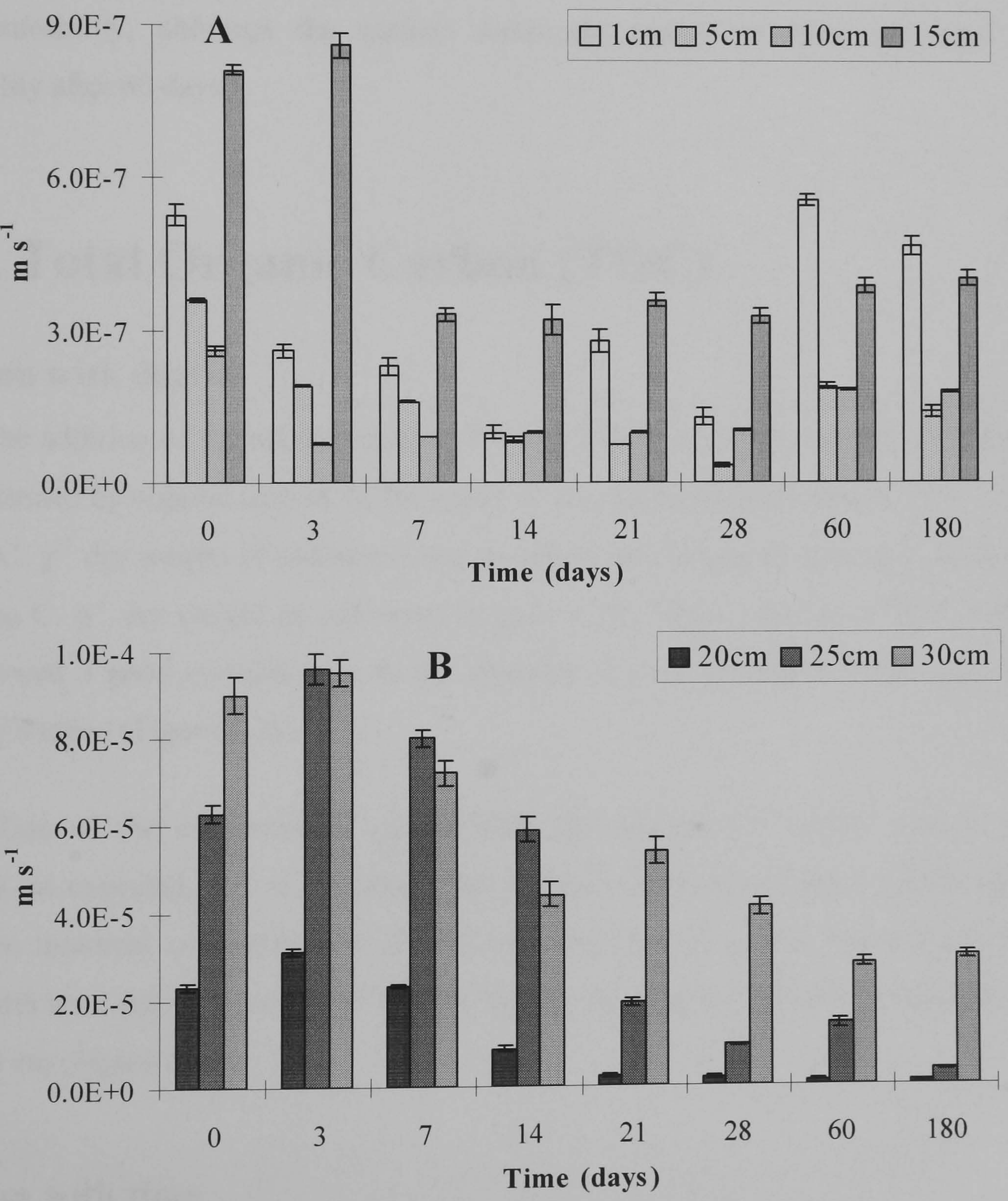


Figure 4.37. Coefficient of permeability (m s^{-1}, ± sd) for the sediment depths 1 to 15 cm (A) and 20 to 30 cm (B) prior to (day 0) and after the addition of sunflower oil.

Temporal variation

The addition of sunflower oil to the sediments reduced their permeability (Figure 4.37). The effects of this addition were observed within 3 days at the top 10 cm, after 7 days in the 15 cm layer, and after 14 days for the remaining depths. In the top 15 cm, after the initial reduction in the permeability, values fluctuated throughout the experiment, but were consistently below those measured on day 0 (Figure 4.37). At day 60, the permeability of these sediment layers appear to have increased, mainly that of the 1 cm depth. For depths between 20 and 30 cm, after the initial decline, permeability remained low until the end of the experiment. These results revealed that sunflower oil reduces both the mud and the sand permeability, although the surface sediments appear to have recovered their permeability after 60 days.

4.5.7. Total Organic Carbon (TOC)

Variation with depth

Prior to the addition of the oil, between 0.17% and 2.08% of the dry weight of sediments was constituted by organic carbon. In the upper 10 cm, the highest amount of TOC (16.5 to 20.8 mg C. g^{-1} dry weight of sediment) was recorded, decreasing continuously with depth (to 1.7 mg C. g^{-1} dry weight of sediment) (Figure 4.38). This variation of TOC with the depth showed a good correlation with the contents in mud present in each layer of the sediments studied (Figure 4.38).

The addition of the oil increased considerably the amount of organic carbon in the sediments, as expected, and at the same time changed the original spatial profile (Figure 4.39). The maximal concentrations of TOC observed at the upper layers became less evident with time and by days 60 and 180 the highest amount had shifted to depths between 15 and 30 cm (Figure 4.39).

Variation with time

An increase in the organic carbon concentrations was observed within 3 days after the addition of the oil at all depths studied, except in the 15 cm layer (14 days) (Figure 4.39).

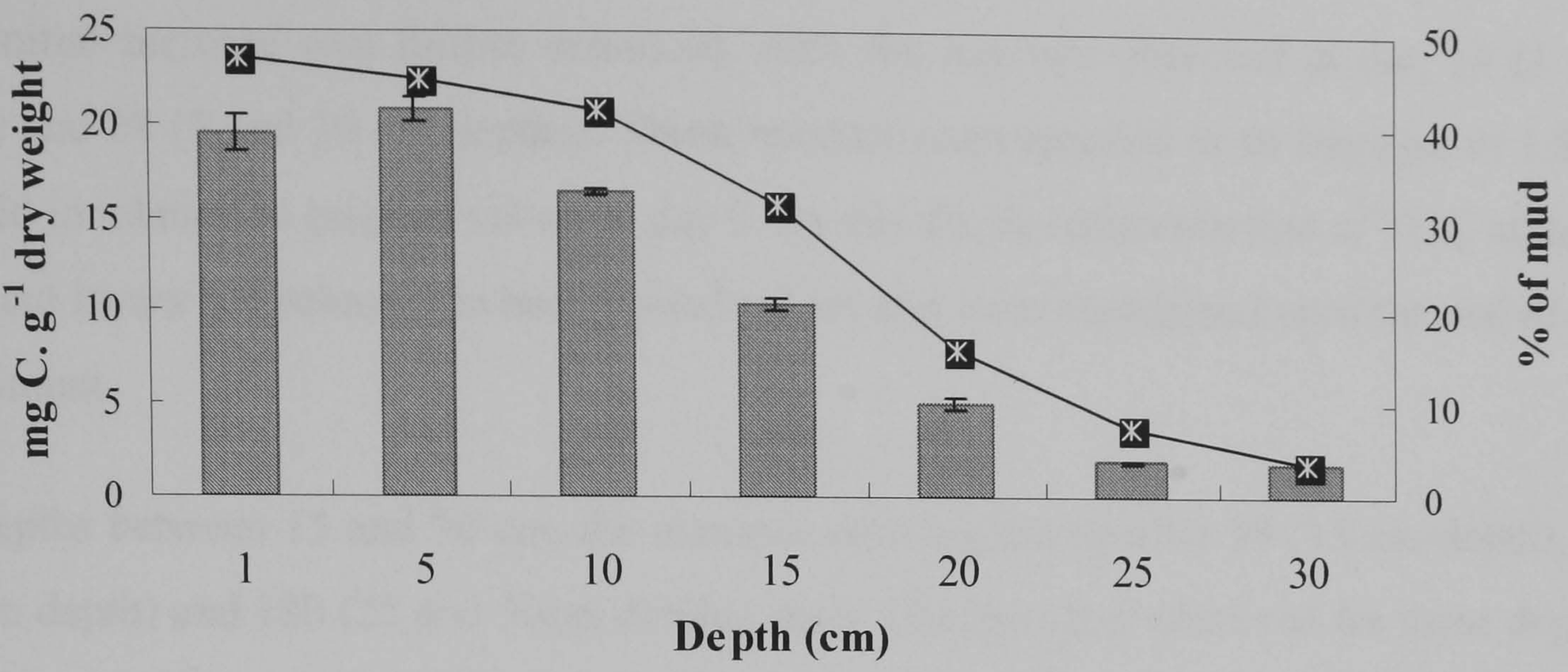


Figure 4.38. Variation with sediment depth of total organic carbon (bar, ± sd) and the percentage of mud (line), prior to the sunflower oil addition (day 0).

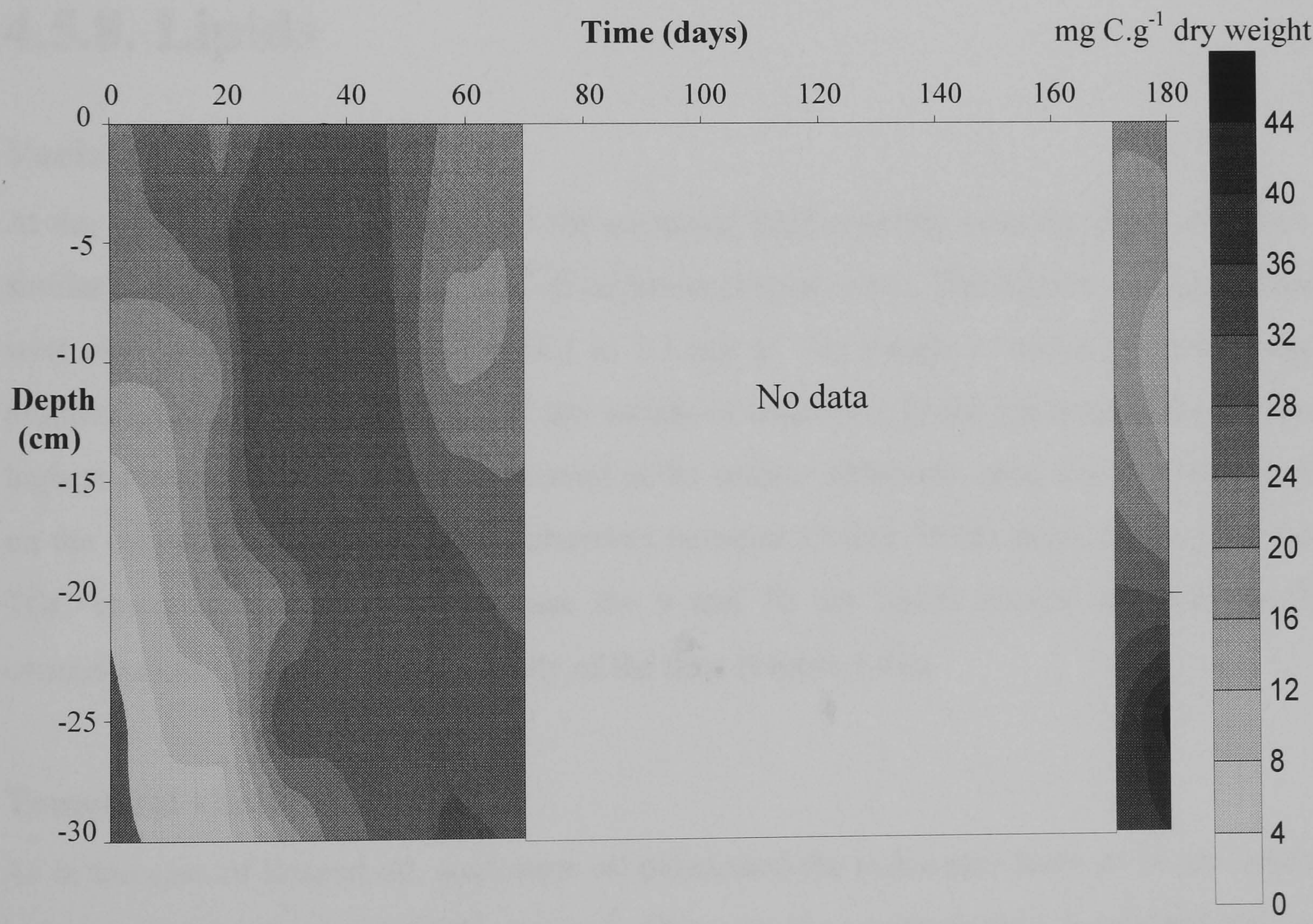


Figure 4.39. Total organic carbon (mg C. g^{-1} dry weight) for the sediment depths prior to (day 0) and after the sunflower oil addition.

This initial increase was further enhanced, with the maxima observed at day 14 (1 cm depth) and 28 (5 and 10 cm depths). These maxima corresponded to an increase of 1.8 to 2.8 fold in relation to original values at day 0. By day 60, the concentration of TOC at these sediment layers had returned to background values and were maintained until the end of the experiment.

For depths between 15 and 30 cm, the maxima were registered after 28 (15 cm depth), 60 (20 cm depth) and 180 (25 and 30cm depths) days. The increases observed for these depths were much higher (up to 24 times) than that in the top 10 cm. Again, contrasting with the upper 10 cm, the concentrations were still high when the experiment finished (especially for the depths between 20 and 30 cm).

4.5.8. Lipids

Variation with depth

At day 0, the profile of variation of the extracted lipid contents with the depth was very similar to that observed for the TOC distribution (Figure 4.40). The highest concentrations were observed in the upper 10 cm (1.1 to 1.3 mg. g^{-1} dry weight of sediment) decreasing progressively with depth (0.2 mg. g^{-1} dry weight of sediment). In the presence of the oil, the highest concentrations were still observed at the surface sediments until day 21. From then on the concentration maxima were observed between 15 and 30 cm depth as seen for the TOC contents. However, in this case the 5 and 10 cm layers always had very small concentrations of lipids, independently of the time (Figure 4.41).

Temporal variation

As in the case of linseed oil, sunflower oil penetrated the sediments down to 30 cm depth in 3 days or less, at a rate of 10^{-7} cm^2 sec^{-1}. This rate of penetration was maintained for the duration of the experiment.

In the top 10 cm, the highest concentration of lipids (21.1, 9.2 and 4.3 mg. g^{-1} dry weight of sediment for 1, 5 and 10 cm depths, respectively) was recorded at day 3. Thus, for these depths there was not a progressive increase in the lipids contents with time (Figure 4.41).

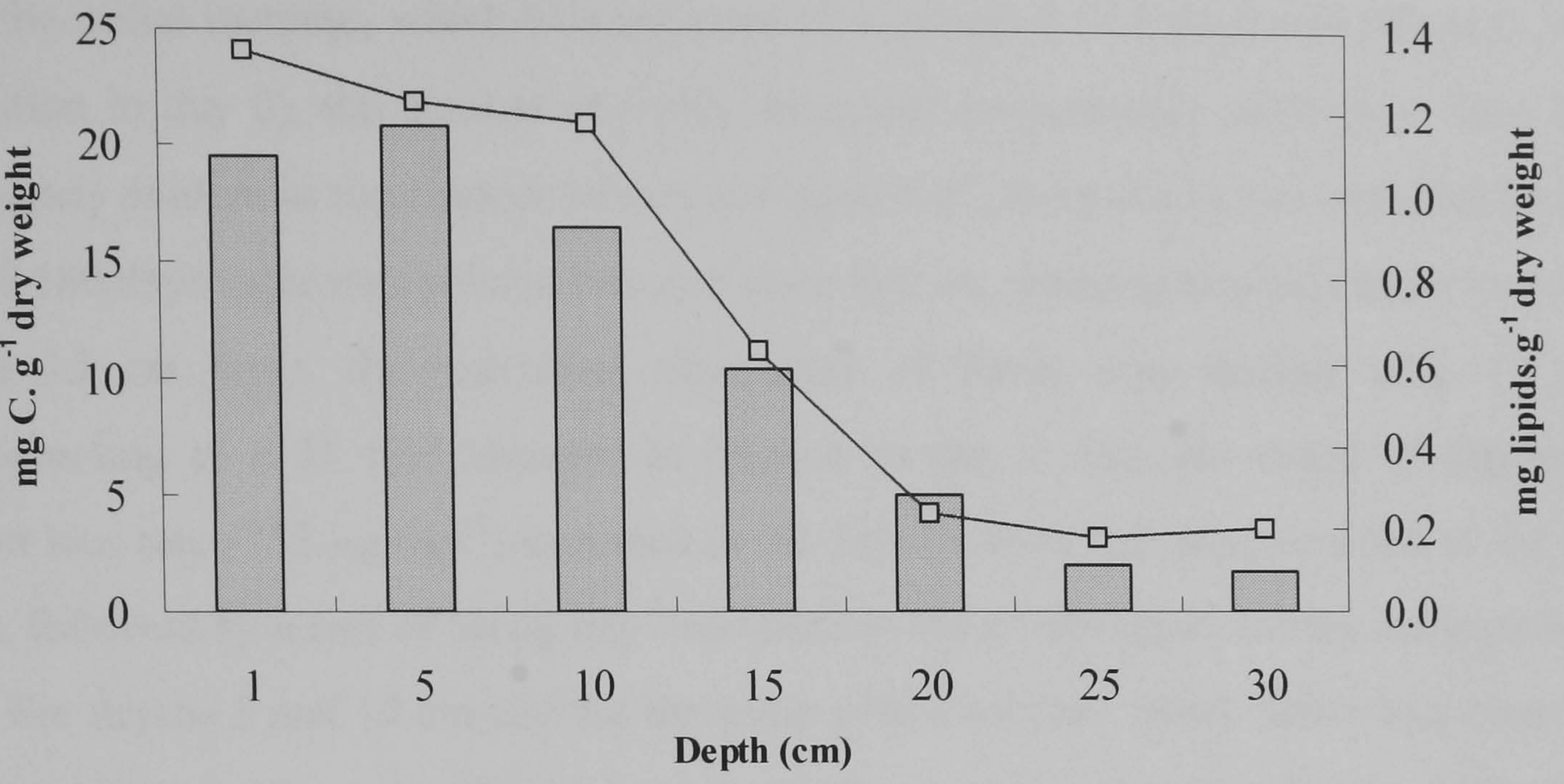


Figure 4.40. Variation with sediment depth of the lipids (line) and the total organic carbon (bars), prior to the addition of sunflower oil (day 0).

mg lipids.g^{-1} dry weight

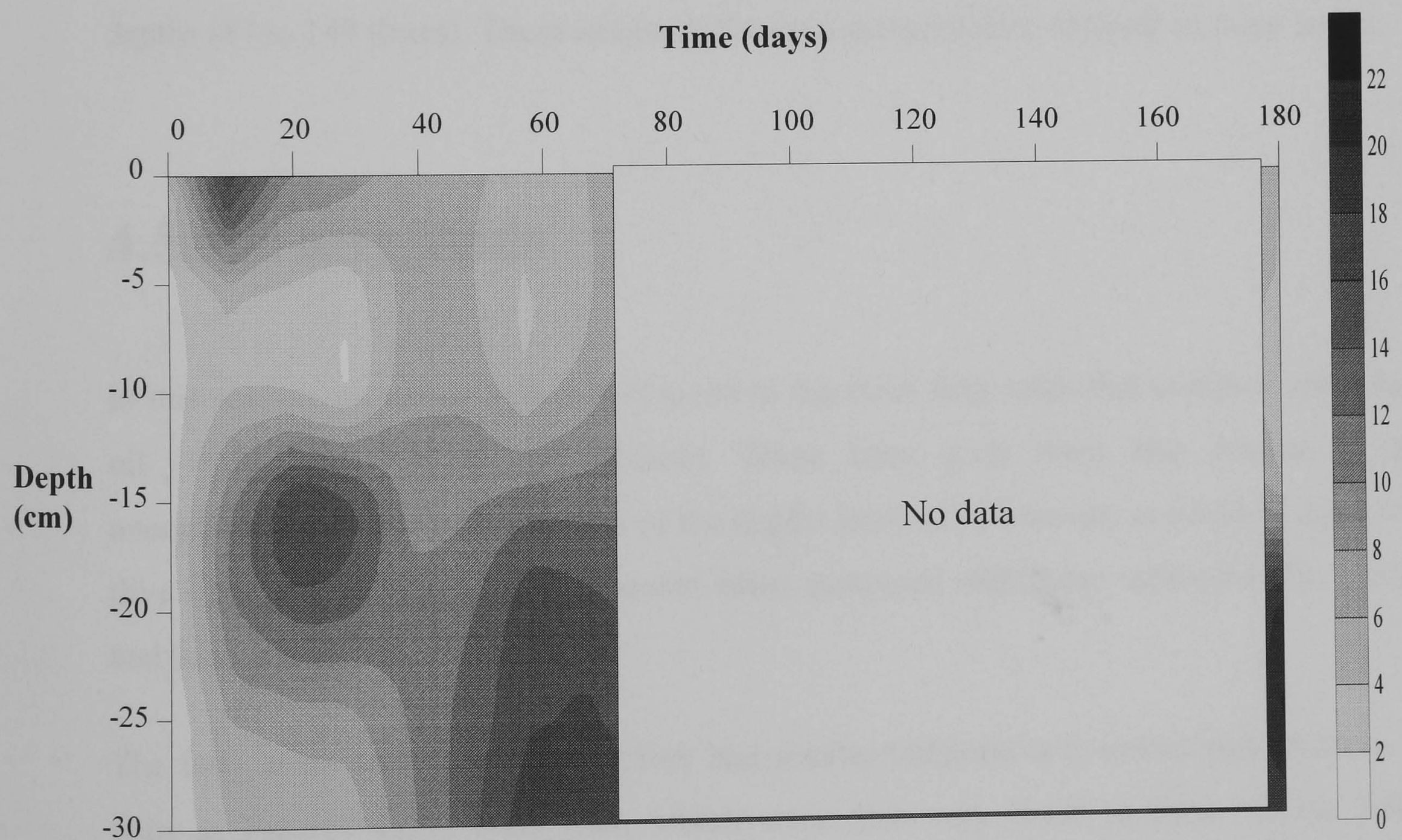


Figure 4.41. Lipids (mg lipids.g-1 dry weight) present at the various sediment depths prior to (day 0) and after the addition of sunflower oil.

After the initial increase, which was not marked at depths 5 (7.5 fold) and 10 cm (3.7 fold in relation to day 0), the amount of lipids decreased continuously with time. This is not particularly evident in the contour shown in Figure 4.41, since the values obtained between 60 and 180 days were interpolated because there was no sampling between these two dates. In the 15 cm layer, the maximum abundance of lipids was reached after 14 days, corresponding to a 31 fold increase in relation to day 0. For this range of depths, the highest loss rate (753 μg day^{-1}) occurred in the first 21 days and was recorded at the 1 cm depth, followed by a rate of 90 μg day^{-1} recorded in the 15 cm depth for the same period of time. For depths 5 and 10 cm and for the same period of time, much lower loss rates were registered (13 to 30 μg day^{-1}) which were similar to those observed for the 1 cm depth, between day 21 and 180.

As in the case of the upper layers, between 20 and 30 cm depth the initial increase in lipids was observed within 3 days of the addition of oil, but in this case the maxima were recorded only after 180 days and corresponded to the highest increase observed for all depths (87 to 149 times). These results indicate an accumulation of lipids in these layers.

4.5.9. Fatty acids

In this section, particular attention is given to the main fatty acids that compose sunflower oil (16:0, 18:0, 18:1ω9 and 18:2ω6). These fatty acids were also present in the uncontaminated sediments for most of the depths analysed. However, as could be expected, they were present in negligible amounts when compared with those measured after the oil had been added.

The fatty acids 16:0, 18:0 and 18:1ω9 had similar temporal and spatial distributions, as seen in Figures 4.42, 4.43, 4.44, which were also very close to those of the lipids distribution profile (Figure 4.41). These fatty acids were recorded down to 30 cm depth within 3 days, and their vertical diffusion was of approximately 10^{-7} cm^2 sec^{-1}. In the surface sediments, the highest concentrations of these fatty acids were recorded between 3 and 14 days, after which a decline with time was observed. From Figures 4.42, 4.43 and 4.44 an increase between the 60^{th} and the 180^{th} day appears to have occurred.

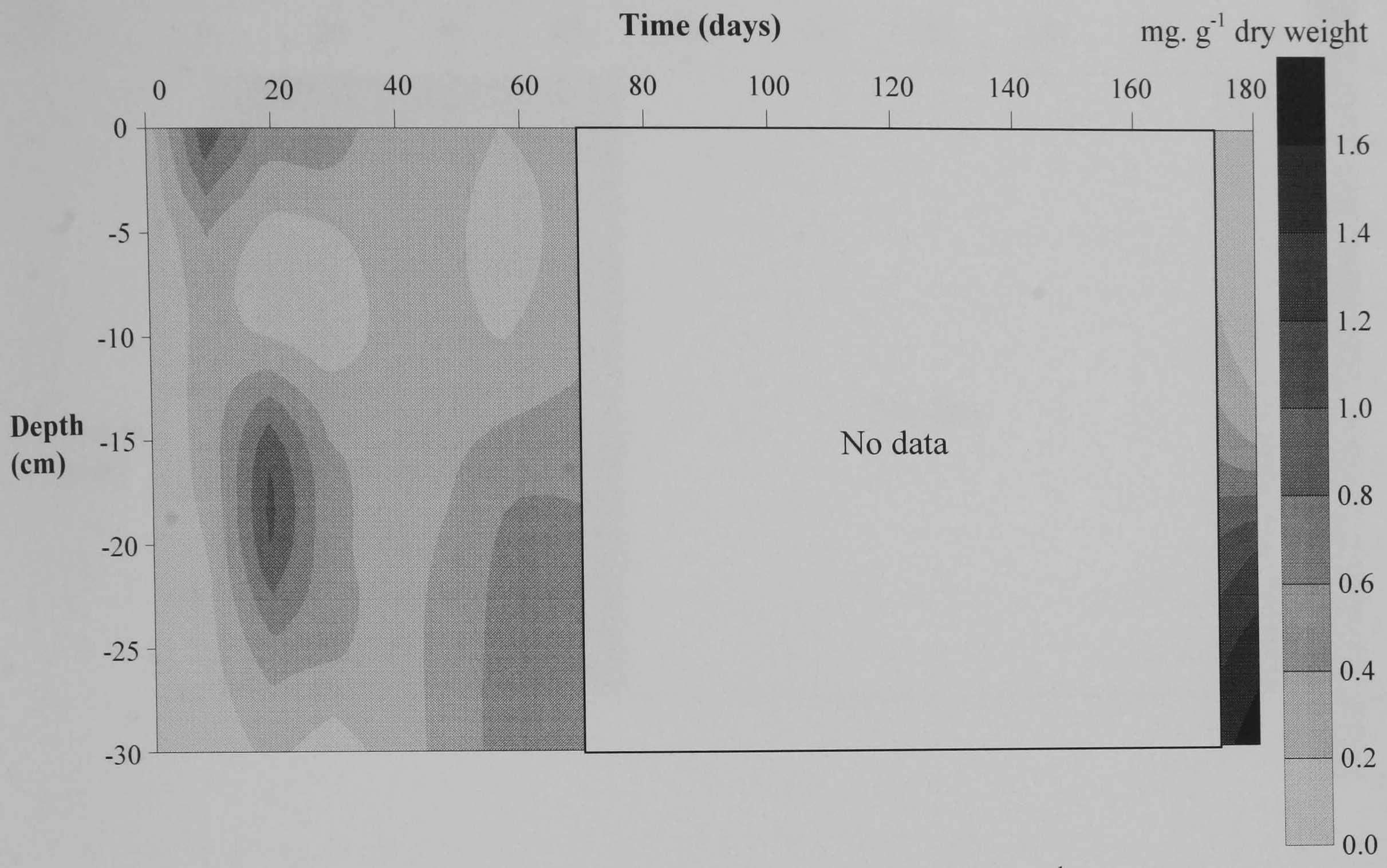


Figure 4.42. Temporal distribution with sediment depth of 16:0 (mg. g^{-1} dry weight of sediment) after the addition of sunflower oil.

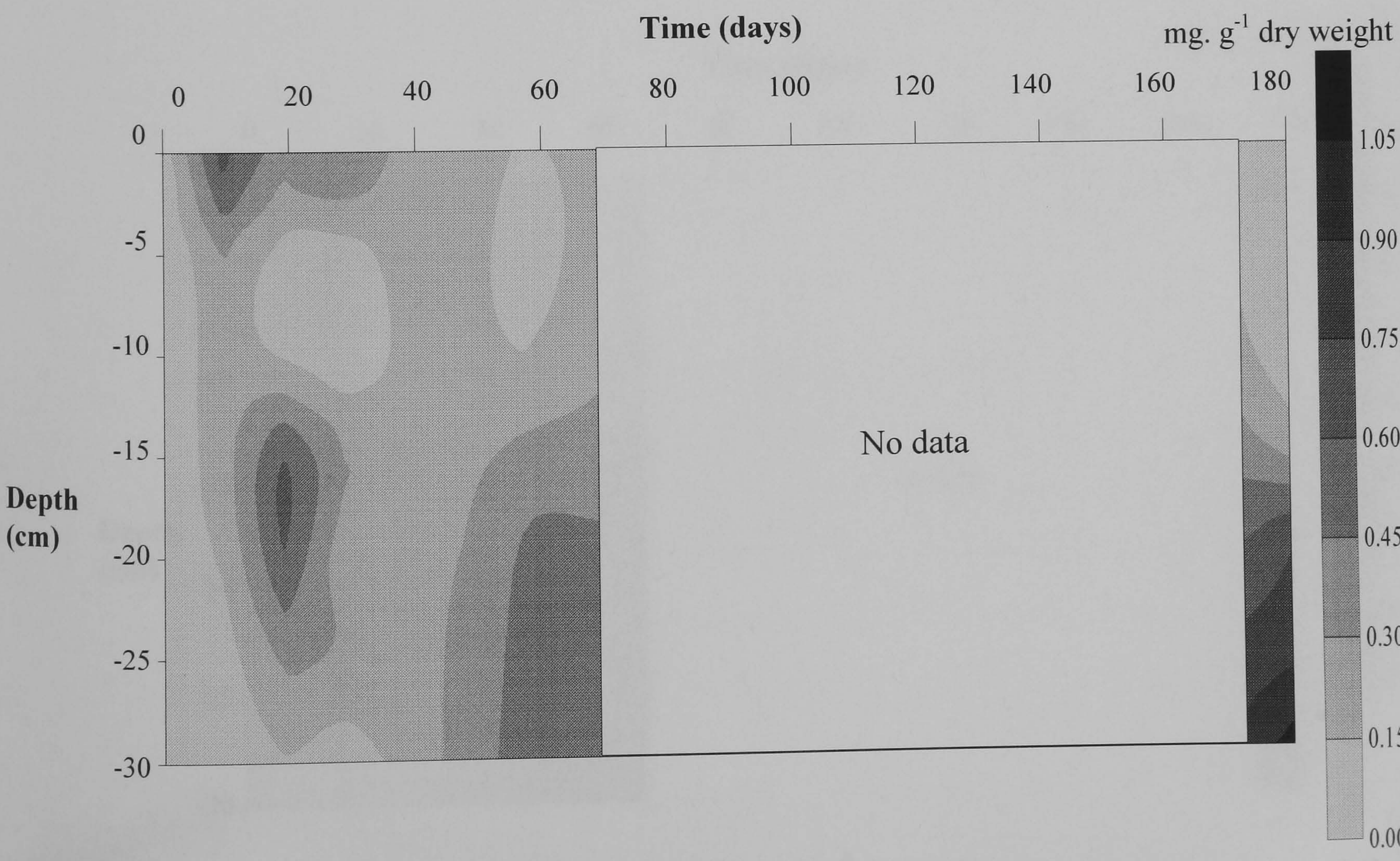


Figure 4.43. Temporal distribution with sediment depth of 18:0 (mg. g^{-1} dry weight of sediment) after the addition of sunflower oil.

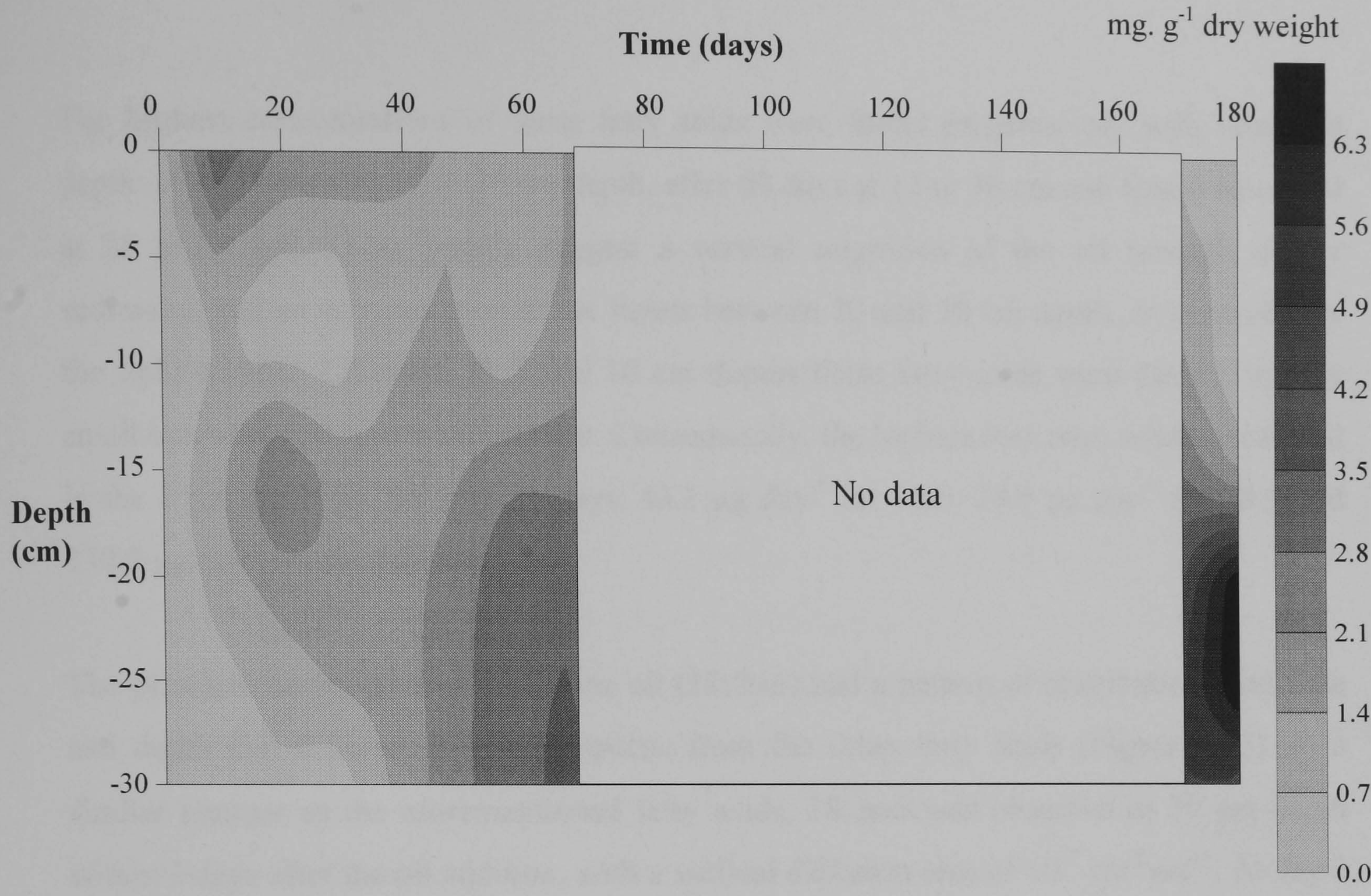


Figure 4.44. Temporal distribution with sediment depth of 18:1ω9 (mg. g^{-1} dry weight of sediment) after the addition of sunflower oil.

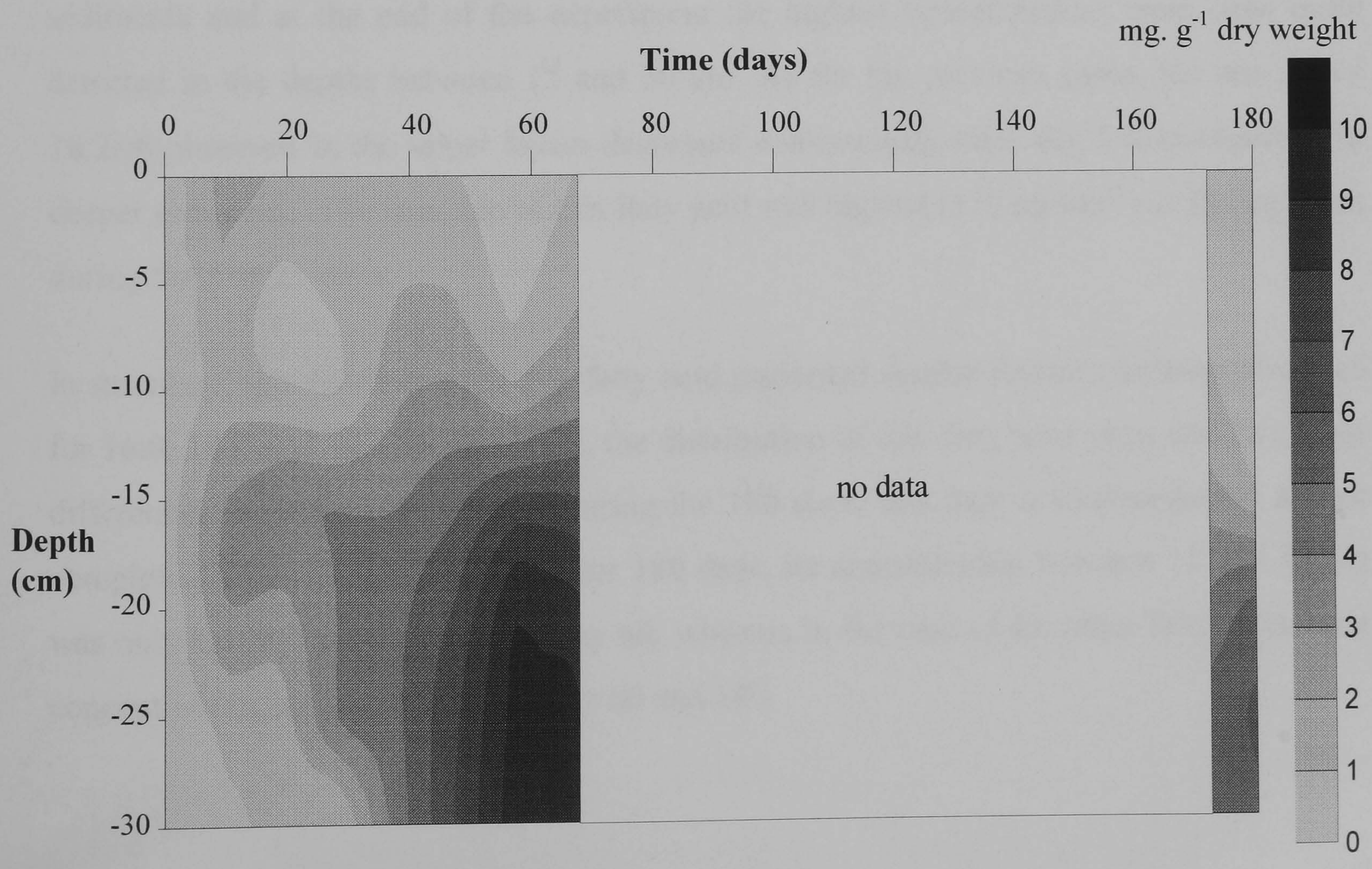


Figure 4.45. Temporal distribution with sediment depth of 18:2ω6 (mg. g^{-1} dry weight of sediment) after the addition of sunflower oil.

The highest concentrations of these fatty acids were found progressively with time and depth: after 21 days at 15 to 20 cm depth, after 60 days at 15 to 30 cm and finally after 180 at 20 to 30 cm. These results suggest a vertical migration of the oil towards deeper sediments and an accumulation at the layers between 20 and 30 cm depth, as observed for the lipid content. Likewise, at 5 and 10 cm depths these fatty acids were present in very small amounts during the experiment. Consequently, the highest loss rates where measured in the 1 cm depth for the first 21 days: 43.1 μg day^{-1} for 16:0; 29.9 μg day^{-1} for 18:0 and 130.7 μg day^{-1} for the 18:1ω9.

The principal component of sunflower oil (18:2ω6) had a pattern of distribution with time and depth that differed, in some aspects, from the other fatty acids (Figure 4.45). In a similar manner as the aforementioned fatty acids, 18:2ω6 was observed at 30 cm depth within 3 days after the oil addition, with a vertical diffusion also of 10^{-7} cm^2 sec^{-1}. At day 3 and until day 7, its maximum concentration was measured in the top 5 cm decreasing continuously with depth. However, after 7 days this pattern changed, and until day 21 the highest amounts were observed at 15 cm depth. This fatty acid penetrated further into the sediments and at the end of the experiment the highest concentrations were once more detected in the depths between 15 and 30 cm. As for the previous cases, the amount of 18:2ω6 observed in the upper layers decreased continuously after day 3 to accumulate in deeper sediments. The loss rate of this fatty acid was highest (110 μg day^{-1}) in the top 5 cm during the first 21 days.

In summary, the distribution of this fatty acid presented similar features to those observed for 16:0, 18:0 and 18:1ω9. However, the distribution of this fatty acid presented two main differences in relation to those: i) during the 180 days, this fatty acid disappeared almost completely from the top 5 cm ii) after 180 days, its concentration between 15 and 30 cm was only half of that registered at day 60, whereas in the case of the other fatty acids their concentrations increased between day 60 and 180.

'New' fatty acids

The fatty acids analysis, in a similar manner as for linseed oil, revealed the presence of octadecenoic and octadecadienoic acids, not original components of the sunflower oil or present in the sediments prior to the addition of sunflower oil.

One octadecenoic acid was identified as 18:1ω7 and the other will be referred as 18:1 (1). 18:1 (1) had a lower retention time than 18:1ω9. These two fatty acids had a different pattern of distribution with time and depth. The 18:1ω7 was recorded in the sediments mainly during the first 14 days, and in very small concentrations at all depths analysed (0.9 to 2.1 % of the total). Between 14 and 180 days this fatty acids was occasionally observed only at the deeper sediment layers (Table 4.8). On the other hand, 18:1 (1) appeared to be present whenever 18:1ω7 was absent. The fatty acid 18:1 (1) was first observed on day 14 at the 1 cm depth and progressively down to 15 cm depth, but never below. The presence of this fatty acid was recorded until the end of the experiment and was prominent between day 28 and 180 in the top 5 cm (Table 4.8). In the top layers, the percentage of 18:1 (1) increased continuously with time.

Table 4.8. Amount of 18:1ω7 (ω7) and 18:1 (1) [(1)] (weight %) present in the sediments after addition of sunflower oil.

Depth	1		5		10		15		20		25		30	
Days	ω7	(1)	ω7	(1)	ω7	(1)	ω7	(1)	ω7	(1)	ω7	(1)	ω7	(1)
3	1.6	-	1.0	-	1.3	-	1.3	-	1.2	-	1.4	-	1.1	-
7	2.1	-	1.6	-	1.5	-	1.5	-	1.2	-	1.9	-	1.3	-
14	0.9	4.9	1.4	-	1.2	-	1.3	-	1.2	-	1.1	-	1.1	-
21	-	4.3	-	8.8	-	2.0	-	-	-	-	-	-	-	-
28	-	24.5	-	12.0	-	4.4	-	2.0	1.2	-	1.4	0.3	-	-
60	-	24.9	-	27.4	-	3.8	-	10.0	1.4	-	1.4	-	1.5	-
180	-	33.0	-	30.6	-	10.7	-	7.0	-	-	-	-	-	-

Once more the GC-MS analysis of the FAMEs was insufficient for the complete identification of the 'new' octadecadienoic acids, thus these fatty acids, will be once more be referred as: 18:2 (1), 18:2 (2), 18:2 (3), 18:2 (4) and 18:2 (5). The fatty acid 18:2 (5) was the only 'new' 18:2 with a retention time lower than that of the 18:2ω6. Of these 5 octadecadienoic acids only two of them ,18:2 (1 and 3) were present in high concentrations, with a maximum of 10.7 and 12% of the total, respectively. The fatty acids 18:2 (5 and 2)

were present in very small amounts (less than 1% of the total), whereas 18:2 (4) was at the most 2.4 % of the total. Some of the 'new' 18:2 fatty acids were first observed at day 14 at the 1 cm layer and from then on they increased in concentration with time and depth [mainly the 18:2 (1 and 5)].

The profile of variation with time and depth of the 'new' octadecadienoic acids is illustrated in Figure 4.46 by the most representative of them 18:2 (3). The highest concentration was registered after 28 days at the 1 cm depth (0.8 mg. g^{-1} dry weight of sediment), after which a decline with time at the top sediment and an increase in the concentration with depth were registered.

Fatty Acids Relative Abundance

The increasing importance of the 'new' fatty acids, as a group, with time, is illustrated in Figure 4.47, as well as the comparison of the percentage of the fatty acids present in the sediments after contamination with sunflower oil and their percentages in the raw oil. In the raw sunflower oil, not only the main fatty acids already mentioned were present but also small amounts of 14:0, 18:3ω3, 20:0, 20:1ω5; 22:0 and 24:0. These fatty acids represented 1.73% of the total fatty acids composition of sunflower oil and in this Figure were represented as a group which is referred as the *others*.

Within 3 days after the addition of the sunflower in the sediments, the oil composition had changed slightly at the top 1 cm whereas at the other depths remained unchanged. These modifications corresponded to a decrease in the 18:2ω6, and an increase in the remaining fatty acids. By day 21, these alterations had extended to all sediment depths and at the depths between 15 and 25 cm the group of *others* appeared to have increased. However, at days 28 and 60 these substantial changes in composition were only observed in the top 5 cm layers. Still, some 'new' 18:1 and 18:2 fatty acids were recorded at the 10 and 15 cm depth. By the end of the experiment these changes had intensified at all sediment depths with a maximum decrease of 18:2ω6 recorded at all depths (53.5% in the 1st cm and 21.7% at the 30 cm depth). To the decrease in 18:2ω6 corresponded an increase in all the other fatty acids, including the 'new', which suggests degradation of 18:2ω6 to form other fatty acids.

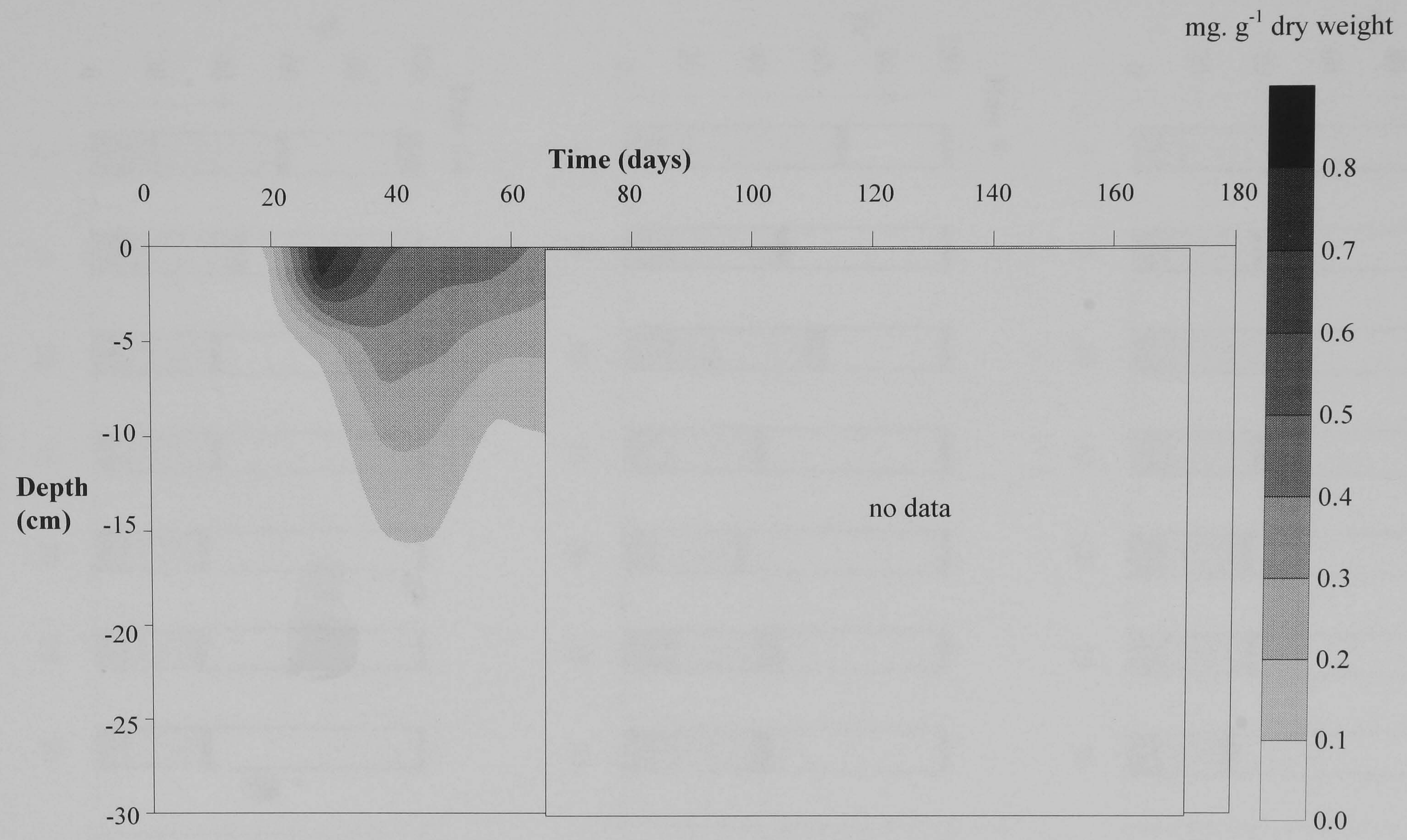


Figure 4.46. Temporal distribution with sediment depth of a 'new' 18:2 (mg. g^{-1} dry weight of sediment) after the addition of sunflower oil.

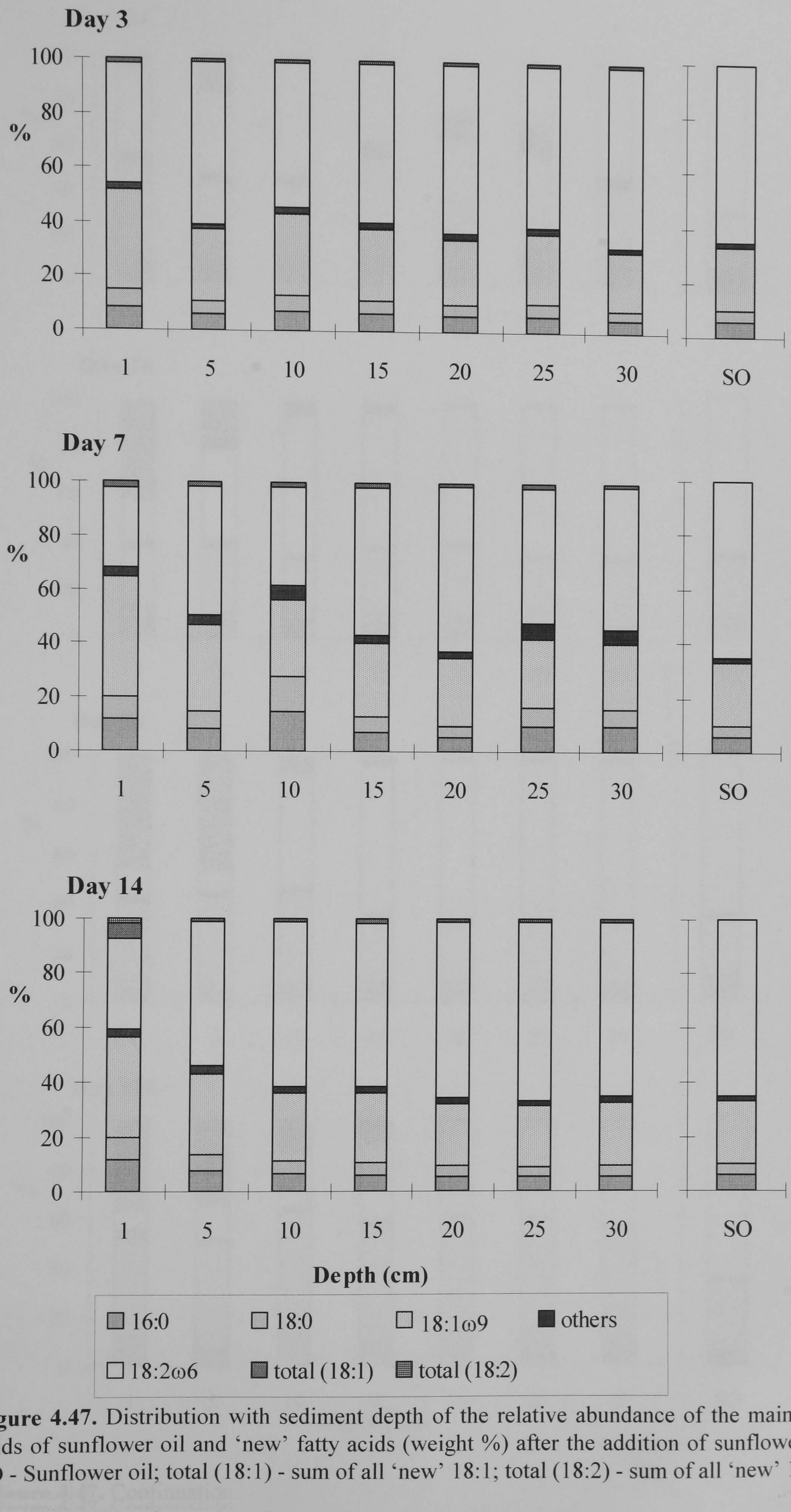


Figure 4.47. Distribution with sediment depth of the relative abundance of the main fatty acids of sunflower oil and 'new' fatty acids (weight %) after the addition of sunflower oil. SO - Sunflower oil; total (18:1) - sum of all 'new' 18:1; total (18:2) - sum of all 'new' 18:2.

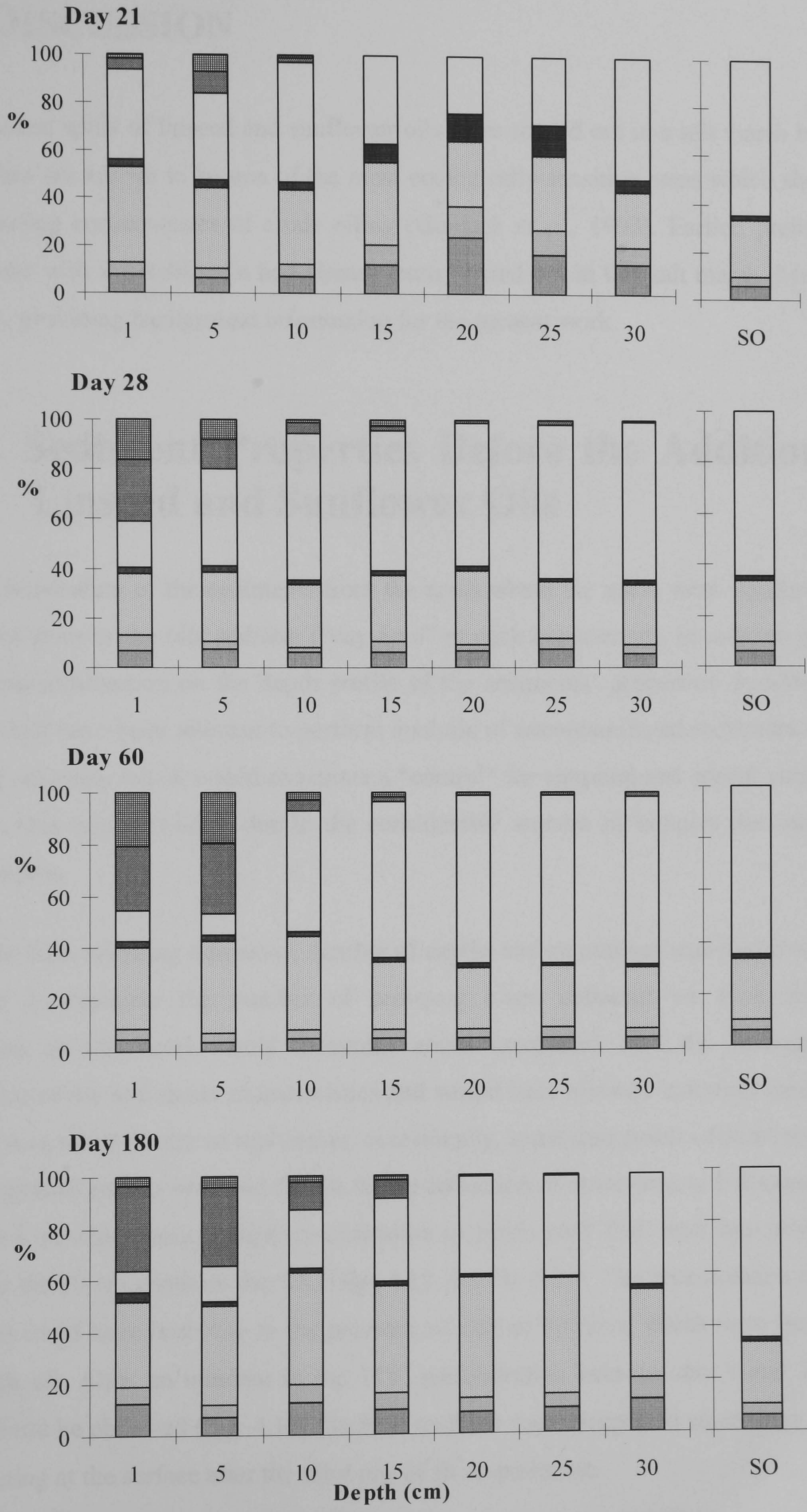


Figure 4.47. Continuation.

4.6. DISCUSSION

The simulated spills of linseed and sunflower oils were carried out in a salt marsh because salt marshes are known to be one of the most ecologically sensitive areas which show the longest lasting consequences of crude oiling (Gunlack *et al.,* 1993). Earlier, preliminary experiments with vegetable oils had already been carried out in this salt marsh (Mudge *et al.,* 1995), providing background information for the present work.

4.6.1. Sediment Properties Before the Addition of Linseed and Sunflower Oils

The characterisation of the sediments from the areas where the spills were simulated was carried out prior to the oils addition ("day zero" of each experiment), in order to provide background information on the depth profile of the sediments' properties. In addition to this, it would have been relevant to perform analysis of uncontaminated sediments at each sampling occasion, which would constitute a "control" for temporal and spatial variability. However, this was unfeasible due to the considerable amount of samples that such task would involve.

Due to the high sampling frequency, number of depths and parameters analysed it was also untenable to replicate the number of sediment cores collected on each occasion. Replication at this level would minimise errors associated with the heterogeneous distribution of the sediments characteristics and would have allowed statistical analysis of the data. As a result of lack of replication, occasionally, some data points contrast markedly with the general pattern observed for the whole collection of observations. For example, in the linseed oil experiment, a high concentration of lipids (and TOC and fatty acids) was evident at the 10 cm depth on day 28 (Figs. 4.15, 4.17 to 4.22). The accumulation of oil at this depth could have been due to the presence of animal burrows, which were frequently filled with oil. Also, an increase in the TOC concentration between day 3 and 7 at the surface could be observed (Fig. 4.15). Such an increase was unexpected since there was no oil remaining at the surface after the third day of the experiment.

The interpretation of the results must also take into account the heterogeneity of the sediments, which was shown in an assay to study the spatial variability of some of the sediments properties in an area of the salt marsh (section 4.3). Variability within the sediments was to be expected since there are a number of orders of heterogeneity in the sediment ecosystem (Jørgensen 1977 a, b): first, the vertical stratification from the normally oxidised upper layers through successively more reducing regions to the most anaerobic zones; second, the region of bioturbation when the activities of burrowing animals leads to oxygen penetration into deeper anaerobic zones; and third, the development of anaerobic microsites in aerobic regions. Is also necessary to consider the temporal heterogeneity (Nedwell & Gray, 1987), which in this particular case will be particularly relevant in the sunflower experiment, as will be discussed later.

Grain size

According to the grain size distribution, both areas where the spills of linseed and sunflower oils were simulated could be separated into two groups: i) from the surface to 15cm depth and ii) from 15 to 30 cm depth (Figs. 4.9, 4.33). The first group was characterised by a higher amount of fine particles than the latter, which contained mainly sand sediments. In the sunflower oil experimental area, the 15 cm sediment layer appeared to have intermediate grain size between these two groups but with values closer to the former (Fig. 4.33).

This separation between upper and lower 15 cm depths could also be drawn from other sediment characteristics studied, accordingly to other works which showed that sediments that differ in grain size also differ in many other properties such as organic matter contents, bacteria numbers, Eh, pH, permeability, *etc.* (Dale, 1974; Parsons *et al,* 1984; Bergamaschi *et al.,* 1997).

Permeability

The permeability of a sediment is influenced by its particle size distribution and especially by the presence/absence of fine particles (Head, 1982). The smaller the particles, the smaller the voids between them, and therefore an increase in the resistance to flow of water with decreasing grain size. As expected, the upper 15 cm sediment layers of the studied area had low to very low permeability (of the order of 10^{-7} m s^{-1}) and between 20 to 30 cm

depth, permeability increased continuously in accordance with the increase in sand contents (Fig 4.48). The permeability values for depths between 20-30 cm in the linseed oil area varied between 10^{-6} to 10^{-5} m s^{-1} (considered to be medium to low permeability) and in the sunflower area around 10^{-5} m.s^{-1} (considered to be medium permeability) (Head, 1982).

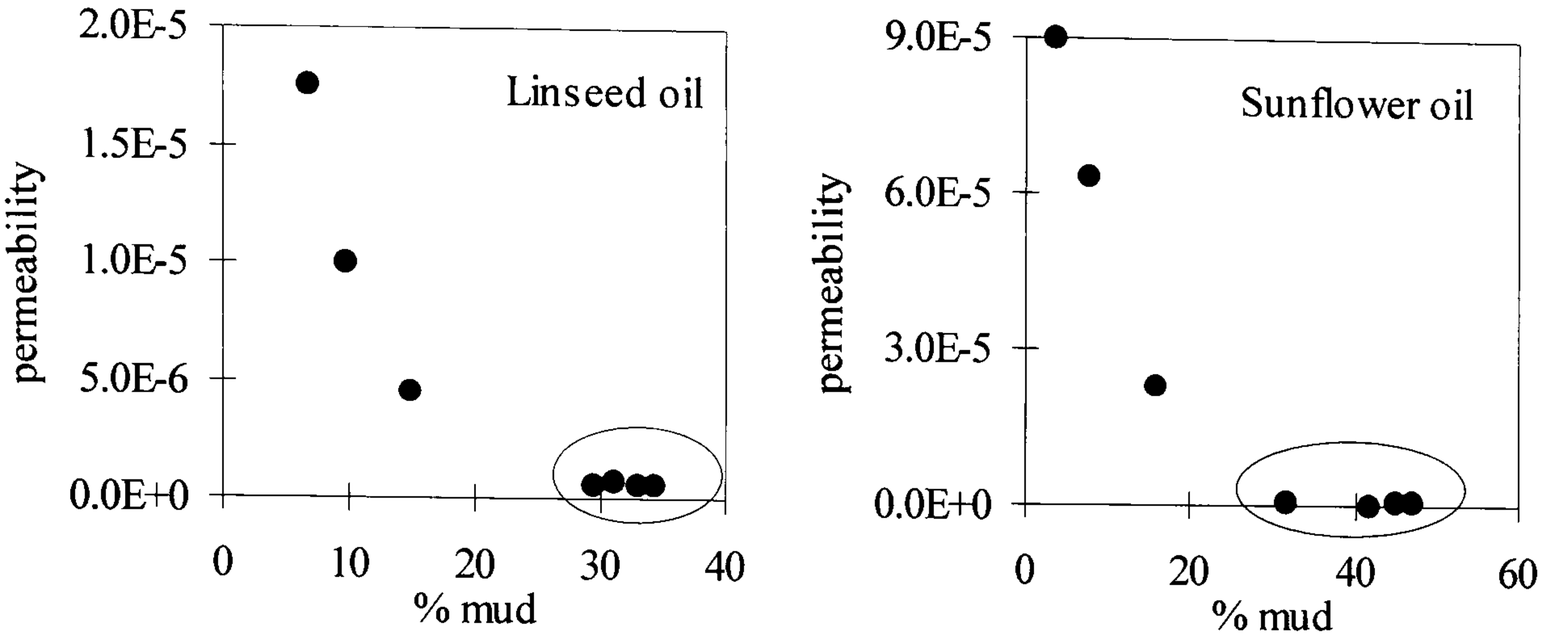


Figure 4.48. Variation of the relationship between grain size (% mud) distribution and coefficient of permeability (m s^{-1}) with sediment depth, prior to the linseed and sunflower oils experiments (day zero). Points enclosed by the ellipse refer to results for depths between 1 to 15 cm.

Organic Carbon

Grain size variations are the principal determinants of the content and distribution of organic carbon in the sediments (Bergamachi *et al.,* 1997). In the two sampled areas, a direct relationship between mud & TOC and mud & lipid contents was found. In Figure 4.49 the relationship between mud contents and TOC is illustrated. The two previously established fractions were maintained, with the 15 cm layer (in the sunflower oil area) located between the upper and the lower groups. In general, the upper fraction (1 to 15 cm - richer in mud sediments) contained 7 times more TOC than the lower one (15 to 30 cm). The presence of high contents of TOC in the upper 15 cm was not unexpected since marshes receive large inputs of organic matter directly into the sediments (Howarth, 1993). Moreover, a greater amount of organic matter was found adsorbed to particles in the muddy than in the sandy sediments (Figs 4.11, 4.35). This is possibly due to the presence of clay particles (in the top 15 cm) which provide surfaces on which solute molecules can be adsorbed. Such adsorption may be reversible or irreversible and provides a buffered store

of material which may be used and mobilised by the sediment microflora (Wimpenny & Peters, 1987).

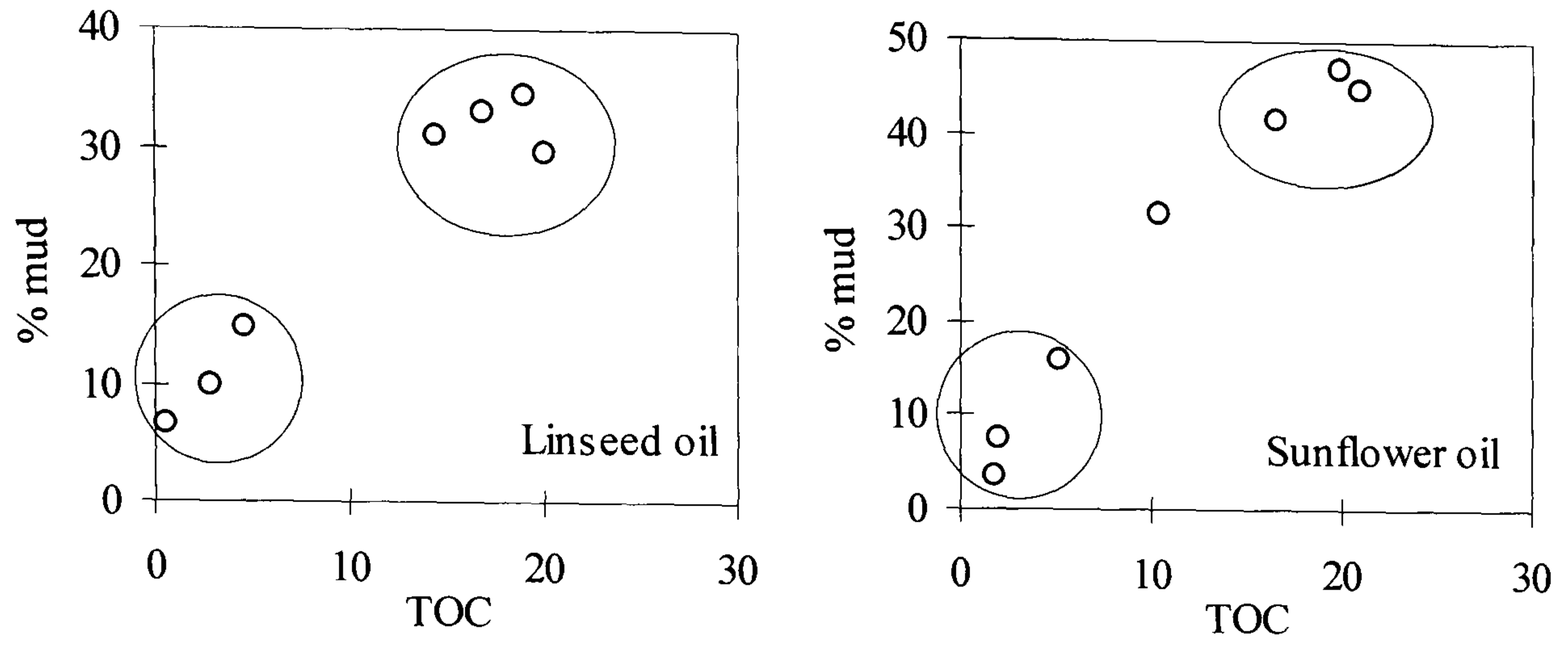


Figure 4.49. Variation of the relationship between grain size (% mud) distribution and total organic carbon (mg C g^{-1} dry weight sediment) with the sediment depth, prior to the linseed and sunflower oils experiments (day zero). The ellipses group the two main fractions observed.

Heterotrophic bacteria

Together, the grain size distribution and organic contents influence the total number of bacteria in sediments. Dale (1974) found a highly significant correlation between bacterial abundance and mean grain size, and between bacterial numbers and carbon concentration.

In general, the number of bacteria obtained in the uncontaminated sediment areas were in agreement with the above cited findings: higher numbers of bacteria were present in the upper than in the lower layers. However, for the bacterial groups enumerated, a separation between upper and lower 15 cm depth was not clear. The greatest abundance of aerobic and anaerobic bacteria was in the surface layer (0-2 cm) (except HAnB in the linseed oil experiment), decreasing invariably with depth. The presence of high numbers of anaerobic bacteria (strict or facultative) at the surface sediments contrast with the conventional predicted profile of vertical zonation from a surface oxidised layer and a subsurface reduced zone. Still, the concepts of aerobic and anaerobic decomposition or simple unidirectional redox succession so often emphasised, have little relevance for bioturbated sediments rich in organic carbon (Aller, 1994). The presence of anaerobic areas (characterised by a dark grey colour) within the aerobic environment in the surface sediments (0-2cm) could account for the co-existence of aerobic and anaerobic bacteria.

Anaerobic patches occur specially when there is a high clay content and a tendency to produce aggregates (Nedwell & Grey, 1987). In the present work, these anaerobic aggregates were observed at the time of sample fractionation but were not detected by the Eh measurements, possibly as a result of the small dimensions of the anaerobic zones compared to the size of the probe used.

Oil degrading bacteria

Mirroring the results obtained for the total heterotrophs, higher numbers of aerobic than anaerobic oil degrading bacteria were present in the sediments. Since a higher percentage of aerobic than anaerobic oil degraders was found, a higher rate of aerobic degradation of the vegetable oils could be expected. Although the results indicated that only a small part of the total heterotrophs (≤ 10%) were oil degraders, they suggest that the autochthonous bacteria have the potential to degrade vegetable oils. The methods used mean that only those bacteria with lipase-like activity will grow. It is likely, however, that there are many more bacteria capable of degrading vegetable oils once the initial cleavage of the fatty acids occurred. Others have also reported increased abundance of hydrocarbonoclastic bacteria resulting from crude oil spills (Floodgate, 1984). Although it is rare to find any natural environment with no hydrocarbonoclastic bacteria at all, usually the crude oil degraders account for less than 10%, and often very much less, of the total prokaryotic population. Dosing with mineral oil greatly increases the number of oil degraders (Crow *et al.,* 1975; Ward & Brock, 1976; Horowitz & Atlas, 1977). As a result Crow *et al.,* (1975) suggest that a proportion of the hydrocarbon degraders to the total heterotrophic population greater than 10% should be used as an indicator of low level chronic pollution.

Sediments Eh and pH

The Eh measurements indicated that the sediments were oxidised in all depths analysed, with oxidation conditions increasing with depth. By contrast, the pH values were highest at the top 6 cm decreasing with depth, but variations occurred within a narrow range. The existence of an extended aerobic layer of 30 or more centimetres deep in this salt marsh area is possibly due to a combination of factors, such as: the sediments were not permanently waterlogged; oxygen diffusion from the plants roots (mainly *Spartina*); bioturbation caused by borrowing organisms; and also the increase in particle size (from 20 to 30 cm depth) and porosity, which enhances transport rates of oxygen (Nedwell & Gray,

1987). When sediments are saturated with water they become anoxic below the top few millimetres because of the high rate of oxygen consumption by marsh sediments biota and the very slow rate of oxygen diffusion through waterlogged pores. *Spartina* oxidises the sediments in which it grows through both passive oxygen release and active metabolic processes; Eh is higher in the root zone of this grass than in sediments below the roots or the unvegetated sediments (Howes *et al.,* 1981). Also, the benthos alters the physical and chemical features of sediments. These effects may induce heterogeneity on a scale comparable to habitat dimensions of the organisms themselves. Reworking, through digging, burrowing and feeding activity (tube building, particle selection, faecal production) creates structures which locally modify sediments (Parsons *et al.,* 1984; Nedwell & Gray, 1987; Aller, 1994).

4.6.2. Alterations in the Sediments after Addition of Linseed and Sunflower Oils

Linseed and sunflower oils penetration rates

After being spilled in adjacent areas of the salt marsh, linseed and sunflower oils penetrated the sediments to at least 30 cm depth in ≤ 3 days. The calculated rate of penetration for both oils, of 10^{-7} cm^2 s^{-1}, was smaller than that stipulated for dissolved organic carbon in sediments, which ranges from 0.8 to $1.9x10^{-6}$ cm^2 s^{-1}, depending on its molecular weight (Burdige *et al.,* 1992). It was, however, undoubtedly higher than the predicted crude oil penetration in muddy sediments. Field and laboratory experiments by Vandermeulen *et al.* (1988), showed that crude oil would be expected to penetrate muddy sediments only to a depth of 2 cm, if the sediments were continuously waterlogged, and also revealed the existence of an inverse relationship between amount of mud and penetration rate. Gundlack *et al.,* (1993) observed that crude oil penetrated 1-12 cm in salt marsh sediments and Floodgate (1996) also observed higher penetration of crude oil into the sediments than that predicted. In the present work, the upper sediment layers (0-15 cm) had a high content of fine particles and a low coefficient of permeability and consequently low percolation was expected for either oil. However, the experimental area, which was located in the upper part of the salt marsh, was not covered by water for a long period of the tidal cycle, being therefore susceptible to a greater penetration of the vegetable oils. The vegetable oils

vertical migration of the vegetable oil could also be greatly influenced by the role played by the activity of benthic organisms. A cursory examination of the sediments showed the presence of burrows through which the oils could have percolated. In this way, the burrows from macro and meiofauna could permit a vertical diffusion of the oils at a much higher rate than the sediments permeability would suggest.

These results contrast with those obtained by Mudge *et al.*, (1995) which revealed different percolation rates for linseed and sunflower oil in sediments located in an identical area of the salt marsh. Mudge and collaborators observed that linseed oil percolated rapidly through the sediments (down to 30 cm in 7 days). However, more than 35 days were necessary for sunflower oil to reach 30 cm depth. As the present work was carried out at lower temperatures (the winter months of 1995 and 1996) than the previous work (summer months of 1992), an increase in the oils' viscosity and consequently a decrease in the oils' percolation would have been expected. These results appear to suggest that the season of the year at which a spill occurs may be an important determinant of the rate at which the oils penetrate the sediments. This is also substantiated by the fact that in the experiments carried out by Mudge *et al.* (1995), sunflower oil polymerised at the surface sediments after 28 days, whereas in the present study such surface polymerisation was not observed during the 180 days of the experiment.

Lipids

The penetration and accumulation of the oils in the sediments is revealed by the amount of extractable lipids. Even though the oils had similar penetration rates, their distribution in the sediments with time and depth, was different (Figs. 4.17 & 4.41). The most fundamental distinction between both oils was the depths at which they tended to accumulate.

If the total amount of lipids extracted from the seven depths analysed is taken into account, in the case of linseed oil, the muddy sediments contained virtually the majority of lipids extracted (93 to 97%). In the case of sunflower oil, 70% of the total lipids where concentrated in the top 15 cm during the initial 7 days of the experiment but with time the concentration of oil in the upper layers decreased continuously with only 30% of the lipids observed in the muddy sediments (upper 15 cm) at day 60; at the end of the experiment (day 180) this value was reduced to 18%. Linseed oil accumulated in the upper 15 cm

depth for the complete duration of the experiment. Sunflower oil also concentrated in the top 15 cm layers during 14 days of the experiment. However, from then on it accumulated in the deeper sandy sediments.

The total amount of lipids extracted from the seven depths revealed that only a small amount of linseed oil was removed from the sediments (0-30 cm) during the first 28 days of the experiment. However, after 60 days only 40% of the added oil remained in those layers (0-30 cm). As the amount of lipids present in the sandy sediments was not markedly altered for the duration of the linseed oil experiment, the decrease in lipid content between day 28 and 60 was the result of changes within the upper 15 cm depth. Conversely, in the case of sunflower oil, the total amount of lipids present in a 30 cm sediment core (as the sum of the 7 depths analysed) was not altered for the duration of the experiment (6 months).

These results suggest retention of linseed oil in the muddy sediments for at least a month. The loss of 60% of the oil between 28 and 60 days from the top 15 cm depth could have been due to its utilisation by heterotrophic organisms (micro-organisms, infauna) or to its percolation to deeper sediment layers. However, the small concentration of linseed oil in the sandy sediments suggest that either only very small amounts of oil penetrated below 15 cm depth or that the vertical migration to these depths occurred but the oil was not retained due to the high permeability of the sandy sediments. In contrast, the sunflower oil appeared to have percolated from the muddy to the more sandy sediments, having been retained there. Advection could also account for some of the losses of linseed and sunflower oils.

In these experiments, and contrary to those of Mudge *et al.,* (1995), sunflower oil did not polymerise at the surface. However, in the sandy layers, the oil remained adsorbed to particles, though not forming concrete-like aggregations as observed by Salgado (1992).

The oil retained in the sediments could be attached to particles or/and within the sediment's pore space. The amount of oils attached to sediment particles can be illustrated by the difference between the percentage of <63μm particles before and after the removal of organic matter (Figs. 4.50A & B). These data also reveal the different behaviour of linseed and sunflower oils. In the former case, higher amounts of organic matter were found adsorbed to small particles in the top 15 cm than to the sandy sediments (bottom 15 cm)

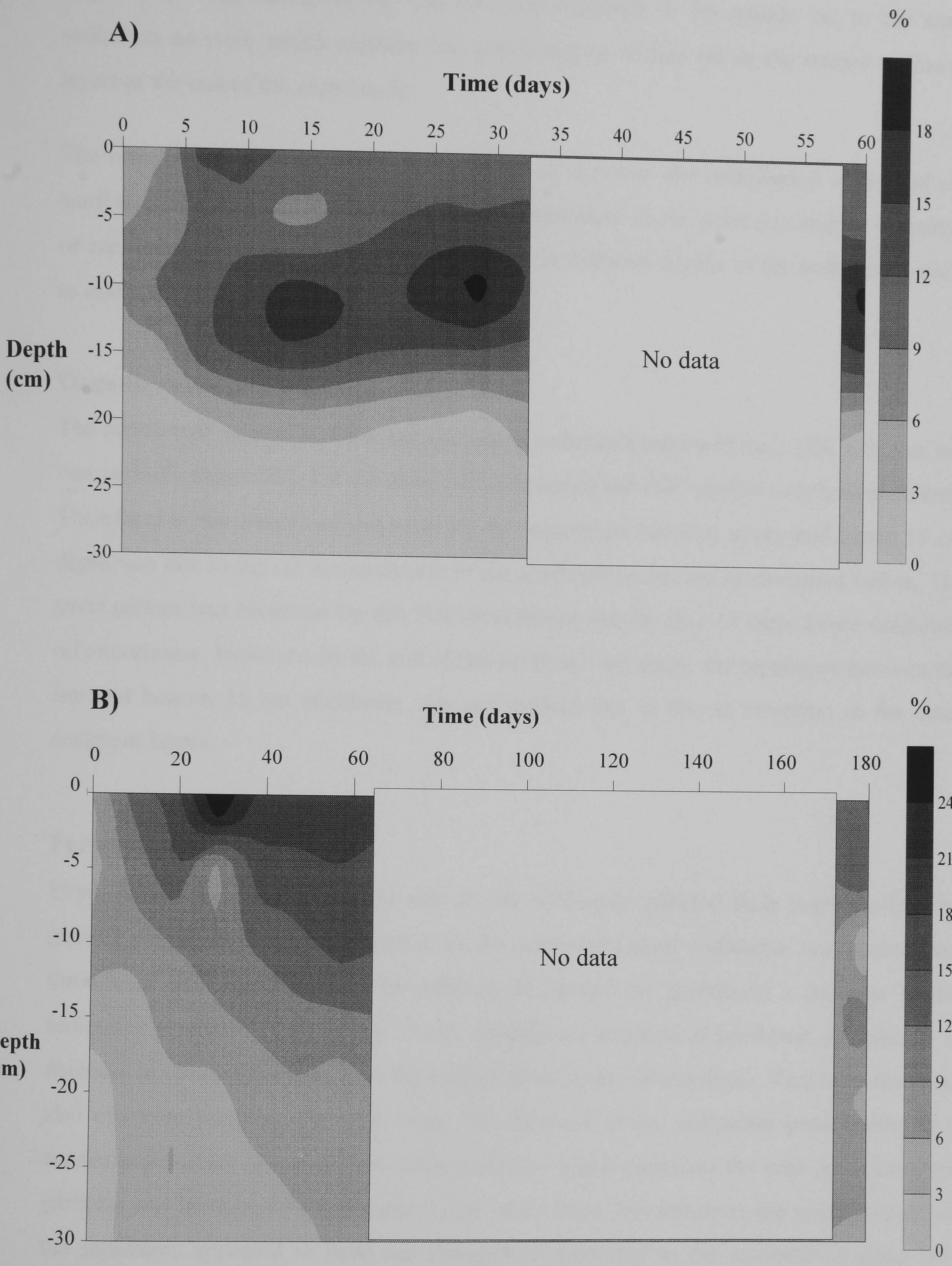


Figure 4.50. Amount of organic matter adsorbed to the sediment particles at the various depths previous to (day 0) and after the addition of A) linseed oil and B) sunflower oil.

(Fig. 4.50). The sunflower oil was not only adsorbed to the muddy but to the sandy sediments as well, which explains the concentration of this oil in the deeper sediments layers at the end of the experiment.

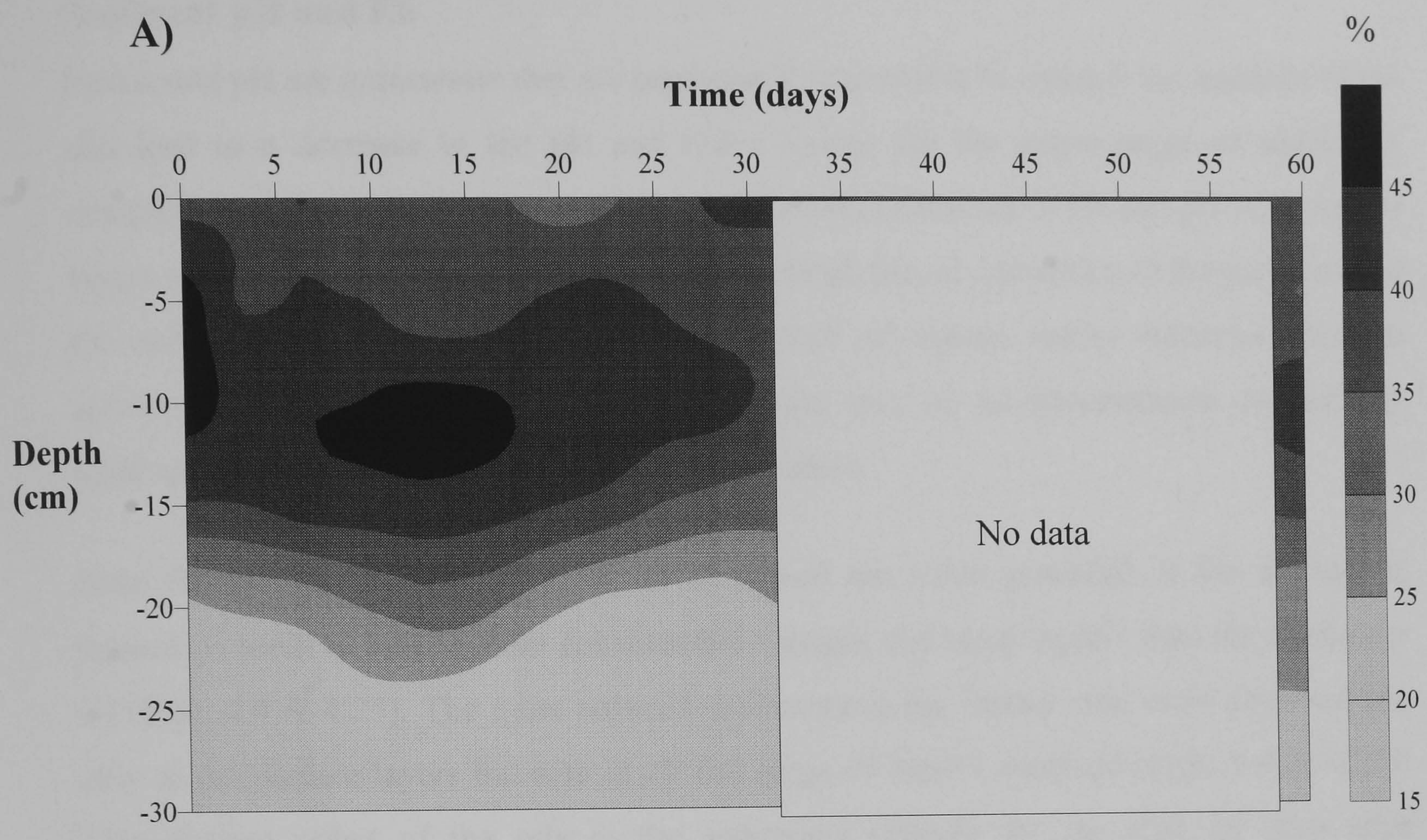
The results suggest that for the same season of the year, the distribution of linseed and sunflower oils with sediment depth is mainly dependent on the grain size and the properties of each oil itself. The accumulation of the oils at different depths of the sediment is going to affect many of the sediment characteristics.

Organic carbon

The addition of vegetable oils to the salt marsh sediments increased their TOC contents and was partially responsible for the observed variation of the TOC profile with time and depth. Therefore, in the linseed oil experiment, the separation between upper and lower 15 cm depth was due to the oil accumulation in the mud-rich sediments as described before. The same pattern was observed for the TOC distribution for the first 14 days, in the sunflower oil experiment. However, by the end of the sunflower oil assay, the separation between the top and bottom 15 cm sediments was not marked due to the oil retention in the latter sediment layers.

Permeability

Even though the presence of the oils in the sediments affected their permeability, the general profile with depth observed in the uncontaminated sediments was maintained throughout both experiments. The addition of linseed oil introduced a decrease in the sediments permeability at the top 15 cm, whereas the addition of sunflower oil induced a decrease in the permeability from the surface down to the 30 cm depth. This reduction was also observed progressively with time. The decrease in the sediments permeability is a consequence of the oil adsorption to the particles, which decreases the void space between particles and increases the resistance to the water flow. Nevertheless, the water content of the sediments appeared to have not changed considerably in the presence of either oil, except maybe for a small decrease at the top 10 cm after the addition of sunflower oil (Figs. 4.51A & B).



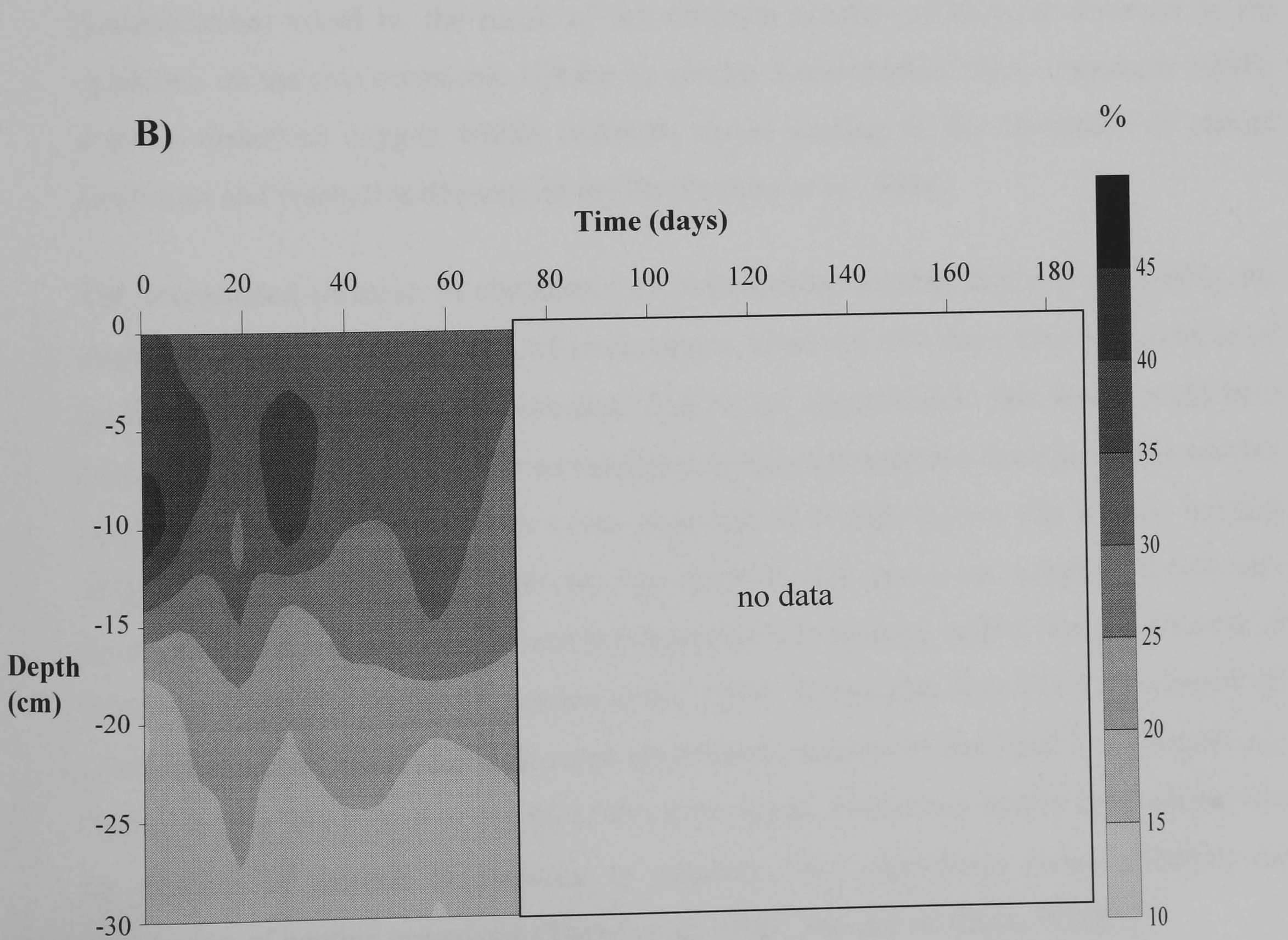


Figure 4.51. Sediment water contents (%) at the various depths previous to (day 0) and after the addition of A) linseed oil and B) sunflower oil.

Sediment pH and Eh

Redox and pH are parameters that are particularly susceptible to change: the addition of the oils lead to a decrease in the pH and redox values for the entire range of sediments analysed, more apparent in the top 6 to 8 cm. The faster decrease of Eh and pH in the upper layers was possibly the result of the high clay content, the oil adsorption to the particles and the existence of anaerobic zones. As the presence of organic matter enhances bacterial activity in localised areas of the sediment, there may be an accumulation of partially oxidised metabolites which reduced the Eh potentials.

Even though the addition of either oil decreased the redox potential of the sediments, linseed oil seem to induce more considerable changes and more rapidly than the sunflower oil (Figs. 4.4 & 4.25). The more reduced sediments in the former case were observed not only at the surface layers but also at all the range of depths analysed (Figs. 4.4 & 4.25). This distinct effect of the oils in the sediments (mainly for the first 28 days after contamination) could be the result of the different number of bacteria observed in the sediments on the two occasions. Uptake by aerobic heterotrophic micro-organisms rapidly depletes dissolved oxygen within sediment layers leading to the formation of anoxic conditions and respective decrease in the Eh (Parsons *et al.,* 1984).

The accentuated increase in abundance of heterotrophic aerobic bacteria is possibly the main reason for the highly reduced environment observed 180 days after the addition of sunflower oil. However, other biological factors, not quantified in this work, could have been responsible for such alterations since during the experiments a decrease in the number of plant roots and animal burrows was observed. It is well known that mobile benthos irrigate and oxygenate sediments as they burrow and move on, creating momentary oxidised conditions and rapidly aerobic/anaerobic successions within feeding pockets of otherwise anaerobic sediment (Jumars *et al.,* 1990). It was also found that the linseed oil could have a deleterious effects in some invertebrate organisms that inhabit sediments, *e.g.* *Hydrobia* was found dead at day 60 of this experiment. Numerous studies have shown that the absence of oxygen or presence of sulphide, is a significant factor affecting the distribution of benthic organisms (Theed *et al.,* 1969; Fenchel & Rield, 1970).

Dramatic alterations in the sediments pH could have a negative influence on the ability of microbial populations to degrade the oils, since most heterotrophic bacteria favour a pH near neutrality (Leahy & Cowell, 1990; Ratledge, 1989, 1994). However, in the present study, the sediments pH decreased only slightly, possibly not having much influence on bacterial growth (Figs. 4.4 & 4.25).

4.6.3. Alterations in the Bacteria Abundance after Addition of Linseed and Sunflower Oils

Heterotrophic Bacteria

As a consequence of the high organic matter availability, an increase in the numbers of bacteria was observed. The rapid increase in the numbers of heterotrophic aerobic and anaerobic bacteria was expected, as heterotrophic bacteria react more quickly to changes in their environment than the other bacteria, and reflect the content of easily degradable organic substances (Rheinheimer, 1977). This also suggests that both oils are more easily degradable than the organic carbon already present in the environment. These results are, nevertheless, in contrast with those obtained by Mudge *et al.* (1995), who observed a lag time of 14 days for the increase in HAB in sediments where linseed oil had been added.

Even though the numbers of heterotrophic aerobes and anaerobes increased quickly, the numbers of aerobic bacteria always surpassed the number of anaerobic ones. These findings are not unexpected as aerobic bacteria can utilise a wider range of organic compounds and also because the oxygen contents of the sediments favour their growth.

Despite the fact that heterotrophic aerobic and anaerobic bacteria responded promptly to the addition of the oils, in the first 28 to 60 days linseed oil appeared to sustain a higher number of bacteria than did the sunflower oil, possibly indicating a higher degradability of the former than the latter. Furthermore, the sediment temperature could have been another factor which influenced the degradation rate of the oils. Although both experiments were carried out at the same time of the year (but in different years), measurements of the sediment temperature indicate that during the sunflower oil experiment values fluctuated

more widely than in the linseed oil. However, these temperature readings provided values only for the sampling time and were not a measure of the range of temperatures between sampling days. Therefore, temperature could have influenced the rate of the oils degradation during the first two months of the experiments but is not believed to have done so in an accentuated manner.

The existence of very high numbers of HAB and HAnB particularly in the sandy sediments after 180 days of the sunflower oil addition, is probably the result of the accumulation of the oil in these sediment layers, which as discussed before, increased considerably the amount of organic matter available for bacterial utilisation. This increase in the bacterial abundance could also have benefited from the rising in the temperature (between winter and summer months), as rates of degradation are expected to increase as a result of the increasing rates of enzymatic activity. Mayer (1994) and Hedges & Keil (1995) have suggested that association with mineral surfaces protects organic carbon from remineralisation. However, in the present work this did not appear to be the case. The present results are in agreement with those of Mudge *et al.,* (1995) who also found that linseed oil sustained a higher number of HAB than sunflower oil, although the values obtained by those authors were much lower (maximum of 650 and 200 colonies g^{-1} wet sediment, respectively for linseed and sunflower oil experiments) than those obtained in the present study.

In the uncontaminated sediments, the highest number of HAB was detected at the surface decreasing with depth in both experimental areas, whereas the highest number of HAnB was detected in the middle depths in the linseed oil area, and in the 1 cm layer in the sunflower area (Figs. 4.7; 4.30). The presence of the oils did not change these depth profiles for the first 60 days of the experiments (even though the redox conditions of the environment changed) revealing the importance of the background number of bacteria present in the sediments previous to the contamination.

Oil Degrading Bacteria

The prompt increase (≤ 3 days) in the abundance of heterotrophic bacteria able to degrade the vegetable oils is not unexpected. In fact, it is well recognised that oil degrading bacteria are widespread (*e.g.,* Ratledge, 1994; Taipa *et al.,* 1992). The increase in numbers of oil degrading bacteria suggest that indigenous bacteria responded to the increase in the oil load

by increasing their oil degradative capability. The fact that AODB responded with an accentuated increase in numbers, whereas AnODB increased only slightly (up to 45 $MPN.g^{-1}$ wet sediment), suggests that the vegetable oil degradation is possibly an aerobic process rather than an anaerobic one. Aerobic conditions may, therefore, be necessary for the route of microbial oxidation of vegetable oils in the environment. The results have also shown that the uncontaminated sediments had more aerobic than anaerobic bacteria with the capability to breakdown these oils.

An increase in the abundance of aerobic heterotrophs and in the percentage of oil oxidisers in relation to the heterotrophic bacteria suggests degradation of the oils. However, the increase in abundance of anaerobic heterotrophic bacteria was not followed by that of anaerobic oil degrading bacteria. In general, the percentage of aerobic oil degraders in relation to the heterotrophic bacteria varied from <1% to <10% in the uncontaminated sediments to 20% in the contaminated ones. Moreover, in the sediment layers between 10 and 30 cm contaminated with sunflower oil, the number of oil degraders surpassed the number of total heterotrophs. These questionable results could be a consequence of the different methods utilised for the enumeration of the HAB and AODB, since viable count media techniques severely underestimate the total bacteria population (Zweifel & Hagström, 1995).

At the end of the experiments, at 1 and 5 cm depths the numbers of AODB present in the sediments contaminated with linseed and sunflower oil were contrasting: in the first case, high bacteria numbers were still present, whereas in the latter the number of bacteria was already decreasing. These results seem to imply that, at this point, sunflower oil had become less degradable than linseed oil and less degradable than it was at the start of the experiment. The decrease in the sunflower oil degradability suggests that the structure of this oil may have been altered, making the bacterial breakdown more difficult.

The accumulation of sunflower oil in the sandy sediments at the later stages of the experiment appear to have induced an increase in the numbers of aerobic bacteria with the capability to breakdown down this oil, revealing that bacteria from these sediments also had the potential to degrade this oil. The profile with depth observed at day zero, consisting of higher numbers of AODB at the top than at the bottom 15 cm depth, was therefore inverted in the sediments contaminated with sunflower oil. The addition of linseed oil did

not modify the depth distribution of AODB, possibly because the amount of oil in the sandy sediments was always low.

Sulphate Reducing Bacteria

The addition of linseed and sunflower oils to the sediments had a different effect on the SRB than that observed for the HAB. In the case of linseed oil, a lag period of 7 or more days occurred before an increase in the number of SRB was observed. In the case of sunflower oil this period was of 21 (1 cm) to 180 days. Since the substrates used for SRB are the fermentation end-products produced by other sedimentary bacteria it was to be expected that this group of bacteria takes longer to respond to the increase of organic carbon than the heterotrophs.

The oxidation-reduction potentials are frequently mentioned as a factor controlling sulphate reducers growth rates in sediments. In the present work a direct relationship between SRB and Eh measurements was not found. These findings are also corroborated by the work of Dale (1974), who did not find a significant correlation between Eh and the number of bacteria in the sediments. The fact that in the sediments contaminated with linseed oil a higher number of heterotrophic bacteria and lower Eh values were found in relation to those in the sunflower oil sediments, suggests that linseed oil promotes more the conditions for the development of this anaerobic group. The accentuated increased in the abundance of SRB, mainly in the sandy sediments between 60 and 180 days after the addition of sunflower oil, could be the response to the accumulation and adsorption of the oil at those depths, the increase in heterotrophic bacteria and consequent formation of a more anoxic environment.

The results from the bacteria enumerations indicated that all studied groups benefited from the extra carbon provided by the oil addition and that the various groups appeared to be present at all depths analysed at the same time, so the typical succession of aerobic layer followed by anaerobic layer was not observed. Periodic re-exposure to oxygen, even brief, results in a more complex and sometimes rapid decomposition of organic matter than is possible under constant or unidirectional redox changes (Aller, 1994).

The addition of vegetable oils provided a carbon source for bacteria to grow. However, nitrogen and phosphorus availability are frequently low in salt marsh sediments (Long &

Mason, 1983). The release of vegetable oils into any environment which contains low concentrations of inorganic nutrients produces an excessively high C/N or C/P ratios, both of which are unfavourable for microbial growth (Lancelot & Billen, 1985). Mudge *et al.,* (1994) found that the vegetable oil degradation rate in seawater increased markedly when inorganic N and P were added to bacteria cultures. It is also well established that addition of inorganic N and P to the environment intensifies the rate of crude oil degradation (Floodgate, 1984, 1995; Coffin *et al.,* 1997). Therefore, the increase in the numbers of bacteria in the presence of the oils could have been intensified, and consequently the degradation rate of the oils increased if the environment had been richer in inorganic nutrients. However, such hypothesis is difficult to substantiate since no measurements of ambient inorganic N or P were carried out.

4.6.4. Transformations in the Fatty Acids Composition of Linseed and Sunflower Oils

From what has been said before, it can be seen that spillages of linseed and sunflower oils altered some of the sediments properties; additionally, the fatty acid composition of both oils also appears to have changed. The changes observed were related to the fatty acid composition and their relative abundances (Figs. 4.24 and 4.47). Since each fatty acid has a different degradation rate (*e.g.* Parker, 1967; Farrigton & Quinn, 1971; Van Vleet & Quinn, 1979), a dissimilar behaviour of the various fatty acids of linseed and sunflower oils would be expected to some extent.

The main fatty acid of linseed and sunflower oils, 18:3ω3 and 18:2ω6 respectively, appear to behave in a different manner from the others present in the oils (Figs. 4.18 to 4.22 and 4.42 to 4.45). For instance, during the first month of the experiment, 18:3ω3 had a faster vertical migration (10^{-6} cm^2 s^{-1}) than the other fatty acids (10^{-7} cm^2 s^{-1}), suggesting its preferential degradation over the other fatty acids in linseed oil. This is in accordance with reports from the literature where polyunsaturated fatty acids are considered more reactive than saturated fatty acids (Parker & Leo, 1965; Farrington & Quinn, 1971; Johnson & Calder, 1973; Van Vleet & Quinn, 1979; Haddad *et al.,* 1992, Sun & Wakeham, 1994; Canuel & Martens, 1996).

The preferential degradation of 18:3ω3 in relation to the saturated fatty acids is probably better illustrated by the ratio between 18:3ω3 and the sum of both saturated fatty acids of linseed oil (16:0 and 18:0) (Fig. 4.52).

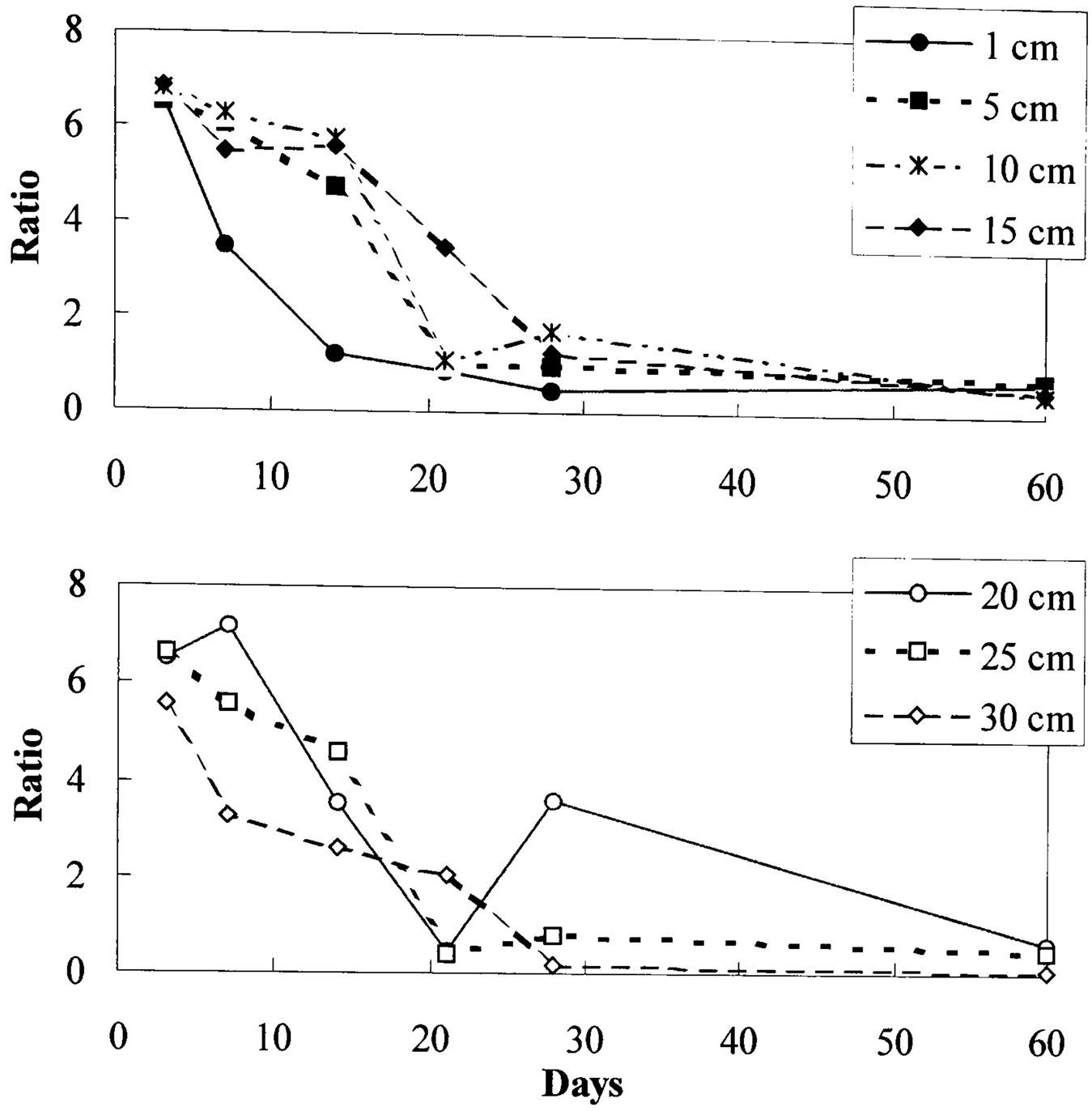


Figure 4.52. Ratio between 18:3ω3 and the sum of the saturated fatty acids (16:0 + 18:0) at various depths, after the addition of linseed oil.

For most of the depths analysed, higher disappearance rate of 18:3ω3 in relation to the saturated fatty acids occurred during the first 21 days (Fig. 4.52). The vertical migration of the oil is possibly responsible for the different lags of time necessary for a change to be registered at each sediment depth. The decrease in oxygen content in the sediments could have been responsible for the slower rate of degradation of 18:3ω3 between days 28 and 60, as various authors have indicated that there are significant differences between degradation rates of fatty acids in aerobic and anaerobic conditions (Sun *et al.,* 1993, 1997; Harvey *et al.,* 1995).

In the case of sunflower oil, a preferential degradation of 18:2ω6 in relation to the saturated fatty acids was also observed (Figure 4.53). However, the ratio between 18:2ω6 and the sum of the 16:0 and 18:0 showed a higher lag time for changes to occur than that observed

for 18:3ω3 in the linseed oil, possibly as a result of the lower bacterial abundance in the sediments contaminated with sunflower oil. Once more, the time necessary for the ratio to decline increased with the depth, from 3 days at 1 cm depth to up to 60 days for the depths between 15 and 30 cm (Fig. 4.53).

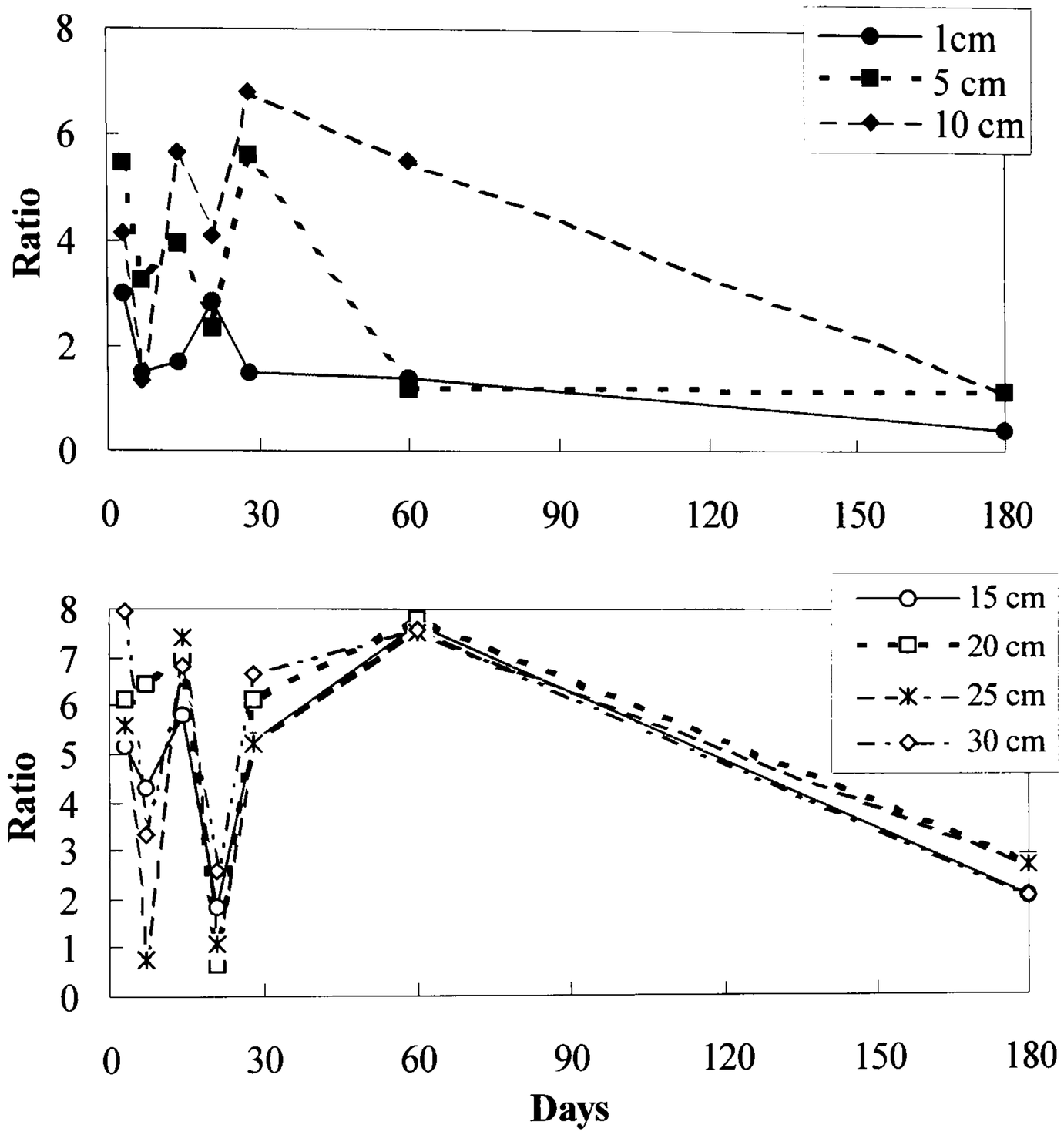


Figure 4.53. Ratio between 18:2ω6 and the sum of the saturated fatty acids (16:0 +18:0) at various sediment depths, after the addition of sunflower oil.

The lag time of 60 days observed for depths between 15 and 30 cm is possibly the result of the time necessary for high concentrations of oil to reach these depths and for bacterial abundance to increase sufficiently to degrade the fatty acids. By day 180, 18:2ω6 had virtually disappeared from the top 5 cm suggesting its complete degradation by bacteria or vertical migration to deeper sediment layers.

In the case of linseed oil, the concentration of the remaining fatty acids also decreased during the first 28 days of the experiment, even though at smaller rates than that of 18:3ω3. Such a decrease, mostly in the upper 15 cm, was probably due to the utilisation of the fatty

acids by bacteria and other benthic organisms, since vertical migration of the oil was not marked. However, after 28 days the concentration of these fatty acids (mainly 18:2ω6 and 18:1ω9) increased. These results can be seen in Figures 4.19 to 4.22 but are better illustrated by the ratio between each fatty acid and the 18:3ω3 (Figure 4.54).

When sunflower oil was added to the sediments, all fatty acids disappeared from the upper 15 cm at a similar rate. The penetration of sunflower oil into deeper sediments was possibly the main reason for the fatty acid's disappearance from the upper sediments and not so much their utilisation by the biota. Even though all fatty acids of sunflower oil accumulated in the sandy sediments, there was a 50% decrease in the concentration of 18:2ω6 between day 60 and 180, while that of the remaining fatty acids was still increasing (Fig 4.42 to 4.45 and 4.55).

The increase in concentrations of 16:0, 18:0, 18:1ω9 and also 18:2ω6 (in the case of linseed oil) could have resulted from the conversion of 18:3ω3 and 18:2ω6 in linseed and sunflower oils, respectively, into all the former. These transformations could occur by two alternative processes: i) by initial reduction of the double bonds, followed by chain shortening *via* loss of C_2 units by β-oxidation; ii) by β-oxidation to acetyl CoA units followed by resynthesis, as observed by Rhead *et al.,* (1971) when studying the degradation of 18:1ω9.

The observed 'new' fatty acids could have been formed by isomerisation (positional and/or geometrical) of 18:3ω3 and 18:2ω6. The 'new' fatty acids could have also been transformed to other 'new' fatty acids by reduction of the double bonds. Furthermore, the presence, in both experiments, of 'new' fatty acids, initially in the upper 5 cm, appears to suggest bacterial degradation since it coincided with the highest spatial bacterial abundance. The presence of 'new' fatty acids was first detected after 21 days of incubation in the linseed oil experiment and after 28 days in the case of sunflower oil, in accordance with the earlier higher bacterial abundance in the former case than in the latter. This is thought to have resulted in more rapid transformations, if indeed these are due to bacterial activity. The action of the various bacteria groups in the transformations of the fatty acids composition of linseed and sunflower oils in salt marsh sediments will be considered in Chapter 5. It is foreseen that the results from the activity of the bacteria indigenous from the salt marsh sediments together with the identification of the 'new' fatty acids will allow

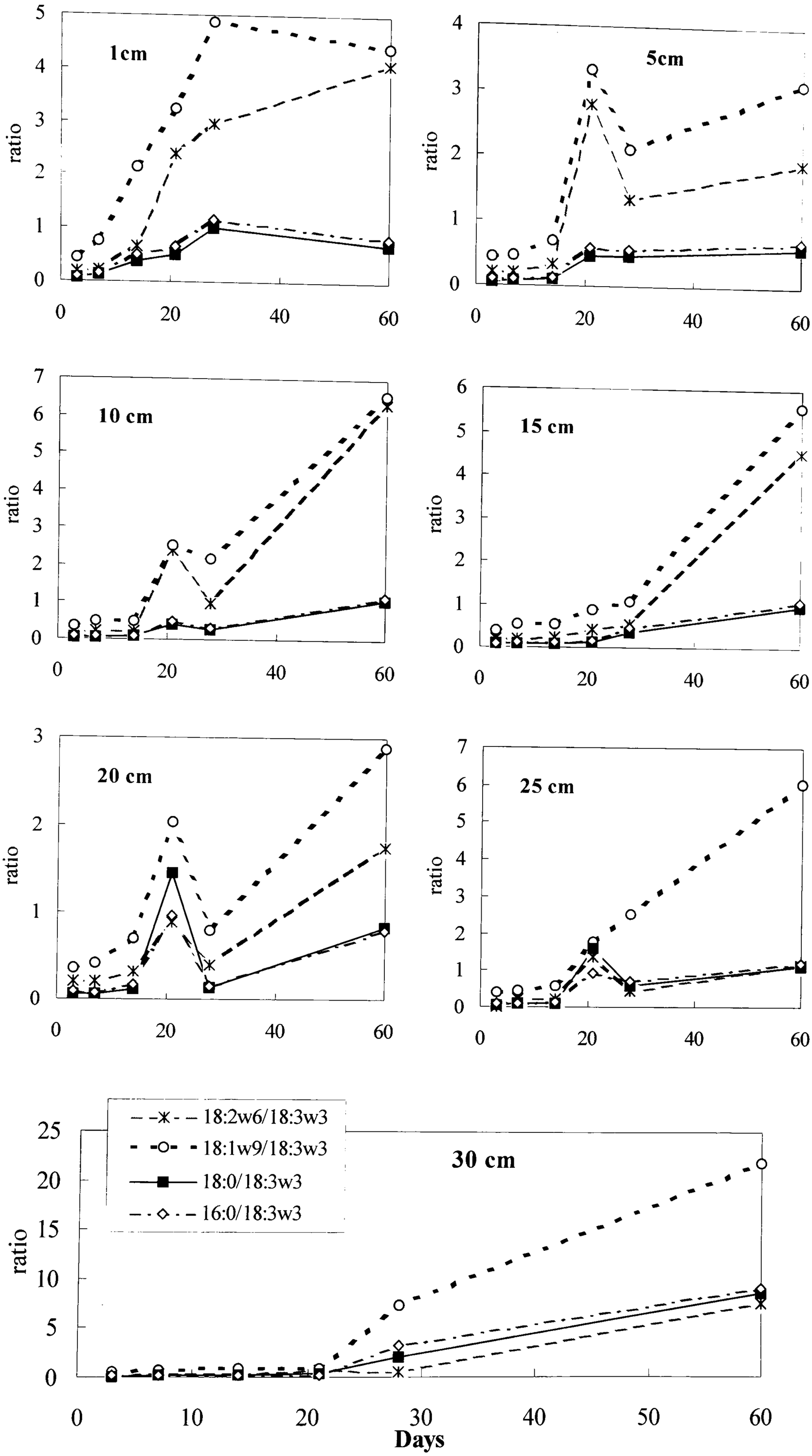


Figure 4.54. Ratio between 16:0, 18:0, 18:1ω9 and 18:2ω6 and 18:3ω3 at the various sediment depths, after the addition of linseed oil.

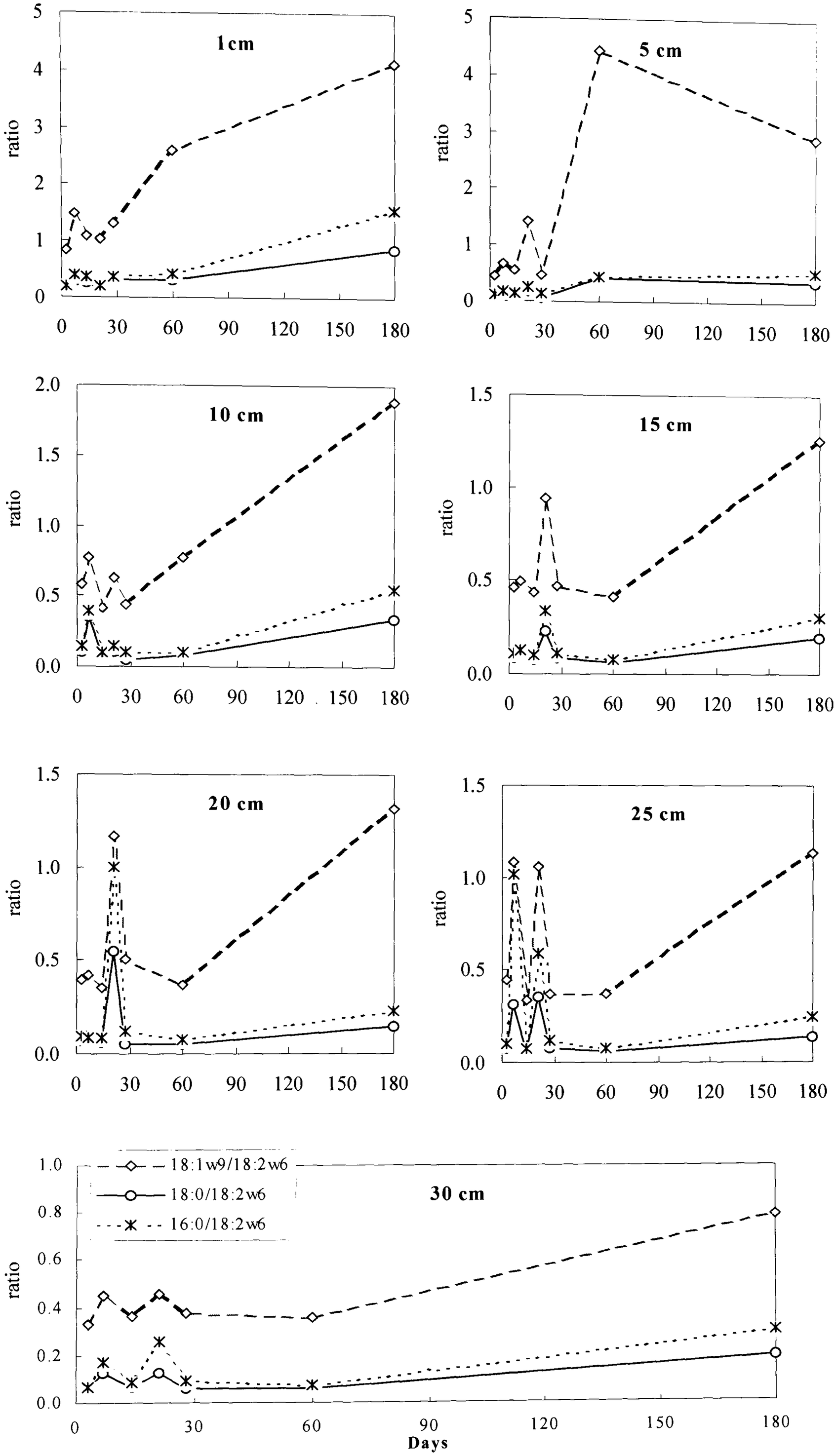


Figure 4.55. Ratio between 16:0, 18:0 and 18:1ω9 and 18:2ω6 for the various sediment depths, after the addition of sunflower oil.

the establishment of the degradative pathway(s) of both oils and the importance of the 'new' fatty acids in this pathway of degradation.

4.7. SUMMARY AND CONCLUSIONS

At the start of the simulation of spills of linseed and sunflower oils in the salt marsh at Foryd Bay, two main hypotheses were put forward and the observations and conclusions reached for each of these hypotheses were as follow:

Hypothesis I) Spills of linseed and sunflower oils in salt marsh sediments affect both the sediments and the oils characteristics.

- Linseed and sunflower oils penetrated to depths of 30 cm in ≤ 3 days, at a rate of 10^{-7} cm^2 s^{-1} but they had dissimilar distributions with depth: i) the majority of the linseed oil remained concentrated in the upper 15 cm sediments (with higher contents of fine particles) than in the sediment layers between 15 and 30 cm depth; ii) most of the sunflower oil was present in the upper 15 cm during the first 14 days of the experiment after which it percolated to deeper and sandier sediment layers (15 to 30 cm) where it concentrated until the end of the experiment (after 6 months).
- The accumulation of linseed oil in the upper sediment layers was thought to be due to its high adsorption to the fine particles, whereas sunflower oil adsorbed onto all range of grain sizes.
- Sunflower oil took longer to be degraded than linseed oil, being retained in the sediments for a longer period of time.
- The adsorption of the oils to the sediment particles reduced the sediments permeability. Linseed oil was responsible for a decrease only in the permeability of the upper 15 cm, whilst sunflower oil reduced the permeability within all the sediment layers analysed (1 to 30 cm).
- The pH, and more markedly, the Eh of the sediments was altered by the presence of the oils. A decrease was first observed in the upper sediments (which contained anaerobic zones at the start of the experiment), extending to deeper layers with time. The sediments contaminated with linseed oil became anaerobic more rapidly than those with

sunflower oil, possibly as a result of the high bacteria numbers observed during the first 28 days in the former.

- There was a decrease in the number of plant roots and infauna in the sediments after the contamination with the oils. In the case of linseed oil, organisms such as *Hydrobia* were found dead at the surface of the sediments. These observations suggest that the vegetable oils have deleterious effects for the flora and macrofauna living in the sediments.
- After 60-180 days, the contaminated area had a foul smell as a result of the development of anoxic conditions.

Changes in the fatty acid composition of both oils and their relative abundances were also observed. These changes were principally:

- A preferential degradation of the main fatty acids of linseed and sunflower oils, 18:3ω3 and 18:2ω6, respectively.
- The decrease in the concentration of 18:3ω3 and 18:2ω6 was accompanied by an increase in the concentration of the other fatty acids of linseed and sunflower oils, which suggest a conversion of the former into the latter.
- 'New' octadecatrienoic, octadecadienoic and octadecadecenoic acids, which were not present in the uncontaminated sediments or in the oils, were observed. These unsaturated fatty acids are possibly products of the degradation of 18:3ω3 and 18:2ω6.

Hypothesis II): Autochthonous aerobic and/or anaerobic bacteria are able to breakdown and utilise these oils.

- After the addition of either oil, the number of heterotrophic aerobic and anaerobic bacteria increased promptly. The increase in the number of bacteria as a response to the supply of organic matter was expected and the prompt increase in numbers attest that the vegetable oils were readily degradable.
- In the presence of both oils, the number of heterotrophic aerobic bacteria was consistently higher than the number of heterotrophic anaerobic bacteria, presumably as a result of the wider range of compounds that the former can utilise as substrates.
- During the first 28 to 60 days of the experiments, the number of heterotrophic aerobic and anaerobic bacteria, was consistently higher in the sediments contaminated with

linseed than sunflower oil, indicating that linseed oil was more readily degradable than sunflower oil.

- An accentuated increase in the number of bacteria in the sandy sediments at day 180 occurred as a result of the accumulation of the sunflower at these depths which was further assisted by the rising of the sediments temperature.
- The proportion of the heterotrophic bacteria able to breakdown the vegetable oils increased promptly. An accentuated increase was observed in the number of aerobic oil degrading bacteria, whilst the number of anaerobic oil degrading bacteria increased very slightly, suggesting that aerobic conditions may be necessary for the breakdown of the vegetable oils.
- Contrasting with the number of total heterotrophs, a higher abundance of aerobic oil degrading bacteria was observed in the sediments containing sunflower than linseed oil, but only for depths between 20-30 cm, where the sunflower oil was concentrated.
- The numbers of sulphate reducing bacteria increased after 7 and 21 days after the addition of linseed and sunflower oils, respectively. The longer lag time for a response to be observed in the presence of sunflower oil is probably a consequence of the longer time for the Eh of the sediments to decrease, indicating that anaerobic conditions were slowly being established.

5. Aerobic and Anaerobic Degradation of Vegetable Oils - Laboratory Experiments

In the field experiments simulating linseed and sunflower oil spills an increase in the number of aerobic and anaerobic bacteria was observed (Chapter 4). This increase in bacteria numbers was accompanied by alterations in the fatty acid compositions of both oils. Therefore, laboratory experiments were carried out to determine the role of aerobic and anaerobic bacteria in the degradation of linseed and sunflower oils. Specifically, experiments were set up with the aim of determining:

- Which bacterial groups were responsible for the degradation of these oils.
- If the bacterial degradation of these oils is a sequential process, and if so, which bacterial group initiated it; which bacterial groups had the capability to continue this degradation.

The experiments were carried out in 1994 (Experiment I) and 1996 (Experiment II) and their practical strategy differed. A detailed analysis of the outcome of these experiments will be given in sections 5.1 and 5.2.

5.1. EXPERIMENT I - LINSEED OIL DEGRADATION BY BACTERIA REMOVED FROM THE DIFFERENT SEDIMENT DEPTHS

5.1.1. Experimental Strategy

The first experiment was carried out in December 1994 and in this case only the degradation of linseed oil was studied. For this assay, natural bacteria populations from the studied salt marsh sediments were used. Two sediment cores of 5 cm diameter and 40 cm length were collected from the sampling area, one of which was kept and transported in air tight conditions for the isolation of anaerobic bacteria. These sediment cores were sliced as described in section 4.1 and samples from every 5 cm (from the surface down to 30 cm) were analysed.

The bacteria populations from each layer were removed from the sediment particles by sonication, as described in section 4.2.4, and inoculated into FSW (Appendix), using the procedure followed for the aerobic oil degrading bacteria (section 4.2.4.1.2.). In the present case, however, 1 cm^3 aliquot of each undiluted sample was inoculated in 6 "universal bottles" containing 9 cm^3 of medium (fortified sea water and 0.2 cm^3 of linseed oil) and in 3 "universal bottles" containing fortified sea water and no oil. This latter group was considered the control, where no bacteria were expected to grow as there was no carbon source available. Samples were incubated at 20°C for 7 days.

This practical approach was also followed for anaerobic bacteria, with the samples treated as described in section 4.2.4.2.2. SRB were also grown following the procedure already given (section 4.2.4.2.3).

After 7 days of incubation, growth was observed in the samples incubated with aerobic and anaerobic bacteria. For each case, 3 replicates of each sediment depth were taken aside for chemical analyses, and the remaining 3 replicates were pooled to eliminate the differences among them that may have occurred during the incubation. The pooled samples were then

filtered through a 0.2 μm Nuclepore polycarbonate membrane (Whatman) to remove the bacteria present in the medium. One problem that arose in the execution of this step was that filtration not only removed bacteria but also large amounts of oil.

After filtration each sample was divided into three fractions and colonies of SRB, that had been previously cultivated from 1 and 5 cm sediment depths, were added to them. Samples that had been incubated in aerobic conditions before, needed to have their redox potential reduced prior to the addition of SRB. This was achieved by the removal of oxygen from the medium, as described in section 4.2.4.2. and by the addition of a reducing agent, sodium thioglycollate (Appendix). Due to the slow growth of these bacteria, samples were incubated for 14 days, at 20°C, under anaerobic conditions, after which their fatty acid composition was analysed.

Colonies of SRB, isolated from sediment depths 1 and 5 cm, were also inoculated in triplicate in FSW and raw oil as described for the anaerobic oil degrading bacteria. In this experiment the variation with sediment depth was not examined.

The practical approach described above for the interaction between aerobic and sulphate reducing bacteria is schematically represented in Figure 5.1.

Chemical Analysis

To extract the oil from the medium, the entire contents of the three replicates of each sample were placed in a glass separation funnel. The oil that remained in the "universal bottles" was rinsed out using chloroform (Rathburns) and then added to the corresponding separation funnel. Chloroform (10 cm^3) was added to the funnel and vigorously shaken. The mixture was allowed to settle and the oil dissolved in chloroform was then transferred to a Florentine flask. This procedure was repeated 4 times after which the organic solvent was evaporated in a rotary evaporator.

Subsequently, the practical procedure followed was identical to that described for the field experiments (4.2.8). In the present case, samples were analysed in a Fisons MD 800 GC-MS and the operating conditions were also those established in the section 4.2.8.

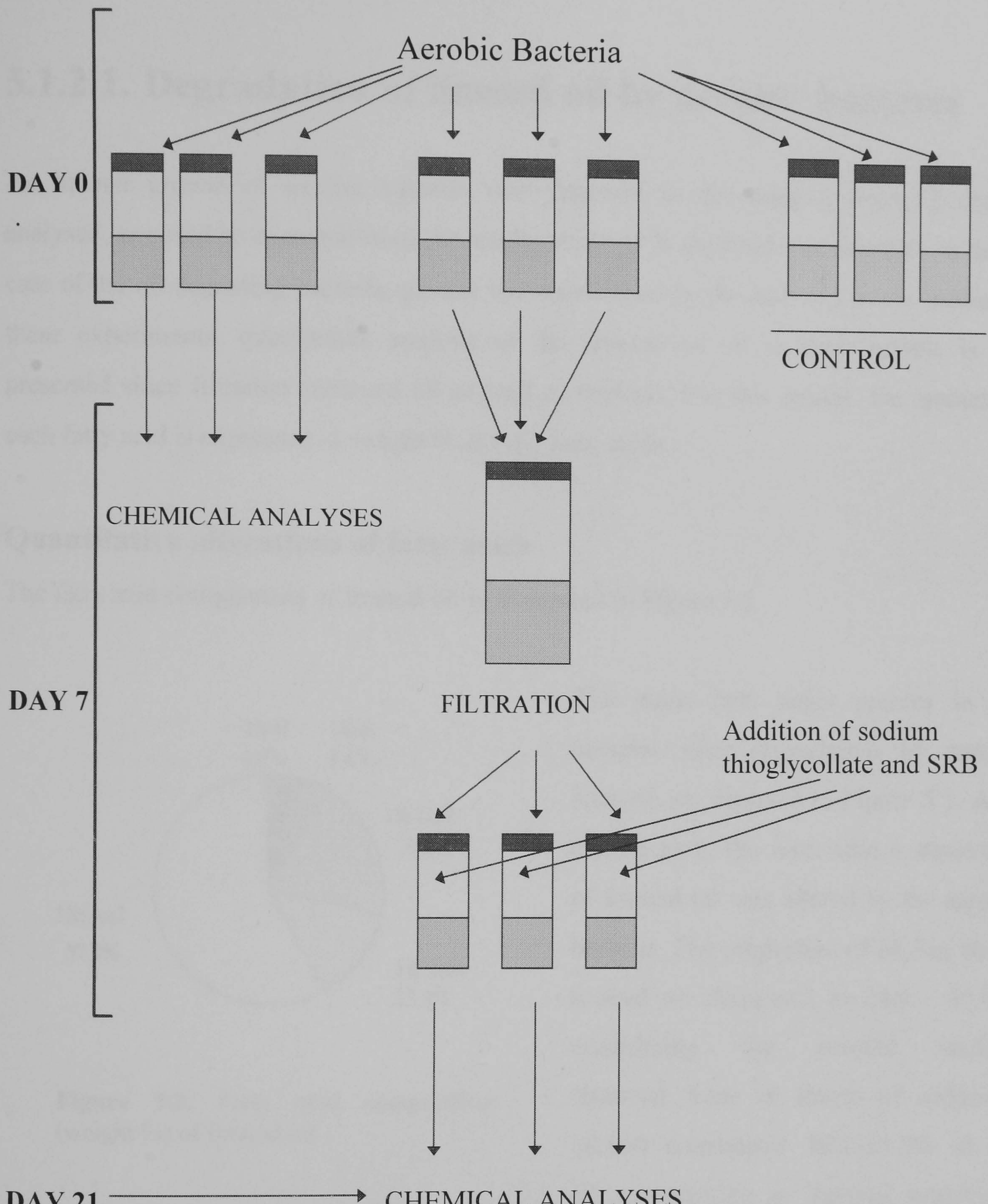


Figure 5.1. Schematic representation of the experimental strategy to test the linseed oil degradation by indigenous aerobic and suphate reducing bacteria from the salt marsh sediments. SRB stands for sulphate reducing bacteria.

5.1.2. Results

5.1.2.1. Degradation of linseed oil by aerobic bacteria

An intense growth of aerobic bacteria was observed in the samples from all depths analysed, as could be expected from the results obtained in the field experiments. As in the case of the oil degrading bacteria, growth was established by the turbidity of the media. In these experiments, quantitative analysis of the amount of oil in each sample is not presented since filtration removed oil as well as bacteria. For this reason, the amount of each fatty acid is expressed in weight % of total fatty acids.

Quantitative alterations of fatty acids

The fatty acid composition of linseed oil is illustrated in Figure 5.2.

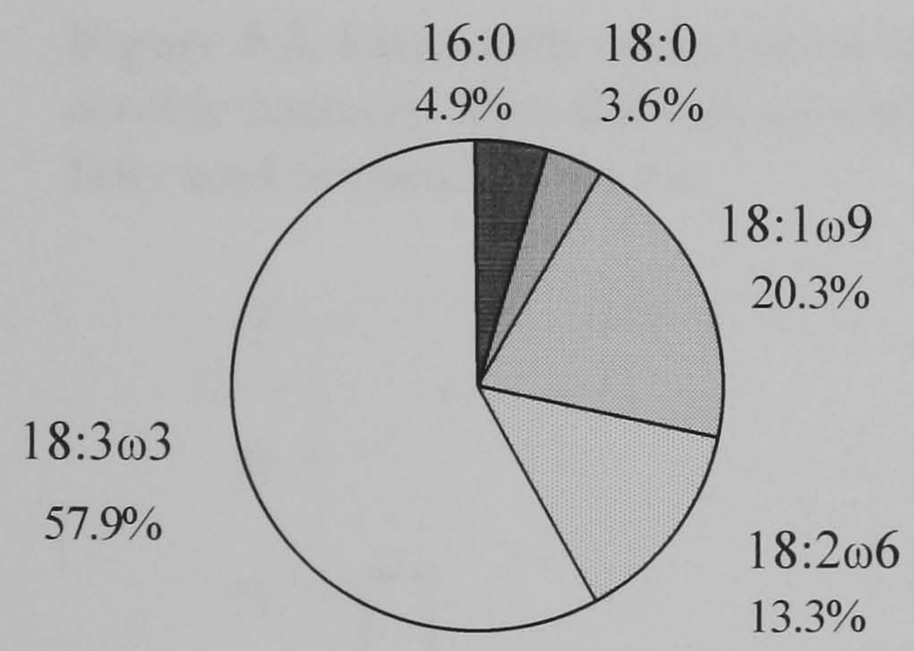


Figure 5.2. Fatty acid composition (weight %) of linseed oil.

The main fatty acids present in the samples after degradation by aerobic bacteria are depicted in Figure 5.3. As it can be seen, the fatty acid composition of linseed oil was altered by the aerobic bacteria. The proportion of 18:3ω3 in the linseed oil decreased to 24.6 - 49.9%, considering the aerobic bacteria removed from all layers of sediment. 18:1ω9 constituted 26.2-39.3% of the oil, representing an increase relative to the raw oil composition (20.3%). The greatest variations were observed with aerobic bacteria from the 1 cm depth layer where an increase in the amount of 18:2ω6 (to 19.7%) was also measured. This last alteration was not carried out by the aerobic bacteria removed from the remaining sediment layers. Furthermore, no modifications were recorded in the quantitative contribution of the saturated fatty acids (16:0 and 18:0) for the linseed oil composition (Figure 5.3).

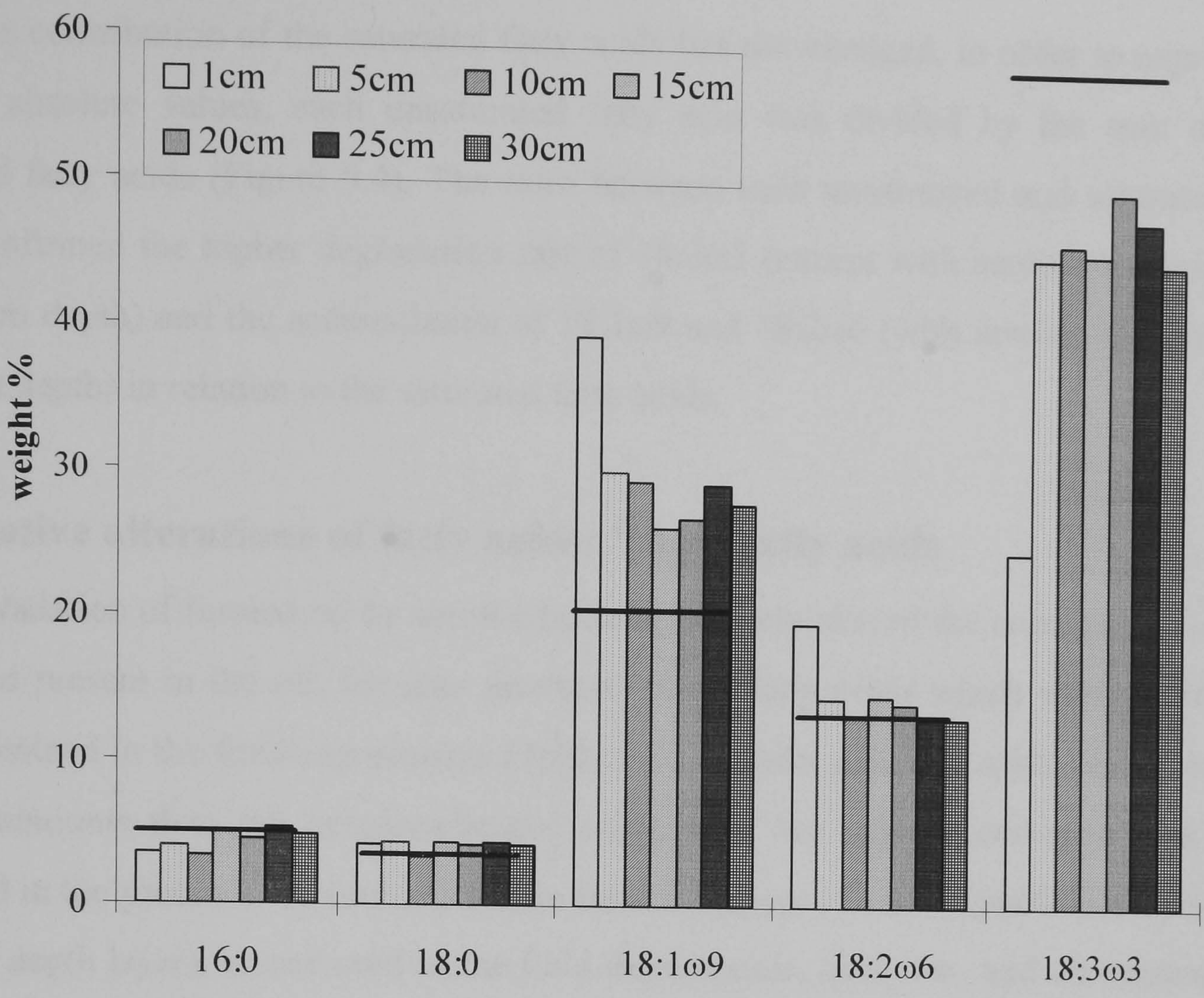


Figure 5.3. Fatty acids composition of the degraded linseed oil incubated with aerobic bacteria from the various sediment depths. ——— percentage of each fatty acid in pure linseed oil.

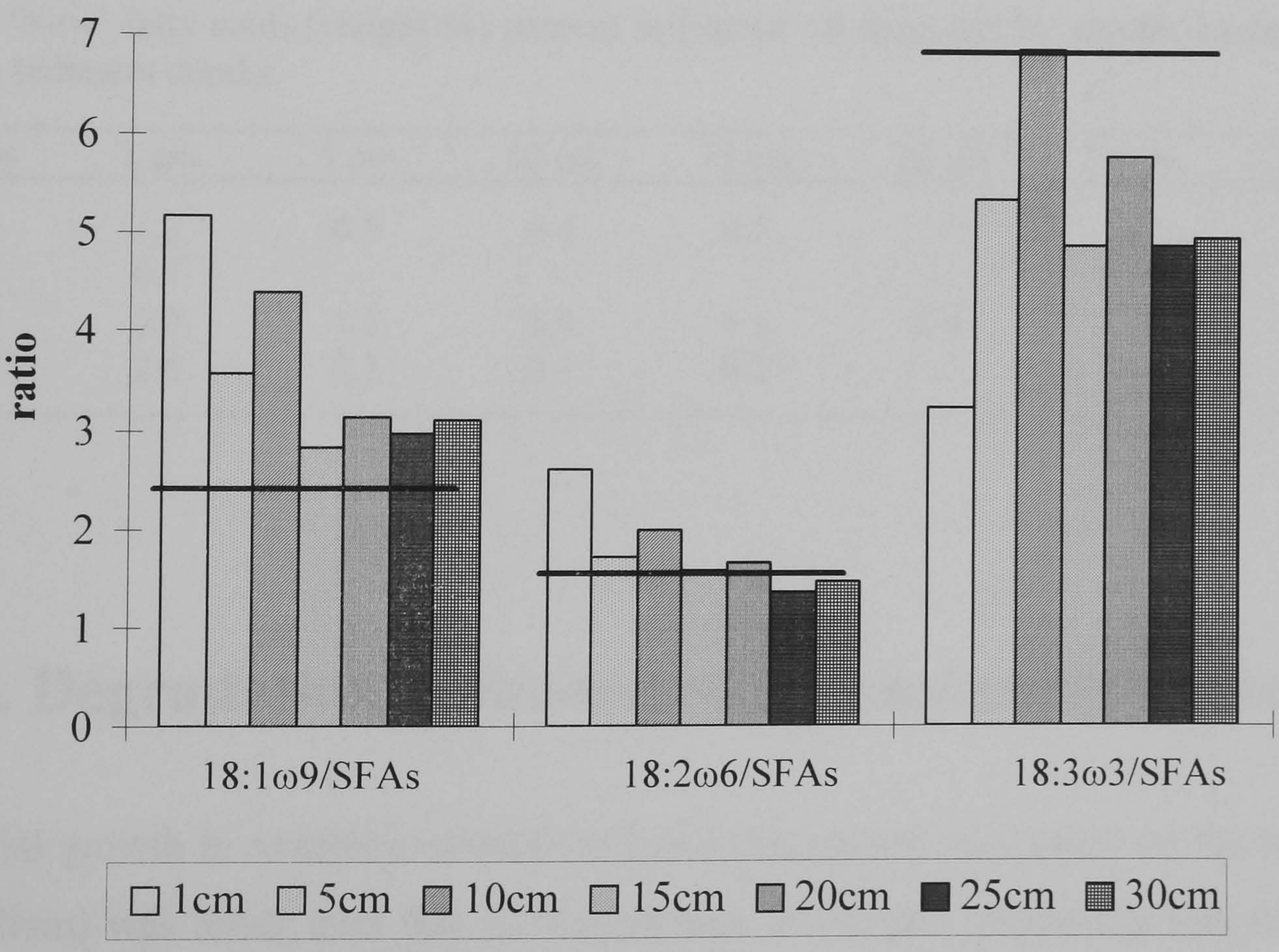


Figure 5.4. Ratio between unsaturated fatty acids and the sum of saturated fatty acids (SFAs, 16:0 +18:0) of linseed oil after degradation by aerobic bacteria from the various sediment depths. ——— values for linseed oil.

Since the contribution of the saturated fatty acids has not changed, in order to express the data as absolute values, each unsaturated fatty acid was divided by the sum of both saturated fatty acids (Figure 5.4). The ratio between each unsaturated and saturated fatty acids confirmed the higher degradation rate of 18:3ω3 (except with aerobic bacteria from the 10 cm depth) and the accumulation of 18:1ω9 and 18:2ω6 (with aerobic bacteria from the 1 cm depth) in relation to the saturated fatty acids.

Qualitative alterations of fatty acids: 'New' fatty acids

The degradation of linseed oil by aerobic bacteria not only altered the contribution of each fatty acid present in the oil, but also produced 'new' fatty acids which were identical to those obtained in the field experiments (Table 5.1). Octadecatrienoic acids were present in greater amounts than the octadecadienoic acids, with the highest concentrations being observed in the presence of aerobic bacteria from the upper 15 cm (in particular those from the 1cm depth layer), as occurred in the field experiments. However, and contrasting with the field experiment results, the most abundant octadecatrienoic acid was the 18:3 (1) instead of the 18:3 (4).

Table 5.1. 'New' fatty acids (weight %) present in linseed oil degraded by aerobic bacteria from the various sediment depths.

Fatty acids	1 cm	5 cm	10 cm	15 cm	20 cm	25 cm	30 cm
18:2 (1)	1.3	0.5	0.4	0.2	-	-	-
18:2 (3)	0.1	-	-	-	-	-	-
18:3 (1)	5.0	1.3	3.9	4.1	0.4	-	2.4
18:3 (4)	2.6	0.2	0.1	0.2	-	-	-

5.1.2.2. Degradation of linseed oil by anaerobic bacteria

The bacterial growth in anaerobic conditions (bacterial growth was based on the turbidity of the medium) was lower than that in the presence of oxygen, producing less extensive alterations to the composition of the linseed oil.

Quantitative alterations of fatty acids

The main fatty acids present in the oil degraded by anaerobic bacteria are depicted in Figure 5.5. A small decrease (to 49.8-53.8 %) in the percentage of 18:3ω3 and an increase (to 23.2-26.7%) in 18:1ω9 were the major changes recorded. The proportion of the remaining fatty acids were not affected by the anaerobic bacteria. These results were similar for all samples.

The ratio between each unsaturated fatty acid and the saturated fatty acids is illustrated in Figure 5.6. These results confirmed the different behaviour of the various unsaturated fatty acids, with18:3ω3 decreasing at a greater rate than the saturated fatty acids; the 18:2ω6 appear to have the same degradation rate as the saturated fatty acids and the 18:1ω9 having increased slightly in relation to the saturated fatty acids.

Qualitative changes of fatty acids: 'new' fatty acids

In anaerobic conditions the only 'new' fatty acid detected was the 18:3 (1). The presence of this fatty acid was observed in the presence of anaerobic bacteria from all depths but only amounting to 2.2 % (w/w) with bacteria from the 15 cm depth, and less than 1% (w/w) with all the other depths.

5.1.2.3. Sulphate reducing bacteria degradation of the oil partially metabolised by aerobic bacteria

The main fatty acids present in the samples 7 days after the addition of SRB to the linseed oil partially degraded in aerobic conditions, are depicted in Figure 5.7. In this case, no data was available for the 30 cm depth sample since all the oil had been removed by filtration. The SRB seem to have increased the changes carried out previously in aerobic conditions, which, once more, were quantitative and qualitative.

Quantitative alterations of fatty acids

A comparison between the results obtained after the addition of SRB and those previously described for the aerobic bacteria showed a further decrease (9.4-42.4%) in the amount of 18:3ω3. The greatest decrease in the amount of this fatty acid was measured when SRB

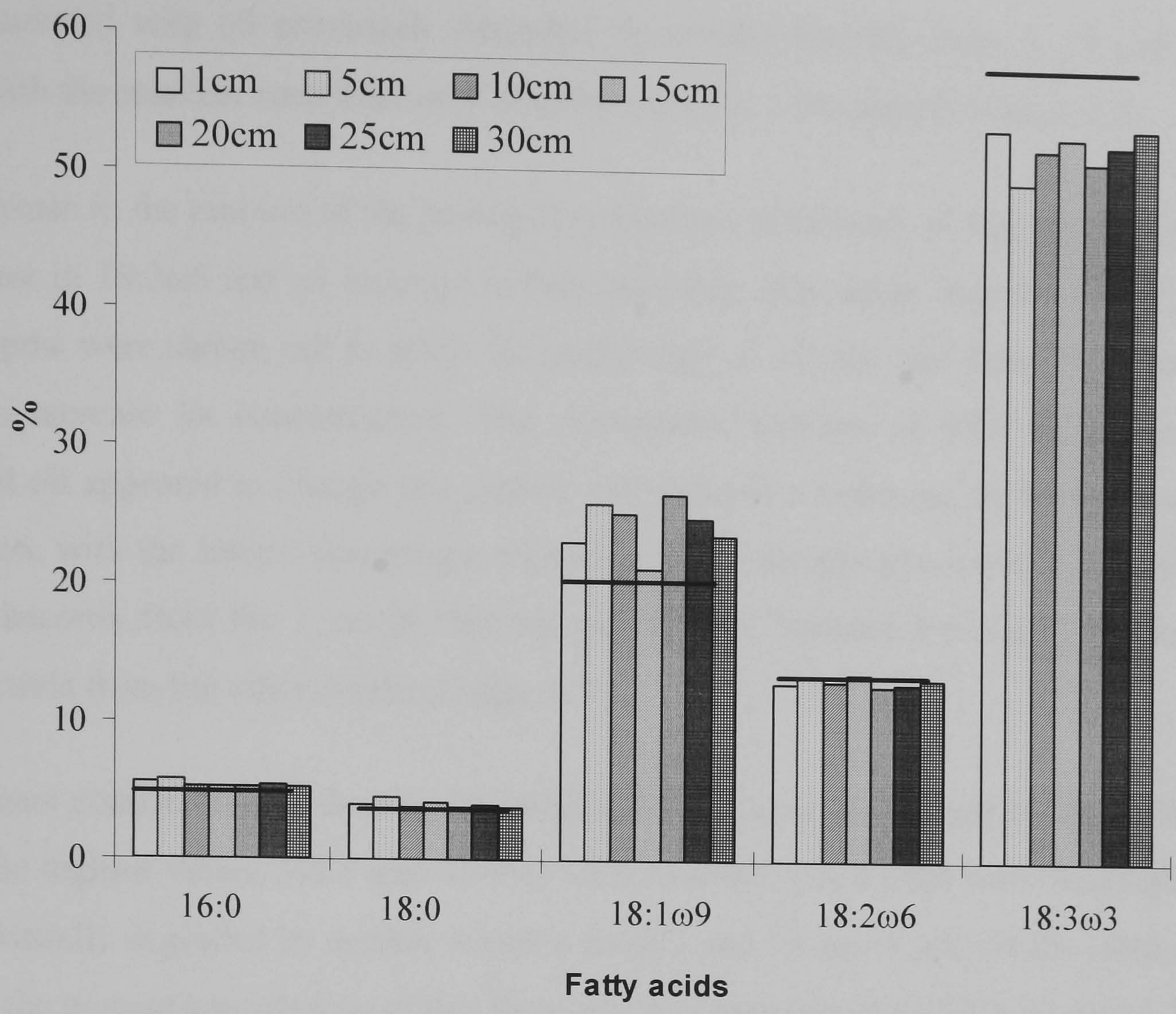


Figure 5.5. Fatty acids composition of the degraded linseed oil incubated with anaerobic bacteria from the various sediment depths. ——— percentage of each fatty acid in pure linseed oil.

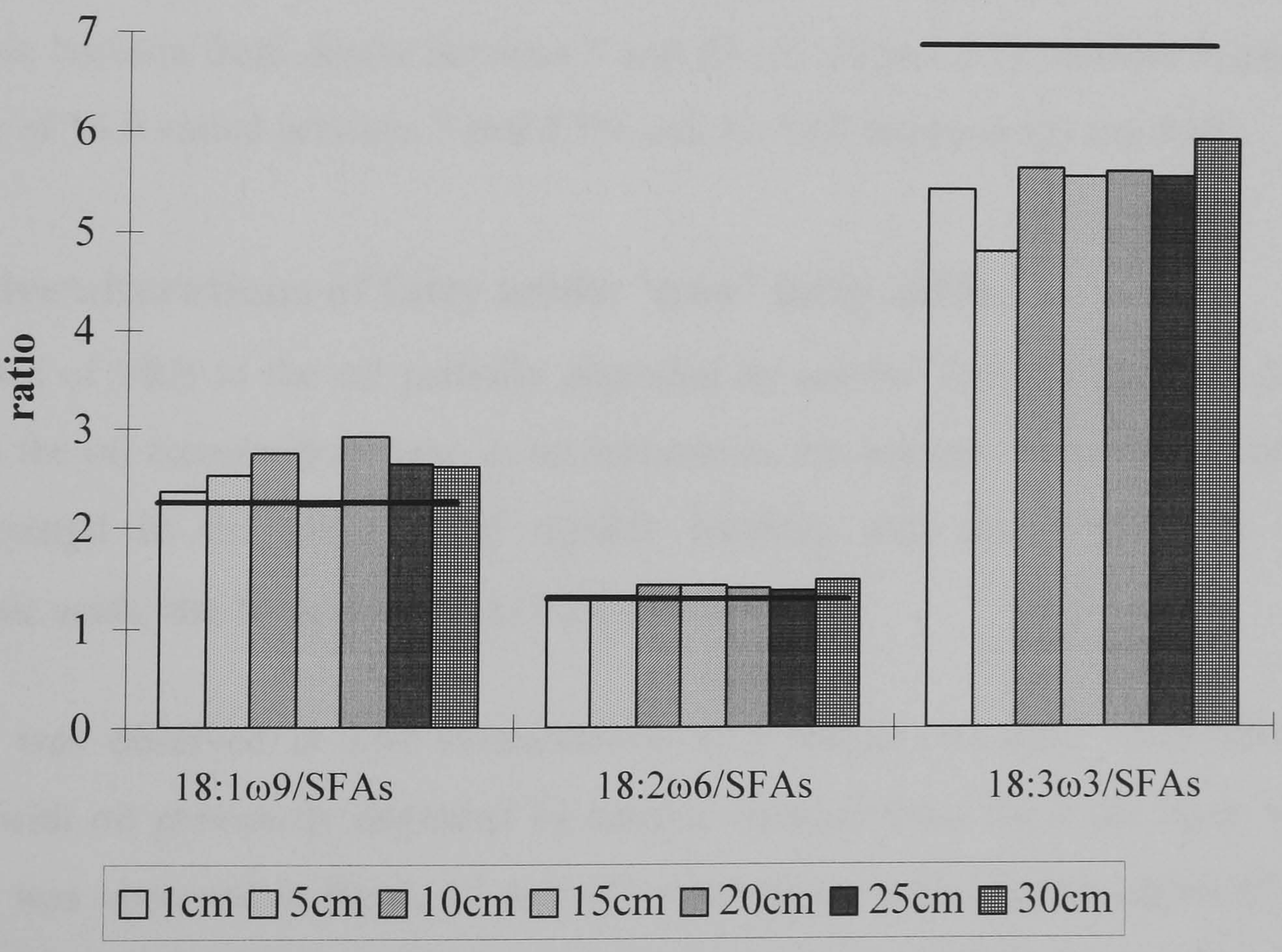


Figure 5.6. Ratio between unsaturated fatty acids and the sum of saturated fatty acids (SFAs, 16:0 +18:0) of linseed oil after degradation by anaerobic bacteria from the various sediment depths. ——— values for linseed oil.

were incubated with oil previously degraded by aerobic bacteria from 1, 15 and 25 cm depth, with the smallest contribution (9.4%) found in the 1 cm sample (Figure 5.7).

The decrease in the amount of the principal component of linseed oil was accompanied by a decrease in 18:2ω6 and an increase in the remaining fatty acids. Aerobic bacteria from most depths were shown not to affect the percentage of 18:2ω6 and those from the 1 cm depth to increase its concentration. The subsequent addition of SRB to this partially degraded oil appeared to change this pattern and induced a reduction in the concentration of 18:2ω6, with the lowest percentage registered in the sample previously incubated with aerobic bacteria from the 1 cm (6.1%) and percentages between 8.6 and 12.6% in those with bacteria from the other depths (Figure 5.7).

The percent contribution of the 18:1ω9 continued to increase as a result of the addition of SRB. The highest values (48.5 and 41.4%) were recorded when SRB were incubated with the oil partially degraded by aerobic bacteria from 1 and 15 cm depth. At the other depths studied, the percent contribution of this fatty acid was approximately 33% (Figure 5.7).

The proportion of the saturated fatty acids which were not altered by the aerobic bacteria, appear to have increased after addition of SRB, mainly in the samples previously incubated with aerobic bacteria from depths between 5 and 25 cm (Figure 5.7). In these samples, the percentage of 16:0 varied between 7 and 8.5% and the 18:0 between 5.6 and 8.6%.

Qualitative alterations of fatty acids: 'new' fatty acids

The addition of SRB to the oil partially degraded by aerobic bacteria introduced further changes to the oil composition, seen as an increase in the amount of the 'new' fatty acids which appeared in the presence of aerobic bacteria, and the introduction of two octadecenoic acids, the 18:1ω7 and the 18:1 (1) (Table 5.2).

The latter was observed at low concentration (0.5 weight %) only when SRB were incubated with oil previously degraded by aerobic bacteria from the 1 cm layer, whereas the former was observed in the 1, 15 and 25 cm samples, with slightly higher (3.7 - 7.1 weight %) abundance than the 18:1 (1). In general, the percentage of octadecadienoic acids increased slightly in relation to the values previously measured in the presence of aerobic bacteria. Still, these fatty acids were present in very small amounts and not in all of

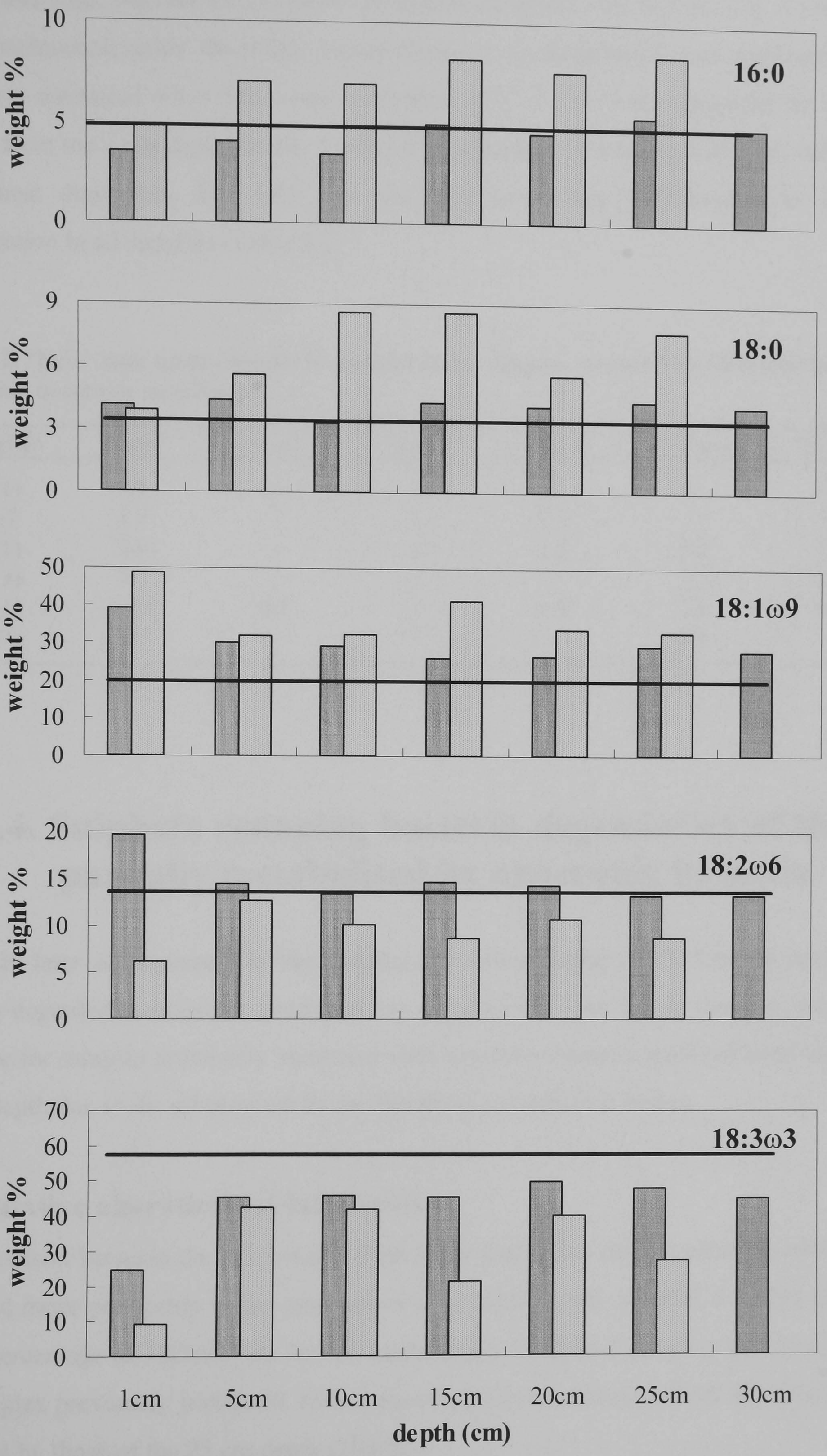


Figure 5.7. Fatty acids composition (weight %) of the linseed oil degraded by aerobic bacteria from the various sediment depths and followed by the addition of SRB Values for linseed oil.

samples analysed, with the 18:2 (1) more frequently observed than the 18:2 (3). As with the 'new' octadecenoic acids, the major contributions of octadecadienoic and octadecatrienoic acids were measured when SRB were incubated with oil previously degraded by aerobic bacteria from the 1 cm depth. In the 5 and 10 cm samples, the presence of 'new' fatty acid was almost negligible. The 18:3 (1) was the 'new' fatty acid present in highest concentration in all samples (Table 5.2).

Table 5.2. 'New' fatty acids (weight %) present in the samples degraded by SRB after previous degradation in aerobic conditions.

Fatty acids	1 cm	5 cm	10 cm	15 cm	20 cm	25 cm
18:1 (1)	0.5	-	-	-	-	-
18:1ω7	3.7	-	-	3.7	-	7.1
18:2 (1)	2.9	-	-	1.5	0.2	1.0
18:2 (3)	0.7	-	-	-	-	1.9
18:3 (1)	17.9	0.3	-	6.8	2.0	5.2
18:3 (4)	1.7	-	-	-	0.3	-

5.1.2.4. Sulphate reducing bacteria degradation of the oil partially metabolised by anaerobic bacteria

The main fatty acids present in the samples after the addition of SRB to the linseed oil partially degraded in anaerobic conditions are depicted in Figure 5.8. In this case, no data is available for samples previously incubated with anaerobic bacteria removed from the 1 and 10 cm depth due to the oil removal by the filtration, as explained before.

Quantitative alterations of fatty acids

A comparison between the proportion of the main fatty acids present after the addition of SRB and those previously in the presence of anaerobic bacteria showed a further decrease in the percentage of 18:3ω3. The lowest contribution of 18:3ω3 (12.3%) was measured in the samples previously incubated with anaerobic bacteria removed from the 5 cm depth, followed by those of the 25 cm depth (20.6%).

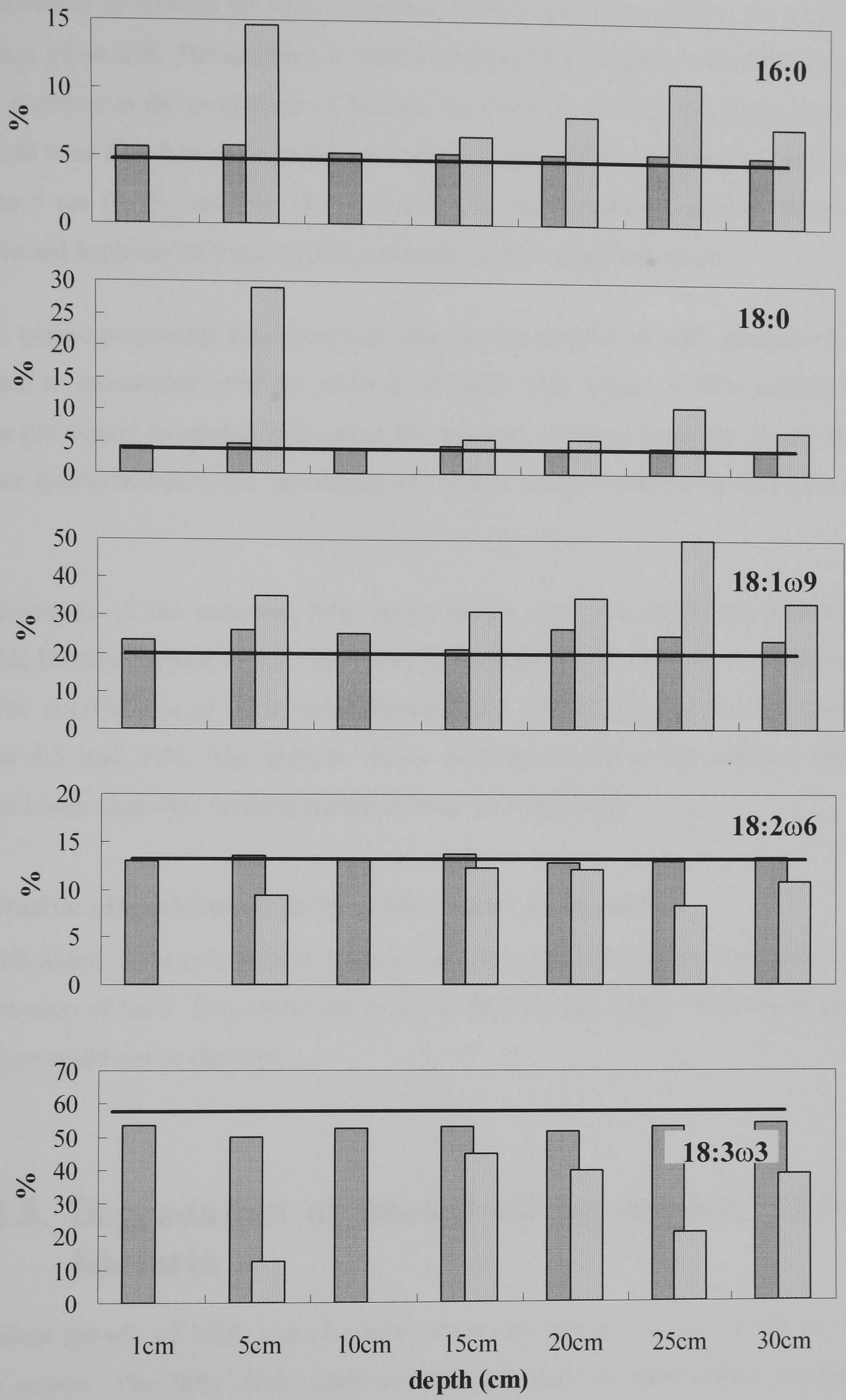


Figure 5.8. Fatty acids composition (weight %) of the linseed oil degraded by anaerobic bacteria from the various sediment depths ▒, followed by the addition of SRB. ▒ —— Values for linseed oil.

The incubation of linseed oil with anaerobic bacteria had been shown not to affect the percentage of 18:2ω6. The addition of SRB to this partially degraded oil seemed to induce a small decrease in the proportion of 18:2ω6. Similarly to 18:3ω3, the lowest percentages of 18:2ω6 were found in the samples previously degraded by anaerobic bacteria removed from the 5 cm (9.3%) and the 25 cm (8.2%). For the remaining samples these relative values varied between 10.9 and 12.3%, indicating a very small reduction.

18:1ω9, whose percentage had increased after the oil incubation with anaerobic bacteria, continued to accumulate after the addition of SRB, with values of 50% recorded in the samples previously incubated with anaerobic bacteria removed from the 25 cm depth. In the other depths samples, the percentage of 18:1ω9 varied between 31 and 35% (Figure 5.8).

The percentage of the saturated fatty acids, which were previously not altered by the anaerobic bacteria, appear to have increased in the presence of SRB, in all samples (Figure 5.8). The contribution of 16:0 varied between 6.6 and 14.6% and that of 18:0 varied between 5.5 and 29%. The highest values were measured in the samples previously incubated with anaerobic bacteria removed from the 5 cm depth.

Qualitative alterations of fatty acids: 'new' fatty acids

The SRB added to the oil partially degraded by anaerobic bacteria were not able to induce the formation of 'new' fatty acids and even the 18:3 (1) previously observed in anaerobic conditions could not be detected.

5.1.2.5. Degradation of linseed oil by sulphate reducing bacteria

No evident growth of SRB was observed when raw linseed oil was given as the only carbon source. The fatty acids analyses revealed that no quantitative or qualitative modifications were carried out by this group of bacteria on the composition of this oil (Table 5.3).

Table 5.3. Fatty acids composition (weight %) of pure and degraded linseed oil by sulphate reducing bacteria.

Conditions	16:0	18:0	18:1ω9	18:2ω6	18:3ω3
Linseed oil	4.9	3.6	20.3	13.3	57.9
After addition of SRB	5.3	3.4	23.4	12.4	55.5

5.2. EXPERIMENT II - LINSEED AND SUNFLOWER OILS DEGRADATION

In experiment I, the breakdown of the linseed oil was shown to be carried out by aerobic and anaerobic bacteria from the salt marsh sediments. SRB were not able to breakdown the raw oil but could continue its degradation subsequent to its partial degradation in aerobic and anaerobic conditions. The main objective of the 1996 experiments (Experiment II) was to further investigate the pathways and rates of degradation of both linseed and sunflower oils by various bacteria groups.

From the results previously obtained, it would be expected that the degradation of the vegetable oils was a sequential process carried out by bacteria belonging to various bacterial groups. To substantiate these observations, three experiments were carried out for each vegetable oil: in Experiment a) the breakdown of the pure oil by aerobic natural populations was followed by that of the anaerobic natural populations and by SRB; in Experiment b), the breakdown of the pure oil by anaerobic bacteria was followed by that of SRB; and in Experiment c) the pure oil degradation by SRB was studied.

To carry out these experiments some changes had to be made to the 1994 protocol (Experiment I), since the previous experiments showed that filtration was not an efficient method for the removal of bacteria, resulting in the loss of a large portion of the oil. Various methods were tested for their effectiveness in the elimination of bacteria: centrifugation, sonication, freezing and a combination of repeated freezing in liquid nitrogen and thawing. Although these methods are known to be effective in causing cell

lysis, in the present circumstances the results were not satisfactory for various reasons: these procedures did not kill the totality of bacteria; they altered the physical and chemical properties of the oil and they removed a significant part of the oil in the sample. Due to the problems encountered in selecting an efficient method to remove bacteria from the medium, it was decided that no other strategies would be tested, instead, the practical procedure of the experiments would be modified by not attempting to remove the bacteria from the media.

5.2.1. Experimental Strategy

Sediment samples were collected in the sampling area following the procedure described in section 5.1.1. In Experiment I (1994), the degradation of linseed oil by the bacteria extracted from the various sediment depths was studied. However, in Experiment II (1996) this procedure was abandoned due to the complexity of this experiment. The present experimental procedure involved the pooling of the sediment from all the studied depths. Bacteria populations from the pooled sediments were removed by sonication and inoculated in fortified sea water, following the same procedure described for the Experiment I. However, in this case, 0.5 cm^3 of linseed and sunflower oils was added to the fortified sea water.

Four replicates of each sample were taken, so statistical analysis of the data could be performed and degradation rates established.

Experiment a): Interaction between aerobic and anaerobic oil degrading bacteria and sulphate reducing bacteria

An aliquot of 1 cm^3 of each undiluted pooled sediment sample was inoculated in 32 "universal bottles" containing 9 cm^3 of medium, and in 4 "universal bottles" with fortified sea water but no oil - "control". Samples were incubated at 20°C. After 14, 28 and 42 days of incubation in aerobic conditions, 4 bottles were taken for chemical analyses: *aero (D14)*, *aero (D28)*, *aero (D42)* respectively.

After 14 days of incubation, the oxygen was removed from the remaining 20 samples and a reducing agent (sodium thioglycollate - ST) was added to ensure anaerobic conditions. The approach followed was the same explained before (Section 4.2.4.2 & Appendix). Four of these samples were incubated at 20°C in anaerobic conditions for 14 days and the other four for 28 days, after which the samples were taken aside for chemical analysis. These samples are referred as: *aero+ST (D28)* and *aero+ST (D42)*, respectively.

The remaining 12 samples were inoculated with anaerobic bacteria which were freshly collected from the various pooled layers of salt marsh sediments, as detailed before. Fresh natural bacteria populations of the salt marsh sediments were used to avoid changes in the characteristics of the population if the samples had been preserved. These samples were incubated in anaerobic conditions at 20°C for 14 days (4 replicates) and 28 days (another 4 replicates), after which chemical analyses were performed. These samples are referred as: *aero+ST+ana (D28)* and *aero+ST+ana (D42)*, respectively.

At day 28, the remaining 4 samples were inoculated with colonies of SRB, that have been grown according to the procedure described in section 4.2.4.2.3. These samples were incubated in anaerobic conditions, at 20°C for 14 days [(*aero+ST+ana+SRB (D42)*], which were then chemically analysed.

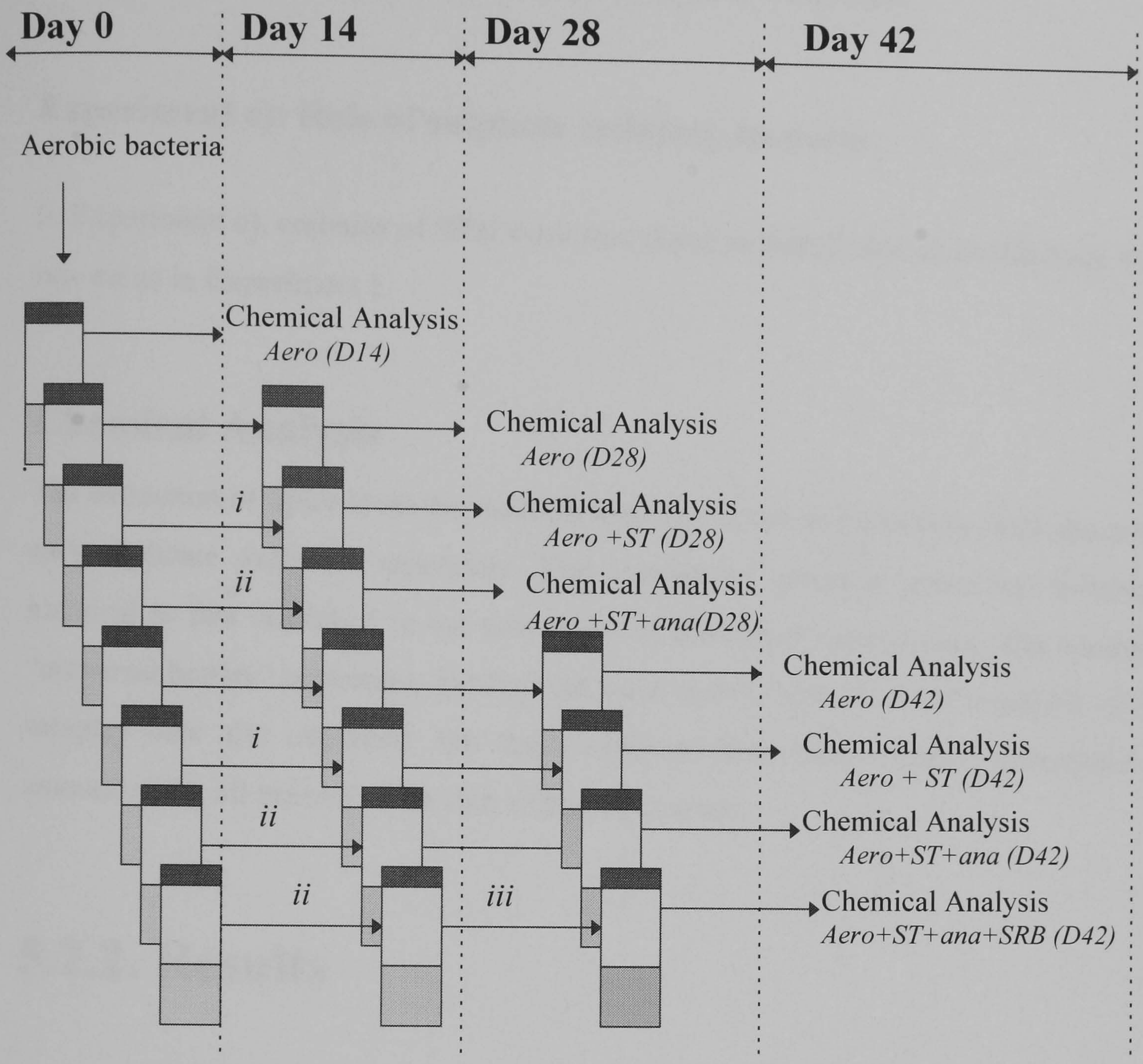
The practical approach just described is represented in Figure 5.9. where one bottle is used to represent 4 replicates.

Experiment b): Interaction between anaerobic oil degrading bacteria and sulphate reducing bacteria

In this experiment, the pooling of the samples and their inoculation was carried out as for Experiment a) but in anaerobic conditions. Twelve samples were incubated in anaerobic conditions at 20°C for 14 days after which 4 replicates [*ana (D14)*] were set apart for chemical analyses; another 4 were kept in the same conditions and to the remaining 4, colonies of SRB were added. These were also incubated in anaerobic conditions at 20°C for 14 days.

After 28 days from the start of the experiment, the remaining 8 samples [*ana (D28)* and *ana+SRB (D28)*] were chemically analysed.

Experiment a)



Experiment b)

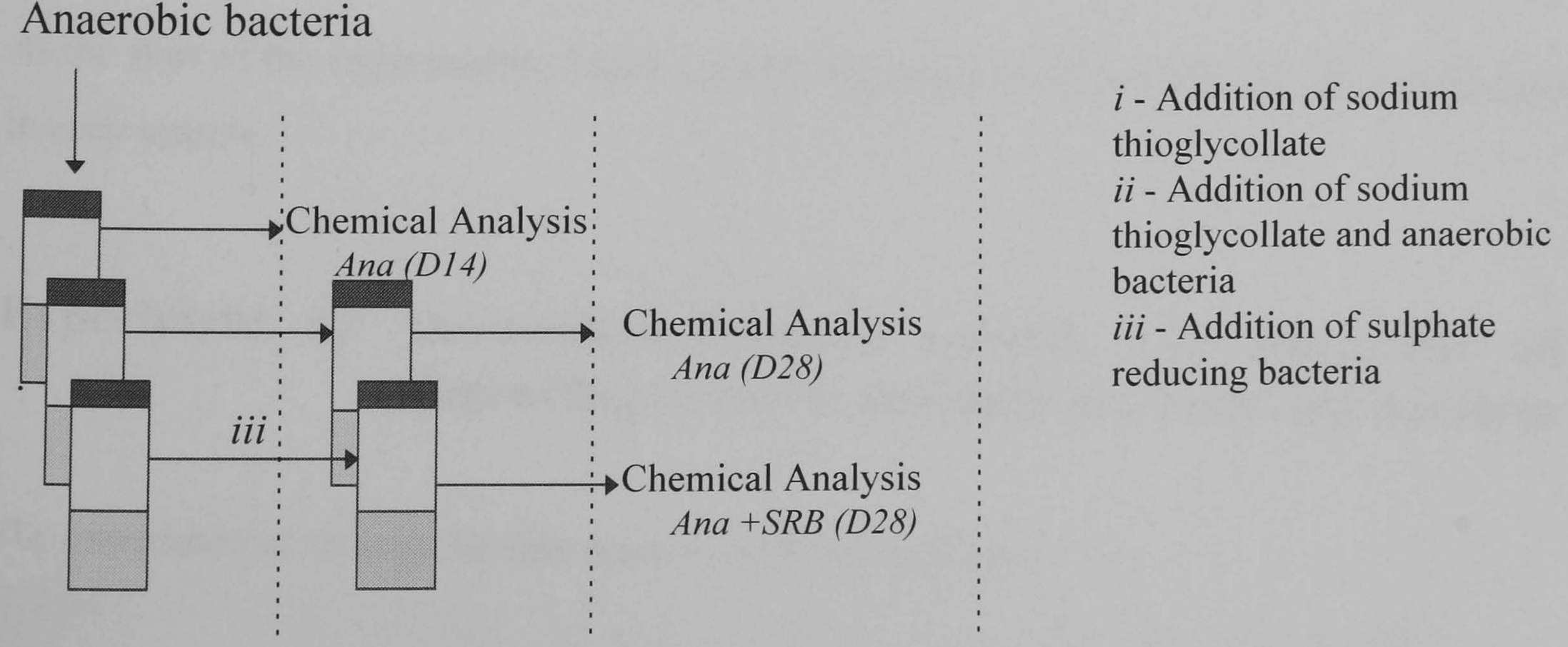


Figure 5.9. Schematic representation of the strategy followed to test *'in vitro'* the vegetable oils degradation by indegenous aerobic, anaerobic and sulphate reducing bacteria [experiment a)] and by anaerobic and sulphate reducing bacteria [experiment b)] from the salt marsh sediments. *Aero*: aerobic bacteria; *ana*: anaerobic bacteria; *ST*: sodium thioglycollate; *SRB*: Sulphate reducing

The practical approach just described is represented in Figure 5.9 and as in the previous case, one bottle is used diagrammatically to represent 4 replicates.

Experiment c): Role of sulphate reducing bacteria

In Experiment c), colonies of SRB were inoculated in 4 replicates in fortified sea water and raw oil as in Experiment I.

Chemical Analysis

The extraction of lipids from the medium was carried out as previously, with the content of each replicate extracted separately. The subsequent practical procedure followed was identical to that described in the former set of laboratory experiments. The contents of 4 "universal bottles" containing fortified sea water and 0.5 cm^3 of either vegetable oil (but no sample) were also extracted. The lipids extracted from these samples correspond to the amount of the oil present at the start of the experiment.

5.2.2. Results

5.2.2.1. Linseed oil

At the start of the experiments, 393.6 ± 13.07 mg (mean ± SD) of linseed oil were present in each sample.

Experiment a): Interaction between aerobic and anaerobic oil degrading bacteria and sulphate reducing bacteria

The experimental strategy for this assay is depicted in Figure 5.10.

In the present case and contrasting with Experiment I (linseed oil degradation by bacteria removed from different sediment depths), a much slower growth (based on medium

turbidity) of aerobic bacteria occurred. For this reason, the initial incubation period was extended to 14 days, even though after this extended time, growth was still less intense than in the previous case.

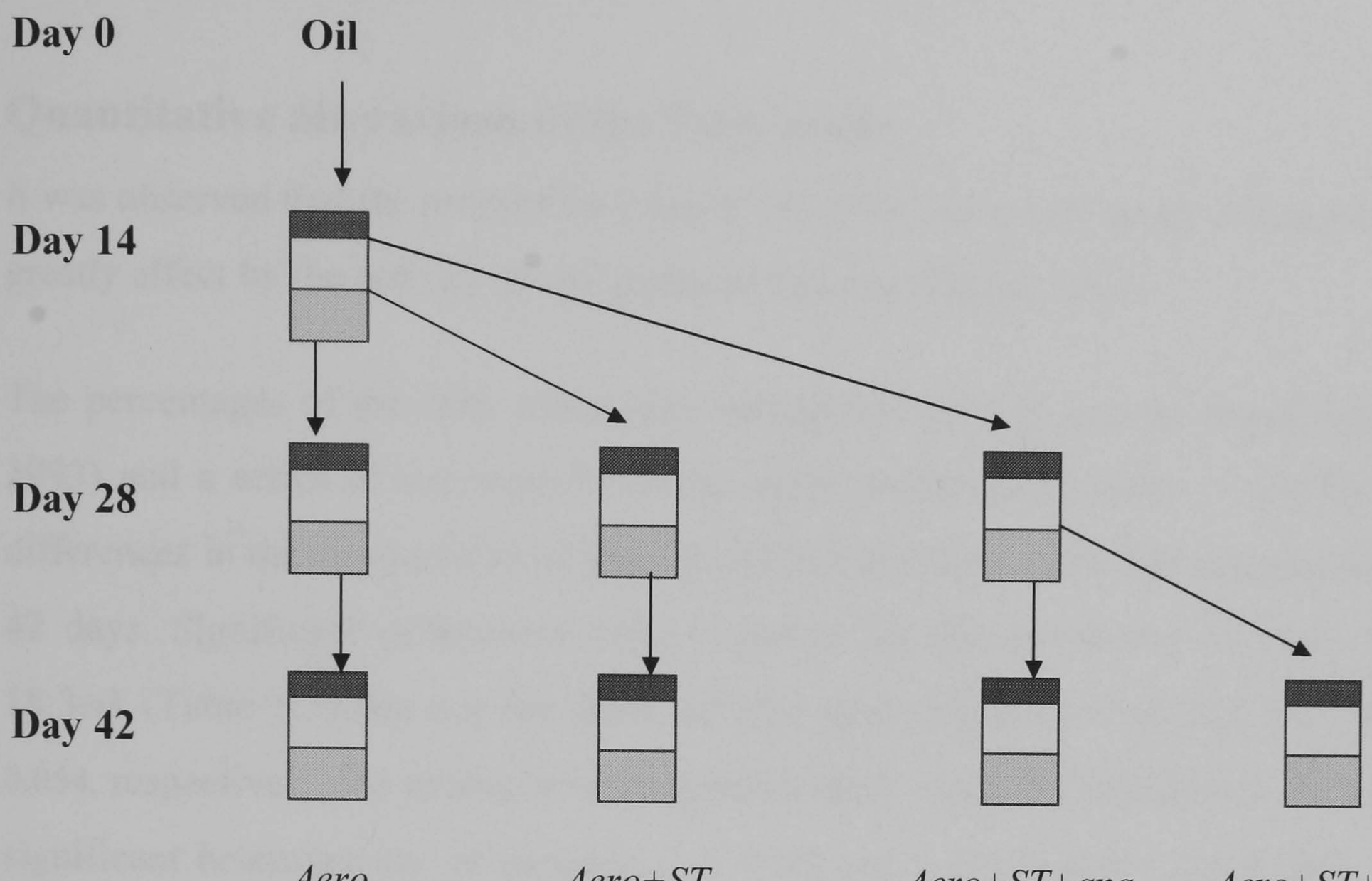


Figure 5.10. Schematic strategy of the laboratory experiment regarding the *'in vitro'* oil degradation by aerobic followed by anaerobic and sulphate reducing bacteria. *aero*: incubation with aerobic bacteria; a*ero+ST*: incubation with aerobic bacteria and reducing agent (sodium thioglycollate-ST); *aero+ST+ana*: incubation aerobic plus anaerobic bacteria and reducing agent; *aero+ST+ana+SRB*: incubation with aerobic, anaerobic plus sulphate reducing bacteria and reducing agent.

The amount of lipids (mg) extracted from each sample, after incubation in the various conditions ranged between 391.2 and 397 mg (Table 5.4.).

Table 5.4. Amount of lipids extracted (mg, mean ± sd) from the various samples after incubation of linseed oil with aerobic, anaerobic and sulphate reducing bacteria.

Aero (D14)	***Aero (D28)***	***Aero +ST (D28)***	***Aero+ST+ana (D28)***
391.2 ± 13.77	395.4 ± 10.35	396.4 ± 8.66	395.6 ± 11.98
Aero (D42)	***Aero+ST (D42)***	***Aero +ST+ana (D42)***	***Aero+ST+ana+SRB (D42)***
397.0 ± 9.59	394.0 ± 14.1	393.4 ± 10.43	392.8 ± 12.28

aero (D14), aero (D28), aero (D42): incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28), aero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively; *aero+ST+ana (D28)*, a*ero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; a*ero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and

A one-way ANOVA indicated no significant differences in the amount of oil extracted from the various treatments or the control ($F=0.08$; $p=0.999$, the residuals were approximately normally distributed and there was no significant heterogeneity of variance $\chi^2 = 0.749$; $p=0.999$) This indicated no significant quantitative degradation of linseed oil over the period of incubation.

Quantitative alterations of the fatty acids

It was observed that the proportion (weight %) of the main fatty acids of linseed oil was not greatly affect by the activity of any group of bacteria (Figure 5.11).

The percentages of the fatty acids were normalised through arcsine transformations (Fry, 1993) and a series of one-way ANOVAs were performed in order to test for significant differences in the composition of pure linseed oil and that of the four samples incubated for 42 days. Significant differences were observed for the proportion of 16:0, 18:2ω6 and 18:3ω3 (Table 5.5) but not for those of 18:0 and 18:1ω9 ($F=2.92$ and 3.05; $p=0.060$ and 0.054, respectively, the residuals were approximately normally distributed and there was no significant heterogeneity of variance $\chi^2 = 1.453$ and 3.316; $p=0.835$ and 0.506). The results obtained showed that neither the proportion of 18:0 nor that of 18:1ω9 was affected by aerobic, anaerobic or SRB during 42 days of incubation, consequently further analysis were not carried out for these two fatty acids.

Table 5.5. F and p values for the three one-way ANOVA applied to the percentage of 16:0, 18:2ω6 and 18:3ω3 (arcsine transformed), in samples incubated in various conditions with aerobic, anaerobic and sulphate reducing bacteria, and in the pure linseed oil.

Various samples tested	**16:0**		**18:2ω6**		**18:3ω3**	
	F	p	F	p	F	p
Oil & *Aero (D42)* & *aero+ST (D42)* & *aero+ST+ana (D42)* & *aero+ST+ana+SRB (D42)*	5.72	0.006	8.18	0.001	23.39	<0.001
Oil & *aero (D14)* & *aero (D28)* & *aero (D42)*	18.14	<0.001	34.99	<0.001	26.17	<0.001
aero (D14) & *aero (D42)* & *aero+ST (D42)* & *aero+ST+ana (D42)* & *aero+ST+ana+SRB (D42)*	0.76	0.569	15.00	<0.001	36.06	<0.001

aero (D14), *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively: a*ero+ST (D28)*, a*ero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively: *aero+ST+ana (D28)*, *aero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; a*ero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

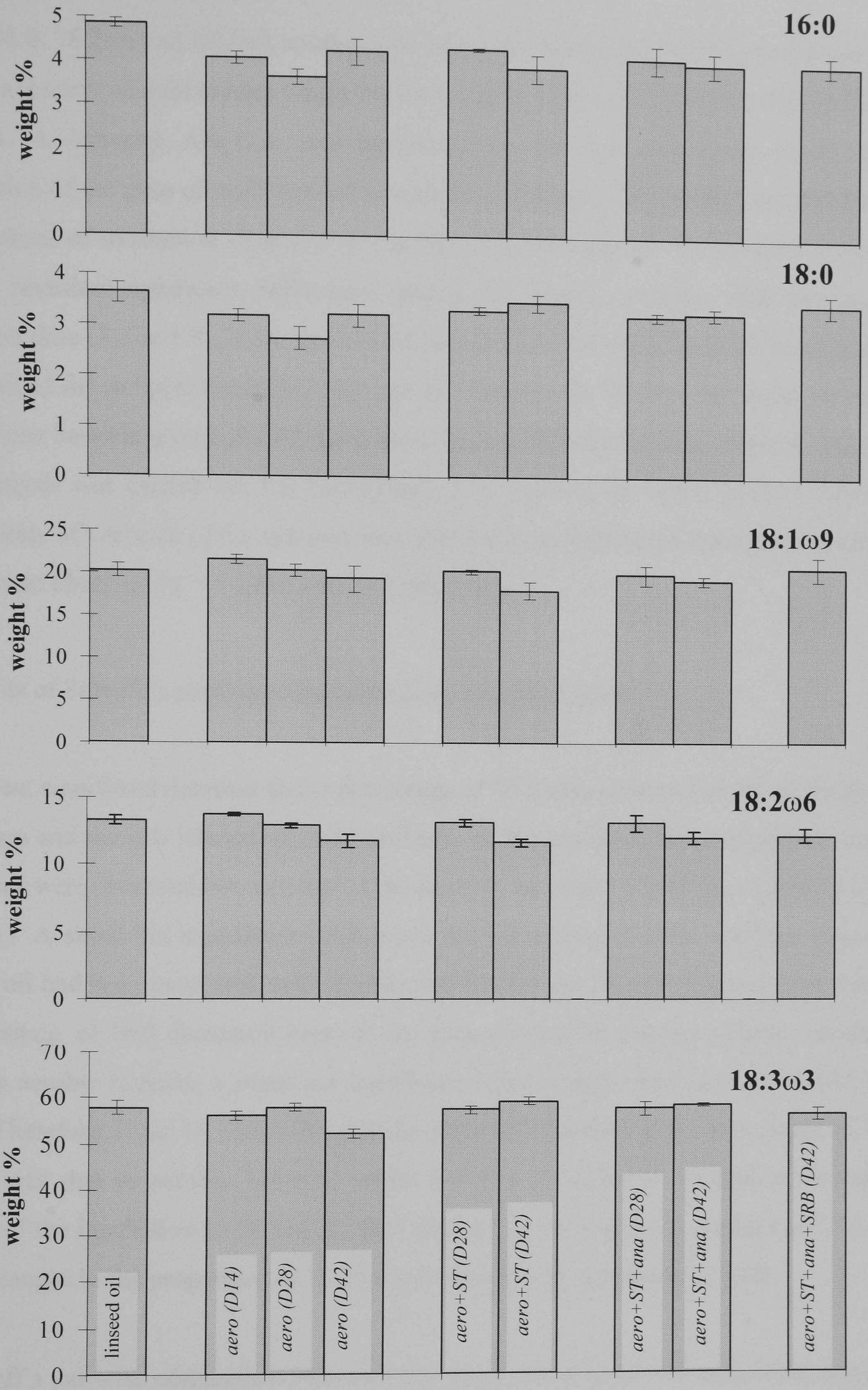


Figure 5.11. Fatty acid composition (weight %, ± sd) of linseed oil incubated with aerobic, anaerobic and sulphate reducing bacteria. *aero (*D*14)*, *aero (*D*28)*, *aero (*D*42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28)*, *aero+ST (*D*42)*; incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days respectively; *aero+ST+ana (*D*28)*, *aero+ST+ana (*D*42):* incubation with aerobic, anaerobic bacteria and reducing agent for 28 and 42 days respectively; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

For the 16:0, 18:2ω6 and 18:3ω3, another two one-way analysis of variance was carried out with the objective of establishing which bacteria groups were responsible for the alterations observed. A one-way ANOVA was performed to test for differences between the composition of the pure oil and that of the samples incubated with aerobic bacteria for the various times of incubation (Table 5.5). For the 16:0, 18:2ω6 and 18:3ω3 fatty acids the analyses revealed significant differences among the various samples with varying the incubation time (Table 5.5). In the case of 18:2ω6 and 18:3ω3, significant differences were also obtained for samples incubated with aerobic bacteria for 14 days and those incubated with various bacteria groups for 42 days; however, no differences were obtained when the same analysis was carried out for 16:0 (Table 5.5). Prior to the series of ANOVAs, the homogeneity of variance of the samples was tested and no significant heterogeneity among samples was observed (χ^2 = 1.289-6.016; p=0.198-0.894).

The results of Scheffe's pairwise comparisons are shown in Table 5.6.

A small but significant decrease in the percentage of 16:0 was observed in the oil incubated for 42 days and the raw linseed oil, independently of the groups of bacteria present, but no differences were observed between any of the samples incubated for 42 days (Table 5.6 and Fig. 5.11). A small but significant decrease in the proportion of 16:0 was also observed after the oil had been incubated with aerobic bacteria for 14, 28 and 42 days. Even though the percentage of 16:0 decreased between the pure oil and the various samples incubated only with aerobic bacteria, a small but significant increase was observed between days 28 and 42. Therefore, it can be concluded that the observed decrease in the percentage of 16:0 to 4.1% was due to aerobic bacteria within the first 14 days of incubation. Increased duration of the incubation to 28 and 42 days seemed to reverse these changes (4.2%). No further changes in the proportion of 16:0 were carried out by anaerobic or SRB.

The Scheff's pairwise comparison indicated that there was a small but significant decrease in the proportion of 18:2ω6 between the pure oil and the various samples incubated for 42 days (Table 5.6 and Fig. 5.11). This test also revealed no significant differences among the samples incubated with the different bacteria groups after 42 days. Samples incubated for different lengths of times with aerobic bacteria only showed a significant decrease in the

percentage of 18:2ω6 after 42 days of incubation (Table 5.6). Therefore, when samples were inoculated with anaerobic bacteria (at day 14) no significant transformations in the percentage of 18:2ω6 had yet occurred. These results indicate that the decrease in the 18:2ω6 percentage (to 11.8 weight %) was due to the activity of the aerobic bacteria after a 42 days period and that a similar decrease resulted from the activity of the anaerobic bacteria (to 12.0 weight %). SRB did not seem to have a role in the degradation of 18:2ω6.

Table 5.6. Results of Scheffe's pairwise comparison (difference of the means ± 95% confidence interval) of the proportion of 16:0, 18:2ω6 and 18:3ω3 (arcsine transformed) between pure linseed oil and the samples in various incubation conditions with aerobic, anaerobic and sulphate reducing bacteria.

pairwise comparison	**16:0**	**18:2ω6**	**18:3ω3**
oil & *aero (D42)*	0.015 ±0.0135 *	0.020 ±0.0159 *	0.058 ±0.0276*
oil & *aero+ST (D42)*	0.023 ± 0.0187*	0.024 ±0.0159 *	-0.013 ±0.0276
oil & *aero+ST+ana (D42)*	0.020 ±0.0187*	0.019 ±0.0159 *	-0.005 ±0.0276
oil & *aero+ST+ana+SRB (D42)*	0.020 ± 0.0187*	0.016 ±0.0159 *	0.0142 ±0.0276
aero (D42) & *aero+ST (D42)*	0.009 ± 0.0173	0.004 ±0.0147	-0.070 ±0.0256*
aero (D42) & *aero+ST+ana (D42)*	0.006 ± 0.0173	-0.001 ±0.0147	-0.062 ±0.0256*
aero (D42) & *aero+ST+ana+SRB (D42)*	0.005 ± 0.0173	-0.004 ±0.0147	-0.043 ±0.0256*
aero+ST (D42) & *aero+ST+ana (D42)*	-0.003 ± 0.0173	-0.005 ±0.0147	0.008 ±0.0256
aero+ST (D42) & *aero+ST+ana+SRB (D42)*	-0.004 ± 0.0173	-0.008 ±0.0147	0.027 ±0.0256*
aero+ST+ana (D42) & *aero+ST+ana+SRB (D42)*	-0.001 ± 0.0173	-0.003 ±0.0148	0.019 ±0.0256
oil & *aero (D14)*	0.019 ±0.0145 *	-0.006 ±0.0099	0.019 ±0.0282
oil & aero (D28)	0.029 ±0.0135 *	0.006 ±0.0092	0.0003 ±0.0264
aero (D14) & *aero (D28)*	0.011 ±0.0135	0.012 ±0.0092*	-0.018 ±0.0264
aero (D14) & *aero (D42)*	-0.004 ±0.0135	0.027 ±0.0092*	0.039 ±0.0264*
aero (D28) & *aero (D42)*	0.015 ±0.0125 *	0.015 ±0.0085*	0.057±0.0244*
aero (D14) & *aero+ST (D42)*	-	0.030 ±0.0150*	-0.032 ±0.0246*
aero (D14) & *aero+ST+ana (D42)*	-	0.025 ±0.0150*	-0.024 ±0.0246
aero (D14) & *aero+ST+ana+SRB (D42)*	-	0.022 ±0.0150*	-0.005 ±0.0246

*significant differences; a*ero (D14)*, *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; a*ero+ST (D28)*, a*ero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively; *aero+ST+ana (D28)*, a*ero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; a*ero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

A small but significant decrease in the percentage of 18:3ω3 between the pure oil and the sample incubated with aerobic bacteria for 42 days was observed, but no significant changes occurred between the oil and the other samples incubated for 42 days (Table 5.6).

Therefore, significant differences were registered between the sample containing aerobic bacteria (day 42) and the others incubated for 42 days. Within 14 days of incubation with aerobic bacteria, a small but not significant decrease in the amount of 18:3ω3 was observed but this change was reversed after 28 days in the same incubation conditions and 42 days were necessary for a significant decrease to be measured. Consequently, at the time of the inoculation with anaerobic bacteria (at day 14), no changes in the percentage of 18:3ω3 had occurred. The pairwise comparison also indicated no significant changes in the proportion of 18:3ω3 between samples containing only aerobic bacteria (day 14) and those containing anaerobic bacteria and anaerobic plus SRB at day 42 (Table 5.6 and Fig. 5.11). Such results suggest that anaerobic bacteria and SRB were not able to change the proportion of 18:3ω3 in the oil. Still, an increase in the amount of 18:3ω3 was registered between the samples containing aerobic bacteria (day 14) and those containing aerobic bacteria and reducing agent (day 42). This increase explains the significant differences observed between the samples *aero+ST+ana* (D42) and *aero+ST+ana+SRB* (D42). Therefore, two different modifications occurred in the percentage of 18:3ω3; the first corresponding to a decrease (to 52.2 weight %) carried out by aerobic bacteria after 42 days of incubation, and the second to an increase (to 58.4 weight %) possibly performed by facultative anaerobic bacteria (as the addition of reducing agent should inhibit the activity of obligate aerobic bacteria).

Qualitative alterations of the fatty acids: 'new' fatty acids

The presence of some 'new' fatty acids observed both in the field and laboratory Experiment I, [18:3 (1 & 4)] and also of fatty acids not seen in the previous experiments [18:2 (5) and 18:3 (5 & 6)] has been detected (Table 5.7). These fatty acids were identified as two octadecenoic acids [18:1ω7 and 18:1 (1)], one octadecadienoic with shorter retention time than that of 18:2ω6, 18:2 (5), and 4 octadecatrienoic acids, two of which (5 & 6) had a shorter retention time than that of 18:3ω3. All of the 'new' fatty acids were observed in the various incubation conditions (Table 5.7). However, they were present in very small amounts, in most of the cases less than 1 weight %, increasing to percentages >1% at day 42 of incubation with aerobic bacteria.

Table 5.7. 'New' fatty acids (weight %) present in the various samples incubated with linseed oil and aerobic, anaerobic plus sulphate reducing bacteria.

Fatty acids	*aero (D14)*	*aero (D28)*	*aero+ST (D28)*	*aero+ST +ana (D28)*	*aero (D42)*	*aero+ST (D42)*	*aero+ST +ana (D42)*	*aero+ST+ ana+SRB (D42)*
18:1(1)	-	0.24	0.10	0.15	0.51	0.12	-	-
18:1ω7	0.09	0.06	0.06	0.09	0.03	0.05	0.19	0.18
18:2 (5)	0.06	0.47	0.07	0.09	0.10	0.12	0.14	0.11
18:3 (1)	0.44	0.05	0.12	0.10	0.11	0.06	0.10	0.13
18:3 (4)	0.04	0.04	0.05	0.05	0.04	0.05	0.04	0.05
18:3 (5)	0.20	1.03	0.63	0.53	2.75	0.97	1.20	1.07
18:3 (6)	-	-	-	-	0.82	0.26	0.27	0.24

aero (D14), *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28)*, a*ero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively; *aero+ST+ana (D28)*, *aero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

Experiment b): Interaction between anaerobic oil degrading and sulphate reducing bacteria

A schematic strategy of this experiment is depicted in Figure 5.12.

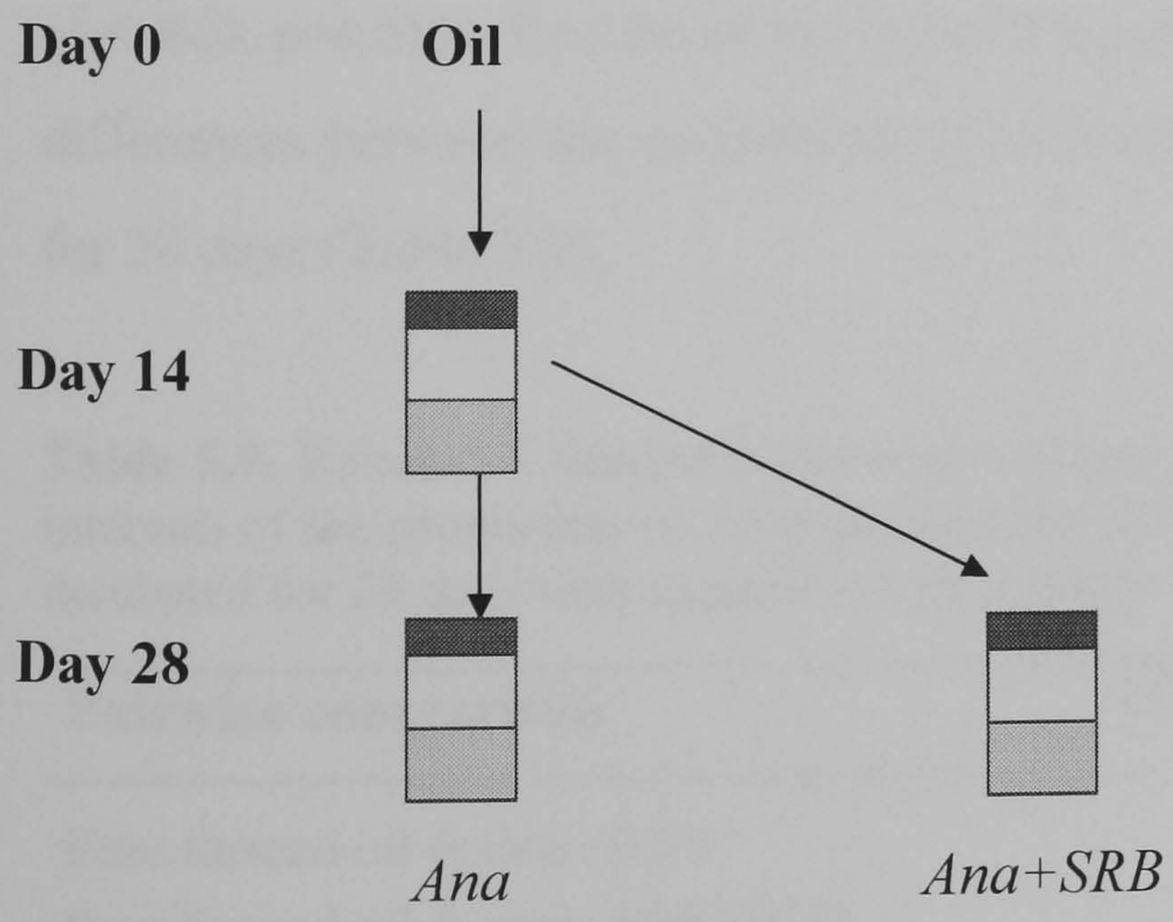


Figure 5.12. Schematic representation of the experimental strategy for the determination of the interaction between anaerobic and sulphate reducing bacteria in the oil degradation. *Ana*: oil incubated with anaerobic bacteria; *Ana+SRB*: oil incubated with anaerobic and sulphate reducing bacteria.

The amount of lipids extracted from the various samples is shown in Table 5.8. A one-way ANOVA indicated no significant differences in the amount of oil extracted from the various treatments or the control ($F=0.87$; $p=0.483$, the residuals were approximately

normally distributed and there was no significant heterogeneity of variance $\chi^2 = 0.302$; p = 0.960). This indicated no significant degradation of linseed oil over the period of incubation.

Table 5.8. Amount of lipids extracted (mg, mean ± sd) after linseed oil incubation with anaerobic and sulphate reducing bacteria.

ana (D14)	*ana (D28)*	*Ana SRB (D28)*
382.3 ± 10.97	386.8 ± 9.52	383.2 ± 10.24

ana (D14), ana (D28): oil incubated with anaerobic bacteria for 14 and 28 days respectively; a*na+SRB (D28)*: oil incubated with anaerobic and sulphate reducing bacteria for 28 days.

Quantitative alterations of the fatty acids

The percentage of the main fatty acids in the pure linseed and in the samples incubated with anaerobic and sulphate reducing bacteria are illustrated in Figure 5.13.

A one-way ANOVA was used to test for differences in the proportion of 16:0 in the pure linseed oil and in the two samples incubated for 28 days [*ana* (D28) & *ana+SRB* (D28)] following the arcsine-transformations of the data. The analysis revealed that at least one of treatment was significantly different from one other (F=4.56; p=0.048, the residuals were approximately normally distributed and there was no significant heterogeneity of variance χ^2=0.860; p=0.651). Results of the Scheffe's pairwise comparison test showed no significant differences between the proportion of 16:0 in the pure oil and the two samples incubated for 28 days (Table 5.9).

Table 5.9. Results of Scheffe's pairwise comparison (difference of the means ± 95% confidence interval) of the proportion of 16:0 (arcsine transformed) between pure linseed oil and the samples incubated for 28 days with anaerobic and sulphate reducing bacteria.

Pairwise comparison	**difference of the means ± 95% confidence interval**
Pure linseed oil & *ana (D28)*	-0.004 ± 0.0094
Pure linseed oil & *ana+SRB (D28)*	0.006 ± 0.0094
ana (D28) & *ana+SRB (D28)*	0.009 ± 0.0087*

*significant differences; *ana (D28)*: oil incubated with anaerobic bacteria for 28 days; *ana+SRB (D28)*: oil incubated with anaerobic and sulphate reducing bacteria for 28 days.

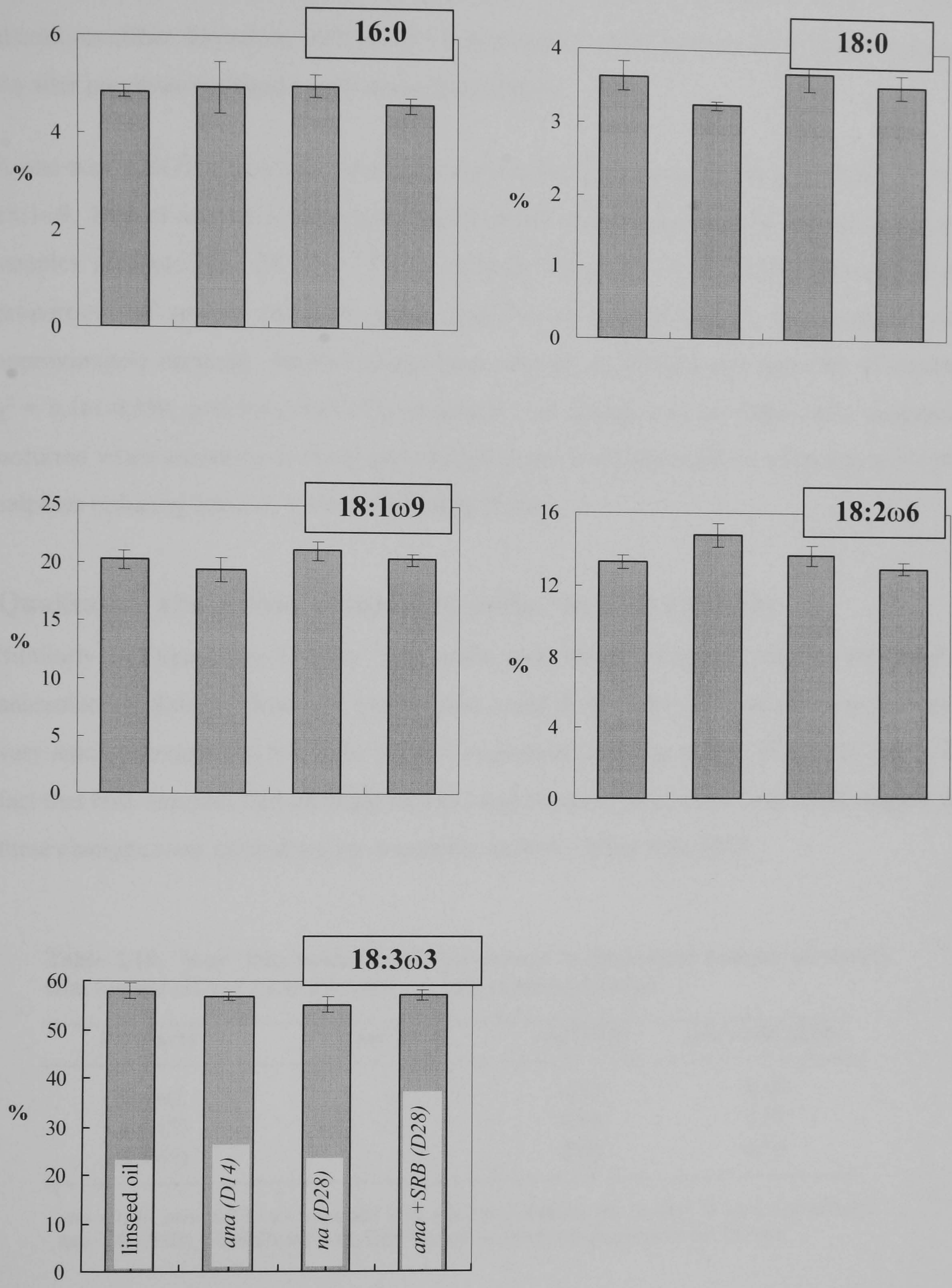


Figure 5.13. Fatty acid composition (weight %, ± sd) of linseed oil incubated with anaerobic and sulphate reducing bacteria. *ana (D14)*, *ana (D28)*: incubation with anaerobic bacteria for 14 and 28 days respectively: *ana+SRB (D28):* incubation with anaerobic and sulphate reducing bacteria for 28 days.

When SRB were added to the medium no changes had occurred in the proportion of 16:0 and the increase in the duration of incubation with anaerobic bacteria did not induce alterations either. However, SRB caused a decrease in the proportion of 16:0 (to 4.6 weight %) after previous incubation with anaerobic bacteria.

A one-way ANOVA was also carried out to test for differences in the proportion of 18:0, 18:1ω9, 18:2ω6 and 18:3ω3 (arcsine transformed) in the pure linseed oil and in the two samples incubated for 28 days. These analyses indicated no significant changes in the proportions of any of the fatty acids ($F=0.67-3.84$; $p= 0.068-0.538$, the residuals were approximately normally distributed and there was no significant heterogeneity of variance $\chi^2 = 0.161-0.539$; $p=0.764-0.867$). Consequently, no changes in the fatty acids proportion occurred when anaerobic bacteria were added to the raw linseed oil or when anaerobic plus sulphate reducing bacteria were present (Fig. 5.13).

Qualitative alterations of the fatty acids: 'new' fatty acids

Similarly to Experiment I, 'new' fatty acids were observed in the samples incubated in anaerobic conditions. However, in this case, only three 'new' fatty acids were present in very small amounts (<0.5 weight %) and exclusively present at day 28 (Table 5.10). The fact that both samples had identical number and percentage of these fatty acids suggest that these changes were carried out by anaerobic bacteria rather than SRB.

Table 5.10. 'New' fatty acids (weight %) present in the various samples incubated with linseed oil and anaerobic plus sulphate reducing bacteria

Fatty acid	*ana (D14)*	*ana (D28)*	*ana+SRB (D28)*
18:1ω7	-	0.35	0.09
18:3 (5)	-	0.18	0.18
18:3 (4)	-	0.06	0.05

ana (D14), *ana (D28)*: oil incubated with anaerobic bacteria for 14 and 28 days respectively: a*na+SRB (D28)*: oil incubated with anaerobic and sulphate reducing bacteria for 28 days.

Experiment c): Role of sulphate reducing bacteria

As in Experiment I, no clear bacterial growth (turbidity of the medium) was observed when SRB were given linseed oil as the only carbon source. A 2-sample t-test assuming equal

variance (Bartlett's test: χ^2=0.001; p=0.979) was used to test for differences between the initial amount of extracted lipids prior to (393.6 ± 13.07 mg) and after 14 days of incubation with SRB (390.8 ± 13.29 mg), and showed no significant differences between the samples (T=0.30; p=0.77; DF=6). Therefore no gross degradation of the oil occurred.

Quantitative analysis of the fatty acids

The proportion of the main fatty acids in linseed oil incubated for 14 days with SRB, is illustrated in the Table 5.11.

Table 5.11. Fatty acids composition (weight %, mean ± sd) of linseed oil after 14 days incubation with sulphate reducing bacteria.

16:0	18:0	18:1ω9	18:2ω6	18:3ω3
4.9 ± 0.18	3.8 ± 0.25	20.4± 0.49	14.6 ± 1.15	55.7 ± 1.12

The differences in the percentage of each fatty acid (arcsine transformed) in the pure linseed oil and in the sample containing SRB was investigated using a series of 2-sample t-tests assuming equal variance (Bartlett's test χ^2 =0.086-1.745; p=0.187-0.769) and no significant differences were found (T=0.01- 1.78; p=0.14-0.99; DF=5)

Qualitative analysis of the fatty acids: 'new' fatty acids

The fatty acid analysis revealed that no qualitative alterations were carried out by the sulphate reducing bacteria. Therefore, SRB were not able to change the fatty acids composition of linseed oil over this time scale.

5.2.2.2. Sunflower oil

At the start of the experiments, 395.5 ± 7.61 mg (mean ± sd) of sunflower oil were present in each sample.

Experiment a): Interaction between aerobic and anaerobic oil degrading bacteria and sulphate reducing bacteria

The amount of lipids (mg) present in each sample, after incubation in the various conditions, ranged between 382.8 and 391.2 mg (Table 5.12). A one-way analysis of variance was used to examine differences between the amount of extractable lipids in the different conditions of incubation, including the control, and showed no significant differences between the samples ($F=0.67$; $p=0.717$, the residuals were approximately normally distributed and there was no significant heterogeneity of variance $\chi^2=4.480$; $p=0.811$). This indicated no significant degradation of sunflower oil by aerobic, anaerobic and sulphate reducing bacteria over the period of incubation.

Table 5.12. Amount of lipids extracted (mg, mean ± sd) from the various samples after incubation of sunflower oil with aerobic, anaerobic and sulphate reducing bacteria.

Aero (D14)	*Aero (D28)*	*Aero +ST (D28)*	*Aero+ST+ana (D28)*
385.7 ± 3.51	386.3 ± 9.16	386.9 ± 7.68	386.5 ± 9.83
Aero (D42)	***aero+ST (D42)***	***Aero +ST+ana (D42)***	***aero+ST+ana+SRB (D42)***
391.1 ± 11.26	385.3 ± 9.84	391.2 ± 11.01	382.8 ± 13.17

aero (D14), *aero* (D28), *aero* (D42): oil incubated with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST* (D28), *aero+ST* (D42): incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days respectively; *aero+ST+ana* (D42): incubation with aerobic, anaerobic bacteria and reducing agent for 28 and 42 days respectively; *aero+ST+ana+SRB* (D42): incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

Quantitative alterations of the fatty acids

The fatty acid composition of sunflower oil is illustrated in Figure 5.14.

In the given experimental conditions, the percentages of the main fatty acids of sunflower oil were not greatly affect by any group of bacteria, as it can be seen in Figure 5.15.

To examine the variation in each fatty acid between the various samples, their percentages were normalised through arcsine transformations (Fry, 1993). A series of one-way ANOVA's was performed to test for differences in the percentage of each fatty acid in the pure sunflower oil and the 4 samples incubated for 42 days. The results indicated significant alterations in the percentage of 16:0; 18:0 and 18:2ω6 (Table 5.13) for the

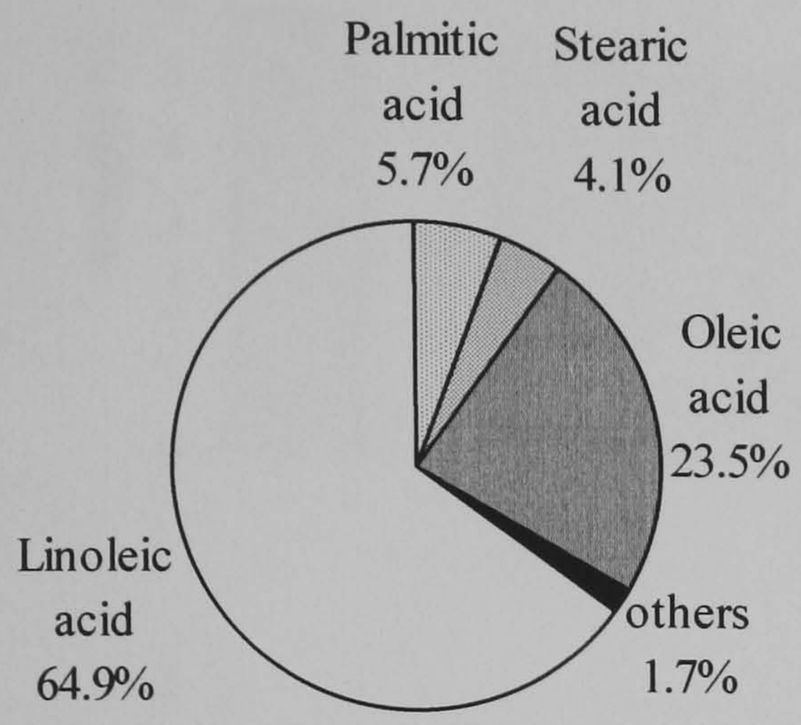


Figure 5.14. Fatty acid composition (weight %) of sunflower oil.

samples tested, but no significant differences were found in the percentage of 18:1ω9 ($F=2.40$; $p=0.100$, the residuals were approximately normally distributed, and there was no heterogeneity of variance $\chi^2 = 1.757$; $p=0.780$). Since no modifications in the percentage of 18:1ω9 were carried out by aerobic, anaerobic or sulphate reducing bacteria after an incubation period of 42 days, no further analysis were carried out for this fatty acid.

Table 5.13. F and p values for the three one-way ANOVAs applied to the percentage of 16:0, 18:0 and 18:3ω3 (arcsine transformed), in samples incubated in various conditions with aerobic, anaerobic and sulphate reducing bacteria, and in the pure sunflower oil.

Various samples tested	**16:0**		**18:0**		**18:2ω6**	
	F	p	F	p	F	p
Oil & *aero (D42)* & *aero+ST (D42)* & *aero+ST+ana (D42)* & *aero+ST+ana+SRB (D42)*	3.44	0.037	13.95	<0.001	26.21	<0.001
Oil & *aero (D14)* & *aero (D28)* & *aero (D42)*	3.23	0.061	15.83	<0.001	37.15	<0.001
aero (D14) & *aero (D42)* & *aero+ST (D42)* & *aero+ST+ana (D42)* & *aero+ST+ana+SRB (D42)*	-	-	2.46	0.094	28.90	<0.001

aero (D14), *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 42 days; *aero+ST+ana (D42)*: incubation with aerobic, anaerobic bacteria and reducing agent for 42 days; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

Even though the analysis of variance showed that significant modifications occurred in the proportion of 16:0 when sunflower oil was inoculated for 42 days with the various bacterial groups, the results of Scheffe's pairwise comparison could not detect any significant differences between any two incubation conditions (Table 5.14). To test further if any alterations occurred in the percentage of 16:0, a one-way ANOVA was carried out for the pure oil and the various samples incubated only with aerobic bacteria for different time periods. This analysis revealed no significant differences between the various samples tested (Table 5.14). Prior to each of the ANOVA, the homogeneity of variance of the

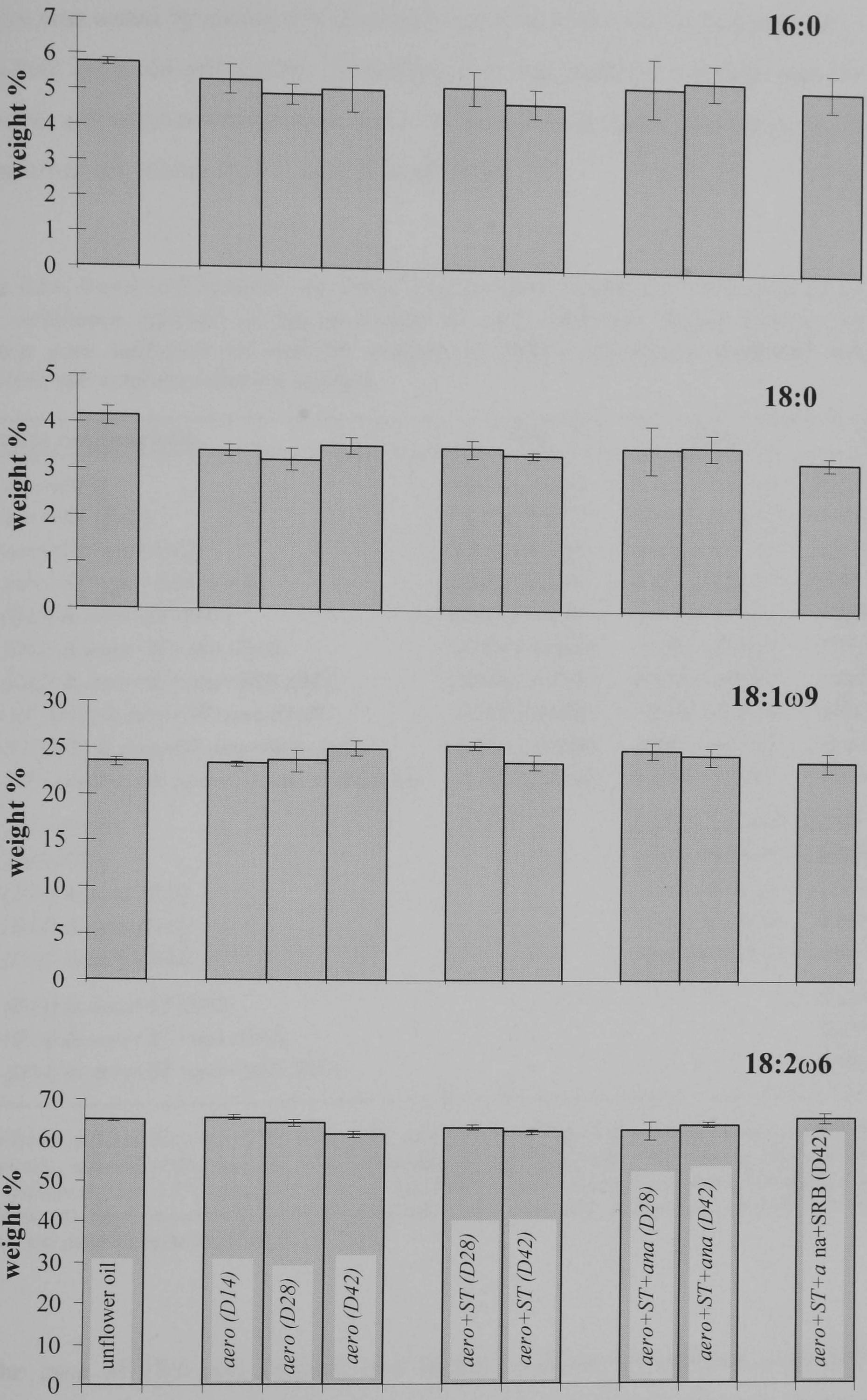


Figure 5.15. Fatty acid composition (weight %, ± sd) of sunflower oil incubated with aerobic, anaerobic and sulphate reducing bacteria. *aero (D14)*, *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28)*, *aero+ST (D42)*; incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days respectively; *aero+ST+ana (D28)*, *aero+ST+ana (D42):* incubation with aerobic, anaerobic bacteria and reducing agent for 28 and 42 days respectively; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

samples was tested by means of a Bartlett's test which showed no heterogeneity (χ^2= 6.582 and 6.860; p= 0.160 and 0.076). Therefore, it is legitimate to conclude that the aerobic, anaerobic and sulphate reducing bacteria did not alter the initial percentage of 16:0 present in sunflower oil, within the 42 days of incubation.

Table 5.14. Results of Scheffe's or Tukey's ($_a$) pairwise comparison (difference of the means ± 95% confidence interval) of the proportion of 16:0, 18:0 and 18:3ω3 (arcsine transformed) between pure sunflower oil and the samples in various incubation conditions with aerobic, anaerobic and sulphate reducing bacteria.

pairwise comparison	**16:0**	**18:0**	**18:2ω6**
oil & *aero (D42)*	0.016 ± 0.0245	0.016 ± 0.0121*	0.038 ± 0.0187*
oil & *aero+ST (D42)*	0.023 ± 0.0245	0.020 ± 0.0121*	0.030 ± 0.0187*
oil & *aero+ST+ana (D42)*	0.007 ± 0.0265	0.015 ± 0.0131*	0.009 ± 0.0202
oil & *aero+ST+ana+SRB (D42)*	0.006 ± 0.0245	0.024 ± 0.0121*	-0.007 ± 0.0187
aero (D42) & *aero+ST (D42)*	0.007 ± 0.0245	0.005 ± 0.0121	-0.008 ± 0.0187
aero (D42) & *aero+ST+ana (D42)*	-0.009 ± 0.0265	-0.001 ± 0.0131	-0.028 ± 0.0202*
aero (D42) & *aero+ST+ana+SRB (D42)*	-0.010 ± 0.0245	0.008 ± 0.0121	-0.045 ± 0.0187*
aero+ST (D42) & *aero+ST+ana (D42)*	-0.016 ± 0.0265	-0.006 ± 0.0131	-0.021 ± 0.0202*
aero+ST(D42) & aero+ST+ana+SRB (D42)	-0.017 ± 0.0245	0.003 ± 0.0121	-0.037 ± 0.0187*
aero+ST+ana(D42) & *aero+ST+ana+SRB(D42)*	-0.001 ± 0.0262	0.009 ± 0.0131	-0.016 ± 0.0202
oil & *aero (D14)*	-	0.019 ± 0.0119*	-0.007 ± 0.0136 $_a$
oil & *aero (D28)*	-	0.024 ± 0.0119*	0.008 ± 0.0136 $_a$
aero (D14) & *aero (D28)*	-	0.005 ± 0.0119	0.016 ± 0.0136*$_a$
aero (D14) & *aero (D42)*	-	-0.004 ± 0.0119	0.045 ± 0.0136*$_a$
aero (D28) & *aero (D42)*	-	-0.009 ± 0.0119	0.029 ± 0.0136*$_a$
aero (D14) & *aero+ST (D42)*	-	-	0.038 ± 0.0196*
aero (D14) & *aero+ST+ana (D42)*	-	-	0.018 ± 0.0208
aero (D14) & *aero+ST+ana+SRB (D42)*	-	-	0.001 ± 0.0193

*significant differences; *aero (D14), aero (D28), aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28), aero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively; *aero+ST+ana (D28), aero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

In the case of 18:0 and 18:2ω6, two further analyses of variance were performed to investigate which group of bacteria was responsible for the transformations observed in the oil and the various samples incubated for 42 days. An one-way ANOVA was performed to test for differences in the pure oil and in the samples incubated for different lengths of time with aerobic bacteria only; for both fatty acids, significant differences were observed (Table 5.13). The second one-way ANOVA tested if any modifications had been carried

out by anaerobic and SRB after the oil had been broken down by aerobic bacteria, so, in this case, to test for differences between the oil inoculated with aerobic bacteria for 14 days, and all the other samples incubated for 42 days. Prior to each one of the ANOVAs, the homogeneity of variance of the samples was tested by means of a Bartlett's test which showed no heterogeneity (χ^2= 1.469 and 5.134; p= 0.291 and 0.690). For each analysis of variance which showed significant differences, a Scheff's (for unequal number of observations) or Tukey's (for equal number of observations) pairwise comparison test was performed and the results are shown in Table 5.14.

A small but significant decrease in the proportion of 18:0 was observed between the pure sunflower oil and the various samples incubated for 42 days. However, no differences were found between the various samples incubated for 42 days (Table 5.14. and Fig. 5.15). The results regarding the oil incubated with aerobic bacteria indicated only that a significant decrease in the percentage of 18:0 occurred within 14 days of incubation, with no further changes after this time. Although changes in the proportion of 18:0 had occurred prior to the addition of anaerobic and sulphate reducing bacteria, these two groups of bacteria were not able to alter the oil further. Therefore, the results suggest that the decrease in the percentage of 18:0 (to 3.4 weight %) occurred during the first 14 days of incubation, due to the activity of aerobic bacteria and neither a longer incubation time nor addition of anaerobic or sulphate reducing bacteria induced more modifications in the percentage of this fatty acid.

A small but significant decrease in the percentage of 18:2ω6 was measured between the raw sunflower oil and the samples incubated for 42 days with aerobic bacteria and between the pure oil and the sample containing aerobic bacteria and reducing agent at day 42 (Table 5.14). However, no changes occurred between the pure oil and the other two samples incubated for 42 days (*aero+ST+ana* & *aero+ST+ana+SRB*) (Table 5.14). Consequently, significant differences existed between the first two groups of samples (*aero* & *aero+ST*) and the second one (*aero+ST+ana* & *aero+ST+ana+SRB*) at the end of this incubation period. When sunflower oil was inoculated with aerobic bacteria only, 28 days were necessary for a significant decrease in the percentage of 18:2ω6 to be registered. The percentage of 18:2ω6 decreased further until 42 days of the incubation. The addition of reducing agent to the sample containing aerobic bacteria (day 14) introduced a significant decrease in the percentage of 18:2ω6, but no alteration was observed when reducing agent

and anaerobic bacteria were added. These results combined with those obtained for aerobic bacteria indicate that the decrease measured in the percentage of 18:2ω6 was carried out by aerobic bacteria (to 61.3 %) and/or facultative anaerobic bacteria (to 62 %), and that anaerobic bacteria were unable to alter the proportion of 18:2ω6 after 42 days of incubation. A 2-sample t-test assuming equal variance (Bartlett's: χ^2=1.025; p=0.311) performed between the samples containing aerobic and anaerobic bacteria (day 28) and the sample containing aerobic, anaerobic and SRB revealed a significant increase in the percentage of 18:2ω6 (arcsine transformed) after the incubation with SRB (T=-2.54; p=0.044, DF=6). Therefore, aerobic and SRB bacteria appear to have changed the percentage of 18:2ω6 in an opposite manner, with the former group responsible for a small decrease (to 61.3 %) and the latter for a small increase.

Qualitative changes of fatty acids: 'new' fatty acids

In good agreement with the field experiment using sunflower oil, 'new' octadecenoic and octadecadienoic acids were observed in the present experimental conditions (Table 5.15).

Table 5.15. 'New' fatty acids (weight %) present in the various samples incubated with sunflower oil and aerobic, anaerobic and sulphate reducing bacteria.

Fatty acids	*aero (D14)*	*aero (D28)*	*aero+ST (D28)*	*aero+ST +ana (D28)*	*aero (D42)*	*aero+ST (D42)*	*aero+ST +ana (D42)*	*aero+ST+ ana+SRB (D42)*
18:1(1)	-	0.35	-	-	-	0.24	0.03	-
18:1ω7	-	-	-	1.17	1.31	-	-	-
18:2 (2)	-	-	-	-	0.05	-	-	-
18:2 (3)	-	-	-	-	-	0.08	0.03	-
18:2 (4)	0.10	-	-	-	0.03	-	-	-
18:2 (5)	0.19	1.02	0.34	0.73	1.32	0.73	0.24	0.27
18:2 (6)	-	0.35	-	-	0.41	0.16	0.03	-

aero (D14), *aero (D28)*, *aero (D42)*: incubation with aerobic bacteria for 14, 28 and 42 days respectively; *aero+ST (D28)*, *aero+ST (D42)*: incubation with aerobic bacteria and reducing agent (ST) for 28 and 42 days, respectively; *aero+ST+ana (D28)*, *aero+ST+ana (D42)*: incubation aerobic, anaerobic bacteria and reducing agent for 28 and 42 days, respectively; *aero+ST+ana+SRB (D42)*: incubation with aerobic, anaerobic, sulphate reducing bacteria and reducing agent for 42 days.

With the exception of the fatty acid 18:2 (6), the 'new' fatty acids were identical to those observed in the field experiments. These fatty acids were consistently present in very small amounts (at the most 1.3 weight %) and were not observed in every sample. It was only

after 42 days of incubation in aerobic conditions that a higher variety of 'new' fatty acids was observed.

Experiment b): Interaction between anaerobic oil degrading and sulphate reducing bacteria

The amount of lipids extracted from the various samples incubated with the different bacteria groups is illustrated in Table 5.16. A one-way ANOVA indicated no significant differences in the amount of oil extracted from the various treatments or the control ($F=0.84$; $p=0.498$, the residuals were approximately normally distributed and there was no significant heterogeneity of variance $\chi^2=0.310$; $p=0.958$). This indicated no significant degradation of sunflower oil over the period of incubation.

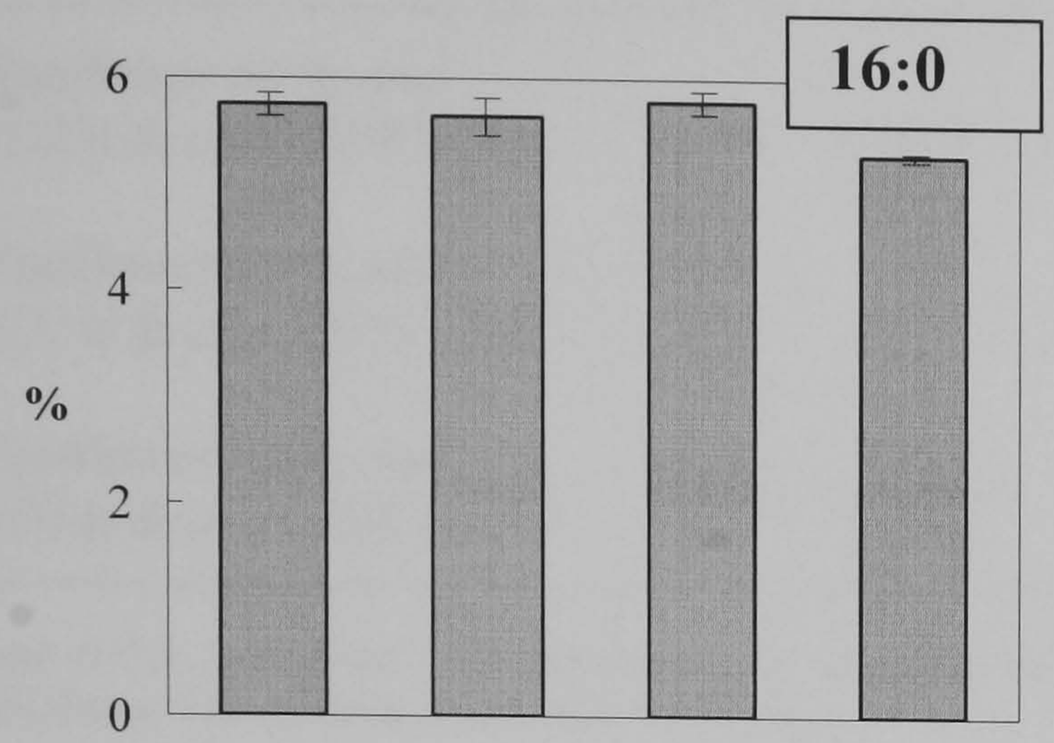
Table 5.16. Amount of lipids extracted (mg, mean ± sd) after sunflower oil inoculation with anaerobic and sulphate reducing bacteria.

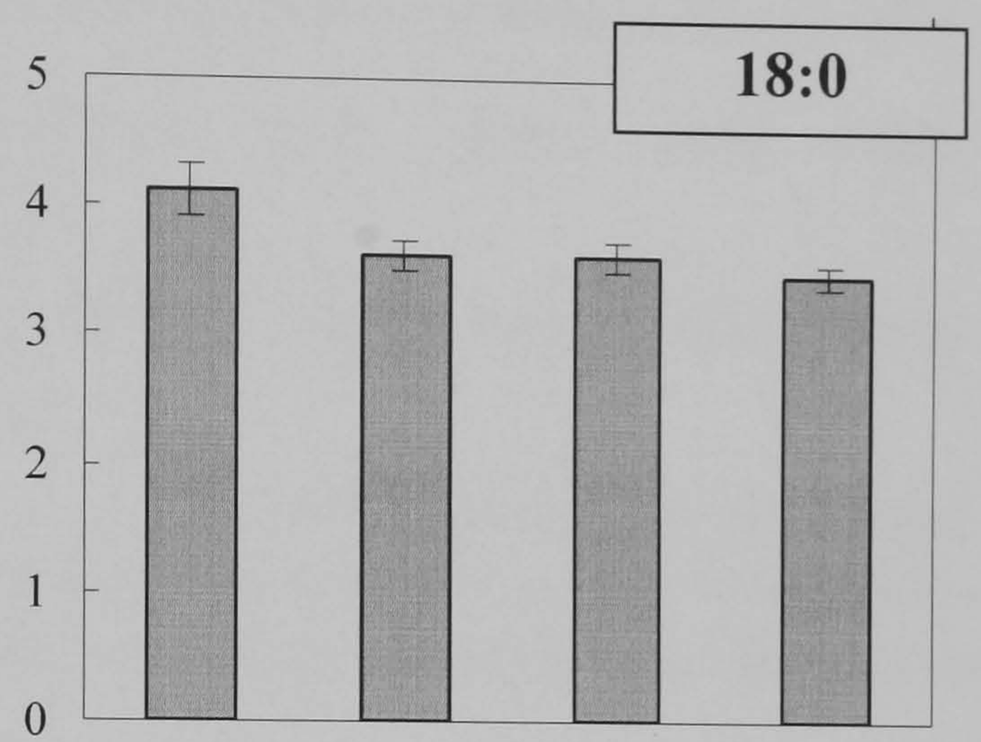
Ana (D14)	*Ana (D28)*	*Ana + SRB (D28)*
397.4 ± 10.45	390.2 ± 8.11	389.1 ± 8.74

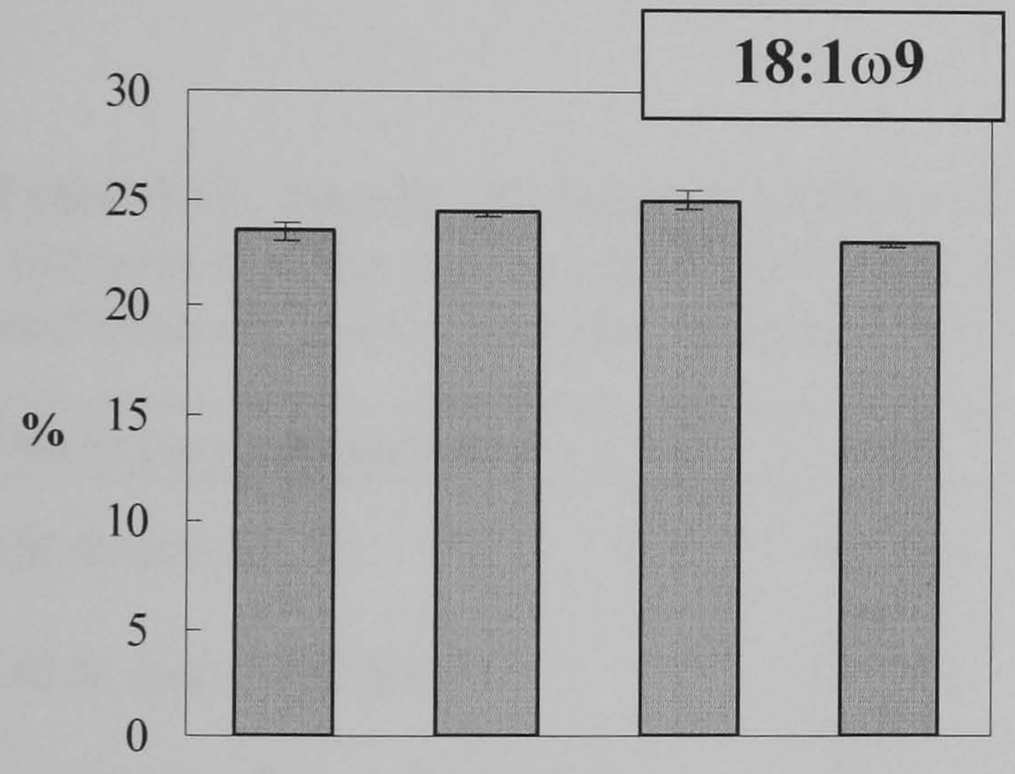
ana (D14), *ana (D28)*: oil incubated with anaerobic bacteria for 14 and 28 days respectively; *ana+SRB (D28)*: oil incubated with anaerobic and SRB for 28 days.

Quantitative analysis of the fatty acids

The percentage of the main fatty acids present in the raw sunflower and in the samples incubated with different bacteria groups is illustrated in Figure 5.16. The percentages of the fatty acids were normalised through arcsine transformations (Fry, 1993) and three one-way ANOVA were performed between: i) pure oil and samples incubated for 28 days; ii) pure oil and samples incubated for 14 and 28 days with anaerobic bacteria; iii) pure oil and bacteria incubated for 14 days with anaerobic bacteria and anaerobic plus SRB (Table 5.17). For all fatty acids, significant differences were measured in the various sets of samples tested (Table 5.17). The Bartlett's test indicated no significant heterogeneity of variance ($\chi^2 = 7.722$ - 10.119; $p = 0.061$ - 0.601).







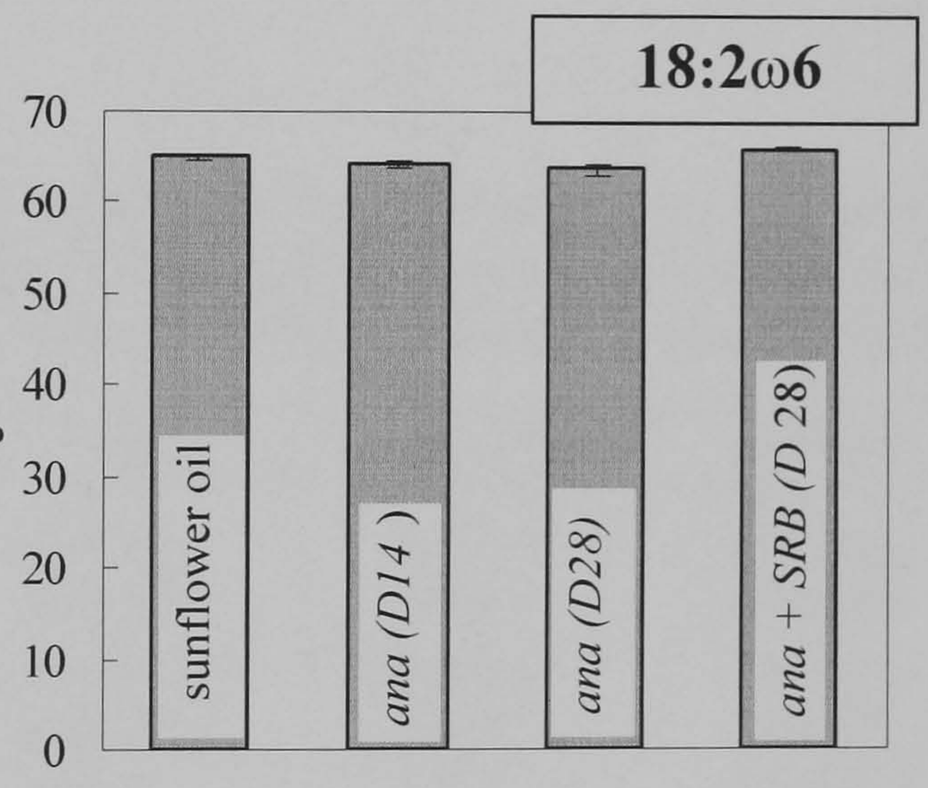


Figure 5.16. Fatty acid composition (weight %, ± sd) of sunflower oil incubated with anaerobic and sulphate reducing bacteria. *ana (D14), ana (D28)*: incubation with anaerobic bacteria for 14 and 28 days respectively: *ana+SRB (D28):* incubation with anaerobic, sulphate reducing bacteria for 28 days.

Table 5.17. F and p values for the three one-way ANOVAs applied to the percentage of 16:0. 18:0. 18:1ω9 and 18:2ω6 (arcsine transformed) between the pure sunflower oil and the samples incubated in various conditions with anaerobic and sulphate reducing bacteria.

Various samples tested	**16:0**		**18:0**		**18:1ω9**		**18:2ω6**	
	F	p	F	p	F	p	F	p
Sunflower oil & *ana (D28)* & *ana+SRB (D28)*	42.99	<0.001	20.89	<0.001	31.45	<0.001	34.78	<0.001
Sunflower oil & *ana (D14)* & *ana (D28)*	-	-	14.21	0.002	15.63	0.001	7.90	0.010
Sunflower oil & *ana (D14)* & *ana+SRB (D28)*	17.18	0.001	-	-	26.77	<0.001	32.19	<0.001

ana (D14), *ana (D28)*: oil incubated with anaerobic bacteria for 14 and 28 days respectively; *ana+SRB (D28)*: oil incubated with anaerobic and SRB for 28 days.

For each analysis of variance which showed significant differences, Tukey's pairwise comparisons were performed and the results are shown in Table 5.18.

Table 5.18. Results of Tukey's (difference of the means ± 95% confidence interval) pairwise comparison of the proportion of 16:0, 18:0, 18:1ω9 and 182:ω6 (arcsine transformed) between pure sunflower oil and the samples incubated for 28 days with anaerobic and sulphate reducing bacteria.

Pairwise comparison	**16:0**	**18:0**	**18:1ω9**	**18:2ω6**
Oil & *ana (D28)*	-0.001 ± 0.0036	0.013 ± 0.0076*	-0.017 ± 0.0085*	0.0125 ± 0.0078*
Oil & *ana+SRB (D28)*	0.010 ± 0.0036*	0.017 ± 0.0076*	0.006 ± 0.0085	-0.011 ± 0.007*
ana (D28) & *ana+SRB (D28)*	0.011 ± 0.0036*	0.004 ± 0.0076	0.023 ± 0.0085*	-0.023 ± 0.0078*
Oil & *ana (D14)*	-	0.013 ± 0.0079*	-0.010 ± 0.0086*	0.006 ± 0.0088
ana (D14) & *ana (D28)*	-	0.0002 ± 0.0079	0.007 ± 0.0086	0.006 ± 0.0088
ana (D14) & *ana + SRB (D28)*	0.008 ± 0.0050*	-	0.017 ± 0.0064*	-0.017 ± 0.0059*

*significant differences: *ana (D14)*, *ana (D28)*: oil incubated with anaerobic bacteria for 14 and 28 days respectively; *ana+SRB (D28)*: oil incubated with anaerobic and sulphate reducing bacteria for 28 days.

Anaerobic bacteria were not able to modify the initial proportion of 16:0 during 28 days of incubation (Table 5.18). However, there was a small but significant decrease in the percentage of 16:0 between the pure oil and the sample containing anaerobic and SRB at

day 28. Consequently, significant differences were observed between the two samples incubated for 28 days. These results suggest that when the SRB were added to the oil, no significant alterations in the percentage of 16:0 had occurred and that the SRB were responsible for the decrease (to 5.3 weight %) in the proportion of this fatty acid after incubation.

A small but significant decrease in the proportion of 18:0 was registered between the pure oil and both samples incubated for 28 days [*ana* (D28) & *ana+SRB* (D28)] (Table 5.18 and Fig. 5.16). In contrast with the results of 16:0, the percentage of 18:0 decreased significantly within 14 days of incubation in anaerobic conditions and was not affected by a longer incubation time with anaerobic bacteria or by the presence of SRB (Table 5.18 and Figure 5.16), indicating that the decrease in the percentage of 18:0 (to 3.6 weight %) was carried out by anaerobic bacteria.

A small but significant increase in the proportion of 18:1ω9 was observed between the pure oil and samples incubated for 28 days with anaerobic bacteria; no differences were measured between the pure oil and the sample incubated for 28 days with anaerobic plus SRB. Therefore, significant differences in the percentage of 18:1ω9 occurred between the two samples incubated for 28 days (Table 5.18). Anaerobic bacteria increased significantly the percentage of 18:1ω9 within 14 days of incubation. However, no further changes occurred with increasing incubation time. Samples containing anaerobic plus SRB, showed a small but significant decrease in the percentage of this fatty acid when compared to the samples containing only anaerobic bacteria, incubated for 14 or 28 days. These results indicate that anaerobic bacteria increased the percentage of 18:1ω9 (to 24.3 weight %) whereas SRB reversed this change (to 22.9 weight %).

A small but significant decrease in the percentage of 18:2ω6 was observed after 28 days incubation with anaerobic bacteria and a significant increase was measured between the pure oil and the sample incubated with anaerobic plus SRB for the same incubation time (Table 5.18 and Fig. 5.16). Consequently, significant differences were registered between the two incubated samples. No significant differences were registered in the percentage of 18:2ω6 between the oil and the sample incubated for 14 days with anaerobic bacteria, indicating that no changes had occurred to this fatty acid when SRB were added. A significant increase was, nonetheless, measured between the sample incubated with

anaerobic bacteria for 14 days and that incubated for 28 days with anaerobic and sulphate reducing bacteria (Table 5.18). Therefore, anaerobic and sulphate reducing bacteria induced contrasting changes in 18:2ω6, with the first group being responsible for a decrease and the second group for an increase (Table 5.18).

Qualitative analysis of the fatty acids: 'new' fatty acids

Only 18:2 (1) was present in every sample analysed, in very small amounts (0.13 to 0.19 weight %).

Experiment c): Role of sulphate reducing bacteria

As observed with the linseed oil, no definite growth of SRB was observed when sunflower oil was the only carbon source present. A 2-sample t-test assuming equal variance (Bartlett's test: χ^2=0.136; p=0.712) was used to investigate the difference between the initial (395.5 ± 7.61 mg) and the final amount of lipids after 14 days of incubation with SRB (397.9 ± 9.59 mg). The t-test revealed no significant differences between the samples (T=-0.38; p=0.71; DF=6).

Quantitative analysis of the fatty acids

The proportion of the main fatty acids present in the sample containing sunflower oil incubated for 14 days in the presence of SRB, is shown in Table 5.19.

Table 5.19. Fatty acids composition (weight %, mean ± sd) of sunflower oil after 14 days incubation with sulphate reducing bacteria.

16:0	18:0	18:1ω9	18:2ω6
5.9 ± 0.23	3.9 ± 0.09	23.6± 0.81	64.3 ± 0.99

The differences in the percentage of each fatty acid (arcsine transformed) in the pure sunflower oil and in the sample incubated with SRB for 14 days were also examined using a 2-Sample t-test and no significant differences were found (T=0.35-2.07; p=0.084-0.74; DF=6, the residuals were approximately normally distributed and there was no significant heterogeneity of variance χ^2 = 0.814-3.441; p = 0.064-0.367).

Qualitative analysis of the fatty acids: 'new' fatty acids

The fatty acids analysis revealed that no qualitative alterations were carried out by the sulphate reducing bacteria. As observed for linseed oil, SRB cannot degrade sunflower oil without it being partially pre-degraded.

5.3. DISCUSSION

5.3.1. Linseed oil degradation

5.3.1.1. Experiment I - Linseed oil degradation by bacteria removed from different sediment depths

Degradation by aerobic and anaerobic bacteria

The results of this experiment showed that indigenous bacteria from the salt marsh sediments at Foryd Bay can grow in the presence of linseed oil using it as the only source of carbon. From the field experiment, it had already been established that these bacteria were able to breakdown linseed oil and that sediment contamination with this oil lead to increased abundance of aerobic bacteria with such capability. Growth was observed by an increase in the turbidity of the medium within ≤ 7 days after incubation with bacteria removed from the surface sediments down to 30 cm depth. A more intense growth was observed when the linseed oil was incubated with aerobic rather than with anaerobic bacteria, which could have been anticipated since the sediments initially contained more aerobic than anaerobic oil degrading bacteria. A more intense growth of aerobic micro-organisms was also observed when they originated from depths of 1 to 15 cm than from those depths between 15 to 30 cm, as a result of higher abundance of bacteria in the muddy sediments than in those with a higher percentage of sandy particles.

An increase in the abundance of aerobic and anaerobic bacteria in the presence of linseed oil implies that bacteria from either group were able to hydrolyse the triacylglycerols into free fatty acids and glycerol before assimilating the fatty acids. Due to the lack of

quantitative measurements of the amount of lipids extracted it was not possible to establish the amount of fatty acids (if any) completely oxidised to CO_2 and H_2O. Still, the results of the fatty acids analysis showed that the lipidic composition of the samples was very different from that of the pure linseed oil, indicating that certain transformations had occurred. In fact, the extracted lipids from the medium containing aerobic and anaerobic cells, revealed changes in the proportion of the main fatty acids of linseed oil as well as the presence of 'new' fatty acids.

Aerobic bacteria not only exhibited a more intense growth, but they also caused more profound changes in the linseed oil composition than the anaerobic micro-organisms, which could be due to the higher abundance of aerobic than anaerobic bacteria or/and to the wider range of substrates used by the former group in relation to the latter (Figure 5.3 and 5.5). Aerobic bacteria, which were removed from the surface sediments (1 cm), were responsible for more changes in the oil composition than the bacteria removed from the other sediment depths, which is in agreement with the results of the field experiment where changes in the oil composition were first observed at the surface, being extended with time to the deeper sediment layers. Moreover, the highest increase in the number of bacteria after the sediments contamination with linseed oil was registered at the 1 cm depth (Figure 4.6).

In terms of quantitative changes of the fatty acids by aerobic and anaerobic bacteria, the most important was the marked decrease in the percentage of 18:3ω3, principal component of linseed oil (57.9 weight % in pure oil) (Figs. 5.3 and 5.5). This is in agreement with the results obtained in the field experiment simulating a linseed oil spill (Chapter 4). In the literature, it is suggested that the recovered fatty acids from micro-organisms that grow on fats and fatty acids are similar to those with which micro-organisms are fed (Bati *et al.*, 1984; Tan & Gill, 1985; Koritala *et al.,* 1987). However, some modifications of the source fatty acids can occur. Koritala and colleagues (1987) observed that when linseed oil was incubated with *Candida lipolytica*, the recovered lipids had only 29% of 18:3ω3 (which is considerably higher than the percentage encountered in any yeast). According to the authors, the yeast was not able to tolerate high proportions of polyunsaturated fatty acids and either failed to incorporate or to reduce it successively to 18:2, 18:1 and even 18:0. Koritala *et al.,* (1987) also incubated soybean oil with *Aspergillus flavus* (Fungi-Ascomycetes) and observed that the percentage of 18:3ω3 decreased from 7.5% after 2

incubation days to 6% after 5 days, whereas the other fatty acids remained the same as in the soybean oil. These authors suggested that *A. flavus* selectively catabolised 18:3ω3; they also argued that the decrease in 18:3ω3 during fermentation was the result of oxidation rather than selective assimilation.

In the present study, the decrease in the percentage of 18:3ω3 in the samples incubated with aerobic or anaerobic bacteria was accompanied by an increase in the proportion of 18:1ω9 (Figs. 5.3 and 5.5), which suggests that the 18:3ω3 was successively reduced to 18:2ω6 and to 18:1ω9 resulting only in an accumulation of the latter. The conversion of 18:3ω3 to other fatty acids was a possible route for the bacteria to eliminate high concentrations of 18:3ω3 from the medium. An increase in the percentage of 18:2ω6 was only observed when linseed oil was incubated with aerobic bacteria from the 1 cm depth sediments. Bacteria removed from the remaining depths did not changed markedly the proportion of 18:2ω6, possibly indicating that if the 18:2ω6 was indeed formed by hydrogenation of the 18:3ω3, it did not accumulate in the medium but was further reduced to 18:1ω9 or isomerised to form 'new' octadecadienoic acids. No accumulation of saturated fatty acids (16:0 and 18:0) occurred when linseed oil was incubated with aerobic or anaerobic bacteria in this experiment, in contrast to the results of the simulated spill of linseed oil in the field. For an increase in the amount of 18:0 and 16:0 to occur, as observed in the field, a longer incubation period with aerobic and anaerobic bacteria may be necessary. It is also possible that a different group of bacteria is responsible for the reactions which will lead to the accumulation of saturated fatty acids.

Aerobic bacteria also appear to have induced the formation of 'new' octadecadienoic and octadecatrienoic acids identical to those observed in the sediments at Foryd Bay after the simulated spill of linseed oil. Although in the linseed oil incubated with aerobic bacteria only two 18:2 (one less than in the field experiment) and three 18:3 (one less than in the field) were observed, these were the same fatty acids as those present at higher concentrations in the field work. Therefore, the pathway of the oil degradation in the laboratory after linseed oil had been incubated during 7 days with aerobic bacteria removed from the salt marsh sediments, seems similar to that suggested to occur in the field. Even though fatty acids are usually assimilated by micro-organisms with minimum amount of modification (Ratledge, 1994), in the present work the 18:3ω3, and perhaps the 18:2ω6 were possibly converted into positional and/or geometric isomers. The isomerisation of

18:3ω3 could account for the decrease in the concentration of this fatty acid. Although anaerobic bacteria were apparently responsible for similar alterations in the percentage of 18:3ω3 and 18:1ω9, only one 'new' octadecatrienoic acid was observed. This appears to suggest that the anaerobic organisms did not need to markedly modify the polyunsaturated fatty acids before they used them; the hydrogenation route probably prevailed over the isomerisation one. The complete pathway of degradation of linseed oil by aerobic bacteria can only be established after the identification of the 'new' fatty acids, which will be discussed in Chapter 7.

Degradation of the pure oil by sulphate reducing bacteria

In addition to the study of the degradation of pure linseed oil by aerobic and anaerobic bacteria, the degradation by SRB isolated from the upper 5 cm depth sediments was also carried out. The fatty acids analysis indicated that SRB cannot breakdown and utilise the pure oil to grow which is in accordance with the fact that SRB are known to use end products of fermentation (Howarth, 1993).

Sulphate reducing bacteria degradation of linseed oil partially metabolised by aerobic and anaerobic bacteria

SRB were able to utilise the products resultant from the oil degradation by aerobic or anaerobic bacteria and intensify the changes in the oil composition initialised by the previous bacteria groups. These observations reveal that the SRB (*Desulfovibrio* and *Desulfotomaculum*) were able to utilise long chain fatty acids. Even though SRB are known to utilise preferentially short chain fatty acids, these results were not completely unexpected since the number of SRB also increased after the addition of the linseed oil to the salt marsh sediments, indicating that they benefited from the addition of the oil (Figure 4.8). Once more, it is not possible to know if the SRB oxidised the fatty acids completely since most of the oil was removed by filtration, as mentioned previously.

SRB performed identical transformations in the main fatty acids independently of whether the oil had been pre-conditioned by aerobic or by anaerobic bacteria (Figure 5.7 and 5.8).

SRB not only accentuated the decrease in the proportion of 18:3ω3 and the accumulation of 18:1ω9, previously initiated by the aerobic or anaerobic bacteria, but also increased the proportion of 18:0 and 16:0. Consequently, SRB appear not only to have used an identical

pathway for the fatty acids degradation as the aerobic and anaerobic bacteria, but also to have gone a step further in the fatty acids metabolism. The increase in 18:0 and 16:0 could have resulted from the reduction of 18:1ω9 to 18:0, followed by a loss of two carbon units by β-oxidation to 16:0. It is also possible that 18:3ω3 could have been completely degraded to acetyl-CoA molecules and was followed by resynthesis of shorter chain fatty acids. However, this latter pathway of degradation seems less probable because no accumulation of other short chain fatty acids was observed. The increase in the saturated fatty acids has to be regarded with caution since the results are expressed in percentage and can, therefore, be linked to the other fatty acids decrease.

Contrasting with the results obtained in the linseed oil field experiment, a decrease was observed in the percentage of 18:2ω6, which can be the result of i) in the field experiment, higher amounts of 18:3ω3 were hydrogenated to 18:2ω6 or ii) in the laboratory experiments, more 18:2ω6 was utilised and possibly converted into other fatty acids. A decrease in the proportion of 18:2ω6 was also observed by Pereira (1993) when studying the degradation of linseed oil in laboratory experiments.

The number of 'new' fatty acids present in the samples incubated with SRB after previous incubation with anaerobic bacteria was much lower than that in samples that had been previously incubated with aerobic bacteria. The 'new' octadecatrienoic acid present after the first 7 days of incubation with anaerobic bacteria, disappeared after the addition of SRB suggesting its degradation by these organisms. The addition of SRB to the oil previously degraded by aerobic bacteria, induced an increase in the proportion of the 'new' fatty acids and introduced two octadecenoic acids identical to those observed in the field experiment. Once again, the presence of 'new' octadecenoic acids, which is thought to have resulted from the reduction of octadecadienoic acids or isomerisation of 18:1ω9, appears to suggest a step further in the pathway of the oil degradation than that observed only in the presence of aerobic bacteria. The presence or absence of 'new' fatty acids in the degradation process, combined with quantitative changes in the main fatty acids, discussed above, suggest that the degradation of oil by SRB can lead or not to the formation of 'new' fatty acids.

The results of this experiment showed that SRB were not able to breakdown pure linseed oil. However, they can grow in the oil partially degraded by aerobic or anaerobic bacteria, which is suggestive of a sequential degradation. The fatty acid modifications observed in

the field experiment were carried out possibly in a similar manner to that observed in the laboratory experiment. Still, in the field experiment the fatty acids alterations could also have been performed exclusively by aerobic or anaerobic bacteria, without the activity of the SRB, because more modifications by the former groups could have occurred by increasing the incubation time. However, degradation of linseed oil is not expected to be carried out by one organism only but by a consortia of organisms belonging to the above mentioned groups.

5.3.1.2. Experiment II

One of the objectives of Experiment (II) was to determine the degradation rates of linseed oil by the various groups of bacteria (aerobic, anaerobic and sulphate reducers). However, this was not achieved due to the very slow growth rates of bacteria, even though aerobic bacteria were incubated for up to 42 days. These results contrasted with those from Experiment I, where intense growth (mainly of aerobic bacteria) was observed within 7 days of incubation. Various factors are necessary for a successful microbial growth in vegetable oils: the optimum pH of the media (6.5 or above); the presence of nutrients (mainly of nitrogen and phosphorus) and the dispersal of the oil within the cultivation vessel, which is usually achieved by mechanical shaking (Ratledge, 1994). In this study, the media used contained nutrients and a pH that should have been favourable to sustain bacterial growth. Mechanical shaking of the media was not performed since it was observed that the oil tended to polymerise when shaken. Although, this set of experimental conditions was identical to that used in Experiment I where growth was obtained, there were two differences in the experimental strategy between these two experiments: i) in Experiment II, bacteria from the various sediment depths were pooled and treated as a single sample, and ii) an increase in the amount of linseed oil from 0.2 cm^3 to 0.5 cm^3 per 10 ml of medium, was introduced. It is possible that the mixture of bacteria from the various depths may have had slower growth. It had been previously observed that transformations in linseed oil were more intense when incubated simply with bacteria from 1 cm depth (mainly aerobic). The mixture of bacteria would have diluted the bacteria with capability to degrade the oil and consequently increase the lag time before growth could be observed. However, in the previous experiment (I) bacteria collected from all sediment

layers down to 30 cm depth did grow and changed the fatty acid composition of linseed oil. Consequently, it was expected that an increase in the incubation period to up to 42 days should have been sufficient for growth to have occurred.

It is well known that micro-organisms can utilise long chain fatty acids as the only source of carbon and that problems of toxicity will arise with fatty acids of C_{10} and shorter, which was not the case in the present work. However, Hunkova & Fencl (1977) suggested that micro-organisms require different concentrations of each fatty acid for an efficient growth, which depends on the micro-organism, fatty acid chain length, medium composition and pH. Since, it had already been observed in the previous experiment that bacteria degraded preferentially 18:3ω3, it is possible that an increase in the oil concentration, and consequently in 18:3ω3, was toxic for the bacteria. Consequently, the combination of the mixture of the bacteria from the various sediments depths and the increase in the oil content could have accounted for the observed slow bacterial growth.

The outcome of the reduced growth of aerobic and anaerobic bacteria was that fewer fatty acids modifications were performed by these bacterial groups when compared with the changes registered in Experiment I.

Experiment a): Interaction between aerobic and anaerobic oil degrading bacteria and sulphate reducing bacteria

During a period of 14 days of incubation, aerobic bacteria induced a small decrease in the proportion of 16:0 in the linseed oil, not altering the percent contribution of any other fatty acid. These results contrast with those obtained in the previous experiment (Experiment I), where aerobic bacteria produced an increase in the percentage of 18:1ω9 and a decrease in the percentage of 18:3ω3. In the present experiment, and within 42 days of incubation, aerobic bacteria reversed the initial changes observed in 16:0, decreasing also the percent contribution of 18:2ω6 and 18:3ω3. The decrease in the percentage of polyunsaturated fatty acids and increase in the proportion of 16:0 were also registered in the Experiment I. However, in Experiment I the decrease in 18:2ω6 and increase in 16:0 were only observed after addition of SRB to the oil partially degraded by aerobic bacteria.

Although there is no data concerning the activity of anaerobic bacteria in linseed oil pre-degraded by aerobic bacteria, it was expected that transformations would occur since it had been observed in Experiment I that anaerobic bacteria can degrade pure linseed oil. In the presence of anaerobic bacteria (at day 42), only a decrease in the proportion of 18:2ω6 was registered. The increase in the proportion of 18:3ω3 was attributed to the facultative anaerobic bacteria, since this alteration was observed in the sample containing aerobic bacteria and reducing agent (which should eliminate all strictly aerobic bacteria). These results reveal that in the given conditions, aerobic bacteria were responsible for a wider range of alterations than anaerobic bacteria.

In Experiment I, SRB decreased the percentages of the 18:2ω6 and 18:3ω3 and increased the proportion of the remaining fatty acids. In this experiment, SRB were not able to carry out any changes in the percentage of the various fatty acids, possibly indicating that the oil had not been sufficiently pre-conditioned.

Although the growth of all bacteria groups studied was small and changes in the fatty acids composition of linseed oil were reduced, the fact that a decrease in the percentage of 18:2ω6 and 18:3ω3 and an increase in the 16:0 were observed after 42 days of incubation with aerobic bacteria may suggest a pathway of degradation similar to that described in Experiment I. However, in Experiment II the route of degradation of polyunsaturated fatty acids did not seem to include hydrogenation, since no accumulation of 18:1ω9 was observed. Isomerisation (geometrical and/or positional) of the 18:2ω6 and 18:3ω3 probably occurred to form 'new' fatty acids. These results appear to indicate that 18:3ω3 and 18:2ω6 were not hydrogenated to form 18:1ω9 but were perhaps isomerised to form 'new' unsaturated fatty acids; therefore, it seems that the route of isomerisation prevailed over that of hydrogenation. The presence of the highest amounts of 'new' fatty acids in the samples incubated with aerobic bacteria, appear also to suggest that the major transformations were carried out by aerobic bacteria after 42 days of incubation. Furthermore, the presence of identical 'new' octadecatrienoic [18:3 (1 & 4)] and of two octadecenoic acids, appears to testify a similar isomerisation of the 18:3ω3 as registered in the previous experiment (I). The presence of 'new' fatty acids 18:2 (5) and 18:3 (5 & 6), (not observed in any other field or laboratory experiment) reveals also that the polyunsaturated fatty acids were isomerised to form different 'new' polyunsaturated fatty acids. The complete identification of the 'new' fatty acids (Chapter 6) will possibly provide

information that will allow a better understanding of the degradative pathway of linseed oil by bacteria from salt marsh sediments. The overall degradative pathway of linseed oil will be presented in Chapter 7.

Experiment b): Interaction between anaerobic oil degrading bacteria and sulphate reducing bacteria

It was observed with the simulated spill of linseed oil that the number of anaerobic bacteria increased slightly after the contamination of the sediments. In Experiment I, it was also established that these bacteria can utilise pure linseed oil. Despite these previous results, in the present experiment anaerobic bacteria did not cause significant changes in the percentage of the fatty acids of linseed oil, possibly as a consequence of the small bacteria abundance. Even though no changes in the proportion of fatty acids were observed after incubation with anaerobic bacteria, it does not mean that this bacterial group did not utilise the oil to grow, but that anaerobic bacteria did not change markedly the oil composition.

The SRB added to the oil previously incubated with anaerobic bacteria were responsible for an increase in the proportion of 16:0. The other fatty acids remained as in the pure linseed oil. These results, once more, reflect the lower number of transformations registered in these experiments when compared with those in Experiment I.

Despite the fact that anaerobic and sulphate reducing bacteria were not responsible for any marked changes in the proportion of the main fatty acids of linseed oil, some small amounts of 'new' fatty acids were present: 18:3 (4) and 18:1ω7, observed in the field and Experiment I; and 18:3 (5) observed only in Experiment II-a). The presence of various 18:3 suggest isomerisation of 18:3ω3, which could have occurred despite no significant decrease in the 18:3ω3. The presence of 18:3 (5), as in Experiment II-a), could be due to a different isomerisation route of the 18:3ω3 from that observed in the field experiment. The fact that the 'new' fatty acids occurred after 28 days of incubation in both samples and in identical amounts, suggest that, even though no quantitative changes of the fatty acids were carried out by anaerobic bacteria, there were qualitative modifications in the composition carried out by this bacteria group.

Experiment c): Role of sulphate reducing bacteria

In agreement with the previous experiment regarding linseed oil degradation (Experiment I), the results of this experiment confirm that SRB cannot grow in pure linseed oil as the only source of carbon.

5.3.2. Sunflower oil degradation

5.3.2.1. Experiment II

The field experiment revealed that autochthonous bacteria from the salt marsh sediments had the capability to degrade sunflower oil and that the contamination of the sediments induced an increase in oil degrading bacteria, mostly aerobic. However, in the present experiment bacteria removed from the salt marsh sediments where not able to grow efficiently in pure or partially degraded sunflower oil, indicating that the factors that influenced the bacterial growth in linseed oil also affected that in sunflower oil. Consequently, the slow growth of bacteria is thought to have been responsible for the small quantitative and qualitative changes observed in the fatty acids composition of the spilt sunflower oil. As in the case of linseed oil Experiment II, no difference between the initial amount of lipids and the amount of lipids extracted from the samples incubated with various bacterial groups was registered, which suggest that the fatty acids were not completely metabolised by the bacteria.

Experiment a): Interaction between aerobic and anaerobic oil degrading bacteria and sulphate reducing bacteria

The very slow growth (until the 42nd day of incubation) of aerobic oil degrading bacteria observed in this experiment had not been foreseen, as a very marked increase in the abundance of these bacteria had been observed in the simulated spill of sunflower oil in the

field. Sunflower oil incubated for 14 days with aerobic bacteria suffered only a small decrease in the proportion of 18:0, with a longer incubation period (up to 42 days) not intensifying this change. This decrease in the proportion of 18:0 suggests preferential degradation of this fatty acid, contrasting with the results obtained in the field work.

Aerobic and facultative anaerobic bacteria incubated for 42 days were responsible for a decrease in the proportion of 18:2ω6. A decrease in the proportion of 18:2ω6 between day 60 and 180 was also observed in the field experiment simulating a spill of sunflower oil, which implies that a long incubation period may be necessary for such alterations to occur. In the field experiment, the decrease in the percentage of 18:2ω6 was accompanied by an increase in that of other fatty acids. In this experiment, oil incubated with aerobic bacteria showed only a decrease in the polyunsaturated fatty acid, without accumulation of any of the other fatty acids. This indicates that the polyunsaturated fatty acid was possibly isomerised to form 'new' octadecadienoic acids. This hypothesis is substantiated by the fact that the highest percentage of 'new' fatty acids was observed after 42 days of incubation in the presence of aerobic bacteria.

Slow growth was possibly the reason why the anaerobic bacteria were not able to cause any changes in the fatty acids composition of the oil that had been initially incubated with aerobic bacteria. Contrasting with this findings, the SRB added to the medium after pre-conditioned with aerobic and anaerobic bacteria, produced an increase in the 18:2ω6, indicating that SRB did not utilise 18:2ω6 but converted other fatty acids into 18:2ω6. The reason for an increase in the concentration of 18:2ω6 is difficult to justify since this fatty was amply available in the medium for bacteria utilisation.

Similarly to the findings in the linseed oil degradation (Experiment II), some of the 'new' fatty acids registered were identical to those from the field experiment and some other were observed for the first time in this experiment. Therefore, 18:2ω6 could have been isomerised by an identical pathway to that described in the field experiment, as well as by other alternative routes. The later identification of the 'new' fatty acids will provide information to establish the degradative pathway(s) of sunflower oil (Chapter 7).

Experiment b): Interaction between anaerobic oil degrading bacteria and sulphate reducing bacteria

In the field experiment it was observed that the abundance of anaerobic oil degrading bacteria would increased after a spill of sunflower oil, although this increase was much smaller than that registered for aerobic bacteria. In the present *'in vitro'* experiment, only a very small number of anaerobic bacteria seem to grow in the pure sunflower oil being, nevertheless, responsible for a decrease in the proportion of 18:0 and an increase in the proportion of 18:1ω9 within 14 days of incubation. These modifications were not intensified when the incubation period was extended to 28 days. The former results appear to suggest a different pathway of degradation from that observed in the field, involving conversion of the saturated 18:0 into the unsaturated 18:1ω9. With the increase in the duration of the incubation to 28 days, no further changes were added to the 18:0 and 18:1ω9, but a decrease in the proportion of 18:2ω6 was observed, which could have been isomerised to form the 'new' octadecadienoic acid observed. It could also have undergone hydrogenation to form 18:1ω9.

Even though there were only a few modifications carried out by anaerobic bacteria in the first 14 days, they were sufficient for the SRB to continue the alterations in the fatty acids composition of sunflower oil. These involved a decrease in the percentage of 16:0 and an increase in 18:2ω6. The accumulation of the latter suggest a reversed reaction from that carried out by anaerobic bacteria, having been also observed in experiment a) after the addition of SRB to the samples containing aerobic and anaerobic bacteria. These results appear to suggest that SRB did not metabolise 18:2ω6. However, the accumulation of 18:2ω6 is difficult to understand for the reason given before. In the field work a decrease in 18:2ω6 was apparent, which does not mean that the SRB were not contributing to increasing the levels of 18:2ω6, but that the effect of these bacteria could have been masked by the presence of a much higher number of aerobic bacteria catalysing the opposite reaction.

Experiment c): Role of sulphate reducing bacteria

As observed in the case of linseed oil, colonies of SRB were not able to grow in pure sunflower oil, which was expected since it is well established that SRB cannot breakdown

polymers. Consequently, no changes in the fatty acids composition of the sunflower oil were observed after the oil inoculation with this type of micro-organisms.

5.4. SUMMARY AND CONCLUSIONS

The experiments described in this chapter had the objective of clarifying the role of indigenous bacteria from salt marsh sediments in the degradation of linseed and sunflower oils. Unfortunately, the low bacterial growth that occurred in Experiment II led to fewer alterations in the fatty acid composition of the oils than that which may have been expected according to the results of Experiment I. Consequently, Experiment II neither provided information about the degradation rates of the oils nor definitive conclusions regarding the role of each type of bacteria in the oil degradation. The observations in these experiments (mainly those involving linseed oil) allowed the drawing of the following conclusions:

- Aerobic and anaerobic bacteria indigenous from the salt marsh sediments, from depths down to 30 cm, can grow and utilise pure linseed and sunflower oil as the only source of carbon.
- SRB were not able to metabolise either oil in its raw state but were able to grow on their degradation products.
- The linseed and sunflower oils' degradation by indigenous bacteria from the studied salt marsh appears to be a sequential process initiated by aerobic or anaerobic bacteria and continued by SRB. Anaerobic bacteria also can utilise the products of aerobic metabolism.
- The various bacteria groups involved in the degradation of the oils and their metabolic products modified the proportion of the fatty acids present in the oils and introduced 'new' unsaturated fatty acids with the larger number of alterations being carried out by aerobic bacteria.
- Aerobic and anaerobic bacteria preferentially degraded 18:3ω3 from linseed oil leading to the accumulation of 18:1ω9 and formation of 'new' unsaturated fatty acids. Sulphate reducing bacteria incubated in oil pre-conditioned by aerobic and anaerobic bacteria also

metabolised preferentially 18:3ω3 as well as 18:2ω6, with accumulation of the remaining fatty acids of linseed oil.

- Aerobic and anaerobic bacteria preferentially metabolised the main fatty acid of sunflower oil, 18:2ω6. In contrast, sulphate reducing bacteria added to the pre-conditioned medium (by aerobic or/and anaerobic bacteria) produced an increase in the concentration of this fatty acid
- The linseed oil and sunflower oils degradation could have been carried out by more than one route involving isomerisation (positional and/or geometrical), hydrogenation and possibly β-oxidation.
- In both oils, when slow bacterial growth occurred, the isomerisation of unsaturated fatty acids seem to prevail over their hydrogenation.
- Linseed and sunflower oils degradation in Experiment II produced 'new' unsaturated fatty acids, not observed in the field experiments or in Experiment I.

6. 'New' Fatty Acids Identification

6.1. Introduction

The information provided by the gas chromatography retention data and the mass spectra of FAMEs, can in some cases, lead to the complete structure identification of a compound. However, the major task in the identification of unsaturated fatty acids is the determination of the position of the double bond. Unfortunately, fatty acids and their simple alkyl esters, such as methyl esters, suffer rearrangements of the double bonds under electron impact ionisation conditions and give mass spectra with extensive fragmentation which cannot be interpreted to locate the original double bond positions (Christie, 1982, 1989a; Murphy, 1993). Nevertheless, important information can be drawn from the GC profile and the mass spectra analysis of the FAMEs as exemplified below:

Gas chromatography (using a polar BPX-70 stationary phase, as in the present work)

i) long chain saturated fatty acids will have an higher retention time than short chain saturated fatty acids.

ii) within the same chain length, the retention time will increase with increasing unsaturation. The order of elution of the C_{18} components will be:

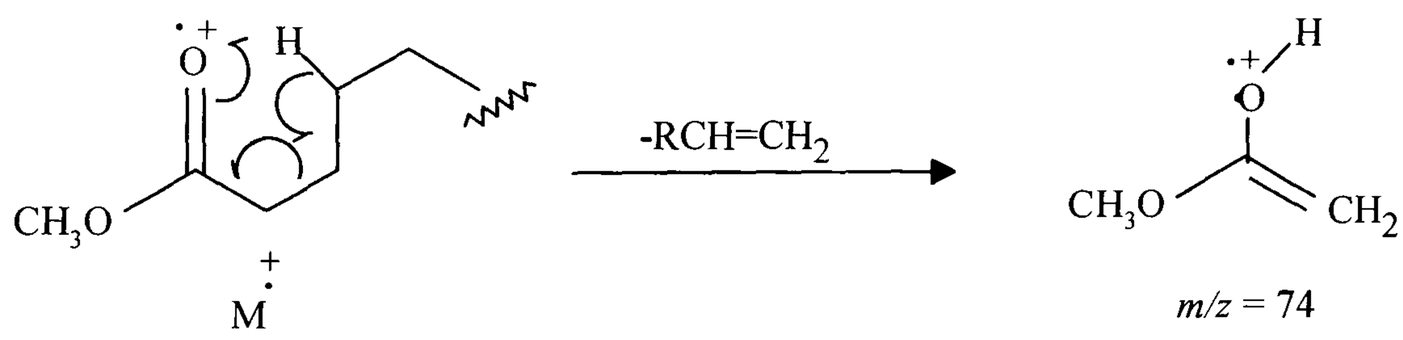
stereate (18:0) < oleate (18:1) < linoleate (18:2) < linolenate (18:3).

iii) when various positional isomers of polyunsaturated fatty acids are found, the shorter the distance between the last double bound and the end of the molecule, the longer the retention time.

iv) when various geometric isomers of unsaturated fatty acids are found, *trans* isomers have a shorter retention time than the *cis*.

v) the presence of a conjugated double bond increases the FAMEs retention time to a larger degree compared to normal methylene interrupted ester (Evershed, 1992).

Mass spectrometry: Long chain saturated methyl esters are easily identified and are characterised by a prominent molecular ion (M^+) and other significant ions at *m/z* = M-31 (loss of methanol) and M-43 (loss of C_2, C_3 and C_4 as a result of a complex rearrangement), together with a series of ions of general formula $-CH_3CO_2(CH_2)_n^+$ (Christie, 1982, 1989a; Murphy, 1993). The base peak at *m/z* = 74 is known as the "McLafferty rearrangement" and is formed in a rearrangement reaction after cleavage of the parent molecule beta to the carboxyl group (scheme 1).



Scheme 1. McLafferty rearrangement.

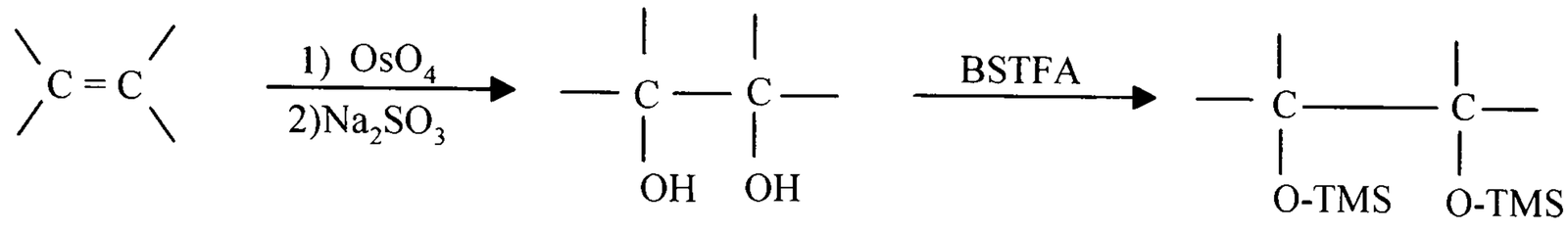
Mass spectra of methyl esters of unsaturated fatty acids differ greatly from those corresponding to saturated compounds, but are less useful for characterisation purposes. A most notable feature is that *m/z* 74 can only be seen in moderate abundance (Murphy, 1993). When three or more double bonds are present in the long chain the most abundant ion is *m/z* = 79 ($C_6H_7^+$) (Murphy, 1993). The molecular ion is often prominent, and there are generally major fragments at *m/z* = M-31 or M-32 and, with monoenes and dienes, at *m/z* = M-74 (loss of the McLafferty rearrangement ion). There are no ions that can be used to locate the position of double bonds, which probably migrate when the molecule is ionised, although there are indications that some positional isomers of polyunsaturated fatty acids may have distinctive but not easy interpreted spectra (Christie, 1982, 1989a; Evershed, 1992; Murphy, 1993).

More complex forms of derivatisation can be used to induce the molecules to fragment in ways that reveal the required structural information. These derivatisations can take two forms: methods in which the structural feature itself is modified and those in which the functional group, such as the carboxylic acid function is altered. The former methods of double bond analysis addressed the problem by fixing the position of the bond using derivatization. This derivatization usually takes the form of hydroxylation followed by permethylation, cyclic acetal or TMS ether formation; it achieves not only stabilisation of the bond, but the introduction of additional oxygen-containing groups which compete with the acid function for the charge (Harvey, 1992). This gives additional fragment ions in the spectrum. With the alternative method, functional-group derivatization, a nitrogenous function is introduced, often in the form of an aromatic ring. This nitrogenous function provides a very favourable charge-site, thus minimising ionisation and subsequent migration of double bonds and, because the charge-site is effectively isolated from the aliphatic chain, it reduces fragmentation produced by α-cleavage or McLafferty rearrangement. Nevertheless, hydrogen migration to the charge-site does occur, not from one very energetically favoured position as in the McLafferty rearrangement, but rather from all positions of the chain with roughly equal probability. The hydrogen migration produces a radical-site at any position of the chain and the resulting radical-site induced cleavages produce spectra in which ions formed by fissions of every carbon-carbon bond are prominent. Unlike the mass of the molecular ion which always occurs at odd mass, these fragment ions are of even mass. The masses and relative abundances of these ions are sensitive to the structure of the chain (Harvey, 1982; 1984; 1992).

This chapter reports the identification of the 'new' fatty acids. These fatty acids were observed in the sediments of the salt marsh at Foryd Bay after simulated spills with linseed and sunflower oils and also in experiments carried out in laboratory conditions (Chapters 4 and 5). These 'new' fatty acids were absent in the sediments and in the raw oils prior to the contamination. For the identification of these fatty acids, information provided by the GC-MS analysis of the FAMEs will be used in conjunction with more complex fatty acid derivatives, as will be described below.

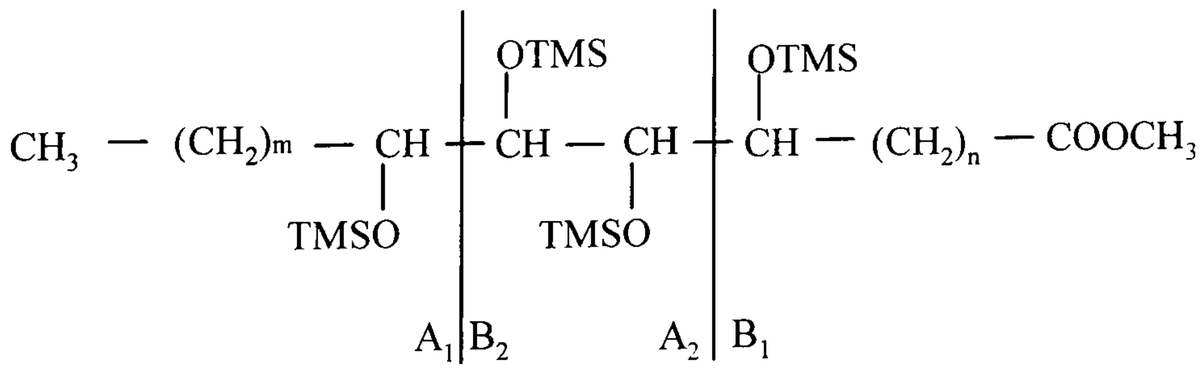
6.2. Derivatisation of the Double Bond to Determine Position

As described before, an approach to locate the unsaturation in fatty acids involves derivatization of the double bonds. In the method followed in the present study, the double bond is first oxidised with osmium tetroxide and the osmate formed is subsequently reduced with Na_2SO_3 to vicinal diols; these are then derivatised to trimethylsilyl (TMS) ethers (Capella & Zorzut, 1968; Sharpless & Alashi, 1976; Schröder, 1980). The outline of the method is schematically represented in scheme 2.



Scheme 2. Formation of trimethylsilyloxy (TMSO) derivatives of unsaturated fatty acids. [BSTFA is bis- (trimethylsilyl) trifluoracetamide]

The double bond in unsaturated fatty acids can be determined from the prevalent mass spectrometric primary cleavage of the bonds located at the positions of the original double bond in the trimethylsilyl ether derivatives of their methyl esters. This cleavage combined with the loss of a molecule of TMSOH for every two vicinal trimethylsilyloxy groups gives the most abundant ions in the mass spectra of monounsaturated and polyunsaturated fatty acids with isolated bonds (Janssen & Parmentier, 1978; Janssen *et al.,* 1985). According to Janssen & Parmentier (1978), this method can also be applied to polyenoic fatty acids with two to four conjugated double bonds. The stability of the resulting fragment ions apparently decreases with an increasing number of TMSO-substituted carbon atoms. Thus, the major fragment ions $[A_1]^+$ and $[B_1]^+$, which contain only one TMSO-substituted carbon atom, are produced by fisson of the bonds located at the positions of the original double bond (Scheme 3).



Scheme 3. Cleavages of the bonds located at the positions of the original double bonds in the trimethylsilyl ether derivatives of a diunsaturated methyl ester.

The complementary ions $[B_2]^+$ and $[A_2]^+$, bearing three TMSO-substituted carbon atoms, are very weak. The α-cleavage also occurs on either side of the original double bond positions. Rupture of the bond connecting the two original double bonds produces the moderately abundant complementary ions $[A_1 + CHOTMS]^+$ and $[B_1 + CHOTMS]^+$. The ions $[A_2 + CHOTMS]^+$ and $[B_2 + CHOTMS]^+$ corresponding to a fission on either side of the tetrol system are not detected (Janssen & Parmentier, 1978; Janssen *et al.,* 1985).

6.2.1. Practical Procedure

The practical procedure followed was based on the method of Janssen & Parmentier (1978).

Hydroxylation and derivatization

To a 1 mg subsample of FAMEs in 1 cm^3 of dioxane (BDH) + pyridine (BDH) (8:1), 0.1 cm^3 of a 5% solution of OsO_4 (BDH) in dioxane was added. The resultant solution was kept at room temperature for 1 hour. Subsequently, 2.5 cm^3 of methanol (Rathburn) and 8.5 cm^3 of a 16% solution of Na_2SO_3 (BDH) in distilled water were added and the mixture was allowed to stand for an additional hour. After centrifugation (1250 rpm for five minutes in a Centaur 1 MSE), the supernatant solution was diluted with four volumes of methanol, filtered (Whatman number 4) and evaporated. The residue was suspended in 2 cm^3 of methanol and 4 cm^3 of chloroform (Rathburn) added. This solution was filtered and the solvent was evaporated. The residue was weighted and dissolved in chloroform in a concentration of 10 mg cm^{-3}.

To 100 µl of the anterior solution, 25 µl of bis-(trimethylsilyl) trifluoracetamide (BSTFA, Sigma) were added. The mixture was heated at 60°C for 30 minutes. The solvent was evaporated and the residue re-dissolved in chloroform.

GC-MS operating conditions

The samples were analysed by GC-MS using a Fisons MD-800 with an on-column injection and secondary cooling. The carrier gas was helium (BOC) with a column flow

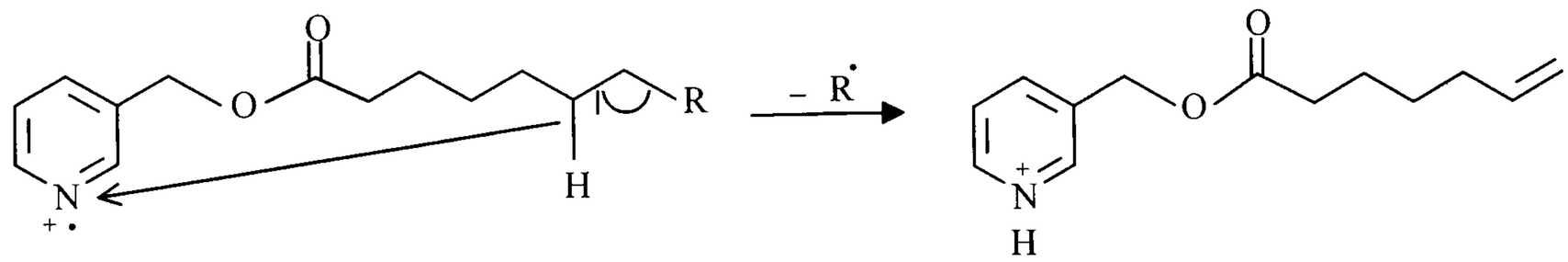
rate of 2 ml min^{-1}. A non-polar capillary column, BPX-5 (SGE) of 30 m x 0.32 mm internal diameter and 0.25 μm film thickness was used. The temperature programme started at 60°C for 1 minute, 40°C min^{-1} to 150°C, 6°C min^{-1} to 320°C and isothermal at 320°C for 5 minutes. The mass spectrometer was configured for electron impact ionisation at 70eV and a mass scan range of 45-585 *m/z*.

6.2.2. Results and Discussion

The results of the mass spectra of the OTMS derivatives were not conclusive; consequently, the determination of the double bond position was not possible. This method had been previously used in identical conditions (Pereira, 1993), having been successfully applied to pure fatty acids (18:1ω9 and 18:2ω6). However, in either occasion (past and current), when applied to a mixture of unknown fatty acids, problems were encountered in the interpretation of the obtained mass spectra and structure elucidation of the compounds was not achieved. The approach of using trimethylsilyl derivatives from polyhydroxy fatty acids that mark double bond positions has been widely used (Schmitz & Klein, 1986) and has been successfully applied to locate either isolated or conjugated double bonds in unsaturated fatty acids (Janssen & Parmentier, 1978; Janssen *et al.,* 1985). However, there are also disadvantages in the use of this technique: the molecular ion is absent and the ions from which the molecular ion can be deduced are of low intensity. The addition of trimethylsilyl groups substantially increases the molecular weight of a molecule and decreases volatility, factors that become particularly important when using GC-MS (Murphy, 1993). As the number of double bonds increase in the polyunsaturated fatty acid, the ions characteristic for the vicinal diols become less abundant and alternative fragmentation pathways increase. This makes structure elucidation of an unknown polyunsaturated fatty acid much more difficult. These limitations of the technique may explain the problems encountered in the present work when identification of unknown fatty acids was attempted.

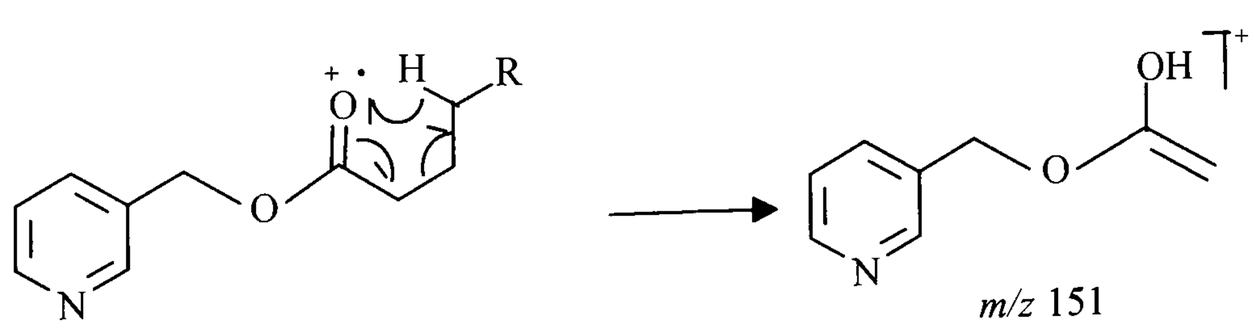
6.3. Derivatisation of the Carboxylic Group for the Determination of the Double Bond Position

An alternative method to establish the double bond location is to form a derivative of the carboxylic group to direct fragmentation. This method was introduced by Anderson & Holman (1974) for the preparation of the pyrrolidide derivatives. This induces cleavages of the aliphatic chain at all positions without isomerisation, and the positions of the double bonds can be determined by the mass increment between the ions. This method led Harvey (1982) to prepare the related picolinyl esters of carboxylic acids. Harvey (1982) proposed the use of picolinyl (3- hydroxymethyl-pyridine) ester derivatives containing double bonds and other functional groups. Like the pyrrolidides, they fragment under electron impact by radical-induced cleavage at each carbon - carbon bond, as shown in scheme 4.



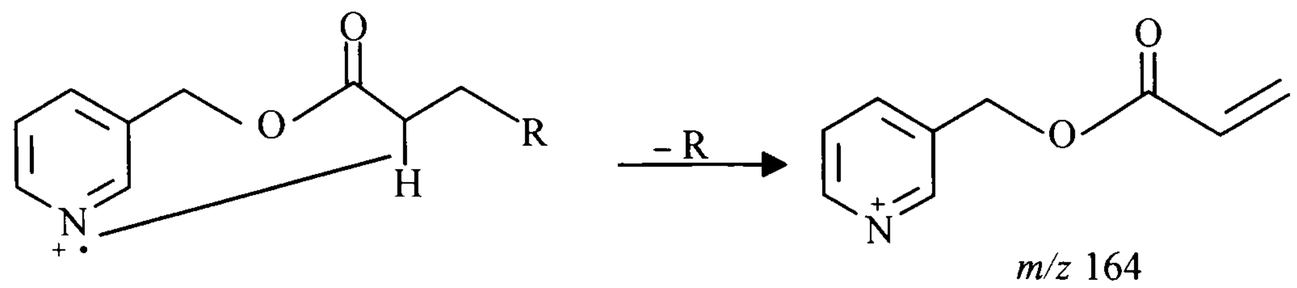
Scheme 4. Mechanism for the formation of the diagnostic, radical-induced cleavage ions for the mass spectra of the picolinyl esters.

The fragments produced are separated by 14 mass units and appear in the region between the molecule ion and the ion *m/z* 151, which is accounted for by the McLafferty rearrangement as shown in scheme 5 (Harvey, 1982).



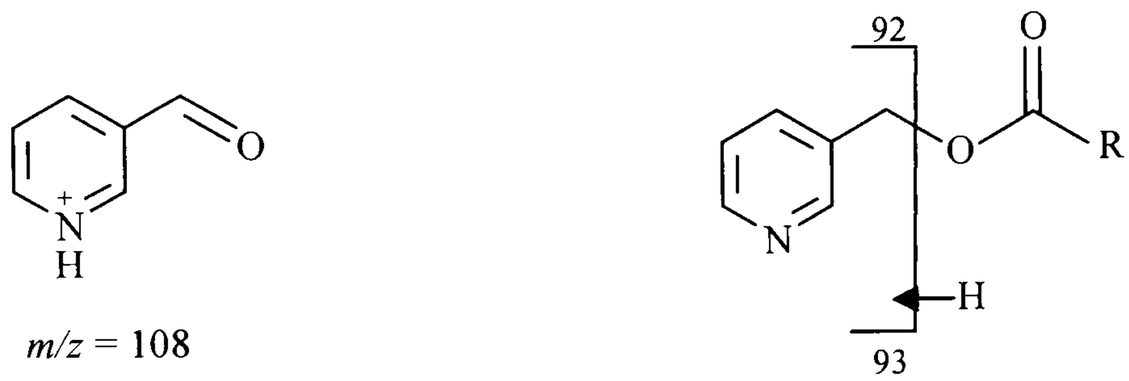
Scheme 5. Formation of the ion at *m/z* 151 by the McLafferty rearrangement.

Another fragment also observed in relatively high abundance in the spectra of picolinyl esters derivatives is the *m/z* 164 which can be rationalised by formation of the conjugated species (Scheme 6) (Harvey, 1982, 1992).



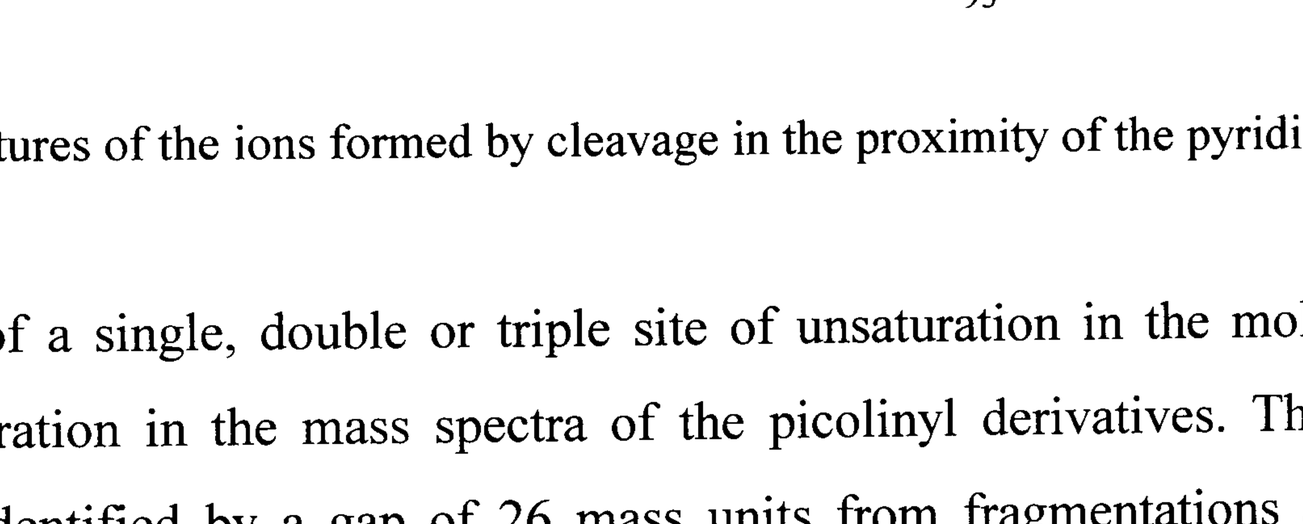
Scheme 6. Mechanism for the formation of the ion at *m/z* 164 in the spectra of the picolinyl esters.

Others also present in high abundance in the spectra of these derivatives are the fragments *m/z* 92, 93 and 108, which are formed by cleavage around the pyridine ester group and whose structures are shown in scheme 7 (Harvey, 1982).



Scheme 7. Structures of the ions formed by cleavage in the proximity of the pyridine ring.

The presence of a single, double or triple site of unsaturation in the molecule causes a significant alteration in the mass spectra of the picolinyl derivatives. The double bond positions are identified by a gap of 26 mass units from fragmentations adjacent to the double bond (Harvey, 1982; 1989; 1992; Christie, 1989b). The mechanism for the fragmentation of the unsaturated fatty acids is shown below (Scheme 8) (Murphy, 1993).



Scheme 8. Mechanism for the fragmentation of picolinyl derivatives of unsaturated fatty acids.

6.3.1. Practical Procedure

Derivatives preparation

The free fatty acids (5 mg) were dissolved in 0.5 cm^3 of trifluoroacetic anhydride (Sigma) and heated at 50°C for 30 minutes in a screw-capped vial. Immediately after cooling and evaporation of the excess reagent under a stream of nitrogen, 20 mg of 3-hydroxymethylpyridine (Sigma) and 4 mg of 4-dimethylaminopyridine (Sigma) as a solution in 0.2 cm^{-3} of dichloromethane (Rathburn) were added. The mixture was allowed to stand for 3 hours at room temperature, after which the solvent was evaporated and the residue dissolved in 8 cm^{-3} of hexane (Rathburn) and washed 3 times with 4 cm^3 of water. The hexane was evaporated to dryness and the residue was dissolved in 1 cm^3 of diethyl ether (Rathburn). To remove the residual free fatty acids the solution was passed through a cartridge containing amino bonded silica (Alltech).

GC-MS operating conditions

The picolinyl esters derivatives were analysed by GC-MS using a Fisons MD-800 with an on-column injection and secondary cooling. The carrier gas was helium (BOC) with a column flow rate of 2 ml min^{-1}. A non-polar capillary column, BPX-5 (SGE) of 30 m x 0.32 mm internal diameter and 0.25 μm film thickness was used. The temperature programme started at 60°C for 1 minute, 15°C min^{-1} to 280°C, 1°C min^{-1} to 310°C and 10°C min^{-1} to 350°C. The mass spectrometer was configured for electron impact ionisation at 70eV and a mass scan range of 45-585 *m/z*.

6.3.2. Results and Discussion

All 'new' octadecenoic, octadecadienoic and octadecatrienoic fatty acids observed in the "*in vitro*" and "*in situ*" experiments using linseed and sunflower oils is summarised in Tables 6.1 and 6.2. Each fatty is characterised by its scan time and also by its relative retention time ($r_{18:0}$), which correspond to its retention time divided by that of 18:0.

Table 6.1. Identification and GC characterisation of the 'new' fatty acids observed in the field and laboratory experiments carried out with linseed oil.

Fatty acid methyl ester	**Retention time** scan (minutes)	**Relative retention time** ($r_{18:0}$)	**Experiment**
18:0	747 (16.453)	1.00	
18:1 (1)	791 (17.186)	1.04	Laboratory Experiment II
18:1ω9	802 (17.370)	1.06	
18:1ω7	822 (17.703)	1.08	Field & Laboratory Experiment I & II
18:2 (5)	890 (18.837)	1.15	Laboratory Experiment II
18:2ω6	923 (19.387)	1.18	
18:3 (5)	1037 (21.287)	1.29	Laboratory Experiment II
18:3 (6)	1068 (21.804)	1.33	Laboratory Experiment II
18:3ω3	1099 (22.321)	1.36	
18:2 (1)	1183 (23.721)	1.44	Field & Laboratory Experiment I
18:2 (2)	1250 (24.905)	1.51	Field
18:2 (3)	1300 (25.672)	1.56	Field & Laboratory Experiment I
18:3 (1)	1330 (26.155)	1.59	Field & Laboratory Experiment I & II
18:3 (2)	1350 (26.505)	1.61	Field
18:3 (3)	1376 (26.939)	1.64	Field
18:3 (4)	1398 (27.305)	1.66	Field & Laboratory Experiment I & II

Table 6.2. Identification and GC characterisation of the 'new' fatty acids observed in the field and laboratory experiments carried out with sunflower oil.

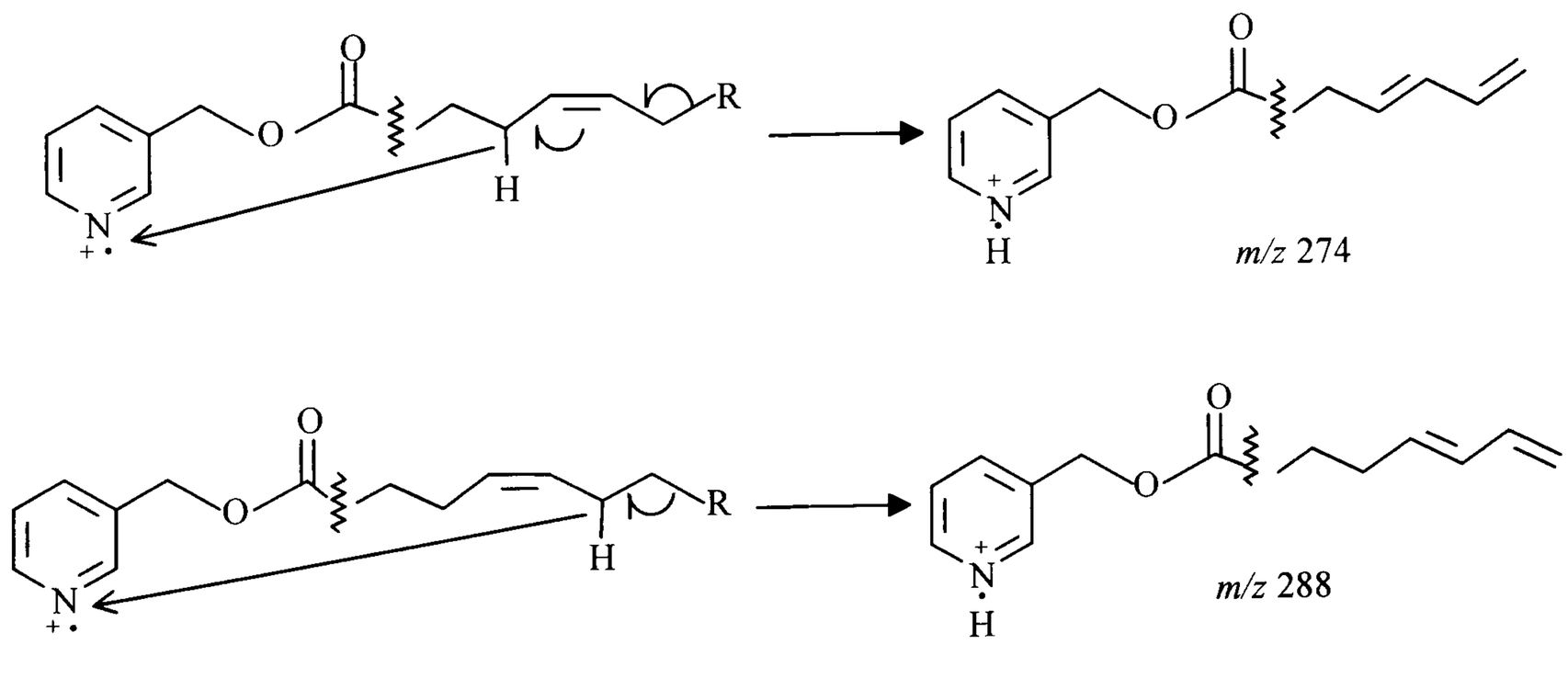
Fatty acid methyl ester	**Retention time** scan (minutes)	**Relative retention time** ($r_{18:0}$)	**Experiment**
18:0	748 (16.470)	1.00	
18:1 (1)	792 (17.203)	1.04	Field & Laboratory Experiment II
18:1ω9	803 (17.386)	1.06	
18:1ω7	824 (17.736)	1.08	Field & Laboratory Experiment II
18:2 (6)	880 (18.670)	1.13	Laboratory Experiment II
18:2 (5)	900 (19.003)	1.15	Field & Laboratory Experiment II
18:2ω6	925 (19.420)	1.18	
18:2 (1)	1180 (23.671)	1.44	Field
18:2 (4)	1200 (24.004)	1.46	Field & Laboratory Experiment II
18:2 (2)	1253 (24.887)	1.51	Field & Laboratory Experiment II
18:2 (3)	1297 (25.604)	1.55	Field & Laboratory Experiment II

Octadecenoic Acids

In the experiments carried out with linseed and sunflower oils, two 'new' octadecenoic acids were observed: one with elution time lower than that of 18:1ω9 and other with greater

retention time than that of this fatty acid (Tables 6.1. & 6.2). According to their relative retention time (RRT) and mass spectra, the 18:1 fatty acids observed in the experiments using linseed and sunflower oil were identical (Table 6.1 & 6.2). The octadecenoic acid with higher elution time was identified as 18:1ω7 (*cis*-11- octadecenoic acid) by comparison with 18:1ω7 in cod liver oil ($RRT_{r18:0}$ = 1.08). The identification of the 18:1ω7 was also confirmed by analysis of the mass spectrum of its picolinyl ester in conjunction with the mass spectrum of the picolinyl ester of *cis*-9 octadecenoic acid (18:1ω9) (Figure 6.1A).

The spectrum of *cis*-9 octadecenoic acid showed the expected general features of a fatty acid picolinyl ester with respect to the ions *m/z* 92, 93, 108, 151 and 164. Several other features are characteristic of the spectra of the picolinyl esters of monounsaturated acids, such as the molecular ion (*m/z* 373) which is more abundant than in the spectra of the picolinyl ester of saturated acids (Harvey, 1982). In the case of the *cis*-9 octadecenoic acid, the position of the double bond is indicated by the 26 mass unit between the ions *m/z* 234 and 260 (Figure 6.1A). Formal cleavage through the double bond gives rise to the group of ions around *m/z* 247. According to Harvey (1982), the elevated abundance of the ions *m/z* 274 and 288 in Figure 6.1A are caused by abstraction of the two allylic hydrogen atoms and formation of the conjugated species shown in scheme 9.



Scheme 9. Mechanism of formation of the ions at m/z 274 and 288 in the mass spectrum of the picolinyl derivative of *cis*-9 octadecenoic acid.

These ions are the most analytically useful for detecting double bonds because of their high abundance and characteristic masses (Harvey, 1982; 1992).

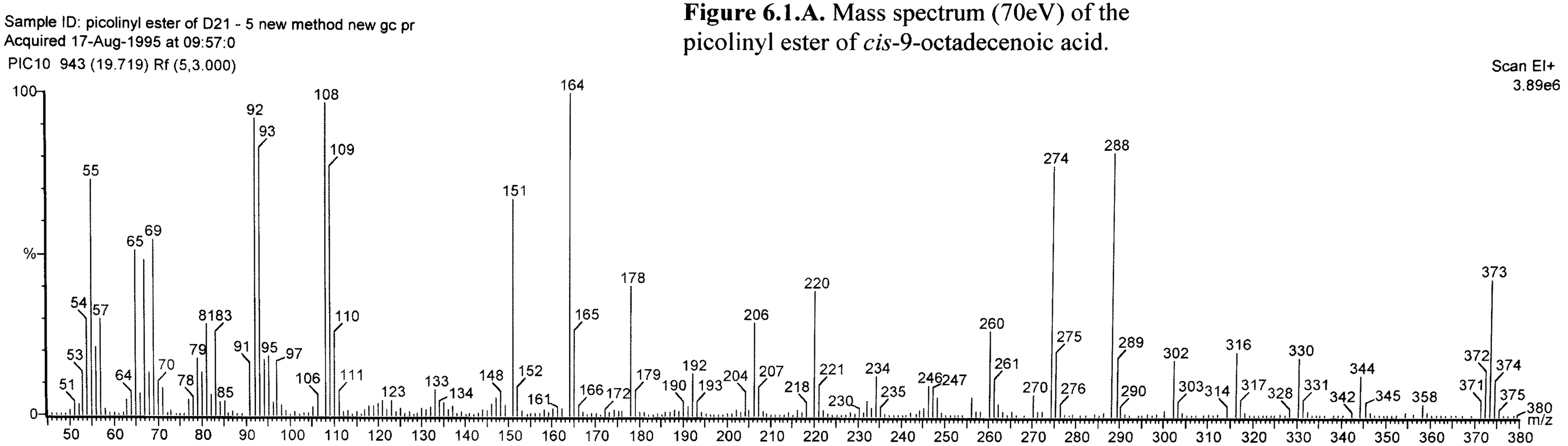


Figure 6.1.A. Mass spectrum (70eV) of the picolinyl ester of *cis*-9-octadecenoic acid.

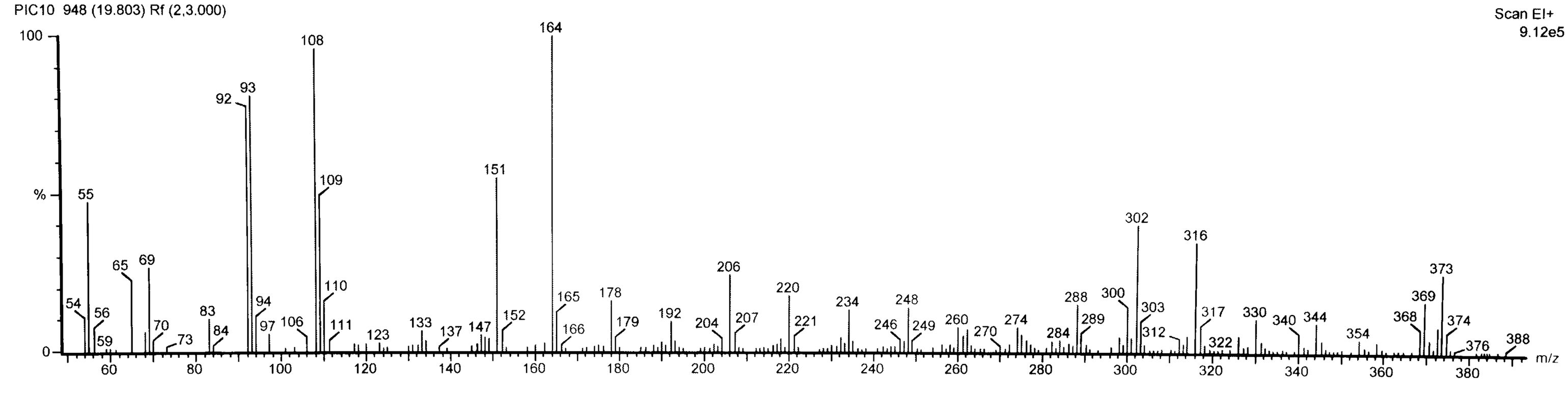


Figure 6.1.B. Mass spectrum (70eV) of the picolinyl ester of 11-octadecenoic acid.

As in the case of 18:1ω9, the spectra of the picolinyl ester of the 18:1ω7 is characterised by the molecular ion (*m/z* 373) and by the 26 mass unit between the ions *m/z* 262 and 288 (Figure 6.1B). This information suggest that the double bond is located in the ω7 position. The high abundance of the ions *m/z* 302 and 316 are caused by the abstraction of the two allylic hydrogen atoms from C-13 and C-14 and formation of the conjugated species shown in scheme 9. These data suggest a Δ^{11} bond. However, they do not provide any information regarding its geometric configuration. The configuration *cis* of the double bond was presumed by the additional information (retention time) provided by the GC analysis of cod liver oil.

The other octadecenoic acid, 18:1(1) had a lower retention time ($r_{18:0}$= 1.04) than 18:1ω9 ($r_{18:0}$= 1.06). This difference in retention times suggest that the 'new' fatty acid is either a *trans*-9 octadecenoic acid or a *cis*-7 octadecenoic acid. A pure standard of *trans*-9 octadecenoic acid (Sigma) showed an identical relative retention time ($r_{18:0}$= 1.04) to that of the 'new' fatty acid. Furthermore, the relative retention time of 18:1ω11 (*cis*-7 octadecenoic acid) present in cod liver oil was $r_{18:0}$= 1.05. Therefore, the 'new' fatty acid, 18:1(1) is suggested to be a ***trans*-9 octadecenoic acid**.

Octadecadienoic Acids

Field and laboratory experiments carried out with linseed and sunflower oils revealed a total of four and six 'new' octadecadienoic acids, respectively (Tables 6.1 and 6.2).

The FAMEs 18:2 (5) and 18:2 (5 and 6) present respectively in the linseed and sunflower oil experiments, had a lower elution time than linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid). According to their RRT and mass spectra, the 18:2 (5) observed in the experiments with both oils were identical compounds (Table 6.1 and 6.2). To identify these two fatty acids, their RRT were compared to those of a standard mixture of geometric isomers of the 9, 12-octadecadienoic acid methyl esters (Supelco) (Figure 6.2). The GC-MS data of these FAMEs and those of the standard mixture suggest that the unknown 18:2 (5) could be the ***cis*-9, *trans*-12 octadecadienoic acid**. The 18:2 (6) observed in sunflower oil experiments could be ***trans*-9, *trans*-12 octadecadienoic acid.**

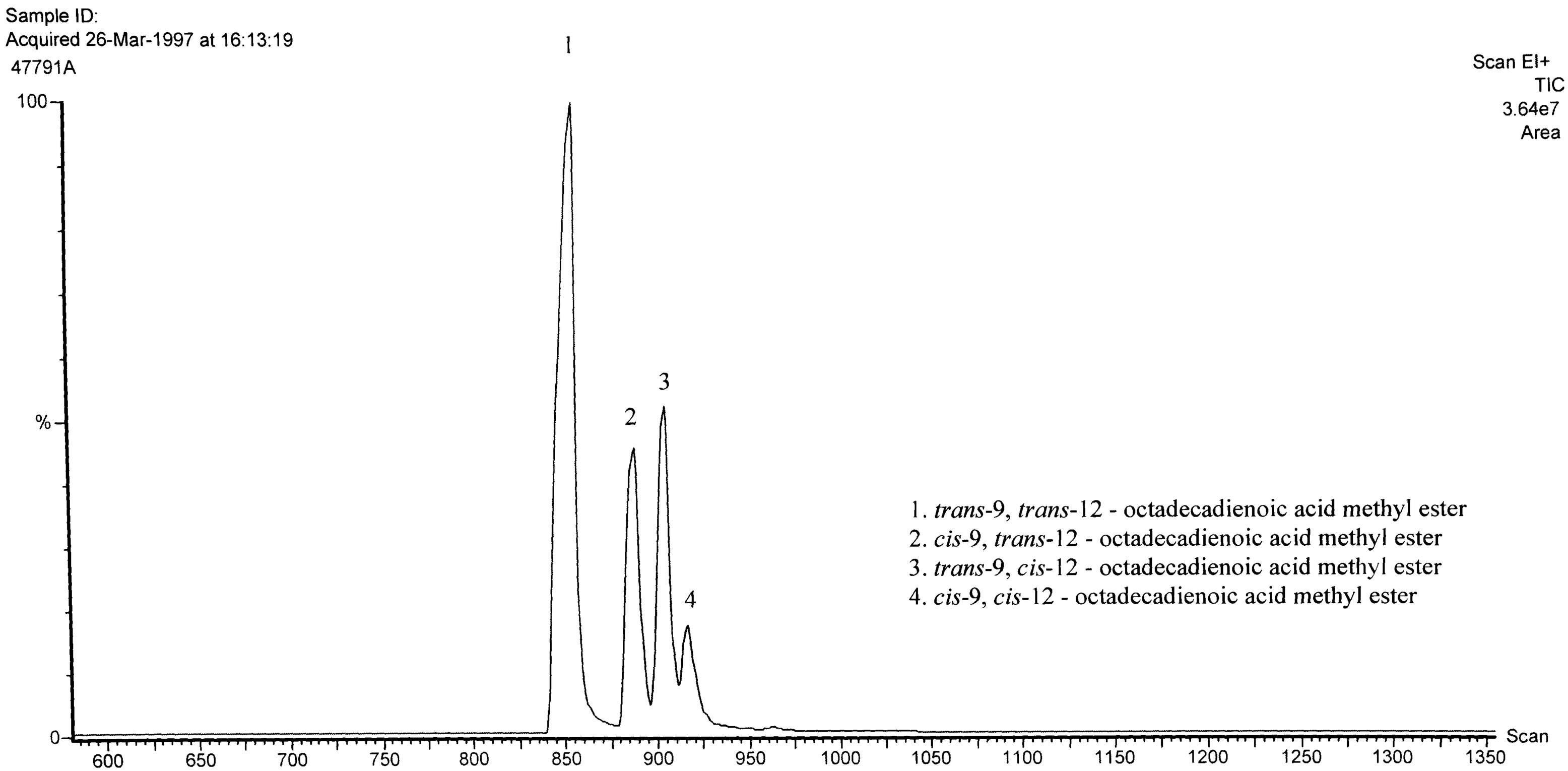


Figure 6.2. Gas Chromatogram of a standard mixture of geometric isomers of 9, 12-octadecadienoic acid methyl esters.

Since the remaining 'new' octadecadienoic acids eluted after the *cis*-9, *cis*-12 octadecadienoic acid, they could not be its geometric isomers. Once more, the comparison of the RRT and the mass spectra of the 'new' fatty acids observed in linseed and sunflower oils revealed that the 18:2 (1), 18:2 (2) and 18:2 (3) found in the experiments using both oils were identical compounds.

The identification of these 18:2 fatty acids was carried out using the analysis of the mass spectrum of their picolinyl esters in conjunction with the mass spectrum of the picolinyl ester of *cis*-9, *cis*-12 octadecadienoic acid (18:2ω6) (Figure 6.3). The spectrum of *cis*-9, *cis*-12 octadecadienoic acid showed the expected general features with respect to the ions *m/z* 92, 93, 108, 151 and 164 but is more complex than that of the monounsaturated acids. The presence of the distal (to the ester function) double bond is indicated by the 26 mass unit gap between the ions at *m/z* 274 and 300 and the second double bond is revealed by the 26 mass unit gap between the ions at *m/z* 234 and 260. The elevated abundance of the ions at *m/z* 314 and 328 correspond to the ions in scheme 9 relating to the Δ^{12}- double bond and the ion *m/z* 274 correspond to the ion in scheme 9 from the Δ^{9}- double bond. The ion m/z 288 (scheme 9) from this bond is absent because the Δ^{12}- double bond is occupying the site of the cleavage (Harvey, 1982, 1984 and 1992). The ion at *m/z* 260 is however, abundant and ions at this relative position appear to be characteristic of methylene-interrupted unsaturation in which the proximal double bond is further from the ester group than C-8 (Harvey, 1992).

The mass spectrum of the picolinyl ester derivative of 18:2 (3) is illustrated in Figure 6.4A. The prominent ion at *m/z* 302 indicates loss of a five carbon fragment containing one double bond. Its even mass indicates that the transferred hydrogen does not come from the leaving fragment. Therefore, cleavage of the bond probably generates a double bond in conjugation with the double bond in the ester-containing fragment. If this original double bond was between carbons 10 and 11, this fragmentation could be accomplished by transfer of the allylic hydrogen from C-12 (Scheme 10) (Harvey, *pers. commun.*). However, a prominent ion would be expected at m/z 288 following the transfer of the other allylic hydrogen at C-9 (Scheme 11) (Harvey, *pers. commun.*).

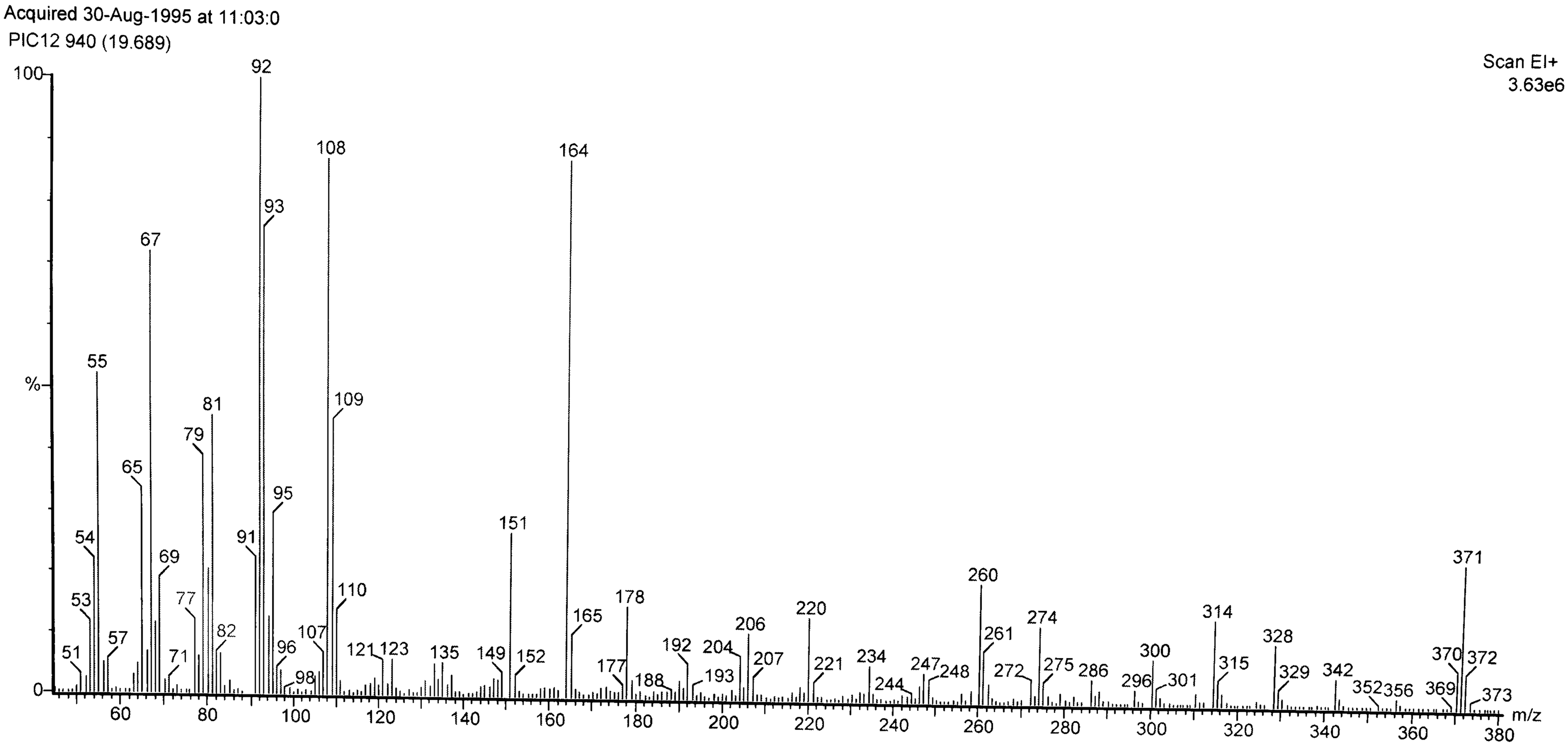
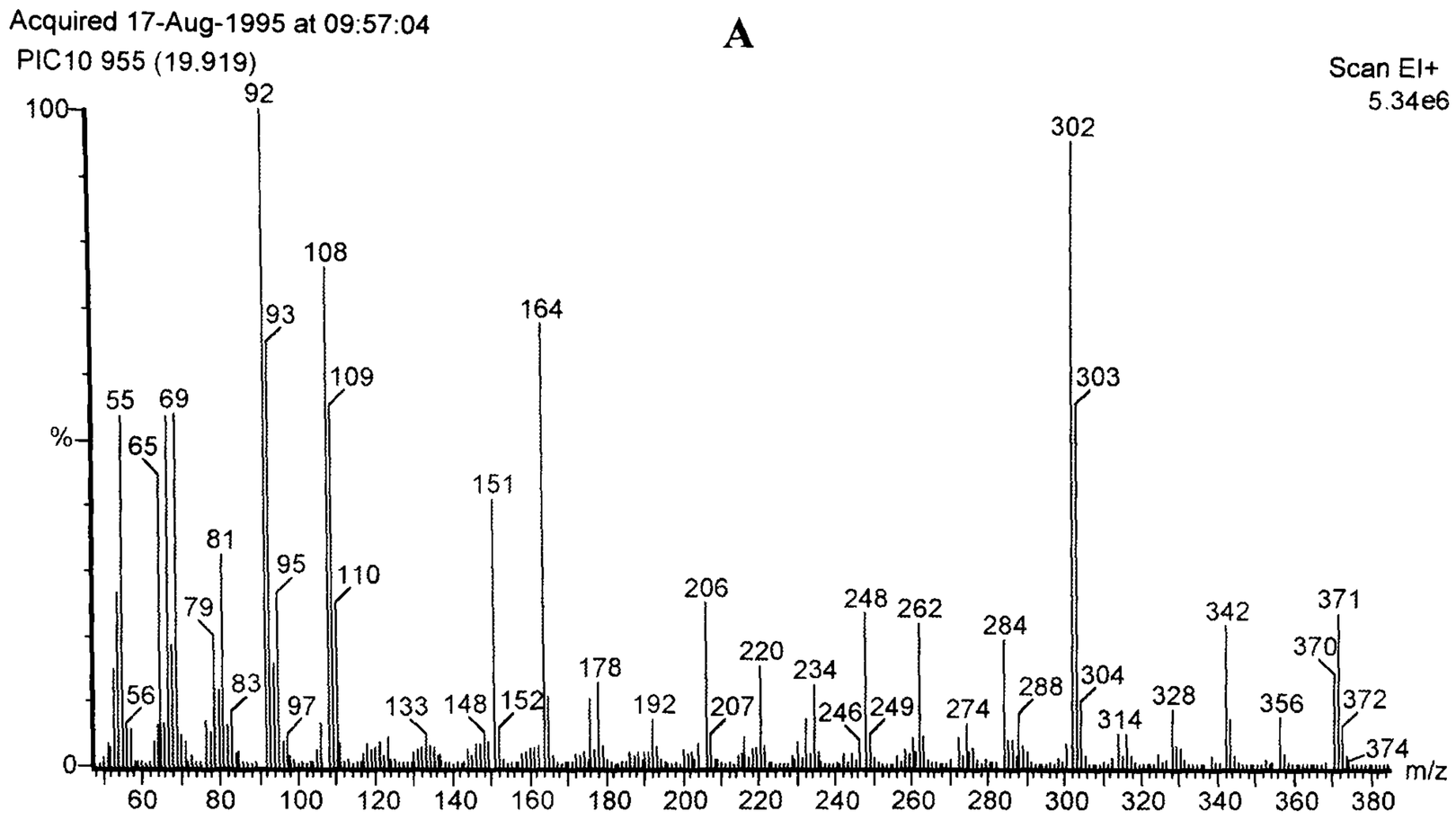
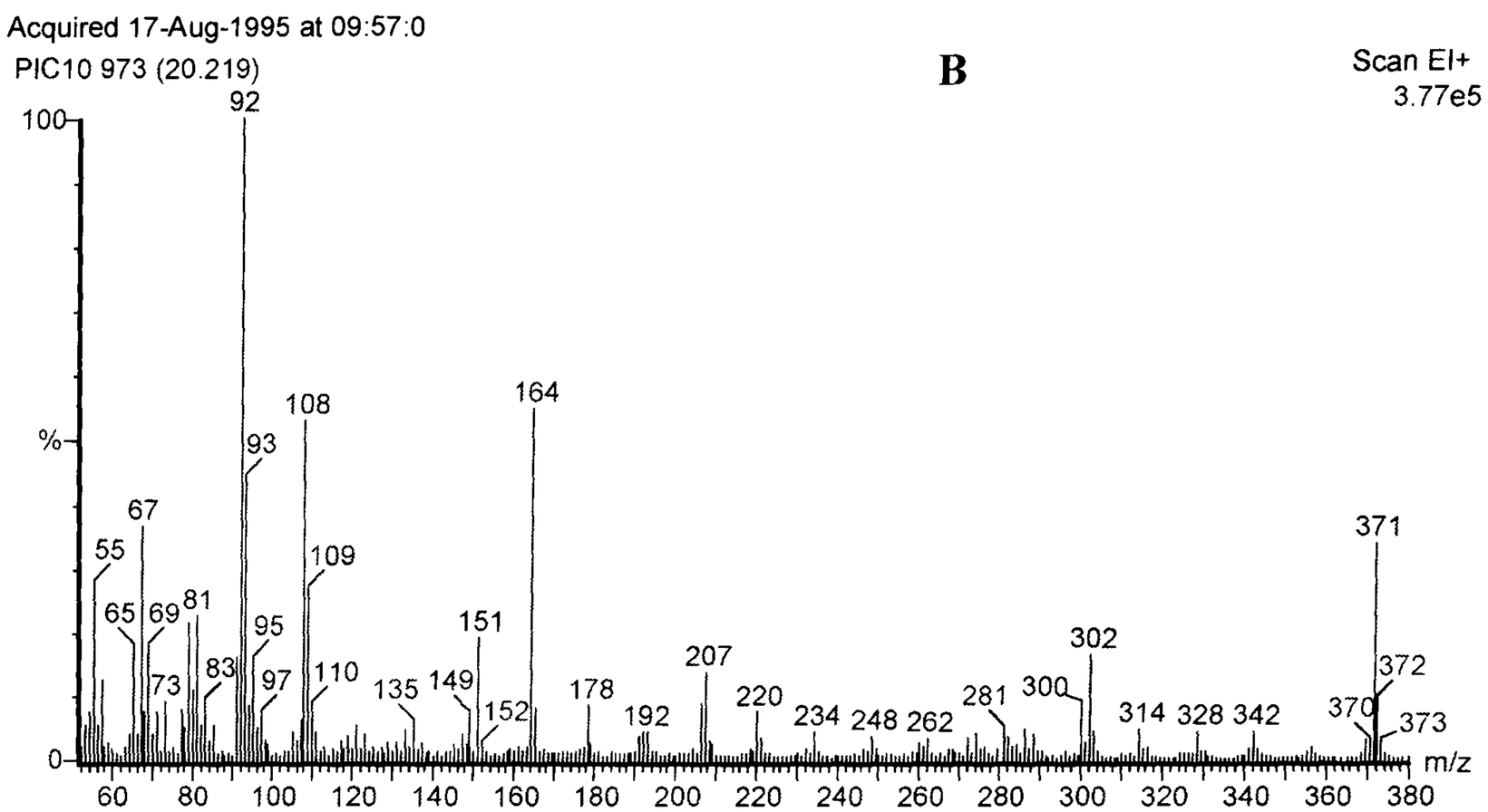


Figure 6.3. Mass spectrum (70eV) of the picolinyl ester of *cis*-9, *cis*-12- octadecadienoic acid.





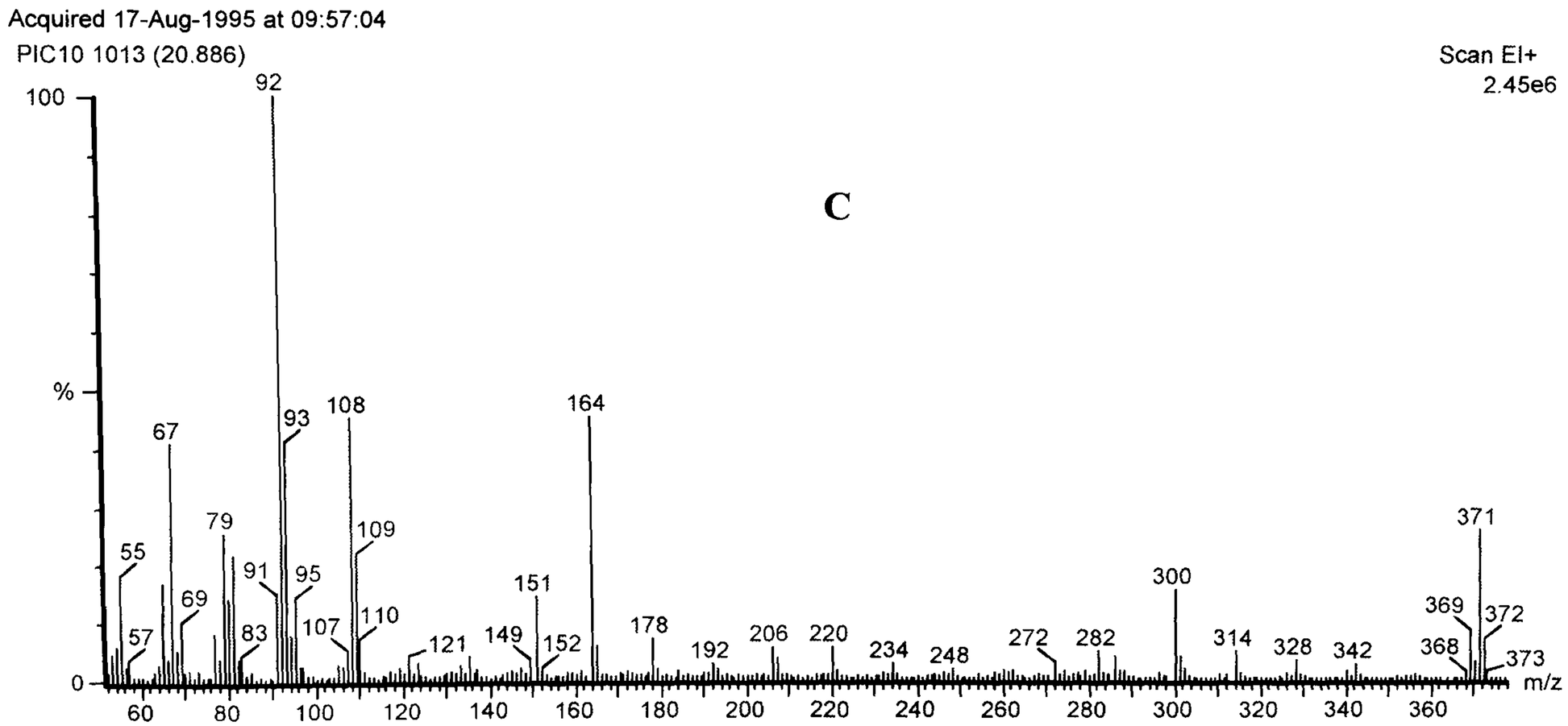
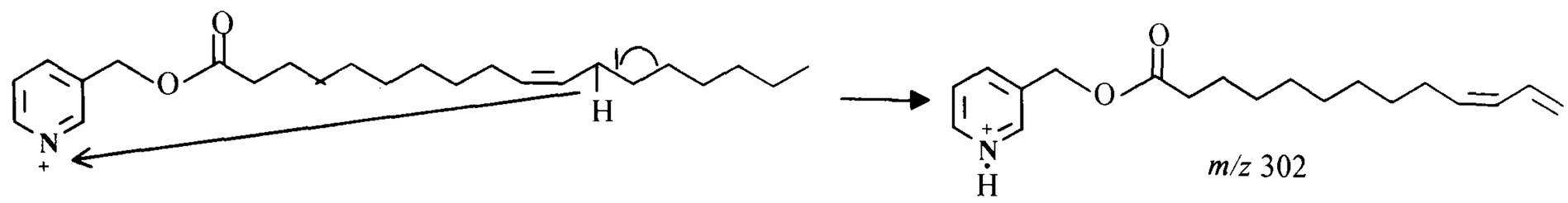
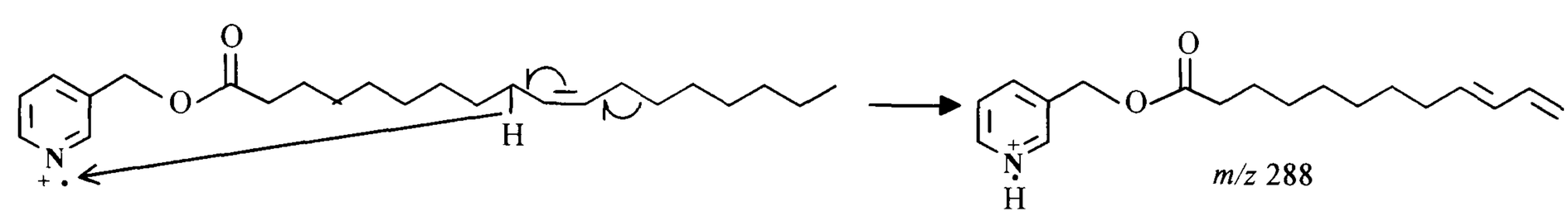


Figure 6.4. Mass spectra (70eV) of the picolinyl esters of the unknown 18:2 (3) (A), 18:2 (2) (B) and 18:2 (1) (C).

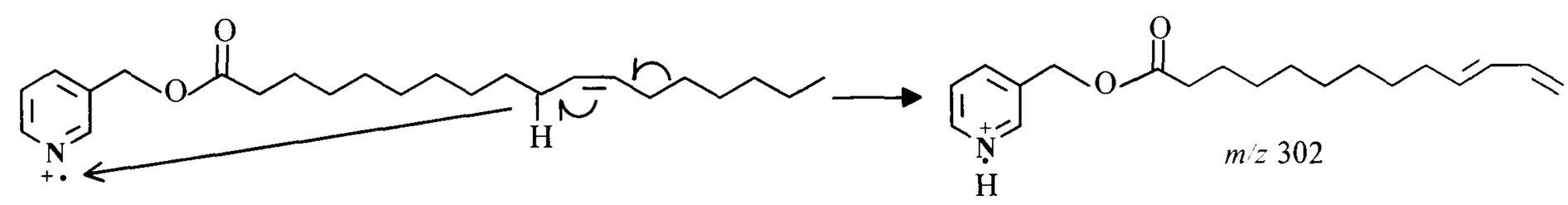


Scheme 10. Mechanism of formation of the ion at *m/z* 302 in the mass spectrum of a picolinyl derivative with a Δ^{10}- double bond (Double bond represented as a *cis*. Second double bond not shown).



Scheme 11. Mechanism of formation of the ion at *m/z* 288 in the mass spectrum of a picolinyl derivative with a Δ^{10}- double bond (Double bond represented as a *cis*. Second double bond not shown).

This ion is not prominent but may be weak because of the influence of the double bond in the 5-carbon fragment (not shown). If the double bond was between carbons 11 and 12, fragmentation could involve transfer of the allylic hydrogen at C-10 followed by rearrangement (Scheme 12) (Harvey, *pers. commun.*).



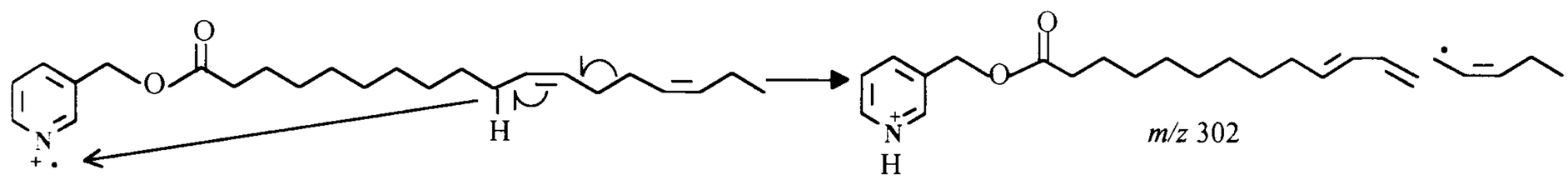
Scheme 12. Mechanism of formation of the ion at *m/z* 302 in the mass spectrum of a picolinyl derivative with a Δ^{11}- double bond (Double bond represented as a *cis*. Second double bond not shown).

The involvement of the other allylic hydrogen at C-13 and cleavage between C-14 and 15 to give *m/z* 316 may again be influenced by the second double bond accounting for its low abundance.

The analysis of the middle portion of the spectrum reveals analogous series of ions from *m/z* 164 to *m/z* 262 (carbons 1 to 10) indicating saturation. In the spectrum of the standard linoleic acid (Figure 6.3), where the double bonds are at C-9 and C-12, this series of ions stops at m/z 234 (carbons 1 to 8). The two extra ions in this spectrum thus locates the

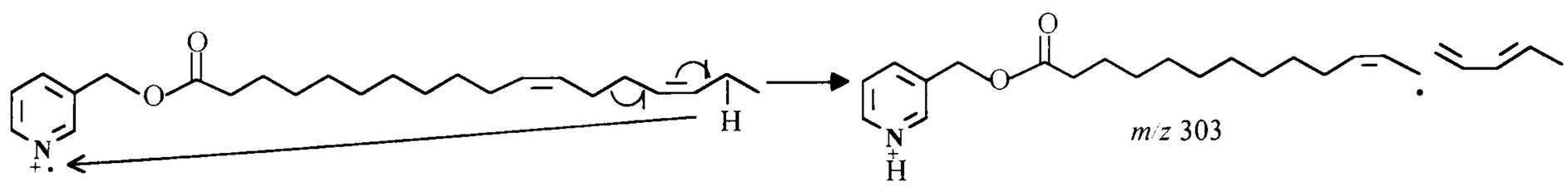
double bond at C-11. The structure in scheme 12 (Δ^{11} unsaturation) is the intermediate in the formation of the major ion at *m/z* 302.

The second double bond cannot be at C-13 as this is the cleavage of bond forming *m/z* 302. Neither can it be at C-14 as the compound would be then a methylene-interrupted fatty acid and would be expected to have a spectrum resembling that of linoleic acid, which it does not. Loss of a methyl and a methylene group to give *m/z* 356 and 342 indicate saturation at carbons 17 and 18. This positions the second double bond at the C-15. Placing the bond at C-15 explains the high relative abundance at *m/z* 302 because, following the cleavage of the C-13- C-14 bond, the radical is left in conjunction with the double bond (Scheme 13) (Harvey, *pers. commun.*).



Scheme 13. Mechanism of formation of the ion at *m/z* 302 in the mass spectrum of a picolinyl derivative of 11, 15-octadecadienoic acid (Double bond represented as a *cis*).

This $\Delta^{11,15}$ structure also accounts for the presence of the abundant ion at m/z 303 whose mechanism of formation must involve transfer of a hydrogen from the neutral fragment to the ester fragment. This is accommodated by transferring the allylic hydrogen from C-17 as below (Scheme 14) (Harvey, *pers. commun.*).



Scheme 14. Mechanism of formation of the ion at *m/z* 303 in the mass spectrum of a picolinyl derivative of 11, 15-octadecadienoic acid (Double bond represented as a *cis*).

The compound thus appears to be 11, 15-octadecadienoic acid, although its geometric configuration was not established.

The inferences made to locate the double bonds in the 11, 15-octadecadienoic acid can also be applied to the mass spectrum of the picolinyl derivative of 18:2 (2) (Figure 6.4B). which

suggest that this fatty acid is also a 11, 15- octadecadienoic acid. The differences between the two spectra can possibly be attributed to a different geometric configuration.

To identify the geometric configuration of these two fatty acids, the RRT of their methyl esters were compared to those of the geometric isomers of linoleic acid (Figure 6.2). It can be expected that the RRT of the various geometric isomers of linoleic acid could be slightly different from those of the observed fatty acids, since the former have methylene-interrupted double bonds and the latter have conjugated double bonds. According to this information it is possible to speculate that 18:2 (3) is ***trans*-11, *cis*-15 octadecadienoic acid** and 18:2 (2) is ***trans*-11, *trans*-15 octadecadienoic acid.**

The mass spectrum of the picolinyl ester 18:2 (1) is illustrated in Figure 6.4C. The prominent ion at *m/z* 300 shows again the loss of 5 carbon fragment but, this time, without a double bond. The saturated series of ions at *m/z* 300, 314, 328, 342 and 356 supports this conclusion. The saturated series of ions from *m/z* 164 to 234 (carbons 1 to 8) suggests that the first double bond is at C-9. The second bond must be at C-11 to account for the *m/z* 300. The position of the double bonds is also confirmed by the 38 mass unit gap between the ions *m/z* 234 and 272, as expected in a conjugated diunsaturated fatty acid (Harvey, 1992). The 18:2 (1) compound thus appears to be 9, 11- octadecadienoic acid. The geometric configuration of the double bonds is unknown. However, from what was found in the mechanism of linoleic acid degradation in rumen organisms (Kellens *et al.,* 1986) it is possible to speculate that it is a ***cis* -9, *trans*-11 octadecadienoic acid.**

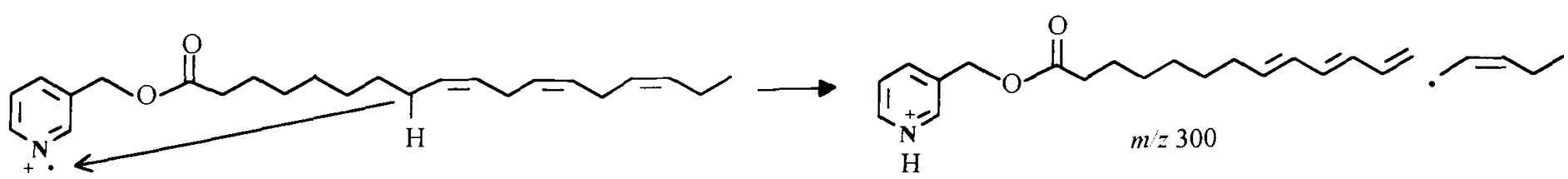
The structure of the 18:2 (4) was not elucidated by the mass spectrum of its picolinyl ester derivative. The methyl ester of 18:2 (4) eluted approximately 20 scans after the *cis*-9, *trans*-11 octadecadienoic acid. The spectrum of the FAME 18:2 (4) was similar to that of the *cis*-9, *trans*-11 octadecadienoic acid. By comparing these data with the standard mixture of geometric isomers of *cis*-9, *cis*-12 octadecadienoic acid it is possible to presume this fatty acid to be ***trans*-9, *cis*-11 octadecadienoic acid.**

Octadecatrienoic Acids

Six 'new' octadecatrienoic acids were observed in the field and laboratory experiments carried out with linseed oil (Tables 6.1). In the GC analysis of the FAMEs, the 18:3 (5 and 6) had an elution time smaller than that of linolenic acid (*cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid). The identification of these two fatty acids was carried out by comparison of the RRT of their methyl esters derivatives with those of a standard mixture of geometric isomers of 9, 12, 15 -octadecatrienoic acid (Supelco) (Figure 6.5). The GC-MS data of these FAMEs and those of the standard mixture indicated that 18:3 (5 and 6) could be, respectively, the ***cis*-9, *cis*-12, *trans*-15 octadecatrienoic acid** and the ***cis*-9, *trans*-12, *cis*-15 octadecatrienoic acid**. No picolinyl ester derivatives of these fatty were carried out due to the small amounts present in the samples.

In the GC-MS analysis, the remaining 'new' octadecatrienoic acids eluted after the *cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid, which indicates that these fatty acids are not geometric isomers of that fatty acid.

The identification of these 18:3s was carried out by analysis of the mass spectrum of their picolinyl esters in conjunction with the mass spectrum of the picolinyl ester of *cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid (18:3ω3) (Figure 6.6). The spectrum of *cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid showed once more the expected general features with respect to the ions *m/z* 92, 93,108, 151 and 164. The spectrum of this compound is more complex in that the diagnostic series of ions tend to exist as groups of peaks rather that as individual ions and thus it is more difficult to establish the spacing of the main peaks (Harvey, 1982). This trend appears to increase with increasing unsaturation. The most diagnostic feature is the abundant ion at *m/z* 300 and its formation can involve a transfer from the allylic hydrogen from C-8 to the pyridine ring and a bond migration as shown in scheme 15 (Harvey, 1984).



Scheme 15. Mechanism of formation of the ion at *m/z* 300 in the mass spectrum of a picolinyl derivative of *cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid.

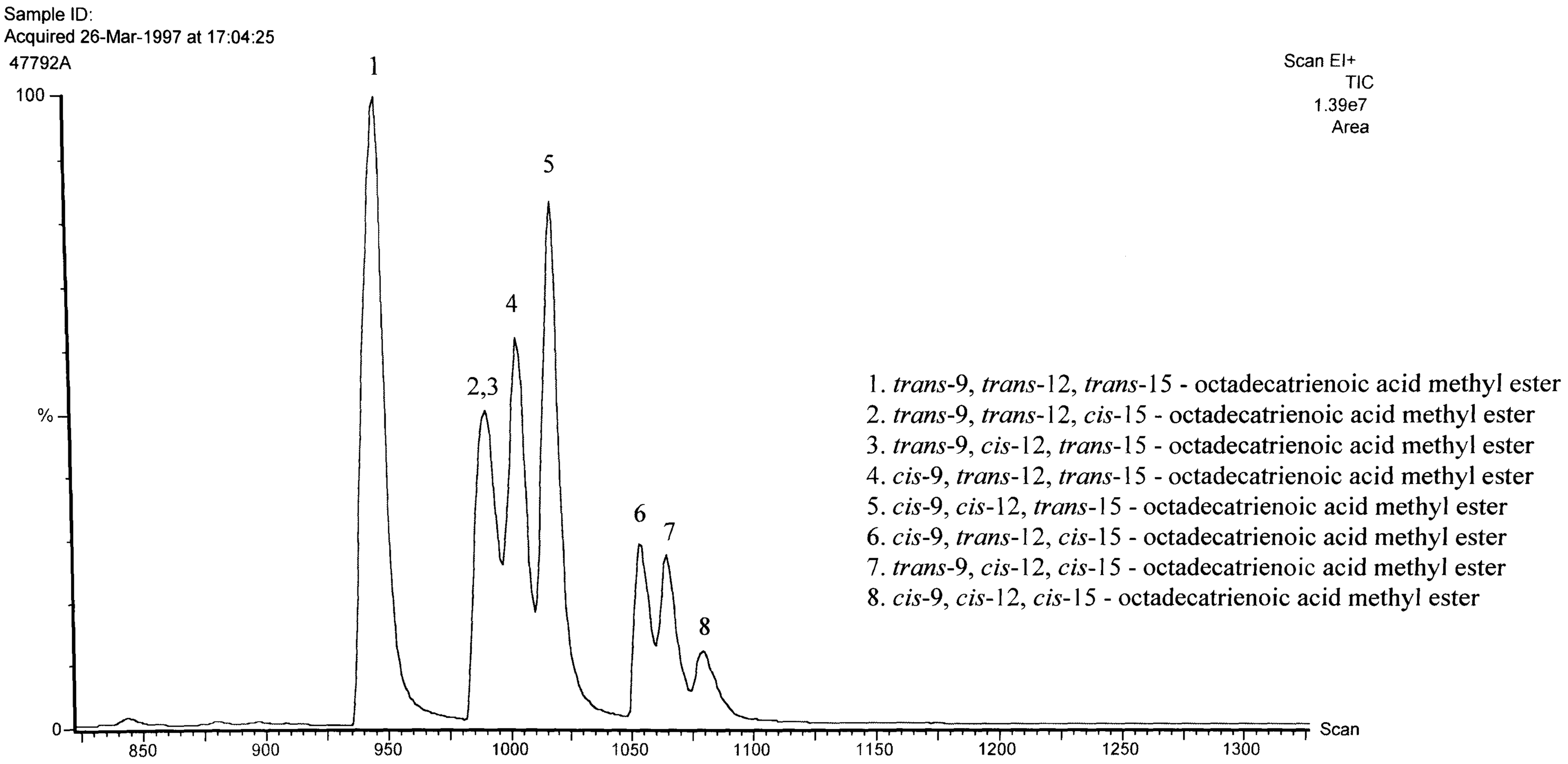


Figure 6.5. Gas Chromatogram of a standard mixture of geometric isomers of 9, 12, 15- octadecatrienoic acid methyl esters.

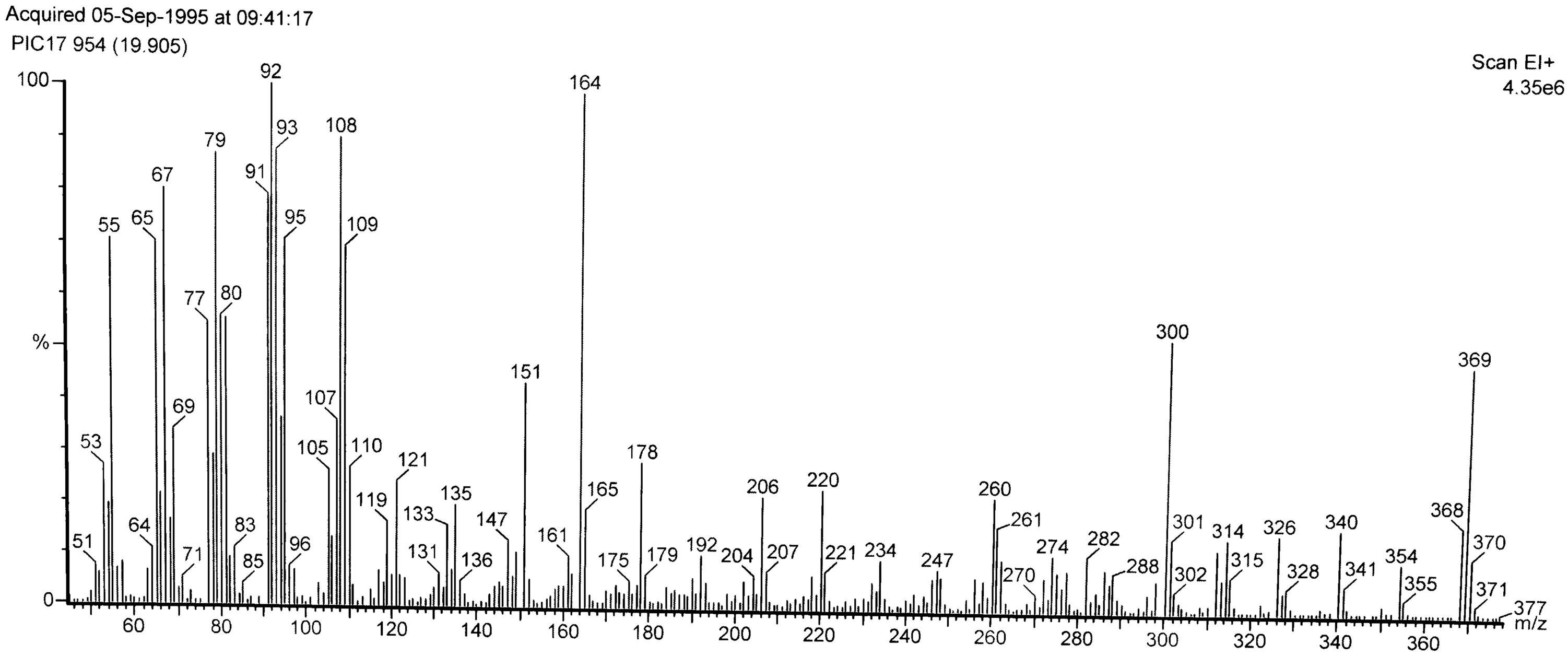
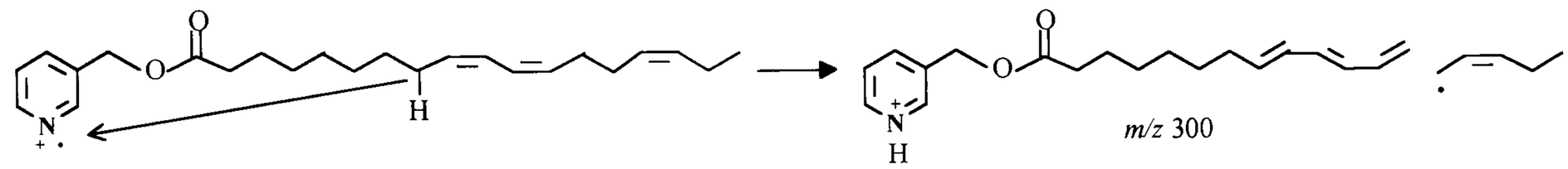


Figure 6.6. Mass spectrum (70eV) of the picolinyl ester of *cis*-9, *cis*-12, *cis*-15- octadecatrienoic acid.

The molecular ion is also very abundant and indicates the extent of unsaturation. A method for double bond location (in methylene-interrupted fatty acids) is indicated by the 14 mass unit gap, followed by a 26 mass unit gap and again by a 14 mass unit gap. The presence of the double bonds is located at *m/z* 234 and 260; at *m/z* 274 and 300 and at *m/z* 314 and 340.

The picolinyl ester derivative of 18:3 (1) is illustrated in Figure 6.7. The similarity of this spectrum with that of the 18:2 (3) (Figure 6.4A) suggests that two of the bonds are at C-11 and C-15. The saturated series of ions from *m/z* 164 to 234 (carbon 1 to 8) suggests that the third double bond is at C-9, as observed in the spectrum of 18:2 (1) (Figure 6.4C). This indicates a fatty acid with conjugated double bonds (C-9 and C-11). The conjugation would explain the absence of other prominent cleavage ions as no additional allylic positions are created. Formation of the major ions at *m/z* 300 could then be drawn as involving transfer of the allylic hydrogen from C-8 to the pyridine ring and bond migrations as shown in Scheme 16 (Harvey, *pers. commun.*).

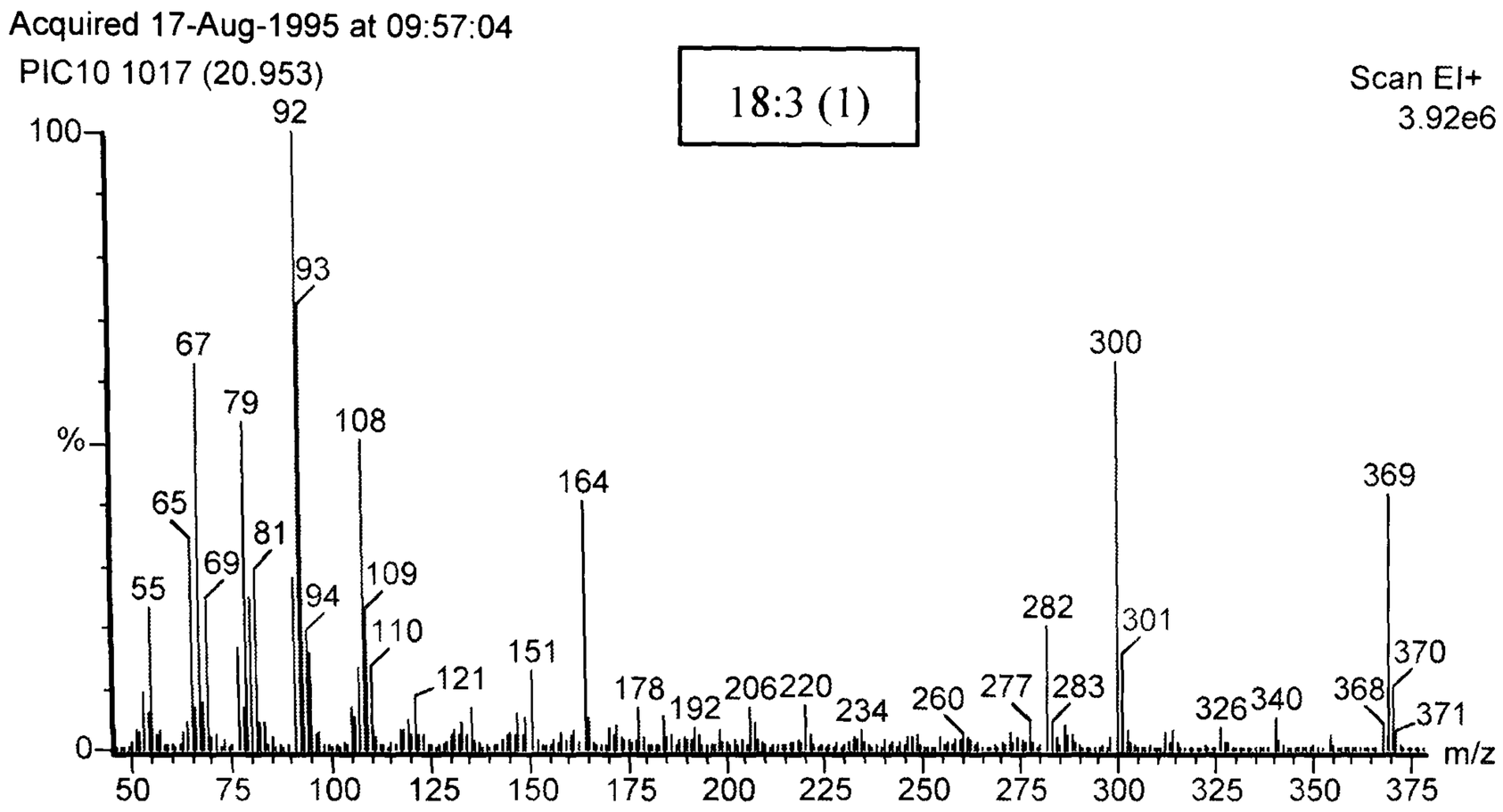


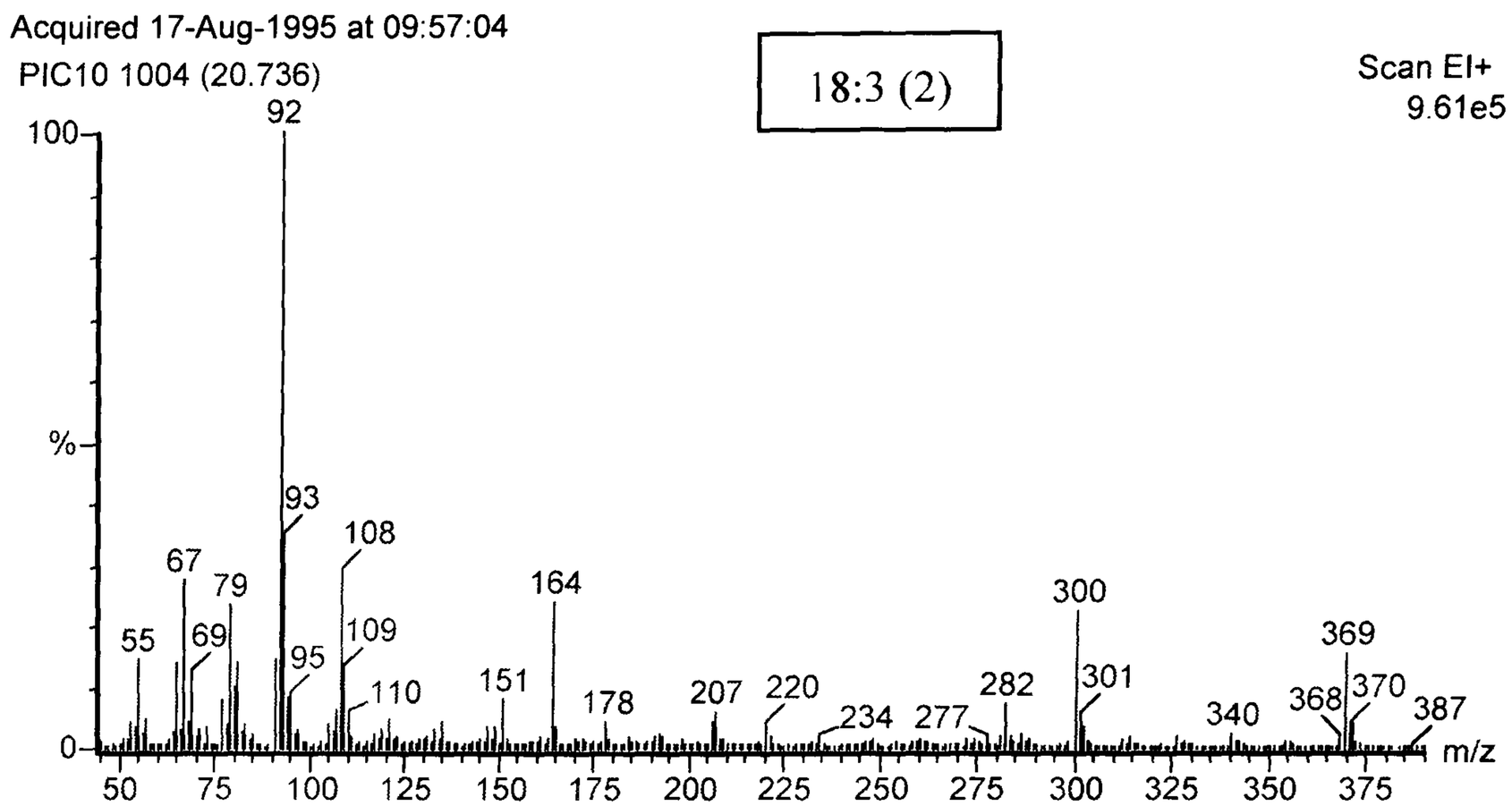
Scheme 16. Mechanism of formation of the ion at *m/z* 300 in the mass spectrum of a picolinyl derivative of 9,11, 15-octadecatrienoic acid (Double bond represented as a *cis*).

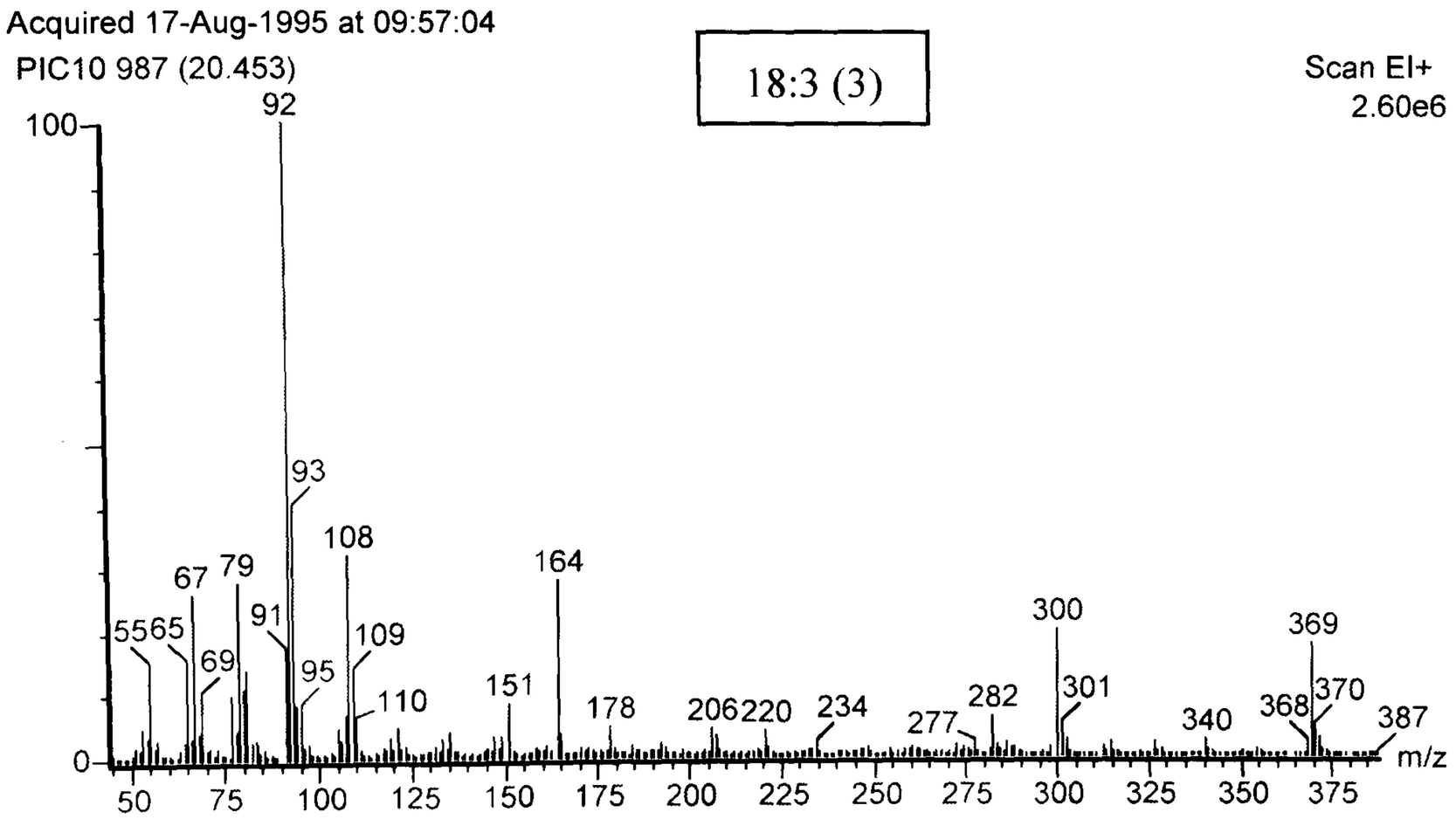
The compound 18:3 (1) appears to be a **9, 11, 15 - octadecatrienoic acid.**

The mass spectra of the picolinyl esters of 18:3 (2, 3, and 4) are also depicted in Figure 6.7. As it can be seen, the pattern of fragmentation of these fatty acids is very similar to that of the 18:3 (1), what suggests that these four fatty acids are possibly geometric isomers of 9, 11, 15- octadecatrienoic acid.

In conclusion, the identification proposed and discussed above of the 'new' fatty acids, up until now denominated by a code number, are presented in Table 6.3.







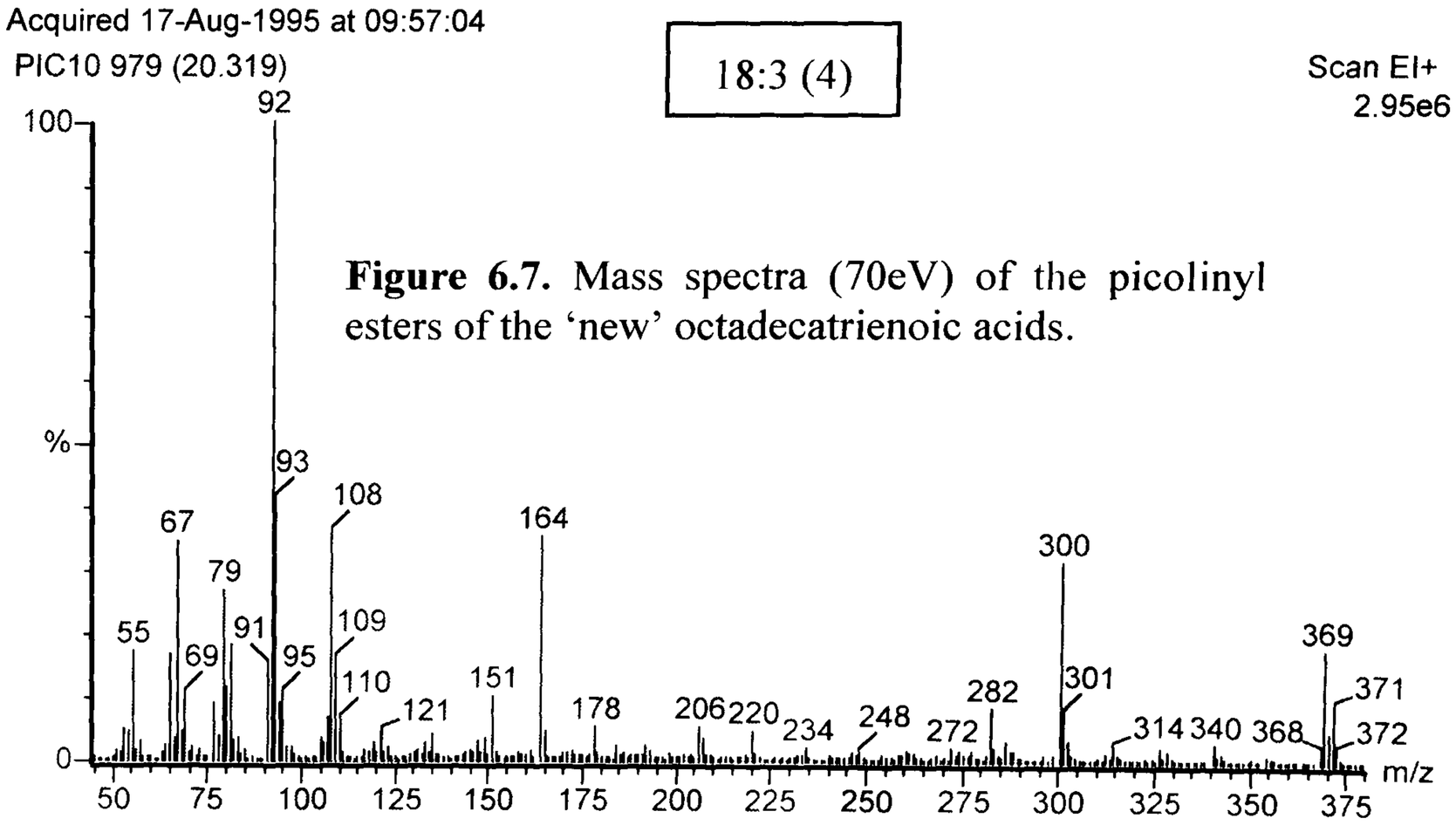


Figure 6.7. Mass spectra (70eV) of the picolinyl esters of the 'new' octadecatrienoic acids.

Table 6.3. Systematic name and code number of the 'new' fatty acids observed in the various experiments using linseed and sunflower oils.

'New' Fatty acids (code number)	Systematic nomenclature	Vegetable oil
18:1 (1)	*trans*-9 octadecenoic acid	Linseed & Sunflower oils
18:1ω7	*cis*-11 octadecenoic acid	Linseed & Sunflower oils
18:2 (1)	*cis*-9, *trans*-11 octadecadienoic acid	Linseed & Sunflower oils
18:2 (2)	*trans*-11, *trans*-15 octadecadienoic acid	Linseed & Sunflower oils
18:2 (3)	*trans*-11, *cis*-15 octadecadienoic acid	Linseed & Sunflower oils
18:2 (4)	*trans*-9, *cis*-11 octadecadienoic acid	Sunflower oil
18:2 (5)	*cis*-9, *trans*-12 octadecadienoic acid	Linseed & Sunflower oils
18:2 (6)	*trans*-9, *trans*-12 octadecadienoic acid	Sunflower oil
18:3 (1)	9,11,15 octadecatrienoic acid	Linseed oil
18:3 (2)	9,11, 15 octadecatrienoic acid	Linseed oil
18:3 (3)	9,11,15 octadecatrienoic acid	Linseed oil
18:3 (4)	9,11,15 octadecatrienoic acid	Linseed oil
18:3 (5)	*cis*-9, *cis*-12, *trans*-15 octadecadienoic acid	Linseed oil
18:3 (6)	*cis*-9, *trans*-12, *cis*-15 octadecadienoic acid	Linseed oil

6.3.3. Conclusions

The use of picolinyl esters derivatives of fatty acids proved to be an efficient method for the determination of the double bonds position, resulting in a relatively simple interpretation of the spectra of the octadecenoic and octadecadienoic acids. The identification of the 'new' octadecatrienoic acids was more complex, although, as Harvey (1982, 1984) states himself, "for fatty acids with three double bonds or more the mass spectra becomes less clear". Even though the determination of the double bond position can be achieved using this derivatisation method, the geometric configuration cannot, since no structural features of these spectra have been found to be characteristic of the double bond configuration (Harvey, 1992). To complement the information given by the mass spectra of the picolinyl esters, geometric isomers of standard fatty acids were used. The problem that can arise from this approach is that the standard isomers had methylene-interrupted double bonds whereas some of the 'new' fatty acids had conjugated double bonds. This is possibly the reason why the exact geometric configuration of the 9,11,15 octadecatrienoic acids could not be established.

Another approach for the determination of the double configuration based on the spectra of picolinyl esters of unsaturated fatty acids and with potential for providing a more conclusive identification is that used recently by Leth (1997). This author showed that it is possible to classify fatty acids picolinyl esters as either *cis* or *trans* by the use of PCA (Principal Component Analysis) and SIMCA modelling (Soft Independent Modelling of Class Analogy), based on data from the mass spectra of the picolinyl esters. Therefore, with the application of chemometric analysis to the spectra of the picolinyl esters of unsaturated fatty acids, this derivatisation method could provide information not only about the double bond position but also about their configuration in future identification of 'new' fatty acids, such as those observed during the course of the present study.

7. General Discussion and Conclusions

7.1. Consequences of Vegetable Oil Spills in Marine Sediments

The consequences of a spill of a potential pollutant in a given environment are determined by the properties of the substance itself and those of the environment where the pollutant is released. In the particular case of a vegetable oil spill in sediments, questions such as how long the oil will remain in the environment, what proportion of the oil will be degraded and at what rate, which products will be formed and where will the oil and its products be transported to and distributed are determined by factors such as sediment pH, sediment temperature, concentration of oxygen in the sediments, dispersal of the oil, the presence of other chemicals in the sediments, soil characteristics (such as particle size, permeability and porosity), ambient nutrient levels and the population of micro-organisms at the location of the spill.

Vegetable oils are generally considered to be not pollutants due to their biodegradability. However, deleterious effects of vegetable oil spills on marine life (birds, fish and other aquatic organisms) have already been reported (Russell & Carlson, 1978; McKelvey *et al.*, 1980; Smith & Herunter, 1989, among others) and have been considered by McKelvey and collaborators (1980) to be more harmful for birds than spills of crude oil.

The broad aim of this study was to investigate the consequences of linseed and sunflower oil spills on salt marsh sediments and the role of bacteria in the degradation of these oils. In order to accomplish this, linseed and sunflower oil spills were simulated and selected sediments properties were examined throughout the duration of the simulated spillages; temporal modifications of the oil's chemical and physical characteristics and those of the indigenous microbial populations were also followed. The results of this work showed that in the event of a spill of linseed or sunflower oils in a salt marsh environment, there is the potential for the oils to be biodegraded. These findings, up to a certain extent, contrast with those reported by Mudge *et al.,* (1995). Mudge and collaborators, found that whilst linseed oil was relatively easily degraded, sunflower oil polymerised at the surface sediments, remaining in the environment for a long period of time being degraded slowly presumably due to the polymerisation of the oil. Reasons for this difference between Mudge's *et al.* work and the present are unknown. It can only be speculated that the variation that occurs in the physical, chemical and microbiological features of the salt marsh sediments between summer and winter were responsible for the discrepancies, since Mudge's *et al.* study was carried out in summer and these were in winter.

Additionally, Mudge *et al.* (1994) observed polymerisation of both sunflower and linseed oils in sea water in laboratory experiments. The authors do not offer an explanation for the trigger for the polymerisation but they suggest that the process involves the degradation of 18:2ω6 and 18:3ω3 to monounsaturated fatty acids. The outcome of the polymerisation was always very slow degradation rates of the oil. During the simulated spills of linseed and sunflower oils carried out in this work, the oils were degraded.

Biodegradation is not without consequences for the environment and to the organisms that inhabit the sediments where the spills occur. The consequences may vary depending on the vegetable oil spilled. The results of this study showed that linseed and sunflower oil spills affected differently (though not markedly) the characteristics of the sediments of the salt

marsh due to the fact that linseed oil appeared to be more readily degradable than sunflower oil, which is in agreement with Mudge *et al.* (1995).

Both oils, but sunflower oil in particular, were degraded by autochthonous bacteria at what appeared to be slow rates which allowed the oils to remain in the sediments for some time. In the case of linseed oil, approximately 60% of the oil was removed from the sediment (0-30 cm) within two months. In contrast, during the sunflower oil experiment no oil appeared to have been removed from the sediment layers analysed within 6 months of the start of the experiment. The longer residence period of sunflower oil in the sediments in relation to linseed oil is possibly a consequence of the longer time necessary for the degradation process to start. Sunflower oil was also retained in the sandy sediments and this could also have contributed to its longer residence since the oil did not penetrate into deeper sediments.

It is also important to take into account that the loss of the oil could not only be resultant from the degradation process but also from lateral movement which was not investigated in this work. Horizontal sampling would have allowed this process to be assessed and perhaps it should be considered in future investigations.

The presence of the oils induced conditions which were unfavourable for the macrofauna and vascular plants, resulting in their death. The biodegradation of vegetable oils also leads to oxygen depletion in the sediments, which could have been exacerbated by the reduction in the sediments permeability. The creation of anaerobic environments lead to the development of anaerobic bacteria and products of degradation such as H_2S with its characteristic smell. In addition, in anaerobic conditions it is expected that the edible oils will remain in the environment for a longer period of time and continue to create a risk to the natural environment, due to the relatively low metabolic rate of the strict anaerobes, as discussed before.

Since this was a preliminary study carried out only with two vegetable oils in one type of marine environment, it is difficult to make generalisations about the consequences of vegetable oils spills in any other situations. However, these experiments showed that different consequences could be expected, varying according to the oil itself, environment where the spill occurred, time of the year and possibly amount of oil spilled.

The degradation or polymerisation of an oil in a natural environment is not well understood as shown by the differences between the results of this study and those of the previous studies. It is therefore difficult to establish in each case which process will prevail. Both degradation and polymerisation may have deleterious effects for the environment and organisms, and polymerisation may bring longer lasting consequences because the oil will remain in the environment for a longer period of time. It is, therefore, important that measures should be taken to avoid spills of vegetable oils and that in the eventuality of an accident, perhaps in certain cases, the removal of the oil from the environment should be considered. The decision should be made case by case considering the oil, the characteristics of the contaminated area and the proximity of the spill to environmentally sensitive areas.

7.2. Role of Bacteria in Linseed and Sunflower Oils Degradation

The role of bacteria in the degradation of vegetable oils in the marine environment has been the subject of a few studies (Mudge *et al.,* 1994, 1995). These studies revealed that bacteria can degrade edible oils unless a polymer is formed. Such degradation occurs as result of the widespread lipolitic activity of bacteria. Thus, bacteria are the ultimate agents of the vegetable oils breakdown, and the means by which some of the carbon of the oil finds its way into upper levels of the food web, by way of protozoa, zooplankton and meiofauna.

The results of these experiments showed that bacteria indigenous to the salt marsh sediments at Foryd Bay can breakdown linseed and sunflower oils and utilise them as source of carbon responding quickly to the environmental changes. The fact that the abundance of oil degrading bacteria increased as a proportion of total heterotrophs is a good indication that bacteria do indeed respond quickly to the presence of new carbon sources and that they can utilise vegetable oils. The breakdown of linseed and sunflower oils appeared to be carried out mainly by aerobic oil degrading bacteria with anaerobic oil

degrading bacteria playing only a minor role. This finding is illustrated by the greater increase in the number of aerobic oil degraders in the field and number of changes in the oil composition observed in the laboratory experiments, compared to the anaerobic bacteria (Chapters 4 & 5). These results suggest that in case of a spill in an environment with low levels of oxygen, the metabolism of the oil will be at much slower rate than in an aerobic environment and the undegraded oil will accumulate.

The enhanced bacterial activity in the sediments due to the addition of organic matter will produce an accumulation of partially oxidised metabolites and will reduce the Eh potentials, thus changing the chemical nature of the sedimentary environment. In the present study, the more reduced sediments allowed the development of anaerobic groups: first there was an increase in the number of heterotrophic anaerobic bacteria followed by increased abundance of sulphate reducers. Despite the similarities in the degradation of both oils, linseed oil generally induced a high increase in the bacteria numbers more promptly than sunflower oil, leading to faster degradation of the former in relation to the latter. As a consequence of the faster rate of degradation, linseed oil also led more quickly to the formation of anaerobic sediments.

The sequential degradation of the vegetable oils, not as much in terms of time because aerobic and anaerobic zones coexist in the sediments but more in terms of degradation products, was identical for both linseed and sunflower oils. It is possible that this sequential degradation is carried out not by one single organism but by a consortia of organisms, where a series of end products resulting from the activity of one group of organisms serve, in turn, as substrates for other metabolic pathways.

Even though bacterial activity increased promptly after the addition of the oils, the rates of degradation could have been raised further by increasing the amount of ambient inorganic nitrogen and phosphorus, normally present in limiting concentrations in the marine environment (Floodgate, 1984, 1995; Coffin *et al.,* 1997). The addition of nitrogen and phosphorus should stimulate microbial activity in environments where these inorganic nutrients are limiting and consequently increase the oil degradation rate (Mudge *et al.,* 1994).

In conclusion, in an eventuality of a spill, if the edible oil does not polymerise an enrichment in oil degrading bacteria should be observed. However, the increase of some bacterial groups will change the composition of the bacterial community in the sediments. Nevertheless, it is important to take into account that the response of micro-organisms to edible oils in the environment is variable and depends on many environmental and biological factors, such as inorganic nutrient availability, temperature, oxygenation of the sediments, *etc.*

The term bacterial degradation of vegetable oils in marine environments means, therefore, the degradation of edible oils by unknown mixed population of bacteria in an erratically changing medium. The elucidation of the degradation pathways by which linseed and sunflower oils were degraded by this consortia of organisms in the salt marsh sediments at Foryd Bay is proposed in the next section.

7.2.1. Pathways of Degradation

The fatty acids of vegetable oils are esterified in triacylglycerols and to be assimilated by organisms these have to be hydrolysed into free fatty acids. The analysis and identification of the fatty acids appeared to suggest multiple pathways of degradation of linseed and sunflower oils. It has been suggested that complete oxidation to CO_2 and H_2O is the main fate of fatty acids in sediments (Gaskell *et al.,* 1976; Harvey *et al.,* 1986; Sun *et al.,* 1997). In the present study it is possible that a certain amount of the fatty acids from linseed and sunflower oils were degraded completely through aerobic metabolism. However, it is not known if complete oxidation did occur, since the vegetable oils could have also penetrated to deeper layers than those studied or could have been lost through lateral movement.

Linseed oil

The results of the experiments simulating a spill of linseed oil, as well as the laboratory experiments with autochthonous bacteria from the salt marsh sediments revealed that 18:3ω3 is the fatty acid preferentially degraded. This is not unexpected as it is well established that polyunsaturated fatty acids are more reactive than saturated fatty acids

(Haddad *et al.*, 1992; Sun & Wakeham, 1994; Canuel & Martens 1996, among others). 18:2ω6 was not as degradable as 18:3ω3, with its concentration actually increasing in the field experiment. In contrast, in the laboratory Experiment I the concentrations of 18:2ω6 decreased due to the metabolic activity of sulphate reducing bacteria.

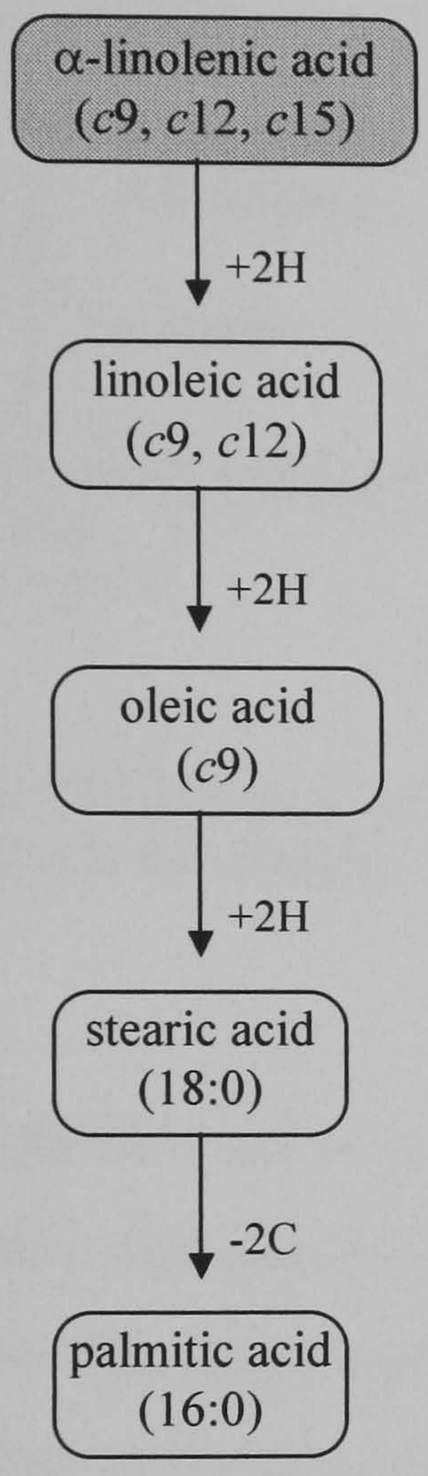


Figure 7.1. Schematic representation of a possible pathway of degradation of linseed oil.

In the field experiment, the decrease in the concentration of 18:3ω3 was accompanied by an increase in the concentrations of the other fatty acids of linseed oil. A likely route of degradation is depicted in Figure 7.1.

In this metabolic pathway, 18:3ω3 is consecutively reduced to form 18:2ω6, 18:1ω9 and 18:0. The sequential reduction of the various double bonds could be followed by chain shortening by β-oxidation, to yield palmitic acid. Further losses of 2-carbon units do not appear to occur, as no significant amounts of smaller chain fatty acids were observed. Despite that, in the pathway illustrated in Figure 7.1., 16:0 is a possible product of the degradation of 18:3ω3.

Accumulation of the intermediate products of this proposed degradation pathway were observed. For instance, in the laboratory Experiment I (linseed oil degradation by bacteria removed from different sediment depths) an accentuated increase in the concentration of 18:1ω9 (*c*9), higher that any of the other fatty acids (results described in chapter 5), occurred. This appears to indicate that either this fatty acid was produced by more than one degradation route or that its hydrogenation to 18:0 is unlikely to have happened under the given circumstances.

Another route for 18:3ω3 degradation could have involved its oxidation to acetyl CoA by β-oxidation and formation of shorter chain fatty acids *via de novo* synthesis (Rhead *et al.*, 1971; Gaskell *et al.*, 1976) followed by elongation and desaturation (Figure 7.2.).

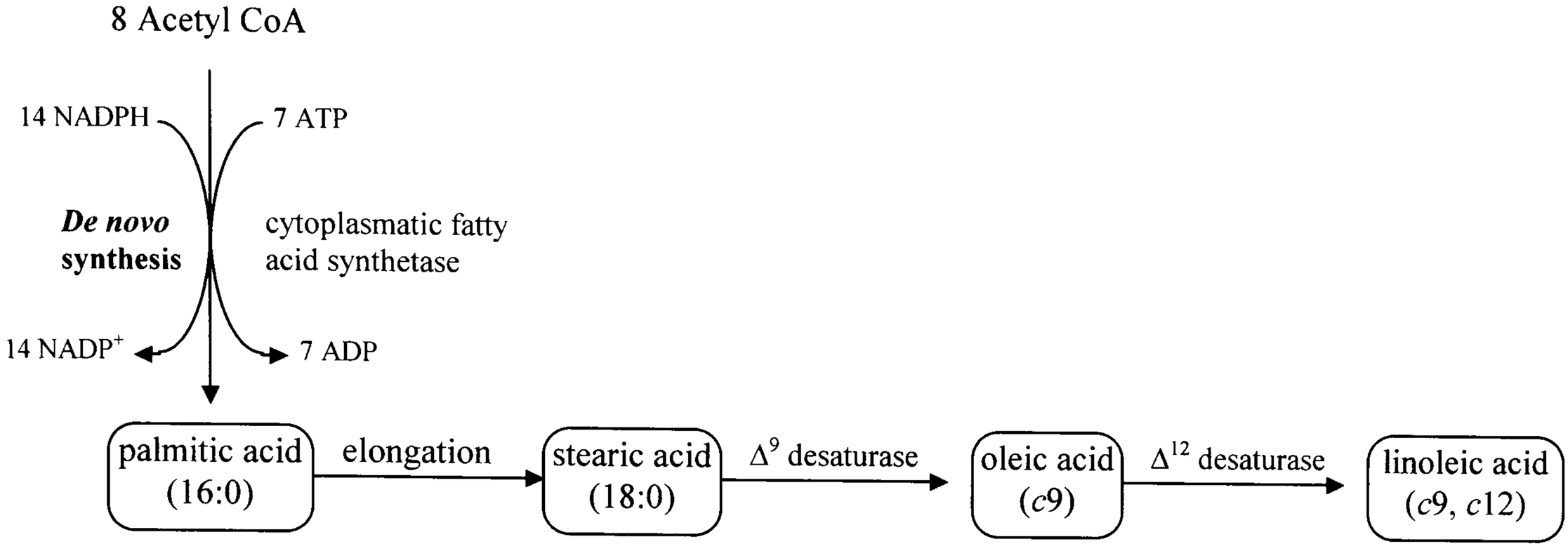


Figure 7.2. Outline of *de novo* synthesis and modification of fatty acids (adapted from Holland, 1978; Kendrick & Ratledge, 1990).

Even though the existence of this pathway of degradation is conceivable, it appears less probable than that described in Figure 7.1, firstly because in the laboratory Experiment I an increase in the concentration of 18:1ω9 was registered prior to the increase in the concentration of the saturated fatty acids and secondly because it implies that the organisms are spending energy to produce fatty acids that are already present at high concentrations in the environment and thus amply available.

The pathways of degradation shown in Figure 7.1. and 7.2 could explain the observed alterations in the concentration of the fatty acids of linseed oil. However, they do not involve the formation of 'new' fatty acids. The appearance of 'new' unsaturated fatty acids observed in the various experiments with linseed oil suggest the presence of metabolic routes, which could have been active (Fig. 7.3) in conjunction with those previously described (Figs. 7.1; 7.2).

The 'new' fatty acids represented in Figure 7.3 were present in the sediment samples at Foryd Bay after the simulated spill of linseed oil and some were also observed in the laboratory experiments with linseed oil degraded by different bacteria groups.

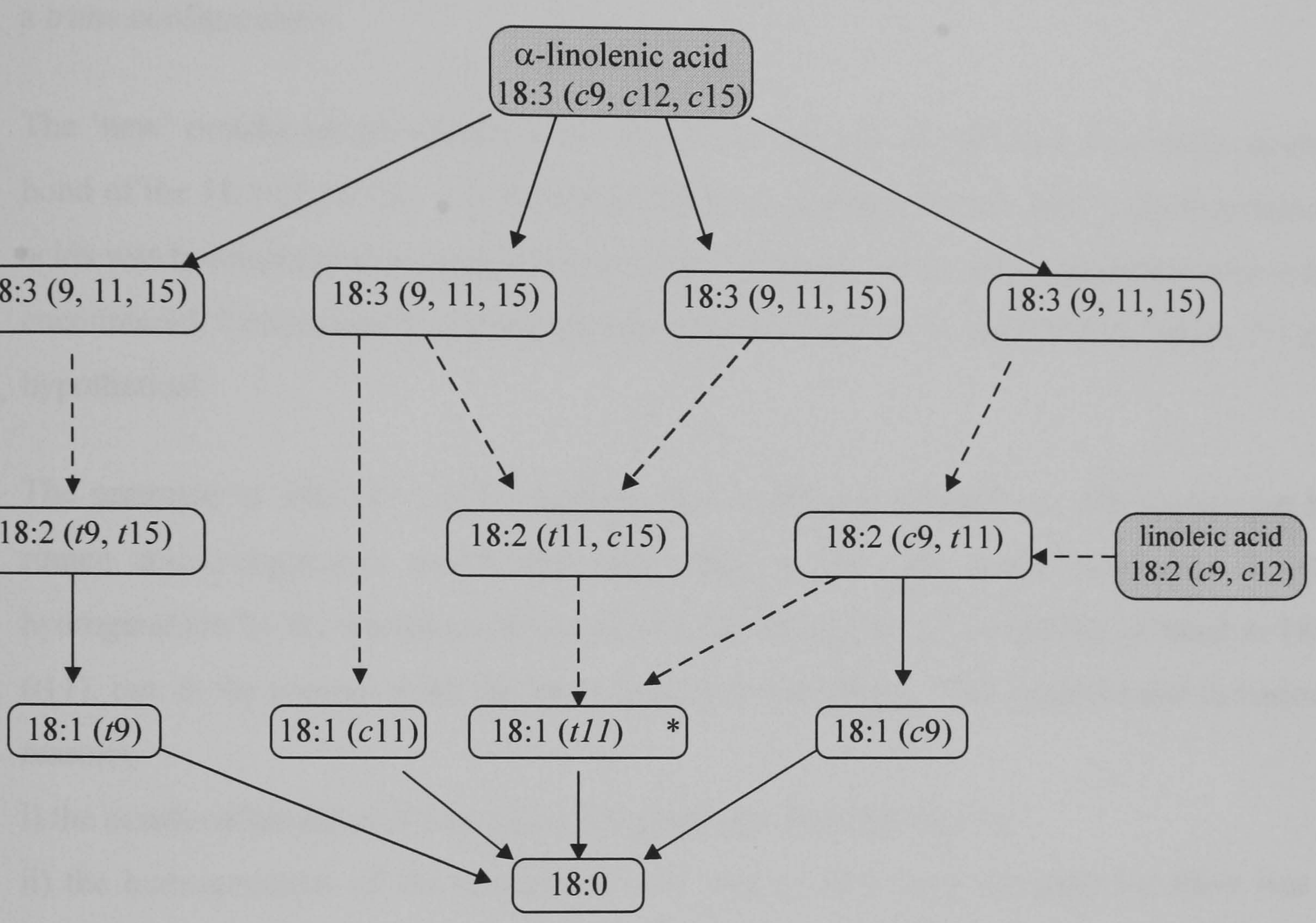


Figure 7.3. Schematic representation of possible pathways of degradation of linseed oil, involving the formation of isomers of linolenic and possibly linoleic acids. * not detected.

The first step in the degradation appears to be the formation of isomers of 18:3ω3 (*c*9, *c*12, *c*15). The double bond configuration of the four isomers, 18:3 (9,11,15) is unknown, but the change of the double bond position from C_{12} to C_{11} without alteration in the configuration of the double bonds in C_9 and C_{15} is identical to the alterations carried out by rumen micro-organisms (Kemp *et al.,* 1975; Kellens *et al.,* 1986; among others). Boon *et al.,* (1978) suggested that a similar pathway of degradation to this could also be present in sediments. Thus, it is legitimate to presume that at least one of the isomers is a 18:3 (*c*9, *t*11, *c*15). It could also be presumed that the four isomers have a *trans* configuration in the

double bond located at the C_{11} and only varied the configuration at the double bonds at C_9 and C_{15}. However, this is not the most probable scenario because in the biohydrogenation in the rumen, the last double bond to be reduced is the *trans* 11, and the presence of the 18:2 (*t*9, *t*15) seems incompatible with such findings. On the other hand, it is unknown if the order of hydrogenation will be affected if the double bond in position 9 and/or 15 have a *trans* configuration.

The 'new' octadecadienoic acids could have been formed by reduction of a single double bond of the 18:3 (9,11,15). Yet, it is impossible to ascertain if each 'new' octadecatrienoic acids was hydrogenated to produce one, various or none of the 'new' octadecadienoic acids encountered. Consequently, the arrows between the 'new' 18:3s and 18:2s in Figure 7.3 are hypothetical.

The presence of 18:2 (*t*11, *c*15) suggests that a pathway identical to that carried out by rumen micro-organisms could also take place in the salt marsh sediments. In the hydrogenation by the rumen micro-organisms the 18:2 (*t*11, *c*15) is further reduced to 18:1 (*t*11), but, in the present work the latter reduction was absent. This could be due to various reasons:

i) the octadecadienoic acid was not hydrogenated to form 18:1 (*t*11);

ii) the hydrogenation of the octadecadienoic acid to 18:1 (*t*11) occurred but there was a rapid conversion to stearic acid. Even though 18:1 (*t*11) has been shown to be a prominent octadecenoic acid in the rumen (Kemp *et al.,* 1984), as 18:1 (*c*9) is hydrogenated more rapidly to stearic acid than is 18:1 (*t*11) (Dawson & Kemp, 1969), it is possible that the bacteria from the salt marsh sediments accumulate other metabolic products.

iii) it is also possible that 18:1 (*t*11) was produced and accumulated in the sediments but wrongly identified as a 18:1 (*c*11).

If 18:1 (*c*11) was indeed present, it would have been formed by hydrogenation of a 18:3 (9, 11,15). This 18:3 had to have a *cis* configuration in the double bond located at C_{11}. However, the intermediate octadecadienoic acid with a *cis* configuration was not found to support the existence of this route.

The octadecadienoic acids (*t*9, *t*15) and (*c*9, *t*11) are likely to be the product of hydrogenation of the 'new' octadecatrienoic acids. However, 18:2 (*c*9, *t*11) could also be

formed by isomerisation of linoleic acid, which is part of the pathway of degradation of 18:2ω6 in rumen organisms. Those two 'new' fatty acids could have been further hydrogenated to octadecenoic acids and even to 18:0.

Although in the experiments concerning linseed oil degradation the focus was placed on the utilisation and modification of 18:3ω3, as this was the fatty acid which decreased in concentration, it should also be taken into account that the 'new' 18:2s and 18:1s could have been formed by isomerisation of 18:2ω6 and 18:1ω9, respectively.

In addition to the 'new' fatty acids mentioned above, in the laboratory Experiment II (linseed oil degradation) two other 'new' octadecatrienoic acids and one octadecadienoic acid were present, which were not observed in any of the other experiments involving linseed oil. They could have been the result of a different degradation route from that observed in the field and laboratory Experiment I (Fig. 7.4).

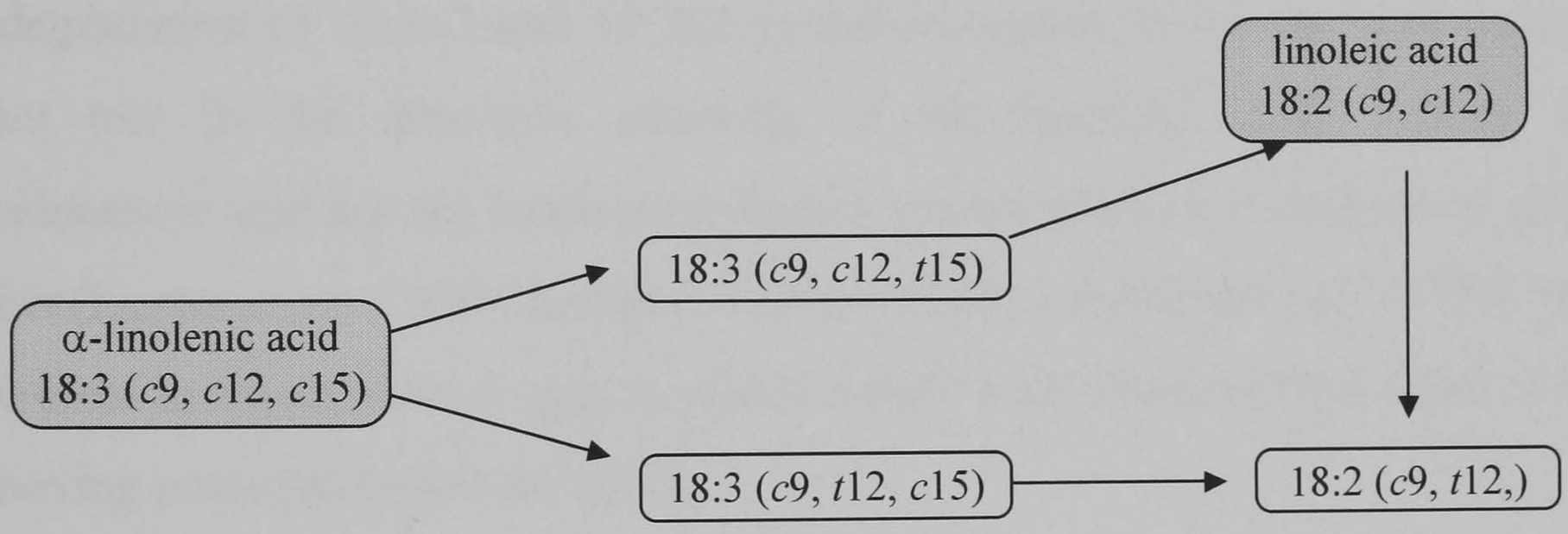


Figure 7.4. Schematic representation of a possible pathway for the degradation of linseed oil, involving the formation of isomers of linolenic and linoleic acids.

This degradative pathway implies that the geometric configuration of the double bond at positions 12 or 15 in linolenic acid changed from *cis* to *trans*, without changing the double bond position. The isomerisation of 18:3 (*c*9, *c*12, *c*15) to 18:3 (*c*9, *t*12, *c*15) could be followed by the reduction of the double bond at C_{15} to form 18:2 (*c*9, *t*12), but the 18:2 (*c*9, *t*12) could have also been produced from 18:2ω6. This octadecadienoic acid (*c*9, *t*12), could have been further hydrogenated to 18:1ω9, but this cannot be confirmed. Rosenfelt & Tove (1971) found that rumen bacteria were able to hydrogenate both *cis* double bonds of 18:3 (*c*9, *t*11, *c*13) as occurred for 18:2ω3; however, no reaction occurred when 18:3 (*c*9,

*t*11, *t*13) was used, suggesting that the configuration *trans* in the double bond at position C_{13} impeded the reduction of the double bond. The 'new' 18:3 (*c*9, *c*12, *t*15) could have been hydrogenated to form 18:2 (*c*9, *c*12) or could have remained as 18:3 without further modification.

It is unknown why these changes occurred in the laboratory Experiment II (Chapter 5). It is possible that the high concentration of 18:3ω3 present in the medium precluded the occurrence of a similar route of degradation as observed in the field and laboratory Experiment I. It could also be the case that the isomerisation of 18:3ω3 into the 'new' fatty acids induced a degree of alterations which prevented further metabolism by bacteria. The reason why so many isomers of 18:3ω3 and 18:2ω6 are produced is also unknown. According to Koritala and colleagues (1987), micro-organisms are so efficient in utilising glycerides for energy and growth, that they are less likely to make intermediate metabolites of degradation which remain stable and accumulate. The scientists that studied the pathway of degradation of 18:3ω3 and 18:2ω6 in rumen organisms do not have a clear idea of its exact role in the metabolic economy of the bacterial cells. Neither the *trans*-11 octadecenoic acid nor the conjugated dienoic intermediate are incorporated into the cellular lipids (Kepler *et al.,* 1970). Kemp & Lander (1984) and Kemp *et al.,* (1984) suggested that microbial hydrogenation of polyunsaturated fatty acids have survival value in the rumen by removing potential toxic fatty acids.

Sunflower oil

The degradation of sunflower oil has presumably taken place by identical pathways of those described for linseed oil. Complete oxidation of the fatty acids could have occurred but again this cannot be confirmed. In the field experiment simulating the spill of sunflower oil, a decrease in 18:2ω6 accompanied by an increase in 18:1ω9, 18:0 and 16:0, was observed. Thus, it is believed that 18:2ω6 was the fatty acid preferentially degraded and its pathways of degradation identical to those suggested for 18:3ω3 (Figs. 7.1; 7.2).

Once more the presence of 'new' unsaturated fatty acids suggests a different route of degradation of 18:2ω6 (Fig. 7.5). A variety of isomers of 18:2ω6 was found in the experiments carried out with sunflower oil, particularly in the field experiment.

In the rumen organisms, the pathway of degradation of 18:2ω6 is initialised by the isomerisation of 18:2 (*c*9, *c*12) to 18:2 (*c*9, *t*11). Therefore, the presence of the 18:2 (*c*9, *t*11) suggests that such pathway of degradation could also be active in the salt marsh sediments. The presence of 18:2 (*t*9, *c*11) appears to indicate that the change in the double bond position from C_{12} to C_{11} could occur without alteration in the double bond configuration. That modification was accompanied by a change in the configuration of the double bond at C_9 position from *cis* to *trans*. Even though the presence of this fatty acid suggest such alterations, it is unknown if they are feasible as apparently no other reports have been published to confirm or dispute such findings.

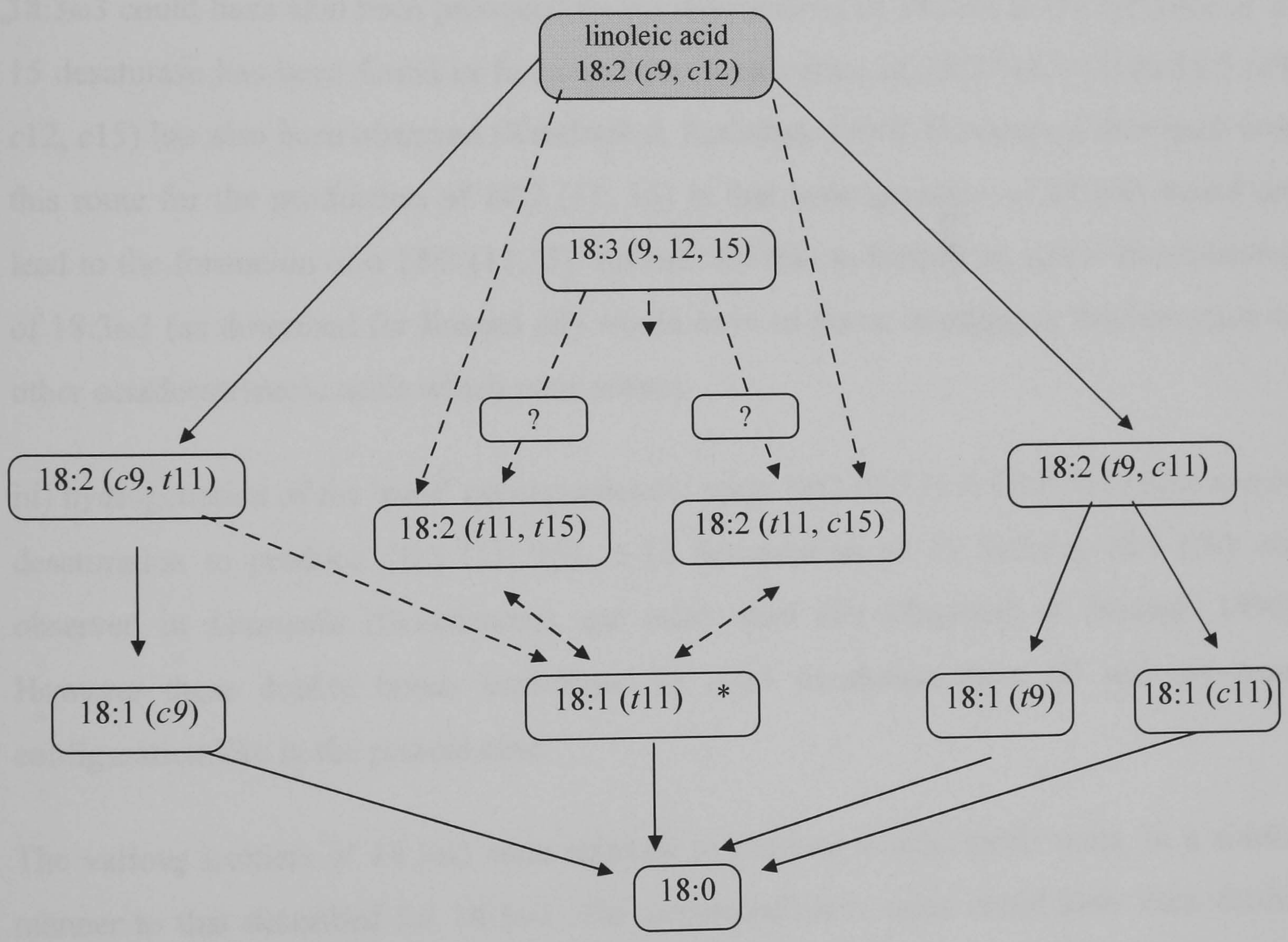


Figure 7.5. Schematic representation of possible pathways of degradation of sunflower oil, involving the formation of isomers of linoleic acid. * not detected.

The two fatty acids with double bonds in position 9 and 11 were those measured at highest concentrations in the field experiment, but small amounts of 18:2 (*t*11, *t*15) and 18:2 (*t*11, *c*15) were also detected. The pathways by which these two fatty acids with double bonds in positions 11 and 15 were produced are more difficult to ascertain than in the previous cases. The following routes are suggested:

i) isomerisation of 18:2 (*c*9, *c*12) to 18:2 (11,15). This does not appear very probable since these alterations are highly costly from a energetic point of view.

ii) formation of octadecadienoic acids (11, 15) as intermediates in the degradation of 18:3ω3 (since this fatty acid is present in very small amounts in sunflower oil- 0.5%). 18:3ω3 could have also been produced by the desaturation of 18:2ω6 as the presence of Δ-15 desaturase has been found in fungi and the desaturation of 18:2 (*c*9, *c*12) to 18:3 (*c*9, *c*12, *c*15) has also been observed (Kendrick & Ratledge, 1990). However, a drawback with this route for the production of 18:2 (11, 15) is that hydrogenation of 18:3ω3 would not lead to the formation of a 18:2 (11,15). Instead, for this to happen an initial isomerisation of 18:3ω3 (as described for linseed oil) would have to occur resulting in the formation of other octadecatrienoic acids which were absent.

iii) hydrogenation of the 'new' octadecadienoic acids 18:2 (9, 11) to 18:1 (*t*11) followed by desaturation to produce 18:2 (11, 15). Δ-15 desaturation of 12 hydroxy-18:1 (*c*9) was observed in *Lesquella* (Brassicacea) and other seed oils (Engeseth & Stymne, 1996). However these double bonds introduced by Δ-15 desaturase have *cis* and not *trans* configuration like in the present case.

The various isomers of 18:3ω3 were reduced to produce octadecenoic acids, in a similar manner to that described for 18:3ω3. The octadecadienoic acids could have been further reduced to 18:0. Chain shortening by β-oxidation to yield 16:0 could have also occurred, which could account for the observed increase in the concentration of 18:0 and 16:0.

Similar to what occurred in the laboratory Experiments (II) carried out with linseed oil, in the experiment regarding the degradation of sunflower oil, two more 'new' octadecadienoic acids were observed: [18:2 (*t*9, *t*12) and 18:2 (*c*9, *t*12)]. In the present case, 18:2 (*c*9, *t*12) was previously observed in the field study, albeit in very small amounts. Once more, it is

possible that other pathways of degradation of linoleic acid occurred in this set of experiments (Fig. 7.6). This route would involve changes in the geometric configuration of the double bonds at position 9 and/or 12, without alteration of the double bond position.

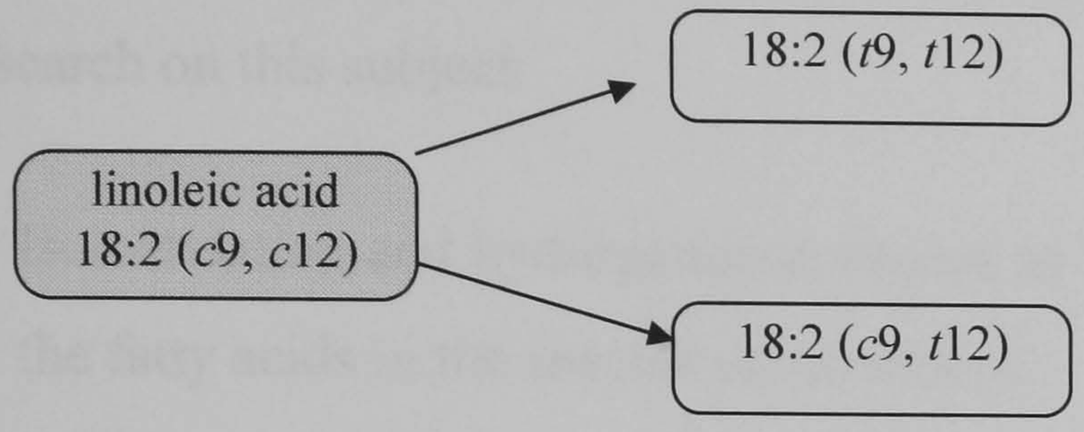


Figure 7.6. Schematic representation of possible pathways for the degradation of sunflower oil, involving the formation of isomers of linoleic acid.

A sample of polymerised sunflower oil from the 1990 wreck of M.V. *Kimya* on the Anglesey coast, was collected in 1997 and fatty acids analyses carried out (T. Dunn, *pers. comun.*). They revealed that the sample contained 4 isomers of 18:2 (9, 12) with the three 'new' isomers 18:2 (*t*9, *t*12); 18:2 (*c*9, *t*12) and 18:2 (*t*9, *c*12) being approximately 50% of the initial amount of 18:2ω6. This polymerised sunflower oil showed very little degradation in the last 7 years. On the other hand, in Experiment II, where these 'new' fatty acids were observed, only a very small bacteria growth was observed. Consequently, it can be presumed that the changes in the double bond configuration of 18:2ω6 impeded the microbial attack and therefore slowed the degradation rate.

Summary and Conclusions

The various pathways of degradation of the main fatty acid components of linseed and sunflower oils described in this chapter are speculative. It is possible that some of these degradation routes, mainly those in which 'new' fatty acids are included, are not completely accurate because the identification of the double bond configuration is subject to error, as discussed before (Chapter 6). To confirm the degradation pathways of the linseed and sunflower oils by the salt marsh bacteria established in here, more work should be carried out. Such work should include a more thorough identification of the 'new' fatty acids, using combined methods of identification and analyses of conjugated fatty acids standards. This identification should be complemented with the use of chemometric

analysis. Radiolabeled fatty acids could also be used to determine in more detail the routes of fatty acids degradation.

The results of this preliminary study showed, nevertheless, a range of alterations of the fatty acids which could have not been anticipated and could provide the basis for further research on this subject:

- Isomerisation and hydrogenation appear to be important reactions in the degradation of the fatty acids in the studied environment.
- β-oxidation could have followed the isomerisation and reduction reactions to produce shorter chain fatty acids.
- α - oxidation appears to be absent since neither hydroxy fatty acids nor odd number fatty acids were observed.
- Isomers of 18:2ω6 and 18:3ω3 identical to those observed in rumen organisms were also observed in these experiments suggesting that a pathway of degradation similar to that observed in those organisms could also occur in the salt marsh sediments.
- In the present study, isomerisation was shown to occur in aerobic as well as in anaerobic conditions, although in the rumen organisms isomerisation of 18:2ω6 and 18:3ω3 was carried out by strictly anaerobic micro-organism.
- In the experiments with bacteria isolated from the rumen organisms it has been shown that isomerisation followed by hydrogenation is only carried out on free fatty acids and neither methyl or ethyl esters nor triacylglycerols will act as substrates (Hughes *et al.*, 1982). In the present study, it is believed that these reactions occurred after hydrolysis of the triacylglycerols, despite the substrate given being a vegetable oil. These results seem to suggest that the salt marsh bacteria can utilise a wider range of substrates for isomerisation than the bacteria isolated from the rumen as long as lipolitic activity is present to yield free fatty acids.
- The presence of a larger number of isomers of 18:2ω6 and 18:3ω3 than observed in the rumen organisms suggests that more alterations occurred to these fatty acids, increasing the number of possible routes through which degradation could have occurred.
- Although the reduction of a double bond on a fatty acid appears to rarely take place in nature, if the pathway of degradation proposed is accurate, hydrogenation of the various

octadecatrienoic, octadecadienoic and octadecenoic acids occurred in the linseed and sunflower oils degradation in the salt marsh sediments at Foryd Bay.

- The results of this study also appear to indicate that the type of isomers formed from the metabolism of 18:3ω3 or 18:2ω6 could mean the difference between the oils being further degraded or not.

The data presented here represented only a first step in unravelling the interaction between the bacteria, the oil and the physical and chemical environment. There is no reason to doubt the potential for the degradation of vegetable oils in the marine ecosystem, but the details may differ from those of other ecosystems.

APPENDIX

BACTERIA MEDIA COMPOSITION

The media utilised for the cultivation of the different, enumerated groups of bacteria is described in this appendix.

Heterotrophic Aerobic Bacteria

The ZoBell medium has the following composition (values in g dm^{-3} of dilution fluid): bacteriological peptone (Lab M), 5; yeast extract (Lab M), 1; ferrous phosphate (BDH), 0.1 and agar (Lab M), 15. The pH was adjusted to 7.6± 0.2 with NaOH. The medium was autoclaved at 121°C, 15 PSI for 15 minutes.

Aerobic Oil Degrading Bacteria

The culture medium, "fortified sea water" (FSW), had the following composition (values in g dm^{-3} of dilution fluid): KNO_3 (Analar®, BDH), 1.01; Na_2HPO_4 (Analar®, BDH), 0.1. The pH was adjusted to 7.2± 0.2 with NaOH. The FSW was autoclaved at 121°C, 10 PSI for 10 minutes to avoid precipitation of the salts. After sterilisation and prior to inoculation, two drops of vegetable oil (0.4%) were added to 9cm^3 of FSW.

Heterotrophic Anaerobic Bacteria

The fluid, pre-prepared thioglycollate medium (Merck), had the following composition (values in g dm^{-3} of dilution fluid): peptone from casein, 15; yeast extract, 5; D(+) glucose, 5.5; L(+) cysteine, 0.5; sodium chloride, 2.5; sodium thioglycollate, 0.5; sodium resazurin, 0.001; agar 0.75.

Solid thioglycollate medium was prepared by adding 14.25g of agar (Lab M) per litre of dilution fluid to the fluid thioglycollate. The pH was adjusted to 7.1± 0.2 with NaOH and the medium was autoclaved at 121°C, 15PSI for 15 minutes.

Anaerobic Oil Degrading Bacteria

The culture medium was composed of oil and FSW similar to that used for the aerobic bacteria. However, in the present case 0.6g sodium thioglycollate (Sigma) was added to one litre of medium to create reducing conditions.

Sulphate Reducing Bacteria

The Postgate medium E had the following composition (values in g dm^{-3} of dilution fluid): KH_2PO_4, 0.5 (Analar®, BDH); NH_4Cl, 1(Analar®, BDH); Na_2SO_4, 1(Analar®, BDH); $CaCl_2.6H_2O$, 1 (Fisons); $MgCl_2.6H_2O$, 2 (Sigma); sodium lactate, 3.5 (Sigma); yeast extract, 1 (Lab M); ascorbic acid, 0.1 (Analar®, BDH); thioglycollic acid, 0.1(Sigma); $FeSO_4.7H_2O$, 0.5 (Analar®, BDH); and agar, 15 (Lab M). The pH was adjusted to 7.6± 0.2 with NaOH. The medium was autoclaved at 121°C, 15 PSI for 15 minutes.

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