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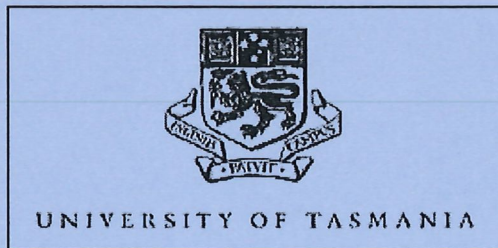
Evaluation of novel polyunsaturated fatty acid (PUFA) producing microheterotrophs for incorporation into aquaculture feeds

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FISHERIES RESEARCH & DEVELOPMENT CORPORATION

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Project No. 1997/329

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DISCLAIMER

Contents

Non-Technical Summary

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OBJECTIVES:

1. to develop targeted screening programs for the isolation and characterisation of PUFA-producing bacteria and other microheterotrophs

2. to maximise PUFA production by manipulation of specific microheterotroph culture conditions

3. to develop product formulations, including PUFA enrichment of live feeds, as a basis for commercial production of suitable strains.

4. to conduct feeding trials, using formulations to be developed during this project, using Atlantic salmon larvae (finfish, manufactured feed), Striped trumpeter – the original plan was to use Greenback flounder - larvae (finfish, live feed) and Prawns (crustacea, manufactured feed) as test species.

NON TECHNICAL SUMMARY:

This project has shown that microorganisms can produce high quality oils, rich in omega 3 polyunsaturated fatty acids, and in sufficient quantity for use in aquaculture diets. This has been a first step towards the development of a sustainable source of PUFA-rich oil for the Australian aquaculture industry. Given the appropriate regulatory and economic environment, it is possible that an industry based on production of PUFA-rich microbial biomass could be established in Australia. If such an industry can be initiated, our current reliance on fish oils as a source of essential PUFA in aquaculture diets will be reduced. This will result in a smaller total environmental footprint, and an increased net production by the aquaculture industry. As well as reducing reliance on oils harvested from wild-caught fish, microbial production of PUFA may help sustain the anticipated increase in aquaculture production. Such an increase in production is predicted to result in an increased demand for PUFA-rich oils, a demand that is unlikely to be satisfied by oil from wild caught fish.

Polyunsaturated fatty acids (PUFA) are natural marine-derived oils that are essential components in aquaculture diets. Commercial sources of PUFA for use within the mariculture industry are currently restricted to certain fish oils and microalgal species which are, respectively, under threat of over-exploitation and expensive to produce. The recent discoveries of non-photosynthetic microorganisms that synthesise PUFA provide a novel and timely opportunity to develop biotechnological processes for sustainable and relatively cheap production of PUFA. The aim of this study was to isolate and characterise PUFA-producing microorganisms from Australian habitats and to evaluate the potential application of selected strains for use in aquaculture feeds.

Objective 1:
To develop targeted screening programs for the isolation and characterisation of PUFA-producing bacteria and other microheterotrophs:

A technique was developed to allow efficient isolation of a particular group of PUFA-producing microorganisms – the thraustochytrids – from marine environments. DNA analysis data showed that the Australian strains were quite diverse. One new Australian strain (ACEM 6063) was found to be closely related to two other thraustochytrid strains that are being commercialised by overseas companies.

Objective 2:
To maximise PUFA production by manipulation of specific microheterotroph culture conditions

The way in which we grow some oil-producing microorganisms influences the amount and type of oil they produce. We therefore looked at the influence of culture conditions on oil production by thraustochytrids. Factors influencing the amount of cells produced by one thraustochytrid, and the oil content of the cells, were examined in detail. We identified culture conditions that allowed some thraustochytrids to produce up to 23 g dry cells L^{-1}, nearly half of which was oil. In general, cell production rates increased with increasing sugar and salt levels and increasing culture temperature. Older cultures contained more oil.

One important omega 3 PUFA is called docosahexaenoic acid (DHA). One of our thraustochytrid strains (ACEM 6063) produced substantial amounts of DHA. The relative level of DHA in the oil in ACEM 6063 decreased in older cells. But because the older cells had a higher oil content, they contained more DHA. The cell and DHA production characteristics of ACEM 6063 are amongst the highest ever reported for thraustochytrids, making further research and development to evaluate its commercial potential attractive.

Objectives 3 & 4:
To develop product formulations, including PUFA enrichment of live feeds, as a basis for commercial production of suitable strains, and

To conduct feeding trials, using formulations to be developed during this project, using Atlantic salmon larvae (finfish, manufactured feed), Striped trumpeter larvae[1] (finfish, live feed) and Prawns (crustacea, manufactured feed) as test species.

As well as learning how to increase oil production by our DHA-producing thraustochytrid, we wanted to see how well its PUFA was taken up by selected aquaculture species.

We selected three test species for this work: Striped trumpeter larvae (representing finfish that eat live feed), Atlantic salmon (representing finfish that eat manufactured feed), and prawns (representing crustaceans that eat manufactured feed).

There were two stages to this part of the project. First, we had to produce feed containing thraustochytrid PUFA in nutritionally significant quantities. Secondly, we had to determine if our test animals could assimilate the thraustochytrid PUFA. We started this process by using standard techniques to enrich rotifers – live feed used for fish larvae –with PUFA from thraustochytrid cells. We also produced manufactured feeds for prawns and Atlantic salmon, in which thraustochytrids were used as a full or partial replacement for the usual PUFA sources in commercial feeds for those species.

In all cases, we showed that the test animals efficiently took up PUFA from the thraustochytrid cells in the live and manufactured feeds. This showed that it is technically feasible to at least partially replace the fish oil currently used in aquaculture feeds with oil-rich thraustochytrid cells. This is a very encouraging result in the search for more sustainable aquaculture production systems.

Oil extracted from wild caught fish is able to meet the current demand for cheap PUFA for aquaculture feeds. However, demand for PUFA-rich products (by a number of industries – not just aquaculture) is predicted to increase dramatically over the 10 to 20 years, as more people become aware of the health benefits to be derived from including PUFA in the diet. As demand starts to outstrip supply, prices for PUFA-rich oils are likely to increase to levels capable of supporting an industry based on sustainable microbial production of PUFA-rich oils. If such an industry is to develop there will be a number of critical scientific steps to negotiate.

The oil (including DHA) production rates achieved by thraustochytrids in this project were high by current standards. However, further improvements will be required if production costs are to approach those required for commercial viability. The culture manipulation studies described in this project were based on small-scale (2 L) fermentation and laboratory-grade nutrients, and have focussed on only a small number of culture variables. One of the biggest challenges to overcome will be achieving similar - if not better - productivity in larger fermenters using cheap culture media. Several studies have shown that ingredients such as corn-steep liquor can be used to grow thraustochytrids. By-products from other commercial processes should also be tested for their ability to support large-scale production of PUFA-rich biomass.

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[1] the original plan was to use Greenback flounder

A number of novel thraustochytrid strains from Australian marine environments were isolated and characterised during this study. Factors influencing biomass and lipid production by one strain - ACEM 6063 - were examined, leading to establishment of culture conditions promoting the laboratory-scale production of substantial quantities of DHA-rich biomass. The potential for two of the new Australian thraustochytrids to be used to enrich live feed organisms with sufficient PUFA to meet currently recognised dietary requirements of some aquaculture species was also demonstrated. It now remains for the commercial potential of these and other thraustochytrid strains to be evaluated in large-scale facilities. If such evaluations show that production of these Australian strains is economically viable, the goal of a sustainable, large-scale PUFA production facility in Australia will be another step closer.

Acknowledgments

Background

During the past 10 years, a strong collaborative link has developed between marine oil specialists from the Marine Products Project of the CSIRO Division of Marine Research and microbiologists from the Microbial Processes Program of the Antarctic CRC. A strong team of researchers is presently examining the production of polyunsaturated fatty acids (PUFA) by Antarctic microorganisms, in particular bacteria isolated from seaice. To date, this research has been aimed primarily at understanding the eco-physiological role of Antarctic microorganisms. However, the potential for biotechnological exploitation of this newly discovered and largely unknown resource clearly exists

Polyunsaturated fatty acids (PUFA are essential components in mariculture diets. where an artificial food chain must be established (Bottino, 1974; Rimmer *et al.*, 1994). For many larval, or fingerling mariculture species, the provision of PUFA (especially the omega-3 fatty acids eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3), and the omega-6 fatty acid arachidonic acid (AA, 20:4n6) is critical. These essential fatty acids must be provided from either a "live" diet, usually via rotifers (e.g. *Brachionus plicatilis*) or brine shrimp (*Artemia* sp.) as intermediates (Ostrowski and Divakaran, 1990; Mourente and Tocher, 1993a; Mourente and Tocher, 1993b; Bell *et al.*, 1995; Southgate and Lou, 1995) or a manufactured diet. As adults, many species of finfish are reared on manufactured (pelletised) foods that must also contain PUFA.

Only certain microheterotrophs, together with unicellular microalgae, can synthesise PUFA *de novo* (Watanabe *et al.*, 1983; Lubzens *et al.*, 1985; Nichols *et al.*, 1992; Nichols *et al.*, 1993). At present, however, only selected fish oils and microalgal species are utilised as industrial sources of PUFA.

While microheterotrophs have previously been considered for use in mariculture feeds (Rieper, 1978; Kiessling and Askbrandt, 1993), their perceived lack of PUFA was seen as a major drawback (Phillips, 1984). With the discovery of microheterotrophs capable of synthesising PUFA, the application of such PUFA production, either from extracts or via direct insertion into aquaculture feed chains, is now an area of expanding interest (Watanabe *et al.*, 1992; Intriago and Jones, 1993). The recent discoveries of Antarctic bacteria which produce EPA (Nichols and Russell, 1996), DHA (D. Nichols unpublished data) and the omega-6 fatty acid AA [Nichols *et al.*, 1997 - the first report of a non-deep-sea (eg DeLong and Yayanos, 1986) bacteria producing this compound] provide a novel and timely opportunity to develop biotechnological processes for the production of these essential dietary components. Detailed studies on the biosynthesis of PUFA (eg. Nichols and Russell, 1996) provide fundamental knowledge as a foundation for this strategic and applied research proposal

Preliminary experiments in which PUFA-producing bacteria were fed to rotifers (Nichols *et al.*, 1996) resulted in a higher enrichment of PUFA (i.e. ratio of final % PUFA in rotifers : % PUFA in the bacteria) than has been reported for many studies using microalgae as the source of PUFA.

In addition to this preliminary result, microheterotroph-derived PUFA possess two distinct advantages over microalgae or fish oils. Firstly, the majority of algal species generally require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation, carbon dioxide and other parameters. These factors can result in considerable expense. Microalgal production in mollusc hatcheries is currently estimated to be 30-50% of total operating costs (M John, Shellfish Culture Pty Ltd, Tasmania, *pers comm*). While few hatcheries (either finfish and mollusc) quantitate costs for microalgae production, it has been estimated that most Australian hatcheries would incur costs of greater than \$120 kg^{-1} (dry weight) (Ass Prof Michael Borowitzka, *pers comm*). In contrast, most microheterotrophs are not fastidious, and often can be grown on the waste products of other agricultural or industrial processes. Indeed, the PUFA-producing strains currently held at the University of Tasmania, have been shown to produce PUFA from relatively simple growth media (eg. single amino acids as sole carbon sources), which contained no supplementary PUFA (D.Nichols, unpublished data). Secondly, fish oils represent a finite and often unreliable resource (New and Csavas, 1995). Bacteria, because of their ability to be cultured and maintained almost indefinitely, can be considered as an easily renewable resource for the production of PUFA (Nichols *et al.*, 1993).

The use of bacterial single cell (BSC) products in fish feeds has been investigated for a number of years. Recently new products have become available and there has been renewed interest in their potential as protein sources in salmonid feeds. One co-investigator (C.Carter: see Perera *et al.*, 1995b; Perera *et al.*, 1995a) recently investigated these for rainbow trout. It is now apparent that the Scandinavians are ready to build a commercial scale plant to produce bacterial products for Atlantic salmon feeds (Dr T. Storebakken *pers comm*). However, as PUFA-producing strains are not yet commercially available, other sources of these key dietary components will still be required.

Need

Polyunsaturated fatty acids (PUFA) are essential components in aquaculture diets, where an artificial food chain must be established (Bottino, 1974; Rimmer *et al.*, 1994). For many larval, or fingerling aquaculture species, the provision of PUFA (especially the omega-3 fatty acids EPA, DHA, and the omega-6 fatty acid AA is critical, and must be provided from either a "live" diet, usually via rotifers (e.g. Brachionus plicatilis) or brine shrimp (Artemia sp.) as intermediates (Ostrowski and Divakaran, 1990; Mourente and Tocher, 1993a; Mourente and Tocher, 1993b; Bell *et al.*, 1995; Southgate and Lou, 1995) or a manufactured diet. As adults, many species of finfish are reared on manufactured (pelletised) foods that must also contain PUFA

Commercial sources of PUFA for use within the mariculture industry are currently restricted to certain fish oils and microalgal species which are, respectively, under threat of over-exploitation and expensive to produce (New and Csavas, 1995; Tacon, 1995). The recent discoveries of bacteria and fungi that synthesise PUFA provide a novel and timely opportunity to develop biotechnological processes for sustainable and relatively cheap PUFA production.

Particular opportunities arise from the recent isolation of the following organisms.

1) Antarctic bacteria that produce the n3 fatty acids EPA and DHA, and the n6 fatty acid AA (Antarctic CRC and University of Tasmania)

2) Marine fungi that produce high levels of both DHA and EPA. (CSIRO Division of Marine Research.

Research combining skills and expertise in microbiology, cell culturing and manipulation, marine oils and lipid chemistry, biotechnology and aquaculture nutrition are required to take advantage of the industrial opportunity presented. Scientific advances can be made in each of these areas.

In microbiology there is a need to develop targeted, intelligent screening protocols to optimise recovery of bacteria with biotechnologically useful traits such as PUFA production. There is also a need to integrate current knowledge of factors that affect microheterotroph growth and metabolic processes into the development of techniques to optimise production of desired compounds. Research integration is expected to lead to the development of technology with which high productivity can be achieved while using cheap culture media

The application of state-of-the-art techniques in lipid chemistry will be applied to qualitatively and quantitatively evaluate PUFA production by microheterotrophs. The biotechnological challenge will involve devising stable formulations of whole cells and/or their extracts, and to transfer this technology from laboratory-scale trials through pilot-scale to commercial production systems.

As discussed above, the potential Australian Bacterial Single Cell (BSC) product/s in this application should be able to meet some or all of the requirement for n-3 and n-6 fatty acids of larval and adult aquaculture species. In addition, the BSC products should be also able to provide a good protein source, and may have the potential to improve the fatty acid profile of product flesh. Thus, the proposed Australian product may have the potential to replace a significant proportion of the fish meal and fish oil currently used.

Industrial advantage will be gained from the application of the scientific knowledge developed during this Project, in the incorporation of PUFA-producing bacteria or products derived there from into aquaculture food chains.

Objectives

1. to develop targeted screening programs for the isolation and characterisation of PUFA-producing bacteria and other microheterotrophs

See sections relating to: Isolation, maintenance and characterisation of novel Australian thraustochytrids

2. to maximise PUFA production by manipulation of specific microheterotroph culture conditions

See sections relating to: Biomass and lipid production by thraustochytrid strain ACEM 6063: influence of physical and chemical parameters

3. to develop product formulations, including PUFA enrichment of live feeds, as a basis for commercial production of suitable strains

See sections relating to: Aquaculture Feeding Trials

4. to conduct feeding trials, using formulations to be developed during this project, using Atlantic salmon larvae (finfish, manufactured feed), Striped trumpeter – the original plan was to use Greenback flounder - larvae (finfish, live feed) and Prawns (crustacea, manufactured feed) as test species

See sections relating to: Aquaculture Feeding Trials

Chapter 1   Materials and Methods

1.1   Isolation, maintenance and characterisation of novel Australian thraustochytrids

1.1.1   Isolation and maintenance

Sediment and seaweed were collected from marine environments around the southern coast of Tasmania (Australia). Initial samples were incubated at 20°C on solid Thraustochytrid Culture Medium (TCM) supplemented with antibiotics (TCMA). TCM was prepared by autoclaving all the ingredients shown in Table 1 (except the vitamin solution) at 108°C for 20 minutes, cooling the mixture to about 50°C and then aseptically adding 1 mL L^{-1} of the vitamin solution (Table 2). An antibiotic solution (Table 3; 20 mL L^{-1}) was aseptically added to TCM to prepare TCMA. It should be noted that sodium glutamate was selected as the defined nitrogen source in TCM, as this compound has been found to support relatively high biomass and 22:6n3 production by a variety of thraustochytrid strains (e.g. Goldstein, 1963; Bahnweg, 1979; Bajpai et al., 1991a; Bajpai et al., 1991b; Singh et al., 1996).

Inoculated TCM agar plates were examined daily by light microscopy. Colonies considered to be neither bacteria, yeast nor filamentous fungi were aseptically transferred to fresh TCMA plates and incubated at 20°C. All colonies growing on the secondary (TCMA) plates were streaked onto TCM (without antibiotics) agar plates to check for purity. Pure cultures were again subcultured onto TCM plates and incubated at 20°C for 7 days. An early maintenance protocol used in this study involved storing cultures at 2°C. However, storage at this temperature was found to be associated with the loss of viability of many strains. Subsequently, cultures were stored at 10°C.

Cultures of thraustochytrid type strains were obtained from the American Type Culture Collection (ATCC) (USA) and maintained on TCM plates as above. These strains were *Thraustochytrium* sp. ATCC 26185, *Schizochytrium aggregatum* Goldstein et Belsky ATCC 28209, *Thraustochytrium roseum* Goldstein ATCC 28210 and *Thraustochytrium aureum* Goldstein ATCC 34304.

Table 1   Thraustochytrid Culture Medium (TCM [1])

Component	Amount
Bacteriological peptone (L37; Oxoid, UK)	5 g
Glucose	10 g
Sodium glutamate	5 g
Yeast extract (L21; Oxoid, UK)	2 g
KH_2PO_4	100 mg
$NaHCO_3$	100 mg
$MnCl_2.4H_2O$	8.6 mg
$FeCl_3.6H_2O$	3.0 mg
$ZnSO_4.7H_2O$	1.3 mg
$CoCl_2.6H_2O$	0.3 mg
$CuSO_4.5H_2O$	0.2 mg
Sea salts (S9883: Sigma, USA)	32 g
Distilled H_2O	1000 mL
Agar (Grade 3: Leiner Davis, Australia) 2	10 g
Vitamin solution (see Table 2)	1 mL

1:    adapted from Iida et al. (1996) and Singh and Ward (1996)

2:    Agar only used for solid media

Table 2   Vitamin solution [1] used in Thraustochytrid Culture Medium (Table 1)

Component	Amount
Pyridoxine hydrochloride	20 mg
Thiamin hydrochloride	10 mg
Calcium pantothenate	10 mg
p-Aminobenzoic acid	10 mg
Riboflavin	10 mg
Nicotinamide or nicotinic acid	10 mg
Biotin	4 mg
Folic acid	4 mg
Vitamin B_{12}	0.2 mg
Distilled H_2O	100 mL

1:    Stirred until dissolved, filter sterilised and stored at –20°C (adapted from Staley et al., 1992)

Table 3   Antibiotic solution [1] added to TCM (20 mL L^{-1},Table 1) for initial isolation of presumptive thraustochytrids

Component	Amount
Penicillin	100,000 units
Streptomycin	250 mg
Distilled H_2O	100 mL

1:    Filter sterilise into sterile bottle, store in freezer (from Raghukumar, 1992)

1.1.2 Characterisation

1.1.2.1 Preparation, extraction and analysis of thraustochytrid fatty acid methyl esters (FAME)

The fatty acid profiles of thraustochytrids are known to vary considerably with variations in culture media and conditions. The following standard culture protocol was therefore used for the 74 newly isolated Australian strains and 4 ATCC type strains examined in this study. Actively growing cells from pure thraustochytrid colonies were used to inoculate freshly prepared TCM agar plates. Following culture at 20°C for 5 days, cultures were checked for purity by light microscopy. A sample of fresh cells from each plate was then aseptically transferred into clean, 10 mL screw-top test tubes containing a fresh transesterification solution, consisting of methanol, hydrochloric acid and chloroform (10:1:1 v/v/v, 3 mL). Cells were suspended in this solution by vortex mixing and transesterified at 90°C for 60 min, after which the tubes were removed from heat and cooled. Water (Milli Q Plus, Millipore SA, France; 1 mL) was then added to each tube and the FAME extracted using a hexane:chloroform 4:1 (v/v) solution.

FAME analyses were obtained using a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column, an HP7673A autosampler, a split/splitless injector, and flame ionization detector (Nichols et al., 1996). Peak areas were recorded and quantified using Millenium 32 v3.05.01 (Waters Corporation, USA). Mass spectrometric data was obtained using either a Fisons MD800 (Fisons Instruments, UK) or a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS), operated as described in Nichols et al. (1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards

Blank controls (i.e. containing no sample) were incorporated into this study. No fatty acids were recovered from any of the blank controls.

1.1.2.2 Extraction, purification, amplification and sequencing of thraustochytrid 18S ribosomal RNA genes

Sufficient biomass for extraction of 18S ribosomal RNA genes (18S rDNA) was obtained from 15 of the 74 Australian strains originally isolated for this study, and from one non-viable sample of the commercially available thraustochytrid-based aquaculture feed Algamac 2000 (Aquafauna BioMarine, USA). All viable strains were grown in TCM broth for 7-10 days at 20°C, harvested by centrifugation, washed in 1% (w/v) NaCl, freeze-dried and stored at -20°C prior to extracting the genomic DNA. All materials that came into contact with cells, DNA and DNA products during the process of extracting, amplifying and sequencing the 18S rDNA were sterile. Total genomic DNA was extracted by a modification of the method developed by Murray and Thompson (1980). Freeze-dried cells from each strain were dispersed in 700 μL extraction buffer [0.7 M NaCl, 1% (w/v) cetyltrimethylammonium bromide, 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% (w/v) ß-mercaptoethanol] in a 2.0 mL bead beating tube. Glass beads (0.1 mm diameter, ~0.5 g) were added to the cell suspension, and the mixture shaken (Mini Bead Beater, BioSpec Products, USA) at 3,800 rpm for 4 x 10 s. Tubes were placed on ice for at least 10 s between each pulse. The mixture was then incubated at 55°C for 30 min. Extracts were emulsified, using gentle inversion, with an equal volume of 24:1 (v/v) chloroform:iso-amyl alcohol (24:1 CHCl$_3$:IAA) and centrifuged at 10,000 x g for 10 min. The upper (aqueous) layer was transferred by pipette into a 1.5 mL eppendorf tube, to which an equal volume of 24:1 CHCl$_3$:IAA and a 0.1 volume of a saline CTAB solution [10% (w/v) CTAB, 0.7M NaCl] were added. This mixture was emulsified by gentle inversion and centrifuged at 10,000 x g for 10 min. The upper layer was transferred by pipette into a new tube, to which an equal volume of phenol:chloroform (Amresco, USA) was added. This mixture was emulsified by gentle inversion and centrifuged at 8,000 x g for 10 min. The upper layer was transferred by pipette into a new tube, to which an equal volume of isopropanol was added, and mixed by gentle inversion. To precipitate DNA, this solution was held at -20°C overnight before centrifugation at 20,000 x g for 25 min at 4°C. Following removal of the supernatant, a further 800 μL ethanol (-20°C) was added and the sample centrifuged at 20,000 x g for 25 min at 4°C. The supernatant was again removed using a pipette, leaving the DNA precipitate in the tube. The precipitate was air-dried at room temperature, resuspended in 50 μL 4% (w/v) RNAse (Sigma, USA) and incubated at 37°C for 30 min before storing at -20°C.

Standard polymerase chain reaction (PCR) protocols were followed to amplify 18S rDNA from the genomic DNA extracts, using the oligonucleotide PCR primers 18S001 and 18S13 (Table 4). PCR reactions were performed using the HotStart Taq Master Mix kit (Qiagen, USA) according to the manufacturer's instructions. Each PCR reaction mixture contained 25 μL HotStart Master Mix, 1 μL of a 50 pM μL^{-1} solution of each primer, 2 - 8 μL of genomic DNA extract and sterile water (Milli Q Plus, Millipore SA, France) to a final volume of 20 μL. The PCR amplification was performed using a DNA Engine PCT-200 (M J Research Inc, USA) with the following thermocycling conditions: 95°C for 15 min, 30 cycles of (94°C for 1 min, 52°C for 1 min and 72°C for 1 min), 72° for 10 min, then holding at 4°C. Purity of PCR products were examined on electrophoresis gels containing 1% (w/v) agarose in TAE buffer (40 mM Tris, 40 mM glacial acetic acid, 1 mM

disodium EDTA, pH 8) and 1 µg mL^{-1} ethidium bromide. Where necessary, PCR product was re-amplified to provide sufficient amounts for sequencing. In these cases, 0.1 µL of the original PCR product was used with the same reaction mixture and thermocycling profile as described above. Unincorporated dNTP and primers were removed from PCR products using QIAquick-spin purification columns (Qiagen, Germany).

Table 4   Oligonucleotide primers [1] used for amplification and sequencing

Code	Synthesis direction	Sequence	Reference
18S001	forward	5´-AACCTGGTTGATCCTG CCAGTA-3´	Honda *et al.* (1999)
18SmidF	forward	5´-CAAGTCTGGTGCCAGC AGCC-3´	this study
18S13	reverse	5´-CCTTGTTACGACTTCAC CTTCCTCT-3´	Honda *et al.* (1999)

1:    Primers were obtained from Life Technologies Pty Ltd, Australia

PCR sequencing reactions were performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Each sequencing reaction contained purified PCR product (1-5 µL); primer (1 µL); BigDye buffer (4 µL); BigDye reaction mix (4 µL) and sterile Milli Q water to a final volume of 20 µL. Primers used for sequencing reactions were 18S001, 18SmidF and 18S13 (Table 4). Sequencing reactions were performed using a DNA Engine PCT-200 (M J Research Inc, USA) with the following thermocycling conditions: 96°C for 10 min, 26 cycles of (50°C for 5 min and 60°C for 4 min), then holding at 4°C.

Each sequencing reaction mix was transferred to a 1.5 mL tube, to which 4 µL of 3M sodium acetate (pH 4.6) and 60 µL of absolute ethanol (-20°C) were added. Tubes were placed on ice for 20 min and centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was removed with a pipette and the remaining pellet rinsed with 250 µL 70% ethanol (-20°C), followed by centrifugation at 20,000 x g for 10 minutes at 4°C. After removing the supernatant with a pipette, the precipitate was dried in a vacuum centrifuge (DNA mini, Heta, Denmark), and stored at -20°C. Electrophoresis and analysis was performed on an automated sequencer (A377, Applied BioSystems, USA).

1.1.3 Statistical analyses

The fatty acid profiles of the thraustochytrid strains were analysed using the software TAXON PC (v1.0 beta 3). A thraustochytrid strain dissimilarity matrix was generated using a Standardised Euclidean Distance analysis of all fatty acids occurring at ≥ 0.1% of the total fatty acids (%TFA) (Kohring *et al.*, 1994). This matrix was clustered into 10 groups using an Incremental Sum of Squares sorting strategy, allowing a chemotaxonomic dendrogram of thraustochytrid strains to be generated.

Sequence electrophoretograms were examined using the software Sequence Navigator v1 (PE Applied Biosystems, USA) to resolve ambiguous base positions. Corrected sequences were manually aligned against published 18S rDNA thraustochytrid sequences (Honda *et al.*, 1999; Lopez-Garcia *et al.*, 2001). Phylogenetic analysis of aligned sequences was done using the software suite PHYLIP v 3.57c (Felsenstein, 1993). Evolutionary distances were determined using the Maximum Likelihood algorithm in the module DNADIST, and the phylogenetic tree constructed using the neighbour-joining method in the module NEIGHBOR.

1.2   Biomass and lipid production by thraustochytrid strain ACEM 6063: influence of physical and chemical parameters

A series of culture experiments were performed to examine the influence of physical and chemical culture parameters on biomass and lipid production by ACEM 6063. Initial experiments to examine the influence of culture age, media composition and inoculum characteristics were performed in 500 mL screw top Erlenmeyer flasks. Subsequent experiments to examine the influence of glucose concentration, physical culture conditions and dissolved oxygen levels were performed in a BioFlo IIC fermenter (New Brunswick, USA). Methodology for these experiments is detailed in Sections 1.2.1 and 1.2.2, below.

1.2.1   Flask culture experiments

Inocula for all flask culture experiments were prepared using the following method. A maintenance culture of strain ACEM 6063 was inoculated onto TCM agar and incubated at 20°C for 5 days. Once strain purity had been confirmed, actively growing cells were used to inoculate 150mL of TCM, modified as appropriate, in 500 mL screw top Erlenmeyer flasks. Flasks were incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm. During incubation, flask lids were kept loose to facilitate gas exchange. After incubation for the appropriate time, culture purity was confirmed by light microscopy, and the culture immediately used to inoculate experimental media.

Experimental cultures were grown in flasks as described above. All cultures were sampled as required by aseptically drawing 10 mL from each flask and transferring the sample directly into sterile, 15 mL screw-top plastic centrifuge tubes (Greiner, Germany). Cells were immediately centrifuged (5000 x g, 10 min: EasySpin, Sorval Instruments, USA), resuspended in 10 mL 1.0% (w/v) NaCl, and recentrifuged. Cell pellets were frozen, freeze-dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure $< 7 \times 10^{-1}$ mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15-20 h, weighed and then stored at -30°C.

Biomass, calculated as g dry weight L^{-1}, was determined using the following equation, which allowed for the dry weight of the small volume of NaCl wash solution still associated with the centrifuged cells.

$$Biomass\ (g\ dry\ weight\ L^{-1}) = \{[T_{dw}\ ((T_{ww}-T_{dw})*W_{dw})]/S_{vol}\}$$

Where: T_{dw} = total dry weight of sample (g)
T_{ww} = total wet weight (centrifuged) of sample (g)
W_{dw} = dry weight of 1 mL of the NaCl wash solution (g)
S_{vol} = sample volume (mL)

1.2.1.1 Flask Experiment 1: Initial glucose concentration and culture age

Ten flasks (5 treatments, in duplicate) containing 150 mL of TCM supplemented to contain 10, 50, 100, 150 and 200 g L^{-1} glucose were inoculated with 5% (v/v) inoculum, prepared as described on page 6. Flasks were incubated on a rotary shaker (200 rpm) at 20°C. Cells were sampled regularly until peak biomass had been reached.

1.2.1.2 Flask Experiment 2: Sea salt concentration

Four flasks containing 150 mL of TCM, modified to contain 1, 8, 15 and 30 g L^{-1} sea salts (S9883: Sigma, USA), were inoculated (5% v/v) as described on page 6, and incubated on a rotary shaker (200 rpm) at 20°C. Each flask was sampled regularly until peak biomass had been reached.

1.2.1.3 Flask Experiment 3: Glucose and sodium glutamate concentration

Thirty two flasks (16 treatments, in duplicate) containing 150 mL of TCM, modified to contain all combinations of 50, 100, 150 and 200 g L^{-1} glucose and 1, 2, 5 and 10 g L^{-1} sodium glutamate, were inoculated with 5% (v/v) inoculum. For this experiment, two flasks of inoculum were prepared as described on page 6. The contents of these flasks were combined prior to use to provide a homogenous inoculum. Experimental flasks were incubated on a rotary shaker (200 rpm) at 20°C for 10 days.

1.2.1.4 Flask Experiment 4: Inoculum size and age

Twenty four flasks (12 treatments, in duplicate) containing 150 mL of TCM, modified to contain 100 g L^{-1} glucose, were inoculated with 1, 5 and 10% (v/v) of 1-, 2-, 5- and 10-day old inocula, prepared as described on page 6. Two inoculum cultures were used for this experiment. Each inoculum culture was used to inoculate one of the duplicate flasks for each treatment on each day. Flasks were incubated on a rotary shaker (200 rpm) at 20°C for 10 days.

1.2.2 Fermenter culture experiments

Fermenter culture experiments were conducted in a BioFlo IIC fermenter (New Brunswick, USA). Two, four-bladed paddle impellers (tip diameter 6.2 cm), were used to mix the fermenter cultures. Both impellers were attached to a central axle, which in turn was connected to the stirring motor. Impellers were situated on the axle at $^1/_3$ and $^2/_3$ the depth of the culture medium in the fermenter chamber.

Inocula for fermenter culture experiments were prepared as described for flask culture experiments in Section 1.2.1 (page 6). Inocula (5% v/v) were aseptically transferred into the fermenter using sterile disposable syringes fitted with large bore needles (18 gauge, Terumo, USA). Inocula were drawn into, and expelled from, the syringes very slowly, to minimise the possibility of cell damage due to pressure changes and/or shear forces. Culture temperatures were controlled to within 1 °C using a microprocessor-controlled water jacket. Dissolved oxygen levels were controlled semi-automatically, using a combination of impeller revolution speed (via microprocessor-controlled feedback from the oxygen probe) and air/oxygen supply (manual control). Aeration, at a maximum flow rate of 1.0 L min^{-1}, was supplied using 0.2 μm-filtered air (Midisart 2000, Sartorius, Germany) for low DO cultures, or with a manually-controlled mixture of air and oxygen (Industrial Grade O_2, BOC Gases, Australia) for high DO cultures. All gas supplied to the fermenter was bubbled through sterile distilled water, to minimise evaporative losses from the culture media, before delivery to the fermenter culture chamber via four 0.5 mm diameter holes situated at the base of the culture vessel. Sterile antifoam solution (Foamaster LQ217, Henkel, Australia) was added automatically as required. A maximum of about 1 mL of antifoam was used in each fermenter culture.

Culture conditions for each fermenter experiment are shown in Table 5. It should be noted that the fermenter culture system used was not able to supply sufficient oxygen to maintain high DO levels in ACEM 6063 cultures grown at 25°C.

During each fermenter experiment, samples (typically 10 mL) were aseptically collected through the fermenter sample port. Cells were centrifuged, freeze-dried, weighed and stored as described in Section 1.2.1.

Table 5   Culture conditions used in ACEM 6063 fermenter culture trials

Treatment code	Initial glucose concentration (g L^{-1})	Dissolved oxygen [1]	Temperature (°C)	Maximum impeller speed [2]
10H20H	10	High	20	High
50H20H	50	High	20	High
50L20L	50	Low	20	Low
50H20L	50	High	20	Low
100L15L	100	Low	15	Low
100H15L	100	High	15	Low
100L20L	100	Low	20	Low
100H20L	100	High	20	Low
100L25L	100	Low	25	Low

1:    Dissolved oxygen: High (H) = > 40% saturation, Low (L) = < 5% saturation
2:    Maximum impeller speed: High (H) = 750 rpm, Low (L) = 200 rpm

1.2.3 Thraustochytrid lipid analyses

Cells were harvested by centrifugation (J2-21M/E centifuge, JA 14 rotor, Beckman USA) at 8000 x g for 15 minutes in 250 mL polyethylene centrifuge tubes. Following centrifugation, the supernatant was discarded, the cell pellet resuspended in 100 mL 1.0% NaCl (w/v), and re-centrifuged. The second supernatant was discarded and the cell pellet frozen overnight at -30°C. Frozen biomass was freeze dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure
< 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15 hours and subsequently stored in a sealed glass container at -30°C. Preliminary experiments showed that freeze-drying ACEM 000A and ACEM 6063 cells prior did not influence the amount of lipid recovered from the biomass, nor the fatty acid profile of the extracted lipid.

1.2.3.1 Lipid extraction, fractionation and analysis

Two procedures were used to extract lipids from freeze-dried thraustochytrid biomass: the extraction of lipids using a modified (White et al., 1979) Bligh and Dyer (1959) solvent extraction; and the direct transesterification of biomass to produce fatty acid methyl esters (FAME) (i.e. without the initial extraction step). Direct transesterification was used to obtain fatty acid data. Total lipid extracts obtained using the modified Bligh and Dyer extraction method were used to obtain lipid class data.

1.2.3.2 Fatty acid analyses

Freeze-dried cells were weighed into clean 10 mL screw-top test tubes to which a fresh solution of the transesterification reaction mix [methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL)] was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90°C for transesterification. Tubes were removed from heat after 60 minutes and cooled. Water (1 mL) was then added to each tube, and the FAME extracted (hexane:chloroform, 4:1 v/v, 3 x 2 mL). For gas chromatographic (GC) analysis, samples were diluted with chloroform containing a known concentration of 19:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter, cross-linked, HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionisation detector (Nichols et al., 1996). Peak areas were recorded and quantified using the software package Millenium 32 v3.05.01 (Waters Corporation, USA). Confirmation of FAME identity was performed on MD 800 (Fisons, United Kingdom) or GCQ (Thermoquest, USA) GC-mass spectrometers (GC-MS) (Nichols et al., 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

Blank controls (i.e. containing no sample) were used in each experiment. No fatty acids were recovered from any of the controls.

1.2.3.3 Lipid class analyses

Lipids were extracted from freeze-dried biomass using a one-phase chloroform-methanol-water (1:2:0.8 v/v/v) extraction. Solvents were added to the freeze-dried biomass in order of increasing polarity, as this technique increased lipid recovery from ACEM 6063 biomass. The biomass-solvent mixtures were left overnight. Phase separation was achieved the next day by adding chloroform and water to obtain a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). Lipids were recovered from the lower chloroform phase. Solvents were removed under vacuum prior to the extracted lipids being stored in chloroform under N_2 at -20°C.

Lipid class data was determined by analysing a portion of the total lipid extract using thin layer chromatography – flame ionisation detection (Iatroscan Mk V TH-10, Iatron Laboratories, Japan). Aliquots (1 μL) from a known volume of each extract were applied onto duplicate Iatroscan chromarods using disposable micro-pipettes. Chromarods were developed using a hexane - diethyl ether- acetic acid (60:17:0.2 v/v/v) solvent system in a sealed glass tank containing pre-extracted filter paper. After development, the chromarods were oven-dried at 60°C for 10 min and analysed immediately. Peak areas were recorded and quantified using the chromatography software DAPA (Scientific Software, Kalamunda, Western Australia).

1.3   ACEM 6063 Storage trials

Biomass from flask cultures of ACEM 6063 was harvested by centrifugation, washed in sterile (autoclaved) 1% (w/v) NaCl and re-centrifuged. Wet biomass (1.0 mL) was transferred into sterile 1.5 mL Eppendorf tubes. Half the tubes were stored overnight at 2°C, while the other half were frozen and freeze-dried as described in section 1.2.1 (page 6). Wet (centrifuged) cells (WC) and freeze-dried cells (FD) were stored, in darkness, at – 20, 2, 10 and 20 °C. At each sampling time, 8 tubes (2 cell treatments, 4 storage temperatures) were selected, frozen at –20°C overnight, freeze-dried and analysed as described in section 1.2.3 (page 9).

1.4   Aquaculture Feeding Trials

1.4.1   Enrichment of rotifers (*Brachionus plicatilis*) with eicosapentaenoic acid and docosahexaenoic acid produced by bacteria

1.4.1.1 Bacterial strains

Bacterial strains ACAM 456 and ACAM 605 (*Shewanella gelidimarina* and *Colwellia psychroerythrus* respectively) are facultatively anaerobic, Gram-negative, non-sporulating pleomorphic rods (Bowman *et al.*, 1997; Bowman *et al.*, 1998). Both strains are psychrophilic, with optimum growth temperatures of 16.5 °C and 12 °C respectively. For this experiment they were grown at 10 °C, with generation times of 184 and 334 minutes. These strains were selected for this feeding experiment due to their

ability to synthesise relatively high amounts (compared to other bacteria) of EPA (ACAM 456) or DHA (ACAM 605).

1.4.1.2 Bacterial culture

The two bacterial strains were grown in 50 mL of Zobell's broth (ZB) (Zobell, 1946) for 2 days (ACAM 456) and 3 days (ACAM 605) at 10 °C. Four 10 mL aliquots of each 50 mL broth culture were used to inoculate each of four sealed 2 L glass flasks containing 1.8 L of ZB. These cultures were incubated at 10 °C for 2 days (ACAM 456) and 3 days (ACAM 605), with agitation and aeration provided by the addition of ~ 2 L min^{-1} of 0.2 μm-filtered air to each flask. All cultures were harvested on the same day by centrifugation (3500 x g, 15 min). Cell pellets were pooled in minimal volumes of sterile 0.85% (w/v) saline. Total bacterial cell numbers were calculated using light microscopy and a glass counting chamber (Weber Scientific, England). Cell concentrates were stored overnight at 2 °C.

1.4.1.3 Rotifer culture

Rotifers were from a population continuously cultured, with aeration and overhead fluorescent lighting, in 2000 L fiberglass cylindrical containers. Culture water was maintained at 21 to 25‰ salinity and at 21 °C. Rotifers were fed three times daily with compressed bakers yeast (*Saccharomyces cerevisiae*) at the rate of 0.6 gm yeast 10^{-6} rotifers. Supplemental feeding of the rotifer culture, with *Tetraselmis suecica*, occurred every second day, with the microalga added to a final concentration of about 1 x 10^5 cells mL^{-1}. Rotifer density was determined from the mean count of five 1 mL aliquots from each culture.

Twelve hours prior to the start of the feeding trial, sufficient rotifers were removed from culture, washed with clean culture water and placed in clean, aerated culture water at a concentration of about 300 rotifers mL^{-1}. These rotifers were not fed again before the start of the feeding trial.

1.4.1.4 Rotifer feeding and harvest

The feeding trial was performed using twelve 2 L glass flasks, maintained at 21 °C, and gently aerated. Flasks were randomly positioned within the experimental system. Bacteria, microalgae, yeast (6 duplicate treatments), were added to experimental flasks containing 1.4 L of 25‰ seawater at 21 °C. A control treatment of starved rotifers was also included. Finally, 100 mL of a suspension of the starved rotifers was added to each flask, to yield a final rotifer concentration of 200 rotifers mL^{-1}. A 9 mL aliquot was obtained from each flask 24 hours after addition of rotifers. Aliquots were added to 10 mL of neutral buffered formalin (NBF) (Ajax Chemicals, USA) in clean test tubes and stored at 4 °C prior to counting. Final counts of rotifers still actively swimming in each flask were obtained by counting rotifers from five x 1 mL aliquots of each NBF fixed sample.

Twenty four hours after the addition of rotifers, a 1 L sample was obtained from each flask. Each sample was transferred onto a clean silk screen (mesh size 63 μm), washed 3 times with ~ 1 L 25‰ seawater and transferred to a clean beaker using a minimal amount of

25‰ seawater. Washed rotifers were harvested by filtration onto premuffled (450 °C, 24 h) glass-fibre filters (Whatman GF/F) and stored at -20 °C. Two 100 mL samples of the initial unfed rotifer culture and five 5 mL samples of the original bacterial concentrates were harvested prior to the start of the feeding trial. These samples were filtered and frozen as described above.

1.4.1.5 Extraction, fractionation and analysis of FAME

Lipids were extracted from bacteria and rotifer samples using a modified one-phase chloroform:methanol:water (1:2:0.8 v/v/v) extraction (White *et al.*, 1979). A portion of the total-lipid extract recovered from the lower chloroform phase was transmethylated by reaction for 1 h at 80 °C with methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL). Fatty acid methyl esters (FAME) were extracted and diluted with chloroform containing a known concentration of either 19:0 or 23:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionization detector (Nichols *et al.*, 1996). Fatty acid percentages represent mean values of duplicate samples. Fatty acid concentration data (expressed as percent of total rotifer dry weight) were calculated using the final abundance of rotifers in each culture along with values previously obtained for dry weight of both fed- and starved-rotifers. Confirmation of FAME identity was performed on a Fisons MD 800 (Manchester, United Kingdom) GC-mass spectrometer (GC-MS) (Nichols *et al.*, 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

1.4.2 Enrichment of rotifers with PUFA from thraustochytrids

1.4.2.1 Thraustochytrid culture

Biomass of ACEM 000A, containing low oil levels (total fatty acids about 85 mg g^{-1}) was used in Trial 1. Biomass of ACEM 000A and ACEM 6063, containing high oil levels (total fatty acid levels about 550 and 340 mg g^{-1} respectively), was used in Trial 2. Biomass was produced in 500 mL, screw-topped Erlenmeyer flasks, containing 150 mL of Thraustochytrid Culture Medium broth (TCM; Chapter 2) supplemented to contain initial glucose concentrations of either 50 or 100 g L^{-1}. Cultures were incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm for 7 (ACEM 6063) and 10 (ACEM 000A) days.

Cells were harvested by centrifugation (J2-21M/E centifuge, JA 14 rotor, Beckman USA) at 8000 x g for 15 minutes in 250 mL polyethylene centrifuge tubes. Cell pellets were washed in 100 mL 1.0% NaCl (w/v), re-centrifuged and the second supernatant discarded. Cell pellets for each strain were combined and then divided into sub-samples, each of which was exposed to one of the four different treatments described below.

- Fresh (FS) cells: stored overnight in a clean glass container at 2°C;

- Frozen (FZ) cells: stored in a clean glass container at -30°C;

- Freeze-dried (FD) cells: frozen at -30°C, freeze-dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure < 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15-18 hours and stored in a sealed glass container at -30°C.
  These cells were weighed before and after drying to determine the dry-weight equivalent of the FS and FZ cells;

- Air-dried (AD) cells: spread thinly in a 20 cm diameter glass dish, dried overnight at 60°C, and stored in a sealed glass container at -30°C.

1.4.2.2 Rotifer culture

Rotifers were from a population continuously cultured as described in Section 1.4.1.3 (page 11).

1.4.2.2.1 Rotifer feeding and harvest

The same experimental protocol was used for Trials 1 and 2.

Eighteen hours prior to the start of each feeding trial, rotifers were removed from their culture tanks, washed with clean culture water and placed in clean, aerated culture water at a concentration of about 300 rotifers mL^{-1}. These rotifers were not fed again before the start of the feeding trial.

Each feeding trial was performed in 2 L glass flasks, maintained at 21 °C, and gently aerated. Flasks were randomly positioned within the experimental system. Biomass of ACEM 000A, ACEM 6063, a commercial thraustochytrid-based enrichment diet (Algamac-2000, Aquafauna BioMarine, USA), microalgae (*Isochrysis* sp, clone T. Iso) and yeast (*Saccharomyces cereviseae*) (Table 6 and Table 7) were added to the flasks, which were then filled to 1.5 L with 25‰ seawater at 21°C. Finally, 100 mL of a concentrated suspension of the starved rotifers was added to each flask, to yield a final rotifer concentration of 200 rotifers mL^{-1}.

Six hours after the addition of rotifers (T6), a 10 mL aliquot of actively swimming rotifers was obtained from the water column of each flask. Aliquots were added to 10 mL of neutral buffered formalin (NBF) (Ajax Chemicals, USA) in clean test tubes and stored at 4 °C prior to counting. Rotifer density was determined from the mean of 5 x 1 mL aliquots from each NBF fixed sample.

Table 6   Initial concentrations of enrichment diets used in Trial 1

	Enrichment diet						
	ACEM 000A (mg dry weight equivalent L^{-1})				Algamac (mg L^{-1})	T. Iso[1] (cells mL^{-1})	Yeast[2] (mg L^{-1})
Treatment[3]	Fresh	Frozen	Freeze-dried	Air-dried			
A FS	100	0	0	0	0	0	0
A FZ	0	100	0	0	0	0	0
A FD	0	0	100	0	0	0	0
A AD	0	0	0	100	0	0	0
Algamac	0	0	0	0	100	0	0
T. Iso	0	0	0	0	0	5×10^5	0
Yeast	0	0	0	0	0	0	100
Starved	0	0	0	0	0	0	0

1:     T. Iso = *Isochrysis* sp, clone T. Iso

2:     Yeast = Saccharomyces cerevisiae

3:     A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells

At the same time, a 350 mL sample was obtained from each flask. Each sample was washed (3 x ~500 mL 25‰ seawater) on a clean silk screen (mesh size 63 μm), and transferred to a clean beaker using a minimal amount of 25‰ seawater. Washed rotifers were harvested by filtration onto pre-weighed and muffled (450 °C, 24 h) glass-fibre filters (GF/F 47 mm, Whatman, USA) and stored at -20 °C. Duplicate samples of the initial unfed rotifer culture (100 mL each) and of each component of the enrichment diets were collected prior to the start of each feeding trial. These samples were filtered and frozen as described above.

Flasks were then refilled to 1.6 L with 25‰ seawater and a second ration of feed (40% of the original ration) was added.

Eighteen hours later (T24), a second 350 mL sample (for fatty acid analysis) and one 10 mL aliquot (for counting) were obtained from each flask, and processed as described above.

Table 7   Initial concentrations of enrichment diets used in Trial 2

| | Enrichment diet | | | | | | | | |
| | ACEM 000A (mg dry weight equivalent L^{-1}) | | ACEM 6063 (mg dry weight equivalent L^{-1}) | | | | Algamac (mg L^{-1}) | T. Iso[1] (cells mL^{-1}) | Yeast[2] (mg L^{-1}) |
Treatment[3]	Frozen	Freeze-dried	Fresh	Frozen	Freeze-dried	Air-dried			
A FZ	100	0	0	0	0	0	0	0	0
A FD	0	100	0	0	0	0	0	0	0
A FD x2	0	200	0	0	0	0	0	0	0
A FD + T. Iso	0	100	0	0	0	0	0	5 x 10^5	0
A FD + 6063 FD	0	100	0	0	100	0	0	0	0
6063 FS	0	0	100	0	0	0	0	0	0
6063 FZ	0	0	0	100	0	0	0	0	0
6063 FD	0	0	0	0	100	0	0	0	0
6063 FD x2	0	0	0	0	200	0	0	0	0
6063 AD	0	0	0	0	0	100	0	0	0
6063 FD + T. Iso	0	0	0	0	100	0	0	5 x 10^5	0
Algamac	0	0	0	0	0	0	100	0	0
Algamac x2	0	0	0	0	0	0	200	0	0
T. Iso	0	0	0	0	0	0	0	5 x 10^5	0
Yeast	0	0	0	0	0	0	0	0	100
Starved	0	0	0	0	0	0	0	0	0

1:     T. Iso = *Isochrysis* sp, clone T. Iso

2:     Yeast = Saccharomyces cerevisiae

3:     A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1})

After obtaining these samples, rotifers from 1.2 L from each flask were washed as described above and transferred into a clean flask, which was then filled to 1.6 L with isothermal 25‰ seawater. No food was added at this time.

Twenty four hours later (T48), a final 500 mL sample (for fatty acid analysis) and one 10 mL aliquot (for counting) were obtained from each flask, and processed as described above.

All filters were freeze-dried, weighed and stored at –20 °C prior to analysis.

1.4.2.3 Lipid analyses

Lipids were extracted, fractionated and analysed as described in Section 1.4.1.5 (page 12), except that confirmation of FAME identity was achieved using either an MD 800 (Fisons, United Kingdom) or a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS).

1.4.2.4 Statistical analyses

Statistical analyses of data were performed using the General Linear Models Procedure of the software package SAS System for Windows v 6.12 (SAS Institute Inc. USA). Differences between individual means of replicate samples were deemed to be significant at $\alpha = 0.05$.

1.4.3 Atlantic salmon feeding trial

1.4.3.1 Experimental diets

Three diets were formulated to compare canola oil (CO) as the only source of oil with supplementation by either the Thraustochytrid meal (CTH) or fish oil (CFO). The Thraustochytrid meal (Table 8) contained protein as well as oil so the casein and soybean meal contents were reduced to account for this and the diets formulated to be isonitrogenous and isoenergetic (Table 9). The lipid class and fatty acid composition of experimental diets are shown in xx. Chilean fish meal was defatted twice using a 2:1 mixture of hexane and ethanol (400 mL 100 g^{-1} fish meal). Soybean meal (ADM Bioproducts, Beenleigh, Qld, Australia); lactic casein (New Zealand Dairy Board, Wellington, New Zealand); wheat gluten (Starch Australasia, Tamworth, NSW, Australia), pre-gelatinised starch (Goodman Fielder, Summer Hill, NSW, Australia) were also used. Fish oil from jack mackeral (Pivot Aquaculture, Cambridge, Tas., Australia) and a domestic source of pure Canola oil were used. Stay-C and Rovimix E50 were supplied by Roche Vitamins Australia (Frenchs Forest, NSW, Australia), A and D by ICN (Seven Hills, NSW, Australia) and the remaining ingredients were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). The diets were manufactured as 3 mm diameter pellets using a California Pellet Mill (CL-2 Lab Mill). Diets were then dried and stored at –20°C.

Table 8   Chemical composition of the Thraustochytrid meal

Nutrient	g.kg^{-1}
Dry matter	979
Crude protein	207
Total fat	589
Ash	85
GE (MJ.kg^{-1})	20.7
Essential amino acids	
Arginine	14.6
Histidine	5.3
Isoleucine	9.1
Leucine	16.9
Lysine	13.0
Methionine	5.5
Phenylalanine	10.4
Threonine	10.9
Tryptophan	2.6
Valine	20.9
Essential fatty acids	
18:2n-6	0.14
20:4n-6	0.53
18:3n-3	0.23
20:5n-3	2.63
22:6n-3	92.21

Table 9   Ingredient and chemical composition of experimental diets

	Diet		
	CO	CTH	CFO
Ingredient composition (g.kg^{-1})			
Defatted fish meal	250.0	250.0	250.0
Soybean meal	56.2	36.5	56.2
Casein	143.2	132.9	143.2
Wheat gluten	100.0	100.0	100.0
Canola oil	169.6	110.5	78.2
Fish oil			91.4
Thraustochytrid meal		100.0	
Pre-gel starch	150.0	150.0	136.2
Vitamin mix (ASV4)[1]	3.0	3.0	3.0
Stay C[2]	3.0	3.0	3.0
Choline chloride	2.0	2.0	2.0
Mineral mix (TM4)[3]	5.0	5.0	5.0
Ca (H$_2$PO$_4$)$_2$	21.8	23.0	21.8
α-Cellulose	36.2	24.2	50.0
Bentonite	49.0	49.0	49.0
CMC	9.0	9.0	9.0
Cholestane	1.0	1.0	1.0
Yttrium Oxide[4]	1.0	1.0	1.0
Chemical composition (g.kg^{-1} DM)			
Dry matter (g.kg^{-1})	940 ± 2	960 ± 1	901 ± 2
Crude protein	398 ± 3	391 ± 3	410 ± 2
Total lipid	206 ± 4	207 ± 12	194 ± 3
Ash	121 ± 1	130 ± 1	124 ± 3
Gross energy (MJ.kg^{-1} DM)	20.9 ± 3.9	20.8 ± 0.6	20.7 ± 0.6

[1]   Vitamin mix (ASV4) to supply per kg feed: 2.81 mg thiamin HCl; 10.0 mg riboflavin; 9.15 mg pyridoxine HCl; 25 mg nicotinic acid; 54.35 calcium D-pantothenate; 750 mg myo-inositol; 0.38 mg d-biotin; 2.5 mg folic acid; 0.03 mg cyanocobalamin; 6250 IU retinol acetate; 2800 IU cholecalciferol; 100 IU DL α-tocopherol acetate; 5 mg menadone sodium bisulphate.

[2]   L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia) , Frenchs Forest, NSW, Australia)

[3]   Mineral mix (TM4) to supply per kg feed: 117 mg CuSO$_4$ 5H$_2$O; 7.19 mg KI; 1815 mg FeSO$_4$ 7H$_2$O; 307 mg MnSO$_4$ H$_2$O; 659 mg ZnSO$_4$ 7H$_2$O; 3.29 mg Na$_2$SeO$_3$; 47.7 mg CoSO$_4$ 7H$_2$O.

[4]   Yttrium oxide was replaced with bentonite outside of the digestibility experiment

Table 10   Lipid class and fatty acid composition of experimental diets

	CO	CTH	CFO
Lipid class composition[1] (g.kg^{-1} DM)			
TAG	199	196	186
PL	4.0	8.3	4.0
Free fatty acid	1.0	1.1	2.0
Cholesterol	0.4	0.6	1.0
Fatty acid composition (% fatty acids)			
14:0	0.50	5.55	4.06
16:0	6.64	12.52	11.90
18:0	2.13	1.58	2.78
Total saturates[2]	10.22	21.28	21.62
16:1n-7	0.44	1.37	4.29
18:1n-7	3.13	2.86	3.15
18:1n-9	54.82	39.42	34.25
20:1n-9	1.20	0.73	1.04
Total monosaturates[3]	60.15	44.28	43.39
18:2n-6	22.19	16.27	11.84
20:4n-6	0.04	0.09	0.61
Total n-6[4]	22.39	18.44	12.71
18:3n-3	6.09	4.38	3.81
18:4n-3	0.45	0.14	1.05
20:5n-3	0.51	0.81	9.58
22:5n-3	0.03	0.20	1.01
22:6n-3	0.65	9.93	4.90
Total n-3[5]	7.83	15.61	21.61
n-3/n-6	0.35	0.85	1.70
20:4n-6/20:5n-3	0.05	0.11	0.06
22:6n-3/20:5n-3	1.28	12.26	0.51

1   calculated from % composition and total lipid

2   includes

3   includes

4   includes

5   includes

1.4.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) parr (36.5 ± 5.7 g) were obtained from Springfield Salmon Hatchery (Tas., Australia) and stocked into 300L tanks at 30 fish per tank. These fish were acclimated for 2 weeks. The tanks were held in a constant environment room

(temperature, 15.7 ± 0.8 °C; photoperiod, 12:12) and part of a partial freshwater recirculation system (Carter & Hauler, 2000). Water was treated through physical and biofilters with a continuous replacement of approximately 20% day^{-1}. Water quality parameters (DO, pH, ammonia, nitrate and nitrite) were monitored to ensure water quality remained well within limits recommended for Atlantic salmon (Wedemeyer, 1996).At the start of the experiment fish were anaesthetised (50 mg L^{-1} Benzocaine) and weight and fork length measured. A group of fifteen fish was used for assessment of initial chemical composition of whole-body and tissues: white muscle, liver, heart and eye (see below). Twenty five fish were returned to each of nine tanks and distributed to ensure there were no significant differences between initial group mean weight for triplicate groups to be fed one of the 3 diets. The fish were re-weighed every 14 days and ration adjusted accordingly. A ration of 1.25 mg g^{-1} initial body weight was supplied twice a day in the morning and afternoon and dispensed over 1 h by automatic belt feeders. The fish were fed in this way each day. Total feed consumption (kg DM) was estimated every fourth day approximately (on 13 days over the experiment) from the amount of feed that was not eaten and was collected from the effluent standpipe. A mean value for feeding efficiency (feed consumed / feed supplied) was calculated for each tank and applied to the total feed fed over the experiment.

The experiment continued for 51 days, at which time the fish had more than doubled in weight. At the end of the experiment the fish were not fed for a day and all individual fish weights were then measured and tank means calculated. Fish were killed by transection of the spinal cord after immersion in anaesthetic and five removed from each tank to determine whole-body chemical composition. A further five fish were removed, dissected to determine liver, heart and eye weights and samples of white muscle, heart, liver and eyes taken for fatty acid analysis. Tissue samples were frozen in liquid nitrogen.

1.4.3.3 Apparent digestibility

Apparent digestibility coefficients were measured during the experiment using all tanks. Faecal samples were collected by settlement (Cho et al., 1982) in settlement collectors attached to the tanks described above (Carter & Hauler, 2000). Groups of salmon were fed the experimental diets containing digestibility markers cholestane and yttrium oxide (10 g kg^{-1}) for 7 days. On days 5,6 and 7 faecal samples were removed from the settlement collector between 1700 to 0900 h, freeze-dried and used in the analysis of the digestibility markers and nutrients (see below). The apparent digestibility coefficients (ADC) were calculated using the standard formula:

ADC (%) = 100 - [100. (% Idiet / %Ifaeces) x (%Nfaeces / %Ndiet)
(Maynard & Loosli, 1969), where I is the inert marker and N the nutrient.

1.4.3.4 Chemical Analysis

Standard methods were used to determine dry matter (freeze dry to constant weight); crude protein (N x 6.25. Kjeldahl using a selenium catalyst); total lipid (Bligh & Dyer, 1959) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid), ash

(AOAC, 1995). Yttrium was analysed using inductively coupled plasma emission spectrometry (ICP-OES) following digestion with nitric acid at 180°C for up to 48 h. Cholestane was determined during the fatty acid analysis (see below).

Representative samples of all diets used in the Atlantic salmon trial were analysed in duplicate. Faeces and tissue samples were collected from each tank and stored at –20°C before analysis. Lipids were extracted from all samples using the modified Bligh and Dyer method described in Section 1.2.3.3. The fatty acid and lipid class content of all samples were analysed as described in Sections 1.2.3.2 and 1.2.3.3 respectively.

1.4.3.5 Immune function and blood chemistry

Whole blood was used for the measurement of neutrophil activity by the reduction of nitroblue tetrazolium (NBT) to formazan, with values presented as the optical density at 540 nm after reduction (Anderson & Siwicki, 1996). Osmotic erythrocyte fragility (OEF) was measured by modifications of those methods described by [Hansen, 1998 #1093] and (Siddall et al., 1995). Briefly, a series of phosphate buffered saline (PBS) solutions were prepared with salt concentrations between 0.15 to 0.90% in 0.05% increments except between 0.4-0.6% where increments were 0.025%. Ten µl of whole blood was placed into wells of round-bottomed microtiter plates, and 190 µl of the PBS series added. Plates were incubated at room temperature for 1 hr, centrifuged (1000 ´ g, 10 min) and supernatants removed and transferred to flat-bottomed microtiter plates. Supernatant absorbance was read at 540 nm. Optical densities (ODobs) were converted to proportional haemolysis values (H) using the equation described by (Siddall et al., 1995): $H_i = ODobs - ODblank / OD0.15$ where OD0.15 is the maximal optical density for that fish at 0.15% PBS, and ODblank is the optical density of 10µl of haemoglobin-free plasma plus 190 µl of the 0.90% PBS. Proportional haemolysis was plotted against PBS concentration, and each curve fitted with the function: $H = (1 + e -\alpha + \beta [PBS])-1$. The logistic positional and rate parameters (α and β, respectively) were used for statistical comparisons of OEF, whilst the value where 50% haemolysis occurred (C50) was used for descriptive purposes only.

Remaining blood not used for neutrophil activity or OEF was centrifuged (1000 x g, 5 min) to obtain plasma. Plasma was used for measurement of lysozyme activity by determining the rate of lysis of a Micrococcus lysodeikticus suspension and using hen egg white lysozyme (Sigma-Aldrich, Castle Hill, NSW, Australia) as standard as described by (Thompson, 1994). The volume of plasma required to inhibit 50% of a standard trypsin activity was used to quantify antiprotease activity (Ellis, 1990). The difference in plasma protein concentration (measured according to (Lowry et al., 1951)) before and after precipitation with polyethylene glycol was considered to be the plasma immunoglobulin fraction (Siwicki et al., 1994). Finally, plasma glucose was measured using a commercial diagnostic kit (Sigma-Aldrich, Castle Hill, NSW, Australia).

1.4.3.6 Statistical analysis

Mean values are reported ± standard error of the mean (S.E.M.). Percentage data were arcsin transformed prior to analysis. Normality and homogeneity of variance were confirmed and comparison between means was by one-way ANOVA. Multiple comparison was by Tukey-Kramer HSD. Statistical analyses were performed using J.M.P. version 3.2.1 (SAS Institute Inc.). Significance was accepted at probabilities of 0.05 or less.

1.4.4 Striped trumpeter feeding trial

1.4.4.1 Rotifer enrichment

The stock rotifer culture was maintained as described in Section 1.4.1.3. Rotifers for use in the trial were removed from the stock culture, washed and kept in clean seawater with no food for > 12 hours prior to enrichment. Seven hours prior to being fed to the larvae, the starved rotifers were divided into three clean, aerated buckets. The rotifers in one bucket was enriched with the 100 mg L^{-1} DHA-Selco (prepared as per manufacturers instructions) and the rotifers in another bucket was enriched with wet cells of ACEM 6063 (2-15 days post harvest, prepared as described in section 1.2.1 (page 6); 100 mg L^{-1} dry weight equivalent). The rotifers in the third bucket were not fed.

One hour prior to feeding to the larvae, the rotifers in the "enrichment" buckets were washed and counted. Washed rotifers were transferred into the appropriate tanks at a rate of 5 rotifers mL^{-1}. Rotifer samples were collected on pre-muffled and weighed glass fibre filters (Whatman GF/F), washed with ammonium formate (5 % w/v) and stored at $-20°C$ prior to analysis.

1.4.4.2 Larval culture

The Striped trumpeter larvae feeding trial was conducted in twelve 200 L hemispherical tanks using a recirculated seawater system (3 diets, 4 replicates). Biofiltration and buffering was achieved using 100 L of pre-conditioned plastic media (Bioballs) and about 2 kg of aged oyster shells. Water temperature was maintained between 16.0 and 17.0°C and between 33 and 35 ppt salinity throughout the trial. Fresh seawater (~ 10% v/v) was exchanged with the recirculated seawater twice each day. Each tank received a flow of 50 L h^{-1}. The tanks were maintained on a 14:10 light:dark cycle, with lights on at 1500hrs and off at 0500hrs.

Final stage yolk-sac larvae were transferred into each tank the day prior to first feeding. Larvae were provided with 5 rotifers mL^{-1} between 1500 hrs and1530 hrs, and again between 2100hrs and 2130 hrs. Rotifers that were washed from the tanks through the outflow were caught in a 63 μm mesh bag to remove them from the recirculating seawater. The collection bag was emptied and cleaned twice each day. Care was taken to keep the surface of the tank water as free from oil as possible. Protein skimmers were placed in each tank. Skimmers, and at times the whole water surface, were cleaned regularly (4 to 5 times

each day) with paper towelling. Particular attention was paid to oil removal in the 1 to 2 hours after feeding. Dead rotifers, faeces, dead larvae and other sediment were siphoned from the tanks daily.

At the end of the trial, larvae used for size and lipid analyses were collected starting at 1500 hrs (i.e. at the end of their "night"). This was to minimise the presence of undigested rotifers in the larvae. Microscopic examination (data not shown) revealed that < 1% of the larvae had a trace of food in their stomachs. All other larvae had empty stomachs. Each tank was carefully siphoned and then stirred vigorously (using a vertical action to avoid concentrating the larvae in the tank centre). Sufficient larvae (> 25) for measuring and lipid analyses were then siphoned from each tank. The remaining larvae were then netted from each tank, and stored in neutral buffered formalin for counting.

Twenty larvae from each tank were measured using light microscopy. Larvae to be used for lipid analyses were carefully washed over a 250 μm screen to separate them from rotifers remaining in the water column. Larvae were then collected on pre-muffled and weighed glass fibre filters (Whatman GF/F), washed with ammonium formate (5 % w/v) and stored at –20°C prior to analysis.

1.4.4.3 Lipid analyses

Freeze-dried rotifers (used as feed for the Striped trumpeter larvae) and larvae were directly transesterified by placing the filter containing the rotifers or larvae into 5 mL of transesterification solution as described in Section 1.2.3.2. The resultant FAME were recovered and analysed also as described in that section.

1.4.4.4 Statistical analyses

Statistical analyses of the Striped trumpeter data were performed using the 1-way ANOVA and Tukey's pair-wise comparison modules in the MS Excel Add-in Analyze-it (Analyse-it Software Limited, UK). Differences between individual means of replicate samples were deemed to be significant at $\alpha = 0.05$.

1.4.5 Prawn feeding trial

Prawns used in the growth assay were obtained as post-larvae (PL15) from a commercial hatchery and reared in 10 tonne tanks at the CSIRO Marine Laboratory, Cleveland until they had grown to about 2 g. At this stage they were sorted by size, transferred to 2.5 t tanks and held at 28 to 30°C until the experiment started.

The experiment consisted of a 6-week growth assay of four dietary treatments, in a randomised balanced design with seven replicates for each treatment. Prawns with an initial mean (± SD) weight of 2.8 ± 0.27 g were used. The diets, except for a reference diet, were all variants of a basal diet, but contained the same concentration of crude protein and total lipid (Table 1). The microheterotroph (ACEM 6063) biomass that was included

in the diet was added at the expense of squid oil and some of the defatted fishmeal and wheat flour. In addition, the MCT diet was supplemented with additional lipid in the form of canola oil. The reference diet was a commercial prawn diet, CP 4004 (Charoen Pokphand, Thailand).

Five prawns were placed into each tank (90 L fibreglass tanks, 0.6 x 0.5x 0.3 m deep) and acclimatised to the experimental conditions for 1 week prior to the start of the experiment, during which time they were fed to excess with the reference diet twice daily. The tanks were supplied with flowing seawater (500 mL min^{-1}) and individual aeration, and were maintained at a mean temperature of 29°C (range: 27.2 to 31.2°C). Prawns were weighed individually at fortnightly intervals throughout the experiment, although the tank was the experimental unit. Losses due to mortality or escape were replaced with a tagged prawn of similar weight to maintain stocking density but the data obtained from these prawns were excluded from the calculation of treatment response. At the end of the trial, all prawns were removed from tanks, weighed and stored at –20°C until analysis.

Prawns were fed twice daily (nominally at 0830 and 1600 h) with the amount of feed give to each tank adjusted daily in response to demand, with the goal of obtaining an estimate of feed intake. At the completion of the experiment, the apparent dry matter (DM) intake of prawns within each tank was calculated and used to determine the apparent feed conversion ratio (FCR).

Diets were prepared by mixing the dry ingredients thoroughly, adding the lipids and mixing further. Sufficient water (a volume equivalent to about 40% of the weight of the ingredients) was added to the diets to form a crumbly dough. The dough was then extruded through a 3 mm die, using the mincer attachment on a commercial food mixer (Hobart Corporation, Ohio, USA). The extruded material was passed through the mincer twice to produce the desired level of mixing and compaction. Following the final extrusion, the spaghetti-like strands were spread onto mesh trays, steamed in an atmospheric steamer for 5 minutes, and air-dried at ambient temperature for 18 hours. The dried strands were broken into pellets of about 8mm in length and stored at -4°C until used.

1.4.5.1 Statistical analyses

Statistical analyses of the prawn data were performed using the 1-way ANOVA and Tukey's pair-wise comparison modules in the MS Excel Add-in Analyze-it (Analyse-it Software Limited, UK). Differences between individual means of replicate samples were deemed to be significant at α= 0.05.

Table 11   Experimental diets prepared for prawn feeding trial.
             Quantities are on an as used basis.

	Diet		
	Basal	MCT [1]	6063 [2]
Ingredient	%	%	%
Fishmeal (defatted)	32.89	32.89	31.25
Crustacean meal	5.00	5.00	5.00
Soybean meal (defat)	15.00	15.00	15.00
Cholesterol	0.10	0.20	0.20
Lecithin	1.00	1.00	1.00
Squid oil	6.17	-	-
ACEM 6063	-	-	6.00
MCT	-	4.06	-
Canola oil	-	2.00	2.56
Flour	30.45	30.46	29.60
Gluten (wheat)	6.00	6.00	6.00
Banox E	0.02	0.02	0.02
Binder 1	3.00	3.00	3.00
Carophyll Pink	0.05	0.05	0.05
Choline Chloride	0.02	0.02	0.02
Vitamin C	0.10	0.10	0.10
Vit premix	0.20	0.20	0.20
Total	100.00	100.00	100.00

Calculated Composition (as used basis)

Dry Matter	90.30	90.30	90.45
Ash	8.78	8.78	8.97
Digest. Energy	13.80	13.80	13.66
Gross Energy	17.38	17.39	17.47
Crude Protein	40.00	40.00	40.00
Digest Protein	35.31	35.31	35.01
Total Lipid	8.50	8.50	8.50
Cholesterol	0.21	0.23	0.23
Phospholipid	1.44	1.44	1.42
Total SFA	1.92	0.63	1.59
Total MUFA	2.11	1.48	1.87
Linoleic acid	0.53	0.82	0.93
Linolenic acid	0.12	0.20	0.23
PUFA n3	2.16	0.18	1.05
PUFA n6	0.14	0.01	0.36
PUFA n3/n6 ratio	3.40	0.46	0.99

1:     MCT diet contained lipids in the form of mid-chain length triacylglycerols instead of the squid oil used in the Basal diet

2:     6063 diet contained biomass of the thraustochytrid strain ACEM 6063 instead of the squid oil used in the Basal diet

1.4.5.2 Lipid analyses

Prawns were prepared for fatty acid extraction and analysis as follows. Prawns were removed from the freezer and allowed to part thaw. All prawns from each individual tank were homogenised using a food blender. About 2 g of well mixed, blended prawn mix from each tank were wrapped in a labelled foil pouch and re-frozen. When all samples had been re-frozen, they were autoclaved for 30 minutes at 121°C. A known weight (about 1.5g, accurate to 0.00g) of each autoclaved sample was then transferred into separatory funnels and the lipids extracted and analysed as described in Sections 1.2.3.2 and 1.2.3.3.

Chapter 2   Results

2.1   Isolation and characterisation of novel Australian thraustochytrids

2.1.1 Fatty acid data

The fatty acid profiles of the newly isolated thraustochytrids and thraustochytrid type strains are shown in Table 12. Strains in Table 12 are ordered in the 10 fatty acid groups formed by the Taxon cluster analysis (described in section 1.1.2.2, page 4). A dendrogram depicting the cluster analysis is shown in Figure 1. Those fatty acids exerting ≥ 10% of the relative influence of all fatty acids on each separation (S1-S9, Figure 1) are shown in Table 13. Variations in the relative proportions (%TFA) of DHA and AA in all strains were the major factors influencing the first separation (S1). Mean levels of DHA and AA in strains grouped in the upper branch of the first separation (S1, Figure 1) were 56 and 0.7 %TFA respectively. Comparative values for strains grouped in the lower branch of S1 were 17 and 8.6 %TFA respectively (Table 13). The mean EPA level of strains in the upper and lower branches of S1 were 5.0 and 12 %TFA respectively.

There was no overlap between the groups of fatty acids exerting ≥ 10% of the relative influence of all fatty acids on separations S3, S5, S8, S9 within the upper branch of S1 in Figure 1 and those exerting similar influence on separations S2, S4, S6, S7 within the lower branch of S1 (Table 13). Separations amongst strains within the upper branch of S1 (S3, S5, S8, S9) were caused by differences in relatively minor (< 10 %TFA) components of the TFA in those strains. The only PUFA substantially influencing separations within the upper branch of S1 (18:4n3 and C_{22} PUFA) were present at very low levels. Mean levels for 18:4n3 in the upper and lower branches of S8 were < 0.1% and 0.3 %TFA respectively, and for the upper and lower branches of S9 were 1.2% and < .1 %TFA respectively. Mean levels for C_{22} PUFA in the upper and lower branches of S5 were 0.9% and < 0.1 %TFA respectively (Table 13).

Thus only small differences in the means of 18:4n3 at S8 and S9 and C_{22} PUFA at S5 influenced the observed separations (Table 13). Similarly, the mean levels of most of the fatty acids exerting substantial influence on the separations within the lower branch of S1 (S3, S4, S6, S9) occurred at < 10 %TFA. Of the fatty acids that exerted substantial influence on the additional separations within the lower branch of S1, only DPAn6 (14 and 0.8 %TFA in the upper and lower branches of S6 respectively) and 16:0 (31 and 18 %TFA in the upper and lower branches of S7 respectively) occurred at mean levels of > 10 %TFA. Several PUFA substantially influenced one or more of the additional separations (S2, S4, S6, S7) within the lower branch of S1 (Table 13). However, apart from DPAn6, all these PUFA were relatively minor (i.e. < 10 %) components of the TFA in these groups.

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Table 12 — Fatty acid composition (% of total fatty acids [1]) of thraustochytrid strains

Fatty acid / Strain[2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C22 PUFA
Fatty acid group 1 [3]																											
6063	0.2	9.6	2.2		3.2		0.2	27.8	0.5			0.1	7.3	0.8	1.0	2.0	0.1	0.4					5.2	38.1	0.1	0.7	
ATCC 26185	0.1	5.3	3.1		1.2		0.2	23.6	1.0	0.1		0.2	5.5	0.6	1.6	3.1	0.2	0.4					9.2	41.9	0.3	0.9	
Fatty acid group 2																											
0007		1.8					2.4	21.5									3.2						1.3	67.2		0.7	1.9
0011		1.7					2.8	20.4									4.1						5.5	65.6			
0012		1.6	0.4				2.6	22.1									2.5						1.1	67.8		0.7	1.3
0013		2.5	0.4				2.9	26.3									1.7						1.1	63.7		0.7	0.8
0019		0.9	0.6			1.5	1.6	21.0							0.6	0.3	0.5						16.8	53.4	0.5	1.5	0.7
Fatty acid group 3																											
0020		2.1						21.8		0.5	0.4	0.2	1.1	0.5	4.5	0.3	0.5						2.7	64.1		1.4	
0021		2.8						25.8		0.8	0.8		1.4	0.5	4.0	0.4	0.3						2.1	60.3		0.8	
6000	0.4	3.7			0.2		0.1	25.7		0.4	0.3	0.4	0.6	0.3	4.4	0.2	0.6					0.1	1.9	59.4	0.2	0.7	
6001	0.4	3.9			0.1		0.2	29.6	0.2	0.6	0.7	0.3	1.0	0.4	3.7	0.3	0.4					0.1	2.1	54.5	0.3	1.0	
6002	0.5	4.1			0.3			26.7	0.2	0.7	0.6	0.5	1.0	0.4	5.6	0.2	0.4					0.1	1.6	55.6	0.3	0.9	
6003	0.5	3.9		0.1	0.2		0.2	29.5	0.7	3.2	2.9	0.2	3.4	1.0	7.6	1.3	0.6	0.2		0.2		0.2	1.2	41.4	0.3	1.0	
6004	0.5	4.4			0.2		0.2	26.6	0.2	0.9	0.9	0.4	1.2	0.5	6.0	0.3	0.5					0.1	1.3	54.6	0.1	0.6	
6005	0.3	3.5	0.3					25.7		0.2	0.2		0.7	0.3	3.7	0.6	0.4					0.1	2.3	60.1	0.4	0.7	
6006	0.5	4.5	0.1		0.2		0.1	32.5	0.3	0.8	1.0	0.2	1.8	0.6	5.8	0.3	0.6					0.1	1.3	48.0	0.2	0.6	

Table 12 (continued)

Fatty acid Strain[2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C22 PUFA
6007	0.5	4.3	0.1		0.2			31.7	0.3			1.3	1.2	0.2	2.3	0.6	5.6	0.5	0.5			0.2	1.5	47.5	0.3	0.8	
6008	0.4	3.7	0.2		0.2			24.1	0.1			0.3	0.3	0.3	0.6	0.3	4.5	0.3	0.5				2.0	60.5	0.3	0.9	
6009	0.3	3.6	0.1		0.2			30.3	0.2			0.7	1.1		1.0	0.4	4.4	0.4	0.6			0.2	1.4	53.7	0.3	0.7	
6010	0.4	3.5	0.1				0.1	28.3	0.1			0.6	0.6	0.2	1.5	0.4	5.7	0.2	0.5			0.1	1.2	54.5	0.2	0.9	
6011	0.5	4.2			0.1			25.1	0.2			0.5	0.4	0.5	0.7	0.3	6.3	0.2	0.4			0.1	1.3	57.7	0.2	0.8	
6012	0.5	3.3			0.3			21.5	0.2			0.9	0.5	0.7	0.6	0.4	8.6	0.2	0.5				1.2	59.3	0.2	1.0	
6013	0.5	3.2	0.1		0.3			21.4	0.2			0.9	0.5	0.7	0.6	0.5	8.6	0.4	0.5				1.2	59.0	0.2	1.0	
6016	0.4	3.4	0.1		0.2		0.1	27.4	0.4			1.2	1.1	0.3	1.6	0.7	5.3	0.8	0.7			0.1	1.9	52.7	0.3	1.0	
6019	0.3	3.4			0.2		0.3	28.7	0.1			0.3	0.2	0.3	0.6	0.2	3.1	0.2	0.5			0.1	1.6	58.5	0.1	0.7	
6024	0.4	3.6			0.2		0.3	25.8	0.1			0.4	0.3	0.4	0.6	0.3	4.1	0.2	0.6			0.1	1.4	60.1		0.6	
6025	0.4	3.6	0.1		0.2		0.2	26.2	0.2			0.6	0.3	0.3	0.8	0.4	4.6	0.4	0.7				1.5	58.1		0.9	
6026	0.4	3.6		0.1	0.2		0.3	25.6	0.3			1.4	0.8	0.4	1.1	0.7	6.9	0.8	0.7			0.2	1.3	53.8	0.1	0.8	
6027	0.4	3.0			0.1			21.9				0.3	0.2	0.7	0.5	0.2	5.9		0.4				1.3	63.7		0.9	
6028	0.4	2.7					0.3	22.0				0.3	0.2	0.4	0.4	0.3	4.5	0.1	0.6				1.7	64.3		1.0	
6030	0.4	3.2	0.1		0.1		0.3	27.2	0.1			0.6	0.5	0.3	0.7	0.4	4.1	0.3	0.5			0.1	1.4	58.6		0.6	
6031	0.4	3.3					0.5	25.8	0.1			0.4	0.3	0.3	0.8	0.3	3.9	0.2	0.5			0.1	1.5	60.0		1.2	
6033	0.5	3.2			0.2		0.3	22.6	0.2			0.9	0.6	0.4	0.9	0.6	7.8	0.5	0.7				1.0	58.0		1.0	
6034	0.4	3.6			0.2		0.5	25.6	0.2			0.8	0.5	0.3	1.1	0.4	4.2	0.3	0.5			0.1	1.3	59.1		0.6	
6035	0.4	4.3			0.1		0.8	28.8	0.2			0.5	0.4	0.3	0.9	0.3	3.5	0.1	0.3			0.2	1.3	56.6		0.6	
6036	0.7	4.6		0.1	0.3		0.7	25.3	0.5			1.5	1.1	0.3	1.4	0.8	7.3	0.5	0.5			0.2	1.0	51.7		1.1	
6037	0.5	4.3			0.1		0.5	31.3	0.2			0.7	0.8	0.2	1.3	0.3	4.2	0.2	0.3			0.2	1.3	52.2	0.1	0.8	
6038	0.4	3.6			0.1		0.5	26.1	0.2			1.2	0.7	0.3	1.4	0.5	4.6	0.2	0.4			0.2	1.1	57.1		0.7	
6039	0.4	3.4					0.2	26.3	0.1			0.5	0.4	0.3	0.9	0.3	4.9	0.2	0.5			0.1	1.3	58.9	0.1	0.8	

Table 12 (continued)

Fatty acid / Strain[2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C22 PUFA
6041	0.4	4.3			0.2		0.5	30.7		0.2		0.7	0.6	0.3	1.3	0.3	3.6	0.3	0.5			0.2	1.4	53.3		0.7	
6042	0.5	4.4	0.1				0.4	27.8		0.1		0.3	0.3	0.1	0.7	0.3	3.2	0.2	0.4				1.4	58.7		0.8	
6043	0.5	4.2			0.2		0.4	25.7		0.2		0.6	0.5	0.3	0.8	0.3	4.6	0.2	0.5			0.1	1.0	58.5	0.1	0.7	
6045	0.4	3.1	0.1		0.1		0.1	20.1		0.2		0.9	0.4	0.5	0.6	0.5	7.0	0.2	0.4				1.6	62.4	0.2	0.9	
6046	0.5	4.5			0.2		0.2	31.1		0.5		1.2	1.1	0.4	1.5	0.6	6.3	0.5	0.5			0.2	1.2	48.3	0.2	0.8	
6047	0.3	3.0			0.1			24.1		0.1		0.3	0.3	0.4	0.6	0.2	4.4	0.1	0.4				2.0	62.2	0.1	0.7	
6048	0.3	3.4			0.1		0.5	26.4		0.1		0.3	0.3	0.3	0.7	0.2	3.4	0.2	0.5			0.1	1.6	60.4		0.7	
Fatty acid group 4																											
6040	0.5	3.2	0.2		0.3	0.1	0.6	20.2		0.2	1.2	0.4	1.0	1.6	1.0	0.4	5.4	0.1	0.4	0.9			1.3	60.0	0.1	0.8	
Fatty acid group 5																											
6050		1.9	1.5	0.1	1.1		0.3	24.0	0.5			1.0	3.3	0.3	2.6	6.5	12.4	2.4	1.5	2.6		0.2	4.0	29.2	1.6	3.0	
ATCC 28209		1.2	1.1		0.5		0.1	23.6	0.3	0.1		0.2	1.3	1.1	0.2	2.3	5.5	1.0	0.3	0.1			5.2	54.2	0.5	0.9	
ATCC 28210		1.1	0.7		0.2	0.1		24.2	0.2	0.2	0.1	0.4	0.9	0.8	0.6	3.3	6.3	0.6	0.3				7.0	51.2	0.7	0.9	
ATCC 34304		1.3	0.4		0.3			25.2	0.2	0.1	0.2	0.2	0.4	0.7	1.1	3.4	7.8	0.4	0.5				7.0	49.3	0.5	0.8	
Fatty acid group 6																											
6029		0.7	0.1	6.8	0.4		0.1	17.6	0.2		0.1	1.6	9.0	0.2	0.6	3.4	15.3	1.3	2.2	8.5	2.3		0.3	24.4	0.2	4.0	0.4
6032		0.7	0.4	8.0	0.4		0.1	16.0	0.3		0.1	1.3	12.9	0.2	0.5	1.6	15.4	1.0	3.2	7.1	4.5		0.1	22.6		3.3	0.2
Fatty acid group 7																											
0005		1.1	0.4	0.3	1.4			21.5		1.3		6.7	22.4	1.2	4.0	3.0	5.6	0.7		0.6			3.2	21.5	3.2	1.5	0.5
0006		2.9	0.3					22.8		0.5		4.5	10.5	1.4	3.1	9.1	5.5	1.3	0.2	1.2			20.2	10.7	4.3	0.7	0.7

Results

Table 12 (continued)

Fatty acid Strain[2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
0017		1.5		0.4				25.5	0.3			1.4	15.5	1.4	5.1	5.9	5.9	0.9	0.2	0.5	0.5	0.2	15.8	14.1	3.7	1.2	
6018		0.6			0.3			33.4	0.5		0.3	2.2	11.3	1.5	4.0	3.6	5.0	0.8	0.1	0.4	0.1	0.1	17.0	16.4	1.5	0.4	
6020		1.2		0.6	0.1			21.8	0.5		0.3	0.8	9.4	1.4	4.2	5.4	9.6	0.5	0.3	0.1	0.1		20.9	18.7	3.0	1.0	
6049	0.1	3.1		0.2	0.3		0.5	32.0	0.9		0.3	10.3	12.7	1.3	3.6	3.1	3.8	5.6	0.6	0.7		0.4	6.5	9.9	3.3	0.8	
Fatty acid group 8																											
0008		2.4	0.5	0.7	0.6	1.6		15.5				1.5	0.9	0.3	0.5	12.1	17.6	3.0	0.7	1.2			17.8	20.8	0.3	2.1	
0009		3.2	0.5	0.3	0.5	3.2		25.6				0.8	0.7	0.4	1.5	7.2	17.4	1.8	0.7	1.0			13.9	19.0	0.4	2.1	
0014		2.2	0.4	0.8	0.7	1.3		16.2				2.4	2.8	0.3	0.8	14.7	13.1	3.7	0.7	1.4			14.4	20.7	0.5	3.1	
0015		2.3	0.4	0.4	0.7	1.3		15.6				1.4	1.7		1.1	9.9	16.6	2.7	0.9	1.2			13.8	27.2	0.7	2.1	
0016		2.7	0.5	0.4	0.9	1.4		19.4				1.2	3.6	0.3	2.6	10.1	10.8	2.6	0.6	0.9			15.3	21.7	0.6	4.5	
6014	0.2	3.8	0.5	0.5	0.9			23.4				1.7	7.0	0.1	2.7	7.0	12.2	1.4	1.2	0.9	0.4	0.1	11.5	20.4	0.4	3.2	
6015	0.2	3.9	0.4	0.5	0.6			20.0	0.2			1.8	1.4	0.1	1.1	12.4	13.9	1.5	0.8	0.7	0.1		10.6	26.6	0.4	2.2	
6017		3.9	0.6	0.6	0.7		0.2	17.7	0.1	0.1		2.7	1.7	0.2	0.7	12.4	16.8	1.9	1.1	1.0	0.2		12.8	20.6	0.4	2.9	
6021		3.6	0.5	0.5	0.5			19.7	0.2			1.6	1.1		0.7	12.6	19.8	1.5	0.9	0.8			12.0	20.9	0.4	2.3	
6023		3.4	0.7	0.5	0.5			17.3	0.2			2.1	0.7	0.1	0.8	15.5	14.2	2.3	1.1	1.1			15.3	21.6	0.4	1.8	
6044		3.2	0.6	0.3	0.4		0.1	20.6	0.2			1.1	0.6	0.2	0.8	11.0	17.3	1.6	1.1	1.1			13.6	23.3	0.3	2.2	
Fatty acid group 9																											
000A		4.1			1.9			26.4				4.0	4.9		8.7	5.8	17.0	2.8	1.8	3.9			0.3	9.5	0.7	8.1	
0001		4.5			3.3			30.1				2.3	5.4		8.6	6.6	13.5	1.7	0.4	3.9			0.3	11.0	0.9	7.4	
0002		4.7			3.5			28.6				1.3	3.7		8.2	5.2	13.9	1.8	0.6	3.7			0.3	13.0	0.9	10.6	
0003		5.1			6.3			23.5				1.2	5.4		5.2	11.3	15.3	1.2	0.2	2.4			0.4	12.8	1.4	8.4	
6022	0.3	7.5	0.1		4.2	0.2		46.3				3.3	3.0	0.3	6.8	13.6	0.6	1.1		2.1		0.2	4.1	3.4	2.2	0.4	

Table 12 (continued)

Fatty acid / Strain[2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C22 PUFA
Fatty acid group 10																											
0004				3.1				14.5			2.1	43.0		5.9		15.5	5.3	3.0	0.2	5.5	1.9						
0010		11.4						16.9			0.8	25.3	0.6		2.4	1.2	9.2	1.1	1.0	1.8	2.1	0.3		20.6	0.3	4.9	
0018		1.2			0.2			21.1			2.0	3.1	4.7			13.1	13.2	3.7	0.6	9.7	0.3		1.1	22.8	1.3	1.9	

1: Only components comprising ≥ 1% of total fatty acids are shown

2: Strains ATCC 26185, 28209, 28210 and 34304 were obtained from the American Type Culture collection as *Thraustochytrium* sp., *Schizochytrium aggregatum* Goldstein et Belsky, *Thraustochytrium roseum* Goldstein and *Thraustochytrium aureum* Goldstein, respectively. All other strains were isolated during the course of this study.

3: Group numbers are those shown in Figure 1.

```
GP 1
*************************
                        *
                        *
GP 2                    *S3
********************  **********************************************************
                 *    *                                                      *
                 *    *                                                      *
    GP 3         *S5 *                                                       *
    ******       *****                                                       *
         *          *                                                       *
         *S8        *                                                       *
GP 4     *************                                                       *
*********  *                                                                 *
        *  *                                                                 *
        ***                                                                  *S1
    GP 5 *S9                                                                 *
    ******                                                                   *
                                                                            *
                                                                            *
GP 6                                                                        *
*****************************                                                *
                          *                                                 *
                          *                                                 *
    GP 7                  *S2                                               *
    *****************  *********************************************************
                  *   *
                  *   *
GP 8              *S4 *
*****************  ******
               *     *
               *S6  *
    GP 9       *****
    ******        *
          * S7    *
          **********
        GP10*
        *****

|                   |                   |                   |
0.00                4.00                8.00                12.00
                      Dissimilarity index
```
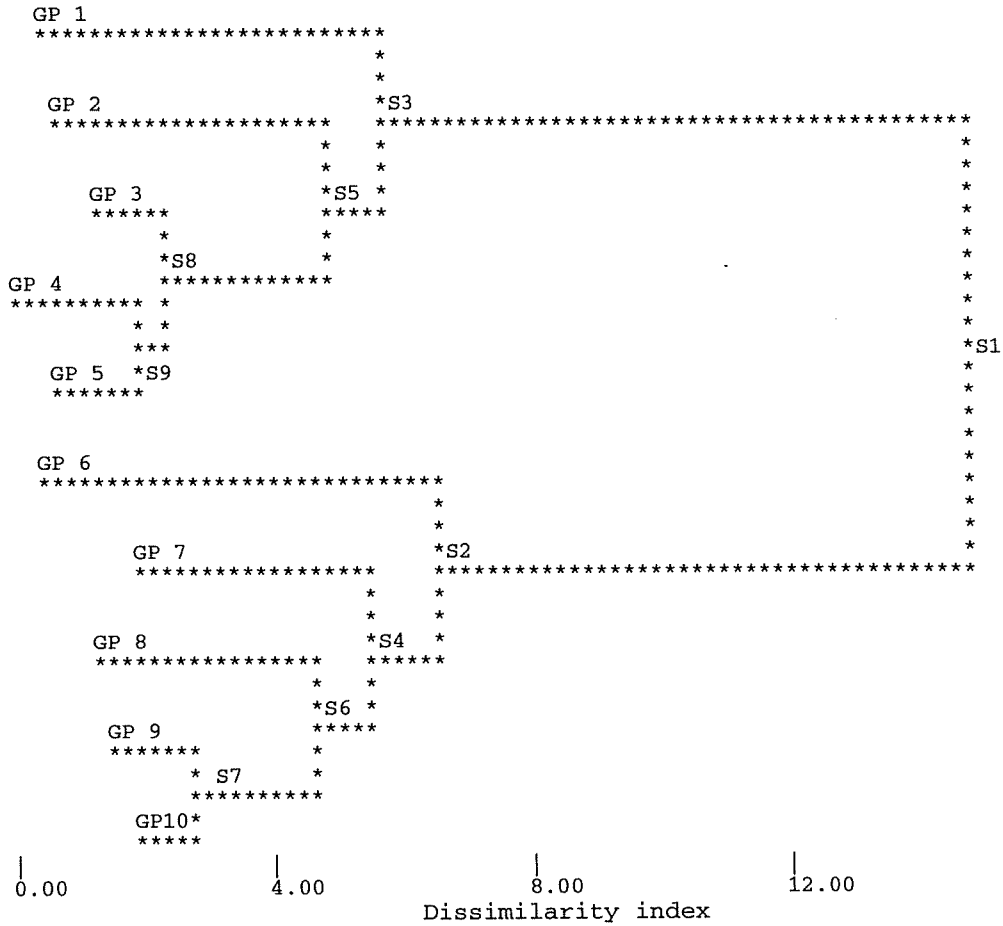

Figure 1   New Australian thraustochytrids and type strains clustered into 10
            groups based on incremental sum of squares sorting of a standard
            euclidean distance dissimilarity matrix of fatty acid profiles (created
            using TAXON v 1.0 beta)

            Separations (S1 – S9) are numbered according to decreasing dissimilarity index;
            see Table 12 for information regarding fatty acid profiles for strains in fatty acid
            groups 1-10; and Table 13 for information regarding which fatty acids
            substantially influenced separations

Table 13   Fatty acids that exerted substantial [a] influence on separations S1 - S9 in Figure 1

Separation	Fatty acid	Relative contribution to each separation (%)	Mean level in upper [b] branch (%TFA)	Mean level in lower [b] branch (%TFA)
S1	22:6n3	15	56	17
	20:4n6	11	0.7	8.6
Upper branch of S1 in Figure 1				
S3	18:1n7	30	6.4	0.4
	15:0	29	2.7	0.1
	17:0	26	0.8	0.1
S5	16:1n5	41	2.5	0.2
	C_{22} PUFA	30	0.9	<0.1
S8	18:4n3	26	<0.1	0.3
	17:0	17	<0.1	0.2
	15:0	15	<0.1	0.8
	12:0	13	0.4	0.1
S9	18:4n3	75	1.2	0.1
Lower branch of S1 in Figure 1				
S2	16:1n9	33	7.4	0.3
	20:1n9+11	22	3.4	0.2
	20:4n3	17	2.7	0.6
S4	18:3n6	22	0.7	0.1
	22:4n6	21	3.2	0.7
S6	18:0	17	1.2	5.7
	22:5n6	16	14	0.8
	16:1n7	13	0.7	2.8
S7	18:1n9	18	4.5	24
	16:0	16	31	18
	16:1n7	14	3.8	1.1
	22:5n3	13	7.0	2.3
	18:0	13	7.5	2.8
	20:1n9+11	11	<0.1	1.4

a: fatty acids which each contributed to ≥ 10% of the total difference at each separation

b: "upper" and "lower" refer to the relative positions of the two branches of each separation in Figure 1

2.1.2 18S rDNA data

A phylogenetic tree generated from 18S rDNA sequence data from this study (15 Australian strains and a sample of Algamac-2000), and 18 thraustochytrid strains (Honda *et al.*, 1999) and 1 thraustochytrid clone (Lopez-Garcia *et al.*, 2001) from the literature is shown in Figure 2. Sequences for two additional Australian strains - ACEM 6003 and ACEM 6030 - were omitted as they contained many unresolved base positions, therefore distorting the final tree. The partial sequences for these two strains (data not shown), grouped them within Cluster A (Figure 2).

The 15 newly isolated Australian strains from which 18S rDNA was extracted were distributed across most of the thraustochytrid phylogenetic tree (Figure 2). The majority (9) of the Australian strains examined were grouped in Cluster A (Figure 2). The nearest known relative of Cluster A was *Thraustochytrium kinnei* KMPB 1694b. Strain ACEM 0005 clustered nearest to *Japonochytrium* sp. ATCC 28207. Strains ACEM 6018 and 6063 clustered closely with both *Schizochytrium limacinum* SR21 and the *Schizochytrium* sp. used in the production of Algamac-2000. Strain ACEM 6032 clustered closest with *Schizochytrium minutum* KMPB N-BA-27. Strains ACEM 000A and ACEM 0004 clustered closely with each other, but were widely separated from the other strains contained in this tree (Figure 2).
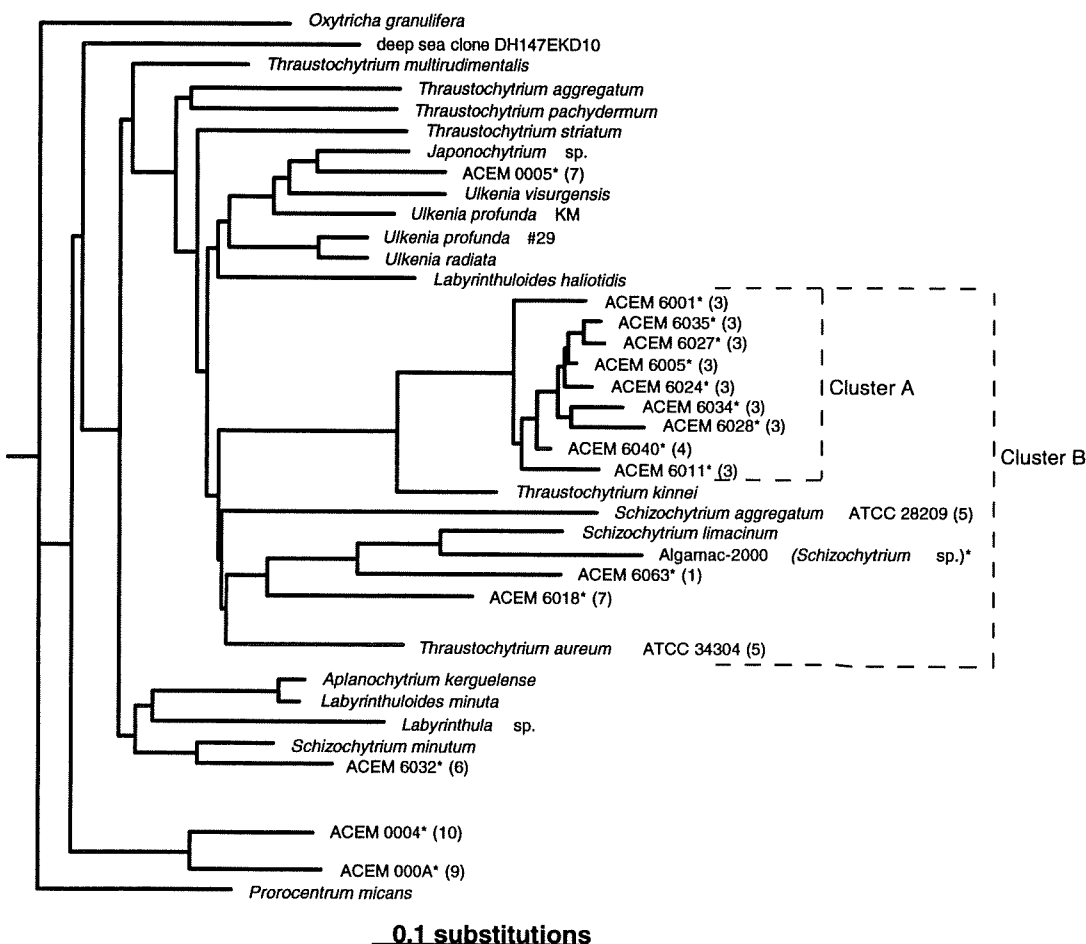
Oxytricha granulifera
deep sea clone DH147EKD10
Thraustochytrium multirudimentalis
Thraustochytrium aggregatum
Thraustochytrium pachydermum
Thraustochytrium striatum
Japonochytrium sp.
ACEM 0005* (7)
Ulkenia visurgensis
Ulkenia profunda KM
Ulkenia profunda #29
Ulkenia radiata
Labyrinthuloides haliotidis
ACEM 6001* (3)
ACEM 6035* (3)
ACEM 6027* (3)
ACEM 6005* (3)
ACEM 6024* (3)
ACEM 6034* (3)
ACEM 6028* (3)
ACEM 6040* (4)
ACEM 6011* (3)
Thraustochytrium kinnei
Schizochytrium aggregatum   ATCC 28209 (5)
Schizochytrium limacinum
Algamac-2000   (Schizochytrium   sp.)*
ACEM 6063* (1)
ACEM 6018* (7)
Thraustochytrium aureum   ATCC 34304 (5)
Aplanochytrium kerguelense
Labyrinthuloides minuta
Labyrinthula sp.
Schizochytrium minutum
ACEM 6032* (6)
ACEM 0004* (10)
ACEM 000A* (9)
Prorocentrum micans

Cluster A

Cluster B

0.1 substitutions

Figure 2   Thraustochytrid 18S rDNA tree, with *Prorocentrum micans* and *Oxytricha granulifera* used as outgroups. Evolutionary distances were determined using a maximum likelihood algorithm. The tree was constructed using a neighbour-joining strategy (created using PHYLIP v 3.5c)

Notes:
- asterisks indicate strains for which 18S rDNA sequences were determined for this study
- numbers in brackets indicate the groups to which strains were assigned, based on fatty acid profiles (Figure 1, Table 12)
- scale bar = maximum likelihood distance for 0.1 substitutions / 100 nucleotides

2.2 Biomass and lipid production by thraustochytrid strain ACEM 6063: influence of physical and chemical parameters

Profiles of the major fatty acid components of ACEM 6063 oil have been provided in Section 2.1.1 (page 27) and Appendix 3:   Publications – page 125). Profiles were largely unchanged by the culture conditions described in this Chapter. Hence, the fatty acid data presented is the proportions of SFA, MUFA, PUFA and DHA (%TFA), and the total fatty acid content (mg g^{-1} dry weight) of ACEM 6063 cultures.

2.2.1 Flask culture experiments

2.2.1.1 Flask Experiment 1: Initial glucose concentration and culture age

Maximum initial growth rates of flask-cultured ACEM 6063 were very similar to each other when the initial glucose concentration was 10, 50, 100 or 150 g L^{-1} ($\sim$2 g L^{-1} d^{-1}), while that of the culture containing an initial glucose concentration of 200 gL^{-1} was slightly lower ($\sim$1.7 g L^{-1} d^{-1}) (Figure 3).
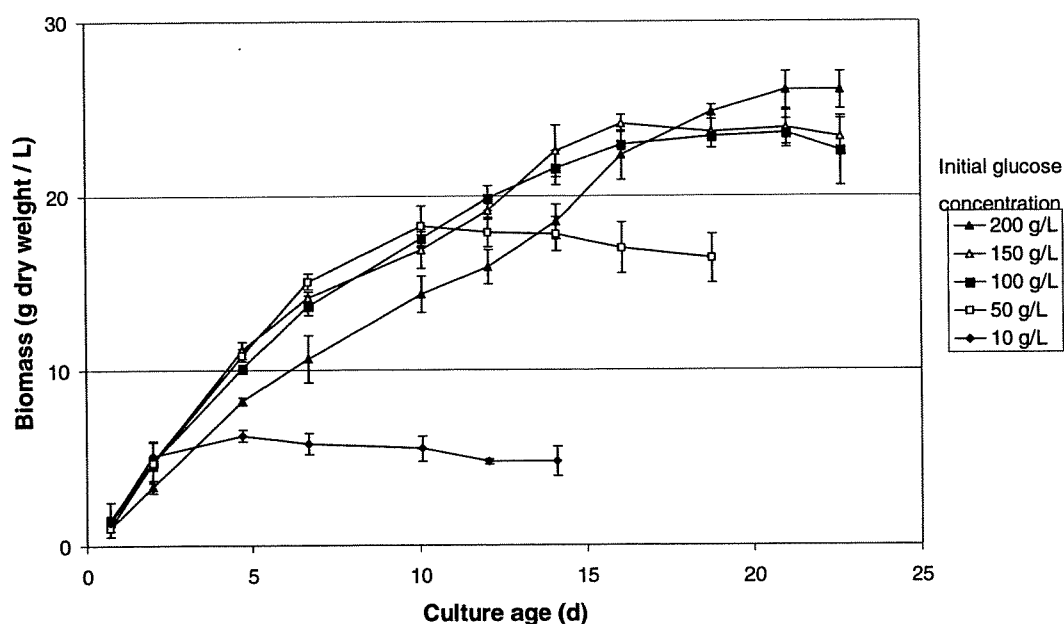
Figure 3   Biomass production by flask-cultured ACEM 6063 grown in TCM broth in response to variations in initial glucose concentration (mean±SD: n=2)

Initial glucose concentration substantially influenced the peak biomass obtained by the flask cultures (Figure 3). Mean (n=2) peak biomasses for cultures in which the initial glucose concentration was 10, 50, 100, 150 and 200 g L^{-1} were 6, 18, 23, 24 and 26 g dry weight L^{-1} respectively. The mean (n=2) age of these cultures when they reached peak biomass were 5, 10, 16, 16 and 21 days respectively (Figure 3). There was little variation in either biomass or fatty acid content between duplicate flasks.

The relative proportions of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in flask-cultured ACEM 6063 cells at peak biomass were not substantially influenced by initial glucose concentration (Table 14). The proportions of these fatty acid classes in ACEM 6063 biomass were, however, influenced by culture age (Table 14).

Table 14   Fatty acid content and proportion of fatty acid classes and DHA of duplicate flask-cultured ACEM 6063 as influenced by initial glucose concentration and culture age

Initial glucose concentration (g L^{-1})	Culture age (d)	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)
		sum SFA	sum MUFA	sum PUFA	DHA	
10	2	24, 26	2.1, 2.2	68, 70	59, 63	72, 78
	5*	36, 41	1.7, 2.0	57, 60	38, 42	87, 93
	10	38, 44	1.4, 1.6	54, 59	35, 39	81, 89
50	2	24, 28	3.7, 4.9	66, 70	55, 57	82, 88
	5	32, 38	0.5, 1.3	57, 61	40, 43	118, 136
	10*	42, 46	0.8, 1.7	51, 53	34, 36	225, 241
	14	43, 47	1.5, 2.0	50, 53	32, 36	272, 283
100	2	23, 28	1.3, 1.4	58, 66	53, 57	102, 117
	5	43, 47	0.7, 1.1	52, 57	35, 39	268, 284
	12*	39, 44	1.5, 2.0	54, 60	34, 39	366, 382
	16	43, 47	1.0, 1.8	51, 56	29, 33	407, 424
150	2	25, 33	1.1, 2.2	66, 71	56, 65	78, 92
	5	39, 48	1.9, 2.3	46, 58	34, 46	217, 233
	16*	36, 45	1.3, 2.1	48, 59	26, 36	379, 400
	19	40, 49	1.1, 2.0	47, 53	22, 34	393, 409
200	2	28, 39	0.6, 1.0	58, 72	54, 62	83, 95
	5	41, 52	0.1, 0.9	43, 53	37, 46	255, 276
	21*	39, 48	0.8, 1.4	47, 57	27, 36	386, 404

*:   day on which peak biomass was reached

1:    unidentified fatty acids have not been included

The proportion of SFA increased from 25-33% of the total fatty acids (TFA) in 2-day old cultures, to 38-44 %TFA on the day each culture reached peak biomass. The proportion of MUFA was very low in all samples, and varied between 0.5 and 4.2 %TFA. There was no apparent correlation between the proportion of MUFA in the samples and culture age (Table 14). The relative proportions of PUFA and DHA decreased with culture age, but did not appear to be influenced by initial glucose concentration. PUFA levels decreased from 62-69 %TFA in 2-day old cultures to 52-58 %TFA at peak biomass. Similarly, DHA levels decreased from 55-61 %TFA in 2-day old cultures to 31-40 %TFA at peak biomass. Higher levels of DHA at peak biomass were seen in those cultures that reached peak biomass earlier (Table 14). Fatty acid content of ACEM 6063 biomass was influenced by both culture age and initial glucose concentration. The TFA content of biomass increased with culture age, ranging from 75-109 mg g^{-1} dry weight in 2-day old cultures to 90-417 mg g^{-1} dry weight at or just after reaching peak biomass. Fatty acid content at peak biomass increased as initial glucose concentrations increased from 10-100 g L^{-1}, with the maximum of 417 mg g^{-1} occuring at 100 g L^{-1}. The TFA content then plateaued, with no further increase observed at initial glucose concentrations of 150 and 200 g L^{-1} (Table 14).

2.2.1.2 Flask Experiment 2: Sea salt concentration

Maximum growth rates of flask-cultured ACEM 6063 were very similar to each other (~ 2 g L^{-1} d^{-1}) when the sea salt concentration in the culture medium was 4, 8, 16 and 32 g L^{-1} (Figure 4). Sea salt concentration, however, clearly influenced the peak biomass obtained by these cultures. Peak biomass achieved by cultures in which the sea salt concentration was 4, 8, 16 and 32 g L^{-1} were 4, 12, 18 and 23 g dry weight L^{-1} respectively. The age of these cultures when they reached peak biomass were 2, 6, 12, and 14 days respectively (Figure 4).

The proportion of SFA in cultures at peak biomass tended to increase (32-49 %TFA) with increasing sea salt concentration, while the proportions of PUFA and DHA tended to decrease (64-44 %TFA and 41-29 %TFA respectively) (Table 15).

The fatty acid content of ACEM 6063 biomass at the time each culture reached peak biomass also increased with increasing sea salt concentration (85-341 mg g^{-1} dry weight) (Table 15). These trends are consistent with those observed in relation to culture age in Flask Experiment 1.
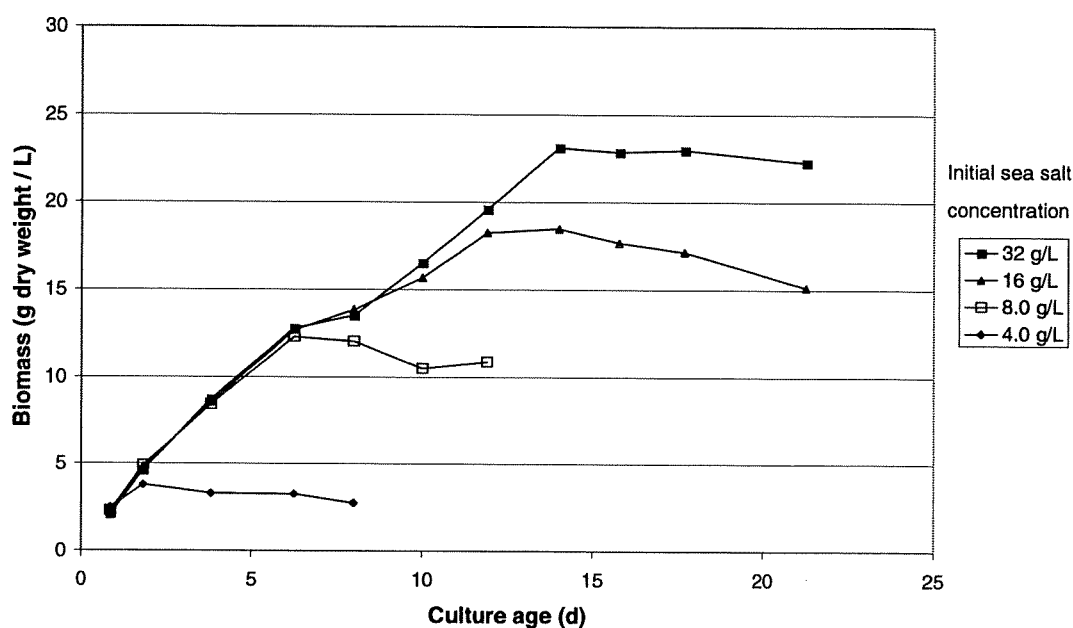
Figure 4   Biomass production by flask-cultured ACEM 6063 grown in TCM
              broth in response to variations in initial sea salt (S9883: Sigma,
              USA) concentration

Table 15   Fatty acid content, proportion of fatty acid classes and DHA, and
              biomass at peak biomass in flask-cultured ACEM 6063 in response
              to different sea salt concentrations

Initial sea salt [1] concentration $(g\ L^{-1})$	Culture age (d)	Fatty acids (% total fatty acids [2])				Total fatty acids $(mg\ g^{-1}$ dry weight)
		sum SFA	sum MUFA	sum PUFA	DHA	
4	2	32	2.0	64	41	85
8	6	44	0.5	52	30	237
16	12	38	8.0	52	31	317
32	14	49	2.9	44	29	341

1:     S9883: Sigma, USA
2:     unidentified fatty acids have not been included

2.2.1.3 Flask Experiment 3: Glucose and sodium glutamate concentration

The relative proportions of SFA, MUFA, PUFA and DHA (%TFA), the fatty acid content (mg g^{-1} dry weight), and the absolute biomass (g dry weight L^{-1}) in 10-day old flask cultures of ACEM 6063, grown with different initial concentrations of glucose and sodium glutamate, are shown in Table 16. Initial glucose concentration had a slight, but statistically significant, effect on the relative proportions of SFA and MUFA in the biomass. The influence of initial glucose concentration on the absolute biomass of these 10-day old flask cultures was also significant (Table 17). There was, however, no obvious trend in the relationship between SFA levels and initial glucose concentration. The relative proportion of MUFA in cultures that received an initial glucose concentration of 200 g L^{-1} was significantly higher than in the other cultures (3.3 %TFA c.f. ~1.5 %TFA). The absolute biomass in 10-day old cultures grown with an initial glucose concentration of 200 g L^{-1} was significantly lower than in the other cultures (9.0 g dry weight L^{-1} c.f. ~15 g dry weight L^{-1}) (Table 17). Statistical analysis detected no significant interaction between the effects of initial glucose and sodium glutamate concentrations on any of the production parameters shown in Table 17.

Initial sodium glutamate concentration had a slight, but statistically significant, effect on the relative proportions of SFA, MUFA, PUFA and DHA in ACEM 6063 biomass. Initial sodium glutamate concentration also influenced the absolute fatty acid content of the biomass and absolute biomass in each flask (Table 18). The relative proportion of SFA in the biomass in 10-day old cultures with an initial sodium glutamate concentration of 1 g L^{-1} (60 %TFA) was higher than in those with an initial sodium glutamate concentration of 5, 10 and 50 g L^{-1} (51-56 %TFA). The proportion of MUFA in biomass varied between 1.3 and 2.3 %TFA, but with no obvious trend in relation to initial sodium glutamate concentration.

PUFA levels did not vary greatly between treatments, but were significantly higher in 10-day old cultures with an initial sodium glutamate concentration of 5 and 50 g L^{-1} (41 %TFA) than in cultures with an initial sodium glutamate concentration of 1 and 10 g L^{-1} (35 and 38 %TFA, respectively). Similarly, DHA levels did not vary greatly, but were slightly higher in cultures with initial sodium glutamate concentrations of 5 and 10 g L^{-1} (~ 25 %TFA respectively) than in cultures with initial sodium glutamate concentrations of 1 and 50 g L^{-1} (21 and 23 %TFA, respectively). Fatty acid content of biomass of 10-day old ACEM 6063 cultures decreased from 232 mg g^{-1} dry weight in cultures with an initial sodium glutamate concentration of 1 g L^{-1}, to 119 mg g^{-1} weight in cultures with an initial sodium glutamate concentration of 50 g L^{-1}. Total biomass increased from 12 g dry weight L^{-1} in cultures with an initial sodium glutamate concentration of 1 g L^{-1}, to 17 g L^{-1} in cultures with an initial sodium glutamate concentration of 10 g L^{-1}, and decreased again to 11 g L^{-1} in cultures with an initial sodium glutamate concentration of 50 g L^{-1} (Table 18).

Table 16   Fatty acid content, proportion of fatty acid classes and DHA, and biomass of duplicate 10-day old flask cultures of ACEM 6063 in response to different initial glucose and sodium glutamate concentrations

Initial glucose (g L^{-1})	Initial sodium glutamate (g L^{-1})	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
		sum SFA	sum MUFA	sum PUFA	DHA		
50	1	59, 63	1.6, 1.7	33, 34	20, 21	189, 225	11, 14
50	5	51, 53	1.2, 1.4	42, 44	25, 27	213, 231	12, 18
50	10	52, 55	1.0, 1.1	39, 40	24, 26	176, 203	16, 20
50	50	54, 56	1.4, 1.6	38, 40	21, 23	124, 127	14, 15
100	1	58, 59	2.2, 2.3	36, 38	22, 23	248, 257	13, 14
100	5	49, 52	1.3, 1.4	42, 44	27, 28	205, 217	16, 17
100	10	53, 53	1.1, 1.2	39, 40	23, 26	188, 215	14, 17
100	50	53, 54	1.3, 1.7	39, 40	21, 25	126, 143	12, 18
150	1	58, 61	1.1, 1.3	35, 37	20, 22	207, 240	10, 12
150	5	57, 58	1.7, 1.8	36, 37	22, 24	191, 224	13, 18
150	10	56, 57	1.2, 1.5	38, 39	22, 25	201, 207	18, 21
150	50	54, 54	1.7, 1.7	38, 40	22, 25	135, 144	10, 12
200	1	60, 61	3.6. 3.9	31, 32	19, 20	224, 271	8.1, 11
200	5	47, 55	2.1, 3.9	36, 44	22, 27	183, 205	5.8, 11
200	10	58, 61	1.5, 1.8	33, 34	19, 21	174, 186	11, 14
200	50	38, 41	4.3, 4.6	46, 49	23, 26	72, 82	4.4, 6.2

1:      unidentified fatty acids have not been included

Table 17   Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in initial glucose concentration (mean±SD; n=8)

Initial glucose concentration ($g\,L^{-1}$)	Fatty acids (% total fatty acids [1])				Total fatty acids ($mg\,g^{-1}$ dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
50	58±4.2 [ab]	1.4±0.3 [a]	36±4.2	23±2.9	186±42	14±2.3 [a]
100	56±2.4 [bc]	1.5±0.4 [a]	37±2.7	25±2.0	200±49	15±2.4 [a]
150	59±2.0 [a]	1.5±0.3 [a]	36±1.5	23±1.3	194±37	14±3.7 [a]
200	55±8.9 [c]	3.3±1.2 [b]	34±6.6	22±2.5	174±71	9.0±2.8 [b]

values in each column with different superscripts are significantly different ($\alpha = 0.01$)

1:    unidentified fatty acids have not been included

Table 18   Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in initial sodium glutamate concentration (mean±SD; n=8)

Initial sodium glutamate concentration ($g\,L^{-1}$)	Fatty acids (% total fatty acids [1])				Total fatty acids ($mg\,g^{-1}$ dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
1	60±2.1 [a]	2.2±1.1 [ab]	35±2.1 [a]	21±1.7 [a]	232±21 [a]	12±1.9 [a]
5	53±3.0 [b]	1.9±0.8 [b]	41±2.8 [b]	25±2.1 [b]	209±12 [b]	14±3.7 [ab]
10	56±2.7 [b]	1.3±0.3 [c]	38±3.0 [c]	24±2.2 [bc]	194±11 [b]	17±3.1 [b]
50	51±7.1 [b]	2.3±1.5 [a]	41±3.9 [b]	23±1.0 [c]	119±29 [c]	11±3.9 [a]

values in each column with different superscripts are significantly different ($\alpha = 0.01$)

1:    unidentified fatty acids have not been included

2.2.1.4 Flask Experiment 4: Inoculum size and age

The proportions of SFA, MUFA, PUFA and DHA (%TFA), the fatty acid content (mg g^{-1} dry weight) of, and the amount of biomass (g dry weight L^{-1}) in 10-day old flask cultures of ACEM 6063 cultured using inocula of different proportions (relative to the volume of the media being inoculated) and different ages are shown in Table 19. There were no significant differences in any of these parameters attributable to differences between the two cultures used as the experimental inocula for this experiment.

The proportion of inoculum, relative to the volume of medium being inoculated, did not significantly influence any of the parameters shown in Table 19. The age of the inoculum (i.e. the time after the inoculum itself was inoculated) significantly influenced the relative proportions of SFA, PUFA and DHA in ACEM 6063 biomass, as well as the absolute amount of biomass in each flask (Table 20). The relative proportion of SFA in the biomass was highest in cultures for which 1- and 2-day old inocula were used (~70 %TFA) and was significantly lower in cultures for which 5- and 10-day old inocula were used (65 and 60 %TFA respectively) (Table 20).

Table 19   Fatty acid content, proportion of fatty acid classes and DHA, and biomass of duplicate 10-day old flask cultures of ACEM 6063 in response to variations in inoculum size and age

Inoculum		Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
Proportion (% v/v)	Age (d)	sum SFA	sum MUFA	sum PUFA	DHA		
1	1	67, 76	1.5, 3.3	19, 30	10, 19	167, 220	14, 20
5	1	63, 62	1.3, 1.9	34, 34	20, 21	184, 203	13, 18
10	1	82, 78	2.1, 2.4	14, 18	7.2, 9.6	155, 157	13, 19
1	2	63, 68	2.4, 2.7	28, 33	17, 19	210, 238	17, 20
5	2	64, 68	2.5, 2.9	24, 27	17, 17	179, 217	18, 19
10	2	69, 70	2.0, 2.1	25, 26	15, 15	182, 210	19, 21
1	5	62, 68	1.8, 2.0	27, 34	17, 21	231, 248	18, 19
5	5	61, 63	2.0, 2.1	32, 33	19, 19	204, 221	17, 19
10	5	61, 64	2.0, 2.1	29, 35	22, 24	138, 189	15, 18
1	10	55, 59	1.3, 1.9	38, 39	23, 25	201, 290	17, 19
5	10	59, 63	2.2, 2.7	34, 37	21, 23	244, 248	18, 21
10	10	59, 61	3.1, 3.9	30, 33	20, 22	130, 195	16, 18

1:    unidentified fatty acids have not been included

Table 20　　Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in inoculum age (mean±SD; n=6)

Inoculum age (d)	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
1	72±8.5 [a]	2.1±0.7	23±8.8 [a]	15±6.2 [a]	181±26	16±3.7 [a]
2	68±2.8 [a]	2.4±0.3	25±2.8 [a]	17±1.5 [ab]	206±22	21±1.1 [b]
5	65±2.3 [b]	2.0±0.1	30±3.0 [b]	20±1.9 [bc]	205±39	18±1.4 [ab]
10	60±2.6 [c]	2.5±0.9	32±3.3 [b]	22±1.7 [c]	218±55	18±1.7 [ab]

values in each column with different superscripts are significantly different ($\alpha = 0.01$)

1:　　unidentified fatty acids have not been included

The relative proportions of PUFA and DHA in 10-day old flask cultures of ACEM 6063 were lowest in cultures for which 1-day old inocula were used (23 and 15 %TFA respectively), and highest in cultures for which 10-day old inocula were used (32 and 22 %TFA respectively). The absolute amount of biomass was lowest in cultures for which 1-day old inocula were used (16 g L^{-1}), and highest in cultures for which 2-day old inocula were used (21 g L^{-1}). Relative to the use of 2-day old inoculum, there was a slight but insignificant decrease in biomass production in cultures for which 5- and 10-day old inocula were used (18 g L^{-1} for both) (Table 20).

2.2.1.5 Cell morphology

Cell morphology in all flask cultures shared the following pattern. About 24 h after inoculation, cultures consisted of clumps containing up to several hundred 10-12 μm diameter cells. Many motile cells (ovoid: ~5 μm long, ~ 2 μm wide) were often seen at or around this time. Between this stage and when the cultures reached peak biomass, the cultures were predominantly composed of spherical clusters (up to 75 μm diameter) of 10-12 μm diameter cells, with smaller clumps and single cells also being present. As the cultures reached peak biomass, the spherical clusters began to break up into smaller clumps and single cells. After peak biomass was reached, > 95% of the cells were present either singly or in clumps of < 5 cells. Individual cell size also tended to decrease with culture age, with most cells at the time peak biomass was reached having a diameter of about 5 μm.

2.2.2 Fermenter Culture

2.2.2.1 Biomass production

Cultures grown using high impeller speeds (cultures 10H20H and 50H20H: see Table 5) grew at similar rates to each other (~3.5 g dry weight L^{-1} d^{-1}) for the first 2 days following inoculation (Figure 5). Culture 10H20H reached peak biomass (5.5 g dry weight L^{-1}) at this time (Figure 5). Culture 50H20H grew more slowly (~ 1 g dry weight L^{-1} d^{-1}) after this time, and reached peak biomass (11 g dry weight L^{-1}) after a total of 5 days incubation (Figure 5).
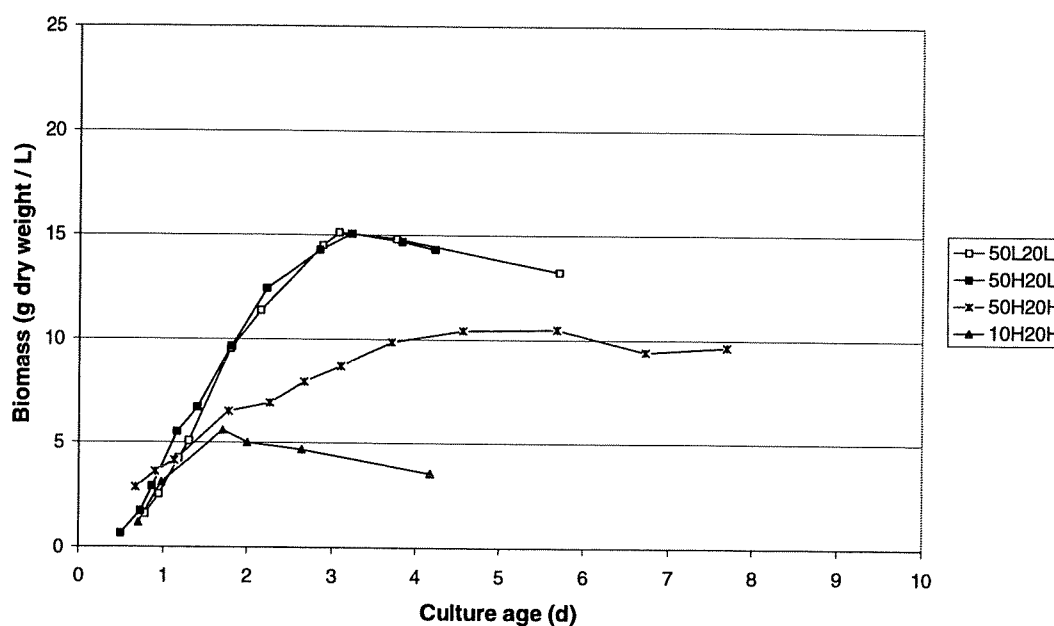
Figure 5   Biomass production by fermenter-cultured ACEM 6063 grown under treatments 10H20H, 50H20H, 50H20L and 50L20L

Treatment notation: 10, 50: initial glucose concentration (g L^{-1}); L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium; 20: incubation temperature (°C); L, H: low (max 200), high (max 750) impeller speed (rpm)

The maximum growth rates of cultures grown with an initial glucose concentration of 50 g L^{-1} and low impeller speed (cultures 50L20L and 50H20L) were very similar to each other (~6.5 g dry weight L^{-1} d^{-1}) (Figure 5), with peak biomass (15 g dry weight L^{-1}) being reached in 3 days.

The maximum growth rates of cultures grown with an initial glucose concentration of 100 g L^{-1}, at 20 and 25°C, and with low impeller speeds (cultures 100L20L, 100H20L and 100L25L) were also similar to each other (~4.5 g dry weight L^{-1} d^{-1}), with peak biomass (21-23 g dry weight L^{-1}) reached in 7-8 days (Figure 6).

Biomass (g dry weight / L) — y-axis: 0, 5, 10, 15, 20, 25

Culture age (d) — x-axis: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

Legend:
- —o— 100L25L
- —▲— 100H20L
- —△— 100L20L
- —■— 100H15L
- —□— 100L15L
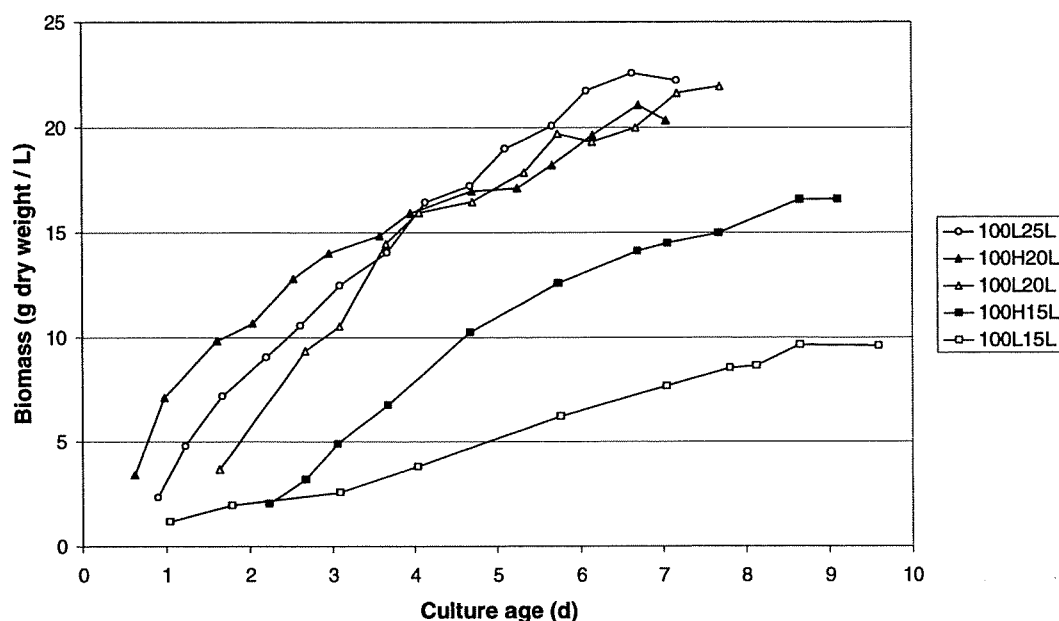
Figure 6   Biomass production by fermenter-cultured ACEM 6063 grown under treatments 100H15L, 100H15H, 100L20L, 100H20L and 100H25L

Treatment notation: 100: initial glucose concentration (g L^{-1}); L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium; 15, 20, 25: incubation temperature (°C); L: low (max 200), high (max 750) impeller speed (rpm)

The maximum growth rate of culture 100H15L (initial glucose concentration 100 g L^{-1}, grown at high DO levels, at 15°C and with low impeller speeds) was similar to those of cultures 100L20L, 100H20L and 100L25L (Figure 6). Culture 100H15L, however, appeared to undergo a longer lag time following inoculation than cultures 100L25L, 100H20L and 100L20L, and reached a peak biomass of 17 g dry weight L^{-1} (c.f. peak biomasses of 21-23 g dry weight L^{-1} for cultures 100L25L, 100H20L and 100L20L) after 8-9 days incubation. Culture 100L15L (initial glucose concentration 100 g L^{-1}, grown at low DO levels, at 15°C and with low impeller speeds) exhibited a similar increased lag time to culture 100H15L, but grew more slowly. Culture 100L15L grew at a maximum rate of ~1.1 g dry weight L^{-1} d^{-1}, and reached a peak biomass of 10 g dry weight L^{-1} after 9 days (Figure 6).

2.2.2.2 Fatty acid and lipid production

The proportions of SFA, MUFA, PUFA and DHA (%TFA), and the fatty acid content (mg g^{-1} dry weight) of fermenter cultures of ACEM 6063 grown under a range of treatments involving initial glucose concentration, dissolved oxygen level, temperature and impeller speed are shown in Table 21. These data indicate that increasing the initial glucose concentration did not substantially influence the relative proportions of SFA, MUFA,

PUFA or DHA in fermenter-cultured ACEM 6063 biomass. Increasing the initial glucose concentration from 10 to 100 g L^{-1} did, however, result in an increase in fatty acid content from 81 to about 450 mg g^{-1} dry weight.

Increasing the level of dissolved oxygen in the culture media from < 5% saturation (termed "low") to > 40% saturation (termed "high") influenced fatty acid production by ACEM 6063. The major changes were that SFA levels decreased from 54-61 %TFA to 33-38 %TFA and that MUFA levels increased substantially from 1.9-5.2 %TFA to 28-35 %TFA. In addition to these major changes in fatty acid composition, PUFA levels decreased from 31-41 %TFA to 27-30 %TFA; DHA levels decreased from 24-30 %TFA to 20-23 %TFA and fatty acid content increased from 60-282 mg g^{-1} dry weight to 123-457 mg g^{-1} dry weight (Table 21).

The fatty acid content (mg g^{-1} dry weight) of ACEM 6063 biomass for cultures 100L15L, 100L20L and 100L25L (grown at 15, 20 and 25°C respectively, at low DO levels, with initial glucose concentration of 100 g L^{-1} and at low impeller speed) were 60, 282 and 404 mg g^{-1} dry weight, respectively.

Table 21   Fatty acid content of fermenter-cultured ACEM 6063 at peak biomass under different culture conditions

Treatment [1]	Fatty acids (% total fatty acids [2])				Total fatty acids (mg g^{-1} dry weight)
	sum SFA	sum MUFA	sum PUFA	DHA	
10H20H	32	33	33	20	81
50H20H	31	36	30	24	102
50L20L	57	2.3	39	27	121
50H20L	38	28	30	23	155
100L15L	54	1.9	41	30	60
100H15L	35	34	27	20	123
100L20L	61	5.2	31	24	282
100H20L	33	35	27	20	457
100L25L	56	4.9	34	22	404

1:    Treatment notation:
      10, 50, 100: initial glucose concentration (g L^{-1})
      L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium
      15, 20, 25: incubation temperature (°C)
      L, H: low (max 200), high (max 750) impeller speed (rpm)
2:    unidentified fatty acids have not been included

Similarly, the total fatty acid content (mg g^{-1} dry weight) of ACEM 6063 biomass for cultures 100H15L and 100H20L (grown at 15 and 20°C respectively, at high DO levels, with initial glucose concentration of 100 g L^{-1} and at low impeller speed) were 123 and 457 mg g^{-1} dry weight, respectively (Table 21). The only direct comparison that could be made of the effects of impeller speed on lipid production was between cultures 50H20H and 50H20L. In this case, lower impeller speed appeared to result in a higher absolute level of total fatty acids (155 c.f 102 mg g^{-1} dry weight). Impeller speed did not appear to substantially influence the relative proportions of SFA, MUFA or PUFA in ACEM 6063 biomass (Table 21).

The predominant lipid classes produced by ACEM 6063 were triacylglycerols and phospholipids (Table 22). The relative proportion of triacylglycerols increased from 38-76% to 69-93% of the total lipids during the four days immediately prior to cultures reaching peak biomass. The relative proportion of phospholipids decreased from 23-59% to 5-29% of the total lipids during the same time. Neither culture temperature nor DO levels had any obvious influence on triacylglycerol or phospholipid levels in ACEM 6063 biomass. Wax esters, sterols and free fatty acids were present in all samples, but at levels less than 3, 2 and 2% of the total lipids respectively (Table 22). The levels of wax esters and free fatty acids showed no obvious trends in relation to culture age, temperature or DO levels. The relative proportion of sterols in the lipid fraction tended to decrease with culture age.

Table 22   Lipid-classes produced by fermenter-cultured ACEM 6063 biomass when grown under different culture conditions (duplicate analyses of single samples)

Treatment [1]	Sampling time [2]	Lipid class (% total lipids)				
		triacylglycerol	phospholipid	wax ester	free fatty acid	sterol
100L15L	4	38, 43	54, 60	1.7, 2.0	0.2, 0.2	1.0, 1.2
	2	67, 72	23, 29	2.7, 3.5	0.1, 0.2	0.9, 1.0
	0	68, 69	28, 30	1.7, 2.1	0.0, 0.1	0.5, 0.9
100H15L	4	38, 37	59, 60	0.8, 0.9	0.0, 0.0	1.8, 1.8
	2	77, 79	19, 21	1.4, 1.8	0.0, 0.0	0.6, 0.7
	0	80, 81	15, 17	2.1, 2.3	0.2, 0.3	0.7, 1.1
100L20L	4	56, 60	34, 36	2.4, 3.2	1.9, 1.9	1.8, 2.0
	2	94, 95	4.3, 4.4	0.6, 0.8	0.0, 0.3	0.5, 0.7
	0	92, 94	4.3, 5.6	0.9, 1.0	0.1, 0.4	0.6, 0.8
100H20L	4	64, 65	33, 33	0.9, 1.6	0.0, 0.8	0.9, 1.1
	2	68, 82	15, 28	1.6, 2.7	0.1, 0.5	0.6, 0.8
	0	71, 81	16, 26	2.1, 2.8	0.2, 0.2	0.5, 0.6
100L25L	4	74, 78	21, 25	0.4, 0.5	0.0, 0.1	0.5, 0.5
	2	84, 88	11, 16	0.5, 0.5	0.0, 0.0	0.3, 0.4
	0	90, 93	5.4, 8.3	0.7, 0.8	0.0, 0.0	0.4, 0.4

1:    Treatment notation:
     100: initial glucose concentration (g L^{-1})
     L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium
     15, 20, 25: incubation temperature (°C)
     L: low (max 200) impeller speed (rpm)
2:    days prior to the culture reaching peak biomass

2.2.3 Cell morphology

Cell morphology for all fermenter cultures except 10H20H and 50H20H followed the pattern described for flask culture in Section 2.2.1.5 (page 45). In cultures 10H20H and 50H20H, however, the spherical clusters of cells typically seen in actively growing flask cultures of ACEM 6063 were evident only during the first 1-2 days. The spherical clusters in both these cultures broke up well before peak biomass was reached - an occurrence not seen in other cultures of ACEM 6063.

2.3 ACEM 6063 Storage Trials

The AA, EPA and DHA content of stored ACEM 6063 cells are shown in Figure 7 (expressed as % TFA) and Figure 8 (expressed as mg g^{-1} dry weight).
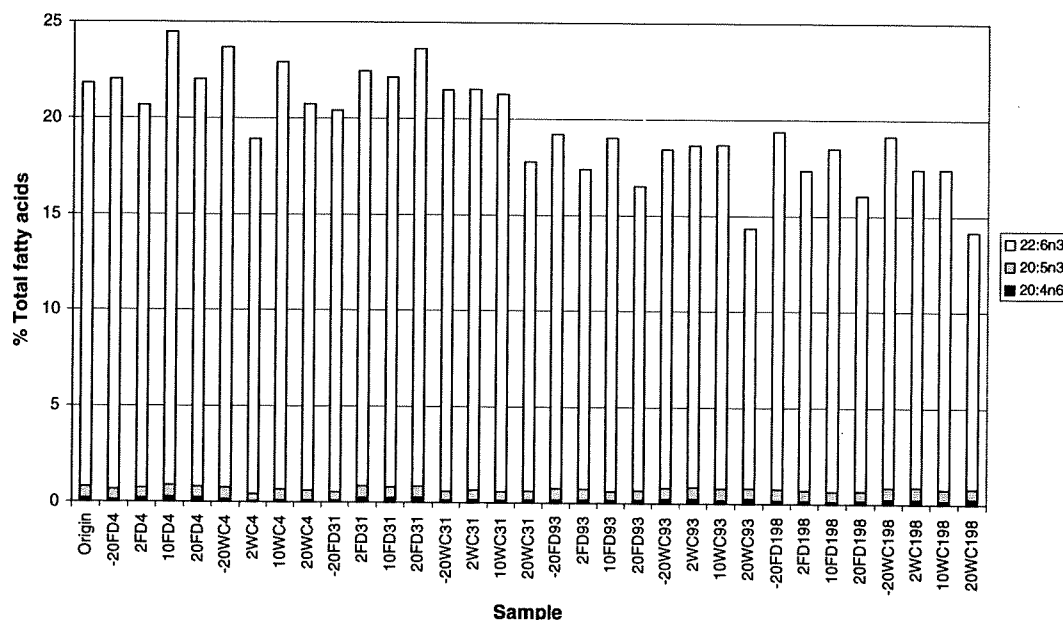
Figure 7   Proportion of AA, EPA and DHA (% total fatty acids) in stored
              ACEM 6063 cells

Sample notation: Origin: freshly harvested cells; All other samples: suffix denotes storage temperature (-20,
    2, 10, 20 °C); cell treatment prior to storage: FD (freeze dried cells), WC (wet cells): suffix denotes
    storage duration (4, 31, 93, 198 days). e.g. -20FD31 denotes freeze-dried cells stored at -20°C for 31
    days
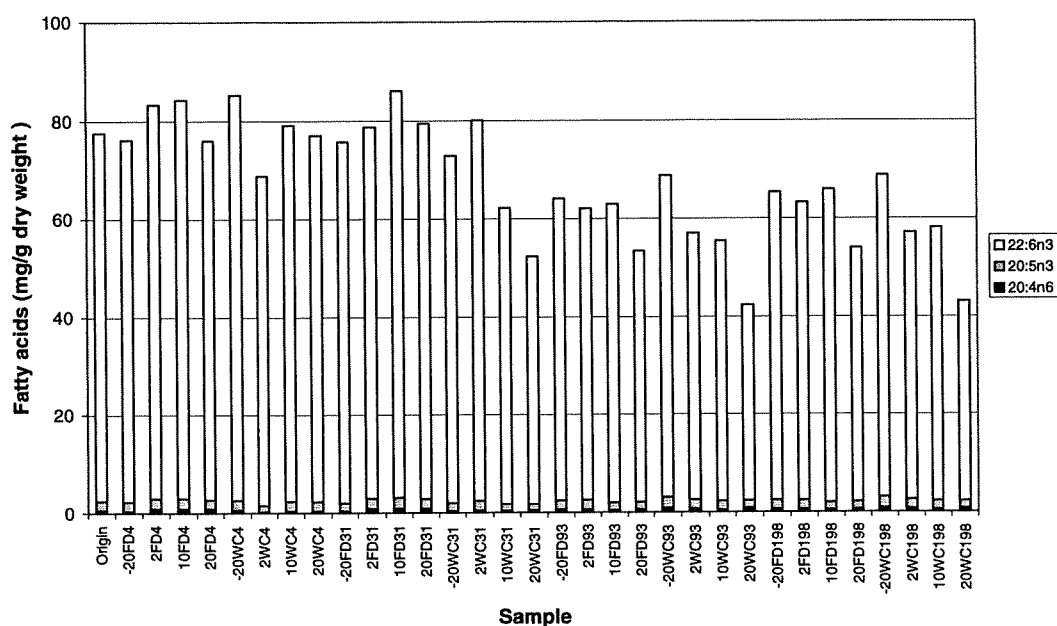
Figure 8   Absolute amount of AA, EPA and DHA (mg g^{-1} dry weight) in stored ACEM 6063 cells

Sample notation: Origin: freshly harvested cells; All other samples: suffix denotes storage temperature (-20, 2, 10, 20 °C); cell treatment prior to storage: FD (freeze dried cells), WC (wet cells): suffix denotes storage duration (4, 31, 93, 198 days). e.g. -20FD31 denotes freeze-dried cells stored at -20°C for 31 days

Statistical analysis of the relative proportions and absolute amounts of fatty acids in stored ACEM 6063 cells indicated that the only treatment variable related with significance change was storage duration ($\alpha = 0.05$). Summaries of the relative and absolute amounts of AA, EPA, DHA, DPAn6, SFA, MUFA and PUFA and the absolute TFA content of ACEM 6063 cells following storage for 4, 31, 93 and 198 days are shown in Table 23 and Table 24 respectively.

The relative proportions (% of total fatty acids) of AA, EPA and SFA in ACEM 6063 cells were not significantly influenced by storage duration. The relative proportion of DHA decreased slightly with storage duration, from 21 %TFA after 0 days storage, to 17 %TFA after 198 days storage. During the same storage interval, the relative proportion of DPAn6 decreased from 5.3 to 3.8 %TFA, MUFA increased from 35 to 39 %TFA, and PUFA decreased from 28 to 23 %TFA.

The absolute amounts of AA, EPA, SFA, MUFA and TFA in ACEM 6063 cells were not significantly influenced by storage duration. The absolute amount of DHA decreased significantly with increasing storage duration, from 75 mg g^{-1} dry weight after 0 days

storage, to 56 mg g^{-1} dry weight after 198 days storage. During the same storage interval, the absolute amount of DPAn6 decreased from 20 to 13 mg g^{-1} dry weight.

There was a trend for a reduction in the absolute amounts of total fatty acids in the cells over time. This decrease, however, was not statistically significant ($\alpha = 0.05$).

Table 23   Proportion of fatty acids (% total fatty acids) in ACEM 6063 cells, as influenced by storage duration (mean ± sd, n=8)

Storage duration (d)	AA	EPA	DHA	DPAn6	SFA	MUFA	PUFA
0	0.2±0.1	0.6±0.2	21±2 [a]	5.3±0.5 [a]	34±2 [a]	35±2 [a]	28±2 [a]
4	0.2±0.1	0.5±0.1	21±2 [a]	5.4±0.9 [a]	35±2 [a]	36±1 [a]	29±3 [a]
31	0.2±0.1	0.5±0.1	21±2 [a]	5.1±0.6 [a]	36±2 [ab]	36±1 [a]	28±2 [a]
93	0.2±0.1	0.6±0.1	17±2 [b]	3.8±0.4 [b]	38±1 [bc]	39±1 [b]	23±2 [b]
198	0.2±0.1	0.6±0.1	17±2 [b]	3.8±0.4 [b]	37±2 [ab]	39±1 [b]	23±2 [b]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For storage descriptions – see section1.3, page 10

Table 24   Absolute amounts of fatty acids (mg g^{-1} dry weight)) in ACEM 6063 cells, as influenced by storage duration (mean ± sd, n=8)

Storage duration (d)	AA	EPA	DHA	DPAn6	SFA	MUFA	PUFA	TFA
0	0.6±0.2	1.9±0.2	75±6 [a]	20±2 [a]	125±6	128±5	100±4 [a]	354±13 [a]
4	0.6±0.3	1.9±0.3	76±5 [a]	20±3 [a]	127±13	130±8	103±9 [a]	359±21
31	0.6±0.3	1.8±0.3	71±11 [a]	18±3 [a]	123±12	124±12	96±14 [a]	343±35
93	0.7±0.3	1.9±0.3	56±8 [b]	13±2 [b]	123±10	128±11	76±10 [b]	327±28
198	0.7±0.1	1.9±0.3	56±7 [b]	13±2 [b]	122±9	128±11	76±9 [b]	326±24

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For storage descriptions – see section1.3, page 10

2.4 Aquaculture Feeding Trials

2.4.1 Enrichment of rotifers (*Brachionus plicatilis*) with eicosapentaenoic acid and docosahexaenoic acid produced by bacteria

Under the culture conditions used, ACAM 456 produced EPA to a level of 14.8±1.1% (mean±SD) of total fatty acids, while ACAM 605 produced 5.9±0.5% and 0.7±0.03% DHA and EPA respectively. Major components of the fatty acid fractions from rotifers are shown in Table 25. Statistical analysis (ANOVA) of final levels of EPA and DHA in rotifers, expressed as either %TFA or %dw, showed significant differences between treatments at $\alpha = 0.0001$ (EPA %TFA; DHA %TFA; DHA %dw) and $\alpha = 0.005$ (DHA %dw). Treatment means were grouped according to Fishers LSD test (SAS System for Windows, version 6.12, SAS Institute Incorporated, USA) (Table 25).

EPA content (%TFA and %dw) in those rotifers fed ACAM 456 only was significantly greater than in all other rotifers in this trial. Rotifers fed *T. suecica* contained either significantly lower (%TFA) or similar (%dw) levels of EPA than those fed a mixture of ACAM 456 and 605. Rotifers from these three treatments contained significantly higher EPA (%TFA and %dw) than in any of the remaining treatments.

DHA content (%TFA and %dw) in those rotifers fed a mixture of ACAM 456 and 605 was significantly higher than for all other rotifers in this trial. DHA levels (%dw) in rotifers from all other treatments were not significantly different from each other. Except as noted above, rotifers fed ACAM 605 only had significantly higher levels of DHA (%TFA) than rotifers from remaining treatments.

The numbers of healthy rotifers (i.e. those remaining in the water column at the conclusion of the feeding trial) varied considerably (Table 25). However, those rotifers exposed to ACAM 605 only, had a significantly lower survival rate than all other groups ($\alpha = 0.05$).

2.4.2 Enrichment of rotifers with PUFA from thraustochytrids

2.4.2.1 Enrichment diets

The major fatty acids in each component of the enrichment diets used in Trials 1 and 2 are shown in Table 27 and Table 28. The different treatments (fresh, frozen, freeze-dried, air-dried) of ACEM 000A and ACEM 6063 biomass did not significantly alter the fatty acid profiles of either strain. The fatty acid profile of ACEM 000A, however, was markedly different between the 2 trials. The proportion of saturated fatty acids (SFA) decreased from 61% of total fatty acids (TFA) in Trial 1 to 23 %TFA in Trial 2, while the proportion of monounsaturated fatty acids (MUFA) increased from 12 to 54 %TFA.

Table 25   Initial cell counts, final rotifer counts and EPA and DHA levels in duplicate samples of rotifers either starved or fed bacteria, microalgae or yeast for 24 h (means of values within each column with the same superscript letter are not significantly different)

Treatment	Initial cell count (mL^{-1})	Rotifer count (post-trial)	% total fatty acids		% dry weight (w/w)	
			EPA	DHA	EPA	DHA
Starved (pre-trial)		-	0.7, 0.7 [ef]	0, 0 [c]	0.1, 0.1 [c]	0, 0 [b]
Starved (post-trial)		173, 138 [a]	0.9, 0.7 [e]	0, 0 [c]	0.1, 0.1 [c]	0, 0 [b]
ACAM 456	1.4×10^9	181,155 [a]	6.8, 6.9 [a]	0, 0 [c]	1.1, 1.3 [a]	0, 0 [b]
ACAM 605	1.0×10^9	17, 102 [b]	1.2, 1.0 [d]	1.1, 1.6 [b]	0.1, 0.1 [c]	0.1, 0.1 [b]
ACAM 456 + 605	$7 \times 10^8 + 5 \times 10^8$	166, 168 [a]	3.5, 3.3 [b]	1.7, 2.1 [a]	0.6, 0.4 [b]	0.4, 0.2 [a]
T. suecica	4.3×10^5	206, 198 [a]	2.0, 2.0 [c]	0.1, 0.1 [c]	0.4, 0.4 [b]	0, 0 [b]
S. cerevisiae	2×10^8	196, 207 [a]	0.5, 0.5 [f]	0, 0 [c]	0.1, 0.1 [c]	0, 0 [b]

Table 26   Major[1] fatty acid composition (mean % total fatty acid; n=2) of the feed bacterial cultures and rotifers either starved or fed bacteria, microalgae or yeast for 24 h

	Bacterial strain		Rotifer diet						
Fatty acid	ACAM 456	ACAM 605	Starved (pre-trial)	Starved	ACAM 456 only	ACAM 605 only	ACAM 456 and 605	*T. suecica*	*S. cerevisiae*
16:0	13.6	24.5	6.2	6.0	7.1	10.1	8.1	11.2	6.1
18:0	0.5	0.6	3.9	3.8	2.6	3.2	2.5	3.2	3.4
16:1(n-7)c	16.2	17.2	23.5	23.4	20.5	19.4	20.4	12.5	26.4
18:1(n-9)c	5.0	5.5	29.2	28.6	14.9	13.6	13.2	19.0	28.9
18:2(n-6)	1.7	1.9	6.6	7.0	3.9	3.0	3.6	15.7	6.5
20:4(n-3)	0.0	0.0	1.8	2.0	1.4	0.8	1.3	2.4	1.4
20:5(n-3)	14.6	0.2	0.7	0.8	6.9	1.1	3.4	2.0	0.5
22:6(n-3)	0.0	5.9	0.0	0.0	0.0	1.4	1.9	0.1	0

1:     Fatty acids detected but not included in this Table: 12:0, 13:0, 14:0, 15:0, 17:0, i13:0, i14:0, i15:0, a15:0, 14:1, i15:1, i16:0, br17:1, i17:1, br19:1, 15:1n6, 16:1n9c, 16:1n7t, 16:1n5c, 17:1n6c, 17:1n8c, 18:1n5c, 18:1n7c, 19:1, 20:1n7c, 20:1n9c, 18:2n9, 22:1, 24:1, C_{16} PUFA, 18:3, 18:4n3, 20:2n6, C_{20} PUFA, 20:4n6, 22:5n3

Table 27   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of thraustochytrid, algal and yeast enrichment diets used in Trial 1

Fatty acid	Enrichment diet						
	A FS[2]	A FZ	A FD	A AD	Algamac	T. Iso	Yeast
14:0	11	12	11	6.0	17	12	
16:1n7	2.9	3.5	2.9	2.8	4.9	5.5	27.1
16:0	38	38	37	40	38	15	14
18:3n6						3.0	
18:4n3						13	
18:2n6						11	20
18:1n9	6.4	6.5	6.1	7.1		23	30
18:0	13	13	14	15			4.9
20:4n6	6.2	6.3	6.3	7.0			
20:5n3	3.5	3.7	3.7	3.9			
20:1n9+11	2.1	2.1	2.1	2.1			
22:5n6					13		
22:6n3	4.8	4.6	4.8	4.3	21	8.2	
22:5n3	7.2	6.4	7.4	5.9			
sum SFA[3]	61	62	61	61	56	27	19
sum MUFA[4]	12	12	12	12	5.9	31	59
sum PUFA[5]	26	26	27	26	37	41	22
sum other	0.7	0.5	1.1	0.9	1.0	0.5	0.3
TFA[6] mg g^{-1}	82	96	81	63	440	84	6.8

1:    fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, 16:1n9, 17:1, 17:0, 18:3n3 (co-eluted with 18:1n9), 18:1n7, 19:1, 20:2, 20:3n6, 20:4n3, 20:2n6, 20:1n7, 20:0, 22:4n6, C22PUFA

2:    A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3, 4, 5, 6:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids

Table 28   Mean (n=2) fatty acid[1] composition of thraustochytrid, algal and yeast enrichment diets used in Trial 2 (% total fatty acids except where noted)

Fatty acid	Enrichment diet								
	A FZ[2]	A FD	6063 FS	6063 FZ	6063 FD	6063 AD	Algamac	T. Iso	Yeast
14:0	3.9	3.4	14	14	13	14	15	16	
16:1n9								2.6	
16:1n7	3.4	3.1					4.9	4.8	30
16:0	15	14	34	33	35	38	42	12	14
18:3n6								2.4	
18:4n3								22	
18:2n6	2.4	2.3						7.4	20
18:1n9	20	21						15	29
18:1n7	25	26						2.7	2.1
18:0	4.2	4.8							5.4
20:4n6	4.8	5.0							
20:5n3	5.2	4.7							
20:1n9+11	4.9	4.9							
22:5n6			11	13	12	12	12		
22:6n3			28	29	28	26	21	7.7	
22:5n3	5.2	5.0							
sum SFA[3]	23	22	52	50	50	54	58	29	19
sum MUFA[4]	53	55	2.7	1.8	2.2	2.4	5.9	27	60
sum PUFA[5]	23	23	45	48	47	43	36	43	20
sum other	0.6	0.6	0.5	0.5	0.8	1.0	0.4	1.0	0.9
TFA[6] mg g^{-1}	557	544	346	342	330	353	533	84	5.7

1:   fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 17:1, 17:0, 20:3n6, 20:4n3, 20:2n6, 20:0, 22:4n6, C22PUFA

2:   A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = Isochrysis sp, clone T. Iso; Yeast = Saccharomyces cerevisiae

3, 4, 5, 6:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids

The proportion of PUFA was essentially the same in both trials. These changes in fatty acid profile were due to modifications of culture conditions. The fatty acid profile of ACEM 6063 biomass, used in Trial 2 only, comprised about 50% SFA, 2% MUFA and 48% PUFA. The fatty acid profile of the commercially-available Algamac cells used in both trials was 58% SFA, 6% MUFA and 36% PUFA. The proportions of SFA, MUFA and PUFA in the T. Iso cells were 29%, 27% and 43% respectively. The proportions of SFA, MUFA and PUFA in the yeast cells were about 19%, 60% and 20% respectively.

In Trial 1, the absolute level of TFA (mg g^{-1} dry weight) in air-dried cells of ACEM 000A was significantly lower than in the other ACEM 000A cells (63 mg g^{-1} c.f 81-96 mg g^{-1}). The absolute TFA content of ACEM 000A used in Trial 2 was about 6-fold higher (550 mg g^{-1}) than that used in Trial 1. This increase in lipid content coincided with the marked shift in the relative proportions of SFA and MUFA mentioned earlier. The TFA content of ACEM 6063 cells was between 330 and 353 mg g^{-1}. The absolute amount of TFA in Algamac, T. Iso and yeast cells was about 530, 84 and 6 mg g^{-1} respectively.

2.4.2.2 Trial 1

The fatty acid compositions of rotifers sampled during Trial 1 are shown in Table 29 and Table 30. Although there were slight differences in the proportions of SFA, MUFA and PUFA in rotifers fed the different ACEM 000A treatments at different time points, the same general trends were evident. The proportion (%TFA) of SFA in all rotifers fed ACEM 000A increased between the start of the trial (T0) and T6, and then decreased slightly between T6 and T48. The proportion of MUFA decreased between T0 and T6, and did not vary substantially between T6 and T48. The proportion of PUFA in ACEM 000A-fed rotifers increased slightly between T0 and T6, and again between T6 and T48.

The proportion of SFA in the Algamac-fed rotifers increased markedly between T0 and T6, decreased between T6 and T24, and was stable between T24 and T48 (Table 30). The proportion of MUFA decreased substantially between T0 and T6, was stable between T6 and T24, and increased between T24 and T48. The proportion of PUFA in these rotifers rose between T0 and T6, and between T6 and T24, and decreased between T24 and T48.

Table 29   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers before enrichment, and after 6 and 24 h enrichment with thraustochytrid ACEM 000A and a further 24 h starvation [Trial 1]

| | | Enrichment diet | | | | | | | | | | | |
| | | A FS[2] | | | A FZ | | | A FD | | | A AD | | |
Fatty acid	TO[3]	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0		5.9	4.4		5.1	4.1	2.3	5.3	4.2	2.6	4.8	3.6	2.0
16:1n7	15	12	9.1	8.5	11	9.8	8.4	12	8.6	8.9	11	9.4	8.6
16:0	9.5	19	16	16	18	18	14	18	17	15	18	17	16
18:2n6	13	8.1	7.1	8.1	8.1	7.8	7.7	8.4	7.0	8.0	8.2	7.3	8.0
18:1n9	26	16	15	16	17	15	15	17	14	16	17	15	16
18:1n7	5.5	3.2	3.3	4.2	3.2	3.5	3.9	3.3	3.2	3.9	3.2	3.6	4.0
18:0	5.2	7.4	6.2	6.3	7.5	6.8	5.8	7.1	6.6	6.3	7.4	6.9	6.4
20:4n6		4.9	6.8	7.4	5.7	7.0	7.7	5.6	7.2	7.3	5.6	7.0	7.3
20:5n3		2.7	4.0	4.4	3.1	3.9	4.3	3.0	3.7	3.8	3.0	3.7	3.8
20:4n3	2.8												
20:1n9+11	3.4	2.2	2.8	3.1	2.3	2.4	3.0	2.1	2.6	3.2	2.5	2.8	2.9
22:6n3		2.4	3.8	3.0	2.8	3.1	3.3	2.5	3.4	2.8	3.1	3.0	2.6
22:4n6				2.3			2.6			2.2			2.3
22:5n3		3.9	7.6	7.5	4.2	5.9	8.1	3.6	6.9	7.3	4.3	6.6	7.2
sum SFA[4]	19^a	34^b	28^c	25^c	32^b	30^b	24^a	32^b	29^{ab}	26^{ab}	32^b	29^b	26^{ab}
sum MUFA[5]	55^a	35^b	33^b	34^b	35^b	33^b	34^b	36^b	31^b	35^b	36^b	34^b	34^b
sum PUFA[6]	26^a	30^a	38^b	40^b	32^{ab}	36^{bc}	41^c	31^{ab}	37^{ab}	39^b	32^{ab}	37^{bc}	39^c
sum other	1.1	0.8	0.8	0.6	0.7	1.6	0.7	0.7	2.5	0.9	0.6	0.6	1.0
TFA[7] mg g^{-1}	34^a	48^b	39^b	22^c	44^b	33^a	28^c	52^b	33^b	32^c	48^b	38^b	28^c

1:   fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, i17:0, 17:0, 18:3n6, 18:4n3, 18:3n3 (co-eluted with 18:1n9), 19:1, 20:3n6, 20:1n9+11, 20:2n6, 20:0, 22:5n6

2:   A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells

3:   TO = before enrichment; T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for TO all have the superscript "a")

Table 30   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of starved rotifers and those fed Algamac, Isochrysis sp (clone T. Iso) or yeast for 6 and 24 h and starved for a further 24 h [Trial 1]

| | Enrichment diet | | | | | | | | | | | |
| | Algamac | | | T Iso[2] | | | Yeast | | | Starved | | |
Fatty acid	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	11	4.9	2.0	8.2	8.8	5.2						
16:1n7	7.5	7.3	8.4	11	8.9	7.8	22	25	20	16	15	12
16:0	25	15	20	10	9.0	11	9.1	7.8	7.9	10	8.1	11.2
18:3n6				2.7	2.8	2.4						
18:4n3				8.7	12	6.6						
18:2n6	3.0	3.2	4.5	13	13	15	10	7.2	7.9	14	14	14
18:3n3				2.6	3.9	3.2						
18:1n9	5.8	6.4	11	18	15	16	29	30	29	25	26	24
18:1n7		3.0	4.9	3.3	3.5	4.2	5.1	5.5	5.7	5.5	5.0	6.5
18:0	2.0	2.8	5.2	2.6	2.0	3.6	5.2	4.3	5.2	5.6	5.9	7.4
20:4n6		2.7	3.6								2.4	2.6
20:5n3		3.4	4.0			2.6						2.1
20:4n3					3.8	5.7				2.4	3.2	2.7
20:1n9+11				2.0	2.6	3.2	3.4	3.8	4.2	3.6	4.4	3.8
22:5n6	15	11	4.7									
22:6n3	19	30	17	5.0	6.1	4.2						
sum SFA[4]	38[b]	23[c]	29[c]	22[a]	21[a]	21[a]	18[b]	15[b]	16[b]	20[a]	17[a]	17[a]
sum MUFA[5]	17[b]	19[b]	28[c]	37[b]	31[c]	34[c]	61[b]	68[b]	62[b]	53[a]	52[a]	53[a]
sum PUFA[6]	45[b]	54[c]	42[b]	40[b]	48[c]	44[b]	20[b]	16[c]	20[b]	27[a]	30[a]	29[a]
sum other	0.4	4.2	1.4	0.7	0.5	1.0	1.0	1.1	1.8	1.0	0.8	0.7
TFA[7] mg g^{-1}	128[b]	66[b]	23[c]	59[a]	90[b]	55[a]	32[a]	34[a]	30[a]	33[a]	19[b]	11[b]

1:   fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, i17:0, 17:0, 19:1, 20:3n6, 20:1n9+11, 20:2n6, 20:0, 22:5n6, 22:4n6, 22:5n3

2:   T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3:   T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for T0 all have the superscript "a")

The proportion of SFA in the T. Iso-fed rotifers did not change substantially between T0 and T48. The proportion of MUFA decreased between T0 and T6, and between T6 and T24, and was stable between T24 and T48. The proportion of PUFA rose between T0 and T6, and T6 and T24, and decreased slightly between T24 and T48. The proportion of SFA in the yeast-fed rotifers decreased slightly between T0 and T6 and was relatively stable between T6 and T48. The proportion of MUFA increased substantially between T0 and T6, and was stable between T6 and T48. The proportion of PUFA in these rotifers decreased between T0 and T6, decreased again between T6 and T24, and increased slightly between T24 and T48. There were no significant variations in the proportions of SFA, MUFA or PUFA in the starved rotifers during the course of this trial.

Absolute TFA levels in all treatments except yeast-fed and starved rotifers increased between T0 and T6. At T6, all rotifers fed ACEM 000A contained similar amounts of TFA ($44 - 52$ mg g^{-1} dry weight). Algamac- and T. Iso-fed rotifers contained significantly more TFA (128 and 59 mg g^{-1} respectively) than the other treatments. TFA levels in yeast-fed and starved rotifers (32 and 33 mg g^{-1} respectively) were similar to those in rotifers at T0.

At T24, absolute TFA levels in ACEM 000A-fed rotifers had decreased slightly and were all between 33 and 39 mg g^{-1}. TFA levels in Algamac-fed rotifers had decreased to 66 mg g^{-1}, while those in T. Iso-fed rotifers had increased to 90 mg g^{-1}. Total fatty acid levels in yeast-fed and starved rotifers were 34 and 19 mg g^{-1} respectively.

At T48, TFA levels in rotifers fed ACEM 000A, Algamac and yeast were all similar ($22 - 32$ mg g^{-1}), while T. Iso-fed rotifers contained 55 mg g^{-1}. The TFA level in starved rotifers had decreased to 11 mg g^{-1}.

At T6, the highest AA levels ($2 - 3$ mg g^{-1}) were found in rotifers enriched with ACEM 000A and Algamac (Figure 9). AA levels in all other rotifers varied between 0.5 mg g^{-1} (yeast-fed rotifers) and 1 mg g^{-1} (T. Iso-fed rotifers). Highest EPA levels (2 mg g^{-1}) were found in Algamac-fed rotifers, and varied between 0.4 mg g^{-1} (yeast-fed rotifers) and 1.5 mg g^{-1} [ACEM 000A (freeze-dried)-fed rotifers] for the other treatments. Highest DHA levels (25 mg g^{-1}) were found in Algamac-fed rotifers. DHA levels in all other rotifers varied between 0.1 mg g^{-1} (yeast-fed rotifers) and 3 mg g^{-1} (T. Iso-fed rotifers).

At T24, the highest AA levels ($2 - 3$ mg g^{-1}) were found in rotifers enriched with ACEM 000A (Figure 9). AA levels in all other rotifers varied between 0.4 mg g^{-1} (yeast-fed rotifers) and 2 mg g^{-1} (Algamac-fed rotifers). Highest EPA levels (2 mg g^{-1}) were found in Algamac-fed rotifers. EPA levels in all other rotifers varied between 0.3 mg g^{-1} (yeast-fed rotifers) and 2 mg g^{-1} [ACEM 000A (fresh)-fed rotifers].

At T48, the highest AA levels ($2 - 3$ mg g^{-1}) were found in rotifers enriched with ACEM 000A (Figure 9). AA levels in all other rotifers varied between 0.3 mg g^{-1} (starved rotifers) and 1 mg g^{-1} (T. Iso-fed rotifers). Highest EPA levels (ca 1 mg g^{-1}) were found in ACEM 000A- and Algamac-fed rotifers. EPA levels in yeast-fed and starved rotifers were 0.4 mg g^{-1} and 0.2 mg g^{-1} respectively. Highest DHA levels (4 mg g^{-1}) were found in Algamac-fed rotifers. DHA levels in all other rotifers varied between < 0.1 mg g^{-1} (starved rotifers) and 2 mg g^{-1} (T. Iso-fed rotifers).

The density of rotifers in each flask was assessed at each sampling time (Figure 10). At T6, rotifer numbers had decreased markedly (c.f. 200 mL^{-1} at T0) for all treatments. At this time, the density of starved rotifers and T. Iso-fed rotifers (117 and 96 rotifers mL^{-1} respectively) were significantly higher than for all other treatments (59 - 84 rotifers mL^{-1}).

At T24, rotifer numbers had again decreased, although this was only significant for the starved rotifers and those fed Algamac and T. Iso. The density of Algamac-fed rotifers (31 rotifers mL^{-1}) was significantly lower than for all other treatments (50 – 75 rotifers mL^{-1}). At T48, rotifer density had decreased significantly (c.f. counts at T24) in treatments fed ACEM 000A or yeast. Differences in rotifer density between treatments at T48 were not significant.
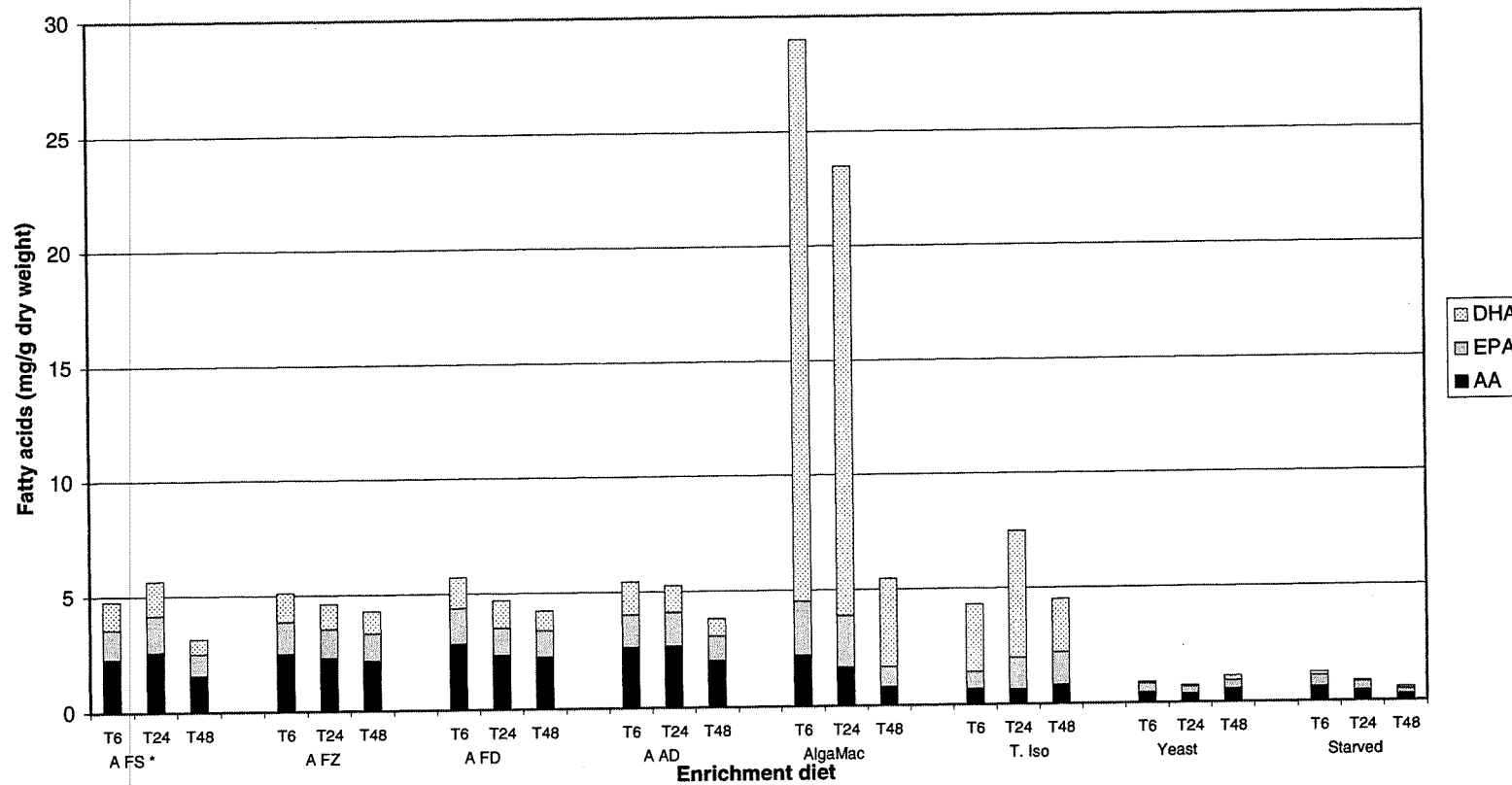
Figure 9   Mean amounts (mg g^{-1} dry weight; n=2) of 20:4n6 (AA), 20:5n3 (EPA) and 22:6n3 (DHA) in rotifers following 6 (T6)
and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 1]

*:   A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*
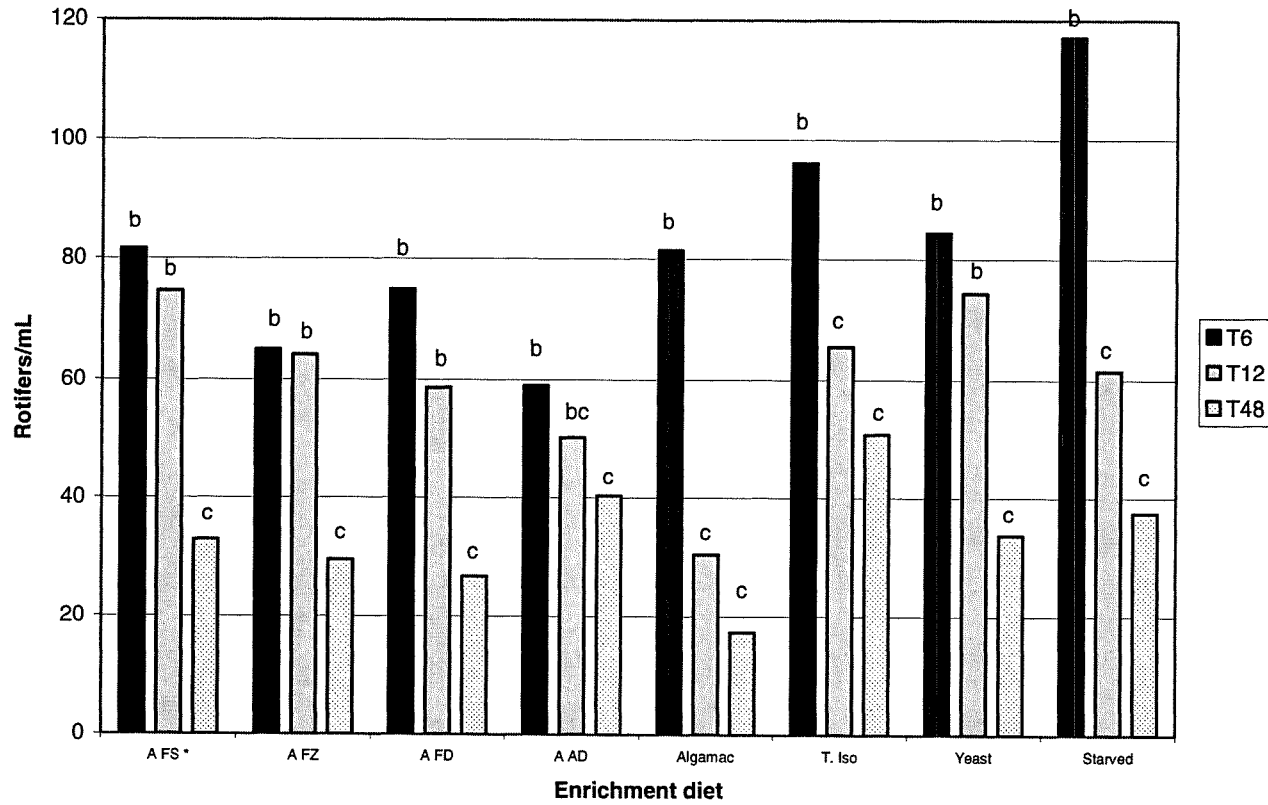
Figure 10   Mean rotifer density (number mL^{-1}; n=2) following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 1]

*:   A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

Values with different letters within each treatment are significantly different (p < 0.05) [values for T0 (200 rotifers mL^{-1}) all have the letter "a"]

2.4.2.3 Trial 2

The fatty acid compositions of rotifers sampled during Trial 2 are shown in Table 31, Table 32 and Table 33. The proportion (%TFA) of SFA in all rotifers fed frozen and freeze-dried ACEM 000A biomass at 100 mg L^{-1} dry weight equivalent did not vary substantially between T0 and T48. The proportion of MUFA in these rotifers was variable, but did not change substantially when compared to levels at T0. The proportion of PUFA in rotifers fed frozen ACEM 000A cells did not vary substantially between T0 and T48. The proportion of PUFA contained in rotifers fed freeze-dried ACEM 000A cells at 100 mg L^{-1} was stable between T0 and T6, increased between T6 and T24, and was stable between T24 and T48.

The proportion of SFA in rotifers fed freeze-dried cells of ACEM 000A at 200 mg L^{-1} remained stable between T0 and T24, and increased between T24 and T48. The proportion of MUFA in these rotifers was stable between T0 and T24, and decreased between T24 and T48. The proportion of PUFA did not vary substantially between T0 and T48. The proportions of SFA, MUFA and PUFA in rotifers fed the ACEM 000A-T. Iso and ACEM 000A-ACEM 6063 mixtures showed similar trends to those for rotifers fed only freeze-dried ACEM 000A cells at 100 mg L^{-1}.

The proportions of SFA, MUFA and PUFA in rotifers fed either ACEM 6063 only, or the ACEM 6063-T. Iso mixture were similar to each other. Between T0 and T6, the proportions of SFA increased, MUFA decreased and PUFA increased. The fatty acid profiles of these rotifers remained quite stable between T6 and T48. The proportions of SFA, MUFA and PUFA in Algamac-fed rotifers showed similar trends to those of ACEM 6063-fed rotifers. The proportion of SFA in T. Iso-fed rotifers did not vary substantially between T0 and T6, and increased slightly between T6 and T48. The proportion of MUFA decreased slightly, and the proportion of PUFA increased slightly, between T0 and T6. Neither varied substantially between T6 and T48. The proportions of SFA and MUFA in yeast-fed rotifers did not vary substantially between T0 and T48. The proportion of PUFA decreased slightly between T0 and T6, and did not vary substantially between T6 and T48. There were no substantial variations in the proportions of SFA, MUFA or PUFA in starved rotifers between T0 and T48.

Table 31   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers before enrichment, and after 6 and 24 h enrichment with thraustochytrids ACEM 000A and ACEM 6063 and a further 24 h starvation [Trial 2]

		A FZ[2]			A FD			A FD x2			A FD+ T. Iso			A FD + 6063 FD		
Fatty acid	TO[3]	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0		3.5	2.9	2.4	3.4	3.0	2.1	3.1	2.5		3.6	3.5	2.7	3.2	3.3	2.8
16:1n7	16	6.4	6.3	6.9	6.4	6.1	5.7	4.9	3.8	4.6	6.4	5.9	5.9	6.4	6.4	6.1
16:0	11	14	13	11	13	13	9.2	13	17	18	12	12	10	12	12	11
18:2n6	9.3		11	11		8.2	12		7.4	6.4		17	12		11	12
18:1n9	27	15	33	33	11	22	31	15	37	29	19	24	32	14	31	31
18:1n7	3.6	29		2.2	30	2.5	2.0	29		2.9	27	2.7	2.2	28		
18:0	5.0	4.3	4.4	4.8	4.3	4.2	3.8	4.0	6.3	11.9	3.8	3.8	3.7	3.8	3.4	3.4
20:4n6		6.6	5.9	6.5	6.8	9.6	7.8	7.0	4.7	3.5	5.5	5.8	5.9	6.3	5.6	6.0
20:5n3	3.0	6.6	5.2	4.9	6.8	8.8	6.3	7.5	3.8	2.9	5.3	5.4	4.7	6.9	5.7	5.1
20:4n3	3.0															
20:3n6						2.3	2.2						2.0			
20:2n6			3.5	2.9	2.0	4.4	3.3		2.9			3.6	3.3		2.9	2.8
20:1n9+11	4.9	2.8			2.8			4.9	3.3		4.6			2.9		
20:0	3.4			2.8			2.6			3.6			2.5			2.8
22:6n3						2.5								2.8	3.2	
22:5n3		3.6	3.1	3.4	4.1	5.6	4.4	4.3	2.9	2.7	3.5	3.7	4.0	3.8	3.1	3.3
sum SFA[4]	20[a]	21[a]	20[a]	21[a]	21[a]	20[a]	18[a]	20[a]	26[a]	37[b]	20[a]	20[a]	20[a]	19[a]	19[a]	21[a]
sum MUFA[5]	53[a]	54[a]	44[a]	45[a]	51[a]	34[b]	41[a]	55[a]	47[a]	40[c]	58[a]	36[b]	42[a]	52[a]	42[b]	41[b]
sum PUFA[6]	26[a]	24[a]	35[a]	34[a]	28[a]	45[b]	40[b]	25[a]	27[a]	23[a]	22[a]	43[b]	37[b]	29[a]	38[a]	38[a]
sum other	0.6	1.0	0.9	0.5	1.0	1.0	0.6	1.0	1.0	0.7	1.0	0.9	0.5	1.0	1.0	0.8
TFA[7] mg g⁻¹	27[a]	142[b]	111[b][a]	43[a]	136[b]	107[b]	46[a]	104[b]	111[b]	19[a]	148[b]	100[b]	80[c]	157[b]	52[c]	48[c]

1:     fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 18:4n3, 20:3n6, 20:4n3, 22:5n6, 22:4n6, C22 PUFA

2:     A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso

3:     TO = before enrichment; T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for TO all have the superscript "a")

Table 32   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers after 6 and 24 h enrichment with thraustochytrid ACEM 6063 and a further 24 h starvation [Trial 2]

Fatty acid	6063 FS[2]			6063 FZ			6063 FD			6063 FD x2			6063 AD			6063 FD + T. Iso		
	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	5.2	5.9	3.7	6.0	8.4	4.3	5.1	4.4	3.3	5.7	5.4	3.7	5.5	4.5	3.3	6.6	6.9	5.3
16:1n7	8.5	7.4	6.3	7.6	9.2	6.6	9.3	7.3	7.9	8.1	8.2	8.1	8.6	6.9	7.8	9.8	7.9	7.7
16:0	18	22	19	21	19	18	18	17	18	19	16	17	20	18	17	17	18	17
18:4n3																5.6	3.8	2.1
18:2n6	5.1	3.1	3.0	4.7	2.9	3.3	6.0	3.9	4.6	4.9	3.7	4.5	5.4	3.7	4.5	6.8	5.0	5.8
18:1n9	13	12	9.9	12	12	11	15	11	14	13	11	13	14	11	13	16	12	13
18:1n7		2.9	4.1		4.3	4.0		3.5	4.9		3.5	4.5		3.2	4.1	2.2	3.6	5.0
18:0	2.9	4.9	4.2	2.9	4.6	3.9	3.0	3.3	4.5	2.9	3.2	4.5	3.0	3.1	4.0	3.1	3.1	4.0
20:4n6			2.8			2.9		2.7	3.2		2.7	3.4		2.4	3.1		2.2	3.0
20:5n3	3.4	2.9	3.9	3.0		4.0	3.4	4.5	4.3	3.3	5.0	4.7	3.1	3.9	4.2	3.1	4.1	4.6
20:4n3	2.2	3.0		2.1			2.3	2.2	2.2	2.2	2.3	2.6	2.1		2.1	2.7	3.4	4.2
20:1n9+11	2.5	2.2	2.7	2.3	2.8	2.4	3.0	2.0	2.6	2.4	2.4	2.9	2.7	2.1	2.7	2.9	2.2	2.9
22:5n6	6.9	6.7	7.6	7.1	3.7	7.5	5.7	7.5	5.3	7.0	6.9	4.9	6.2	7.9	6.3	3.5	4.8	4.1
22:6n3	19	16	19	19	17	19	16	21	13	20	20	13	17	21	16	11	14	12
22:4n6			2.4			2.4			2.0						2.1			
sum SFA[4]	28[b]	34[b]	29[b]	31[b]	35[b]	28[b]	27[a]	26[a]	27[b]	29[b]	26[b]	28[c]	29[b]	27[b]	26[b]	28[b]	30[c]	28[c]
sum MUFA[5]	28[b]	26[b]	26[b]	26[b]	32[b]	25[b]	30[b]	25[b]	32[b]	27[b]	26[b]	31[b]	29[b]	24[b]	30[b]	33[b]	27[c]	31[c]
sum PUFA[6]	44[b]	39[b]	45[b]	42[b]	32[b]	46[b]	42[b]	49[c]	40[b]	44[b]	47[b]	41[c]	41[b]	48[c]	44[b]	38[b]	42[b]	41[b]
sum other	0.8	0.7	0.2	0.8	0.6	0.6	0.8	0.8	0.4	0.8	0.8	0.5	0.8	0.8	0.5	0.8	0.8	0.5
TFA[7] mg g^{-1}	68[b]	53[b]	38[a]	55[b]	37[b]	26[a]	77[b]	52[b]	29[a]	85[b]	40[c]	22[a]	76[b]	53[c]	25[a]	79[b]	46[c]	27[a]

1:    fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 20:3n6, 20:2n6, 20:0, 22:5n3, C22PUFA

2:    6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso

3:    T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for T0 all have the superscript "a")

Table 33   Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of starved rotifers and those fed Algamac, Isochrysis sp (clone T. Iso) or yeast following 6 and 24 h enrichment and a further 24 h starvation [Trial 2]

| | Enrichment diet | | | | | | | | | | | | | | |
| | Algamac | | | Algamac x2[2] | | | T. Iso | | | Yeast | | | Starved | | |
Fatty acid	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	9.2	7.8	4.2	9.2	7.4	3.9	4.3	4.6	3.9		2.1				
16:1n7	7.2	7.0	5.5	6.5	6.6	4.1	13.4	11.9	11.1	20.1	20.2	18.3	15.8	15.0	14.4
16:0	23.9	28.0	24.0	24.2	21.6	22.0	9.6	10.9	10.8	10.5	9.4	9.1	10.5	10.5	10.5
18:4n3							8.6	7.0	5.8	2.1	2.4	2.7		2.5	2.7
18:2n6	4.2	3.1	3.0	3.8	3.0	2.4	10.4	9.8	9.8	8.0	3.6	6.9	10.1	9.9	9.3
18:1n9	9.9	10.9	9.7	9.6	10.5	9.4	24.1	23.7	22.7	30.3	31.0	29.3	28.4	28.3	26.3
18:1n7		2.9	3.1		2.8	2.3	2.7	4.0	5.5	3.4	4.5	5.5	3.4	4.3	6.2
18:0	2.5	3.9	6.0	2.4	3.3	7.0	3.7	4.1	4.2	4.9	4.9	4.8	4.9	4.9	5.1
20:4n6		2.7			2.8										
20:5n3	2.2	2.2	3.0	2.3	2.8	2.7	2.9	2.8	3.1	2.4	2.6	2.4	3.1	3.0	2.8
20:4n3							3.2	3.7	3.5	2.6	2.2	2.4	3.1	2.5	2.4
20:1n9+11		2.1	4.2		2.4	5.5	4.1	3.5	3.7	4.3	4.3	4.0	4.5	3.9	4.3
20:0															
22:5n6	7.4	5.5	4.5	6.8	7.4	4.5									
22:6n3	19.9	14.9	10.7	21.7	19.9	11.0	2.4	2.2	2.2						
22:4n6			5.4			8.3									
sum SFA[4]	37^b	41^b	38^b	37^b	34^b	37^b	19^a	22^b	22^b	19^a	19^a	18^a	19^a	20^a	20^a
sum MUFA[5]	22^b	25^b	26^b	21^b	23^b	25^c	47^b	46^b	46^b	60^a	62^a	60^a	55^a	54^a	54^a
sum PUFA[6]	40^a	33^a	37^a	41^b	42^b	39^a	34^b	32^b	32^b	20^b	19^b	22^b	26^a	26^a	25^a
sum other	0.9	0.8	0.2	0.9	0.9	0.5	0.7	0.6	0.3	0.7	0.6	0.3	0.6	0.5	0.6
TFA[7] mg g^{-1}	111^b	32^a	17^a	94^b	57^a	13^c	43^a	25^a	27^a	31^a	28^a	18^a	29^a	27^a	12^b

1:    fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 20:3n6, 20:2n6, 22:5n3, C22PUFA

2:    x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3:    T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7:   SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for T0 all have the superscript "a")

At T6, all fed rotifers contained higher absolute levels of TFA (mg g^{-1} dry rotifer weight) than at T0. Rotifers fed only ACEM 000A contained TFA levels of between 104 and 142 mg g^{-1}. Rotifers fed the ACEM 000A-T. Iso or ACEM 000A-ACEM 6063 mixtures contained 148 and 157 mg g^{-1} respectively. Rotifers fed ACEM 6063 (alone or mixed with T. Iso) contained TFA levels between 55 and 85 mg g^{-1}. Algamac-fed rotifers contained TFA levels of about 100 mg g^{-1}. The lowest TFA levels at T6 were found in T. Iso-fed, yeast-fed and starved rotifers (43, 31, and 29 mg g^{-1} respectively).

At T24, highest absolute TFA levels were found in rotifers fed only ACEM 000A or the ACEM 000A-T. Iso mixture (100 – 111 mg g^{-1}). TFA levels in all other treatments were quite variable, and ranged from 25 – 57 mg g^{-1}.

At T48, absolute TFA levels in all rotifers had decreased markedly from those recorded at T24. The highest TFA level was found in rotifers fed the ACEM 000A-T. Iso mixture (80 mg g^{-1}). TFA levels in all other treatments were again quite variable, ranging between 13 and 48 mg g^{-1}.

The absolute levels of AA, EPA and DHA (mg g^{-1} dry weight) in rotifers at the three time points are shown in Figure 11. At T6, the highest AA levels (7 – 10 mg g^{-1}) were found in ACEM 000A-fed rotifers. All other rotifers contained AA levels between 0.4 mg g^{-1} (yeast-fed rotifers) and 1 mg g^{-1} [ACEM 6063 and Algamac-fed rotifers]. Similarly, the highest EPA levels (8 – 11 mg g^{-1}) were found in ACEM 000A-fed rotifers. Other rotifers contained EPA between 0.8 mg g^{-1} (yeast-fed rotifers) and 3 mg g^{-1} [ACEM 6063 (freeze-dried)-fed rotifers]. The highest DHA levels at T6 (~ 20 mg g^{-1}) were found in Algamac-fed rotifers. Rotifers fed only ACEM 6063, and the ACEM 6063-T. Iso mixture, contained DHA levels of between 9 and 17 mg g^{-1}. Levels of DHA in the other samples varied between < 0.1 mg g^{-1} (yeast-fed rotifers) and 4 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture).

At T24 (Figure 11), the highest absolute AA levels (5 – 10 mg g^{-1}) were found in rotifers fed only ACEM 000A. AA levels in all other samples were between 0.4 mg g^{-1} (T. Iso-fed rotifers) and 3 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture). Absolute EPA levels were highest in rotifers fed only ACEM 000A or the ACEM 000A-T. Iso mixture (4 – 9 mg g^{-1}).
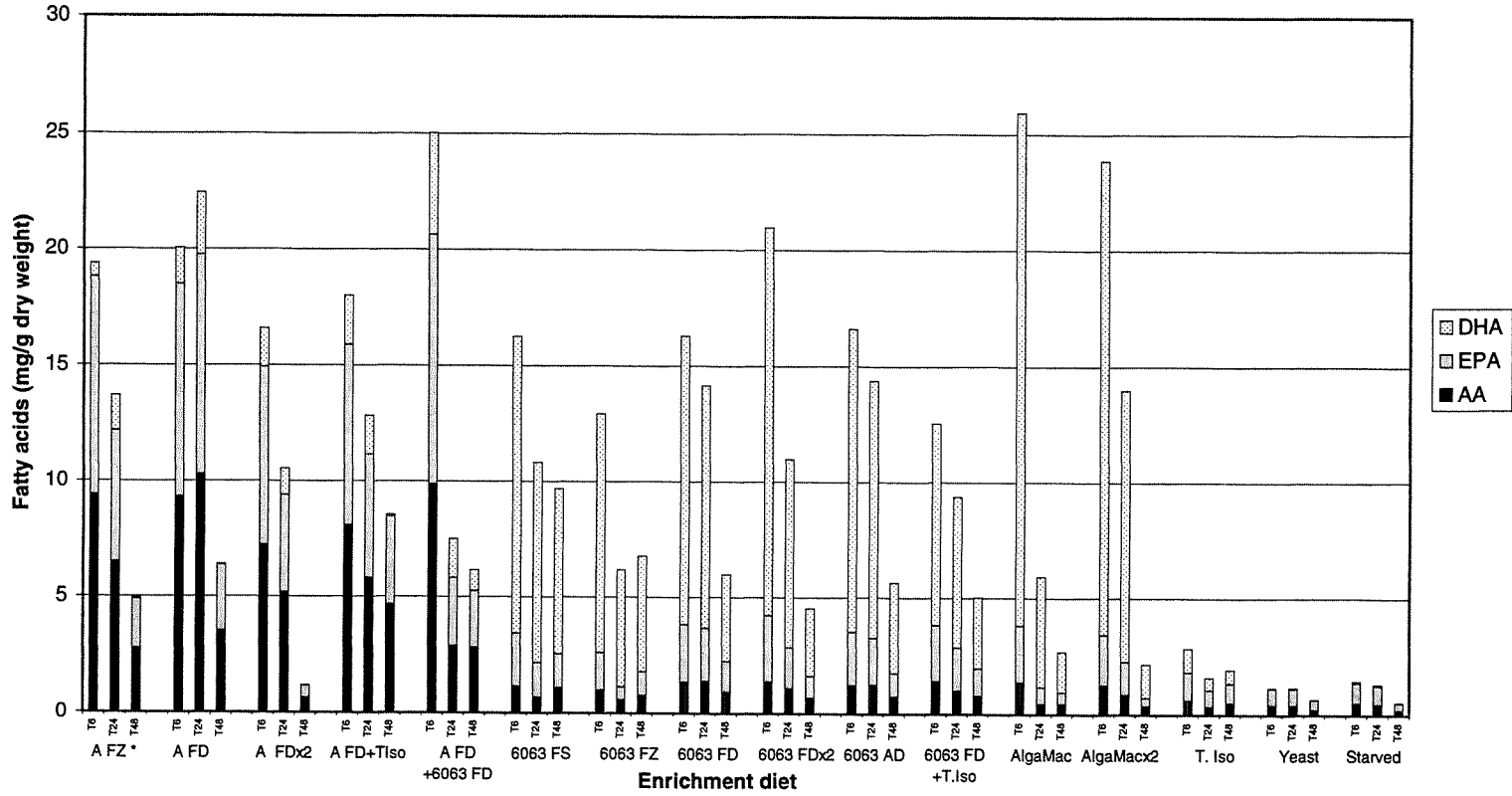
Figure 11   Mean amounts (mg g^{-1} dry weight; n=2) of 20:4n6 (AA), 20:5n3 (EPA) and 22:6n3 (DHA) in rotifers following 6
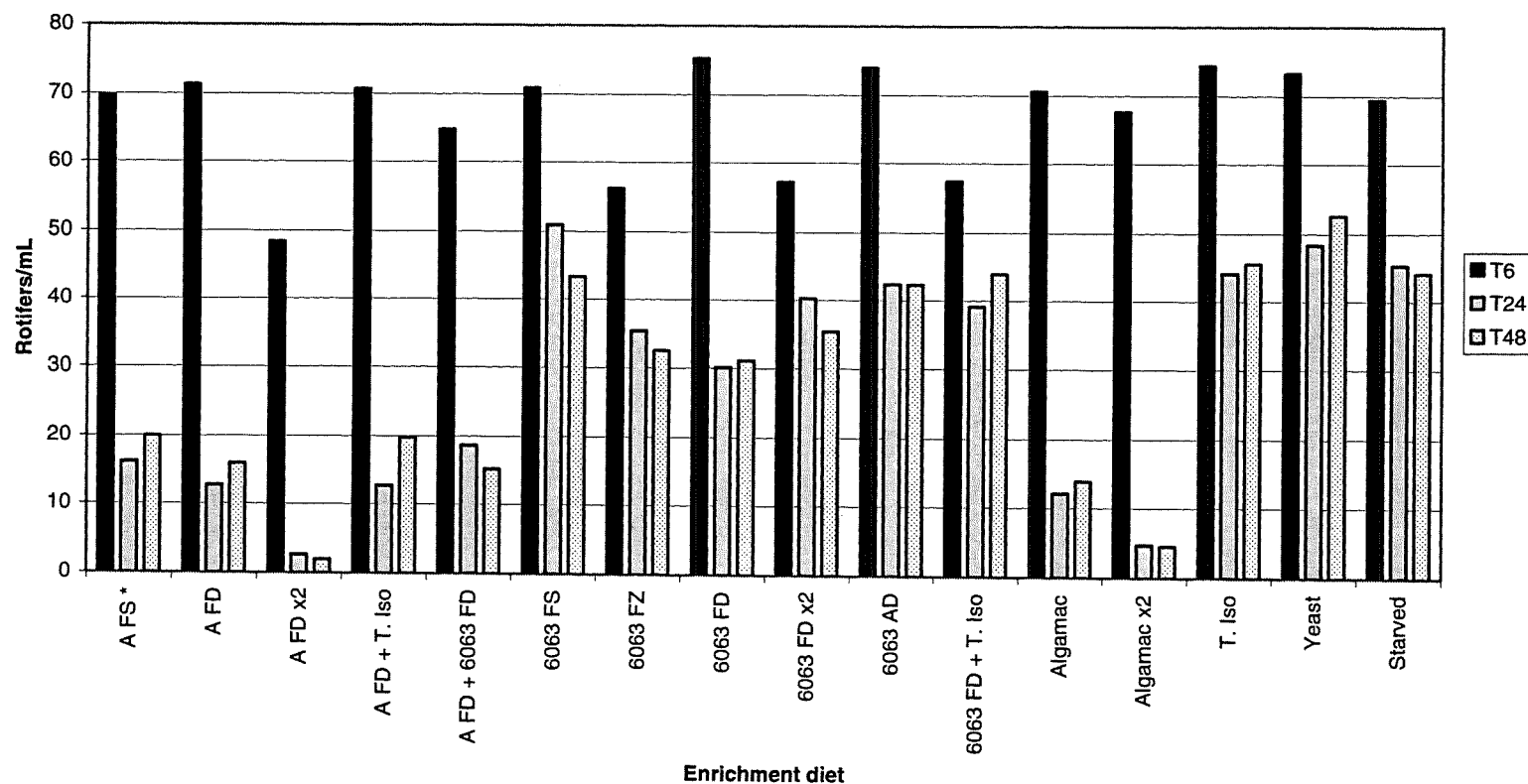
(T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 2]

*:   A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate
(200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

EPA levels in other rotifers varied between 0.7 mg g^{-1} (yeast- and T. Iso-fed rotifers) and 3 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture). Absolute DHA levels were highest in rotifers fed only ACEM 6063, the ACEM 6063-T. Iso mixture, and Algamac (7 – 11 mg g^{-1}). DHA levels in all other rotifers varied between 0.1 mg g^{-1} (yeast-fed, T. Iso-fed and starved rotifers) and 3 mg g^{-1} [ACEM 000A (freeze-dried)-fed rotifers].

At T48 (Figure 11), absolute AA, EPA and DHA levels had all fallen from those at T24. The highest AA levels were found in rotifers fed frozen or freeze-dried ACEM 000A at 100 mg L^{-1} dry weight equivalent, the ACEM 000A-T. Iso mixture, and the ACEM 000A-ACEM 6063 mixture (3 – 5 mg g^{-1}). All other rotifers contained AA levels between 0.2 mg g^{-1} (Algamac-fed rotifers) and 1 mg g^{-1} [ACEM 6063 (fresh)-fed rotifers]. The highest EPA levels at T48 were in rotifers fed frozen or freeze-dried ACEM 000A at 100 mg L^{-1} dry weight equivalent, the ACEM 000A-T. Iso mixture, and the ACEM 000A-ACEM 6063 mixture (2 – 4 mg g^{-1}). All other rotifers contained EPA levels of < 2 mg g^{-1}. Absolute DHA levels were highest in rotifers fed only fresh ACEM 6063 (7 mg g^{-1}). Rotifers fed only frozen, freeze-dried or air-dried ACEM 6063, or the ACEM 6063-T.Iso mixture contained between 3 and 5 mg g^{-1}. All other rotifers contained DHA at levels between < 0.1 mg g^{-1} and 2 mg g^{-1} (yeast- and Algamac-fed rotifers respectively).

The density of rotifers was assessed at each sampling time (Figure 12). At T6, rotifer densities had decreased markedly (48 – 75 mL^{-1} c.f. 200 mL^{-1} at T0) in all treatments. There were no significant differences between rotifer densities in any of the treatments at T6. Rotifer densities in all treatments decreased significantly between T6 and T24. At T24, density was highest amongst starved rotifers and those fed ACEM 6063 (excluding rotifers fed the ACEM 000A-ACEM 6063 mixture), yeast and T. Iso (30 – 51 rotifers mL^{-1}). Counts for the other treatments varied between 3 and 19 rotifers mL^{-1}. Rotifer counts for all treatments at T48 showed no significant change from those at T24. At T48, density was highest for starved rotifers and those fed ACEM 6063 (excluding rotifers fed the ACEM 000A-ACEM 6063 mixture), yeast and T. Iso (31 – 53 rotifers mL^{-1}). Counts for the other treatments varied between 2 and 20 rotifers mL^{-1}. The lowest counts were seen in treatments fed either freeze-dried ACEM 000A or Algamac at 200 mg L^{-1} (2 and 5 mL^{-1} respectively).

Figure 12   Mean rotifer density (number mL^{-1}; n=2) following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 2]

*:   A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*
For all treatments, rotifer densities at T0 (200 rotifers ml^{-1}) were significantly higher (p<0.05) that at T6, densities at T6 were significantly higher that at T24, and densities at T24 were not significantly different from values at T48

2.4.3 Atlantic salmon feeding trial

There were no significant differences between the dietary treatments in growth performance as measured by final weight, weight gain or survival (Table 34). Total feed consumption and weight specific feed consumption were not different between the diets either and this meant that diet had no effect on FER or PPV (Table 34). Diet had no effect on whole body chemical composition (Table 35).

Diet had an effect on the distribution of lipid in tissues and the tissue profiles tended to reflect the dietary fatty acid profiles (Table 36). In the liver the PL accounted for more of the lipid than TAG and PL fraction was significantly higher for CTH. The proportion of FA as OA and LNA were higher in CO, EPA was highest in CFO and DHA higher in CTH than CO. There were no differences in n-6 FA. In the heart TAG were signficantly higher in CO than CTH but there were no differences between PL. As in the liver OA and LNA were highest for CO and EPA highest for CFO. LA was significantly different for all diets and highest in CO, there were no differences in DHA or AA. In the muscle TAG and PL were approximately similar and there were no differences between diets. OA, LA, AA and LNA were highest in CO, EPA highest for CFO and DHA highest for CTH. In the eye OA, LA and LNA were higher for CO compared with CFO. The n-3/n-6 ratio was significantly higher for CFO for all tissues although the magnitude of the ratio differed between tissues and was highest in muscle (Table 36).

Apparent digestibility coefficients calculated from the yttrium marker showed there were no differences in crude protein or energy digestibility (Table 37). ADC for total lipid, TAG and PL were affected by diet and were lower for CTH (Table 37). Digestibility of fatty acids were high at over 99% and there were few major differences between the diets (Table 37).

No significant differences ($P > 0.05$) were detected between diets for any of the immune function or blood chemistry parameters measured (Table 38). Similarly, no significant differences were detected between osmotic erythrocyte fragility curves between the different diets (Fig. 1). Mean concentrations of NaCl where 50% haemolysis occurred were 0.44 ($\pm$ 0.01), 0.47 ($\pm$ 0.01) and 0.44 ($\pm$ 0.01) for diets CFO, CO and CTH, respectively.

Table 34   The performance of Atlantic salmon fed experimental diets
              containing different oil sources

Parameter		Diet		
		CO	CTH	CFO
Initial weight	(g)	36.4 ± 0.1	36.7 ± 0.5	36.5 ± 0.2
Final weight	(g)	107.6 ± 2.8	105.8 ± 0.4	105.0 ± 2.4
Weight gain	(g)	71.1 ± 2.8	69.2 ± 0.7	68.5 ± 2.4
Total feed consumption	(g DM)	1499 ± 41	1562 ± 41	1423 ± 10
FC	$(mg\ DM.g^{-1}.d^{-1})$	16.3 ± 0.3	17.1 ± 0.4	16.2 ± 0.4
FER	$(g \cdot g^{-1})$	1.19 ± 0.03	1.10 ± 0.04	1.16 ± 0.05
PPV	(%)	49.1 ± 4.6	52.6 ± 2.3	51.9 ± 2.0
Overall survival	(%)	100	98.7 ± 1.3	98.7 ± 1.4

Each value is the mean ($\pm$ SEM) of three replicates

FC.   feed consumption = total feed consumption (g DM) / Σ individual mid-weight (g) / 51 days

FER.   feed efficiency ratio = (total weight gain (g) / total feed consumption (g DM))

PPV.   productive protein value = 100.(fish protein gain (g CP) / total protein consumption (g CP))

Table 35   Chemical composition of Atlantic salmon fed experimental diets
              containing different oil sources

Parameter		Diet		
		CO	CTH	CFO
Dry matter	(%)	26.9 ± 1.5	29.4 ± 0.5	27.7 ± 0.9
Crude protein	(%)	16.0 ± 0.9	17.3 ± 0.1	16.6 ± 0.5
Total lipid	(%)	2.4 ± 0.3	2.9 ± 0.2	2.8 ± 0.3
Ash	(%)	1.9 ± 0.1	2.2 ± 0.1	1.9 ± 0.1

Each value is the mean ($\pm$ SEM, n=3, 3 fish pooled per replicate)

Initial group (mean $\pm$ sd; n = 6): 25.9 ± 1.0 % DM; 15.1 ± 0.1 % crude protein; 2.2 ± 0.1 % total lipid; $\pm$ % ash.

Table 36 Organ somatic indices and tissue content of triacylglycerol (TAG), phospholipid (PL) and selected fatty acids (% total fatty acid) of Atlantic salmon fed experimental diets containing different oil sources

Parameter	Diet			P
	CO	CTH	CFO	
Liver (% wet weight)	1.64 ± 0.08	1.46 ± 0.05	1.40 ± 0.12	ns
TAG (mg.g^{-1})	2.3 ± 1.4	5.3 ± 1.6	8.6 ± 2.6	ns
PL (mg.g^{-1})	$6.5^b \pm 1.1$	$24.0^a \pm 3.9$	$13.2^b \pm 3.1$	<0.05
18:1n-9	$49.50^a \pm 1.30$	$42.06^b \pm 0.13$	$40.35^b \pm 1.28$	<0.005
18:2n-6	8.83 ± 0.19	8.73 ± 0.52	7.93 ± 0.43	ns
20:4n-6	1.60 ± 0.30	1.77 ± 0.16	0.97 ± 0.17	ns
18:3n-3	$5.50^a \pm 0.15$	$4.67^b \pm 0.01$	$4.48^b \pm 0.14$	<0.005
20:5n-3	$1.17^b \pm 0.19$	$1.30^b \pm 0.15$	$3.37^a \pm 0.15$	<0.0001
22:6n-3	$3.26^b \pm 0.69$	$7.40^a \pm 0.55$	$6.10^{ab} \pm 0.95$	<0.05
n-3/n-6	$1.02^b \pm 0.06$	$1.10^b \pm 0.03$	$1.55^a \pm 0.11$	<0.005
Heart (% wet weight)	0.18 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	ns
TAG (mg.g^{-1})	$0.8^a \pm 0.1$	$0.3^b \pm 0.1$	$0.4^{ab} \pm 0.1$	<0.05
PL (mg.g^{-1})	4.4 ± 0.3	3.7 ± 0.1	4.4 ± 0.6	ns
18:1n-9	$43.17^a \pm 0.57$	$33.66^b \pm 0.44$	$30.45^c \pm 0.25$	<0.0001
18:2n-6	$10.27^a \pm 0.14$	$7.33^b \pm 0.27$	$5.57^c \pm 0.41$	<0.0001
20:4n-6	0.83 ± 0.09	0.87 ± 0.09	0.80 ± 0.06	ns
18:3n-3	$4.80^a \pm 0.06$	$3.74^b \pm 0.05$	$3.38^c \pm 0.03$	<0.0001
20:5n-3	$0.70^b \pm 0.10$	$0.63^b \pm 0.14$	$1.80^a \pm 0.20$	<0.005
22:6n-3	2.23 ± 0.28	4.36 ± 0.87	3.93 ± 0.48	Ns
n-3/n-6	$0.78^b \pm 0.02$	$0.95^b \pm 0.07$	$1.46^a \pm 0.01$	<0.0001
White muscle				
TAG (mg.g^{-1})	2.0 ± 0.4	2.3 ± 0.7	2.9 ± 1.6	ns
PL (mg.g^{-1})	3.7 ± 1.3	2.5 ± 0.7	3.4 ± 0.6	ns
18:1n-9	$34.65^a \pm 0.36$	$26.91^b \pm 1.89$	$24.03^b \pm 1.41$	<0.005
18:2n-6	$11.73^a \pm 0.26$	$8.80^{ab} \pm 0.74$	$7.23^b \pm 0.53$	<0.005
20:4n-6	$1.30^a \pm 0.06$	$1.10^{ab} \pm 0.10$	$0.93^b \pm 0.03$	<0.05
18:3n-3	$3.85^a \pm 0.04$	$2.99^b \pm 0.21$	$2.67^b \pm 0.16$	<0.005
20:5n-3	$3.17^b \pm 0.12$	$2.60^b \pm 0.06$	$6.80^a \pm 0.30$	<0.0001
22:6n-3	$12.33^b \pm 0.32$	$19.43^a \pm 1.83$	$17.00^{ab} \pm 1.53$	<0.05
n-3/n-6	$1.59^b \pm 0.04$	$2.05^b \pm 0.19$	$3.37^a \pm 0.33$	<0.005
Eye (% wet weight)	0.50 ± 0.01	0.50 ± 0.01	0.49 ± 0.02	ns
TAG (mg.g^{-1})	27.5 ± 3.7	17.9 ± 3.0	23.4 ± 4.1	ns
PL (mg.g^{-1})	$7.6^b \pm 2.5$	$11.9^b \pm 3.0$	$23.7^a \pm 2.5$	<0.05
18:1n-9	$41.58^a \pm 1.31$	$35.58^b \pm 1.07$	$32.04^b \pm 0.32$	<0.005
18:2n-6	$9.63^a \pm 0.81$	$7.33^{ab} \pm 0.82$	$5.97^b \pm 0.19$	<0.01
20:4n-6	0.43 ± 0.03	0.33 ± 0.03	0.27 ± 0.09	Ns
18:3n-3	$4.62^a \pm 0.15$	$3.95^b \pm 0.12$	$3.56^b \pm 0.04$	<0.005
20:5n-3	1.93 ± 0.33	1.80 ± 0.42	2.37 ± 0.12	Ns
22:6n-3	3.73 ± 0.93	4.73 ± 0.90	3.77 ± 0.13	Ns
n-3/n-6	$0.91^c \pm 0.01$	$1.21^b \pm 0.03$	$1.56^a \pm 0.01$	<0.0001

Each value is the mean ($\pm$ SEM) of three replicates (3 fish per replicate)

Initial group (mean $\pm$ sd; n = 9) liver = $1.03 \pm 0.16\%$BW; heart = 0.19 ± 0.04 %BW; eye = 0.79 ± 0.12 %BW

Table 37   Apparent digestibility coefficients (ADC) for crude protein (N), total lipid (TL), energy (kJ), phosphorous (P) and fatty acids for diets containing different oil sources

Parameter	Diet			p
	CO	CTH	CFO	
ADC_N (%)	$93.2^a \pm 0.2$	$92.4^b \pm 0.1$	$93.2^a \pm 0.5$	ns
ADC_{kJ} (%)	85.9 ± 0.2	84.1 ± 1.1	84.0 ± 1.4	ns
ADC_{TL} (%)	$95.9^a \pm 0.3$	$94.1^b \pm 0.2$	$95.5^{ab} \pm 0.6$	<0.01
ADC_{TAG} (%)	$99.9^a \pm 0.0$	$99.6^b \pm 0.0$	$99.9^a \pm 0.0$	<0.0001
ADC_{PL} (%)	79.3 ± 1.9	73.3 ± 1.9	67.1 ± 7.6	ns
$ADC_{16:0}$ (%)	$99.5^a \pm 0.1$	$97.6^c \pm 0.1$	$99.0^b \pm 0.1$	<0.0001
$ADC_{18:1n-9}$ (%)	$99.7^{ab} \pm 0.1$	$99.8^a \pm 0.1$	$99.6^b \pm 0.1$	<0.05
$ADC_{18:2n-6}$ (%)	$99.8^{ab} \pm 0.1$	$99.9^a \pm 0.0$	$99.7^b \pm 0.1$	<0.05
$ADC_{18:3n-3}$ (%)	99.7 ± 0.1	99.8 ± 0.1	99.6 ± 0.1	Ns
$ADC_{20:5n-3}$ (%)	99.4 ± 0.3	99.7 ± 0.1	99.9 ± 0.0	ns
$ADC_{22:6n-3}$ (%)	99.6 ± 0.1	99.5 ± 0.1	99.7 ± 0.1	ns
ADC_P (%)	$38.1^{ab} \pm 1.9$	$42.7^a \pm 0.2$	$34.2^b \pm 1.2$	< 0.05

Each value is the mean (± SEM) of three replicates

Means with the same letter are not significantly different (Tukey-Kramer HSD multiple comparison)

Table 38   Immune response of Atlantic salmon fed diets containing different oil sources

Parameter	Diet		
	CO	CTH	CFO
Neutrophil (mg mL^{-1})	0.465 ± 0.026	0.519 ± 0.021	0.485 ± 0.080
Lysozyme (µg mL^{-1})	3.45 ± 0.29	3.36 ± 0.43	3.24 ± 0.41
Antiprotease (µl plasma for 50% inhibition)	1.14 ± 0.08	1.40 ± 0.09	1.29 ± 0.16
Total protein (mg.mL^{-1})	43.9 ± 4.2	45.0 ± 4.7	54.6 ± 1.2
Total immunoglobulin (mg.mL^{-1})	14.1 ± 2.9	20.5 ± 4.3	21.3 ± 4.6
Glucose (µmol.mL^{-1})	7.87 ± 1.27	6.97 ± 1.11	8.41 ± 1.16

Each value is the mean (± SEM) of three replicates

Initial samples (mean ± SEM, n=4): neutrophil 0.405 ± 0.038 mg.ml^{-1}; lysozyme 3.43 ± 0.57 µg.ml^{-1}; antiprotease 1.10 ± 0.09 µl serum for 50% inhibition; total protein 44.2 ± 3.8 mg.ml^{-1}; total immunoglobulin 19.0 ± 3.1 mg.ml^{-1}; glucose 6.82 ± 0.9 µmol.ml^{-1}.
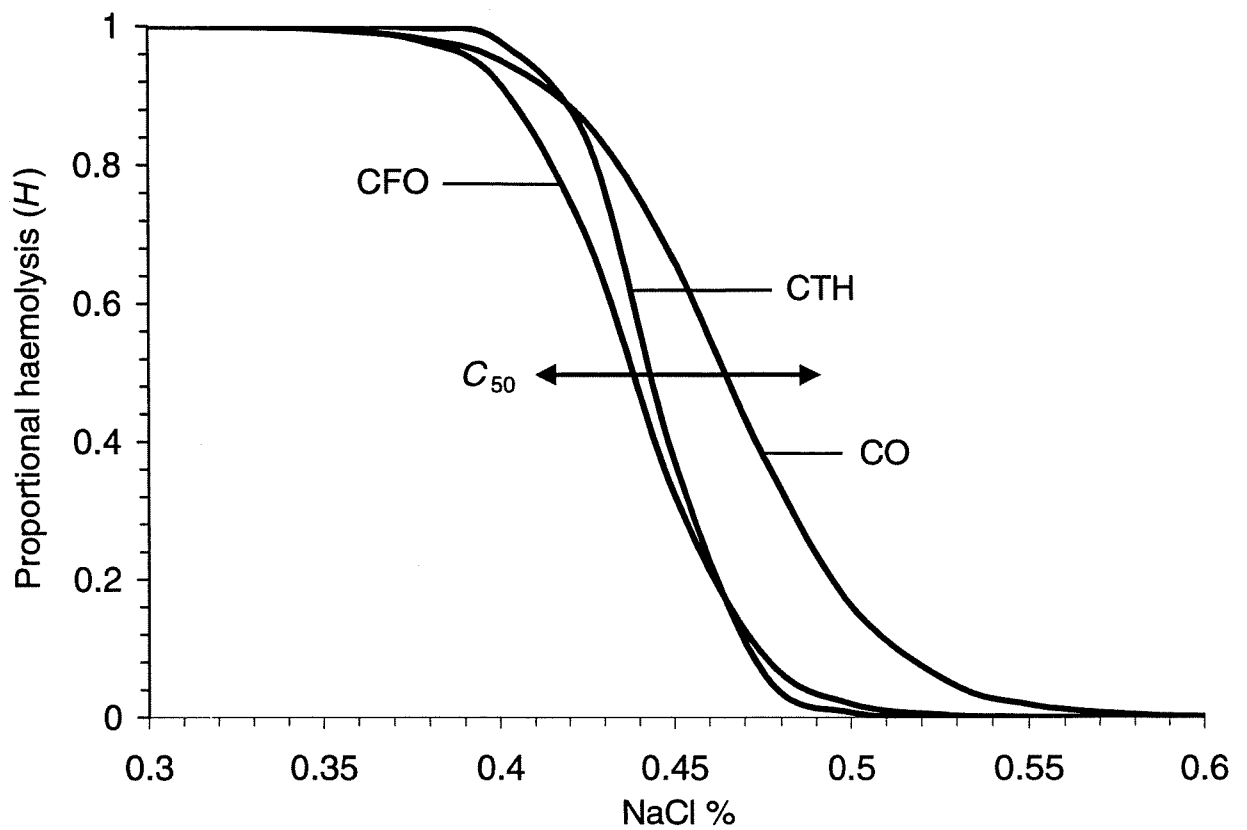
Figure 13   Osmotic erythrocyte fragility curve of Atlantic salmon fed diets
             containing different oil sources. Curves are marked for each diet
             (CFO, CO, CTH), with the point where 50% haemolysis occurs
             marked (C_{50}). No significant differences ($P > 0.05$) were found
             between diets.

2.4.4 Striped trumpeter feeding trial

The total fatty acid profile (%TFA) and absolute amounts of fatty acids (mg g^{-1} dry weight) in rotifers either starved for 12 hours or enriched with DHA-Selco or ACEM 6063 are shown in Table 39 and Table 40. The proportions of AA, EPA, MUFA and PUFA in rotifers enriched with ACEM 6063 were significantly lower than in starved rotifers and those enriched with DHA-Selco. The proportion of DHA and SFA in the starved rotifers was significantly lower than in rotifers enriched with DHA-Selco or ACEM 6063 Table 39. The absolute amounts of AA, EPA, MUFA and PUFA were significantly higher in rotifers enriched with DHA-Selco than in starved rotifers or those enriched with ACEM 6063. The absolute amounts of DHA, SFA and TFA were significantly lower in starved rotifers than in rotifers enriched with DHA-Selco or ACEM 6063.

Table 39   Proportion of fatty acids (% total fatty acids) in rotifers (mean ± sd, n=5)

Diet [1]	AA	EPA	DHA	SFA	MUFA	PUFA
STV	1.5±0.3 [a]	4.0±0.8 [a]	0.7±0.2 [a]	19±1 [a]	48±4 [a]	33±4 [ab]
Selco	1.7±0.3 [a]	4.7±0.2 [a]	13.6±0.9 [b]	28±2 [b]	35±3 [b]	37±3 [a]
6063	0.6±0.1 [b]	1.7±0.2 [b]	10.5±2.3 [b]	56±4 [c]	20±1 [c]	24±4 [b]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For diet descriptions – see section 1.4.4.1, page 22

Table 40   Absolute amounts of fatty acids (mg g^{-1} dry weight) in rotifers (mean ± sd, n=5)

Diet [1]	AA	EPA	DHA	SFA	MUFA	PUFA	TFA
STV	0.3±0.0 [a]	0.9±0.0 [a]	0.2±0.1 [a]	4.2±0.8 [a]	10.6±2.9 [a]	7.1±0.6 [a]	21.9±4.1 [a]
Selco	0.9±0.1 [b]	2.6±0.1 [b]	7.3±0.5 [b]	14.9±0.6 [b]	18.9±2.4 [b]	20.0±1.4 [b]	53.9±2.1 [b]
6063	0.3±0.0 [a]	0.9±0.1 [a]	5.5±1.3 [b]	29.2±1.9 [c]	10.2±0.7 [a]	12.7±2.3 [c]	52.1±0.5 [b]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For diet descriptions – see section 1.4.4.1, page 22

Mean treatment data for larval length, weight and total numbers are shown in Figure 14. There was no significant difference due to diet detected for any of these attributes. Larval length increased significantly in all treatments during the trial. Total larval numbers decreased markedly in all treatments during the feeding trial. Individual larval weights did not vary significantly during the trial, but the average larval weight in each treatment at the end of the trial were less than at the start of the trial.
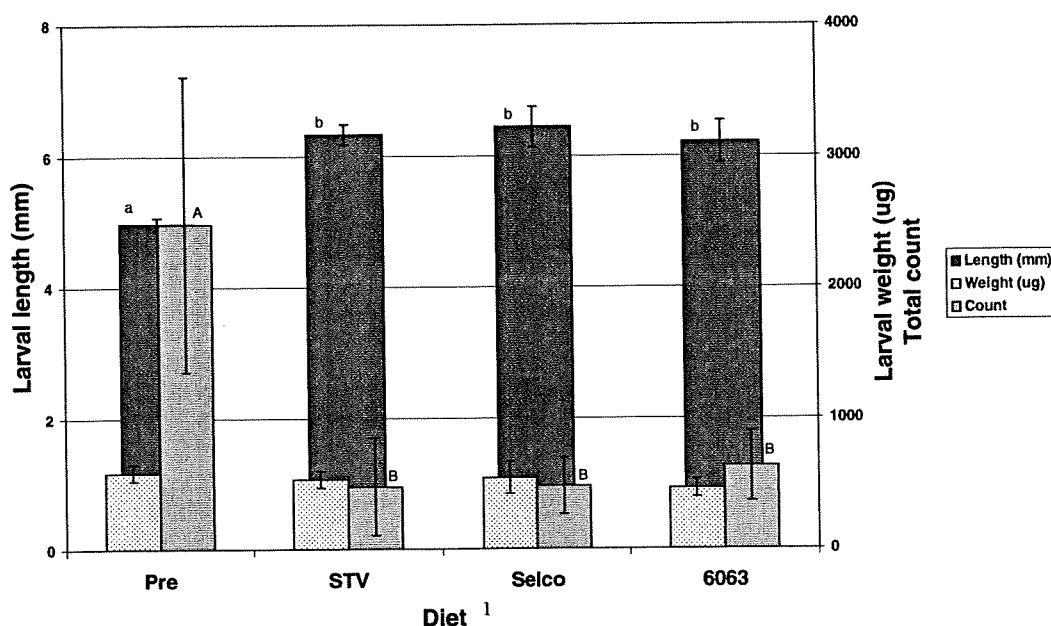
Figure 14   Mean length, weight and total numbers of striped trumpeter larvae at the start and end of the trial (tank mean±sd, n=4)

Values for each attribute with different superscripts are significantly different ($\alpha = 0.05$)

1: For diet descriptions – see section 1.4.4.1, page 22

The total fatty acid profile (%TFA) and absolute amounts of fatty acids (expressed as mg g^{-1} dry weight and ng individual^{-1}) in larvae at the start of the trial (T_0), and larvae fed rotifers either starved for 12 hours or enriched with DHA-Selco or ACEM 6063 are shown in Table 41, Table 42 and Table 43. The proportions of AA and MUFA in the larvae did not vary markedly between any of the treatments and were similar to those in larvae at T_0. The proportions of EPA and SFA in the larvae did not vary markedly between any of the treatments, but were lower and higher, respectively, than those in larvae at T_0. The proportion of DHA in larvae fed the ACEM 6063 enriched rotifers were not significantly different, but were both significantly higher than in larvae fed starved rotifers, or rotifers enriched with DHA-Selco. The proportion of PUFA in larvae at T_0 and in larvae fed rotifers enriched with DHA-Selco or ACEM 6063 were not significantly different, but were all significantly higher than in larvae fed starved rotifers.

Table 41   Proportion of fatty acids (% total fatty acids) in striped trumpeter larvae fed rotifers enriched by different methods (mean ± sd, n=4)

Diet[1]	AA	EPA	DHA	SFA	MUFA	PUFA
Pre	2.6±0.1 [b]	8.5±0.3 [c]	23.9±1.1 [a]	30±1 [b]	26±1 [a]	44±2 [c]
STV	2.9±0.1 [a]	4.7±0.1 [a]	10.8±0.4 [b]	30±1 [b]	35±1 [b]	34±1 [b]
Selco	3.3±0.1 [c]	4.7±0.1 [a]	18.4±0.8 [c]	29±1 [b]	31±1 [b]	40±1 [a]
6063	3.1±0.1 [a]	3.5±0.3 [b]	22.1±1.7 [a]	33±2 [a]	27±1 [a]	40±2 [a]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For diet descriptions – see section 1.4.4.1, page 22

The absolute amounts of fatty acids in the larvae showed similar trends when expressed in terms of either mg g^{-1} dry weight or µg individual^{-1}. The absolute amounts of AA, SFA, MUFA and TFA were similar between treatments, but were all generally greater than the absolute levels in larvae at T_0. Absolute levels of EPA were similar between larvae at T_0 and larvae from all treatments. Absolute levels of DHA and PUFA were higher in larvae fed ACEM 6063 or Selco-DHA enriched rotifers than in larvae at T_0 or fed starved rotifers.

Table 42   Absolute amounts of fatty acids (mg g^{-1} dry weight) in striped trumpeter larvae fed rotifers enriched by different methods (mean ± sd, n=4)

Diet[1]	AA	EPA	DHA	SFA	MUFA	PUFA	TFA
Pre	0.4±0.1 [a]	1.2±0.2	3.3±0.5 [a]	4.1±0.3 [a]	3.6±0.3 [a]	6.1±0.9	13.8±1.5 [a]
STV	0.7±0.1 [ab]	1.2±0.2	2.7±0.5 [a]	7.5±1.2 [ab]	9.2±1.9 [b]	8.8±1.7	25.6±4.9 [ab]
Selco	1.0±0.3 [b]	1.4±0.4	5.5±1.3 [b]	8.7±2.2 [b]	9.5±2.9 [b]	12.2±3.3	30.4±8.4 [b]
6063	0.8±0.2 [ab]	0.9±0.2	5.6±1.8 [b]	8.3±2.6 [ab]	6.7±2.0 [ab]	10.3±3.5	25.3±8.0 [ab]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1: For diet descriptions – see section 1.4.4.1, page 22

Table 43   Absolute amounts of fatty acids (μg individual^{-1}) in striped trumpeter larvae fed rotifers enriched by different methods (mean ± sd, n=4)

Diet [1]	AA	EPA	DHA	SFA	MUFA	PUFA	TFA
Pre	0.2±0.01 [a]	0.7±0.02 [a]	1.9±0.1 [ab]	2.4±0.1 [a]	2.1±0.1 [a]	3.5±0.1 [a]	7.9±0.1 [a]
STV	0.4±0.04 [bc]	0.6±0.08 [a]	1.4±0.1 [b]	4.0±0.3 [b]	4.8±0.7 [b]	4.6±0.5 [ab]	13.4±1.6 [ab]
Selco	0.5±0.10 [b]	0.8±0.15 [a]	2.9±0.5 [a]	4.6±0.8 [b]	5.0±1.2 [b]	6.4±1.3 [b]	16.0±3.2 [b]
6063	0.4±0.09 [ac]	0.4±0.11 [b]	2.5±0.7 [a]	3.8±1.0 [ab]	3.1±0.8 [b]	4.7±1.5 [ab]	11.6±3.3 [ab]

Data within each column with different superscripts are significantly different (α = 0.05)

1: For diet descriptions – see section 1.4.4.1, page 22

2.4.5 Prawn feeding trial

The mean weights of prawns during this feeding trial are shown in Figure 15. At each measurement time, the mean size of prawns fed the commercial diet was greater than the mean size of prawns fed the diet incorporating ACEM 6063, which in turn was greater than those of prawns fed the basal diet and that incorporating MCT's (for details of diet contents see Table 11, page 25).
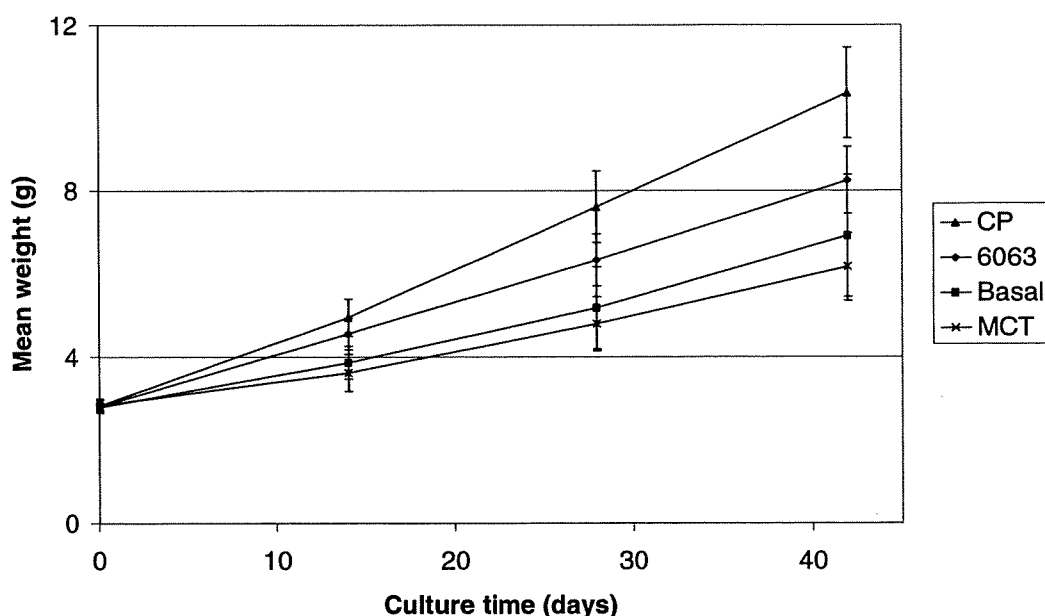
Figure 15   Weights of prawns fed different diets (mean ± sd, n=7 [2]) during feeding trial

1:    Diets: CP: CP 4004 (Charoen Pokphand, Thailand); 6063: containing ACEM 6063; Basal: CSIRO Basal diet; MCT: containing mid-chain triacylglycerols (see Table 11, page 25)

2:    Data is the mean from each of the 7 tanks receiving a given diet

Prawn performance data (final weight; feed conversion ratio – FCR: dry feed intake: weight gain; specific growth rate – SGR: % weight increase d^{-1}; survival) at harvest are shown in Table 44. The survival of prawns in this feeding trial was variable, which meant that no significant differences in survival attributable to diet were detected. Mean survival was greatest for the 6063 diet, followed by the CP diet, then the Basal and MCT diets.

In all cases except survival, prawns fed the 6063 diet performed significantly better or similarly to prawns fed the Basal diet, and performed significantly better than prawns fed the MCT diet. Prawns fed the CP diet performed significantly better than those fed the Basal and MCT diets. There was no significant difference between the apparent FCR's recorded for prawns fed the CP and 6063 diets. There were no significant differences detected in final weight, SGR and apparent FCR between prawns fed the 6063 and Basal diets. The performance of prawns fed the 6063 diet, measured as either final weight, SGR or apparent FCR, were all significantly better than those fed the MCT diet.

Table 44   Weight, SGR [1], apparent FCR [2] and survival of prawns at harvest (mean ± sd, n=7 [3])

Diet [4]	Final weight (g)	SGR (% bw d^{-1})	Apparent FCR	Survival
CP	10.4±1.1 [c]	3.2±0.3 [c]	2.4±0.2 [c]	86±10
6063	8.3±0.8 [b]	2.6±0.2 [b]	3.0±0.5 [bc]	91±11
Basal	6.9±1.5 [ab]	2.2±.0.6 [ab]	4.2±1.5 [ab]	77±18
MCT	6.2±0.8 [a]	1.9±.0.3 [a]	4.6±0.9 [a]	71±20

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1:    specific growth rate - % weight increase d^{-1}

2:    feed conversion ratio - dry feed intake: weight gain

3:    Data is the mean from each of the 7 tanks receiving a given diet

4:    Diets: CP: CP 4004 (Charoen Pokphand, Thailand); 6063: containing ACEM 6063; Basal: CSIRO Basal diet; MCT: containing mid-chain triacylglycerols (see Table 11, page 25)

The lipid profiles of whole prawns fed the different diets are shown in Table 45. The levels of polar lipid, triacylglycerol and free fatty acids in prawns fed the CP diet were significantly different than those fed the MCT diet. No other significant differences due to diet were detected.

Table 45   Total lipid profile (% total lipid) of whole prawns at harvest (tank
            mean ± sd, n=7 [1])

Diet [2]	Polar lipid	Triacylglycerol	Free fatty acid	Sterol
CP	73±5 [a]	11±4 [a]	8±2 [a]	8±1
6063	77±4 [ab]	8±3 [ab]	8±1 [ab]	8±1
Basal	75±7 [ab]	8±5 [ab]	8±3 [ab]	10±2
MCT	82±2 [b]	4±2 [b]	5±1 [b]	9±2

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1:    Data is the mean from each of the 7 tanks receiving a given diet

2:    Diets: CP: CP 4004 (Charoen Pokphand, Thailand); 6063: containing ACEM 6063; Basal: CSIRO
      Basal diet; MCT: containing mid-chain triacylglycerols (see Table 11, page 25)

A summary of the total fatty acid profile (%TFA) of prawns fed the different diets is
shown in Table 39. There were minor, but significant differences due to diet detected in the
relative proportion of all the fatty acids and fatty acid classes, except for PUFA levels,
listed in Table 39.

Table 46   Proportion of fatty acids (% total fatty acids) in whole prawns at
            harvest (tank mean ± sd, n=7 [1])

Diet [2]	AA	EPA	DHA	SFA	MUFA	PUFA
CP	3.8±0.4 [c]	7.2±0.7 [b]	14.5±0.6 [a]	34±1 [ab]	24±1 [b]	42±1
6063	3.5±0.2 [bc]	5.0±0.6 [cd]	12.3±0.4 [c]	33±1 [ab]	25±1 [ab]	42±1
Basal	2.9±0.6 [ab]	10.5±2.0 [a]	14.2±.8 [a]	32±1 [a]	27±3 [a]	41±3
MCT	2.5±0.6 [a]	6.6±1.0 [bd]	8.3±1.0 [b]	34±2 [b]	23±1 [ab]	43±3

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1:    Data is the mean from each of the 7 tanks receiving a given diet

2:    Diets: CP: CP 4004 (Charoen Pokphand, Thailand); 6063: containing ACEM 6063; Basal: CSIRO
      Basal diet; MCT: containing mid-chain triacylglycerols (see Table 11, page 25)

A summary of the total fatty acid content (mg g^{-1} wet weight) of prawns fed the different
diets is shown in Table 47. There were no significant differences due to diet detected in the
absolute amounts of DHA, SFA, MUFA, PUFA and TFA in the prawns fed the CP, 6063
and Basal diets. Absolute amounts of DHA, SFA, MUFA, PUFA and TFA in the prawns
fed the CP, 6063 and Basal diets were all significantly greater than those in prawns fed the
MCT diet. Absolute amounts of AA were greatest in prawns fed the CP and 6063 diets and

least in prawns fed the MCT diet. Absolute amounts of EPA were greatest in prawns fed the Basal and CP diets and least in prawns fed the MCT diet.

Table 47   Fatty acids (mg g^{-1} wet weight) in whole prawns at harvest (tank mean ± sd, n=7 [1])

Diet [2]	AA	EPA	DHA	SFA	MUFA	PUFA	TFA
CP	1.5±0.1 [a]	2.8±0.3 [b]	5.7±0.8 [a]	13.4±1.7 [a]	9.4±1.6 [a]	16.5±2.1 [a]	39.3±5.3 [a]
6063	1.4±0.3 [a]	2.0±0.4 [a]	4.9±1.1 [a]	13.0±3.2 [a]	10.0±2.6 [a]	16.6±3.8 [a]	39.6±9.4 [a]
Basal	1.0±0.1 [b]	3.6±0.4 [c]	4.9±0.8 [a]	11.3±2.0 [a]	9.5±2.5 [a]	14.1±1.8 [a]	34.8±6.2 [a]
MCT	0.6±0.2 [c]	1.6±0.5 [a]	2.0±0.6 [b]	7.9±1.7 [b]	5.54±1.2 [b]	10.0±2.8 [b]	23.3±5.6 [b]

Data within each column with different superscripts are significantly different ($\alpha = 0.05$)

1:    Data is the mean from each of the 7 tanks receiving a given diet

2:    Diets: CP: CP 4004 (Charoen Pokphand, Thailand); 6063: containing ACEM 6063; Basal: CSIRO Basal diet; MCT: containing mid-chain triacylglycerols (see Table 11, page 25)

Chapter 3   Discussion

3.1   Isolation and characterisation of novel Australian thraustochytrids

As outlined in Lewis *et al.* (1999) (see page 129 of this report), some thraustochytrid strains have the potential to be used as sustainable commercial sources of DHA and other PUFA. The relative level of DHA was one of the most influential factors in separating thraustochytrids strains based on whole cell fatty acid profiles of 5 day old thraustochytrid plate cultures. The mean levels of the nutritionally important PUFA AA, EPA and DHA in the strains grouped in the upper branch of S1 were 0.7, 5.0 and 56 %TFA respectively (Figure 1). Corresponding values in the lower branch of S1 were 8.6, 12.0 and 18 %TFA respectively (Figure 1). From a nutritional point of view, this is potentially important information. It is becoming clear that not only is the total amount of n3 PUFA in, for example, human and finfish diets important, but it is considered beneficial for the diets to contain high ratios of n3 : n6 PUFA and DHA : EPA (e.g. Takahata *et al.*, 1998; Sargent *et al.*, 1999). On average, PUFA derived from strains in the upper branch of S1 would be better suited to provide these requirements than strains in the lower branch of S1 (Figure 1).

Strains ACEM 6063 and ATCC 26185 formed a two-member group based on the presence of DHA and DPAn6 at levels of ≥ 5 %TFA (Table 48). These two strains also formed a two-member group based on their overall fatty acid profiles (Table 12). However, the relative level of DPAn6 in these strains did not substantially influence the separation of this group from other strains (Table 13, Figure 1).

Fatty acid, including PUFA, profiles have been used in attempts to distinguish microorganisms for some time (e.g. Korn *et al.*, 1965; Shaw, 1966). Separation of taxa was largely based on the presence or absence of specific fatty acids. Ellenbogen *et al.* (1969) reported that the major fatty acids of *Thraustochytrium roseum* and *Schizochytrium aggregatum* were 16:0, 18:0 and DHA, while those for *Thraustochytrium aureum* were 16:0, DPAn6 and DHA. More recently, it has been suggested that thraustochytrids can be mainly divided into 6 categories, based on their PUFA profiles (Yokochi *et al.*, 1997). As mentioned in Lewis *et al.* (1999) (see page 131 of this report) these categories were defined by the occurrence of LA, AA, EPA, DPAn6 and DHA in the total fatty acids. In the present study, only three of these five PUFA (AA, DPAn6, DHA) were substantially involved with any of the separations observed in the fatty acid profile-derived dendrogram (Figure 1, Table 13). The lack of correlation between the categories put forward by Yokochi *et al.* (1997) and the results presented in this study suggests that more work is required before a scheme for categorising thraustochytrid strains on PUFA content can be routinely applied.

The intrinsic variability of fatty acid profiles of thraustochytrids grown under various culture conditions makes comparison of fatty acid data provided by different studies

difficult. Such comparisons will be possible only when all strains being compared are cultured under the same conditions. Yokochi *et al* (1997) state that the strains they used when forming their PUFA category concept were not all cultured using the same medium. This information raises some doubts about the validity of the six categories suggested by those authors. During the present study, care was taken to culture all strains under the same conditions (e.g. media composition, light levels, temperature, culture duration) to reduce the influence of variables that are known to influence fatty acid profiles of thraustochytrids. No strict criteria were used to define the categories proposed by Yokochi *et al* (1997) (D. Honda, Konan University, Japan, *pers. comm.*), making direct relation of these categories to the data in this study difficult. When the Australian and type strains were grouped depending on the presence of one or more specific PUFA at levels of ≥ 5 %TFA, a pattern different to that described by Yokochi *et al.* (1997) appeared (Table 48). Out of a total of 13 PUFA categories determined by this method, only 4 of the 6 categories proposed by Yokochi *et al* (1997) were represented.

Findlay *et al.* (1986) suggested that the fatty acid 22:5n6 had the potential to be a signature marker for thraustochytrids. However, the presence of high levels of 22:5n6 in thraustochytrid whole cell fatty acids is by no means a standard trait. The range of 22:5n6 contained in the Australian strains cultured for this study ranged from < 0.1 to 21 %TFA.

These comparisons highlight the potential difficulties with trying to categorise thraustochytrids by their PUFA profiles. There is a similar lack of correlation between many conventional thraustochytrid taxonomic characters and their 18S rDNA sequences, although thraustochytrid 18S rDNA data correlated better with cell wall sugar compositions than with other parameters (Honda *et al.*, 1999).

In this study, there was some correlation between the groups formed using 18S rDNA sequence data and whole cell fatty acid data. However, the low number of strains for which both fatty acid profiles and 18S rDNA sequences were available must temper this observation to some degree. The largest grouping from the 18S rDNA data (Cluster A, Figure 2) all belonged to fatty acid group 3, except for one strain that belonged to fatty acid group 4. There was very little difference between the mean fatty acid profiles of these two groups. The fatty acids that were largely responsible for the separation of the two groups (18:4n3, 17:0, 15:0, 12:0) all occurred at levels of < 1.0 %TFA in both groups (Table 13, S8). No strains from fatty acid groups 3 and 4 occurred anywhere else in the 18S rDNA tree, indicating the relative specificity of the fatty acid profiles of those strains. Other correlations between the 18S rDNA and fatty acid profile trees were less apparent when viewed on the scale in which Figure 1 is presented (*i.e.* a 10-group solution). However, when Figure 1 is viewed as a 2-group solution (*i.e.* with strains grouped on the basis of S1 only), all strains found within the upper arm of S1 were also found in Cluster B in Figure 2. Only one strain found within the lower arm of S1 in Figure 1 (strain ACEM 6018 - fatty acid group 7) was present in Cluster B. The presence of this strain in Cluster B is an interesting anomaly. Strain ACEM 6018 was morphologically similar to strain ACEM 6063 (data not shown), but it's fatty acid profile contained only 16% DHA (c.f. 38% DHA for strain ACEM 6063). Other strains from the lower arm of S1 (Figure 1) were distributed more widely amongst the 18S rDNA tree (Figure 2). Strain ACEM 0005 (fatty acid group 7) was widely separated from strain ACEM 6032 (fatty acid group 6). Strains ACEM 000A

and ACEM 0004 (fatty acid groups 9 and 10 respectively), clustered together in a 2-member group that was widely separated from all other strains (Figure 2).

Table 48   Distribution of strains that contained only the nominated PUFA at levels of $\geq$ 5% of total fatty acids after culture on TCM at 20°C for 5 days

PUFA	Strain
22:5n6, 22:6n3 [1]	ACEM 6063, ATCC 26185
20:5n3, 22:6n3 [1]	0010, 0011, 0019, 6002, 6003, 6004, 6006, 6007, 6010, 6011, 6012, 6013, 6016, 6026, 6027, 6033, 6036, 6040, 6045, 6046
20:5n3, 22:5n6, 22:6n3 [1]	6018, ATCC 28109, ATCC 28210, ATCC 34304
20:4n6, 20:5n3, 22:6n3 [1]	6050
20:4n6, 20:5n3, 22:5n6, 22:6n3	0006, 0008, 0009, 0014, 0015, 0016, 0017, 6020, 6014, 6015, 6017, 6021, 6023, 6044
18:2n6, 20:3n6, 22:5n6, 22:6n3	6049
20:4n6, 20:5n3, 20:2n6, 22:6n3	0018
20:4n6, 20:5n3, 22:6n3, 22:5n3	000A, 0001, 0002, 0003
20:4n6, 20:5n3, 20:2n6	0004
18:2n6, 20:5n3, 22:6n3	0005
20:5n3, 20:2n6, 22:6n3	6029, 6032
22:6n3	0007, 0012, 0013, 0020, 0021, 6000, 6001, 6005, 6008, 6009, 6019, 6024, 6025, 6028, 6030, 6031, 6034, 6035, 6037, 6038, 6039, 6041, 6042, 6043, 6047, 6048
20:4n6	6022

1:    Groups which correspond to categories proposed by Yokochi et al. (1997).

Despite the correlations between fatty acid profiles and 18S rDNA profiles found in this study, scope exists for closer agreement between the two data sets. It is likely that the clusters formed by a given set of thraustochytrid strains based on fatty acid or PUFA profiles will change depending on the conditions under which the strains are cultured. Thus, the possibility exists that a specific set of culture conditions could give rise to fatty acid or PUFA profiles that more closely fit phylogenetic data. However, at present, there appears to be insufficient understanding of the influence of environmental conditions on thraustochytrid fatty acid profiles to achieve this goal. Compilation and careful analysis of such data will be necessary before fatty acid profiles can be used to provide a detailed picture of the evolutionary relationships between different thraustochytrid strains.

Comparison of 18S rDNA sequence data from the new Australian thraustochytrid strains, with that currently available from the literature reveals that the Australian strains represent a wide cross-section of the currently described thraustochytrid taxon (Figure 2). This indicates that the isolation protocol used in this study was sufficiently non-selective to allow a wide diversity of thraustochytrids to be isolated. Of the 74 thraustochytrid strains originally isolated for this study, only 15 were still viable when work towards DNA sequence analysis started. No strains from two of the 10 fatty acids groups (groups 2 and 8) shown in Table 12, and only limited numbers of strains from the other groups, could be grown for 18S rDNA sequence analysis. Loss of strain viability during long-term maintenance of thraustochytrid cultures by continual subculture is a recognised problem amongst thraustochytrid researchers (D. Honda, Konan University, Japan; C. Leander, University of Georgia, USA; D. Frampton, CSIRO Marine Research, Australia; *pers. comm.*).

A close phylogenetic relationship apparently exists between strain ACEM 6063, *Schizochytrium limacinum* SR21 and the DNA extracted from a sample of the commercial product Algamac-2000 (which is based on a proprietary *Schizochytrium* sp. Strain). Honda *et al.* (1999) suggested that *Schizochytrium limacinum* SR21 represents a separate lineage from all other thraustochytrids. The data presented in the present study suggest that strain ACEM 6063, the Algamac-2000 *Schizochytrium* sp. strain, and possibly strain ACEM 6018 belong to the same lineage. This relationship places strains ACEM 6063 and ACEM 6018 in a group of organisms showing particular potential as sources of 22:6n3-rich biomass. Fermenter culture studies have shown *Schizochytrium limacinum* SR21 has produced biomass up to 48 g dry weight L^{-1}, and up to 270 mg 22:6n3 g^{-1} dry weight (Nakahara *et al.*, 1996; Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998). The *Schizochytrium* sp on which Algamac-2000 is based is presumably undergoing continued development as a commercial source of 22:6n3. The apparent correlation between members of the *Schizochytrium limacinum* SR21 lineage and high rates of 22:6n3-rich biomass suggests that other strains from the same lineage could have potential as commercially viable sources of 22:6n3. It remains to be determined how more strains from this lineage can be targeted for isolation and development.

Strain ACEM 6063 was isolated from a single colony, from one of a large number of plates used to isolate the original suite of strains obtained during this study. Similarly *Schizochytrium limacinum* SR21 was just one of about 400 thraustochytrid strains isolated during a sampling trip to the Yap Islands in Micronesia (Nakahara *et al.*, 1996). In both

cases, all strains had to be screened for fatty acid and biomass production before strains worthy of closer examination could be identified. Targeted isolation strategies, as discussed by Bull *et al.* (1992), could markedly improve the efficiency of isolating additional closely related strains. Analysis of the 18S rDNA sequences of thraustochytrid strains notable for their high production of DHA-rich biomass could reveal specific oligonucleotide sequences. If that were the case, it would be feasible to construct appropriate DNA probes, enabling rapid screening of environmental samples for the presence of these patterns. Isolation and screening of viable strains need only proceed for those samples yielding positive results for the DNA probes. If this technique proves effective for strains related to *Schizochytrium limacinum* SR21, it should also work for other thraustochytrid lineages. Accumulation of sufficient DNA sequence and fatty acid profile data would be a first step towards development of strategies to target the isolation of thraustochytrids that produce other fatty acids of interest.

Data presented in this Chapter demonstrate that samples obtained from Australian marine environments contained a wide diversity of thraustochytrids. Preliminary data (see section 2.1.1) indicated that two new strains - ACEM 000A and ACEM 6063 – were able to produce substantial quantities of lipid-rich biomass. Of these, strain ACEM 6063 was of primary interest due to its ability to produce substantial amounts of the nutritionally important PUFA 22:6n3. Subsequent research in this project describes the development and application of these 2 strains.

3.2   Biomass and lipid production by thraustochytrid strain ACEM 6063: influence of physical and chemical parameters

This study demonstrated that changes to both physical and chemical culture conditions substantially influence biomass and lipid production by thraustochytrid strain ACEM 6063. [2]

Biomass levels in both flask and fermenter cultures increased at linear rates with increasing culture age, until peak biomass was reached, and then decreased slightly after that time. Similar growth curves have been reported for *T. aureum* ATCC34304 (Bajpai *et al.*, 1991b), *T. roseum* ATCC 28210 (Singh and Ward, 1996), *Thraustochytrium* sp ATCC 20892 (Singh *et al.*, 1996) and *Schizochytrium* sp SR21 (Yaguchi *et al.*, 1997; subsequently identified as *Schizochytrium limacinum* SR21 by Honda *et al.*, 1998). These data indicate that, unlike bacteria and other microheterotrophs, thraustochytrids growing in liquid media do not replicate in truly exponential fashion during times of maximum growth rate. The linear growth rate observed in thraustochytrid cultures could be due to the

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[2] A companion study, describing the influence of physical and chemical culture parameters on sterol and squalene production by ACEM 6063 (Lewis, T.E., Nichols, P.D. and McMeekin, T.A. (2001). Sterol and squalene content of a DHA-producing thraustochytrid: influence of culture age, temperature and dissolved oxygen. *Marine Biotechnology* in press.) is appended (page144).

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apparent complexity of the life history of thraustochytrids (e.g. Booth and Miller, 1968; Bahnweg and Jackle, 1986; Azavedo and Corral, 1997; Honda *et al.*, 1998).

As cultures of ACEM 6063 aged, the total fatty acid content of the biomass increased, and the relative proportion of DHA decreased. This trend continued past the time when peak biomass was reached in all cultures, except for one flask culture grown with an initial glucose concentration of 10 g L^{-1}. The lipid contents of three thraustochytrid strains have all been reported to increase, and the proportion of DHA to decrease, as the cultures approached peak biomass. Following attainment of peak biomass these trends reversed substantially [*T. aureum* ATCC 34304 (Bajpai *et al.*, 1991b), *T. roseum* ATCC 28210 (Singh and Ward, 1996) and *Thraustochytrium* sp. ATCC 20892 (Singh *et al.*, 1996)]. Unsaturated fatty acid content of many microorganisms has been reported to decrease with culture age (Ratledge, 1989).

The type of culture vessel, and the physical conditions within culture vessels, also influenced growth rate, peak biomass and lipid content of ACEM 6063 cultures. Maximum growth rates in fermenter cultures were about 2-3 greater than those in flask cultures. The reasons for this disparity are unclear, but do not appear to be related to dissolved oxygen levels in the media. Growth rates of flask and fermenter cultures of *T. aureum* ATCC 34304, grown under conditions comparable to the present study, were reported to be similar to each other (Bajpai *et al.*, 1991b), indicating that responses to different culture conditions by thraustochytrids are strain specific. The fatty acid content and the relative proportions of SFA, MUFA and PUFA in flask cultures (which had low DO levels, data not shown) and the fermenter cultures grown at low DO levels were quite similar. This indicates that the type of culture vessel did not substantially influence lipid production by ACEM 6063 cells.

Dissolved oxygen levels in fermenter cultures substantially influenced lipid production by ACEM 6063. Increasing DO levels from < 5% saturation to > 40% saturation resulted in absolute fatty acid levels increasing by > 30%, and the proportions of SFA, MUFA and PUFA decreasing by > 30%, increasing by > 700% and decreasing by > 20% respectively. These findings suggest that DO levels will be important for the optimisation of oil production by ACEM 6063. To the author's knowledge, no other reports on the influence of DO levels on fatty acid production by thraustochytrids have been published. Increased oxygen availability has, however, been shown to increase the degree of unsaturation in the fatty acids produced by other eukaryotic microorganisms (Erwin, 1973; Beach, 1973 cited in Jiang and Chen, 2000).

Maximum impeller rotation speed influenced the peak biomass reached by fermenter cultures of ACEM 6063. Rotation speeds of up to 200 rpm did not appear to impede growth or disrupt the normal morphological development of ACEM 6063. Impeller speeds of up to 750 rpm did, however, result in a decrease in growth rate, a lower peak biomass and abnormal morphological development of the cultures. Impeller type and rotation speed have also been reported to influence growth rate and peak biomass of *Schizochytrium* sp. SR21 cultures, and DHA production by *T. aureum* cultures (Yaguchi *et al.*, 1997), indicating that these strains are also susceptible to the high shear forces which can be generated in fermenters. Peak biomass reached by ACEM 6063 cultures grown using

comparable media in flasks or in the fermenter with low impeller rotation speeds, were similar to each other. Other studies have found that peak biomass reached in fermenter cultures were different to those reached in flask cultures [fermenter biomass < flask biomass for *T. aureum* ATCC 34304 (Bajpai *et al.*, 1991b; Iida *et al.*, 1996); fermenter biomass > flask biomass for *Schizochytrium* sp. SR21 (Yaguchi *et al.*, 1997)].

Culture temperature also influenced biomass and lipid production by ACEM 6063. Maximum growth rates and peak biomass reached by fermenter cultures grown at 20°C and 25°C were very similar, and were higher than those of fermenter cultures grown at 15°C. ACEM 6063 grows substantially slower at 35° than at 25°C (data not shown). Other thraustochytrid strains have been shown to grow fastest between 20°C and 30°C, with growth rates declining rapidly at super-optimal temperatures (Bajpai *et al.*, 1991b; Li and Ward, 1994; Singh *et al.*, 1996; Yokochi *et al.*, 1998). The fatty acid content (mg g^{-1}) of ACEM 6063 increased with culture temperature up to 25°C. Culture temperature did not appear to influence the proportions of SFA or MUFA in the lipid, while the proportions of PUFA and DHA appeared to decrease slightly with increasing culture temperature. Higher incubation temperatures have been shown to decrease the content of unsaturated fatty acids in other microheterotrophs (Ratledge, 1989; Nichols *et al.*, 1994). Lipid content of two other thraustochytrids has also been shown to increase with increasing culture temperature (Bajpai *et al.*, 1991b; Singh *et al.*, 1996). These two studies also reported that lipid content decreased in cultures grown at super-optimal temperatures, and it is likely that ACEM 6063 would follow this trend.

The initial concentration of sea salts used in the culture media influenced the peak biomass reached by cultures of ACEM 6063, but not the growth rate. Maximum peak biomass was reached in cultures grown at the highest salt concentration tested – the equivalent of 100% seawater. Yokochi *et al.* (1998) reported that *Schizochytrium limacinum* SR21 could grow at 0% seawater, with maximum growth being reached at 50% seawater and being maintained up to 200% seawater. The salinity tolerance of *T. aureum* ATCC 34304 (Iida *et al.*, 1996) was markedly different to *Schizochytrium limacinum* SR21 and ACEM 6063. *T. aureum* ATCC 34304 growth was completely inhibited at 0% seawater, peaked at 50%, decreased at 100%, and was again completely inhibited at 200% seawater. As cultures of *Schizochytrium limacinum* SR21 and *T. aureum* ATCC 34304 were all harvested after a set period (5 and 4 days respectively), it is not possible to tell whether variations in seawater concentration influenced only their growth rate, only their peak biomass, or both. Data for ACEM 6063 culture suggests that peak biomass in these cultures is being limited by depletion of a trace nutrient/s provided by the sea salt mixture. Identification of such limiting micronutrient/s could help increase peak biomass in ACEM 6063 cultures. Lipid content at peak biomass of flask-cultured ACEM 6063 cells appeared to be related to the initial sea salt concentration in the media. These differences, however, are consistent with changes related to the culture age at peak biomass, and may not be a direct response to the actual sea salt concentration. This argument is supported by the observation that there was no significant difference in the DHA yields from *T. roseum* ATCC 28210 when sodium chloride levels in the culture media were decreased from 25 g L^{-1} to 10 g L^{-1} (Singh and Ward, 1996).

Inoculum age was found to have significant effects on both biomass and lipid production by 10-day old ACEM 6063 cultures. Increasing inoculum age was associated with decreases in the proportion of SFA, increases in the proportions of PUFA and DHA, and increases in absolute biomass in these cultures. This is in contrast to data reported for *T. aureum* ATCC 34304, which indicated that lipid content of the biomass decreased markedly when 2- or 3-day old inocula were used instead of a 1-day old inoculum (Bajpai *et al.*, 1991b).

Although the influence of initial culture pH was not examined in this study, the initial pH of TCM was about 6.6, and rose to 7.1-7.4 during fermentation (data not shown). The initial pH of TCM is within the range reported to be optimal for biomass and DHA production by other thraustochytrids (Bajpai *et al.*, 1991b; Li and Ward, 1994), and other DHA producing microheterotrophs (Jiang and Chen, 2000).

Initial glucose concentration influenced peak biomass and lipid production in ACEM 6063 cultures, although differences in lipid content became more apparent after cultures reached peak biomass. Peak biomass levels increased, but the increments decreased, as initial glucose concentrations increased from 10 g L^{-1} to 200 g L^{-1}. This indicates that other factors were limiting biomass production at the higher glucose concentrations. Similar data has been presented for thraustochytrid strain G13 (Bowles *et al.*, 1999). ACEM 6063 grew well in media with an initial glucose concentration of 200 g L^{-1}, whereas other thraustochytrids have been reported to have maximum growth at glucose concentrations of only 20 g L^{-1} (*T. aureum* ATCC 34304: Bajpai *et al.*, 1991a) and 40 g L^{-1} (*Thraustochytrium* sp. ATCC 20892: Singh *et al.*, 1996; strain G13: Bowles *et al.*, 1999). Measurements for these three strains were made after 6, 4 and 4 days respectively, and it may be that cultures provided with higher initial glucose concentrations would have reached higher biomass given more time. *Schizochytrium limacinum* SR21 is, to date, the only thraustochytrid reported to obtain peak biomass levels substantially greater than those reached by ACEM 6063 in this study (Yaguchi *et al.*, 1997). *Schizochytrium limacinum* SR21 is, to our knowledge, the only thraustochytrid strain reported to grow well at the high initial glucose concentrations (i.e. ≥100 g L^{-1}) preferred by ACEM 6063 (c.f 30-40 g L^{-1} for several other strains: e.g. Kendrick and Ratledge, 1992; Singh *et al.*, 1996; Bowles *et al.*, 1999). It is possible that the *Schizochytrium* sp. strain from which Algamac-2000 is produced would also thrive under such high glucose concentrations, given its apparent phylogenetic relationship with ACEM 6063 and *Schizochytrium limacinum* SR21 (section 2.1.2, page 35).

Modifications of initial glucose levels resulted in only minor changes in the relative proportions of SFA and MUFA in ACEM 6063 biomass, and no significant changes in the proportions of PUFA or DHA. Glucose concentration has also been reported to have little or no influence on the proportion of DHA in biomass of *T. aureum* ATCC 34304 (Bajpai *et al.*, 1991a), *Thraustochytrium* sp. ATCC 20892 (Singh *et al.*, 1996), *T. roseum* ATCC 28210 (Singh and Ward, 1996), *Schizochytrium limacinum* SR21 (Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998) and strain G13 (Bowles *et al.*, 1999). In all cases, however, glucose concentration had a substantial effect on both peak biomass and lipid content of the biomass, consequently influencing overall DHA production by these strains.

The initial concentration of sodium glutamate influenced biomass and lipid content of 10-day old flask cultures of ACEM 6063. Biomass increased when the initial sodium glutamate concentration increased from 1 to 10 g L^{-1}, and decreased with a further increase of the initial sodium glutamate concentration from 10 to 50 g L^{-1}. This is in contrast to biomass production of *T. aureum* ATCC 34304, which was highest with an initial sodium glutamate concentration of 20 g L^{-1} (Iida *et al.*, 1996). Initial sodium glutamate concentration had only a minor influence on the relative proportions of SFA, MUFA, PUFA and DHA in ACEM 6063 lipid, although total absolute lipid levels decreased substantially as initial sodium glutamate concentration increased. There was no apparent interaction between initial concentrations of glucose and sodium glutamate on either biomass or lipid production by ACEM 6063. Although sodium glutamate was not the only source of nitrogen in the media, this result suggests the carbon:nitrogen ratio in the media had no significant effect on either biomass or lipid production by ACEM 6063. A high C:N ratio has been reported to be generally favourable for microbial growth and lipid production, the latter usually being triggered by N depletion (Yongmanitchai and Ward, 1989; Singh *et al.*, 1996; Yokochi *et al.*, 1998; Bowles *et al.*, 1999).

The lipid of ACEM 6063 biomass was dominated by triacylglycerol and phospholipid, with small amounts of free fatty acid, wax ester and sterol (see Lewis *et al.* 2001, page 144 of this report) for a description of sterol production by ACEM 6063). The proportion of triacylglycerols in the total lipids of ACEM 6063 increased with culture age, and reached > 90% of the total lipid in some cultures at peak biomass. These results, when considered in conjunction with decreases in the proportion of DHA in the total fatty acids with age, indicate that the triacylglycerol fraction of ACEM 6063 lipids contained a relatively lower proportion of DHA than the phospholipid fraction. This characteristic has also been reported for *Schizochytrium* SR21 (Yaguchi *et al.*, 1997). Thraustochytrid strain G13 was also found to have a predominantly triacylglycerol-based oil (Bowles *et al.*, 1999).

Conditions in which thraustochytrid strain ACEM 6063 cultures have produced biomass up to 23 g dry weight L^{-1} biomass and triacylglycerol-rich oil up to 450 mg g^{-1} dry weight have been ascertained. Cell biomass contained up to 130 mg g^{-1} dry weight DHA, allowing potential production of 3 g DHA L^{-1}. This level of DHA production is amongst the highest reported for thraustochytrids (c.f. 2.2 g L^{-1}: Bowles *et al.*, 1999; 13.3 g L^{-1}: Yaguchi *et al.*, 1997).

An important consideration when assessing the efficiency of thraustochytrid production facilities is the dry weight content of DHA in the biomass. If thraustochytrid biomass is to be used as a source of DHA for aquaculture feeds it is important for the biomass to contain as much DHA as possible. In these cases, it could well be important to choose culture conditions that maximise biomass DHA content, even if this compromises overall biomass production and/or DHA production as measured on a volumetric basis.

It is likely that further changes to culture conditions, including other combinations of the initial types and concentrations of macro- and micro-nutrients and sea salts and/or additional doses of nutrient (e.g. Singh and Ward, 1996) will result in further enhancement of growth rates and/or peak biomass for ACEM 6063. The results for laboratory-scale production bolster the argument that thraustochytrids are amongst the most productive

known sources of microbial PUFA [see Lewis *et al.* (1999), page 129 of this report]. If and when culture conditions, including media, can be optimised to allow production of PUFA-rich oils and/or biomass at a quality and price acceptable to market demands, then thraustochytrids will become a viable source of these products. Indeed, several DHA-rich, thraustochytrid-based products have already appeared on the aquaculture market, and research into additional applications is continuing. As research into this group of organisms continues, it is likely that such strains will be further developed to expand their use as commercially viable sources of PUFA-rich oils for use in nutraceuticals and functional foods.

In section 3.1, it was suggested that ACEM 6063 is closely related to the highly productive *Schizochytrium limacinum* SR21 and also to the *Schizochytrium* sp used in the commercial product Algamac-2000. This relationship, together with the biomass and DHA production data reported for ACEM 6063 in this Chapter, support the argument that ACEM 6063 may be amenable to culture in less defined media and conditions, to provide DHA-rich biomass and/or oil a price sufficient to allow entry into the PUFA market.

3.3   ACEM 6063 Storage Trial

Results from the storage trial performed during this project indicate that of the three cell and/or storage treatments examined (biomass preparation, storage temperature, storage duration), only storage duration had a significant influence on the fatty acid profile and content of ACEM 6063 biomass. When assessed in either proportional (% of total fatty acids) or absolute terms, the major effect of storage on ACEM 6063 cells was a reduction in the amount of the two major PUFA (DHA and DPAn6) and total PUFA levels. The data suggests that 1) the PUFA profile of ACEM 6063 biomass was stable for up to 30 days, and 2) to gain maximum advantage from the initial PUFA and DHA content, biomass should not be stored for more that 30 days from harvest.

With the data currently to hand, there are two possible interpretations of these results: 1) the proportion and absolute amounts of total PUFA and DHA in stored ACEM 6063 cells decreased with storage duration > 30 days; 2) the recovery efficiency of total PUFA and DHA from ACEM 6063 cells decreased with storage duration > 30 days.

The first interpretation suggests that freeze-drying ACEM 6063 cells and/or storing the cells at low temperatures do not protect the cells from a reduction of PUFA, specifically DHA, over time. This interpretation is perhaps somewhat surprising, as storage of microbial and other PUFA-rich cells at $-20°C$ is recognised as the standard method for maintaining lipid and fatty acid content and profiles. If DHA and DPAn6 levels did reduce over time, associated reductions in the relative and absolute amounts of AA and EPA would also have been expected.

The second interpretation suggests changes to cell structures and/or binding of lipid within the cells during storage, regardless of cell treatment or storage temperature. If such changes did occur, this could have implications for the bioavailability of PUFA from the cells stored for > 30 days.

Although it appears that PUFA content/recovery from ACEM 6063 cells was quite stable between 93 and 198 days storage, the reasons for the observed reduction between 31 and 93 days storage are unclear. Further research is required to determine the mechanisms that led to the observed decrease in recovery of PUFA and DHA from ACEM 6063 biomass over time.

3.4 Aquaculture Feeding Trials

3.4.1 Rotifer enrichment using PUFA-rich bacteria

Interest in microorganisms, including bacteria and yeasts, for use in aquaculture diets is increasing (Watanabe *et al.*, 1992; Intriago and Jones, 1993; Nichols *et al.*, 1996). Brown *et al.* (1996) investigated 7 strains of marine bacteria and 6 strains of yeast as food for bivalves. One major finding from Brown *et al.* (1996) and similar work (Phillips, 1984) was that, although most strains provided high levels of good quality protein, they lacked detectable levels of essential PUFA, including EPA and DHA.

Maximum EPA incorporation by rotifers in this study (6.9 %TFA; 1.2 %dw) was similar to results presented by other studies in which EPA-producing bacteria were used as feed-stock for rotifers (Watanabe *et al.*, 1992). These authors reported EPA incorporation to a level equivalent to 1.8 %dw after exposing rotifers to 10^9 cells mL^{-1} of the marine bacterium SCRC-6370 for 12 hours. Nichols *et al.* (1996) reported a maximum EPA enrichment equivalent to 1.4 %dw for rotifers following 24 h exposure to 10^8 cells mL^{-1} of ACAM 456.

EPA enhancement of rotifers in the present study decreased significantly, from 1.2 %dw to 0.5 %dw, as the concentration of ACAM 456 to which rotifers were exposed decreased from 1.4 x 10^9 cells mL^{-1} to 7 x 10^8 cells mL^{-1}. These data support conclusions reached by Watanabe *et al.* (1992) and Nichols *et al.* (1996) that there is a close relationship between the concentration of EPA-producing bacteria and the level of EPA incorporation by rotifers exposed to those bacteria.

It should be noted that the maximum EPA enhancement of rotifers reported by Nichols *et al.* (1996) was equivalent to 5.8 %dw. However, differences in protocols for estimating bacterial concentrations are evident (*i.e.* direct microscopic cell counts in this study; colony forming units in Nichols *et al.* (1996)). Comparison of the two techniques reveals that the concentration of 10^8 cells mL^{-1} of ACAM 456 as measured by Nichols *et al.* (1996) was equivalent to the concentration of 10^9 cells mL^{-1} used in the present study (T. Lewis and D. Nichols, University of Tasmania, unpublished data).

The efficiency of incorporation of DHA increased when the concentration of DHA-producing bacteria to which rotifers were exposed decreased from 1 x 10^9 cells mL^{-1} to 5 x 10^8 cells mL^{-1} (0.1 %dw c.f. 0.3 %dw; Table 25). The survival of rotifers exposed to 1.0 x 10^9 cells mL^{-1} of ACAM 605 for 24 h was significantly lower than for any other treatment ($\alpha = 0.05$) (Table 25). These results suggest that conditions in flasks containing ACAM 605 only (at the higher cell concentrations) were detrimental to rotifer survival, and may have

led to the low uptake of bacterial DHA by rotifers. Barclay and Zeller (1996) reported DHA enrichment of rotifers to 1.4 %dw using a spray-dried *Schizochytrium* sp. This result is about 5-fold higher than the 0.3 %dw achieved in the present study.

The present study provides the first report of trophic transfer of DHA from bacteria to rotifers. The successful simultaneous transfer of DHA and EPA, both derived from bacteria, to rotifers has also been demonstrated. These results represent a unique step in the development of bacteria-based feeds for the aquaculture industry. Although thraustochytrids are considered to be a richer source of microbial PUFA than bacteria, there still may be a role for bacterial PUFA producers in the area of rotifer enrichment. Some aquaculture species assimilate PUFA more readily if it is provided in the form of phospholipid rather than triacylglycerol (Izquierdo *et al.*, 2000). Bacterial lipids are primarily phospholipid (Harwood and Russell, 1984), while thraustochytrid lipids are mainly triacylglycerol (Bowles *et al.*, 1999; section 2.2 of this report). It may be that PUFA-producing bacteria will be more suitable as a source of PUFA-rich phospholipid in some areas of the aquaculture industry.

3.4.2 Rotifer enrichment using new Australian PUFA-rich thraustochytrids

This study has also shown that the microheterotroph strains ACEM 000A and ACEM 6063 can be used to enhance PUFA content of rotifers to levels nutritionally significant for many aquaculture species. It is probable, however, that production costs would inhibit the use of fresh thraustochytrid biomass for enriching rotifers in all but the largest facilities. A more likely scenario would be a central production facility supplying preserved biomass to a diversity of customers. Preservation techniques that could be used include freezing and spray-drying (Barclay and Zeller, 1996). In this study, no significant differences were found in the fatty acid profile of either ACEM 000A or ACEM 6063 biomass treated in four different ways (fresh, frozen, freeze-dried, air-dried). In Trial 1, the absolute amount of fatty acids detected in the air-dried ACEM 000A biomass was slightly, but significantly, lower than in the other treatment forms of this strain. In Lewis *et al.* (2000) (see Appendix 3:   Publications, page 163 of this report) it was demonstrated that the amount of fatty acids extracted from fresh ACEM 000A and ACEM 6063 biomass was influenced significantly by the extraction method. It is likely that the method by which ACEM 000A (or other microheterotroph) biomass is preserved would also influence fatty acid extraction efficiency.

Changes in the relative proportions of fatty acids in rotifers during the first 6 h of enrichment reflected the fatty acid composition of their enrichment diets. Changes in fatty acid profiles between T6 and T24 were generally minor, and were considered more likely to be affected by prevailing culture conditions than by the enrichment diets. These observations support the views that lipid content of rotifers correlates well with the lipid composition of their food (Ben-Amotz *et al.*, 1987; Frolov *et al.*, 1991; Rainuzzo *et al.*, 1994), and that all major changes to fatty acid profiles of enriched rotifers occur during the first 6-24 h of enrichment (Olsen *et al.*, 1989; Rainuzzo *et al.*, 1994; Rodriguez *et al.*, 1996; Yoshimatsu *et al.*, 1997). No consistent changes in fatty acid profiles were observed during the 24 h starvation period (T24 - T48) for any of the treatments in this study. It has

previously been reported that the fatty acid profile of rotifers enriched with lipids from easily digestible diets (e.g. yeast, fish oil and/or some microalgae) remained fairly stable during starvation periods of up to 5 days (Lubzens et al., 1985; Olsen et al., 1993). This indicates that rotifers do not preferentially catabolise either total SFA, total MUFA or total PUFA during starvation periods. Gut passage times for B. plicatilis have been reported to be in the order of 30-40 minutes at 20-22°C (Korstad et al., 1989). It is therefore likely that, in the present study, any undigested food would have been excreted well before the end of the 24 h starvation period. Thus, if the diets used in the current trial had proved indigestible, rotifer fatty acid profiles would have been expected to return to pre-enrichment levels following 24 h starvation, as undigested food was excreted. The stability of the fatty acid profiles of rotifers fed ACEM 000A and ACEM 6063 during the post-enrichment starvation period indicates that fatty acids from these microheterotrophs had been digested and assimilated by the rotifers.

The absolute amounts of fatty acids accumulated by rotifers during the enrichment period also reflected those in the enrichment diets. There were no significant differences in the absolute TFA levels in rotifers fed biomass from single strains that had been exposed to different post-harvest preservation treatments. This indicates that freezing, freeze-drying or air-drying did not alter the fatty acid content of the microheterotroph biomass, even though analyses indicated that air-dried ACEM 000A contained a lesser absolute amount of fatty acids than the other treatments. When the increase in average absolute TFA levels in starved rotifers fed single-component (including the high- and low-TFA ACEM 000A biomass) enrichment diets for 6 h were plotted against absolute TFA levels in their diet, a linear correlation was found ($R^2 = 0.90$; Figure 16). Barclay and Zeller (1996) used spray-dried Schizochytrium sp. biomass, which contained about 320 mg g^{-1} TFA, to enrich rotifers. The increase in absolute TFA level in rotifers after 8 h enrichment (75 mg g^{-1}) in that study also correlated well with the linear line fitted to the single component enrichment diet data. A data point for rotifers fed the EPA-producing Antarctic bacterium ACAM 456 for 24 hours (derived from data presented in 1.4.1) has also been included in Figure 16. This data point lies above the trendline in Figure 16. However, it should be noted that this data was for rotifers that had been enriched for 24 hours, as compared with the 6-8 hours enrichment for other rotifers represented in Figure 16. A previous study (Nichols et al., 1996) demonstrated that the absolute amount of EPA in rotifers increased by a factor of about 3.5 between 6 and 24 hours exposure to ACAM 456. It would therefore be expected that, after 6 hours enrichment, the absolute TFA levels in the rotifers examined in 1.4.1 would have been lower than was observed after 24 hours enrichment – thus correlating more closely with the derived trendline in Figure 16.

This analysis suggests that, using knowledge of the fatty acid content of enrichment diets, changes in the fatty acid content and profile of rotifers following short-term enrichment with single component diets can be predicted.
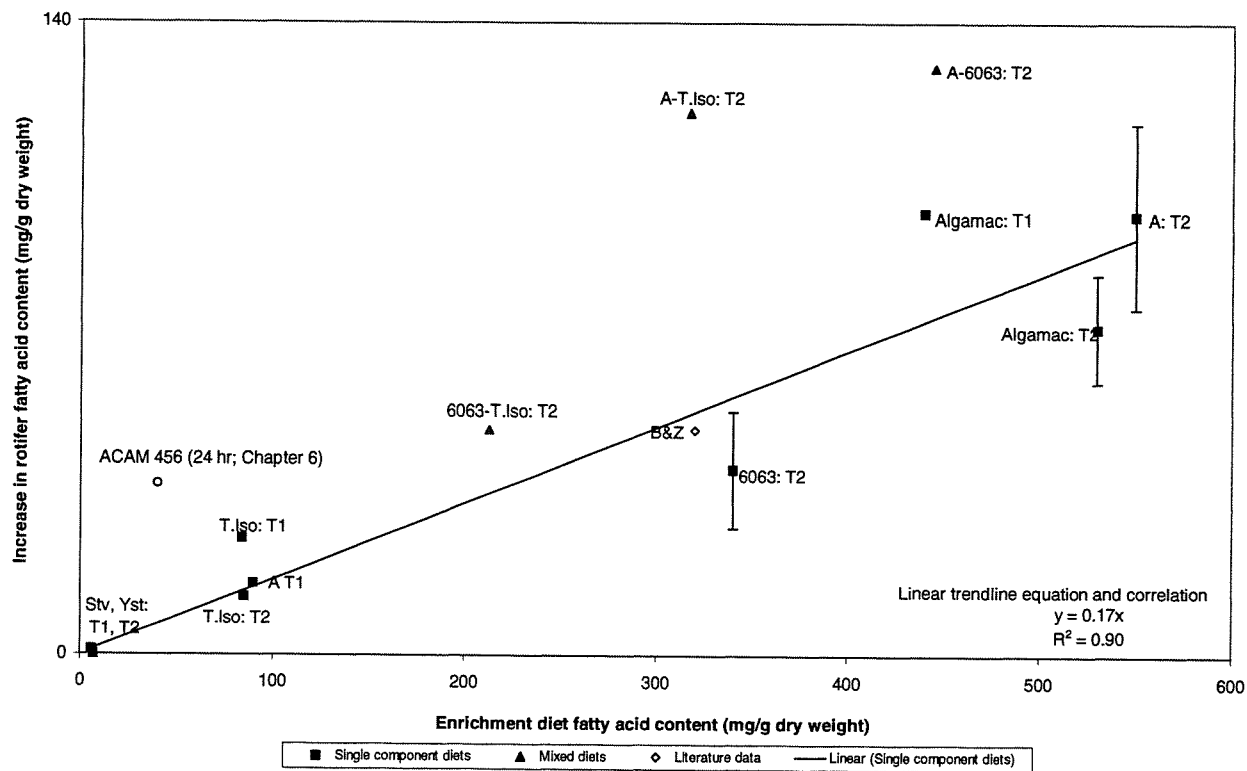
Figure 16   Relationship between total fatty acid content (mg g^{-1} dry weight) of rotifers (starved or fed for 6-8 h) and total fatty acid concentration of single component enrichment diets. [Literature data and that for bacterial or mixed diets were not used for calculating the fitted line; error bars ($\pm$ SD) are shown for data points that are the mean of several samples].

A = ACEM 000A; 6063 = ACEM 6063; T. Iso = *Isochrysis* sp, clone T. Iso; Yst = *Saccharomyces cerevisiae*; T1 = Trial 1; T2 = Trial 2; B&Z = data from Barclay and Zeller (1996); ACAM 456 – EPA-producing bacterial strain fed to rotifers for 24 hours (see Chapter 6)

Data for rotifers fed mixed diets did not correlate as consistently with the fitted line in
Figure 16. To calculate the "average" absolute TFA content of the mixed diets, it was
assumed that the rotifers ingested an equal weight of each of the components of their diet.
The value, based on these "average" TFA contents, for rotifers fed the ACEM 6063-T. Iso
mixture generally fitted the correlation for single diets. The data for mixed diets containing
ACEM 000A, however, lie somewhat above the linear fitted line, indicating that the
"averaging" model did not fit well for diets containing ACEM 000A. Absolute levels of
AA, EPA and DHA detected in rotifers fed mixed diets containing ACEM 000A (Figure
11) also do not support the concept that ingestion of equal amounts of the components
occurred with these particular diets. It may be that these rotifers preferentially ingested
ACEM 000A ahead of ACEM 6063 and T. Iso, to levels similar to rotifers fed ACEM
000A only, and also ingested small amounts of ACEM 6063 and T. Iso. It therefore seems
likely that, in this study, there was some degree of selective ingestion of different dietary
components by the rotifers. Selective intake of food by rotifers has been reported
previously—based on size (Heerkloss and Hlawa, 1995), or whether the food particles are
alive or not (Chotiyaputta and Hirayama, 1978). Reasons for the apparent selectivity in the
present study are not clear, although particle size may have influenced ingestion efficiency.
ACEM 000A and ACEM 6063 cells used in this study were about 10-15 μm and 5-10 μm
in diameter respectively, while T. Iso cells were about 5 μm long. If particle size did
influence ingestion rates, then it would appear that the rotifers favoured the larger particles.
However, this suggestion does not agree with results from a study of particle ingestion by
B. plicatilis (Heerkloss and Hlawa, 1995), which showed a preference for smaller particles
(2 μm) ahead of larger particles (3-10 μm). One implication of feed selectivity by rotifers is
that the degree of enrichment elicited by different combinations of dietary components
must be determined by direct experimentation, rather than by extrapolation from data
obtained from single component diets.

Absolute TFA levels in rotifers fell substantially during the 24 h starvation period (T24 –
T48), with the rate of loss generally being proportional to the TFA level at T24. Similar
results were reported by Olsen et al. (1993). Absolute TFA levels at T48 were generally
similar to those at T0, notwithstanding the levels reached at T6 and T24. The main
exceptions to this trend were rotifers fed 200 mg L^{-1} of either ACEM 000A or Algamac,
which had very low absolute TFA levels at T48. Comparison of rotifer density at T48
strongly suggests that the health of these rotifers had been severely compromised by these
two diets.

The potential of new diets for enriching rotifers with PUFA for aquaculture must be
evaluated with species-specific requirements for these essential fatty acids in mind. The
absolute dietary requirements for total or specific PUFA has been assessed for a number of
marine finfish larvae, and are often about 1.5-2 % of feed dry weight [e.g. Gilthead
seabream ~2.1% n3 PUFA (Rees et al., 1994); cod ~2.1% DHA (Zheng et al., 1996); Red
sea bream ~1.6% DHA (Furuita et al., 1996a); Yellowtail ~2% DHA (Furuita et al.,
1996b)]. Similar levels were achieved in many of the ACEM 000A and ACEM 6063-based
treatments in Trial 2 of this study.

Recent developments in the understanding of essential PUFA requirements of fish have
been reviewed by Sargent et al. (1999). Evaluation of larval requirements for AA, EPA

and DHA in terms of relative, as well as absolute, amounts is now considered necessary. For example, optimum dietary ratio of DHA:EPA:AA for sea bass larvae is about 2:1:1 (w/w/w), while that for turbot and halibut larvae is about 2:1:0.1 (w/w/w) (Sargent et al., 1999). It appears from these data that rotifers enriched for 6 h with ACEM 000A and ACEM 6063 will provide appropriate levels of PUFA for many larvae, and that ACEM 6063 can provide what are currently accepted as preferred DHA levels and DHA:EPA ratios for many fish and prawn larvae. It may be that further research will point to increased requirement of some larvae for AA or other fatty acids. For instance, adult prawns generally have higher AA levels than many fish (Yearsley et al., 1999), and AA has been found to be essential for some, but not all, prawn species (Deering et al., 1997). Larval prawns, therefore, may require more AA in their diet than the levels generally accepted as necessary for fish larvae. If provision of AA and other fatty acids in larval diets is seen as necessary, then organisms such as ACEM 000A could well be used as a reliable source. Further manipulation of absolute and relative PUFA levels in enriched rotifers to provide optimum fatty acid nutrition can be achieved, as required, through the use of enrichment diets consisting of single or mixed microheterotrophs with different PUFA profiles.

Changes in the levels of AA, EPA and DHA in rotifers between T24 and T48 reveal an interesting trend. The ratio of DHA to both AA and EPA decreased during this starvation period, especially in rotifers containing higher levels of DHA at T24. This indicates that DHA was being preferentially catabolised by these rotifers. Results from earlier studies (Olsen et al., 1993; Rainuzzo et al., 1994; Barclay and Zeller, 1996) also indicated that rotifers may preferentially catabolise DHA before EPA or AA, and that rotifers may be able to retroconvert some longer chain PUFA to their shorter chain counterparts. Similar data has also been reported for a number of strains of Artemia sp. – another live-feed species used by aquaculturists (Han et al., 2000). This ability could have significant implications in cases where a low proportion of AA is required and when rotifers are provided with diets containing high DHA levels for several days prior to being fed to larvae. For short-term enrichments, however, it is unlikely that significant retroconversion would occur.

An important consideration when evaluating the potential of any new rotifer enrichment diet is its influence on rotifer health. Although the experimental system used in this study did not support high rotifer survival, comparison against standard rotifer diets (yeast, T. Iso) allows some assessment of the influence of the enrichment diets on rotifer health. The data indicate that feeding ACEM 000A at 100 mg L^{-1} did not unduly influence rotifer survival during the first 6 h enrichment period. Longer enrichment periods resulted in more variable results, with rotifer survival in Trial 2 being lower than in Trial 1. The reason for this difference could be related to initial rotifer health and/or differences in the ACEM 000A biomass used. The fatty acid levels in ACEM 000A were very different in both trials (~85 mg g^{-1} in Trial 1; ~550 mg g^{-1} in Trial 2), and there could also have been differences in other properties of the biomass. In Trial 2, rotifers fed only ACEM 000A and the ACEM 000A-ACEM 6063 and ACEM 000A-T. Iso mixtures all showed poor survival after T6. This suggests that the ACEM 000A biomass used in Trial 2 may have adversely influenced rotifer health. Survival amongst rotifers fed ACEM 6063 at all sample times was

essentially the same as that for rotifers fed yeast and T. Iso, indicating that the ACEM 6063 biomass did not influence rotifer health during short term enrichments.

Many countries appear to be increasing quarantine restrictions on the importation and use of exotic microorganisms (for example the two Antarctic bacterial strains used to enrich rotifers in 1.4.1) in aquaculture diets. It is likely that such restrictions will be tightened further, making it important for affected countries to have access to native organisms with which to supply the needs of their local industries. In this Chapter, it has been shown that the new Australian thraustochytrid strains ACEM 000A and ACEM 6063 can be used to enrich rotifers with nutritionally significant levels of PUFA. Indeed, enrichment with these thraustochytrid strains increased rotifers PUFA content to levels higher than were achieved using the PUFA-producing bacteria discussed in 1.4.1. It also appears that fatty acids from these two thraustochytrids can be assimilated by rotifers, and therefore should be available to any larvae that subsequently eat rotifers enriched with these thraustochytrids.

The predominant lipid class produced by specific microheterotrophs could be an important consideration when selecting a strain to use for rotifer enrichment. Incorporation of dietary n3 PUFA has been found to be more efficient in some aquaculture species if the PUFA are contained in phospholipid rather than triacylglycerol (Izquierdo et al., 2000). Bacterial lipids are predominantly phospholipid (Harwood and Russell, 1984), whereas the predominant lipid class of some thraustochytrids is triacylglycerol (section 2.2, Bowles et al., 1999). This indicates that, while thraustochytrids may generally have the potential to enrich rotifers to higher PUFA levels than bacteria, bacterial PUFA may be more nutritionally available to some aquaculture species. The possibility of using a mixture of PUFA-rich bacteria and thraustochytrids to provide PUFA in the form of both phospholipids and triacylglycerols could also be considered.

The observation that fatty acid content of thraustochytrids can be modified by manipulation of culture conditions (2.2) potentially adds further flexibility to rotifer enrichment systems. Mixtures of enrichment diets can be used to tailor fatty acid content of rotifers, which may then be used to meet specific absolute and relative PUFA requirements of different larvae. The fatty acid profiles exhibited by PUFA-producing thraustochytrids reported to date are quite diverse (Bowles et al., 1999; Chapter 2). This indicates the significant potential of these organisms to be used as a new source of PUFA for aquaculture (amongst other potential uses), providing a potential alternative to the more traditional sources of PUFA. As additional strains are isolated, and their production of PUFA optimised, it seems likely that the PUFA requirements of many aquaculture species could be met by thraustochytrid production systems.

3.4.3 Aquaculture species feeding trials

Three feeding trials, using formulations developed during this project were conducted to test the hypothesis that PUFA from ACEM 6063 could be taken up by Atlantic salmon (finfish, manufactured feed), Striped trumpeter (finfish, live feed), and prawns (crustacea, manufactured feed).

These trials are, to our knowledge, among the first recorded in which growth performance parameters and fatty acid profiles of aquaculture species fed live or manufactured feed containing thraustochytrid biomass were assessed. The formulations used for the Atlantic salmon and Prawn manufactured feeds were designed by Chris Carter (UTas) and David Smith (CSIRO Marine Research) respectively. In both cases, the animals fed ACEM 6063-based diets performed very well. The Striped trumpeter larvae feeding trial built on the results from the rotifer enrichment studies, discussed in section 3.4.1.

3.4.3.1 Atlantic salmon

The diets were designed so that CO would be marginal in terms of meeting the essential n-3 fatty acid requirements and the added Thraustochytrid would increase the essential fatty acid content. The addition of 10% Thraustochytrid doubled the essential n-3 fatty acid content due to its high content of DHA. However, the total n-3 content was still lower than added by fish oil and the CTH diet had an n-3/n-6 ratio intermediate between CO and CFO. The growth experiment showed that canola oil could be successfully used to replace fish oil in salmon parr diets having no effect on growth and growth efficiency or on other measures of performance such as survival, measures of immune response or nutrient digestibility. The experiment represented a significant part of the salmon freshwater stage where by the salmon almost tripled their wet weight and achieved smolt status. The different distribution of fatty acids in the diets affected fatty acid composition in the salmon and it is suggested that this explained the different response to the combined stressors of sea water and disease challenge (data not shown here). Salmon fed the diet containing the Thraustochytrid meal showed similar mortality to those fed the canola oil diet. Consequently, the assessment of the possible importance of differences in fatty acid composition focused on values that were similar for CO and CTH but both different from CFO.

Several studies have demonstrated the potential for replacement of substantial amounts of fish oil with plant oils in diets for salmon during their freshwater parr stage (Bell et al., 1997; Dosanjh et al., 1998; Tocher et al., 2000). For example, the performance of two feeds, predominantly fish meal and fish oil (with some plant meal) or predominantly plant meals and oil (with low fish meal), were compared in Atlantic salmon parr (Bell et al., 1997). There were no differences in growth performance over the winter freshwater phase with parr growing from around 20 g to a final weight of 35 g (Bell et al., 1997). Diets containing added oil (accounting for 68% of total dietary lipid) from either fish oil, rapeseed oil or linseed oil produced equivalent winter growth performance in growing 30 g parr to exceed 40 g after 19 weeks (Tocher et al., 2000). The higher temperatures used in the present study allowed much faster growth and 40 g parr reached over 100 g after less

than eight weeks. It is of interest that the CO diet performed well despite a marginal essential n-3 fatty acid content. This is likely to be explained by the high residual levels in the fish carcasses even though the fish were fed on a low oil diet prior to the experiment.

In the present experiment salmon were transferred to seawater and then fed a commercial fish oil based feed (data not reported here). Similarly, some studies changed from plant oil diets to commercial diets containing fish oil for the seawater period (Bell et al., 1997; Tocher et al., 2000) whereas other studies fed plant oils to seawater salmon (Bell et al., 1996; Dosanjh et al., 1998; Torstensen et al., 2000). Neither Bell et al. (1997) nor Tocher et al. (2000) observed any difference in the growth of salmon when transferred to seawater and fed a normal commercial feed if previously fed diets containing either a 1:1 rapeseed linseed oil mix (Bell et al., 1997) or rapeseed oil (Tocher et al., 2000). Canola oil successfully replaced 47% of the fish oil in 28% lipid diets fed to seawater Atlantic salmon parr growing from 145-180 g to around 400 g (Dosanjh et al., 1998). Although diets containing high amounts of plant oil are successful in terms of growth performance they result in significant differences in fatty acid composition compared with fish oil diets. These differences have different consequences at different stages in the salmon life / production cycle.

It has been proposed that fish oil replacement during the parr stage may be beneficial for smoltification due to a reduction in a high dietary content of n-3 PUFA from fish oil (Bell et al., 1997). The study by Bell et al. (1997) was significant because it showed desaturation activity (in isolated hepatocytes) increased towards the time of seawater transfer and that this activity was higher on the plant oil diet. Desaturation is inhibited by high n-3 PUFA levels and desaturation along n-3 pathways occurs in preference to n-6 pathways. Thus, high n-3 PUFA levels from the fish oil diet were thought to inhibit desaturation of both n-3 and n-6 fatty acids. This is of importance because AA acts as the main precursor for prostaglandins important in smoltification. Bell et al. (1997) found liver phospholipid AA/EPA ratios were over 1 and 50 percent higher on a plant oil diet than on a fish oil diet and reflected by higher synthesis of $PGF_{3\alpha}$, a prostaglandin derived from AA. In the present study the AA/EPA ratios in the liver (total lipid) were over 1.3 for the CO and CTH diets compared with 0.3 on the CFO diet. Although there were no significant differences in the relative concentration of AA in liver, eye or heart tissue between dietary treatment muscle concentrations were significantly lower for CFO. This may have been a consequence of differences in desaturation activity especially in view of the considerably higher AA content in CFO.

The overall distribution of FA generally reflected the FA distribution in the different diets. For example, oleic acid (18:1n-9) increased with increasing canola oil in the diet and this was reflected in the liver, heart and muscle. Similar trends were noted for LNA and EPA in all tissues and for LA in the heart and muscle but not the liver. The very high relative concentration of DHA in the CTH diet was not reflected by a large difference in tissue concentrations between the CFO and CTH diets. However, it should be noted that interpretation of the data is made more difficult by the use of total FA composition rather than of the phospholipid fraction.

Few studies record FA concentrations in fish eyes and these were investigated to determine whether the eye was a potential indicator of nutritional status. There were large differences in the relative size of different lipid class in the eye in the present study. There was no difference in the absolute TAG concentration between dietary treatment and this was the major lipid class from the CO and CTH diets and present at similar concentrations (25-30 mg g^{-1}) to other studies (Brodtkorb et al., 1997). However, the PL content for the CFO diet was significantly higher and equal to the TAG content. The influence of dietary fatty acid composition on its concentration in the eye appears to depend on the fatty acid (Brodtkorb et al., 1997). In small juvenile salmon (<5 g) the relative concentrations of EPA and LA were affected by dietary composition whereas DHA was not (Brodtkorb et al., 1997). Similarly, in the present study, large differences in dietary concentrations of fatty acids were reflected by relative concentrations in the eye for OA and LA but not for AA, LNA, EPA or DHA. The n-3/n-6 ratios were significantly higher for CFO for all tissues and this difference may have had implications in terms of the challenge to seawater / disease that occurred following this experiment.

Differences in the n-3/n-6 ratio of between 0.84 and 1.70 in the present trial did not significantly alter immune function or blood chemistry in Atlantic salmon after seven weeks. When Thompson et al. (1996) supplied two feeds containing either sunflower or fish oil and with n-3/n-6 ratios of 0.3 or 5.2, respectively, they also failed to find any alteration to nonspecific defense mechanisms including lysozyme, antiprotease, macrophage respiratory burst and phagocytosis. Similarly, there was no effect on serum protein, lysozyme activity or kidney phagocyte respiratory burst when different oils (sunflower, linseed or two fish oils) and n-3/n-6 ratios of between 0.3 to 10.6 were fed to Atlantic salmon (Bell et al., 1996). In the present trial immune parameters were measured at the conclusion of a growth trial and values probably represented resting values (Thompson et al., 1996). Further, rainbow trout (*Oncorhynchus mykiss*) with a 'history' of adequate nutritional status did not show signs of immunomodulation when fed diets which would normally compromise younger fish (Kiron et al., 1995). Prior to their use in the present trial, salmon were supplied a commercial feed that would have provided all essential nutrients including EFA. The use of smaller, younger salmon, or a longer growth period may have been required to elicit any negative impacts on the immune system. Muscle mass in salmon is approximately 50% of the wet weight and fatty acid concentrations in this tissue indicated that fish contained large amounts of all essential fatty acids. Nevertheless evidence presented herein suggest these oils and the n-3/n-6 ratio used do not alter those immune mechanisms quantified.

As indicated by the concentration of sodium chloride required to cause 50% haemolysis of erythrocytes, there was a trend whereby increasing quantities of dietary n-3 increased erythrocyte cell wall strength. This is similar to observations made in Atlantic salmon with increased cell wall strength with higher concentrations of dietary n-3 (Salte et al., 1988; Erdal et al., 1991). Improved rigidity of erythrocytes may indicate improved resistance to certain diseases, particularly those associated with fluctuating water temperatures such as 'cold-water vibriosis' (*V. salmonicida*) (Salte et al., 1988).

This trial taken in isolation demonstrated the robustness of salmon parr to diets with varying quality in terms of meeting the essential FA requirements. Differences between the

diets were reflected in tissue FA concentrations but the consequence only became apparent when the fish were put under extreme conditions. The Thraustochytrid meal proved vialbe and later failure of this diet was attributable to the FA composition and this could have been controlled by a larger inclusion (15-20%) in a diet.

3.4.3.2 Striped trumpeter

Striped trumpeter larvae that had been fed rotifers enriched with ACEM 6063 also exhibited enhanced DHA levels when compared to larvae fed starved rotifers. Larvae fed ACEM 6063-enriched rotifers contained levels comparable to those in larvae fed rotifers enriched with a commercial enrichment product (DHA-Selco). When measured on an absolute basis (mg g^{-1} dry weight or ng individual^{-1}), PUFA and fatty acid levels were significantly higher than those in larvae fed starved rotifers.

This indicates that PUFA, and notably DHA, is efficiently transferred from ACEM 6063 cells to rotifers, and thence to the finfish larvae. It should be noted that the rotifers used in this trial did not achieve the levels of PUFA enrichment achieved during the rotifer enrichment trial (section 3.4.1). This may have been due to the different systems used to enrich the rotifers in the two trials. It is therefore likely that, with refinement of ACEM 6063 culture systems (to manipulate PUFA levels and the SFA:MUFA ratio if required) and rotifer enrichment techniques, ACEM 6063 can be a useful source of DHA-rich oil for finfish larvae.

3.4.3.3 Prawns

The ACEM 6063 biomass included in the prawn diet was added at the expense of the squid oil and some defatted fishmeal and wheat flour included in the Basal diet. Prawns fed the ACEM 6063 diets performed as well or better than those fed the Basal diet in all aspects shown in Table 44. Lipid and fatty acid profiles (proportional or absolute) of prawns fed the ACEM 6063 diet were also very similar to those of prawns fed the commercial and Basal diets. The lipid composition and growth performance and survival data for prawns are very encouraging, and indicate that ACEM 6063 can be used as a substitute for squid oil, and hence a source of PUFA, in manufactured prawn diets.

Benefits and Conclusions

Benefits

Longer-term projections indicate that demand for PUFA-rich oils for inclusion in live and manufactured aquaculture feeds may outstrip current sources of supply. This project has been a successful early step in the development of Australian technology that will provide a sustainable supply of PUFA-rich biomass and oils to the local aquaculture industry.

It is not currently possible to provide accurate projections to quantify the benefits that the adoption of the research results will make. Such estimates will need to evaluate difference in terms of prices (*eg* quality, market penetration, user satisfaction) and costs (*eg* productivity). However, the provision of a sustainable source of PUFA-rich components for aquaculture diets is of fundamental importance to many marine aquaculture species. Data from this and subsequent projects could therefore provide technology that underpins sustainable production of live and manufactured feed for many of Australia's marine aquaculture species.

These projected benefits are in direct accordance with those identified in the original application. Should uptake of the organisms, technology and applications developed during this project occur, the opportunity for further application into agrifeeds and functional foods will also be available.

Further developments

This project has demonstrated the technical feasibility of using specific microheterotrophs as a source of PUFA in aquaculture feeds. To commercially exploit the results of this research the following process should be followed:

- Attract further funding for pilot-scale production of new Australian thraustochytrids.

- Conduct a thorough examination of existing IP in this area, to determine mechanisms by which this project can be pursued in a commercial setting.

- Commence Phase 2 of the Thraustochytrid Research Program, which could be divided into three Projects, each to be conducted over a two-year period:

  Project I: Optimisation and scale-up of DHA production by thraustochytrid strain ACEM 6063

  Project II: Targeted isolation of PUFA (e.g. AA and DHA)-producing thraustochytrids, including development of molecular probes and screening techniques

Project III: Incorporation of PUFA-rich biomass and oil from Projects I and II into aquaculture feeds, to be used in longer term feeding trials and/or feeds for species other than those included in the current project

Planned outcomes

The major outputs of this project have been:

- generation of considerable IP relating to sustainable microbial production of PUFA-rich biomass and oils derived from new Australian thraustochytrids.

- application of this technology for the production of aquaculture diets

If these outputs can be utilised to their full potential, substantial long-term benefits for the Australian aquaculture industry, resulting from increased net production efficiency, are possible. Some leading international experts have expressed the view that the predicted increase in worldwide aquaculture production is likely to be retarded by a static or diminishing supply of PUFA-rich oils. Most of the fishmeal and fish oil presently used by the Australian aquaculture industry is imported. Therefore, sustainable production of new PUFA sources could ease this potential limitation on Australian aquaculture production, and thus help add to Australia's economic, environmental and social resources.

Conclusions

The aim of this study was to isolate PUFA-producing thraustochytrids from Australian habitats, to characterise these strains, and to evaluate the potential application of selected strains for use in aquaculture feeds. The project had four main objectives, all of which were successfully achieved, as follows.

1   to develop targeted screening programs for the isolation and characterisation of PUFA-producing bacteria and other microheterotrophs

Methods to target the isolation of thraustochytrids (known for their production of PUFA) were developed and refined. This technology is now available to underpin any continuation or expansion of this project.

2   to maximise PUFA production by manipulation of specific microheterotroph culture conditions

The influence of physical and chemical culture conditions on biomass, lipid and PUFA production of a promising DHA-producing thraustochytrid strain – ACEM 6063 – were studied in detail. Variations in culture conditions substantially influenced biomass and lipid production by ACEM 6063. Conditions were identified under ACEM 6063 cultures achieved up to 23 g dry weight L^{-1}, of which up to 45% and 10% (w/w) were total lipid and DHA respectively. In general, biomass production rates (g dry weight L^{-1} d^{-1}) were higher in fermenter cultures than in flask cultures. Biomass production increased with initial glucose concentration, initial sea salt concentration, culture temperature, and fermenter impeller speed. Lipid content increased with culture age. The dominant lipid classes in ACEM 6063 oil were triacylglycerol and phospholipid. The proportion of triacylglycerol increased to as cultures reached peak biomass. Increasing DO levels substantially decreased the proportion of saturated fatty acids and substantially increased the proportion of monounsaturated fatty acids in ACEM 6063. The proportion of DHA in the total fatty acids of ACEM 6063 fermenter cultures decreased with culture age. The levels of biomass, lipid and DHA production by ACEM 6063 are amongst the highest for thraustochytrids reported to date. ACEM 6063 is therefore worthy of further research and development to evaluate its commercial potential.

3   to develop product formulations, including PUFA enrichment of live feeds, as a basis for commercial production of suitable strains.

Preliminary strategies for adding PUFA-rich ACEM 6063 biomass to manufactured feed for prawns and Atlantic salmon resulted in encouraging growth and fatty acid profile results for both test species. Similarly, nutritionally significant amounts of PUFA have been successfully transferred from these new thraustochytrids to rotifers. Substantial variations in the types and amount of PUFA incorporated into rotifer biomass were achieved by using different amounts and types of thraustochytrid strains in the enriching medium. This is the first report regarding manipulation of rotifer fatty acid profiles with diets containing different thraustochytrid strains and different proportions of thraustochytrid biomass. These results show that if thraustochytrid strains with appropriate

PUFA production characteristics can be cultured commercially, rotifers could be enriched to meet specific PUFA requirements of different aquaculture species. Such tailoring of diets to meet specific dietary PUFA requirements could result in increased survival, growth and/or production efficiencies for selected aquaculture species and/or life stages.

4   *to conduct feeding trials, using formulations to be developed during this project, using Atlantic salmon larvae (finfish, manufactured feed), Striped trumpeter – the original plan was to use Greenback flounder - larvae (finfish, live feed) and Prawns (crustacea, manufactured feed) as test species.*

Three feeding trials, using formulations developed during this project were conducted to test the hypothesis that PUFA from ACEM 6063 could be taken up by the following test species: Atlantic salmon (finfish, manufactured feed), Striped trumpeter (finfish, live feed), and prawns (crustacea, manufactured feed).

These trials are among the first recorded in which growth performance parameters and fatty acid profiles of aquaculture species fed live or manufactured feed containing thraustochytrid biomass were assessed. The formulations used for the Atlantic salmon and Prawn manufactured feeds were designed by Chris Carter (UTas) and David Smith (CSIRO Marine Research) respectively. The Striped trumpeter larvae feeding trial built on the results from the rotifer enrichment studies.

In all cases, results showed the technical feasibility of using thraustochytrid biomass as a source of essential PUFA in the diets of these species. All animals performed on a par with those fed standard commercial or experimental reference diets. Refinement of enrichment techniques and feed formulations could well result in an improvement on the already encouraging results obtained. These results indicate the need for further research and development to assess the commercial viability of thraustochytrids as a source of PUFA in aquaculture diets. If such evaluations show that production and use of these Australian strains is economically viable, the goal of a sustainable, large-scale PUFA production facility in Australia will be another step closer.

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Appendix 1:   Intellectual Property

Techniques and results pertaining to isolation, optimisation, maintenance and storage of PUFA-producing microheterotrophs and live-feed enrichment techniques developed during this project may be subject to intellectual property restrictions.

It is therefore also suggested that all data contained in the *Material and Methods* and *Results* sections of this report be treated as commercial-in-confidence until IP implications and future developments of the project outputs are clarified.

Appendix 2:   Staff

Tom Lewis	University of Tasmania
Peter Nichols	CSIRO Marine Research
Mina Augerinos	CSIRO Marine Research
Ben Mooney	CSIRO Marine Research
Chris Carter	University of Tasmania
Tom McMeekin	University of Tasmania
Jenny Kettlewell	University of Tasmania
David Smith	CSIRO Marine Research
Simon Tabrett	CSIRO Marine Research
David Morehead	University of Tasmania
Stephen Battaglene	University of Tasmania
Bill Wilkinson	University of Tasmania
Piers Hart	University of Tasmania
Andrew Trotter	University of Tasmania
David Nichols	University of Tasmania
Ortwin Bode	Clover Corporation Pty Ltd

Appendix 3:   Publications

The following peer-reviewed publications have been either wholly or partially derived from work associated with this project. Data from the publication marked # is included in the body of this report. Publications marked * are included in this Appendix.

*Lewis, T., Nichols, P.D. and McMeekin, T.A. (2001). Sterol and squalene content of a DHA-producing thraustochytrid: influence of culture age, temperature and dissolved oxygen. *Marine Biotechnology* **in press.**

*Lewis, T., Nichols, P.D. and McMeekin, T.A. (2000). Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *Journal of Microbiological Methods* **43**:107-116.

*Lewis, T.E., Nichols, P.D. and McMeekin, T.A. (1999). The biological potential of thraustochytrids. *Marine Biotechnology* **1**:580-587. (Invited review).

#Lewis, T.E., Nichols, P.D., Hart, P.R., Nichols, D.S. and McMeekin, T.A. (1998). Enrichment of rotifers (*Brachionus plicatilis*) with eicosapentaenoic acid and docosahexaenoic acid produced by bacteria. *Journal of the World Aquaculture Society* **29**:313-318.

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Nichols, D., Bowman, J., Sanderson, K., Mancuso Nichols, C., Lewis, T., McMeekin, T. and Nichols, P.D. (1999). Developments with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes [Review]. *Current Opinion in Biotechnology*, **10**(3), 240-246.

The following conference papers and posters and media interviews were also presented as part of this project:

Presentations and Posters:

Lewis, T.E., Nichols, D.S., Nichols, P.D., Hart, P.R. and McMeekin, T.A. (1997) Novel bacteria as alternate sources of polyunsaturated fatty acids in aquaculture. 6th International Tasmanian Aquaculture Exchange, Hobart, Tasmania, 25-28 July 1997.

Nichols, P.D., Nichols, D.S., Lewis, T. Bowman, J.P., Brown, J., Skerratt, J.H. and McMeekin, T. A. (1997) Novel bacteria as alternate sources of polyunsaturated fatty acids for use in aquaculture and other industries. Marine Microorganisms for Industry Conference. Brest, France, 17-19 September, 1997.

Lewis, T., Mooney, B., McMeekin, T. and Nichols, P. (1998). New Australian microbial sources of polyunsaturated fatty acids *Chemistry in Australia* 65:37-39.

Lewis TE, Nichols PD, Nichols DS, Mooney B, Hart P, McMeekin TA. (1998) Marine microheterotrophs as alternative sources of polyunsaturated fatty acids in aquaculture feeds.

- Invited symposium lecture at Australian Society of Microbiology Annual Scientific Meeting, 28 September- 2 October 1998, Hobart, Tasmania.

- Marine Bioprocess Engineering International Symposium, 8-11 November 1998, Noordwijkerhout, The Netherlands.

Nichols PD, Nichols DS, Lewis T, Bowman JP, Brown J, Skerratt JH and McMeekin TA (1998). Novel bacteria as alternate sources of polyunsaturated fatty acids for use in aquaculture and other industries. In: LeGal, Y. and Muller-Feuga, A. (Eds), *Marine Microorganisms for Industry*, Editions IFREMER, Plouzane, pp 26-32.

Lewis TE, Nichols PD, Mooney, BD, McMeekin TA (1999) Polyunsaturated fatty acids from Australian thraustochytrids: the potential to reduce our reliance on fish oils?

- World Aquaculture '99, 26 April – May 2 1999, Sydney, Australia.

- AMSA '99, 7 – 9 July 1999, Melbourne, Australia.

Lewis TE, Nichols PD, McMeekin TA (1999). Production of polyunsaturated fatty acids by Australian Thraustochytrids.

- 8th International Conference on Applied Algology. Sept 26 – Oct 2 1999, Montecatini Terme, Italy.

- FRDC Aquaculture Diet Development Scientific Meeting, December 16 1999, Salamander Bay, Australia

Lewis, T.E., Nichols, P.D. and McMeekin, T.A. (2000) New Australian marine
      microheterotrophs: an alternative source of polyunsaturated fatty acids in
      aquaculture feeds?

- International Marine Biotechnology Conference 2000. Sept 28 – Oct 4 2000,
  Townsville, Australia.

- Aquafest Australia 2000, October 6-8 2000, Hobart, Australia

- American Oil Chemists Society (Australian Chapter) annual meeting.
  October 12 2000, Werribee, Australia

- Inaugural Australian Omega Discussion Group workshop, February 11 2001,
  Wollongong, Australia

Media interviews:

July 1997	Media interest following the 6th International Tasmanian Aquaculture Exchange resulted in several interviews on commercial television and ABC radio.
19-1-99	Interview regarding novel sources of PUFA for aquaculture and industry on Tasmanian Country Hour, ABC Radio.
14-5-99	Interview regarding novel sources of PUFA for aquaculture on ABC TV.
22/23-5-99	"Health saviour may lie in mud off Tasmania" *Weekend Australian*
27-5-99	Interview regarding novel sources of PUFA for aquaculture and industry on ABC Radio.
16-7-99	Interview regarding novel sources of PUFA for aquaculture and industry for ABC Radio *Country Hour*.
16-7-99	Interview regarding novel sources of PUFA for aquaculture and industry for ABC Radio *Country Hour* and *AM*.
Nov-1999	"Where the good oil comes from". Sci-Files 99-11. Interview with Dr Peter Nichols, produced by CSIRO Marine Research and Pegasus Media and distributed to ABC Radio.
Oct 2000	Interviews regarding microbial sources of PUFA for industry. ABC TV and Radio News.

The biotechnological potential of thraustochytrids

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Summary

Thraustochytrids are common marine microheterotrophs, taxonomically aligned with heterokont algae.

Recent studies have shown that some thraustochytrid strains can be cultured to produce high biomass, containing substantial amounts of polyunsaturated fatty acid (PUFA)-rich lipid. It is also evident that cell yield and/or PUFA production by some thraustochytrid strains can be varied by manipulation of physical and/or chemical culture parameters.

At present, fish oils and cultured phototrophic microalgae are the main commercial sources of PUFA. The possible decline of commercial fish stocks, and the relatively complex technology required to commercially produce microalgae, has prompted research into possible alternative sources of PUFA. The culture of thraustochytrids and other PUFA-producing microheterotrophs is seen as one such alternative. Indeed, several thraustochytrid-based products are already on the market, and research into additional applications is continuing.

Many fish and microalgal oils currently available have relatively complex PUFA profiles, increasing the cost of preparation of high-purity PUFA oils. In contrast, some of the thraustochytrids examined to date have simpler PUFA profiles. If these or other strains can be grown in sufficient quantities and at an appropriate cost, the use of thraustochytrid-derived oils may decrease the high expense currently involved with producing high purity microbial oils.

As more is learned about the health and nutritional benefits of PUFA, demand for PUFA-rich products is expected to increase. Results to date suggest that thraustochytrids could form an important part in the supply of such products.

Introduction

Thraustochytrids are common marine microheterotrophs that feed as saprobes or occasionally as parasites (Porter 1990). Thraustochytrids have a wide geographic distribution, with strains isolated from Antarctica (Bahnweg and Sparrow 1974), the North Sea (Raghukumar and Gaertner 1980), India (Raghukumar 1988), Micronesia (Honda et al. 1998), Japan (Naganuma et al. 1998) and Australia (Lewis et al. 1998a).

Originally thought to be primitive fungi, thraustochytrids have more recently been assigned to the sub-class Thraustochytridae (Chromista, Heterokonta), aligning them more closely with the heterokont algae (e.g. brown algae and diatoms) (Cavalier-Smith et al. 1994).

Following an early description of thraustochytrids (Sparrow 1936), little research concerning this group of organisms occurred until a number of descriptive and ecological studies in the 1960's (e.g. Goldstein 1963; Gaertner 1968). Findlay et al. (1986) discussed the use of fatty acids as biochemical indicators for thraustochytrids within mangrove detrital systems. Since then, several studies (which will be referred to later in this paper)

have catalogued the ability of some strains of thraustochytrid to produce 1) a relatively high biomass in culture, 2) a high proportion of lipid as part of this biomass, and/or 3) a high proportion of polyunsaturated fatty acids (PUFA) in the lipid.

Interest in the nutritional importance of PUFA has increased markedly during the past decade. As PUFA are necessary constituents of cell membranes and of many cell signaling systems, deficiencies in PUFA can be associated with defects in cellular function, which may lead to disease. A number of studies have further demonstrated that PUFA are essential dietary components for humans (reviewed by Simopoulos 1989; Takahata et al. 1998) and also in aquaculture operations for marine finfish and crustacean larvae (Sorgeloos and Leger 1992; Castell et al. 1994; D'Abramo 1997).

PUFA are generally classified into two main groups: the omega-6 (ω6 or n-6) and omega-3 (ω3 or n-3) series. Of the n-6 PUFA, arachidonic acid [AA; 20:4 (n-6)] is of particular importance, as it is a major precursor of many prostaglandins and eicosanoids. Eicosapentaenoic acid [EPA; 20:5 (n-3)] and docosahexaenoic acid [DHA; 22:6 (n-3)] are two n-3 PUFA which are currently receiving much attention and have been termed "essential" fatty acids. The n-3 PUFA are known to decrease the incidence of coronary heart disease, stroke and rheumatoid arthritis (Kinsella 1987). Evidence on the benefits and risks of n-3 PUFA for human health has recently been reviewed by Takahata et al. (1998). DHA is essential for normal development of neural tissue in infants, especially in the eyes and brain. The possible role of these PUFA against other disorders (e.g. asthma, dyslexia, depression, some forms of cancer) is also becoming increasingly recognised, although further research is required (Simopoulos 1989; Takahata et al. 1998).

As the importance of the presence and proportions of various PUFA in the diet of both man and beast becomes better understood, the value of these dietary components to a range of industries also increases.

At present, selected fish oils and microalgal species are utilised as the main industrial sources of PUFA. However, fish oil sources may be unreliable due to the failure or variability of various fisheries. There is concern that insufficient fish oil will be available in the future to meet the expected growth in world demand for n-3 oils (Tacon 1995; Ward 1995). Phototrophic microalgae are also used to provide PUFA for aquaculture operations (Volkman et al. 1989), with additional application in the production of nutraceuticals (dietary supplements).

In comparison, the de novo synthesis of n-3 and n-6 PUFA by thraustochytrids and other heterotrophic microorganisms may provide a cheaper and easier means of producing PUFA-rich biomass and oils. In recent years, interest in the use of microheterotrophs as a source of PUFA has increased (Ratledge 1993). Microheterotrophs do not require some of the elements necessary for the culture of autotrophs (e.g. light, carbon dioxide), and are seen by some as a potential alternative to traditional commercial sources of PUFA. Arachidonic acid has been produced in quantity by some fungi (Sajbidor et al. 1990; Gandhi and Weete 1991). Certain bacteria have been shown to produce EPA and DHA (Nichols et al. 1993; Jøstensen and Landfald 1997). The recognised need in aquaculture for alternate sources of PUFA for feeding to both larvae and adults has seen PUFA-producing

bacteria successfully demonstrated as a means to enrich rotifers (Brachionus plicatilis; a live-feed organism for finfish larvae) with these fatty acids (Watanabe et al. 1992; Nichols et al. 1996; Lewis et al. 1998b).

An increasing body of research into microheterotrophic PUFA production has concentrated on the thraustochytrids. This review is a brief synthesis of that work.

PUFA production by thraustochytrids

The importance of DHA in human and animal nutrition has received a great deal of research attention during the past decade (Simopoulos 1989; Takahata et al. 1998). This has prompted many studies into alternative sources of PUFA to focus on this particular fatty acid. Most reports concerning the production of PUFA by thraustochytrids have dealt almost exclusively with DHA production (Table 1), as this compound is often the most abundant PUFA produced by strains of thraustochytrids reported to date.

Data presented in Table 1 demonstrates the large variation in biomass, lipid and maximum DHA yields obtained for different thraustochytrid strains. For example, Schizochytrium aggregatum produced a biomass of 0.9 gL^{-1} after 10 days (Vazhappilly and Chen 1998), while a biomass of 48 gL^{-1} after 4 days was achieved using Schizochytrium sp. SR21 (Yaguchi et al. 1997). Perhaps more importantly, PUFA production by a single strain (T. roseum ATCC 28210) cultured under different conditions also showed marked differences. For this strain, a fed-batch flask culture yielded 2100 mg DHA L^{-1} (Singh and Ward 1996), as compared to an unsupplemented flask culture, which yielded 650 mg DHA L^{-1} (Li and Ward 1994).

It is beyond the scope of this review to explore in detail the many physicochemical manipulations of culture conditions that have been used to influence PUFA production by different thraustochytrid strains. However, Table 2 gives an indication of how variations in culture conditions can influence the biomass and amount of PUFA produced by various thraustochytrid strains. It is apparent from these results that changes to culture conditions do not have uniform effects on PUFA production by different thraustochytrid strains. Optimisation and manipulation of culture conditions to produce the amounts and types of PUFA required for specific applications are definitely areas that will require extensive research for each strain taken towards commercial production.

Although production of DHA has been the main focus of recent attention, it is evident that some thraustochytrid strains also produce other PUFA. Yokochi et al. (1997) suggested the fatty acid profiles of DHA-producing thraustochytrids could be used to classify them into 6 separate categories namely (a) DPA (22:5 n-6)/DHA, (b) EPA/DHA, (c) EPA/DPA/DHA, (d) AA/EPA/DHA, (e) LA (18:2 n-6)/AA/DPA/DHA and (f) LA/AA/EPA/DHA. Lewis et al. (1998a) isolated a number of thraustochytrid strains with a range of different PUFA profiles, including one strain (ACEM E) which produced AA to 30% of total fatty acids (TFA), with no other PUFA exceeding 10% TFA (Figure 1).

Given the diversity of PUFA profiles seen for thraustochytrids examined to date, we think it is likely that other, more unusual, PUFA will also be discovered from this group of organisms.

The market for PUFA

It is anticipated that the market in which thraustochytrid-based oils could have the most impact will be that currently occupied by PUFA-rich oils derived from marine fish. As such, some consideration of this market is warranted. Market data included in this section has been obtained from published papers, where available, and also from industry association and company websites and from market research information provided by Australian industry (Clover Corporation Pty Ltd, New South Wales, Australia).

The current and potential world market for fish oil-based products spans a number of sectors: from unprocessed, oil-rich biomass for animal feeds, to high quality food-grade oils for use as food additives and nutraceuticals and to very high purity oils and even individual fatty acids for use in the pharmaceutical industry.

At present, the major commercial source of PUFA-rich oils is fish oil. Annual worldwide production of fish oil has remained stable at about 1.3 million t for the past 10 years or so, and is unlikely to rise (Tacon 1997). The International Fishmeal and Oil Manufacturers Association (www.ifoma.com) estimates that inclusion of fish oil in aquaculture feeds will rise from 380,000 t in 1994 to 582,000 t in 2001 and 1,133,000 t in 2010. This could well result in a worldwide under-supply of fish oil, leading to increased demand for fish oil alternatives.

Fish oil is included in aquaculture feeds as a source of both dietary energy and PUFA (New and Csavas 1995). There is considerable research occurring worldwide in an effort to find alternatives to fishmeal and fish oil in aquaculture feeds. However, this research is tempered by the obligate dietary requirement of many marine finfish species for long chain PUFA (LCPUFA: e.g. EPA and DHA). It seems likely that cheap, plant- or animal-derived oils, which often contain low levels of LCPUFA, will be used increasingly as an alternative source of energy in some aquaculture feeds. If such substitution does occur, sufficient LCPUFA to meet the dietary requirements of cultured aquaculture species may be required from other sources. Typically, many cultured marine species require around 1-2% w/w LCPUFA in their diets (e.g. Rees et al. 1994; Salhi et al. 1994). Pike (1999) estimated that marine aquaculture finfish species will require about 2×10^6 t of feed in 2010. These figures point to a potential demand, for these species alone, for at least 20,000 t of LCPUFA per annum.

The imprecise boundaries surrounding the nutraceutical market make estimating the size of this market sector more difficult. Sales of marine supplement oils were in the order of US$55 million in the USA in 1996 (Molyneaux and Chong 1998), and represented 20% of sales from health food retail outlets. In the United Kingdom, fish oils account for approximately 29% (US$140 million) of the total annual market for nutraceuticals (Mukherjee 1999). Analysis of consumer awareness and knowledge indicates n-3 PUFA rate highly in both market perception and potential market success.

The western European market for infant formula increased from 81,500 t in 1988 to 103,933 t in 1994. There is an increasing trend for infant formula manufacturers to include PUFA-rich oils in their products. Typical inclusion levels of PUFA-rich oils are designed to achieve a final DHA concentration in dry infant formula of 0.1-0.2% w/w. Extrapolating these figures suggests a potential annual demand in the European infant formula market for up to 100-200 t of DHA.

There is already a number of food and beverage products, enriched with DHA or other PUFA, on the market. Mukherjee (1999) reported the availability of products such as enriched spreads, breads, eggs and soft drinks in Europe and Japan. Bread enriched with refined tuna oil as a source of LCPUFA is achieving substantial market penetration in Australia. As awareness, by both consumers and regulators, of the importance of adequate levels of PUFA in our diet increases, it can be assumed that demand for a greater range of PUFA-enriched products will increase.

The potential of thraustochytrids

Large-scale culture of thraustochytrids has real potential to be developed further as a commercial source of PUFA. If and where thraustochytrid-derived products are to fit into the market will be determined by our ability to produce, refine and/or enrich the oils to meet market specifications.

Thraustochytrids are already being used for commercial production of PUFA-rich products. A Schizochytrium strain is the basis for two products marketed for enriching rotifers (Brachionus spp) and brine shrimp (Artemia spp) with PUFA, prior to feeding these organisms to cultured finfish larvae (Barclay and Zeller 1996; www.aquafauna.com; www.sandersbshrimp.com). These products have entered the market in direct competition with microalgal and fish-oil based products. It is possible, however, that thraustochytrids may offer some advantage over other oils as sources of PUFA for aquaculture. Many aquaculture species require proportionally more DHA than EPA in their diet (Narciso et al. 1999). The PUFA profiles of many thraustochytrids fit this criterion, while most oils from the fish meal industry contain more EPA than DHA.

Other uses for thraustochytrid oil are being actively explored. Monsanto (www.monsanto.com) is producing Schizochytrium sp.-derived oil under a cooperative technology agreement with OmegaTech (www.omegadha.com). This oil is currently being used as a feed ingredient for laying hens to produce DHA-enriched eggs, and is under investigation for other food applications (Fitch Haumann 1999).

Ratledge (1993) stated that an increase in demand for pure PUFA preparations (e.g. oils containing EPA or DHA only) is likely to be the main driving force behind any commercial success of microbial oils. Oils derived from fish and microalgae generally have a complex fatty acid (total and polyunsaturated) profile, and do not readily lend themselves to the isolation of high purity (>98%) fatty acids. On the other hand, oils produced by some thraustochytrids have relatively simple fatty acid profiles (e.g. Schizochytrium limacinum; Yokochi et al. 1998) and may well be more amenable to cost effective refinement.

Development of economically viable technologies for the production of microbial PUFA
for aquaculture, livestock and human diets is the subject of intense worldwide research at
present. Given the potential for significant economic gain by those funding the research,
much of the data and results being generated have not been released to the scientific
community. However, selected information is available via the Internet (e.g.
www.aquafauna.com; www.omegadha.com; www.zenecalsm.com; www.monsanto.com).

Thraustochytrids are clearly a new and potentially competitive player in the PUFA market.
Considerable work is required before the production of oil from these organisms
significantly increases its share of the market for PUFA-rich products. To achieve this
aim, the following key stages need to be negotiated:

Collection, screening and maintenance of PUFA-producing strains: Several strains with
potential for the commercial production of DHA-rich oils have been already isolated.
However, if thraustochytrids are isolated and optimised that produce higher yields, more
attractive PUFA-profiles or other less common but sought after PUFA, demand for these
isolates and compounds may well increase.

Optimisation of efficiency of PUFA production: The types and amounts of PUFA
produced by individual strains of thraustochytrids are susceptible to manipulation by
varying culture conditions. Enhancement of PUFA profiles using molecular techniques
may be also considered. Different markets will provide demand for strains that produce
high levels of PUFA measured either in terms of biomass (i.e. PUFA production w/w cell
mass) or volume (i.e. PUFA production w/v fermentation medium).

Determination of appropriate conditions for long-term storage of microbial cells and/or
their products: The form and stability of thraustochytrid biomass and/or oils will be major
factors in determining the suitability of these products for use as food additives.

Development of oil extraction and refinement technologies to meet market demands for
cost-effective and safe trophic transfer of PUFA to the target consumer/s. The bottom line
for the biotechnological future of thraustochytrid-oils will be their competitiveness against
other PUFA-rich oils. Examples given above indicate large-scale culture of
thraustochytrids for commercial purposes is, or will soon be, economically feasible.
However, the commercial success of value-added oil products, for which the market is
willing to pay, is yet to be proven.

In summary, recent research has clearly demonstrated thraustochytrids as either an already
useable or potential source of PUFA-containing biomass and oils. Although this short
review has concentrated on PUFA production, the high growth rates and biomass already
achieved with some thraustochytrid strains suggests that these organisms could have wider
use as cell factories for the generation of other products. Further research and
development with the PUFA-producing thraustochytrids is necessary to allow increased
transfer of this knowledge to the biotechnology and associated industries. Similarly,
increased market acceptance of, and demand for, specific omega-3 products, including

within a range of nutraceuticals and functional foods, will require increased knowledge of specific nutritional and health benefits of these oils.

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Table 1   DHA production by thraustochytrids

Organism	Culture conditions				Biomass (g/L)	Total lipid (% dw)	DHA production			Reference
	Age (d)	Temp (°C)	Vessel	Other			%TFA	mg/g	mg/L	
Schizochytrium sp SR21	2.5	28	fermenter	pH 4	21	50	35	224	4700	Nakahara et al. (1996)
Schizochytrium sp SR21	4		fermenter 300 rpm	-	48	77	36	277	13300	Yaguchi et al. (1997)
S. limacinum SR21	5	25	flask	-	38	37	33	110	4200	Yokochi et al. (1998)
S. aggregatum ATCC 28209	10	25	flask 200 rpm	dark	0.9	-	1.7	30	0.4	Vazhappilly and Chen (1998)
Thraustochytrium aureum ATCC 34304	6	25	flask 300 rpm	light	3.8	16.5	49	70	270	Bajpai et al. (1991a)
T. aureum ATCC 34304	6	25	flask 300 rpm	light	4.9	20.3	51	104	511	Bajpai et al. (1991b)
T. aureum ATCC 34304	2.5	25	flask	light	5.7	8.1	40	-	-	Iida et al. (1996)
T. aureum ATCC 28211	6	25	flask 200 rpm	dark	0.8	-	3.7	50	4.0	Vazhappilly and Chen (1998)
T. roseum ATCC 28210	5	25	flask 250 rpm	light	7.6	18.2	50	87	650	Li and Ward (1994)
T. roseum ATCC 28210	12	25	flask 200 rpm	fed batch	17.1	25	49	115	2100	Singh and Ward (1996)
Thraustochytrium sp. ATCC 20892	4	25	flask 200 rpm	-	2.7	7.3	35	25	68	Singh et al. (1996)
Thraustochytrium sp. ATCC 26185	6	28	flask 120 rpm	light	7.5	32	25	-	-	Weete et al. (1997)

Table 2   Some factors which have been shown to effect cell yield and PUFA production by thraustochytrids.

| Organism	Culture vessel	Culture conditions, which when varied resulted in changes in cell yield and/or PUFA production	Reference
Schizochytrium sp SR21	fermenter	Medium composition Impeller speed (300 rpm; 500 rpm) Impeller shape (propeller-shaped, turbine-shaped) Culture age (62 – 125 h)	Yaguchi et al. (1997)
S. limacinum SR21	flask	Medium composition Incubation temperature (10 - 35°C) Seawater concentration (0 – 200% seawater)	Yokochi et al. (1998)
T. aureum ATCC 34304	flask fermenter	Medium composition Incubation temperature (15 - 35°C) Initial medium pH (4 – 9) Illumination (light; dark) Inoculum age (24 – 72 h) Culture vessel type (shake flask; fermenter) Culture age (0 – 12 d)	Bajpai et al. (1991b)
T. aureum ATCC 34304	flask fermenter	Medium composition Salinity (0 – 200% seawater) Vitamins Culture vessel type (shake flask; fermenter)	Iida et al. (1996)
T. roseum ATCC 28210	flask	Medium composition Incubation temperature (5 - 37°C) Initial medium pH (4 – 9) Culture age (0 – 7 d)	Li and Ward (1994)
T. roseum ATCC 28210	flask	Medium composition Batch feeding (4 – 12 d after inoculation) Culture age (2 – 8 d)	Singh and Ward (1996)
Thraustochytrium sp. ATCC 20892	flask	Medium composition Incubation temperature (15 – 35 °C) Incubation temperature shift (initial 25°C; final 15°C) Culture age (1 – 7 d)	Singh et al. (1996)
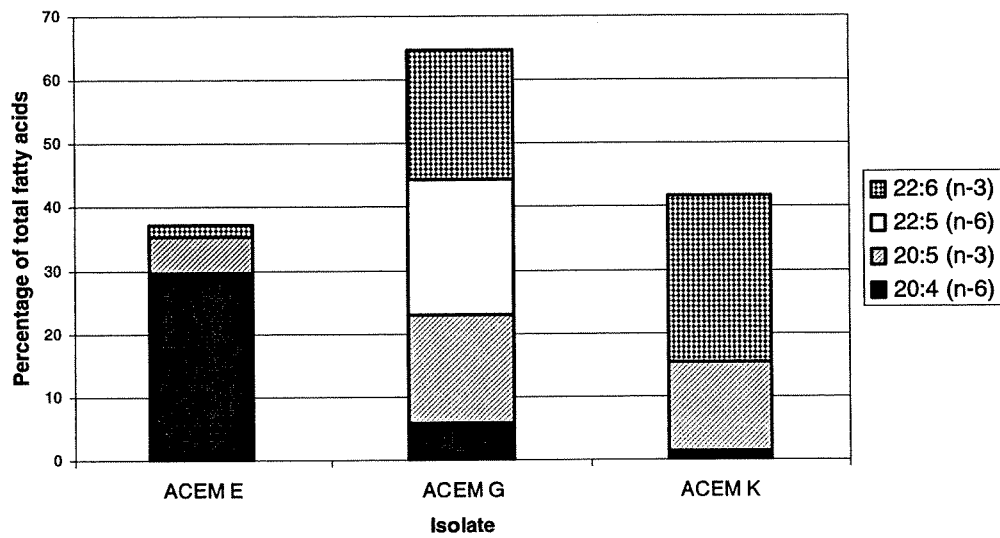
Figure 1   Composition of PUFA in new Australian thraustochytrids
            [expressed as % of total fatty acids; after Lewis et al. (1998a)]
            [Key: 22:6 (n-3) - docosahexaenoic acid;
            22:5 (n-6) - docosapentaenoic acid (n-6);
            20:5 (n-3) - eicosapentaenoic acid;
            20:4 (n-6) - arachidonic acid]

Sterol and squalene content of a DHA-producing thraustochytrid: influence of culture age, temperature and dissolved oxygen

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Running title:

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Summary

Thraustochytrid strain ACEM 6063, rich in omega-3 polyunsaturated fatty acids, was cultured at 15°C and 20°C in high (> 40%) and low (< 5%) dissolved oxygen (DO), and at 25°C in low DO media. Samples were taken 4, 2 and 0 days before each culture reached peak biomass (T_{-4}, T_{-2} and T_p respectively). Twenty sterols, 13 of which were identified, were detected. Predominant were: cholest-5-en-3-ol, 24-ethylcholesta-5,22E-dien-3-ol, 24-methylcholesta-5,22E-dien-3-ol, and two co-eluting sterols – one of which was 24-ethylcholesta-5,7,22-trien-3-ol. These 4 sterols comprised 50-90% of total sterols. Cultures grown at high DO contained simpler sterol profiles than those grown at low DO. Only the 4 sterols mentioned above were present at >3% of total sterols in high DO cultures. In low DO cultures, up to 6 additional sterols were present at >3% of total sterols. Culture age, temperature and DO influenced squalene and sterol content. Total sterols (as a proportion of total lipids) decreased with increasing culture age. If organisms such as ACEM 6063 are to be used for commercial production of lipid products for human consumption, both their sterol content and factors influencing sterol production need to be characterised thoroughly.

Introduction

Interest in the nutritional importance of long chain polyunsaturated fatty acids (PUFA) has increased markedly in recent years. As the importance of the presence and proportions of various PUFA in human and animal diets becomes better understood, demand for, and hence the value of, these dietary components is expected to increase (Mukherjee, 1999). This potential increase in the demand for PUFA, coupled with the predicted stasis or decline in the major current commercial source of PUFA-rich oil (fish oil) (New and Csavas, 1995), has led to an increase in research into novel, sustainable sources of these compounds.

One area receiving considerable interest at present is the culture of PUFA-producing microheterotrophs. One group of microheterotrophs, the thraustochytrids, is considered to hold particular potential as the basis of a viable industry to supply PUFA-rich biomass and oils (see review by Lewis et al. (1999)). Thraustochytrids are common marine microheterotrophs taxonomically aligned with heterokont algae. Recent studies (e.g. Nakahara et al., 1996; Yaguchi et al., 1997; Vazhappilly and Chen, 1998; Yokochi et al., 1998) have shown that some thraustochytrid strains can be cultured to yield substantial amounts of PUFA-rich biomass. It is also evident that cell yield and PUFA production by some thraustochytrid strains can be varied by manipulation of physical and chemical culture conditions.

Although PUFA production by several thraustochytrid strains has been well documented, there has been, to our knowledge, only one report discussing the production of sterols by these organisms (Weete et al., 1997). Sterols are membrane lipids that are produced by all microeukaryotes, as well as by a limited number of bacteria (e.g. Jones et al., 1994; Volkman et al., 1998; Schouten et al., 2000). The first major step in sterol synthesis is the oxygenation of squalene to form (3S)-squalene epoxide (3S-squalene-2,3-oxide), which is then cyclised by one of two enzymes to form either lanosterol or cycloartenol. These two compounds then undergo a series of additional reactions to yield further sterol products (Torssell, 1983; Jones et al., 1994).

Those sterols commonly found in food plants (phytosterols: e.g. brassicasterol, sitosterol, campesterol, stigmasterol: Harwood and Russell, 1984) are generally regarded as safe for human consumption, and are even believed to confer some positive health benefits (e.g. Moghadasian and Frohlich, 1999). Regulatory authorities, however, treat the presence of uncommon and/or unknown sterols in food products very warily. This wariness is based on knowledge that, when ingested, sterols may influence metabolic functions (Nichols et al., 1998), and therefore evidence confirming the safety of novel sterols is required.

In this study, we have examined in detail the sterol composition of the DHA-producing thraustochytrid ACEM 6063. We have also determined the effects of culture temperature, dissolved oxygen concentration and culture age on the squalene and sterol content of biomass of this strain. This was a first step towards understanding the factors that influence sterol production by a PUFA-producing thraustochytrid.

Materials and Methods

Analytical reagents

All reagents used were of analytical standard. Chloroform, methanol, hexane and hydrochloric acid were obtained from Mallinckrodt (USA). Deionised water was produced using a Milli Q Plus (Millipore SA, France) water treatment system.

Microheterotroph culture and cell preparation

A thraustochytrid (strain ACEM 6063), notable for producing high levels of the n3 fatty acid docosahexaenoic acid [DHA, 22:6n3], was isolated from coastal marine sediment in southeastern Tasmania (Australia). Preliminary 18S rDNA sequence data suggests this strain may be a member of the genus *Schizochytrium* (T. Lewis, unpublished data, Honda et al., 1999). Strain ACEM 6063 can produce up to 400 mg fatty acids g^{-1} dry weight (Lewis et al., 2000), with DHA levels of about 30% total fatty acids (T. Lewis, unpublished data). Biomass for lipid analysis was produced under ambient light conditions in a BioFlo IIC fermenter (New Brunswick, USA) containing an initial volume of 2000 mL of a glucose-rich culture medium (Staley et al., 1992; Iida et al., 1996; Singh and Ward, 1996). Culture parameters varied were temperature and dissolved oxygen (DO). Culture temperatures (15°C; 20°C; 25°C) were controlled to within 1°C using a microprocessor-controlled water jacket. Dissolved oxygen levels (low: 0-5% saturation; high: 40-60% saturation) were controlled semi-automatically, using a combination of impeller revolution speed (via microprocessor-controlled feedback from the oxygen probe) and air/oxygen supply (manual control). The maximum impeller speed in all fermenter cultures was limited to 200 rpm, as some thraustochytrids may be susceptible to higher impeller speeds (Yaguchi et al., 1997). Aeration was supplied using 0.2 μm-filtered (Midisart 2000, Sartorius, Germany) air for low DO cultures, or a mixture of air and oxygen (Industrial Grade O_2, BOC Gases, Australia) for high DO cultures. The air/oxygen mixture was delivered via 0.5-mm diameter holes situated at the base of the culture vessel. The culture system used was not able to supply sufficient oxygen to maintain high DO levels in cultures grown at 25°C.

Fermenter cultures were sampled daily from inoculation until 4 days after peak biomass (mg dry weight L^{-1}) was reached. Samples (10 mL) were aseptically drawn from the culture chamber directly into sterile, 15 mL screw-top plastic centrifuge tubes (Greiner, Germany). Cells were immediately centrifuged (5000 x g, 10 min: EasySpin, Sorval Instruments, USA), resuspended in 10 mL 1.0% (w/v) NaCl, and recentrifuged. Cell pellets were frozen, freeze-dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure < 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15 hours, weighed and then stored at -30°C. Culture biomass (mg dry weight L^{-1}) was determined daily using freeze-dried samples. The small amount of NaCl from the wash water remaining in the samples was calculated from the amount of weight lost during the freeze-drying process, and was subtracted from the dry sample weight to provide the true culture biomass (mg dry weight L^{-1}).

Sterol extraction, fractionation and analysis

Analyses were performed on ACEM 6063 samples collected on the day each culture reached peak biomass, and 2 and 4 days prior to that day (T_p, T_{-2} and T_{-4} respectively). Lipids were extracted, in duplicate from each sample, using a one-phase chloroform-methanol-water (1:2:0.8 v/v/v) extraction (White et al., 1979). Solvents were added to the freeze-dried biomass in order of increasing polarity, as this technique led to an increased lipid recovery from ACEM 6063 (Lewis et al., 2000). The biomass-solvent mixtures were left in darkness overnight. Phase separation was achieved the next day by adding chloroform and water to obtain a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). Lipids were recovered from the lower chloroform phase. Solvents were removed under vacuum prior to the extracted lipids being stored in chloroform under N_2 at -20°C.

The sterol content of the various ACEM 6063 biomass samples was determined by analysing a portion of each total lipid extract using thin layer chromatography – flame ionisation detection (TLC-FID) (Iatroscan Mk IIITH-10, Iatron Laboratories, Japan). Aliquots from a known volume of each extract were applied onto duplicate chromarods using disposable micropipettes. Chromarods were developed using a hexane-diethylether-acetic acid (60:17:0.2 v/v/v) solvent system in a sealed glass tank containing pre-extracted filter paper. After development, chromarods were oven-dried at 100°C for 10 min and analysed immediately. Peak areas were recorded and quantified using DAPA (Scientific Software, Kalamunda, Western Australia).

To obtain the non-saponifiable lipids (which included the squalene and sterol fractions), portions of the total lipid extracts were transferred into 10 mL, screw-top test tubes. A fresh solution of 5% (w/v) potassium hydroxide in methanol-water (4:1 v/v) was added to each tube for reaction (saponification) at 60°C for 3 hours. After the addition of water (1 mL), the resultant non-saponifiable neutral lipids were extracted with hexane-chloroform (4:1 v/v, 3 x 2 mL) (Bakes and Nichols, 1995). Sterols in the non-saponifiable neutral lipid fraction were analysed by gas chromatography of their trimethylsilyl ether (TMS) derivatives. Steryl-TMS derivatives were prepared by reacting a portion of the non-saponifiable neutral lipid with an excess of N,O-bis(trimethylsilyl) trifluoroacetamide (Alltech, USA) at 60°C for one hour (Bakes and Nichols, 1995). Gas chromatographic data was obtained with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 µm film thickness) fused-silica capillary column, an HP7673A autosampler, a split/splitless injector and flame ionization detector (Nichols et al., 1996). Peak areas were recorded and quantified using Millenium 32 v3.05.01 (Waters Corporation, USA). Mass spectrometric data was obtained using a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS), operated as described in Nichols et al. (1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards, including lipid from *Methylosphaera hansonii* (Schouten et al., 2000).

Blank controls (i.e. fresh, cell-free culture medium) were incorporated into this study. No sterols were detected in any of the controls.

Results

Twenty sterols, 13 of which have been identified, were recovered from ACEM 6063 biomass (Figure 2, Table 2). Only those sterols exceeding 3% of the total sterol fraction are shown in Table 3. The two major sterols in all samples were cholest-5-en-3β-ol and 24-ethylcholesta-5,22E-dien-3β-ol (sterols B and J respectively, Table 3). These two sterols accounted for 35–80% of the total sterols.

Cultures grown at 15°C-high DO and 20°C-high DO contained only sterols B, J, E (24-methylcholesta-5,22E-dien-3β-ol) and M (a mixture of two sterols co-eluting at a relative retention time of 1.58– one of which was 24-ethylcholesta-5,7,22-trien-3β-ol) at levels > 3% of the total sterols (Table 3). Cultures grown at 15°C-low DO, 20°C-low DO and 25°C-low DO also contained these sterols, but at slightly lower levels than in the high DO cultures. The low DO cultures also contained up to 6 of the following sterols at levels of > 3% of total sterols: cholest-8(9)-en-3β-ol, 5α-cholest-7-en-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-ethylcholesta-5, 24(28)E-dien-3β-ol, 24-ethylcholesta-5, 24(28)Z-dien-3β-ol, and 4 unidentified sterols.

Absolute squalene content (expressed as mg g^{-1} dry weight; mean, n=2) in ACEM 6063 biomass varied under different temperature and dissolved oxygen parameters, and with time (Figure 3). Cultures grown at high DO contained lower levels of squalene than those grown at low DO. Cultures grown at 15°C-high DO all contained $\leq$ 0.01 mg g^{-1} squalene. Cultures grown at 20°C-high DO contained 0.01, 0.02 and 0.01 mg g^{-1} squalene at T$_{-4}$, T$_{-2}$ and T$_p$ respectively. Squalene content of cultures grown at 15°C-low DO decreased sequentially from T$_{-4}$ to T$_{-2}$ to T$_p$ (1.8, 1.5 and 1.2 mg g^{-1} respectively). At each sample time (T$_{-4}$, T$_{-2}$ and T$_p$), the culture grown at 20°C-low DO contained less squalene (0.1, 0.7 and 0.2 mg g^{-1} respectively) than that grown at 15°C-low DO. Similarly, at each sample time (T$_{-4}$, T$_{-2}$ and T$_p$), the culture grown at 25°C-low DO contained less squalene (0.02, 0.1 and 0.01 mg g^{-1} respectively) than that grown at 20°C-low DO. The three cultures grown at 20°C and 25°C all contained the highest squalene concentrations at T$_{-2}$, whereas the two cultures grown at 15°C contained the highest squalene concentration at T$_{-4}$.

Total sterol content for ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters, and sampled at T$_{-4}$, T$_{-2}$ and T$_p$ is shown in Figure 4. The relative level of sterols in the total lipid extracted from ACEM 6063 decreased between T$_{-4}$ (0.5-2.4 % total lipids) and T$_p$ (0.4-0.9 % total lipids). Sterol concentrations in cultures grown at 15°C-low DO were lower than for those grown at 15°C-high DO (0.4-0.6 mg g^{-1} c.f. 0.8-1.2 mg g^{-1}). Sterol concentration in cultures grown at 20°C increased markedly between T$_{-4}$ and T$_{-2}$, and then decreased slightly between T$_{-2}$ and T$_p$. At T$_{-4}$, both cultures grown at 20°C contained a similar amount of sterol to those grown at 15°C-high DO ($\sim$1.0 mg g^{-1}). However, at T$_{-2}$, sterol content in cultures grown at 20°C had increased markedly (reaching 2.2 mg g^{-1} for low DO cultures, and 3.2 mg g^{-1} for high DO cultures). Sterol levels for both low and high DO cultures grown at 20°C at T$_p$ (1.7 mg g^{-1} and 2.2 mg g^{-1} respectively) were slightly lower than those at T$_{-2}$. Absolute sterol concentration in the 25°C-low DO culture increased substantially between T$_{-4}$ and T$_{-2}$, and increased slightly between T$_{-2}$ and T$_p$ (0.6, 1.4 and 1.5 mg g^{-1} respectively).

Cultures grown at 20°C and 25°C reached a peak biomass of 21-23 g dry weight L^{-1} after incubation for 7-8 days. Cultures grown at 15°C reached a peak biomass of 17 and 10 g dry weight L^{-1} (high DO and low DO cultures respectively) after incubation for 9 days.

Discussion

This study has demonstrated that changes to both physical and chemical culture conditions can influence squalene and sterol production by the thraustochytrid ACEM 6063. Changes in sterol levels, measured as a proportion of the total lipid fraction, were observed in cells cultured at different temperatures and dissolved oxygen concentrations. The proportion of sterols in the total lipid fractions of all treatments decreased with culture age, even when absolute sterol levels increased. Although there was no clear pattern to these changes, it is likely that they were brought about by variations in the relative amount of other lipid classes, in particular triacylglycerols, produced by ACEM 6063. As sterols are associated with cell membranes (Harwood and Russell, 1984), this result indicates ACEM 6063 produced proportionally less membrane lipids with increasing culture age. Similar findings have been reported for microalgae, some of which are thought to be the closest relatives of thraustochytrids (Cavalier-Smith et al., 1994; Honda et al., 1999). In a review on microalgal biomarkers, Volkman et al. (1998) reported that the proportion of microalgal membrane lipids generally increases during logarithmic growth, while neutral lipids (free fatty acids, triacylglycerols, ketones and hydrocarbons) are highest during the stationary phase (i.e. after achieving peak biomass).

The concentration of dissolved oxygen in the culture media influenced the squalene and total sterol concentration in ACEM 6063. A clear inverse relationship was seen between the squalene concentration in the different cultures and the DO levels at which those cultures were grown. Squalene requires oxygenation to form (3S)-squalene epoxide (3S-squalene-2,3-oxide), before being cyclised to form either lanosterol or cycloartenol (Jones et al., 1994). We propose that at low DO levels, the efficiency of the conversion of squalene into sterols by ACEM 6063 was reduced. This observation could also explain the high squalene levels (63% of total non-saponifiable lipids) in another thraustochytrid (*Thraustochytrium* sp ATCC 26185) reported by Weete et al. (1997). The cultures described by Weete et al. (1997) were grown in Erlenmeyer flasks (100 mL medium in 250 mL flasks) on a rotary shaker at 120 rpm. These conditions are similar to those known to produce very low oxygen levels (< 5% DO) in actively growing thraustochytrid cultures (T. Lewis – unpublished data). Cultures grown at high DO contained higher total sterol concentrations than equivalent cultures grown at low DO.

Incubation temperature also influenced the absolute amount of squalene and total sterols in ACEM 6063 cells. An inverse relationship between absolute squalene content and culture temperature was observed. This was especially evident for cultures grown at low DO levels. While there also seemed to be an inverse correlation between squalene and sterol content at different oxygen levels (as discussed earlier), no correlation was evident at different culture temperatures. As was the case for culture DO, variations in culture temperature had a greater impact on total squalene content than on total sterol content.

The influence of culture age on squalene and total sterol content of ACEM 6063 biomass varied with incubation temperature. Squalene content of cells grown at 15°C did not vary substantially between T_{-4} and T_p. However, squalene content of cells grown at 20°C and 25°C did vary with culture age, and all showed the same pattern: squalene content increased between T_{-4} and T_{-2}, and decreased between T_{-2} and T_p. Total sterol concentration in the various ACEM 6063 cultures followed a similar trend to squalene content, especially for samples collected at T_{-4} and T_{-2}. In cultures grown at 15°C, sterol content at T_p was similar to those at T_{-2} and T_{-4}. In cultures grown at 20°C and 25°C, the total sterol content increased between T_{-4} and T_{-2}, and either decreased slightly or remained the same between T_{-2} and T_p.

Our data indicate a relationship between the absolute levels of squalene and total sterols in ACEM 6063 biomass. However, the degree of variation in squalene content between low and high DO cultures was much greater than that for sterols. There was also some discrepancy between concentration of squalene and total sterols in samples incubated at 20°C and 25°C and collected at T_{-2} and T_p, with the substantial decreases in squalene content not coinciding with similarly substantial decreases in total sterols. Although further work is required, the following sequence could at least partially explain these observations: a) from inoculation until T_{-2}, squalene production is sufficient to meet demands for sterol production; b) between T_{-2} and T_p, squalene production cannot meet this demand, resulting in a decrease in squalene reserves.

Culture DO appears to have had a greater influence on the relative amounts of individual sterols (expressed as a % of total sterols) than culture temperature or age. Although all sterols listed in Table 1 were detected in all samples, many were often present at very low levels (e.g. $\leq 1\%$ of total sterols), especially in the high DO cultures. Cultures grown at high DO had a less diverse profile of those sterols comprising > 3% of the total sterols than low DO cultures. The high DO cultures were dominated by 4 or 5 sterols: cholest-5-en-3β-ol, 24-ethylcholesta-5,22E-dien-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, and a mixture of two sterols co-eluting at a relative retention time of 1.58– one of which was 24-ethylcholesta-5,7,22-trien-3β-ol. These 5 sterols were present in similar proportions in all high DO cultures and comprised about 90% of the total sterols in each culture. The low DO cultures also contained these same 4 or 5 sterols, but in lower proportions, together with several other sterols – again combining to a total of about 90% of the total sterols. One consistent difference between the sterols in high and low DO cultures was the relatively high levels of an unidentified sterol (sterol S: a mixture of two unidentified sterols, co-eluting at the relative retention time of 1.81) in all low DO cultures. These two sterols were also observed, but at much lower levels, in the high DO cultures. In all low DO cultures, the relative abundance of these two sterols was highest at T_{-2}.

Although we did not examine the influence of culture media on sterol production, it is likely that sterol content of ACEM 6063 would also be influenced by changes to the media. Weete et al. (1997) reported that by omitting basal salts from the culture medium, the non-saponifiable lipid content of *Thraustochytrium* sp. ATCC 26185 decreased by 68%. Unfortunately, no data was presented on the sterol profiles obtained under these different culture conditions.

The individual sterol profiles of ACEM 6063 were similar to those reported in the only other study (to our knowledge) of sterol production by a thraustochytrid. Weete et al. (1997) reported that *Thraustochytrium* sp. ATCC 26185 contained cholest-5-enol, 24-methylcholesta-5,22-dienol, 24-methylcholesta-5,24,(28)-dienol, 24-ethyl-cholesta-5,22-dienol and 24-ethyl cholesta-5,7,22-trienol at levels of 41.3%, 7.6%, 1.5%, 12.4% and 21% respectively. In the present study, the same sterols (sterols B, E, H, J and a proportion of M) were detected at levels of 22-55%, 3-10%, <3-5%, 13-28% and a proportion of 5-14% respectively. Weete et al. (1997) also reported 8 unknown sterols to a total of 17%. It is interesting to note that neither lanosterol nor cylcoartenol, the two primary sterols formed by the cyclisation of 3S-squalene-2,3-oxide (Torssell, 1983), were detected in the present study or that of Weete et al. (1997). Assuming that one or both of these primary sterols would have been produced, it appears that, in these organisms, lanosterol and/or cylcoartenol occurred only as intermediates during the formation of other sterols.

Microalgae and other eukaryotic microorganisms produce a wide variety of sterols, some of which have been used as chemotaxonomic markers (e.g. Volkman et al., 1989). However, Volkman et al. (1998) also offered a caveat on comparisons of microalgal sterol compositions for taxonomic purposes, citing environmental conditions as a source of significant changes in many species. Given the results presented in our study, it appears that variations in some environmental conditions can also significantly influence the sterol profile of thraustochytrids. This indicates that taxonomic comparisons of thraustochytrids using sterol profiles should only be made with care. Notwithstanding these comments, the similarities of the sterol profiles of *Thraustochytrium* sp. ATCC 26185 presented by Weete et al. (1997) and those of ACEM 6063 in this study indicate a degree of taxonomic similarity between these two organisms. This inference is strengthened by the comment made by Weete et al. (1997) that unpublished sterol compositions for four other *Thraustochytrium* spp. were very similar to that of *Thraustochytrium* sp. ATCC 26185. It may be that the minor sterols will provide further taxonomic insight into the relationships between members of this group of organisms.

We have presented, for the first time, an examination of sterol and squalene content of thraustochytrid biomass produced under different culture conditions. Our results indicate that total squalene and sterol content were influenced to varying degrees by culture age, temperature and dissolved oxygen concentration. An interaction between incubation temperature and culture age was also noted; in that sterol content (mg g^{-1} dry weight) increased markedly between T_4 and T_{-2} in cultures grown at 20°C and 25°C, but did not increase in cultures grown at 15°C. This study represents an early step in the process of understanding sterol production by thraustochytrids. Further research will be needed to more carefully examine any interactions between the influence of major environmental variables on sterol content and composition. Some of the sterols identified in ACEM 6063 and in *Thraustochytrium* sp. ATCC 26185 (Weete et al., 1997) are the same as those found in many common food plants (Harwood and Russell, 1984), and, as such, could be regarded as safe for human consumption. However, these two thraustochytrids also contain low levels of other unusual or unidentified sterols, which will require further characterisation and study before their nutritional effects can be determined. Whether our

understanding of thraustochytrid sterol production is required for taxonomic or nutraceutical purposes, the importance of describing the effect/s of physical and chemical culture parameters on sterol production by these organisms is likely to increase.

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Acknowledgements

Table 2   Sterols recovered from the thraustochytrid, strain ACEM 6063

Code	Name	Trivial name [1]	RRT [2]	M+	Base peak	Major ion fragments (m/z)
A	cholesta-5, 22-dien-3β-ol	22-dehydrocholesterol	0.87	456	366	351, 327, 255, 129
B	cholest-5-en-3β-ol	cholesterol	1.00	458	368	443, 353, 329, 255, 247
C	cholest-8(9)-en-3β-ol	24-dihydrozymosterol	1.06	458	458	443, 368, 353, 255, 229, 213
D	cholesta-5, 24-dien-3β-ol	desmosterol	1.11	456	207	441, 366, 343, 253
E	24-methylcholesta-5, 22E-dien-3β-ol	brassicasterol / crinosterol	1.14	470	255	445, 380, 365, 340, 129
F	5α-cholest-7-en-3β-ol	lathosterol	1.18	458	458	443, 368, 353, 255, 229, 213
G [3]	4-methylcholest-8(14)-en-3β-ol		1.29	472	472	457, 382, 367, 227
H	24-methylcholesta-5,24(28)-dien-3β-ol	24-methylenecholesterol	1.32	470	296	455, 386, 341, 253
I [4]	Unknown 1		1.34	472	472	457, 382, 367, 227
J	24-ethylcholesta-5, 22E-dien-3β-ol	stigmasterol / poriferasterol	1.45	484	394	379, 355, 255, 129
K	Unknown 2		1.47	484	227	470, 394, 365
L [5]	Unknown 3		1.50	470	343	455, 386, 253
M [6]	Unknown 4, 24-ethylcholesta-5,7,22-trien-3β-ol		1.58	484/ 482	211	394, 377, 351, 253, 225
N [7]	4,4-dimethylcholesta-8(14),24-dien-3β-ol		1.60	484	241	469, 379, 357, 227
O	24-ethylcholesta-5, 24(28)E-dien-3β-ol	fucosterol	1.63	484	296	386, 371, 355, 281, 257
P [8]	Unknown 5		1.67	484	241	469, 394, 379, 357, 227
Q	24-ethylcholesta-5, 24(28)Z-dien-3β-ol	isofucosterol	1.68	484	296	386, 371, 281, 257
R	Unknown 6		1.78	470	357	400, 379, 267, 227
S [9]	Unknown 7		1.81	484/ 498	343	469, 409, 393, 386, 365, 253

1:   C_{24} stereochemistry not determined for sterols E and J
2:   Relative Retention Time – calculated using cholesterol (1.00) and fucosterol (1.63) (Jones et al., 1994)
3:   structure IV from Schouten et al. (2000)
4:   possibly the Δ8(9) analogue of G
5:   shares major ion fragments with Unknown 7
6:   mixed peak containing a C_{29} sterol with 2 double bonds [M+: 484] and 24-ethylcholesta-5,7,22-trien-3β-ol.
7:   structure I from Schouten et al. (2000)
8:   possibly the Δ8(9),24 analogue of N
9:   mixed peak, shares major ion fragments with Unknown 3

Table 3   Sterol composition [1] of thraustochytrid strain ACEM 6063 cultured under different temperature and dissolved oxygen parameters (expressed as % of total sterols)

| Treatment [3] | Sterol [2] (mean, n=2) | | | | | | | | | | | | | Total [4] |
	B	C	E	F	H	I	J	M	O	P	Q	R	S	
15LT$_{-4}$	25		3.2			4.7	14	13	9.4	4.1	3.9	3.1	12	92
15LT$_{-2}$	22					4.4	13	14	8.2	5.2	3.2		19	89
15LT$_p$	27	6.9				3.3	18	11	11				11	88
15HT$_{-4}$	51	9.5					22	5.6						88
15HT$_{-2}$	54	8.1					25	4.7						92
15HT$_p$	55	7.9					25	5.2						93
20LT$_{-4}$	41	5.0	8.5				21	5.0	3.3				7.3	91
20LT$_{-2}$	42	3.1		3.2			16	4.5	4.2				12	85
20LT$_p$	50	3.8		5.4			19	4.3	4.3				7.2	94
20HT$_{-4}$	52	6.3					28	5.4						92
20HT$_{-2}$	55	6.9					26	5.0						93
20HT$_p$	53	6.5					26	5.2						91
25LT$_{-4}$	44						19	13	5.2				7.7	89
25LT$_{-2}$	41						18	13	3.5				13	89
25LT$_p$	47		4.6				19	13	3.2				4.3	91

1:    Sterols comprising > 3% of the total sterol fraction (mean data, n=2)
2:    see Table 2 for sterol identifications
3:    Treatment notation:
       15, 20, 25: incubation temperature (°C);
       L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;
       T$_{-4}$, T$_{-2}$, T$_p$: sampled 4, 2 and 0 days prior to peak biomass being reached
4:    Cumulative total of major sterols as a % of total sterols

Figure legends:

Figure 2   Representative partial gas chromatogram of squalene and sterols (as OTMS ethers) recovered from thraustochytrid strain ACEM 6063 (HP5 capillary column; peak labels refer to Table 2)

Figure 3   Squalene content of thraustochytrid strain ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters (expressed as mean±SD (n=2) mg g^{-1} of dry weight)

[Treatment notation:
15, 20, 25: incubation temperature (°C);
L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;
T-4, T-2, Tp: sampled 4, 2 and 0 days prior to peak biomass being reached]

Figure 4   Total sterol content of thraustochytrid strain ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters (expressed as mean±SD (n=2) of % of total lipid and mg g^{-1} of dry weight)

[Treatment notation:
15, 20, 25: incubation temperature (°C);
L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;
T-4, T-2, Tp: sampled 4, 2 and 0 days prior to peak biomass being reached]

Figure 1

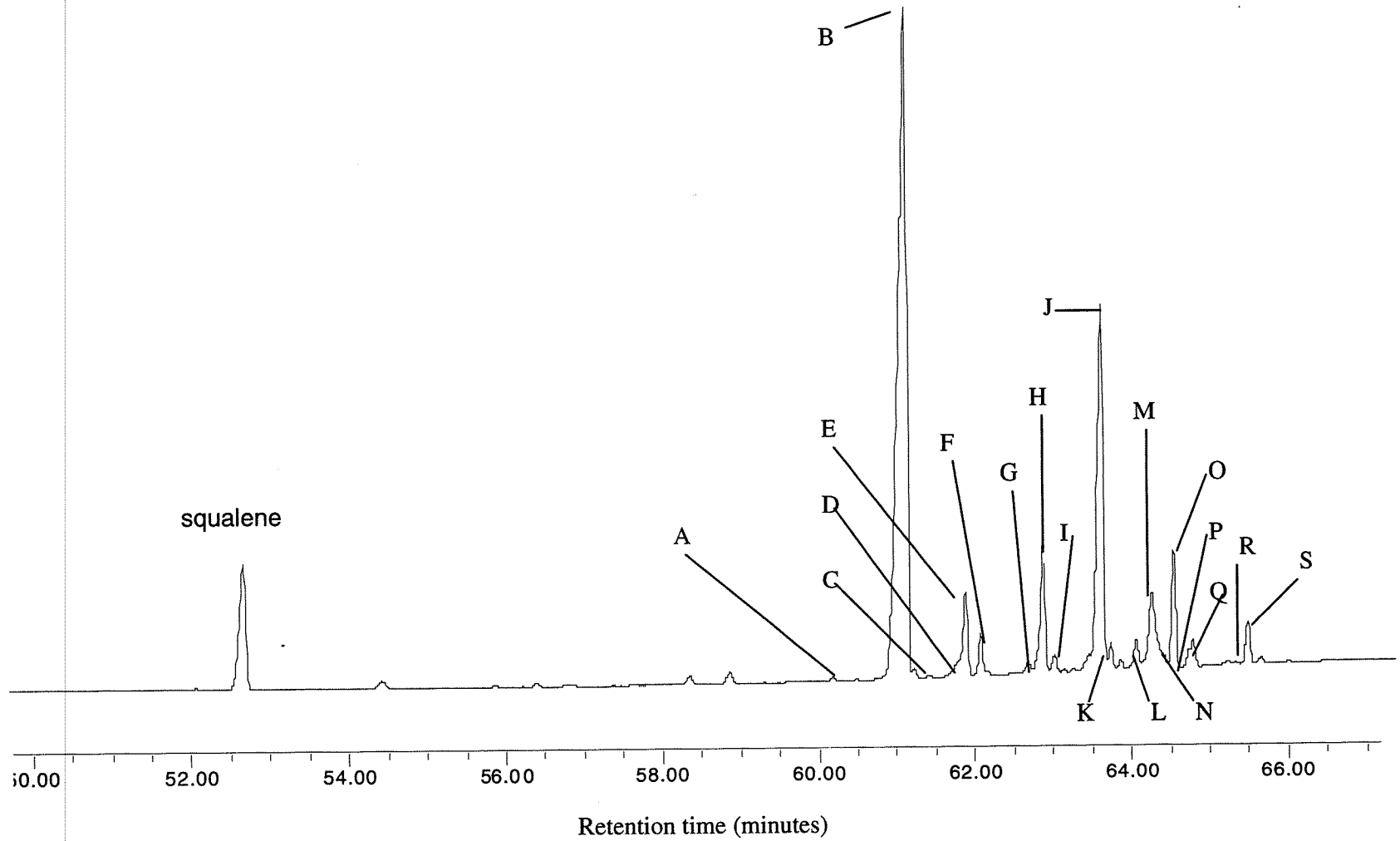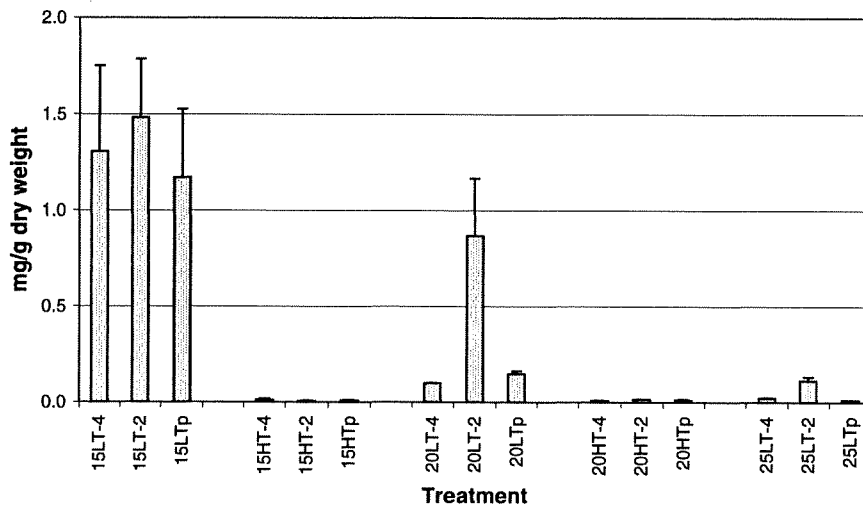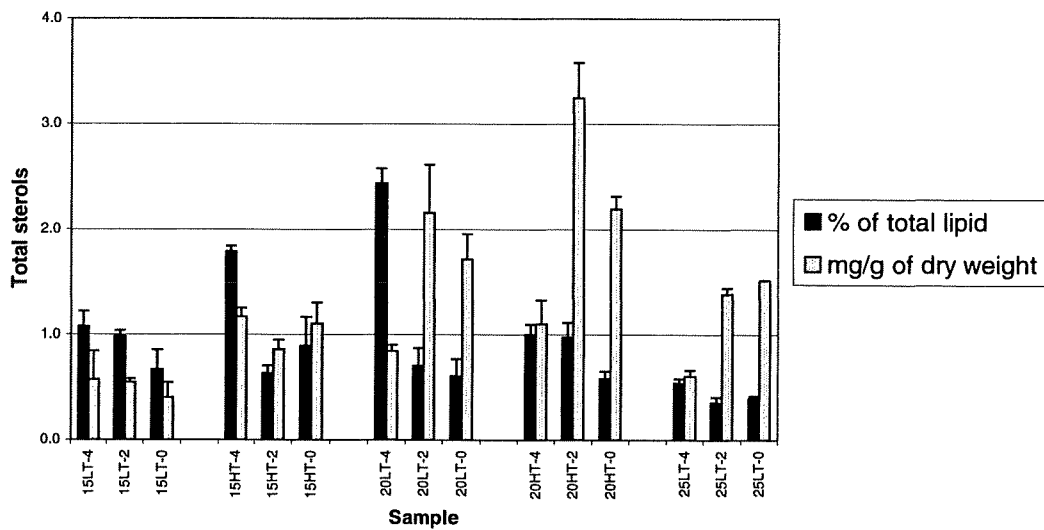Retention time (minutes)

Figure 2

Figure 3

Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs

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Summary

We examined the effect of different extraction techniques on the recovery of fatty acids from freeze-dried biomass of two lipid-producing microheterotrophs. Two procedures were used: the extraction of lipids from biomass followed by transesterification of the fatty acids (extraction-transesterification); and the direct transesterification of biomass to produce fatty acid methyl esters (i.e. without the initial extraction step). Variable factors in the extraction-transesterification experiment were the sequence in which solvents were added to the samples, the relative amount of methanol in the solvent mix, and sonication of biomass while in the solvent mix. Variable factors in the direct transesterification experiment were sample size, and reaction duration. Statistical analysis of data (level of significance $p < 0.05$) showed that: 1) extraction of total fatty acids prior to transesterification was significantly more efficient when solvents were added in the order of increasing polarity; 2) neither sonication nor increasing the proportion of methanol in the extraction solvent significantly affected extraction of fatty acids prior to transesterification; 3) efficiency of direct transesterification of fatty acids increased significantly with reaction time; 4) efficiency of direct transesterification of fatty acids was not significantly affected by sample size; 5) the most efficient method for extraction of fatty acids prior to transesterification yielded significantly less fatty acids than the most effective direct transesterification method. While the study examined only two strains, our results suggest that fatty acid analysis methodology for microheterotrophs under consideration for biotechnological exploitation requires optimisation and validation.

Key words

Lipid extraction; direct transesterification; fatty acids; lipid analysis;

Introduction

During the past decade, interest in the ability of some heterotrophic microorganisms to produce substantial quantities of lipids has increased. Microbial lipids [in particular polyunsaturated fatty acids (PUFA), but also monounsaturated (MUFA) and saturated (SFA) fatty acids] have potential commercial applications as nutraceuticals, pharmaceuticals, and feed ingredients for aquaculture. Considerable effort has gone into the isolation of, and optimisation of lipid production by, oleaginous strains (Ratledge, 1993; Leman, 1997).

To determine the quantity and types of lipid produced by microheterotrophs, the lipids need to be extracted from the biomass in a form that can be analysed quantitatively. A common lipid extraction method is that of Bligh and Dyer (1959), in which lipids are extracted from biological material using a combination of chloroform and methanol in a ratio such that a single phase is formed with the water in the sample. Dilution of the solvent mixture with water and chloroform causes separation into two phases, with the lower (chloroform) phase containing all the extracted lipids. Further modifications of this method, including addition of water to the solvent mixture when extracting lipids from dry samples (White et al., 1979), sample sonication (Dunstan et al., 1992), adding water to lyophilised samples before the non-polar solvents (Dunstan et al., 1993) and increasing the proportion of methanol in the extraction solvent mixture (Smedes and Askland, 1999) have also been used.

Other solvent systems and methods have also been evaluated for prokaryotes and eukaryotes. While a hexane-isopropanol solvent system was found to be effective for the bacterium Pseudomonas atlantica (Guckert and White, 1988), this system gave lower recovery of lipids in the green alga Chlorella (Guckert et al., 1988). Pressurised accelerated hot solvent extraction (ASE) of soil, air and water samples has been tested using a modified Bligh and Dyer solvent mix (Macnaughton et al., 1997). Compared to the Bligh and Dyer extraction performed at ambient temperature, recovery of lipids using ASE was similar for vegetative biomass, soil and water samples, but was higher for spores and air-borne biomass.

Extracted lipids are usually transesterified, to form fatty acid methyl esters, allowing identification and quantification of fatty acids using gas-chromatographic techniques. This process has been used regularly to quantify lipid content of microheterotroph cells (e.g. Sajbidor et al., 1990; Bajpai et al., 1991; Gandhi and Weete, 1991; Iida et al., 1996).

In studies concerning lipid production by microheterotrophs, some researchers have dispensed with the initial lipid extraction step, in favour of single-step (extraction-transesterification) methods (e.g. Eroshin et al., 1996; Nakahara et al., 1996; Yokochi et al., 1998; Allen et al., 1999). A sequential direct saponification-acid hydrolysis-esterification procedure has also been used to recover more effectively cyclopropane and hydroxylated fatty acids from bacterial samples (Mayberry and Lane, 1993). The concept of direct transesterification of fatty acids in samples has received attention over the years,

primarily due to the substantial reduction in both time and solvents that this technique offers. Several studies have discussed the relative efficiency of direct transesterification and lipid extraction followed by transesterification. Increased recovery of fatty acids (from adipose tissue and milk, and lactic acid bacteria respectively) by direct transesterification techniques has been reported (Lepage and Roy, 1984; Dionisi et al., 1999). However, direct transesterification did not change fatty acid recovery from plant tissue and infant formulae respectively (Garces and Mancha, 1993; Bohnert et al., 1997).

These results strongly suggest that before true comparison of data from different sources can be made, it is important to consider the methods used to extract and analyse fatty acids from biological samples, including microorganisms. If deviations from a standard method give rise to significantly different results, as has been suggested by Smedes and Askland (1999), then direct comparisons of lipid production will not be possible. Similarly, it is important to determine the most effective extraction and analysis method for microheterotrophs, so that lipid content and composition can be determined accurately.

In this study, we examined the effect of some variations of both the Bligh and Dyer method (at ambient temperature) and direct transesterification procedures on the efficiency of extraction of fatty acids from freeze-dried biomass of two lipid-producing microheterotrophs. This was a precursor to a larger project examining fatty acid (in particular polyunsaturated fatty acid) production by selected, recently isolated Australian microheterotrophs.

Materials and Methods

Analytical reagents

All reagents used were of analytical standard. Chloroform, methanol, hexane and hydrochloric acid were obtained from Mallinckrodt (USA). Deionised water was produced using a Milli Q Plus (Millipore SA, France) water treatment system.

Microheterotroph culture and cell preparation

Two lipid-producing microheterotrophs (strains ACEM 6063 and ACEM A - tentatively identified as thraustochytrids), isolated from coastal marine sediment in southeastern Tasmania (Australia), were used. Biomass was produced in 500 mL, screw topped Erlenmeyer flasks, containing 150 mL of liquid culture medium, incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm. Cells of ACEM 6063 were harvested after 10 days incubation. Cells of ACEM A were harvested after 3 and 5 days incubation.

Cells were harvested by centrifugation (J2-21M/E centifuge, JA 14 rotor, Beckman USA) at 8000 x g for 15 minutes in 250 mL polyethylene centrifuge tubes. Following

centrifugation, the supernatant was discarded, the cell pellet resuspended in 100 mL 1.0% NaCl (w/v), and re-centrifuged. The second supernatant was discarded and the cell pellet frozen overnight at -30°C. Frozen biomass was freeze dried (chamber temperature - 110°C, Mini Ultra Cold, Dynavac Australia; pressure $< 7 \times 10^{-1}$ mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15 hours and subsequently stored in a sealed glass container at -30°C. Biomass used in the experiments described below came from a single batch of each strain.

Preparation of fatty acid methyl esters (FAME)

Two procedures were used to prepare FAME from freeze-dried biomass: a) extraction followed by transesterification; b) direct transesterification (Figure 1). The two procedures and variants are described below.

Extraction-transesterification

Freeze-dried ACEM 6063 cells were weighed (100 ± 10 mg) into clean, 100 mL screw-top test tubes. A total of 80 mL of solvent was then added in a predetermined sequence, with each sample being vortex mixed for 15 seconds following the addition of each solvent (Table 1). Selected samples were sonicated (Labsonic 1510 Ultrasonicator, Braun, Germany) by immersing a clean probe directly into the cell-solvent mixture and supplying the probe with 100 W (peak) for 2 x 1 minute (Table 1).

ACEM 6063 biomass-solvent mixtures were left in the tubes for about 18 hours, after which they were quantitatively transferred into clean glass separatory funnels. Each tube was rinsed (2 x 5 mL) with a mixture containing the same ratio of extraction solvents as was used for that tube, with the rinse mixture being added to the appropriate separatory funnel.

Freeze-dried ACEM A cells, from 3- and 5-day old cultures, were weighed (100 ± 10 mg) into glass separatory funnels, to which a total of 114 mL solvents were added in the sequence: chloroform, methanol, water to achieve a final chloroform:methanol:water ratio of 1:2:0.8. Samples were shaken for 15 seconds immediately following the addition of each solvent, and allowed to stand for about 18 hours, with occasional shaking by hand.

Phase separation of the biomass-solvent mixtures in the separatory funnels was achieved by adding chloroform and water to each separation funnel to obtain a final chloroform:methanol:water ratio of 1:1:0.9. A known portion of each total lipid extract recovered from the lower chloroform phase in the separatory funnel was used for FAME preparation and analysis.

Portions of the total lipid extracts were transferred into clean 10 mL, screw-top test tubes, to which a fresh solution of methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL) was added for reaction at 90°C for 60 minutes.

Direct transesterification

Freeze-dried ACEM 6063 cells were weighed (desired weight ± 10%) into duplicate clean, 10 mL screw-top test tubes (Table 2), to which a fresh solution of the transesterification reaction mix [methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL)] was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90°C for transesterification. Reaction times varied between 15 and 120 minutes (Table 2).

Freeze-dried ACEM A cells were weighed (50 ± 5 mg) into duplicate, clean 10 mL, screw-top test tubes, to which a fresh solution of the transesterification reaction mix was added. Cells were suspended in this solution by vortex mixing and reacted at 90°C for 60 minutes.

Extraction and analysis of FAME

Transesterification reaction tubes were removed from heat after the appropriate reaction time, and cooled. Water (1 mL) was then added to each tube and the FAME extracted (hexane:chloroform, 4:1 v/v, 3 x 2 mL). For gas chromatographic (GC) analysis, samples were diluted with chloroform containing a known concentration of 19:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionization detector (Nichols et al., 1996). Peak areas were recorded and quantified using the software package Millenium 32 v3.05.01 (Waters Corporation, USA). Confirmation of FAME identity was performed on a MD 800 (Fisons, United Kingdom) or GCQ (Thermoquest, USA) GC-mass spectrometers (GC-MS) (Nichols et al., 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

Blank controls (i.e. containing no sample) were used in each experiment. No fatty acids were recovered from any of the controls.

Statistical analyses

Statistical analyses of data were performed using the General Linear Models Procedure of the software package SAS System for Windows v 6.12 (SAS Institute Inc. USA). Differences between individual means were deemed to be significant at $p < 0.05$.

Results

Extraction-transesterification experiment - strain ACEM 6063

The amount of fatty acids extracted from ACEM 6063 by various extraction-transesterification treatments is shown in Table 1.

The sequence in which solvents were added to samples resulted in significant differences in the amount of total fatty acids (TFA), SFA and PUFA extracted. The amount of MUFA extracted was not affected significantly by changing the solvent addition sequence (Table 1).

Neither sonication (2 x 1 minute) nor doubling the relative amount of methanol in the extraction solvent mixture resulted in significant differences between any of the parameters examined (Table 1). Similarly, there were no significant differences in the proportions of SFA, MUFA or PUFA extracted by any of the treatments (Table 1).

Direct transesterification experiment - strain ACEM 6063

The amount of fatty acids extracted from ACEM 6063 by direct transesterification is shown in Table 2. Variations in sample size had no significant influence on the amount of TFA, SFA, MUFA or PUFA extracted from freeze-dried biomass of ACEM 6063. Reaction time significantly influenced the amount of fatty acids extracted from the biomass. Significant increases in the amount of TFA, SFA, MUFA and PUFA recovered from the biomass were observed between 15 and 30, and 30 and 60 minutes reaction time (Table 3). There was also a significant increase in the amount of MUFA extracted between 60 and 120 minutes reaction time. However, the amounts of TFA, SFA and PUFA extracted did not increase further nor vary significantly between 60 and 120 minutes reaction time.

The proportions of SFA and PUFA extracted from ACEM 6063 biomass by direct transesterification were not significantly affected by reaction time (Table 3). The proportion of MUFA extracted by this method was significantly affected by reaction time, although no clear trend was evident (Table 3).

Comparison of extraction-transesterification and direct transesterification

methods

Strain ACEM 6063

Pooled data for all samples extracted by direct transesterification with a reaction time of 60 minutes was compared with that for all extraction-transesterification samples from which fatty acids were extracted by adding solvents in the sequence chloroform-methanol-water (N.B. the latter were also transesterified at 90°C for 60 minutes). The amounts of major fatty acids (those comprising > 2% TFA in most samples) recovered from ACEM 6063 are shown in Table 4. The amounts of TFA, SFA and PUFA extracted by direct transesterification were all significantly higher than those extracted by extraction-transesterification. Neither the amount of MUFA extracted, nor the

proportions of SFA, MUFA or PUFA in the extracts varied significantly between these two methods (Table 4).

Co-efficients of variation (CV) of data obtained from the two groups of pooled data showed that the amounts of TFA, SFA and PUFA extracted were less variable than the amounts of MUFA extracted. The CV for MUFA extracted by direct transesterification was less than that for MUFA extracted by extraction-transesterification. The CV's for TFA, SFA and PUFA were all slightly higher in the direct transesterification experiment than in the extraction-transesterification experiment (Table 4).

Strain ACEM A

The amounts of major fatty acids (those comprising > 2% TFA in most samples) recovered from ACEM A are shown in Table 5. Samples from both 3-day old (low lipid) and 5-day old (high lipid) extracted by direct transesterification yielded significantly more TFA, SFA, MUFA and PUFA than those from which lipid was extracted prior to transesterification.

There were also significant differences in the proportion of MUFA and PUFA, but not SFA extracted from ACEM A by the two methods. Fatty acids recovered from ACEM A by direct transesterification contained proportionally more MUFA and less PUFA than those recovered by extraction-transesterification (Table 5).

Discussion

Results from this study indicate that deviations from a standard lipid extraction method (Bligh and Dyer, 1959) significantly influenced the amount of fatty acids recovered from ACEM 6063 and ACEM A biomass.

The amount of TFA, SFA and PUFA extracted from ACEM 6063 by various extraction-transesterification methods was significantly affected by the sequence in which solvents were added to the samples. Adding solvents in the order chloroform-methanol-water resulted in an increase in extraction efficiency when compared to adding the solvents in the reverse order. Similar results have been reported by Smedes and Askland (1999). These authors suggested that increased efficiency by some multi-step extractions (i.e. those in which the non-polar component/s of a solvent system are mixed with samples before the polar components) could be explained in terms of cell disruption prior to lipid extraction. Initial contact with the non-polar solvents could weaken the association between lipids and cell structures, prior to lipid dissolution in the mono-phasic system. Dunstan et al. (1993) reported an increased lipid recovery from freeze-dried oyster tissue following rehydration of the tissue prior to extraction by the Bligh and Dyer method.

In our extraction-transesterification experiment, using heterotrophic microorganisms, neither sonication nor doubling the proportion of methanol in the solvent mixture significantly affected the amounts of SFA or PUFA extracted. In contrast, Smedes and

Askland (1999) found the effect of solvent addition sequence on extraction of lipid from fresh fish was over-ridden by increasing the proportion of methanol in the extraction mixture. This difference could be due to differences in cell structure of the two types of biomass and/or differences in sample treatment before analysis [i.e. freeze-dried microheterotroph biomass in our study; fresh fish flesh in Smedes and Askland (1999)].

The amount of MUFA recovered in our extraction-transesterification experiment was not affected significantly by any of the variations we used. This lack of correlation appears to be due to the relatively high variability in the MUFA data. However, the proportion of MUFA in the sample was relatively minor and had little effect on the overall lipid yield. For experiments where the proportion of MUFA in the sample is of critical importance to the interpretation of results, similar variability in MUFA extraction efficiency, if it occurs, could have serious implications.

When freeze-dried cells of strain ACEM 6063 were directly transesterified, variations in sample size (within the range examined in this study) did not significantly influence the total amount or the relative proportion of fatty acids recovered. This also indicated the freeze-dried biomass was quite homogenous.

It is apparent, however, that the duration of the transesterification reaction had a significant effect on the amount of fatty acids extracted. Our data indicated that a reaction time of 60 minutes at 90°C was sufficient to extract the vast majority of SFA and PUFA from these samples. Although a reaction time of 60 minutes has been used frequently (e.g. Bohnert et al., 1997; Sonnichsen and Muller, 1999), it is evident that optimum reaction time is sample specific. Rodriguez-Ruiz et al. (1998) reported similar lipid recovery after transesterification reaction times of 60 and 10 minutes, for both cod-liver oil and freeze-dried macroalgae. However, Garces and Mancha (1993) increased lipid recovery from sunflower seeds with increasing reaction times up to two hours.

The proportion of SFA and PUFA, and individual fatty acids from the two groups, extracted from ACEM 6063 by direct transesterification did not vary significantly, indicating that there was no selective extraction of these two fatty acid classes attributable to different reaction times.

The recovery of MUFA from strain ACEM 6063 by direct transesterification increased steadily between 15 and 120 minutes reaction time, and did not plateau at 60 minutes reaction time. The proportion of MUFA also increased significantly between reaction times of 15 and 120 minutes. This indicated that the MUFA-containing lipid in these samples was not as easily extracted as the SFA- or PUFA-containing lipid, and that a reaction time of at least 120 minutes was necessary to allow full transesterification of MUFA from these samples.

For the two strains of microheterotroph examined, greater recovery of fatty acids was achieved using direct transesterification than by using extraction-transesterification. These observations support the suggestion by Smedes and Askland (1999) that cell disruption plays a role in the efficiency of lipid extraction from some samples. The transesterification reaction mix used in this study contained about 8% (v/v) chloroform

and was highly acidic: conditions which could be expected to cause severe disruption to cell integrity.

Conclusion

We have shown that the total amount of fatty acids recovered from two lipid-producing microheterotrophs increased by about 30% by adding solvents to the biomass in order of increasing, as opposed to decreasing, polarity.

We also demonstrated that by directly transesterifying the microheterotroph biomass, instead of transesterifying a portion of extracted lipid, the total amount of fatty acids extracted was significantly greater than for the most efficient extraction-transesterification extraction method. This increase in extraction efficiency differed between the two microheterotroph strains examined, and varied from 15-20% for strain ACEM 6063 and about 150% for strain ACEM A.

One consequence of using direct transesterification for fatty acid analyses is that lipid class data can not be obtained. For samples where lipid class data is required, extraction of the lipid fraction prior to transesterification is obviously required. Biotechnologists and analysts should be aware, however, that if a portion of the total lipid extract is subsequently used for transesterification and fatty acid analysis, the data could underestimate the true fatty acid content of the biomass.

Our results show that the efficiency of lipid recovery from different microheterotrophs can vary substantially between extraction techniques. This is an important observation for researchers involved in the field of microheterotrophic fatty acid production and analysis. We suggest that, given the wide variation in extraction efficiencies revealed by our results, meaningful data for other microheterotrophs under consideration for biotechnological applications will require careful choice and validation of analysis methodology.

Acknowledgements

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Table 1.   Fatty acid recovery from ACEM 6063 by extraction-transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

Treatment	Solvent addition sequence	Solvent ratio (v/v) C : M : W	Fatty acid recovery sum TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	%PUFA
WMC	W-M-C [2]	1 : 2 : 0.8	261.2 ,256.2 [a]	121.2, 117.9 [a]	3.6, 3.3	131.3, 140.1 [a]	47.3, 45.1	1.4, 1.2	51.3, 53.6
WMCS [1]	W-M-C	1 : 2 : 0.8	289.8, 234.7 [a]	125.0, 100.4 [a]	6.9, 2.7	157.8, 131.6 [a]	43.1, 42.8	2.4, 1.2	54.5, 56.1
CMW	C-M-W [3]	1 : 2 : 0.8	348.3, 351.6 [b]	150.4, 150.4 [b]	4.3, 4.4	186.4, 196.8 [b]	45.3, 42.8	1.2, 1.3	53.5, 56.0
CMWS	C-M-W	1 : 2 : 0.8	333.0, 352.7 [b]	142.8, 161.7 [b]	8.3, 4.9	181.9, 186.1 [b]	42.3, 45.9	2.5, 1.4	54.6, 52.8
C2MW	C-M-W	1 : 4 : 0.8	336.4, 326.5 [b]	156.8, 147.3 [b]	4.7, 4.8	174.9, 174.4 [b]	46.6, 45.1	1.4, 1.5	52.0, 53.4
C2MWS	C-M-W	1 : 4 : 0.8	327.8, 363.3 [b]	150.6, 169.8 [b]	6.3, 11.3	170.9, 182.3 [b]	45.9, 46.7	1.9, 3.1	52.1, 50.2

Values in each column with different superscripts are significantly different ($p < 0.05$)

1:     W: water; M: methanol; C: chloroform; S denotes sample sonicated for 2 x 1 minute

2:     water then methanol then chloroform

3:     chloroform then methanol then water

Table 2.   Fatty acid recovery from ACEM 6063 by direct transesterification
             (expressed as mg g^{-1} dry weight)

Treatment	Reaction time (minutes)	Nominal sample size (mg)	Fatty acid recovery sum SFA	sum MUFA	sum PUFA
15/5	15	5.0	46.4, 52.6	1.3, 1.7	44.8, 52.3
30/1	30	1.0	150.4, 135.5	3.6, 3.3	157.4, 152.6
30/5	30	5.0	122.5, 118.1	3.7, 3.9	135.7, 130.6
30/20	30	20.0	120.1, 114.3	3.3, 3.2	116.6, 112.2
60/1	60	1.0	177.9, 192.1	4.3, 5.6	200.9, 217.8
60/3	60	3.0	188.4, 178.9	5.2, 4.9	225.4, 208.5
60/5	60	5.0	201.3, 151.1	7.8, 5.6	187.5, 209.9
60/10	60	10.0	213.8, 198.4	5.5, 5.4	220.2, 203.7
60/20	60	20.0	198.9, 204.3	5.7, 6.0	182.4, 208.0
120/1	120	1.0	194.8, 210.3	6.7, 5.3	156.9, 205.4
120/5	120	5.0	237.6, 206.7	9.0, 8.7	156.9, 220.2
120/20	120	20.0	206.4, 224.2	5.8, 6.4	205.7, 213.3

Table 3.   Fatty acid recovery from ACEM 6063 by direct transesterification
             (expressed as both mg g^{-1} dry weight and % total fatty acids)

Reaction time	Mean fatty acid recovery						
	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
15 (n=2)	99.5 [a]	49.5 [a]	1.5 [a]	48.6 [a]	49.8	1.5 [ab]	48.8
30 (n=6)	264.5 [b]	126.8 [b]	3.5 [b]	134.2 [b]	48.0	1.3 [b]	50.6
60 (n=10)	402.5 [c]	190.5 [c]	5.6 [c]	206.4 [c]	47.3	1.4 [b]	51.3
120 (n=6)	413.4 [c]	213.3 [c]	7.0 [d]	193.1 [c]	51.8	1.7 [a]	46.5

Values in each column with different superscripts are significantly different ($p<0.05$)

Table 4.   Fatty acid recovery from strain ACEM 6063 by extraction-transesterification and by direct transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

	Major [a] fatty acids								Mean fatty acid recovery						
	14:0	15:0	16:0	18:0	20:4 n6	20:5 n3	22:5 n6	22:6 n3	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
extraction-transesterification [b]															
Mean	35.5	3.8	109.0	4.2	3.9	5.0	44.9	115.4	342.4 [A]	154.6 [A]	6.1	181.7 [A]	45.1	1.8	53.1
CV [c]									3.9	5.6	40.5	4.6	3.4	38.3	3.3
direct transesterification [d]															
Mean	48.1	4.8	129.7	4.8	4.8	6.0	49.7	131.4	402.5 [B]	190.5 [B]	5.6	206.4 [B]	47.3	1.4	51.3
CV									5.3	9.3	16.4	6.6	6.4	17.1	6.1

Values for mean fatty acid content in each column with different superscripts are significantly different ($p < 0.05$)

a: fatty acids comprising >2% total fatty acids in most samples. Other fatty acids detected were: 12:0, 14:1, i15:1, i15:0, a15:0, 15:1, i16:0, 16:1n9, 16:1n7, 16:1n7t, 16:1n5, 17:0, 18:3n6, 18:4n3, 18:2n6, 18:1n9, 18:1n7, 20:3n6, 20:4n3, 20:2n6, 20:0, 22:4n6, 22:5n3, C22PUFA

b: combined data for all extraction-transesterification samples extracted by adding solvents in the sequence chloroform:methanol:water (n=8)

c: Coefficient of Variation (mean/standard deviation)

d: combined data for all direct transesterification samples reacted for 60 minutes (n=10)

Table 5. Fatty acid recovery from 3- (low lipid) and 5- (high lipid) day old cultures of ACEM A by extraction-transesterification and by direct transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

Culture age (days)	Major [a] fatty acids (mean, n=2)											Fatty acid recovery						
	14:0	16:1 n7	16:0	18:2 n6	18:1 n9	18:0	20:4 n6	20:5 n3	20:2 n6	22:6 n3	22:5 n3	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
extraction-transesterification																		
3	0.65	0.55	7.65	0.6	0.85	1.5	1.2	5.2	0.65	3.2	1.3	25.0, 23.5 [c]	10.6, 9.0 [c]	1.5, 1.3 [d]	12.9, 13.2 [c]	42.6, 38.1 [ab]	5.8, 5.7 [d]	51.6, 56.2 [c]
5	2.25	1.95	14.15	5.15	8.1	2.9	2.35	11.2	1.6	5.5	2.85	55.7, 65.7 [b]	17.9, 21.2 [b]	9.8, 11.2 [b]	28.0, 33.4 [b]	32.2, 32.3 [a]	17.5, 17.0 [b]	50.3, 50.7 [bc]
direct transesterification																		
3	3.25	1.25	19.05	1.75	3.1	5.55	1.95	8.6	2.2	6.4	4.25	57.7, 63.0 [b]	26.6, 29.7 [b]	4.2, 6.2 [c]	27.0, 27.1 [b]	46.1, 47.2 [b]	7.2, 9.8 [c]	46.7, 43.0 [ab]
5	7.7	4.6	38.45	16.25	28	10.55	4.25	19.5	7.35	8.6	7.9	181.0, 136.8 [a]	66.2, 49.2 [a]	38.2, 28.4 [a]	76.5, 59.2 [a]	36.6, 36.0 [a]	21.1, 20.8 [a]	42.3, 43.2 [a]

Values for mean TFA, SFA, MUFA and PUFA content in each column with different superscripts are significantly different (p<0.05)

a: fatty acids comprising >2% total fatty acids in most samples. Other fatty acids detected below this value were: 12:0, 15:0, 16:1n9, 16:1n5, 16:1, 17:0, 18:3n6, 18:3n3, 18:1n7, 20:3n6, 20:4n3, 20:1n11,9, 20:1n7, 20:0, 22:5n6, 22:4n6, C22PUFA

Extraction-Transesterification   Direct Transesterification

BIOMASS

Lipid Extraction (Bligh and Dyer)

Treatments:

a) Solvent addition sequence

C-M-W: $CHCl_3$ then MeOH then H_2O

(1:2:0.8 v/v/v)

or

W-M-C: H_2O then MeOH then $CHCl_3$

b) MeOH concentration

C-2M-W: 2 x standard concentration

c) Sonication

C-M-W-S, W-M-C-S, C-2M-W-S: 2 x 1 minute

Acid Transesterification   **Acid Transesterification**

($MeOH:HCl:CHCl_3$; 10:1:1 v/v/v)   ($MeOH:HCl:CHCl_3$; 10:1:1 v/v/v)
60 minutes, 90°C   *Treatments:*
                                                  a) Sample size
                                                       1,3, 5, 10, 20 mg
                                                  b) Reaction duration
                                                       15, 30, 60, 120 min, 90°C
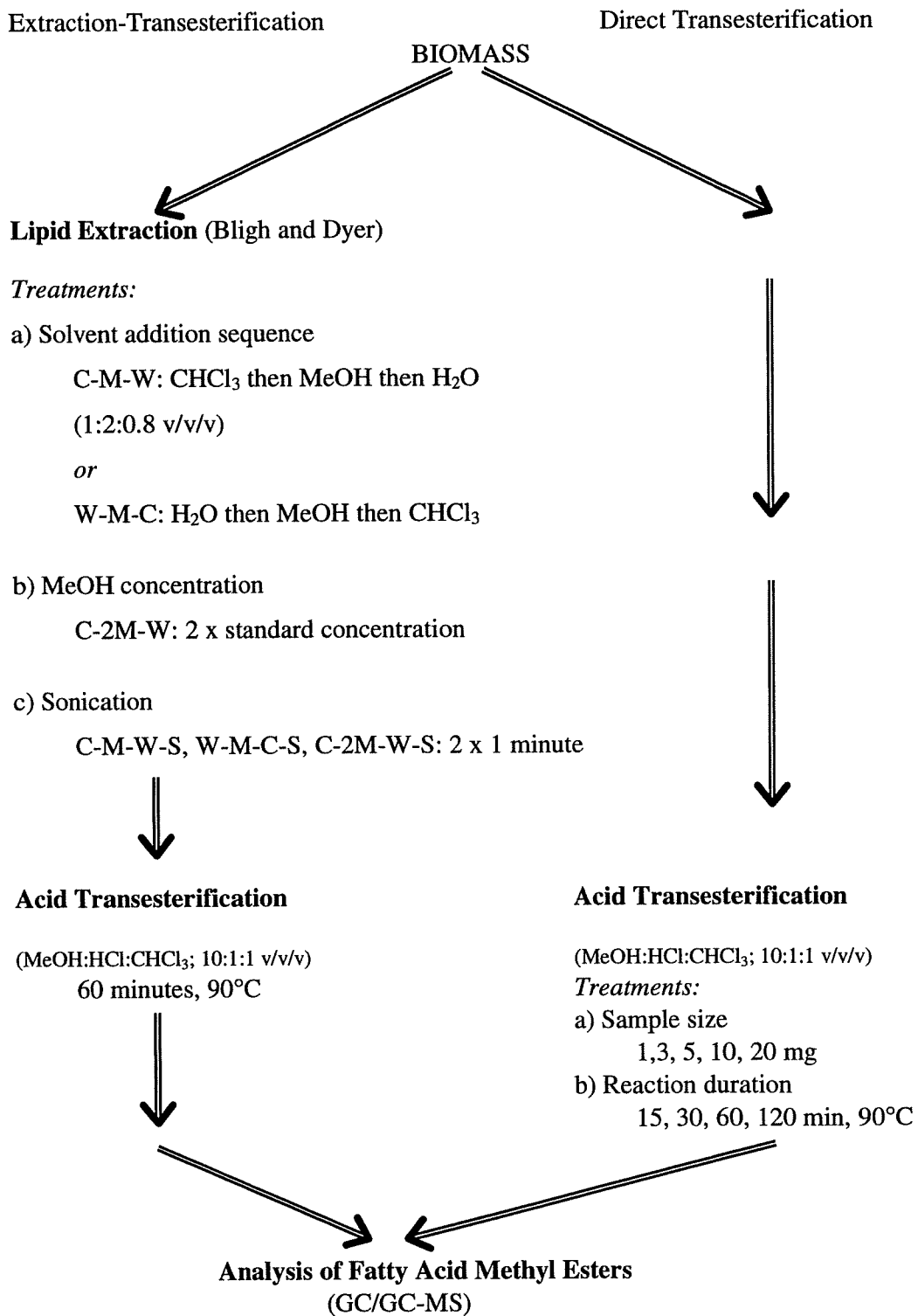
Analysis of Fatty Acid Methyl Esters
(GC/GC-MS)

Figure 1   Flow diagram of lipid extraction methods