Can fatty acids, found in sediments from three coastal B.C. Inlets, be used as biomarkers to hindcast harmful algal blooms of *Heterosigma akashiwo*?

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Oceanography)

THE UNIVERSITY OF BRITISH COLUMBIA

July 2007

Abstract

This study presents fatty acid profiles of the harmful algae *Heterosigma akashiwo.* These profiles were generated to assess the variability in *H. akashiwo* fatty acid proportions and to compare the results with past research on this and other species of marine algae.

Past research has identified several important fatty acids that are used as biomarkers for microbial functional groups including: diatoms, dinoflagellates, bacteria, zooplankton and terrestrial plants. Can the fatty acids found in sediments be used to trace the historical bloom events of *H. akashiwo*? This question was the focus of this study.

Key fatty acids for this species were selected and used as probes to search for similar fatty acid proportions in marine sediments collected from three British Columbian Inlets near the Strait of Georgia.

The sediments from Howe Sound, Hotham Sound and Malaspina Inlet showed considerable variation in fatty acid abundance. The latter Inlet had the highest concentration of acids whereas Howe Sound had the least.

The fatty acid biomarkers for *H. akashiwo* were incorporated into a multivariate analysis of the sediment samples to locate the particular depths in the sediment that the proportions of these fatty acids matched the *H. akashiwo* profiles. The results were tested by comparing DNA counts for *H. akashiwo* that were extracted concurrently by another researcher using the same sediment samples from Hotham Sound and Malaspina Inlet.

The DNA and fatty acid data were not in close agreement. Degradation limits the interpretive power of both DNA and fatty acid biomarkers found at depth in sediments. Further analysis using multi biomarkers including pigments and sterols could be incorporated into the method of analysis presented in this two part study.

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Acknowledgements

I first wish to thank the members of my thesis committee: Dr's. Doug Bright, Roger Francois, Curtis Suttle and Philippe Tortell, for their generous support and guidance.

I also gratefully acknowledge the help and advice I received from the following: Dr. Matt Dodd, at Royal Roads University, Dr.'s Assit Mazumder, Director of the Fresh Water Institute at the University of Victoria and Lab Manager Sergei Verenitch , Amy Chan and Dr's. Azeem Ahmad, Steve Calvert, Mike Henry, Janice Lawrence and Al Lewis at the University of British Columbia, Dr Robbie Macdonald at the Institute of Ocean Sciences-Pat Bay and Dr. Ian Whyte at the Pacific Biological Station-Nanaimo.

Co-Authorship Statement

All work for papers done, at this point, by Helen Drost

2.2 Methods and Materials

2.2.1 Cultures and culture conditions

The nutrient replete strains of *H. akashiwo* (500, 503, 278, and 522) were provided by Amy Chan at the University of British Columbia (UBC). The strains in this study were isolated from the Northwest Pacific; 522 were isolated from near Vancouver, BC, Canada; # 503, 500 & 278 from WA, USA). Cultures were maintained in F/2-Si medium made with seawater that was collected at 9 m depth near Port Sidney, BC (49°17 N 122°23.1 W). The seawater was filtered using Millipore MF™ - Membrane 0.45μm HA filters prior to adding F/2 stock solutions. After adding solutions the seawater was autoclaved and refrigerated.

Semi-continuous cultures (1 L) (Harrison 1990) were grown for 9 generations in 2 L Erlenmeyer flasks in a Revco incubator maintained at 20°C ±0.5°C, under ~75μmol $m^{-2}s^{-1}$ illumination, on a 12:12h light:dark regime. The cells were then filtered onto combusted glass-fibre GF/F filters (Whatman), wrapped in tinfoil and stored in a -70°C freezer until the fatty acids were extracted. As well, one field sample was collected during an intense bloom in Port Moody, Indian Arm (49°17 N 122°51.6 W) and kept frozen at -70°C until analysis 14 months later.

H. akashiwo (#522) cells were also grown under either nitrogen (N) or phosphate (P)-limited conditions by PhD. student Mike Henry. Cultures were grown in 2L polycarbonate bottles using the artificial seawater medium ESAW (Berges 2001). Cultures were grown with 200 uE $m^{-2}s^{-1}$ on a 16:8 light:dark cycle at a temperature of 18°C. Cell densities were monitored by in vivo fluorescence and by counting cell numbers on a coulter counter. For nutrient limitation cultures two different N:P ratios; 50 (P limited) and 5 (N limited) were used. Samples were also collected during exponential growth and two days after stationary phase.

2.2.2. Fatty acid analysis

2.2.2.1. Extraction Technique

Glassware was cleaned with phosphate-free Decon lab Contrad 70 soap, rinsed in distilled water, placed in 10% HCl for 12 h, rinsed again and dried before being wrapped in

Al foil and combusted at 450 °C for 8 h. Nitrogen (N_2) was added during all stages of the extraction process. VWRcan (GC ultra pure grade) reagents and standards were used for GC/FID analyses.

The following procedures were all done under N_2. Sample filters were placed in Pyrex 50 mL test tubes. Following Bligh and Dyer (1959), as modified by Canuel and Martens (1993), extractions were done by sonicating the samples in 5 mL methanol (CH_3OH) for 1 h followed by 3 more extractions in 2:1 (4:2 mL) methylene dichloride:methanol (CH_2CL_2: CH_3OH), for 1 h each. Extractions were done in a Branson 5210 sonicator in which a continuously circulating water bath kept the temperature below 20°C. The organic phase (top layer) from each extraction was transferred to a separatory funnel, saturated NaCl was added and the organic phase transferred to glass-stoppered flat-bottom glass vials. Anhydrous Na_2SO_4 crystals (~3.0 g) and N_2 were added and the samples left overnight.

Following the procedure of Whyte (1988), the extract was transferred to 5 mL glass micro-reaction vials and concentrated by a gentle continuous N_2 stream, while being stirred with a Reacti-Vial magnetic stirrer. Once concentrated, N_2 was added and the samples were sealed with mininert valves (Pierce Chemical). Methanolic KOH (0.5N) was purged in N_2 for 5 min then 1.0 mL of this solution was injected into the samples using Luer-Lok glass syringes (one for each sample). Vials were lightly vortexed and placed in a Pierce Reacti-therm heating stirring module at 85 °C for 30 min. Once cooled (15 min), 1 mL of hexane was added and the samples vortexed. The hexane layer and non-polar material was then discarded; this step was repeated until all the unsaponified material was removed as indicated by near colourless hexane. Boron triflouride in methanol (12%BF_3/MeOH, 2 mL) was added and allowed to react for 15 min at 85°C. Once cooled (15 min), 1 mL Hexane and 0.5 mL saturated NaCl (dissolved in de-ionized water) were added and the samples again vortexed. Once the emulsion settled, the aqueous layer was discarded. The remaining sample was washed with 1 mL saturated aqueous sodium bicarbonate ($NaHCO_3$) and centrifuged using an IEC clinical centrifuge (115 VAC, 50/60HZ) at speed setting 2 (~2,000 x g) for 2 min. The solvent layer was transferred to a 5 mL flat bottom vials with glass lids and placed under a gentle N_2 stream to evaporate the solvent. Using sterilized glass disposable pipettes, the oily residue was washed into labelled and pre-weighed chromatography vials

with hexane. A glass insert was added to the vial when concentrations were low. The hexane was removed by N_2-assisted evaporation, the vials re-weighed and carrier liquid, ethyl acetate was added (Whyte 1988). Samples were again frozen, under nitrogen at -70°C until injection into the GC/FID.

2.2.2.2. Gas Chromatographic Analysis

Fatty acids were analyzed as methyl esters (FAMEs) using a Varian CP-3800 gas chromatograph (GC) equipped with a flame ionization detector (FID). The FAMES were separated on a Supelco 2560 capillary column (100 mm x 0.25 mm ID, 0.2 μm film). The column temperature was 100°C for 2 min then increased at 3° C min^{-1} to 230°C, and held at this temperature for 16.67 min for a total run time of 62 minutes. The injector (10μL syringe, flushed 3x's, split ratio 20,) was isothermal at 260°C and the FID detector set at 300°C. N_2 (make-up gas), air (combustion) and He_2 (carrier gas) had flow rates of 30, 300 and 20 mL min^{-1}, respectively.

FAMEs were identified by comparison of their retention times with a known library of standards (Mazumder 2003) and cod liver oil chromatograms (Whyte 1988). The cod liver oil standard (CLO) was included in each batch extraction and used to track drift in the FA elution time on the chromatogram (Ackman 1965, Chuecas 1969, Whyte 1988).

2.2.2.3. Quantification

The base line for quantifying peak area was initially drawn automatically by the Star Chromatography Workstation (Varian) version 5.51 software program and then manually refined. The % relative abundance of each fatty acid was calculated using the formula

$$\% \text{ relative abundance} = (X / Y) \times 100$$

where X = area of fatty acid peak and Y = total area of all peaks

The percent recovery of sample fatty acids was calculated based on the known concentrations of the surrogate standard C19:0, and internal standards C21:0 and C13:0; the latter two standards were added before saponification. These pure standards, of known concentration, were added to the samples during the extraction process. The surrogate

standard (C19:0) was added to all the Haka samples at the very beginning of the extraction process and any sample with less than 50% C19:0 recovery was discarded.

2.2.2.4. Error and statistical analysis

Fatty acid values < 0.5% were considered trace. The standard deviation (SD) for replicate analyses (n= 2) of Haka strains was ≤ 12.0%. The average SD (n= 3) was ≤ 25% in the nutrient and growth samples. One sample (Exp2-a) was removed from analysis, as there was appreciable error. This sample was spilled during extraction and the recovery of the internal standard (C19) was the lowest of all extractions (41%).

Excel (Microsoft), SYSTAT (SPSS Inc.) and MINITAB (E-academy) were used to perform normality and variance testing, regressions, ANOVA and Principle Component Analysis (PCA).

2.3. Results and Discussion

2.3.1 *H. akashiwo* strains (522, 503, 500, 278) and past research results

Chromatographic analysis of the various fatty acid components of algae generate unique profiles that can be used to help assign taxonomic categories to controversial species (Nichols 1987). In this study the FA profiles of 4 *H. akashiwo* (Haka) strains and one field isolate (wild) sample were analyzed (Figure 1.), and compared with data from past research (Figure 2.). Comparing FA profiles based on percent of total FA must be done with caution, as all peak values are affected by an unusually high amount of just one FA (Budge 2003).

The FA 14:0 is a common fatty acid found primarily as a component of triglycerides for energy storage (Henderson 1991). The percent of total fatty acids (% relative abundance) of this saturated fatty acid (SFA) remains consistent in all profiles and with past research results (Marshall 2002, Mostaert 1998, Nichols 1987).

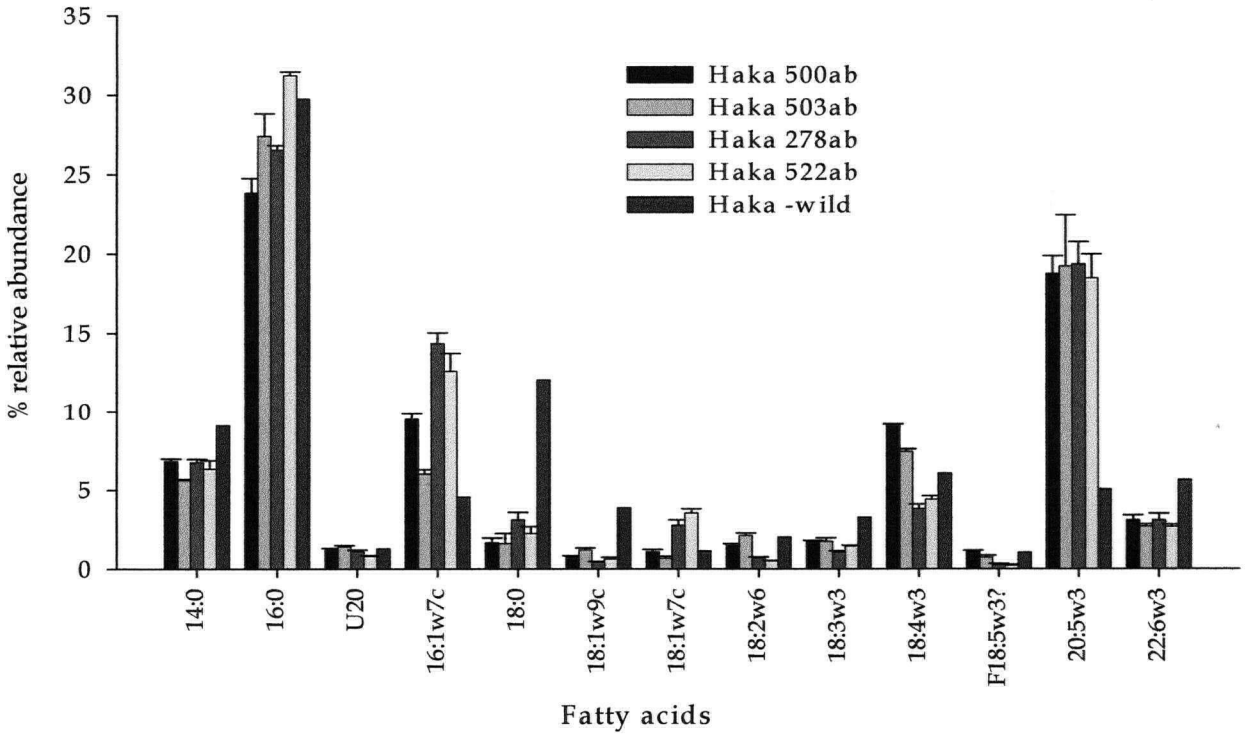

Figure 1. *Heterosigma akashiwo* strains - stationary phase (n=2). Haka-wild (n=1). Note: The ω symbol is replaced with the letter n. U20 and F 18:5ω3 are tentatively identified. Fatty acids with W as a prefix were identified using cod liver oil comparison chromatograms. All others are identified by known standards

The dominant FAs in the Haka isolates were 16:0, 20:5ω3, 16:1ω7, 14:0, 18:4ω3 with lower levels of 22:6ω3, 18:0 and 18:3ω3. FA 16:1ω7 was significantly different ($p<0.001$ *1 way ANOVA & Tukey comparison) among strains, especially between strains 503 and the more similar 522 and 278. The wild sample collected from the field during a Haka bloom had a high %RA of 18:0, yet less overall 20:5ω3. This wild sample also has the greatest %RA of 18:1ω9c and 22:6ω3 and the least 16:1ω7. This sample was not included in ANOVA testing due to lack of replication (n=1) and the undefined composition and physiological state of the bloom where the wild sample was collected.

In this study, the fatty acid labelled U20 is likely to be 16:1ω13 based on retention time (relative to known standards). This acid was not found in the cod liver oil standards but was present in all the *H. akashiwo* samples (Figure 1. & 4.). The mono-unsaturated fatty acid (MUFA) 16:1ω13 has been reported to occur as phospholipids in the chloroplasts of Haka (Nichols 1987), and as a neutral, triglyceride lipid in the sea ice diatom *Nitzschia cylindrus* (Nichols 1991).

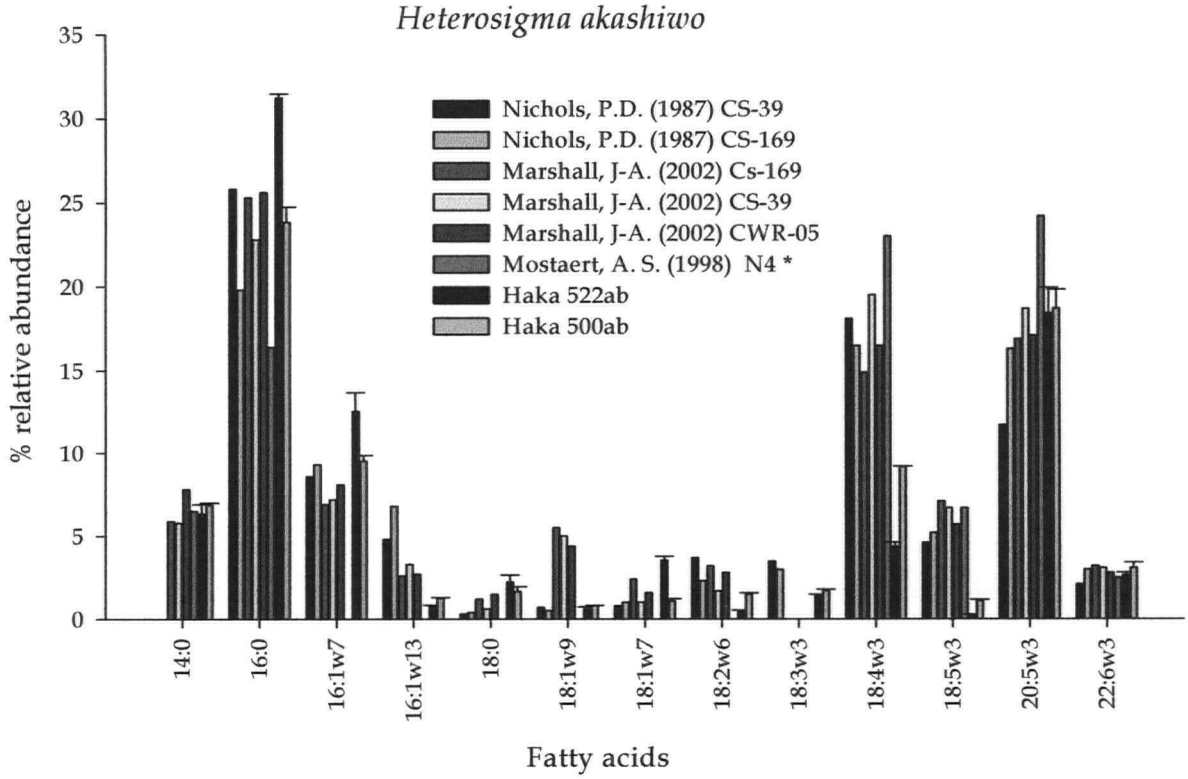

Figure 2. *Heterosigma akashiwo* fatty acid profiles -literature and study values. Mostaert's profile excluded 16:1 and 18:1 FAs, as the double bond position was not reported.

Regardless of its cellular source, this acid, according to Nichols, widely occurs in microalgae (Nichols 1991); yet few studies report it. Besides being present in most raphidophytes (0.5<%RA<6.8) (Nichols 1987, Marshall 2002), it has only been reported to occur in similar abundance in chlorophytes and prasinophytes (0.9 - 6.4 %RA). Trace amounts (0.5%RA) have also been found in a few species of diatoms including *Nitzshia cylindrus* (1.8%RA), *Stauroneis amphioxys* (1.14%RA) and *Thalassiosira oceanica* (0.5%RA) (Dunstan 1992, Gillan 1981, Volkman 1988). Also trace amounts were reported in a symbiotic prochlorophyte (Johns 1981). This acid does not appear to be ubiquitous. It was not detected in the diatom *Skeletonema costatum* (Nichols 1983), or in any prymnesiophytes (Berge 1995, Nichols 1991) other than trace amounts in *Pavlova* sp. (Volkman 1991). As well, only two dinoflagellates (*Gymnodinium wilczeki* and *Prorocentrum cordatum*) have been reported to contain trace amounts (0.1 and 0.2%RA, respectively) of this acid (Nichols 1984), despite extensive investigations (Jones 1983, Mansour 1999). Consequently, this FA is potentially a good marker to discriminate between raphidophytes (e.g. Haka) and most dinoflagellates.

The fatty acid, 18:1ω7 is a widely used bacterial marker that constitutes less than 5 %RA, in all *H. akashiwo* samples. Nichols (1983, 1984) also found this acid in 2 of 4 species of dinoflagellates studied and concluded that this acid is present in algae as well as bacteria. Others disagree, and conclude that 18:1ω7 is only found in bacteria and its presence in algal cultures is evidence of bacterial contamination (Mansour 1999). This FA was significantly ($p<0.001$ *1 way ANOVA with Tukey comparison) higher in *H. akashiwo* 278 & 522, than in strains 500 and 503, although the cultures were grown under identical conditions. This suggests that either 2 strains of Haka produced more 18:1ω7 or there were more bacteria in the culture.

All the strains in this study had only traces of the poly-unsaturated fatty acid (PUFA) 18:5ω3, whereas in other studies it ranged from 4.6-7.5 %RA (Bodennec 1998, Marshall 2002, Mostaert 1998, Nichols 1987). The marker is thought to be primarily synthesized by a 2 carbon shortening of 20:5ω3 (Joseph 1975), and has been used to distinguish *H. akashiwo* from other raphidophytes, and to indicate the presence of toxic algae (Bodennec 1998). It is

found in high proportions in toxic dinoflagellates, including *Scrippsiella* CS-259 and *Gynmnodinium* CS-389 which have %RA of 43.1 and 20.8, respectively (Mansour 1999).

Although toxicity decreases in *H. akashiwo* in late exponential phase, 18:5ω3 has still been found in appreciable amounts (4-5%) (Nichols 1987). The *H. akashiwo* strains, analyzed in this study, were all collected at stationary phase to simulate the end of blooms when sedimentation is likely important. It is possible that 18:5ω3 is depleted at stationary phase; however, the other cultures, harvested during exponential growth, showed no significant increase in this marker. It was suggested that the production of 18:5ω3 can be affected by the type of culture medium (Joseph 1975). In this study there was no difference in %RA of 18:5ω3 in trial cultures grown in ESAW (data not presented) and F/2 seawater media.

The PUFA 18:5ω3 is a component of the glycolipid class (Bodennec 1998, Kobayashi 1992, Pond 1998), in which the amide linkage that binds the fatty acid to the parent molecule (ceramide) is not readily saponified (Lehninger 1993). Some methods, including the one used in this study, still employ 0.5 to 1.0 N methanolic KOH; whereas, Ishiwatari (1987) found more lipids were extracted at 2.0 N methanolic KOH. It has also been reported that there is poor resolution of this acid on certain GC columns (Napolitano 1990); hence, the abundance of this FA may have been underestimated in this study.

2.3.2. Nutrient limited and growth phase comparison

Example chromatograms of replicate phosphate limited stationary stage Haka FA are shown in Figure 3. Samples (Figure 4.) were collected at both nutrient limited stationary and exponential growth phases. Figure 4. includes *H. akashiwo* strain #522 as a reference point. As previously mentioned, the strain samples are from different cultures thus no specific comparison in fatty acid abundance, between strain and nutrient limited fatty acids, can be made.

The bacterial marker, 18:1ω7, although low in concentration, is significantly lower in StatNl than in both StatPL ($P = 0.012$) and Exponential (Exp) growth cultures ($P=0.001$). As mentioned above, its presence in algal cultures is considered, by some researchers, to be evidence of bacterial contamination.

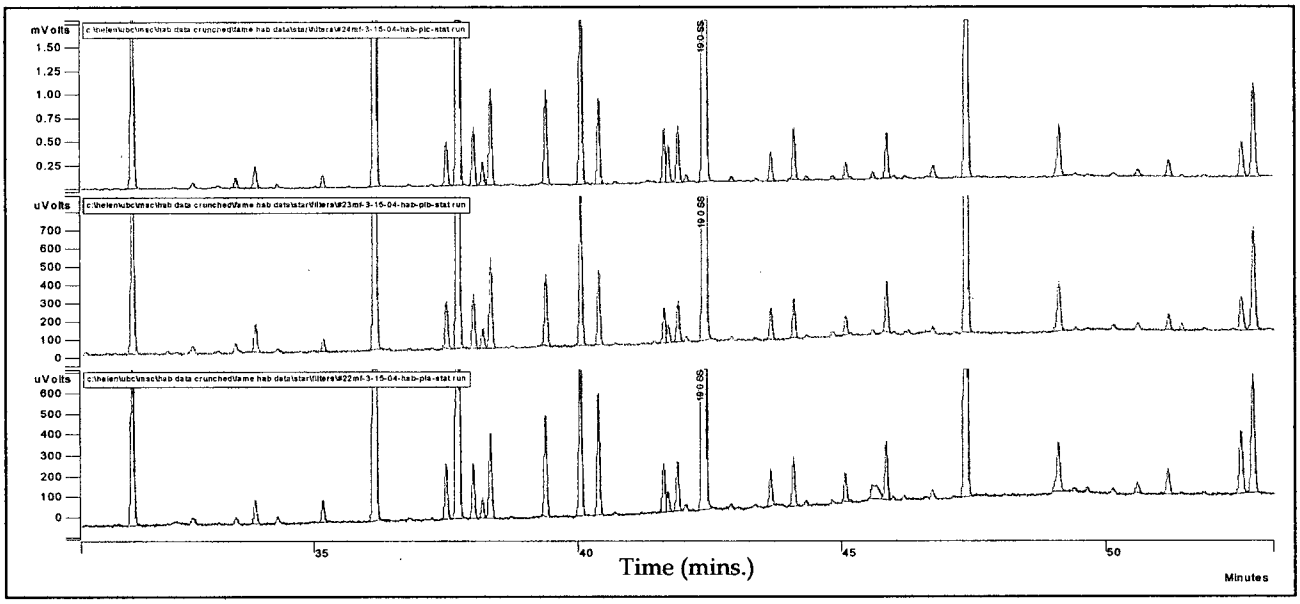

Figure 3. StatPl abc replicates *Heterosigma akashiwo* fatty acid chromatograms.
X axis = retention time (minutes) and Y axis = μVolts, mVolts

The interpretation of these fatty acid profiles is complex due, in part, to the many individual fatty acids that are found in several fatty acid classes. Three major classes are the phospholipids, the triacylglycerides (triglycerides) and the glycolipids.

Phospholipids are polar molecules which contain ~90% poly-unsaturated fatty acids and comprise ~75 % of cell membranes (Thompson 1990). The triglycerides (3 fatty acids attached to glycerol by ester linkage) store excess energy (Henderson 1991, Lehninger 1993, Thompson 1990). The glycolipids are specific to photosynthetic organelle membranes (Kobayashi 1992).

Among the triglycerides, the saturated fatty acids (SFAs) 16:0 and 14:0 are a common component (Thompson 1990, 1993). Studies on diatoms and dinoflagellates found more triglycerides under N and P limitation (Harrison 1990, Lombardi 1991); whereas, less phospholipids tend to be produced (Harrison 1990, Henderson 1991, Lombardi 1991, Mansour 1999, Sukenik 1991, Wood 1995). However, with or without nutrient limitation, algae generally increase their energy storage fatty acids (triglycerides) as they age (Laureillard 1997, Mansour 1999).

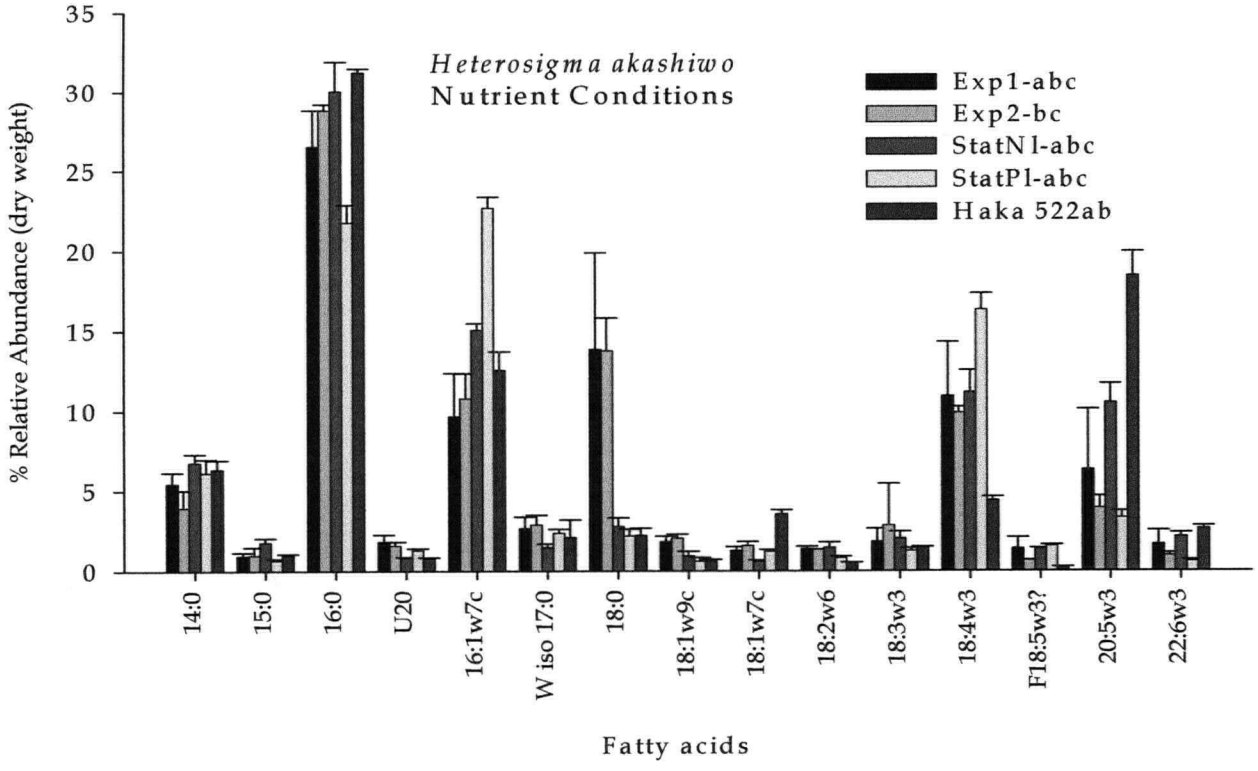

Figure 4. Nutrient limited Haka cultures at stationary and exponential growth phase. Standard deviation from sample size n=3 except for StatPl & Haka 522 (n=2) & no deviation for wild strain (n=1)

In this study both the SFA 14:0 and 16:0 %RA values increase in aging N-limited (StatNl) cultures, while only the abundance of 14:0 increases under stationary P-limited (StatPl) conditions. Although not significantly different, there was an overall trend towards greater abundance of the FA14:0 when nutrients were limited in stationary cultures.

The differences in FA abundance in exponential cultures 1&2 were found to be insignificant ($P>0.1$) so these two cultures were pooled together (n=5) when comparing with nutrient limited (n=3) cultures (Table 3.). Exponential Haka cultures had significantly higher values of 18:0 and 18:1ω9c and lower values of 16:1ω7 than the nutrient limited cultures. The 18-carbon fatty acid series is usually associated with membrane lipids and 16:1ω7 is considered a triglyceride (Lehninger 1993); hence, the pattern agrees with past research that shows an increase in a storage triglyceride in older algal cells as mentioned above.

ANOVA GLM statistical significance (P value < 0.05) with Tukey Simultaneous Tests										
	14:0	**16:0**	**U20**	**16:1ω7**	**18:0***	**18:1ω9**	**18:2ω6**	**18:4ω3**	**20:5ω3**	**22:6ω3**
Exp & NL	0.056	0.182	*0.005*	*0.007*	*0.000*	*0.005*	0.786	0.894	*0.031*	0.179
Exp & PL	0.22	*0.007*	0.131	*0.000*	*0.000*	*0.001*	*0.012*	*0.009*	0.458	0.169
NL & PL	0.69	*0.001*	0.171	*0.001*	0.568	0.359	*0.009*	*0.028*	*0.010*	*0.018*

Table 3. ANOVA - general linear model and Tukey comparison differences in nutrient and exponential growth phase Haka cultures. The fatty acid 18:0* was log transformed to meet normal distribution requirements. With unequal sample size, coefficients are based on weighted means for each factor level rather than the arithmetic mean (sum of the observations divided by n).

The reduced levels of 16:0 in StatPl is not expected based on past research. Pearson correlation confirms there is a significant inverse relationship between 16:1ω7 and 16:0 in StatPl (data not shown). The reduction in 16:0 and 20:5ω3, coupled with the dramatic increase in 16:1ω7 and significantly higher levels of 18:4ω3, separates StatPl cultures from all the other groups.

Sukenik (1991) found that with N-starved *Isochrysis galban,* a prymnesiophyte algae used in aquaculture feed, 18:4ω3 will increase but 16:0 and 18:1ω9 will decrease. This was not found for the StatNl samples in this study, but StatPl samples followed this exact trend.

The abundance of 18:4ω3 in StatPl is curious as it is generally found that chloroplasts per cell decline as they reach stationary phase (Cattolico 1976, Geider 1993). A decrease in photochemical efficiency was noted in N-limited cultures of the diatom *Phaeodactylum tricornutum* (Geider 1993). Yamochi (1984) proposed that nitrogen was primarily used for photosynthetic activity in *H. akashiwo*. Yet Cattolico did find a dramatic increase in chloroplasts per cell in B_{12} deprived samples. If 18:4ω3 does exist primarily in chloroplast membranes then an increase in its abundance in StatPl cultures shows a marked production shift towards the photosynthetic apparatus relative to Exponential and Stationary N-limited samples.

The poly unsaturated fatty acid 18:4ω3, like 16:1ω13, is thought to be a glycolipid, primarily associated with photosynthetic membranes (Kobayashi 1992, Sukenik 1991). *H. akashiwo* are known for their abundant number, 10-20 per cell, of chloroplasts. If the two acids are from the photosynthetic complex it is assumed that there are similar trends in

production (Throndsen 1997, Cattolico 1976). However, Pearson Correlation (data not shown) shows an inverse, but not significant relationship between these two fatty acid profiles. Glycolipids are one of three distinct functional groups of galactolipids that are found in chloroplast membranes. The groups share the same FA but in different concentrations (Bodennec 1998). According to Bodennec (1998), the fatty acid 18:5ω3 is primarily found in the same class as 18:4ω3. Yet, as discussed above, only trace amounts were found of 18:5ω3. Perhaps *H. akashiwo* glycolipids 16:1ω13, 18:5ω3 and 18:4ω3 are from different functional groups in the photosynthetic complex.

Significant changes in relative abundance of the FA 22:6ω3 was found between stationary Nl and PL. The latter had less. The results for 20:5ω3 and 22:6ω3 are remarkably similar. Both are significantly lower in abundance in StatPl- than in the StatNl samples. The 20:5ω3 was significantly lower in abundance in Exponential Haka than StatNl cultures. Bodennec (1998) partitioned the various lipid classes in *H. akashiwo*. From this work we can, with certainty, place 22:6ω3 in the phospholipid, cell membrane, class. It can be considered a proxy for growth. As predicted it is low in StatPl samples but surprisingly, not in StatNl cultures (when compared with Exponential Haka). This result is contrary to past research outlined above, which finds phospholipid reduction with N-limitation.

The complex interpretations regarding the changes in fatty acid production can be simplified when the chemical nature of fatty acids and the general biology of *H. akashiwo,* are both included in the analysis. Triglycerides provide insulation and a way in which some aquatic organisms regulate their buoyancy (Lehninger 1993). A triglyceride will increase in density with increasing number of saturated acid chains (Lehninger 1993). This is due to the tight packing ability of hydrogen saturated carbon chains. Saturated fatty acids increase the melting point of the parent molecule (Lehninger 1993). An increase in cell density would aid in downward migration.

H. akashiwo migrates vertically daily and can obtain nutrients from deeper waters at night. Intense Haka blooms are often associated with an increase in concentration of nitrogen (N) and phosphorus (P) in surface waters (Honjo 1992). In Japan, the availability of iron is also considered a key trigger (Yamochi 1983). Yamochi (1984) considered migration a crucial factor in bloom formation. *H. akashiwo* start their ascent just before dawn; spend the

day at the top of the euphotic zone and then return to the nutrient rich depths after dark (Taylor 1993, Wada 1985, Wood 1995). It is important to note that *H. akashiwo* migration is not regulated chiefly by phototaxis (Wada 1985, Hershberger 1997). The mechanism is more related to specific gravity, buoyancy and centre of gravity within the cell (Fukui 1980, Wada 1985).

Does the increase in the SFA proportion found in triglycerides, which occurs when *H. akashiwo* are N-limited, increase cell density and thus aid in downward migration? This could be considered a negative feedback cycle. N-limitation triggers a reduction in photosynthetic related fatty acids. Fatty acid production shifts towards energy storage in the form of a high percent of SFA triglycerides. This shift increases the cell density which influences migration. Descent to nutrient rich waters allows for a replenishment of N, P and trace metals.

Field observations indicate that *H. akashiwo* blooms suddenly end (Honjo 1992). As they approach senescence (old age) they stop migrating (Itakura 1996). The collapse of *H. akashiwo* blooms is likely attributable to a variety of factors (Honjo 1992, Itakura 1996). Some research suggests nutrient limitation by iron, phosphorus or nitrogen can be important, despite *H. akashiwo's* diel migration pattern (Wada 1985, Watanabe 1982, Yamochi 1983, 1984). Other researchers claim the sudden disappearance of blooms results from wind induced surface mixing (Iwata 1989). Finally, infection by viruses may be associated with bloom collapse (Lawrence 2002, Nagasaki 1994b).

Rontani (1998) predicts, with a model of photodegradation, that MUFA and PUFA %RA increases as cells reach senescence. Figure 5. shows fatty acids grouped together based on the number of double bonds present in the carbon chain. Samples harvested at exponential growth phase have a higher proportion of SFA (saturated) relative to MUFA (one double bond - mono) and PUFA (two or more double bonds – poly).

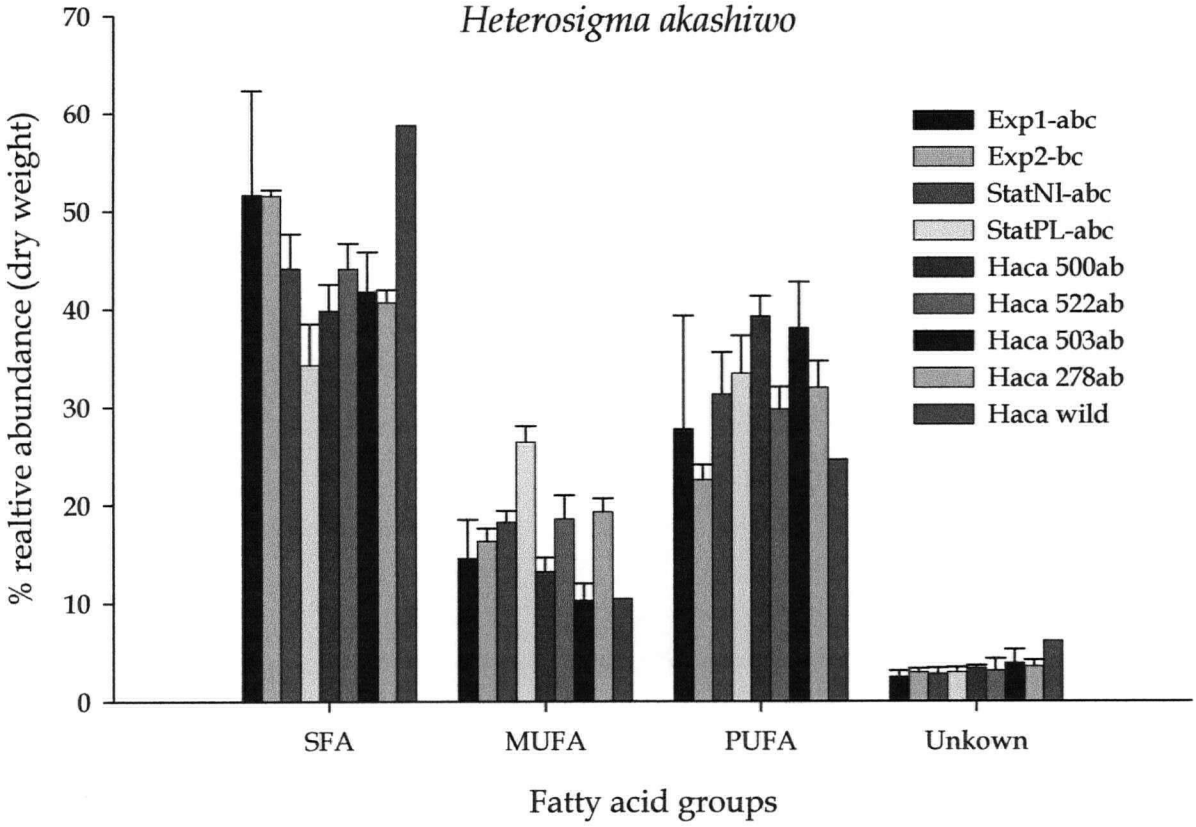

Figure 5. The total abundance of saturated (SFA) and unsaturated (Mufa and Pufa) FAs. Peaks not identified are designated as unknown.

Despite the differences in growth conditions, the StatNl and StatPl fatty acid profiles resemble the *H. akashiwo* strain samples (also harvested at stationary phase) more than the exponential samples except in amount of MUFA, where they are all less than StatPl.

Both stationary NL and PL tend to have higher MUFA and PUFA concentrations than exponential phase Haka. Yet they are distinct in that the StatNL cultures do not follow the large decline in the 16:0/16:1ω7 ratio as seen in StatPL cultures.

The single Haka wild sample appears to be more similar to the exponential samples than the stationary ones. This field sample was collected at the beginning of a Haka bloom that formed in Port Moody, B.C. and was not likely to be in the stationary phase.

2.3.3. Multivariate analysis of all samples

This study contains an inherent bias towards increased variation between strains, nutrient limited and wild samples (due to the different culture conditions). However, multivariate analysis of all 20 samples using a sub sample of 10 key fatty acids: 14:0,16:0,U20, 16:1ω7, 18:0, 18:1ω9, 18:2ω6, 18:4ω3, 20:5ω-3 and 22:6ω-3 reveals distinct cluster relationships (Figure 6.).

The initial 3 components explain 79% of the variation. PCA 1&2 components show the previously noted differences in the StatPl fatty acid profiles, primarily due to changes in %RA of 16:0, 16:1ω7, 18:4 ω3 and 20:5ω3. The wild sample is also very distinct. Using different fatty acids changes the positions of the various samples on the x/y axis but the general grouping outlined in Figure 6. is resilient. However, unlike the other samples, when different fatty acids are used in PCA computations, the wild *H. akashiwo* changes associations. This malleable nature of the wild strain warrants further investigation. Some fatty acids tie it closely with exponential samples, others place it very close to *H. akashiwo* #503 and still others cluster it with #522 (data not presented).

The proximity of StatNl PCA scores to the 4 strain nutrient replete scores is noteworthy. The N:P ratio of *H. akashiwo* is reported to be 15.2 (Watanabe 1982). The F/2 seawater medium, which the *H. akashiwo* strains were cultured in, has a ratio of 24:1 (Harrison 1990).

These strains were collected at late stationary phase, after replenishing many generations with fresh seawater medium. Phosphate limitation would be the expected result based on the N:P ratio proposed by Watanabe.

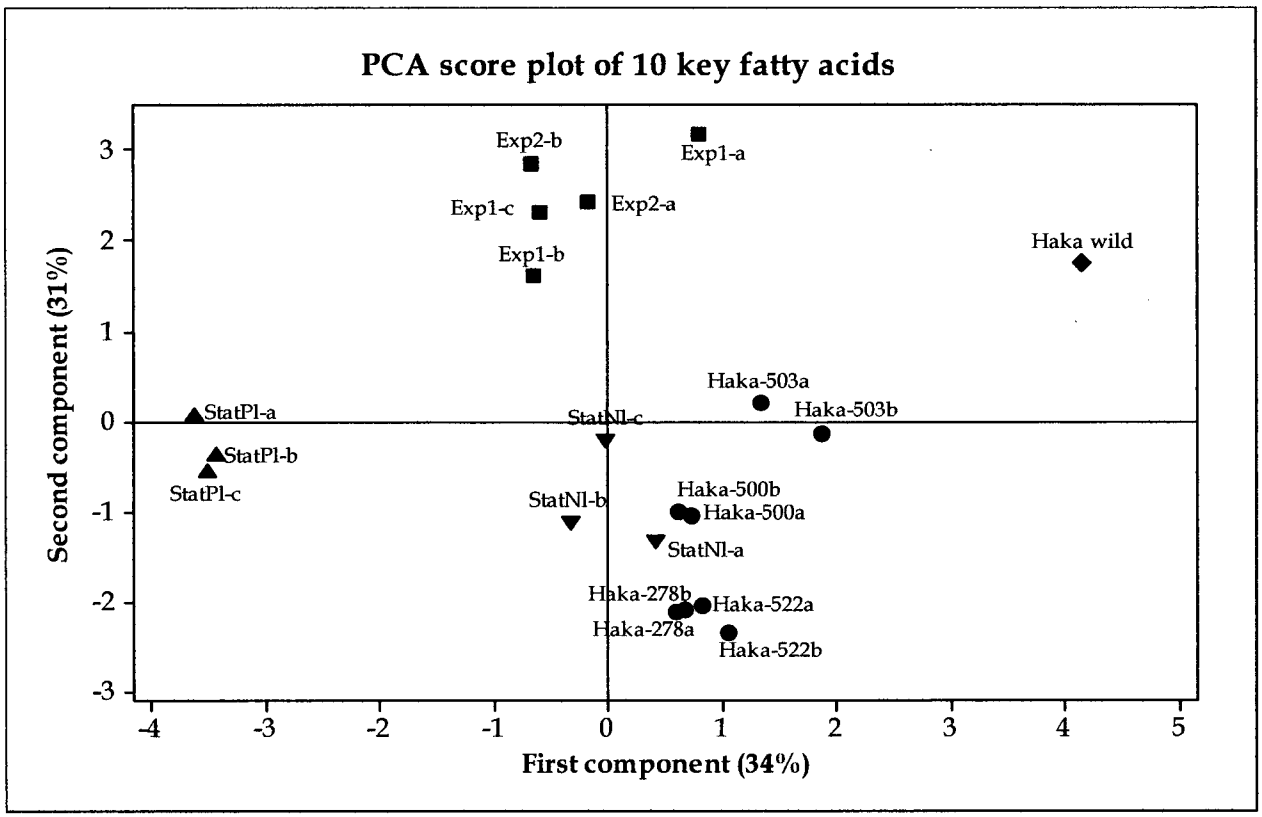

Figure 6. PCA of *Heterosigma akashiwo* cultures with 10 key fatty acids. Replicates designated a,b,c. Limiting the number of variables (fatty acids) increases the power of principle component analysis - PCA (Bright per. comm., Harris 2001).

The association of StatNl with Haka strain cultures, as depicted in Figure 6, can be explained if the range of N:P in *H. akashiwo* is greater than the ratio (24:1) found in the F/2-si medium. La Roche et al. (1993) show, in their study of proteins, that there is a wide range in possible N:P ratios within a single species. Wood (1995) found *H. akashiwo* can accumulate pools of both excess N and P; the proportion of which changes based on what form N and P are available in. This complexity in the nutrient N:P requirements in algae is the focus of recent field studies (Fong 2003).

There is a spread in the position of exponential sample replicates. This spread accounts for the higher than average error, as previously noted, in growth and nutrient limited samples. The higher error associated primarily with exponential may indicate more than just extraction efficiency. Perhaps the fatty acid production is more variable during rapid growth?

2.4. Conclusion

There is significant variation in the fatty acid profiles of *Heterosigma akashiwo*. This variation was expected as past research has shown how complex these profiles are. The strains 522 and 278 are more similar, primarily due to the similar abundance of 16:1ω7, than the 500 and 503 strains. The latter samples have higher levels of 18:4ω3.

Past research shows trends in fatty acid production. Several predictions, such as the reduction of the phospholipids Un20, 18:0, 18:1ω9c and 22:6ω3 as they age, are supported by results from this study. The results of 14:0 are remarkably similar within strains and compared with all samples and literature values.

Fatty acids such as 16:0, 16:1ω7 and 18:4ω3 display complex reactions to various conditions. The former two 16-carbon chain fatty acids are considered primarily triglycerides and the latter, a glycolipid, is assigned specifically to chloroplast membranes. The changes in abundance of these fatty acids did not mirror results from previous investigations. This difference in StatPl, StatNl and Exponential growth phase cultures requires further study. The FA analysis could be broadened to include viral infected, B_{12} depleted and iron limited Haka cultures.

When lipid chemistry and *H. akashiwo* biology are combined to interpret these results a tentative hypothesis helps to explain the usual StatPl *H. akashiwo* fatty acid profile. Fatty acids, specifically triglycerides, are key components in buoyancy regulation. An investigation comparing cell density and fatty acid production would be interesting.

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Chapter 3. Fatty Acids in Marine Sediments

3.1 Introduction

This study quantifies fatty acids to a maximum depth of 30 cm in marine sediments from three inlets near the Georgia Strait Basin, British Columbia. Sediment cores were collected from Malaspina Inlet, Hotham Sound and Howe Sound.

Several marine algal groups can be tracked using fatty acid biomarkers extracted from sediments. Species that have large blooms and consequently a large amount of debris sinking to the ocean floor may, by sheer bulk, enhance preservation of a unique species signature downcore in sediments.

Although few unique fatty acids exist as proxies for marine algae, if a local taxonomic record is available then greater confidence can be given to selected suits of fatty acids used to hindcast bloom events.

This study uses the proportions of a suite of key fatty acids that are found in all the *H. akashiwo* strains (see Chapter 2.). These unique proportions are considered a species signature and thus act as a proxy for *H. akashiwo* in marine sediments.

Fatty acids are subject to degradation and the signal becomes weaker with age. Yet the persistence of labile poly unsaturated fatty acids at depth in sediments indicates the potential for these biomarkers to hindcast bloom events.

Algal fatty acid biomarkers reveal changes in algal group composition and bloom frequencies at the three sites. This is the first inventory of fatty acid data from sediments in British Columbia inlets.

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[2] A version of this chapter will be submitted for publication. Drost, H. Fatty Acids in Marine Sediments

3.2. Materials and Methods

3.2.1. Site Descriptions

Three sediment cores were collected in Malaspina Inlet (A; 49°58.73 N 124°41.31 W), Hotham Sound (B; 49°49.68 N 124°04.78 W) and Howe Sound (C; 49°27.34 N 123°17.01 W) (Figure 7.), at depths of 37 m, 169 and 251 m, respectively.

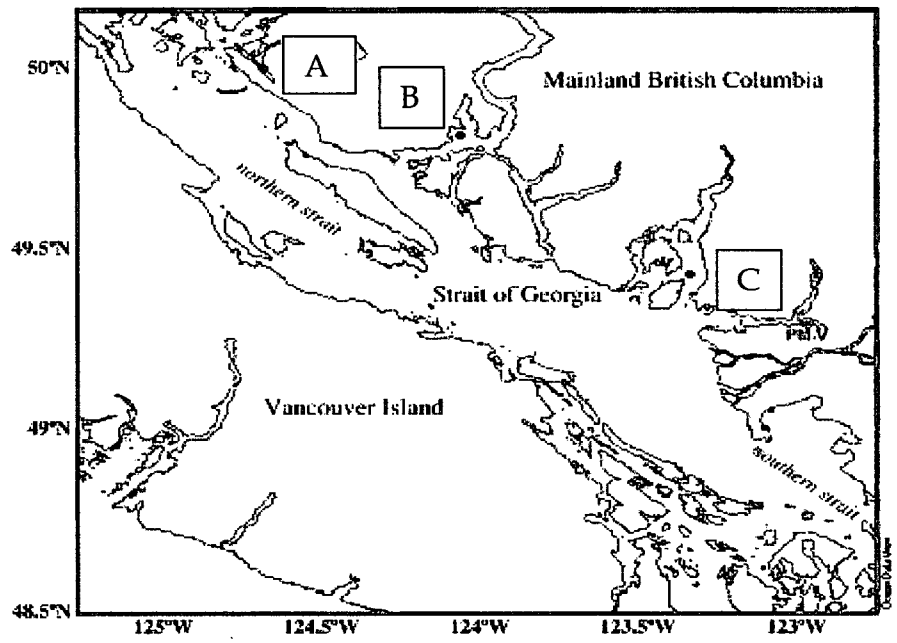

Figure 7. Location of sediment core samples (circle) near the Strait of Georgia, British Columbia

3.2.2. Sample collection and storage

Sediment cores (30 cm max length: 8 cm o.d.) were collected by J. E. Lawrence using a triple-barrelled gravity corer (Rigosha & Co.) during the CCGS Vector cruise in August 2002 as described in Lawrence et al. (2002). Briefly, sediment was taken from the centre of each core; the outer 2 cm was discarded to avoid contamination from smearing. The 1 cm cakes from the 3 cores were combined and then partitioned for 4 different research projects. The samples for lipid analyses were placed into acid-washed, combusted jars with Al-foil lined lids. Nitrogen gas (N_2) was added and they were immediately frozen at -20°C on board. Once ashore, the sediment samples were transferred to acid washed plastic tubes (Sartstedt

screw cap 50mL) and dried for 4 days using a Labconco Freezedryer (vacuum 111x10-3 mbar, collector -47°C, shell 28°C) and then stored at -70°C under N_2 gas until analysis.

3.2.3. Cultures of *Heterosigma akashiwo*

See Chapter 2.2. Materials and Methods for details.

3.2.4. Loss on ignition (LOI)

Using a muffle furnace, samples were combusted at 550 °C (4 h) with the weight of the samples measured before (~1.0 g) and after drying and combustion (Heiri et al. 2001). LOI can reportedly overestimate total organic carbon (TOC) concentrations (CSIRO 2000), thus this proxy was only used to compare the relative organic matter at the three study sites. The loss was determined by:

$$LOI_{550} = (DW_{105}-DW_{550})/DW_{105})*100$$

where LOI_{550} represents total organic matter (percent), DW_{105} = dry weight of the sample before combustion (g) and DW_{550} the dry weight of the sample after heating to 550 °C (g).

3.2.5. Fatty acid analysis

3.2.5.1. Extraction

Chapter 2. outlines, in detail, the method employed to extract and quantify fatty acids (FAs). The only difference, when extracting FAs from sediments rather than filters, is that the sediments were gently homogenized, using sterilized glass stir rods in a glove box, before weighing (~400 mg) and transferred to a test tube.

3.2.5.2. Fatty acid quantification

A library of 32 fatty acid standards acquired from SUPELCO where weighed, dissolved in hexane and then, to increase temperature volatility, methylated using BF_3. Chromatogram profiles for each fatty acid standard were created using a range of 7 concentrations. These profiles generated the known concentration vs. peak area line equation

$$Y_1 = 484.52(X) + 2.1049$$

where: X = known concentration of fatty acid standards and

Y_1 = peak area

Fatty acid concentration was corrected for total weight of sediment used and final dilution of the purified fatty acid extract with ethyl acetate by the following equation:

$$Y_2 = X\ \mu g\ FA/g\ * D * 1000\ (mg/g) / S$$

where: D = dilution in (mL)

S = dried sediment sample weight (mg)

Y_2 = Corrected concentration (μg of fatty acid/g of sediment dry weight).

The percent recovery of sample fatty acids was calculated based on the known concentrations of the surrogate standard C19, and internal standards C21 and C13; the latter two standards were added before saponification. These pure standards (SUPELCO), of known concentration, were added to many of the samples during the extraction process. The surrogate standard (C19) was added to several sediment samples at the very beginning of the extraction process and was used to assess extraction efficiency.

3.2.5.3. Error and statistical analyses

The average (μg FA/g sediment dry weight) variation between triplicate samples was 11.3% (n = 3). Before log transformation zero values (missing peaks) and fatty acid concentrations less than 0.5 μgFA/g sediment dw were considered trace for statistical analysis and given a random value less than 0.5. Changes in FA concentration due to length of sample storage (up to 7 months) and how different laboratory techniques impact fatty acid concentration were tested for this study (Appendix 3.6.1). Analysis of standard deviation includes changes due to long term storage.

For multivariate analysis, the data (Y) were transformed using the following equation:

$$Y = Ln\ (X_i / \Sigma X_n) * 100.$$

where X_i is the fatty acid concentration (μgFA/g sediment dry weight) and

X_n is the total concentration of all fatty acids used in the analysis.

When log transforming data for multivariate analysis, zero and trace values (0.5 μg) were assigned a random number, where $0 < X < 0.5$ μg FA/g sediment dry weight. Data were also transformed when normality or variance requirements were not met for ANOVA and sample t-tests. Microsoft Excel, SYSTAT (SPSS Inc.) and MINITAB (e-academy version) were used to perform normality and variance testing, regressions, ANOVA and Principle Component analysis.

3.2.6. ^{210}Pb analysis

^{210}Pb was measured in an effort to determine depth of mixing and approximate dates of fatty acid deposition in sediments from Hotham Inlet. ^{210}Pb values were determined on 2-5g of duplicate sediment samples (Flett Research Ltd., Winnipeg, MB) from Hotham Sound. Background levels of ^{210}Pb were not reached at depth in the sediment core thus a slope regression model was used to estimate a value. This value is used to correct the total activity that is measured as disintegrations per minute (DPM). This estimate, coupled with a well defined 10 cm mixed layer in Hotham Sound sediments, allows for only an approximate depositional history estimate. A steady-state two-layer advection-diffusion model was used to interpret ^{210}Pb profiles (Macdonald, pers. com.). The ^{210}Pb values, entered in the model, were corrected for detector background and sediment porosity. The age of sediments was estimated using the Intrinsic Time Resolution Equation,

Date of sediment = depth in core (cm) / sediment velocity (cm/yr)

The sediment velocity was calculated from the model described above. Error estimate included the depth mixed sediment, range in ^{210}Pb dpm/cm^3 values (n=3) and cummulative mass (g/cm^2) variation. The sediment velocity value was selected based on the lowest value of the sum of squares difference of the corresponding values between ^{210}Pb measured in sediments and those derived from the model.

3.2.7. DNA results

Researcher Azeem Ahmad, from the University of British Columbia, was also provided with sediment samples from the same cores collected from Hotham Sound and Malaspina Inlet. He extracted *Heterosigma akashiwo* cyst DNA at selected depths (Ahmad pers. com.). The DNA results were compared with the multivariate analysis of 5 key fatty acids found in both sediment and *H. akashiwo*. The distance of sediment samples from *H.*

akashiwo profiles on PCA plots was calculated using the Pythagorean Theorem, $d=\sqrt{((X_2-X_1)^2 + (Y_2-Y_1)^2)}$.

This value was then transformed by subtraction of a constant to display a positive slope in regression analysis.

Close proximity of certain FAs from sediment samples to *H. akashiwo* FA profiles was considered to represent past bloom events. This assumption was tested by comparison with *H. akashiwo* DNA abundance data.

3.2.8. Abbreviation Key

FA	Fatty acid
T	Hotham Sound
H	Howe Sound
M	Malaspina Inlet
abc	Single sample extracted 3x's concurrently
ab*	Single sample extracted at different time (up to 7 months apart)
%RA	Percent of total fatty acids (dry weight)
$\mu g\ gdw^{-1}$	µg fatty acid/g sediment dry weight
LOI	Loss on ignition
OM	Organic matter
Haka	*Heterosigma akashiwo*

Table 4. Abbreviations used in paper

3.3. Results and Discussion

Malaspina Inlet and Hotham Sound sediments were a rich golden brown colour, when suspended in solvents, compared with the pale yellow colour of samples from Howe Sound sediments and consistently required more hexane washes to remove organic traces. Colour differences can indicate a range of organic content in the samples.

Loss on ignition (LOI) is used to assess the organic content in sediment samples (Heiri 2001). The OM^{LOI} values are similar for Hotham Sound (T) and Malaspina Inlet (M) samples (Figure 8.) but are much lower and show little vertical change downcore in Howe Sound (H) samples.

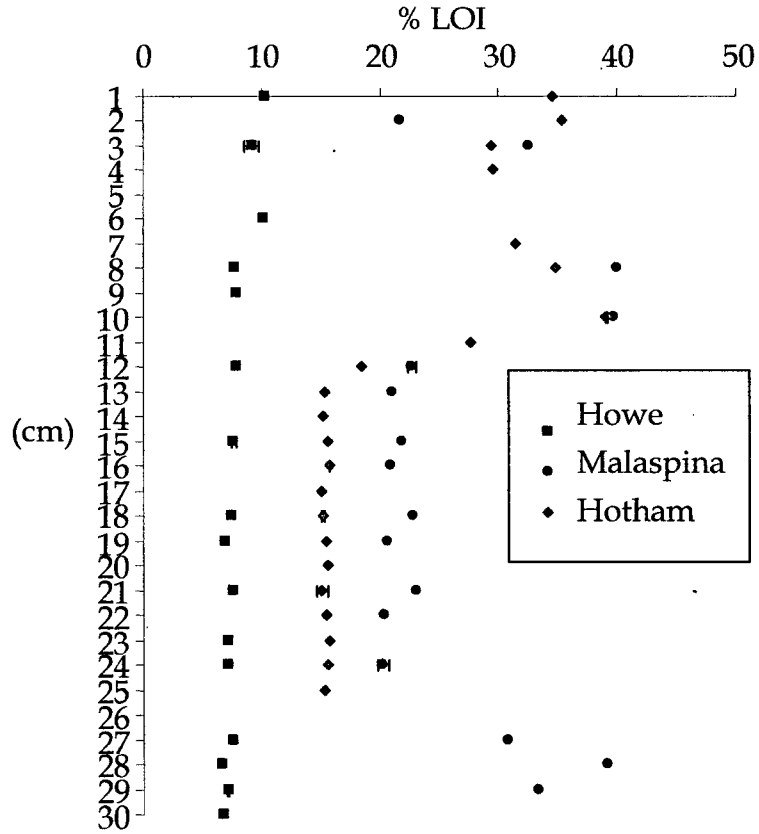

Figure 8. Loss on Ignition (LOI 550°C) used as a proxy for organic carbon. Standard deviation is based on n=3 replicates.

Malaspina Inlet sediments had more organic content to burn off than Howe Sound sediments, consistent with the colour differences observed initially. Reference to the results

in Figure 8. will use the short hand OM^{LOI} to differentiate this proxy from other methods of estimating OM.

The overlap in Hotham Sound and Malaspina Inlet OM^{LOI} values is not reflected in the total concentration of fatty acids (μgFA/g sediment dry weight) found at the three sites (Figure 9abc). It is possible that high organic carbon content does not necessitate a corresponding increase in fatty acid concentration. For instance, Hotham Sound may have had log booms tied up near where the sediment cores were collected. Another reason could be experimental. The percent loss of organic carbon vs. carbonates under different temperature and time regimes can be highly variable (Bisutti 2004). Yet the researchers, who outlined the method used in this study, verified the reproducibility and accuracy of the OM^{LOI} estimate by having several laboratories test samples with known OM concentrations (Heiri 2001).

3.3.1. Fatty acid concentration and downcore patterns

The fatty acids are separated into identified peaks and unknown peaks. The unkown peaks range in concentration from 10-30% at the 3 sites. The ubiquitous palmitic fatty acid 16:0, which is used as a proxy for the total community biomass, is also presented in Figure 9abc.

The concentrations of FAs are highest at Malaspina Inlet, moderate in Hotham Sound and low in Howe Sound. The average concentrations of surface sediments versus the deepest sediment sections were significantly different ($p < 0.05$) at all 3 sites. All three sites show downcore pulses in total FA concentration.

The concentration of FAs in sediments reported in other studies ranges from 1.9 off the west coast of Scotland on the Hebridean Shelf to 756.5 μg FA/ g sediment (dry weight) from Peruvian continental shelf sediments (Carrie 1998). The FA concentrations measured in these 3 coastal BC inlets are within the range measured at other locations, and indicate high productivity in Malaspina Inlet compared with Howe Sound.

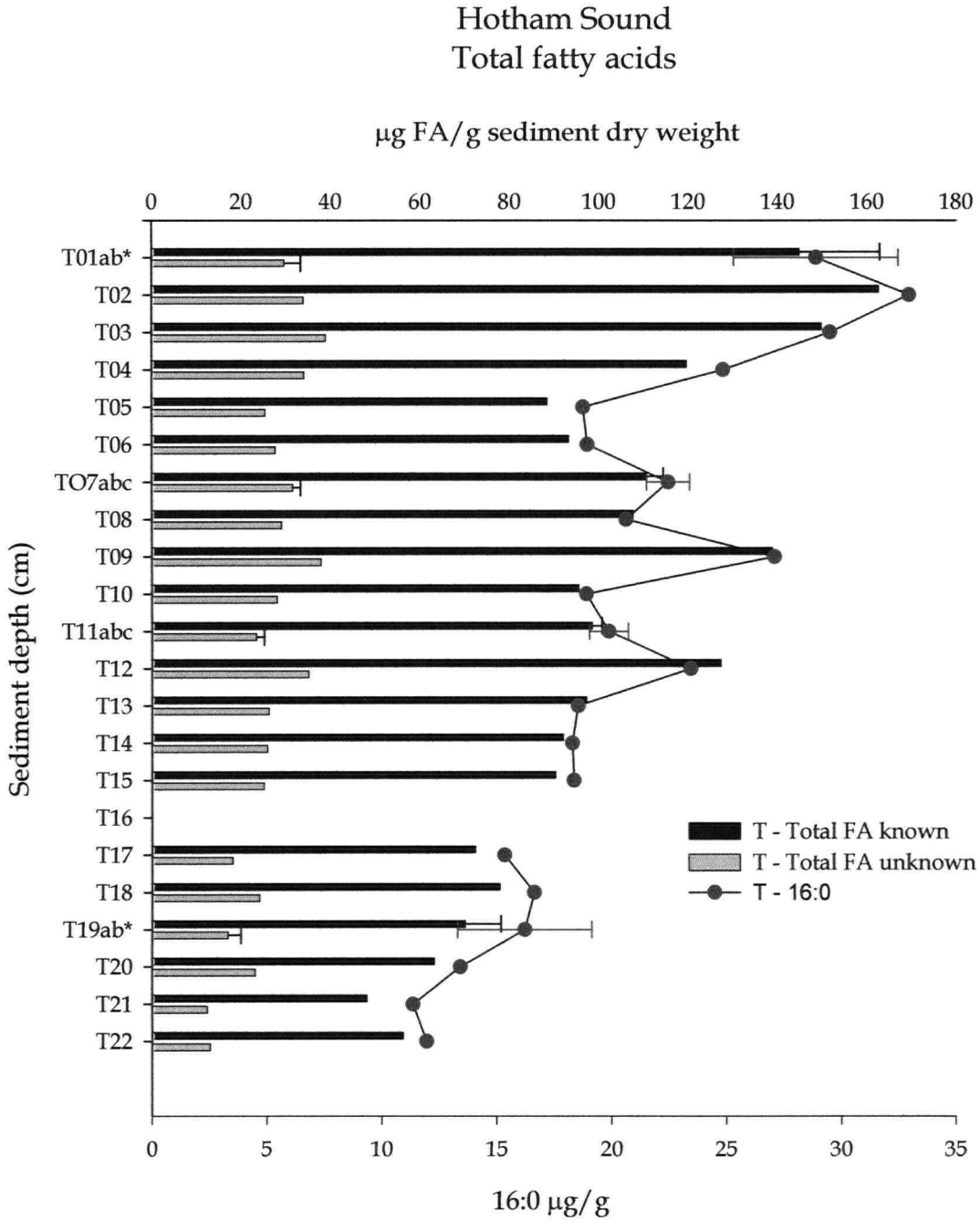

Figure 9a. Total known and unknown FAs from coastal marine sediment cores

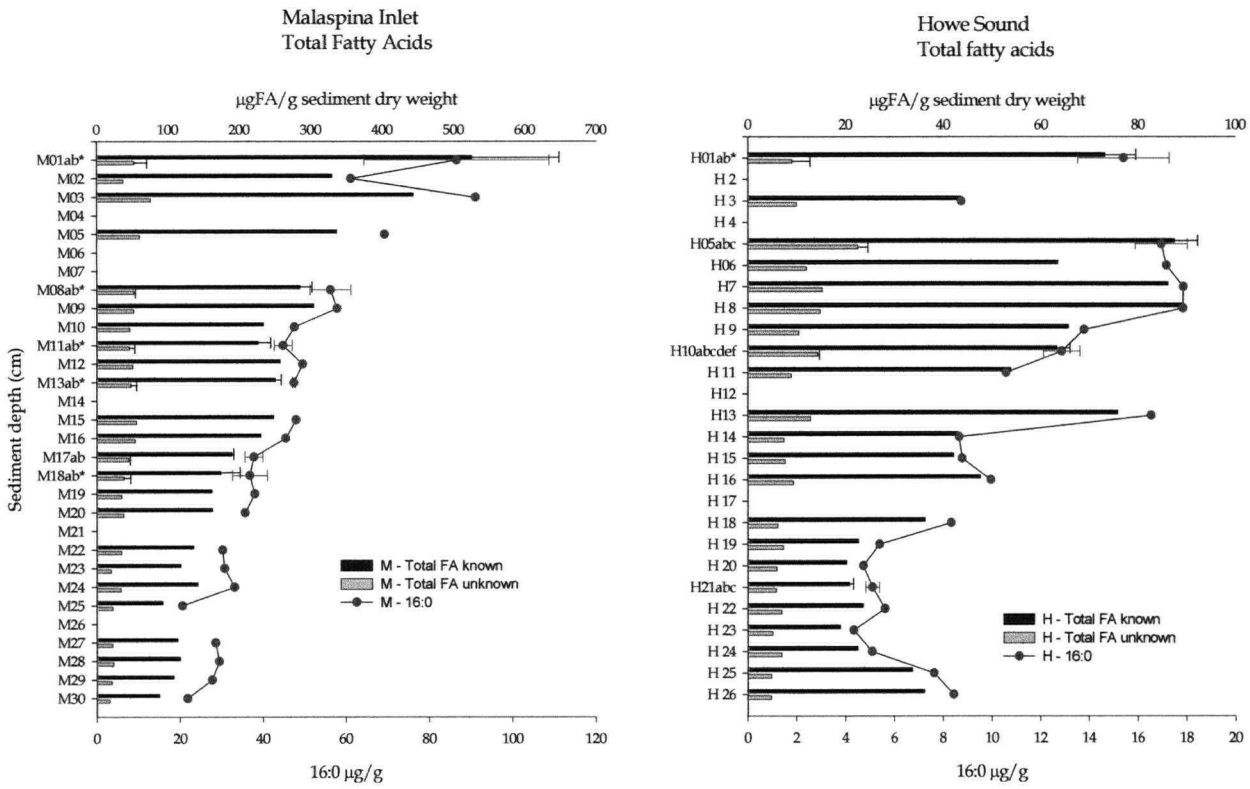

Figure 9(bc). Total known and unknown FAs from coastal marine sediment cores. Standard deviation values are derived from sediment sample replicates and represent the reproducibility of the FA extraction method. Palmitic FA 16:0 is used as a proxy for total community biomass.

The high concentration in Malaspina Inlet could be due to the shallow depth (37m) at which the sediment core was collected when compared to the much deeper Hotham Sound (169m) and the deepest (251m) at Howe Sound. The shallow water column in Malaspina Inlet reduces the exposure time to degradation. However, in past studies it is estimated that the loss of FA in the water column, as they sink to the bottom, is relatively insignificant compared with the loss in the surface sediments (Parrish 1998). Malaspina Inlet also differs from both Howe and Hotham Sound due to extensive shellfish farming (MSRM 2004). Bivalves contain a large proportion of 16:0 FAs with the largest proportion (57-66%) found in the shells (CoBabe & Pratt 1995), which could cause enrichment of 16:0 in M sediments. In general, clams and oysters can affect water quality and influence the type of OM matter buried in sediments (Canuel 1995, Boesch 2001).

Other studies suggest that salinity, which drives the type and abundance of phytoplankton communities, indirectly influences FA concentrations (Canuel 2001, Shi 2001). Canuel, 2001 found that there are more algal blooms at lower salinities ~25psu than found in the water column of ~30psu. The average salinities at the locations of this study, ranged from 24psu in Malaspina Inlet to 31psu in Hotham Sound, with the latter only slightly higher, on average, than in Howe Sound (DFO-OSAP 2000).

Only Hotham Sound has a distinct subsurface FA concentration maximum (submax). This term refers to the immediate increase in concentration just below the sediment surface. Other researchers report the existence of a FA submax (Belicka 2002, Conte 1995, Shi 2001). This distinct feature of a biomarker profile in sediment is attributed to two important types of advective mixing processes that occur at the sediment/water interface (Conte 1995).

Horizontal advective mixing can have a big impact on biomarker preservation. Mesoscale eddies that can be detected near the sea floor can move at speeds of 7-9 cm s^{-1}. These eddies can disrupt and resuspend deposited organic material into the nepheloid layer (Conte 1995). This layer, found below the mid-water transparency maximum, has elevated levels of OM, bacteria and viruses compared to higher in the water column (Carrie 1998, Cochlan 1993). This type of mixing can alter the short term biomarker patterns in surficial sediments (Shi 2001).

The second type of advective mixing is vertical. This mixing is facilitated by bioturbation, the reworking of OM by benthic organisms. Biomarkers are preserved either virtually unaltered due to local depositional focusing, or markers undergo chemical alteration due to consumption of material and subsequent egestion (Conte 1995). The downcore profile of 210Pb indicates that Hotham Sound sediments have a distinct mixed layer (Figure10.).

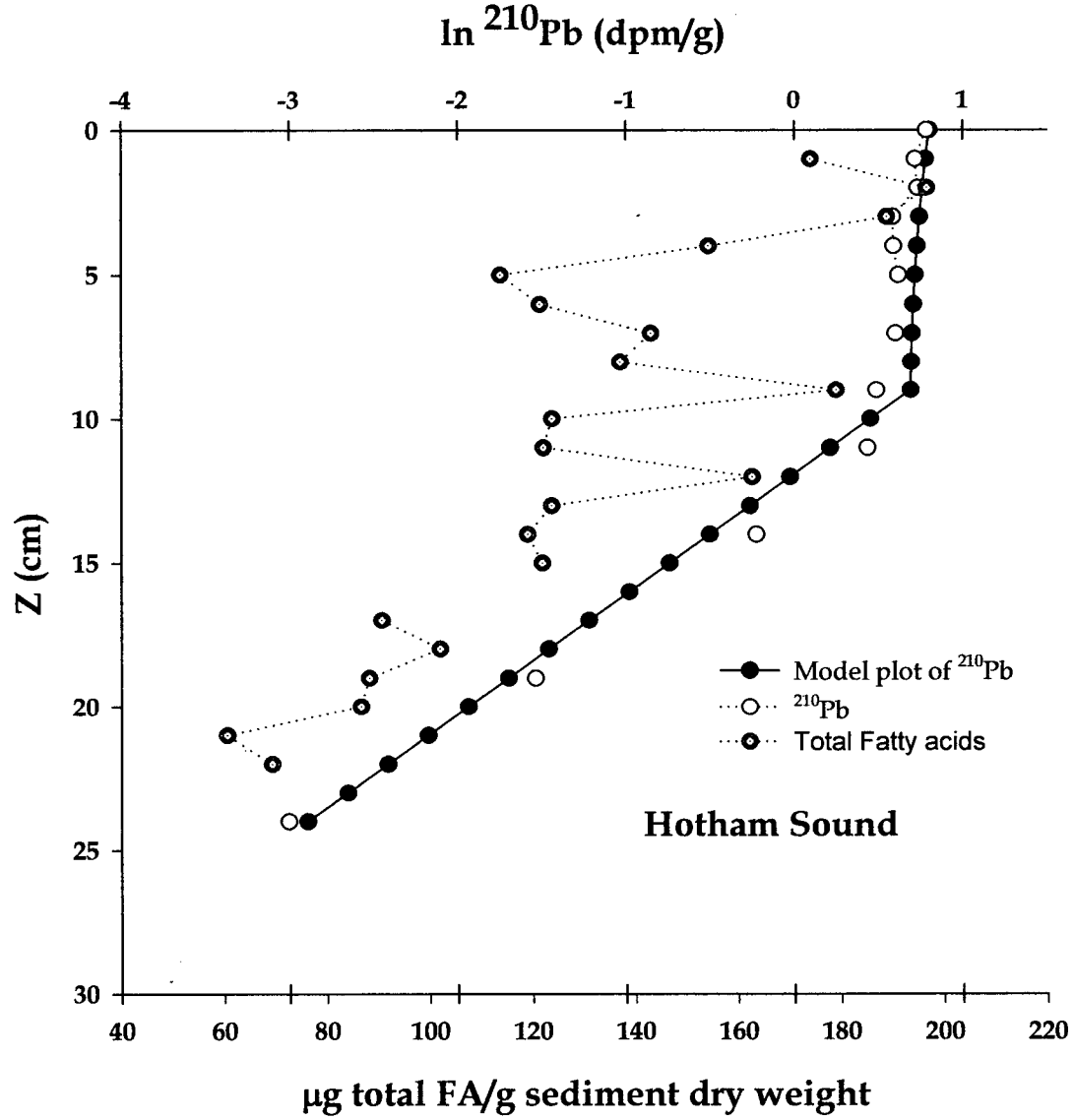

Figure 10. 210Pb downcore concentration profile, extrapolated 210Pb values from model and total FAs in Hotham Sound sediments

The ^{210}Pb profile shows bioturbation extending to ~10 cm in Hotham Sound sediments, which smears the source FA signature over several years. Also, the background level of ^{210}Pb was not reached at the bottom of the core. For these reasons, a steady-state two-layer advection-diffusion model was used that assumes the ^{210}Pb and sedimentation rates are constant over time. The calculated overall sedimentation rate is 0.117cm yr^{-1}, which, using the basic time resolution calculation, translates to 188 ± 90 years at 22 cm depth in Hotham Sound sediments. Thus, ~88 µg total FA/ g sediment dw was lost in Hotham Sound over 188 y and ~25 µg FA/ g sediment dw over 30 y. This is similar to the total FA

loss of 32 μg FA/g sediment dw over 30 y that was calculated for sediments from Buzzards Bay, MA (Farrington 1977).

^{210}Pb was also used to date sediments at two other locations near Hotham Sound. The sites were only 1 km apart yet the estimated MAR in 1995 and 1989 were 1,500 g $m^{-2}y^{-1}$ and 650 g $m^{-2}y^{-1}$ (Timothy 2001), respectively, while in this study the rate was 530 g $m^{-2}y^{-1}$. Timothy reported a sedimentation rate of 0.6 cm y^{-1} at the location of his study near Hotham Sound, which is much greater than calculated (0.117cm yr^{-1}) in this study. The wide range in values may reflect errors in the ^{210}Pb dating or Timothy (2001) suggests it may reflect true variation in deposition rates. For example, deposition rates would appear to be much higher in a localized zone of depression (OM depot zone).

The similarity of LOI^{OM} between Malaspina Inlet and Hotham Sound sediment samples, despite the difference in FA total concentrations, was also depicted by Principle Component Analysis using 31 known FAs extracted from 67 marine sediment samples (Figure 11.a).

Samples follow a depth gradient from right to left on the PCA plot in Figure 11a as shown by the size of symbol, which decrease in size with depth. The PCA loading plot of FAs (Figure 11.b) shows how the FA are grouped based on similar variation patterns downcore. The grouping shows that degradation and species composition (source material) from the overlying water column are the two main components that explain 36% of the variation found in 31 of the sediment fatty acid profiles. Degradation and source material was also found to be important factors in a FA multivariate study conducted by Canuel in 2001.

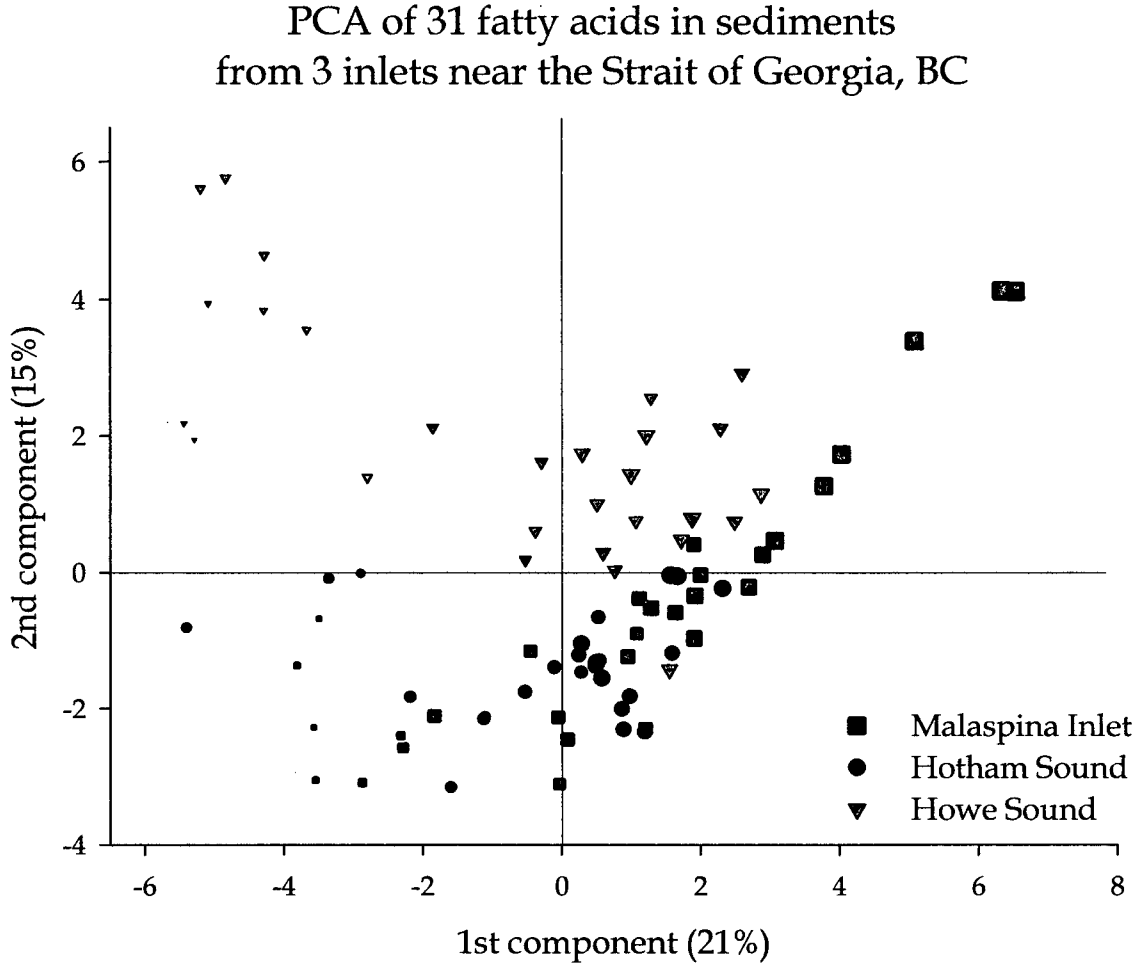

Figure 11a. Principal Component Analysis of fatty acids in Sediment. All FA values transformed (log10).The components cumulative values were 0.221, 0.369, and 0.496.

The Howe Sound sediments with the lowest FA concentration are situated in the left upper corner of the PCA plot. The blend in Hotham Sound and Malaspina Inlet PCA values may indicate a similar rate of FA degradation at these two locations. FA degradation will be reviewed in further detail in section 3.3.4.

The PCA loading plot shows which of the 31 FAs used in the analysis clustered together (Figure 11.b). These clusters show distinct patterns of fatty acids. Degradation and the source organisms explain 36% of the variation in fatty acids downcore in marine sediments.

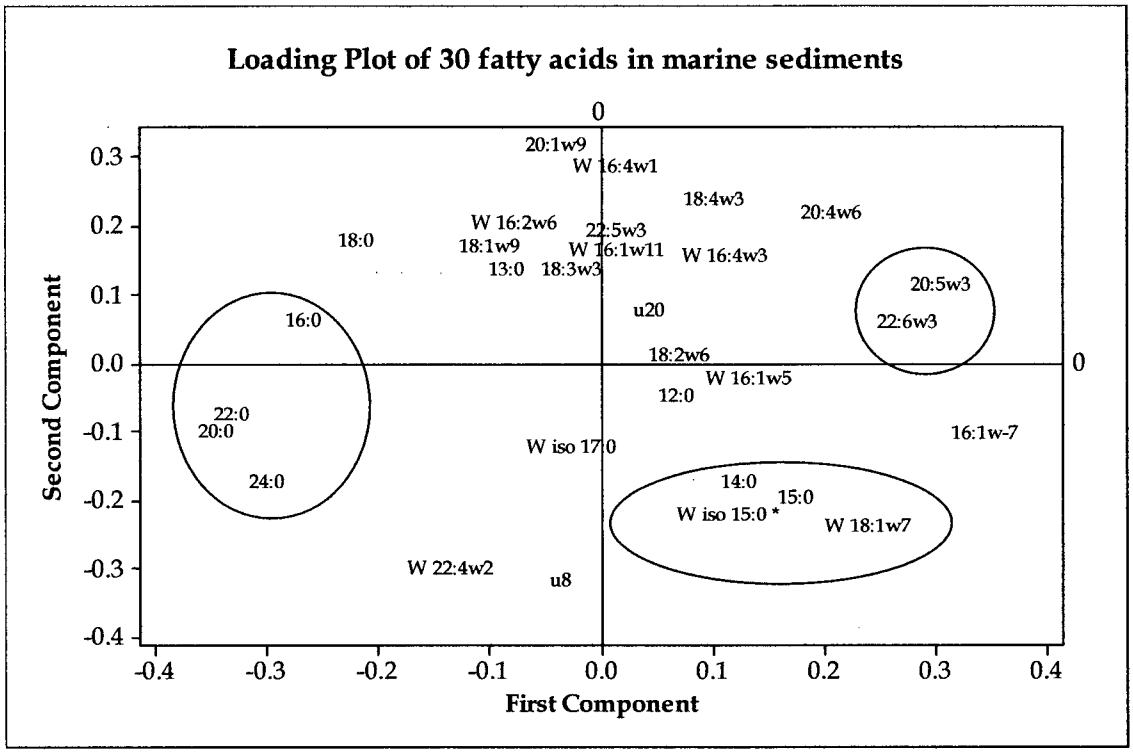

Figure 11b. Loading plot of the fatty acids used in PCA analysis of sediment samples

Degradation is shown by the cluster of saturated FAs, 24:0, 20:0, 22:0 and 16:0 loaded negatively for both components whereas the FAs with 4 or more double bonds were positively loaded. A cluster of mono and double unsaturated FAs was positive for component 2 and negative for component 1. These loading plot fatty acid clusters indicate that degradation, based on hydrogen desaturation (carbon double bonds), is an important factor that explains downcore variation of FA in sediments.

Source organisms show a bacterial and algal component. The former indicated by FAs iso-17:0 & 15:0 and 18:1ω7. The fatty acid 14:0 also loaded positively for the first component and negative for the second. This saturated fatty acid is common to many organisms but perhaps the contribution from bacteria dominates in the sediment. This may reflect a significant contribution of fatty acids by bacteria and helps to explain some of the variation in fatty acid concentrations found in sediments.

The loading plot also shows a distinct algal FA cluster namely the biomarkers for diatom (22:5ω3) and dinoflagellate (22:6ω3) groups. This indicates a significant algal

contribution. The abundant 16:1ω7 fatty acid is situated between 20:5ω3 (diatom indicator) and 18:1ω7 (bacteria indicator). The association between 16:1ω7 and 18:1ω7 was noted in earlier research, but only as a possibility as it is well known that some diatom species have 16:1ω7 in abundance (Nishimura 1987). Recently other researchers are including 16:1ω7 in the bacterial group (Pearson 2001). The position of this fatty acid on the loading plot indicates that both diatoms and bacteria are significantly contributing to the pattern of 16:1ω7's variation in sediments.

The fate of labile organic matter during sediment burial is complex, but the source organisms are recorded in FA signatures in the sediments. Degradation and species composition was also found to be an important explanation of the variation in FA concentrations attached to particulate OM in San Francisco and Chesapeake Bays (Canuel 2001).

3.3.2. Fatty acid biomarkers: proxies for source organisms

Correlation can be used to infer the relationship between algal and bacterial FA biomarkers in the sediment cores (Conte 1995). When algal debris increases, bacterial abundance also typically increases (Shi 2001, Jasti 2005, Cochlan 1993). The relationship between bacterial FAs and un-saturated ω3 acids, which are only found in algae was best described, in Figures 12abc, by an exponential curves in Hotham Sound ($r^2 = 0.77$) and in Malaspina Inlet sediments ($r^2 = 0.88$). In Howe Sound sediments the r^2 was higher for a linear regression.

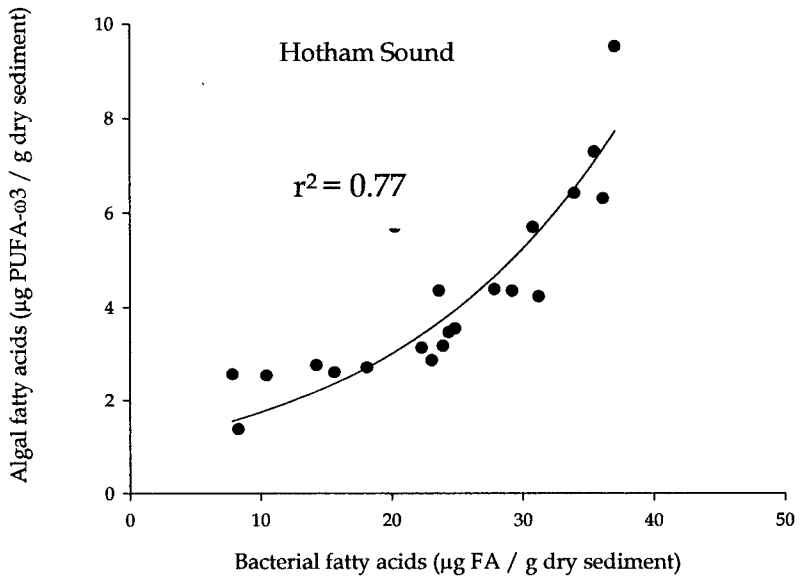

Figure 12a. Regression analysis between bacteria and algae in Hotham Sound

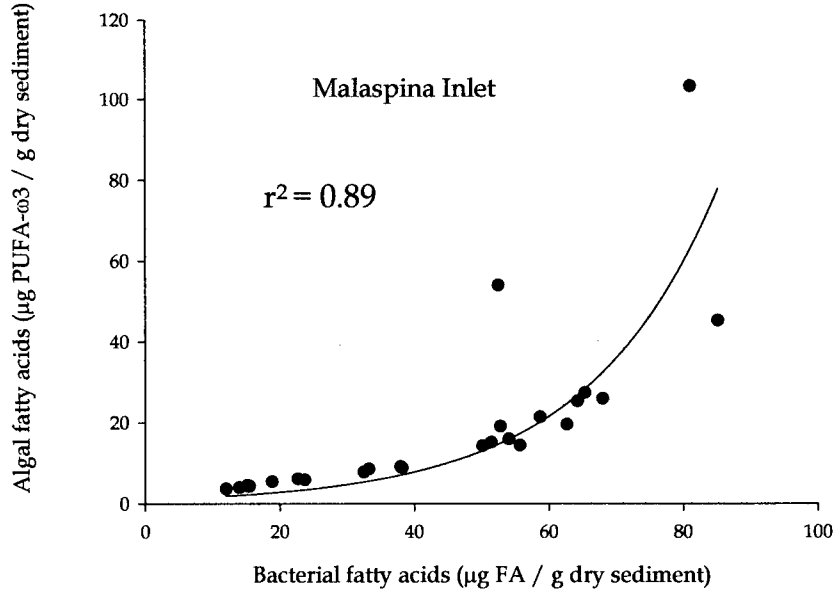

Figure 12b. Regression analysis between bacteria and algae in Malaspina Inlet

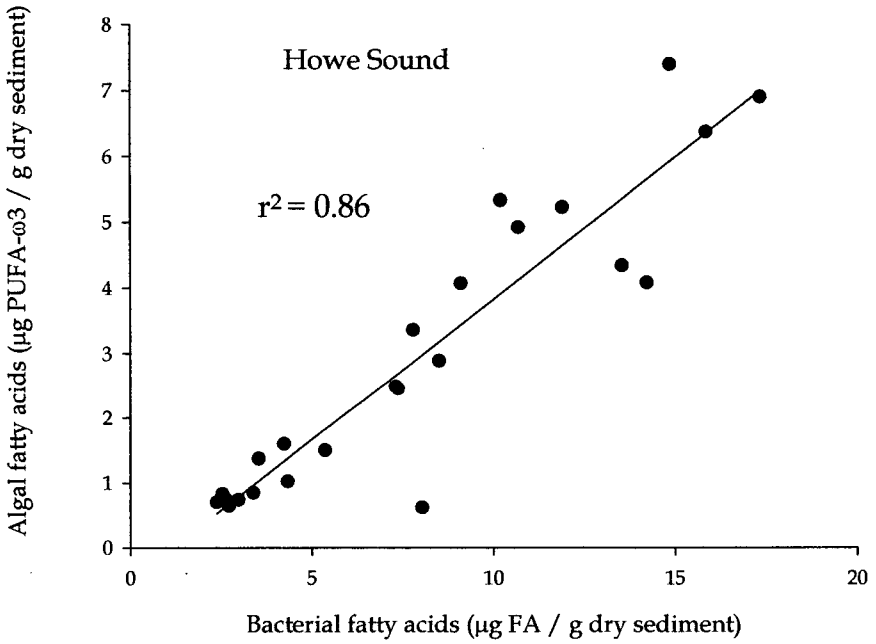

Figure 12c. Regression analysis between bacteria and algae in Howe Sound

An even stronger relationship emerges between algal and bacterial biomarkers when a suite of FA ratios is used to separate the algal group into diatoms and dinoflagellates (Figure 13 a-f). The value of the fatty acid ratios are used to assess the relative contribution of diatoms and dinoflagellates (Budge & Parrish 1998, Parrish 2000). The sum of bacteria and terrestrial fatty acid markers also provide quantitative values that can be used to indicate if the groups supply a significant source of OM (Budge 1998, Claustre 1990). The ratio and sum values that indicate a significant source of diatoms, dinoflagellates, bacteria and terrestrial organic matter are 1.6 (for the ratio Σ16:0/Σ18:0), 0.5, ~5 and 2.5 respectively (Budge 1998). These values have been verified in field experiments. The second diatom proxy (Σ16:1/16:0) does not have an assigned value to estimate abundance. The two proxies for diatoms are similar in Hotham Sound sediments but less so in Howe Sound and they de-couple with depth in Malaspina Inlet sediments.

As mentioned above, the bacterial FA profile is more similar to the diatom than dinoflagellate profile. For instance, in Malaspina Inlet sediments there is a spike in diatom and bacterial (proxy) markers at 3-5 cm (Figure 13 e&f). Dinoflagellate markers are low at these depths in Malaspina Inlet sediments. In the bottom part of the H sediments there is a

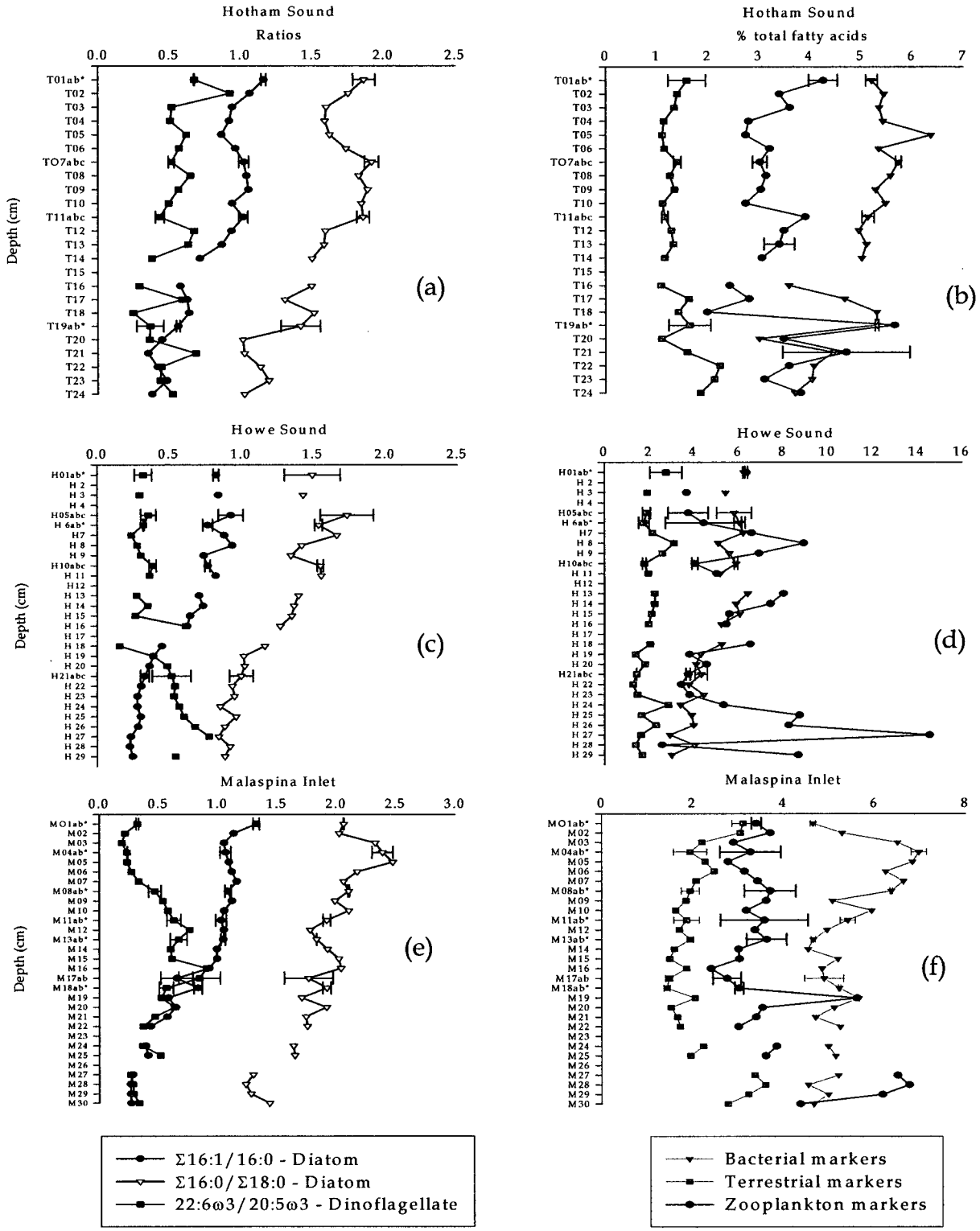

Figure 13(a-f).Comparison of ratios and percent abundance of FA biomarkers found downcore at the 3 study sites. Bacterial markers are the sum of iso-15:0, 15:0 and iso-17:0. Terrestrial markers include 18:2ω6cis + 18:3ω3 Zooplankton markers are the sum of 18:1ω9, and 20:1ω9c. Note: The sum of all 16:1 FAs include: 16:1ω11, 16:1ω7 and 16:1ω5. Note Howe Sound at 28 cm depth missing data point due to peak anomaly

higher proportion of markers for dinoflagellates but not for bacteria.

Other studies have shown a range in the sum of bacterial biomarkers from different locations including the water column (Bianchi 2002), particles collected from suspended traps (Derieux 1998) and marine sediments (Perry 1979). For example, Budge (1998) found 9.8 ± 1.8% bacterial FAs in offshore surficial sediments near Trinity Bay, Newfoundland, but only 2% on sinking particles. In this study (Figure 13.b,d & f), total bacterial FAs in sediments ranged from ~3 to 7% at the 3 sites. The highest values (≥6.2) were between 3 and 8 cm in Malaspina Inlet sediments, 5 cm in Hotham Sound sediments and 1, 7 and 13 cm in Howe Sound sediments.

The profiles suggest that in Hotham Sound there is a long-term balance of diatom and dinoflagellate source material. Hotham Sound has the greatest proportion of FAs associated with dinoflagellates although they never exceed 1.0% of the total. This is contrary to studies that found dinoflagellates made up only a small component of the total algal biomass in Hotham Sound during 1985 to 1987 (Sancetta 1989).

An interesting feature of the sediment profiles (Figure 13.) are the fluctuations in the signals from diatoms and dinoflagellates, which begin in the top few cm and continue to the bottom of the cores. The dinoflagellate ratio value of 0.5 indicates a significant source of this group. Hotham Sound appears to have a significant supply of dinoflagellates throughout the sediment core. Yet in Malaspina Inlet and Howe sound sediments there appears to be a shift in species composition. Dinoflagellate markers become more prominent with respect to diatom markers in the deeper sediments. As each cm represents years rather than seasons, these fluctuations are not the result of spring bloom of diatoms followed by nutrient depletion and dinoflagellates (Canuel 1993, Parrish 2000).

Some of these fluctuations may result from the physical characteristics of these sites. For example, strong stratification in Hotham Sound can prevent nutrient turnover that drives the fall diatom bloom (Sancetta 1989). This would cause a relative increase in the percent of total FAs from dinoflagellates in the sediments. In turn, large diatom blooms can mask dinoflagellate markers (Budge 2001). The high value of Malaspina Inlet diatom markers indicates an abundance of this group compared with Hotham and Howe Sound locations.

Terrestrial inputs were highest in H sediments at the surface, 8, 9 and 24 cm and even exceeded those in Malaspina Inlet sediments, which have a value greater than 2.5 only at surface (1&2 cm) and bottom (27-30 cm) samples. Hotham Sound appears to only have a significant source of terrestrial plants at 22cm depth. Howe Sound is the closest site to the Fraser River, which has flow rates ranging from ~ 1000 m^3s^{-1} in winter to ~9000 m^3s^{-1} in early summer (Morrison 2004). Rivers, especially ones with flow rates like the Fraser River, deposit large amounts of terrestrial organic matter into coastal marine systems. Howe Sound is the closest of the 3 sites to the Fraser River.

The zooplankton FA ratio profile is independent of the other profiles. The biomarkers 18:1ω9 and 20:1ω9 increased with depth at the three sites; however, the error also increased with depth. Also 20:1ω9c may have occasionally co-eluted with the 18:3ω3 fatty acid. A third biomarker used to indicate zooplankton, 22:1ω11, often did not elute from the GC column independently (column interactions), thus was not included in this study.

Each of the study sites had different FA profiles. The FA markers distinguished both the type and proportions of source organic material, and revealed pulses of inputs from diatoms and dinoflagellates. Although the zooplankton and bacteria groups could have been partitioned into finer taxonomic resolution, the focus of this investigation was whether FA biomarkers can be used as proxies for the presence and abundance of *Heterosigma akashiwo*.

3.3.3. Harmful Algae species analysis

Heterosigma akashiwo (Haka) from the family Raphidophyceae, as described in Chapter 2., forms harmful blooms that affect aquaculture in British Columbia (Taylor 1993) and Japanese coastal waters (Yamochi 1984).

There are a number of FAs used as proxies for phytoplankton (see Table 1. .in Chapter 1.). FA 20:5ω3 is a diatom marker that can be used as a proxy for many locally abundant *Thalassiosira* spp. including *T. decipiens, T. aestivalis and T. rotula.* Another fatty acid marker, 22:6ω3, is used as a general proxy for dinoflagellates and would occur in many locally abundant species including: *Goniodoma pseudogonyaulax, Protoceratium reticulatum, Ceratium fusus* and *Alexandrium catenella* (Haigh 1992). The MUFA 16:1ω7 is used as both a

diatom and bacterial marker as discussed above. The saturated FA 14:0 is a common mixed acid, such as 16:0, and is widely distributed in microalgae; including the Haka strains used in this study (see Chapter 2).

All 5 Haka strains and wild isolate, analysed for this study, had the unidentified acid (U20) in low abundance (average 1.27 ± 0.44% of total acids), which eluted near other unsaturated 16 carbon FAs and is assumed to be 16:1ω13. This fatty acid is also found, in centric diatom *Thalassiosira* spps. and Prasinophyte alga *Tetraselmis* spps. (Berge 1995, Rhodes 2003, Volkman 1988). The genus *Tertaselmis* is not present in local waters, according to local taxonomic studies, yet several *Thalassiosira* species are (Haigh 1992, Sancetta 1989, Taylor 1993). Thus although this acid appears to be specific to a few genus, it cannot be used, on its own, to indicate the presence or absence of *Heterosigma akashiwo*. *Thalassiosira* species contribute only trace amounts, certainly less than the reported values in Haka. Persistent contribution of this acid by Haka is possible. The long term residence of Haka is considered likely based on observations of viruses infecting Haka recovered from depths of up to 40 cm below the sediment-water interface in nearby inlets (Lawrence 2002).

In order to interrogate sediments for evidence of Haka blooms, the suite of 5 fatty acid markers described above (14:0, U20, 16:1ω7, 20:5ω3 and 22:6ω3), were selected based on three main datasets including: eight FA profiles of 4 different strains of Haka, a table of the FA profiles of marine organisms culled from past research, and taxonomic records of this region (Haigh 1992, Sancetta 1989, Taylor 2002). Multivariate analysis of these five key FA shows similar patterns of variation between Haka fatty acid profiles and sediment FAs (Figure 14.).

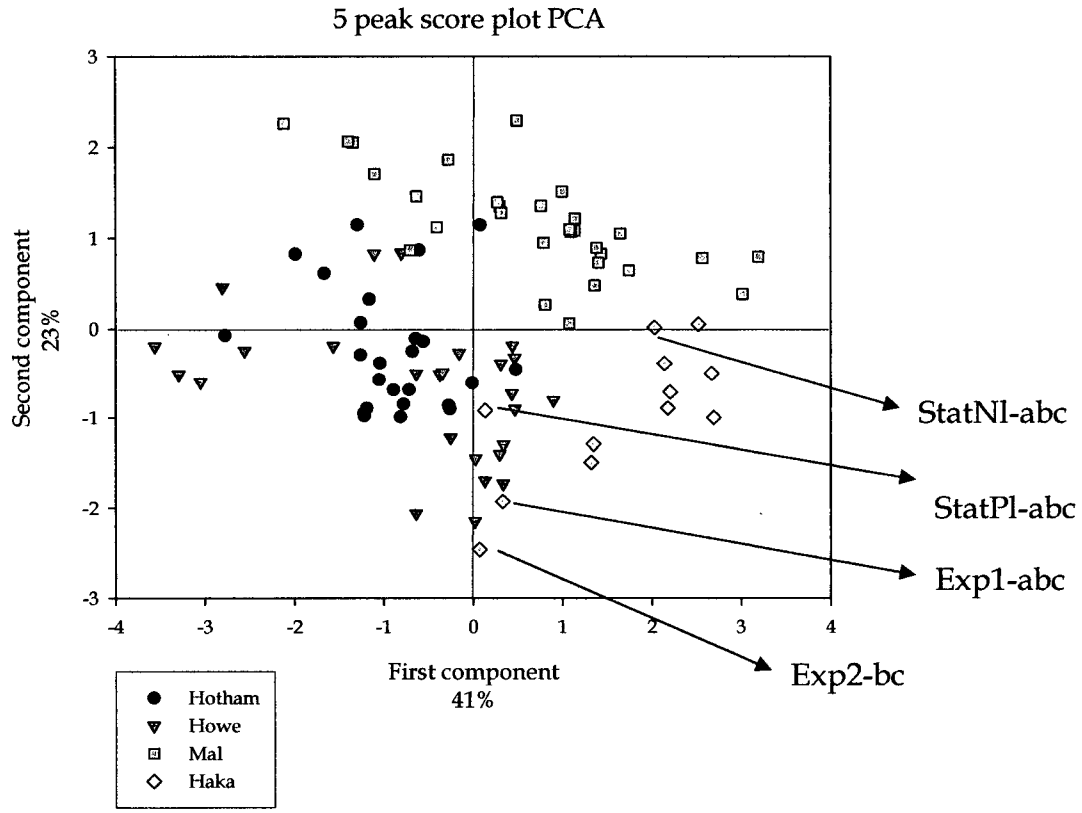

Figure 14. Principal Component Analysis used to contrast sediment samples with Haka strains and Haka nutrient limited cultures. 5 FAs - 14:0, u20, 20:5ω3, 22:6ω3, 16:1ω7 See Appendix 3.6.3 for sample identification

The multivariate analysis (Figure 14.) consistently placed certain Hotham and Howe Sound sediment samples close to the phosphate limited stationary (StatPl) Haka FA profiles, while Malaspina Inlet sediments were closer to the nitrogen limited stationary (StatNl) Haka FA profiles. The differences between P and N-limited Haka FA profiles are also discussed in Chapter 2.

Again, as in Figure 11a., the samples show a distinct depth gradient in the PCA plot (Figure 14.). However, this time the deeper sediment samples are farthest away from the Haka FA samples (see Appendix 3. for sample depths).

The Hotham and Malaspina sediment samples, that were analysed for Haka DNA (Figure 15.), were ranked according to their distance from the StatPl and StatNl Haka

positions on the PCA plot respectively. It was assumed that this distance reflected the relative abundance of Haka in the sample.

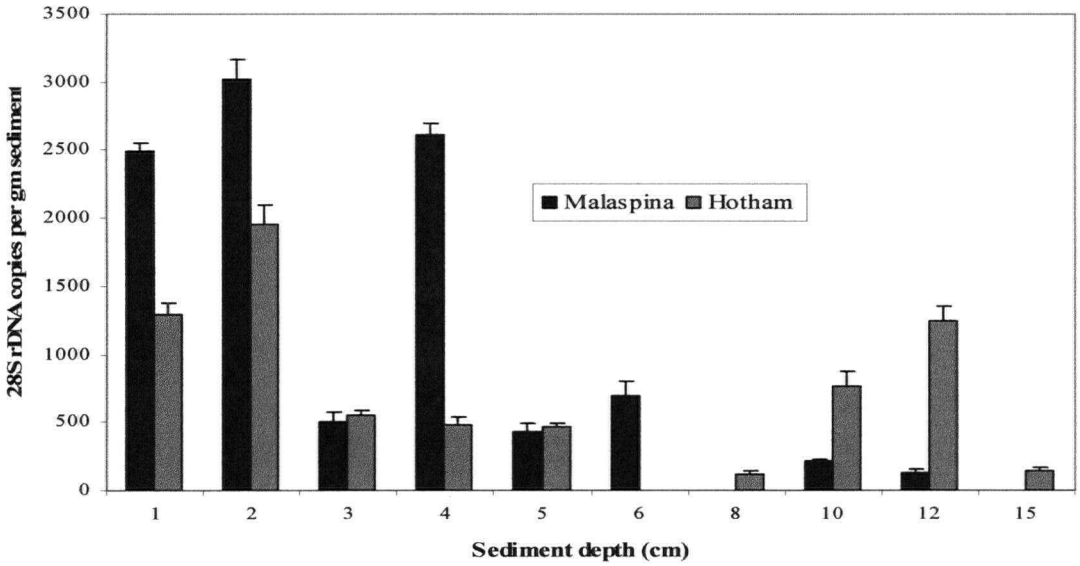

Figure 15. DNA (counts/g sediment) of *Heterosigma akashiwo* from the same Hotham Sound and Malaspina Inlet sediment samples. This graph was provided by Dr. A. Ahmad.

The rank value of abundance in sediments was compared to the empirical abundance of Haka DNA (counts/g sediment) to test for correlation (Figure 16.ab). The r^2 value for Hotham Sound and Malaspina Inlet was 0.46 and, when DNA counts were log10 transformed, 0.15 respectively.

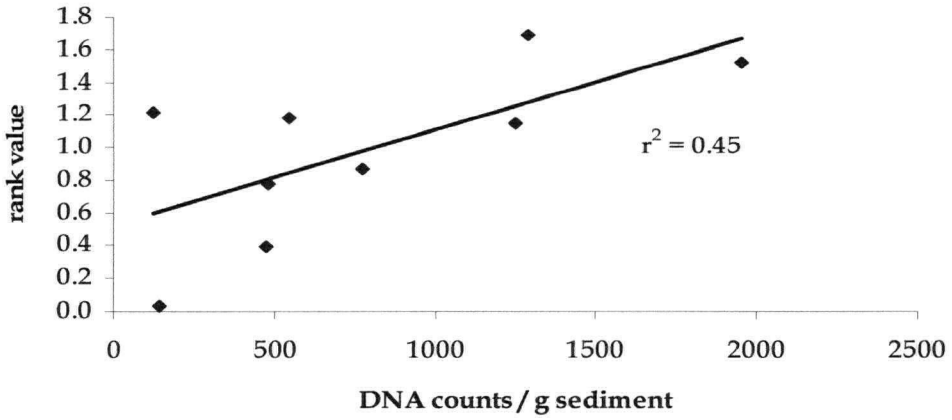

Figure 16a. Correlation between DNA counts and distance of Hotham Sound sediment samples on the multivariate analysis of sediments and Haka FA profiles

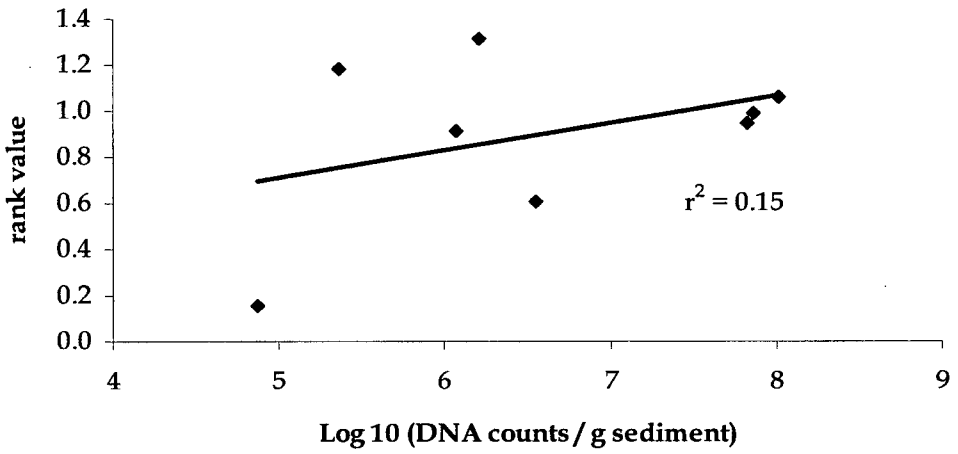

Figure 16b. Correlation between DNA counts and distance of Malaspina Inlet sediment samples on the multivariate analysis of sediments and Haka FA profiles

Searching for a FA pattern in the cores using a suite of Haka FAs, which also exist in other marine organisms, assumes that a Haka signal is large enough to overcome the input of these fatty acids from other sources. A similar assumption is made when the fatty acid 18:2ω6 is used as a biomarker for terrestrial organic matter. In one study the justification for combining these two fatty acids, as proxies for terrestrial plants, was based on the low values of δ^{13} C in samples collected in coastal waters and sediments off Newfoundland (Budge 2001). The researchers recognized the marine source for 18:2ω6, but felt justified combining the markers because of the known high input of allochthonous material in the area.

A second assumption is that FAs degrade at the same rate, thus a signal based on proportions will remain unaltered throughout the sediment core. Haka FAs can be preserved unaltered in sediments when encapsulated in a dormant and durable cysts (Imai 1993). A FA profile of Haka cysts was not generated for this study, thus an assessment of this possible contribution of Haka FAs in sediment is not possible. If a degradation correction value was known for each FA, it could be applied before multivariate analysis, and would increase the accuracy of the results.

3.3.4. Degradation

FA profiles (Figure 17.a-f) from T, H and Malaspina Inlet sediments were grouped together based on the number of double bonds in the carbon chain. The FAs are either saturated with hydrogen (SFA), have one (mono) double bond and are unsaturated (MUFA), or have more than one (poly) double bond (PUFA). Past research show diverse fatty acid degradation rates and possible preservation states in sediments, yet generally the labile PUFAs are expected to degrade first (Budge 2001, Eglinton 1979, Canuel 1996, Ganeshram 1999, Sun 2002).

Comparing the concentration (μg FA/ g sediment dw) and percent total FAs (Figure 17.a-f), yields different, but complimentary stories. Although the concentration of FAs declines with depth in all sediment cores, the percent of SFA significantly increases at all three sites compared to PUFAs, which remain relatively constant downcore in Hotham Sound, and MUFAs, which show an erratic but significant decline (see Table 5. for regression tests).

	Hotham RA	Trend	Howe RA	Trend	Mal RA	Trend		Hotham μg/gdw	Howe μg/gdw	Mal μg/gdw	Trend
SFA	0.002*	↑	0.003	↑	0.000*	↑		0.000	0.000	0.000*	↓
MUFA	0.000	↓	0.002	↓	0.000*	↓		0.000	0.001	0.000*	↓
PUFA	*0.228*	–	0.000	↓	0.000*	↓		0.000	0.000	0.000*	↓
Uknown	*0.838*	–	*0.118*	–	0.001*	↑		0.001	0.001*	0.000*	↓

P value in italics indicates accepted null hypothesis (no significant difference downcore)
* when normality not met data log transformed
Ho = there is no difference in fatty acid concentration with depth

↓ Decreasing concentration
↑ Increasing
– no change

Table 5. P values from regression test of downcore changes in FA groups based on the number of double bonds (hydrogen de-saturation)

The increase in the SFA percent total FA downcore compared with percent MUFA, is consistent with results from past research (Farrington 1977, Rhead 1971). Using isotopic labels to track the fate of 18:1 in sediments, Rhead et al. (1971) found that degradation and re-synthesis through acetyl-CoA hydrogenated and shortened the carbon chains from 18:1 to 16:0. His results showed that a small amount of the MUFA 18:1 is converted into SFAs.

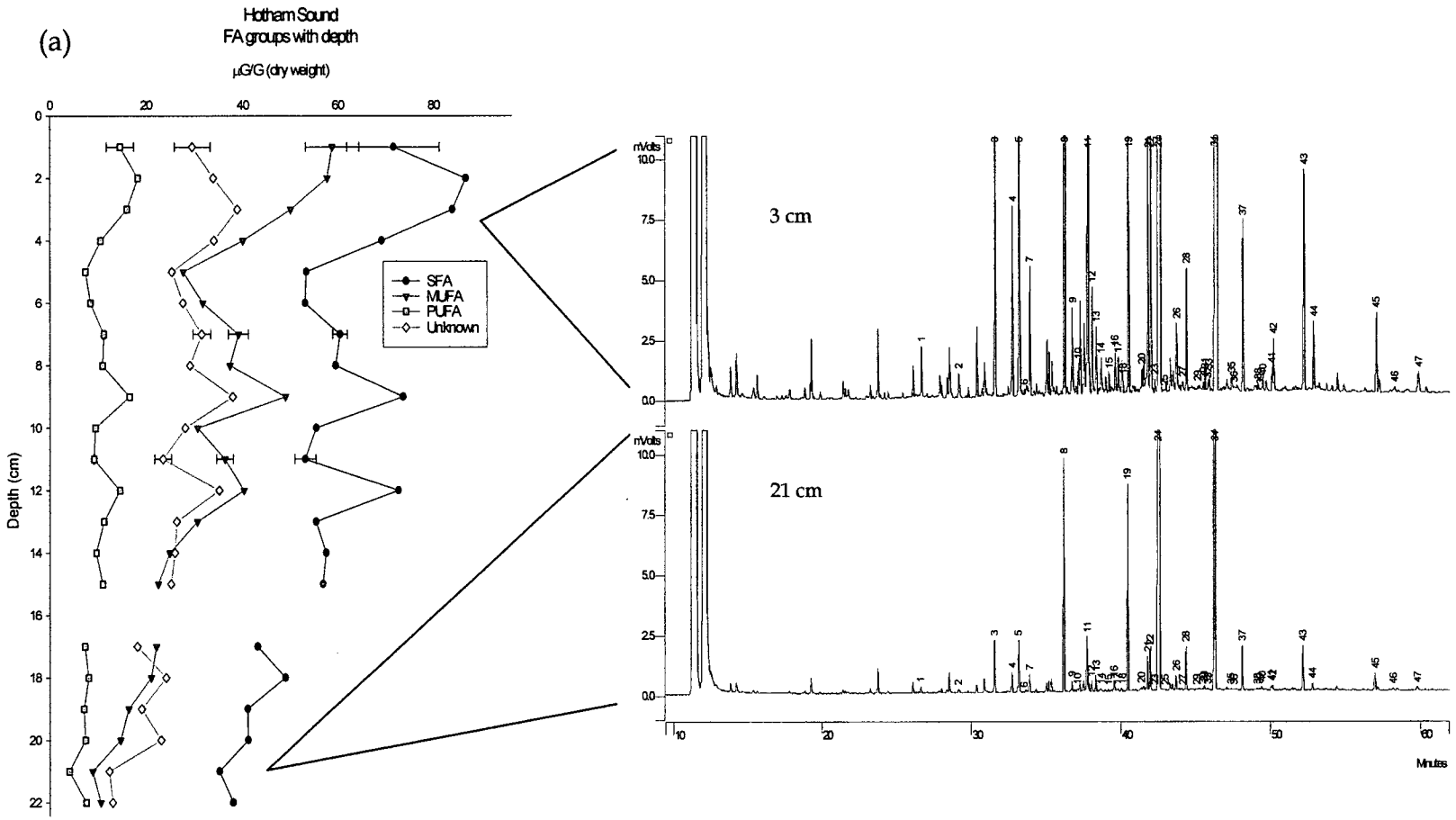

Figure 17a. Fatty acid hydrogen de-saturation groups with sample chromatograms. Standard deviation was calculated from replicate samples, n=2 at 1 & 9 cm and n=3 at 7 & 11 cm for Hotham Sound. Chromatograms (3 cm & 21 cm) produced from GC/FID are shown to the right of the graph. SFA saturated fatty acids; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids. Relative abundance (percent total of all fatty acids peaks integrated from each chromatogram) is compared with concentration (µg FA/g dry weight).

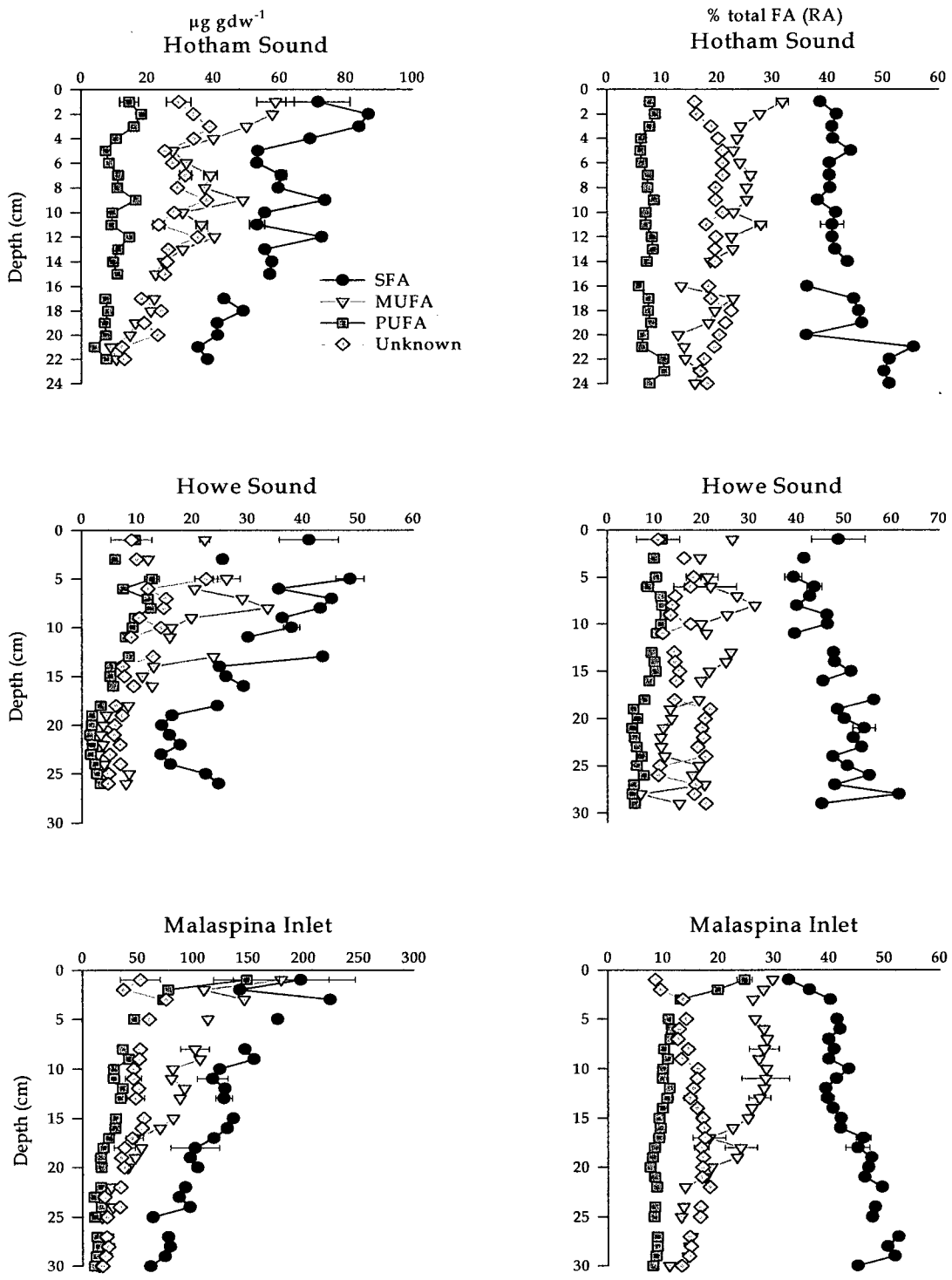

Figure 17(a-f). Fatty acid hydrogen de-saturation groups. Note percent total fatty acid and µgFA/g dry weight sediment values have different replicate and omitted samples.

The reduction in concentration of FAs with sediment depth is highly variable due to chemical structure, redox conditions, source material, physical characteristics of the sediment and dilution by other sedimentary constituents (Canuel 1996, Francois pers. com., Hoefs 2002). For example, Hotham sound sediments are oxic to at least 5 cm depth (Calvert 1993), and Malaspina Inlet has a well oxygenated (9-12 mg/L) water column (Freyman 2004). In contrast, Howe Sound bottom water, since the 1980's, can be anoxic (Appendix 2.), which would result in less degradation of FAs, especially PUFAs (Sun 1999). Yet it is the Hotham Sound sediments, not Howe Sound, that show no significant decline in PUFA relative abundance downcore.

Hindcasting harmful algal blooms, using fatty acid biomarkers extracted from sediments depends on the pattern of abundance of a suite of key FAs. As different FAs degrade at different rates, this can affect the interpretation of FA signatures in sediments. Calculating degradation rates requires accurate estimates of FA concentrations and sediment age (Lewis 1993). If the degradation rates of the different FAs are known, they can be used to infer the FA composition of the original source material in deeper sediment samples.

3.4. Conclusion

This study presented various combinations of key fatty acid patterns that highlight potential differences in the three inlets. The main differences are:

- Malaspina Inlet sediments have the greatest concentration of FA compared with Hotham and Howe Sound.
- Dinoflagellates are not as significant a portion of the OM in recent sediments in Malaspina Inlet and Howe Sound compared with past elevated values, whereas Hotham Sound appears to have a constant fluctuating supply of both dinoflagellates and diatoms.
- Hotham Sound has an unusually high level of poly-unsaturated fatty acids (PUFA) at depth in sediments. Mono-unsaturated fatty acids (MUFA), contrary to the expectation that multiple bond FAs degrade first, show a greater loss in concentration.
- The phosphate limited *H. akashiwo* FA signal had the closest association with sediment FA in Hotham and Howe Sound. The fatty acids from Malaspina Inlet sediments have a closer association with nitrogen limited *H. akashiwo* profiles.
- Stationary growth phase *H. akashiwo* FA profiles have a closer association with sediment *H. akashiwo* FAs than exponential growth phase *H. akashiwo* FA profiles.
- The abundance of *H. akashiwo* DNA, extracted from the same 2 sediment cores, had a better correlation with the *H. akashiwo* signature found in Hotham Sound sediments than to Malaspina Inlet
- Changes in fatty acid concentrations downcore are variable due to their chemical structure (including number of double bonds) and sediment conditions (location of burial).

This study shows that unsaturated algal fatty acid biomarkers, considered highly labile, persist at depth in marine sediments. Important traces of algal blooms are preserved in sediments. The power of fatty acid biomarkers to hindcast harmful algal bloom events would increase if the concentrations were corrected for degradative loss in downcore sediment samples. Combining other key biomarkers such as DNA, isotopes, pigments and

other lipid classes including sterols into the analysis would help to identify species when taxonomic records are not available.

Interpreting the biochemical clues, preserved in sediments, can help describe long term changes in coastal marine ecosystems and ultimately aid in understanding the key triggers for harmful algal bloom formation and collapse.

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Chapter 4. Overall Conclusion

This study investigates whether fatty acids are good biomarkers for *Heterosigma akashiwo* (Haka), a harmful species of marine algae common in B.C. coastal waters. At present the only method used to identify this species in sediment is by counting the cysts, formed during the dormant stage of Haka's life cycle. These cysts are microscopic and non-descript in shape and colour. Can fatty acids replace the arduous task of counting cysts?

Fatty acids are a common component in all life forms but the abundance varies. Results from Chapter 2. show that fatty acid abundance varies not only by species but also by growth conditions. Past studies have also shown that nutrient limitation directly impacts fatty acid production. The results indicate that P-limitation is more likely than N-limitation to lead to cell death for Haka based on the idea that their buoyancy could be altered by the amount of saturated fatty acids produced. These hypotheses are based on past studies that have described the biological and chemical components of this species including details about how this motile algae stops migrating and decrease their production of membrane fatty acids before death.

Future research could verify if there are predictable changes in fatty acid production due to the migration patterns of this species. If fatty acids are a vital part of migration then the P-limited profiles presented in this study could be the death profile of this species. This would explain why it is found in Chapter 3. that the P-limited Haka profile, when using multivariate analysis, associates with sediment fatty acid profiles more closely than all the other profiles generated for this study. It would be interesting to include Haka fatty acid profiles for cysts and viral infected cells in a future study.

This study is the first report on fatty acid concentrations in three B.C. Inlets near the Strait of Georgia. The Haka fatty acid profiles all included 5 key fatty acids that were used as probes to search, using multivariate analysis, for a similar proportion of the same fatty acids in marine sediments.

The results were tested by comparing the DNA of Haka extracted from the same sediment samples. The DNA traces, contained in the dormant cysts, are not found in deeper sediment sections nor do they match the fatty acid results in the surface sediments. Both

DNA and fatty acids degrade over time. The degradation of fatty acids is variable based on number of double bonds, length of carbon chain and type of material they are preserved in. This variability in degradation changes the original fatty acid proportions that were laid down in surface sediments. A unique profile, based on fixed proportions of key fatty acids, will be lost as degradation increases.

This study shows that the deeper sediment samples are removed from the Haka fatty acid profiles depicted in multivariate analysis PCA plots. It is suggested that future studies, with dated sediment cores, could apply a degradative correction value to the fatty acid proportion profiles in order to improve the ability to trace the historical bloom events of Haka and other marine species.

Appendices

Appendix 1. Graphs of percent standard deviation

The following graphs of error values examine the effects of long term storage (Figure 1. & 2.) and the effects of different laboratory techniques (Figure 3. & 4.). Each data point represents the standard deviation of fatty acid abundance between replicate samples.

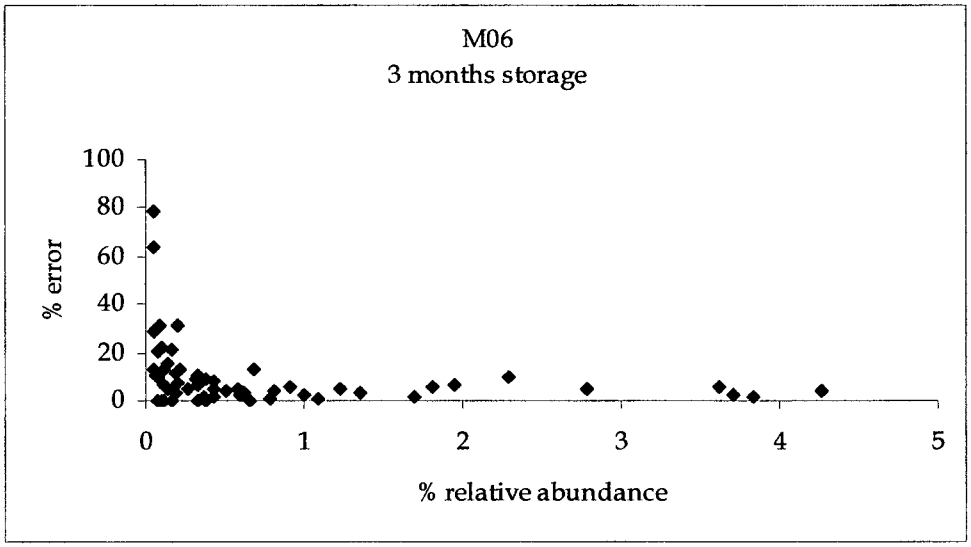


Figure 1. Extracted sample stored in -70°C freezer with nitrogen added (n=2)

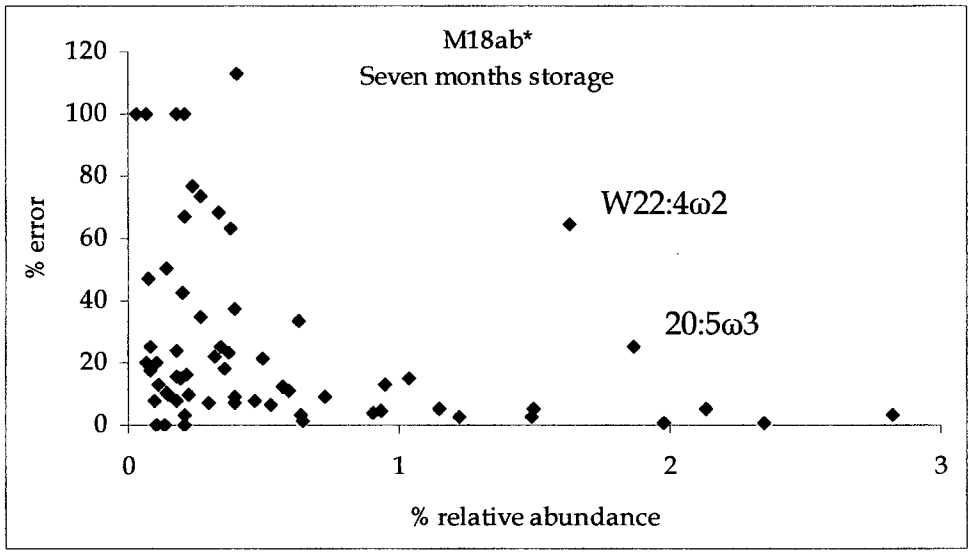


Figure 2. Sediment sample re-extracted after 7 months (n=2)

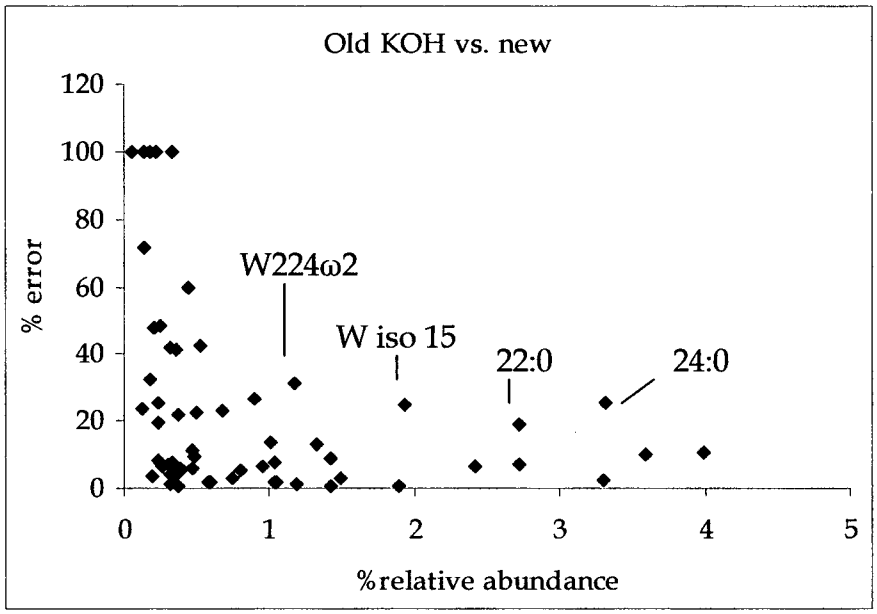


Figure 3. The KOH, used for saponification, was replenished during the study and the new stock was compared to see if there was a difference in fatty acid concentration (n=3).

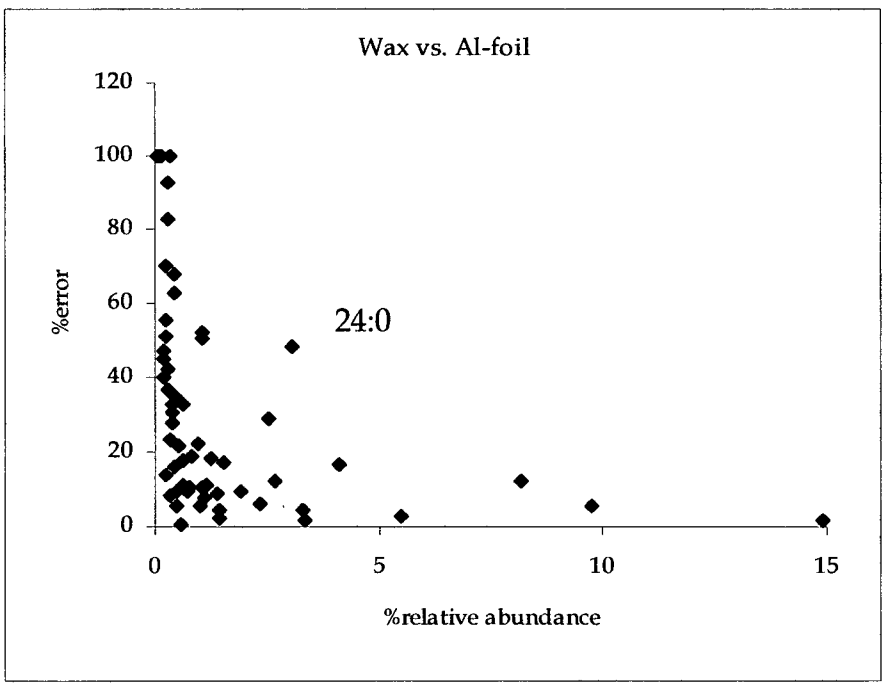


Figure 4. At the beginning of the study the sediment was weighed using wax paper. This was changed to aluminium foil to reduce the risk of any contamination (n=2).

Extraction blanks, with no sample added, were also conducted (Figure 5.). Peak artefacts (16:0 and 18:0) concentrations, present in the blank runs, were considered insignificant.

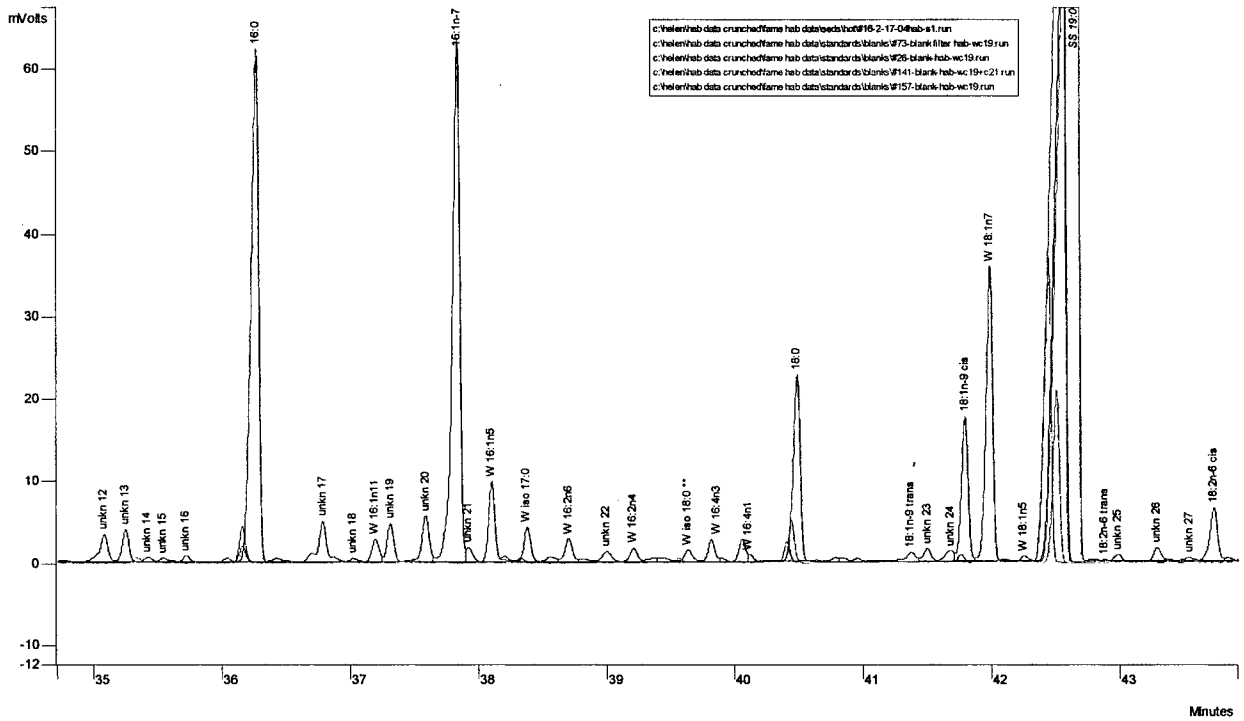
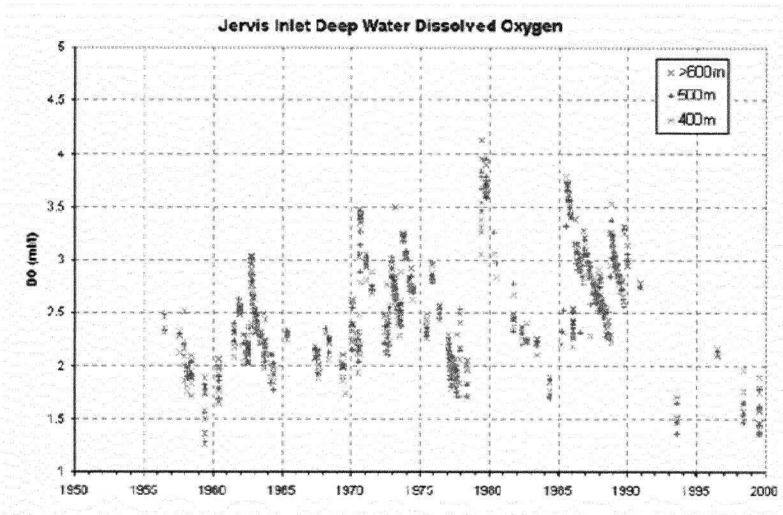


Figure 5. Chromatograms with all blanks and one sediment sample (black). The largest peaks, not including surrogate standard C19, are 16:0 and 18:0.

Appendix 2. Dissolved oxygen in Hotham Sound and Howe Sound



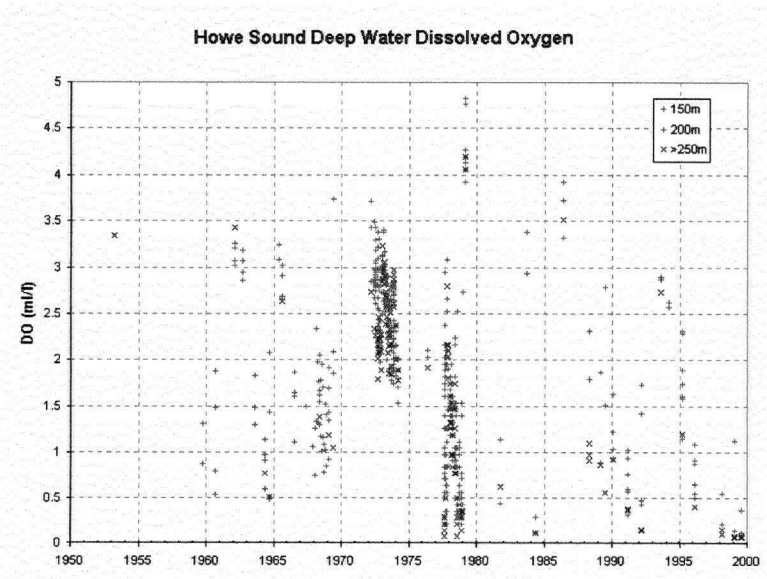


Figure 6(ab). Dissolved oxygen in a) Jervis Inlet and b) Howe Sound. Graphs available at http://www.pac.dfo-mpo.gc.ca/sci/osap/projects/bcinlets/default_e.htm

Appendix 3. Comparing *Heterosigma akashiwo* with sediment fatty acid profiles

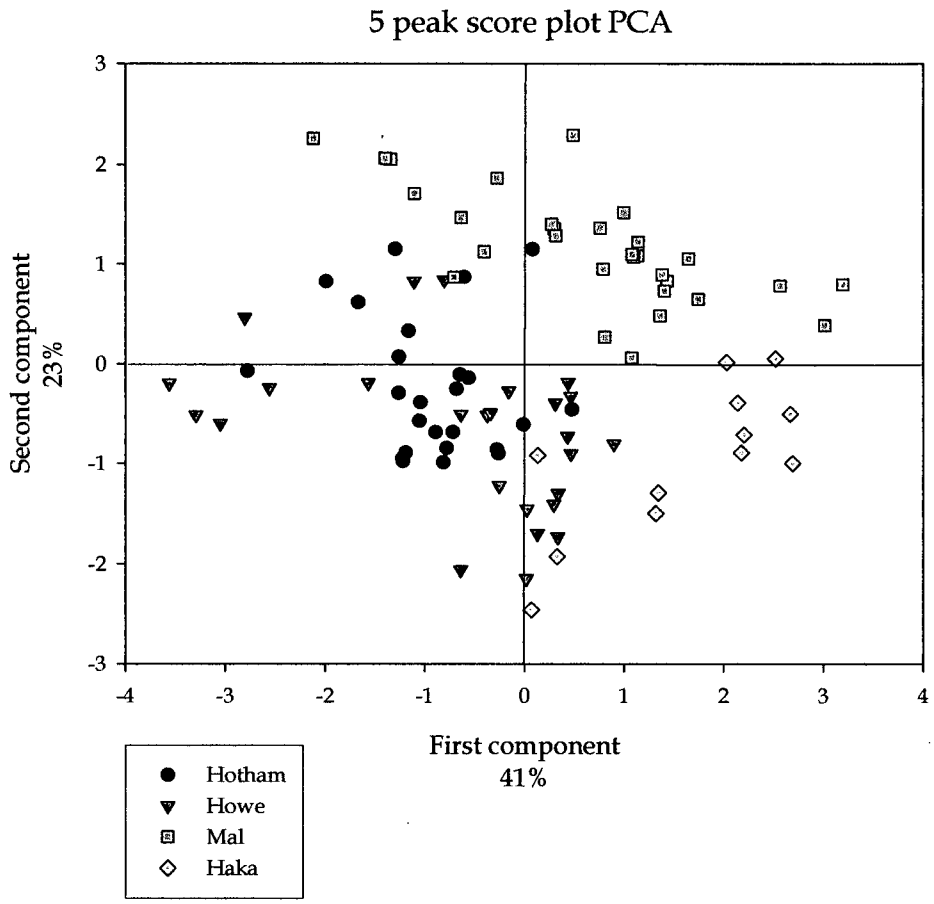
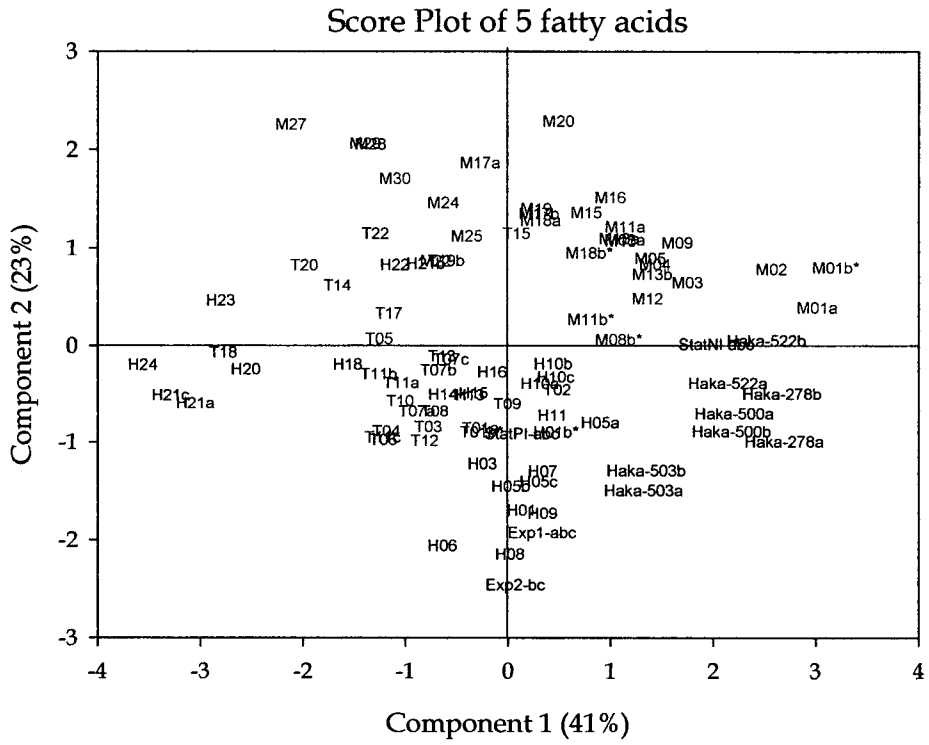


Figure 7a. Principal Component Analysis: 5 fatty acids - 14:0, U20, 20:5n-3, 22:6n-3, 16:1n7
Cumulative components: 1 (41%) 2 (64.3%) 3 (81.9%).



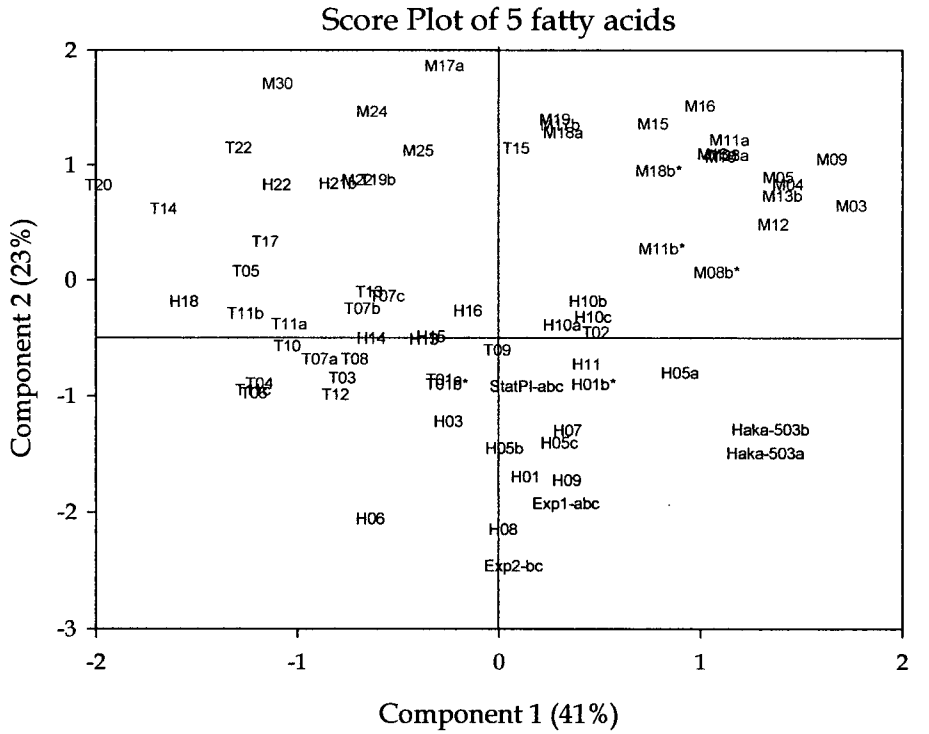


Figure 7(bc). b) Identification of sediment samples from Figure 7a. c) X-axis magnified

Appendix 4. Ranking of sample distance from Haka for DNA comparison.

The distance formula was used to calculate distance of sediment samples from Haka fatty acid profiles on the PCA plot (Table 1). The distance was compared with DNA (counts/g) of Haka concentration data to test for correlation.

Distance formula	d=sqrt((x2-x1)^2 + (y2-y1)^2)			
5 peak test - U20, 16:1n-7, 20:5n-3, 22:5n-3, 22:6n-3				
Sediment location and depth (cm)	Score plot values		Distance from =	
Labels_1	**x**	**y**	**StatPlabc**	**StatNlabc**
T01a	-0.27226	-0.86073	0.4117	
T02	0.47899	-0.4534	0.5783	
T03	-0.7785	-0.84566	0.9168	
T04	-1.18893	-0.89188	1.3246	
T05	-1.25721	0.06973	1.7076	
T06	-1.21645	-0.97858	1.3531	
T08	-0.71602	-0.68242	0.8835	
T10	-1.05115	-0.56989	1.2367	
T12	-0.81196	-0.99032	0.9500	
T15	0.08333	1.14461	2.0638	
M01a	3.01477	0.38586		1.0519
M02	2.5666	0.7826		0.9357
M03	1.74682	0.64489		0.6897
M04	1.43409	0.82776		1.0070
M05	1.38446	0.89294		1.0889
M08a	1.13861	1.08288		1.3902
M10	1.09718	1.06839		1.4063
M12	1.36029	0.47985		0.8146
M15	0.76365	1.35575		1.8435

Table 1. Sediment sample distance on PCA plot from Haka Cultures (StatPl and StatNl).