Using Sterol Biomarkers in Paleoecological Studies

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ABSTRACT

Using Sterol Biomarkers in Paleoecological Studies

Maggy Mouradian

Sediments from five contrasting stations in the western Mexican margin (Pacific Ocean) were investigated for sterol distribution and organic-walled dinoflagellate cysts (dinocysts). Sub-samples were taken from depths of 3-6cm and 16-27cm, representing different environmental conditions. In general, the sterol concentrations examined from these sites showed more advanced degradation in the deeper sediments compared to samples near the sediment-water interface. Using a molecular-level gas chromatography (GC) approach, 23 sterols of marine and terrigenous sources were identified in the natural samples. The major sterol components detected in most of these sediments were cholest-5-en-3β-ol (cholesterol), 24-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol or dihydrobrassicasterol) and 24-Ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol). This method also enabled the identification of a specific biomarker of a eukaryotic organism, 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol), indicating dinoflagellate inputs to marine sedimentary organic matter. The sterol content in the sediments (GC method) was positively and linearly correlated with the dinocyst counts measured for each sample by optical microscopy. It thus appears that the molecular GC approach used in this project constitutes a less tedious and more widely available alternative to the lengthy microscopy technique, at least when detailed information about the dinocyst assemblages is not required.

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Abbreviations

-BP: before present; i.e. before 1950

-BSTFA: N,O bis[trimethylsilyl]trifluoroacetamide

-"dino"sterols: dinosterol + dinostanol

-ESI: electrospray ionization

-FID: flame ionization detector

-GC: gas chromatography

-HPLC: high performance liquid chromatography

-LOD: limit of detection

-MS: mass spectrometry

-m/z: mass to charge ratio

-NIST: National Institute of Standards and Technology

-OC: organic carbon

-R^2: correlation coefficient

-RRT: relative retention time

-RSD: relative standard deviation

-SAM: S-adenosylmethionine

-SIM: single ion monitoring

-Std mix: standard sterol mixture

-TMS: trimethylsilyl

1 Introduction

Reflecting upon the times of our ancestors, it is obvious that through science life has greatly evolved. However, the basic scientific concepts that our progenitors applied in their everyday lives, unaware of the fact that they were doing so, should not be left unrecognized. Over centuries, science has developed considerably, nevertheless based on premature scientific ideas. The use of geochemistry also goes as far back as our ancestors. However, the term geochemistry as we know it today is a fairly new concept. In the world of science, geology only began to be accepted as its own field in the late 18th century.[1-2] The idea of inclusion of chemistry to this "new" discipline was born out of mainly economical and environmental concerns. Nowadays, geochemistry is a broad discipline with several areas of specialization and has ramifications for various complementary fields such as, for example, paleontology.

Adopting two different perspectives, such as geochemistry and paleontology, to study an important environmental question can lead to a synergistic advance of scientific knowledge. Accordingly, multidisciplinary studies have been quickly expanding throughout all fields of science. With the knowledge and expertise of different scientific backgrounds, this approach enables the application of two individual techniques to a specific case analysis. In this research, a paleoecological problem was tackled with a chemical perspective: a molecular approach commonly used in Chemistry was compared to an optical microscopy method exploited by paleontologists. The main focus of this project was the quantitative comparison of these two techniques, which both estimate the contribution of a class of seawater planktons, dinoflagellates, to organic matter in marine sediments. While cysts produced by dinoflagellates are individually counted in the

microscopy approach, the chemical approach involves the measurement of lipid biomarkers specific to dinoflagellates, the dinosterols.

1.1 Lipids in the environment

Over the years, many organic compounds have been identified in marine sediments. A major source of lipids in marine environments has been attributed to algae. Research has shown a wide variety of lipids that have been isolated from these organisms (see for example references 3-8; Figure 1-1). Some of these lipids are considered important biomarkers that are specific for different organisms or reaction pathways. They can thus be used for estimating the contribution of specific algal species to total sedimentary organic carbon.[9]

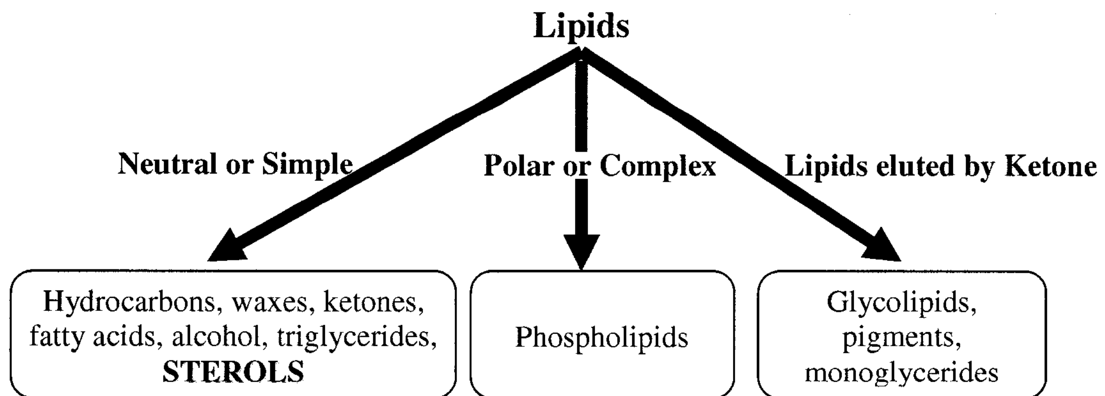


Figure 1-1: Overview of the lipid classes.

Lipids are carbon rich compounds that are important biochemical components of living organisms.[10] They are extractable with organic solvents and are usually identified by means of chromatography.[11]

In marine ecosystem studies, neutral and polar lipids are the two major classes that are of particular interest (Figure 1-1). Within these two groups, different compounds

can be used as specific biomarkers for given families or even organisms.[10] In this research, a series of sterols were used as indicators of the eukaryotic dinoflagellate community.

1.1.1 Sterols

Analyzing the sterol composition and concentration in sediments containing lipids from various organisms can be a challenging task. The relative contribution of the different sterols varies considerably between organisms and even amongst the same genus.[12] In addition, the *in situ* oxidation of sedimentary organic matter can strongly modify the distribution and relative proportions of the different sterols produced by marine organisms.

Sterols are found in all animal and vegetal tissues. They are non-saponifiable lipids that are predominantly unsaturated alcohols of the steroid group and have a 3β-hydroxy moiety and an aliphatic side chain of eight or more carbon atoms at position 17 (Figure 1-2) [13].

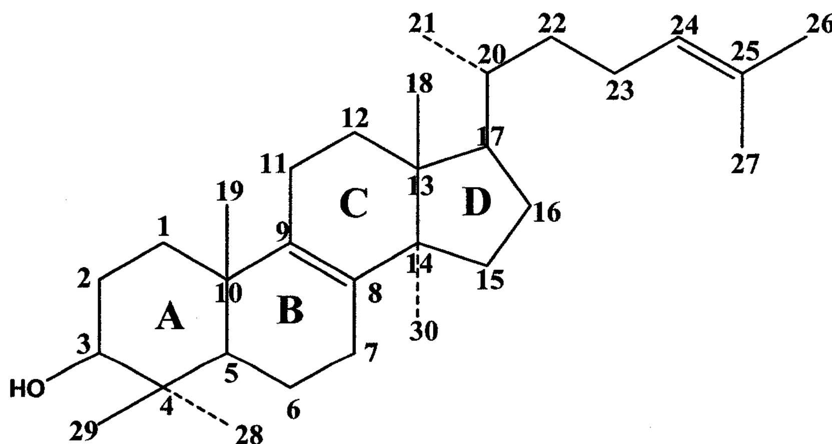


Figure 1-2: Numbering of the carbon atoms in a sterol molecule (lanosterol).

The main sterol found in the tissues of vertebrates is the well known cholesterol. This is probably why cholesterol was the first sterol to be isolated from gall stones (by Poulletier de La Salle in 1770). In 1815, it was named cholesterine by M.E. Chevreul who achieved the isolation of this component from animal fats. The correct formula ($C_{27}H_{46}O$) was only proposed in 1888 by F. Reinitzer. Most importantly, structural studies were carried out from 1900 to 1932. The two main leaders in this effort were H.O. Wieland "on the constitution of the bile acids and related substances"[14] and A.O.R. Windaus on "the constitution of sterols and their connection with the vitamins".[14-15] The two were merited Chemistry Nobel Prizes in 1927 and 1928, respectively. Through these investigations, an accurate steric representation of cholesterol was obtained. History shows that it took over a century of hard work to achieve a structural conformation of cholesterol.[16]

The main sterol found in higher plants is stigmasterol. Originally, it was termed phytosterine by O. Hesse who isolated this substance from Calabar beans in 1878. Stigmasterol was only accepted as the proper name for this sterol in 1906 by A.O.R. Windaus and J.C. Hauth after the plant genus (*Phytostigma venenosum*). Since 1897, all sterols of vegetal origin are called phytosterol, as proposed by H. Thoms.[17]

Although certain sterols can be synthesized only by specific organisms, all these lipids are derived through the cyclization of squalene (triterpene). Squalene, a C_{30} hydrocarbon, is made up of six isoprene units (C_5). The first step in the synthesis of squalene is the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate (Figure 1-3).[13]

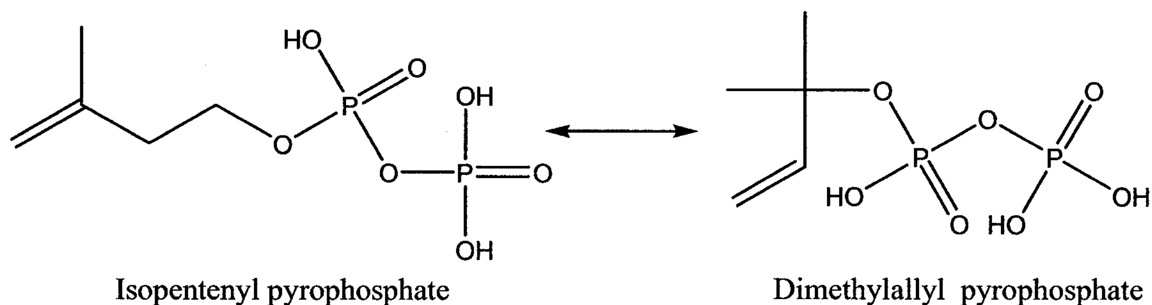


Figure 1-3: Two isomers - isopentenyl pyrophosphate and dimethylallyl pyrophosphate.

The reaction proceeds with the condensation of these isomeric C_5 molecules, first into geranyl pyrophosphate (C_{10}) and next into farnesyl pyrophosphate (C_{15}). These condensations through the attack of the allylic carbonium ion are in a head-to-tail manner. The last step involved in the synthesis of squalene is the joining of two C_{15} molecules via tail-to-tail condensation (Figure 1-4).[13]

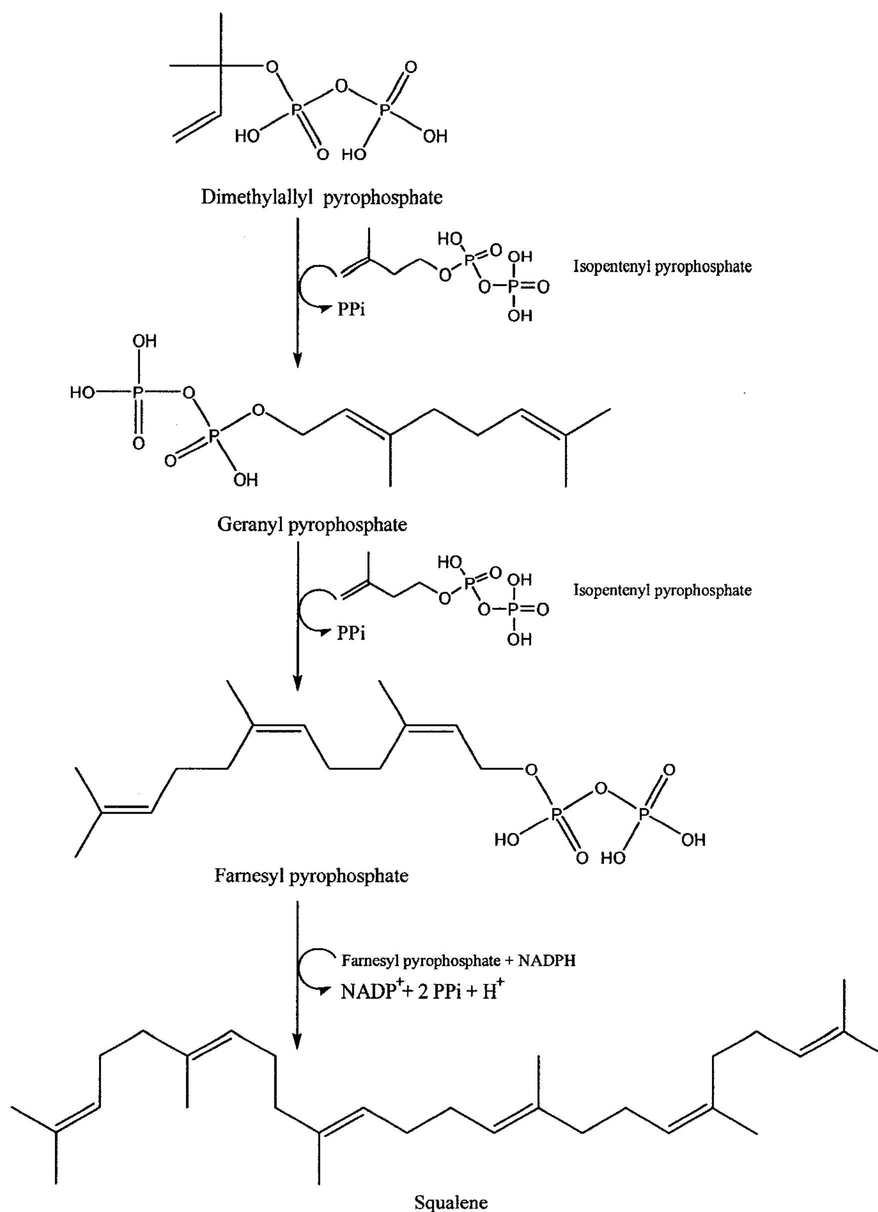


Figure 1-4: Synthesis of squalene.

Finally, squalene undergoes cyclization to yield triterpenoids found in bacteria and eukarya. However, the biosynthetic pathways are quite different from one another since bacteria produce hopanoids (triterpenoids biosynthesized anaerobically, where substitution of the side chain occurs through hydroxyl and amino groups), whereas eukaryotic organisms synthesize sterols (substitution of the side chain is by methyl and

ethyl groups). Pathways for bacterial hopanoid and eukaryotic sterol biosynthesis are shown in Figure 1-5.[18]

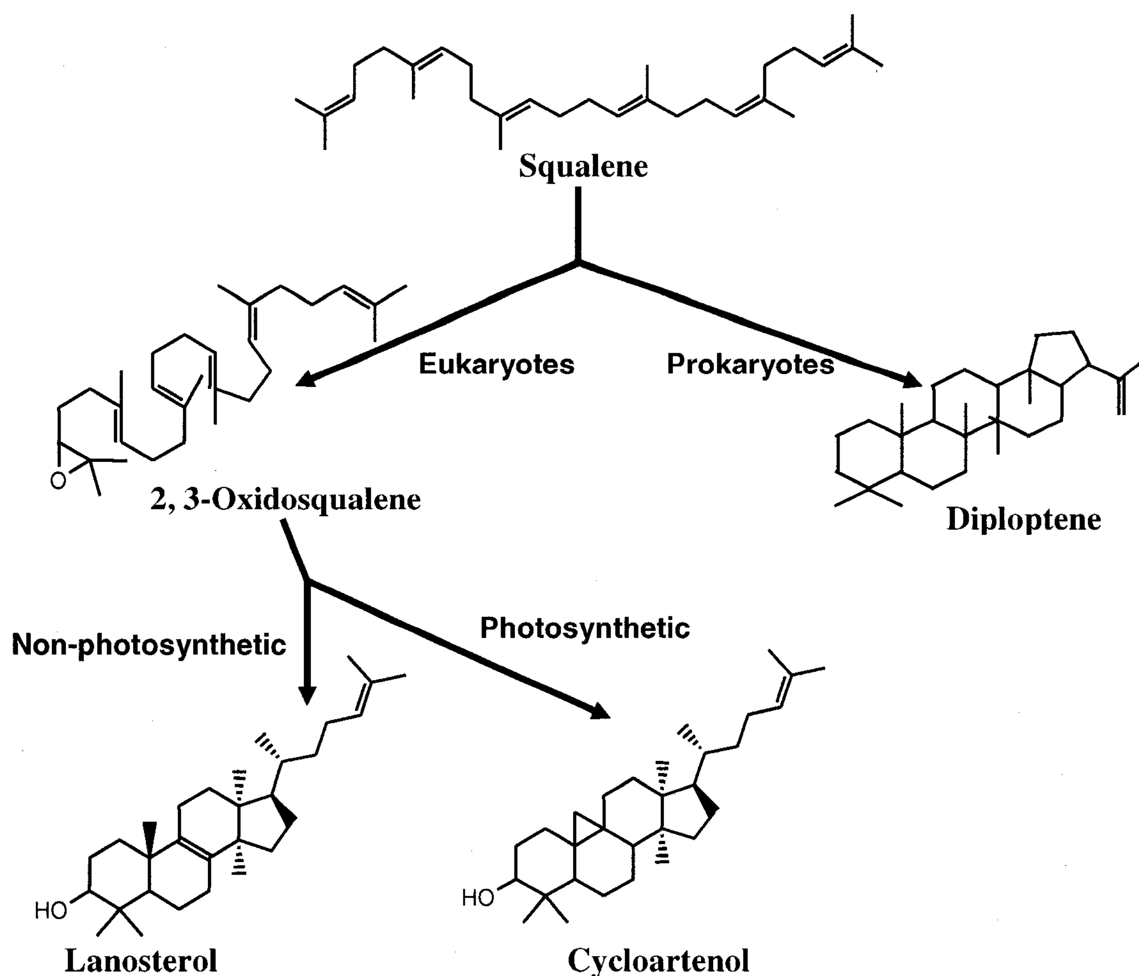
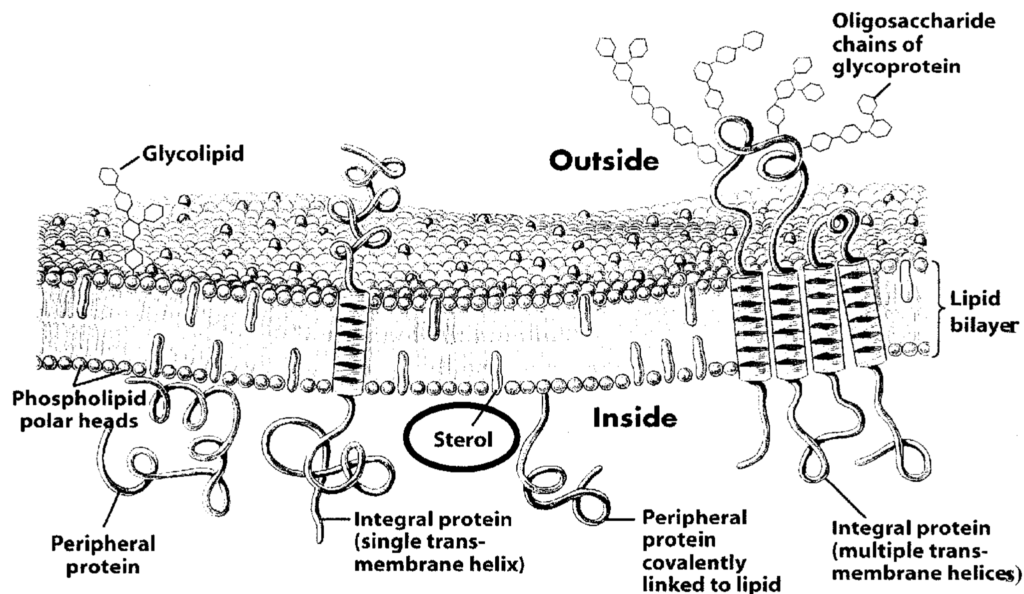


Figure 1-5: Pathways for bacterial hopanoid and eukaryotic sterol biosynthesis.

Sterols are vital constituents of the membranes of all eukaryotic organisms. Consequently, they have many important functions within the cells depending on the organisms synthesizing these molecules. One of the key roles of sterols is the regulation of membrane fluidity and permeability by condensing the lipid bilayer.[19-20] The hydroxyl group at one end of the sterol binds to a carbonyl oxygen atom of a phospholipid head group through hydrogen bonding, while the flexible hydrocarbon tail at the other

extremity of the sterol is inserted in the hydrophobic core of the bilayer. In this fashion, the sterol molecule prevents the crystallization of fatty acyl chains within the bilayer, preventing phase transition and keeping it in a fluid state. On the contrary, a bulky sterol molecule may sterically hinder the movement of fatty acyl chains leading to a decrease in membrane fluidity, thus rigidifying the bilayer (Figure 1-6).[13]



Source: http://faculty.uca.edu/~march/bio1/scimethod/8.5_The_detailed_structure_o.JPG

Figure 1-6: Integration of a sterol molecule within the membrane lipidic bilayer.

Besides their fundamental role of controlling the membrane fluidity, sterols also have an important purpose in the regulation of various systems. For example, cholesterol is involved in the embryonic development of vertebrates. Also, the interaction of cholesterol with caveolin induces the formation of microdomains. In short, these processes may trigger signalling pathways for many biological reactions.[19]

Furthermore, sterols are also precursors of hormones. For instance, brassinosteroids are important hormones involved in the regulation of higher plant

growth.[19] More familiar hormones would be androgens (such as testosterone) and estrogens, which are the male and female sex hormones, respectively. They are both derived from cholesterol.[13]

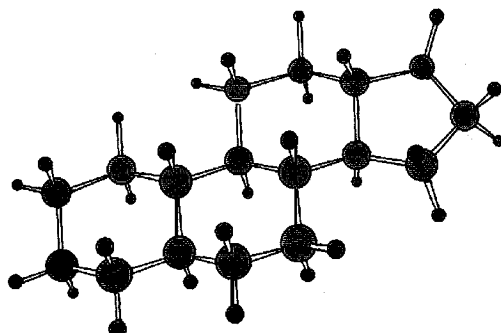
1.2 Sterols in the environment

Along with the biological roles that sterols undertake, they also have important uses in the environment. Many lipid compounds found within sediments have been used to trace past paleoecological changes in the environment. Amongst the wide variety of these organic compounds, several biomarker lipids are used as source indicators of organic matter inputs in sediments.[21] By combining these biomarkers to relevant paleontological data, it is possible to obtain a more complete understanding of environmental conditions prevailing in ancient ecosystems.

In order for a biomarker to be useful in paleontological and paleoecological studies, it should either remain unaltered, or retain important structural characteristics in their stable altered form, such that the precursor molecule can be identified. Due to their stability and biosynthetic specificity with regards to various organisms, lipid tracers constitute informative indicators of organic matter inputs in sediments.[22] With the improvement of analytical techniques, the number of compounds identified as biomarkers for specific families of organisms have tremendously increased in recent years.[23] Despite the fact that the specificity of several proposed microalgal biomarkers is still debated,[23] they are being used with the full understanding that minor non-specific contributions can occur. Such is the case for certain sterols.

Many studies have shown that within the different classes of lipids, sterols may be most suitable biomarkers of organic matter input in sediments.[24-26] Sterols are ubiquitous

constituents in all eukaryotic organisms. They bear particular structural differences that are specific to various organisms (e.g., number of unsaturated bonds and their positions within the side chain, along with the patterns of side chain alkylation).[27] Sterols are relatively resistant to degradation in sediments due to the stability of their bulky aliphatic backbone, cyclopentanoperhydrophenanthrene ring (sterane), shown in Figure 1-7.



source: http://holivo.pharmacy.uiowa.edu/46131/steroids/ch2/presentation2.pdf

Figure 1-7: Sterol backbone - cyclopentanoperhydrophenanthrene ring (sterane).

Sterols are also characteristically distributed in various organisms leading to specific signatures of high stability.[28] Some examples of sterol biomarkers for various eukaryotic families are shown in Figure 1-8.

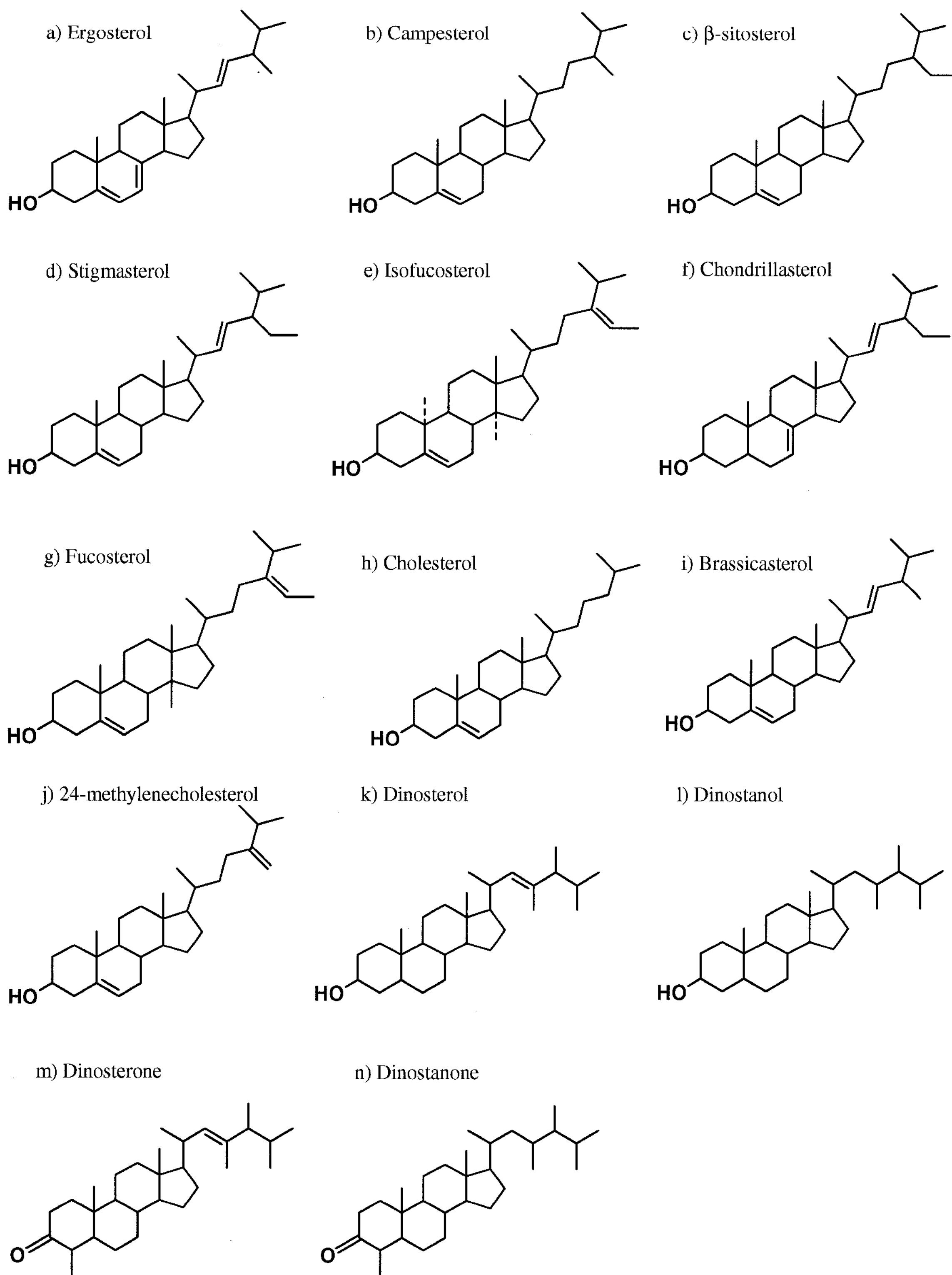


Figure 1-8: Biomarker and sources – a) fungi; b, c, d) higher plants (terrestrial); e, f) green algae; g) brown algae; h) ubiquitous; i, j) diatom; k, l, m, n) dinoflagellates.

In order to identify some of these sterol biomarkers and, in particular, sterols specific to dinoflagellates (Figure 1-8), sediment samples with contrasting organic matter contents were studied. Validation of the methodological approach was done using samples from the Saguenay Fjord (Figure 1-9).

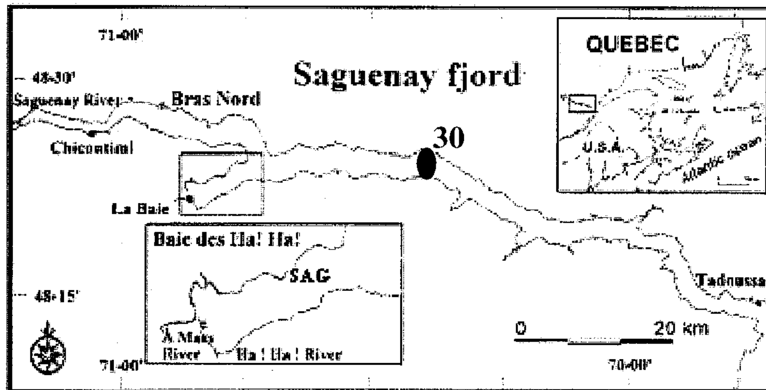


Figure 1-9: Saguenay fjord – station Sag 30.

The samples used in this work were collected in the eastern Pacific Ocean on the Mexican margin (near Mazatlan). Near-surface and deep sediments from five stations with different environmental conditions were analyzed for sterol content (Figure 1-10).

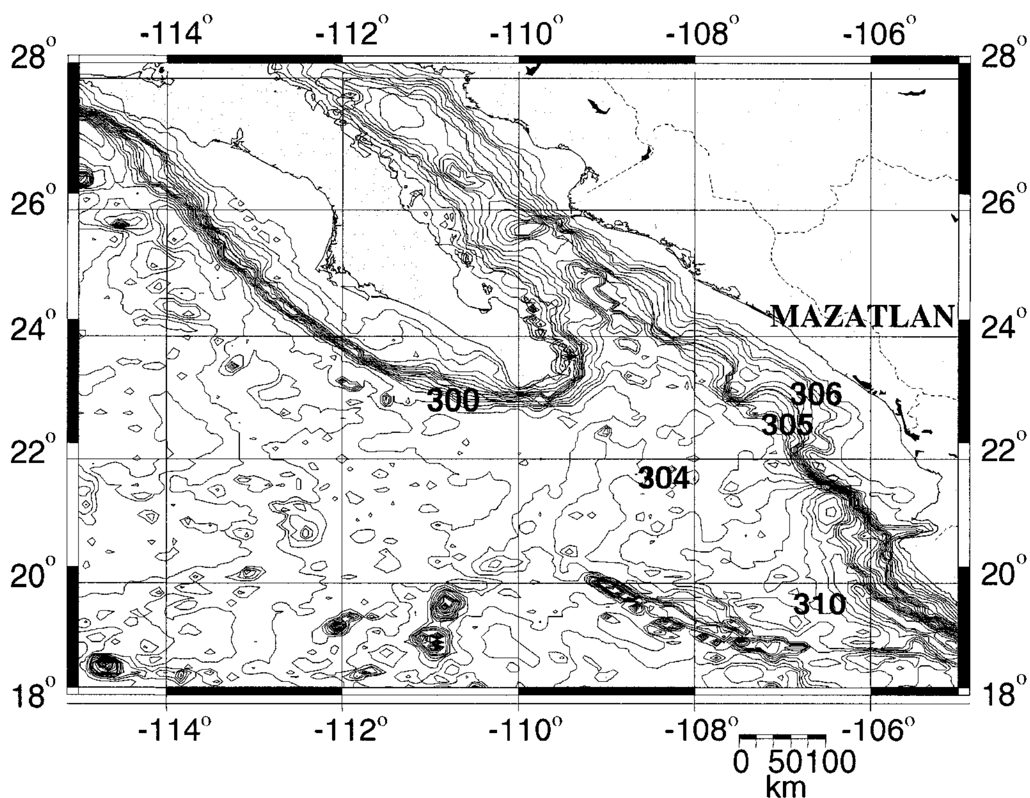


Figure 1-10: Five sampling stations from the Mexican margin (Mazatlan).

With the intention of understanding how redox conditions in the sediment may affect sterol content and distribution, we selected sediments from a wide range of contrasting environments (e.g., organic carbon content between 2-8 %OC and redox potential, which may influence the degradation patterns of the sterols), covering almost the entire range of environmental conditions that may exist in marine sediments. Under oxic conditions, aliphatic and cyclic molecules, such as sterols, are degraded more rapidly than in the absence of oxygen.[29] Since the degradation patterns of dinocysts in these contrasting redox conditions are not known, any assessment of the correlation between dinocysts and dinosterols must be done under the whole range of organic carbon contents and conditions. Furthermore, by looking at near-surface and deep sediment

layers at each station, the robustness of any correlation found between dinocysts and dinosterols will be tested on time scales of several millennia.

While studying the impact of contrasting environmental conditions on the degradation of sterols is interesting, the major thrust of this research consisted in the comparison of two independent techniques for assessing the relative contribution of dinoflagellates to sedimentary organic matter. Particular attention was paid to measuring a suite of specific sterols exclusively synthesized by the dinoflagellate family, namely dinosterol and dinostanol (referred to as "dino"sterols), along with the corresponding steroidal ketones, dinosterone and dinostanone. The concentration of these sterols was quantified by gas chromatography and compared to the abundances of dinocysts counted by optical microscopy.

1.3 "Dino"sterols and dinocysts

"Dino"sterols and dinocysts have one essential point in common: they are both synthesized by dinoflagellates. The Dinophyceae is a branch of the Pyrrhophyta division and the members of this class have been considered as "primitive eukaryotes", mainly because their chromosomes remain attached to their cellular membrane.[30-31] They are a group of microscopic flagellated protists, commonly known as algae.[32] They are mostly unicellular forms with two distinct flagella: longitudinal and transverse. The latter enables the creatures to move in whirling motion, which gives the name dinoflagellate (Greek *dinos*, whirling). These organisms are both producers (autotrophic) and consumers (heterotrophic). They are usually marine planktonic species but some also live in fresh water habitats.[33] Dinoflagellates can reproduce at a fast rate if the conditions are appropriate (light, temperature, nutrients) and these explosions are referred to as blooms.

When in high abundance, these organisms are responsible for red tides, which make the water appear brownish red.[34] They produce toxins in increased concentrations and may be lethal to marine life. Humans may also be affected by these toxins if they consume poisoned shellfish.[35-39] In unfavorable conditions, the dinoflagellates form different types of cysts.

In this research, the studied palynomorphs were organic-walled dinocysts. Palynomorphs are defined as microfossils of refractory organic membranes composed of chitin or sporopollenin, a polymer with cross-linkage derived from the polymerization of polyunsaturated fatty acids.[40] They are resistant to hydrochloric and hydrofluoric acids.[41] The cysts of dinoflagellates are the capsules that protect the cell during the dormancy phase of variable length in relation with the sexual reproduction. The cyst stage is often referred to as the "hypnozygote" phase. Among dinoflagellate population about 15 to 20% of the species are known to produce fossilisable organic-walled cysts.[42]

Dinocyst assemblages, in which the relative proportions of dinocysts from different species are numbered, have been a hot topic for palynologists for decades. Assemblages can be useful when probing the effects of chemical and physical environmental conditions such as temperature, deep water upwelling, salinity or ice coverage on productivity.[43] Dinocysts are also good tracers of hydrographic conditions and salinity.[44] Consequently, they are a great tool for reconstruction of environmental processes.[45-47] By definition, all palynomorphs are well preserved. However, dinocysts are being studied in this project as opposed to other palynomorphs, since they dominate marine environments, whereas in terrestrial deposits, pollen and spores are much more abundant.

Most dinoflagellates synthesize mainly 4α-methyl sterols, including the C_{30} dinosterol (4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol)[48-49] and its stanol counterpart, dinostanol (4α,23,24-trimethyl-5α-cholestan-3β-ol). These molecules are rarely synthesized by other algae (Figure 1-8) and can therefore, be used as biomarkers for dinoflagellate input to marine sediments.[50] This specificity is due to many uncommon structural traits in the dinoflagellate sterols, such as the presence of 4α-methyl group, a saturated ring system instead of the usual Δ^5 in ring B (eg. Figure 1-8), and the existence of a double bond at positions 8,14 in ring C or 14,15 in ring D.[48]

Dinoflagellates use the animal/fungal route of sterol biosynthesis, such that they cyclize oxidosqualene to lanosterol (Figure 1-5).[51-52] The pathway suggested for the synthesis of dinosterol in the organism is shown in Figure 1-11.

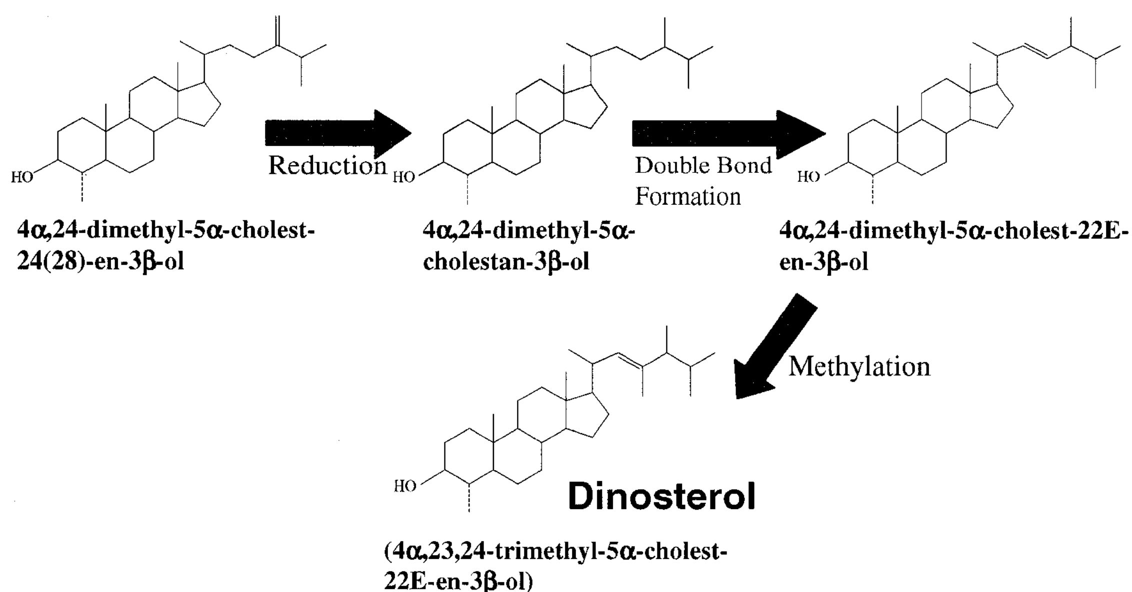


Figure 1-11: Dinosterol biosynthesis pathway.

Dinosterol is synthesized *in vivo* through a series of side chain alkylation reactions. Initially, 4α,24-dimethyl-5α-cholest-24(28)-en-3β-ol is formed from lanosterol. It is then reduced to 4α,24-dimethyl-5α-cholestan-3β-ol and a Δ^{22} double bond is introduced to yield 4α,24-dimethyl-5α-cholest-22E-en-3β-ol. Finally, methylation occurs at C-23 to synthesize dinosterol.[23] For a complete schematic representation of the biosynthetic pathway of dinosterol, please refer to Appendix A.

1.4 Application of techniques

The aim of this research is to obtain quantitative information about dinoflagellate contributions to the sedimentary organic matter from the eastern Pacific Ocean (Mexican margin) through two independent methods: a molecular-level method based on the chromatographic separation and quantification of species-specific "dino"sterols, and the bulk method in which individual cysts produced by dinoflagellates are individually counted under a microscope.

Detailed experimental procedures of the two optimized methods will be presented and discussed in chapter two, while the third chapter, written as an article (to be submitted to *Marine Geology* in August 2005), will present the main findings derived from the comparison of the results generated by the two independent methods. Finally, the last chapter of the thesis will highlight the general conclusions obtained from this project, and describe future work that should be undertaken on this topic.

2 Experimental Methods and Analysis

2.1 Overall methodology

This project involves the development of an analytical method involving wet chemical extraction, separation and gas chromatography (GC), to identify and quantify the sterols found within sediment samples. We tested the efficiency of this approach as an alternative to the tedious and potentially subjective optical microscope counting method that is currently being used in paleoecological laboratories. By developing this approach, we wish to be able to reduce the time involved in the microscopic analysis of individual samples, as well as to minimize subjectivity issues that may arise in the identification of palynomorphs by different individuals, leading to increased variability in the results. By directly measuring the concentration of molecules that are specific to dinoflagellates, a better precision and accuracy may be obtained. This molecular-level approach could complement the widely used microscopy method and, if successful, it should considerably reduce the time and money involved in paleoecological studies based on the counting dinoflagellate cysts.

To achieve our goal, we selected undisturbed sediment samples from five contrasting stations of the eastern Pacific Ocean on the Mexican margin close to Mazatlan (collected in February 1999 using a multicorer, *R/V New Horizon* cruise). These samples were analyzed with an elemental analyzer (Costech) to obtain wt% organic carbon concentrations with a precision of ±2%.[53] The variety of these sediments characterize a range of environments enriched with organic carbon closer to the shore, where oxygen is depleted, to areas where there is deficiency in organic matter due to oxygen enhanced conditions existing offshore. At each station, we analyzed sub-samples

from just below the surface of the sediment, at a sediment depth of between 3-6cm, as well as deeper in the sediment between 16-27cm. These samples were analyzed in parallel in our laboratory using the GC-based method described below, as well as by microscopic counting of dinoflagellate cysts at the laboratory of Dr. Anne de Vernal at the Université du Québec à Montréal (Département des Sciences de la Terre et de l'Atmosphère).

The lyophilized sediment samples were first chemically treated (total lipid extraction using non-polar solvents), purified and separated to collect the neutral lipids fraction containing the sterols of interest. After derivatization, the samples were ready for analysis using GC-FID and GC-MS. The overall methodology is depicted in Figure 2-1.

In order to develop and optimize the quantification method, standard mixtures of sterols, purchased from Aldrich Chemicals, were prepared. The mixtures contained cholest-5-en-3β-ol (cholesterol, 95% purity), 5α-cholestan-3β-ol (dihydrocholesterol, 95% purity), 24-methyl-5α-cholest-22-en-3β-ol (ergosterol, 95% purity), 24-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol, 93% purity) and 24-ethylcholest-5-en-3β-ol (β-sitosterol, 40% purity; containing 20-30% campesterol, 10-30% dihydrobrassicasterol, 10-30% stigmasterol). The method was validated (detection limits and reproducibility) using either standard mixtures or homogenized and spiked sediment samples collected using a boxcore in the Saguenay Fjord in May 2002 (station Sag 30, *R/V Alcide Horth* cruise). Differences in the dinoflagellate contribution to total organic carbon in the sediment samples from the eastern Pacific Ocean were studied using both the molecular sterol approach as well as the microscopic method, which involves counting various palynomorphs including dinocysts. The analysis of these samples thus

enabled us to compare the molecular method, using the “dino”sterols concentrations, with the microscopy technique, in which dinocysts are individually counted.

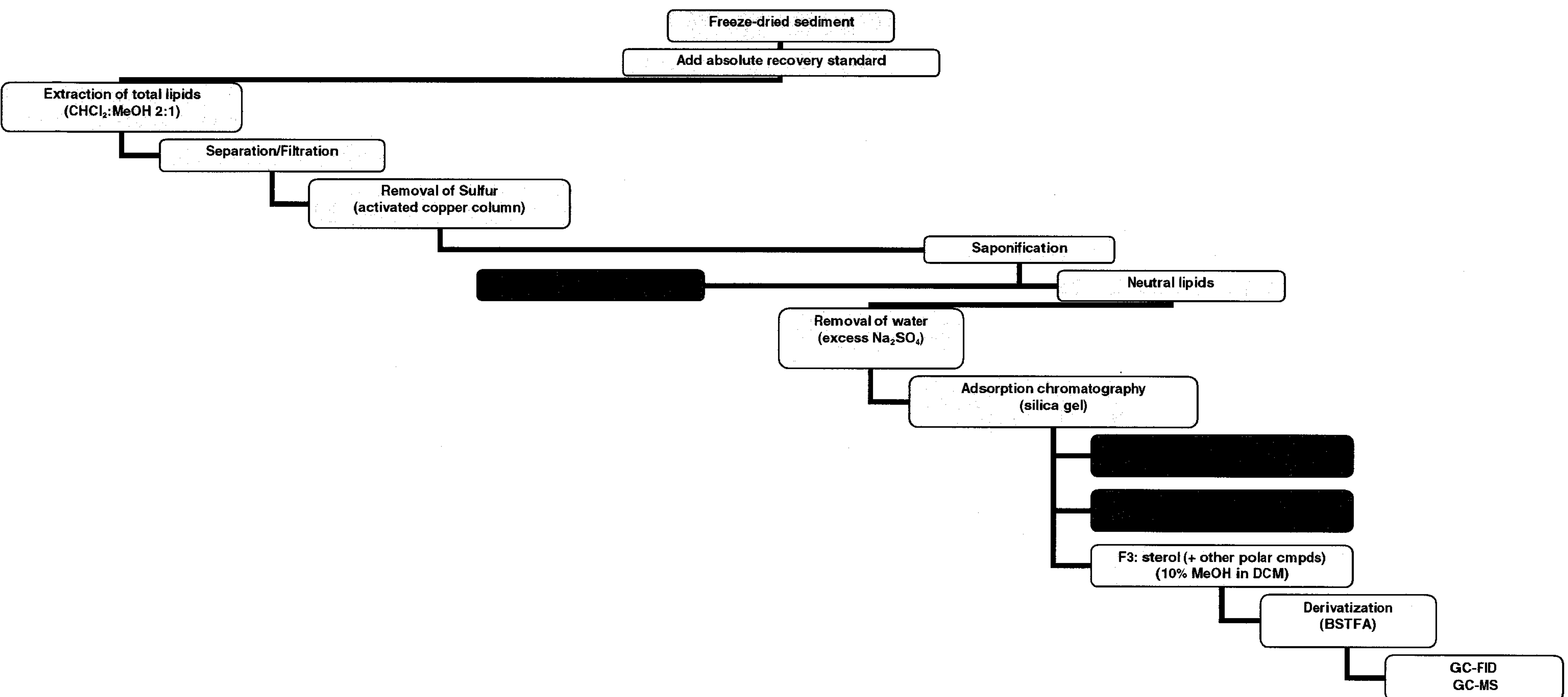


Figure 2-1: Overall method flow chart.

2.1.1 Sample preparation

All sediment samples were lyophilized prior to lipid extraction using a freeze-drier from Labconco. The samples were accurately weighed (approximately 5g) and then spiked with 100μL of a 1000μg/mL solution of absolute recovery standard solution, cholesteryl linoleate (Sigma, 98+%), which was stored frozen at -80°C and protected from light. The sediment samples were then extracted using 200mL of a 2:1 solution of HPLC-grade dichloromethane/methanol.[54] Prior to extraction, samples were ultra-sonicated (model FS60H, Fisher Scientific) for 30 minutes. In addition to decreasing the time necessary for complete extraction, sonication lyses cell membranes and allows optimal recovery of the lipids. Earlier work has shown that more than 95% of the lipids are extracted using sonication.[55]

The samples were then transferred to separatory funnels and about 100mL of water were added in order to form two distinct liquid phases: water-methanol and dichloromethane. The bottom, lipid-containing dichloromethane organic phase was collected in filtration flasks and two more extractions were performed on the aqueous layer, to assure that the majority of the lipids were recovered.

The organic phase was then vacuum-filtered using either 4.7-cm (Whatman) or 11-cm (Fisher) filter paper to remove residual solid particles from the liquid organic phase. The extracts were then roto-evaporated (model RE111 from Büchi) to dryness and recovered in 5mL of HPLC-grade chloroform.

Finally, sulfur was removed from the chloroform extracts by using activated copper columns.[56] Pasteur pipettes were plugged using glass wool and filled with copper powder 40 mesh size (Aldrich, 99.5%). The pipettes were rinsed with clean 3N

hydrochloric acid to dissolve any copper (II) oxide present. The columns were then rinsed with water, acetone and dichloromethane to neutralize the pH and remove organic impurities that may be present. Once the copper column had been activated, the samples were then added and eluted in a round bottom flask using chloroform. The solvent was then evaporated under a gentle nitrogen stream.

2.1.2 Sample purification and separation

Saponification is a procedure by which glycerol and fatty acid salts called "soap" are liberated from mono-, di- or tri-substituted parent acylglycerols (Figure 2-2). Saponification of the lipid extracts was performed using 25mL of methanolic KOH (0.5N) at a pH of 14 followed by the addition of 5mL of distilled water.[54] Methanol is very good at solubilizing polymeric organic materials, but some carboxyl groups may be converted to methyl esters. Adding a small amount of water to the reaction can help limit their formation. The reaction mixture was then put in a round-bottom flask fitted with a condenser, heated and brought to boiling in a water bath in order to speed up the reaction.

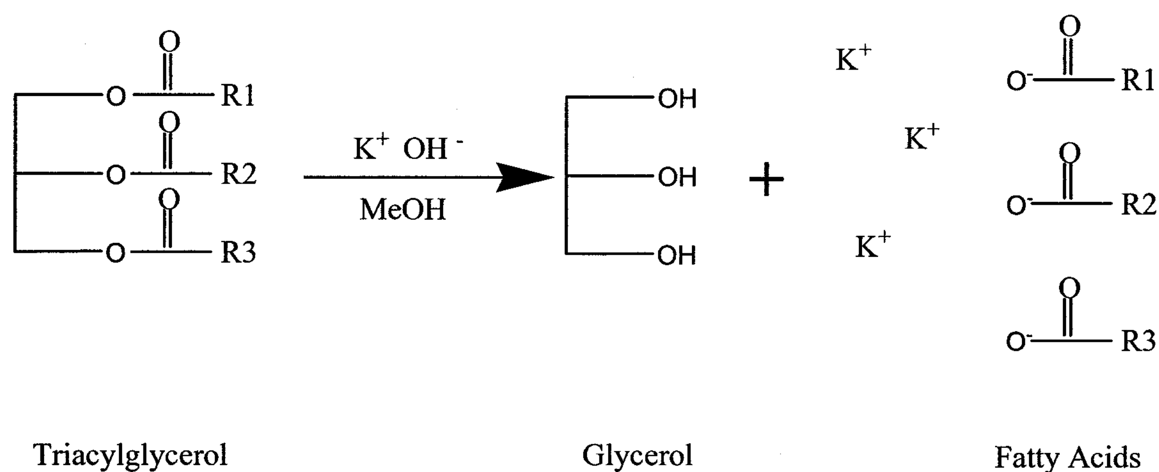


Figure 2-2 : Saponification of triacylglycerol.

The hydroxide base drives the reaction forward by attacking the carbonyl group of the acylglycerol, which carries a partial positive charge in its carbonyl carbon atom. Glycerol is a good leaving group, therefore the reaction yields various fatty acid salts. The pH must remain highly basic in order to carry out this reaction efficiently. The reaction reached completion after refluxing for two hours. The product was then extracted with three aliquots of 30mL HPLC-grade dichloromethane and 10mL of a 5% NaCl solution to form a two-phase water-methanol/dichloromethane system. The organic layer was washed one more time with 30mL of a 5% NaCl solution. The dense dichloromethane phase was then drained and combined with the previously collected organic fraction in a round bottom flask. Clean sodium sulfate (Na_2SO_4) was added to the round bottom flasks containing the organic neutral lipids to remove traces of water. The aqueous phase was then returned to the separatory funnel and acidified to a pH of 2 with concentrated HCl. This phase, containing the acidic lipids, was then back-extracted three times with 30mL dichloromethane and stored refrigerated at 4°C for further studies.

After saponification, the neutral lipids were separated from the polar lipids (including the sterols of interest) based on differences in their polarities by performing long column silica gel chromatography.[54] A 25-cm glass column was filled with approximately 7g of clean, deactivated silica gel (60-100 mesh size, Fisher) that was rinsed with 40mL of hexane. The sample was then loaded on the column and different fractions of lipids with similar polarities were sequentially eluted from the column (from the least to the most polar) with organic solvent mixtures of increasing polarity. Straight chain hydrocarbons were first eluted from the silica gel column with 12mL of hexane. The slightly more polar branched hydrocarbons were then eluted with 24mL of a 1:1

solution of dichloromethane/methanol. The last fraction collected was the polar lipid fraction containing the sterol compounds, which was eluted using 24mL of a 10% solution of methanol in dichloromethane.[56] The straight chain and the branched hydrocarbons fractions were stored for future work, while the polar lipid fraction was rotary evaporated at 40°C and redissolved in 5mL of chloroform.

In order to deactivate silica for the above described separation step, it was first extracted by sonication (30 min.) in a 2:1 solution of dichloromethane/methanol and left at room temperature overnight covered with aluminium foil to avoid contamination. After baking at 150°C for 48 hours to remove traces of solvent, the silica was hydroxylated with 5% w/w water to form silanol groups in order for separation to occur[54].

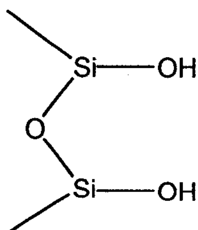


Figure 2-3: Silanol groups on the surface of silica particles.

2.1.3 Sample derivatization and GC analysis

Derivatization is widely used in gas chromatography whereby polar compounds are chemically modified to make them more amenable to analysis in the gas phase. The sterol fraction was derivatized through a silylation reaction using N,O-bis[Trimethylsilyl]trifluoroacetamide (BSTFA; Figure 2-4). It involves the replacement of acidic hydrogens on the compound with an alkylsilyl group such as trimethylsilyl (TMS), as shown in Figure 2-5.

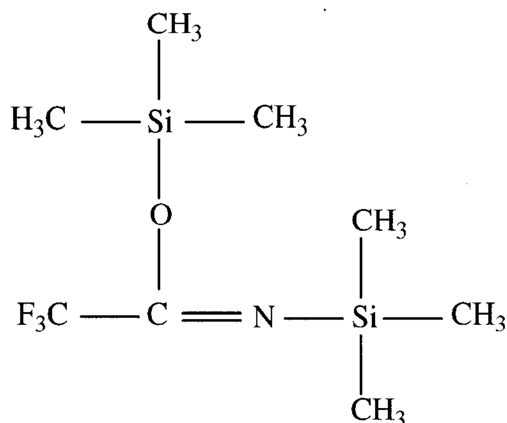


Figure 2-4: Derivatizing agent: BSTFA (N,O-bis[Trimethylsilyl]trifluoroacetamide).

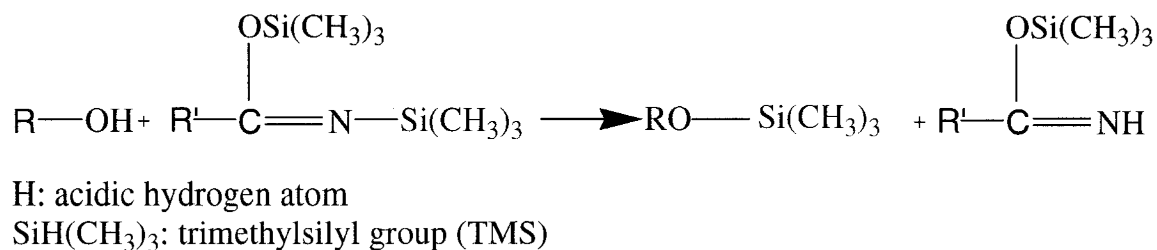


Figure 2-5: General derivatization reaction.

Silylation occurs through a nucleophilic attack (SN2 reaction). A good leaving group ensures the silylation reaction proceeds efficiently. Derivatized molecules become less polar, more volatile and more thermally stable than their non-derivatized counterparts.[57]

The samples suspended in chloroform were first evaporated under N_2 to remove traces of water that can quench the reaction. The derivatizing reagent, BSTFA (Supelco), was then added to the samples (300μL), with 300μL of N_2-flushed pyridine (Fisher).

Pyridine is the most commonly used solvent in this reaction because it is an efficient acid scavenger that drives the reaction forward. The solutions were then purged with N_2 and the vials were sealed tightly before heating at 70°C for 2 hours. After this last reaction step, the reagents were evaporated under N_2, followed by an addition of about 0.5mL of HPLC-grade hexane to the samples that were also blown off with nitrogen gas. The samples were then suspended in 200μL of HPLC-grade chloroform and filtered through glass pipettes plugged with clean glass wool in order to remove remaining particles and avoid blockage of the capillary GC column.

Compound identification of the derivatized polar lipid extracts was carried out by GC-MS using the National Institute of Standards and Technology (NIST) mass spectrum database, while GC-FID was used for quantifying a suite of sterols. In a first step, chromatographic parameters were optimized to ensure complete separation of the sterols in the standard mixture. Calibration curves (25 to 250μg/mL) were then obtained to assess linearity and reproducibility of the sterol standard measurements. Natural sediment samples from the Mexican margin were also analyzed for their total sterol content. It was possible to positively identify a total of 23 sterols with concentrations varying with sediment depth and location. Within these samples, specific biomarkers of dinoflagellates, namely dinosterol and dinostanol, were identified and precisely quantified (GC-MS) in order to compare their total concentrations (μg/g sediment) to the dinocyst concentrations (cysts/g sediment) calculated using the microscopic technique.

2.2 Microscopy technique[41]

Palynology is the scientific study of microfossils consisting of refractory organic membranes composed of chitin or sporopollenin. These microfossils include mainly

pollen, spores and dinoflagellate cysts, amongst other palynomorphs. Although about 1/5 of dinoflagellate species produce fossilisable organic-walled cysts, it is assumed that the total dinocyst concentrations found in sediments is proportional to the dinoflagellate densities in the surface ocean[58] and that the relative proportions of each dinoflagellate species can provide clues on environmental and ecological conditions prevailing in the surface ocean at the time when these cysts were produced.[44] Dinocysts thus represent an extremely valuable tool in paleoceanography and biological oceanography.

In order to remove the sedimentary minerals and concentrate the palynomorphs, our samples had to undergo physical separations (sieving) as well as chemical treatments (with HCl and HF), as described below. These preparation techniques enabled the observation and counting of palynomorphs, such as dinocysts in this project (Figure 2-6), using an optical microscope. The counts presented below were acquired at the laboratoire de micropaleontology of GEOTOP at Université de Québec à Montréal (UQAM).

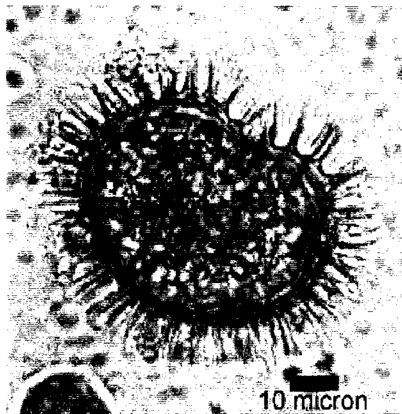


Figure 2-6: Microscopic image of a dinocyst (*Protoperidinium conicum*, heterotrophic species).

For marine samples, $5cm^3$ of sediment were measured in a graduated cylinder and transferred into labelled beakers. The samples were then spiked with a capsule of marker grains of lycopode spores, known as *Lycopodium clavatum* (10679 spores/capsule). Each sample was then filtered through two sieves of 120μm and 10μm that were superimposed and agitated using a magnetic stirrer. The fraction between 120μm and 10μm which contained the dinocysts (along with other palynomorphs) was recuperated and transferred into previously labelled tubes. The tubes were then centrifuged at 2000rpm for 10 minutes and the supernatant was removed. To remove carbonate minerals, a few millilitres of 10% HCl were added to the samples progressively and the tubes were placed on a hot plate (50-60°C) for 20 minutes for the reaction to complete. Silicate minerals were removed with a few millilitres of a second acid using 49% HF and left to react overnight. Finally, the samples were treated with 10% HCl one more time as above to dissolve the minerals that re-precipitated due to the supersaturation of the HF solution. The samples were then rinsed with distilled water in order to remove traces of acid and the tubes were centrifuged to discard the liquid supernatant. One last sieving procedure was performed on the treated samples to collect the fraction between 120μm and 10μm, followed by centrifugation (10 min.) and removal of the supernatant. The pellet was finally ready to be mounted on a microscopic slide embedded in glycerine jelly. The counting of palynomorphs was done using an optical microscope with a magnification power of 250X to 1250X. Depending on the cysts' density in the samples, areas of varying sizes on the slide were counted according to the differences in morphologies of various species. Final cyst concentrations were calculated on the basis of the following equations:

Equation 1

$$Np = (Ne \times np) / ne$$

Np: number of palynomorphs in initial sample
Ne: known concentration of marker grains
np: number of counted palynomorphs
ne: number of counted marker grains

Equation 2

$$Cysts\ concentration = Np / sample\ \ volume\ (cm^3)$$

This method has been previously estimated to be reproducible at approximately 10% for a confidence level of 0.95.[41]

2.3 GC Method development

Because optimal chromatographic parameters must be used to ensure an efficient separation of the different sterols and since optimal conditions might vary from lab to lab and from instrument to instrument, the first part of this project involved the optimization of a GC-based method for the identification and quantification of the compounds of interest. Identification of certain molecules was achieved using a Varian Saturn CP-3800/2200 ion trap GC-MS fitted with a split/splitless injector set at 320°C. The column used for the MS was a DB-5 (95% dimethylpolysiloxane with 5% phenyl groups), 30m x 0.32mm I.D. with a film thickness of 0.25μm (RESTEK by Fisher).

In developing our method, consideration was given both to optimizing the separation of the different sterol compounds (which all elute within a narrow retention time window because of the similarity in their chemical structures), and to minimizing the total analysis time. The samples were injected in the splitless mode, and the carrier gas flow rate was kept constant at 1.25 mL/min. The final temperature ramp program was as follows: initial temperature of 45°C rising to 255°C at a rate of 30°C/min., followed by

a hold time of 10 minutes, and a second ramp to 257°C at a rate of 1°C/min. The temperature was then increased to 271°C at 4°C/min and finally to 310°C at a rate of 20°C/min. The GC oven was held at this temperature for 10 minutes. Using these parameters, the majority of the sterols detected in our samples were well resolved, and the major sterols were identified using reference mass spectra from the NIST library database. Several more sterols could be identified based on their relative retention times and comparison with published chromatograms acquired using the same column and a similar temperature ramp program.

A second GC fitted with a flame ionization detector (FID) was exploited to quantify the sterols of interest. The instrument used for this purpose was an Agilent 6890N also fitted with a DB-5 (polysiloxane with 5% phenyl groups), 30m x 0.32mm I.D. column with a film thickness of 0.25μm (RESTEK by Fisher). On this instrument, an inlet split ratio of 50:1 and a constant carrier gas flow rate of 2mL/min were used. The oven temperature was set at 45°C and increased to 281°C with a ramp of 30°C/min. The temperature was then increased to 310°C at a rate of 4°C/min and held there for 10 minutes. The FID was set at 315°C and the injector had an initial temperature of 65°C. It was held there for 1 minute and then increased to 305°C at 1°C/sec and held for another 10 minutes. This program allowed good separation of most of the sterols.

2.4 Method Validation

Prior to the analysis of the natural samples, the GC method was validated using a 100μg/mL mixture of sterol standards purchased from Aldrich, consisting of, cholest-5-en-3β-ol (cholesterol), 5α-cholestan-3β-ol (dihydrocholesterol), 24-methyl-5α-cholest-22-en-3β-ol (ergosterol), 24-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol) and 24-

ethylcholest-5-en-3β-ol (β-sitosterol). The retention time of each standard was first determined through compound identification on the GC-MS and then the concentration for each sterol was calculated using the GC-FID. Calibration curves were constructed in order to assess linearity and quantitative detection limits, and reproducibility assays were performed to validate the overall methodology used in this project.

2.4.1 Qualitative analysis – GC-MS

In a first step, identification of the standard sterols within the 150μg/mL mixture solution was done using the optimized GC method and MS detection. All the components of the mixture were positively identified using the NIST library database. A typical example of a chromatogram for the standard solution is given in Figure 2-7(**a**)).

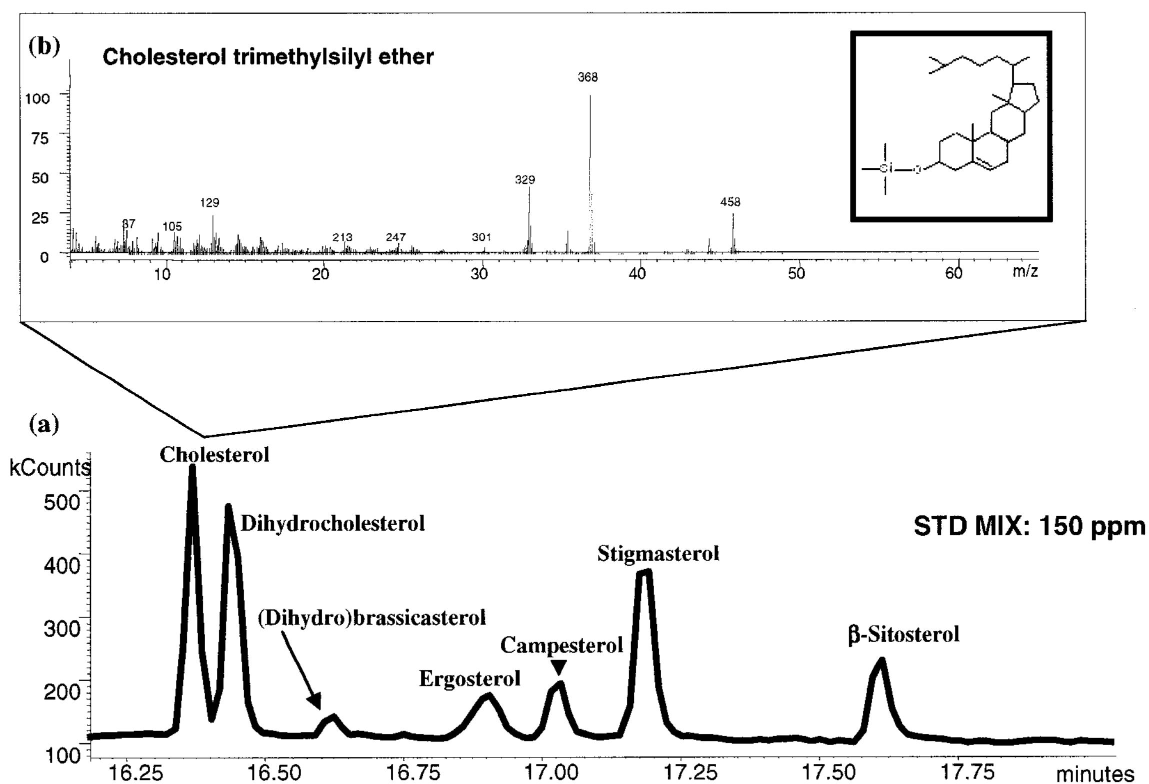


Figure 2-7: (a) Standard sterols chromatogram, (b) mass spectrum of cholesterol.

The mass spectra of individual standard sterols showed the fragmention patterns of single components found within the mixture. Each analyte is broken down into a set of characteristic charged fragments which are then subjected to a magnetic field and detected based on mass-to-charge ratios. An example of the mass spectrum of cholesterol is shown above in Figure 2-7**(b)**, displaying the masses of each fragment. Since each sterol displays fragments of specific mass-to-charge (m/z) ratios (Table 2-1), quantification could also be done through the single ion monitoring (SIM) method, in which a series of m/z ratios, each one of them specific to a different sterol, are monitored, rather than the total ions count of a wide m/z window. Although it has not been used in this project, SIM could considerably lower the quantitative detection limits for the different sterols by minimizing the background noise due to the presence of non-sterol compounds in the sample.

2.4.2 Quantitative analysis – GC-FID

Following qualitative identification, quantification of the different sterols was best accomplished using a conventional GC-FID approach.

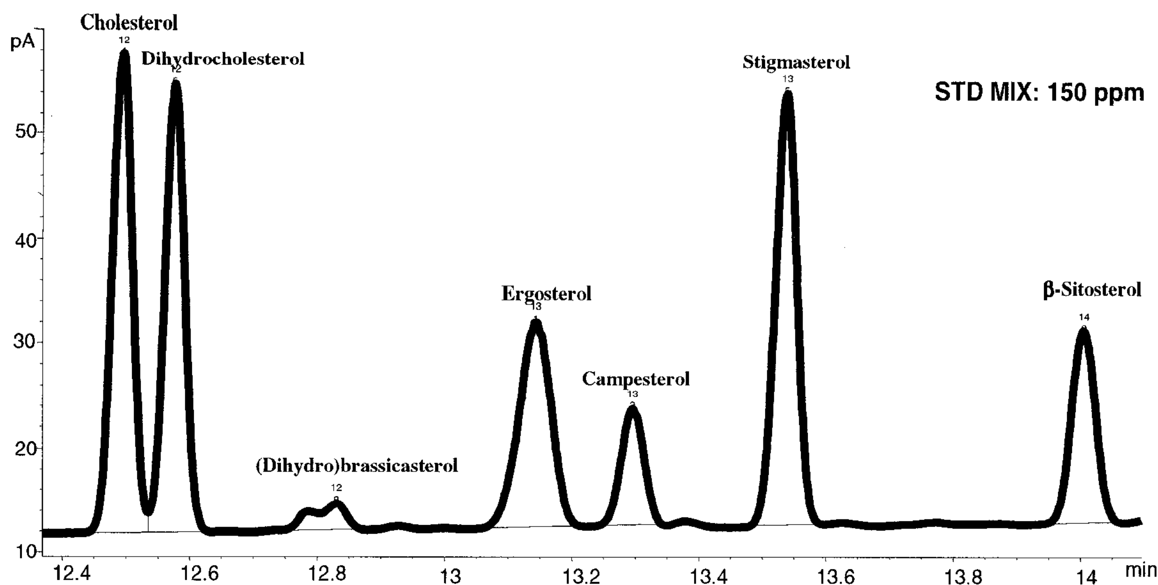


Figure 2-8: Typical chromatogram of the standard sterol mixture using FID detection.

In gas chromatography, sample components are vaporized and separated based on their physical and chemical properties. In an ideal separation, each analyte elutes to give its own distinct chromatographic peak. The retention time (tr) is the time it takes for the analyte to reach the detector, whereas the time it requires for the unretained molecules of the mobile phase to travel through the column is called the dead time (tm). Retention (k'_A) and selectivity (α) factors of the solution influence the migration rates of the analytes, enabling the differentiation of compounds found in a mixture (Equation 3 and Equation 4). When the retention factor is < 2, accurate determination of retention times becomes difficult; and a retention factor > 10 leads to extremely long elution times. Thus, optimized separations occur when retention factors are within a range of 2 to 10.

Equation 3

$$k'_A = (tr - tm) / tm$$

Equation 4

$$\alpha = k'_B / k'_A = ((tr)_B - tm) / ((tr)_A - tm)$$

A: compound A (elutes first)
B: compound B (elutes later)

The efficiency of a column of any length (L) increases with increasing plate number (N) and decreasing plate heights (H). The chromatographic peaks become narrower by lengthening the column (or increasing N), however it would be at the expense of elution time. The column efficiency is described in Equation 5 .

Equation 5

$$N = L / H$$

The resolution (R_s) is a measure of the ability of a column to separate two analytes (Equation 6)[59]. An optimal method is achieved by maximizing the resolution of the column, while minimizing the time involved in separation.

Equation 6

$$R_s = (((tr)_B - (tr)_A) / (tr)_B) \times (\sqrt{N} / 4) = (\sqrt{N} / 4)((\alpha - 1) / \alpha)(k'_B / (1 + k'_B))$$

A typical chromatogram, such as that shown in Figure 2-8, indicates that the programmed method for the standard sterols provided well resolved separations. An important aspect of GC-FID analysis lies in the shape of the peaks. The chromatograms obtained throughout the analysis showed symmetrical Gaussian type peaks, confirming good analytical conditions and efficient separation of the components. In general, there were no overlapping peaks further indicating that the designed program was suitable for sterol analysis on the GC-FID (Table 2-1).

Table 2-1: Main mass fragments for the standard sterols and relative retention times (RRT) for individual sterols in the standard mixture using GC-MS.

STEROL	RRT	m/z
Cholesterol	1.00	329, 369, 401, 458
Dihydrocholesterol	1.09	215, 257, 306, 371, 403, 460
(Dihydro)brassicasterol	1.31	255, 340, 365, 380, 455, 470
Ergosterol	1.69	338, 363, 437, 468
Campesterol	1.85	255, 343, 367, 382, 472
Stigmasterol	2.12	256, 351, 394, 442, 484
β-sitosterol	2.62	256, 283, 357, 397, 444, 486

2.4.3 Method validation assays

The reproducibility of the complete method (extraction, purification, derivatization and GC analysis) was tested by analyzing 3 aliquots of a homogenized stock of sediment from station Sag 30 (Saguenay Fjord) spiked with the standard sterol mixture (equivalent to a concentration of 100μg/mL). A portion of the chromatograms showing the peaks of cholesterol and dihydrocholesterol from the spiked and natural samples is shown in Figure 2-9.

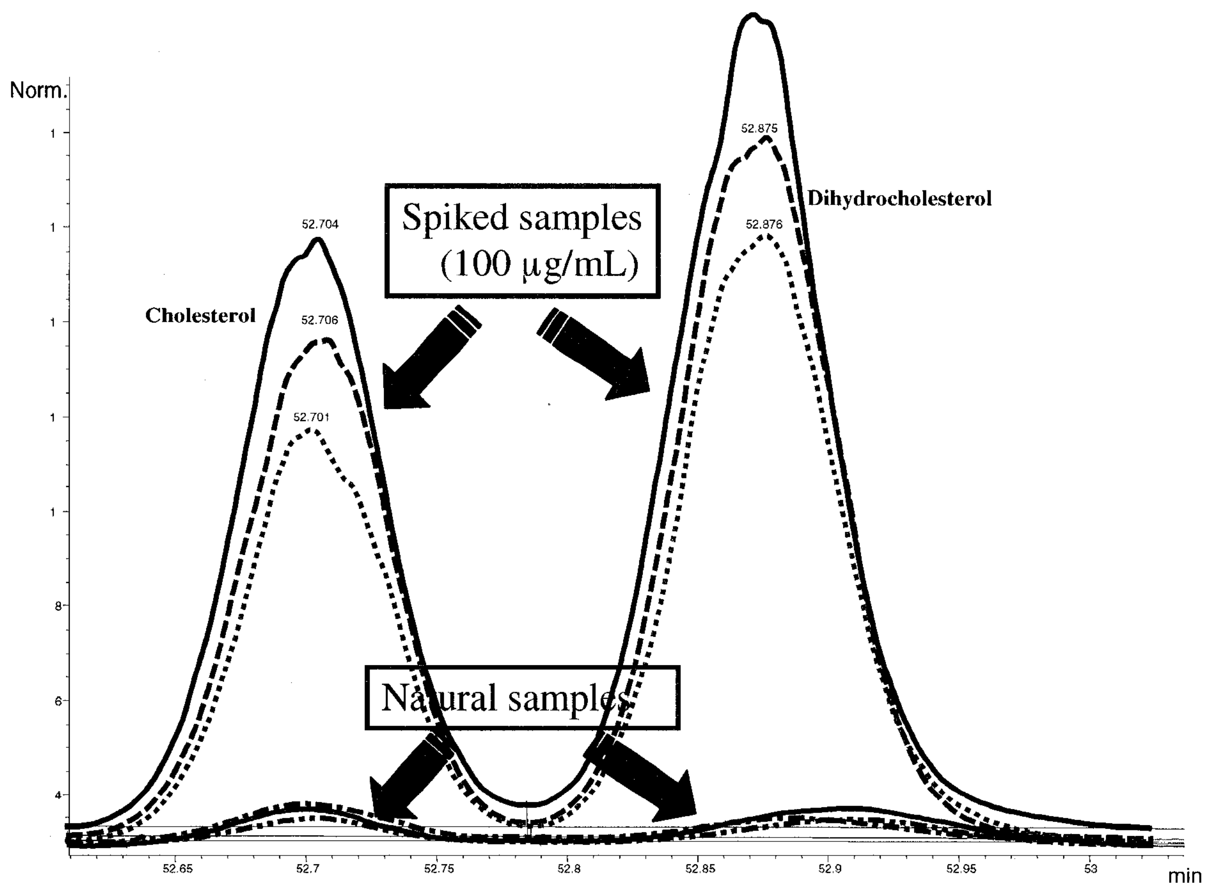


Figure 2-9: Portion of GC-FID chromatograms for the spiked and natural Saguenay Fjord sediment sample.

The reproducibility of the whole method is expressed in terms of relative standard deviation (RSD, Table 2-2).

Table 2-2: Reproducibility of the overall analytical procedure for a spiked sediment sample (Sag 30).

STEROL	Rep 1 (µg/mL)	Rep 2 (µg/mL)	Rep 3 (µg/mL)	Average (µg/mL)	SD (µg/mL)	RSD (%)
Cholesterol	109.9	142.4	137.8	130.0	17.6	13.5
Dihydrocholesterol	175.3	205.1	205.3	195.2	17.3	8.8
Ergosterol	72.1	91.6	83.1	82.2	9.8	11.9
Stigmasterol	395.8	479.2	496.3	457.1	53.8	11.8

Note: Concentrations of the spiked samples have been corrected using the average concentrations of the triplicate natural samples; Rep = replicate; SD = standard deviation; RSD = relative standard deviation

The overall method appears highly reproducible with results falling within the RSD range of ±15%.

The linearity and quantitative detection limits of the method were assessed using a standard sterol mixture at three or four concentrations varying between 25 and 250μg/mL. A calibration plot of the response versus the concentration has been drawn out in order to fit the best linear regression and minimize the square of the differences between measured and calculated values (Figure 2-10).

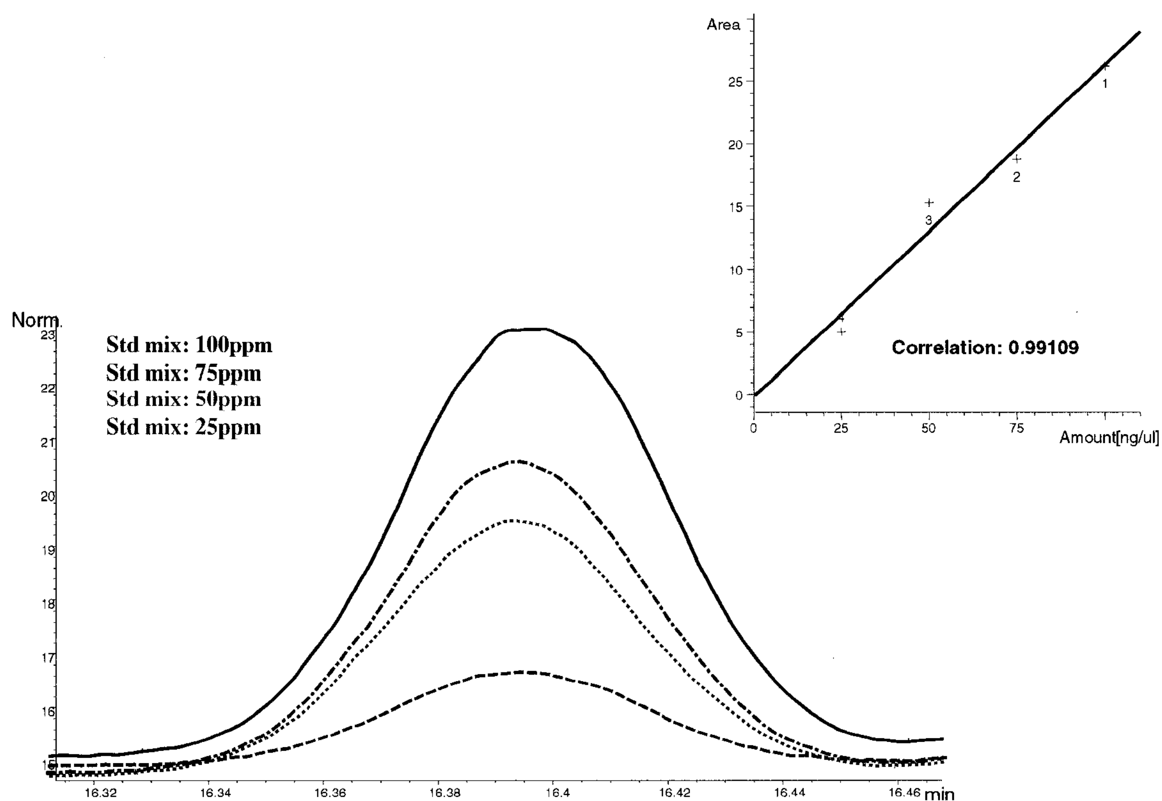


Figure 2-10: Chromatograms and corresponding calibration curve for cholest-5-en-3β-ol (cholesterol).

All sterols showed a correlation coefficient (R^2) greater than 0.98 over this concentration range. The absolute lower limit of detection (LOD), defined as three times the background noise, was 25ng, equivalent to a concentration of 25µg/mL in a volume of 50µL (the smallest volume that can be used with the GC autosampler). This is considered to be the smallest concentration that can reliably be detected using the GC-FID.

2.5 Natural Samples Analysis

Once the molecular method was developed and validated, natural sediment samples were analyzed using the optimized analytical parameters. Samples were taken from five stations along the western Mexican margin, offshore from the city of Mazatlan. This suite of samples represented a steady continuum between organic carbon rich sediments deposited in an oxygen depleted environment (closer to shore), to more organic depleted sediments that accumulated in normal, oxygen rich conditions (offshore). Cores from each station were subsampled just below the sediment-water interface (3-6cm) and deeper in the sediment (16-27cm). The general characteristics of the sediment samples are summarized in Table 2-3.

Table 2-3: Characteristics of samples from the western Mexican margin used in this study.

Sampling Station	Coordinates	Water Column Depth (m)	Sediment Depth (cm)	Organic Carbon (%)	Porewater Redox Conditions
300	Lat.: 25°19.71 N Long.:112°46.02W	387	3-6	6.39	Suboxic
			16-27	7.49	Anoxic
306	Lat.: 22°43.23 N Long.:106°28.91W	375	3-6	7.70	Suboxic
			16-27	7.86	Anoxic
305	Lat.: 22°11.41 N Long.:107°19.08W	2990	3-4	3.27	Suboxic
			19-22	3.20	Suboxic
304	Lat.: 21°34.34 N Long.:108°18.53W	3070	3-4	2.38	Oxic
			16-19	2.30	Suboxic
310	Lat.: 19°06.79 N Long.:106°14.61W	3500	3-4	1.96	Oxic
			16-19	1.87	Suboxic

There is a general trend between organic carbon (OC) contents in the sediment and redox conditions, where more oxidizing conditions lead to lower OC contents. Such a trend can be explained by the presence of a significant fraction of organic compounds that can only be degraded in the presence of oxygen and are thus preferentially preserved when conditions become suboxic (no dissolved oxygen) or anoxic (no dissolved oxygen, nitrate and sulphate in the porewater).[60] This series of samples encompasses the entire range of organic matter degradation 'harshness' in margin sediments, and thus allows probing for the effect of contrasting sedimentary environments on the comparison between the molecular level (dinosterols) and microscopy approaches for estimating the dinoflagellates contribution to total organic carbon in the sediments. Because sterols and dinocysts are considered highly resistant to degradation and will only be degraded under oxic conditions (albeit at potentially different rates), such redox effect should be minimal.

The sterol concentrations were measured in these samples to evaluate the degradation patterns of these potentially important biomarkers, and to compare sterol

concentrations with the dinocysts counts. Particular attention was mainly paid to sterols that are used as biomarkers for dinoflagellates, namely dinosterol and its stanol counterpart, dinostanol. While most sterols found in sedimentary environments have different sources, both terrestrial and marine, “dino”sterol are specific to dinoflagellates and thus are expected to be highly correlated to dinocysts counts.

2.5.1 Molecular approach

The analysis of the sterol content of our suite of samples was carried out using the GC-FID. Note that because of limited amounts of sediment at our disposition and the high mass of sample needed for a single analysis (about 5g), it was impossible to analyze more than one replicate per sample. Based on our previous results on the spiked sediment samples, it is assumed that the precision was equal to or better than ±15%. A total of 23 sterols were identified and quantified in our samples. A portion of the chromatogram for the sediment sampled at station 305 (3-4cm) on the western Mexican margin is shown in Figure 2-11. An instrument blank and an aliquot of the sterol standards mixture (100µg/mL) was included in each GC run to detect small shifts or drifts in baseline intensity, as well as small shifts in sterol retention times due to the slow degradation of the column stationary phase.

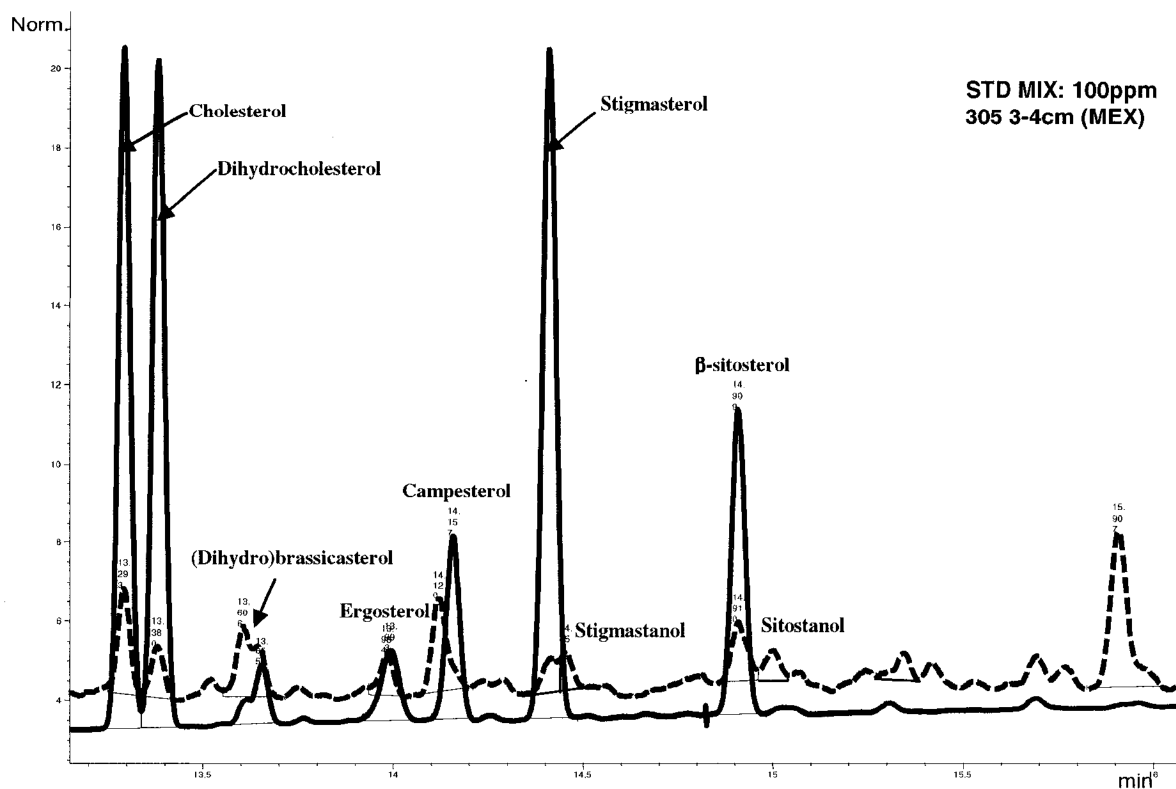


Figure 2-11: GC-FID chromatograms of sediment from the Mexican margin station 305 (3-4 cm, blue trace), and of a sterol standards mixture (red trace).

The area under each peak is proportional to the concentration of a particular molecule present in the solution being injected on the column. The concentration of the individual sterols was calculated using the following equations:

Equation 7

$$[sample]\ (\mu g / mL) = (sample\ \ area \times [std]) / std\ area$$

The values calculated in µg/mL using Equation 7 were then normalized to the organic carbon (OC) content of the sediment sample:

Equation 8

$$sterol_{extract}\ (\mu g) = ([sterol]_{sample} \times final\ volume) / 1000$$

Equation 9

$$[sterol]_{sample}\ (\mu g / g\ OC) = sterol_{extract} / OC\ weight$$

Equation 10

$$OC\ weight\ (g) = (sample\ weight \times OC\%) / 100$$

The final concentrations are reported using equation 5 and are normalized to OC to account for dilution by inorganic materials in the sediment (Table 2-4). For most sterols, the results agree with the expected trend of lower concentrations for samples taken deeper in the sediment. Such a decrease in concentration is due to the slow degradation of sedimentary organic matter with time (sediments are about 5000 years old at 20 cm). The constant inputs of fresh organic matter produced in the surface waters or close to the sediment-water interface are responsible for the higher concentrations of sterol measured at 3-6 cm.

The subsurface sediment redox conditions at these sites differed considerably from fully oxic (offshore) to suboxic (inshore). Because the degradation rate of sterols in oxic conditions is much higher than in anoxic environments and since organic matter inputs to the sediments should be similar at all stations (which are all located within a radius of about 100 km), we therefore expected to find trends in sterol contents, with higher concentrations in suboxic and anoxic sediments. However, no spatial pattern in sterol concentrations was found in this set of stations. The absence of a trend could be due to several factors including differences in seawater surface productivity (abundance and diversity), presence of different populations of organisms in the water-sediment boundary layer (fungi in anoxic sediments vs. benthic invertebrates in oxic sediments), and age of the sediment at the shallower depths (about 1000 years old at 4 cm). More detailed profiles (several depths, including the surface 0-1 cm layer) should be obtained

to identify trends or discriminate between the above factors; since this issue was not one of the parameters of our study, it was not pursued further.

Table 2-4: Sterol concentrations (μg/g OC) in sediments from the western Mexican margin.

Stations[1]	300 top	300 bot	306 top	306 bot	305 top	305 bot	304 top	304 bot	310 top	310 bot
Coprostanol	ND	ND	ND	ND	ND	*1.35*[2]	1.71	*3.13*[2]	ND	ND
n-C28-alcohol	2.68	0.98	4.20	3.18	ND	*14.0*[2]	4.11	*34.9*[2]	1.68	1.62
Occelasterol	3.40	1.90	2.72	1.27	29.9	6.28	ND	ND	ND	*4.93*[2]
Dehydrocholesterol	1.28	ND	1.16	0.69	9.05	9.08	7.92	*18.0*[2]	1.23	*1.97*[2]
Dehydrocholestanol	33.0	6.33	42.1	16.7	39.3	6.28	6.34	*9.09*[2]	2.72	*3.33*[2]
Cholesterol	143	19.3	194	46.4	635	17.1	140	67.5	616	*1057*[2]
Dihydrocholesterol	20.5	2.17	21.3	6.69	28.8	8.59	55.1	25.6	ND	ND
n-C29-alcohol	7.72	ND	8.92	2.80	4.61	*14.0*[2]	ND	*2.76*[2]	ND	ND
(Dihydro)brassicasterol	1008	158	1364	514	708	229	600	*1431*[2]	ND	ND
Ergosterol	78.4	ND	124	66.3	232	56.1	54.2	*85.4*[2]	116	18.6
24-methylenecholesterol	30.2	11.9	45.9	37.1	42.5	13.8	15.7	*30.0*[2]	114	37.0
Campesterol	96.5	47.2	53.2	*55.2*[2]	218.3	14.7	166	81.7	540	256
Ergostanol	21.3	21.3	3.88	*22.4*[2]	26.0	0.60	13.2	6.45	205	63.1
Stigmasterol	67.5	*171*[2]	70.1	*125*[2]	80.6	13.8	192	71.3	1712	87.0
Stigmastanol	16.7	*22.8*[2]	20.4	*30.0*[2]	78.8	7.63	30.5	13.9	317	14.6
Dinosterone	23.7	*1.96*	32.1	16.3	21.2	8.08	11.4	8.48	0.39	*2.28*
β-sitosterol	17.0	5.38	30.4	13.4	85.2	6.19	128	40.9	14.7	13.5
Sitostanol	29.7	3.68	18.7	11.4	38.2	3.16	37.2	15.1	14.1	*24.2*[2]
Fucosterol	22.7	3.64	16.8	16.2	71.0	0.79	25.0	8.54	5.51	4.78
Dinostanone	27.6	1.51	57.2	9.18	24.7	5.33	5.69	*21.5*[2]	4.28	*9.00*[2]
Dinosterol	9.60	2.02	17.1	6.37	21.2	7.45	15.1	*18.4*[2]	4.66	3.81
4α,23,24-Trimethyl-5α-cholest-4(14)-en-3β-ol	32.5	13.0	30.3	23.6	83.6	13.4	41.4	14.0	ND	ND
Dinostanol	7.39	1.20	10.5	4.50	11.3	4.20	10.6	9.34	2.37	*2.42*[2]

[1]Stations are listed in order of increasing oxicity
[2]Values in *italic* show disagreement with expected trends
ND: Not detectable

The main thrust of this study was the identification and quantification of sterols that are specific dinoflagellate biomarkers for comparing their abundances with dinocysts counts. These source biomarkers (dinosterol and dinostanol) were first characterized by GC-MS and their presence was further confirmed by ESI-MS before quantification by GC-FID Figure 2-12.

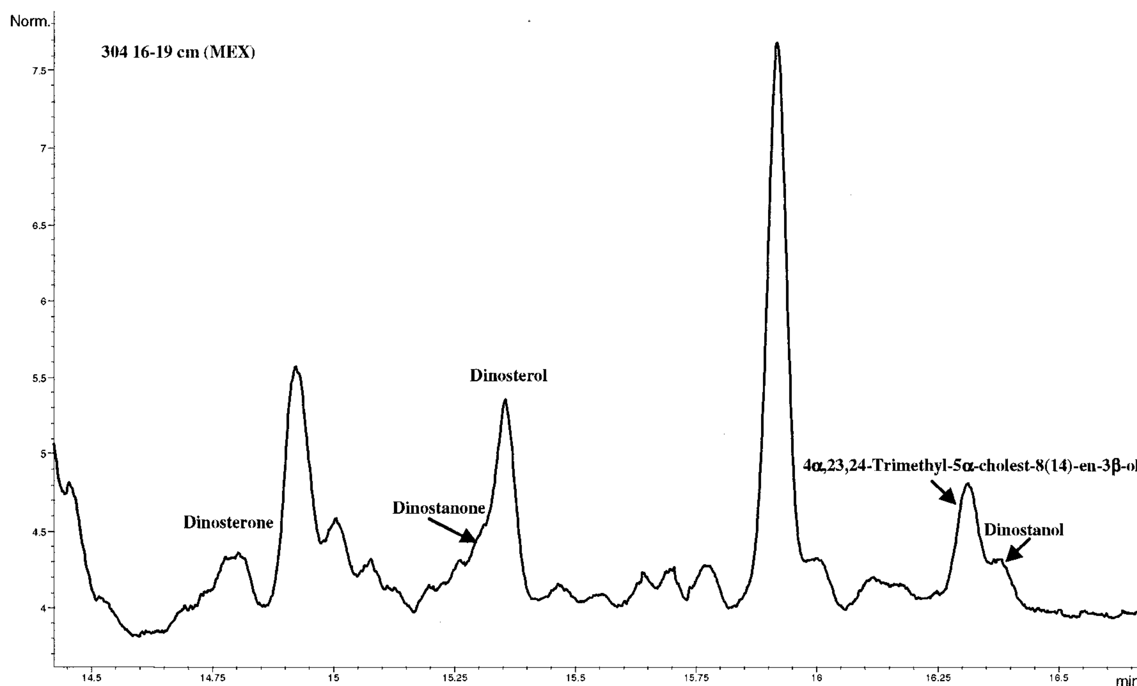


Figure 2-12: GC-FID chromatogram of sample from station 304 (western Mexican margin, 16-19 cm) used for quantification of dinoflagellate sterols.

The corresponding steroidal ketones (dinosterone and dinostanone) as well as 4α,23,24-Trimethyl-5α-cholest-4(14)-en-3β-ol were also identified (Figure 2-12), further indicating the contribution of dinoflagellates to the pool of sedimentary organic matter. Since these sterols are minor components (low in abundance), they were not taken into account when correlating the total "dino"sterols to the dinocysts concentrations.

Identification of many sterols found in the sediment samples was done based on chromatographic data published on the sterol content in marine sediments.[9,18,27,34,51,61]

The concentrations of the sterols were calculated by averaging the areas of the standard sterols (Equation 7) and then they were normalized to the OC content (Equation 9). The results obtained for the different samples are compiled in Table 2-4 and Table 2-5.

The correlation coefficient (R^2) between dionosterol and dinostanol was greater than 0.9 (Figure 2-13) further suggesting that the identification work was successfully performed.

Table 2-5: Concentrations of "Dino"sterols by GC-FID.

Station	Dinosterol (µg/g OC)	Dinostanol (µg/g OC)	Total "Dino"sterols (µg/g OC)	Total "Dino"sterols (µg/g sediment)
300 top	9.60	7.39	16.99	1.08
300 bot	2.02	1.20	3.22	0.24
306 top	17.1	10.49	27.59	2.12
306 bot	6.37	4.50	10.87	0.86
305 top	21.18	11.33	32.51	1.04
305 bot	7.45	4.20	11.65	0.38
304 top	15.12	10.61	25.73	0.61
304 bot	18.39	9.34	27.73	0.64
310 top	4.66	2.37	7.03	0.14
310 bot	3.81	2.42	6.23	0.12

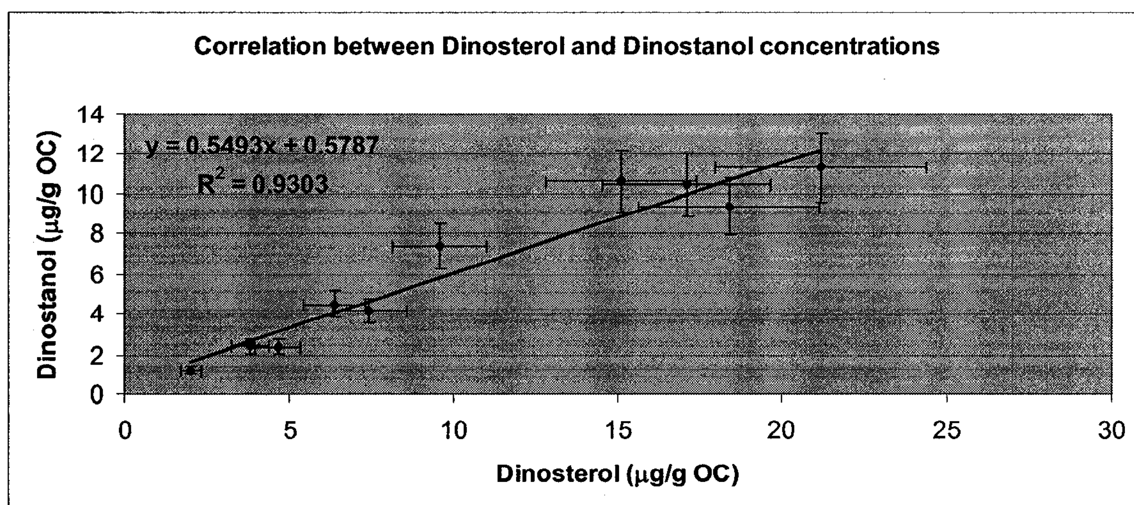


Figure 2-13: Correlation between dinosterol and dinostanol.

Using the molecular GC-FID and GC-MS methods, it was possible to identify and quantify 23 sterols including the specific markers for dinoflagellates, enabling the comparison of "dino"sterols and dinocysts concentrations.

2.5.2 Comparison of the molecular approach to the optical microscopy method

The results obtained using this optimized molecular-level analytical procedure, were compared to the dinocyst concentrations calculated by the microscopy technique. The dinocysts for the same samples as those presented in the above section (western Mexican margin sediments) were counted under an optical microscope at UQAM. Since the "dino"sterols are almost exclusively synthesized by dinoflagellates and the dinocysts are representative of this eukaryotic family, it is possible to observe a good correlation in terms of concentrations calculated using either laboratory method. Table 2-6 cumulates

the determined concentrations using each experimental means. Both values are normalized to the sediment weight (g).

Table 2-6: Concentrations of "dino"sterols (GC-FID) and dinocysts (optical microscope).

Station	Total "Dino"sterols (µg/g sediment)	Dinocysts (dinocysts/g sediment)
300 top	1.08	957
300 bot	*0.24*	*6468*
306 top	2.12	2174
306 bot	0.86	984
305 top	1.04	848
305 bot	0.38	504
304 top	0.61	76
304 bot	0.64	402
310 top	0.12	52
310 bot	0.14	292

Values in *italic* show disagreement with expected correlation; sample needs to be repeated and is excluded from correlation graph (chapter 3, Figure 4)

By analyzing the results obtained analytically, it was noticeable that stations 310 and 304 followed the inverse trend in terms of the expected course of degradation. The sediments from deeper end of the core (16-27cm) showed increased concentrations of total "dino"sterols compared to the amount found at the sub-surface (3-6cm). However, the microscopic analysis displayed similar inclination concerning the variations in dinocyst concentrations with respect to the depth of the sediment, which confirmed the unexpected results obtained by GC-FID. A positive linear correlation was observed between the two techniques and the detailed findings are further discussed in the next chapter.

In the paleoecological laboratories, the counting of palynomorphs is not limited to that of the dinocysts. Other fossils preserved in the samples were counted as well, such as pollen and spores, amongst others. Unexpectedly, similar degradation trends were

observed between the “dino”sterol and pollen concentrations (normalized to gram of sediments; Table 2-7) with a correlation coefficient greater than 0.96 (Figure 2-14).

Table 2-7: Concentrations of palynomorphs (dinocyst and pollen) and “dino”sterols.

Station	Dinocysts (dinocysts/g sediment)	Pollen (pollen/g sediment)	Total "Dino"sterols (µg/g sediment)
304 top	76	1236	0.61
304 bot	402	1385	0.64
305 top	848	3002	1.04
306 top	2174	4988	2.12

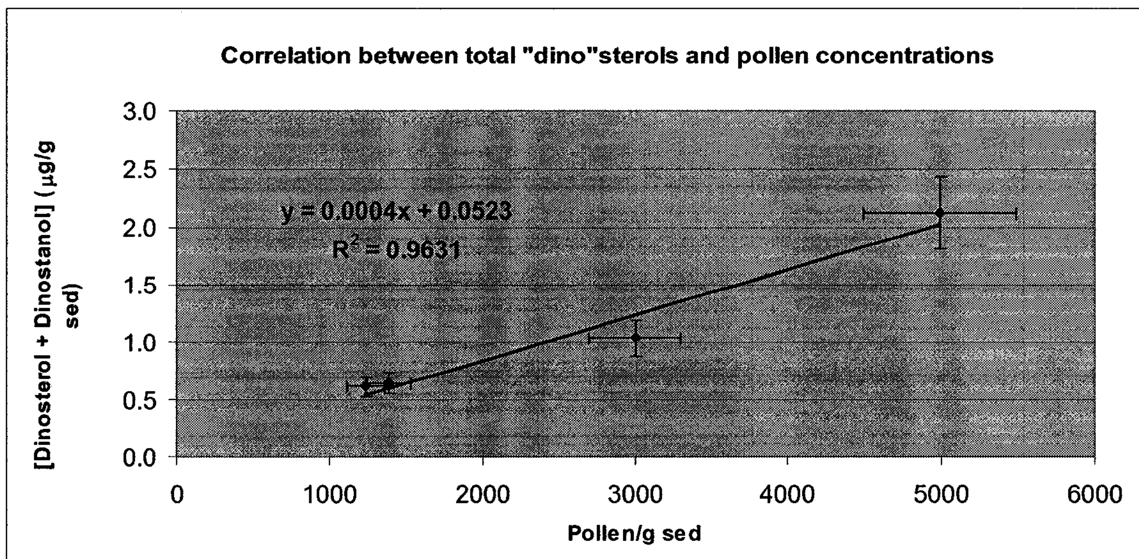


Figure 2-14: Correlation between pollen and “dino”sterols.

In order to verify the potential of pollen as a source of “dino”sterols, a fresh pollen sample collected from the Saguenay Fjord in May 2002 was analyzed. As mentioned previously, “dino”sterols are specific biomarkers for marine dinoflagellates, whereas pollen is produced by certain terrestrial plants. Therefore, such a good correlation between pollen counted microscopically and the “dino”sterol concentrations calculated analytically was unlikely.

Consequently, the analysis of pollen showed that the "dino"sterol molecules were not present in this sample and validated the specificity of the dinoflagellate biomarkers as shown in Figure 2-15. As expected, the tracers of terrestrial input were found in the pollen sample with great intensities (Figure 2-15).

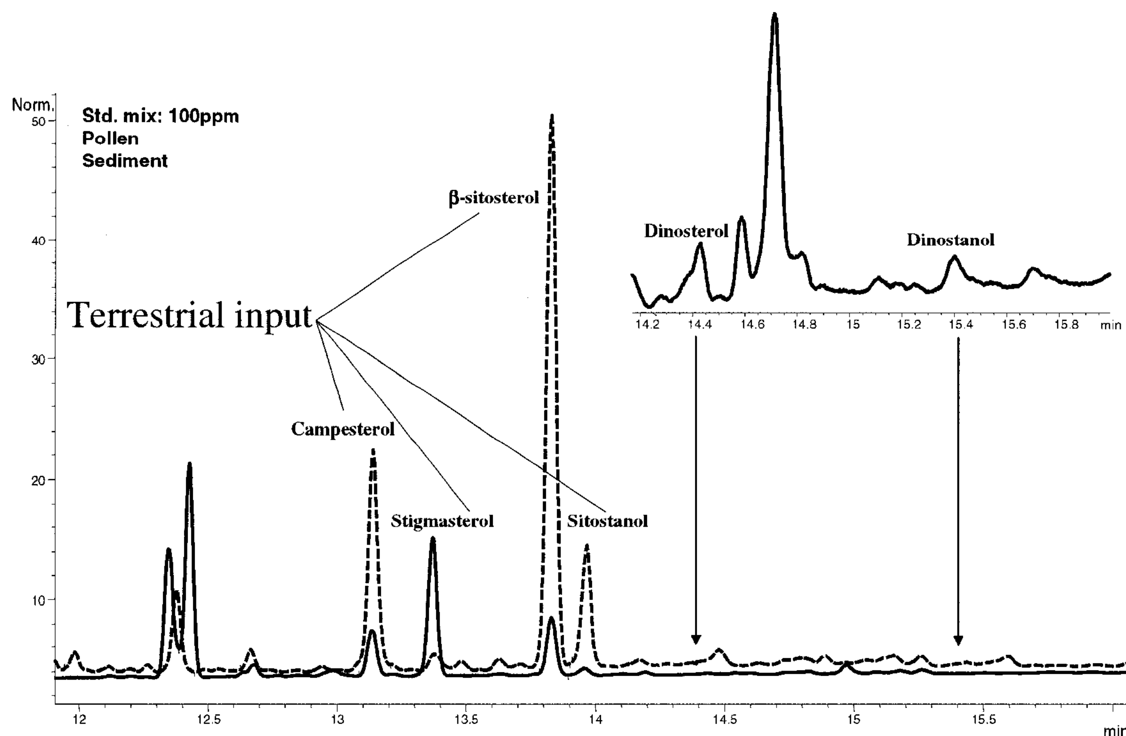


Figure 2-15: GC-FID analysis of pollen to verify sources of dinosterol and dinostanol.

In the next chapter, the findings of the comparative study will be further discussed in the form of an article. The results obtained using the two individual techniques, GC-FID and optical microscopy, will be correlated.

3. Using dinosterol to estimate dinoflagellates contribution to marine sedimentary organic matter: A comparison with total dinocysts counts

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3.1 Abstract

Sterol distribution and organic-walled dinoflagellate cysts (dinocysts) were studied in sediments from the western Mexican margin, from 375 to 3500 meters of water depth. Samples were taken from sub-surface (3-6cm) and from the depth of the sediment core (16-27cm) and differed in their organic carbon content and redox conditions. Using analytical tools (GC-MS), major sterol components were identified in sediment samples including the biomarker of interest, 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol), indicating dinoflagellate input to marine sedimentary organic matter. Along with sterol assessment in the sediments, the dinocyst concentrations were also measured for each sample. Evidence of positive correlation was observed between the molecular approach and the microscopy technique for sediment samples analyzed from the same marine environment.

3.2 Introduction

Extractable lipids compose a very small fraction of molecularly-characterizable sedimentary organic matter (Hedges *et al.*, 2000). However due to their structural diversity and specificity, an increasing number of lipids are now being used as biological markers for indicating organic matter sources and reaction pathways (Brassell and Eglinton, 1981; Lee and Wakeham, 1988; Wakeham and Lee, 1993; Hedges and Prahl, 1993; Birgel *et al.*, 2004; Nash *et al.*, 2005), and as diagnostic tools to probe variations in paleoecological (e.g. Brassell, 1993; Schefuβ *et al.*, 2004) and environmental conditions (Sikes *et al.*, 2005). Sterols in particular have attracted a lot of attention and have been used extensively as source markers for seawater particulate and sedimentary organic

matter (Mackenzie, 1982; Brassell and Eglinton, 1983; Nash *et al.*, 2005; Volkman 2005). Owing to their relative stability in marine sediments, sterols can provide a long-term sedimentary record of the relative importance of organic matter inputs from a variety of sources (Volkman, 1986). They are produced by a wide range of marine and terrestrial biological organisms, and because they are often exclusive to specific families of biota, they are extensively used as indicators for sedimentary contributions of their specific biological sources (Volkman, 1986; Volkman *et al.*, 1987; Hudson *et al.*, 2001; Nash *et al.*, 2005).

Sterols are ubiquitous in eukaryotic organisms (Jones *et al.*, 1994). They represent a significant fraction of the lipid pool in marine algae, and comprise a series of structurally similar molecules with differences in the number and position of unsaturation units, side-chain and ring methylations, as well as stereochemistry (Volkman, 1986). A subset of the sterol pool, the 4α-methyl sterols (particularly, dinosterol, or 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol), have been proposed as tracers of the dinoflagellate contribution to sedimentary organic matter (Boon *et al.*, 1979; Withers, 1983, 1987; Robinson *et al.*, 1984; Volkman *et al.*, 1998, 1999; Giner *et al.*, 2003) since they are rarely synthesized by other organisms (Volkman *et al.*, 1993).

Dinocysts are also extensively used as a proxy for the contribution of dinoflagellates to sedimentary organic matter. They consist in organic-walled capsules that protect the cell during the dormancy stage, or "hypnozygote" phase, which characterize the life cycle of many dinoflagellate species (Head, 1996). Because they are well preserved in marine sediments, dinocysts are particularly useful for reconstructing paleoenvironmental variables and processes (de Vernal *et al.*, 2001, 2005; Huber *et al.*, 2005; Stickley *et al.*,

2005). The "assemblages", or relative proportions of cysts from an array of dinoflagellate species in a sample, constitute powerful indicators of physical and chemical conditions such as nutrient availability, temperature, salinity, seasonality, or glacial coverage in the past ocean (e.g., de Vernal *et al.*, 2001, 2005).

Precise and accurate counting of different dinocysts species in a sample is tedious and requires an expertise that can be acquired only through lengthy training. Moreover, the time to prepare the sediment and to make the microscope observations represents about one day per sample. This drawback strongly limits the number of samples that can be analyzed in any given research project. Furthermore, the dinocyst recovered in sediments represent only a fraction of the original dinoflagellate populations since only 15 to 20% of the species produce fossilisable organic-walled cysts (e.g., Head, 1996). In cases where total dinoflagellate contribution to sedimentary organic carbon is needed in lieu of detailed dinocysts assemblages, using a less tedious and more widely available method would be advantageous. In this respect, while a dinosterol method using gas chromatography showed early promises (Schmitz, 1978; Djerassi *et al.*, 1979; Djerassi, 1981), recent publications report either a poor and non-linear (Marret and Scourse, 2002) or even an absence of significant correlation (Sangiorgi *et al.*, 2005) between dinocysts counts and dinosterol concentrations in marine sediments.

The main goal of this article is thus to test whether specific sterols, and in particular dinosterol and dinostanol, could be used as a proxy to estimate the total dinocyst counts, and therefore the contribution of dinoflagellates to sedimentary organic carbon, in sediments from the western Mexican margin (Pacific Ocean). The study site extends over a small geographical area (<1000 km^2) and is characterized by widely contrasting

sedimentary redox conditions (from fully oxic to sulfidic). The distribution of 23 sterols was determined in near-surface and deep layers of five sediment cores using lipid extraction and gas chromatography analysis, and their concentration was compared to total dinocysts counts obtained from the same samples using the traditional optical microscopy method.

3.3 Materials and methods

3.3.1 Sampling sites

Sediment samples were collected using a multicorer during the *R/V New Horizon* cruise in February 1999 at five stations on the western Mexican margin in the Pacific Ocean (Figure 1). These samples covered a wide range of redox conditions and organic carbon contents, from fully oxic near-surface sediments to organic-rich and sulfidic deep sediments (Table 1). Each core was sub-sampled right below the surface (3-6cm) and at a depth of 16-27cm. At an average sediment accumulation rate of about 10 mg cm^{-2} y^{-1} in the investigated area (Hartnett *et al.*, 1998), these depths correspond to ages of about 1000 and 5000 years BP, respectively. Upon collection, all samples were sliced in a N_2-purged glove box, centrifuged to remove porewater, and stored frozen at -80°C. Each subsample was homogenized and split in two parts for sterol analysis and dinocyst counting.

3.4 Analysis of sterols

3.4.1 Sample treatment

Approximately 5g of lyophilized sediment was used for the extractions. The samples were ultra-sonicated for 30 minutes and extracted three times using 200mL of a 2:1 solution of HPLC-grade dichloromethane/methanol (Hanisch *et al.*, 2003). The organic phase was collected and vacuum-filtered to remove residual solid particles from the liquid organic phase.

The extracts were roto-evaporated, recovered in HPLC-grade chloroform and passed through an activated copper column to remove elemental sulfur (Carreira *et al.*, 2002). Saponification of the eluant was performed using 25mL of methanolic KOH (0.5N) at a pH of 14 followed by the addition of 5mL of distilled water (Hwang *et al.*, 2003). The reaction reached completion after refluxing for two hours, and the product was extracted with three aliquots of 30mL dichloromethane and 10mL of a 5% NaCl solution. Sodium sulfate was added to the organic neutral lipids to remove traces of water.

The polar lipids, including the sterols of interest were isolated from the non polar ones by performing long column silica gel chromatography (Carreira *et al.*, 2002). A 25-cm column was filled with approximately 7g of deactivated silica (60-100 mesh) that was rinsed with 40mL of hexane. Straight chain and branched hydrocarbons were first eluted with hexane and 1:1 solution of dichloromethane/methanol, respectively (Carreira *et al.*, 2002). Polar lipids were then recovered using a 10% solution of methanol in dichloromethane (Carreira *et al.*, 2002) and transformed into their trimethylsilyl derivatives using a 1:1 solution of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)

and pyridine 70°C for 2 hours. Following evaporation of the resulting solution to dryness under nitrogen gas stream, the derivatized polar lipids were dissolved in 200μL HPLC-grade chloroform before analysis by GC-MS or GC-FID.

3.4.2 GC-MS analysis

Sterols identification was achieved using a Varian Saturn CP-3800/2200 ion trap GC-MS. The instrument was fitted with a split/splitless injector set at 320°C and a 30m × 0.32mm I.D. DB-5 column (95% dimethylpolysiloxane with 5% phenyl), with a film thickness of 0.25μm. A constant carrier gas (helium) flow rate of 1.3mL/min was used throughout the analysis. The initial oven temperature was set at 45°C and then ramped at a rate of 30°C/min. to 255°C where it was held for 10 minutes. Three additional ramps followed (to 257°C at 1°C/min., then to 271°C at 4°C/min, and finally to 310°C at 20°C/min.) before the last hold time of 10 min. Electron-impact mass spectra (70eV) were recorded between 50 and 600 m/z at 1 scan s^{-1}.

Quantification was done using an Agilent GC-FID model 6890N also fitted with a DB-5 column. A constant carrier gas (helium) flow rate of 2mL/min and an inlet split ratio of 50:1 were used throughout. The initial oven temperature was set at 45°C and increased to 281°C at 30°C/min, followed by a 4°C/min ramp to 310°C where it was held for 10 min. The FID was set at 315°C and the injector temperature was initially programmed at 65°C (1 min.), and then ramped to 305°C at 1°C/sec, where it was held for 10 min.

3.4.3 Quantitation

Standard mixtures of sterols purchased from Aldrich Chemicals were prepared and contained: cholest-5-en-3β-ol (cholesterol, 95% purity), 5α-cholestan-3β-ol (dihydrocholesterol, 95% purity), 24-methyl-5α-cholest-22-en-3β-ol (ergosterol, 95% purity), 24-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol, 93% purity) and 24-ethylcholest-5-en-3β-ol (β-sitosterol, 40% purity; containing 20-30% campesterol, 10-30% dihydrobrassicasterol, 10-30% stigmasterol). Identification of many sterols found in the sediments was done based on sedimentary sterol chromatographic data published elsewhere (Volkman, 1986; Jones *et al.*, 1994; Volkman *et al.*, 1998; Leblond and Chapman, 2002, 2004; Sangiorgi *et al.*, 2005).

Sterol quantification was achieved through calibration of peak areas (GC-FID trace) for a series of standard solutions (25 to 250 μg/mL) containing the sterols listed above. Typical correlation coefficients were above 0.98, and the absolute limit of detection (LOD) was estimated at 25ng. The response factors of sterols for which no commercial standards are available were calculated by averaging the areas of the standard sterols. The method was validated (linearity, detection limits and reproducibility) using either standard sterol mixtures of known concentrations or spiked and natural samples. Reproducibility of the overall procedure (extraction, purification, derivatization and GC analysis) was determined by analyzing three aliquots of natural and spiked (100mg/L of the standard sterol mixture) sediments collected in the Saguenay Fjord in May 2002 (station Sag 30). The average relative standard deviation was ±15%. There was no carry over of sterols detected in procedural blanks at the end of each run. Due to limited sample

availability and to the large quantity of sediments required for the accurate quantification of sterols and dinocysts (about 5g each), each sample could only be analyzed once.

3.5 Analysis of dinocysts

Dinocysts were counted by graduate students at the Université du Québec à Montréal (Département des Sciences de la Terre et de l'Atmosphère) following the method detailed in de Vernal *et al.* (1996). Briefly, about 5cm^3 of wet sediment was spiked with a capsule of marker grains of lycopode spores, belonging to the species *Lycopodium clavatum* (10679 spores/capsule). Each sample was passed through two stacked sieves (120µm and 10µm), and the fraction between 120µm and 10µm was recovered and centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and the sample was treated with about 2mL of 10% HCl (20 min. at 50-60°C). The HCl supernatant was removed by centrifugation and about 2mL of 49% HF was added (overnight, room temperature). The samples were finally treated with about 2mL of 10% HCl, after removing the HF supernatant by centrifugation, and sieved once more to collect the fraction between 120µm and 10µm, and centrifuged at 2000 rpm for 10 min. to recover the pellet. Sub-samples of the pellet were mounted on microscopic slides embedded in glycerine jelly. Depending on the cyst density, a few lines to the entire slide area (22 X 45 mm) was scanned using an optical microscope with a magnification power of 250× to 1250× to identify and count dinocysts, *Lycopodium* spores and other palynomorphs. Usually, a minimum of 200 dinocysts is counted in each sample for further calculation of species percentages.

3.6 Results and discussion

3.6.1 Sterols fingerprint and concentrations

Because all sterols have a similar 4-ring backbone and a side chain of 8-10 carbon atoms, they all elute within a narrow time window during GC analysis, i.e., the 'sterol window'. More than 35 peaks were found in most samples within the sterol eluting region, and more than 20 of them were identified either through GC-MS analysis or comparison with literature data (Figure 2 and Table 2). No further attempt was made at identifying the remaining peaks since this was not within the scope of this study. Sterols that were successfully identified comprised most of the more intense peaks, namely cholest-5-en-3β-ol (peak #6, cholesterol), 24-methylcholesta-5,22*E*-dien-3β-ol (peak #9, brassicasterol or dihydrobrassicasterol) and 24-ethylcholesta-5,22*E*-dien-3β-ol (peak #14, stigmasterol). The 5α(H)-stanol counterparts of the above sterols (peaks # 7, 13 and 15, respectively) also generated quantifiable signals.

In the majority of the investigated samples, the peak for the C_{30}-sterol 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol (peak #21, dinosterol) is intense, indicating a significant contribution of dinoflagellates to these sediments. Dinosterol is the most reliable biomarker for dinoflagellates even though it is a minor component in some dinoflagellate species and completely absent in others (Withers, 1987; Volkman *et al.*, 1993; Leblond and Chapman, 2002; Sangiorgi *et al.*, 2005). Also present at lower abundances are the corresponding stanol, 4α,23,24-trimethyl-5α-cholestan-3β-ol (peak #24, dinostanol), the steroidal ketones 4α,23,24-trimethyl-5α-cholest-22-en-3-one and 4α,23,24-trimethyl-5α-cholestan-3-one (peaks #16 and #20, dinosterone and

dinostanone, respectively), as well as a $C_{30}\Delta^8$-sterol, 4α,23,24-trimethyl-5α-cholest-8(14)-en-3β-ol (peak #23). These minor components also are all indicative of dinoflagellate inputs to the sediments.

The concentrations of the 23 sterols identified in these samples are presented in Table 3. Similar trends were found for most stations with higher concentrations in the near-surface than at depth, suggesting slow degradation or alteration of sterols with time. Because a large fraction of the organic matter pool is more efficiently degraded in oxygenated, rather than anoxic, conditions (Gélinas *et al.,* 2001) we also compared sterol concentrations in sediments accumulating under an oxygen-depleted water column (stations 300 and 306), to those in sediments exposed to oxygenated bottom water (stations 304, 305 and 310). No general trend was found, suggesting that redox conditions do not represent a major constraint on sterol preservation in this environment.

The major compounds, namely cholest-5-en-3β-ol (cholesterol), 24-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol or dihydrobrassicasterol), 24-Ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol) and 4α,23,24-Trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol), accounted for 62 to 75% of the total sterol content measured in the samples. The concentration of 24-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol), a widely used biomarker for diatoms and other algae (Volkman, 1986), is high in all samples but station 310, representing more than 40% of the total sterol content and suggesting a relatively high, diatomaceous dominated productivity in the surface waters. A small contribution of terrigenous organic matter is also suggested by the presence of the $29\Delta^{5,22}$ sterol 24-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol) in all the sediments, with a relative contribution to the total sterol content that decreases from stations closer

to shore (300 and 310, >20%), to stations further offshore (304 and 305, <10%). Stigmasterol is the major sterol compound in higher plants (Volkman, 1986), although it can also be found in some diatoms (Wardroper *et al.*, 1978; Volkman *et al.*, 1998).

The sterol fingerprints in sediments from the different stations thus suggest that the major fraction of the organic carbon pool is autochthonous and dominated by diatom inputs to the sediments, while terrestrial sources might be significant, especially at station 310.

3.6.2 Dinocysts

Thirty four species of dinocysts were identified in the samples, with fairly uniform relative proportions of each species at the different sampling sites. The most abundant dinocysts were those of the heterotrophic taxa *Brigantedinium* spp. and *Selenopemphix quanta*. These are cysts of *Protoperidinium* spp. and *Protoperidinium conicum*, respectively. The most abundant autotrophic species were *Operculodinium centrocarpum* (cyst of *Protoceratium reticulatum*), *Spiniferites* spp., as well as *Bitectatodinium spongium* (cyst of *Gonyaulax* spp.), *Lingulodinium machaerophorum* (cyst of *Lingulodinium polyedrum*), *Pentapharsodinium dalei* and *Polysphaeridium zoharyi*. On average, the above species contributed at least 70% of the total counts of dinocysts in these samples (Figure 3).

3.6.3 Comparison of dinosterol and dinocysts distributions

We found a positive linear correlation ($R^2 = 0.88$) between total dinocyst counts and [dinosterol + dinostanol] concentrations when both are normalized to gram of sediment (Figure 4). Although this coefficient falls to 0.65 when the near-surface sample

from station 306 is excluded, the slope of the regression remains the same, suggesting that the data point for station 306 top is not an outlier. This strong correlation is in disagreement with recent results published by Marret and Scourse (2002), who suggest an exponential relationship between dinocysts and dinosterols in samples from the Celtic and Irish Seas. They also disagree with the findings of Sangiorgi *et al.* (2005) who failed to find any significant correlation between dinocyst counts and dinosterol concentrations for sediments of the northern Adriatic Sea close to the mouth of the Po River.

A potential reason for these discrepancies might be the large geographical area covered in the first study (the Irish and Celtic Seas): because not all dinoflagellates synthesize dinosterols (Withers, 1987; Volkman *et al.*, 1993; Leblond and Chapman, 2002; Sangiorgi *et al.*, 2005), and produce organic-walled cysts (Dale, 2001), differences in surface seawater conditions or in productivity may alter the relationship between dinocysts and dinosterol. Such potential limitation makes difficult direct comparisons between very different environments, such as the Mexican margin, the Celtic or Adriatic Seas.

In the study of Sangiogi *et al.* (2005), the absence of a correlation is simply due to the fact that dinocyst counts and dinosterol concentrations were not normalized in the same way (dinocyst counts per gram of sediment and μg dinosterol per gram of organic carbon, respectively). Hydrodynamic sorting of particles, which is particularly important in the vicinity of large river mouths (the Po River mouth in this case), probably lead to contrasting mineral dilution at each station. The decoupling between dinosterols and dinocysts in this case is due to the fact that the total sediment normalized parameter (dinocysts counts) is affected by mineral dilution, while the OC normalized one

(dinosterols) is not. With the exception of one sample (CH50, a probable outlier), the best fit for the regression between dinocysts counts and dinosterol concentrations for all the Adratic Sea samples is linear with a correlation coefficient of 0.78.

The positive linear correlations found in our, and Sangiorgi's, studies thus suggest that (*i*) dinosterol is a good proxy for estimating variations in dinoflagellate inputs to marine sediments, in agreement with early studies reporting on the specificity of dinosterol for dinoflagellate organisms (Schmitz, 1978; Djerassi *et al.*, 1979; Djerassi, 1981) and (*ii*) the two independent methods for measuring dinocysts and dinosterols provide internally consistent data. The rate of degradation of dinosterols and dinocysts appears similar in oxic and anoxic environments (at least on the time scales covered by this study, i.e. ~5000 years), as suggested by the similar dinocysts-dinosterol relationship in highly oxidized vs. non-oxidized samples (sample 304 16-19 cm was exposed to oxic conditions for >1000 years, while sediments from stations 300 and 306 accumulated in anoxic bottom water conditions). Although still speculative at this point, it thus appears that dinosterol is a robust proxy for estimating time-dependent changes of dinoflagellate inputs into sediments over small scale geographical areas, but that biases may arise when comparing samples from large or widely contrasting geographical areas. These biases could be due to differences in sea surface productivity or conditions which may induce variations in the relative contribution of the different dinoflagellate species, or modify the proportions of cells entering the cyst-producing dormant stage of their life cycle. Such differences in productivity might also have played a role in the scattering of the data points in Figure 4.

3.7 Conclusions

The internal consistency between dinocyst counts and [dinosterol + dinostanol] concentrations data suggests that both methods provide a fairly robust, semi-quantitative proxy for the total contribution of dinoflagellates to marine sediments collected over small geographical areas and time scales of a few thousands of years. In cases where detailed dinocyst assemblages are not needed, the more commonly available and less tedious GC-based method could thus replace the microscopy-based cyst counting method used in paleoecological laboratories. More work should be undertaken to estimate the sensitivity of the dinocyst-dinosterol relationship to longer time scales and larger geographical scales, and to determine which proxy, dinosterol or dinocysts, provides better estimates of dinoflagellates contributions to sedimentary organic matter in these conditions. Although promising for studies such as this one, the relationship between dinoflagellates and dinosterol or total dinocyst counts most likely is not universal and thus, it should be used with caution.

3.8 Acknowledgements

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Table 1: Characteristics of samples from the western Mexican margin used in this study

Sampling Station	Coordinates	Water Column Depth (m)	Sediment Depth (cm)	Organic Carbon (%)	Porewater Redox Conditions
300	Lat.: 25°19.71 N Long.:112°46.0W	387	3-6	6.39	Suboxic
			16-27	7.49	Anoxic
306	Lat.: 22°43.23 N Long.:106°28.9W	375	3-6	7.70	Suboxic
			16-27	7.86	Anoxic
305	Lat.: 22°11.41 N Long.:107°19.0W	2990	3-4	3.27	Suboxic
			19-22	3.20	Suboxic
304	Lat.: 21°34.34 N Long.:108°18.5W	3070	3-4	2.38	Oxic
			16-19	2.30	Suboxic
310	Lat.: 19°06.79 N Long.:06°14.61W	3500	3-4	1.96	Oxic
			16-19	1.87	Suboxic

Table 2: Compound assignment for GC-MS peaks reported in Figure 2

Peak #	Compound	Common name
1	5β-Cholestan-3β-ol	Coprostanol
2	n-C28-alcohol	~
3	27-*nor*-24-Methylcholesta-5,22*E*-dien-3β-ol	Occelasterol
4	Cholesta-5,22*E*-dien-3β-ol	Dehydrocholesterol
5	5α-Cholest-5,22*E*-en-3β-ol	Dehydrocholestanol
6	Cholest-5-en-3β-ol	Cholesterol
7	5α-Cholestan-3β-ol	Cholestanol
8	n-C29-alcohol	~
9	24-Methylcholesta-5,22*E*-dien-3β-ol	(Dihydro)Brassicasterol
10	24-Methyl-5α-cholest-22-en-3β-ol	Ergosterol
11	24-Methylcholesta-5,24(28)-dien-3β-ol	24-methylenecholesterol
12	24-Methylcholest-5-en-3β-ol	Campesterol
13	24-Methyl-5α-cholestan-3β-ol	Dihydrobrassicastanol
14	24-Ethylcholesta-5,22*E*-dien-3β-ol	Stigmasterol
15	24-Ethyl-5α-cholest-22-en-3β-ol	Stigmastanol
16	4α,23,24-Trimethyl-5α-cholest-22-en-3-one	Dinosterone
17	24-Ethylcholest-5-en-3β-ol	β-sitosterol
18	24-Ethyl-5α-cholestan-3β-ol	Sitostanol
19	24-Ethylcholesta-5,24(28)-dien-3β-ol	Fucosterol
20	4α,23,24-Trimethyl-5α-cholestan-3-one	Dinostanone
21	4α,23,24-Trimethyl-5α-cholest-22*E*-en-3β-ol	Dinosterol
22	Unknown?	~
23	4α,23,24-Trimethyl-5α-cholest-8(14)-en-3β-ol	~
24	4α,23,24-Trimethyl-5α-cholestan-3β-ol	Dinostanol

Table 3: Sterol concentrations in sediments from the western Mexican margin

Stations[1]	300 top	300 bot	306 top	306 bot	305 top	305 bot	304 top	304 bot	310 top	310 bot
Coprostanol	ND	ND	ND	ND	ND	1.35	1.71	3.13	ND	ND
n-C28-alcohol	2.68	0.98	4.20	3.18	ND	14.0	4.11	34.9	1.68	1.62
Occelasterol	3.40	1.90	2.72	1.27	29.9	6.28	ND	ND	ND	4.93
Dehydrocholesterol	1.28	ND	1.16	0.69	9.05	9.08	7.92	18.0	1.23	1.97
Dehydrocholestanol	33.0	6.33	42.1	16.7	39.3	6.28	6.34	9.09	2.72	3.33
Cholesterol	143	19.3	194	46.4	635	17.1	140	67.5	616	1057
Dihydrocholesterol	20.5	2.17	21.3	6.69	28.8	8.59	55.1	25.6	ND	ND
n-C29-alcohol	7.72	ND	8.92	2.80	4.61	14.0	ND	2.76	ND	ND
(Dihydro)brassicasterol	1008	158	1364	514	708	229	600	1431	ND	ND
Ergosterol	78.4	ND	124	66.3	232	56.1	54.2	85.4	116	18.6
24-methylenecholesterol	30.2	11.9	45.9	37.1	42.5	13.8	15.7	30.0	114	37.0
Campesterol	96.5	47.2	53.2	55.2	218	14.7	166	81.7	540	256
Ergostanol	21.3	21.3	3.88	22.4	26.0	0.60	13.2	6.45	205	63.1
Stigmasterol	67.5	171	70.1	125	80.6	13.8	192	71.3	1712	87.0
Stigmastanol	16.7	22.8	20.4	30.0	78.8	7.63	30.5	13.9	317	14.6
Dinosterone	23.7	1.96	32.1	16.3	21.2	8.08	11.4	8.48	0.39	2.28
β-sitosterol	17.0	5.38	30.4	13.4	85.2	6.19	128	40.9	14.7	13.5
Sitostanol	29.7	3.68	18.7	11.4	38.2	3.16	37.2	15.1	14.1	24.2
Fucosterol	22.7	3.64	16.8	16.2	71.0	0.79	25.0	8.54	5.51	4.78
Dinostanone	27.6	1.51	57.2	9.18	24.7	5.33	5.69	21.5	4.28	9.00
Dinosterol	9.60	2.02	17.1	6.37	21.2	7.45	15.1	18.4	4.66	3.81
4α,23,24-Trimethyl-5α-cholest-4(14)-en-3β-ol	32.5	13.0	30.3	23.6	83.6	13.4	41.4	14.0	ND	ND
Dinostanol	7.39	1.20	10.5	4.50	11.3	4.20	10.6	9.34	2.37	2.42

[1]Stations are listed in order of increasing oxicity
ND: Not detectable

Figure captions

Figure 1: Sampling area showing the five stations used in this study (western Mexican margin).

Figure 2: Partial GC-MS chromatogram showing the elution window for sterol TMS-esters extracted from a typical sample. Numbers correspond to compounds listed in Table 2.

Figure 3: Relative abundances (% dinocysts) of the major dinoflagellate species in the sediment samples.

Figure 4: Correlation between [dinosterol + dinostanol] concentrations (μg/g sediment) and dinocyst concentrations (cysts/g sediment).

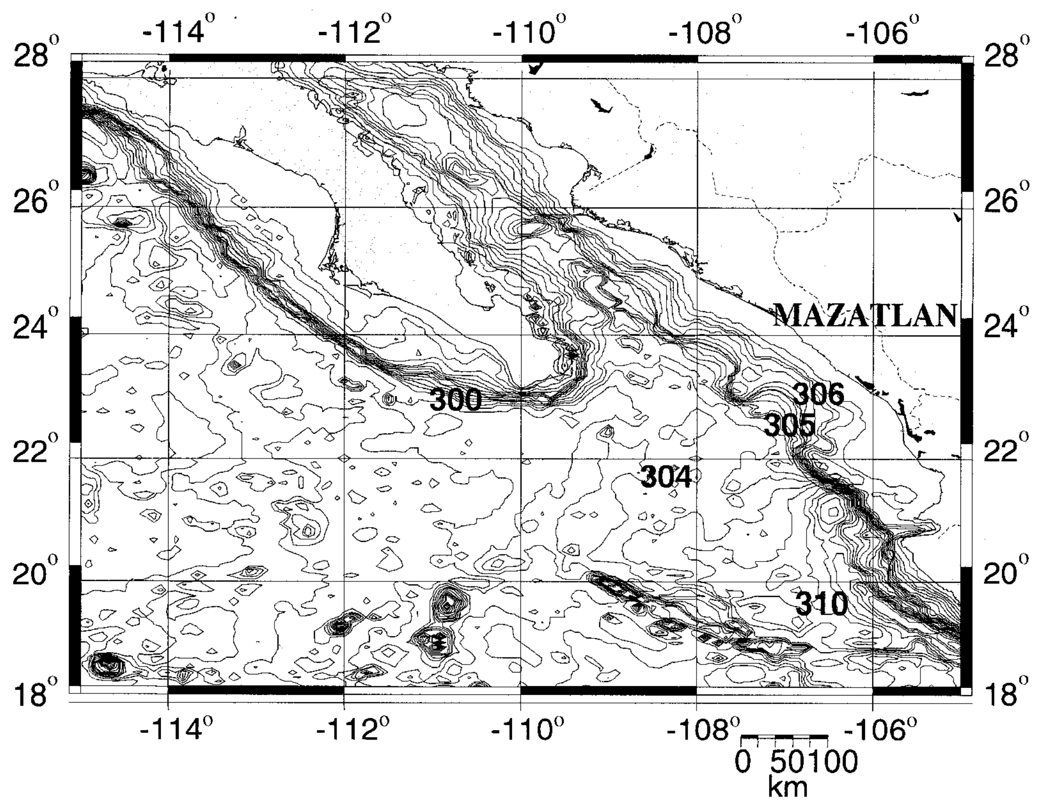

Figure 1.

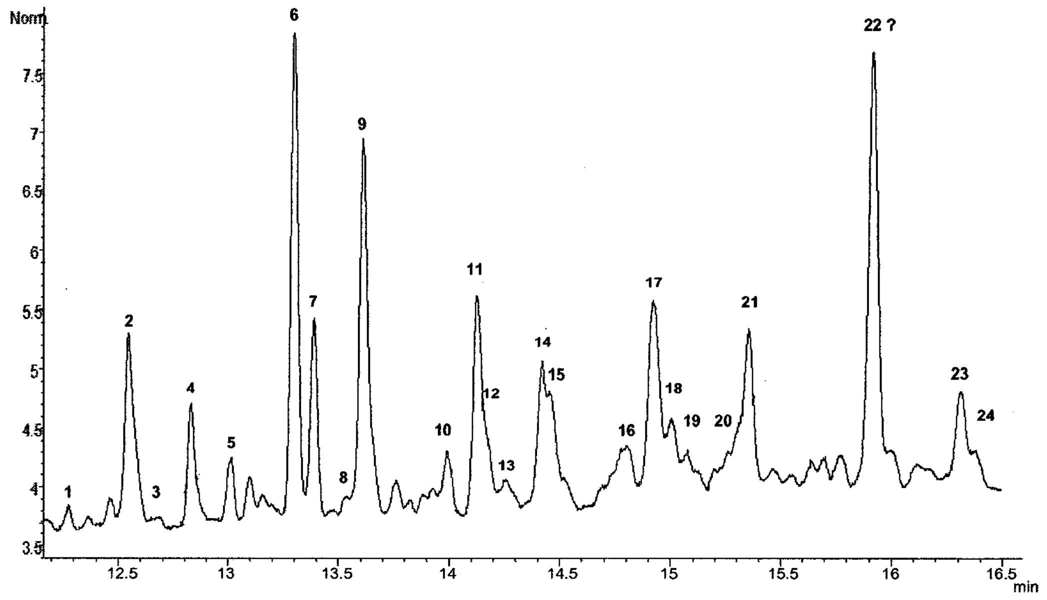

Figure 2.

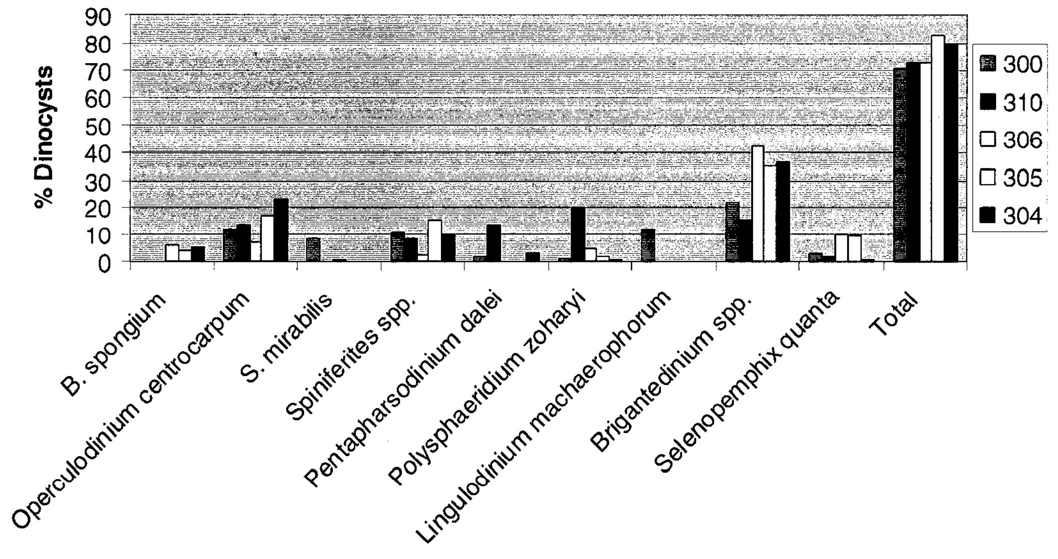

Figure 3.

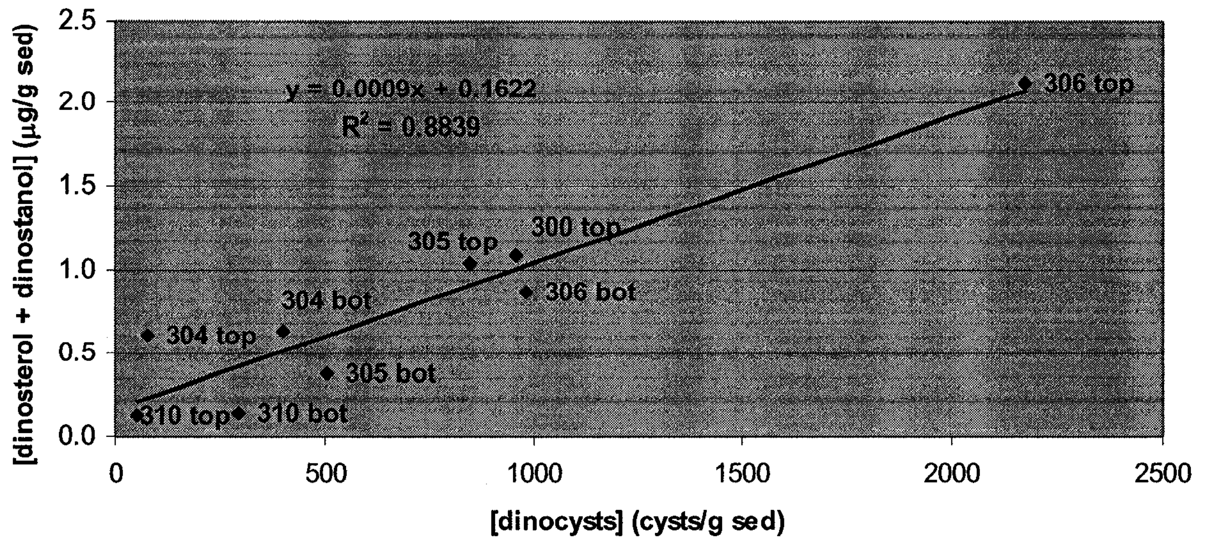

Figure 4.

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4 General Conclusions and Future Work

4.1 General Conclusions

The optimized GC-MS method allowed the identification and quantification of 23 sterols found in natural samples from five contrasting stations (within a radius of ~100km) of the Mexican margin, near Mazatlan (Pacific Ocean). These sediments represent an array of environments ranging from organic carbon enriched zones closer to the shore (bottom water depleted in oxygen) to offshore areas where organic matter contents are low (bottom water enriched in oxygen). At each station, samples were analyzed from just below the surface of the sediment (3-6cm), as well as deeper in the sediment (16-27cm).

In general, the abundance of sterols examined in the investigated stations followed the expected trend, such that increased degradation was observed in the deeper sediments due to the slow degradation rate of sedimentary organic matter (5000 years old at 20cm). Whereas, enhanced primary productivity in the surface waters lead to steady inputs of fresh organic matter close to the sediment-water interface accounting for increased sterol concentrations in the subsurficial samples.

The "dino"sterols were quantified and the concentrations were then compared to the measures of dinocyst assemblages obtained by counting them microscopically. A positive linear correlation (R^2=0.88) between the two dinoflagellate tracers was observed in the investigated environment. Hence, this internal consistency suggests that both methods provide a semi-quantitative proxy for dinoflagellate contributions to marine sedimentary organic matter over a restricted geographical area. The molecular GC

method could replace the tedious microscopy counting technique, only when extensive information about dinocyst assemblages is not necessary. Although this positive relationship between dinosterol and dinocysts shows promising results for future studies of similar nature, it is most likely not widespread and therefore should be used with care.

4.2 Future Work

Masters are short-term research projects in which answering questions that arise from lab work unfortunately cannot all be explored and answered. In this case, two levels of information should be sought before being able to say that the project is finalized: further optimization of some analytical aspects and better understanding of the biogeochemical potential of the method developed here.

Analytically, more work has to be done on the identification of dinoflagellate-derived sterols. For instance, samples of cultured dinoflagellates or sediments enriched with these planktonic organisms could be analyzed using either chemical ionization-mass spectrometry (CI-MS) and tandem mass spectrometry (MS/MS) to obtain the exact molecular weight and chemical structure of the sterol. ESI-MS techniques should also be explored to determine whether quantitative information on dinosterol concentrations could be obtained from complex solutions such as sediment extracts.

Also, the analysis of replicate samples from the same environment is essential in order to better assess the precision associated with the positive linear correlation observed between the [dinosterol + dinostanol] and the dinocysts, and to determine whether the spread observed in the graph showing the relationship (Chapter 3, Figure 4) is due to variability in dinocyst counts, dinosterol concentrations, or both. Choosing an appropriate absolute recovery standard for the extraction and sterol analysis is also desirable in order

to correct for systematic and random errors which may have occurred but that unfortunately could not be quantified in this work.

Biogeochemically, a survey of surface sediments all over the world ocean would be needed to assess the extent to which the correlation identified here can be extrapolated to other environments (wide geographical scale). The hypothesis of a decoupling of dinocyst counts and dinosterol concentrations at varying productivity intensities should also be tested. Parallel to this investigation, it should also be determined which one of the two proxies, dinocysts or dinosterols, provide a better estimate of dinoflagellate inputs to sediments when such decoupling occurs. Samples from several depths, including the surface layer (0-1cm) should also be analyzed at selected stations to obtain more detailed profiles and identify particular trends in sterol content and degradation.

Biomarkers have great potential for studying the past environments in biogeochemistry. Unfortunately, fully understanding the factors that affect their behaviour, and thus the information that they provide, is a very complicated task. This work helped advance our comprehension of dinosterols (particularly in relation with dinocysts) in ways that hopefully will prove useful in the near future.

Appendix A

Research has shown that amphisterol, 4α-methyl-24-methylenecholest-8,14-en-3β-ol **(4)**, is an intermediate in the biosynthesis of dinosterol (Figure A-1). The 24-methylene is a precursor in the dinosterol side chain formation.[62]

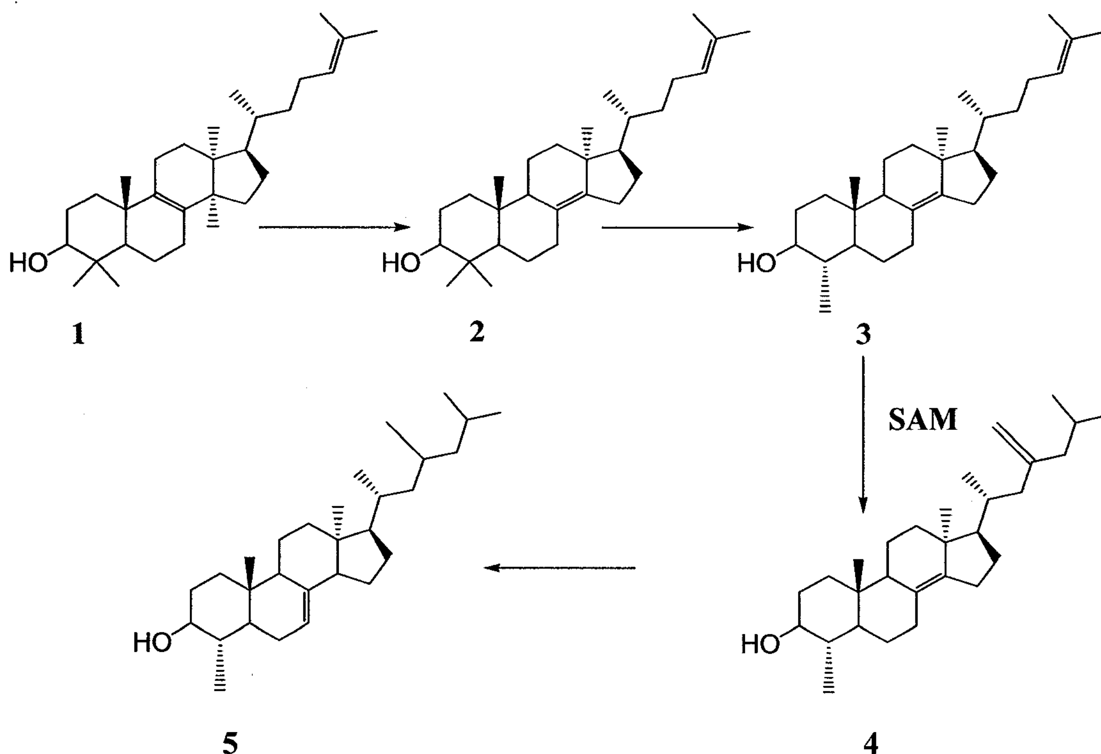

Figure A-1: Postulated scheme for partial biosynthetic sequence of dinoflagellate sterols.

The first two steps are demethylation steps at C-14 and C-4, respectively. The next step involves the side chain alkylation using SAM (S-adenosylmethionine). Finally, the 8,14 double bond is hydrogenated and migrated to position 7,8.[48]

The following mechanisms have been broken down into the ring saturation from $\Delta^{7,8}$ precursor (Figure A-2) and the alkylation of the side chain in dinosterol synthesis (Figure A-3), for clarity purposes.

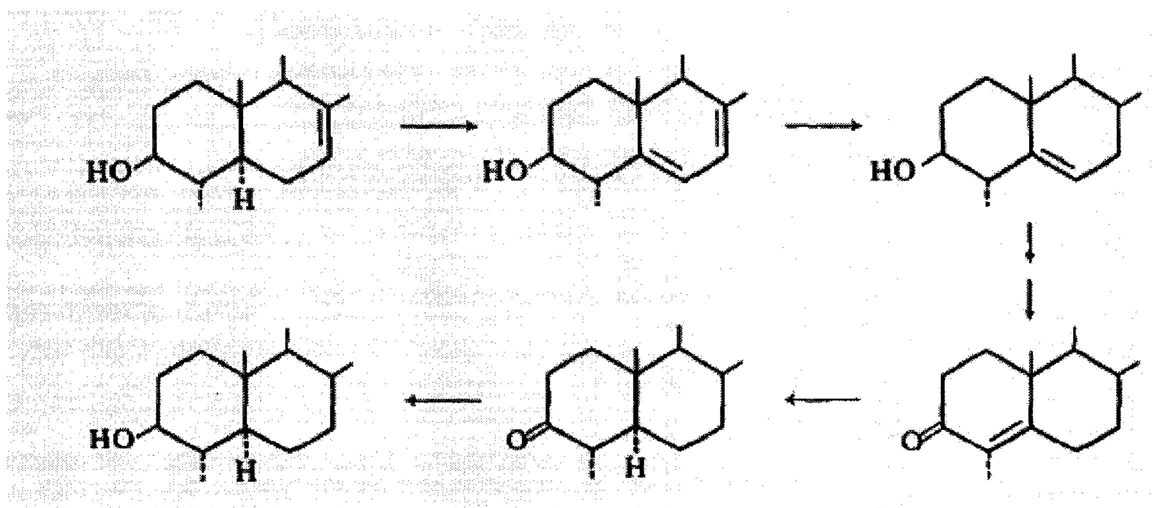

Figure A-2: Possible route for the saturation of the sterol ring system in dinosterol production.[63]

The isolation of Δ^5-dinosterol and dinosterone from *Crypthecodinium cohnii* led to the pathway shown in Figure A-2. Goad postulated that Δ^7-unsaturated sterol, 4α,24S-Dimethyl-5α-cholest-7-en-3β-ol (**5**: Figure A-1) is a precursor for the ring saturation mechanism in dinosterol production (Figure A-2).[48]

Withers *et al.* investigated the biosynthesis pathway of dinosterol from *Crypthecodinium cohnii.* They showed the sequence of side chain alkylation by conducting an experiment using methionine-[CD_3][23] (Figure A-3). The heterotrophic dinoflagellate was cultured in a nutrient medium containing methioine-[CD_3], which enabled the identification (MS) of the incorporated deuterium into the synthesized dinosterol molecule. Three deuterium atoms are transmethylated directly from methionine to the C-23 methyl group, whereas only two deuterium atoms are shown in the C-24 methyl group. However, this observation is consistent with the formation of a 24-methylenesterol intermediate which is then reduced to form the 24-methyl side chain found in the dinosterol (Figure A-3).[62]

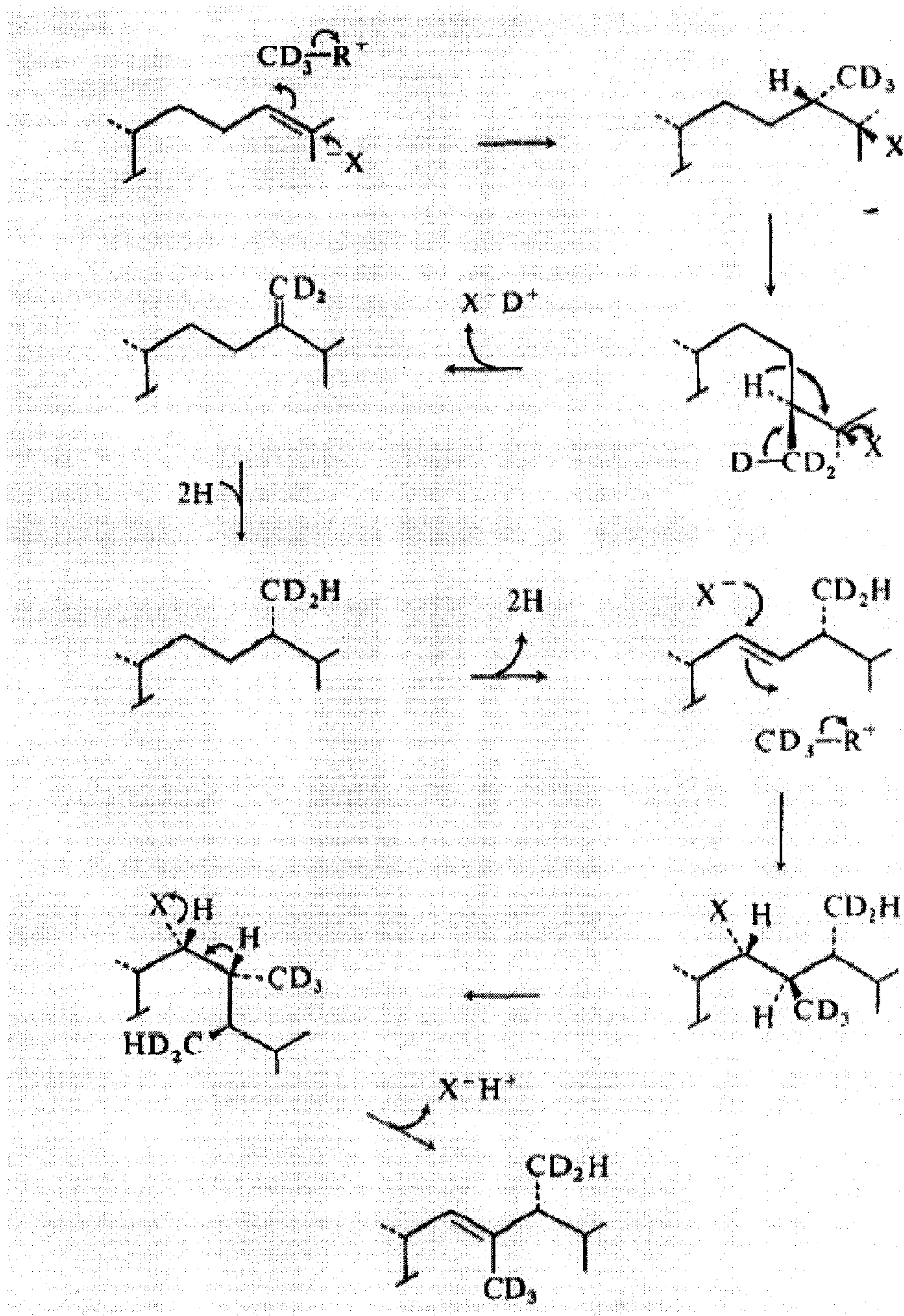

Figure A-3: Postulated mechanism for biosynthetic side chain alkylations at C-23 and C-24 in dinosterol production in *C. cohnii*.[62]

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