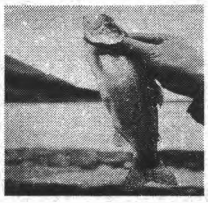
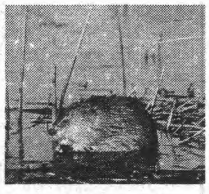
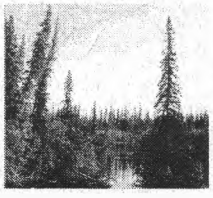
Canada Alberta Northwest Territories

Northern River Basins Study



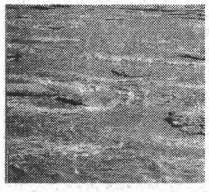


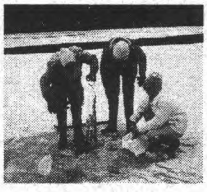
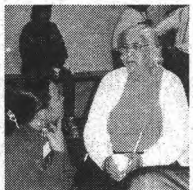




NORTHERN RIVER BASINS STUDY PROJECT REPORT NO. 60

EVALUATION OF SMALL VOLUME TECHNIQUES FOR BROAD SPECTRUM ANALYSIS OF BIOFILM MATERIALS AND BLEACHED KRAFT MILL EFFLUENTS



Prepared for the
Northern River Basins Study
under Project 2617-C1

by

John V. Headley, Patricia A. Chambers,
Joseph M. Culp and Kerry M. Peru
National Hydrology Research Institute

NORTHERN RIVER BASINS STUDY PROJECT REPORT NO. 60

EVALUATION OF SMALL VOLUME TECHNIQUES FOR BROAD SPECTRUM ANALYSIS OF BIOFILM MATERIALS AND BLEACHED KRAFT MILL EFFLUENTS

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PREFACE:

The Northern River Basins Study was initiated through the "Canada-Alberta-Northwest Territories Agreement Respecting the Peace-Athabasca-Slave River Basin Study, Phase II - Technical Studies" which was signed September 27, 1991. The purpose of the Study is to understand and characterize the cumulative effects of development on the water and aquatic environment of the Study Area by coordinating with existing programs and undertaking appropriate new technical studies.

This publication reports the method and findings of particular work conducted as part of the Northern River Basins Study. As such, the work was governed by a specific terms of reference and is expected to contribute information about the Study Area within the context of the overall study as described by the Study Final Report. This report has been reviewed by the Study Science Advisory Committee in regards to scientific content and has been approved by the Study Board of Directors for public release.

It is explicit in the objectives of the Study to report the results of technical work regularly to the public. This objective is served by distributing project reports to an extensive network of libraries, agencies, organizations and interested individuals and by granting universal permission to reproduce the material.

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Whereas the above publication is the result of a project conducted under the Northern River Basins Study and the terms of reference for that project are deemed to be fulfilled,
IT IS THEREFORE REQUESTED BY THE STUDY OFFICE THAT;
this publication be subjected to proper and responsible review and be considered for release to the public.

F. J. Wrona — 14 Sept 95
(Dr. F. J. Wrona, Ph.D., Science Director) (Date)

Whereas it is an explicit term of reference of the Science Advisory Committee "to review, for scientific content, material for publication by the Board",
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P. A. Larkin — 18 Sept 95
(Dr. P. A. Larkin, Ph.D., Chair) (Date)

Whereas the Study Board is satisfied that this publication has been reviewed for scientific content and for immediate health implications,
IT IS HERE APPROVED BY THE BOARD OF DIRECTORS THAT;
this publication be released to the public, and that this publication be designated for: **[ ] STANDARD AVAILABILITY [ ] EXPANDED AVAILABILITY**

Lucille Partington — 14 Sept. 95
(Lucille Partington, Co-chair) (Date)

Robert McLeod — 14/09/95
(Robert McLeod, Co-chair) (Date)

EVALUATION OF SMALL VOLUME TECHNIQUES FOR BROAD SPECTRUM ANALYSIS OF BIOFILM MATERIALS AND BLEACHED KRAFT MILL EFFLUENTS

STUDY PERSPECTIVE

An important initiative of the Northern River Basins Study is the development of methods for assessing the effects of industrial effluents and nutrients on the natural production of algae and other biofilm materials. At present, studies of organic contaminants in biofilm materials are severely hampered by the requirement for a relatively large sample size (5-20 g) and multi-step sample preparation techniques with long turn-around times of several weeks or months. Although a recently developed non-destructive technique for biofilm study (using laser microscopy) is well suited for understanding the spatial arrangement of microorganisms within biofilms, complementary studies are required for the identification of degradation products.

Related Study Questions

2)	*What is the current state of water quality in the Peace, Athabasca and Slave river basins, including the Peace-Athabasca Delta?*
4a)	*What are the contents and nature of the contaminants entering the system and what is their distribution and toxicity in the aquatic ecosystem with particular reference to water, sediments and biota?*
5)	*Are the substances added to the rivers by natural and man-made discharges likely to cause deterioration of the water quality?*

The objectives of this study were: (1) to assess the utility of small volume extraction techniques for Broad Spectrum Analyses of biofilm from riverine environments, and (2) to compare the results obtained with those obtained by conventional extraction techniques of pulp-and-paper mill effluents.

Conventional extraction (500 ml samples) with gas chromatography/mass spectrometry detection was used to obtain a fingerprint of organic contaminants present in pulp-and-paper mill effluent from the bleached kraft mill at Hinton. These fingerprints were compared with data obtained from small volume (2µL, 5mg) samples of biofilm exposed to effluent from the same mill. An advanced tandem mass spectrometry (MS/MS) technique was used to obtain fingerprints of organic contaminants in biofilm. The technique provided rapid analysis, minimal sample extraction steps and sensitive detection of target analytes. Preliminary results indicated that the biofilm contained classes of contaminants matching those in the raw effluent.

This study reports on the application of a MS/MS procedure for the study of very small volumes of biofilm from riverine environments. The results suggest that the MS/MS scans are selective and sensitive for identifying classes of contaminants from pulp mill effluents in small quantities of biofilm. Further refinement of the technique is required for routine quantification of specific contaminants using authentic standards.

REPORT SUMMARY

Conventional liquid/liquid extractions (500 ml samples) with gas chromatography/mass spectrometry detection was used to obtain a fingerprint of organic contaminants present in pulp-and-paper mill effluent from the Weldwood of Canada Ltd. bleached kraft mill. These fingerprints were compared with data obtained from small volume (2µl, < 5mg) samples of biofilm exposed to effluent from the same mill. For the biofilm material, advances in tandem mass spectrometry (MS/MS) were used to obtain fingerprints of organic contaminants. The MS/MS technique was amenable to rapid analysis (< 35 min for sample preparation and instrumental analysis), minimal sample extraction steps and provided sensitive detection of target analytes. Preliminary results indicated that the biofilm contained classes of contaminants matching those in the raw effluent. These results suggest that the MS/MS product-ion scans are selective and sensitive for identifying classes of contaminants from pulp mill effluents in small quantities of biofilm. Further refinement of the technique is required for routine quantification of specific contaminants using authentic standards.

ACKNOWLEDGEMENTS

Technical assistance for sample extractions was provided by Brenda Headley. This research was funded by the Northern Rivers Basin Study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

This brief report covers the work performed for a small Northern Rivers Basin Study (NRBS) project ($3K) in which some preliminary studies were conducted. The results of this investigation should be considered in the context of a feasibility study, facilitating an informed decision on the likelihood of a successful end-product based on more extensive study.

1.1 OBJECTIVES

The objectives of this study were:

(a) To assess the utility of small volume extraction techniques for Broad Spectrum Analysis of biofilm from riverine environments.

(b) To compare the results obtained for (a) with those obtained by conventional extraction techniques of pulp-and-paper mill effluents.

1.2 RATIONALE AND SCOPE

One of the objectives of the Northern River Basin Study is the development of models and other predictive tools for assessing the effects of industrial effluents and nutrients on the natural production of algae and other biofilm materials. At present, studies of organic contaminants in biofilm materials are hampered severely by the requirement for relatively large sample size (5-20 g) and multi-step sample preparation techniques with long turn-around times of several weeks or months.

In recent years, scanning confocal laser microscopy (SCLM) has been used to study biofilms (Caldwell et al. 1992, Wolfaardt et al. 1993). Although this non-destructive technique is well suited for study of the spatial arrangement of microorganisms within biofilms, complementary studies are required for the identification of degradation products. This report describes the application of a MS/MS procedure for broad spectrum analysis of biofilm (< 5 mg, ~2µl volume)(Headley and Harrison 1985, Vaughan and Zakrevsky 1988, Headley et al. 1992) from riverine environments with a thickness of 5-40 µm and detection of analytes at sub-femtomole levels. The MS/MS technique is based on procedures developed for degradative bacterial biofilms (Headley et al. 1995a; Wolfaardt et al; 1994) and lipid-rich tissue (Headley et al. 1995b, 1995c).

2.0 EXPERIMENTAL

2.1 MATERIALS

For this preliminary study, authentic standards were not available for confirmation of the identification of the analytes. This was deferred to follow-up studies if warranted. Tentative identification was limited to interpretation of GC/MS and MS/MS spectra obtained for the biofilm materials and bleached kraft mill effluents.

2.2 EXTRACTION OF BLEACHED KRAFT MILL EFFLUENT

The extraction procedure for conventional sample sizes was based on the US. EPA method 8270. In brief, a volume of effluent (500 ml) was spiked with surrogate standards (20µl of 10ng/µl per component mixture in methanol containing nitrobenzene-d_5, 2-Fluorobiphenyl and p-Terphenyl-d_{14}). Extractions of the base/neutrals organics were performed by adjusting the pH of the sample to > 12 using a 10N NaOH solution, and performing 3 serial extractions with dichloromethane (3 x 60 ml). The combined extracts were dried using anhydrous Na_2SO_4, collected in a round bottom flask, and concentrated to approximately 3ml using a rotary evaporator. Following quantitative transfer to a volumetric tube (2 ml) using glass pasteur pipettes with rinses of dichloromethane, the extract was further concentrated using nitrogen to a final volume of 2 ml. For GC/MS analysis, an aliquot (0.5 ml) was transferred to a GC vial to which 1µl of internal standard solution (5ng/µl mixture in dichloromethane containing acenaphthene-d_{10}, phenanthrene-d_{10}, and chrysene-d_{12}) was added. The vial was then capped for subsequent instrumental analysis.

For the acid fraction, the pH of the aqueous phase was adjusted to < 2 using sulphuric acid (H_2SO_4:H_2O 1:1). The sample was then extracted using the same procedure described above. However, the final extraction volume (2 ml) was divided into two sub-fractions. One set was subjected to derivatization (methylation) using diazomethane and the other aliquot was retained without derivatization. Both the non-methylated fraction and the methylated fractions were analyzed using gas chromatography/mass spectrometry.

2.3 PREPARATION OF BIOFILM MATERIALS

The biofilm was grown at a field location in re-circulating streams containing river water (Athabasca River upstream of Hinton, Alberta), to which effluent from the Weldwood of Canada Ltd. bleached kraft mill had been added to simulate in-river concentrations under fully-mixed conditions (corresponding to ~ 2% volume:volume dilution). The biofilm was produced over a 5 week period during September-October, 1994, collected in 500 ml glass jars with teflon-lined lids and kept frozen (-75 °C) until instrumental analyses. For laboratory analysis, sub-samples (2 µl) of

the biofilm were subjected directly to instrumental analysis with no sample extraction, cleanup or preconcentration steps.

2.4 INSTRUMENTAL ANALYSIS

2.4.1 GC/MS Analysis of Extracts of Pulp-And-Paper Mill Effluents

Electron impact GC/MS analysis was used for identification of the components detectable in the extracts of the pulp-and-paper mill effluents. The GC/MS experiments utilized a Fisons Autospec-Q mass spectrometer with EBEQ geometry. All identifications were based on: (a) mass spectral library searches and (b) fundamental principles of interpretation of mass spectra (McLafferty 1980; Heller and Milne 1978). GC/MS experimental conditions were established as per Method 8270 guidelines (US EPA 1990): 30 m DB-5 column, internal diameter 0.25 mm, film thickness 0.25 μm, helium carrier gas 1 ml/min, injector 285°C, transfer line 290°C, temperature program 39°C/2 min at 10°C/ min - 290°C/10 min.

2.4.2 Probe/MS of Biofilm

Preliminary Probe/MS experiments of biofilm were conducted using a Fisons AutospecQ mass spectrometer (EBEQ geometry) equipped with a 4000-60 VAX data system and Opus 3.1X Software. Samples were pre-dried at room temperature, in shallow cups of the direct insertion probe for 30 minutes, prior to introduction to the ion-source. This was necessary to avoid tripping the vacuum protection system of the mass spectrometer. Heating of the direct insertion probe was limited to radiant heat from the ion-source. The ion-source was operated under electron impact conditions at 70 electron volts, 250°C and, trap current 250 µA. The mass spectrometer was operated at scan speed 1 s/decade, mass range 50 - 600 daltons and at 1300 resolution.

2.4.3 Probe/MS/MS of Biofilm

For the Probe/MS/MS experiments, the precursor ion-beam (m/z 331) was reduced to 50 percent transmission using perfluorokerosene as the reference standard. Xenon was used as the collision gas. The collision cell was located in the fourth field free region. Experiments were performed for low energy collisions in which the collision cell was held at 12 eV (laboratory frame of reference). Product-ions were detected by scanning the quadrupole at unit resolution.

3.0 RESULTS & DISCUSSIONS

3.1 GC/MS

Qualitative data obtained from the GC/MS analyses for the pulp-and-paper mill effluents investigated was used to select diagnostic ions for subsequent tandem mass spectrometry studies. The organic compounds observed were consistent with observations reported earlier in the literature (Headley, 1987), with evidence of artifacts arising from phthalate esters. For the purpose of this investigation, selected components characteristic of kraft mill effluent, were used to evaluate the application to riverine biofilm. A summary of the selected classes of components are given in Table 1, including the diagnostic m/z values for possible chlorinated sulphones, thiophenic compounds, phenolic compounds, resin acid derived compounds and their related terpenoid derived compounds. Subsequent fingerprinting was therefore based on the ability to detect these classes of compounds using limited quantities of the biofilm. It is noted that for a rigorous study, full identification of specific components would require the use of authentic standards for confirmation purposes. The latter was outside the scope and funding of the present feasibility study, and thus the identification of specific components will not be discussed further in this report.

3.2 PROBE/MS

An example of a total-ion chromatogram obtained for biofilm is illustrated in Figure 1. Little spatial separation was achieved using the direct-insertion probe for introduction of the biofilm material to the ion-source. This is illustrated in Figure 2a, in which a representative example of the full scan mass spectrum of the biofilm is given. For clarity the display of the mass range has been expanded in Figure 2b-d. For convenience, a summary of diagnostic ions observed and their possible compound classes are given in Table 1. There is thus evidence of "kraft mill effluent" components present in the full scan mass spectrum. For the higher molecular weight classes of components (e.g resin acid derived compounds with m/z 257, 272), there is less chemical noise than for the lower molecular weight components, such as the possible chlorinated sulphones (m/z 83, 85). However, below m/z 200, the fingerprint data contains significant overlapping contributions from the various components. This region of the full scan mass spectra is therefore not well suited for confirmation of the presence of components within the biofilm material. For the latter, further mass resolution or selective mass fragmentation with adequate spatial separation of the analytes is required.

Figure 1: Example of a Total Ion Chromatogram Obtained from Biofilm Material

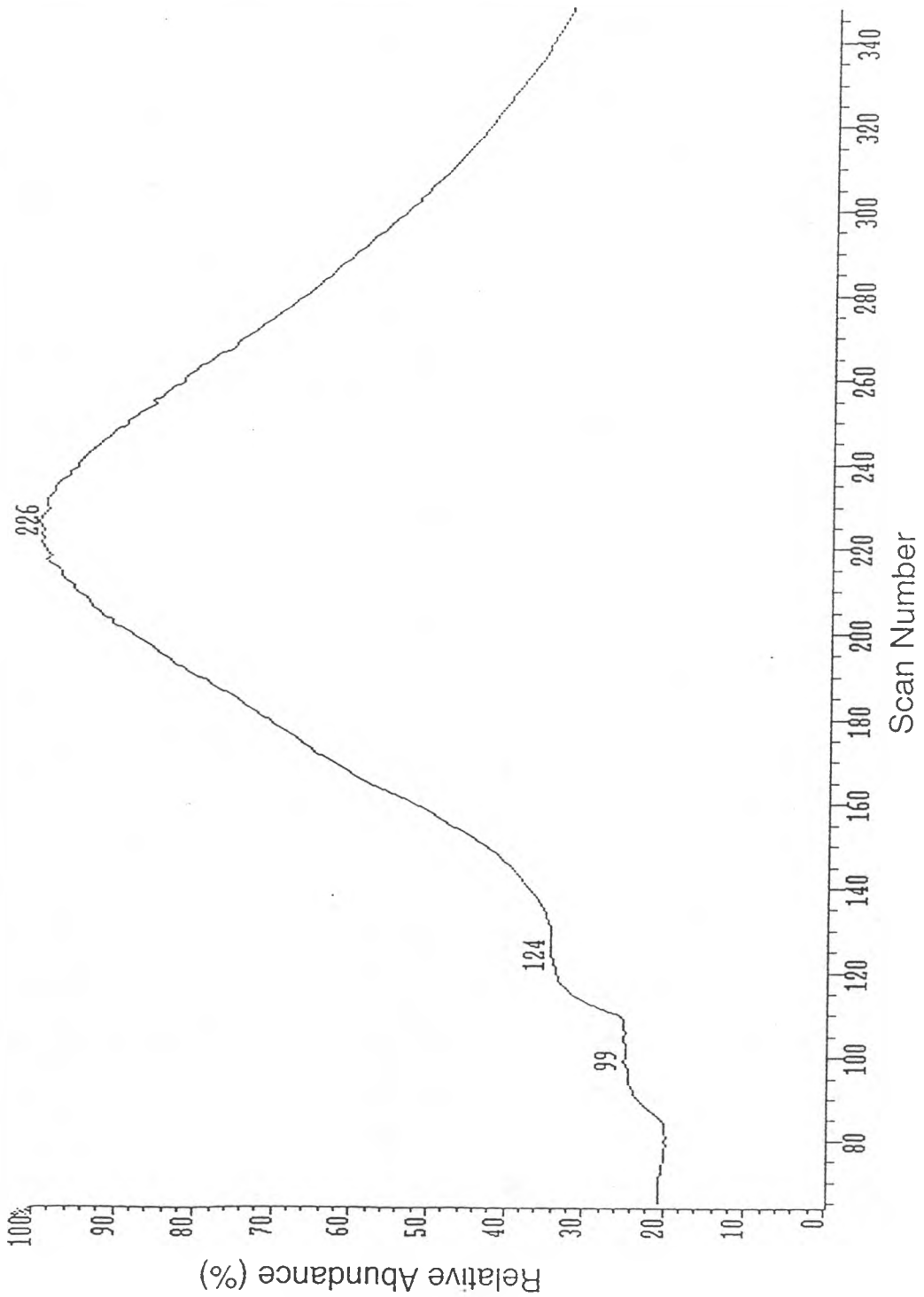


Figure 2a: Example of the Full Scan Mass Spectra of Biofilm Material
(* indicates diagnostic ions)

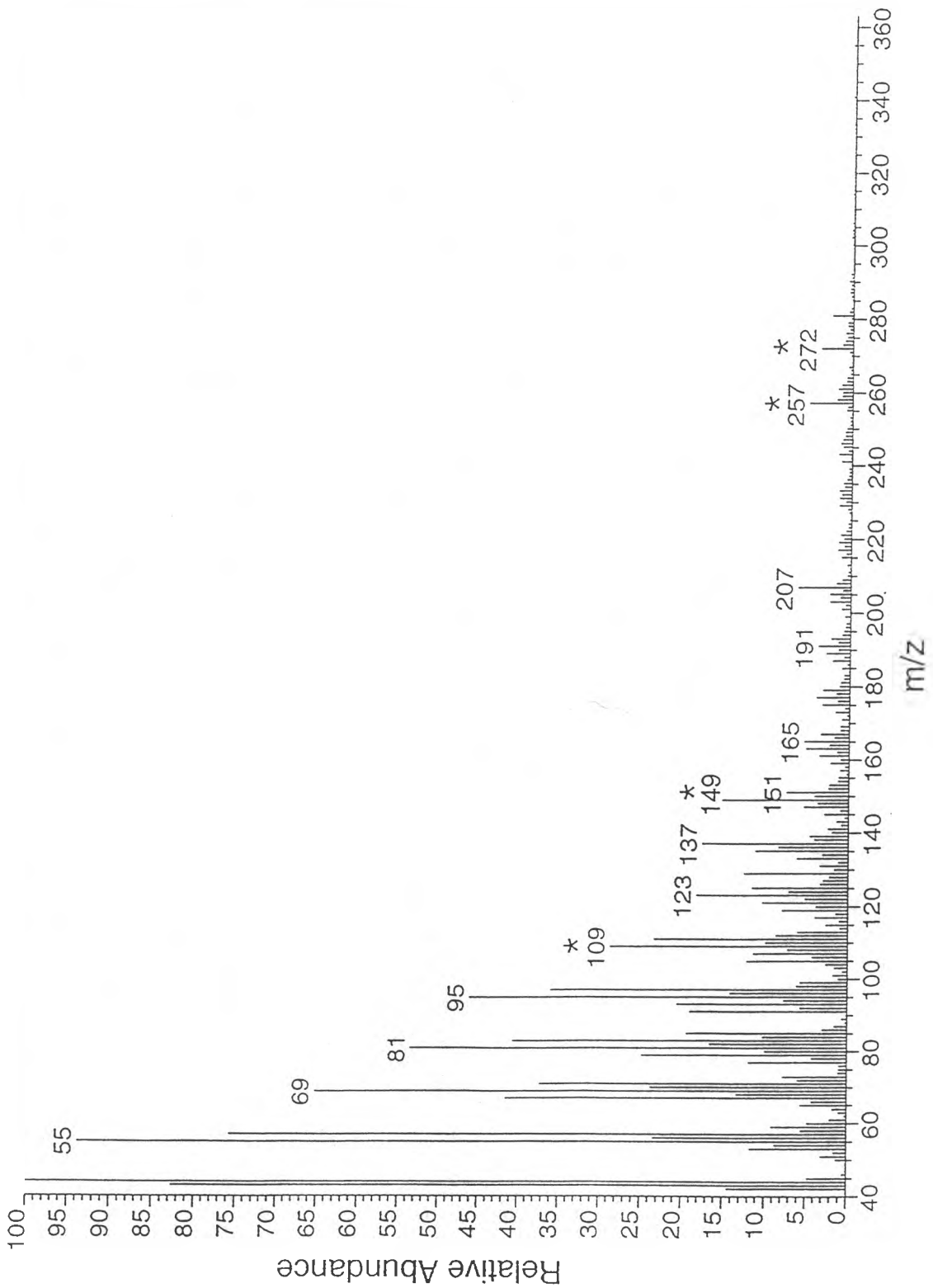


Figure 2b: Expanded Display of the Mass Range of Full Scan Mass Spectra of Biofilm Material Given in Figure 2a (* indicates diagnostic ions)

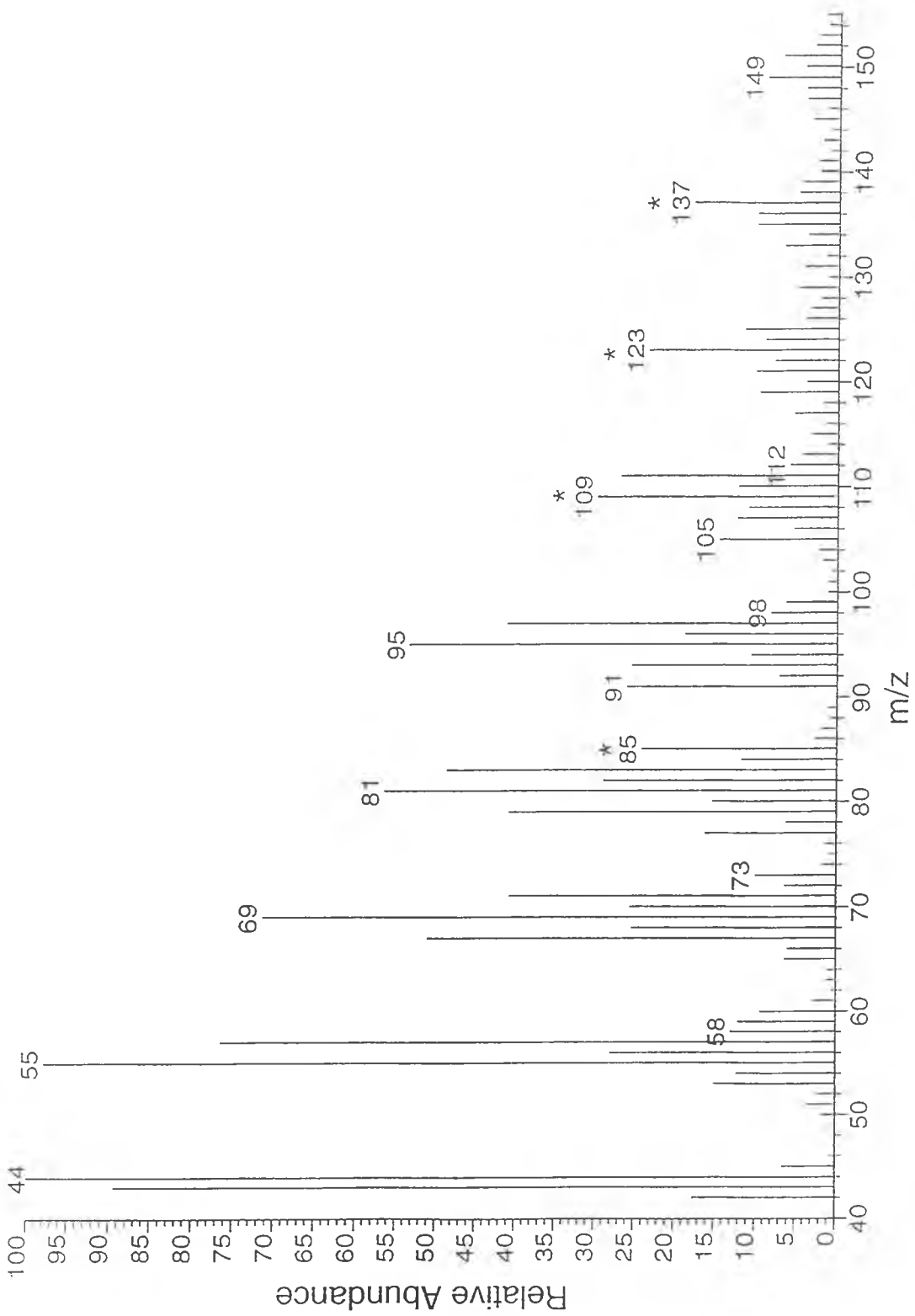


Figure 2c: Expanded Display of the Mass Range of Full Scan Mass Spectra of Biofilm Material Given in Figure 2a (* indicates diagnostic ions)

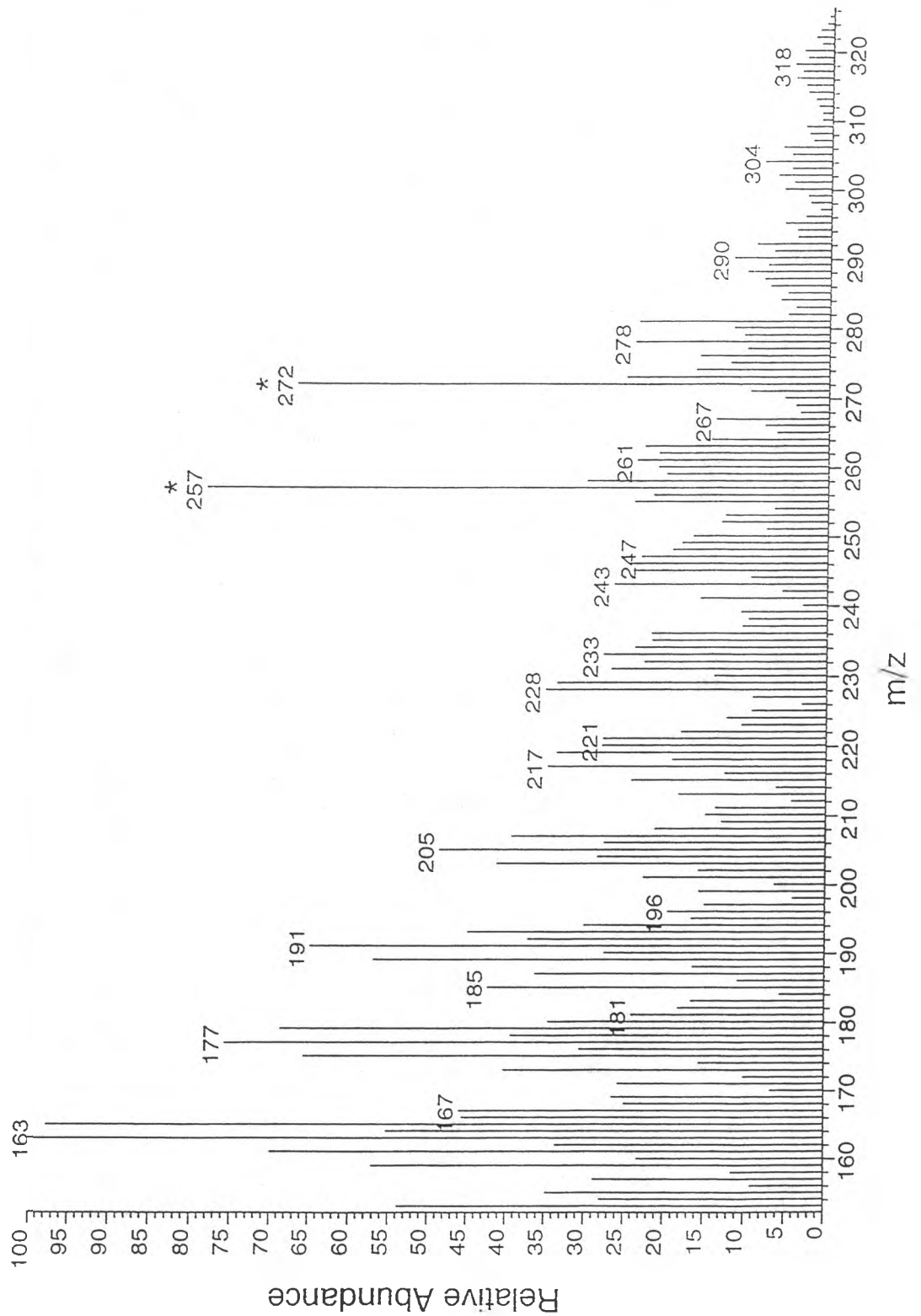


Figure 2d: Expanded Display of the Mass Range of Full Scan Mass Spectra of Biofilm Material Given in Figure 2a (* indicates diagnostic ions)

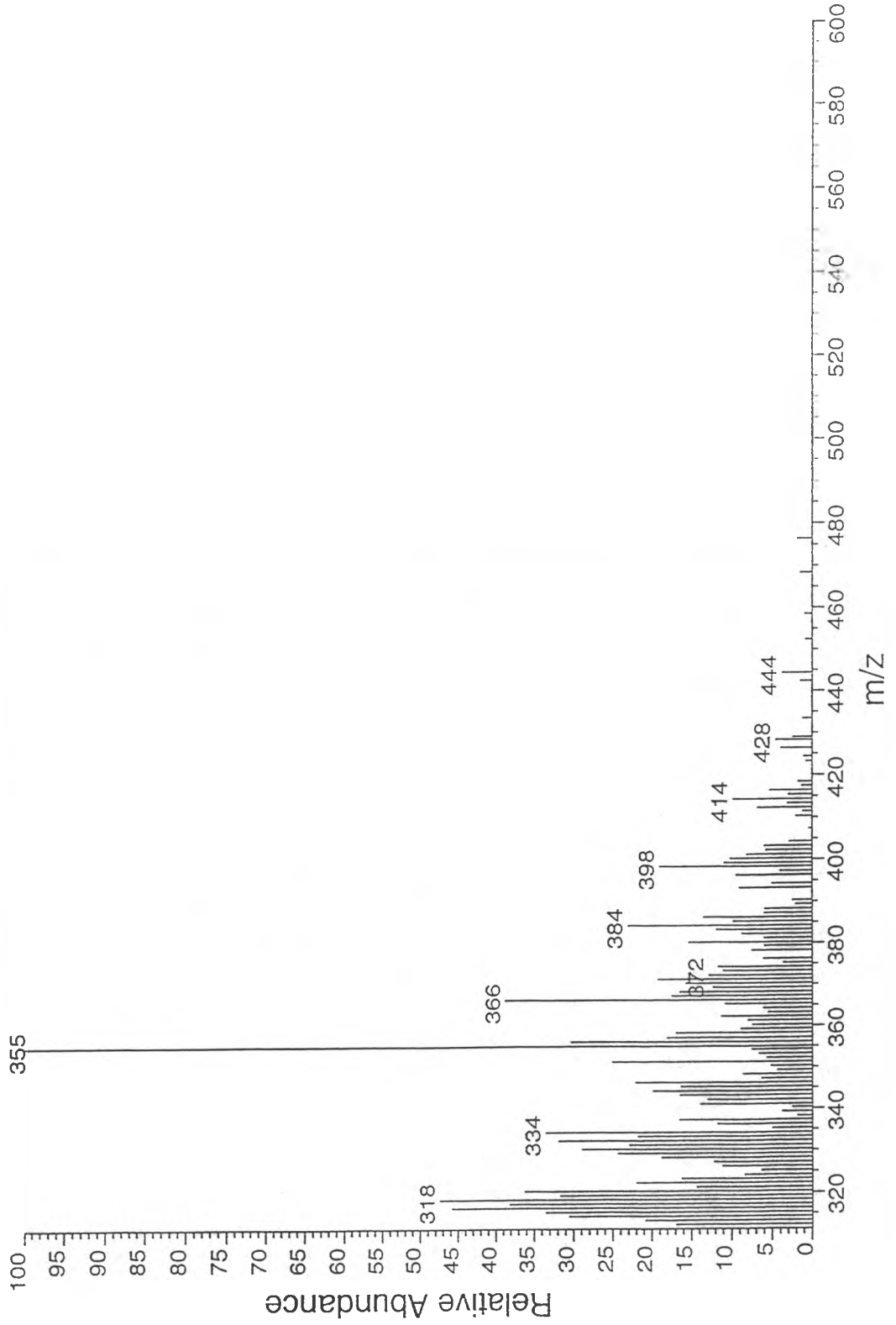


Table 1. A summary of diagnostic ions observed in the full scan mass spectra of biofilm and their possible compound classes.

m/z	Compound Classes
83, 85	dichlorodimethyl sulphone, and/or -$CHCl_2$ functional group
109, 257	resin acid derived product
123	phenolics
129	adipate
137	phenolics
149	phthalate ester (artifact)
152	phenolics
257	resin acid derived products
272	resin acid derived products

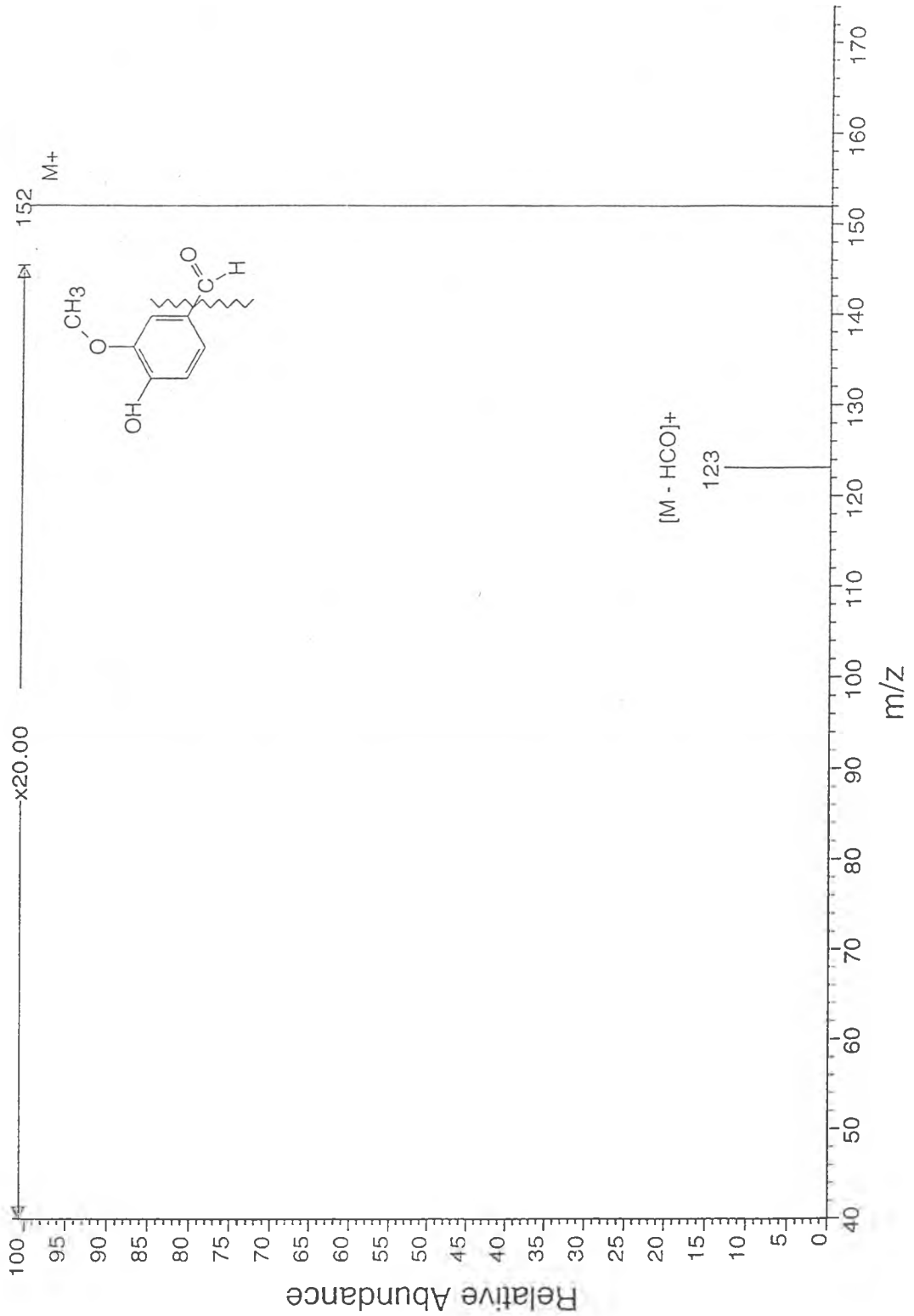
These results indicate the presence of classes of contaminants occurring within the biofilm material based on comparison with reference mass spectra of effluent streams from kraft pulp-mills, and mass spectra from GC/MS analysis of effluent obtained in this work. Thus, the ability to detect diagnostic ions of various compound classes in small volumes of biofilm was demonstrated. Further work, however, is required to obtain fingerprint data for biofilm from control streams with no addition of effluent. For example, dichlorodimethylsulphone and other chlorinated sulphones are not known to occur naturally in the environment and are linked to the chlorination of sulphones in the kraft process. It is therefore anticipated that the chlorinated sulphone and related compounds from the kraft process would not be observed in biofilm from control streams.

3.3 MS/MS PRODUCT-ION SCANS

The application of MS/MS product-ion scans for confirmation of specific components in complex mixtures is well documented in the literature. In this preliminary work, MS/MS was first performed using a GC/MS scan to achieve spatial separation of the components in the extract of the raw effluent. The MS/MS product-ion scan of the base-ion (most abundant ion in the mass spectrum of the compound) for a given component in the effluent can be used as an "in-house" library. This library, however, must be validated using authentic standards of the individual components as required. The latter is a subject of future studies. For follow up work, the product-ion scans are anticipated to provide confirmation of the identification of specific contaminants in the biofilm without the need for spatial separation prior to mass analysis (Headley et al. 1995b,c).

In this investigation, preliminary work was conducted to determine the utility of MS/MS product-ion scans for the confirmation of a phenolic compound, vanillin. An authentic standard of vanillin was not available, and thus the component detected in the GC/MS scan of the raw effluent at 12.52 minutes (tentative identification as vanillin) was utilized. As shown in Figure 3, the

Figure 3: MS/MS Product-ion Scan a Phenolic Compound, Vanillin



molecular-ion of vanillin (m/z 152) was resistant to collision induced dissociation under the conditions investigated. The loss of HCO was the only dissociation observed for the molecular-ion. This is in contrast to the electron impact mass spectrum for which there was a wide range of fragmentation (Figure 4). Ideally, the relative abundances of a minimum of three product-ions is desirable for full confirmation. For thermodynamically stable aromatic ions (such as vanillin) this ideal situation is not expected to be attained at low collision energies.

3.4 SENSITIVITY

To investigate the usefulness of the product-ion scans for filtering chemical noise in a biofilm matrix, a subsample of biofilm (~ 5 mg) was spiked with 2 ul of the extract of the raw effluent. The product-ion scan of vanillin, provided a diagnostic fingerprint of the compound in this complex mixture. For similar work with lipid-rich tissue the tandem mass spectrometry technique was suitable for confirmations at levels of approximately 20 pg of the target analyte. Based on the observed product-ion scans in the present work, similar levels of detection are anticipated for target analytes in riverine biofilms. Product-ion scans of key components in biofilm should be the focus of follow up work, in which biofilm materials should be collected from different aquatic environments.

3.5 FUTURE DEVELOPMENTS AND RECOMMENDATIONS

There are many areas for future development of the MS/MS procedure for obtaining fingerprint information of biofilm materials. The following key areas of research are recommended for future work:

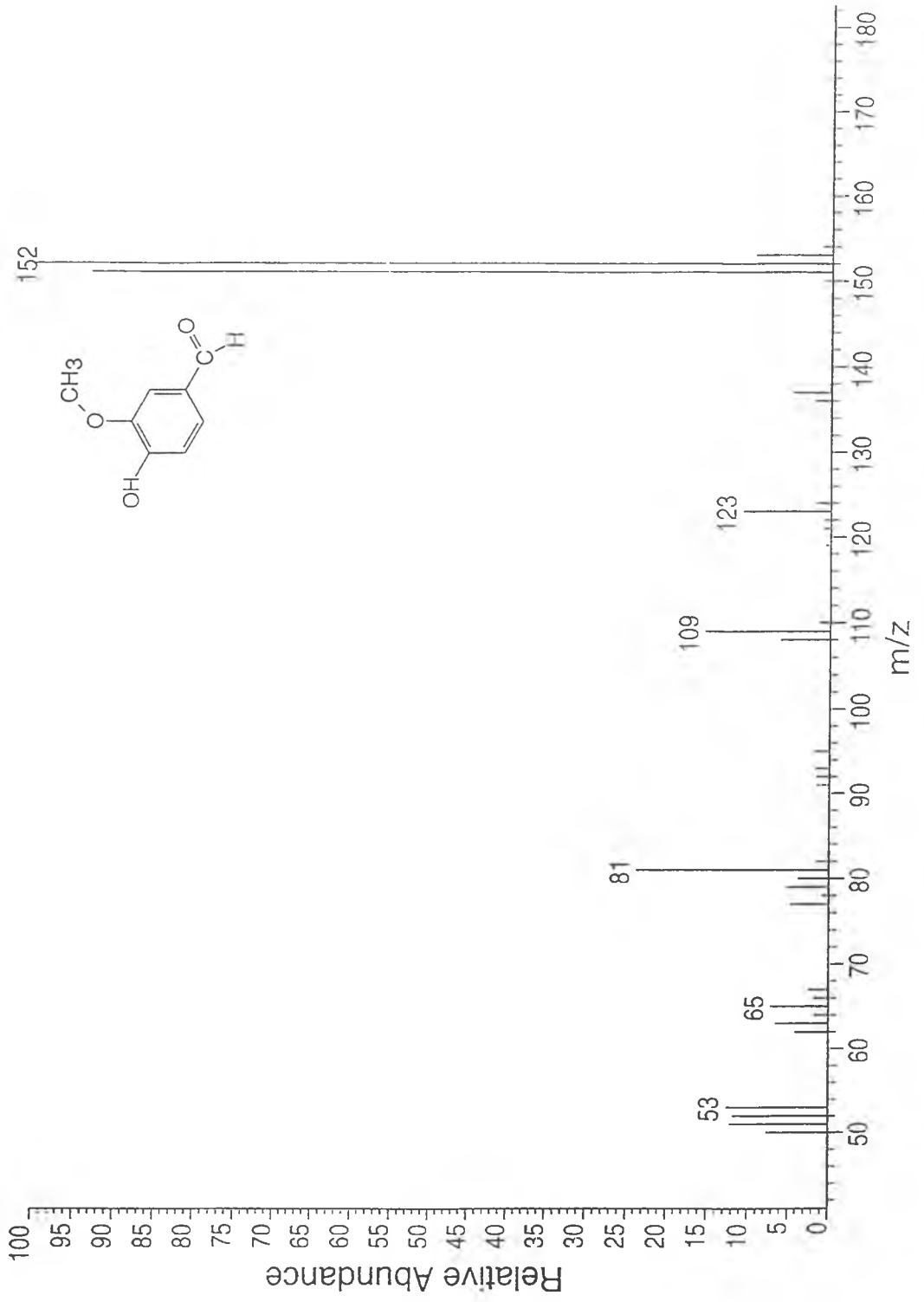
- Perform crude chromatographic separation of components in the biofilm materials prior to sample introduction to the ion-source. This step can be fast (1-3 minutes) and will eliminate or reduce mass spectral interference from coeluting contaminants. Chromatographic separation may be possible with high transfer efficiency using electrospray/LC/MS, with minimal sample preparation.

- Utilize lower ion-source temperatures and lower electron energies to extend the diagnostic ions observed in the full scan data to lower molecular weight contaminants.

- Develop MS/MS product-ion spectra of selected indicator compounds for fingerprinting biofilm from riverine environments.

- Develop an internal standard method for quantitative analyses of target analytes in biofilms using selected reaction-monitoring. This mode of tandem mass spectrometry is well suited to quantitative analyses.

Figure 4: Electron Impact Mass Spectrum of Vanillin



- Subject to evaluation of the results of a more detailed study, the technology should be transferred to less expensive methods of tandem mass spectrometry, including ion-traps or quadrupole instrumentation.

4.0 CONCLUSIONS

1. The preliminary results indicate that classes of pulp-and-paper related contaminants were detectable within limited quantities of biofilm (<5 mg) cultivated in river water to which pulp mill effluent at river concentrations had been added (~ 2% volume:volume dilution).

2. MS/MS techniques warrant further development for selective and sensitive fingerprinting of riverine biofilms.

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APPENDIX A: Terms of Reference

TERMS OF REFERENCE NORTHERN RIVER BASIN STUDY

Introduction

One of the major initiatives of the Northern River Basin Study is the development of models and other predictive tools, for assessing the effects of industrial effluents and nutrients on the natural production of algae and other biofilm materials. Present studies of biofilm materials are hampered severely by the requirement for relatively large sample size (20 g) and multi-step sample preparation techniques with long turn-around times.

Objective

(a) Assess the utility of small volume extraction techniques for Broad Spectrum Analysis of biofilm materials and pulp and paper-mill effluents.

(b) Compare the results obtained for (a) with those obtained by conventional extraction techniques.

Requirements

(a) Sub-sampling of representative samples of biofilm materials and pulp and paper-mill effluents in the laboratory.

(b) Purchase of reagents and consumables for extraction procedures.

(c) Extraction of representative samples using conventional base-neutral/acid extraction techniques with for example, acetylation and\or methylation of the acid fractions.

Upon completion of the extraction procedures, extracts will be submitted to NHRI for Broad Spectrum Analyses employing advances in mass spectrometry techniques for biofilm materials.

Project Organization

The project will be managed by Dr. Patricia Chambers, National Hydrology Research Institute. Payment of $3031.67 is required in advance to cover the expenses of the extraction procedures, during February 14 - March 31, 1994.

APPENDIX B: Reviewer's Comments

REVIEWER'S COMMENTS:

This report contains several deficiencies in basic chemistry, some of which are enumerated below. Please refer to the marked manuscript for the others.

1. *The GC-MS results on the conventional extracts of bleached kraft mill effluent (BKME) are puzzling:*
   - *a) the acid fraction contains several neutral compounds, most notably phthalates.*
   - *b) the methylated acid fraction contains several peaks assigned as ethoxy compounds indicating a problem with the diazomethane as suggested by the authors.*
   - *c) For the base/neutral fraction there is some confusion regarding resin "acids", e.g, the formula $C_{20}H_{34}O$ is assigned to a resin acid --- resin acids contain two oxygen atoms.*
   - *d) Phthalate esters were observed in all fractions. These are common laboratory contaminants so it is unclear if they are coming from the BKME or laboratory contamination*

The reviewer's comments are endorsed and the text has been revised accordingly. Table 1 has been deleted. In the revised manuscript, the results and discussion pertaining to conventional extractions is limited to the selection of **target compound classes** for subsequent tandem mass spectrometry of the biofilm.

2. *The purpose of the GC-MS work on BKME extracts was to construct a list of diagnostic ions for subsequent probe-MS and probe-MS-MS work on BKME-exposed biofilm samples. Due to the equivocal nature of the GC-MS results a lot of "interpretation" is required to determine which diagnostic ions are meaningful, ie., application of a priori knowledge about compounds to be expected in BKME. Also, the authors should perhaps have been more critical in arriving at structural assignments based on library searchers as presented in Table 1.*

Agreed. The technique is limited to target analysis. Table 1 has been deleted in the revised report.

3. *If one accepts some of the diagnostic ions as being useful, then the probe-MS results (Figure 2a-2d) do indicate the presence of BKME-related compounds in the exposed biofilm. However, the conclusion that "... a wide range of pulp-and-paper related contaminants were detectable...." is unsupported.*

The text has been revised accordingly, in which the phrase "wide range" has been eliminated.

4. *In the final step, a sample of biofilm to which BKME extract was added, was analyzed by probe-MS-MS. The product ion scan showed two ions attributable to vanillin thus confirming the presence in the sample. However, the "spiking" level corresponds to 0.5 ml of effluent per 5 mg of biofilm. I'm not sure how realistic this is. The utility of these results would be more convincing if they were for a BKME-exposed biofilm sample.*

Agreed , the text has been revised accordingly.

5. *Ultimately this work has to be evaluated as a feasibility study. Does it show promise as a rapid and sensitive method for broad spectrum analysis of small samples of biofilm for BKME-related compounds? I think alot of method development is needed before probe-MS-MS will achieve those goals. An alternative which should be considered is a small scale extraction and cleanup method followed by GC-MS with conventional quadropole or ion trap mass spectrometer. Before going further with probe-MS-MS the authors should make, as a paper exercise, a comparative study of the advantages of the two approaches (probe-MS-MS without extraction and cleanup vs. conventional GC-MS with extraction and cleanup).*

Agreed. The text has been revised accordingly to put more emphasis on the complementary nature of the technique compared to conventional methods, with reference to recent applications described in the literature. The latter applications address the pros and cons of conventional methods versus the tandem mass spectrometry procedure for bacterial biofilms and lipid- rich tissue.

Reviewer 2.

I do not believe the basic objectives of comparing small extraction techniques with conventional techniques were met (see Terms of Reference Objective 7.1.2). The biofilm was grown in the laboratory, and analyzed by direct probe MS/MS, which is information in itself, but not a comparison of extraction techniques.

The text has been revised to more clearly state that the biofilm samples were actually grown in the field using river water. The probe/MS/MS method was based on the on-line volatilization of semivolatile organics from small volumes of biofilm and was thus complementary to conventional extraction methods, as per the Terms of Reference.

As to the suitability of the technique for the intended purpose, I have some reservations as to whether it will succeed. The authors note in their future developments and recommendations that they should "perform crude chromatographic separation of components in the biofilm materials prior to sample introduction to the ion-source." To get to this stage, an extraction step is required.

The suitability of the method is subject to debate. Some relevant applications of the procedure, are only now appearing in the recent scientific literature as indicated in the revised text.

Presently, the procedure is being further developed as part of the German-Canada Bilateral Cooperation, and the collaborators anticipate a positive outcome of the application to riverine biofilm.

> *The sensitivity of the method as demonstrated by spiking the biofilm with 2μ l of the 2.0 ml effluent is good, but similar results would be expected using less expensive low resolution quadrupoles or ion traps.*

Agreed. The text has been revised accordingly. There is clearly a need to adapt the tandem MS procedure, if warranted, to less expensive instrumentation. This development is presently under investigation as part of the Germany-Canada Bilateral

> *There are a surprising number of compounds in the acid and base fractions that one would expect to be found in the other fraction. For example, in the acid fraction that is not derivatized, only compounds 2, 3, and 4 would be expected. The authors refer to all compounds with molecular formulas containing C_{20} as resin acids. These are diterpenoids as they do not contain the carboxylic acid moiety. The exception is compound 10 in the base/neutral fraction that is not described as a resin acid. In fact, there are no resin acids confirmed in the effluent or the biofilm. Most mill (either kraft mill or chemical/mechanical) effluents contain some residues of resin acids.*

The concerns and comments pertaining to the tentatively identified compounds have been addressed in the revised manuscript, as per action outlined above (see Reviewer 1).

> *Regarding the identification of diclorodimetyhylsulphone, I would make these comments. The diagnostic ions m/z 83 and 85 are the dominant ions in chloroform, trichlorodimethylsulphone, and tetrachlorodimethylsulphone. All of these compounds have been detected in kraft mill effluents.*

Agreed. Table 1 in the revised manuscript has been revised accordingly.

> *I would not recommend funding further work that involves direct probe analysis of the biofilm. The authors's recommendations to use LC/MS and using lower ion source temperatures and lower electron energies might be of some use, specially if this is combined with MS/MS work, and specific target compounds are sought.*

The development of the probe-MS/MS procedure is subject to many concerns and several problems or challenges are anticipated. The response from the scientific community for a similar application (lipid-rich tissue) is regarded by some reviewers as a "success story". We anticipate a successful application to riverine biofilm.