

The Spatial Distribution of Lead in Teeth as a Biomarker of Prenatal and Neonatal Lead Exposure

Manish Arora

BDS, MPH

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

The Cellular and Molecular Pathology Research Unit

Department of Oral Pathology and Oral Medicine

Faculty of Dentistry

The University of Sydney

August 2005

**DENTISTRY LIBRARY
UNIVERSITY OF SYDNEY**

This thesis is dedicated to my parents, Arjun and Indra Arora

TABLE OF CONTENTS

Table of Contents	i
Declaration	x
Acknowledgements	xi
List of Publications Arising from this Study	xiii
List of Figures	xiv
List of Tables	xvii
Abbreviations	xix
Summary	xxi

Chapter 1

Environmental Lead Exposure: A Literature Review

1.1	Introduction	2
1.2	Historical Perspective of Lead Exposure	2
1.3	Epidemiology of Lead Exposure	3
1.3.1	Global Distribution of Lead Exposure	4
1.3.1.a	<i>Developed Countries</i>	4
1.3.1.b	<i>Developing Countries</i>	5
1.3.2	Socioeconomic Determinants of Lead Exposure	6
1.3.3	Sources of Lead Exposure	6
1.3.4	Global Burden of Disease Due to Lead Exposure	7
1.4	Lead Absorption, Distribution and Excretion	8
1.4.1	Absorption	8
1.4.1.a	<i>Absorption after Ingestion</i>	8
1.4.1.b	<i>Absorption after Inhalation</i>	9
1.4.1.c	<i>Percutaneous Absorption</i>	9
1.4.2	Distribution	10
1.4.3	Excretion	11
1.5	An Overview of the Health Effects of Lead Exposure	12
1.5.1	Acute Effects	12
1.5.2	Chronic Effects	13
1.5.2.a	<i>Neurological Effects</i>	13

1.5.2.b	<i>Haematological Effects</i>	16
1.5.2.c	<i>Renal Effects</i>	17
1.5.2.d	<i>Hypertension</i>	18
1.5.2.e	<i>Oral Health Effects</i>	19
1.5.2.f	<i>Cancer</i>	21
1.5.2.g	<i>Other Effects</i>	21
1.6	Biological Monitoring of Heavy Metal Toxins	22
1.6.1	Biological Monitoring of Lead	23
1.6.1.a	<i>Blood and Plasma</i>	24
1.6.1.b	<i>Urine</i>	26
1.6.1.c	<i>Bone</i>	27
1.6.1.d	<i>Hair</i>	28
1.6.1.e	<i>Saliva</i>	30
1.6.1.f	<i>Nails</i>	32
1.6.1.g	<i>Sweat</i>	33
1.6.1.h	<i>Teeth</i>	34
1.6.1.h.i	Lead Levels in Whole Teeth and Environmental Exposure to Lead	35
1.6.1.h.ii	Lead Levels in Enamel and Dentine of Human Teeth, and Environmental Exposure to Lead	39
1.6.1.h.iii	Tooth-Lead Levels and Health Effects	43
1.6.1.h.iv	Incorporation of Lead in Enamel and Dentine	44
1.6.1.h.v	Alternative Approaches in Using Tooth-Lead Levels	47
1.7	Questions Arising from the Literature	48
1.8	Tooth Development (Odontogenesis)	50
1.8.1	Cellular Changes during Tooth Development	50
1.8.2	Enamel and Dentine Apposition during Tooth Development	51
1.8.3	Mineralisation of Enamel and Dentine	54
1.8.4	Rationale behind the Use of Tooth-Lead Levels as a Biomarker of Lead Exposure	55
1.9	Aims	55

Chapter 2

Determination of Lead in Biological Tissues: Principles of the Methods Used in this Study

2.1	Introduction	57
2.2	Inductively Coupled Plasma Mass Spectrometry	58
2.2.1	Principles and Components of ICP-MS	58
2.2.1.a	<i>Sample Introduction in ICP-MS</i>	58
2.2.1.b	<i>Torch and Plasma Generation</i>	60
2.2.1.c	<i>Ion Extraction Interface</i>	61
2.2.1.d	<i>Ion Lens System</i>	61
2.2.1.e	<i>Quadrupole Mass Analyser</i>	61
2.2.1.f	<i>Ion Detectors and Data Acquisition</i>	63
2.2.2	Advantages and Limitations of ICP-MS	63
2.3	Inductively Coupled Plasma Atomic Emission Spectrometry	65
2.3.1	Principles of Atomic Emission Spectrometry	65
2.3.2	Advantages and Limitations of ICP-AES	66
2.4	Laser Ablation Inductively Coupled Plasma Mass Spectrometry	67
2.4.1	Principles and Components of LA-ICP-MS	67
2.4.2	Advantages and Limitations of LA-ICP-MS	70
2.5	Nuclear Microprobe	71
2.5.1	Principles and Components of a NMP	71
2.5.2	Advantages and Limitations of NMP Analysis	73
2.6	Autometallography	75
2.6.1	Principle of Autometallography	75
2.6.2	Advantages and Limitations of Autometallography	76
2.7	Synchrotron Radiation Induced X-ray Emission	77
2.7.1	Principles and Components of a Synchrotron	77
2.7.2	Advantages and Limitations of Synchrotrons	80

Chapter 3

The Spatial Distribution of Lead in Enamel and Coronal Dentine of Wistar Rats

3.1	Introduction	82
3.2	Materials and Methods	83
3.2.1	Mating and Animal Husbandry	83
3.2.2	Housing and Environment	83
3.2.3	Diet, Water and Lead Administration	84
3.2.4	Preparation of Sample Teeth for NMP Analysis	85
3.2.5	Nuclear Microprobe Analysis of Sample Teeth	89
3.2.6	Lead Levels in Reagents, Consumables and Glassware	90
3.2.7	Statistical Analysis	90
3.2.8	Lead Levels in Whole Teeth, Blood and Organs	90
3.3	Results	91
3.3.1	Health of Animals and Lead Levels in Reagents, Consumables and Glassware	91
3.3.2	Spatial Distribution of Lead in Enamel and Dentine of Rat Pups	91
3.3.3	Spatial Distribution of Other Trace Elements	97
3.4	Discussion	98
3.4.1	Distribution of Lead in Enamel	98
3.4.2	Distribution of Lead in Dentine	99
3.4.3	Similarities in Lead and Zinc Distribution in Sample Teeth	101
3.4.4	Relevance of the Findings of this Experiment in using Teeth as a Biomarker of Lead Exposure	101
3.4.5	Limitations, Future Directions and Conclusion	105

Chapter 4

The Spatial Distribution of Lead in Teeth as a Biomarker for Prenatal and Neonatal Lead Exposure in a Wistar Rat Model

4.1	Introduction	107
4.2	Materials and Methods	108
4.2.1	Lead Administration and Harvesting of Tissues from Wistar Rats	108
4.2.1.a	Mating and Animal Husbandry	108

4.2.1.b	<i>Diet, Water and Lead Administration</i>	109
4.2.1.c	<i>Harvesting of Blood, Teeth and Organs</i>	111
4.2.2	Inductively Coupled Plasma Mass Spectrometry Analysis	112
4.2.2.a	<i>Calibration of the ICP-MS Instrument used in this Study</i>	112
4.2.2.b	<i>Detection Limits of ICP-MS</i>	113
4.2.2.c	<i>Method for Tooth-Lead Analysis by ICP-MS</i>	114
4.2.2.d	<i>Method for Blood-Lead Analysis by ICP-MS</i>	114
4.2.2.e	<i>Method for Organ Lead Analysis by ICP-MS</i>	116
4.2.2.f	<i>Method for Bone Lead Analysis by ICP-MS</i>	117
4.2.3	Preparation of Sample Teeth for NMP Analysis	117
4.2.4	Nuclear Microprobe Analysis of Sample Teeth	117
4.2.5	Validation of NMP data with LA-ICP-MS	118
4.2.6	Identification of Prenatal and Postnatal Regions in Enamel and Dentine	118
4.2.7	Statistical Analysis of Data	122
4.2.7.a	<i>Analysis of Tooth-, Blood-, Bone- and Organ-Lead Data</i>	122
4.2.7.b	<i>Comparison of Spatial Distribution of Lead and Dose of Lead Administered</i>	122
4.3	Results	123
4.3.1	Health of Animals Exposed to Lead	123
4.3.2	Analysis of Standard Reference Materials	123
4.3.3	Lead Levels in Teeth, Blood and Organs	126
4.3.4	The Spatial Distribution of Lead in Teeth and Pattern of Lead Exposure	132
4.3.5	Results of LA-ICP-MS Analysis of Rat Teeth	137
4.4	Discussion	138
4.4.1	Lead Levels in Whole Teeth and Body Burden of Lead	138
4.4.2	The Spatial Distribution of Lead in Teeth and the Intensity and Pattern of Lead Exposure	139
4.4.3	Limitations, Future Directions and Conclusion	143

Chapter 5

The Spatial Distribution of Lead in Human Primary Teeth as a Biomarker for Prenatal and Neonatal Lead Exposure – A Pilot Study

5.1	Introduction	146
5.2	Materials and Methods	146
5.2.1	Participant Recruitment and Selection Criteria	146
5.2.2	Preparation of Sample Teeth	147
5.2.3	Identification of the Prenatal and Postnatal Regions in Enamel and Dentine	148
5.2.4	NMP Analysis of Sample Teeth	149
5.2.5	LA-ICP-MS Analysis of Sample Teeth	150
5.2.6	Statistical Analysis	151
5.2.7	Lead Levels in Reagents, Consumables and Glassware	151
5.3	Results	152
5.3.1	Analysis of Standard Reference Materials	152
5.3.2	Details of Participants Recruited	154
5.3.3	Comparison of Lead Levels in Enamel and Dentine with Blood-Lead Levels	155
5.3.3.a	<i>Enamel- and Dentine-Lead Levels in Participants with Blood-Lead Levels Measured at Birth and One Year of Age</i>	155
5.3.3.b	<i>Enamel- and Dentine-Lead Levels in All Ten Participants</i>	163
5.4	Discussion	165
5.4.1	Limitations, Future Directions and Conclusion	167

Chapter 6

The Spatial Distribution of Lead in the Roots of Human Primary Teeth

6.1	Introduction	169
6.2	Materials and Methods	169
6.2.1	Participant Recruitment and Selection Criteria	169
6.2.2	Preparation of Sample Teeth for ICP-MS Analysis	172
6.2.3	ICP-MS and ICP-AES Analyses of Teeth	173

6.2.4	Statistical Analysis	174
6.3	Results	175
6.3.1	Analysis of Standard Reference Materials	175
6.3.2	Lead, Calcium and Phosphorus Concentrations in Tooth Roots	176
6.4	Discussion	180
6.4.1	Limitations, Future Directions and Conclusion	181

Chapter 7

The Intracellular Localisation of Lead during Tooth Development in a Wistar Rat Model

7.1	Introduction	183
7.1.1	Uptake of Lead in Teeth	183
7.1.2	Light Microscopic Identification of the Different Stages of Ameloblast and Odontoblast Development	184
7.1.2.a	<i>Stages of Ameloblast Development in Rat Mandibular First Molar Tooth Germs</i>	184
7.1.2.b	<i>Stages of Odontoblast Development in Rat Mandibular First Molar Tooth Germs</i>	185
7.2	Materials and Methods	186
7.2.1	Methods Overview	186
7.2.2	Lead Administration and Preparation of Tissue Sections	187
7.2.2.a	<i>Mating and Animal Husbandry</i>	187
7.2.2.b	<i>Diet, Water and Lead Administration</i>	187
7.2.2.c	<i>Processing of Tooth Germs</i>	188
7.2.2.d	<i>Sectioning of Embedded Tooth Germs</i>	189
7.2.2.e	<i>Identification of Cellular Structures in Sectioned Tooth Germs</i>	189
7.2.3	Autometallographic Analysis	189
7.2.3.a	<i>Preparation of Physical Developer</i>	189
7.2.3.b	<i>Preparation of Stop Bath</i>	190
7.2.3.c	<i>Procedure for Physical Development</i>	190
7.2.3.d	<i>Lead Levels in Reagents, Consumables and Glassware</i>	190
7.2.3.e	<i>Controls used during Autometallography</i>	191
7.2.4	Synchrotron Radiation Induced X-ray Emission Analysis	191
7.2.4.a	<i>Sample Preparation for SRIXE</i>	191

7.2.4.b	<i>Sample Analysis using SRIXE</i>	192
7.3	Results	193
7.3.1	Ameloblasts and Odontoblasts at Different Stages of Development were Readily Identified	193
7.3.1.a	<i>Stages of Development in Ameloblasts Identified in this Study</i>	193
7.3.1.b	<i>Stages of Development in Odontoblasts Identified in this Study</i>	193
7.3.2	Results of Autometallography	205
7.3.2.a	<i>90 min Developing Time was Optimal for AM</i>	205
7.3.2.b	<i>Differentiating Ameloblasts and Differentiating Odontoblasts (Pre-odontoblasts)</i>	208
7.3.2.c	<i>Secretory Odontoblasts and Presecretory Ameloblasts</i>	213
7.3.2.d	<i>Secretory Ameloblasts and Secretory Odontoblasts</i>	213
7.3.2.e	<i>Maturation-Stage Ameloblasts</i>	218
7.3.3	Results of Synchrotron Induced X-ray Emission Analysis	222
7.3.3.a	<i>Secretory Ameloblasts</i>	222
7.3.3.b	<i>Maturation-Stage Ameloblasts</i>	229
7.3.3.c	<i>Presecretory Ameloblasts</i>	233
7.3.3.d	<i>Secretory Odontoblasts</i>	237
7.3.3.e	<i>Control</i>	240
7.4	Discussion	243
7.4.1	Autometallographic Analysis of Tooth Germs	243
7.4.2	Synchrotron Radiation Induced X-ray Emission Analysis of Tooth Germs	245
7.4.3	Limitations, Future Directions and Conclusions	248

Chapter 8

General Discussion

8.1	The Main Findings of the Present Study	251
8.1.1	The Spatial Distribution of Lead in Teeth	251
8.1.2	Tooth-Lead Levels as a Biomarker of Prenatal and Neonatal Lead Exposure in a Wistar Rat Model	252
8.1.3	The Spatial Distribution of Lead in Human Primary Teeth as a Biomarker of Prenatal and Neonatal Lead Exposure – A Pilot Human Study	254
8.1.4	Intracellular Distribution of Lead during Tooth Development	255
8.1.5	The spatial Distribution of Lead in the Roots of Human Primary Teeth	256

8.2	Direction of Future Work	256
8.3	Significance of the Findings of the Present Study	257
Appendix		
A.1	Energy Dispersive X-ray Analysis	260
A.1.1	Principles of EDAX	260
A.1.2	Methods for EDAX used in this Study	260
A.2	Secondary Ion Mass Spectrometry	262
A.2.1	Principles of SIMS	262
A.2.2	Methods for SIMS used in this Study	262
A.3	Weight of Wistar Rats (Chapter 3)	264
A.4	Lead Levels in Reagents, Consumables and Glassware	265
A.4.1	Method	265
A.4.2	Results	267
A.5	ICP-MS and ICP-AES Instrument Parameters	269
A.6	Bland-Altman Test	270
A.7	Weight of Wistar Rats (Chapter 4)	273
A.8	Spatial Distribution of Lead in Rat Teeth Measured by NMP – Tabulated Data	274
A.9	NMP Analysis of a Human Primary Tooth	276
References		277

DECLARATION

This is to certify that all of the material presented in this thesis comprises original work conducted by the candidate with the exception of Figures 3.1 and 4.2 drawn in part by Ms Minh Huynh and operation of the 2-ID-D synchrotron beamline at the Advanced Photon Source, USA, by Dr Barry Lai and Dr Zhonghou Cai. Due acknowledgement has been made in the text of all other materials used.

This thesis is less than 100,000 words in length, exclusive of the tables, lists, figures, references and appendix. The thesis has not been submitted for the award of any other degree or diploma at any other institution.

Manish Arora

August, 2005

ACKNOWLEDGEMENTS

Firstly, I wish to express my deepest gratitude to my primary supervisor, Dr Sheena Chan, for her guidance and support during the past four years. I am also indebted to my co-supervisors Associate Professor Brendan Kennedy and Professor Murray Walker for their constant advice and encouragement. Similarly, my sincerest thanks to Associate Professor Hans Zoellner for sharing his wealth of knowledge and the time he has devoted to helping me prepare this thesis.

My research would not have been possible without the help and guidance of many researchers from institutions in Australia and overseas. My sincerest thanks to Dr Chris Ryan (CSIRO), Ms Ashwini Sharma and Mr Dante Crisante (Division of Analytical Laboratories, ICPMR), Mr Ross Boadle and Ms Levina Dear (Electron Microscope Unit, ICPMR), Ms Suzy Elhlou and Dr Norman Pearson (GEMOC, Macquarie University), Mr Stephen Kum-Jew (Department of Pathology, University of Sydney), Dr Ross Mathews and his staff (Westmead Animal Holding, Westmead Hospital), Dr Cathy Harland, Dr Barry Lai, Dr Zhonghou Cai, Dr Stephen Vogt (Advanced Photon Source, USA), and Ms Mara Cvejic (Institute of Dental Research, Westmead Hospital).

I would also like to thank my friends and colleagues from the Department of Oral Pathology for their support. Thank you Ite, Minh, Deborah, Rainier, Xiao Yang, Christine, Yin, William and Dave for your companionship. Also, a big thank you to Ms Janice Matthews and Ms Carolyn Bruce for all the help they have given me.

I would like to extend my love and gratitude to my partner, Catherine, for always believing in me and for all her patience and support over the years.

I received financial support from the Australian Dental Research Foundation, Dental Board of NSW, Australian Institute of Nuclear Science and Education, Faculty of Dentistry,

University of Sydney, Australian Synchrotron Research Program, International Postgraduate Research Scholarship, International Postgraduate Award and the Westmead Millennium Stipend Enhancement Grant. Experiments at the Advanced Photon Source, Argonne National Laboratories, USA, were supported by the US Department of Energy, Basic Energy Sciences, Office of Science, under contract no. W-31-109-Eng-38.

J.B.W.

LIST OF PUBLICATIONS ARISING FROM THIS STUDY

Arora M, Chan SWY, Kennedy BJ, Sharma A, Crisante D, Walker DM (2004). Spatial distribution of lead in the roots of human primary teeth. *Journal of Trace Elements in Medicine and Biology* 18:135-9.

Arora M, Chan SWY, Ryan CG, Kennedy BJ, Walker DM (2005). Spatial distribution of lead in the enamel and coronal dentine of Wistar rats. *Biological Trace Element Research* 105: 159-70.

Arora M, Chan SWY, Kennedy BJ, Ryan CG, Sharma, A, Crisante D, Byth K, Walker DM. Dental lead levels as biomarker of prenatal and neonatal lead exposure in a Wistar rat model. *Biomarker* (Accepted, April 2005).

Arora M, Chan SWY, Kennedy BJ, Elhlou S, Pearson N, Walker DM. Spatial distribution of lead in human primary teeth as a biomarker of prenatal and neonatal lead exposure. *Environmental Research* (Submitted May, 2005).

LIST OF FIGURES

1.1	Diagram showing enamel and dentine apposition in a human mandibular molar tooth	53
2.1	Schematic diagram of the cross-section of a concentric nebulizer in an inductively coupled plasma mass spectrometer	59
2.2	Schematic diagram of the cross-section of an inductively coupled plasma torch	60
2.3	Schematic diagram of the sampler and skimmer cones comprising the ion extraction interface, the ion lens system and the quadrupole mass filter of an inductively coupled plasma mass spectrometer	62
2.4	Diagram of the layout of a laser ablation inductively coupled plasma mass spectrometer	69
2.5	Schematic diagram of the layout of the nuclear microprobe used in the present study	74
2.6	Diagram outlining the principle of autometallography	76
2.7	Diagram of the layout of a synchrotron	80
3.1	Diagram of a right mandibular first molar tooth from a 15-day-old Wistar rat pup	87
3.2	Photomicrographs of a polished section of the lingual aspect of the crown of a right mandibular first molar tooth from a 15-day-old Wistar rat pup	88
3.3	Typical lead distribution map from a single NMP scan of the lingual aspect of a mandibular first molar tooth from a rat pup exposed to lead during tooth development	94
3.4	The mean total lead concentration, mean peak lead concentration and mean width of the four zones of lead distribution in the enamel and coronal dentine of lead-exposed rat pups	96
3.5	Distribution of lead and zinc in a mandibular first molar tooth of a lead-exposed rat	97
4.1	Typical calibration graph of the inductively coupled plasma mass spectrometer used in the present study	113
4.2	Diagram of the sites of analysis on the lingual aspect of the mesio-lingual cusp of a mandibular first molar tooth, sectioned vertically in the mesio-distal plane, of a 15-day old Wistar rat	121
4.3	The spatial distribution of lead in enamel and coronal dentine of a lead-exposed rat pup measured using laser ablation inductively coupled	134

	plasma mass spectrometry	
4.4	Maternal lead consumption and tooth-lead levels in one pup from each of the Exposure groups (Groups 2-5)	136
4.5	Average daily maternal lead consumption and tooth-lead levels of pups from Exposure groups (Groups 2-5)	137
5.1	Diagram of a human primary mandibular central incisor	149
5.2A-B	Spatial distribution of lead in enamel (▲) and coronal dentine (■) of primary mandibular incisors of Participants 1 and 2	159
5.2C-D	Spatial distribution of lead in enamel (▲) and coronal dentine (■) of primary mandibular incisors of Participants 3 and 4	161
6.1	Diagram of the root of a human primary incisor	174
6.2	The distribution of lead, calcium and phosphorus in the roots of different types of teeth	177
6.3	Lead, calcium and phosphorus concentrations in the apical, middle and cervical section of tooth roots	178
6.4	Lead concentrations in the apical, middle and cervical section of incisors, canines and molars	179
7.1	Methylene blue stained longitudinal section of an undecalcified mandibular first molar tooth germ from a newborn Wistar rat	196
7.2	Methylene blue stained longitudinal section of an undecalcified mandibular first molar tooth germ from a newborn Wistar rat showing differentiating ameloblasts and odontoblasts	198
7.3	Methylene blue stained, undecalcified, longitudinal section of a mandibular first molar tooth germ from a newborn Wistar rat showing presecretory ameloblasts and secretory odontoblasts	200
7.4	Methylene blue stained longitudinal section of an undecalcified mandibular first molar tooth germ from a 7-day old Wistar rat showing secretory ameloblasts	202
7.5.	Methylene blue stained longitudinal section of an undecalcified mandibular first molar tooth germ from a 7-day old Wistar rat showing secretory and maturation-stage ameloblasts	204
7.6	Autometallographic analysis of secretory ameloblasts and secretory odontoblasts from the Control group at extended development times	207
7.7	Autometallographic analysis of differentiating ameloblasts and differentiating odontoblasts	210
7.8	Autometallographic analysis of a section of a mouse spinal cord (positive control)	212

7.9	Autometallographic analysis of presecretory ameloblasts and secretory odontoblasts	215
7.10	Autometallographic analysis of secretory ameloblasts and secretory odontoblasts	217
7.11	Autometallographic analysis of maturation-stage ameloblasts	220
7.12	The spatial distribution of zinc, lead, calcium, phosphorus, copper and nickel in secretory-stage ameloblasts	226
7.13	The spatial distribution of calcium, phosphorus, zinc, lead, copper and nickel in maturation-stage ameloblasts	232
7.14	The spatial distribution of calcium, phosphorus, lead, copper and nickel in presecretory-stage ameloblasts	236
7.15	The spatial distribution of calcium, phosphorus, zinc, lead, copper and nickel in secretory-stage odontoblasts	238
7.16	The spatial distribution of calcium, phosphorus, zinc, lead, copper and nickel in a control tooth germ	242
A.1	Typical energy dispersive X-ray analysis scan	261
A.2	The spatial distribution of lead (^{208}Pb and ^{207}Pb) and calcium (^{42}Ca) measured in the dentine of a human mandibular molar tooth using secondary ion mass spectrometry	263
A.3	The difference between tooth-lead and bone-lead concentrations plotted as a function of the average of the tooth-lead and bone-lead measurements	271
A.4	The difference between tooth-lead and kidney-lead concentrations plotted as a function of the average of tooth-lead and kidney-lead concentrations	272
A.5	The difference between log transformations of tooth-lead and kidney-lead as a function of the average of the log transformations of tooth-lead and kidney-lead	272
A.6	A nuclear microprobe scan of a human mandibular central incisor tooth	276

LIST OF TABLES

1.1	Key studies reporting lead levels in whole primary and permanent human teeth collected from individuals residing in industrial, urban, suburban and rural areas	37
1.2	Key studies reporting lead levels in enamel and dentine of primary and permanent human teeth collected from individuals residing in industrial, urban, suburban and rural areas	41
3.1	Key studies reporting the spatial distribution of lead in human teeth.	103
4.1	Concentration of lead nitrate in maternal drinking water and time periods of exposure for the five experimental groups of Wistar rats	110
4.2	Analysis of standard reference materials using inductively coupled plasma mass spectrometry (ICP-MS)	125
4.3	Dose of administered lead and lead levels in maternal blood as well as teeth, blood, bone and organs of pups	129
4.4	Statistical analysis of tooth-, blood-, bone- and organ-lead data for rat pups in all groups	131
5.1	Analysis of standard reference material NIST SRM-1486 (Bone Meal) using laser ablation inductively coupled plasma mass spectrometry	152
5.2	Analysis of standard reference material NIST SRM-610 using laser ablation inductively coupled plasma mass spectrometry	153
5.3	Details of the ten participants recruited from Broken Hill	154
5.4	Lead levels in enamel, dentine and blood of the four participants (ID:1-4) with two blood-lead measurements	162
5.5	Lead levels in enamel, dentine and blood of the six participants (ID :5-10) with one blood-lead measurement	163
5.6	Comparison of lead levels in postnatally formed enamel and dentine of participants with high (> 10 µg/dL) and low blood-lead (< 10 µg/dL) levels	164
6.1	Participant age at the time of dental extraction, the type of tooth collected and number of roots for each tooth type	171
6.2	Analysis of standard reference material SRM-1486 (Bone Meal) using inductively coupled plasma mass spectrometry	175
7.1	Summary of the autometallographic findings from tissue sections of mandibular molar teeth of rats in the lead-exposed group	223

7.2	The intracellular concentrations of selected elements in secretory-stage ameloblasts	226
7.3	The intracellular concentrations of selected elements in maturation-stage ameloblasts	232
7.4	The intracellular concentrations of selected elements in the presecretory-stage ameloblasts	236
7.5	The intracellular concentrations of selected elements in the secretory-stage odontoblasts	239
A.1	Weight of the Wistar rat neonates and maternal rats in the Control and Exposure groups	264
A.2	Lead levels in reagents, consumables and glassware measured by ICP-MS	267
A.3	Instrument parameters used during ICP-MS and ICP-AES analysis	269
A.4	Weight of the Wistar rat neonates and maternal rats in the five experimental groups	273
A.5	Tabulated data of the lead levels in enamel and coronal dentine of Wistar rat pups from the four Exposure groups measured using the NMP	274

ABBREVIATIONS

Ad	differentiating ameloblasts
Ag	silver deposits
AIHW	Australian Institute of Health and Welfare
ALA	δ-aminolevulinic acid
ALAD	δ-aminolevulinic acid dehydratase
AM	autometallography
Am	maturation ameloblasts
Aps	presecretory ameloblasts
As	secretory ameloblasts
ASRP	Australian Synchrotron Research Program
ATSDR	Agency for Toxic Substances and Disease Registry
CDC	Centers for Disease Control and Prevention
CEJ	cemento-enamel junction
CSIRO	Commonwealth Scientific and Industrial Research Organi sation
D	dentine
DALY	disability-adjusted life year
DEJ	dentino-enamel junction
DL	distolingual
DMT1	divalent metal transporter 1
DP	dental papilla
E	enamel
EDAX	energy dispersive X-ray analysis
EDTA	ethylene di-amine tetra acetic acid
EFA	enamel free area
EG	electron gun
EPA	Environmental Protection Authority
FMN	facial motor neuron
GEMOC	Geochemical Evolution and Metallogeny of Continents
GF-AAS	graphite furnace atomic absorption spectrometry
IARC	International Agency for Research on Cancer
ICP-AES	inductively coupled plasma atomic emission spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
IDL	instrument detection limit
IQ	intelligence quotient
LA-ICP-MS	laser ablation inductively coupled plasma mass spectrometry

laser	light amplification by stimulated emission of radiation
LINAC	linear accelerator
MDL	method detection limit
ML	mesiolingual
MOPS	3-(N-morpholino)-propane-sulphonic acid buffer
n	nucleus
NAG	N-acetyl- β -D-glucosaminidase
Nd:YAG	neodymium: yttrium aluminium garnet
NIST	National Institute of Standards and Technology
NMP	nuclear microprobe
Od	differentiating odontoblasts
Os	secretory odontoblasts
P	pulp chamber
PD	predentine
PIGE	proton induced γ -ray emission
PIXE	proton induced X-ray emission
PoZ	postnatal zone
ppb	parts-per-billion
ppm	parts-per-million
ppt	parts-per-trillion
PQL	practical quantitation limit
PrZ	prenatal zone
QC	quality control
RF	radiofrequency
RSE	relative standard error
SD	standard deviation
SEM	scanning electron microscopy
SE	standard error
SI	stratum intermedium
SIMS	secondary ion mass spectrometry
SR	stellate reticulum
SRIXE	synchrotron radiation induced X-ray emission
SRM	standard reference material
UK	United Kingdom
US	United States of America
WHO	World Health Organisation

SUMMARY

Lead is amongst the most hazardous toxins in our environment and continues to pose a significant health risk. At present there is no suitable biological marker to measure lead exposure experienced during the prenatal and neonatal periods of development. In the present study it was hypothesised that the spatial distribution of lead in teeth would provide temporal information regarding the pattern and intensity of lead exposure experienced during the prenatal and neonatal periods of development.

A Wistar rat model was firstly used to establish a suitable experimental methodology and study the spatial distribution of lead in teeth at a fixed level of lead exposure. A high-resolution map of lead distribution was developed and served as the basis for a larger animal experiment where female Wistar rats were exposed to different predetermined doses of lead in drinking water during the periods of gestation and lactation. Lead concentrations in the prenatally and postnatally formed enamel and dentine of rat pups were measured using a nuclear microprobe, and were compared to maternal intake of lead during the corresponding time periods. The spatial distribution of lead in coronal dentine adjacent to the dentino-enamel junction showed a strong positive correlation with the maternal lead intake ($r > 0.68$). Lead levels in enamel, however, did not mirror the pattern of maternal lead consumption ($r < 0.42$).

In addition to mapping the spatial distribution of lead in enamel and coronal dentine, lead concentrations were also determined in whole first mandibular molar teeth, blood, bone and key organs (liver, kidney and brain) using inductively coupled plasma-mass spectrometry. Tooth-lead levels consistently showed a positive correlation with the organ-lead levels ($r > 0.83$), which was greater than the correlation between blood- and organ-lead levels ($r < 0.73$).

This methodology was subsequently applied in a pilot human study. Primary teeth, blood-lead and environmental data were collected from ten children living in the Broken Hill region of NSW, Australia. The lead concentrations in prenatally and postnatally formed enamel and dentine, measured using laser-ablation inductively coupled plasma-mass spectrometry, were compared with blood-lead levels taken at birth and at the age of one year. Similar to observations in the animal experiments, lead concentrations in dentine reflected the blood-lead concentrations. Enamel-lead levels, however, did not agree with the lead concentrations in blood or coronal dentine.

To understand the biological mechanisms behind the observed heterogeneity of lead distribution in dental hard tissues attempts were made to determine the intracellular distribution of lead in ameloblasts and odontoblasts during tooth development using autometallography, energy dispersive X-ray analysis and synchrotron radiation induced X-ray emission. Intracellular deposits of lead, however, were not localised using these techniques.

Overall, this thesis illustrates the potential value of dentine-lead measurements as an effective biomarker for prenatal and neonatal lead exposure and also establishes the necessary methodology.

CHAPTER 1

Environmental Lead Exposure: A **Literature Review**

1.1 Introduction

Lead is amongst the most hazardous substances in our environment (Agency for Toxic Substances and Disease Registry (ATSDR), 2003). While blood-lead levels have declined in developed countries over the past decade, those living in developing countries are experiencing an unprecedented increase in environmental lead exposure (Tong *et al.*, 2000a). In addition, emerging evidence indicates that blood-lead levels previously considered safe may have a significant influence on human health, particularly during the period of early development (Canfield *et al.*, 2003).

The determination of the health risk to individuals and groups due to environmental lead exposure requires the use of accurate biological markers. Current methods of biological monitoring that rely primarily on blood-lead levels suffer from several drawbacks that limit their use in epidemiological research and public health surveillance. Lead levels in blood, due to a short half-life of 28-36 days (World Health Organisation [WHO], 1995), are not a reliable marker of long-term exposure and body burden of lead. As a consequence, there exists a need to develop and refine alternative biological markers to measure the degree of exposure and better understand the health effects of environmental lead exposure. This chapter provides relevant background information on the global epidemiology and health effects of lead exposure. The issues of biological monitoring and the kinetics of lead uptake and absorption are also addressed. The aims of the present study are presented in the final section.

1.2 Historical Perspective of Lead Exposure

Woolley (1984) describes lead poisoning as one of the earliest occupational diseases experienced by humans. Lead is believed to have been discovered as early as 3500 BC, and was used by most major ancient civilisations including the Egyptians, Phoenicians, Hebrews, Romans and Greeks (Lin-Fu, 1980). The toxic effects of lead were well

recognised by both the Romans and the Greeks, however, its use was widespread in those communities. In the 16th and 17th centuries, the exploitation of lead-silver mines continued during the Spanish expeditions into Mexico and Peru and, at times, the volume of lead production was similar to that seen in Roman times (Woolley, 1984). The Industrial Revolution in the mid-18th century saw an unprecedented increase in the production of lead resulting in numerous cases of lead poisoning in occupationally exposed workers and their families. With increasing amounts of lead being released into the environment, incidences of neonatal lead exposure began to increase (Lin-Fu, 1980). Paediatric lead poisoning that was unrelated to parental exposure first gained recognition in Australia in 1892 when Gibson and his colleagues from the Brisbane Hospital for Sick Children reported ten cases of lead related palsy (Lin-Fu, 1980).

In 1921, the discovery of the 'anti-knock' effect of tetraethyl lead at the Kettering Research Laboratories of General Motors proved to be one of the most significant events in the history of lead exposure (Kitman, 2000). The highly toxic organo-lead compounds, due to their volatile nature, became the primary contributors to global lead emissions (Kitman, 2000). In the late 1970s and early 1980s, with the recognition of the adverse effects of leaded petrol, the use of unleaded petrol was made mandatory for new automobiles in the United States of America (US). Other countries, including Australia, have since taken similar steps.

In most developed countries improvements in industrial hygiene have resulted in a rapid decline in the incidence of acute lead poisoning. In developing countries, however, the incidence of adult and paediatric lead poisoning has remained high (Tong *et al.*, 2000a).

1.3 Epidemiology of Lead Exposure

The prevalence of lead poisoning has shown marked variations between developed and developing countries. This section provides an overview of the global distribution of lead

poisoning, the socioeconomic determinants of excessive lead exposure, the major environmental sources of exposure, and comparisons of the impact of lead exposure on health with that of other diseases.

1.3.1 Global Distribution of Lead Exposure

1.3.1.a Developed Countries

Since the 1980s there has been a steady decline in the blood-lead levels in developed countries that has been attributed to the reduction in the use of leaded petrol and better industrial practices (Tong *et al.*, 2000a). In the US, data from successive National Health and Nutrition Examination Surveys showed declines of greater than 70% in blood-lead levels in children and adults from 1974 to 1994 (Pirkle *et al.*, 1994 and 1998). In 1994, an estimated 4.4% of American children aged 1-5 years had blood-lead levels above the Centers for Disease Control and Prevention's (CDC) recommended limit of 10 µg/dL (CDC, 1991). However, due to recent concerns of the health effects at even lower blood-lead levels (Canfield *et al.*, 2003), a re-examination of the data from the third National Health and Nutrition Examination Survey was undertaken and revealed that 25.6% of American children had blood-lead levels above 5 µg/dL (Bernard and McGeehin, 2003).

In the United Kingdom (UK), Delves *et al.* (1996) reported a similar decline in blood-lead levels across individuals of all ages over an 11-year period from 1984 to 1995. In 1995, 97% of the people surveyed had blood-lead concentrations below the recommended limit of 10 µg/dL. Similarly, in New Zealand, data on a birth cohort of 799 individuals showed a 53% decline in mean blood-lead levels during a 10-year period (Fawcett *et al.*, 1996). In Australia, the national survey of lead in Australian children conducted in 1995 showed that only 7.3% of the children had blood-lead levels above 10 µg/dL (Australian Institute of Health and Welfare [AIHW], 1996). Evidence of country-wide trends in blood-lead levels of

both adult and younger populations is not available as no national survey has since been conducted in Australia. Evidence from the surveillance of high-risk populations in Broken Hill, New South Wales, and Port Pirie, South Australia, however, indicated that blood-lead levels in children have been steadily declining in these regions (Burke *et al.*, 2003; Maynard *et al.*, 2003).

Overall, blood-lead levels have continually declined in most developed countries (Tong *et al.*, 2000). As a result, acute high-level lead exposure is now rare and chronic low-level lead exposure poses the primary risk to populations residing in developed countries (Tong *et al.*, 2000).

1.3.1.b *Developing Countries*

In contrast to developed countries, surveys undertaken in developing countries have reported significantly higher blood-lead levels. For example, in India, Patel *et al.* (2001) reported elevated blood-lead levels in 67% of the 297 children surveyed. Another Indian study reported a similar prevalence of chronic lead toxicity and also an increase in the number of children with elevated blood-lead levels during the period of 1985-1998 (Kaul, 1999). In China, Qi *et al.* (2002) surveyed over 6000 children from major cities and reported that 29.91% of those children had elevated blood-lead levels. The authors also reported an increase in blood-lead levels over a 10-year period.

In South Africa, a cross-sectional survey of 433 school children reported that 78% of children had blood-lead levels exceeding 10 µg/dL (Mathee *et al.*, 2002). In a study in Nigeria, elevated blood-lead levels were reported in 95.3% of the 86 participants at risk from occupational lead exposure (Adeniyi and Anetor, 1999). Interestingly, in that study, measurements of blood-lead levels in the control group (n= 51) consisting of individuals not occupationally exposed to lead revealed an 82% prevalence of chronic lead toxicity.

Similar rates of elevated blood-lead levels were reported in other developing countries (Tong *et al.*, 2000).

Fewtrell *et al.* (2004) have compiled literature on blood-lead levels of individuals from different geographical regions and their summary analysis indicated that developing countries in Eastern Europe, Africa, Asia and South America have a 20-30% prevalence of elevated blood-lead levels in both adults and children. Overall, while blood-lead levels have declined in developed countries, the limited data from developing countries indicates that blood-levels are significantly higher and are increasing in some regions.

1.3.2 Socioeconomic Determinants of Lead Exposure

Closer inspection of the data from the US National Health and Nutrition Examination Surveys revealed that non-Hispanic African American children and those from low income families had significantly higher blood-lead levels than their non-Hispanic white counterparts (Pirkle *et al.*, 1994 and 1998). Likewise, in Australia, a cross-sectional survey of 164 children reported that Aboriginal children had higher blood-lead levels than non-Aboriginal Australian children (Willis *et al.*, 1995). It was reported that elevated blood-lead levels were also more prevalent in those residing in older housing. Similar associations have been reported in some developing countries, including South Africa and China (Shen *et al.*, 1997; Von Schirnding *et al.*, 1991). In summary, while there exist geographical differences in the prevalence of chronic lead toxicity between developing and developed countries, the socioeconomic status of sub-groups within countries is also an important determinant of lead exposure.

1.3.3 Sources of Lead Exposure

Chronic low-level lead exposure is generally associated with multiple sources of pollution. The major sources of lead exposure for the general population include leaded petrol, industrial processes, lead containing paint, lead solder in canned food, leaded water

pipes, hobbies (e.g. shooting and certain types of pottery), and in some countries traditional herbal remedies (Fewtrell *et al.*, 2004; Tong *et al.*, 2000). Occupational lead exposures were mainly associated with industrial operations including, automobile manufacturing, lead battery recycling, panel beating, lead alloy production, soldering, and various processes in the plastics, ceramics, printing and paint industries (Tong *et al.*, 2000).

The major sources of exposure in Australia identified by the Environmental Protection Authority (EPA) include, leaded petrol, lead containing paint in old houses, lead containing piping and fixtures, and consumer products, for example imported cosmetics, alloys, hobby material and old paint. In addition, automotive and construction items, and sporting or hobby activities, including shooting, pottery glazing, painting, and automobile-related activities, have also been identified as potential sources of lead exposure (EPA, 2003).

1.3.4 Global Burden of Disease Due to Lead Exposure

Environmental lead exposure is one of many factors that influence human health. As such, it is important to place environmental lead exposure in the context of the wider spectrum of disease and ill health. A brief discussion is provided here on the global burden of disease and disability attributed to environmental lead exposure. Fewtrell *et al.* (2004) studied the summary measure 'disability-adjusted life year' (DALY) for 'mental retardation' and cardiovascular disease caused by environmental lead exposure world-wide. The authors concluded that 90% of all children having blood-lead levels above 10 µg/dL live in developing countries and that environmental lead exposure accounted for 0.9% of the total global burden of disease. This places lead in the 16th position in terms of the leading risk factors for health globally (Fewtrell, 2004). In a similar analysis limited to Europe, Valent *et al.* (2004) concluded that lead exposure accounted for 4.4% of the total DALYs. The outcome of these two analyses confirmed that environmental lead exposure remains an important global public health issue.

1.4 Lead Absorption, Distribution and Excretion

1.4.1 Absorption

1.4.1.a Absorption after Ingestion

Lead is absorbed through the gastrointestinal tract after ingestion. On average, lead absorption and retention after ingestion are approximately 4-20% and 5% respectively (Goyer, 1988; Chamberlain, 1985; Blake and Mann, 1983; Gross, 1981; Rabinowitz *et al.*, 1980). In children, the uptake of lead is higher than in adults. Alexander (1974) observed absorption and retention rates of 53% and 18% respectively from ingested lead in children.

Fasting significantly increases lead uptake, while certain dietary constituents inhibit lead absorption (Rabinowitz *et al.*, 1980; Blake and Mann, 1983). For example, during fasting, gastrointestinal lead absorption was $35 \pm 13\%$ (Rabinowitz *et al.*, 1980). In another study, fasting subjects absorbed approximately 65-70% lead, but co-ingestion of calcium and phosphorus reduced the absorption to approximately 14-18% (Blake and Mann, 1983). Similar results have been reported in rodent models where lead absorption was decreased by a factor of two when 6% calcium was administered simultaneously (Varnai *et al.*, 2001).

Divalent metal transporter 1 (DMT1) is a transmembrane protein that has been shown to regulate the absorption of lead in the gastrointestinal tract (Bannon *et al.*, 2003). High concentrations of DMT1 are found in the duodenum where it can bind to iron, lead and other metal ions facilitating their transport into the blood (Gunshin *et al.*, 1997). Consistent with the fact that DMT1 secretion is regulated by iron intake, dietary iron deficiency results in increased lead absorption in humans (Kwong *et al.*, 2004).

1.4.1.b *Absorption after Inhalation*

The amount of lead deposited in the lungs is reliant on several factors including the airborne concentration of lead, an individual's ventilation rate, and the size of the lead-containing particles (Chamberlain, 1985). Atmospheric lead-containing particles with a diameter less than 0.5 μm may be transported to the alveolar region where exchange of lead with blood is possible (Skerfving, 1988). Chamberlain *et al.* (1975) exposed human volunteers to ^{203}Pb labelled petrol exhausts from automobiles under controlled conditions and observed that at a respiration rate of 15 breaths/min, the deposition of lead in the lungs was $35 \pm 2\%$. In addition, at an interval of 50 hr the authors observed no difference between lead concentrations in blood after inhalation of tetraethyl lead and lead chloride injections. From this, they concluded that all lead taken into the lungs was transferred to the blood. Evidence in support of this finding was provided by Barry (1975) in a study that compared lead levels in the different tissues of deceased lead workers. The lungs at autopsy did not show any significant lead accumulation, indicating that inhaled lead was almost completely transferred from the lungs to the blood. Although not retained in the lungs, inhaled lead has been shown to affect lung function in occupationally exposed groups where the maximum voluntary ventilation, forced expiration flow and the first forced expiration volume measurements were significantly worse than the non-exposed individuals in the control group (Bagci *et al.*, 2004).

1.4.1.c *Percutaneous Absorption*

While it is known that lead can be absorbed through the skin, this is not regarded as a major route of lead uptake. Stauber *et al.* (1994) applied lead nitrate and lead acetate to the skin of human volunteers and observed a significant increase in the lead content of sweat and saliva, but no increase was noted in blood-lead concentrations. It was argued that lead absorbed through the skin is largely restricted to the extracellular compartments including sweat and saliva. In that study, 29.4% of lead applied to the skin was absorbed

during the first 24 hr. Additional studies exploring the use of lead levels in sweat as an indicator of exposure are discussed in Section 1.6.1.g.

1.4.2 Distribution

Over 90% of absorbed lead is deposited in the bones (Tsuchiya, 1979). Within the skeletal compartment, cortical bone contains the larger proportion of lead, approximately 69%, while trabecular bone contains approximately 23% of the total lead body burden. Soft-tissues and blood account for approximately 8% and 0.4% of the lead body burden respectively (Skerfving *et al.*, 1993). The half-life of lead in the various compartments varies considerably. Lead deposits in cortical bone have the longest half-life in the order of 10 years, while the half-life in trabecular bone is approximately 1 year. This contrasts with soft tissue and blood cells (approximately 1 month), as well as plasma (less than 1 hr) (Skerfving *et al.*, 1993; Goyer, 1988).

Several models for the kinetics of lead body burden have been published with a focus on the largest pool of lead present in the skeleton (Brito *et al.*, 2005; Mason, 2000; O'Flaherty, 1998; Leggett, 1993; Rabinowitz, 1991; Bert *et al.*, 1989; Steenhout, 1982). Essentially, in these models lead deposits from recent exposure were considered to be present primarily in the blood, soft tissues (including internal organs) and to some extent in the trabecular bone. Cortical bone-lead was thought to represent long-term stores. It was proposed that after cessation of exposure, the lead deposits in cortical bone may constitute an endogenous source of long-term exposure, as lead may be released from cortical bone over many years (Hu *et al.*, 1998). Lead stores from bones may also be mobilised more rapidly during periods of increased physiological bone remodelling such as pregnancy and lactation (Gulson *et al.*, 1999).

Mobilisation of lead from various compartments during pregnancy and lactation may pose a significant health risk to the developing foetus and neonate (Gulson *et al.*, 1999). It is

well established that lead from the mother readily crosses the placenta and is taken up by the developing foetus (Goyer, 1990). Korpela *et al.* (1980) compared lead levels in maternal blood and umbilical cord blood of 19 women and found a significant positive correlation between the two variables ($r = 0.77$). Also of interest was the finding that amniotic fluid had a significantly higher mean lead concentration than the other tissues studied. Similar results have been reported in other studies (Wang *et al.*, 2004; Osman *et al.* 2000; Ernhart *et al.*, 1986). The lack of a significant difference in lead concentrations between maternal blood and cord blood in these studies indicates the absence of an effective placental barrier for lead.

Aside from the transplacental route, lead may also be transferred from mother to infant through maternal milk. Ettinger *et al.* (2004) measured lead levels in maternal blood and milk of 255 participants. Mean lead levels in breast milk were significantly lower than both maternal and infant mean blood-lead concentrations. Significant positive correlations were observed between infant blood-lead levels and maternal milk-lead at the age of one year ($r = 0.32$). The authors concluded that maternal milk-lead levels, although low, exerted a significant influence on infant blood-lead concentrations. Other studies have also reported a positive association between maternal blood-lead and breast milk-lead levels (Nashashibi *et al.*, 1999; Ong *et al.*, 1985).

1.4.3 Excretion

Following ingestion, approximately 90% of the unabsorbed lead is excreted through faeces (Tsuchiya, 1979). Faecal lead levels are considered to be indicative of dietary lead intake but are not considered a good indicator of lead body burden because they mainly represent unabsorbed lead (Skerfving, 1988). Isotopic analysis of lead in faeces may, however, be useful for the determination of sources of exposure (Gwiazda *et al.*, 2005). Details of lead excretion in urine and sweat, and their applications in estimating environmental lead exposure are discussed in Section 1.6.1.b and 1.6.1.g respectively.

1.5 An Overview of the Health Effects of Lead Exposure

Environmental lead exposure can adversely affect many systems in the human body. These effects may be broadly classified as acute and chronic effects. Chronic effects are discussed in greater detail as the present study is concerned with the biological monitoring of long-term lead exposure.

1.5.1 Acute Effects

The acute effects of lead poisoning are generally encountered at blood-lead levels above 80 µg/dL (Tsuchiya, 1979). Acute lead poisoning is characterised by anorexia, dyspepsia, constipation and gastrointestinal colic with diffuse paroxysmal abdominal pain. Pale skin, slow pulse and hypertension are also known to occur. Acute lead encephalopathy may develop with cerebral oedema, sudden onset of seizures, vomiting, ataxia, drowsiness and in some cases coma and cardiac arrest (Goyer, 1988; Tsuchiya, 1979). Nephrotoxicity characterised by aminoaciduria, phosphaturia and glycosuria is also observed in acute lead poisoning (Goyer, 1992). Numerous recent case studies of lead poisoning in children and adults have described the aforementioned symptoms (Coyle *et al.*, 2005; Gustavsson and Gerhardsson, 2005; Maslinski and Loeb, 2004; VanArsdale *et al.*, 2004; van der Klooster, 2004; Gordon *et al.*, 1998; al Khayat *et al.*, 1997; Goodheart and Dunne, 1994; Woolf *et al.*, 1990; Sharma *et al.*, 1990). The appearance of a purple-blue discolouration of the gingiva, known as Burton's line, and excessive salivation accompanied with a persistent metallic taste may also occur in some cases of acute lead poisoning (Neville *et al.*, 2002). The clinical presentations of acute lead poisoning may, however, show variations between individuals as documented by Gordon *et al.* (2002) in a report of three adults having similar blood-lead levels in the range of 85 µg/dL but different clinical symptoms.

1.5.2 Chronic Effects

The incidence of acute lead exposure has declined significantly in developed countries. Chronic low-level lead exposure, however, continues to pose a major health risk. The major health effects of chronic lead exposure are discussed in this section.

1.5.2.a Neurological Effects

The neurological effects of long-term low-level lead exposure have received considerable attention over the years. Several large cross-sectional and prospective cohort studies of children have been conducted to explore the effects of lead exposure on neurological development.

Studies published in the late 1970s and early 1980s by Needleman and co-workers (Needleman, 1982a and 1982b; Needleman *et al.*, 1982; Needleman *et al.*, 1979; Needleman, 1979; Needleman and Leviton, 1979) reported inverse correlations between dentine-lead levels and neurobehavioral development. Needleman *et al.* (1979) showed that children, with no overt clinical symptoms of lead poisoning, who had dentine-lead levels higher than 24 parts-per-million (ppm) were more likely to suffer from neuropsychological deficits measured using a battery of intelligence and class-room behaviour tests. Compared to children with low dentine-lead levels (defined as <6 ppm), children with high dentine-lead concentrations consistently performed poorly on tests measuring intelligence, speech processing abilities and non-adaptive class-room behaviour. In an 11-year follow-up study of this cohort, Needleman *et al.* (1990) reported that those participants who had high dentine-lead levels in childhood showed a higher risk of not completing high school, lower reading skills and vocabulary, poor hand-eye coordination and had a lower class standing in high school. A subsequent analysis of the same cohort at the ages of 19-20 years showed that neurological deficits persisted in those with high dentine-lead levels (Bellinger *et al.*, 1994).

The Port Pirie Cohort studies in South Australia recorded maternal blood-lead levels during pregnancy, and subsequently the blood-lead levels of the participating children at birth, 1, 2, 5 and 7 years of age (Baghurst *et al.*, 1995; McMichael *et al.*, 1988; Wigg *et al.*, 1988; Baghurst *et al.*, 1987; McMichael *et al.*, 1985). The main outcomes of these studies showed that postnatal blood-lead concentrations were inversely related to cognitive development in childhood. Subsequent analyses revealed an inverse relationship between tooth-lead levels and intelligence quotient (IQ) (McMichael *et al.*, 1994). Interestingly, the blood-lead levels of these individuals showed a strong inverse relationship with visual-motor skills at the age of 7 years (Baghurst *et al.*, 1995). Follow-ups of the participants at the ages of 11-13 years demonstrated that the blood-lead levels taken during early childhood continued to show an inverse relationship with intelligence, emotion and behaviour ratings (Burns *et al.*, 1999; Tong *et al.*, 1996). Another significant finding that arose from this cohort study was the observation that declines in blood-lead levels due to public health intervention and environmental risk management partially reversed the cognitive deficits associated with exposure to environmental lead in early childhood (Tong *et al.*, 1998).

A number of cross-sectional studies have also found similar significant deficits in the neurobehavioural development of children exposed to environmental lead, when compared with the children in the non-exposed control groups (Rabinowitz *et al.*, 1991; Fulton *et al.*, 1987; Smith *et al.*, 1983; Winneke *et al.*, 1983).

A systematic review and meta-analysis of five cohort studies reported that a doubling of the blood-lead levels from 10 to 20 $\mu\text{g/dL}$ is associated with a deficit of 1-2 IQ points (Pocock *et al.*, 1994). A more recent meta-analysis by Schwartz *et al.* (1994), however, showed that the negative correlation between blood-lead and IQ increases in magnitude at blood-lead levels below 15 $\mu\text{g/dL}$. Results of recent cohort studies have also shown that the effect of low-level lead exposure on intellectual impairment may be more pronounced

than that shown in earlier research (Ris *et al.*, 2004; Canfield *et al.*, 2003). For example, Canfield *et al.*, (2003) found that blood-lead levels below 10 µg/dL were strongly related with declines in IQ in children and that a number of children previously thought to be safe from the effects of environmental lead exposure under the CDC guidelines of 10 µg/dL of lead in blood may suffer from an increased health risk.

In addition to the substantial epidemiological evidence supporting the toxic effects of lead on neurobehavioural outcomes, there are several published reviews exploring the biological mechanisms of lead-induced neurotoxicity. Lead has been reported to exert its neurotoxic actions thorough a number of mechanisms including the induction of apoptosis, excitotoxicity, interference with neurotransmitter storage and release, effects on mitochondria, altered second messenger activity, and effects on astroglia and oligodendroglia (Lidsy and Schneider, 2003). Lead substitutes for calcium in many cellular processes and, as a consequence of this, lead is able to cross the blood-brain barrier (Silbergeld, 1992). Lead exposed cells show a marked accumulation of calcium and lead also stimulates the release of calcium from mitochondrial stores (Silbergeld, 1992). The increased concentration of calcium was found in turn to induce apoptosis or programmed cell death (Bressler *et al.*, 1999).

Lead can accumulate in mitochondria and the damage sustained by these organelles has a cascading effect on neuronal functions resulting in disruption of synaptic transmissions in the brain and neuronal death through excitotoxicity (Lidsky and Schneider, 2003). In addition, lead can activate second messengers like calmodulin and protein kinase C that are normally activated by calcium, and participate in important neuronal functions including neuronal proliferation, differentiation and potentiation (Lidsky and Schneider, 2003). In a similar manner, by substituting for calcium, lead can disrupt the calcium-dependent release of neurotransmitters including acetylcholine and dopamine (Bressler *et al.*, 1999). The effects of lead exposure on oligodendroglia and astroglia include delayed

maturation of the progenitor glial cells, hypomyelination and demyelination (Coria *et al.*, 1984). Thus, from a biological standpoint, there is evidence that lead exposure during early neurodevelopment can disrupt the structural development of the central nervous system, while exposure in later life can interfere with the mechanisms of neurotransmission (Silbergeld, 1992).

1.5.2.b Haematological Effects

Environmental lead exposure has been linked with iron deficiency anaemia. The body of epidemiological evidence for this association is, however, considerably smaller than that in support of lead-induced neurotoxicity. Wright *et al.* (2003a) conducted the first longitudinal study on this issue and compared iron deficiency status with the blood-lead levels of 1050 urban children. Children with iron deficiency anaemia had greatly increased risk for developing high blood-lead levels in comparison to children with normal blood-iron levels (Odds ratio = 4.12). A number of cross-sectional studies have also reported finding a link between blood-lead levels and iron deficiency anaemia (Choi and Kim, 2003; Bradman *et al.*, 2001; Hammad *et al.*, 1996).

The biological mechanisms responsible for the association between environmental lead exposure and iron deficiency anaemia have been reviewed by Kwong *et al.* (2004). As mentioned in Section 1.4.1.a, lead and iron absorption are linked to the gastrointestinal transcellular metal transporter, DMT1 (Bannon *et al.*, 2003). Lead uptake via DMT1 is increased during iron deficiency, while iron has a higher affinity for DMT1 than lead and, as a consequence, iron supplementation can reduce the gastrointestinal uptake of lead absorbed (Kwong *et al.*, 2004). In the red blood cells, during heme synthesis, the combined effect of iron deficiency and lead poisoning result in the incorporation of zinc instead of iron into protoporphyrin resulting in an increase in zinc-protoporphyrin (Kwong *et al.*, 2004). Lead also inhibits δ -aminolevulinic acid dehydratase (ALAD) causing an increased excretion of δ -aminolevulinic acid (ALA) via urine. ALA is necessary for heme

synthesis and excessive excretion of this compound is also believed to play a role in the onset of anaemia. Other mechanisms of lead induced anaemia include suppression of erythropoietin release from the kidneys and reduction in the number of erythroid progenitor cells. These mechanisms, however, have not as yet received detailed investigation.

1.5.2.c Renal Effects

Chronic environmental lead exposure has been linked with tubular cell atrophy, interstitial fibrosis and glomerular sclerosis (Goyer, 1988). Yu *et al.* (2004), in a prospective epidemiological study, demonstrated increases in serum creatinine levels with increasing blood-lead levels. Similarly, Muntner *et al.* (2003) in an analysis of data from the third National Health and Nutrition Examination survey in the US found that lead exposure in participants with hypertension was related to elevated serum creatinine and chronic kidney disease characterised by a glomerular filtration rate of less than 60 mL/min. A cross-sectional survey of occupationally exposed workers in a lead battery plant found a positive association between blood-lead levels and urinary N-acetyl- β -D-glucosaminidase (Ehrlich *et al.*, 1998). In another cross-sectional survey, a number of parameters including urinary-albumin, - α 1 microglobulin, - β 2 microglobulin, and -retinol-binding protein, increased significantly when blood-lead levels exceeded 40 μ g/dL (Lim *et al.*, 2001). These recent studies indicate that low-level environmental lead exposure may accelerate progressive renal insufficiency in patients, and such effects may be enhanced by other systemic conditions such as hypertension. However, as acknowledged by Gidlow (2004), the full clinical significance of the effects of lead exposure on renal function remains to be explored.

The biological mechanisms of lead-related nephrotoxicity have been discussed in detail by Nolan and Shaikh (1992). The histopathological features of lead poisoning were found to be periglomerular and interstitial fibrosis, atrophy of renal tubules and glomeruli, and the

presence of characteristic cytoplasmic and nuclear inclusion bodies which were more common in acute lead poisoning than in low-level chronic lead exposure. By affecting glomerular filtration, renal clearance and tubular reabsorption, lead exposure may lead to renal failure (Nolan and Shaikh, 1992).

Cytoplasmic and nuclear inclusion bodies commonly seen in lead-related nephropathy have received much attention. These inclusion bodies are believed to play a protective role by sequestering the lead (Shelton and Egle, 1982), and have been shown to contain a specific protein of molecular weight 32,000 and isoelectric point of 6.3 (Shelton and Egle, 1982). Qu *et al.* (2002) showed that metallothionein also played an important role in the formation of these inclusion bodies in lead poisoning. Other proteins that have received attention include α_2 -microglobulin (Nolan and Shaikh, 1992), thymosin, acyl-CoA binding protein (also known as diazepam binding inhibitor) (Smith *et al.*, 1998a) and lead-thionein-like protein (Ikebuchi *et al.*, 1986). The clinical significance of lead interaction with these proteins has not been fully elucidated.

1.5.2.d Hypertension

Hypertension has long been regarded as a health effect of long-term lead exposure (Tsuchiya, 1979) and recent epidemiological studies have provided evidence in support of such an association. Glenn *et al.* (2003), in a longitudinal study of 496 adult participants, found a positive association between increased systolic blood pressure and lead levels in bone and blood. Likewise, Cheng *et al.* (2001) examined 833 participants in a longitudinal study and found a positive association between hypertension and bone-lead levels. No significant association, however, was found between blood-lead levels and blood pressure. Glenn *et al.* (2001) while studying the relationship of alleles of the sodium-potassium adenosine triphosphatase α_2 gene with blood pressure and lead exposure found that those individuals with a particular polymorphism of this gene were more likely to show a positive association between blood-lead and hypertension. These results were

indicative of the possible genetic predisposition of certain individuals to the toxic effects of lead which has also been suggested by other researchers (Chia *et al.*, 2004; Long *et al.*, 2002; Qu *et al.*, 2002).

Animal models have been developed to explore the biological mechanisms behind the association between chronic lead exposure and hypertension. These studies have suggested that lead may exert a number of effects including interference with the renin-angiotensin system, induction of sympathetic hyperactivity and increased sensitivity of cardiac and vascular β and dopaminergic receptors (Gildow, 2004; Boscolo and Carmignani, 1988).

1.5.2.e Oral Health Effects

Numerous cross-sectional epidemiological studies have explored the association between environmental lead exposure and dental caries. Early studies by Curzon and Bibby (1970) compared caries prevalence in populations having high lead levels in soil and water with control populations. Children living in the high-lead environment showed a 40% greater prevalence of caries than the children in the control group. More recent studies have compared blood-lead levels and dental caries scores and have found a weak to moderate association (Gemmell *et al.*, 2002; Moss *et al.*, 1999). Gil *et al.* (1996) studied the association of whole tooth-lead levels with various 'dental health' factors including dental caries, dental plaque, salivary pH as well as levels of *Salivarius lactobacilli* and *Mutans streptococci*, tooth brushing frequency, tooth discoloration and tooth abrasion. This study showed a positive correlation between tooth-lead levels and all but one of the dental health factors listed above. An inverse relationship was seen between lead levels in teeth and the pH of saliva, however, salivary lead levels were not measured in their study. Controlled prospective human studies measuring the effect of lead exposure on dental caries could not be found at the time of this review.

The biological mechanisms behind these findings have only had limited investigation using experimental animal models. Watson *et al.* (1997) exposed rats to 34 ppm of lead in drinking water during gestation and lactation and found a 40% increase in the incidence of caries in the lead-exposed pups compared to the pups in the control group. They also observed a 30% decrease in the stimulated salivary gland function of the rat pups. It was proposed that the reduced salivary secretions may partly explain the increase in caries prevalence (Watson *et al.*, 1997). In turn, it was thought that lead exposure may adversely affect salivary gland function. However, as no lead could be detected in the saliva of the pups, the authors suggested that exposure to lead during gland development can alter salivary gland function. Unfortunately, in the absence of histological evidence demonstrating glandular pathology and an incomplete salivary analysis, other than salivary flow rate, the authors' hypothesis cannot be confirmed.

In a similar manner, Abdollahi *et al.* (1997) exposed adult male rats to varying concentrations of lead through drinking water and observed a decrease in salivary calcium and protein concentrations. A decrease in the salivary secretion of lysozomal enzyme N-acetyl- β -D-glucosaminidase was also seen. Contrary to the findings of Watson *et al.* (1997), the animals in this experiment showed no statistically significant change in salivary flow rate. The additional evidence presented here appears to support the proposition that lead exposure during the prenatal period, when the salivary glands are forming can not only alter salivary gland function, but perhaps also development. Exposure during adulthood, however, appears to primarily affect the quality of saliva rather than the quantity.

Other studies exploring the influence of socio-demographic and environmental factors on salivary lead levels have not found any significant associations (Wilhelm *et al.*, 2002, Gonzalez *et al.*, 1997). Furthermore, limited evidence has been presented for the effects of lead exposure on tooth development and tooth eruption (Gerlach *et al.*, 2002; Gerlach

et al., 2000a and 2000b; Lawson *et al.*, 1971). Gerlach *et al.* (2002) demonstrated, in a rodent model, that lead exposure can delay enamel maturation, while Gerlach *et al.* (2000a) showed that lead exposure resulted in delayed tooth eruption in hypofunctional rat incisors that were removed from occlusion. Gerlach *et al.* (2000b) provided preliminary evidence that lead exposure inhibited the activity of enamel matrix proteinases in rats.

1.5.2.f *Cancer*

The role of lead as a carcinogen remains unclear. In a recent review of documented evidence, the International Agency for Research on Cancer (IARC), France, concluded that, “inorganic lead compounds are probably carcinogenic to humans”. A similar consensus could not be reached for organic lead compounds (IARC, 2004). Fu and Boffetta (1995) conducted a meta-analysis of several epidemiological studies that had explored the link between lead exposure and stomach, brain, kidney and lung cancer. When confounding variables were controlled, no clear association could be established between environmental lead exposure and the occurrence of cancer (Fu and Boffetta, 1995).

Although epidemiological studies have not provided convincing evidence for the potential carcinogenicity of lead, results from experimental models do support the proposition that lead exposure can cause cancer (Silbergeld *et al.*, 2000). The various mechanisms proposed include direct DNA damage, inhibition of DNA synthesis or repair, generation of reactive oxygen species, and possibly altered gene expression (Silbergeld *et al.*, 2000).

1.5.2.g *Other Effects*

Lead exposure has been implicated as a reproductive toxin. Very high blood-lead levels have been associated with miscarriages and low birth weights (Bellinger, 2005; Gidlow, 2004). Men exposed to high levels of lead have reduced sperm counts (Hernandez-Ochoa *et al.*, 2005; Bonde *et al.*, 2002). Lead has also been linked to immune suppression,

however, the evidence for this effect is limited (Goyer, 1988). Finally, it has been suggested that lead may also delay the healing of bone fractures (Carmouche *et al.*, 2005).

1.6 Biological Monitoring of Heavy Metal Toxins

Aitio (1988) defined biological monitoring as “a systemic or repetitive measurement and assessment of agents or their metabolites either in tissues or secretions, to evaluate the uptake of a chemical and the health risk involved, with the purpose of prevention of health effects by corrective actions as needed”. The primary objectives of biological monitoring for heavy metal toxins, as outlined by Elinder *et al.* (1988), are to measure the current and past exposure to toxins, identify at-risk individuals and groups, follow trends in exposure over time, assess body burden of a particular toxin or groups of toxins, provide data on dose-response relationships for risk assessment and to evaluate the effectiveness of medical treatment.

Biological monitoring requires appropriate media that may be collected and analysed to provide the necessary information regarding the dose of exposure, the body burden of the toxin under investigation and risk of adverse health effects. Such media are usually referred to as biological markers or biomarkers. Silbergeld and Davis (1994) defined biological markers as “physiological signals that reflect exposure, early cellular response or inherent or acquired susceptibilities, which provide a new strategy for resolving some toxicological problems”. Alternatively, the National Academy of Sciences, USA, defined a biological marker as “a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample” (ENTOX/TIWET, 1996).

An ideal biomarker, according to Grandjean *et al.* (1994), is one that can be easily collected and reliably analysed. It should also be specific for a particular type of toxin,

should reflect a subclinical and reversible change, assist in the implementation of suitable intervention or preventive strategies and be ethically acceptable.

Biomarkers are generally classified in terms of their primary role as markers of exposure, effect or susceptibility (Walker *et al.*, 1996; Grandjean *et al.*, 1994). Biomarkers of exposure are primarily indicative of the internal dose or the amount of toxin present in the whole body or a specific compartment of the body. Biomarkers of exposure may reflect short-term or chronic cumulative exposure. Biomarkers of effect are reflective of the biochemical changes within an organism as result of exposure to a toxin. Ideally the biomarkers of effect should permit early detection while the pathological changes remain reversible and are easily treated. Biomarkers of susceptibility indicate an individual's increased sensitivity to a toxin. These comprise various factors of inherent or acquired abilities, including genetic markers of chromosomal aberrations that may predispose an individual to an increased susceptibility to certain toxins (Gil and Pla, 2001).

Overall, biomarkers can be used to classify and quantify environmental exposures and their related effects, and their use serves an important role in toxicological experiments and public health (Grandjean, 1995).

1.6.1 Biological Monitoring of Lead

Although all biomarkers share common fundamental principles, biomarkers of specific toxins, like lead, have unique properties that govern their use in environmental surveillance and epidemiological research. This section provides a review of the various biomarkers for environmental lead exposure that have been reported in the literature. The advantages and limitations of each approach are discussed.

1.6.1.a *Blood and Plasma*

Blood-lead concentrations are the most commonly used biomarker and diagnostic index of lead exposure. The CDC has recommended that for children a blood-lead level of 10 µg/dL or above requires preventative intervention, while levels above 20 µg/dL require medical intervention (CDC, 1991).

Over 95% of the lead in blood is present in the erythrocytes where it is in dynamic equilibrium with the plasma lead (Baloh, 1974). Lead in the erythrocytes is primarily bound to the haemoglobin and, to a lesser extent, with other proteins (Lolin and O’Gorman, 1988; Ong and Lee, 1980; Bruenger *et al.*, 1971). The half-life of lead in blood is in the order of 28-36 days (WHO, 1995). Comparatively, the half-life of lead in plasma is considerably shorter (<1 hr) (Sakai, 2000; DeSilva, 1991). Lead levels in plasma are believed to be in equilibrium with the extracellular compartments and are, therefore, regarded as important indicators of lead in the soft tissues. Due to the low levels of lead in plasma, the use of plasma-lead as a biomarker for environmental exposure has been hampered by limitations in available analytical techniques. However, recent developments in the use of inductively coupled plasma mass spectrometry have enabled accurate measurement of lead in plasma (Apostoli, 2002).

The relationship between blood-lead and plasma-lead is complex. At levels below 40 µg/dL, a near linear relationship exists (Apostoli, 2002). However, at higher blood-lead levels the relationship between blood and plasma-lead concentrations is curvilinear (Apostoli, 2002; Schutz *et al.*, 1996). Apostoli (2002) has suggested that the correlation between blood-lead and plasma-lead is logarithmic due to the saturation of lead binding sites in the erythrocytes at high concentrations.

The majority of the epidemiological studies have relied on blood-lead levels as the primary biological marker of exposure. Associations have been observed between blood-lead

levels and health effects including neuropsychological deficits (McMichael *et al.*, 1986 and 1988), anaemia (Wright *et al.*, 2003a), renal function (Yu *et al.*, 2004) and hypertension (Glenn *et al.*, 2003). Additional details of these studies are provided in Section 1.5.2.

Several limitations and advantages have been identified in the use of blood-lead levels as a biomarker for long-term environmental lead exposure. Lead in whole blood has the advantage of being convenient to sample with a simple laboratory analysis. However, the half-life of lead in blood is short (28-36 days) and therefore a single blood-lead measurement can only provide limited information regarding long-term exposure. Blood-lead exhibits a nonlinear relationship with lead exposure and uptake from inhalation and gastrointestinal ingestion (Skerfving *et al.*, 1993). Furthermore, a nonlinear relationship was also observed between blood-lead and different metabolic and toxic effects including altered heme and nucleotide synthesis (Skerfving *et al.*, 1993). Finally, because of the reversible exchange of lead between blood, bone and soft tissues, a single blood-lead measurement cannot be used to differentiate between a low-level chronic exposure and a short high-level exposure (WHO, 1995). To overcome this limitation, serial blood-lead measurements have been collected in some studies and the data used to calculate the average blood-lead level over a lifetime, termed the 'lifetime average integrated blood-lead level' (WHO, 1995).

In addition to serving as a biomarker of lead exposure, several parameters in blood may be used as biomarkers of effect. As previously mentioned in Section 1.5.2.b lead is known to inhibit the enzyme ALAD that is crucial for heme synthesis (Kwong *et al.*, 2004). As a result of this, the blood and urine concentrations of ALA may be increased. Lead exposure may also disrupt other stages of heme synthesis resulting in an increase in blood levels of zinc protoporphyrin and urine concentrations of coproporphyrin. Determination of the latter components in blood and urine can be used to determine the extent of exposure (Sakai, 2000). The concentrations of these components in blood, except ALAD activity, were

found to be altered only at blood-lead levels above 30 µg/dL. ALAD was, therefore, considered a better marker of effect. However, it is known that ALAD activity reaches a steady state at blood-lead levels above 40 µg/dL and does not increase at higher blood-lead levels. It, therefore, cannot be used in cases of high-level lead exposure (Sakai, 2000; Skerfving *et al.*, 1993).

1.6.1.b Urine

Lead is excreted in urine and thus urinary lead levels are a potential biomarker of lead exposure. After a period of environmental lead exposure, lead levels in urine rise rapidly to reach a steady state after some weeks (Tola *et al.*, 1973). The use of urine lead levels suffers from several drawbacks and urine-lead is generally considered less reliable than blood-lead in the assessment of lead exposure. Skerfving *et al.* (1993) have outlined the drawbacks of using urine-lead as a biomarker of environmental lead exposure. Urinary lead levels are significantly lower than blood-lead concentrations and are, therefore, more difficult to measure accurately. Furthermore, urine sampling carries a higher risk of contamination. With considerable variation in the urine sample matrix, the density and/or creatinine levels of the urine sample must be checked to account for any potential matrix interferences. In addition, urinary lead levels are considered to primarily represent recent exposure and thus are not considered a reliable indicator of long-term exposure (Skerfving *et al.*, 1993).

Although use of urinary lead levels for biological monitoring of environmental lead exposure has been criticised (Fukui *et al.*, 1999; Skerfving *et al.*, 1993), the chelatable lead excreted in urine is regarded as an important marker of the toxic effects of lead and is known to positively correlate with the symptoms of lead intoxication (Lee *et al.*, 2000; Sakai, 2000). Chelatable lead may be determined in urine 24 hr after administration of chelating agents such as calcium disodium ethylenediamine tetraacetic acid or dimercaptosuccinic acid (Skerfving *et al.*, 1993). Various other markers have been

measured in urine in response to lead exposure including ALA, α 1 microglobulin, coproporphyrin and N-acetyl- β -D-glucosaminidase (NAG) (Oktem *et al.*, 2004; Lee *et al.*, 2000; Fukui *et al.*, 1999; Chia *et al.*, 1995; Pergande *et al.*, 1994). NAG and α 1 microglobulin exhibit a positive correlation with blood-lead levels in epidemiological studies (Oktem *et al.*, 2004; Chia *et al.*, 1995; Pergande *et al.*, 1994). However, the reliability of these markers has not been fully established.

1.6.1.c Bone

In the human body, approximately 90% of the absorbed lead is deposited in the skeletal compartment (Tsuchiya, 1979). Determination of lead in bone is, therefore, of importance in estimating the body burden of lead. Ahlgren (1976) reported amongst the first studies on the use of X-ray fluorescence for *in vivo* determination of lead concentrations in bone. In this technique lead atoms in a bone are excited by an incident beam of γ -rays. The excitation of lead atoms results in electron transfers in the K-shell or L-shell such that energy is released in the form of characteristic X-rays which can be measured in a detector to obtain quantitative data on bone lead concentrations (Todd *et al.*, 1992). The source of the incident γ -rays is an important determinant of the sensitivity of the technique. Cobalt (^{57}Co) and cadmium (^{109}Cd) are the primary sources of the incident γ -rays for fluorescing the K-shell electrons, while iodine (^{125}I) is used for fluorescing L-shell electrons (Nilsson and Skerfving, 1993). The cobalt source, which is more commonly used, was reported to have a minimum detection limit for lead in the range of 3-15 $\mu\text{g/g}$ of bone (Ambrose *et al.*, 2000).

Trabecular and cortical bone are known to have different rates of turnover and thus measurements are made at sites where fluorescence readings from both types of bone could be collected separately. The cortical bone of the tibia and phalanx, and the trabecular bone of the patella and calcaneus are commonly used in studies (Ambrose *et al.*, 2000).

Several epidemiological studies have been conducted in occupationally exposed groups and non-exposed general populations with the aim of comparing bone-lead levels with other biomarkers and health outcomes. Hu *et al.* (1994) measured blood-lead, bone-lead, haematocrit and haemoglobin levels in 250 subjects involved in the construction industry. A significant negative correlation was reported between bone-lead levels and haematocrit and haemoglobin levels. Blood-lead levels, however, did not show any significant correlation with either of the latter variables. Other studies have also shown positive associations between bone-lead levels and various health outcomes including, hypertension (Cheng *et al.*, 2001; Hu *et al.*, 1996) and deficits in cognitive performance (Wright *et al.*, 2003b) (see Section 1.5.2 for details).

In a long-term cohort study, Bellinger *et al.* (1994) measured lead levels in dentine, blood and bone (tibia and patella) and compared these measurements with neuropsychological test scores in 79 subjects aged 19-20 years. Only dentine-lead levels showed an inverse relationship with the test scores. The authors concluded that one of the key shortcomings of the use of X-ray fluorescence was its limited precision. Another study using the same cohort of participants reported that dentine-lead levels were positively associated with the body mass index while bone-lead levels showed no significant correlation with body mass index (Kim *et al.*, 1995). Results from both these studies were influenced by the limited sensitivity of X-ray fluorescence for *in vivo* bone-lead determination. Other disadvantages of the X-ray fluorescence technique include an inability to provide retrospective chronological information of lead exposure, or isotopic data to identify the sources of exposure.

1.6.1.d Hair

It has been suggested that lead may be incorporated into growing hair from the circulating blood (Wibowo *et al.*, 1986). The use of hair-lead levels has, therefore, been suggested as a biomarker of lead exposure and body burden. Although hair samples can be collected

with relative ease, the use of hair-lead concentrations has several drawbacks. Esteban *et al.* (1999) conducted a study to determine whether hair-lead levels could be used to identify children with blood-lead levels greater than 10 µg/dL. Lead concentrations in hair and blood samples were measured for 189 children. Mean blood-lead levels were higher than mean hair-lead levels. In addition, hair-lead levels were found to have a low sensitivity (57%) and showed high false-negatives (18%). In view of this, the authors concluded that determination of hair-lead levels was not sufficiently sensitive for the screening of children. Finally, washing of hair, on the scalp or during laboratory analysis procedures, can cause a loss of lead (Sen and Das Chaudhuri, 2001).

Despite these limitations, estimation of hair-lead levels has been used in several epidemiological studies (Sanna *et al.*, 2003; Wilhelm *et al.*, 2002; Nowak and Chmielnicka, 2000; Nowak, 1996; Sukumar and Subramanian, 1992). Studies that have measured hair-lead levels in conjunction with other established biomarkers of lead exposure are discussed in this section.

Wilhelm *et al.* (2002) compared lead levels in hair, whole blood, and saliva in 245 healthy children. Blood-lead and hair-lead values showed a weak correlation ($r = 0.217$). Similarly, Ahmed *et al.* (1989) in another cross-sectional study of 200 school-aged boys, found a weak and statistically insignificant correlation between hair-lead and blood-lead values ($r = 0.13$). In contrast, a moderate positive correlation ($r = 0.50$) was observed between blood-lead and hair-lead concentrations in a cross-sectional study of 222 children (Sanna *et al.*, 2003). Laker (1982) offered a possible explanation for such weak correlations suggesting that hair-lead concentrations were a measure of cumulative exposure while blood-lead levels, are only indicative of short-term exposure. This view, however, was not supported by the results from a rodent model developed by Hac and Krechniac (1996). In their model, rats were administered lead via drinking water. Hair-lead and bone-lead showed a strong positive correlation ($r = 0.87$) during the period of exposure. However, nine weeks

after the cessation of exposure, the hair-lead levels fell to pre-exposure levels while bone-lead levels remained high. The authors concluded that it was unlikely that hair-lead levels are an accurate index of cumulative long-term exposure.

Finally, in a study comparing lead levels in autopsy samples of kidney, liver, brain and lung tissue in 32 deceased lead-smelter workers and 10 non-exposed controls, Gerhardtsson *et al.* (1995) found a significant difference in lead concentrations of the various organs and blood samples between the two groups. No significant difference, however, could be found between hair and nail samples further highlighting the limitations in predicting body burden of lead using lead concentrations in hair and nails.

Results from such studies have indicated that, firstly, exogenous contributions to the total lead-content of hair may limit the use of hair as an index of body burden and, secondly, that lead levels in hair may only be indicative of recent absorption and not total cumulative lead body burden. Recent studies using sophisticated technologies, including synchrotron-related techniques, however, have attempted to map the elemental composition of hair at sub-micron resolutions, in an attempt to separate endogenous and exogenous contributions of lead (Martin *et al.*, 2005; Bertrand *et al.*, 2003). Future developments of such methodologies may increase the utility of hair-lead levels in biological monitoring.

1.6.1.e Saliva

A number of studies have compared levels of lead in human saliva with lead concentrations in blood and other tissues (Wihlem *et al.*, 2002; Gonzalez *et al.*, 1997; Brodeur *et al.*, 1983; Cleymaet *et al.*, 1991a; Brudevold *et al.*, 1977). Cleymaet *et al.* (1991a) studied lead levels in saliva, blood and surface enamel in 23 children. The mean lead level in the saliva of those children was found to be approximately 10% of the mean blood-lead levels. A weak correlation was observed between saliva-lead and blood-lead ($r = 0.14$). In a study of 266 adult males, however, P'an (1981) found a strong positive

correlation between saliva-lead and blood-lead ($r = 0.72$). Also, lead levels in saliva rose exponentially and exceeded blood-lead concentrations when lead levels in blood rose above 500 parts-per-billion (ppb). No physiological mechanism explaining the rapid increase of salivary lead levels was forthcoming. Wilhelm *et al.* (2002) measured lead levels in the saliva, blood and hair of 245 children using atomic absorption spectrometry. Lead levels in 89% of the saliva samples were below the detection limit of $1.5 \mu\text{g/L}$. Lead levels in blood could, however, be measured reliably and the authors concluded that because lead levels in saliva were too low to be measured accurately, it was not a suitable biomarker for low-level lead exposure.

The half-life of saliva-lead is known to be shorter than that of blood-lead (Brodeur *et al.*, 1983). These authors observed a rapid decline in salivary lead levels in five occupationally exposed workers, while the blood-lead levels showed a more gradual decline. They reported that the half-life of lead in human saliva was in the range of 5-7 days. Similarly, Cleymaet *et al.* (1991a) observed that salivary lead levels correlated with blood-lead levels for short time periods only. They suggested that saliva-lead was most likely derived from the diffusible compartment of lead in plasma, which itself has a very short half-life (<1 hr) (Sakai, 2000; DeSilva, 1981).

In a rodent model, Mobarak and P'an (1984) found strong positive correlations between saliva-lead and lead concentrations in whole blood and plasma ($r = 0.78$ and 0.80 respectively). These experiments, however, were conducted over a relatively short time period of 21 days, and utilised high doses of lead ($100 \mu\text{g/g}$). Such a scenario would only be comparable to high-level acute lead exposure in humans and would not be reflective of chronic low-level exposure, which is currently considered as the primary pattern of lead exposure in developed countries.

Overall, although saliva offers a convenient non-invasive means of sample collection, detectable salivary lead levels appear to be indicative of short-term exposure and can be reliably measured only when exposure is of a high magnitude. Furthermore, recent studies have also shown that trace element concentrations in saliva may be confounded by the presence of metallic dental restorations and intra-oral orthodontic appliances (Gursoy *et al.*, 2005; Garhammer *et al.*, 2004). Consequently, saliva appears to be of limited use for the biological monitoring of chronic low-level lead exposure.

1.6.1.f Nails

Fingernails and toenails offer an easily accessible and non-invasive means of sample collection for trace element analysis. A number of studies have reported the use of nails for the purposes of estimating environmental lead exposure and body burden (Mehra and Juneja, 2005; Mortada *et al.*, 2002; Nowak and Chmielnicka, 2000; Gulson, 1996a; Gerhardsson *et al.*, 1995; Garland *et al.*, 1993; Hayashi *et al.*, 1993; Gammelgaard *et al.*, 1991). Although there is a large volume of cross-sectional data, very few longitudinal studies have been conducted to examine the uptake of lead in nails, and the relationship of nail-lead concentrations with other indices of body lead burden. Two key epidemiological studies are discussed here. Gulson (1996a) monitored lead concentrations and lead isotopic levels in the urine and blood samples of a 48-year old subject on a weekly and monthly basis respectively over an 18-month period. Fingernail and toenail samples were also collected at regular intervals and analysed for lead content. Blood-lead and urine-lead concentrations of this subject remained fairly constant (300-400 ppb). The fingernail- and toenail-lead concentrations, however, showed large and erratic variations ranging from 100 to 700 ppb, with no apparent discernable pattern. From that study, it appears that nail-lead levels are not linearly related to lead levels in blood and urine and do not accurately reflect the extent of exposure experienced by the individual.

In another study, as described earlier (Section 1.6.1.d), Gerhardsson *et al.* (1995) compared lead levels in autopsy tissue from 32 occupationally exposed male workers and 10 non-exposed adult males. Lead levels in the kidney, liver, brain and lung samples showed statistically significant differences between the two groups. However, nail and hair samples did not show any significant difference, further highlighting that hair-lead and nail-lead concentrations are of limited value as an index of lead body burden. Overall, from the literature, it appears that until the kinetics of lead uptake in nails are further explored, the use of nail-lead as a biomarker of lead exposure offers limited advantage.

1.6.1.g Sweat

Sweat is considered an important mode for eliminating toxins from the body (Stauber and Florence, 1988). Lilley *et al.* (1988) compared lead levels in the sweat and blood of nine occupationally exposed workers and found significantly higher levels of lead in the sweat than in blood. The authors subsequently placed lead nitrate on the left arm of a human volunteer daily over four days. Sweat was collected at daily intervals from the left (exposed) arm at the same location where lead was applied, and from the right (unexposed) arm. Sweating was induced in this area using pilocarpine iontophoresis and the sweat was collected on a membrane filter. Lead levels in the sweat of the exposed arm increased from a pre-exposure level of 43 to 339 $\mu\text{g/L}$ one day after exposure and to a maximum value of 4765 $\mu\text{g/L}$ on the fifth day after exposure. In the unexposed arm a smaller increase from a pre-exposure level of 34 to 94 $\mu\text{g/L}$ was observed one day after exposure and the maximum value of 108 $\mu\text{g/L}$ was reached five days after exposure. Interestingly, no increase in the blood-lead or urine-lead concentrations was observed. Lead levels in saliva however, showed a three-fold increase. The authors suggested that lead absorbed through the skin was transported through plasma but was then removed rapidly to the extracellular fluid compartment thus affecting both saliva and sweat with minimal amounts of lead entering the erythrocytes. Support for this theory comes from

Chamberlain (1985), who had earlier reported that lead could be transported from plasma to the extracellular fluid in approximately one minute.

Likewise, Stauber *et al.* (1994) applied lead nitrate on the forearms of one human volunteer for 24 hr. Lead was absorbed through the skin and could be detected in sweat within one hr. No appreciable increase in the total concentration of lead in blood or urine was observed. In addition, application of a stable lead isotope (^{204}Pb) to the forearm of the same volunteer confirmed the earlier results; a small increase (approximately 0.3 $\mu\text{g/L}$) in the isotope levels could also be detected in the blood of the volunteer, although total lead concentrations did not increase in blood. Based on their results, the authors proposed a model similar to that postulated by Lilley *et al.* (1988) where lead absorbed through the skin is rapidly transported from plasma to the extracellular fluid compartments including sweat and saliva.

It appears that absorption of lead through skin is an important route of exposure. In addition, sweat-lead levels respond rapidly to this mode of exposure and are a potentially valuable indicator in cases of acute percutaneous exposure where blood-lead levels may not be reliable.

1.6.1.h Teeth

It is known that the half-life of lead in calcified tissues including teeth is of the order of 10-20 years (Skerfving *et al.*, 1993; Tsuchiya, 1979). Teeth have, therefore, been used as a biomarker of long-term lead exposure and cumulative body burden of lead in a number of studies. This discussion provides an overview of the different methodologies applied in using tooth-lead levels for the purposes of biological monitoring. Details of major studies that have explored the association of lead levels in teeth and health outcomes, as well as the limitations and advantages of using dental lead levels are discussed.

1.6.1.h.i *Lead levels in Whole Teeth and Environmental Exposure to Lead*

Monitoring of the lead exposure experienced by populations living in different geographical regions, particularly in terms of the proximity to industrial sources of lead emission, has received considerable attention. A number of studies have used lead levels in extracted permanent or naturally exfoliated primary teeth to compare the differences in lead exposure experienced by individuals living in rural, suburban, urban and industrial environments (Table 1.1). Results from some of these studies indicate that individuals residing in close vicinity to lead-related industries are likely to have significantly higher tooth-lead levels than those living in suburban or rural areas (Steenhout and Pourtois, 1981; Needleman *et al.*, 1972). However, there are other studies that have reported only minor differences amongst such populations (Bu-Olayan and Thomas, 1999; Brockhaus *et al.*, 1988; Khandekar *et al.*, 1986). Small differences in tooth-lead levels have also been reported

when comparing urban, suburban and rural areas (Steenhout and Pourtois, 1981; Kaneko *et al.* 1974; Needleman *et al.*, 1972).

Comparison of tooth-lead levels between such studies are hampered by a number of issues including the different criteria of classifying populations as urban, suburban, rural or industrial, and the different analytical techniques employed in various cases. Furthermore, the lead levels in whole teeth may be affected by the type of tooth, the arch (maxillary or mandibular) from which the tooth was derived and, in the case of primary teeth, the extent of physiological root resorption (Fergusson and Purchase, 1987).

Mackie *et al.* (1977) measured lead concentrations in human deciduous teeth and found that mean lead levels in first and second incisors (21.3 and 19.2 µg/g, respectively) were higher than those observed in the canines, first molars and second molar teeth (12.7, 11.3, 9.2 µg/g, respectively). Similarly, differences in lead concentrations were reported between maxillary and mandibular teeth. Smith *et al.* (1983) found that, within the same

mouth, human mandibular primary central and lateral incisor teeth had lower lead concentrations than their maxillary counterparts by a mean difference of 1.31 $\mu\text{g/g}$ and 1.74 $\mu\text{g/g}$ respectively. It was suggested that the age of the tooth may partly account for these observed variations in lead concentrations amongst different types of teeth, with lead levels being higher in teeth that were among the first to erupt into the mouth (Fergusson and Purchase, 1987).

The effect of physiological root resorption on the lead content in crowns of primary human teeth has not been studied in detail. Rabinowitz *et al.* (1993) compared serial blood-lead levels of 82 children with the lead concentrations in sections of coronal dentine from naturally exfoliated deciduous teeth. A weak, but statistically significant, correlation was observed between blood-lead levels taken at 57 months of age ($r = 0.18$) and dentine-lead levels. Blood-lead measurements at earlier time points did not show any statistically significant correlations with the dentine-lead concentrations. The author suggested that physiological root resorption may have 'reset' the coronal dentine-lead levels to the then current blood-lead levels, and that this effect may operate at sites 2-3 mm away from the resorption front. However, since dentine is deposited and mineralised incrementally, a more probable explanation for this data may be that lead is incorporated in dentine at the time of mineralisation, and lead concentrations in specific anatomical regions of dentine may only be related to the lead levels in blood at the time of dentine development.

Table 1.1. Key studies reporting lead levels in whole primary and permanent human teeth collected from individuals residing in industrial, urban, suburban and rural areas.

Abbreviations: AAS = atomic absorption spectrometry. DPSAV = differential pulse stripping anodic voltammetry. DPP = differential pulse polarography.

Reference [Tooth Type]	Region	Analytical Method	Sample size	Mean Lead (µg/g)
Bu-Olayan and Thomas, (1999) [Primary teeth]	• Industrial • Suburban	AAS	• 108 • 108	• 2.38-2.50 • 2.21-2.30
Brockhaus <i>et al.</i> (1988) [Primary teeth]	• Suburban/rural • Urban/industrialised • High-risk area (lead smelter)	AAS	• 485 • 2401 • 118	• 1.8-2.3 • 2.3-2.8 • 3.5-4.5
Khandekar <i>et al.</i> (1986) [Primary teeth]	• Suburban • Urban/industrial	DPSAV	• 106 • 21	• 2.27-2.77 • 4.52
Van Wyk and Grobler, (1983) [Primary teeth]	• Urban (light industry) • Urban (lead-smelter)	AAS	• 27 • 21	• 16.55 • 20.41
Ewers <i>et al.</i> (1982) [Primary teeth]	• Rural • High-risk area	AAS	• 86 • 302	• 3.9 • 6.0
Steenhout and Pourtois, (1981) [Permanent teeth]	• Rural • Urban • Industrial	AAS	• 38 • 42 • 51	• 21.45 • 17.72 • 35.35
Ewers <i>et al.</i> (1979) [Primary teeth]	• Rural • Urban	AAS	• 40 • 690	• 2.74 • 4.53
Moore <i>et al.</i> (1978) [Primary teeth]	• New houses • Old houses with lead plumbing	DPP	• 39 • 19	• 8.4 • 20.3
Kaneko <i>et al.</i> (1974) [Permanent teeth]	• Urban • Rural • Fishing • Industrial (Non-lead)	AAS	• 24 • 20 • 17 • 35	• 11.61 • 4.65 • 8.64 • 6.27
Needleman <i>et al.</i> (1972) [Primary teeth]	• Suburban • Urban • High-lead area	AAS	• 40 • 30 • 69	• 11.1 • 11.2 • 51.1

1.6.1.h.ii *Lead Levels in Enamel and Dentine of Human Teeth, and Environmental Exposure to Lead*

Many researchers have measured lead levels in enamel and dentine separately to estimate environmental lead exposure. Methods of sampling enamel included acid-etch enamel biopsies (Gomes *et al.*, 2004; Cleymaet *et al.*, 1991a,b,c), enamel fragments (Frank *et al.*, 1990) and gravitational separation of enamel (Anttila and Anttila, 1987; Haavikko *et al.*, 1984). Similar methods, except acid-etch biopsies, have been used for dentine (Grobler *et al.*, 1985; Johansson *et al.*, 1976). Table 1.2 lists important studies that have measured lead levels in enamel and dentine in an attempt to compare exposure amongst individuals from rural, suburban, urban and industrial regions around the world..

These studies indicate that, in some instances, there are marked differences in the dentine- and enamel-lead levels between participants from industrial and non-industrial areas (Gomes *et al.*, 2004; Cleymaet *et al.*, 1991). Smaller differences, however, were observed between rural, suburban and urban areas (Anttila and Anttila, 1987; Johansson *et al.*, 1976).

Comparisons between such studies are hampered by the lack of uniformity with regard to the classification of living environments and also the inherent differences in the levels of lead in different parts of enamel and dentine. Grobler *et al.* (2000) reported that median lead levels in 48 naturally exfoliated human primary teeth, collected from children who had lived permanently in one rural area, varied markedly between enamel, dentine and circumpulpal dentine (defined by the authors as approximately 300 µm of dentine immediately adjacent to the pulp). The ratio of lead concentrations between these three defined zones was 1 : 8.16 : 26.84 respectively. Contrasting results were reported by other researchers. Purchase and Fergusson (1986) measured mean lead levels in the corresponding parts of a series of permanent teeth and found a ratio of 1 : 2 : 6 for the lead concentrations in enamel, dentine and circumpulpal dentine. In an earlier study,

Grobler *et al.* (1985) reported that the relative proportion of lead between these dental zones varies amongst rural, suburban and urban areas. Because of this, comparisons cannot be made between studies measuring lead in different parts of enamel and dentine. Furthermore, lead levels in one part of enamel or dentine cannot be used to accurately predict concentrations of lead in other parts of teeth.

In addition to these studies, other researchers have reported high concentrations of lead ($> 100 \mu\text{g/g}$) in the surface enamel using acid-etch biopsies (Gomes *et al.*, 2004; Cleymaet *et al.*, 1991a). Acid-etch biopsies of the enamel surface may be affected by exchange of lead from the saliva. It is known that the demineralisation-remineralisation cycles that occur continually on the enamel surface can transport calcium, phosphorus and fluoride ions reversibly between the enamel surface and saliva (McIntyre, 1998). It is, therefore, possible that lead deposits on the surface of enamel may be affected by similar mechanisms. Indeed, Cleymaet *et al.* (1991a) found a significant negative correlation between etch depth and saliva-lead levels. It is conceivable that if lead ions in saliva are exchanged with the enamel surface, that lead levels in the superficial layers of enamel removed in the earlier etch-biopsies would correlate better with saliva-lead than lead levels in the deeper layers of enamel sampled by subsequent etch-biopsies.

In summary, while the observed lead concentrations in teeth may reflect the extent of lead exposure experienced by an individual, other factors such as tooth-type, location of the tooth in the mouth, physiological root resorption, and region of tooth-sample analysed, may have considerable influence on the measurable lead concentration.

Table 1.2. Key studies reporting lead levels in enamel and dentine of primary and permanent human teeth collected from individuals residing in industrial, urban, suburban and rural areas.

Abbreviations: AEB = acid etch biopsy. AAS = atomic absorption spectrometry. DPSAV = differential pulse stripping anodic voltammetry. PIXE = proton induced X-ray emission. EDXRF = energy dispersive X-ray fluorescence. ^aDetailed method given in Manly and Hodge, (1939). ^bMedian values. ^cInner enamel adjacent to the dentino-enamel junction. ^dOuter enamel close to surface of enamel.

Reference [Tooth Type]	Region	Enamel/Dentine [Analytical Method]	Sample Size	Mean Lead (µg/g)
Gomes <i>et al.</i> (2004) [Primary teeth]	• Industrial • Non-industrial	Surface enamel by AEB [AAS]	• 132 • 197	• 275.5 • 183.1
Cleymaet <i>et al.</i> (1991a) [Permanent teeth]	• Belgium (Industrial) • Kenya (Rural)	Surface enamel by AEB [AAS]	• 252 • 60	• 1236 • 145
Frank <i>et al.</i> (1990) [Permanent teeth]	• Urban (Mexico) • Urban (Strasbourg) • Rural (Alsace)	Enamel punch biopsy [EDXRF]	• 7 • 7 • 7	• 21.8 ^c , 17.4 ^d • 8.8 ^c , 6.9 ^d • 8.7 ^c , 7.8 ^d
Anttila and Anttila, (1987) [Primary teeth]	• Urban • Rural	Whole enamel by centrifugal separation ^a [PIXE]	• 19 • 9	• 6.7 • 10.2
Grobler <i>et al.</i> (1985) [Primary teeth]	• Urban • Suburban • Rural	300 µm of sectioned dentine adjacent the pulp [AAS]	• 12 • 11 • 12	• 74.5 • 44.3 • 26.5
Haavikko <i>et al.</i> (1984) [Primary teeth]	• Urban • Rural	Whole enamel by centrifugal separation ^a [PIXE]	• 49 • 47	• 4.2-9.1 ^b • 4.4-4.5
Johansson <i>et al.</i> (1976) [Permanent teeth]	• Urban • Urban • Suburban	Dentine in tooth section [PIXE]	• 26 • 38 • 23	• 4.5 • 3.4 • 3.2
Shapiro <i>et al.</i> (1973) [Primary teeth]	• Lead poisoned • Urban • Rural/suburban	300 µm of sectioned dentine adjacent the pulp [DPASV]	• 9 • 20 • 17	• 54.8 • 16.9 • 5.4

1.6.1.h.iii *Tooth-Lead Levels and Health Effects*

Tooth-lead levels have been used by researchers to study the association between chronic low-level lead exposure and health outcomes. Longitudinal cohort studies measuring tooth-lead levels have primarily focused on the effects of lead on neurobehavioural development. While data on blood-lead levels from some of these cohorts has been reported by the authors, this section focuses solely on the tooth-lead data. Where available, blood-lead data from these studies has been described in brief in Section 1.5.2.a.

Needleman *et al.* (1979) compared dentine-lead levels amongst first and second grade school children who had no clinical signs of lead poisoning. A battery of neurobehavioural tests administered to 270 children revealed significant differences between participants with high dentine-lead ($>24 \mu\text{g/g}$) and those with low dentine-lead levels ($<6 \mu\text{g/g}$). Children with higher dentine-lead levels also rated poorly on teacher-reported measures of classroom behaviour. Bellinger *et al.* (1986) re-evaluated this group of children after an interval of approximately 4 years and found lower IQ scores and a higher exam-failure rate in those with high dentine-lead when compared to those belonging to the low dentine-lead group. In an eleven year follow-up of this group, 132 of the original 270 participants were examined for neurobehavioural ability (Needleman *et al.*, 1990). Participants in the high dentine-lead group (revised to $>20 \mu\text{g/g}$) had a higher risk of dropping out of school, lower reading ability, vocabulary, poorer hand-eye coordination and lower class standing in high school. A subsequent follow-up of 79 of the original 270 participants at the ages of 19-20 years found that differences in neuropsychological performance persisted in those with high-dentine lead levels (Bellinger *et al.*, 1994).

Another cohort study conducted in the Port Pirie region of South Australia, compared serial blood-lead levels for approximately 600 children with neuropsychological performance, visual-motor integration and emotional development at different ages (Tong

et al., 1998; Baghurst *et al.*, 1995; Wigg *et al.*, 1988). As part of this study, lead levels were measured in naturally exfoliated deciduous maxillary central incisors (McMichael *et al.*, 1994). An inverse relationship was observed between IQ and tooth-lead levels.

Several cross-sectional studies have reported similar associations between tooth-lead/dentine-lead levels and neurobehavioural development (Rabinowitz *et al.*, 1991; Hansen *et al.*, 1989; Winneke *et al.*, 1983). While there is substantial evidence in support of the association between lead exposure and deficits in neurological development, some cross-sectional studies have found no association between tooth-lead levels and measures of IQ after controlling for parental and social confounders (Winneke *et al.*, 1994; Pocock, *et al.*, 1987). Overall, the use of dentine and whole tooth-lead levels serves as a useful biomarker for studying the effects of long-term sub-clinical, low-level lead exposure on neurobehavioural development.

Comparatively fewer studies have measured tooth-lead levels in relation to other health outcomes associated with long-term lead exposure. Studies have observed associations between tooth-lead levels and chronic renal failure (Scharer *et al.*, 1991), dental caries (Gierat-Kucharzewska *et al.*, 2003; Tsuji *et al.*, 2003; Gil *et al.*, 1996), and gastrointestinal ulceration (Bercovitz and Laufer, 1991). However, a causal association between lead exposure and health outcome could not be established in any of these studies possibly due to issues relating to sample size, cross-sectional study designs, and limited control of confounding variables.

1.6.1.h.iv Incorporation of Lead in Enamel and Dentine

Epidemiological studies have shown that lead is incorporated into enamel and dentine of primary and permanent teeth. In this section the microscopic and ultrastructural aspects of lead deposition into enamel and dentine are discussed. It is known that lead accumulates preferentially in calcified tissues, including teeth (Barry, 1975). Kato *et al.* (1977) showed

that intravenous injections of lead produced a 'lead-line' in bone and dentine of rats within a time period of 30 s. There was also an accompanying increase in serum calcium and phosphorus levels with maximum values observed at 1 hr and 30 min respectively. The authors suggested that the replacement of calcium with lead in the hydroxyapatite could have changed the configuration of the crystal lattice resulting in phosphorus and calcium being released into the blood to restore stability of the hydroxyapatite. Appleton (1991) administered a single intravenous injection of 30 mg/kg body weight of lead acetate to adult rats and reported a hypomineralised 'lead-line' within the dentine. Using electron microscopy the author observed incomplete fusion of calcospherites in the lead-line, as well as irregular tubular structure and uneven mineralisation in the dentine deposited immediately after the lead-line. No lead could be detected within the lead-line using energy dispersive X-ray analysis. The absence of lead in the region of the lead-line was attributed to the poor sensitivity of this technique, which is generally above 0.2% (Watt, 1997). Based on the results of this study, Appleton (1991) suggested that lead ions may act directly on odontoblasts interfering with their ability to regulate calcium transport. Further information on lead uptake by odontoblasts is limited. Studies have, however, shown that lead may interfere with the function of osteoblasts during bone formation (Puzas *et al.*, 1991; Pounds *et al.*, 1991). According to Pounds *et al.* (1991) the effects of lead on the osteoblast physiology and function include interference with DNA and RNA polymerases, impairment of collagen matrix synthesis and inhibition of the synthesis of several proteins, for example, osteocalcin, osteopontin and osteonectin. It is, therefore, possible that odontoblasts may be similarly affected by lead during dentine matrix deposition.

Eisenmann and Yaeger (1969) studied the effect of subcutaneous lead injections on rat enamel and dentine. They observed that lead primarily affects dentine resulting in the formation of hypomineralised zones. The effect in enamel was less pronounced, and only a few enamel sections showed a single zone of hypomineralisation. Other studies,

however, have described more pronounced effects of lead on enamel. Gerlach *et al.* (2002) exposed adult rats to lead via drinking water at doses ranging from 34-170 mg/L and found that, compared to control animals, the immature enamel in lead-exposed rats showed higher amounts of protein matrix and lowered microhardness. These differences were, however, not present in fully developed enamel. The authors concluded that lead exposure delays enamel maturation. Gerlach *et al.* (2000b) also showed that lead exposure may interfere with amelogenesis due to alteration in normal proteolysis in a rodent model.

At an ultrastructural level, lead may displace calcium from hydroxyapatite (Kato *et al.*, 1977). Calcium hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) forms the primary inorganic constituent of enamel and dentine. Lead hydroxyapatite ($\text{Pb}_{10}(\text{PO}_4)_6(\text{OH})_2$) can adopt the same structure (Sugiyama *et al.*, 1999). It has been established that lead can replace the calcium ions in synthetic calcium hydroxyapatite crystals at high temperatures ($>700^\circ\text{C}$), resulting in the formation of the mixed metal hydroxyapatites of the form $\text{Ca}_{10-x}\text{Pb}_x(\text{PO}_4)_6(\text{OH})_2$ (Sugiyama *et al.*, 1999). These compounds can also be formed at lower temperatures by direct reaction of suitable calcium and lead salts. The substitution of calcium by lead is facilitated by the similarities in the size and charge of the two cations. At a 7-coordinate a calcium ion has an ionic radius of $1.09 \text{ \AA}$ compared to that of lead of $1.23 \text{ \AA}$ (Shannon, 1976). This incorporation of lead into the hydroxyapatite lattice can result in the distortion of the unit cell. The extent of the distortion was found to be greater at an intermediate lead content, while complete substitution of lead with calcium resulted in a structure more similar to calcium hydroxyapatite (Bruckner *et al.*, 1995). In the apatite structure, the lead (or calcium) atoms may occupy different sites and can bind to six oxygen atoms or to six oxygen atoms and one hydroxyl ion (Bruckner *et al.*, 1995). Weatherell and Robinson (1982) suggested that many lead ions may also be found on the surface of and in any defects within the hydroxyapatite crystals.

1.6.1.h.v *Alternative Approaches in Using Tooth-Lead Levels*

While lead levels in whole teeth, and fragmented enamel and dentine are commonly used in epidemiological studies, alternative approaches in the use of teeth for biological monitoring of lead exposure have recently been developed. The determination of stable lead isotopes in dental hard tissues has been used in the identification of the source of exposure (Gulson *et al.*, 2004; Gulson, 1996; Gulson and Wilson, 1994). It is known that the lead deposits from major ore bodies in different continents have specific relative proportions of stable lead isotopes* (namely ^{204}Pb , ^{206}Pb , ^{207}Pb , ^{208}Pb) that serve as a signature to the identity of the source of lead. In a similar manner, the common sources of lead exposure in the environment may be identified by comparing their unique isotopic signature with the relative concentrations of different isotopes in teeth or other biological markers.

Gulson and Gillings (1997) used stable lead isotope data of European immigrants to Australia to determine the rate of exchange of lead in deciduous and permanent teeth. Their results showed that there was a minimal exchange of lead with the environment (approximately $1 \pm 0.3\%$ per year) within circumpulpal dentine (defined as the dentine immediately adjacent to the pulp) while by comparison enamel and the remaining dentine did not show any significant exchange of lead with the environment.

Gulson and Wilson (1994) have also suggested using enamel fragments from primary teeth as a biomarker of prenatal lead exposure. They proposed that because enamel from primary human incisor teeth is formed prior to the crawling stage in human development, that deciduous enamel-lead levels reflect *in utero* exposure. However, it is known that enamel continues to mature after birth (Deutsch and Shapiro, 1987) and, therefore, the method proposed by Gulson (1996) may serve as a biomarker for early development, but not necessarily an exclusive marker of prenatal exposure.

* while ^{204}Pb is of primordial origin and ^{206}Pb , ^{207}Pb , ^{208}Pb are products of radiogenic decay from uranium and thorium, in this thesis they are collectively referred to as 'stable isotopes'

Recent studies have proposed the use of the spatial distribution of lead in teeth to provide chronological information regarding the intensity and pattern of lead exposure experienced by an individual. Evans *et al.* (1995) measured trace elements including lead in the 'growth rings' of walrus teeth using laser ablation inductively coupled plasma mass spectrometry to study the variations in environmental exposure experienced over time. Similar approaches were used in the mapping of growth rings of trees and mussel shells (Bellis *et al.*, 2002; Watmough *et al.*, 2002, Nystrom, 1995). Ericson (2001) measured the spatial distribution of lead in the enamel of three longitudinally sectioned primary human mandibular incisors using ion mass spectrometry and proposed that the observed distribution of lead was reflective of the exposure experienced during the periods of tooth development. No comparisons were made, however, with blood-lead measurements taken at birth or before the age of 1 yr, to confirm if the observed enamel-lead levels were indicative of exposure experienced during the prenatal and neonatal periods. Prozesky *et al.* (1995) measured the spatial distribution of zinc and strontium in elephant ivory using a nuclear microprobe and suggested that the respective variations in trace element content may be due to seasonal variations in rainfall and vegetation. The approach used in these latter studies presents a useful alternative to the use of whole teeth and fragmented enamel and dentine for the purposes of biological monitoring, wherein, not only can the cumulative lead body burden be estimated, but the pattern of lead exposure during the prenatal and neonatal periods may be established as well.

1.7 Questions Arising from the Literature

This section discusses the key research questions arising from the reviewed literature and outlines the main aims of the present study. As the experiments in this thesis describe the analysis of lead concentrations in dental hard and soft tissues, relevant information on tooth development is also provided.

Despite the considerable progress that has been made towards the use of tooth-lead levels as a biomarker for chronic lead exposure, several key areas remain unexplored. It is known that susceptibility to lead is increased during early periods of human development (Lidsky and Schneider, 2003). However, current approaches to measuring lead concentrations in whole teeth and fragmented enamel and dentine do not permit the specific estimation of prenatal exposure. Fergusson and Purchase (1986), in their review, stated that while teeth provide a historical record of lead exposure it is not possible to determine the time period of significant exposure. This limitation arises primarily because current methodologies in the use of teeth for biological monitoring have not taken advantage of the incremental formation of enamel and dentine. It is known that the formation of primary human teeth commences prenatally and proceeds in a well-defined incremental manner (Ten Cate, 1998). It is, therefore, possible that the spatial distribution of lead in enamel and dentine of primary teeth may be used to determine the pattern and intensity of exposure experienced during the prenatal and neonatal periods. Although a similar argument was made by Ericson (2001), no animal models with controlled doses of lead exposure have been documented in the literature. Furthermore, in the absence of data validating the spatial distribution of lead in teeth against an established biomarker of lead exposure, it is not possible to ascertain whether lead concentrations at specific anatomical points in enamel and dentine reflect the intensity of lead exposure experienced during the period of tooth development.

Research on the biological mechanisms of lead uptake in teeth has provided information on the deposition of lead in the enamel and dentine of developing and fully mineralised teeth. However, the role of ameloblasts and odontoblasts in transporting lead into the mineralising enamel and dentine matrix is not fully understood at present. From the literature, it is evident that calcium transport into the enamel and dentine of developing teeth is largely regulated by ameloblasts and odontoblasts respectively (Hubbard, 2000; Smith, 1998; Lundquist, 2002). In view of the fact that lead has been shown to substitute

for calcium in many biological processes (Pounds, 1984), it is plausible that lead transport in the developing tooth germ may be influenced by ameloblasts and odontoblasts in a manner similar to that observed in the uptake of calcium during odontogenesis. Research into the aforementioned issues will allow further improvement in the use of tooth-lead levels as a biomarker of chronic lead exposure.

1.8 Tooth Development (Odontogenesis)

As the experiments in this thesis describe the uptake and distribution of lead in dental hard and soft tissues, a brief discussion is provided here on the development of teeth with particular emphasis on the formation of enamel and dentine. This information will serve as the basis for the development of the study aims described in Section 1.9.

1.8.1 Cellular Changes during Tooth Development

The development of primary teeth in humans commences prenatally at approximately the sixth week of development (Berkovitz *et al.*, 1992). The development of the tooth germ is categorised into the bud, cap, bell and advanced bell stages based on the histological appearance of the epithelial component of the tooth germ (*ie.* the enamel organ) (Berkovitz *et al.*, 1992). In the bud stage, the enamel organ is a spherical mass of cells that are poorly differentiated. The tooth germ continues to enlarge and enters the cap stage in the 11th week of development which is characterised by the appearance of an invagination in the enamel organ giving it a cap-like appearance. As this invagination deepens the enamel organ appears bell shaped and has epithelial cells arranged in the shape of the crown of the tooth. The inner layer of these cells, the inner enamel epithelium, gives rise to the columnar ameloblast cells that deposit the enamel matrix. Similarly, cells of the dental papilla adjacent to the inner enamel epithelium give rise to the odontoblast cells which are involved in dentine formation. The junction between the ameloblasts and odontoblasts is the site of the dentino-enamel junction once enamel and dentine deposition commences.

1.8.2 Enamel and Dentine Apposition during Tooth Development

At approximately the 18th week of development, the enamel organ enters the advanced bell stage characterised by the appearance of enamel and dentine at the DEJ on the cusp tip (Berkovitz *et al.*, 1992). The apposition of enamel and dentine may be visualised in two directions. Starting at the cusp tip, enamel and dentine deposition proceeds down the DEJ towards the cervical end of the tooth (Figure 1.1A). Enamel and dentine deposition in this direction is termed as extension (Shellis, 1984). Enamel extension in human primary teeth occurs at the rate of $23.8 \pm 5.6 \mu\text{m/day}$ (Shellis, 1984). Furthermore, it is known that the rate of incremental dentine formation is strongly correlated with that of enamel (Dean and Scandrett, 1996). Dentine extension, therefore, occurs at almost the same rate as enamel.

In addition to extension, enamel and dentine apposition may be visualised as deposition away from the DEJ as shown in Figure 1.1B. Because enamel and dentine deposition occurs in a rhythmic manner, incremental lines akin to growth rings can be seen in enamel and dentine. The rate of enamel and dentine deposition is believed to be at a rate of approximately $4 \mu\text{m}$ per day during the period of crown formation. Enamel apposition stops once the crown is complete, however, dentine continues to be deposited adjacent to the pulp at slower rate throughout the life of the tooth (Ten Cate, 2003).

1.8.3 Mineralisation of Enamel and Dentine

There are significant differences in the pattern of mineralisation between enamel and dentine. When the enamel matrix is first deposited, the inorganic constituents comprise only 15% of the total volume. By the time the entire thickness of enamel has been deposited in a part of the tooth germ, the enamel is approximately 30% mineralised (Ten Cate, 2003). During the subsequent phase of maturation, much of the protein content of

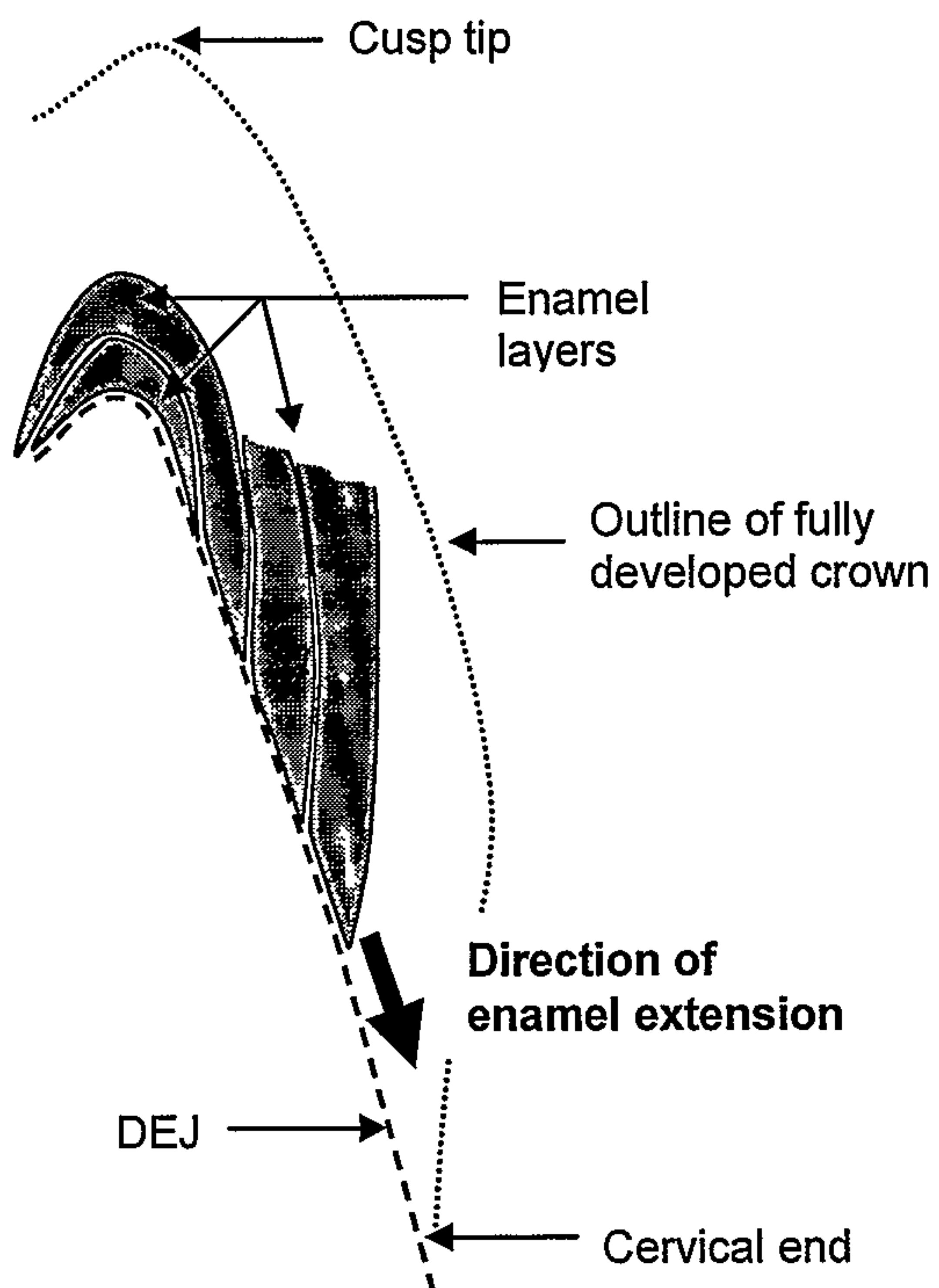
enamel is lost resulting in a final structure that is approximately 96% inorganic. The maturation of enamel follows a pattern that is different to the initial deposition of the enamel matrix. Maturation commences at the cusp tips and passes to the DEJ. It then spreads along the DEJ, before extending to the superficial enamel (Berkovitz *et al.*, 1992). The mineralisation of dentine occurs in globular (calcospheric) or linear patterns of calcification. Unlike enamel, dentine is mineralised to its final state of approximately 70% immediately after the deposition of the dentine matrix (Berkovitz *et al.*, 1992).

Current literature indicates that once teeth are mineralised, inorganic constituents of enamel and dentine may be exchanged with saliva and blood. The surface of enamel can release calcium, phosphorus and fluoride ions into the saliva at a pH below 4.5 (McIntyre, 1998). At a higher pH, these ions may be incorporated into the enamel surface from the saliva. Such a reversible exchange of mineral ions has been termed the demineralisation-remineralisation cycle (McIntyre, 1998). In a similar manner, it is possible that other ions, including lead, may be lost from the enamel surface. However, *in vivo* experiments dealing specifically with the stability of lead in the surface of enamel have not been reported.

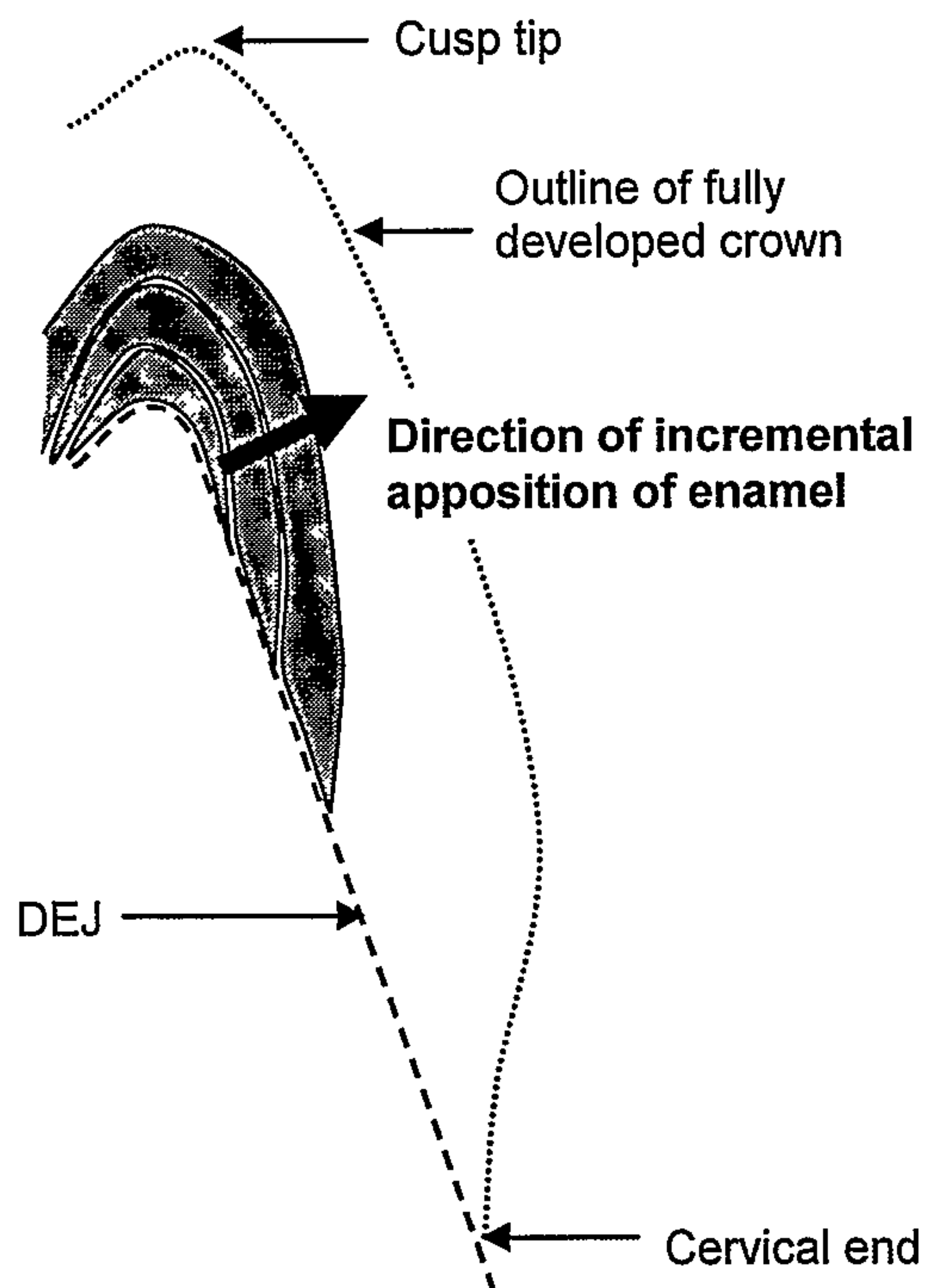
In dentine, using lead isotopic data of European migrants, Gulson and Gillings (1997) found that dentine immediately adjacent to the pulp exchanged lead at a rate of approximately $1 \pm 0.3\%$ per year. However, other parts of dentine did not show any significant exchange of lead.

Figure 1.1. Diagram showing enamel and dentine apposition in a human mandibular molar tooth. Enamel and dentine apposition begins at the cusp tip and proceeds towards the cervical end of the tooth. The apposition of enamel and dentine may be viewed in two planes as shown in A and B. In A, the extension of enamel along the dentino-enamel junction (DEJ) from the cusp tip towards the cervical end of the tooth is shown. In B, the incremental apposition of enamel away from the DEJ can be seen. Dentine apposition, although not shown in this figure, occurs in a similar manner.

A



B



1.8.4 Rationale behind the Use of Tooth-Lead Levels as a Biomarker of Lead Exposure

Based on the above discussion, it is clear that the extension and incremental apposition of enamel and dentine occurs at well-defined rates. It may, therefore, be possible that lead levels measured in teeth may reveal temporal information regarding the intensity of lead exposure experienced during the prenatal and postnatal periods of development. Towards this end, lead levels in enamel and dentine may be mapped in the incremental zones at approximately right angles to the DEJ. Alternatively, lead levels may also be measured adjacent to the DEJ in the direction of enamel and dentine extension.

1.9 Aims

The present study describes a series of experiments designed to test the applicability of tooth-lead levels in determining prenatal and neonatal lead exposure. The specific aims of the present study were:

1. To determine the spatial distribution of lead in teeth at a controlled level of exposure, using a rodent model.
2. To study the relationship of the spatial distribution of lead in enamel and dentine with the pattern and intensity of lead exposure, using a rodent model.
3. To compare the spatial distribution of lead in primary human teeth with blood-lead measurements.
4. To study the intracellular distribution of lead in the ameloblasts and odontoblasts during tooth development.

CHAPTER 2

The Determination of Lead in Biological Tissues: Principles of the Methods Used in this Thesis

2.1 Introduction

A number of different analytical techniques were used to measure the concentration of lead and other elements in the sample tissues analysed in this thesis. Because these methods are not universally well known, a discussion is provided on the relevant background information, including basic principles, advantages, limitations and procedural issues, with particular reference to the specific objectives of the study. Methods that proved unsuccessful in measuring lead concentrations in sample tissues are described only briefly.

The analytical techniques used included:

- Inductively coupled plasma mass spectrometry
- Inductively coupled plasma atomic emission spectrometry
- Laser ablation inductively coupled plasma mass spectrometry
- Nuclear microprobe/proton induced X-ray emission
- Synchrotron radiation induced X-ray emission
- Autometallography
- Energy dispersive X-ray analysis (Appendix A.1)
- Secondary ion mass spectrometry (Appendix A.2)

2.2 Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS), introduced by Houk *et al.* (1980), is a highly sensitive technique for multi-element and isotopic analysis of samples with detection limits for most trace elements ranging from parts-per-trillion (ppt) to ppb. In the present study, ICP-MS was used to measure the lead content in the teeth, blood and organs of rats in Chapter 4 and in the roots of human teeth in Chapter 6.

2.2.1 Principles and Components of ICP-MS

ICP-MS may be used to analyse samples that are in solid, liquid or gaseous states. Solid samples are vaporised using lasers or heated cells, while liquid samples are introduced as aerosols using a nebulizer. Gaseous samples may be introduced directly into the instrument. Samples are subsequently carried into a plasma comprising an inert gas, commonly argon, at a temperature varying from 5000 to 10,000 K. The high temperature of the plasma converts the sample atoms into ions which are focused using an ion lens, and introduced into a mass spectrometer. Ions are separated by mass spectrometry and are counted in a detector. Computerised data acquisition systems are used to convert the measured signal intensities into concentrations for each element (Montaser *et al.*, 1998; Aggarwal *et al.*, 1994; Jarvis *et al.*, 1992).

2.2.1.a Sample Introduction in ICP-MS

The ICP-MS requires that samples be introduced into the system as a gas, vapour or aerosol of fine droplets of liquid or solid particles. In the present study, the samples analysed were in a liquid state. In most systems, a pneumatic nebulizer is used to convert sample solutions into aerosols. High velocity gas streams are produced at an angle to the liquid-containing tube, resulting in a fine droplet dispersion of the analyte solution. The droplets then pass through a spray chamber that only permits droplets less than 8 µm in size to pass into the plasma (Jarvis *et al.*, 1992). Although many different types of

nebulizers are available, the cross-flow nebulizer and the concentric nebulizer, as described in Figure 2.1, are most commonly used in commercially available systems (Jarvis *et al.*, 1992). A concentric nebulizer was used in the present study.

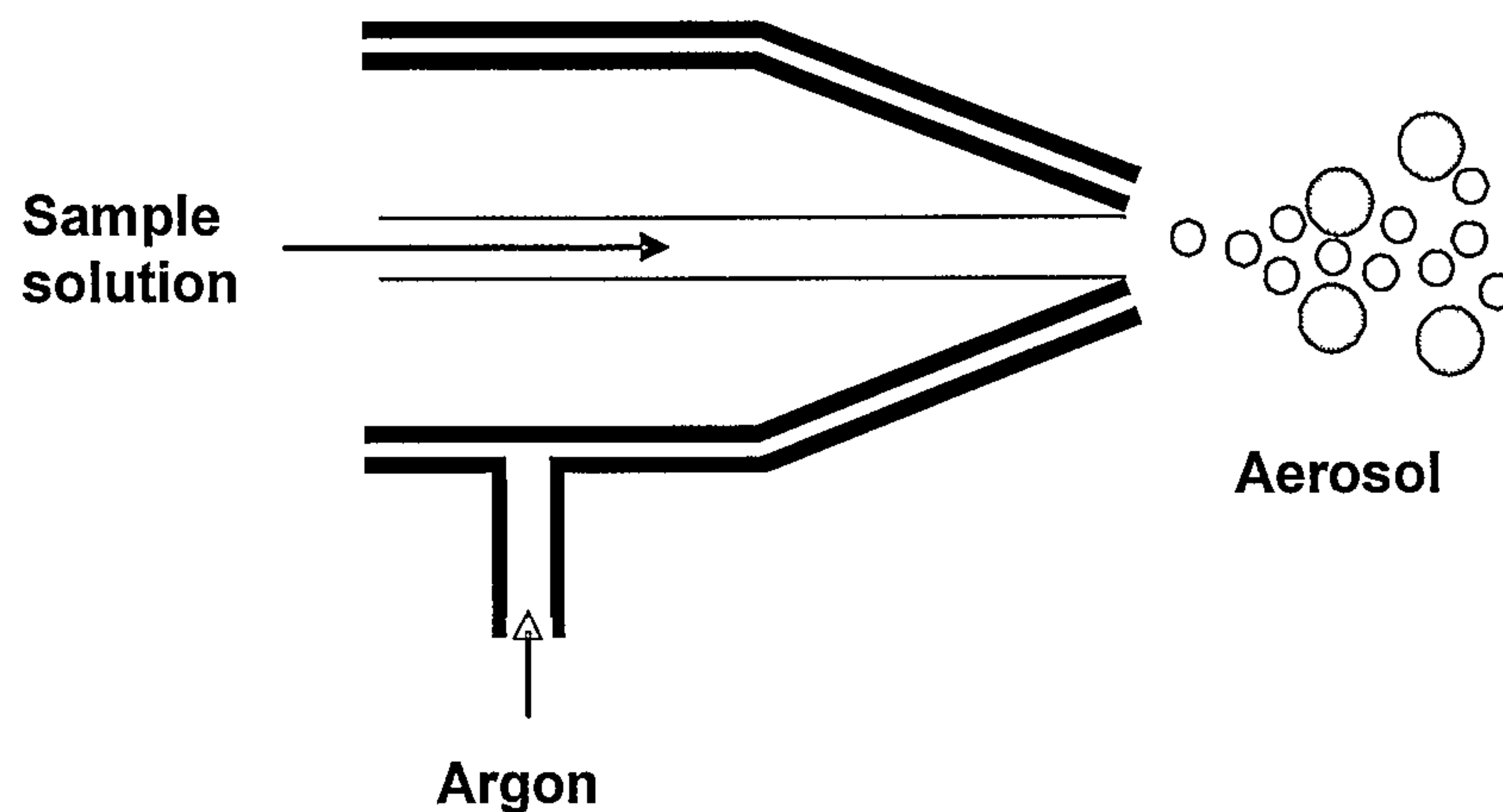


Figure 2.1. Schematic diagram of the cross-section of a concentric nebulizer in an inductively coupled plasma mass spectrometer (modified from Montaser *et al.*, 1998). Liquid samples are passed through a central tube encased within a hollow cylindrical tube containing argon. The liquid emerges from the tube, hitting the high velocity argon gas at an angle resulting in aerosol formation.

2.2.1.b Torch and Plasma Generation

In ICP-MS, a plasma is generated inside an assembly of quartz tubes known as the torch (Jarvis *et al.*, 1992). At one end, the torch is surrounded by a radiofrequency (RF) generator made of lead and copper coils. Energy passed via the RF generator heats the argon gas in the torch to a temperature in the range of 5000 to 10,000 K. As sample aerosols are passed through the plasma, the sample matrix and elements are initially dried to a solid and then heated to a gas. Finally, as more energy is absorbed, the sample atoms lose electrons and become ions. Figure 2.2 describes the different components of the torch.

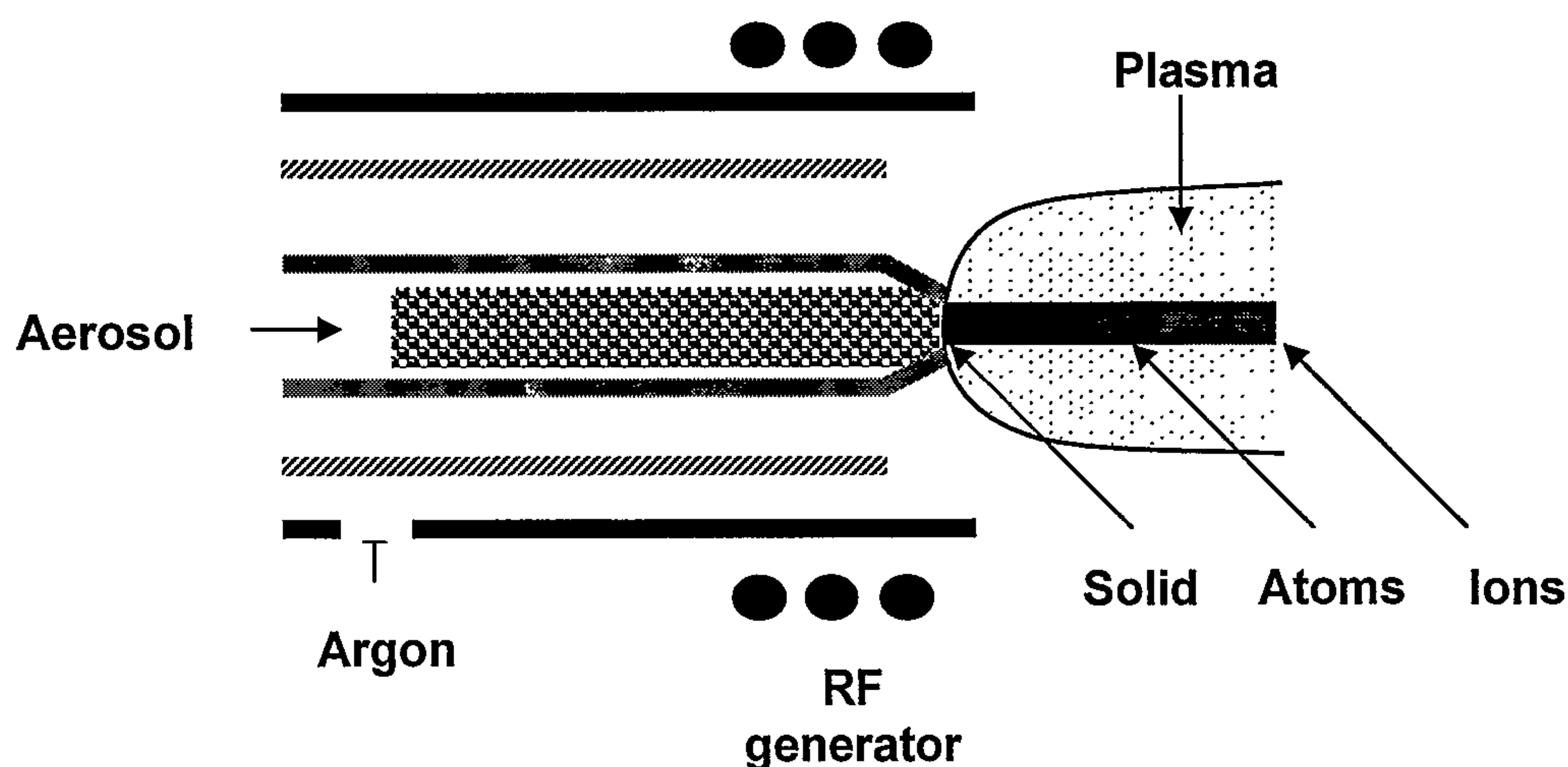


Figure 2.2. Schematic diagram of the cross-section of an inductively coupled plasma torch (modified from Jarvis *et al.*, 1992). The sample aerosol travels through a central tube encased within an argon containing concentric cylinder. The radiofrequency (RF) generator heats the argon gas to a temperature of 5000-10,000 K, forming a plasma where the sample aerosol is dried into a solid and then converted into atoms and finally ions.

2.2.1.c Ion Extraction Interface

The plasma in the ICP-MS system is at atmospheric pressure (760 Torr) while the detector is housed in a high vacuum at a pressure of approximately 10^{-6} Torr (Thomas, 2001). The transfer of ions across this large pressure differential is achieved by an ion extraction interface. In brief, ions generated in the plasma are extracted into a chamber maintained at a pressure of approximately 2 Torr through a sampling orifice approximately 1 mm in diameter. The inverted funnel-shaped sampling orifice, as shown in Figure 2.3, is called the sampler cone and is generally made of nickel or platinum (Thomas, 2001; Jarvis *et al.*, 1992). As ions from the plasma pass through the sampler cone into the vacuum chamber, a supersonic jet of ions is produced due to the pressure differential between the plasma and the vacuum chamber. Another cone of similar shape, called the skimmer cone is located a few millimeters from the sampler cone. The sample ions pass through the latter to reach the ion lens system (Aggarwal *et al.*, 1994).

2.2.1.d Ion Lens System

The ion lens system is placed immediately behind the skimmer cone and focuses the ion beam into the mass analyser (Figure 2.3). This lens system comprises a metallic cylinder that is maintained at a positive charge. Since ions generated in the plasma are mostly positively charged, they are repelled from the similarly charged wall of the cylinder and are focused into an ion beam. A shadow stop, located between the cylinder and the skimmer cone, prevents the un-ionised particles and photons from passing through the lens.

2.2.1.e Quadrupole Mass Analyser

Different types of mass analysers are used in ICP-MS including the quadrupole mass filter, multiple collector magnetic sector, time-of-flight and collision-reaction cell type (Turner *et al.*, 1998). The ICP-MS used in the present study contained a quadrupole mass filter, which separates ions based on their mass-to-charge ratio. This type of filter consists of four metallic rods approximately 20 cm in length and 1 cm in diameter (Figure 2.3)

(Jarvis *et al.*, 1992). A direct current is applied to one pair of the rods and a RF field to the opposite pair of rods. As the sample ions are channelled through the four rods, they are deviated from their trajectory under the influence of the voltage potential and radiofrequencies across the rods. The deviation experienced by an ion is a function of the charge and mass of the ion. The parameters can be adjusted so that only the ions of interest pass through to the detector, while ions with different mass-to-charge ratios are deviated to the side and are either ejected from the quadrupole or collide into the rods.

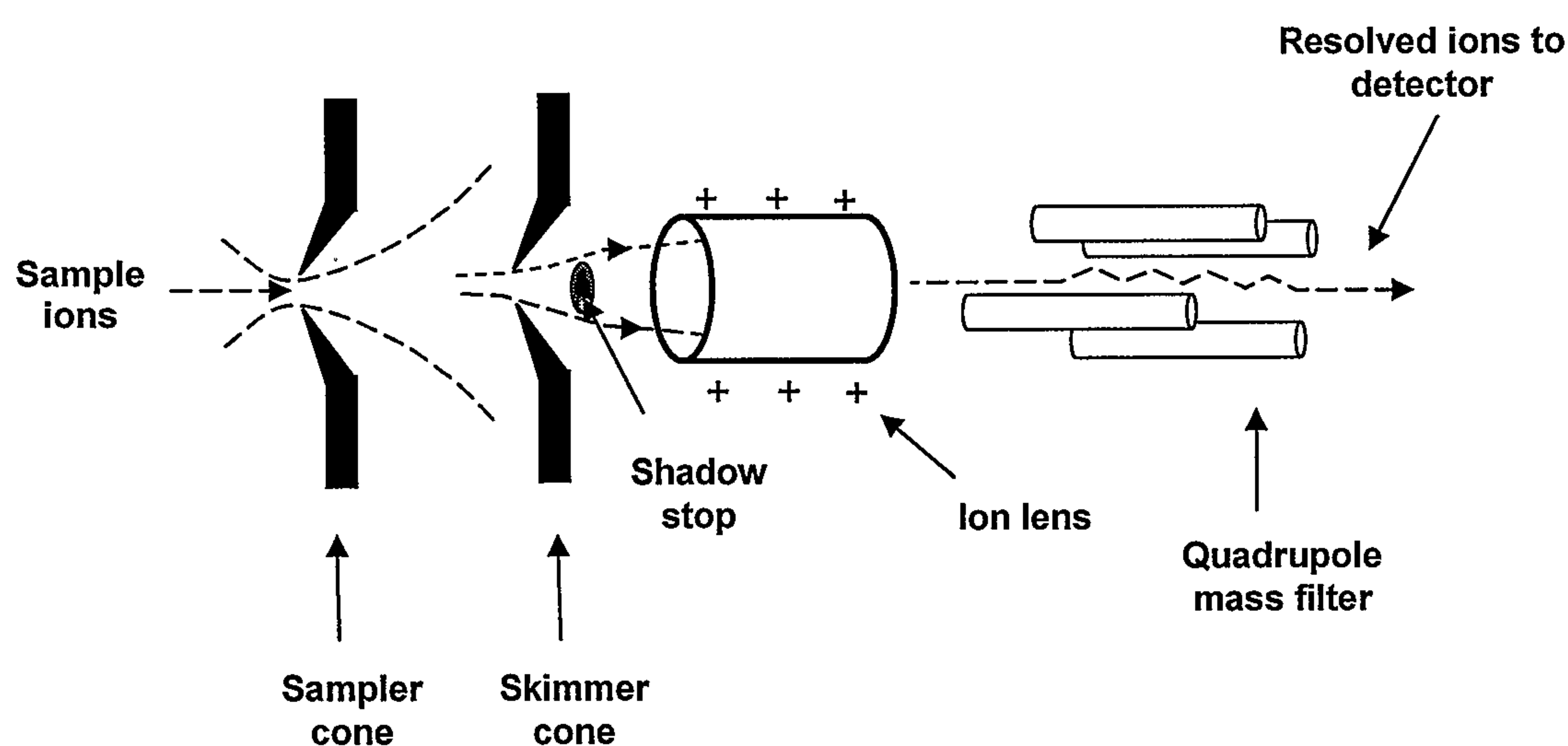


Figure 2.3. A schematic diagram of the sampler and skimmer cones comprising the ion extraction interface, the ion lens system and the quadrupole mass filter of an inductively coupled plasma mass spectrometer (modified from Jarvis *et al.*, 1992). Sample ions emerging from the plasma pass through the sampler and skimmer cones and are focused into a beam in the ion lens. The shadow stop prevents the passage of unionised particles and photons into the ion lens. The quadrupole mass filter separates the ions based on their mass-to-charge ratio.

2.2.1.f Ion Detectors and Data Acquisition

Channeltron electron multipliers are the most common type of ion detectors used in ICP-MS instruments (Jarvis *et al.*, 1992). This type of detector consists of an open glass tube which is coated with lead oxide. Ions exiting the quadrupole strike the active surface of the tube thereby releasing an electron at every collision and generating electric pulses. By counting the number of pulses generated, the concentration of the ions of interest can be calculated with appropriate software. In the present study, data acquisition was performed using the Winmass for Ultramass ICPMS software package, version 1.1b54 (Varian, Australia).

2.2.2 Advantages and Limitations of ICP-MS

The ICP-MS is a highly sensitive method for simultaneous or rapid sequential determination of multiple elements in biological samples. The sensitivity of the ICP-MS is better than that of comparable techniques including, graphite furnace or flame atomic absorption spectrometry. The reported sensitivity of ICP-MS for lead is in the ppb to ppm range (Jarvis *et al.*, 1992). ICP-MS can also be used for isotopic analysis. Sample introduction into the system is automated and relatively easy. Compared to other methods such as nuclear microprobes, ion mass spectrometers and synchrotrons, ICP-MS machines are more readily accessible and are relatively inexpensive to maintain and operate.

Several disadvantages of the ICP-MS have been reported in the literature (Aggarwal *et al.*, 1994; Evans and Giglio, 1993; Jarvis *et al.*, 1992). ICP-MS analysis is destructive since it generally requires that biological samples be liquefied by dissolution in acid. The most important limitation of ICP-MS is the potential for interferences. Interferences may be classified into two main categories, namely, spectroscopic and non-spectroscopic, sometimes referred to as matrix effects (Jarvis *et al.*, 1992). Spectroscopic interferences may be due to isobaric overlap, where isotopes of two different elements have the same

mass, and also due to the formation of short lived polyatomic ions originating from argon, hydrogen and oxygen that are present in the plasma. Major elements like nitrogen, sulphur and chlorine in the solvents and acids used during sample preparation may also contribute to the formation of polyatomic ions. Spectroscopic interferences due to polyatomic species are mainly significant in the analysis of elements with a mass-to-charge ratio of 82 and below.

Other sources of spectroscopic interferences include refractory oxides and doubly charged ions. Refractory oxides may result from incomplete dissociation of the sample matrix or from recombination of ions in the plasma tails. Doubly charged ions may be produced, particularly in the case of elements where the second ionisation energy is lower than the first ionisation energy of argon (16 eV). Lead has a second ionisation energy of 14-16 eV and, therefore, lead analysis may potentially be affected by this interference (Jarvis *et al.*, 1992).

Non-spectroscopic interferences can be broadly classified into physical effects from high quantities of dissolved solids and suppression or enhancement effects of the analyte (Jarvis *et al.*, 1992). A high volume of total dissolved solids may cause progressive blockage of the sampling aperture, resulting in a drift of the observed counts of the element of interest. Suppression or enhancement of an analyte may occur due to the presence of easily ionisable elements in the matrix. Beauchemin *et al.* (1987) reported enhancement of the signal of various analyte elements by elements such as calcium, sodium, magnesium and potassium in the matrix. These interferences are greatest for elements with an atomic mass number less than 80 (Evans and Giglio, 1993). Aggarwal *et al.* (1994) suggested internal standard dilution, standard addition, matrix matching and isotope dilution, as methods available for correcting for these interferences.

2.3 Inductively Coupled Plasma Atomic Emission Spectrometry

Inductively coupled plasma atomic emission spectrometry (ICP-AES) is a technique similar to ICP-MS. The ICP-AES relies on an optical spectrometer for the detection of elements, rather than mass spectrometry as is the case in ICP-MS. In the present study, the ICP-AES was used for the detection of calcium and phosphorus in tooth-root samples (Chapter 6). ICP-AES is the preferred technique for the detection of calcium and phosphorus (Brown *et al.*, 2004), both of which are present at high concentrations in teeth. ICP-MS may also be used for the detection of calcium and phosphorus, however, the sample solution would have to be diluted several times and this may potentially introduce dilution errors. The sample preparation, sample introduction and plasma generation in ICP-AES are similar to ICP-MS. The principles of optical spectrometry and the advantages and limitations of ICP-AES are discussed in the following sections.

2.3.1 Principles of Atomic Emission Spectrometry

When the sample atoms are energised by the plasma to an unstable state, excess energy is released in the form of radiation. The wavelengths and intensities of these emissions are characteristic of the nature and concentration of the elements present in the sample (Miller, 1992). The spectrometer resolves the radiation from the plasma into its component wavelengths by diffraction and the light intensity is measured by the detector at a wavelength specific for the elements of interest and converted into an electrical signal that can be quantified to reveal the concentration of the particular elements.

In the present study, the ICP-AES instrument used contained an Echelle polychromator, which produces a three-dimensional spectrum by directing the diffracted light from a fixed low resolution grating to a dispersing prism. The prism separates the spectrum into its component wavelengths. A charge transfer device detector converts incoming photons of

light into an electric current. A computerised data acquisition system is used to convert the measured signal intensities into concentrations of each element (Greenberg, 2000).

2.3.2 Advantages and Limitations of ICP-AES

ICP-AES shares many of the advantages of the ICP-MS. It provides better detection limits than graphite furnace or flame atomic absorption spectrometry. The reported detection limits for ICP-AES are higher than those of ICP-MS at 0.01 µg/mL for calcium and 0.042 µg/mL for lead (Greenberg, 2000). Sample preparation for ICP-AES is relatively simple and compared to nuclear beam methods of analysis, ICP-AES is comparatively less expensive and more easily accessible.

The most prominent source of error in ICP-AES analysis is spectral interference arising from the overlap of spectral lines of the element of interest with spectral lines of other atomic or molecular species (Zander, 1992). Other sources of spectral interferences include variations in the background signal of the argon plasma and stray light entering the detector. Additional limitations relevant to the present study are that ICP-AES is destructive and cannot provide high resolution spatial information on the distribution of trace elements in biological samples.

2.4 Laser Ablation Inductively Coupled Plasma Mass Spectrometry

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was first described as a technique for the analysis of solid samples using mass spectrometry by Gray (1985). The combination of a laser (light amplification by stimulated emission of radiation) with ICP-MS results in a technique that has high sensitivity and also allows the analysis of the spatial distribution of trace elements in samples. ICP-MS has been discussed in detail in Section 2.2. The principles, applications, advantages and limitations of laser ablation only are discussed in the following sections.

2.4.1 Principles and Components of LA-ICP-MS

Jackson (2001), Gunther *et al.* (1999) and Durrant (1999) have provided detailed discussions of the principles and application of lasers in mass spectrometry. A laser is a device that produces a high intensity beam of photons that has a very pure wavelength (Jackson, 2001). The neodymium: yttrium-aluminium-garnet (Nd:YAG) laser, which was used in the present study, contains a yttrium-aluminium-garnet rod that has approximately 1% neodymium. A high intensity light from a flash lamp excites the electrons of neodymium to a higher energy state known as the metastable state (Jackson, 2001; Durrant, 1999). A population inversion is said to occur when the number of electrons in the higher energy state exceeds the number in the ground state. Due to the instability of such a configuration, the electrons return to their original energy state resulting in the release of photons. Interactions between these photons and any unstable electrons, known as stimulated emission, results in the release of additional photons. Mirrors are employed to reflect the photons through the rod several times to amplify the stimulated emission. The final result of this process is the release of photons that have the same energy, wavelength, direction, polarisation and phase (Jackson, 2001). Durrant (1999) provides an overview of other laser types that have been used in mass spectrometry.

The Nd:YAG laser emits photons in the infrared wavelengths. Emissions in the ultraviolet wavelengths are, however, required to guarantee a high degree of absorption by samples, thus ensuring reliable ablation. A harmonic generator is used to convert the infrared laser emissions to ultraviolet wavelengths. In the present study, a wavelength of 213 nm (also referred to as the 5th harmonic) was used (Jackson, 2001).

The laser beam is directed at the samples using mirrors and prisms, and focused using a focusing objective, which may be of a refracting or reflecting type. In Nd:YAG lasers, the spot size is controlled by adjusting the laser focus and incident energy in combination (Gunther *et al.*, 1999). An interchangeable aperture in the laser beam path may also be used to control spot size (Jackson, 2001). The power of the laser is controlled by an optical attenuator that consists of a polariser and a rotatable crystal quartz half-wave plate. Rotation of the wave plate changes the plane of polarisation and alters the proportion of beam transmitted to the sample (Jackson, 2001; Gunther *et al.*, 1999). The energy of the laser used in the present study was 0.10 mJ/pulse. Other means of controlling power include the use of apertures, filters and adjustment of the flash lamp voltage (Gunther *et al.*, 1999).

As the Nd:YAG laser requires considerable time to attain thermal equilibrium and output stability, a shutter is inserted in the beam path to initiate and terminate ablation (Jackson, 2001). In the present study, the beam was fired for approximately 3 s before the shutter was removed from the beam path and sample ablation commenced.

The sample to be analysed by LA-ICP-MS is placed in an air-tight cell that has a window transparent to the laser wavelength, and also has ports for the entry and exit of the carrier gas that transports the ablated sample material to the ICP-MS (Jackson, 2001). Good gas flow dynamics are achieved by having a small chamber and high gas velocity. The carrier

gas is usually argon or helium. Ablation in helium is preferred as it minimises the deposition of ablation products in the sample cell (Jackson, 2001).

Finally, a viewing system comprising a television camera and monitor is used to select the ablation site and also observe the ablation process. Sophisticated systems with microscopes are also available allowing high resolution viewing of samples. A diagram of the LA-ICP-MS system is shown in Figure 2.4.

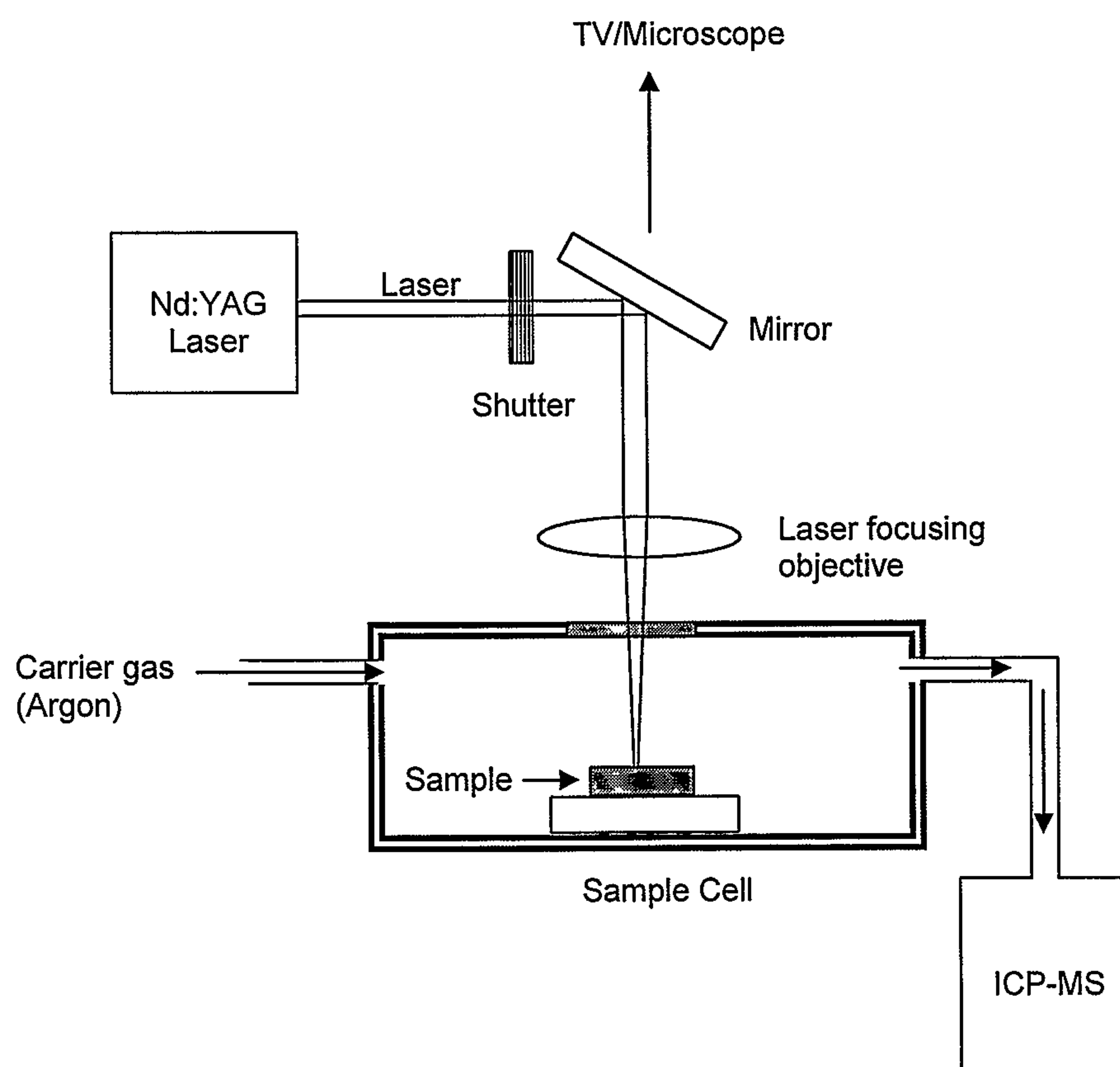


Figure 2.4. Diagram of the layout of a laser ablation inductively coupled plasma mass spectrometer (adapted from Gunther *et al.*, 1999 and Durrant, 1999). The neodymium: yttrium-aluminium-garnet (Nd:YAG) laser beam passes through a shutter and is reflected by a mirror to a focusing objective, which directs it at the sample surface. The sample is held in an air-tight cell. Ablated sample material is carried by an argon gas stream to the inductively coupled plasma mass spectrometer.

2.4.2 Advantages and Limitations of LA-ICP-MS

The primary advantage of LA-ICP-MS, in the context of this study, is the ability to map the spatial distribution of trace elements in solid samples at low detection limits. Detection limits of LA-ICP-MS vary with the size of the ablation, improving with an increase in the diameter of the ablation crater. The LA-ICP-MS provides ppb detection limits for lead and can also provide information on isotopes of lead and other elements. This method is less expensive than the nuclear microprobe, an alternative method used in this thesis, and is therefore more accessible. Gunther *et al.* (1999) stated that for sets of elements with similar ablation behaviour and properly selected internal standards, a high degree of accuracy can be achieved without matrix matching.

The primary disadvantage of LA-ICP-MS lies in the destructive nature of the ablation process. Also, unlike the nuclear microprobe, energy dispersive X-ray analysis and synchrotron radiation induced X-ray analysis, LA-ICP-MS cannot be used to map the spatial distribution of trace elements in soft tissues. In addition, the spatial resolution of the LA-ICP-MS is inferior to these three techniques. Finally, the general limitations of ICP-MS also apply to this technique and are discussed in Section 2.2.2.

2.5 Nuclear Microprobe

The nuclear microprobe (NMP) is a surface analytical technique for mapping multiple trace elements at micron resolutions. The NMP has evolved from the proton induced X-ray emission (PIXE) and proton induced γ -ray emission (PIGE) techniques commonly used in geology and material sciences.

2.5.1 Principles and Components of a NMP

In a NMP analysis, a proton beam is generated in a tandem accelerator and is systematically scanned across the sample surface. Collision of the high-energy protons with atoms on the sample surface results in several reaction products including characteristic X-rays, backscattered and transmitted protons, secondary electrons and gamma rays (Watt *et al.*, 1991). The ejection of core electrons from a sample atom during a NMP analysis de-stabilises the atom. To restore the stability of the sample atom, an electron from an outer shell moves to occupy the vacancy in the lower shell which results in the release of X-rays that are characteristic for each element present in the sample. The wavelength of the X-rays reveals the identity of the elements present and the intensity of the X-rays generated determines the concentration of the elements (Traxel, 1988).

Because the components and configuration of NMPs vary markedly in different laboratories, the present discussion is limited to the major components of the instrument used in the present study, namely the CSIRO-GEMOC NMP developed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), and the Australian Research Council's National Key Centre for Geochemical Evolution and Metallogeny of Continents (GEMOC).

In this instrument the proton beam is delivered by a 3 MeV tandem accelerator and collimated by object and divergence slits. The object slits are formed by 5 mm tungsten

rods in a “V” shaped configuration and the slit-gap can be continuously varied from 0-1200 μm . The divergence slits are located 2.6 m from the object slits (Figure 2.5). These are made of 1 mm thick tantalum plates and are motorized to allow linear movement. The slit gaps and the distance between object and divergence slits can be adjusted to control the degree of aberration in the focused beam-spot at a given beam current (Ryan *et al.*, 1999).

A lens system is used to focus the protons into micron dimensions ready for scanning across the sample. The CSIRO-GEMOC NMP uses a unique quintuplet magnetic quadrupole lens system. This system offers the advantage of achieving high demagnification despite the short overall system length of 4.7 m. Using this lens system, a beam spot of approximately 1.0 μm has been reported (Ryan *et al.*, 1999).

As the 3 MeV proton beam is scanned across the sample surface, the resultant X-rays are detected using a germanium solid-state detector (Ultra LE Ge, Canberra, USA), located 16 mm from the target, which has a 100 mm² aluminium filter that is 10 μm thick, and a 32 μm beryllium window. A second X-ray detector is also present to measure major elements (Ryan *et al.*, 1999). The co-ordinates of each individual emission are marked in two dimensions, which enables elemental distribution images to be recovered.

The detection limits of the NMP are determined by the magnitude of the background radiation produced. In this technique the protons produce the Bremsstrahlung or braking radiation through target nuclei scattering. This is, however, several magnitudes lower than the background radiation produced by an electron and consequently the NMP has significantly better detection limits compared to an electron probe (Nystrom, 1995; Cookson, 1991). Method Detection Limit (MDL) was calculated using Poisson statistics with the equation: $\text{MDL} = 3.26 \sqrt{2B}$, where B is the background

2.5.2 Advantages and Limitations of NMP Analysis

The NMP provides detection limits in the range of 1-2 ppm for lead which are better than other techniques including the electron probe and energy dispersive X-ray analysis. The NMP is capable of spatial resolution of 1-2 μm and is non-destructive as opposed to other techniques requiring prior acid dissolution or laser ablation. This technique also has the added advantage of providing quantitative multi-element data. Furthermore, sample preparation of teeth for the NMP is relatively simple.

The limitations of the NMP arise primarily from the high expense and limited accessibility. Of note is the fact that the NMP is less sensitive than LA-ICP-MS and cannot be used for isotopic analysis. This is a major limitation as lead levels in human teeth are often found to be below 1 ppm. Furthermore, interferences from neighbouring elements may contaminate the signal of the element of interest. For example, the L- β peak of iridium may overlap with the L- α peak of lead in the PIXE spectrum. Additionally, a high level of operator expertise is required to operate a NMP.

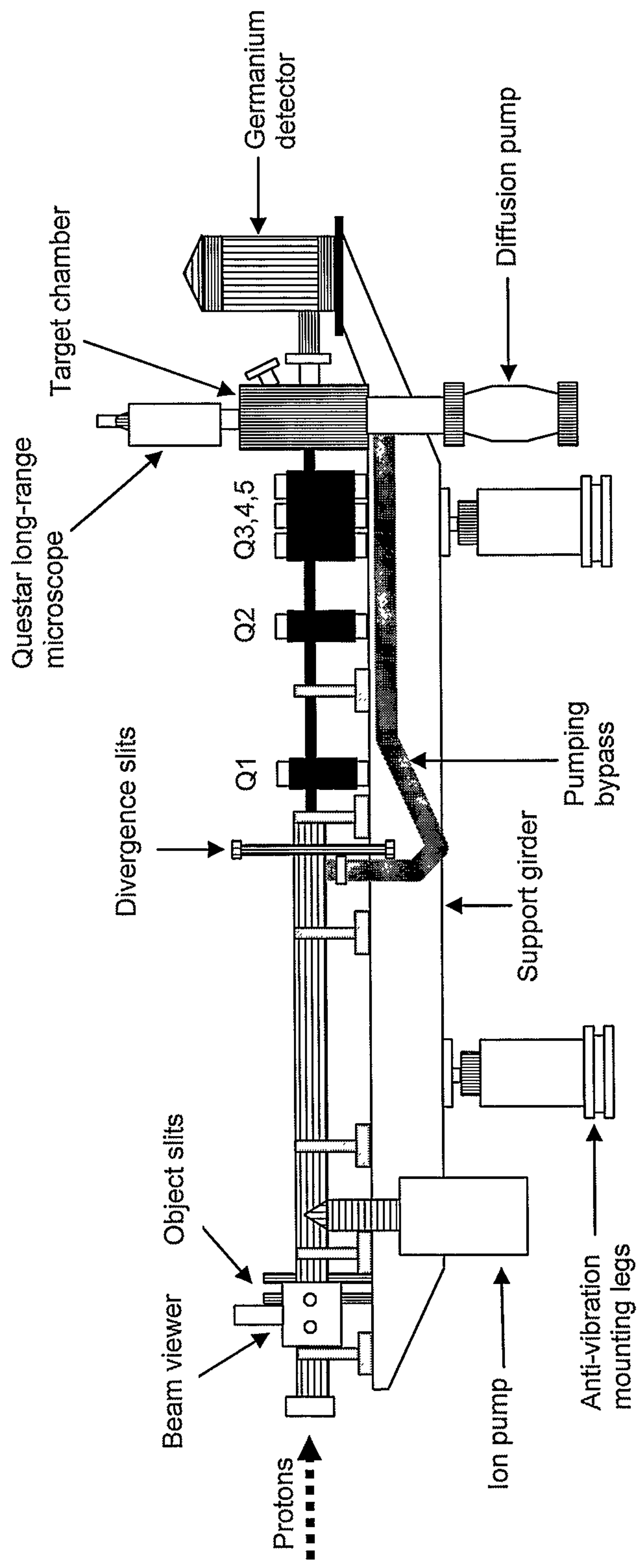


Figure 2.5. Schematic diagram of the layout of the nuclear microprobe used in the present study (adapted from Ryan *et al.*, 1999). Protons from the accelerator are collimated by the object and divergence slits. A quintet quadrupole lens system (Q1-Q5) focuses the proton beam to a spot size of approximately 1.0 μm . Collision of the proton beam and the sample, mounted in the target chamber, releases characteristic X-rays that are measured by the germanium detector.

2.6 Autometallography

Autometallography (AM) is a histochemical method for the microscopic visualisation of trace metals in biological tissues which utilises a similar process as that of physical development in photography. AM was introduced by Danscher in a series of publications in the early 1980s (Danscher and Norgaard, 1985; Danscher, 1984 and 1981).

2.6.1 Principle of Autometallography

Sections of the sample tissue to be analysed are placed in a physical developer containing silver ions and a reducing agent, such as hydroquinone. Transfer of electrons from the reducing agent converts silver ions to metallic silver. Trace metal ions present in the sample tissue act as a catalyst for this reaction and the reduced silver accumulates around the trace metal ions (Figure 2.6) (Danscher, 1991; Danscher *et al.*, 1987; Danscher, 1984 and 1981). As this process continues, trace metal and elemental silver deposits increase in size becoming visible by light microscopy. The reaction is stopped by immersing the sample tissue into a stop bath. The process of physical development is primarily dependant on the temperature of the solutions used and the duration of development. Danscher (1981) recommended that the temperature should be kept constant and the time period should be varied such that silver deposits are clearly visible in the sample tissue, while the control tissue sections remain clear. In order to determine the appropriate duration of development, serial sections should be developed from 30-150 min. The thickness of the tissue sections is also a factor that may affect the reaction and should be kept constant.

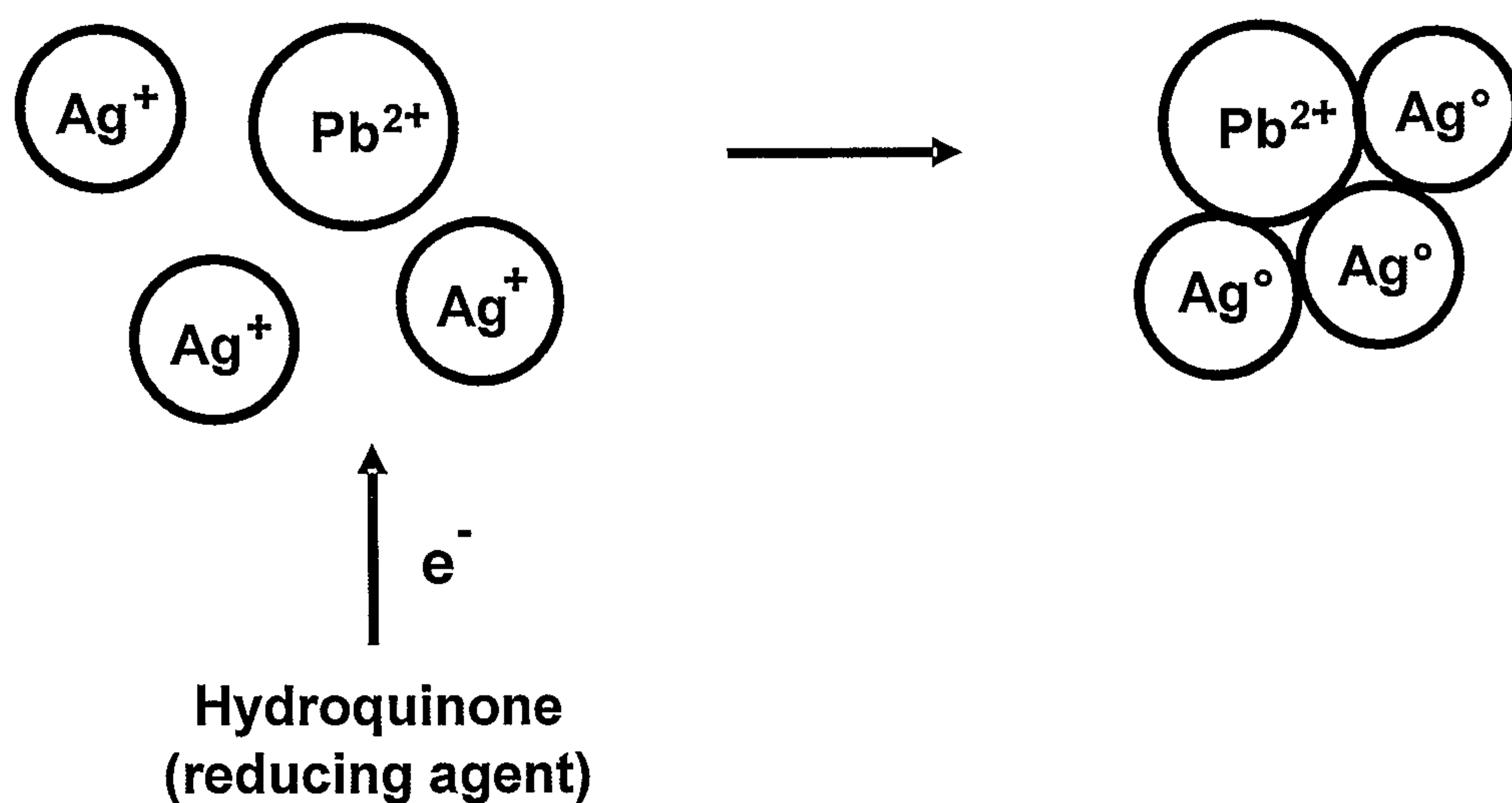


Figure 2.6. Diagram outlining the principle of autometallography (modified from Danscher and Norgaard, 1985). Silver ions (Ag^+) are reduced to metallic silver (Ag°) by receiving electrons (e^-) from the reducing agent, hydroquinone. Lead ions (Pb^{2+}) act as catalysts for the reduction of silver ions, resulting in the aggregation of silver around the lead which can be visualised by light microscopy.

2.6.2 Advantages and Limitations of Autometallography

AM is a procedurally simple and relatively inexpensive technique. It allows visualisation of trace metal deposits in biological tissues at light and electron microscopic levels. However, the identity of the trace metal ions cannot be distinguished using AM. In addition, the concentrations of the trace elements cannot be measured by AM. For samples containing multiple trace elements, high concentrations of the element of interest need to be present for detection by AM.

2.7 Synchrotron Radiation Induced X-ray Emission

The first practical synchrotron was put into operation in 1947 at the General Electric Research Laboratory, USA (Thompson *et al.*, 2001) and since this time synchrotrons have become an essential tool in many areas of scientific and medical research. A synchrotron is a source of intense tunable electromagnetic radiation that can be adjusted into a number of wavelengths which have numerous research applications including, a growing importance for, the determination of trace elements in biological tissues (Australian Synchrotron Research Program (ASRP), 2003).

2.7.1 Principles and Components of a Synchrotron

The following is a brief introduction to the operation of a synchrotron. Detailed discussions of the principles and components of synchrotrons are provided in American Institute of Physics, 1997, Craievich, 1990 and Ternov *et al.*, 1985. The operation of the synchrotron relies on the principle that electromagnetic radiation is emitted when any particle is accelerated. The radiation is most intense for particles with small masses, such as electrons, and is emitted perpendicular to the direction of acceleration (Jones *et al.*, 1988). In a synchrotron, high energy electrons are accelerated to a velocity nearing the speed of light and directed in a circular path using bending magnets. During the circular movement of the electrons, photons are emitted along a direction tangential to the path of the electrons. These photons are emitted in short pulses and have certain unique characteristics including high intensity, wide spectrum of energy, and a high degree of polarisation (ASRP, 2003).

A schematic layout of a typical synchrotron is presented in Figure 2.7. The electrons in a synchrotron are generated in a linear accelerator using an electron gun that operates in a similar manner to a cathode ray tube. A metal filament is heated to high temperatures resulting in the emission of electrons which are subsequently accelerated across a

potential difference of approximately 100 keV. At this stage the electrons are traveling at approximately half the speed of light. To prevent interaction with atomic and molecular particles in air, the electrons are generated and maintained in a vacuum (ASRP, 2003).

Focusing elements are used to ensure that the electrons do not collide with the walls of the vacuum chamber. Further acceleration of the electrons is produced in cylindrical radiofrequency cavities, where an electromagnetic field is applied perpendicular to the path of the electrons resulting in acceleration of the electrons. The radiofrequency cavities also result in the bunching of electrons, in effect producing highly intense electron pulses (ASRP, 2003).

From the linear accelerator the electron beam is passed into the booster ring, where the energy of the electrons is further increased by using radiofrequency cavities. Bending magnets are used to keep these energised electrons in an approximately circular orbit. From the booster ring, the electrons are passed into a larger storage ring from where the electromagnetic radiation emitted tangential to the electron beam path is collected. Radiofrequency generators are used to maintain the energy of the electrons and insertion devices are used to increase the intensity of the synchrotron radiation at selected wavelengths (ASRP, 2003).

Two types of insertion devices are commonly used. A multipole wiggler produces multiple pronounced deflections of the electron beam. At the peak of each deflection, a beam of light is emitted. Superimposition of the cones of emitted light results in the increase of the intensity. An undulator is another type of insertion device which produces gentler deviations of the electron beam. The emitted light is enhanced by approximately 10,000 times (ASRP, 2003).

The radiation emitted from the storage ring can be accessed by several beamlines, each operating independently. A beamline contains several key components including the optics, experiment room and control room. The optics comprise slits to change the shape of the beam, attenuators to control the intensity of the beam, mirrors to focus the beam, and a rotating crystal monochromator to select the specific wavelength of interest. Each beamline is optimised for a particular class of measurement. In the present study the sample to be analysed is placed in an aluminium holder in line with the beam in the experiment room. A computer console in the adjacent control room is used to control the analysis parameters (ASRP, 2003).

The incident photons excite the sample atoms, which results in electron transitions from higher energy to lower shells with accompanying emissions of X-rays. The energy of the X-rays is characteristic of the type of element present and the intensity is proportional to the concentration of the element present. These X-rays are measured using a suitable detector (Jacobsen and Kirz, 1998).

2.7.2 Advantages and Limitations of Synchrotrons

A synchrotron radiation induced X-ray emission (SRIXE) beamline on a third generation synchrotron is capable of providing sub-micron spatial resolution with ppm detection limits. At photon energies of approximately 10 keV synchrotrons provide spectral brightness 10^5 - 10^{10} times greater than conventional sources of X-rays (Thompson *et al.*, 2001). SRIXE is a non-destructive technique capable of mapping the distribution of elements at a cellular level in both hard and soft tissues. In addition, SRIXE provides quantitative data.

Due to the high demand across a range of disciplines and lack of a synchrotron facility in Australia at present, only a limited number of samples prepared in this study could be analysed by SRIXE. The detection limits of SRIXE are higher than techniques such as ICP-MS and LA-ICP-MS. SRIXE analysis requires a high level of operator expertise.

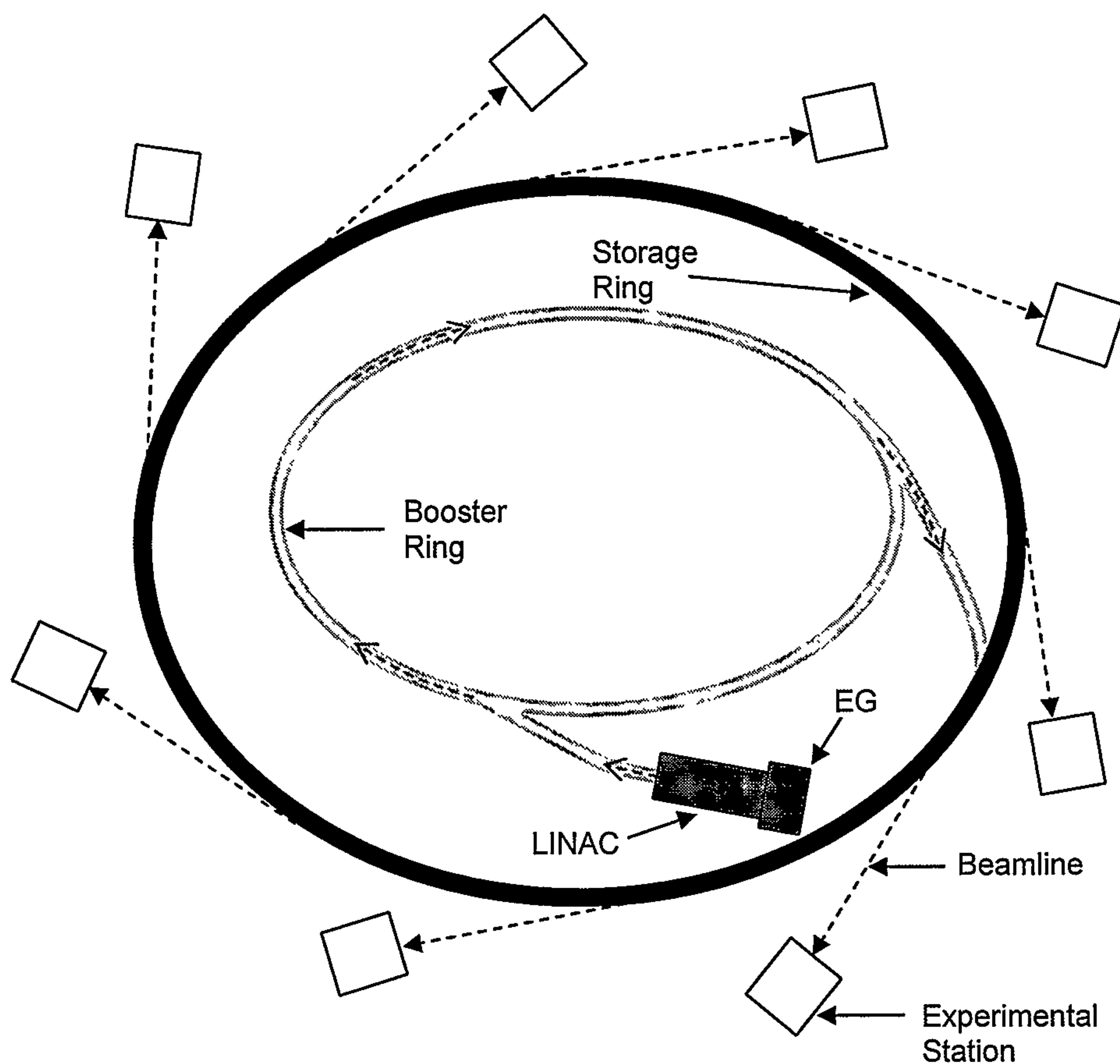


Figure 2.7. Diagram of the layout of a synchrotron (adapted from ASRP, 2003). Electrons generated in the electron gun (EG) are accelerated in the linear accelerator (LINAC) and the booster ring. The electrons are transferred to the storage ring and beamlines comprising photons travelling tangential to the electron beam path are tapped in the experimental stations. The wavelength of the emitted photons can be adjusted to suit the requirements of the experiment.

CHAPTER 3

The Spatial Distribution of Lead in Enamel and Coronal Dentine of Wistar Rats

3.1 Introduction

Although dental lead levels have been shown to be a useful marker of lead exposure, very limited information is available on the temporal and spatial incorporation of lead in dental tissues. The majority of studies that have examined lead levels in teeth at controlled exposures have been limited to the analysis of whole teeth. Grobler *et al.* (1985 and 1991) and Kaplan *et al.* (1980) measured lead levels in whole teeth in rodents and dogs respectively under controlled lead exposure. Other studies, including those by Gdula-Argasinska *et al.* (2004), Glab *et al.* (1995), Smith *et al.* (1992), Sileo and Beyer (1985), and Witkowski *et al.* (1982), measured lead levels in the whole teeth of animals without quantifying the levels of lead exposure.

Comparatively, a smaller number of studies have reported the spatial distribution of lead in enamel and coronal dentine of human and animal teeth (Kang *et al.*, 2004; Budd *et al.*, 1998; Evans *et al.*, 1995; Malik and Fremlin, 1974). However, as the intensity of lead exposure was not quantified, it cannot be ascertained whether variations in lead concentrations within different regions of enamel and dentine were due to the physiological differences in lead uptake by enamel and coronal dentine or due to changes in the intensity of exposure during the period of tooth development and mineralisation.

Overall, the spatial distribution of lead in enamel and dentine at a controlled level of lead exposure remains unexplored, making it impossible to establish a quantitative relationship between the intensity of exposure and lead levels in specific regions of enamel and dentine. This chapter describes experiments characterising the distribution of lead in enamel and coronal dentine of Wistar rats exposed to a fixed concentration of lead.

3.2 Materials and Methods

3.2.1 Mating and Animal Husbandry

Wistar rats were obtained from the Westmead Animal Holding, Westmead Hospital, NSW, Australia. Four female Wistar rats aged 12-14 weeks of normal weight (150-270 g) were divided into two groups (Exposure and Control) with two animals in each group. The rats in these groups were mated with male Wistar rats aged 12-14 weeks of normal weight (200-400 g). The adult animals were examined prior to mating by a trained animal technician for any external signs of ill health including raised fur, restricted movement, reduced intake of water or low body temperature. One male and one female animal were housed in a single cage. After mating, which was confirmed by a positive vaginal smear, the male rats were removed from the cage. After the birth of the litters, each female rat and her pups were housed in a single cage. All animals were weighed weekly and checked daily for any signs of ill health. In accordance with the regulations of the Westmead Animal Holding, any rat pups failing to gain weight or any adult rats losing more than 10% of their body weight were culled immediately.

3.2.2 Housing and Environment

The rats were kept in standard plastic rat cages (Mascot Wireworks, Mascot, NSW, Australia) that were 19.5 cm high, 60 cm long and 39 cm wide. Sterile wood chips, which were analysed for lead content, were used as bedding material. The room housing the cages was kept at a temperature of 21-24°C and humidity of between 40-60%. A 12 hour light/dark cycle was employed, using an automatic timing device. All personnel entering the room were required to wear clean gowns, shoe covers and latex gloves.

3.2.3 Diet, Water and Lead Administration

The adult rats from both groups were fed a standard rodent diet *ad libitum* with a lead concentration less than 0.006 µg/g (AIN93G, Glen Forrest Stockfeeders, Glen Forrest, Western Australia) (Appendix A.4). The rat feed was located where it was only accessible to the adult rats. The maternal rats in the Control group were given ultrafiltered deionised water, while the maternal rats in the Exposure group were given a constant dose of 40 mg/L of lead as lead nitrate (Asia Pacific Specialty Chemicals Ltd. NSW, Australia) through drinking water commencing five days before the expected birth of the pups. This timing for the commencement of lead exposure was chosen because mineralisation of the first mandibular molar tooth in the pups begins approximately two days prenatally (Hoffman and Schour, 1940) allowing a margin of at least two to three days for the lead to be transferred across the placenta from the mother to the developing foetus. Lead exposure was continued until day-11 after birth, via maternal milk. At day-15 after birth, the maternal rats and pups were culled by carbon dioxide asphyxiation. In Wistar rat first molars, root formation has started by day-15, which permits easy identification of the anatomic crown.

The concentration of lead used in this study is similar to the dosage previously employed in rodent dental-caries models and is considered to be a moderate level of exposure (Watson *et al.*, 1997). For the entire duration of the experiment, the only source of lead for the rat pups in the Exposure groups was through maternal milk. The experimental protocol for this study was approved by the Animal Ethics Committee, Westmead Hospital, NSW, Australia (Ethics clearance No. 124.06).

3.2.4 Preparation of Sample Teeth for NMP Analysis

From the four litters generated, 20 Wistar rat pups (Exposure group: five males, four females and Control group: five males, six females) were randomly selected for the study. The high cost of accessing the NMP prevented analysis of all teeth from the four litters.

To avoid any potential bias due to inter-arch differences and variations between different types of teeth, the analysis was restricted to mandibular right first molar teeth. Mandibular first molar teeth were selected in preference to incisors for their greater similarity to human molars in morphology and development, and were also used in subsequent work detailed in Chapter 4. Crown completion in rat mandibular first molar teeth occurs by the age of approximately 11 days (Hoffman and Schour, 1940) and all 20 teeth included in the study were unerupted at the time of culling. It was considered important to analyse unerupted teeth to avoid any incorporation of lead from saliva into the enamel surface.

Following euthanasia, the right mandibular first molar teeth were removed by dissection under microscopy using sterile surgical instruments. The teeth were cleaned mechanically to remove all soft tissue from the pulp chamber and the external surface. Subsequently the teeth were cleaned in an ultrasonic bath of 5% v/v hydrogen peroxide solution (Sigma-Aldrich, NSW, Australia) for 5 min to remove any soft tissue remnants, followed by further cleaning in an ultrasonic bath of ultrafiltered deionised water for 10 min. The sample teeth were dried at 60°C for 2 hr.

The 20 sample teeth were embedded in Spurr's resin (Taab Laboratories Equipment Inc. Berkshire, UK) at a curing temperature of 60°C for 12 hr. The lingual aspect of each embedded tooth was ground, under microscopy, using sandpaper (Grades 220, 400 and 1000. Matador, Wasserfest, Germany) to a plane that passed close to the tips of the

lingual cusps (Figures 3.1 and 3.2A). Due to the morphology of the coronal lingual surface, the tips of the three cusps are not present in the same plane and therefore it was not possible to polish the entire lingual aspect to the same depth. Consequently, the mesiolingual cusp was selected as the primary region of interest and the sample teeth were further ground to a plane that passed through the tip of this cusp. Please note that the anatomical nomenclature in this section serves only to identify the structures described. The designated names do not imply homologies with teeth in other mammals.

The embedded samples were subsequently polished with a 3 μm diamond paste (Presi Tavernolles, Breil, France) to create a smooth surface and placed in an ultrasonic bath of ultrafiltered, deionised water for 10 min, dried at 60°C for 2 hr and sputter-coated with carbon under vacuum (Blazers Union FL 9496, Germany).

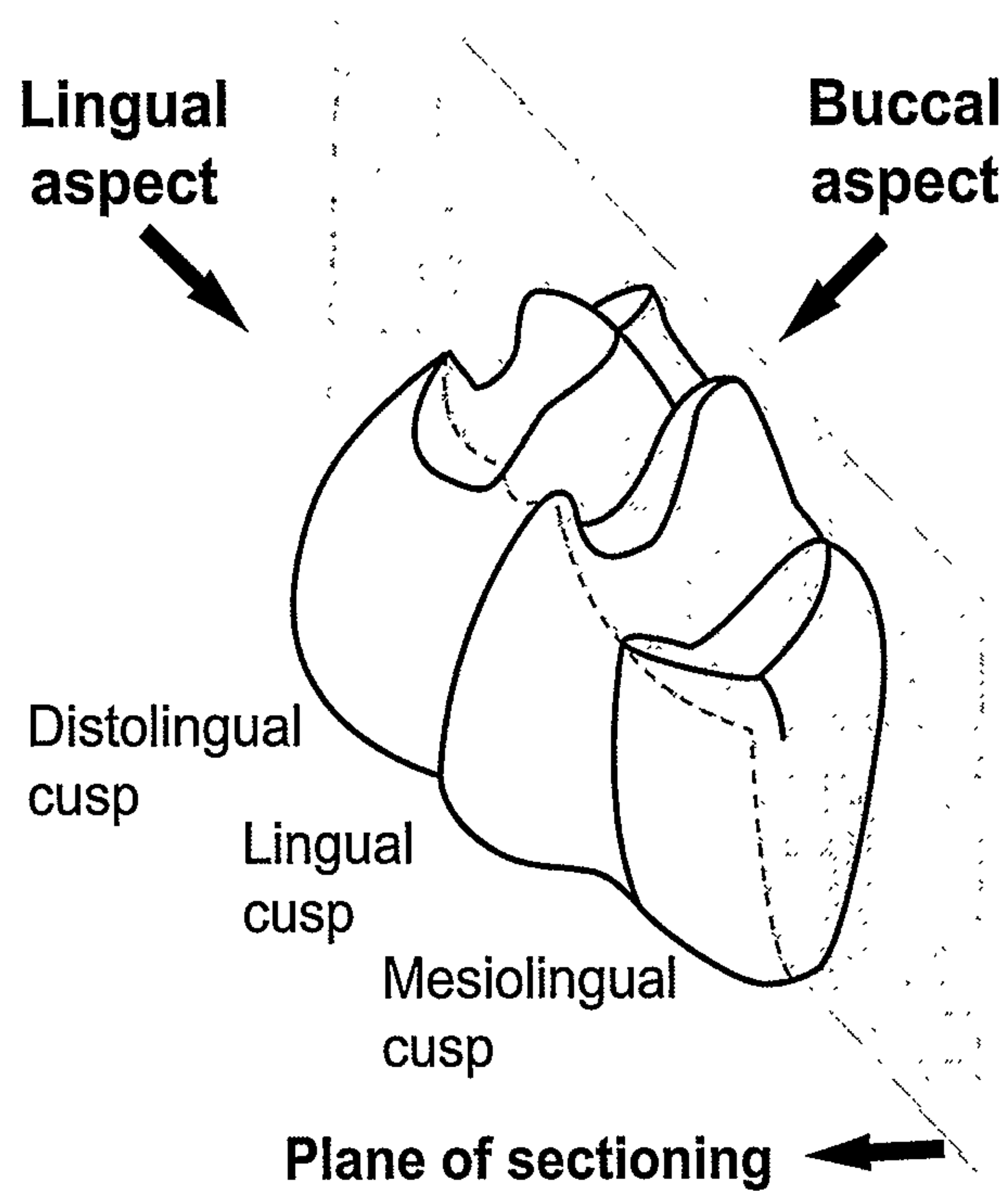


Figure 3.1. Diagram of a right mandibular first molar tooth from a 15-day-old Wistar rat pup. Mesiolingual, lingual and distolingual cusps are marked on the lingual aspect of the tooth. Sample teeth were ground to a plane that passed close to the tips of the cusps on the lingual aspect as indicated by the grey area. Please note that the anatomical nomenclature in this figure serves only to identify the structures described. The designated names do not imply homologies with teeth in other mammals.

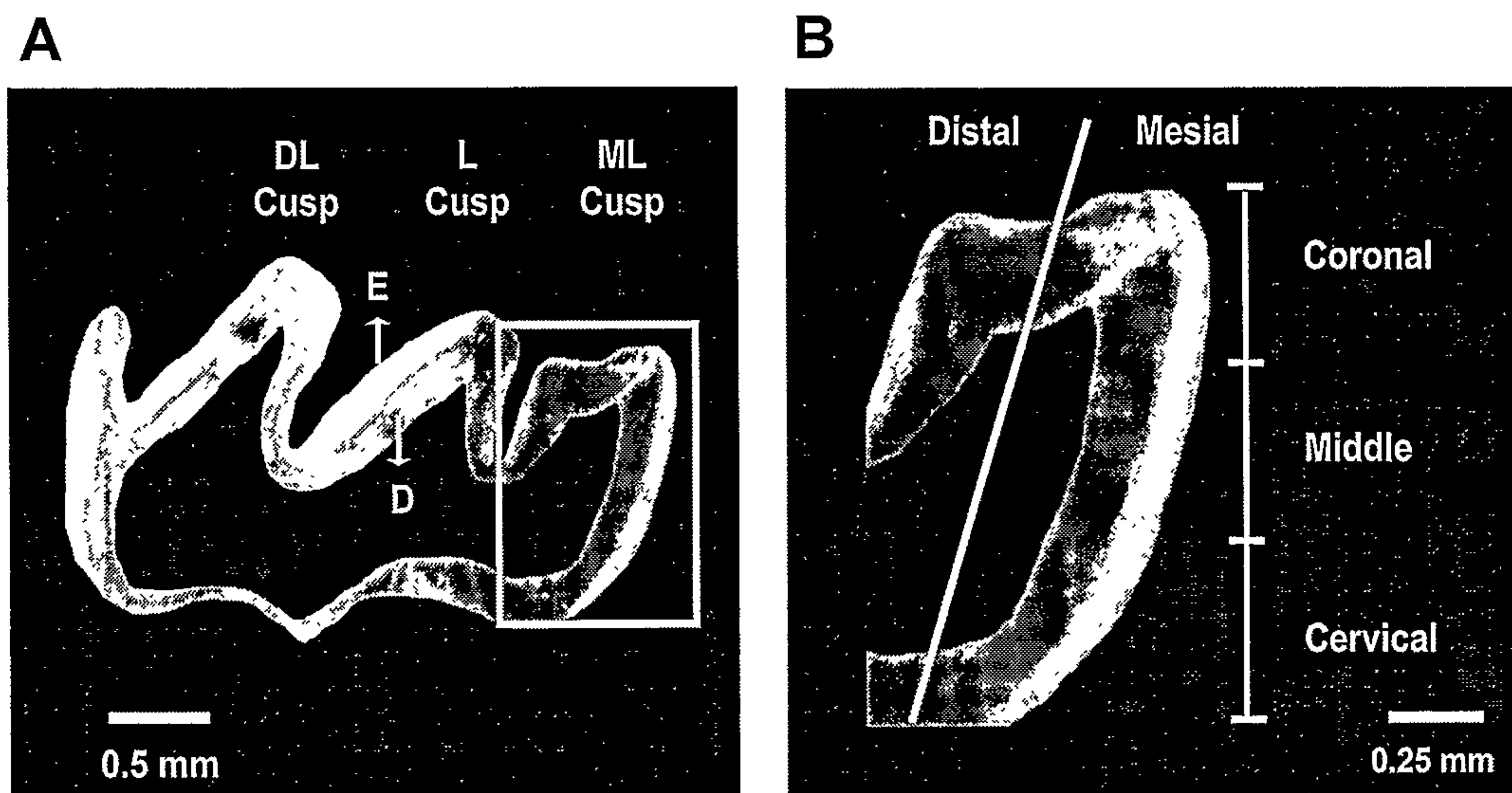


Figure 3.2. Photomicrographs of a polished section of the lingual aspect of the crown of a right mandibular first molar tooth from a 15-day-old Wistar rat pup. Distolingual (DL), lingual (L) and mesiolingual (ML) cusps with enamel (E) and dentine (D) are shown in Panel A. The tooth was ground in the mesio-distal plane until the tip of the mesiolingual cusp was reached. Panel B is a higher magnification of the area shown in the rectangle in Panel A. Mesial and distal halves as well as the coronal, middle and cervical regions of the mesiolingual cusp are demarcated by the lines in Panel B.

3.2.5 Nuclear Microprobe Analysis of Sample Teeth

The spatial distribution of lead was determined using the CSIRO-GEMOC NMP developed at the Commonwealth Scientific and Industrial Research Organisation, Australia (Ryan and Jamieson, 2001). The principles of the NMP have been described in detail in Section 2.5, Chapter 2.

In the present study, the NMP provided a focused beam of 3 MeV protons for trace multi-element microanalysis and imaging. The beam was focused into a spot size of 1.5-2 μm using a quintuplet magnetic quadrupole lens system and was systematically scanned across the sample. The resultant X-rays were detected using a germanium solid-state detector and the XY co-ordinates of each individual emission were marked, which enabled elemental distribution images to be recovered. Images were generated using the Dynamic Analysis algorithm in the GeoPIXE software (CSIRO, Australia) (Ryan, 2001; Ryan and Jamieson, 1993). These images were explored interactively in GeoPIXE to extract concentration profiles and averages. In this study the NMP was operated at a beam current of 6 nA to provide a spatial resolution of 1.8 μm and detection sensitivities for lead of 1.4 $\mu\text{g/g}$ (over 100 μm^2).

Several standard reference materials were used during the calibration of the NMP prior to sample analysis. These reference materials include BCR-1 (lead concentration = 15 $\mu\text{g/g}$), AGV-1 (lead concentration = 35 $\mu\text{g/g}$) and GSP-1 (lead concentration = 54 $\mu\text{g/g}$) (US Geological Survey rock standards, Department of Interior, USA). A detailed discussion of the components of the NMP used in the present study is given in Section 2.5, Chapter 2, and by Ryan *et al.* (1999).

3.2.6 Lead Levels in Reagents, Consumables and Glassware

All reagents, consumables, animal diet and drinking water used in this study were analysed for lead concentration using ICP-MS. The methods used to determine levels of lead in these items are given in Appendix A.4. The detailed description of the analytical method used during the ICP-MS analysis is described in Section 4.2.2, Chapter 4, where it is the primary analytical method used.

3.2.7 Statistical Analysis

The mesial and distal halves of the mesiolingual cusp of the sample teeth were considered separately for statistical analysis. Lead concentrations were assessed for the coronal, middle and cervical regions in the mesial halves of the cusps, whereas only the coronal and middle regions could be assessed in the shorter distal half (Figure 3.2B). The mean and highest lead concentrations of different regions were compared using 95% confidence intervals. Data analysis was done in Microsoft Excel 2000 (Microsoft Corporation, USA).

3.2.8 Lead Levels in Whole Teeth, Blood and Organs

Lead levels in blood, whole teeth, liver, kidney, brain and bone samples of the animals described here were also analysed using ICP-MS. The detailed results of these analyses are described in Chapter 4 as part of a larger experiment which compared lead levels in teeth, with the body burden of lead.

3.3 Results

3.3.1 Health of Animals and Lead Levels in Reagents, Consumables and Glassware

The weight of the adult female Wistar rats and their pups fell within the weight range mentioned in the literature (Pass and Freeth, 1993). Comparisons between Wistar rat neonates in Control and Exposure groups did not reveal any statistically significant differences ($p>0.05$) (Appendix A.3).

Less than 0.03 ppm of lead was detected in the reagents and no leaching of lead could be detected from the instruments or glassware (Appendix A.4).

3.3.2 Spatial Distribution of Lead in Enamel and Dentine of Rat Pups

No lead was detected using the NMP in the right mandibular first molar teeth of the 11 pups in the Control group. The lead distribution maps in the enamel and coronal dentine of the right mandibular first molar teeth from the rat pups in the Exposure group showed a distinct pattern as illustrated in Figure 3.3A. High lead concentrations were consistently observed in certain areas, while other regions of enamel and coronal dentine showed significantly lower lead levels. Such major variations in lead concentration, which were consistently observed in all the sample teeth, were designated into zones and can be visualised in Figures 3.3A and 3.3B. A peak in lead concentration was observed in the outer enamel (Zone A), followed by relatively low lead concentrations in the inner aspect of enamel (Zone B). In comparison to Zone B, the lead levels in dentine were generally higher with an increase in lead concentration at the dentino-enamel junction (DEJ) that extended for 25-57 μm into dentine (Zone C). Lead levels increased further in the region

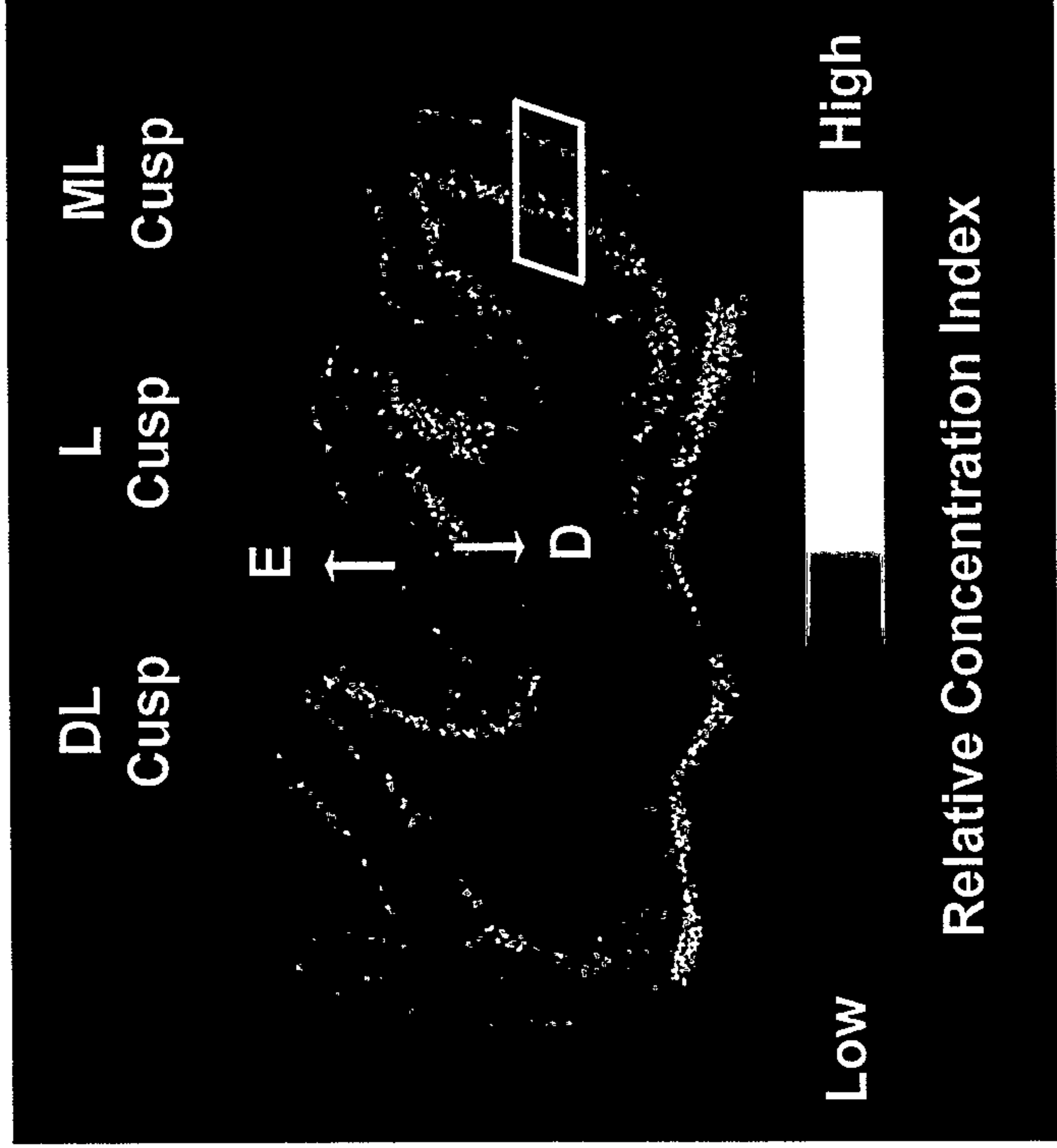
immediately adjacent to the dentine-pulp margin (Zone D) and in five of the nine sample teeth from the exposure group, this was the site of the highest lead concentration. However, in the remaining four of the nine teeth examined, Zone A was the site of the highest lead concentration as illustrated in Figure 3.3B. Zone D was not observed in the coronal third of the cusp as the analysis was conducted using a horizontal traverse, which did not extend into the pulp chamber in this region.

Figure 3.4 summarises the mean and peak lead concentrations, as well as the average dimensions, of the designated four zones on the mesial and distal aspects of the sample teeth. Furthermore, in eight of the nine teeth from the exposure group, a small peak in lead concentrations was observed in dentine adjacent to the DEJ (as indicated by the arrow in Figure 3.3B). The mean lead concentration of this peak was $67 \pm 19 \mu\text{g/g}$.

Comparisons between the four designated zones (A to D) of the coronal, middle and cervical regions of the mesiolingual cusps revealed that in each region the mean lead levels between all zones, except Zones A and C, were significantly different ($p < 0.05$) (Figure 3.4). The peak lead concentrations of Zone A, however, were consistently higher than the peak concentrations observed in Zone C. No differences in lead concentrations were observed between corresponding zones in the mesial and distal halves of the cusps. Although, in each zone (A-D), the mean width showed large variations between coronal, middle and cervical regions, no statistically significant difference could be observed when comparing the width of a zone between different regions at a 5% level of significance.

Figure 3.3. Typical lead distribution map from a single NMP scan of the lingual aspect of a mandibular first molar tooth from a rat pup exposed to lead during tooth development. Panel A shows the distolingual (DL), lingual (L) and mesiolingual (ML) cusps, as well as enamel (E) and dentine (D) of the tooth in Figure 3.2. Light colours indicate high concentrations and dark colours represent low concentrations of lead (as described by the relative concentration index). Graph B shows the variation in lead concentration with distance from the enamel surface in the area highlighted in Panel A. Four zones (A – D) corresponding to major changes in lead concentration are designated. The arrow in Graph B indicates a small peak in lead concentration at the dentino-enamel junction. High concentrations of lead were seen in the outer layers of enamel and the dentine immediately adjacent to the pulp.

A



B

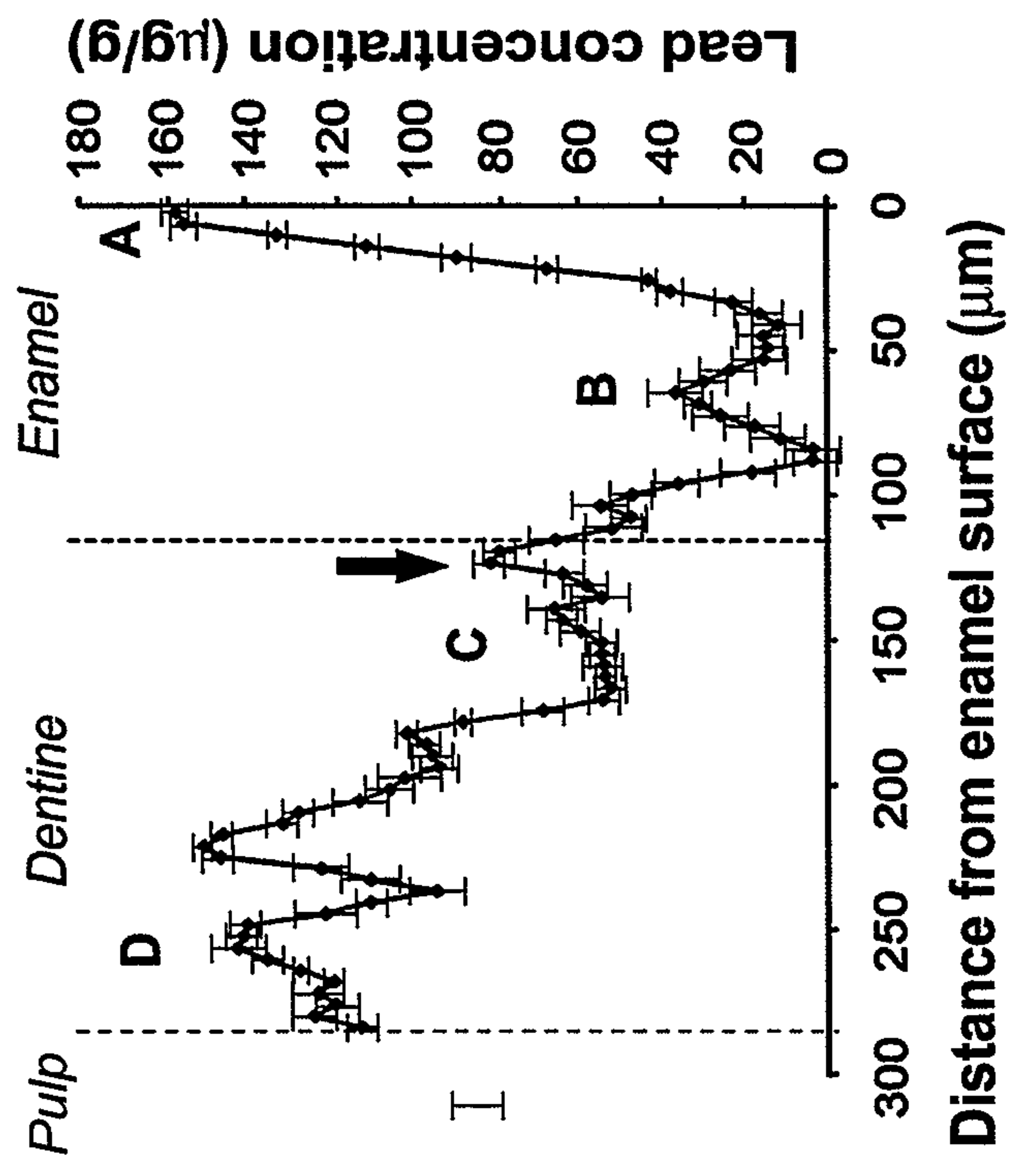
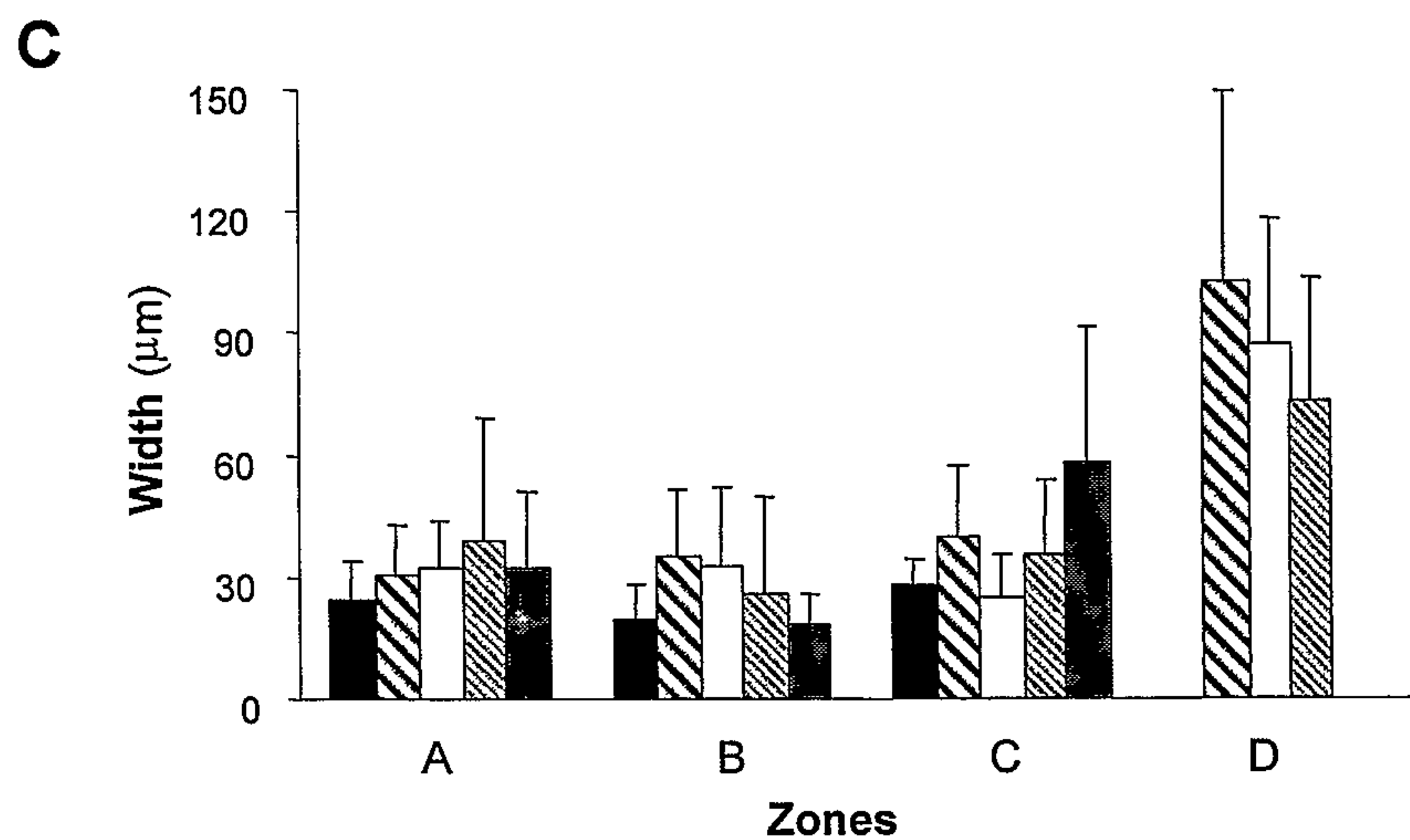
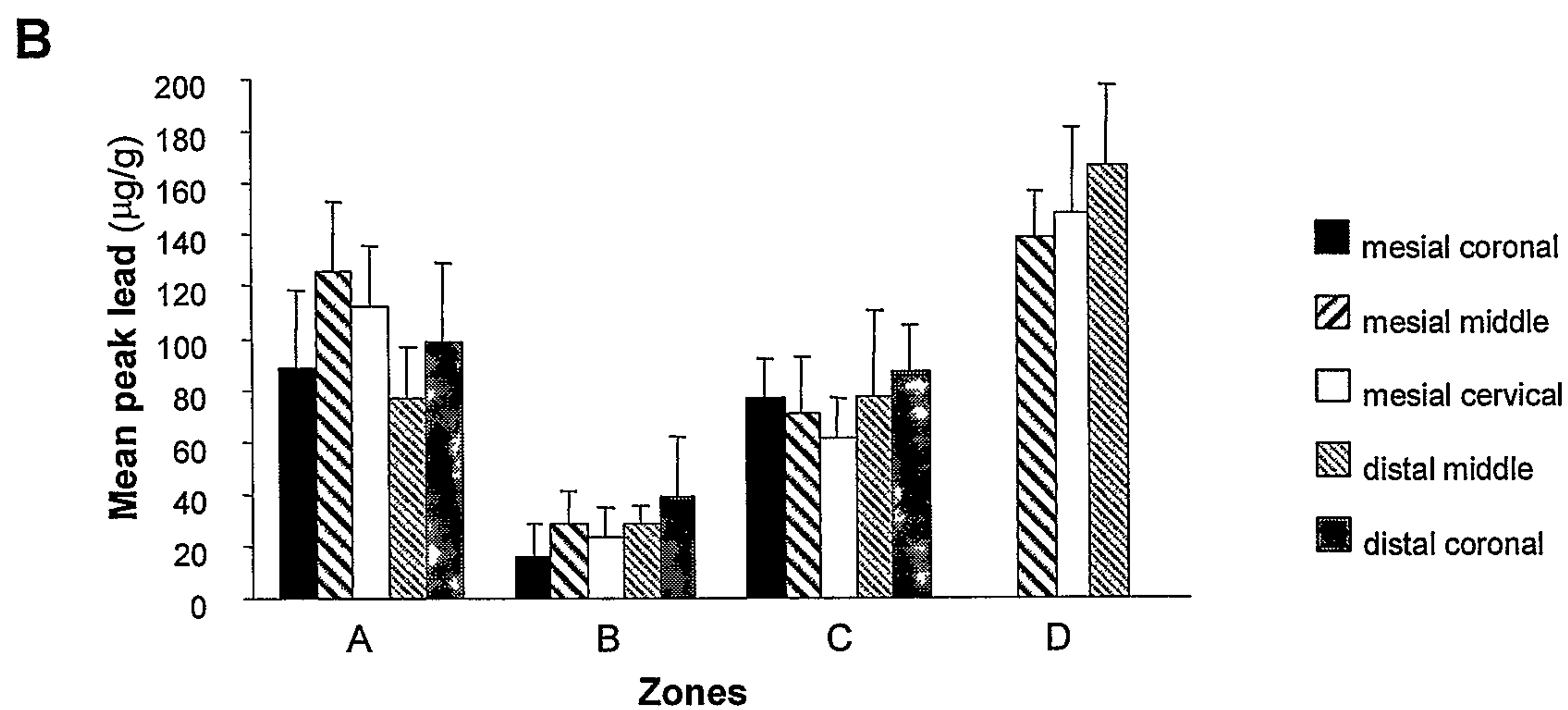
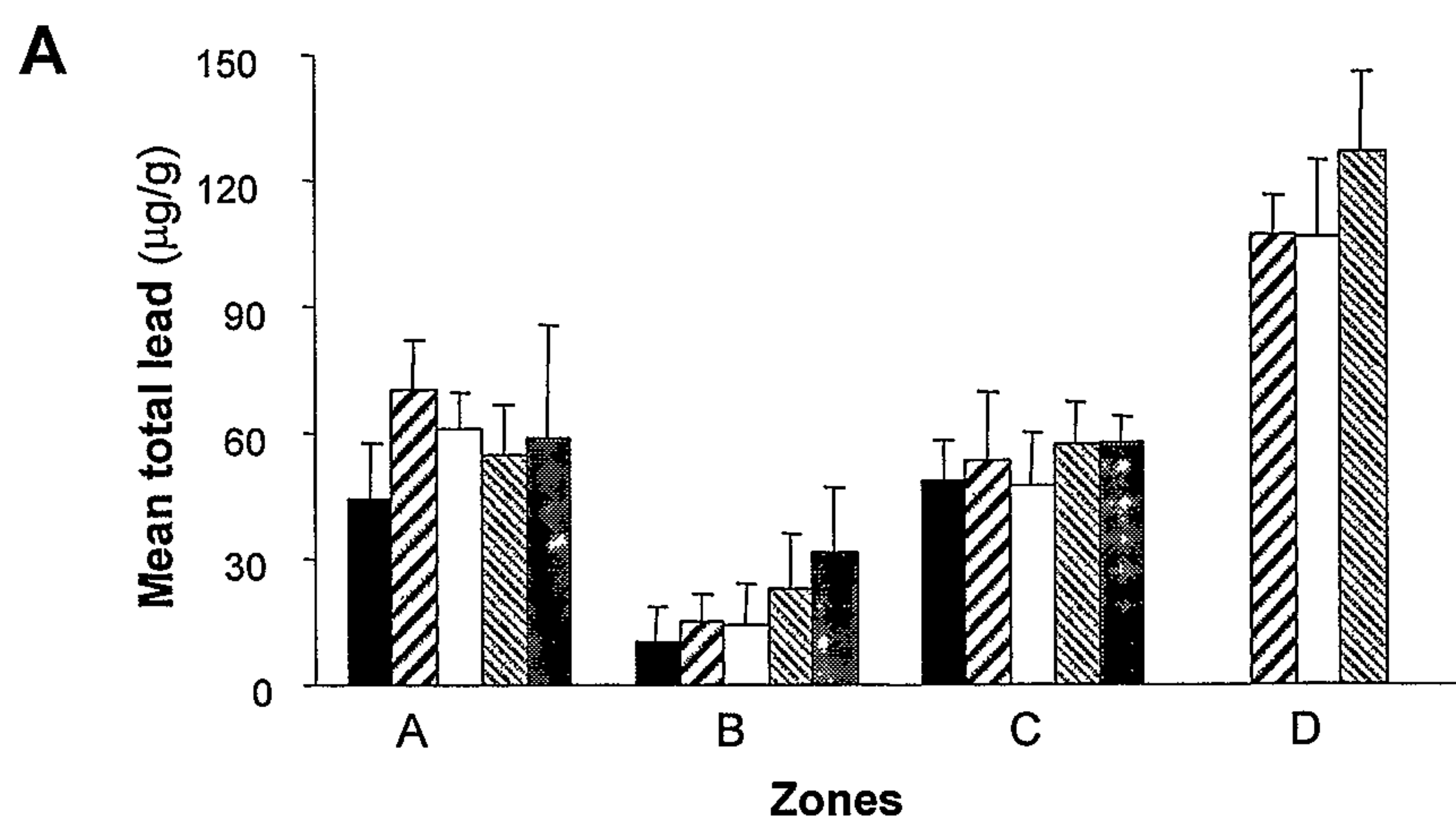


Figure 3.4. The mean total lead concentration, mean peak lead concentration and mean width of the four zones of lead distribution in the enamel and coronal dentine of lead-exposed rat pups. Data are means $\pm$ SE. The coronal, middle and cervical regions of the mesial half, as well as the coronal and middle regions of the distal half of the mesiolingual cusp of the right first mandibular molar teeth were analysed. Zone D showed the highest mean total lead and peak lead concentrations, while Zone B showed the lowest values of these parameters (Graphs A and B). Zone D was also wider than other zones (Graph C). These patterns were consistently observed in the coronal, middle and cervical regions of all teeth analysed. The location of zones is shown in Figure 3.3.



While the primary focus of this study was the distribution of lead in enamel and dentine, the NMP provided data on the concentrations of other elements in the sample teeth as well. While most elements displayed a dissimilar distribution to that of lead, it was noticed that the spatial distribution of zinc was very similar to that of lead (Figure 3.5). Preliminary analyses of the relationship between lead and zinc concentrations in the sample teeth revealed a positive correlation ($r = 0.4 - 0.80$) between the two variables.

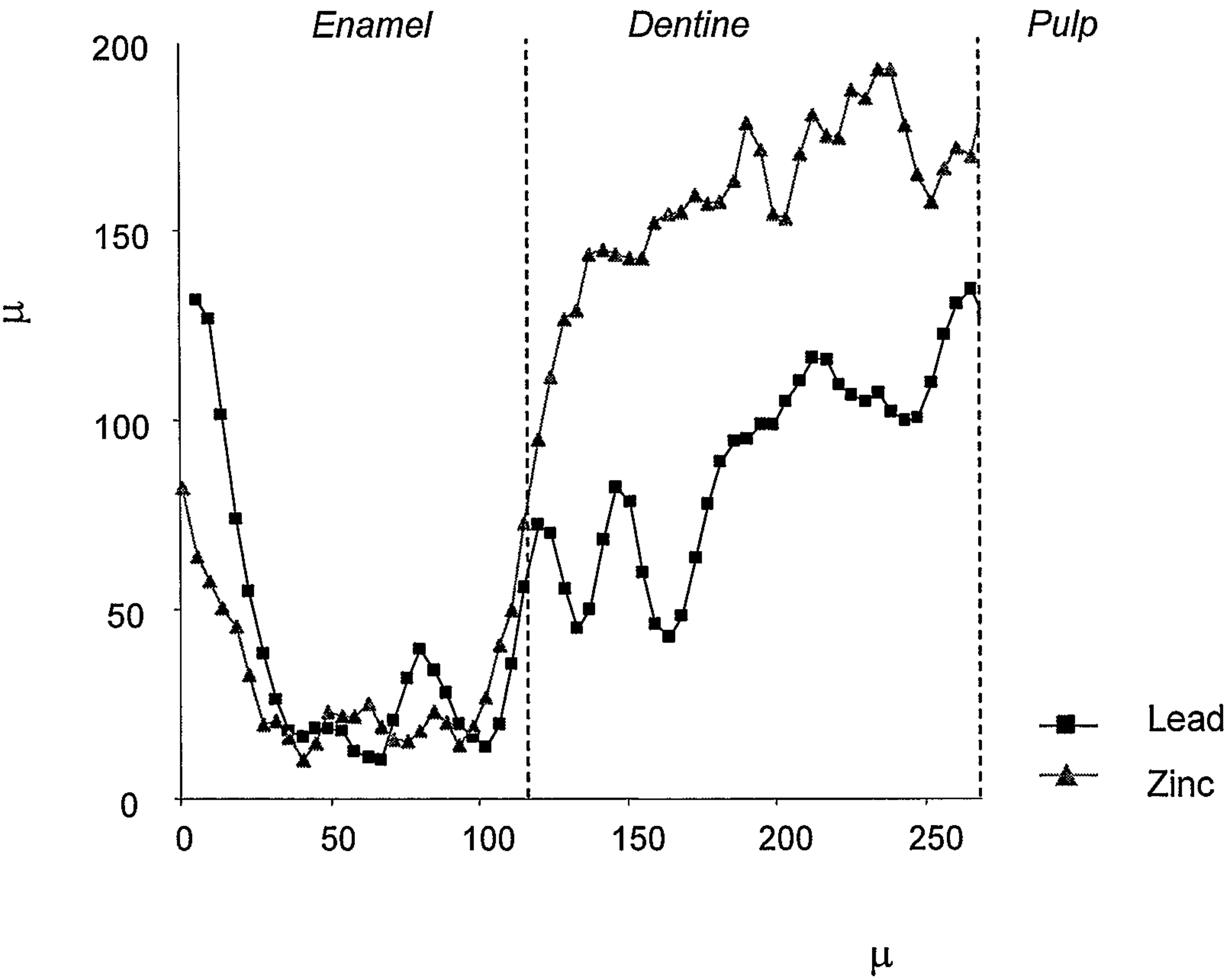


Figure 3.5. Distribution of lead and zinc in a mandibular first molar tooth of a lead-exposed rat. Lead and zinc show a similar pattern of distribution in enamel and coronal dentine.

3.4 Discussion

The distribution of lead in the crowns of rodent molar teeth from lead exposed rat pups followed a distinct and reproducible pattern with clear differences in the concentration of lead between enamel and coronal dentine. Within the enamel and dentine compartments, considerably larger deposits of lead were seen in the surface enamel and the dentine immediately adjacent to the pulp.

3.4.1 Distribution of Lead in Enamel

The reasons for the high enamel concentration of lead near the tooth surface remain unclear. In a previous study on human teeth, it was proposed that these high surface enamel-lead levels arise due to the passive diffusion of lead from saliva (Malik and Fremlin, 1974). The present work does not support this hypothesis as all the teeth included in this study were unerupted and as such would not be in direct contact with saliva. Alternatively, lead uptake by surface enamel from the soft tissues of the dental follicle might continue after completion of crown formation. Furthermore, the concentration of lead in the enamel matrix secreted by the ameloblasts may also vary according to the stage of tooth development, analogous to the variations observed in the uptake of calcium at different stages of tooth development (Aoba and Moreno, 1990; Crenshaw and Takano, 1982). The uptake of lead by ameloblasts and odontoblasts remains unexplored and could provide information on the variations of lead concentration observed in different parts of enamel and dentine.

The lead concentration in enamel and dentine formed at different times in the development of the animal will also depend on the mode of intake of the heavy metal. Prenatal exposure of the rat foetus will occur via the placenta and reflect maternal blood-lead concentrations, whereas postnatally, the lead will be acquired by the pups by suckling and exposure will depend on the lead concentration in their mother's milk.

Differences between prenatal and postnatal lead concentrations in enamel and dentine in the present rat model and in human teeth have been examined in other sections of the present study and are reported in Chapters 4 and 5 respectively.

There are only limited published data on the differences in lead concentrations between the surface and the interior regions of enamel. Purchase and Fergusson (1986) studied the levels of lead in the surface of human enamel to a depth of approximately 40 μm using serial etchings with hydrochloric acid followed by analysis of the solution by graphite furnace atomic absorption spectrometry. A sharp decline in the concentration of lead was reported with increasing depth from the surface of enamel. In the present study, a similar rapid decline in lead concentration was observed to a depth of approximately 50 μm from the enamel surface. At greater depths, the concentration of lead in enamel remained low.

3.4.2 Distribution of Lead in Dentine

In dentine, the highest levels of lead were observed near the dentine-pulp margin, which may be attributed to the exchange of lead from the pulpal blood vessels. Alternatively, it is known that the rate of dentine apposition declines after crown completion (Nanci, 2003a) which would result in a longer time period for lead to be incorporated into the mineralising matrix of the secondary dentine adjacent to the pulp. This in turn would result in a higher concentration of lead in this region.

The remainder of dentine showed considerably lower levels of lead with a small peak in concentration near the DEJ, in the region of mantle dentine. There is considerable disagreement in the literature on the mineral content of mantle dentine. Herr *et al.* (1986) reported that the mineral density of human mantle dentine was 4% (by volume) lower than that of circumpulpal dentine. Stratmann *et al.* (1997), however, found no significant difference in the mineral content of mantle and circumpulpal dentine in rat molar teeth. This conflicting data from these studies have not assisted us in further determining

whether the degree of mineralisation in the region of mantle dentine has a role to play in the observed differences in lead concentrations.

There were clear differences between the concentration and distribution of lead in enamel and coronal dentine, with mean lead levels in dentine being higher than in enamel. This finding is in agreement with that of Grobler *et al.* (2000) who studied lead levels in fragments of human enamel, dentine and circumpulpal dentine by dissolution in acid and analysis using graphite furnace atomic absorption spectrometry. Dentine was reported to have approximately eight times more lead than enamel, while circumpulpal dentine had 26 times the concentration of lead in enamel. However, the method employed in their study could not provide precise data relating to the spatial distribution of lead in teeth.

Other studies that have reported the spatial distribution of lead in enamel and dentine of human teeth are outlined in Table 3.1. In those studies the absolute concentrations of lead in enamel and dentine showed a high degree of variability between studies, most likely representing the different exposure histories experienced by the respective sample populations. In addition, the studies outlined in Table 3.1 were unable to quantify the exposure to lead during tooth development, and consequently, the significance of lead concentrations in different anatomical regions of a tooth cannot be fully interpreted. Future prospective studies in human populations that record the history of exposure through periodical blood-lead and environmental sampling and correlate these exposure variables to lead levels in exfoliated teeth would allow better quantitation of the relationship between lead levels in different compartments of teeth and the intensity of exposure.

3.4.3 Similarities in Lead and Zinc Distribution in the Sample Teeth

The similarity in the distribution of lead and zinc may be due a similarity in the mode of absorption of the two elements in dental tissues. In such a scenario, administration of higher doses of zinc may inhibit the uptake of lead in teeth and other organs. Flora *et al.* (1994) provided some preliminary evidence in support of this suggestion, where zinc administration increased the excretion of lead during chelation therapy in a rat model. Experiments where varying doses of lead and zinc are administered concomitantly would provide useful data on this issue.

3.4.4 Relevance of the Findings of this Experiment in using Teeth as a Biomarker of Lead Exposure

The findings of the present study will be of significance when using tooth-lead levels to obtain temporal information regarding lead exposure. If longitudinal sections of teeth are mapped in the direction of enamel and dentine increment (as described in Figure 1.1B, Section 1.8.2, Chapter 1), the results are likely to indicate an increase in lead concentration from the inner enamel, adjacent to the DEJ, to the enamel surface. A similar increase will be observed when measuring lead levels in dentine from the DEJ towards the pulp. This, however, does not reflect a temporal increase in lead exposure, but rather the normal variations in lead levels in different parts of enamel and coronal dentine. In light of this, the mapping of lead concentrations in the direction of enamel and dentine extension from cusp tip to the cervical end (Figure 1.1A, Section 1.8.2, Chapter 1) is more likely to reflect temporal patterns in lead exposure.

As discussed earlier in Section 1.6.1.h.ii, Chapter 1, lead deposits in the surface of enamel (Zone A) may not be stable. Similarly, the lead levels in dentine immediately

adjacent to the pulp (Zone D) may be influenced by short-term variations in pulpal blood-lead levels. From this it appears that lead levels in enamel and dentine adjacent to the DEJ (Zones B and C respectively) are the more suitable zones for the determination of temporal variations in lead exposure. This issue is addressed in Chapter 4.

Table 3.1.

Authors	Sample	Method	Key findings
Grobler <i>et al.</i> (2000)	48 primary teeth from 48 school children (21 males, mean age 9.2 years; 27 females, mean age 8.5 years). Blood collected by finger-prick.	Teeth embedded in resin and 70µm thick sections made using slow-speed saw. Pieces of enamel, dentine and circumpulpal dentine were dissected, ground and analysed by graphite furnace atomic absorption spectrometry (GF-AAS).	• Highest lead levels observed in 'circumpulpal' dentine with lowest lead levels in enamel. • Mean lead levels: Blood 0.031± 0.022 µg/g, enamel 0.33±0.265 µg/g, dentine 2.23±1.32 µg/g, 'circumpulpal' dentine 9.88±8.291 µg/g.
Purchase and Fergusson (1986)	8 extracted permanent teeth and naturally exfoliated primary teeth from 30 children*.	Teeth were sectioned longitudinally. Segments of enamel, dentine and circumpulpal dentine were removed and analysed by GF-AAS. For some teeth, surface enamel was etched using 1.6 M HCl and subsequently washed. The resulting solution was analysed by GF-AAS.	• Highest lead levels observed in circumpulpal dentine, followed by root dentine, coronal dentine and bulk enamel. • Mean lead levels: circumpulpal dentine 99.0 µg/g, root dentine 24.2 µg/g, coronal dentine 20.7 µg/g, enamel 7.9 µg/g.** • Lead levels in surface enamel ranged from 230-2400 µg/g and fell rapidly in the first 1-2 µm followed by a gradual decline up to a depth of 40 µm from the surface of enamel.
Budd <i>et al.</i> (1998)	3 'modern' teeth (1 permanent, 2 primary teeth). 6 archaeological permanent teeth.	Teeth embedded in resin and sectioned longitudinally. Spatial distribution of lead determined using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS).	• High lead concentration in outer 30 µm of enamel (modern teeth: 67.31-141.56 µg/g, archaeological teeth: 6.00-123.78 µg/g). • Low lead concentrations in 'core enamel' (modern teeth: 0.16-0.41 µg/g, archaeological teeth: 0.04-1.64 µg/g). • Mean dentine lead concentrations generally higher than core enamel lead concentrations (modern teeth: 0.22-1.00 µg/g, archaeological teeth: 0.06-4.77 µg/g).

Petersson <i>et al.</i> (1978)	2 permanent premolars, 1 deciduous canine and 1 primary molar.	Teeth were sectioned longitudinally and spatial distribution of lead recorded using secondary ion mass spectrometry.	• Lead concentrations ranged from less than 10 µg/g to 2000 µg/g. • Highest lead concentrations observed in outer enamel and 'innermost' dentine close to the pulp.
Kang <i>et al.</i> (2004)	1 exfoliated primary maxillary central incisor.	Tooth embedded in resin and sectioned longitudinally. Lead concentrations analysed by LA-ICP-MS.	• High lead concentrations observed near surface enamel and dentine adjacent to the pulp. • Lead levels in different regions: from DEJ to surface enamel: 2.22±14.3 µg/g, from DEJ to neonatal line in enamel: 0.40±0.87 µg/g, DEJ: 0.26±0.69 µg/g and 0.39±0.57µg/g, dentine near pulp: 12.8±3.9 µg/g and 15.4±6.2 µg/g.

* Exact sample size was not explicitly stated and additional teeth were analysed for lead levels in root dentine.

** Standard deviations not reported.

Table 3.1. Key studies reporting the spatial distribution of lead in human teeth.

3.4.5 Limitations, Future Directions and Conclusion

The current study is restricted by the small number of teeth analysed which is a consequence of the high cost of accessing the NMP. The dimensions of the different zones of lead distribution showed a degree of variability between samples and between different sites in the same sample. This may be attributed to the minor differences in the plane of sectioning of different samples and also due to the normal variations in the morphology of teeth between individual rats.

In conclusion, the results of this study have provided a detailed high-resolution map of the spatial distribution of lead in the enamel and coronal dentine of mandibular first molar teeth of Wistar rats administered a controlled dose of lead. A specific reproducible pattern of lead distribution has been shown to exist with key differences in the levels of lead in different regions of enamel and dentine. The reproducibility of the four zones in our sample of nine teeth suggest that it will be possible to focus on discrete regions in teeth where lead concentrations are best able to reflect the lead exposure experienced by an individual during the period of tooth development. These data have provided valuable information towards the development of an animal model as detailed in Chapter 4.

Results of this study have highlighted a need for additional research in two areas. Firstly, the intracellular uptake of lead during tooth development needs to be explored to further elucidate the mechanisms behind the observed differences in the distribution of lead in enamel and coronal dentine. Secondly, the spatial distribution of lead in the roots of teeth remains unexplored. Lead concentrations in radicular dental hard tissues may be useful in determining the history of lead exposure in grossly decayed teeth or teeth with damaged crowns. Work in subsequent chapters addresses these issues.

CHAPTER 4

The Spatial Distribution of Lead in Teeth as a Biomarker for Prenatal and Neonatal Lead Exposure in a Wistar Rat Model

4.1 Introduction

As discussed in Section 1.5, Chapter 1, childhood exposure to lead has severe health effects. Current biomonitoring approaches to long-term lead exposure rely primarily on blood sampling, which cannot provide key information regarding chronic exposure due to the short half-life of lead in blood (WHO, 1995). Additionally, lead levels in blood are easily influenced by the mobilisation of lead from the skeletal compartment during periods of bone remodelling, making them unreliable for estimating exposure and body burden during these periods.

In light of the shortcomings associated with blood-lead sampling, there is a need for alternative approaches to biological monitoring of long-term environmental lead exposure. Because lead accumulates preferentially in calcified tissues, including primary teeth, with a half-life of the order of 10 - 20 years (Skerfving *et al.*, 1993; Tsuchiya, 1979), tooth-lead levels have the potential to significantly improve biomonitoring of environmental lead exposure. Lead deposits in teeth are relatively stable and exchange with the environment is very limited (Gulson and Gillings, 1997). Furthermore, formation of the crowns of primary teeth commences prenatally and proceeds in a well-defined incremental pattern, making naturally exfoliated primary teeth a potentially valuable marker for prenatal and neonatal lead exposure.

Primary teeth have been widely used to estimate cumulative lead exposure and in some studies comparisons between industrial and rural populations have revealed significant differences in tooth-lead levels (Tables 1.1 and 1.2, Chapter 1). In addition, tooth-lead levels correlate with decreased IQ and cognitive function (Bellinger *et al.*, 1994; Needleman *et al.*, 1990). The use of teeth in such studies, however, has been limited to the analysis of lead in whole teeth or fragments of enamel and dentine. Such an approach is problematic because lead is not distributed homogeneously in enamel and dentine. Work described in Chapter 3 has shown that the surface of enamel and the dentine immediately

adjacent to the pulp have large deposits of lead. It is also known that the surface of enamel exchanges calcium, phosphorus and fluoride ions with saliva (McIntyre, 1998) and, therefore, lead deposits in this region of the tooth may not be stable *in vivo*. A similar exchange of lead with blood may account for the high lead concentrations seen in dentine adjacent to the pulp. From this information it appears lead levels in enamel and dentine adjacent to the DEJ offer a more appropriate region of analysis.

Lead levels in dental hard tissues have not been used to obtain specific information of prenatal and neonatal exposure. In addition, the relationship between lead levels in teeth and key organs of the body has not been quantified. The aims of the work described in this chapter were to determine the relationship between lead levels in teeth and lead levels in blood, bone, kidney, liver and brain, in an experimental rodent model. In addition, this chapter describes work that compares the distribution of lead along the extension zones in enamel and coronal dentine of rodent teeth with the pattern and intensity of lead exposure.

4.2 Materials and Methods

4.2.1 Lead Administration and Harvesting of Tissues from Wistar Rats

4.2.1.a Mating and Animal Husbandry

Ten female Wistar rats, aged 12-14 weeks, of normal weight (150-270 g) were divided into five groups of two, numbered Groups 1 to 5, and mated with male Wistar rats, aged 12–14 weeks, of normal weight (200-400 g) (Pass and Freeth, 1993). Mating and animal husbandry as well as housing and environment were identical to that described in Sections 3.2.1 and 3.2.2, Chapter 3. The experimental protocol used had the approval of

the Animal Ethics Committee, Westmead Hospital, NSW, Australia (Ethics clearance No.124.06).

4.2.1.b *Diet, Water and Lead Administration*

The animals were given ultrafiltered deionised water and fed a standard rodent diet (AIN93G, Glen Forrest Stockfeeders, Western Australia) *ad libitum* which contained less than 0.006 µg/g lead (Appendix A.4). Maternal rat exposure to lead nitrate (Asia Pacific Speciality Chemicals, NSW Australia) was via drinking water. The pups were housed with their mothers and were fed only by lactation and received no drinking water directly.

Details of the dose and timing of lead nitrate administration are given in Table 4.1. Group 1 served as a control and was not given any lead nitrate. Group 2 received lead only during the prenatal period, while Groups 3 and 4 were given lead in an increasing and decreasing pattern respectively. Group 5, comprised the animals used in Chapter 3 which were given a fixed daily dose of lead during the experimental periods. These different patterns of lead exposure were used to mimic possible lead exposure events in a typical human environment. Drinking water bottles containing the ultrafiltered deionised water or lead nitrate were changed daily. The volume of water consumed was measured to determine the lead intake by the mother in each cage.

Exposure to lead nitrate occurred over three time periods, which included the prenatal period and two postnatal periods. The prenatal period of exposure started approximately five days before the birth of the pups, anticipating the commencement of crown formation in the first mandibular molar teeth approximately two days before birth (Berkovitz *et al.*, 1992b). The margin of approximately three days was given to allow transplacental transfer of lead from the mother to the developing foetus. The postnatal period of exposure extended to 11 days after the birth of the pups, as the crown of the first mandibular molar tooth is completely formed at that time (Hoffman and Schour, 1940). The long postnatal

period of tooth development provided the opportunity to administer two varying doses of lead nitrate. The postnatal period of lead exposure was divided into two time periods that extended from day-1 to day-6 and from day-7 to day-11 respectively.

All the experimental animals were culled by carbon dioxide asphyxiation four days after the second postnatal period, on day-15. The designated doses of lead nitrate are not considered toxic and are substantially lower than the established fatal dose (Holck, 1962). Previous studies that have used similar or higher doses did not report any observable signs of acute toxicity (Dearth *et al.*, 2002; Roels *et al.*, 1977).

Time period	Concentration of lead (mg/L)				
	Group 1	Group 2	Group 3	Group 4	Group 5
	No lead	Prenatal dose	Increasing dose	Decreasing dose	Steady dose
Prenatal					
Day –5 to birth	0.00	40.00	10.00	40.00	40.00
Postnatal					
Day 1 to day 6	0.00	0.00	20.00	20.00	40.00
Day 7 to day 11	0.00	0.00	40.00	10.00	40.00

Table 4.1. Concentration of lead nitrate in maternal drinking water and time periods of exposure for the five experimental groups of Wistar rats. Lead was administered *via* drinking water in one of five patterns: no lead (Group 1), lead only during the prenatal period (Group 2), an increasing dose (Group 3), a decreasing dose (Group 4) and a steady dose (Group 5). The doses were divided into three experimental time periods, one prenatal and two postnatal as indicated.

4.2.1.c *Harvesting of Blood, Teeth, Bone and Organs*

Ten rat pups were selected randomly from each of the five groups. Blood, organ, bone and tooth samples were harvested at appropriate time points from these animals as detailed below.

Blood was taken from the maternal rats and pups by cardiac puncture immediately after euthanasia. The skin of the chest was cleaned using ultrafiltered deionised water and detergent (7X, ICN Biomedicals Inc., USA), and a sterile 2 mL syringe with a 25 gauge needle (Precision Glide, Becton Dickinson, Singapore) was used to withdraw blood from the heart. The collected blood was stored in sterile metal-free 2 mL tubes (EZ Micro Test Tubes, Bio-Rad Laboratories, USA) at -20°C.

Following blood collection, the unerupted left and right mandibular first molar teeth, the liver, right kidney, brain and the right femoral bone were removed from each rat pup, using clean, sterile surgical instruments in a biological safety cabinet (Email Westinghouse Ltd. NSW, Australia) to avoid any contamination from the laboratory environment. Organs were stored in sterile metal-free tubes (EZ Micro Test Tubes, Bio-Rad Laboratories, USA) at -20°C. Analysis was restricted to first mandibular molar teeth, as these are the only molar teeth in Wistar rats that form enamel and dentine prenatally and can therefore be used to assess prenatal exposure to lead.

Molars were preferred over incisors in the present study because of the greater anatomical similarity between rodent and human molar teeth. Rat incisors lack enamel on the lingual aspect and do not have a root (Berkovitz *et al.*, 1992b). In addition, an iron pigment is present on the surface of rat incisors (Berkovitz *et al.*, 1992b), which may potentially affect the lead deposition in the enamel. Finally, the orientation of enamel rods in rat molar enamel is similar to that in humans, which is not the case in rat incisor enamel (Skobe and Stern, 1978).

4.2.2 Inductively Coupled Plasma Mass Spectrometry Analysis

ICP-MS was used to measure total lead levels in organs, bone and left mandibular molar teeth. This section provides the details of the ICP-MS method used in the present study. The principles of ICP-MS are described in Section 2.2, Chapter 2.

4.2.2.a Calibration of the ICP-MS Instrument used in this Study

In the present study, the ICP-MS instrument used was a Varian UltraMass-700 (Varian, Australia), located at the Division of Analytical Laboratories, Institute of Clinical Pathology and Medical Research, Lidcombe, NSW, Australia. The first step in calibration of this instrument involved the use of calibration standards ranging in lead concentration from 2 ppb to 500 ppb, made from a stock standard solution of 10 ppm of lead (Lot no. B1075023, Merck, Australia). Five standards of varying concentrations were analysed and the results plotted to produce a calibration graph. Figure 4.1 shows the calibration graph from one such run. The calibration curve was verified by analysing a certified reference material or quality control (QC) material that contained 80 ppb of lead (Merck, Australia). $^{209}\text{Bismuth}$ (^{209}Bi) was used as an internal standard to correct for any drift in the observed lead counts. For all calculations the readings obtained for $^{208}\text{lead}$ (^{208}Pb) were used as this is the most abundant lead isotope and its use is commonly accepted in other studies measuring lead in biological samples (Boer *et al.*, 2004). Method blanks and matrix matched standards were employed to ensure the quality of the ICP-MS data. The details of these procedures are described in subsequent sections (Sections 4.2.2.c, d and e). The instrument parameters used during the ICP-MS analysis are shown in Appendix A.5.

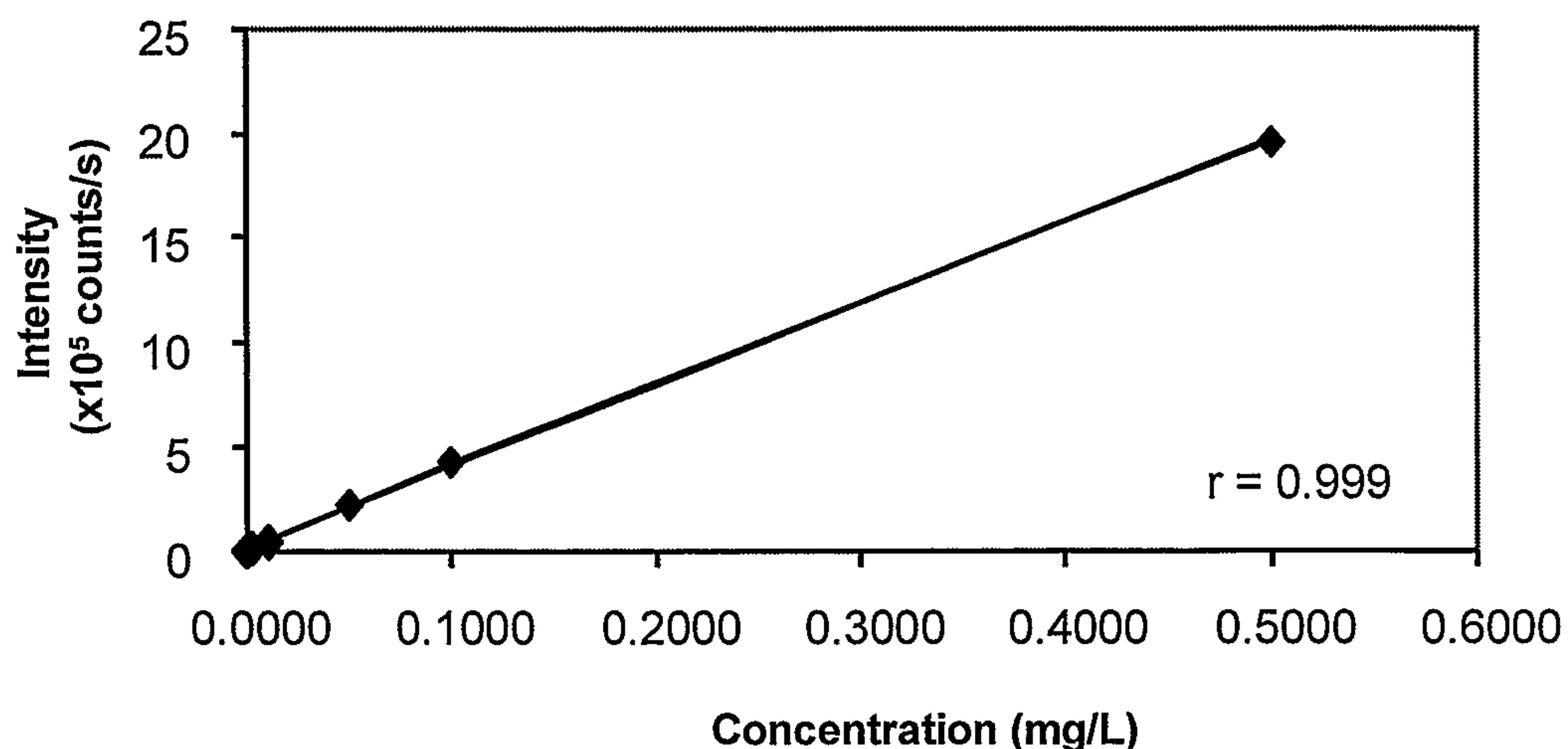


Figure 4.1. Typical calibration graph of the inductively coupled plasma mass spectrometer used in the present study. Five standards varying in concentration from 2 to 500 ppb of lead were analysed. The correlation coefficient (r) for the concentration and intensity is shown.

4.2.2.b Detection Limits of ICP-MS

Instrument detection limits (IDL) for ICP-MS were determined by analysing the calibration standards seven times. A calibration curve based on the raw counts was generated and the standard deviation at each point was calculated. At a 99.8% confidence level, the IDL is calculated by the equation:

$$\text{IDL} = 3 \times \sigma \quad (\text{Standards Australia, 2004})$$

where σ is the standard deviation of the lowest data point on the calibration curve.

Practical quantitation limits (PQL) are calculated by the equation:

$$\text{PQL} = 10 \times \sigma \quad (\text{Standards Australia, 2004})$$

Method detection limits (MDL) that incorporate the dilution factor and any spectral interferences and drift (Standards Australia, 2004), were calculated using matrix matched standard reference materials. In the present study, the MDL for lead varied from 0.005 to 0.02 µg/mL, which are similar to the limits for lead in blood and urine (0.001-0.002 µg/mL) reported by Boer *et al.* (2004).

4.2.2.c *Method for Tooth-Lead Analysis by ICP-MS*

Whole left mandibular first molar teeth were cleaned mechanically under a dissecting microscope to remove any visible soft tissue. Samples were then placed in an ultrasonic bath of 5% v/v hydrogen peroxide for five min to further remove any adherent soft tissue. The samples were then treated in an ultrasonic bath of ultrafiltered deionised water for 10 min and dried at 60°C for 2 hr. The samples were weighed using a calibrated digital balance and placed in a volumetric flask. Two mL double-distilled ultrafiltered 65% v/v nitric acid was added to the flasks and the samples were left overnight for digestion. Sample solutions were then heated at 60°C for up to 2 hr to ensure complete digestion and were diluted to 10 mL with ultrafiltered deionised water. The sample solutions were finally homogenised in an ultrasonic bath for 10 min and analysed by ICP-MS.

NIST SRM-1486 (Bone Meal) (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA) was used as the external standard in each analytical run. One gram of the SRM-1486 was dissolved in 2 mL of double-distilled ultrapure nitric acid and processed as for teeth described above. Two to three standard solutions were analysed in each analytical run.

4.2.2.d *Method for Blood-Lead Analysis by ICP-MS*

In the present study, when establishing the method for the preparation of blood samples, several issues were considered, including the method for dilution and digestion, use of

whole blood or plasma, and the use of matrix matched standards or standard addition (spiking) to control for any matrix effects.

Several methods for sample dilution have been reported in the literature. Apostoli (2002), Boer *et al.* (2004) and Rodushkin *et al.* (1999 and 2000) used a simple method of diluting blood samples in nitric acid. Other researchers have, in addition to nitric acid, used ethylene diamine tetra acetic acid (EDTA) and/or Triton X-100, a non-ionic detergent, to facilitate dilution (Barany *et al.*, 2002; Smith *et al.*, 1998; Schultz *et al.*, 1996). In the present study, however, the simple approach of dilution in nitric acid only was used as this minimised the potential for contamination from the addition of other chemical reagents. Nitric acid is preferred over other mineral acids in the analysis of biological samples as it introduces less interference (Evans and Giglio, 1993).

Whole blood samples were weighed using a calibrated digital balance before addition of 2 mL double distilled ultrapure 65% v/v nitric acid (Rhone Poulenc, France). Sample solutions were subsequently microwave-digested in Teflon containers (Ethos, Milestone Microwave Laboratory Systems, USA) for 10 min at 180°C. Microwave digestion is preferred to digestion at room temperature because it simplifies the sample composition by reducing the amount of organic matter in the sample solution and also reduces possible matrix effects and spectral interferences (Boer *et al.*, 2004). Samples were then diluted to 10mL with ultrafiltered, deionised water.

It is known that more than 95% of the lead in blood is contained in the red blood cells, but it is the lead in plasma that exchanges with the different biological compartments (Baloh, 1974). It is also known that lead concentrations in whole blood show strong positive correlations with plasma-lead levels (Apostoli, 2002). For the present study, it was decided that lead levels in whole blood would be analysed as this allowed for the measurement of the total lead content in all fractions of blood and also facilitated easier

detection of lead by making a larger concentration of lead available for measurement by ICP-MS.

Method blanks were prepared by diluting 2 mL of double-distilled ultrapure 65% v/v nitric acid with ultrafiltered, deionised water to a final volume of 10 mL. Method blanks were analysed in every run with five blanks for every 15-20 samples analysed. Matrix matched survey validated standard reference materials for blood, QC 410448 and QC02407 (Quality Control Technologies Pty. Ltd. Australia) were analysed in each run. The standards were prepared in an identical manner to the blood samples.

4.2.2.e *Method for Organ Lead Analysis by ICP-MS*

In the present study, whole liver, kidney and brain samples were removed by dissection immediately after the animals were culled. Organs were stored in sterile metal-free 2 mL tubes (EZ Micro Test Tubes, Bio-Rad Laboratories, USA) at -20°C. At the time of analysis, the organs were weighed individually, transferred to Teflon beakers and 2 mL each of double-distilled ultrapure 65% v/v nitric acid and 30% v/v hydrogen peroxide (Biolab, Australia) was added. The mixture was subjected to microwave digestion for 10 min at 180°C and was subsequently diluted to 10 mL with ultrafiltered deionised water. Sample solutions were homogenised in an ultrasonic bath for 10 min and analysed by ICP-MS. To facilitate the digestion of organs, some researchers have advocated, in addition to nitric acid, the use of perchloric acid or hydrochloric acid (Ek *et al.*, 2004; Takahashi *et al.*, 2000). This was not considered necessary in the present study as complete digestion was achieved by microwaving.

Method blanks were prepared by diluting 2 mL double-distilled ultrapure 65% v/v nitric acid and 2 mL 30% v/v hydrogen peroxide with ultrafiltered deionised water to final volumes of 10 mL. Method blanks were analysed in every run with approximately five blanks for every 15-20 samples analysed. The standard reference material NIST SRM-

1577b (Bovine Liver) obtained from NIST, Gaithersburg, MD, USA, was used as an external standard. This particular standard has been used as a reference material in other studies measuring trace element concentrations in liver, kidney and brain samples (Takahashi *et al.*, 2000; Gelinas *et al.*, 1997; Saito and Saito, 1996). Two mL each of double-distilled ultrapure nitric acid and hydrogen peroxide were added to 1 g of SRM-1577b. The solution was processed as for organs describe above. Two standard solutions were placed in each analytical run.

4.2.2.f *Method for Bone Lead Analysis by ICP-MS*

The analysis of the bone samples (right femurs) was essentially identical to the tooth-lead analysis that has been described in Section 4.2.2.c. Soft tissue was removed mechanically from the external surface of the bone sample. The samples were placed in an ultrasonic bath of 5% v/v hydrogen peroxide for 5 min followed by cleaning in an ultrasonic bath of ultrafiltered deionised water for 10 min. The samples were dried at 60°C for 2 hr and dissolved in 2 mL of double-distilled ultrapure 65% v/v nitric acid. The digest was subsequently diluted to 10 mL with ultrafiltered deionised water and analysed by ICP-MS. NIST SRM-1486 (Bone Meal) was used as the external reference standard for bone-lead analysis.

4.2.3 Preparation of Sample Teeth for NMP Analysis

The preparation of sample teeth for NMP analysis was done as described in Section 3.2.4, Chapter 3.

4.2.4 Nuclear Microprobe Analysis of Sample Teeth

The spatial distribution of lead in enamel and coronal dentine was determined using NMP as described in Section 3.2.5, Chapter 3. Due to the cost of NMP analysis only 39 tooth samples were analysed.

4.2.5 Validation of NMP data with LA-ICP-MS

To validate the results obtained using the NMP, two first mandibular molars from Group 3 (increasing dose) and one first mandibular molar from Group 1 (control group) were analysed using LA-ICP-MS. The procedure for LA-ICP-MS is described in detail in Chapter 5 where it is the main analytical method used. The procedure for analysis of rodent teeth was identical to that described in Section 5.2.5, Chapter 5.

4.2.6 Identification of Prenatal and Postnatal Regions in Enamel and Dentine

The procedure used to identify prenatally and postnatally formed dentine and enamel in the first mandibular molar teeth of the Wistar rat pups is described in this section. Relevant background information on enamel and dentine formation in Wistar rat first molar teeth is also provided.

The onset of mineralisation in the first mandibular molar of a Wistar rat commences *in utero*, on day 20-21 of gestation (Berkovitz *et al.*, 1992b; Hoffman and Schour, 1940). In a newborn rat both dentine and enamel are present (Hoffman and Schour, 1940; Berkovitz *et al.*, 1992b). However, since dentine apposition precedes enamel formation, a smaller amount of enamel is likely to be present at birth. Because this finding relies on a normal gestation period of 22-23 days (Baker, 1979), in the present study any litter that was delivered before 22 days of gestation was excluded. This was considered necessary to ensure that prenatal onset of mineralisation had taken place. In the present study, two litters were excluded in this way.

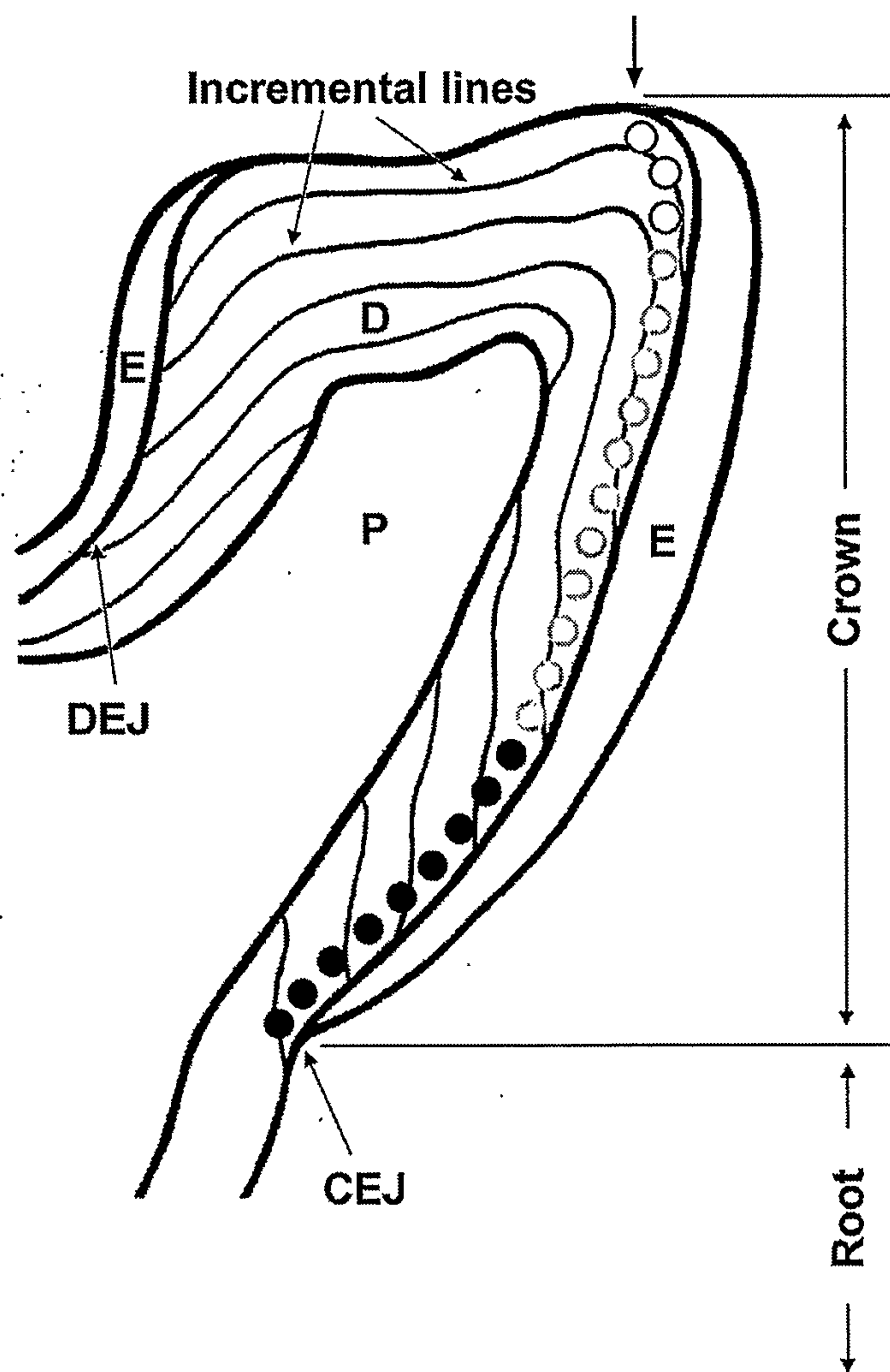
A diagram of the lingual aspect of the mesiolingual cusp of a mandibular first molar tooth of a Wistar rat is shown in Figure 4.2. Formation of the crown commences approximately two days before birth, at the DEJ on the cusp tip. Enamel and dentine matrix deposition

proceeds towards the root of the tooth and reaches the cemento-enamel (CEJ) junction by day-11 (Hoffman and Schour, 1940). As in the case of human teeth (Figures 1.1A and B, Section 1.8.2, Chapter 1), the enamel and dentine apposition in rats may be visualised as extension towards the cervical end of the tooth or as an incremental deposition away from the DEJ. The incremental formation of enamel and dentine results in the formation of incremental lines akin to growth rings (Nanci, 2003a,b). These lines are shown in dentine only in Figure 4.2, although similar lines are also seen in enamel.

The rate of dentine deposition shows only minor variations during the first two weeks of life in rats (Hoffman and Schour, 1940). Similar results are reported in the enamel of human permanent mandibular molars, where only small variations are observed in enamel extension rates, with increasing distance from the cusp tip, while extension rates in primary human teeth remain fairly constant (Shellis, 1984).

In the present study, the distance from the cusp tip, where tooth formation begins, to the CEJ, where crown formation terminates, was measured along the DEJ of the crown. This distance was divided into one prenatal and two postnatal zones based on the mean times of prenatal and postnatal enamel and dentine formation. A mean time interval of two days was taken for prenatal tooth formation. Time intervals of five and six days, respectively, were used to demarcate the two postnatal zones. Mean lead concentrations in the enamel and dentine adjacent to the DEJ in these zones, as shown in Figure 4.2, were compared with the concentration of lead nitrate administered through drinking water.

Figure 4.2. Diagram of the sites of analysis on the lingual aspect of the mesio-lingual cusp of a mandibular first molar tooth, sectioned vertically in the mesio-distal plane, of a 15-day old Wistar rat. Enamel (E), dentine (D), pulp chamber (P), dentino-enamel junction (DEJ), cemento-enamel junction (CEJ) and the crown and root of the tooth are marked. Incremental growth lines are shown in dentine only, although similar lines exist in enamel. Dots in dentine indicate points of analysis of lead concentration in the prenatal and postnatal zones with the nuclear microprobe. A similar analysis was also conducted in the adjacent enamel.



- Prenatal period
- Postnatal period (day 1 to day 6)
- Postnatal period (day 7 to day 11)

4.2.7 Statistical Analysis of Data

4.2.7.a Analysis of Tooth-, Blood-, Bone- and Organ-Lead Data

Lead levels in whole left first mandibular molar teeth of the rat pups were correlated with lead levels in the bone, kidney, liver and brain respectively using Pearson's correlation analysis. In addition, the Bland-Altman test was used to establish a quantitative relationship between lead levels in teeth, and the various organs studied (Bland and Altman, 1999). For the Bland-Altman test, logarithmic transformations were applied when comparing lead concentrations in teeth with lead levels in kidney, liver and brain, because the difference in lead concentrations between teeth and the organs increased with increasing tooth-lead levels. The rationale behind this transformation is discussed in detail in Appendix A.6. The Kolmogorov-Smirnov test was used to confirm the normal distribution of lead concentration in teeth, bone and key organs using SPSS version 11.0 (SPSS Inc., USA). 95% confidence intervals were used to compare the mean lead levels in tissue samples between different groups, using Microsoft Excel 2000 (Microsoft Corporation, USA). Outliers were identified as any variable falling outside two standard deviations of the mean and were excluded from the analysis.

4.2.7.b Comparison of Spatial Distribution of Lead and Dose of Lead Administered

Lead concentrations in enamel and dentine as measured by the NMP were correlated with the pattern and intensity of lead exposure using Pearson's correlation coefficient analysis. Comparisons were made between the total amount of lead consumed by the mothers during each experimental period, and the lead levels in the teeth and blood of the pups.

4.3 Results

4.3.1 Health of Animals Exposed to Lead

Body weights of all animals were within the reported normal range (Pass and Freeth, 1993) and there were no significant differences in body weights between rat pups exposed to lead and those in the control group ($p>0.05$). Details of the weights of the adult female Wistar rats and the neonates are given in Appendix A.7. Two rat pups belonging to Group 2 appeared to be rejected by the mother and showed physical signs of distress in the first week after birth, including low body temperature, wrinkled skin and low body weight. These animals were immediately culled by carbon dioxide asphyxiation. A total of 39 Wistar rat pups were included in the study.

4.3.2 Analysis of Standard Reference Materials

Standard reference materials for blood (QC 410448 and QC 02407), organs (NIST SRM-1577b) as well as bone and teeth (NIST SRM-1486), were analysed in each ICP-MS run. Percentage recovery of lead from these standards was within 7% of the expected value for blood and organs, and 5% of the expected value for teeth (Table 4.2), confirming validity of ICP-MS analysis.

Table 4.2. Analysis of standard reference materials by inductively coupled plasma mass spectrometry (ICP-MS). To confirm the validity of the ICP-MS results, standard reference materials for blood, organs, bone and teeth were analysed. Two standard reference materials for blood, QC410448 (certified concentration of lead = 0.052 ± 0.009 $\mu\text{g/g}$) and QC02407 (certified concentration of lead = 0.412 ± 0.032 $\mu\text{g/g}$), an organ reference material, SRM-1577b (certified concentration of lead = 0.129 ± 0.004 $\mu\text{g/g}$) and a tooth and bone reference material, SRM-1486 (certified concentration of lead = 1.335 ± 0.014 $\mu\text{g/g}$) were analysed. The results from multiple runs showed that measured lead concentrations were within 7% of the expected value for blood and organs and within 6% of the expected value for teeth and bone thus confirming the validity of results obtained from ICP-MS.

Reference Materials									
Run	QC410448		QC02407		SRM-1577b		SRM-1486		
	Lead concentration (µg/g)	Percentage recovery (%)	Lead concentration (µg/g)	Percentage recovery (%)	Lead concentration (µg/g)	Percentage recovery (%)	Lead concentration (µg/g)	Percentage recovery (%)	Percentage recovery (%)
1	0.05	96.15	0.43	106.06	0.13	103.1	1.28	96.2	
2	0.04	94.23	0.42	103.64	0.11	92.2	1.29	96.9	
3	0.05	96.15	0.41	100.72	0.13	107.7	1.40	105.4	
4	0.05	98.07	0.42	102.18	0.13	101.5	1.30	98.0	
5	0.05	98.07	0.41	100.48	0.12	96.1	1.38	103.9	
6	-	-	-	-	0.12	94.5	1.30	97.5	
7	-	-	-	-	0.12	96.8	1.32	99.3	
8	-	-	-	-	0.12	93.7	1.39	104.7	
9	-	-	-	-	0.13	100.7	1.35	101.3	
10	-	-	-	-	0.12	98.4	1.26	94.9	
11	-	-	-	-	-	-	1.39	104.4	

4.3.3 Lead Levels in Teeth, Blood and Organs

Table 4.3A gives the total amount of lead consumed by maternal rats in each group and their mean blood-lead levels measured at the termination of the experiment. Group 5 had the highest mean maternal blood-lead levels, followed by Groups 3, 4 and 2 in decreasing order of mean blood-lead concentrations. This pattern reflects the amount of lead consumed by the respective maternal rats.

Table 4.3B shows mean lead levels in whole teeth, blood, bone and key organs (liver, kidney and brain) of the Wistar rat pups in Groups 1-5, measured by ICP-MS. Notably, teeth and bone had the highest concentrations of lead, while brain samples showed the lowest lead levels in every group.

Mean tooth-lead levels of the pups were significantly different between all five groups ($p<0.05$) and showed the same pattern as the mean maternal blood-lead levels. Pups in Group 5 had the highest mean tooth-lead levels, followed by pups in Groups 3, 4 and 2 in decreasing order of mean tooth-lead concentrations. Additionally, the mean tooth-lead levels were generally ranked in the same order as the mean lead levels in the key organs in the exposure groups, with the one exception of the lead levels in livers of the pups in Group 3 which were lower than those of Group 2. Mean blood-lead levels of the pups also mirrored the maternal mean blood-lead levels, but significant differences between blood-lead levels of the pups were only observed between Groups 5 & 4, 5 & 2 and 2 & 3 ($p<0.05$).

The Pearson's correlation coefficient analysis of lead levels in the kidney, liver, brain and bone with lead concentrations in teeth and blood are given in Table 4.4A. When compared with blood-lead, tooth-lead levels correlated more strongly with lead levels in each of the organs studied and this difference was statistically significant for liver, brain and bone ($p<0.05$). The Bland-Altman analysis further allowed a quantitative relationship to be

established between the tooth-lead levels and lead concentrations in the different organs studied (Table 4.4B). With the exception of bone, lead levels in teeth were substantially higher than the lead concentrations of the other organs.

Table 4.3. Dose of administered lead and lead levels in maternal blood as well as teeth, blood, bone and organs of pups. Lead levels of blood from the two maternal rats in each group at the time of culling reflected the total dose consumed (A). Higher concentrations of lead were found in the teeth and bone of pups compared with the blood and organs of pups (B) and the difference in tooth-lead levels between groups was significant for all groups ($p<0.05$). N=number of rat pups in each group; n=number of samples analysed, excluding outliers.

A

	Group 1	Group 2	Group 3	Group 4	Group 5
Total Maternal Dose (mg)	0.00	1.68 ± 0.28	12.50 ± 1.83	7.41 ± 0.41	15.79 ± 1.76
Maternal Blood-Lead (µg/g)	0.01 ± 0.02	0.19 ± 0.04	0.36 ± 0.03	0.32 ± 0.07	0.61 ± 0.08

B

	Group 1 N = 11	Group 2 N = 5	Group 3 N = 7	Group 4 N = 7	Group 5 N = 9
Teeth (n = 37)	1.29 ± 1.52	11.68 ± 2.74	27.11 ± 3.41	20.67 ± 4.04	50.10 ± 7.19
Blood (n = 34)	0.00 ± 0.00	0.26 ± 0.08	0.78 ± 0.44	0.34 ± 0.14	0.71 ± 0.15
Kidney (n = 38)	0.02 ± 0.08	0.45 ± 0.10	1.60 ± 0.56	1.30 ± 0.31	2.41 ± 0.22
Liver (n = 37)	0.02 ± 0.07	0.97 ± 0.22	0.67 ± 0.07	1.23 ± 0.19	2.76 ± 0.25
Brain (n = 34)	0.00 ± 0.00	0.08 ± 0.02	0.21 ± 0.09	0.18 ± 0.04	0.43 ± 0.03
Bone (n = 38)	0.64 ± 0.20	21.40 ± 4.78	24.89 ± 2.30	21.44 ± 4.79	55.88 ± 10.23

Table 4.4. Statistical analysis of tooth-, blood-, bone- and organ-lead data for rat pups in all groups. Lead concentrations in teeth and blood, were compared with lead concentrations in kidney, liver, brain and bone samples using Pearson's correlation coefficient analysis (A). Tooth-lead levels correlated more strongly with organs and bone-lead levels than blood-lead levels indicating that, in the present model, tooth-lead levels were a better predictor of body burden than blood-lead levels. The Bland-Altman analysis of the tooth-lead and organ-lead concentrations of the Wistar rat pups from the five experimental groups (B) indicated that lead-levels in teeth were up to 79.6 times higher than lead levels in organs while bone-lead levels were more similar to tooth-lead levels.

*For the Bland-Altman analysis, logarithmic transformations were applied when comparing lead concentrations in teeth, kidney, liver and brain. Consequently, results are reported as ratios of lead concentration in teeth and the respective organ. For comparisons between tooth-lead and bone-lead levels, logarithmic transformations were not required and results are reported as difference in mean lead concentration ($\mu\text{g/g}$) (Bland and Altman, 1999). Details of the Bland-Altman analysis are presented in Appendix A.6.

A

Organ	Tooth-lead	Blood-lead	<i>p</i> value
Kidney (n = 38)	0.82	0.73	0.35
Liver (n = 37)	0.90	0.38	0.001
Brain (n = 34)	0.91	0.60	0.001
Bone (n = 38)	0.89	0.56	0.001

B

Tooth-lead vs Organ-lead	Mean	95% Confidence Interval
Tooth-lead : Kidney-lead	14.60	11.81, 18.04
Tooth-lead : Liver-lead	22.81	18.36, 28.34
Tooth-lead : Brain-lead	79.60	63.82, 99.30
Tooth-lead minus Bone-lead (µg/g)*	-2.25	-5.40, 0.88