

**DISINFECTION BY-PRODUCTS IN
DRINKING WATER AND
GENOTOXIC CHANGES IN
URINARY BLADDER EPITHELIAL
CELLS**

Geethanjali Piyawadani Ranmuthugala

A thesis submitted for the degree of Doctor of Philosophy

The National Centre for Epidemiology and Population Health
The Australian National University

January 2001

This is to confirm that I have made the following contributions to the conduct of the study presented in this thesis. The works was undertaken through the National Centre for Epidemiology and Population Health of the Australian National University. Contributions made by external individuals or organisations have been acknowledged in the methods chapter and in the acknowledgements of this thesis.

Geethanjali P. Ranmuthugala

Candidate's contributions to the conduct of the study

- Major role in re-designing the study
- Designed, planned and coordinated pilot studies
- Submitted amended ethics application
- Modified questionnaire
- Selection of study sample
- Planning recruitment of study subjects to study
- Planning and involvement in fieldwork
- Construction of data entry methods
- Data cleaning
- Data analysis

Acknowledgments

I wish to thank the following people for their contributions and assistance.

Professor Louis Pilotto, Dr Wayne Smith, and Professor Robert Douglas, for their unstinting support, supervision, guidance and encouragement over the last four years. With the optimistic attitude and enthusiasm shared by all, there was no way but forward with this research project. I would like to mention Professor Pilotto in particular for giving me the opportunity to undertake this research, and for getting me enthusiastic in water quality and health effect research.

Dr Keith Dear for his supervision and advise in matters relating to statistical analysis. Ms Robyn Attewell from INSTAT provided statistical advice prior to the arrival of Dr Dear and I would like to thank her for the foundation knowledge she provided, and for her encouragement and guidance.

Dr Alan Wade and Dale Weber from ACTEW, and Dr Titus Vimalasiri, Penny Laver, Carmel Boatwright, Ronn Hogg, Carl Magyar, Greg Ryan, and Steve Hure from Ecowise Environmental for their time, advice and assistance in undertaking this study. Dr Vimalasiri and Ms Laver, in particular, spent a great deal of time on the pilot studies, and water sampling and analysis aspects of the cohort study.

Professor Nina Titenko-Holland from the Division of Environmental Health Sciences, School of Public Health, Berkeley, USA, for her advice and assistance with this study. Professor Holland, in addition to training me in micronuclei scoring, provided me with advice in all matters dealing with the micronuclei assay. The micronuclei scoring for this study was undertaken by her team at Berkeley.

Professor Christopher Parish, Ms Erin O'Neil, Mr Goeffrey Osborne, and Ms Virginia McPhun from the John Curtin School of Medical Research (JCSMR, ANU) for their advice and assistance in laboratory work undertaken for this study. It was the initiative of Professor Parish and Ms O'Neil to test the suitability of flow cytometry to assess DNA damage in epithelial cells. I would also like to thank Professor Doe, Professor Board and Ms Jan Bateman of JCSMR for providing the lab space and equipment necessary for this study.

Mr Malcolm Mearns and staff at DATACOL for undertaking the recruitment, interviewing, data entry, and for their assistance in fieldwork. Mr Mearns contributed greatly towards the study by advising on questionnaire design and other practical aspects of research conduct, and was extremely generous with his time towards this project.

Mr Keith Hayes, Dr Bret Robinson, Dr Brenton Nicholson and other staff at SA Water for their invaluable advice and assistance in conducting the Adelaide component of this study.

Mr George Levay of Levay and Co. Environmental Services at the Ian Wark Research Institute for his assistance and advice in testing of water for organic halogens.

Mr Rob Howell of Jeir Creek Winery, Murrumbateman, for his time and assistance in the water packaging pilot study.

Study participants in Bungendore, Canberra and Adelaide for their contribution towards this research. The several research assistants and technical officers who worked diligently on weekends and after hours to obtain results for this study. Clinpath Laboratories (Adelaide) and Macquarie Pathology (Canberra) for blood collection and analysis.

Fellow students and all at NCEPH (too numerous to name) whose friendship and support made my time at NCEPH very special. I would like to specially thank the administrative and IT staff at NCEPH who were very helpful and supportive at all times. Ms Kaye Devlin, NCEPH's finance officer, spent a lot of time with me on budget issues and I learnt a great deal on budget planning and management from her. My colleague and office mate Dr Clare McGuinness needs special mention for putting up with a cluttered office space for over three years.

The Cooperative Research Centre for Water Quality and Treatment for granting me a scholarship towards PhD studies, for funding this research project, and for their assistance in implementing this research project.

My brother Devapriya Ranmuthugala for his encouragement. He has been a great role model to me. Siva Nalliah for being so understanding, and for his care and encouragement particularly towards the end of the program.

My son Ushantha for his love. With his birth during the PhD program I was faced with quite a challenge, but I knew at the end of a long day there was a big cheerful smile and warm hug awaiting me. My parents Janaki and Douglas Ranmuthugala for their love, encouragement, support and tolerance, not only during the last four years, but throughout my working and studying days. Without them, this thesis would not have been possible.

Abstract

There is much debate on the carcinogenic potential of disinfection by-products (DBP) in chlorinated water supplies. Until recently, epidemiological studies have been limited in their ability to examine accurately the risk of cancer with exposure to environmental carcinogens. This has largely been due to the long latency periods associated with cancer development, and the difficulties in accurately estimating chronic exposure. Although there is evidence, from predominantly case-control studies, of increased bladder cancer with exposure to chlorinated water supplies, the evidence is inconclusive.

In an attempt to determine the carcinogenic potential of trihalomethanes (THMs) in chlorinated water, this study utilises DNA damage to bladder cells, evident as micronuclei, as a pre-clinical outcome measure. Using a pre-clinical marker helps overcome some of the limitations associated with long latency periods. The study improves on previous studies by estimating exposure to DBP at an individual level, and takes into consideration ingestion, inhalation and dermal exposure.

A cohort study was undertaken in three Australian communities. The Bungendore (NSW) water supply was not chlorinated thereby providing a community unexposed to DBPs from chlorinated water. Canberra (ACT) and Adelaide (SA) had intermediate and relatively higher (but still within NHMRC guideline levels) of DBPs in the reticulation system. Trihalomethane levels in reticulated water (external dose) and in urine (internal dose) were used as exposure indices. As well, intake dose was computed by adjusting external dose for individual variations in ingestion and bathing. The primary outcome measure was the prevalence of micronuclei in bladder epithelial cells. A DNA index derived from flow cytometry was also used to estimate DNA damage in bladder cells. Associations between exposure and outcome were estimated using Poisson regression models, having identified and adjusted for interaction effects and confounders.

A total of 529 participants were eligible to participate, of which 348 (65.8%) completed all aspects of the study. Analysis was limited to the 228 participants

(65.53% of those who completed the study) who had slides suitable for micronuclei scoring. One hundred and forty three (63%) of the 228 participants were from the exposed communities, while 85 (37%) were from the unexposed community. This sample exceeded the estimated 50 per group required to detect a relative risk of 1.4, with a significance level of 0.05 and 80% power.

External dose for total THM for the two chlorinated (exposed) communities ranged from 37.75 to 157.25 $\mu\text{g/l}$. Intake dose estimated by fluid intake diary ranged from 2.9 to 469.5 $\mu\text{g/l}$, while a retrospective questionnaire estimated intake dose to range from 0 to 409.4 $\mu\text{g/l}$. Internal dose (urine levels) of total THM for the same two communities ranged from 0 to 6.82 $\mu\text{g/l}$. Adjusted risk estimate for DNA damage to bladder cells (using the micronuclei assay) when total THM was assessed by available dose was 1.0002 (0.997 to 1.003), by intake dose estimated by fluid intake diary was 1.0001 (0.998 to 1.002), by intake dose estimated by questionnaire was 1.001 (0.999 to 1.003), and by internal dose was 1.05 (0.89 to 1.24). Using DNA index from flow cytometry as the outcome measure also did not identify significant associations, except when exposure was assessed as available dose of total THM (RR=1.0042; 1.0003 to 1.0081).

The results suggest that THM levels are not significantly associated with DNA damage to bladder cell. This supports suggestions of THMs being non-genotoxic. Further work is required to assess the relationship between THM and the more mutagenic compounds, and to assess the carcinogenicity of the more mutagenic compounds at concentrations occurring in drinking water.

TABLE OF CONTENTS

| | |
|--|-----------|
| CHAPTER 1. INTRODUCTION | 1 |
| 1.1 Disinfection by-products..... | 1 |
| 1.2 Factors influencing DBP formation..... | 6 |
| 1.3 Safe levels for human consumption..... | 7 |
| 1.4 Significance of this study..... | 9 |
| 1.5 Objective of study..... | 10 |
| 1.6 Structure of thesis..... | 10 |
| CHAPTER 2. LITERATURE REVIEW | 11 |
| 2.1 Experimental and toxicological studies..... | 11 |
| 2.2 Epidemiological studies..... | 13 |
| 2.3 Dose response relationship..... | 17 |
| 2.4 What does this mean for Australia?..... | 19 |
| 2.5 The need for further studies..... | 20 |
| CHAPTER 3. BIOMARKERS, AND THE USE OF MICRONUCLEI AS BIOMARKERS OF GENOTOXICITY | 22 |
| 3.1 Biomarkers..... | 24 |
| 3.2 Advantages and disadvantages of using biomarkers..... | 27 |
| 3.3 Micronuclei..... | 30 |
| 3.4 Micronuclei, a biomarker of genotoxicity..... | 31 |
| CHAPTER 4. EVOLUTION OF PROJECT: PILOT STUDIES | 34 |
| 4.1 Pilot study 1: Packaging of drinking water..... | 35 |
| 4.2 Pilot study 2: Second attempt to package water..... | 46 |
| 4.3 RCT not feasible..... | 49 |
| CHAPTER 5. METHODS | 52 |
| 5.1 Selection of study groups within the three populations..... | 52 |
| 5.2 Sample size calculation..... | 56 |
| 5.3 Eligibility criteria..... | 56 |
| 5.4 Exposure measurement..... | 57 |
| 5.5 Sampling and analysis for DBP..... | 63 |
| 5.6 Outcome measure..... | 66 |
| 5.7 Sample collection for urine 2 and urine 3..... | 72 |
| 5.8 Questionnaire..... | 72 |
| 5.9 Respondent burden and study procedure..... | 73 |
| 5.10 Other testing..... | 75 |
| 5.11 Data entry and statistical analysis:..... | 76 |
| 5.12 Ethics approval..... | 81 |

| | |
|--|------------|
| CHAPTER 6. RESULTS | 82 |
| 6.1 Comparison of characteristics of participant with scorable slides and non-scorable slides | 83 |
| 6.2 Characteristics of participants with micronuclei score, by exposure status | 87 |
| 6.3 Exposure levels | 92 |
| 6.4 Outcome – Micronuclei frequency | 106 |
| 6.5 Association between exposure and outcome | 107 |
| 6.6 Association of potentially confounding variables with exposure and outcome..... | 107 |
| 6.7 Bladder cancer incidence in the three study communities | 108 |
| | |
| CHAPTER 7. ESTIMATION OF FLUID INTAKE BY DIARY RECORDS AND BY RETROSPECTIVE QUESTIONNAIRE | 110 |
| 7.1 Aim | 110 |
| 7.2 Methods | 110 |
| 7.3 Results..... | 114 |
| 7.4 Discussion | 121 |
| | |
| CHAPTER 8. RISK ASSESSMENT ANALYSIS | 127 |
| 8.1 Results part I: Identifying variables to be included in the final models for assessing relationships between exposure to THM and micronuclei frequency | 128 |
| 8.2 Results part II: Relative risks for the final models..... | 138 |
| | |
| CHAPTER 9. FLOW CYTOMETRY | 141 |
| 9.1 Characteristics of participants in the flow cytometry analysis..... | 142 |
| 9.2 DNA Index..... | 142 |
| 9.3 Association between THM and DNA index | 143 |
| | |
| CHAPTER 10. DISCUSSION | 151 |
| 10.1 Precision and validity of study..... | 152 |
| 10.2 Implications for the water industry | 157 |
| 10.3 Conclusion | 160 |
| | |
| APPENDICES | 161 |
| | |
| REFERENCES | 263 |

List of tables

| | |
|--|-----|
| Table 1-1. Australian drinking water guidelines and WHO recommendations for disinfection by-products. | 4 |
| Table 4-1 Meeting of Australian Food Standard Code in pilot study 1, where water was analysed and discarded on day one of opening..... | 42 |
| Table 4-2. Meeting of Australian Food Standard Code in pilot study 1, where all bottles were opened on day 1 and retained for analysis intermittently | 43 |
| Table 4-3. Evaluation of UV unit at various flow rates | 45 |
| Table 4-4. Meeting of Australian standards for packaged water by number of bottles | 49 |
| Table 5-1. Interaction terms for exposure variables..... | 78 |
| Table 6-1. Study response rate..... | 82 |
| Table 6-2. Mean age of participants, by slide scorability | 83 |
| Table 6-3. Smoking status of participants, by slide scorability | 84 |
| Table 6-4. Proportion of participants completing high school, by slide scorability | 85 |
| Table 6-5. Highest level of post-secondary school education, by slide scorability | 85 |
| Table 6-6. Available dose and fluid intake, by slide scorability | 86 |
| Table 6-7. Proportion of smokers by region of residence | 88 |
| Table 6-8. Proportion of smokers by exposure status | 89 |
| Table 6-9. Proportion of participants completing high school, by region of residence | 90 |
| Table 6-10. Highest level of completed post-secondary school education obtained by study participants, by region of residence | 91 |
| Table 6-11. Fluid consumption, by region of residence..... | 92 |
| Table 6-12. Unadjusted prevalence of micronuclei in bladder epithelial cells, by region | 106 |
| Table 6-13. Bladder cancer incidence rates for the study communities..... | 108 |
| Table 7-1. Beverage consumption pattern during the study period, as estimated by diary records and by questionnaire | 115 |
| Table 7-2. Level of agreement between fluid intake diary and questionnaire | 120 |
| Table 7-3. Repeatability of the retrospective fluid intake questionnaire | 121 |
| Table 8-1. Correlations between available dose for THM compounds..... | 129 |
| Table 8-2. Unadjusted relative risks for the association between available dose of THMs (exposure) and frequency of micronuclei in bladder epithelial cells | 129 |
| Table 8-3. Correlations between interaction terms of available dose for THM compounds | 130 |
| Table 8-4. Correlation between intake dose for the THM compounds..... | 131 |
| Table 8-5. Crude and adjusted relative risks for the association between intake dose of THMs (exposure) as estimated by fluid intake diary, and frequency of micronuclei in bladder epithelial cells | 132 |

| | |
|---|-----|
| Table 8-6. Correlation between interaction terms of intake dose as estimated by fluid intake diary..... | 132 |
| Table 8-7. Correlations between available dose for THM compounds..... | 133 |
| Table 8-8. Unadjusted relative risk for the association between intake dose of THMs (exposure) as estimated by questionnaire, and frequency of micronuclei in bladder epithelial cells..... | 133 |
| Table 8-9. Correlations between interaction terms for intake dose of THM compounds, as estimated by questionnaire..... | 134 |
| Table 8-10. Correlations between internal dose for THM compounds..... | 135 |
| Table 8-11. Unadjusted relative risk for the association between internal dose of THMs (exposure), and frequency of micronuclei in bladder epithelial cells..... | 136 |
| Table 8-12. Correlations between interaction terms of internal dose for THM compounds..... | 137 |
| Table 8-13. Unadjusted relative risks for DNA damage to bladder cells with exposure to total THM in community water supplies, by exposure indices..... | 138 |
| Table 8-14. Relative risk estimates for DNA damage to bladder cells with exposure to THMs in community water supplies, by exposure measure, adjusted for interaction and confounding..... | 140 |
| Table 9-1. Characteristics of participants with flow cytometry results compared with all study participants..... | 141 |
| Table 9-2. Characteristics of participants with flow cytometry results, by region of residence..... | 142 |
| Table 9-3. DNA index (untransformed), by region of residence..... | 143 |
| Table 9-4. Unadjusted relative risks for the association between available dose of THMs (exposure) and DNA index from flow cytometry..... | 145 |
| Table 9-5. Unadjusted relative risks for the association between intake dose of THMs as estimated by fluid intake diary (exposure), and DNA index from flow cytometry..... | 146 |
| Table 9-6. Unadjusted relative risks for the association between intake dose of THMs as estimated by questionnaire (exposure) and DNA index from flow cytometry..... | 147 |
| Table 9-7. Unadjusted relative risks for the association between internal dose of THMs (exposure) and DNA index from flow cytometry..... | 148 |
| Table 9-8. Unadjusted relative risks for the association between total THM and DNA index from flow cytometry, by exposure index..... | 149 |
| Table 9-9. Relative risks for DNA damage to bladder cells (estimated by DNA index from flow cytometry), with exposure to THMs in community water supplies, by exposure index, adjusted for interaction and confounding..... | 150 |

List of figures

| | |
|--|-----|
| Figure 3-1. Evolution of the detailed continuum for molecular epidemiological research | 26 |
| Figure 4-1. Bottling process used for packaging water..... | 37 |
| Figure 4-2. Testing regime for bottled water | 40 |
| Figure 4-3. Disinfection and bottling process for the second pilot study | 48 |
| Figure 5-1. Map of ACT showing the 17 selected sampling points..... | 54 |
| Figure 5-2. Map showing the 10 selected sampling points in Adelaide | 55 |
| Figure 5-3. Re-graphing of water and air concentrations of chloroform presented by Jo et al ... | 62 |
| Figure 5-4. An example of a flow cytometry output showing the distributions of cells in terms of DNA content..... | 70 |
| Figure 5-5. Flow cytometry output from a study participant. | 71 |
| Figure 5-6. Requirements from study participants..... | 75 |
| Figure 6-1. Age (in quartiles) of participants by region of residence | 88 |
| Figure 6-2. Distribution of available dose of total THM, by region of residence..... | 93 |
| Figure 6-3. Distribution of available dose of THMs, by compound and region of residence.... | 95 |
| Figure 6-4. Proportionate representation of mean total THM concentrations in Canberra and Adelaide reticulated water (Available dose)..... | 96 |
| Figure 6-5. Mean AOX concentration in quartiles for reticulated water, by region of residence..... | 97 |
| Figure 6-6. Distributions of intake dose of THM as estimated by fluid intake diary and retrospective questionnaire, for the three study communities | 99 |
| Figure 6-7. Distribution of internal dose of THM concentrations (in quartiles), by compound and region of residence | 102 |
| Figure 6-8. Proportionate representation of mean and total THM concentrations in urine of Canberra and Adelaide participants (Internal dose) | 105 |
| Figure 7-1. Scatter plots of diary versus questionnaire intake estimates | 116 |
| Figure 7-2. Plots of differences against average readings for diary records and questionnaire estimated intake, with mean and 95% confidence limits | 118 |

List of Appendices

| | |
|--|-----|
| Appendix 1: Scatter plots (with smoothers) examining the relationship between available dose and frequency of micronuclei in bladder epithelial cells | 163 |
| Appendix 1a: Scatter plots (with smoothers) examining the relationship between available dose and DNA index | 165 |
| Appendix 2: Scatter plots examining the relationship between intake dose and frequency of micronuclei in bladder epithelial cells..... | 167 |
| Appendix 3: Scatter plots examining the relationship between internal dose and frequency of micronuclei in bladder epithelial cells..... | 169 |
| Appendix 3a: Scatter plots examining the relationship between internal dose (excluding outliers) and DNA index from flow cytometry | 171 |
| Appendix 4: Correlation coefficients between available dose of THMs and potentially confounding variables..... | 173 |
| Appendix 5: Correlation coefficients between intake dose of THM (from diary) and potentially confounding variables..... | 174 |
| Appendix 6: Correlation coefficients between intake dose of THMs (from questionnaire) and potentially confounding variables..... | 175 |
| Appendix 7: Correlation coefficients between internal dose of THMs and potentially confounding variables..... | 176 |
| Appendix 8: Relative risks for the associations between the potentially confounding variables and frequency of micronuclei..... | 178 |
| Appendix 9: Relative risks in the assessment of confounding in the association between available dose of chloroform and bromoform, and frequency of micronuclei | 180 |
| Appendix 10: Relative risks in the assessment of confounding in the association between intake dose of chloroform and bromoform as estimated by fluid intake diary, and frequency of micronuclei..... | 182 |
| Appendix 11: Relative risks in the assessment of confounding in the association between intake dose of chloroform and bromoform as estimated by questionnaire, and frequency of micronuclei | 184 |
| Appendix 12: Relative risks in the assessment of confounding in the association between internal dose of chloroform, bromodichloromethane, and bromoform, and frequency of micronuclei..... | 186 |
| Appendix 12a: Relative risks in the assessment of confounding in the association between internal dose (excluding outliers) of chloroform, bromodichloromethane, and bromoform, and frequency of micronuclei | 188 |
| Appendix 13: Relative risks in the assessment of confounding in the association between available dose of total THM and frequency of micronuclei | 190 |
| Appendix 14: Relative risks in the assessment of confounding in the association between intake dose of total THM as estimated by fluid intake diary, and frequency of micronuclei | 191 |
| Appendix 15: Relative risks in the assessment of confounding in the association between intake dose of total THM as estimated by questionnaire, and frequency of micronuclei | 192 |

| | |
|--|-----|
| Appendix 16: Relative risks in the assessment of confounding in the association between internal dose of total THM and frequency of micronuclei | 193 |
| Appendix 16a: Relative risks in the assessment of confounding in the association between internal dose of total THM (excluding outliers) and frequency of micronuclei | 194 |
| Appendix 17: Relative risks in the assessment of confounding in the association between available dose of chloroform and bromoform, and DNA index from flow cytometry | 195 |
| Appendix 18: Relative risks in the assessment of confounding in the association between intake dose of chloroform and bromoform as estimated by fluid intake diary, and DNA index from flow cytometry..... | 197 |
| Appendix 19: Relative risks in the assessment of confounding in the association between intake dose of chloroform and bromoform as estimated by questionnaire, and DNA index from flow cytometry | 198 |
| Appendix 20: Relative risks in the assessment of confounding in the association between internal dose of chloroform, bromodichloromethane, and bromoform, and DNA index from flow cytometry..... | 199 |
| Appendix 21: Relative risks in the assessment of confounding in the association between available dose of total THM and DNA index from flow cytometry..... | 201 |
| Appendix 22: Relative risks in the assessment of confounding in the association between intake dose of total THM as estimated by diary, and DNA index from flow cytometry.. | 202 |
| Appendix 23: Relative risks in the assessment of confounding in the association between intake dose of total THM as estimated by questionnaire, and DNA index from flow cytometry..... | 203 |
| Appendix 24: Relative risks in the assessment of confounding in the association between internal dose of total THM and DNA index from flow cytometry | 204 |
| Appendix 25: Environmental Health Questionnaire 1 | 205 |
| Appendix 25a: Environmental Health Questionnaire 2 | 230 |
| Appendix 26: Fluid intake diary | 241 |
| Appendix 27: Introductory letter..... | 246 |
| Appendix 28: Information brochure | 248 |
| Appendix 29: Consent form..... | 250 |
| Appendix 30: Interviewers declaration | 251 |
| Appendix 31: Instructions for collecting urine sample 1 | 252 |
| Appendix 32: Instructions for collecting urine sample 2 | 253 |
| Appendix 33: Randomised controlled trial | 254 |
| Appendix 34: Abbreviations | 260 |
| Appendix 35: Glossary | 261 |