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THE APPLICATION OF ELEMENTAL FINGERPRINTING TECHNIQUES TO IDENTIFY POPULATION CONNECTIVITY USING *AUSTROVENUS STUTCHBURYI* RECRUITS

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ABSTRACT

The density, distribution and dynamics of benthic bivalve populations are believed to be largely determined by the planktonic larval phase of the life history. As the hard parts of larvae, such as the prodissoconch (larval shell), develop and grow, ambient environmental conditions are recorded as chemical signatures (elemental fingerprints). If the chemical signals of reference sites are known, the larval signatures can be matched to reference sites, hereby reconstructing the origin of the larvae. The collection and identification of larval bivalves is extremely difficult, however, previous studies have shown that the prodissoconch is retained into the juvenile phase, thus enabling juveniles to maintain a record of the larval movement. Before elemental fingerprinting can be used as a larval tracking tool, site specific signatures must be evident in the shells. As a precursor study to test the application of elemental fingerprinting to track bivalve larvae, the presence of spatial variability in shell signatures as well as the scale at which these variations occurred, were established for New Zealand conditions. Furthermore, temporal stability of the shell signatures was explored, as temporal stability is crucial if the signals of shells collected at one time are to be used as predictors of unknown samples collected at a different time.

The venerid bivalve *Austrovenus stutchburyi* is a common and widespread constituent of New Zealand estuarine communities and were therefore selected as the study species. The chemical signatures of entire *Austrovenus stutchburyi* shells were examined to determine the inter-site spatial differences in elemental fingerprints of shells and also to characterise the temporal stability of the signatures. Furthermore, shells were ablated at two reference points (the prodissoconch and most recently formed shell material) to determine the intra-shell variation in the chemical signatures. Juvenile individuals were collected from 19 sites in the North Island of New Zealand as part of the whole shell spatial study. One site (in Tauranga Harbour) was examined for the temporal study, whilst four sites were used to compare intra-shell chemical variation. Whole shells were digested and analysed as solution based samples using inductively coupled

plasma-mass spectrometry (ICP-MS) for the spatial and temporal studies, and by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) for the point ablation intra-shell variation study.

Results showed that shell concentrations were sufficiently different to yield a classification success of 68% over 19 sites, however the classification success markedly increased as the number of sites included in the analysis decreased (e.g. 12 sites = 75%, 10 sites = 84%, 5 sites = 90%, 3 sites = 95%). Shells were successfully classified across all of the spatial scales that were tested (approximately 10 km to 1150 km). Temporal stability in chemical signatures was observed over a 44 day period. The chemical signatures were not correlated with ambient temperature or salinity, however more vigorous sampling is needed to accurately assess the relationship between shell elemental incorporation and environmental conditions. Intra-shell variation was also observed for some of the shells analysed from two of the four sites. These results were promising and indicated that there may be chemical variations between the larval shells and the more recently formed shell material, thus suggesting the possibility of external recruitment.

The results from this study emphasised the potential for the application of elemental fingerprinting techniques to track and better understand the larval transport and population connectivity for New Zealand invertebrate species, however more research is required before elemental fingerprinting can effectively be applied to New Zealand invertebrate species. With a fundamental understanding of the origin of bivalve recruits, restoration efforts following estuarine disturbance events can be effectively employed.

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TABLE OF CONTENTS

Abstract ..... **ii**
Acknowledgements ..... **iv**
Table of Contents ..... **vi**
List of Figures ..... **viii**
List of Tables ..... **x**
List of Equations ..... **xiii**
1.0 Chapter One: Introduction **14**
1.1 Metapopulation dynamics 14
1.2 Tracking marine larvae 16
1.2.1 Artificial markers 16
1.2.2 Genetic markers 16
1.2.3 Environmental markers 17
1.3 Elemental fingerprinting 18
1.3.1 Principles of elemental fingerprinting 18
1.3.2 Applications of elemental fingerprinting 20
1.3.2.1 Fish otoliths 20
1.3.2.2 Invertebrate shells/hard parts 21
1.3.3 Factors that influence elemental uptake in invertebrates 22
1.4 Objectives and hypotheses 23
2.0 Chapter Two: Materials and Methods **24**
2.1 Study species 24
2.2 Sampling locations 25
2.3 Sample collection 28
2.4 Cleaning procedures and sample preparation 29
2.4.1 Solution based ICP-MS 29
2.4.2 Point ablation LA-ICP-MS 29
2.4.3 Water and sediment samples (ICP-MS) 31
2.4.3.1 Water samples 31
2.4.3.2 Sediment samples 31
2.5 Sample analysis 32

2.6 Statistical analysis 35
3.0 Chapter Three: Results 39
3.1 Spatial variation in elemental fingerprints (shell digestion) 39
3.1.1 Variation over different spatial scales 44
3.1.1.1 Large spatial scale 44
3.1.1.2 Small spatial scale 46
3.1.2 Shell, water and sediment elemental comparisons 48
3.1.3 Environmental factors contributing to variation 50
3.2 Temporal variation in elemental fingerprints (shell digestion) 52
3.3 Intra-shell elemental variation (point ablation) 55
4.0 Chapter Four: Discussion 60
4.1 Spatial variation in whole shell chemistry 60
4.1.1 Effect of the number of sampling sites on discriminating power 60
4.1.2 Spatial scale of discriminating power 61
4.2 Factors responsible for the spatial variation 63
4.3 Temporal variation in whole shell chemistry 65
4.4 Point ablation ICP-MS 68
4.5 Limitations and future directions 70
5.0 Chapter Five: Conclusions 72
References 74
Appendices 86
A.1 Shell size and weight data 86
A.2 Exact sediment weights 96
A.3 The influence of the number of sites on DFA 97
A.4 HSD post-hoc results (19 sites) 101
A.5 HSD post-hoc results (point ablation study) 106

LIST OF FIGURES

FIGURE 2.1. A map of the central and upper North Island of New Zealand indicating the 20 collection sites. 26

FIGURE 2.2. View of a 1 mm thick shell prepared for LA-ICP-MS. 30

FIGURE 2.3. A scatterplot showing the effect that the number of sites included in the analysis had on the classification success of the DFA. 38

FIGURE 3.1. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles collected from 12 sites 41

FIGURE 3.2. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles collected from PAR, HRK, WHR and WHT 42

FIGURE 3.3. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles collected from PAI, AOT and KAW.. 43

FIGURE 3.4. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles as part of the large spatial scale study 46

FIGURE 3.5. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles as part of the small spatial scale study 48

FIGURE 3.6. Scatterplots showing the correlation between the mean concentrations of elements in the shells to the concentrations of elements in the water and sediment 49

FIGURE 3.7. Discriminant function scores of element (Sr, Ba, Zn) to Ca ratios in shells of *Austrovenus* juveniles analysed as part of the temporal study 54

FIGURE 3.8. The mean difference in elemental concentrations between the shell edge and the prodissoconch 57

FIGURE 3.9. A scatterplot of the canonical scores of the shell edge (E) and the predicted prodissoconch (P) values calculated from the unstandardised root 1 and 2 canonical coefficients of the shell edge..... 59

LIST OF TABLES

TABLE 2.1. The study estuaries, study sites and their geographical locations listed from north to south. 27

TABLE 2.2. The laser operating conditions for juvenile *Austrovenus* shells and NIST-612 standard material analysis by LA-ICP-MS. 33

TABLE 2.3. A list of previous studies of elemental fingerprinting applied to invertebrates 34

TABLE 2.4. The elemental ratios and concentrations used during ANOVA and DFA analyses of the shells 35

TABLE 3.1. Mean (± 1 SE) element to calcium ratios in juvenile *Austrovenus* shells collected from the North Island of New Zealand 39

TABLE 3.2. Classification success using the chemistry of *Austrovenus* shells collected from 12 sites 40

TABLE 3.3. Classification success using the chemistry of *Austrovenus* shells collected from four sites: PAR, HRK, WHR and WHT 42

TABLE 3.4. Classification success using the chemistry of *Austrovenus* shells collected from three sites: PAI, AOT and KAW 43

TABLE 3.5. Classification success using the chemistry of *Austrovenus* shells collected from three sites situated over a large spatial scale 45

TABLE 3.6. Classification success using the chemistry of *Austrovenus* shells collected from three sites situated over a small spatial scale 47

TABLE 3.7. A summary of the correlation of discriminant function scores with environmental variables 51

TABLE 3.8. Mean (± 1 SE) elemental to calcium ratios in juvenile *Austrovenus* shells collected as part of the temporal study 52

TABLE 3.9. Classification success using the chemistry of *Austrovenus* shells collected as part of the temporal study 53

TABLE 3.10. Mean (± 1 SE) standardised (to ^{42}Ca) elemental concentrations of juvenile *Austrovenus* shells collected from four sites 55

TABLE 3.11. Mean (± 1 SE) standardised (to ^{42}Ca) elemental concentrationsof the edge (*E*) and prodissoconch (*P*) regions of juvenile *Austrovenus* shells 56

Appendices

TABLE A.1. The sizes and weights of all the shells used in the whole shell digestion study. 86

TABLE A.2. The exact weights of the sediment that was digested to determine the sediment chemistry. 96

TABLE A.3.1. Classification success using the chemistry of *Austrovenus* shells collected from three sites 97

TABLE A.3.2. Classification success using the chemistry of *Austrovenus* shells collected from five sites 98

TABLE A.3.3. Classification success using the chemistry of *Austrovenus* shells collected from ten sites..... 99

TABLE A.4.1. Unequal *N* HSD post-hoc test investigating the difference in Sr:Ca concentrations in *Austrovenus* juvenile shells collected from 19 sites 101

TABLE A.4.2. Unequal N HSD post-hoc test investigating the difference in Ba:Ca concentrations in Austrovenus juvenile shells collected from 19 sites..... 102

TABLE A.4.3. Unequal N HSD post-hoc test investigating the difference in Mn:Ca concentrations in Austrovenus juvenile shells collected from 19 sites..... 103

TABLE A.4.4. Unequal N HSD post-hoc test investigating the difference in Mg:Ca concentrations in Austrovenus juvenile shells collected from 19 sites..... 104

TABLE A.4.5. Unequal N HSD post-hoc test investigating the difference in Zn:Ca concentrations in Austrovenus juvenile shells collected from 19 sites..... 105

TABLE A.5.1. Unequal *N* HSD post-hoc test investigating the difference in Sr concentrations in *Austrovenus* juvenile shells collected from four sites..... 106

TABLE A.5.2. Unequal N HSD post-hoc test investigating the difference in Ba concentrations in Austrovenus juvenile shells collected from four sites 106

TABLE A.5.3. Unequal N HSD post-hoc test investigating the difference in Mg concentrations in Austrovenus juvenile shells collected from four sites 106

TABLE A.5.4. Unequal N HSD post-hoc test investigating the difference in Mn concentrations in Austrovenus juvenile shells collected from four sites 106

LIST OF EQUATIONS

EQUATION 3.1. The unstandardised canonical root 1 and root 2 scores from the juvenile *Austrovenus* DFA 58

1.0 CHAPTER ONE: INTRODUCTION

1.1 Metapopulation dynamics

A fundamental question in estuarine ecology, and one which has long been debated, is whether estuaries are 'open' or 'closed' systems (Roughgarden et al., 1988; Cowen et al., 2000; Hellberg et al., 2002). Most marine species have at least one potentially widespread dispersive stage in their life histories. In non-brooding benthic species, this stage is generally a pelagic larval stage. The existence of pelagic larvae has resulted in many scientists hypothesising that estuarine systems are in fact open (or well mixed) on an ecological and evolutionary scale, with the capability of attaining new recruits over significant distances (Cowen et al., 2000). This hypothesis infers that the process of dispersal is driven by only two factors: ocean current trajectories; and the duration of the planktonic larval stage prior to settlement (Cowen et al., 2000; Bradbury & Snelgrove, 2001; Treml et al., 2008). Larvae, however, are not passive particles and can potentially influence their dispersal through behavioural adaptations including 'swimming', burrowing or vertical migration (Bradbury & Snelgrove, 2001; Hunt, 2004). These behaviours, along with vertically stratified currents, may result in nearshore larval retention (Cowen et al., 2000), and subsequent closed systems.

Larval dispersal and recruitment of invertebrates has been a common area of biological study since the discovery of the planktonic larval stage in the 19th century (Levin, 2006). However it was not until recently (late 1980's) that the importance of the planktonic larval stage, in shaping the distribution and abundance of marine benthic populations, has been recognised (Roughgarden et al., 1988; Young, 1990; DiBacco & Levin, 2000; Levin, 2006). Despite many studies conducted in this field of research, some fundamental questions regarding the movement of larvae remain unanswered, thus making it difficult for scientists to confirm the importance of larval supply in structuring adult populations (Becker et al., 2005). An understanding of larval exchange among populations of

marine benthic organisms is vital for the study of population dynamics, the management of stocks, and also in designing and implementing marine reserves (Caley et al., 1996; Hedgecock et al., 2007).

Attempts have been made to examine the role of larval exchange in shaping benthic marine populations by applying the metapopulation theory, a theory originally derived by Levins (1969; 1970, as cited in Strasser (2008)) to describe terrestrial population dynamics. The application of the metapopulation theory has only frequented marine literature since the 1990s, in which time it has proven useful in highlighting important processes that shape marine populations (Hanski & Gilpin, 1991; Grimm et al., 2003; Kritzer & Sale, 2004; 2006).

A metapopulation can be defined as several spatially separate populations of the same species which interact at some level, thus it is "a population of populations" (Hanski & Gilpin, 1991). Although individual populations may be subject to extinction due to stochastic events, metapopulations are much more stable, as individuals from one population can re-colonise habitats that were left vacant by the extinction of another (Hanski, 1998; Grimm et al., 2003). The metapopulation theory therefore emphasises migration to be a vital process in shaping marine populations with the extent of migration determining the degree of connectivity among populations (Strasser, 2008). Quantifying the migration (or dispersal) of populations where the dispersing life stage is very small (i.e. planktonic larvae for benthic invertebrates) is the challenge facing marine ecologists.

In other environments (e.g. terrestrial) the degree of migration, dispersal and connectivity is easily quantified through sophisticated tracking techniques (e.g. mark-recapture). Such techniques enable researchers to systematically track individuals over different spatial scales and through different developmental and life stages, thus gaining an insight into their movement and behaviour (Thorrold et al., 2002). However, in aquatic environments, particularly marine systems, many organisms release millions of propagules during a single spawning event. The larvae are typically very small and have high mortality rates, meaning that the recapture probability of marked individuals is low. These factors, along with other physical (i.e. oceanographic conditions) and biological (i.e. planktonic duration)

factors that affect the potential for a species to disperse, are responsible for the lack of empirical data on larval movement and retention, and ultimately the population connectivity of marine species (Thorrold et al., 2002). Nevertheless, considerable efforts have been made to develop and implement techniques to address this problem.

1.2 Tracking marine larvae

The need to quantify population connectivity in marine organisms has led to the development and implementation of three main tagging techniques; (1) artificial, (2) genetic and (3) environmental markers.

1.2.1 Artificial markers

Artificial tagging is a simple method to track organisms and uses practices like fluorescent compounds (e.g. Norkko et al., 2001), elemental tags (e.g. Levin et al., 1993), radioactive isotopes or thermal marks (e.g. Thorrold et al., 2002). Although all these techniques have shown some promise, sufficient numbers of tagged individuals need to be recovered for reliable results to be attained, thus making this technique labour and resource intensive. In a field study conducted by Jones et al. (1999), coral reef fish larvae were tagged with a fluorescent compound. Of ten million larvae that were tagged, only 15 individuals were retrieved. These results emphasise the effort involved with carrying out artificial marking studies.

1.2.2 Genetic markers

Genetic markers take advantage of natural genetic variation that may exist among source populations or make use of genetic tags produced through artificial breeding. Natural genetic variation, however, is often marginal among marine populations (Hellberg et al., 2002). Despite this problem, the underlying principles of genetic markers have been used to develop successful tagging

approaches that rely on natural variation in spawning success (natural cohorts) as well as artificial incorporation of rare natural and transgenic markers (Thorrold et al., 2002). The disadvantages of genetic markers are that they can require considerable effort, and may have significant environmental consequences. Genetic markers have the potential to alter the genetic make-up of individuals that are released into natural populations and can have long term evolutionary impacts (Thorrold et al., 2002).

1.2.3 Environmental markers

Environmental markers hold significant advantages over artificial and genetic markers available for tracking and studying larval dispersal. Naturally induced tags are expressed in each individual within an experimental system; therefore each individual collected contains the marker without any expense to the investigator. Additionally, environmental markers do not impose unnecessary stress on the organisms; they are not hazardous to the environment; and larval behaviour is not changed as a result of experiments being carried out in the laboratory (DiBacco & Levin, 2000).

Environmental markers are naturally incorporated as individuals experience variations in the physical and chemical environment. These variations can be expressed in the phenotype of the larval form or growth rate (developmental markers), and/or recorded as trace elements or isotopic chemistry (elemental fingerprints or geochemical signatures) in the hard parts (i.e. skeleton or shell) of organisms (Thorrold et al., 2002). Developmental markers have limited applications as such markers are usually short lived and disappear rapidly after settlement. Developmental markers also rely on prior knowledge of the spatial variation of a multitude of biological (e.g. food resources) and physical (e.g. temperature) factors, which are not always easily measured (Thorrold et al., 2002). In contrast, the preservation of geochemical signatures in the hard parts of individuals are longer lasting and should provide a time series of the environment experienced by the larvae.

Analysing the geochemical signatures in calcified structures, a process referred to as elemental fingerprinting, has been widely used to accurately track fish movement and migration (e.g. Campana, 1999; Gillanders & Kingsford, 2003; Elsdon & Gillanders, 2006). The application of elemental fingerprinting to track invertebrates such as bivalves has been less well explored (DiBacco & Levin, 2000; Zacherl et al., 2003a; 2003b; Zacherl, 2005; Becker et al., 2005; 2007); however, results of such studies have shown considerable promise.

The following section will investigate the principles and application of elemental fingerprinting as well as highlight the factors that may influence the uptake of elements into shells, with a particular focus on molluscs.

1.3 Elemental fingerprinting

1.3.1 Principles of elemental fingerprinting

The chemical characteristics (or chemical signatures) of water masses can be vastly different among various locations. Such differences are a function of both natural (i.e. geographical location and atmospheric deposition) and anthropogenic causes (i.e. proximity to urban centres and sources pollution). Variations in water chemistry are exacerbated within estuarine systems, due to substantial salinity (a function of freshwater inputs) and temperature differences (Thorrold et al., 2007). The principles of elemental fingerprinting are based on these location specific chemical signatures derived from the chemical and physical environment.

As the carbonate matrix of the hard parts (i.e. shells or otoliths) of marine organisms incrementally forms, a chemical time series is incorporated from the ambient environment in the form of non-calcium elements, thus keeping a record of movement (Zacherl et al., 2003a; 2003b). As this is an ongoing process throughout the individual's development, the elemental fingerprints recorded in the hard parts are likely, and have been shown, to log changes in the physico-chemical properties of the surrounding water as the individual moves across

gradients in seawater composition (Stecher et al., 1996). An organism's chemical signature should therefore correspond to the environmental conditions or water chemistry experienced by that individual during its development (Thorrold et al., 2002; Zacherl et al., 2003b; Levin, 2006). If the chemical signatures of a group of organisms from known origins can be established, then the origin of another group of organisms from unknown locations can be determined by comparing and matching the chemical signatures of the individuals (Becker et al., 2005).

Elemental fingerprinting requires that the water masses in which organisms are developing have sufficiently different chemical signatures at different locations (spatial scales), whilst remaining stable over time (temporally) at each location (Thorrold et al., 2002). By analysing the hard parts, it is possible to determine the location where various stages of the hard parts of an organism were formed (Becker et al., 2005). Similarly, the microchemistry of the larval structures that are retained after settlement (e.g. the prodissoconch in bivalves), provide a record of larval movement, which can also be analysed to determine the larval origin of settled juveniles (Becker et al., 2007). By comparing the chemistry of the prodissoconch and the shell edge (the most recently formed shell material), conclusions can be drawn regarding the larval origin. If the prodissoconch and shell edge chemistries are similar, then it can be concluded that both areas of the shell formed in the same water mass (internal supply or closed system). If the chemistry of the shell edge and the prodissoconch differ, it is probable that the larvae came from a different estuary or water mass (open system). Elemental fingerprinting techniques could potentially allow scientists to infer the spatial extent of dispersal and recruitment for organisms with hard structures that record chemical signatures from their ambient environment (Becker et al., 2005).

Hard parts can easily be analysed using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). This method allows the researcher to read the chemistry at specific points along the shell or otolith. Hard parts can also be completely dissolved and analysed by solution based inductively coupled plasma-mass spectrometry (ICP-MS) as liquid samples to obtain an indication of the overall chemistry of the shell or otolith (Gillanders & Kingsford, 2000; Lazareth et al., 2003; Elsdon & Gillanders, 2006).

1.3.2 Applications of elemental fingerprinting

Elemental fingerprinting was introduced to the marine environment in an attempt to quantify population dynamics (i.e. migration and recruitment) and to track dispersal trajectories of organisms (DiBacco & Levin, 2000). Much of the initial work has focussed on application of elemental fingerprinting in fish otoliths (ear bones), however more recent applications have also included invertebrate larvae, such as crabs (DiBacco & Levin, 2000) and bivalves (Becker et al., 2005; 2007).

1.3.2.1 Fish otoliths

Elemental fingerprinting of fish otoliths has been hugely successful in examining the connectivity of coastal and estuarine fish populations (e.g. Secor et al., 1995; Campana, 1999; Swearer et al., 2003; Elsdon & Gillanders, 2004; Begg et al., 2005; Kellison & Taylor, 2007). Common applications of the technique include: tracking fish movements and/or migration patterns which can be used to assess population connectivity (e.g. Gillanders & Kingsford, 1996; Secor et al., 2002; Hobbs et al., 2005; Elsdon & Gillanders, 2006); discriminating between fish stocks (Edmonds et al., 1991; 1992; Campana et al., 2000); and tracking dispersal trajectories of anadromous fish (Kalish, 1990; Secor et al., 1995; Thorrold et al., 1998). For example, Secor et al. (2002) found variations in otolith microchemistry of juvenile blue fin tuna that confirmed two distinct nursing grounds, whilst Gillanders and Kingsford (2003) reported significant differences in the otolith chemistry of juveniles (analysed by solution based ICP-MS) of three different fish species (snapper, tarwhine and bream) collected from different estuaries.

Elemental fingerprinting is fast becoming a commonly used tool for marine and fishery biologists and ecologists to answer questions regarding the life histories of fish species. It is hypothesised that a similar technique may also hold the key to determining the origins and movement of mollusc and other invertebrate larvae.

1.3.2.2 Invertebrate shells/hard parts

Although elemental fingerprinting has shown great promise for tracking fish species for over a decade, its application to invertebrate larvae was first explored by DiBacco and colleagues (DiBacco & Levin, 2000; DiBacco & Chadwick, 2001), and was not applied specifically to bivalve shells until 2003 (Lazareth et al., 2003). Studies by DiBacco and Levin (2000) and DiBacco and Chadwick (2001) explored the microchemistry of crab zoeae to distinguish if larvae were spawned inside or outside of San Diego Bay, whilst Zacherl et al. (2003a) found significant spatial variability between three sites in Chile when analysing the statoliths (larval shells) of gastropods. These three studies demonstrated the effectiveness of using the elemental fingerprinting technique on a variety of invertebrate species.

Very few studies have applied elemental fingerprinting specifically to bivalve invertebrates (e.g. Lazareth et al., 2003; Becker et al., 2005; 2007). The larval shell (prodissoconch) in many venerid bivalve species forms within 24 hours of the veliger larva hatching (Mouëza et al., 1999; 2006), and is followed by an approximate four week growing period in the plankton (pelagic stage), before the bivalves settle into the benthos as juveniles (Tompa et al., 1984). Throughout the development of the prodissoconch and juvenile shell, environmental conditions are recorded as signatures. As juveniles retain their prodissoconch after settlement (Levin, 2006), the prodissoconch can be used to infer the larval origin (i.e. using the site specific signature of the larval shell). Lazareth et al. (2003) explored the applicability of the elemental fingerprinting on the mangrove bivalve *Isognomon ephippium* to record spatio-temporal environmental variations. Significant variations in Mn and Ba concentrations in shells were recorded between three study sites. Mn and Ba concentrations were found to be strongly correlated to the hydrodynamic properties and nutrient inputs of the sites (Lazareth et al., 2003).

Becker et al. (2005; 2007) examined the application of elemental fingerprinting to determine the movement and dispersal of open coast mytilid mussel species. Becker et al. (2005) found there to be significant spatial variability in the chemical signature of individuals, whilst temporal variation was minimal. Becker et al.

(2007) showed that two closely related species previously thought to be part of demographically open populations, had substantially different connectivity patterns. Both studies exhibited the usefulness of using the technique to track open coast bivalve species; however, the application of elemental fingerprinting has not been tested on large spatial scales for estuarine bivalve species to date. It has been reported that variations in chemical signatures are probably more pronounced for estuarine species compared to open coast species due to extensive anthropogenic inputs into estuarine environments (Thorrold et al., 2007).

1.3.3 Factors that influence elemental uptake in invertebrates

Many papers have reported factors that could influence the elemental uptake in invertebrates. Understanding and mitigating these factors when possible is important to ensure accurate results are obtained.

A number of studies have reported a relationship between water temperature and elemental uptake in bivalves and gastropods (Lazareth et al., 2003; Zacherl et al., 2003b; Zacherl, 2005), whilst phytoplankton blooms were recorded as distinct Ba peaks (Lazareth et al., 2003). Gillikin et al. (2005) found biological processes such as growth rate to have significant correlations with elemental concentrations in aragonic bivalve shells. Carré et al. (2006) supported the findings of growth rate effects but concluded, from their results, that environmental parameters like salinity and temperature had minimal impacts on elemental uptake. Klein et al. (1996) and Strasser et al. (2008) also warned against the confounding impacts that growth rate may have on results from biochemical studies, and identified age to influence and bias results.

1.4 Objectives and hypotheses

The objectives of this study were to test elemental fingerprinting techniques over both large and small spatial scales and to determine if the elemental fingerprints remained temporally stable. The outcomes will allow conclusions to be drawn regarding the applicability of elemental fingerprinting for New Zealand bivalve species and environmental conditions. This study can provide the groundwork for future larval tracking studies in estuarine environments.

In order to test the applicability of elemental fingerprinting as a means of measuring connectivity between estuaries, three hypotheses were examined. The first two hypotheses were tested by analysing the shells using solution based ICP-MS, whilst the third and final hypothesis was tested using point ablation LA-ICP-MS. Understanding population connectivity of estuarine bivalves will enhance the management and conservation of estuarine ecosystems in New Zealand. The venerid bivalve *Austrovenus stutchburyi* was used as the study species as this bivalve is a common, widespread and important constituent of New Zealand estuarine communities.

For my first hypothesis, I tested if (1a) the chemical signatures of *Austrovenus stutchburyi* shells varied significantly between and within estuaries and (1b) if the signals correlated with the water and/or sediment chemistry. I also attempted to describe the spatial patterns of the data using the geographical location of the sites, as well as other estuarine classification parameters. My second hypothesis tested whether the chemical signatures of individuals collected bi-weekly from one location (e.g. Tauranga Harbour) remained constant over time, as this is essential for the applicability of elemental fingerprinting. Lastly, I tested whether, for some sites, the shell chemistry varied between two reference points on the shell. The two reference points used were the prodissoconch and the most recently formed shell edge material. The overall aim of this research was to act as a precursor study to test the application of elemental fingerprinting to quantify larval dispersal, and to distinguish between shell microchemistries of juvenile bivalve species subject to New Zealand conditions.

2.0 CHAPTER TWO: MATERIALS AND METHODS

2.1 Study species

The common New Zealand cockle, *Austrovenus stutchburyi* (hereafter referred to as *Austrovenus*), was selected as the study species as it is a common and important constituent of the soft sediment estuarine ecosystems of New Zealand (Pridmore et al., 1990). *Austrovenus* is a suspension-feeding venerid bivalve that lives in the surface sediment of intertidal habitats (Powell, 1979). *Austrovenus* beds can reach densities of up to 3000 individuals m^{-2} (Stephenson & Chanley, 1979). The growth rates and maximum age of *Austrovenus* are extremely variable, however populations in excess of 20 years old with lengths greater than 60 mm have been recorded (Stephenson & Chanley, 1979). Adults spawn during the warmer summer months, with gonadal development peaking in January (Stephenson & Chanley, 1979). The life cycle consists of a planktonic larval phase (where the shell begins to form), whilst the juveniles and adults are restricted to the sediment surface layer (Cummings et al., 1995). Although the planktonic larval phase is capable of dispersing over vast distances, the juvenile and adult phases primarily disperse in the bedload (usually < 1 km) (Norkko et al., 2001; Lundquist et al., 2004; Petuha et al., 2006). Juveniles retain their larval shell (prodissoconch) after settlement (Levin, 2006), thus it is hypothesised that juveniles can be used to infer larval origins and movements.

For the purposes of this study, juveniles between the sizes of 0.5 – 3.5 mm were collected as they were easy to identify under a compound microscope, but still had a visible larval shell component, thus making juveniles an ideal study specimen. The small size range increased the probability that individuals of a similar age were collected, thus mitigating the effect age and growth rate may have on elemental incorporation into the shell.

2.2 Sampling locations

A total of 20 sites, from 16 different estuaries in the upper and central North Island of New Zealand were sampled (Figure 2.1 and Table 2.1). Nineteen of these sites, located across all 16 estuaries, were sampled during a seven week period as part of the spatial study (Figure 2.1). This enabled spatial variability in bivalve chemical signatures to be determined. By using a short (seven week) time frame between 15 January 2007 and 8 March 2008 (months of generally relatively stable weather patterns with an abundance of juvenile bivalves), potential differences due to temporal variation were minimised. This sampling period further ensured that inter-annual variation would not confound the results.

Study sites selected for the spatial study encompassed a range of geographical, physical, oceanographical and geological characteristics. Sites ranged from: the far north (e.g. PAR) to central North Island of New Zealand (KAW); pristine (e.g. WHP) to urbanised (e.g. ARP); well flushed (e.g. PAI) to poorly flushed (e.g. TIN), as well as large estuaries (e.g. HOK) to small estuaries (e.g. HRK). The spatial variability of the microchemistry signals of juvenile *Austrovenus* shells were determined by comparing the signatures of samples collected from all of the estuaries (large spatial scale), whilst the two estuaries with multiple sample sites (e.g. Manukau and Tamaki) were used to determine if variation existed within estuaries (or over smaller spatial scales).

Tuapiro Point (TUA), located in the northern basin of the Tauranga Harbour (Figure 2.1), was continuously sampled every 2 – 3 weeks over the summer months to determine if temporal variation existed in the elemental signatures of the bivalves collected from that site. The collection period (22 November 2007 – 30 January 2008) coincided with that of the spatial study to test the temporal stability of a site over the summer (months of peak recruitment) (Table 2.1).

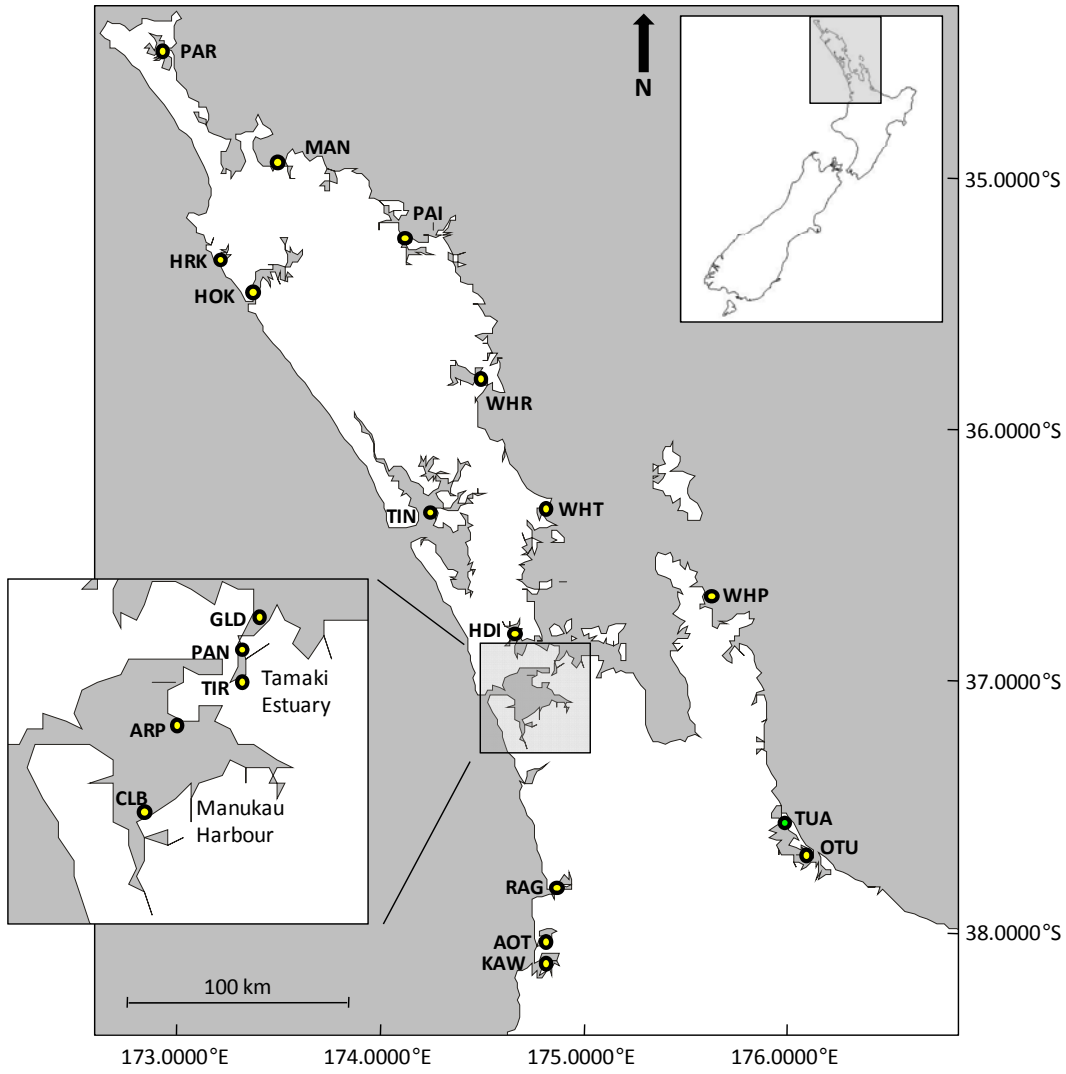


FIGURE 2.1. A map of the central and upper North Island of New Zealand indicating the 20 collection sites. PAR = Parengarenga; MAN = Mangonui; PAI = Paihia; HRK = Herekino; HOK = Hokianga; WHR = Whangarei; TIN = Tinopai; WHT = Whangateau; WHP = Whangapoua; HDI = Herald Island; GLD = Glendowie; PAN = Panmure; TIR = Tiraumea; ARP = Auckland Airport; CLB = Clarks Beach; TUA = Tuapiro Point; OTU = Otumoetai; RAG = Raglan; AOT = Aotea; KAW = Kawhia. Yellow circles indicate the sites used in the spatial study, whilst the green circle indicates the site used for the temporal component of the study. Samples from KAW, AOT, TIN and PAI were used in the point ablation study.

TABLE 2.1. The study estuaries, study sites and their geographical locations listed from north to south.

Estuary	Site	Date Sampled	LAT : LONG
Parengarenga Harbour	PAR	19 Jan 2008	34°31.163' S 172°54.810' E
Mangonui Inlet	MAN	17 Jan 2008	34°59.961' S 173°32.666'E
Herekino Harbour	HRK	21 Jan 2008	35°16.636' S 173°12.036' E
Paihia Inlet	PAI	16 Jan 2008	35°18.035' S 174°06.176'E
Hokianga Harbour	HOK	16 Jan 2008	35°23.786' S 173°30.070' E
Whangarei Harbour	WHR	15 Jan 2008	35°46.798' S 174°23.820' E
Kaipara Harbour	TIN	21 Jan 2008	36°15.206' S 174°14.894' E
Whangateau Harbour	WHT	20 Feb 2008	36°18.937' S 174°46.550' E
Whangapoua Estuary	WHP	13 Feb 2008	36°43.930' S 175°37.014' E
Waitemata Harbour	HDI	08 Mar 2008	36°46.621' S 174°39.497' E
Tamaki Estuary	GLD	31 Feb 2008	36°51.623' S 174°52.984' E
	PAN	31 Feb 2008	36°54.215' S 174°51.917' E
	TIR	31 Feb 2008	36°55.472' S 174°51.801' E
Manukau Harbour	ARP	15 Feb 2008	37°01.111' S 174°49.150' E
	CLB	15 Feb 2008	37°07.695' S 174°42.719' E
Tauranga Harbour	TUA	22 Nov 2007 – 30 Jan 2008	37°29.248' S 175°56.957' E
	OTU	08 Feb 2008	37°39.561' S 176°08.537' E
Raglan Harbour	RAG	10 Feb 2008	37°48.094' S 174°52.048' E
Aotea Harbour	AOT	09 Feb 2008	38°00.990' S 174°49.606' E
Kawhia Harbour	KAW	09 Feb 2008	38°03.838' S 174°49.492' E

2.3 Sample collection

Juvenile *Austrovenus*, water and sediment samples were collected an hour either side of low tide from each site during each visit. Collection always occurred during this time period, as this was when the estuary contained the least amount of oceanic water inputs and also showed the most terrestrial influence. Juvenile bivalves were collected by sieving the upper 1 – 2 cm of sediment at the sample sites (usually located around known *Austrovenus* beds) with a 500 μm mesh sieve. Using a hand lens, small juvenile *Austrovenus* were picked out of the material left in the sieve. These juveniles were then placed in an eppendorf tube filled with seawater from the site. Samples were immediately chilled on ice and transported back to the laboratory for analysis. All equipment (glassware, pipette tips and sample containers) was appropriately sterilised prior to use by undergoing a 5% HCl acid wash for at least 24 hours before being soaked and rinsed three times in Milli-Q water.

In the laboratory, samples were placed in acid-washed Petri dishes, where they were measured and sorted using fine-tipped forceps under a microscope with an attached micrometer. At this time, the identification of each individual was also confirmed. Individuals that measured $\geq$ 3.5 mm in size were discarded. Individuals that were $\leq$ 3.5 mm in length were frozen in local seawater and retained for further analysis.

One water sample and three sediment samples were collected at each site. Water samples were obtained at a depth of 15 – 30 cm below the water surface from the channel situated closest to the collection site. Sediment samples were collected from within five meters of where bivalves were collected using a 50 ml syringe core. All the plastic ware used during sample collection and storage were first acid washed to avoid sample contamination (Gasparon, 1998). The water temperature and salinity was also recorded at each site during each visit.

2.4 Cleaning procedures and sample preparation

Fifteen individuals (ranging between 1 – 3 mm in size) from all of the sites were defrosted. Each individual bivalve was split open using sterilised (acid-dipped) fine-tipped forceps and the flesh was removed (for exact shell sizes and weights see Appendix A.1). Both valves were manually cleaned of as much debris as possible and individually stored in eppendorf tubes containing Milli-Q water. The right valves were used for solution based whole shell digestion purposes, whilst the left valve was used in the point ablation study.

2.4.1 Solution based ICP-MS

The right valves of all the individuals were completely digested to carry out solution based analysis. The cleaning and digestion methods were based on those described by Strasser et al. (2008), as this study digested individuals of a similar size. First, the shells were meticulously cleaned to eliminate contamination. The shells were briefly sonicated (30 – 35 seconds) to remove organic matter. Shells were removed from the tubes and rinsed three times in quartz-distilled Milli-Q water and soaked for ten minutes at 80 °C in 1% H_2O_2 solution buffered in 1 N ultrapure NaOH to further remove organic matter. Shells were rinsed another three times in quartz-distilled Milli-Q water and individually transferred to clean acid washed vials. Shells were then left to dry overnight in a laminar flow hood. After the shells were dry, each shell was individually weighed to the nearest 1 μg and dissolved in 2% ultrapure HNO_3 to achieve an approximately 20,000 fold dilution of calcium carbonate for each shell based on weight.

2.4.2 Point ablation LA-ICP-MS

The left valve of each individual was cleaned following the decontamination steps described by Arslan & Secor (2008) (except that deviations were made specific for bivalve shells), as this method had been proven to reduce surface contamination. The shells were firstly soaked in 5% H_2O_2 for ten minutes to

dissolve any biological material on the shell, including the thin layer of organic matter that covers the shell (periostracum). Next, the shells were submerged in 1% HNO_3 acid for five minutes to remove any surface contamination, and rinsed three times in Milli-Q water to remove all traces of the acid. Finally, the shells were dried in a laminar flow hood. Once dry, the left valve of each individual was mounted in two-part epoxy resin. Latex moulds were half filled and left to set overnight. The valves were then positioned and orientated identically in the moulds and the remaining half of the mould was filled with resin, thus embedding the shell. Care was taken during this step to eliminate any bubbles that may have form in the resin. The resin was left for three days to harden.

The embedded shells were then examined under a dissecting microscope and a cross section of each shell was lightly marked as a line using a scalpel. The shell was cut into a 1 mm section (0.5 mm either side of the marked line) using a Buehler Isomet low speed diamond saw. Any excess resin was cut away with scissors and the section was mounted onto a microscope slide using double sided tape. The mounted sections were then ready for laser ablation (Figure 2.2). Ten sectioned shells were mounted on each slide. This reduced the need to open the sampling chamber on the ICP-MS machine between samples. The prepared samples were rinsed three times with Milli-Q water and dried overnight. They were then stored in dust free containers until ablation.

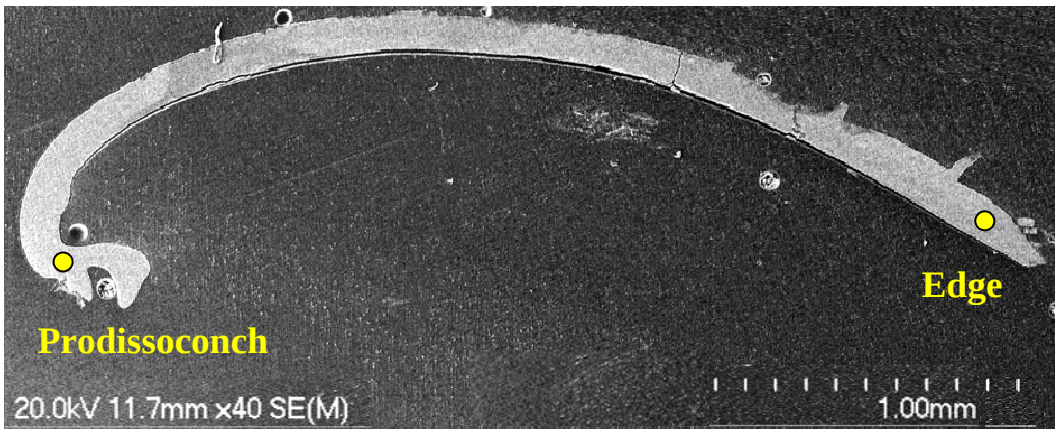


FIGURE 2.2. View of a 1 mm thick shell prepared for LA-ICP-MS (Scanning electron microscope (SEM) image taken at The University of Waikato). The prodissoconch and the shell edge are indicated, with the yellow dots showing the regions that were ablated.

2.4.3 Water and sediment samples (ICP-MS)

The water and sediment sample preparation was based on the EPA 200.2 methods described by Martin et al. (1994) and Trolle et al. (2008) and are outlined below. These methods best suited the University of Waikato laboratory equipment and conditions (D. Trolle, pers. comm., 2008).

2.4.3.1 Water samples

The water samples were centrifuged at 4000 revolutions min^{-1} for ten minutes to concentrate any suspended material in the bottom of the 50 ml falcon tubes. Subsequently, 1 ml of the sample was pipetted into a 15 ml falcon tube (with care being taking not to pipette any settled material that had accumulated in the bottom of the tube) and was diluted with 9 ml Milli-Q water to achieve a 1:10 dilution. One drop of concentrated hydrochloric acid (HCl) was then added to the sample to dissolve any metals. The lids were closed until finger tight and the tubes were inverted to mix the contents. Tubes were then stored for ICP-MS analysis.

2.4.3.2 Sediment samples

The preparation of sediment samples was more complex than that of the water. Firstly, nitric acid (HNO_3) and HCl were diluted to achieve a 1:2 and a 1:5 dilution, respectively. Approximately 0.5 g of dry sediment from each site was added to 50 ml falcon tubes, and 5 ml of the diluted HNO_3 and 15 ml of the diluted HCl were added to extract and dissolve metals from the sediment (for exact sediment weights see Appendix A.2). The lids were closed until finger tight and the tubes were agitated to mix the contents well. The closed tubes were transferred to a rack and left in the fume hood until the following day.

The next day, a water bath was preheated to 90 °C and the racks containing the samples were placed in the water bath for 2 h. After 2 h, the samples were removed from the water bath and were placed in the fume hood for approximately

24 h. This allowed the samples to cool to room temperature and also gave the suspended material time to settle to the bottom of the tube. Subsequently, 0.25 ml of liquid was extracted from each sample and transferred to a 15 ml falcon tube (care was taken to ensure no sediment or suspended material was transferred during this process). These samples were diluted with 9.75 ml Milli-Q water to create 1:40 dilution. Samples were then stored for ICP-MS analysis.

2.5 Sample analysis

The analysis of the digested whole shells, the point ablation, the water and the sediment samples were performed using a Perkin Elmer Elan SCIEX DRC II mass spectrometer unit at the University of Waikato. Digested shells, water and sediment samples were analysed by solution based ICP-MS, whilst the shells used in the point ablation study were analysed by laser ablation ICP-MS.

Laser technology has only recently developed the ability to ablate tiny fragments from specific locations on the shell instead of obtaining mean elemental signatures from digested whole shell samples. Each part of the shell corresponds to a different stage of the individual's development. With the recent advances in laser technology various parts of the shell can be analysed separately. For the purposes of this study, two regions of each shell were analysed: the prodissoconch (or larval shell); and the edge of the shell (most recently formed material). Small fragments from these two regions were ablated and subsequently vaporised. The vaporised shell material was then transported from the sample chamber to the high resolution ICP-MS by a carrier gas consisting of a mixture of argon and helium. The ICP-MS hereby obtained the chemical signatures of precise areas of the shell.

The laser was optimised and warmed up for approximately half an hour prior to analysis, which allowed the laser energy to stabilise. A glass standard treated with trace elements according to National Institute of Standards and Technology Standard Reference Material 612 (NIST-612) was run twice before, during and after each run as an external standard. NIST-612 was run to optimise the ICP-MS and to correct for any machine drift. Accepted concentrations of NIST-612 were

taken from Pearce et al. (1997). The calcium isotope ^{42}Ca was used as an internal standard to correct for instrumental drift (Vander Putten et al., 2000). Vander Putten et al. (1999) showed that a precision of < 10% relative standard deviation is achieved when using NIST-612 as an external and ^{42}Ca as an internal standard. The laser operating conditions varied slightly between the shells and the external standard (Table 2.2).

TABLE 2.2. The laser operating conditions for juvenile *Austrovenus* shells and NIST-612 standard material analysis by LA-ICP-MS.

Sample	Spot size (μm)	Laser output (%)	Laser dwell time (s)	Repetition rate (Hz)
Juvenile shells	30	60	40	10
NIST-612 external reference	60	60	60	10

Previous studies have highlighted a range of isotopes that are useful for elemental fingerprinting analyses of invertebrates (Table 2.3). After optimisation, a laser ablation method was set up which comprised a suite of 14 isotopes (^{25}Mg, ^{42}Ca, ^{43}Ca, ^{52}Cr, ^{55}Mn, ^{66}Zn, ^{75}As, ^{85}Rb, ^{87}Sr, ^{88}Sr, ^{133}Cs, ^{137}Ba, ^{208}Pb, ^{238}U). This suite was selected because the isotopes had minimal isobaric interference with the carrier gas (Ar). For the spatial whole shell digestion and the temporal study, isotopic values were converted to element:Ca ratios. The isotopic values for the water and sediment samples were converted to elemental concentrations (ppb).

For the ablation study, each shell was ablated at the dorsal apex (where the prodissoconch had formed) and subsequently on the outer ventral margin of the shell (where the most recently formed shell material occurs) (see Figure 2.2). The elemental concentrations in the shells (in ppm) were calculated using GLITTER LA-ICP-MS data reduction software (Version 4.4.1., Macquarie Research Limited © 1991 – 2000). The concentration conversion used concentrations of NIST-612 (both published concentrations and concentrations obtained during ablation within this study), as well as the calcium oxide (CaO) composition of aragonitic material (56.03%) (Mineralogy Database, 2005), as juvenile shells largely consist of aragonite (Vander Putten et al., 1999).

TABLE 2.3. Previous studies of elemental fingerprinting applied to invertebrates, indicating the class and developmental phase of the study species (L = larvae; J = juveniles; A= adults), the number of collection sites, the spatial distance covered in the study and the elements that were used. The study in bold indicate the current study.

Reference	Class of study species	Number of sites	Spatial distance covered	Elements used
Lorens & Bender, 1980	Bivalvia	Lab culture	Lab culture	Mg, Sr, Na, Ca, S
Boyden & Phillips, 1981	Bivalvia (A)	3	Unknown	Cd, Co, Cu, Zn, Fe, Pb, Mn, Ni
Stecher et al., 1996	Bivalvia (A)	2	Unknown	Sr, Ba, Ca
Vander Putten et al., 1999; 2000	Bivalvia (A)	1	0 km	Mg, Mn, Sr, Ba, Pb, Ca
DiBacco & Levin, 2000	Malacostraca (L)	15	80 – 100 km	Cu, Zn, Mn, Sr, Ca, Al, Mg
Richardson et al., 2001	Bivalvia (A)	2	~ 60 km	Cu, Zn, Pb
Lazareth et al., 2003	Bivalvia (A)	3	150 km	Mg, Mn, Sr, Ba
Zacherl et al., 2003a	Gastropoda (L)	Lab culture	Lab culture	Ba, Sr, Ca
Zacherl et al., 2003b	Gastropoda (L)	3	Unknown	Ba, Zn, Pb, Ca
Richardson et al., 2004	Bivalvia (A)	3	~ 50 km	Mg, Sr, Ca
Zacherl, 2005	Gastropoda (L)	7	~ 360 km	Mg, Sr, Ba, Ca, Pb, Mn, Ce, Cd, U, Zn
Gillikin et al., 2005	Bivalvia (A)	9	~ 10 km	Ca, Sr
Becker et al., 2005	Bivalvia (J)	7 (+1 for spatial)	~ 50 km	Mn, Sr, Pb, Ba, U, Mg, Ca, Zn
Carré et al., 2006	Bivalvia (A)	3	~ 2000 km	Sr, Mg, Ba, Mn, Ca
Becker et al., 2007	Bivalvia (L,J)	13	~ 60 km	Ca, Mn, Co, Sr, Ba, Pb, U, Sn
Strasser et al., 2007	Bivalvia (J)	Lab culture	Lab culture	Ba, Ca
Strasser et al., 2008	Bivalvia (J)	Lab culture	Lab culture	Mn, Mg, Ba, Pb, Sr, Ca
Klünder et al., 2008	Bivalvia (A)	N/A	N/A	Mn, Pb, Ba, Mg, Ca
This study	**Bivalvia (J)**	**19**	**~ 1500 km**	**Ca, Sr, Mn, Mg, Ba, Zn**

Different combinations of elements were used for the solution based whole shell digestion, the temporal and the point ablation studies, depending on which elements yielded detectable results and showed variability among the sites (Table 2.4).

TABLE 2.4. The elemental ratios and concentrations used during ANOVA and DFA analyses of the shells for the whole shell digestion, the temporal and the point ablation studies.

Study	ANOVA	DFA
Whole shell digestion	Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, Zn:Ca	Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, Zn:Ca
Temporal variation	Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, Zn:Ca	Sr:Ca, Ba:Ca, Zn:Ca
Point ablation	Sr, Ba, Mn, Mg	Sr, Ba, Mn, Mg

2.6 Statistical analysis

All statistical analyses were carried out using Statistica 8 software. Prior to any analysis, outliers were removed independently for each study. Concentrations of the five main elements analysed (Sr, Ba, Mn, Mg, Zn) were used to detect outliers. Elemental ratios and concentrations that fell outside two standard deviations of the mean for each sample site in each study were considered outliers and the entire shell was excluded from any further statistical analysis; values that fell within this margin were included in all further analyses (Strasser et al., 2008). Initially, 15 shells were analysed from each site for the spatial and temporal solution based digestion studies. Once outliers were excluded, 4 – 13 shells remained per site. Thus, sites where only a small number of shells remained (e.g. CLB = 4, WHP = 6 (see Table 3.1)), exhibited considerable variation in chemical signatures. For the point ablation study, only ten shells were analysed from each site. Each shell was ablated once at the edge and once at the prodissoconch. Only the edge concentrations were used to identify outliers, as the edge material should be consistent for all shells collected from any given site. The chemistry of the prodissoconch may vary between individuals from the same site if the shells formed in different water masses. It was these variations that were of interest and therefore the prodissoconch was not used to determine outliers.

Parametric statistical tests, including analysis of variance (ANOVA) and Tukey's HSD (honestly significant differences) post-hoc tests were used to investigate if the variation in the chemistry of shells collected from different sites was significant. Where element to calcium ratios or elemental concentrations did not satisfy the assumptions of normality or homogeneity of variance (HOV), the data were transformed. Consequently, all the ratios, with the exception of Sr:Ca (Shapiro-Wilks $W = 0.99$; $p = 0.06$), were $\log(x + 1)$ transformed for the spatial whole shell digestion study to improve normality. Log_{10} transformations of the temporal data did not result in normality or HOV (Shapiro-Wilks W = 0.51 – 0.97; $p = < 0.001 – 0.049$; Levene's test $F = 5.52 – 36.10$; $p < 0.0005 – 0.001$). Thus, normal one-way ANOVA's could not be calculated and an alternative non-parametric ANOVA (Kruskal-Wallis) was used to establish if significant variation existed among collection dates. For the point ablation study, the elemental concentrations were calculated for each element using GLITTER LA-ICP-MS data reduction software. All data were log_{10} transformed to ensure that the elemental concentrations satisfied the assumptions of normality (Shapiro-Wilks W = 0.92 – 0.98; $p = 0.051 – 0.902$). Sr, Ba and Mg also satisfied the assumption of HOV (Levene's test $F = 0.29 – 2.76$; $p = 0.069 – 0.834$), but Mn did not display homogeneous variance (Levene's test $F = 9.04$; $p = 0.001$).

To further analyse the variation between sites, a multivariate multistep discriminant function analyses (DFA) was used to examine the elemental concentrations of shells. Forward stepwise DFA was carried out for all the elemental ratios and the least significant variable, as determined by the F to remove statistic, was eliminated. The DFA was then run again, and the next-least significant variable was removed. This process was repeated until the F to remove statistic for each of the elemental ratios included was > 3.5 (Becker et al., 2005). For the spatial whole shell digestion study Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca and Zn:Ca ratios were included in the analysis, whilst for the temporal study, only Sr:Ca, Ba:Ca and Zn:Ca had F to remove ratios > 3.5, and were subsequently included in the DFA. The point ablation study only included Sr:Ca, Ba:Ca, Mn:Ca and Mg:Ca (Table 2.4) as Zn was only present in very small quantities.

Previous studies that have employed DFA have used a smaller number of classifying variables (i.e. number of sites), with most studies comparing the chemistries of shells collected from < 10 sites (Table 2.3). In this study, the effect of the number of sites on the discriminating power of DFA was tested. Initially, DFA was used in an attempt to classify all 19 sites against each other. The returned results showed little variability among the sites and the discriminating power was low, with only 68% of the shells correctly classifying to their collection site (Figure 2.3). Poulsen and French (2003) stated that DFA requires the sample size (n) for the smallest group (i.e. site) to at least exceed the number of predictor variables (i.e. elemental ratios) used, which in this case was five (Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, Zn:Ca). This requirement resulted in the elimination of site CLB ($n = 4$) from the DFA analysis.

It was also reported that it is desirable, but not essential, for the sample size to be four or five times greater than the number of predictor variables (Poulsen & French, 2003). This was not possible as no sites had $n \geq 20$ shells. Using these criteria as a guideline, 12 of the original 19 sites had at least twice the number of predictor variables (i.e. $n \geq 10$ shells) and were subsequently selected for DFA to examine the variation across sites. The DFA analysis resulted in 75% of the shells correctly classifying to their collection sites (see results). To examine the effect that the number of sites used in each analysis had on the classification success, DFA was carried out using ten, five and three sites. For each analysis, the sites were obtained by random selection, and five replications were performed for each given number of sites (see Appendix A.3). As the number of sites decreased the proportion of shells that correctly classified to their collection site increased (Figure 2.3). Where the number of sites was ≤ 5, the average classification success was greater than 90%, indicating a high discrimination power. Consequently, five or less sites were used for the spatial and temporal comparisons, except where all 12 sites with $n \geq 10$ shells were presented.

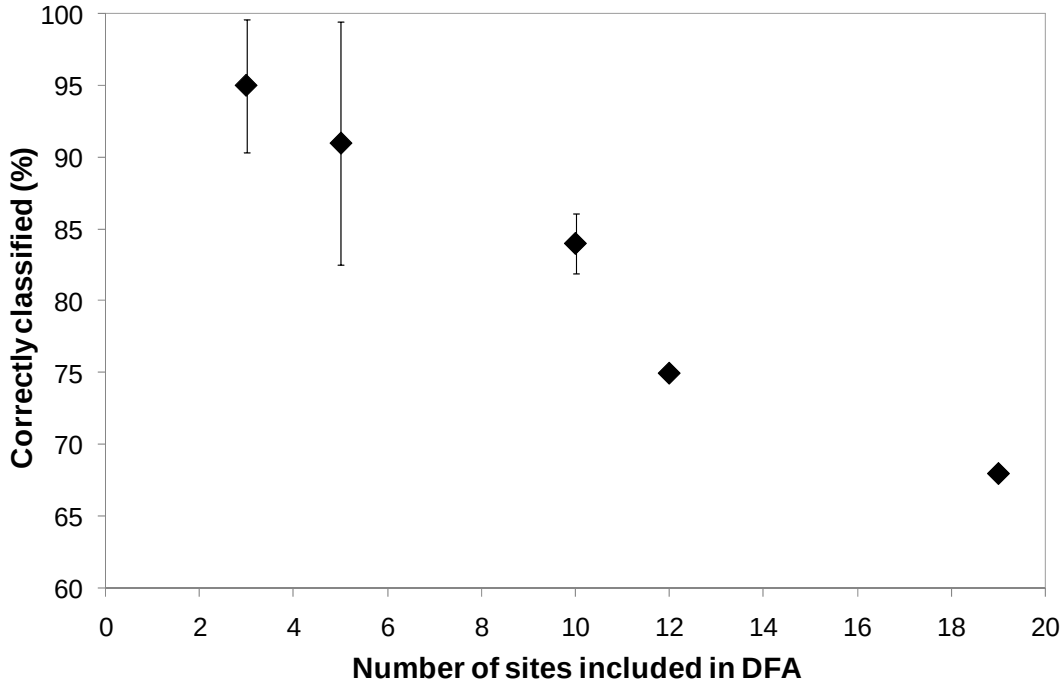


FIGURE 2.3. A scatterplot showing the effect that the number of sites included in the analysis had on the classification success of the DFA. Error bars indicate 95% confidence intervals, and were absent from 12 and 19 sites as no replicates were carried out for these number of site comparisons.

3.0 CHAPTER THREE: RESULTS

3.1 Spatial variation in elemental fingerprints (shell digestion)

Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca and Zn:Ca consistently yielded detectable concentrations and showed variability among the sites sampled. The average concentrations of five elemental:Ca ratios were calculated at each site (Table 3.1). One-way ANOVA results indicated significant differences existed in elemental ratios among sites (ANOVA Sr:Ca $F_{18.174} = 38.76$, $p < 0.001$; Ba:Ca $F_{18.174} = 40.70$, $p < 0.001$; Mn:Ca $F_{18.174} = 23.93$, $p < 0.001$; Mg:Ca $F_{18.174} = 21.06$, $p < 0.001$; Zn:Ca $F_{18.174} = 36.26$, $p < 0.001$). HSD post-hoc tests outline where the variation between sites occurred (Appendix A.4).

TABLE 3.1. Mean (± 1 SE) element to calcium ratios in juvenile *Austrovenus* shells collected from the North Island of New Zealand. For site names and locations refer to Figure 2.1 and Table 2.1. Sites in bold had $n > 10$ shells and were included in the analysis presented in Figure 3.1 and Table 3.2.

Site	*n*	Sr:Ca (mmol mol^{-1})	Ba:Ca (μmol mol^{-1})	Mn:Ca (mmol mol^{-1})	Mg:Ca (mmol mol^{-1})	Zn:Ca (mmol mol^{-1})
PAR	12	2.40±0.05	0.59±0.01	0.00±0.00	0.26±0.00	0.01±0.00
MAN	9	2.50±0.03	0.69±0.02	0.03±0.02	0.25±0.01	0.10±0.01
HRK	12	2.47±0.04	0.65±0.01	0.02±0.01	0.24±0.00	0.02±0.00
PAI	12	3.35±0.06	0.77±0.01	0.01±0.00	0.28±0.00	0.05±0.00
HOK	7	2.42±0.06	0.84±0.03	0.04±0.01	0.24±0.00	0.05±0.01
WHR	12	2.64±0.06	0.61±0.01	0.04±0.01	0.26±0.01	0.01±0.00
TIN	13	2.50±0.05	0.95±0.01	0.08±0.02	0.27±0.01	0.02±0.00
WHT	13	2.72±0.02	0.86±0.03	0.00±0.00	0.29±0.01	0.01±0.00
WHP	6	2.99±0.07	1.03±0.04	0.00±0.00	0.31±0.01	0.01±0.00
HDI	11	2.92±0.02	0.04±0.04	0.01±0.00	0.26±0.01	0.09±0.02
GLD	7	3.03±0.04	0.53±0.04	0.07±0.02	0.32±0.01	0.02±0.00
PAN	9	2.80±0.07	0.74±0.02	0.03±0.01	0.29±0.01	0.03±0.01
TIR	13	3.08±0.04	0.65±0.06	0.40±0.06	0.39±0.02	0.03±0.00
ARP	13	2.72±0.03	0.40±0.07	0.02±0.00	0.30±0.01	0.17±0.01
CLB	4	3.11±0.05	0.27±0.07	0.01±0.00	0.31±0.00	0.03±0.00
OTU	10	2.76±0.04	0.72±0.01	0.00±0.00	0.32±0.01	0.04±0.01
RAG	8	2.45±0.05	0.62±0.01	0.02±0.01	0.22±0.01	0.07±0.00
AOT	12	3.05±0.02	0.47±0.03	0.02±0.00	0.30±0.01	0.02±0.00
KAW	10	3.15±0.06	0.43±0.05	0.05±0.01	0.30±0.01	0.03±0.01

After outliers were removed, 12 sites had more than ten shells that remained available for the analyses (Table 3.1), and were included in the DFA. On average, shells from these 12 sites had a successful discrimination of 75% (Table 3.2). Both PAI and OTU had a 100% classification success, with eight out of the 12 sites showing > 82% classification success. PAR, HRK and WHR primarily misclassified to each other, whilst KAW only misclassified to AOT. The latter two sites are geographically close to one another, whilst PAR, HRK and WHR are separated by considerable stretches of coastline.

TABLE 3.2. Classification success using the chemistry of juvenile *Austrovenus* shells collected from 12 sites. Rows list the actual sites, whilst the columns list the sites predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.1.

Actual	Predicted site												Total	% Correctly
site	PAR	PAI	HRK	WHR	TIN	WHT	HDI	TIR	ARP	OTU	AOT	KAW	per site	classified
PAR	7		1	4									12	58
PAI		12											12	100
HRK	2		7	3									12	58
WHR	2		5	3							2		12	25
TIN					11	2							13	85
WHT						11				2			13	85
HDI							9		1			1	11	82
TIR								11			1	1	13	85
ARP									11	2			13	85
OTU										10			10	100
AOT											10	2	12	83
KAW											5	5	10	50
Total	**11**	**12**	**13**	**10**	**11**	**13**	**9**	**11**	**12**	**14**	**18**	**9**	**143**	**75**

To investigate the groupings indicated by the classification matrix, the discriminant function scores were plotted as a scatterplot (Figure 3.1A). Score 1 and score 2 represented 47.5% and 23.7% of the variation, respectively. Sites did not appear to group based on their geographical location (e.g. east coast/west coast). The mean scores for the analysis were also plotted, highlighting two distinct groups of sites where individuals overlapped on both the first and second scores (Figure 3.1B). Group 1 consisted of four sites (PAR, HRK, WHR and WHT); Group 2 comprised three sites (PAI, AOT and KAW). Within each group, the chemical signatures of shells from the three or four different sites appeared to be very similar, thus resulting in a less accurate classification power and considerable misclassification among the sites (Table 3.2 and Figure 3.1B).

The DFA scores for the first group was interesting, as the three sites that previously misclassified to each other in the classification matrix (PAR, HRK and WHR, see Table 3.2) were not the same sites that grouped very close together when the discriminant scores were plotted (PAR, HRK, WHT, see Figure 3.1B). The two groups were investigated separately from the rest of the sites (Group 1: Table 3.3 and Figure 3.2; Group 2: Table 3.4 and Figure 3.3).

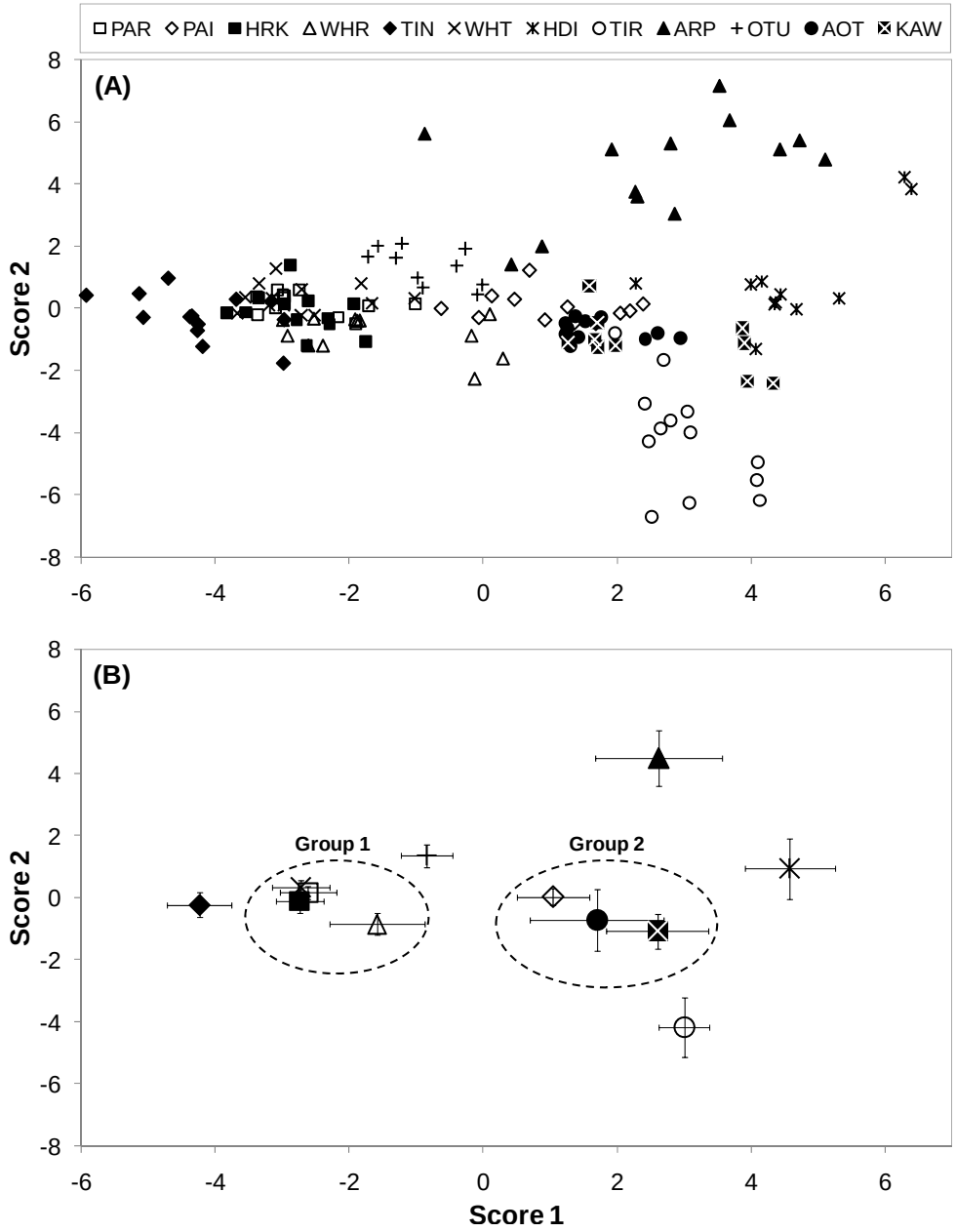


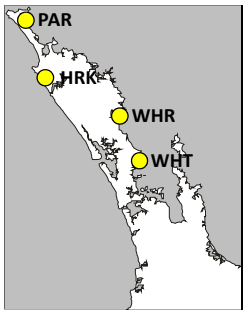
FIGURE 3.1. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles. Sites are listed from north to south in the legend. Filled shapes denote west coast sites, whilst unfilled shapes and crosses signify east coast sites. (A) Scatterplot of DFA scores; (B) data from (A) plotted as averages (± 95% confidence intervals).

After running the DFA using only the four sites from Group 1, it became apparent that these sites could be successfully classified (86% overall classification success). HRK and WHR were both classified with a 75% success rate. Interestingly, both of these sites misclassified to each other and to PAR, but PAR only slightly misclassified to HRK. WHT shells had a classification success of 100%, suggesting that the elemental signatures of shells from this site are unique compared to the three sites tested in this sub-analysis (Table 3.3).

The discriminant scores show similar trends to the classification matrix and reiterate the close grouping between HRK and WHR (Figure 3.2A and B). Within this smaller group, WHT appeared to be distinctly different from the other three sites (Figure 3.2A and B).

TABLE 3.3. Classification success using the chemistry of juvenile *Austrovenus* shells collected from four sites: PAR, HRK, WHR and WHT (Group 1, Figure 3.1B). Rows list the actual sites, whilst the columns list the sites predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.2.

	Predicted site				Total per site	% Correctly classified
	PAR	**HRK**	**WHR**	**WHT**		
Actual site						
PAR	11	1			12	92
HRK	1	9	2		12	75
WHR	2	1	9		12	75
WHT				13	13	100
Total	**14**	**11**	**11**	**13**	**49**	**86**



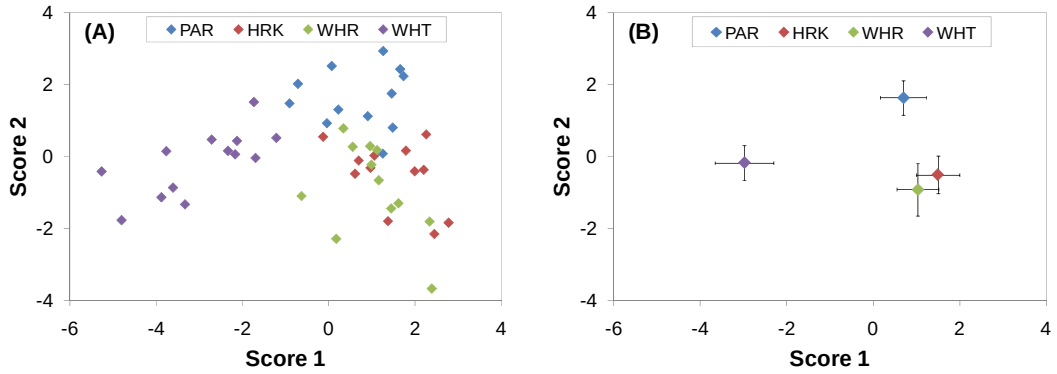


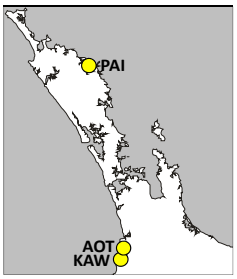
FIGURE 3.2. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles. Sites are listed from north to south in the legend, with each colour denoting a specific site. (A) Scatterplot of DFA scores; (B) data from (A) plotted as averages ± 95% confidence intervals.

Group 2 (PAI, AOT, KAW) also showed that shells from these sites could be successfully classified, with an overall classification success of 91% (Table 3.4 and Figure 3.3). Both PAI and AOT showed 100% classification success, whilst KAW correctly classified 70% of the time. Interestingly, all three of the individual shells that were misclassified for KAW were misclassified to AOT, the most closely neighbouring estuary to KAW (Table 3.4).

The discriminant function scores produced for both PAI and AOT showed that these sites had very different chemical signatures, with little variation in the shell chemistries at both sites when compared in a smaller sub-group (Figure 3.3A and B). The shell chemistries from the individuals collected at KAW however, did not have a tight grouping and showed considerable variation about the mean (Figure 3.3B). The misclassification of the KAW shells to AOT is again reiterated in Figure 3.3A.

TABLE 3.4. Classification success using the chemistry of juvenile *Austrovenus* shells collected from three sites: PAI, AOT and KAW (Group 2, Figure 3.1B). Rows list the actual sites, whilst the columns list the sites predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.3.

	Predicted site			Total per site	% Correctly classified
	PAI	**AOT**	**KAW**		
Actual site					
PAI	12			12	100
AOT		12		12	100
KAW		3	7	10	70
Total	**12**	**15**	**7**	**34**	**91**



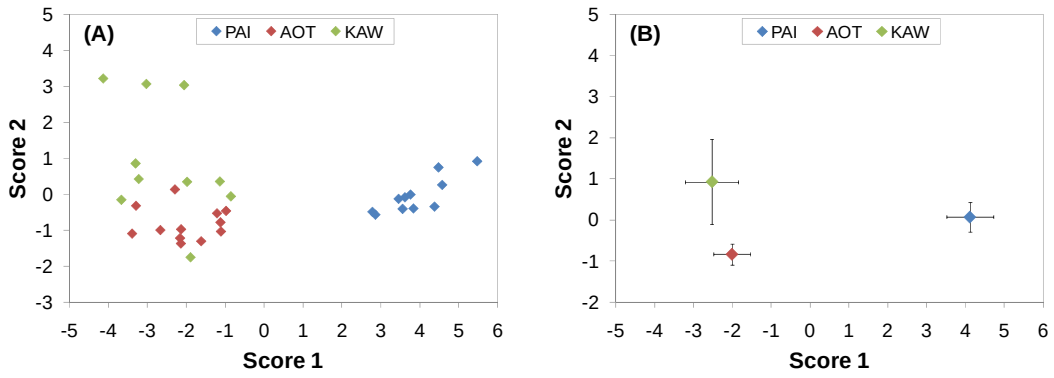


FIGURE 3.3. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles. Sites are listed from north to south in the legend, with each colour denoting a specific site. (A) Scatterplot of DFA scores; (B) data from (A) plotted as averages ± 95% confidence intervals.

3.1.1 Variation over different spatial scales

In order to investigate the distances over which elemental fingerprinting may be applicable, two spatial scales (large and small) were investigated. The large spatial scale covered estuaries separated by at least 520 km of coastline, whilst the small spatial study investigated the signatures of three sites within a single estuary, as well as three sites situated less than 50 km apart. Three randomly chosen sites were used for each analysis and sites from both the east and west coast were included.

3.1.1.1 Large spatial scale

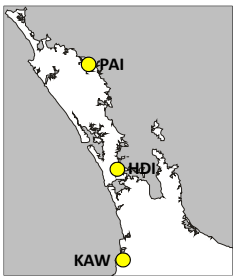
The first group of three sites investigated for the large spatial scale variation was PAI, HDI and KAW. These sites were situated over roughly 1150 km of coastline, with PAI and HDI located on the east coast, and KAW on the west coast. The classification success for these three sites was very good, with an overall success rate of 97%. Both PAI and KAW had 100% classification success, whilst only one of the eleven shells collected from HDI were misclassified and grouped with KAW (Table 3.5A; Figure 3.4A and B).

The second group of sites (MAN, WHT and OTU) were situated over a 770 km distance, with all three sites located on the east coast. The third group of sites (PAR, TIN and AOT) covered approximately 520 km of coastline, with PAR being situated on the east coast, whilst TIN and AOT were both located on the west coast. All of the sites from both the second and third groups had a 100% classification success, suggesting marked differences among the shell chemistries of the shells found at these locations (Table 3.5B and C and Figure 3.4C – F).

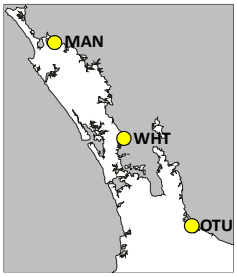
A total of 102 shells were analysed across all three groups. Overall, estuaries showed an exceptionally high average classification success over large spatial scales (99%), with eight out of the nine sites having a 100% success rate (Table 3.5A – C).

TABLE 3.5. Classification success using the chemistry of juvenile *Austrovenus* shells collected from three sites situated over a large spatial scale. (A) PAI, HDI and KAW; (B) MAN, WHT and OTU; and (C) PAR, TIN and AOT. Rows list the actual sites, whilst the columns list the sites predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.4A – F.

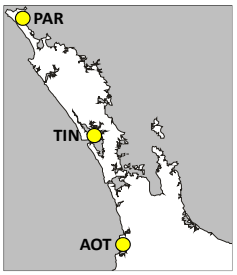
(A)	Predicted site			Total per site	% Correctly classified
	PAI	HDI	KAW		
Actual site					
PAI	12			12	100
HDI		10	1	11	91
KAW			10	10	100
Total	**12**	**10**	**11**	**33**	**97**



(B)	Predicted site			Total per site	% Correctly classified
	MAN	WHT	OTU		
Actual site					
MAN	9			9	100
WHT		13		13	100
OTU			10	10	100
Total	**9**	**13**	**10**	**32**	**100**



(C)	Predicted site			Total per site	% Correctly classified
	PAR	TIN	AOT		
Actual site					
PAR	12			12	100
TIN		13		13	100
AOT			12	12	100
Total	**12**	**13**	**12**	**37**	**100**



The scatterplots showing the resulting DFA scores are presented in Figure 3.4 and reiterate the clear distinction in the chemical signatures of juvenile *Austrovenus* shells over large spatial distances. There was little variation about the mean for all of the sites, with very little or no crossover between shells collected from different sites. HDI was the only site that showed any misclassification, with a single shell that misclassified to KAW (Table 3.5A and Figure 3.4A). PAR, TIN and AOT showed the greatest distinction between sites and the smallest overall variation within sites (indicated by the error bars) (Figure 3.4E and F).

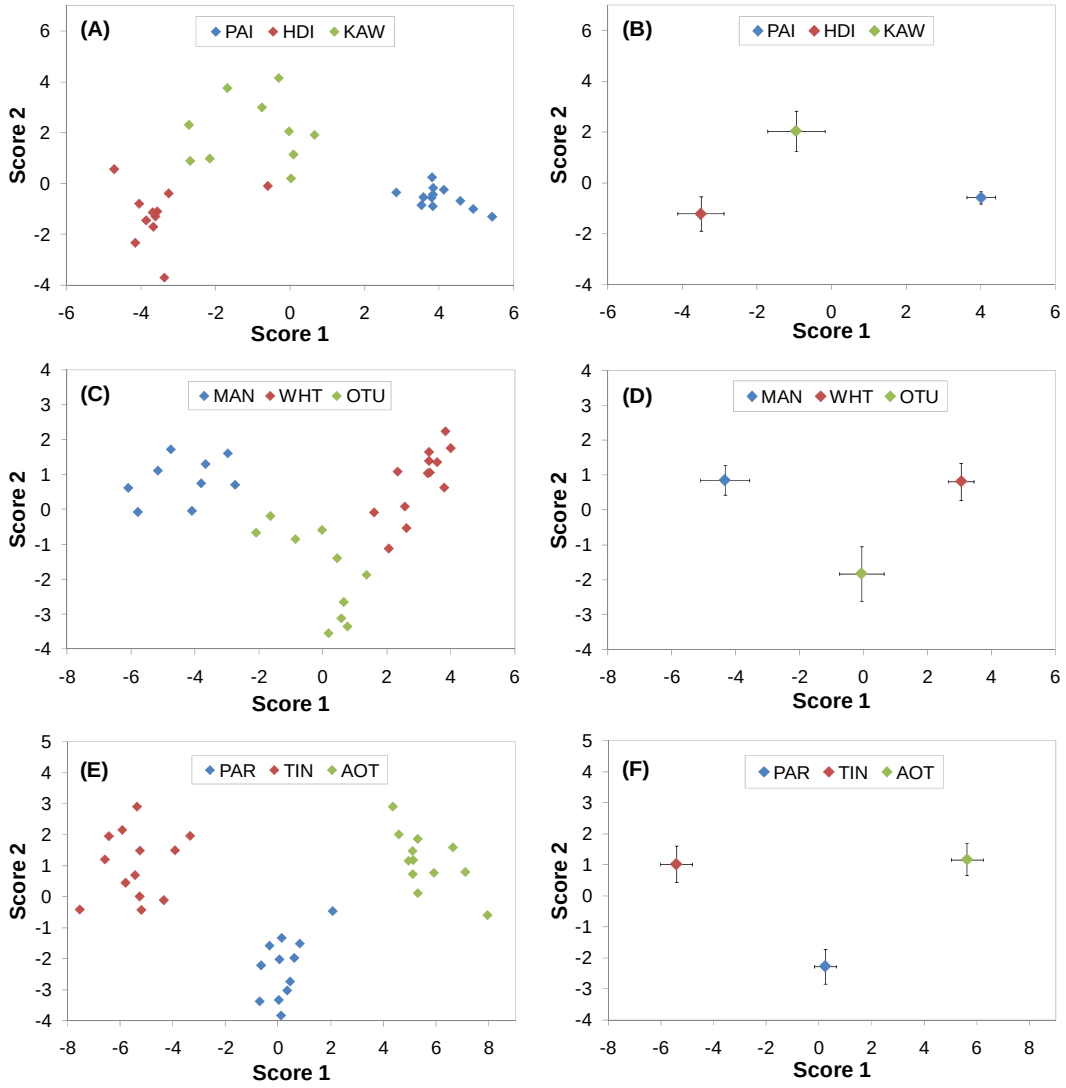


FIGURE 3.4. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles. (A) Scatterplot of DFA scores for PAI, HDI and KAW; (B) data from (A) plotted as averages ± 95% confidence intervals; (C) scatterplot of DFA scores for MAN, WHT and OTU; (D) data from (C) plotted as averages ± 95% confidence intervals; (E) scatterplot of DFA scores for PAR, TIN and AOT; (F) data from (E) plotted as averages ± 95% confidence intervals.

3.1.1.2 Small spatial scale

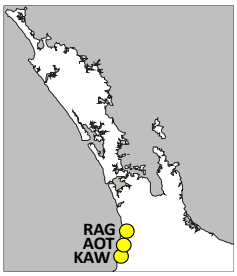
Sites used to determine the feasibility of using elemental fingerprinting techniques over small spatial scales were selected based on their close proximity to each other. Of the 19 sites sampled, only two groups (consisting of three sites each) were located close enough to one another to be compared as part of the small spatial scale study.

RAG, AOT and KAW are situated along approximately 50 km of coastline on the west coast of New Zealand. The classification success for these three sites was very good, with an overall success rate of 90%. Both RAG and AOT had a 100% classification success. KAW had a lower classification success of 70% (Table 3.6A). As previously found in the 12 site analysis and the group of three analysis of sites with similar discriminant scores, all three of the shells that were misclassified from KAW, misclassified to AOT (refer to Table 3.4 and Table 3.6A).

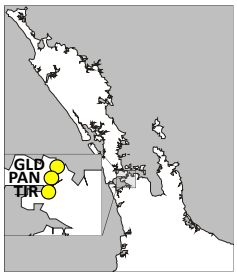
Within estuary comparisons were made using the three collection sites in Tamaki Estuary (GLD, PAN and TIR) which is situated on the east coast of New Zealand. The distance between GLD (most northern) and TIR (most southern) is approximately 10 km. For GLD, PAN and TIR the overall classification success was 89% (Table 3.6B). PAN had a 100% classification success, whilst GLD and TIR had an 86% and 83% classification success respectively, with both sites only misclassifying to each other (Table 3.6B).

TABLE 3.6. Classification success using the chemistry of juvenile *Austrovenus* shells collected from three sites situated over a small spatial scale (A) RAG, AOT and KAW (three nearby estuaries); and (B) GLD, PAN, TIR (within the same estuary). Rows list the actual sites, whilst the columns list the sites predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.5A – D.

(A)	Predicted site			Total per site	% Correctly classified
	RAG	AOT	KAW		
Actual site					
RAG	8			8	100
AOT		12		12	100
KAW		3	7	10	70
Total	**8**	**15**	**7**	**30**	**90**



(B)	Predicted site			Total per site	% Correctly classified
	GLD	PAN	TIR		
Actual site					
GLD	6		1	7	86
PAN		9		9	100
TIR	2		10	12	83
Total	**8**	**9**	**11**	**28**	**89**



The resulting DFA scores for comparisons of small spatial scales highlighted the variation in the chemical signatures of shells collected from KAW, and the

misclassification of KAW shells to AOT (Figure 3.5A and B). Similarly, Figure 3.5C and D indicate the misclassification between GLD and TIR juvenile shells, with these two sites being noticeably different from PAN along score 1. This was surprising, as PAN is situated in between GLD to the north and TIR to the south.

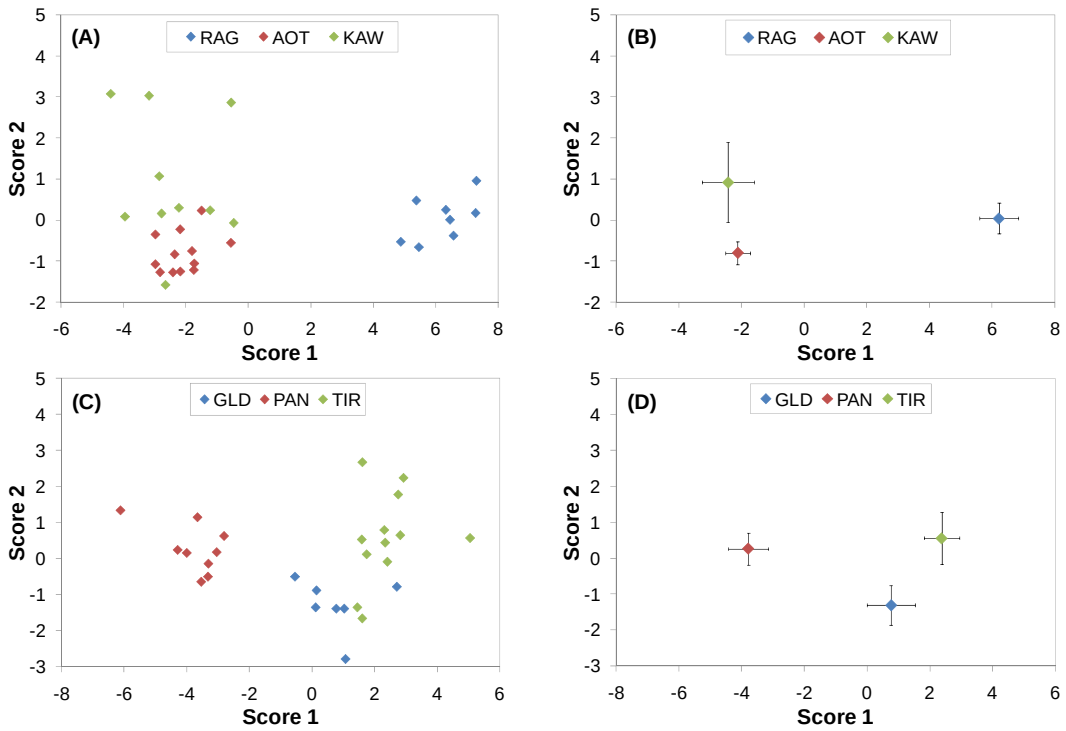


FIGURE 3.5. Discriminant function scores of element (Sr, Ba, Mn, Mg, Zn) to Ca ratios in shells of *Austrovenus* juveniles. (A) Scatterplot of DFA scores for RAG, AOT and KAW; (B) data from (A) plotted as averages (± 95% confidence intervals; (C) scatterplot of DFA scores for GLD, PAN and TIR; (D) data from (C) plotted as averages ± 95% confidence intervals.

3.1.2 Shell, water and sediment elemental comparisons

Average shell elemental concentrations were plotted against the concentrations of elements in both the water and sediment to determine if any correlation between the elemental composition of the shells and the environment existed (Figure 3.6). There were no significant correlations ($p < 0.05$) between the elemental concentrations of shells compared to that of water or sediment. This indicated that neither the water nor sediment chemistries had any significant relationship with the shells, however only a single water and sediment sample was analysed for each site, which may have confounded the results.

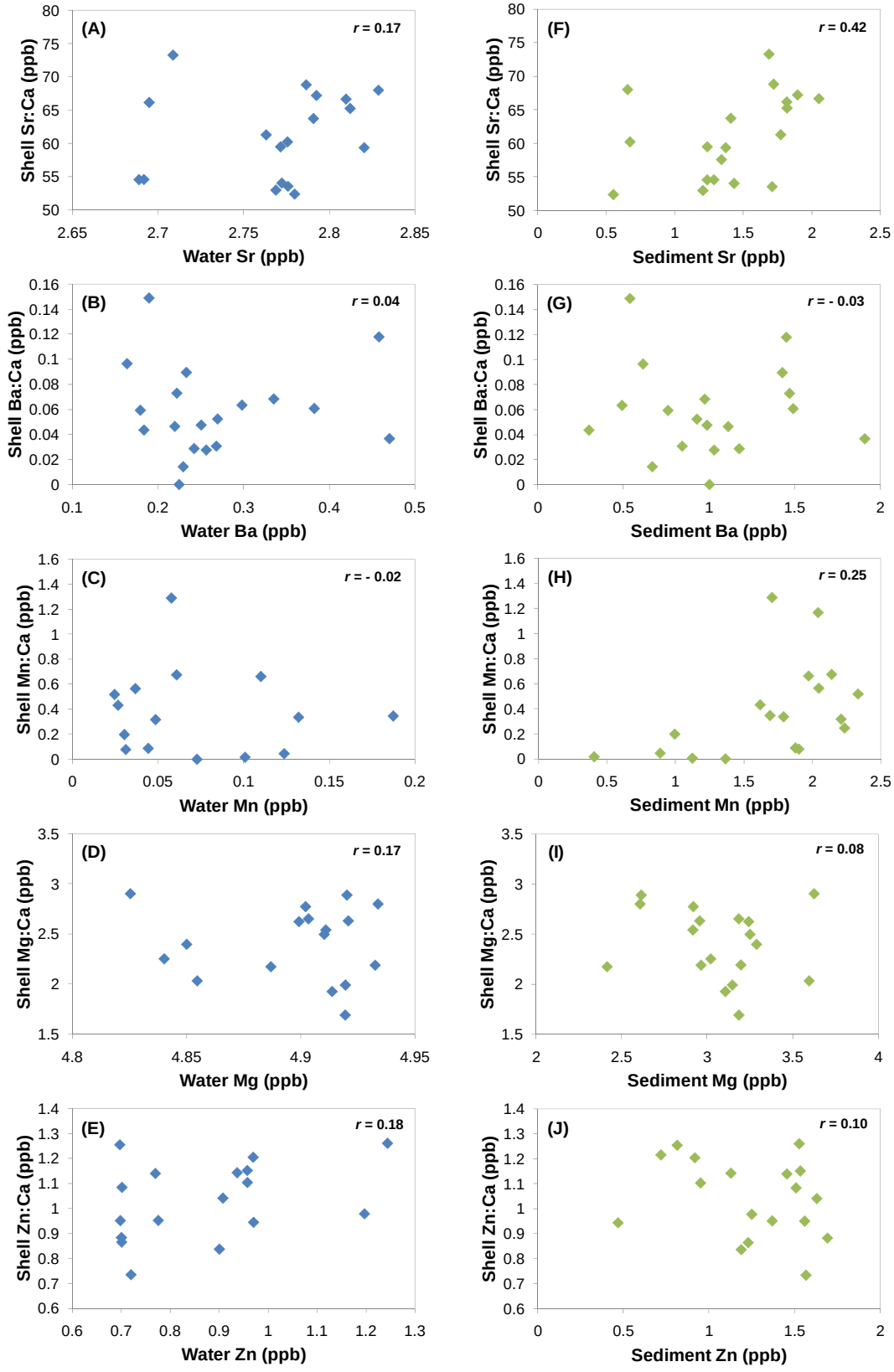


FIGURE 3.6. Scatterplots showing the correlation (r = Pearson correlation coefficient) between the average concentrations of elements in the shells (parts per billion) analysed at each site and the concentrations of elements (parts per billion) in the water and sediment. Plots (A) Sr, (B) Ba, (C) Mn, (D) Mg and (E) Zn compare shell and water concentrations; plots (F) Sr, (G) Ba, (H) Mn, (I) Mg and (J) Zn compare shell and sediment concentrations. None of the correlations were significant ($p < 0.05$).

3.1.3 Environmental factors contributing to variation

Shell chemistries did not group based on geographic location (east coast/west coast) (see Figure 3.1), proximity to one another (see Table 3.3), or water and sediment chemistry (see Figure 3.6). It has been reported that shells should incorporate the chemistry of the ambient environment, particularly the water chemistry (e.g. Thorrold et al., 1997; Bath et al., 2000; Vander Putten et al., 2000; Elsdon & Gillanders, 2003). As only one water and one sediment sample were collected, it may not have been enough to accurately represent the true signatures of the sediment and the water. A different approach for assessing the contribution of environmental factors to *Austrovenus* shell chemistry was therefore needed.

Many environmental variables will directly affect the water and sediment chemistry. These variables were investigated as a final attempt to explain the variability and similarities among shell chemistry. Flint and Rabalais (1980) used a simple linear correlation to determine which environmental conditions made up the niche of polychaete worms. These methods were followed in an attempt to determine which, if any, environmental variables may be responsible for the variation and the groupings observed for discriminant scores 1 and 2 among the sites (i.e. Figure 3.1).

Several studies have outlined a suite of environmental conditions that are specific to my 12 study sites (Heath, 1976; McLay, 1976; Hume et al., 2007; T. Hume pers. comm., 2008). Of all the environmental variables, only two of the catchment land cover variables (urban development and planted forest) were significantly correlated with discriminant score 1 showing a positive and negative correlation, respectively. Discriminant score 2 was not significantly correlated to any environmental variables (Table 3.7).

TABLE 3.7. A summary of the correlation of discriminant function scores with environmental variables that potentially characterise the variation in shell chemistry of *Austrovenus* juveniles. Bold values indicate significant correlations at $p < 0.05$. Data used for this analysis was obtained from T. Hume (pers. comm., 2008).

Environmental variable	**Linear correlation coefficient (*r*)**	
	Discriminant score 1	**Discriminant score 2**
Area (m^2)	0.02	0.44
Perimeter length (m)	0.06	0.33
Estimated mean estuary depth (m)	0.36	0.01
Mean annual river discharge (m^3s^{-1})	-0.04	0.35
Tidal vs river forcing	0.32	0.15
Estuary mouth width (m)	0.18	0.00
Sand (% of high water area)	-0.22	0.19
Mud (% of high water area)	0.03	0.05
Mangrove (% of high water area)	-0.38	-0.12
Land catchment area (km^2)	-0.04	0.36
Largest stream order	0.00	0.27
Mean catchment rainfall ($mm.yr^{-1}$)	-0.01	0.04
Mean runoff ($mm.yr^{-1}$)	0.08	0.05
Urban development (% of catchment)	**0.64**	-0.23
Pasture (% of catchment)	-0.32	0.39
Indigenous forest (% of catchment)	0.04	0.14
Planted forest (% of catchment)	**-0.59**	0.21
Scrub (% of catchment)	-0.55	0.25

3.2 Temporal variation in elemental fingerprints (shell digestion)

The temporal stability of elemental signatures of the shells was assessed to determine if shells collected at different times can be compared with one another. If temporal variations exist then differences observed in shells collected from different sites at different times, may simply be a result of temporal variation, thus making it impossible to quantify the degree of spatial variation. A total of five samples were collected on five different sampling dates between 22 November 2007 and 9 March 2008 (22.11.07, 17.12.07, 09.01.08, 30.01.08, 09.03.08).

Non-parametric statistical tests (Kruskal-Wallis) revealed that Sr:Ca, Ba:Ca, Mn:Ca and Zn:Ca ratios were all significantly different among collection dates (Kruskal-Wallis statistic $p < 0.0001$), whilst Mg:Ca did not vary significantly (Kruskal-Wallis statistic $p < 0.26$). DFA could be computed even when the assumptions of normality and HOV were violated (Manly, 1990). Averaged molar to calcium ratios were used for the remaining analysis of temporal variation in shells.

Between four and 24 shells were analysed for each date and the means (± 1 standard error) are presented in Table 3.8. There were noticeably higher concentrations of Sr:Ca, Ba:Ca and Mn:Ca in the shells of juveniles collected on 22 Nov 2007 compared to the other collection dates. Conversely, shells collected on the final collection date (9 Mar 2008) had the lowest concentrations of Sr:Ca, Ba:Ca and Mn:Ca, but had unusually high concentrations of Zn:Ca.

TABLE 3.8. Mean (± 1 SE) elemental to calcium ratios in juvenile *Austrovenus* shells collected from TUA, grouped by date. Collection spanned over a 15 week period between 22 November 2007 and 9 March 2008.

Collection Date	n	Sr:Ca (mmol mol^{-1})	Ba:Ca (μmol mol^{-1})	Mn:Ca (μmol mol^{-1})	Mg:Ca (mmol mol^{-1})	Zn:Ca (mmol mol^{-1})
22 Nov 2007	19	2.78±0.03	8.27±0.38	24.09±6.34	1.06±0.08	0.02±0.00
17 Dec 2007	10	2.59±0.05	3.04±0.35	8.55±2.69	1.05±0.04	0.04±0.01
09 Jan 2008	17	2.41±0.06	2.43±0.33	4.55±0.78	0.90±0.05	0.03±0.01
30 Jan 2008	24	2.28±0.03	2.44±0.09	3.04±0.19	0.99±0.02	0.09±0.02
09 Mar 2008	4	2.33±0.04	2.21±0.17	1.42±0.55	0.90±0.04	0.34±0.01

Initial DFA revealed that only Sr:Ca, Ba:Ca and Zn:Ca had *F* to remove ratios > 3.5. These three elemental ratios were therefore used in the DFA to determine the temporal stability of juvenile *Austrovenus* shells at TUA. The classification matrix revealed the dates that resulted in the Kruskal-Wallis ANOVA results concluding that there was significant temporal variability. The earliest (22.11.07) and latest (09.03.08) collection dates were both 100% correctly classified, although it should be noted that only four shells were analysed on 09.03.08 (Table 3.9). The three collection dates that fell between the first and the last date (17.12.07, 09.01.08, 30.01.08), however, had low classification success (41– 63%) and usually misclassified to the collection dates either side of the given date (i.e. 09.01.08 misclassified to 17.12.07 and 30.01.08; and 30.01.08 misclassified to 09.01.08 and 09.03.08). The low classification success achieved for these three dates suggest very little temporal variation across these three dates (17.12.07, 09.01.08, 30.01.08; a 44 day period).

TABLE 3.9. Classification success using the chemistry of juvenile *Austrovenus* shells collected at TUA on five different dates during the summer of 2007/2008. Rows list the actual dates, whilst the columns list the dates predicted using the discriminant function analysis (DFA) model. Individual DFA scores are shown in Figure 3.7.

	Predicted date					**Total per**	**%Correctly**
	22.11.07	**17.12.07**	**09.01.08**	**30.01.08**	**09.03.08**	**date**	**classified**
Actual date							
22.11.07	19					19	100
17.12.07		5	4	1		10	50
09.01.08	1	3	7	6		17	41
30.01.08			7	15	2	24	63
09.03.08					4	4	100
Total	**20**	**8**	**18**	**22**	**6**	**74**	**68**

The resulting DFA scores are presented as scatterplots in Figure 3.7A and B. Figure 3.7B clearly shows the three groups described in Table 3.9, with 22.11.07 and 09.03.08 forming two distinct groups and the other three sites assembling together to constitute the third group.

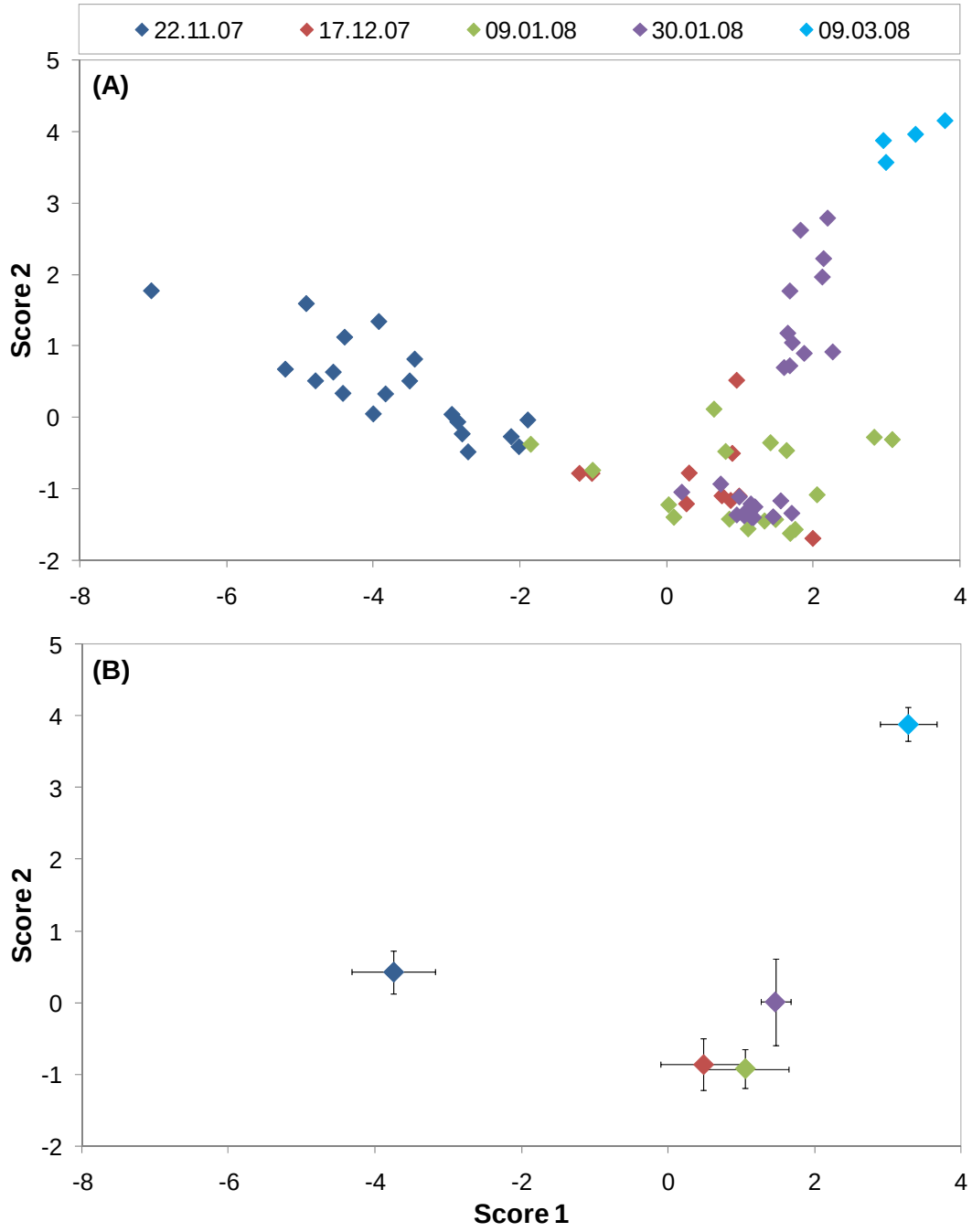


FIGURE 3.7. Discriminant function scores of element (Sr, Ba, Zn) to Ca ratios in shells of *Austrovenus* juveniles. (A) Scatterplot of DFA scores for shells collected on five different dates. (B) Data from (A) plotted as averages ± 95% confidence intervals.

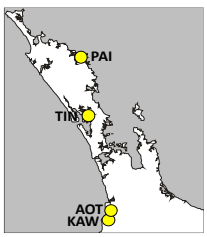
3.3 Intra-shell elemental variation (point ablation)

Four isotopic counts (^{88}Sr, ^{137}Ba, ^{25}Mg and ^{55}Mn) from four sites (PAI, TIN, AOT, KAW) were converted to elemental concentrations using GLITTER software, which were used to examine the trends for the LA-ICP-MS study. PAI was selected as it was situated in an exposed bay close to the estuary mouth, thus having a high chance of being an 'open' site, whilst TIN was situated a significant distance from the estuary mouth, thus increasing the probability of being a 'closed' site. AOT and KAW were selected due to their close proximity to one another and thus the possibility of larval movement between these two sites.

Average elemental concentrations were established for each shell, using the shell edge and prodissoconch concentrations (Table 3.10). These average concentrations were then used to determine if significant variations in the shell chemistry occurred between sites. Sr, Ba, and Mg were all significantly different between sites (ANOVA Sr: $F_{3.20} = 7.48$, $p = 0.002$; Ba: $F_{3.20} = 3.87$, $p = 0.02$; Mg: $F_{3.20} = 4.32$, $p = 0.02$), whilst Mn was not significant between sites (ANOVA Mn: $F_{3.20} = 1.24$, $p = 0.32$). HSD post-hoc tests highlighting where differences occurred are presented in Appendix A.5. Mn was still included in the analysis as it was found to hold a significant discriminating power (F to remove > 3.5) in the DFA. A summary table of the average elemental concentrations of the ablated shells for the four respective sites, as well as the number of shells that were included in the analyses, are presented in Table 3.10. TIN had low concentrations of Sr and Mg. PAI showed high concentrations of all of the elements, but also had large standard errors. Only three of the ten shells ablated from KAW were not considered outliers and thus were included in the analyses.

TABLE 3.10. Mean (± 1 SE) standardised (to ^{42}Ca) elemental concentrations (parts per million) of juvenile *Austrovenus* shells, grouped by site.

Site	n	Sr (ppm)	Ba (ppm)	Mg (ppm)	Mn (ppm)
PAI	7	3.17±0.02	0.59±0.09	2.19±0.07	0.71±0.19
TIN	8	3.04±0.01	0.49±0.05	2.00±0.02	0.71±0.05
AOT	6	3.10±0.01	0.40±0.08	2.17±0.06	0.54±0.17
KAW	3	3.11±0.02	0.17±0.04	2.05±0.04	0.56±0.07



The average elemental concentrations of the shell edge and prodissoconch were compared to determine the intra-shell variation (Table 3.11). Paired *t*-test results indicated that the Sr concentration at TIN was the only elemental concentration that was significantly different between the shell edge and prodissoconch (p = 0.02). Ba was significant to p = 0.1 for AOT. This was surprising, as when examining the data, intra-shell variation appeared obvious (i.e. Ba concentrations for all the sites; and Mn concentrations for PAI and TIN (Table 3.11)).

A possible explanation for lack of significance in the *t*-test results could be that the difference between the concentrations of elements for the shell edge and the prodissoconch is not always positive (i.e. for one shell at a site the edge may have a higher concentration of an element compared to the prodissoconch, whilst for another shell from the same site the prodissoconch may have the highest concentration). Therefore, whilst each pair (the edge versus the prodissoconch of one shell) might differ from each other, it is possible that different pairs cancel each other out, therefore making the overall comparison insignificant.

TABLE 3.11. Mean (± 1 SE) standardised (to ^{42}Ca) elemental concentrations (parts per million) of the edge (*E*) and prodissoconch (*P*) regions of juvenile *Austrovenus* shells.

Site and region of the shell	n	Sr (ppm)	Ba (ppm)	Mg (ppm)	Mn (ppm)
PAI (*E*)	7	3.15±0.03	0.43±0.07	2.18±0.04	0.51±0.12
PAI (*P*)	7	3.18±0.02	0.74±0.16	2.20±0.14	0.91±0.35
TIN (*E*)	8	3.03±0.01	0.51±0.08	1.99±0.04	0.64±0.04
TIN (*P*)	8	3.06±0.01	0.48±0.06	2.02±0.03	0.79±0.10
AOT (*E*)	6	3.09±0.02	0.25±0.08	2.16±0.04	0.43±0.03
AOT (*P*)	6	3.10±0.01	0.54±0.10	2.17±0.13	0.65±0.36
KAW (*E*)	3	3.12±0.02	0.12±0.03	2.07±0.08	0.56±0.11
KAW (*P*)	3	3.10±0.03	0.22±0.06	2.03±0.02	0.56±0.10

In an attempt to overcome the problems associated with using a paired *t*-test to compare intra-shell variation, differences between elemental concentrations of the two regions of the shells (edge and prodissoconch) were recorded as absolute values. The objective of this analysis was simply to determine if variations occur between these regions, and not which of the two regions had higher or lower elemental concentrations. The average elemental differences between the two regions of the shells from each site are presented in Figure 3.8.

The intra-shell variation was greatest for PAI for three of the four elements (Ba, Mn, Sr), with KAW having the least amount of intra-shell variation for three of the four elements (Ba, Mg, Sr) (Figure 3.8A, B, C and D). In general, the intra-shell variation for KAW and TIN was lower than AOT and PAI. This pattern is represented in three of the four elements analysed (Ba, Mg, Mn) (Figure 3.8A, B and C) and is particularly prominent for Ba and Mg (Figure 3.8A and B).

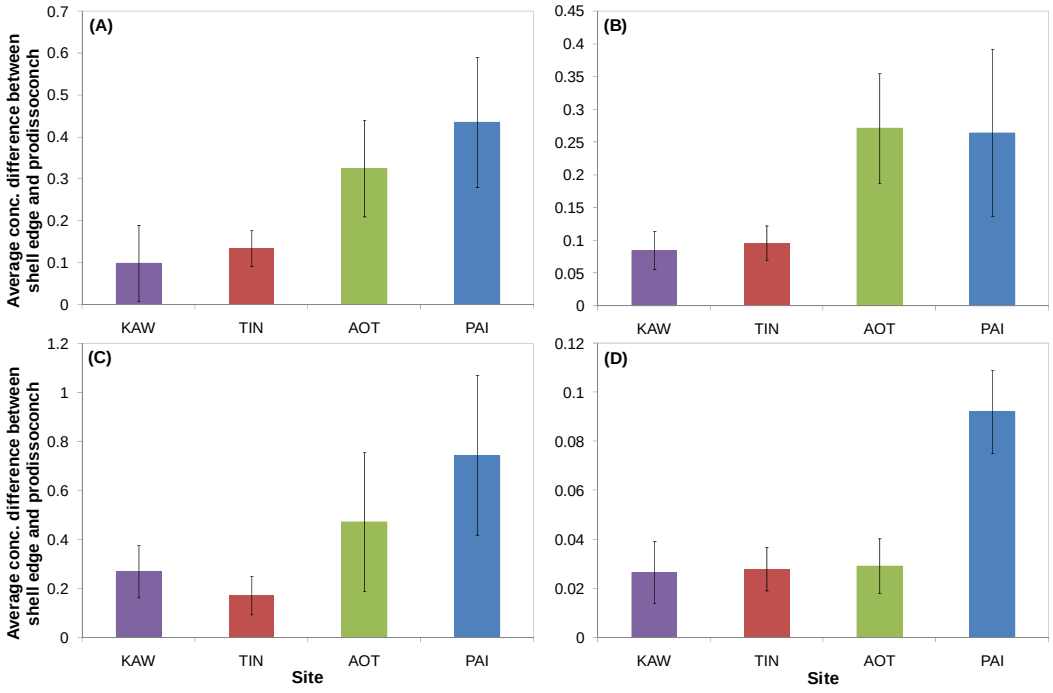


FIGURE 3.8. The average (± 95% confidence intervals) difference in elemental concentrations between the shell edge and the prodissoconch. (A) Ba, (B) Mg, (C) Mn, (D) Sr.

The results presented in Figure 3.8 suggested that for at least two of the sites (AOT and PAI) the elemental concentrations of the shell edges and prodissoconchs were quite variable. The elemental concentrations of the shell edge (most recently formed shell material) should be representative of the collection site as the juveniles are unlikely to move great distances after settlement (Lundquist et al., 2004). The equation that is generated by the DFA to calculate the canonical root scores of the shell edges should thus represent the elemental conditions at PAI, TIN, AOT and KAW (Equation 3.1).

EQUATION 3.1. The unstandardised canonical (A) root 1 and (B) root 2 scores from the juvenile *Austrovenus* DFA, grouping individuals by site using elemental concentrations of Sr, Ba, Mg, and Mn at the shell edge.

(A) Unstandardised root 1 canonical score:

$$= -65.903 - \left(20.030 \times {}^{88}Sr\right) - \left(4.075 \times {}^{55}Mn\right) - \left(1.651 \times {}^{137}Ba\right) + \left(3.229 \times {}^{25}Mg\right)$$

(B) Unstandardised root 2 canonical score:

$$= -9.494 + \left(0.775 \times {}^{88}Sr\right) - \left(1.858 \times {}^{55}Mn\right) + \left(5.069 \times {}^{137}Ba\right) + \left(2.961 \times {}^{25}Mg\right)$$

The intra-shell variation was evaluated by substituting the concentrations of Sr, Ba, Mg and Mn of the prodissoconchs into the unstandardised canonical coefficients equation generated through the DFA for the shell edges (i.e. Equation 3.1). If the variation between the shell edge and prodissoconch was minimal, then the predicted root scores for the prodissoconch should closely match the root scores of the shell edge. Conversely, if significant variation existed, then the root scores for the two regions of the shells would vary greatly. This method allowed every shell edge and prodissoconch to be evaluated individually. The results are presented as scatterplots in Figure 3.9A – D.

Three outliers were identified: two from PAI and one from AOT (Figure 3.9). Interestingly, all three of the outliers were from the prodissoconch elemental concentration analysis, and all the outliers were considerable. The variation between the TIN shell edge and prodissoconch was small, with most of the data points clustering close together (i.e. small 95% confidence intervals). PAI, AOT and KAW showed considerable variation between the edge and prodissoconch discriminant function scores, with large confidence intervals, particularly for score 1. The prodissoconch concentrations were particularly variable for PAI and AOT (large 95% confidence intervals along score 1).

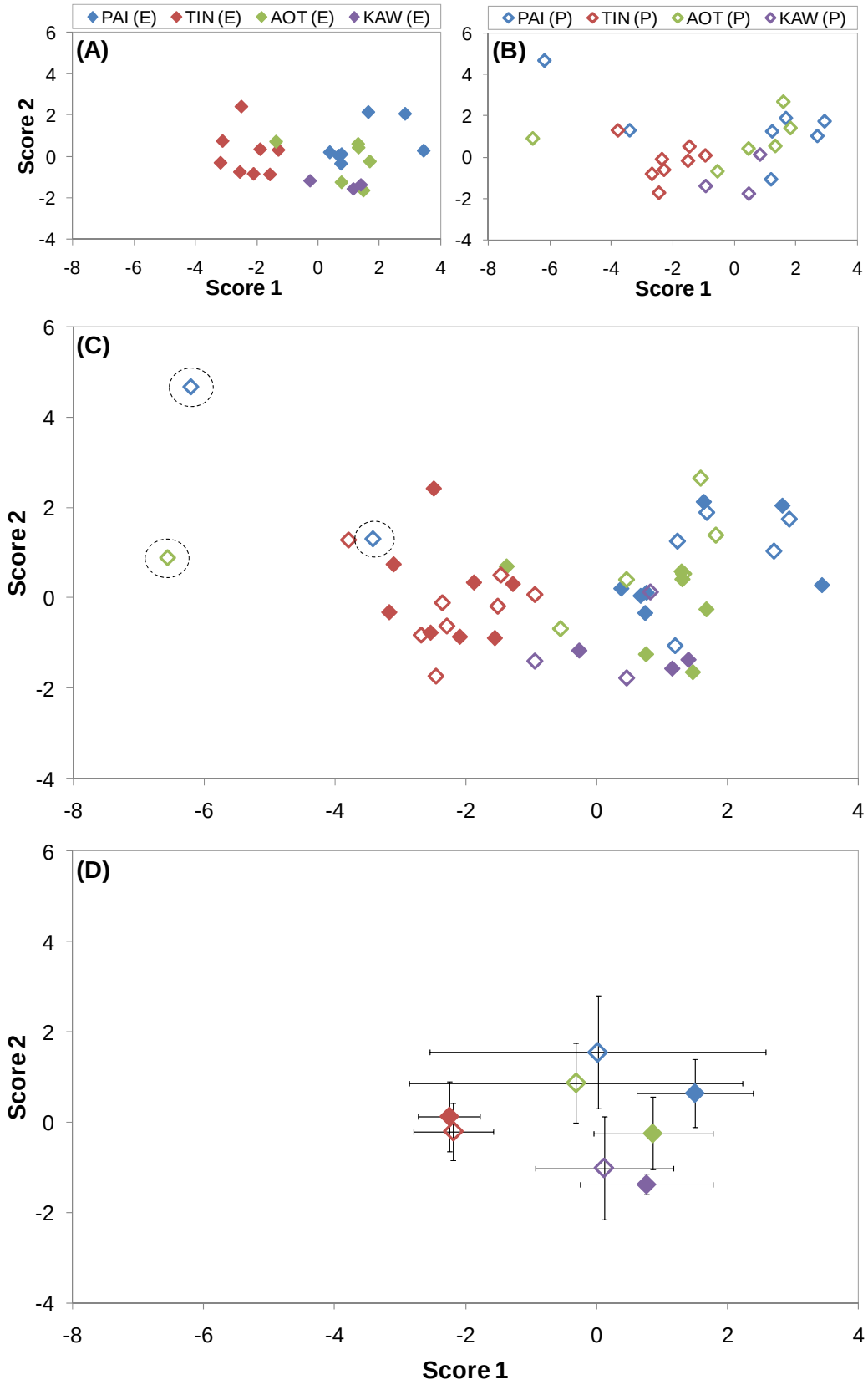


FIGURE 3.9. A scatterplot of the canonical scores of (A) the shell edge (E); and (B) the predicted prodissoconch (P) calculated from the unstandardised root 1 and 2 canonical coefficients (Equation 3.1) for four sites (PAI, TIN, AOT, KAW) using the elemental concentrations (Sr, Ba, Mg, Mn) of the shell edge. (C) Plots (A) and (B) combined on one graph, and (D) is the data from (C) plotted as mean values (± 95% confidence intervals). Outliers are encircled by a dotted line.

4.0 CHAPTER FOUR: DISCUSSION

4.1 Spatial variation in whole shell chemistry

4.1.1 Effect of the number of sampling sites on discriminating power

To test the feasibility of using elemental signatures to track the pre-settlement movement of *Austrovenus*, the spatial scale at which the signatures were unique were assessed (Becker et al., 2005). Newly settled juvenile *Austrovenus* were collected from 19 sites along 1500 km of coastline and the chemical signatures of their shells were compared. Significant differences in the chemistry of shells collected from different sites were apparent, however, the accuracy with which individuals could be assigned to their collection sites was only 68%. When the number of sites analysed was reduced from 19 to 12, the discriminating power increased markedly to 75%. These results were promising, as a study carried out by Becker et al. (2007) around San Diego Bay, California, which compared differences in the shell chemistry of *Mytilus californianus* collected from 12 study areas, had a much lower classification success of 49%.

The results further emphasise that the classification success of the DFA may be influenced by the number of sites that are analysed simultaneously. For example, the sites where misclassification occurred were compared in two smaller sub-groups. When 12 sites were compared, WHR had a very low classification success (25%) and was largely misclassifying to PAR and HRK (Table 3.2). However, when WHR was compared with only PAR, HRK and WHT (Table 3.2) the classification success was considerably higher (75%). Similarly, AOT and KAW misclassified to each other in the analysis of 12 sites with the sites yielding classification successes of 83% and 50%, respectively. When the analysis was reduced to three sites (AOT, KAW and PAI) the resulting classification success was again higher than that of 12 sites. All AOT individuals were correctly

assigned, whilst KAW individuals were successfully classified 70% of the time. This hypothesis was also supported when DFA was carried out for 19, 12 and five replicates of ten, five, and three sites, randomly chosen from the pool of 19 sites. Three sites could be classified with a much higher success compared to 12 or 19 sites, and it was found that the fewer the sites included in the analysis, the stronger the discriminating power. Comparable studies using DFA also achieved a high discriminating power when using between three and seven sites (Becker et al., 2005; 2007). Caution should be exercised when interpreting the success of a study and comparing it to other studies that have used a different number of sites in the DFA, as the observed classification success may simply be a function of the number of sites included in the analysis.

A limitation of the spatial study was that the number of shells included in the final analysis at each site was small, usually ranging between 10 – 13 individuals. Due to resource constraints, for this study, the decision was made to cover a larger spatial area and analyse fewer individuals, rather than analysing larger sample sizes over a smaller spatial scale. Although the number of shells analysed were comparable to other studies (e.g. Zacherl, 2005; Becker et al., 2005; 2007), increasing the number of analysed shells would greatly increase the accuracy of the results by increasing both the statistical power, as well as the likelihood of detecting and removing true outliers. Now that spatial variation has been established for New Zealand estuaries, future studies may wish to be more selective, analysing more shells from fewer sites, to gain a more accurate account of the spatial variation in the chemistry of bivalve shells found in New Zealand estuaries.

4.1.2 Spatial scale of discriminating power

The results from the study illustrated a high classification success at both large and small spatial scales. Firstly, the shell chemistry compared between sites over a large spatial scale (> 520 km of coastline) yielded a high classification success (99±1.0%) compared to other similar studies (Becker et al., 2005 (84%); Zacherl, 2005 (89%)).

The small spatial scale comparisons also showed a high classification success. Small spatial scale comparisons were made for three nearby estuaries (~ 50 km of coastline) as well as for three sites situated within a single estuary, Tamaki Estuary (~ 10 km), and yielded a 90% and 89% classification success, respectively. The ability to assign individuals to sites within a single estuary (i.e. the three Tamaki sites) was particularly surprising and interesting, considering the close proximity of the samples. A study along the San Diego open coast has reported a high classification success (90%) for mussel shells over about 40 km (Becker et al., 2005), however no studies to my knowledge have been able to successfully discriminate between sites that cover a spatial scale of approximately 10 km. A high classification success over such a small spatial scale (which indicates strong intra-site variation) makes it more difficult to confidently determine inter-site variations, as intra-site variations need to be taken into account. Within estuary variation may make it difficult, if not impossible, to distinguish between juveniles originating from nearby estuaries or juveniles originating from different sites within the same estuary.

Tamaki estuary, however, is a unique example, as significant pollution gradients have been reported (Abrahim & Parker, 2002; Auckland Regional Council, 2004). These pollution gradients may contribute to the strong signatures of the shells collected from closely situated sites. Tamaki Estuary may not be a 'typical' New Zealand estuary, and is therefore an unsuitable site from which to draw general conclusions regarding the applicability of the elemental fingerprinting technique for all New Zealand estuaries. TIR is the most southern site and also the most polluted, being surrounded by industrial development. PAN, the middle site, is slightly less polluted compared to TIR, whilst GLD is the most northern and least polluted site of the three. GLD is situated closest to the open coast and undergoes the most tidal flushing. Interestingly, GLD and TIR misclassified to each other, despite being separated by PAN. PAN, on the other hand, had a 100% classification success. These results suggested that the pollution gradient was possibly not the most dominant factor influencing the shell signatures in the Tamaki Estuary. Future studies should explore the factors that are driving the incorporation of chemical signatures into bivalve shells. The sample sizes for the three Tamaki sites, however, were small (7, 9 and 12 shells) and this may have

confounded the results. Future studies should focus on within estuary variation using multiple estuaries with multiple sample sites, as well as larger sample sizes, to establish if small scale variation in shell chemistry is widespread, or simply a function of an unusual environmental condition (e.g. a pollution gradient).

The results suggested that the elemental signatures expressed by *Austrovenus* juveniles are considerably variable, over both large and small spatial scales, and indicate that elemental fingerprinting may hold potential to be successfully applied to New Zealand estuarine environments.

4.2 Factors responsible for the spatial variation

The primary purpose of the whole shell digestion study was to assess if general spatial variation existed between shells collected from different sites, and noticeable differences were detected at all spatial scales examined (from approximately 10 km to 1150 km). The next step was to determine the factors responsible for the similarities and differences in the elemental signals of shells from different sites. A number of studies have suggested multiple factors that may be driving the observed differences in shell chemistry among shells collected from different estuaries, and some of these factors were investigated. These factors include geographical location, water and/or sediment chemistry and environmental variables such as temperature and salinity (Klein et al., 1996; Lazareth et al., 2003; Zacherl, 2005; Gillikin et al., 2005).

Firstly, geographical location was investigated as an explanation for similarities in the chemical signatures of shells. There was no clear distinction in shell chemistries between shells collected from the east and west coasts (Figure 3.1). AOT and KAW were closely situated and did misclassify to each other (and thus had similar signatures), however, many other sites that displayed similar shell signatures were situated considerable distances from one another and were a mix of both east and west coast sites (e.g. PAR, HRK and WHR). These results indicate that New Zealand sites cannot be grouped primarily on their geographical location, unlike the findings of other studies (i.e. Becker et al., 2005; 2007).

Secondly, if the shells obtain their unique signatures by incorporating the chemistry of the surrounding environment, as previously reported (see Thorrold et al., 1997; Bath et al., 2000; Vander Putten et al., 2000; Elsdon & Gillanders, 2003), then it can be expected that there should be some significant correlations between the elemental compositions of the shell and the chemistry of the water and/or sediment. No significant correlations were found, however, this may be due to a number of possible factors. Firstly, water and sediment samples were collected during low tide. Whilst the sediment would remain indifferent between high and low tides, the chemistry of the water may change based on tidal stage. Water samples were thus only representative of the low tide water signals. *Austrovenus*, however, feed during the high tide and it is likely that their shell signatures may reflect these water conditions to a greater extent. Also, only one water and one sediment sample was taken at each site as these sites were not readily accessible and logistical difficulties prevented further sampling. A single sample is probably not representative of the conditions at these sites over the time that the shells were growing and incorporating their chemical signature from the surrounding environment. Collecting multiple water and sediment samples over the whole summer (for at least three or more sites) may provide a better record for the chemical properties of the water and sediment, enabling more robust conclusions to be achieved. It has also been reported that the elemental:Ca ratios in the shells can be influenced by environmental conditions, like temperature (Carré et al., 2006) or physiological processes like metabolism (Vander Putten et al., 2000). The lack of a correlation between shell and water and/or sediment chemistry may be attributed to a combination of the aforementioned factors (i.e. the timing and frequency of sampling, temperature and physiological processes).

Finally, environmental variables that directly influence the water and/or sediment chemistry were investigated. Environmental variables specific to each estuary (obtained from the literature), were correlated to the discriminant scores by a simple linear correlation. This method examined the contribution of environmental variables to the chemical signals of the juvenile shells. Two catchment land cover variables (‘urban development’ and ‘planted forest’) were the only variables that significantly influenced the discriminant scores. Catchment land cover directly impacts a range of catchment processes, including

hydrological characteristics (e.g. rainfall interception and evapotranspiration potential) and erosion rates, which subsequently affect the physico-chemical properties of estuaries. Fluxes of sediment as well as pollutants such as pathogens, heavy metals and organic compounds, are directly derived from these catchment processes, and can severely alter the water and sediment chemistry of estuaries (Hume et al., 2007). However, whilst landcover may be associated with the chemical signatures of shells, it can vary significantly over both small and large spatial scales and it will therefore be difficult to use landcover parameters to evaluate chemical signatures of shells.

4.3 Temporal variation in whole shell chemistry

In order to use the signatures of shells collected at one time from a site to infer the collection sites of unknown samples, knowledge of if, and how, the signals are changing over time, is crucial (Becker et al., 2005). Similarly, if shells collected from different sites over a period of time are to be compared with one another, it is vital for the signals at each site to be temporally stable, as temporal variation may confound any similarities or differences reported between sites.

Temporal stability was examined at a single site (TUA) located in Tauranga Harbour, during the summer of 2007/2008 (22.11.07 – 09.03.08), with juveniles being collected on five different dates over this period. ANOVA results reported significant differences, however the differences were only associated with the first and last collection dates, and were primarily attributed to unusual Mn:Ca and Zn:Ca ratios on 22.11.07 and 09.03.08, respectively.

The differences at these two collection dates may be due to different cohorts of juveniles in TUA. *Austrovenus* generally spawn during the summer months, but spawning may occur throughout the year (Booth, 1983; Norkko et al., 2006). The planktonic larval phase lasts for approximately 20 days (or between one and four weeks), after which the larvae settle as juveniles (Stephenson & Chanley, 1979; Tompa et al., 1984; Carré et al., 2006). The time that it takes for larvae to mature and settle allows for the possibility of multiple cohorts settling every summer,

with each cohort potentially having slightly different chemical signatures. The juveniles collected on the first date may have belonged to an early cohort, with spawning occurring sometime during mid to late October. The following three collection dates (17.12.07, 09.01.08, 30.01.08) occurred over the course of 44 days, and may have been part of multiple overlapping cohorts if the larval period or growth rates varied for the different cohorts. As individuals of a similar size were collected, these three collection dates may have incorporated slower and the faster growing individuals, from earlier and later cohorts, respectively, thus incorporating individuals across cohorts. The mid-date (09.01.08) provides the link between these three sampling times (17.12.07, 09.01.08, 30.01.08), and is illustrated by the majority of the misclassification from both the 17.12.07 and 30.01.08 classifying to 09.01.08 (Table 3.9). However, as the larval shell only comprises a small proportion of the whole shell, the majority of the shell signatures can be attributed to the conditions of the collection site (which is where the largest proportion of the shell formed), and may not be representative of the conditions where the larval shell formed. Thus, multiple cohorts are unlikely to be responsible for the temporal stability observed at TUA.

Another explanation for the observed temporal stability from 17.12.07 to 30.01.08 may be the stability in environmental factors (i.e. temperature and salinity) during this period (Greig et al., 1988). These three sampling dates (17.12.07, 09.01.08, 30.01.08) represent the middle of the summer and thus the period when temperature and salinity is relatively stable. As previously mentioned, environmental factors (i.e. temperature and salinity) as well as physiological processes (i.e. growth rate) have been shown to influence the elemental uptake in mollusc shells. A positive relationship between Sr incorporation and temperature was reported for the protoconch of the marine gastropod *Kelletia kelletii*, whilst an inverse relationship was reported between temperature and the Sr (statolith) and Ba (protoconch and statolith) incorporation (Zacherl, 2005). Conversely, Klein et al. (1996) and Carré et al. (2006) found that environmental variables only had minor influences on the elemental signatures of bivalve species (*Mytilus trossulus, Mesodesma donacium* and *Chione subrugosa*). These studies highlighted growth rate (which can be co-dependent on temperature) as the major factor influencing the elemental incorporation into shells. These results suggest

that the relationships are often species, shell region and site specific and the relationships cannot be generalised (Gillikin et al., 2005). As detailed temperature and salinity data were not recorded as part of this study, it is difficult to estimate the effect that these environmental factors may have on the elemental signature of *Austrovenus* shell. Future studies should examine temperature and salinity records throughout the temporal study sampling period so that accurate conclusions can be drawn. Furthermore, future studies should investigate the effect other physiological processes (i.e. growth rate and age effects) may have on elemental uptake of *Austrovenus* shells.

In general the temporal stability of the site was good, with an overall classification success of 68% (the lower the classification success, the less variability among dates, therefore the higher the temporal stability). Comparatively, five randomly chosen sites in the spatial study yielded (on average) a 90% classification success, thus confirming that the temporal stability is real and not a function of the number of classifying variables (in this case, sampling dates). The temporal stability found at TUA was greater than that reported for *Mytilus edulis* mussels at Scripps Pier (89%), however the study looked at seasonal variation over a year (Becker et al., 2005). Similarly, other studies have reported significant seasonal or annual variation in elemental concentrations of shells; for example, in Sr, Ba, Mg in the gastropod *Kelletia kelletii* (Zacherl, 2005), and in Sr and Ba in the bivalve *Mercenaria mercenaria* (Stecher et al., 1996). DiBacco and Levin (2000) stated that the three month collection period used in their study sufficed to ensure that temporal variation did not confound their results. However, based on the analysis of juvenile *Austrovenus* collected from TUA, temporal variation may exist even within a three month time frame. These results also suggest that inter-annual variation is highly likely, however, due to the time constraints of this study, inter-annual temporal variation was not tested.

Although some temporal variation was evident, multiple sites would need to be tested before any firm conclusion may be drawn to confirm the extent at which temporal variation may confound results over small temporal scales (months) and for New Zealand estuaries and conditions. Likewise, sites will need to be sampled monthly and annually to determine the probability of inter-annual variation. For

example, seasonal variations in shell chemistries are likely to be due to upwelling or rain seasons contributing large amounts of freshwater to the system. These temporal variations need to be established to accurately interpret results. Future research should focus on the factors that influence and contribute to different scales of temporal variation.

4.4 Point ablation ICP-MS

Solution based whole shell digestion procedures (ICP-MS) result in average signatures across entire shells. An advance in laser technology has enabled LA-ICP-MS analysis. This method allows for very high precision ablation of materials (e.g. carbonate matrices), thus obtaining signatures for specific regions (Levin, 2006). Fish otolith cores (representing the juvenile phase) and edges (representing the current phase, usually adult), have shown to hold information about two different developmental phases in fish lifecycles. Scientists have used this method to compare the otolith core and edge elemental fingerprints to infer migration patterns of many fish species (Secor et al., 1995; Jessop et al., 2002; Arai et al., 2007). I used LA-ICP-MS to determine if elemental fingerprinting could be used to determine variations in the signatures of different developmental phases of bivalve life histories.

The prodissoconch of many venerid bivalves start to form within 24 hours of spawning (Mouëza et al., 1999; 2006) and the shells are retained after settlement (Levin, 2006). These shells thus provide a record of the chemical signature of the environment where the prodissoconch formed. The prodissoconch is still present in recently settled juveniles, and, using LA-ICP-MS, can be analysed separately from, and thus compared to, the chemical signature of the most recently formed shell material. The prodissoconch and shell edge signatures can therefore be compared to establish if these two regions of the shell were formed in the same water mass. As *Austrovenus* is unlikely to disperse over large distances (e.g. < 1 km) after settlement (Petuha et al., 2006), the most recently formed shell material should represent the chemistry of the water mass from which the bivalve was collected.

A comparison of the chemistries of the edge and prodissoconch of *Austrovenus* juvenile shells collected from four estuaries highlighted differences in the chemistries for at least two (PAI and AOT) of the four sites (Figure 3.8). These differences suggest that at least some of the *Austrovenus* juveniles that were collected had been recruited from a different water mass, and possibly a different estuary. When an estuary is receiving larval recruits from outside of the system (i.e. external recruitment), the estuary is considered demographically 'open'. Conversely, when all new recruits originate from within the system (i.e. internal recruitment), the estuary is considered demographically 'closed'. The findings from this study suggest that PAI and AOT may possibly be open estuaries. The variation between the edge and prodissoconch of juvenile shells collected from KAW and TIN was consistently low across all elements analysed, thus proposing that both regions (prodissoconch and edge) of the shell were formed under similar environmental conditions, and quite possibly in the same water mass, thus inferring self-recruitment (or a closed estuary).

Another analysis further supported the idea that PAI and AOT may be 'open' estuaries, whilst KAW and TIN may be closed. It was expected that the shell edge chemistry of all shells collected from a specific site would be similar, as the shell edge chemistries should represent the chemistry of the collection site. This trend was observed, with small 95% confidence intervals about the mean for the shell edges (Figure 3.9D). If the prodissoconch was formed at the collection site, it is expected that, when the concentration of elements from the prodissoconch is substituted into the unstandardised canonical coefficients equation generated by the edge DFA, the two regions should yield similar results. Although this was true for most of the shells, three prodissoconchs (two from PAI, and one from AOT) were clear outliers (Figure 3.9D). These results suggested that the prodissoconchs for those three shells were formed in a different water mass, thus implying that the larvae had been recruited from elsewhere.

Temporal variation reported for TUA (see section 4.3) raises the possibility that the variation observed in the signatures between the three edges and prodissoconchs could also be a result of temporal variation within sites. Temporal variation for four sites (PAI, TIN, AOT, KAW) was examined based on the

temporal variation observed for TUA. Sampling at these four sites occurred from 16.01.08 – 09.02.08 (see Table 2.1). Studies for the clam, *Chione subrugosa*, have reported growth rates of up to 120 μm/day (Carré et al., 2006). Although no specific daily growth rates for juvenile *Austrovenus* could be found, it is not unreasonable to assume *Austrovenus* would grow at a similar rate, as both species are venerid bivalves. Assuming a growth rate of between 80 – 120 μm/day, it would take 20 – 32 days for *Austrovenus* juveniles to reach 2.5 mm. Thus, the prodissoconch for each shell would have formed sometime between the 15.12.07 and the 19.01.08. The edge material probably formed within a week prior to the collection, i.e. between 09.01.08 – 02.02.08.

The period from when the prodissoconch was formed to when the edge material was laid down (15.12.07 – 02.02.08) coincided with the period of maximum temporal stability in the shell signatures at TUA (17.12.07 – 30.01.08). Using TUA as a reference site for expected temporal variation at other sites, the temporally stable period covers most of the growing and development times for the collected bivalves, with the exception of the edges for the AOT, KAW and TIN sites. There was, however, a large gap in the temporal study in the month of February, and therefore temporal variability for this month could not be confirmed. Assuming the temporal stability observed at TUA is representative of temporal stability throughout New Zealand estuaries, then the differences observed between the chemistry of the prodissoconch and the shell edge is unlikely to be a function of temporal variation.

4.5 Limitations and future directions

Although the potential of using elemental fingerprinting techniques to investigate connectivity in New Zealand bivalve populations was demonstrated, some limitations became evident throughout this study and improvements will enhance the quality of future studies. Firstly, comparisons were made using relatively few shells (< 15 individuals). Although this appeared to be sufficient to determine the spatial and temporal variation of New Zealand estuaries, analysing more shells would strengthen the conclusions that can be drawn from this research.

As previously reported, environmental and physiological factors can have significant impacts on the uptake of different elements and can significantly confound results (see section 4.3). Larval culture studies under different environmental conditions and in ambient water, containing known quantities of elemental concentrations, may aid in our understanding of the effect that the environmental conditions may have on the elemental integration capabilities of *Austrovenus* shells. Furthermore, these studies will quantify the extent to which elements are incorporated by *Austrovenus* juveniles. Such studies are essential, as the elemental integration capabilities of *Austrovenus* have not previously been studied and cannot be inferred from the literature, as the literature have shown the integration capabilities of shells to be very species specific.

Whilst this was a useful preliminary study in demonstrating the potential of elemental fingerprinting for New Zealand conditions, conclusions are limited to a single species. Similar studies should be conducted across a range of bivalve and gastropod species to improve the interpretation for New Zealand invertebrates as a group. Future studies should aim to track the dispersal of bivalve shells using shells that showed different chemical signatures between the prodissoconchs and more recently formed material. To do this, reference signatures would need to be established for sites to allow the larval shell signature to be matched to a surrounding estuary. Once dispersal distances for bivalves have been established, researchers and estuarine managers will be better equipped to determine connectivity between and within estuarine bivalve populations, and thus ensure more effective management approaches in the conservation of estuarine ecosystems.

5.0 CHAPTER FIVE: CONCLUSIONS

The primary goal of this thesis was to establish if spatial variation existed between the elemental signatures of shells collected from different sites whilst the signals of the shells remain temporally stable. Both of these aims were achieved, with considerable spatial variability confirmed and the elemental signature for one New Zealand bivalve species showing temporal stability at a single site for over a month. Differences were also detected between the prodissoconch and most recently formed shell material for some sites, suggesting the possibility of demographically 'open' systems.

The major findings and implications of this study were:

- Shell signatures showed significant spatial variability at all scales, however sites did not group based on geography as I had predicted. Results also revealed that the number of sites analysed simultaneously had a considerable influence on the classification success (i.e. the fewer the sites analysed the higher the classification success), thus suggesting that the results may not be a true representation of actual classification success. Caution should be exercised when interpreting and comparing results between/among studies.

- Chemical signatures of the shells did not correlate with the ambient water and sediment chemical signatures, however there was a significant relationship between the catchment land cover (i.e. urban versus plantation forested catchments) and shell signatures. It is important to note, however, that too few water and sediment samples were collected for adequate analyses and conclusions. Future studies should incorporate a more vigorous sampling regime to address the relationship between water, sediment and shell chemistries.

- Shell signatures were temporally stable for the majority of the summer (i.e. over a 44 day period from mid-December to late-January), however some temporal variation was observed for one collection date either side of the 44-day 'stable' summer period. Whilst this temporal stability is a promising result for the use of chemical signatures, future studies should test temporal variation over longer periods using more sites, to improve our understanding of temporal variability for New Zealand estuaries.

- Point ablation analysis confirmed the hypothesis that a difference in the chemical signature between the prodissoconch and outer shell edge material occurred. This was true for some, but not all of the shells analysed. These results were promising as they confirmed that some shells may have been recruited from outside of the collection estuary thus supporting the hypothesis that at least some estuaries are 'open' systems (i.e. external recruitment).

- Although this study showed significant promise, results can only act as a precursor for more detailed future studies. Such studies should explore larger sample sizes, longer sampling periods, investigate chemical uptake rates into shells and consider the role of environmental and physiological factors in the incorporation of elements into developing shells.

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APPENDICES

A.1 Shell size and weight data

TABLE A.1. The sizes and weights of all the shells used in the whole shell digestion study.

Location	Sample number	Size (mm)	Weight (g)
MAN			
	A1	1.9	0.000585
	A3	1.7	0.000480
	A5	1.9	0.000590
	A7	1.9	0.000570
	A9	1.5	0.000355
	A11	1.5	0.000360
	A13	1.7	0.000500
	A15	1.8	0.000585
	A17	1.7	0.000465
	A19	1.8	0.000550
	A21	2.0	0.000605
	A23	1.8	0.000600
	A25	1.8	0.000525
	A27	1.9	0.000600
	A29	1.6	0.000565
	A31	1.9	0.000570
HOK			
	B1	2.2	0.001150
	B3	2.1	0.001000
	B5	2.3	0.001300
	B7	2.2	0.001200
	B9	2.1	0.001050
	B11	1.8	0.000580
	B13	1.8	0.000765
	B15	1.9	0.000545
	B17	1.9	0.000760
	B19	1.7	0.000520
	B21	2.5	0.001595
	B23	2.2	0.001120
	B25	2.3	0.001200
	B27	2.1	0.001170
	B29	2.4	0.001430

RAG			
	C1	1.6	0.000455
	C3	1.7	0.000555
	C5	1.7	0.000600
	C7	1.9	0.000755
	C9	1.7	0.000570
	C11	2.0	0.000915
	C13	1.8	0.000745
	C15	1.7	0.000565
	C17	1.9	0.000750
	C19	1.7	0.000615
	C21	1.9	0.000865
	C23	1.8	0.000805
	C25	1.6	0.000480
	C27	1.7	0.000610
	C29	1.8	0.000820
ARP2			
	D1	1.4	0.000320
	D3	1.8	0.000620
	D5	1.0	0.000120
	D7	1.8	0.000630
	D9	1.4	0.000330
	D11	2.0	0.000845
	D13	2.1	0.000990
	D15	1.9	0.000600
	D17	1.9	0.000705
	D19	2.0	0.000760
	D21	2.3	0.001225
	D23	1.2	0.000210
	D25	1.6	0.000425
	D27	1.5	0.000375
	D29	1.9	0.000705
	D31	1.6	0.000470
PAN			
	E1	2.2	0.001300
	E3	2.4	0.001450
	E5	2.0	0.000900
	E7	2.5	0.001600
	E9	2.2	0.001200
	E11	2.3	0.001240
	E13	3.0	0.002530
	E15	3.2	0.003415
	E17	3.2	0.002990
	E19	1.6	0.000470
	E21	1.8	0.000565
	E23	2.7	0.001985
	E25	3.0	0.002945

	E27	2.6	0.001725
	E29	2.8	0.002225
OTU			
	F1	2.1	0.001250
	F3	2.5	0.001715
	F5	2.4	0.001400
	F7	2.3	0.001210
	F9	2.3	0.001300
	F11	2.3	0.001105
	F13	2.1	0.000800
	F15	2.8	0.002175
	F17	3.0	0.002980
	F19	2.3	0.001505
	F21	2.3	0.001400
	F23	2.3	0.001220
	F25	3.0	0.002515
	F27	3.2	0.002890
	F29	2.8	0.002200
	F31	2.9	0.001910
	F33	2.8	0.001725
ARP			
	G1	1.9	0.000835
	G3	1.4	0.000330
	G5	2.0	0.000690
	G7	1.3	0.000210
	G9	1.4	0.000295
	G11	1.6	0.000410
	G13	1.4	0.000300
	G15	1.3	0.000215
	G17	1.8	0.000510
	G19	1.4	0.000340
	G21	1.4	0.000260
	G23	1.4	0.000350
	G25	1.2	0.000200
	G27	1.3	0.000260
	G29	1.2	0.000190
PAI			
	H1	2.7	0.001840
	H3	2.5	0.001390
	H5	2.9	0.002270
	H7	2.7	0.001975
	H9	2.6	0.001650
	H11	2.3	0.001080
	H13	2.2	0.001090
	H15	2.3	0.001355
	H17	2.2	0.000930
	H19	2.3	0.001375

	H21	2.3	0.001160
	H23	2.3	0.001165
	H25	2.1	0.000840
	H27	2.5	0.001400
	H29	2.2	0.001050
HDI			
	I1	1.1	0.000145
	I3	1.3	0.000210
	I5	1.6	0.000395
	I7	1.1	0.000120
	I9	1.4	0.000300
	I11	1.5	0.000340
	I13	1.3	0.000180
	I15	1.7	0.000500
	I17	1.4	0.000245
	I19	1.8	0.000540
	I21	1.3	0.000240
	I23	1.5	0.000285
	I25	1.5	0.000300
	I27	1.2	0.000155
	I29	1.4	0.000300
WHT			
	J1	2.4	0.001475
	J3	2.4	0.001380
	J5	2.0	0.000880
	J7	2.1	0.001000
	J9	2.5	0.001425
	J11	2.0	0.000790
	J13	2.1	0.000920
	J15	2.1	0.001050
	J17	2.0	0.000745
	J19	1.9	0.000695
	J21	2.2	0.001100
	J23	1.9	0.000835
	J25	2.2	0.001110
	J27	2.0	0.000820
	J29	1.9	0.000640
WHP			
	K1	2.9	0.002540
	K3	2.1	0.001070
	K5	1.7	0.000590
	K7	1.9	0.000930
	K9	2.1	0.001200
	K11	2.2	0.001290
	K13	3.0	0.002475
	K15	1.7	0.000615
	K17	2.2	0.001305

	K19	2.1	0.000965
	K21	1.5	0.000435
	K23	2.5	0.002050
	K25	2.6	0.002045
	K27	3.4	0.004255
	K29	3.1	0.003535
CLB			
	L1	1.8	0.000445
	L3	1.7	0.000510
	L5	2.1	0.000850
	L7	1.9	0.000690
	L9	3.0	0.002020
	L11	2.0	0.000885
	L13	2.0	0.000975
	L15	2.4	0.001085
	L17	1.7	0.000515
	L19	1.7	0.000480
	L21	2.2	0.001140
	L23	2.4	0.001390
	L25	1.8	0.000570
	L27	1.5	0.000320
	L29	1.7	0.000550
AOT			
	M1	2.1	0.001270
	M3	2.2	0.001145
	M5	2.0	0.000930
	M7	2.5	0.001750
	M9	2.5	0.001720
	M11	2.3	0.001340
	M13	2.7	0.002080
	M15	1.8	0.000610
	M17	2.6	0.001540
	M19	2.8	0.002480
	M21	2.7	0.002345
	M23	2.9	0.002720
	M25	3.0	0.003265
	M27	2.9	0.002270
	M29	2.4	0.001630
KAW			
	N1	2.2	0.001080
	N3	2.3	0.001385
	N5	3.0	0.002435
	N7	2.9	0.002460
	N9	2.3	0.001450
	N11	2.9	0.002925
	N13	2.8	0.002665
	N15	2.2	0.001270

	N17	2.9	0.002595
	N19	2.3	0.001205
	N21	2.5	0.001555
	N23	3.0	0.002460
	N25	3.7	0.005170
	N27	1.8	0.000785
	N29	1.9	0.000865
GLD			
	O1	2.6	0.002030
	O3	2.1	0.000990
	O5	1.4	0.000385
	O7	2.4	0.001490
	O9	2.6	0.001905
	O11	3.3	0.003370
	O13	3.3	0.003360
	O15	2.0	0.000915
	O17	2.6	0.001795
	O19	2.6	0.001585
	O21	N/A	0.000000
	O23	N/A	0.000000
	O25	N/A	0.000000
	O27	N/A	0.000000
	O29	N/A	0.000000
TIR			
	P1	2.4	0.001425
	P3	2.9	0.002610
	P5	3.1	0.003245
	P7	3.0	0.002350
	P9	2.8	0.002390
	P11	2.4	0.001490
	P13	2.7	0.002055
	P15	3.1	0.003515
	P17	2.5	0.001645
	P19	2.9	0.002370
	P21	2.6	0.001770
	P23	2.8	0.002350
	P25	3.1	0.003320
	P27	3.3	0.003155
	P29	2.2	0.001050
WHR			
	Q1	2.9	0.002120
	Q3	2.7	0.001860
	Q5	2.7	0.001670
	Q7	3.1	0.002060
	Q9	3.1	0.002665
	Q11	2.8	0.001670
	Q13	2.9	0.001920

	Q15	2.8	0.002250
	Q17	2.6	0.001370
	Q19	2.8	0.001860
	Q21	2.7	0.001960
	Q23	2.7	0.001620
	Q25	2.7	0.001760
	Q27	2.9	0.001540
	Q29	2.8	0.001980
PAR			
	R1	2.6	0.001735
	R3	3.1	0.002505
	R5	3.0	0.002320
	R7	3.0	0.002395
	R9	2.8	0.002010
	R11	2.7	0.001725
	R13	2.7	0.001840
	R15	2.9	0.002165
	R17	3.0	0.002700
	R19	2.7	0.002030
	R21	2.7	0.001765
	R23	2.6	0.001795
	R25	2.7	0.001510
	R27	3.0	0.002280
	R29	2.7	0.002030
TIN			
	S1	2.5	0.001655
	S3	2.0	0.000890
	S5	2.5	0.001595
	S7	2.5	0.001430
	S9	2.4	0.001500
	S11	2.4	0.001310
	S13	2.5	0.001505
	S15	2.0	0.000985
	S17	2.6	0.001930
	S19	2.4	0.001300
	S21	2.5	0.001685
	S23	2.4	0.001320
	S25	2.5	0.001420
	S27	2.5	0.001585
	S29	2.0	0.000925
HRK			
	T1	2.3	0.001305
	T3	2.8	0.002320
	T5	2.3	0.001545
	T7	2.1	0.001010
	T9	1.9	0.000925
	T11	2.8	0.002160

	T13	2.6	0.001960
	T15	2.1	0.001090
	T17	2.8	0.002440
	T19	2.7	0.002530
	T21	2.6	0.001870
	T23	2.9	0.002540
	T25	2.8	0.002380
	T27	2.8	0.002300
	T29	2.7	0.002460
TUA1 (22.11.07)			
	U1	2.9	0.002640
	U3	1.7	0.000460
	U5	2.3	0.000950
	U7	2.4	0.001530
	U9	3.0	0.003140
	U11	2.7	0.002070
	U13	1.8	0.000600
	U15	2.1	0.001070
	U17	1.9	0.000770
	U19	1.6	0.000430
	U21	1.2	0.000220
	U23	2.6	0.001695
	U25	3.0	0.002555
	U27	2.0	0.000665
	U29	2.2	0.001115
TUA2 (22.11.07)			
	V1	2.7	0.002145
	V3	2.7	0.002095
	V5	1.0	0.000110
	V7	1.8	0.000600
	V9	1.6	0.000490
	V11	1.4	0.000325
	V13	1.9	0.000715
	V15	2.1	0.000995
	V17	1.4	0.000380
	V19	0.9	0.000120
	V21	0.8	0.000065
	V23	1.8	0.000665
	V25	N/A	0.000000
	V27	N/A	0.000000
	V29	N/A	0.000000
TUA1 (17.12.07)			
	W1	1.6	0.000495
	W3	1.6	0.000425
	W5	1.4	0.000355
	W7	1.4	0.000360
	W9	1.3	0.000310

	W11	1.5	0.000450
	W13	1.5	0.000370
	W15	1.5	0.000410
	W17	1.3	0.000220
	W19	1.3	0.000290
	W21	1.3	0.000235
	W23	1.3	0.000235
	W25	1.3	0.000320
	W27	1.2	0.000185
	W29	1.1	0.000200
TUA1 (09.01.08)			
	X1	1.9	0.000720
	X3	1.7	0.000600
	X5	1.9	0.000955
	X7	2.0	0.000780
	X9	1.9	0.000770
	X11	1.9	0.000680
	X13	1.7	0.000525
	X15	2.3	0.001615
	X17	2.0	0.000925
	X19	2.3	0.001365
	X21	2.1	0.000990
	X23	2.4	0.001425
	X25	2.2	0.001220
	X27	2.4	0.001365
	X29	2.5	0.001750
TUA2 (09.01.08)			
	Y1	1.6	0.000445
	Y3	1.9	0.000750
	Y5	1.8	0.000665
	Y7	1.7	0.000475
	Y9	1.9	0.000780
	Y11	1.6	0.000440
	Y13	1.7	0.000575
	Y15	1.8	0.000775
	Y17	1.6	0.000445
	Y19	1.5	0.000350
	Y21	1.4	0.000340
	Y23	1.4	0.000315
	Y25	1.3	0.000275
	Y27	1.3	0.000240
	Y29	1.3	0.000240
TUA1 (30.01.08)			
	Z1	2.5	0.001870
	Z3	2.3	0.001260
	Z5	2.3	0.001145
	Z7	2.3	0.001190

	Z9	2.0	0.000785
	Z11	2.3	0.001260
	Z13	1.9	0.000810
	Z15	2.9	0.002375
	Z17	3.0	0.002950
	Z19	3.0	0.002600
	Z21	2.7	0.001855
	Z23	2.7	0.002300
	Z25	2.6	0.001765
	Z27	2.6	0.001960
	Z29	3.1	0.003495
TUA2 (30.01.08)			
	AA1	2.4	0.001650
	AA3	2.4	0.001585
	AA5	2.1	0.001050
	AA7	2.3	0.001630
	AA9	2.3	0.001340
	AA11	1.9	0.000925
	AA13	1.9	0.000865
	AA15	2.4	0.001420
	AA17	2.4	0.001590
	AA19	2.4	0.001535
	AA21	2.1	0.001040
	AA23	2.1	0.001000
	AA25	2.4	0.001585
	AA27	1.9	0.000815
	AA29	2.4	0.001480
TUA2 (09.03.08)			
	AC1	1.7	0.000560
	AC3	1.8	0.000590
	AC5	1.9	0.000665
	AC7	1.7	0.000420
	AC9	1.5	0.000320
	AC11	1.9	0.000670
	AC13	1.4	0.000270
	AC15	1.2	0.000190
	AC17	1.3	0.000225
	AC19	1.3	0.000220
	AC21	1.3	0.000215
	AC23	1.5	0.000360
	AC25	1.1	0.000120
	AC27	1.4	0.000305
	AC29	1.1	0.000165

A.2 Exact sediment weights

TABLE A.2. The exact weights of the sediment that was digested to determine the sediment chemistry.

Site	Weight (g)
PAR	0.5054
MAN	0.5023
HRK	0.5008
PAI	0.5057
HOK	0.5070
WHR	0.5059
TIN	0.5047
WHT	0.5124
WHP	0.5018
HDI	0.5008
GLD	0.5016
PAN	0.5020
TIR	0.5028
ARP	0.5021
CLB	0.5046
TUA 1 (22.11.07)	0.5087
TUA 2 (22.11.07)	0.5018
TUA 1 (17.12.07)	0.5022
TUA 2 (17.12.07)	0.5025
TGA 1 (09.01.08)	0.5039
TUA 2 (09.01.08)	0.5032
TUA 1 (30.01.08)	0.5076
TUA 2 (30.01.08)	0.5039
TUA 2 (09.03.08)	0.5034
OTU	0.5096
RAG	0.5075
AOT	0.5036
KAW	0.5054

A.3 The influence of the number of sites on DFA

TABLE A.3.1. Classification success using the chemistry of *Austrovenus* shells collected from three sites. For each analysis, three random sites were selected, using a random number table, from the 19 sites sampled. This process (analysis) was repeated five times (A – E), to determine the average classification success when comparing three sites.

(A)	Predicted site			Total per site	% Correctly classified
	HOK	GLD	TIR		
Actual site					
HOK	7			7	100
GLD		6	1	7	86
TIR		2	11	13	85
Total	**7**	**8**	**12**	**27**	**89**

(B)	Predicted site			Total per site	% Correctly classified
	PAR	MAN	WHT		
Actual site					
PAR	12			12	100
MAN		9		9	100
WHT			13	13	70
Total	**12**	**9**	**13**	**34**	**90**

(C)	Predicted site			Total per site	% Correctly classified
	GLD	ARP	AOT		
Actual site					
GLD	5		2	7	71
ARP		13		13	100
AOT			12	12	100
Total	**5**	**13**	**14**	**32**	**94**

(D)	Predicted site			Total per site	% Correctly classified
	TIN	TIR	ARP		
Actual site					
TIN	13			13	100
TIR		13		13	100
ARP			13	13	100
Total	**13**	**13**	**13**	**39**	**100**

(E)	Predicted site			Total per site	% Correctly classified
	PAR	HRK	GLD		
Actual site					
PAR	12			12	100
HRK		12		12	100
GLD			7	7	100
Total	**12**	**12**	**7**	**31**	**100**

TABLE A.3.2. Classification success using the chemistry of *Austrovenus* shells collected from five sites. For each analysis, five random sites were selected using a random number table from the 19 sites sampled. This process (analysis) was repeated five times (A – E), to determine the average classification success when comparing five sites.

(A)	Predicted site					Total per site	% Correctly classified
	GLD	CLB	OTU	AOT	KAW		
Actual							
GLD	5			2		7	71
CLB		3		1		4	75
OTU			10			10	100
AOT	1			10	1	12	83
KAW		2		4	4	10	40
Total	**6**	**5**	**10**	**17**	**5**	**43**	**74**

(B)	Predicted site					Total per site	% Correctly classified
	HOK	HDI	TIR	OTU	KAW		
Actual							
HOK	7					7	100
HDI		10			1	11	91
TIR			11		2	13	85
OTU				10		10	100
KAW		1			9	10	90
Total	**7**	**11**	**11**	**10**	**12**	**51**	**92**

(C)	Predicted site					Total per site	% Correctly classified
	HRK	HOK	HDI	TIN	KAW		
Actual							
HRK	12					12	100
HOK		5		2		7	71
HDI			10		1	11	91
TIN		1		12		13	92
KAW					10	10	100
Total	**12**	**6**	**10**	**14**	**11**	**53**	**92**

(D)	Predicted site					Total per site	% Correctly classified
	TIN	WHT	ARP	CLB	AOT		
Actual							
TIN	12	1				13	92
WHT		13				13	100
ARP			12		1	13	92
CLB				2	2	4	50
AOT					12	12	100
Total	**12**	**14**	**12**	**2**	**15**	**55**	**93**

TABLE A.3.2 continued.

(E)	Predicted site					Total per site	% Correctly classified
	PAI	HRK	WHT	HDI	TIR		
Actual							
PAI	12					12	100
HRK		12				12	100
WHT			13			13	100
HDI				11		11	100
TIR					13	13	100
Total	**12**	**12**	**13**	**11**	**13**	**61**	**100**

TABLE A.3.3. Classification success using the chemistry of *Austrovenus* shells collected from ten sites. For each analysis, ten random sites were selected using a random number table from the 19 sites sampled. This process (analysis) was repeated five times (A – E), to determine the average classification success when comparing ten sites.

(A)	Predicted site										Total per site	% Correctly classified
	PAR	TIN	WHT	HDI	PAN	TIR	ARP	OTU	AOT	KAW		
Actual site												
PAR	11				1						12	92
TIN		11	2								13	85
WHT			12					1			13	92
HDI				9			1		1		11	82
PAN		1	1		5			2			9	56
TIR						11			1	1	13	85
ARP					1		11	1			13	85
OTU					1			9			10	90
AOT									10	2	12	83
KAW									5	5	10	50
Total	**11**	**12**	**15**	**9**	**8**	**11**	**12**	**13**	**17**	**8**	**116**	**81**

(B)	Predicted site										Total per site	% classified
	PAR	HOK	WHR	TIN	WHP	PAN	TIR	ARP	RAG	KAW		
Actual site												
PAR	8		4								12	67
HOK		4		2					1		7	57
WHR	2		9			1					12	75
TIN		1		12							13	92
WHP					5	1					6	83
PAN				1		7			1		9	78
TIR							11			2	13	85
ARP						2		11			13	85
RAG									8		8	100
KAW						1				9	10	90
Total	**10**	**5**	**13**	**15**	**5**	**12**	**11**	**11**	**10**	**11**	**103**	**82**

TABLE A.3.3 continued

(C)	Predicted site										Total	%
	PAI	HRK	WHR	WHT	HDI	TIR	CLB	OTU	RAG	KAW	per site	classified
Actual site												
PAI	12										12	100
HRK		9	3								12	75
WHR		5	6					1			12	50
WHT				11				2			13	85
HDI					10					1	11	91
TIR						11				2	13	85
CLB							3			1	4	75
OTU								10			10	100
RAG									8		8	100
KAW							2			8	10	80
Total	**12**	**14**	**9**	**11**	**10**	**11**	**5**	**13**	**8**	**12**	**105**	**84**

(D)	Predicted site										Total	% Correctly
	MAN	HOK	PAI	WHP	PAN	TIR	ARP	CLB	OTU	AOT	per site	classified
Actual site												
MAN	8	1									9	89
HOK		7									7	100
PAI			12								12	100
WHP				5	1						6	83
PAN	1	1	1		4				2		9	44
TIR						11				2	13	85
ARP					1		11		1		13	85
CLB								2		2	4	50
OTU					1				9		10	90
AOT										12	12	100
Total	**9**	**9**	**13**	**5**	**7**	**11**	**11**	**2**	**12**	**16**	**95**	**85**

(E)	Predicted site										Total	% Correctly
	PAR	MAN	HRK	HOK	TIN	WHT	HDI	ARP	CLB	KAW	per site	classified
Actual site												
PAR	10		1			1					12	83
MAN		8			1						9	89
HRK	2		10								12	83
HOK				6	1						7	86
TIN				2	10	1					13	77
WHT						13					13	100
HDI							9		1	1	11	82
ARP						1		12			13	92
CLB									3	1	4	75
KAW									1	9	10	90
Total	**12**	**8**	**11**	**8**	**12**	**16**	**9**	**12**	**5**	**11**	**104**	**87**

A.4 HSD post-hoc results (19 sites)

TABLE A.4.1. Unequal *N* HSD post-hoc test investigating the difference in Sr:Ca concentrations in the shells of *Austrovenus* juveniles collected from 19 sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	MAN	HOK	RAG	PAN	OTU	ARP	PAI	HDI	WHT	WHP	CLB	AOT	KAW	GLD	TIR	WHR	PAR	TIN	HRK
MAN		1.000	1.000	***0.002***	***0.026***	0.121	***0.000***	***0.000***	0.154	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.897	0.996	1.000	1.000
HOK	1.000		1.000	***0.000***	***0.004***	***0.022***	***0.000***	***0.000***	***0.029***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.439	1.000	1.000	1.000
RAG	1.000	1.000		***0.000***	***0.006***	***0.031***	***0.000***	***0.000***	***0.041***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.570	1.000	1.000	1.000
PAN	***0.002***	***0.000***	***0.000***		1.000	1.000	***0.000***	0.986	0.999	0.829	0.256	0.051	***0.000***	0.328	***0.015***	0.623	***0.000***	***0.002***	***0.000***
OTU	***0.026***	***0.004***	***0.006***	1.000		1.000	***0.000***	0.627	1.000	0.421	0.076	***0.001***	***0.000***	0.069	***0.000***	0.949	***0.000***	***0.013***	***0.003***
ARP	0.121	***0.022***	***0.031***	1.000	1.000		***0.000***	0.185	1.000	0.181	***0.026***	***0.000***	***0.000***	***0.016***	***0.000***	0.996	***0.000***	***0.015***	***0.006***
PAI	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***		***0.000***	***0.000***	***0.003***	0.712	***0.000***	0.178	***0.006***	***0.001***	***0.000***	***0.000***	***0.000***	***0.000***
HDI	***0.000***	***0.000***	***0.000***	0.986	0.627	0.185	***0.000***		0.144	1.000	0.939	0.832	0.054	0.997	0.552	***0.001***	***0.000***	***0.000***	***0.000***
WHT	0.154	***0.029***	***0.041***	0.999	1.000	1.000	***0.000***	0.144		0.150	***0.021***	***0.000***	***0.000***	***0.012***	***0.000***	0.998	***0.000***	***0.022***	***0.008***
WHP	***0.000***	***0.000***	***0.000***	0.829	0.421	0.181	***0.003***	1.000	0.150		1.000	1.000	0.927	1.000	1.000	***0.006***	***0.000***	***0.000***	***0.000***
CLB	***0.000***	***0.000***	***0.000***	0.256	0.076	***0.026***	0.712	0.939	***0.021***	1.000		1.000	1.000	1.000	1.000	***0.001***	***0.000***	***0.000***	***0.000***
AOT	***0.000***	***0.000***	***0.000***	0.051	***0.001***	***0.000***	***0.000***	0.832	***0.000***	1.000	1.000		0.994	1.000	1.000	***0.000***	***0.000***	***0.000***	***0.000***
KAW	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.178	0.054	***0.000***	0.927	1.000	0.994		0.992	1.000	***0.000***	***0.000***	***0.000***	***0.000***
GLD	***0.000***	***0.000***	***0.000***	0.328	0.069	***0.016***	***0.006***	0.997	***0.012***	1.000	1.000	1.000	0.992		1.000	***0.000***	***0.000***	***0.000***	***0.000***
TIR	***0.000***	***0.000***	***0.000***	***0.015***	***0.000***	***0.000***	***0.001***	0.552	***0.000***	1.000	1.000	1.000	1.000	1.000		***0.000***	***0.000***	***0.000***	***0.000***
WHR	0.897	0.439	0.570	0.623	0.949	0.996	***0.000***	***0.001***	0.998	***0.006***	***0.001***	***0.000***	***0.000***	***0.000***	***0.000***		***0.012***	0.731	0.422
PAR	0.996	1.000	1.000	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.012***		0.980	0.999
TIN	1.000	1.000	1.000	***0.002***	***0.013***	***0.015***	***0.000***	***0.000***	***0.022***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.731	0.980		1.000
HRK	1.000	1.000	1.000	***0.000***	***0.003***	***0.006***	***0.000***	***0.000***	***0.008***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.422	0.999	1.000	

TABLE A.4.2. Unequal *N* HSD post-hoc test investigating the difference in Ba:Ca concentrations in the shells of *Austrovenus* juveniles collected from 19 sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	MAN	HOK	RAG	PAN	OTU	ARP	PAI	HDI	WHT	WHP	CLB	AOT	KAW	GLD	TIR	WHR	PAR	TIN	HRK
MAN		0.641	0.999	1.000	1.000	***0.000***	0.997	***0.000***	0.204	***0.000***	***0.000***	***0.006***	***0.000***	0.399	1.000	0.992	0.914	***0.000***	1.000
HOK	0.641		***0.038***	0.988	0.895	***0.000***	1.000	***0.000***	1.000	0.248	***0.000***	***0.000***	***0.000***	***0.000***	0.174	***0.024***	***0.006***	0.959	0.179
RAG	0.999	***0.038***		0.801	0.969	***0.027***	0.474	***0.000***	***0.005***	***0.000***	***0.002***	0.488	0.086	0.993	1.000	1.000	1.000	***0.000***	1.000
PAN	1.000	0.988	0.801		1.000	***0.000***	1.000	***0.000***	0.840	***0.002***	***0.000***	***0.000***	***0.000***	***0.049***	0.972	0.596	0.295	***0.018***	0.974
OTU	1.000	0.895	0.969	1.000		***0.000***	1.000	***0.000***	0.406	***0.000***	***0.000***	***0.000***	***0.000***	0.161	0.999	0.840	0.536	***0.001***	0.999
ARP	***0.000***	***0.000***	***0.027***	***0.000***	***0.000***		***0.000***	***0.000***	***0.000***	***0.000***	0.974	0.998	1.000	0.906	***0.000***	***0.002***	***0.014***	***0.000***	***0.000***
PAI	0.997	1.000	0.474	1.000	1.000	***0.000***		***0.000***	0.933	***0.008***	***0.000***	***0.000***	***0.000***	***0.011***	0.541	0.079	***0.016***	***0.015***	0.552
HDI	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***		***0.000***	***0.000***	0.270	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
WHT	0.204	1.000	***0.005***	0.840	0.406	***0.000***	0.933	***0.000***		0.415	***0.000***	***0.000***	***0.000***	***0.000***	***0.001***	***0.000***	***0.000***	0.862	***0.002***
WHP	***0.000***	0.248	***0.000***	***0.002***	***0.000***	***0.000***	***0.008***	***0.000***	0.415		***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.999	***0.000***
CLB	***0.000***	***0.000***	***0.002***	***0.000***	***0.000***	0.974	***0.000***	0.270	***0.000***	***0.000***		0.571	0.908	0.136	***0.000***	***0.004***	***0.011***	***0.000***	***0.000***
AOT	***0.006***	***0.000***	0.488	***0.000***	***0.000***	0.998	***0.000***	***0.000***	***0.000***	***0.000***	0.571		1.000	1.000	***0.016***	0.220	0.543	***0.000***	***0.015***
KAW	***0.000***	***0.000***	0.086	***0.000***	***0.000***	1.000	***0.000***	***0.000***	***0.000***	***0.000***	0.908	1.000		0.984	***0.002***	***0.042***	0.152	***0.000***	***0.002***
GLD	0.399	***0.000***	0.993	***0.049***	0.161	0.906	***0.011***	***0.000***	***0.000***	***0.000***	0.136	1.000	0.984		0.882	0.998	1.000	***0.000***	0.877
TIR	1.000	0.174	1.000	0.972	0.999	***0.000***	0.541	***0.000***	***0.001***	***0.000***	***0.000***	***0.016***	***0.002***	0.882		1.000	0.999	***0.000***	1.000
WHR	0.992	***0.024***	1.000	0.596	0.840	***0.002***	0.079	***0.000***	***0.000***	***0.000***	***0.004***	0.220	***0.042***	0.998	1.000		1.000	***0.000***	1.000
PAR	0.914	***0.006***	1.000	0.295	0.536	***0.014***	***0.016***	***0.000***	***0.000***	***0.000***	***0.011***	0.543	0.152	1.000	0.999	1.000		***0.000***	0.998
TIN	***0.000***	0.959	***0.000***	***0.018***	***0.001***	***0.000***	***0.015***	***0.000***	0.862	0.999	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***		***0.000***
HRK	1.000	0.179	1.000	0.974	0.999	***0.000***	0.552	***0.000***	***0.002***	***0.000***	***0.000***	***0.015***	***0.002***	0.877	1.000	1.000	0.998	***0.000***	

TABLE A.4.3. Unequal *N* HSD post-hoc test investigating the difference in Mn:Ca concentrations in the shells of *Austrovenus* juveniles collected from 19 sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	MAN	HOK	RAG	PAN	OTU	ARP	PAI	HDI	WHT	WHP	CLB	AOT	KAW	GLD	TIR	WHR	PAR	TIN	HRK
MAN		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	***0.000***	1.000	1.000	0.987	1.000
HOK	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	***0.000***	1.000	1.000	0.999	1.000
RAG	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.992	***0.000***	1.000	1.000	0.946	1.000
PAN	1.000	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.999	***0.000***	1.000	1.000	0.975	1.000
OTU	1.000	1.000	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.995	0.850	***0.000***	0.996	1.000	0.398	1.000
ARP	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.995	***0.000***	1.000	1.000	0.746	1.000
PAI	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	1.000	1.000	0.998	0.883	***0.000***	0.994	1.000	0.284	1.000
HDI	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	1.000	0.998	0.891	***0.000***	0.997	1.000	0.382	1.000
WHT	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	0.989	0.795	***0.000***	0.974	1.000	0.123	1.000
WHP	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	0.886	***0.000***	1.000	1.000	0.794	1.000
CLB	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	0.998	***0.000***	1.000	1.000	0.995	1.000
AOT	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	0.979	***0.000***	1.000	1.000	0.613	1.000
KAW	1.000	1.000	1.000	1.000	0.995	1.000	0.998	0.998	0.989	1.000	1.000	1.000		1.000	***0.000***	1.000	0.992	0.999	1.000
GLD	1.000	1.000	0.992	0.999	0.850	0.995	0.883	0.891	0.795	0.886	0.998	0.979	1.000		***0.000***	1.000	0.816	1.000	0.993
TIR	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***		***0.000***	***0.000***	***0.000***	***0.000***
WHR	1.000	1.000	1.000	1.000	0.996	1.000	0.994	0.997	0.974	1.000	1.000	1.000	1.000	1.000	***0.000***		0.980	0.997	1.000
PAR	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.992	0.816	***0.000***	0.980		0.193	1.000
TIN	0.987	0.999	0.946	0.975	0.398	0.746	0.284	0.382	0.123	0.794	0.995	0.613	0.999	1.000	***0.000***	0.997	0.193		0.766
HRK	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.993	***0.000***	1.000	1.000	0.766	

TABLE A.4.4. Unequal *N* HSD post-hoc test investigating the difference in Mg:Ca concentrations in the shells of *Austrovenus* juveniles collected from 19 sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	MAN	HOK	RAG	PAN	OTU	ARP	PAI	HDI	WHT	WHP	CLB	AOT	KAW	GLD	TIR	WHR	PAR	TIN	HRK
MAN		1.000	0.670	0.173	***0.000***	***0.011***	0.574	1.000	0.073	***0.010***	0.083	***0.006***	***0.012***	***0.000***	***0.000***	1.000	1.000	0.993	1.000
HOK	1.000		0.991	0.076	***0.000***	***0.005***	0.306	0.986	***0.032***	***0.001***	***0.016***	***0.003***	***0.006***	***0.000***	***0.000***	0.985	0.992	0.897	1.000
RAG	0.670	0.991		***0.000***	***0.000***	***0.000***	***0.000***	0.081	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	0.078	0.104	***0.021***	0.849
PAN	0.173	0.076	***0.000***		0.630	1.000	1.000	0.878	1.000	0.996	0.999	1.000	1.000	0.777	***0.000***	0.883	0.832	0.987	0.076
OTU	***0.000***	***0.000***	***0.000***	0.630		0.983	0.136	***0.001***	0.769	1.000	1.000	0.994	0.980	1.000	***0.000***	***0.001***	***0.000***	***0.004***	***0.000***
ARP	***0.011***	***0.005***	***0.000***	1.000	0.983		0.974	0.134	1.000	1.000	1.000	1.000	1.000	0.996	***0.000***	0.093	0.065	0.242	***0.000***
PAI	0.574	0.306	***0.000***	1.000	0.136	0.974		0.990	1.000	0.912	0.974	0.937	0.992	0.375	***0.000***	0.985	0.968	1.000	0.129
HDI	1.000	0.986	0.081	0.878	***0.001***	0.134	0.990		0.501	0.172	0.449	0.085	0.211	***0.009***	***0.000***	1.000	1.000	1.000	0.993
WHT	0.073	***0.032***	***0.000***	1.000	0.769	1.000	1.000	0.501		1.000	1.000	1.000	1.000	0.915	***0.000***	0.423	0.340	0.723	***0.003***
WHP	***0.010***	***0.001***	***0.000***	0.996	1.000	1.000	0.912	0.172	1.000		1.000	1.000	1.000	1.000	***0.000***	0.176	0.142	0.375	***0.004***
CLB	0.083	***0.016***	***0.000***	0.999	1.000	1.000	0.974	0.449	1.000	1.000		1.000	1.000	1.000	***0.013***	0.454	0.404	0.670	***0.045***
AOT	***0.006***	***0.003***	***0.000***	1.000	0.994	1.000	0.937	0.085	1.000	1.000	1.000		1.000	0.999	***0.000***	0.056	***0.038***	0.216	***0.000***
KAW	***0.012***	***0.006***	***0.000***	1.000	0.980	1.000	0.992	0.211	1.000	1.000	1.000	1.000		0.995	***0.000***	0.217	0.166	0.512	***0.001***
GLD	***0.000***	***0.000***	***0.000***	0.777	1.000	0.996	0.375	***0.009***	0.915	1.000	1.000	0.999	0.995		***0.000***	***0.010***	***0.007***	***0.037***	***0.000***
TIR	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.013***	***0.000***	***0.000***	***0.000***		***0.000***	***0.000***	***0.000***	***0.000***
WHR	1.000	0.985	0.078	0.883	***0.001***	0.093	0.985	1.000	0.423	0.176	0.454	0.056	0.217	***0.010***	***0.000***		1.000	1.000	0.988
PAR	1.000	0.992	0.104	0.832	***0.000***	0.065	0.968	1.000	0.340	0.142	0.404	***0.038***	0.166	***0.007***	***0.000***	1.000		1.000	0.995
TIN	0.993	0.897	***0.021***	0.987	***0.004***	0.242	1.000	1.000	0.723	0.375	0.670	0.216	0.512	***0.037***	***0.000***	1.000	1.000		0.856
HRK	1.000	1.000	0.849	0.076	***0.000***	***0.000***	0.129	0.993	***0.003***	***0.004***	***0.045***	***0.000***	***0.001***	***0.000***	***0.000***	0.988	0.995	0.856	

TABLE A.4.5. Unequal *N* HSD post-hoc test investigating the difference in Zn:Ca concentrations in the shells of *Austrovenus* juveniles collected from 19 sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	MAN	HOK	RAG	PAN	OTU	ARP	PAI	HDI	WHT	WHP	CLB	AOT	KAW	GLD	TIR	WHR	PAR	TIN	HRK
MAN		0.015	0.618	***0.000***	***0.000***	***0.000***	***0.001***	1.000	***0.000***	***0.000***	***0.003***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
HOK	***0.015***		0.986	0.988	1.000	***0.000***	1.000	0.170	0.096	0.167	0.998	0.255	0.769	0.210	0.963	0.094	0.118	0.722	0.614
RAG	0.618	0.986		0.071	0.449	***0.000***	0.919	0.986	***0.000***	***0.001***	0.514	***0.000***	***0.006***	***0.001***	***0.039***	***0.000***	***0.000***	***0.005***	***0.002***
PAN	***0.000***	0.988	0.071		1.000	***0.000***	0.988	***0.000***	0.908	0.982	1.000	0.992	1.000	0.996	1.000	0.904	0.936	1.000	1.000
OTU	***0.000***	1.000	0.449	1.000		***0.000***	1.000	***0.000***	0.263	0.710	1.000	0.587	0.985	0.814	1.000	0.257	0.316	0.975	0.937
ARP	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***		***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
PAI	***0.001***	1.000	0.919	0.988	1.000	***0.000***		***0.005***	***0.007***	0.258	1.000	***0.041***	0.626	0.324	0.866	***0.006***	***0.010***	0.381	0.258
HDI	1.000	0.170	0.986	***0.000***	***0.000***	***0.000***	***0.005***		***0.000***	***0.000***	***0.028***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***	***0.000***
WHT	***0.000***	0.096	***0.000***	0.908	0.263	***0.000***	***0.007***	***0.000***		1.000	1.000	1.000	0.999	1.000	0.835	1.000	1.000	0.997	1.000
WHP	***0.000***	0.167	***0.001***	0.982	0.710	***0.000***	0.258	***0.000***	1.000		1.000	1.000	1.000	1.000	0.994	1.000	1.000	1.000	1.000
CLB	***0.003***	0.998	0.514	1.000	1.000	***0.000***	1.000	***0.028***	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
AOT	***0.000***	0.255	***0.000***	0.992	0.587	***0.000***	***0.041***	***0.000***	1.000	1.000	1.000		1.000	1.000	0.992	1.000	1.000	1.000	1.000
KAW	***0.000***	0.769	***0.006***	1.000	0.985	***0.000***	0.626	***0.000***	0.999	1.000	1.000	1.000		1.000	1.000	0.999	1.000	1.000	1.000
GLD	***0.000***	0.210	***0.001***	0.996	0.814	***0.000***	0.324	***0.000***	1.000	1.000	1.000	1.000	1.000		0.999	1.000	1.000	1.000	1.000
TIR	***0.000***	0.963	***0.039***	1.000	1.000	***0.000***	0.866	***0.000***	0.835	0.994	1.000	0.992	1.000	0.999		0.871	0.916	1.000	1.000
WHR	***0.000***	0.094	***0.000***	0.904	0.257	***0.000***	***0.006***	***0.000***	1.000	1.000	1.000	1.000	0.999	1.000	0.871		1.000	0.998	1.000
PAR	***0.000***	0.118	***0.000***	0.936	0.316	***0.000***	***0.010***	***0.000***	1.000	1.000	1.000	1.000	1.000	1.000	0.916	1.000		0.999	1.000
TIN	***0.000***	0.722	***0.005***	1.000	0.975	***0.000***	0.381	***0.000***	0.997	1.000	1.000	1.000	1.000	1.000	1.000	0.998	0.999		1.000
HRK	***0.000***	0.614	***0.002***	1.000	0.937	***0.000***	0.258	***0.000***	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

A.5 HSD post-hoc results (point ablation study)

TABLE A.5.1. Unequal *N* HSD post-hoc test investigating the difference in Sr concentrations in the shells of *Austrovenus* juveniles collected from four sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	TIN	PAI	AOT	KAW
TIN		***0.000***	***0.028***	0.064
PAI	***0.000***		***0.003***	0.162
AOT	***0.028***	***0.003***		0.958
KAW	0.064	0.162	0.958	

TABLE A.5.2. Unequal *N* HSD post-hoc test investigating the difference in Ba concentrations in the shells of *Austrovenus* juveniles collected from four sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	TIN	PAI	AOT	KAW
TIN		0.777	0.797	0.157
PAI	0.777		0.289	***0.040***
AOT	0.797	0.289		0.450
KAW	0.157	***0.040***	0.450	

TABLE A.5.3. Unequal *N* HSD post-hoc test investigating the difference in Mg concentrations in the shells of *Austrovenus* juveniles collected from four sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	TIN	PAI	AOT	KAW
TIN		0.059	0.177	0.977
PAI	0.059		0.988	0.573
AOT	0.177	0.988		0.716
KAW	0.977	0.573	0.716	

TABLE A.5.4. Unequal *N* HSD post-hoc test investigating the difference in Mn concentrations in the shells of *Austrovenus* juveniles collected from four sites. Significant values ($p < 0.05$) are highlighted in red and italicised.

Site	TIN	PAI	AOT	KAW
TIN		1.000	0.830	0.954
PAI	1.000		0.844	0.958
AOT	0.830	0.844		1.000
KAW	0.954	0.958	1.000	