The Growth and Reproduction of the Freshwater Limpet *Burnupia stenochorias* (Pulmonata, Ancylidae), and An Evaluation of its Use As An Ecotoxicology Indicator in Whole Effluent Testing

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

DOCTOR OF PHILOSOPHY
of
RHODES UNIVERSITY

by

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September 2001

ABSTRACT

For the protection of the ecological Reserve in South Africa, the proposed introduction of compulsory toxicity testing in the licensing of effluent discharges necessitates the development of whole effluent toxicity testing. The elucidation of the effects of effluent on the local indigenous populations of organisms is essential before hazard and risk assessment can be undertaken. The limpet *Burnupia stenochorias*, prevalent in the Eastern Cape of South Africa, was chosen to represent the freshwater molluscs as a potential toxicity indicator. Using potassium dichromate (as a reference toxicant) and a textile whole effluent, the suitability of *B. stenochorias* was assessed under both acute and chronic toxicity conditions in the laboratory. In support of the toxicity studies, aspects of the biology of *B. stenochorias* were investigated under both natural and laboratory conditions.

Using Principal Component and Discriminant Function Analyses, the relative shell morphometrics of three feral populations of *B. stenochorias* were found to vary. Length was shown to adequately represent growth of the shell, although the inclusion of width measurements is more statistically preferable. Two of the feral populations, one in impacted water, were studied weekly for 52 weeks to assess natural population dynamics. Based on the Von Bertalanffy Growth Equation, estimates of growth and longevity were made for this species, with growth highly seasonal. Age is not easily discerned from shell size. Egg laying occurred all year round, with early summer (peak egg lay), mid summer (a second, smaller peak in egg lay), and winter (limited presence of eggs) phases. In toxicity testing, consideration is given to the choice of the test organism based on age and sexual development. Consequently, the sexual development of *B. stenochorias* relative to shell length was determined with the aid of histological examinations of transverse sections of limpets, of all sizes, collected over one year. Limpets less than 3mm shell length were found to be immature in the development of the oocytes

and spermatozoa, and were later chosen for acute toxicity tests. A laboratory diet was developed, for both culturing and maintaining of the limpets during toxicity tests; however, the diet requires optimisation. Under laboratory conditions, growth was linear, and individual fecundity highly variable.

Successful methods for the collection of limpets from naturally occurring populations, and their acclimation to the laboratory were developed. Three *B. stenochorias* populations, representing different hydrological and water quality conditions, were compared to a laboratory population (maintained for three years) in their responses to the textile whole effluent and potassium dichromate. Under acute conditions, variability of mortality between limpet populations and between seasons was consistent with acceptable international standards. However, seasonal differences between feral limpets were apparent, with early summer limpets significantly more susceptible to both potassium dichromate and textile effluent than winter limpets. Although mortality occurred within the effluent at all concentrations, no 96 hour LC_{50} values were obtained.

The chronic toxicity effects of the textile whole effluent were assessed over the entire life cycle of *B. stenochorias*, based on survival, growth and reproductive effects. Lower concentrations of effluent (# 10%) gave greater variability of responses and toxicity than higher concentrations, with a 43 day LC_{50} of 3.9% effluent. The No Observed Effect Concentrations for survival (over 43 days) were calculated in consecutive years as 0.1% and 1% effluent. Survival is considered a useful tool for determining toxicity endpoints using *B. stenochorias*. Limpet growth remained linear in effluent, with an apparent stimulation of growth at the 3-10% effluent concentration, confusing the toxicity and variability assessments. The possible addition of nutrients from the effluent points to either a potential inadequacy of the food quality provided in the chronic assessment, or the presence in the effluent of growth stimulants. Growth was also found to be too variable to allow adequate statistical conclusions

about the toxicity of the effluent, although it is suggested that growth may be useful in the assessment of single compounds. Despite large individual variability in fecundity, statistical differences were discernible between effluent concentrations. The application of fecundity of *B. stenochorias* in hazard assessment therefore warrants further investigation. It was concluded that an assessment of textile whole effluent toxicity to *B. stenochorias* over an entire life cycle, and an F1 generation, is unnecessary. The development of the bucket/plastic bag method for both acute and chronic toxicity assessment of *B. stenochorias* was useful.

In the final assessment of the usefulness of *B. stenochorias* as a toxicity indicator, toxicity endpoints were compared with those of the standard laboratory organism *Daphnia pulex*. Both in acute and chronic toxicity, *B. stenochorias* was found to be more sensitive. *B. stenochorias* is therefore considered valuable as a South African freshwater molluscan ecotoxicological indicator, with a place in hazard assessment, although further development and research is necessary before the limpet can be effectively used.

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ACKNOWLEDGEMENTS

" This enquiry, I must confess, is a groping in the dark;
but although I have not brought it into a clear light;
yet, I can affirm that I have brought it from an utter darkness to a thin mist."
John Aubrey, Brief Lives

There are lots of folk to thank for helping me attain the thin mist stage in my investigations into this freshwater limpet. Lil Haigh, who inadvertently gave me the opportunity to study further, encouraging and concerned at every step. My supervisors Professors Tally Palmer and Alan Hodgson to whom I am greatly indebted for opportunities, advice and not least of all, the motivations for funding to keep me financially afloat! Financial assistance was received via National Research Foundation and ESKOM Tertiary Education Support Programme studentships, and from the Rhodes University Graduate Assistance Programme. The other members of the Institute for Water Research team to whom I extend my thanks are Drs Patsy Scherman, Nikite Muller and Gay Youthed, and Lynn Marajh who were always willing to help. Special thanks go to Lora Pakhomova for the *Daphnia pulex* (plenty of neonates whenever I needed them); Valerie Hodgson for setting up the bacterial plates; and Robin Cross and Shirley Pinchuck of the Electron Microscopy Unit, for help with microscopy techniques and procedures. Thanks also to other members of the Rhodes University academia who contributed to valuable discussions on statistics, in particular, Dr Martin Villet (thanks especially *swaar*), Professor Sarah Radloff and Dr Tony Booth. I must also acknowledge Sebastian Jooste (Department of Water Affairs and Forestry) for acting as an enlightening source of information. Finally, but by no means least, thanks to my husband Mike and children Greg and Jodie, ever patient and understanding and so encouraging. Thanks be to God for you especially. This thesis is dedicated to you.

CHAPTER 1. GENERAL INTRODUCTION

Global water consumption is likely to double in the next twenty years, and industrialization and urbanization have dramatically increased environmental pollution from toxic contaminants (Naiman *et al*., 1994). Hence it is imperative for governments to possess sound information on the availability and state of health of water resources; to institute comprehensive legislation to cope with maintaining and restoring the health of these resources; and to increase ecological awareness amongst their electorate (Naiman *et al.*, 1994; Palmer *et al.*, 2000). In South Africa, water resources are considered scarce in global terms (Davies *et al*., 1995), and critically needing legislative attention for their long term preservation (Palmer *et al*., 1996; Liu and Dutka, 1999).

Aquatic ecosystems are complex and diverse. They are structured by the climatic regime, the physical (light, temperature, flow, habitat and others) and chemical environments (organic and inorganic carbon, nutrients and oxygen) with which they interact, as well as the biological interactions that occur within them (Hart *et al*., 1999). Conditions in South Africa such as drought, floods and erosion as well as the human related stressors[1], all have consequences on the numbers and types of biota present at any one time. Water quality, which includes physical, chemical, biological and aesthetic properties, determines the aquatic system's fitness for a variety of water users including industry, agriculture, domestic supply, and [human] recreation (DWAF [Department of Water Affairs and Forestry], 1995). Any deterioration in freshwater quality, especially in urban and industrial areas, is recognised as a serious threat to the freshwater ecosystems which form the resource base on which water users depend. Accordingly, freshwater ecosystems need to be protected and maintained in a state of ecological integrity where biological processes remain functional, as this is a pre-requisite for meeting most other user requirements (Palmer *et al*., 1991). A compromise has to be made between the human demands and the need for protection and rehabilitation (if necessary) of freshwater systems. Therefore, there is an imperative need to focus on the provision of sound and well-managed information which will effectively allow us to improve the protection of water resources and aquatic species in South

[1] Stressors - the physical, chemical or biological factors that can cause an adverse effect on an aquatic ecosystem. Toxic stressors include heavy metals and toxic organic compounds, salinity and pH. Non-toxic stressors include nutrients, turbidity and suspended particulate matter, organic matter, flow and habitat (Hart *et al.*, 1999).

Africa (Breen *et al.*, 1998; Palmer, 1999).

1.1. Water Quality Monitoring

Ecosystems can function at a number of levels of health. In South Africa, different levels of ecosystem health have been recognized in a classification system which grades water quantity, instream habitat, riparian habitat, biota present and (of primary concern to this thesis) water quality into classes A (pristine) to D (maximum sustainable use) (Palmer, 1999). In addition, several relevant monitoring and management programmes are currently under development. They include guidelines for in-stream biological monitoring (Uys *et al.*, 1996), modelling estimations of in-stream flow requirements (King and Louw, 1998), and toxicological assessments of chemicals and whole effluent (Slabbert *et al.*, 1998). Only the successful integration of these programmes and the correct application of the resulting information will ensure the sustainable use of the country's water resources (Roux *et al.*, 1996).

South Africa has a long history of chemical water quality monitoring, based on a network of chemical monitoring sites. Water quality standards have been previously expressed as chemical concentrations (general and specific standards) (DWAF, 1995). However, characterizing and setting chemical standards for complex effluents is difficult, because of the chemical interactions which occur. The resulting chemical species and concentrations and the consequent effects on aquatic life are difficult to predict (Blaise *et al.*, 1988). Toxicology provides a link between chemical concentration and biotic response (Rand, 1995); therefore, whole effluent toxicity testing in particular, enables an integrated, cost-effective assessment of potential hazardous impacts in the aquatic environment. Most international water quality guidelines for ecosystem protection are based on toxicological data (CCME, 1991; ANZECC, 1992; DWAF, 1996).

Whereas toxicology addresses the responses of biota to toxic stressors, *eco*toxicology seeks to link these responses to ecosystem processes (Pritchard, 1993; Chapman, 1995). Ecotoxicology and toxicology (defined in more detail in Chapter 8) can provide information for the development of both resource quality objectives and effluent discharge licence conditions. In the last decade it has become increasingly necessary for South Africa to develop its own database on the toxic effects of chemicals and effluents on indigenous freshwater fauna and flora (Moore, 1990). It is envisaged that future legislation regarding licences for the discharge of chemically complex wastes will require toxicity

testing (Palmer and Jooste, 2000). The latest edition of the South African Water Quality Guidelines for Aquatic Ecosystems (DWAF, 1996) were based primarily on international toxicity data bases, with the imposition of safety factors traditionally used in the formulation of water quality guidelines where few data are available. A data base on the response of South African invertebrates could contribute to more relaxed site specific guidelines by providing additional data and decreasing the need for safety factors (Scherman and Palmer, 2000). This study therefore contributes to the development of whole effluent toxicity testing in South Africa, using the indigenous freshwater gastropod *Burnupia stenochorias* as a test organism.

1.2. The Choice of Toxicity Indicators

Many taxa have been used as biomonitors of the aquatic environment, including algae (*e.g.* Trama, 1984), protozoa and bacteria (Liu and Dutka, 1984); macroinvertebrates (France, 1993), fish (Mayer *et al.*, 1994), amphibians (Cooke, 1981) and birds (Anderson and Hickey, 1997). Freshwater macroinvertebrates have been widely used as indicators of water quality in river management because they are affected not only by changes in the natural features of rivers, but also by changes induced by human activities (Boudou and Ribeyre, 1989; Bargos *et al.*, 1990; Palmer *et al.*, 1991; Merritt and Cummins, 1996; O'Keeffe, 1999). Although there are many arguments both for and against the use of benthic macroinvertebrates (Rosenberg and Resh, 1993; Chapter 8), there are considerable data available that indicate their value in toxicity testing (*e.g.* Mayer and Ellersieck, 1986; Larson and Hyland, 1987). Approximately 50 macroinvertebrates have been or are recommended for acute toxicity testing in the U.S.A. (Buikema and Voshell, 1993), Australia (Chapman *et al.*, 1999), Canada (Blaise *et al*., 1988) and in European countries (OECD, 1987; Jäger *et al.*, 1996).

Guidelines for the selection of organisms to be used in toxicity tests have been suggested by many authors (*e.g.* USEPA, 1979a; Winner *et al.*, 1980; Sheehan, 1980; Mayer and Ellersieck, 1986; Hilsenhoff, 1987). Few potential test organisms meet all of the criteria used in the selection of an 'ideal' test organism and thus the species choice may be difficult (Buikema *et al.*, 1982; Buikema and Voshell, 1993; Chapter 1). Cairns (1986b) addresses the concept of the most sensitive species as a choice for toxicity testing, with the "erroneous perception that responses from sensitive species can be extrapolated to a wider array of species at different levels of biological organization". Firstly, the sensitive species may not be resident in the ecosystem for which management decisions are to be taken,

with the consequent setting of incorrect criteria. Secondly, sensitivity to a variety of chemicals is known to differ between species (Rand *et al.*, 1995; Liu and Dutka, 1999) with variability of response demonstrated by feral populations compared to laboratory bred populations (Cairns,1986a; Calow, 1989; Dillon, 2000). Toxicity test data acquired from feral, local, riverine invertebrates are particularly recommended where site specific recommendations, guidelines and licence criteria are required (Buikema and Voshell, 1993; Scherman and Palmer, 2000).

One of the key criteria of particular concern in this study includes the necessity for end-points that are easily identified and measured (USEPA, 1979a). The assessment of toxicity, the control of effluent discharges, or the registration of a new chemical require the collection of consistent and comparable toxicity response data (APHA, 1992; Rand, 1995; Slabbert, 1996). In these cases, data from the use of a standard laboratory organism such as *Daphnia* species are preferable as they provide benchmark toxicity data (APHA, 1992; Chapter 12). However, there is always the question of whether standard test organisms which inhabit lentic waters reflect the responses of lotic dwelling, indigenous organisms.

The Mollusca are possibly the most numerous and successful group of animals after the arthropods, with twice as many species (approximately 110 000 still living) as the vertebrates (Seed, 1983). Their biomass dominates the lower trophic levels of many of the marine and freshwater ecosystems (Brown, 1994). Molluscs represent an important base component of the food web in aquatic organisms (Lodge *et al*., 1986; Mance, 1987) and their link to the human diet in Africa because of the dependence of the inland fisheries on the molluscan group underlines their ecological and economic importance (Brown, 1994). In fresh waters they are either grazers (Gastropoda, the snails and limpets) or collectors of fine particulate matter and phytoplankton (Bivalvia, the bivalve molluscs, in South Africa numbering half as many species as the gastropods [Appleton, 1996]). By scraping algae and other organic layers from the benthic surfaces including macrophytes, gastropods can play a prominent role in the bioenergetics of an ecosystem (Brönmark, 1989; Huryn *et al.*, 1994). However, in Africa many of the freshwater snail species, including pulmonates belonging to the family Ancylidae, are believed to be endangered and vulnerable to extinction (Brown and Kristensen, 1998). Over a period of 40 to 50 years, pulmonates from North American lakes have been shown to disappear completely as a result of eutrophication caused primarily from human pollution of one sort or another (Russel-Hunter, 1978). The need to pay attention to mollusc conservation lends weight to consideration of their use as

indicators of water quality.

Molluscs have been used as indicators of contamination in aquatic systems since at least the 1960s (Keller and Lydy, 1997). Freshwater molluscs are sedentary, and therefore reflect past and present environmental conditions at a particular site. They are tolerant of a wide range of pollutants but these pollutants tend to bioaccumulate (Walsh *et al.*, 1994; Keller and Lydy, 1997), and in the case of pulmonates, this is primarily through their grazing habit (Brown, 1991). Freshwater pulmonates, with their rapid reproductive cycle, are therefore a group of organisms which can provide useful information on the quality of the environment and act as bioindicators of contaminated aquatic ecosystems (Mouthon, 1996a).

The toxicity of molluscicides in the [human] fight against schistosomiasis has been a long standing research endeavour in Africa (de Kock and Wolmarans, 1998). However, snails have been shown to be useful models for estimating ecological significance of various types of pollution (Gomot, 1998). Like bivalves, the gastropods may act as indicators of sediment contamination (Tessier *et al.*, 1993). They are generally sensitive to many metals, including cadmium, copper, chromium, nickel, silver, mercury and zinc (Khangarot and Ray, 1988). Current water quality criteria for the U.S.A. were developed based on toxicity data that included very little information on molluscs (Williams *et al.*, 1974; Stephan, 1977; Jacobson, 1990; Naimo *et al.*, 1992). However, long term exposure to sub-lethal concentrations affecting such aspects as changes to shell structure and tissues in molluscs, in turn influencing the ability to deal with additional stresses, are more difficult to assess if surrogate, laboratory organisms such as *Daphnia pulex* are used (Walsh *et al.*, 1994; Keller and Lydy, 1997).

Toxicity tests using molluscs include exposure to sub-lethal concentrations of organics, metals and pesticides (Ravera, 1991; Walsh *et al.*, 1994). Death, metabolic disturbances and tissue necrosis (Gardner *et al.*, 1981), changes in enzyme activity (Farris *et al.*, 1989), behavioural changes, growth disruptions (Metcalfe-Smith and Green, 1992), reproduction effects and other responses have all been recorded (Walsh *et al.*, 1994). The various factors that affect bioaccumulation of metals and organics in molluscs are covered in Keller and Lydy (1997). Pulmonates have been used to test the toxicity of pesticides (Bluzat and Seugé, 1983; Baturo *et al.*, 1995), insecticides (Woin and Brönmark, 1992), detergents (Tarazona and NuÁez, 1987) and polyhalogenated biphenyls (Gomot, 1998). In the

development of toxicity biomarkers (described in Chapter 8), techniques have been developed for determining the presence and level of various enzymes in molluscs (Chirombe *et al*., 1998). These levels of enzymes have been related to the toxic presence of pesticides (Dauberschmidt *et al*., 1997; Chirombe and Naik, 2001) and other pollutants (Giesy and Graney, 1989). Yet information on the relationship between specific water quality factors and changes in health, abundance and distribution of freshwater molluscs lags behind that of other invertebrate taxa (Williams *et al.*, 1974; Dillon, 2000). Reproductive strategies amongst the freshwater molluscs are varied, and the lack of biological information has had a major impact on the acquisition of toxicity data (Keller and Lydy, 1997). The eggs of freshwater pulmonates are generally less hardy than the adults (Machin, 1975) and so their susceptibility to toxicants could play a role in chronic toxicity testing.

1.3. The Choice of *Burnupia stenochorias* as an Ecotoxicological Indicator in South Africa

The selection and investigation of any South African indigenous organism for toxicity testing will provide two ways in which the toxicological results could be used: firstly, to set site-specific licence conditions for waste disposal (i.e. used in source directed control); and secondly, to set and audit instream resource quality objectives (*i.e*. used in resource directed measures) (Palmer *et al.*, 1996; DWAF, 1999). At present, no toxicity data are available for a southern African mollusc, although in Zimbabwe, research continues on enzyme analyses as biomarkers within *Lymnaea natalensis* and *Helisoma duryi* (Chirombe and Naik, 2001). Unionid mussels, snails, sphaeriid clams and *Corbicula* species have been used internationally in toxicity tests with *Amnicola limosa* (Maltby and Calow (1989), *Biomphalaria glabrata*, *Physa acuta*, *L. stagnalis* and *Radix auricolaria japonica* the dominant species of snails (Gomot, 1998). *P. acuta*, an invasive species tolerant of polluted water (Brown, 1994; Appleton, 1996), has been used in pesticide toxicity tests in Australia where it is also invasive (A. Colville, pers. com.). However, in South Africa, only indigenous species have been recommended for the setting of South African water quality guidelines (Section 1.2).

The freshwater limpet *Burnupia stenochorias* (Melvill and Ponsonby, 1903, *Ancylus*) was therefore one of the invertebrate freshwater species (representing the freshwater Mollusca) chosen for use in toxicity tests at the Centre of Aquatic Toxicology, Institute for Water Research (CAT-IWR; Rhodes University, Grahamstown) (Haigh and Davies-Coleman, 1997, 1999). *B. stenochorias* is a gastropod belonging to the subclass Pulmonata, order Basommatophora, of the family Ancylidae (sometimes

referred to as the Ancyloplanorbidae) which includes all the freshwater limpets (Seed, 1983; Brown, 1994). Although the ancylids are represented world-wide, the genus *Burnupia* is generally confined to Africa (Figure 1.1; Brown, 1994), with the exception of some recent records of this genus occurring in Brazil, which suggest a Gondwanaland origin (Santos, 1990, 1999; Lanzer, 1991, 1996).

The genus *Burnupia* is widespread in South Africa (Figure 1.1), where the species are generally found in well-oxygenated fresh waters, especially on stones in streams and at the edges of lentic water bodies (Brown, 1994). Ancylids in general have a very reduced mantle cavity and are therefore totally dependent for their respiration needs on oxygen dissolved in the water. This contributed to its choice as a candidate freshwater mollusc indicator, as opposed to representatives from the Planorbid, Lymnaeid and other snail families. Although the taxonomy of *Burnupia* species is based on shell differences, including shell shape, it has been suggested that shell differences may be influenced by microhabitat (Brown, 1994; Appleton, 1996). The classification of the genus is therefore in need of revision (Brown, 1994; Appleton, 1996). Limpets collected from the study sites and sent in 1996 to the British Museum of Natural History were positively identified, pending revision of the genus, as *Burnupia stenochorias* (D. Brown, pers. com.). *B. stenochorias* will, for the purposes of this thesis, be considered a distinct and recognizable species, as referred to by Walker (1923, 1926), Pilsbry and Bequaert (1927), and Mandahl-Barth (1968). A full description of the shell of *B. stenochorias* is given in Chapter 3.

Toxicological procedures must be founded on defensible methods, and therefore the role of variability of response of the test organism must be addressed when considering the quality assurance of regular toxicity testing (USEPA, 2000). Factors contributing to variability of response of the test organism include season, with associated changes in water flow and temperature, and developmental stage of the animal (size, age, sexual development, and in the case of most invertebrates, ecdysis) (De Graeve *et al*., 1992; Warren-Hicks and Parkhurst, 1992; Cairns and Pratt, 1993; Warren-Hicks and Moore, 1998). Results of toxicity tests will, unfortunately, reflect the variability that is inherent in a feral population, causing problems in the analysis and interpretation of results (Kooijman, 1981; Hickey and Martin, 1995). However, variability may also provide information as to the degree to which the feral population may be more or less resilient to stressors. The use of feral populations in site specific toxicity tests therefore offers the chance to take advantage of natural resilience, while not

compromising resource protection (DWAF, 1997). Most complex effluents are sublethally toxic, and consequently, there is the need for sublethal testing. The most commonly used criteria for determining sublethal testing (described in Chapter 8) are effects on the growth and reproduction of the test organism (Buikema and Voshell, 1993; Rand, 1995).

The species chosen for research have been determined to a large degree by the ease with which they are kept under laboratory conditions (Persoone and Janssen, 1993). *B. stenochorias* appeared to be an excellent candidate for South African toxicological investigations using an indigenous animal. All life history stages of *B. stenochorias* are aquatic, circumventing the problem of an aerial phase often encountered in maintaining or culturing aquatic species in the laboratory. It is easy to feed, robust with careful handling, does not appear to be prone to disease, infection or physical damage compared to other soft bodied invertebrates, and breeds easily in the laboratory (Haigh and Davies-Coleman, 1997). Freshwater pulmonates in general are likely to be susceptible to changes in water quality because of the high proportion of water within their bodies (80-92%; Machin, 1975). The surface area in contact with the aquatic exterior and therefore exposed to toxicants is large. The gut, mantle cavity and superficial mucus all contain water (Blinn, 1964) as does the open blood system where the body tissues are bathed in haemolymph (Burton, 1983). The other genus of the Ancylidae represented in southern Africa, *Ferrissia*, is generally smaller than *Burnupia* (Brown, 1994) and is considered less suitable for use in artificial streams developed for the purposes of toxicity testing at CAT-IWR (Palmer *et al*., 1996).

With the decision to test the effects of a textile whole effluent on a mollusc, it also seemed appropriate that a species found upstream from the effluent end-of-pipe be chosen. The textile mill's end-of-pipe is into the Mlakalaka Stream, immediately flowing into the Buffalo River (Figure 10.1). The upper reaches of the Buffalo River are inhabited by *B. stenochorias* (pers. obs.). Palmer and Uys (1994) completed invertebrate surveys of Mlakalaka Stream and the Buffalo River up and down stream of entry of the Mlakalaka Stream (Figure 10.1). *Burnupia* species were absent from the stream but present elsewhere; Palmer and Uys suggested further testing of the textile effluent was necessary before conclusions could be drawn as to the impact the effluent had on the invertebrates, and this included the limpets.

The primary disadvantage in the choice of *B. stenochorias* was the lack of information about its ecology, life history parameters, behaviour and genetics. Very few ecological or life history studies of the Ancylidae in southern Africa had been previously completed (Brown, 1994; Haigh and Davies-Coleman, 1997). Background knowledge was based on only one *B. mooiensis* paper which deals with limited histology and morphology (Oberholzer and van Eeden, 1969). An understanding of growth and reproduction of *B. stenochorias* and the innate variability of these parameters is essential before a study of its responses at a chronic (long term, sublethal) level to a complex effluent can be investigated. Only then can contributions be made to an understanding of the ecotoxicological effects of a given effluent. This biological information would not only contribute to toxicity methodology development and the capacity to monitor the chronic effects of the effluent chosen, but also help in the development of methods for culturing *B. stenochorias* in the laboratory for future use in toxicity testing. It has been suggested that bioassay regulations in South Africa could be industry or source specific *i.e.* mining effluents and runoff, specific industrial effluents such as pulp and paper or textile, municipal sewage, wine industry and garbage dump runoffs; or chemically grouped, such as hydrocarbons or pesticides and so on (Liu and Dutka, 1999). A textile effluent already being investigated for acute toxicity to mayfly nymphs (Zokufa, 2001) was chosen in this study for further toxicological studies using *B. stenochorias* as the test organism.

1.4. Aims and Summary of Remaining Thesis Structure

The overall aims of this study were to describe some of the responses of *B. stenochorias*, an indigenous freshwater organism, to a complex effluent, and to assess its use as an ecotoxicological indicator, based primarily on the nature of the variability of response to selected toxicants. The ecotoxicological investigations included both acute (short term) and chronic (long term) tests. Because effluents themselves are complex and variable, the responses of *B. stenochorias* were also tested against a reference toxicant (potassium dichromate) and compared to a standard laboratory test organism (*Daphnia pulex*).

This is a broadly based study with two main lines of enquiry: the growth and reproductive biology of *B. stenochorias,* which provided supportive information for the investigation into the lethal and sublethal responses of *B. stenochorias* from exposure to textile effluent. This thesis is therefore presented as Part One, which includes the biological issues examined; Part Two, where ecotoxicology

is examined; and Part Three, a concluding discussion. Figure 1.2 provides a diagrammatic plan of the study, and shows the linkages between each line of investigation. Introductory and method chapters are not included in the diagram as individual boxes, although they form important explanatory components of the first two parts of the thesis; a link between the two parts, rather, is given in Figure 1.2, underlining some of the reasoning behind this investigation.

Part One

The first part (Chapters 2 to 7) deals with growth and reproduction investigations which provided the basis from which to undertake the chronic toxicity testing using *B. stenochorias.* The methodology for these biological investigations is given in Chapter 2. One of the most commonly used parameters in sub-lethal toxicology is growth, measured, in the case of limpets, by assessing changes in shell length. A comparison of shell morphometrics from different feral populations (discussed in Chapter 3) was therefore necessary to decide whether shell length was indeed adequate for describing the growth of individual *B. stenochorias* specimens, or whether further dimensions needed to be included. Chapter 4 considers the suggestion by the malacologist Russel-Hunter (1982) that an initial investigation of any ecological problem can occur in the field, in the laboratory, or as a theoretical, modelling concept; there is no "best" starting method. However, all three methods should eventually revert to field conditions for verification, where the crucial dynamics of naturally age-structured populations in their natural environment can be monitored. On the basis of this concept, two feral populations of *B. stenochorias* were surveyed fortnightly for 52 weeks. The number of egg capsules and seasonality of lay were monitored, and age structure of each population, growth curves and longevity were estimated.

With the concept that pre-sexually developed invertebrates in general are usually considered more susceptible to toxicants (Gomot, 1998), Chapter 5 investigates the relationship of shell length of *B. stenochorias* to its sexual development. Natural population data form a vital baseline for later interpretation of the chronic toxicological investigations of *B. stenochorias* which are described in Part Two. However, because the toxicity tests would be performed in the laboratory, Chapter 6 investigates laboratory growth and reproduction, and since diet is a fundamental determinant of growth and reproduction (Lamberti and Steinman, 1993), the effect different food sources has on these parameters was investigated. A summary of the biological investigations and the innate variability observed is

presented in Chapter 7.

Part Two

The second part of the thesis, as depicted in Figure 1.2, deals with toxicological investigations. An introduction to the principles of toxicology, and the terms and methods used, is given in Chapter 8. The general methodology used in the toxicity tests is introduced in Chapter 9, including the collection of limpets from feral populations, their acclimation to laboratory conditions, and their transfer to the containers used in toxicity tests. The development of these methods forms an important component of the investigation into using *B. stenochorias* as a toxicity test organism. Chapter 10 introduces the acute toxicity tests with *B. stenochorias* using both potassium dichromate and textile whole effluent. The variability of response of *B. stenochorias* over 96 hours is compared between replicates and concentrations of both toxicants. Because instream dilutions of an effluent must be considered when estimating the effective toxicity of an effluent to a test organism, the longer term (chronic) effects of the textile whole effluent are also investigated (Chapter 11). Toxic effects were measured in terms of growth, fecundity and mortality of *B. stenochorias*, with the variability demonstrated within these parameters statistically analysed and discussed.

There are many advantages to using standard laboratory organisms instead of feral populations of indigenous organisms in toxicity studies. Using *Daphnia pulex*, presently cultured in the CAT-IWR laboratories as a standard laboratory organism, acute effluent and potassium dichromate toxicity tests were completed, and results from two 21 day chronic tests where toxicity of the effluent to *D. pulex* in terms of reproduction and growth are given (Chapter 12). The final chapter to Part Two (Chapter 13) discusses the toxicological results, particularly relating to *B. stenochorias*, and the variability of response to the toxicants used. General conclusions are given as to the part toxicological investigations using *B. stenochorias* may play in hazard and finally risk assessment.

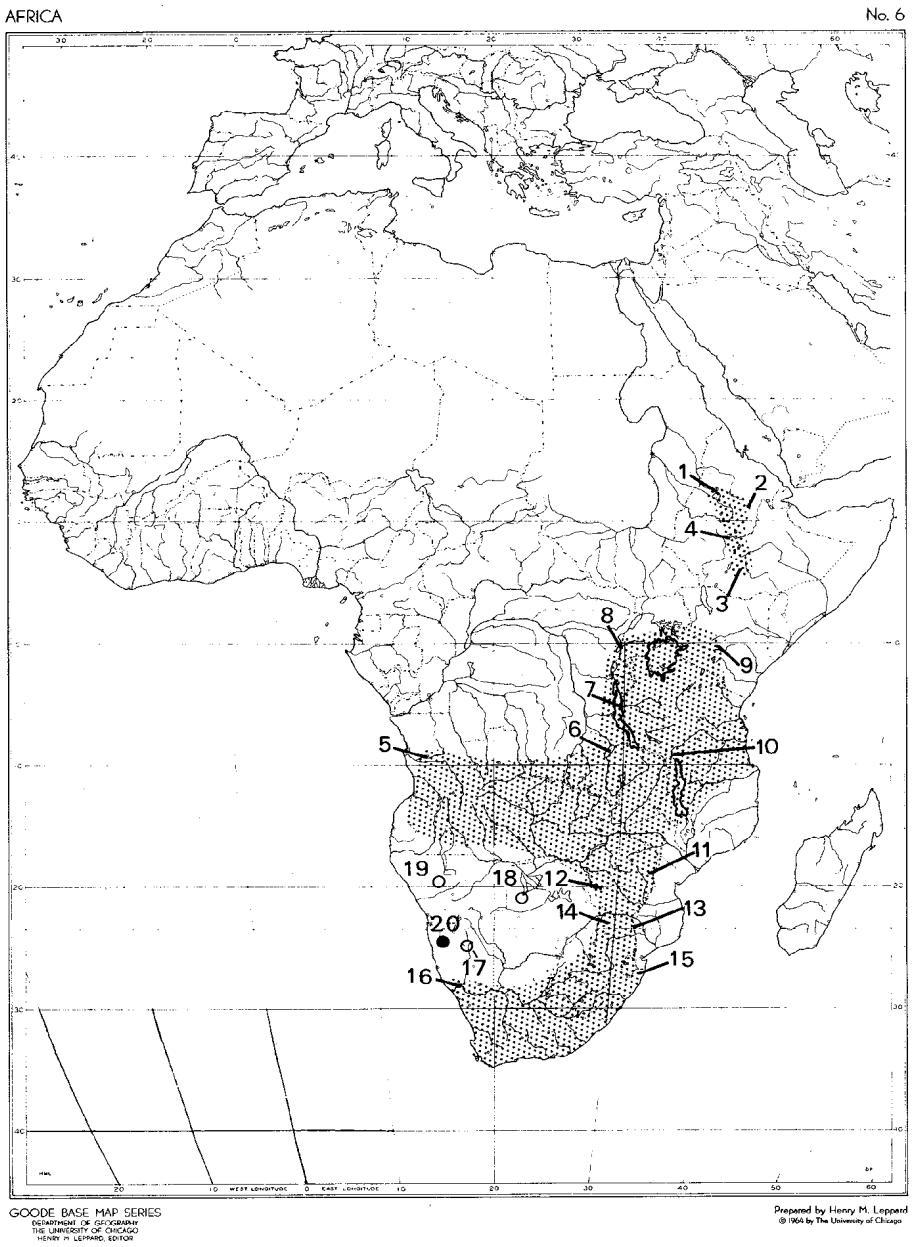


Figure 1.1. Distribution of *Burnupia* in Africa (Brown, 1994).
1-4: Ethiopia - Gorgora, Debra Berhan, Adolla and Wolliso 5: Angola - Salazar 6: Lake Mwera at Lukonzolwa 7: Lake Tanganyika 8: Lake Edward 9: Kenya - highlands. 10: Tanzania - Mbeya. 11,12: Zimbabwe, Nyanga and Matopos. 13,14: Mpumalanga, Kruger National Park and Soutpansberg 15: Natal, Lake Sibaya 16: lower Orange River 17-19: Great Namaqualand, Ngamiland and Kamanyab (*B. trapezoidea*). 20: Namibia - Naukluftberge (State Museum, Windhoek).

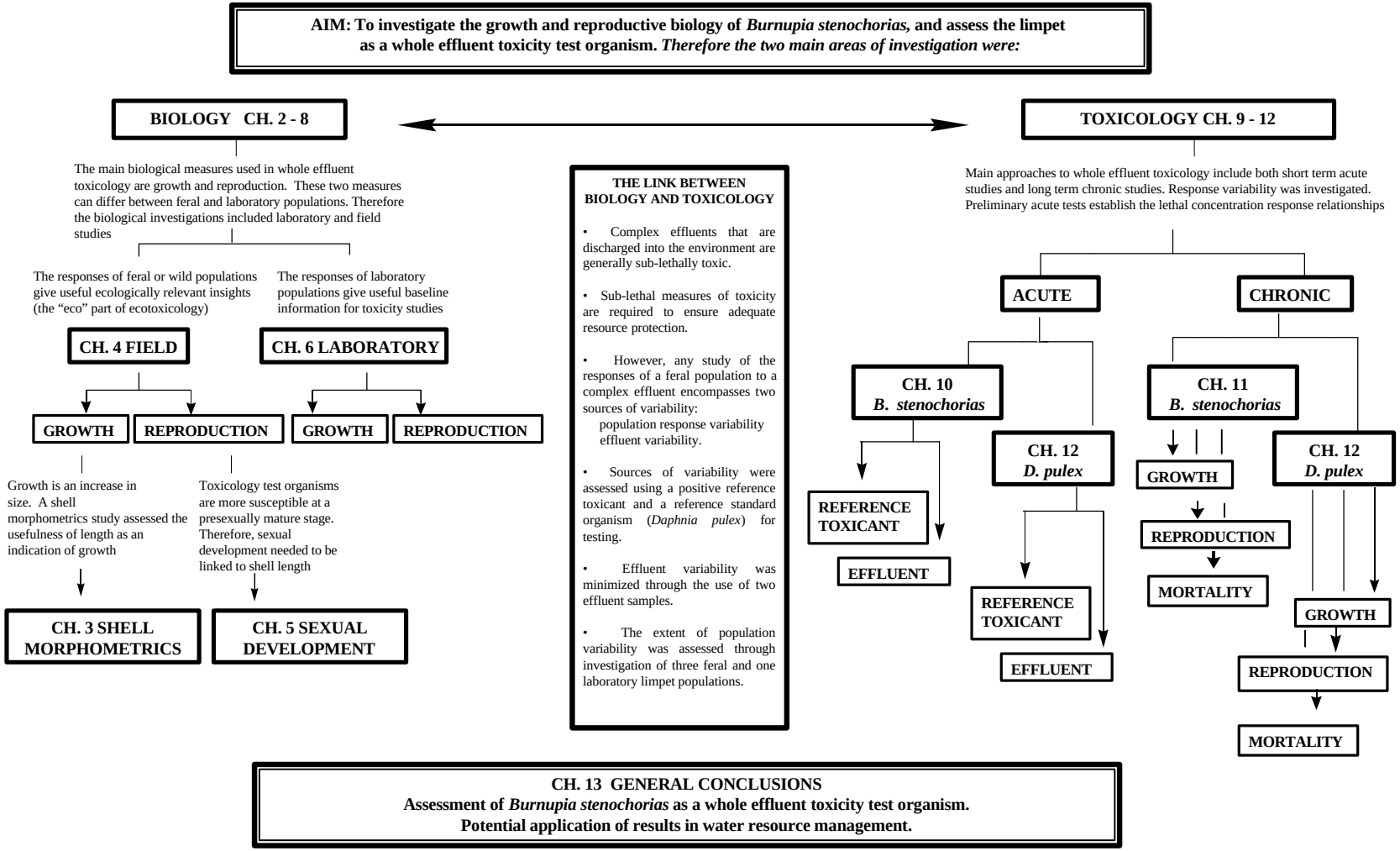


Figure 1.2. Schematic diagram of thesis structure. The introductory and summarizing chapters (1, 7 and 8) are not included.

PART ONE

INVESTIGATIONS INTO THE BIOLOGICAL PARAMETERS OF *BURNUPIA STENOCHORIAS* OF USE IN TOXICITY TESTING

We need to appreciate that a species of whatever form is life, is a unique manifestation of life, the final product of some evolutionary development.

Irston R. Barnes
Conservation Foundation letter, 1968

CHAPTER 2. METHODS USED IN THE BIOLOGICAL INVESTIGATIONS

Although information about animal morphology, histology and life history patterns is sought for a variety of reasons, the objective of each study largely determines the method(s) used. Similarly, statistical analyses and interpretation of the data accumulated through a study may be different for each objective. The four chapters which follow consider various aspects of the biology of *Burnupia stenochorias*. Each of the methods used is related to the aims of each chapter. However, much of the data deals with comparisons of length. In Chapter 3, shell morphology of the limpets is analysed in terms of length, width and height. In Chapter 4, the relationship between size (as measured by shell length) and sexual development (as determined by histological sectioning and staining of limpets of known size) is considered, with the aim of selecting pre-sexually developed *B. stenochorias* for toxicity testing. Chapter 5 considers a natural population study, including the estimation of growth under natural conditions, based on shell length. Reproductive activity, as indicated by the presence of egg capsules, formed part of the study. Finally, a comparison of the natural growth and reproduction is made with growth and reproduction under laboratory conditions, with an analysis of the effects of diet on these parameters (Chapter 6).

The general methods used in all the biological investigations of *B. stenochorias* are reported within this chapter; specific methods and data analyses are reported later within the relevant chapters.

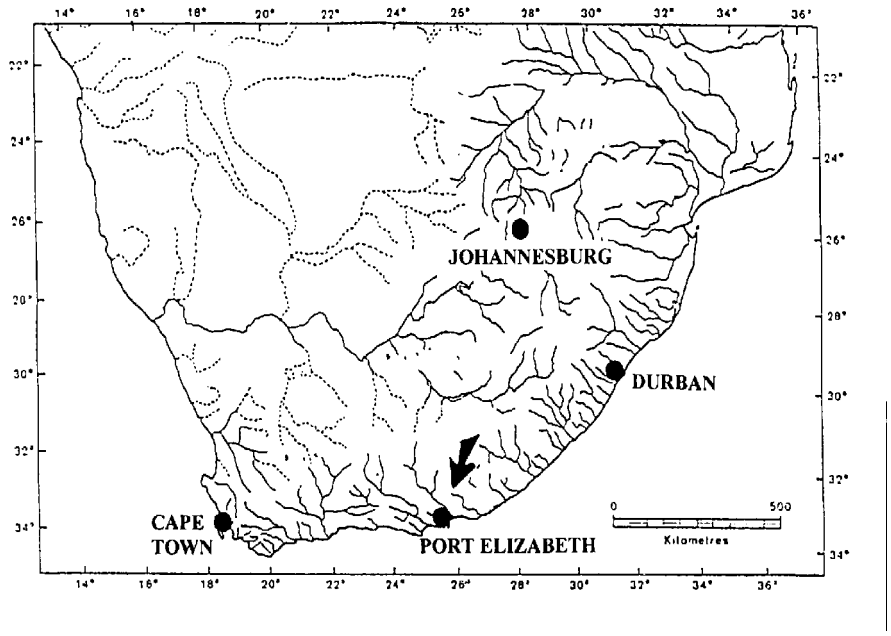
2.1. Source of *Burnupia stenochorias* Specimens

The three sites used for collection of specimens in this section are in the Eastern Cape Province of South Africa (Figure 2.1). The Botha River population represents a unimpacted, non-flowing source; the Belmont River a flowing impacted source; and the Manley Flats a less impacted but not pristine, flowing source of *B. stenochorias.*

2.2. Method of Collection

Collecting, handling and transporting toxicity test animals should be done in a manner that minimizes injury and physiological stress. *B. stenochorias* is particularly sensitive to handling,

a)



b)

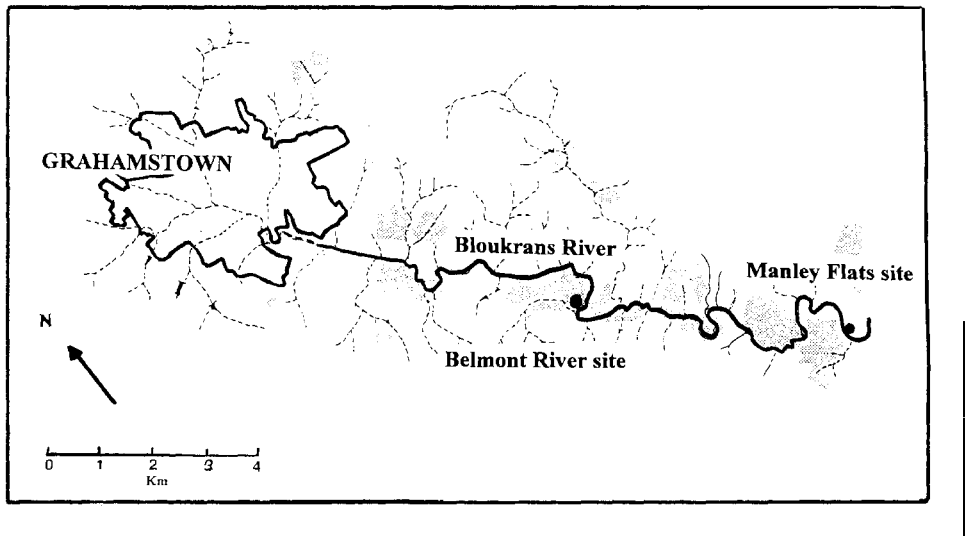


Figure 2.1.
a) Map of southern Africa, indicating (arrow) approximate area from which all *Burnupia stenochorias* specimens were observed or collected.
b) Diagrammatic presentation of the Belmont River site (33E19,400'S, 26E35,988'E), from where the Bloukrans river flows through a series of weirs and small dams, bound by natural vegetation, until it reaches the Manley Flats site (33E19,344S, 26E36,441E) in a more pristine condition (Chapters 4 and 6). The Bloukrans River has its headwaters in Grahamstown and immediately surrounding areas. Water within the Bloukrans River consists of town and informal settlement runoff, Grahamstown sewage effluent, natural runoff from the valley (the area approximately shown in the diagram), and agricultural runoff from irrigated fields within the valley. The Botha River site (Chapters 4, 10 and 11) is approximately 10 kms north of Grahamstown (33E13'S, 26E30'E).

(Haigh and Davies-Coleman, 1997) and the following method for collecting feral limpets was developed and used. Stream rocks, on which the *B. stenochorias* were found, were lifted out of the water and held either in, or over, a 5 litre bucket lined with a plastic bag, holding approximately 2 litres of river (collection site) water. The limpets were brushed off the rock by the gentle brushing of a wet hand over the rock surface, and were rinsed off the hand by splashing the hand gently in the water. When each bag contained approximately 60 limpets, the majority of the water was poured off to leave approximately 400 ml. The bag was then sealed with a knot so that only a small pocket of air was enclosed. A number of bags were transported thus in the bucket, sealed with a lid so that no movement of bags occurred when transporting. If necessary, the bucket was filled with water to ensure no movement occurred. No aeration was necessary.

Later, either at the collection site or in the laboratory, each bag was opened and laid as flat as possible on a sorting tray. Limpets which had not already attached themselves to the plastic bag were separated from other limpets or debris by 'blowing' the water with a plastic dropper with a 2-3 mm nozzle width. Debris and any other obviously damaged limpets (including those with chipped shells) were discarded. For greatest survival, these separated limpets were placed on their feet. However, if left overnight, the majority of limpets attached themselves to the plastic surface. The bags were filled to the brim with river water collected at the same time.

After transportation to the laboratory, at least one unglazed 7x7 cm ceramic tile was placed in each bag. These tiles had been seeded with rock scrapings of diatoms and other periphyton from the river source, and cultured for two weeks in aerated dechlorinated tap water, in either artificial channels or buckets. The choice of tap water was based on a general decision for all toxicology studies (Palmer *et al*., 1996). To confirm diatoms were the dominant form of algal growth, samples of tiles were examined using an Olympus BX40 compound light microscope.

Because dechlorinated tap water was the diluent for the toxicity tests, approximately 10% of the river water was replaced daily with dechlorinated tap water until the limpets were in 100% tap water (approximately 10 days). Each bag was gently aerated each day. Acclimation to the laboratory conditions of 20-23EC, and 12 hours light/dark artificial biolux lighting of photon flux density of 120 mol/m^2/sec occurred at the same time.

2.3. General Methodology

Shell measurements in the field were made by means of a graded template made from undeveloped film, which was flexible, cheap, did not tear, and was easily replaceable if lost. Holes from 1.0 mm (larger than the newly hatched size of approximately 0.75 mm shell length; pers. obs.) to 7.5 mm (maximum size personally observed) at 0.5 mm intervals were drilled into the film using drill bits accurate to within 0.01mm width. Measurements within the laboratory were either taken with the aid of Vernier calipers to an accuracy of 0.1 mm; or with the aid of a Nikon bifocal microscope at x10 magnification, using an ocular graticule calibrated with a slide graticule. The measurements used in the calculations included shell length (greatest distance of the anterior and posterior axis), shell height (greatest vertical distance from the apex of the shell to the plane of the aperture), and shell width (greatest distance perpendicular to the anterior/posterior axis).

The methods used in reproductive research are recorded separately in each chapter.

2.4. Statistical Analysis

All statistical analysis and interpretation was based on Zar (1984) and Sokal and Rohlf (1981) unless otherwise stated. Most statistical analyses and other calculations are used in a single case in this research and are therefore discussed in the appropriate chapter.

Before parametric analyses of variance and other related procedures could be applied (such as used in Chapters 3 and 6, and then later in Chapters 10, 11 and 12), certain basic assumptions had to be met: the data had to be normally distributed, with equal variances. When length data of *B. stenochorias* were to be compared, normality was assessed based on Shapiro-Wilk's test (Conover,1980), with the introduction of the Kolmogorov "D" statistic (Stephans, 1974) where there were more than 50 data points. The testing of homogeneity of variance was based on Bartlett's test (Snedecor and Cochrane, 1980). If the data did not display either of these criteria, transformation of data by log transformation alleviated the problem. Either $\log_{10}$, $\log_{10}(x + 1)$, or $\log_e$ was used, whichever transformation ensured log normality and homogeneity of variances. The Shapiro-Wilk's / Kolmogorov calculations were made with the use of Statgraphics 5.0.

Simple linear regression models were used to compare the relationships between length, width, height,

and weight samples (Chapter 4), and change in growth (shell length) over time. The regression coefficient given with each linear regression line is a measure of quantitative change of the one variable with respect to the other variable. The correlation coefficient, on the other hand, is the unitless measure of the intensity of association between the two variables. There are various assumptions of the regression line (Zar, 1984), particularly relating to normal distributions of the two data sets, and these assumptions were assumed to be correct as covered by the tests for normality and homogeneity of variances. The slopes of the regression lines were compared using ANOVA (analysis of variance) where the variability of morphometric measurements within and between populations sampled was compared.

Where growth data were non-parametric (not normally distributed), the Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to compare differences in growth over time between different groups (the groups were based on diets; Chapter 7). Analyses were completed using Statgraphics 5.0.

Other statistical packages used, and described within the appropriate chapters, include Principal Component and Discriminant Function Analyses (Statgraphics 5.0) which were used to compare the distribution of morphometric samples between the three populations studied (Chapter 4). Bhattacharya, NORMSEP and ELEFAN programmes were used to analyse length frequency data over time, based on the von Bertalanffy Growth Equation (Gayanilo *et al*., 1996).

CHAPTER 3. SHELL MORPHOMETRICS OF THREE NATURAL POPULATIONS

Key issues: Do shell dimensions vary between populations, and can shell length be used to indicate size? (Figure 1.2)

3.1. INTRODUCTION

The shell of a gastropod grows with its body, new shell being laid down at the shell aperture (Russel-Hunter, 1961b). If the body stops growing or grows more slowly because of an unsuitable environment, for example, shell growth usually ceases or slows (Meglitsch, 1972). Many methods have been developed for both freshwater and marine limpets to estimate the growth using the shell as an indicator. These include periodic measurements of labelled individuals (Russel-Hunter, 1978), checks in growth produced by known changes of environmental conditions (Russel-Hunter, 1961b; Vahl, 1971), annual growth rings where microgrowth band analysis can yield data on both growth and age of the limpet (Picken, 1980), and mean shell lengths of cohorts (Lutz,1976; Peterson *et al.*, 1983).

Although the presence or absence of growth rings in freshwater limpets is not well documented (Vermeij, 1980), many of the calcareous skeletons of both living and fossilised marine molluscs show evidence of periodic deposition in their shells in the form of regular external microgrowth ridges or internal banding (Ekaratne and Crisp, 1982) forming annual rings (Cerrato and Keith, 1992), spring tidal bands (Richardson, 1988) or summer and winter banding (Thiesen, 1973). Many studies have recorded the presence and periodicity of daily growth bands in marine molluscs, but few have used the data to generate aging and growth curves (Crisp *et al.*, 1990). This is primarily because variability in the degree of daily banding also depends on whether conditions are favourable to growth, whether spawning occurs, *etc.*, in turn leading to inaccuracies of age estimation (Richardson, 1988). Broad growth rings on the periostraca of the freshwater pulmonates *Ancylus fluviatilis* and *Acroloxus lacustris* are said to demonstrate differential growth rates of summer and winter, and to allow estimates of the length of the life cycle (Russel-Hunter,1953).

The Ancylidae, including *B. stenochorias*, possess a shell which undergoes no metamorphosis or change in differential gradient in growth throughout the life cycle. As an alternative to growth rings,

therefore, change in shell length (the greatest dimension along the anterior-posterior axis) is considered a possible means of representing growth and age (Caquet, 1993; Gayanilo *et al.*, 1995).

Both inter- and intra-population variation in shell shape has been regularly observed, however, in both marine (Moore, 1934; Branch, 1974) and freshwater (Calow, 1975a) limpets, with the pulmonates as no exception *e.g. Lymnaea* spp. (Hubendick, 1970; Hunter, 1975), *Ferrissia* spp. (Burky, 1971), *A. fluviatilis* (Sutcliffe and Durrant, 1977) and *Physella* spp. (Burnside and McMahon, 1997). The roundness index (Width / Length) results from the fixed growth gradients of the mantle-edge, is isometric, highly specific to each population and appears to be genetically determined. It is suggested that its range within a population is much less than that for the species as a whole (Russel-Hunter, 1978). The steepness index (3 Height / Length + Width) which reflects local growth patterns as modified by trophic conditions (Vermeij, 1971), is allometric, and may even be used in assessments of changes in nutritional history of the individual limpet or of the population. Interpopulation variation in shell form may, consequently, be either broadly ecophenotypic, or more rigidly genetically controlled (Russell-Hunter, 1978). Walker's descriptions of *Burnupia* (1923) clearly indicated the possibility of habitat conditions influencing their shell dimensions, claiming that the narrow type specimen is not the normal form. He referred particularly to the South African ancylids, mentioning *B. stenochorias, B. gordonensis* and *B. trapezoidea*. As the typical form frequently occurs with the normal form, he suggested that they be regarded as individuals rather than as exemplifying racial differences.

This variation of morphological measurements may, however, influence the estimates of, and calculations of change in, growth between populations, particularly if change in shell length was the only parameter used. The aims of this chapter were therefore twofold:

1. To find an accurate means of describing growth of *B. stenochorias.* The presence of shell growth rings was initially considered, visible as a part of either the external or internal shell sculpture. As an alternative, shell morphometric measurements were considered as a means of measuring growth, in the form of length, width and/or height. Consequently, the shell dimensions of *B. stenochorias* collected from three natural populations were compared, firstly by means of geometric mean ratios, then by linear regression analyses.
2. Once shell dimensions proved to be reasonably consistent amongst individuals within the three populations studied, the relationships between length, width and height measurements between

populations were considered, to determine whether *B. stenochorias* could, in future toxicity tests, be sampled from any one of a number of allocations. Comparisons were made with the use of the multivariate analyses programmes Principal Component Analysis and Discriminant Function Analysis.

3.2. MATERIALS AND METHODS

3.2.1. External Shell Sculpture

To describe external shell sculpture, eight shells of *Burnupia stenochorias*, 2.8mm to 6.5mm in length and collected from the Manley Flats site (Section 3.1), were cleaned by immersion for 5 minutes in 10% sodium hypochlorite. They were then rinsed in distilled water, mounted on brass stubs and sputter coated with gold for examination under a Jeol JSM 840 Scanning Electron Microscope. Specimens representing those collected are stored in the British Natural History Museum, London [Invertebrate 1 collection registration numbers 19990449 - 19990452].

3.2.2. Internal Growth Bands

To determine the presence of internal growth bands, the soft-bodied parts of 10 limpets, 2.8mm to 6.5mm in shell length, were removed and the remaining shells placed in 10% sodium hypochlorite solution for approximately one minute, to remove all organic debris. The shells were allowed to harden in polyester resin (metaserv sw 137/12742) thinned with one drop of 0.01M hydrochloric acid. Each limpet shell was cut in half along the radial axis with a diamond blade, the cut edge ground with increasingly fine abrasive paper (340,120 wet and dry trimiter paper) and finally polished with household metal polish "Brasso". Each shell was then etched for varying lengths of time (25-40 minutes) with cold 0.01M hydrochloric acid. Agar Scientific Ltd No G255 strips of acetate replicating material, used to obtain replicas of the polished and etched surfaces, were left on the sections for 5 minutes. Each peel was removed, kept flat by holding between coverslip and microscope slide, and examined for signs of growth bands or growth increments under both a Nikon bifocal microscope, and a phase contrast microscope.

3.2.3. Use of Weight or Morphometric Measurements as an Indication of Growth

Using Vernier calipers (± 0.05 mm), shell length (greatest distance of the anterior and posterior axis), shell height (greatest vertical distance from the apex of the shell to the plane of the aperture), and shell

width (greatest distance perpendicular to the anterior/posterior axis) of limpets were measured, to the nearest 0.5 mm, sampled from each of three sites. Two sites were sampled in the Bloukrans River near Grahamstown, referred to as the Belmont River and Manley Flats populations (number of limpets = 173 and 175 respectively), and the third from a different catchment, referred to as the Botha River population (number of limpets = 21) (Section 3.1). All were sampled in September 1997. Limpets were rejected if the shell edges were damaged in any way during collection.

Geometric means were measured, including the roundness and steepness indices (Section 3.1). Although dry weights of individuals, as opposed to wet weights, are preferred (Calow, 1975a), too many *B. stenochorias* were needed in each sample relative to the number available in the field, to warrant using this method. Only wet weights of Belmont River and Botha River individuals were therefore taken. However, confidence limits cannot be assigned to these ratios. Consequently, logarithmic regressions were used to compare individuals and populations using $\log_{(10)}$ transformed data (Sokal and Rohlf, 1981). According to Fischer-Piette (1948), volume^{a} provides a better representation of the overall shape of each individual limpet. Volume (V) was also therefore calculated from its shell parameters by treating the shell as an elliptical cylinder, using the following formula:

$$V = Đ/4 \times \text{length} \times \text{width} \times \text{height}, \qquad \text{where } Đ = \text{pi } (22/7).$$

Linear regressions obtained were compared by analysis of covariance (ANCOVA) to test for homogeneity of slopes.

Whether growth is allometric or isometric relative to length and height can be determined by the equation $H = cL^{\%}$, where '%' (the constant of allometry) and 'c' values are substituted from regression analyses of the measured length and height data (Snedecor and Cochran, 1980). The '%' values less than 1 indicate shell length grows faster than shell height, and *vice versa* (Branch, 1981). In this way, the relationship between height and length was compared between the three natural populations studied, although this method of substitution is said to be less sensitive than the linear regression analysis (Sutcliffe and Durrant, 1977).

Two further, multivariate tests were employed to examine the intricate relationships between the variables measured, namely Principal Component Analysis (PCA), and Discriminant Function Analysis (DFA) (within Statgraphics 5.0). PCA was used with the aim of ordering the variables in a small

number of dimensions, emphasizing the major patterns of variation between them. This method works on ungrouped data, reducing the set of variables by linear transformation so that a minimum of information is lost. The principal components obtained are independent of each other and account for as much of the variation as possible. Principal Component 1 (PC1) accounts for as much variation as possible in the original data, PC2 a progressively smaller amount and so on. DFA was then applied, to classify observations into two or more groups based on the variables used. In this way, a set of discriminant functions was produced by which a specimen was allotted to one of the groups on which the analysis was based. The procedure assumes that the variables are drawn from populations with multivariate normal distributions and that the variables have equal variances. It is based on the difference between the between-group and the within-group co-variances, and aims to find the discriminant functions that maximize the ratio between the between-group and within-group variations (Huberty, 1994).

3.3. RESULTS

3.3.1. Shell Sculpture

Various external aspects of the shells of *Burnupia stenochorias* are shown in Figure 3.1. At the apex of each shell is a regularly shaped pit surrounded by a smooth area with a radius of about 0.1 mm (Figure 3.1b, c). From the distal edge of the smooth area radiate rows of characteristic pits or punctae (Figure 3.1c, d) which have been used to differentiate *B. stenochorias* from other *Burnupia* species (Walker, 1923). After approximately 0.7mm, these rows abruptly stop and are replaced with spiral striae or threads which appear to represent growth rings running transversely and distally, radiating from the apex (Figure 3.1a).

3.3.2. Growth Rings

Although ridges and rings of apparently variable deposition of calcium can be seen on the shell (Figure 3.1a), no internal or external growth rings were found in the shell structure. This suggests that, unlike the marine patellogastropods, there is no cyclical deposition of the *B. stenochorias* shells which would enable growth estimates to be made.

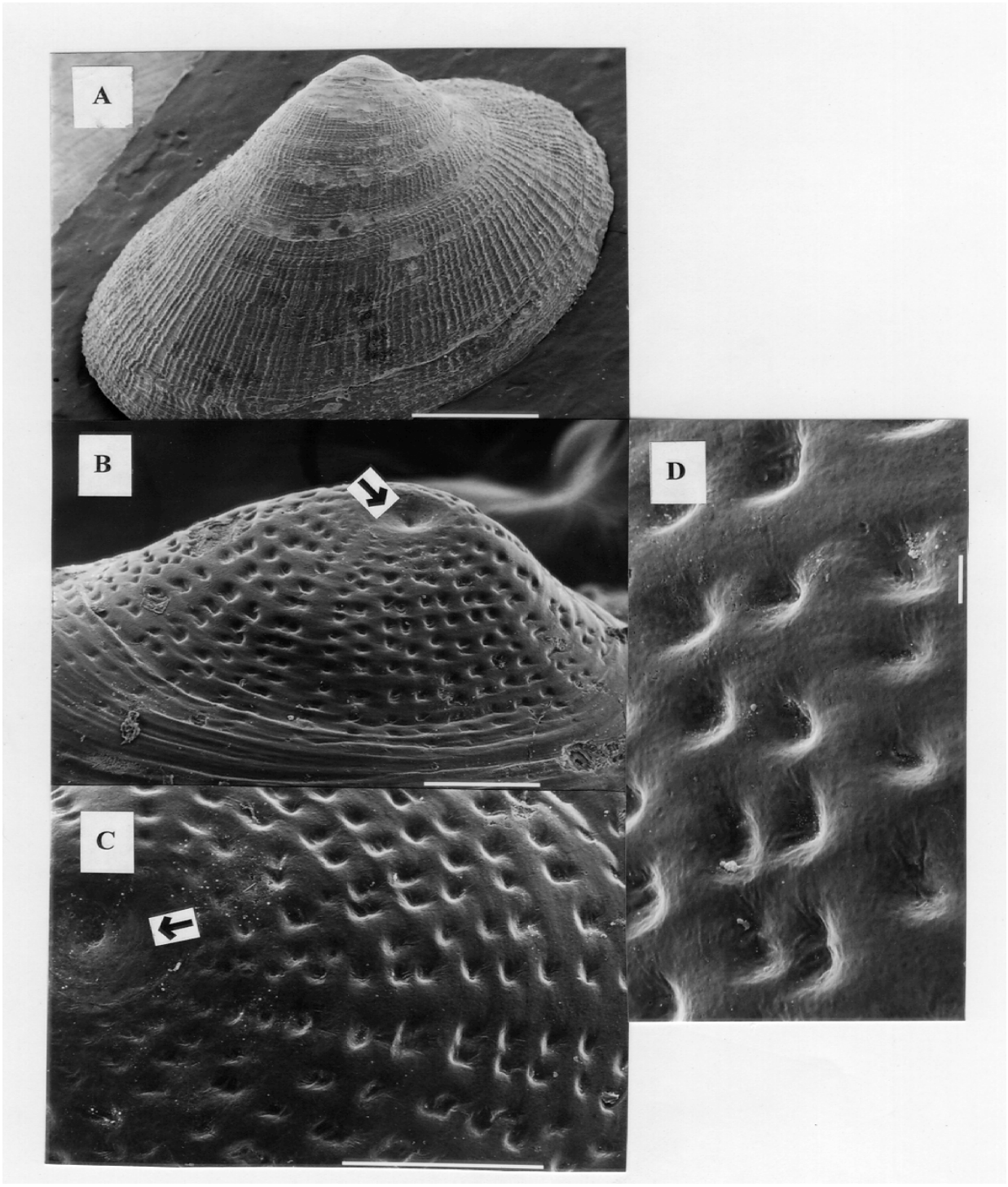


Figure 3.1. Scanning electron microscope photographs of the shell of *Burnupia stenochorias*. **A**) anterior view, complete shell showing spiral and radiating striae; scale = 1 mm. **B**) apex of first mm (º) with rows of punctae, radiating from a central pit devoid of sculpturing, abruptly ceasing after approximately 200 Fm. Posterior view; scale = 100 Fm. **C**) apex (³), with central pit and whorls of punctae (showing their characteristic square or rectangular shape); scale = 100 Fm. **D**) variation in shape of punctae closest to apex of shell; scale = 10 Fm.

3.3.3. Morphometric Measurement Ratios, Correlations and Regressions

Compared to specimens from Belmont River and Manley Flats, Botha River limpets were slightly narrower and flatter in shape, with a consequent lower mean steepness ratio (Table 3.1). Manley Flats limpets displayed the largest steepness ratio suggesting height played a greater role in the shape of the limpet than at the other two sites. When calculating regressions of the morphometric measurements at each site (normalised as $\log_{(10)}[x + 1]$), together with correlation coefficients, comparisons showed significant correlations ($p = 0.001$; Figure 3.2) between length and width, with less significant relationships between length and height (although more so with Manley Flats limpets; Figure 3.2), and width and height (Figure 3.3). ANCOVAs of combined values for length versus width for all three sites, and for Manley Flats versus Belmont River, and Belmont River versus Botha River, revealed significant differences between slopes ($F = 9.5$; 9.8; and 39.8 respectively; $p < 0.005$), suggesting the relationship between length and width was different for each population.

Table 3.1. Comparisons of morphological data presented by Walker (1923)* of the type specimen and the "normal" form of *B. stenochorias* with those measured from the Belmont River, Botha River and Manley Flats sites near Grahamstown, and with *Ancylus fluviatilis* (Sutcliffe and Durrant, 1977). Shell morphometric measurements are expressed in terms of average ratios of mean width to mean length (roundness index), mean height to mean length, and 3H/L+W (steepness index), where H = height, L = length, and W = width. The roundness index given for the original type specimen is smaller indicating it is narrower than the remainder of the specimens. n/a indicates data not available.

Specimen	sampled	width/length	height/length	3H/L + W
Belmont River	173	0.72	0.40	3.67
Botha River	49	0.69	0.33	3.34
Manley Flats	175	0.73	0.39	4.11
Type specimen*		0.56	0.37	n/a
'Normal' form*	n/a	0.75	0.44	n/a
Ancylus fluviatilis	171	0.78	0.47 (lake) 0.55 (river)	n/a

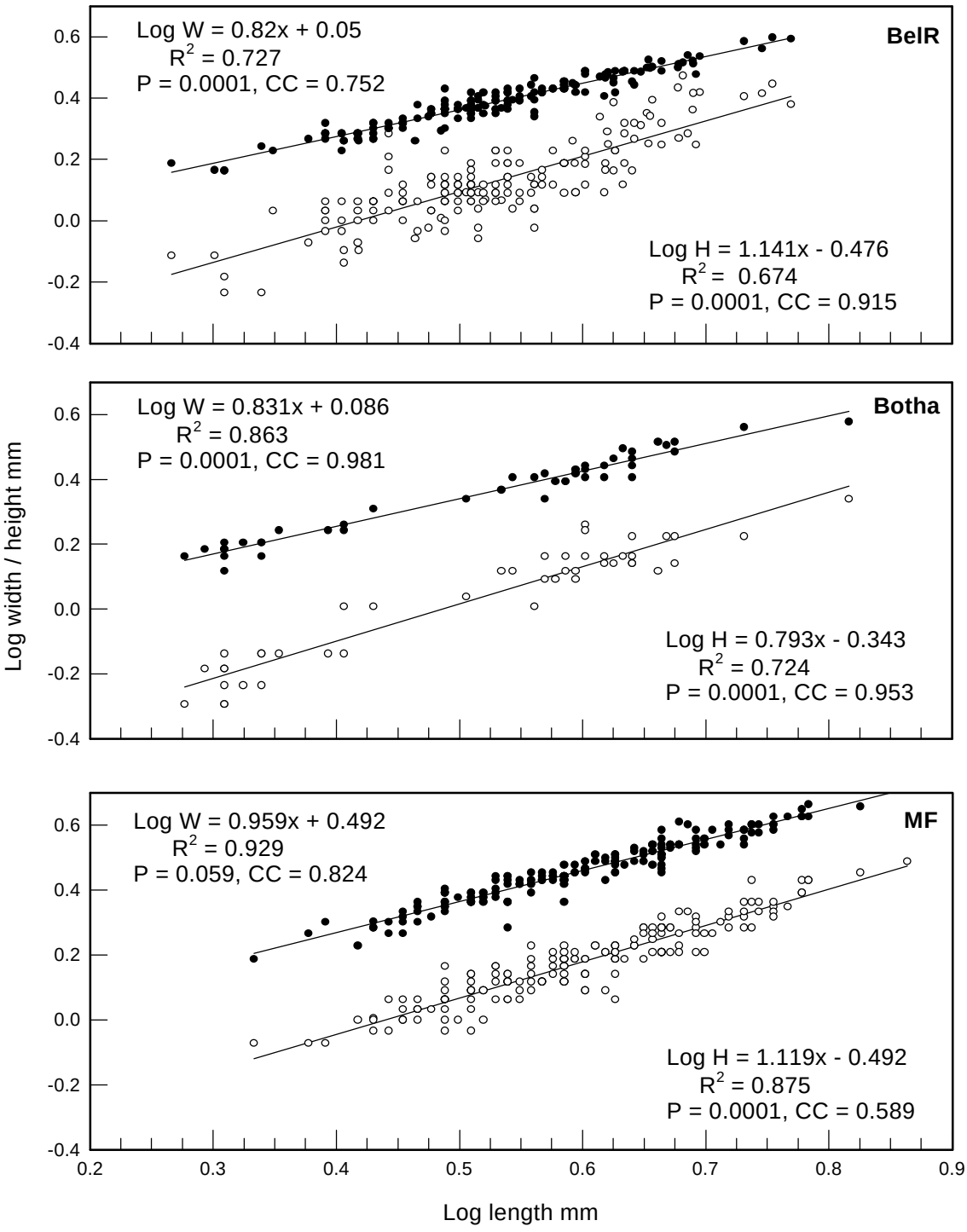


Figure 3.2. Comparison of the relationships between shell length and width (!, W) or height (", H) of *Burnupia stenochorias* specimens collected from the three populations (Belmont River [BelR], Botha River [Botha], and Manley Flats [MF]), represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. Although not strong, the correlation coefficient value for height in relation to length suggests height plays a more dominant role than width in determining change in shape.

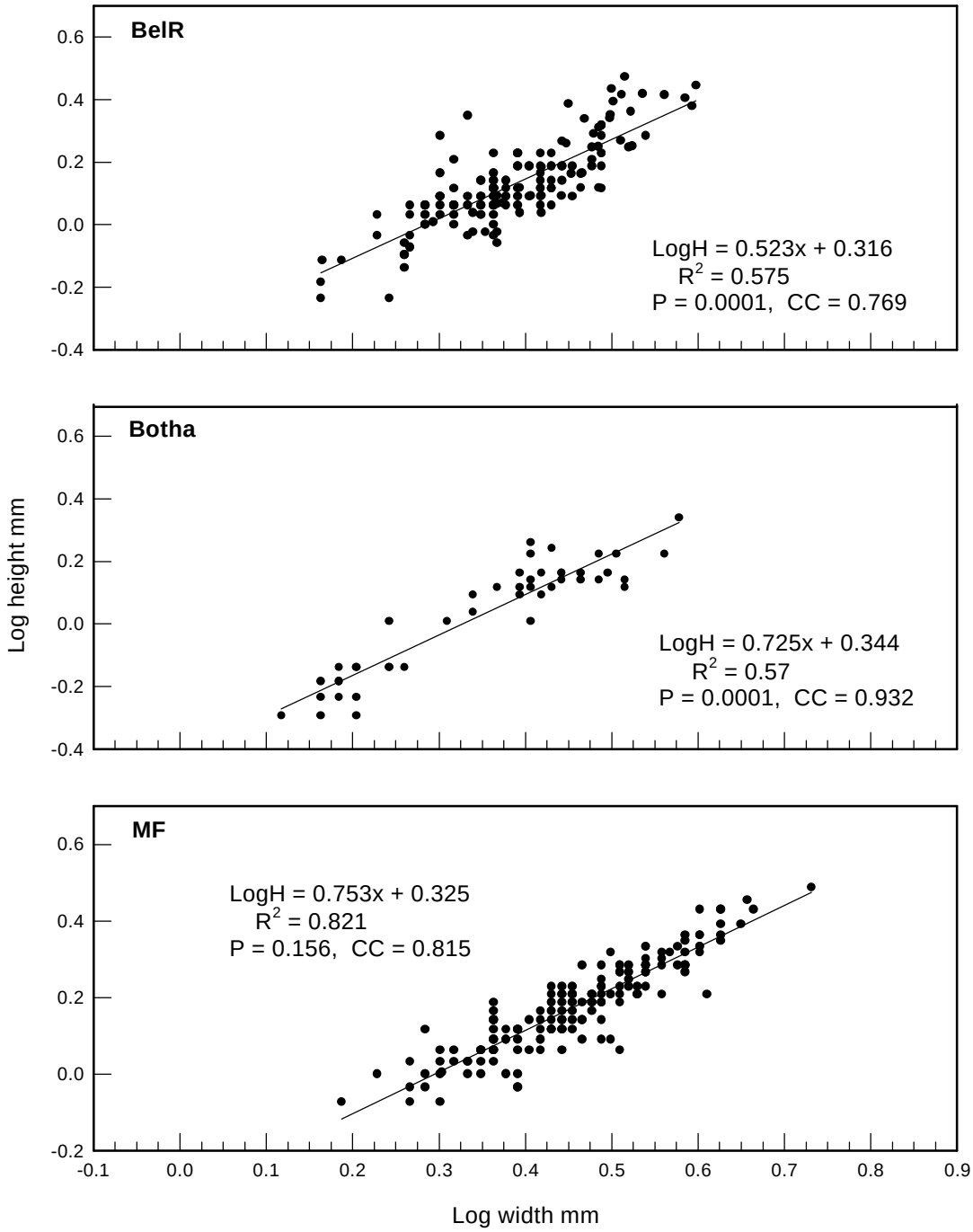


Figure 3.3. Comparison of the relationships between shell width and height of *Burnupia stenochorias* specimens collected from the three populations (Belmont River [BelR], Botha River [Botha], and Manley Flats [MF]) represented as regression equations. R^2= coefficient of determination. CC = correlation coefficient. P = significance value. W = width. H = height. The relationship between change in width with height is generally poor, particularly in the case of the Manley Flats limpets.

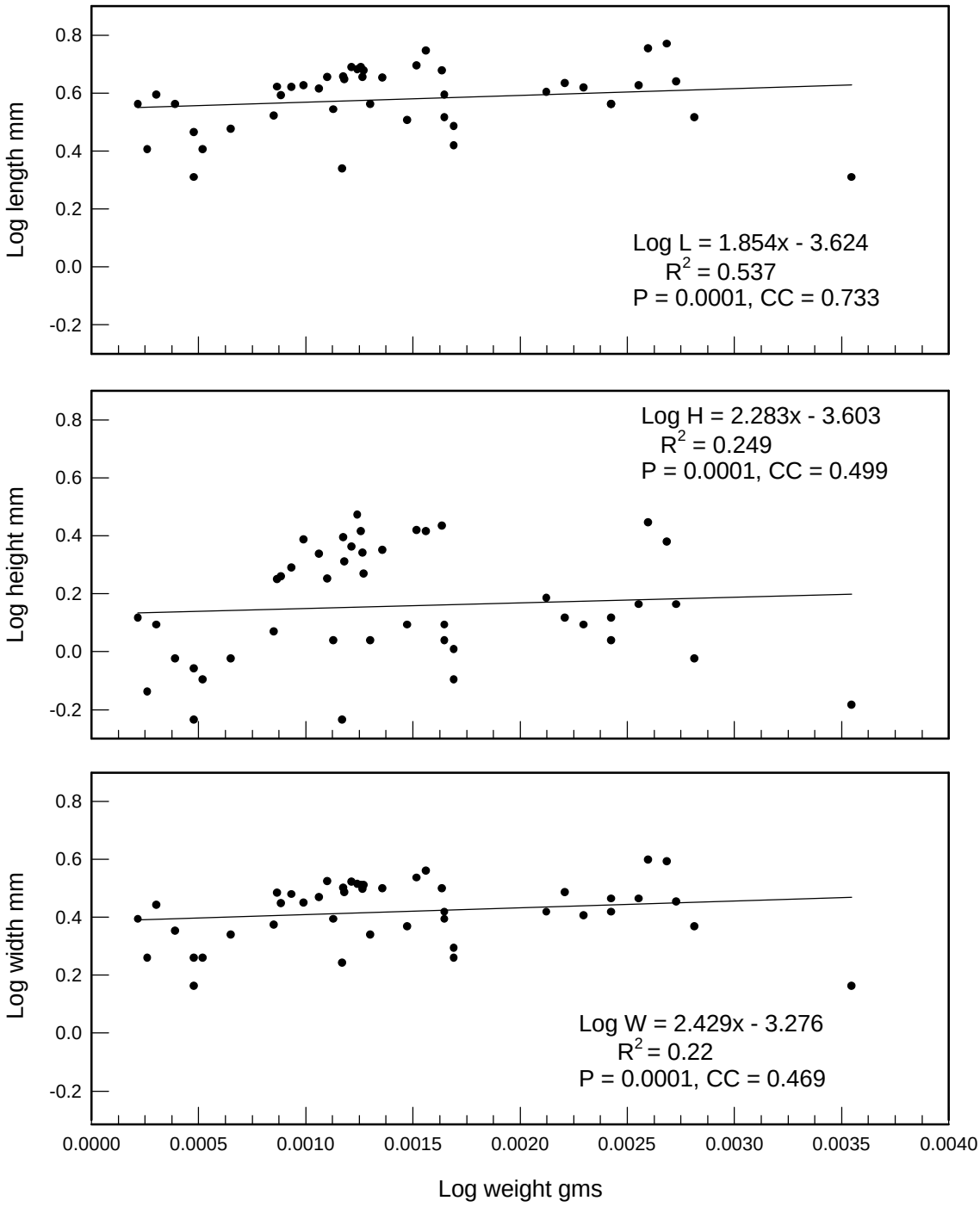


Figure 3.4. Comparison of the relationships between shell weight (wet) with length, width and height of *Burnupia stenochorias* specimens collected from the Belmont River population, represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. L = length. W = width. H = height. With the large scatter and small changes in weight values relative to shell measurements, it appears there is little relationship between the wet weights of Belmont River limpets and length, width or height.

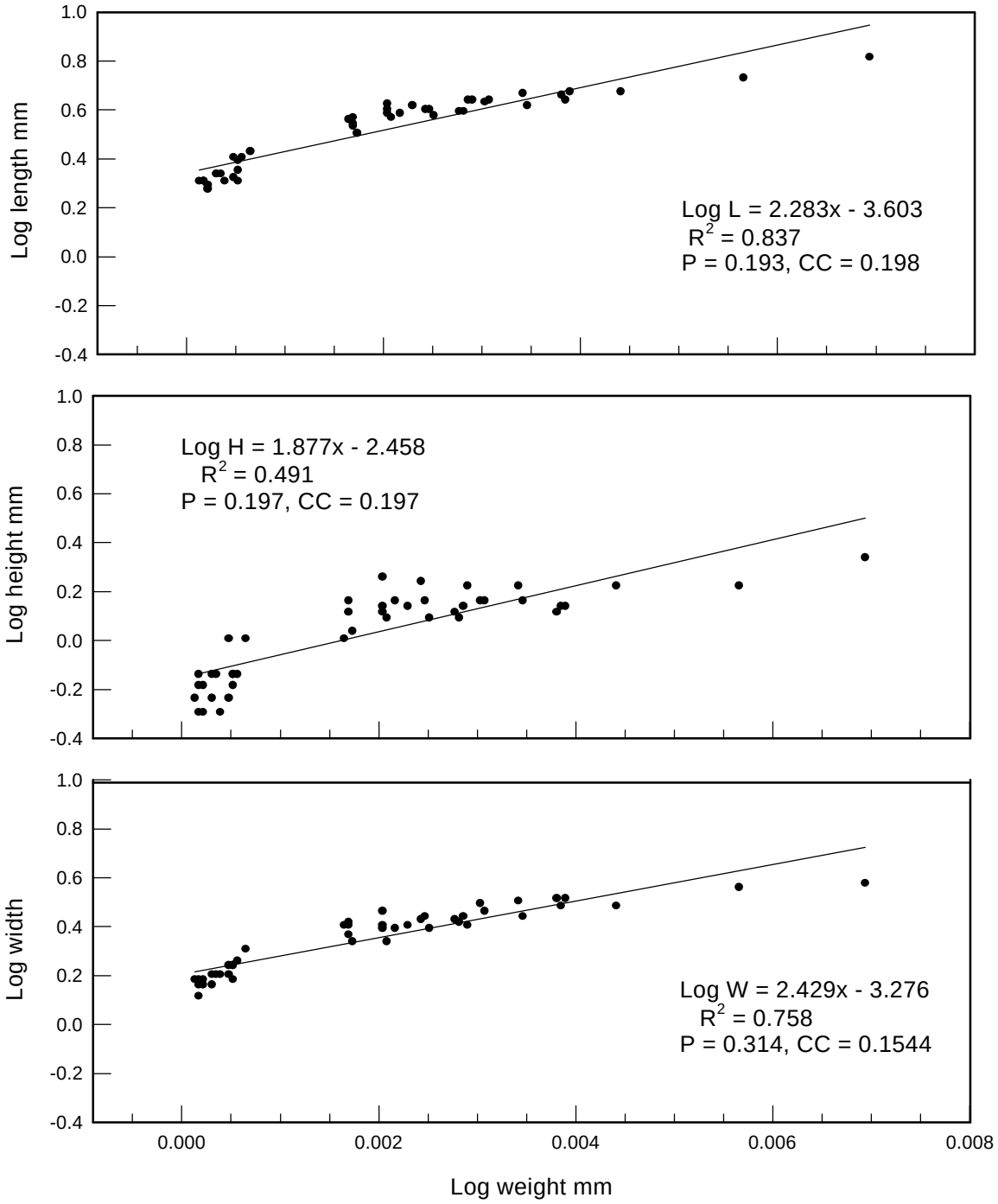


Figure 3.5. Comparison of the relationships between shell weight (wet) with length, width and height of *Burnupia stenochorias* specimens collected from the Botha River population, represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. L = length. W = width. H = height. Scatter around the regression equation is smaller than found with the Belmont River limpets (Figure 3.4). However, correlation coefficient values are weak suggesting length, width or height are not closely related to wet weights for Botha River limpets.

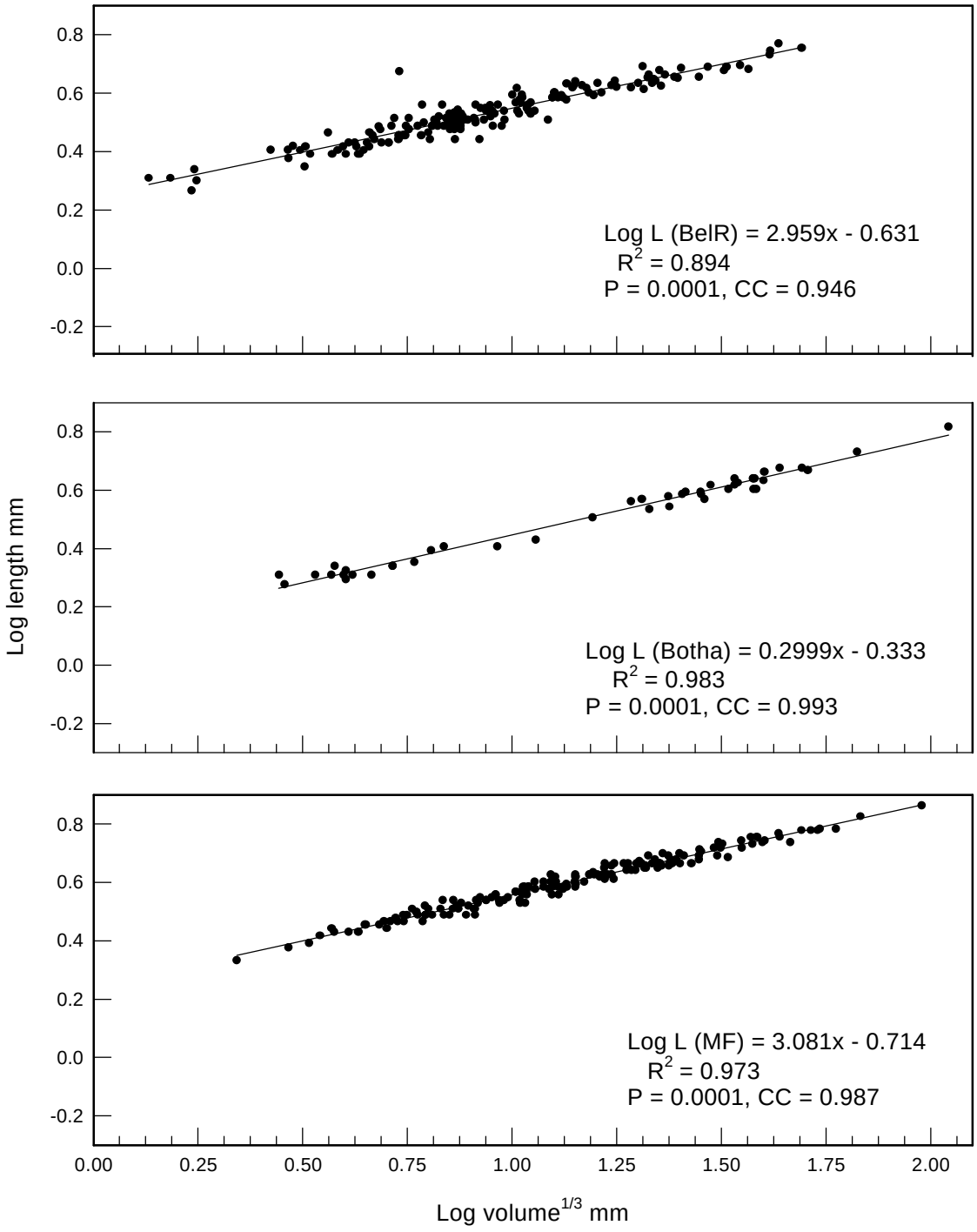


Figure 3.6. Comparison of the relationship between shell volume$^{1/3}$ and length of *Burnupia stenochorias* specimens collected from the three populations (Belmont River [BelR], Botha River [Botha], and Manley Flats [MF]) represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. L = length. A significant relationship exists between length and volume$^{1/3}$ with limpets from all three sites, although with more variability evident with limpets from the Belmont River site.

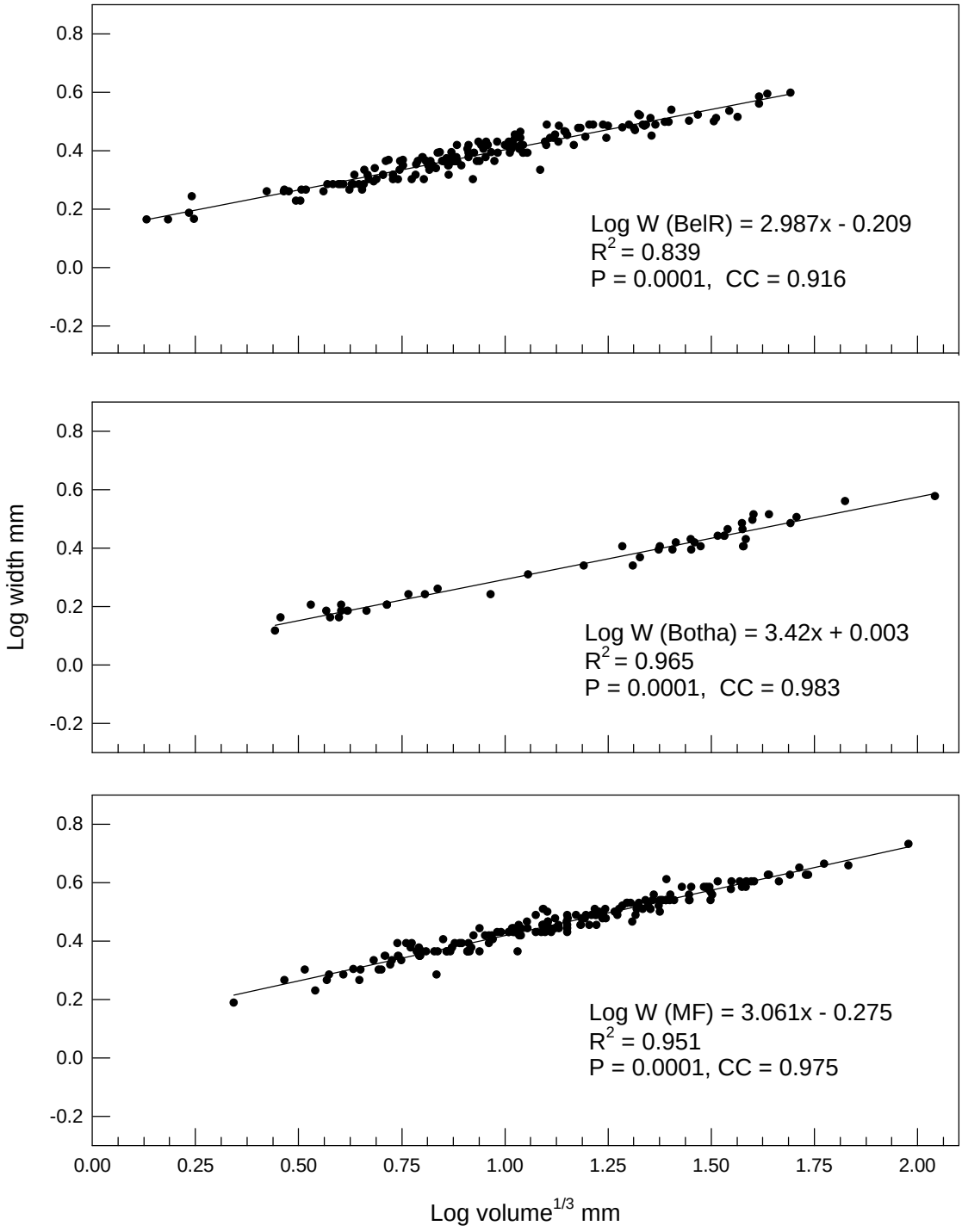


Figure 3.7. Comparison of the relationship between shell volume$^{1/3}$ and width of *Burnupia stenochorias* specimens collected from the three populations (Belmont River [BelR], Botha River [Botha], and Manley Flats [MF]) represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. W = width. A significant relationship exists between volume$^{1/3}$ and width with limpets from all three sites.

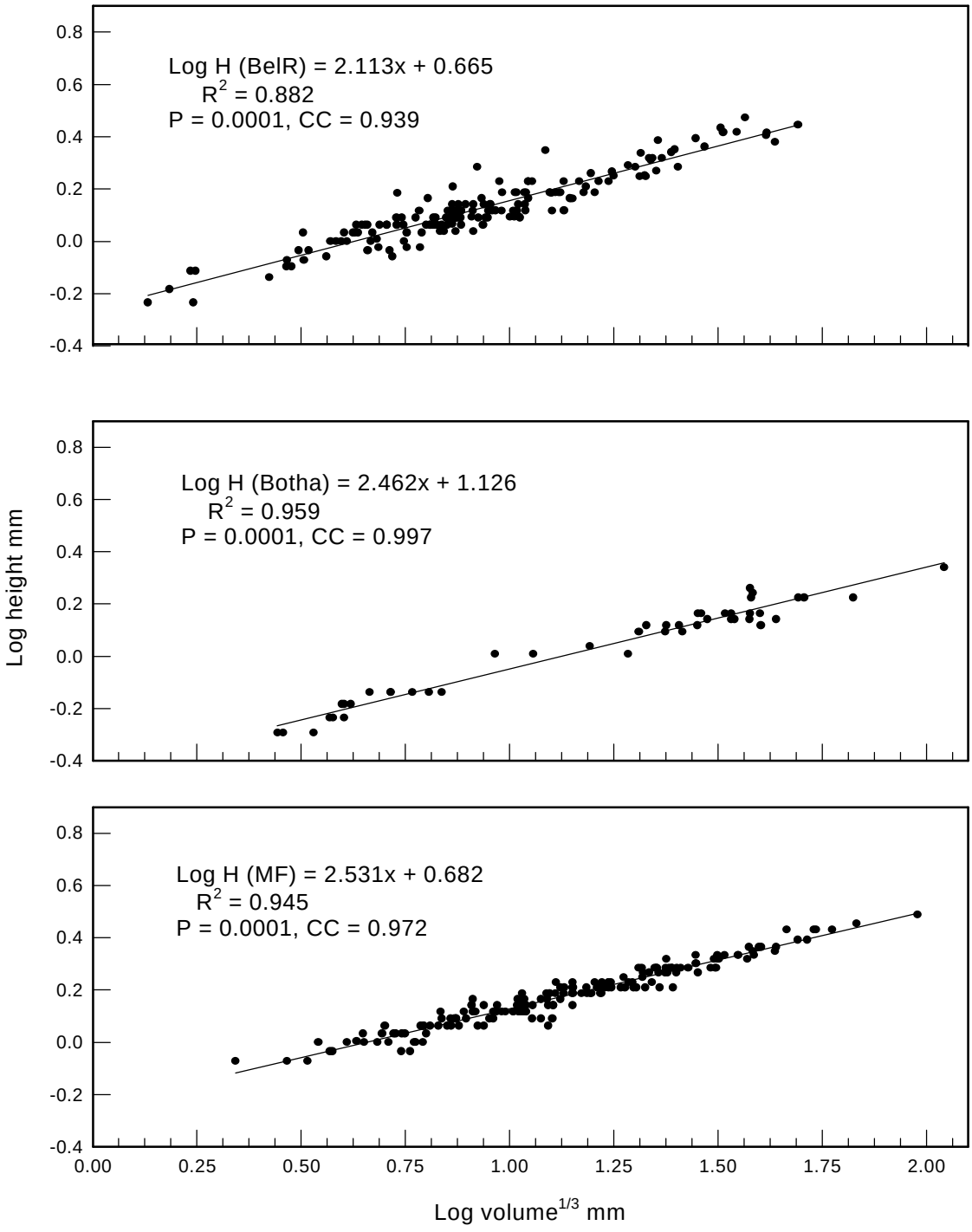


Figure 3.8. Comparison of the relationship between shell volume$^{1/3}$ and height of *Burnupia stenochorias* specimens collected from the three populations (Belmont River [BelR], Botha River [Botha], and Manley Flats [MF]) represented as regression equations. R^2 = coefficient of determination. CC = correlation coefficient. P = significance value. L = length. A significant relationship exists between volume$^{1/3}$ and height with limpets from all three sites, although more variability was evident with the Belmont River limpets.

3.3.4. Relationship Between Growth and Morphometric Measurements

$Log_{(10)}$ length to $log_{(10)}$ height comparisons within the relationship defined by $H = cL^{\%}$ (Table 3.2) showed growth was universally negatively allometric (Figure 3.2). The slopes of the regressions of $log_{(10)}$ length against $log_{(10)}$ volume ^{a} (change in volume representing growth) for the three populations combined (ANCOVA) showed significant differences ($F = 171.3$; $P < 0.001$). There was also a significant relationship between individually tested length, width and height to volume at all three sites (Figures 3.6 to 3.8), suggesting all influenced volume, although more variability and therefore less influence was seen with the Belmont River population length, width or height. Change in wet weights, however, were less influenced by individual morphometric measurements (Figures 3.4 and 3.5).

Table 3.2. Based on regression analyses of the original length and height data for the three *Burnupia stenochorias* populations, where log H = %log L + log c, values for '%' and 'c' are obtained for the equation $H=cL^{\%}$. Botha River limpets had the smallest % value.

Site	Equation
Belmont River	$H = 13.85\ L^{0.334}$
Botha River	$H = 14.003\ L^{0.277}$
Manley Flats	$H = 13.138\ L^{0.322}$

3.3.5. Principal Component Analysis

Based on the scatter plots from the Principal Component Analysis, the first two principal components for *B. stenochorias* demonstrate limpets from the three sites overlap in their distribution of morphometric measurements (Table 3.3). In all three sites, length and width strongly influence the size and shape of *B. stenochorias* relative to height (Figure 3.9).

3.3.6. Discriminant Function Analysis

When measurements for Belmont River, Botha River and Manley Flats were amalgamated, there was a stronger relationship between length and width relative to height (Table 3.4), as shown by the previous regressions. The low covariance values indicate several principal components are preferable in explaining the variation between sites, contrary to the results obtained with Principal Components Analysis (Section 3.3.4). Statistics derived from the Discriminant Function Analysis give eigen values

below 1 (Table 3.6). However, component 1 was still greater than component 2, *i.e.* 62.74% of the variation was explained by the first component (Table 3.6). The canonical correlation values were both high indicating both components dominate in the differences between the limpets and their consequent distribution, with high chi-squared values suggesting group differences were significant (Tables 3.5 and 3.6).

A stepwise Discriminant Function Analysis classified the limpets into predicted groups, based on their discriminant functions and determined from the three morphometric characters measured. The proportion of individuals correctly assigned into each population sampled is given (Table 3.7). The discriminating power is shown to be weak when the data are treated by populations (Belmont River, Botha River and Manley Flats), particularly regarding the Belmont River site where little more than half of the limpets (56.07%) are "correctly" identified.

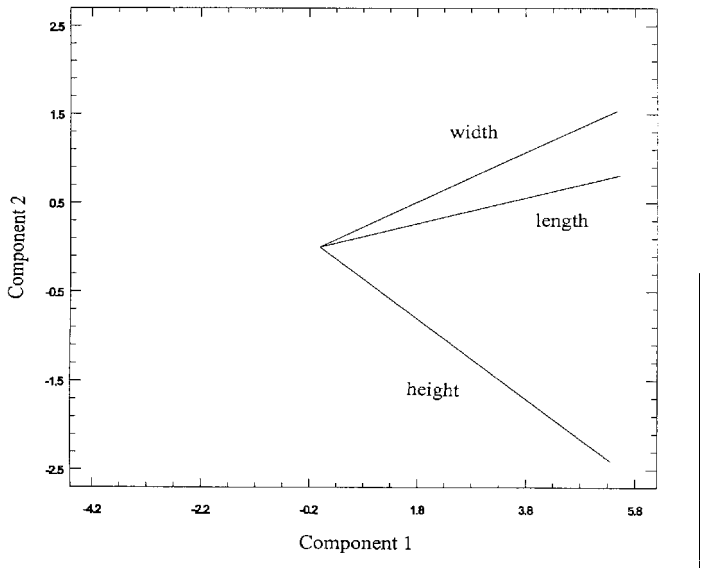


Figure 3.9. Biplot for the first two principal components of the analyses for all length, width and height measurements of *Burnupia stenochorias* combined. The variables width and length positively weight component 1, height negatively weights component 2. The length of the lines (vectors) are each proportional to their contribution to the principal components, and the angle between any two lines is proportional to the correlation between them.

Table 3.3. Summarized results of the Principal Component Analysis based on shell length, width and height characters from the three populations of *Burnupia stenochorias*. The percentages of variance indicate the variation in the data sets are described mainly by the primary component, and the variances of the indices of the other two components are low *i.e.* the variation in the variables length, width and height are accounted for by the components chosen.

Component Number	% of Variance	Cumulative %
1	92.86161	92.86161
2	5.22702	98.09863
3	1.90137	100

Table 3.4. Within-group correlation matrix ([a]) and within-group covariance matrix ([b]) for *Burnupia stenochorias* with length, width and height character measurements combined for the Belmont River, Manley Flats and Botha River sites.

	length[a]	height[a]	width[a]	length[b]	height[b]	width[b]
length	1.0000	0.8872	0.9335	0.0065	0.00537	0.00527
height	0.8872	1.0000	0.8520	0.00537	0.00568	0.00451
width	0.9335	0.8520	1.0000	0.00527	0.00451	0.00493

Table 3.5. Standardized discriminant function coefficients from the Discriminant Function Analysis. The first function coefficient predicts tall, narrow or flat, wide limpets, suggesting a possible correlation between hydraulic conditions and shell shape for *Burnupia stenochorias*.

	1	2
length	0.07128	-2.37663
height	0.81436	1.9842
width	-1.65862	0.82882

Table 3.6. Classification of statistics of observations derived from Discriminant Function Analysis for *Burnupia stenochorias* length, height and width frequencies from the Belmont River, Botha River and Manley Flats sites. DF = degrees of freedom. The variance of each component of the discriminant functions is expressed by the Eigenvalue (based on the eigenvectors which indicate how much each morphometric character influences each principal component), and the Wilks Lambda value (indicating the proportion of variation explained by each component).

Discriminant Function	Eigenvalue	Relative %	Canonical Correlation	
1	0.2266281	62.74	0.42983	
2	0.1345633	37.26	0.34439	
Functions Derived	Wilks Lambda	Chi Squared	DF	P
0	0.7185523	128.571	6	<0.0001
1	0.8813964	49.1104	2	<0.0001

Table 3.7. Classification of *Burnupia stenochorias* into the three predicted population groups based on their actual morphometric measurements, with differing degrees of discrimination obtained from the differential ordering of the data. Results are presented as predicted counts, (and as percentages), for the Belmont River, Botha River and Manley Flats sites sampled.

	Predicted	Groups		
Actual Group	1	2	3	Total
1 (Belmont River)	97 (56.07%)	40 (23.12%)	36 (20.81%)	173 (100%)
2 (Botha River)	8 (17.78%)	28 (62.22%)	9 (20%)	45 (100%)
3 (Manley Flats)	42 (24%)	25 (14.29%)	108 (61.71%)	175 (100%)
Total	147 (37.4%)	93 (23%)	153 (38.9%)	393 (100%)

3.4. DISCUSSION

A practical means of monitoring growth is necessary, if chronic toxicity tests using *Burnupia stenochorias* are to be used by water quality management in South Africa. The use of internal shell growth rings quantified to certain, stipulated conditions is a theoretically accurate method of assessing growth. However, clear external or internal shell growth rings were lacking in *B. stenochorias* with a possible explanation found in Crisp's review (1989). He gave various lines of evidence to suggest that harder and more perfectly crystalline parts of the shell comprised the bands; and that these formed when the body fluids were temporarily at a lower pH due to an accumulation of carbon dioxide and perhaps organic acids during immersion. All shell-secreting invertebrates exposed to the air and closed temporarily to avoid water loss would be likely to experience acidosis, and this would slow down or prevent secretion of calcium carbonate. The greater the aerial exposure, the stronger the banding. The three sample sites for *B. stenochorias* did not undergo temporary dry or exposed periods [I observed that *B. stenochorias* seems intolerant of dry conditions]. Thus, the shell structure would be unlikely to undergo acidosis, if this is indeed the form in which the daily growth rings are laid. The rings on the outer shell of *B. stenochorias* of striae radiating transversely from the apex may rather represent an evolved shell form adapted to cope with turbulence. The rings on the striae are likely to reduce turbulence behind the limpet, in turn minimizing the drag on the animal (Branch, 1981).

It is generally accepted, however, that instead of by rings, growth is best measured by increase in weight, wet or dry, as a consequence of change in the morphological dimensions (namely shell length, width and height) (Calow, 1975a). The correlation between weight and either length, width or height was poor for *B. stenochorias* (Figures 3.4. and 3.5), possibly due to variable wetness of the shell when weighed. Volume, as an ideal overall measurement of size, and therefore potential source of monitoring growth, also was significantly correlated with length, width or height depending on the population studied either after regression (Figures 3.6 to 3.8) or ANCOVA analyses ($F = 171.3$, $p < 0.001$). This suggests that any one of these measurements was suitable as an absolute measure of growth, as represented by the change in the volume of the limpet.

The relationship between the morphometric measurements also varied between sites. In attempting to explain differences in shell shape proportions, Sutcliffe and Durrant (1977) examined *Ancylus fluviatilis* (European fresh water limpet) shells from riverine and lacustrine habitats. They found the

roundness index (W/L) was the same for limpets in either riverine or lacustrine conditions, but the steepness index (3H/L+W) indicated river limpets were (non-significantly) taller. Durrant (1975) suggested that riverine limpets might respond to high current conditions by increasing the downpull of the peripheral foot musculature, resulting in a narrower base with a taller shell, which in turn is more efficiently streamlined (Warburton, 1976). The discriminant function coefficient values for *B. stenochorias* (Table 3.5) predict tall narrow, or flat wide *B. stenochorias*. However, although Botha River limpets, living in a lacustrine condition, were rounder and flatter (Table 3.1), a comparison of Manley Flats with Belmont River limpets (where the latter lived in the highest flow conditions of the three sites studied; Figure 4.1) showed the steepness and therefore potential drag did not conform to this notion (Table 3.1). Tallness (height) may reside in the genotype and not just be a phenotypic response to flowing water (Lam and Calow, 1989); this appears to be the case with the three *B. stenochorias* populations studied around Grahamstown. To confirm the effects of flow rate on the growth and eventual shape of *B. stenochorias*, current flow would have to be measured on a microscale, then compared to the area of the cross section of the shell, representing the drag resistance against current velocity (Branch, 1981). However, boundary layer effects, geometric dimensions, water movement; and daily activities of the limpets; and hydrochemistry (Table 10.2) all complicate the issue.

Variations in shell shape may also be related to water chemistry, as found with differences between *Ferrissia* species (Sutcliffe and Durrant, 1977). Unfortunately, the functional relationship of gastropod shells with their environment is not well understood (DuPouy *et al.*, 1993). For example, there is little relationship between shell amino acid deposition and shell shape (Dussart, 1983); and Evans (1989) found little relationship between calcium content of freshwater gastropod shells and the calcium concentration of the local environment.

Although length and width geometric means can be used for inter and intraspecific comparisons, confidence limits are not provided in such comparisons (Sutcliffe and Durrant, 1977). Regressions do provide values for quantitative comparisons between populations. Using the regression and correlation analyses of the combined data for *B. stenochorias* shells, a more significant relationship was found between length and width, relative to height (Table 3.4). This was confirmed for *B. stenochorias* by the Principal Component Analysis and Discriminant Function Analysis results where length and width were shown to have a strong influence on shell size and shape (Figure 3.9; Table 3.6). Using length,

width and height measurements within the Principal Component and Discriminant Function Analyses, populations can be compared sufficiently to determine their degree of morphological separateness. The discrimination between the three *B. stenochorias* populations predicted by the Discriminant Function Analysis was weak, however, particularly with individuals from the Belmont River population (Table 3.7). This signifies a degree of overlap in shell shape between individuals from the three sites chosen, suggesting they are only geographically separated populations. Any differences are probably due to phenotypic variability across a mean, as found in species of *Patella* (Corté-Real, 1992; Vat, 2000). Genotypic analyses may confirm this origin of variability.

The results of this study indicate that *B. stenochorias* could be chosen from any population for use in growth trials, whether these be for general life history studies, or chronic toxicology tests. Individuals portray similar patterns of allometric growth of length to width or height, so that the use of length measurements would satisfactorily describe growth. However, with the sometimes confusing correlation and regression results, the use of both length and width would be statistically more acceptable as an indication of growth.

CHAPTER 4. RECRUITMENT, GROWTH AND LONGEVITY WITHIN NATURAL POPULATIONS

Key issues: When does egg lay occur, what is an individual's growth rate, and what is its estimated longevity under natural conditions? (Figure 1.2)

4.1. INTRODUCTION

Life history studies quantify the variation in fundamental demographic parameters such as birth rate and survival, as well as the relationship between age and size, from immature to adult (Dillon, 2000). Considerable intraspecific inter-population variation in growth, reproductive rates and cycles, and survival after reproduction has been shown amongst freshwater gastropods, studied primarily from the European and North American areas. The main freshwater life cycle patterns encountered in natural populations of gastropods have been proposed by Russel-Hunter (1961b, 1978) and reviewed and modified by Calow (1978) and Dillon (2000). Up to nine general patterns of life cycles have been identified, with the time of egg lay determining the life cycle pattern shown. The immediate responses of an organism to the environmental conditions in which it is living are based on its genetics (McMahon, 1975a, b). Some authors believe it is these responses that determine life cycle patterns (Begon *et al.*, 1990); others (*e.g.* Brown, 1985) feel life history variation is the result of phenotypic plasticity. Dillon (2000) proposed that life cycles of freshwater molluscs in general appear to be primarily a function of phylogeny controlled by size at sexual maturity. Size is determined by growth rates; and survivorship determines the number of individuals reproducing. A study of the growth and reproduction of populations under natural conditions over an extended period of time would therefore help to illuminate the life cycle pattern of a species.

The effects of environmental conditions on the life cycle patterns of freshwater molluscs are well documented (Fretter and Peake, 1975; Geraerts and Joosse, 1984; Dillon, 2000). Water temperature is considered the most influential factor, affecting metabolic and feeding rates (Shiff, 1964; Duncan, 1975; McMahon, 1975a, 1983; Russel-Hunter, 1978; Heller, 1990). Time of onset and intensity of spermatogenesis and egg-laying have been directly correlated with temperature (Geraerts and Joosse, 1984; Heller, 1990) with critical temperatures for oviposition reported for various pulmonates (*e.g.* Duncan, 1975; Streit, 1985). Growth rates in gastropods have, in general, been directly correlated to

temperature, subsequently affecting the time of first lay and thus life cycle pattern (McMahon, 1975a, b; Streit, 1975; Raut *et al*., 1992). The time of year when recruitment occurs in turn strongly influences patterns of body growth because of the prevailing temperatures (Cloern and Nichols, 1978). A variation in the ambient temperature of a few degrees may result in an order of magnitude difference in survivorship and reproduction (Dillon, 2000). Other abiotic factors which influence life cycles include water chemistry (reflecting water quality; Macan, 1961; Williams, 1970), current velocity, water depth and stream geology (Appleton, 1978), and photoperiodicity (Geraerts and Joosse, 1984). The salt marsh snail *Melampus bidentatus* (Price, 1979) and marine pulmonates often show breeding cycles timed by lunar-related cues. However, lunar effects have not been recorded in freshwater pulmonates.

Biotic factors affecting life cycles are numerous but are closely linked to the abiotic conditions experienced by each population. Evidence is strong that populations of the same species may exhibit differences in growth and maximum length attained, depending on the environmental conditions experienced (Calow, 1973; Russel-Hunter, 1978), and population genetic variability (Jarne *et al.*, 1993). Temperature also affects food quality by influencing algal growth rates (Ward and Stanford, 1982), with food availability influencing population growth (Russel-Hunter, 1961a, b; Byrne *et al.*, 1989; Caquet, 1993). Annual patterns in populations of the genus *Physa* have been shown to be strongly influenced by temperature and the effects it had on quality and quantity of food; the number of generations varied from one to three years (Duncan, 1959; Girod, 1969; Eckblad, 1973). *Elimia fascinans* populations were reported to be virtually identical at different sites, but individuals from three populations of *E. cahawbensis* grew at markedly different rates (Huryn *et al.*, 1994). Brown *et al.* (1985) found that shell length of *Lymnaea elodes* at maturity was a function of habitat productivity; and that snails matured and reproduced at smaller sizes in the less productive sites [and had higher fecundities]. Costil and Daguzan (1995a, b) proved various sizes were attained by cohorts of *Planorbis planorbis* and *Planorbarius corneus* (both Planorbidae) from different sites and times of the year, related to temperatures and food quality.

Density dependent control mechanisms may also influence the life cycle displayed by a population of a freshwater pulmonate (Hill, 1992; Hershey, 1992). Many different mechanisms have been linked to density, including trophic limitations (van der Steen, 1967; Eisenberg, 1970; Mooij-Vogelaar *et al*.,

1973; Hill *et al*., 1992), pheromone-like substances (Berrie and Visser, 1963; Thomas *et al*., 1976; Chaudhry and Morgan, 1987), metabolite accumulation in the environment (Hiruta and Kawabata, 1954), removal of dissolved minerals from the environment (Thomas and Benjamin, 1974) and the frequency of tactile contact between snails (Chernin and Michelson, 1957a, b; Eversole, 1974).

The intraspecific variation seen in life history patterns is considered of fundamental selective value in a freshwater species' evolution within its environment (Russel-Hunter, 1961b; Calow, 1978). However, this plasticity causes problems with population studies. Several natural populations of freshwater pulmonate species have been extensively studied, but only three species of Ancylidae have been and continue to be studied in the northern hemisphere temperate regions and U.S.A.. They are *Ancylus fluviatilis* from Great Britain (*e.g.* Geldiay, 1956; Hunter, 1953, 1961; Willis, 1983); *Ferrissia rivularis* (*e.g.* Burky, 1971; Nickerson, 1972; Keating and Prezant, 1998) and *Laevapex fuscus* (*e.g.* McMahon, 1975b) from the U.S.A.. Similar in-depth studies have been made on three populations each of the more tropical Texan species *Hebetancylus excentricus* and *Laevapex fuscus* (McMahon, 1976). In southern Africa, however, gastropod research has been confined to species of either medical importance (primarily bilharzia-carrying snails; *e.g.* Shiff, 1964; O'Keeffe, 1982) or invasive significance (*e.g.* Brackenbury and Appleton, 1993). An independent investigation was therefore considered necessary to determine recruitment patterns and growth rates of *Burnupia stenochorias*.

Growth of pulmonates is considered sigmoid with an initial linear phase followed by an exponential phase, after which size increases to a steady state (Calow, 1981). Interpretation of data depicting this type of growth may be complex. Numerous models for the estimation of growth are available which fit curves to data, based on either a reductionistic or an holistic approach, depending on the effects variables have on growth (Calow, 1973; Castro, 1992). Samples from natural populations are often based on :

- a grab and remove system, where individuals are removed from the aquatic system and measurements taken; or
- mark and recapture techniques, for example by painting the animals (Dazo *et al.*, 1966) or using numbered microtags (Freilich, 1989); or
- the collection of a series of length frequency data collected over time. This is possibly the cheapest and easiest method to use, with a plethora of literature of length-based methodology

available in the literature (Pauly, 1984; Isaac, 1990; Mytilineou and Sarda, 1995).

The Von Bertalanffy Growth Equation (Von Bertalanffy, 1934) is probably the most commonly used growth function. It represents asymptotic growth as described above with linear, exponential and steady state phases, and is based on a relationship between L_t (length at time 't'), L_4 (asymptotic length but usually represented by mean or maximum length of oldest individuals), ê (rate at which L_4 is reached), and time. Based on this type of growth curve, there are many length frequency distribution analyses available (Longhurst and Pauly, 1987). Although originally developed for fish, many of the techniques can be applied to invertebrates without modification (Tomalin, 1995). Length frequency data analysis can be divided into parametric (MacDonald, 1987) and non-parametric (Pauly and Morgan, 1987) classes. The latter identify modes in some way and uses a goodness of fit test to determine the 'best' set of growth parameters.

Within this chapter, results from a year's field survey of two natural populations of *B. stenochorias* are reported. Reproductive activity was determined, as exemplified by the presence of newly laid egg capsules, with estimations of growth rates and lifespan of any one cohort[2], based on length frequency distributions. Although changes in length *and* width are considered a more accurate measure of changes in shell morphometrics over time (Chapter 4), a programme using both these criteria was not available. Therefore estimates were based on change in shell length only. The package chosen for length frequency data analysis of *B. stenochorias* is FiSAT, an FAO stock assessment tool developed by Gayanilo, Sparre and Pauly (1995). It is a BASIC programme incorporating modal class progression analysis, and the ELEFAN I programme (*E*lectronic *L*ength *F*requency *A*nalysis) (Pauly and Morgan, 1987; Pauly, 1988).

Knowledge of *B. stenochorias* life cycle data, accumulated through population studies, has facilitated more efficient culturing in the laboratory, but has also provided useful data for chronic toxicity tests (Chapter 12). Although no attempt was made to complete the information necessary for a complete life table analysis of *B. stenochorias*, a suggestion as to the life cycle type is made.

[2]A "cohort" is defined by the Oxford Reference Dictionary (1986) as having a common statistical characteristic. In the case of the limpets, it is a group of limpets all of the same age belonging to the same stock.

4.2. MATERIALS AND METHODS

4.2.1. Sampling

Shell lengths of *Burnupia stenochorias* were measured fortnightly from February 1996 to March 1997 at two local sites along the Bloukrans River (Manley Flats and Belmont River; Figure 2.2). A graded plastic template was used (Section 2.3). An initial rock with large numbers of limpets was chosen at each of the two sites, with the remainder of rocks sampled within one metre circumference. Thereafter, the same rocks were sampled where possible (selection technique followed after Gardefors and Orrhage, 1968). Sampling always occurred between 09h00 and 11h00. Each rock was removed from the river and all limpets measured with minimal disturbance before being replaced into the river. This continued until at least 250 limpets were measured, although this number was not reached during some months due to a lack of limpets (Figures 4.4 and 4.5). Reproductive output was measured by counting egg capsules within the same unit area of rock.

4.2.2. Environmental Data

Total Dissolved Salts (TDS), and pH were measured when sampling using M90 hand-held probes. Some fortnightly maximum and minimum water temperatures were measured at Manley Flats with a mercury thermometer tied to vegetation in the stream; thermometers at the Belmont River site were continually washed away or stolen. Temperatures at the time of sampling were noted. An Ott flow meter was used to measure flow rate (m/sec). Detailed water chemistry was not undertaken because of time constraints.

4.2.3. Analysis

A progression with the analysis of data was made:

(a) Histograms.

Time of egg-laying and numbers of egg capsules laid at each site were determined using size-frequency histograms of all data collected over the 52 week period.

(b) Probability Paper Method.

A very common method of differentiating cohorts from each other is with the probability paper method of Harding (1949) (further developed by Cassie, 1954). Any group of observed length frequency data (LFD) will fall into a normal distribution, and Harding's method relies on the fact that it is difficult to assess by visual inspection whether points within a set of length-frequency data conform to a bell-

shaped or a sigmoid curve. The probability paper method is a graphical means of constructing an easy-to-read straight line showing the cumulated frequency distribution, with a mixture of several normal distributions providing a more complex line with inflexions (Sparre *et al.*, 1993). Although polymodal curves are generally not considered difficult to analyse, it was, however, found with this data set that the component populations had considerable overlap, and a more refined method of analysis was necessary.

(c) FiSAT Package.

The grouping of sampled individuals into age classes does facilitate population studies (Southwood, 1978) but as it is often difficult to determine ages (Gillespie, 1969), various methods of length frequency distribution analysis (LFDA) have been developed to estimate growth parameters. The FiSAT package provides this opportunity.

Using the Modal Class Progression Analysis, an attempt was made to translate the length frequency data into age scales, firstly using the Bhattacharya's method and then the NORMSEP method. The results were statistically unacceptable, based on the Chi squared test, for each sample date for both the Belmont River and Manley Flats data.

The ELEFAN I programme was then used to estimate the growth parameters, based on the Von Bertalanffy Growth Equation (von Bertalanffy, 1934). It is based on length-at-age-data and incorporates the "tracing" of growth curves through length-frequency samples sequentially arranged. The non-parametric method used by the ELEFAN programme (as opposed to parametric, reviewed in MacDonald, 1987) has a history (Pauly, 1987) of successful use on short-lived animals (*e.g.* Peruvian anchoveta; Hilborn and Walters, 1992). The Von Bertalanffy Growth Equation is represented by the equation:

$$L_t = L_4[1 - \exp(-\hat{e}(t-t_0)]$$

where L_t is the length at time t (mm), L_4 is the asymptotic length (mean length of oldest individuals) in mm, $\hat{e}$ is the exponential rate at which length approaches the asymptotic length (per year) and t_0 is the age at length 0 (years). Biologically, t_0 has no significance as it is present purely as a result of the mathematical modelling procedure (Ricker, 1975). Growth is considered to start at the time of hatching and not at the value 0 mm.

C ***Maximum Length***

ELEFAN provides a means of predicting the maximum length, with confidence intervals provided at 95% probability where the lower limit is less than the observed maximum length. In determining the growth parameters, both the observed and the predicted maximum lengths were used and compared to each other, for seasonal and non-seasonal growth rates.

C ***Growth Parameters***

The seasonalized von Bertalanffy Growth Equation produces seasonal oscillations of the growth rate by changing t_0 during the year (Pitcher and MacDonald, 1973; Cloern and Nichols, 1978; Pauly and Gaschütz, 1979):

$$L_t = L_4 \, (1 - \exp(-ê(t - t_o)-(Cê/2Ð) \sin(2Ð(t-t_s))))$$

The parameter 't_s' is called the 'summer point' and takes the value between 0 and 1. At the time of the year when the fraction t_s of the year has elapsed, the growth rate is highest. At time $t_w = t_s + 0.5$, the 'winter point' (WP), the growth rate is lowest. The parameter C, the 'amplitude', also usually takes values between 0 and 1, the higher its value, the more pronounced the seasonal oscillations.

To test the variability of shell length data from different seasons, for predicting growth curves, the Manley Flats and the Belmont River shell length data were split into three groups, based on the appearance of the recruitment phase from approximately September, as below, which were then compared with each other.

1) With recruitment (September 1996 to February 1997; no winter phase).
2) Without recruitment (March 1996 to September 1997; no summer phase).
3) Twelve months' data (March 1996 to February 1997; recruitment and a winter phase).

Each set of data was analysed for growth twice, using either seasonalized or non-seasonalized Von Bertalanffy growth parameters, and compared using the growth curves based on the growth parameters obtained.

C ***Rn Values and Phi Prime (Ö')Test***

A so-called Rn value is used to determine the best fit of the length frequency data (LFD), by either decrementing or incrementing the seeded values of L_4, ê, C and WP until the Rn value reaches a maximum. The values for Rn can be compared within data sets, but are not usually used to compare sites or populations (Pauly and David, 1981). To compare growth between sites (Pauly and Munro, 1984) the ELEFAN programme provides the so-called "phi prime (Ö') test" (Munro and Pauly, 1983;

Pauly and Munro, 1984), a statistic which relies on the inverse correlation and relationship between L_4 and ê, and which is known as Pauly's growth performance index:

$$Ö'(\text{phi prime}) = \ln ê + 2\ L_4.$$

Although originally described for fish, the Ö' has been shown to fit observed correlations in a yellow clam (Defeo *et al.*, 1992) and other invertebrates (Longhurst and Pauly, 1987). The Ö' value represents and quantifies the energetics of a given habitat or niche because it is directly related to the growth performance as represented by change in length, and hence metabolism and food consumption (Munro and Pauly, 1983).

4.3. RESULTS

4.3.1. Site Details

The Belmont River site (Figure 4.1a) had a continual annual flow of water (Figure 4.2), fed primarily by the run-off from the city of Grahamstown (in particular treated waters from the sewage works) and its immediately surrounding areas. The Manley Flats site (Figure 4.1b) experienced no flow and pool conditions at certain times of the year (Figure 4.3). Water depth was always 50 cm or less at both sites, with the exception of very occasional floods after heavy rains where the level of water rose by 60 cm at both sites for 48 hours. Scouring of the bed material, and consequently depletion of the limpet population, occurred at the Belmont River site during these times.

No *Burnupia stenochorias* were found on sand or silt sections of the river bed, and only a few on the vegetation growing at the river edge, but always submerged in water. The majority were distributed in a contiguous manner on the submerged stones and rocks (Table 4.1). Although of some interest, absolute densities of limpets were not considered because of the heterogeneity of rock size and consequent limpet distribution in the river. During low or no flow periods at the Manley Flats site (Figure 4.4), silt buildup occurred with stones covered with up to 1 mm of silt at times with *B. stenochorias* of all sizes immersed beneath the silt for nearly 4 weeks.

4.3.2. Environmental Data

The Bloukrans River and its catchment area receives rain in all seasons, although predominantly during spring and autumn (Schulze, 1986). Thunderstorms are comparatively rare, and there is little frost because of cool sea breezes. Winds are strong in spring, with hot “berg” winds (38EC) during the

summer months. Average daily maximum temperatures are 28EC (January) and 19EC (July), and minimum temperatures 15EC (January) and 6EC (July) with cloudy conditions 50% of the year. The climate is therefore considered temperate to warm (Schulze, 1986).

Practical problems caused insufficient maximum/minimum temperature data to be recorded from either site to estimate average daily temperatures according to the method used by Macan (1958). Eckel and Reuter (1950) and Fry and Watt (1957) suggest the average temperature of small streams is not greatly different from that of the air, although Crisp (1990) disagrees. Regression of temperatures recorded fortnightly at 09h00 at each site against minimum air temperatures (Statgraphics 5.0) revealed a linear relationship ($p < 0.001$). However, multiple regressions of recorded temperatures versus minimum and maximum air temperatures did not correlate, and it can be concluded, therefore, that although these recorded temperatures give a general indication of the seasonal temperature cycle, they cannot be used to estimate the average daily temperatures at the two sites.

There were no significant differences between the environmental conditions of pH, temperature measured at the time of sampling, and TDS at the two sites studied (Figures 4.2 and 4.3). However, flow rates were significantly lower at the Manley Flats site, possibly contributing to a pond effect at Manley Flats, and therefore daily temperature fluctuations not experienced at the Belmont River site where continual flow occurred. The activity of cattle at the Manley Flats site during low flow periods contributed to an apparent poorer quality water than found at higher flow periods or at the Belmont River site.

Table 4.1. Size distribution of rocks from which *Burnupia stenochorias* were sampled from February 1996 to March 1997. All figures are in cm; numbers in brackets indicate range. Surface area of the rocks were approximated according to Calow (1972):

Surface area = 2.22(± 0.26)x (where x = longest perimeter).

Site	Average perimeter	Average depth	Average surface area (cm^2)	Average number used at sampling
Belmont River	37 (15-90)	28 (12 - 40)	82	11 (4-27)
Manley Flats	44 (25-76)	16 (9 - 30)	98	4 (3-7)

Table 4.2. Recorded environmental data for the Manley Flats and Belmont River sites. Student's one sample 't' tests (Statgraphics 5.0) comparing the two sites revealed no significant differences in recorded temperatures, pH and TDS between Manley Flats and Belmont River ($p > 0.1$), but a significant difference in flow rates* ($p < 0.001$).

Recorded data	Manley Flats			Belmont River			Student's
	Max.	Min.	Avg.	Max.	Min.	Avg.	't' test
Flow rates, m/sec	0.283	0.006	0.165	0.815	0.084	0.375	-4.9502*
TDS, mg/litre	842	385	598	723	264	550	-1.3207
pH	8.76	5.66	6.98	8.25	6.16	6.91	-1.899
Recorded temp, EC	22.5	9.5	15.9	23	9.5	16.4	1.2039

Figure 4.1 (a) The Belmont River site, described (after King and Tharme, 1994) as a meandering section bound within a confined narrow plain, pool and riffle with large cobble bed material with small (3.5cm length) stones at the edges, and silt drapes with cobbles embedded. Bank material is of silt/clay, and riparian vegetation of reeds and grasses (in channel), and grasses, shrubs and trees (bank). Erosion within the channel is limited with bed scouring from moving rocks. In-channel modification is a causeway (closest to the camera).

Figure 4.1 (b) The Manley Flats site, described (after King and Tharme, 1994) as a straight channel bound within a confined narrow plain, pool and riffle (furthest from the camera) with bed material of large gravel (3.2cm length) to large cobble (51cm length) and silt drapes with cobble embedded, particularly dominant in the pool area. Bank material is of sand and riparian vegetation of grasses and reeds (in channel) and shrubs and trees (bank). Erosion within the channel is very limited.

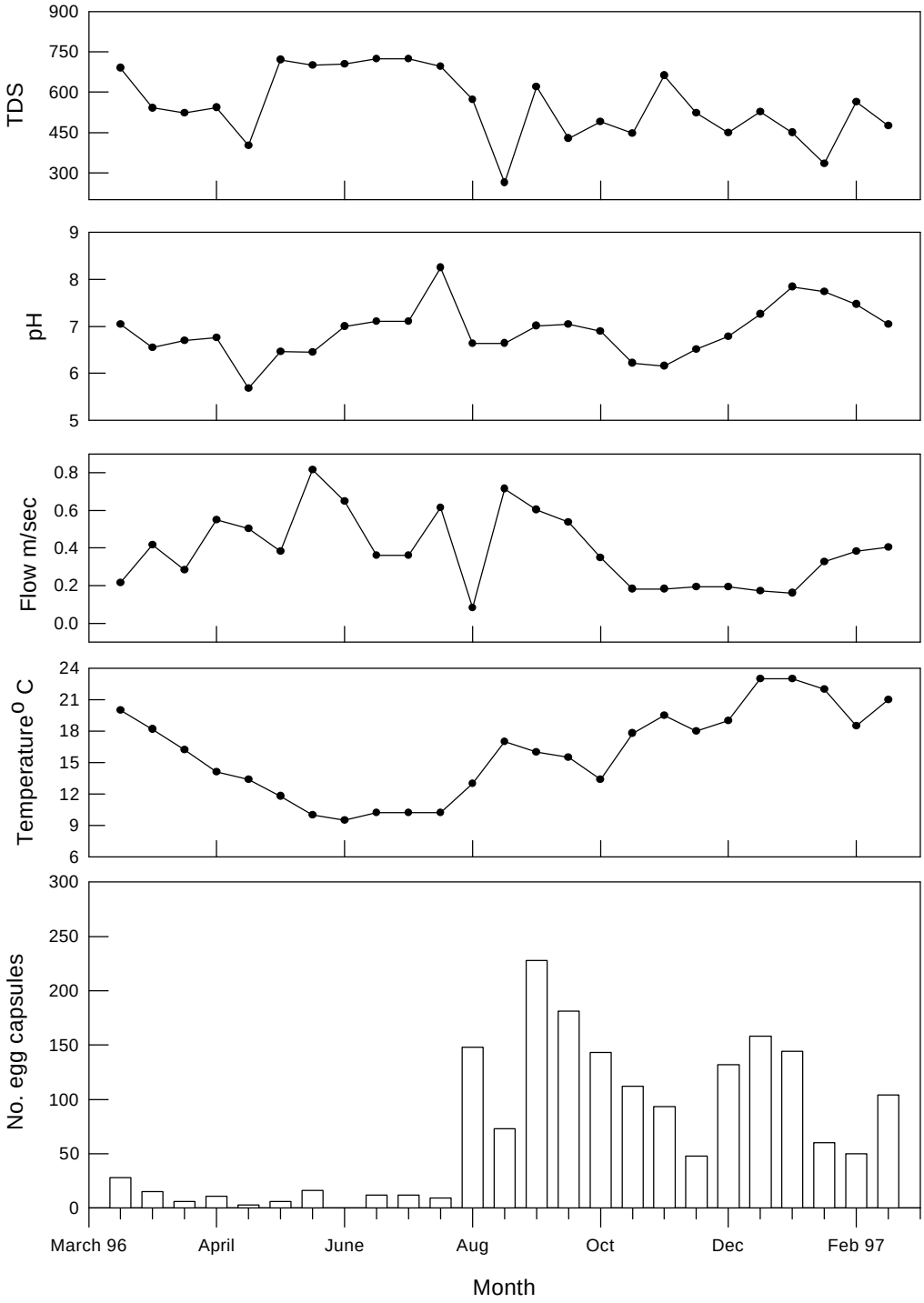


Figure 4.2. Total dissolved solids (mg/l), pH, flow rate (m/sec), temperature (EC) and number of egg capsules recorded fortnightly at the Belmont River site on the Bloukrans River from March 7, 1996 to March 13, 1997. The number of egg capsules are those recorded from the rocks sampled for limpet shell length frequencies, usually within an area of $4m^2$.

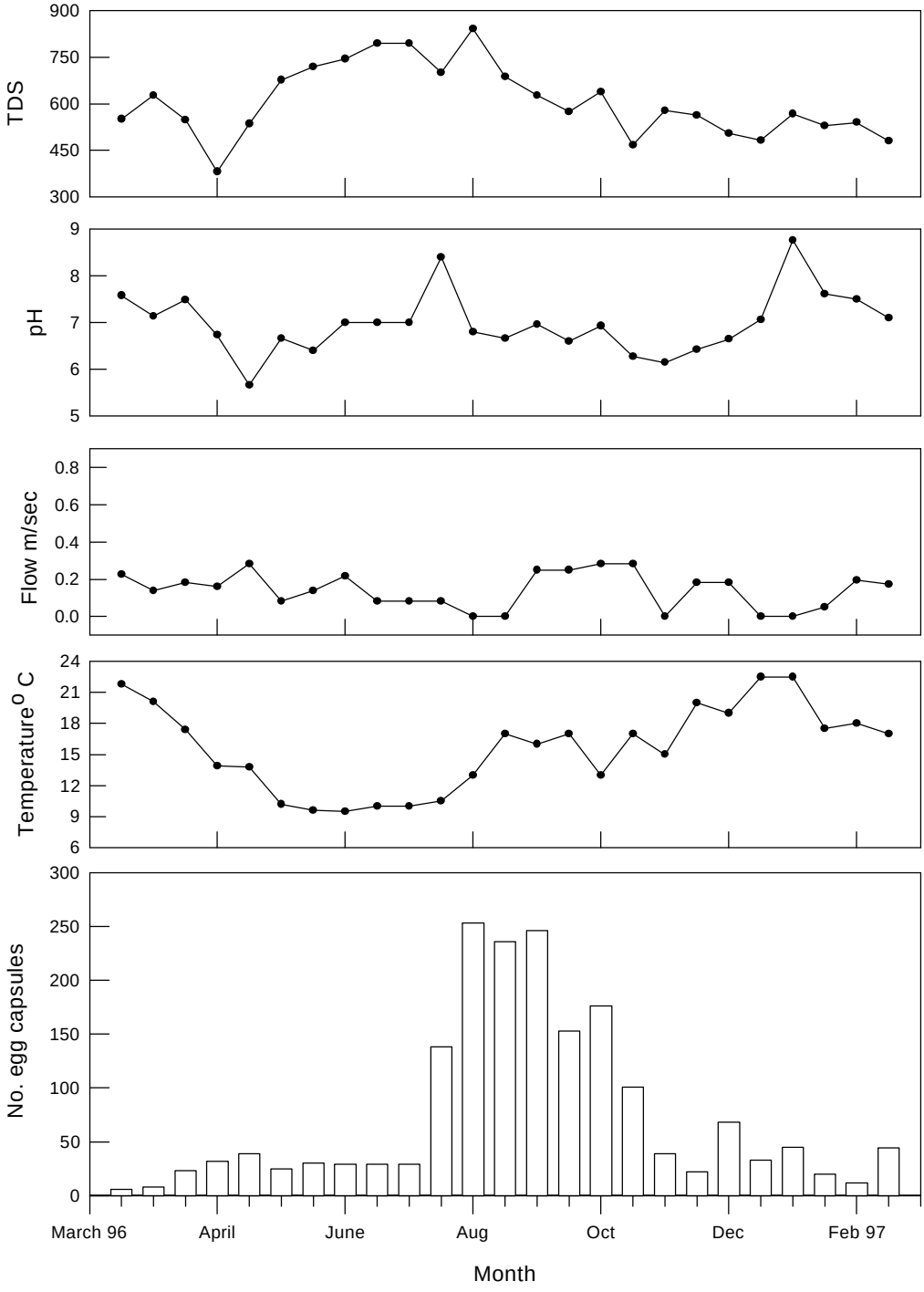


Figure 4.3. Total dissolved solids (mg/l), pH, flow rate (m/sec), temperature (EC) and number of egg capsules recorded fortnightly at the Manley Flats site on the Bloukrans River from March 7, 1996 to March 13, 1997. The number of egg capsules are those recorded from the rocks sampled for limpet shell length frequencies, usually within an area of $4m^2$.

4.3.3. Egg Capsules

The majority of egg capsules (1.75-2 mm diameter) were found on the underside of the stones or rocks, primarily on the lower extremes but often on the underside of protruding edges or indentations on the stones large enough to hold a capsule; none were found on upper surfaces. When a rock was lying in silt, egg capsules were in a concentrated ring around the closest edge to the bottom of the rock, never overlapping but often with edges touching. Occasionally capsules were found on either vegetation or on smooth parts of natural or artificial debris wedged between rocks. Eggs were laid all year round at both sites (Figures 4.2 and 4.3) but with an obvious substantial peak beginning in August continuing until early November (Manley Flats) or the end of January (Belmont River). Capsules ranged from 2 - 13 eggs per capsule, although the practical difficulties in determining the lower numbers suggest there may also have been a number of capsules containing just one egg, as found in the laboratory.

4.3.4. Measurements of Limpets

The growth of *B. stenochorias* has already been shown to be negatively affected by disturbance (Haigh and Davies-Coleman, 1997). Marking limpets individually with either red paint or white nail polish (as described by Dazo *et al.*, 1966), or by using numbered microtags (Freilich, 1989) were not considered viable options, since (i) the paint or polish needed approximately 30 minutes to dry, which was considered too stressful for the limpets as they have not been found to occur naturally out of the water for any period; (ii) many were covered in green algae which would have to be removed first, causing further stress and possible damage to the shell itself; (iii) the newly hatched limpets up to 1 mm in shell length were considered too small either to remove satisfactorily from the rock (when seen), or to be effectively marked for the duration of the sampling time.

Using length frequency data collected over 53 weeks, it was found that with the continual egg laying and consequent recruitment over a period of three to four months, the progression of modes using histograms (Figures 4.4 and 4.5) is unclear and insufficient to show the number, longevity or growth of any cohorts. However, spring recruitment can be clearly seen on September 29 (Belmont River site) and October 12 (Manley Flats site) with an increase in the number of limpets approximately 1 mm shell length. The majority of limpets appeared to be within the 2.5 to 3.5 mm category of shell

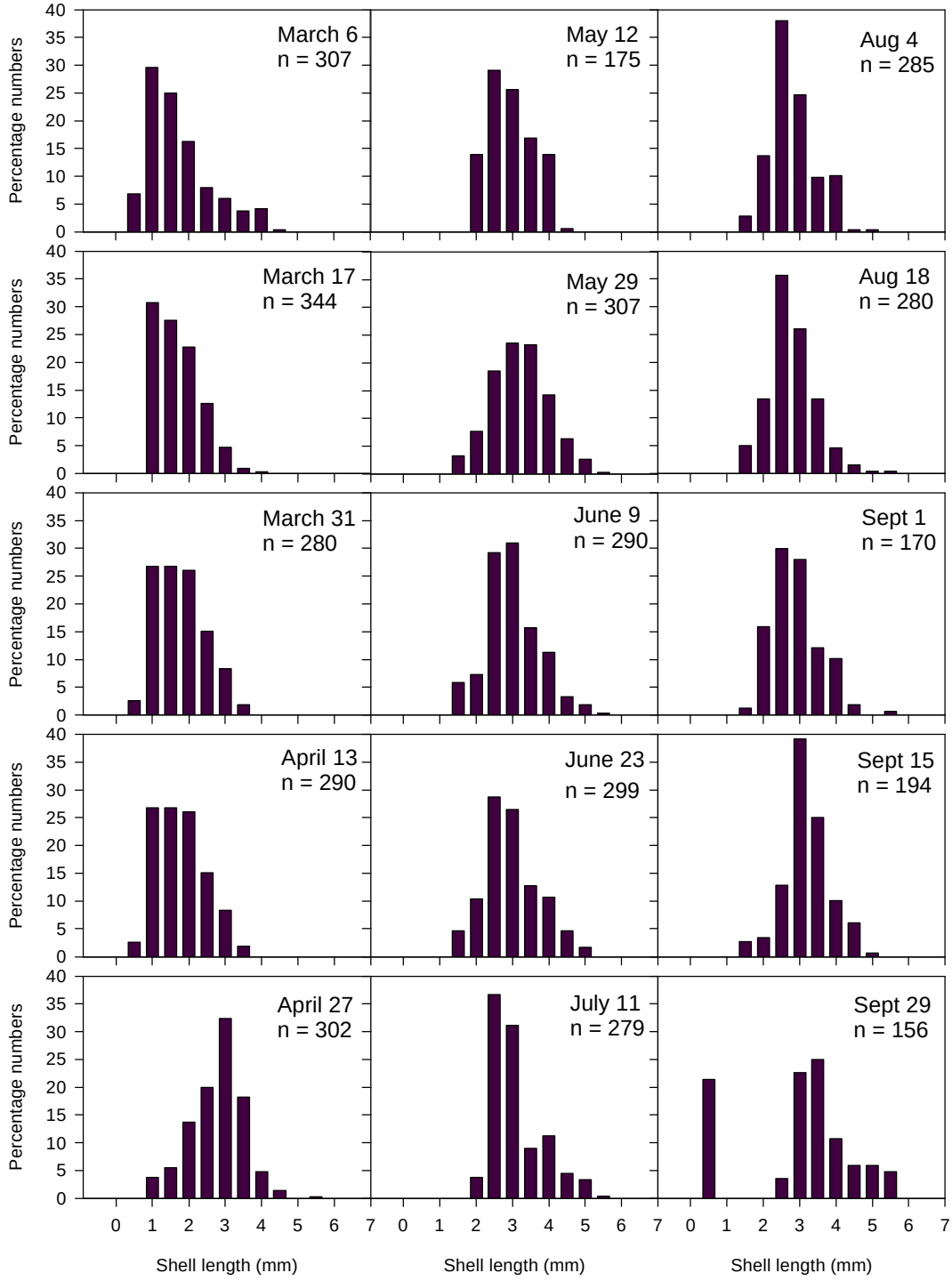


Figure 4.4. Histograms of the number of limpets measured for shell length at the Belmont River site from March 6, 1996 to March 1, 1997. Percentage numbers indicates the percentage of measurements (n = actual number) taken each fortnight which fall into each particular size category, as represented by size (mm); size (mm) refers to shell length, in increments of 0.5 mm, but with the smallest category at approximately 0.75 mm shell length representing newly hatched limpets.

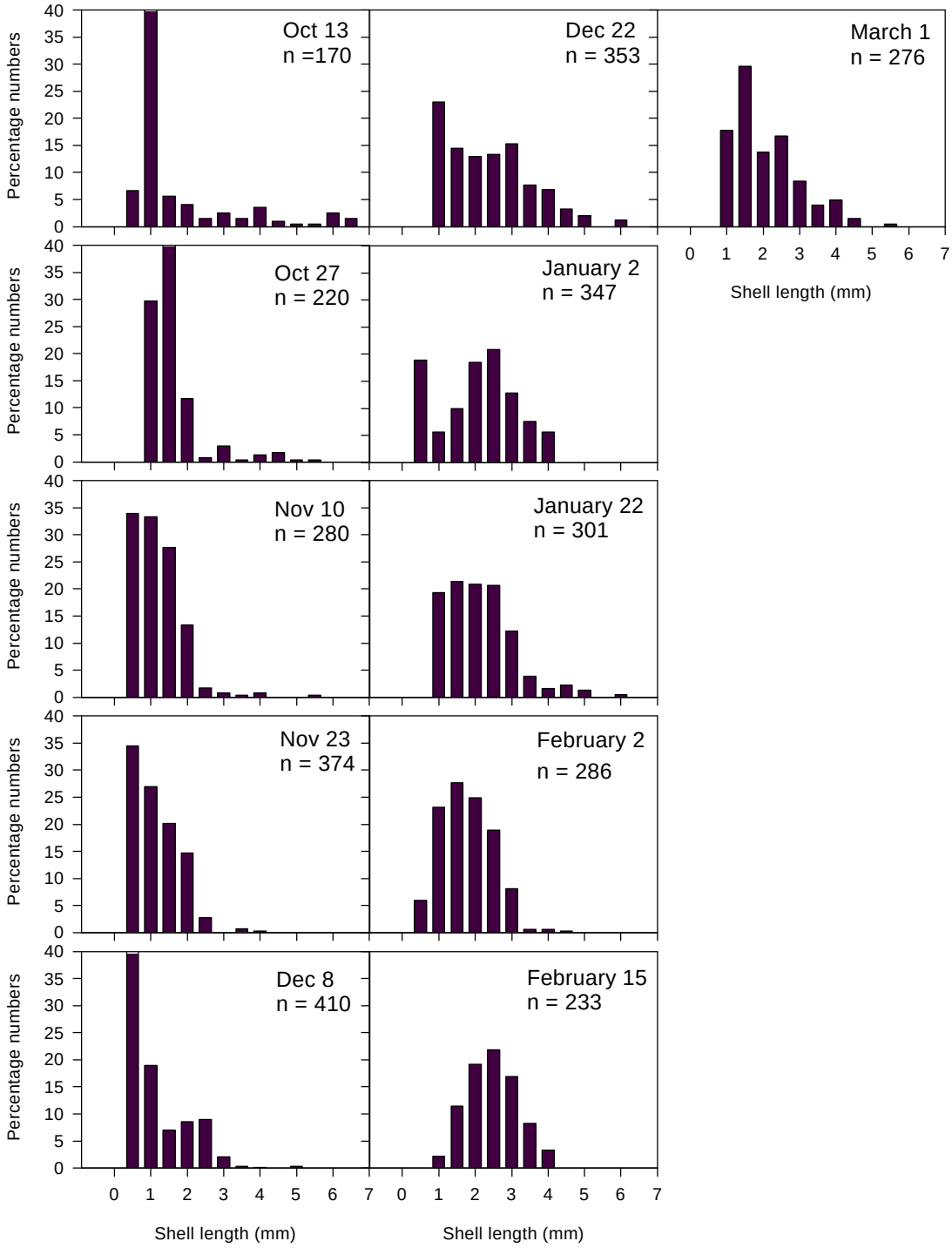


Figure 4.4. continued.

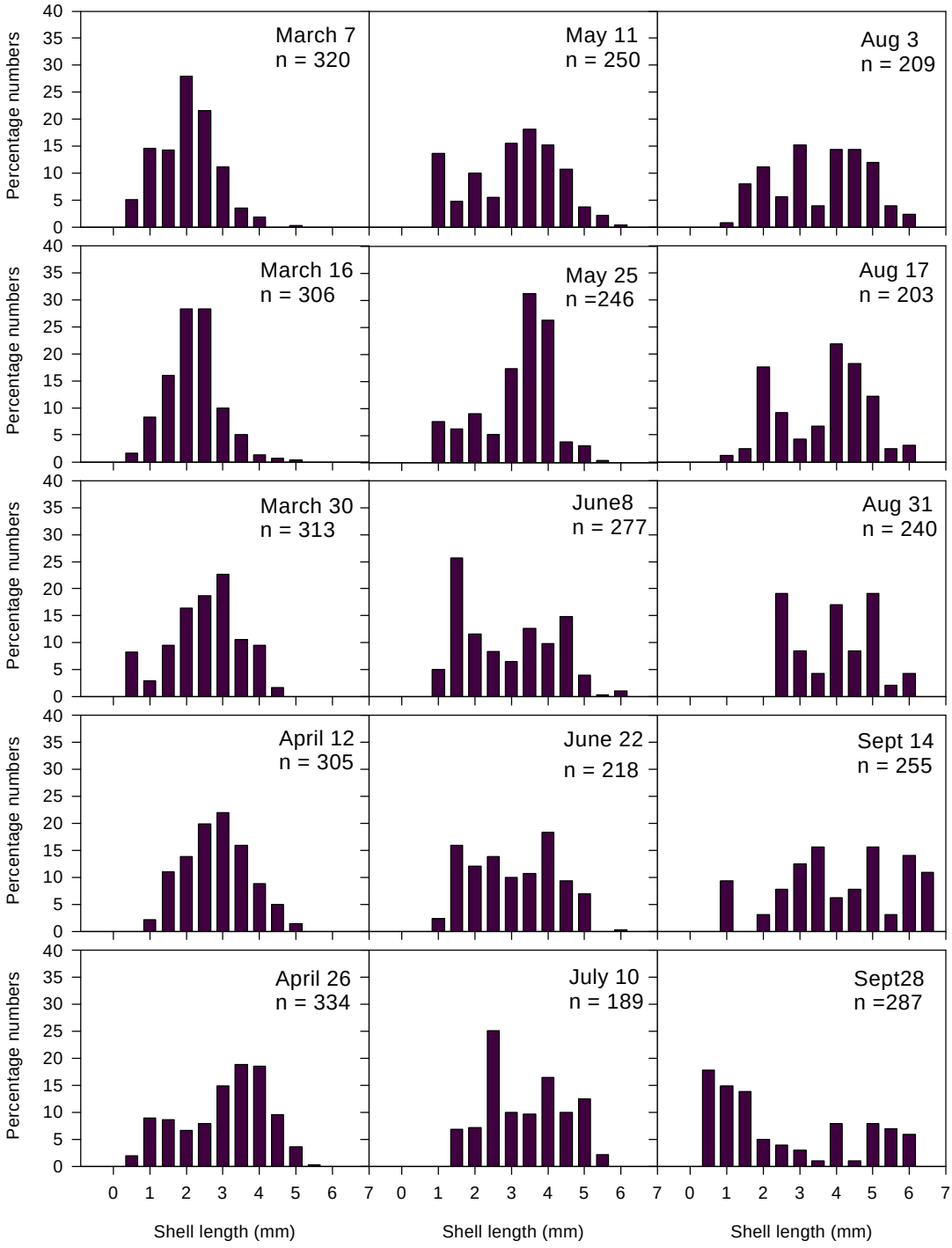


Figure 4.5. Histograms of the number of limpets measured for shell length at the Manley Flats site from March 7, 1996 to March 13, 1997. Percentage numbers indicates the percentage of measurements (n = actual number) taken each fortnight which fall into each particular size category, as represented by size (mm); size (mm) refers to shell length, in increments of 0.5 mm, with the smallest category at approximately 1 mm.

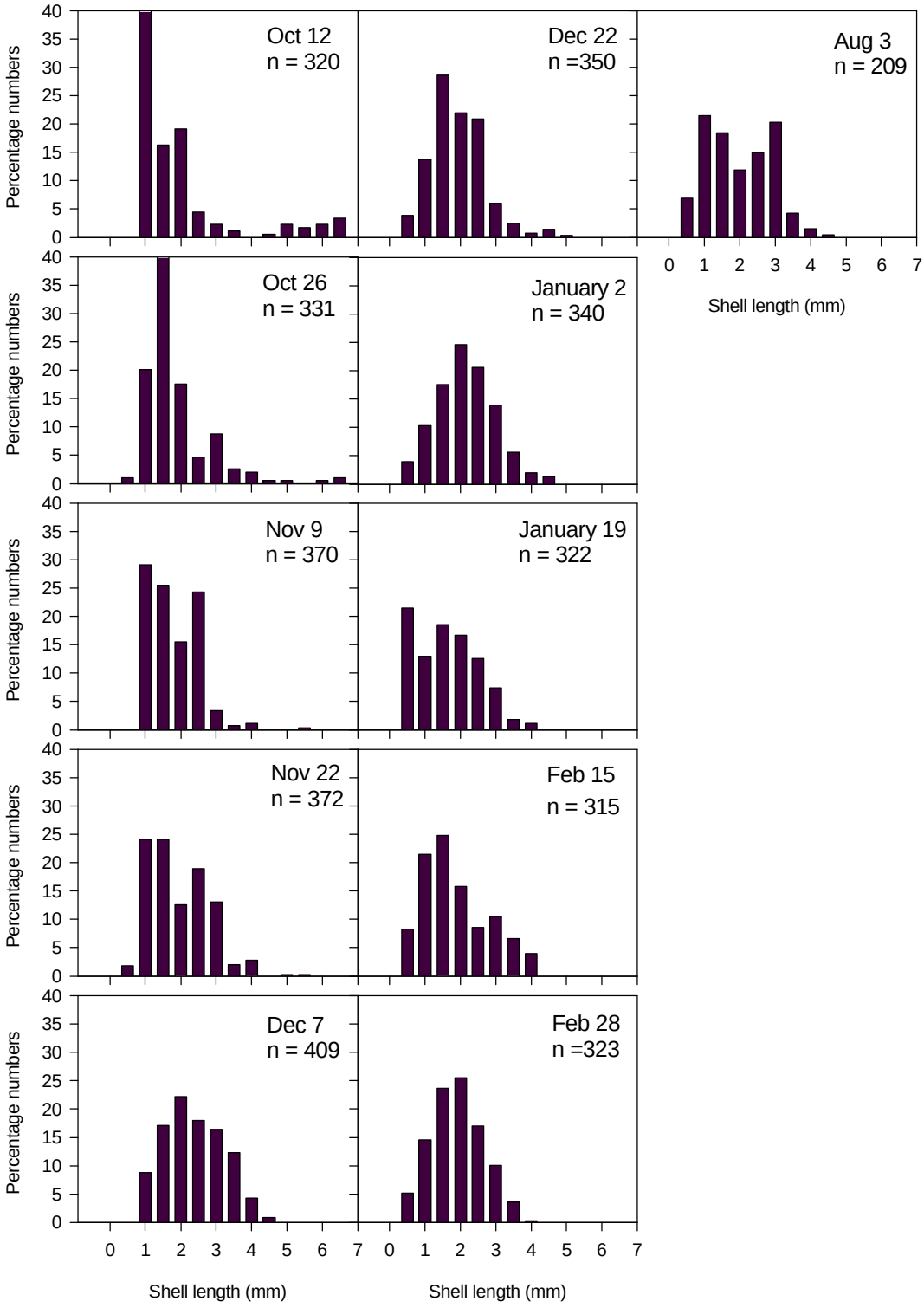


Figure 4.5 continued.

length throughout the year, at both sites, with the exception of the pre-spring recruitment period when limpets were distributed across all size categories.

4.3.5. Growth and Cohort Analysis Using ELEFAN

c *Maximum Predicted Lengths*

There was little difference between the observed and predicted maximum lengths, irrespective of the group of data used in the calculations (Table 4.3). The predicted maximum lengths for each group of data were then used to identify the "best" growth curves for the two sites (based on the Rn values; Section 4.2.3(c)), and to predict the growth performance indices (phi prime values; Section 4.2.3(c)).

Table 4.3. Observed and predicted values for L_4 (maximum shell length), using the summer data only, the winter data only, and all data combined, for *B. stenochorias* at the Belmont River (BelR) and Manley Flats (MF) sites.

Period of observation	Observed maximum length	Predicted maximum length	Confidence interval (95% prob.)
BelR Sept to Feb (summer, with recruitment)	7	7.29	6.45 - 8.13
BelR March to Sept (winter, with no recruitment)	6	6.68	6.05 - 7.31
BelR all year	7	7.34	6.66 - 8.03
MF Sept to Feb (summer, with recruitment)	6.95	7.52	6.57 - 8.47
MF March to Sept (winter, with no recruitment)	7	7.19	6.64 - 7.74
MF all year	7	7.61	6.82 - 8.39

c *Optimum Parameter Combinations*

Rn values (Table 4.4) for the Belmont River data showed better fits (0.523 and 0.591) for the seasonalized data compared to the non-seasonalized data sets (0.258 and 0.301) [Section 4.2.3(c)], when using the winter data set, than when the summer or all-year data sets were used. This period also had the smallest maximum length measured and predicted (Table 4.3). Differences between the Rn values for Manley Flats data were not as pronounced. Comparisons of all phi prime values

Table 4.4. Comparison of growth parameters and Rn values (estimating the goodness of fit of the growth parameters) for *Burnupia stenochorias* from the Belmont River and Manley Flats sites. Seasonal and non-

seasonal growth (dependent on the C and WP values) were analysed using data collected from one of three periods of the year; September 1996 to February 1997 (with recruitment), March to September 1996 (without recruitment), and March 1996 to February 1997 (all year). L_4 = maximum* (observed) or asymptotic (predicted) shell length; ê = exponential rate at which L_4 is approached; C = amplitude of growth curve, indicating seasonality; W = winter period; Ö' = phi prime value, the growth performance index.

Data set used	L_4	ê	C	WP	Rn	Ö'
Belmont River	7*	1.8	0.9	0.1	0.252	1.95
September - February	7*	1.1	0	0	0.206	1.77
[with recruitment]	7.29	0.89	0.9	0.2	0.208	1.67
	7.29	1.0	0	0	0.206	1.73
Belmont River	6*	1.2	1.0	0.6	0.523	1.64
March - September	6*	1.1	0	0	0.258	1.60
[w/out recruitment]	7	1.1	1.0	0.5	0.591	1.73
	7	0.71	0	0	0.301	1.54
Belmont River	7*	1.7	1.0	0.4	0.355	1.92
All year	7*	0.87	0	0	0.203	1.63
	7.34	1.3	1.0	0.42	0.351	1.85
	7.34	1.3	0	0	0.143	1.85
Manley Flats	7*	1.4	0.3	0.9	0.275	1.84
September - February	7*	1.5	0	0	0.219	1.87
[with recruitment]	7.52	1.2	0.45	1.0	0.275	1.83
	7.52	1.0	0	0	0.260	1.75
Manley Flats	7*	1.9	0.22	0.41	0.355	1.97
March - September	7*	1.7	0	0	0.337	1.92
[w/out recruitment]	7.19	1.8	0.3	0.3	0.355	1.97
	7.19	1.5	0	0	0.350	1.89
Manley Flats	7*	1.3	0.7	1.0	0.263	1.8
All year	7*	1.9	0	0	0.242	1.97
	7.85	1.0	0.84	0.97	0.293	1.79
	7.85	1.0	0	0	0.208	1.79

Table 4.5. Results of analysis of variance (Statgraphics 5.0) on the Ö' values for *Burnupia stenochorias* from Belmont River and Manley Flats. Data from both sites (from Table 4.4) have been

amalgamated and analysed for differences in Ö' values. Data were grouped according to either recruitment (with, without, or all year recruitment; n = 6); or for all values of L_4 (both predicted and observed*, n = 7); or for seasonality (when non-seasonal, C and W = 0). The Ö' values were not significantly different from each other indicating no significant differences in growth between the two populations, irrespective of the data set used.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	Probability (significant at 5%)
Data set used	0.12	5	0.02	3.08	0.055
Seasonality	0.01	1	0.02	2.31	0.157
L_4	0.05	6	0.01	0.98	0.481
Residual	0.08	11	0.01		

showed only small differences (Table 4.5). The Belmont River population 'C' values (Section 4.2.3.(c); the amplitude of the growth curve) suggest growth rate was zero or nearly zero at the winter point; a pronounced seasonal variability in growth.

C Growth Curves and Monthly Predicted Lengths

The data in Table 4.4 can be used to draw growth curves for each set of von Bertalanffy Growth parameters as exemplified by those in Figure 4.6, from which monthly growth rates can be predicted (Table 4.6). There is little difference between curves when either the observed or the predicted L_4 values are used. Personal observations have shown hatching to occur when shell length is between 0.65 and 0.78 mm which correlates well with the pre-hatch sizes predicted by the analysis of the length frequency data (Table 4.6). Small differences in growth rates were seen between the two sites:

- During May (early winter) measured (early morning) temperatures dropped from 14EC to the winter low of 10EC. The Manley Flats limpets increased from 0.49 mm to 0.56 mm per month; Belmont River limpet growth rates decreased from 0.3 mm to 0.06 mm shell length per month (for limpets reaching sexual maturity).
- Winter growth at Manley Flats (0.31-0.59 mm per month for maturing limpets; and 0.19-0.22 mm for older limpets) was greater than at Belmont River (0.01-0.34 mm and 0-0.06 mm respectively).
- Mid summer growth was lower for Manley Flats (0.11-0.24 mm for maturing limpets) than

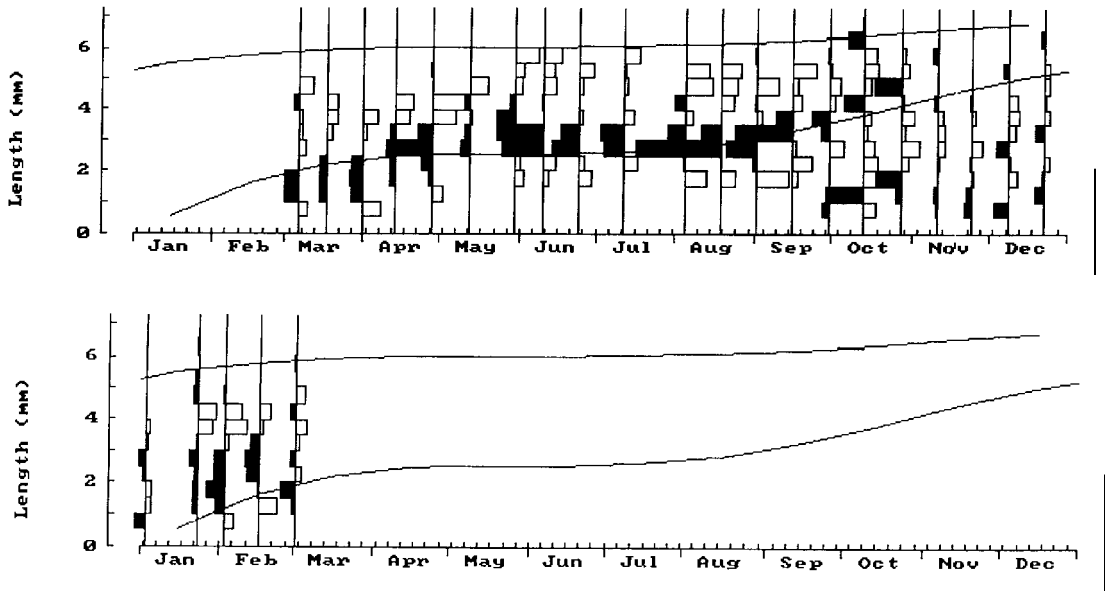
for Belmont River (0.42-0.65 mm shell length).

It is predicted that, based on a January hatching, Belmont River limpets reached sexual maturity in 8 months (September). Manley Flats limpets, hatching in September, reached sexual maturity in 9 months (May). Longevity is estimated to be approximately 24 months. Two generations and possibly a third occur each year. The increase in shell length of *B. stenochorias* after 30 days is compared to that of other freshwater limpets in Table 4.7. The percentage changes in shell length for *Burnupia stenochorias* (added to Table 4.7) are estimated from the Von Bertalanffy Growth models for the Manley Flats and Belmont River sites and possibly reflect the more advantageous conditions for initial growth in the early summer period (Manley Flats) than mid to late summer (Belmont River).

Table 4.6. Predicted shell lengths (mm) of *Burnupia stenochorias* at the 15th of each month, using the seasonalized optimal growth parameters (as given in Figure 4.7) and shell length data from all year. The vertical sequence of lengths is followed in accordance with the month, beginning with the pre-hatched size given in bold font.

Month	BelR site		MF site		
	year 1	year 2	year 1	year 2	year 3
January	**0.53**	5.76		2.12	5.74
February	1.62	6.02		2.30	5.89
March	2.22	6.13		2.59	6.10
April	2.52	6.18		3.08	6.35
May	2.58	6.20		3.64	6.59
June	2.59	6.20		4.23	6.73
July	2.71	6.22		4.73	6.89
August	3.05	6.31	**0.47**	5.14	7
September	3.62	6.50	1.23	5.42	
October	4.29	6.71	1.68	5.58	
November	4.94	6.89	1.92	5.67	
December	5.36	7	2.03	5.71	

a)



b)

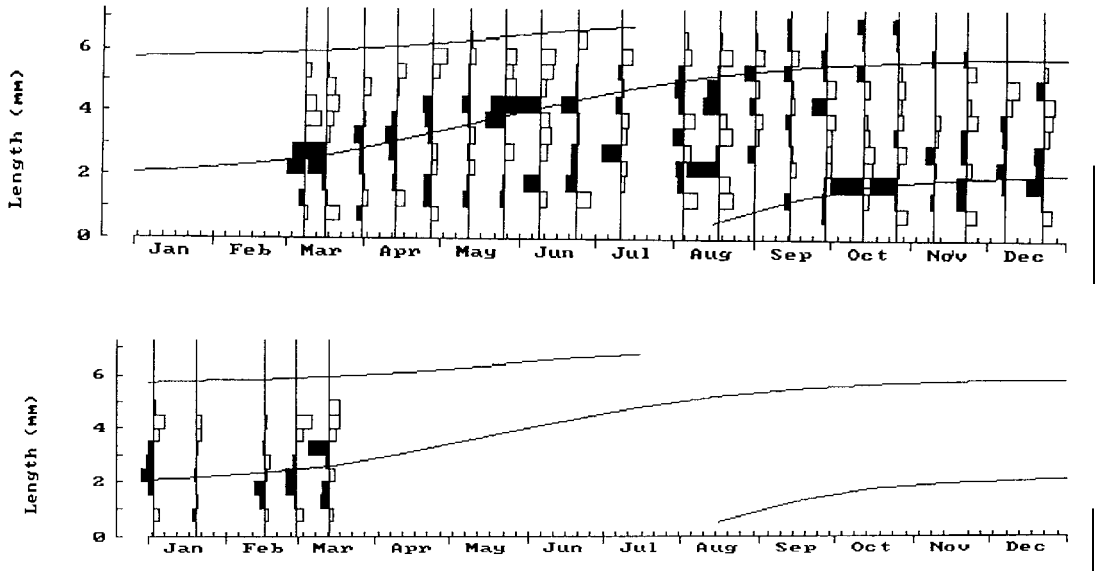


Figure 4.6. Growth curves for predicted cohorts of *Burnupia stenochorias*, based on the length frequency data obtained from the Belmont River and Manley Flats sites over 53 weeks. The observed L_4 values are used in the von Bertalanffy Growth Equation. Seasonality is included (represented by the values C and WP). a) Belmont River population growth curve. $L_4 = 7$, ê = 1.7, C = 1, WP = 0.4, Rn = 0.335, Ö' = 1.92. Recruitment time is given as mid January. A three to four month winter dormancy is predicted. b) Manley Flats population growth curve. $L_4 = 7$, ê = 1.3, C = 0.7, WP = 1, Rn = 0.293, Ö ' = 1.8. Recruitment time is given as mid September. Growth in winter and mid summer were predicted as slow.

Growth Model

The differences between the observed L_4 values and the L_4 predicted by the length frequency analysis for the Belmont River and Manley Flats populations can be seen in Figure 4.7. Initially growth was calculated to be linear, but ceased during a winter period (with three of the models predicting a decrease in size). The pattern was repeated through a second and possibly a third growing period until maximum size was reached after approximately 700 days.

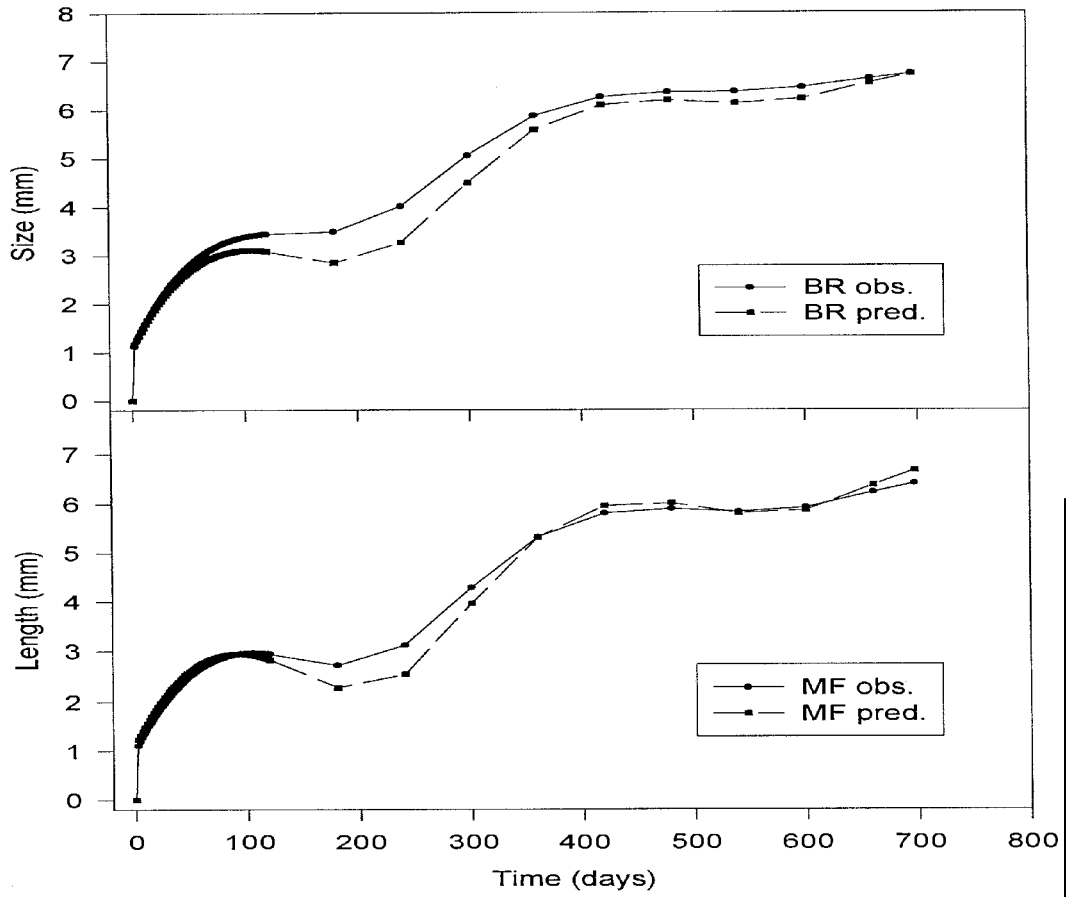


Figure 4.7. Growth models predicted from hatching to maximum shell length for the two populations of *Burnupia stenochorias* studied, based on the Von Bertalanffy Growth Function. The initial 120 days appear bold due to the incorporation of the many points used by the programme on the graph. BR = Belmont River population; MF = Manley Flats population. Obs. = model based on L_4 equal to the maximum observed shell length; Pred.= model based on L_4 equal to the asymptotic shell length.

Table 4.7. Percentage changes in shell aperture lengths of different populations of freshwater limpets after 30 days, demonstrating the large variability of growth rates within species (McMahon, 1976).

Limpet	Percentage change in size
Hebetancylus sp	48; 58; 69; 116; winter growths of 9 and 15.
Laevapex sp	64; 77; 73; 129; 138; 169.
Ferrissia rivularis	66; 74; 87; 96; 174.
Ancylus fluviatilis	35; 105; 114; 147.
Burnupia stenochorias	120 (Manley Flats); 112 (Belmont River).

4.4. DISCUSSION

With the circumspection around the correct identification of *Burnupia stenochorias* to species level, the question arose as to whether the limpets from the two populations studied were all one species and not species occurring sympatrically (as occasionally occurs within the freshwater Mollusca *e.g.* the *Elimia* species; Huryn *et al.*, 1994). It was assumed all individuals were *B. stenochorias*.

Distribution

The contagious distribution of *B. stenochorias* amongst the rocks is typical of freshwater molluscan populations, where aggregation seems to be the rule rather than the exception (Elton, 1966). Although negative correlations between depth and density have been recorded for *Ancylus fluviatilis* (Geldiay, 1956; Macan, 1970), distribution of *B. stenochorias* at the two sites did not differ from the deepest (50 cm) to the most shallow edges (1 cm water flow) [personal observations of *B. stenochorias* in non-flowing pools (Botha River; Section 2.1) found the limpets to be absent deeper than 75 cm]. Distribution of *B. stenochorias* across the width of the river bed was governed to a degree by the obvious aversion to sediment, corroborating Verdcourt's (1949) suggestion that it is the absence of sediment, rather than the presence of flowing conditions, which determines the distribution of the Ancylidae. The vertical distribution of *B. stenochorias* may also be influenced by the reduced density of microalgae at increased depths (Calow, 1974). The feeding habits and requirements of *B. stenochorias* need further investigation to corroborate this notion.

Sampling

One of the main concerns of sampling theory and practice is whether or not the sample represents the population, with reasons for concern being the heterogeneity of habitats and the distribution of organisms within habitats (Pielou, 1974). Sampling in stony streams was reviewed by Macan (1958), Albrecht (1959), Longhurst (1959), Cummins (1962) and Kajak (1963); and it is clear from these reviews that any sampling method will cause a disturbance of the system, which in itself will affect measurements taken. As a rule, the more rigorous the measurements, the greater the disturbance and the less likely the results will apply to a natural system (O'Keeffe, 1982).

Sample size and class interval affect the analyses of length-frequency methods (Mytilineou and Sarda, 1995). MacDonald and Pitcher (1979) suggested that sample size should consist of at least 50 specimens for each age group. A total sample size of over 1500, collected over a period of four months, is said to be a "very good" sample for the application of ELEFAN I (Pauly, 1984). Approximately 4000 *B. stenochorias* were measured for shell length. Based on a method by Wolff (1989) for estimating class interval, 0.5 mm was considered sufficiently small for the analysis of *B. stenochorias* cohorts.

There is substantial variation in choice of time interval for measuring freshwater molluscs for growth determinations. Aldridge (1982) measured *Leptoxis carinata* monthly (bimonthly in winter); fortnightly to monthly intervals were used by Streit (1985) with *Ancylus fluviatilis*; and weekly sampling was used by Calow (1973) with *Planorbis contortus*, and by Eisenberg (1966) with *Lymnaea elodes*. The choices of sample size and class interval for *B. stenochorias* were considered adequate.

Recruitment

With continual (although sporadic) egg laying through the winter months at both the Manley Flats and Belmont River sites, the range of minimum winter temperatures (10-13EC) did not appear to be below a minimum egg laying threshold. However, an increase in post-winter egg laying occurred at both sites, possibly related to a rise in temperatures (14-16EC). The Ancylidae have adapted to a variety of temperatures influencing spring egg lay. Temperatures recorded for *A. fluviatilis* include

7EC (Bondesen, 1950), 8.5EC (McMahon, 1976), and 11EC (Geldiay, 1956); 10EC for *Ferrissia rivularis* (Nickerson, 1972); and for the more tropical populations of *Heteronychus excentricus* and *Lymnaea fuscus*, egg laying occurs when water temperatures increase from winter lows of 7-12EC to 14-18EC (McMahon, 1976). Post-winter egg laying may also be related to an increase in photoperiod, although it is generally agreed that the effects of photoperiod on growth and reproduction are confined to the most temperate climatic regions (Geraerts and Joosse, 1984).

Although Ancylidae lay their eggs on the rock on which they are feeding (Calow, 1974), the first increase in *B. stenochorias* hatchlings from first spring egg lay was only observed approximately 10 weeks later. Personal observations in the laboratory have shown eggs to take no longer than 18 days to develop to hatching. There are possibly two reasons for this lag:

i) High mortalities after hatching are common in freshwater snails (Calow, 1978). Environmental conditions may initially not have been suitable for survival of *B. stenochorias*.

ii) O'Keeffe (1985) suggests, without direct evidence, that hatchlings of *Bulinus* species undergo a free-floating dispersal phase so that this phase is not recorded during field sampling. Although not recorded for other Ancylidae (Dillon, 2000), this remains a possibility for *B. stenochorias* which needs investigation.

A second, midsummer egg laying period, was present only at the Belmont River site. Poor water quality, particularly low oxygen levels, is known to curtail egg deposition (Timmermans, 1959; ter Maart *et al*., 1983; Boyle and Yoshino, 2000). Conditions during summer at Manley Flats included a no-flow period when cattle excrement was present. Thus, it is suggested that two peaks for egg production are the norm, the first at the end of winter/spring and the second four to five months later during summer.

Estimations of Growth

The lack of direct aging techniques (Vermeij, 1980) such as growth rings (Chapter 3), and problems encountered in mark and recapture or cages kept in flowing conditions, makes the estimation of growth and longevity of *B. stenochorias* under natural conditions difficult. It was assumed in the case of *B. stenochorias* that the length frequency field data collected were in accordance with the

assumptions necessary for the successful use of the ELEFAN programme (Pauly and David, 1981). Two assumptions give rise to problems:

C All individuals in the set of samples had the same length at the same age (Stiven and Walton, 1967), and that differences in length could therefore be attributed to differences in age.

For animals whose generations overlap significantly, the grouping of sampled individuals into age classes does facilitate population studies (Southwood, 1978). However, this does not take into account differences that occur between individuals, such as with age or season. Those *B. stenochorias* laid during the latter half of summer will have a shorter optimal growth rate period than those laid earlier in summer. Although the graphs produced by the Von Bertalanffy Growth Equation (Figures 4.6 and 4.7) are extrapolated to give predicted monthly lengths, they do not indicate the expected lengths for those limpets laid either in earlier or later summer (the Belmont River and the Manley Flats populations respectively).

C The Von Bertalanffy Growth Equation describes the average growth of the investigated population.

Estimates of growth rate are based on the assumption that limpets of a given size grow at the same rate under the same conditions in order to achieve identical asymptotic sizes (Richardson and Martin, 1994; Stiven and Walton, 1967). Sainsbury's paper (1980) on the "Effect of Individual Variability on the Von Bertalanffy Equation" made some profound mathematical judgements as to the use of the equation. Particular emphasis was placed on the possibility of individual growth, as described by the Von Bertalanffy Growth Equation differing from that of the population as a whole. This concept was first mooted by a number of authors (*e.g.* Chapman, 1961; Frank, 1965) who intimated problems occurring in the estimation of the population's growth parameters where there was individual variation within one cohort. This would, over time, cause a change in the age composition of that cohort, and consequently, results of cohort analysis completed in this way are sometimes considered unrealistic (Sainsbury, 1980; Vermeij, 1980; Hilborn and Walters, 1992; Huryn *et al.*, 1994). The overlapping cohorts, as is found with *B.stenochorias* with its continual summer recruitment, obscured temporal patterns of growth and development.

Individual variability is further supported by evidence from numerous observations of freshwater pulmonates both in the field and in the laboratory, which show that growth rates become lower at

the onset of female sexual maturation and at the time of increased egg production (Russel-Hunter, 1978). The problem is compounded by the non-availability of direct aging techniques, and the decreasing growth rate with age, combined with mortality. Overlaps are produced in the size of individuals in the older age groups, and consequently the detection of well-defined age groups is very difficult (Mytilineou and Sarda, 1995).

Roff (1980, 1986) and Charnov (1989) have suggested that where there is indeterminate growth, a model based on resource allocation is preferable to one based on the Von Bertalanffy Growth Equation. This is primarily because growth rate in the original Von Bertalanffy Growth Equation was formulated to result from the difference between catabolism and anabolism, with no account being taken of a change in energy allocation at maturity. Day and Taylor (1997) mathematically compared the Von Bertalanffy Growth Equation to an example of an alternative life history model which specifies growth rate as a power function (PF) of mass (*e.g.* Roff, 1986; Kozlowski, 1992, 1996):

PF: $dw/dt = kw$ where 'k' is a habitat quality parameter.

They suggest an exponential decline in resource allocation to growth after maturity should be incorporated into the power function model, assuming the organism can continue to acquire resources at a rate proportional to a power function of its size. Their results showed predictions of L_4 and size at maturity to be substantially altered. However, in the case of *B. stenochorias*, predictions of L_4 remain very close to the observed maximum shell lengths using the Von Bertalanffy Growth Equation (Figure 4.7).

Seasonalized Growth

Although the growth results of *B. stenochorias* from the ELEFAN programme should be viewed with some caution, the predicted Von Bertalanffy Growth curves, with the mathematical adaptation made for seasons, correlate well with Kozlowski's (1992; 1996) explanation of an animal displaying indeterminate seasonal growth. Von Bertalanffy Growth models predicted for *B. stenochorias* do not display asymptotic growth but a highly seasonal double sigmoid growth curve. It is suggested that growth almost ceases for *B. stenochorias* during winter periods at the Belmont River site, continuing (although at a slower rate) in the second optimal growth period (summer months) after maturity has been attained (Chapter 3) to reach maximum length. This closely resembles the growth

curve of *Ancylus fluviatilis* (Durrant, 1975) and appears to be typical of temperate freshwater pulmonates (Russel-Hunter, 1961).

A slow or null growth in length for the winter months has been reported for many freshwater snails (Russel-Hunter, 1961a; Calow, 1973; Vincent *et al.*, 1981), resulting primarily from low temperatures which also affect food quality and availability. However, Figure 4.8, based on length calculations, reflects an unrealistic predicted condition of a decrease in shell length during the winter no-growth. There have been negative growth rates for the marine limpets *Patella oculus* and *P. longicosta* because the shell was worn away by wave action (Branch, 1974). In the case of *B. stenochorias*, the negative growth rates may directly result from the original data collection, where death of larger limpets gave a significant overall decrease in average shell length. Pitcher and MacDonald (1973) maintain this unrealistic shrinkage during winter is the main disadvantage of the sine wave Von Bertalanffy Growth Equation model.

An alternative explanation to the decrease in size over winter is that the weight of *B. stenochorias* is dependent on shell length to a degree (Figures 3.4 and 3.5). True biomass growth, physiologically in individuals or ecologically in populations, can be defined as an increase in unit mass of structural proteins. Similarly, any decrease in mass of structural proteins is "degrowth" and can be reflected in terms of weight loss, or degradation of tissues or organs, particularly of gonads or secondary sexual structures (Russel-Hunter and Eversole, 1976). This degrowth has been shown to occur in several species of freshwater molluscs (Russel-Hunter and Eversole, 1976; Calow, 1977).

Population Differences

When considering the differences in growth rates seen between the Manley Flats and Belmont River populations of *B. stenochorias*, a significant difference was found in flow rates between the two sites. In the light of this, with the absence of continual temperature monitoring, it seems possible that the daily temperature changes were also significantly different. The pond effect at Manley Flats with potentially warmer conditions would have favoured growth in winter compared to Belmont River, but been detrimental in mid summer when cattle activity affected water quality. Consequently, a difference in growth rates between the two populations would be expected. Depending on the

"choice" of recruitment time made by ELEFAN, differences in environmental conditions and therefore potential growth rate will be reflected in the growth curves drawn. *B. stenochorias* hatchlings laid during the latter half of summer will have a shorter optimal growth rate period than those laid earlier in summer.

The predicted growth models do appear to have incorporated the environmental conditions experienced by each of the *B. stenochorias* populations. However, when testing the predicted growth rates using the phi prime (Ö') test (Section 4.3.2), no significant differences were found between the populations, even when data collected from different months of the year were used to predict the growth parameters of one cohort (Table 4.5). Phi prime test values have been found to be similar within related taxa, having narrow normal distributions with minimum variance (Pauly, 1979; Tomalin, 1995). Non-inclusion of the smallest size class, as occurred with the *B. stenochorias* data collected, does not appear to affect the estimate of the phi prime value (Defeo *et al.*, 1992). However, the use of the phi prime statistic in length frequency analysis should be used cautiously, as the statistic estimates mean population growth rates (averaged over all survivors) and not the mean individual growth rates (of all limpets emerging) (Ricker, 1975). If there is differential mortality between individuals displaying different growth rates, the estimates of L_4 may be biased and therefore so will those of ê (Lee's phenomenon, Ricker, 1975); because the phi prime values are a function of the L_4 and ê parameters, they will also be affected.

Life Cycle

Although the life history parameters of *B. stenochorias* which have been investigated or calculated in this study are not exhaustive, it does seem appropriate that, based on the observations made, speculation can be made about its life cycle pattern. Gastropods vary in the time to reach sexual maturity and the number of generations produced per year (McKillop, 1985; Jokinen, 1985; Dillon, 2000).Thirty two percent of pulmonates display a generation time of less than a year, most have one or two generations per year, and rarely one generation every two years is seen (Brown *et al.*, 1985; Dillon, 2000).

The life cycle strategies given by Russel-Hunter (1978) and Calow (1978) (Figure 4.8) are based on

two types of reproductive strategies, the *iteroparous*[3] and the *semelparous*[4] conditions. The freshwater pulmonates demonstrate semelparity (Calow, 1978; Dillon, 2000) which is considered an evolutionary trend from the iteroparous state of the marine prosobranchs, and is also found in the primitive saltmarsh pulmonate *Melampus bidentatus* (Russel-Hunter *et al.*, 1972). Calow (1978) gives a review of lifecycle stategies in various ancylids, where *Ancylus fluviatilis* is thought to show a type 'A' pattern (Bondesen, 1950; Geldiay, 1956; Calow, 1972); *A. lacustris* type 'A' (Russel-Hunter, 1953); *Ferrissia rivularis* types 'A' and 'C' (Burky, 1971), and also type 'B' (Nickerson, 1972); *Hebetancylus excenticus* type 'C' (McMahon, 1976); and *Laevapex fuscus* types 'A', 'D' and 'E' (McMahon, 1976). The pattern of lifecycle demonstrated by *B.stenochorias* fits types 'B' and 'D', but personal observations both in the field and in the laboratory suggest that *B.stenochorias* is not usually semelparous, but always demonstrates iteroparity.

The iteroparity of *B.stenochorias* is a possible indication that in its evolutionary history it can be considered closer to the more primitive state than other ancylids. An alternative thought is that the iteroparous state rather reflects a life history change when moving from the poles to the tropics. A move is made towards a 'k' life strategy selection (slower development with greater competitive ability) which is supposed to become more intense and where iteroparity becomes more frequent when compared to the "r" life strategy (rapid rate of development and increase in numbers) (Pianka, 1970; Krebs, 1978). Tropical pulmonate studies are few and mostly confined to the bilharzia-carrying snails (*e.g.* Shiff, 1964; O'Keeffe, 1982, 1985) but they do point to opportunism and recklessness, as the iteroparous state is considered to demonstrate (Calow, 1978; Heller, 1990).

[3] Iteroparous (iteroparity): parents live on after reproduction to reproduce again.

[4] Semelparous (semelparity): parents die after reproduction. (Calow, 1978).

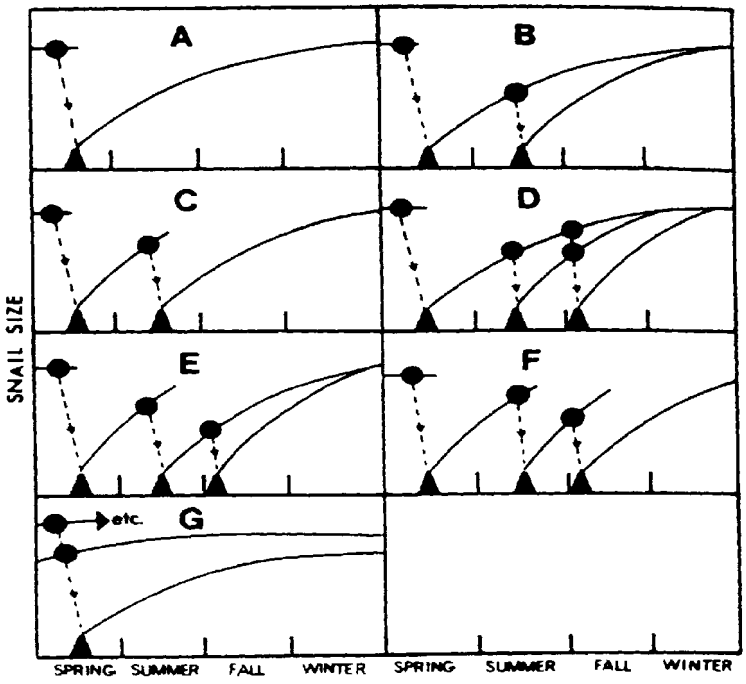


Figure 4.8. Patterns of life cycle found in freshwater gastropods. Ž reproduction begins; • egg capsules appear. Lifecycle 'G' is the true semelparous condition; 'B' and 'D' are 'quasi-iteroparous' in that they occur in special circumstances in species which are usually semelparous.
(Russel-Hunter, 1978, reproduced with permission from Calow, 1978).

4.5. CONCLUSIONS

Reproduction occurred throughout the year at the two sites investigated. The Bloukrans River is a permanent habitat, with water available all year, and because all year round breeding occurred, it is suggested that the winter period for *Burnupia stenochorias* is not as stressful as in temporary habitats which dry out or have very cold winter temperatures (Appleton, 1978). However, a substantial increase in egg production at the end of winter for *B. stenochorias* (Figures 4.2 and 4.3) appeared to be a function of either increase in temperature or photoperiod. Eggs were laid on the undersurface of rocks, possibly because the eggs and young are affected by sedimentation, as with other Ancylidae (Durrant, 1976). The spring egg production continued through the summer with a second midsummer peak visible in the Belmont River population.

Growth and longevity are difficult to determine with the lack of external or internal growth rings, and the inability to differentiate cohorts through length frequency changes over time. However, the ELEFAN I programme, based on the Von Bertalanffy Growth Equation, appeared to produce acceptable growth curve models for the two *B. stenochorias* populations, despite the question against its suitability for use with freshwater limpet length frequency data. It successfully linked the predicted growth of the two limpet populations to the environmental conditions prevalent at the time.

The phi prime statistic, provided by the ELEFAN programme, should be used cautiously to compare growth rates, as the statistic estimates mean population growth rates (averaged over all survivors) and not the mean individual growth rates (of all limpets emerging). However, if used to compare Manley Flats and Belmont River populations, comparison of phi prime values suggests there is no significant difference in growth rates. It is therefore suggested that the two growth curves (Manley Flats and Belmont River) represent two arbitrarily selected cohorts of a representative population of *B. stenochorias*. Growth under natural conditions is predicted to be in the form of a highly seasonal, double sigmoid curve. A rapid initial growth is demonstrated, decreasing with age. The time taken to reach maximum size (represented by shell length) is also the age at death. Observed maximum size at both populations was 7 mm shell length. Time to sexual maturity is estimated to take 8-9 months, and longevity to be approximately 24 months. Two generations occur each year, with slow or nil winter growth.

Considerable intraspecific interpopulation variation in life cycle pattern is known to occur in the pulmonates (Calow, 1978; Dillon, 2000). Further studies of other populations of *B. stenochorias* will clarify the general life cycle strategy it adopts as a species.

CHAPTER 5. SEXUAL DEVELOPMENT, RELATED TO SHELL LENGTH

Key issues: Does the production of sperm and ova within each individual vary through the year, and can shell length be consistently used to choose pre-sexually developed individuals? (Figure 1.2)

5.1. INTRODUCTION

Pulmonates are adapted to various habitats, and have developed numerous strategies with regard to sexual development and life cycles in order to deal with the challenges of local conditions (Dillon, 2000). All pulmonates are hermaphrodites (Geraerts and Joosse, 1984), and an ability to reproduce by self-fertilisation may be advantageous in freshwater, particularly where individuals may become isolated or are in low densities (Jarne *et al*., 1993). Pulmonates have a single gonad, an ovotestis, in which simultaneous production of eggs and sperm is usual (Heller, 1993). Protandry (the development of sperm before ova) does occur in the primitive pulmonates of the Ellobiidae, but also in some of the higher pulmonates, such as in *Physa fontinalis* (Duncan, 1959), *Lymnaea stagnalis* (Duncan, 1975), *Siphonaria* species (Branch, 1981), *Bulinus globosus* (Rudolph, 1983) and *Biomphalaria glabrata* (Trigwell and Dussart, 1998). *Laevapex fuscus* is a highly evolved, temperately distributed, ancylid freshwater limpet, entirely aquatic in its development as observed through the development of its respiratory system (Russel-Hunter and McMahon, 1976); it too shows protandry, indicating that protandry is not necessarily a condition found only in the more primitive species. The possibility of protandry occurring in *B. stenochorias* is therefore feasible. Female fertility preceding male fertility may also occur (Wethington and Dillon, 1993).

Control of the reproductive cycle in pulmonates is both internal (in the form of endocrine control) and external (in the form of short and long term stimuli) (Geraert and Joosse, 1984). These internal and external forms of control are directly linked, but are also interdependent with the growth of the limpet (Geraert and Joosse, 1984). Short term stimuli usually revolve around water quality, often with water hardness, but primarily with levels of dissolved oxygen (van der Steen, 1967). Significant increases in ovulation and oviposition have been seen in a number of pulmonates with a change from dirty to clean or well oxygenated waters (ter Maat *et al.*, 1983; Boyle and Yoshino, 2000). Long term stimuli include

temperature, food, mating, crowding, photoperiod (although this refers to those pulmonates in the temperate regions) and lunar cues (for marine pulmonates) (Geraert and Joosse, 1984).

Temperature is an important factor determining growth, and therefore time of onset of the breeding season (Duncan, 1975; Russel-Hunter, 1978; Branch, 1981; Dillon, 2000). Charnov's theory (1991) predicts that survivorship (including growth and the site specific factors which influence growth) determines size at reproductive maturity; and size at maturity then determines reproductive effort. The maturation rate of the reproductive tract (Thomas and McClintock, 1990), the ratio of development, and consequently the initiation of oviposition and the intensity of breeding, are all directly influenced by the ambient temperature (Appleton, 1978). Critical lower temperatures for initiation and subsequent control of spermatogenesis (Joosse, 1964) and oviposition have been reported for various basommatophorans (*e.g.* Duncan, 1959). McMahon (1975a) suggests this critical temperature is 10-12EC in temperate regions. Tropical *Biomphalaria* in Brazil have a critical temperature of 15EC (Duncan, 1975), although Geraert and Joosse (1984) suggest this critical temperature may be when ripe oocytes are present but have not ovulated.

In considering the choice of limpet size or development for use in toxicity tests, pre-sexually developed individuals are usually considered to be more susceptible to toxicants (Calow, 1989). Similarly, if protandry exists in some form, male fecundity may also be related to small size (Russel-Hunter and McMahon, 1976). Differential sexual development, related to season or age and size, may also affect a hermaphrodite's susceptibility to toxicants (Gomot, 1998). Consequently, it was necessary for the relative presence and stage of development of sperm and ova within the ovotestis to be identified, and related to shell length, before *B. stenochorias* could be used in toxicity tests. Although the organization of the pulmonate genital system is well documented (Fretter and Peake, 1975), investigations relating to ancylid life cycles and gonad development in particular have been few in the Northern hemisphere, and non-existant in the Southern, or in Africa. Oberholzer and van Eeden (1969) gave details of the ovotestis of *B. mooiensis*, but with no indication as to whether seasonal development occurred. Similarly, only the sperm structure of *B. stenochorias* has been described by Hodgson and Healy (1998), with no further studies of presence or absence of sperm in individuals throughout the year.

In trying to identify the stages of development of *B. stenochorias*, the problem arose that age and size

at reproduction are a function of prevailing environmental conditions, particularly temperature and quantity and quality of food (Dillon, 2000). This leads to inter-population variability in size at first reproduction in particular (Vianey-Liaud, 1996). Consequently, collections of *B. stenochorias* were made from two sites; with stage of sexual development related to shell length. Pre-sexually developed *B. stenochorias* could therefore be selected for toxicity tests.

5.2. MATERIALS AND METHODS

Specimens of *Burnupia stenochorias* were collected at fortnightly intervals over a period of 52 weeks from the Belmont River and Manley Flats sites (Section 2.1; Chapter 4). At the same time, temperatures, pH and Total Dissolved Salts of the water at each site were measured using hand held probes. A complete range of sizes were collected with each sample, from 2 mm shell length to the maximum size present (usually from 5 to 6 mm shell length). All limpets were measured for shell length using an ocular graticule calibrated against a stage micrometer (Section 3.3), and preserved in Bouins Fluid for later analysis of each ovotestis (Hodgson and Bernard, 1992). A cross section of 84 limpets were sampled from this collection, drained of the Bouins Fluid, and processed for light microscopy by embedding in paraplast. Transverse sections were cut 10 µm thick, and stained in haematoxylin and eosin (Hodgson and Bernard, 1992). Sections were mounted on glass slides with Haupt's gelatine and sealed with DPX mounting medium.

Assessment of the development of the ovotestis relative to early and late spermatogenesis and developing and mature ova was made subjectively according to a scoring index, and compared between population source, shell length, and season collected.

5.3. RESULTS

Temperatures, pH and Total Dissolved salts (Figures 4.2, Belmont River population; Figure 4.3, Manley Flats population) were not significantly different between sites (Section 4.3.2). Observations suggested the densities of *Burnupia stenochorias* on the sampled rocks varied between sites, with those at Manley Flats approximately five times greater in number per rock than the Belmont River site.

The transverse histological sections confirmed *B. stenochorias* to be a simultaneous hermaphrodite. Although accurate counts of the number of acini in the sections could not be made with any surety

because of their vast numbers, the description given by Oberholzer and van Eeden (1969) for *B. mooiensis* accurately describes the gonad morphology and histology of *B. stenochorias*. The ovotestis is situated in the posterior quadrant of the body, is spherical in shape and consists of a number of elongated follicles or acini. Proceeding from the base of the acinus towards the apex, the germinal epithelium shows various stages in the production of male and female elements, such as oogonia, nurse cells, sperm cells and Sertoli cells. Male and female zones in the acinus cannot be differentiated. In the final stages of sperm development, the Sertoli cells appear to no longer be attached to the wall of the acinus, floating unattached in the lumen of the ovotestis. It is the Sertoli cells which liberate the mature sperm as the cells disintegrate (Oberholzer and van Eeden, 1969).

The results of the ovotestis development of a selection of the limpets collected are given in Tables 5.1 and 5.2. A scoring index estimating the proportion of each of the stages of development relative to each other was used. Oocytes and spermatozoa were clearly visible within the transverse sections. A penis was not differentiated in the sections; although absent in some pulmonates (Dillon, 2000), a penis has been recorded in *B. mooiensis* (Oberholzer, 1963).

The temperatures measured at the two sites studied during 1995 and 1996 (Figures 4.2 and 4.3) indicate a continual drop in temperature from May to a minimum below 10EC through the winter months of June and July, rising again in August. All overwintering limpets (as depicted by those sampled in May to July) which were greater than 3 mm shell length displayed developing ova, with the majority displaying late egg development. Those limpets less than 3 mm shell length did not appear to exhibit early or late spematogenesis or ova development through the whole winter period; mature sperm were only present in those limpets greater than 4.2 mm shell length in June. Although not quantified, many limpets during the winter period had ovotestes occupying approximately 80% of the total area of transverse sections for each limpet; for a number of other limpets, the ovotestis was only 15% of the total area for each limpet.

As temperatures warmed into August and September (Figures 4.2 and 4.3), individuals varied in their development relative to their shell length (Table 5.2). The number of ova did not differ noticeably between individuals from the two sites, with mature ova never absent from limpets greater than 3.15mm shell length. Sperm development, however, differed, with late spermatogenesis absent from smaller limpets (approximately 3.5 mm shell length) (Table.5.2). Large limpets (> 5.5 mm shell length) had few mature sperm, and early spermatogenesis was absent; this period of sampling (mid September)

coincided with peak egg laying (Figures 4.2 and 4.3). Early or late spermatogenesis could not be discerned in limpets below 3 mm shell length during this spring time, although ova very early in development were seen.

As the year progressed through the summer (late 1995; Table 5.2), all stages of gamete development were present in the sections of the majority of limpets, with the ovotestis occupying up to 50% of the body size. However, from January to March (1996) limpets had fewer late spermatogenesis and particularly few mature ova, coinciding with a decrease in egg laying (Figures 4.2 and 4.3). Both oogenesis and spermatogenesis were present in the limpets with shell length < 3 mm collected from both sites, but at a very early developmental stage.

Table 5.1. The presence (indicated by star) or absence (a) of sperm and ova within a selection of transverse sections of *Burnupia stenochorias* collected during early winter and winter, 1996, showing the great diversity in development relative to shell length, season and site of collection. Date = the collection date; MF = Manley Flats site; BR = Belmont River site; E.S.= early spermatogenesis; L.S.= late spermatogenesis; D.O.= developing ova; M.O.= mature ova. The relative abundance of sperm and ova is indicated by *** = dominates the ovotestis; ** = present in large numbers but not dominating; * = present in small numbers.

Season	Month	Length (mm)	E.S.	L.S.	D.O.	M.O.	Site
Early	May	3.0	**	a	a	a	BR
Winter		3.25	**	a	**	a	MF
(1996)		3.45	**	a	**	a	BR
		3.8	**	*	***	**	MF
		3.9	**	a	**	a	BR
		4.6	a	*	***	***	MF
Winter	June	2.7	*	a	a	a	MF
(1996)		3.1	**	a	**	**	MF
		3.4	**	a	**	a	BR
		3.65	**	a	**	a	BR
		4.1	**	**	***	**	MF
		4.25	***	**	**	**	BR
		4.6	**	**	**	**	MF

Table 5.2. The presence (indicated by star) or absence (a) of sperm and ova within a selection of transverse sections of *Burnupia stenochorias* collected during spring and summer 1996 and summer 1997. The large diversity in development relative to shell length, season and site of collection is shown. Date = the collection date; MF = Manley Flats site; BR = Belmont River site; E.S.= early

spermatogenesis; L.S.= late spermatogenesis; D.O.= developing ova; M.O.= mature ova. A comparison is made between the relative abundance of sperm and ova by *** = dominates the ovotestis; ** = present in large numbers but not dominating; * = present in small numbers.

Season	Month	Length (mm)	E.S.	L.S.	D.O.	M.O.	Site
Spring	August	3.15	**	**	**	**	BR
(1996)		3.25	**	a	**	**	BR
		5.55	**	*	**	**	MF
	September	3.6	**	**	***	***	MF
		5.4	**	**	**	**	MF
		6.0	a	*	**	**	MF
Summer	October	3.85	**	**	**	**	BR
(late		4.3	**	**	**	**	BR
1996)		5.0	**	*	**	**	MF
		5.0	**	*	a	a	MF
	December	3.2	**	*	*	*	MF
		3.5	**	**	**	**	MF
		3.8	**	**	*	**	BR
(early	January	2.95	**	a	**	a	MF
1997)		2.95	**	a	**	a	BR
		3.85	**	a	**	a	MF
		4.95	**	**	**	**	BR
	March	2.8	**	a	*	a	MF
		2.85	**	*	**	a	BR
		3.2	*	*	*	a	BR
		3.5	*	*	**	*	MF

5.4. DISCUSSION

Charnov (1991) put forward a model for mammals of life history evolution, the principles of which

Dillon (2000) suggests apply equally well to freshwater molluscs. A key component of this model is that survivorship determines size at first reproduction; the better the chance of survival, the smaller the size at maturity and *vice versa*. This model would usually be applied to species and the lifestyle each evolved over time. Within species, population differences in size at first reproduction also arise, seemingly linked to genetic differences with possibly little correlation between age, and size at first reproduction (Richards and Merrit, 1975; Jarne and Städler, 1995). Ideal conditions early in a season may encourage quicker maturation and therefore the opportunity to produce a second generation when 'normally', this may not occur; a few pulmonate species demonstrate this type of life cycle strategy (Calow, 1978, 1981). O'Keeffe (1982) and Anderson *et al*. (1982) have all demonstrated that onset of reproduction in pulmonates is more closely related to size than to age. Temperature is usually considered the primary cue for reproduction, closely linked to the growth rate (Russel-Hunter, 1978; Wethington and Dillon, 1993).

When random sampling of sexual development of individuals over a number of seasons is made, such as that completed of *Burnupia stenochorias,* it is difficult to relate environmental factors to age and thus sexual development. Interpretation of the percentage makeup of the ovotestis were also subjective. Corroboration of results with repetitions of the study would therefore be difficult. The simplest indicator of reproductive state is the gonad index, where the ratio of the weight of the gonads to the total animal weight is made (Grant and Tyler, 1983). However, the aim of the research was rather to show with certainty that limpets below a certain size were sexually immature, irrespective of shell length or time of year (season). The aim was ultimately to be able to select with confidence pre-sexually mature *B. stenochorias* for acute toxicity tests. Gonad indices do not indicate differential development when the gonads are ovotestes (Grant and Tyler, 1983).

Variables such as differences in season would be very likely to change this minimum size, depending on the quality of conditions (temperature, food and light, for example) and their effects on growth, for example. A study of *Physa acuta* noted that field temperatures affected the sexual maturity of the cohort, with summer maturation more rapid, so that individuals were younger and smaller; winter individuals had delayed maturity with poorer fecundity, although larger individuals produced eggs through the winter phase (Perrin, 1986). In similar studies of *P. acuta,* it was found that the better the environmental conditions, the earlier the maturation, the greater the number of large, sexually mature individuals (Aboul-Ela and Biddiny, 1969; Brackenbury, 1989).

Sexual development of *B. stenochorias* did appear to be seasonal, but with some degree of reproductive activity all year. In winter, all limpets with shell length greater than 4 mm possessed both developing and mature ova, with limited spermatogenesis present. It is presumed that it is these larger limpets which account for the eggs laid through the winter months. With one exception, most of those below 4 mm did not have mature ova. Late spermatogenesis was also limited, usually absent irrespective of size of limpet. Immature sperm were found to dominate the sexual development of limpets of less than 3 mm shell length, with only one exception possessing ova.

As temperatures rose in August and September, most limpets, small and large, showed increased sexual development with the development of mature spermatozoa and ova. This increased oogenesis explains the significant increase in egg laying periodicity from the winter into the summer months found in a previous survey (Figures 4.2 and 4.3). However, the larger limpets ($6 mm shell length) did not demonstrate early spermatogenesis. Despite the potential for increased growth reducing the size of sexually mature limpets, those below 2.8 mm shell length possessed only very immature sperm and ova. Variability in sexual development relative to shell length was greatest in the summer, with no apparent differences between limpets from the two sites. Samples of limpets from late January and March showed a decrease in the number of mature sperm and ova seen in the transverse sections of the limpets. Those below 2.8 mm shell length again possessed very immature sperm and ova.

Temperature changes are a possible explanation for these differences, but other possible causes include photoperiod and food quality and availability. There is substantial evidence, particularly with *Lymnaea elodes* (Brown *et al*., 1985) and with *L. peregra* (Calow, 1981), that reproductive output is strongly related to the quality of food available (Chapter 7). It has been shown that 70% of organic carbon is consumed by pulmonates for reproductive output (Britton and McMahon, 1997). Quality and quantity of food between the Manley Flats and Belmont River sites were not investigated. However, no differences in the development of sperm and ova within the ovotestis of *B. stenochorias* between the sites could be discerned. Although density differences occurred between the sites (Figures 4.4 and 4.5), it is suggested that a greater number of *B. stenochorias* would need to be examined before this could be substantiated as having affected development of sperm and ova, as was found with *L. peregra* (Calow, 1981).

Jarne and Städler (1995) emphasise that once sexually mature, freshwater pulmonates are essentially simultaneous hermaphrodites, and *B. stenochorias* appeared to be no exception. Many molluscs

demonstrate protandry, although this condition does not seem to be the norm (Hoagland, 1978; Brackenbury, 1989; Dillon, 2000). Environmental conditions are responsible for mediating sex change in a number of pulmonates (Hoagland, 1978). Geraerts and Joosse (1984) suggest that most freshwater snails hatching in spring go through a short protandric period before entering simultaneous hermaphroditism, although many small oocytes may already be present. However, this present study suggests that *B. stenochorias* does not appear to exhibit transient protandry.

Occasionally, it was seen in *B. stenochorias* that mature sperm were present in the sections, presumably in the spermatheca, and not in the ovotestis. These sperm may have been donated. Sperm can be stored in some pulmonates for up to a few months (Jarne *et al*., 1993). For any freshwater animal, whether passively dispersed to establish new populations or not, there are obvious advantages if a sustained period of production of fertilised eggs can follow a single copulation. Copulatory patterns and the extent of self-fertilisation differ among the Basommatophora freshwater families (Russel-Hunter and McMahon, 1976); although observations were made only from approximately two hours after first light, copulation was seldom observed in the two populations. Selfing can occur in *B. stenochorias* (pers. obs. within laboratory cultures), while some noteworthy exceptions to outcrossing have been found in *Biomphalaria* and *Bulinus* (Jarne and Städler, 1995). However, outcrossing appears to be the rule in natural populations (Jarne *et al*., 1993). Irrespective of whether a limpet at any one stage of development was selfing or outcrossing, those limpets possessing mature sperm and developing and mature eggs were all larger than 3.15 mm shell length.

It is concluded that *B. stenochorias* individuals below 3 mm shell length do not display sexual maturity, irrespective of the season or population sampled. Below 3 mm shell length was therefore chosen as the choice of size of *B. stenochorias* used in the acute toxicity testing. However, as differences in the presence of developing ova were found between seasons, toxicological differences in susceptibility may occur, depending on the time of year *B. stenochorias* were sampled for toxicity tests.

CHAPTER 6. EFFECTS OF DIET ON THE GROWTH AND REPRODUCTION OF LABORATORY POPULATIONS

Key issues: What is the growth and reproduction of *Burnupia stenochorias* when subjected to different laboratory diets, and maintained under the constant conditions to be used in chronic toxicity testing? (Figure 1.2)

6.1. INTRODUCTION

The effects of a toxicant on the growth and reproduction of an organism usually forms the basis of chronic toxicity testing. Growth and reproductive data are also useful in providing a constant laboratory supply for toxicology studies. Any toxicity test organism must be in a 'fit' state of health before being used (USEPA, 1991). There are many factors which influence freshwater molluscan growth (Section 4.1; Geraerts and Joosse, 1984; Dillon, 2000), and fecundity in terms of time of maximum spawning, mean number of egg capsules per adult, and the necessity for cross-fertilization (Section 6.1; Russel-Hunter, 1961b; Streit, 1975). Abiotic factors such as the quantity (cumulative) and quality (rising and falling) of temperature, day length and water quality (van der Steen, 1967), particularly with regard to calcium and magnesium (Appleton, 1978), all influence the reproduction of freshwater molluscs. Biotic factors include density and mating opportunity (Eversole, 1974), and diet (Aldridge, 1982) with many different mechanisms implicated, including trophic limitations (van der Steen, 1967; Mooij-Vogelaar *et al.*, 1973), and metabolite accumulation in the environment (Hiruta and Kawabata, 1954).

Food is generally accepted as a major component of a population's environment (Eisenberg, 1970; de Kock and van Eeden, 1980). Choice of food is proportional to the size of the animal and its jaw, and its habitat (Dillon, 2000). Substrate preferences have been demonstrated in a number of groups of gastropods, varying from macrophytes, filamentous algae and macro and micro diatoms to protozoans, detritus and sand particles (Sheldon, 1987; Lamberti and Steinman, 1993; Thomas, 1998). Of all the pulmonate families, it is the planorbids which have been studied the most for dietary preferences, because of their medical importance (Liang, 1974; Dillon, 2000). Chemoreception research has shown some plants

are more attractive to planorbids than others (Sterry *et al*., 1983; Thomas, 1987; Madsen, 1992), while other plants repel planorbids, forming the basis for the search for new molluscicides (Brackenbury and Appleton, 1997). One of the major problems encountered in successful maintenance and breeding of freshwater gastropods in the laboratory has been the selection of a suitable quantity and quality of food, satisfying the basic requirements of a well-balanced diet (de Kock and van Eeden, 1980). As with temperature, diet is also considered to be a control parameter with significant interactions with growth rate; and differences in growth rate directly affect duration of life-cycles, size attained at maturity, and survivorship (Anderson and Cummins, 1979; Waters, 1977).

The dietary components and preferences of a pulmonate may therefore play a major role in the success of cultured or studied laboratory populations, particularly as adults and juveniles have been found to consume different resources in some species (Thomas *et al*., 1985). Freshwater snails actively transport across their general body surfaces a proportion of their food requirements, including amino acids and short chain carboxylic acids, sugars, and particulate matter (Gilbertson and Jones, 1972; Zylstra, 1972; Thomas, 1998). Therefore, water quality may also play a role in influencing the dietary requirements of *Burnupia stenochorias*. Although artificial diets have been used in the past to maintain snails in the laboratory (Vieira, 1967), they may not reflect the true nature of foodstuffs in the wild, with consequent inadequacies present (Calow, 1975b). McMahon (1975b) reported a clear correlation between quality of food and eggs per capsule in the Physidae. The higher the protein content of the periphyton, the greater the number of eggs per capsule. Similar results were obtained for the ancylids *Ferrissia rivularis* (Hunter, 1975) and *Ancylus fluviatilis* (Russel-Hunter, 1953; Geldiay, 1956).

The diet of southern African freshwater Ancylidae has not been studied. Generally, freshwater limpets are considered herbivores, scraping the algal/detrital/bacterial complex from the substratum with their radulae (Hunter, 1961; Calow, 1973), with diatoms the major food component (Streit, 1975). However, comparatively little is known of the actual diets of most species, even the most common gastropods (Brönmark,1989) including *B. stenochorias*, or their ability to select from within the spectrum of algal foods available to them (Dillon, 2000). Many herbivorous invertebrates in freshwater habitats are considered to be generalist feeders (Cummins, 1979) and algae consumption is usually dependent upon

the relative availability of foods (Gregory, 1983). However, discrimination between algae has been described according to algal class (Calow, 1970; Imrie *et al.*, 1990), food particle size (Levinton and DeWitt, 1989), taste (Daldorph and Thomas, 1988) and carbon to nitrogen energy values (Eisenberg, 1970; McMahon *et al.*, 1974). Gut content analyses of certain ancylid species indicated they grazed and fed on diatoms and possibly fungi, lichens and bacteria (Hunter, 1953; Geldiay, 1956). Calow (1973) maintains that in the absence of epilithic algae, lichen may be used but it results in less rapid growth and must be considered as a less rich energy source. *A. fluviatilis* actively seeks out algae in the form of diatoms, in preference to, although also consuming, lichen, bacteria, detritus and fungi (Calow, 1973). Sand particles have been observed in the gut of Ancylidae (Geldiay, 1956), and it has been proposed that bacterial colonies, in association with the sand, are intricately involved with the digestive processes (Thomas, 1998). *B. mooiensis* has the same radula structure as *A. fluviatilis* (Oberholzer, 1963) and it was presumed that *B. mooiensis* and also *B. stenochorias* followed the same preference for unicellular diatoms.

Polyethylene sheeting is often used to culture algae and snails as it is non-toxic to both (Cattaneo and Amireault, 1992). Rearing of *B. stenochorias* in the laboratory from hatching to full maturity is successful in 5 litre plastic buckets lined with polyethylene plastic bags (Haigh and Davies-Coleman, 1999). As growth and mortality of *B. stenochorias* are affected when the limpets are handled (Haigh and Davies-Coleman, 1997), the plastic sheeting facilitates handling. Periphyton, particularly diatoms, are cultured on this plastic by seeding the water in containers with scrapings from natural stream rocks. However, provision of food in the form of relatively constant and uniform growth of diatom communities on the plastic sheeting is difficult. Diatoms have a carbon content per unit weight lower than found in green algae (Calow, 1975b), and the consequent lower ratio of C:N levels (interpreted as a measure of relative protein content) is considered indicative of a good food source. At certain times trace element content, vitamins, essential amino acids, and other factors may, however, be more important than overall organic content alone (McMahon *et al.*, 1974). In *Lymnaea elodes*, food additives have been shown to alleviate the inverse relationships between snail density and mean size, mean number of eggs per egg mass, and total number of eggs (Eisenberg, 1970). Survivorship also improved.

An additive to the periphyton grown in the laboratory was tested as a more suitable feeding regime,

acceptable to both *B. stenochorias* hatchlings and adult limpets. Tetramin® for young fish has been successfully used in the feeding of newly hatched *Biomphalaria* species (Jennings *et al.*, 1970). Greater numbers of eggs were produced and quicker growth rate and mass increase with *Biomphalaria pfeifferi*, *Lymnaea natalensis* and a number of *Bulinus* species compared to four other, well used snail foods, including lettuce and Standen's snail food (Mahoney, 1966). Tetramin B® is also the standard food source for the mass cultures of the snail *Helisoma trivolvis* (Lawrence, 1981) and *Hexagenia* mayflies, used in toxicology testing in Canada (Flannagan and Cobb, 1979) and the U.S.A. (Giberson and Rosenberg, 1992) respectively. Subsequent algal growth acts as a supplement. This prompted an investigation into the effect of Nutrafin Fry Food®, of similar constituents to Tetramin® but in a powder form, as a food additive for the culture of *B. stenochorias.*

In this study, the effects of the feeding regimes were addressed in terms of growth (represented by an increase in shell length), reproductive biology (age and size at sexual maturity, reproductive period as a proportion of life span, and egg laying patterns) and fecundity (total number of eggs per limpet, and mean number of eggs/capsule/limpet/treatment). With the provision of both algae and Nutrafin Fry Food®, an improved technique for maintaining *B. stenochorias* cultures in the laboratory was developed. The data was also, later, used as a comparison for the chronic toxicity studies under the same laboratory conditions, where the effects of the textile whole effluent on the growth and reproduction of *B. stenochorias* were examined.

6.2. MATERIALS AND METHODS

6.2.1. Growth Study

Twelve 5 litre buckets, lined with new polyethylene plastic bags, filled with carbon filtered tap water and gently aerated, were maintained in a controlled environment (CE) room, at 20EC (the ideal temperature choice for maintaining *Burnupia stenochorias* in the laboratory; Haigh and Davies-Coleman, 1999). A light regime of 12 hours light/dark and photon flux density of 120 mol/m^2/s were constantly maintained. Carbon-filtered tap water was used for topping up when necessary, after evaporation. Water quality in terms of pH, dissolved oxygen (DO) and Total Dissolved Salts (TDS) was monitored twice weekly using M90 hand held probes. Adequate silicon levels for diatom growth were maintained by pouring 2 ml of

sodium silicate solution (100 mg/l) into each container at weekly intervals (Stein, 1973). Calcium, necessary for shell development, was added in the form of portions of calcium carbonate (chalk) sticks (C.C. Appleton, pers. com.). Tap water samples were analysed for calcium, magnesium and sodium approximately every 4 weeks, using a Merck spectroquant spectrophotometric system; it is preferable that ratios of sodium to calcium remain high, and calcium to magnesium low for maximum calcium availability (Appleton, 1978). Silicon in the form of silicate was measured from the same samples by the Port Elizabeth Municipality Water Quality Laboratories. Copper levels, as possible reticulation system contamination, were also measured.

B. stenochorias, collected from the Manley Flats site on the Bloukrans River (Figure 2.2) were maintained in containers as above, and allowed to lay eggs. From the first generation, twenty newly hatched limpets were measured for shell length to the nearest 0.5 mm using Vernier calipers, and then placed in each of the twelve plastic lined buckets. Four of these buckets were then subjected to one of the following diets:

Diet 1) Nutrafin Fry Food®, 0.02 gm fed twice weekly. Nutrafin Fry Food® consists of crude protein, 46%, fat 5%, and fibre 8% [replicates are referred to as N].

Diet 2) A combination of the Nutrafin Fry Food® fed twice weekly, combined with cultivated periphyton primarily in the form of diatoms, as for Diet 3 [replicates are referred to as N & A].

Diet 3) Periphyton in the form of diatoms (Section 2.3), seeded on the plastic at the start of the experiment from a mixture of rock scrapings sampled from where populations of *B. stenochorias* naturally flourish, and allowed to grow unimpeded [replicates are referred to as A]. The periphyton was examined under a light compound microscope to determine the presence of diatoms and other algal growth, fungal mycelia and bacteria.

Hatchlings were allowed to grow to adult size (approximately 3.5 mm, 53 days). Curvilinear and linear models using the least squares method were fitted to limpet size over the 53 days to test for linearity. Growth rate models were drawn for each feed, and individual ANCOVA's tested for any significant differences between slopes (Statgraphics 5.0).

6.2.2. Reproductive Study

The Nutrafin diet was omitted from the (later) reproductive phase of the investigation because of the algal

growth. At first sign of egg lay between all replicates, 9 pairs of limpets from the Nutrafin and Algae, and 8 pairs of limpets from the Algae diets were taken from those replicates that had no capsules, and placed as pairs in separate aerated containers, maintaining the same diet from which they were removed. Then after the first egg capsule was laid within each of these pairs, the individuals within each pair were separated again (based on research by Lam and Calow, 1989) into containers as before. All bags were inspected daily for egg capsules, and eggs counted using a stereo-microscope. The experiment continued for a further 5 months until the death of the last limpet.

Fecundity was calculated as number of eggs per capsule; number of eggs per limpet per day; and total number of eggs per limpet per reproductive period. Statistical analyses (Statgraphics 5.0) included comparisons of the number of eggs/capsule for individual limpets and for treatments (ANOVA). Life span, reproductive period, total number of eggs and total number of capsules were compared by ANOVA and Kruskal-Wallis tests.

6.3. RESULTS

Observations of *Burnupia stenochorias* feeding showed that a limpet clears the periphyton from a feeding path as it sweeps its head from side to side in a rasping action, consuming adnate but not (visually) filamentous algae. Diatoms dominated the periphyton growth, with some fungal mycelial threads and colonies of bacteria also present. Although not examined under the scanning electron microscope, the colour and texture of the algae grown suggested the diatom populations were dominated by *Navicula, Gomphonema, Achnanthes,* and *Cocconeis* species, as occurred in previous investigations where the water source and laboratory conditions were the same (Haigh and Davies-Coleman, 1999).

Generally, water quality did not vary between replicates or diets, although total dissolved salts generally increased through time (Table 6.1). An increase in concentration of sodium, magnesium and calcium can be seen over the five months. Sodium was significantly different in the samples from the first three to the last four samples (Student's 't' test, $p < 0.05$); however ratios of sodium to calcium remained high, and

Table 6.1. Range in sodium, magnesium, calcium, and silicon in the form of silicate (SiO^2 mg/litre) found

in the carbon-filtered dechlorinated tap water used as test water. Samples (numbered 1 - 7) were taken approximately every 3 - 4 weeks. n/a = not available [the samples were lost by the analytical laboratories].

Sample number	Sodium	Magnesium	Calcium	SiO^2
1	44 - 72	9 - 15	12 - 17	10 - 11
2	44 - 72	9 - 12	13 - 17	9 - 14
3	52 - 104	10 - 14	18 - 26	9 - 25
4	100 - 194	8 - 20	16 - 32	n/a
5	96 - 152	14 - 28	24 - 34	6 - 25
6	104 - 174	13 - 25	32 - 52	6 - 23
7	108 - 154	15 - 25	30 - 50	9 - 25

calcium to magnesium low. Calcium remained above 10 mg/l and the hard condition of the shells suggests calcium was not limiting. Silicon levels remained sufficiently high for diatom growth (Stein, 1973). Total dissolved salts (maximum 583, minimum 239, mean 364) and pH (maximum 7.91, minimum 6.66, mean 7.35) levels did not differ significantly between replicates or diets ($p < 0.001$). Copper was not significant in significant quantities (Tables 10.2 and 11.2).

6.3.1. Growth study

On a diet of Nutrafin, 46.5% mortality occurred in the first 18 days, with the rate of mortality decreasing so that after 53 days only 53.5% had died in total (Figure 6.1). Mortality in the Nutrafin & Algae and Algae diets was approximately 7.5% in the first 18 days, which increased to 30% and 35.4% respectively after 53 days. Irrespective of diet, variability was seen in growth and maximum size attained by individuals (Figure 6.1). The average growth rate within the 53 days was 0.485 mm (Nutrafin), 0.404 mm (Nutrafin and Algae) and 0.379 mm (Algae) shell length per week (Figure 6.1). Regressions of size attained in the three diets, and subsequent ANCOVAs to test for equality of slopes, gave the following results indicating linear growth (R_2 values; Figure 6.2) and a significant difference between slopes:

	Model	R^2
Nutrafin (Diet 1)	y = 0.061x + 1.108	0.794
Nutrafin & Algae (Diet 2)	y = 0.059x + 1.142	0.746
Algae (Diet 3)	y = 0.049x + 1.389	0.713

($F_{(2,331)} = 10.97$, $p < 0.05$).

When compared between diets, there was no statistical significance between growth of limpets in a Nutrafin diet compared to a Nutrafin & Algae diet ($F_{(1,213)} = 2.956$, $p > 0.05$); but a significant difference between growth in a Nutrafin compared to an Algae diet ($F_{(1,199)} = 74.3$, $p < 0.05$) and in a Nutrafin & Algae compared to an Algae diet ($F_{(1,253)} = 54.04$, $p < 0.05$). Cross-correlations of change in pH and TDS with weekly growth, within each replicate, indicated no correlation ($p > 0.1$).

Based on the growth of the limpets, the diets can be classified (from most to least advantageous) as:

Nutrafin diet > Nutrafin & Algae diet > Algae diet;

but based on mortality, the diets can be classified (from most to least advantageous) as:

Nutrafin & Algae > Algae > Nutrafin.

6.3.2. Fecundity and Reproductive Biology

Mating only occurred in pairs and not simultaneously with more individuals as seen in other pulmonates (Bondesen, 1951). Eggs were first laid when limpets were between 70 and 84 days old when fed a diet of either Algae or Nutrafin & Algae. Most capsules, with few exceptions, were laid near or at the bottom of each plastic bag. Numbers of eggs per capsule varied from 1 to 13 in both diets with no abnormal development occurring. The largest percentage of total egg numbers were laid within the first four weeks (81%) after which the proportion of eggs laid declined rapidly with both diets.

Fecundity (Table 6.2) and time between lays (Table 6.2; Figure 6.3) were variable. Laying frequency was characterized by spurts during which at least one and sometimes more capsules were deposited every night followed by rest periods (Figure 6.3). Laying periods were from 1-15 days without rest, and time between lays from 1-40 days, with no apparent correlation to diet. The largest percentage of the total egg numbers were always laid in the first four weeks, after which the proportion of eggs laid declined rapidly (Figure 6.3). Limpets with life spans in excess of 196 days (only in Nutrafin & Algae) also produced the

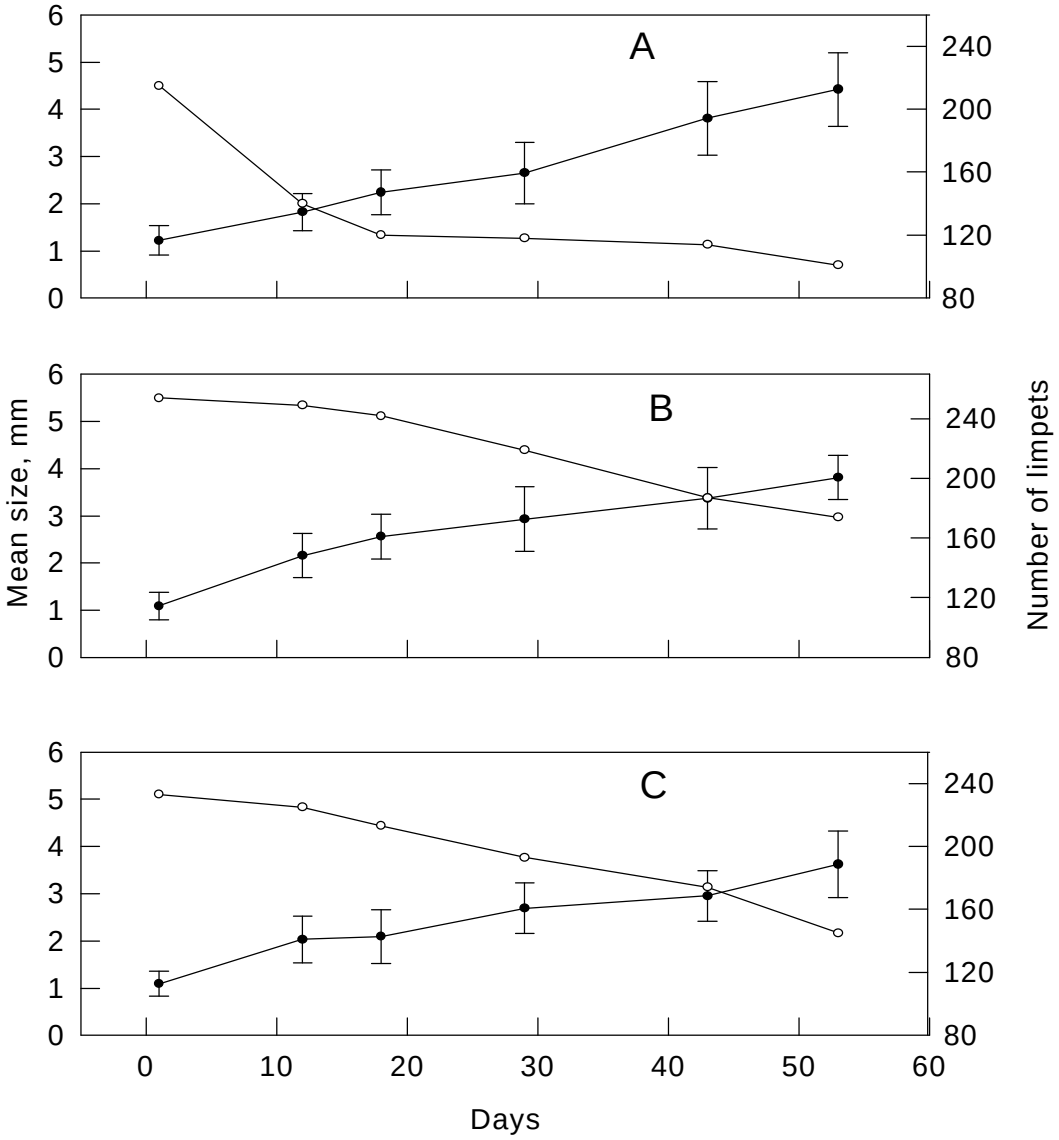


Figure 6.1. Mean size (mm, ± standard deviations) (ž) and number of live limpets (·) of *Burnupia stenochorias* over 53 days, when maintained on a diet of either Nutrafin (A), Nutrafin & Algae (B), or Algae (C).

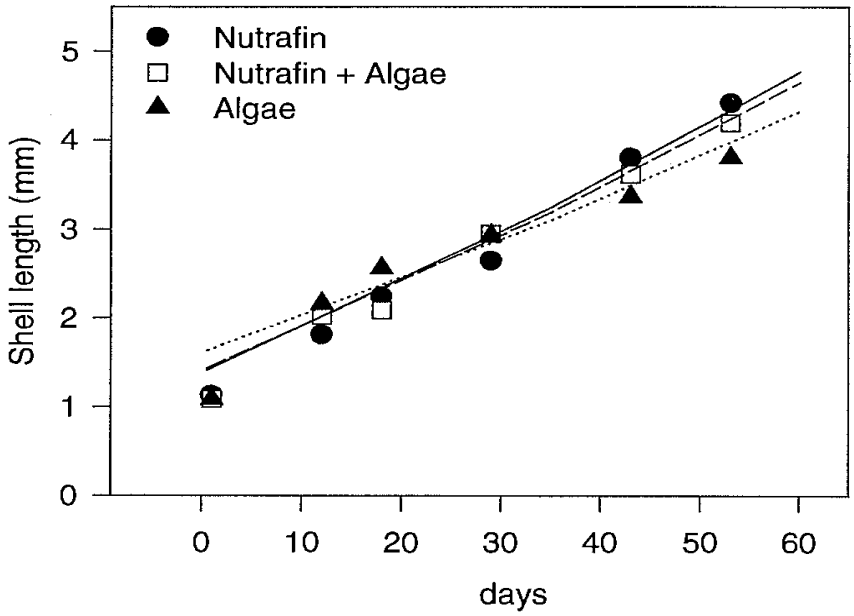


Figure 6.2. Regression lines comparing 53 days of growth of individuals between the three diets of Nutrafin, Nutrafin & Algae, and Algae (ANCOVA's). **xxx** Nutrafin; ---- Nutrafin & Algae; ..... Algae.

largest numbers of eggs. However, although also fed Nutrafin & Algae, the animal with the highest intrinsic fecundity (7.58 eggs/day) and fecundity rate calculated as number of eggs laid by an individual divided by its reproductive period (8.8 eggs/day), lived for only 154 days. Multiple range analyses to compare the effects of the two diets on the limpets, showed that Nutrafin® increased longevity by an average of 21 days (16%; $F = 4.2$, $p = 0.047$ [ANOVA, represented as A] $p = 0.03$ [Kruskal-Wallis, represented as K-W]).

This significantly increased laying period (98%; $F = 4.6$, $p = 0.037$ [A] $p = 0.039$ [K-W]), total number of capsules laid (140%; $F = 5.77$, $p = 0.022$ [A] $p = 0.025$ [K-W]) and total number of eggs produced (136%; $F = 4.9$, $p = 0.033$ [A] $p = 0.024$ [K-W]). Non-layers constituted 6% (1 out of 18) when fed Nutrafin & Algae, and 25% (4 out of 16) when fed Algae. When non-layers were excluded from the data set, the intrinsic fecundity and fecundity rates (eggs/capsule of 5.37 and 5.32, and eggs/day of 2.8 and 2.2 for Diets N&A and A respectively) were not significantly different ($p = 0.16$ [K-W]). However, as

the laying period and life span were both longer in limpets with a diet of Nutrafin & Algae (Table 6.2), these limpets had higher overall fecundities. Fewer than 50% of the replicate populations survived after 63 days under the conditions provided. Of these, the mean life span for limpets with a diet of Algae was 125 days (maximum 147; minimum 98); and 146 days (maximum 224, minimum 77) for a diet of Nutrafin & Algae.

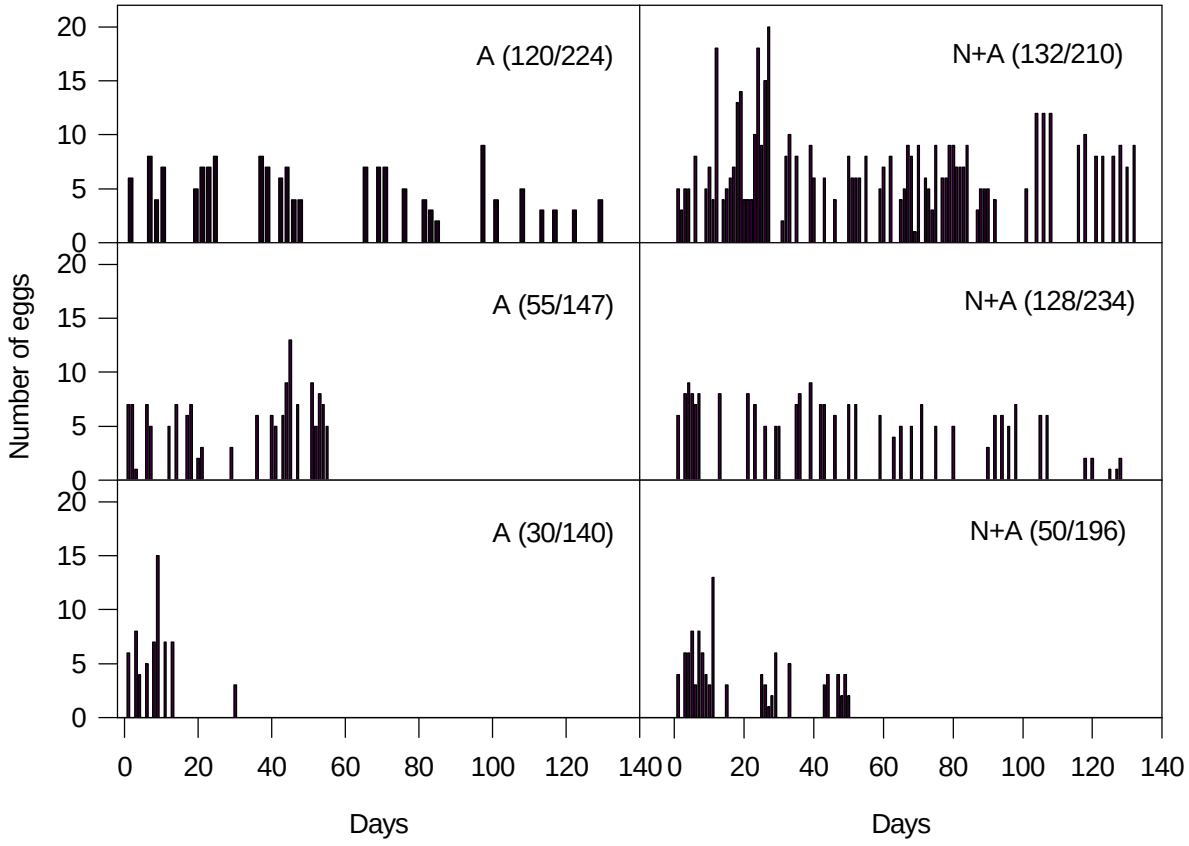


Figure 6.3. Pattern in egg laying periodicity in a selection of *Burnupia stenochorias* with greatest longevity. A = algae diet, N+A = Nutrafin & Algae diet. Numbers in brackets refer to the laying period or lifespan.

Table 6.2. Fecundity values recorded for *Burnupia stenochorias* fed Nutrafin & Algae or Algae. Figures in [ ] represent mean figures for each treatment with 'infertile' limpets excluded. The lowest number of eggs laid by an individual was 5 eggs in 5 capsules (Nutrafin & Algae) and the largest, 562 eggs in 81 capsules (Nutrafin & Algae). No. = number of; Std. dev. = standard deviation.

	Nutrafin & Algae			Algae		
	Mean	Range	± Std dev	Mean	Range	± Std dev
Number of limpets	18			16		
Size (shell length, mm) at first lay	4.5			4.8		
Size (shell length, mm) at death	5.3 [5.4]	4.0-6.0		4.9 [5]	4.0-5.5	
Longevity	147 [152]	84-210	± 8.9	126 [123]	98-154	± 4.7
Reproductive period, days (percentage of lifespan)	47 (32%) [43.5]	14-132	± 8.5	23.6 (18.7%) [31.6]	5-74	± 6.1
Time between lays (days)		1-40			1-40	
Total number of capsules	24 [25.4]	5-81	± 4.55	10.8 [14.3]	4-28	± 2.49
Total number of eggs	141 [149.6]	5-326 & 562	± 32.17	59.6 [79.6]	11-167	± 14.41
Number of eggs/capsule (intrinsic fecundity)	5.37	1-8.8	± 0.52	5.32	2.8-6.4	± 0.63
Number of eggs/day (fecundity rate)	2.8	0 & 0.3-7.6		2.2	0 & 1.8-5.6	

6.4. DISCUSSION

The feeding action of *Burnupia stenochorias*, where a path is cleared through the periphyton, is similar to, for example, *Ancylus fluviatilis* and *Bulinus tropicus* (Streit, 1985). This is in contrast to the combing action used by *Physa acuta* where particles loosely attached or deposited on the substratum are collected (Brackenbury, 1989), or the preferential scraping that the limpet *Ferrissia fragilis* displays (Blinn *et al.*,1989). Food choices made by a freshwater gastropod through its lifespan may be related to hunger levels (Daldorph and Thomas, 1988), or may change over time with age (Skoog, 1979). For example, immature limpets are presumed to grow at a faster rate and therefore demand more food (Calow, 1973). The estimated growth rates of *B. stenochorias* under natural conditions (Figure 4.7) suggests this occurs, yet growth was linear in all three diets in the laboratory. A comparison of the inter-cusp gaps of teeth on the radulae of *B. stenochorias* with other gastropods, and gut contents over the life span of *B. stenochorias*, would clarify the food choices made by *B. stenochorias*. However, it was presumed, in the limited time available for study, that diatoms constituted the majority component of the periphyton consumed.

Under natural conditions, the 'clearing of the path' by *B. stenochorias* may facilitate the picking up of small sand grains (mineral particles) which allow greater trituration of food (Hunter 1980; Thomas, 1998), of particular importance in the digestion of diatoms (Calow, 1970). The sand particles would also act as a source of cations and epilithic bacteria, and thus are likely to enhance the growth of *B. stenochorias*, as was found with *Biomphalaria glabrata* (Thomas, 1998). In the laboratory, where conditions for the growth and fecundity studies of *B. stenochorias* were relatively simple compared to the natural environment, sand particles were lacking. However, the addition of Nutrafin® to the diet of *B. stenochorias* in the laboratory significantly increased growth, longevity and fecundity. Its contents of crude protein, fat and fibre provide an ideal medium for the growth of bacteria. Bacteria are always present in water, even treated tap water, although in small quantities (Paperna, 1980). These bacteria will not only be an important source of Vitamin K and other essential vitamins (S. Daya, pers. com.) but will break down the crude protein of the Nutrafin® into its constituent amino acids, making the amino acids available to the limpets (Thomas, 1998; Dillon, 2000).

At the start of the growth study with *B. stenochorias*, those replicates with only Nutrafin® began with relatively sterile containers, with no other food source and presumably low bacterial counts, although this was not confirmed. Under these conditions, mortality was initially high (Figure 6.1). However, bacteria would have flourished profusely with nutrients freely available from the fish food and no competing algae to drain available supplies. After the second week, algae dominated by diatoms then began to grow, and the mortality rate decreased below that of the other diets. This suggests that either:

a) an algal (plant) diet is essential to the initial survival of *B. stenochorias (*as found with *Biomphalaria pfeifferi*; Jennings *et al*., 1970); or

b) that, rather than the consumption of Nutrafin® itself, it was the bacteria which played a role in one of two ways. The first was in the growth of the periphyton, dominated by algae; the second was in the feeding and growth of the limpets.

Despite the initial differences in mortalities between the diets, mortality was generally high in this pre-sexually mature phase of development, with approximately 33% loss of the populations. Although it is traditionally difficult to control water quality when the water medium is regularly changed (de Kock and van Eeden, 1980), low ratios of calcium/magnesium, and high ratios of sodium/calcium were generally maintained throughout the replicates. These ratios are ideal for growth (Harrison and Shiff, 1966) and snail reproduction (Appleton, 1978) respectively. Calcium, necessary for shell growth, remained at a level sufficiently high (>10 mg/l) for easy absorption (van der Borght and Puymbroeck, 1966). Silicon levels for diatom growth remained adequate within each replicate (Stein, 1973). Temperatures were also controlled, and there was no correlation between changes in pH or TDS, and growth.

As with other Ancylidae, deposition of egg masses occurred primarily during the night (Berg *et al.*, 1958), favouring the lower extremities of the containers (under natural conditions this would represent the lower extremities of rocks; Geldiay, 1956). Mating occurred in pairs as opposed to the stacked mating observed with some other freshwater limpet species (Bondesen, 1950). The innate capacity for increase of *B. stenochorias* is difficult to determine because it is hermaphroditic and able to self fertilize (pers. obs.; Haigh and Davies-Coleman, 1999). The gonadosomatic index and fecundity can be established for single event spawners from sacrificed animals. However, the establishment of fecundity

in serial spawners such as *B. stenochorias* necessitates the collection or examination of live eggs as they are deposited, as completed in this study.

Irrespective of diet, a large variation was seen in all aspects of the reproductive biology and fecundity of *B. stenochorias,* despite the controlled conditions. This may reflect the genotypic plasticity demonstrated by freshwater gastropods in general, in response to changing environments (Jarne *et al*., 1991). Streit (1985) gave a useful overall synopsis of *Ancylus fluviatilis,* and this is compared to *B. stenochorias* (Table 6.3). However, within this reproductive study of *B. stenochorias*, early mortality of many of the limpets contributed to the variation seen in reproductive effort, by reducing the reproductive period, and hence, number of capsules and eggs produced. Similarly, variations in the number of eggs per capsule, and number and frequency with which capsules were deposited, contributed to the large daily variation in fecundity rate. This is significant if *B. stenochorias* is to be used in long term chronic toxicity studies. [Despite the expected high mortalities with newly hatched *B. stenochorias*, chronic studies were completed over an entire life cycle in order to obtain data on fecundity of those limpets surviving low effluent dilutions; Chapter 11]. The intrinsic fecundity (number of eggs per capsule) was not significantly different between the Nutrafin and Algae and Algae diets. However, the addition of Nutrafin® to the diet increased longevity by 16% which constituted a mean increase in reproductive period of 98%,. The number of limpets that did not lay egg capsules was decreased from 25% to 6%. This suggests the effect of Nutrafin® and its provision of nutrients, either as mineral elements, or as amino acids and vitamins via bacterial production, is an ideal additive to the culture of *B. stenochorias* in the laboratory. Longevity under these controlled laboratory conditions and diets was not greater than six months, compared to the predicted longevity of 24 or more months under natural conditions (Chapter 4).

It was assumed that limpets mated before first egg lay and consequent separation. However, 25% of *B. stenochorias* under these conditions did not lay; this was reduced to 6% with the addition of Nutrafin® to the diet. The question arises as to whether the ‘male’ (Jarne and Charlesworth, 1993) of the pair mated as a female itself, receiving sperm from its mate? Was being mated as a ‘female’ necessary to

initiate egg production? Although pulmonates are simultaneous hermaphrodites, freshwater mating systems are highly heterogeneous (Jarne *et al.*, 1991; Städler *et al.*, 1995). Most freshwater pulmonates cross-fertilize (Jarne, 1995; Dillon, 2000) but often resort to self-fertilization when old or weak (Duncan, 1975). Mating is not always necessary, however (*e.g. Lymnaea* and *Biomphalaria* species; Salenddin *et al.*, 1997). Fifty pulmonate species have been shown by Jarne *et al.* (1991) to be self-fertile, although with outcrossing the preferred mode of reproduction. *A. fluviatilis* is polyploid and there is evidence of a high degree of self-fertilization, even when allosperm are available, with a minimum population outcrossing rate of 10.3% (Städler *et al.*, 1995). A comparison of basic differences and similarities of fecundity and reproductive biology between *B. stenochorias* and *A. fluviatilis* is given in Table 6.3.

Although self-fertilization occurs in *B. stenochorias*, intrinsic fecundity and fecundity rates are reduced when mating does not occur (Haigh and Davies-Coleman, 1999). The influence that the utilization of allosperm or autosperm has on the energy allocation during the reproductive process is not clear (Jarne *et al.*, 1993). The results from this study of diet and reproduction of *B. stenochorias* suggest there is a possibility that the paired limpets did not mate before final separation. However, irrespective of whether allosperm or autosperm were utilized in egg fertilization, the addition of Nutrafin Fry Food® to the diet increased growth, longevity and overall intrinsic fecundity and fecundity rates.

Temperature is a deciding factor in reproductive effort (Dillon, 2000). A repeat of this experiment under different temperature regimes may alter the fecundity rates of *B. stenochorias*; a change from 20EC to 14EC altered the number of eggs/adult/day in *Lymnaea stagnalis* from 2.8 to 1.5 (Dillon, 2000). This should be considered if the data presented here for *B. stenochorias* are to be used in a comparative manner with future chronic toxicity tests where reproduction is to be monitored. In the application of these fecundity results, and of those data from chronic toxicity tests, it would be useful to know how fecundity under these laboratory conditions compares to that found under natural conditions.

Table 6.3. Basic differences and similarities of fecundity and reproductive biology between *Burnupia stenochorias* and *Ancylus fluviatilis*. *B. stenochorias* demonstrates a greater production potential due to greater total numbers of capsules laid.

Character	*B. stenochorias*	*A. fluviatilis*	Reference (*A.f.*)
Mean size of egg capsule	0.75 mm	1.2 mm	Bondeson, 1950
Total number of capsules laid	5-81 (mean 35)	10-20 (mean 15)	Russel-Hunter, 1953; Geldiay, 1956; Lamberet, 1966
Number of eggs per capsule	1-13 (mean 5)	1-13 (mean 5)	Geldiay, 1956
Hatching	crawling snails shell length 0.75 mm	crawling snails shell length 0.8-1 mm	Lamberet, 1966
Earliest size when egg laying occurs	3.2-3.5 mm (± 50% maximum) shell length	5 mm (± 50% maximum) shell length	Streit, 1975
Spring temperature change	10-14EC	7-10EC	Lamberet, 1966; Streit, 1976

6.5. CONCLUSIONS

Growth to sexual maturity and eventual longevity and death occurred under the laboratory conditions in less than six months. This is significantly faster than the predicted life cycle for individuals under natural conditions, as seen in Chapter 4. The significance of this is to be discussed in Chapter 7. There are many unanswered questions regarding the role that both biotic and abiotic factors play in the growth and reproduction of *B. stenochorias* under laboratory conditions. The effects of water quality variables on the growth and reproductive biology and fecundity of *B. stenochorias* ideally require further

investigation, particularly in the light of chronic toxicity tests where water quality is under scrutiny. Although typical of pulmonates, the numbers of capsules and eggs produced and the laying patterns of individual *B. stenochorias* vary considerably, irrespective of diet. However, the technique of algal growth combined with regular additions of Nutrafin® (which increased growth and longevity) provides a feeding scheme for *B. stenochorias* which is simple and practical. Age at which egg production commenced did not differ between diets, but the increased longevity significantly increased total egg production. The number of *B. stenochorias* that did not produce egg capsules under these conditions was also reduced by the addition of Nutrafin®. Increased fecundity is obviously advantageous when maintaining laboratory cultures of *B. stenochorias*. Similarly, any animal chosen for toxicity tests has to be in a healthy state (USEPA,1991) and the increased longevity and fecundity observed in *B. stenochorias* suggests that the state of each limpet was healthier than when maintained on a diet of periphyton alone, even when this diet was dominated by diatoms. A diet of periphyton, dominated by diatoms, plus regular additions of Nutrafin® will therefore be used in the culture of *B. stenochorias* and also in the long term, chronic toxicity testing of textile whole effluent on *B. stenochorias.*

CHAPTER 7. SUMMARY OF PART ONE AND FUTURE RESEARCH

Life history data are essential in longer term (chronic) toxicity testing (Rand and Petrocelli, 1985; Persoone and Janssen, 1993) and growth and reproduction are the primary life history parameters used in chronic toxicity tests (Rand, 1995). These parameters, both under natural and laboratory conditions, were unknown for *Burnupia stenochorias* which was to be investigated as a possible toxicity test organism.

Significant differences in life history parameters of *B. stenochorias* were found between feral and laboratory bred populations (Table 7.1). Based on shell length, feral populations were estimated to show slower growth (which varied between summer and winter) and to live about six times longer than laboratory limpets. In addition, the feral limpets produced fewer eggs per capsule (Table 7.1).While growth is an important fitness characteristic of individuals, an apparent impairment of growth does not necessarily imply a decrease in the overall fitness. It is therefore desirable to measure other parameters

Table 7.1. Summary of biological parameters measured or estimated for the feral and laboratory populations of *Burnupia stenochorias*. * will vary depending on the time of year hatching occurred, and prevailing conditions such as temperature, water quality and flow rate; all growth rates here are those estimated for limpets hatching in early summer.

	Units	Feral population	Laboratory population
Growth form		Sigmoid	Initially linear, slowing in last approximately 14 days
Maximum length	mm	6.5-7.0	6.0
Growth rate mm/month			
Summer period *	mm	0.675	2.25 & 1.5
Winter period *	mm	0	not applicable
Post winter period *	mm	0.087	not applicable
Longevity	months	24	4.8
Eggs per capsule		2 - 13 (average 4.6)	1 - 9 (average 5.37)

(usually reproduction) in addition to growth to provide a more accurate assessment of the effect of the toxicant involved. Within the two natural populations studied, egg lay occurred throughout the year, with a distinct increase in August/September (spring), and again in January (mid summer). With differences in egg capsule quantities observed in mid summer being attributed to water quality in this study (Sections 4.31 and 4.4), the usefulness of the presence of egg capsules within *B. stenochorias* populations in long term, on-site biomonitoring should not be overlooked. Although time of egg lay may influence susceptibilities to toxicants, fecundity is the most useful reproductive parameter in toxicology (Rand, 1995). The fecundity of pulmonates (clearly described in Smith and Stanisic, 1998; and reviewed in Dillon, 2000) is difficult to unravel, largely because of their simultaneous hermaphroditism and ability to self-fertilize. The fecundity of *B. stenochorias* was not determined under natural conditions.

However, chronic toxicological research is primarily based in laboratories. A diet of periphyton (principally in the form of diatoms, fungal mycelia and bacteria) and Nutrafin® fish food was most successful in terms of fecundity in the laboratory, and this diet was used in the *B. stenochorias* chronic toxicological studies (Chapter 11). Egg production in the laboratory began when limpets were 4.5 mm shell length or longer (Table 6.2). Generally amongst the pulmonates, differences observed between individuals selected from the same population in age and size at first egg lay are variable and may be indicative of stressors (Lam and Calow, 1989; Wethington and Dillon, 1993). However, the verification of the cause of variable size-at-first-reproduction may be weak or difficult (Lam and Calow, 1989). It is therefore suggested that size at first lay is not a good criterion to use in toxicity testing; and that age at first lay is difficult to estimate accurately.

The individuals of *B. stenochorias* grown in the laboratory displayed large ranges in frequency of lay and fecundity (total number of egg capsules and eggs per capsule, and number of eggs per limpet per day: Section 6.3.2), suggesting it would be difficult to statistically differentiate toxicant effects. The data, however, served as a baseline study for later toxicological investigations.

It is possible that the differences in life history parameters exhibited by feral and laboratory populations were primarily a result of either food abundance or quality, linked to the conditions of temperature. The

laboratory populations of *B. stenochorias* were maintained on a mixed diet of periphyton (principally in the form of diatoms, fungal mycelia and bacteria) and Nutrafin® fish food. Personal observations of the natural food supply (made by simple scrapings of the rocks and examination under a compound microscope), compared to the food available in the laboratory experiments, suggest quantity of food under natural conditions may have been limiting. The situation may have been exacerbated by the higher feral densities of limpets observed (approximately 206/100 cm^2) compared to the densities in the laboratory (35/100 cm^2). Increased food availability has been shown to result in an increase in growth rate and maximum size attained of prosobranch limpets (Bosman and Hockey, 1988; Vat, 2000). Although not examined, the differences in energy content and quality of the diets may have also contributed to differences in growth rates between limpets under either natural or laboratory conditions (Calow, 1973; Streit, 1975). Although the ancylids may have the capacity to absorb nutrients from the water body itself, the majority of nutrients come from the consumption of food. It appears *B. stenochorias* has the capacity for increased scope for growth, in turn suggesting the limpet demonstrates phenotypic plasticity; that its metabolism is not genetically fixed as in some marine limpets (Gray, 1996; Vat, 2000). Scope for growth would possibly be a more accurate measure of growth in *B. stenochorias* than change in size.

The constant temperature of 20EC in the laboratory may also have allowed optimal metabolism and therefore growth (Leighton, 1974). Thus, growth was linear in the laboratory but sigmoid under natural conditions. Longevity is generally negatively correlated to growth rate (Ebert, 1975; Branch, 1981). Thus rapid growth is associated with early maturity and a shorter lifespan. The ability of *B. stenochorias* to demonstrate an increase in growth rate suggests a capacity to change from an 'r' to a 'K'[1] selected life strategy (Krebs, 1978). Future research based on the physiological and genetic responses of feral individuals, including dietary preferences and their energetic components, may not only clarify the optimal capacity for growth demonstrated by *B. stenochorias*, but may also help to interpret the impact of

[1] 'r' species which live in an unpredictable environment and are subject to density-independent forces.
'k' species which live in a favourable environment and are subject to density-dependent forces. (Krebs, 1978)

industrial discharges on local populations. Given the variation between individuals and populations, and the effect of time of year of emergence, it is suggested that a given shell length of *B. stenochorias* cannot be used to accurately estimate the age of a limpet.

Despite the apparent more favourable conditions in the laboratory, initial mortalities were higher (33%) than the more acceptable 10% to 20% advocated within toxicity tests after more than 10 days (USEPA, 2000). Cohort analysis of fortnightly samples of feral populations cannot be used to give an accurate estimate of mortalities when frequent egg lay occurs (Gosselin and Qian, 1997). However, examination of the length frequency histograms of the natural populations over the year gives indirect evidence to suggest mortalities, particularly within the newly hatched limpets, are also naturally high (up to 90% or more, estimated from the delay in appearance of limpets of approximately 1 mm shell length; Figures 4.4 and 4.5) so that a small percentage of the limpets hatching naturally reach maximum size. The first day after hatching is a critical period for many benthic freshwater and marine invertebrates, with subsequent cumulative mortalities to the adult stage varying from 24% to > 99% (Gosselin and Qian, 1997; Hunt and Scheibling, 1997). Factors responsible for this mortality are many (Gosselin and Qian, 1997; Dillon, 2000) but it is suggested that these differences between the natural and laboratory population mortalities of *B. stenochorias* in the first few days after hatching reflect the natural variation within this trend, largely due to differences in intensity of mortality factors such as food, temperature and flow rate. Other factors, such as disease, migration of hatchlings (Martel and Chia, 1991), and internal causes (energy depletion, and developmental and physiological defects), also potentially contribute to early mortalities.

The assessment of the mortality rates within natural populations, and therefore the potential recruitment rates, and the relative importance of recruitment rate compared to (subsequent) mortality of later life stages, forms a valuable tool for future biomonitoring and toxicological tests. A better understanding of the early juvenile mortality should contribute to an understanding of how the population parameters of *B. stenochorias* are regulated. The definition of recruitment, it should be noted, varies within the ecological literature (Hunt and Scheibling, 1997). Recruitment may refer to individuals which can be quantified at a certain period of observation (which is the definition loosely used in the field study of *B. stenochorias*), or the term may be used to quantify the addition of new individuals to the adult (breeding) population.

This latter would require knowing the size (or age) at sexual maturity; examinations of sectioned ovotestes from *B. stenochorias* of different lengths sampled throughout the year contributed to this knowledge (Chapter 5).

Mollusc size is also known to influence contaminant accumulation and susceptibility (Wier and Walter, 1976; Gomot, 1998), and the young are generally considered more sensitive than the adult stage (*e.g.* Cairns, 1986b). The examinations of the sectioned ovotestes from *B. stenochorias* revealed fluctuations in the proportion of mature ova and sperm throughout the year. However, limpets less than 3 mm shell length did not have mature sperm or ova. Consequently, only limpets smaller than 3 mm shell length were chosen for the acute toxicological investigations of *B. stenochorias*.

Collection of feral populations and their acclimation to laboratory conditions is an important process in toxicological testing (Buikema and Voshell, 1993). The method development for the collection of *B. stenochorias*, their transport to the laboratory and their acclimation which follows has been valuable (Scherman and Palmer, 2000). Similarly, the apparatus developed in the laboratory study serves as an ideal system for both acute and chronic toxicity studies; it is easily assembled, cheap and practical to use and move around.

The results of the preceding chapters have undoubtedly left many questions regarding the biological processes of *B. stenochorias* unanswered. The variability in shell morphometrics also suggests taxonomic clarity of the *Burnupia*, presently based on shell form, is necessary. Although the growth and reproduction of newly hatched limpets under laboratory conditions provided a baseline study, it would be preferable to either increase the database because of the large ranges observed, or refine the feeding regimes provided. It was felt, however, that the aim of supplying sufficient data, methods and a viable laboratory population with which to assess acute and chronic toxicity of a whole effluent had been fulfilled.

PART TWO

INVESTIGATIONS INTO THE VARIABILITY OF RESPONSES OF *BURNUPIA STENOCHORIAS* TO TOXICANTS AND CONCLUSIONS AS TO THE USEFULNESS OF *B. STENOCHORIAS* AS AN ECOTOXICOLOGICAL INDICATOR

> What makes it so hard to organize the environment sensibly is that everything we touch is hooked up to everything else.
>
> Isaac Asimov, 1988

`CHAPTER 8. INTRODUCTION TO ECOTOXICOLOGY

Ecotoxicology is concerned with the toxic effects of chemical and physical agents on populations and communities within ecosystems, and includes the transfer pathways of those agents and their interactions with the environment (Newman, 1995). Ecotoxicological studies provide a valuable tool for defining the acceptable levels of generally harmful chemicals in the environment, as they are the means by which the effects of chemicals on biota can be quantified (Pritchard, 1993; Connell *et al*., 1999). Practical ecotoxicology therefore applies available knowledge and procedures to specific problems and incorporates biological reality and applicability as key concepts (Newman, 1995; Chapter 14). Within riverine ecology, toxicology is concerned with the effects of selected pollutant levels on single organisms, for defined periods of time, under controlled conditions (Moriarty, 1983). A multitude of toxicity tests have been developed to estimate the short and long term effects of industrial and sewage whole effluents and emissions including among others, pesticides, and mining waste and land fill leachates (OECD, 1987; USEPA, 1979a; Hunt *et al*., 1992).

In Part Two of this study (Figure 1.2), wild populations of *Burnupia stenochorias* are used to assess the toxicity of a complex effluent. Because of the complexity of using a wild population of test organisms, with season, size, and collection site and conditions possibly affecting responses, as well as a variable and complex effluent, the study was conducted in several parts. Chapter 10 includes the acute testing of *B. stenochorias* using textile whole effluent, comparing responses to those seen when subjected to a reference toxicant. Chapter 11 includes the effects of the effluent on survival, growth and reproduction over an extended period; the third part of the study tests the responses of a standard laboratory organism (*Daphnia pulex*) to a reference toxicant and to short and long term exposure to textile effluent. The responses of *B. stenochorias* are then compared to those of *D. pulex* (Chapter 13), with an assessment of the acceptability of *B. stenochorias* as a toxicity indicator.

A*cute Toxicity Testing*

The acute toxicity test provides information about the relative lethality of a waste material, and is designed to determine the highest concentration of a waste that will affect a given percentage (*e.g.* 50%) of a limited number of test organisms (Buikema *et al.*, 1982; Section 9.3). This critical concentration is estimated by exposing organisms to a graded, logarithmic series of concentrations of

waste and then observing their responses, usually death, after one to four days (Rand *et al.*, 1995). When all types of toxicity tests were evaluated in their assessment of chemicals to aquatic life, acute tests were considered ecologically significant, most scientifically and legally defensible, modest in predictive capability, the simplest and most cost effective to perform, and having the greatest utility (Buikema *et al.*, 1982). In some species where death is difficult to determine, data are expressed as an effective concentration (EC) rather than a lethal concentration (LC). Much of the documentation on acute toxicity testing discusses the need to use sensitive life history stages in toxicity tests, with immature stages generally considered more sensitive to toxicants than the adults (Harman, 1974; Wier and Walter, 1976; Økland and Økland, 1986; Timmermans, 1993).

In this study, the variability in survival between three natural (feral) populations and a laboratory bred population were compared in 96 hour toxicity tests conducted in early summer (representing peak egg lay period) mid summer and winter.

Chronic Toxicity Testing

Some authors believe that death is a crude index of stress, being of limited relevance in ecosystem protection, and providing little information about bioaccumulation and life cycle disruption (Timmermans, 1993). Chronic or sub-chronic effects are usually the result of long term exposures to low levels of persistent chemicals, alone or in combination (Rand *et al.*, 1995). The effects may be lethal or sub-lethal (Rand *et al.*, 1995). The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the 'safe' or 'no effect' concentration of these substances, defined as the concentration which will permit normal propagation of plant and animal species in the receiving waters (USEPA, 1992). The quantitative data from exposure to toxicants in chronic tests are compared to those in the controls to determine whether any differences are statistically significant (Section 9.3). Chronic toxicity test endpoints are usually addressed in terms of the Lowest Observed Effect Concentration (LOEC) *i.e.* the lowest concentration in which values for the measured response show a statistically significant difference from those in the control; and the No Observed Effect Concentration (NOEC) *i.e.* the highest toxicant concentration in which the values for the measured response do not show a statistically significant difference to the control (Hoekstra and van Ewijk, 1993).

There are many levels of organization which can be affected by contaminants at a level which may cause lethality only after a long period (Giesy and Graney, 1989; Keller and Lydy, 1997; Liu and Dukta, 1999), leading to the possibility of a wide variety of biological endpoints to be used in chronic toxicity tests (Clark, 1994; Warne, 1998). These include histological, pathological, biochemical, haematological and behavioural criteria (Nerbert and Gelboin, 1968; Lloyd and Orr, 1969; Sprague, 1971; Payne *et al*., 1987; Giesy and Graney, 1989; Liu and Dukta, 1999; Chirombe and Naik, 2000). Although these biochemical methods are generally very sensitive, it is not always feasible to detect and/or measure the endpoints. It is also difficult to link the biochemical or physiological responses with their ecological significance (Abel, 1989) and therefore these responses are more useful in elucidating modes of toxic action (Giesy and Graney, 1989; Chirombe and Naik, 2001).

Full life cycle chronic toxicity tests with fish and invertebrates have also been used, where the test populations are continuously exposed to toxicants for sufficiently long enough to grow, become sexually mature and produce an F1 generation (Arthur, 1970; Biesinger and Christensen, 1972; Petrocelli, 1985; Rand *et al.*, 1995). Survival, growth and development of the F1 generation may also be monitored. With the demand for rapid, simple tests, the use of microorganisms such as luminescent bacteria, together with enzyme assays (Rindelberg and Kersting, 1978; Jäger *et al*., 1996); tests to detect mutagenicity and genotoxicity (Hawkins *et al.*, 1995; Shugart, 1995 and 1996); and mammalian and fish cell culture assays, as well as cyst-based tests using cryopreserved biological material, have all been recently pursued as toxicity tests, although the latter are usually used in acute toxicity testing (Cooney, 1995; Jagoe, 1996; Slabbert *et al*., 1998). Partial or early life cycle stages have been explored as a shorter, less expensive alternative to chronic toxicity testing, using the most sensitive stage of the life cycle of the chosen species (Birge *et al*., 1989; USEPA, 1990; Rand *et al*., 1995). This was considered in the chronic toxicity tests using *B. stenochorias.*

Observations in chronic toxicity tests have generally been limited to survival or mortality, and effects on growth and reproduction (Holdway, 1996), with the latter considered unequivocal criteria of sub-lethal toxic effects because of their ecological significance (Suter *et al*., 1987; OECD, 1992; Connell *et al*., 1999). Survival is the most reliable endpoint and is the easiest and least expensive to measure (Mayer *et al.*, 1986). However, exposure of aquatic organisms to stress frequently causes reduced growth rates (Giesy and Graney, 1989; Sibly, 1996) and impaired reproduction (Suter *et al*., 1987).

Therefore growth and reproduction form the focus of the chronic investigation in this study.

Growth is usually estimated as an increase in body size or mass which is easily measured (Geraerts and Joosse, 1984). Slow individual growth is likely to cause a delay in sexual maturity and embryonic development (Ravera, 1991). Alternatives to change in size or mass provide indirect information on the growth performance of individual organisms and include scope for growth (Streit, 1979, 1985; Lai and Lam, 1994; Connell *et al*., 1999), and the quantification of ribonucleic acid, deoxyribonucleic acid and protein (Sower *et al*., 1983; McKee and Knowles, 1986). Disadvantages to their use include the difficulty and expense in the techniques; the variability which arises from differing nutritional status with growth not always affected; and finally that growth may not be the most sensitive indicator of toxicity (Giesy and Graney, 1989). In many invertebrates a significant amount of energy is channeled into reproduction during certain periods, with a significant decrease in growth (Connell *et al*., 1999).

Reproduction is biologically meaningful as a toxicity tool as toxicity interferes with the production of future generations (Suter *et al*., 1987). Effects on reproduction may range from delayed sexual maturity to reduced fecundity, decreased hatchability and hatching rate of eggs, and lower offspring survival (Petrocelli, 1985; Connell *et al*., 1999). The effects may be temporary with an interruption in gametogenesis, or more permanent because of cellular damage of reproductive tissues (Connell *et al*., 1999). Fecundity is often more sensitive than survival (*e.g*. Borgmann *et al*., 1978; Suter *et al*., 1987), and is not well predicted from acute exposures, necessitating chronic toxicity studies (Kulshrestha *et al*., 1986).

Although survival, growth and reproduction are the parameters most frequently used, sensitivities either vary amongst different species' life history stages (Woltering, 1984; Green, 1987) or stimulation of growth and reproduction by sub-lethal concentrations of poisons may occur (Pickering, 1968; McLeay and Brown, 1974; Mitchell, 1992). Method development in chronic toxicological investigations has to therefore include not only an understanding of the biological parameters of the test species (Part One of this thesis), but also the relative sensitivities of each parameter to a given toxicant or toxic mixture.

The Selection of a Test Organism

Ecotoxicological investigations have added complications to the choice of a test species (Cairns,1986a; Scherman and Palmer, 2000; Chapter 1). Cairns (1986b) dismissed the selection of the most sensitive species. Rather, a suite of species is recommended because of differences in sensitivity (Rand *et al.*, 1995; Liu and Dukta, 1999), and because of differences with variability of response demonstrated by feral populations compared to laboratory bred populations (Calow, 1989). Toxicity test data acquired from feral riverine invertebrates are particularly recommended where site specific recommendations, guidelines and licence criteria are required; this concept, and the choice of *B. stenochorias*, are discussed further in Chapter 14.

Factors That Modify Toxicity of Effluents

Although chemical characterisation of water or effluents gives an idea of chemical changes which may occur, it does not indicate the toxicological[2] or environmental[3] bioavailability, but only the potential availability of each chemical (Rand *et al.*, 1995). There are many factors which affect toxicity of a chemical or effluent, related to either the duration, kind and frequency of exposure and concentration of the toxicant, or to factors related to the organism itself (Walsh *et al*., 1980; Abel, 1989; Sprague, 1995). The environmental conditions at end-of-pipe are the main factors affecting responses to exposure to a given toxicant (Abel, 1989). Instream toxicity is influenced by the natural chemistry of the receiving waters (O'Keeffe *et al*., 1996) and toxin bioavailability is often controlled by the processes of chemical speciation (Rand and Petrocelli, 1985; Abel, 1989; Chapman, 1995).

Species responses are also influenced by differing rates and patterns of metabolism and excretion (Rand *et al.*, 1995); tolerance due to genetic factors (Baird *et al*., 1989) or previous exposures (Crane, 1995); nutritional status, which affects physiological and biochemical body functions (Rand *et al.*, 1995); and/or age of the test organism, where immatures are considered potentially more susceptible

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[2] Toxicological bioavailability is the fraction of the exposure concentration which ultimately reaches the site(s) of toxic action. The fraction that reaches the target is dependent on such processes as the uptake, metabolism and elimination of the concentration (Rand *et al*., 1995).

[3] Environmental bioavailability is the fraction of the environmentally available material that an organism actually accumulates when processing or encountering a given environmental medium. It is the ratio of uptake rate to the rate at which an organism encounters a particular contaminant (Rand *et al*., 1995).

for a variety of reasons (*e.g.* Timmermans, 1993; Cairns, 1986b). Because of differences in modifying factors, it is important that indigenous species be included in the revisions of South Africa's water quality guidelines for protecting aquatic ecosystems, particularly as international literature is based primarily on temperate species (Chapman, 1995).

Interpretation of Toxicity Test Results

Fundamental to aquatic toxicology is the concentration-response relationship which relies on the assumption that the response in question is a result (direct or indirect) of the exposure of the organism to the toxic substance being examined, and that a relationship exists between the concentration of toxicant and the intensity of exposure (Rand *et al.*, 1995).

Variability of response is of concern in both acute and chronic toxicity, and is the focus of the research in the chapters which follow, where the responses of *B. stenochorias* to textile whole effluent are considered, compared to responses to a reference toxicant, and to the responses of a standard laboratory organism. On the basis of response variability, the usefulness of *B. stenochorias* as a toxicity test organism is discussed (Chapters 13 and14). There are two types of variability. The first, innate variability, relates to observed differences attributable to true heterogeneity or diversity in a population or exposure parameter (SETAC, 1997; USEPA, 2000). This innate variability is the result of natural random processes and stems from environmental, lifestyle and genetic differences. Examples include physiological variation, weather variability, and differences in contamination concentrations in the environment. Considerable differences in sensitivity to toxicants in general can occur between families, genera or species of the same genus (Appleton *et al.*, 1992). The true variability is fixed, when considering statistical sampling theory, but the sample estimate of the variance can be reduced with further measurements (SETAC, 1997; USEPA, 2000). The second type of variability relates to uncertainty, defined as imperfect knowledge of the present or future state of the system under consideration; a component of risk resulting from imperfect knowledge of many kinds including the degree of hazard or its spatial and temporal pattern of expression (SETAC, 1997; USEPA, 2000). Generally, uncertainty can be reduced with further information and knowledge, usually with the use of a reference toxicant (Warren-Hicks and Moore, 1998; USEPA, 2000; Chapter 10). Both types of variability (innate variability and uncertainty) give rise to within (or inter-) test and inter- and intra-laboratory variability (SETAC, 1997; USEPA, 2000). Numerous factors can affect the variability of

any toxicity test method. These factors include the variability of the effluent itself, in whole effluent toxicity tests, the number of test organisms, the number of treatment replicates, randomization techniques, the source and health of the test organisms, the type of food used, dilution water quality and the environmental conditions in the laboratory (Schindler, 1996; USEPA, 2000). The experience of the analyst performing and analyzing the test may also affect variability (Grothe *et al*., 1996; Fulk, 1996).

Examining variability for each effect concentration of each biological endpoint, for each test method is essential to investigations of toxicity (USEPA, 2000). The biological endpoints may differ in sensitivity to different toxicants. Consequently, the variance of the endpoint response, the confidence intervals of point estimate (to quantify the uncertainty of the *e.g.* LC_{50} values), and the standard deviation or standard error of the mean of the replicate response at each concentration within each test (USEPA, 2000) are all calculated. Because of the link between the type of response and the choice of statistical method used in the analysis of toxicity and variability, the analyses of variable responses shown by *B. stenochorias* to the toxicants tested are discussed further in Sections 9.3.1 and 9.3.2.

Textile Effluents

Toxicants enter aquatic ecosystems from two sources:

(ii nonpoint (diffuse) sources such as agricultural or urban runoff, and contaminated ground water, and

(ii point (discrete, localized) sources such as discharges (effluent) from industry or municipal wastewater treatment plants (Slabbert *et al*., 1998).

The effluent chosen for toxicological examination using *B. stenochorias* is a point source post-irrigation textile effluent collected from an Eastern Cape Province textile factory (Section 9.2.3). The choice of a textile effluent was partially made on the basis that the textile industry in South Africa and elsewhere is a major water user and polluter (Rutherford *et al.*, 1992; Steffen and Kirsten, 1993; Jäger *et al*., 1996; Gravelet-Blondin *et al*., 1997). All types of fibres used in the textile industry go through three main processes: fibre pretreatment, dyeing and finishing. These processes require the use of fabric modifying agents such as starch, fats, lignin, sodium hydroxide, acetic and sulphuric acids, and calcium hypochlorite; dyes, detergents, wetting agents, emulsifiers, smoothing, colour fixing and antistatic agents, softeners, surfactants made of organic compounds, and finishing agents. Many of these

chemicals are not retained in the final product but are discarded in liquid effluent discharges (Pagga and Brown, 1986; Rutherford *et al*., 1992). High concentrations of chemical and biological oxygen demand, suspended solids, heavy metals, extreme pH, and elevated temperatures all contribute to textile effluent's negative environmental impact (Chen, 1989, in Rutherford *et al*., 1992; Steffen and Kirsten, 1993; Dallas and Day, 1993). In South Africa, chemical oxygen demand, colour and salinity (total dissolved salts) are the most problematic in textile discharges (Gravelet-Blondin *et al*., 1997). The Eastern Cape textile mill, from where samples were sourced for toxicity testing, together with tannery and sewage effluents, contributes to a high salt load in Laing Dam, 7 kms downstream from the factory and a major water source for East London and surrounding areas (Figure 10.1; O'Keeffe *et al*., 1996).

The South African textile industry has lagged in the implementation of pre-treatment technologies compared to European and North American countries (Rutherford *et al*., 1992; Lal, 1996; Bahorsky, 1997), partly because of inadequate legislation (Gilfillan *et al*., 1997). Europe, potentially the main market for South African textiles, requires a European product guarantee, entailing the whole textile product to be ecologically (amongst other facets) acceptable, including its initial manufacture (Mohr, 1992). The South African textile industry is now faced with increasingly tight legislation regarding water discharges [as well as its air emissions and other waste production], with recommendations from the Water Research Commission that the industry also needs to attempt to reduce their water usage (Steffen and Kirsten, 1993).

The majority of South African textile effluent discharge is primarily to sewage works; the remainder is treated for discharge, or, rarely, for re-use in the factory. There are many processes by which water can be saved from discharge (Hefler, 1975; Mohr, 1992; Bahorsky, 1997) but few factories either in South Africa (Gilfillan *et al*., 1997) or abroad (Hazel, 1991a and b) can economically justify the wholesale treatment of effluent for re-use, as opposed to treatment for effluent discharge. Effluent discharge in South Africa therefore remains a problem in terms of the pollution control mechanisms employed.

Relatively few studies have been completed on textile effluents (Steffen and Kirsten, 1993). Generally, because of the diversity of industrial processes and chemicals used, worldwide variability is seen in the

ecotoxicity of textile effluents (*e.g*. Rawlings and Samfield, 1979; Jäger *et al*., 1996). Acute toxicity has been observed (USEPA, 1979b; Chen 1989), particularly in luminescent bacteria and daphnids (Walsh *et al*., 1980; Rutherford *et al.*, 1992; Jäger *et al*., 1996) and to a lesser extent, fish (less than 10% effluent; Walsh *et al*., 1980), frogs, mussels and crabs (Barton and Metcalfe-Smith, 1992; Shrinivas *et al*., 1984) and algae (Walsh *et al*., 1980; Rutherford *et al.*, 1992). The 96 hour LC_{50} of untreated or minimally treated effluent to rainbow trout ranged from 2.8% to 46.3% (Ernst, 1999). Chronic toxicity tests completed include decreased protein content and increased glucose and liver cholesterol in fish (Nisha and Shuka, 1986), changes in blood characteristics of fish (Riva and Flos, 1993), reduction in tissue oxidative enzyme activity and respiration in fish (Sakthivel *et al*., 1991) and food utilization in fish (*e.g.* Murugesan and Haniffa, 1994).

Toxicological Investigations in this Study

B. stenochorias was chosen to represent South African indigenous freshwater molluscs, as a possible ecotoxicological indicator, partly because of the extensive distribution of the genus in South Africa, but also because of the ease with which *B. stenochorias* is transported and cultured in the laboratory (Chapter 1). Whole effluents are known to be toxic to invertebrates and plants at both acute and chronic levels, depending on the dilution rates of the receiving waters at end-of-pipe (Gilfillan *et al*., 1997). Consequently, both acute and chronic toxicity tests were completed on *B. stenochorias*, using survival rates, growth (represented by change in shell size) and reproduction (represented primarily in terms of fecundity) as the primary indicators of toxicity (Figure 1.2). Because this was an initial investigation into the responses of the species, controlled laboratory conditions were used to quantify the variability in response to a textile whole effluent. Samples of the effluent were kept to a minimum to reduce the added variability of different chemical compositions of whole effluent in the interpretation of the toxicity results. The results suggest *B. stenochorias* would be a useful ecotoxicological test organism in the setting of site specific licence conditions, and in the setting and auditing of instream resource quality objectives (Figure 1.2; Chapter 13).

Although standard laboratory organisms provide benchmark toxicity data, the question arises as to the suitability of, for example, the commonly used lentic cladocerans (in this study: *D. pulex*) representing the responses of molluscs from primarily lotic riverine conditions. Responses of *D. pulex* to a textile whole effluent and the reference toxicant potassium dichromate were therefore compared at an acute

and chronic toxicity level to those of *B. stenochorias*, to assess the potential for *D. pulex* to represent the indigenous mollusc in routine toxicity tests (Chapter 13).

CHAPTER 9. METHODS USED IN THE TOXICOLOGICAL INVESTIGATIONS

The primary aim of this study was to assess the use of *Burnupia stenochorias* in toxicity testing, using a post-irrigation textile effluent as the primary toxicant (Section 9.2.3), based on acute and chronic toxicity test results (Figure 1.2). It is essential in any toxicological investigation that good quality test organisms be used, *i.e.* the organism's health and age and its inter- and intra-laboratory variability of response should be known. There are two types of variability in toxicity test results, as discussed in Chapter 8: uncertainty and variability (Warren-Hicks and Moore, 1998). However, both the response of the limpet and the effluent toxicity were potentially variable. Therefore the variability of toxicity results from effluent exposures was compared firstly with *B. stenochorias* tested with a reference toxicant (potassium dichromate; Section 9.2.3); and secondly with tests using the standard laboratory organism *Daphnia pulex* (Figure 1.2; Chapter 13). The methodology for *D. pulex* is discussed in Chapter 12.

9.1. Source and Collection of *Burnupia stenochorias*

Long term use of an ecotoxicology indicator will rely on either its constant supply from laboratory cultures or feral populations. Point source toxicology, used to set site-specific criteria, requires the use of local, feral populations of the test organism (Scherman and Palmer, 2000). Consequently, innate variability in acute responses of *Burnupia stenochorias* to a reference toxicant and an effluent were quantified by comparing individual limpet responses from different feral sources, collected at different seasons of the year.

Three sources of feral *B. stenochorias* were used in the acute testing, two of which were previously investigated (Section 2.1; Figure 2.1), namely the populations of Belmont River (representing a flowing, impacted water source) and Botha River (a non-flowing, pristine water source). The third represented a population from a flowing, pristine source (Kubusi River, Figure 9.1). The method of collection and acclimation to laboratory conditions is recorded in Section 2.2. A fourth, laboratory, source of *B. stenochorias* was used in the acute tests, cultured for approximately 2 years in artificial streams in the CAT-IWR laboratory (Haigh and Davies-Coleman, 1997 and 1999). Food in the laboratory was in the form of periphyton seeded from a local river where *B. stenochorias* are

abundant. Unglazed ceramic tiles (70 mm x 70 mm x 3 mm) lined the channel and, because they grew the same periphyton as the channels (dominated by diatoms, Section 6.2), the limpets tended to congregate on the tiles which then served as a readily movable supply of *B. stenochorias.*

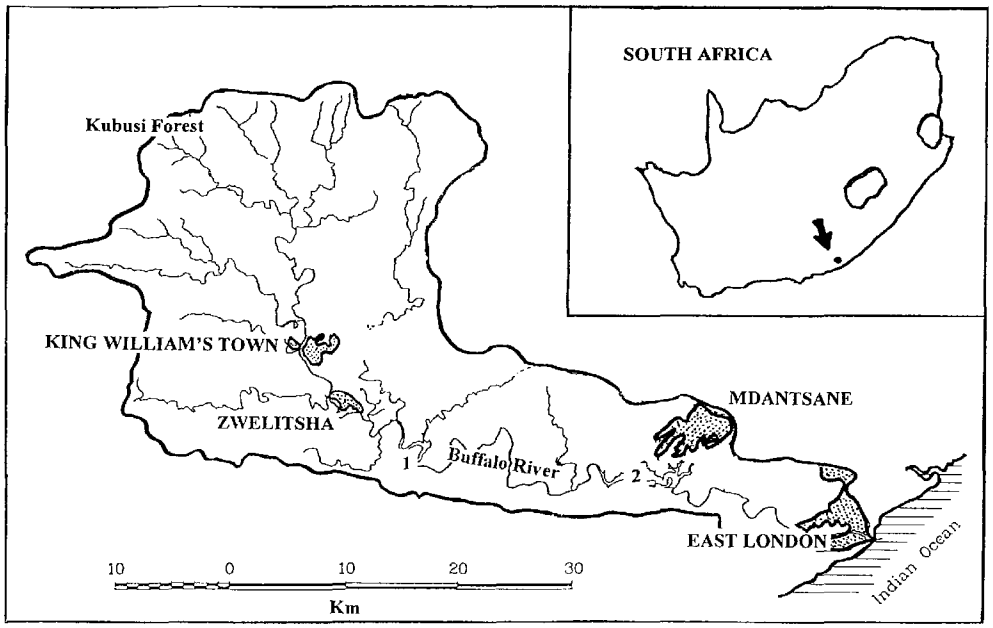
9.2. Experimental Design of Toxicity Tests

9.2.1. Apparatus

There are four basic techniques within which testing of riverine invertebrates occur: static, recirculation, renewal and flow-through tests (Rand *et al*., 1995). As *Burnupia stenochorias* inhabits both lentic and lotic waters, and with the view to developing simple, cost effective apparatus, easy to maintain and assemble, a static system was chosen for the acute, and a renewal system for the chronic toxicity testing of *B. stenochorias.* The apparatus was refined (Haigh and Davies-Coleman, 1999) and is recorded in Part One of this thesis, primarily in Chapter 6. It consisted of replicates of five litre white plastic buckets, filled with carbon filtered, dechlorinated tap water, gently aerated. White plastic lids were used to prevent evaporation.

All toxicity tests were completed in a controlled environment room maintained at 20EC (monitored daily using a rotating thermograph). Lighting was provided by biolux fluorescent tubing giving a photon flux density of 25 FE/m^2/s in a 12 hours L/D cycle (considered sufficient for diatom growth, necessary for the chronic test; Drew, 1983, cited in Lobban and Harrison, 1994). The buckets were randomly placed within the controlled environment room. Each bucket was lined with a clear plastic bag, filled with carbon filtered, dechlorinated tap water, and left to stand for 24 hours. On day one of each test, the water was then discarded, and replaced with the appropriate test solution. The plastic bags were discarded after each acute test, but remained for the duration of the chronic test for two reasons. The first was to allow algal growth to occur; the second, so that the limpets were not disturbed, as this has been found to detrimentally affect their growth rates (Haigh and Davies-Coleman, 1999). Polyethylene plastic bags have, until recently, been considered non-toxic (Sadik and Witt, 1999; Maczka *et al*., 2000). However, their plasticisers are soluble in water and will therefore leach into the solutions tested in this study.

a)



b)

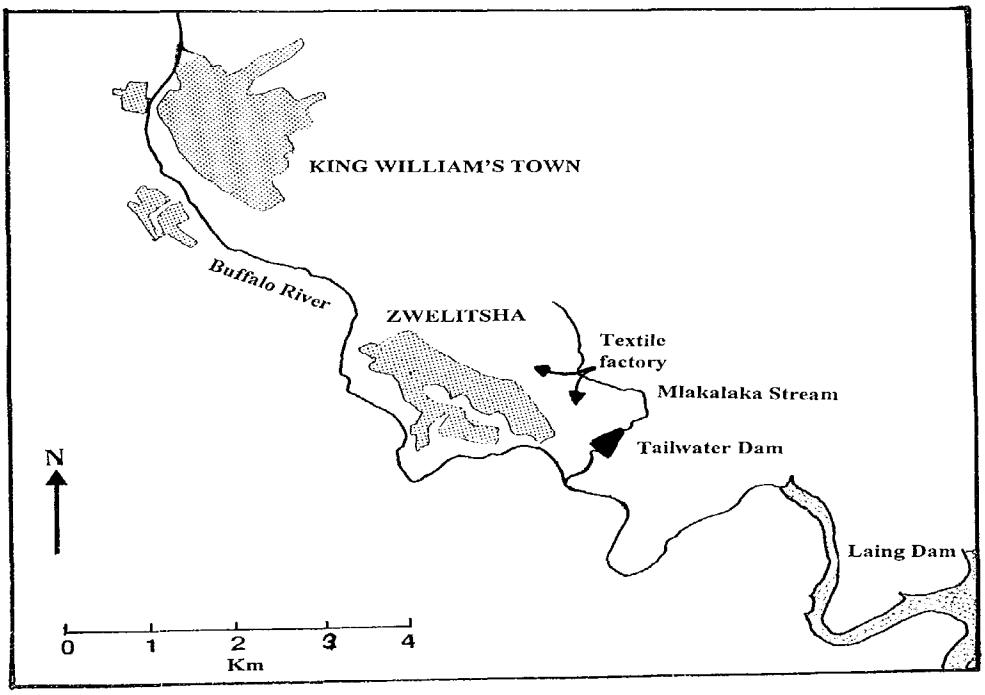


Figure 9.1.
a) The Buffalo River catchment, including the headwaters in the Kubusi Forest region, source of *Burnupia stenochorias* from a pristine flowing stream (27E20' latitude, 32E33' longitude; referred to as the Kubusi River population). The Buffalo River feeds the Laing (1) and Bridle Drift (2) Dams.
b) The proximity of the textile factory and Tailwater Dam are shown relative to the Mlakalaka Stream, which flows directly into the Buffalo River.

In the last decade, evidence has suggested that the plasticisers may mimic or interfere with mechanisms of naturally occurring hormones responsible for regulating reproduction and developmental bodily processes (Sadik and Witt, 1999). It has been suggested the chemicals as a whole can also dramatically alter normal physiological functions of the endocrine system and can affect the synthesis of hormones (Maczka *et al*., 2000). However, the use of plastic bags within the toxicity studies in this thesis was continued, primarily because it was felt their usefulness presently outweighed any potential toxicity by the plasticisers (Maczka *et al*., 2000).

Throughout the chronic tests, light aeration was provided to maintain oxygen levels at 95-100% saturation, measured during the day. This was not provided for the acute tests, and neither was food (USEPA, 1991, 1993). Food was provided for the chronic toxicity tests as the tests were only terminated after a complete life cycle. Based on the results of Chapter 6, the most suitable diet of algae grown on ceramic tiles (dominated by diatoms), plus a twice weekly portion (approximately 0.02 gm) of Nutrafin® fish food were chosen as the initial food sources. One tile per replicate was initially provided, and was not replaced at any stage of the chronic test; the further development of algal growth was dependent on the environmental conditions prevalent in each replicate bucket.

9.2.2. Dilution Medium

The source of dilution waters is dependent on the objectives of the study (Cooney, 1995). In effluent toxicity testing, it is usual for the diluent to be taken from as close to the point of effluent discharge as possible, so that dissolved oxygen, pH, hardness, salinity and other water qualities vary as naturally as possible with those of the receiving waters (APHA, 1992). In the case of the textile effluent, this would have been many kilometres upstream because of the presence around the textile factory of industrial and other contamination sources within the vicinity of King William's Town (Figure 9.1).

However, four sources of limpets from four water systems were being tested for variability of response to both the effluent and to potassium dichromate. The laboratory population was already acclimated to tap water; it was known therefore that *B. stenochorias* would survive, grow and reproduce in tap water. Carbon filtered, dechlorinated tap water was therefore chosen as diluent in all experiments, with the exception of one acute test where tap and receiving waters were compared (Tables 9.1 and 10.2). Tap water is the commonly used alternative to river water and carbon filtering removes chlorine,

metals and organic compounds, and helps to control hardness and alkalinity (Rand and Petrocelli, 1985; APHA, 1992). The primary advantage of using tap water as opposed to receiving water is that it is readily available. A disadvantage is that it is prone to potential change through time, with fluctuating metal ions, particularly copper and zinc, as well as total residual chlorine (Cooney, 1995).

9.2.3. Choice of Toxicants

Textile Effluent

The Eastern Cape Province textile factory from which the effluent used in this study was collected (Figure 9.1) disposes of its end-of-pipe effluent in a number of ways. Part of the main effluent is used in the irrigation of kikuyu grass pastures. Excess water, in the form of both irrigation runoff and ground seepage, returns to a post-irrigation dam (Tailwater Dam). The level of this dam is controlled with further irrigation of pastures, and it has an estimated minimum 14-day holding capacity. However, excessive rains and seepage through the dam wall cause a flow into the Mlakalaka Stream (Figure 9.1). Within approximately 1.5 km, this water mixes with Zwelitsha sewage effluent and reaches the Buffalo River. The Laing and Bridle Dams are a major source of water for the urban areas of East London and surrounding areas (van Ginkel *et al.*, 1996).

Collections of textile effluent were made from the Tailwater Dam wall on 18/8/1998 and 5/2/1999. The first collection gave sufficient effluent for use in all the acute tests, and one chronic toxicity test. However, with the need for a second chronic test, more was collected early in 1999; factory production and environmental conditions had changed little in the six months in between collections (samples of effluent were analyzed with each usage to check for differences in chemical composition; Section 9.2.5). Collection of post-irrigation effluent was made in a number of 25 litre plastic polypropylene containers filled to the brim to exclude air, and immediately transported (1.5 hours) to a -4EC freezer. When effluent was required for toxicity testing (usually twice weekly), one 25 litre container was defrosted in the controlled environment room (20EC) over two days and mixed thoroughly before use. The containers were not re-used.

Potassium Dichromate

Because of the nature of whole effluents, it is not always possible to identify whether observed toxicity is caused by the effluent, by receiving water contributions to the effluent, by animal health, or by

variations in the laboratory operator technique (Lee, 1980). Consequently, a reference toxicant was chosen to determine the general health, viability and sensitivity of the test organisms and assess the consistency in testing protocol implementation (USEPA, 2000). The choice of potassium dichromate was made because it is recommended both in South Africa and internationally, and because chromium ions are usually present in high concentrations in textile effluent (Brown, 1981). Justification for its use and further background information are contained in Appendix I.

9.2.4. Introduction of Limpets to Toxicity Tests

For all acute tests, once the feral *Burnupia stenochorias* were acclimated to tap water and laboratory conditions, they were randomly transferred onto disposable petri dishes, each containing a few millilitres of carbon filtered, aerated tap water [to keep the limpets wet before final placement in the test dilutions]. Laboratory bred limpets cultured in the artificial streams were already acclimated to the conditions to be used in the toxicological tests; thus ceramic food tiles from within the artificial streams were removed and limpets randomly placed onto the plastic petri dishes. When all individuals (20 in summer per petri dish and 10 in winter, limited by availability) were securely attached, one petri dish was randomly assigned to each replicate dilution. Limpets were within the range of 2.4 mm to 2.8 mm shell length.

Chronic studies, however, used newly hatched limpets approximately 1 to 4 days old (1 mm shell length or less). They were reared in the laboratory from feral population adults collected from the Botha River population (Section 2.1). These adults were acclimated to the water and laboratory conditions, and maintained at 20EC in the controlled environment room. The containers and food source used to maintain them were as described in Section 9.1. Adult samples were sent to the Natural History Museum, London (reference number not allocated to date) for future examination. Samples previously sent from this population to the Natural History Museum were positively identified as *Burnupia stenochorias*, notwithstanding the present question mark over the taxonomic identifications of the *Burnupia* species (D. Brown, pers. com., email dated 3 September,1999). Egg capsules were laid on the plastic lining of each container, providing the newly hatched limpets for use in the chronic toxicity tests. These limpets were sorted for each test by placing individuals randomly on the food tiles (described in Section 9.2.1). They were covered with a few millilitres of carbon filtered, aerated tap water. When all individuals (10 per tile) were securely attached, one tile was assigned to each replicate

within each dilution tested.

9.2.5. Environmental Conditions Monitored

Total Dissolved Salts (TDS), pH and oxygen levels were monitored before and immediately after each effluent/water change using M90 hand held probes and a Hanna Dissolved oxygen metre. In the event of supersaturation of oxygen in the effluent after defrosting to 20EC, moderate aeration was given for a few minutes with an airstone when the effluent was completely defrosted. This allowed the dissolved oxygen to reach 6 mg/l (100%), in equilibrium with the ambient air. The dissolved oxygen was measured every 2 hours for the first 8 hours after effluent was added with each experiment to guard against oxygen depletion.

Samples of defrosted effluent and carbon filtered, aerated tap water were collected with each test solution change (biweekly), frozen and couriered overnight to the Department of Water Affairs and Forestry Institute for Water Quality Studies (Pretoria), where full analyses of the major inorganic determinands and trace metals were completed. The former included total alkalinity, nitrogen and phosphate, and dissolved organic carbon. Chemical oxygen demand and biological oxygen demand were assessed by LIRI Technologies Laboratory Services (Grahamstown).

Growth of algae was not monitored over each of the 96 hour acute tests. However, as an ancillary observation to the 1999 chronic test, and to assist in understanding the effects of effluent on the limpets, differences in periphyton and bacterial growth (potential food sources) were described, although not quantified. Analogue images of each tile were recorded (as per Section 7.2.1) approximately 50 days after the start of the test. Digitized images were saved on Agfa APX100/120 film using a JEOL Winstor image archiving system. This was linked to Rhodes University's intranet backbone through which images using proprietary software (Adobe Photoshop and Paintshop Probe) were examined for the presence of diatoms, bacteria and fungal spores.

To check whether the defrosted effluent and tap water were sources of bacteria, samples were sent (November 1999) to the Eastern Cape Medical Laboratories for total plate counts of colony forming units. Similarly, an indication of differences in bacterial counts between concentrations and replicates after 60 days of growth was obtained by culturing scrapings from each container bag on agar plates.

Three scrapings from each replicate were microcentrifuged in 1.5 ml sterile distilled water;100 Fl were then plated out on agar plates, previously made from bacteriological agar mixed with equal quantities of 100% textile effluent and control water. This encouraged development of the same bacteria as were growing in the toxicity tests.

9.3. Test Procedure and Statistical Analyses

Glass beakers, cylinders, rod and pipettes were used to mix the appropriate proportions of effluent or potassium dichromate and diluent; then cleaned with a series of acid/detergent/rinsing waters (Slabbert, 1994). The following analyses were completed:

9.3.1. Acute Toxicity Tests

Acute tests over 96 hours were completed for *Burnupia stenochorias* using textile whole effluent as the toxicant with the different feral population groups (Belmont River, Botha River and Kubusi River) and the laboratory population (Table 9.1; tests using *Daphnia pulex* are given in Table 12.1). Only the Botha River limpets were compared to the Kubusi River limpets using potassium dichromate as the reference toxicant due to inadequate numbers of limpets of the appropriate size, and insufficient space in the controlled environment room (Table 9.1).

On the basis of a range finding toxicity test using *B. stenochorias*, all acute effluent toxicity tests used six concentrations of effluent diluted in a series x0.5. The dilutions used were:

e) 100%, 50%, 25%, 12.5%, 6.25% and 3.13% whole effluent, made up to 2 litres with carbon filtered, aerated tap water, with a control of carbon filtered, aerated tap water. There were 3 replicates per concentration.

Based on a range finding acute toxicity test with potassium dichromate, all tests included seven concentrations diluted with carbon filtered, dechlorinated tap water were used (diluted at x0.5). The dilutions were:

C 1.1, 2.3, 4.6, 9.2, 18.5, 37.5 and 75 ppm (mg/l) potassium dichromate, made up to 2 litres, including a control dilution of carbon filtered, aerated tap water, with 3 replicates per dilution.

Two of these concentrations gave partial mortalities. After each test the potassium dichromate solutions used in the test and the plastic bags lining the buckets were discarded as toxic waste. The stock solution of potassium dichromate was stored refrigerated at 4EC (Slabbert, 1996). Every two

hours for the first 12 hours, and thereafter at 24 hourly intervals to 96 hours, the number of limpets surviving was monitored. When a limpet was unable to attach itself to the substratum, it's foot was examined under a x10 magnification (Nikon bifocal microscope); if no movement occurred, even with mechanical stimulus (Duncan and Sturrock, 1987), it was considered dead.

Model assumptions were too stringent to use either Probit Analysis and Trimmed Spearman-Karber in the calculations of the LC_{50} values of *B. stenochorias* in effluent over 96 hours, based on too much variability in the 50% mortality of limpets between replicates and between concentrations. Chronic toxicity was considered the only alternative for estimating the toxicity of the effluent. The Probit analysis and Trimmed Spearman-Karber programmes were used to assess the toxicity of potassium dichromate to *B. stenochorias*. Calculating the seasonal or population differences between the potassium dichromate LC_{50} data was based on comparisons of the 95% upper and lower confidence limits (CL) (APHA,1992). The LC_{50} data are considered statistically different when their confidence limits do not overlap. When confidence limits do overlap, statistical differences are calculated, based on the following formula (APHA, 1992):

$$f_{1.2} = \text{antilog } \%[(\log f_1)^2 + (\log f_2)^2] \qquad \text{Equation 9.1}$$

where f_1 is calculated as the ratio of (upper 95% CL of $LC_{50}[1]$)/($LC_{50}[1]$),

and f_2 is calculated as the ratio of (upper 95% CL of $LC_{50}[2]$)/($LC_{50}[2]$).

If the ratio (upper LC_{50})/(lower LC_{50}) exceeded the value for $f_{1.2}$, the LC_{50} values were considered significantly different (APHA, 1992).

In order to quantify the innate variability of response to either potassium dichromate or effluent, the number of limpets surviving and the LC_{50} values for each replicate of each concentration were compared with each other using multivariate analysis of variance (MANOVA; Statistica, 1999). Percentage data were either arcsine or logarithm transformed, and tested for normality (Shapiro-Wilk's test; Conover, 1980), and homogeneity of variance (Bartlett's chi squared test; Snedecor and Cochrane, 1980). The mean number of limpets surviving at each concentration were also compared for seasonal and limpet source differences, based on results from MANOVA and Tukey's (parametric or non-parametric) Honest Significant Difference pair wise comparison test (Sokal and Rohlf, 1981). The programmes Statgraphics v 5.0 and Statistica (1999) were used.

9.3.2. Chronic Toxicity Tests

There were two aspects to the analyses in the chronic toxicity tests; variability of response between individuals, and the toxic effects of the effluent. Survival, growth and reproductive effort (including fecundity) were the parameters monitored over two toxicity tests, the duration of which were over a complete life cycle (Table 9.1). Mortality results within the laboratory had been previously shown to be poor over 53 days (Section 6.3.1 and Figure 6.1). However, one of the aims of testing long-term exposure to sublethal toxicants is to see the possible effects of the toxicants on the next generation. Despite the expected mortalities, therefore, chronic tests were completed until all limpets eventually died; those surviving beyond 53 days were monitored for their reproductive effort, and the F1 generation were also tested for survival and growth (see below). The first and second tests involved dilutions of:

- 100%, 10%, 3%, 1% and 0.1% effluent, made up to 2 litres with carbon filtered, aerated tap water, with a control of carbon filtered, aerated tap water. There were 4 replicates of each.
- 20%, 10%, 3% and 1% effluent, made up to 2 litres, and a control of carbon filtered, aerated tap water, with 4 replicates each.

More than four concentrations are ideal in determining toxic effects after any given time; however, due to the practicalities of insufficient newly hatched limpets less than 4 days old, a compromise was made between sufficient concentrations for toxicity effects, and sufficient replicates for adequate within-concentration comparison of responses (Gelber *et al*., 1985).

Measurements Used in the Chronic Tests

As with the acute toxicity tests, when a limpet was unable to attach itself to the substrate, its foot was examined under a x10 magnification; if no movement occurred, even with mechanical stimulus, it was considered dead.

It was suggested (Section 3.3) that both shell width and shell length be used to describe change in size (growth). The ratio of width/length best described differences between individuals. However, it was found on analysis of growth data that these ratios did not change over time suggesting an equal change in both. As a compromise between the results of Section 3.3 and 11.3.2, and as the shape of the limpet's foot area is elliptical, the most suitable relationship between length and width was chosen as the area of an ellipse: π x L/2 x W/2, where π = pi (22/7), L = shell length, W = shell

width. These dimensions were measured twice weekly, to indicate shell growth, as was mortality.

When the first egg capsules were laid in the controls in 1998, all limpets still remaining were preserved in Bouins' Fluid, and mounted in wax for microtoming into sections (Chapter 5; Hodgson and Bernard, 1992). Sexual development was to be compared on the basis of the development of the ovotestis. However, the limpets were very rubbery in texture, and despite the use of Mollifex to soften the tissue (Narsai, pers. com.), were impossible to section cleanly. The chronic test was therefore repeated in 1999/2000, and terminated when the last limpet died (100 days). Reproductive effort was instead monitored using fecundity in terms of number of egg capsules laid; number of eggs per capsule; and time to hatching. In 1998, 100% effluent gave a high mortality, and it was felt that a lower concentration (20%) should be tested, amongst other lower concentrations, in order to obtain fecundity results which more accurately reflected chronic toxicity (Suter *et al.*, 1987).

The effects of the effluent may be longer term than one generation, and may alter if the animals are returned to a non-toxic situation. To test survival and growth of the F1 generation, sixty randomly chosen newly hatched juveniles (< 1 mm shell length) from at least one replicate from each effluent concentration were then divided into two groups. One group was subjected to the same effluent concentration; the other to carbon filtered, aerated tap water (control water). Plastic 500 ml jars lined with plastic bags were used as containers, with 10 limpets per container (3 replicates). Survival, and shell length and width were monitored for 3 weeks. Food was the same as for the P1 generation.

Variability of Response of Burnupia stenochorias in the Chronic Tests

The innate variability of *Burnupia stenochorias* will be reflected in the within-concentration differences seen in survival, growth and reproduction. In order to explore these differences, survival data were first plotted from 1998 and 1999 for each concentration, and each replicate. Survival data among replicates at 10, 50 and 100% mortality, and at 21 days (chosen as a possible future end point for subchronic effect) and 43 days (approximately 10% of the *B. stenochorias* life span in the laboratory; Section 10.3.1) were converted to percentages. The percentage data were then arcsine transformed in an attempt to normalize the distribution; then tested for normality of residuals (Shapiro-Wilk's test; Statistica, 1999) and homogeneity of variances of sample means (Bartlett's test; Zar, 1984). Variability was established using either Multiple Analysis of Variance (MANOVA) if

data were normally distributed (Statistica, 1999) or Steel's Many-One Rank test if not normally distributed or with heterogeneity of variances (USEPA, 1994).

Secondly, growth was calculated as change in area of the shell aperture (ellipse). Regression lines of $Log_{(10)}$ (A + 1), where A = area of the shell aperture, versus time (to 43 days) were drawn. Data distribution around each observation time demonstrated variability within concentrations. To compare growth rate over time, between replicates, regression lines were compared statistically using Analysis of Covariance (ANCOVA, Graphpad Prism 3.0). Areas of shell apertures at 25 and 43 days were tested for normality (Shapiro Wilk's test) and homogeneity of variances (Bartlett's test); and their distributions were plotted.

Lastly, innate variability in reproductive effort between replicates was demonstrated by the fecundity values of time of first lay; number of egg capsules and eggs per capsule laid; and rate of lay as capsules/day/limpet. Student 't' tests (Sokal and Rohlf, 1981) and the coefficients of variance and standard deviations were compared for differences between replicates (Statistica, 1999).

Toxicity of Effluent to Burnupia stenochorias in the Chronic Tests

To estimate the sublethal effects of the effluent, the following estimates were made:

C the Lethal Concentration (LC) values using the point estimation techniques of Probit and Trimmed Spearman-Karber analysis (APHA, 1992) for all data at 21, 30 and 43 days (LC_{50}), and at first (2.5% = $LC_{2.5}$) and 10% (LC_{10}) mortality in the controls.

(x) the NOEC and LOEC values (No Observed Effect Concentration, the maximum dose that does not produce a statistically significant harmful effect, also expressed as the No Observed Adverse Effect Concentration; and Least or Lowest Observed Effect Concentration, the minimum dose used in a test which produces a statistically significant deleterious effect, also expressed as the Least or Lowest Observed Adverse Effect Concentration, Figure 9.2) using hypothesis testing by way of Analysis of Variance (ANOVA) and Steel's Many-One Rank test (Snedecor and Cochrane, 1980; APHA, 1992; USEPA, 1994).

Parametric statistical methods require data from each test to be normally distributed with homogeneity of variance. In order to normalize data, percentage survivals were arcsine transformed and analyzed for normality (Shapiro-Wilk's test; Zar, 1974) and homogeneity of variances (Bartlett's

chi squared test; Zar, 1974). Steel's Many-One Rank test (USEPA, 1994) was used to find the NOEC using survival data at 43 days. Similarly, effects of the effluent on growth were analyzed. Growth regression lines for each concentration used were compared between concentrations using ANCOVAs (Graphpad Prism 3.0). Sizes of limpets (based on the area of the shell aperture) at 21 and 43 days were compared between concentrations and the control (ANOVA; Statistica, 1999). The effects of effluent on the fecundity were analyzed using two sample Student's 't' tests, assuming unequal variances (Statistica, 1999). The rate of egg capsule lay was calculated as follows :

$$\frac{\text{(number of capsules laid per concentration [four replicates])}}{\text{(number of lay days) x (number of limpets alive each day)}}$$

This is equivalent to the number of capsules laid per limpet day. Finally, the F1 generation were used to consider the potential long term effect of the textile effluent. Survival data were plotted through time (1 to 21 days). Regression equations of the growth of these limpets were calculated for each concentration, and compared between effluent and tap water, and between concentrations of effluent and the control (ANCOVA, Sokal and Rohlf, 1981; Graphpad Prism 3.0).

Table 9.1. List of toxicological tests completed with *Burnupia stenochorias* in 1998 and 1999. The effluent used refers to the defrosted textile effluent. * represents what would be, under natural conditions, the peak breeding period in early summer, with F1 generations laid in approximately the mid summer period.

Type of toxicity test	Toxicant	Diluent	Duration of test	Population Source	Starting Date	Season represented
Acute	effluent range finding	tap	96 hours	Belmont River	31 / 8 / 98	early summer
	effluent	tap	96 hours	Belmont River	7 / 9 / 98	
	effluent	tap	96 hours	Laboratory	7 / 9 / 98	
	effluent	tap	96 hours	Botha River	14 / 9 / 98	
	effluent	tap	96 hours	Kubusi River	23 / 9 / 98	
	effluent	tap / receiving	96 hours	Botha River	14 / 12 / 98	mid summer
	effluent	tap	96 hours	Kubusi River	9 / 2 / 99	
	effluent	tap	96 hours	Laboratory	9 / 2 / 99	
	effluent	tap	96 hours	Botha River	16 / 2 / 99	
	effluent	tap	96 hours	Belmont River	21 / 2 / 99	
	effluent	tap	96 hours	Botha River	2 / 8 / 99	winter
	effluent	tap	96 hours	Belmont River	2 / 8 / 99	
	effluent	tap	96 hours	Kubusi River	9 / 8 / 99	
	effluent	tap	96 hours	Laboratory	9 / 8 / 99	
Chronic	effluent	tap	100 days	Botha River	16 / 10 / 98	early to mid summer
	effluent	tap	122 days	Botha River	2 / 9 / 99	
Acute	$K_2Cr_2O_7$ range finding	tap	96 hours	Botha River	1 / 9 / 98	early summer
	$K_2Cr_2O_7$	tap	96 hours	Botha River	14 / 9 / 98	
	$K_2Cr_2O_7$	tap	96 hours	Kubusi River	23 / 9 / 98	
	$K_2Cr_2O_7$	tap	96 hours	Botha River	16 / 2 / 99	mid summer
	$K_2Cr_2O_7$	tap	96 hours	Botha River	16 / 7 / 99	winter

CHAPTER 10. ACUTE TOXICITY TESTS

Key issues: How variable is the response of *Burnupia stenochorias* to the effluent compared to a reference toxicant? Can *B. stenochorias* be used to test the toxicity of textile whole effluent over 96 hours? (Figure 1.2)

10.1. INTRODUCTION

Acute toxicity tests are designed to evaluate the relative toxicity of various concentrations of a chemical or whole effluent over a short-term exposure period (Rand, 1995). Death is the commonly used criterion for endpoint determination.

Although sources of inconsistencies in test results include both inter- and intra-laboratory and inter-and intra-test factors (Section 9.2.3), of concern in this study is the inter-and intra-test variability in lethal response. Sources of end point variability are many, including both uncertainty and innate variability (Ausley, 1995; Chapter 8), but consensus still needs to be reached as to how much variability is acceptable, before data can be used to define water quality criteria. The natural genetic heterogeneity inherent in any natural population may be responsible for variability in toxicity test results (Moore, 1990; Naylor *et al*., 1990; Møller *et al*., 1994). Toxicity responses may be based on selective breeding in response to certain chemical and physical conditions, age, previous exposure, or other stressors present in the system (Ausley, 1995; Palmer and Scherman, 2000). Inherent variation is considered greater in feral than in laboratory bred populations (Snyder *et al.*, 1991; Ausley, 1995; Hickey and Martin, 1995).Where wild populations are used as test organisms, the degree of variability demonstrated in the various physiological responses between populations to a toxicant at different times of the year is generally unknown: Dillon (2000) suggests there is a great deal of genetic variance for fitness traits hidden in any sample of 30 week old gastropods. Laboratory bred molluscs have shown lower resistance towards toxicants, for example molluscicides (Yousif, in Brackenbury, 1997), than those collected from the field, giving rise to the question of response differences between the choice of feral and laboratory populations as test organisms. The tolerance of feral populations to toxicants, as with variability of response, is also usually unknown and is likely to vary between species (Chapman, 1996). Feral populations of *Burnupia stenochorias* have been chosen as toxicity test organisms for a number of reasons, primarily because indigenous fauna are representative of local conditions, and are therefore more useful in site specific toxicity tests. Response differences between

feral and laboratory responses were therefore examined.

The choice of animal species used in characterizing the acute toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test (USEPA, 1993). However, it is essential that good quality test organisms be used (Lanno *et al.*, 1989). This refers to not only the health and age of the organism, which should be known, but also its variability of both inter- and intra-laboratory responses. One of the main problems in ecotoxicology is the observed high variability of results, even with standard species in standard laboratories (Masters *et al.*, 1991; Blok and Balk, 1995; Cooney, 1995; USEPA, 2000). It has frequently been demonstrated that differences as large as orders of magnitude in toxicity exist between different life history stages (Hoekstra, 1993; Timmermans, 1993). Size of test animal (Newman *et al.*, 1994), in relation to different age and reproductive development, potentially related to seasonal differences, may affect responses, depending on the mode of toxicant action (Appleton *et al.*, 1992; Lam, 1996a). In the case of *B. stenochorias*, larger, sexually mature limpets represent those expending more energy into reproductive effort than growth. Smaller, pre-sexually developed limpets (Chapter 5) expend energies into both growth and the development of the reproductive organs themselves. Sexual developing life stages are usually considered more susceptible to toxicants (USEPA, 1994; Rainbow and Dallinger, 1993; Chapter 5), and this stage was therefore selected for the acute toxicity tests.

If an indigenous organism is to be used for site specific toxicity testing, for both source and resource directed measures (Chapter 8), the organism should, amongst other criteria, exhibit a constant response to the same test chemical. It is therefore a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results for a toxicity test species with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes (USEPA, 1994). To meet this requirement, the intra-laboratory precision, expressed as coefficients of variation, of each type of test to be used in the laboratory should be determined, using the reference toxicant at the same concentration with the same test organism species (Lee, 1980; USEPA, 1993). A reference toxicant is described as a chemical which deliberately causes known adverse effects (Rand, 1995; Appendix A). Information collected is used to determine the general health, viability and sensitivity of the test organisms and to assess consistency in testing protocol implementation (Rand, 1995). It is acknowledged that reference chemicals prepared from pure chemicals may not always be representative of effluents, but there is too

much variability in effluents to specify a representative effluent (Lee, 1980; USEPA, 1994). The regular testing with reference toxicants is a concept followed in all laboratories in South Africa where test organisms are cultured, particularly with the standard laboratory organism *Daphnia pulex* (Slabbert *et al*, 1998; Chapter 12).

Defining the variability in response of *B. stenochorias* to both a reference toxicant and the textile whole effluent was a pre-requisite for interpretation of effluent effects on mortality and subchronic toxicity. Two populations were compared in their responses to acute levels of potassium dichromate over 96 hours, with seasonal differences also examined (Figure 1.2). The acute responses to textile whole effluent were then compared between four populations, sampled and tested during three seasons (Figure 1.2).

10.2. MATERIALS AND METHODS

Test limpets were sourced from a laboratory population (Section 9.1) and three feral populations, namely the Belmont River representing a population from an impacted flowing environment (Section 2.1 and Figure 2.1); the Botha River representing a population from a pristine non-flowing environment (Section 2.1 and Figure 2.1); and the Kubusi River representing a population from a pristine flowing environment (Section 9.1 and Figure 9.1). The methods of collection and acclimation to laboratory conditions where applicable are recorded in Sections 9.1 and 9.2.2. The experimental design (Section 9.2), including the apparatus used (Section 9.2.1), dilution medium (Section 9.2.2), the introduction of the limpets to the test chambers (Section 9.2.4) and the environmental conditions monitored (Section 9.2.5) did not differ between tests. The collection of the textile whole effluent was made once for all acute toxicity tests, from the post-irrigation dam, frozen and sub-samples defrosted when needed (Section 9.2.3). The full composition of each defrosted effluent sample was analysed, as was the (potential) degradation of effluent over a four day period (the duration of an acute test) as this may contribute to variable toxicity results. Acute toxicity tests were completed using all four populations of limpets. Collections of each population were tested in three seasons were compared, namely early summer (when egg lay appears to be at a peak; Figure 4.3), mid summer and winter (Table 9.1). Only the two pristine populations (Botha and Kubusi Rivers) during early summer, and Botha River limpets in winter (Table 9.1, Section 9.2.3) were tested against potassium dichromate. LC_{50}s were calculated for the whole effluent and the potassium dichromate, and the variability of

toxicity response of *B. stenochorias* between populations during each season and between seasons was compared (Section 9.3.1).

10.3. RESULTS

During the entire period of acclimation, and within hours of placing food tiles in the plastic bags in which they were held, *Burnupia stenochorias* fed on the periphyton provided. This suggests they were in a reasonable state of health before use (after 10 days acclimation to laboratory conditions) in the toxicity tests. The mean shell lengths of limpets used from each population and within each concentration were not significantly different to each other (paired Student's 't' tests, $p > 0.05$; Table 10.1).

Table 10.1. Initial sizes (shell length, mm) of *Burnupia stenochorias* selected from four populations for use in the textile whole effluent and potassium dichromate 96 hour toxicity tests. St. Dev. = ± standard deviation; St. Error = ± standard error.

Population	Mean	Range	± St. Dev.	± St. Error
Botha River	2.7	0.5	0.26	0.08
Kubusi River	2.6	0.8	0.32	0.11
Belmont River	2.6	0.8	0.27	0.09
Laboratory	2.4	0.6	0.23	0.07

10.3.1. Textile Whole Effluent

The Botha River (non-flowing, pristine) had significantly higher levels (from 4 to 10 times the amounts; Student's 't' test, $p < 0.05$) of nitrates and nitrites, chloride, sodium, potassium, dissolved organic carbon and alkalinity, than the tap water (diluent) (Table 10.2)[4]. The Kubusi River (flowing,

[4]TWQR = Target Water Quality Range (Table 10.2) is not a water quality criterion but a management objective which has been derived from quantitative and qualitative criteria. This is the range of concentrations or levels within or below which no measurable adverse effects are expected on the health of aquatic ecosystems (DWAF, 1996). The Chronic Effect Value (CEV) is defined as that concentration or level of a constituent at or below which there is expected to be a significant probability of measurable chronic effects to up to 5% of the species in the aquatic community (DWAF, 1996). The Acute Effect Value (AEV) is defined as

pristine), however, had significantly lower (approximately 5 times less) electrical conductivity and sodium than the tap water, but three to four times the nitrates, nitrites, and silicon (Table 10.2). Defrosted textile effluent had significantly higher levels of most metals and inorganic ions (in particular sodium and chloride) than in the Botha and Kubusi River water (Table 10.2). Effluent sub-samples did not show change in chemical composition over four days (the duration of the acute tests).

Toxicity

Because the data did not possess Probit characteristics (Appendix A), Kruskal-Wallis and Tukey's Honest Significant Difference (non-parametric) tests were primarily used in comparisons. Data were logarithmically (Ln) transformed.

There was a significant difference in *B. stenochorias* mortalities between concentrations when all data at 96 hours were compared (Kruskal-Wallis $chi^2 = 49.33$, df = 6, $p < 0.001$). Limpet mortalities compared within each source population (location) and season were also significantly different (p # 0.01), with mortality generally increasing with increasing concentration of textile whole effluent (Figures 10.1, 10.2 and 10.3). 100% mortality did not occur in any replicate after 96 hours. With the exception within some replicates in the higher concentrations of effluent, 50% mortality did not occur, and therefore no acute LC_{50} values were calculated. Only occasionally did monotonicity of response occur, but this was not correlated with source of limpet or season (Figure 10.1). Mortality in the controls was never above 10%.

The 20-40 hour period of toxicity tests and after 72 hours appeared to be important periods for increased toxicity of *B. stenochorias* amongst many of the replicates during mid and early summer, when mortality rates generally increased (Figure 10.3). Mortality rates within each dilution of effluent or the controls over time were calculated by converting the cumulative mortalities to regression lines. Regression line analysis (Graphpad Prism 3.0) showed no differences between locations or between

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that concentration or level of a constituent at or below which there is expected to be a significant probability of measurable acute toxic effects in up to 5% of the species in the aquatic community (DWAF, 1996). The TWQR, CEV and AEV values represent stringent South African water quality values based on safety factors of international criteria, and are used in effluent and chemical hazard assessment in South Africa, where rivers are classified according to their state of health (Palmer, 1999).

seasons at each location (p = 1) with the exception of 100% effluent tested during the mid summer and winter periods ($F_{(1,14)}$ = 5.77, p = 0.028), and 50% effluent during early summer and winter ($F_{(1,14)}$ = 5.03, p = 0.031).

Variability of Response

Replicates within concentrations at each season and location did not differ, although the most variability in mortality between replicates was most consistently seen with limpets from the Kubusi River (Figure 10.2). However, when amalgamating sources of limpet population [location] data at 96 hours with concentrations as covariates, significant differences were seen between the early summer and winter mortalities (highest and lowest respectively) at 96 hours (chi^2 = 28.81, df = 3, p < 0.001; Figures 10.1 to 10.4). A comparison of limpet mortalities between locations gave non-significant differences (chi^2 = 8.31, df = 3, p = 0.04). When 96 hour data from each location were amalgamated and compared for seasonal differences using Kruskal-Wallis multiple comparisons and Tukey's non-parametric honest significant difference test**,** very significant seasonal differences were seen for the Botha River (non-flowing, pristine; chi^2 = 13.28, p = 0.001), Kubusi River (flowing, pristine; chi^2 = 9.72, p = 0.008) and Belmont River (flowing, non-pristine; chi^2 = 21.59, p = < 0.001) populations. The mortalities of the Laboratory populations showed a weakly significantly difference between seasons (chi^2 = 2.33, p = 0.05) (Figure 10.1). Seasonal limpet mortalities were compared within each location, and with other locations (Tukey's non-parametric honest significant difference test, where á = 0.05; and Contingency Tables [Sokal and Rohlf, 1981; Zar, 1984]). Seasonal differences were shown to be mainly due to low winter mortalities with limpets from the Botha River and Belmont River populations (Table 10.3; Figure 10.1).

10.3.2. Potassium Dichromate

pH levels of the potassium dichromate solutions did not range beyond 0.8 units between replicates or concentrations. Total dissolved salts however, were significantly different in range (Student's 't' test, p < 0.5) between samples from the Botha River in early summer and winter, although not significantly different between concentrations used during each of the respective seasons (Table 10.4). Metal and inorganic ion concentrations in the tap water remained within acceptable limits with the exception of aluminium and chloride ions, and ammonium and nitrate levels, which were highest during the winter toxicity test (Table 10.5). Oxygen levels remained at 95-100% saturation, measured during the day,

and so aeration was not considered necessary.

Limpets immediately crawled out of the petri dishes with higher concentrations of potassium dichromate [this was not observed when effluent was tested as a toxicant]. Control mortalities never went above 10% (Figure 10.5). Mortalities within the potassium dichromate dilutions displayed monotonicity (Figure 10.5) and analyses using Trimmed Spearman-Kärber gave LC_{50} values for all populations tested (Table 10.6). However, percentage trims were high (13.3-33.3%; Hamilton *et al*., 1977). Kubusi early summer limpets were the most inconsistent in mortalities between replicates, as per with effluent. Botha River limpets demonstrated the least monotonicity in mortalities, but winter Botha River limpets were the most consistent in mortalities and therefore the LC_{50} calculations. The upper and lower 95% confidence limits obtained with each calculation of the LC_{50} overlapped with each other between locations and seasons, suggesting no significant differences (Figure 10.6). However, the intervals were large compared to each LC_{50} value (Table 10.6).

The LC_{50} values for potassium dichromate were mathematically compared between test populations and between replicates of the same test population, using Equation 9.1. The only significant difference was found between the LC_{50} values of the test populations Botha River early summer and winter (Table 10.6). Although the LC_{50} values are predictions based on the toxicity curve and concentrations tested and not actual numbers, a Tukey's honest significant difference test (ANOVA; Statistica, 1999) gave the same results ($p = 0.011$). The LC_{50} for potassium dichromate using *B. stenochorias* as the test organism can therefore be said to be within the range of 7.6 parts per million (ppm) potassium dichromate in early summer, and 16.9 ppm in winter (Table 10.6).

Comparing arcsine transformed mortalities of Botha River *B. stenochorias* populations at each concentration (Figure 10.5), there was a significant difference between early summer, mid summer and winter mortalities ($F_{(2,68)} = 3.62$, $p = 0.03$). A Tukey's honest significant difference test suggested differences in mortalities occurred between the early summer and the winter populations ($p = 0.027$), possibly at the 1.1 ppm or 18.5 ppm dilutions (Figure 10.5). However, if season and concentration are compared in a two way comparison with arcsine transformed mortalities, then $F_{(14,48)} = 1.02$, $p = 0.45$ (not significant). Comparing Botha River with Kubusi River limpets, a non-significant result in mortalities

Table 10.2. Major inorganic determinands and trace metals in carbon filtered tap water (diluent), Botha River and Kubusi River samples, and defrosted textile effluent. Number of samples tested = 4 (river), and 24 (effluent and tap water). Cobalt, beryllium, nickel, molybdenum, cadmium, mercury and lead were not detectable. TWQR = Target Water Quality Range; CEV = Chronic Effect Value; AEV = Acute Effect Value (DWAF, 1996). [1] refers to the USA recommended sodium content of drinking water (Bunce, 1994). [a] = significantly different to tap water (Student's 't' test, $p < 0.5$).

Determinand	mg/litre	Tap water	Botha River	Kubusi River	Effluent tested	TWQR mg/l	CEV mg/l	AEV mg/l
pH (in pH units)	Range	7.8-8.4	8.4-8.6	7.7-7.9	8.1-8.9			
	Mean	7.9	8.5	7.8	8.6			
Electrical Conductivity (mS/m)-	Range	33.8-54.1	89.2-119.9	9.4-9.7	203.1-372.3			
	Mean	49.5	95.6	9.6	276.5 [a]			
Ammonium as N	Range	0.04-0.1	0.04-0.1	0.04-0.07	0.21-7.13	0.007	0.015	0.1
	Mean	0.06	0.07	0.06	1.01			
Nitrate and Nitrite as N	Range	0.04-0.07	0.04-0.30	0.13-0.27	0.04-0.17	0.007	0.015	0.1
	Mean	0.04	0.15 [a]	0.19 [a]	0.08			
Kjeldahl Nitrogen as N	Range	0.22-0.28	1.00-1.01	0.13-0.25	8.28-11.28	0.007	0.015	0.1
	Mean	0.24	1.01	0.21	10.12 [a]			
Fluoride as F	Range	0.1-0.2	0.1-0.2	0.1	0.3-0.4	#0.75	1.5	2.54
	Mean	0.2	0.2	0.1	0.4			
Chloride as Cl	Range	10.0-15.5	217.0-291.0	<10	104.0-318.0	#0.0002	0	0
	Mean	13.9	242.5 [a]	<10	192.0 [a]			
Sulphate as SO4	Range	17-31	9-20	4-9	177-515			
	Mean	26	15	7	307 [a]			
Alkalinity as Calcium Carbonate	Range	14.0-42.0	70.0-111.0	27.0-28.0	680.0-1250.0			
	Mean	28.1	85.0 [a]	27.4	820 .0[a]			
Total Phosphate	Range	0.020.05	0.04-0.09	0.03	2.67-4.0			
	Mean	0.03	0.06	0.03	3.52 [a]			
Ortho Phosphate as P	Range	0.009-0.014	0.018-0.060	0.019-0.024	1.900-5.600			
	Mean	0.012	0.036	0.022	2.211			
Sodium as Na	Range	35-65	129-162	7-9	403-879	[<100 ppm][1]		
	Mean	44	136	8	648 .0[a]			

Table 10.2 continued

Determinand	mg/litre	Tap water	Botha River	Kubusi River	Effluent tested	TWQR mg/l	CEV mg/l	AEV mg/l
Calcium as Ca	Range	11.0-18.0	17.0-20.0	5.0-7.0	3.8-55.0			
	Mean	14.7	19.0	5.7	19.2			
Magnesium as Mg	Range	6.0-12.0	18.0-27.0	2.0-3.0	17.0-32.0			
	Mean	8.3	22.5	2.7	25.7 [a]			
Potassium as K	Range	1.4-2.3	8.0-14.6	0.9-1.3	15.7-26.2			
	Mean	1.6	10.7 [a]	1.0	20.3 [a]			
Silicon as Si	Range	1.4-4.5	0.5-1.4	10.5-11.1	8.6-19.2			
	Mean	2.9	0.8	10.7	11.6			
Aluminium, acid soluble	Range	0.020-0.230	<0.020	0.085-0.266	0.056-0.133	#0.01	0.01	0.1
	Mean	0.07	<0.02	0.19	0.04			
Titanium, acid soluble	Range	<0.003	<0.003	<0.003	0.007-0.3			
	Mean	<0.003	<0.003	<0.003	0.024			
Chromium, acid soluble	Range	0.005-0.022	<0.005	<0.003	<0.007-0.032	#0.007	0.014	0.2
	Mean	0.011	<0.005	<0.003	0.024			
Manganese, dissolved	Range	<0.001-0.207	<0.001	<0.001	0.201-0.4	#0.18	0.37	1.3
	Mean	0.013	<0.001	<0.001	0.279 [a]			
Iron, acid soluble	Range	<0.005-1.094	0.095-0.181	0.196-0.257	0.295-0.817			
	Mean	0.239	0.132	0.224	0.598			
Copper, acid soluble	Range	<0.005	<0.005	<0.005	<0.011-0.073	#0.0014	0	0.01
	Mean	<0.005	<0.005	<0.005	0.025			
Zinc, acid soluble	Range	<0.005-0.12	<0.005	0.005-0.036	0.026-0.16	#0.002	0.036	0.36
	Mean	0.036	<0.005	0.02	0.065			
Barium, acid soluble	Range	0.002-0.042	<0.002	0.004-0.01	<0.004-0.058			
	Mean	0.029	<0.002	0.007	0.044			
Dissolved Organic Carbon as C	Range	3.4-5.6	12-12.1	0.4-2.5	51.7-75.3			
	Mean	4.9	12.1 [a]	1.6	62.3 [a]			

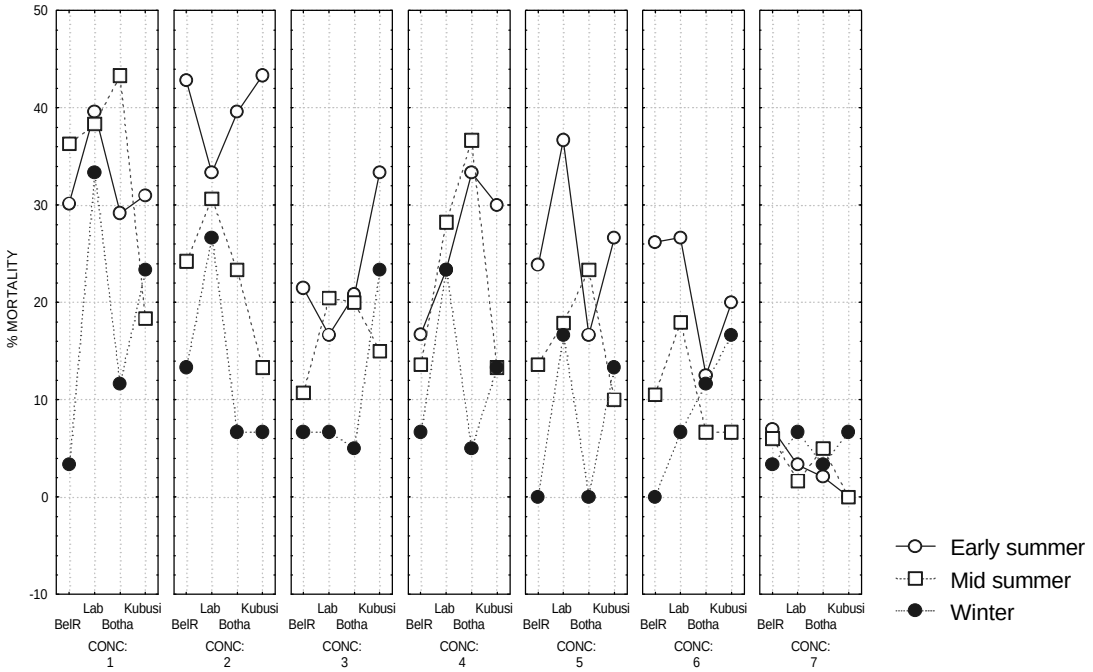


Figure 10.1. Mean (n = 4) percentage mortalities of *Burnupia stenochorias* after 96 hours' exposure to different concentrations of textile whole effluent, showing the variation between responses of each population source within each dilution of effluent. Concentrations of effluent are represented by the numbers 1 (100% effluent), 2 (50% effluent), 3 (25% effluent), 4 (12.5% effluent), 5 (6.25% effluent) and 6 (3.3% effluent) with 7 the control of dechlorinated tap water. BelR = Belmont River, Lab = Laboratory, Botha = Botha River, and Kubusi = Kubusi River populations.

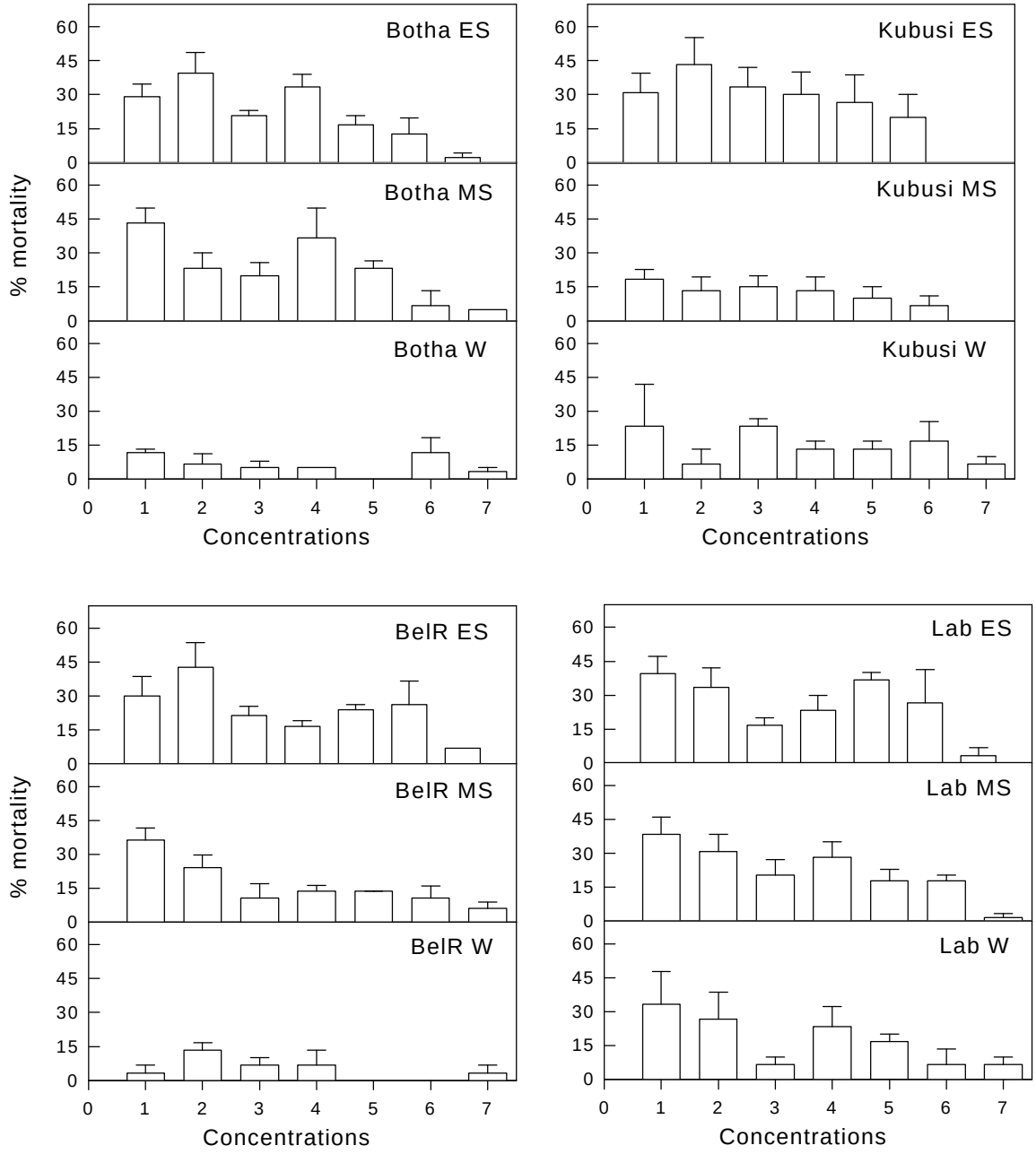


Figure 10.2. Mean ± standard error (n = 4) percentage mortalities of *Burnupia stenochorias* after 96 hours' exposure to different concentrations of textile whole effluent. Concentrations of effluent are represented by the numbers 1 (100% effluent), 2 (50% effluent), 3 (25% effluent), 4 (12.5% effluent), 5 (6.25% effluent), and 6 (3.3% effluent) with 7 the control of dechlorinated tap water. Graphs are grouped according to the location from which limpets were collected; Botha = Botha River, Kubusi = Kubusi Forest stream, BelR = Belmont River, Lab = Laboratory bred population. Seasons tested are represented by ES (early summer), MS (mid summer) and W (winter).

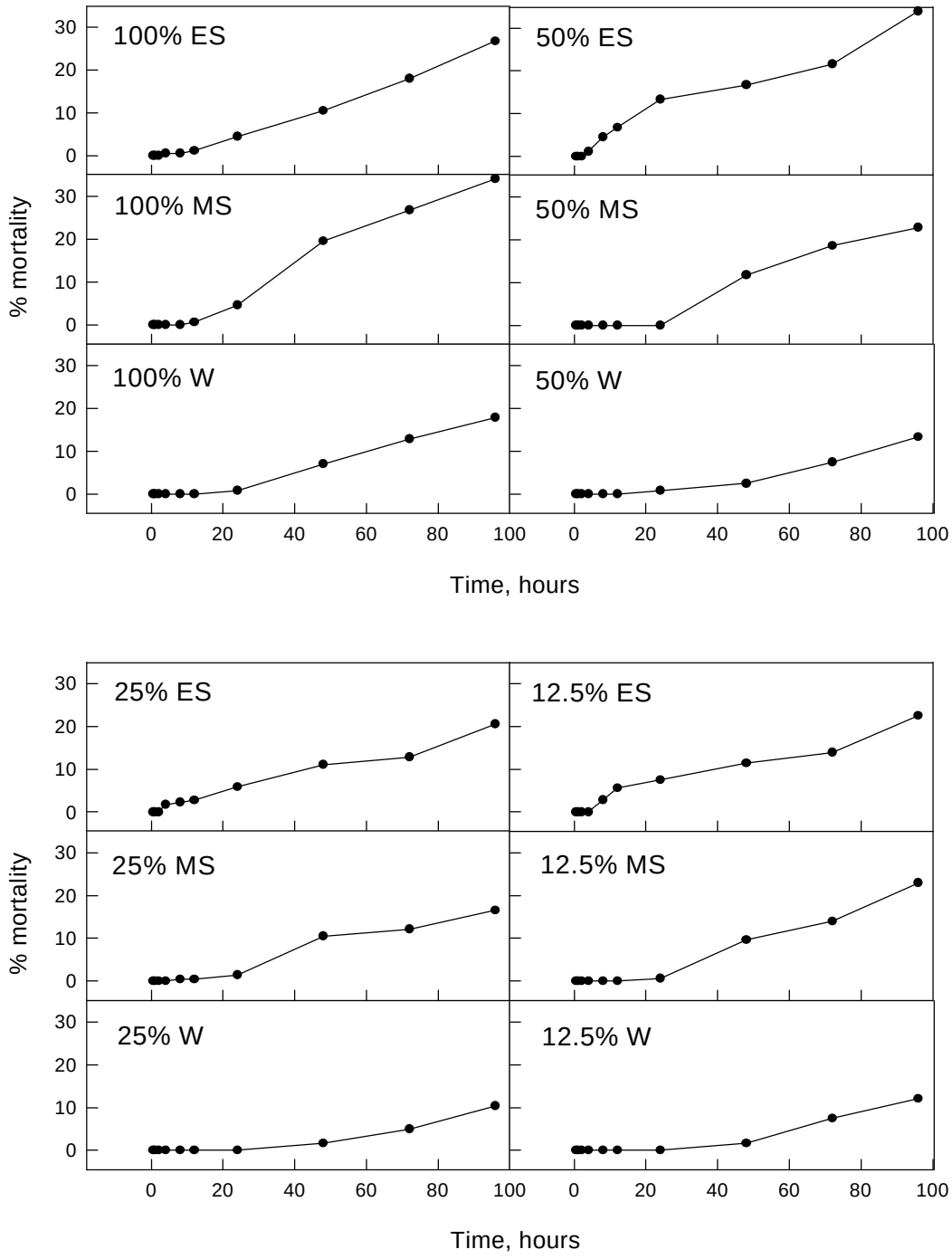


Figure 10.3. Mean (n = 12) cumulative percentage mortality of *Burnupia stenochorias* over 96 hours when maintained in textile whole effluent (100%, 50%, 25%, 12.5%, 6.5% or 3.3%) or in the controls of dechlorinated tap water. Seasons are represented by ES (early summer), MS (mid summer) and W (winter). Data for location and replicates are combined.

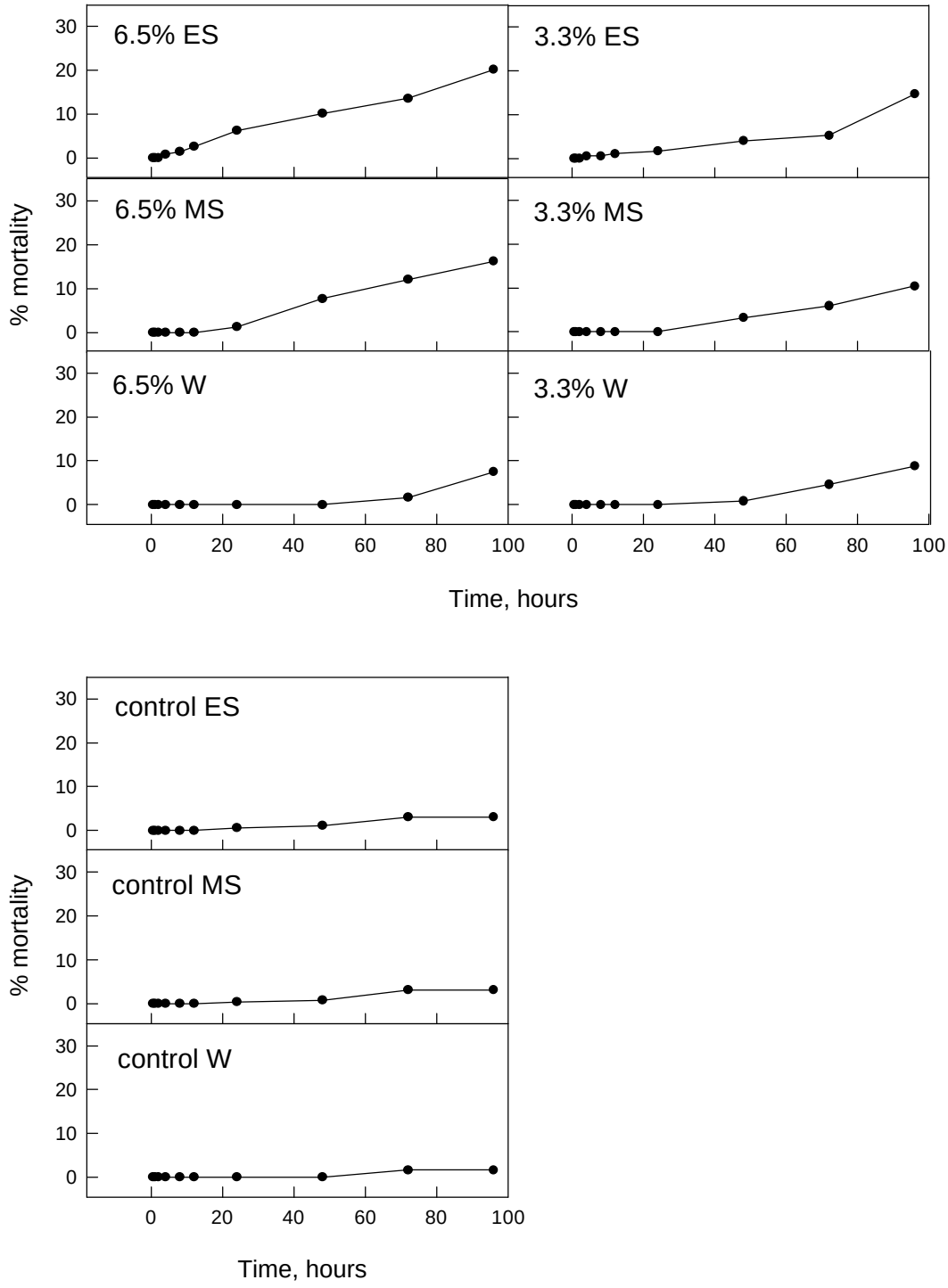
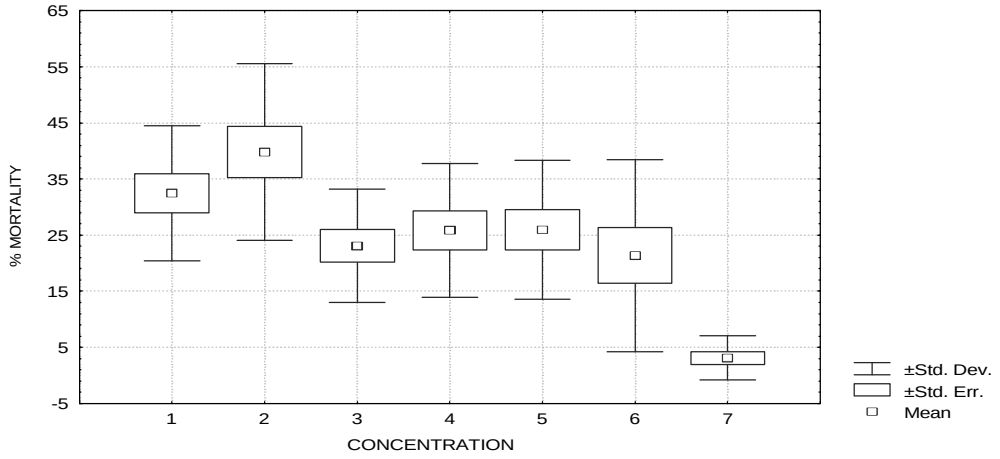


Figure 10.3. continued.

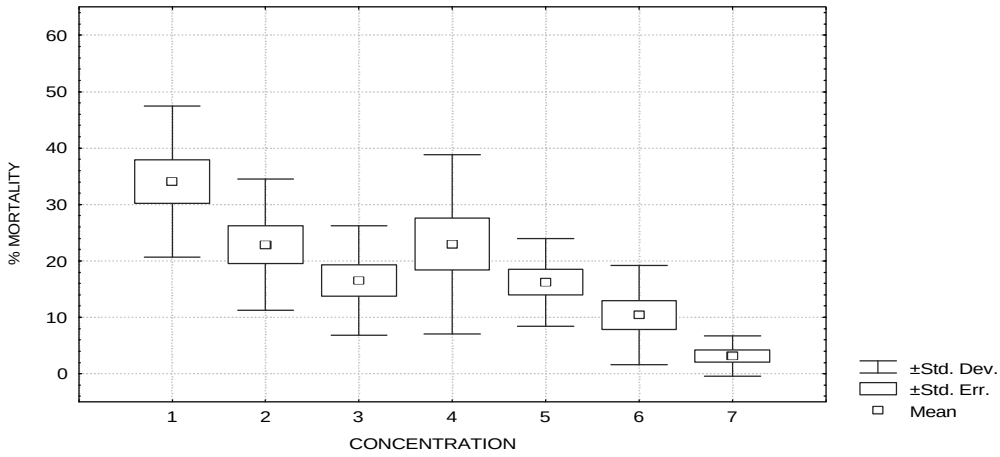
Table 10.3. Significant differences between mortalities of *Burnupia stenochorias* from the four sources of limpet, with toxicity tests completed in early summer (ES), mid summer (MS) and winter (W). Botha = Botha River, Kub = Kubusi River, BelR = Belmont River and Lab = Laboratory populations. x indicates degree of significance where xxx = $p < 0.001$, xx = $p < 0.01$, x = $p < 0.05$.

	Botha ES	Botha MS	Botha W	Kub ES	Kub MS	Kub W	BelR ES	BelR MS	BelR W	Lab ES	Lab MS	Lab W
Botha ES			xxx						xxx			
Botha MS			xxx						xxx			
Botha W	xxx	xxx					xx				x	
Kub ES									xxx			
Kub MS									xx			
Kub W									xx			
BelR ES			xx						xxx			
BelR MS									xxx			
BelR W	xxx	xxx		xxx	xx	xx	xxx	xxx		xxx	xxx	xxx
Lab ES									xxx			
Lab MS			x						xxx			
Lab W									xxx			

a)



b)



c)

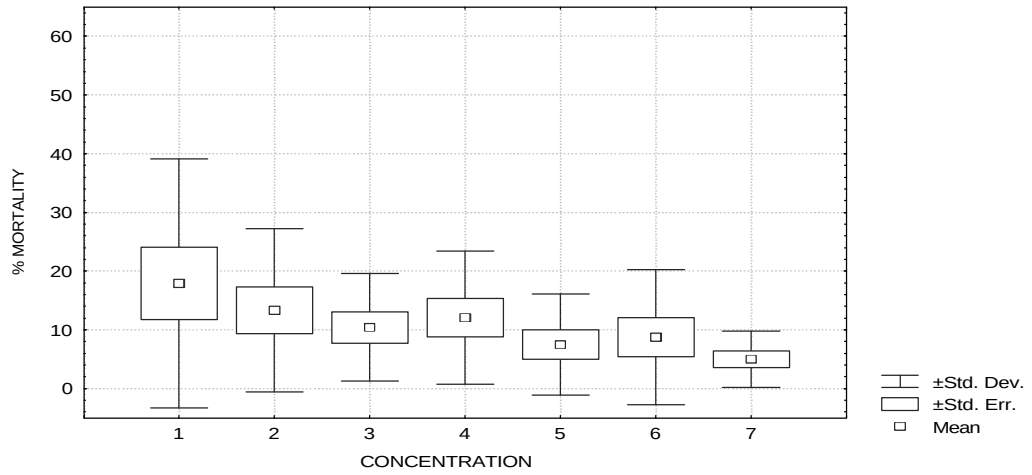


Figure 10.4. Mean (n = 16) percentage mortalities of *Burnupia stenochorias* after 96 hours' exposure to different concentrations of textile whole effluent tested during each of the seasons, with the data from the four limpet populations combined. a) = early summer, b) = mid summer, c) = winter. Concentrations of effluent are represented by the numbers 1 (100% effluent), 2 (50% effluent), 3 (25% effluent), 4 (12.5% effluent), 5 (6.25% effluent) and 6 (3.3% effluent) with 7 the control of dechlorinated tap water.

Table 10.4. pH values and total dissolved salts (TDS, mg/l) monitored over the 96 hours of the potassium dichromate ($K_2Cr_2O_7$) toxicity tests using *Burnupia stenochorias*. Mean and range $K_2Cr_2O_7$ refer to the test dilutions used, and are not specified for each concentration as they did not differ significantly between replicates or concentrations with each population of limpets tested. Oxygen levels did not go below 95% and are therefore not recorded here. [a] = significantly different to early summer TDS values (Student's 't' test, $p < 0.05$); [b] = ranges were within 0.01 pH unit between replicates.

	Population	Season	Mean, $K_2Cr_2O_7$	Range, $K_2Cr_2O_7$	Mean, tap water [b]
pH	Botha 14/9/98	early summer	7.34	7.30 - 7.60	7.38
	Botha 16/2/99	mid summer	7.75	7.60 - 7.90	7.9
	Botha 7/8/99	winter	8	7.80 - 8.20	8.12
	Kubusi 23/9/98	early summer	7.35	7.28 - 7.49	7.49
TDS	Botha 14/9/98	early summer	287	282 - 304	290
	Botha 16/2/99	mid summer	256	251 - 266	252
	Botha 7/8/99	winter	210 [a]	188 - 225	201[a]
	Kubusi 23/9/98	early summer	290	284 - 302	291

Table 10.5. Major inorganic determinands and trace metals in the carbon filtered tap water used as diluent for the potassium dichromate tests. Metals and inorganics not listed were either below the detectable levels, or below the CEV values. TWQR = Target Water Quality Range; CEV = Chronic Effect Value; AEV = Acute Effect Value (DWAF, 1996). [a] = considered a mesotrophic condition (Dallas and Day, 1993).

Determinand	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
Ammonium as N	0.05-3.2	1.4	0.007	0.015	0.1
Nitrate and Nitrite as N	0.04-0.07	0.04	0.007	0.015	0.1
Kjeldahl Nitrogen as N	0.23-0.29	0.26	0.007	0.015	0.1
Chloride as Cl	10-15.5	13.9	#0.0002	0	0
Sulphate as SO4	30-35	32.14			
Calcium as Ca	18-23	20			
Magnesium as Mg	6-15	14.5			
Alkalinity as Calcium Carbonate	23-42	38.6			
Total Phosphate	0.01-0.05	0.01[a]			
Aluminium, acid soluble	0.056-0.516	0.14	#0.01	0.01	0.1
Copper, acid soluble	<0.011	<0.011	#0.0014	0	0.01
Dissolved Organic Carbon as C	3.5-4.2	3.95			

Table 10.6. Potassium dichromate: summary of the statistical analyses of LC_{50} values at 96 hours using

the Trimmed Spearman-Kärber method for *Burnupia stenochorias* collected from the Botha and Kubusi Rivers. ppm = parts per million potassium dichromate. 95% LCL = 95% lower confidence limit; 95% UCL = 95% upper confidence limit; Std. dev. = standard deviation (replicates); C.V.% = percentage coefficient of variation (replicates). [a] significantly different (Student's 't' tests, $p < 0.05$).

Population	Replicate(s) analysed	% trim	LC_{50} ppm	95% LCL ppm	95% UCL ppm	± Std. dev.	C.V. %
Botha River	all	33.3	7.6 [a]	4.3	13.4	2.7	36.7
14/9/98	replicate 1	40	4.2	0.6	28.9		
early summer	replicate 2	30	8.7	3.5	21.8		
	replicate 3	20	8.8	6.0	13.0		
Botha River	all	17.24	11.5	8.0	16.5	2.3	20.7
16/2/99	replicate 1	10	9.8	6.3	15.3		
mid summer	replicate 2	0	9.9	6.5	15.0		
	replicate 3	22.2	13.8	7.9	24.3		
Botha River	all	13.3	16.9 [a]	12.9	22.3	0.9	5.76
7/8/99	replicate 1	5	17.0	11.9	24.3		
winter	replicate 2	10	17.0	11.0	26.4		
	replicate 3	20	15.4	8.6	27.5		
Kubusi River	all	25.93	10.0	6.0	16.6	3.8	37.5
23/9/98	replicate 1	33.33	14.4	5.8	35.5		
early summer	replicate 2	22.2	8.3	4.3	16.0		
	replicate 3	22.2	7.5	3.7	15.1		
All four tests	all		11.5			3.9	34.5

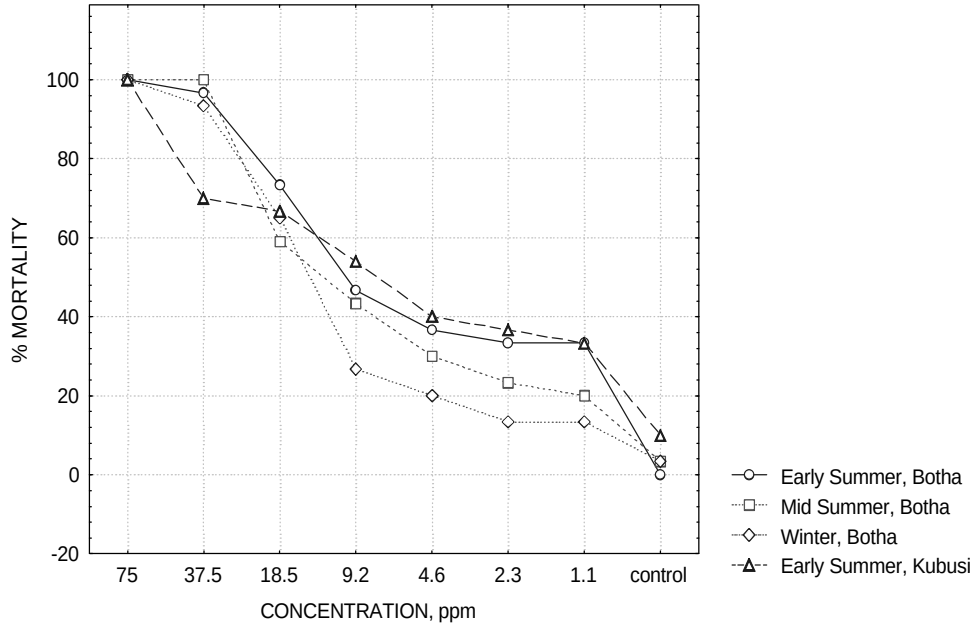


Figure 10.5. Mean (n = 4) percentage mortalities of *Burnupia stenochorias* at each of the concentrations of potassium dichromate (replicates combined; ppm = parts per million). The control was of dechlorinated, carbon filtered tap water. Botha = Botha River population, Kubusi = Kubusi River population.

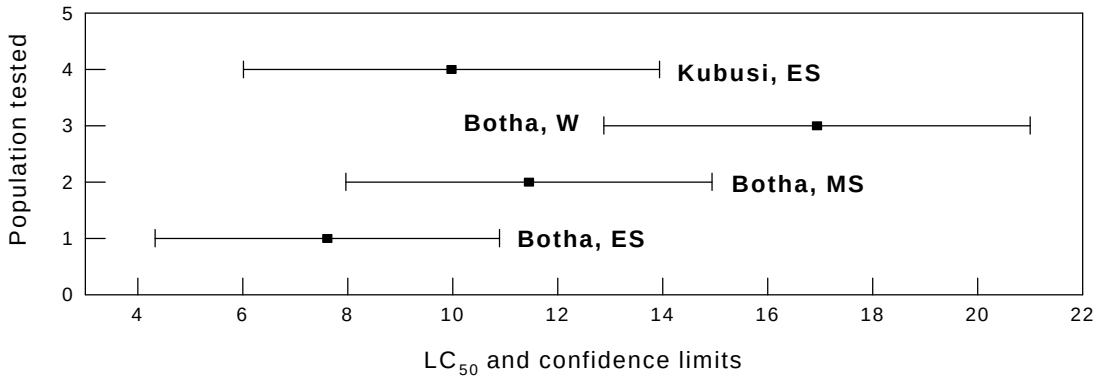


Figure 10.6. Potassium dichromate LC_{50} (parts per million) values for each of the *B. stenochorias* populations sampled, with 95% confidence limits included. Population 1 = Botha River, early summer; 2 = Botha River, mid summer; 3 = Botha River, winter; 4 = Kubusi River, early summer.

was obtained ($F_{(1,32)}$ = 0.008, p = 0.931), although a two way comparison of location and concentrations gave a significant difference between the populations at the 37.5 ppm dilution of potassium dichromate, which, however, is above the LC_{50} values for either population ($F_{(7,32)}$ = 3.23, p = 0.011; Figure 10.5).

10.4. DISCUSSION

Possible seasonal and population differences in response to a toxicant may contribute to variable toxicity results. Consequently the responses of *Burnupia stenochorias* to textile whole effluent were assessed and compared to responses to potassium dichromate, a standard reference toxicant (Section 9.2.3). Toxicity to potassium dichromate was found within the ranges of 7.6 (early summer population) to 16.9 parts per million (ppm) (winter population) potassium dichromate. These results are comparable with other molluscan LC_{50} values (Table 10.7). The largest differences in survival between populations tested occurred at the 37.5 ppm and 18.7 ppm dilutions, above the LC_{50} estimations but within the 95% confidence limits. Further multiple tests would establish the LC_{50} value with a higher degree of confidence. Acute toxicity, as defined in Chapter 8, was not evident from exposure to the whole effluent, although a trend of increasing mortality with increasing concentration of effluent could be seen. The lack of an LC_{50} value is in contrast to other tests with textile whole effluent using molluscs (Chapter 8). Consequently, the results pointed to the necessity for the completion of a chronic textile effluent toxicity study to describe more accurately the effects of the effluent on the limpet (Chapter 11; Figure 1.2). Increasing variability of survival of *B. stenochorias* between replicates was found with increasing copper concentrations in an acute toxicity test (Gerhardt and Palmer, 1998) and this would be expected as concentrations rose to a toxic level (Warren-Hicks and Parkhurst, 1992). However, increased variability of survival of *B. stenochorias* with increasing concentrations of effluent was not obvious under these conditions (Figure 10.1).

The innate variability in responses of *B. stenochorias* to the toxicants was the primary concern of the tests discussed in this chapter. Seasonal differences in susceptibility to both potassium dichromate and the textile whole effluent were apparent. Early summer limpets were most susceptible to both potassium dichromate (represented by a low LC_{50} value; $p < 0.05$) and effluent. Winter populations generally displayed significantly fewer mortalities. Early summer *B. stenochorias* represent pre-sexually developed limpets that have hatched during the previous winter or early spring and are

Table 10.7. Ranges of potassium dichromate LC_{50} values for various molluscan species, compared to *Burnupia stenochorias.* ppm = parts per million. Time refers to the duration of the test.

Species	LC_{50} mg/l (ppm)	Time, days	Reference
Biomphalaria glabrata	37.3 - 147.0	0.5	Bellavere & Gorbi, 1981
Elimia livesens	2.4 - 10.0	1	Cairns *et al*., 1976
Helisoma trivolvis	11	4	Ewell *et al*., 1986
Hyriopsis cumingii	8.8 - 128.0	2	Chin & Chou, 1978
Lymnaea acuminata	6.7 - 9.7	3	Khangarot *et al*., 1982
Lymnaea emarginata	34.8 - 52.0	3	Cairns *et al*., 1976
Lymnaea luteola	4.1 - 16.5	3	Khangarot & Ray, 1988
Physa integra	6.6	2	Cairns *et al*., 1976
Physella heterostropha	17.3	4	Patrick *et al*., 1968
Viviparus bengalensis	2.2 - 17.0	1	Gupta *et al*., 1981
Burnupia stenochorias	7.6 - 16.9	4	This study

potentially undergoing a flush in growth with the increasing temperatures and light (Chapter 4). Such an increase has been shown with other Ancylidae to rapidly drain stores of carbohydrates and lipids (Streit, 1978) which, in the case of *B. stenochorias*, appears to have increased susceptibilities to the toxicants tested

Coefficients of variation for between-replicate comparisons were higher (36.68% and 37.46%) for the early summer limpets than the mid summer (20.73%) or winter (5.76%) populations in response to potassium dichromate. Winter limpets are potentially stressed from cold temperatures and reduced oxygen consumption (*e.g.* Berg *et al*., 1958; Burky, 1971). Reduced light and possibly food quality or quantity may contribute to a reduction in metabolism (Burky, 1971; Streit, 1978). However, a reduced winter metabolic rate not only means a reduced intake of nutrients from the aqueous medium in which the limpets live, but also possibly toxicants. *B. stenochorias* does remain relatively active in winter, as demonstrated by the egg capsules laid, but with reduced reproductive effort (Figures 4.3 and 4.4), predicted slower growth rates through winter (Figure 4.8) and fewer limpets found in the streams in general (Figures 4.5 and 4.6). It would seem, therefore, that winter is a less favourable period for *B. stenochorias*. Physiological studies of winter and summer populations of *B. stenochorias* are needed

to confirm differences in rate of metabolism. Prior to the toxicity tests, the winter limpet populations were acclimated to the laboratory temperatures which were higher (20EC) than found in the field (Figure 4.2). However, seasonal variations in anabolic rate functions are not necessarily a function of temperature changes (Marshall and McQuaid, 1994). Consequently, it is likely that a lower metabolism is the cause for the lower winter susceptibilities to both potassium dichromate and effluent. Greater fluctuations in response to potassium dichromate in the early summer may be due to variation in sexual development and therefore susceptibility to toxicants (Chapter 5)

Responses also differed between populations. Considering the populations from each source separately, both the Botha River (pristine, non-flowing) and Belmont River (non-pristine, flowing) populations showed significantly lower mortalities in winter than in the summer months, with the potassium dichromate LC_{50} values with Botha River limpets giving the same results. Early summer mortalities with Kubusi River (pristine, flowing) limpets were, however, significantly higher than both the mid summer and winter Kubusi River limpet mortalities when subjected to effluent, but replicates from the Kubusi population were most variable of all populations, although not significantly so (Figure 10.2). However, Kubusi River and Botha River early summer effluent mortalities and potassium dichromate LC_{50} values did not differ significantly (Table 10.6). Laboratory bred limpets, already acclimated to approximately 20EC, did not display significant seasonal differences, although the trend of high to low mortalities in the early and mid summer to winter periods was seen. Comparing each of the seasonal results from each population with all other results showed significant differences, resulting primarily from the lower winter mortalities (Table 10.3).

The provision of 95% upper and lower confidence limits about the LC_{50} describes the distribution of the susceptibilities of the individual test organisms (Stephan, 1977). The confidence limits describe the variability of the LC_{50} under the specific conditions used and no other (Stephan, 1977; Chapman, 1996; Fulk, 1996). However, there are some species where variability in survival is much larger than that based on the LC_{50} Coefficients of Variation (C.V.) (Warren-Hicks and Parkhurst, 1992). Thus the C.V. values associated with a calculated end point may not be appropriate for representing the variability of survival measurements at single concentrations, and therefore the variability of the test organism response (Anderson and Norberg-King, 1991). However, with identical laboratory conditions and stock solutions used in the potassium dichromate tests, the LC_{50} values can be legitimately compared using

the formula suggested by APHA (1992) (Section 9.2.). The 95% confidence limits were not significantly different between replicates or concentrations within each test. Coefficients of variation averaging 20-44% or more (with ranges 18-143%) can be expected between proficient inter- and intra-laboratory tests using standard laboratory organisms and procedures with single chemical tests (Anderson and Norberg-King, 1991; USEPA, 1985; USEPA, 1991; Warren-Hicks and Parkhurst, 1992; Chapman, 1996). Similarly, acute toxicity values may differ by a factor of 2-3 without due concern (T. Norberg-King, pers. com.). Some population toxicity studies indicate physiological adaptation to toxicants, which would imply genetic variation as a source of differences in tolerance (*e.g.* Reed and Moffat, 1983; Khan and Weis, 1987); other studies give the opposite results (Soares *et al*., 1992). Although the responses of *B. stenochorias* to both effluent and potassium dichromate indicate significant seasonal differences in toxicity tests occur, it is possible that the results reflect acceptable 'reproducability', i.e. the mortalities form the basis of the 'real world' variation expected when using a wild population, but with equal variability to that shown by a standard laboratory organism.

There are further sources of variability besides the innate responses demonstrated by *B. stenochorias*. The decision as to whether there are sufficient test organisms of the same size and age per concentration is one of the factors determining whether wild or laboratory cultures are used for toxicity tests (Rand, 1995). Increasing the number of test organisms is bound to decrease the standard error around the LC_{50} (Jensen, 1972; Hayes, 1987). However, the use of ten animals per replicate (with three replicates), plus the inclusion of seven concentrations and a control, satisfy the need for both a reasonable initial estimation of the LC_{50} value for the potassium dichromate, and testing of within-concentration variability of response (Jensen, 1972; Zar, 1974; Stephan, 1977; Yanagimoto and Yamamoto, 1979; WHO guidelines for molluscicide testing 1983, in Brackenbury, 1997; APHA, 1992; Hoekstra, 1993). Variability was partially alleviated in these tests by the choice of pre-sexually developed specimens as test individuals, with limpet size [length] not varying significantly within or between tests. Similarly, the general good health of the limpets was demonstrated by the low mortalities within the controls over 96 hours (APHA, 1992; USEPA, 1993).

Method choices also influence response variability. There are both advantages and disadvantages in using a static system for the toxicity (Parkhurst *et al*., 1992). Particularly with a whole effluent, there is potential for a change over the 96 hours in dilutions within each concentration (either as the effluent

volatilizes or degrades), but a breakdown of the constituent textile whole effluent components suggested this did not occur. The use of tap, as opposed to receiving, water as a diluent has several limitations (USEPA,1991; APHA, 1992; Rand,1995; Slabbert *et al.*, 1998). Brackenbury and Appleton (1997) showed significant ($p < 0.05$) and very significant ($p < 0.01$) differences in the effect of a crude plant extract as a molluscicide, depending on the choice of water source as diluent. However, the choice of diluent may alter the 95% confidence limits but not the LC_{50} (Stephan, 1977) and the LC_{50} values were therefore compared. In the case of potassium dichromate, the range of dilutions was made on the basis of an initial range finding test, and a dilution rate of x0.5, with the aim of obtaining at least two partial mortalities, and a 0% and a 100% mortality rate in each of one of the concentrations (Finney, 1971; USEPA, 1993). However, low mortalities still occurred in the lowest concentration of potassium dichromate tested (Figure 10.7), suggesting a longer term or chronic toxicity test would define this tail end of the toxicity curve with more accuracy.

Human error also plays an important role in variability of endpoints (USEPA, 1991; Fulk, 1995; USEPA, 2000), as does toxicant variability. Effluent components were not significantly different between seasons in this study. The toxicity of potassium dichromate is generally affected by pH, hardness and alkalinity (Chapman, 1995; Sprague, 1995; Appendix A). There is a pH-dependent balance between chromate and dichromate (Berglind and Dave, 1984); and sulphate ions and the ratio between calcium and magnesium have been shown to directly affect toxicity of potassium dichromate to test organisms such as *Daphnia magna* and *D. similis* (Hosokawa *et al.*, 1991) and diatoms (Riedel, 1985). Both sulphate and magnesium ions were high in the tap water used (Table 10.2; Dallas and Day, 1993). Further toxicity testing to assess whether differences in the ionic balances of the tap water between winter and early spring were associated with the LC_{50} values would be ideal. However, it is beyond the scope of this investigation, and so it is considered sufficient at this stage to provide the ionic data and pH values (Table 10.4).

If temperature is an important controlling factor of metabolism, it can have a marked effect on the toxicity of heavy metals (Møller *et al.*, 1994), thus influencing the LC_{50} values obtained for *B. stenochorias*. The constant temperature of 20EC for test conditions was based on research which concluded it as an optimal temperature for *B. stenochorias* (Haigh and Davies-Coleman, 1997). Chromium as dichromate is generally considered uninfluenced by test temperature used within a range

of 10-28EC (Cairns *et al*., 1975; Møller *et al*., 1994). The mechanism of chromium^{6+} toxicity would have to be determined in *B. stenochorias* before assessment could be made as to the cause of toxicity, such as rate of metabolism, oxygen consumption, structural changes at membrane interfaces *etc*., were responsible for the limpet's death, and subsequently the effects different temperatures may then have on the toxicity of potassium dichromate. It is interesting to note that there is a relative lack of available data on the biology of trace metals in freshwater molluscs, compared to their marine counterparts (Rainbow and Dallinger, 1993). It is suggested that a 10 day period of acclimation to the controlled environment room temperature may be a minimum requirement to stabilize metabolism rates in *B. stenochorias* before toxicity testing occurs (Lam, 1996).

10.5. CONCLUSIONS

The method of collection and acclimation of *Burnupia stenochorias* described in this chapter can be considered successful, particularly because of the low mortalities of control limpets. The method is compliant with the Standard Methods for the Examination of Waste Water (APHA, 1992), and has been included in the South African Protocol for Acute Toxicity Testing for The Use of Indigenous Invertebrates (Scherman and Palmer, 2000). The method for completing the acute toxicity testing was also successful and will be discussed further in Chapter 13. This set of reference toxicant data forms the beginnings of the reference database for intra-laboratory precision for *B. stenochorias*.

The suitability of *B. stenochorias* as a test organism can be assessed from two perspectives. The first is its variability, both within concentrations (individuals) and between seasons and populations, in reaction to both a reference toxicant, potassium dichromate, and a textile whole effluent. Confidence limits around the LC_{50} values and percentage coefficients of variation suggest broad differences in mortality of individuals, but which were consistent between replicates. Winter limpets were less variable in mortality, and significantly less susceptible to either potassium dichromate or textile effluent than early and mid summer limpets. Generally, early summer limpets gave significantly higher mortalities, although the potassium dichromate results over the whole year fall well within the range of results demonstrated by other species in intra-laboratory toxicity testing in general, and within the ranges obtained with other molluscan LC_{50} values. Populations differed in mortalities, primarily because of the low winter responses of the feral populations. The laboratory population demonstrated the least seasonal variability, showing a 'summer' response all year round, and would afford the most protection

to the species if toxicity results were applied to permit levels. However, when compared to other species inter-laboratory results, the range of LC_{50} potassium dichromate values obtained, together with the variability of responses to textile whole effluent, appear acceptable and not significantly different in ecological terms; rather a reflection of the natural range of responses by the limpets to the chemical components of the toxicants.

Responses of *B. stenochorias* in acute toxicity tests should be obtained side-by-side with a recommended or standard laboratory organism before it can be assessed as a useful toxicity indicator under acute conditions. Results with the standard laboratory organism *Daphnia pulex* (Chapter 12) are compared with those for *B. stenochorias* (Chapter 13), where the suitability of *B. stenochorias* as a toxicity test organism is also discussed further. With no LC_{50} values obtained for *B. stenochorias* for the post-irrigation effluent, this led to the conclusion that chronic studies should be completed. With death a crude index of stress within an ecological system, it is considered of limited ecotoxicological value, so that more information on sub-lethal effects is required.

CHAPTER 11. CHRONIC TEXTILE EFFLUENT TOXICITY TESTS

Key issues: How variable, and consequently how useful in ecological assessment, are the responses of *Burnupia stenochorias* over its entire life cycle to a sub-lethal dilution of a textile whole effluent? How toxic is the effluent in terms of survival, growth and reproduction, and survival and growth within the F1 generation? (Figure 1.2)

11.1. INTRODUCTION

The ultimate goal of toxicity testing is to monitor or predict the effects of single compounds, elements or mixtures, such as are found in waste water and whole effluents, on the long term health of individuals, populations, communities and ecosystems (Giesy and Graney, 1989). Acute (short term) tests are not, however, necessarily adequate for this. Fertility or fecundity effects, for example, are not well predicted from acute exposures (Giesy and Graney, 1989). There is no single acute or chronic test which will allow the prediction of ecosystem-level effects of chemicals, thereby protecting systems from toxic waste (Warne, 1998). Chronic (longer term) studies aim to provide information on sub-lethal or cumulative effects of toxic substances (Chapter 8). There is some dispute as to the definition of a chronic toxicity test, but it is generally considered to last over a period of more than 10% of the organism's life span (Rand, 1995). Short-term chronic tests, suitable for effluents (Rand, 1995), are for periods less than 10% of the lifespan; often 7-14 days (Chapter 8). Chronic whole effluent toxicity testing is useful in water quality management, as most effluents are discharged at sub-lethal levels (Jooste, 2000). Results of whole effluent testing give a clearer picture of the hazard the effluent may pose to the test organism (and the environment) than if the effluent's constituent chemicals were examined separately in toxicology tests (Rand, 1995; Chapters 8 and 14).

A battery of standard freshwater acute and chronic toxicity tests have been evaluated and modified for use in South Africa (Slabbert, 1996; Slabbert *et al.*, 1998). However, there have been few whole effluent toxicity studies using South African indigenous invertebrates (Everitt, 1999; Zokufa, 2001; Muller and Palmer, 2001). It is ideal if the test organism(s) represent(s) the system being subjected to potentially toxic waste, so that results from the tests can be applied with a reasonable degree of confidence (Cairns and Pratt, 1993). The ancylid *Burnupia stenochorias* has been chosen to represent freshwater molluscs in South African toxicity tests. The species also forms part of the aquatic invertebrate community of the upper and middle reaches of the Buffalo River, eventual receiving

waters of the textile whole effluent via the Mlakalaka Stream (pers. obs.; Palmer and Uys, 1994; Figure 9.1). It was previously determined that an Eastern Cape Province textile mill effluent was not acutely toxic to *B. stenochorias* (Chapter 10) or to Baetid mayflies (Zokufa, 2001) over 96 hours.

Despite, or because of, their complexity and variability of chemical components, few textile effluents have been assessed for toxicity (Steffen and Kirsten, 1993; Mouthon, 1996a, b). Those that have been tested have generally shown both acute and chronic toxicity to benthic macroinvertebrates, but this is dependent on the source of whole effluent *i.e.* end-of-pipe, holding dam or post-irrigation (Barton and Metcalfe-Smith, 1992). The dyes used and their accompanying surfactants, with consequent high salinity levels (McKee and Wolf, 1963; Timofeeva, 1991), metals (Muley and Yelpale, 1993; Wells *et al*., 1994) or chloride ions (DiGiano *et al*., 1992) were the primary toxicants. Biological oxygen demand, chemical oxygen demand, pH and nitrates, all of which are usually at high levels within textile effluent (Rutherford *et al.*, 1992), all play a major role in the toxicity of effluents in general to molluscs (Mouthon, 1996b). Sub-lethal effects of textile effluents have been seen with *Physa acuta* and *Hydrobia jenkinsi* (Brown, 1981; Rutherford *et al*., 1992). Toxicity testing in Africa has mostly been limited to the molluscs and the effects of molluscicides on a number of the bilharzia snail hosts and introduced snail species, but no previous African ecotoxicology studies have been completed using textile whole effluents and an indigenous freshwater mollusc (Chapter 8).

Receiving water chemistry will affect the bioavailability of chemicals from an effluent, and in whole effluent toxicity testing, choice of diluent (distilled, tap, synthetic or receiving) is important (Abel, 1989). Tests using receiving waters provide site specific water quality criteria. However, preliminary laboratory tests are essential for understanding the amount of uncertainty in the measured end points (Schindler, 1996). The greater the variability in response, the less confidence in the results and their applicability (Chapter 8). Although sources of test result inconsistencies include both inter- and intra-laboratory factors, of concern in this study is the intra-laboratory variability. The natural genetic response inherent in any natural population may be responsible for variability of toxicity test results. The response may be based on genetics, age, previous exposure, or other stressors present in the system (Scherman and Palmer, 2000). Where wild populations are used as test organisms, the degree of variability demonstrated between populations in the various physiological responses to the toxicant is unknown: Dillon (2000) suggests there is a great deal of genetic variance for fitness traits hidden

in any sample of 30 week old gastropods. The influence that this innate variability has on the endpoints should be measured, if possible, for later interpretation and application, and forms the crux of this investigation. Variability from the effluent samples was brought to a minimum by the use of only two effluent samples (Section 9.2.3).

Introducing effluent into a river can modify the physical, chemical and biological parameters of the receiving waters to a lesser or greater degree. This modification may affect any or all stages of an organism's life history. An investigation into the growth and reproduction effects on *B. stenochorias* would indicate possible long term effects of a toxicant on its life cycle. The investigation into growth and reproductive aspects of *B. stenochorias* (Figure 1.2; Part One of this thesis) provided data relevant to a chronic toxicity investigation. However, reaction at a sub-lethal level to a textile effluent was unknown. Similarly, methodology involved in monitoring growth and reproduction was described but needed further development. With few mollusc species found in Europe, North America and Australasia, compared to other invertebrates, fewer studies in general have been completed on chronic toxicant effects on molluscs, particularly gastropods, and thus information from the literature was scarce (Mouthon, 1996b).

The ultimate aim of using *B. stenochorias* as a toxicity test organism is resource protection (Chapters 1 and 14). Chronic toxicity testing of *B. stenochorias* was completed with two primary aims:

a) To evaluate whether survival, growth or reproduction of *B. stenochorias* are suitable as chronic or sub-chronic toxicity endpoints.

b) If any one or more of these endpoints are suitable, to use them to evaluate the chronic toxicity of the textile effluent.

11.2. MATERIALS AND METHODS

Two series of replicated textile effluent concentrations (in 1998 and 1999) were used to assess the toxicity of the effluent to, and the response variability of, *Burnupia stenochorias*. The methods which were used are recorded in Chapter 9, and relate to the collection and acclimation of test organisms (Section 9.1), test conditions (Section 9.2), provision of food (Section 9.2.5), chemical analyses of tap water and effluent samples (Section 9.2.5), and growth and reproductive criteria measured throughout the experiment (Section 9.3.2). Data analyses allow the evaluation of variability in growth, fecundity

and survival between replicates within each dilution of textile whole effluent, and the differences between dilutions of effluent in the responses (*i.e.*, the toxicity of the whole effluent) (Section 9.3.2). In assessing variability of response and toxicity of effluent, comparisons were made of survival, growth (represented as a regression line over time) and size at 21 and 43 days, with the ultimate aim of finding an early period after which time chronic toxicity effects could be quantified. The 1998 chronic test (100%, 10%, 3%, 1% and 0.1% effluent dilutions) revealed the need for a concentration greater than 10% but less than 100% to be tested, and in 1999, 20% replaced 100% effluent (0.1% was omitted because of a lack of newly hatched test organisms) (Section 9.3.2 and Table 9.1).

11.3. RESULTS

11.3.1. Experimental Conditions

In order to evaluate the responses of limpets between replicates (reflecting individual responses), the variability that occurred in the experimental conditions relating to food supply and water chemistry are considered first.

Food

Despite the culture of food tiles in one laboratory stream, it could be seen with the naked eye that before the tiles were randomly chosen for use in the toxicity tests, colour differences occurred *i.e.* different diatom species colonised different tiles. Food choice for the limpets therefore potentially differed between replicates from the start of the experiments. Although results were not quantified, examination of the tiles after 40 days showed 100% effluent had much organic deposition (black in colour) which encouraged a slimy black / fungal growth, with very few diatoms visible which were possibly an *Achnanthes* species (Round, 1991). For the remainder of the effluent concentrations, bacterial colonies and diatoms were always present (*Achnanthes, Cocconeis* and a few stalked *Nitzschia* species). Numbers differed but were always greater than on tiles found in 100% effluent, and more extensive on the upper tile surfaces (facing the light), particularly in the 10% and 3% effluent concentrations. Filamentous algal threads could be seen growing on at least one replicate of each test concentration, including the controls, but particularly at 1% concentration where fungal growth was also present.

In 1999 the presence of bacteria as a potential food source or beneficiator (Section 5.4) was

considered. The colony-forming bacterial units in the defrosted effluent amounted to approximately ten times those found in the carbon filtered, aerated tap water. The numbers of bacteria present after three months' growth, sampled from the plastic bags and cultured on agar plates, varied between concentrations at a ratio of 1 (20%, 10% and 1% effluent) : 1.5 (control) : 2 (3% effluent). The type of bacteria present, and hence their potential benefit to the limpets (Section 5.4), was not investigated.

Despite differences in the numbers of bacteria and algal growth on each tile, as well as on the plastic linings, the limpets were evenly distributed around each container.

Properties of Effluent and Tap Water

The effluent was a dark blue/black colour pre- and post-defrosting, attributed to the dye particles which deposited and adhered to the walls of the containers. Total dissolved salts (TDS) and pH levels of each replicate did not change over the 3 or 4 days between solution changes. Small differences did occur with each change in solution, but the coefficients of variation were small in both years (Table 11.1). No significant differences ($p > 0.05$) occurred between replicates within concentrations. However, the TDS of 1999 tap water was approximately 50-60% higher than in 1998, contributing to significant differences in the same test solution between years (Students 't' test, $p < 0.05$).

Although 100% effluent determinands and metals are compared with the TWQR, CEV and AEV values[1] in Table 11.2 which are derived after dilution, potential sources of toxicity can be seen. Large ranges of concentration were regularly observed, despite the effluent samples being defrosted sub-samples of the same sample. Levels of carbon, oxygen, and nitrogen expressed as Kjeldahl nitrogen were high, and these were attributed to the presence of organic compounds which were said to be dominant in the whole effluent, namely starch, urea-formaldehyde based organic nitrogen compounds, various unnamed dyes, soaps, thickeners and some emulsions (C. Mayalo, pers. com.). Similarly, the effluent dissolved organic carbon (62.25 mg/l), and the chemical and biological oxygen demands (740

[1] (See Section 10.3.1)
TWQR = Target Water Quality Range
CEV = Chronic Effect Value
AEV = Acute Effect Value (DWAF, 1996).
The TWQR, CEV and AEV values represent stringent South African water quality values based on safety factors of international criteria, and are used in effluent and chemical hazard assessment in South Africa, where rivers are classified according to their state of health (Palmer, 1999).

and 131 mg/l respectively) were excessively high compared to other Buffalo River data (Rutherford *et al*., 1992; O'Keeffe *et al*., 1996). Dissolved organic carbon within the effluent was not significantly different between 1998 and 1999. Total nitrogen to total phosphorus ratios, and chloride and sulphate ions were significantly higher than tap water, with large ranges.

Chromium and copper levels in the effluent were both greater than the CEV values (Table 11.2). The effluent values for calcium, magnesium and potassium remained fairly constant over the test period and relative ratios of sodium > calcium/magnesium > potassium ions were in accordance with acceptable water quality criteria (DWAF, 1996). Sodium and potassium, however, were almost tenfold that found in the tap water (averaged over both years) with sodium originating from the many reducing compounds (sodium hydrosulphides, sodium sulphates, and sodium bisulphides) and bleach (sodium hypochlorite) used in the factory production of the textiles (C. Mayalo, pers. com.). Although considered a micronutrient, iron also fluctuated in the effluent (from 0.295 mg/l to 0.817 mg/l), exceeding the USEPA recommended value of 0.001 mg/l (Dallas and Day, 1993; South African values are not available).

The toxicants of greatest concern were therefore sodium, potassium and chromium ions, sulphates and chlorides, total nitrogen, dissolved organic carbon, and chemical and biological oxygen demand.

Table 11.1. Variability in pH and total dissolved salts (TDS; mg/l) found within each effluent dilution and the controls, including range, mean, standard error, standard deviation and coefficient of variation, as measured over the entire chronic (parent generation) study for 1998 (n = 26) and 1999 (n = 32). Oxygen levels are not individually noted as they were never below 98% saturation. s.error = ± standard error; s.dev. = ± standard deviation; c.v. = coefficient of variation. [x] Significantly different to same test solution from other test year (students 't' test; Statistica, 1999); [a] significantly different to all other test solutions within the same year, including the control; [b] significantly different (1999) to 1% effluent; [c] significantly different to each other.

		1998 100%	10%	3%	1%	0.1%	control	1999 20%	10%	3%	1%	control
pH	range	7.9-8.5	7.7-8.72[x]	8.0-8.8[x]	7.7-8.8[x]	8.0-8.8	8.0-8.8[x]	8.5-9.4[c]	8.2-9.5[x]	8.1-9.4[cx]	8.1-9.6[cx]	8.2-9.4[ax]
	mean	8.3	8.3	8.3	8.3	8.3	8.3	9.1	9.1	9.0	9.0	8.9
	s.error	0.04	0.07	0.07	0.07	0.07	0.06	0.02	0.06	0.06	0.08	0.06
	s.dev.	0.16	0.32	0.27	0.27	0.27	0.26	0.13	0.26	0.26	0.35	0.29
	c.v.	1.88	3.80	3.25	3.24	3.25	3.13	1.39	2.87	2.90	3.89	3.21
TDS	range	1430-1840[ab]	382-489[abx]	301-354[x]	276-326[x]	272-308	264-303[ax]	698-910[ab]	455-650[abx]	455-585[abx]	390-585[x]	390-533[x]
	mean	1638.0	412.0	322.0	300.0	286.0	284.0	739.0	574.0	502.0	474.0	461.0
	s.e.	29.1	8.0	4.7	3.5	3.2	3.3	19.9	6.7	9.2	5.1	9.2
	s.dev.	99.9	27.6	16.4	12.0	11.1	11.4	89.1	30.1	41.4	23.0	40.9
	c.v.	6.1	6.7	5.1	4.0	0.4	4.0	12.1	5.3	8.2	4.2	8.9

Table 11.2. Major inorganic determinands and trace metals in carbon filtered tap water (diluent) and defrosted textile effluent. The 'Factory' source refers to the analyses of the post-irrigated effluent [the same source as sampled for chronic testing] completed by the textile factory chemistry department from 1996 to 1998. Number of samples (tap water and effluent) = 26. Cobalt, beryllium, nickel, molybdenum, cadmium, mercury and lead were not detectable. TWQR = Target Water Quality Range; CEV = Chronic Effect Value; AEV = Acute Effect Value (DWAF, 1996). [a] refers to the USA recommended sodium content of drinking water (Bunce, 1994). [bcd] refer to typical figures for upland stream, lowland stream and large lowland river (Dallas and Day, 1993). * indicates value measured within effluent above the CEV.

Determinand	Source	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
* Ammonium as N	Tap water	0.05-4.20	1.40	0.007	0.02	0.10
	Effluent	0.21-7.13	1.01			
	Factory	12.00-29.00	18.00			
* Nitrate and Nitrite as N	Tap water	0.04-0.23	0.12	0.007	0.02	0.10
	Effluent	0.04-0.17	0.08			
	Factory	2.00-16.00	4.00			
* Kjeldahl Nitrogen as N	Tap water	0.23-0.29	0.26	0.007	0.02	0.10
	Effluent	8.28-11.28	10.12			
* Fluoride as F	Tap water	0.6-0.1	0.2	#0.8	1.5	2.5
	Effluent	0.3-0.4	0.4			
* Chloride as Cl	Tap water	10.0-15.5	13.9	#0.0002	0.0035	0.0050
	Effluent	104.0-318.0	192			
Sulphate as SO4	Tap water	30-35	32			
	Effluent	177-1515	307			
Alkalinity as Calcium	Tap water	23-42	39			
Carbonate	Effluent	498-1196	820	[very hard]		
Total Phosphate	Tap water	0.09-0.11	0.10			
	Effluent	2.67-4.02	3.52	[Hypertrophic]		
	Factory	1.93-16.09	4.95			
Ortho Phosphate as P	Tap water	0.006-0.051	0.019			
	Effluent	1.901-5.600	2.211			
* Sodium as Na	Tap water	30-84	74	[<100 ppm][a]		
	Effluent	403-844	648			
	Factory	326-840	555			
Calcium as Ca	Tap water	18.0-23.0	20.0			
	Effluent	3.8-55.0	19.2			
Calcium as Ca	Factory	4.4-20.9	10.1			

Table 11.2 continued

Determinand	Source	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
Magnesium as Mg	Tap water	15.0-20.0	15.5			
	Effluent	17.0-32.0	25.7			
	Factory	1.2-40.7	6.2			
Potassium as K	Tap water	2.0-2.8	2.2			
	Effluent	15.7-26.2	20.3			
Silicon as Si	Tap water	0.4-2	1.3			
	Effluent	8.6-19.2	11.6			
Electrical Conductivity (mS/m)	Tap water	46.8-67.3	63.7			
	Effluent	203.0-372.0	276.5			
* Aluminium, acid soluble	Tap water	0.06-0.52	0.14	#0.01	0.01	0.1
	Effluent	0.06-0.13	0.08			
Titanium, acid soluble	Tap water	0.007	0.007			
	Effluent	0.007-0.300	0.024			
Chromium, acid soluble	Tap water	<0.007	<0.007	#0.007	0.01	0.2
	Effluent	<0.007-0.032	0.024			
* Manganese, dissolved	Tap water	<0.003	0.008	#0.18	0.37	1.3
	Effluent	0.201-0.400	0.279			
Iron, acid soluble	Tap water	<0.02-0.09	0.03			
	Effluent	0.30-0.82	0.60			
* Copper, acid soluble	Tap water	<0.011	<0.011	#0.0014	0.0028	0.012
	Effluent	<0.011-0.073	0.025			
* Zinc, acid soluble	Tap water	<0.01-0.06	0.02	#0.002	0.04	0.36
	Effluent	0.03-0.16	0.07			
Barium	Tap water	0.01-0.02	0.02			
	Effluent	<0.01-0.06	0.04			
Dissolved Organic Carbon as C	Tap water	3.5-4.2	4.0			
	Effluent	51.7-75.3	62.3			
* Biological Oxygen Demand	Effluent	75.0-135.0	102.0	[0.5-2.0][b]	[2.0-5.0][c]	[3.0-7.0][d]
	Factory	129.0-341.0	197.0			
Chemical Oxygen Demand	Effluent	610-734	629			
	Factory	698-2427	1125			

11.3.2. Variability of Limpet Reaction

In order to evaluate the variability of reaction to effluent, mortality/survival, growth and reproductive effort (fecundity) were compared within and between replicates. Because of the variability in response, each parameter is considered separately below.

Survival

A limpet was considered dead when it released its foot hold, and the foot did not respond to probing. At the time of limpet death, neither excessive production of mucus, abnormal swelling of the tentacles, or extension of the cephalopedal mass from the shell were observed. The first death in the controls (2.5% mortality) occurred after 14 days (1998 and 1999); 10% mortality in the controls was between 21 and 25 days (1998) and 18 and 21 days (1999); and 20% mortality in the controls was between 28 and 31 days (1998) and 21 and 24 days (1999). As an overview of limpet numbers in each concentration, survival data were plotted for each year (Figure 11.1). When considered separately, each concentration showed large variability between replicates in the number of limpets still alive over the duration of each test (Figures 11.2 to 11.5), exemplified at the selected times of 21 and 43 days (Table 11.3; Figures 11.6 and 11.7). Variances were generally large in the time taken to reach 10%, 50% and 100% mortality with the exception of the controls in 1999 (50% and 100% mortality did not occur in 1998) (Table 11.4). However, variability was not due to data outliers resulting from measurement error or transcription of data.

Growth

Growth data were similarly variable although with large scatter around the regression lines (Figure 11.8 and 11.9). The r^2 values suggest poorer fitting curves for growth of *Burnupia stenochorias* in 100% effluent (1998; Figure 11.8) and 3% and 1% effluent and the control (1999; Figure 11.9). Individual replicates within each concentration were not significantly different from each other (Graphpad Prism 3.0, $p > 0.05$), with the exception of the 1999 10% effluent where the slope of one of the replicates was significantly different to the other three ($F_{(1,175)} = 18.99$, $F_{(1,154)} = 10.98$, and $F_{(1,169)} = 6.45$, $p < 0.001$).

As a further representation of growth differences between replicates, sizes of limpets (area of shell aperture) at 21 days and at 43 days were compared (Figures 11.10 to 11.13), although comparison of variability of size within each replicate was influenced by the number of surviving limpets, particularly

low in 100% effluent (Figures 11.11 and 11.13). At 21 days, the 1998 and 1999 data were not normally distributed (Shapiro-Wilk's test, $W = 0.942$ and 0.9563 respectively, $p < 0.05$). In 1998, individuals in 10% and 1% effluent displayed the most variability in size (Figures 11.10). In 1999, size was consistently the same when grown in 1% effluent, but not in 3% effluent (Figure 11.12). At 43 days, only the 1999 data was normally distributed ($W = 0.9814$, $p < 0.244$). The 1998 limpets in 10% and 1% continued to display variability in size and therefore response to the effluent (Figure 11.11).

In summary, growth and therefore size attained within replicates was not consistent within any of the effluent dilutions tested, with variability increasing in the higher effluent concentrations of 10% and 100% effluent.

Fecundity

The third interpretation of variability of reaction within concentrations to the potential toxic effects of the textile effluent lay in reproductive observations. In 1999, fecundity data were accumulated (Table 11.4). The practicality of monitoring eggs within all egg capsules laid was difficult with so many capsules laid daily. Instead, the number of eggs per capsule and time to hatching were monitored in only a selection of capsules within each replicate. However, the counting of eggs and observations of their development was easily accomplished with the aid of a x5 eye magnification, because plastic bags were used to line the containers and egg capsules were only laid on sections of the plastic bags where very little algal growth occurred or which had been grazed clear of algal growth.

Statistics became problematic because the limpets were not kept individually in the containers, individuals were not tagged or labelled, and differential mortalities occurred throughout the experiment. The mean reproductive period per individual was therefore not calculated. However, the results that were obtained (Table 11.4) clearly demonstrate the high innate variability between replicates. Out of a total of four replicates, two of the 10% and 1% effluent and one of the 20% effluent replicates had no egg capsules laid, giving rise to small degrees of freedom in fecundity calculations. Large standard deviations occurred with the number of lay days per replicate, and the total number of capsules and eggs per capsule laid. Time to hatching was more consistent throughout, with the greatest variability occurring when limpets were in 20% effluent.

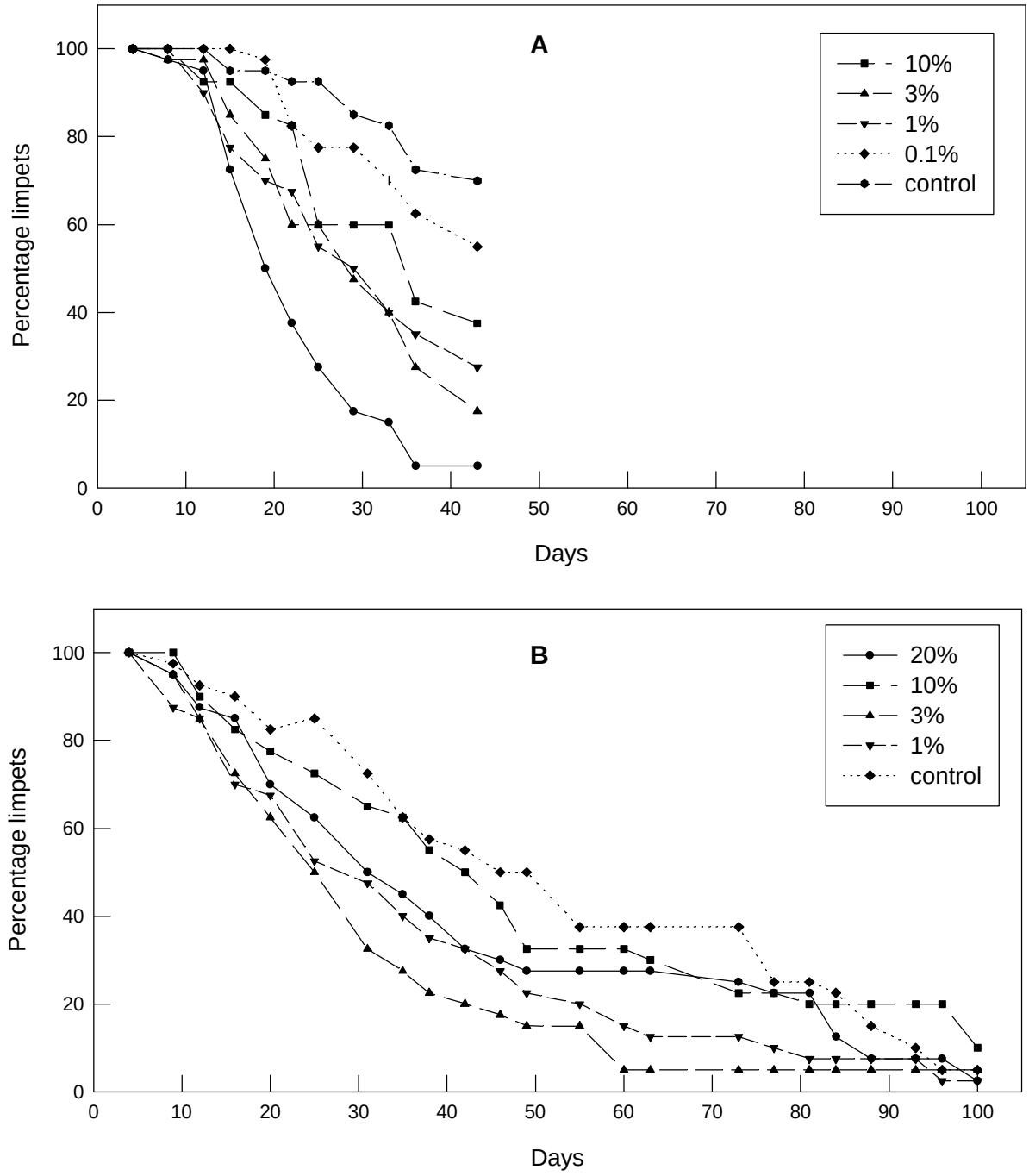


Figure 11.1. An overview of the number of *Burnupia stenochorias* surviving over the entire chronic toxicity period in **1998 (A)** and **1999 (B)**. Each concentration of effluent is represented as a percentage (%) averaged over four replicates, and the controls were of tap water. The 1998 chronic test was discontinued after 43 days.

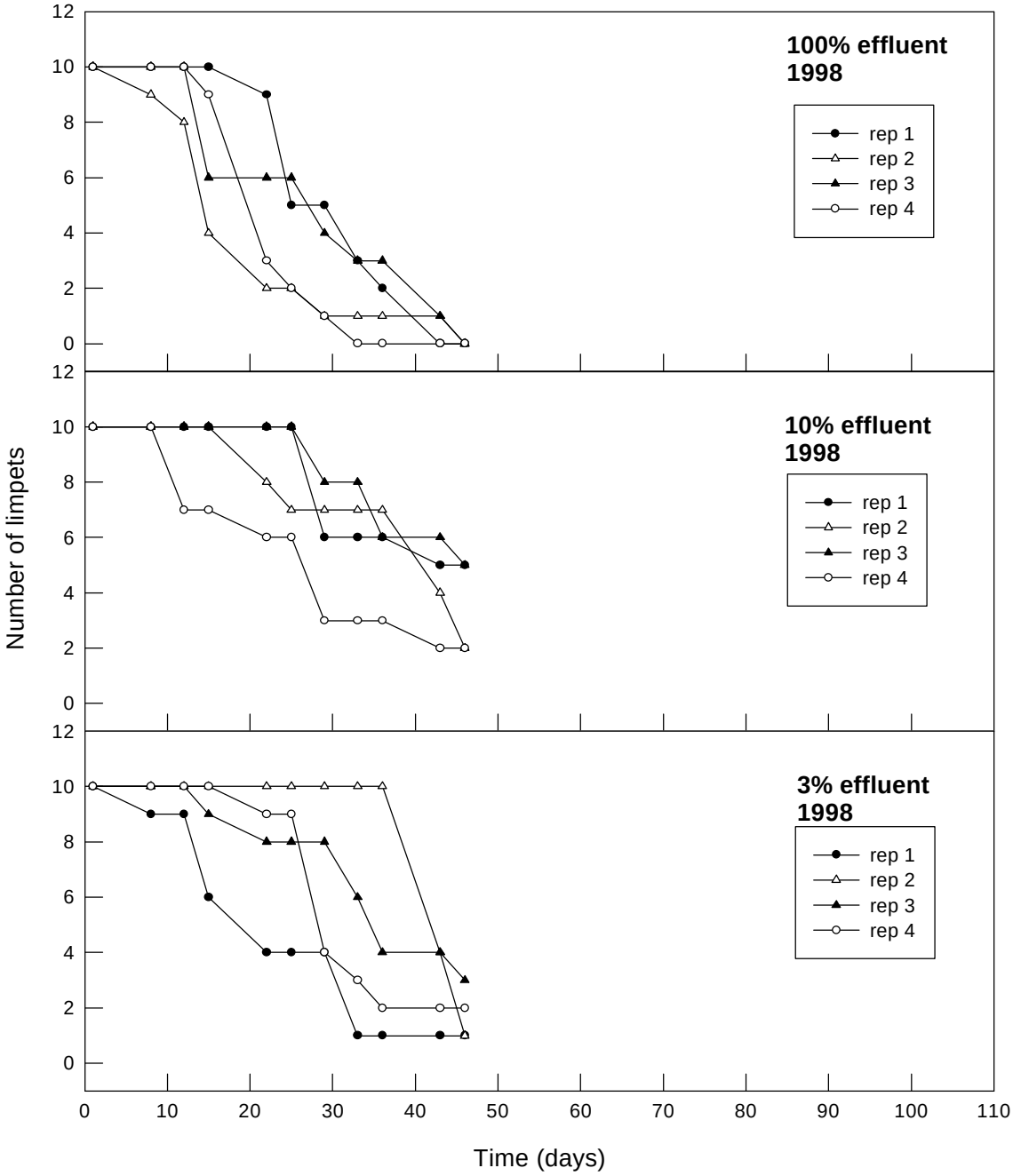


Figure 11.2. **1998** survival of *Burnupia stenochorias* within each replicate grown in the 100%, 10% and 3% textile whole effluent concentrations. Variability in response between replicates is shown.

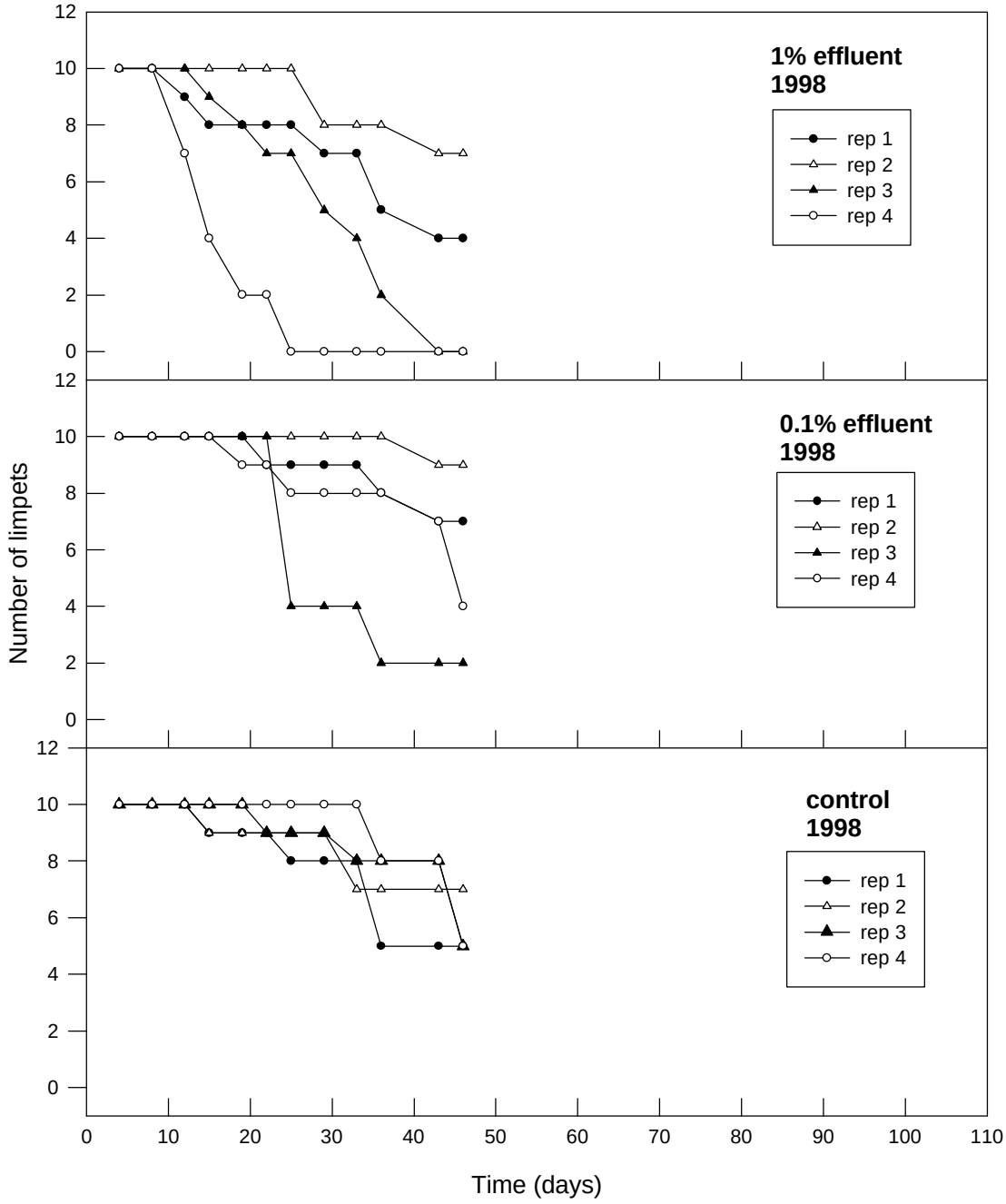


Figure 11.3. **1998** survival of *Burnupia stenochorias* within each replicate grown in the 1% and 0.1% textile whole effluent concentrations and the control of tap water. Variability in response between replicates is shown.

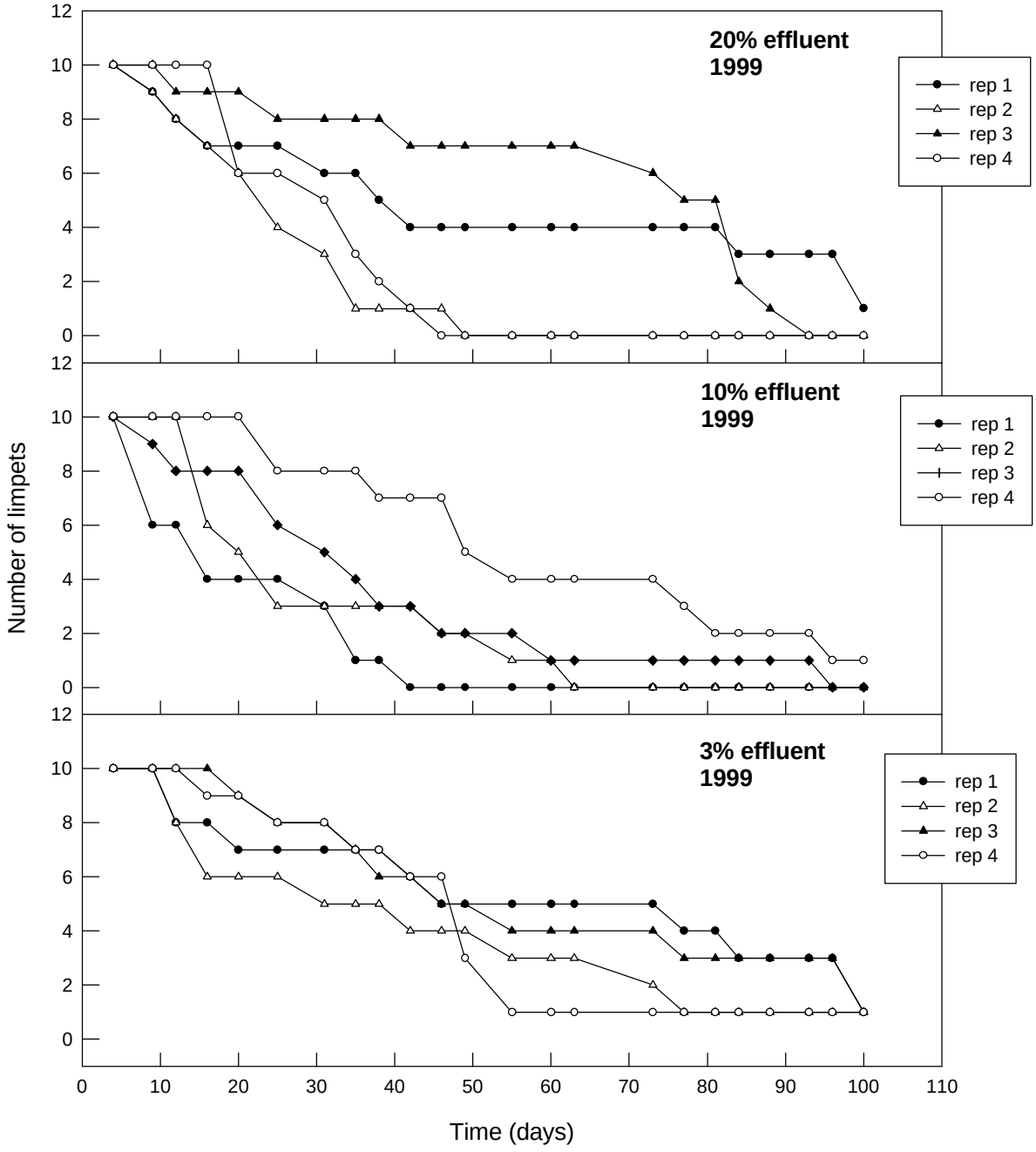


Figure 11.4. **1999** survival of *Burnupia stenochorias* within each replicate grown in the 20%, 10% and 3% textile whole effluent concentrations. Variability in response between replicates is shown.

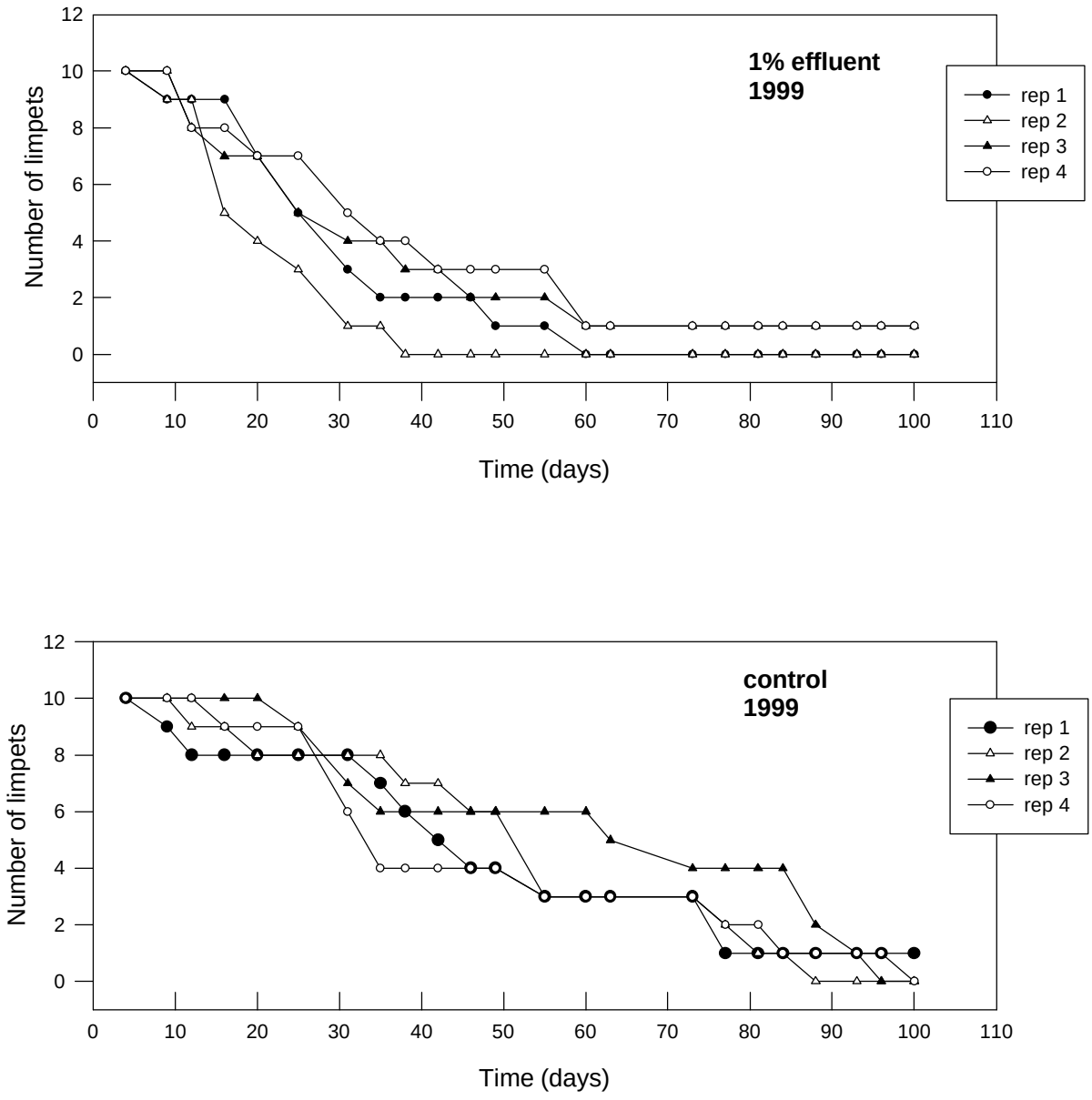


Figure 11.5. **1999** survival of *Burnupia stenochorias* within each replicate grown in the 1% textile whole effluent concentration and the control of tap water. Variability in response between replicates is shown.

Table 11.3. Variability in the chronic toxicity tests in 1998 and 1999, in the number of limpets surviving between replicates at each concentration (= Conc.) at 21 and 43 days (where n = 10 per replicate). S.dev. = ± standard deviation; S.error = ± standard error; C.V. = % coefficient of variation.

	Conc.	Mean	Variance	S. dev.	S. error	Range	C.V.
@21 days, 1998	100%	5.0	10.0	3.2	1.6	7	63.2
	10%	8.5	3.7	1.9	1.0	4	22.5
	3%	7.8	6.9	2.7	1.3	6	33.9
	1%	6.8	11.6	3.4	1.7	8	50.4
	0.1%	8.3	8.3	2.9	1.4	6	34.8
	Control	9.5	0.3	0.6	0.3	1	6.1
@ 21 days, 1999	20%	6.3	2.9	1.7	0.9	4	27.3
	10%	5.3	4.9	2.2	1.1	5	42.2
	3%	7.3	0.9	1.0	0.5	2	13.2
	1%	5.0	2.7	1.6	0.8	4	32.7
	Control	8.5	0.3	0.6	0.3	1	6.8
@ 43 days, 1998	100%	0.5	0.3	0.6	0.3	1	115.5
	10%	4.3	2.9	1.7	0.9	4	40.2
	3%	2.8	2.3	1.5	0.8	3	54.6
	1%	2.8	11.6	3.4	1.7	7	123.8
	0.1%	6.3	8.9	3.0	1.5	7	47.8
	Control	7.0	2.0	1.4	0.7	3	20.2
@ 43 days, 1999	20%	3.0	10.0	3.2	1.6	7	105.4
	10%	2.8	8.9	3.0	1.5	7	108.6
	3%	5.0	0.7	0.8	0.4	2	16.3
	1%	1.8	1.6	1.3	0.6	3	71.9
	Control	5.0	1.3	1.2	0.6	2	23.1

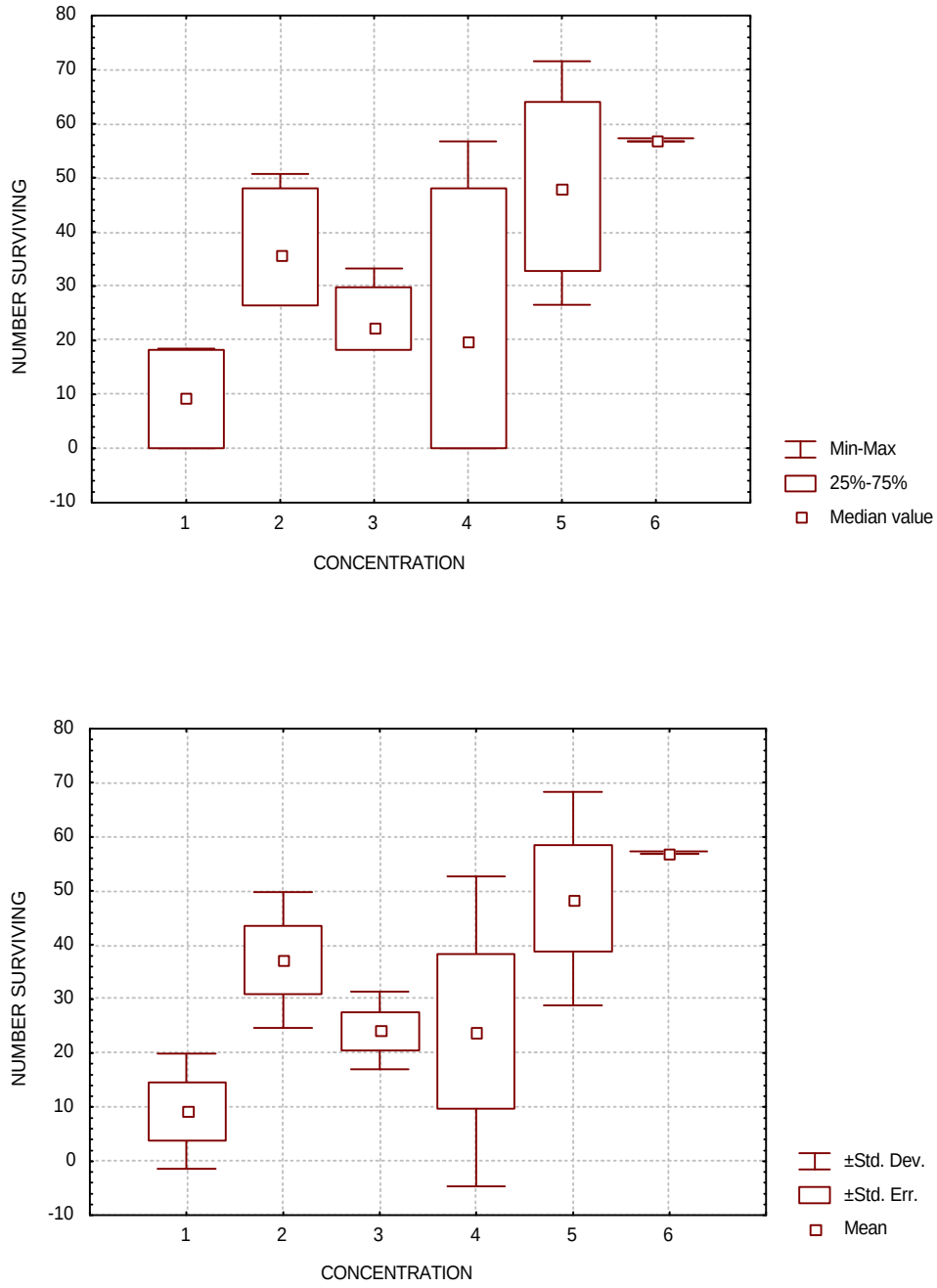


Figure 11.6. Variability in percentage survival (arcsine transformed) of *Burnupia stenochorias* (**1998 at 43 days**), with minimum, maximum, and median values illustrated for each replicate, and means, standard deviations and standard errors shown separately. Concentration 1 = 100% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = 0.1% effluent; 6 = Control of tap water

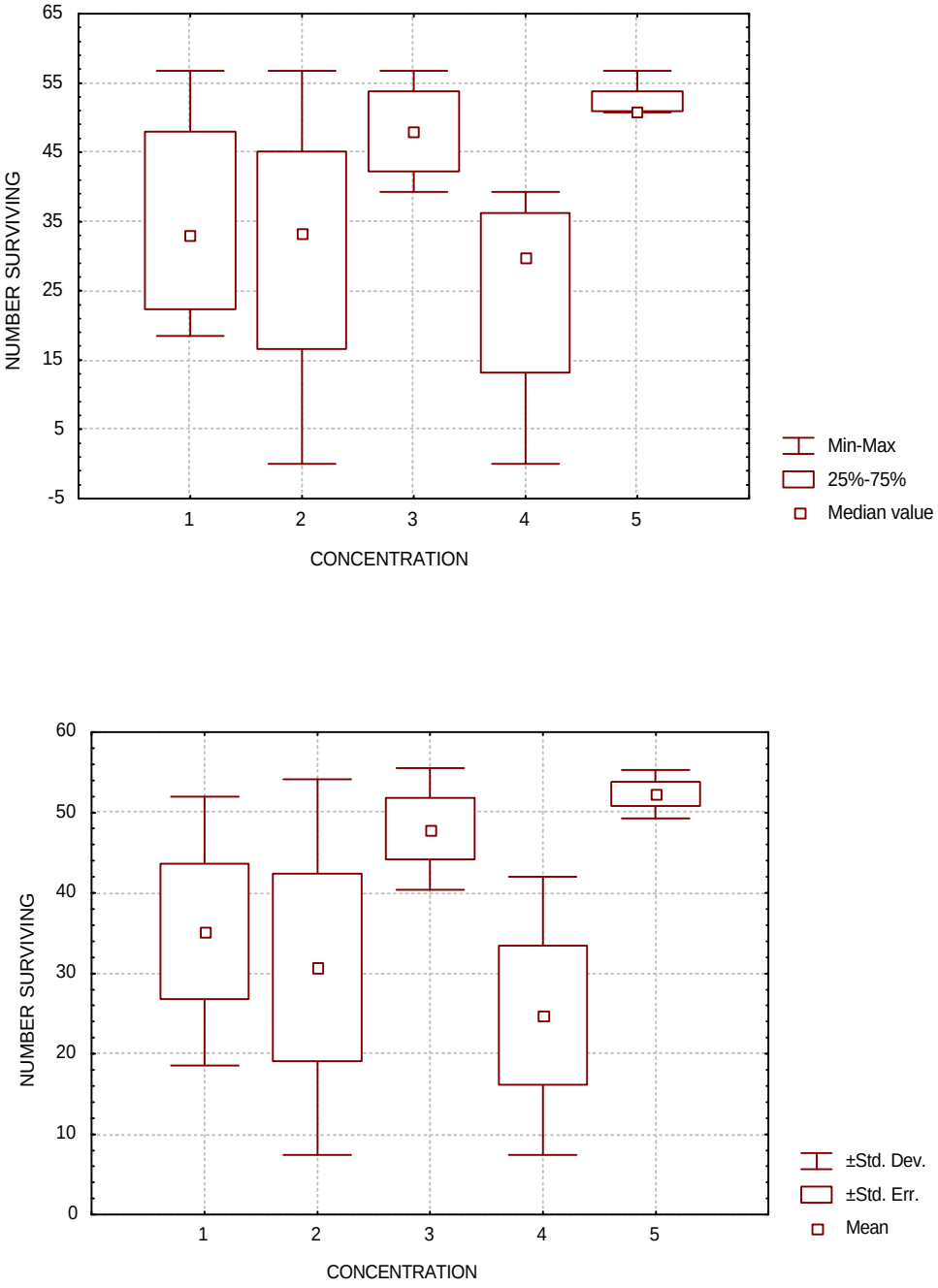


Figure 11.7. Variability in percentage survival (arcsine transformed) of *Burnupia stenochorias* (**1999 at 43 days**), with minimum, maximum, and median values illustrated for each replicate, and means, standard deviations and standard errors shown separately. Concentration 1 = 20% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = Control of tap water.

Table 11.4. Variability of time (days) taken to 10%, 50% and 100% mortality between replicates at each concentration (= Conc.), for 1998 and 1999. S.dev. = ± standard deviation; S.error = ± standard error; C.V. = coefficient of variation. N/A refers to data where not all replicates had 50% mortality. 100% mortality was not considered for 1998 due to early termination of the experiment.

Mortality	Conc.	Mean	Variance	S.dev.	S.error	Range	C.V.
10%, 1998	100%	13.6	33.7	5.8	2.9	14	40.0
	10%	18.6	70.3	8.4	4.2	19	41.4
	3%	15.4	54.2	7.4	3.7	17	44.0
	1%	15.7	50.9	7.1	3.6	16	42.6
	0.1%	24.9	112.2	10.6	5.3	23	40.4
	Control	20.2	8.9	9.4	4.7	20	43.9
10%, 1999	20%	12.1	11.0	3.3	1.7	7	26.5
	10%	11.9	36.6	6.1	3.0	14	46.6
	3%	16.3	18.3	4.2	2.1	9	25.5
	1%	9.5	1.0	1.0	0.5	2	10.5
	Control	14.9	59.6	7.7	3.8	18	47.5
50%, 1998	100%	21.8	43.9	6.6	3.3	15	30.3
	10%	38.8	69.6	8.3	4.2	19	21.5
	3%	30.0	103.3	10.2	5.1	24	33.9
	1%	N/A					
	0.1%	N/A					
	Control	N/A					
50%, 1999	20%	56.8	670.9	25.9	13.0	50	45.6
	10%	29.0	221.3	14.9	7.4	34	51.3
	3%	41.0	38.0	6.2	3.1	14	15.0
	1%	24.0	44.0	6.6	3.3	16	27.6
	Control	44.8	170.3	13.1	6.5	31	29.2
100%, 1999	20%	67.6	810.0	28.4	14.2	54	39.6
	10%	71.40	744.00	27.2	13.6	57	36.1
	3%	80.90	462.00	21.5	10.8	45	25.8
	1%	68.00	997.30	31.6	15.8	64	42.7
	Control	96.40	22.30	4.7	2.4	10	4.9

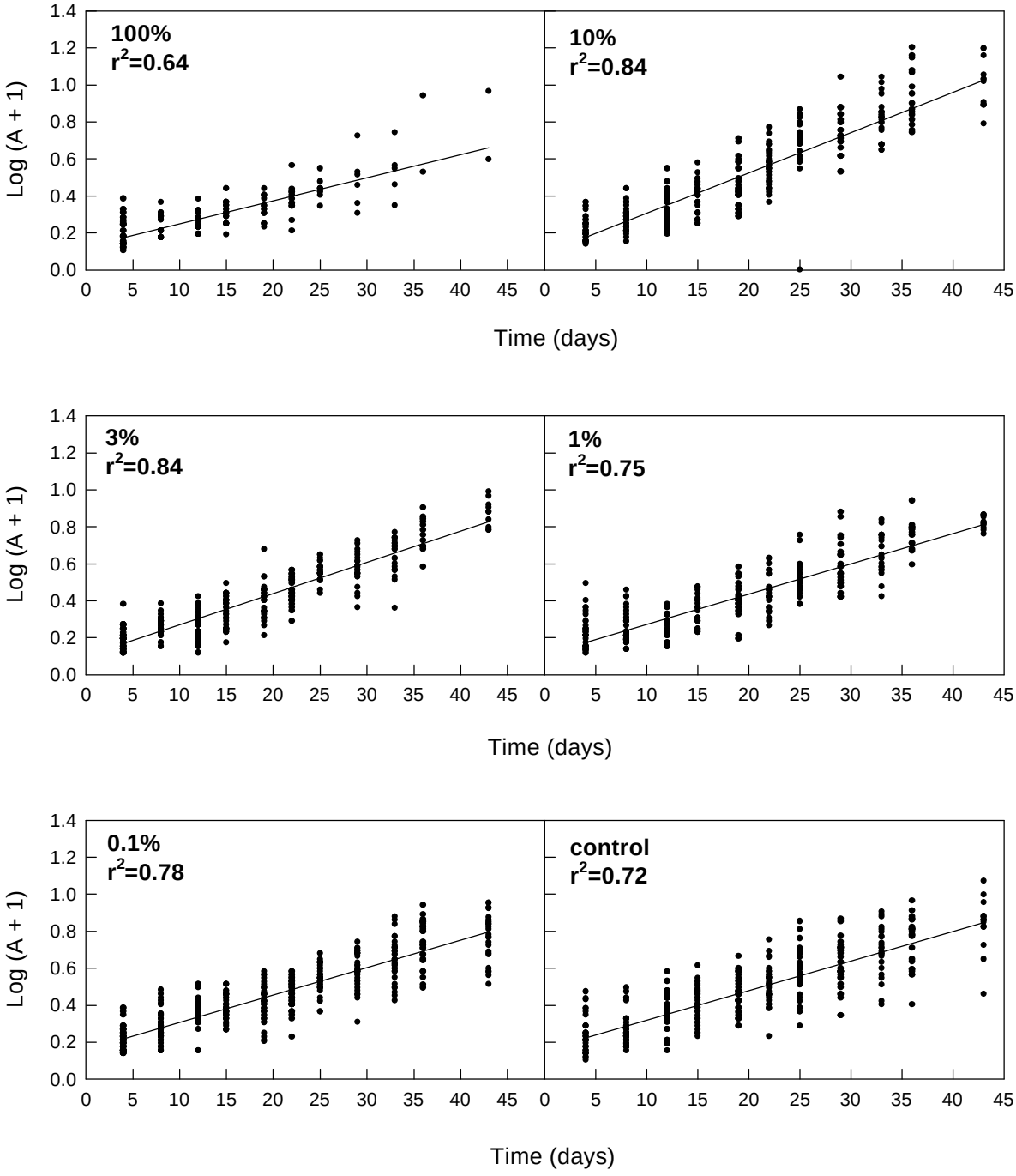


Figure 11.8. **1998** regression lines for the first 43 days' growth of *Burnupia stenochorias* within each test dilution (100%, 10%, 3%, 1% and 0.1% effluents and the controls). The data points indicate individual sizes with all replicates for each dilution pooled. Distribution around the regression line is an indication of the variability in size (as indicated by change in the logarithms of shell area).

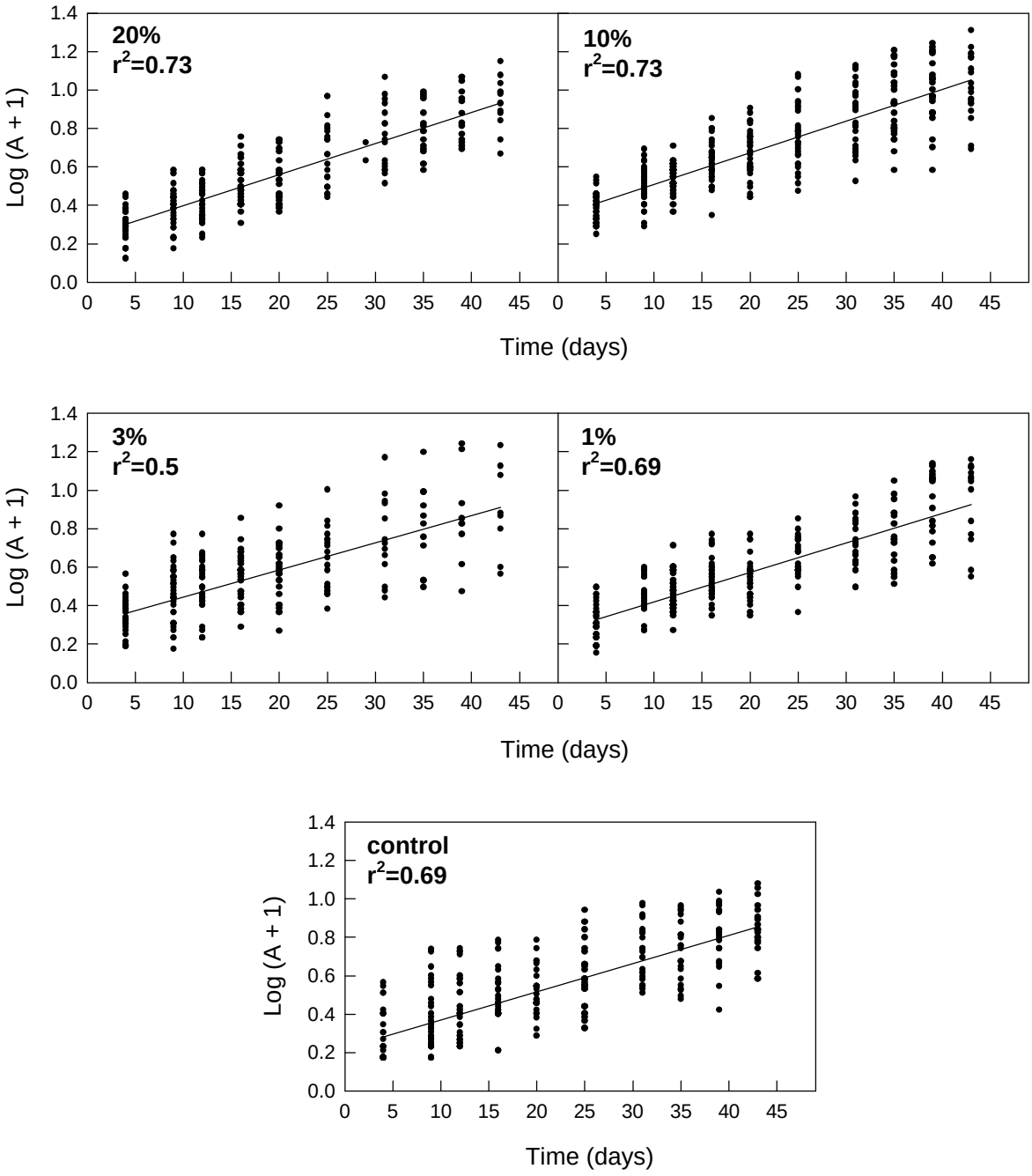


Figure 11.9. **1999** regression lines for the first 43 days' growth of *Burnupia stenochorias* within each test dilution (20%, 10%, 3% and 1% effluent and the controls). The data points indicate individual sizes with all replicates from each dilution pooled. Distribution around the regression line is an indication of the variability in size (as indicated by change in the logarithms of shell area).

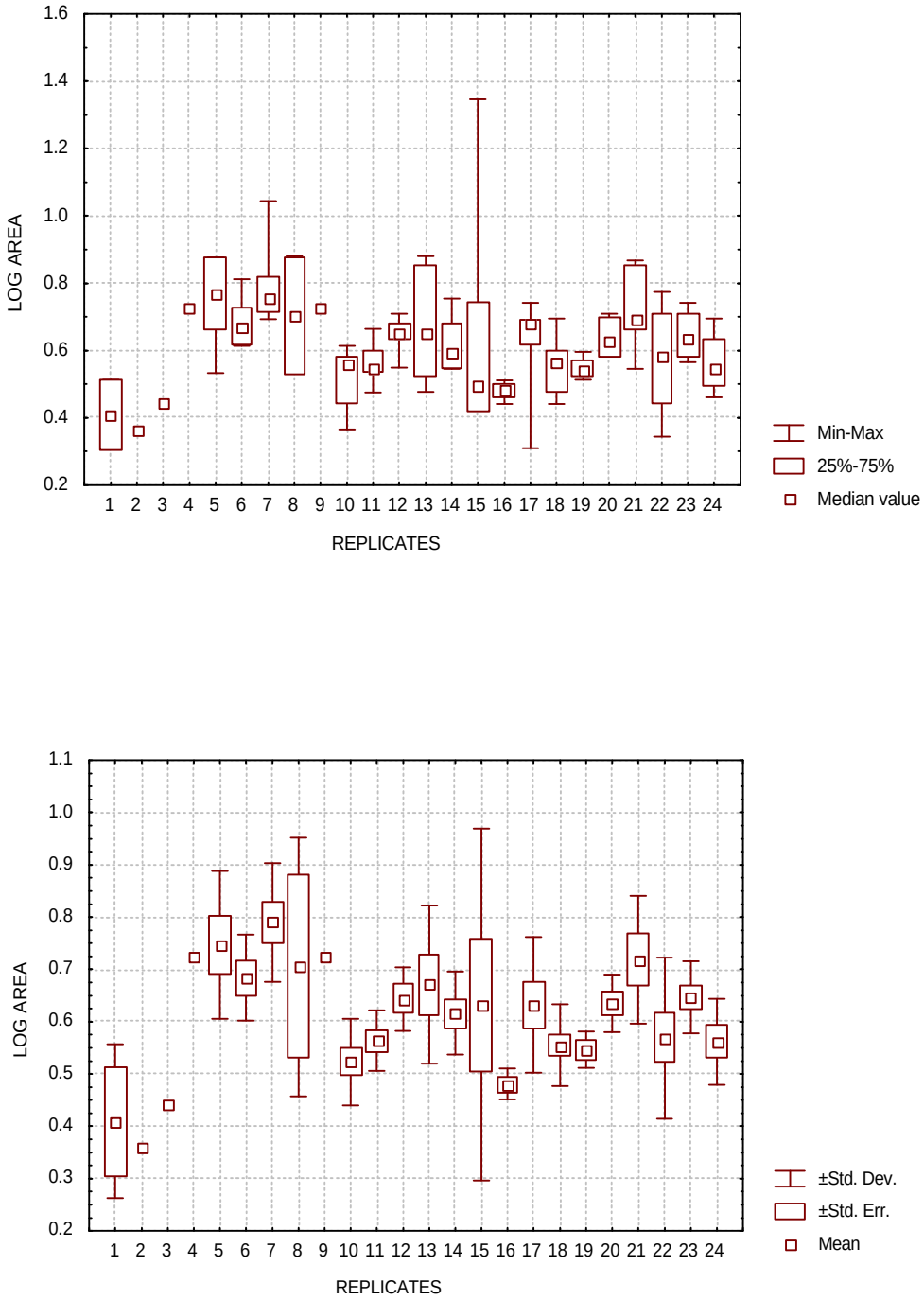
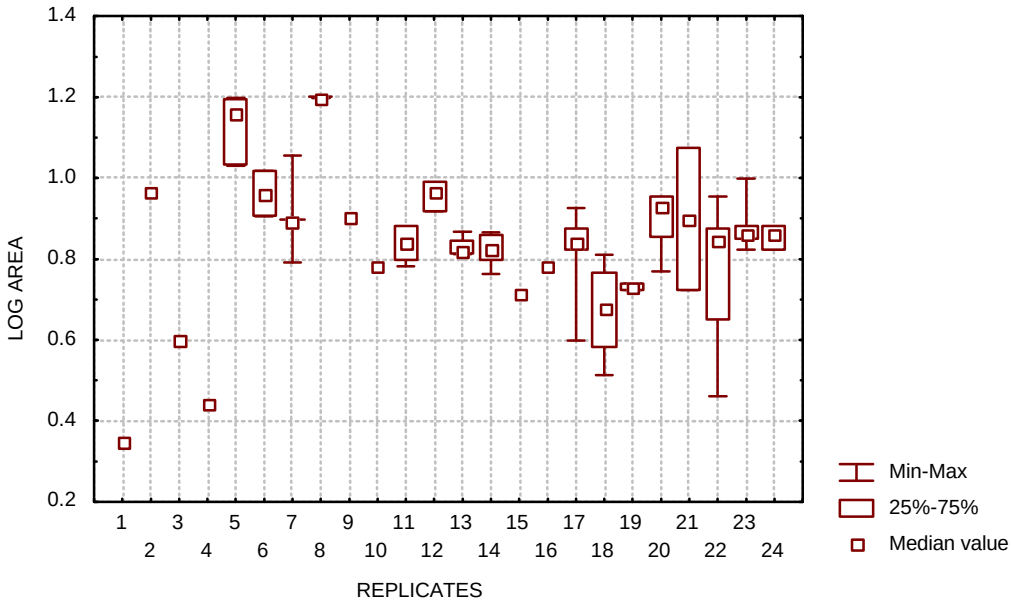


Figure 11.10. **1998, 21 day** minimum and maximum sizes [$\log_{10}$ (area +1)] of *Burnupia stenochorias* within each replicate, with means, standard deviations and standard errors presented separately. Replicates 1-4 = 100% effluent; 5-8 = 10% effluent; 9-12 = 3% effluent; 13-16 = 1% effluent; 17-20 = 0.1% effluent; 21-24 = control (tap water).



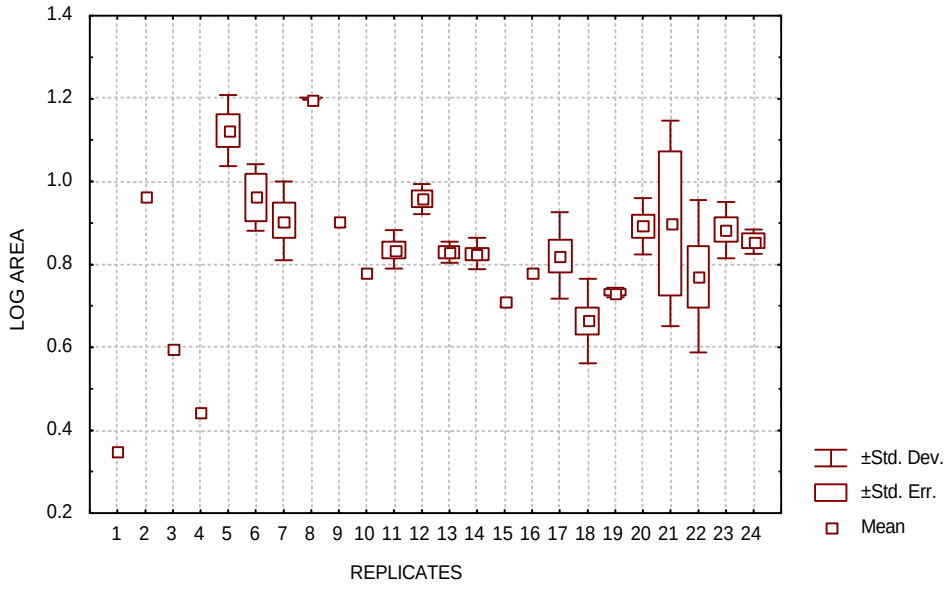


Figure 11.11. **1998, 43 day** minimum and maximum sizes [$\log_{10}$ (area +1)] of *Burnupia stenochorias* within each replicate, with means, standard deviations and standard errors presented separately. Replicates 1-4 = 100% effluent; 5-8 = 10% effluent; 9-12 = 3% effluent; 13-16 = 1% effluent; 17-20 = 0.1% effluent; 21-24 = control (tap water).

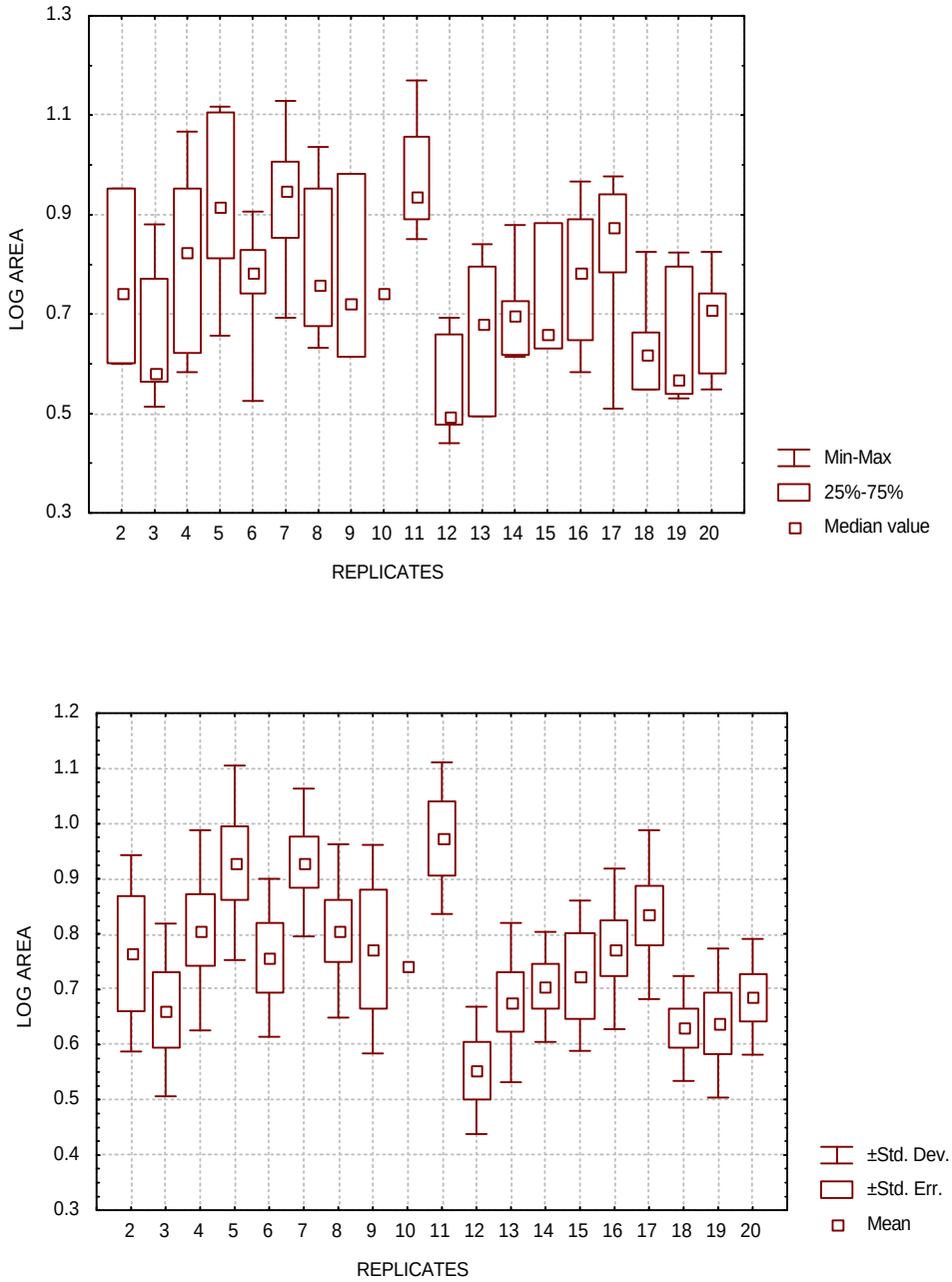
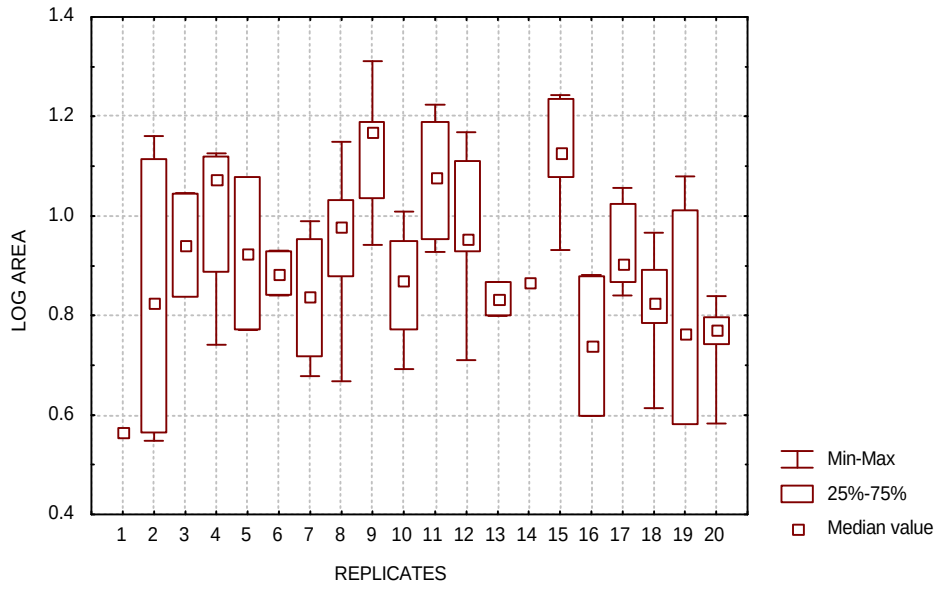


Figure 11.12. **1999, 21 day** minimum and maximum sizes [$\log_{10}$ (area +1)] of *Burnupia stenochorias* within each replicate, with means, standard deviations and standard errors presented separately. Replicates 1-4 = 20% effluent; 5-8 = 10% effluent; 9-12 = 3% effluent; 13-16 = 1% effluent; 17-20 = control (tap water).



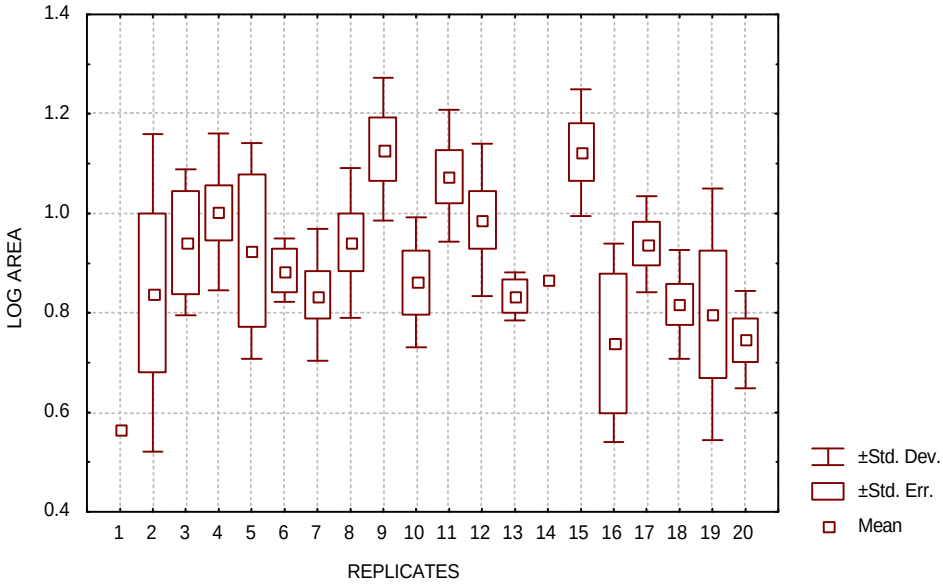


Figure 11.13. **1999, 43 day** minimum and maximum sizes [$\log_{10}$ (area +1)] of *Burnupia stenochorias* within each replicate, with means, standard deviations and standard errors presented separately. Replicates 1-4 = 20% effluent; 5-8 = 10% effluent; 9-12 = 3% effluent; 13-16 = 1% effluent; 17-20 = control (tap water).

Table 11.5. Fecundity data recorded for the P1 generation (1999) subjected to the various concentrations of textile effluent, compared to the control. N = total number of replicates. [1] Mean total number of capsules is used as an indication of a potential change to the population and not for individual fecundity rates; [2] calculated from the total number of capsules per concentration ÷ number of limpet days from first lay; [3] one capsule did not develop. [a] Significant difference between concentration and the control; [b,c,d] significant difference between concentrations (Student's 't' test, $p < 0.05$).

Concentration	Statistic	20% effluent	10% effluent	3% effluent	1% effluent	Control
Number of replicates with egg capsules laid	n/N	3/4	2/4	4/4	2/4	4/4
Age at first lay (days)	age	47	65	48	69	55
	range	46-49	46-84	42-55	42-96	46-73
	C.V.	8.7	47.1	13.5	55.3	23.1
	± s.dev	4.2	29.7	6.5	38.2	12.7
Maximum number of lay days [per replicate, not individual]	mean	31.3	27.5	52.3	29.5	30.3
	range	1-54	16-54	41-62	1-58	7-5
	C.V.	71.1	135.55	18.9	132.0	71.1
	± s.dev	22.4	37.4	9.8	39.6	21.5
Mean total number of capsules[1] [averaged per replicate and not per replicate where laying occurred]	mean	80.2[a]	45.8	198.2[a]	16.5	47.3
	range	0-260	0-129	31-330	0-61	5-106
	C.V.	86.1	133.6	104.2	80.5	89.6
	± s.dev	52.7	61.1	86.1	29.8	42.6
Number of capsules/ limpet lay day[2]		0.57	0.98	0.54	0.87	0.59
Mean number of eggs per capsule	mean	7.8[ab]	7.5[ac]	7.45[ad]	4.6[bcd]	5.4
	range	2-12	4-13	2-13	1-9	1-11
	C.V.	30.0	38.2	35.6	34.2	45.4
	± s.dev	1.9	2.9	2.4	1.6	2.3
Mean time to hatching	mean	12.5[a]	12.7[ab]	11.6[ab]	12.2[a]	14.1
	range	10-15	12-14	9-13	11-14[3]	13-15
	C.V.	12.8	5.9	10.4	9.8	5.8
	± s.dev	1.6	0.8	1.2	1.2	0.8

11.3.3. Toxicity of Effluent

Survival

Percentage data were arcsine transformed to normalize their distributions. Statistically, survival of limpets at different concentrations generally had high coefficients of variance (Table 11.3 and 11.4). Monotonicity of dose response did not occur. In 1998, 43 day arcsine transformed data were not normally distributed (Shapiro-Wilks, $W = 0.871$, $p < 0.001$). In 1999, survival data were normally distributed (Shapiro-Wilks, $W = 0.885$ $p < 0.018$) but variances were not homogenous (Bartlett's Test; $\div^2 = 7.815$, $p < 0.05$). The main results were as follows:

- The rate at which mortality of limpets occurred at 100% effluent (Figure 11.1) was significantly greater ($p < 0.05$) than when limpets were maintained in 20% or less effluent.
- Survival at 43 days (Figure 11.6 and 11.7) could be expressed as:

  $100\%^a < 3\%^a < 1\%^a < 10\%^a < 0.1\%^a <$ control (1998), and

  $1\%^b < 10\% < 20\% < 3\% <$ control (1999),

with ^{a} and ^{b} significantly different to the controls (Steel's Many-One Rank Test). The toxicological interpretation is a 43 day No Observed Effect Concentration (NOEC) at < 0.1% (1998) and < 1% (1999) effluent, and Lowest Observed Effect Concentration (LOEC) at 0.1% (1998) and 1% (1999) effluent. At 100 days (1999) there was no significant difference between survival in the controls versus effluent concentrations (100% effluent was not tested in 1999).

Based on mortalities, Probit analyses to determine LC_{50} values for 21, 30 and 43 days were unsuccessful because of high chi-squared values. Trimmed Spearman-Karber analyses for 1999 mortality data at 21, 30 and 43 days were too high (78.6%, 72.4% and 59.1% respectively) to calculate any LC_{50} values. The 1998 data gave no LC_{50} value at 21 days, because of high trim values (52.6%). At 30 days, the LC_{50} value was calculated as 20.35% effluent but with a large 95% confidence interval of 7.49-55.28. However,

- at 43 days the LC_{50} value was 3.87% effluent (95% confidence interval of 2.3-6.49).

No $LC_{2.5}$ (first death in the control replicates) and LC_{10} values could be calculated using Probit (chi squared values high) or Trimmed Spearman-Karber (trim too large).

Growth

Regressions of the first 43 days' growth of limpets, represented as change in size [$\log_{10}$(area of shell

aperture + 1)], at the different test solutions (Figures 11.8 and 11.9) indicated a linear relationship between increase in shell aperture and time. Comparison of the limpet growth regression lines (ANCOVA, Graphpad Prism 3.0) up to 21 days gave no significant differences in slopes between the effluent solutions ($p > 0.21$), or between the effluent solutions and the control ($p \geq 0.8$). However, over 43 days:

- Slower growth occurred in 100% effluent (1998) compared to all other solutions including the control, except 0.1% effluent; and slower in 0.1% effluent compared to 3% and 1% but not from the control (1998) (Table 11.6).
- More rapid growth occurred in 10% effluent than in all other solutions (1998) and than 3% effluent and the control (Table 11.6).
- In 1999, growth rates within the 20%, 3%, 1% and controls were not significantly different (Table 11.6).

Contrary to the growth rates to 21 days, Steel's Many-One Rank Test ($p < 0.05$) suggested:

- Limpet sizes at 21 and 43 days in 100% effluent were significantly smaller than the control and other effluent concentrations (Figures 11.14 and 11.15).
- In 1999, mean limpet size at 21 days in all effluent concentrations was larger than when in control conditions (Figure 11.14).
- At 43 days however, size data was not normally distributed in 1998 (Shapiro-Wilks Test, $W = 0.9563$, $p < 0.007$) but normally distributed with homogeneity of variances in 1999 (Shapiro-Wilks Test, $W = 0.9814$, $p < 0.244$; Bartlett's Test, $\chi^2 = 27.815$, $p < 0.05$) (Figure 11.15). When all concentrations from 1999 were compared, significant differences in size of limpets at 43 days were seen ($F_{(4,82)} = 3.78$, $p < 0.007$).
- However, only 3% effluent growth was significantly different (greater than) the control (Tukey's honest significance test for data not normally distributed, $p < 0.003$) (Figure 11.15).

Combining the results from growth rates and size attained at 43 days, notwithstanding the effects of mortality through time on average sizes, a loose pattern as to the effects of effluent can be defined, in relation to growth and size:

$$10\% > 3\% = \text{Control} = 1\% > 0.1\% > 100\%^{a} \quad (1998), \quad \text{and}$$

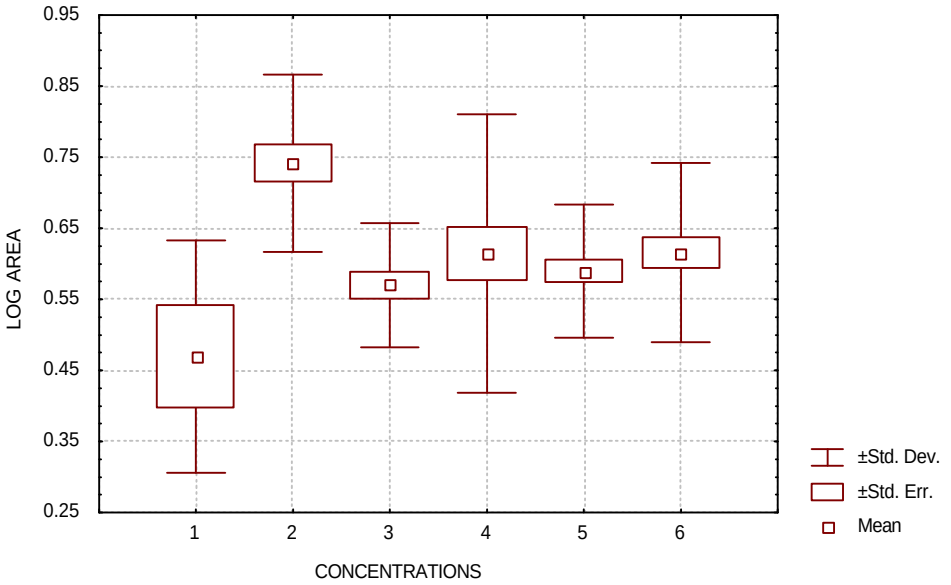
$$3\%^{a} > 1\% = 10\% > 20\% > \text{Control} \quad (1999),$$

where ^{a} is significantly different in growth and size attained to limpets in the remainder of the dilutions.

Table 11.6. Comparison between the combined test solution growth regression lines after 43 days within each dilution of effluent and the controls, in 1998 and 1999. df = degrees of freedom; P = probability; a = slopes significantly different; b = slopes and intercepts not significantly different ($p < 0.05$).

Year	Test solution	Comparison with	F	df, df error	P	Significance
1998	100%	10%	80.2	1,502	<0.001	a
		3%	32.12	1,518	<0.001	a
		1%	17.7	1,474	<0.001	a
		0.1%	8.03	1,610	0.004	b
		Control	11.91	1,538	<0.001	a
	10%	3%	48.3	1,654	<0.001	a
		1%	43.57	1,612	<0.001	a
		0.1%	108.5	1,746	<0.001	a
		Control	56.3	1,674	<0.001	a
	3%	1%	0.33	1,628	0.562	b
		0.1%	13.49	1,762	<0.001	a
		Control	2.19	1,690	0.139	b
	1%	0.1%	6.82	1,720	0.009	a
		Control	0.64	1,648	0.422	b
	0.1%	Control	2.82	1,782	0.093	b
1999	20%	10%	0.084	1,588	0.771	b
		3%	3.81	1,485	0.051	b
		1%	1.06	1,533	0.302	b
		Control	3	1,568	0.083	b
	10%	3%	5.39	1,567	0.02	a
		1%	1.91	1,615	0.166	b
		Control	4.69	1,650	0.031	a
	3%	1%	1.06	1,530	0.302	b
		Control	3	1,568	0.08	b
	1%	Control	0.86	1,642	0.355	b

a) 1998



b) 1999

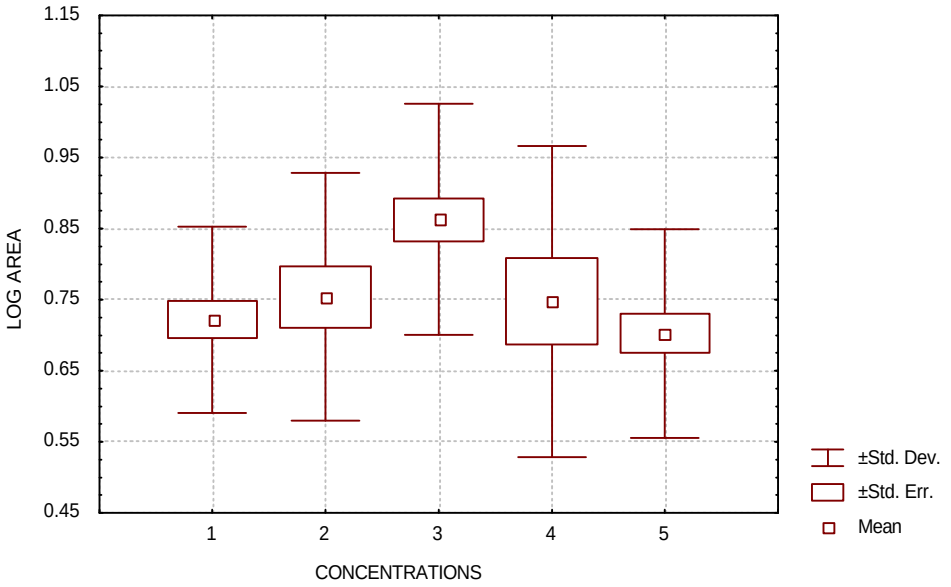
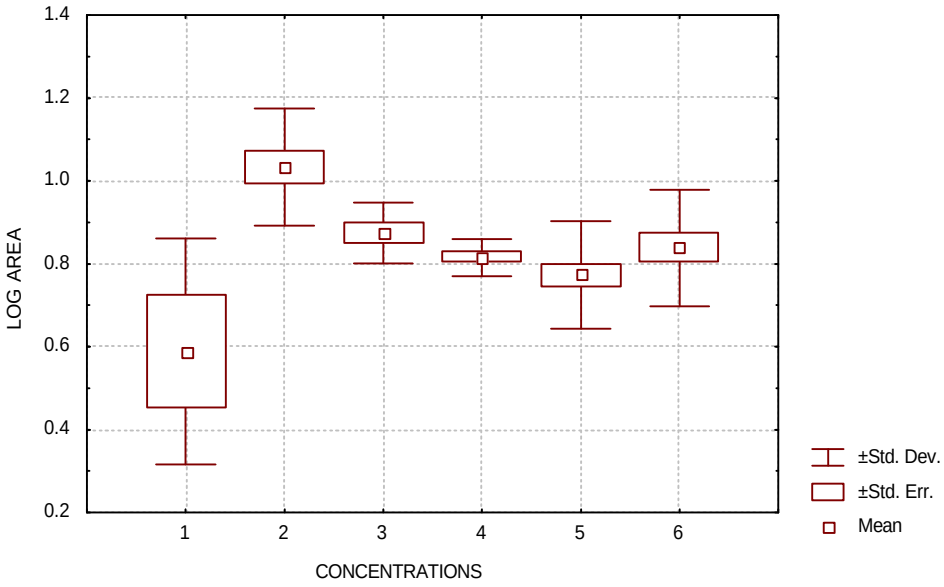


Figure 11.14. Categorized plots of size (Log [A+1]) of *Burnupia stenochorias* **after 21 days**, in each concentration.
a) **1998**: concentration 1 = 100% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = 0.1% effluent; 6 = control (tap water).
b) **1999**: concentration 1 = 20% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = control (tap water).

a) 1998



b) 1999

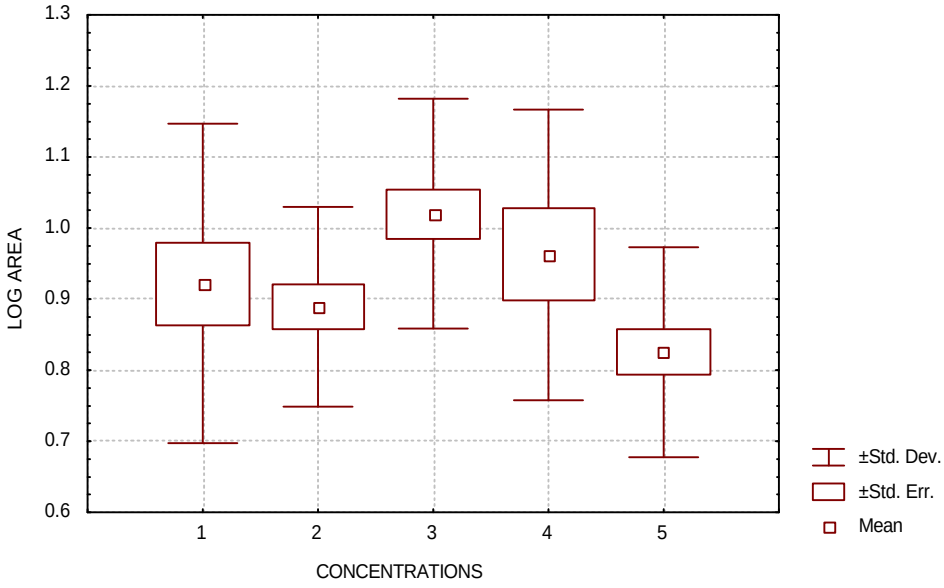


Figure 11.15. Categorised plots of size (Log [A+1]) of *Burnupia stenochorias* **after 43 days**, in each concentration.
a) **1998**: concentration 1 = 100% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = 0.1% effluent; 6 = control (tap water).
b) **1999**: concentration 1 = 20% effluent; 2 = 10% effluent; 3 = 3% effluent; 4 = 1% effluent; 5 = control (tap water).

Fecundity

The majority of limpets in 100% effluent (1998) died at a young age and the few remaining did not reproduce. In 1999, of four replicates in each test solution, two had no egg capsules laid in 10% and 1% effluent; and one in 20% effluent, giving small degrees of freedom (Table 11.5). This, combined with generally large coefficients of variability (Table 11.5), and varying numbers of limpets per test solution (Figures 11.4 and 11.5), gave non-statistically significant differences ($p < 0.05$) between solutions for age at first lay. Similar non-significant differences were found for the maximum number of lay days, where the range is based on replicates and not individuals within the replicates. Although the range in maximum number of lay days varied little between each of the concentrations, the range within the 3% effluent was narrower and higher than limpets in the other solutions. Because of high mortalities, the effect of effluent on the rate of reproduction was calculated as number of capsules per limpet lay day. Both 10% and 1% effluent increased this rate compared to the control (Table 11.5). However, despite large ranges, the number of eggs per capsule was significantly less than the control (Student's 't' test, $p < 0.05$) when *B. stenochorias* were exposed to 1% effluent, and significantly more ($p < 0.05$), but not different from each other, when exposed to 20%, 10% and 3% effluent. Time to hatching was more consistent between replicates and test solutions (coefficients of variation 5.91-12.82; Table 11.5). Hatching times were less in effluent, with those in 3% effluent hatching significantly earlier than those in 10% effluent and the control (Student's 't' test, $p < 0.05$).

As a summary, fecundity of limpets in the tests dilutions could be depicted as:

3% > 20% > 10% > control > 1%.

F1 generation

Long term effects of effluent can be judged by the effects on the parent generation continuing through the F1 generation. Newly hatched limpets were all less than 1mm shell length, and were from 1-4 days old. Food tiles supplied had the same [colour] differences as found with the parent generation, suggesting diatom species were different and therefore food supply also differed between replicates. The colours did not change over the duration of the test. The survival and growth of the F1 generation monitored in the three replicates of either the same effluent to which the parent generation had been subjected, or the control solution (dechlorinated carbon filtered tap water), gave larger variability in response than seen in the P1 generation (the initial generation used in each test).

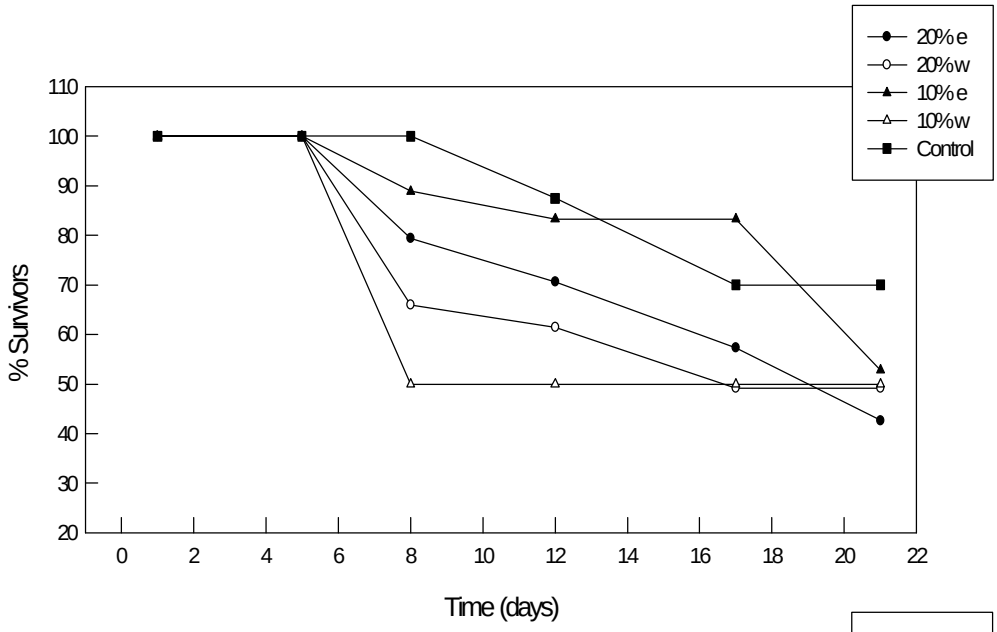
- Survival was significantly higher after 21 days in the control replicates than for limpets previously maintained in effluent (MANOVA, $p < 0.01$; Figure 11.16).
- Survival was significantly lower after 21 days in the 3% effluent[P1] & water[F1] regime than all other solutions ($p < 0.01$).

Comparisons of the regressions of growth data for 21 days (Graphpad Prism 3.0), based on a change in shell aperture size (Log_{10} [area + 1]), gave no significant differences in slopes (growth rates) between the F1 generation of limpets subjected to the different concentrations ($p > 0.21$), or between limpets in either effluent or carbon filtered, aerated tap water ($p \geq 1$). However, r^2 values for the regression lines were very poorly fitting for those limpets from 20%, 10% and 3% effluent (Table 11.7).

Table 11.7. R^2 values, indicating goodness of fit, from the growth regression lines of *Burnupia stenochorias* F1 generation maintained in either effluent or dechlorinated, carbon filtered tap water. Limpets originated from the P1 generations which were grown in either effluent (solution) or tap water (control). Growth was over 21 days. n.t. = not tested.

Solution	20% effluent	10% effluent	3% effluent	1% effluent	control (tap water)
effluent	0.44	0.244	0.204	0.64	n.t.
water	0.59	0.222	0.166	0.599	0.751

a)



b)

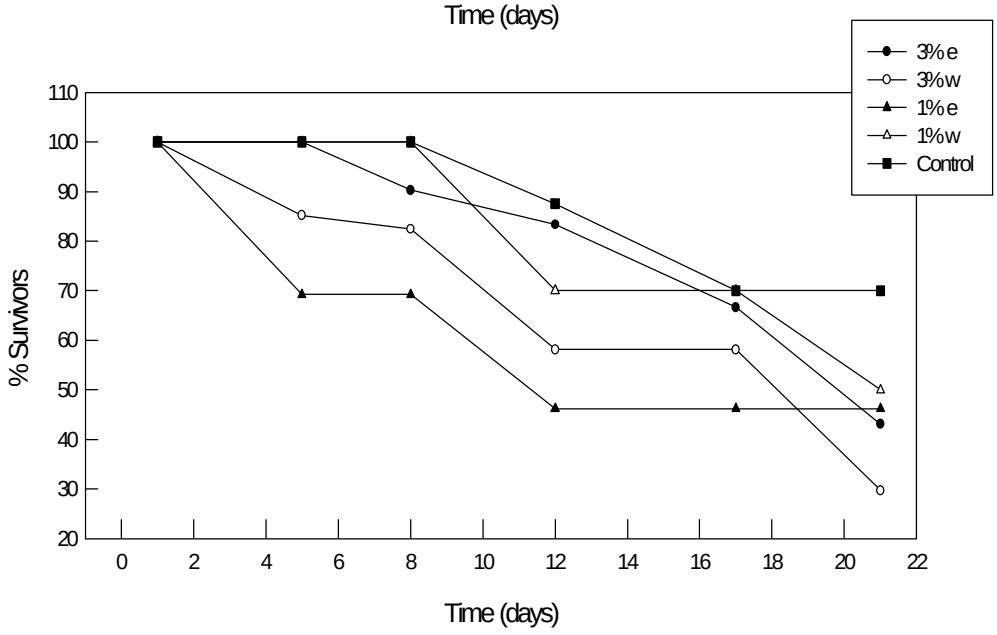


Figure 11.16. 1999 survival over 21 days of F1 generation of *Burnupia stenochorias*, maintained in either the same effluent (e) after hatching or control solution (tap water; w).
a) limpet survival when parent generation from 20% or 10% effluent, and control.
b) limpet survival when parent generation from 3% or 1% effluent, with control included as a comparison.

11.4. DISCUSSION

The value of chronic whole effluent toxicity testing, compared to acute toxicity testing either with individual chemicals or with whole effluent, is in the holistic assessment of the potential hazard to the test species; and that the potential longer term effects on a species life cycle are more clearly defined. The objective of chronic whole effluent testing is to estimate a concentration which will allow normal propagation of the species within the ecosystem. There are many responses which can be tested at a chronic level (Blok and Balk, 1995; Freidig *et al*., 1999; Roses *et al*., 1999). Survival has been the endpoint most frequently monitored (Mayer *et al*., 1986). However, other key life history parameters include reproductive effort, growth (together with reproduction, perceived as more sensitive and ecologically relevant than survival which is too-extreme a endpoint [Suter *et al*., 1987]), and pattern of energy allocation (e.g. scope for growth [Maltby *et al*., 1990; Lai and Lam, 1994; Lam, 1996b]). In this study, survival, growth and reproduction of *Burnupia stenochorias* were initially selected for examination.

Whole effluent is complex in nature and variable with time (Slabbert *et al*., 1998). The textile effluent samples used in the chronic tests were dominated by dye colour, combined with organic compounds, salts and heavy metals, with high oxygen demands. The chronic effects on an aquatic benthic organism are likely to be complex, disrupting osmoregulation, respiration, and other metabolic processes (Levin *et al*., 1989).The toxicological testing of the textile effluent using *B. stenochorias* gave rise to both positive and negative effects and large variability in survival, growth and reproduction, making the results difficult to unravel and summarize.

11.4.1. Variability Introduced by the Materials and Methods

Development of methodology, after biological studies, is the first step towards a successful predictive chronic toxicity test. The basis of this chronic investigation was to monitor the limpets from hatching to 100% mortality, and in order to do this, field collected adults (Section 9.1) were used to ensure sufficient hatchlings of the same age were available at the same time. The field limpets therefore acted as the source of variability to be studied (on the presumption that wild populations have larger phenotypic and genotypic variation than laboratory populations). Although criticized as not being environmentally real (USEPA, 1994), the static system for the chronic toxicity tests (as opposed to flow-through; USEPA, 1993) was easy, cheap, reliable and could be used in any field or laboratory

situation if required. It was also considered suitable for *B. stenochorias* which had been sampled from a non-flowing water source where habitat and therefore exposure to toxicants are potentially more easily compared. There is the possibility that the plastic bags used to line the containers may have released plasticisers into the solutions, but whether the plasticisers are toxic has still to be proved (Section 9.2.1). The bags facilitated an easy transfer of hatchlings from where the egg capsules were laid, to the test containers; provided an ideal medium from which to examine limpet size, and to monitor egglay and development; and reduced the known stress of handling (Haigh and Davies-Coleman, 1997).

The nutritional status of the test organisms is a vital part of a chronic toxicity test (Lanno *et al*., 1989; Lamberti and Steinman, 1993). Dietary factors are also known to modify the toxicity of various compounds (Dahlgren, 1988). The feeding regime used was initially considered adequate for this investigation (Section 6.2.1), particularly as the ceramic tiles have been used successfully in other chronic toxicity tests (Lowell *et al*., 1995). The time taken to reach sexual maturity of the control limpets compared favourably (47 - 69 days; Table 11.5) with previous laboratory bred populations of *B. stenochorias* (40 - 55 days; Chapter 5), compared to 240 - 270 days under natural conditions (Section 4.3). Although the quantity of algae did not appear to be limiting, the quality was of concern however, primarily because of the inevitable variability that occurred in algal, fungal and bacterial growth on the food tiles used, and eventually on the plastic bags themselves. As sand or silt particles naturally occur in riverine systems, their addition may improve the ability of the limpets to deal with the effluent toxicity (Thomas, 1998).

Over the entire test period however, food variability was also a product of the effluent, as the change in nutrients with each mixture of effluent, particularly the high nitrogen levels, would have affected the growth and composition of algal communities (Mulholland *et al*., 1991) and therefore the diet of *B. stenochorias*. These changes, however, would also be expected in the receiving waters of the whole effluent. Similarly, the effect of the effluent, in particular the dyes, on the presence of the potentially nutritionally valuable bacteria (Section 4.4) is unknown, but the bacterial results suggest the effect may be varied and difficult to describe. Many dyes have antimicrobial properties, depending on the types of compounds used in the process (Timofeeva, 1991). However, the particular compounds present in the effluent are undeclared, and the presence of bacteria in the test was only monitored as

presence or absence of colonies and not as type (gram positive or negative). A similar situation exists with the growth of algae: most surfactants (found in dyes, many of which are sodium compounds) are absorbed by algae and are considered regulators of algal photosynthesis (Timofeeva, 1991). Limpets would therefore also accumulate potential toxins through the concentration of surfactants in the algae.

The renewal of the effluent twice weekly had a two-fold effect. The renewal reduced some of the factors which may have affected the effluent toxicity, such as biodegradation of the effluent or chemical transformation of the toxicants; volatilization; and uptake and metabolism of the toxicants by the test organism (APHA, 1992). Renewal of effluent also renewed depleted dissolved oxygen, calcium and other nutrients. With only ten limpets per container, calcium was unlikely to have limited limpet growth, however, as a volume of 40 ml of solution per limpet is considered sufficient for molluscicide tests (WHO, 1965); and a density of 25ml solution per limpet was previously found to be acceptable for laboratory growth and reproduction (Haigh and Davies-Coleman, 1997), compared to the 300 ml per limpet used in the chronic tests. Effluent renewal however, is more likely to reflect the field conditions of effluent variability after dilution with river water.

The renewal of effluent also introduced variables into the chronic tests, as its chemical composition varied over time, despite the use of sub-samples of the effluent being transported, frozen and defrosted according to USEPA protocols (1984; Section 9.1). Preserving the samples to be analysed with mercuric chloride, before delays in analysis at the analytical laboratories, discredits the idea that bacterial activity may have changed the water chemistry to the degree seen between samples (P. Wade, pers. com.). Insufficient mixing of the defrosted effluent before re-sampling for analysis may also have contributed to the variability in chemical composition. Precipitation of toxic metals may occur during freezing, decreasing their bioavailability (Pretorius, 1996). Similarly, copper, zinc and other essential trace elements will adsorb onto solids such as the dye particles. Human error in the analytical laboratory should also not be discounted as a cause of variability in the effluent analyses. The tap water used as diluent also varied over time, contributing to the significant differences between 1998 and 1999 effluent dilution TDS and pH recordings (Table 11.1). Comparisons of the toxic effects of the effluent between the 1998 and 1999 limpets can therefore only be made with caution.

11.4.2. Toxicity of Effluent and the Variability of Response of *Burnupia stenochorias.*

Studying the effects of the textile whole effluent on the complete *B. stenochorias* life cycle (from egg to egg) has provided baseline data for future toxicological use of the limpet. However, the results suggest that growth and reproductive responses give a confusing level of variability, both innate and toxicological, and it was difficult to discriminate whether these parameters were biologically significant with negative effects when exposed to the effluent.

Size of Test Organism

Sensitivity of newly hatched limpets has been shown to vary within the first few days, underlining the importance of same-age test organisms (Gomot, 1998). Mollusc size is also known to influence contaminant accumulation and susceptibility (Wier and Walter, 1976; Gomot, 1998). The young are generally considered more sensitive that the adult stage (*e.g.* Millington and Walker, 1983). The use of newly hatched in the chronic tests is therefore useful in determining toxicological effects of the effluent, as well as reducing variability of response. Growth regression line intercept comparisons confirmed that all *B. stenochorias* hatchlings used in the chronic tests were not statistically significantly different in size.

Survival

It was difficult to discuss any general trends in survival over time and with increasing effluet concentration, based on coefficients of variation within effluent solutions and the controls (Tables 11.3 and 11.4). However, few *B. stenochorias* survived in the undiluted effluent, and those that did were small and did not reproduce. The 0.1% and 1% effluent dilutions were defined as the Lowest Observed Effect Concentrations but with large ranges (LOECs, Section 9.3.2). The use of LOECs (together with No Observed Effect Concentrations, NOEC) is questionable but an alternative is not yet available (Section 10.4). An LC_{50} value of 20.4% effluent was calculated after 30 days, although with large 95% upper and lower confidence intervals; and 3.9% at 43 days.

The control mortalities of 20% or more after 43 days were higher than normally acceptable for a test organism (USEPA, 1991; Greve *et al.*, 1998). However, it was considered feasible to compare survival of limpets between the controls and effluent dilutions because initial survival of immature snails at an early stage can be poor (Section 4.3.4; Dillon, 2000). Longevity in the controls was approximately 100 days for the toxicity tests, compared to previous laboratory cultures of 84 to 210

days (Section 6.3; Chapter 7). Fungal growth and the proliferation of bacteria on individuals were not apparent, although they have been recorded as responsible for variability in survival (SETAC, 1999).

Growth

Growth was linear, as previously demonstrated in the laboratory (Section 6.3.1), with large variability between individuals (Figures 11.8 to 11.13), common amongst limpets (Kozlowski, 1996). Growth was monitored for 43 days before adequate statistical differences were found; 14 days is usually considered sufficient with other pulmonates (Skoog, 1979). Limpets in 100% effluent were significantly smaller than all other limpets (Steel's Many One Rank Test). However, stimulation of growth and fecundity also occurred, particularly at the lower dilutions of effluent, although mortality was also greater. At 43 days, limpets in 3% effluent were significantly larger than the controls (Tukey's Honest Significant Difference Test, $p < 0.05$).

In assessing the usefulness of *B. stenochorias* growth in chronic toxicity testing, where good quality control between duplicated tests is required, smaller growth variation in the controls would be expected (USEPA, 2000). Coefficients of variation for *Ceriodaphnia* species and fathead minnow larvae, for example, are typically 10-20% and 6-15% respectively (Stewart and Loar, 1994). Similarly, mortality rates below those in the NOEC effluent dilution are usually considered necessary before toxic effects on growth can be significantly assessed (Rand and Petrocelli, 1985; Green, 1987; Grothe *et al.*, 1996). In this study, the NOEC (mortality) was estimated to be below the effluent concentrations tested. Therefore, an assessment of a toxicity endpoint of the whole effluent on the growth of *B. stenochorias* can only be made with some circumspection.

Reproduction

Assumptions were made when determining the effect of the effluent on the fecundity rate. Firstly, the rate of lay would not change with the age of the adult after first reproduction. Secondly, birth rate of the eggs would not change over time under ideal conditions. It was presumed, therefore, that any effects seen reflected the influence of the textile effluent.

Fertility is generally more sensitive to toxic metals than fecundity (Ravera, 1991). No eggs were laid in one or two of the four replicates subjected to 20%, 10% and 1% effluents compared to 6%

previously obtained (Section 6.3.2). This was possibly a combination of reduced food quality (Section 11.3.1) and accumulated toxic effects of the effluent (Humphrey *et al.*, 1995). Embryonic deformity, common when freshwater gastropods are exposed to heavy metals (Ravera, 1977; 1991), was not observed. A naturally large range in fecundity appears to be a regular phenomenon amongst pulmonates, including *B. stenochorias* (Table 6.3; Duncan, 1975; Dillon, 2000). The ranges in fecundity data for the controls in the chronic test (Table 11.4) were as broad as previously found in reproductive investigations (Table 6.2). Similar standard deviations have occurred with other species of snails in toxicity tests, but this large innate variability tends to hide toxicological effects (Watton and Hawkes, 1984; Baturo *et al.*, 1995; Gomot, 1998). Different mortality rates and possibly dietary differences between replicates may have contributed to uncertainty of fecundity results of *B. stenochorias*.

Limpets in 1% effluent had reduced fecundity compared to those in the controls. Fecundity was higher for limpets maintained in 3% to 20% effluent, but not monotonically, suggesting a complexity of reactions occurred. Any increases in fecundity were possibly related to earlier sexual maturity resulting from the increased growth. Although no individual fecundity data were obtained, the overall results suggest the potential for negative population effects. Both primary toxic effects and secondary compensatory effects are often expressed in the same response variable, but in opposite directions. Thus the increase in the measured variable, like growth and reproduction, does not necessarily imply that the organism is performing better, but rather performing differently to the control. Hence, negative longer term effects of reproductive stimulation should not be ruled out (Mitchell, 1992). The further development of techniques using these criteria for monitoring the effects of effluent on the reproduction of *B. stenochorias* would be useful, but should include the rate of lay over time, and the development and hatching rate of the eggs, the latter potentially providing a shorter term method of monitoring toxicity effects. Monitoring rate of lay would rely on a more refined feeding regime than was used to date.

In many invertebrates and algae, it is not uncommon to see stimulation of reproduction and growth, relative to controls, occur at low test concentrations and inhibition at high test concentrations (Walsh *et al.*, 1980; Masters *et al.*, 1991), referred to as hormesis (Stebbing, 1982). However, the stimulatory effects are generally seen at a dose 30%-60% greater than the control, but 4-5 fold *below* that of the

NOEC, depending on the number of doses used (Calabrese and Baldwin, 1998). Although this is not a hard and fast rule, the stimulation of aspects of the growth and reproductive effort in *B. stenochorias* were unlikely to be hormetic responses. Similar non-hormetic complexities of growth and fecundity responses were observed with *Lymnaea stagnalis* subjected to acetone (Bluzat *et al*., 1979). It appears that, in these particular effluent samples, the difference between the level of concentration of chemical(s) which provided stimulation and the level at which that concentration, perhaps through necrosis (Verhaar *et al*., 1999), became toxic to *B. stenochorias* occurred within the 0.1%-20% range of effluent over 30 to 43 days.

F1 generation

Physiological adaptation is said to be the most common cause of inter-population divergences in metal tolerance in aquatic organisms (Lam, 1996c). Although there is the possibility that inter-population divergences would not be genetically based, Lam (1996c) has suggested that a pulmonate F1 generation would loose their differences in sensitivity to metals after 1 week. The long term effects of the textile effluent dilutions on *B. stenochorias*, as depicted in survival and growth effects observed in the F1 generation for three weeks, were not significantly different from each other or from the control limpets. However, far greater variability was seen in growth rates than observed in the parent generation, and growth was not linear with time. Survival was least in 3% effluent (compared to 1% for the parent generation). Some acclimation to toxicant does appear to have occurred.

An F2 study (*e.g.* Flannagan, 1974; Lam and Calow, 1989) would be useful but time consuming, expensive in terms of human resources and therefore unlikely to be of use in molluscan methods developed for future toxicological application in South Africa. Although continuing the toxicology tests to an F1 generation showed the consequences of long term exposure, the time taken to reach the endpoints, in themselves vague, suggests P1 toxicological tests using *B. stenochorias* would be more practical and useful.

Water Quality Variables Contributing to Toxicity

It is possible to speculate on the effects of the water quality and effluent variables (Tables 11.1 and 11.2) on the toxicity responses of *B. stenochorias*. Although whole effluents are characteristically chemically complex, an overview of the potentially toxic water quality variables may be of use in future recommendations to the industry concerned. Toxicity Identification Evaluations would reduce the speculation (Dallas and Day, 1993; Correia *et al*., 1994).

The colour (Meyer *et al.*, 1992), and high chemical oxygen demand in the whole effluent reflect the high load of dyes and fibre particles found in the effluent. Dyes are considered potent inhibitors of various enzymes, also interfering with the transport of calcium and magnesium ions which are responsible for muscle contraction (Chhaya *et al.*, 1997). It is questionable, therefore, whether the recognition of death (which relied on no movement of the foot after mechanical stimulation) was accurate. The inability to attach to the substrate is recognized as a distress symptom, however, characteristic of molluscicide effects for example (Harry and Aldridge, 1973). Continued paralysis, if maintained under the same conditions, would have resulted in death. Non-movement of the foot therefore remains a reasonable method of recognizing a survival, toxicity endpoint for *B. stenochorias.*

It is recommended in South Africa that a pH change of more than 1 unit would be undesirable in a natural situation, but little is known about the effects of elevated pH levels on freshwater invertebrates in general in South Africa (Dallas and Day, 1993; DWAF, 1996). Gastropods are generally sensitive to pH (Mouthon, 1996a, b). The chronic tests produced statistically significant differences in pH between the test solutions occurred (Student's 't' test, $p < 0.05$), but with ranges of 1.5 or less. The greatest range measured over 12 months in pH measurements from local rivers where *B. stenochorias* proliferate was 2.8 (Figure 4.4). This suggests *B. stenochorias* is tolerant (although not necessarily free from effect) of such a range in pH. The effects of pH changes on the life history parameters of *B. stenochorias* require further investigation before the full implications for chemical and effluent toxicity can be assessed.

The fluctuations in total dissolved salts (TDS) exceeded the 15% acceptable limit in the100%, 20% and 10% effluent solutions (Table 11.1; Dallas and Day, 1993; DWAF, 1996). The TDS also differed from natural conditions (Table 4.2). Furthermore, many organic compounds, as found in textile effluent, do not dissociate when dissolved in water and therefore TDS measurements do not reflect their presence (Dallas and Day, 1993). The ancylid limpet *Ancylus fluviatilis* is considered tolerant to organic pollution measured in terms of COD, relative to other molluscs (Macan, 1970; Umwelt, 1972, in Mouthon, 1996a), with populations proliferating with moderate loads (Durrant, 1976). Only separate toxicity evaluations of the organics in the effluent would ascertain the tolerance of *B. stenochorias*. Waters rich in dissolved salts are generally preferred by snails (*e.g.* Macan, 1961;

Appleton, 1978; Deaton and Greenberg, 1992), although pulmonates appear to be less tolerant of very saline waters than other snail families prevalent in South Africa, such as the Planorbidae (Madsen, 1990). It is likely, however, the salts accumulated in *B. stenochorias* to a toxic level (De With, 1977; Burton, 1983; Freidig *et al*., 1999). Further studies of salinity effects on *B. stenochorias* would be advisable, to particularly include calcium, sodium and chloride ions which were at high concentrations (Macan, 1961; Madsen, 1990; Dillon, 2000). Copper was not detectable in the tap water at the ranges presently considered ecologically significant; however, copper (present in the dyes) averaged an acutely toxic level in the effluent (Table 11.2). Molluscs in general are known to be very sensitive to copper, hence coppers historically broad scale use in molluscicides (Cheng and Sullivan, 1974; Ishak and Mohamed, 1975; Österberg, 1987, in Clark and Appleton, 1996; Clark and Appleton, 1996). There is reason to believe that copper from the effluent accumulated in the algae, further contributing to toxic effects (Reed-Judkins *et al*., 1997).

Dyk and Dykova (1973) considered the use of *A. fluviatilis* as a water quality indicator because of its sensitivity to oxygen levels. Similarly, in the studies completed by Mouthon (1996a, b), intolerances by the ancylid genera *Ancylus*, *Ferrissia* and *Acroloxus* were found with the levels of COD (>30 mg/l *cf* maximum effluent values of 734 mg/l in this study), BOD (>8 mg/l, *cf* 102 mg/l) and nitrogen levels (Kjeldahl >4.5 mg/l, *cf* 11.28 mg/l). Thus the COD, BOD and nitrogen levels also potentially played a major role in the toxic effects of the textile whole effluent to *B. stenochorias*.

There are a number of causes of variability in survival, growth or reproduction besides those due to either the innate variability of the test species or the toxicant (USEPA, 1991). It appears, however, that *B. stenochorias* displayed the typically high natural statistical 'noise' in the key life history parameters seen in other molluscs (Fretter and Peake, 1975; Dillon, 2000). Trends in variability in response with increasing textile effluent are difficult to describe or suggest an increase in stress levels for *B. stenochorias* in terms of survival, growth and reproduction. Because laboratory toxicity tests are performed at a totally different spatial and temporal scale and ecological complexity to that of field conditions, the *B. stenochorias* chronic toxicity tests offer only a preliminary estimate of the potential hazard of the effluent (Jooste, 2000). The conclusion in determining a suggested 'safe' dilution of effluent for this particular textile effluent source (based on the LOEC values; Warne, 1998) will differ from Zokufa's (2001) results with a mayfly. Zokufa suggested a 3-5% concentration of the same

textile whole effluent would still adequately protect a Class 'A' river. This is discussed further in Chapter 13, but the results underline the importance of both chronic toxicity testing and the use of a diversity of test organisms in whole effluent toxicity assessment.

11.5. CONCLUSIONS

The difficulty in unravelling the toxic components of the textile effluent underlined the importance of whole effluent, as opposed to single substance, toxicity testing. Responses of *Burnupia stenochorias* to the textile effluent in terms of survival, growth and reproduction were variable. Consequently, the calculation of statistical endpoints, was not always possible. Large innate variability in growth and reproductive parameters suggests *B. stenochorias* may not be a suitable toxicity indicator. However, this variability is characteristic of freshwater pulmonate molluscs, including *B. stenochorias*, and consequently should not discriminate against their use when assessing toxicity of whole effluents and single compounds.

Despite the large innate variability and non-monotonicity seen in toxic effects, increased variability in survival and growth was observed with increasing effluent concentration. An LC_{50} (43 days) of approximately 4% textile effluent was calculated, although large ranges in survival occurred. Short-term survival (21 days) gave no significant differences between survival in 20% effluent or greater dilution and the controls. The Lowest Observed Effect Concentrations were calculated as 0.1% (1998) and 1% (1999) effluent dilutions after 43 days. In 1999, fecundity of limpets in 1% was also significantly less than in the controls. Decreased tolerance to the effluent in the F1 generation occurred in terms of survival and growth effects, but with the possibility of some individuals showing an acclimation to the effluent conditions.

The monitoring of fecundity is easy, using the methods suggested within this chapter, and ecologically sound in terms of the effects seen on a given population. Further research into the reproductive effects of toxicants on *B. stenochorias* is suggested. As a measurement of toxicity, changes in growth measured as a change in aperture size calculated from shell length and width, gave variable results leading to data not normally distributed or with heterogeneity of variances. Compared to the ease with which survival and fecundity were monitored, and their statistical acceptability, it is suggested that growth is not a suitable whole effluent toxicity indicator for *B. stenochorias*.

Conclusions drawn from the chronic results using *B. stenochorias* should be viewed with some caution. In most toxicity tests, it is assumed that optimal conditions are provided. Although the basic methodology (described in Chapter 9) can be recommended, test conditions require further attention, primarily from a dietary perspective. The use of river water as opposed to tap water may alleviate some of the dietary problems encountered, and possibly give more accurate applied extrapolations to conclusions drawn. Toxicity identification evaluations would ascertain toxic components of the textile whole effluent, but it is suggested there may be a number of components, so that whole effluent toxicity tests are more valuable in this case. However, further research into the tolerance of *B. stenochorias* to salts, and pH combined with temperature changes would not only contribute to future toxicity evaluations, but also assist in the culturing and maintenance of the limpets in the laboratory.

CHAPTER 12. *DAPHNIA PULEX* ACUTE AND CHRONIC TESTS

Key issues: How variable is the response of *Daphnia pulex* when subjected to a reference toxicant such as potassium dichromate? Is textile whole effluent toxic to *D. pulex* over 48 hours? Are growth and reproduction affected under low effluent concentrations, and what long term effects does the effluent have, expressed in terms of mortality and growth within the F1 generation? What variability is shown in the responses? (Figure 1.2)

12.1. INTRODUCTION

The cladocerans, or daphnids, are recognised as useful species for acute and chronic toxicity tests (Anderson, 1944; OECD, 1981; Dorn *et al*., 1987; Oris and Bailer, 1993; USEPA, 1994; Anderson-Carnahan *et al*., 1995; Chapman, 1995). They are considered more sensitive to chemicals and effluents than many other animals (Persoone and Janssen, 1993), and have been chosen as test organisms because of their small size, short life cycle, ease of culture, high sensitivity to toxicants, and their clonal method of reproduction which lends itself to genetically homogeneous culture populations (Baird *et al*., 1989; Rand, 1995; Versteeg *et al*., 1997). Because of their short life span, acute and chronic toxicity tests are quick to complete, and endpoints such as immobility, mortality and reproductive output are easy to observe (Buikema *et al.*, 1976). The volumes of dilutions and thus toxicants used in toxicity tests are small, making the use of cladocerans practical and inexpensive. *Daphnia pulex* (De Geer) has been chosen as a standard laboratory test organism in South African water quality testing because it is a cosmopolitan species found in South Africa (Slabbert, 1994; Slabbert *et al*., 1998). It is envisaged that in South Africa the use of a standard laboratory test organism will provide the primary basis for general toxicity management, with the use of an indigenous organism focusing on site-specific toxicity management (Palmer and Jooste, 2000). For this reason, and as a standard against which to compare toxicity results using *Burnupia stenochorias*, the following study was made.

D. pulex is principally a pond dweller, not usually found in lotic waters (USEPA, 1993), and forms an important link in food chains (Gulatti, 1978). The life span, from the release of eggs into the brood chamber until death of the adult is variable and dependent on environmental conditions (Pennak, 1978). The mean life cycle at 20EC is 50 days. Usually, clutches of 6-10 eggs are released into the brood chamber where they hatch after 2 days into juveniles or neonates. The time to reach sexual maturity and production of the first offspring is 6-10 days and depends on conditions and body size of the

mother. *D. pulex* has 3 to 4 juvenile instars, moulting between instars. The adolescent stage is only one instar after which neonates are produced with each moult (approximately 18-25). Moulting is usually every 48 hours at 20EC. The less favourable the conditions, the longer the instars and the fewer the number of neonates produced, although a decrease does occur in the number of neonates after approximately the tenth instar (Anderson and Zupancic, 1937). *D. pulex* attains a maximum length of 3.5 mm.

Two types of cladoceran toxicity tests are commonly used. Acute tests over 48 or 96 hours where end results are based on mortalities; and 21 day chronic toxicity tests, which involve reproductive effects and long term survival (Biesinger and Christensen, 1972; Winner and Farrel, 1976; Adema, 1978; Maki, 1979) (although culture techniques and conditions during the chronic tests have been considered problematic; Buikema *et al*., 1979; Stephenson and Watts, 1984). Although South African standard procedures for acute toxicity testing using *D. pulex* as the test organism have been developed (Slabbert, 1994; Slabbert *et al*., 1998), those for chronic toxicity testing are still under development (S. Jooste, pers. com.). The results of these *D. pulex* chronic tests will therefore contribute to a national database for *D. pulex* chronic toxicity tests.

As with all biological processes, a degree of variability is associated with each toxicity test undertaken (Section 9.3). Genetic heterogeneity amongst the test organisms, which contributes to variability of response, is less of a concern with cladocerans because of their clonal reproduction with consequent genotypic homogeneity (although this does not preclude the possibility of mutations or sexual recombination occurring). Environmental heterogeneity may also influence response to toxins (Falconer, 1981). Maternal reserves are known to play a role in juvenile growth (Tessier *et al*., 1983) and response of their offspring (Baird *et al*., 1989) suggesting long term effects of toxicants can occur. Complete standardisation of culture methods and experimental conditions, both presently available for *D. pulex*, does alleviate part of the problem of environment influencing response (USEPA, 2000). In this study, *D. pulex* was periodically tested against a reference toxicant (Section 9.2.3) with the purpose of assessing the expected variability of reaction of the *D. pulex* cultures maintained at the Centre for Aquatic Toxicology, Institute for Water Research (CAT-IWR), Rhodes University, to a toxicant over time (Section 10.1). The choice of potassium dichromate as a reference toxicant was made on the basis that it is used internationally (USEPA, 1993). With low variation between clones

in reaction to potassium dichromate seen with other cladocerans (Baird *et al*., 1991), the usefulness of potassium dichromate as a reference toxicant is emphasised.

Acute toxicity of textile effluent to *D. pulex* has been demonstrated on a number of occasions, although the source of effluent samples tested in toxicity trials relative to the textile mill processing, or end product manufactured (which will change the chemicals used; Correia *et al*., 1994), is often not clearly defined in the toxicology literature (Rutherford *et al*., 1992). Although using a different species, Zokufa's (2001) acute toxicity test results using pre- and post-irrigated effluent, sourced from the same Eastern Cape Province textile mill as reported on in this thesis, underline the potential for sample differences in acute responses; Zokufa's results were acutely (pre-irrigation) and not acutely (post-irrigation) toxic. *D. pulex* has generally been found to be more sensitive to textile effluent than fish (Maki, 1979; Walsh *et al*., 1980; Rutherford *et al*., 1992). In tests conducted on a number of textile mill effluents from across Canada, acute responses of *D. magna* and *C. dubia* were found (Rutherford *et al*.,1992). LC_{50} values varied from 24.5% to 68.7% textile effluent. Toxicity of the surfactants used in the application of the textile dyes depends on the chemicals used, but most are found to be toxic to *Daphnia* species at the acute level, and retard egg and embryo development at the chronic level (Timofeeva, 1991).

Within chronic toxicity tests, reproduction is generally considered the most sensitive criterion in cladocerans (Biesinger and Christensen, 1972), and more accurate and reliable endpoint for monitoring toxic effects (Canton and Adema, 1978). The most frequently used criterion is the total number of neonates produced over 21 days (van Leeuwen *et al*., 1987; APHA, 1992; Rutherford *et al*., 1992), although toxicity over 7 and 14 days has also been recommended (Gersich and Milazzo, 1990; USEPA, 1994). Growth changes in cladocerans, measured as body length or caudal spine length, have also been used as sensitive toxicity parameters, although the ecological significance of such changes is not always clear (van Leeuwen *et al*., 1985a, b). Geiger *et al*. (1980), however, found that body length was a good predictor of later success in reproduction. In many other cases, survival of the parent population has been considered the most sensitive parameter to use in toxicity tests (Kühn *et al*., 1989).

Acute and chronic textile whole effluent toxicity tests were completed in this study to compare the

responses of *D. pulex* (representing a standard laboratory organism) to those of *Burnupia stenochorias* (Figure 1.2). The aims were to investigate the responses of *D. pulex* to the textile whole effluent at an acute and chronic level of toxicity, where survival, growth, and reproduction in terms of time to first lay and number of neonates produced were monitored. It is hypothesised that *D. pulex* will provide less ambiguous whole effluent toxicity test results than *B. stenochorias*, and their respective responses will be compared in Chapter 13. However, the question arose as to whether the organism response variability precluded the discrimination of a response to a (variable) whole effluent. Consequently, variability of response of *D. pulex* to potassium dichromate, representing a reference toxicant, was assessed. These results will also be compared to those of *B. stenochorias* (Chapter 13).

12.2. MATERIALS AND METHODS

12.2.1. Culture Techniques

Daphnia pulex cultures have been maintained for a number of years by the Centre for Aquatic Toxicology, Institute for Water Research (CAT-IWR), according to the Methods Manual produced by the South African Department of Water Affairs and Forestry (DWAF, 1996, primarily adapted from the USEPA, 1993). The cultures are kept in a controlled environment room at 20EC, with a light regime of 16 hours light/8 hours dark. The cultures are maintained from neonates parthenogenetically produced; if any ephippia (sexually produced eggs) are produced, they are discarded to ensure the cultures are as genetically homogeneous as possible. The culture medium is an aerated synthetic water consisting of deionized (Millipore) water to which is added 1 litre hydrous calcium sulphate (1.2 g/litre deionized water), 10 ml potassium chloride (8 g/litre deionized water), 10 ml hydrous magnesium sulphate (24.576 g/100 ml deionized water), and 20 ml sodium bicarbonate (96 g/litre deionized water), all made up to 25 litres. This mixture ensures a moderately hard water hardness of 80-90 mg/l $CaCO_3$ and a pH of 8.1 ± 0.4 units (*D. pulex* can survive over a wide pH range but the optimum range is within 7.0-8.6; Lewis and Weber, 1985). This culture medium was also the diluent used in all toxicity tests.

The cultures of *D. pulex* are fed daily an artificial diet of trout pellets, dried yeast and dried alfalfa, prepared as a filtered solution with culture medium. This was also the food source for the chronic toxicity tests. Glassware used in culturing and in all tests was cleaned by rinsing in a series of acid/detergent/tap and deionized waters according to the procedure used by DWAF (1996).

12.2.2. Acute Toxicity Tests

Forty eight hour screening (range finding) and definitive toxicity tests were completed in the controlled environment room as described above, according to the test methods used by the USEPA (1993) and DWAF (1998). The maximum period cladocerans can be deprived of food without increased mortality occurring is 48 hours (Adema, 1978). The tests are described as being static non-renewal with no aeration. The day before each test, approximately 50 females with embryos nearing hatching were separated from the main cultures and placed into 150 ml sterile glass beakers filled with fresh culture medium (Section 12.2.1). The following day, the newly hatched neonates were then randomly divided into the test replicates, with 5 neonates per replicate. The neonates were not fed during the acute toxicity tests.

Test replicates consisted of 40 ml glass beakers, filled with 25 ml of test dilutions. Various dilutions of reagent grade potassium dichromate diluted to a 1% solution with deionized (Millipore) water (representing a reference toxicant; Section 9.2.3.) and defrosted textile effluent (sampled from the 25 litres defrosted twice weekly for *Burnupia stenochorias* tests; Section 9.2.3) were tested. Each test had four replicates per test solution (Table 12.1).

Death of a neonate was described as the neonate lying immobile at the bottom of the beaker, not responding to gentle probing. The neonate was confirmed dead by removing the neonate to clean culture medium and observing for a few seconds to confirm no gut contraction occurred (Persoone and Janssen, 1993). Mortalities were recorded after 1, 2, 4, 8, 12, 24 and 48 hours when the tests were terminated. LC_{50} values were calculated, when possible, using the Probit (Finney, 1971) and Trimmed Spearman-Karber (Hamilton *et al*., 1977) methods (Section 9.3.1).

12.2.3. Chronic Toxicity Tests

These static renewal tests with no aeration were based on the protocols according to OECD (1987) and Persoone and Janssen (1993). Neonates less than 24 hours old (collected as above) were exposed for 21 days (Adema, 1978) to various textile effluent dilutions (Table 12.1), using the culture medium as diluent. The effluent was renewed every two or three days to remove excreta of the test animals, to ensure the oxygen supply did not diminish (Adema, 1978), and to attempt to maintain the constituents of the effluent as constantly as possible over the entire test period. Sub-samples of effluent

were defrosted for each renewal; but were originally sampled and frozen at the same time as those samples collected for the *B. stenochorias* toxicity tests (18/8/98 and 5/2/99). Oxygen levels, pH and Total Dissolved Salts of each effluent dilution and the culture medium (controls) were monitored with each renewal. Metal and inorganic analyses, however, relied on 100% effluent samples sent simultaneously from the *B. stenochorias* tests. At the time of renewal of effluent, each glass beaker was replaced with a clean beaker, previously rinsed out with diluent solution (Kühn *et al.*, 1989). The beakers were covered with transparent plastic to prevent evaporation.

Each 50 ml glass beaker (15 per dilution) had one neonate. Individual reproductive effort could therefore be monitored. Feeding consisted of one drop (approximately 100 Fl) per day of the artificial food used in the culturing of *D. pulex*, which was considered not limiting. The time to first reproduction, the number of neonates produced daily, and mortality were noted. The number of embryos (as opposed to neonates born) per female was not monitored, although this criterion has been used to assess toxicity (Stephenson and Watts, 1984). Newly hatched neonates were removed daily during the 21 days. The size of each individual at the end of 21 days' exposure was recorded as the length and width of the caudal spine, measured using a Nikon bifocal microscope with a calibrated ocular graticule at magnification x40.

12.2.4. Statistical Analyses

In analysing for differences in survival between concentrations of effluent, actual data were compared for statistical differences between diluents and concentrations using the Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) following the method stipulated by the USEPA (1994). To determine differences between concentrations in time to first death, Student's two sample 't' tests assuming equal and unequal variances (Statistica 1999) were used. The time to first brood production data were $\log_{10}$ transformed to normalize data and analysed for differences between 1998 and 1999 using Analysis of Variance (ANOVA) (Statistica 1999). Growth (change in caudal tail size) was compared using the Tukey's Honest Significance Test (ANOVA, $p < 0.05$).

The longer term effects of the effluent were investigated in 1999 only, by subjecting neonates produced under the chronic conditions (F1 generation) to effluent for 48 hours (acute test). Forty neonates less than 24 hours old were randomly selected from each of the chronic dilutions and placed in equal

numbers into one of eight replicates of the same effluent dilution as the chronic dilutions from which they had been selected (P1 generation). No feeding occurred. Mortalities were recorded as per the acute toxicity tests, and the test was terminated after 48 hours. Mortalities were then compared to those obtained for the parent acute tests (Section 12.2.2).

12.3. RESULTS

12.3.1. Acute Toxicity Tests

Reaction to potassium dichromate proved to be non-monotonic over 48 hours, with high chi-squared values in Probit analysis, so that the LC_{50} values were calculated using Trimmed Spearman-Karber. Percentage trim was small (5%), LC_{50} values were within a three fold range of each other (Table 12.2), and the mean LC_{50} value was 1.41 ppm potassium dichromate. Control mortalities did not occur during the potassium dichromate tests. pH (range of 7.03 - 7.55 throughout all dilutions) and Total Dissolved Salts (range of 308 - 357 throughout all dilutions) were not significantly different between tests, between concentrations or within concentrations of potassium dichromate over the 48 hours (Student's 't' tests, $p > 0.05$).

In testing the effects of the textile effluent, it was found that the dye particles within the effluent adhered to the glass sides of the test containers. However, as the effluent dilutions were changed regularly into clean containers, the accumulation of the dye particles over the entire test period did not occur as it did in the plastic-lined buckets used to test *Burnupia stenochorias*. Mortalities of *Daphnia pulex* in textile effluent over 48 hours was not greater than 5% (1 out of 20 neonates) in any of the effluent concentrations, and a control mortality of 5% occurred in only one of the effluent toxicity tests (18/9/98, Table 12.1). The textile effluent was therefore considered not acutely toxic over 48 hours to *D. pulex.*

Table 12.1. List of acute and chronic toxicity tests completed using *Daphnia pulex* as a toxicity test organism. The diluent used was reconstituted water, described in Section 12.2, with the exception of * which used a mixture of reconstituted water and receiving water. Control dilutions (not listed in the table) consisted of culture medium.

Test solution	Test type	Period of exposure	Concentrations used	Date started
Effluent	Acute	range finding	100%, 50%, 25%, 12.5%, 6.25%	22 / 8 / 98
		48 hours		
		48 hours	100%, 50%, 25%, 12.5%, 6.25%	18 / 9 / 98
		48 hours	100%, 50%, 25%, 12.5%, 6.25%	18 / 2 / 99
		48 hours	100%, 50%, 25%, 12.5%, 6.25%	5 / 8 / 99
		48 hours *	100%, 50%, 25%, 12.5%, 6.25%	10 / 12 /99
	Chronic	21 days	100%, 50%, 25%, 12.5%, 6.25%	17 / 11 / 98
		21 days	100%, 20%, 10%, 3.33%, 1%	15 / 9 / 99
	Acute F1	48 hours	100%, 20%, 10%, 3.33%, 1%	29 / 9 / 99
$K_2Cr_2O_7$	Acute	range finding	5, 2.5, 2.2, 1.25, 1.1, 0.625, 0.55,	22 / 8 / 98
		48 hours	0.312, 0.156 and 0.078 ppm	
		48 hours	5, 2.5, 1.25, 0.625, 0.312, 0.156	27 / 8 / 98
			and 0.078 ppm	
		48 hours	5, 2.5, 1.25, 0.625, 0.312, 0.156	27 / 10 / 98
			and 0.078 ppm	
		48 hours	5, 2.5, 1.25, 0.625, 0.312, 0.156	2 / 2 / 99
			and 0.078 ppm	

12.3.2. Chronic Toxicity Tests

Inorganic and metal ion analyses of the textile effluent are as per samples used in the chronic toxicity tests with *Burnupia stenochorias* (Table 11.2). When comparing total dissolved salts (TDS) and pH values of the 1998 and 1999 100% effluent samples (Table 12.3), significant differences were found (Student's two sample 't' test for means, t = -6.98 [TDS] and -4.79 [pH], $p < 0.01$). The 1998 and 1999 responses of *D. pulex* should not therefore be compared. No significant differences occurred between the TDS and pH control values.

Because each individual *D. pulex* was in a separate beaker, no within treatment testing of variability was possible. Survival of neonates over 21 days was generally greater in 1999 than 1998 (Figure 12.1), with survival in 100% textile effluent equal to or greater than survival in the controls for each year. The times to first death were not significantly different between 1998 and 1999 (Student's 't' test, t = 0.312, p = 0.38). No LC_{50} data over the 21 days were obtainable as 50% mortality did not occur. Toxicity of the defrosted textile effluent could be loosely defined as follows:

100% = 12.5% < control = 6.25% = 50% = 25% [1998]

100% = control < 1.1% < 10% < 20% = 3% [1999].

However, differences in survival in each effluent dilution compared to the controls were not significant (Fisher's Exact Test, $p > 0.05$), with the consequent allocation of 12.5% textile whole effluent as the No Observed Effective Concentration (NOEC). When re-tested at 14 days, survival in 1999 was only significantly different (greater) at 100% effluent compared to the control (Fisher's Exact Test, $p > 0.05$). Because the range of tested effluent concentrations was not at a factor of 0.5 or more (USEPA, 1994), and the dilution below 100% was only 20% effluent, it seemed appropriate to allocate the NOEC value between 10% and 20% textile whole effluent (Hoekstra and van Ewijk, 1993).

Reproductive effort, calculated as the total number of neonates produced, is dependent on the number of broods produced. Because *Daphnia* neonates are usually produced every second day (Persoone and Janssen, 1993), those produced on two consecutive days are often counted as one brood (Stephenson and Watts, 1984). However, the *D. pulex* studied here regularly produced broods on a daily basis for a number of days. Consequently, each day that *D. pulex* neonates were produced was considered an independent brood, irrespective of the pattern of brood production within each solution tested. No statistical differences between 1998 and 1999 occurred in the mean time to first production within each

dilution, including between the controls (ANOVA, $p > 0.05$). Comparing each dilution with the control of the same year, all 1998 concentrations significantly decreased the time to first reproduction by approximately 2 days (Student's 't' test, $p < 0.01$), where the controls had a mean time of 11.07 days (Table 12.4). However, in 1999 only 20% effluent significantly decreased the time to first production from a mean control time of 11.23 days (Student's 't' test, $p < 0.05$; Table 12.4). A significantly greater number of total neonates were produced per test solution in 1999, both in the control and effluent solutions (Figure 12.2). The number of neonates per cladoceran day and the size of the first three broods generally increased with increasing effluent concentration (Table 12.4).

The use of the caudal spine as a measure of growth was not satisfactory, as many of the caudal spines had been broken off. However, comparison of the tails of the remaining cladocerans gave a significant difference in size (longer) between cladocerans in100% effluent and the controls (Tukey's Honest Significance Test, ANOVA, $p < 0.05$; Figure 12.3). Variability in length between the cladocerans within each dilution is demonstrated by the spread of data which were always within a range of two standard deviations at each dilution (USEPA, 2000).

Mortalities in the F1 generation of *D. pulex* subjected to textile effluent were not significantly different to those in the parent acute toxicity tests, with only a 5% mortality occurring in 100%, 20% and 1% effluent, and 10% in 1% effluent, with no control deaths. The effluent was therefore not acutely toxic to the F1 generation, although an increase in mortality did occur, compared to the parent generation.

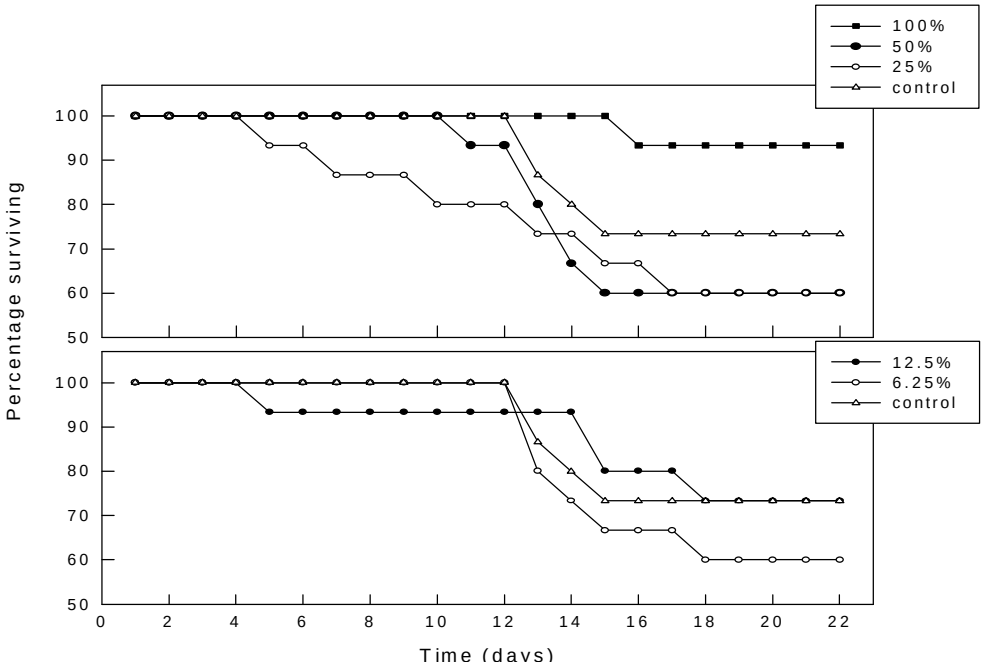
Table 12.2. *Daphnia pulex* Probit and Trimmed Spearman-Karber (TSK) results of potassium dichromate acute toxicity tests after 48 hours. $\tilde{a}^2$ = chi-squared statistical value ($p < 0.05$); CL = 95% confidence limits. Mean LC_{50} = 1.41 ppm.

Date	Probit $\tilde{a}^2$	Probit $\tilde{a}^2$ for hetero-geneity	TSK LC_{50} estimation	Upper CL for LC_{50}	Lower CL for LC_{50}	TSK % trim	LC_{50} compared to mean LC_{50}
22 / 8 / 98	221.8	11.07	0.65 ppm	0.137 ppm	2.31 ppm	5	46.09%
27 / 10 / 98	243.6	14.07	1.75 ppm	1.49 ppm	2.04 ppm	5	124.11%
2 / 2 / 99	187.3	9.48	1.83 ppm	1.73 ppm	1.94 ppm	5	129.78%

Table 12.3. Total dissolved salts (TDS) and pH readings for the two chronic textile effluent *Daphnia pulex* toxicity tests. ± Std.dev. = ± standard deviation; C.V. = percentage coefficient of variation.

Year	Effluent conc	TDS mean	± Std. dev.	C.V.	pH mean	± Std. dev.	C.V. %
1998	100.0%	1480	158.1	10.7	8.4	0.3	3.1
	50.0%	849	83.1	1.0	8.6	0.2	2.2
	25.0%	587	81.1	13.8	8.6	0.2	2.0
	12.5%	388	70.6	18.2	8.6	0.2	2.2
	6.25%	297	19.8	6.7	8.7	0.2	2.2
	[control]	210	29.7	14.2	8.7	0.2	2.8
1999	100.0%	1709	66.6	39.0	9.2	0.2	2.6
	20.0%	618	4.9	7.9	9.2	0.2	2.2
	10.0%	425	3.3	7.7	9.1	0.2	2.2
	3.33%	305	6.2	19.8	8.9	0.3	3.8
	1.0%	272	4.3	15.5	8.7	0.3	2.5
	[control]	248	4.9	17.6	8.7	0.2	3.0

a) 1998



b) 1999

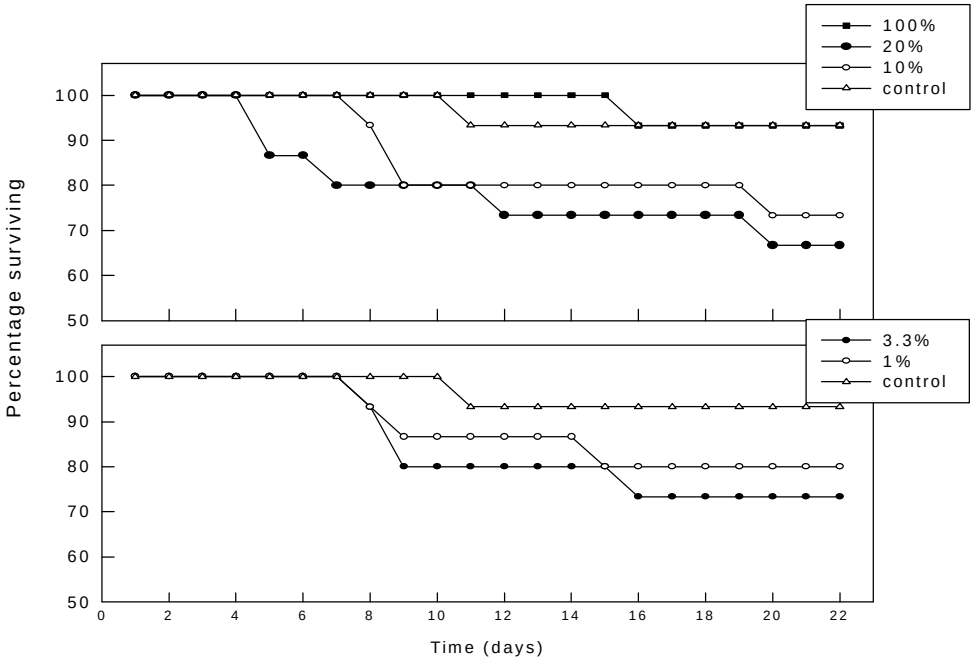


Figure 12.1. 1998 and 1999 survival of 15 *Daphnia pulex* per concentration of effluent compared to the controls over 21 days, expressed as a percentage of survival at each concentration. Day 1 refers to the first day neonates were added to the dilutions. Each year's control is duplicated for comparison.

Table 12.4. Survival and reproduction rates of *Daphnia pulex*, after 21 days' exposure to varying concentrations of effluent. Ephippia were not produced in any of the beakers. * numbers in brackets refer to actual number of neonates dead; ** = total percentage increase relative to the controls; Conc. = concentration; no. = number of; ± St. dev. = ± standard deviation; reprodn = production of neonates; prodn = production. Daily mortality rate was calculated as the total number of neonates dead ÷ the total number of observation days (21). Production rate / cladoceran day was calculated from the total number of cladocerans produced per day per concentration ÷ the total number of days limpets survived. [a] significantly different ($p < 0.01$) to 1998 control; [b] significantly different ($p < 0.05$) to 1999 control.

Year	% conc. effluent	Total % dead *	Time at first death (days)	Daily mortality rate	Mean time first reprodn (days)	Mean no. neonates / adult	St. dev. / adult	Prodn rate / cladoc-eran day	Total % increase in no. neonates **	Mean size first 3 broods
1998	100	26.7 (4)	7	0.11	9.4[a]	27.1	16.5	1.5	73	5.6
	50	40.0 (6)	12	0.29	8.9[a]	27.1	11.8	1.5	72	4.7
	25	40.0 (6)	7	0.29	8.9[a]	24.4	15.3	1.5	55	6.1
	12.5	26.7 (4)	7	0.19	9.7[a]	32.9	15.8	1.7	109	6.1
	6.25	33.3 (5)	12	0.24	9.1[a]	26.9	17.2	1.5	71	3.8
	Control	33.3 (5)	12	0.24	11.1	15.7	13.1	0.8	-	3.5
1999	100	6.7 (1)	16	0.05	10.7	48.3	13.5	2.2	105	8.0
	20	33.3 (5)	5	0.24	9.3[b]	44.5	28.4	2.6	88	8.7
	10	26.7 (4)	6	0.14	10.1	43.0	24.2	2.4	82	8.0
	3.33	33.3 (5)	5	0.24	10.4	32.4	19.4	1.8	37	5.7
	1.1	20.0 (3)	9	0.14	10.9	30.7	21.6	1.6	30	4.7
	Control	6.7 (1)	12	0.05	11.2	23.6	16.6	1.2	-	4.4

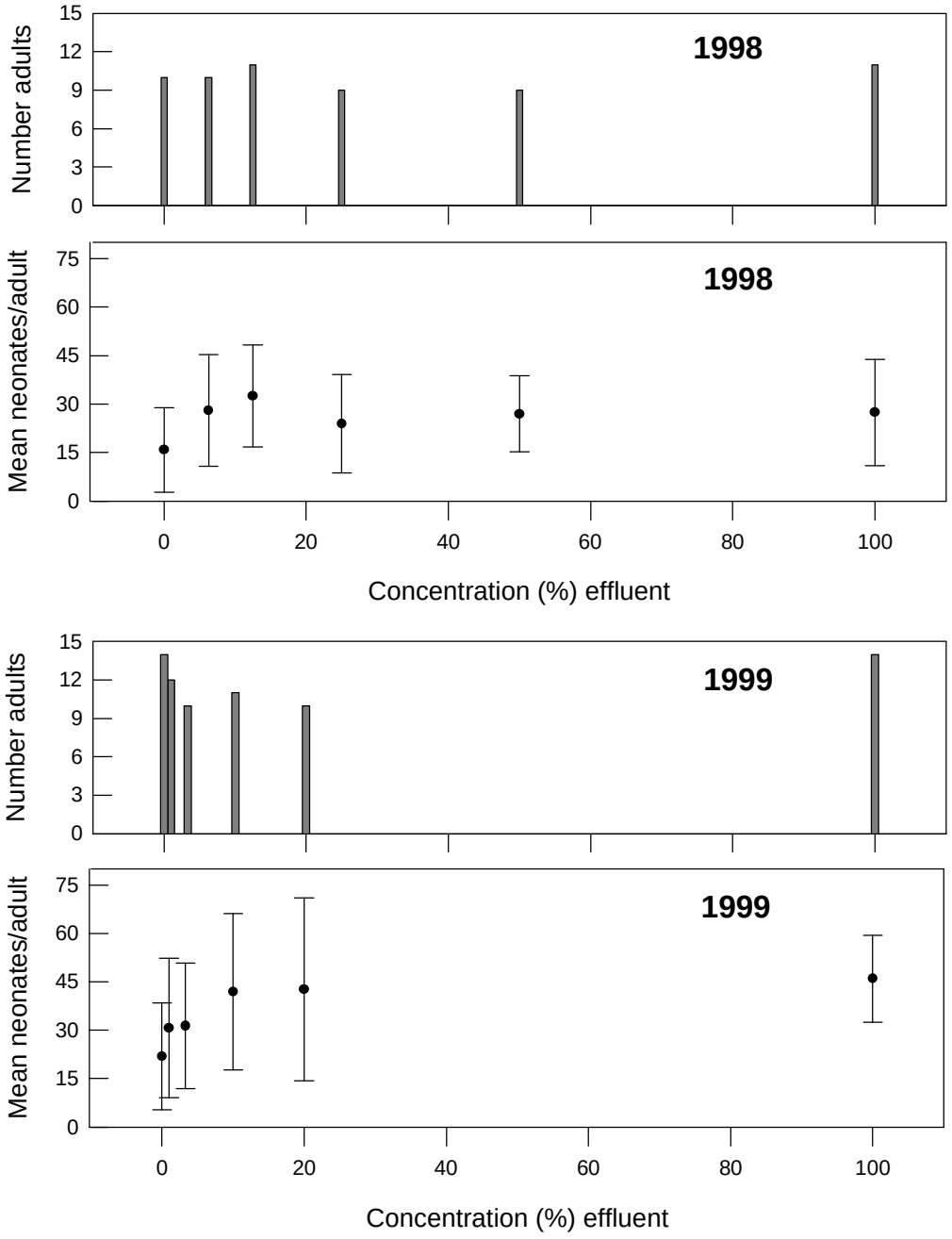


Figure 12.2. The total number of adults within each concentration of effluent at the end of 21 days, out of an initial total of 15. The differences between 1998 and 1999 in the mean aggregate of neonates per adult at each concentration of textile effluent are also shown. Concentration (%) effluent 0 = control of culture medium.

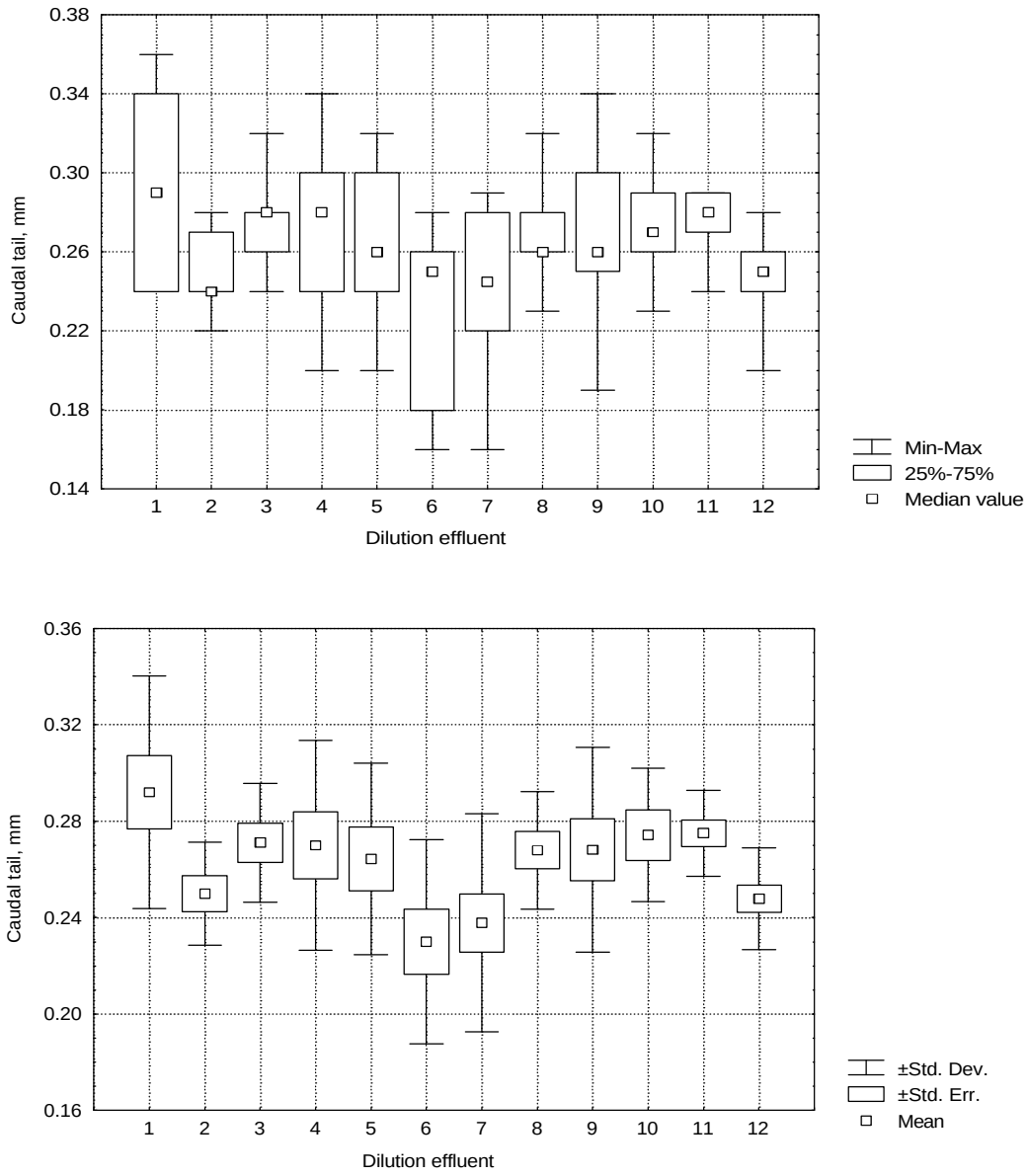


Figure 12.3. Respective lengths of *Daphnia pulex* caudal tails for each dilution, after 21 days. Cladocerans with damaged tails are excluded. The dilution effluent figures on the x axis represent the following dilutions: 1 to 6 represent the 1998 effluent concentrations, where 1 = 100%, 2 = 50%, 3 = 25%, 4 = 12.5%, 5 = 6.25%, 6 = control; 7 to 12 represent the 1999 effluent concentrations, where 7 = 100%, 8 = 20%, 9 = 10%, 10 = 3.33%, 11 = 1%, 12 = control.

12.4. DISCUSSION

The *Daphnia pulex* tests were not undertaken to define thoroughly the acute and chronic responses to the textile whole effluent and to potassium dichromate, but rather to provide comparative data with those obtained for *Burnupia stenochorias* (Chapters 10 and 11; Figure 1.2). It is clear from the responses obtained with the chronic *D. pulex* toxicity tests that many parameters, including food quality, need further investigation in both CAT-IWR, from where the cultures were obtained, and other South African laboratories as a whole before chronic protocols and expected results are defined. However, an assessment can still be made (Chapter 13) as to the suitability of *B. stenochorias* as a toxicological indicator, based on a comparison of the limpets' variability of responses and the acute and chronic endpoints obtained, with the results of the standard laboratory organism *D. pulex* recorded within this chapter.

The acute LC_{50} results for potassium dichromate (0.65, 1.75 and 1.83 ppm) from this study are higher than generally found. Internationally, LC_{50} values range from 0.086 ppm (Dorn *et al*., 1987) to 0.2-0.45 ppm (Stackhouse and Benyon, 1988; Jop *et al*., 1990) to 2.0 ppm (Müller, 1980). Results have also varied with *Daphnia magna* (Cairns *et al*., 1978; Stephenson and Watts, 1984; van Leeuwen *et al*., 1987; Enserink *et al*., 1993) although some higher LC_{50}s (2.4 and 2.8 ppm) have been recorded (Sorvari and Sillanpaa, 1996). The 48 hour LC_{50} of 0.65 ppm obtained in this study has inn other studies only given a fifty percent reproductive impairment (Biesinger and Christensen, 1972). Lowest Observed Effect Concentrations (Section 9.3.2) have been calculated after 21 days as 0.6 ppm (van Leeuwen *et al*., 1987b) and 0.94 ppm (Enserink *et al*., 1993), respectively close to or higher than the LC_{50} value (0.65 ppm) calculated for *D. pulex* on 22/8/98 in this study. Coniglio and Baudo (1989) found that as chromium^{6+} increased in concentration from 0.02 to 0.1 ppm, there was an increase in fecundity per *Daphnia obtusa* neonate, but a decrease in the number of neonates that survived, giving an overall population decrease over 45 days. Variability of results is therefore prominent amongst the literature.

However, it is essential that a standardized laboratory organism such as *D. pulex* gives consistent results when repeated through time or by different analysts (Baird *et al*., 1989; USEPA, 2000). Variability of response of this culture of *D. pulex* to potassium dichromate was within a range of

approximately three fold (equivalent to approximately 30% coefficient of variation [Norberg-King, pers. com. e-mail dated 14 October 1998]). Depending on the reference toxicant and test species used in acute toxicity tests, coefficients of variation have previously been calculated between 6% and 124% (Nebeker, 1982; Parkhurst *et al*., 1992). Averages of 23% coefficient of variation were obtained in a 3-laboratory study for *D. magna* and potassium dichromate (Buikema, 1983); and 39% in a 47-laboratory study (Cabridene, 1986). These latter inter-laboratory differences were attributed to food differences; no food was used in the acute toxicity tests with *D. pulex* discussed in this thesis.

Because of the effects water quality may have on the availability of the chromate ion and therefore its toxicity (Müller, 1980; Hosokawa *et al*., 1991; Appendix A), toxicity test results may vary from one test to another if these parameters differ between tests. The culture medium used in these tests as diluent did not differ significantly between the potassium dichromate (or effluent) tests in pH and Total Dissolved Salts (Section 12.3.1) and these water quality variables were therefore considered not to have contributed to the variability in potassium dichromate LC_{50} values obtained with *D. pulex*.

In the textile whole effluent study, the effluent was not acutely toxic to *D. pulex* after 48 hours. Acute toxicity of textile effluent to cladocerans has been found, but is dependent on the textile mill processing (Correia *et al*., 1994). *D. pulex* has been used in a number of textile effluent toxicity identification evaluations (Rutherford *et al*., 1992; Wells *et al*., 1994). In the latter case, the LC_{50} was calculated as 74% but zinc was found to be the primary toxicant; the speciated forms of calcium and magnesium were also important in overall toxicity. However, in this study, calcium and magnesium were in low concentrations (Table 11.2), as is generally the case in textile effluent (Wells *et al*., 1994).

Moulting is thought to influence LC_{50} values obtained in acute toxicity tests, with cyclic changes in susceptibility occurring, depending on the time of moulting (Lee and Buikema, 1979). It is the opinion of Lee and Buikema (1979) that ideally, acute toxicity tests should last long enough for all neonates used in the tests to moult at least once. In the acute tests reported here, the use of newly hatched neonates (which do not moult within the 48 hours duration of the acute test) results in two issues. The first is that non-synchronization of moulting does not affect (increase) the variability of the endpoint, although laboratory (Lee and Buikema, 1979) and natural populations (Green, 1956; Hovenkamp,

1990) usually display similar synchrony. The second issue is that the endpoints may not reveal the wider responses of *D. pulex* to the toxicant. Neonates less than 24 hours old would have to be compared with moulting or recently moulted juvenile instars or adults for a full assessment of the acute toxicity of the potassium dichromate or effluent.

Temperature also affects *D. pulex* growth rates (and therefore time to maturity), time between broods, the number of eggs, and the longevity of the adults (Lewis and Horning, 1991). A constant temperature of 20EC was maintained in this study, which has been recommended as optimal for survival and reproduction (Goss and Bunting, 1983; Westlake *et al*., 1983; Lewis and Horning, 1991; APHA, 1992).

The role the dye particles play in the toxicity of the effluent to *D. pulex* is unclear. The dye particles could be removed by centrifugation and tested separately for toxicity (Burkhard and Ankley, 1989; Timofeeva, 1991). In this study, dye particles adhered to the sides of the beakers, which were exchanged for clean beakers every two or three days. If the dye was the primary toxic component of the effluent, its adherence to the sides of the beakers potentially removed it as a toxicant from the cladocerans, whose behaviour seldom diverged from continually moving through the solutions. It may therefore be considered questionable whether the results can be compared to *B. stenochorias,* where the plastic lining of the test containers was not exchanged throughout the chronic tests but to which *B. stenochorias* remained continually attached. However, under natural situations, the lifestyles of neither *B. stenochorias* nor *D. pulex* are unlikely to be different to those described here (attached to substrate and free floating, respectively), and therefore the tests can be compared. This difference in lifestyle perhaps underlines the difference between the frequent use of a test organism such as *D. pulex*, which will act as a short term, effective analytical tool to predict the potential effects of a toxicant on the aquatic life in receiving waters; and the use of indigenous, feral test organisms representing a different order, reflecting site-specific, realtime, possibly different effects of the toxicants.

Chronic LC_{50} values have ranged from 9.3% to 23% (Rutherford *et al*., 1992), with No Observed Effect Concentration values ranging from 0.63% to 6.3% textile effluent (Biesinger and Christensen,

1972). As with this study, stimulation of reproduction in the cladocerans with small metal additions to a test solution are known to occur (Naudin *et al*., 1995). The number of neonates produced over 21 days increased relative to the controls in this study, generally with increasing concentrations, from 55% to 109% [1998] and 29% to 105% [1999] (Table 12.4; Figure 12.2). In addition, time to first production of neonates was significantly decreased at all effluent dilutions in 1998 and at 20% effluent in 1999, relative to the controls. This suggests either that the culture medium (used as control medium) was not optimal, or that stimulants were provided by the effluent. Without further experimental data, the question as to which occurred (increased nutrients or stimulation) is difficult to answer. The food particles may also have adsorbed portions of the effluent toxicants, thus influencing the toxicant uptake. The provision of additional nutrients may have allowed an increase in energy expenditure until the maximum genetically determined rate of reproduction and growth was reached (scope for growth or reproduction) (Winner, 1976; van Leeuwen *et al*., 1985b; Chapter 8). The synthetic diet used in the feeding of these neonates is a well-tried method, successfully used in the cultures of *D. pulex* (Slabbert, 1994; Slabbert *et al*., 1998). However, food maintains the condition of the animals, and because it influences the animals' physiology, it also influences uptake and metabolism of toxicants (Adema, 1978). The ability of neonates to withstand toxicants and starvation is said to be closely linked to food (Persoone and Janssen, 1993), although others disagree (Stephenson and Watts, 1984; Norberg-King and Schmidt, 1993). Both the quantity (Baird *et al*., 1989) and quality (Belanger *et al*., 1989) of food potentially affect the maternal production of neonates. In static tests such as described in this chapter, food is more likely to be present in unrealistically high quantities (Buikema and Voshell, 1993). Its quality is, however, questionable. *Daphnia magna* have been shown to produce many small neonates under favourable conditions, and few, stress-resistant neonates when food is scarce (Enserink *et al*., 1993). In this investigation of *D. pulex,* although many more neonates were produced in the effluent dilutions than in the controls, the size of neonates relative to neonates from the controls was not monitored. More concentrated research on food quality, monitored over many generations of *D. pulex,* is suggested before conclusions as to the chronic toxicity of whole effluents using *D. pulex* as a toxicity indicator can be made.

If stimulation, as opposed to added nutrients, was the cause for increased growth and reproduction, it would not be unexpected if, under such effluent conditions, the population continued to increase in

reproductive output (and potentially also growth) over many generations, until a crash in numbers caused the population to be significantly reduced in numbers compared to the controls (Price, 1975; Mitchell, 1992).

Because of the lack of South African data, the data are loosely compared (as different species have been used) to those stipulated in the guidelines of the OECD (1981) and USEPA (1982b). In the acute tests in this study, *D. pulex* gave less than 10% mortalities, which is internationally acceptable (Rutherford *et al*., 1992). The acceptable reproductive performance using *Ceriodaphnia dubia,* which includes the production of three or more broods and 15 or more neonates in 60% of the surviving females in the controls (USEPA, 1994), was seen in the controls of the *D. pulex* tests completed in this study. The reproductive criteria stipulated by the OECD to be important in the reproduction of *D. magna* are the production of the first brood which must be within 9 days, and two more within the next 5 days; plus the production of 20 neonates per adult. Within this study of *D. pulex*, control individuals did not generally fulfil these three criteria. The buildup of a database for the South African *D. pulex* will, in time, provide a range of data within which to compare the reproductive results obtained in the controls, thus determining the acceptability of each test's results.

Where reproductive effects are studied, Leonhard (1991) suggested the total number of young produced in the 10 days following the first birth gives a measure of fertility. Geiger *et al*. (1980) suggested that 21 day reproductive data were moderately predictive of long term effects. van Leeuwen *et al*. (1985b) believes calculations based on 21 days underestimates long term hazard assessments. From an ecological perspective, it is more realistic to study the effects on the population as a whole. The life table approach has been used to a limited degree (*e.g.* Winner and Farrel, 1976; Bertram and Hart, 1979; Daniels and Allen, 1981; van Leeuwen *et al*., 1985b), but it is not generally used nor does it form part of the general protocols for cladoceran toxicity tests (USEPA, 1993). Population modeling of the reproductive data combined with the survival data may give a more meaningful conclusion as to the effect of textile whole effluent on the population as a whole, although this is also not generally a pre-requisite for interpretation of toxicity data (van Leeuwen *et al*., 1985b; USEPA, 1993). However, age or stage classified models require a greater number of replicates per dilution than the fifteen used in these experiments, with the duration of the tests completed over the entire life cycle and

not just 21 days preferred (Verhaar *et al*., 1999). The required statistical bootstrapping is therefore difficult (Verhaar *et al*., 1999). The observations over 21 days are therefore considered sufficient for an assessment of the toxic effects of the effluent. However, the chronic tests of *D. pulex* reported here began with unexposed animals. There is the possibility that a necrotic effect occured, so that effects will either be shown after a few broods of neonates are produced (chronic toxicity on oogenesis and early embryogenesis), or after a number of generations (acute or chronic toxicity with toxicant transfer from generation to generation). The F1 generations tested here appeared to display a very small, but non-significant increase in susceptibility to the effluent and toxicity was still not sufficiently high to obtain any lethal concentration values. An expansion of the chronic test to include further generations would verify whether necrosis of toxicants occurred.

A comparison of these results is made with those from acute and chronic whole effluent and potassium dichromate toxicity tests using *B. stenochorias* in Chapter 13.

CHAPTER 13. GENERAL CONCLUSIONS

The continual discharge of complex effluents into riverine systems suggests that the elucidation of their effects on local, indigenous organisms is essential before hazard and risk assessments can be made (Munkittrick and Dixon, 1989; Suter, 1995). Future legislation in South Africa regarding licences for the discharge of chemically complex wastes will introduce compulsory toxicity testing (Palmer and Jooste, 2000). In South Africa there is a lack of toxicity databases for both chemicals and effluents and their effects on indigenous freshwater fauna and flora (Moore, 1990; DWAF, 1996). The overall aims of this study were to describe some of the toxicological responses of the freshwater gastropod *Burnupia stenochorias* to a complex effluent, and to assess its suitability as an ecotoxicity indicator (Figure 1.2).

Part One of this study led to the development of methods for the successful culture of a laboratory population, and for the reliable collection and acclimation to laboratory conditions of feral populations, of *B. stenochorias*. Differences in reproductive and growth parameters between natural and laboratory were also examined. Although *B. stenochorias* were chosen from habitats differing in flow rate and water quality, shell size did not vary significantly between populations, with shell length and width adequately representing growth. It was suggested, however, that although age cannot be accurately estimated from shell size, shell size could still be used to choose sexually immature limpets for acute toxicological experiments. Monitoring of egg capsule production, and development of the eggs, provided useful methods for estimating toxic effects. Similarly, a method for feeding limpets in the laboratory was developed, although the ideal nutritional (quality and quantity) requirements of *B. stenochorias* were not elucidated. Large individual variability was seen in growth and fecundity of *B. stenochorias*, in both natural and laboratory populations. However, although good toxicological practice requires the collection of consistent and comparable, legally defensible data (APHA, 1992; Rand, 1995; USEPA, 2000), the possible use of *B. stenochorias* as an ecotoxicological indicator organism was pursued because of the necessity for the freshwater Mollusca to be represented in South African toxicity testing (DWAF, 1996). As algal grazers on stones and rocks, the limpets are an important component of the benthic freshwater community.

13.1. Summary of Part Two and Future Research

Results from Part One of this thesis contributed to method development in the:

- acclimation and transfer of limpets to test containers;
- measurement of survival, growth and fecundity; and
- observation of egg development.

Since it is the magnitude of uncertainty that is important in the application of toxicity test data to hazard and risk assessment (Hickey and Martin, 1995; Suter, 1995), responses of *B. stenochorias* were compared to those of the standard test taxon *Daphnia pulex* (Chapter 12).

D. pulex is presently used extensively as a standard laboratory toxicity test organism in South Africa. The daphnids' clonal reproduction lends itself to genetically homogenous populations and theoretically to reduce variability of response endpoints (Rand, 1995; Section 12.1). There is the question, however, regarding the suitability of such standard test organisms which inhabit lentic waters, and whether they reflect the responses of lotic dwelling, indigenous organisms (Hoekstra and van Ewijk, 1993; Lamberti and Steinman, 1993; Palmer *et al.*, 1996). *Daphnia* species also tend to dominate in conditions which indicate meso- or eutrophic conditions rather than indicating conditions that are just changing from oligotrophy (Gray, 1989). Laboratory cultures in general are considered insufficient for hazard evaluation, particularly in representing freshwater snails which are known to exhibit great inter-population and inter-specific variability in life cycle patterns and physiology (Russel-Hunter, 1961b; Calow, 1978; Brown, 1985; Cairns, 1988; Dussart, 2001).

Methods and Variability

Variability is inherent in any repeated analytical procedure (Parkhurst *et al.*,1992; USEPA, 2000). Although there are various procedures which can be used to account for toxicity using laboratory compared to site-water, the use of tap water as a diluent has several limitations which contribute to differences in intra- and inter-laboratory variability of responses to toxicants (Roux *et al.*, 1998). Interpretation of the variability in responses of *B. stenochorias* and *D. pulex* was further complicated by temporal changes in composition of the tap water used as diluent in the limpet toxicity tests, and the effluent during the acute and chronic toxicity tests (Tables 10.2, 11.1, 11.2 and 12.3). Despite this variability, tap water is an accepted standard diluent (APHA, 1992) and Stephan (1977) suggests toxicity data endpoints can still be compared.

Although 20EC is considered an ideal temperature for the maintenance of *B. stenochorias* in the laboratory (Haigh and Davies-Coleman, 1997), temperature effects on growth and fecundity in *B. stenochorias* must be considered in refining the endpoints used in future toxicity tests, and requires further investigation.

Acute Toxicity Tests

The use of the reference toxicant potassium dichromate (Chapter 10; Appendix A) introduced a means of characterizing method variability between consecutive acute tests, with results forming the initial South African database for *B. stenochorias* to be used in future intra- and inter-laboratory comparisons. Monotonicity of response to potassium dichromate by both *B. stenochorias* and *D. pulex* was observed, suggesting consistency of response of both species towards the reference toxicant. Although six is the accepted minimum number of individual laboratory tests that should be completed to determine test method precision (three and four for the daphnids and limpets, respectively, were completed with potassium dichromate), a comparison of the LC_{50} values (1.4 and 11.5 parts per million potassium dichromate respectively) and coefficients of variation values (30% and 35% respectively) suggest both species gave consistent, and comparable, endpoints (Parkhurst *et al*, 1992; USEPA, 2000).

LC_{50} values for neither the limpets nor the daphnids were determined in the effluent acute tests because of low mortalities. *D. pulex* was less sensitive to the effluent with infrequent, low mortality (5%) over 48 hours. This compares with the values in the international literature (Chapman *et al.*, 2000; Laughton *et al*., 2000). Variability in mortality figures of *B. stenochorias* was observed with increasing effluent concentration, suggesting *B. stenochorias* may, like other pulmonates (Patrick and Palavage, 1994), be considered pollution-tolerant. Although limpet mortalities in both effluent and potassium dichromate were within wide ranges, the ranges and rates of mortality did not vary significantly between replicates within each dilution tested.

Unlike that of the daphnids, limpet response was seasonal, with significantly lower mortalities in winter to both effluent ($p < 0.001$; Table 10.3) and potassium dichromate ($p < 0.05$; Table 10.6). Further physiological research may determine whether this was due to a lower winter metabolism. Population differences in mortalities also occurred, but these were due to significantly low winter mortalities in

effluent for those limpets sampled from the pristine, non-flowing and impacted, flowing fresh water. This may be related to flow rate and a consequent physiological adaptation to increased salt concentrations (Lam, 1996a, b); the non-flowing site from where the limpets were selected for testing experienced high winter evaporation, and the rate of flow at the impacted site, the second limpet source, was very low to zero during winter (Figure 4.3).

Other sources of variability influencing the acute *B. stenochorias* tests included the age and sexual development of the limpets. This was reduced by the selection of *B. stenochorias* below 2.8 mm shell length (pre-sexually developed; Chapter 5), but not eliminated as differences in the sexual development between individual limpets may still have contributed to the greater variability in response to potassium dichromate seen in the early summer (Table 10.6). The definition of a toxicological endpoint is therefore more uncertain with *B. stenochorias* compared to the asexually producing *D. pulex*. Despite the uncertain variability in responses displayed by *B. stenochorias*, and the rapidity of the 48 hour *D. pulex* test, *B. stenochorias* should be considered an acceptable acute textile whole effluent toxicity test organism, in South African hazard and risk assessment. The limpets are also preferable as a toxicity indicator compared to *D. pulex*, as they represent freshwater molluscs found in lotic conditions, with a greater sensitivity to the textile whole effluent. Studies of the effects of a variety of organic and inorganic pollutants on *B. stennochorias* should now be undertaken by the Water Research Commission and other research bodies and industries within South Africa.

Chronic Toxicity Tests

Most complex effluents are sub-lethally toxic, particularly after instream dilution (Holdway, 1996; Lai and Lam, 1994). As a 96 hour LC_{50} value for the textile effluent could not be calculated, this confirmed the need for a chronic toxicity investigation. Survival, growth and reproduction were the three responses investigated with both *B. stenochorias* and *D. pulex*.

The measurement of growth for both species was both time consuming and statistically and practically unsatisfactory in revealing toxicological effects of the whole effluent. Stimulation in growth, possibly due to either the provision of extra nutrients or growth stimulants, was generally seen with limpets maintained in effluent concentrations up to 20% effluent. This is not an uncommon occurrence with invertebrates in effluents (*e.g.* Lowell *et al.*, 1995). However, growth stimulation may just reflect a

shift in physiological response rather than a positive effect on the limpet life cycle (Lowell *et al.*, 1995); it needs to be carefully linked to reproductive efforts and longevity (Giesy and Graney, 1989; Ravera, 1991; Sibly, 1996), before a decision as to the advantage of this increase in growth is made. This is particularly so where the growth factors ê (rate at which L_4 is reached) and L (longevity) in the Von Bertalanffy Growth Equation are known to be negatively correlated in indeterminate growers such as *B. stenochorias* (Chapter 4). The uncertainty in the appropriateness of growth as a chronic toxicity indicator is also underlined by the dependency growth rates of *B. stenochorias* display on season (Chapter 4) and nutrition (Chapter 6).

With the lack of monotonicity of response, coupled with poorly fitting growth regression lines in some dilutions, it is suggested that growth is not an ideal whole effluent toxicity indicator in *B. stenochorias.* However, the growth rates of individual replicates were not significantly different, suggesting growth should not be completely discarded as an indication of toxic effects. Growth may be most useful in single chemical chronic toxicity tests where there is no problem with added nutrients or growth stimulants initially hiding the negative responses of the toxicant, as was the case in this study. The differences in quality of food provided during the tests, combined with low growth and higher mortalities, suggested differences in the health of individual limpets may have contributed to variability of response of limpets between dilutions, and between tests. A study of the nutritional requirements of *B. stenochorias*, and an improved method of food provision would be essential before their use in further toxicity tests.

Stimulation of growth in *D. pulex*, in many of the replicates throughout all effluent concentrations, also occurred. The synthetic water used in the culture of *D. pulex*, now standardised for South Africa (Slabbert, 1996), may not be optimal and may need further refining before chronic toxicity tests using *D. pulex* can be introduced in South Africa. However, *D. pulex* is a filter feeder, not a grazer. This suggests the increased growth was less likely to be because of a change in nutrient supply alone.

The fecundity of *B. stenochorias* is a useful ecotoxicological measure. Fecundity has been used in biological monitoring with other pulmonate species (*e.g.* Bedova and Kolupaev, 1998; Humphrey *et al.*, 1999). Despite the high innate variability in growth and reproduction of *B. stenochorias* (Chapters 4 and 6; Table 11.5), statistically significant differences in fecundity data became clear (Table 11.5).

Significantly increased fecundity was seen in the 3% effluent, compared to 20%, 10%, 1% and the controls of dechlorinated tap water, possibly related to the increased growth seen when in 3% effluent, and therefore earlier sexual maturation. Stimulation of reproduction by sub-lethal concentrations of poisons has previously been recorded in various invertebrates (Pickering, 1968; McLeay and Brown, 1974; Mitchell, 1992; Lowell *et al*., 1995). Egg lay was possibly inhibited in half the 1% effluent replicates, where significantly increased age at first lay, and significantly fewer eggs per capsule and total number of capsules occurred. Future tests would require temperature and density effects on fecundity would be required, as well as the testing of receiving waters as the diluent, before hazard assessments can be made from laboratory studies. Possibilities for reproductive criteria include calculations of the number of eggs laid versus hatching, and egg development, both of which are easy to observe using a bifocal microscope (Chapter 11). The egg capsules of *B. stenochorias*, unlike other pulmonates (Bondesen, 1950), are laid with a quaternary layer sealing the capsule (Haigh and Davies-Coleman, 1997). The eggs may therefore not be as susceptible to toxicants as other South African pulmonates. The capsules adhere to the surface, and eggs and embryos within the capsule do not move around and therefore individual development could be monitored.

Although the investigation of fecundity was practically easier to monitor in *D. pulex,* its sensitivity to toxic effects was significantly lower than *B. stenochorias*. The No Observed Effect Concentration (NOEC) was 12.5% effluent (*D. pulex*), compared to < 0.1% and <1% (*B. stenochorias*). The use of *D. pulex* is therefore questionable for effectively monitoring the chronic effects of textile whole effluent tests, based on reproductive effects, compared to the results obtained with *B. stenochorias*. The NOEC value plays a major role in recent procedures for hazard and risk assessment, but its derivation relates directly to the precision within the experiment (Wagner and Løkke, 1991; Aldenberg and Slob, 1993). There are, therefore, arguments against the NOEC approach for risk assessment (Sokal and Rohlf, 1981; Hoekstra and van Ewijk, 1993; Noppert *et al.*, 1994; Chapman *et al.*, 1996; Sparks, 2000), but with no general agreement as to which endpoints to choose. Alternatives to the NOEC are listed and discussed in Hoekstra (1993).

Although the practicalities of completing an F1generation test with *D. pulex* were significantly easier (shorter period) than with *B. stenochorias*, the value of the F1 generation results with either species did not justify its use in estimating the toxic effects of textile whole effluent, and its usefulness in future

applications is limited.

On the basis of survival and fecundity in particular, *B. stenochorias* has clearly shown an ecologically significantly toxic effect of the textile whole effluent at dilutions of < 1% and < 0.1%, using tap water as the diluent. The complexity of results obtained with growth and reproductive endpoints should not be discounted for the future use of *B. stenochorias*, but rather seen as an indication of the variability of responses of a species which will show the same complexity of responses in the natural environment. Site specific whole effluent toxicity tests using feral populations of *B. stenochorias* therefore offer the opportunity for examining natural resilience, resulting in adequate and accurate resource protection (Draggan and Giddings, 1978; Slabbert *et al*., 1998; Liu and Dutka, 1999; Shukla, 2000). The keys to predictive utility of toxicity tests are interpretation and extrapolation to the receiving system in terms of estimated hazard of the toxicant; the sensitivity and variability of the responses of the test organisms used in the toxicity assessments; and the replicability by a wide variety of quality control personnel (Cairns and Pratt, 1989). Future research into the use of *B. stenochorias* should therefore be aimed with these ideal points in mind, with the development of a practical, robust, unambiguous toxicity test method (or methods).

13.2. Application of *Burnupia stenochorias* Toxicological Data in the South African Context

Water quality management in South Africa began, from a legislative perspective, with the Union Health Act 36 of 1919 which dealt primarily with sewage disposal. The promulgation of the Water Act, 54 of 1956, and later amendments broadened water quality management. Industrial effluent and other sources of effluent, such as waters arising from agricultural, industrial and mining activities, were made subject to pollution control regulation. The present National Government recognizes that water is a scarce and unevenly distributed resource, belonging to all people. The principle behind the South African water policy states that “The objective of managing the quantity, quality and reliability of the nation’s water resources is to achieve optimum, long-term, environmentally sustainable and economic benefit for society from their use.” (DWAF, 1997; National Water Act No. 36, 1998). The National Water Act seeks to balance resource protection with resource use for optimal social and economic good. This aim is achieved through the application of resource directed measures (resource/ecosystem guidelines and quality objectives) and source directed controls (licences for abstraction or waste disposal, and national end-of-pipe water quality standards) (Jooste, 2000).

Aquatic invertebrates are presently used in South Africa in the assessment of resource directed measures as indicators of river conditions, in particular organic pollution, habitat availability and changed patterns of flow (Uys *et al*., 1996; Chutter, 1998; DWAF, 2000). At present, regulation and control of effluent quality is accomplished primarily through the chemical analyses of water samples using single substance chemical standards (DWAF, 1995). This approach has many limitations (Roux, 1994). Toxicity test data have and are being used in South Africa, in the development of guidelines and resource quality objectives (Palmer and Rossouw, 2001) as well as in licence requirements, based on methodology developed and data accumulated in other countries (CCEM, 1991; ANZECC, 1992; USEPA, 1992, 1994). The inclusion of data on South African species will enhance the application of toxicity test data in South Africa (Roux *et al*., 1996; Slabbert *et al*., 1998b).

Single species toxicity tests have been most widely used for deriving numerical, national water quality guidelines for ecosystem protection, although there are inherent difficulties in applying the results to the environment (Boyle, 1983; Cairns, 1983; Moriarty, 1983; Kimball and Levin, 1985; Lawrence and Williams, 1991; Hunt *et al.*, 1992). Results of both acute and chronic tests are used in the guidelines development (Roux *et al*., 1996). Presently in South Africa, the toxicity test data requirements include defensible data for a minimum of eight species representing different taxa, life forms and trophic levels (Roux *et al*., 1996). South African-derived data are, however, very limited and lacking for many indigenous species, including the Mollusca (Slabbert *et al*., 1998b). At a chronic level, it is primarily only Crustacea (*Daphnia* species) that have been investigated and are being used in South Africa, and there is a desperate need for chronic toxicity methods to be developed (Slabbert *et al*., 1998b; Palmer and Jooste, 2001). The water quality guidelines continue to be updated and refined as tolerances of the indigenous organisms inhabiting South African freshwater ecosystems are investigated and understood (Palmer *et al*., 1996, 2000). The results from the *Burnupia stenochorias* acute and chronic toxicity tests will therefore contribute to the updating of the guidelines.

The implementation of the National Water Act (1998) depends, however, on the licenced control of waste water disposal as well as the provision of resource quality objectives (DWAF, 1997; Scherman and Palmer, 2000). At the most general level, waste water disposal is licensed by a general authorization, which lists those conditions under which a specific licence is not required. The disposal of complex industrial waste water is explicitly excluded from the general authorization, and these

discharges will have to be licensed (Palmer and Jooste, 2001). In South Africa, many complex effluents are discharged via the sewage system. However, the toxicity of some substances may not be ameliorated by the sewerage treatment processes, thus posing a potential ecological hazard[2] (Hunt *et al*., 1992; Tonkes *et al*., 1999). Policy and regulations are therefore being developed in South Africa which are based on a tiered system of toxicity testing, from the simplest single species standard organism tests through to site testing using organisms from the receiving waters (Palmer and Jooste, 2000, 2001). The licence specifications derived from acute and chronic whole effluent toxicity tests (also referred to as direct toxicity assessment) are then in terms of a toxicity test endpoint, rather than a chemical concentration (Tonkes *et al*, 1999; Palmer and Jooste, 2001). This is considered an acceptable means of complex industrial waste water management (Blaise *et al*., 1988; Hunt *et al*., 1992; Tonkes *et al*, 1999; USEPA, 1998, 1999; Warne, 1998), applicable in both ecological hazard and risk[3] assessment procedures. However, no single direct toxicity assessment best assesses all ecological effects (Liu and Dutka, 1999; Jooste, 2000; Palmer and Jooste, 2001), exemplified by the inadequacy of the *D. pulex* results in this toxicological study, compared to the toxicity endpoints obtained using *B. stenochorias*. More realistic decisions such as those based on chronic toxicity results may be more appropriate under certain conditions, where risk-based decisions, rather than hazard based, may be called for (van Leeuwen *et al*., 1992; Warne, 1998, ANZECC and ARMCANZ, 2000). To date, chronic methods and parameters for representative species and trophic levels are very limited both in South Africa (Slabbert *et al*., 1998; Palmer and Jooste, 2001) and internationally (MacKay *et al*., 1989; USEPA, 2000).

The role of indigenous organisms, and hence *B. stenochorias,* in reflecting the receiving water impact with site specificity is ideal, as their responses are generally more variable than laboratory bred species (Cairns,1986a; Calow, 1989; Dillon, 2000). This approach is within the discipline of ecotoxicology rather than toxicology (DWAF, 2000; Chapter 8). The toxicity based ecological hazard assessment methodology, with the inclusion of the use of indigenous organisms such as *B. stenochorias*, is

[2]Hazard: A state that may result in a undesired event, the cause of risk. In environmental toxicology, the potential for exposure of organisms to chemicals at potentially toxic concentrations constitutes the hazard (Murray and Claassen, 1999).

[3] Risk: Concerned with estimating the probabilities and magnitudes of ecological events on a specified target organism or other ecological entity (Suter, 1995; SETAC, 1997).

therefore a first step towards more realistic and more informative ecological risk assessment (Palmer and Jooste, 2001). The methods used must, however, be rigorously defined (Calow, 1989; Warne, 1998). In the case of *B. stenochorias*, the endpoints selected and their rigorousness require further investigation before the species can be effectively used in hazard or risk assessment.

With the vast range of textile dyes and processes used and applied in the textile industry, the importance of individual (factory) environmental risk assessments is emphasized (Brown and Anliker, 1988 in Gilfillan, 1997). As an immediate example of the application of the chronic whole effluent toxicity tests using *B. stenochorias*, the hazard-based guidelines for the disposal of textile whole effluent given by Zokufa (2001) can be examined. Post-irrigated effluent (the same source as used in the *B. stenochorias* tests) was not acutely toxic, defined in terms of an LC_{50} value, to mayflies (Zokufa, 2001), which contrasts with the results from this study of *B. stenochorias* (Chapter 10). However, on the basis of both acute and 7-day chronic (survival) toxicity tests, using general (pre-irrigated) textile effluent from the same factory source, Zokufa (2001) calculated that a 3% effluent concentration would protect Class A water resources *i.e.* those resources most natural in character (Table 13.1). However, 43 day toxicity tests with *B. stenochorias* gave No Observed Effect Concentrations (NOECs), also based on survival, of less than 0.1% (1998) and less than 1% (1999) post-irrigation textile whole effluent (Section 11.3.3). Subtle toxic complexities, potentially cumulative to a toxic level, were therefore not found at the acute level tested by Zokufa (2001). This clearly demonstrates the necessity, in the South African context, for chronic toxicity data with a variety of indigenous organisms, before resource protection through ecological risk assessment is ensured. A precautionary note is made that some of the present literature currently questions the validity of using NOEC data for resource protection (Chapter 11; Section 13.1).

A further use for *B. stenochorias* would be in the monitoring of the effects of molluscicides, when applied to natural water resources, on non-target indigenous freshwater molluscs. Brackenbury and

Table 13.1. The state of health of a river or reach of a river can be grouped according to various classes (below, loosely based on Palmer, 1999). Palmer and Scherman (2000) have developed a method which relates toxicity test data (which describes the hazard) to this resource classification.

Class	Degree of change from the natural ecosystem condition
A	Natural
B	Slightly changed
C	Moderately changed
D	Considerably changed
E, F	Seriously / critically changed

Appleton *et al*. (1992) suggest molluscicide toxicity tests should be modified and adapted by national regulatory bodies to incorporate non-target species indigenous to South Africa, allowing for the more realistic prediction of the impact of the molluscicides.

The results of this study suggest *B. stenochorias* has many attributes that make it suitable for ecotoxicological studies, representing the grazing freshwater molluscs of South Africa. However, variability in the results recorded in this study suggest a number of points need further development and research before *B. stenochorias* can be effectively used. The accumulation of biological data is generally a long term process before there is any confidence in suggestions of life cycle patterns. Much more detail of dietary preferences and the effects of different water chemistry is needed for the effective culturing of *B. stenochorias* in the laboratory. Effective long term toxicity tests and the role diluents play in toxicity will also rely on this information. Understanding the mechanisms underlying the patterns of toxicity would contribute to more effective use of *B. stenochorias* as a toxicological indicator. However, variability of toxic responses compared favourably with the standard laboratory organism *D. pulex*, using both the textile whole effluent and the reference toxicant potassium dichromate. The chronic toxicity studies have also pointed to worthwhile possibilities within the life cycle which warrant further investigation in the search for practical, reproducible chronic effect methods to be introduced in site specific hazard and risk assessments. Future research should examine the effects of a wide variety of organic and inorganic toxicants. Results from the chronic toxicity tests in particular, have underlined the necessity for consideration to be given to a wide number of species representing different taxonomic groups at sub-acute levels of toxicity, to maintain ecological integrity and sustainability in South African water resources.

APPENDIX A. BACKGROUND INFORMATION AND JUSTIFICATION FOR THE METHODS EMPLOYED IN THE TOXICOLOGICAL TESTS

A.1. The Use of Potassium Dichromate as a Reference Toxicant

A reference toxicant is described as a chemical used in an aquatic toxicity test as a positive control (*i.e.* deliberately causes known adverse effects) in contrast to the negative control provided by exposure water without the test chemical (USEPA, 2000). The criteria used in the selection of a reference toxicant have been discussed by several authors (*e.g.* Fogels and Sprague, 1977; Lee, 1980). Two important aspects to the properties of the reference toxicant are those pertaining to the nature of the materials, and those dealing with the material's effects on the biological systems (Dorn *et al*., 1987). A reference toxicant should be readily available in purified form, easy to handle, easy to analyse, and fully dissociated at test pH (Dorn *et al*., 1987). Toxicological characteristics should include toxicity at low concentrations, consistency in response, non-selective toxicity, rapid attainment of effect, and known mode of action or metabolic stressor which should be clearly defined (Lee, 1980). Changes in water pH, hardness and alkalinity should not affect the speciation and therefore toxicity of the reference toxicant (Dorn, *et al*., 1987). There are many chemicals which can be used as a reference toxicant (Lee, 1980), with potassium dichromate fulfilling all of the necessary criteria (AQUIRE, 1994). Potassium dichromate is therefore a suitable choice as a reference toxicant for this study.

The hexavalent form is not easily adsorbed onto the vessel walls or onto particulate matter, thus a constant concentration can be easily maintained during the period of exposure (Guglielmucci *et al*., 1981). Dorn *et al*. (1987) showed potassium dichromate's consistency as a reference toxicant under varying water quality conditions with different test organisms, although there is some dispute as to its stability under changing hardness and alkalinity levels (Müller, 1980; Hosokawa *et al*., 1991). Potassium dichromate is now used in South Africa (E. Truter, pers. com.) and internationally (Di Giulio and Malloy, 1995), including its election by Canadian scientists as a suitable reference toxicant in the control of toxicity test precision for fish, algae and invertebrates in both acute and chronic tests (Environment Canada, 1990). Chromium is present in many types of waste water, including sewage treatment works, petroleum refineries, metal finishing plants, iron and steel plants, leather tanning manufacturers, inorganic chemical manufacturers, porcelain enamelling, and timber

producers (Brown, 1981). Trivalent and hexavalent chromium are also regularly found in textile effluents as they make up many of the dye constituents (Brown, 1981). This confirms the suitability of potassium dichromate as a reference toxicant in this study.

A.2. Statistics Used in The Analysis of Acute Toxicity Data

In establishing a dose response relationship, it is necessary to distinguish between the concentration of the chemical in the environment and the concentration that reaches the target tissue, whether potentially resulting in biochemical, physiological or behavioural responses (Connell *et al.*, 1999). However, measuring the actual amount of a chemical reaching the target tissue is difficult. In general, the biological effect of a toxicant is assumed to be related to the dose administered. In determining the dose response relationship, the response must be easily quantified, identified as a possible endpoint, and reproducible in a method that is relevant to the toxic process under investigation (Connell *et al*., 1999). A critical factor in the dose response relationship is the duration of toxin exposure, with mortality the most common endpoint in acute toxicity tests (Rand and Petrocelli, 1985). A post exposure observation (recovery) period is often included in a toxicity test, where the test organisms are placed in a 'clean' environment, usually the diluent, and observed for a sufficiently long period to ensure the organisms are indeed dead and not anaesthetized (Brackenbury *et al*., 1997). However, as this investigation with *B. stenochorias* deals with a whole effluent continually produced and not of a sporadic or episodic nature, it was felt the provision of a recovery period was not necessary.

When cumulative mortalities are plotted against dose concentration, a sigmoid curve usually results (Figure A.1). The 50% effect level falls on the straightest portion of the curve, and exhibits least variability of all responses. 95% confidence limits are given with each LC_{50} estimation. There are numerous methods used in the calculation of an LC_{50} value for a given set of concentrations, with advantages and disadvantages to whichever method is used and the interpretation of the results (Hamilton *et al.*, 1977; Stephan, 1977; Gelber *et al.*, 1985; APHA, 1992). The analysis of the results also depends on the experimental design chosen. Because it is unlikely that mortality within the test will be exactly 50% at a given concentration, the greater the number of concentrations tested, the more likely the accuracy of the LC_{50} value (Stephan and Rogers, 1985). A regression design and analysis is therefore usually chosen, where more concentrations are selected (6 or more) with only

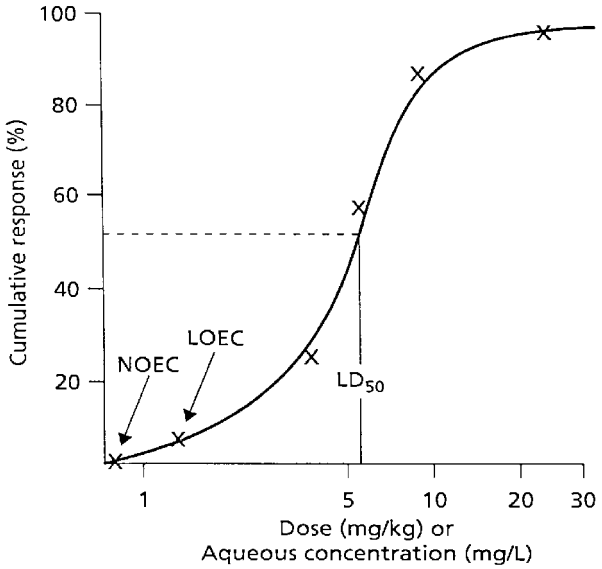


Figure A.1. A theoretical cumulative dose response curve. The cumulative percentage mortalities of the test organisms from a toxicity test are plotted against the dose concentration, usually measured in mg/l or part per million in aqueous toxicity testing. The lethal dose or concentration where 50% death occurred is indicated. Below this response are the Least Observed Effects Concentration (LOEC) and the No Observed Effects Concentration (NOEC), both of which are criteria used in chronic toxicity test analyses (Hoekstra, 1993). Redrawn from Donnell *et al*., 1999.

2 replicates, to assist in interpolation of effect at untested concentrations. However, part of the aim of investigating the responses of *B. stenochorias* was to quantify its variability of response within concentrations. The number of replicates was therefore increased to four (Sokal and Rohlf, 1981).

Probit or logit methods are usually used for parametric data (Finney, 1971); and Spearman-Karber or Trimmed Spearman-Karber analysis for non-parametric data (Hamilton *et al*., 1977; USEPA, 1994). The Probit method assumes a normal frequency of tolerance distribution. Mortality data are consequently probit transformed from the sigmoid curve to a straight line. Unless two partial mortalities occur within the series of concentrations tested, the model assumptions are not met, with the production of high chi squared values suggesting heterogeneity of results (Finney, 1971; USEPA, 1994). The non-parametric Spearman-Karber analysis does not require test data with partial mortalities

within the series of concentrations, but must exhibit 0% and 100% mortalities (Hamilton *et al.*, 1977). The calculations are based on interpolation of results, assuming a symmetrical distribution of tolerance around the distribution curve. Should the data not exhibit 0% and 100% data, the Trimmed Spearman-Karber programme 'trims' the data to fit the model. Should the response data be non-monotonic (response not increasing with concentration), the Trimmed Spearman-Karber smooths the data accordingly in its calculation of the LC_{50}. Most data within this study were trimmed and smoothed. However, a disadvantage to this is the alteration of the variances that occurs (Hoekstra, 1993).

As response to a toxicant is always variable around a mean at any one concentration under stipulated conditions, increasing the number of test organisms per concentration should increase the precision of results. In the case of all acute toxicity tests using *B. stenochorias*, 40 or more individuals per concentration should have sufficiently reduced the standard error around the LC_{50} values (Jensen, 1972), and therefore variability of responses towards the toxicant could be examined. The addition of further limpets was curtailed primarily because of practical difficulties pertaining to time management and insufficient limpets of the same size.

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