The Development of a Production Facility for Standard Laboratory Test Organisms for Ecotoxicological Research

EH Haigh • HD Davies-Coleman

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THE DEVELOPMENT OF A PRODUCTION FACILITY FOR STANDARD LABORATORY TEST ORGANISMS FOR ECOTOXICOLOGICAL RESEARCH

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EXECUTIVE SUMMARY

1 INTRODUCTION AND PROJECT RESEARCH OBJECTIVES

Water is Life.

The Department of Water Affairs and Forestry, as custodian of the water resources of South Africa, must manage both water quantity and quality to ensure sustainable development in South Africa. One of the prime aspects of protection of this scarce resource, which is now recognised as a **national** asset, must be through ensuring that the correct water quality for use is maintained. Quality assurance, which is resource directed, entails *inter alia*, the establishment of guidelines for water quality for the natural environment. The setting of these guidelines involves both the reviewing of established guidelines from around the world (Kempster *et al* 1980), and experimental investigation of those suitable for indigenous riverine organisms (Palmer *et al* 1996). Both the Artificial Stream Project and the Standard Laboratory Organisms Project (WRC Projects K4/475 and K5/545) were initiated to satisfy this need. In the Artificial Stream Laboratory at Rhodes University, which is now operational, toxicological work is undertaken which requires a steady supply of indigenous macro invertebrates as test subjects. This has emphasised that if this facility in which a large investment has been made, is to function adequately, the potential bottleneck of an inadequate supply of test animals should be avoided. Therefore, a building dedicated to the breeding and rearing of the selected invertebrate species is essential. This facility will ensure an uninterrupted supply of test organisms of known quality and origin for ecotoxicology research.

Little information is available concerning the life history, biology and ecological role of southern African aquatic invertebrates and this knowledge will enable researchers to make informed decisions about collecting times and the potential availability of animals of the best size and stage of development. In addition, the impact of proposed environmental change cannot be accurately predicted unless information of this type is available. The understanding of the physiology and functioning of test animals under natural conditions is needed if results of functional impairment due to contaminants and toxins are to be accurately assessed.

During the standard laboratory organisms project, invertebrates ranging from mayflies to crabs and flatworms were screened and several species were selected as candidates suitable for laboratory cultivation. Two species, *Burnupia stenochorias*, a limpet, and *Adenophlebia auriculata*, a mayfly, were chosen for in-depth investigation (Haigh and Davies-Coleman 1997).

Furthermore, the long term effect of contaminants on the physiology of the test species can only be critically evaluated if information is available on baseline responses under normal and optimal laboratory conditions. Information gained from the biological investigations to determine optimal maintenance and rearing conditions, therefore, have a wider application. The expertise being developed in this and during the earlier project can be applied to other areas of environmental management such as the rehabilitation of damaged rivers and streams.

1.1 Aims of the proposed project

- To conduct experimental and field investigations on the selected test species which will support the information already available:

  Burnupia stenochorias. Field and associated laboratory studies on the reproduction. Continuing laboratory studies on growth and feeding.

  Adenophlebia auriculata. Laboratory studies on artificial fertilization, and the rearing of hatchlings.

- To test the experimental techniques so developed at a larger production scale.

- To investigate and design features and costing for a laboratory dedicated to the large-scale production of the selected species. The suitability of various biological filtration systems to ensure optimal water quality conditions and light conditions for the culture of both the test species and selected diatom species (periphyton) as feed for the test species, will be investigated.

1.2 Background to project: Summary of previous project conclusions.

Burnupia stenochorias.

Although a substantial body of information had already been obtained by the end of 1995 certain areas of investigation was incomplete and we realised that another years of experiential work would yield information which would potentiate the work done already. The field investigation into the reproductive biology of the limpet which had been going on for six months needed a full cycle of seasons for interpretable results. A second large group of adult was discovered in a pristine river and afforded the opportunity for a comparative rearing and feeding experiment as well as a subsequent experiment using the laboratory reared adults to verify the fecundity questionable values obtained in 1994. Field collected individuals of known age and size still had to be sectioned to determine the onset of sexual maturity under natural conditions. This would augment information on reproduction gained from laboratory work. The analysis and interpretation of the results was completed in August and papers are being prepared for

submission to journals.

Adenophlebia auriculata.

The field investigation of this species in the Palmier River needed six more months of data collection for the observations to have been carried on for a full two year cycle. The artificial fertilisation had only been accomplished once and additional work to standardise the method and on the rearing of the hatchlings would hopefully yield a useful result.

2. SUMMARY OF PROGRESS: Development of the rearing facility

Some of the major constraints in optimising the culture of the invertebrate have been due to the lack of a suitable facility with good quality water on tap and the correct light quality to grow diatoms and algae for food. The design of a dedicated facility took into consideration the type of structure which would be most suitable to the purpose and cost effective in terms of thermal control and the light requirements for algal cultivation. The building, for which funds will be be raised, will serve as both rearing facility for test animals and as experimental test laboratory. The overall size is 25M x 6.5M with a pitched roof.

It will consist of:

1. **A breeding and rearing** area, divided into an area with recirculating streams (3M x 0.4M) and tanks (5-25l) in which algae will be cultivated and the animals will be reared and an adjacent small cool room with smaller streams (1.5 x 0.25M).
2. **Two offices and a storeroom** in the central part of the building with computer facilities, filing systems and kitchen / ablution facilities.
3. **A toxicology laboratory** which is air-conditioned of a similar size to the rearing room, with shelving and work benches.

This type of building has specific requirements.

The thermal stability of the building will be important and attempts will be made to achieve this without having to resort to the use for electrical cooling which can be very expensive. Firstly, the walls will be cavity walls filled with insulation and the roof space will be insulated. Secondly, fast-growing deciduous trees will be planted at strategic positions to provide shade and additional cooling in summer. The proposed building should be sited adjacent to the new hatchery at the Department of Ichthyology and Fisheries Science, Rhodes University, which is close to the existing Artificial Stream Laboratory.

Light. The correct light for growing algae as food i.e. as close to natural light as possible. In addition to large, shuttered window spaces in the rearing room, some artificial light which provides the correct wave lengths for algal growth will be needed.

Water. Good quality natural water from a sunken water reservoir should be delivered to the recirculating units by gravity feed. Bio-filtration should take place at the channels. Adequate provision for waste and toxic water disposal through exit channels into the municipal drainage. Good quality sloping floor of sealed cement for drainage. Should the water from the rearing section be recirculated to the tank, a sand filter outside the building will be needed.

Temperature. Temperature control through insulation of cavity walls, and ceiling, air conditioning for summer and heaters in winter. The roof space will be supplied with 'whirly-gig' extractors.

Hygiene. Each recirculating system must be separate to prevent contamination spreading through the laboratory. The toxicology and chemical lab separate from the rearing area to prevent poisoning. An ultra-violet sterilising unit in the water supply to ensure removal of pathogens. Hot water available in sluice room for washing equipment.

Air. Ducted air supply for rearing juveniles in tanks and buckets.

3. SUMMARY OF RESULTS - INVESTIGATION OF *BURNUPIA STENOCHORIAS*

3.1 Introduction

Manipulation of *Burnupia* populations in the laboratory could possibly be considered the bottom line to culturing effectively under any given laboratory conditions. The productivity of any species and its population structure can only be determined with a knowledge of growth rates, age structure, longevity and mortality rates. There are many ways in which growth estimates have been made. Aquaculturists typically use one of three measures when reporting growth: absolute growth rate, relative growth rate, and specific growth rate. Less frequently, von Bertalanffy Growth Functions are used. Growth of Ancylidae in southern Africa has not been studied to date, so that no background knowledge was available. The methods to determine growth in this project were aimed at providing a spectrum of data, from field (in which there was no control of the environment and measuremnets were of natural populations), to enclosed field studies (where some control was provided but under almost natural conditions), to laboratory studies (in which the inputs were rigorously controlled and actual growth rates of the same small populations of limpets were obtained).

3.2 Natural Populations

3.2.1 Field

A field investigation of 52 weeks was completed, where at least 300 limpets were measured for shell length, at fortnightly intervals from two sites in the Bloukrans River below Grahamstown. Any egg capsules present were counted and number of eggs inside noted, if possible. Through these observations it was seen that eggs are laid all year round but with a substantial peak of laying from the start of the spring months, mainly September, to early January. As direct aging techniques are not available for limpets, and no growth rings were found in the shell, a series of steps were taken within the programme FiSAT, to determine the longevity of a limpet and to predict a growth model for the two populations studied. Histograms of frequency of sizes, and Probability Paper did not give any results as to number of cohorts per year, or longevity. ELEFAN analysis, a programme within FISAT based on the von Bertalanffy Growth Equation, suggests the longevity time to reach the maximum shell length of 7.5mm is nearly two years, however, in reality this figure could be much less, depending on the time of year and conditions prevailing in the river at that time, this has been borne out with laboratory trials. Monthly and daily predicted growth rates are provided within the programme, where for any one cohort, there is a very strong winter phase of no-growth. The rates are compared to growth rates of known-aged limpets, in Section 3.3.3.

Temperature, pH and TDS differences between the two sites were found to be non-significant. However, flow rate was very significantly different and this in turn influenced the predicted growth rates.

3.2.2 Morphometric Analyses

Both wet weights and shell length, width and height were measured in three local populations of limpets (Manley Flats, Belmont River and Botha River respectively), and compared using regressions, correlations, ANCOVAs, Principal Component Analysis and Discriminant Analysis. The results indicate the significant relationship between length and width, with a greater variation in height measurements when related to the former two. Similarly wet weights were most significantly correlated with length at the two sites in the flowing water (Manley Flats and Belmont River), not surprising as length is the largest Morphometric measurement. However, in the non-flowing Botha River site, poor correlations were found between weight and length, height or width. As weight changes are generally considered an indication of growth, any analyses of growth in the future should measure height and width, and not just length, particularly if limpets from Botha River are used for ecotoxicity trials.

However, if individuals from all three sites are compared, the three populations cannot be separated on the basis of their morphometric measurements alone.

3.2.3 Habitats.

The investigation of the habitat preference of *B. stenochorias* was conducted as a field study by Lisa Pretorius, a third year Zoology student to confirm casual observations made to date on the habitat occupation of *B stenochorias*. Three local populations in the Bloukrans, Botha and Palmiet Rivers, in the Grahamstown area, were investigated during summer and winter.

Results indicate a preference for the following:

- Substrate - a firm surface to which the limpets can attach, lay it's eggs and feed on algal growth; these surfaces can include stones or rocks (from as small as 2mm in length), or less commonly, submerged vegetation. No preference was shown for size of rock relative to size of limpet. Only the occasional limpet was found in soft sediment, and this may have been as a result of error on the part of the researcher.
- Flow rate - stones-out-of-current had higher densities than stones-in-current; non-flow conditions in Botha River maintained high densities of limpets.
- River site - Palmiet River had significantly fewer (p,0.03) individuals. It was noted that from the results of the one water sample analysed in detail for ppm of sodium, calcium and magnesium, all three chemicals were substantially less in the Palmiet River than in either the other two rivers.
- Temperature - between 10° and 25°C was tolerated. However, winter sampling revealed fewer, large limpets present.
- Water chemistry - all rivers averaged 500mg/l TDS, and did not fluctuate outside the range pH 6.8-8.04, where 8.04 represented the Palmiet River site.

3.3 Experimental Work

3.3.1 Laboratory Growth Conditions.

A new development was that the streams were lined with plastic for ease of handling limpets and sampling for diatoms on a regular basis. Dechlorinated tap water was used, and calcium and silica were added regularly for the growth of the limpet shells, and the ditaom fustules respectively. Water chemistry was analysed regularly (pH, TDS, phosphates, nitrates, nitrites, alkalinity, and various metals). Growth of periphyton was monitored by means of chlorophyll samples, and diatoms identified by Scanning Electron Microscopy. Results have given a good indication of the diatoms which can be expected over a long term, in the streams under the given conditions of water and light, and the effect of the limpets grazing.

3.3.2 Effects of Feeds on Growth and Reproduction.

In order to improve the growth of the limpets in the laboratory cultures, Nutrafin, a fish food with various proteins included, was tested against the usual diatom/periphyton layers as a feed for newly hatched limpets of known age, and the effects of the different feeds on growth and fecundity were analysed. Nutrafin had a significant positive effect on the growth (substantially increased) and longevity (extended) of the limpets, although initial mortalities were similar to the other feeds. Enormous individual variation in fecundity in all feeds occurred, with laying times relative to age of limpets, with rest periods between laying, with number of capsules laid, with numbers of eggs in capsules, and with fertility of eggs laid. The largest percentage of eggs was laid in the first four weeks of the reproductive period, which was 10 to 12 weeks after hatching. This confirms previous results of 1995. The extended longevity also realised a greater output of eggs. 27% of the limpets laid egg capsules showed one or more developmental abnormalities, and this, together with the enormous individual variability, makes predictions as to expected numbers of offspring very difficult.

3.3.3. Field Baskets

Limpets of known age (from the same source as the above) were placed on rocks in baskets in the Bloukrans River, and growth and mortalities were monitored weekly. These were compared to the growth of the limpets from Section 3.3.2 by regression and ANCOVA, and found to be similar. The results were also found not to be significantly different to the growth rates predicted by the von Bertalanffy Models for the two sites previously analysed using length frequency data (Section 3.2.1), thereby confirming the authenticy of the models.

3.3.4 Sexual Development

Limpets of various sizes were collected and preserved in Bouins Fluid each fortnight for 52 weeks from the two sites in the Bloukrans River (Section 3.2.1) mounted in wax, sectioned and stained. The results showed the description of the gross gonadal morphology of *B. mooiensis*, published by Oberholzer and Van Eeden (1969), to apply to *B.stenochorias*. Male and female sex cells are intermingled in the acinus. All overwintering limpets larger than 3mm shell length contained mature ova. Smaller limpets showed only early Spermatogenesis. The only limpets in the overwintering population which showed late Spermatogenesis were greater than 4.08mm shell length. With increase in water temperatures in spring the sexual development appears to accelerate. By midsuumer the ovotestes occupies up to 50% of the body cavity in most limpets and both male and female sexual development was present. Spermatogenesis and oogenesis declines during late summer and autumn.

3.4 Conclusions

With the adverse effects of handling on the growth of the limpets, extensive use was successfully made of plastic sheeting, in both the streams and the aerated bucket system, as well as in the movement of limpets from one locality to another for the purposes of toxicology experiments. The aerated buckets give the most ideal growth and fecundity results of all the containers investigated. However, the limpets will have to be conditioned to running water during some stage in their cultivation where they should be held until required, as they should represent the natural populations of limpets being tested for effects of toxicants in the toxicology programme. Plastic sheeting with limpets attached is an ideal means of transferring the limpets from the buckets to the streams presently used for their cultivation.

Attempts to successfully cultivate *Burnupia* on a larger scale included the refining of the use of the large streams. Plastic strips packed into the sumps acted as a successful means of maintaining water quality for both the diatoms and the limpets, via the incidental growth of a layer of bacteria on the plastic strips acting as a biofilter. Nitrogen and phosphate levels remain low under these circumstances. Visual surveillance indicated the addition of calcium in the form of calcium carbonate maintained the strength of the limpet shells, a problem previously encountered. Similarly, sodium silicate was added to the streams for the growth of the diatoms (Section 3.5). The additional sodium and calcium did not appear to disrupt the balance between calcium, sodium and magnesium which could have possibly affected the fecundity in particular. The growth of the periphyton, in particular the species of diatoms present over time, was monitored in the streams. Results have shown under the given conditions of light and dechlorinated tap water, with limpets grazing and therefore affecting the periphyton storey and composition, that unicelluar and stalked diatoms are successfully grown, with bacteria and fungal spores acting as a further food source. Photographs of the species identified, taken with a Scanning Electron Microscope, are provided. Both the literature survey regarding those conditions considered necessary for the growth of the periphyton, and the variation in growth of periphyton seen by the project researchers under the conditions provided by the University, have all been considered in the designs for the new laboratory.

Comparisons by various statistical means have revealed that using the morphometric measurements of length, width and height, three local populations of *Burnupia* cannot be separated with ease. This is encouraging should it be decided in future that further genetic stock is necessary for the cultures. Similarly, it confirmed that should test individuals be directly taken from a natural population for ecotoxicology trials, all three measurements were necessary to identify the chronic effects of potential toxicants on the limpets.

4. SUMMARY OF RESULTS - INVESTIGATION OF *ADENOPHLEBIA AURICULATA.*

4.1 Introduction

Ephemeroptera form a large portion of the population of rivers and streams world-wide and are an important link in the food web as they are detritus and algal consumers. The family Leptophlebiidae had previously been identified as having species which are suitable for laboratory culture. The family is widely distributed on southern Africa and most species are large enough for easy of handling. Furthermore the family may prove to be valuable as bio-indicators of water quality conditions. Previous investigations had yielded information on the laboratory requirements and life history of the species *Adenophlebia auriculata.*

4.2 Autecology

4.2.1 Life history.

The life history style of *A. auriculata* in the Palmiet River is one of repeated emergences throughout the year except from mid-winter to early spring. There are major emergences ain spring ans summer but lesser emergences occur between these major events and this makes the analysis of the captured samples to determine the life cycle extremely complex. The life cycle is most probably annual or semi-annual in that it may fall a few weeks short of 52. The energy budget required the nymph to complete this life cycle is between 1800 and 2000 DD. A final answer about the life cycle will only be available once a field rearing experiment has been done.

4.2.2 Population Distribution between biotopes in two Rivers

The distribution of *A. auriculata* amongst biotopes is strongly influenced by the size of the nymphs. The initial distribution is random, with hatchlings occurring throughout the stream. Subsequently there is a redistribution to the optimal ecological biotopes stones-out-of-current and marginal vegetation, corresponding with the hydraulic biotopes slackwater, backwater and pool. This redistribution occurs in the small nymphs and is either active or passive. Passive locomotion makes use of current flow and explains the transient occupation of high current biotopes by small size class nymphs.

The optimal conditions for the majority of nymphs of this mayfly appear to be slow currents, large substrate size and vegetation, and large quantities of detritus. An important feature for the large nymphs (pre-emergent) is that they have suitable structures for emergence. This is provided in marginal areas by vegetation, rock and the bank itself.

4.3 Laboratory Culture

The initial success in 1994 with the artificial fertilisation of eggs had to optimised and a method had to be developed to produce larger numbers of hatchlings by this method. Dr de Bisthoven reared nymphs in a constant environment room inside an emergence cage to investigate *in vivo* reproduction which did not occur. He also attempted *in vitro* (AI) fertilisation which was unsuccessful. Two further trials on *in vitro* fertilisation using two additional new methods were attempted and although fertilisation was achieved no nymphs hatched. This has been the greatest disappointment of the project as several authors have reported success using artificial fertilisation.

4.4 Conclusions.

The life history of *A. auriculata* makes it suitable invertebrate for use in toxicological experiment where field caught specimens are used. The species responds well to laboratory maintenance and will emerge successfully after lengthy rearing. The growth rate can be doubled if the diet of detritus and decayed leaves is enhanced with the addition of a protein rich supplement although mortalities may increase if hygiene is not scrupulous.

The distribution of *A. auriculata* amongst biotopes is strongly influenced by the size of the nymphs. The initial distribution is random, with hatchlings occurring throughout the stream. Subsequently there is a redistribution to the optimal ecological biotopes stones-out-of-current and marginal vegetation which are areas with slower current. The redistribution occurs in the small nymphs and is either active or passive. Passive locomotion makes use of current flow and explains the transient occupation of high current biotopes by small size class nymphs. The optimal conditions for the majority of nymphs of this mayfly appear to be slow currents, large substrate size factors which should be considered when toxicology experiments are designed.

The scale of the river does not affect the distribution pattern of the nymphs. In both the Palmiet and the Buffalo Rivers the nymphs are found under the similar conditions except when vastly different current velocities are found in similar ecological biotopes.

5. ACHIEVEMENT OF AIMS

i *To conduct experimental and field investigations on the selected test species which will support the information already available:*

Burnupia stenochorias. Field and associated laboratory studies on the reproduction. Continuing laboratory studies on growth and feeding.

Adenophlebia auriculata. Laboratory studies on artificial fertilization, and the rearing of hatchlings.

- All the field work was completed as well as some additional investigations, due to the availability of students with an interest in our investigations. All the laboratory work was accomplished but the results were not always positive. However the life history and laboratory requirements of both species are now fully understood.

ii *To test the experimental techniques so developed at a larger production scale.*

- Some trials were conducted to satisfy this aim. Additional biolux tubes were installed to test the suitability of the light sources for the proposed building. The data are available for the calculation of numbers to ensure optimal supply of test animals on an ongoing basis.

iii. *To investigate and design features and costing for a laboratory dedicated to the large-scale production of the selected species. The suitability of various biological filtration systems to ensure optimal water quality conditions and light conditions for the culture of both the test species and selected diatom species (periphyton) as feed for the test species, will be investigated.*

- This aim has been fully accomplished. The design is included in the report and the trials on diatom growth are reported in Appendix 3.6. The biofilter systems have been successfully operational for up to 20 months. However , the fundraising for the building needs the undivided attention of a fundraiser for the building to become a reality.

Presentations

1) Haigh, EH and HD Davies-Coleman, 1997. Presentations by both researchers on the development of methods for laboratory culture of aquatic invertebrates, including the success to date with *Burnupia stenochorias* and *Adenophlebia auriculata*. Zoology Dept, University of Port Elizabeth.

2) Davies-Coleman, HD and EH Haigh, 1997. Life cycle and Growth of Two Natural

Populations of a South African Ancylid, *Burnupia stenochorias*. Poster presentation by HD Davies-Coleman, North American Benthological Society 45th Annual Meeting, San Marcos, Texas, USA.

* also presented June 1997 at the SASAQS (South African Society for Aquatic Scientists) Conference, Mtinzini, Natal.

3) Haigh, EH and HD Davies-Coleman, 1997. Investigations into the Reproductive Biology of *Burnupia stenochorias* (Mollusca, Ancylidae). Paper presented by EH Haigh, SASAQS Conference, Mtinzini, Natal.

4) HD Davies-Coleman, EH Haigh and A Booth*, 1997. Growth Analyses of Natural and Laboratory-Reared Populations of the Freshwater Limpet *Burnupia stenochorias* (Ancylidae), 1997. Paper presented by HD Davies-Coleman, SASAQS Conference, Mtinzini, Natal.

* Dept. Ichth. Fisheries Services, Rhodes Univ.

5) Haigh, EH and B Hunt, 1997. Population Distribution of the Mayfly *Adenophlebia auriculata* in Two Eastern Cape Rivers. Poster presented by B. Hunt (Zoology Hons), SASAQS Conference, Mtinzini, Natal.

6) HD Davies-Coleman contributed to the paper to be presented by Prof K deKok, Potchefstroom University, as part of a review of freshwater malacological work currently being undertaken in South Africa, September 1997. African Medical Malacology Conference, Harare.

Proposed publications

As this project (K5/755) will be completed in December 1997, we submit a list of papers in preparation. These papers are to be submitted to various journals, both national and international, from November 1997. Their titles may change on final submission to the chosen journal.

Burnupia stenochorias

1) The breeding biology and fecundity of *Burnupia stenochorias* (Pulmonata, Ancylidae).

2) The embryology of *Burnupia stenochorias*. Melvill and Ponsonby (Mollusca, Pulmonata, Ancylidae).

3) Life cycle and growth of two natural populations of a South African freshwater limpet, *Burnupia stenochorias* (Pulmonata, Ancylidae).

4) The effects of density and temperature on the growth rate and mortality of a freshwater limpet, *Burnupia stenochorias* (Pulmonata, Ancylidae).

5) The effects of diet on growth and mortality of *Burnupia stenochorias* (Mollusca, Pulmonata, Ancylidae) in the laboratory.

* co-authored with A.Booth.

6) The effects of container size on the growth and survivorship of *Burnupia stenochorias* (Basommatophora, Ancylidae) when cultured in the laboratory.

7) Sexual development of *Burnupia stenochorias*, Melvill and Ponsonby (Mollusca, Basommatophora, Ancylidae).

8) Early colonisation of bare surfaces by epilithic diatoms in artificial streams, and the impact of grazing limpets (*Burnupia stenochorias*, Ancylidae) on the diatom assemblage. * co-authored with M.Balarin, 1998.

Adenophlebia auriculata

9) The effect of different diets on the growth of *Adenophlebia auriculata* (Ephemeroptera, Leptophlebiidae) in the laboratory.

10) The effect of temperature on instar period and mortality of *Adenophlebia auriculata* (Ephemeroptera, Leptophlebiidae).

11) Population distribution of nymphs of *Adenophlebia auriculata* (Ephemeroptera, Adenophlebia) in two Eastern Cape rivers.

* co-authored with Brian Hunt (Honours student, Zoology)

12) Life history of *Adenophlebia auriculata* (Ephemeroptera, Adenophlebia) in a small Eastern Cape river.

ACKNOWLEDGEMENTS

As project principal researcher I wish to acknowledge my colleague Heather Davies-Coleman for her support and devotion to the work we undertook over the past two years. I have observed her development from that of an assistant in the previous project to a fully fledged and most competent researcher. She has grasped the opportunities afforded her to develop skills she did not have, and capitalised on them and I wish her well in the pursuit of her doctoral qualification.

We as a team would like to acknowledge again the support and assistance of the all staff of the Institute for Water Research, and we offer particular thanks to Gaye Youthed our senior technical officer and Margi Rogers our secretary. We thank our editorial assistant of the past few weeks, Dr. Nikite Muller. Her sharp eyes and well developed sense of science were greatly appreciated. Colleagues from the University, namely Prof. Sarah Radloff, Dr. Martin Villet and Dr. Tony Booth are thanked for analytical and statistical help. To our students Marian Balarin, Brian Hunt and Lisa Pretorius goes our grateful thanks for the valuable information we have gained from their work.

The Water Research Commission was once more generous in their support and our project manager, Dr Steve Mitchell, was as ever kind, helpful and encouraging, besides giving insightful advice.

Finally, our thanks must go to our Steering Committee: Professors Chris Appleton, Johan van Vuren and Tom Hecht, Drs. Ferdy de Moor and Anton Bok and Ms Chirsta Thirion for their input and time.

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CHAPTER 1.
OBJECTIVES AND BACKGROUND

1.1 INTRODUCTION AND PROJECT RESEARCH OBJECTIVES

Water is Life.

The Department of Water Affairs and Forestry, as custodian of the water resources of South Africa, must manage both water quantity and quality to ensure sustainable development in South Africa. One of the prime aspects of protection of this scarce resource, which is now recognised as a **national** asset, must be through ensuring that the correct water quality for use is maintained. Quality assurance, which is resource directed, entails *inter alia*, the establishment of guidelines for water quality for the natural environment. The setting of these guidelines involves both the reviewing of established guidelines from around the world (Kempster *et al* 1980), and experimental investigation of those suitable for indigenous riverine organisms (Palmer *et al* 1996). Both the Artificial Stream Project and the Standard Laboratory Organisms Project (WRC Projects K4/475 and K5/545) were initiated to satisfy this need. In the Artificial Stream Laboratory at Rhodes University, which is now operational, toxicological work is undertaken which requires a steady supply of indigenous macro invertebrates as test subjects. This has emphasised that if this facility in which a large investment has been made, is to function adequately, the potential bottleneck of an inadequate supply of test animals should be avoided. Therefore, a building dedicated to the breeding and rearing of the selected invertebrate species is essential. This facility will ensure an uninterrupted supply of test organisms of known quality and origin for ecotoxicology research.

Until the building is a reality the test animals must be collected from local rivers. Little information is available concerning the life history, biology and ecological role of southern African aquatic invertebrates and the previous project has contributed towards augmenting this knowledge. This has enabled researchers to make informed decisions about collecting times and the potential availability of animals of the best size and stage of development. In addition, the impact of proposed environmental change cannot be accurately predicted unless information of this type is available. The understanding of the physiology and functioning of test animals under natural conditions is needed if results of functional impairment due to contaminants and toxins are to be accurately assessed.

During the standard laboratory organisms project, invertebrates ranging from mayflies to crabs and flatworms were screened and several species were selected as candidates suitable for laboratory cultivation. Two species, *Burnupia stenochorias*, a limpet, and *Adenophlebia auriculata*, a mayfly, were chosen for in-depth investigation (Haigh and Davies-Coleman 1997).

Research on the limpet had largely been confined to replicated experiments investigating the effect of

variables such as temperature, density, hydraulic conditions and various handling techniques on growth and survival. The embryology and fecundity had been investigated and we had a fairly good understanding of the laboratory requirements of the limpet. Six months of field investigations to establish the breeding biology, had been conducted at the outset of this project we believed that to understand the environmental cues which drive the reproductive biology.

A field study, to attempt to understand the population dynamics of the mayfly, had been conducted which had resulted in a good understanding of the ecology of this species. However, a further six months of data collection was needed to complete two seasonal cycles. A number of laboratory experiments had been completed to ascertain responses to hydraulic as well as food and temperature variables. Initial experiments on the methods for artificial fertilisation of eggs were done as we were unable to induce the mating of the mayflies in the laboratory. Cues for this behaviour are poorly understood at present. The artificial fertilization work required further detailed experimental investigation to optimise the technique and establish methods for large scale rearing of hatchlings.

Optimal feed provision for a laboratory population is always a major part of the development of an aquaculture project. Literature surveys have revealed periphyton to be a suitable feed. The cultivation of periphyton had been on a trial and error basis but optimal growth conditions, particularly water quality, needed to be investigated experimentally. This information was felt to be a prerequisite in the designing of a production laboratory.

Furthermore, the long term effect of contaminants on the physiology of the test species can only be critically evaluated if information is available on baseline responses under normal and optimal laboratory conditions. Information gained from the biological investigations to determine optimal maintenance and rearing conditions, therefore, have a wider application. The expertise being developed in this and during the earlier project can be applied to other areas of environmental management such as the rehabilitation of damaged rivers and streams.

1.2 AIMS OF THE PROPOSED PROJECT

1.2.1 To conduct experimental and field investigations on the selected test species which will support the information already available:

Burnupia stenochorias. Field and associated laboratory studies on the reproduction. Continuing laboratory studies on growth and feeding.

Adenophlebia auriculata. Laboratory studies on artificial fertilization, and the rearing of hatchlings.

1.2.2 To test the experimental techniques so developed at a larger production scale.

1.2.3 To investigate and design features and costing for a laboratory dedicated to the large-scale production of the selected species. The suitability of various biological filtration systems to ensure optimal water quality conditions and light conditions for the culture of both the test species and selected diatom species (periphyton) as feed for the test species, will be investigated.

1.3 TARGETS

Gantt chart

TASKS	1996				1997			
	1	2	3	4	1	2	3	4
Burnupia. Field work	•	•						
Experimental rearing in streams	•	•	•		•	•		
Experimental rearing in pots			•	•	•	•	•	
Establishment of breeding cues			•	•	•			
Sectioning			•	•	•			
Experimental anaesthesia.						•	•	
Adenophlebia. Field work	•	•						
Artificial fertilisation		•	•	•				
Establishment of breeding cues		•	•	•				
Rearing experiments			•	•	•	•		
DIATOM Experiments and identification		•	•	•				
Supervision of student projects		•	•					
Collecting for ecotox	•	•	•	•	•	•	•	•
Analyses of results			•	•	•	•	•	
Reports	•	•					•	•
Publications			•	•		•	•	•
BUILDING Literature search and information gathering	•		•	•	•	•		
Drafting plans	•		•	•				

1.4 BACKGROUND TO PROJECT: Summary of previous project conclusions.

Burnupia stenochorias.

Although a substantial body of information had already been obtained by the end of 1995 certain areas of investigation was incomplete and we realised that another years of experiential work would yield information which would potentiate the work done already. The field investigation into the reproductive biology of the limpet which had been going on for six months needed a full cycle of seasons for interpretable results. A second large group of adult was discovered in a pristine river and afforded the

opportunity for a comparative rearing and feeding experiment as well as a subsequent experiment using the laboratory reared adults to verify the fecundity questionable values obtained in 1994. Field collected individuals of known age and size still had to be sectioned to determine the onset of sexual maturity under natural conditions. This would augment information on reproduction gained from laboratory work. The analysis and interpretation of the results was completed in August and papers are being prepared for submission to journals.

Adenophlebia auriculata.

The field investigation of this species in the Palmier River needed six more months of data collection for the observations to have been carried on for a full two year cycle. The artificial fertilisation had only been accomplished once and additional work to standardise the method and on the rearing of the hatchlings would hopefully yield a useful result.

Development of the rearing facility

Some of the major constraints in optimising the culture of the invertebrate have been due to the lack of a suitable facility with good quality water on tap and the correct light quality to grow diatoms and algae for food. The design of a dedicated facility should consider the following aspects:

a) type of structure in terms of suitability and cost effectiveness
b) cost effective ways of thermal control;
c) light requirements for good periphyton cultivation and the ability to vary day-length, should this prove to be an important cue in controlling the reproductive behaviour in the limpets;
d) water quality control both in adjusting municipal water to the needs of the various species as well as in the control of pathogens and metabolic byproducts.

A fund-raiser will have to be engaged to obtain funding for the building project as the project staff do not have time do engage in this type of activity.

Finally the large volume of data generated during the course of the previous project warrant publication and time made available to the researchers would enable then to achieve this.

CHAPTER 2.

DEVELOPMENT OF BUILDING PLANS

2.1 THE PURPOSE AND REQUIREMENTS OF THE BUILDING.

The building for which funds are being raised will have a twofold purpose, that of rearing riverine invertebrates as test animals, and as an experimental test laboratory, with three operational areas as follows:

1. **A breeding and rearing** area, divided into an area with recirculating streams (3M x 0.4M) and tanks (5-25l) in which algae will be cultivated and the animals will be reared and an adjacent small cool room with smaller streams (1.5 x 0.25M).
2. **Two offices and a storeroom** in the central part of the building with computer facilities, filing systems and kitchen / ablution facilities.
3. **A toxicology laboratory** which is air-conditioned of a similar size to the rearing room, with shelving and work benches.

Description of the building

Breeding and rearing rooms

The largest area should be devoted to breeding and rearing invertebrates, housing both recirculating channels, and plastic buckets and baths with water aerated from a central compressor. The hatchlings will be reared in these containers which will be placed on benches under the window. Once the limpet juveniles reach a size of 2-3mm, they will be transferred to independent recirculating streams (3m x 0.4m), made from 400mm PVC industrial pipe which is halved both in length and width. The sumps of these channels will contain a bio-filter of plastic strands, and a submersible aquarium pump recirculating the water. One of these channels will be reserved exclusively for the cultivation of diatoms on plastic sheeting and tiles, as a food supply.

Adjacent to the breeding and rearing room will be a smaller cool room in which the animals which have reached an optimum size for experimental research will be stored in smaller streams (1.5m x 0.25m). These organisms are maintained at an optimal size by lowering the room temperature, which will have the effect of retarding their growth rate.

Office / laboratories

The building must have two office / laboratories, one adjacent to the breeding / rearing area, and the other adjoined to the toxicology laboratory. Both offices will house analytical equipment and computing

facilities, which require sufficient bench and cupboard space.

The office of the breeding / rearing section must be well insulated to exclude the high moisture air from the breeding area, and will house a microscope, fridge and telephone. The office of the ecotoxicology section must have a lockable cupboard for the storage of chemicals, a window in the north wall, and shelves against the walls for storing equipment.

Store room / sluice room

A store room / sluice room is needed in which to clean, sterilize and store equipment. A small geyser or solar heater is therefore needed to supply hot water. Chemicals will not be used for cleaning, as this could introduce contamination into the breeding area. A lavatory and hand basin is also needed.

Ecotoxicology laboratory

The toxicology laboratory must be completely separate from the breeding section to prevent contamination. The room must be air-conditioned to maintain a temperature of 20°C during toxicity experiments, and must have biolux lighting operating on a timer. Requirements include

(i) a double door in the east wall,

(ii) a deep double stainless steel washbasin with draining rack against the east wall next to the door,

(iii) benches around both the northern and southern walls with sufficient plug points,

(iv) shelves for storing equipment above the benches, and against the wall adjacent to the office / laboratory area, and

(v) a window with reflective roller-blinds in the north wall. A 4 000 litre rain tank will be housed on a concrete base outside the double door for waste storage.

Specifications for the laboratories

The following details give specific requirements deemed necessary in a successful invertebrate breeding facility.

The thermal stability of the building will be important and attempts will be made to achieve this without having to resort to the extensive use of expensive electrical cooling. Firstly, the cavity walls and roof space will insulated. Secondly, fast-growing deciduous trees will be planted at strategic positions to provide shade and additional cooling in summer. The proposed building should be sited adjacent to the new hatchery and the present Artificial Stream Laboratory at the Department of Ichthyology and

Fisheries Science, Rhodes University. The overall size is 25M x 6.5M with a pitched roof.

Light provision

The basic food for most invertebrates is a complex of unicellular diatoms, algae and bacteria which grow on substrates in streams. The correct light for growing algae is close to natural light. In addition to large, shuttered window spaces in the rearing room, some artificial light from biolux tubes which provides the correct wave lengths for algal growth will be needed. Lighting control should be automated.

Water supply and removal / recirculation of waste water

Good quality 'naturalised' water from a 5000 litre sunken water reservoir should be delivered to the recirculating units from a header tank in the roof by gravity feed. Bio-filtration should take place at the channels, but an ultraviolet sterilizer should be installed at the outlet of the header tank.. The water pipe into the building must be mounted on the surface of the wall on brackets, with taps at 1.75 metre intervals along both walls of the rearing room. The floor should be of high density sealed cement, sloping to central drainage channel down the centre of the entire building to carry any spillages out of the building. This channel will void either into the reservoir via a sand filtration sump from the breeding laboratory or will return waste water to a tank outside the toxicology laboratory. Adequate provision must be made for waste water disposal through exit channels into the municipal drainage. Polluted waste water from the ecotoxicology laboratory will drain directly from each channel into the outside storage tank from which it can then be diluted and drained in to the municipal drainage system or disposed of by a contractor.

Electrical supply

The electric wiring of the entire building should be chased. All plugs must be waterproofed and above the level of the water pipes. As many of the systems in both the rearing and ecotoxicology laboratories will be recirculating and run off aquarium pumps, plug points will be needed at 1.75 metre intervals. Provision should be made for a stand-by power source and / or an alarm to alert staff of power cuts.

Temperature control

The building must have efficient roof insulation and roof extractors (whirligigs), as well as insulated cavity walls. The breeding area will be divided into two sections, one at ambient with the option of warming the water in the header tank, and the other (cool room for animal maintenance) at between 15 and 18 °C. It may be possible to reduce the temperature below 15 °C by installing a large air conditioner and cooling coils in a separate header tank used specifically by the cool room. Various options will be

investigated. The toxicology laboratory must be cooled to a standard 20°C.

Ducted air

The breeding laboratory requires ducted air for the aeration of water. This can be supplied from a compressor already on the premises. Air pipes should be exposed on the walls.

Prevention of contamination

Contamination into the breeding area will be prevented by the following:

(i) Each recirculating system must be a separate unit to prevent contamination from spreading through the laboratory.

(ii) there should be no direct access between the toxicology laboratory and the breeding area to prevent potential contamination.

(iii) an ultra-violet sterilising unit in the water supply to ensure removal of pathogens, and (iv) hot water available in the sluice room for washing and sterilizing equipment.

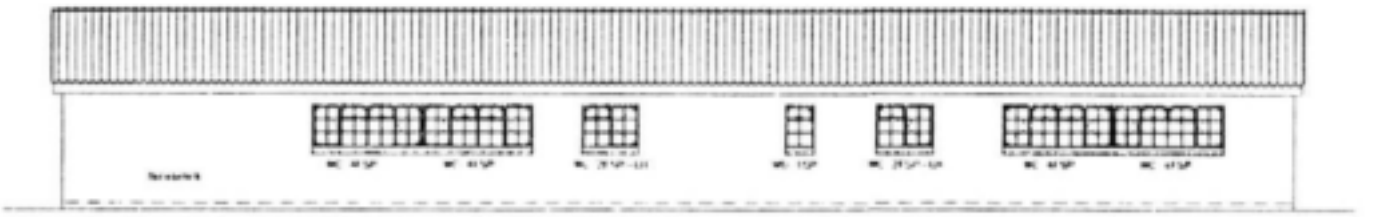
2.2 TABLE OF ESTIMATED COSTS:

Building	- 163m² @ R1 500/m²	R 262 500.00
Equipping the building	- with coolroom	R 104 582.00
Equipping the building	- with air-conditioners	R 70 582.00

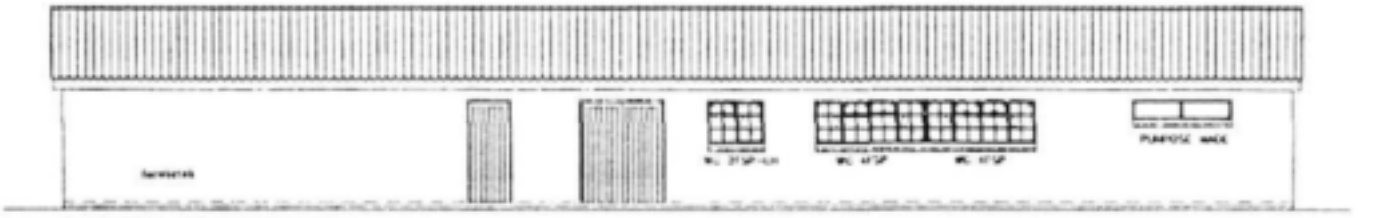
Equipment	Unit cost	Cost
Water supply		
Filtration sump to handle ± 200 litres / day		2000.00
25mm piping for inflow 55m	R3.00 x 55	165.00
25mm elbows (10)	R4.00 x 10	40.00
25mm T junctions (10)	R15.00 x 10	150.00
25mm Taps (20)	R90.00 x 20	1 800.00
Overhead tank + 1500 litres (40cm channels) + 500 litres (25cm channels) + 250 litres (baths)		
Total capacity = 2250 litres	(10% = 225 litres) (D200 of D250)	350.00
Water tank (Thinvac 5000 litres)		2 000.00
Submersible pump lift ± 3m		600.00
	Sub total	7105.00
Channels		
8 Channels (capacity 150 litres) 3 m in length - PVC Industrial piping	2 x 6 x 0.4m @ R260.00/m +VAT	3 557.00
6 Sumps (capacity 150 litres)		
10 pumps	@ R300.00	3 000.00
30 x 25mm hosepipe		
10 plastic baths (25 litres)		
20 x 5 litre plastic buckets		
	Sub total	9677.00

Sub totals carried forward		16 782.00
	Unit cost equipment	**COST**
	Sub totals brought forward	16 782.00
Office equipment		
Lockable cupboard		600
2 tables on rollers	@ R700.00	1 400.00
4 chairs	(R350.00 or R450.00)	1 600.00
2 filing cabinets	@ R600.00	1 200.00
2 Computers		11 000.00
Printer		2 000.00
Fridge		2 000.00
Basic stereoscopic microscope		2 000.00
Merck Spectroquant		20 000.00
	Sub total	41 800.00
Cool room		
Installation of cool room inside existing building plus condenser and compressor (5000 x 3000 x 24000)		40 000.00
Air-conditioned (for accedes lab) - 1 unit		±6 000.00
	Sub total	46 000.00
	TOTAL	104 582.00

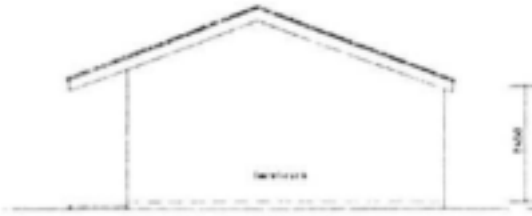
TOTAL COST FOR BUILDING (1997) R333 082.00 - 370 082.00



SOUTH ELEVATION



NORTH ELEVATION



WEST ELEVATION

SECTION XX

EAST ELEVATION

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drawing title
INSTITUTE FOR WATER RESEARCH - PROPOSED LABORATORY

Figure 1.1 (a) Plan for the proposed invertebrate rearing and toxicology laboratory for the Institute for Water Research, Rhodes University. External views and elevations.

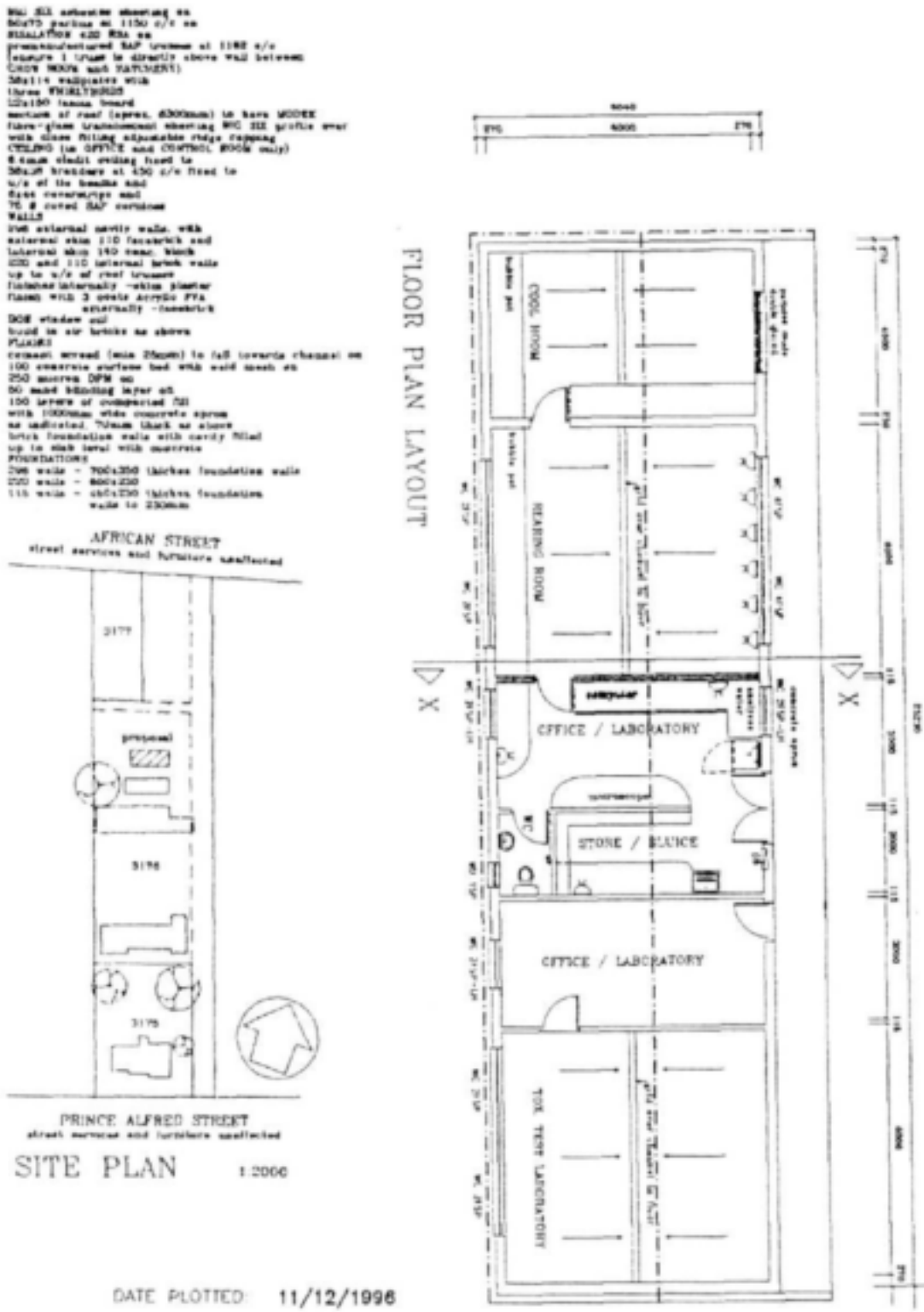


Figure 1.1 (b) Plan for the proposed invertebrate rearing and toxicology laboratory for the Institute for Water Research, Rhodes University. Floor plans.

CHAPTER 3.

THE INVESTIGATION OF THE FRESHWATER LIMPET *BURNUPIA STENOCHORIAS* MELVIL & PONSONBY

3.1 INTRODUCTION

Manipulation of *Burnupia* populations in the laboratory could be considered one of the fundamental issues to be addressed in the effective laboratory culture. Branch (1974) suggests that the productivity of any species and its population structure can only be determined with a knowledge of growth rates, age structure, longevity and mortality rates. Chant (1963, p vii) too, says "The ecology of (pest) populations should be studied to gain understanding of the dynamics of the populations in the hope that its mechanisms may be revealed. We hope this will enable us to manipulate the populations and ability to manipulate surely is the aim of all attempts to control animal populations". Both the reference to the ecology, and the productivity in nature of any species applies to maintaining cultures in laboratory conditions.

There are many ways in which growth estimates have been made either on marine or freshwater limpets. These include periodic measurements of labelled individuals; checks in growth produced by known changes of environmental conditions (Vahl, 1971); animal growth rings where microgrowth band analysis yields data on both growth and age of the limpet (Picken, 1980), and mean shell lengths of cohorts (Ekaratne and Crisp, 1982; Crisp *et al*, 1990). Aquaculturists typically use one of three measures when reporting growth: absolute growth rate, relative growth rate, and specific growth rate. Less frequently, von Bertalanffy Growth Functions are used. Each of these is a numerical representation of growth which can be used for various purposes including: i) statistical evaluation of the effects of various treatments on growth; ii) presentation of growth data in a standard format which allows experimenters to compare growth in different experiments; and iii) providing the basis for management decisions (e.g. estimating how long it will take for a limpet to reach maturity) (Hopkins, 1992).

No growth studies have been made of any of the Ancylidae in southern Africa. Growth has, however, been studied extensively for three species in northern hemisphere temperate regions: *A. fluviatilis* from Great Britain (Geldiay, 1956; Hunter, 1953, 1961); *Ferrissia rivularis* (Burky, 1971; Nickerson, 1972) and *Laevapex fuscus* (McMahon, 1975) both from the USA. Similar in-depth studies have been made on each of three populations of the more tropical Texan species *Hebetancylus excentricus* and *Laevapex fuscus* (Burky, 1971).

Freshwater snails present great intraspecific variation in life history patterns (Calow, 1978; Brown, 1985), and this plasticity has been considered to be of fundamental selective value in their evolution (Russel-

1985), and this plasticity has been considered to be of fundamental selective value in their evolution (Russel-Hunter, 1961). Despite the difficulty in identifying *Burnupia* to species level (Brown, 1994), and the fact that additional species have been known to occur sympatrically within the freshwater Mollusca (e.g. *Elimia* species as described by Huryn *et al*, 1994), it was presumed in these studies that all individuals were the same species.

Any sampling method will cause a disturbance of the system, which in itself will affect the measurements that are being taken. The growth of *Burnupia* has already been shown to be negatively affected by disturbance (Haigh and Davies-Coleman, 1995), and, as a rule, the more rigorous the measurements the greater the disturbance and the less likely the results are to apply to a natural system (O'Keeffe, 1982). The methods to determine growth in this project were aimed at providing a spectrum of data, from field (in which there was no control of the environment and measurements were of natural populations), to enclosed field studies (where some control was provided but under almost natural conditions), to laboratory studies (in which the inputs were rigorously controlled and actual growth rates of the same small populations of limpets were obtained).

3.2 NATURAL POPULATIONS

3.2.1 FIELD STUDIES

Introduction

Population studies allow investigation of those factors which influence the development, growth and mortality of the subject. Life-cycle data are needed for the efficient culture of the limpet in the laboratory. Questions about the longevity, seasonal growth rate, mortality rates under natural conditions require answers and comparisons with result from other species by authors such as Calow, (1978) should be made to determine the effect of geographic area and climate. Natural responses must be compared to laboratory reponses in order to determine the efficacy of culture methods.

The study of growth essentially means the determination of the body size as a function of age (Sparre *et al*, 1993). The growth of a limpet is continuous until death, although varying between populations according to non-genetic ecophenotypic environmental influences, and in particular varying from habitat productivity (Russel-Hunter, 1961; Byrne *et al*, 1989; Caquet, 1993). Shell length (the greatest dimension along the anterior-posterior axis) is considered an adequate means of representing growth and age in any limpet, and in particular *Burnupia* (representing the Ancylidae), because it possesses a shell which undergoes no metamorphosis or change in differential gradient in growth throughout it's life cycle, unlike e.g. Patellidae where the larval shell grows as a coil and the adult as a cone (Russel-Hunter, 1953). Neither is the life cycle of *Burnupia* represented by different phases or larval forms of distinctly different morphology, so that any study of its growth from hatching to death can, from a morphological

perspective, be easily accomplished.

Direct ageing techniques are not currently available for either *Burnupia*, or any other Gastropod (Vermeij, 1980). Various methods of length frequency distribution analysis (LFDA) have been developed to determine growth parameters for animals whose age cannot easily be determined, and although these were initially developed for fish, Longhurst and Pauly (1987) showed that many of the techniques can be applied to invertebrates without modification. It is very easy to collect large, unbiased samples of length frequency distributions, leading to the conclusion that a field survey would be the most feasible means of determining growth over a year, and lifespan and mortality rates of any one cohort. 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. The cohort is said to demonstrate two major elements in its dynamics, primarily, the average growth in body length and weight, but also the death process (Sparre *et al*, 1993). Figure 3.2.1.1. shows the theoretical relationship between changes in body length and numbers of individuals (Calow 1978). All of the individuals in one cohort are presumed to be the same age at any given time so that they all attain the "recruitment age" at the same time. Recruitment can be considered the age at which the limpets are seen and counted in any survey, theoretically demonstrated by "A1" in Figure 3.2.1.1.

Length frequencies were obtained regularly over a 52 week period from two natural populations in a stream close to, and having its headwaters in, Grahamstown, Eastern Cape. Using various mathematical means (mainly based on the ELEFAN programme [Pauly and David, 1981]) a number of basic life history facts were elucidated, namely: that major recruitment in the form of egg laying occurs in the spring period but that eggs are laid all year round; an average cohort can live from 18 to 24 months, if the limpets reach the maximum size; growth rates vary at different times of the year, primarily with winter and summer phases; and growth performance indices were calculated, which can be compared to those for laboratory cultures.

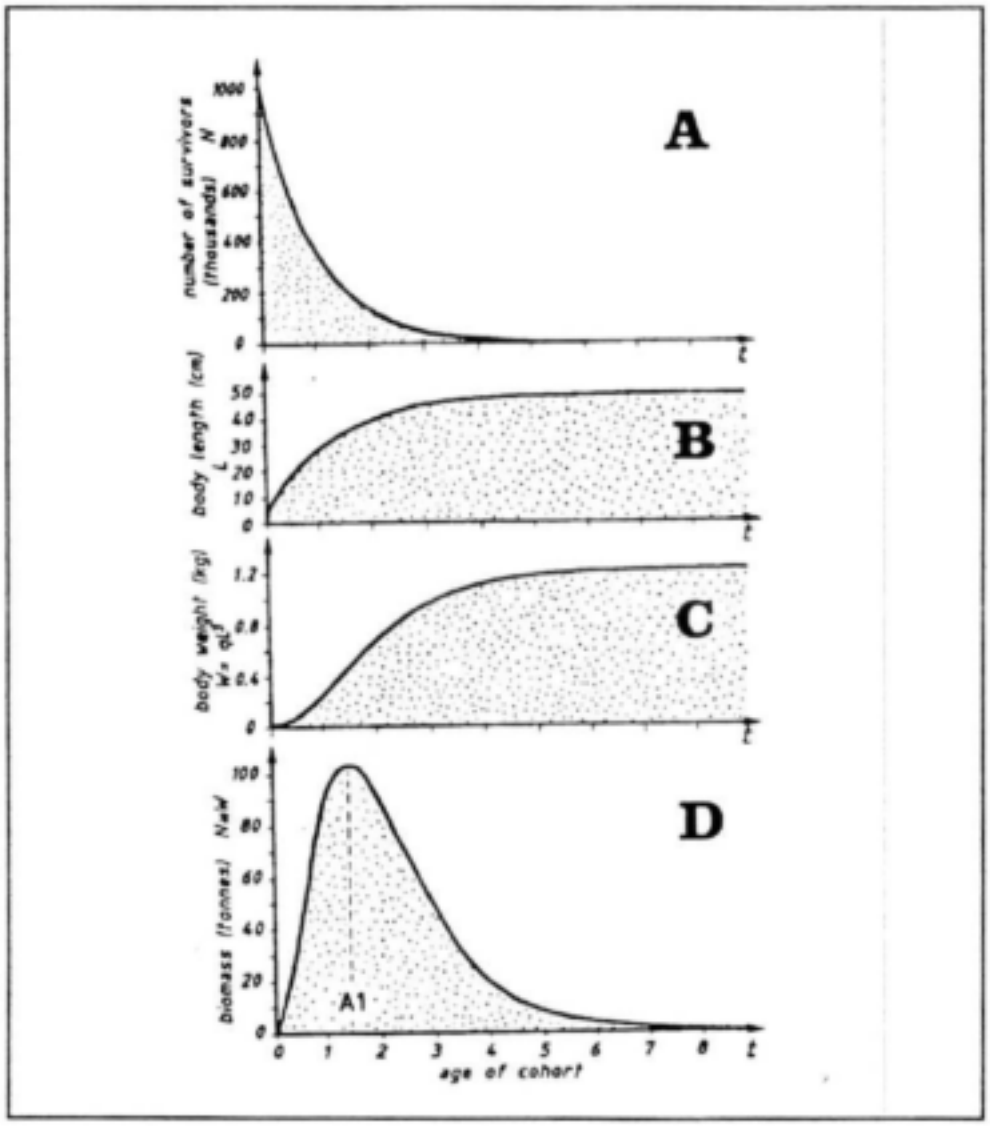


Figure 3.2.1.1 The dynamics of a cohort. (Reproduced with permission from Calow 1978)

Method

1. *Site*

A preliminary investigation was carried out at two sites along the Bloukrans River in January 1995 to investigate the possibility of measuring populations of limpets from week to week at the same site, without disturbance. Site (1) is called Belmont River (BR) and site (2), Manley Flats (MF). Daily measurements were taken of all limpets on various sized rocks to determine the daily recruitment or loss (are these animals sessile, as suspected?), the sizes and distribution of rocks on which the limpets were found; and the approximate number per rock.

2. *Sampling*

(a) Very little, if any, movement onto or from the rocks was found, and consequently the method of sampling was chosen as follows. The initial selection of rocks was made based on their having large numbers of limpets to measure, the first rock being randomly chosen within the stream with others a fixed distance from it; thereafter the same rocks were used where possible. This is the method recommended by Gardefors and Orrhage (1968) for benthic faunas, and was maintained throughout. The time of day when sampling occurred was always the same. Each rock was removed from the river and all limpets measured without disturbing them in any way before being replaced into the river, continuing with rocks until a minimum of 300 limpets was reached (this number included any egg capsules present). The longest perimeter of each rock was measured in order to calculate the surface area of the rock.

(b) Shell lengths were measured fortnightly at from February 1995 to March 1996. Class intervals were at 0,5mm intervals using a home-made template.

3. *Environmental data*

Total Dissolved Salts (TDS), and pH were measured at the time of 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 due to time constraints, despite the knowledge that the availability of calcium, for example, can be a major component affecting the growth of freshwater molluscs (McMahon, 1983). Day length data was obtained from the Port Elizabeth Meteorological Department, and air temperatures from the Grahamstown Weather Station maintained by the Geography Department, Rhodes University.

4. *Analysis*

The data analyses followed a sequence as follows:

(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*, 1989). However, although polymodal curves are generally not considered difficult to analyse, it was found with this data set that the component populations had considerable overlap, and a more refined method of analysis was necessary.

(c) *FiSAT package.*

Using the package FiSAT, an FAO stock assessment tool by Gayanilo, Sparre and Pauly (1996), the duration of life cycle, estimation of growth parameters both with and without seasonal oscillations, and age were all determined. All methods of analysis of growth were based on the mathematical model developed by von Bertalanffy (1934) called the Von Bertalanffy Growth Equation (VBGE), represented by the equation:

$$L_t = L_\infty[1 - \exp(-\kappa(t-t_0)]$$

where L_t is the length at time t (mm), L_∞ is the asymptotic length (mean length of oldest individuals) in mm, κ 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 0mm.

(i) *Modal Class Progression Analysis*

The first main step taken within the FISAT package was to attempt to translate the LFD into age scales, using Modal Class Progression Analysis (MCPA). FiSAT provides two means by which this can be done, the Bhattacharya's method (BHATTA) and the NORMSEP method. The results produced by the BHATTA and NORMSEP methods were statistically unacceptable, according to the chi squared test, for each sample date for both the MF and the BR length-frequency data, and thus further analysis was necessary.

(ii) *ELEFAN I*

Growth parameters

The second major step taken within the FiSAT package was to use the ELEFAN I programme (*E*lectronic *L*ength *F*requency *A*nalysis) to estimate the growth parameters from length-at-age-data. ELEFAN I is an alternative to the above-mentioned MCPA methods and is a BASIC programme (Pauly and David, 1981; Pauly, 1988) which incorporates the "tracing" of growth curves through length-frequency samples sequentially arranged in time. Steps within the programme are as follows:

1) It initially restructures the length-frequency samples such that small, clearly identifiable peaks are attributed a number of points.
2) It calculates the maximum sum of points "available" in a set of length-frequency samples ("available" refers to points which can possibly be accumulated by one single growth curve). This sum is termed "Available Sum of Peaks" (ASP).

3) It traces through the set of length-frequency samples sequentially arranged in time, for any arbitrary seed input of L_∞ and κ, a series of growth curves started from the base of each of the peaks, and projected forwards and backwards in time to meet all other samples of the same set.

4) It accumulates the points obtained by each growth curve when passing through peaks (positive points) or through the troughs separating peaks (negative points).

5) It selects the curve which, by passing through most peaks and avoiding most troughs, best explains the peaks in the set of samples and therefore accumulates the largest number of points. This new sum is called the "Explained Sum of Peaks" (ESP).

6) It decrements or increments the seeded values of L_∞ and κ until the ratio ESP/ASP (called the Rn value) reaches a maximum, and gives the growth parameters corresponding to this optimum ratio. This Rn value is used to determine the best fit of the LFD, and replaces the SI value used in the BHATTA and NORMSEP methods (Pauly and David, 1981).

The opportunity is available within the ELEFAN programme to use the Seasonalised VBGE, which is the usual VBGE but with an extra term (Pitcher and MacDonald, 1973; Cloern and Nichols, 1978; Pauly and Gaschutz, 1979):

$$L_t = L_\infty (1 - \exp(-\kappa(t - t_0)-(C\kappa/2\Pi) \sin(2\Pi(t-t_s))))$$

This extra term produces seasonal oscillations of the growth rate by changing t_0 during the year. 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", the growth rate is lowest. The parameter C, the "amplitude", also usually takes values between 0 and 1. If $C = 0$ (which implies no seasonality of the growth rate) the seasonalised VBGE is reduced to the ordinary VBGE. The higher the value of C the more pronounced the seasonal oscillations. If $C = 1$, the growth rate becomes zero at the winter point. The procedure involved in determining seasonality characteristics results in not just two parameters (L_∞ and κ) but 4 parameters (L_∞, κ, WP and C). These were obtained for both the MF and the BR sites.

In order to compare the growth rates between the winter and the non-winter phases of the two sites, the individual growth parameters cannot be compared, but instead the resulting growth curves were compared. Based on the VBGE, where $t_0 = 0$, and t (time) was given a daily value, the growth curves were drawn, giving estimated daily lengths.

Phi Prime Test

A second test to compare sites is the so-called "phi prime test" (Munro and Pauly, 1983; Pauly and Munro, 1984), a statistic which relies on the inverse correlation and relationship between L_∞ and κ, and which is known as Pauly's growth performance index (Φ'):

Φ'(phi prime) = ln κ + 2 L_∞

Maximum length

Lastly, based on Wetherall (1986), 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.

Results

Site details

It was found that the BR site has a continual annual flow of water, fed primarily by the run-off from Grahamstown City and its immediately surrounding areas. The treated sewage waters of the city which flow into the river particularly ensure the continual flow during times of no rainfall. The Bloukrans River flows through a series of weirs and dams, and is bound by natural vegetation until it reaches the MF site approximately 7kms downstream.

No limpets were found on the sandy parts of the river bed, and only a very few on the vegetation growing at the edge of the river. The vast majority were distributed in a contiguous manner on the submerged stones and rocks. Calow (1974) organised his stones into classes according to their length, and on the basis of this, the stones on which limpets were found at both sites fell into all classes (namely very small of length 0-5cm, to very large of length 21-25cm). Calow (1974) found with *A. fluviatilis*, however, that the snails were absent from stones of length less than 6cm. This was not the case with *Burnupia*, personal observations of which revealed the smallest of pebbles (1,5cm in length) to be occupied by limpets. Similarly with *Burnupia*, there seemed to be no preference for any aspect of the stone. No limpets were ever found on an exposed, dry surface either on stones or vegetation. Egg capsules were found on surfaces that could be considered the underside aspects of the stones. Although not numerically analysed, the majority were on the bottom of the stones, many on the sides and a number were also present on the underside of those surfaces that either formed part of the stone that jutted out in a horizontal plane (from 3mm or more), or that were part of indentations in the stone.

At the MF site, approximately four rocks consistently had sufficient numbers of limpets for analysis throughout the year, and these rocks varied little from each other in size compared to those sampled at BR (Table 3.2.1.1). Surface area (S.A.) of the rocks can be approximated according to the equation set out by Calow (1972), where

$$S.A. = 2.22(\pm 0.26)x, \quad \text{and} \quad x = \text{longest perimeter.}$$

In this study it was decided that sampling need not provide absolute densities. Although these would be of some interest, they would be difficult to compare considering the heterogeneity in the river, particularly with regard to the size of the rocks and the consequent variability in limpet distribution. The depths of stones and rocks varied, depending on the flow rate, as seen in Table 3.2.1.1.

Table 3.2.1.1 Dimensions (cm) of the rocks used in sampling limpets. Numbers in brackets indicate range.

Site	Average perimeter	Av. surface area	Av. no. used at sampling	Av. depth of stones
Belmont River	37 (17-90)	82,14	10,8 (3-27)	28 (12-40)
Manley Flats	44 (30-76)	97,65	3,7 (2-7)	16 (9-30)

Table 3.2.1.2 Recorded environmental data for MF and BR. "t" values reveal results of comparison between the two sites. * significantly different ($p < 0.001$).

	M F			Bel. R			
	Max.	Min.	Avg.	Max.	Min.	Avg.	t test
Flow rates (m/s)	0.283	0.006	0.165	0.815	0.084	0.375	-4.9502*
TDS	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 °C	22.5	9.5	15.9	23	9.5	16.4	1.2039

Environmental data

Graphs of flow rates, temperature, pH and TDS are shown in Figures 3.2.1.2 and 3.2.1.3, with maximums, minimums and averages shown in Table 3.2.1.2. One sample "t" tests on STATGRAPHICS 5.0 revealed no significant differences between MF and BR with regard to recorded temperatures, pH and TDS, but a significant difference (see Table 3.2.1.2) between flow rates.

Although temperatures were monitored at fortnightly intervals, such data is considered to have little value, mainly because of the large diel fluctuations of temperature which can occur in streams and rivers (Crisp, 1990). However, Eckel and Reuter (1950) and Fry and Watt (1957) all state that small streams warm up and reach a point of equilibrium in quite short distances from their sources, and that the average temperature is not greatly different from that of the air. In the light of this it is not surprising that a

Figure 3.2.1.2). Multiple regressions of recorded temperature versus minimum and maximum air temperatures did not correlate, and it can be concluded, therefore, that although the recorded temperatures measured here give a general indication of the seasonal temperature cycle, they cannot be used to estimate the average daily temperatures at the two sites.

When maximum/minimum thermometers are read every two weeks to estimate average daily temperature, it has been successful within 0,5°C, when compared to daily maximum and minimum temperature and thermograph readings (Macan, 1958). However, this would have to be over a period of at least 26 weeks, according to Macan (1958); collection of sufficient data from MF was not possible, so that these readings cannot be used to estimate the average daily temperatures of the two sites.

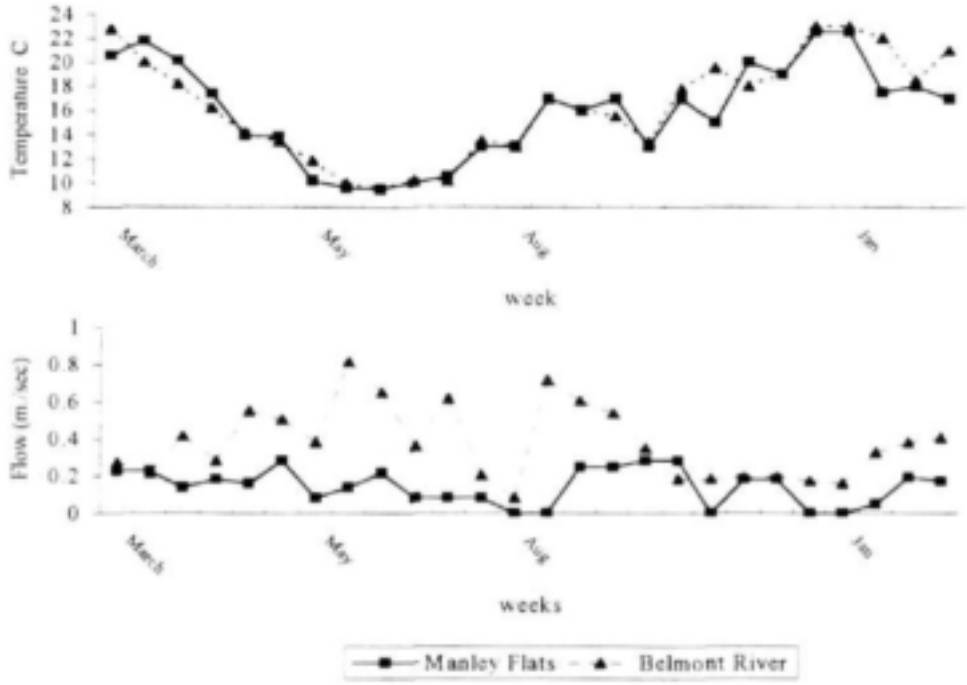


Figure 3.2.1.2. Temperature °C (top) and Flow rate m/sec (bottom) measured at the Manley Flats and Belmont River sites during from 7.3.1995 to 13.3.1996.

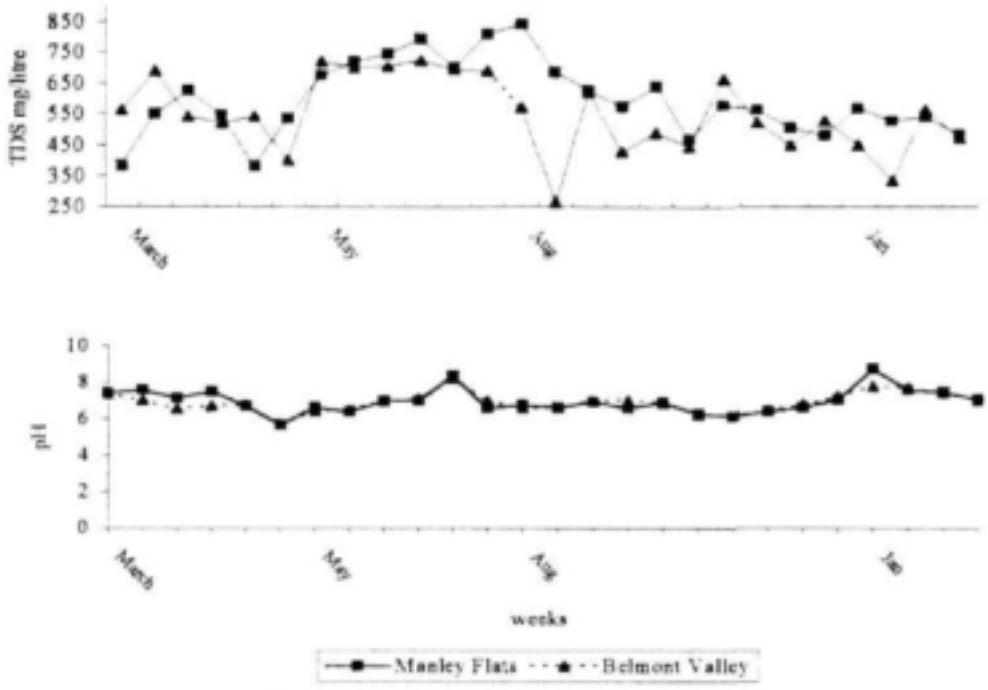


Figure 3.2.1.3 Total dissolved salts in mgm/litre (top) and pH (bottom) measured at the Manley Flats and Belmont River sites of the Bloukrans river from March 1995 to April1996.

Measurements of limpets

Figures 3.2.1.4 to 3.2.1.11 reveal the number of limpets and egg capsules found at each sample date at thetwo sites. The total number sampled at each sample date varies between 220 and 450 limpets, and the differences in these numbers necessitated, in the graphs, the use of percentages of each sample. A clear progression can be seen in the makeup of each population through the year, with a peak egg laying period beginning in August (post-winter), continuing until mid November (MF) or the end of January (BR). At this time, the number of adults larger than 2,5 mm in length decreases substantially in number while large numbers of hatched limpets form the majority of the limpets present. Although these presumably grow in length, increasing the average shell length, mortality decreases their numbers until the winter phase from approximately May, when fewer, large limpets overwinter.

Eggs capsules were laid on the sides or bottom of the rocks, or on the underside of any protruding ledge on the rougher rocks that was large enough to hold a capsule (1.5 - 2mm diameter). Eggs were laid all year round at both sites, but with an obvious substantial peak beginning in August (post winter) which was responsible for the annual recruitment (i.e. that age when the limpets can first be seen).

It was found that with the continual egg laying and consequent recruitment over a period of three to four months, the progression of modes within the histograms is unclear and insufficient to show the number,

longevity or growth of any cohorts. For the purposes of further analyses and discussions, all individuals from this peak recruitment period will be classified as a single cohort, demonstrating continual recruitment and possessing the same two major elements of a single cohort (namely, growth in body length and weight, and death). This is despite the fact that there will be variation in individual growth rates, and variation between, for example, a limpet hatched in October compared to one hatched in December, when measuring in January.

4. ***Identification of Components Using Probability Paper and the Bhattacharya and NORMSEP Methods.***

Clear identification of the component populations at any one sample date proved impossible for both the Probability Paper Method and the two methods of Modal Class Progression Anaylsis. Egg capsule data were excluded from these analyses.

Based on the appearance of the recruitment phase from approximately September, analysis of the length-frequency data using the BHATTA and NORMSEP methods was split into three groups, and analysed on this basis. They were:

1) With recruitment (September 1995 to February 1996. This has no winter phase).
2) Without recruitment (March 1995 to September 1995).
3) Twelve months' data (March 1995 to February 1996. Recruitment and a winter phase).

Results of a sample, initially analysed with BHATTA and then NORMSEP, are shown in Figure 3.2.1.12 and 3.2.1.13. The standard deviation refers to the variation around the mean of the selected groups; "Class" or "mm" refers to the midlength of each class. Different length class sizes were tested by amalgamating classes, since it often happens that the structure of the points on the BHATTA plot emerge only for an optimal length class size (Sparre *et al*, 1989). Similarly a two week interval was found to give the optimum SI and standard deviation values. However, despite the optimisation of both the length of shell, and sampling time intervals, the choices which have to be made within the BHATTA programme in plotting mean lengths of the components against time and connecting those mean lengths that appeared to be connected to the same cohort, was found to be very subjective.

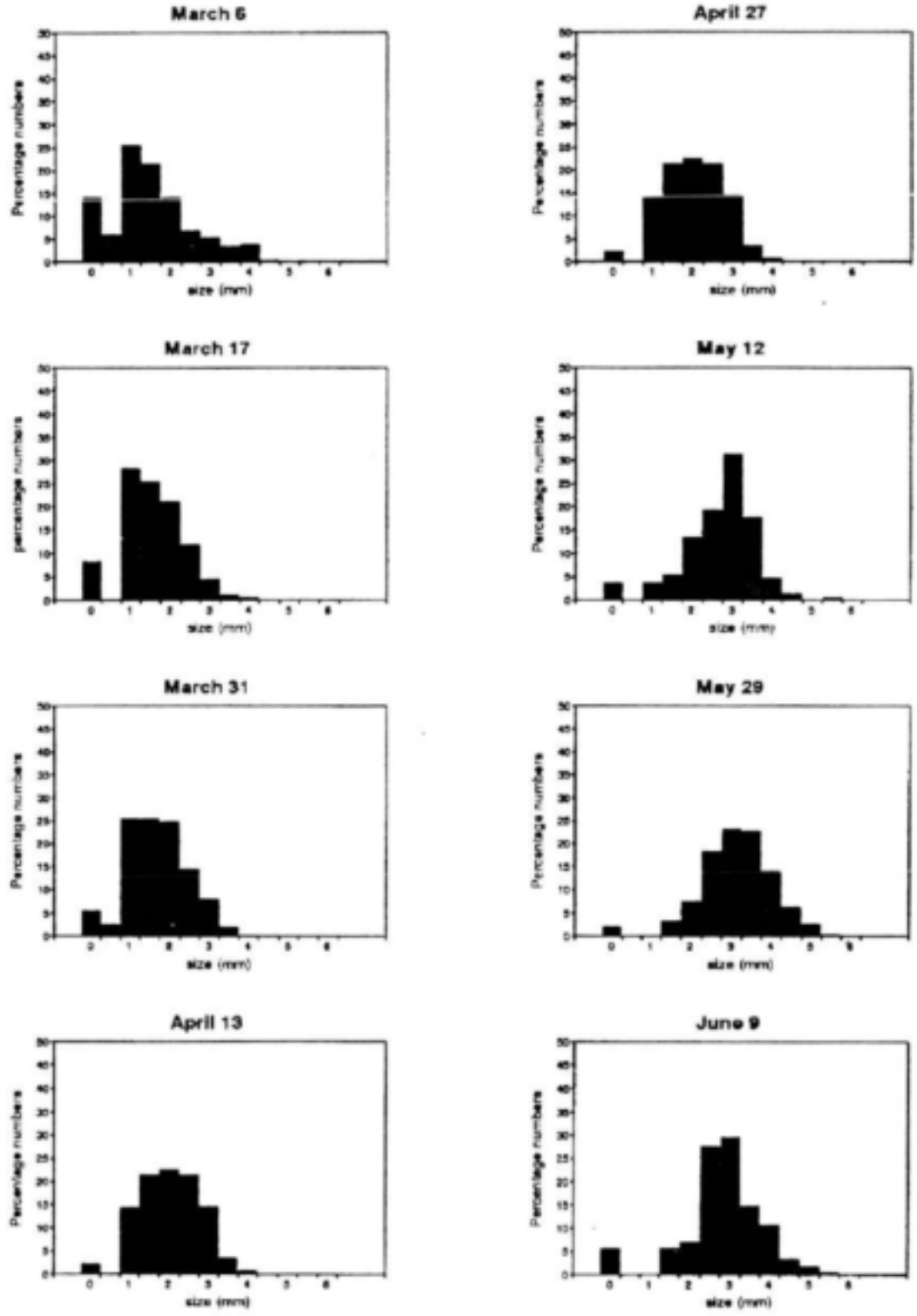


Figure 3.2.1.4

Figures 3.2.1.4 to 3.2.1.7 represent histograms of the size range of limpet population collected from the Belmont river over 52 weeks

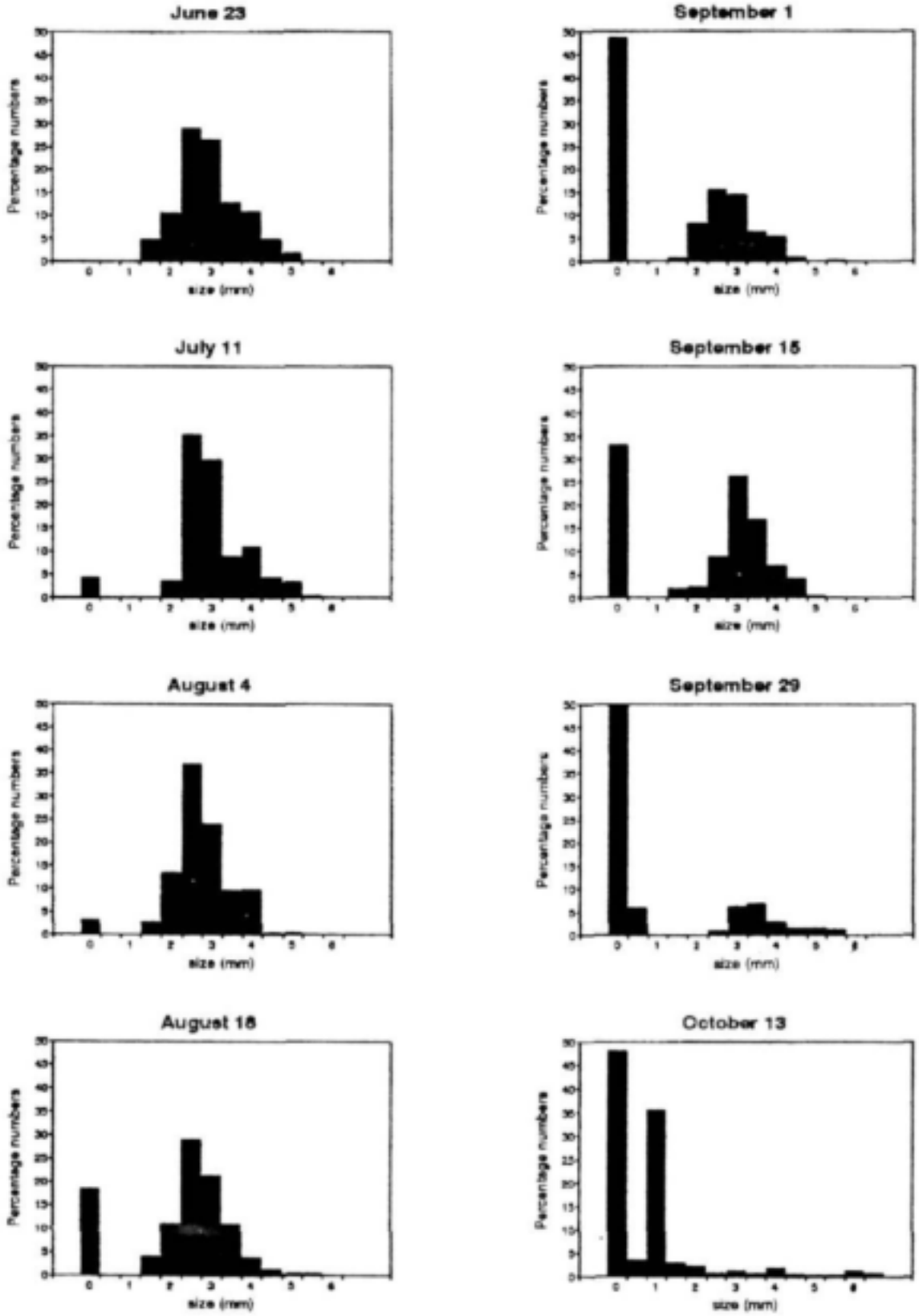


Figure 3.2.1.5 Histograms of the size range of limpet population collected from the Belmont river

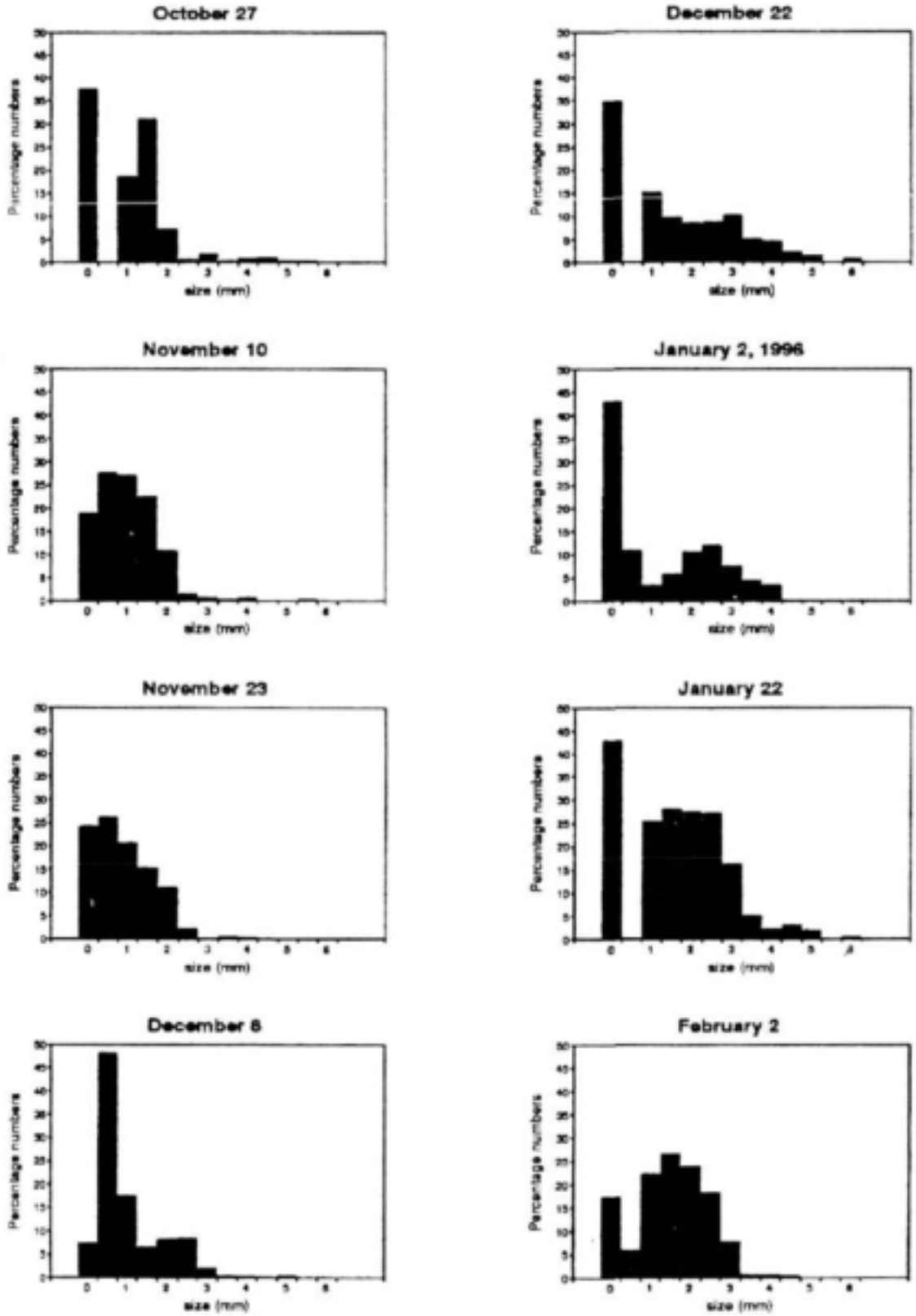


Figure 3.2.1.6 Histograms of the size range of limpet population collected from the Belmont river

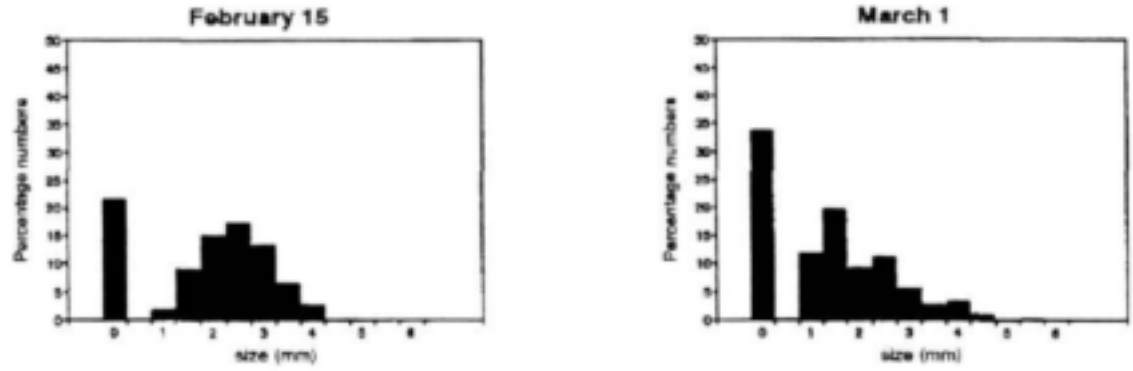


Figure 3.2.1.7 Histograms of limpets from the Belmont river

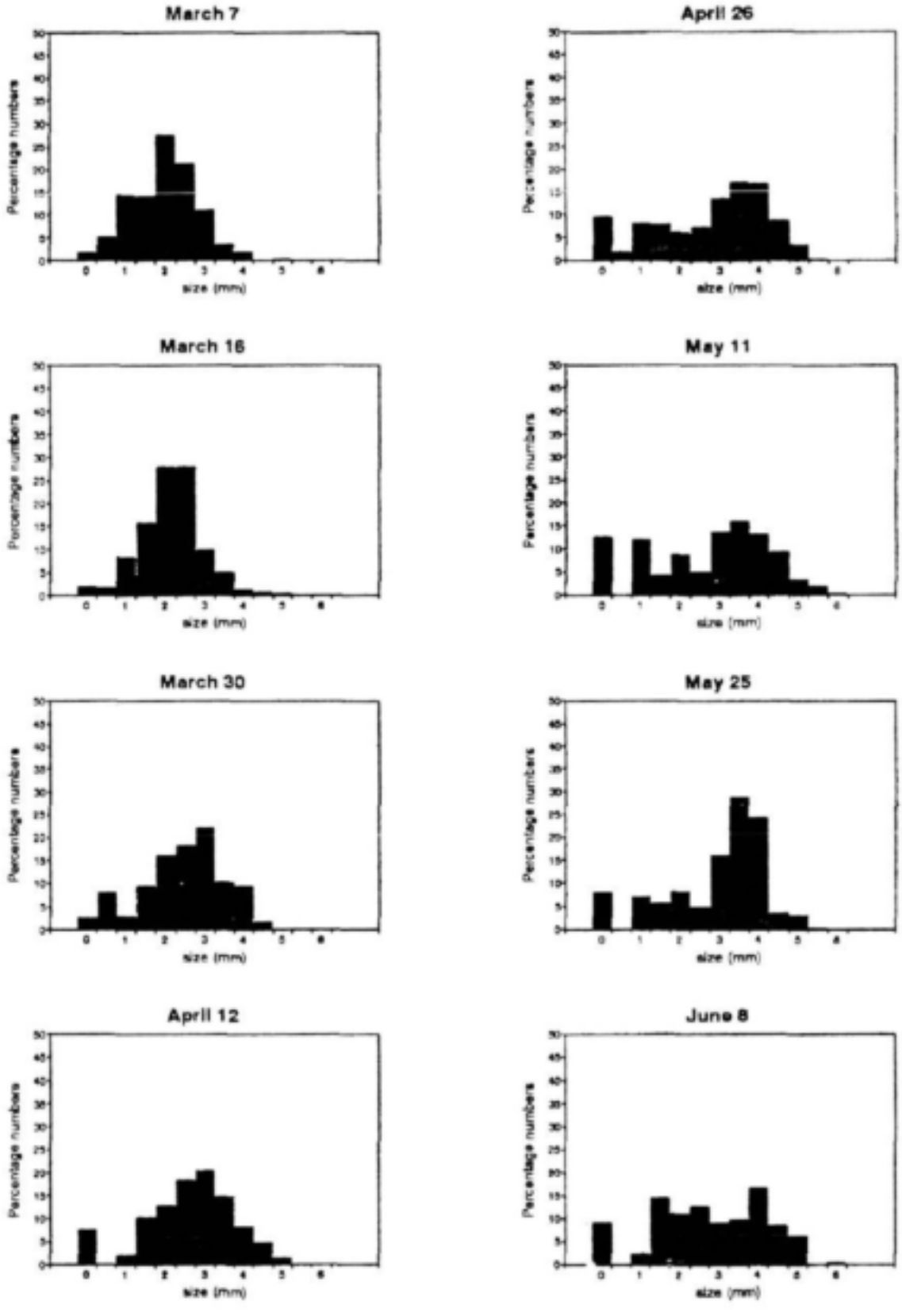


Figure 3.2.1.8

Figures 3.2.1.8 to 3.2.1.11 Histograms of the size range of limpet population collected from the Manley Flats over 52 weeks.

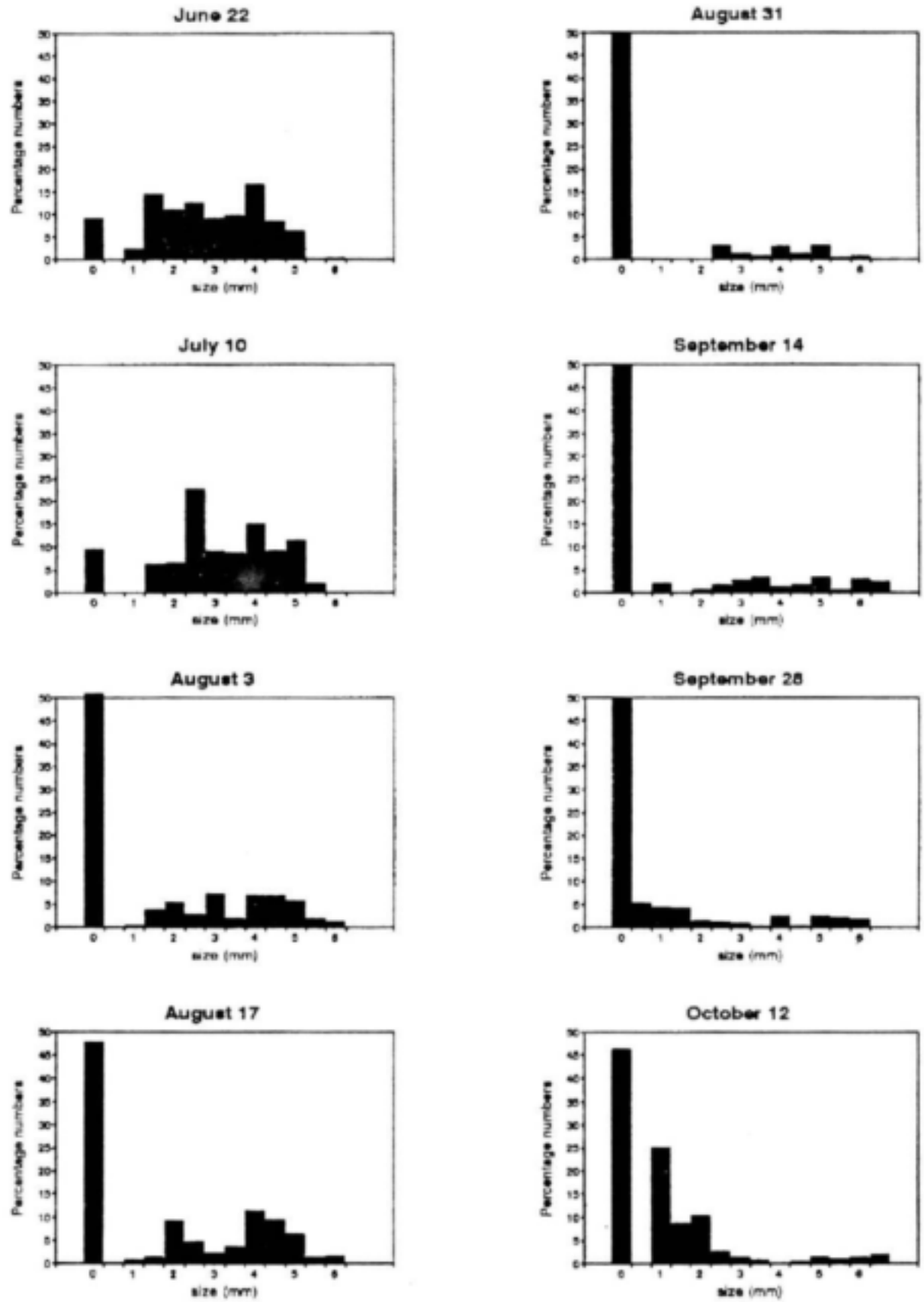


Figure 3.2.1.9 Histograms of the size range of limpet population collected from the Manley Flats site

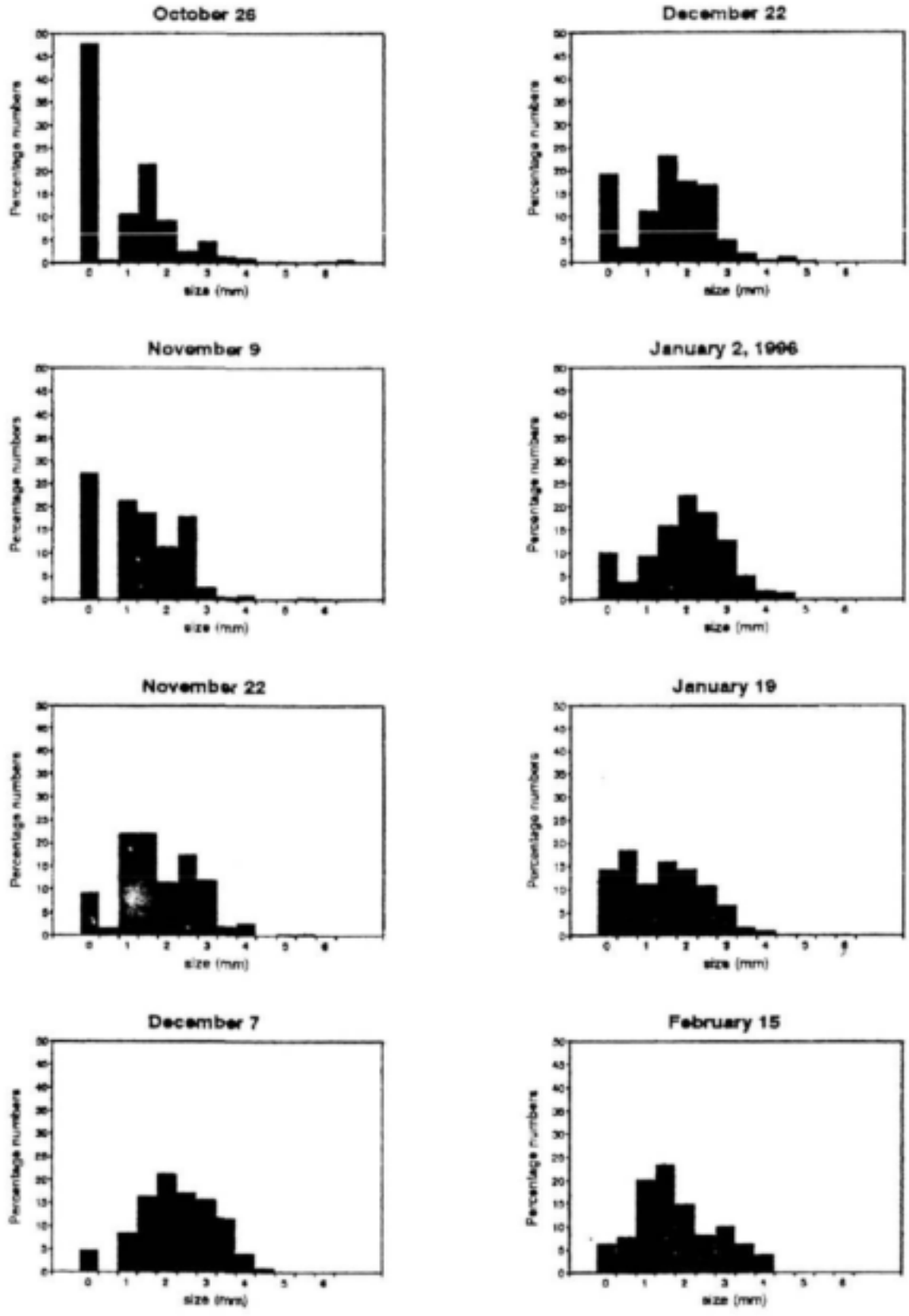


Figure 3.2.1.10 Histograms of the size range of the limpet population from the Manley Flats site

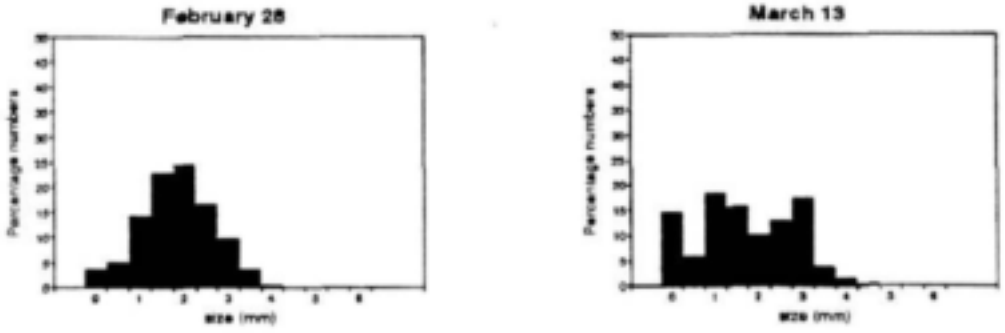


Figure 3.2.1.11 Histograms of the limpet population at Manley Flats

In the "choice" of cohort mean lengths, SI values less than 2 were ignored, as designed by the programme. Those which were all 2 or more gave one, two or three cohorts for any sample time, but all sample dates had SI values with chi squared values significantly different between the observed and the predicted population length frequencies. Generally, once the first two mean lengths of cohorts have been estimated, it is possible to roughly estimate the von Bertalanffy parameter κ, provided it is possible to estimate the age difference between the two cohorts. However, in this case with the high chi-squared values making the predictions not statistically significant, further analysis beyond the BHATTA and NORMSEP methods was necessary.

Growth and Cohort Analysis using ELEFAN 1

As a result of all the facilities provided by the ELEFAN programme, the three different phases of the year (as above with the BHATTA and NORMSEP methods) were compared between the two sites, each with Seasonalised and Non-Seasonalised Growth data, to produce the following results:

Maximum predicted lengths

Table 3.2.1.3 shows the predicted maximum lengths from the observed maximum lengths at BR and MF, for the periods with recruitment (September to February), with little recruitment (March to September) and all year. These were then used to identify the "best" growth curves for the two sites, and to make predictions of the phi prime values.

Table 3.2.1.3 Observed and predicted values for L_∞ for seasonalised and non-seasonalised growth at the Manley Flats and Belmont River sites.

	Observed max. length	Predicted max. length	Conf. interval (95% prob)
BR Sept to Feb	7	7.29	6.45 - 8.13
BR March to Sept	6	6.68	6.05 - 7.31
BR all year	7	7.34	6.66 - 8.03
MF Sept to Feb	6.95	7.52	6.57 - 8.47
MF March to Sept	7	7.19	6.64 - 7.74
MF all year	6.95	7.61	6.82 - 8.39

Optimum parameter combinations

Table 3.2.1.4 shows all the values estimated for the various combinations of growth parameters, for seasonalised and non-seasonalised growth at BR and MF. The values for Rn give an indication of the best fit for each set of data, and can be compared between both sites.

Comparing the C values ("amplitude" of the growth curves) it can be seen that, in the case of BR, they are estimated to be either 1 or 0.9, i.e growth rate is zero or nearly zero at the winter point. MF, however, varies between 0.22 and 0.84, where the latter is the value for the observed data from all year. WP gives the estimated period of lowest growth, and its calculation depends on which data set is chosen (i.e. from which month the data set was started, either March or September).

Comparison of the Rn values reveals the best fit for the data set. They are best compared within the same data set: for example, the all-year data for BR shows better fits (0.335 and 0.351) for the seasonalised data when compared to the non seasonalised.

Comparison of all phi prime values, representing as they do a unified parameter of growth performance, shows very little differences (see Figure 3.2.1.12). The average for the 12 month data set for BR (1.81) is particularly close to that of the average MF (1.84), and from this we can deduce that although the two sites are separated by some distance, the variations between them are such that they cannot be considered separate populations with regard to their growth parameters. Small differences between the growth performances can be considered due to differences in the respective environmental factors.

Growth curves

The data in Table 3.2.1.4 can be used to draw growth curves for each set of VBG parameters. Figures

3.2.1.13 to 3.2.1.16 show growth curves using 12 months' data, for both sites with and without the seasonalised growth, using the observed values of L_∞. Predicted L_∞ values give little change in growth curves from those of observed, and are not presented here. The BR non-seasonalised Rn value indicates the poor fit of the curves, and this can be seen in Figure 3.2.1.16, particularly through the months of July, August and September. With the difficulty of fitting the data, prediction of the expected longevity, and number of cohorts at any one time of the year becomes inaccurate with these two sets of data. Here, 4 cohorts are predicted.

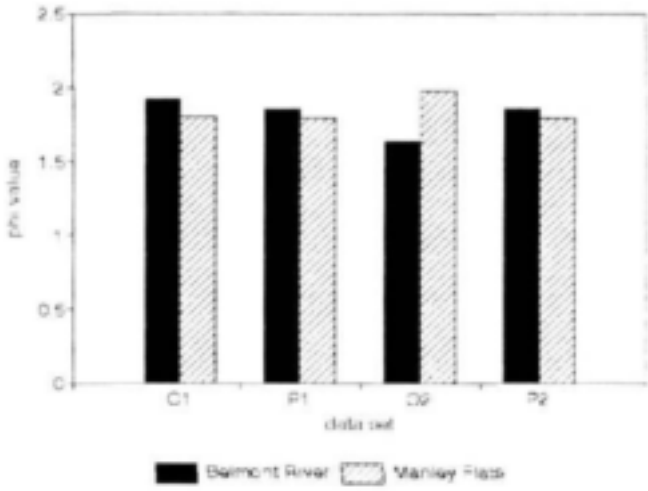


Figure 3.2.1.12 Comparison of Manley Flats and Belmont River phi prime values for predicted (P) and observed (O) values for seasonal (1) and non-seasonal (2) data sets.

We can conclude that *Burnupia* can be expected to live for 24 months in the conditions found at BR at the time of sampling, before reaching the maximum predicted shell length of 7,34mm. The programme has drawn a theoretical growth curve where the initial size is not 0, but the 0,65mm average hatching size, and the cohort begins mid January. The growth rates are presented below in the form of a table giving the monthly predicted sizes for this theoretical population growing at BR (Table 3.2.1.5) and at MF (Table 3.2.1.6), and shows the long period of little or no growth through the winter months. By the end of October, *ie* after approximately 5 months of growth (and approximately 4 months of lying dormant), a limpet would be expected to reach the period of sexual maturation (Section 3.3.4). It would take another 12 months to reach the predicted full size. Determination of the time needed for those limpets laid in the September/October period to reach sexual maturity is difficult, based on the data accumulated for the 1995/1996 sampling.

The population at MF did not show the same winter dormancy in growth rate found at BR, but maintained growth through this period, the programme predicting a little less time to reach the predicted maximum size of 7,85mm in shell length. However, it predicts a slower rate through the early months of the summer. In 1995 flow rates were slow to nil (Figure 3.2.1.3) and cattle activity in the area increased simultaneously. This may have had a negative effect on water quality in the area.

Table 3.2.1.4 Growth parameters and Rn values for the BR and MF sites, analysed for three times of the year; September 1995 to February 1996 (with recruitment, no winter phase; c=0and WP=0), March to September 1995 (without recruitment, winter phase), and March 1995 to February 1996 (with recruitment and winter phase). * indicates the observed values while the remaining values for L_∞ are the predicted values.

Site	L_∞	k	C	WP	Rn	phi
BR	7*	1.8	0.9	0.1	0.252	1.95
Sept to	7*	1.1	0	0	0.206	1.77
February	7.29	0.89	0.9	0.2	0.208	1.67
	7.29	1.0	0	0	0.206	1.73
BR	6*	1.2	1.0	0.6	0.523	1.64
March to	6*	1.1	0	0	0.258	1.60
September	7	1.1	1.0	0.5	0.791	1.73
	7	0.71	0	0	0.301	1.54
BR	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
MF	7*	1.4	0.3	0.9	0.275	1.84
Sept to	7*	1.5	0	0	0.219	1.87
February	7.52	1.2	0.45	1.0	0.275	1.83
	7.52	1.0	0	0	0.260	1.75
MF	7*	1.9	0.22	0.41	0.355	1.97
March to	7*	1.7	0	0	0.337	1.92
September	7.19	1.8	0.3	0.3	0.355	1.97
	7.19	1.5	0	0	0.350	1.89
MF	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

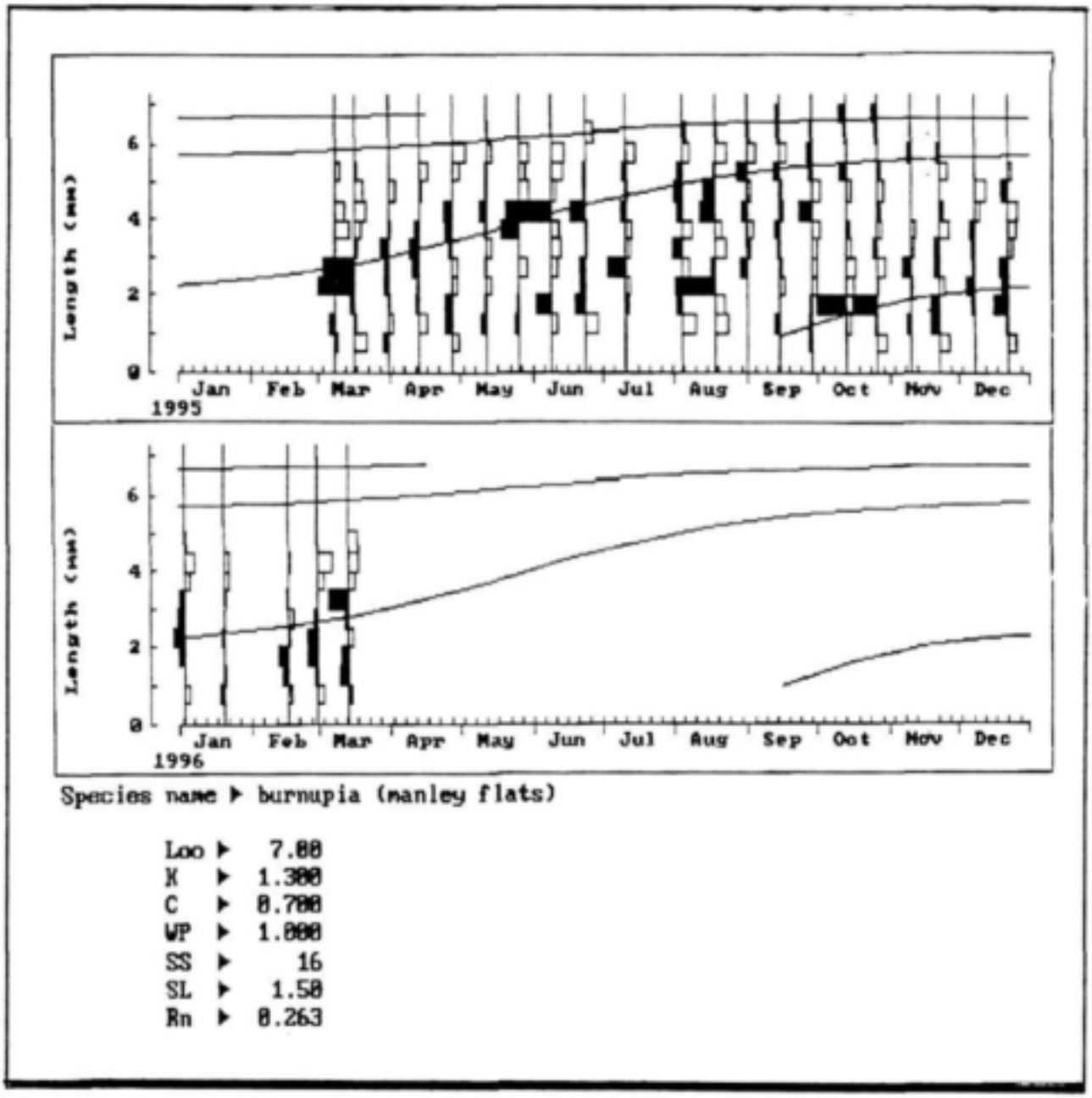
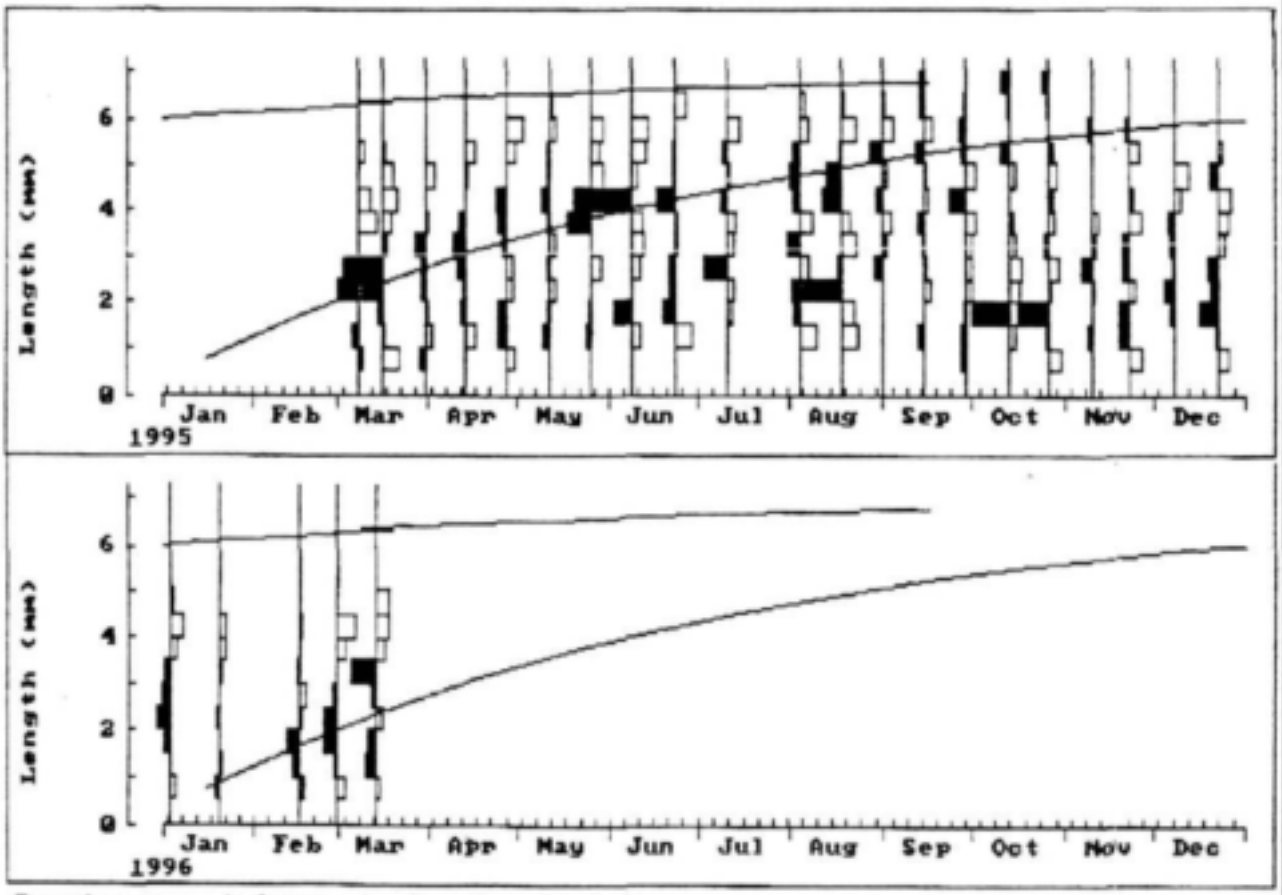


Figure 3.2.1.13 Predicted growth curves for cohorts from the Manley Flats site with a winter period (C=0.7, WP=1.0). Recruitment is calculated to begin in September.



Species name ▶ burnupia (manley flats)

Loo	▶	7.00
K	▶	1.900
C	▶	0.000
WP	▶	1.000
SS	▶	4
SL	▶	3.00
Rn	▶	0.242

Figure 3.2.1.14. Predicted growth curves for cohorts from Manley Flats, with no winter period calculated. Recruitment is calculated to begin in January.

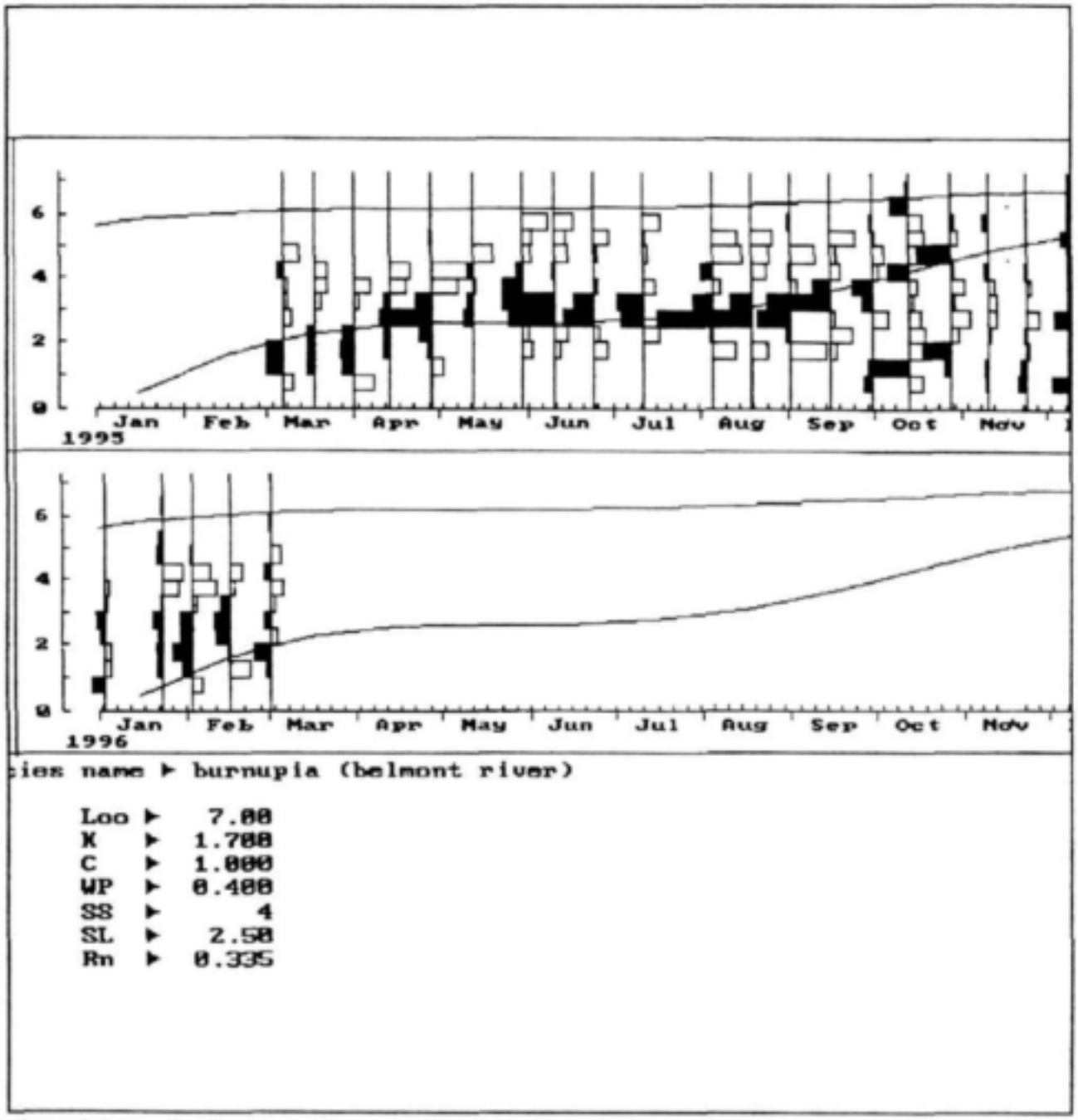


Figure 3.2.1.15 Predicted growth curves for cohorts from the Belmont River site, with a winter period, C=1.0, WP=0.40. Recruitment time is calculated to start in January

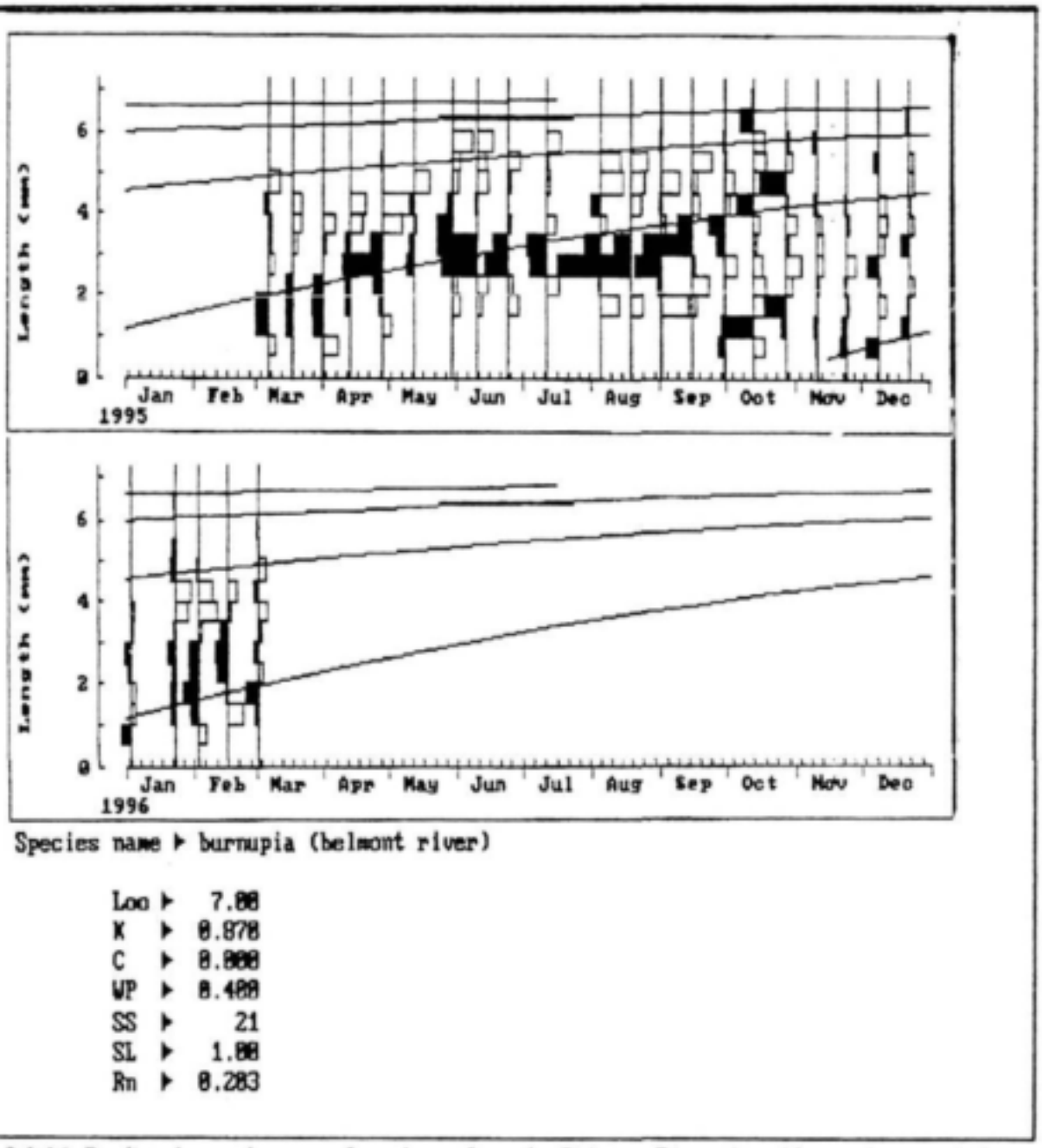


Figure 3.2.1.16. Predicted growth curves for cohorts from the Belmont River site with no winter period (C=0, WP= 0.4). Recruitment is calculated to begin in January.

Table 3.2.1.5. Predicted lengths at the 15th of each month, for limpets from the Belmont River site, using the growth parameters from the ELEFAN programme.

a) $L_\infty = 7$, $\kappa = 1,7$, C = 1, WP = 0,4, Rn = 0,335, $\Phi' = 1,92$.

b) $L_\infty = 7,34$, $\kappa = 1,3$, C = 1, WP = 0,42, Rn = 0,351, $\Phi' = 1,85$.

The sequence of lengths and then the sequence of months is followed.

BELMONT RIVER SITE (a)		
JAN	0.41	5.8
FEB	1.62	6.02
MARCH	2.22	6.13
APRIL	2.52	6.18
MAY	2.58	6.20
JUNE	2.59	6.20
JULY	2.71	6.22
AUG	3.05	6.28
SEPT	3.62	6.39
OCT	4.29	6.51
NOV	4.94	6.63
DEC	5.44	6.72

BELMONT RIVER SITE (b)		
JAN	0.53	5.49
FEB	1.58	5.77
MARCH	2.15	5.93
APRIL	2.45	6.01
MAY	2.54	6.04
JUNE	2.55	6.04
JULY	2.61	6.05
AUG	2.85	6.12
SEPT	3.30	6.24
OCT	3.89	6.40
NOV	4.52	6.58
DEC	5.06	6.72

Table 3.2.1.6 Table to show the predicted lengths at the 15th of each month, for limpets from the Manley Flats site, using the growth parameters from the ELEFAN programme.

a) $L_\infty = 7$, $\kappa = 1{,}3$, $C = 0{,}7$, $WP = 1$, $Rn = 0{,}263$, $\Phi' = 1{,}8$.

b) $L_\infty = 7{,}85$, $\kappa = 1{,}0$, $C = 0{,}84$, $WP = 0{,}97$, $Rn = 0{,}293$, $\Phi' = 1{,}79$.

The sequence of lengths and then the sequence of months is followed.

MANLEY FLATS SITE (a)			
JAN	2.31	5.73	6.66
FEB	2.52	5.78	6.67
MARCH	2.80	5.86	6.69
APRIL	3.23	5.98	6.72
MAY	3.73	6.11	
JUNE	4.26	6.26	
JULY	4.71	6.38	
AUG	5.08	6.48	
SEPT	0.93	5.35	6.55
OCT	1.55	5.52	6.60
NOV	1.94	5.62	6.63
DEC	2.15	5.68	6.64

MANLEY FLATS (b)		
JAN	2.12	5.74
FEB	2.30	5.81
MARCH	2.59	5.92
APRIL	3.07	6.10
MAY	3.64	6.30
JUNE	4.23	6.52
JULY	4.73	6.71
AUG	0.47	5.14
SEPT	1.23	5.42
OCT	1.68	5.58
NOV	1.92	5.67
DEC	2.03	5.71

Monthly and Daily Predicted Lengths

Based on the predicted growth parameters and curves, monthly lengths are predicted in Tables 3.2.1.5 and 3.2.1.6 for both sites, for observed and predicted L_∞ of the year's data, with seasonal growth. Similarly, daily sizes are predicted for both observed and predicted L_∞ values, with seasonal growth, but are not presented here.

Growth Model

Figures 3.2.1.19 and 3.2.1.20 show the total growth models to the L_∞ value with estimates of limpet shell length at any given period. Regressions of the first 43 days will be used to compare the predicted growth of limpets in the field to that of actual growth of limpets in the CE Room and limpets enclosed in baskets in the field (Section 3.3.3).

Discussion

Sampling

Sampling in stoney streams was reviewed by Usinger and Needham (1954), Macan (1958), Albrecht (1959), Longhurst (1959), Cummins (1962) and Kajak (1963).

Southwood (1978) underlines three important components to sampling i) the sampling unit must lend itself to conversion to unit areas ii) the sampling unit must be easily delineated in the field iii) the sampling unit must not be too small in relation to the animal's size as this increases the edge effect errors.
The sample size and the class interval both have an effect on the analyses of length-frequency methods (Mytilineou and Sarda, 1995). The collection of 300 length frequency samples at fortnightly intervals over 52 weeks satisfies three suggestions by different authors as to the choice of sample size and class interval:

1) MacDonald and Pitcher (1979) suggest that sample size should consist of at least 50 specimens for each age group, and substantiate this mathematically.
2) Pauly (1984) suggests that a total sample size of over 1500, collected over a period of four months, is a "very good" sample for the application of ELEFAN I.
3) Wolff (1989) proposed a simple method for the estimation of the class interval:

$$\text{Class interval} = (0.1)(L_{max})/(\text{Number of assumed modal groups}).$$

Although this was not initially used to determine the class interval to be used in the field, when the ascertained values for L_{max} and number of modal groups are inserted into the equation, the result confirms the choice of 0,5mm class interval is sufficiently small for the analysis of the limpet cohorts.

The method of sampling the rocks proved a sampling method of least disturbance to the limpets. Marking the 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 due to i) the paint or polish needing approximately 30 minutes to dry, considered stressful for the limpets as they do not naturally occur for any length of time out of the water ii) many were covered in green algae which would have to be removed first, causing further stress iii) the newly hatched limpets up to 1mm in length were considered too small to either remove from the rock (when seen) or to be effectively marked for the duration of the sampling time.

For the purposes of growth analyses, there is substantial variation in choice of time interval for measuring freshwater molluscs in the field. For example, 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*.

Distribution

The contagious type of dispersion demonstrated by *Burnupia* is typical in natural populations where aggregation seems to be the rule rather than the exception (Elton, 1966). Although depth of the water in this river system did not seem to be an issue with regard to the distribution, personal observations of *Burnupia* in non-flowing pools, also in close proximity to Grahamstown, showed the limpets to be absent after a depth of 75cm. Negative correlations between depth and density have been recorded for *A. fluviatilis* (Geldiay, 1956; Macan, 1970). Similarly their distribution may be governed to a degree by their obvious sensitivity to sediment. Verdcourt (1949) suggests it is the absence of sediment, rather than the presence of flowing conditions which determines the distribution of *A. fluviatilis*. As well as sediment, heterogenieties in the distribution of food may influence vertical dispersion. *Burnupia* is a herbivore which ingests, in particular, unicellular algal periphyton, which in turn is found in reducing densities as the depth of the water increases (Calow, 1974). *Burnupia* apparently prefers smooth stone surfaces and this may be correlated to the fact that its shell and foot widths are generally greater than the crevice widths found on rough stones. A smooth surface may also be necessary to provide uninterrupted contact between the shell margins and the substrate during periods of high flow rate. It should be noted, however, that *Burnupia* (and also *A. fluviatilis* and *Acroloxus lacustris* Linn) have a thin, flexible uncalcified edge to their shells which allows them to conform to the irregularities of the surfaces in which they live (Fretter and Peake, 1978). Nevertheless, in *Burnupia* this skirt is only a millimeter wide and so it cannot conform to extreme stone irregularities. Personal observations too showed that eggs were laid on the smooth surfaces, also a requirement for example, for egglaying by *A. fluviatilis* and *Planorbis contortus* (Calow, 1974).

In his work on *A. fluviatilis*, Calow (1974) found no limpets on stones less than 6cm in longest length, not the case with *Burnupia*. However, none of these stones at MF or at BR were found in the mainstream, but rather at the quiet edges (particularly at BR where there was less of a pool effect than at MF) which is not surprising as these stones have greater mobility during times of fast flow, initiating crushing or scouring of the limpets present.

Recruitment

A post-winter surge in egg laying was very prominent at both sites, presumably related to the rise in temperatures. Temperatures for spring laying of eggs in other Ancylids varies. For *A. fluviatilis* estimates have been made at 7°C (Bondeson, 1950), 8,5°C (McMahon, 1976), and 11°C (Geldiay, 1956); 10°C for *F. rivularis* (Nickerson, 1972); and for the more tropical populations of *H. excentricus* and *L. fuscus* egg laying occurs with the spring rise in water temperatures to 14-18°C from winter lows of 7-12°C (McMahon, 1976). As Ancylidae lay their eggs on the rock on which they are feeding (Calow, 1974) we can presume that the egg capsule counts accurately reflected the number laid by the limpets within the sampling areas. However, it has proved difficult explaining the lag in time (approximately 10 weeks) from the initial egg laying to the time of first recruitment. The measured temperatures for this period are from 14 - 16°C. Controlled Environment (CE) Room experiments at 15°C (Haigh and Davies-Coleman, 1995) as well as personal observations in the laboratory have shown eggs to take no longer than 18 days to develop to hatching, and with a growth rate which would increase their shell length to that of the recruitment phase in less time than is shown by the sampling, from initial time of egg lay to time of recruitment. There are possibly two reasons for this lag:

i) High mortalities after hatching are common for freshwater snails (Calow, 1978), indicating the susceptibility of juveniles, when compared to adults, of any adverse conditions. Flow rates at this time vary between the two sites, but remain higher than later in the season; presumably oxygen content of the water would therefore not be limiting during this pre-recruitment period.

ii) O'Keeffe (1985) suggests, although there is no direct evidence, that for *Bulinus* species the hatchlings undergo a free-floating dispersal phase during which they would not have been recorded during field sampling. The question arises as to whether this occurs with *Burnupia*? It was not observed in the laboratory and neither is this phenomenon recorded in the literature. If the newly hatched limpets do undergo a free-floating phase, did this affect the numbers of observed hatchlings sampled from the chosen rocks, and could the numbers taken from the populations on each rock mathematically affect the analysis of the cohort growth. In Section 3.3.3, where the field trial with plastic baskets held in a stream is described, it was observed that despite the baskets being placed in a stretch of stream where there were no limpets upstream or downstream for approximately 50 metres either way, large limpets were found on the outside of the baskets after 3 to 4 weeks in the stream, and as these did not come from the experimental limpets, they must have migrated from another site. Certainly migration must occur from the top to the sides or bottom of the rocks as this is where egg capsules are laid. The conclusion, therefore, is that despite the initial feelings (from the first set of observations of limpets on rocks in the streams) that there was no movement from or onto each rock, based on the fact that the numbers and size groups on each of the respective rocks remained approximately the same, it appears that migration does occur to a certain extent under natural conditions. Perhaps, then, the possibility that the hatchlings do also migrate is not so far fetched. Detailed field observations would have to be made to substantiate this suggestion.

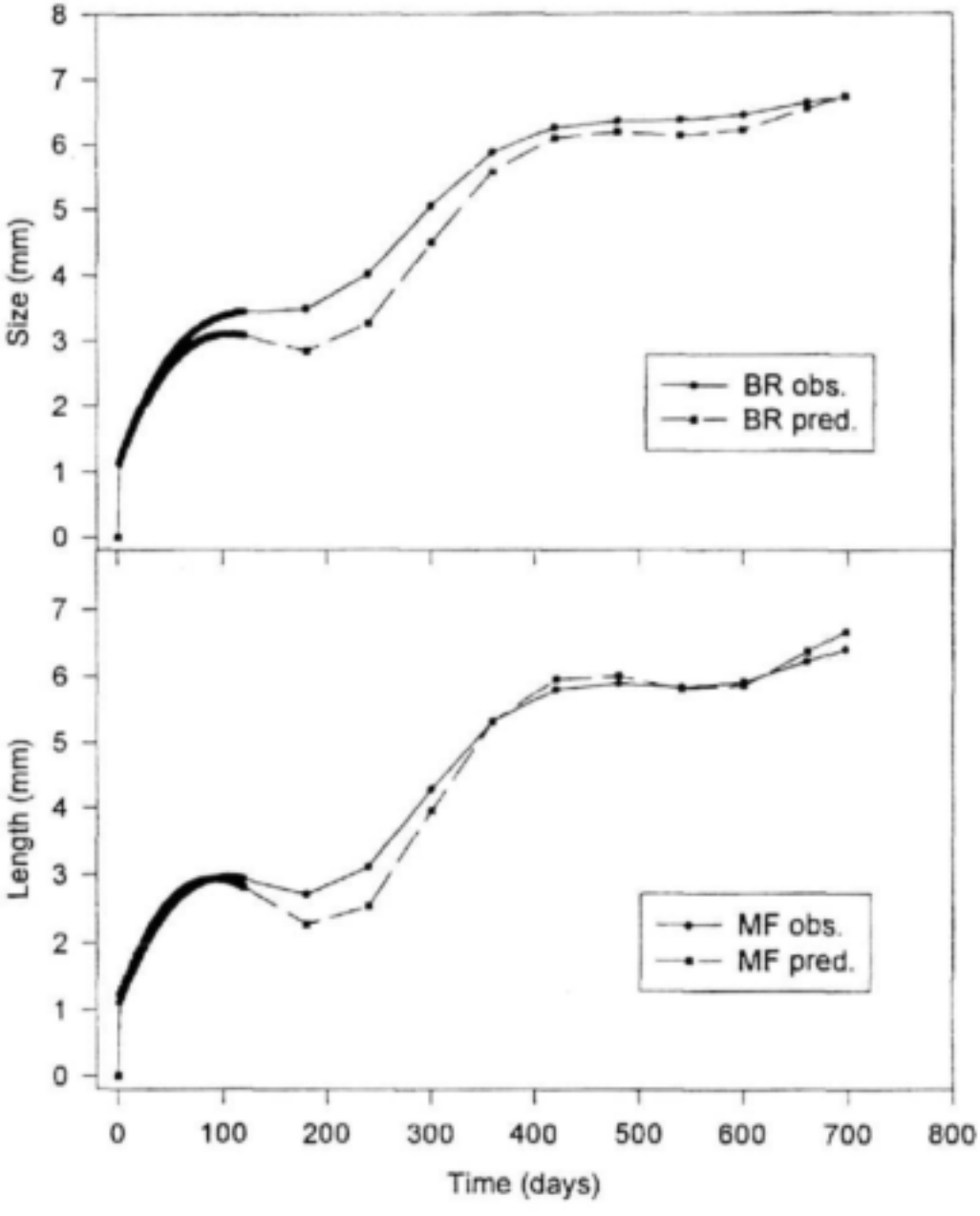


Figure 3.2.1.17. Growth models based on the VBGF, using the observed and the predicted L_∞ values, for Belmont River and Manley Flats sites.

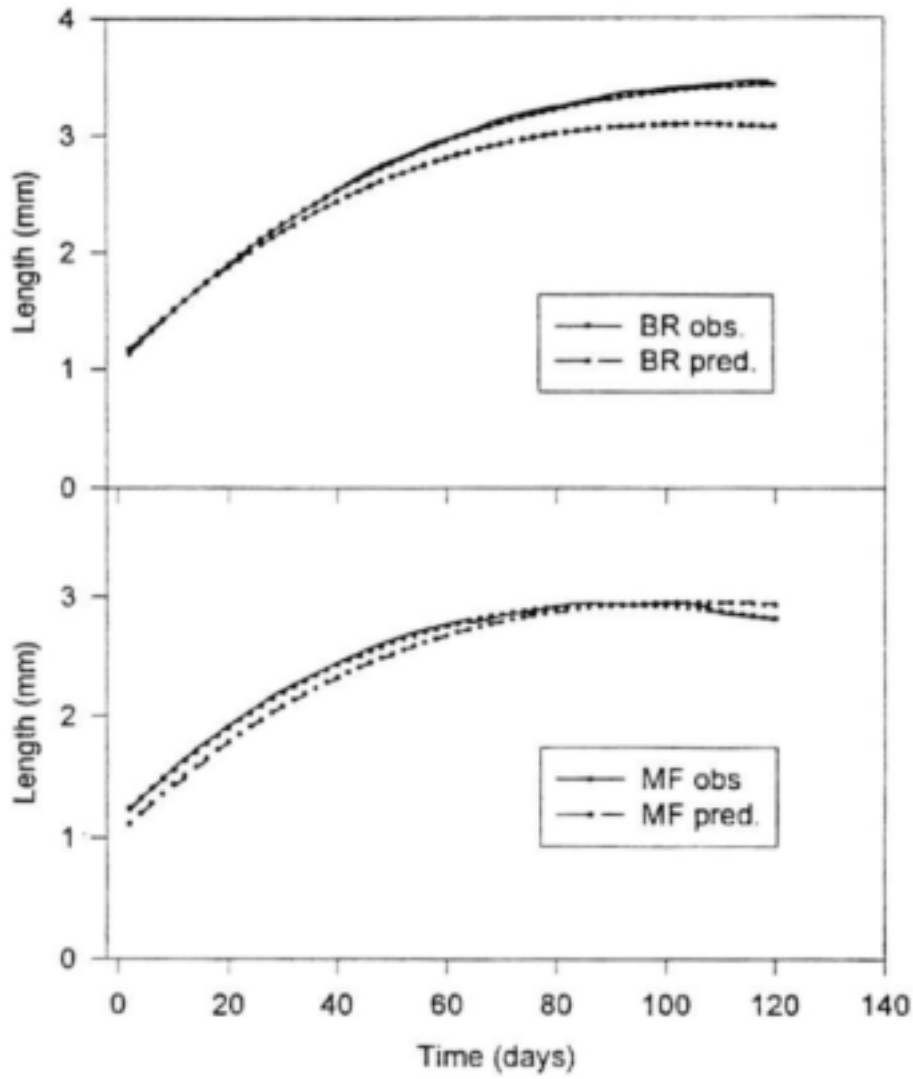


Figure 3.2.1.18 The first 120 days of the growth models predicted for Belmont River and Manley Flats data, using both predicted and observed L_∞ values.

on the outside of the baskets after 3 to 4 weeks in the stream, and as these did not come from the experimental limpets, they must have migrated from another site. Certainly migration must occur from the top to the sides or bottom of the rocks as this is where egg capsules are laid. The conclusion, therefore, is that despite the initial feelings (from the first set of observations of limpets on rocks in the streams) that there was no movement from or onto each rock, based on the fact that the numbers and size groups on each of the respective rocks remained approximately the same, it appears that migration does occur to a certain extent under natural conditions. Perhaps, then, the possibility that the hatchlings do also migrate is not so far fetched. Detailed field observations would have to be made to substantiate this suggestion.

Environmental factors and their effects

Water quality and diatom analyses would have underlined any differences between the sites, possibly explaining some of the variation seen between the two sites, on either growth or period of egg-laying.

However, of all the factors influencing life within a body of water, temperature is generally considered by ecologists (e.g. MacMahon, 1973) to have the most profound effect, affecting not only the geographical distribution of a species but also the metabolic processes of individuals. Many authors have demonstrated the effects of temperature on the growth of the individual and this has been discussed in depth in Haigh and Davies-Coleman (1995).

The flow rates would have made a significant difference to the conditions found at the two sites, primarily because the flow rate at MF was significantly less or even zero at times in comparison to BR. As a consequence of this, temperatures on a day to day basis would have varied, and because of the pond effect created at MF, it is presumed the temperatures would have been higher at MF, significantly affecting (increasing) the rate of growth of the limpets. However, at the times when there was an extended period of cattle activity, this lack of flow would be detrimental to the limpets as an accumulation of excrements would have occurred. Surprisingly, this was not reflected in the pH or TDS values.

Analysis of Growth

Harding (1949) says that neither the graphical method nor any other will give a complete and unequivocal solution, but fortunately the simplest solution is likely to be the most significant biologically, as well as statistically.

BHATTA and NORMSEP

There are two sources of bias in the analysis with BHATTA which will affect the results:

(a) if the number of newly-hatched was substantially underestimated, for example, poor field methods resulting in their not being seen, and

(b) if any large limpets migrated away from the site examined, their "loss" could have affected the results of component cohorts of any one sample date, particularly as at certain times of the year they were in very low numbers.

Identification of Modes

Within the methods of LFD Analysis, there are two classes used to estimate growth parameters: parametric methods, which assume that a length frequency distribution is a finite mixture of normal distributions (reviewed in MacDonald, 1987); and non-parametric methods which identify modes in some way and use a goodness-of-fit function to determine the best set of growth parameters (reviewed in Pauly and Morgan, 1987). Matrix progression methods which attempt to determine growth rate (e.g. Sullivan *et al*, 1990) are also non-parametric but do not identify modes in the LFD. The estimates obtained by following the progression of modes (= cohorts) in the length frequencies is considered superior to the method based on one single sample, e.g. by Harding's method. The latter methods are also considered unsuitable where the species is short-lived and there are only one or two cohorts in a single length frequency sample. However, non-parametric methods have a history (Pauly, 1987) of successful use on short-lived animals (e.g. Peruvian anchoveta, Hilborn and Walters, 1992).

The validity of the procedure used by ELEFAN relies on the following assumptions:

1. That the samples used represented the population investigated.
2. That the growth pattern in the population is the same from year to year.
3. That the VBGE describes the average growth of the investigated population.
4. That all individuals in the set of samples have the same length at the same age, and that, therefore differences in length can be attributed to differences in age (Pauly and David, 1981).
5. That continuous mortality occurs at a uniform, instantaneous rate (Wetherall, 1984).

Within the definition of the VBGE, asymptotic length is an extrapolation and hence will be poorly determined when large animals are rare, particularly in short-lived species or those with a high mortality. It results in an inverse correlation between estimates of L_∞ and the curvature parameter (rate at which L_∞ is attained, κ) over a banana-shaped region of the $L_\infty\kappa$ parameter space (Xiao, 1994), i.e. several combinations of L_∞ and κ can be equally well fitted to the data to produce similar lengths at age. This is where the ELEFAN method has an advantage over other non-parametric LFDA methods because it "restructures" the data so that modes representing large numbers of animals are de-emphasised relative to modes representing few animals (Pauly, 1987). It appears that variability in κ is common amongst the molluscs (e.g. Feare, 1970; Paul and Feder, 1973; Taylor and Venn, 1978; Poore, 1972). However, L_∞ is not usually expected to have biological meaning, i.e. to be observed in the population. Wetherall (1986) provides a statistically valid method for constraining L_∞ to lie within a "meaningful" range so that estimates of length at age within the range of data are not greatly affected. Variability in L_∞ would not influence the mean growth increment or mean length at age of a group because it occurs as a linear term in the original VBGE (Sainsbury, 1980).

Individual Growth

Southwood (1978) states that for animals whose generations overlap significantly, the grouping of sampled individuals into age classes does facilitate population studies. However, one problem when using length-based analysis and the subsequent placing of groups of limpets into cohorts, is the decreasing growth rate with age, combined with mortality, which produces overlaps between the older age groups, and consequently disallowing the detection of well-defined age groups (Mytilineou and Sarda, 1995).

A second problem in using length frequency analysis is the differences that occur in the growth rates through the year; a limpet laid during the latter half of summer will possibly have less of an optimum growth rate over a shorter period before the onset of winter, than a limpet laid in an earlier part of summer. The difficulty in estimating the growth rate of any given limpet is exemplified by the graphs in Figures 3.2.1.13 to 3.2.1.16. Although each graph is extrapolated to give predicted monthly lengths, it does not give an idea of the expected lengths for those limpets laid in the early parts of the summer, for example with the BR site, or in the later months, for example January and February, at the MF site. The restructured data in these figures and the histograms indicate a continual recruitment of hatched limpets through the summer months. Originally, the whole recruitment period from September to January was considered as one cohort, although with continual recruitment due to continual egg laying within this period. In retrospect, it is difficult to define the growth of an individual, on the basis of the growth model derived for each site, for any given size at any given period through the year. The question arises, therefore, as to whether the length of a limpet can be used to accurately determine its age, the fourth basic assumption of the ELEFAN programme as previously mentioned?

Individual Variability

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 on the "Effect of Individual Variability on the von Bertalanffy Equation" (1980) made some profound mathematical judgements as to the use of the VBGE, in particular with the possibility of individual growth, as described by the VBGE, not being the same as 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, which would, over time, cause a change in the age composition of that cohort. Consequently any results should be considered unrealistic (Vermeij, 1980). The effect of individual variability is particularly important when interpreting growth increment data in predicting yield and mortality estimates, as the data can result in serious overestimations of the mean length at age of a cohort (Sainsbury, 1980).

Huryn *et al* (1994) suggest that because of variation in growth rates and asymptotic size among individuals, size-at-age analysis and iterative models such as the ELEFAN 1 are *not* appropriate for estimates of absolute

or maximum age for organisms that exhibit asymptotic growth. *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). The overlapping cohorts, as is found with *Burnupia*, obscured temporal patterns of growth and development. The problem is compounded by the non-availability of direct aging techniques for natural populations (Vermeij, 1980), and the lack of growth rings.

Individual variability is further supported by evidence from numerous observations of freshwater pulmonates both in the field and in the laboratory which show that the growth rates become lower at the onset of female sexual maturation and at the time of increased egg production (Russel-Hunter, 1978). Presiding environmental conditions at a given time of year will have a direct influence growth rate and on the availability and quality of food which in turn will also influence the growth rate,

Winter growth

A slow or null growth in length for the winter months has been reported for many freshwater snails (Russel-Hunter, 1961b; Calow, 1973; Vincent *et al*, 1981). Growth rates for *Bithynia tentaculata*, found in temperate zones, fluctuated from 0,17mm/week to 0,8mm/week with the seasonal conditions (Pinel Alloul and Magnin, 1971). However, Figure 3.2.1.17 reflects a decrease in size of the shell length during the periods of no-growth (winter), and the question arises as to how this can be explained, and is the model curve accurate? Pitcher and MacDonald (1973) suggest that the main disadvantage of the sine wave VBG model is that it can unrealistically generate animal shrinkage during the winter. However, it can be of value when dealing with weights which can easily decrease, and can therefore be used to simulate periods of negative production over the winter. Weight can be expressed as a power function of length:

$$W_t = a\,(L_t)^b \text{ and therefore, in the VBGE,}$$

$$W_t = W_\infty[1-\exp\kappa(t-t_0)]^b$$

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 of degradation of tissues or organs, particularly of gonads or of 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).

Variations Between Populations

The growth rates of *Burnupia* can perhaps be compared to those of other ancylids reported in McMahon (1976). He states that field growth rates can be compared when the percentage increase in the mean aperture length of a generation 30 days after spat are collected in the field. Although absolute shell length and not aperture size was used for *Burnupia*, the percentages in change can be compared to the various examples given by McMahon (1976). For example, *Hebetancylus* populations studied had growth rates of 58%, 116%, 48%, 69% in the first

30 days, and two winter growth rates of 9% and 15%, similarly various *Laevapex* populations had 77%, 73%, 138%, 169%, 59%, 124% and 64% growth rates; *A. fluviatilis* demonstrated 35%, 105%, 114%, and 147%; and lastly McMahon gives examples of *Ferrissia rivularis* which had increases in aperture size of 66%, 74%, 96%, 87%, and 174%, demonstrating the large inter-population variation. The percentage increase in size of *Burmupia* in the first 30 days, estimated from the VBGM, is 184%.

Russel-Hunter and Eversole (1984) indicate some general features applicable to the majority of gastropods.
(1) There is no definite adult size, and the processes of somatic growth are to some extent independent of the processes of maturation (Russel-Hunter and Eversole, 1976; Calow, 1978; Russel-Hunter, 1978). This contrasts with the hormonal close coupling of sexual maturation and growth pattern in arthropods and higher vertebrates.
(2) Any size parameters that can be defined are more likely to be characteristic of each interbreeding population rather than to the species as a whole. There may be intraspecific differences in mean size at first reproduction, or mean size at death, with very little variation within each population and highly significant differences between populations (in many sets of cases with no overlap).
(3) Many gastropod populations exhibit an environmental cutoff at a certain size (equivalent to a certain age for that specific population only) and this prevents the detection of any endogenous senescence (Russel-Hunter, 1978).
These gastropod features result in great interpopulation plasticity of pattern of growth and of life cycle (Calow, 1978; Russel-Hunter, 1978) and Huryn *et al* (1994) suggest that any longevities calculated are likely to be baized.

I conclude that in reality the longevity of *Burmupia* is less than the two years predicted, and that a given shell length of *Burmupia* cannot be used to estimate the age of any individual limpet, given the variation between individuals, effect of time of year of emergence, and inter-population variation.

Comparison of Phi Prime Values

Can the MF and BR limpet populations be considered the same population in terms of growth parameters? Pauly (1979) discovered that Φ' values he developed within the Φ' test are very similar within related taxa and have narrow normal distributions with minimum variance. Although originally described for fish, the Φ' has been shown to fit observed correlations in a bivalve (Defeo *et al*, 1987) and other invertebrates (Longhurst and Pauly, 1987).

Defeo *et al* (1987) in their analysis of yellow clams, found that inclusion of the smallest size class not originally measured and included in the analysis, did not affect the estimate of phi prime. In the case of the MF and BR field samples, a similar scenario is assumed where the lack of the newly-hatched limpets will have no effect on the values of the respective phi prime values.

The usefulness of the Φ' test can be extended to the comparison of species of similar shell shape (Pauly and Munro, 1984). However, comparisons between the Ancylidae and e.g. the Planorbidae would not be possible. Another downfall of the phi prime statistic in length frequency analysis is that it 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_∞ may be baized and therefore so will those of κ (Lee's phenomenon, Ricker, 1975), with ultimately the Φ' values being affected.

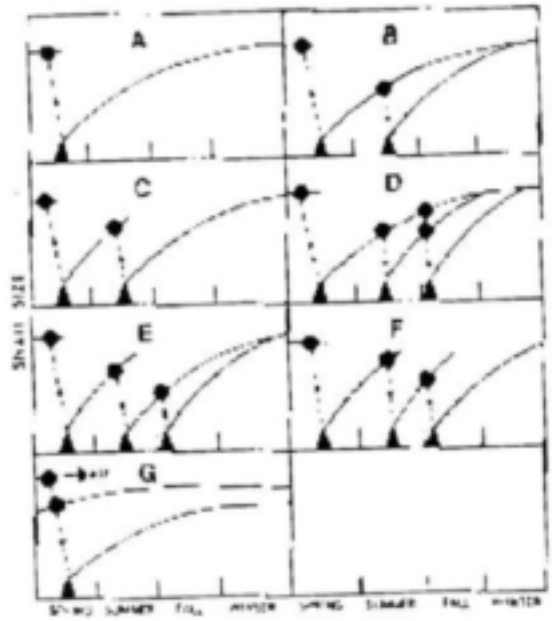


Figure 3.2.1.19 Patterns of life cycle to be found in freshwater gastropods. Circles, reproduction begins; triangles, egg capsules appear (Calow 1978).

Predation

According to Lodge *et al* (1987) the major predation on freshwater snails is exerted by vertebrates, while those invertebrates known to consume snails are insects and leeches that pierce the shell (Costil and Daguzan, 1995). No pierced shells of dead limpets were observed during this investigation, although it must be stated that the shells disintegrate rapidly after death. At these site other potential predators such as stoneflies were absent. This suggests that predation is unlikely to be a major influence on mortality of this species at these sites.

Life history

The main life cycle patterns encountered in natural populations of freshwater gastropods have been described by Russel-Hunter (1961b, 1978) and reviewed by Calow (1978). The time of egg-laying will determine the life cycle pattern shown. When considering the egg-laying at the two sites studied, it can be seen there is a period where egg laying activity increase abruptly after the winter periods. The effects of the environmental conditions on the time of egg-laying and thus on the life cycle are well documented. Water temperature is considered the most critical factor determining growth and time of onset and intensity of the breeding season (Duncan, 1975; McMahon, 1975a; Russel-Hunter, 1978). Critical temperatures for the initiation and subsequent control of oviposition have been reported for various pulmonates, for example, 15 °C for *Biomphalaria* in Brazil (Duncan, 1975). Spermatogenesis is also temperature dependent (Geraerts and Joosse, 1984). Possibly related to this is the availability and quality of food. Marine pulmonates often show breeding cycles timed by lunar-related cues,

as does the salt marsh snail *Melampus bidentatus* (Price, 1979). However, this does not occur in the freshwater pulmonates. Similarly, it is generally agreed that the effects of photo period on growth and reproduction are confined to the temperate climatic regions (Geraerts and Joosse, 1984). Considering the continual egg-laying through the winter months at both the MF and BR sites, allbeit sporadic, it appears not to be a determining factor in relation to *Burnupia*. Mating was not observed or considered here, but it has been shown that freshwater pulmonates can be induced to mate by various stimuli, and for example, *Lymnaea stagnalis* shows a peak in mating activity at sunset (Geraerts and Joosse, 1984). However, not all interpopulation differences can be considered to be explained by environmental factors, rather that they are genetically determined (McMahon, 1975a).

Russel-Hunter (1978) suggests various life cycle strategies for temperate zones, reproduced (from Calow, 1978) in Figure 3.2.1.21 for the sake of clarity. They are based on two types of reproductive strategies, the *iteroparous* condition where parents live on after reproduction to reproduce again, and the *semelparous* condition where parents die after reproducing. He suggests B and D are "quasi-iteroparous" in that they occur in special circumstances in species which are usually semelparous; and that life cycle G is the true semelparous condition. The pattern of life cycle shown by *Burnupia*, according to the above, would possibly be demonstrated by types B and D, but it would appear from all personal observations both in the field and in the laboratory, that *Burnupia* is not usually semelparous, but demonstrates iteroparity. Calow (1978) gives a review of life cycle strategies 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, B and C (Burky, 1971); *Hebetancylus excenticus* type C (McMahon, 1976); and *Laevapex fuscus* types A, D and E (McMahon, 1976). Calow points out that most of the freshwater pulmonates demonstrate semelparity, considered an evolutionary trend from the iteroparous state of the marine prosobranchs and which is also found in the primitive saltmarsh pulmonate *Melampus bidentatus* (Russel-Hunter *et al*, 1972). This iteroparous state shown by *Burnupia* could possibly be a reflection of its evolutionary history, considered by some (e.g. Hodgson *et al*, ***in prep***) to be closer to the more primitive state than other ancylids.

An alternative thought is that this iteroparous state rather reflects a life history change when moving from the poles to the tropics, moving towards a "K" selection which is supposed to become more intense and where iteroparity becomes more frequent when compared to the "r" life strategy (Pianka, 1970). Tropical pulmonate studies are few and mostly confined to the bilharzia-carrying snails (e.g. Shiff, 1964; O'Keeffe, 1982) but they do point to opportunism and recklessness, as the iteroparous state is considered (Calow, 1978).

When explaining life history traits, Byrne *et al* (1989) suggested the extended adult survival and oviposition of *Lymnaea palustris* allowed the snail to survive in marginal, unstable habitats (this trend was also seen in a number of planorbids not in conditions which would appear marginal (Costil and Daguzan, 1995). Despite the

continual flow of the higher reaches of the Bloukrans River, with the treated sewage waters of Grahamstown feeding it in times of drought, damming along parts of the river cause a cessation in flow at times, particularly at the MF site. Consequently, these populations of *Burnupia* could be considered to be in a marginal area, thus showing similar life history patterns to the *Lymnaea* species.

The maximum size attained by animals inhabiting a given site depends on many factors, such as the environmental conditions, but also genetic makeup and (although not seen in *Burnupia*) parasitic infections. The environmental conditions are considered of the highest importance in the life history strategies of freshwater gastropods (Russel-Hunter, 1978) but it is difficult to know to what extent the environmental conditions influence the maximum size attained by any snail of a given species. Costil and Daguzan (1995) showed various sizes attained by cohorts of *Planorbis planorbis* and *Planorbarius corneus* (both Planorbidae) from different sites and times of the year, and they suggest only a small proportion of the limpet populations attain maturity. Brown *et al* (1985) found that shell length of *Lymnaea elodes* at maturity was a function of habitat productivity. Snails matured and reproduced at smaller sizes in the less productive sites, and had higher fecundities. With the variation between BR and MF, perhaps this was also the case here. The full analysis of the sexual development of individuals has still to be completed (section 3.3.4).

To conclude, there can be considerable infraspecific interpopulation variation in the life cycle pattern, and Calow (1978) lists many of these. Studies of other populations of *Burnupia* will be necessary to clarify the general life cycle stategy it adopts as a species.

3.2.2 MORPHOMETRIC ANALYSES

Introduction

The shape of the shell plays a dominant role as a taxonomic character. Walker (1923) describes the shell of *B. stenochorias* as "Broadly oval, slightly wider posteriorly, quite elevated, rather thick for the genus, opaque, not shining,...surface with..very fine radiating striae..; apex prominent, small, subacute, slightly deflected at the tip, situated in the posterior fourth of the length, strongly inclined towards the right and nearly overhanging the margin; anterior slope long, regularly curved, but slightly flattened towards the apex; posterior slope short and concave; left slope convex; right slope somewhat concave". Shell size plays a part in the description of each limpet (Brown, 1994). However, a revision of the taxonomy of the genus *Burnupia* would probably reveal cases of synonomy among the large number of nominal forms recognised by Walker (1923) and Connolly (1939), mainly because it is only the descriptions of the shell and radula characteristics that have been used to describe the species, with a total lack of knowledge of the soft anatomy (Brown, 1967; 1994). Yet the relative proportions of the length, breadth and height of the shell, for example, vary considerably (Brown, 1961).

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. However, the presence or absence of growth rings in freshwater limpets is not well documented. Russel-Hunter (1953) indicates the presence of broad growth rings on the periostraca of *A. fluviatilis* and *Acroloxus lacustris*, demonstrating growth rates of summer and winter, and allowing estimates of the length of the life cycle. The presenceor absence of any growth rings in *Burnupia* was investigated.

It is generally accepted, however, that 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) and body mass (Calow, 1975b). In previous sections, length has been used to define growth and this appears to be justified (Section 3.2.1). However, we need to ascertain the variability of the morphological dimensions between sites. Similarly, if *Burnupia* is to be used as an ecotoxicology indicator species, there is the possibility that limpets from different local rivers could be collected either for use in chronic toxicological tests (where the effects of toxicant on growth are monitored) or for replenishing genetic stock within laboratory cultures (although the necessity of this latter is still a debated question). The variation between populations therefore needs to be considered.

Method a)

To determine the presence of daily growth bands:

1) All soft-bodied parts of 10 limpets were removed and the remaining shells placed in 10% sodium hypochlorite solution to remove all organic debris.
2) The shells were placed into polyester resin, with one drop of 0,01M hydrochloric acid to thin the resin, and allowed to harden.
3) Each limpet shell was cut in half with a diamond blade, the cut edge ground with increasingly finer abrasive paper (340,120 wet and dry trimiter paper) and finally polished with household metal polish "Brasso".
4) Each shell was etched for varying lengths of time (25–40 minutes) with cold 0,01M hydrochloric acid.
5) Agar Scientific Ltd No G255 strips of acetate replicating material were used to obtain replicas of the polished and etched surfaces. The strips were left on the sections for 5 minutes, each peel removed and kept flat by holding between cover slip and microscope slide.
6) Each peel was examined for any sign of growth bands or growth increments under a Nikon bifocal microscope up to 10x40 magnification, and with a low power phase contrast microscope.

Method b)

To determine the possibility of using morphometric measurements to calculate growth, and to investigate this from three populations.

1) Using 0.5mm vernier calipers, measurements of shell length (greatest distance of the anterior and the posterior axes), 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) were taken from three sites, two in the Bloukrans River (Belmont River [BR] site ± 7kms upstream from the Manley Flats [MF] site), and one in Botha River (Botha), in a large deep pool. Wet weights were also taken of those individuals from BR and Botha.

2) To consider the relationship between growth and morphometric measurements, simple linear regressions (Sokal and Rohlf, 1981) were initially calculated for each population using log-transformed data. According to Fischer-Piette (1948) volume$^{1/3}$ provides a better representative of the overall shape of each individual. The volume (V) of each individual was calculated from its shell parameters by treating the shell as an elliptical cylinder, using the following formula:

$$V = \Pi/4 \times \text{length} \times \text{width} \times \text{height}$$

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

3) Two further, multivariate tests were employed to examine the intricate relationships between the variables measured: principal component analysis (PCA), and discriminant function analysis (DFA) (within STATGRAPHICS 5). PCA was carried out with the aim of ordering the variables in a small number of dimensions, emphasising the major patterns of variation between them. This method works on ungrouped data reducing the set of variables by linear transformations 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. PC1 accounts for as much variation as possible in the original data, PC2 a progressively smaller amount and so on (Manly, 1994).

4) 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 are produced by which a specimen can be allotted to one of the groups on which the analysis is based. A way of evaluating the group differences is then to compute the proportion of correctly identified specimens in each group. This ultimately minimises the probability of wrong assignment of unknown individuals. 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 maximise the ratio between the between-group and within-group variations (Huberty, 1994).

Results and Discussion

1) *Growth rings.*

No daily growth rings were found, suggesting that, unlike the marine patelloid archegastropods, there is no periodic deposition of the limpet shells displayed as ridges or bands. Crisp (1989) in reviewing the phenomenon

of growth rings, 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. Temporary dry and exposed periods did not occur in the case of the limpets chosen for analysis, and thus, they would be unlikely to undergo acidosis, if this is indeed the form in which the daily growth rings are laid.

2) *Ratios of Morphometric Measurements*

The variability within these ratios is obvious, although difficult to test further. Walker (1923) claims that the original type specimen is not the normal form; that in a considerable number of species of Ancylidae there have been recorded instances of abnormally narrow forms, possibly resulting from some peculiar local condition which has affected the growth of the limpet.

He refers particularly to the South African ancylids, and mentions *B. stenochorias*, with *B. gordonensis* and *B. trapezoidea* also affected in this way. He suggests that as the typical form frequently occurs with the normal form, they should be regarded as individuals rather than as a racial difference.

Table 3.2.2.1. Ratios of width to length, and height to length. Comparisons with type specimens after Walker (1923), normal form and from the 3 sites investigated here, are presented.

	width/length	height/length
Type specimen	0.56	0.37
Normal form	0.75	0.44
Belmont River	0.72	0.40
Botha River	0.69	0.33
Manley Flats	0.73	0.39

Linear Regressions and Correlations.

The regressions of the logged (base 10) weight against volume show a very significant relationship between the values for those limpets from Botha River (Table 3.2.2.2), but not for those from Belmont River. Comparing the regressions and correlations (Table 3.2.2.3) between weight and individual morphometric measurements indicates very generally a poorer relationship. The correlation values of BR suggest length can be used to measure weight (0.7326), although with a degree of uncertainty. There is no weight data for MF to compare these results with a third site. However, all three sites showed a very significant relationship ($p < 0.0001$; Table 3.2.2.2) between length and width, with less significant relationships between length and height, and height and

weight. ANCOVAs of length against width for all three sites, and for MF against BR, and BR against Botha, however, reveal significant differences between slopes ($F = 1,9.5; 1,9.8; 1,39.8$ respectively; $P < 0.005$).

In determining whether the volume of a limpet depends largely on the length, regression and correlation analyses of the log length, width and height against log volume$^{1/3}$ showed shell length plays a greater role in determining the volume$^{1/3}$ (Table 3.2.2.4), and therefore the shape of the limpet, compared to shell width or height. This is not surprising as length is always the greatest dimension. However, testing the slopes of the regressions of log length against log volume$^{1/3}$ by means of ANCOVA showed significant differences between the slopes within the three sites ($F = 1, 171.3; P < 0.0005$).

In general, therefore, individuals portray similar patterns of allometric growth of length with respect to width and height, although different populations show relative differences in these variables. Corte-Real (1994) had similar results in her studies of the marine limpet *Patella*.

Table 3.2.2.2 Calculated relationships for length, height and width in each population tested. Also included are the weight regressions for the Belmont and Botha sites.

Location (Site)	Values Compared	Regression Equation	N	R^2
Belmont River	length v weight	y = 1.854x - 3.624	49	0.537
	height v weight	y = 0.711x - 2.662	49	0.249
	width v weight	y = 1.072x - 2.992	49	0.22
	length v height	y = 1.142x - 0.476	173	0.674
	length v width	y = 0.819x - 0.05	173	0.727
	height v width	y = 0.523x + 0.316	173	0.575
Botha River	length v weight	y = 2.283x - 3.603	21	0.837
	height v weight	y = 1.877x - 2.458	21	0.491
	width v weight	y = 2.429x - 3.276	21	0.758
	length v height	y = 0.793x - 0.343	21	0.724
	length v width	y = 0.831x - 0.066	21	0.863
	height v width	y = 0.725x + 0.344	21	0.57
Manley Flats	length v height	y = 1.119x - 0.492	175	0.875
	length v width	y = 0.959x - 0.115	175	0.929
	height v width	y = 0.753x + 0.325	175	0.821

Table 3.2.2.3 Correlation matrices of the logarithms of weight and shell length, height and width values from the three sites. Significance values are represented in brackets.

1) Belmont River				
	weight	length	height	width
weight	1.0000 (0.000)			
length	0.7326 (0.000)	1.0000 (0.000)		
height	0.499 (0.000)	0.9154 (0.000)	1.0000 (0.000)	
width	0.469 (0.000)	0.7521 (0.000)	0.7694 (0.000)	1.0000 (0.000)
2) Botha River				
	weight	length	height	width
weight	1.0000 (0.000)			
length	0.1975 (0.193)	1.0000 (0.000)		
height	0.1968 (0.197)	0.953 (0.000)	1.0000 (0.000)	
width	0.1536 (0.314)	0.9812 (0.000)	0.9324 (0.000)	1.0000 (0.000)
3) Manley Flats				
	length	height	width	
length	1.0000 (0.000)			
height	0.5885 (0.000)	1.0000 (0.000)		
width	0.2835 (0.059)	- 0.215 (0.156)	1.0000 (0.000)	

Table 3.2.2.4 Simple regression equations of the limpet volumes logged against the log of weights for Belmont River and Botha River.

	$y = mx + c$	N	R^2	P	Corr. Coeff.
Belmont River	$y = 0.4061x + 2.973$	48	0.34	0.0001	0.5858
Botha River	$y = 1.0561x - 3.824$	44	0.94	0.0001	0.9673

Table 3.2.2.5 Regression and correlation results comparing the relationship between shell volume and shell length, width or height of the limpets at the Belmont Rover, Manley Flats and Botha river sites.

	y = mx + c	N	R^2	Probability	Corr. Coeff
Length					
Belmont	y = 2.9599x - 0.6309	174	0.894	0.0000	0.9455
Manleys	y = 3.0808x - 0.7135	175	0.973	0.0000	0.9865
Botha	y = 0.2999x - 0.3333	46	0.983	0.0000	0.9916
Width					
Belmont	y = 2.987x - 0.2094	174	0.839	0.0000	0.9158
Manleys	y = 3.0616 - 0.2748	175	0.951	0.0000	0.9751
Botha	y = 3.4201x + 0.0027	46	0.965	0.0000	0.9825
Height					
Belmont	y = 2.113x + 0.665	174	0.882	0.0000	0.9389
Manleys	y = 2.5308x + 0.6815	175	0.9454	0.0000	0.9723
Botha	y = 2.462x + 1.1268	46	0.959	0.0000	0.9794

Principal Component Analysis

Scatter plots of the first two principal components for limpets from the three sites demonstrates the overlap of their distributions. The PCA shows that length and width strongly influence the size and shape of a limpet relative to height; that there are differences between the three sites based on these morphometric measurements; and that on the bases of shell shape, limpets from Belmont River site can be distinguished from limpets from the Botha and Manley Flats sites. To ascertain whether the variables measured could be used confidently to separate samples, Discriminant Functional Analysis was performed.

Table 3.2.2.6 Summarised results of the principal component analysis based on the three morphometric characters of the three populations of *B. stenochorias*.

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

The percentage of variance indicates that the variation in the data set is described mainly by the primary component and that 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.

Discriminant Function Analysis

Within-group correlations are given by the correlation and covariance matrices (Tables 3.2.2.7 and 3.2.2.8). When all measurements are amalgamated (Table 3.2.2.7), once again there is a strong relationship between length and width relative to height. The low numbers in Table 3.2.2.8 indicate several principal components are preferable in explaining the variation between sites. According to the first function coefficient (Table 3.2.2.9), the analysis predicts tall narrow limpets or flat wide limpets, suggesting a possible correlation between hydraulic conditions and the shape of a limpet.

The variance of each component of the discriminant functions (Table 3.2.2.10) is expressed by the eigenvalue, based on the eigenvectors which indicate how much each morphometric character influences each principal component. The proportion of variation explained by each component is given by the Lambda value. Although the eigenvalues are below 1, component 1 is still greater than component 2, *i.e.* 62.74% of the variation is explained by the first component. The canonical correlation values are both high indicating both components dominate in the differences between the limpets and therefore their distribution, with high chi-squared values underlining that the group differences are highly significant. In the DFA approach, the results are finally classified into predicted groups on the basis of their discriminant functions determined from the three morphometric characters measured, with the proportion given (Table 3.2.2.11) of correctly assigned individuals into each population sampled.

The results of Table 3.2.2.11 show the discriminating power is weak when the data are treated by populations (BR, Botha and MF), particularly with BR where little more than half of the limpets are correctly identified.

Table 3.2.2.7 Discriminant function analysis of within-group correlation matrix, where all values for length height and width from the three sites have been amalgamated

	length	height	width
length	1.0000	0.8872	0.9335
height	0.8872	1.0000	0.8520
width	0.9335	0.8520	1.0000

Table 3.2.2.8. Discriminant function analysis of within-group covariance matrix where all values for length height and width from the three sites have been amalgamated

	length	height	width
length	0.00645	0.00645	0.00527
height	0.00537	0.00568	0.00451
width	0.00527	0.00451	0.00493

Table 3.2.2.9. Discriminant function analysis of Standardised Disc Function Coefficients, predicting tall, narrow limpets or flat wide limpets.

	1	2
length	0.07128	-2.37663
height	0.81436	1.95842
width	-1.65862	0.82882

Table 3.2.2.10. Classification of Statistics of Observations, given by the discriminant functions analyses.

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	Signif. level
0	0.7185523	128.571	6	<0.0001
1	0.8813964	49.1104	2	<0.0001

Table 3.2.2.11. Classification of limpets, with differing degrees of discrimination obtained from the differential ordering of the data. Results are presented as actual counts, and as percentages.

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

Conclusion

Morphometric characteristics are not sufficient to establish taxonomic affinities, particularly with *Burnupia* (Brown, 1994), and should be combined with techniques such as karyology (Nakamura, 1987), sperm morphology (Hodgson *et al*, 1991), and allozyme electrophoresis analysis (Corte-Real, 1992). However, using the length, width and height measurements within the Principal Component and Discriminant Function Analyses, populations can be compared sufficiently to determine whether or not they can be considered separate populations, a question considered here. Variation in shell shape is a common occurrence in both freshwater (Calow, 1975) and marine limpets (Moore, 1934), and Walker (1923) clearly indicated the possibility of habitat conditions influencing the shell dimensions of *Burnupia*. The question arises as to whether *Burnupia* can be selected from any one of the three local sites presently used, for the purposes of ecotoxicological research. Similarly, can shell length be used to determine its growth?

The results of the Principal Component and Discrimination Function Analyses suggest that growth can be predicted by using measurements of shell length at the Belmont River and Manley Flats sites, although with a degree of uncertainty. This is mainly due to the significant relationship between length and width, and length and height. Height was less closely related to width, although this was not as significant with limpets from the Botha River site. Weight, any increase of which is considered a means of determining growth, was found to be closely correlated to volume$^{2/3}$ and shell length. However, those limpets from the Botha River site were very poorly correlated with either length, height or width, so that length, width and height should all be used to estimate growth.

The inability to separate the populations by PCA and DFA (Table 3.2.2.11) indicates that *B. stenochorias* sampled from the three sites can be considered geographically separated populations only, varying in their proportion of morphometric characters, but insufficiently to consider them as separate populations. We can therefore have confidence in sampling limpets from the three sites for testing growth effects within toxicology trials, although measurements of all three morphological characters (*i.e.* length, width and height) should be used. The DFA scatter plots suggest that samples form the Belmont has less overlap with Manley Flats and Botha River sites. The hydraulics conditions prevalent at each site may offer an explanation for this, with disc function coefficient values (Table 3.2.2.9) suggesting that limpets from the Belmont River are longer and thinner where the current velocity was higher (Section 3.2.1) than at the other two sites. Here the limpets are possibly flatter and broader. Testing the effects of the flow rates on the growth and eventual shape of a limpet would be difficult in the field, current flow would have to be measured on a microhabitat scale, then compared to the area of the cross section of the shell, representing the drag resistance against current velocity (this would presume limpets confine themselves to the same position in the microhabitat).

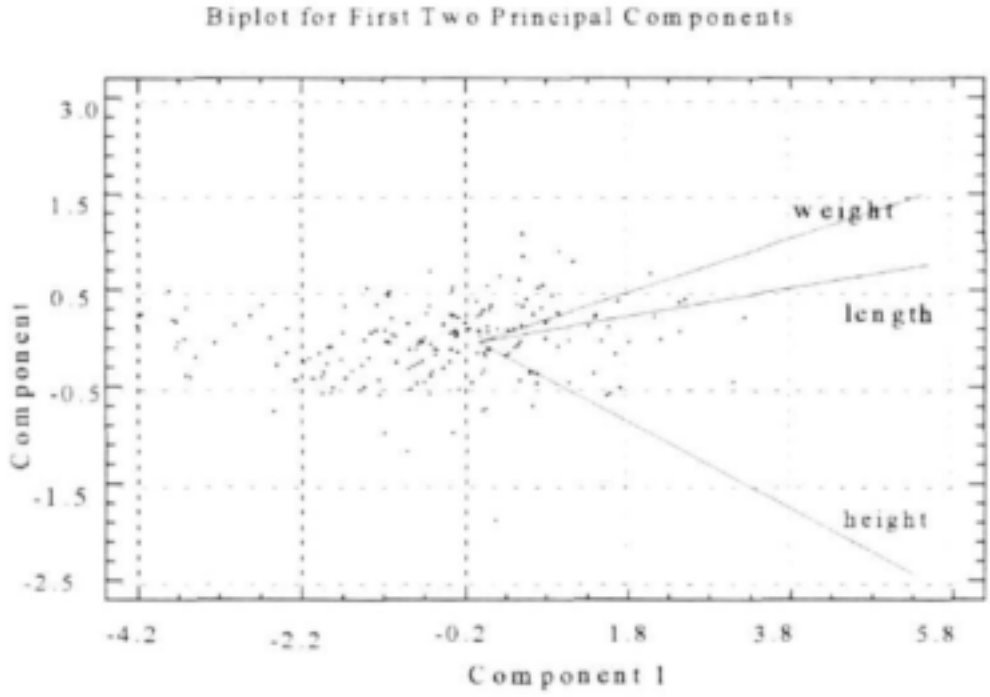


Figure 3.2.2.1 The length of the lines (vectors) are each proportional to its contribution to the principal components. The angle between the lines is inversely proportional to the correlation between them. This confirms the regression analyses.

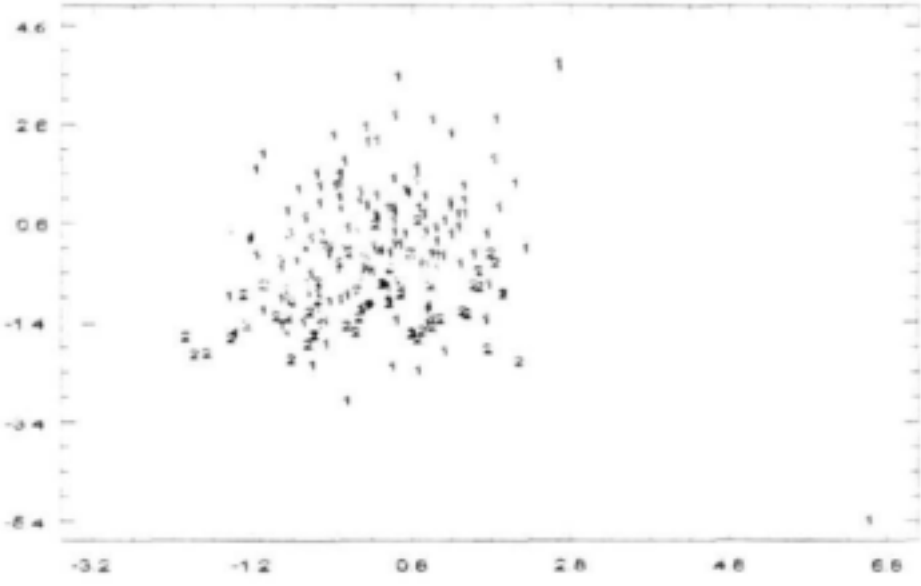


Figure 3.2.2.2. Discriminant function plot for all three site 1=Belmont river, 2= Botha river, 3=Manley Flats.

3.2.3. HABITAT

A field investigation by Lisa Pretorius (third year Zoology student) into the habitat requirements and distribution of *B. stenochorias* in selected Eastern Cape rivers. This report has been edited to a certain extent.

Introduction

The habitat requirements of this species have not been investigated, and as it is a key organism to be developed as an ecotoxicological indicator, it is necessary to know what conditions are optimal for its survival and can be recreated in artificial stream channels for culturing purposes.

Burnupia are thought to occur in well oxygenated water as they are almost entirely confined to small, stony streams and the shores of lakes which are exposed to wave action (Brown 1980). The genus is widely distributed throughout South Africa with the exception of the Western Cape, and have been recorded in biotopes such as stones-in-current, stones-out-of-current and on vegetation (F. de Moor, Albany Museum, *pers.comm*). Appleton (1978) describes abiotic factors influencing the distribution of a bilharzia intermediate host snail (a different family to that of *Burnupia*) as temperature, turbidity, salinity, current velocity and water depth amongst others. Of these, temperature and current velocity are factors thought to be of major importance in determining the distribution of limpets. Although highly variable in fresh water, water chemistry (concentrations of chemicals such as Mg, K, Cl, NO_3, PO_4 etc.) only affects the community structure extensively under extreme conditions (Allan 1995, Dussart 1979).

Just as the environment changes, so the numbers of animals change, reflecting the equilibrium natural populations have with the environment (Eisenberg 1966). The time scale over which population measures are taken is important because a temporary change in conditions may affect the numbers in the population but will not give an accurate estimation of density and distribution under normal conditions, thus a longer time period is required when doing population studies.

A habitat is defined as the place where an organism normally lives (Southwood, 1978). The habitat requirements are factors which enable the organism to make an area its habitat. To know what these factors are, it is necessary to make comparisons of abiotic and biotic conditions occurring in those areas where *B. stenochorias* is present. The factors which are similar can be assumed as being habitat requirements i.e. not limiting to the occupation of the habitat, and can be further manipulated in the laboratory to achieve optimum fecundity and growth rates.

Method

1. Three Eastern Cape rivers were investigated and are referred to as sites in this experiment. The sites were visited once in summer (March) and again in winter (July). The sites were :

   a) Site A - Bloukrans River, an organically polluted river receiving effluent from the Grahamstown sewage farm, agriculture and rural areas Same as Belmont river in section 3.2.2.

   b) Site B - Botha River, a pool in a pristine, unimpacted temporary river in which extremely low flows were observed while under study.

   c) Site C - Palmiet River, a pristine unimpacted fourth order stream.

2. In each site four biotopes were selected. The biotopes included :

   a) stones out of current

   b) stones in current

   c) marginal, submerged vegetation

   d) soft sediment

3. In each biotope limpets present were measured for length using a template with holes of known diameter. Samples of submerged marginal vegetation and soft sediment were taken back to the laboratory and seived. Limpets found were measured by the same method and results recorded.

4. At each site a time / catch per unit effort was done (adopted from Southwood, 1978) i.e. limpets were counted for one minute, to give an indication of the number of limpets present in each biotope in the river.

5. Abiotic factors which may influence the distribution of the limpets were measured once at each site during March and July. These included

   a) water quality determined by measuring total dissolved salts (TDS), calcium, sodium and and magnesium in parts per million, and pH.

   b) flow rate

   c) temperature

6. Statistics which included an analysis of variance and multiple range analysis test were used to compare the number, and size variance between sites, seasons and biotopes.

Results

Figure 3.2.3.1 shows the number of *B. stenochorias* found at each site during summer and winter 1996. Site B (Botha River) has the highest number of *B.stenochorias*, whereas site A (Bloukrans River) was intermediate and at site C (Palmiet River) very low numbers were present. It must be noted that the Botha River had no stones-in-current biotope as it was a pooled trickle stream with intermittent flow.

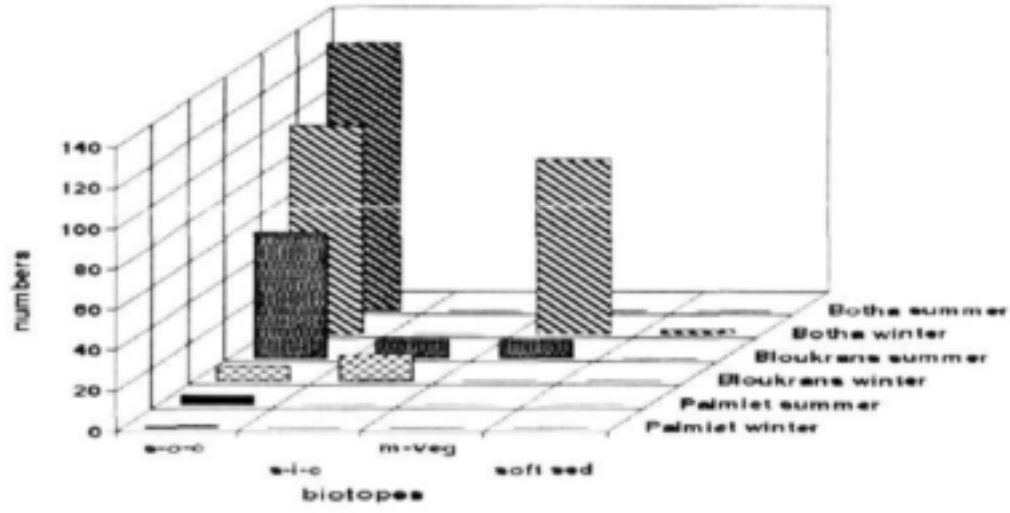


Figure 3.2.3.1 Number of limpets found at each site during summer and winter sampling

Figure 3.2.3.2 a) to f) show the size variations of the limpet in the different biotopes at each site during each season. At each site there is an increase in average size between summer (1.66mm) and winter (3.4mm). During winter, Site B shows the most variation in size ranging from shell lengths of 0.9mm to 6.5mm.

ANOVAs completed on STATGRAPHICS 5.1 revealed the following:

a) A significant difference in the number of limpets ($p = 0.0212$), but no obvious significant difference in the size ($p = 0.0658$) of limpets found at each site.

b) A significant difference in number ($p = 0.0174$) and size ($p = 0.001$) of limpets in each biotope.

c) No significant difference in the number ($p = 0.9701$) but a difference in the size ($p = 0.0104$) of limpets between seasons.

A multiple range analysis test, comparing the numbers in the biotopes (regardless of site) showed that stones-out-of-current was the most different biotope while soft sediment, marginal vegetation and stones-in-current were still comparable. The same test with sites showed that numbers in site B were most different from the other sites.

A multiple range analysis comparing sizes in different biotopes revealed that limpets found in soft sediment had the most diverse sizes compared with limpets in the other biotopes, with very few found in soft sediment. Stones-

in-current limpets had some similarities with limpets in marginal vegetation and limpets in marginal vegetation in turn had similarities with limpets in stones-out-of-current.

Table 3.2.3.1. Variation in the abiotic factors at time of sampling during 1996 at sites A= Bloukrans river, B=Botha River, and C=Palmiet River.

	SUMMER	WINTER	SUMMER	WINTER	SUMMER	WINTER
	A		B		C	
Temperature°C	18	9.7	20.2	10	23.6	11
Flow rate m/sec	34	14	0	0	16.5	4
TDS mg/l	596	67.3	449	552	74	88.4
pH	7.2	8.1	7.4	7.35	6.8	8.04

Table 3.2.3.2. Variation in water chemistry between the three sites on 7/03/1996.

	Na (ppm)	Ca (ppm)	Mg (ppm)
Botha River	110	16	18.6
Bloukrans River	147	30	23
Palmiet River	23	1	3

Discussion

The habitat of an organism and its dispersion pattern may be defined at various observational levels, the habitat definition (i.e. simple or complex) depending on the degree of precision and the ecological question being asked (Calow 1974). In this investigation, the focus is placed on an overall comparison rather than more involved complex explanations of habitats. *B. stenochorias* occured in different biotopes and was present during winter and summer. To understand what conditions affect their distribution, and appear to determine habitat requirements, it is necessary to compare sites, and abiotic factors that occurred during the time samples were taken. Biotope comparisons are of importance because they indicate a narrow range of factors which will affect the distribution of this limpet.

Sites

Botha River had the highest number of limpets during winter and summer (Figure 3.2.3.1 c and d). The area sampled was in a pool in a temporary river and no stones-in-current biotope existed. Because the river is temporary and low inflow of water occurred at the time of sampling, the pool of water evaporated as no rain fell, with a consequent shrinking of the habitat. Under these conditions the limpets move into a smaller area and thus have to exploit all habitats, possibly those they would normally not inhabit under optimal conditions. The no-flow conditions allowed the marginal vegetation to be exploited, including submerged sticks and leaves which had fallen from the overhanging trees. These had grown a layer of algae on the surface, providing the limpets with a food source. Egg capsules were also observed on these leaves. The size of the limpets varied with the range

Bloukrans River had the second highest population of limpets. In summer a very small number were found on stones-in-current. It has been observed that stones-in-current is a dominant habitat elsewhere, suggesting the low number may have resulted from a high rainfall just before sampling which had disturbed the habitat and washed the limpets downstream. In the winter months there was a comparatively low density of limpets in all biotopes, presumably as a result of abiotic factors in particular temperature. At the Botha river 11% of limpets were found on vegetation during summer, compared to 45% during the winter months.

The Bloukrans River is rich in organic pollution caused by agriculture runoff and human waste from Grahamstown. This high organic concentration provides nutrients for algal growth which in turn provides food for the limpets. There is a distinct size difference during winter (ave. size 3.26mm) and summer (ave. size 1.89mm) (see Figure 3.2.3.2 e and f). Limpets in the winter tended to be much larger than those in the summer as explained above. There is a narrower size range of limpets at Bloukrans site when compared to the limpets found in the Botha River.

The Palmiet River is a pristine natural river which flows all year round. Only 4 individuals were found here in March, and 1 in July (Figure 3.2.3.1 c). The size of the individual found in the winter sampling (3.5mm) was bigger than the size (ave. size 1.25mm) of the limpets found during summer.

Statistics show that there were no significant difference between the number of limpets found during change in seasons, between sites, but that there was a significant difference between sizes (Figure 3.2.3.2 b), d) and f)).

Biotopes

Stones-out-of-current were most densely populated with limpets in all sites. It can thus be assumed that limpets prefer this type of habitat. Stones-out-of-current provided the limpet with a solid surface, presumably enough oxygen and a protected, smooth surface to lay its eggs on. The last factor seems to be of importance because a species may be absent from a place because conditions, although not unfavourable, may be unattractive to the ovipositing female (Macan 1961).

Stones-in-current biotope had considerably fewer limpets (Fig 3.2.3.1). It must be kept in mind that the Botha River had no stones-in-current and that heavy rainfalls before sampling at Bloukrans River could have influenced the distribution of limpets; Eisenberg (1965) stresses that weather should not be ignored when determining the number of animals in their natural environments. Although this biotope provides a surface on which the limpets

live, graze, and lay their eggs, limpets were only found in this biotope in the Bloukrans River. This may have been due to higher flow rate limiting this habitat (Appleton, 1978; Macan, 1960). The water in this biotope was very well oxygenated. Oxygenated water was considered a key habitat requirement which affected *B. stenochorias* distribution. However, experiments done in the laboratory found that these limpets can survive well in aerated containers with no flow (H Davies-Coleman *pers.com.*). The stones-out-of-current biotope at Botha River contained the most limpets. Thus, it appears that if there is enough oxygen in a habitat it will survive despite the absence of flow.

Marginal vegetation was not a common biotope for *B.stenochorias* to live on, but during the winter as the pond at Botha River started to dry, the number of individuals on the vegetation increased as stones in the pond became fewer in number. A few individuals were found on vegetation at the Bloukrans River site but this was not usually observed. Costil and Daguzan (1995) revealed that fresh macrophytes were rarely eaten by snails but that the macrophytes supported growth of periphyton, which is a food source for limpets, and provided them with shelter from predators, egg-laying sites and a substratum on which limpets could crawl. Ndifon and Ukoli (1989) revealed that macrophytes with smooth surfaces were favoured for egg deposition and resting while plants with hairy surfaces were seldom used for either function. Thus, gastropods in general appear to prefer whichever smooth surface is available for certain activities. The limpets that did occur on these sticks were smaller than those that lived on stones.

Soft sediment appeared to be an unfavourable habitat for *Burnupia*. The only limpets found in this biotope were in the Botha river during winter. During this time limpets were at very high densities and it was possible that the limpets found in soft sediment could have fallen off the substratum they were attached to and recorded as occurring in this biotope. From direct observation limpets did not occur in soft sediment. If compared to other biotopes the reason for this is that no solid surface is available for egg-laying, feeding and movement of the limpet. Brown and Lodge (1993), in an experiment on gastropod diversity and abundance in areas of high and low macrophyte biomass, found that in the laboratory (given a choice of sand, macrophytes and rocks) gastropods preferred cobble (first choice) and macrophytes but avoided sand.

Abiotic factors

Various abiotic factors were measured in each site (Table 3.2.3.1). It must be stressed that these factors are generally not constant through the year and by only measuring them twice, no conclusive evidence as to why *B. stenochorias* has included or excluded a site as its habitat is revealed. Future research into the habitat

requirements of this species should include replicate sampling over the whole year, to get an accurate idea of conditions prevailing in a given site. If physico-chemical factors are not optimal a species could find itself in competition with others which are better adapted to the conditions (Dussart 1979).

Appleton (1978) found that bilharziasis intermediate host snails (Planorbidae) have a broad tolerance range to field water temperatures. *B. stenochorias* occurs in the whole of South Africa with the exception of the Western Cape (F de Moor, Albany Museum *pers. comm.*). The wide distribution of this limpet could indicate a broad tolerance of temperature as Northern parts of South Africa have warmer rivers when compared to the Southern parts i.e. the Cape. This suggests temperature was not a limiting factor in any of the sites. Macan (1960) gives numerous reasons why organisms can not invade warmer or cooler waters than what they are used to, e.g. warm water never reaches low enough temperatures to stimulate reproduction. It was observed that *B. stenochorias* laid eggs at the end of winter suggesting low temperatures are necessary for egg laying. To investigate this further, a countrywide survey will have to be done to compare number of limpets in rivers with varying temperatures.

Macan (1960) and Appleton (1978) explain that snails have a remarkably low tolerance to a range of current velocity. The reason for this may be that snails cannot remain attached to a substratum when exposed to a flow rate greater than 0.3 m/s. Allan (1995) believes that water velocity and the associated physical forces collectively represent the most important physical factor affecting animals in rivers. This suggests that current velocity (flow rate) may influence the distribution of *B. stenochorias* in a river. Botha River had a very low flow at the time of sampling. Table 3.2.3.1 shows that the Bloukrans River had a faster flow in both seasons than that of the Palmiet River. The summer flow was very high due to a high rainfall before sampling in the Bloukrans River and this suggests that current velocity does affect the distribution of limpets because observations have shown that under a slower water velocity there are more limpets present in this river on stones-in-current (H. Davies-Coleman *pers com.*). The flow rate during summer in the Palmiet River was also higher than in the winter but this did not affect the number of limpets found, because during both seasons, no limpets were found in stones-in-current. Thus, the low number of *B. stenochorias* found in this river may be due to another factor. This suggests that the habitat requirement for the limpet in terms of current strength is a slow current, which does not wash the limpet off its substratum, or even a pond with no current as in the Botha River.

Allan (1995) explains that the total dissolved salts (TDS) content of fresh water is the sum of the concentrations of the dissolved major ions. TDS in the Botha and Bloukrans River was relatively high at an average of 500mg/l. The Palmiet River had a very low TDS in comparison to this with an average of 81mg/l. TDS of 360mg/l did not affect the snails themselves but prevented development and hatching of eggs. *B. stenochorias* appears to be much more tolerant to a higher TDS. The fact that the Palmiet has such a low TDS may be the reason for the low number of limpets found at this site. TDS also affects the growth of aquatic plants and by direct observation the

Palmiet River seemed to have much less algal growth on stones than the other two rivers. The habitat requirement in terms of TDS is one which is relatively high i.e. 400-500mg/l.

The water chemistry results (Table 3.2.3.2) indicate that Botha River and Bloukrans River are similar in relation to the sodium and magnesium concentration, with calcium concentration being almost double in the Bloukrans River. The Palmiet River has much lower concentrations of ions. Macan (1960) explains that while calcium is a necessity for the shell of the limpets, little is known about how calcium affects distribution. Shells are thinner in softer water. However, the Palmiet River is very low in calcium compared to the other sites where limpets were found. It can be assumed that calcium is a requirement. Macan (1960) could only find a correlation between calcium and mollusc populations, the other ions appear to have little influence on the distribution of the mollusc.

This is not surprising as the pH of a river does not vary extensively because only when the pH is below 5 that biological consequences are serious (Allan 1995). The pH did not vary among sites (range 6-8) with the average pH being neutral.

Conclusion

After comparing the sites, the biotopes and abiotic factors measured at these sites the following similarities were found in places were *B. stenochorias* occurred :

- substrate - a firm surface to which the limpet can attach, lay its eggs and feed on algae; these surfaces can include stones or less commonly vegetation.
- flow rate - the limpets in this investigation were found mainly on stones-out-of-current, thus as low flow or even no flow is suitable.
- temperature - between 10 and 25°C (the extreme tolerances are not known).
- TDS - many limpets occur where TDS is 500mg/l.
- pH - neutral pH is favourable.

These criteria can be seen as habitat requirements for *B. stenochorias*. Future studies on the habitat of these limpets can include water chemistry, and biotic factors such as competition and predation which may influence the distribution of this organism.

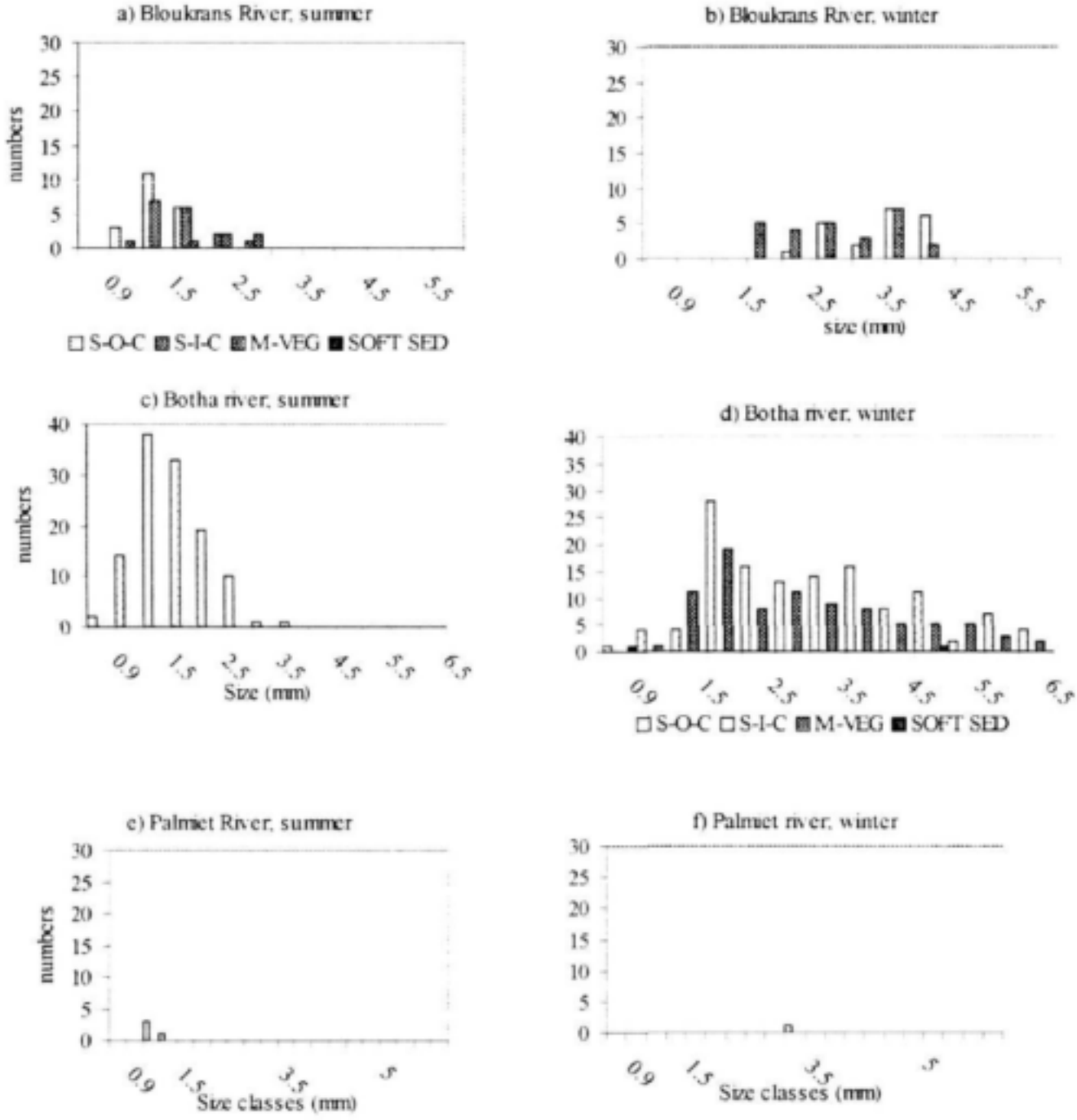


Figure 3.2.3.2 (a-f) The range of sizes of *B.stenochorias* limpets occurring in each of four biotopes in three Eastern Cape rivers during the summer and winter months in 1996

3.3 EXPERIMENTAL

3.3.1 LABORATORY GROWTH CONDITIONS

The investigation into the laboratory conditions necessary for optimal growth of limpets, initiated in 1994 and reported in Haigh and Davies-Coleman (1996), was continued. Water conditions are particularly relevant to the growth of the diatoms, considered the primary source of food for many groups of benthic macro-invertebrates, An investigation into the growth of diatoms in artificial streams was completed by Mrs M. Balarin in partial completion of her Botany Honours year and is reported in section 3.5. Results from the growth of the limpets during this experiment and discussion are included below, together with a summary. Due to the extent of the work involved and the time constraints of an Honours student, the day to day maintenance, water chemistry and chlorophyll analysis, as well as measuring of limpets was undertaken by H Davies-Coleman.

Introduction

To date, the food preference of *B. stenochorias* is unknown, but it is thought that it grazes on filamentous algae, diatoms and possibly fungi, lichens and bacteria. Hunter and Russel-Hunter (1953) report that analysis of the gut content of certain limpet species of the family Ancylidae indicated a micro herbivorous habit. Observations within the laboratory show *Burnupia* sweeping their radulae over the surface and collect apparently a large percentage of the periphyton growing on the surface (as explained in Section 3.3.2). This study investigated the effects on algal assemblages of these grazers at a high density, as well as the suitability of these laboratory conditions for the growth of the algae as a food source for *B. stenochorias*.

Much of the literature refers to the effects that grazers have on the periphyton community (*e.g.* Bronmark, 1989; Calow, 1974; DeNicola *et al.*, 1990; Lamberti *et al.* 1984), being either negative or positive, depending on the herbivore species or the algal species consumed (Steinman *et al.* 1989). Lamberti and Moore (1984) summarise the effects of grazing on biomass and productivity of periphyton in a generalised conceptual model. They suggest at very low grazing pressures the biomass of the periphyton is high and productivity moderate. When the densities of grazers increase, the biomass declines, whereas the productivity increases to a maximum at the intermediate grazing pressure. Further increases in grazing pressure result in overgrazing and both the biomass and productivity decreases to a minimum.

This project sought to clarify the process within 6 of our laboratory streams, with 3 streams containing limpets to monitor the effects of the grazing. Diatoms in samples used for inoculation of the artificial streams were enumerated according to the Utermohl technique. Diatoms growing on the plastic substrate in the streams were counted and identified to species level, where possible, using scanning electron microscopy. Results were analysed using the programme PRIMER.

Results and Discussion

The majority of results pertaining to diatom growth is reported in section 3.5, but other results, a summary and discussion are given below.

The sequence of colonisation from bacteria through to the stalked and filamentous diatoms was illustrated, with a slight decrease in species richness (Margalef) and diversity (Shannon-Weaver Diversity Index) over the 40 day period of grazing. *Burnupia* did not have a discernable effect on the periphyton assemblage. The variability in the diatom data was such that there was no significant relationships between the control streams (< 14 days) and grazed and ungrazed streams (> 14 days). Dominant genera throughout the whole experiment were *Achnanthes*, *Cocconeis* and *Navicula*.

Water chemistry, and in particular pH, plays a vital role in the assemblage of diatoms. At very acid or very alkaline pH values, the flora may be restricted in terms of numbers of species, although not necessarily in terms of abundance of species. Diatoms not only adapt themselves to an optimum pH, but also exhibit varying degrees of tolerance to pH levels on either side of their optimum. Cholnoky (1968) in particular has pointed out the importance of pH fluctuations as a limiting factor in the distribution of diatoms. Any drop in pH will change the CO_2 to CO_3^-, unavailable for the diatom growth. All streams in this experiment showed a 1 point drop in pH over a 7 day period, and this must be avoided when culturing limpets for use in the toxicology experiments. It is suggested this can be achieved by changing 10% by volume of the water in each rearing vessel, also recommended by Pieterse (*pers com*) as a means of supplementing nutrients for the diatom growth.

Rainwater is not considered suitable as the water source by Pieterse (*pers com*), as it probably would contain spores of unwanted species picked up from the air. Pieterse suggests that dechlorinated tap water is sufficient for the growth of the diatoms, with no further nutrients being necessary, except for the addition of silica in the form of sodium silicate. This was achieved in the diatom trial by dissolving the silicate in tap water and pouring small quantities into each channel at weekly intervals, according to the recommendations by Stein (1973). The silicate results of samples taken throughout the experiment, and analysed by Port Elizabeth Municipality, indicate sufficient quantities present in the streams' water for successful diatom growth (Table 3.3.1.1) Silicates contribute to the alkalinity of the water, and although alkalinity has no effect, *per se*, on the growth of animals or plants, it is important in determining the effect and concentrations of other water quality criteria, and therefore the general suitability of the water source for culturing.

Decrease in the total number of cells through the six channels may have been related to an unfavourable change in the chemical conditions of the streams, as indicated by the increase in all streams of the phosphate levels to 10x the South African recommended levels for freshwater habitats (Dallas *et al*, 1994); see Table 3.5 3), although these did decrease in concentrations by the end of the experiment. Although filamentous algae

were not seen during the diatom experiment, high nitrogen (not seen in these streams) and phosphorus concentrations are known to encourage filamentous algal growth, unsuitable for the limpets. Balarin found no correlation between chlorophyll *a* and the level of orthophosphates ($R^2 = < 0.5$), total dissolved salts ($R^2 = < 0.3$), and pH ($R^2 = < 0.3$) in any of the channels, using standard regression analyses.

Data not included in Balarin's writeup are the metal ion analyses, and the limpet growth and mortality, given here in Table 3.3.1.1 and Figure 3.3.1.1.

Table 3.3.1.1. Range of approximate metal ion concentrations in the 6 channels, in mg/litre3.5 month period. * refers to two extreme values of 21 and 23mg/litre silicon oxide.

Date	Potassium	Sodium	Magnesium	Calcium	SiO_2
11/4	4 - 12	44 - 72	9 - 15	12 - 17	10 - 11
19/4	6 - 10	44 - 72	9 - 12	13 - 17	9 - 14
30/4	6 - 10	52 - 104	10 - 14	18 - 26	9 - 25
29/5	4 - 10	100 -194	8 - 20	16 - 32	
14/6	11 - 12	96 - 152	14 - 28	24 - 34	6 - 26
8/7	10 - 12	104 - 174	13 - 25	32 - 52	1 - 23*
31/7	11 - 12	108 - 154	15 - 25	30 - 50	1 - 21*

The ratios of metals ideal for the growth and particularly reproduction of limpets must be low for calcium/magnesium (Harrison and Shiff, 1966) and high for sodium/calcium (Appleton, 1978), with the level of calcium ideal at approximately 10mg/l, below which calcium fails to be absorbed by the majority of snails (van der Borght and Puymbroeck, 1966). The metal ion levels in these streams remained at these suitable ratios. Calcium was added to the streams in the form of chalk (calcium carbonate) dissolved in tap water at irregular intervals (on the advice of Appleton, *pers com*).

The biological filters, in the sumps and made of compacted masses of fine strands of plastic, were found to be very successful in filtering out sloughed-off algae and other debris from the water. Unicellular cells such as *Chlamydomonas* species are not collected by the filters, but they are not considered to be a problem to the systems, or to the limpets which very likely scrape them off the surface and possibly use them as a food source as well.

The light levels reported by Balarin in her project writeup are possibly too low for the successful growth of either the diatoms or the limpets. Since then, the channels now have double the lighting previously available,

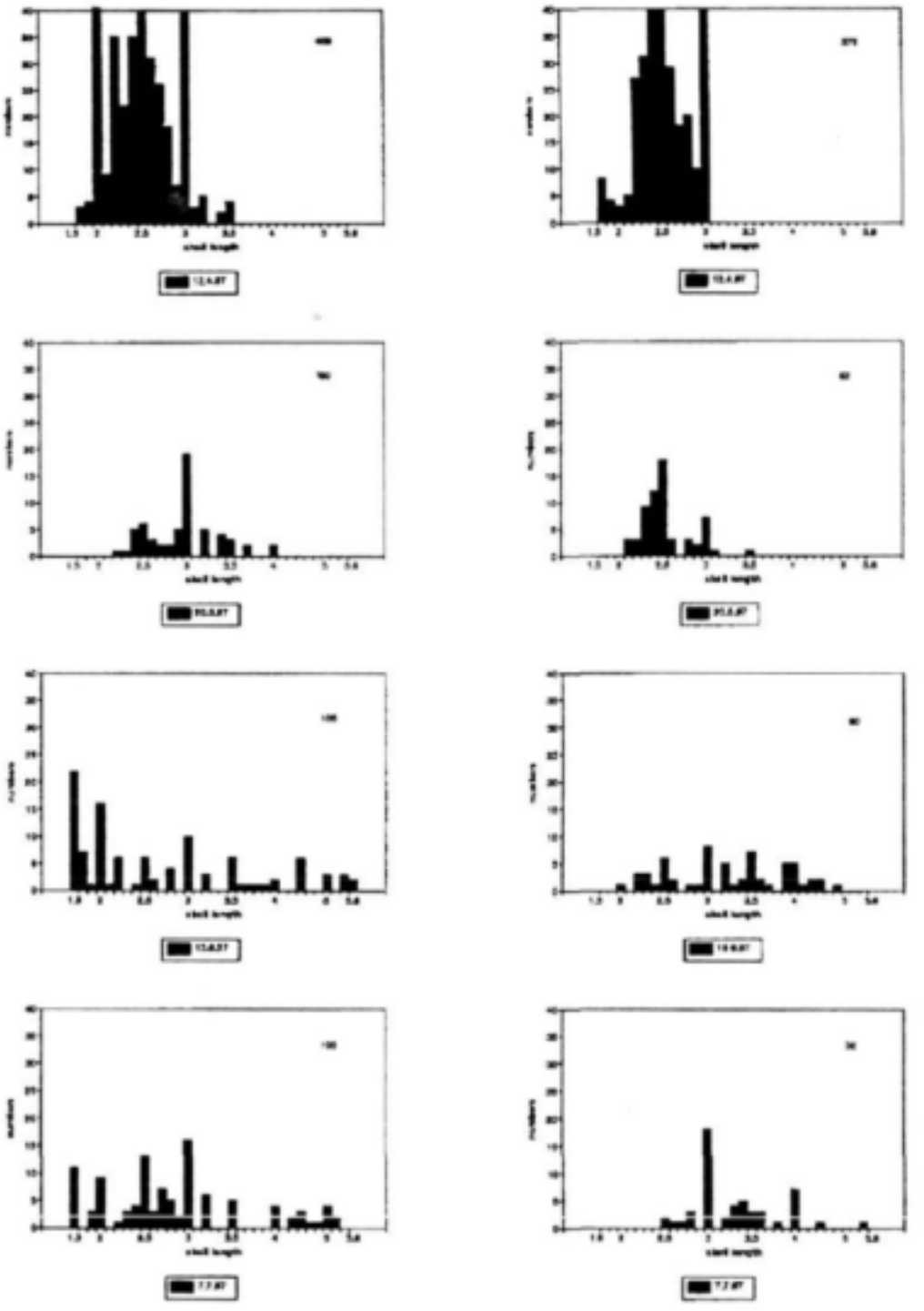
reading at an approximate photon flux density of 120mol/m^2/s. It has been suggested by Pieterse (*pers com*) that the dark colour of the channels will decrease the likelihood of filamentous algae. This would be a disadvantage of the white 5 litre plastic pots used extensively in the laboratory. Darkening the outside of the pots to decrease the outside incidence of light intensity is to be done where the limpets are to be cultured.

The plastic surface of the channels (and any other container used) should not curtail the growth of the diatoms. At initial seeding, the diatoms would have moved from the water to the sides of the channel within 6 hours (Round, *pers com*), and remained attached to the plastic. Similarly, any bacteria would be unlikely to remain in the water body itself, but attached to the plastic sides.

There are a number of points to be noted with regard to the channels with limpets added as part of the grazing experiment, seen also in Figures 3.3.1.1 and 3.3.1.2:

1) The numbers of limpets displayed in the corner of each graph give an indication of the high mortality experienced in all three channels. This is as a consequence of the unavoidable handling of the limpets, *ie* removal from the original place of hatching, being measured and then placed in the (new) channels.
2) Initially, the densities of limpets within each channel had to be high, to see any effects of grazing. However, 1200 limpets (400 in each channel) of small sizes was extremely difficult to organise and find all at one time: consequently, there was a large variation in size of those initially placed into the channels. However, most were below 3mm *ie* pre-egg-laying.
3) Variable growth was seen, with the first eggs hatching from newly laid egg capsules approximately 8 weeks later. All channels had limpets which reached the large size of 5-6mm in length by the end of the experiment, indicating the suitability of the conditions provided. However, despite the large number of capsules laid, very few hatchlings reached a size in excess of 1.5 mm, which was easy to measure, without touching the limpets in the large streams. This confirms previous attempts to rear young limpets in these streams, indicating that, for a high success rate of hatched limpets reaching adult size, an alternative means of mass rearing the limpets from hatching will be necessary.

Over a two year period the maintenance of the channels consisted of the addition of distilled and conditioned tap water once a week, the addition of chalk every month and the occasional addition of a few drops of water plant fertiliser. The amount and type of algal growth varied through time waxing and waning but there was never and excessive growth of filamentous algae. Toward the end of the second year sewerage fungus appeared. The channels were then emptied, washed out with clean water and the biofilter rinsed through and exposed to sunlight for a few hours. After that they were assembled again with tiles from one of the other channels as seed. Within a week, diatom growth was sufficient to sustain a new limpet population.



a)

b)

Figure 3.3.1.1. Number of limpets in channels 1 (a) and 3 (b), where the effects of grazing on the diatoms was monitored

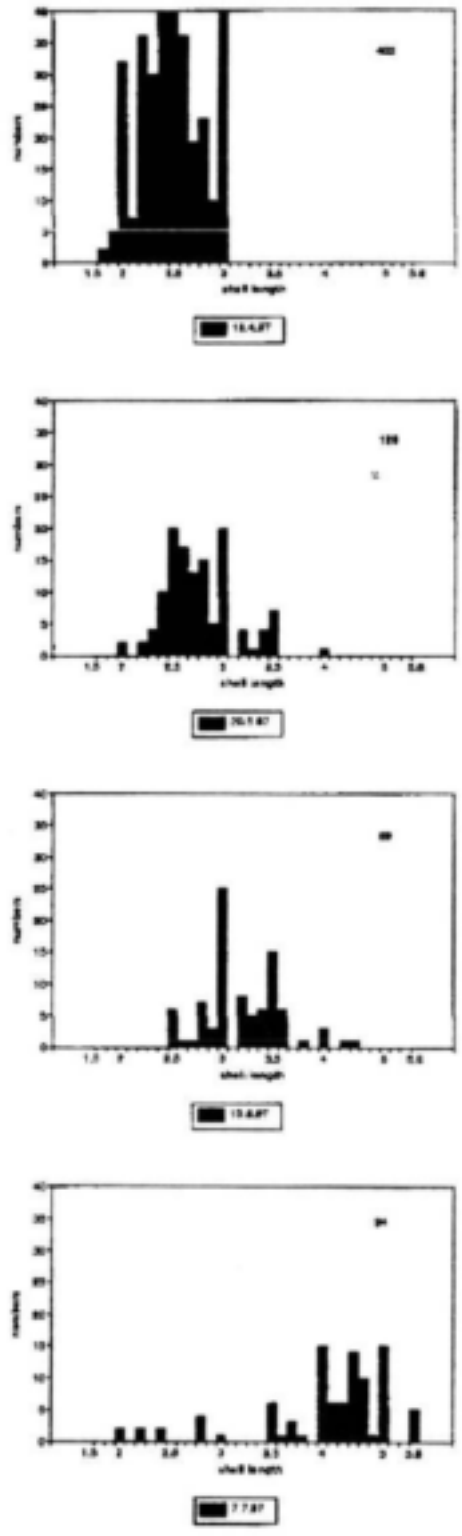


Figure 3.3.1.2 Number of limpets in channel 5, the third replicate where the effects of limpets grazing on the diatoms was monitored.

3.3.2 EFFECTS OF DIET ON GROWTH AND REPRODUCTION

(the assistance of Dr A. Booth with statistical analyses of the growth data is gratefully acknowledged)

Aim

To assess the effects of three feeding regimes on the growth rates and fecundity of *B. stenochorias*, grown in the laboratory.

Introduction

One of the major problems encountered in the successful maintenance and breeding of freshwater gastropods in the laboratory has been the selection of suitable foods satisfying the basic requirements of a well-balanced diet.

Food is generally accepted as a major component of a population's environment. As with temperature, it is also considered to be a control parameter with significant interactions on rate of growth, and differences in growth rate directly affect duration of life-cycles, size attained at maturity (and therefore fecundity) and survivorship (Anderson and Cummins, 1979; Waters, 1979). The problems of quality and quantity of food are likely to arise within laboratory cultures, particularly where there may be maximum densities, with the possibilities of food shortages occurring. At two field sites, where separate populations of limpets were closely monitored for a year, it became clear that dissimilar sizes were achieved by the resident populations. Examination of the stones on which the limpets fed showed a difference in the dominating species of algae (namely unicellular diatoms versus diatoms and filamentous algae). This gives an indication of the possibility of inadequate nutrient supply with the laboratory-cultivated periphyton for the limpets' sustained growth and energy requirements for growth and reproduction; hence, it was decided to find a more suitable feeding regime that would be readily acceptable to both hatchlings and adult limpets with maximum egg production.

Eisenberg (1970) showed very clearly with *Lymnaea elodes* that the inverse relationships between snail density and mean size, mean number of eggs per egg mass, as well as total eggs were found to disappear with food additives. Food additives appear to also have improved the survivorship of the snails, and he presents evidence which indicates that the food limitation in this snail is one which probably involved accessory growth factors. Similarly, Tetramin for young fish has been successfully used in the feeding of newly hatched *Biomphalaria* in the snail breeding unit at Potchefstroom University (Jennings *et al*, 1970).

Burnupia is presumed to be hermaphroditic, as with all other Ancylidae, and consequently the innate capacity for increase is difficult to determine. Unlike the case for single event spawners, where the determination of the gonadosomatic index and fecundity can be established from sacrificed animals, the establishment of fecundity

in serial spawners necessitates the collection of live eggs as they are deposited. The matter is further complicated by the ability for self fertilisation to take place, with both partners theoretically able to lay eggs, because the limpets are hermaphroditic.

In this experiment the effects of the feeding regime on fecundity and breeding biology were addressed in terms of:

1. the fecundity:
    - ♦ total number of eggs per limpet/ average number of eggs per treatment
    - ♦ average number of eggs/ capsule/ limpet/ treatment
2. the breeding biology
    - ♦ age at sexual maturity and reproductive period
    - ♦ reproductive period as a proportion of life span
    - ♦ egg-laying patterns

Method

1) 12 five litre aerated buckets lined with new plastic bags and filled with de-chlorinated tap water, were set up in the Controlled Environment room at 20°C, 12 hours L/D. Algae seeded from one mixed source, taken from numerous scrapings of rocks in the local river where limpets are known to flourish, were cultivated on the plastic. Dechlorinated tap water was also used as the top-up water source.
2) Into each were placed 20 newly hatched limpets, previously measured for shell length. Four replicates of each were subjected to one of the following feeding regimes:
    a) diatoms cultivated previously from the river water.
    b) Nutrafin Fish Food. The ingredients are fish meal, fish liver, roe, milt, brine shrimp, egg yolk, aquatic plants, kelp, day fly eggs, oatflour, wheatgerm oil and cod liver oil. The guaranteed manufacturer's analysis is:

crude protein	46%
fat	5%
fibre	8%.

    c) previously cultivated diatoms and Nutrafin Fish Food.

The limpets were second generation limpets from field collected parents. The parent limpets were allowed to lay eggs in the laboratory for a two week period.

3) All limpets were grown to adult size, approximately 3,5mm (53 days), and shell length measured weekly. Food was not a limiting factor during this time. Mortality rates and water quality in terms of pH and TDS were monitored.

4) When the first egg capsules were laid, paired limpets were taken from those replicates that had no capsules, and placed on one tile (previously seeded from the same source as above) per pair in separate buckets, suspended within the water with fine netting. This was to discourage the movement of the limpets from the tiles. The feeding regime previously used for each pair was maintained, with the exception of the Nutrafin Only (as algae had grown to approximately the same amounts in the original containers as in the Nutrafin and Algae replicates). As soon as an egg capsule was observed for each pair of limpets, the limpets were separated again and one limpet was placed in a plastic bag in the same bucket as the partner. These plastic bags had periphyton previously cultured on the surface. All tiles and bags were inspected for capsules every two days under a stereo-microscope. In the bags the eggs could be counted immediately. The capsules on the tiles were marked by date and the eggs were counted when the embryos became visible (usually 3-4 days).

5) The experimental duration was 5 months, considered complete when the last limpet died.

6) Curvilinear and linear models using the least squares method were fitted to the limpet sizes over time, up to the point of mating and separation, to test for linearity. Growth rate models were drawn for each feed, and individual ANCOVA's tested for any significant differences between the slopes.

7) For each treatment the fecundity was calculated as eggs per limpet per day; average eggs per limpet per week and total number of eggs per limpet per reproductive period. Average population fecundity figures include all limpets from each treatment. The intrinsic fecundity is taken to be the number of eggs per capsule. The fecundity rate is the number of eggs deposited in a given period. The number of eggs/capsule for individual limpets and for treatments were compared by ANOVA. The life span, reproductive period, the total number of eggs and the total number of capsules were compared by ANOVA and Kruskal-Wallis test for each treatment. The pattern of egg deposition was analysed by periodogram and autocorrelation. All statistical analyses were conducted using STATGRAPHICS 5.

Results

Water quality.

Although time constraints disallowed the nutrient analysis of water to be regularly completed, calcium did not appear to be a limiting factor in terms of growth, judging by the condition of the shells. Dissolved oxygen (DO) levels were not monitored as previous experience has shown the level of aeration provided was sufficient to maintain the DO levels above 85%.

Table 3.3.2.1 Maximum, minimum and average pH and TDS values for each treatment, replicates combined.

	pH (1)	TDS (1)	pH (2)	TDS (2)	pH (3)	TDS (3)
maximum	7.73	550	7.83	583	7.91	548
minimum	7.03	244	6.81	239	6.66	236
average	7.33	351	7.36	372	7.36	364

Cross-correlations of pH and TDS with weekly growth, within each replicate, indicate no correlation ($p > 0.1$), suggesting variation of the pH or TDS within the time of growth had no significant influence on growth.

Algae. Sampling of the periphyton growing on the plastic bags or on the tiles for identification or cell counts was not carried out. However, superficially the periphyton appeared to be very similar to that grown in a different experiment, seeded from the same source and grown under relatively similar conditions, completed by the Honours student Mrs M.Balarin. Her investigations showed diatoms dominated the periphyton both at the source of seed and in the laboratory, the dominant genera being *Navicula, Gomphonema, Achnanthes,* and *Cocconeis*. Some fungal mycelial threads and colonies of bacteria were also present in her trials. With the addition of the Nutrafin within this feed trial, and the likelihood of substantial bacterial growth, as indicated previously, we assume that diatoms and bacteria played the major roles in food source for the limpets.

Growth and Size.

Table 3.3.2.3 shows the average size of limpets reared on the three diets, with replicates amalgamated. The standard deviation reveal the large degree of variability in growth and ultimate size attained .

Table 3.3.2.2 Mean size and standard deviation (s.d.)of limpets over time (days) within the three diets (replicates amalgamated). n = number of limpets.

Time	Diet 1		Diet 2		Diet 3	
	n	$\bar{x} \pm$ s.d.	n	$\bar{x} \pm$ s.d.	n	$\bar{x} \pm$ s.d.
1	215	1.22 ± 0.31	249	1.09 ± 0.29	233	1.09 ± 0.26
12	140	1.82 ± 0.39	254	2.16 ± 0.47	225	2.03 ± 0.5
18	120	2.24 ± 0.47	242	2.56 ± 0.47	213	2.09 ± 0.57
29	118	2.65 ± 0.65	219	2.93 ± 0.68	193	2.69 ± 0.54
43	114	3.81 ± 0.78	187	3.37 ± 0.65	174	2.95 ± 0.54
53	101	4.42 ± 0.78	174	3.81 ± 0.47	145	3.62 ± 0.71

The initial curvilinear and linear model testing showed the linear model to give the best fit for the data especially over 53 days. Regressions of the sizes attained in the three feeding regimes, and subsequent ANCOVA's to test for equality of slopes (with a significant $F_{(obs)}$ value) indicate a significant difference in the slopes:

	Model	r_2
Nutrafin (Diet 1)	y = 0.0611x + 1.1075	0.794
Nutrafin & Algae (Diet 2)	y = 0.0586x + 1.1418	0.746
Algae (Diet 3)	y = 0.049x + 1.3889	0.713
	(F_{obs} = 10.97, $p < 0.05$, df = 2,3311)	

The r^2 values confirm that the growth of the limpets within each diet is linear(Figure 3.3.2.2). If the growth of individuals within each diet is compared with those in the other two diet using ANCOVAS the results of the F ratios given below indicate growth 1(Nutrafin only) > growth. 2 (Nutrafin +algae) >3 (algae only). Results of the individual ANCOVA's conclude that Diet 3 was significantly different in the growth of the limpets to Diets 1 and 2:

1 - 2	F = 2.956	p > 0.05	(1,2130 df)
1 - 3	F = 74.3	p < 0.05	(1,1988 df)
2 - 3	F = 54.04	p < 0.05	(1,2525 df)

Mortality

A large variation in the mortality rates is shown in Figure 3.3.2.1when the numbers of survivors in the three feeding regimes are compared. Initially the NUTRAFIN only diet shoed a higher mortality rate but this decreased after 2weeks. This did not occur on the other two diets

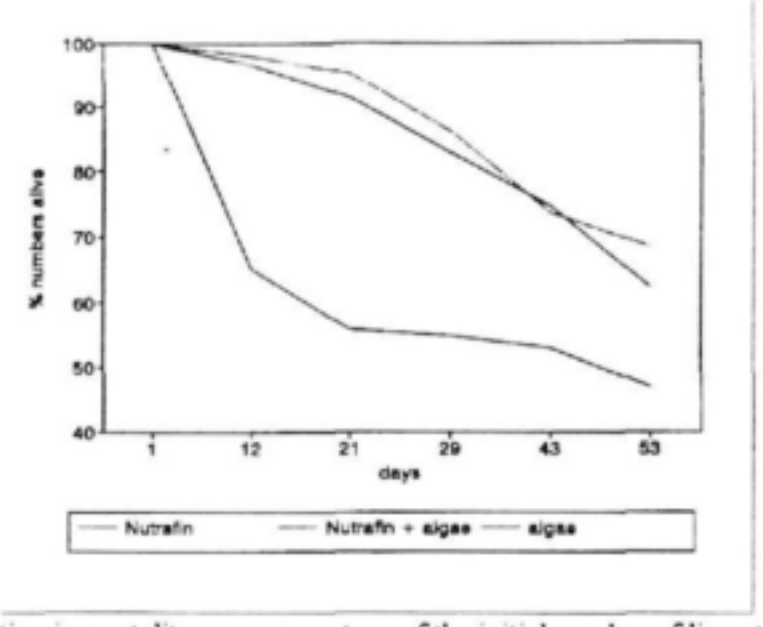


Figure 3.3.2.1 Variation in mortality, as a percentage of the initial number of limpets, between the three feeding regimes.

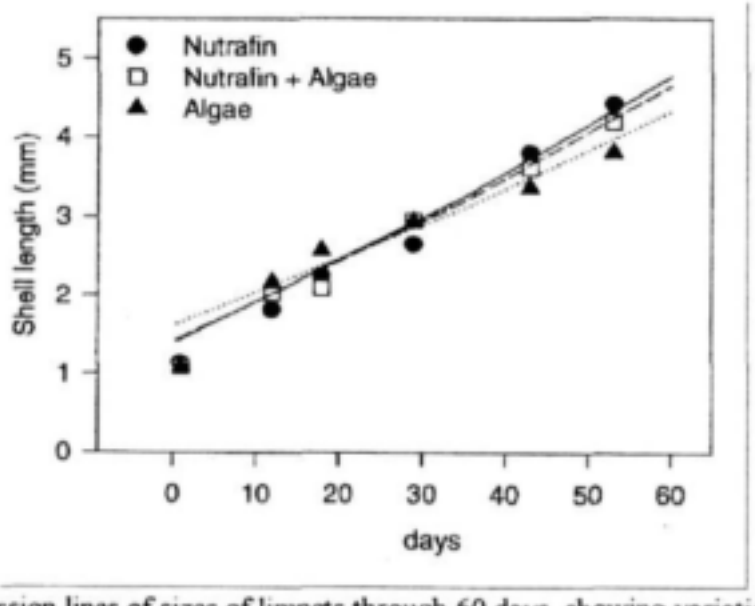


Figure 3.3.2.2 Regression lines of sizes of limpets through 60 days, showing variation between the three feeding regimes.

Fecundity

Table 3.3.2.3 emphasises the extreme variability in the fecundity of this species under experimental conditions resulting from both intrinsic variation and early mortality. Intrinsic variation of such aspects as the numbers of eggs per capsule, and the number of, and frequency with which capsules are deposited, resulted in a wide variation in the number of eggs per day (0.3-7.58), while early mortality accounted for fewer eggs due to shortened reproductive periods.

It is evident from Figure 3.3.2.3. that the laying activity is not consistent among limpets. Some limpets (2a2, 2a4, 2b4 and 2b11) demonstrate greater laying towards the end of the life span, while others decrease the number of capsules laid with time. Fewer than 20% of the population survived after nine weeks and all limpets in treatment 3 (algae) died by the end of the experiment. If the infertile animals and limpet 2a3 (562 eggs) are disregarded (n=28), limpets with a lifespan of between 12 and 32 weeks can lay between 5 and 326 (avg 105 (14.52)) eggs in a period of 5-128 (avg 38 (4.95)) days at a deposition rate of between 1.8 and 7.6 eggs per day. Inclusive calculations (n=34) result in an average lifespan of 154 (5.0) days, with an average of 103 (19.4) eggs in an average reproductive period of 36 (5.63) days (taking into account that the mortality rate was high).

Table 3.3.2.3. Fecundity values recorded for all the limpets used in the experiments. All decimal places rounded up to one and standard error to two. RP = reproductive period (days), LS = life span (days), se = standard error. #eggs/day = # eggs divided by reproductive period.

Rep	size (mm)	RP # days	# eggs	# caps	avg eggs/cap	# eggs/day	LS(days)
2a1	5.5	28	70	16	5.0(.63)	2.5	143.5
2b1	6	42	114	21	5.4(.51)	2.7	154
2a2	5.3	38	134	24	5.4(.28)	3.5	147
2b2	5.0	0	0	0	0	0	77
2a3	5.8	132	562	81	7.0(.26)	4.3	210
2b3	5.5	70	191	45	4.2(.36)	2.8	154
2a4	4	39	56	14	4.0(.25)	1.4	154
2b4	5.3	27	40	11	3.6(.47)	1.5	140
2a5	5.5	29	91	13	7.0(.37)	3.1	140
2b5	6.0	70	184	34	5.4(.39)	2.6	154
2a7	5.8	40	178	27	6.6(.48)	4.5	154
2b7	5.3	43	326	37	8.8(.43)	7.6	154
2a8	5.3	14	32	5	6.4(.75)	2.3	119
2b8	5.8	44	189	24	8.0(.68)	4.3	147
2a10	5	17	5	5	1(.63)	0.3	91
2b10	5.3	17	31	7	4.3(.88)	2.1	84
2a11	6.0	128	234	40	5.9(.33)	1.8	224
2b11	5.5	50	106	27	4.2(.41)	2.1	196
3a1	5	28	86	15	5.7(.32)	3.7	112
3b1	4.8	20	36	7	5.1(.40)	1.8	98
3a2	5	74	149	28	5.3(.39)	2.0	154
3b2	5.5	0	0	0	0	0	154
3a3	5.3	42	105	20	5.3(.53)	2.5	147
3b3	4.5	0	0	0	0	0	119
3a4	5.5	55	146	26	5.8(.48)	2.7	147
3b4	5	13	34	7	5.0(.26)	2.6	105
3a5	5.5	64	167	28	6.0(.29)	2.6	140
3b5	5	30	62	10	6.2(.53)	2.1	112
3a6	5.3	26	90	14	6.4(.46)	3.5	119
3b6	5.5	11	40	8	5.0(.78)	3.6	105
3a7	4	6	11	4	2.8(.11)	1.8	119
3b7	4.3	0	0	0	0	0	140
3a8	4	5	28	5	5.6(.68)	5.6	119
3b8	4.3	0	0	0	0	0	112

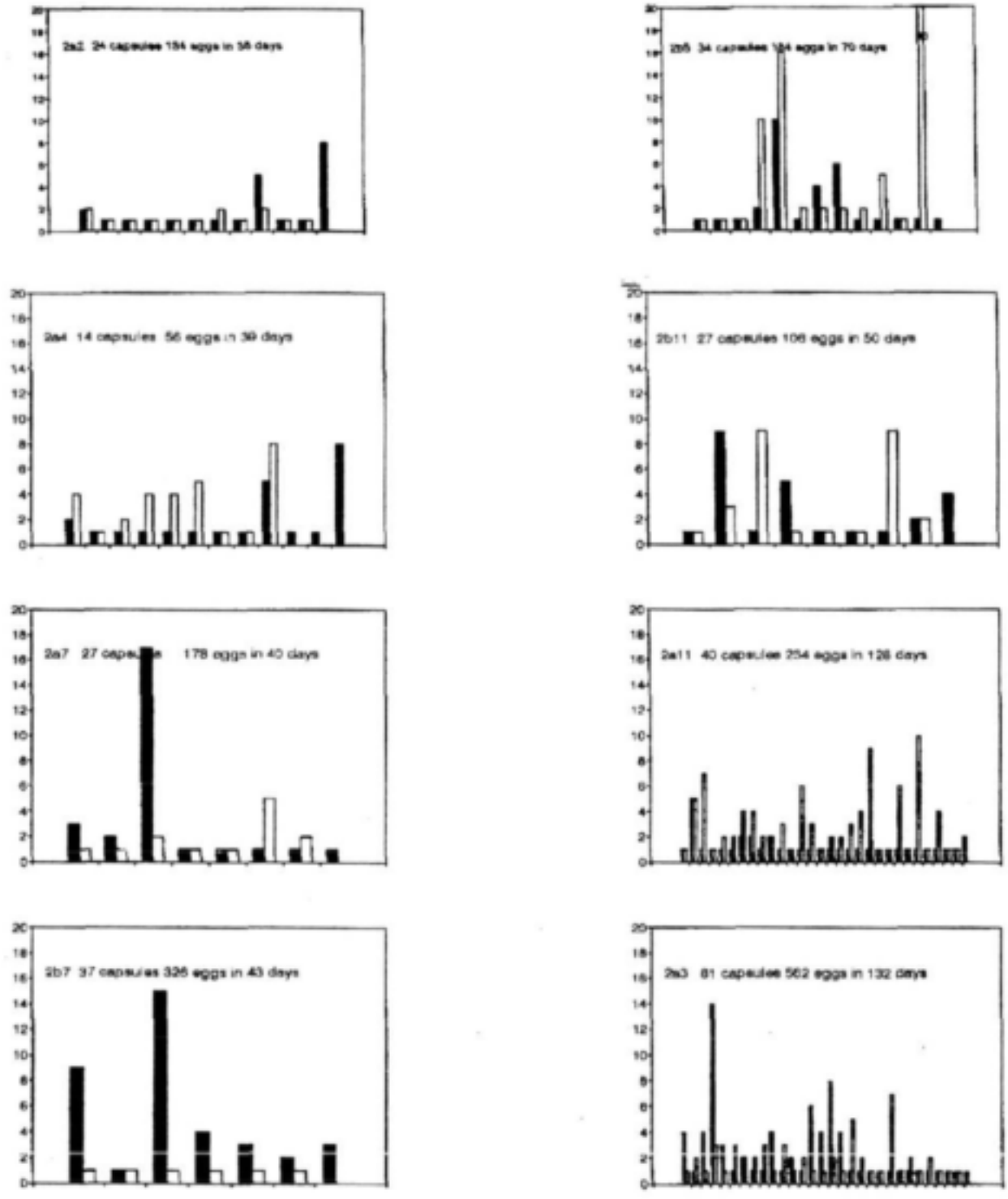


Figure 3.3.2.3. Pattern in egg laying periodicity of individual limpets in treatment 2. The active days are black and the rest days clear.

Table 3.3.2.4. Average fecundity values for the two treatments with all limpets included.

RP = reproductive period. se = standard error.

Treatment	avg.RP(se)	min/max RP	ave # eggs(se)	max/min # eggs	avg.# cap.(se)	min/max cap.	eggs/ cap.(se)	min/max eggs/cap
2 (n = 18)	46.8(8.5)	14/132 days	141(32.17)	5/562	23.6(4.55)	2/81	5.19(.52)	1.0/8.8
3 (n = 16)	23.6(6.1)	5/74 days	59.6(14.41)	11/167	10.7(2.49)	4/27	4(.63)	2.8/6.4

Table 3.3.2.5. Average fecundity in two treatments with outliers excluded.

Treatment	RP	min/max	# eggs	min/max	#capsules	min/max
2 (# 16)	43.5(6.9)	27/132	123.8(21.89)	40/326	21.9(3.1)	11/81(40)
3 (# 12)	31(6.6)	11/74	79(15.29)	36/167	14.3(2.6)	7/28

Table 3.3.2.6. Average population response in the two treatments.

Treatment	Ave. longevity	% Infertile	% low fertility	% of lifespan reproductive
2 (# 18)	21(1.28) weeks	5%	16%	28.5
3 (# 16)	18(.67) weeks	25%	31%	24.7

Breeding biology

Mating was observed infrequently and usually took place early in the day. No stacked mating (Bondesen, 1950) has ever been observed in this species in any of the experiments. In this experiment sexual maturity was reached 10 to 12 weeks after hatching and the mode for life span was 22 weeks; eggs are therefore possibly produced for about 50% of the life span of a limpet. The average productive period recorded and calculated in this experiment is between 24% and 27% of the recorded lifespan. Limpets (2a11, 2a3) with life spans in excess of 28 weeks also reproduced the largest numbers of eggs (562, 234) although the animal with the highest intrinsic fecundity rate (7.58 eggs/day and 8.8 (0.43) eggs/capsule) lived for 22 weeks, which was closer to the average (Table 3.3.2.3.).

The laying frequency and pattern of the limpets is characterised by spurts during which at least one and sometimes more capsules are deposited every night followed by rest periods. Although an attempt was made to establish the periodicity by periodograms, it was not successful. Figure 3.3.2.3 gives an indication of the laying pattern which was recorded in treatment 2 at 20°C. The number of sequential laying days are

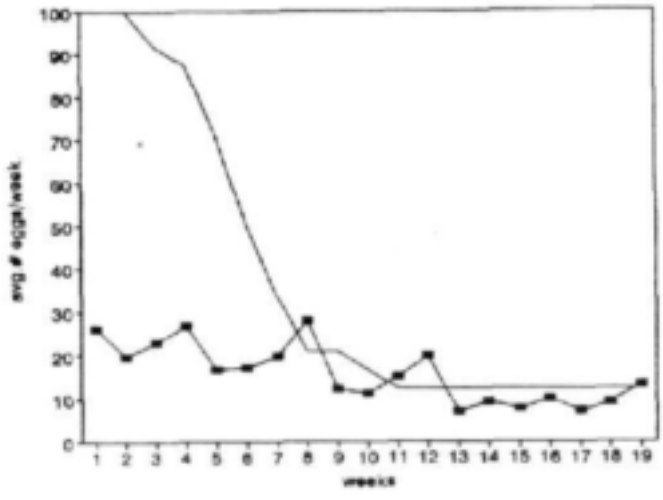


Figure 3.3.2.4. Average number of eggs per week for all fertile limpets, with the percentage of survivors each week (solid line)

contrasted with the following number of rest days. As can be seen, the pattern is not universal and some limpets show periods of high activity early on in the laying period while others seem to peak immediately prior to death. If the average number of eggs per week are calculated for all the fertile limpets in this experiment, a fluctuation in number with time can be discerned (Figure 3.3.2.3). The laying periods varied from 1 - 15 days and rest periods from 1 - 40 days. The average number of eggs per week declines with time. In Figure 3.3.2.4 it can be seen that the largest percentage of the total eggs numbers were laid in the first four weeks of the reproductive period.

Influence of the rearing methods

The addition of Nutrafin to the rearing containers (treatment 2) of the limpets had a clear effect on the overall fecundity of the limpets (Table 3.3.2.4 and 3.3.2.5). As the variances were not homogeneous and the data set was non-parametric, a Kruskal-Wallis test was conducted in addition to the ANOVA. When all limpets were included in the two tests, the differences between lifespan ($f=4.2$, $p=0.047$(A) $p=0.03$(K-W)), laying period ($f=4.6$, $p=0.037$(A) $p=0.039$(K-W)), total number of eggs laid ($f=4.9$, $p=0.033$(A) $p=0.024$(K-W)) and the numbers of capsules ($f=5.77$, $p=0.022$(A) $p=0.025$(K-W)) proved to be significantly different when a multiple range analysis was performed. The intrinsic fertility rate

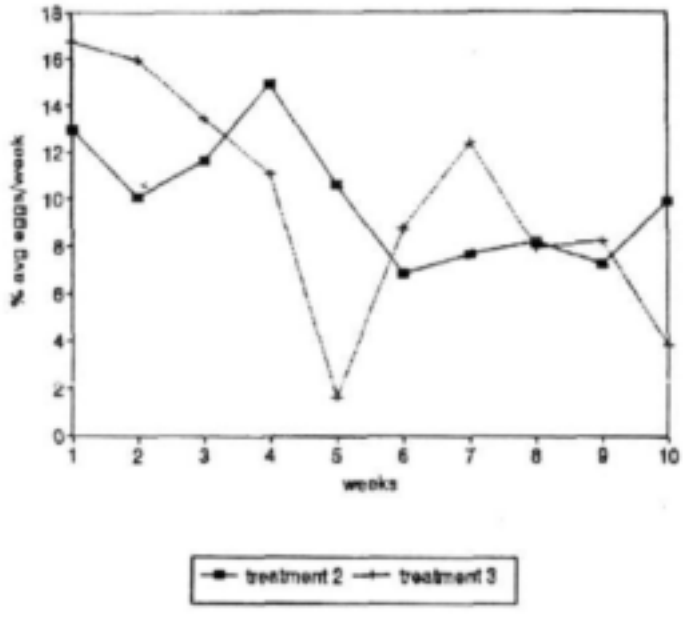


Figure 3.3.2.5 Average number of eggs per week in treatments 2 and 3

(eggs/capsule of 5.2 and 4.0 and eggs/day of 2.68 and 2.10 for treatments 2 and 3 respectively, surprisingly did *not* show significant differences (p=0.16(K-W)). However, as the laying period was longer in treatment 2 (Table 3.3.2.5) and the animals lived longer, treatment 2 (Table 3.3.2.6) had higher overall fertility.

If the outliers such as limpets 2b2, 2a3, 3b2, 3b3, 3b7 and 3b8 are removed from the data set, the significant differences disappear. The average fecundity data can be found in Table 3.3.2.5. These figures can be taken as the fecundity of second generation, fertile from a pristine river, when cultivated in the laboratory. These data were then used to determine the reproductive periodicity of the limpets. Figure 3.3.2.5 shows that on the average, fewer eggs per week were produced in treatment 3 than in treatment 2. However, in treatment 3 the reproductive activity increased in weeks 7 and 8; in treatment 2 the increased activity can be seen in week 4, although there was an increase in average egg numbers in week 8.

Fertility.

Practically, hatching rates were difficult to determin. However, it was noted that 27% of the limpets laid either one or more capsules which showed some form of developmental abnormality, frequently laid towards the end of the reproductive period. Some capsules were sterile while others showed partial sterility, and yet others showed differential development of the embryos with some embryos lagging as much as 10 days behind the most advanced in any one capsule. Limpet 2a2, which showed the greatest abnormalities in eggs laid, had 13 out of 24 capsules develop unevenly. These capsules were laid in the middle of the laying period for 20 days after which normally developing eggs were laid.

The average numbers of eggs per week decreased later in the laying period. Figure 3.3.2.5b) gives an indication of the percentage of eggs deposited in the various stages of the reproductive period.

Discussion and Conclusions

Food

Choice of food

Food choices of freshwater gastropods are not constant but may change over time or with the age of the animal (Skoog, 1978). Food preference may be related to hunger levels (and where young are presumed to grow at a faster rate and therefore demand more food) (Calow, 1973), and selection may also be based on the quantity (Daldorph and Thomas, 1988) and quality of food (Calow, 1970; Eisenberg, 1970; MacMahon *et al*, 1974).

Little is known about the ability of freshwater pulmonates to select from within the spectrum of algal foods available to them. Many herbivorous invertebrates in freshwater habitats are considered to be generalist feeders (Cummins, 1973) 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 (MacMahon *et al*, 1974). Work by Calow (1973) shows very clearly that *Ancylus fluviatilis* (Ancylidae), which has the same method of rasping action in order to acquire its food as *Burnupia*, actively seeks out algae in preference to, although also consuming, lichen, bacteria, detritus and fungi. Calow 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. In 1973(a) he showed that the preferred algae were the diatoms. *Burnupia* is very likely a micro-herbivore grazer with the same preferences.

There *is* some evidence that certain gastropod grazers select algae relative to the position, mode of attachment, and size of the algal cells. Blinn *et al* (1989) suggests from studies that the freshwater limpet *Ferissia fragilis* (reaching maximum shell length of 4mm) preferentially scrapes off the adnate understory species rather than the larger over story diatom species accessible to the larger gastropods and preferred by many insects (Lamberti and Resh, 1983; Steinman *et al*, 1987). The choices made by *Ferrissia* may be a function of both shell morphology and the radular architecture of the limpet. Observations of live *Ferrissia* revealed that the front edge of the limpet shell was so close to the leaf substratum that upright and stalked diatoms were frequently clipped off or pushed aside by the front edge as the limpet moved forward. The adnate cells remaining were scraped by the small, stout radular teeth. *Burnupia* did not demonstrate this behaviour, but clearly clears a path as it sweeps its head from side to side, a rasping action similar to, for example, *A. fluviatilis* and *Bulimus tropicus*, as opposed to the combing action used by *Physa acuta* where particles loosely attached or deposited on the substratum are collected (Brackenbury, 1989). A "clearing of the path" would also allow small sand grains (mineral particles) to be picked up, potentially important as they allow greater trituration of food (Hunter 1980), of particular importance in the digestion of diatoms which Calow (1970) suggests are less efficiently assimilated than green filamentous algae due to their high ash content and protective exoskeleton. A comparison of the inter-cusp gaps of teeth on the radulae of *Burnupia* with *Physa* and other gastropods would clarify the potential food intake of *Burnupia*, as would gut analyses.

Quality of food

It is acknowledged that bomb calorimeter analyses of samples of limpets would determine energy values, and provide a more accurate measure of the suitability of the different feeds and subsequent growth of the limpets. However, too many newly hatched limpets were required to obtain a great enough weight for the bomb calorimeter to show results, and consequently this method was not used.

Diatoms are characterised by their frustules that consist mainly of silica. Their carbon content per unit weight is known to be lower than in green algae (Calow, 1970), and the consequent lower ratio of C:N levels (considered a measure of relative protein content) is considered a good food source (MacMahon *et al.* 1974). However, at certain times trace element content, vitamins, essential amino acids, and other factors may be more important for an animal than overall organic content alone. Nutrafin's content of crude protein, fat and fibre provides an ideal medium for the growth of bacteria, and these bacteria will be an important source of Vitamin K in particular, but also other essential vitamins needed for the growth of the limpets (Daya, *pers com*). Some of these bacteria will also break down the crude protein into its constituent amino acids, making the amino acids available for use by the limpets. Bacteria are always present in the water, even treated tap water (Paprener, 1980), but in small quantities. This buildup in bacteria in the buckets would have taken some time with very likely a low bacterial count in the first few days. The Nutrafin-only replicates began with relatively sterile containers, with Nutrafin as the only food source. Mortality in these replicates was very high to begin with (Figure 3.3.2.1) suggesting bacteria played a greater part in the feeding and growth of the limpets than the Nutrafin in the first 12 days of the experiment. Mortality was lower in the Nutrafin replicates after the initial loss, when compared to the other feeds. A Microbiology III student will attempt to analyse the bacterial counts in the containers and possibly in the limpets' guts in 1998, in a repeat of the feeding regimes.

Jennings *et al* (1970) found Tetramin staple food showed greater number of eggs produced and quicker growth rate and mass increase with *Biomphalaria pfeifferi*, *Bulinus spp* and *Lymnaea natalensis*, compared to 4 other, well used snail foods, including lettuce and Standon's snail food (Mahoney, 1966). Tetramin B is also the standard food source for the mass cultures of *Hexagenia* mayflies used extensively for toxicology testing in the U.S.A. (Lawrence, 1981), where subsequent algal growth, as found in the above tests, acts as a supplement.

From the condition of the shells, which appeared normal, it seems that calcium was not limiting.

Individual variability

The figures in Table 3.3.2.2 reveal the variation of growth within individuals over time, under the same conditions of temperature, water quality and feed availability, and underline the difficulty in analysing for growth from length frequency data alone.

Reproductive biology and fecundity

Ancylidae deposit their egg masses primarily during the night-time (Berg *et al.*, 1958) beneath stones (Geldiay, 1956), observations within this experiment once again confirmed *Burnupia* to do the same, continually depositing on the underside of the tiles.

Factors which influence fecundity

Both abiotic and biotic factors influence the fecundity. Abiotic factors such as the quantity (cumulative) and quality of temperature (rising and falling), day length and water quality, particularly with regard to calcium and magnesium (Appleton, 1978), have all been shown to influence the reproduction of molluscs. Among biotic factors, diet (Aldridge, 1982), density and mating opportunity (Eversole, 1978) seem to be the principal factors influencing reproduction, with many different mechanisms implicated, including trophic limitations, and metabolite accumulation in the environment (van der Steen, 1967). McMahon (1975) reported a clear correlation between quality of food and eggs per capsule. The higher the protein content of the periphyton, the greater the number of eggs per capsule. Similar results were obtained by Hunter (1975) and Burky (1971) for *F. rivularis*, and by Russel-Hunter (1953) and Geldiay (1956) for *A. fluviatilis*. Interpopulation variations of ecophysiological characteristics also occur and have been studied in North America and Europe by Russel-Hunter *et al* (1970), Burky (1971) and McMahon (1973). Such traits as mean number of egg capsules per adult, time of maximum activity and spawning, and the necessity for cross-fertilization vary between populations (Russel Hunter, 1961; Streit, 1976).

Reproductive biology

Streit (1985) gives a useful synopsis of the available knowledge of the European river limpet, which we regard as the basommatophoran which most closely resembles *B. stenochorias*. *A. fluviatilis* is widely distributed throughout Europe and North Africa (Hubendick, 1970) and usually has an annual life-cycle (Russel-Hunter, 1953, 1961; Lamberet, 1966; Streit, 1976). Egg laying in spring is initiated when ambient temperatures reach 7-10°C. Roughly 10 to 20 egg capsules are deposited by each individual over the entire life-span (Russel-Hunter, 1953; Geldiay, 1956; Lamberet, 1966). Eggs are laid within capsules containing 1-13 eggs, but most frequently 4-6 (e.g. Geldiay, 1956), and they are considered very large in size when compared to other gastropods of similar size (Bondeson, 1950). The limpets hatch in an advanced stage of development as crawling snails, with a shell aperture length (AL) of 0.8-1.0mm (Lamberet, 1966). Egg-laying often starts at an AL of 5mm, 50% of maximum length reached (Streit, 1975), but populations differ slightly in this respect.

The results of this trial indicate *Burnupia* to have a very similar reproductive biology to the European limpet *A. fluviatilis*. Differences in lifecycle and number of generations per year are discussed in Section 3.2.1.

Variability

It has been observed in this experiment that some capsules are sterile while others show partial sterility, and yet others differential development of the embryos with some embryos lagging as much as 10 days

behind the most advanced in a capsule. This had no correlation with feeding regime. The natural variation in the intrinsic fecundity is 1-13 eggs per capsule. The variability of fecundity observed in this experiment was related to this intrinsic variation, and the incidence of early mortality, which in turn lowered average fecundity. Variation of other aspects, such as the number and frequency with which capsules are deposited, contributed to the variation in the daily fecundity rate of 0.3-7.58 eggs per day.

Mating systems

Like most pulmonates, the Basommatophora, to which the family Ancylidae belong, are simultaneous hermaphrodites. Fifty species have been shown to be self-fertile (Jarne *et al*, 1991) although outcrossing seems to be the preferred mode of reproduction. However, the ability to self-fertilise has been demonstrated in *B. stenochorias* (Haigh and Davies-Coleman, 1997). *A. fluviatilis* is polyploid and there is evidence of a high degree of self-fertilisation, even when allosperm is available, with a minimum population outcrossing rate of 10.3%. The mating systems of the Basommatophora are highly heterogeneous, however, and in the populations investigated by Städler (1995), some families showed an outcrossing rate above 50% while in other families selfing was predominant or high. Multiple paternity appears to be limited.

Jarne & Delay (1990) examined the effects of self fertilisation and cross fertilisation in *Lymnaea pereger*. Cross fertilisation resulted in a larger number of eggs laid, and young hatching and reaching sexual maturity. Jarne *et al* (1991) provided similar evidence with *Bulinus globosus* where selfing snails and snails produced by selfing were less fit in terms of fecundity and the hatchability of their eggs. Data on other Basommatophora have also provided evidence for an inbreeding depression. Boycott *et al* (1930) showed that with pulmonates, allosperm can be stored and remain viable after copulation, but as it becomes exhausted or close to dying, the self-fertilisation rate gradually increases (Duncan 1975). Vianey-Liaud (1976) observed a decrease of 40% in the number of eggs laid in *Biomphalaria glabrata* when comparing snails during a grouping period with the same snails one month after isolation. It was suggested that snails had exhausted their allosperm and had progressively switched to self fertilisation. In this case, previous copulations did not prevent an inbreeding depression (estimated by the number of eggs laid). Vianey-Liaud (1976) reported a decrease in both eggs/clutch and clutch deposition rate in isolated *Austrolorbis glabrata*. The influence that the utilisation of allosperm and autosperm have on the energy allocation during the reproductive process is not clear.

Whereas fecundity is the reproductive capacity of an individual or population, the fertility is the actual number of live births per female members of the population. In this experiment, where the limpets were split from the pairs into isolation, it was found a number of these isolated limpets (5 out of 34) were infertile until death. The question arises, despite each pair being separated after mating, as to whether

the "male" of the pair mated as a female itself, *ie* did *it* receive sperm from the other limpet? If not, did this in turn have an effect on its ability to produce offspring? Was being mated as a "female" necessary to initiate egg production? The suggestion that it is, is contrary to previous experiments (Haigh and Davies-Coleman, 1995) where although there was a decrease in fecundity among limpets reared in isolation, they nontheless self-fertilised. Did the food quality also influence this inability to produce eggs (4 of the 5 were fed on algae only)? It appears it could have influenced fecundity.

From this study of *B. stenochorias*, protandrous hermaphroditism cannot be determined. Field collected snails have been sectioned in an attempt to determine the maturation sequence of the gonads, but these have still to be analysed and will be published (Davies-Coleman *et al*, in prep.).

The effects of diet on the fecundity rates in this experiment were not significantly different but the influence of diet on other aspects which influence total fecundity, such as extended lifespan and laying period, resulted in significantly higher numbers of eggs from those animals reared with the addition of "Nutrafin". The results obtained in these experiments can be used as a guideline to the possible fecundity of *Burnupia* cultured under these conditions in the laboratory. The possibility of using the intrinsic fecundity as a biomonitoring tool requires further investigation.

3.3.3 GROWTH OF LIMPETS IN BASKETS PLACED IN THE BLOUKRANS RIVER.

Introduction

Through analysis of the year's field data accumulated through 1995 and 1996 from monitoring two natural populations, information on the number of cohorts expected each year, the egg laying periods, and the calculated VBG Model were acquired (Section 3.2.1). Similarly growth data was obtained from the C.E. Room feeding experiment, with its uncertainties and downfalls of analysis of growth considering the artificial conditions of 20°C and 12 hours L/D (Section 3.3.2). In order to link these two sets of data, it was considered necessary to compare the actual growth of a group of limpets of known age, under natural conditions. Limpets of known age were placed into replicated baskets in Bloukrans River in an area close to the Belmont River site and their growth was analysed, and compared to that of the C.E. Room and the VBG Model calculated from the year's data.

Method

1) Limpets of the same parent group as those used in the C.E.Room experiment, and therefore of known age, were hatched (September 1996) in 6 plastic bags already seeded with algae from the river. These limpets were measured for shell length, and excess numbers removed so that an equal number were left in each bag.
2) Bags were then turned inside-out, a rock was placed into each one and the bag was tied, so that the bag acted as a skin over the rock. Each bagged rock was then placed into a large plastic laundry basket, with its open sides covered in 350µm mesh to prevent limpets from either entering or leaving the basket. The tops of the baskets remained open to the air. The baskets were placed in the Bloukrans River, anchored to riparian vegetation. Flow of water still occurred through the mesh.
3) Limpet shell lengths, with pH, temperature and TDS of the water were measured weekly. Debris which had collected around the mesh was cleared to ensure continued water circulation.
4) Resulting growth curves were compared to those of the C.E.Room by means of regressions, with ANCOVAs testing the equality of the growth equations. The observed average length for the limpets is compared to that predicted by the models for the two natural populations (Section 3.2.1) after the same number of days' growth.

Results

Out of season flooding turned the baskets onto their sides and consequently some limpets were swept away; the trial was therefore abandoned after 35 days. At 400µE light was adequate when tested early in the morning and late in the afternoon, despite the heavy riparian vegetation. Table 3.3.3.1 shows the average, maximum and minimum pH, TDS and temperature values recorded. Table 3.3.3.2 gives the regression models for each of the feeds and the baskets when compared to each other, with ANCOVA

results on all equations indicating the significant differences between the growth rates. Figure 3.3.3.1 graphically represents these regression lines. Table 3.3.3.3 shows the results of the ANCOVA test comparing the individual regression equations of the limpet growth in field baskets and the three feeding regimes, indicating where the growth is considered significantly different. It can be seen that a diet containing Nutrafin result in growth rates which as not significant different, as previously shown, Those limpets reared in the river under semi-natural conditions showed significantly less growth than any limpets cultured in the laboratory.

If the VBGE is used to draw a growth model for the first 40 days' growth, on the basis of the predicted sizes from Section 3.2.1, where $t_0 = 0$, and this model is compared to that of the baskets (Statgraphics 5), the differences between the slopes ($F = 2,13251$; $p = 0,1462$) and the intercepts ($F = 0.950776$; $p = 0.331$) are not significant. This suggests that the VBGM has accurately predicted the growth of the limpets for the first approximately 40 days' growth, as a result of the length frequency data analyses when compared to the growth of the limpets in the baskets. Both the baskets and the predicted growth model analyse growth at the beginning of the summer *ie* beginning in the latter half of September. It was presumed that the food provided in the form of seeded algae (mainly diatoms) on the plastic bags in the CE room varied little from one bag to the other. However, as growth relies heavily on the quality of food, as previously indicated, all replicates were combined before comparing with the three feeds and the VBG model.

Table 3.3.3.1. Average, maximum and minimum temperatures, pH and TDS recorded over the 35 days.

	Maximum	Minimum	Average
Temperature	16°C	13°C	14.1°C
pH	7.31	6.89	7.04
TDS	707	589	620

Table 3.3.3.2 Regression models and result of ANCOVA, indicating a significant difference between the growth rates of the three feeds and the baskets

	n	model	R^2
Nutrafin	707	$y = 0.059x + 1.127$	0.74
Nutrafin & Algae	1151	$y = 0.059x + 1.335$	0.64
Algae	1038	$y = 0.053x + 1.335$	0.66
Basket	559	$y = 0.046x + 1.367$	0.50

ANCOVA $F(3,3448\ df) = 2623,5$, $p < 0,05$.

Table 3.3.3.3 ANCOVA results comparing the growth equations of the baskets with that of the three feeding regimes of the C.E.Room. 1 = Nutrafin only, 2 = Nutrafin and Algae, 3 = Algae, 4 = Baskets.

Comparison	F values	P values	df
1 - 2	0.0059	> 0.05	1.1858
1 - 3	12.33	< 0.05	1.1745
1 - 4	27.607	< 0.05	1.1266
2 - 3	12.52	< 0.05	1.2189
2 - 4	54.69	< 0.05	1.1710
3 - 4	7.29	< 0.05	1.1597

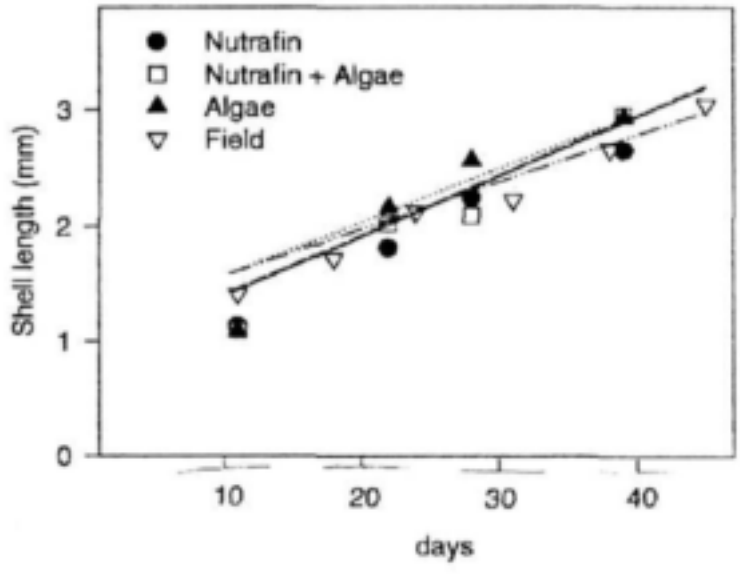


Figure 3.3.3.1. Comparison of regressions of the growth of the limpets on the three feeding regimes with the growth in the field baskets, combining all replicates. Days refer to the age of the limpets, 35 of which were spent in the river

Discussion

The F ratios (Table 3.3.3.2) indicate only the diets containing Nutrafin have similar growth rates. Even though the baskets are almost the same as the Algae feed, there is still some significant difference in the growth rates, possibly explained by the difference in the length of the experiment, as well as the environmental variability with the baskets: the experiment with the baskets was too short, and was overridden by the variation in the environment. It is not surprising that the growth under the constant conditions of temperature and light gave faster growth rates to that found in the baskets. For the purposes of culturing the limpets, the C.E.Room must be more ideal than in natural conditions of fluctuating temperatures, particularly when the degree days accumulated over the growth period are considered (Figure 1.3.3.2). The CER had accumulated double the the amount of degree days to the natural environment , over similar period.

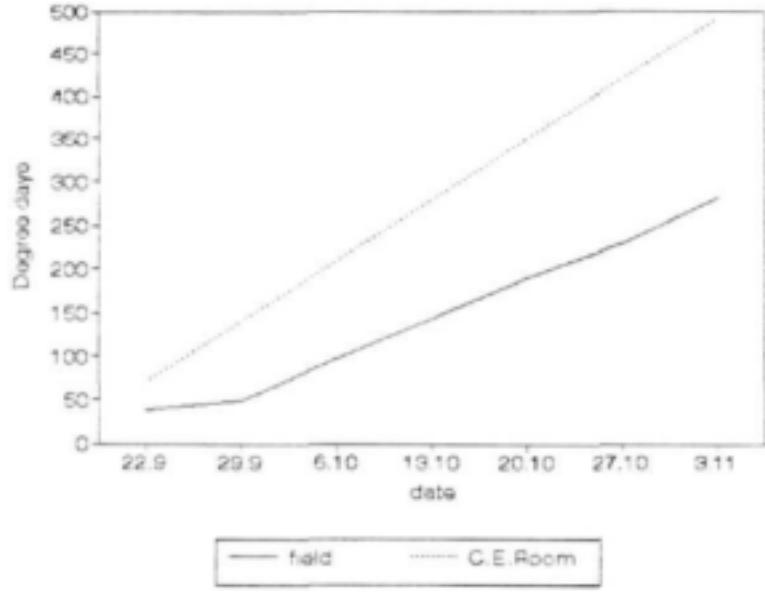


Figure 3.3.3.2 Accumulated weekly degree days for the limpets grown in baskets compared to those grown in the C.E.Room, for the period of growth the limpets were in the field.

3.3.4 SEXUAL DEVELOPMENT

Introduction

Basommatophorans 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 that the local conditions portray. Hermaphroditism is the only method of reproduction in the Basommatophora, and is advantageous to the freshwater situation, particularly where individuals may become isolated or are in low densities, as they are capable of reproducing by self-fertilisation (Geraerts and Joosse, 1984). The pulmonates have a single gonad, an ovotestis, in which there is simultaneous production of eggs and sperm. Slight protandry does occur in the primitive pulmonates of the Ellobiidae, but also in some of the higher pulmonates, such as the *Physa* spp (Physidae). *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 less evolved species. Protandry may therefore occur in *Burnupia stenochorias*.

During the investigations of *Burnupia stenochorias* it has been observed that, below a certain shell length, the limpets do not lay any eggs. If *B.stenochorias* is to be cultured in the laboratory for the purposes of toxicology, and individuals are to be used for tests, information as to what size (*ie* shell length) the individuals can be used, having already laid some egg capsules for the continued production of the culture within the laboratory, is needed. If protandry exists in some form, male fecundity may be related to size and therefore mobility: Russel-Hunter and McMahon, (1976) and information as to the average shell length of newly fertile males is needed.

The organisation of the pulmonate genital system is well documented (Fretter and Peake, 1975). However, investigations relating to pulmonate lifecycles and gonad development have been few in the Northern hemisphere, and none in the Southern, or in Africa. Oberholzer and van Eeden (1969) give details of *B. mooiensis*, but with no indication as to the seasonal development of the gonads; similarly, only the sperm structure of *B. stenochorias* has been described by Hodgson and Healy (1997, *in press*), with no further studies of presence or absence of sperm in individuals throughout the year.

Method

1) A range of sizes of *B.stenochorias* were collected each fortnight for 52 weeks from both the Belmont River and the Manley Flats sites (Section 3.2.1), preserved in Bouins Fluid for later analysis.
2) A sample of 62 limpets were drained of the fluid, embedded in paraplast, sectioned in TS form to 10µm; and stained according to the Haematoxylin and Eosin Method (Hodgson and Bernard, 1992).

3) Sections were mounted on Haupt's gelatine and sealed with DPX mounting medium.

4) Analysis of the development of the ovotestis relative to early and late spermatogenesis and developing and mature ova were made, comparing results relative to source of limpet, size (relative to shell length, width and height) and season collected.

Results

The sections confirm *B.stenochorias* to be a simultaneous hermaphrodite. Although accurate counts of the number of acini in the sections could not be made, the description given (below) 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, being a roughly spherical gland composed of up to 28 elongated follicles or acini, more or less separated from each other at their apices. These acini are also found behind the posterior shell muscle. 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. There is no differentiation between male and female zones in the acinus, and both kinds of sexual elements occur intermingled. During the early stages of sperm development the Sertoli cells, to which the developing sperm are attached, are closely associated with the wall of the acinus. During the final stages of this process, the Sertoli cells seperate from the wall of the acinus and float freely in the lumen until they finally disintegrate, thus liberating the mature sperm.

The results of the ovotestis development of a selection of the limpets collected from March 1995 to February 1996 are given in Table 3.3.4.1. With the difficulty in estimating the percentage of body mass or volume of the ovotestis to the remainder of the body,only estimations of the proportion of each of the stages of development relative to each other are given. In some limpets, approximately 80% of the cross sections of the limpet were filled with ovotestis and developing sperm; in others, this was very small (approximately less than 15%, even when there were mature ova present). Oocytes were clearly visible within the transverse sections. We were not able to discern the penis, which, although absent in some of the Ancylidae, was described in *B.mooiensis* by Oberholzer, (1963).

Table 3.3.4.1. Collection data and gonadal condition in a sample of some of the transverse sections of *B.stenochorias*. Date refers to 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. *** = dominates the gonadal development; ** = present in large numbers but not dominating; * = present in small numbers.

Season	Date	Site	Length	E.S.	L.S.	D.O.	M.O.
early	11/05	MF	3.23	**		**	
Winter	11/05	MF	3.77	**	*	***	**
(1995)	11/05	MF	4.62		*	***	***
	12/05	BR	3	**			
	12/05	BR	3.46	**		**	
	12/05	BR	3.92	**		**	
	22/06	MF	2.69	*			
	22/06	MF	2.92	**		**	**
Winter	22/06	MF	4.08	**	**	***	**
(1995)	22/06	MF	4.62	**	**	**	**
	23/06	BR	3.62	**		**	
	23/06	BR	3.39	**		**	
	23/06	BR	4.23	***	**	**	**
	4/08	BR	2.85	**	**	**	**
Spring	17/08	MF	5.54	**	*	**	**
(1995)	18/08	BR	3.23	**		**	**
	14/09	MF	3.62	**	**	***	***
	14/09	MF	5.39	**	**	**	**
	14/09	MF	6		*	**	**
	26/10	MF	5	**	*	**	**
Summer	26/10	MF	5	**	*		
(late	27/10	BR	3.85	**	**	**	**
1995)	27/10	BR	4.31	**	**	**	**
	21/12	MF	3.07	**	*	*	*
	21/12	MF	3.5	**	**	**	**
	22/12	BR	3.8	**	**	*	**
(early	19/01	MF	2.39	**		**	
1996)	19/01	MF	3.85	**		**	

	22/01	BR	2.92	**		**	
	22/01	BR	4.92	**	**	**	**
	1/03	BR	2.8	**	*	**	
autumn (1996)	1/03	BR	3.2	*	*	*	
	13/03	MF	2.8	**		*	
	13/03	MF	3.5	*	*	**	*

All overwintering limpets (as depicted by those sampled in May to July) which were greater than 3mm shell length displayed ovarian maturation. Development varied, but all displayed early, and the majority displayed late, egg development. Those limpets less than 3mm shell length had only immature spermatogenesis, with the exception of a limpet with shell length 2.92mm which also had developing ova. There appears to be a correlation between increase in the presence of early and then also late spermatogenesis with increase in size of limpet, with the exception of the individual of 4.62mm shell length which had no early spermatogenesis and very little mature sperm. During this overwintering period, late spermatogenesis was only present in those limpets with shell length > 4.08mm. The development of sperm appeared to increase in June in those limpets 4.23mm shell length and larger. The limpet of length 2.92mm (preserved 22/06/95) was an exception in that it displayed both early spermatogenesis, and developing and mature ova. The ovotestis in all limpets was, as a whole, rarely larger than 25% of the body mass through the winter months, unlike *Physa fontinalis* where the ovotestis occupies approximately 50% of the body mass (Duncan, 1959).

As temperatures rose in August and September (Figure 3.2.1.2.a), individuals varied in their development relative to their shell length. Despite the average shell length of limpet originally sampled at the MF site generally being longer than at the BR site (Figures 3.2.1.5 and 3.2.1.9; Table 3.3.4.1), the number of ova did not appear to differ noticeably between the MF and BR sites. Sperm development differed substantially, however. The MF limpet of 6mm shell length (collected 14/09/95) had no early and very little late spermatogenesis in September, the peak of egglaying (Section 3.2.1, Figures 3.2.1.9). Variation was also seen with those limpets of approximately 3.3mm to 3.6mm, where there was either a dominance of developing or mature egg development, or fewer eggs and no late spermatogenesis. Again, a limpet of shell length < 3mm (2.85mm) showed both developing and mature eggs and sperm.

As the year progressed through the summer (late 1995), all stages of gamete development were equally as dominant in the sections of the majority of limpets, with the ovotestis occupying up to 50% of the body mass. However, from January to March (1996) limpets had fewer late spermatogenesis and particularly few mature ova, not surprising when the egg laying decreases considerably in the field

(Figures 3.2.1.6; 7; 10 and 11). Both oogenesis and spermatogenesis were present in the limpets with shell length < 3mm collected from both the BR and MF sites.

Discussion

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). These internal and external forms of control are directly linked, but are also interdependant 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; ter Maat *et al* (1983) demonstrated a 90% increase in ovulation and oviposition of *Lymnaea stagnalis* with a change from dirty to clean or well oxygenated waters. 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).

Temperature is the most important factor determining growth, and therefore time of onset of the breeding season (Duncan, 1975; Russel-Hunter, 1978). 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. 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 10-12°C in temperate regions. Tropical *Biomphalaria* in Brazil has a critical temperature of 15°C (Duncan, 1975), although Gereart and Joosse (1984) suggest this critical temperature may be where ripe oocytes may be present but not necessarily ovulated. The temperatures measured at the two sites studied during 1995 and 1996 (Figure 3.2.1.2a) indicate a continual drop in temperature from May to a minimum below 10°C through the winter months of June and July, rising again in August. However, it is only in those limpets less than 4mm shell length that primary spermatocytes persisted in winter, with some mature sperm. Limpets greater than 4mm shell length continued in their development of both eggs and sperm in June and July, presumably accounting for the eggs laid through the winter months. A similar study of *Physa acuta* (Perrin, 1986) noted that field temperatures affected the sexual maturity of the cohort, with summer maturation more rapid, hence individuals were younger and smaller. Winter individuals delayed maturity with poorer fecundity, although larger individuals produced eggs through the winter phase.

Temperatures may have again been critical during the early parts of 1996, where temperatures measured at 9am rose above 22°C. Generally, rate of oviposition increases with increase in temperature until it reaches an optimum, decreasing again with higher temperatures until it ceases altogether (McMahon, 1975). Although maximum temperatures were not consistently monitored in the Bloukrans

field study, it is likely the temperatures could have risen above 25°C, a maximum for many freshwater molluscs (Brackenbury, 1989).

The list of molluscs that have been proven protandrous hermaphrodites is given by Hoagland (1978) and Brackenbury (1989), although this condition does not seem to be the norm. Environmental conditions are responsible for mediating sex change in a number of basommatophorans (Hoagland, 1978). Geraerts and Joosse (1984) suggest that most 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.

As temperatures rose in August and September, most limpets, small and large, showed increased gonadal activity with the development of mature spermatozoa and eggs. This trend of development is substantiated by the egg laying periodicity increasing significantly from the winter into the summer months (Figures 3.2.1.5 and 3.2.1.9). Doubt as to the validity of the observation that some of the limpets acted as simultaneous hermaphrodites can be cast where only mature sperm were present in the sections, and not in the ovotestis. These sperm may have been donated. Sperm can be stored in some Basommatophora for 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 only made 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), and some noteworthy exceptions to outcrossing have been found in *Biomphalaria* and *Bulinus* (Jarne and Stadler, 1995). However, outcrossing appears to be the rule in natural populations (Jarne *et al*, 1993). Irrespective of the season, those limpets possessing mature sperm and developing and mature eggs were all large in size (> 4,9mm shell length). It is difficult to judge from these sections of *B.stenochorias* whether the mature sperm were exogenous or endogenous.

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 (Section 3.3.2). It has been shown that 70% of organic carbon is consumed for reproductive output (Britton and McMahon, 1997). Similarly, the size of limpet relative to its population average may affect the number of eggs produced by the limpet, as found with *Physa acuta* (Aboul-Ela and Biddiny, 1969). The better the conditions are, the earlier the maturation, the greater the number of large limpets, and the greater the number of eggs produced. Quality and quantity of food between the MF and BR sites were not investigated. However, no differences in the ovotestis development were seen between the sites.

Neither did density differences between the sites appear to affect stage of development of the ovotestis, contrary to that found with *L. peregra* (Calow, 1981).

When considering life cycle strategies, most pulmonates are semelparous and annual, but Calow (1978) has shown that a few species can transform to a life cycle where two generations can lay eggs in one year. Considerable infraspecific, interpopulation variations in lifecycles can occur (Geraert and Joosse, 1984). This may depend on the conditions prevalent at the time, or may be a genetically determined interpopulation response (Jarne and Städler, 1995). Earlier time of maturity for any one population could result in earlier and possibly higher reproductive effort, as was found with *Lymnaea peregra* (Calow, 1981). Both the results from this sectioning and from the field study (Section 3.2.1) corroborate this is very likely the case with the *B.stenochorias* populations studied here *ie* those eggs laid early in the spring would have had conditions suitable to grow and reach maturity within the summer or pre-winter months; those laid later in the year would not have done so.

Jarne and Städler (1995) emphasise that once sexually mature, freshwater pulmonates are essentially simultaneous hermaphrodites, and *B.stenochorias* bears this out. Previously, it has been suggested (Haigh and Davies-Coleman, 1995) that sexual maturity occurred in those limpets 3mm shell length or longer only. However, clearly there are a number of exceptions to this, at any time of the year.
The question arises as to what difference this would make to any toxicity test, and would the tests have to be made at various times of the year, to understand the susceptibility of limpets to toxins at all stages of sexual development? This will be investigated by H Davies-Coleman during 1998 and 1999.

3.3.5 TRANSFERENCE FROM LABORATORY TO LABORATORY

Introduction

As part of the ongoing development of expertise in using *Burnupia* for toxicological purposes, brief experiments have to be conducted when opportunities arise. One of these was the testing of techniques for transferring the limpets from one laboratory to another, completed by Dr WJ Muller.

Method

Limpets randomly chosen from artificial streams within the laboratory were transferred into one of three small (500ml) plastic bags filled with de-chlorinated tap water, which were subsequently placed in 500ml plastic lidded bottles. An approximately equal number were placed into one larger plastic bag filled with 1500ml de-chlorinated tap water and tied at the top.

The pots and bag were packaged into a cool box with ice and left for 36 hours, at which time they travelled by road for 12 hours. Each container was then aerated overnight, and mortality checked the next day, when algae-covered tiles were placed into each container for the first time.

Results

Table 3.3.5.1 Survival and mortality data for *Burnupia* travel.

	Total at arrival	Survivors #'s	Mortality #'s	% Survival
Pot 1	25	18	7	72
Pot 2	29	29	0	100
Pot 3	30	28	2	93.3
Plastic Bag	61	57	4	93.4
Total	145	132	13	91

Discussion

The technique used here had a high success rate in our opinion, despite the handling as the limpets were placed into the bags, the time spent in the bags with no aeration, and the distance travelled by road. The technique is to be used in all future handling of *Burnupia* over longer distances.

3.4 CONCLUSIONS

Handling

The technique of handling the limpets has been refined to a level where there is an absolute minimum mortality: how to collect limpets from their natural habitats, and to move them to the laboratory; then how to keep them in the laboratory; and how to move them once they have been living in the laboratory for a generation or two, to the test laboratory elsewhere for toxicity testing. The underlining factor is to touch the limpets as little as possible; they have higher mortality and are affected in their growth rate if handled. Collections should be made directly into plastic bags which have already had a layer of algae growing in the laboratory. This puts the limpets straight onto their primary food source. Similarly, handling within the laboratory, and then to the toxicity tests, must be while they are attached to the plastic. The plastic, therefore, must line all buckets and channels used. If long distances are travelled, the limpets should be kept as cool as possible, preferable with ice, and aerated on arrival.

Conditions within the Laboratory

Dechlorinated tap water is suitable for both the growth of the limpets, and growth of the diatoms. Limpets appear to grow and reproduce better in an aerated bucket system, although the channel design submitted in Haigh and Davies-Coleman (1997) is satisfactory and would act as a means of holding limpets until required by toxicology. The water within the containers should be changed by 10% volume daily to maintain the nutrient levels at a satisfactory level, although calcium in the form of dissolved calcium carbonate, and silica in the form of dissolved sodium silicate, should be added on a weekly basis to maintain the limpet shells, and the diatom frustules respectively. Water quality is maintained to a large degree in the channels by the addition of plastic threads packed into the sumps. These act as very effective filters, particularly for nitrogen in the form of ammonia, plus excess phosphates produced as excrement by the limpets, and removed by the action of a bacteria film which develops on the plastic threads. River water can be added to increase nutrient levels occasionally, but it is essential that a sudden drop of 1 in the pH does not occur, as this will cause the death of the periphyton growing in the containers.

Diatoms appear to be the primary food source, with the ideal surface provided by the plastic sheeting in the containers, allowing easy grazing and growth of diatoms; however, substantial increases in growth are found with the addition of Nutrafin^{R} . The diatoms can be easily seeded from scrapings of rocks where limpets are known to occur. Light conditions should be in the lower range (± 100µm/sec) for optimal periphyton growth, and under these conditions, unicellular diatoms are prominent, ideal for grazing by the limpets.

Field and laboratory investigations revealed the necessity for, and the suitability of, flat unglazed ceramic tiles as a refuge for egg laying, and as an ideal surface for the growth of periphyton. These can

be easily removed from the channels and cleaned, should the layer of algae grow unsuitable species (such as filamentous algae). Similarly, water temperature is considered the most critical factor determining growth and onset and intensity of the breeding season; temperatures of approximately 20°C and high densities (400/m^2 equivalent) give maximum production for the culturing of the limpets.

Life history

Through natural population studies, egg laying periods were shown to extend primarily during the spring and early summer months, although capsules are laid all year round. Mortalities of hatchlings appear to be high, both in the laboratory (approximately 20%) and under natural conditions (difficult to determine but literature indicates it is greater than 20%). To reach the maximum size of 7mm shell length (The longevity of a limpet) is predicted to be up to two years, although the majority do not seem to live longer than one year. However, as shown in other species studied in detail in Europe and North America, there is considerable individual and interpopulation variability, mainly due to the different growth rates attained, determined by local conditions.

The actual growth rates of limpets under various conditions are now known, with approximate mortalities. As aging techniques do not exist for the Ancylidae, and there are no growth rings to count, analysis of growth is very difficult, relying on trials with known aged limpets. Although baskets designed to hold the limpets gave some answers as to growth rates, changing river conditions continually cause difficulties in any long-term trial. Winter conditions prevailing around Grahamstown seem to discourage any limpet growth, and thus these conditions should be avoided in limpet culture.

Burnupia is hermaphroditic and has the ability to self-fertilise. Approximately 10 to 20 egg capsules are laid by each individual over its entire lifespan; 1 to 13 eggs are laid per capsules, although most frequently 5, in a gelatinous capsule with a thick extra (quaternary) layer for protection. They take 14 to 18 days to hatch, although there is variability shown within each capsule, with a small percentage non-viable. Fecundity can be increased by the addition of Nutrafin®, as the high quality food results in an extended lifespan and laying period, leading to in significantly higher numbers of eggs laid.

Toxicology trials

Φ' values, calculated from the FiSAT programme and based on one years' data from two sites, indicate that there is little difference between the two populations in terms of growth. Similarly Principal Component Analysis and Discriminant Analysis (STATGRAPHICS) also show that limpets from the three populations sampled (namely in the Bloukrans and Botha Rivers close to Grahamstown) for shell length, width and height, would make ideal sources of limpets for toxicity trials, should laboratory cultures not be used. They cannot be separated easily on the basis of their morphometric measurements which are the main criteria presently used for species identification, it is therefore presumed they are one species.

3.5 EARLY COLONIZATION OF BARE SURFACES BY EPILITHIC DIATOMS AND THE IMPACT OF GRAZING GASTROPODS ON THE ASSEMBLAGE IN ARTIFICIAL FRESHWATER STREAMS

Botany Honours Project completed by Marianne Balarin; June 1996 under supervision of Prof. R Lubke and Mrs. H Davies-Coleman..

Introduction

Periphyton provides a rich food source for most invertebrate grazers associated in high abundances with freshwater macrophytes (Brönmark 1989) and snails are a prominent feature of this fauna. Their grazing activities may be expected to strongly influence the dynamics of the periphyton system.

Historically, the study of potential interactions between periphytes (macrophytes, epiphytes) and invertebrates, such as freshwater limpets, has applied a reductionistic approach, separately investigating such aspects as food choice, grazing effects or host-specificity of microalgae. Recently a more holistic view has been applied to the study of interactions in freshwater macrophyte habitats. This has spawned most hypotheses that take into consideration both the direct effects and the more indirect interactions taking place in the system (Brönmark 1989, MacLulich 1986).

Invertebrate/periphyton interactions

Freshwater limpets are generally considered herbivores. Their main feeding method is scraping the algae/detritus/bacteria complex from the substrate with their radulae which have rows of chitinous teeth. Although studies on freshwater limpet ecology are many, comparatively little is known of the actual diets of most species, even the most common ones (Brönmark 1989).

Studies of most species have revealed that the main part of snails diets consists of detritus (50-90% of the volume), algae (< 25%) and living macrophytes and animal remains constituted a negligible part of the diet (< 1%). Calow (1970) reported that the gut contents indicated that snails are indiscriminate browsers on the periphyton unless the algae were patchily distributed.

Indicator species - Burnupia stenochorias

In ecotoxicological work use is made of freshwater molluscs as indicator species to monitor pollution levels in natural streams. As few local indicator species are readily available in sufficient numbers to carry out standardised toxicity tests in streams in South Africa, the Institute for Water Research, Rhodes University (Grahamstown), is developing a method to culture the limpet, *Burnupia stenochorias*. This indicator species could provide an adequate and constant supply. This study, conducted in collaboration with Heather Davies-Coleman of the Institute for Water Research, investigates suitable conditions for the growth of algae as a food source for *B. stenochorias*.

To date, the food preferences of the limpet, *Burnupia stenochorias*, are unknown, but it is thought that it grazes on filamentous algae, diatoms and possibly fungi, lichens and bacteria. Hunter (1953) reports that analysis of the gut content of certain limpet species of the family Ancylidae indicated a microherbivorous habit. As *Burnupia stenochorias* is closely related to *Ancylus fluviatilis* (Family Ancylidae), it is presumed that *B. stenochorias* grazes on the same periphyton assemblages.

B. mooiensis has been found to have the same radular structure as *A. fluviatilis* (Oberholzer 1963). It is thought that *B. stenochorias* will be a general feeder with a preference for diatoms, like *A. fluviatilis*, and will feed in the same way.

Periphytic community changes

The most obvious effect of herbivory is the removal of biomass which may or may not affect the standing crop. Much of the literature refers to the effect that *A. fluviatilis* and other limpets have on the periphyton stream algal assemblages (Calow 1974, Lamberti 1984, Brönmark 1989, DeNicola *et al.* 1990).

Manipulations of snail densities in the laboratory stream channels have shown that snails reduce the standing crop (Sumner and McIntire 1982, Steinman *et al.* 1987a, Lamberti *et al.* 1987). However, not all studies show a negative effect of grazing on the standing crop (Kedhe and Wilhm 1972, cited in Brönmark 1989).

Grazing may also change the species composition of the periphytic community. A general pattern seems to be that the large overstorey species, such as the stalked diatoms and filamentous species, are more susceptible to grazing and are reduced in abundance at high grazing pressures, whereas the tightly adherent species increase in relative abundance (Gregory 1973, cited in Brönmark 1989). These findings are supported by the studies of Steinman *et al.* (1987a) and Lowe and Hunter (1988). They found that the small prostrate diatoms (e.g. *Cocconeis* and *Achnanthes*) increase when the periphyton is grazed. Sumner and McIntire (1982) found a significant reduction in the proportion of large diatoms and the subsequent increase in the relative abundance of small, adherent diatoms. They suggest that this was due to either a higher growth rate of smaller diatoms or a greater vulnerability of larger diatoms to ingestion and dislodgement during grazing.

Successional patterns

Grazing may also affect the successional pattern of periphyton. Succession in periphytic communities usually proceeds from a monolayer community of bacteria to small, adnate diatoms, such as *Achnanthes* and *Cocconeis*. Then a more structurally complex community with stalked diatoms and short, filamentous algae take over and as a final stage a community dominated by long, filamentous algae (Steinman *et al.* 1987a).

Succession in laboratory streams

In natural streams, two types of succession are observed (H.T. Odum 1956): (1) Longitudinal upstream-downstream succession, and (2) the short term response of a stream section to variations in import and to seasonal changes in such physical factors as light, temperature and current velocity.

Studies of ecological succession in laboratory streams have been concentrated almost exclusively on short term succession. Seasonal changes in import and local physico-chemical conditions produce a pronounced response in the structure and metabolism of any given stream section. If these conditions remain constant, the stream will come to a steady state, relative to the conditions (Guillard and Kilham 1977).

Most studies in succession investigated the sequence of colonization of freshwater periphyton on perspex or glass and natural substrates. Belanger and Cardinal (1977) observed development of microfloral populations on glass slides.

Laboratory streams

The early laboratory streams were closed systems (e.g. Odum and Hoskin 1957) where the same water was recirculated over the community. More realistic lotic communities were developed later, often by diverting natural stream flow into a holding tank and thence through the laboratory streams (e.g. McIntire 1966a). In this way, water chemistry, temperature, turbidity, organic load, and other factors vary with the natural stream. The investigator controls light intensity, photoperiod and current velocity. In a closed system, such as was used in this study, careful monitoring of water chemistry and nutrients is required.

Aim

The aim of this study is to investigate the early successional trends of the periphytic community and plant-herbivore interactions in artificial, recirculating streams in the laboratory.

Key objectives

1. To determine the successional pattern of periphyton on bare substrata.
2. Identification of the diatoms present in the artificial, recirculating stream on the surface of clear, plastic sheeting.
3. To determine whether over an extended period the laboratory conditions are suitable for diatom growth, and therefore also limpets.
4. To determine the effect of grazing pressure, resulting from high limpet density, on the primary production and abundance of diatoms, and the community structure.

Methods

Experimental design

Experiments were conducted in laboratory streams consisting of six U-shaped PVC channels, each 0.25m wide and 2m long. Water was supplied and recirculated by a submersible aquarium pump, placed in a 100L plastic storage tank. This tank contained a biofilter of plastic strips.

Water was replaced at 0.13L s^{-1} which resulted in a total recirculation in two hours. Irradiance was supplied by four Biolux (75 W) lamps that provided a photon flux density between 20 - 60 μmol.m^{-2}.s^{-1}

measured with a handheld PAR metre at the water surface at top, middle and end of the stream. A normal daily photoperiod was employed. A double layer of clear plastic, sealed in strips, 3cm apart, was used to line the streams and served as substrate for periphyton colonization and units for sampling.

To inoculate the streams the biofilm was scraped from the rocks at twelve different positions in the natural stream (Bloukrans River). The scrapings were homogenised and brought to a volume of 6.0L with river water. A 1.0L subsample of the algal suspension was added to each laboratory stream. Algae were allowed to become established in the streams without limpets for 14 days. This period was sufficient for obvious establishment of algae but was before the exponential growth phase of algae that could prevent the grazer influence on those assemblages. The experiment was shortened from 65 to 37 days due to excessive rainfall and flooding of the Bloukrans River at the proposed sampling time, which prevented collection of limpets and suitable water for the artificial streams.

Two treatments were imposed on the six channels, giving three replicate streams per treatment:

1. Limpets and recirculated flow through biofilter,
2. Recirculated flow without limpets through biofilter.

Initially unfiltered Bloukrans River water was used to fill the channels. Subsequently a mixture of 50% river water and 50% dechlorinated tap water was used to top up the system to account for evaporation.

As diatoms have a very low phosphate and/or nitrate requirement the only nutrient enrichment used was sodium silicate salt ($NaSiO_3$) at a concentration of $0.21 mgL^{-1}$, which was added once a month. Temperature throughout the experiment was ambient. The limpet treatment consisted of adding *Burnupia stenochorias* to the three streams at a density of 400 which approximated natural densities as found in the Bloukrans River (H. Davies-Colenan, pers. comm.). The other three streams contained no limpets, but microflora may have been present. The experiment lasted 37 days, which included inoculation and colonization period (14 days) and grazing period (23 days).

Sampling strategy and analytical methods

Periphyton

Algal assemblages were sampled on day 14 just before limpets were introduced and on day 37. Chlorophyll-*a* was sampled on days 17 and 36, waterchemistry was measured on days 17 and 28, taxonomic structure and physiognomy were examined on days 14 and 37.

Chlorophyll-a

Five sets of random samples ($1cm^2$ each) were collected from each stream on each sampling date for determination of *chlorophyll-a*. The cold extraction technique, as described by Holm-Hansen and Rieman (1978), was used. The samples were placed in 10mL 0f 100% methanol, were kept in darkness and refrigerated

for 6 - 12 hours. Absorption readings were taken at 665nm and 750nm using a Shimadzu UV-1201 ultraviolet spectrophotometer. Chlorophyll-*a* concentrations (μgcm^{-2}) were calculated from:

$$\frac{A \times v \times 13.9}{d \times V} \quad \mu gcm^{-2}$$

Where A = (absorbance at 665nm) - (absorbance at 750nm)

V = area of initial sample ($4cm^2$) d = cell path length (5cm)

13.9 = constant v = volume of solvent in ml

Water chemistry

Physico-chemical conditions (temperature, pH, total dissolved salts (TDS)) in each stream were monitored monthly. A Checkmate CCA475627 kit was used for pH measurements. Nutrient concentrations of ammonium, nitrate, nitrite and phosphate were monitored spectrophotometrically using a Spectroquant 118 photometer. Water samples were taken from each stream, chilled and analysed within three hours. Ammonium was analysed by the indophenol blue method, nitrate by reacting samples with nitrospectral in concentrated H_2SO_4 to produce a dark red-coloured nitro compound, nitrite by reacting samples with sulphanilic acid and N-1-naphthylethylenediamine dihydrochloride to produce a magenta azo dye (Griess' Reaction), and phosphate was measured by the phosphomolybdenum blue (PMB) method (Merck Manual Photometer SQ118). Levels of detection, or measuring ranges, were determined by the dilution (river) water used for each experiment.

Taxonomic structure

Natural stream

Samples of epilithic diatoms for cell counts and identification were collected from the natural stream, Bloukrans River, on the farm "Rockwood", in Manley Flats. The biofilm was scraped off the rocks, taken randomly from eight different sites and depth zones (15-60cm depth). Scrapings of $4cm^2$ of the cover material from different stone aspects were removed with a scalpel and were stored in $10cm^3$ distilled water with three drops Lugol's fixative (Saraceni and Ruggui 1969) for enumeration and identification.

For the quantitative analysis of species composition, the diatom frustules were first cleaned by boiling in concentrated HNO_3. After this acid preparation of the diatoms, using the method described by Archibald (1981), the number of siliceous frustules, that were remaining, were counted and identified. The prepared samples (10mL) were placed into a counting chamber, allowed to settle for at least two hours, and counted with a Nikon MS inverted microscope at x450 magnification (Utermöl.l 1958). During this procedure, diatoms were identified to genus level and, were possible, to species level. An algal unit was an individual cell or valve, if the taxon was a unicellular form, or an individual cell of a filament if it was a filamentous or colonial form. A maximum of 500 cells was counted in each sample. The proportion of the diatom taxa in each sample was used to estimate the abundances of these taxa in the corresponding count of 500 algal units.

The final concentrations of cells were calculated as follows:

$$\frac{N_A}{2} = \frac{N_B}{f} = \frac{N_C \times 2500}{4cm^2} = C_C$$

Where N_A = Total counts of valves

N_B = Total number of cells

f = Total number of fields

N_C = Number of cells per field

2500 = Total number of fields per chamber

$4cm^2$ = Area sampled

C_C = Cell concentration cm^{-2}

Artificial streams

Randomly located samples (5 x 3 mm^2) were taken from each stream, one from the top, one from the middle and one from the bottom of the stream and prepared for the scanning electron microscope (SEM) examination. The samples were fixed directly on the pieces of plastic in 2.5% Gluteraldehyde solution for 12 hours, washed for 5 minutes in a buffer solution of NaCacodylate (pH ± 7%), dehydrated in a series of alcohol (30, 50, 70, 80, 90 and 100%), placed in 1,1,1,3,3,3,Hexamethyldisilazan ($C_6H_{19}NSi_2$) solution and allowed to air-dry. The air-dried samples were glued onto brass stubs and sputter coated for one minute with a thin layer of Au in the Biorad Polaron Equipment coating unit at 19.4 $g.cc^{-1}$ film thickness. Each plastic strip was scanned 30 or more times with a JEOL JSM-840 electron microscope at a fixed magnification of x500 (single field of view = 0.04 mm^2). The cells were counted and identified to genus level and, where possible, to species level. All SEM work was conducted at an accelerated voltage of 12KV at magnifications up to x20 000. Settlement surfaces were examined before and after grazing by the limpets, *Burnupia stenochorias*. To obtain micrographs for later use and identification, photographs were taken of the communities and specific individual cells using Agfa Agfapan Professional film (film type AP x 100).

Statistical analysis

The computer software package PRIMER (Plymouth Routines in Multivariate Ecological Research) was used to analyse the data. This program was developed for ecological studies of community structure and interpretation of data on abundance and/or biomass. Treatment effects on biomass accumulation were examined with a 2-way analysis of variance (Clarke and Warwick 1994). Diversity was calculated and graphically presented using Shannon-Wiener's Diversity Index. This index is not biased by rare or abundant species and is, therefore, useful for describing diatom assemblages dominated by a few species (Begon *et al.* 1990). Tests for differences between the two groups of samples (grazed and ungrazed) were computed from Bray-Curtis similarities for $\sqrt{\sqrt{}}$ transformed species abundances and represented graphically in a dendrogram and ordination graphs. All above tests were carried out using the PRIMER program routines.

Diatom flora

Diatoms sampled from the Bloukrans River were members of the Order Pennales (classification according to the system of Hustedt, 1930, cited in Werner 1977), and were represented by species of *Achnanthes, Amphora, Cocconeis, Gomphonema, Gyrosigma, Navicula* (the most common diatom), *Nitzschia, Surirella* and *Synedra*. The Order Centrales were represented by the genera *Cyclotella, Melosira* and *Thalassiosira* (Refer to Table 3.5.1 for a complete list of identified species and Appendix 3.5.1 for SEMs). Most of the genera found in the natural stream were represented in the artificial stream. Three taxa were absent, i.e. *Gyrosigma, Melosira* and *Synedra*.

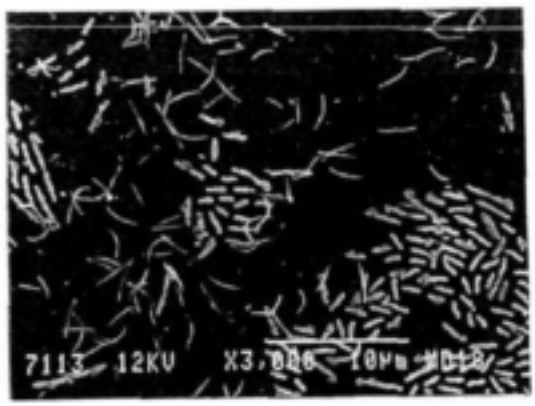


Figure 3.5.1 Fourteen days after innoculation colonies of bacteria dominated the streams.

After fourteen days, the settlement surfaces in the artificial streams were dominated by colonies of bacteria (× 18 colonies, 0.04mm^{-2}) (Fig. 3.5.1) and patches of thin mycelial filaments of soil bacteria, possibly filamentous *Actinomycetes* (Brock and Madigan 1988).

The low-growing, prostrate diatoms *Cocconeis scutellum* and *Achnanthes lanceolata*, which were the dominant species in all streams, surrounded the colonies of bacteria (Figure 3.5.2). The diatom distribution was heterogeneous and patchy, and different diatom assemblages were apparent in the same stream.

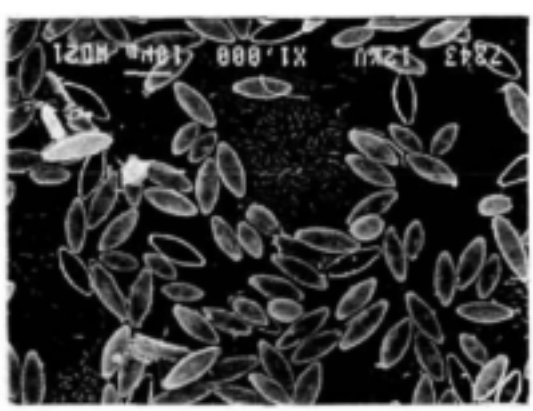


Figure 3.5.2 After 14 days a monolayer of adnate diatoms *Achnanthes & Cocconeis* surround the patches of bacteria

After thirty-seven days successional changes had taken place. The dominant species in stream numbers 1, 2 and 6 was *Achnanthes lanceolata*, in stream 4 *Cocconeis scutellum* was dominant, (Figure 3.5.3), followed by *Amphora pediculus* (Figure 3.5.4).

The stalked, overstorey diatoms, which were initially rare, became more numerous. In stream number 3 patch of an unidentified algae , possibly a blue-green, became apparent, interspersed with many pedunculate diatoms (Figure 3.5.5). This may constitute a successional change.

The relative density of the adnate diatom, *Cocconeis scutellum* increased from 58.59% (range 0.19% - 58.59%) at Time 1 (14 days after inoculation) to 65.83% (range 2.90% - 65.83%) at Time 2 (37 days after inoculation).

Similarly *Achnanthes lanceolata* showed a maximum increase from 22.68% (range 8.99% - 22.68%) at Time 1 (T1) to 83.26% (range 0.21% - 83.26%) at Time 2 (T2). *Gomphonema parvulus*, a pedunculate diatom, increased from 4.40% (range 0.28%- 4.40%) at time 1 to 9.5% (range 0.09% - 9.5%) at Time 2. The increases, represented in a histogram, could not be correlated to any particular stream. The streams 4, 5 and 6, which were subjected to grazers after fourteen days of diatom growth, showed no discernable effect due to the limpets' presence (Fig. 3.5.6).

When the samples taken from the top, middle and bottom of the streams were compared, using abundance (actual total number of cells counted), again no pattern was distinguishable (Fig. 3.5.7).

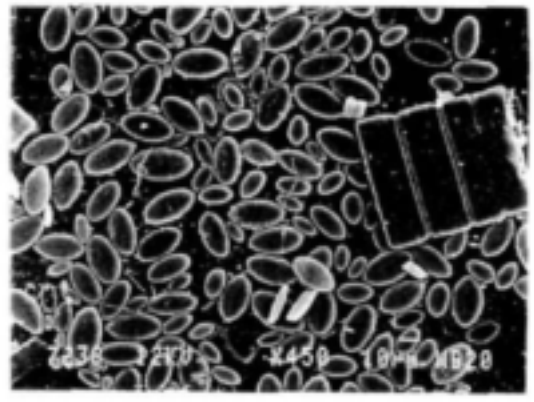


Figure 3.5.4 Thirty days after colonisation the density of the diatom assemblage increased. Patches were dominated by *Amphora pediculus* Interspersed with *Gomphonema parvulus* and fungal mycelia.

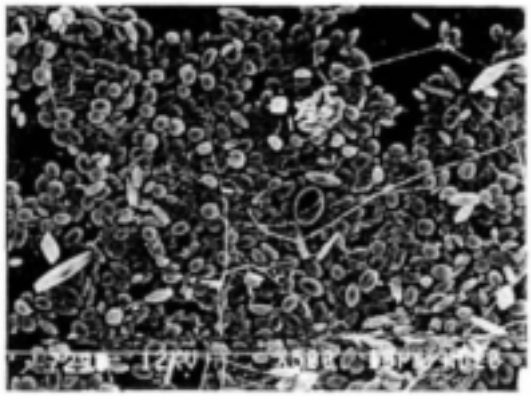


Figure 3.5.3 SEM of a patch of dominant *Cocconeis scutellum* in stream 4 with a few pedunculate *Gomphonema parvulus* (white). Some bacteria visible below the diatoms

Figure 3.5.5 A patch of filamentous algae interspersed with many stalked diatoms in stream 3, indicating a successional stage. Note the agglomeration of diatoms in the lower right corner

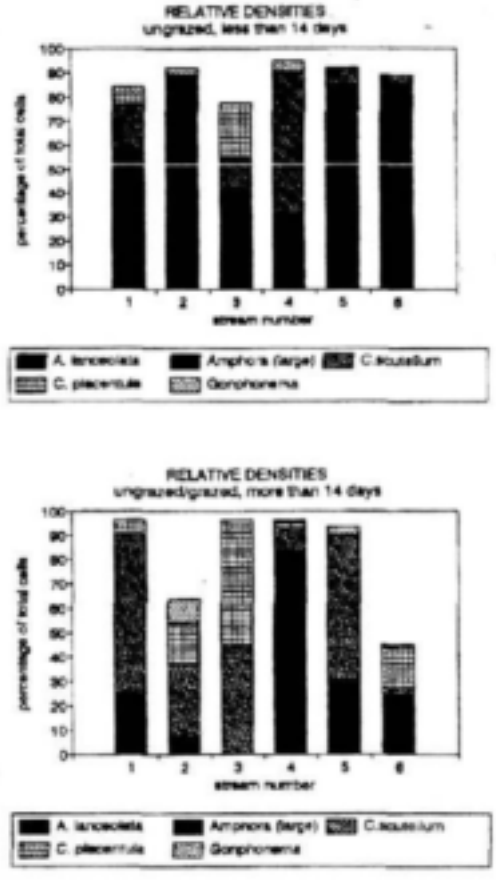


Fig.3.5.6 Histograms representing Relative Density (%) of the most dominant diatom species in the artificial streams (1 - 6), before 14 days on top, and after 14 days, bottom.

Chlorophyll-a

Although notorious for poor correlation with algal growth (Appleton *pers. com)* the average biomass accumulation as determined by the chlorophyll-*a* extractions indicated a high variability in each stream. Stream number 1 (grazed), which showed an increase in the total number of cells from 4.12 (log of mean total of cells cm^{-2}) at Time 1 (T1) to 5.85 at Time 2 (T2), had a corresponding increase in chlorophyll-*a* from 0.0556$\mu g.cm^{-2}$ at T1 to 0.7923$\mu g.cm^{-2}$ at T2. In stream 2 (ungrazed, control), however, the increase in number of cells (5.22 - 5.69) showed a decline in chlorophyll-*a* from 1.501 to 0.2224$\mu g.cm^{-2}$. Variable results were also obtained for the other streams (Table 3.5.2). The results could not be correlated to the presence or absence of grazers, but seemed to correspond to the total number of cells present in each stream (Fig. 3.5.9).

Water chemistry

The soluble nitrates, nitrites and ammonia concentrations remained below 1.0 $mg.L^{-2}$ throughout the experiment. The orthophosphates (range 0.79 - 1.22 $mg.L^{-1}$) increased sharply after 37 days to between 3.21 - 3.54 $mg.L^{-1}$ in the streams (Table 3.5.3). pH ranged in the different streams from 7.7 - 8.4 at the beginning of the experiment to 6.7 - 7.7 after 28 days. At the end of the experiment the pH had increased ranging between 7.65 - 8.3 with the greatest fluctuation shown in channel 1 (Fig. 3.5.10). The temperature fluctuated between 17 °C and 21 °C during the course of the experiment, dropping to 14.5 °C towards the end (Fig. 3.5.10). The total dissolved salts (TDS) showed a steady increase, sharply rising towards the end of the experiment, which correlated with the overall increases in all the streams.

Statistical analysis

The treatment effects on the biomass accumulation, using a two-way ANOVA indicated no significant difference between the grazed and the ungrazed streams ($p < 0.5$). No correlation between chlorophyll-*a* and orthoposphates ($r^2 < 0.5$), total dissolved salts ($r^2 < 0.3$) and pH ($r^2 < 0.3$) were detected using standard regression analyses. Similarly, using the Shannon Diversity Index, no clear trends were apparent (Fig. 3.5.15).

The tests for differences between the two groups of samples (grazed and ungrazed) computed from Bray-Curtis similarities for species abundances indicated that at the 45% level 5 groups separated out, but no clear differences were seen in the community composition (Figure 3.5.16). In the data analysed using ordination analysis, the control data (ungrazed streams) were clustered entirely to the left indicating a small difference between the ungrazed group (control, before 14 days) and grazed/ungrazed group (after 14 days) (Fig. 3.5.17). The level of stress was high at 0.22, but still gives a potentially useful 2-dimentional picture. This suggests that there is little difference between the streams and any difference present is as a result of the time within the experiment and not due to the effect of grazing

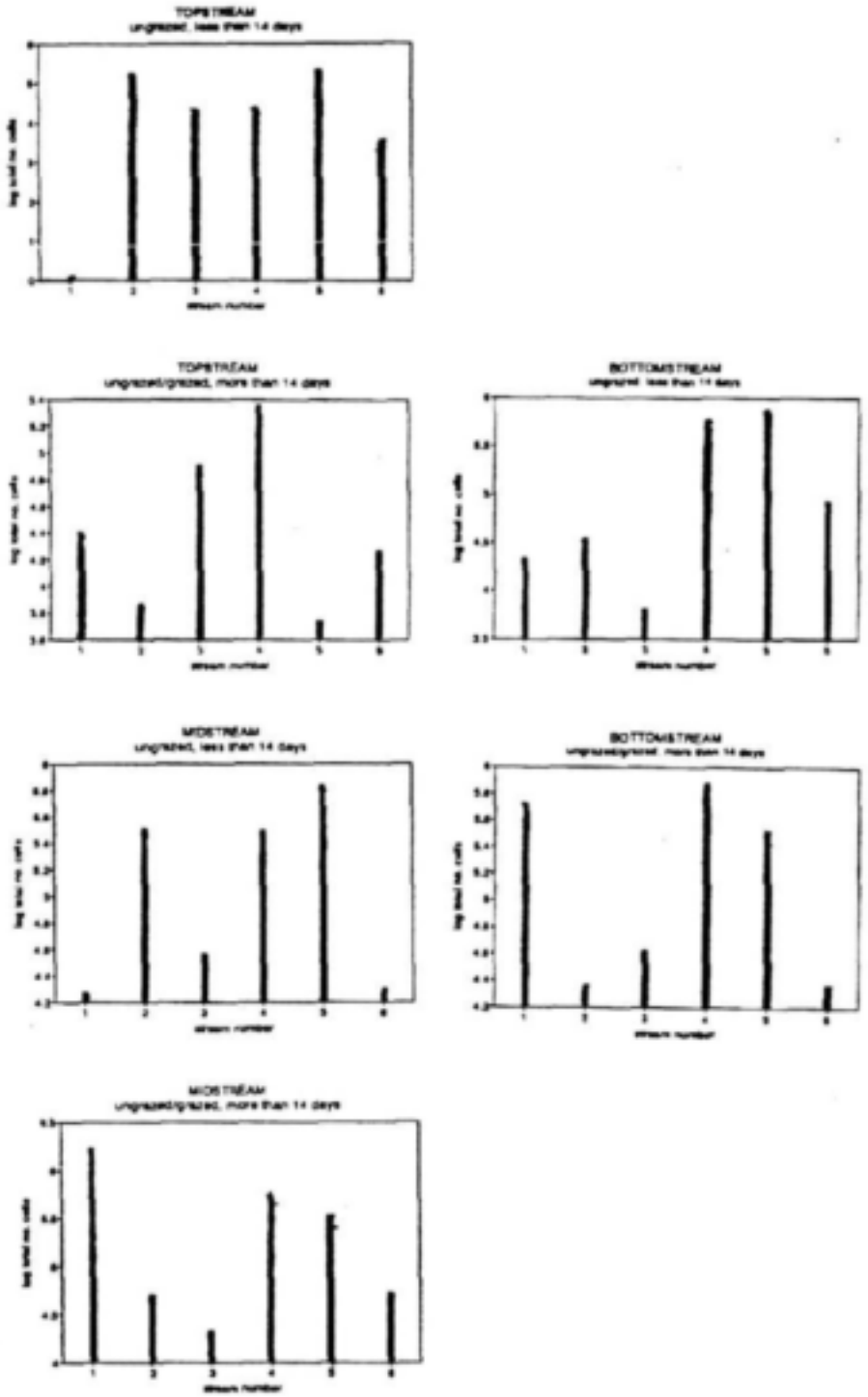


Figure 3.5.7 Histograms representing total number of cells in the top, middle and bottom of the streams at time1 compared to time 2. Grazer effect on diatom abundance is not apparent.

Table 3.5.1. List of diatom taxa present in the natural stream, Bloukrans River, and the artificial streams in the laboratory.

Diatoms in the river	**Diatoms in artifical streams**
Order Pennales	
Achnanthes spp.	*Achnanthes exigua*
	A. inflata
	A. lanceolata
Amphora spp.	*Amphora copulata*
	Syn. *A. ovalis*
	A. libyca
	A. ovalis var. *affinis* or *libyca*
	A. pediculus
Cocconeis spp.	*Cocconeis placentula*
	Cocconeis scutellum
	Cocconeis spp.
	Diatoma sp.
	Fragelaria sp.
Gomphonema sp.	*Gomphonema parvulum* (?)
Gyrosigma spp.	
Navicula spp.	*Navicula gregaria* Donkin
	N. minima (?)
	N. pupula Kützing
Nitzschia spp.	*Nitzschia acuminata* (?) Tryblionellae group
	N. debilis (Arnott) Grunow
	N. Lanceolatae group
	N. Tryblionellae group
Surirella sp.	
Synedra sp.	*Synedra* sp.
Order Centrales	
Cyclotella meneghiniana	*Cyclotella meneghiniana*
Melosira sp.	
Thalassiosira sp.	*Thalassiosira* sp.

Table 3.5.2. Results of spectro-analysis of chlorophyll-*a* samples taken at 665nm and 750nm. *ch* refers to channel number and results are compared to methanol 99.8% purity. Figures with * represent the values converted to $\mu g\ cm^{-2}$.

Date 1996	Wavelength	Methanol	ch 1	ch 2	ch 3	ch 4	ch 5	ch 6
19/4	665nm	0.008	0.004	0.108	0.009	0.046	0.048	0.004
			*0.0556	*1.501	*0.125	0.6394	*0.6672	*0.0556
	750nm	0.001	0.001	0.01	0.004	0.002	0.005	0.004
			*0.0139	*0.139	*0.0556	*0.0278	*0.0645	*0.556
8/5	665nm	0.001	0.057	0.016	0.01	0.004	0.013	0.004
			*0.7923	*0.2224	*0.139	*0.0556	*0.1807	*0.0556
	750nm	0.001	0.001	0.009	0.005	0.004	0	0.006
			*0.0139	*0.1251	*0.0695	*0.0556		*0.0834
12/6	665nm	0.001	0.002	0	0	0	0.051	0
			*0.0278				*0.7089	
	750nm	0	0	0.011	0	0	0	0
				*0.1529				

Table 3.5.3. Chemical analyses of water. "*ch*" refers to channel number. Amounts are in mg L^{-1}.

Date	Chemical	ch 1	ch 2	ch 3	ch 4	ch 5	ch 6
17/3	NO_3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	NH_4	0	0	0	0	0	0.02
	NO_2	0.06	0.03	0.03	0.05	0.03	0.02
	ortho-PO_4	0.79	0.99	1.1	1.03	1.09	1.22
19/4	NO_3	<0.17	<0.18	0.62	<0.14	<0.03	0.87
	NH_4	0	0	0	0	0.04	0
	NO_2	0.02	0.06	0.04	0.04	0.10	0.05
	ortho-PO_4	>3	>3	>3	>3	>3	>3
30/4	NO_3	0.37	<0.2	0.38	0.83	0.85	1.53
	NH_4	<0.01	<0.01	0	<0.01	0.03	0.04
	NO_2	0	0	0	0	0	0
	ortho-PO_4	3.54	3.73	3.21	4.21	3.54	3.43
29/5	NO_3	<0.02	0.3	0.42	0.12	0.19	0.47
	NH_4	0	<0.01	0	<0.01	0.04	0.01
	NO_2	<0.01	<0.01	<0.01	0.02	<0.01	0.02
	ortho-PO_4	0.08	0.28	1.14	3.74	0.10	1.14

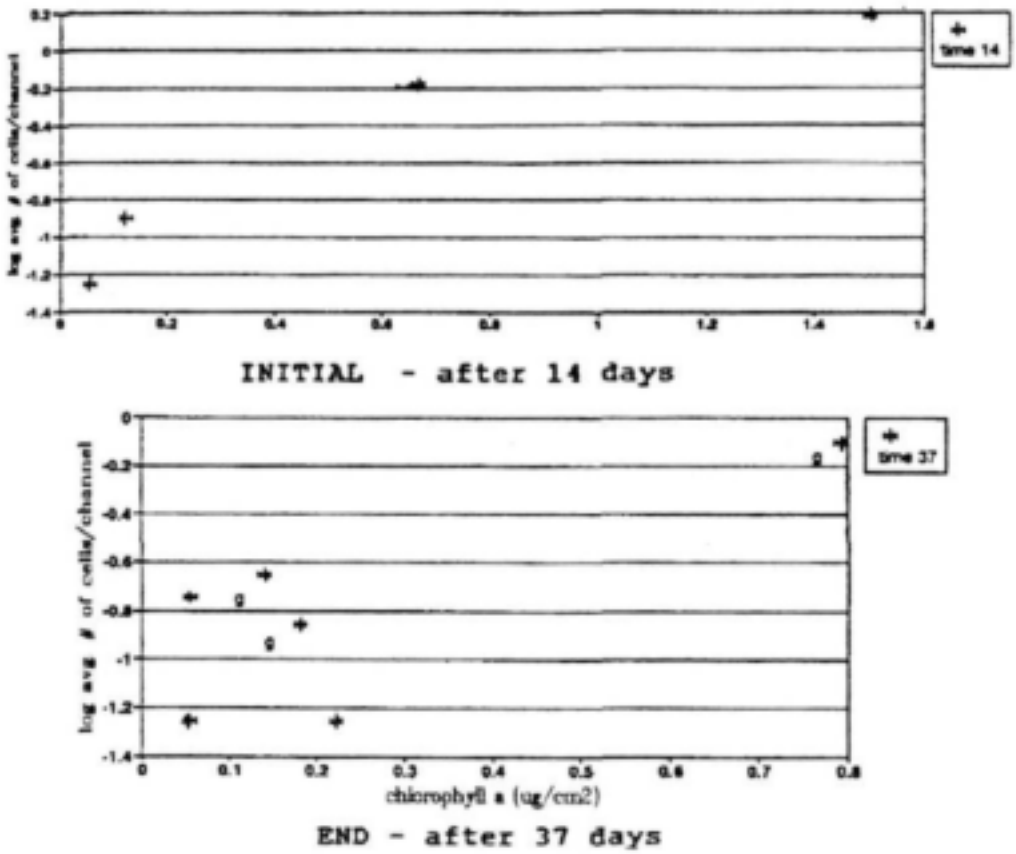


Figure 3.5.8 Scatterplot of chlorophyll-*a* accumulation 14 days after inoculation of artificial streams(top). Chlorophyll-*a* accumulation after 37 days, at the end of the experiment (bottom). The "g" indicates "grazed" streams.

Discussion

Periphyton

The heterogeneous distribution of epilithic algal assemblages in the streams may be partly the result of hydrological differences created when water flows over a ruffled substrate such as found in the artificial streams. DeNicola and McIntire (1990) reported similar results. For instance *Amphora* species seem to favour areas of decreased water velocity aggregating in greater densities in the depressions where the two plastic sheets were sealed together, and among the tangled filaments of chain-forming diatoms (Fig. 3.5.8 and 3.5.22).

Succession and taxonomy

Results of earlier SEM studies have suggested that periphyton in artificial stream assemblages follow a predictable series of seral stages after colonization, resulting in a successional sequence that is analogous to patterns in terrestrial ecosystems (Korte and Blinn, 1983, cited in Steinman and McIntire, 1986). The hypothesis corresponding to these studies suggests a sequence of stages consisting of (1)an organic matrix and bacteria: (2) relatively small adnate diatoms with a low vertical profile: (3) short vertically-orientated diatoms (often stalked): and (4) green filamentous algae.

In the present study, SEM indicated that many bacterial colonies were present during early colonization (stage 1). Bacterial presence is well documented in past studies and further analyses seem well warranted (e.g. epifluorescence microscopy) to assess the quantity of bacteria in laboratory streams.

The mode by which the diatoms were attached to the substrate fell into two categories:

i) Adnate, i.e. the cells were closely appressed to the substrate as in *Cocconeis* and *Achnanthes* and were not colonial;

ii) Pedunculate, i.e. the cells were attached to the substrate by pads or stalks, e.g. *Gomphonema* and some *Nitzschia* species.

The adnate forms are rarely colonial and single cells are held onto the substrate with mucilagenous fibrils as shown in the SEM of *Cocconeis placentula* (Fig. 3.5.18). Many diatoms form colonies and are linked together in different ways. Cells may be held together by interlocking siliceous spines (*Staurosira construens*) (Fig. 3.5.19). In this way short chains are formed. Linkages between cells are also produced by diatoms forming mucilagenous pads. Adhesion is thought to be mediated by a polysaccharide material (Round 1990). The cells are connected at the poles and colonies thus formed are starlike (*Nitzschia* sp.) (Fig. 3.5.20). Others, e.g. *Diatoma* sp. are connected over the whole of the single valve face (Fig. 3.5.20). *Gomphonema parvulum* produces mucilagenous stalks, which arise from areas of special pores at one or either pole (Round 1990). The stalks are frequently unequal in length (Fig. 3.5.21 a, b and c).The development of the pedunculate forms on the surface transformed the structure of the community from two-dimensional to three-dimensional. Within the microscopic "forest" produced, some species could be seen to

be canopy-formers, e.g. long-stalked species of *Gomphonema*, whereas others formed the "shrub" and "field" layers (*Cocconeis* and *Achnanthes* spp.).

The epilithic community, which is very similar to epiphytic community, is distinct from other diatom assemblages, e.g. episammic or epipelic communities, but they are never pure as other algae are intermingled (Fig. 3.5.10) such as blue-green algae. Together, the diatoms produce copious amounts of mucilage in which other non-attached algae live including several diatom species, e.g. *Amphora pediculus*, usually a motile species, which seem to attach themselves to the larger *Diatoma* species (Fig. 3.5.20).

Artificial substrata

Many workers have experimented with a variety of artificial substrates on which periphyton assemblages can develop for quantitative and qualitative studies. But none refer to clear plastic sheeting. In the review of Cattanea and Amireault (1992), it is reported that polyethylene plastic sheets have become very popular as it proved to be nontoxic (Dyer *et al.* 1962, Blankley 1973). A good agreement between biomass and taxonomic composition of the diatom assemblages (82%) was seen in comparisons between natural epilithon and artificial substrata. It was noted that the representation of blue-green algae on artificial substrata was very low initially (47% and 38% respectively), but improved after 60 days of colonization (67% and 40%). A similar increase in growth of filamentous blue-green algae was taking place in the artificial streams used in this study after 37 days (Fig. 3.5.10). Wetzel, however, warned in 1983 that artificial substata are too artificial resulting in a possible distortion of natural periphyton.

Water chemistry

The abundant growth of blue-green algae is considered dependant on low N:P availability in water. It has also been reported that relative high P concentrations are required for them to become abundant (Steinman *et al.* 1991). Assessing the cause of the increased growth of the undesirable, blue-green algae in the artificial streams is difficult, as the initial N:P concentrations were low. However an increase in biomass may occure without and increase in recorded phosphate levels, because of a very high turnover of phosphates. Elevated orthophosphate concentrations were recorded over time, but the nitrate concentrations remained low throughout. A distinct, visual change took place in the grazed and ungrazed channels over time. The water in the grazed channels turned yellowish and a thicker dark brown mat of periphyton became apparent on the substrate. Lesser growth and no colour change of the water was noted in the ungrazed channels. Further investigations as to the causes of these changes have not been carried out due to time constraints.

Light requirements

Light intensity must be sufficient to allow diatoms to grow. If it is too high, filamentous algae will dominate. Many fluorescent tubes can be used for irradiance in culture studies where artificial light is employed. "Growlux" tubes are favoured by horticulturists, but Drew (1983) (cited in Lobban and Harrison, 1994), reports that in work on algae "daylight" tubes are best, because they have a spectral

distribution close to that of natural light at a few metre depths in clear coastal waters. Diatoms irradiance requirements are reported to be very low. Marine diatoms can reach a maximum rate of photosynthesis when light intensity is less than 5% full sunlight, and they can maintain a net production at less than 1% (Taylor 1964, cited in E.P. Odum 1971). The very slow increase in algal biomass in the grazed or ungrazed channels may be due to the low irradiance (photon flux density 20 - 60 μmol.m^{-2}.s^{-1}) experienced in the laboratory. Lamberti *et al.* (1989) report that growth stimulation was provided by a shift from low to intermediate irradiance (20 - 100μmol.m^{-2}.s^{-1}) which resulted in a significant increase in biomass plus an increased herbivore growth rate.

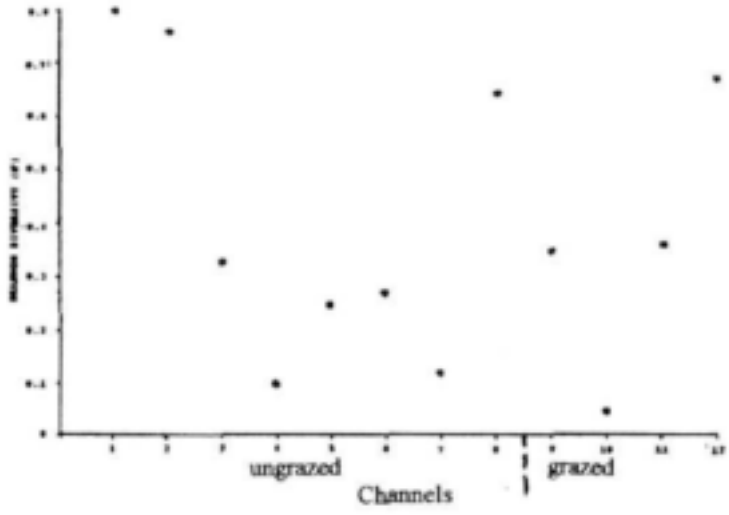
Effect of grazers on periphyton

The grazed streams were stocked with 400 limpets per stream which is equivalent to 330 limpets per m^2. Limpet densities were not maintained throughout the experiment, and mortality accounted for circa 50% of the population over the duration. No reproduction by the snails was observed. It is therefore not surprising that the grazers effect was minimal and could not be established with certainty. It is known that the morphological features of algae influence grazing. Steinman *et al.* (1992) reported that studies with snails (*Elimia*) showed that gelatinous and unbranched filamentous forms were avoided. The unbranched *Fragilaria* was found to be susceptible to grazing, which may be due to its nutritive quality. *Gomphonema* and other stalked diatoms, which provide vertical extentions from the substrate, also were susceptible. Those organisms which have a true "prostrate" growth form, usually not higher than a few micrometers high, are not grazed if they are next to the substrate, but once these organisms begin to accumulate and form clumps, extending to 0.50 μm in height, the snails' radulae are able to harvest those cells (Steinman *et al.* 1992). The initial dominance shift from *Achnanthes lanceolata* to *Cocconeis scutellum* and *C. placentula* observed in the beginning of the experiment when the grazer density was still high, may possibly be due to food preference electivity of the limpets.

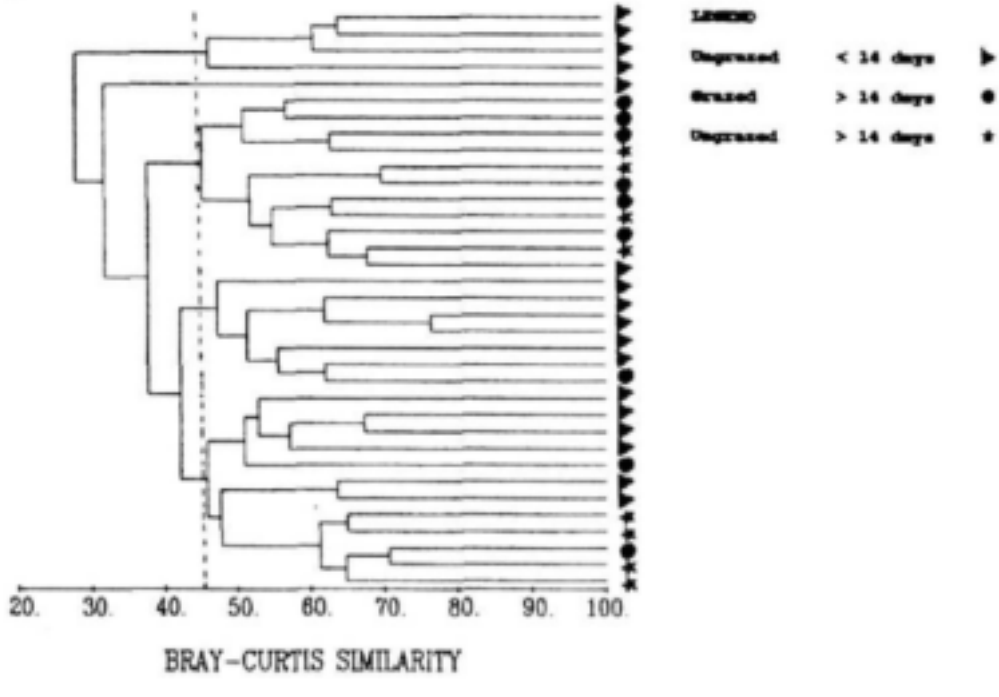
Conclusion

The duration of this study, unfortunately, had to be limited and analysis of a third set of samples was impossible due to time constraints. Further analyses would have given a better insight into the seral stages taking place in the streams during succession as only the beginning of a possible shift to dominance by blue-green algae was observed. Low irradiation and nutrient concentrations may have caused the slow increase in periphyton biomass, and 37 days gave too little time to see full successional changes.

The reason for the high mortality experienced in the limpet community *Burnupia stenochorias* could not be established. Those limpets that survived were growing well, however, showing an increase in length. The lack of any significant effect of grazers on the periphyton assemblages was presumable caused by the high mortality rate and the resulting low density of the limpets, but this could not be established with certainty. The role of diatoms as a food source in these limpets deserves further attention and it is recommended that the gut contents be analysed in future studies.



a



b

Figure 3.5.11 a) Scatterplot of Shannon's Diversity of the species in two treatments. (b) Diatom count of grazed and ungrazed streams. Dendrogram for hierarchical clustering using group-average linking of Bray-Curtis similarities matrix. Abundance data are $\sqrt{\sqrt{}}$ - transformed.

Figure 3.5.12. Multi-Dimentional Scaling (MDS) of species abundances from the 6 streams (grazed and ungrazed/control) (Stress = 0.22).

APPENDIX Illustrating diatoms found during this Project

Scanning Electron Micrographs of the diatoms observed on samples cut out of the plastic lining in the artificial streams.

Figure 3.5.13	*Achnanthes exigua*
Figure 3.5.14.	*Achnanthes inflata* (Round pp 502)
Figure 3.5.15.	*Achnanthes lanceolata*
Figure 3.5.16.	*Amphora copulata* (syn. *A.ovalis var affinis or libyca*)
Figure 3.5.17a,b	*Amphora pediculus*
Figure 3.5.18.	*Cocconeis scutellum* Ehlenberg
Figure 3.5.19.	*Cocconeis placentula*
Figure 3.5.20 a and b.	*Cocconeis* spp
Figure 3.5.21	*Navicula gregaria* Donkin.
Figure 3.5.22.	*Navicula minima*(?)
Figure 3.5.23.	*Navicula pupula* Kützing
Figure 3.5.24.	*Nitzschia debilis* (Arnott) Grunow
Figure 3.5.25.	*Nitzschia* Lanceolatae group example
Figure 3.5.26.	*Nitzschia* Tryblionellae group
Figure 3.5.27	*N. elegantula*(?) Tryblionellae group
Figure 3.5.28	*N. acuminata* (?) Tryblionellae group
Figure 3.5.29	*Gomphonema parvulus*
Figure 3.5.30.	*Fragilaria construens* renamed *Staurosira construens* (Round, 1990).

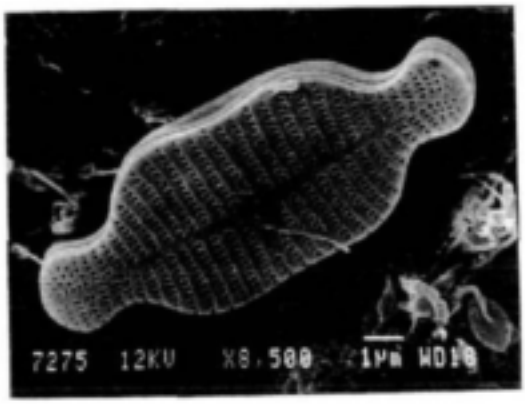


Figure 3.5.13 *Achnanthes exigua*

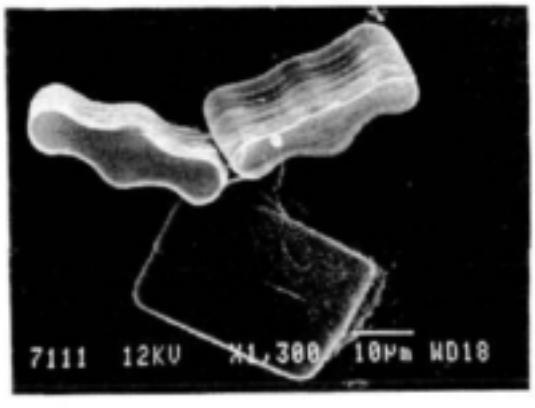


Figure 3.5.14. *Achnanthes inflata* (Round pp.502)

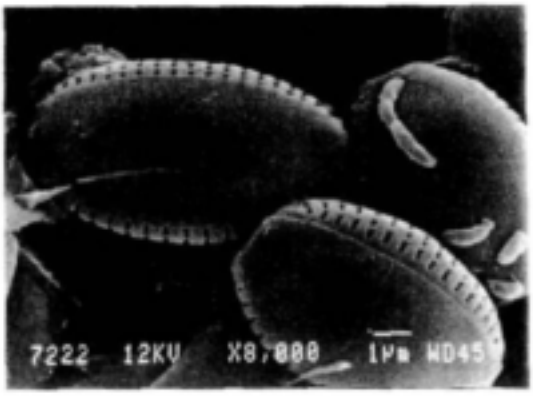


Figure 3.5.15. *Achnanthes lanceolata*

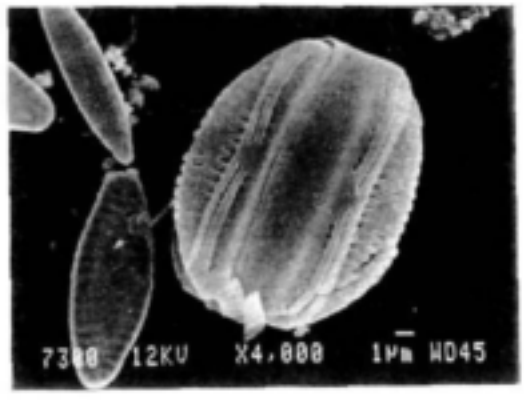


Figure 3.5.16. *Amphora copulata* (syn. *A.ovalis var affinis or libyca*)

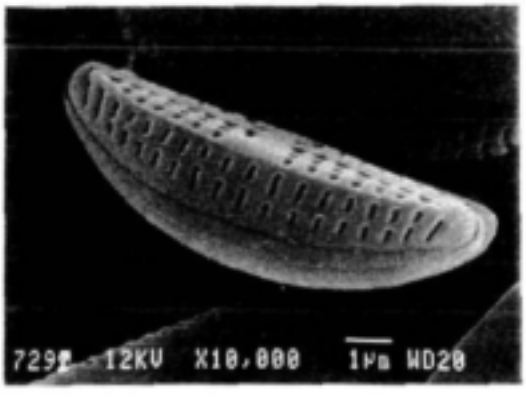


Figure 3.5.17a. *Amphora pediculus*

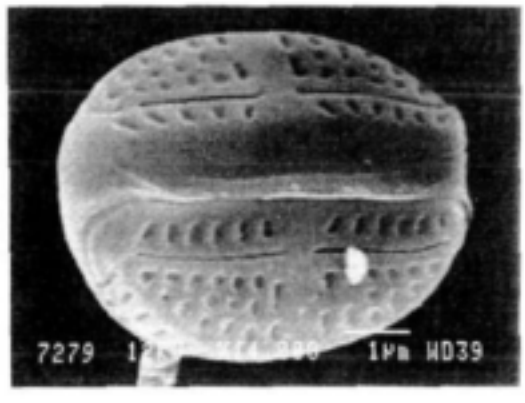


Figure 3.5.17 b. *Amphora pediculus*

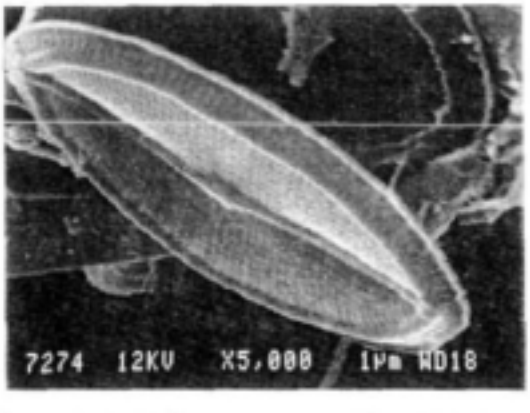


Figure 3.5.18 *Cocconeis scutellum* Ehlenberg

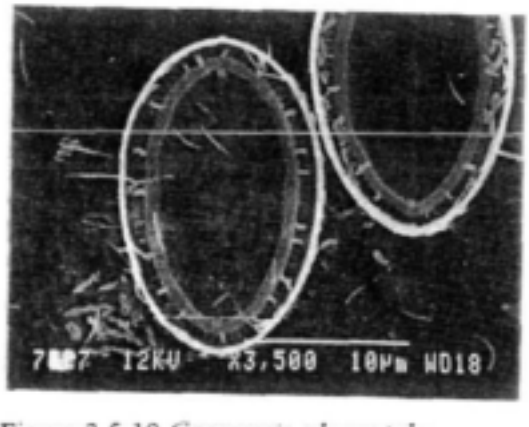


Figure 3.5.19 *Cocconeis placentula*

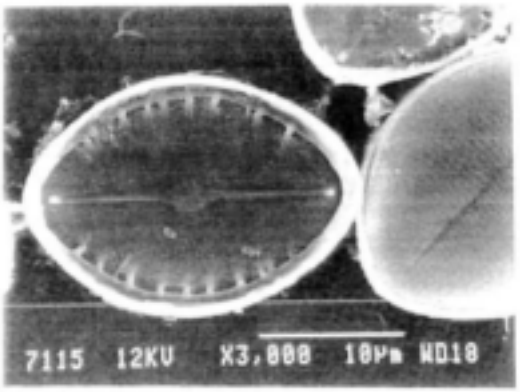


Figure 3.5.20a. *Cocconeis* spp

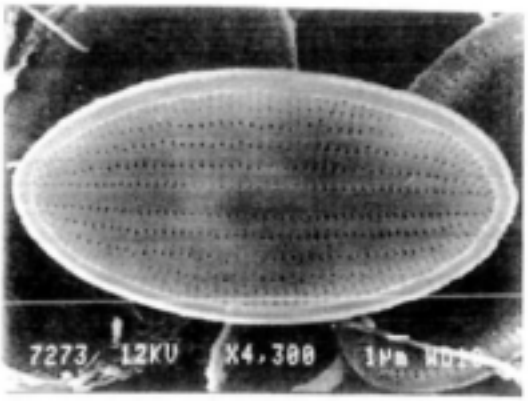


Figure 3.5.20 b. *Cocconeis* spp

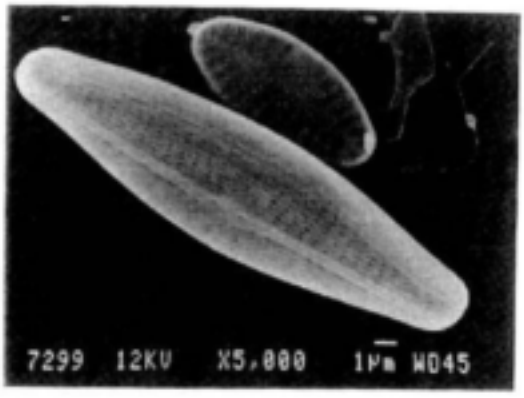


Figure 3.5.21 *Navicula gregaria* Donkin

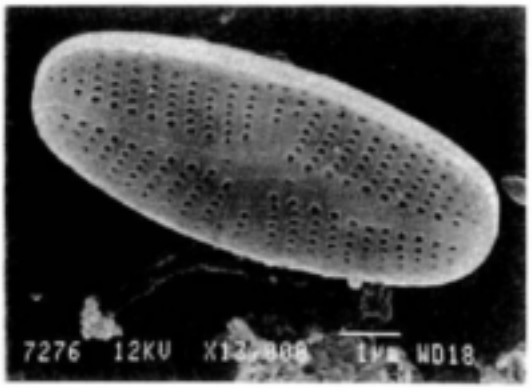


Figure 3.5.22 *Navicula minima*(?)

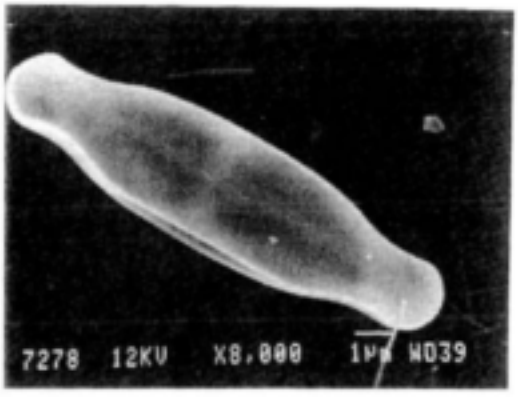


Figure 3.5.23 *Navicula pupula* Kützing

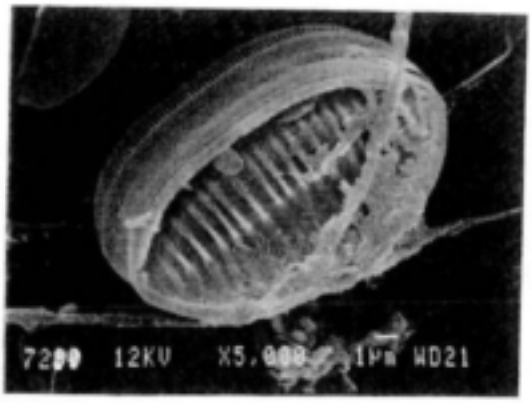


Figure 3.5.24. *Nitzschia debilis* (Arnott) Grunow

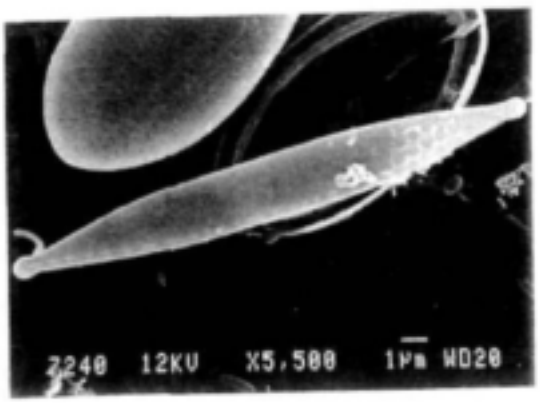


Figure 3.5.25 *Nitzschia* Lanceolatae group

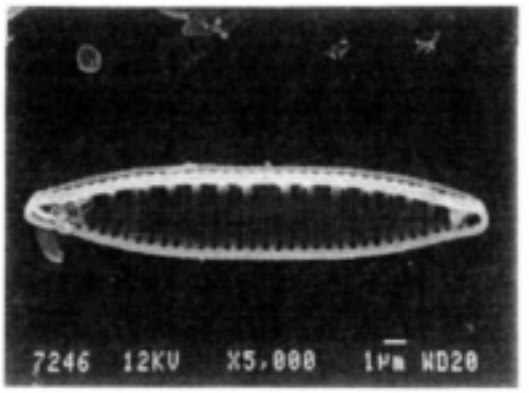


Figure 3.5.26 *Nitzschia* Tryblionellae group

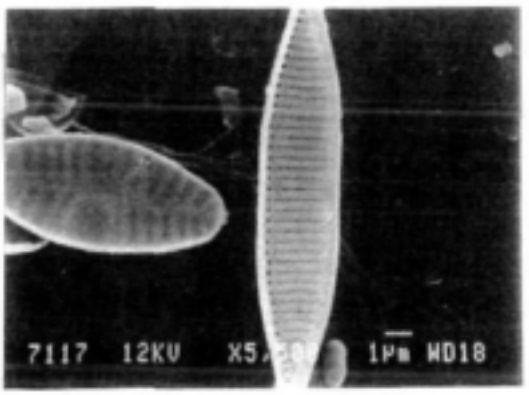


Figure 3.5.27 *Nitzschia elegantula*(?) Tryblionellae group

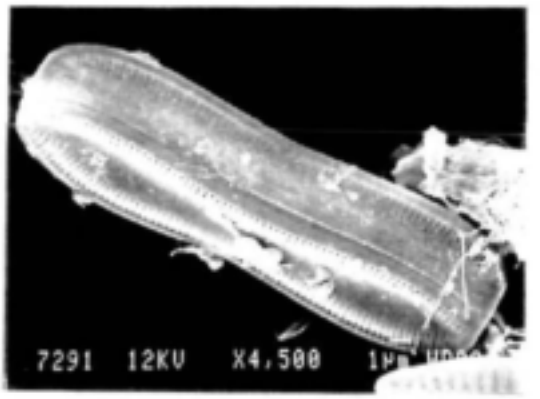


Figure 3.5.28 *Nitzschia acuminata* (?) Tryblionellae group

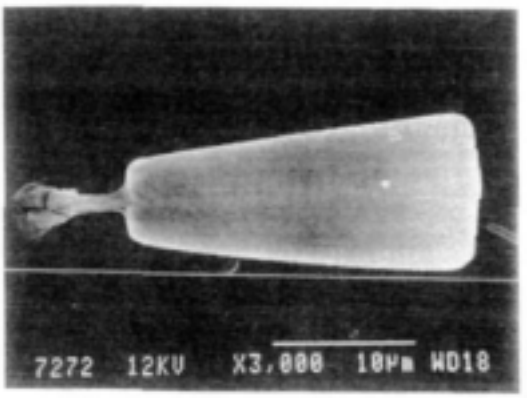


Figure 3.5.29 *Gomphonema parvulus* can have stalks of various lengths

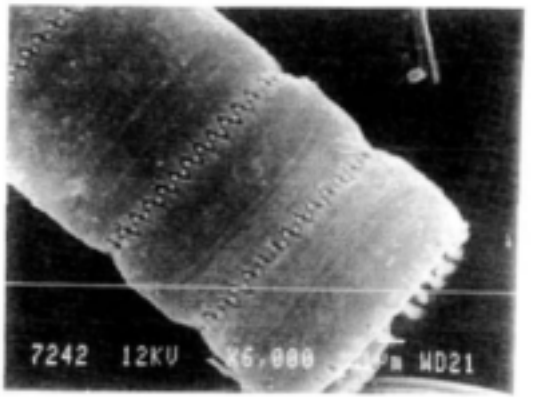


Figure 3.5.30 *Fragilaria construens* renamed *Staurosira construens* (Round 1990) Cells adhere through interlocking siliceous spines

CHAPTER 4
INVESTIGATIONS OF THE MAYFLY *ADENOPHLEBIA AURICULATA* (EATON), 1871

4.1 INTRODUCTION

We have identified genera from the family Leptophlebiidae as mayflies which may be suitable ecotoxicology test subjects since members of this family are widespread and fairly large in size, robust and easy to identify. According to Johnson *et al* (1993) the most useful life history indicators of environmental stress are survival, and alterations to growth and reproduction. This emphasises that information about the life history growth, reproduction and responses of the mayfly to laboratory conditions is essential as a baseline against which to judge results from toxicological experiments. Buikema & Benfield (1979) reviewed a hundred publications on toxicology and found that 50% used no life history information in the analysis of the results. Buikema and Benfield (1979) discussed the importance of using both life cycle and life history information in designing and assessing toxicity tests. For many reasons, different life stages, such as immatures and gravid females, have been found to be more sensitive to toxicants. Reproductive impairment and moulting frequency are good measures of stress. Therefore, if the selected test species is to become a successful laboratory animal, its responses to test conditions should be accurately interpretable against adequate 'natural' life history information. To fulfil some of these requirements, the field population should be studied for at least a year if not longer. *Adenophlebia auriculata* is the species we decided to focus our attention on. Collection records from the Albany Museum in Grahamstown shows the known distribution of this species to follow the Drakensberg escarpment, to the Amatola range in the eastern Cape and extending to the southern Cape (Everitt 1996). In the Palmiet River in the eastern Cape, *A. auriculata* is an abundant component of the aquatic macroinvertebrate fauna, and has potential as an important pollution indicator species as it is fairly widespread and inhabits upper reaches of rivers and streams. Detailed information on its response to laboratory conditions as well as knowledge of its population distribution, seasonality and habitat preference is needed to determine whether *A. auriculata* will be a suitable subject for laboratory culture for ecotoxicological testing.

The previously completed experimental work on *A. auriculata* yielded information on the responses of the mayfly to:

a) Habitat variables such as hydraulic conditions and substrate type. An essential prerequisite was found to be a solid substrate and that, although the species is found in riffles and runs, the

survival rate in static aerated water is better than in experimental channels. This concurs with observations in the field that *A. auriculata* is seldom captured in the full current but tends to be found under stones on the edges of runs where detritus collects (See section 4.2.3)

b) Diet and temperature variables. In this investigation it was found that the natural diet of decaying leaves and periphyton could be successfully supplemented by the addition of TETRAMIN, a commercial fish food, and provide optimal conditions for growth provided that good water quality was maintained. The instantaneous growth rate observed are detailed in Table 4.1.1.

Table 4.1.1 Growth rates (mm/day) pre sub-adult nymphs calculated from all experiments in channels and bubblepots in the laboratory

REP. CHAPTER EXPERIMENT NO.	AVE INSTANT. GROWTH RATE MM/DAY	TEMPERATURE	HYDRAULIC CONDITIONS & DIET
K/545 5.2.1	0.019-0.023	19-22°C	Flowing, periphyton
K/545 5.2.2	0.014-0.042	17-22 °C	Static; leaves; TETRAMIN
K/545 5.2.5	0.005	15 °C	Static, leaves
	0.009	20 °C	
	0.015	25 °C	
RANGE	0.005-0.04	15-25°C	

4.2 THE AUTECOLOGY OF THE MAYFLY *ADENOPHLEBIA AURICULATA* (EATON) (EPHEMEROPTERA, LEPTOPHLEBIIDAE).

4.2.1. LIFE HISTORY IN A SUBTROPICAL STREAM IN SOUTHERN AFRICA.

Introduction

The order Ephemeroptera dates from Carboniferous and Permian times and today their highest diversity is in lotic habitats where they are an important component of the ecosystem. They consume organic matter such as leaves and fine particulate organic matter as well as algae, and this energy in then made available to predators in the system (King *et.al* 1987 a & b). These processes are essential in the upper reaches of rivers where the majority of energy in the system is imported from outside in the form of leaves and detritus.

Mayflies are unique among the insects in that they have two winged adult stages, the subimago and the imago. There is much debate as to whether this is a primitive or derived state (Edmunds & McCafferty 1988). The presence of long dark wingbuds on nymphs usually indicates imminent emergence. Males which have large double eyes can be easily distinguished from females, which tend to be larger. In leptophlebiids the pharate subimago crawls a few centimetres out of the water usually onto a rock or up vegetation and moults into the subimago. The subimago may then fly or walk a short distance to cover before resting. Subimagoes are generally not strong fliers and seldom fly far. After 24-48 hours the subimago moults into an imago which differs from the subimago in that the dark opaque wings with a fringe of fine cilia become clear and reflective and the cerci and forelegs are longer. The only function of adult mayflies is reproduction and the adults have no functional digestive system. Hence, adult mayflies seldom survive for more than one week and usually die due to desiccation (Edmunds 1972). The general breeding behaviour of mayflies involves the males forming a swarm, and any female flying through the swarm is usually mated with immediately (Needham & Traver 1972; Brittain 1982; Edmunds & McCafferty 1988).

Environmental Influences

Investigations into the environmental factors which govern the life cycle of stream macro-invertebrates are numerous and mayflies in particular, have received much attention in recent years. The total development time is governed by external factors such as temperature, available food and photo period, which all influence the expression of the genotype, of which temperature may be the most important (Sweeney 1978 & 1984). Investigations have revealed similar traits in large numbers species from

similar biomes (Wiggins, 1977; Clifford, 1982; Newbold *et.al.* 1994; Jackson & Sweeney, 1995) while differences in life history are also found between occupants of the same biome (Sweeney & Vannote, 1981; Jacobi & Benke, 1991; Sweeney *et al.* 1995). This leads one to the conclusion that the genotype will determine the life history style: how the species apportions the available energy between growth and reproduction is often determined by local conditions.

To determine the life history, life cycle and population dynamics, a population should be sampled repeatedly at either one site or at several sites in different climatic regimes. The correlation of environmental cues (photic = (day length and moon phases), thermal = (water temperature and degree-day) and partial pressure with emergence periodicity requires a data set which cover more than one year to ensure replication of seasonal variations. Only if similar responses occur over a number of seasons, can conclusions be drawn about the effect of climate on the life history . Experimental growth rates can be used, with caution, to calculate possible generation periods.

Sweeney (1978), investigating the metabolism of *Isonychia bicolor* showed that there is little metabolic compensation or acclimation to thermal fluctuations within this species and that summer and winter generations will exhibit the same metabolic rate and response to thermal changes. It was found that respiration rates of both winter and summer nymphs were the same when measured at 15 °C. Similar responses were recorded from gastropods by Berg *et al* (1958) & Calow (1975). It is therefore not surprising that several studies (Wise, 1980; Giberson & Rosenberg, 1994; Sweeney *et al*, 1995; Pritchard & Zloty, 1994) have revealed that intra-specific voltinism may be affected by the thermal regime of the environment. This results in a uni or semi-voltine life cycle gradually becoming bi-voltine as the thermal regime of the environment increases. Furthermore it has become clear that as the ambient temperature of an aquatic system increases with a concomitant decrease in seasonal fluctuation, the life history styles of the assemblages of invertebrate inhabitants tend toward multivoltinism (Jackson & Sweeney 1995; Jacobi & Benke 1991). Under these circumstances the development speeds up with increases in temperature.

However, adult size and fecundity, which are positively correlated (Anderson & Cummins 1979), and developmental period can be adversely affected by temperatures outside the optimal range for a given species. Sweeney & Vanotte (1978) hypothesized that this is due to a disequilibrium between larval growth rate and the timing of metamorphoses caused by a shift in the energy partitioning.

Aims

1) To determine the seasonal population densities, habitat preferences, life history style (uni/bi/multivoltine) and the peak emergence periods of *A. auriculata* in the Palmiet river.

2) To attempt to correlate the rate of growth determined experimentally for *A. auriculata* nymphs and from the natural population using a computer analyses program in order to estimate the life cycle.

Materials and Methods

Study site and field data

1) The study site is a stretch about 0.8km upstream of the confluence of the Berg and Palmiet rivers at the national road and comprises a series of riffles, runs and pools (Wadeson 1994) (Figure 4.2.3.1). During a preliminary study in 1993 a stretch of about 0.5 km consisting of 2 riffles, 2 runs and a pool is sampled at monthly intervals from February to September. In 1994-96 the stretch, which was sampled weekly, is the run where the highest densities of *A. auriculata* were recorded throughout the year in 1993. During the weekly field samples, the specimens which were collected, were measured as described below and immediately returned to the upstream area of the study site in order to minimize the impact of the investigation. Animals which were captured and returned to the laboratory were collected downstream from the study site. Water temperature, conductivity and pH were taken weekly and averaged for each quarter (Table 4.2.1.1) and the water level of the stream was also noted. Daily maximum and minimum air temperature as well as rainfall is recorded automatically and was used to calculate degree days (DD) for the area using the formula DD = mean T-To.). where To = 10°C, chosen from the literature as the temperature below which no development may take place. DD was summed for weekly periods to coincide with the sampling strategy.

Table 4.2.1.1 Average water quality information from the Palmiet River 1994-1994.

YEAR	SUMMER			AUTUMN			WINTER			SPRING		
Condition	*pH*	*TDS*	*℃*	*pH*	*TDS*	*℃*	*pH*	*TDS*	*℃*	*pH*	*TDS*	*℃*
1994	5.81	68.4	24.8	7.2	84	21	6.0	86	17.6	7.0	71	17.2
1996	6.0	73.5	20.1	7.0	71.3	19.4	8.0	86	12.1	7.0	96	20

2) Collecting was by handnet with 80μm mesh which was downstream from a rock which was lifted and washed into the net. The contents were placed in a 5 litre bucket containing stream water. At the site the nymphs were sorted in a shallow tray and measured. For the derivation of quantitative data, the collection method was for the same collector to collect for a standard time period. These samples were preserved and returned to the laboratory. The sub-imaginal shucks, observed on rocks at the edges of the stream, were counted each week, as were adults, either resting on the edges of the stream, drowned, or caught in spider webs or emergent traps.

3) The dorsal head-widths (HW) across the widest points at the eyes were measured in the laboratory using a calibrated microscope eyepiece at 250 times magnification or on a calibrated 'V' on graph paper. The head widths show a closer correlation to the instar than does body length which can vary considerably, as discussed below in section 4.3. During the weekly sampling of 1994-95 the head-widths (n=50) were measured in the field. The smallest nymphs below 0.8mm are more difficult to capture, and the largest sizes are comparatively scarce. The two groups were purposefully sought, while the other sizes were collected randomly. During 1996 the entire sample was measured and collections were only done at fortnightly intervals. Once measured the insects were placed in a holding bucket to prevent re-sampling and were released into the same site once the sample was complete. The smallest individuals (<0.4mm HW) were never captured alive, which leads to an underestimation of the proportion of hatchling nymphs in the population as well as difficulty in estimating hatching period and the duration of the earliest part of the post embryonic period.

4) Analysis. The samples collected were grouped in two types of size classes. To ascertain the population dynamics the first division was rather coarse, those below 0.9mm HW were grouped and represent the recruits from the hatchlings of the previous laying. The sizes between 1.0 and 1.6 mm HW were considered the standing stock of the population. The sizes above 1.6mm, which have wingbuds visible to the naked eye, represent the sub-adult maturation phase. The mean size and standard deviation of this group and of the entire sample was calculated. For the purpose of ascertaining the population growth rate the size classes were smaller, being 0.4mm apart. This grouping was used with the program FiSAT in an attempt to determine growth rate and lifespan in the population (see Chapter 3.2.2).

Results

Temporal variation in population.

The head width (HW) of *Adenophlebia auriculata* ranges from 0.04mm to 2.7mm, which was the largest sub-adult female ever recorded from the Palmiet. Figure 4.2.1.1displays the mean size of the population for the entire sampling period. The most striking feature is the degree of similarity between samples, between seasons and between years. The mean size of the samples collected was between 0.7 and 1.7 with a grand mean of 1.2mm. A mean head width of less than 1mm was only recorded for five samples. The smallest mean size recorded was in April 1995, (0.70±0.386mm) a sample collected after a period of adult emergences. A drop in the mean recorded size generally follows adult emergence and can be ascribed to both the large adults leaving and the hatchling nymphs beginning to appear in the collections. The mean size of the nymphs in the summer and autumn of 1994-95 followed a trend of declining mean size after the early spring flight due to this occurrence. Towards the end of summer the mean size

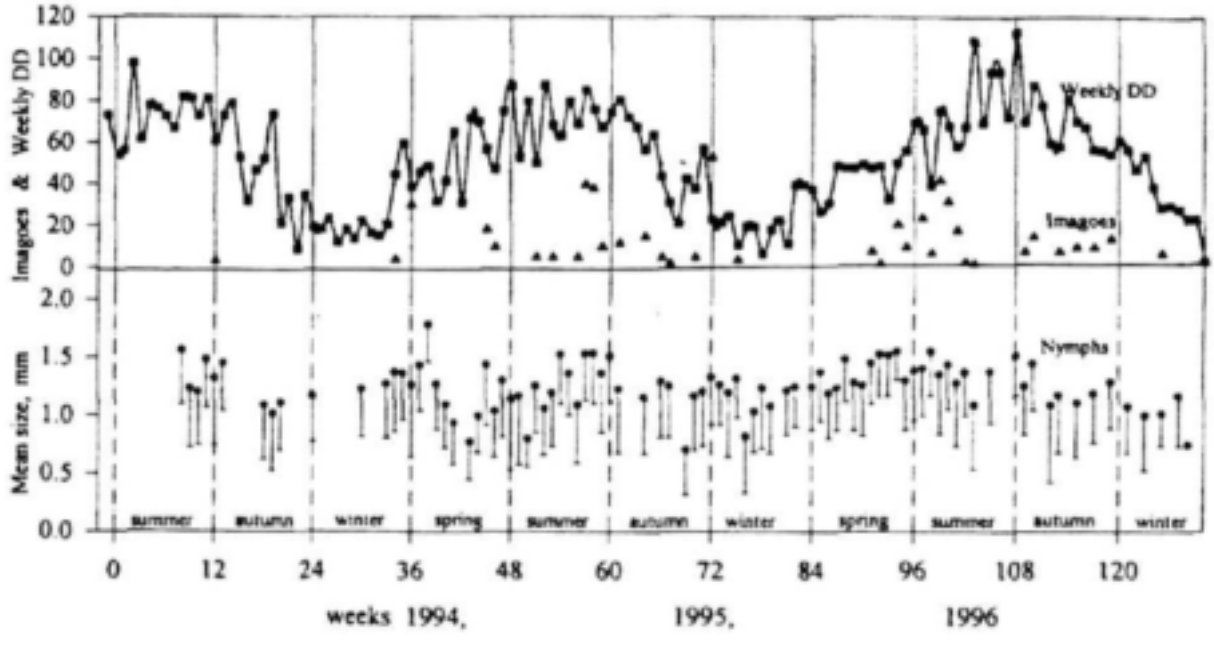


Figure 4.2.1.1 The mean head width ●(-STD) of weekly sample of nymphs of *A. auriculata* for a two and a half year period, together with the numbers of emerged adults (▲) observed during the weekly collections in the Palmiet River, overlaid with weekly aerial cumulative DD(-■-).

increases again (weeks 48-60) then declines. The same trend was again observed in the 1995-1996 from weeks 84 to 108.The effect of the summer hatchlings entering the population can be seen in late autumn before week 24, 72, and 120 when mean size of successive samples show a decline. A large group of small nymphs were measured before week 72. The largest mean sizes are recorded at the beginning end of summer (just before week 60 and 96).

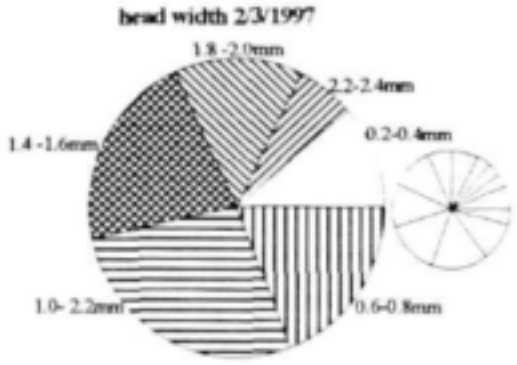


Figure 4.2.1.2 Proportion of the population which is found in each size class at the end of the summer emergence. The small pie gives an indication of the fine divisions in each class. This sample was preserved and measured under magnification.

The principal reason for the consistency in mean size can be found in the composition of the population throughout the year. Figure 4.2.1.2 depicts the composition of a sample of 812 nymphs collected from the Palmiet River, preserved and measured under a binocular microscope. This gives an accurate reflection of the population structure at the height of summer and also shows which proportion of the sample are true hatchlings (the clear slice). As can be seen about 50% of the sample comprises the middle range of sizes. If this pie chart is compared to Figure 4.2.1.3, the similarity of the population composition is immediately obvious. A notable feature of the population profile for 1994 is the standing stock of middle size nymphs (1 - 1.6 mm HW) the cross hatched slice forms the upper end of this group) which is usually about 50% of the sample except for brief periods. The numbers of recruits in the samples fluctuate considerably but it is clear from Figure 4.2.1.3 that recruitment continues throughout the year. Another feature worthy of note is the consistent presence of sub-mature nymphs the slice immediately above the crosshatched segment. Right through winter (16/6/94 to1/9/94) this proportion remains present and consistently comprising more than 50% of the sample. The hatchlings make a significant appearance in summer 27/10/94. To show the consistency of the type of population composition across years Figure 4.2.1.4 was compiled from samples from similar time periods from all three years during which investigation took place. If one considers the single sample from 1997 (Figure 4.2.1.2) as well, it seems that Figure 4.2.1.3 gives an accurate picture of the population profile for *A. auriculata* nymphs in the Palmiet River.

The dynamics of the size class from 1.6 to 2.4mm HW was investigated by separating this group from the smaller nymphs which meant that the mean size was unaffected by the recruitment of hatchlings (Figure 4.2.1.5). In the period before emerging, these nymphs, which we term sub-adults, metamorphose, developing wingbuds, testes and ovaries. The nymphs of this size class were present in all but a few weeks during the year (Figure 4.2.1.2, and Figure 4.2.1.3). In Figure 4.2.1.5, the emerged adults are indicated by arrows above the mean size of the sub-adult nymphs of that week, which are displayed in conjunction with the weekly degree days. What becomes apparent is that there are extended periods of emergence during which the mean size of this group remains above 1.75mm, while during winter it drops below that. Eighty three percent of the samples from the winter/spring period

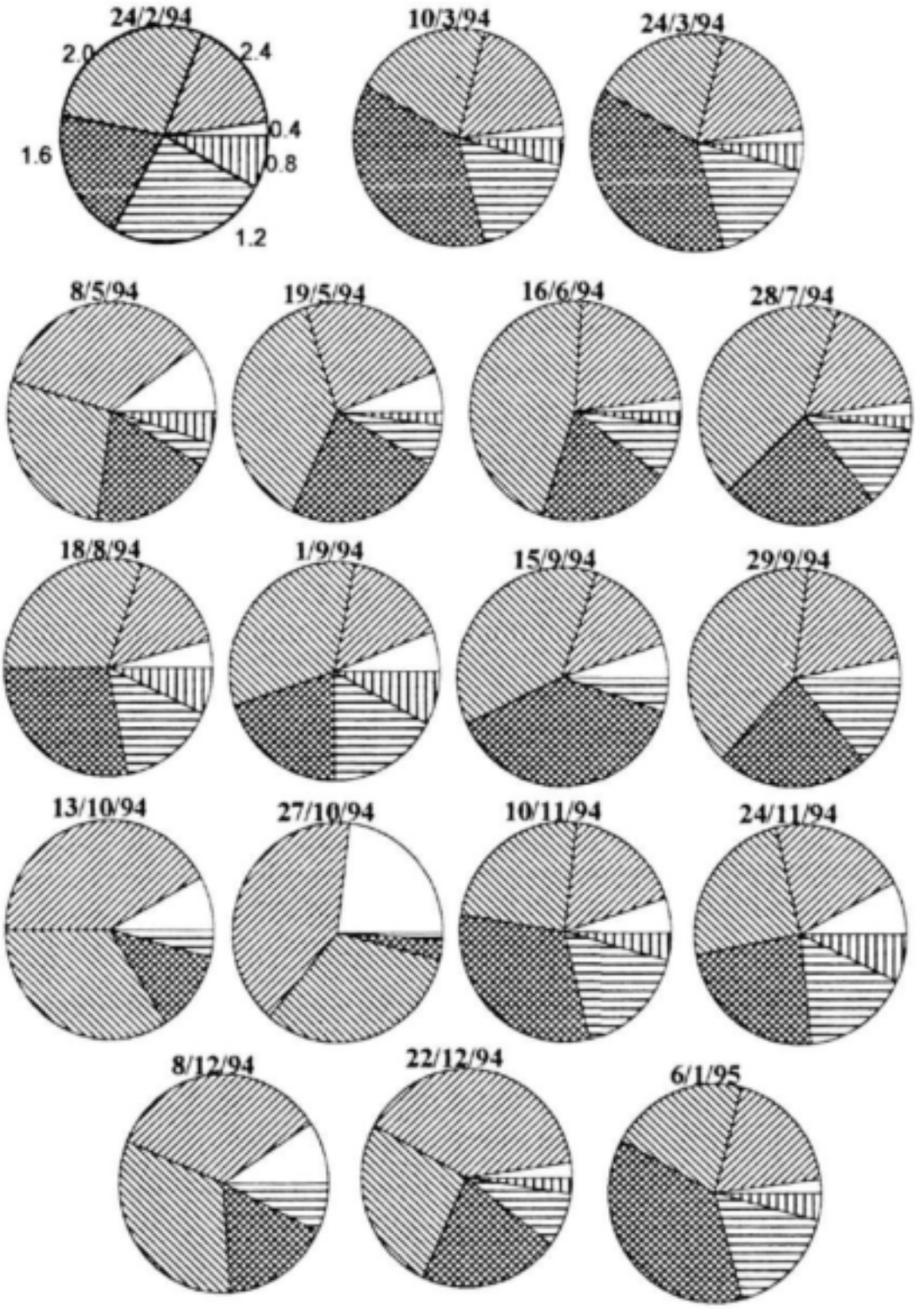


Figure 4.2.1.3 Proportion of the various sizes classes in samples of nymphs of *A. auriculata* collected from the Palmiet River during 1994. The clear wedge represents the class 0.2-0.4mm HW which is the smallest group collected. Classes are grouped in 0.4mm intervals and the numeral on each pie in the first graph indicates the upper size limit. Sample dates are given above each graph. The consistency of the population profile throughout the year is clearly demonstrated in this figure. An indication is also given of the proportional changes between size classes.

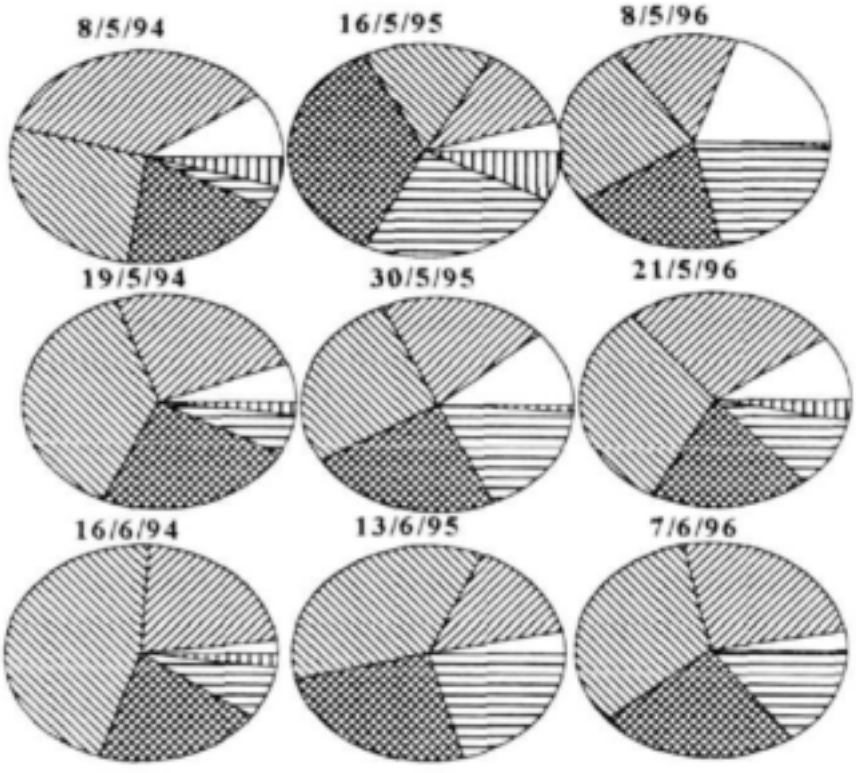


Figure 4.2.1.4 Proportions of each size-class in late autumn at the time of the autumn emergence, in three years. The consistency of proportion across the years is the major feature. The clear wedge represents the smallest size class 0.2-0.4mm HW.

(31/5-4/10) had mean HW <1.75mm., while in the preceding summer/autumn (2/3-31/5) and spring/summer period following (11/10-24/1) only 16% to 33% of the subadult samples were <1.75mmean HW (Figure 4.2.1.5). The last two samples before the spring emergence both have a mean size of 1.75mm which may be an indication of the imminent spring emergence. The considerable drop in mean size following the spring flight is clearly as a result of a batch of large adults leaving the population. During summer the mean size of the nymphs do not appear to have any effect on the emergence rate. The small mean size of the overwintering sub-adult could be as a result of both the emergence of the remainder of the large mature nymphs in spring and the retarded growth rates during the winter period when the maximum weekly temperature recorded in the river seldom exceeds 20°C.

From Figure 4.2.1.1 and Figure 4.2.1.5 it is clear that there are extended periods of emergence during the year but that at times more adults were seen than at others, which indicate that there are periods of intense ovipositing. *A. auriculata* adults usually live for no more than four days. This would indicate that emergence aught to be closely synchronized. During the periods of high levels of emergence this could be so but during those periods when fewer adults emerge mating opportunities may be more chancy. The

periods of intense emergence occur in early spring when the overwintering mature nymphs emerge (before weeks 48 and 96) in summer and late autumn. The summer emergence of 1994, started at a low level. No adults were observed in October for a few weeks. Then the number of emergences gradually increased until a large number were observed during February just before week 60. As the summer progresses, flights became less frequent with a hiatus occurring in autumn during April and early May. The summer emergence in 1995/6 started earlier and was more regular for a longer and the peak occurred earlier than 1994 at around weeks 99-100 (November to January).

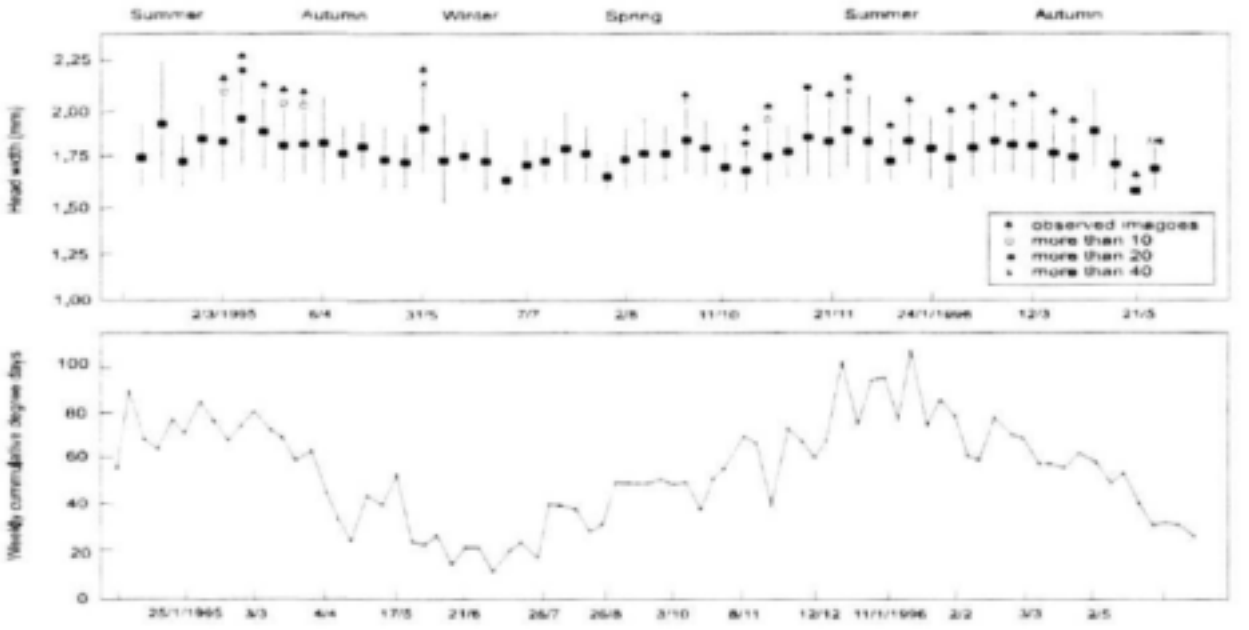


Figure 4.2.1.5 The mean±STD of the sub-adult nymphs in the Palmiet River from March 1995 to May 1996. The arrows indicate flights which are coded for number. The cumulative DD for the area during the same period is depicted on the lower chart.

In 1994 (Figure 4.2.1.1) autumn emergences occurred in late April early May (weeks16-18), when about 30 adults and final instar shucks were observed. In the pie charts dated 24/3/94 to 8/5/94 of Figure 4.2.1.3 the large proportion of subadults bear further testimony to the possibility of flights. During 1995 this period was closely monitored. The samples from weeks 69 to 72 collected from the 2/5 to the 31/5/1995 indicate a similar pattern for 1995. During the autumn of 1996 (weeks 108 to120) flights showed a similar pattern to 1995 and the autumn hiatus was pushed into early winter the last flight taking place after week 120 in early June (Figure 4.2.2, Figure 4.2.1.5).

The spring emergence which occurred in weeks 35-37 and 5 adults and 22 final instar shucks were observed. The relatively short period of this emergence is most likely an artifact as there was a rainstorm the following week which could have washed the shucks off the rocks. This deduction is supported by the

fact that the number of sub-adult were still high in the nymphal population despite the large emergences the preceding weeks. The spring of 1995 bears this out as emergences were recorded for the whole of October and November (weeks 92-100).

During winter and early spring (weeks 24-36 and 74-86) no shucks were recorded although the sub-adults were present (Figure 4.2.1.3; pie 28/7 to 18/8/94). The early spring emergence expected between week 84 and 86 did not happen and the first shucks were counted in early October (week 90). The biggest differences between autumn 1995 and 1996 (weeks 60-72 and 108-120) is the extended period of emergences in 1996 when the autumn hiatus was half of that in 1995.

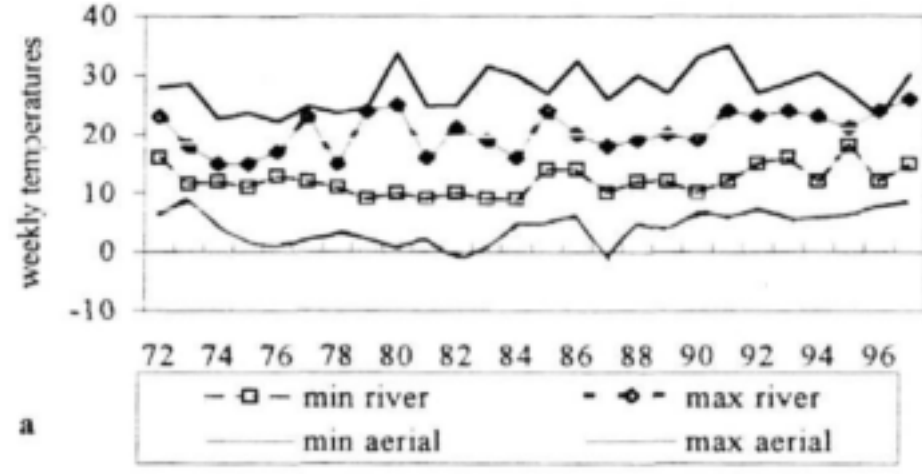
We feel that this can be directly attributed to the greater heat input of 1996. The heat summation in total DD for that year was about 2800 while for the previous year it was 2500 (Figure 4.2.1.6b). Although the observation of adults in the field is not quantitative nor totally accurate (it can be masked by the river flooding and washing shucks off the rocks and drowning sub-imagoes, as well as observer failure) we feel that it gives as reliable indication of emergence periodicity.

A. auriculata has a complex life history in the Palmiet River which is rather difficult to interpret. Successful interpretation depended on discovering if the species is multivoltine or univoltine. To establish this, the life span of one cohort *A. auriculata* needs to be determined. We have attempted to accomplish this by three different methods: a) Deductively by attempting to establish the heat summation between major emergence periods b)By using growth rates obtained experimentally c) By using a computer program, FiSAT developed for the purpose of analyzing overlapping cohorts in fish.

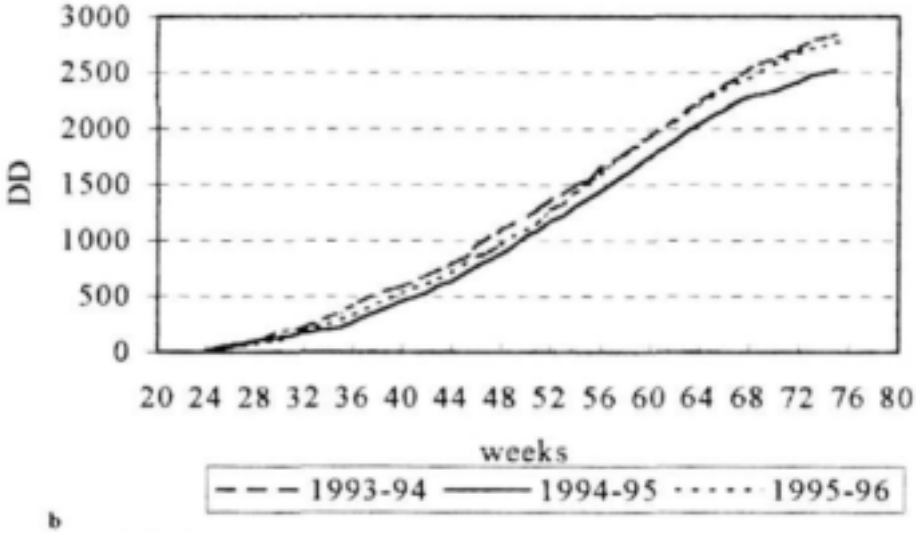
Beside available food, daily temperature input into the area and the climatic region has a major influence on the emergence periodicity of poikilothermous. Therefore we give two charts which show the thermal conditions of the Eastern Cape during the study period. Figure 4.2.1.6 a) shows the temperature recorded in the Palmiet River by a max/min thermometer for a period of a year and the aerial temperature were recorded at a weather station 20km from the river. One or two of the recording appear anomalous such as the 24°C max river temperature at about week 77 or the 15°C recorded the following week when there is no apparent aerial correspondence

Cohort analysis.

In Chapter 3 we give a detailed description and discussion on the various methods and techniques which are available to assess cohort growth and to distinguish multiple cohorts. Length frequency distribution analyses is most commonly used. Separating overlapping cohorts remain problematic, despite the mathematical model developed to deal with such populations. In *A. auriculata* the problem is further complicated by the continual recruitment of hatchlings throughout the winter from the eggs laid during the autumnal flights. The extended emergence periods give further indications that in the Eastern Cape,



a



b

Figure 4.2.1.6 a) Weekly maximum and minimum water and aerial temperatures for the Palmiet River and its geographical area. b) Cumulative DD for each year from 1993 to 1996 was summed from the coldest midwinter day each year.

recruitment to the population of this mayfly is continuous. When Figure 4.2.1.3 and Figure 4.2.1.4 are examined again it becomes clear just how closely the cohorts overlap. The population can undergo differential growth rates depending on the ambient temperature and quality of available food as can be seen from Table 4.1.1 This can result in the overwintering standing stock experiencing growth retardation to some degree which will extend the life span for these cohorts relative to those which hatch from the spring spawning. So the question about length of the life cycle remain difficult to resolve, for although it is now known when the mating periods are, we have not yet been able to accurately predict the period between hatching and when the 0.4mm HW nymphs appear in the samples. We also have no idea wether autumn eggs hatch or wether they remain in diapause and hatch near or simultaneously with the spring eggs.

In Figure 4.2.1.6 the observed adults are plotted against the cumulative DD. As no emergence were recorded for the winter period we feel safe to assume that no eggs were laid from mid June to end of August (weeks 24-34 and 74-86). The autumn emergence at weeks 12-24 and 70-74 was used as suitable starting point from which to calculate possible generation periods. The start and end of the winter period where there are large numbers of flights was used as an estimated cut off point. That being so, it appears that 2200-2500 DD may be the requisite amount of energy for *A. auriculata* to complete its life cycle. If 2000 DD were sufficient then emergence would have had to take place in week 84 which is midwinter. The largest number of suitable termination points occurs where the recorded emergences coincide with 2500 DD in Figure 4.2.1.7. In terms of time, the generation period can be anything between 48 and 50 weeks. So why was there not an emergence in week 84? It can be that the 1994 spring generation delayed their emergence due to a cold early spring in 1995. From Figure 4.2.2. it can be seen that the early spring in 1995 was indeed cooler for an extended period. Compare the weekly DD profile between weeks 31-39 and 79 - 87 in Table 4.2.1.2. The * indicate those weeks which were cooler in1995. The total thermal input for that period was also less.

Table 4.2.1.2 Comparison the cumulative weekly Degree Day in like weeks in early spring in1994 and 1995

WEEK 1994	DD	DD	WEEK 1995
31	17	19	79
32	15	23	81
33	21	12*	82
34	45	40	83
35	60	38*	84
36	39	38	85
37	46	27*	86
38	48	31*	87
39	32 **total 323**	49 **total 277**	88

However a further calculation from observed data can be made to corroborate this evidence. The laboratory growth data obtained previously (Table 4.1.1) indicated that instantaneous growth rates ranging from 0.014 to 0.045mm/day could be expected, depending on both temperature and food quality. The highest growth rates were obtained at a steady ambient temperature of 25 °C and on a diet supplemented with TETRAMIN. These conditions are both abnormal and these high growth rates can therefor be ignored for

the purpose of calculating lifespan and growth in the field under natural conditions. Nymphs needed between 10 and 14 weeks to grow from 0.6mm HW to maturity at2.4mm HW at 20 °C under laboratory conditions at ambient fluctuating temperatures.

From the egg fertilization experiment in 1994 it was established that the eggs hatch in 17 to 20 days (2-3 weeks) at average ambient temperature of 20°C. If the instantaneous growth rates obtained experimentally are extrapolated to weekly growth and the divided by the possible HW achieved by males and females respectively, the possible generation periods vary from 12 to 38 weeks (Table 4.2.1.3). Add the two to three weeks for hatching to take place and the generation period then ranges from 14 to 40 weeks. If this period is applied to Figure 4.2.1.7 the thermal input needed to complete the life cycle is reduced to 1800 to 2000 DD. This seems perfectly feasible, as no flights would then coincide with the winter period. Unfortunately it is not possible to get an accurate DD calculation for laboratory conditions as the temperature was not measured daily.

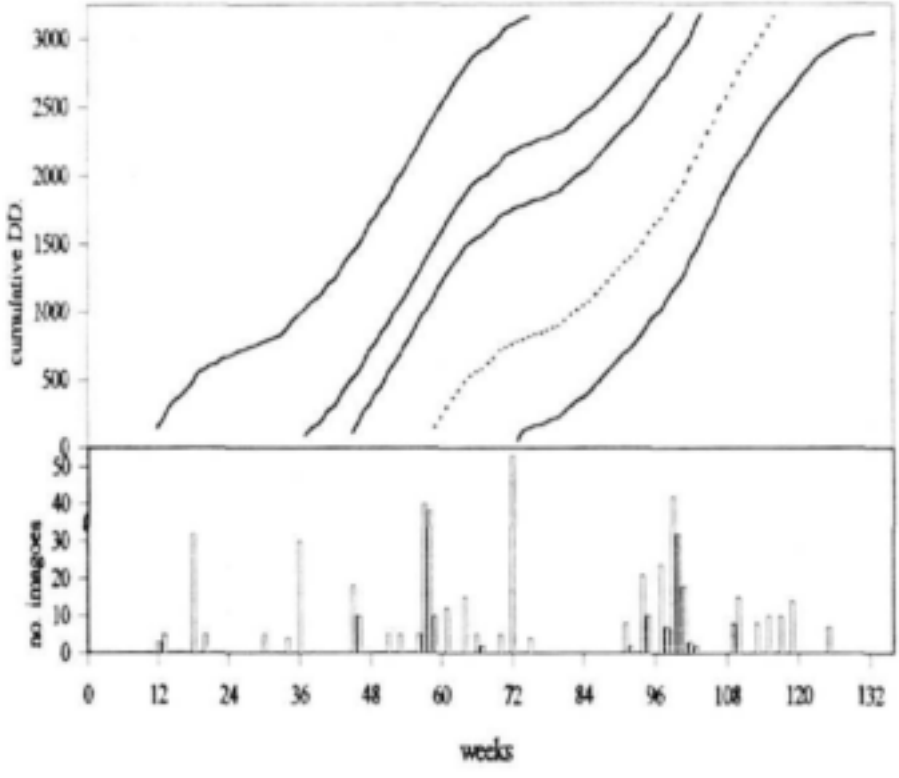


Figure 4.2.1.7 Emergences observed at the Palmiet river plotted with the cumulative DD summation calculated from each emergence period. The winter period is around weeks 24 and 84 and these periods were used as starting point for estimating the generation period.

Table 4.2.1.3 Generation times calculated from laboratory-obtained growth rates.

Instantaneous growth rate	weekly growth rate	weeks to achieve 1.8mm HW	weeks to achieve 2.4mm HW
0.009	0.063	28.57	38.1
0.014	0.098	18.36	24.5
0.02	0.14	12.85	17.14

The discrepancy in the life cycle between males and females can be partially explained if Figure 4.2.1.8 is examined. As sub-adults mature, the relationship between head-width and length, head-width and wingbud length changes, and the measurement of HW as indicator of size should be viewed with caution. Regressions of these body proportions were plotted with head- width as the independent variable, separately for males and females. What is clear from these regressions is that males are shorter and with smaller heads than females and that wingbud development escalate after 1.5mm head-width is reached. However this does not indicate if the males of the cohort will emerge five to ten weeks earlier than the females or if the male growth is slower than that of the females. It has been observed by De Bisthoven (section 4.3) and previously by us, that males do emerge earlier than females in the laboratory.

Further corroboration for the life cycle was the sought by using the program FiSAT. For the principles underlying the methods employed by FiSAT of Gayanilo *et.al* (1995) see Chapter 3.2. The samples for every two week were combined, placed in 0.2mm size classes but had to be separated into two sequential sets as the data set was too large for the program to handle. (Figure 4.2.1.9). Only one calculation for the procedure is shown here. Modal Class Progression Analyses yielded no statistically acceptable results for the data set. The ELEFAN program to estimate growth by tracing growth curves through a time sequence population sample was then used. In Figure 4.2.1.9 both the seasonalised and non seasonalised curves are shown. The amplitude C=1 allows for zero growth during the winter period. The results from the best fit Rn values indicate that the mayfly has an annual life cycle, at a growth rates of κ 0.045 mm/day.

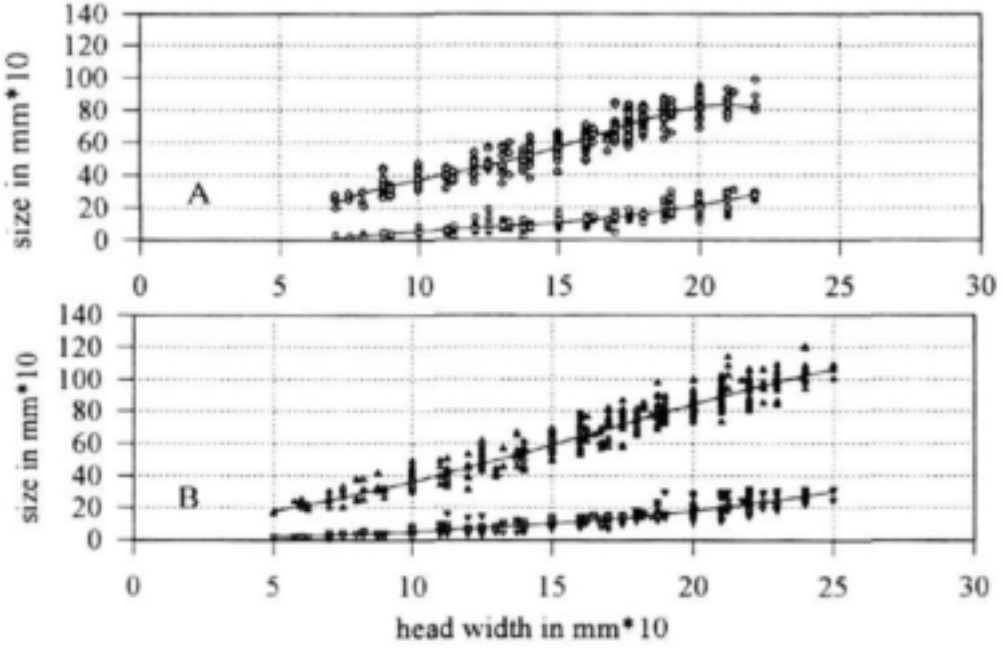
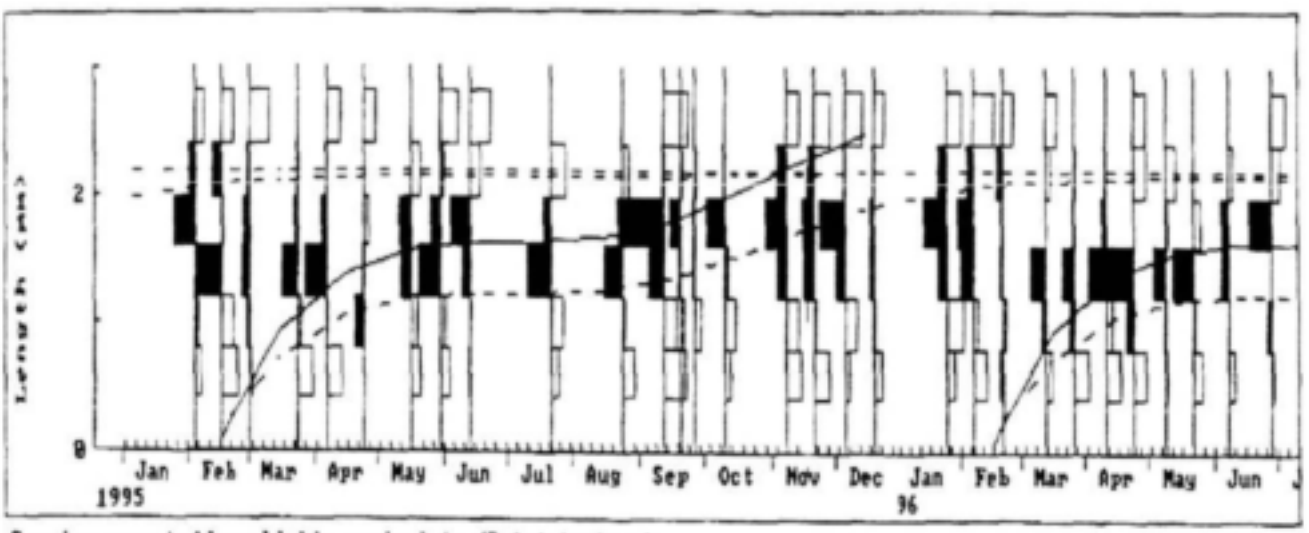


Figure 4.2.1.8 Regressions of body length and wingbud length against HW as the independent variable. The regressions were fitted to the 4th order. A. Males B. Females. The smaller size recorded for the younger females is most probably an artefact as in small males the differential eye development would not be distinguishable.



Species name ▸ Adenophlebia auriculata (Palmiet river)

	(1 ———)	(2 — —)
Loo ▸	2.00	2.20
K ▸	3.200	2.000
C ▸	1.000	1.000
WP ▸	0.500	0.500
SS ▸	29	29
SL ▸	1.600	1.200
Rn ▸	0.292	0.200

Figure 4.2.1.9 Growth model predicting the lifespan and growth rate of a natural population of *A. auriculata* using FiSAT and the von Bertalanffy growth model. 1.seasonalised —— 2. non seasonalised- - growth curve-

Discussion

Sampling method

In designing a field sampling protocol the dilemma is to choose a sampling procedure which will yield the most comprehensive and representative insight into the dynamics of the life history. The problem areas as far as this investigation is concerned were an extended emergence period and a short lived adult phase poorly documented behaviour and an apparent overlap in size classes. We concur with Malicky (1989) when he questions the validity of using the captured aerial phases of aquatic insect to deduce quantitative measures such as secondary production. To counter this problem, the presence or absence of the adult and subimaginal shucks in the surrounds of the stream was used as an indication of emergence. However an absence of the shucks could not be taken as an indication that no emergence activity was taking place as rain and rising river levels could wash the shucks off the rocks. As the investigation proceeded the pre-adult nymphs were more carefully scrutinized and documented..

Cues for population changes

Studies have shown that flooding in unstable rivers, changes in water temperature and pH, pollution and food availability are amongst the factors that affect survival and distribution of other leptophlebiid mayflies (Collier & Winterbourn, 1990; Dudgeon, 1989, 1990; Graesser, 1988, Hall *et al*, 1988, Jowett *et al*, 1991; King *et al*, 1988; Scrimgeour *et al*, 1988, Scrigneour and Winterbourn, 1989; Scrimgeour, 1991). During the period of investigation several flooding periods occurred when the river level rose dramatically and sampling was somewhat difficult. These data have not yet been combined to ascertain coincidence. Figures 4.2.2 and Figure 4.2.1.4 appear to show coincidence between rising aerial temperature and flights when the ambient temperature has either been falling, such as at the end of summer, or has been low for an extended period of time, such as during winter. A peak of temperature at week 36 coincided with a flight. At week 72 after a short hiatus, the rise in ambient temperature resulted in a large flight and again at week 89-90. However emergence does continue after the temperature has dropped such as at week 75.

Temporal population dynamics and emergence periodicity

Brittain (1976) has shown that another leptophlebiid, *Leptophlebia vespertina,* in the northern hemisphere, has an adult emergences in late autumn and early winter. However it has become increasingly clear that the voltinism of aquatic insects in temperate areas are quite different from those in sub-tropical areas. This is largely due to the fact that temperature seems to be the major controlling factor influencing emergence periodicity as previous studies have shown (Brittain, 1976,1982, Sweeney *et al*,1978, Newbold *et al* 1994, Giberson & Rosenberg, 1994).

From the first investigation during 1993 it was possible to deduce the nature of the population structure and to predict possible adult emergence periods for *A. auriculata* in spring and autumn. This complemented the findings of Sweeney (1978) who showed that *Isonychia bicolor* winter generations underwent a fairly synchronous emergence in the first ten days of spring in response to higher water temperatures in Pennsylvania. That the emergence in spring is fairly synchronous, is implied by the appearance of large numbers of late instars in August (Weeks 35-37 and 88-96) (Figures 4.2 4 & 5.) and the appearance of large numbers of shucks in the stream. In the subsequent extended and intensive sampling it became clear that the emergence of *A. auriculata*, is not confined to spring and autumn, but that extended and the major flight period s occur in summer (in some years starting in October, in other years in November) through to late summer. The weekly sampling regime revealed that small numbers of nymphs emerge repeatedly, but that large numbers will emerge in a more synchronized fashion in spring , late summer and autumn (weeks 24,36,58,72 98-102). According to Needham & Traver (1972) this trend of a few general emergence periods with a few isolated emergences throughout the year is fairly widespread in the more common species of mayflies. Contrary to the finding of Wise (1980), the mean size of the final instar nymphs during the spring emergence was not greater than the mean size of the nymphs emerging in summer or autumn. The samples have a slightly larger mean size.

Conclusions

The life history of the mayfly is characterized by repeated emergences throughout the year except from mid-winter to early spring. This life history style is often characteristic of highly changeable environments as well as a climate which is not strongly seasonal. The Eastern Cape is a typical environment of this type. For long periods of the year the weekly DD summation can fluctuate widely and is very unpredictable. The seasons are not totally distinct and although the winters are cold, the duration is quite short. The fact that emergences occur between these major events makes the analysis of the captured samples to determine the life cycle extremely complex. The life cycle is most probably annual or semi-annual in that it may fall a few weeks short of 52. The energy budget required the nymph to complete this life cycle is between 1800 and 2000 DD. A final answer about the life cycle will only be available once a field rearing experiment has been done.

4.2.2 POPULATION SIZE DISTRIBUTION OF THE NYMPHS OF *ADENOPHLEBIA AURICULATA* IN TWO EASTERN PROVINCE RIVERS, AND THE APPLICATION OF THE BIOTOPE CONCEPT TO ECOLOGICAL STUDIES.

Brian Hunt. Honours project Zoology (1997) under the supervision of E.H. Haigh.

Introduction

Many environmental factors interact to affect the habitat distribution of lotic benthic macro invertebrates. These include abiotic factors such as substratum type (Moss *et al*, 1987), substratum stability (Cobb *et al*, 1992), current velocity (Egglishaw, 1967), pH and temperature (Vannote and Sweeney, 1980) and light (Brittain, 1982). Biotic factors include predation (Collier, 1994), competition (Lancaster *et al*, 1988) and food supply (Wilson, 1975).

Numerous studies have indicated that nymphal size is an important factor in determining the distribution of animals amongst biotopes (Cummins, 1964; Hall *et al.*, 1980; Holomuzki and Messier, 1993; Lancastar and Hildrew, 1993). This is a direct result of differential interactions between nymphs of different sizes and the above mentioned environmental factors. Interactions that have been investigated include predation (Lancastar and Hildrew, 1993), competition (Hildebrand, 1974) and differential resource utilisation (Palmer, 1991). Life history characteristics also play an important role in determining nymphal distribution. For example, the presence of small nymphal *Paraleptophlebia* sp. in riffles have been attributed to adults laying their eggs in this biotope (Needham, *et al* 1935). In the case of *Adenophlebia auriculata* the occurrence of large nymphs in slackwater has been linked to their requirement for emergence structures providing them with access to the terrestrial environment (Haigh and Davies-Coleman, 1997; Collier, 1994). A study conduced by Hunt (1996) indicated that the distribution of *A. auriculata* is affected by the size of the nymphs and one of the principal aims of this project was to investigate this further.

The scale on which a study is conducted has important implications for the variables recognised as affecting the system under study (Minshall, 1988). To be of use in ecological studies habitat units must be of a scale relevant to the animals of interest. However as Pringle *et al.* (1988) stated,

"Patchiness within streams at scales perceived and/or exploited by stream organisms is a reality that has often been ignored" (p 504).

A literature search reveals the biotope concept has been used extensively in ecological studies. This is due to the intuitive knowledge that biotope characteristics affect the distribution of macro invertebrate assemblages in streams (Palmer *et al.*, 1991). However, a review by Wadeson (1994) showed that biotopes are usually subjectively defined, and where the same terminology has been used there is a considerable lack of

consistency, with different terms applied to the same features and the same term applied to different features.

One system widely used in South Africa is the system which is referred to as the ecological biotope concept, described in (Table 1). Rowntree and Wadeson (1996) have developed a new biotope classification based on stream hydraulics - the hydraulic biotope concept. The characterisation of hydraulic biotopes is based on the assumption that: "Surface flow characteristics can be used to distinguish habitat classes which are relatively distinctive in terms of both near bed and mean flow conditions", (Rowntree and Wadeson, 1996, p. 281).

In Table 2 the hydraulic biotopes are defined in terms of physical characteristics and flow types. As yet the ecological significance of hydraulic biotopes is unproven (Rowntree and Wadeson, 1996). However, stream hydraulics are believed to be one of the fundamental abiotic factors in determining the distribution of lotic invertebrates (Statzner *et al.*, 1988) and collective experience indicates that they will indeed be ecologically relevant (Rowntree and Wadeson, 1996). This study will investigate the significance of the hydraulic biotope to *A. auriculata*, and compare it to the presently used ecological biotope classification.

Aims

1. To determine the size distribution of *A. auriculata* nymphs amongst lotic biotopes.

2. To determine whether flow rate and substrate type affect the size distribution of nymphs.

3. To investigate the effect of river scale on size distribution.

4. To investigate the application of the biotope concept to animal distribution, with particular reference to the hydraulic biotope concept.

Study sites

The Palmiet River (26°26'E 33°22'S) is an upper tributary of a coastal river, the Bushmans River, and arises about 70 kilometres from the coast. The sample sites were situated approximately 14 kilometres south west of Grahamstown at an altitude of 526 metres (Figure 4.2.2.1). The river has characteristics of a perennial mountain stream with clear water and a turbulent flow. It is never more than 2.5 metres wide and is unimpacted by impoundment in the area of the study sites.

The Buffalo River is a steep coastal river system characteristic of those draining the eastern escarpment of South Africa (Figure 4.2.2.1). From its headwaters in the Amatola Mountains, at an altitude of 1300 metres, it flows in a southerly direction for 125 km before discharging into the Indian Ocean at East London. The sample sites for this study were situated in the upper reaches of the river where it is between 4 and 6 metres wide. Here it is described as a "steep mountain or foothills type boulder or cobble stream" (Rowntree and Wadeson, 1996). It flows through indigenous yellowwood forest, is unimpacted by impoundment and has a perennial flow.

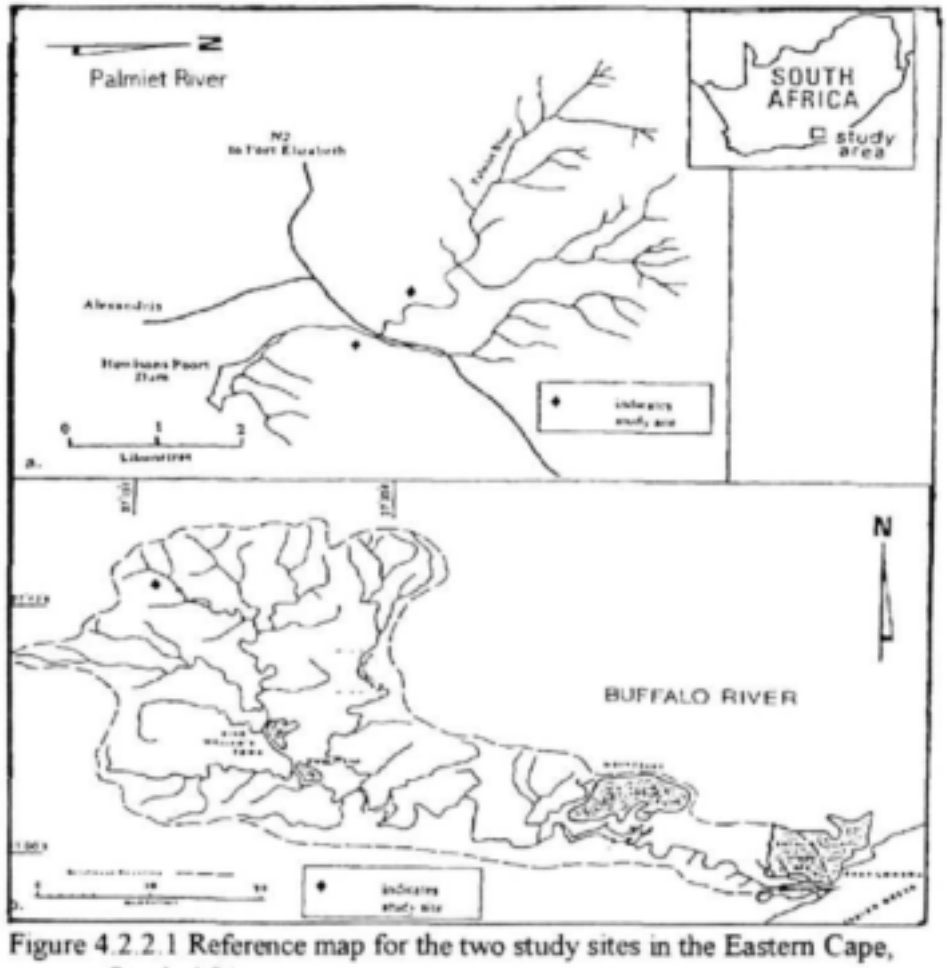


Figure 4.2.2.1 Reference map for the two study sites in the Eastern Cape, South Africa.

Sampling

Three sampling sites which encompassed all six ecological biotopes (Table 4.2.3 1) were selected in each river. Two representative samples were made from each ecological biotope at each sampling site using an 80 micro metre handnet, yielding 36 samples per sampling period. Each river was sampled on three occasions at six-week-intervals (March, April and June 1997).

Table 4.2.2.1 Table of the ecological biotopes sampled and their descriptions.

ECOLOGICAL BIOTOPES	DESCRIPTIONS OF ECOLOGICAL BIOTOPES
Stones-in-current (SIC)	The presence of gravels, cobbles or boulders in any body of water in which flow velocity is strong enough to prevent the settlement of fine sediment or detritus.
Stones-out-of-current (SOC)	The presence of gravels, cobbles or boulders in any body of water in which flow velocity is low enough to allow the settlement of fine sediment or detritus.
Marginal vegetation (MV)	Emergent vegetation arising from anywhere within the stream, and occurring at any current velocity.
Leaf-packs-in-current (LPIC)	Leaf packs occurring at any current velocity high enough to prevent the settlement of fine sediment or detritus. Often occur in snags created by a fallen branch.
Leaf-packs-out-of-current (LPOC)	Leaf packs occurring at any current velocity low enough to allow the settlement of fine sediment or detritus.
Sandy bottoms (SB)	Characterised by fine substrates, either silt or sand, which are allowed to settle because of low current velocities. This biotope is usually associated with pools.

For each sample the following abiotic variables were measured and recorded:

1. Hydraulic biotope (Table 4.2.2.2). The hydraulic biotope rapid occurred in the Buffalo River but no *A. auriculata* nymphs were recorded from it so it is excluded from the analysis.
2. Flow rate (average measured over a 30-second period), was recorded 5cm above the substratum, for each biotope in each sample with a Marsh-McBirney model 2000 portable electromagnetic water flow metre.
3. Depth

4. Substrate type (Table 4.2.2.3)

5. Temperature

6. pH

7. TDS

Table 4.2.2.2 The hydraulic biotopes with general description and flow type (sensu Rowntree and Wadeson, 1996).

HYDRAULIC BIOTOPE	GENERAL DESCRIPTION	FLOW TYPE
1a. Rapid	occur over fixed substrate such as bolder and bedrock	undulating or breaking standing waves
1. Riffle	occur over coarse alluvial substrate from gravel to cobble; relative deb roughness high	undulating or breaking standing waves
2. Run	occur over any substrate except silt; relative roughness low	rippled flow
3. Chute	occur in boulder and bedrock channels where flow is funnelled between macro bed elements; exhibit flow acceleration.	Smooth boundary turbulent flow exhibiting flow acceleration
3. Glide	occur over any substrate as long as the depth is sufficient to minimize relative roughness.	Clearly perceptible flow without any surface disturbance
4. Slackwater	hydraulically detached from the main flow but within the main channel, occur over any substrate	barely perceptible flow
5. Backwater	area physically separated from the main channel, connected to it at downstream end. Occur over any substrate.	barely perceptible flow
6. Pool	direct hydraulic contact with upstream and downstream water; occur over any substrate.	barely perceptible flow

All samples were preserved in 10% Formaldehyde in the field. Samples were sorted in the lab and all the *A. auriculata* nymphs removed and transferred to 90% ethanol. Nymphs greater than 0.5 mm head width were removed without the help of magnification. Nymphs smaller than this were extracted under a dissecting microscope after being brought to the surface by flotation in a saturated magnesium sulphate solution. It

was not possible to identify the hatchling *A. auriculata* nymphs below 0.3 mm head width in the Buffalo River as other leptophlebiid mayflies, with which they can be confused, occur here.

Head width, to the nearest hundredth of a micrometer, was determined by micrometer eyepiece and is used as a measure of nymph size. The head width is a more conservative measure of size than body length which tends to vary at sexual maturity, and between sexes (Haigh, see section 4.2.2. & personal communication). Once measurements had been completed the nymphs were placed in size classes (Table 4.2.2.4).

Table 4.2.2.3. List of substrate types and particle size (mm).

Substrate type	Substrate particle size
1. Silt	< 0.125 mm
2. Sand	0.125 - 0.05 mm
3. Gravel	0.5 - 2.0 mm
4. Small cobble	64-128 mm
5. Large cobble	128-250 mm
6. Small boulder	250-500 mm
7. Medium boulder	500-1000 mm
8. Large boulder	1000-4000 mm.
9. Vegetation	-

Table 4.2.2.4 Size classes used for the categorical analysis of *A. auriculata* nymphal distribution.

Size class categories	Size class (mm)
1. Hatchlings	0.1 mm - 0.5 mm
2. Small nymphs	0.5 mm - 1 mm
3. Medium nymphs	1 mm - 1.5 mm
4. Sub-mature nymphs and mature males	1.5 mm - 2 mm
5. Mature males and females	2 mm - 2.5 mm

Data analysis

Initially a separate analysis was conducted on the March, April and June samples. However, not all biotopes were sampled in each month and in some instances the samples from biotopes were too small to be

used in the analysis. The data sets for the three months were therefore pooled to increase the sample size and to include all biotopes so that nymph distribution patterns could be compared between the two rivers. *Adenophlebia auriculata* has an asynchronous life history (Haigh, personal communication) which is consistent with the life history pattern usually shown by animals in unpredictable environments which most South African rivers are (O'Keeffe, 1995). A similar pattern is shown by the New Zealand Leptophlebiidae of the genus *Deleatidium* (Death, 1995). Consequently the seasonal variation in size distribution should be minimal, particularly as sampling was only conducted over a four month period.

ANOVA was used to test the relationship between mean head width and ecological and hydraulic biotopes in the Palmiet and Buffalo rivers. ANOVA was also used to investigate the relationship between mean head width and substrate.

To obtain greater clarity of the distribution patterns, size class frequency distributions were plotted for the two biotope classifications tested, for current velocity and substrate. These were obtained by determining the percentage of the total number of nymphs per size class, for each of these variables. The following formula was used:

$$\% = [(N_b) / (N_t)] \times 100$$

Where: N_b is the total number of nymphs from a single size class.

N_t is the total number of nymphs in a size class.

Contingency tables were used to analyse the distribution of size classes amongst current velocities. Current velocities recorded were pooled into 0.1 m/s intervals. The samples in reach river were polled for the analysis. Standardised residuals were calculated from observed and expected frequencies. For the purpose of this study standardised residuals of > 2.00 and > -2.00 were considered greater than and lower than the expected frequencies respectively.

Results

Size distribution

Ecological biotopes: Mean head width

Figure 4.2.2.2 illustrates the mean head width and standard error for all the nymphs in the ecological biotopes in the Palmiet and Buffalo River.

In the Palmiet River the smallest mean nymph size was recorded in sandy bottoms, and these nymphs were significantly smaller ($p<0.01$) than in any other biotope. The mean nymph size in stones-in-current was significantly larger than in sandy bottoms but significantly smaller than nymphs in any other biotope. In leaf-packs-out-of-current the mean size of the nymphs were significantly larger than those in sandy bottoms and stones-in-current but significantly smaller than those in leaf-packs-in-current, marginal vegetation and stones-out-of-current. There was no significant difference in mean nymph size between leaf-packs-in-current, marginal vegetation and stones-out-of-current.

In the Buffalo River nymphs with the smallest mean size was recorded in stones-in-current. Nymphs in leaf-packs-in current were significantly larger ($p<0.01$) than those in stones-in-current. There was no significant difference in mean nymph size between stones-out-of-current, marginal vegetation or leaf-packs-out-of-current. No nymphs were recorded from sandy bottoms. However it must be noted that we were unable to identify the hatchling nymphs below 0.3 mm head width in the Buffalo River, as they were indistinguishable from those of other members of the family. In the Palmiet River these smaller hatchling nymphs made up a large part of the sandy bottom sample.

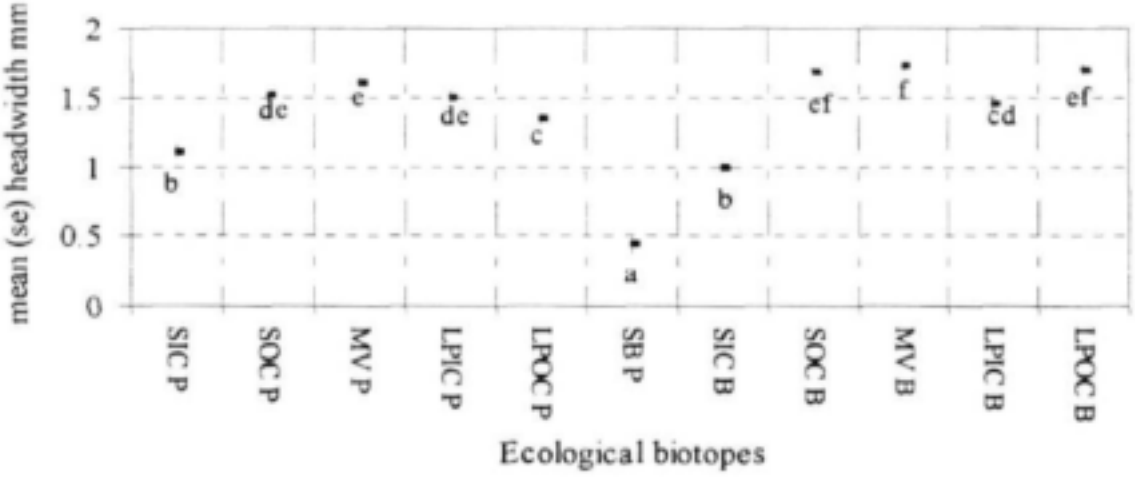


Figure 4.2.2.2 Mean headwidth in mm with standard error of the *A.auriculata* nymphs recorded in the various ecological biotopes in the Palmiet (P) and Buffalo (B) Rivers. Significant differences are indicated by letter ($p<0.01$). Biotope codes are described in Table 4.2.2.1.

Size class frequency distribution

Figures 4.2.2.3 and 4.2.2.4. illustrate the percentage of the total number of nymphs, in each size class, found in each ecological biotope for the Palmiet and the Buffalo Rivers. In the following and subsequent discussion of size frequency a high percentage of nymphs will be considered as > 20 % and a low percentage as < 20 %.

All size classes were found in all ecological biotopes but in different proportions. In stones-in-current (SIC) in the Palmiet River a low percentage of nymphs in all size classes occurred, except in size class 2 (28 %). From size class 2 to size class 5 there was a pattern of decreasing percentage of nymphs with increasing size class in this biotope. This pattern is very distinct in the Buffalo River The reverse of this occurs in stones-out -of-current (SOC), with larger percentages of the larger size classes In the Palmiet (24 % to 27 % of the total number of nymphs per size class in all size classes except the smallest and in the Buffalo the percentage of nymphs increased with increasing size from class 2 to 5. The highest percentage of nymphs in this biotope was recorded in size class 5 (50 %).

In marginal vegetation (MV) in the Palmiet River there were a relatively low percentage of nymphs in size classes 1 and 2. However, from size class 2 to 5 there was a pattern of increased percentage with increasing size class (5 % to 39 %). In the Buffalo River a similar distribution pattern is seen with the highest percentage of nymphs to be found was in size class 5 (28 %).

In the Palmiet River in leaf-packs-in-current (LPIC) size class 4 was relatively well represented (20%), as were size classes 2 and 5 (17 %), while in leaf-packs-out-of-current (LPOC) all size classes were relatively well represented (17 % to 25 %)

All size classes were poorly represented in leaf-packs-in-current and leaf-packs-out-of-current in the Buffalo River.

In the Palmiet River on sandy bottoms (SB) a relatively high percentage of size class 1 nymphs (32 %) occurred but there were few nymphs from size classes 2 to 5.

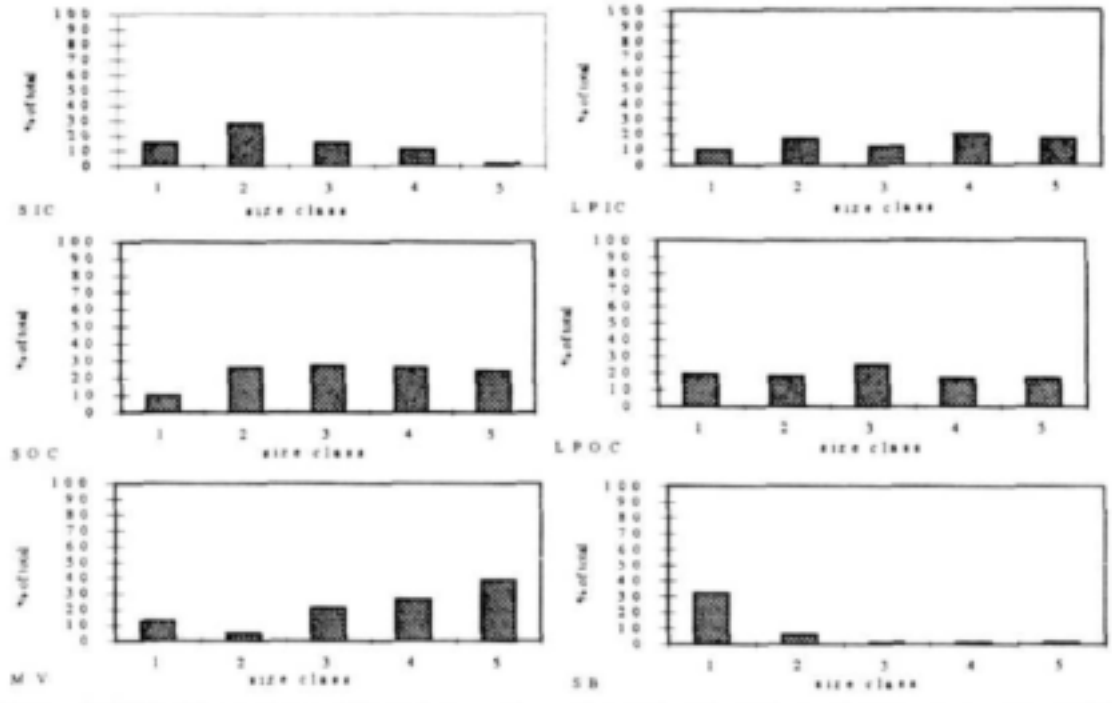


Figure 4.2.2.3. Percentage of the total number of nymphs found in each size class, recorded in each ecological biotope in the Palmiet River. The biotopes are described in Table 4.2.2.1.

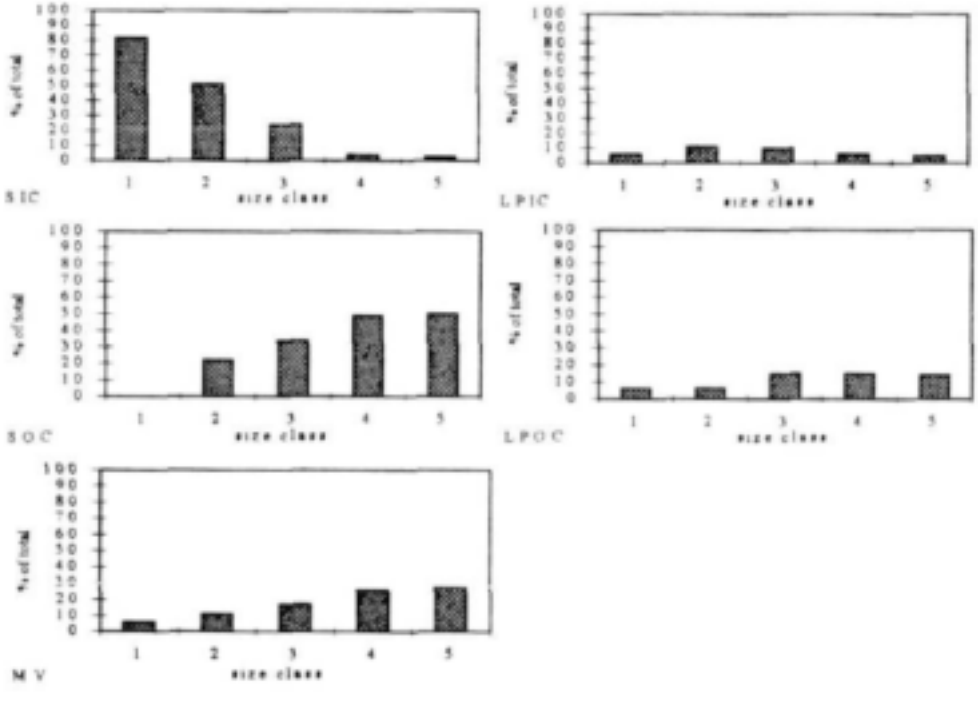


Figure 4.2.2.4. Percentage of the total nymphs in each size class, recorded in each ecological biotope in the Buffalo River.

Hydraulic biotopes: Average head width

Figure 4.2.2.5 illustrates the results of a multivariate analysis of the relationship between head width and hydraulic biotope

In the Palmiet River the smallest nymphs were found in chutes and the largest in glides and backwaters. There was no significant difference ($p<0.01$) in the mean size of nymphs found in riffles, pools, runs, and slackwater. The nymphs from riffles were significantly different from those in chutes (larger) and the mean size of nymphs found in glides and backwater were significantly larger than those in rffles and pools. The nymphs from runs, glides, backwater and slackwater were not significantly different.

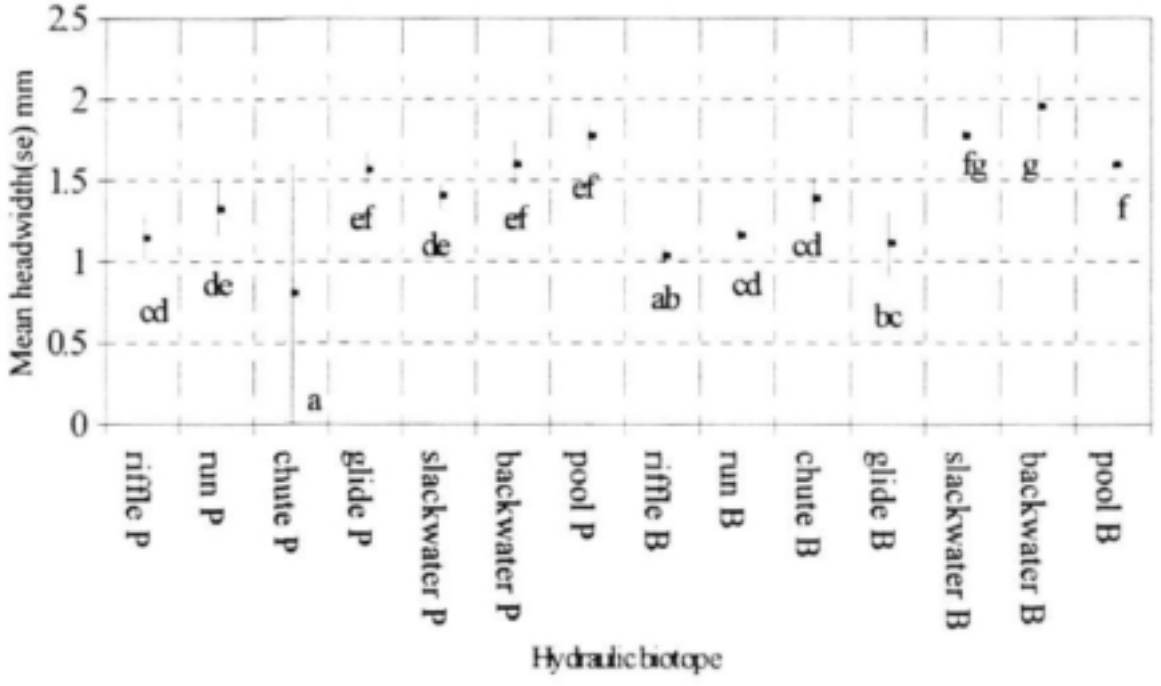


Figure 4.2.2.5 Mean headwidth and standard error of the nymphs of *A. auriculata* recorded in the hydraulic biotopes in the Palmiet (P) and Buffalo (B) Rivers. The significant differences are indicated by letters ($p<0.01$).

In the Buffalo River, nymph of the smallest mean size was recorded in riffles and the largest mean size in backwater. There was no significant ($p<0.01$) difference between the mean nymphal sizes recorded from riffles and glides, runs and chutes, or slackwater and backwater. However the nymphs found in riffles were significantly different from those in runs and chutes but not found in glides. The nymphs from slackwaters, backwaters and pools were significantly larger than those from any of the other biotopes and those from backwaters were significantly different from those in pools.

Size class frequency distribution

From Figure 4.2.2.6, which illustrates the percentage of the total number of nymphs, in each size class, in the Palmiet River, it is clear theat the smallest percentage of nymphs were recorded in chutes and a low percentage of nymphs in riffles, runs and backwaters. In riffles 20 % of class 2 nymphs were found. The distribution pattern in glides and backwaters is increasing percentage of nymphs with increasing size class (5% in size class 1 to 15 % in size class 4 in backwater and 11 % in class 1 to 25 % in class 5 in glides). The largest portion of nymphs, in all size classes, were found in slackwaters and pools (17% - 44 %).

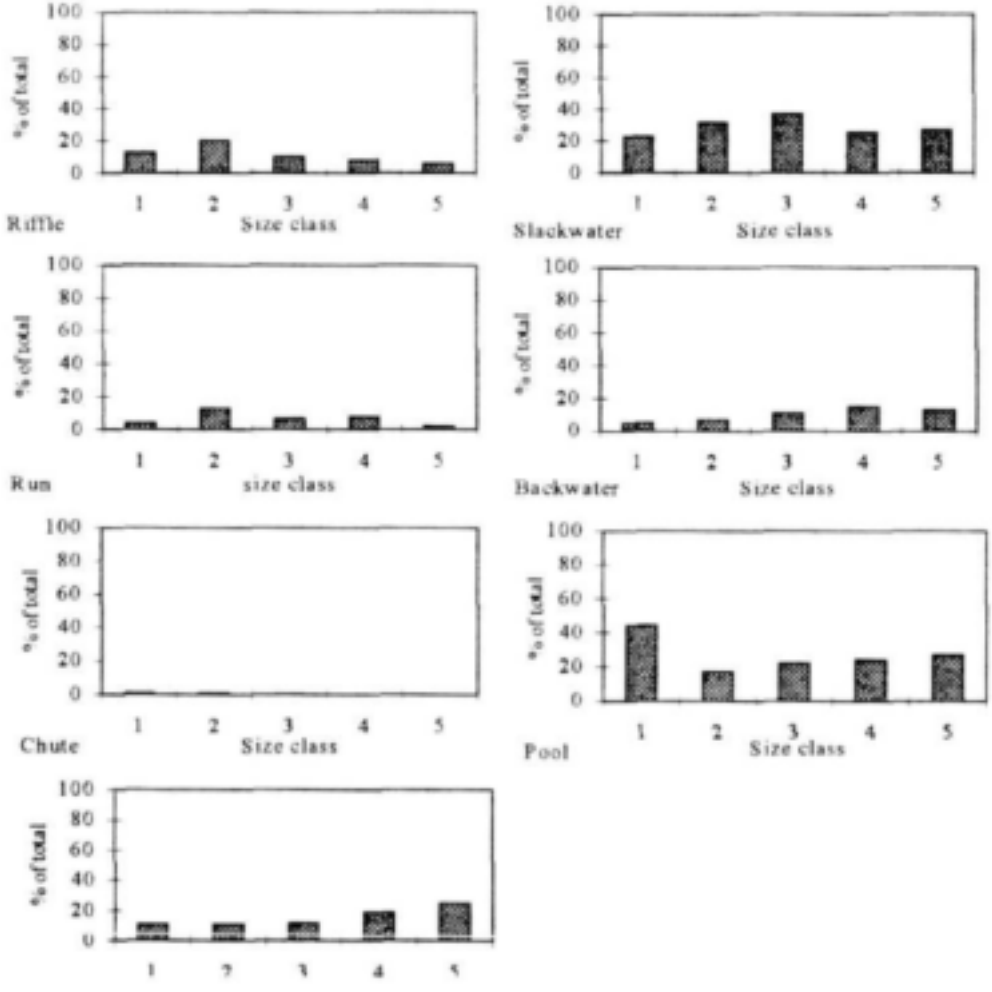


Figure 4.2.2.6. Percentage of the total nymphs in each size class, found in each hydraulic biotope in the Palmiet river

.Figure 4.2.2.7 illustrates the distribution pattern of nymphs in the hydraulic biotope recorded in the Buffalo River. Chutes, backwaters and glides had the lowest portion of nymphs. In riffles and runs a relatively high percentage of nymphs in size classes 1, 2 and 3 were recorded, with smaller portion of the

larger size classes. Pools and slackwaters showed a strong increase in the percentage of nymphs with increasing size class, class 5 having the largest representation, 67 % in slackwaters. Nymphs of all size classes were found in pools.

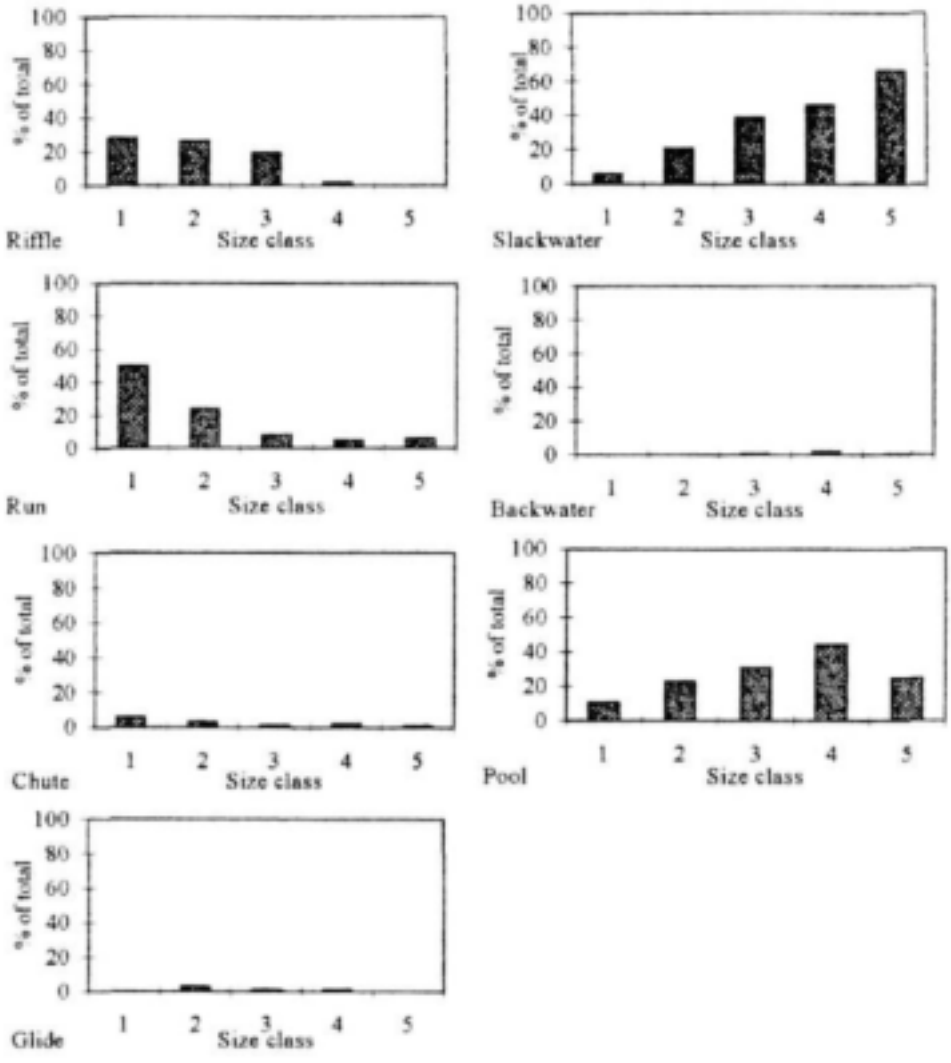


Figure 4.2.2.7. Percentage of the total nymphs in each size class, found in each hydraulic biotope in the Buffalo River.

Comparison of biotope concepts

A range of hydraulic biotopes were recorded for each ecological biotope and *vice versa* (Table 4.2.2.5.a and b). In stones-in-current the hydraulic biotopes recorded included riffle, run and chute. Leaf-packs-in-current corresponded with the fast flowing biotopes riffle, run, glide and chute. Stones-out-of-current and leaf-packs-out-of-current only corresponded with the slow and no flow biotopes slackwater, backwater and pool. Marginal vegetation corresponded with these same slow flow areas as well as glides. Sandy bottoms corresponded with pools and with glides.

Table 4.2.2.5 a. The hydraulic biotopes corresponding with the ecological biotopes in the Palmiet River.

	HYDRAULIC BIOTOPES		
ECOLOGICAL BIOTOPES	March	April	June
1. Stones-in-current	run, chute	riffle, run	riffle
2. Stones-out-of-current	slackwater, backwater	slackwater, backwater	slackwater, backwater
3. Marginal vegetation	glide, slackwater, pool	glide	pool
4. Leaf-packs-in-current	riffle, run, glide	glide	glide
5. Leaf-packs-out-of-current	slackwater, backwater, pool	slackwater, pool	
6. Sandy bottoms	pool	pool, glide	pool

Table 4.2.2.5 b The hydraulic biotopes corresponding with the ecological biotopes in the Buffalo River.

	HYDRAULIC BIOTOPES		
ECOLOGICAL BIOTOPES	March	April	June
1. Stones-in-current	riffle, run	riffle, run	riffle
2. Stones-out-of-current	slackwater, pool	slackwater, pool	slackwater, pool
3. Marginal vegetation	slackwater, pool	slackwater, pool	pool
4. Leaf-packs-in-current	run, chute	riffle, chute, glide	riffle, glide
5. Leaf-packs-out-of-current	glide, slackwater, backwater, pool	slackwater, pool	slackwater, pool

Comparison between rivers

Figure 4.2.2.2 displays the results of a multivariate analysis of head width and ecological biotope for the Palmiet and Buffalo rivers.

The mean nymph size recorded in sandy bottoms was significantly smaller than that in any other biotope. There was no significant difference between the nymphal sizes recorded from stones-in-current, leaf-packs-in-current and stones-out-of current in the two rivers. In both marginal vegetation and leaf-packs-

out-of-current in the Palmiet River the mean nymph size was significantly smaller than those in the marginal vegetation of the Buffalo River.

Figure 4.2.2.5 illustrates the results of a multivariate analysis of head width and hydraulic biotope for the Palmiet and Buffalo rivers.

In the Palmiet river the smallest mean nymph size occurred in chutes and was significantly smaller than the mean nymph size recorded in chutes in the Buffalo River. There was no significant difference in mean nymph size between riffles and runs in the two rivers. Glides had a significantly larger mean nymph size in the Palmiet River than in the Buffalo River while the nymphs from slackwater, backwater and pool were significantly smaller in the Palmiet river than those recorded from the corresponding biotopes in the Buffalo River.

Variables affecting size class distribution

Substrate

Figure 4.2.2.8 illustrates the results of a multivariate analysis of substrate and head width in the Palmiet and Buffalo Rivers.

In the Palmiet River the smallest mean nymph size was recorded in silt. Sand, small cobble and small boulder recorded significantly ($p<0.01$) larger mean nymph sizes than silt but significantly smaller mean nymph sizes than large cobble and vegetation. There was no significant difference in mean nymph size between large cobble and vegetation.

In the Buffalo River the smallest mean nymph size was found in sand and the largest mean nymph size was recorded in vegetation. There was no significant difference in mean nymph size between any of the substrates.

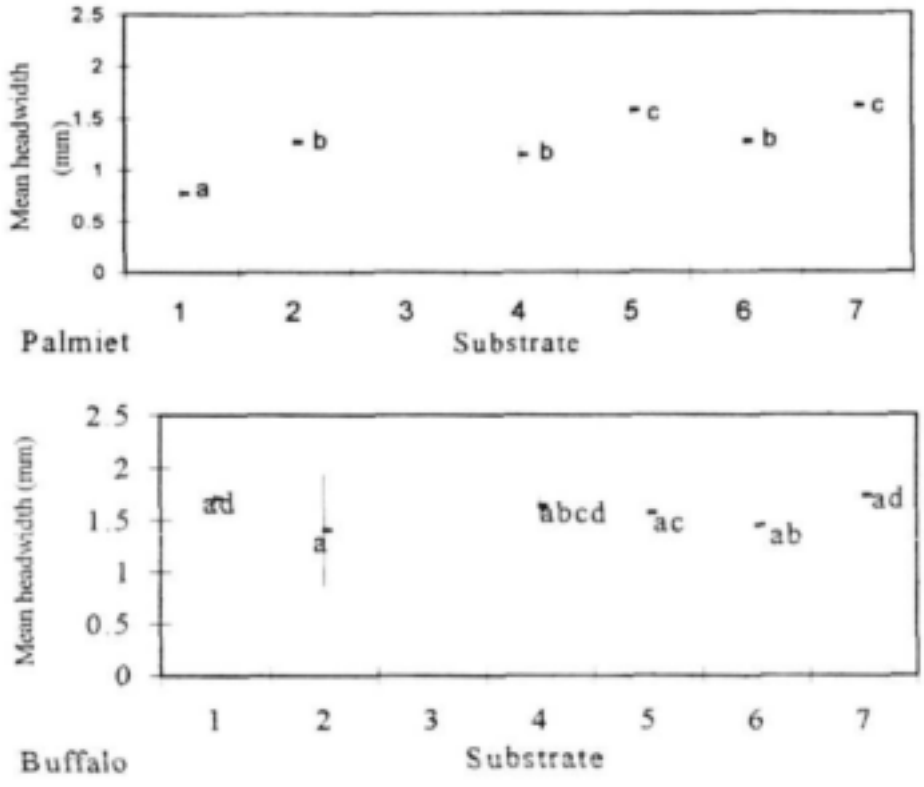


Figure 4.2.2.8 Multivariate analyses of the mean headwidth with standard error of the nymphs recorded on substrates in the Palmiet and Buffalo Rivers. Significant differences are indicated by different letters ($p<0.01$)
Substrates: 1=Silt; 2=Gravel; 3=Gravel; 4=Small cobble; 5=Large cobble; 6=Small boulder; 7=Marginal vegetation.

Figure 4.2.2. 9 illustrates distribution pattern of nymphs according to the percentage of the total number of nymphs, in each size class, occurring on each substrate type, for both the Palmiet and the Buffalo Rivers.

Apart from size class 1 nymphs in the Palmiet river, a low percentage of all size classes of nymph was recorded in silt in both the rivers. However it must be remembered that the smallest nymphs were only identified from the Palmiet River samples. This fact is reflected in the proportion of nymphs found in sand where less than 20% of all size classes in the Palmiet River, and only a few class 3 nymphs in the Buffalo River, was found.

In both rivers very few nymphs were recorded from small cobbles but a high percentage of nymphs in all size classes were found in larger cobble and small boulders.

In marginal vegetation a similar distribution pattern were found in both the Palmiet and the Buffalo Rivers, the percentage representation of size classes increasing with increasing size class, size classes 4 and 5 being best represented.

Current velocity

The standardised residuals obtained using contingency table analysis of size class distribution and current velocity for the Palmiet River is given in Table 4.2.2.6. In all size classes a greater than expected frequency was found in the current velocities 0 m/s, 0.1 m/s and 0.2 m/s. A lower than expected frequency of size classes 1, 3, 4 and 5 was found in most current velocities > 0.5 m/s. The frequency of size class 2 nymphs was neither greater than or less than expected in current velocities ≥0.3 m/s.

Table 4.2.2.6. Standardised residuals obtained using contingency table analysis of size class distribution and current velocity for the Palmiet River. Light shading indicates standardised residuals that are greater than expected and dark shading indicates standardised residuals that are less than expected.

	Size class:				
Current velocity	1	2	3	4	5
0	2.31	1.55	3.68	4.96	1.88
0.1	21.33	14.31	33.89	45.73	17.38
0.2	12.74	8.55	20.47	27.31	10.38
0.3	0.72	0.48	1.15	1.55	0.59
0.4	-0.57	-0.38	-0.92	-1.24	-0.47
0.5	-1.78	-1.19	-2.83	-3.82	-1.45
0.6	-0.57	-0.38	-0.92	-1.24	-0.47
0.7	-2.65	-1.78	-4.21	-5.69	-2.16
0.8	-0.43	-0.29	-0.69	-0.93	-0.35
0.9	-1.15	-0.77	-1.84	-2.48	-0.94
1	-2.77	-1.45	-3.45	-4.65	-1.77
1.2	-2.5	-1.68	-3.98	-5.38	-2.04

$X^2 = 226.21$

Table 4.2.2. 7 illustrates the standardised residuals obtained using contingency table analysis of size class distribution and current velocity for the Buffalo River where a lower than expected frequency of size class 1 nymphs was found at 0.1 m/s. However, the frequency of size class 1 nymphs was neither greater than or less than expected in any other current velocity. A greater than expected frequency of size class 2, 3, 4 and 5 nymphs was found in the current velocities 0 m/s and 0.1 m/s. For these same size classes a lower than expected frequency was found in the current velocities 0.3 m/s and 0.8 m/s.

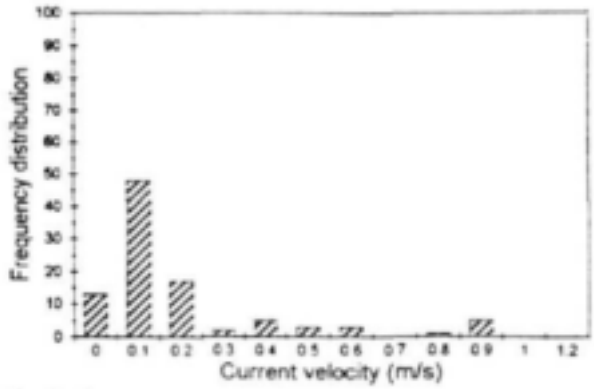
Table 4.2.2.7 Standardised residuals obtained using contingency table analysis of size class distribution and current velocity for the Buffalo River. Light shading indicates standardised residuals that are greater than expected and dark shading indicates standardised residuals that are less than expected.

	Size class:				
Current velocity	1	2	3	4	5
0	-1.09	4.36	5.25	4.42	4.23
0.1	-3.89	15.57	18.76	15.81	15.1
0.2	0.07	-0.3	-0.36	-0.31	-0.29
0.3	0.53	-2.1	-2.57	-2.17	-2.07
0.4	-0.26	1.04	1.26	1.06	1.01
0.5	0.16	-0.65	-0.78	-0.66	-0.63
0.6	0.26	-1.04	-1.26	-0.06	-1.01
0.7	0.4	-1.61	-1.94	-1.63	-1.56
0.8	0.53	-2.1	-2.57	-2.17	-2.07

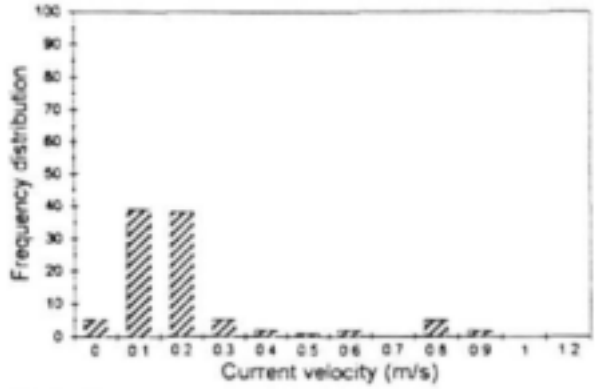
$X^2 = 330.41$

Figure 4.2.2.10 illustrates the percentage of nymphs in each size class recorded for each current velocity recorded in the Palmiet River. All size class occurred at relatively high percentages in the lower current velocities 0 m/s and 0.1 m/s. Relatively high percentage of nymphs in size classes 1, 2, 4 and 5 occurred at 0.2 m/s and a high percentage of size class 5 nymphs occurred at 0.3 m/s. All size classes occurred at low percentages at current velocities greater than 0.4 m/s but the lowest percentage of class 5 nymphs was recorded at these higher current velocities.

In the Buffalo River (Figure 4.2.2.11) the highest percentage of nymphs of size class 1 was found at 0.3 m/s, 0.4 m/s and 0.6 m/s; high percentages of class 2 nymphs were found at 0.1 m/s, 0.4 m/s and 0.5 m/s and the size classes 3, 4 and 5 nymphs were the most abundant (> 50%) at 0.1 m/s, and 0 m/s (18% - - 25%).



Size class 1



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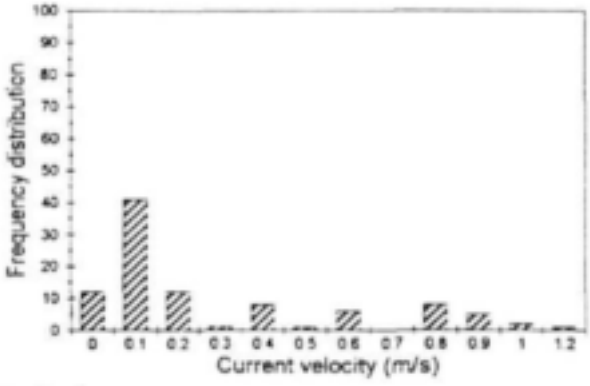

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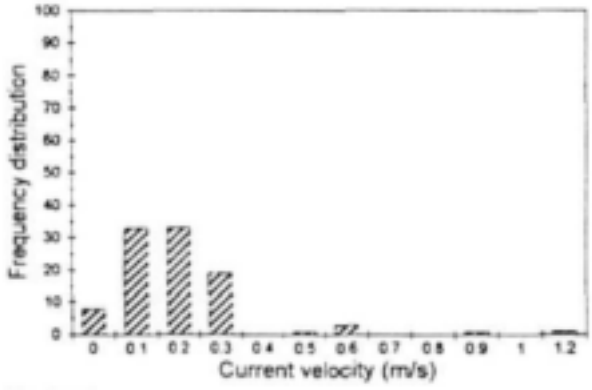

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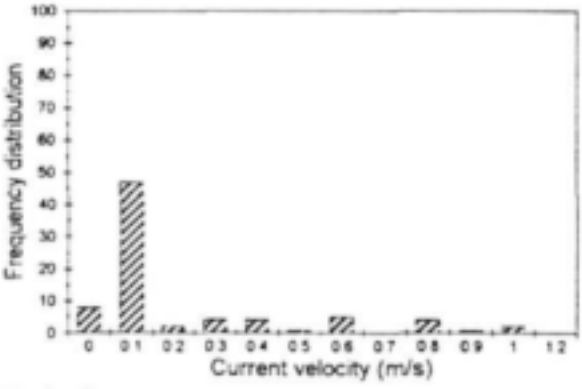
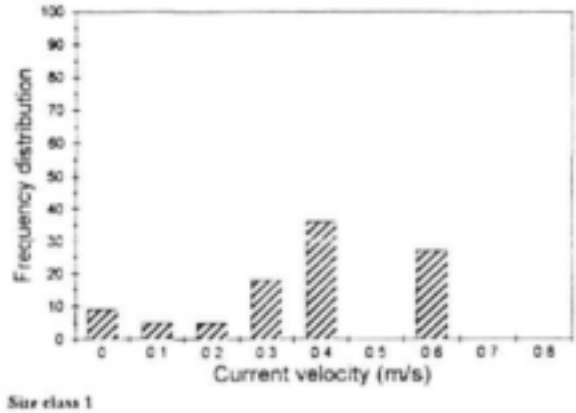
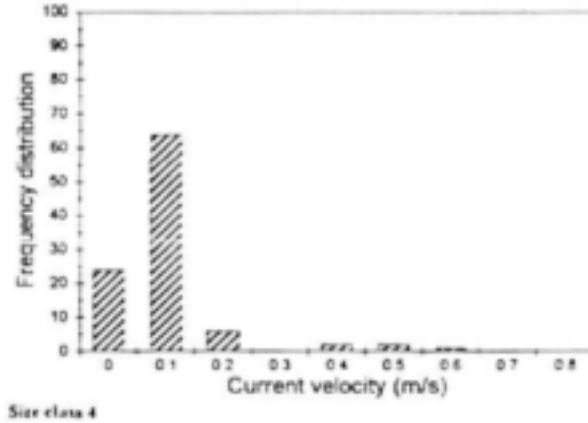


Size class 3

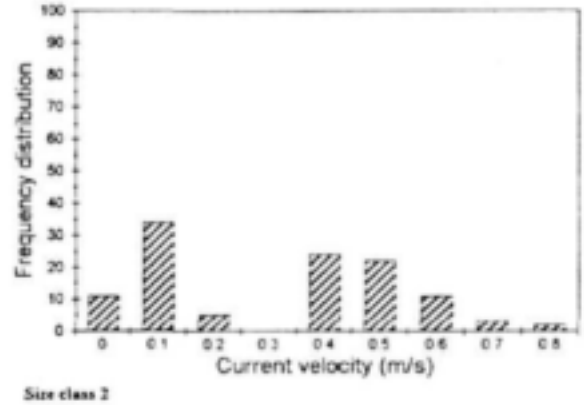
Figure 4.2.2.10 The percentage of nymphs in each size class occcurring in each current velocity recorded in the Palmiet River.



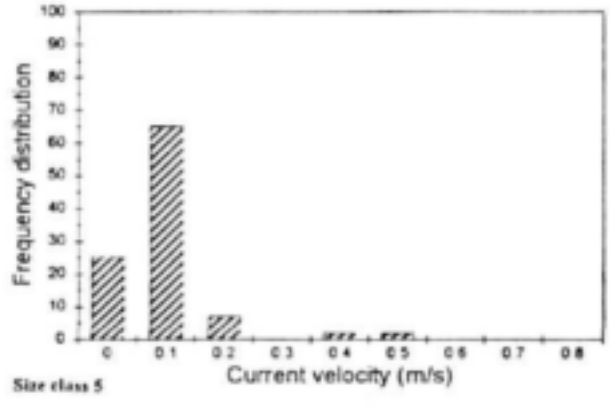
Size class 1



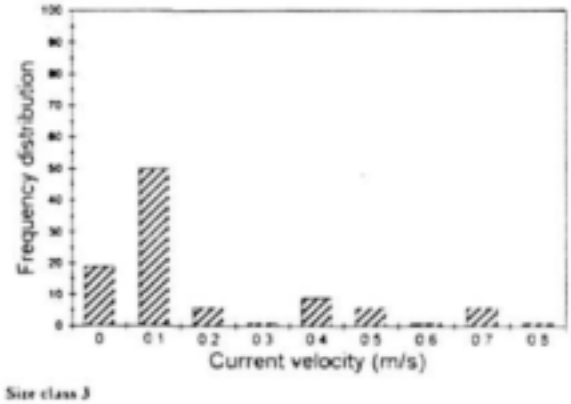
Size class 4



Size class 2



Size class 5



Size class 3

Figure 4.2.2.11 The percentage of nymphs in each size class occurring at each current velocity recorded in the Buffalo River.

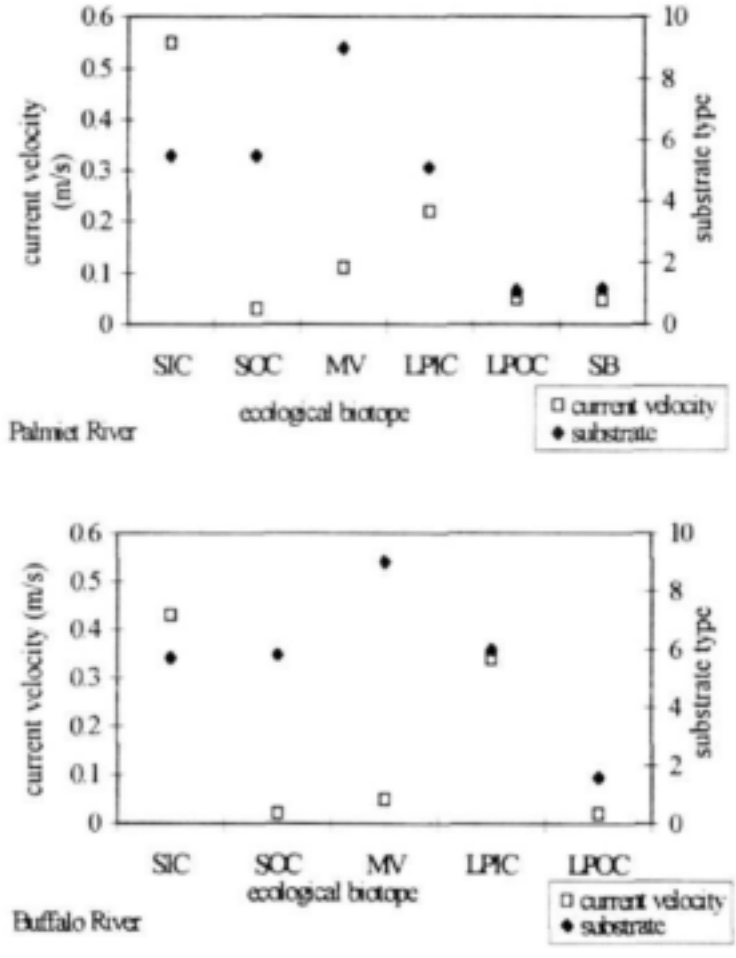


Figure 4.2.2.12 Mean current velocity and modal substrate type in ecological biotopes in the Palmiet and Buffalo Rivers

Figure 4.2.2.12 illustrates the mean current velocities and modal substrate type recorded for ecological biotopes in the Buffalo and Palmiet Rivers respectively. All the ecological biotopes show a strong correspondence between current velocity and substrate, which is similar in both the Buffalo River and Palmiet River. Stones-in-current recorded the highest average current velocity in both rivers. In the Palmiet River the average current velocity in this biotope was approximately 0.2 m/s higher than in the Buffalo River. Leaf-packs-in-current in the Palmiet had a relatively low current velocity in comparison with the Buffalo River. Stones-out-of-current, leaf-packs-out-of-current and sandy bottoms were always associated with low or no current flows. Stones-in-current, leaf-packs-in-current and stones-out-of-current were associated with the substrates large cobble and small boulders. Leaf-packs-out-of-current and sandy bottoms were always associated with silt and sand. No values are shown for sandy bottoms in the Buffalo River as for reasons mentioned already.

Figure 4.2.2.13 illustrates the mean current velocities and modal substrate sizes associated with the hydraulic biotopes in the Buffalo and Palmiet Rivers respectively. Both rivers have a similar pattern of current flow. The hydraulic biotopes pool, slackwater and backwater are all associated with no or very slow flow. The hydraulic biotopes riffle, run, chute and glide are all characterised by higher flows. The average current velocity in run and chute are higher in the Palmiet than the Buffalo River. Glides in the Buffalo River have a higher average current velocity than those in the Palmiet River, the former being 0.37 m/s and the latter being 0.22 m/s.

The substrate characteristics of the ecological and hydraulic biotopes in the two rivers are very similar (Figures 4.2.2.12 and 4.2.2.13). Riffles, runs, chutes, glides and slackwater were associated with the substrates large cobble and small boulder. Backwater in the Buffalo River were associated with silt while in the Palmiet River it was associated large cobble and small boulder. Pools in the Buffalo River have a high modal substrate size of 7 due to its association with vegetation and large cobble and small boulder. In the Palmiet River pools are associated with silt.

The water chemistry of the rivers did not differ widely in terms of TDS and pH in any of the samples (Table 4.2.2.8). Temperature did differ with the Buffalo River being colder on average than the Palmiet River. The effect of temperature on distribution was not studied.

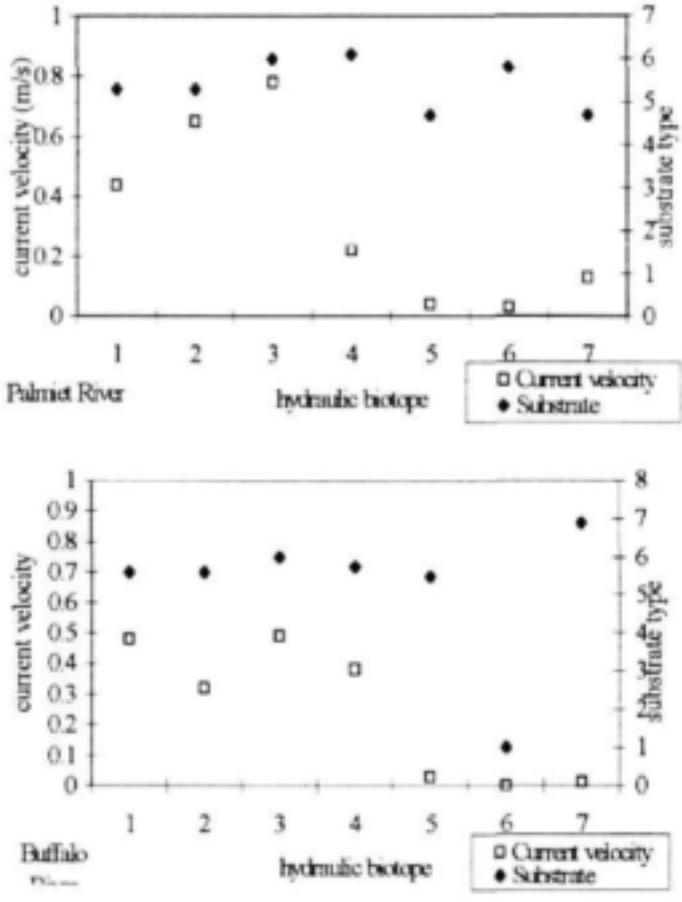


Figure 4.2.2.13 Mean current velocity and modal substrate types in the hydraulic biotopes in the Palmiet and Buffalo Rivers. 1=riffles 2=run 3=chute 4=glides 5=slackwater 6=backwater 7=pool

Table 4.2.2.8 TDS, pH and temperature recorded in the Palmiet and Buffalo Rivers in the March, April and June samples.

	Palmiet River			**Buffalo River**		
	March	April	June	March	April	June
TDS (mg/l)	835	878	853	822	732	
pH	7.4	7	7.1	7.36	6.52	6.8
Temp. °C	21	20.3	13.5	17	15	9.8

Discussion

Size distribution

It was only possible to identify the smallest nymphs in size class 1 (between 0.05 mm and 0.3 mm head width) from the Palmiet River and not from the Buffalo River. Consequently the samples from the Palmiet River give the most accurate reflection of the distribution patterns of entire size class 1 nymphal group, including hatchlings.

The hatchlings are found in all biotopes, independent of classification (Figures 4.2.2.3, 4.2.2.4, 4.2.2.6 and 4.2.2.7). This could be construed as evidence of the adults being random spawners (not laying eggs in any particular biotope), contrary to the case with many other mayflies (Lehmkuhl and Anderson, 1972; Brittain, 1982; Collier, 1994), and/or the eggs/ hatchlings being distributed by current. The high percentage of hatchlings in depositional areas such as sandy bottoms (EB) and pools/slackwater (HB) indicates that current plays an important role in determining their distribution. These two hydraulic biotopes include the ecological biotopes sandy bottoms, marginal vegetation and leaf-packs-out-of-current (Table 4.2.2.5) in which the current is slow or absent. The low percentage of hatchlings in backwaters is indicative of this biotope being detached from the main body of the river (Table 4.2.2.2), and consequently receiving little input. The distribution of size class 1 nymphs amongst biotopes with high current velocities, indicate their tolerance of higher current conditions, a characteristic which disappears with increasing size (Figure 4.2.2.2 , stones-in-current).

In the Buffalo River the smallest nymphs (0.05-0.3 mm) were not identifiable and so the size class 1 nymphs are a larger average size (in the range 0.3 mm - 0.5 mm) than those in the Palmiet River. The fact that the small nymphs were not identifiable may be the reason why no nymphs were recorded from sandy bottoms in the Buffalo. This may also account for the fact that the highest percentage of size class 1 nymphs in the Buffalo River was found in stones-in-current (Figure 4.2.2.4).

In both the Buffalo River and the Palmiet River a relatively high percentage of size class 2 nymphs occur in stones-in-current. This may be indicative of size class 1 nymphs already in this biotope remaining there, as well as recruitment from other biotopes. The decline in the percentage of size classes 3, 4 and 5 nymphs occurring in stones-in-current corresponds with the increase in the percentage representation of these size classes in the ecological biotopes stones-out-of-current and marginal vegetation (Figures 4.2.2.3 and 4.2.2.4), and the hydraulic biotopes slackwater and pool (Figures 4.2.2.6 and 4.2.2.7). This is further supported by the large mean nymph size recorded in these biotopes (Figures 4.2.2.2 and 4.2.2.5).

Therefore, with increasing size, nymphs in high current velocities move into biotopes associated with slow or no currents.

The size distribution pattern of nymphs gives some indication of the life history of *A. auriculata*. Initially the distribution of nymphs is random, as indicated by the occurrence of hatchlings in all biotopes. Subsequently the nymphs are redistributed through drifting or active migration (Ladle and Ladle, 1992). It is important at this point to realise the stream is a mosaic of a number of biotopes, all of which are interlinked in a dynamic fashion and in a continual state of flux (Pringle *et al*, 1988). Transport from one biotope to another is either via active or passive locomotion. Passive locomotion by drifting in the current seems the most likely common mode of locomotion for the smaller size classes. The biotopes with current are thus a vital link between different areas of slow, or no current. The relatively high percentage of size class 2 nymphs in fast currents indicates that the passive redistribution process occurs predominantly amongst the small nymphs. A high propensity to drift in small nymphs has been observed in numerous aquatic invertebrates (Waters, 1972). Once the nymphs have reached areas of slow current there is active locomotion through swimming or walking to the optimal areas, e.g. within the ecological biotopes stones-out-of-current and marginal vegetation (Figures 4.2.2.3 and 4.2.2.4), occurring in the hydraulic biotopes slackwater and pool (Figures 4.2.2.6 and 4.2.2.7). Not all nymphs necessarily go through a redistribution process. The high percentage of nymphs in all size classes in the ecological biotope stones-out-of-current and the hydraulic biotope slackwater and pools indicates that many nymphs reach these biotopes early in their life history and could subsequently remain there.

Based on percentage representation, the optimum biotopes appear to be the ecological biotopes stones-out-of-current and marginal vegetation, which correspond to the hydraulic biotopes slackwater and pool. These biotopes all have slow currents and larger substrates in common (Figure 4.2.2.12 and 4.2.2.13).
The question of which factors affect distribution begs an answer.

Factors affecting distribution

The possible factors affecting distribution include:

- Current velocity
- Substrate
- Detritus and position of biotope in the stream.

Current velocity

The current velocity has a twofold effect on the biotope occupation of *A. auriculata* nymphs. The first effect will be through the transport of eggs and nymphs through drift as already discussed and the second effect will be by the degree of current tolerance displayed by the species. The transport function has already been discussed above so in this section we will concentrate on the tolerance of the species.

A. auriculata appears to have lowered tolerance of high current with increase in size. Class 1 to 3 nymphs were frequently found at velocities above 0.3m/s but nymphs from class 4-5 occur seldom (Figure 4.2.2.10 and 4.2.2.11). Overall, in the higher current velocities the representation of nymphs is lower than expected (Table 4.2.2.6. and Table 4.2.2.7), indicating that high current velocity or some factor associated with high current velocity is sub-optimal for *A. auriculata*.

The distribution of size class 5 nymphs differ in the Buffalo River and the Palmiet River in that in the Buffalo River size class 5 nymphs occur at relatively high frequencies at the current velocities 0 m/s and 0.1 m/s while in the Palmiet River they occur at high frequencies at higher velocities of between 0.1 m/s to 0.3 m/s. This is due to the occurrence of marginal vegetation which occur in glides, an hydraulic biotope which recorded a mean current velocity of 0.38 m/s (Figure 4.2.2.13). In the Buffalo River the marginal vegetation sampled was confined to slackwater and pools (Table4.2.2.5. b). This may indicate that substrate type and size plays an important role in determining population distribution. Marginal vegetation in glides provides an interesting case in that there are large nymphs in an area of relatively high current velocity. It must be recognised that the strength of the current, which was measured alongside the vegetation, would be negated within it. Thus, the current velocity measured for this biotope is not necessarily what the nymphs are experiencing.

The substrates large cobble and small boulder are found over the full range of current velocities measured (Figures 4.2.2.12 and 4.2.2.13) and are occupied by a large percentage of nymphs from all size classes. When these substrates are divided into regions of high and low flow (this is done by examining the ecological and hydraulic biotopes in which these substrates occur) it is found that where they coincide with high flow (*e.g.* Stones-in-current) they are dominated by small size classes and where they coincide with low flow (*e.g.* Stones-out-of-current) they are dominated by large size classes. Current velocity, or some variable associated with current velocity, is thus more important than substrate in determining the distribution of nymphs. An important factor, resulting from current velocity is detritus distribution. Detritus is concentrated in depositional zones, and hence slow current zones have a higher detrital content, of all types, than high current zones. Stones-in-current and stones-out-of-current thus not only differ in current flow but also in the amount and type of associated detritus.

The effect of detritus can be removed by examining the size class distribution in the ecological biotope leaf-packs-in-current in the Buffalo and Palmiet rivers. In the Palmiet River nymphs of a large mean size were found in this biotope while in the Buffalo River a small mean nymph size was found. Leaf-packs-in-current differs between the two rivers in terms of current velocity with the Buffalo River recording an mean current velocity approximately 0.2 m/s faster than that in the Palmiet River. In this instance substrate size (Figure 4.2.2.12) and detritus content are the same, and so current velocity must be seen as the most important factor in determining the distribution of the nymphs.

The role that current velocity plays in determining biotope occupation thus appears to be influenced by the type ans size of substrate and detritus in any particular biotope.

Substrate

A high percent of the total nymphs in all size classes were found in the substrates large cobble and small boulders in both rivers (Figure 4.2.2.9). Large substrates are thus optimal for all nymphal size classes. These substrates occur in a range of biotopes and consequently have a range of associated conditions (Figures 4.2.2.12 and 4.2.2.13). When the associated size classes and biotopes are considered it is apparent that the nymphal size distribution among these substrates varies according to the associated conditions. For example, large substrates occur in both stones-in-current and stones-out-of-current and the size distribution patterns in these biotopes are mirror images of each other (Figures 4.2.2.3 and 4.2.2.4). The biggest difference between these two biotopes is current velocity, a factor which has already been shown to have a strong effect on size distribution.

Vegetation shows a distinct pattern, in both rivers, of increased percentage of the total nymphs with increasing size class (Figures 4.2.2.3 and 4.2.2.4). Unlike the substrates large cobbles and small boulders, low percentages of small nymphs occur on vegetation. This indicates that vegetation is a substrate in which conditions are suitable for later instars. This is supported by the high percentage of size class 5 nymphs on this substrate as well as the large mean nymph size recorded in the ecological biotope marginal vegetation (Figure 4.2.2.2).

Detritus and position in the stream

Detritus is an important factor in determining the distribution of *A. auriculata* as this animal is a detritivore (Palmer, 1991).

The different biotopes show large differences in the amount and type of detritus that occur within them. Stones-in-current contains predominantly fine detritus (Haigh, personal communication; personal observation). Stones-out-of-current on the other hand has a large amount of detritus and a larger average detritus particle size (personal observation). Palmer (1991) found that the gut content of small *A. auriculata* comprised only fine detritus, whereas the foreguts of larger nymphs contained more material, a larger average particle size and a wider variety of food types. It has been suggested that this dietary change is a causative factor in determining the distribution of *A. auriculata* nymphs (Hunt, 1996). However, it would seem more probable that this dietary shift is more a reflection of increasing mouthpart size making the nymphs physically capable to make use of a more varied diet.

The biotopes leaf-packs-in-current and leaf-packs-out-of-current both have high detritus contents and a large average detritus particle size. However, they have a low percentage of the total nymphs in all size classes, in relation to stones-out-of-current and marginal vegetation. In these biotopes detritus practically constitutes the substrate and so cannot be the limiting factor. The effect of detritus quality cannot be taken into account as it was not measured for this study. However, Drake (1984) indicated that detrital particle size may have an important affect on the distribution of aquatic invertebrates. The biotope leaf-packs-in-current is associated with a high average current velocity, an important factor in determining the small mean nymph size in this biotope. Leaf-packs-out-of-current, however, have a high detritus content and low current velocities yet still recorded a medium mean nymph size in both rivers. An important difference between leaf-packs-out-of-current, marginal vegetation and stones-out-of-current is that marginal vegetation and stones-out-of-current are found on stream margins. *Adenophlebia auriculata* nymphs need an emergence structure for the transition from the aquatic to the terrestrial environment (Haigh and Davies-Coleman, 1997; personal observation) and so large animals become concentrated in these biotopes. This reiterates what has been observed by Brittain (1982), Hall *et al* (1980), Holomuzki and Messier (1993) and Collier (1994) who found that during the final stages of nymphal life there is a movement into, and concentration in, the shallower areas of rivers. Emergence structures include emergent vegetation and rock, as well as the river bank itself. These are all found in marginal habitats which incorporates the ecological biotopes stones-out-of-current and marginal vegetation and the hydraulic biotopes slackwater, backwater and pools.

Comparison between the rivers

The size distribution pattern of nymphs in ecological biotopes was similar in the two rivers (Figure 4.2.2.2). The only differences were that marginal vegetation and leaf-packs-out-of-current in the Buffalo River had a significantly larger mean nymph size than the corresponding biotopes in the Palmiet River. However, both of these biotopes recorded high mean nymph sizes in the respective rivers.

In terms of the hydraulic biotopes the same pattern of small nymphs in fast current and large nymphs in slow currents is shown in both rivers (Figure 4.2.2.5). However, the mean nymph sizes recorded in the same hydraulic biotope differed significantly between the rivers. In the Palmiet River chutes recorded a significantly smaller mean nymph size than chutes in the Buffalo River. However, chutes in the Palmiet River recorded a mean current velocity approximately 0.2 m/s faster than chutes in the Buffalo River and smaller nymph sizes have already been shown to occur at higher current velocities. In the case of glides and pools the differences between the two rivers may be attributed to different substrates being sampled and are explained by the analysis of the associated ecological biotopes. The large mean size of the nymphs in glides in the Palmiet is due to the presence of marginal vegetation in this biotope. The low mean nymph size in pools in the Palmiet River is due to this hydraulic biotope including the ecological biotope sandy bottoms in which small nymphs predominate. The hydraulic biotopes slackwater and backwater recorded

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The size distribution pattern of nymphs between biotopes is similar in the two rivers. Where differences do occur they may be attributed to different physical conditions being associated with the same biotopes. The general similarity in the size distribution of nymphs between rivers indicates that the nature of the biotopes, and their associated physical variables, are generally consistent. This is important as consistency across rivers is vital for the comparison of research, and the application of research to different river systems.

A significant feature of Figure 4.2.2.9 is that although the size distribution patterns between biotope were similar the mean nymph size recorded in depositional biotopes in the Buffalo River were significantly larger than those in the Palmiet River in a number of cases.

Summary of the size distribution of A. auriculata

The size distribution of *A. auriculata* nymphs differs significantly between ecological biotopes as well as between hydraulic biotopes sampled. This distribution pattern reflects the life history of the animal and is consistent between the two rivers indicating that river scale does not effect size distribution.

The optimal habitat for *A. auriculata*, in all size classes, but particularly size classes 4 and 5, appears to combine a number of variables, none of which are mutually exclusive. These include slow currents, large substrate sizes, marginal vegetation and high detritus content. Similar preferences were found for the mayfly *Paraleptophlebia guttata* (Leptophlebiidae) by Holomuzki and Messier (1993). An important

Lancastar and Hildrew (1993) and Dudgeon (1993) cite predation as a major factor influencing local densities of invertebrates. Teague *et al* (1985) discuss how size selective behaviour by predators contributes to the prevalence of smaller grazing caddisfly larvae in shallower, faster water. This, however, does not explain why the highest percentage of small *A. auriculata* nymphs occur in fast currents. Another factor is inter- and intraspecific competition for food and space which may lead to smaller larvae being excluded from preferred biotopes by larger larvae (Collier, 1994; Buffagni *et al*, 1995). Once again, this cannot explain the high percentage of small nymphs in the same biotopes as large nymphs. Furthermore, it has been observed that where disturbance is frequent or intense the importance of biotic interactions can be reduced (Feminella and Resh, 1990, Resh *et al* 1988, Poff and Ward, 1989).

Size related shifts in aquatic invertebrate distribution may reflect changing oxygen requirements (Collier, 1994). As individuals grow they develop larger surface area to volume ratios, decreasing the efficiency of diffusion in supplying sufficient oxygen to meet the animals requirements. Larger nymphs are thus found in areas of high current velocity which promote oxygen transfers. This pattern of large nymphs in high current velocities is found in some mayflies such as *Deleatidium* species (Collier, 1994). However, *A. auriculata* shows the opposite pattern, with large nymphs being concentrated in slow currents. Oxygen therefore does not seem to be an important factor in determining the distribution of these mayflies within the stream.

Comparison of the two Biotope Concepts

The range of hydraulic biotopes recorded per ecological biotope, and *vice versa*, indicates that the two biotope classifications are based on very different criteria. The hydraulic biotopes major determinant is the nature of the surface flow or disturbance. They thus give a good indication of current velocity and flow type. They give an indication of the underlying substrate and slope of the stream (Table 4.2.2.2), as this determines flow characteristics. However, no substrate type is specific to any one hydraulic biotope. All hydraulic biotopes are potentially associated with numerous substrate types. Substrate predictions can therefore not be made categorically based on the biotope names. Furthermore, they give no indication of ecologically relevant information such as detritus content and the presence of marginal vegetation.

Ecological biotopes (Table 4.2.2.1), on the other hand, give an indication of substratum type, current velocity (although not with the clarity of the hydraulic biotopes), detritus content, and to some extent the position in the river eg. marginal, pool, riffle etc.

When the distribution of *A. auriculata* is examined purely in terms of hydraulic biotopes a different impression is obtained from that when considering only the ecological biotopes. Hydraulics now become the most important factor affecting distribution, in terms of both the velocity and the nature of the flow. In

most cases the hydraulic biotopes *riffle, chutes and run* are associated with small average head widths. The hydraulic biotopes glide, slackwater, backwater and pool are dominated by animals of large average head width. One can therefore say that these animals favour areas of low or no current velocity.

The division in size class distribution between areas of slow flow and areas of fast flow may be due to the secondary effects of substrate composition and detritus deposition. Slow currents are indicative of depositional areas and hence should be associated with high detritus content, a factor favouring inhabitation by *A. auriculata* nymphs. However, this is by no means a safe assumption. Since the flow in these rivers fluctuates quite dramatically, low current velocity areas could be areas that were riffle in the recent past and contain little in the form of deposited organic matter. The amount of deposited material depends on the age of the biotope. Another factor is that some ecological biotopes, such as sandy bottoms, do not have a high detritus content despite being characterised by slow or no current. Furthermore, hydraulic biotopes give no idea of the type of detritus. Hydraulics was not the fundamental determinant of *A. auriculata* size distribution, although certainly an indirect cause through its influence on other abiotic factors such as substrate type and detrital distribution. By not giving insight into many ecologically relevant variables the hydraulic biotopes had a low resolving power in terms of the size distribution of *A. auriculata* nymphs. The hydraulic biotopes therefore need to be augmented with other habitat data. This can be done by:

- breaking them down into their constituent ecological biotopes. This gives greater insight into the substrate characteristics and detritus content, two important factors in the distribution of this mayfly species.
- using measured current velocity, substrate and detritus characteristics.

The explanatory power of the ecological biotope is illustrated by examining the size distribution patterns in the hydraulic biotopes in terms of their corresponding ecological biotopes. For example, glides recorded a large mean nymph size in the Palmiet River and a low mean nymph size in the Buffalo River (Figure 4.2.2.5). The difference between the two rivers was that in the Palmiet River glides correlated with both marginal vegetation and leaf-packs-in-current while in the Buffalo River glides only corresponded with leaf-packs-in-current.

Value of the hydraulic biotope concept

Ecological sampling strategies need to be relevant to animals being studied and practical for the biologist. As such, biotopes are useful ecological units. However, the inconsistency of biotope classifications, as highlighted by Wadeson (1994), prevents the comparison of data from different studies and consequently hinders the development of stream ecology. What is required is a standardisation of habitat units. The hydraulic biotope concept would be useful in this regard because the biotope definitions are simple and unambiguous. The hydraulic biotope could be seen as a fundamental habitat unit, and abiotic (substrate

type, size, current velocity etc.) and biotic data built upon it. The hydraulic biotope concept demonstrates the heterogeneity/complexity of stream flow. As such it is likely to be particularly relevant to the population distribution of current, and flow-type dependant invertebrates such as Simuliidae.

Traditionally, aquatic ecologists have designated riffles, pools and runs as relatively homogenous units of study (Pringle *et al*, 1988). Sampling in these "biotopes" takes place over relatively large areas which may include several hydraulic as well as ecological biotopes. Studies therefore lose resolution, possibly to the extent that the scale of sampling exceeds the range relevant to the study animal. However, even within the biotopes used for the purpose of this study, which consider small habitat units, there was considerable inconsistency in associated variables including substrate, current velocity and water depth. The function of the ecological and hydraulic biotope concepts should therefore be that of a descriptive tool, used to demarcate ecologically relevant habitat units. As such they can play an important role in river management. However, in order to determine the factors effecting the distribution of invertebrates it is necessary to make precise measurements of abiotic and biotic variables.

River management, insect life histories and the biotope concept.

In South Africa, significant gaps in the ecological understanding of river ecosystem processes and the absence of target species has led to the focus on habitat maintenance rather than species management (O'Keeffe, 1995). The basis of habitat conservation as a tool is the assumption that species richness follows from habitat richness, subject to limits imposed by chemical water quality (Harper and Ferguson, 1992). But what is meant by habitat units that are to be preserved? Of prime importance is that these include habitats that are recognised by animals that use the stream. As already indicated, the larger spatial scales generally used, included a range of biotopes. By conserving biotopes, either hydraulic or ecological, one is preserving a range of habitat types that are relevant to the animal.

By classifying the size distribution of this species of mayfly it becomes apparent that it is not sufficient to just consider specific habitats or biotopes in ecological studies. *Adenophlebia auriculata* uses a suite of biotopes, both ecological and hydraulic, in order to complete its life cycle. It would appear important for river management that the life history of animals is fully understood so that those features of the environment that are pertinent to the organism can be maintained. If an organism requires a number of different biotopes for its persistence then conservation of a subset of these will have the same affect as conserving none. This is recognised by Harper and Ferguson (1992). The distribution of a species among habitats should be viewed very carefully. The presence of species A in habitat X may be dependant on habitat Y (Harper and Ferguson, 1992). The stream must be viewed as a mosaic that is dynamic in nature. Ecosystem functioning and insect life cycles require that all biotopes be maintained for their life cycle to

be completed. Instream flow requirements (IFR's) should consider this particularly since lotic macro invertebrates play a vital role in nutrient cycling, and hence ecosystem functioning.

Recommendations.

In order to accurately assess the distribution pattern of stream invertebrates the sampling programme should consider the entire stream cross section as a dynamic mosaic. In this way a three-dimensional picture of distribution can be established. However, it is not sufficient to record only the associated conditions at a point. It is important to be able to place a point sample in the context of the entire stream. Recording both the hydraulic and ecological biotope with every point sampled performs this function. Although biotopes are distinct areas, they form a continuum with the areas above, below and along side them.

For ecological studies the following sampling strategy is suggested:

Sampling should be conducted along a transect, running across the stream, which should be set at intervals that enable extrapolation of conditions between transects. The number of transects is determined by the nature and extent of the study and to provide sufficient replicates for statistical analyses. For example, if one were investigating the distribution patterns of a particular species, a reach incorporating all of the biotopes would be sufficient, as this should include the full range of conditions available to the animal. Points will be sampled at intervals along the transect. At each point the relevant abiotic variables should be sampled, the biotope in which the sample is made recorded, as well as detritus. In this way a clear picture of the variables effecting the distribution of an animal/ or population can be established.

Conclusions

1. The nymphal size *A. auriculata* influences the distribution amongst biotopes. The initial distribution is random, with hatchlings occurring throughout the stream. Subsequently there is a redistribution to the optimal ecological biotopes stones-out-of-current and marginal vegetation, corresponding with the hydraulic biotopes slackwater, backwater and pool. This redistribution occurs primarily in the small nymphs and is either active or passive. Passive locomotion makes use of current flow and explains the transient occupation of high current biotopes by small size class nymphs.

2. The optimal conditions for *A. auriculata* nymphs appear to be slow currents, large substrate size and vegetation, and large quantities of detritus. An important feature for the large nymphs (pre-

emergent) is that they have suitable structures for emergence. This is provided in marginal areas by vegetation, rocks and the bank itself.

3. The scale of the river does not affect the distribution pattern of the nymphs. The size distribution pattern of nymphs between biotopes is similar in the two rivers. Where differences do occur they may be attributed to different physical conditions being associated with the same biotopes.

4. The different biotope concepts are based on different criteria and as such give different insights into the factors affecting the size class distribution patterns of *A. auriculata*. Although considering useful ecological units the abiotic and biotic conditions associated with them show a great deal of variation. The function of ecological and hydraulic biotopes should therefore be limited to that of a descriptive tool.

4.3 LABORATORY CULTURE METHODS FOR *A. AURICULATA*

4.3.1. INTRODUCTION

At present most animals used in laboratory experiments are collected from a river in the vicinity of the laboratory. This is not only time consuming and labour intensive, but may lead to over sampling in the field. Hence, an artificial breeding program in the laboratory would be invaluable with regard to saving time and conserving field populations. This program would also provide a stock of animals of known background and in time, may reduce the degree of variation in the captive population.

Artificial fertilization of the eggs of Ephemeroptera has been reported by several authors as part of a program of investigating growth rates and life histories. Sweeney (1978) reports combining the sperm and eggs of *Isonychia bicolor* directly on a glass slide without any other solution, leaving the mixture together for 5 minutes and then washing the eggs into a container of filtered stream water. Giberson & Rosenberg (1992) on the other hand stripped the eggs from female sub imagoes into Yeagar's solution and then macerated the terminal segments of the males and placed these with the eggs for 10 minutes. They also reported that the fertilized eggs of *Hexagenia limbata* could be stored at 8°C for extended periods and will nevertheless develop normally when returned to higher temperatures.

In the family Leptophlebiidae, female imagos lay eggs in suitable habitats by descending to the water and releasing a few eggs at a time when the tip of their abdomen is dipped into the water (Needham & Traver 1972; Brittain 1982). The eggs are covered with fine spring-like hairs which uncoil on contact with water and become adhesive (Needham & Traver 1972). The eggs presumably adhere to the first solid object that they come into contact with. The time taken for the eggs to hatch is strongly influenced by temperature. Most mayfly eggs may hatch in two to three weeks but those laid in cold streams (<10°C) may diapause over winter (Needham & Traver 1972; Wise, 1980 and Brittain, 1982). The effect of temperature on hatching also appears to be linked to the natural environmental regime of the species.

Suter & Bishop (1989) incubated eggs of *Atalophlebia australis*, *Nousia inconspicua*, *N. fuscula* & *Baetis soror* from South Australia under constant temperature conditions in the laboratory (Range 4-24°C). Embryonic development of *N. inconspicua* occurred at all temperatures, but hatching did not occur below 12°C for *A. australis*, below 15°C for *N. inconspicua* or below 5°C for *N. fuscula* & *B. soror*. Photo period length had no effect on the incubation period. The hatch rate of eggs of *Potamanthus formosus* which was 34-53% over a temperature range of 15 - 30°C, dropped abruptly below 15°C to almost zero at 13°C (Watanabe 1992). Brittain & Campbell (1991) incubated the eggs of *Coloburiscoides* sp. in the laboratory at constant temperatures in 5°C intervals between 5°C and 30°C.

Hatching success was high (> 80%) at temperatures between 10°C and 25°C. No eggs hatched either at 5°C or 30°C. Artificially fertilized eggs had low (< 10%) hatching success. Beside temperature, the pH of the water in which the species evolved must be considered when developing methods of artificial incubation. Rowe *et al* (1988) reared *Leptophlebia cupida, Habrophlebia vibrans, Stenonema femoratum* & *Baetis flavistriga* at different pH levels (4.0, 4.5, 5.0, and 6.5) in the laboratory and the proportion of eggs undergoing eclosion did not vary with pH. Hatching rate was affected at the three lower pH values for *H vibrans, & B. flavistriga* but was unaffected in the other three species which occur in low pH waters. Punzo & Thompson (1990) investigated the combined effects of pH and temperature on hatching and hatchling survival of *Caenis diminuta* & *C. hilaris* and found high mortality at pH 3.5 over a temperature range of 10-30°C with best hatching and survival success at 20°C over a pH range of 4.0-7.2.

Review of previous experiments.

In previous experiments similar methods of collection and treatment of the pre-adult nymphs to those described below, were used. A variety of ways in which to fertilize the eggs were also tried: these included:

a) Pinning a male and removing the head and then attempting artificial copulation with a female by holding their genitalia together. This method has proved successful with mosquitoes (WHO Report 1975). This did not work.

b) Dissecting the eggs out of a female in insect Ringers, Yeager's solution and a mixture of insect Ringers and Yeager's solution immediately dissecting out the seminal vesicles of the male and mixing the sperm and eggs. Both sperm and eggs were examined microscopically to check viability.

c) Dissecting a male in insect Ringers and then stimulating a female to release its eggs onto the sperm by removing its head and dipping the tip of its abdomen in the Ringers above the sperm.

d) Fertilization was also attempted between;

i. a male imago and a female subimago,

ii. between a male subimago and a female imago and

iii. between male and female subimagoes to determine whether the subimagoes contained viable eggs or sperm.

Eggs were then left in the sperm/Ringers mix for 5, 10, 15, 20 and 30min before being transferred onto ground glass slides in stream water to determine the minimum time that the eggs and sperm should be

mixed to yield a sufficiently high fertilization rate. The most successful method involved dissecting the seminal vesicles out of a male imago in a fresh solution of freshly prepared insect Ringers.

During the previous series of experiments development was poor in the first few trials but full development was eventually achieved. The length of time that the sperm and eggs were in contact influenced the proportion of fertilized eggs with approximately 50% fertilized after 5min, 70% fertilized after 10min, 80% fertilized after 15min and 85% fertilized after 20 and 30min. It was also found that subimagoes did contain viable sperm or eggs and fertilization rates were no different when male and female imagoes, a male imago and a female subimago, a male subimago and a female imago and male and female subimagoes were used for the fertilization experiment. As was found by Giberson (pers. comm.) the hatch rate of artificially fertilized eggs was less than 15%. All three successful experiments involved a male imago and a female imago. The first and second successful experiments yielded a hatch rate of 5% to 6% but when the female was induced to release her eggs, a hatch rate of approximately 15% occurred. The eggs hatched between 16 and 22 days at 25°C, at 19°C none hatched but many appeared to still be developing at the termination of the trial.

The perivitelline space formed in approximately 24 hours. After five days a small embryo was visible and the body had differentiated by the tenth day. After 14 days the embryo had developed an eye patch, antennae and legs and by day 17 hatching began and took place through a longitudinal slit orientated near one pole of the egg. The first instar nymphs were approximately 0.35mm long and had pentagonal shaped heads, no filamentous gills, simple tarsi and three equally sized ocelli. The hatchlings fed by brushing the bottom of the petri dishes. Gills began to develop in the later instars and these instars were more active.

The second series of experiments during which the eggs were incubated in shallow petri dishes was more successful. Algal growth was suppressed by covering the petri dishes with a semi-transparent lid, thus reducing the light intensity. In her successful experiments with a different species of mayfly, Giberson (pers. comm.) used Yeager's solution. Yeager's solution, however, triggered the adhesive 'hairs' prematurely and resulted in the eggs not adhering when placed in water. Thereafter, a fresh insect Ringers solution and a combination of the two were used and both proved successful. The reasons for the high fertilization rate and low hatch rates are not clear and can only be determined with further experimentation. Many eggs could have succumbed to bacteria or other microorganisms so it may be necessary to use filtered stream water. Further, 25°C may have been too high and the eggs may have been oxygen deprived. A possible method for this would be to fertilize a large number of eggs as described above and 'seed' them onto a fine mesh screen in still water. Once the eggs adhere, which takes a few minutes, a current can be directed over the screen to ventilate the eggs.

4.3.2 OPTIMISATION OF PREVIOUS METHODS AND DEVELOPMENT OF MASS REARING TECHNIQUES.

Report by Dr L Janssens de Bisthoven & EH Haigh.

The aim of the project is to realize a standing stock of several hundreds of individuals of middle-sized age (head width 1.0 - 1.5 mm) mayflies bred in the laboratory. Two alternatives to achieve this will be explored, namely the induction of reproduction in a CE room (in vitro) and secondly artificial insemination (AI). These are complementary investigations: in order to achieve reproduction, a large number adults have to be available for swarming, mating and oviposition. Some of these adults can be used for artificial insemination trials. Hatching success and hatchling survival in the in vivo trial can be used as a control for the same parameters in the in vitro trial.

Main questions: in vitro reproduction

(1) How to achieve synchronised emergence of males and females?

(2) How to induce mating?

(3) How to induce oviposition?

(4) How to handle the eggs and subsequently the rearing of hatchlings?

(5) How to produce more than a hundred individuals a week on a continuous basis?

Artificial fertilisation

(1) Dissection of male and female gonads

(2) Medium: Ringer, Yeager or combination

(3) Temperature and timing

Subsidiary questions: Do they lay eggs individually or in batches? Do they swarm before mating? Do they mate while swarming or on the ground or vegetation? Does the oviposition occur under water, on the water or on substrate at the edge of the water? Is there a density-dependent territorial behaviour of the nymphs?

Growth and Mortality Under Two Food Conditions of *Adenophlebia Auriculata*

Introduction

The reproduction and laboratory rearing is under continuing investigation as part of the project. The initial success that we had with the artificial fertilisation of eggs in 1994 now had to be attempted on a large scale and standard methods established. A method for rearing hatchlings had to be developed. From previous experience we know that large numbers of sub-adult nymphs should be kept in captivity to ensure that sufficient numbers of males and females to effect fertilisation should emerge at the same time. The first step was therefore to collect large numbers of late instar nymphs and rear them through the final stages before emergence, and to attempt fertilisation using these adults.

Materials And Methods

Table 4.3.2.1. Design of experiment, Mayflies were collected from the Palmiet river on 23 May 1995.

Bubble pots	no. Nymphs	diet	day 1	Origin
A,B,C,	20	detritus ad. lib.	35575	Palmiet River
D,E,F,	20	Tetramin ad. lib.	35608	Palmiet River

Nymphs were kept under fluctuating laboratory conditions of daily estimated 15°C and 23°C, and fluctuating photo period. This is of importance for the nymphs of tanks D, E, F which remained there for 54 days with detritus as food, prior to the experiment. Per bubblepot (inner diameter 17 cm): one litre Palmiet water strained through 85 micron mesh: detritus from the Palmiet River was pre-dried. Aeration with Pasteur pipette through nylon netting covering the pot. The substrate in A, B, C was plastic netting covering 4/5 of the bottom surface and one 5x5cm tile on top of it but after 26 days this was replaced with a larger stone which acted as natural emergence structure. D, E, F: one stone per bubblepot. All stones were higher than the water level.

Measurements of each nymph were of length L without procerci and head width HW (0.1 mm) :
Measurements were made under binocular microscope on plastified mm-paper, graded up to 0.1 mm for the head widths by means of crossed lines method.
Measuring schedule: **A, B, C**; day 1, 12, 26, 55, 81. **D, E, F**: day 1, 22, 52.
Nymphs were manipulated with a wet paint brush.
Food and Palmiet River water was added weekly.

Subsequent to this experiment a sample of nymphs (n => 500) was collected from the Palmiet river, preserved and measured under binocular microscope. The regression between HW and L was calculated for this sample.

Homogeneity of replicates

Table 4.3.2.2 Differences in L and W tested with Kruskal-Wallis among A, B and C and among D, E, F at each measurement day. Only the significant differences ($p<0.05$) are given.

Tanks	Day	Variate	H	p
A, B, C	1	W	H(2, 60)=8.41	0.02
A, B, C	1	L	H(2, 60)=6.45	0.04
A, B, C	26	L	H(2, 49)=6.78	0.03
A, B, C	55	L	H(2, 24)=9.98	0.007

The nymphs in tank B caused the significant differences as at day 1, B contained two very small nymphs with HW=0.4-0.5 mm (size classes), and two larger outliers with HW=1.5-1.6 mm. There is one smaller nymph with L=9-10 mm, and two larger outliers with L=21-22 mm. At day 26, B contained 7 nymphs which grew more slowly, with L=12-14 mm. At day 55, B contained 4 nymphs which were larger, with L=22-23 mm. These outlying nymphs may be seen in Fig. 3.3.2.1, below.

Emergence cage

The presence of pre-adults and adults was monitored daily. They were transferred to an aerated glass aquarium containing a large stone, topped by a one metre high cage, consisting of an unpainted wooden frame covered by white mosquito netting, in order to induce mating and oviposition. Two dry *Phragmytes* stems the height of the cage were placed inside and rolled up green plastic netting and a large stone to facilitate emergence and mating. However mating and oviposition was never observed.

Results

Growth and development

Linear regressions were constructed between length and head width for the nymphs in the rearing containers and for a large sample collected from the same river. The regressions give the range and the ratio of both variates at a certain time or size. In that respect they contain information about the development of the nymphs, since head width is related to the stage of moult (Figure 4.3.2.1, Table 4.3.2.3). Note that 'day 1' refers to the first day of the experiment and not the age of the nymphs, neither is

it the same date as the nymphs reared in respectively detritus and TETRAMIN were collected at different periods. Although both experimental populations probably belong to the same Palmiet generation of the beginning of autumn, the TETRAMIN population had one and a half months to develop in the laboratory prior to the experiment. This gave that population an advantage at the onset of the experiment.

Because the width of the head capsule is widely used and recognised as a conservative measure of development, but not condition, its relationship with both body length and wingbud development is of interest. Figure 4.3.2.1 and Figure 4.3.2.2 both show the relation hip between HW and L. In the smaller sizes the r^2 values show a closer correlation between the two measures than the larger sizes where r^2 values decrease below 50%.

Table 4.3.2.3 Linear regression between HW and body length of *A. auriculata* in the growth experiment

Food	day	r^2	SE	F	df	p	int	SE	t	p	r
Detritus	1	0.82	0.12	273.3	158	0	-0.57	0.97	-5.8	0	0.91
	12	0.77	0.14	193.2	157	0	-0.25	0.12	-0.2	0.1	0.88
	26	0.63	0.18	82.05	147	0	-0.1	0..17	-0.37	0.7	0.8
	55	0.59	0.13	11.51	122	0	0.92	0.28	3.34	0	0.59
TETRA MIN	1	0.69	0.16	132.7	158	0	0	0.13	-0.27.	0.8	0.83
	22	0.31	0.17	21.49	145	0	0.88	0.26	3.39	0	0.57

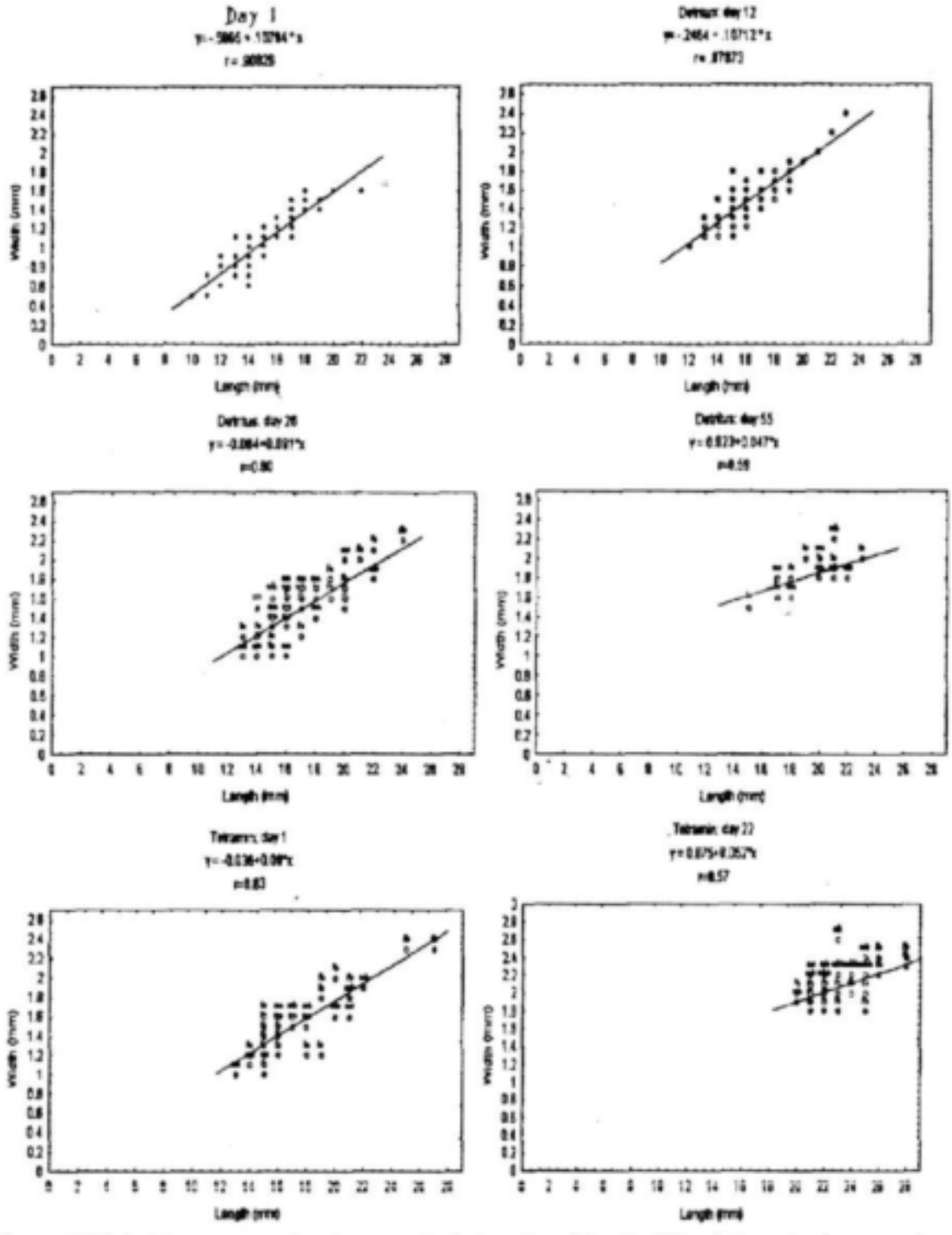

Figure 4.3.2.1. Linear regressions between body length and head width of *A. auriculata* nymphs on the diets detritus and TETRAMIN, at different days. Each regression contains nymphs of tanks A,B,&C. The different graphs represent the same nymphs which survived at that time of measurement.
m=male f=female, n=no wingbuds, b=well developed wingbuds, s=small wingbuds, bb=dark brown before pre-adult. Not enough nymphs survived to day 81(detritus) & 52(TETRAMIN.)

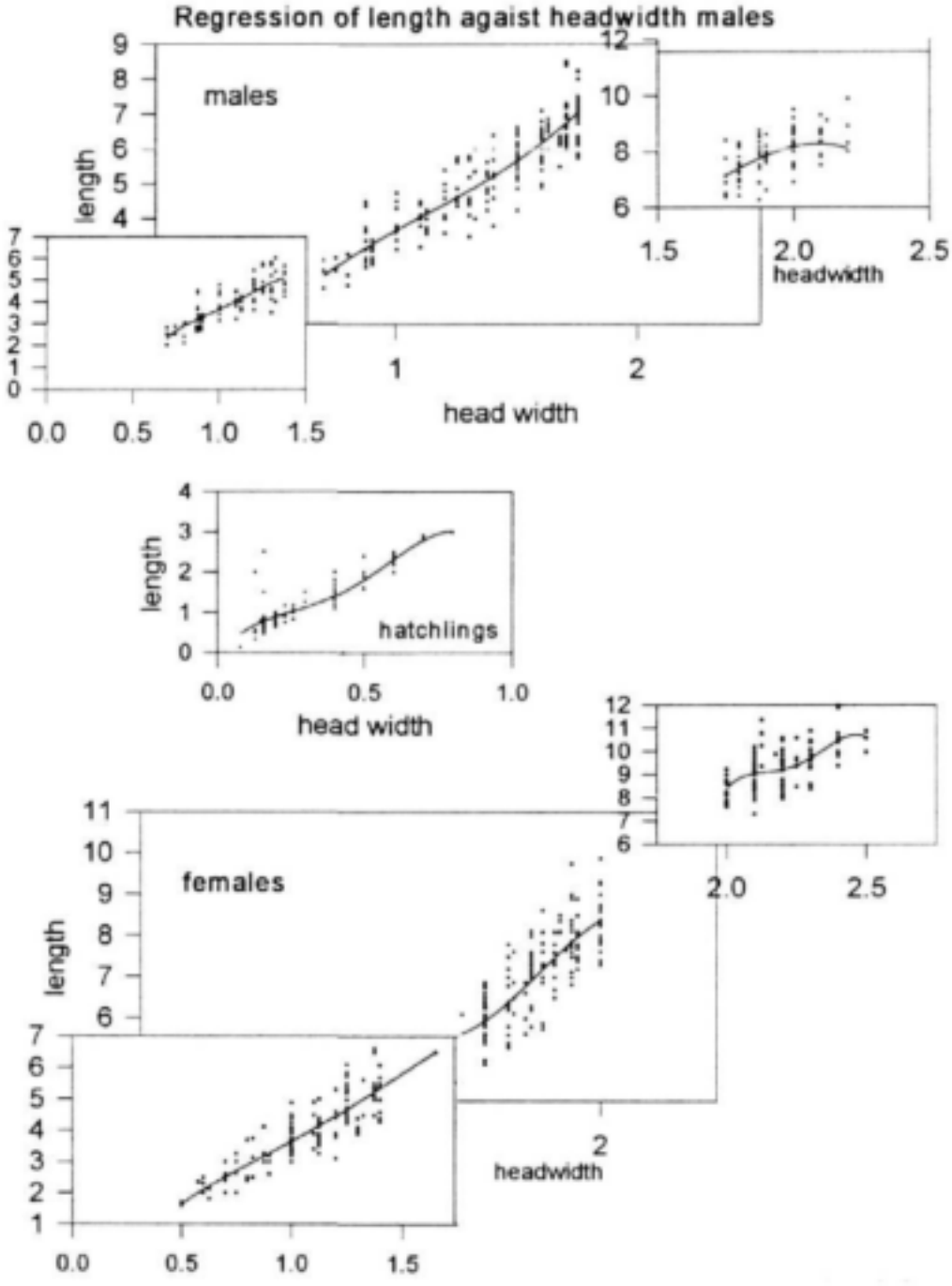

Figure 4.3.2.2 Regression (4th order) between head width and body length, for natural population of nymphs of *A. auriculata* from the Palmiet river.

Males: Small class 0.5-1.5mm; r^2 0.688, b=25.3768 (order 4) Medium size; 1.5-1.6mm; r^2=0.8180 b= -0.744086; Mature nymphs 1.6-2.2 r^2=0.11 b= -10.67331. Hatchlings; r^2= 0.70332, b= -39.2211. Females: Small 0.5-1.2mm; r^2 =0.7772; b=-1.7280, Medium 1.4-2.0mm ; r^2=0.655 b=-10.6252 and matures at 2-2.5mm; r^2=0.3915, b=-8.18723 (or r^2= 0. 3936 order10)

Linear regressions were calculated between time and head width and body length in each replicate of both food conditions. The slopes of the regressions are the growth rates for the respective variates (Table 4.3.2.4 and Figure 4.3.2.3). There is a significant time effect for head width in detritus (ANOVA $F=68.4$, $p=0$) in TETRAMIN ($F=68.4$, $p=0$) and length in detritus ($F=26.4$ $p=0$) and TETRAMIN ($F=83.0$, $p=0$). Post hoc Tukey tests for unequal N show ($p<0.05$) significant differences in the growth rate on the detritus diet for head width and length between days 12, 26 and 55 but not between day 12 and 26: on TETRAMIN for width and length between day 1 and 22.

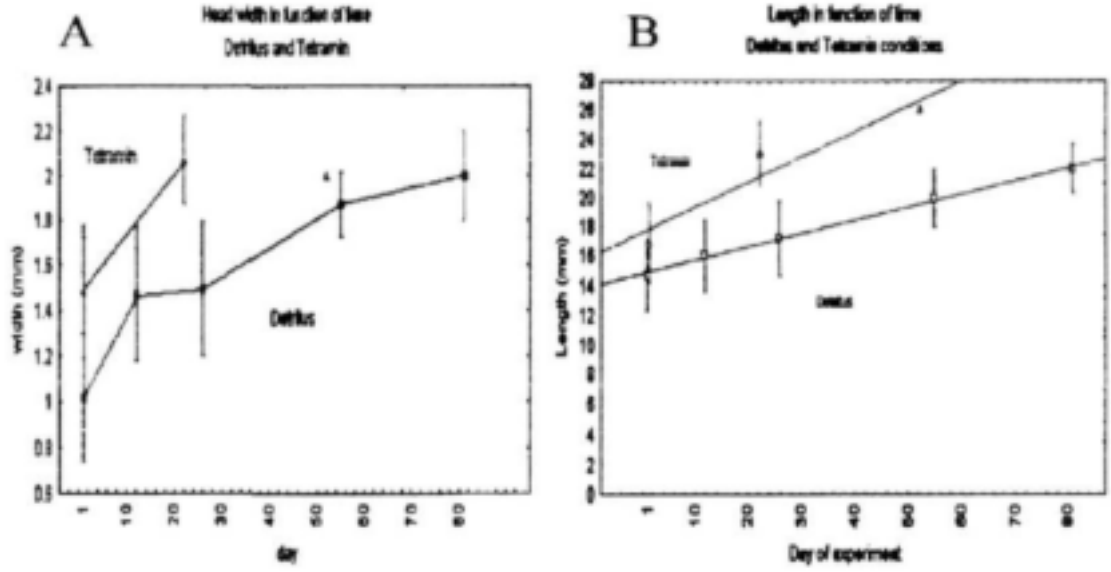

Figure 4.3.2.3 Head width (A) and length (B) of *A. auriculata* in function of time (mean±SD) for the TETRAMIN and the detritus conditions. The third data point of the TETRAMIN series represents only one nymph, since all other nymphs either died or emerged at that time. The length graphs are linearly fitted. The regression equations were calculated on the original data. TETRAMIN: $L=16.7+0.27Day$, $R^2=0.59$, $r=0.77$, $p<0.05$; Detritus: $L=14.7+0.09Day$, $R^2=0.35$, $r=0.60$, $p<0.05$.

Table 4.3.2.4 Growth rates (mean±SD) for length and width in detritus and TETRAMIN.

Food	Variate	N	mean (mm day^{-1})	STD
Detritus	Length	3	0.091_a	0.025
TETRAMIN	Length	3	0.283_b	0.063
Detritus	Width	3	0.014_c	0.0012
TETRAMIN	Width	3	0.026_d	0.0062

t-tests ab: $t=-5.75$, $p=0.005$ cd: $t=-4.35$, $p=0.01$

The addition of TETRAMIN to the diet has a doubling effect on the head width growth rate and a tripling effect on the length growth rate (Table 4.3.2.4).

Emergence, sex ratio, development of wingbuds, mortality

In each replicate of both food conditions (A, B, C and D, E, F) the percentages of emergence, males and females, animals with wingbuds and mortality were calculated.

No differences in the proportion of males were found amongst the different days and food conditions (Table 4.3.2.5), repeated measures ANOVA on Arcsin($x^{0.5}$) transformed percentages).

Table 4.3.2.5 Proportion of males (mean ± SD)

Day	Food	% males
1	TETRAMIN	48.3±9.4
22		42.3±6.6
26	Detritus	51.0±9.8
55		46.3±16.1

No differences in development were found amongst the days and food groups (repeated measures ANOVA on Arcsin($x^{0.5}$) transformed percentages, (*t*-test for dependent samples on Arcsin($x^{0.5}$) transformed percentages) or between males and females. What was noted was the preponderance of males with larger wingbuds. The tendency for males to emerge earlier than females, which was observed previously in the laboratory, was confirmed on the TETRAMIN diet (Fig. 4.3.2.4). This observation needs further quantitative confirmation. The mortality curves in function of time were similar in both food conditions (Fig.4.3.2.5).

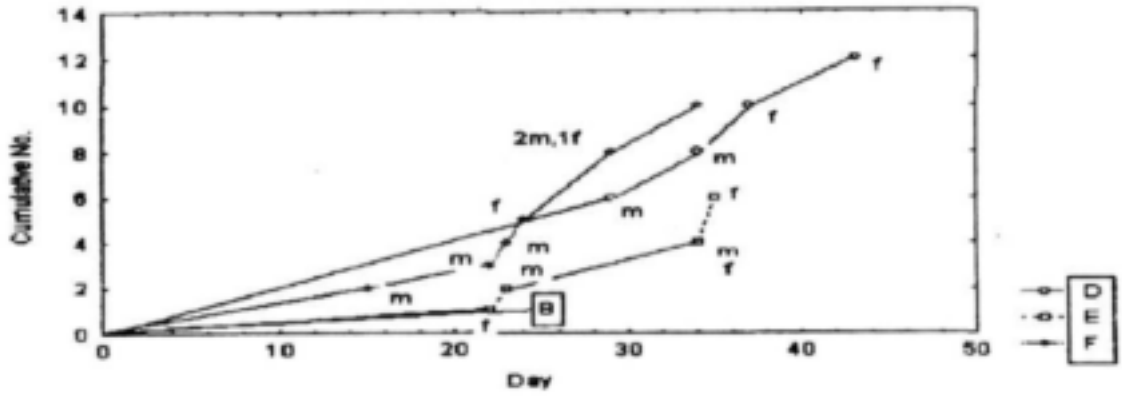

Figure 4.3.2.4 Cumulative number of emerged animals in both food conditions in function of time. No emergence was noted in the tanks A and C (detritus). Maximum number of animals per tank is 20. m=male, f=female.

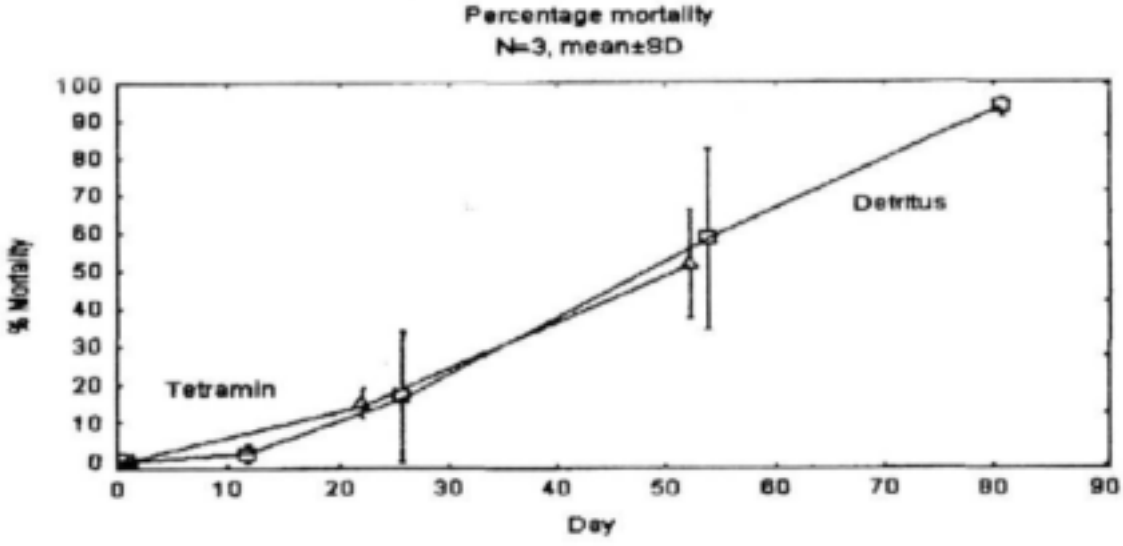

Figure 4.3.2.5 Mortality percentages (N=3, mean±SD) in both food conditions in function of time. The data were corrected for emergence.

Conclusions

The range of L and W and their ratio in the TETRAMIN population at day 1 is very similar to the same parameters in the Detritus population at day 26 (Fig ure 4.3.2.1). Therefore, we may conclude that the TETRAMIN population at day 1 was in a stage of development equal to the detritus population at day 26. In other words, although both populations originate from the same field generation, the TETRAMIN population gained approximately 25 days of development during its 52 days in the lab. (food: detritus)

prior to the experiment, compared to the Detritus population. This could most probably be ascribed to the elevated temperature in the laboratory relative to the river.

As attested by the diminishing slopes of the L-W regressions, the L-W dependence and the predictability of W from L decrease with time (Table 4.3.2.3, Figure 4.3.2.2 - decreasing r^2). The width does not increase linearly with time, but levels off to about 2 mm, while the length increases linearly until emergence (Fig. 4.3.2.3). This is most likely an effect of the maturation of the gonads, as females especially, effectively become bags of eggs toward the end of the final instar, while the eyes of males become proportionally large. In the regressions performed on field collected nymphs the changes in the r^2 values for the different size classes give an indication of the changes in body proportion with age.

During the experiment, the TETRAMIN population grew faster than the detritus population, 2 times faster for the width and 3 times faster for the length (Table 4.3.2.4). This is based on linear curve fit and is thus a rather conservative estimate.

The sex ratio was about 1 at the onset of the experiment. The proportion of males decreased a little (not significant), because of male emergence in the TETRAMIN population. There was definitely a development inhibition in the detritus population (Fig. 4.3.2.3) compared with the TETRAMIN population. High within-group variances prevented significant differences. Emergence occurred in the TETRAMIN population for about 60% of the animals while the other 40% died after 50 days. At that stage, the mortality was similar in the detritus population. However, the surviving animals failed to mature and eventually died within 30 days (Figure 4.3.2.5). We suspect an inadequate quality and quantity of detritus as the cause of the cessation of development and the mortality. In previous experiments (Haigh & Davies-Coleman, 1997), decayed leaves had provided sufficient balanced nutrient for successful emergence. It was decided in future to keep late instar on a combination of leaves and TETRAMIN to ensure good growth and egg quality.

Trials of Artificial Fertilisation and Estimate of Female Fecundity of *Adenophlebia auriculata*

Six trials were conducted to optimize previously established methods of artificial fertilization and of rearing the hatchlings *A. auriculata* nymphs on a large scale. The mature nymphs and the imagoes which were used in the trials were collected from the Palmiet River in May and September 1996.

Materials and methods for trials one to five.

The general procedure was to dissect the males and females in a suitable solution, either Ringer's or Yeager's, and to allow the eggs and sperm to remain together for 5, 15 , 30 or 45 minutes, after having been agitated with a glass pipette. After the fertilisation time had elapsed the eggs were then transferred by Pasteur pipette to 300 ml of Palmiet River water in triplicate plastic beakers. The eggs were counted to estimate the fecundity of the females. Five experiments were conducted each three replicates, A,B,C.

Table 4.3.2.6 The layout for the artificial fertilisation experiment. (Trial number; replicate; time)

Time	5 min	15 min	30 min	45 min
Replicates in	1A5-5A5	1A15-5A15	1A30-4A30	1A45
each experiment	1B5-5B5	1B15-5B15	1B30-5B30	
	1C5-5C5	1C15-5C15	1C30-5C30	

Results

Trial 1.

Dissection of 1female subimago and 1 male adult in old Ringer's solution in petri dish

Distribution of eggs: replicate; number of eggs

1A5; 120	1A15; 160	1A30; 180	1A40; 500
1B5; 150	1B15; 135	1B33; 170	
1C5; 155	1C15; 105	1C30; 240	

Day 2; The following containers were monitored 1A40, 1B5, 1C15, 1A30.

The eggs showed a shrunk appearance in the Ringer's, but inflated again once placed in the water. Most eggs were white but a few appeared opaque.

Day 4; Control. *Paramecium* sp. were observed in the containers and many eggs had died and disintegrated. The remaining eggs show a granular appearance with a faint darker longitudinal stripe in the middle.

Day 6; IA15 dried out so distilled water and Palmiet water were added to all containers.

Day 8; In IA5 only filamentous fungi were left, in IC15. 1C30 a few brown granular eggs were left and fungi.

Day 12; Water was added In 1A40 1B30, 1C30, 1A30, 1C15, 1B15, 1B5, 1C5, no eggs were left, only brown sludge, a few Oligochacta, Copepoda and green filamentous algae were observed.

Day 26; All eggs in lC30 and 1A5 have disappeared.

Trial 2

A new batch of Ringer's medium was made up from tablets: 2 tablets + 250 ml distilled water,(New Ringer's). One pre-adult female and two adult males were dissected as before. The eggs retained their shape in the new Ringer's. The beakers with eggs in Palmiet water were now aerated via Pasteur pipettes.

Egg distribution

2A5; 200	2A15; 110	2A30; 160	2A45; 300
2B5; 270	2B15; 130	2B33; 120	
2C5; 230	2C15; 110	2C30; 90	

Day 2: 2B5; eggs normal, granular with fine translucent membrane around which may be the perivitelline space and an indication of fertilisation.

Day 5; Distilled and Palmiet water were added to all containers. In 2B5 many eggs were observed to have with a fine darker spot at one end and were less white than day 1 and very granular. Fewer fungi were observed than in the first experiment. In 2B15 the medial black stripe was more obvious in some eggs but some eggs were brown and considered dead. In 2A40 the appearance of the eggs was similar to those in 2B15 but a smaller proportion was dead (opaque or brown).

Day 18; In 2B5, 2A15 and 2B40 few eggs remained while in all other replicate there were no eggs.

Day 24; No eggs left

Trial 3

For this trial Yeager's solution was used as medium. From one pre-adult female 30% of the eggs were mixed with the sperm from two adult males which had much more sperm than in those in experiment II. Smaller numbers of eggs were placed in the beakers which were aerated.

Egg distribution

3A5; 54	3A15; 60	3A30; 65	3A40; 125
3B5; 62	3B15; 150	3B30; 100	3B40; 105
3C5; 80	3C15; 37	3C30; 57	3C40; 60

Trial 4

30% of the eggs from female in the above experiment was blended with sperm of 2 new adult m. (one good, one bad) with some sperm in old Ringer's and only left for 15 minutes before being distributed into the three replicates. Same shrinking of eggs as observed in trial one

Egg distribution

4A5; 73	4A45; 100
4B5; 130	
4C5; 150	

Trial 5

The remainder of the eggs from female 3 was blended with sperm of 1 pre-adult male (very few sperm)and 1 adult male in fresh Ringer's (medium quantity of sperm), left for 15 minutes and placed in 5A15; 39 (not enough eggs left for multiple replicates).

Results of Trials 3-5

Day 14; 3A40 some eggs left

Day 20; No eggs left

Table 4.3.2.7 Estimate of female fecundity

Experiment	No. of eggs per female
1	2915
2	1720
3	1447
mean +- SD: 2027+-647.5	

Conclusions after trials one to five

The artificial fertilisation methods employed above could not produce hatchlings, since all eggs died within 25 days. Either the eggs were not fertilised, or the fertilised eggs died because of an unsuitable environment. Fresh medium should be made up - The old Ringer's was unsuitable, as it caused shrinking of the eggs (trials 1 and 4). The new Ringer's and the Yeager's solutions did not alter the shape of the eggs.

From the visual observation of ontogeny of egg morphology it appears that live fertilized eggs are translucent white and a perivitelline space appears as a membrane. The egg becomes more granular in appearance and a faint longitudinal medial stripe appears with a faint dark distal spot. In trial 1 mortality was already >80% (estimate) at day 4 and only a few eggs remained in two tanks at day 12.

Trial 2 looked more promising, since mortality was still low at day 5 and at day 18, 3 beakers contained some eggs. However, at day 24 and 26 respectively, both experiments 1 and 2 showed 100% egg mortality: brown granular eggs (dead) disintegrating in brown sludge.

In Trial 3, 4 and 5 at day 14, only the eggs in the Yeager's solution survived in only one tank (3A40). The duration of mixing eggs in the respective media (5 min, 15 min, 30 min) did not seem to affect egg survival; for example, 1C30 and 1A5 are the tanks with longest living eggs; 2B5, 2A15, 2C40. Due to Palmiet water,a typical succession of undesirable organisms appeared from day 4 onwards, *e.g.*, *Paramecium*; on day 8, fungi and on day 12, green algae, Copepoda and Oligochaeta. Aeration of the tanks considerably diminished the growth of fungi and algae.

Fecundity: considerable differences in fecundity exist amongst males (visual qualitative observation of sperm cloud) and amongst the females (count of eggs). Release of sperm or eggs by decapitating the (pre)adults did not work.

Recommendations

Use smaller volume than standard Petri-dish to mix gametes. Use female adults instead of pre-adults. Pre-filter all the Palmiet water through 45 μm filter paper to avoid seeding with undesirable organisms, which probably also prey on the eggs. Use larger volumes than 300 ml plastic beakers to keep the fertilised eggs, because (1) the water evaporates too fast, (2) it is difficult to control the aeration, (3) it is often too strong or too weak for such volume and (4) the weight of tubing and pipette is too heavy for that kind of plastic beaker. Aeration keeps the fungi in check, so it is necessary. Glass beakers are recommended. Check regularly the water volume of the beakers. Add only distilled water. Try to incubate the eggs on a fine layer of clean sand and a bit of detritus, instead of a bare beaker bottom.

Trial 6

Acting on some of these recommendations, a more elaborate experimental procedure was devised to pursue the elusive success in fertilising mayfly eggs. Mr. Simon Burton who was the previously successful fertiliser of mayfly eggs was employed to assist in this attempt.

Materials and Methods

Two new types of incubation apparatus were constructed. Both were devised in an attempt to increase the aeration of water past the eggs without the danger of drying out.

Flow through Chambers. (Figure 4.3.3.1) Based on the principle that eggs are laid in the river and that the normal hatching condition is in flowing water, a flow through system was devised . A submersible "Micra" aquarium pump was connected to the ventral part of eight 500 ml plastic jars *via* plastic tubing. The lids of the jars were modified by cutting out the central part and gluing in fine mesh. Water was pumped into the jar and flowed out through the mesh in the lids where the eggs were placed after fertilisation. The jars and the pump were placed in a 30 litre glass aquarium, with a mixture of municipal and strained river water.

Aerated chambers. In a similar fashion, eight 500ml jars modified as described above were connected to an air supply instead of an aquarium pump and placed in a 30 litre glass aquarium. A small air-stone was suspended in each jar.

Control. The third method was to place the eggs in petri-dishes which were kept in a larger container. These containers were monitored daily for the first 10 days and then weekly, while the flow through and aerated containers were monitored weekly.

All the incubators were placed in a Constant Environment Room at 20°C and 14hour photo-period.

The adults were collected from the field as sub-mature nymphs and kept in the laboratory until emergence. They were fed on decayed leaves collected from the site. Altogether, 37 adults were used in the trial one female to every two males when available. The male was decapitated, and the seminal vesicles were removed by pulling the claspers from the body gently withdrawing the testes. The sperm was freed by teasing the testes apart in an excavated slide or watch glass in 5ml freshly made Ringer's solution. The females were induced to release their eggs by grasping the wing-bases in one pair of forceps while with a second pair the end of the abdomen was sharply twisted upward. As the vent is on a subterminal segment this forces the eggs out and into the sperm solution. The last few eggs were forced out by gently compressing the abdomen against the side of the dish. The eggs were left in the sperm mixture for 10 minutes. Three ground glass slides were placed in each incubator with strained river

water, to act as adherence structure for the eggs. The eggs were left for 20 minutes in the river water for the development of the adherence coils before being counted. Each fertilisation was dispensed in approximately equal proportions to all three methods. After day 3 the fertilisation rate was estimated by counting sub-samples for each of the petri dishes. The incubation chambers were inspected every week but the petri dishes every two days. The fecundity of the females were ascertained.

Results

Although 30% fertilisation was achieved in most of the containers no hatching took place.
After one week the Petri dishes were colonised by many miro-organisms and algae as was observed in the earlier trials.

The incubators worked very well but they are quite cumbersome to open in order to check the progress of the eggs. Fecundity of the female used. Range 960-2020 mean 1440 eggs/female

Conclusions

In vitro fertilisation of the eggs of these mayflies is possible but it does not seem to provide a satisfactory method for bulk rearing of nymphs. The reported fertilisation rates from other workers are always low and given the small size and difficulty in handling the eggs I would suggest that field collection of either gravid females or 0.4-0.6mm HW nymphs for rearing in the laboratory is a more cost effective and failsafe method. During the period of this project attempts to collect adults by using a light trap were unsuccessful.

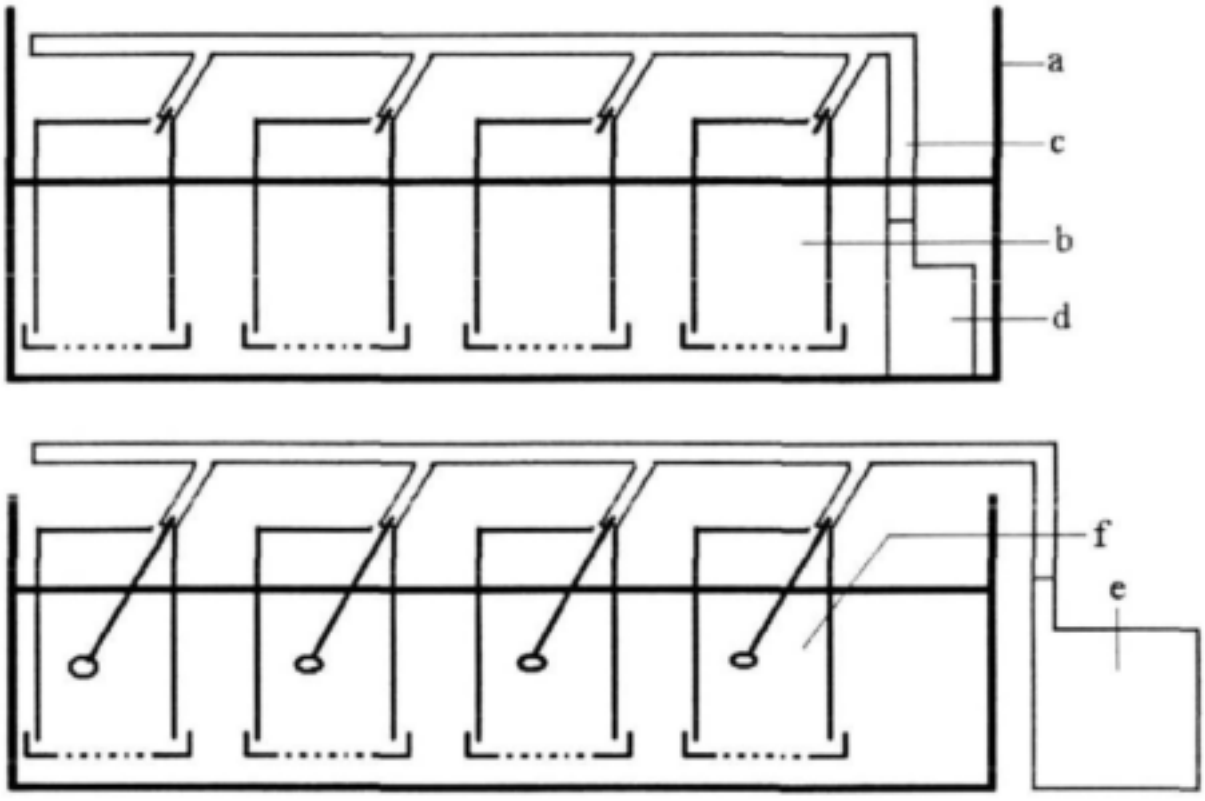

Figure 4.3.2.6 Diagram of the two types of incubation tanks developed for mayfly eggs. The top diagram is of the flow through type and the bottom diagram is of the aerated type. a) glass aquarium 50l. b Transparent plastic incubation chambers with removable bottoms with fine gauze inserts. c) plastic tubing. d) submersible aquarium (Micra) pump e) airpump f) air stones in the incubation chambers.

CHAPTER 5.
CONCLUSIONS

5.1 INTRODUCTION

With the need to investigate the means by which invertebrates and in particular the limpet *Burnupia stenochorias* and the mayfly *Adenophlebia auriculata* should be cultured in the laboratory, a series of questions arose as to the basic life history data, in particular longevity, fecundity together with mortality to estimate production rate, and growth rates. Conditions necessary for the maintenance of these invertebrates under laboratory conditions, including light, water quality, food requirements and the production of that food, all required investigation. Literature surveys revealed that the Ancylidae, the family to which *Burnupia* belongs, had previously only been investigated in Europe, where conditions and therefore life histories, will very likely be different to any local South African population. A similar situation prevails for mayflies, but the literature on this group is more comprehensive and several studies on the life history of Australian mayflies have been published. These are useful for comparison as Australian climatic conditions are reasonably similar to those in southern Africa. Investigations, therefore, had to start at a basic level. The initial investigations were completed in the previous project in 1993-95. However, as the aims of the project indicate, further information was required for the culture of the limpets in the laboratory.

Although growth had already been investigated within certain conditions in the laboratory, with associated fecundities, conditions were obviously not optimal. It was also felt that it was necessary to complete the field investigations to compare growth in the laboratory to that found under natural conditions; to determine reproductive periodicity and to attempt to discover expected longevity. Chapters 3 and 4 provide substantial answers to these investigations. Particularly emphasised within laboratory experiments was the choice of, and the necessary production of, food. Nutrafin, a fish food with high protein content and added as a powder suspended in some water, was found to significantly increase both growth and longevity of the limpets, and consequently, fecundity. Similar experiments have not been able to be done for the mayfly as the method of reproduction is not only different but also less well understood.

5.2 ACHIEVEMENT OF AIMS

The aims are italicised.

1.1 *To conduct experimental and field investigations on the selected test species which will support the information already available:*

Burnupia stenochorias. Field and associated laboratory studies on the reproduction.

Continuing laboratory studies on growth and feeding.

Adenophlebia auriculata. Laboratory studies on artificial fertilization, and the rearing of hatchlings.

5.2.1 INVESTIGATION OF *BURNUPIA STENOCHORIAS*

Several problems peculiar to the limpet presented itself and had to be addressed. With the adverse effects of handling on the growth of the limpets, extensive use was successfully made of plastic sheeting, in both the streams and the aerated bucket system, as well as in the movement of limpets from one locality to another for the purposes of toxicology experiments. The aerated buckets give the most ideal growth and fecundity results of all the containers investigated. However, the limpets will have to be conditioned to running water during some stage in their cultivation where they should be held until required, as they should represent the natural populations of limpets being tested for effects of toxicants in the toxicology programme. Plastic sheeting with limpets attached is an ideal means of transferring the limpets from the buckets to the streams presently used for their cultivation.

Attempts to successfully cultivate *Burnupia* on a larger scale included the refining of the use of the large streams. Plastic strips packed into the sumps acted as a successful means of maintaining water quality for both the diatoms and the limpets, via the incidental growth of a layer of bacteria on the plastic strips acting as a biofilter. Nitrogen and phosphate levels remain low under these circumstances. Visual surveillance indicated the addition of calcium in the form of calcium carbonate maintained the strength of the limpet shells, a problem previously encountered. Similarly, sodium silicate was added to the streams for the growth of the diatoms. The additional sodium and calcium did not appear to disrupt the balance between calcium, sodium and magnesium which could have possibly affected the fecundity in particular. The growth of the periphyton, in particular the species of diatoms present over time, was monitored in the streams. Results have shown under the given conditions of light and dechlorinated tap water, with limpets grazing and therefore affecting the periphyton storey and composition, that unicelluar and stalked diatoms are successfully grown, with bacteria and fungal spores acting as a further food source. Photographs of the species identified, taken with a Scanning Electron Microscope, are provided. Both the literature survey regarding those conditions considered necessary for the growth of the periphyton, and the variation in

growth of periphyton seen by the project researchers under the conditions provided by the University, have all been considered in the designs for the new laboratory.

Comparisons by various statistical means have revealed that using the morphometric measurements of length, width and height, three local populations of *Burnupia* cannot be separated with ease. This is encouraging should it be decided in future that further genetic stock is necessary for the cultures. Similarly, it confirmed that should test individuals be directly taken from a natural population for ecotoxicology trials, all three measurements were necessary to identify the chronic effects of potential toxicants on the limpets.

5.2.2 INVESTIGATION OF *ADENOPHLEBIA AURICULATA*

The life history of this mayfly is characterized by multiple emergences throughout the year except for very short period s, the longest being for about 10 weeks during the winter period. The growth rate can be doubled in the laboratory on protein enriched food. It is possible to fertilize the eggs artificially but it seems unlikely that successful hatching will be brought about as matter of course. Given all this information, it seems most feasible that the small mayflies should be brought in from the river at regular intervals and kept under standard laboratory conditions to acclimate and grow to a standard size of 1.2-1.4 mm HW when they are to be used in toxicology experiments. The large mature nymphs with wing buds should be left in the river to minimize the danger of depleting stock.

The distribution of *A. auriculata* amongst biotopes is strongly influenced by the size of the nymphs. The initial distribution is random, with small nymphs present throughout the stream. Subsequently there is a redistribution to the optimal biotopes which have slow currents, large substrate size and vegetation, and large quantities of detritus in common. An important feature for the large nymphs (pre-emergent) is that they have suitable structures for emergence. This is provided in marginal areas by vegetation, rocks and the bank itself. The scale of the river does not affect the distribution pattern of the nymphs except when different current velocities occur in the same biotopes from the different rivers.

The ecological and hydraulic biotope concepts are based on different criteria and thus give different insights into the factors affecting the size class distribution patterns of *A. auriculata*. The unambiguous nature of the hydraulic biotype descriptions lends support to the adoption as a unifying classification system. Although based on the nature of the surface flow and measurable it

is necessary to augment the classification with ecologically relevant information to achieve a true reflection of the factors determining the distribution of the population.

1.2 *To test the experimental techniques so developed at a larger production scale.*

The achievement of this aim has been covered under Section 5.2.1, 5.2.2 and 5.2.3.

1.3 *To investigate and design features and costing for a laboratory dedicated to the large-scale production of the selected species. The suitability of various biological filtration systems to ensure optimal water quality conditions and light conditions for the culture of both the test species and selected diatom species (periphyton) as feed for the test species, will be investigated.*

5.2.3 BUILDING DESIGN

The plans for the building are presented in Chapter 2 and there has been extensive investigation of equipment and costing to equip the building. The area which has not received sufficient attention is the fund-raising activity. The fund-raising manager of the University has been unavailable for extended periods. I have always felt that an outside agency should be engaged to take this on and I am still of the opinion. However, the Director of Marketing is opposed to this suggestion. We shall attempt to resolve this issue.

The testing of water quality maintenance was accomplished by using shredded plastic waste as biofilter in the sump of each of the recirculating channels with great success. Two of the channels have been running uninterruptedly for twenty months.

5.3 PRESENTATIONS AND PUBLICATIONS

Presentations

1) Haigh, EH and HD Davies-Coleman, 1997. Presentations by both researchers on the development of methods for laboratory culture of aquatic invertebrates, including the success to date with *Burnupia stenochorias* and *Adenophlebia auriculata*. Zoology Dept, University of Port Elizabeth.

2) Davies-Coleman, HD and EH Haigh, 1997. Life cycle and Growth of Two Natural Populations of a South African Ancylid, *Burnupia stenochorias*. Poster presentation by HD Davies-Coleman, North American Benthological Society 45th Annual Meeting, San

Marcos, Texas, USA.

* also presented June 1997 at the SASAQS (South African Society for Aquatic Scientists) Conference, Mtinzini, Natal.

3) Haigh, EH and HD Davies-Coleman, 1997. Investigations into the Reproductive Biology of *Burnupia stenochorias* (Mollusca, Ancylidae). Paper presented by EH Haigh, SASAQS Conference, Mtinzini, Natal.

4) HD Davies-Coleman, EH Haigh and A Booth*, 1997. Growth Analyses of Natural and Laboratory-Reared Populations of the Freshwater Limpet *Burnupia stenochorias* (Ancylidae), 1997. Paper presented by HD Davies-Coleman, SASAQS Conference, Mtinzini, Natal.

* Dept. Ichthyology and Fisheries Services, Rhodes Univ.

5) Haigh, EH and B Hunt, 1997. Population Distribution of the Mayfly *Adenophlebia auriculata* in Two Eastern Cape Rivers. Poster presented by B. Hunt (Zoology Hons), SASAQS Conference, Mtinzini, Natal.

6) HD Davies-Coleman contributed to the paper to be presented by Prof K deKok, Potchefstroom University, as part of a review of freshwater malacological work currently being undertaken in South Africa, September 1997. African Medical Malacology Conference, Harare.

Papers

As this project (K5/755) will be completed in December 1997, we submit a list of papers in preparation. These papers are to be submitted to various journals, both national and international, from November 1997. Their titles may change on final submission to the chosen journal.

Burnupia stenochorias

1) The breeding biology and fecundity of *Burnupia stenochorias* (Pulmonata, Ancylidae).

2) The embryology of *Burnupia stenochorias*, Melvill and Ponsonby (Mollusca, Pulmonata, Ancylidae).

3) Life cycle and growth of two natural populations of a South African freshwater limpet, *Burnupia stenochorias* (Pulmonata, Ancylidae).

4) The effects of density and temperature on the growth rate and mortality of a freshwater limpet, *Burnupia stenochorias* (Pulmonata, Ancylidae).

5) The effects of diet on growth and mortality of *Burnupia stenochorias* (Mollusca,

Pulmonata, Ancylidae) in the laboratory.

* co-authored with A.Booth.

6) The effects of container size on the growth and survivorship of *Burnupia stenochorias* (Basommatophora, Ancylidae) when cultured in the laboratory.

7) Sexual development of *Burnupia stenochorias*, Melvill and Ponsonby (Mollusca, Basommatophora, Ancylidae).

8) Early colonisation of bare surfaces by epilithic diatoms in artificial streams, and the impact of grazing limpets (*Burnupia stenochorias*, Ancylidae) on the diatom assemblage.

* co-authored with M.Balarin, 1998.

Adenophlebia auriculata

9) The effect of different diets on the growth of *Adenophlebia auriculata* (Ephemeroptera, Leptophlebiidae) in the laboratory.

10) The effect of temperature on instar period and mortality of *Adenophlebia auriculata* (Ephemeroptera, Leptophlebiidae).

11) Population distribution of nymphs of *Adenophlebia auriculata* (Ephemeroptera, Adenophlebia) in two Eastern Cape rivers.

* co-authored with Brian Hunt (Honours student, Zoology)

12) Life history of *Adenophlebia auriculata* (Ephemeroptera, Adenophlebia) in a small Eastern Cape river.

CHAPTER 6

FUTURE RESEARCH DIRECTIONS.

6.1 CAPTIVE BREEDING

It became clear during the course of the five years which this project has been in operation that the establishment of a successful rearing programme depends largely on the selection of APPROPRIATE species, and that until the selected species has undergone a series of experimental investigations their suitability cannot be determined. Obviously the ability to induce mating in captivity is the most important feature for the success of a long term laboratory breeding programme. Captive breeding although the first important step, is only one aspect in the process of establishing a suitable laboratory species. The responses to toxicological testing of the selected species also requires investigation. Tolerance ranges should too broad or too narrow are equally unsuitable. The ideal towards which we should be working will remain a multi-species suite of riverine organisms with a range of responses to a variety of pollutants. Now that we have gained a reasonable understanding of the methods to rear freshwater invertebrates, the way is clear to start a series of short-term tests on as yet untried species. In our opinion other groups, such as Platyhelminthes or Thrichoptera have potential for captive breeding.

As far as the existing captive breeding programme is concerned, the failure of the mayfly embryos to complete development after fertilisation, should investigated. At the same time, an attempt should be made to capture gravid females in the field and induce egg deposition in the laboratory for variety of riverine invertebrates. If this technique can be mastered then the captive rearing of nymphs will be accomplished with ease as rearing techniques are now fairly well established.

6.2 LIFE HISTORIES

The aims of this project did not allow the in depth investigation of the all the aspects of the life history of the mayfly. Questions which still require answers include:

- What are the cues which govern emergence and mating?
- How is the instar period controlled?
- What is the growth rate under natural conditions?

Comparison of life histories of both the limpet and the mayfly species from a subtropical summer rainfall area and a southern winter rainfall area would throw valuable light on the reponses of these species to changes in climate and geographical region. This research would provide 2 MSc theses.

The establishment of the life history styles of other aquatic species will add to the current understanding of South African river ecology in terms of the ecological requirements of species resident in rivers in different biogeographic regions. This will refine the river management expertise being developed at present to a degree where sustainable utilisation of the freshwater reserve will be guaranteed.

6.3 TAXONOMY

The taxonomy of the genus *Burnupia* is outdated and inadequately described. Consequently, we suggest the following:

- a re-visit of all the collections made of *Burnupia*, particularly those held in the National Freshwater Mollusc Collection, Potchefstroom University of Christian Higher Education, at the Albany Museum and at Rhodes University in Grahamstown and in the Pietermaritzburg Natural History Museum. However, there are problems associated with the records of some of these collections and the accessibility and conditions of the specimens.
- an extensive collection be made from the places cited in species descriptions where possible, at least country wide or within southern Africa to provide full morphological descriptions and to address the current problems in extant collections.
- on the basis of these descriptions, a comparison with the descriptions provided by Brown (1994), with a reconciliation being made of the 14 described species of *Burnupia* in southern Africa alone. Voucher specimens can be obtained from the British Natural History Museum.
- employment of new genetic techniques to determine the sytematics of the group.

6.4 DIETARY PREFERENCES.

A full investigation into the dietary preferences of *B. stenochorias* is necessary, by analysis of gut contents. This was suggested by Prof. Round of Surrey University (*pers com*) to be sufficient material for an MSc project.

The addition of Nutrafin or Tetramin fish food to the containers where *B.stenochorias* and *A. auriculata* is cultured, in Controlled Environment Rooms, result in an increase in the growth rate. At present we presume the increased growth of the limpet to be due to increases in bacterial population, as the Nutrafin supplies essential amino acids to the bacteria. Bacterial counts both on the plastic bags lining the pots, and within the gut of the limpets, would throw light on this matter, and support the above investigation. The reasons for the accelerated growth of the mayfly can be investigated by calorific and component analysis of the body as well as an assesment of the influence of the added amino acids on the fecundity of the females.

CHAPTER 7

REFERENCES

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